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Black fungal extremes

Edited by G.S. de Hoog and M. Grube

CBS Fungal Biodiversity Centre,
Utrecht, The Netherlands

An institute of the Royal Netherlands Academy of Arts and Sciences

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Cover: Images of various habitats on black yeast-like fungi. Top from left to right: flying foxes at temple of Chachoengsao province, Thailand, the hypothesized natural niche of *Exophiala dermatitidis*. Saltpans at Sečovlje, Slovenia, the natural habitat of *Hortaea werneckii*. Gas station, The Netherlands, human-made habitat of *Cladophialophora immunda*. Bottom from left to right: rock formations in the McMurdo Dry Valley, Antarctica, one of the natural habitats of *Recurvomyces mirabilis*. Human foot with lesions of *Coniosporium epidermidis*. Sea ice from Kongsvegen glacier in Spitsbergen, natural habitat of *Aureobasidium pullulans var. subglaciale*.

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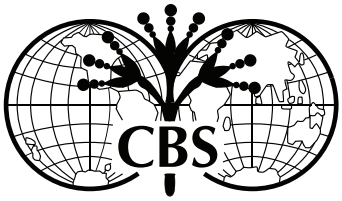

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PREFACE

The terms "black fungi" or "dematiaceous fungi" are practical tags that recall the early days of mycological classification. At the beginning of the 19th century the grouping of anamorphic fungi relied on characters that were easy to observe with minimal optical equipment, such as colour mycelial. Since these times, and till today, hyphomycetes producing olive-grey, brown or black pigment in their cell wall or conidia are classified as "dematiaceous fungi" (referring to the meanwhile obsolete genus *Dematium*, originally introduced for black, clumpy fungi). The dark pigments are presumed to be DHN-like (dihydroxynaphthalene) melanins, although biochemical and structural characterisation has not always been achieved and may involve various precursor molecules.

It has now become clear that black fungi do not comprise a single phylogenetic lineage, but stem from divergent branches of the fungal tree of life. The lineages share production of melanin-like pigments, which has had profound evolutionary consequences for these groups. In human- and phytopathogenic fungi melanins are linked to increased virulence. Melanins also provide protection from a broad range of environmental stress conditions.

With their adaptive potential to uncommon habitats, black fungi have raised increasing interest of mycologists in medical sciences as well as in environmental ecology. It has become clear that a comprehensive understanding of black fungal evolution, ecology and functionality requires a synergic interdisciplinary approach, supported by a cooperative effort among specialists. A first step to bundle the interests in black fungi and to create a common forum was the foundation of a Working Group "Black Yeasts" under auspices of the International Society for Human and Animal Mycology (ISHAM). A small-scale meeting was held in Graz, Austria (May 30–June 1, 2006), and focused on the extremophilic ecology of black fungi. The growing interest in the topic was reflected in the second workshop held in Utrecht, The Netherlands (April 26–28, 2007), jointly with the ISHAM-affiliated Working Group on "Chromoblastomycosis", with 55 participants from 19 countries.

The workshops helped to further establish the scientific network among fundamental scientists, clinicians and workers in applied fields. Oral contributions at the Utrecht workshop covered such diverse topics as human infections, diseases on cold-blooded animals, fungi growing in lichens or on rock under extreme climatic conditions, fungal use in bioremediation of polluted environments, and black yeasts in drinking water, but also susceptibility testing, and molecular evolution. Nearly all contributions included new material and quickly the idea was born that this information would be valuable to be published in a coherent fashion. For the majority of the medical papers we refer to the journal "Medical Mycology", issue 46(1), 2009. The present issue of *Studies in Mycology* entitled "Black Fungal Extremes" reveals unexpected types of ecology, such as growth in Arctic glaciers, Mediterranean rock, in lichens, in pure acid, and in nearly saturated salt solutions. Other papers investigate the evolutionary origins of black fungi, expression of relevant genes, medical aspects, and technical advances in culturing techniques.

This interdisciplinary blend of approaches gives an insight in current research on black fungi. We hope that the present issue will attract the interest of more mycologists, who will join in our initiative to shed more light on the fascinating biology of extremophilic and pathogenic black fungi.

The Editors

November 2008

The papers in this issue of *Studies in Mycology* emerged from a workshop organised by the Working Groups on Black Yeasts and on Chromoblastomycosis, under auspices of the non-profit organisation "International Society for Human and Animal Mycology" (ISHAM). All authors report no conflicts of interest. The authors alone are responsible for the content and writing of the papers.

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Drought meets acid: three new genera in a dothidealean clade of extremotolerant fungi

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Abstract: Fungal strains isolated from rocks and lichens collected in the Antarctic ice-free area of the Victoria Land, one of the coldest and driest habitats on earth, were found in two phylogenetically isolated positions within the subclass *Dothideomycetidae*. They are here reported as new genera and species, *Recurvomyces mirabilis* **gen. nov.**, **sp. nov.** and *Elasticomyces elasticus* **gen. nov.**, **sp. nov.** The nearest neighbours within the clades were other rock-inhabiting fungi from dry environments, either cold or hot. Plant-associated *Mycosphaerella*-like species, known as invaders of leathery leaves in semi-arid climates, are also phylogenetically related with the new taxa. The clusters are also related to the halophilic species *Hortaea werneckii*, as well as to acidophilic fungi. One of the latter, able to grow at pH 0, is *Scytalidium acidophilum*, which is ascribed here to the newly validated genus *Acidomyces*. The ecological implications of this finding are discussed.

Key words: Acidophilic fungi, Antarctica, black fungi, extremotolerance, halophilic fungi, ITS, lichens, phylogeny, rock-inhabiting fungi, SSU, taxonomy.
Taxonomic novelties: *Recurvomyces* Selbmann & de Hoog, gen. nov.; *Recurvomyces mirabilis* Selbmann & de Hoog, sp. nov.; *Elasticomyces* Zucconi & Selbmann, gen. nov.; *Elasticomyces elasticus* Zucconi & Selbmann, sp. nov.; *Acidomyces* Selbmann, de Hoog & De Leo, gen. nov.; *Acidomyces acidophilus* (Sigler & J.W. Carmich.) Selbmann, de Hoog & De Leo, comb. nov.

INTRODUCTION

Contrary to expectations, bare rocks in arid and semi-arid climates may harbour a bewildering biodiversity of black fungi. Many species have been reported from the Mediterranean basin (Sterflinger *et al*. 1997, Wollenzien *et al*. 1997, Bogomolova & Minter 2003, De Leo *et al*. 1999, 2003, Bills *et al*. 2004, Ruibal *et al*. 2005, Ruibal *et al*. 2008). These extremotolerant fungi live and even thrive on surfaces that are too harsh to support growth of competing microorganisms; they shelter in small depressions in the marble surface, called micropits (Sterflinger 1998). Similar extremotolerant fungi were discovered in the extremely cold and ice-free McMurdo Dry Valleys, a desert area in the Antarctic (Nienow & Friedmann 1993), where temperatures are only occasionally above zero, dropping to about –50 °C in winter. Onofri *et al*. (1999) and Selbmann *et al*. (2005) even reported on the existence of possibly endemic genera, *Friedmanniomyces* Onofri and *Cryomyces* Selbmann, de Hoog, Mazzaglia, Friedmann & Onofri in these habitats, which apparently show active evolution under conditions of near-permanent frost and extreme dryness (Friedmann *et al*. 1987). These fungi may escape prohibitive environmental conditions by colonising air spaces in rocks, living in association with lichens and algae in cryptoendolithic communities (Friedmann & Ocampo 1976, Friedmann 1982).

In the present paper we describe three new fungal genera and species; their novelty is supported by molecular phylogeny, taking a clearly separate position within the *Dothideomycetidae*. One genus includes two strains isolated from rocks in the Antarctic desert, one strain from rocks collected in Monte Rosa in the Alps, Italy, and an unidentified rock fungus from Puebla de la Sierra, Spain; the other genus includes three strains isolated from different thalli of Antarctic lichens, one from cryptoendolithic Antarctic communities and one from rocks collected in Aconcagua in the Argentinian Andes. In contrast to most rock-inhabiting black fungi, which are generally scarcely differentiated, they show peculiar and distinguished morphological traits.

Fungi may also be encountered in extremely acidic environments. Some are able to grow at pH values down to pH 0 (Starkey & Waksman 1943, Harrison *et al*. 1966, Gould *et al*. 1974, Ivarsson & Morita 1982, Gimmler *et al*. 2001). Sigler & Carmichael (1974) compared four strains from an acidic soil (pH 1.4–3.5) with the ones previously isolated by Starkey & Waksman (1943) and Ivarsson & Morita (1982), referring them to the genus *Scytalidium* Pesante on the basis of scarcely differentiated brown arthroconidia. Our SSU and ITS comparison proved these fungi also to be members of a clade within the *Dothideomycetidae,* amidst rock-inhabiting fungi from cold and semi-arid climates.

MATERIALS AND METHODS

Strains

Black fungi were isolated from rock samples harbouring a cryptoendolithic lichen-dominated community and from epilithic lichens collected in different locations of Northern and Southern Victoria Land, Antarctica, in the framework of the Italian expedition

Table 1. List of strains studied.

Species	Strain no.	Source	Geography	ITS	Reference
Acidomyces acidophilum	CBS 335.97	Acidophilic algae *Dunaliella acidophila* pH 1.0	Germany	AJ244237	Gimmler *et al.* 2001
Acidomyces acidophilum (deposited as *Scytalidium acidophilum)*	CBS 270.74 T (ATCC 26772; UAMH 3460; IMI 183518)	Soil near acidic elemental sulphur pile pH 1.1	Canada	–	Sigler & Carmichael 1974
Acidomyces acidophilum (deposited as *Botryomyces caespitosus*)	CBS 899.87	Pyrite ore acidic drainage pH 2.0	Germany	–	–
	dH 13081 = det 106/2003	2N Sulphuric acid pH 1	Danmark (supplied by GC Frisvad)	–	Starkey & Waksman 1943
	dH 11526 = det 237-1999	Volcanic soil	Iceland (supplied by S Gross, Berlin)	–	–
	dH 12881 = det 142-AF1			–	–
Acidomyces sp.	dH 13119	Acidic industrial process water pH 1.5	Emmen, The Netherlands	–	–
Batcheloromyces proteae	CBS 110696; CPC 1518	*Protea cynaroides*	South Africa	–	Crous *et al.* 2007
Capnobotryella renispora	CBS 214.90 T (CBS 176.88; IAM 13014; JCM 6932)	*Capnobotrys neessii*	Japan	–	–
Catenulostroma abietis	CBS 290.90	Man, skin lesion	The Netherland	AY128698	Crous *et al.* 2007
	CBS 145.97 (dH 15396)	sandstone of cathedral	Zeitz, Germany	AY128699	Butin *et al.* 1996
	CBS 300.81	*Juniperus communis* (*Cupressaceae*), needle	Graubünden, Grüsc, Switzerland	AJ244264	–
	CBS 279.86		Kiel, Germany	–	Butin *et al.* 1996; Sterflinger *et al.* 1999
	dH 12687 = det 396/2001	Painted wall	Sweeden	–	–
	TRN 128	Limestone	Mallorca	AY559363	–
	dH 12697 = det 373/2001 RMF N113	Desert soil	Namibia	–	–
	dH 13593	See snail	Italy	–	–
	CBS 618.84	*Ilex* sp. leaf	Germany	AY128696	–
	CBS 118765 (TRN 127; dH 14531)	Limestone	Cala San Vincenc, Mallorca	AY559362	–
Catenulostroma elginense	CBS 111030 (CPC 1958)	*Protea grandiceps*	South Africa	AY260093	Crous *et al.* 2007
Catenulostroma germanicum	CBS 539.88	Stone	Germany, former West-Germany	EU019253	Crous *et al.* 2007
Catenulostroma macowanii	CBS 110756 (CPC 1872)	*Protea nitida*	South Africa	–	Crous *et al.* 2007
	CPC 1488			–	–
Elasticomyces elasticus	CBS 122538 (CCFEE 5313)	*Lecanora fuscobrunnea*	Kay Island, Northern Victoria Land, Antarctica	FJ415474	–
	CBS 122539 (CCFEE 5319)	*Lecanora* sp.	Inexpressible Island, Northern Victoria Land, Antarctica	FJ415475	–
	CBS 122540 (CCFEE 5320)	*Usnea antarctica*	Edmondson Point, Northern Victoria Land, Antarctica	FJ415476	–
	Da-004-06	Rock	Mount Aconcagua, Andes, Argentina	–	–
	CCFEE 5474 (D007-06)	Sandstone	Tarn Flat, Northern Victoria Land, Antarctica	–	–
Friedmanniomyces endolithicus	CBS 119423 (CCFEE 5208)	Sandstone	Northern Victoria Land, Antarctica	–	Selbmann *et al.* 2005
	CBS 119424 (CCFEE 5195)	Rock	Northern Victoria Land, Antarctica	–	Selbmann *et al.* 2005
	CBS 119429 (CCFEE 5193)	Sandstone	Timber Peak, Northern Victoria Land, Antarctica	–	Selbmann *et al.* 2005

Table 1. (Continued).

Species	Strain no.	Source	Geography	ITS	Reference
Friedmanniomyces endolithicus	CBS 119428 (CCFEE 5001)	Sandstone	Timber Peak, Northern Victoria Land, Antarctica	–	Selbmann *et al.* 2005
Friedmanniomyces simplex	CBS 116775 T (CCFEE 5184)	Sandstone	Battleship Promontory, Southern Victoria Land, Antarctica	DQ028271	Selbmann *et al.* 2005
Hobsonia santessonii		*Peltigera scabrosa*	Sweden	–	Sikaroodi *et al.* 2001
Hortaea acidophila	CBS 113389 (dH 11932)	Lignite pH 1.0	Germany	–	Hölker *et al.* 2004
Hortaea werneckii (preserved as *Pseudotaeniolina globosa*)	CBS 110352 (dH 12843 = VPCI 176)	Hollow tree	Sudan	–	–
Hortaea wemeckii	dH 12322; Poonwan 13-44-08648	–	–	–	–
	CBS 373.92 (dH 15813)	Ceach soil	La Palma, Spain	AJ238474	–
	CBS 359.66 (dH 15803)	Can, tinea nigra palmaris	Suriname, Paramaribo	AJ244249	–
	CBS 122.32 (dH 15340)	Can, tinea nigra palmaris	–	AJ238473	–
	CBS 117.90 (UAMH 4978; dH 15327)	Salted fish, *Osteoglossum bicirrhosum*	Brazil	AJ238472	Mok *et al.* 1981
	CBS 116.90 (ATCC 52681; UAMH 5389; dH 15311)	*Cantharus cantharus*, eye infection, black sea bream in aquarium	Italy	AJ238471	Todaro *et al.* 1983
	CBS 115.90 (UAMH 4985; dH 15303)	*Bufo granulosus*, kidney	Brazil	AJ238470	–
	CBS 111.31 (dH 15284)	Man, keratomycosis nigricans palmaris	Brazil	AJ238679	–
	CBS 100455 (MZKI B-675)	Coral, sea water	Croatia	AY128704	Zalar *et al.* 1999
	CBS 107.67 (dH 15206)	Man, tinea nigra	Lisboa, Portugal	AJ238468	McGinnis 1979
	MZKI B-987	Ipersaline water	Spain	–	–
	dH 13416	Angelfish	–	–	–
	CBS 117931 (TRN 122, dH 14528)	Limestone	Cala San Vincenc, Mallorca	AY559357	–
	BMU00057	Patient's foot	China	–	–
Mycocalicium victoriae	CBS 109863	Soil, garden museum	Messina, Italy	AJ312123	–
Mycosphaerella tasmaniensis	CBS 114556 (CMW 14663; STE-U 1556)	*E. nitens*	Australia	DQ267592	–
	CBS 111687 (CMW 14780; STE-U 1555)	*E. nitens*	Australia	AY667578	–
Pseudotaeniolina globosa	CBS 110353 (dH 12840; det M108/2002)	58-yr-old man, aorta, at autopsy	Würzburg, Germany	–	Kurzai *et al.* 2003
	CBS 109889 T (MC 769)	Church roof	Sicily, Italy	AY128700	De Leo *et al.* 2003
	CBS 113249 (dH 13060)	–	–	–	–
	CBS 303.84	Wood	Germany	AJ244268	de Hoog *et al.* 1999
	CBS 119923 (dH 16905)	Window	Japan	–	–
Recurvomyces mirabilis	CBS 119434 (CCFEE 5264; dH 14759)	Sandstone	Battleship Promontory, Southern Victoria Land, Antarctica	FJ415477	–
Recurvomyces mirabilis	CCFEE 5480 (D016-02)	Sandstone	Battleship Promontory, Southern Victoria Land, Antarctica	–	–
	CCFEE 5391	Rock	Mount Rosa, P.ta Indren, Alps, Italy	–	–
Recurvomyces sp.	CBS 117957 (TRN 491; dH 14566)	Quarzite	Puebla de la Sierra, Madrid, Spain	AY1843175	–
Teratosphaeria cryptica	TC 0.56	*E. globulus*	Australia	DQ665661	–

Table 1. (Continued).

Species	Strain no.	Source	Geography	ITS	Reference
Teratosphaeria mexicana	CBS 110502 (CMW 14461)	*E. globulus*	Manjimup, Darling View, Plantation, Western Australia	AY725558	Crous *et al.* 2007
Teratosphaeria microspora	CBS 110890 (CPC 1832)	*Protea* leaf	South Africa	AY260097	Taylor *et al.* 2003
Teratosphaeria molleriana	CBS 111164 (CMW 4940; STE-U 1214)	*E. globulus*	Abrantes, Portugal	AF309620	Crous *et al.* 2007
	CPC11842	*Eucalyptus* sp.	Portugal	DQ302989	Crous *et al.* 2006
	CPC11845	*Eucalyptus* sp.	Portugal	DQ302990	Crous *et al.* 2006
	CBS 116370 (CPC 10397)	*E. globulus*	Spain	AY725561	
	CPC 12056			DQ302991	Crous *et al.* 2006
Teratosphaeria nubilosa	CBS 116005 E (CMW 3282; CPC 937)	*E. globulus*	Austria	AY725572	Crous *et al.* 2007
	CPC 4661	*E. globulus*	Spain	AY725570	Crous *et al.* 2004
	CBS 111445 (CPC 4660)	*E. globulus*	Spain	AY725569	Crous *et al.* 2004
	CPC 3722	*E. globulus*	Spain	AY725568	Crous *et al.* 2004
	CPC 1099	*E. globulus*	Tanzania	AY725567	Crous *et al.* 2004
	CBS 111969 (CPC 1078)	*E. globulus*	Kenia	AY725563	Crous *et al.* 2004
	CPC 4663	*E. globulus*	Spain	AY725571	Crous *et al.* 2004
	CPC 11882	*E. globulus*	Portugal	DQ302999	Crous *et al.* 2006
	CPC 11723			–	
	CPC 11487	*E. globulus*	Spain	DQ302994	Crous *et al.* 2006
	CPC 11249	*E. globulus*	Spain	DQ302993	Crous *et al.* 2006
	CPC 11246	*E. globulus*	Spain	DQ302992	Crous *et al.* 2006
	CBS 114419 (CPC 10497)	*Eucalyptus globulus* (*Myrtaceae*)	New Zealand	AY725574	Crous *et al.* 2007
	TC 0.42	*E. globulus*	Australia	DQ665659	–
	TC 0.40	*E. globulus*	Australia	DQ665657	–
	TC 0.47	*E. globulus*	Australia	DQ665658	–
	CBS 116283 (CPC 10495)	*E. globulus*	New Zeland	AY725573	Crous *et al.* 2004
Teratosphaeria nubilosa (	CPC 11885	*E. globulus*	Portugal	DQ303000	Crous *et al.* 2006
Teratosphaeria ohnowa	CPC 1005	–	–	AF309605	Crous *et al.* 2001
	CBS 112896* (CMW 4937; CPC 1004)	*E. globulus*	South Africa	AF309604	Crous *et al.* 2001
		–	–	AF173299	–
	CMW 9103	–	–	AF468881	–
	CBS 110949 (STE-U 1006)	*E. grandis* (*Myrtaceae*), leaves	Hazyview, South Africa	AY725575	–
Teratosphaeria toledana	CBS 113313 H (CMW 14457)	*Eucalyptus* sp., leaves	Toledo, Spain	AY725580	–
	CPC 10840	*Eucalyptus* sp.	Spain	AY725581	Crous *et al.* 2004

Abbreviations used: ATCC – American Type Culture Collection, Manassas, VA, U.S.A.; CBS – Centraalbureau voor Shimmelcultures, Utrecht, The Netherlands; dH – GS de Hoog private collection, CBS, Utrecht, The Netherlands; CCFEE – Culture Collection of Fungi From Extreme Environments, Università degli Studi della Tuscia, Viterbo, Italy; CMW – Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; CPC – Culture collection of P Crous, housed at the CBS; IMI – International Mycological Institute, U.K.; MC – Collection of Istituto di Microbiologia di Messina, Italy; MZKI – Microbiological Culture Collection, National Institute of Chemistry, Ljubljana, Slovenia; STE-U – University of Stellenbosch fungal culture collection, Stellenbosch, South Africa; TRN – T Ruibal private collection; UAMH – The University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada.

*Ex-type cultures.

2003/2004, and from rock samples collected in Mount Aconcagua, Andes, Argentina, and Monte Rosa in the Alps, Italy, as reported in Table 1. The samples were collected using a sterile chisel, sterlilised in the field before each sampling, and preserved in sterile bags at –20 °C. To remove potential contaminants, rocks collected in other environments than Antarctica were washed 15 min in sterile physiological solution added with 0.1 % Tween 20 (Sigma - Aldrich, Munich, Germany) and rinsed 4 times with physiological solution to remove any trace of the detergent. Isolations from rocks were performed by powdering the samples and seeding fragments in triplicate Petri dishes filled with 2 % malt extract agar (MEA, AppliChem, GmbH) and dichloran-rose bengal agar (DRBC, Oxoid Ltd., Basingstoke, Hampshire, U.K.). Media were supplemented with chloramphenicol 100 ppm to prevent bacterial growth. Fungi from lichens were isolated as follows: small fragments of thalli were sterilised by washing with H_2O_2 (8 %) for 5 min, washed with sterile deionised water to remove any trace of H_2O_2 and finally seeded on MEA and DRBC. Plates were incubated at 5 and 15 °C and inspected every 15 d until no new colonies appeared. Colonies were transferred to MEA slants and incubated at 15 °C. Strains analysed for comparison are listed in Table 1; they were taken from reference collections of the Culture Collection of Fungi from Extreme Environments (CCFEE, Viterbo, Italy) and the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), including a large set of strains sampled by P.W. Crous from plants with leathery leaves in a semi-arid climate.

Morphology

Hyphal maturation and conidiogenesis were studied using both light and scanning electron microscope (SEM). Slide cultures were seeded onto MEA, incubated for 10 wk and mounted in lactic acid. Samples for SEM observations were prepared according to methods described by Onofri *et al*. (1980).

DNA extraction and sequencing

DNA was extracted from mycelial fragments taken from 6-mos-old MEA slants grown at 10 °C, using Nucleospin Plant kit (Macherey-Nagel, Düren, Germany) following the protocol optimised for fungi. PCR reactions were performed using BioMix (BioLine GmbH, Luckenwalde, Germany). In each 25 µL reaction tube 5 pmol of each primer and 40 ng on template DNA were added. The amplification was carried out using MiniCycler™ (MJ Research, Waltham, Massachusetts, U.S.A.) equipped with a heated lid. The first denaturation step at 95 °C for 3 min was followed by: denaturation at 95 °C for 2 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s. The last three steps were repeated 35 times, with a last extension 72 °C for 5 min. The products were purified using Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). Primers NS1, NS2, NS3, NS4, NS5, NS8, ITS1, ITS4 (White *et al.* 1990), SR10R (Bruns *et al.* 1992), ITS5, and ITS4a (Larena *et al*. 1999) were employed to amplify SSU and ITS rDNA portions. Sequencing reactions were performed according to the dideoxynucleotide method (Sanger *et al*. 1977) using the TF Big Dye Terminator 1,1 RR kit (Applied Biosystems). Fragments were analysed using an ABI 310 Genetic Analyser (Applied Biosystems). Sequence assembly was done using the software Chromas (v. 1.45 1996–1998, Conor McCarthy School of Health Science, Griffith University, Southport, Queensland, Australia).

Alignment and tree reconstruction

SSU sequences were aligned with ARB beta-package (v. 22-08-2003, Ludwig *et al*. 2004; www.mikro.biologie.tu-muenchen.de/pub/ARB). The SSU alignment spanned positions 141–2512, which corresponds to 1515 bp with reference to *Saccharomyces cerevisiae*. Trees based on SSU sequences were reconstructed with neighbour-joining in ARB.

ITS sequences were aligned iteratively with Ward's averaging (Van Ooyen 2002) in a research data base of black yeasts present at CBS using the BioNumerics package (Applied Maths, Kortrijk, Belgium). Due to gaps necessary for alignment, the ITS1 domain spanned 187 positions (real lengths 147–154 bp), the 5.8S gene 156 positions and the ITS2 domain 184 positions (real lengths 143–155 bp). The alignments were based on the positions 28–481, the initial and the final parts were cut off to compare fragments with the same length. Alignments were exported and the best-fit substitution model was determined using Modeltest MrAic.pl 1.4.3 (Nylander 2004, program distributed by the author) estimated using PhyML (Guindon & Gascuel 2003) through hierarchical likelihood ratio tests. MrAic calculates the Akaike Information Criterion (AIC), corrected Akaike Information Criterion (AICc) and Bayesian Information Criterion (BIC); Akaike weights for nucleotide substitution model and model uncertainty. All 56 models implemented in Modeltest were evaluated. Phylogenetic trees were reconstructed by Maximum Likelihood, using Treefinder (Jobb *et al*. 2004) and the resulting tree was displayed using Treeview v. 1.6.6 (Page 1996). The robustness of the phylogenetic inference was estimated using the bootstrap method (Felsenstein 1985) with 100 pseudoreplicates generated and analysed with Treefinder.

As alignment over the entire complex was highly ambiguous, an algorithm for tree reconstruction without alignment, the DNA-walk Divergence method (DNAWD, Licinio & Caligiorne 2004; Caligiorne *et al*. 2005) was used, involving the entire spacer region. DNA-walks are defined by incrementing walk steps for each nucleotide in the sequence (for example a positive step for purines, and negative for pyrimidines). It makes simultaneous comparisons of the three-dimensional walks (representing three composition skews): AG-TC, AC-TG, and AT-CG for each pair of sequences. One sequence slides against the other until the minimum squared walk difference is found, corresponding to a global alignment. This is then taken as a measure of their distance since statistically independent mutations and indels increase the mean square walk differences linearly. The resulting distance matrices are then fed into the Kitsch program of the Phylip package (v. 3.572c, Felsenstein 1996).

Cultural preferences

Cultural characteristics and growth rates were recorded on Potato-Dextrose Agar (PDA), MEA, Czapek Dox Agar (CzA) and Oatmeal Agar (OA). Strain CBS 119434 was incubated at 10 °C, strains CBS 122538, 122539 and 122540 at 20 °C and strains CBS 899.87, CBS 335.97, dH 12881, dH 11526 and dH 13081 at 25 °C. The diameter of the colonies was recorded monthly. Tests were performed in triplicate.

Temperature preferences

Temperature preferences for the strains CBS 119434, 122538, 122539 and 122540 were tested by incubating them on MEA,

Table 2. Physiological profiles of Antarctic strains.

Species	Strain no.	Cultural preferences				Thermal preferences (° C)							
		PDA	MEA	CzA	OA	0	5	10	15	20	25	30	35
Recurvomyces mirabilis	CBS 119434	1.5±0.14	1.8±0.14	0.5±0.14	1.65±0.07	0.45±0.07	0.8±0.2	1.5±0.26	1.9±0.26	0.78±0.2	–	–	–
Elasticomyces elasticus	CBS 122538	1.7±0.2	1.5±0.14	0.5±0.14	1.5±0.14	0.82±0.064	0.64±0.079	1.2 ±0.2	1.6 ±0.26	1.54 ±0.09	0.95 ±0.15	–	–
	CBS 122539	1.5±0.1	1.43±0.15	0.3±0.02	1.23±0.2	0.77±0.1	0.6±0.2	1.1±0	1.5±0.14	1.2±0.2	0.6±0.2	–	–
	CBS 122540	1.0±0.2	1.1±0.1	0.2±0.02	1.0±0.2	0.72±0.1	0.5±0.14	1.13±0.2	1.4±0.17	1.2±0.2	0.8±0.08	–	–

Cultural and thermal preferences reported as diameter of the colonies (cm), after two mos of incubation. The values represent the average of three different tests. Plates for cultural preferences were incubated at 10 °C for strain CBS 119434 and at 20 °C for strains CBS 122538, 122539 and 122540; plates for temperature preferences were seeded on MEA; – = no growth.

Table 3. Cultural preferences and salt tolerance of acidophilic strains.

Species	Strain no.	Cultural preferences				NaCl %						
		PDA	MEA	CzA	OA	1.2	1.5	3	5	7	10	12
Acidomyces acidophilum	CBS 899.87	1.17±0.1	1.33±0.15	0.3±0.02	1.17±0.1	1.3±0.02	0.7±0.02	0.6±0.03	0.6±0	0.5±0	0.2±0.02	–
	CBS 335.97	1.08±0.1	1.9±0.14	0.6±0.2	1.0±0.2	1.5±0.02	0.5±0.02	0.4±0.04	0.3±0.02	0.2±0.02	–	–
	dH 12881	2.0±0.14	1.5±0.14	1.2±0.2	1.5±0.1	1.5±0.02	1.5±0.02	1.5±0.02	0.7±0.03	0.5±0.04	–	–
	dH 11526	2.5±0.2	3.0±0.2	1.5±0.14	1.2±0.2	2.5±0.02	2.0±0.04	2±0.04	0.8±0.03	0.6±0.008	–	–
	dH 13081	1.8±0.14	2.2±0.2	1.1±0.1	1.0±0.2	1.0±0.04	0.8±0.02	0.8±0.02	0.6±0.02	0.3±0.02	–	–

Growth on different media and salt concentration expressed as diameters of the colonies (cm); – = no growth.

Table 4. Thermal and pH preferences of acidophilic strains.

Species	Strain no.	Thermal preferences (° C)						pH				
		4	10	18	25	30	37	1	3	5	7	9
Acidomyces acidophilum	CBS 899.87	–	–	+	++	+	–	+	+	++	+	±
	CBS 335.97	+	+	++	+	+	–	+	++	++	+	–
	dH 12881	+	+	++	++	+	–	+	++	+	±	–
	dH 11526	+	+	+	+	++	–	+	++	++	+	–
	dH 13081	+	+	+	++	+	–	±	++	++	+	–

++ = maximum growth recorded; + = growth; ± = weak growth; – = no growth.

in Petri dishes at 0–35 °C (in 5° intervals) ± 1 °C. The diameter of the colonies was recorded monthly. Tests were performed in triplicate. Optimum temperatures for growth and development of the strains CBS 899.87, CBS 335.97, dH12881, dH11526 and dH13081, were determined by seeding 25 mL flasks containing 2 % Malt Extract Broth (MEB) with 0.25 mL of a 10^5 cells/mL suspension and incubating in shaken culture at 70 r.p.m. After 30 d of incubation at temperatures of 4, 10, 18, 25, 30, 37 °C, cultures were filtered and the biomass dry-weighed. The test was performed in duplicate.

Growth at different salt concentrations

The ability to grow at different salinities was tested in duplicate on plates of MEA amended with 1.2, 1.5, 3, 5, 7, 10 or 12 % NaCl. Strains were inoculated in three spots on each plate and incubated at 25 °C for one mo, when the colony diameter was recorded. Colonies with a diameter >2 mm were considered positive (Kane & Summerbell 1987).

Growth at different pH

The ability to grow at different pH values for the strains CBS 899.87, CBS 335.97, dH12881, dH11526 and dH13081 was tested in duplicate using MEB2 % medium at pH 1, 3, 5, 7 and 9. Values of pH 5 were obtained by the addition of 1N HCl; remaining pH values were obtained according to Küster & Thiel (1990) as follows: McIlvaine solution for pH 2–7, Clark & Lubs solution for pH 8–9; buffer HCl/KCl for pH 1. Strains were incubated at 25 °C in shaken culture at 70 r.p.m. for one mo, cultures were filtered and the biomass dry-weighed.

RESULTS

Physiology

Temperature relations and cultural features of the strains CBS 119434, 122538, 122539 and 122540 are shown in Table 2. Physiological data for the strains CBS 899.87, CBS 335.97, dH12881, dH11526 and dH13081 are reported in Tables 3 and 4. All fungi tested were able to grow on natural media showing clearly visible growth on MEA as well as on PDA and OA, whilst there was a marked reduction of ultimate colony diameter when seeded on CzA. CBS 119434 was able to grow in the range 0–20 °C, with an optimum at 15 °C, whereas strains CBS 122538, 122539 and 122540 grew in a wider range of temperatures between 0 and 25 °C, with an optimum at 15–20 °C. Strains CBS 899.87, CBS 335.97, dH12881, dH11526 and dH13081 grew best in the range of 18–30 °C, with the optimum temperature at 18 °C for the strains CBS 335.97 and dH 12881, 25 °C for the strains CBS 899.87 and dH 13081, and 30 °C for the strain dH 11526. Since almost all strains (except CBS 899.87) were still able to reproduce at 4 °C, they can be referred as mesophilic-psycrotolerant (Zucconi *et al*. 1996). None of them grew at 37 °C. Furthermore, they were able to grow in very acidic conditions (pH 1) and most of them grew best at pH 5 or below. In particular the optimum was pH 5 for the strain CBS 899.87, around pH 3–5 for the strains CBS 335.97, dH11526 and dH 13081 and pH 3 for the strain dH 12881. All the strains studied showed a decreasing growth at pH 7 and stopped to grow at pH 9, with the exception of strain CBS 899.87 which was able to grow in a very wide range of pH values showing a weak growth even at pH 9. The strains demonstrated also to be moderately halophilic being able to grow in up to 7 % NaCl (strains CBS 335.97, dH11526, dH 13081, dH 12881) and in up to 10 % for the strain CBS 899.87.

Phylogeny

SSU sequences were analysed for 71 strains of ascomycetous black yeasts and relatives belonging to the orders *Capnodiales*, *Dothideales*, *Myriangiales* as well as the recently proposed new order *Botryosphaeriales* (Schoch *et al*. 2006). The recently described taxon *Baudoinia compniacensis* (Richon) J.A. Scott & Unter. (Scott *et al*. 2007) with an SSU similarity around 97 % with some capnodialean/dothidealean strains, was not included in this comparison. Fig. 1 shows a Neighbour Joining tree based on the SSU comparison where the outgroup is represented by *Aliquandostipite khaoyaiensis* (*Jahnulales*).

Three Antarctic rock strains (CBS 122538, 122539 and 122540), with nearly identical sequences, were included in a group paraphyletic to *Friedmanniomyces endolithicus* Onofri. The strains shared a remarkable morphology, with straight fertile hyphae of which fragments detached, sometimes by basauxically expanding connectives.

Strain CBS 119434 clustered in a clade composed of mainly meristematic species, which included the Antarctic rock-inhabiting genus *Friedmanniomyces*. This strain also had characteristic morphology, showing a unique pattern of recurved hyphal branching at conidiation. Another strain with nearly identical sequence, CBS 117957 from Mediterranean rocks, showed a simple, undiagnostic meristematic micromorphology. The lichenicolous fungus *Hobsonia santessonii* Lowen & D. Hawksw. belonged to the same group, together with the black fungus *Mycocalicium victoriae* (C. Knight ex F. Wilson) Tibell.

The acidophilic species *Hortaea acidophila* Hölker *et al.* and *Acidomyces acidophilum* as "*Acidomyces richmondensis*" B.J. Baker *et al.* composed a sister clade to the *Friedmanniomyces* complex. The halophilic species *Hortaea werneckii* (Horta) Nishimura & Miyaji was found at a larger distance, in a heterogeneous clade with *Pseudotaeniolina globosa* De Leo *et al.*, *Catenulostroma abietis* (Butin & Pehl) Crous & Braun and *Coccodinium bartschii* A. Massal. As the backbone of the tree shows low bootstrap values for most clades, the exact phylogenetic positions of the newly added Antarctic and acidophilic species are difficult to determine.

The ITS tree shown in Fig. 2 was generated on the basis of the manually optimised alignment containing 95 sequences of halophilic, acidophilic, rock and plant-pathogenic fungi; it is based on a length of 452 characters, including alignment gaps. The AICc selected HKY+G (Hasegawa *et al*. 1985) as the best model. The base frequencies was as follows: T = 0.2293, C = 0.3120, A = 0.2009, G = 0.2574, TC = 0.5413, AG = 0.4583. The entire ITS region showed too many polymorphisms to allow alignment with a sufficient degree of confidence. For this reason, DNAWD was applied, which is insensitive to alignment. Topology of this tree was identical (data not shown).

In general the ITS trees showed excellent resolution of entities. Inter-group variability was constant and invariably significantly larger than intra-group variability. The tree contained anamorph taxa from extreme environments, as well as a number of plant-associated species of *Teratosphaeria* Syd. & P. Syd. Three main clades were discernible: an upper clade around *Teratosphaeria microspora* J.E. Taylor & Crous, a clade with *T. nubilosa* (Cook) Crous & U. Braun as central species, and an outgroup with strains isolated at very low pH (Fig. 2).

A single cluster is composed of strains listed as *Teratosphaeria*

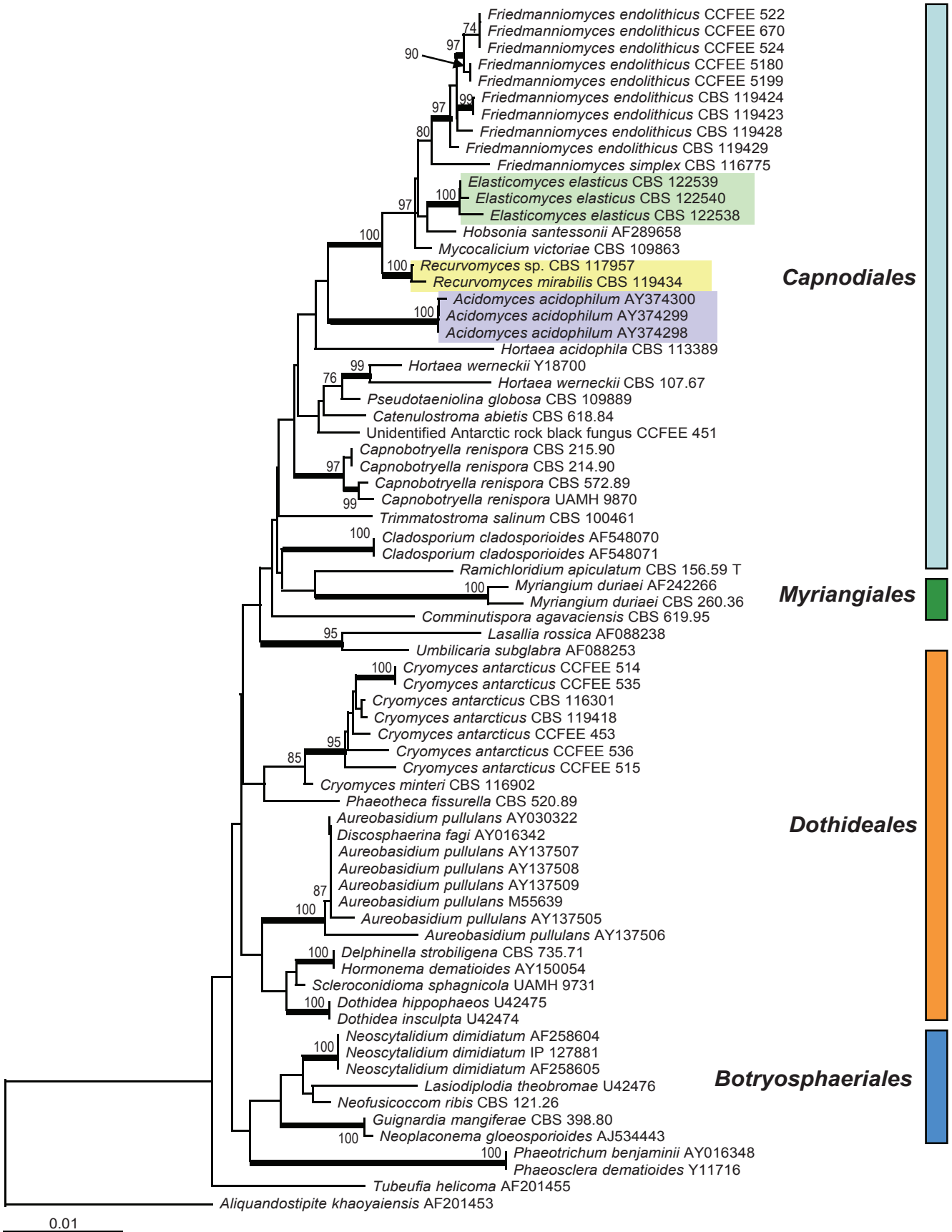

Fig. 1. Molecular phylogeny based on SSU sequences indicating the positions of the clades in *Dothideomycetidae*; the described new genera were highlighted with coloured rectangles. The tree has been built with neighbour-joining algorithm in ARB package with 100 replications. Branches of the clades supported by a bootstrap value above 95 % are in bold.

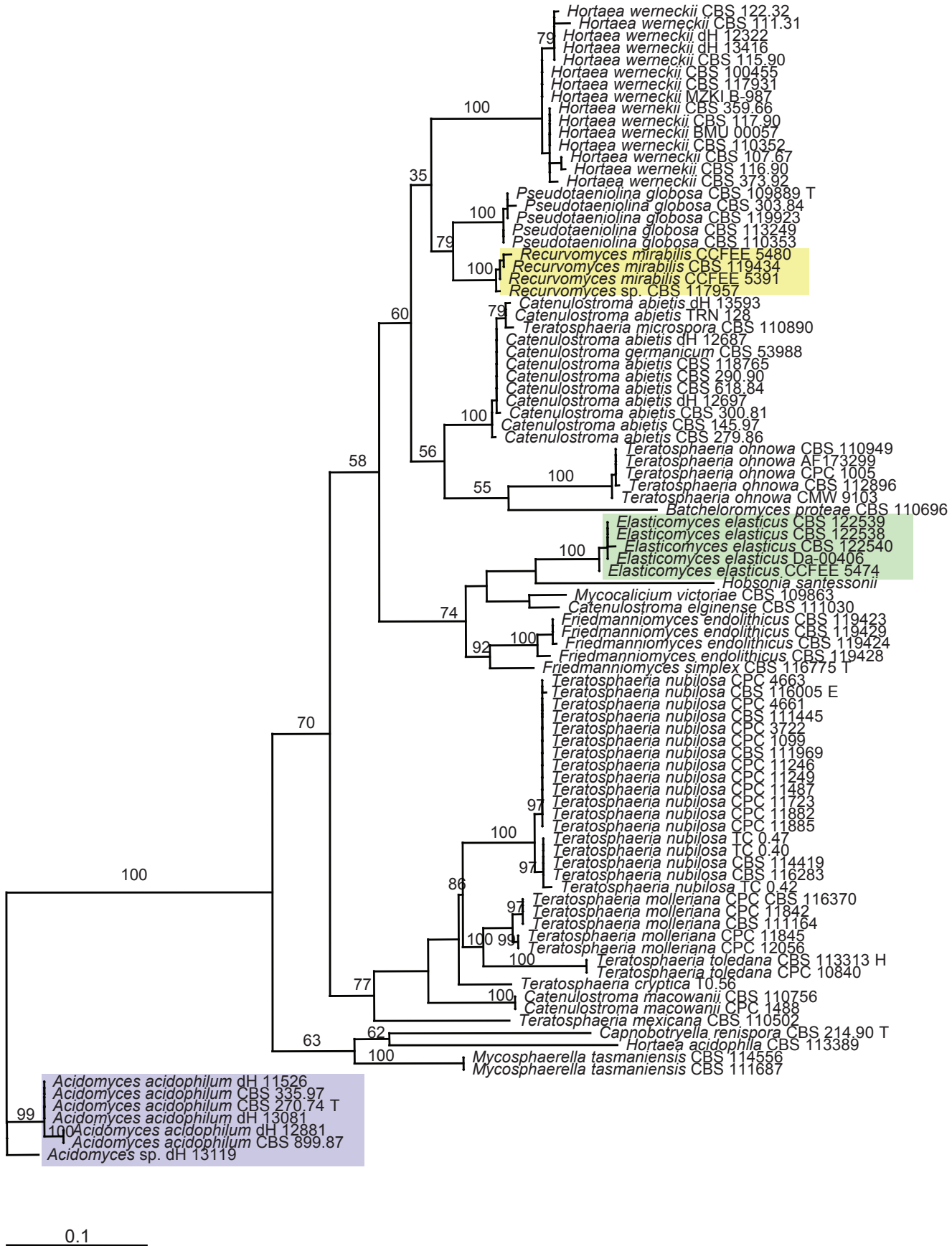

Fig. 2. Molecular phylogeny of the analysed ITS rDNAs showing the relationship of the new genera described, highlighted with coloured rectangles, in the *Capnodiales*. The neighbour-joining tree, based on 95 sequences and 452 nucleotide positions, has been generated using HKY+G model; the model was calculated using ML in MrAIC software. Bootstrap values from 100 resampled data sets are shown.

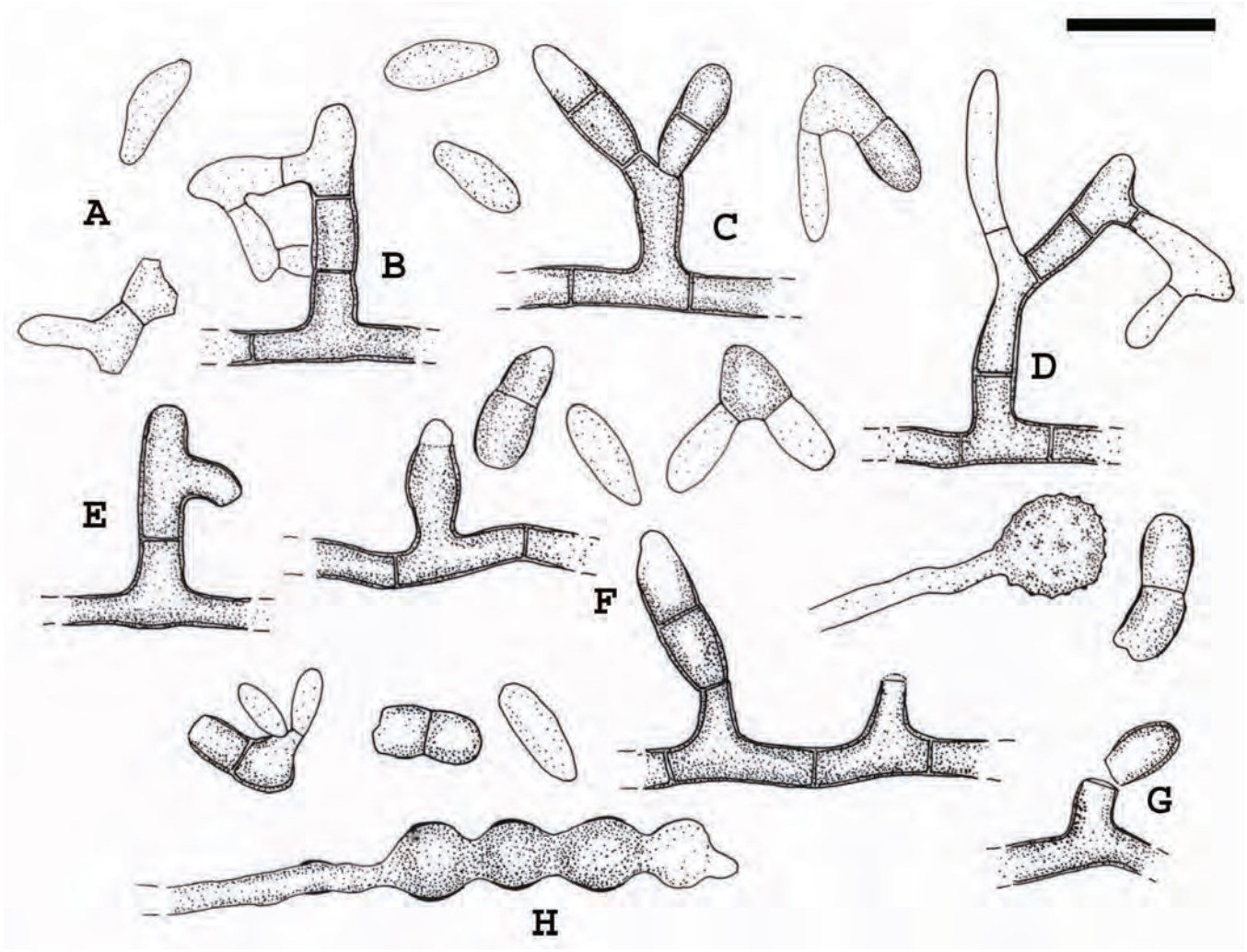

Fig. 3. *Recurvomyces mirabilis*. A. 1- celled conidia and ramoconidia. B–D. Septate and branched conidiophores; branches forming right angle and bent down. E. Conidiophore with recurved hyphal branching. F. Conidiogenous cells producing conidia by enteroblastic proliferation. G. Schyzolytic conidial secession. H. Swelling cells. Scale bar = 10 µm.

microspora, Catenulostroma abietis (Butin & Pehl) Crous & U. Braun and *C. germanicum* Crous & U. Braun. The strain TRN 128 from Mediterranean rock is found in a paraphyletic position. *Pseudotaeniolina globosa* is a member of the same clade, while the endemic Antarctic *Friedmanniomyces* species are members of a neighbouring clade which includes *Mycocalicium victoriae* CBS 109663 and the lichenicolous fungus *Hobsonia santessonii*, without any known teleomorph. Five further undescribed Antarctic strains, among which was CBS 122539, constituted a sister clade (Fig. 2), and phenetically all showed conidial dehiscence with basauxically expanding connectives; we here describe a new genus, *Elasticomyces* Zucconi & Selbmann for this group. Another, separate cluster of four strains from Antarctic and Mediterranean rocks was found around CBS 119434, strains microscopically either showing recurved conidial branches or a reduced, meristematic morphology; for which we describe a new genus, *Recurvomyces* Selbmann & de Hoog. The halo- respectively acidophilic *Hortaea* species, *H. werneckii* and *H. acidophila*, were located at clearly separate positions. The same clade included also the plant-pathogenic teleomorph species *Teratosphaeria ohnowa* (Crous & M.J. Wingf.) Crous & U. Braun occurring on leaves of *Myrtaceae*. A basal cluster comprised some strains, among which *Scytalidium acidophilum* CBS 270.74 was selected as outgroup. The clade contained the plant-pathogenic *Mycosphaerella nubilosa* (Cooke) Hansf. and, as sister clade, a group of acidophilic fungi which included the ex-type strain of *Scytalidium acidophilum* Sigler & J.W. Carmich. CBS 270.74, isolated from acidic soil, was found to be identical to that species. No teleomorph relationships are known for this group. *S. acidophilum* was found to be identical to strains of the the invalidly described genus "*Acidomyces"* B.J. Baker *et al*. That genus is validated below as *Acidomyces* B.J. Baker *et al.* ex Selbmann *et al*.

DESCRIPTIONS

Recurvomyces Selbmann & de Hoog, **gen. nov.** – MycoBank MB511293.

Ad fungos anamorphos, hyphomycetes pertinens. *Coloniae* in agaro maltoso lente crescentes, compactae, velutinae, nigrae vel olivaceo-nigrae. *Mycelium* ex hyphis longis, levibus, pallide brunneis et crassitunicatis compositum. Hyphae torulosae interdum productae, brunneae, crassitunicatae, enteroblastice proliferantes. *Conidiophora* macronematosa vel semi-macronematosa, saepe ramosa, cum ramis lateralibus saepe angulo recto dispositis deorsumque inflexis. *Conidia* enteroblastica, schizolytice secedentia. *Ramoconidia* interdum producta. Teleomorphosis ignota.

Species typica: *Recurvomyces mirabilis* Selbmann & de Hoog, sp. nov.

Anamorphic fungi, hyphomycetes. *Colonies* very slowly growing, compact, heaped, black or olive-black. *Mycelium* composed of long, smooth, yellowish brown and thick-walled hyphae. Torulose hypae sometimes present, brown, thick-walled, smooth to verrucose, enteroblastically proliferating. *Conidiophores* macronematous or semi-macronematous, often branched with lateral branches mostly at roughly right angle and bent down. *Conidia* produced by enteroblastic proliferation released by schizolytic secession. *Ramoconidia* sometimes present.

Teleomorph: Unknown; phylogenetic affinity to the ascomycete order *Capnodiales*.

Type species: *Recurvomyces mirabilis* Selbmann & de Hoog, sp. nov.

Recurvomyces mirabilis Selbmann & de Hoog, **sp. nov.** – MycoBank MB511294, Figs 3–5.

Coloniae in agaro maltoso acervatae, compactae, margine regulari atque plano, velutinae, nigrae vel olivaceo-nigrae, reversus nigrum, lente crescentes, post tres menses usque ad 18 mm diam. *Mycelium* ex hyphis longis, levibus, pallide brunneis et crassitunicatis, apicem versus gradatim pallidioribus et subtilius tunicatis, subhyalinis, interdum anastomoses ferentibus, septatis, ramosis compositum. Torulosae hypae interdum productae, brunneae, crassitunicatae, leves vel verrucosae, partibus inflationis 4.5–7.5 µm crassis, proliferatione apicali enteroblastica ortae. *Conidiophora* ex mycelio superficiali orientia, macronematosa vel semi-macronematosa, mononematosa, erecta, brevia, septata, levia, crassitunicata, brunnea, apicem versus gradatim pallidiora et tenuitunicata, ex proliferatione enteroblastica percurrente orientia, saepe ramosa, cum ramis lateralibus saepe angulo recto dispositis, deorsum inflexis. *Cellulae conidiogenae* polyblasticae, integratae, terminales vel intercalares, tenuitunicatae, brunneae, enteroblasticae, post schizolyticam secessionem conidiorum tenui cicatrice praeditae. *Conidia* enteroblastica, sicca, solitaria, 0–1-septata, subhyalina vel dilute brunnea, tenuitunicata, levia, ellipsoidea vel obovoidea, raro modice ad septum constricta, ad apicem rotundata, plano hilo basilari incospicuo praedita post schizolyticam secessionem, 7.2–11.2 × 2.5–4.7 µm. Propagula liberata e partibus terminalibus conidiophororum vel cellulis conidiogenis orientia; ramoconidia etiam producta, saepe 1-septata, brunnea, crassitunicata, forma irregulare, quae a latere enteroblastica conidia gignere possunt. Teleomorphosis ignota.

Holotypus: CBS H-20178, cultura ex-typo CBS 119434 = CCFEE 5264, in Promontorio Pugnae Navalis, Terra Victoriae Meridionalis, Antarctica, isolatus ex arenari saxo. L. Zucconi legit, 24.I.2004.

Cultural characteristics: Description based on strain CBS 119434 at 10 °C.

Colonies on MEA and PDA heaped, with flat and regular margin, on OA remaining almost completely flat; growth very slow: diameter after two mos 18 mm on MEA2, 16.5 mm on OA, 15 mm on PDA, and 5 mm on CzA. Colony obverse olive-black, compact, felty; reverse black. *Mycelium* composed of long, smooth, yellowish brown and thick-walled hyphae, paler and thinner-walled towards the subhyaline apices, septate, branched, with occasional anastomoses. Torulose hyphae sometimes present, brown, thick-walled, smooth to verrucose, with swellings 4.5–7.5 µm wide, formed by enteroblastic apical proliferation. *Conidiophores* erect, semi-macronematous, mononematous, short, septate, smooth, thick-walled, brown, paler and thinner towards the apex, developing from percurrent enteroblastic proliferations of short simple conidiophores, repeatedly branched, with lateral branches mostly at roughly right angle and bent downward. *Conidiogenous cells* polyblastic, integrated, terminal or intercalary, thin-walled, brown, producing conidia by enteroblastic proliferation, with slight abscission scars remaining after schizolytic conidial secession. *Conidia* enteroblastic, dry, solitary, 0–1-septate, subhyaline to yellowish brown, thin-walled, smooth, ellipsoidal to obovoidal, sometimes slightly constricted at the median septum, rounded at the apex and with a flat scar at the base, 7.2–11.2 × 2.5–4.7 µm; conidial secession schizolytic. Terminal parts of conidiophores or apical conidiogenous cells generally breaking off producing propagules; ramoconidia also present, formed by schizolitic secession, mostly 1-septate, brown, thick-walled, irregular in shape, laterally producing conidia by enteroblastic proliferation.

Teleomorph: Unknown.

Holotype: CBS H-20178, culture ex-type CBS 119434 = CCFEE 5264, Battleship Promontory, McMurdo Dry Valleys, Southern Victoria Land, Antarctica, isolated from sandstone. Leg. L. Zucconi, 24 Jan. 2004.

Strains examined: CBS 119434; CCFEE 5480; CCFEE 5391; CBS 117957.

Elasticomyces Zucconi & Selbmann, **gen. nov.** – MycoBank MB511296.

Ad fungos anamorphos, hyphomycetes pertinens. *Coloniae* compactae, coactae, cumulatae, nigrae, lente crescentes. *Mycelium* ex hyphis longis, septatis, ramosis, tenuitunicatis, hyalinis vel pallide pigmentatis compositum**.** *Hyphae fertiles* obscuriores et magis crassitunicatae, repetite ramosae, septatae, fissione ad septa in conidia disarticulantes. *Arthroconidia* catenata, uni- vel pluricellularia, cylindrica, utrimque secessione schizolytica truncata. Teleomorphosis ignota.

Species typica: *Elasticomyces elasticus* Zucconi & Selbmann, sp. nov.

Anamorphic fungi, hyphomycetes. *Colonies* compact, felted, clumped, black, slow growing. *Mycelium* composed of long, branched, septate, thin-walled, colourless or yellowish to pale brown hyphae. Fertile hyphae more pigmented and thicker-walled, repeatedly branched at roughly right angle, septate, forming conidia by fragmentation. *Arthroconidia* catenate, one or pluricellular, cylindrical, with truncated ends due to schizolytic secession.

Teleomorph: Unknown.

Phylogenetic affinity to the ascomycete order *Capnodiales*.

Type species: *Elasticomyces elasticus* Zucconi & Selbmann, sp. nov.

Elasticomyces elasticus Zucconi & Selbmann, **sp. nov.** – MycoBank MB511297, Figs 6, 7.

Coloniae in agaro maltoso compactae, coactae, partim immersae, margine plerumque irregulari, cumulatae, obversum reversumque nigra, lente crescentes, post duos menses usque ad 15 mm diam. *Mycelium* ex hyphis longis, septatis, levibus, tenuitunicatis, luteolis vel pallide brunneis, ramosis, anastomosantibus, 3.5–5.3 µm latis compositum**.** Hyphae fertiles brunneae, crassitunicatae, ad angulo recto repetite ramosae, septatae, primum leves, demum crenulatae, fissione ad septa in numerosa brevia segmenta, ex uno vel pluribus conidiis composita, disarticulantes, interdum connectivo coniuncta. *Arthroconidia* catenata, plerumque bicellularia, rarius unicellularia, levia vel crenulata, cylindrica, truncata et magis crassitunicata ad apices secessione schizolytica, modice ad septum constricta, 12.5–16 × 3.5–5.3 µm.Cellulae intercalares chlamydosporarum similes interdum orientes, parietibus crassis et brunneis. Teleomorphosis ignota.

Holotypus: CBS H-20177, cultura ex-typo CBS 122538 = CCFEE 5313, Insula Kay, Terra Victoriae Settentrionalis, Antarctica, isolatus ex thallo *Usneae antarcticae*. L. Zucconi legit, 30.I.2004.

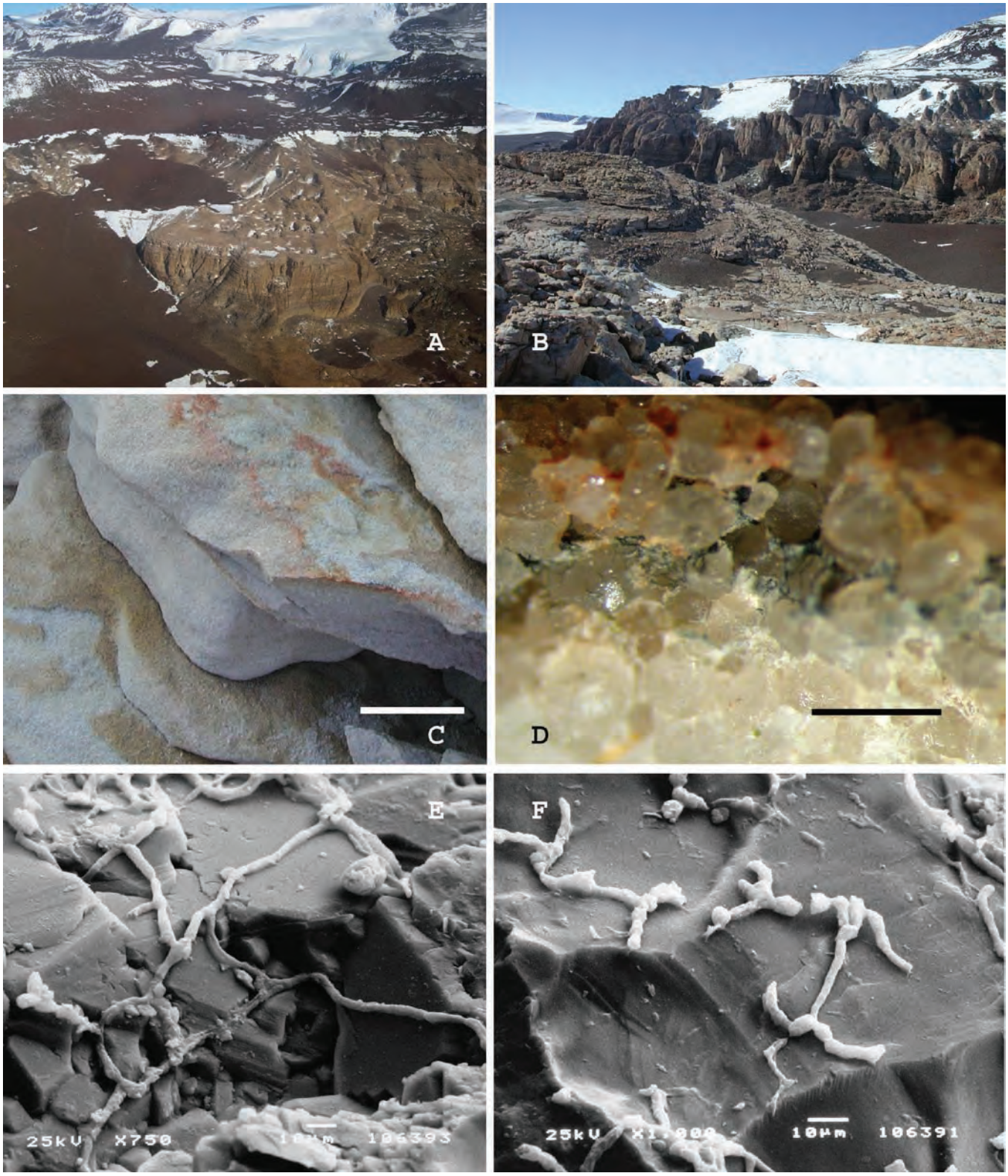

Fig. 4. A. Battleship Promontory, Southern Victoria Land, Antarctica, view from the helicopter. B. Battleship Promontory, Southern Victoria Land, Antarctica, landscape. C. Sandstone sample from which *Recurvomyces mirabilis* CBS 119434 has been isolated. The surface is weathered by the activity of cryptoendolithic microorganisms. Scale bar = 5 cm. D. Magnification at the dissecting microscope of the black fungi endolithic colonization inside the sample of sandstone used for the isolation. Scale bar = 2 mm. E, F. Rock colonised by the fungus observed at the Scanning Electron Microscope.

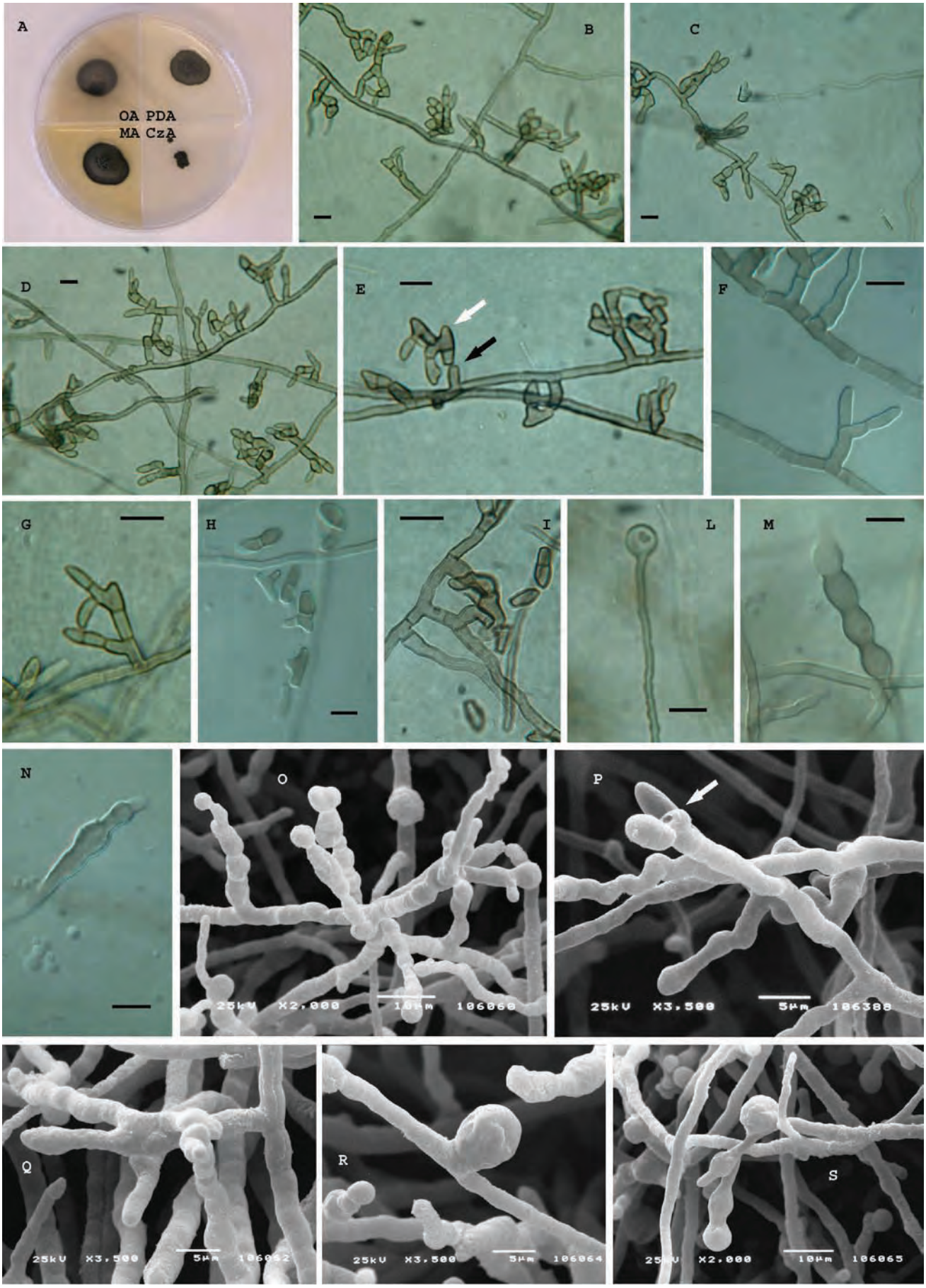

Fig. 5. *Recurvomyces mirabilis*, CBS 119434 (= CCFEE 5264). A. Strain grown on different media after two mo of incubation at 15 °C. B–D. Hyphae with branched and unbranched conidiophores producing 0–1 septated conidia. E. Curved branched conidiophores schyzolytically seceding (black arrow) showing enteroblastic elongation (white arrow) at the apex. F, G. high magnification of branched conidiophores producing 1-celled conidia. H, I. 1- and 2-celled conidia and ramoconidia. L. Terminal swelling cell. M, N. swelling hyphae showing enteroblastic elongation. O, P. Unbranched conidiophores producing 1-celled conidia, scar is visible after schizolytic secession (P, arrow). Q. Branched conidiophore. R, S. swelling cell at intercalary (R) and terminal position (S). B–N. Light microscopy; Scale bars = 10 µm. O–S. SEM.

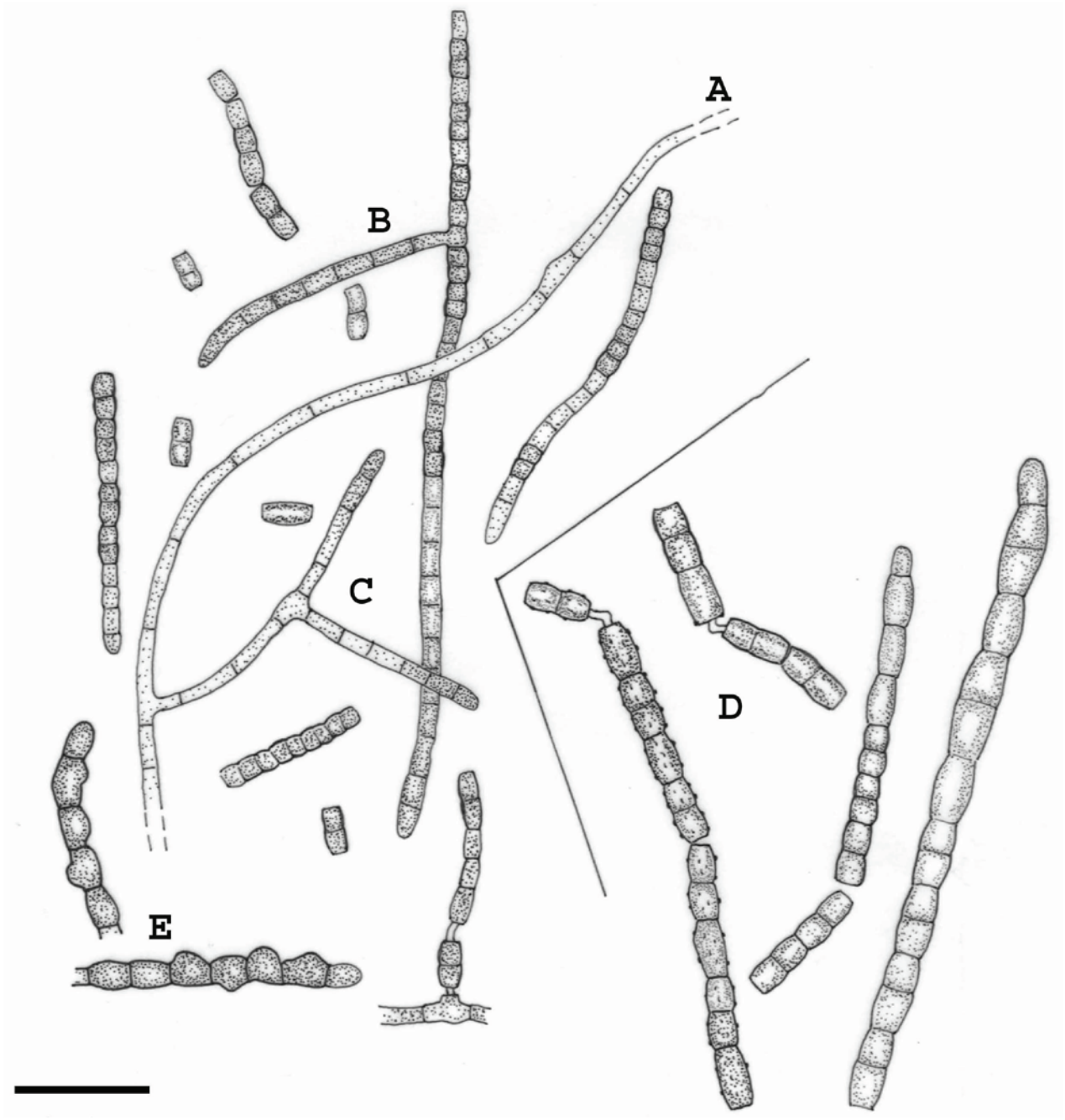

Fig. 6. *Elasticomyces elasticus*. A. Vegetative hyphae. B, C. Branched fertile hyphae producing conidia by fragmentation. D. Disarticulating fertile hyphae where cells remain joint by connectives. E. Swelling hyphae. Scale bar = 20 µm.

Cultural characteristics: Description based on strain CBS 122538 at 20 °C.

Colonies compact, felted, partially immersed in the agar, margin rather irregular, obverse and reverse black, clumped on MEA2 % and PDA, almost completely flat on OA; slow growth: diameter after two mos 15 mm on MEA2 % and OA, 17 mm on PDA, and 5 mm on CzA. *Mycelium* composed of long, septate, branched, smooth, thin-walled, yellowish to pale brown hyphae, 3.5–5.3 µm wide, with anastomoses. Fertile hyphae more pigmented, thicker-walled, at roughly right angle repeatedly branched, septate, at first smooth, then crenulate, forming by fragmentation numerous short segments, composed of one or more conidia, sometimes joined by connectives. *Arthroconidia* catenate, mostly bicellular, rarely aseptate, smooth or crenulate, cylindrical, with thickened and truncated ends due to schizolytic secession, slightly constricted at the septum, 12.5–16 × 3.5–5.3 µm. Intercalary chlamydospore-like cells, with thickened and brown wall, sometimes present.

Teleomorph: Unknown.

Holotype: CBS H-20177, culture ex-type CBS 122538 = CCFEE 5313, Kay Island (75°04'13.7"S, 165°19'0.2.0"E), Northern Victoria Land, Antarctica, isolated from lichen thallus (*Usnea antarctica* Du Rietz).Leg. L. Zucconi, 30 Jan. 2004.

Strains examined: CBS 122538; CBS 122539; CBS 122540; Da-004-06; CCFEE 5474.

Notes: The conidium ontogeny is holoarthric, involving an irregular basipetal maturation of cells and fragmentation of fertile hyphae. Often short portions of fertile hyphae are released by fragmentation

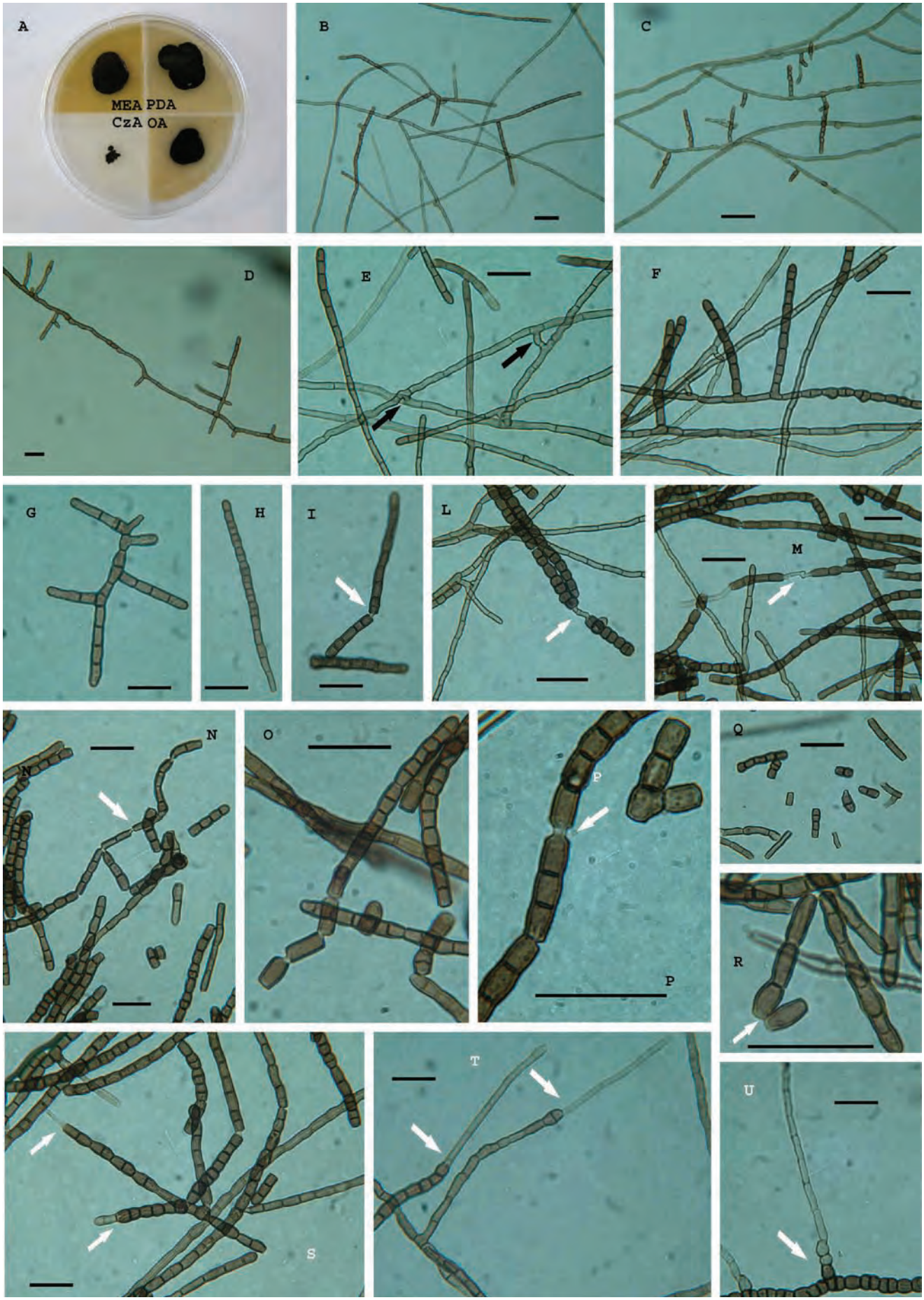

Fig. 7. *Elasticomyces elasticus*, CCFEE 5313. A. Strain grown on different media after 1.5 mo of incubation at 15 °C. B–D. Vegetative and fertile hyphae. E–H. High magnification of fertile hyphae; anastomoses in the vegetative hyphae (black arrows in E). I–O. Uncompleted disarticulation of artic conidia and hyphal fragments remaining joint by connectives (white arrows). P. High magnification of conidia remaining joint after disarticulation. Q, R. 1- and 2-celled conidia produced after schyzolithic secession (white arrow). S–U. Enteroblastic elongation at the apexes (white arrows). Scale bars = 20 µm.

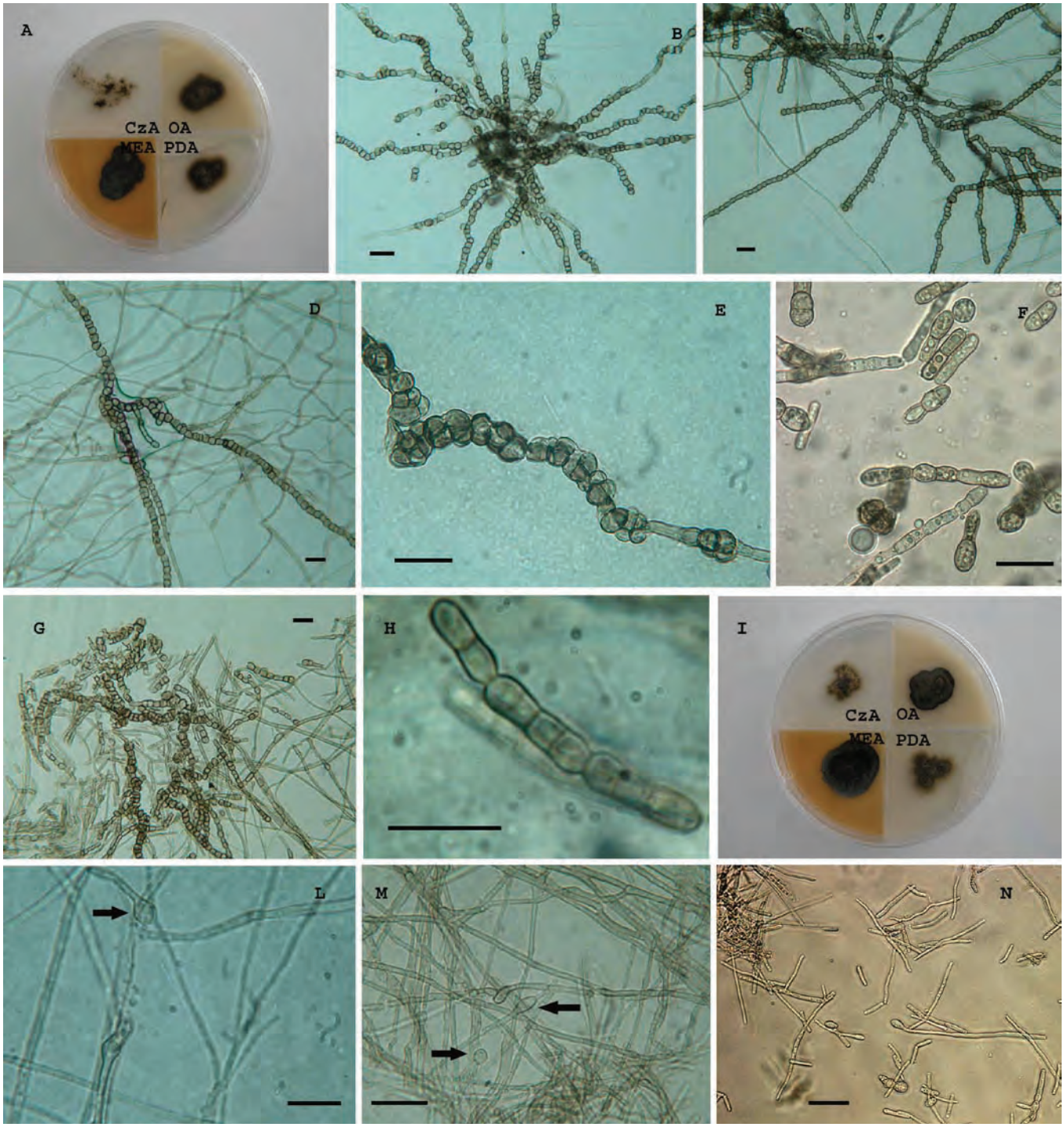

Fig. 8. *Acidomyces acidophilus*. A. Strain CBS 899.87 grown on different media after 1 mo of incubation at 25 °C. B–D, G. Toruloid unbranched hyphae with melanised and thick-walled cells. E. Meristematic development of the hyphae. F. Fungus grown at pH 1 in liquid culture. H. Chain of 1- 2- and 3- celled conidia. I. Strain CBS 335.97 grown on different media after 1 mo of incubation at 25 °C. L, M. Filamentous hyphae with intercalary and terminal swelling cells (black arrows). N. Fungus grown at pH 1 in liquid culture. Scale bars = 20 µm.

of longer hyphae, functioning as propagules. Apical growth of fertile hyphae occurs concomitantly with holoarthric development. A circumscissile scar remains at both ends, after schizolytic secession of adjacent conidia; complete disarticulation is retarded by the presence of thin strands of wall material at the central convexity of septa. Sometimes conidial secession is not completed; new wall material is laid down in the existing septum, and adjacent cells remain connected by narrow and pale connectives. Connectives can eventually elongate to form new pale hyphae, sometimes evolving in new fertile hyphae.

Acidomyces B.J. Baker, M.A. Lutz, S.C. Dawson, P.L. Bond & J.F. Banfield ex Selbmann, de Hoog & De Leo, **gen. nov**. – MycoBank MB511298.

Ad fungos anamorphos, hyphomycetes pertinens. *Coloniae* lente crescentes, celerius in acido agaro, compactae, nigrae. *Mycelium* ex septatis, interdum ramosis, brunneis crassitunicatisque hyphis compositum, demum meristematice increscens. *Conidia* arthrice secedentia. *Teleomorphosis* ignota.

Anamorphic fungi, hyphomycetes. Colonies growing slowly, faster in acidic medium, compact, dark. Mycelium composed of septate, scarcely branched, brown and thick-walled hyphae, eventually

converting into a meristematic mycelium. Conidia produced by arthric disarticulation of hyphae.

Teleomorph: Unknown; phylogenetic affinity to the ascomycete order *Capnodiales*.

Type species: *Acidomyces acidophilus* (Sigler & J.W. Carmich.) de Hoog & De Leo

Acidomyces acidophilus (Sigler & J.W. Carmich.) Selbmann, de Hoog & De Leo, **comb. nov.** – MycoBank MB511856, Fig. 8.

Basionym: *Scytalidium acidophilum* Sigler & J.W. Carmich., Canad. J. Microbiol. 20: 267, 1974.

≡ 'Fungus D', Starkey & Waksman, J. Bacteriol. 45: 512, 1943 (without description, without type).

≡ '*Acidomyces richmondensis*' B.J. Baker, M.A. Lutz, S.C. Dawson, P.L. Bond & J.F. Banfield, Appl. Environm. Microbiol. 70: 6270, 2004 (nom. inval., Arts 36.1, 37.1 ICBN).

Type: UAMH 3460, from field soil, adjacent to elementary sulphur stockpile from natural gas purification plant, Bowden, Alberta, Canada [as *Scytalidium acidophilum*].

Ex-type strain: CBS 270.74 (= ATCC 26772 = IMI 183518 = UAMH 3460).

Additional strains examined: See Table 2.

DISCUSSION

Melanised rock-inhabiting fungi are phylogenetically quite heterogeneous and have been grouped at least in four different fungal orders: *Capnodiales, Dothideales*, *Chaetothyriales* and *Pleosporales* (Sterflinger *et al*. 1997, Selbmann *et al*. 2005, Ruibal *et al*. 2008). Now that suitable isolation and identification techniques have been developed for these fungi (Wollenzien *et al*. 1995, Ruibal *et al*. 2005), they appear to be very common in arid climates, from Polar Regions to the subtropics. Stringent environmental conditions include high solar radiation (Urzì *et al*. 1995), strongly fluctuating temperatures (Nienow & Friedmann 1993), repeated freezing and thawing cycles (Selbmann *et al*. 2002), low water activity (Sterflinger 1998, Zalar *et al*. 1999), acidity (Price 2000), and nutrient deficiency (Sterflinger *et al*. 1999).

The physiological studies indicated that all the strains studied were well adapted to the particular stressing conditions characterising their natural environments. For instance, the strains belonging to the newly validated genus *Acidomyces*, all isolated from very acidic environments (see Table 1), showed an acidophilic profile being able to grow very well at pH 1 and with optimum well below the neutral value (pH 3–5). This means that most probably they are scarcely competitive in other environments and remain trapped in the extremely acidic ones where they have been isolated. Furthermore, they showed only a moderate tolerance to salinity. Similar data have been already reported for other acidophilic fungi phylogenetically distant from *Acidomyces* strains; for instance, *Hortaea acidophila*, was reported to be very sensitive to osmotic stresses being unable to grow at NaCl concentration above 2 % (Hölker *et al*. 2004). These data suggest that the adaptation to acidic environments does not require a concomitant tolerance to osmotic stresses. The situation seems to be different for fungi colonising rocks; Sterflinger (1998) observed a certain degree of halotolerance for some fungal strains in a selected, phylogenetically heterogeneous, group of rock fungi. Halotolerance has been proven to be particularly pronounced in some fungi isolated from the exposed rocks of the Antarctic desert (Onofri *et al*. 2007), among the driest terrestrial ice-free areas on Earth; there, the high evaporation leads also to salt accumulation on rock surfaces and fungi adapted to that environment have to cope with both water deficiency and salinity (Ruisi *et al*. 2007).

All Antarctic strains studied can also be referred to as psychrophilic according to criteria outlined by van Uden (1984) and Vishniac (1987), having an optimum at 15 °C and being unable to grow at temperatures above 20–25 °C. This result confirms what has been recently published for other strains isolated from Antarctic rocks (Selbmann *et al*. 2005). Remarkable is the finding that strains belonging to the genus *Elasticomyces* here described, isolated from Antarctic lichens, showed a wider temperature range for growth with respect to the Antarctic cryptoendolithic strain of *Recurvomyces mirabilis* Selbmann & de Hoog. Their ability to grow at 0 as well as at 25 °C can be an adaptive strategy to withstand not only the very low temperatures characterising their natural environment but also the very wide thermal fluctuations, much more marked in the epilithic rather than in the endolithic environment.

Common features enhancing survival are high degrees of melanisation and thick cell walls (Figueras *et al*. 1996), slow, isodiametric growth, isodiametric expansion ensuring an optimal surface/volume ratio (Wollenzien *et al*. 1995), ability to change cellular polarity (Yoshida *et al*. 1996), and hence little differentiation although with great polymorphism. These characters seem to favour a marked degree of convergent evolution.

In the SSU phylogeny (Fig. 1) a clade is recognisable, containing a large number of melanised fungi that can be isolated from bare rocks, among which there is *Pseudotaeniolina globosa* isolated from rock surfaces in Sicily (De Leo *et al*. 2003). The clade also contains some *Teratosphaeria* species inhabiting leathery plant leaves, mostly in semi-arid climates (Crous *et al*. 2004); this genus was re-established as separate from *Mycosphaerella* because of this ecology and because of its phylogenetic position (Crous *et al*. 2007). The phylogenetic relationship to numerous extremotolerant species is remarkable. Most plant-associated fungi are known with their teleomorph, while for the epi- and endolithic species no teleomorphs are known. *Teratosphaeria microspora* can be found both on rock and plant leaves, suggesting that adaptation to a life at the extreme starts with dispersal under semi-arid conditions.

In general the ITS tree shows excellent resolution of species. Recognised entities were ecologically consistent (Fig. 2), such as the halophilic species *Hortaea werneckii* and the acidophilic *Acidomyces acidophilus*.

Trimmatostroma Corda, with the generic type species *T. salicis* Corda, was recently treated as a genus of *Leotiales*, while the capnodialean species were reclassified in *Catenulostroma* Crous & U. Braun (Crous *et al*. 2007). Teleomorph connections of *Catenulostroma* are in *Teratosphaeria*. Crous *et al.* (2007) mentioned differences in ecology and geography, as *Teratosphaeria microspora* would be an endemic species of *Protea* in South Africa, while other taxa within the *T. microspora* complex were observed on conifer needles and rocks in the Northern Hemisphere. Members of the complex are commonly observed on rocks and other relatively inert surfaces in temperate climates, but on conifer needles they produce well-defined acervuli (Butin *et al*. 1996). This suggests that superficial rock-colonising strains of this group may have originated from leathery plant leaves; we did not find any match between observed ITS polymorphisms and geography or ecology (Table

1). Crous *et al*. (2007) distinguished two additional anamorph species in the complex of *T. microspora*, viz. *Catenulostroma abietis* and *C. germanicum*. ITS sequences of these species are nearly identical (Fig. 2). At reduced water activity, *C. abietis* adapts with a meristematic form (Figueras *et al*. 1996). *Catenulostroma germanicum* was claimed to be different from *C. abietis* in having occasional oblique conidial septa, in contrast to the remaining species. In addition, in *C. abietis* the transformation to meristematic morphology reported above can be reproduced *in vitro* when reaching the stationary phase (Figueras *et al*. 1996, Yoshida *et al*. 1996), leading to the formation of septa in all directions, as in *C. germanicum*.

The genus *Friedmanniomyces* consists merely of species occurring cryptendolithically in rocks in the Antarctic, suggesting a further degree of extremotolerant specialisation (Selbmann *et al.* 2005). The strains belonging to the new genus and species here described, *Elasticomyces elasticus*, are located as a sister group of *Friedmanniomyces*. They have been firstly isolated from Antarctic lichens, but later on also from Antarctic lichen-dominated cryptoendolithic communities, *i.e.* microorganisms living inside rocks in the airspaces between crystals (Friedmann & Ocampo 1976; Friedmann 1982), as well as from Andean rocks (4885 a.s.l.) colonised by epilithic lichens. In this respect lichens seem to be a recurrent element in the environments where these strains have been found. Therefore, *Elasticomyces* seems to be particularly sensitive to the oligotrophic conditions of rocks and the epi- or endolithic lichens could play a pivotal role as nutrient suppliers. The peculiar ability to produce connectives seems a distinctive feature of the genus, being observed both in strains from the Antarctic and the Andes.

The Antarctic strain CBS 119434 is at some distance from *Friedmanniomyces*. The group to which the strain belongs is described as *Recurvomyces mirabilis,* a further cryptoendolithic member of the *Capnodiales*. The ex-type strain and the strain CCFEE 5480 were both isolated from inside sandstone as a member of an Antarctic lichen-dominated cryptoendolithic community. Additional, sterile strains with a nearly identical ITS sequence, CBS 117957 (TRN 491) and CCFEE 5391, were isolated from Spanish rocks (Ruibal 2004) and the Alps (unpublished data) respectively. *Recurvomyces mirabilis* thus is an example of a rock-inhabiting fungus with a distribution spanning both hemispheres. Antarctic and Mediterranean environments have very different temperature regimens, but share high solar radiation and water deficiency at least during part of the yr, suggesting that tolerance to such stresses is promoted by the same set of morphological and physiological factors (Ruisi *et al*. 2007). No cryptoendolithic behaviour is known for Mediterranean rock-colonisers, but their prevalent mode of action is superficial biopitting (Sterflinger *et al.* 1997). Their phylogenetic affinity could be related to the production of easily air-dispersed propagules. Particularly the catenate conidia of *R. mirabilis* resemble *Cladosporium*, which is abundantly present in Antarctic air (Marshall 1997). Otherwise air-dispersed conidia are uncommon among black rock-inhabiting fungi.

Hortaea werneckii was found in derived position in the tree (Fig. 2). The species is one of the most pronounced halophilic fungi known to date (Sterflinger 1998, Zalar *et al*. 1999). It has a growth optimum at 17 % salt and still shows good growth near the saturation point of NaCl (Plemenitaš & Gunde-Cimerman 2005). Its natural niche is in waters of solar salterns worldwide, reaching its optimum distribution during the hot summer period.

Hortaea acidophila is a further related species with a very peculiar ecology. It was isolated from a lignite extract at pH 0.6, using humic and fulvic acids as carbon sources. It was placed in the monotypic genus *Hortaea* which only included the halophilic species *H. werneckii*, in the order *Dothideales*, based on SSU rDNA sequences (Hölker *et al*. 2004).

The outgroup of the tree is composed of the genus *Acidomyces* which includes a single acidophilic species. The name *Acidomyces* was invalidly introduced (Baker *et al*. 2004) for a fungus, named "*Acidomyces richmondensis"*, isolated from warm (35 to 57 °C) pyrite ore mine drainage at pH between 0.5 and 0.9. A fungus with identical properties is *Scytalidium acidophilum* (Sigler & Carmichael 1974), which was invariably isolated from extremely acidic environments (Table 1). Sequencing reference and additional strains of this species, we noticed that "*Acidomyces richmondensis*" is indeed identical to *S. acidophilum*. The phylogenetic position of the latter fungus is far away from that of the generic type species of *Scytalidium, S. lignicola* Pesante. The ex-type strain of that fungus, CBS 233.57 = UAMH 1502, was recently proven to belong to the subclass *Leotiomycetidae* (Hambleton & Sigler 2005), while the present study highlighted that *S. acidophilum* phylogenetically belongs to the *Dothideomycetidae*, order *Capnodiales*. This result justifies the validation of the genus *Acidomyces* and the synonymy of *A. richmondensis* with *S. acidophilum*.

Acidomyces acidophilus has a remarkable ecology. Starkey & Waksman (1943) first found it in extremely acidic, sulphate-containing industrial water. Gould *et al*. (1974) reported the species as the only organism isolated from a sulphur-containing soil at a pH of 1.1, where it occurred at high CFU counts. Harrison *et al*. (1966) found it in uranium mine drain water and Gimmler *et al*. (2001) on an acidophilic moss species. Ivarsson & Morita (1982) showed that acidity is a crucial factor in the ecology of this fungus, obtaining good growth when adjusting the pH to 0.5 with HCl. In pre-molecular times strains of this fungal species – defined by slow-growing cultures producing arthric conidia – frequently were not recognised, because strains tend to convert to meristematic growth, reluctantly disarticulating clumps of cells being produced, or remain entirely hyphal, without conidiation. ITS-sequencing proved the strict identity of all these strains. All positively identified strains originated from environments with pH of 2.0 or below (Table 1).

All the fungi described above are highly melanised. Melanin is frequently viewed as a virulence factor playing a role in fungal pathogenicity to humans (Wheeler & Stipanovic 1985, Schnitzler *et al*. 1999, Paolo *et al*. 2006). Increasing amounts of melanin made *Madurella mycetomatis* (Laveran) Brumpt more virulent, apparently scavenging oxygen radicals (van de Sande *et al*. 2006). Meristematic growth also is a known virulence factor (Matsumoto *et al*. 1984). Nevertheless, members of the subclass *Dothideomycetidae* are only exceptionally encountered as agents of infection (Clark *et al*. 1995, Caporale *et al*. 1996, Kurzai *et al*. 2003). In contrast, a large number of agents of severe mycoses is known in black yeasts belonging to the subclass *Chaetothyriomycetidae*, order *Chaetothyriales* (de Hoog *et al*. 2001). All factors discussed above of melanisation, meristematic morphology, growth at low water activity and at high/low temperature, and acid tolerance, are encountered in *Dothideales* as well as in *Chaetothyriales*, in varying combinations, but only in *Chaetothyriales* they play a role in infection. For example, meristematic morphology, particularly expressed at low pH (Mendoza *et al*. 1993), determines the invasive form in humans with the black yeast-specific skin disease chromoblastomycosis. The natural habitat of one of the agents of this disease, *Cladophialophora carrionii* (Trejos) de Hoog *et al*., was found to be in cactus debris in semi-arid climates (de Hoog *et*

al. 2007), where the same morphology is expressed as prevalent in human skin. This suggests that in *C. carrionii* the extremotolerant morphology directly enhances human invasion. Nevertheless, de Hoog *et al*. (2005) noticed that human pathogenicity is associated with a stress-factor like osmotolerance at the order level, but the two factors are nearly mutually exclusive at the species level. This means that extremotolerance may facilitate pathogenic evolution, but this has to be additive to other factors, such as, in the case of *Chaetothyriales*, oligotrophy with the ability to assimilate monoaromates (Prenafeta-Boldú *et al*. 2006). We therefore consider the characters listed above as primarily suited for growth on exposed surfaces under harsh climatic conditions, rather than for the capacity to evade immune cells.

In summary, we determined a group with pronounced extremotolerance among semi-arid plant-associated fungi. It is probable that all these fungi share elaborate complexes of factors, as an adaptive response to these extreme conditions (Plemenitaš & Gunde-Cimerman 2005). Having acquired a basic set of vitality factors, a shift to a different habitat with a comparable degree of stress seems to be allowed. Phylogeny thus is predictive for ecology in that overall tendencies within a single clade are similar; the shifts to other extreme conditions may be possible provided that they fit the same framework of extremotolerance. In this group of fungi, the winning strategy consists in escaping competitors by colonising selective niches.

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Redefinition of *Aureobasidium pullulans* and its varieties

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Abstract: Using media with low water activity, a large numbers of aureobasidium-like black yeasts were isolated from glacial and subglacial ice of three polythermal glaciers from the coastal Arctic environment of Kongsfjorden (Svalbard, Spitsbergen), as well as from adjacent sea water, sea ice and glacial meltwaters. To characterise the genetic variability of *Aureobasidium pullulans* strains originating from the Arctic and strains originating pan-globally, a multilocus molecular analysis was performed, through rDNA (internal transcribed spacers, partial 28 S rDNA), and partial introns and exons of genes encoding β-tubulin (*TUB*), translation elongation factor (*EF1α*) and elongase (*ELO*). Two globally ubiquitous varieties were distinguished: var. *pullulans*, occurring particularly in slightly osmotic substrates and in the phyllosphere; and var. *melanogenum*, mainly isolated from watery habitats. Both varieties were commonly isolated from the sampled Arctic habitats. However, some aureobasidium-like strains from subglacial ice from three different glaciers in Kongsfjorden (Svalbard, Spitsbergen), appeared to represent a new variety of *A. pullulans.* A strain from dolomitic marble in Namibia was found to belong to yet another variety. No molecular support has as yet been found for the previously described var. *aubasidani*. A partial elongase-encoding gene was successfully used as a phylogenetic marker at the (infra-)specific level.

Key words: Arctic, *Aureobasidium*, black yeasts, elongase, glacier, ITS, LSU, phylogeny, polar environment, rDNA, sea ice, seawater, taxonomy, translation elongation factor, β-tubulin.
Taxonomic novelties: *Aureobasidium pullulans* var. *subglaciale* Zalar, de Hoog & Gunde-Cimerman, var. nov.; *Aureobasidium pullulans* var. *namibiae* Zalar, de Hoog & Gunde-Cimerman, var. nov.

INTRODUCTION

Aureobasidum pullulans (De Bary) G. Arnaud is a black yeast-like species that is particularly known for its biotechnological significance as a producer of the biodegradable extracellular polysaccharide (EPS) pullulan (poly-α-1,6-maltotriose). This component is a promising biomaterial (Rekha & Sharma 2007), and is currently used among others for the packaging of food and drugs (Singh *et al*. 2008). Its biotechnological potential is also seen in the production of a variety of hydrolytic enzymes (Federici 1982, Chi *et al.* 2006, Wang *et al.* 2007, Li *et al.* 2007, Ma *et al*. 2007, Zhiqiang *et al*. 2008).

Aureobasidum pullulans was taxonomically characterised by de Hoog & Yurlova (1994) on the basis of its morphology and nutritional physiology. These authors noted some differences in growth with galactitol, glucono-δ-lactone, creatine and creatinine, and in gelatin liquefaction. Since the species shows considerable variability in its morphological and physiological properties, three varieties have been described during the last decades, viz. *Aureobasidium pullulans* var. *pullulans* (Viala & Boyer 1891), *A. pullulans* var. *melanogenum* Hermanides-Nijhof (1977), and *A. pullulans* var. *aubasidani* Yurlova (Yurlova & de Hoog 1997). The first two of these were distinguishable by culture discolouration, while the latter is unique in its production of aubasidan-like EPS (glucans with α-1,4-D-, ß-1,6-D- and ß-1,3-D-glycosidic bonds). Diagnostically, var. *aubasidani* is unique due to the absence of assimilation of methyl-α-D-glucoside and lactose and by N-source assimilation for the production of EPS. In a further study using PCR ribotyping (rDNA RFLP and UP-PCR/hybridisation), Yurlova *et al.* (1996) divided the *Aureobasidium* strains into four groups, which, however, do not correlate with morphological differences. Yurlova *et al.* (1999) also revealed close relationships between *Kabatiella lini* (Laff.) Karak., the teleomorph species *Discosphaerina (Columnosphaeria) fagi* (H.J. Huds.) M.E. Barr and *Aureobasidium pullulans*.

Aureobasidum pullulans is a ubiquitous and widespread oligotrophe that can be found in environments with fluctuating water activities, such as the phyllosphere (Andrews *et al.* 1994), bathrooms, food and feeds (Samson *et al.* 2004). It can also be found in osmotically very stressed environments, such as hypersaline waters in salterns (Gunde-Cimerman *et al.* 2000), and rocks and monuments (Urzí *et al.* 1999). Due to the production of large quantities of yeast-like propagules, this fungus disperses globally, although thus far it has only rarely been reported in cold environments. This may be because most investigations on the occurrence and diversity of fungi in the cold have been limited to frozen Antarctic soils and Siberian permafrost, where basidiomycetous yeasts prevail (Abyzov 1993, Babjeva & Reshetova 1998, Deegenaars & Watson 1998, Golubev 1998, Ma *et al.* 1999, 2000, 2005, Margesin *et al.* 2002, Onofri *et al*. 2004, Price 2000, Vishniac 2006, Vishniac & Onofri 2003). Thus far, no investigations of mycobiota in ice had been carried out. We recently investigated ice originating from glacial and subglacial environments of three different polythermal Arctic glaciers in Svalbard (Spitsbergen, Norway) (Butinar *et al*. 2007, 2008, Sonjak *et al*. 2006). During these studies, aureobasidium-like fungi were found among the dominant ascomycetous mycota. Given the known adaptive ability of *A. pullulans* to low water activity (a_w) and oligotrophic conditions, it appeared likely that ice from cryocarstic formations and subglacial ice in polythermal glaciers constitute a

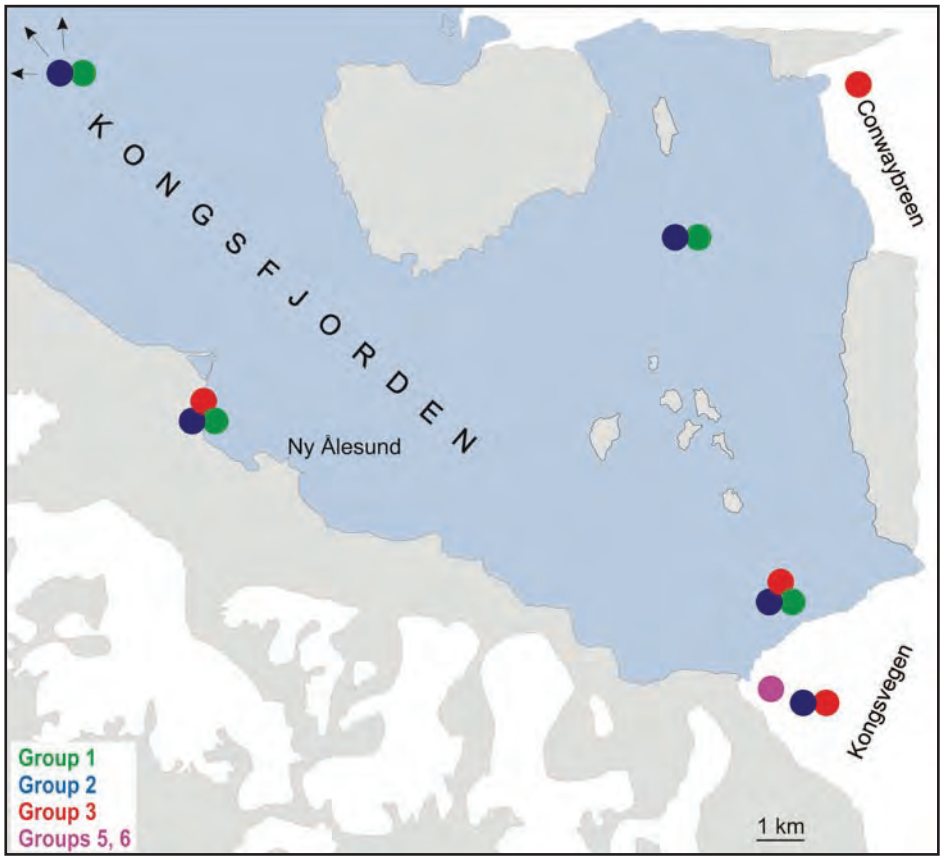

Fig. 1. Detailed map of the sampling area in Svalbard, with sites of retrieved aureobasidium-like isolates marked.

potential natural habitat. Since some of the Arctic aureobasidium-like isolates deviated phenetically from the pan-global population, a taxonomic study into the genus *Aureobasidium* was performed. Isolates obtained from different niches in Arctic, temperate and tropical climates were compared by multilocus analyses of rDNA internal transcribed spacers (ITS), partial large subunit of rDNA (LSU), and partial introns and exons of genes coding β-tubulin (*TUB*), translation elongation factor (*EF1α*) and elongase (*ELO*). The main aims of the study were to describe the total diversity of *A. pullulans*, to redefine its entities, to describe potentially new varieties, and to correlate these with their ecology, focusing on the Arctic sampling area investigated.

MATERIALS AND METHODS

Arctic sampling sites and sample collection

Kongsfjorden is located at 79°N 12°E, and it is one of the larger fjords along the western coast of Spitsbergen, in the Svalbard Archipelago, Norway. The greater part of this drainage basin is covered by glaciers that calve into the fjord. The annual mean temperature is around −5 °C, the mean salinity of the sea water ranges from 34.00 to 35.00 PSU. Twenty-five samples of glacial and subglacial ice were collected aseptically in 2001 and 2003, as described previously (Gunde-Cimerman *et al.* 2003, Butinar *et al.* 2007). These originated from three polythermal glaciers (Copland & Sharp 2001): Conwaybreen, Kongsvegen and austre Lovénbreen. Ice was also collected from a moulin and from a glacial cave at Kongsvegen. The subglacial samples included sediment-rich and overlying clear basal ice. Some ice samples, particularly from Kongsvegen, were rich in gypsum inclusions. During summer, subglacial meltwaters from Kongsvegen and the austre Lovénbreen glaciers were also sampled directly. The supraglacial samples comprised two samples of snow/ ice mixtures from austre Lovénbreen and Kongsvegen, and eight samples of seasonal meltwaters on the glacier surfaces. During the summer season of 2001, samples of seawater and a mixture of snow and ice in the tidal area were collected from six different locations within the fjord.

Physico-chemical parameters (pH, Na^+, Mg^{2+} and K^+ concentrations, and total phosphorus content) were determined for five basal ice samples (originating from Kongsvegen), a sample of subglacial meltwater, and three samples of seawater, as described by Gunde-Cimerman *et al.* (2003).

Isolation and preservation

Ice samples were transported to the laboratory, where they were processed. The surface layer of ice was aseptically melted at room temperature and discarded. The remaining ice was transferred to another sterile container and melted. The resulting water, as well as directly sampled glacier meltwater and seawater, were filtered immediately (Millipore membrane filters; 0.22-µm and 0.45-µm pore sizes) in aliquots of up to 100 mL. The membrane filters were placed on general-purpose isolation media [DRBC: Dichloran (2,6-dichloro-4-nitroanilin) Rose Bengal Agar (Oxoid CM729) and Malt Extract Agar (MEA)], as well as on a medium for the detection of moderate xerophiles [18 % dichloran glycerol agar (DG18; Hocking & Pitt 1980)], and on selective media with high concentrations of salt (MEA with addition of 5 % to 15 % NaCl) or sugar (malt extract yeast extract with 20 %, 35 % and 50 % glucose). For prevention of bacterial growth, chloramphenicol (50 mg/L^{-1}) was added to all of the media. One drop of the original water sample was applied onto a membrane and was dispersed with a Drigalski spatula. For

each sample and medium, at least four and up to 10 aliquots were filtered in parallel, and average numbers of colony forming units (CFUs) were calculated (Gunde-Cimerman *et al.* 2000). The plates were incubated for up to 14 wk at 4, 10 and 24 °C.

Subcultures were maintained at the Culture Collection of Extremophilic Fungi (EXF, Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia), while a selection have been deposited at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). Reference strains were obtained from the CBS, and were selected either on the basis of strain history, name, or on the basis of their ITS rDNA sequence. The strains were maintained on MEA and preserved for long periods in liquid nitrogen or by lyophilisation. The strains studied are listed in Table 1. A detailed map of the sampling area, with the sites of retrieved isolates marked, is shown In Fig. 1.

Cultivation and microscopy

For growth rate determination and the phenetic description of colonies, the strains were point-inoculated onto potato-dextrose agar (PDA; Oxoid CM139), and Blakeslee's MEA (Samson *et al.* 2004), and then incubated at 25 °C for 7–14 d in darkness. Surface colours were rated using the colour charts of Kornerup & Wanscher (1978). For microscopic morphology, MEA blocks of about 1 × 1 cm^2 were cut out aseptically, placed on sterile microscope slides, and inoculated at the upper four edges by means of a conidial suspension (Pitt 1979). Inoculated agar blocks were covered with sterile cover slips and incubated in moist chambers for 2, 4, and 7 d at 25 °C in the dark. The structure and branching pattern of the immersed hyphae were examined under magnifications of 100× and 400× in intact slide cultures under the microscope without removing the cover slips from the agar blocks. For higher magnifications (400×, 1000×) the cover slips were carefully removed and mounted in 60 % lactic acid.

DNA extraction, sequencing and analysis

For DNA isolation, the strains were grown on MEA for 7 d. Their DNA was extracted according to Gerrits van den Ende & de Hoog (1999), by mechanical lysis of approx. 1 cm^2 of mycelium. A fragment of rDNA including ITS region 1, 5.8S rDNA and ITS 2 (ITS) was amplified using the ITS1 and ITS4 primers (White *et al.* 1990). LSU (partial 28 S rDNA) was amplified and sequenced with the NL1 and NL4 primers (Boekhout *et al.* 1995). For amplification and sequencing of the β-tubulin (*TUB*) gene, primers Bt2a and Bt2b were used (Glass & Donaldson 1995). Translation elongation factor EF-1α (*EF1α*) was amplified and sequenced with the primers EF1-728F and EF1-986R (Carbone & Kohn 1999). For amplification and sequencing of the partial elongase gene (*ELO*), the ELO2-F (5'-CAC TCT TGA CCG TCC CTT CGG-3') and ELO2-R (5'-GCG GTG ATG TAC TTC TTC CAC CAG-3') primers were used, designed for *Aureobasidium pullulans*. Reactions were run in a PCR Mastercycler Ep Gradient (Eppendorf) with a profile of initial denaturation of 2 min at 94 °C, followed by 6 cycles of 15 s at 94 °C, 15 s at 58 °C and of 45 s at 72 °C, and 30 cycles of 15 s at 94 °C, 15 s at 56 °C and of 45 s at 72 °C, with a final elongation of 7 min at 72 °C. BigDye terminator cycle sequencing kits were used in sequence reactions (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were obtained with an ABI Prism 3700 (Applied Biosystems). They were assembled and edited using SeqMan 3.61 (DNAStar, Inc., Madison, U.S.A.). Sequences downloaded from GenBank are indicated in the gene trees by their GenBank accession numbers; newly generated sequences are indicated by their strain numbers (see also Table 1).

Phylogenetic analyses

Sequences were automatically aligned using ClustalX 1.81 (Jeanmougin *et al*. 1998). Alignments were adjusted manually using MEGA4 (Tamura *et al*. 2007). Gene trees were generated with MrBayes software, applying Bayesian inference (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Three parallel runs were performed for three million generations with mixed amino-acid models, the default temperature and five chains. The gene trees were sampled every 100 generations. Gene trees sampled before the analysis that reached stationarity of likelihood values, and those sampled before the mean standard deviation of the split frequencies decreased to under 0.5 % were excluded from the final analysis. The stationarity of likelihood values was checked using the Tracer software (Rambaut & Drummond: MCMC Trace Analysis Tool, version 1.4, 2003–2007). In phylogenetic analysis of LSU rDNA the LSU sequence of *Elsinoe veneta* (DQ678060) was selected as an outgroup, according to Schoch *et al.* (2006). Isolates were grouped on the basis of multilocus analyses and representative strains were selected for morphological analyses.

RESULTS

Isolates from Arctic samples

The fresh isolates from Arctic samples are listed in Table 1. Subglacial ice samples without and with gypsum inclusions, incubated on MEA with 5 % NaCl at 10 °C, contained aureobasidium-like propagules in the highest CFU range (>30 CFU/mL). Strains were also isolated from gypsum crystals collected from soil bordering subglacial ice with gypsum inclusions. Lower CFU numbers of *Aureobasidium* (2–3 CFU/100 mL) were detected also in other ice samples: in sea ice and moulin ice, and in specimens from an ice cave.

Phylogenetic analyses

Alignments for the phylogenetic analyses included 599 base pairs for LSU, 488 for ITS, 704 for *ELO*, 323 for *EF1α*, and 425 for *TUB*. Internodes were considered strongly supported if they received posterior probabilities ≥95 % (Lutzoni *et al.* 2004). Good convergence of the runs was reached when constructing all of the gene trees with MrBayes. The likelihood values reached plateaus after approximately 24,000 (LSU), 4,000 (ITS), 6,000 (*TUB*), 7,000 (*EF1α*) and 15,000 (*ELO*) generations, while the mean standard deviations of the split frequencies dropped below 1 % after 600,000 (LSU), 300,000 (ITS), 800,000 (*TUB*), 300,000 (*EF1α*) and 200,000 (*ELO*) generations. The first 6,000 (LSU), 3,000 (ITS), 8,000 (*TUB*), 3,000 (*EF1α*) and 2,000 (*ELO*) trees were discarded as burn-in.

According to the LSU rDNA analysis (Fig. 2), a high level of support was evident for the clade containing *A. pullulans* (groups 1–4) together with *Selenophoma mahoniae* A.W. Ramaley (CBS 388.92), *Kabatiella caulivora* (Kirchn.) Karak (CBS 242.64) and *Kabatiella microsticta* Bubák (CBS 114.64). Group 7, consisting of *Sydowia polyspora* (Bref. & Tavel) E. Müll., *Pringsheimia smilacis* E. Müll., *Delphinella strobiligena* (Desm.) Sacc. ex E. Müll. & Arx and *Dothichiza pithyophila* (Corda) Petr., formed a well supported,

Table 1. List of analysed strains subjected to DNA sequence analyses and morphological studies.

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (*LSU, ITS, BTUB, EF, ELO*)
Group 1: *Aureobasidium pullulans* var. *pullulans*						
(**T**, *Dematoidium nigrescens*)	CBS 146.30	dH 15398	Slime flux of *Quercus* sp.	Germany, Ohlsdorf near Hamburg	–	FJ150916, FJ150902, FJ157871, -, FJ039824
(**T**, *Candida malicola*)	CBS 701.76 = ATCC 11942		*Malus sylvestris*, fruit	–	–	FJ150951, FJ15090, FJ157865, -, FJ039834
(**NT**, var. *pullulans*)	CBS 584.75	dH 16041	*Vitis vinifera*, fruit	France, Beaujolais, Beaujeu	E.J. Hermanides-Nijhof	FJ150942, FJ150906, FJ157869, FJ157895, FJ039835
	CBS 109810	dH 12237	Chernobil radioactive wall	Ukraine, Kiev region	V.A. Zakharchenko	FJ150953, FJ150901, FJ157868, -, FJ039838
(**T**, var. *aubasidani*)	CBS 100524	–	*Betula*, slime flux	Russia, Leningrad Region	J.M. Voznjakovskaja	FJ150952, FJ150905, FJ157867, FJ157900, FJ039839
	CBS 100280	–	Hypersaline saltern water	Slovenia, Sečovlje salterns	P. Zalar	FJ150941, FJ150910, FJ157864, FJ157906, FJ039831
	EXF-915 = CBS 122385	dH 12636	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150947, FJ150911, FJ157870, FJ157899, FJ039830
	EXF-88 MZKI B-985	dH 16414	Hypersaline saltern water	Slovenia, Sečovlje salterns	P. Zalar	FJ150957, -, -, FJ157904, FJ039833
	EXF-150	dH 16416	Hypersaline saltern water	Slovenia, Sečovlje salterns	P. Zalar	FJ150915, FJ150908, -, FJ157905, FJ039832
	EXF-1668	dH 13836	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150949, FJ150900, FJ157875, FJ157898, FJ039827
	EXF-1702B	dH 13844	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150950, FJ150899, FJ157861, FJ157897, FJ039828
	EXF-2449	dH 13859	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150955, FJ150898, FJ157866, FJ157907, FJ039837
	MZKI B-700	dH 16413	Hypersaline saltern water	Slovenia, Sečovlje salterns	P. Zalar	FJ150956, FJ150909, -, FJ157903, FJ039826
	–	dH 13843	Subglacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150954, FJ150904, FJ157876, FJ157896, FJ039829
	–	dH 12637	Glacial ice from sea water	Norway, Svalbard, Conwaybreen, Kongsvegen	N. Gunde-Cimerman	FJ150948, -, FJ157855, FJ157901, FJ039825

Table 1. (Continued).

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (*LSU, ITS, BTUB, EF, ELO*)
Group 2: *Aureobasidium pullulans* var. *melanogenum*						
(**T**, var. *melanogenum*)	CBS 105.22	dH 15197	–	–	–	FJ150926, FJ150886, FJ157858, FJ157887, FJ039812
(**AUT,** *Torula schoenii*)	CBS 123.37	dH 15346	–	–	–	FJ150917, FJ150881, FJ157852, -, FJ039818
	CBS 621.80	dH 16090	Deteriorated army supplies	Russia	–	FJ150921, FJ150885, FJ157859, FJ157890, FJ039813
	CBS 109800	dH 11797	Endoperitoneal fluid	Greece, Athens	–	FJ150925, FJ150880, FJ157851, -, FJ039814
	CBS 100225	dH 15131	Bathroom glass	Netherlands, Hilversum	G.S. de Hoog	FJ150923, FJ150890, FJ157854, -, -
	CBS 110373		Soil	Thailand	M. Sudhadham	FJ150928, FJ150887, -, FJ157888, FJ039810
	CBS 110374		Public fountain	Thailand, Bangkok	M. Sudhadham	FJ150929, FJ150888, -, FJ157886, FJ039808
	EXF-924	dH 13831	Ponds on sea ice	Norway, Svalbard, Kongsfjorden	N. Gunde-Cimerman	FJ150918, FJ150883, FJ157850, FJ157885, FJ039817
	EXF-926	dH 13840 =dH12625	Surface glacier ice	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150922, FJ150884, FJ157853, FJ157894, FJ039816
	EXF-3382	275-1-1	Deep sea (4500 m depth)	Japan	F. Abe	FJ150930, FJ150876, -, -, FJ039807
	EXF-3383	N11	Deep sea (4500 m depth)	Japan	F. Abe	FJ150931, FJ150877, -, FJ157884, FJ039806
	EXF-3384	671-3-PI	Deep sea (4500 m depth)	Japan	F. Abe	FJ150932, FJ150879, -, -, FJ039820
	EXF-3385	671-3-MI	Deep sea (4500 m depth)	Japan	F. Abe	FJ150933, FJ150878, -, -, FJ039821
	–	dH 12640	Fountain	Thailand	M. Sudhadham	FJ150919, FJ150889, FJ157857, FJ157889, FJ039809
	–	dH 12643	Air	Thailand	M. Sudhadham	FJ150924, FJ150882, FJ157856, FJ157892, FJ039815
	–	dH 12676	Soil	Thailand	M. Sudhadham	FJ150927, -, FJ157860, FJ157893, FJ039811
	–	dH 12740	–	Thailand	M. Sudhadham	FJ150920, FJ150874, FJ157862, FJ157891, FJ039819
Group 3: *Aureobasidium pullulans* var. *subglaciale*						
	EXF-2479 = CBS 123388	dH 13860	Glacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150935, FJ150893, FJ157877, FJ157910, FJ039846
	EXF-2480	dH 13880	Subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150934, FJ150891, FJ157879, FJ157909, FJ039841
(**T**, var. *subglaciale*)	EXF-2481 = CBS 123387	dH 13862	Subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150913, FJ150895, FJ157878, FJ157911, FJ039845
	EXF-2491	dH 13865	Subglacial ice	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150936, FJ150894, FJ157880, FJ157902, FJ039844
	EXF-2510 = CBS 123386	dH 13868	Moulin	Norway, Svalbard, Conwaybreen	N. Gunde-Cimerman	FJ150938, -, -, -, -

Table 1. (Continued).

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (*LSU, ITS, BTUB, EF, ELO*)
	EXF-3640	dH 13864	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150939, FJ150896, FJ157882, FJ157913, FJ039842
	–	dH 13876	coastal ponds of melted snow and ice	Norway, Svalbard, Kongsfjorden, Ny Ålesund	N. Gunde-Cimerman	FJ150958, FJ150892, FJ157881, FJ157912, FJ039843
Group 4: *Aureobasidium pullulans var. namibiae*						
(**T,** *var. namibiae*)	CBS 147.97	–	dolomitic marble	Namibia, Namib Desert	U. Wollenzien	FJ150937, FJ150875, FJ157863, -, FJ039822
Group 5, Unnamed						
	EXF-914 = CBS 122350	dH 12626 = 13830	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150961, -, -, -, -
	EXF-3639 = CBS 122359	dH 13832	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150966, -, -, -, -
	EXF-935 = CBS 122362	dH 12635	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150963, -, -, -, -
	EXF-922	dH 12623	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150964, -, -, -, -
	EXF-1934	dH 13842	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150962, -, -, -, -
	EXF-1936	dH 13839	subglacial ice from sea water	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150965, -, -, -, -
Group 6, Unnamed						
	EXF-2500 = CBS 123390	dH 13881	glacial ice	Norway, Svalbard, Kongsvegen	N. Gunde-Cimerman	FJ150914, -, -, -, -
	–	dH 12633			N. Gunde-Cimerman	FJ150960, -, -, -, -
	EXF-938 = CBS 123389	dH 12634	subglacial ice from sea water	Norway, Svalbard, Conwaybreen	N. Gunde-Cimerman	FJ150959, -, -, -, -
Species included for comparison.						
Dothichiza pithyophila	CBS 215.50	–	*Abies concolor*, dead bark	Norway	–	FJ150968, -, FJ157883, -, -
Dothichiza pithyophila	–	dH 12609	Vishniac Y-31	–	–	FJ150969, -, -, -, -

Table 1. (Continued).

Taxon	Accession no.	dH number	Source	Geography	Collector	GenBank accession no. (*LSU, ITS, BTUB, EF, ELO*)
Dothichiza sp.	EXF-3641	dH 13858	sea ice with sediment	Norway, Svalbard	N. Gunde-Cimerman	FJ150967, -, -, -, -
	= CBS 122355					
Kabatiella caulivora	CBS 242.64	dH 15602	*Trifolium incarnatum*	U.S.A., Oregon	–	FJ150944, FJ150871, -, -, FJ039836
Kabatiella microsticta	CBS 114.64	dH 15299	*Hemerocallis* sp.	Netherlands, Wageningen	–	FJ150940, FJ150873, -, FJ157914, FJ039848
	= MUCL 18713					
Kabatiella microsticta	CBS 342.66	dH 15774	*Convallaria majalis*, dying leaf	Germany	W. Gams	FJ150945, FJ150903, FJ157872, -, FJ039823
Kabatiella lini	CBS 125.21 **T**	dH 15350	*Linum usitatissimum*	U.K.	–	FJ150946, FJ150897, FJ157873, FJ157908, FJ039840
	= MUCL 8712					
Pringsheimia smilacis	CBS 873.71 **T**	–	*Smilax aspera*, twig	Italy, Napoli	L. Froidevaux	FJ150970, -, -, -, -
Selenophoma mahoniae	CBS 388.92 **T**	dH 15823	*Mahonia repens*, leaf	U.S.A., Colorado	A.W. Ramaley	FJ150943, FJ150872, FJ157874, FJ157915, FJ039847
Sydowia polyspora	CBS 750.71	–	*Pinus strobus*, twig	Canada, Quebec; Lac Normand	E. Müller	FJ150912, -, -, -, -

* Abbreviations used: ATCC = American Type Culture Collection; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; dH = de Hoog Culture Collection, CBS, Utrecht, The Netherlands; EXF = Culture Collection of Extremophilic Fungi, Ljubljana, Slovenia; MUCL = Mycotheque de l'Universite catholique de Louvain; MZKI = Microbiological Culture Collection of the National Institute of Chemistry, Ljubljana, Slovenia; NT = ex-neotype strain; T = ex-type strain.

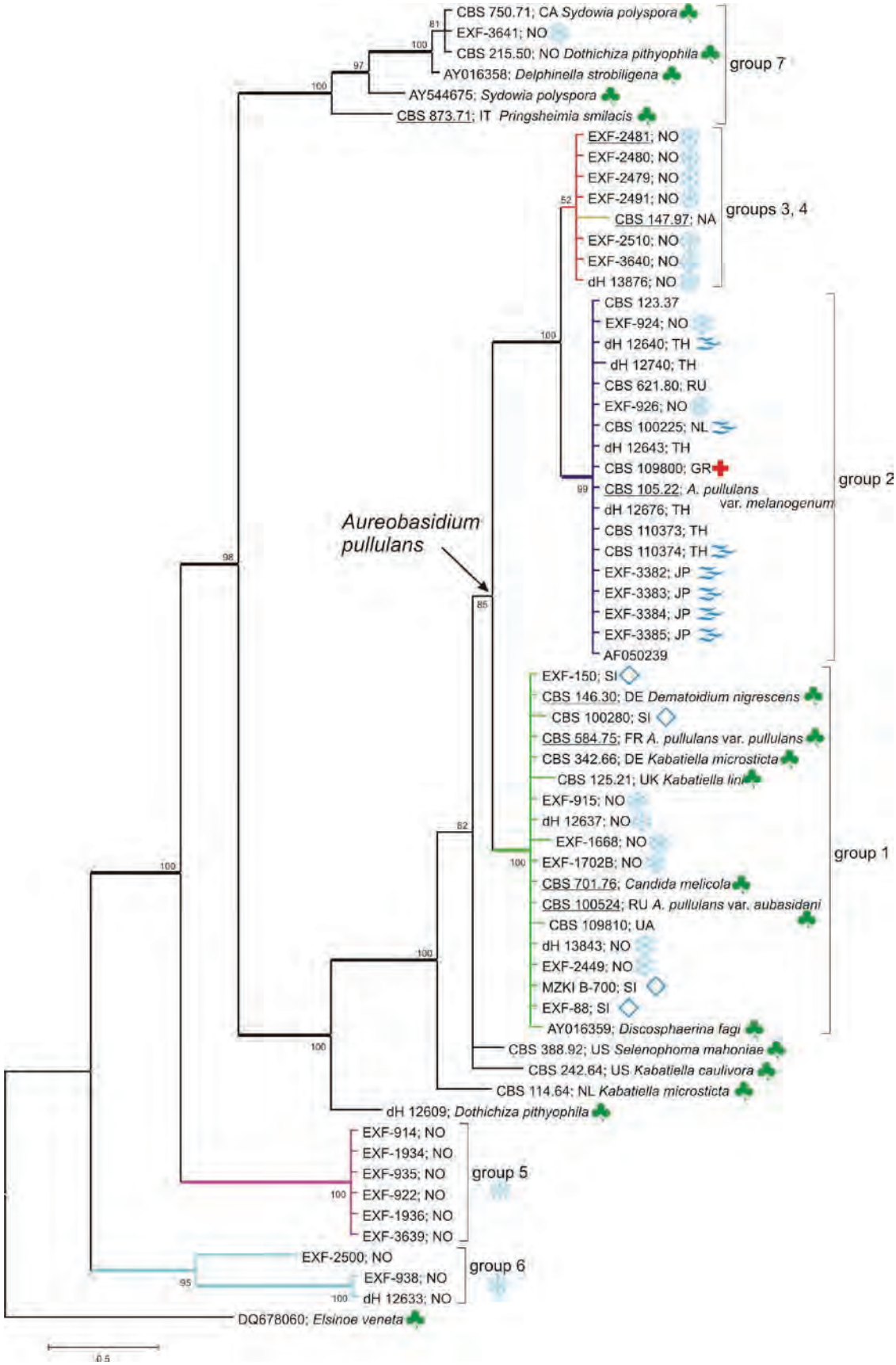

Fig. 2. Consensus phylogram (50 % majority rule) of 24000 trees resulting from a Bayesian analysis of the LSU sequence alignments using MrBayes v. 3.1.2. Bayesian posterior probabilities are indicated at the nodes, branches with posterior probabilities >95 in bold. The tree was rooted to the sequence of *Elsinoe veneta* (DQ678060). Ex-type and ex-neotype strains are underlined; when known origin two digit country codes are listed after strain numbers. The colour marks stand for:

- plant associated;
- originating from Arctic ice;
- originating from hyperosmotic environment;
- clinical strain.
- water.

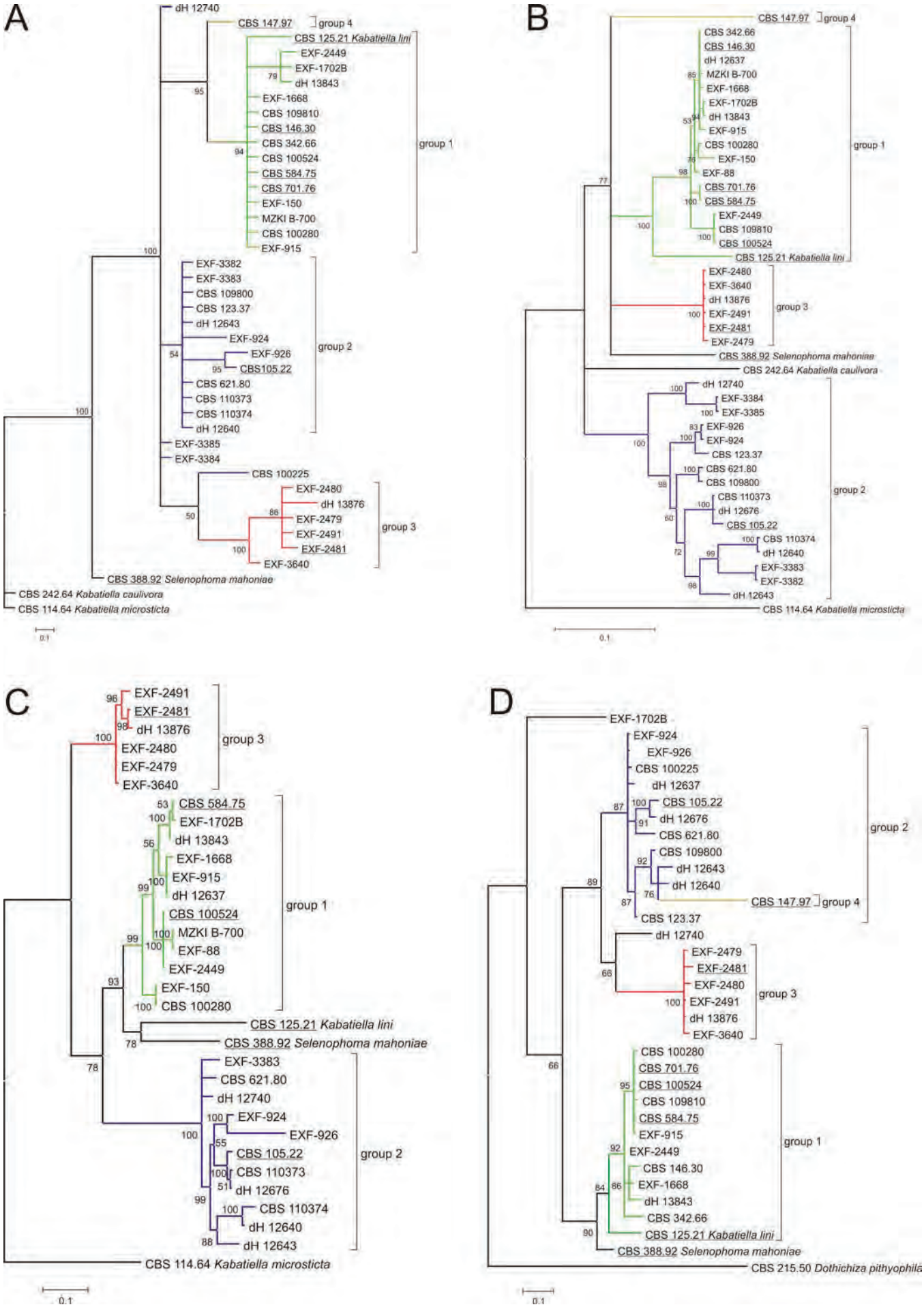

Fig. 3. Consensus phylograms (50 % majority rule) resulting from a Bayesian analysis of: A. ITS rDNA; B. elongase gene; C. translation elongation factor EF-1α gene; D. β-tubulin gene. Bayesian posterior probabilities are indicated at the nodes. The trees are not rooted. Ex-type and ex-neotype strains are underlined.

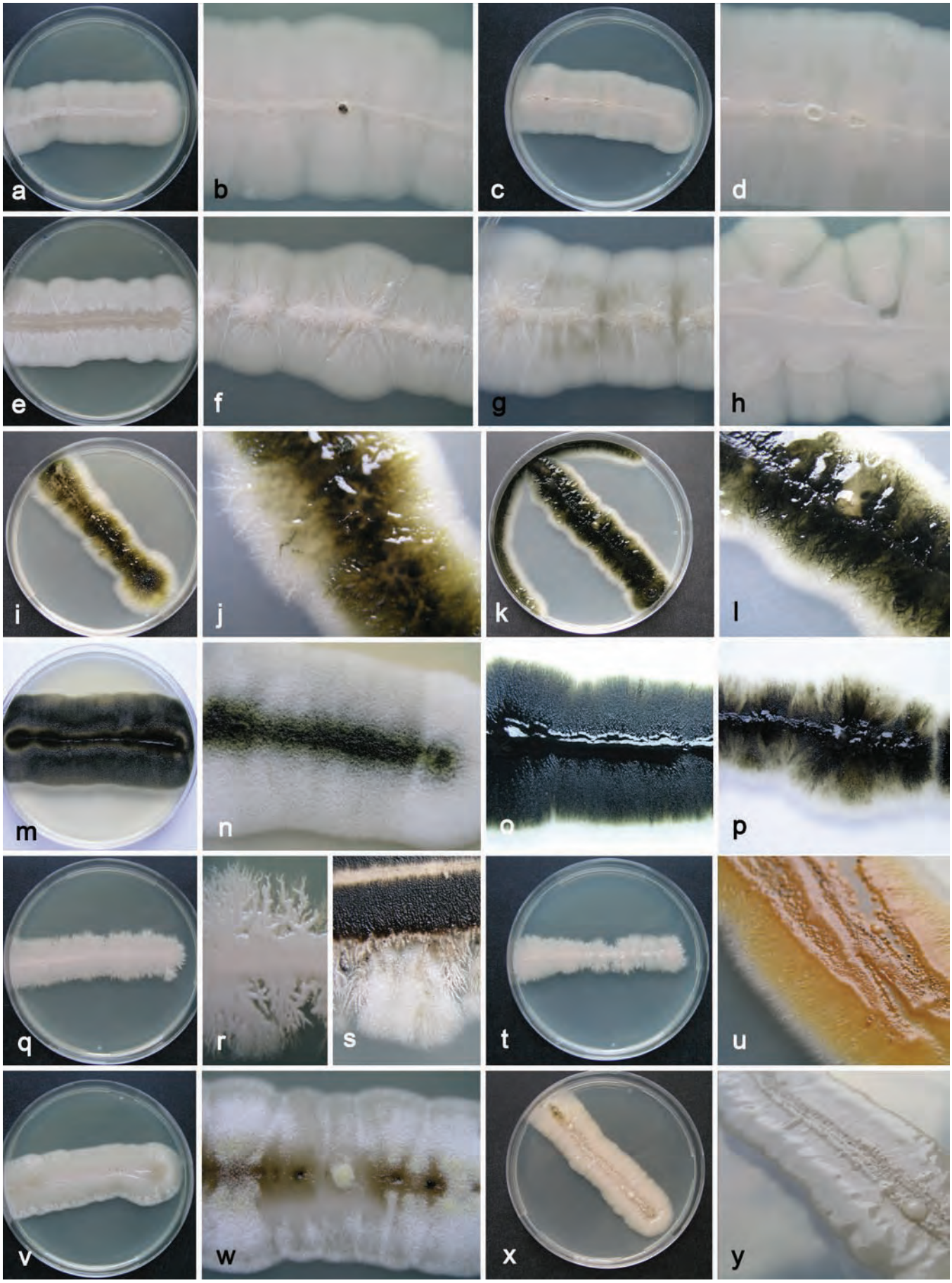

Fig. 4. Macromorphology of different *Aureobasidium pullulans* varieties incubated for 7 d at 25 ºC in the dark on MEA (left 2 columns) and on PDA (right two columns). **a–h.** *A. pullulans* var. *pullulans*: a, b. CBS 584.75 (MEA); c, d. CBS 584.75 (PDA); e. CBS 109810 (MEA); f, g. CBS 701.76 (MEA, PDA); h. MZKI B-700 (PDA). **i–p.** *A. pullulans* var. *melanogenum*: i, j. CBS 105.22 (MEA); k, l. CBS 105.22 (PDA); m. EXF-3382 (MEA); n. CBS 621.80 (MEA); o. EXF-924 (PDA); p. CBS 100225 (PDA). **q–u.** *A. pullulans* var. *subglaciale*: q, r. EXF-2481 (MEA); s. EXF-2481 after 14 d incubation (MEA); t. EXF-2481 (PDA); u. EXF-2479 (PDA). **v–y**. *A. pullulans* var. *namibiae*. v, w. CBS 147.97 (MEA); x, y. CBS 147.97 (PDA).

but separate, clade. Separate well-supported clades (groups 5 and 6) joined arctic strains of no affinity to any of the known taxa. Clade *Aureobasidium pullulans* was badly supported (85 posterior probability). Groups 1 and 2 within this clade were statistically supported, while groups 3 and 4 reached a poor posterior probability value. Group 1 contained the ex-neotype strain of *A. pullulans* var. *pullulans* (CBS 584.75), its supposed teleomorph *Discosphaerina (Columnosphaeria) fagi*, the ex-type strain of *Kabatiella lini* (CBS 125.21), the ex-type strain of *Dematoidium nigrescens* Stautz (CBS 146.30), the ex-type strain of *A. pullulans* var. *aubasidani* (CBS 100524), and a strain of *Kabatiella microsticta* (CBS 342.66). Another strain of *K. microsticta* was placed on the basal branch as the sister taxon of *K. caulivora* (CBS 242.64) and *Selenophoma mahoniae* (CBS 388.92). Group 2 contained the ex-type strain of *A. pullulans* var. *melanogenum*. Group 3 contained exclusively Arctic strains, while group 4 consisted of one strain only (CBS 147.97). Analyses of the more variable ITS spacers (Fig. 3A), and *ELO* (Fig. 3B), *EF1α* (Fig. 3C), and *TUB* (Fig. 3D) introns and exons almost consistently supported the first three groups, with only a few exceptions. For example, several strains of group 2 were dispersed outside the clade of group 2 in ITS analysis, while in other analyses they formed a monophyletic group. In analyses of ITS and *ELO*, group 4 was supported, whereas based on *TUB* it was grouped together with group 2, but on a separate and long branch. The amplification of the *EF1α* gene failed in the only strain of group 4; therefore, its phylogenetic position concerning this gene is unknown.

Morphology

The main difference observed among isolates was pigmentation of cultures (Fig. 4). Strains belonging to groups 1 and 3 remained pinkish for at least 1 w. The majority of strains from group 1 became pigmented only after 3 wk, or even later. The only exception among this group was the ex-type strain of *Dematoidium nigrescens* (CBS 146.30), which was darkly pigmented already after 1 wk of incubation due to melanised septated hyphae. This was also the only strain that was exclusively filamentous and formed no conidia. Strains of group 3 became melanised only at the margin, where dark pigmented, heavily branched hyphae developed, while the colony centre remained pinkish at least for 3 wk. All strains of group 2 were green or black after 7 d of incubation, due to the production of melanised hyphae and conidia. Structures of cultures differed within different varieties, from almost entirely yeast-like to entirely filamentous, and also yeast-like with marginal or central aerial mycelium. Marginal areas of colonies were significantly different in at least two pink pigmented groups, consisting of arachnoid mycelium in group 1 and of thick and undulating hyphae in group 3. Conidiogenesis seen in the groups of *A. pullulans* (groups 1−4) studied was synchronous, either on rather undifferentiated short denticles, intercalary or terminally on hyaline (groups 1, 3, 4) or melanised (group 4) hyphae. This kind of conidiogenesis was also seen on enlarged globose conidiogenous areas that developed laterally on hyphae, giving rise to multiple conidia, or on enlarged yeast cells, which were synchronously budding from both poles. Synchronous conidiogenesis was sometimes difficult to observe due to heavy yeast proliferation. More complex conidiophore-like structures were noted in group 3. Another mode of conidiation seen in all varieties was percurrent conidiogenesis alongside the hyphae. Conidia formed either synchronously or percurrently, and were budding secondarily in all groups; therefore, the size and shape of conidia in *Aureobasidium* in general was very variable. Conidia in groups 1 and 4 were almost exclusively non-pigmented, while in group 2, as well as 1-celled non-pigmented conidia, also melanised 1−2-celled conidia were also abundant. Pigmented conidia were also seen with the strain CBS 100524, the ex-neotype strain of *A. pullulans* var. *aubasidani*. Endoconidia were seen in only some strains of groups 1 and 3.

TAXONOMY

Aureobasidium pullulans (de Bary) G. Arnaud var. ***pullulans*** – Annales École Nat. Agric. Montpellier 16: 39, 1918. MycoBank MB101771). Fig. 5.

Synonyms: *Dematium pullulans* de Bary 1884 (MB 219317; NT = CBS 584.75)
Aureobasidium pullulans (de Bary) Arn. var. *aubasidani* Yurlova in Yurlova & de Hoog 1997 (MB 442903; T = CBS 100524)
Candida malicola D.S. Clark & R.H. Wallace 1955 (MB 294033; T = CBS 701.76)
Dematoidium nigrescens Stautz 1931 (MB 272259; T = CBS 146.30)

Cultural characteristics: Colonies on MEA/PDA at 25 ºC attaining about 40/30 mm diam after 7 d, appearing smooth and slimy due to abundant sporulation, pinkish (pinkish white, 7A2) to yellowish (light yellow, 3A4), reverse yellowish (pale yellow (4A3) to light yellow (4A4)). Black sectors composed of dark pigmented hyphae or conidia develop in some isolates after 14 d. Margin composed of arachnoid mycelium, sometimes in sectors. No aerial mycelium.
Deviations: White aerial mycelium at the edge of cultures present in some strains (CBS 109800, EXF-915), some strains entirely filamentous (dH 12637), some develop white, setae-like mycelial formations in colony centre and marginal leathery mycelium (CBS 701.76). Strain CBS 146.30 was black and filamentous already after 1 wk of incubation.

Microscopy: Vegetative hyphae hyaline, smooth, thin-walled, 4–12 µm wide, transversely septate, in older cultures sometimes locally converted to dark-brown hyphae. Conidiogenous cells undifferentiated, intercalary or terminal on hyaline hyphae. Conidia produced synchronously in dense groups from small denticles, and also formed percurrently on short lateral denticles. Conidia hyaline to dark brown. Hyaline conidia one-celled, smooth, ellipsoidal, very variable in shape and size, 7.5–16 × 3.5–7 µm, often with an indistinct hilum. Dark brown conidia (measured in strain CBS 100524, developed after 2 wk) 1–2 celled, one celled 10–17 × 5–7 µm, two celled slightly constricted at septum, 14–25 × 5–11 µm. Budding of hyaline and dark brown conidia frequently seen, with the secondary conidia being smaller than the primary ones. Conidia in old cultures transfer to globose, brownish structures of 10–15 µm diam. Endoconidia, about 6 × 3 µm occasionally seen in intercalary cells.

Maximum tolerated salt concentration: 15 % NaCl.

Cardinal temperatures: Minimum at 4 °C, optimum at 25 °C, maximum at 30 °C.

Specimens examined: **France**, fruit of *Vitis vinifera*, 1974, coll. and isol. E.J. Hermanides-Nijhof, ex-neotype culture CBS 584.75; for additional specimens, see Table 1.

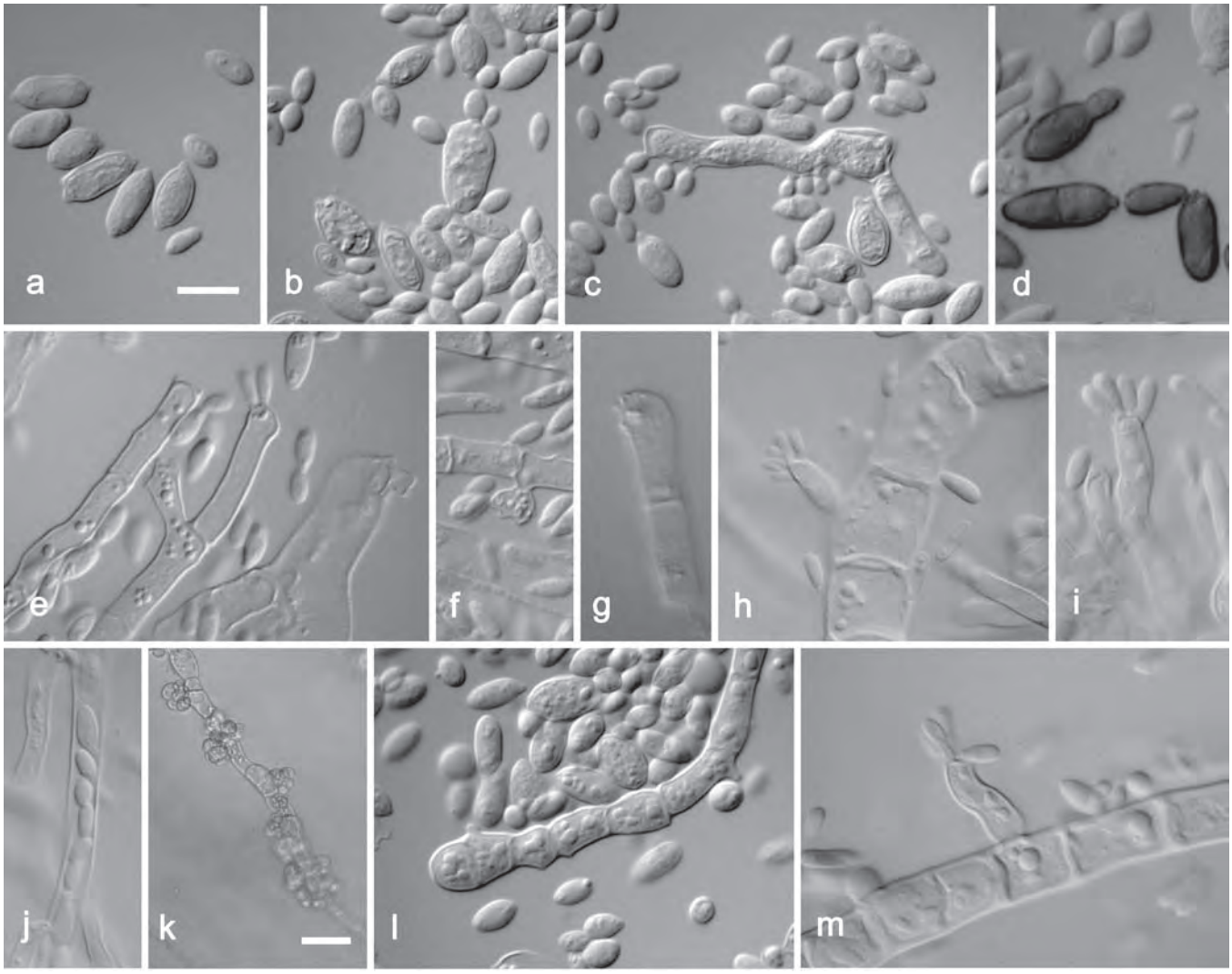

Fig. 5. *Aureobasidium pullulans* var. *pullulans.* a. Liberated conidia transforming to budding cells. b. Synchronous production of conidia on a transformed conidium – yeast cell. c. Short hypha synchronously producing conidia. d. Dark brown conidia. e–i, m. Poorly differentiated, terminal and intercalar conidiophors performing synchronous conidiation. k. Immersed hypha with lateral accumulation of conidia. l. Hypha with lateral scars – conidiogenous loci. j. Endoconidia. a–c, e–g, k–m. CBS 584.75 (ex-neotype strain); d. CBS 100524; h–i, j, m. EXF-1702B. Scale bars: a–j, l–m= 10 µm; k= 20 µm.

Aureobasidium pullulans (de Bary) G. Arnaud var. ***melanogenum*** Hermanides-Nijhof – Stud. Mycol. 15: 161, 1977. MycoBank MB352628. Fig. 6.

Synonyms: *Torula schoenii* Roukhelman 1937 (MB 445735; AUT = CBS 123.37) (Invalid; Art. 37 ICBN)
Pullularia fermentans Wynne & Gott var. *schoenii* (Roukhelman) Wynne & Gott 1956 (MB 352450)
Aureobasidium pullulans (de Bary) G. Arnaud var. *melanogenum* Hermanides-Nijhof 1977 (MB 352628; T = CBS 105.22)

Cultural characteristics: Colonies on MEA/PDA at 25 ºC attaining 25 mm diam after 7 d, appearing smooth and slimy due to abundant sporulation and EPS formation, olive brown (4F3-4F8) to black in centre, towards margin mustard yellow (3B6), margin yellowish white (3A2); reverse olive-grey (3E2) at the centre, towards margin dull yellow (3B4), at the margin yellowish white (3A2). Margin composed of arachnoid to thick undulating hyphae growing into the agar, sometimes sectorial. After 14 d the entire colonies are green to black. Aerial mycelium develops in some parts of the colonies. Deviations: White aerial mycelium present in strain CBS 621.80.

Microscopy: Vegetative hyphae in the central part of colonies, dark brown, smooth to slightly roughened, thick walled, 6–12 µm wide, transversely septate, constricted at septa, embedded in EPS, disarticulating to 1–2-celled, dark brown chlamydospores, one celled 13–16 × 8–12 µm, two celled 17–24 × 10–12 µm. Vegetative hyphae at colony edge hyaline, smooth, thin-walled, 2–10 µm wide, transversely septate, getting thicker and darker with age. Immersed hyphae with multiple lateral pegs. Conidiogenous cells undifferentiated, intercalary or terminal on hyaline hyphae, sometimes grown in the form of an outgrowth with three denticles. Conidia produced synchronously in dense groups from small denticles (1.0–2.5 µm long), and also formed percurrently alongside hyphae and on short lateral branches. Conidia hyaline and dark brown. Hyaline conidia one-celled, smooth, ellipsoidal, very variable in shape and size, 8–30 × 3.5–5 µm, often with an indistinct hilum. Dark brown conidia 1–2-celled, smooth, ellipsoidal when one celled, 7 × 6 µm, slightly constricted at septa when two celled, 12–20 × 4–12 µm. Unilateral and bilateral budding of hyaline conidia frequently seen, with the secondary conidia being smaller than the primary ones. Endoconidia not seen.

Maximum tolerated salt concentration: 10 % NaCl.

Cardinal temperatures: Minimum at 10 °C, optimum at 30 °C, maximum at 35 °C.

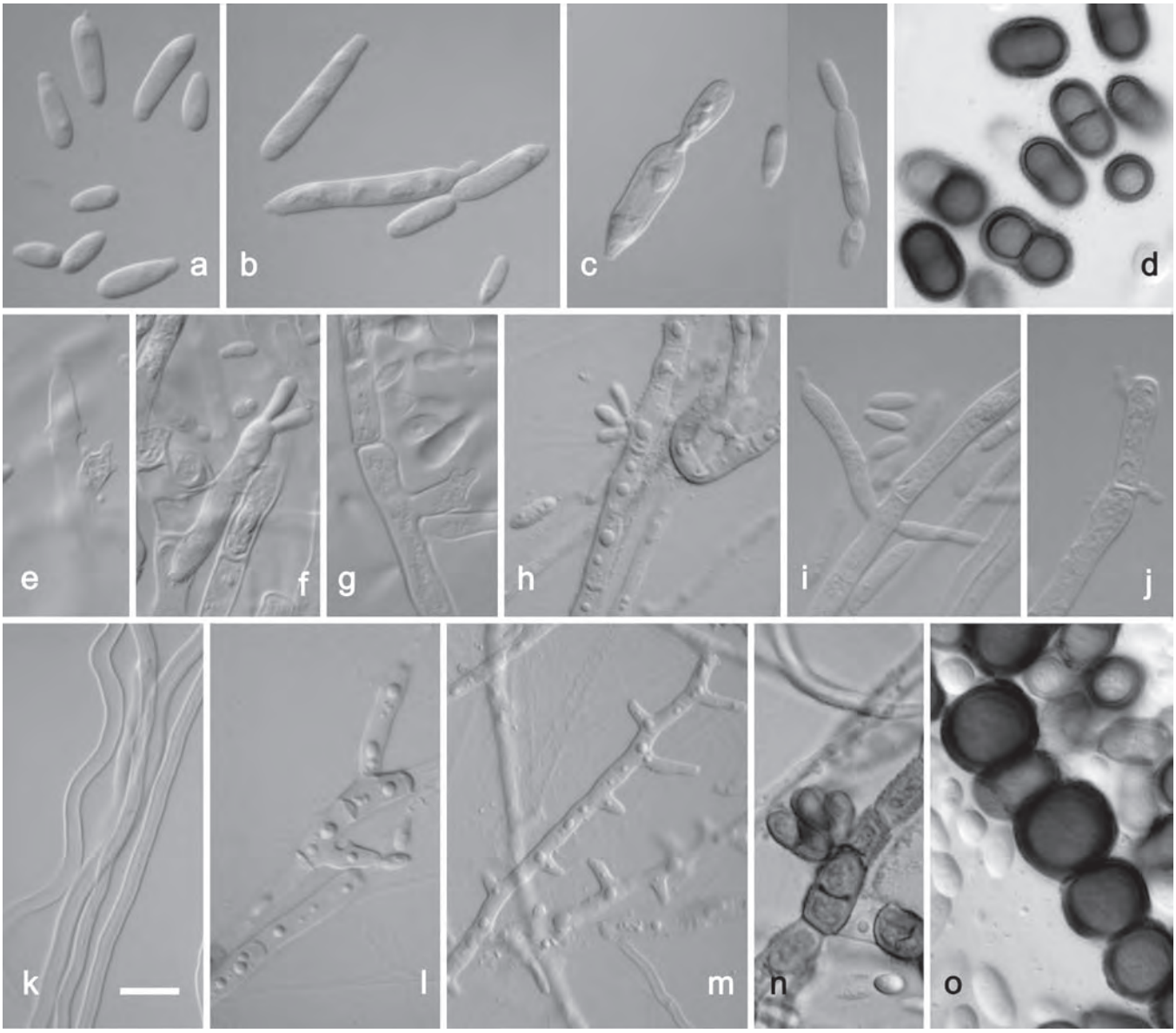

Fig. 6. *Aureobasidium pullulans* var. *melanogenum.* a–c. Liberated conidia transforming to budding cells. d. Dark brown conidia. e–h. Poorly differentiated, terminal and intercalar conidiophors performing synchronous conidiation. i, l. Hypha with long lateral conidiogenous cells. j. Hypha with prolonged lateral pegs. k. Vegetative hyphae. m. Immersed hyphae with multiple lateral pegs. n. Melanized hyphae with intercalar synchronous conidiogenesis. o. Melanized hyphae / chlamydospores. a–c, e–n. CBS 105.22 (ex-type strain); d, o. EXF-926. Scale bar: as marked on k (a–o) = 10 µm.

Specimens examined: **Unknown,** culture ex-type CBS 105.22 = ATCC 12536 = CECT 2658 = IMI 062460 = NRRL Y-7469, isolated by M. Church; additional specimens see Table 1.

Aureobasidium pullulans (de Bary) G. Arnaud var. ***subglaciale*** Zalar, de Hoog & Gunde-Cimerman, **var. nov.** MycoBank MB512380. Fig. 7.

Coloniae in agaro MEA vel PDA 25 °C ad 20 mm diam post 7 dies, leves, haud lucidae, copiose sporulantes, roseae, reverso dilute aurantiaco; post 15 dies in medio roseae, marginem versus obscure brunneae; hyphae marginales superficiales latae, undulantes, nonnumquam sectores formantes; hyphae aeriae absentes. Hyphae vegetativae hyalinae, leves, tenuitunicatae, 2–10 µm latae, in coloniis vetustis nonnumquam hyphae fuscae, crassitunicatae, 5–9 µm latae. Conidia hyalina vel fusca, hyalina unicellularia, levia, ellipsoidea, forma magnitudineque variabilissima, 5.5–28 × 2–6.5 µm, fusca 1- vel bicellularia, 8–16(–25) × 5–9 µm. Conidia saepe gemmantia, secundaria primariis minora; endoconidia circa 8 × 3 µm nonnumque in cellulis intercalaribus formata. Temperatura optima et maxima crescentiae 25 °C.

Holotype: CBS H-20186

Cultural characteristics: Colonies on MEA/PDA at 25 ºC attaining 20 mm (10–35 mm) diam after 7 d, appearing smooth and matt due to abundant sporulation, pinkish (pinkish white, 7A2), reverse pale orange (5A3). After 14 d central areas of colonies remain pinkish, towards the margin becoming dark-brown (greyish brown, 5F3). Margin composed of thick undulating superficial and immersed branched hyphae, sometimes with sectors. Aerial mycelium absent.

Deviations: Culture EXF-2479 develops more intensively pigmented colonies than others, pink in centre and yellowish orange towards the colony margin on MEA, and golden-yellow on PDA.

Microscopy: Vegetative hyphae hyaline, smooth, thin-walled, 2–10 µm wide, transversely septate, in older cultures locally converted to dark brown, thick-walled hyphae of 5–9 µm diam. Conidiogenous cells mostly undifferentiated, intercalary or terminal on hyaline hyphae, sometimes developed in clusters as conidiophore-like structure. Conidia produced synchronously in dense groups from small denticles, and also percurrently on short lateral branches. Conidia hyaline to dark brown. Hyaline conidia one-celled, smooth,

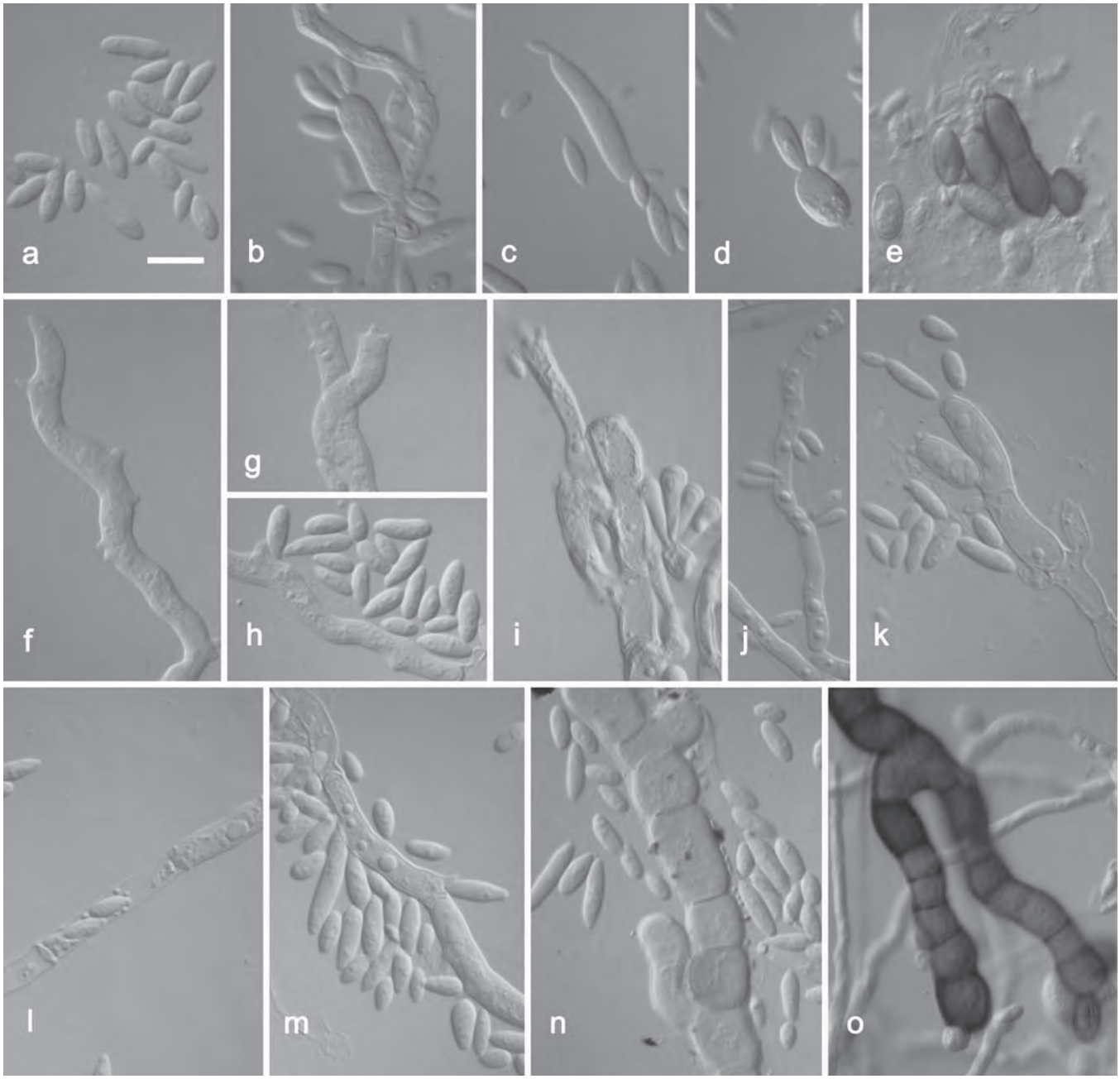

Fig. 7. *Aureobasidium pullulans* var. *subglaciale*. a. Conidia. b–d. Budding conidia. e. Dark brown conidia. f, g. Hyphae with multiple lateral pegs, which develop into synchronous conidiation aparatus. h, m. Hyphae with lateral pegs. i, k. Conidiophore-like structure synchronously producing conidia. l. Endoconidia. n. Hyaline vegetative hyphae. o. Melanized hyphae. a–o. EXF–2481 (ex-type strain). Scale bar: as marked on a (a–o) = 10 µm.

ellipsoidal, very variable in shape and size, 5.5–28 × 2–6.5 µm, often with an indistinct hilum. Dark conidia 1–2-celled, one celled 8–16 ×5–9 µm, two celled 9–25 × 5.5–7.5 µm. Budding frequently seen, with secondary conidia being smaller than the primary ones. Endoconidia, about 8 × 3 µm, sometimes present in intercalary cells.

Maximum tolerated salt concentration: 10 % NaCl.

Cardinal temperatures: Minimum at 4 °C, optimum and maximum at 25 °C.

Specimen examined: **Norway,** Spitsbergen, subglacial ice from sea water, 2003, coll. and isol. N. Gunde-Cimerman, Holotype CBS H-20186, culture ex-neotype EXF-2481 = CBS 123387; additional specimens see Table 1.

Aureobasidium pullulans (de Bary) Arnaud var. ***namibiae*** Zalar, de Hoog & Gunde-Cimerman, **var. nov.** MycoBank MB512381. Fig. 8.

Coloniae in agaro MEA 25 °C ad 25 mm diam post 7 dies, leves, lucidae textura coriacea, roseae, in medio brunneae, margine hyphis aereis albae, reverso griseo-luteo; coloniae in agaro PDA post 7 dies ad 20 mm diam, leves, sporulatione copiosa lucidae, in medio aurantio-albae olivascentes, nonnumquam hyphales et setosae, reverso armeniaco. Hyphae aeriae absentes. Hyphae vegetativae hyalinae, leves, tenuitunicatae, 2–13 µm latae, in coloniis vetustis nonnumquam hyphae fuscae. Conidia hyalina vel fusca, hyalina unicellularia, levia, ellipsoidea, forma magnitudineque variabilissima, 7–17 × 3.5–7 µm, fusca 1- vel bicellularia, 8–13(–24) × 5–9(–10) µm, saepe granulis ectoplasmaticis circumdata. Conidia saepe gemmantia, secundaria primariis minora; endoconidia haud visa. Temperatura optima crescentiae 25 °C, et maxima 30 °C.

Holotype: CBS H-20184

Cultural characteristics: Colonies on MEA at 25 ºC attaining 25 mm

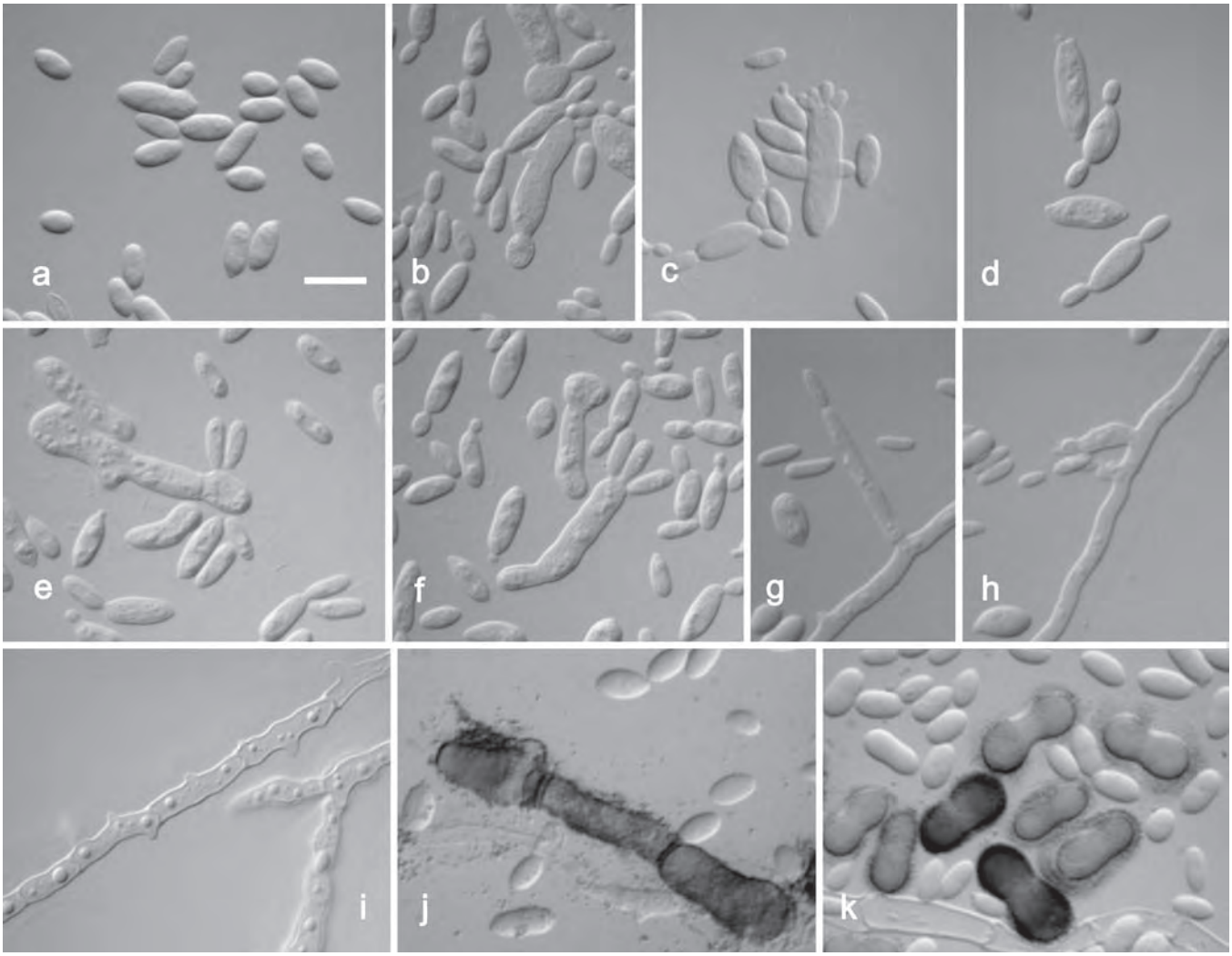

Fig. 8. *Aureobasidium pullulans* var. *namibiae*. a. Conidia. b–f. Liberated conidia transforming to budding cells. g–h. Hypha with long lateral conidiogenous cells. i. Immersed hyphae with multiple lateral pegs. j. Melanized hypha surrounded by EPS. k. Hyaline and dark brown conidia. a–k. CBS 147.97 (ex-type strain). Scale bar: as marked on a (a–k) = 10 µm.

diam after 7 d, appearing smooth and shiny due to the leathery structure of colonies, pinkish (pinkish white, 7A2) with brownish (greyish brown, 5E3) central part, margin white (5A1), reverse yellowish (greyish yellow, 4B4); margin composed of superficial aerial mycelium. Colonies on PDA at 25 ºC attaining 20 mm diam after 7 d, appearing smooth and shiny due to abundant sporulation, orange-white (5A2) with olive-brown (4F3) centre, sometimes hyphal and with setae, reverse apricot (orange white, 5A2). No aerial mycelium.

Microscopy: Vegetative hyphae hyaline, smooth, thin-walled, 2–13 µm wide, transversely septate, locally converted to dark brown, thick-walled hyphae. Conidiogenous cells undifferentiated, intercalary or terminal on hyaline hyphae and on larger transformed conidia. Conidia produced synchronously in dense groups from small denticles, later formed percurrently on short lateral branches. Conidia hyaline and dark brown. Hyaline conidia one celled, smooth, ellipsoidal, very variable in shape and size, 7–17 × 3.5–7.0 µm, often with an indistinct hilum. Dark brown conidia 1-2-celled, one celled 8–13 × 5–9 µm, two celled 8–24 × 2–10 µm, surrounded by granular EP; if two-celled, constricted at the septum. Budding frequently seen, with secondary conidia smaller than the primary ones. Endoconidia not seen.

Maximum tolerated salt concentration: 10 % NaCl.

Cardinal temperatures: Minimum at 10 °C, optimum at 25 °C and maximum at 30 °C.

Specimen examined: **Namibia**, dolomitic marble in Namib Desert, 1997, coll. and isol. U. Wollenzien, holotype CBS H-20184, culture ex-type CBS 147.97.

DISCUSSION

The elongase-encoding gene (*ELO*) was used as phylogenetic marker for the first time. Southern blotting of *A. pullulans* genomic DNA did not suggest the existence of more than one copy of the elongase gene in the genome of *A. pullulans*, while this is the case in other fungi (Gostinčar *et al.* 2008); this would have diminished its value for routine studies. The gene provided excellent resolution of the *Aureobasidium* complex and thus could reliably be used for tree reconstruction.

The anamorph genus *Aureobasidium* phylogenetically belongs to *Ascomycota*, order *Dothideales*, family *Dothideaceae* (Schoch *et al.* 2006). The fungi have been known since the late 19th century, when Viala & Boyer (1891) described *A. vitis* as a common coloniser of the sugary surface of grapes (*Vitis vinifera*). Type material is not known to be preserved. In her revision of the genus, Hermanides-Nijhof (1977) neotypified *Dematium pullulans* De Bary (1884) with CBS 584.75, thus establishing *A. pullulans* as the oldest name for

the type species of *Aureobasidium*. The genus was circumscribed using criteria of conidiogenesis, *i.e.*, synchronous holoblastic conidium production. This feature is also known in sporodochial *Kabatiella* species forming defined leaf spots on specific host plants. When these fungi are cultured, the sporodochia fall apart, and the micromorphology becomes very similar to that of *Aureobasidium pullulans*. For this reason, Hermanides-Nijhof (1977) classified all *Kabatiella* species in *Aureobasidium*, even though most *Kabatiella* species have not been cultured and are only known from the sporodochial anamorph on the host plant. LSU sequences of the few species thus far available for study indeed show affinity to *A. pullulans*.

Kabatiella zeae Narita & Y. Hirats. (Hermanides-Nijhof 1977) is found in isolated positions away from other aureobasidia (de Hoog *et al.* 1999, Yurlova *et al.* 1999). Synchronous conidiation is thus polyphyletic. However, in addition to molecular differences for some species, morphological distinctions may also be possible, since most *Kabatiella* species have sickle-shaped conidia, such as *K. caulivora*, *K. harpospor*a (Bres. & Sacc.) Arx, *K. phoradendri* (Darling) Harvey f. *umbellulariae* Harvey, and *K. zeae*. In *Kabatiella lini,* a species clustering within *A. pullulans* (Fig. 2), the conidia have similar shape, but are slightly larger than in the var. *pullulans*. *Kabatiella microsticta*, *K. caulivora* and another, related pycnidial fungus, *Selenophoma mahoniae* deviate in all of the genes studied at the variety level. Thus, they might be regarded as separate varieties of *A. pullulans*, but the possibility cannot be excluded that unintentially the ubiquitous phyllosphere fungus *A. pullulans* was isolated instead of the pathogen. It appears likely that the plant-invading, host-specific pathogens are consistently different from *A. pullulans*, which on host plants colonises surfaces only, but unambiguously identified strains are needed to prove this.

Our multilocus analysis shows that *Aureobasidium pullulans* consists of three robust main groups, two of which have high statistic support in LSU and show the same topology with all of the genes sequenced. The ex-neotype of the species, CBS 584.75, is in group 1, *A. pullulans* var. *pullulans*. This group also contains CBS 146.30, the ex-type strain of *Dematoideum nigrescens* Stautz, CBS 701.76, the ex-type strain of *Candida malicola* D.S. Clark & R.H. Wallace, and CBS 100524, the ex-type strain of *A. pullulans* var. *aubasidani* Yurlova, which should thus be regarded as synonyms. The production of aubasidan rather than pullulan as the main extracellular exopolysaccharide (Yurlova & de Hoog 1997) is apparently strain dependent. Although the production of EPS and other previously described diagnostic characters for this variety were not evaluated in this study, we believe that used multilocus approach as molecular diagnostic tool would show the difference of var. *aubasidani* to other varieties. The ex-type strain of *Dematoidium nigrescens* (CBS 146.30) was the only initially darkly pigmented strain in group 1, which is probably due to its degeneration. The var. *pullulans*, which is newly defined, is phenetically characterised by rapidly expanding, pinkish cultures that can develop radial dark brown sectors due to the local presence of thick-walled, melanised hyphae. Most isolates attributed to this variety originate from sugary or osmotically fluctuating habitats, such as saline water in the salterns, tree slime flux, fruit surfaces and phyllosphere (Table 1). This well supported variety was obtained pan-globally from temperate to tropical habitats, and was also found trapped in Spitsbergen glaciers and in ice released from these glaciers into the sea water. Its distribution is wide, ranging from the Arctic to the Mediterranean coast. Given the small degree of diversity with *TUB, ELO* and *EF1α*, the taxon can be regarded as being relatively recent.

Group 2, *A. pullulans* var. *melanogenum* contains CBS 105.22, the ex-type strain of this variety, and an authentic strain, CBS 123.37 with the invalid description of *Torula schoenii* Roukhelman. Earlier data (Yurlova *et al.* 1995) suggested that this taxon cannot be distinguished from var. *pullulans*, but current sequence data show that the groups are strictly concordant. Cultures are characteristically black from the beginning. They produce an abundance of dark, ellipsoidal conidia, which can either originate from disarticulating hyphae (arthroconidia) or transfer from hyaline conidia. The hyaline conidia are ellipsoidal and emerge from inconspicuous scars alongside undifferentiated hyphae; the process of conidiogenesis is synchronous in addition to percurrent, the latter being identical to that in the anamorph genus *Hormonema*. The sources of isolation of the strains of this variety, as far as is known, are low-nutrient, mostly low-strength environments, such as moist metal and glass surfaces, showers, fountains, as well as ocean water. Only one strain of this variety was retrieved from a human patient, but it is also possible that this was a culture contaminant, since it is often reported in air, especially in warmer climates (Punnapayak *et al.* 2003). Strains of this variety have a world-wide distribution, from the Arctic to the tropics. Given its marked diversity with *TUB*, *ELO* and *EF1α*, this may be an ancestral taxon, the introns having accumulated more mutations than var. *pullulans*.

Group 3, *A. pullulans* var. *subglaciale* Zalar *et al.* is exclusively known from Kongfjorden glacial and subglacial ice and sea water. Its psychrotolerant nature is in line with its active metabolism under conditions of permanently cold in Arctic glaciers.

Group 4 consists of a single isolate, CBS 147.97, the ex-type of the monotypic variety *A. pullulans* var. *namibiae*, isolated from marble in Namibia, Africa. The strain takes an isolated position with all sequenced genes, but has not drifted far away from the ancestral variety.

Other related groups are 5 and 6, which are aureobasidium-like but consistently different. Strains of these groups thus far have only been recovered from glacial ice in Spitsbergen. The species occurred with very high densities in subglacial ice in microchannels, and in gypsum-rich ice at high pH. During their travel through the glacier, these cells have been subjected to extreme variations in a_w due to ice freezing and thawing. These conditions are highly selective, for which reason this entity is likely to be restricted to small endemic areas, such as Kongsfjorden (Skidmore *et al*. 2005). The description as novel species will be the subject of a later paper.

The overall phylogenetic structure of *A. pullulans* suggests that the species is strictly clonal. A possible teleomorph, *Discosphaerina fagi*, has been suggested on the basis of ITS sequence similarity (de Hoog *et al.* 2000), but this finding awaits confirmation with multilocus analysis and re-isolation from single ascospores.

The varieties of *Aureobasidium pullulans* are markedly different for melanin production. This can be of biotechnological interest, since the organism is highly significant for its pullulan and aubasidan production (Yurlova & de Hoog 1997). Melanin contamination leads to low pullulan quality. Attempts have been made to grow non-pigmented yeast cells, *e.g.* by culturing *A. pullulans* in a two-stage fermentation process in media with a special nutrient combination (Shabtai & Mukmenev 1995), or with melanin-deficient mutants (Gniewosz & Duszkiewicz-Reinhard 2008). From the present study, it is apparent that the use of strains of the variety *pullulans* is recommended.

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The influence of ortho- and para-diphenoloxidase substrates on pigment formation in black yeast-like fungi

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Abstract: Dothideaceous black yeast-like fungi (BYF) are known to synthesise DHN-melanin that is inhibited by the systemic fungicide tricyclazole. The final step of the DHN melanin pathway is the conjoining of 1,8-DHN molecules to form the melanin polymer. There are several candidate enzymes for this step, including phenoloxidases such as tyrosinase and laccases, peroxidases, and perhaps also catalases. We analysed the type polyphenoloxidases that are involved in biosynthesis of BYF melanins. For that purpose we used substrates of o-diphenoloxidases (EC 1.10.3.1.): 4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, pyrocatechol, 3,4-dihydroxyphenylalanine and homogentisic acid, as well as substrates of p-diphenoloxidases (EC 1.10.3.2.): syringaldazine, resorcinol, p-phenylenediamine, phloroglucinol, guaiacol and pyrogallic acid. Fourteen strains of black yeasts originating from different natural biotopes were investigated. The tested strains could be divided into four groups based on their ability to produce dark pigments when cultivated on aromatic substrates of o- and on p-diphenoloxidases. It was established that syringaldazine, pyrogallic acid and 4-hydrophenyl-pyruvic acid, β-phenyllactic acid optimally promote melanin biosynthesis. Average intensity of pigmentation of all strains studied was minimal when guaiacol was used as a substrate. The present investigation indicates that the melanisation process may involve more enzymes and more substrates than those commonly recognised. Black yeasts are likely to contain a multipotent polyphenoloxidase.

Key words: Black yeast-like fungi, *Dothideales*, dothideaceous black yeasts, 1,8-dihydroxynaphthalene-melanin, phenoloxidases, o-diphenoloxidases, p-diphenoloxidases.

INTRODUCTION

Black yeast-like fungi (BYF) are either of basidiomycetous or ascomycetous relationship. The basidiomycetes are classified in the genera *Moniliella* and *Trichosporonoides*, of which a precise phylogenetic position has as yet not been established. Most species of these genera are of industrial significance and are rarely seen in clinical practice. In the ascomycete order *Chaetothyriales,* mainly comprising the family *Herpotrichiellaceae,* the genus *Exophiala* is the preponderant yeast-like anamorph (de Hoog *et al*. 2000). The order contains numerous human pathogens, with a wide spectrum of clinical pictures (Vitale & de Hoog 2002, de Hoog *et al*. 2005). The majority of these infections are cutaneous or mild pulmonary, but rarely they may be devastating and fatal. These infections are very difficult to treat because *in vivo* the species are frequently more resistant antimycotics than *in vitro* (Vitale & de Hoog 2002, de Hoog *et al.* 2005). The pathology of these black yeasts and their relatives is poorly understood (de Hoog *et al*. 2000, 2005).

In contrast, the ascomycete order *Dothideales* (anamorph genus *Aureobasidium* and its relatives) mainly comprises saprobic fungi, which are only exceptionally involved in human disease. *Aureobasidium pullulans* is industrially important because of its production of extracellular polysaccharides (EPS), which are applied in biotechnology (Deshpande *et al*. 1992). The EPS concerned comprise pullulan, a poly-α-1,6-maltotriose, and aubasidan, a related glucan with α-1,4-D, β-1,6-D and β-1,3-D-glycosidic bonds. A separate variety, *Aureobasidium pullulans* var. *aubasidani* was described for the strains producing aubasidan-like components (Yurlova & de Hoog 1997).

Dothidealean black yeast-like fungi were found to be predominant in soils highly contaminated with radionuclides emitted during the Chernobyl accident (Zhdanova *et al.* 1994, 2007). They play an important role in blackening of rock and architectural surfaces, in the destruction of marble and limestone (Sterflinger & Krumbein 1995, 1997). The fungi show active growth in extreme ecological niches, surviving low humidity, high temperature, high solar irradiation, presence of long lived radionuclides, and absence of traditional sources of nutrition and energy. The presence of melanin pigments, which possess a wide protective action, provides the dark-coloured fungi a competitive advantage under harsh environmental conditions. The pigments contain stable organic free radicals (Lyakh 1981). Fungal melanins may occur as electron-dense granules located in the fungal cell wall, polymers in the cytoplasm, as extracellular polymers in the medium surrounding the fungus, or in any combination (Butler & Day 1998). In the scientific literature there is information on biological activity of melanins as radioprotectors, antitumor remedies and as growth stimulators of plant seeds (Lyakh 1981). It had been suggested that these pigments might be useful as topical sunscreens and sunlight-protective coatings for plastics. DOPA melanins (of animal and biotechnological origin) have also been recommended for use in cosmetics (Della-Cioppa *et al.* 1990).

Melanins are produced by a variety of higher organisms including humans, but microbes are the melanin producers of choice in biotechnology. Melanin harvest from mammalian tissues sometimes may reach up to 8–10 mg/kg of raw material, while that of fungi can be 100–1000 times higher (Lyakh 1981). Differences were established between the absorption spectra of

Table 1. Strains examined.

Species	Accession no.	Source
Aureobasidium pullulans	CBS 105.22 = ATCC 11942 = VKM F-179	T of *Pullularia fermentans* var. *fusca*
	VKPM F-370	Metallic equipment
	VKPM F-371	Metallic equipment
	VKM F-1125	Fruitbody of *Inonotus obliquus*
	VKM F-2204	Lake water, Latvia
	VKM 2205	Lake water, Yaroslav region, Russia
	SPChPhA 129(11)	Unknown
	SPChPhA 2320	Soil, Chernobyl district, Ukraine
Aureobasidium pullulans var. *aubasidani* T	VKPM F-448 = CBS 100524	Birch sap, *Betula* sp., Russia
Hormonema macrosporum T	VKM F-2452 = CBS 536.94	*Rutilus rutilus*, Vologda region, Russia
Hormonema dematioides	VKM F-2836	Fruit body of *Mycena* sp., Moscow region, Russia
Kabatiella lini T	CBS 125.21	Leaf, *Linum usitatissimum*
Exophiala nigra T	VKM F-2137 = CBS 535.94	T of *Nadsoniella nigra*, seawater
Exophiala prototropha T	CBS 534.94	Unknown

Abbreviation used: T = ex-type strain, CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; SPChPhA = St. Petersburg State Chemical-Pharmaceutical Academy, St. Petersburg, Russia; VKM = All-Russian Collection of Microorganisms, Pushchino, Russia; VKPM = All-Russian Collection of Industrial Microorganisms, Moscow, Russia.

black yeast melanins and commercial Sepia melanin, which have been kept in the dark and photomodified by daylight irradiation. These data indicate perspectives of some black yeast melanins as photoprotectors and stimulators of skin regeneration (Blinova *et al.* 2003, Turkovskij & Yurlova 2002). Some black yeast melanins have higher UV-defensive activity than commercial melanins and they stimulate human skin regeneration significantly (Yurlova 2001, Turkovskij & Yurlova 2002). The photochemical properties of these melanins were found to be dependent on both the producing strain and the condition of its cultivation.

Melanins are high-molecular weight pigments formed by the oxidative polymerisation of phenolic compounds. The phenolic compounds from which the fungal melanin polymers are derived include tyrosine via 3,4-dihydroxyphenylalanine (DOPA) in various fungi and other microorganisms; γ-glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol in Basidiomycetes, and 1,8-dihydroxynaphthalene (DHN) in Ascomycetes (Bell & Wheeler 1986). Dothideaceous species that have been found to synthesise DHN-melanin include *Aureobasidium pullulans* (Siehr 1981), *Cladosporium cladosporioides* (Latgé *et al.* 1988), *Hortaea werneckii, Phaeotheca triangularis,* and *Trimmatostroma salinum* (Kogej *et al*. 2004). Chaetothyrialean species include *Cladophialophora carrionii, C. bantiana*, *Exophiala jeanselmei* and *E. mansonii* (Taylor *et al*. 1987). The authors mentioned above used the inhibitor tricyclazole to test the fungi for the presence of 1,8-dihydroxynaphthalene (DHN)-melanin biosynthesis.

The final step in the DHN melanin pathway is the conjoining of 1,8-DHN molecules to form the melanin polymer. There are a number of candidate enzymes for this step, including phenoloxidases such as tyrosinase and laccases, peroxidases, and perhaps also catalases (Butler & Day 1998). DHN appears to be polymerised to melanin via a laccase but not much is know about this enzyme and its function in the melanin pathway (Bell & Wheeler 1986). The aim of the present study was to analyze the influence of ortho- and para-diphenoloxidase substrates on pigment formation in black yeasts and to determine the type polyphenoloxidases that are involved in biosynthesis of black yeast melanins.

MATERIALS AND METHODS

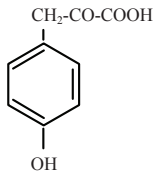
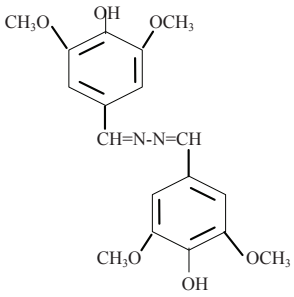
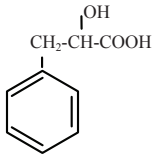
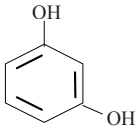
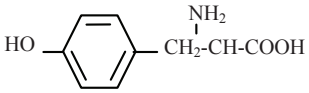
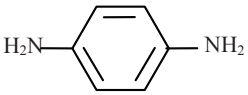
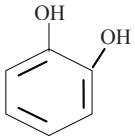
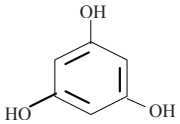
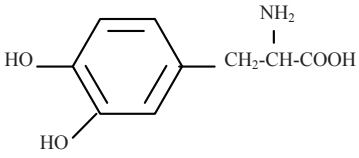
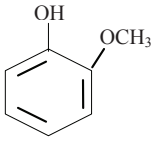
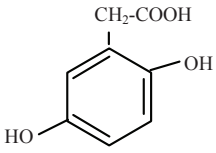
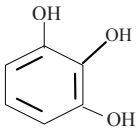
Diphenoloxidase substrates

Stock cultures (Table 1) were maintained on 2 % malt extract agar (MEA) slants. The low molecular weight aromatic compounds tested are listed in Table 2. The formation of melanin from low molecular weight aromatic compounds was determined by a modified auxanographic technique in which plates of Czapek agar (CzA) (in 90 × 15 mm Petri dishes) were divided in half diametrically (Fig. 1). One side of the plate was spread with a suspension of seven-day-old culture cultivated on 2 % MEA at 24 °C. Simultaneously three substrate assay cups were placed on each side. Each cup on each side received 0.1 mL of a solution of aromatic substrate (Table 2) in 0.1 M phosphate buffer (pH 7.0 or 7.2). The other half of the plate served as control for spontaneous oxidation of aromatic compounds. Plates were incubated at 24 °C and observed at intervals for 1 to 7 ds for development of a black-brown colour. The intensity of growth and pigmentation was estimated visually, and the intensity of growth and pigmentation of strain *Aureobasidium pullulans* CBS 105.22 = VKM F-179 (T) cultivated on 4 % MEA was listed as 100 %. The intensity of the pigmentation was represented according to five-grade scale: 100 % (black), 75 % (dark-brown, dark olive-green or dark grey), 50 % (brown or grey), 25 % (light brown or green-brown), and 0 % (yellow or white or pinkish).

Tricyclazole inhibition

Each fungus listed in Table 1 was grown in 90 × 15 mm Petri dishes containing 4 % MEA with tricyclazole, CzA with tricyclazole, and on 4 % MEA (control), and CzA (control). Tricyclazole was first dissolved in 100 % ethanol and then added to cooled medium prior to solidification to produce a concentration of 10–50 μg/mL. The final concentration of ethanol was 1.0 %. Control cultures were established on 4 % MEA and CzA which received only 1.0 % ethanol. All media were adjusted to pH 7.5 prior to dispensing.

Table 2. Substrates of diphenoloxidases tested.

Substrates of o-diphenoloxidases (EC 1.10.3.1.)	Concentration, mM/ml	Substrates of p-diphenoloxidases (EC 1.10.3.2.)	Concentration, mM/ml
4-Hydroxyphenyl-pyruvic acid 	0.02	Syringaldazine 	0.05
L-β-Phenyllactic acid 	0.02	Resorcinol 	0.05
Tyrosine 	0.02	p-Phenylenediamine 	0.05
Pyrocatechol 	0.05	Phloroglucinol 	0.05
3,4-Dihydroxyphenylalanine 	0.005	Guaiacol 	0.05
Homogentisic acid 	0.02	Pyrogallic acid 	0.05

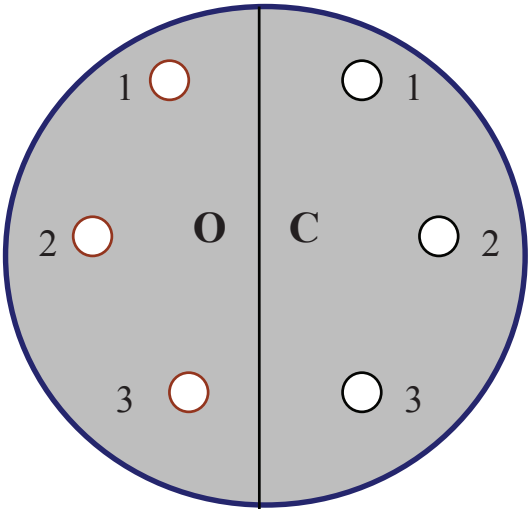


Fig. 1. The effect of the aromatic substrates on pigment production by strains studied. The left half only of the plate was inoculated (O), the right half serving as a control (C) for spontaneous oxidation of aromatic substrates. Cups 1, 2, 3 contained 0.1 mL of a solution of aromatic substrates.

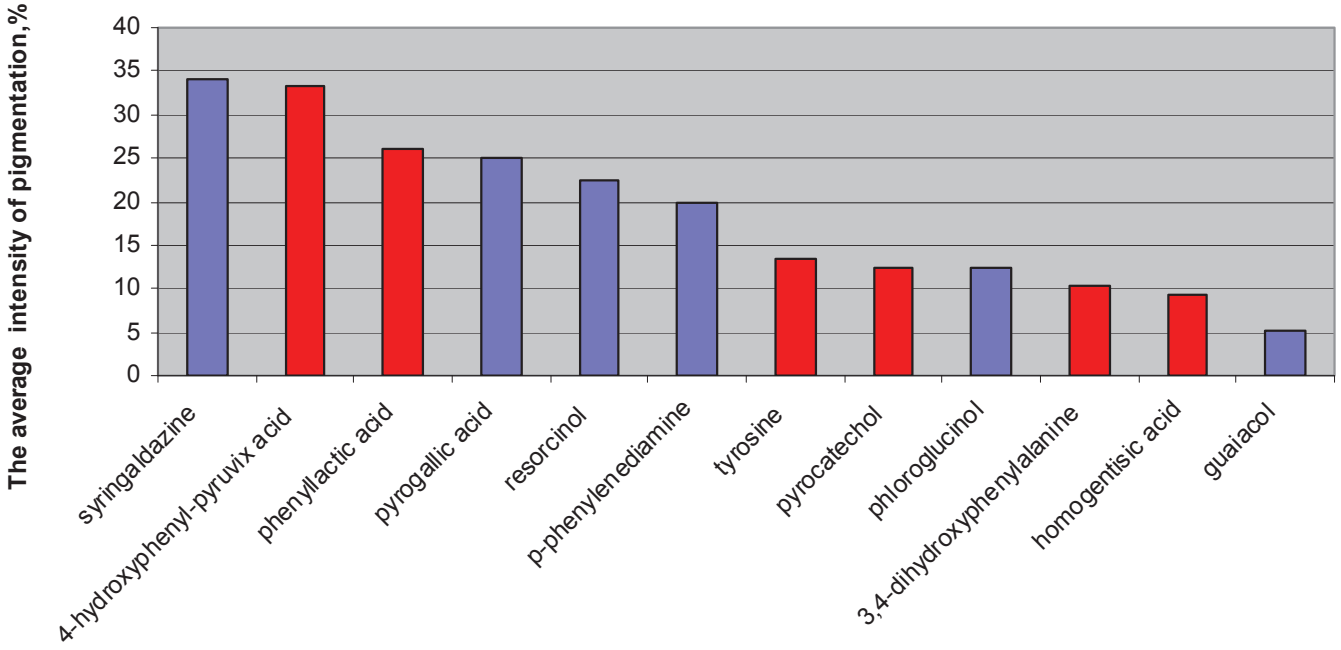


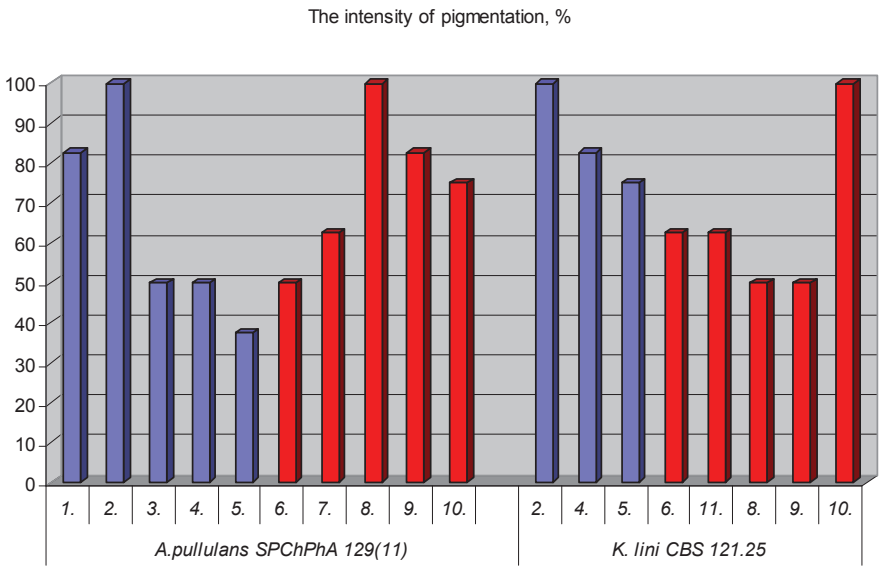
Fig. 2. The average intensity of pigmentation of some strains by presence of different phenolic substracts.
Substrates of o-diphenoloxidases (EC 1.10.3.1.): 4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, pyrocatechol, 3,4-dihydroxyphenylalanine and homogentisic acid are indicated in red colour.

Point inoculation of each fungus was made centrally on the plate in Petri dishes (inoculation was made by a suspension of seven-day-old culture cultivated on 2 % MEA at 24 °C). The cultures were grown in the dark at 24 °C for 21 d. The intensity of growth and pigmentation was estimated visually, and the intensity of growth and pigmentation of strain *Aureobasidium pullulans* CBS 105.22 = VKM F-179 cultivated on 4 % MEA was accepted as 100 %. All tests were performed three times in duplicate.

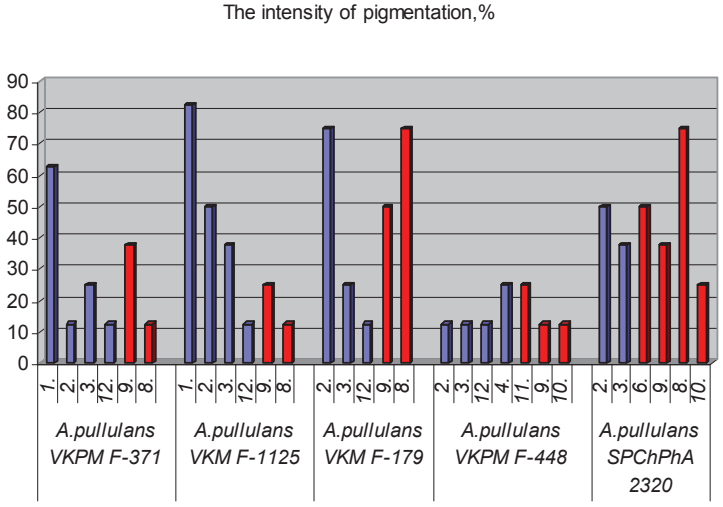
Thin-layer chromatography (TLC)

Fourteen-day-old Petri dish cultures of *A. pullulans* VKM F-179 = CBS 105.22, *A. pullulans* VKM F-370, *A. pullulans* VKPM F-371, *A. pullulans* var. *aubasidani* VKPM F-448, grown on CzA with (10–50 μg/mL) or without tricyclazole, were cut into small fragments (about 1 cm^3) and extracted in 150 mL acetone for 8 h. The extracts were subsequently filtered, evaporated under reduced pressure and the remaining aqueous solutions extracted twice with equal volumes of ethyl acetate. The ethyl acetate fractions were collected, combined, and residual water was removed over $NaSO_4$. After the ethyl acetate was evaporated under reduced pressure, each sample was reconstituted with 1 mL of ethyl acetate to provide concentrated solutions for chromatographic evaluation (Taylor *et al*. 1987, Kogej *et al*. 2004). The concentrated extracts and the standards of flaviolin, 2-hydroxyjuglone (2-HJ), scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-THN), 1,8-dihydroxynaphthalene were spotted on silica gel-coated TLC plates with fluorescent indicator (Merck) and developed with ether-hexane-formic acid (60:39:1). Once separated, metabolites from the extracts were observed in daylight and under ultraviolet (UV) light at 254 and 365 nm for characteristic colours and R_f values. The plates were then sprayed with an aqueous solution of 1 % $FeCl_3$. Once they were dried, they were again evaluated for colours that appeared in daylight (Taylor *et al*. 1987, Kogej *et al*. 2004).

A



B



C

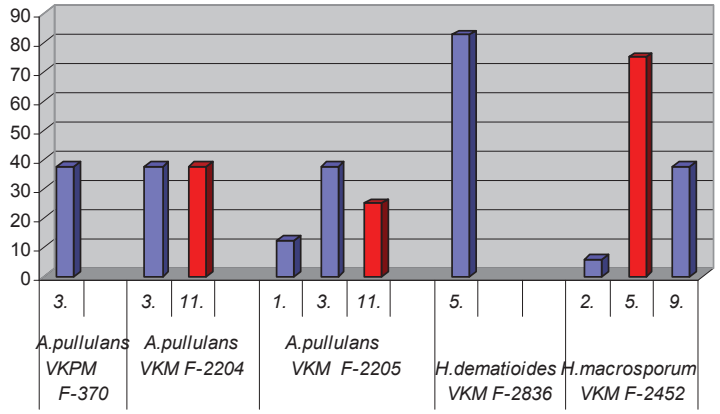


Fig. 3. The intensity of pigmentation of some strains cultivated on media with different aromatic substrates.
Y-axis: intensity of pigmentation, %.
A. Second group; B. Third group; C. Fourth group.
The intensity of pigmentation of strains *Exophiala nigra* F-2137 = CBS 535.94 (T) and *E. prototropha* CBS 534.94 (first group), when cultivated on all 12 aromatic substrates used, was listed as 100 %.
Substrates of o-diphenoloxidases (EC 1.10.3.1.): 4-hydroxyphenyl-pyruvic acid, L- β-phenyllactic acid, tyrosine, pyrocatechol, 3,4-dihydroxyphenylalanine and homogentisic acid are indicated in red colour.

1. phenylenediamine;
2. syringaldazine;
3. pyrogallic acid;
4. phloroglucinol;
5. resorcinol;
6. tyrosine;
7. 3,4-dihydroxyphenylalanine;
8. L-β-phenyllactic acid;
9. 4-hydroxyphenyl-pyruvic acid;
10. homogentisic acid;
11. pyrocatechol;
12. guaiacol.

RESULTS

Fourteen strains of BYF originating from different natural biotopes were investigated (Table 1). It was established that syringaldazine, pyrogallic acid (substrates of p-diphenoloxidases) and 4-hydrophenyl-pyruvic acid, L-β-phenyllactic acid (substrates of o-diphenoloxidases) optimally promoted melanin biosynthesis when compared to other groups of substrates investigated. Average intensities of pigmentation of all strains studied were the lowest when guaiacol (substrate of p-diphenoloxidases) was used as a substrate (Fig. 2).

Diphenoloxidase substrates

Strains investigated were divided into four groups based on their ability to produce dark pigments when they were cultivated on aromatic o- and on p-diphenoloxidase substrates (Fig. 3).

Group 1. *Exophiala nigra* VKM F-2137 and *E. prototropha* CBS 534.94 produced black pigments when they were cultivated on all 12 aromatic substrates used, including o- and on p-diphenoloxidase substrates. The intensity of pigmentation of strains *Exophiala nigra* F-2137 = CBS 535.94 and *E. prototropha* CBS 534.94, cultivated on each of the 12 aromatic substrates, was listed as 100 %.

Group 2. Strains utilising 8−10 aromatic substrates and synthesising dark pigments (Fig. 3A). This group includes two strains. *A. pullulans* SPChPhA 129(11), growing and synthesizing black or dark brown or brown pigments when five substrates of o-diphenoloxidases (4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, 3,4-dihydroxyphenylalanine, homogentisic acid) and five substrates of p-diphenoloxidases (syringaldazine, resorcinol, p-phenylenediamine, phloroglucinol, pyrogallic acid) were used for cultivation. *Kabatiella lini* CBS 125.21 produced black or dark-brown or brown pigment when five substrates of o-diphenoloxidases (4-hydroxyphenyl-pyruvic acid, L-β-phenyllactic acid, tyrosine, pyrocatechol, homogentisic acid) and three substrates of p-diphenoloxidases (syringaldazine, resorcinol, phloroglucinol) were used for cultivation.

Group 3. Strains utilising 5−7 aromatic substrates and synthesizing dark pigments when cultivated on CzA with aromatic substrates (Fig. 3B). This group includes mostly strains of *Aureobasidium pullulans*: VKPM F-371, VKM F-179, VKM F-1125 produced black or dark brown or dark olive-green or dark-grey, brown or grey, light brown or green-brown pigments on two substrates of o-diphenoloxidases (L-β-phenyllactic acid, 4-hydroxyphenyl-pyruvic acid) and on four substrates of p-diphenoloxidases (p-phenylenediamine, syringaldazine, pyrogallic acid, guaiacol). Strain *A. pullulans* var. *aubasidani* VKPM F-448 produced brown or light brown or green brown pigment on three substrates of o-diphenoloxidases (4-hydroxyphenyl-pyruvic acid, pyrocatechol, homogentisic acid) and four substrates of p-diphenoloxidases (syringaldazine, pyrogallic acid, phloroglucinol, guaiacol). Strain *A. pullulans* SPChPhA 2320 formed pigment on four substrates of o-diphenoloxidases (tyrosine, L-β-phenyllactic acid, 4-hydroxyphenyl-pyruvic acid, homogentisic acid) and two substrates of p-diphenoloxidases (syringaldazine, pyrogallic acid).

Group 4. Strains synthesizing dark pigments only on 1−3 aromatic substrates when cultivated on CzA with aromatic substrates (Fig. 3C). Strains included *A. pullulans* VKPM F-370 (light brown pigmentation) and *H. dematioides* VKM F-2836 (dark olive-green pigmentation), producing pigment only when substrates of p-diphenoloxidases (pyrogallic acid, resorcinol) were used. *Aureobasidium pullulans* VKPM F-2204 produced pigment of equal intensity (brown or light brown) on p-diphenoloxidases (pyrogallic acid) and on o-diphenoloxidases (pyrocatechol) substrates. Strain VKPM F-2205 gave light brown pigmentation on two substrates of p-diphenoloxidases (p-phenylenediamine, pyrogallic acid) and on pyrocatechol (substrate of o-diphenoloxidases).

Tricyclazole inhibition

Following the same subdivision:

Group 1. Tricyclazole had no apparent effect on growth of the black yeast strains belonging to the strains of this group, as was observed both on 4 % MEA and on CzA. The strains concerned were blackish or dark brown in colour, when grown on 4 % MEA containing 10–20 µL/mL tricyclazole, and on CzA containing 10–20 µL/mL tricyclazole. We observed reddish pigment only in Group 1 strains (*Exophiala nigra* VKM F-2137 and *E. prototropha* CBS 534.94) when we used higher (40−50 µg/mL) concentrations of tricyclazole. Other groups (Groups 2−4) of strains studied did not form reddish or red-brown pigments even they were cultivated in media with high (30−50 µg/mL) concentrations of tricyclazole.

Group 2. Tricyclazole had no apparent effect on growth and pigmentation of *A. pullulans* SPChPhA 129(11), when grown on 4 % MEA. It slightly inhibited the growth of this strain on CzA and had no effect on pigmentation (Fig. 4). The plant pathogen *K. lini* CBS 125.21 was inhibited by tricyclazole on 4 % MEA and CzA. The intensity of pigmentation was decreased almost in two times, when *K. lini* CBS 125.21 was grown on CzA with tricyclazole (Fig. 4).

Group 3. The growth of the strains belonging to this group was slightly inhibited by tricyclazole both on 4 % MEA and on CzA. Tricyclazole affected intensity of pigmentation of *A. pullulans* VKM F-179, VKM F-1125, SPChPhA 2320, *A. pullulans* var. *aubasidani* VKPM F-448 on CzA. On 4 % MEA an effect was found on pigmentation of only *A. pullulans* var. *aubasidani* VKPM F-448 (Fig. 5).

Group 4. Tricyclazole had no apparent effect on growth of the strains *A. pullulans* VKM F-2204, VKM F-370 of this group, both on 4 % MEA and on CzA (Fig. 6). The intensity of pigmentation of the strains VKM F-2204, VKM F-370 grown on 4 % MEA with tricyclazole was almost the same as on 4 % MEA without tricyclazole. The strains *A. pullulans* VKM F-2204, VKM F-2205, VKM F-370 and *Hormonema macrospora* VKM F-2452 were yellow or light yellow or pinkish, when grown on CzA with or without tricyclazole. *Hormonema dematioides* VKM F-2836 did not grow at all on CzA (Fig. 6). Microscopic comparisons indicated that all strains studied had their normal morphologies in the presence of tricyclazole.

Identification of DHN-melanin intermediates

Metabolites from ethyl acetate extracts of *A. pullulans* VKM F-179 = CBS 105.22, VKM F-370, VKPM F-371 and *A. pullulans* var. *aubasidani* VKPM F-448 were analysed by TLC to determine if DHN-melanin precursors or related metabolites were present. Flaviolin/biflaviolin and 2-HJ were detected in the extracts of 14 ds old cultures of *A. pullulans* VKM F-370 and *A. pullulans* VKPM F-371 when they were grown with tricyclazole; however, they were not found in culture without tricyclazole (Table 3). The TLC results indicated that tricyclazole had blocked the DHN-melanin pathway, causing the accumulation of 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) and 1,3,8-THN, which were autoxidised to flavolin or 3,3-biflaviolin and 2-HJ, respectively (Table 3). Strains *A. pullulans*

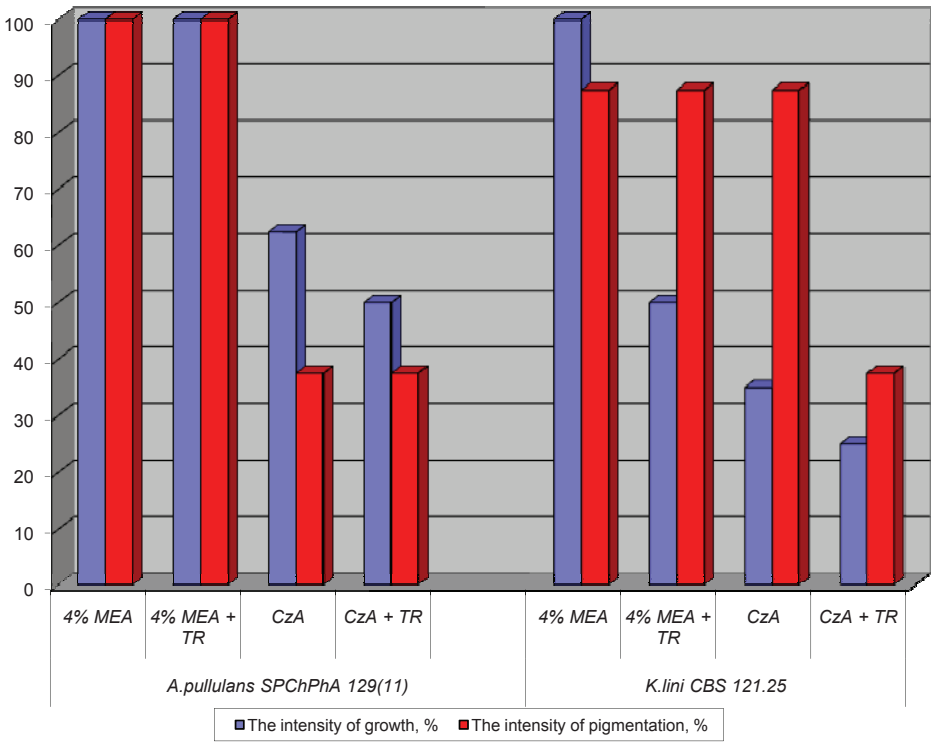


Fig. 4. The influence of tricyclazole on growth and pigmentation of strains belonging to the second group.
Y-axis: intensity of growth, intensity of pigmentation, %.
X-axis: 4 % MEA = 4 % malt extract agar; 4 % MEA +TR = 4 % malt extract agar with 20 µL/mL tricyclazole; CzA = Czapek agar; CzA + TR = Czapek agar with 20 µL/mL tricyclazole.

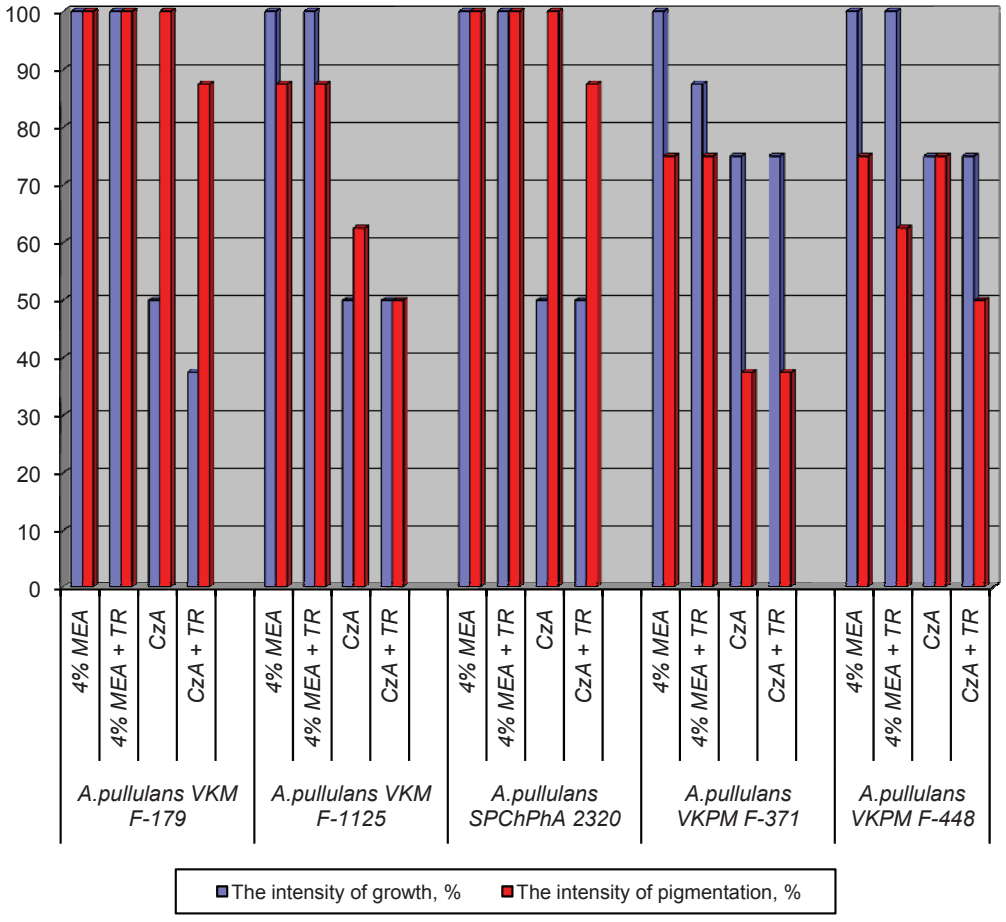


Fig. 5. The influence of tricyclazole on growth and pigmentation of strains belonging to the third group.
Y-axis: intensity of growth, intensity of pigmentation, %.
X-axis: 4 % MEA = 4 % malt extract agar; 4 % MEA +TR = 4 % malt extract agar with 20 µL/mL tricyclazole; CzA = Czapek agar; CzA + TR = Czapek agar with 20 µL/mLtricyclazole.

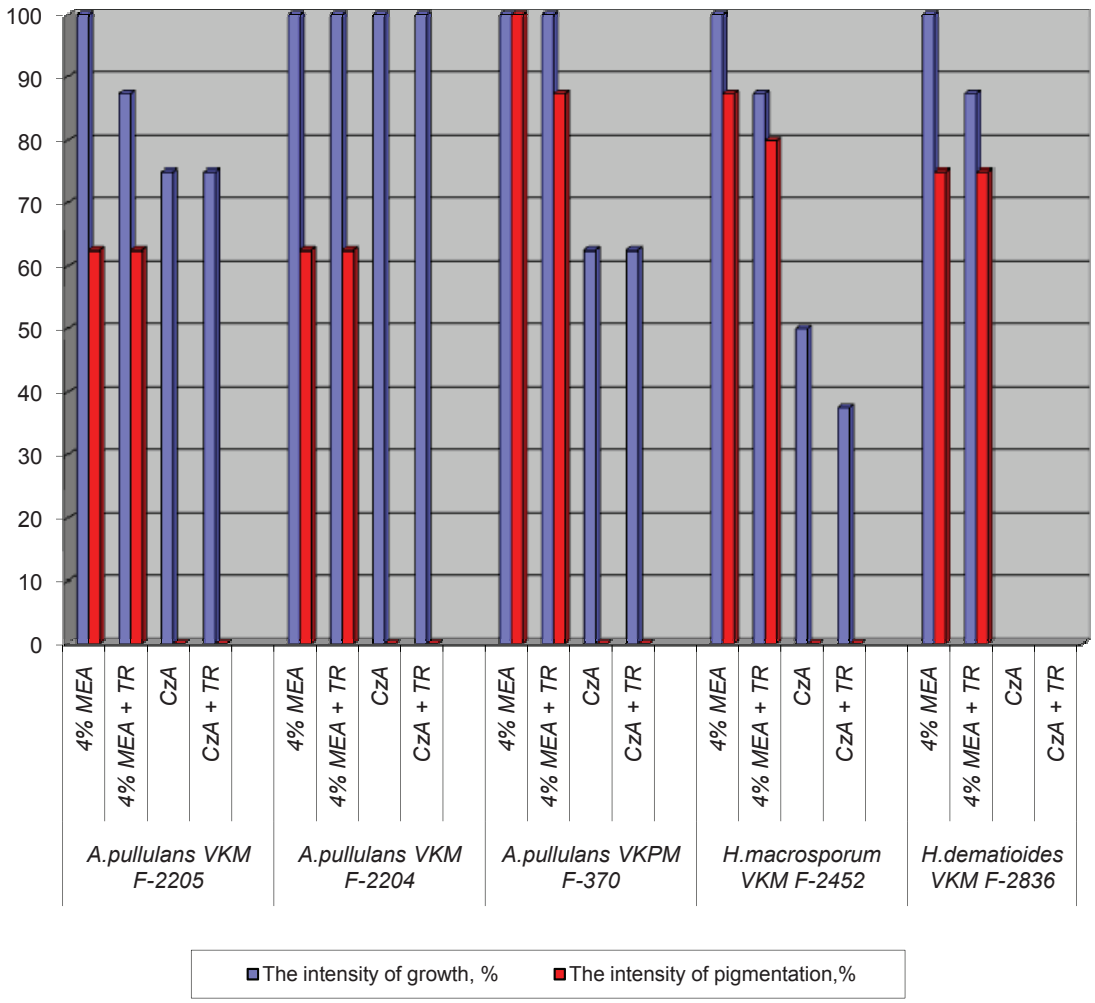


Fig. 6. The influence of tricyclazole on growth and pigmentation of strains belonging to the fourth group.
Y-axis: intensity of growth, intensity of pigmentation, %.
X-axis: 4 % MEA = 4 % malt extract agar; 4 % MEA + TR = 4 % malt extract agar with 20 µL/mL tricyclazole; CzA = Czapek agar; CzA + TR = Czapek agar with 20 µL/mL tricyclazole.

VKM F-179 and *A. pullulans* var. *aubasidani* VKPM F-448 did not secrete 2-HJ and flaviolin both in the presence of tricyclazole and without tricyclazole (Table 3).

DISCUSSION

Three out of four black yeast genera analysed (*Aureobasidium, Hormonema* and *Kabatiella*) (Table 1) belong to the ascomycetous order *Dothideales*, while *Exophiala* is an anamorph of *Chaetothyriales* (de Hoog *et al*. 1999). Many authors (Siehr 1981, Taylor *et al*. 1987, Butler & Day 1998, Butler *et al.* 2004, Kogej *et al.* 2004) indicated that both types of fungi synthesise a DHN-type melanin. Details of the DHN-type melanin pathway have been elucidated using a number of different fungi. Much of what is known about the pathway and its enzymes has come from the use of melanin-deficient strains and compounds, such as tricyclazole, which inhibit specific enzymes in the pathway (Bell & Wheeler 1986, Butler & Day 1998).

The systemic fungicide tricyclazole [5-methyl-1,2,4-thiazolo(3,4,b)-benzothiazile] (TR) is an inhibitor of biosynthesis of melanins, which form via the pentaketide pathway (Bell & Wheeler 1986). For example, it is known to strongly inhibit the enzymatic reduction (reductase enzymes) of 1,3,8-trihydroxynaphthalene (1,3,8-THN) to vermelone. Tricyclazole has also been shown to weakly inhibit the reduction of 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone (Wheeler & Greenblatt 1988).

Flaviolin and 2-hydroxyjuglone (2-HJ) are known as autoxidative products of 1,3,6,8-THN and 1,3,8-THN, respectively (Fig. 7). The presence of flaviolin and 2-HJ in fungal cultures, treated with tricyclazole, is usually accepted as proof that 1,3,6,8-THN and 1,3,8-THN were involved in the synthesis of DHN-melanin (Butler & Day 1998). Once produced, 1,3,8-DHN is reduced to vermelone, which in turn is dehydrated to 1,8-dihydroxynaphthalene (DHN) (Bell & Wheeler 1986, Taylor *et al*. 1987) (Fig. 7). In most cases, these two reactions are carried out by the same reductase and dehydratase enzymes that produce 1,3,8-THN from 1,3,6,8-THN. DHN appears to be polymerised to melanin via a laccase (Butler & Day 1998).

In the present investigation we demonstrated, that the DHN-melanin inhibitor, tricyclazole, inhibited melanin biosynthesis only in some black yeast strains. Four groups were distinguished, differing by their ability to produce pigment with o- and p-diphenoloxidase substrates and to be inhibited by tricyclazole (Table 4).

The effect of tricyclazole on pigment production proved to be more pronounced when strains were grown on CzA. On this medium 53.3 % of the strains were inhibited by tricyclazole, whereas only 26.6 % of the strains decreased their intensity of pigmentation

Table 3. Melanin metabolites analysed by TLC in control cultures and in tricyclazole-inhibited cultures of *A. pullulans* VKM F-179, VKM F-370, VKPM F-371 and *A. pullulans* var. *aubasidani* VKPM F-448.

Accession no.	Tricyclazole[1]	Metabolites[2]		
		2-HJ	flaviolin	3,3'-biflaviolin
VKM F-179	−	−	−	−
	+	−	−	−
VKM F-370	−	−	−	−
	+	+	+	−
VKPM F-371	−	−	−	−
	+	+	−	+
VKPM F-448	−	−	−	−
	+	−	−	−

[1]Tricyclazole concentration in the medium was 0 µg mL^{-1} (−) or 20 µg mL^{-1} (+).

[2] "+ " = metabolite was observed; "− " = metabolite was not observed.

VKM/VKPM = Russian Collection of microorganisms, Puschuno, Russia.

Table 4. Subdivision of the strains into pigmentation groups.

Strains	Pigmentation on substrates of		Influence of tricyclazole on intensity of			
	o-diphenoloxidases	p-diphenoloxidases	pigmentation		growth	
			4 % MEA	CzA	4 % MEA	CzA
Group 1 (12/12)[1]						
Exophiala nigra VKM F-2137	+	+	-	+	-	-
Exophiala. prototropha CBS 534.94	+	+	+	+	-	-
Group 2 (8-10/12)[2]						
A. pullulans SPChPhA 129(11)	+	+	-	-	-	+
K. lini CBS 125.21	+	+	-	+	+	+
Group 3 (5-7/12)[3]						
A. pullulans VKPM F-371	+	+	-	-	+	-
A. pullulans CBS 105.22	+	+	-	+	+	-
A. pullulans VKM F-1125	+	+	-	+	-	-
A. pullulans var. *aubasidani* VKPM F-448	+	+	+	+	-	-
A. pullulans SPChPhA 2320	+	+	-	+	-	-
Group 4 (1-3/12)[4]						
A. pullulans VKM F-2204	+	+	-	-	-	-
A. pullulans VKM F-2205	+	+	-	-	+	-
A. pullulans VKM F-370	-	+	+	-	-	-
H. dematioides VKM F-2836	-	+	-	*	+	*
H. macrosporum VKM F-2452	+	+	+	-	+	+

* *H. dematioides* F-2836 did not grow at all on CzA.

Abbreviations used: "+ " = characteristic was observed; "− " = characteristic was not observed.

[1]Strains produced black pigments when they were cultivated on all 12 aromatic substrates used: both on o-diphenoloxidases and on p-diphenoloxidases substrates;

[2]Strains produced dark pigments when they were cultivated on 8–10 aromatic substrates from 12 used: both on o-diphenoloxidases and on p-diphenoloxidases substrates;

[3]Strains produced dark pigments when cultivated on 5–7 aromatic substrates from 12 used: both on o-diphenoloxidase and p-diphenoloxidase substrates;

[4]Strains produced dark pigments when cultivated on 1–3 aromatic substrates from 12 used: both on o-diphenoloxidases and on p-diphenoloxidases substrates.

when they were cultivated on 4 % MEA with tricyclazole (Table 4). Metabolites from ethyl acetate extracts of biomass from *A. pullulans* VKM F-179 = CBS 105.22, VKM F-370, VKPM F-371 and *A. pullulans* var. *aubasidani* VKPM F-448 were analysed by thin-layer chromatography to determine if DHN-melanin precursors or related metabolites were present. Flaviolin and 2-HJ were detected only in the extracts of 14-d-old cultures of *A. pullulans* VKM F-370, *A. pullulans* VKPM F-371, when they were grown on CzA with 10 and 20 µg/mL tricyclazole. However, flaviolin and 2-HJ were not found in acetone extracts of biomasses of these strains, when they were grown in CzA without tricyclazole (Table 3). Strains *A. pullulans* VKM F-179, *A. pullulans* var. *aubasidani* VKPM F-448 did not secrete 2-HJ and flaviolin, neither in the presence of nor without tricyclazole (TR) (Table 3) and even when they were

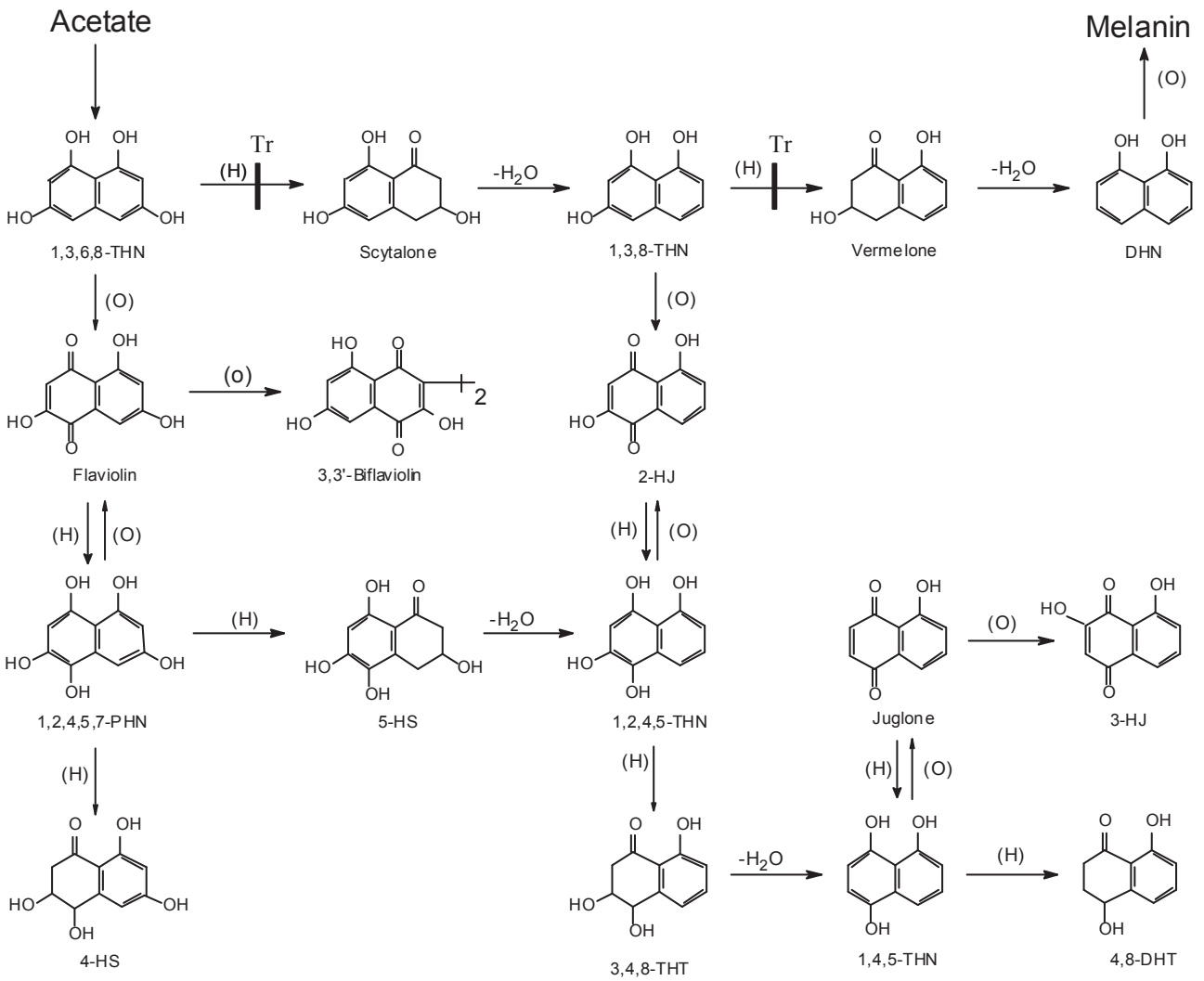


Fig. 7. Biosynthetic pathway of DHN-melanin and related pentaketide metabolites, from the scheme shown by Bell & Wheeler (1986). The first known product of the pathway is l.3.6.8-THN. This metabolite is reduced lo scytalone, which is then dehydrated to 1,3.8-THN. Next, 1.3.8-THN is reduced to vermelone, which is then dehydrated to DHN. The enzyme(s) that catalyze the final polymerization reaction, oxidation of DHN to melanin, have not yet been adequately studied but it appears to be a laccase. Tricyclazole (Tr) inhibits the reduction of 1.3.6,8-THN and 1.3.8-THN to scytalone and vermelone, respectively. Its strongest inhibitory effect is on the reduction of 1.3.8-THN. This results in the accumulation of flaviolin, 2-HJ, and their related shunt products, 1.2.4.5.7-pentahydroxynaphthalene (1.2.4.5.7-PHN), 1.2.4.5-tetrahydroxynaphthalene (1.2.4.5-THN), and 1.4.5-trihydroxynaphthalene (1.4.5-THN) are extremely unstable and have not been isolated from fungi.

cultivated in media with high (30−50 µg/mL) concentrations of tricyclazole. The halophilic ascomycetous black yeasts *Hortaea werneckii, Phaeotheca triangularis* and *Trimmatostroma salinum* accumulated 4,8-dihydroxytetralone (4,8-DHT) in cultures non-inhibited by TR (Kogej *et al*. 2004) (Fig. 7). Small amounts of 4-hydroxyscytalone (4-HS) (Fig. 7) have been reported in wild-type cultures of *Curvularia lunata* non-inhibited by TR (Rižner & Wheeler 2003), as well as of scytalone in *Thielaviopsis basicola* (Wheeler & Stipanovic 1979) and *Sporothrix schenckii* (Romero-Martinez *et al*. 2000). This means that products which are typical for cultures of black yeasts inhibited by tricyclazole (TR) were also found in non-inhibited cultures.

In our earlier investigations (Yurlova & Sindeeva 1996) we proved the presence of intracellular and extracellular laccase activity of 14 above mentioned strains of black yeasts. Tricyclazole decreased laccase activity (Yurlova & Sindeeva 1995). Tyrosinase, which oxidises tyrosine, was not found in any of the strains investigated (Table 1) (Yurlova & Sindeeva 1995). On the basis of the present data we hypothesise that black yeasts contain a multipotent polyphenoloxidase able to oxidise substrates characteristic for o-diphenoloxidases and p-diphenoloxidases. Such kind of multipotent polyphenoloxidase has previously been observed in the marine bacterium *Marinomonas mediterranea* (Fernandez *et al.* 1999). The melanisation process might involve other enzymes and more substrates than those commonly recognised. The mechanism of biosynthesis of black yeast melanins remains to be further elucidated.

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Expression of fatty-acid-modifying enzymes in the halotolerant black yeast *Aureobasidium pullulans* (de Bary) G. Arnaud under salt stress

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Abstract: Multiple tolerance to stressful environmental conditions of the black, yeast-like fungus *Aureobasidium pullulans* is achieved through different adaptations, among which there is the restructuring of the lipid composition of their membranes. Here, we describe three novel genes encoding fatty-acid-modifying enzymes in *A. pullulans*, along with the levels of their mRNAs under different salinity conditions. High levels of Δ^9-desaturase and Δ^{12}-desaturase mRNAs were seen at high salinities, which were consistent with an increased desaturation of the fatty acids in the cell membranes. Elevated levels of elongase mRNA were also detected. Surprisingly, increases in the levels of these mRNAs were also seen following hypo-osmotic shock, while hyperosmotic shock had exactly the opposite effect, demonstrating that data that are obtained from up-shift and down-shift salinity studies should be interpreted with caution.

Key words: *Aureobasidium pullulans*, desaturase, elongase, extremotolerance, halotolerance, salt stress.

INTRODUCTION

The salinisation of irrigated land has become a major agricultural problem, while attempts to breed crops with increased salt tolerance have still not yielded satisfactory results. Studies of the basic adaptation mechanisms of halophilic and halotolerant organisms, and particularly of eukaryotic ones, should help in the breeding of such crops in the future.

Aureobasidium pullulans (de Bary) G. Arnaud (*Dothideaceae, Ascomycota*) is a ubiquitous, saprophytic, black, yeast-like fungus. It is often found in the phyllosphere (Andrews *et al.* 1994), the air (Lugauskas *et al.* 2003) and many other, often extreme, environments, such as hypersaline water in solar salterns around the World (Gunde-Cimerman *et al.* 2000) and Arctic glacier ice (Gunde-Cimerman *et al.* 2003). *A. pullulans* is of great biotechnological interest because of its production of extracellular pullulan (Leathers 2003), and is also a well known pathogen that can cause a variety of localised, and rarely even, systemic infections (Hawkes *et al.* 2005). *A. pullulans* thrives under many different environmental conditions, and it can tolerate a variety of environmental stresses. It can grow from 4 °C up to 35 °C, and although it thrives best without NaCl, it can tolerate up to 17 % NaCl in its growth medium; it even shows an upward shift in its salinity optimum at high temperatures (Torzilli *et al.* 1985, Zalar *et al.* 1999, Gostinčar unpublished data).

Cell membranes have crucial roles in the adaptation of any organism to environmental extremes, such as low temperature and high salinity. The active restructuring of the membrane lipid composition that can occur in response to environmental changes preserves the suitable dynamic state of the membrane bilayer and restores membrane function following environmental insult (Hazel & Williams 1990). This restructuring of the membrane lipid composition in response to environmental cues can occur either through changes in the products of cell lipid biosynthesis, or through the selective degradation of lipid species with inappropriate properties (Hazel & Williams 1990).

Such cell membrane changes have been particularly well studied in salt-sensitive *Saccharomyces cerevisiae*. As in other eukaryotes, the *de-novo* biosynthesis of saturated fatty acids in these yeast requires acetyl-CoA carboxylase and the fatty-acid-synthase complex. Yeast are only able to synthesise mono-unsaturated fatty acids containing a Δ^9 double bond, using a desaturase that is encoded by the *OLE1* gene, the expression of which is severely repressed by unsaturated fatty acids (Trotter 2001). Regulation of this gene's expression occurs at the level of *OLE1* transcription and via mRNA stability (Gonzalez & Martin 1996). In *S. cerevisiae*, several different enzyme systems have been described that can elongate the fatty-acyl-CoAs that are formed from *de-novo* synthesis or are acquired from the growth medium, some of which are essential for cell viability (Trotter 2001).

The halotolerant *A. pullulans* has so far been studied only at the level of its membrane composition and fluidity when exposed to hypersaline conditions (Turk *et al.* 2004, 2007). The levels of unsaturated fatty acids in the cell membranes of *A. pullulans* cells grown in 5 % and 10 % NaCl increase significantly due to their enrichment in C18:2$^{\Delta 9,12}$ fatty acids. Significant changes in the lengths of the fatty acids were not seen, although such changes have been detected in another black, yeast-like fungus, the halophilic *Hortaea werneckii* (Horta) Nishim. & Miyaji (Turk *et al.* 2004). Although *A. pullulans* is more sensitive to salt than *H. werneckii* (but less so than *S. cerevisiae*), it is of interest because of its multiple tolerances to many other types of stress.

The aim of the present study was to find desaturase and elongase genes that are involved in these fatty-acid modifications in *A. pullulans*, to compare them to other known homologous enzymes from different fungi, and to study their differential expression under saline and non-saline conditions. In particular,

we wanted to determine whether these changes in the fatty-acid saturation levels can be explained by the differential expression of the genes encoding desaturases, and whether the expression of the elongase gene changes in cells subjected to salt stress.

MATERIALS AND METHODS

Strain and culture conditions

The EX-F150 strain of *A. pullulans* was originally isolated from the Sečovlje solar salterns (Slovenia) (Gunde-Cimerman *et al.* 2000) and has been maintained in the Extremophilic Fungi (Ex) Culture Collection of the Department of Biology, Biotechnical Faculty, University of Ljubljana (Slovenia), and as MZKI B-802 in the Microbial Culture Collection of the National Institute of Chemistry (Slovenia). *A. pullulans* was grown using the standard Yeast Nitrogen Base (YNB) chemically defined liquid medium, both without NaCl and with different concentrations of NaCl (2.5 %, 5.0 %, 7.5 %, 10.0 % and 13.0 %; w/v). The liquid cultures were grown at 28 °C and under constant shaking (180 rpm).

DNA and RNA isolation

For DNA isolation, *A. pullulans* was grown in YNB, and the biomass was harvested by centrifugation in the mid-exponential growth phase (OD_{600} = 0.8–1.0). The cell pellet was frozen in liquid nitrogen and homogenised using a mortar and pestle. The DNA was then isolated according to the protocol described by Rozman & Komel (1994).

For RNA isolation, *A. pullulans* was cultured in YNB with different concentrations of NaCl; the biomass was harvested by centrifugation in the mid-exponential growth phase (OD_{600} = 0.8–1.0). The cells that were subjected to osmotic shock were initially grown in YNB without NaCl or with 10 % NaCl (w/v). These cells were also harvested in the mid-exponential growth phase, and then subjected to osmotic shock by resuspension in YNB with 10 % NaCl or without NaCl, respectively (at the same pH and temperature) for specific times (5–120 min); they were then harvested by filtration. Adapted cells that were grown constantly at the final concentration of NaCl were used as an additional end-point reference. The biomass was frozen in liquid nitrogen and stored at –80 °C until further analysis, when they were homogenised in liquid nitrogen using a mortar and pestle. The RNA was isolated using TRI REAGENT™ (Sigma), according to the manufacturer instructions. Possible contaminating DNA was degraded with deoxyribonuclease I (Fermentas). The integrity and purity of the RNA was evaluated spectrometrically and with formaldehyde agarose electrophoresis.

Amplification and sequencing of genes

Partial sequences of the genes were amplified by polymerase chain reaction (PCR), which was performed as described by Lanišnik Rižner *et al.* (1999). In all cases, the annealing temperatures were 60 °C. Non-degenerate oligonucleotide primers were constructed on the basis of highly conserved domains in known fungal desaturases: 5'- TAC ACC GAT ACC GAC AAG GAC CCC TA-3' and 5'-GGA ACT CGT GGT GGA AGT TGT GGT A-3' for Δ^9-desaturase, and 5'-CCA TCA AGG AGA TCC GTG ATG CCA T-3' and 5'-ATG TTA CCA GTG GCC TTG TGG TGC T-3' for Δ^{12}-desaturase. Primers specific for part of the elongase gene were designed using the CODEHOP algorithm (Rose *et al.* 1998), from protein sequences of known fungal elongases: 5'-GTC ATC TAC TAC ATC ATC ATH TTY GGN GG-3' and 5'- CGG GCG GAC TGG AAG TAG TAV YAR TAC AT-3'. The amplified fragments were recovered from electrophoresis gels using Perfectprep Gel Cleanup (Eppendorf), and then sequenced. Fragments of the elongase gene were cloned into an *Escherichia coli* plasmid (pGEM®-T Vector System, Promega) prior to sequencing. The same regions were also amplified using cDNA as a template, to confirm the existence of introns indicated by alignment with sequences of homologous genes in the GenBank database. New non-degenerate primers were designed and later used in RT-PCR reactions: 5'-ATC TCC GAC CTC ACG ACG AC-3' and 5'-CTC ACC GAG AGT GAC GAT GG-3 for Δ^9-desaturase gene; 5'-CCG AGA TAC ATT CCC TCG AC-3' and 5'-CCA TGA GAA GTA AGG GAC AAG G-3' for Δ^{12}-desaturase gene; and 5'-TGG TAG GTG TGG AGG AAA GC-3' and 5'-CAT ATT TGG CGG CAG AGA GT-3' for the elongase gene.

Southern blotting was performed as described previously (Turk & Plemenitaš 2002). DNA fragments were amplified as described above (using non-degenerate primers in the case of the elongase gene), and then radioactively labelled and used as probes.

The upstream and downstream sequences of all three of these genes were obtained using the GenomeWalker™ Universal Kit (Clontech), according to the manufacturer instructions, using oligonucleotide primers that were specific for the adapters supplied in the kit, and oligonucleotide primers specific for known fragments of the genes. These were as follows: *ApOLE1* upstream 5'-GGC AAC GAG GGT GGG GAA GAT CAA A-3', upstream nested 5'-TGA GGT AGT TCT TGT GCT GCC AGA CGA CAA-3', downstream 5'-ACT GGC TCG GTG ACC AGC CTT TCG AT-3', downstream nested 5'-CGT CAC TCT CGG TGA GGG CTA CCA CAA CT-3'; *ApODE12* upstream 5'-GTG TCG TTG AGG GTC TTG GAG GGA GAG AA-3', upstream nested 5'-GGT GTA GAG AGC CCA GAG GCC AGC TCT AA-3', downstream 5'-TTC TCT CCC TCC AAG ACC CTC AAC GAC AC-3', downstream nested 5'-CTC CCA CGG CAA GCA CCA CAA CTT CT-3'; *ApELO1* upstream 5'-CAG GGT CAG GTA GAG GTT GTG GAC CTT GAA-3', upstream nested 5'-GCA TGA ACT CTC TGC CGC CAA ATA TGA TG-3', downstream 5'-CAC CGC AGT CTC ATG GGT CCC CAT TA-3', downstream nested 5'-TGA ACT TGA CCG TCC ACG TCG TCA TGT ACT-3'.

Gene phylogeny reconstruction

Homologues of the Δ^9-desaturase and Δ^{12}-desaturase and the elongase were identified by BLAST searches (Altschul *et al.* 1997) against a GenBank non-redundant protein database. In addition to fungal homologues, sequences from other species were added. Protein sequences were aligned using ClustalX (Thompson *et al.* 1997) and edited in BioEdit software (Hall 1999). Gene trees were generated with MrBayes software applying Bayesian inference (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). Runs were performed for two million generations with mixed amino-acid models, default temperature and numbers of chains. The trees were sampled every 100 generations. Trees sampled before the analysis reached stationarity of likelihood values and those sampled before the average standard deviation of the split frequencies lowered under 0.5 % were excluded from the final analysis. The stationarity of likelihood values was checked using the Tracer software (Rambaut & Drummond 2007). Enzymes from non-fungal organisms were used as outgroups: *Vibrio fischeri* (Δ^9-desaturases) and *Arabidopsis thaliana* (Δ^{12}-desaturases and elongases).

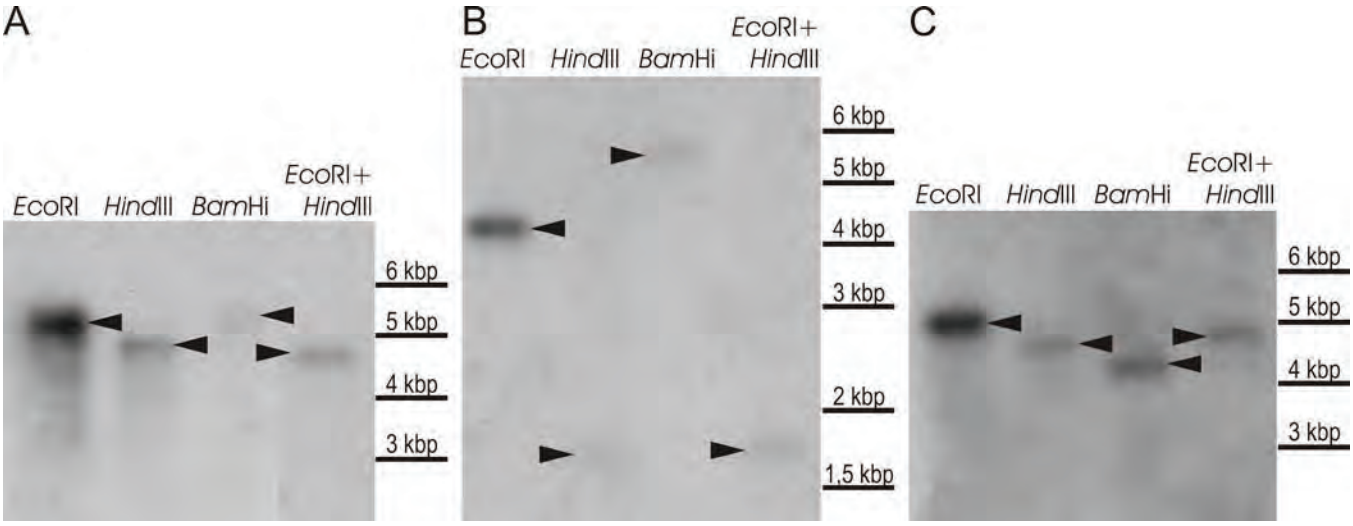

Fig. 1. Determination of the *ApOLE1* (A), *ApODE12* (B) and *ApELO1* (C) gene copies in *A. pullulans*. Southern blotting of genomic DNA digested with different restriction endonucleases (*Eco*RI, *Hind*III, *Bam*HI, *Eco*RI+*Hind*III, as indicated). Southern blots of the gels with separately digested DNAs were probed with radiolabelled fragments, and amplified with oligonucleotide primers specific for parts of the genes in question.

RT-PCR and statistical analysis

Total cDNA was synthesised from the RNA samples isolated as described above, using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Approximately 12.5 ng of the synthesised cDNA was used as a template for multiplex PCR with each pair of the oligonucleotide primers described above and oligonucleotide primers specific for the 28S rRNA (Lanišnik Rižner *et al.* 1999). The PCR consisted of 21, 24 and 22 cycles for the Δ^9-desaturase and Δ^{12}-desaturase and for the elongase, respectively. The primer dropping method was used (Wong *et al.* 1994), with 28S primers added to the reaction for the last 16 PCR cycles. The products of the reactions were separated on agarose gels and stained with ethidium bromide, with the relative abundance of each amplified fragment evaluated by measuring its luminescence with the UN-SCAN-IT software (v. 5.1, Silk Scientific Corporation). The values for the genes of interest were standardised with the amounts of the 28S rRNA fragments, the expression of which remained unchanged under the different environmental conditions. Transcription patterns of given genes were established through comparison of these values between different samples. RT-PCR analyses were carried out in triplicate.

Means and standard deviations were calculated, and the variance due to systematic error was subtracted. The data were analysed for statistically significant differences using a one-way ANOVA test (the variance was checked for homogeneity), followed by the Tukey (HSD) test.

RESULTS

Specific products were successfully amplified using PCR with the oligonucleotide primers specific for parts of the genes encoding a Δ^9-desaturase, a Δ^{12}-desaturase and a fatty-acid elongase. Sequencing of the fragments produced 306 bp, 234 bp and 548 bp partial sequences. Using the GenomeWalker™ Universal Kit, complete coding sequences of three new genes from *A. pullulans* were obtained. Searches with the BLAST programme (Altschul *et al.* 1997) showed similarities with several genes for Δ^9-desaturases and Δ^{12}-desaturases and for fatty acid elongases. The genes were named *ApOLE1*, *ApODE12* and *ApELO1*, respectively. The sequences have been stored in GenBank under the accession numbers DQ901954 (*ApOLE1*), DQ901955 (*ApODE12*) and EF123104 (*ApELO1*).

The coding regions were determined through the alignment of the sequences with known homologous genes obtained from the GenBank database and with cDNA sequences. The deduced ApOle1 protein is 458 amino acids long and the gene contains a 50-bp intron. *ApODE12* contains no introns, while the putative ApOde12 protein is of 480 amino acids. Two 52 and 53 bp introns were discovered in the *ApELO1* fragment, and their sequences were found to be 50 % identical. The hypothetical ApElo1 protein is of 343 amino acids. Southern blotting of the genomic DNA did not suggest the existence of more than one copy of any of the three genes in the genome of *A. pullulans* (Fig.1).

Good convergence of the runs was reached when constructing all three of the gene trees with MrBayes. The likelihood values reached plateaus after approximately 10,000 (Δ^9-desaturases), 8,000 (Δ^{12}-desaturases) and 6,000 generations (elongases), while the average standard deviations of the split frequencies dropped below 0.5 % after approximately 400,000 (Δ^9-desaturases) and 800,000 (Δ^{12}-desaturases and elongases) generations. The first 4,000 (Δ^9-desaturases) and 8,000 (Δ^{12}-desaturases and elongases) trees were discarded as burn-in. The posterior probabilities for the amino-acid models were 1 for the Blosum62 model (Henikoff & Henikoff 1992) for Δ^9-desaturases and Δ^{12}-desaturases, and 1 for the WAG model (Whelan & Goldman 2001) for elongases. All three of the deduced proteins (ApOle1, ApOde12 and ApElo1) clustered with homologous enzymes from fungi belonging to *Pezizomycotina*.

The relative abundances of the *ApOLE1*, *ApODE12* and *ApELO1* genes were studied by RT-PCR. Their profiles for growth at different salinities, and under hyper- and hypo-osmotic shock were analysed separately. One-way ANOVA ($\alpha = 0.05$) showed significant differences ($3.13 \times 10^{-07} < P < 0.003$) in all cases. The results of the Tukey (HSD) *post-hoc* testing are shown in Figures 2–4.

The abundance of *ApOLE1* mRNA (Fig. 5) was significantly higher at 13 % NaCl than at the lower salinities. When *A. pullulans*

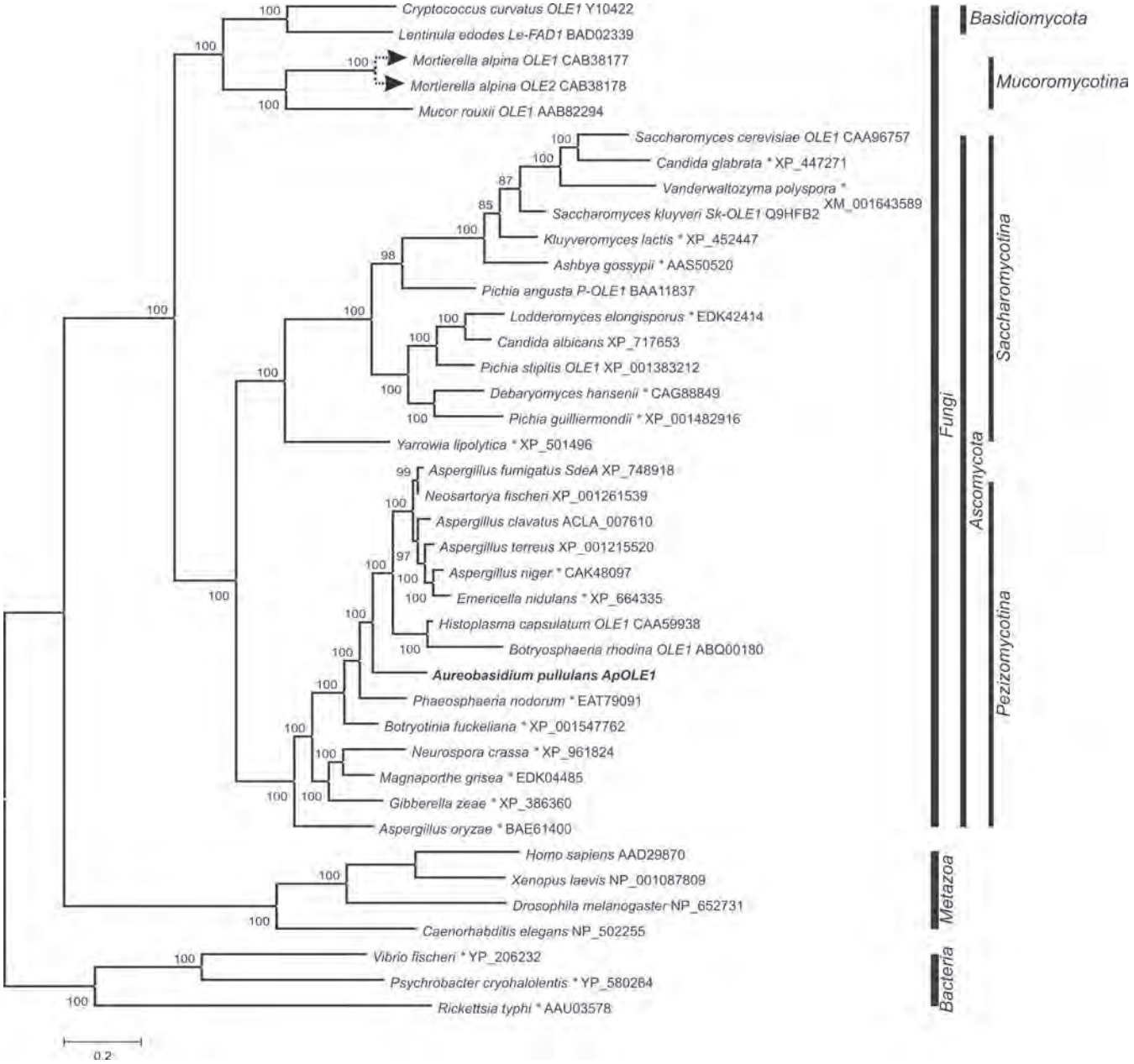

Fig. 2. Phylogeny of the Δ^9-desaturase enzymes. The tree was constructed from the alignment of protein sequences found in the GenBank database with the BLAST programme. Analyses were performed with MrBayes software in two runs with two million generations each. Mixed amino-acid models were used, and the first 20 % of the trees were excluded from the final consensus tree. Full scientific names of source organisms are followed by gene names (if available) and GenBank accession numbers. Entries marked with an asterisk (*) are available in the database as hypothetical proteins without any assigned functions.

cells were subjected to osmotic shock, the responses differed considerably. Surprisingly, after hyperosmotic shock, the *ApOLE1* mRNA level was reduced, although a significant decrease was detected in only one sample (1 h after the up-shift). All other levels, except that initial prior to up-shift, were significantly lower than the final steady-state levels at 10 % NaCl. In contrast, hypo-osmotic shock led to increased levels of *ApOLE1* mRNA. A significant difference was first detected 10 min after the down-shift, and it reached its peak after 30 min. One hour after the down-shift, the *ApOLE1* mRNA decreased again, but two hours after the down-shift it was still significantly higher than prior to the osmotic shock. Nevertheless, the steady-state mRNA levels in the cells not exposed to NaCl was significantly lower than in all of the samples obtained after the hypo-osmotic shock.

The levels of *ApODE12* mRNA were generally higher at higher salinities (Fig. 6): the levels were significantly higher at 13 % NaCl as compared to 0.0 %, 2.5 % and 5.0 % NaCl. As seen for the *ApOLE1* gene, the levels of *ApODE12* mRNA decreased significantly after exposing the cells to hyperosmotic shock. A transient increase in *ApODE12* mRNA levels was seen 10 min after the up-shift, which was significantly higher than after 30 min, and 1 h and 2 h later. Hypo-osmotic shock resulted in transiently increased *ApODE12* mRNA levels. The peak here was reached 30 min after the down-shift. A significant increase was first seen 10 min after exposing the cells to the shock and 2 h later it could not be detected any more.

The levels of *ApELO1* mRNA were highest at 13.0 % NaCl (Fig. 7), and at 7.5 % and 10.0 % NaCl they were also significantly higher than at both 0.0 % and 2.5 % NaCl. After exposing the cells to hyperosmotic shock, the *ApELO1* mRNA levels decreased after 5 min, and the decrease remained significant throughout the shock period, even though the steady-state *ApELO1* mRNA expression levels at 10.0 % NaCl were significantly higher than all others seen during the up-shift. Hypo-osmotic shock resulted in a significant increase in *ApELO1* mRNA levels after 10 min, which then decreased slowly up to 1 h after the shock, when it was still significantly higher than prior to the down-shift.

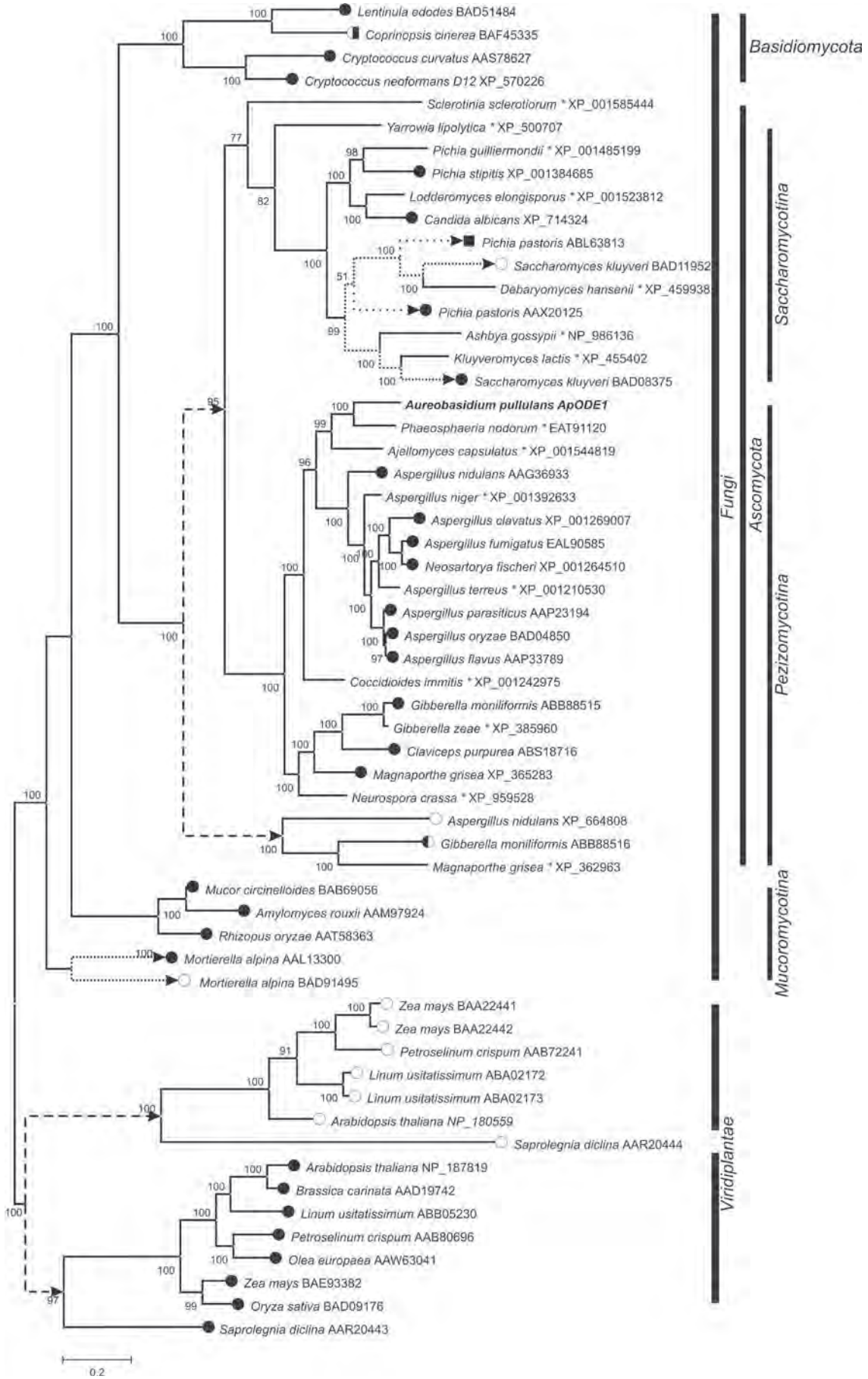

Fig. 3. Phylogeny of the Δ^{12}- Δ^{15}- and ω^3-desaturase enzymes. The tree was constructed from the alignment of the protein sequences found in the GenBank database with the BLAST programme. Analyses were performed with MrBayes software in two runs with two million generations each. Mixed amino-acid models were used, and the first 40 % of the trees were excluded from the final consensus tree. Full scientific names of source organisms are followed by the GenBank accession numbers. the presumed functions of the enzymes are marked with full circles ● (Δ^{12} activity), empty circles ○ (ω^3 activity), squares ■ (Δ^{15} activity), or combinations thereof. Entries marked with an asterisk (*) are available in the database as hypothetical proteins without assigned function. Presumed large duplications of the ancestor genes are marked with arrows and dashed lines. Other gene duplicates are connected with dotted lines.

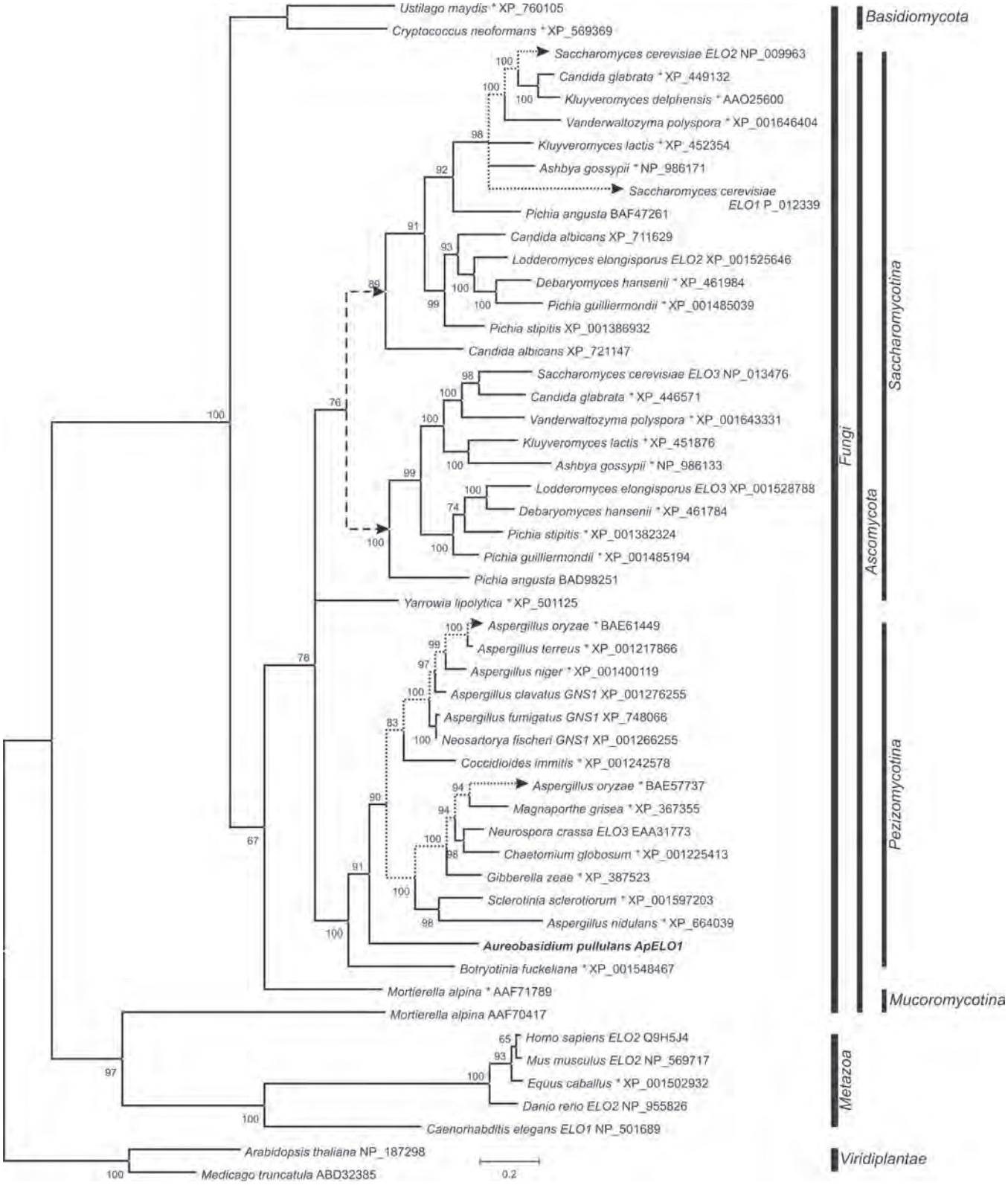

Fig. 4. Phylogeny of elongase enzymes. The tree was constructed from the alignment of the protein sequences found in the GenBank database with the Blast programme. Analyses were performed with MrBayes software in two runs with two millions generations each. A mixed amino-acid model was used, with the first 40 % of trees were excluded from the final consensus tree. Full scientific names of source organisms are followed by gene names (if available) and GenBank accession numbers. Entries marked with an asterisk (*) are available in the database as hypothetical proteins without assigned functions. Presumable large duplication of the ancestor gene is marked with arrows and a dashed line. Other gene duplicates are connected with dotted lines.

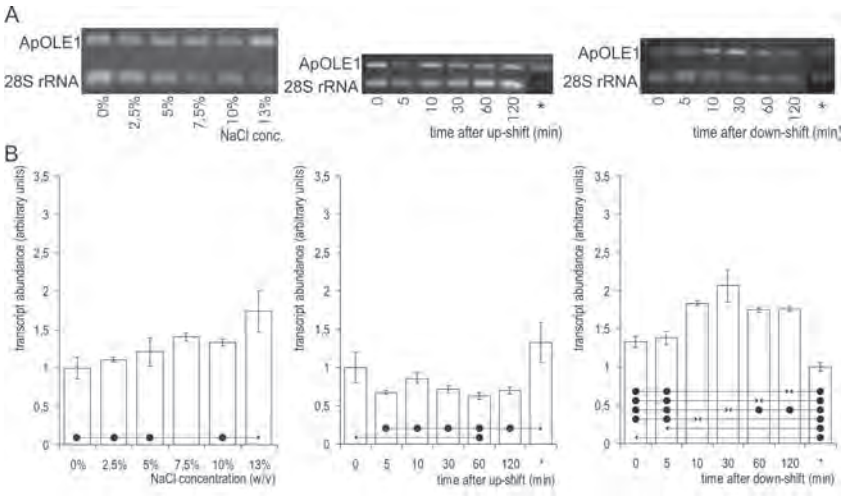

Fig. 5. *ApOLE1* mRNA abundance. A. Semi-quantitative transcriptional analysis by RT-PCR. Samples were prepared from cells grown at different salinities (first column) or subjected to a salinity shift from 0 % to 10 % NaCl (second column) and 10 % to 0 % NaCl (third column). mRNA abundances in cells completely adapted to the final salinity (10 % in the case of an up-shift, and 0 % in the case of a down-shift) are marked with an asterisk (*). Amplified fragments of *ApOLE1* and 28S rRNA genes were run on 1 % agarose gel and stained with ethidium bromide. B. *ApOLE1* gene expression as detected by RT-PCR. Data represent means ±SD of three independent experiments. Significant differences (Tukey's HSD) were seen between the value marked with an arrow-head and each of the samples marked with the dot.

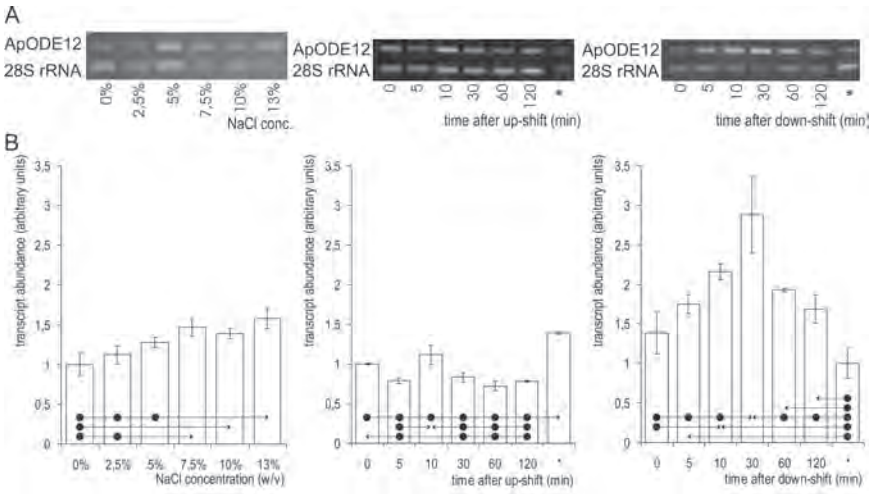

Fig. 6. *ApODE12* mRNA abundance. A. Semi-quantitative transcriptional analysis by RT-PCR. Samples were prepared from cells grown at different salinities (first column) or subjected to salinity shifts from 0 % to 10 % NaCl (second column) and 10 % to 0 % NaCl (third column). mRNA abundances in cells completely adapted to the final salinity (10 % in case of up-shift and 0 % in case of down-shift) are marked with an asterisk (*). Amplified fragments of *ApOLE1* and the 28S rRNA genes were run on 1 % agarose gels and stained with ethidium bromide. B. *ApODE12* gene expression as detected by RT-PCR. Data represent means ±SD of three independent experiments. Significant differences (Tukey's HSD) were seen between the value marked with an arrow-head and each of the samples marked with dots.

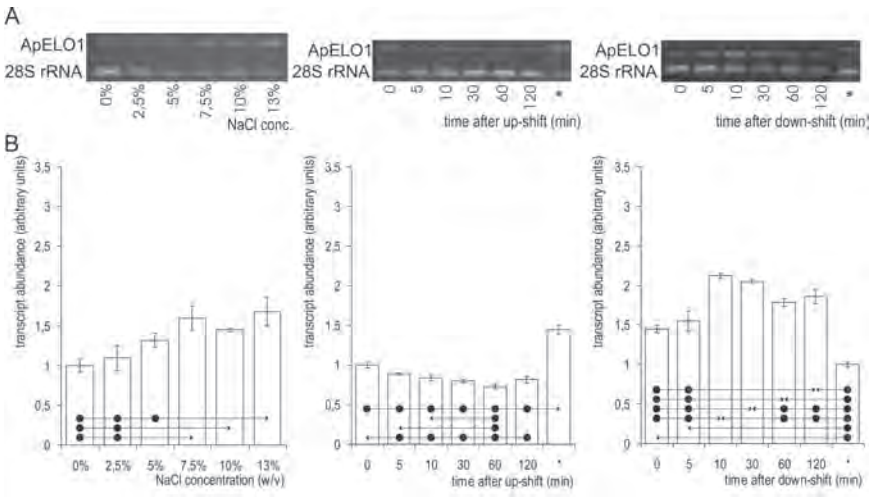

Fig. 7. *ApELO1* mRNA abundance. A. Semi-quantitative transcriptional analysis by RT-PCR. The samples were prepared from cells grown at different salinities (first column) or subjected to salinity shifts from 0 % to 10 % NaCl (second column) and 10 % to 0 % NaCl (third column). mRNA abundances in the cells were completely adapted to the final salinities (10 % in the case of the up-shift, and 0 % in the case of then down-shift) are marked with an asterisk (*). Amplified fragments of *ApOLE1* and the 28S rRNA genes were run on 1 % agarose gels and stained with ethidium bromide. B. *ApELO1* gene expression as detected by RT-PCR. Data represent means ±SD of three independent experiments. Significant differences (Tukey's HSD) were seen between the value marked with an arrow-head and each of the samples marked with the dot.

DISCUSSION

The cell membranes of *A. pullulans* contain long-chain fatty acids with double bonds in positions 9 and 12 (Δ^9, Δ^{12};Turk *et al.* 2004). We thus assumed that the *A. pullulans* genome must encode the corresponding fatty-acid desaturases as well as a fatty-acid elongase. We also wanted to know if changes in the mRNAs levels of these desaturases and elongases correlate with alterations in fatty-acid composition that have previously been seen at different salinities of the surrounding medium (Turk *et al.* 2004).

The proteins coded by the new genes that are involved in fatty-acid modifications in *A. pullulans* did not differ significantly from their homologues in other fungi. Analysis of the *ApOLE1* sequence revealed the presence of an intron with atypical splice acceptor bases at its 3' end (TG instead of AG). The high degree of identity between two introns in the *ApELO1* gene indicated that they might originate from a relatively recent duplication event and could thus be useful in studies of the evolutionary history of this species.

The essential *OLE1* gene was first found in *S. cerevisiae*, and later also in several other fungi (Watanabe *et al.* 2004). A homologue in *A. pullulans* was therefore expected. The Δ^{12}-desaturases are, on the other hand, less common, and have not been extensively studied (Watanabe *et al.* 2004). They introduce a *cis* double bond into fatty acids that already have a *cis* double bond at the Δ^9 position. This additional double bond further increases the membrane fluidity, although its effect is less pronounced than the effect of the first double bond (Los & Murata 1998). Therefore, this Δ^{12}-desaturase in *A. pullulans* may enable the cell to fine-tune its cell-membrane fluidity and may be one of the reasons for its halotolerance, in contrast to the salt-sensitive *S. cerevisiae* which is not capable of Δ^{12}-desaturation. Like the Δ^9-desaturases, elongases are essential for cell growth (Dittrich *et al.* 1998). Three different elongases exist in *S. cerevisiae*, each with a different substrate specificity (Trotter 2001). So far, only one elongase homologue has been identified in *A. pullulans*, using degenerate PCR and Southern blotting.

The evolutionary origins of all three of these enzymes from *A. pullulans* in the *Pezizomycotina* branch as shown by our phylogenetic analysis are not surprising.

Evolution of Δ^9-desaturases also did not show any unusual characteristics with the exception of desaturases from *Mucoromycotina* which clustered with homologous enzymes from *Basidiomycota*.

Comparisons of the deduced amino-acid sequences of the Δ^{12}-desaturases and ω^3-desaturases indicate several independent duplications of their genes, leading to separate Δ^{12} and ω^3 branches; this was previously reported by Damude *et al.* (2006). According to our analysis, the major duplication occurred before the separation of *Pezizomycotina* from *Saccharomycotina*, resulting in a separate ω^3 branch with very few representatives. At least one duplication event could also be detected in *Saccharomycotina* and *Mucoromycotina*.

The phylogenetic analysis of the elongase genes indicates a duplication event early in the evolutionary history of *Saccharomycotina*, which led to two groups of elongase genes, each represented with (at least) one enzyme in most species. Surprisingly, Elo1 and Elo2 from *S. cerevisiae* belong to the same group, and Elo3 to the other one, although Elo2 and Elo3 are more functionally related. Two hypothetical proteins from *Aspergillus oryzae* indicate a possible duplication event in *Pezizomycotina* as well. Another interesting elongase is a long-chain polyunsaturated fatty acid elongation enzyme from *Mortierella alpina* described by Parker-Barnes *et al.* (2000), which proved to be quite different from all of the other elongases, and in our analysis it clustered with animal elongase enzymes.

The structural and functional integrity of biological membranes requires the presence of water. Changes in cellular water activities can have profound influences on membrane stability. Cations from salts interact with membrane constituents and can also affect their conformation. Furthermore, abrupt changes in salinity can expose cell membranes to physical stress because of the changes in osmotic pressure (Hazel & Williams 1990). Phenotypic adjustments in the cell-membrane lipid compositions as a response to altered salinity have been observed, among which there are increases in the relative proportions of anionic lipids. Increased levels of unsaturated acyl chains and longer fatty acids have been seen in yeasts and cyanobacteria at higher salinities (Hazel & Williams 1990, Turk *et al.* 2004), and there is evidence that these changes serve to increase the membrane fluidity at high salt concentrations (Russell 1989, Hazel & Williams 1990).

The high abundance of the desaturase mRNAs seen at high salinities in *A. pullulans* was expected, and it is consistent with an enrichment in the C18:$2^{\Delta 9,12}$ fatty acids in the *A. pullulans* cell membranes at 5 % and 10 % NaCl (Turk *et al.* 2004). These changes may help to sustain sufficient levels of membrane fluidity at high salt concentrations (Russell 1989, Hazel & Williams 1990). Increased proportions of long chain fatty acids would oppose this effect, but when the *A. pullulans* cell membranes were analysed, no shifts in fatty-acid lengths were detected (Turk *et al.* 2004). A higher abundance of *ApELO1* mRNA at high salinities might not therefore be associated with alterations in the fatty-acid composition. The reason for this discrepancy might lie in a faster turnover of the long-chain fatty-acid pool at high salinities, a lower efficiency of *ApELO1* due to the high concentrations of compatible solutes, additional undetected elongases with complementary roles, or the posttranscriptional control of expression.

The changes in the mRNA levels following osmotic shock were contrary to expectations, considering the observations of the mRNA abundances at different salinities. A similar transient decrease in mRNA abundance after exposure of the cells to hyperosmotic shock was seen previously for the *S. cerevisiae OLE1* gene. Expression of the *OLE1* gene diminished 45 min after a shift to high osmolarity (Rep *et al.* 2000), and increased again after 90 min (Yale & Bohnert 2001). In both cases, the changes can probably be associated with the temporary growth arrest caused by the shock, a response that is similar to the previously reported expression of ribosomal proteins (Rep *et al.* 2000).

Another possibility is that these reactions act as part of a stress-response system in *A. pullulans.* By introducing double bonds into these phospholipid fatty-acyl chains and by decreasing the fatty-acyl chain length in artificial membranes, glycerol permeability is increased (Blomberg & Adler 1992). The immediate changes following the osmotic shock could thus be an emergency response, which in the case of hyperosmotic shock protects the cells from compatible solute leakage until other – possibly slower or energetically more demanding – mechanisms can take over, such as synthesis of other compatible solutes and membrane glycerol transporters, or cell wall melanisation. Indeed, in salt-sensitive *S. cerevisiae*, the ORF's encoding many known yeast salinity stress response proteins are either unaffected or down-regulated immediately following the up-shift, and gradually induced only later (Yale & Bohnert 2001). Similarly, a decrease in the saturation levels of the fatty acids, and therefore an increase in the permeability of the membranes for molecules such as glycerol could facilitate export of compatible solutes when they are no longer needed

(Tamas *et al.* 1999), explaining the transient increases in the levels of the mRNAs encoding fatty acid desaturases.

Finally, maybe cells deal with abrupt changes in osmotic pressure caused by changes in salinity in a very different way than when they are grown in an environment with constant water activity with this "turgor shock" response somehow having priority over the "salinity shock" response.

The opposite response patterns in mRNA levels following hyper- and hypo-osmotic shock compared to the slow adaptation to growth at different salinities indicates that these two types of stress represent a fundamentally different challenge to cells.

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HMG-CoA reductase is regulated by environmental salinity and its activity is essential for halotolerance in halophilic fungi

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Abstract: The activity and level of HMG-CoA reductase (HMGR) were addressed in halophilic fungi isolated from solar saltpans. Representative fungi belonging to the orders *Dothideales, Eurotiales* and *Wallemiales* have a specific pattern of HMGR regulation, which differs from salt-sensitive and moderately salt-tolerant yeasts. In all of the halophilic fungi studied, HMGR amounts and activities were the lowest at optimal growth salinity and increased under hyposaline and hypersaline conditions. This profile paralleled isoprenylation of cellular proteins in *H. werneckii*. Inhibition of HMGR *in vivo* by lovastatin impaired the halotolerant character. HMGR may thus serve as an important molecular marker of halotolerance.

Key words: Adaptation; extremophiles; isoprenylation; lovastatin; mevalonate regulation.

INTRODUCTION

Until recently, no true halophilic representatives were thought to exist within the kingdom of Fungi. Reports have, however, emerged arguing that the orders *Dothideales*, *Eurotiales* (Ascomycota) and *Wallemiales* (Basidiomycota) generally include genera and species adapted to growth under hypersaline conditions that represent part of the *mycota* of solar salterns (Gunde-Cimerman 2000, Butinar *et al*. 2005[a,b]; Zalar *et al*. 2005). Many unicellular eukaryotic organisms can adapt to changing environmental osmolarity mainly due to their ability to modify the sterol composition of cellular membranes in response to environmental stress (Horvath *et al*. 1998). 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMGR, EC 1.1.1.34) is the major metabolic flux regulator of the mevalonate pathway for sterol biosynthesis, and it catalyses NADPH-dependent reductive deacylation of HMG-CoA to mevalonate. HMGR is crucial for the biosynthetic production of sterols and other isoprenoids, like protein modifying prenyl groups, in all three domains of life. Regulation of HMGR levels and activities occurs at multiple levels, including transcription, phosphorylation and protein degradation (Goldstein & Brown 1990). Little is known about regulation of HMGR activity in the new ecological group of moderately to extremely halophilic fungi that have adapted to growth in highly saline environments (Prista *et al*. 1997; Gunde-Cimerman 2000). We have previously reported unusual HMGR activity in the halophilic black yeast *Hortaea werneckii* (Petrovič *et al*. 1999, Vaupotič *et al*. 2007): while HMGR activity was highly dependent on environmental NaCl concentrations, the sterol content in *H. werneckii* did not change accordingly (Mejanelle *et al*. 2001, Turk *et al*. 2004), indicating that regulation of HMGR activity influences the metabolic flux of mevalonate differently to the biosynthesis of sterols, possibly at the pre-squalene level.

In this study, we have explored effects of salinity on HMGR regulation in five fungi species from solar salterns: the halotolerant *Aureobasidium pullulans*, and the halophilic *Phaeotheca triangularis*, *Trimmatostroma salinum (Dothideales)*, *Eurotium amstelodami* (*Eurotiales*) and *Wallemia ichthyophaga* (*Wallemiales*). In particular, we have addressed the correlation between their HMGR activity and halophilic character. Two further species were included as additional references: a moderately halophilic yeast *D. hansenii*, and a salt-sensitive (i.e. mesophilic) yeast *Saccharomyces cerevisiae*. We demonstrate here a specific HMGR regulation by environmental salinity that correlates well with the halophilic character of these fungi. Focused on *H. werneckii*, the best characterized of the halophilic fungi from solar salterns, we also provide evidence that HMGR activity is crucial for halotolerance as well as for the changes in protein prenylation in response to changing salinity.

MATERIALS AND METHODS

Strains, media, and growth conditions

Cultures of halophilic fungi were isolated from Sečovlje salterns at the Slovenian Adriatic coast: *H. werneckii* (MZKI B736), *P. triangularis* (MZKI B741), *T. salinum* (MZKI B734), *A. pullulans* (MZKI B802), *E. amstelodami* (MZKI A561), *W. ichthyophaga* (EXF 994). These have been deposited in the culture collections of the Slovenian National Institute of Chemistry (MZKI) or of EXF at the Department of Biology, Biotechnical Faculty, University of Ljubljana. The reference strains were the salt-sensitive *S. cerevisiae* (MZKI K86) and the moderately halophilic *D. hansenii* (CBS 767), from Centraalbureau voor Schimmelcultures (CBS) Utrecht, The Netherlands. The fungi were grown at 28 °C (30 °C for *S.*

cerevisiae) on a rotary shaker at 180 rpm in defined YNB medium adjusted to the indicated NaCl concentrations at pH 7.0. The cells were harvested in mid-exponential phase by centrifugation (4,000× *g*, 10 min), washed in 50 mM Tris-HCl, pH 7, and frozen in liquid nitrogen. The YNB medium agar plates were also prepared with 50 µM lovastatin (Lek). Ten µL of *H. werneckii* liquid culture were spotted onto agar plates and incubated for seven days prior to microscopy studies.

Measurement of HMGR activity

HMGR activity was measured as decribed previously (Petrovič, *et al*. 1999; Vaupotič, *et al*. 2007). Briefly, cell lysates were prepared from exponentially growing cells by disruption with a microdismembranator, in homogenization buffer (50 mM Tris, pH 8.5, 20 % glycerol, 0.5 % NaCl, 0.5 % Triton X-100; or at pH 7.0, without glycerol and NaCl for *S. cerevisiae*) containing fungal protease inhibitors (Sigma). The lysates were fractionated into soluble fraction and cellular debris by centrifugation (600× *g*, 15 min). After following centrifugation at 10,000× *g*, the supernatants were used for HMGR activity assessments. Protein concentrations were measured by spectrophotometry at 590 nm using the Bradford method with Nanoquant reagent (Roth). HMGR activity was assayed with 50 µg total protein with *D*-3-[3-^{14}C]-hydroxy-3-methylglutaryl-CoA and *R*,*S*-[5-^{3}H(N)]-mevalonolactone (NEN) as substrate and internal standard, respectively. HMGR activity was expressed as pmol HMG-CoA converted to mevalonate min^{-1}.(mg protein)$^{-1}$ and are given as means ± standard error from at least three independent experiments.

Western blotting

Cell lysates were prepared, with 20 µg protein boiled for 10 min in 5× protein-loading buffer (Fermentas), separated by SDS-PAGE on 10 % polyacrylamide gels, and transferred to PVDF membranes (Roth). Immunodetection was performed with antibodies against HMGR (Upstate) and β-actin, and secondary antibodies conjugated with HRP (Santa Cruz Biotechnology), using the ECL detection system (Amersham Bioscience).

Metabolic radiolabelling with [3H]-mevalonate

Hortea werneckii was grown in media with the indicated NaCl concentrations, without or with 50 µM lovastatin and with 0.75 µCi/mL [^{3}H]-mevalonate ([^{3}H]-MVA; 50 Ci/mmol) added in the early logarithmic phase. The cells were harvested during the exponential phase by centrifugation and washed several times with PBS. Total protein was isolated from 200 mg of cells using the TRIzol reagent (Invitrogen), and then solubilized in 1 % SDS. Protein concentrations were determined spectrophotometricaly using the BCA method (Pierce). A delipidation procedure was performed to release the [^{3}H]-MVA-derived moiety from 200 µg labelled cellular protein, as described previously (Konrad & Eichler 2002). Briefly, SDS-solubilized proteins were incubated in 0.5 M HCl at 95 °C for 1 h, with vigorous shaking. Samples were extracted twice with chloroform/ methanol (2:1, v/v), and radioactivity released was quantified in the organic fraction by scintillation counting. Incorporation of [^{3}H]-MVA was expressed as pmol of incorporated [^{3}H]-MVA per mg protein, as means ±standard error from three independent experiments.

Microscopy

For morphological analysis of cells, fungi were washed in fresh growth medium, added to glass slides and covered with a coverslip. To prevent evaporation, the coverslip was sealed with nail-polish. Cell morphology was examined under an inverted light microscope (Nikon Eclipse 300) and images taken with a digital camera (Nikon DS-5M).

RESULTS

HMGR activities and protein levels in halophilic fungi depend on environmental salinity

To determine enzyme activities and protein levels of HMGR, the halophilic fungi were grown at three different environmental salinities. The specific HMGR activity was responsive to changes in NaCl concentrations in all of the fungal species (Fig. 1), including *S. cerevisiae* and *D. hansenii* as reference strains. All HMGR activity profiles were similar, showing minimal activity under optimal conditions and a 2-6-fold increase in activity under hyposaline and hypersaline conditions. We also explored HMGR in these fungi at the protein level under these growth conditions. Immunoblotting with an antibody against a conserved catalytic domain of HMGR revealed that according to the enzyme activities, the HMGR protein was also lowest at optimal growth salinity and increased under hyposaline and hypersaline conditions (Fig. 1).

Inhibition of HMGR by lovastatin *in vivo* resulted in the salt-sensitive character of *H. werneckii*

To demonstrate that HMGR activity is connected with the halotolerant character of saltern-inhabiting fungi, the growth of one of the most adaptable and halophilic yeast, *H. werneckii*, was monitored in the presence of sub-lethal concentrations (50 µM) of the specific HMGR inhibitor lovastatin at different NaCl concentrations. There was no effect of lovastatin on the growth curve of *H. werneckii* in salt-free media (Fig. 2). In contrast, lovastatin remarkably reduced growth in the otherwise physiologically optimal medium containing 17 % NaCl, an effect even more pronounced in hypersaline medium containing 25 % NaCl.

Microscopy revealed the effects of lovastatin on the morphology of *H. werneckii* cells (Fig. 3). In hyposaline media (Fig. 3a), the cells were significantly thinner and more elongated compared to those at optimal salinity (Fig. 3c), and most had a hardly visible septum and predominantly one unipolar bud. The only effect of lovastatin treatment was on the bipolar budding of cells (Fig. 3b). At optimal salinity (Fig. 3c), the cells grew as double-celled meristematic clusters that were slightly elongated and separated by a septum. The lovastatin treatment caused an increased number of irregularly shaped meristematic clusters of three, or even four, cells separated by a septum (Fig. 3d). In hypersaline media (Fig. 3e), the double-celled conidia were bulkier and less elongated than at optimal salinity. The effect of lovastatin was most evident under this extreme growth condition, resulting in four-celled meristematic clumps that were irregularly shaped, had a thick cell wall and well developed septa (Fig. 3f). Hyphal growth of *H. werneckii* was also affected by lovastatin, as seen on agar plates. No obvious growth effect of lovastatin on hyphae formation occurred in hyposaline media (Figs. 3g-h). With optimal salinity (Fig. 3i), the hyphae were

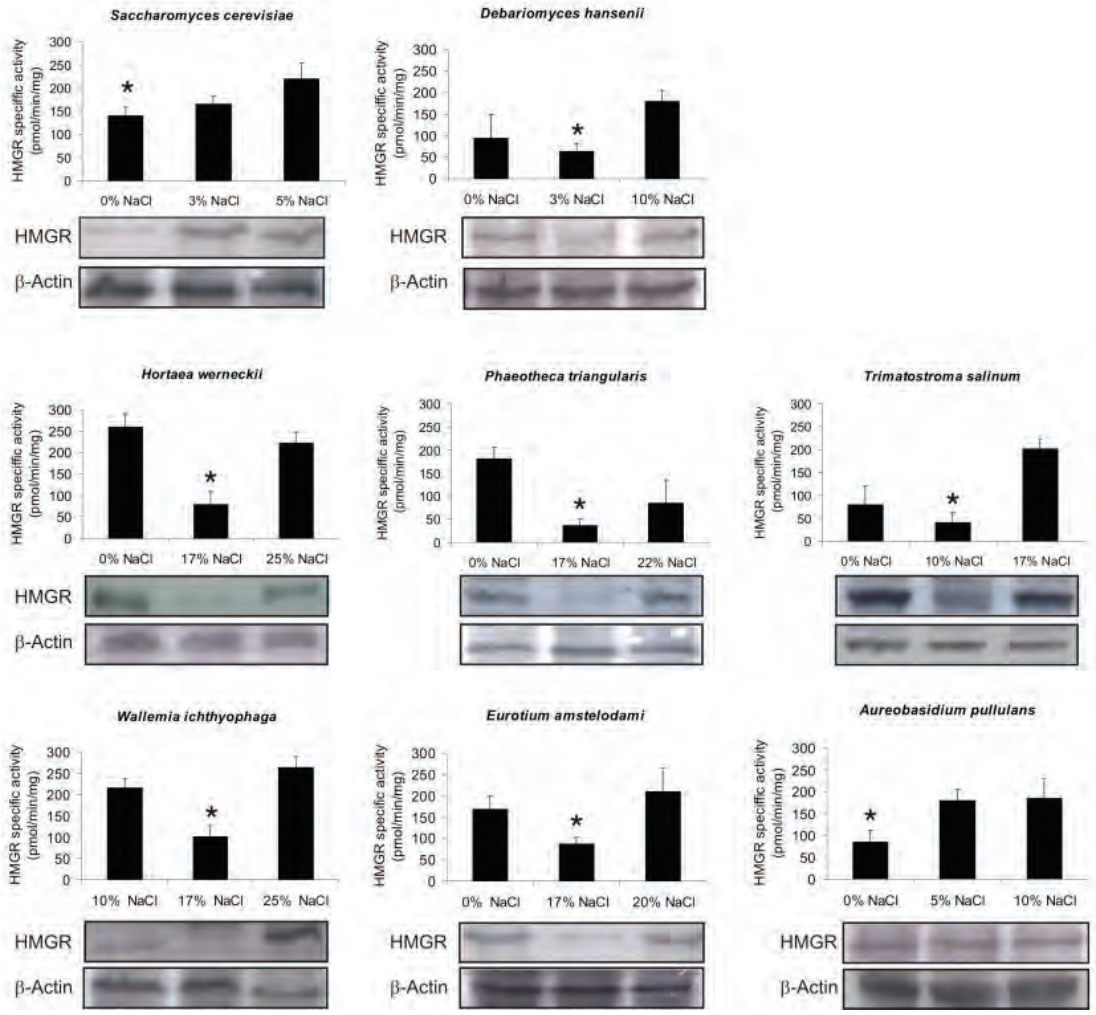

Fig. 1. Regulation of fungal HMG-CoA reductase activity and protein levels by environmental salinity. Different salinities of the medium were chosen as hyposaline (0% NaCl, only for halophiles), optimal, and hypersaline conditions in different fungi. Profiles of HMGR activity and HMGR protein levels of the indicated fungi are shown. β-Actin was used as loading control. *Optimal growth salinities. R. Data represent means ±SD of three independent experiments. Significant differences (Tukey's HSD) were seen between the value marked with an arrow-head and each of the samples marked with the dot.

highly branched and extended and had numerous buds, which were significantly reduced in number by the lovastatin treatment (Fig. 3j). In hypersaline media, the lovastatin treatment completely prevented branching of the hyphae and the formation of buds (Fig. 3k-l).

The mevalonate-derived lipid modifications of proteins correlate with HMGR activity in *H. werneckii*

To determine whether non-sterol mevalonate-derived lipid modifications of proteins accounted for the HMGR activity profile at these different environmental salinities, we investigated the incorporation of radioactively labelled mevalonate derivatives into proteins, as covalently linked lipid moieties. The *H. werneckii* cells were grown under different NaCl concentrations in the presence of [^{3}H]-mevalonate, without or with lovastatin. After harsh acidic delipidation of the isolated proteins, the [^{3}H]-labelled lipids released were assessed using a chloroform/ methanol extraction: both the total radioactivity of the protein fractions and the lipid-derived radioactivity after protein delipidation were lowest at optimal growth salinity (17 % NaCl), and approximately 2.5-fold higher in hyposaline (0 % NaCl) and hypersaline (25 % NaCl) media (Fig. 4), following the HMGR activity profile of *H. werneckii* (Fig. 1). Treatment with lovastatin increased the incorporation of [^{3}H]-mevalonate into the

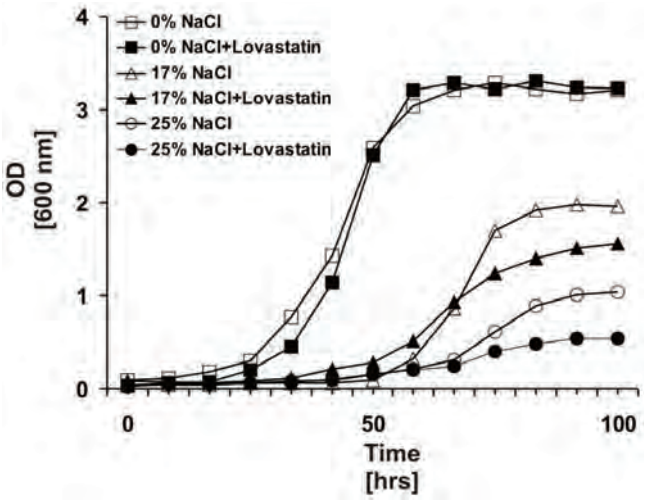

Fig. 2. Lovastatin impaired growth ability of the halophilic *H. werneckii* in NaCl-containing media. Growth curves of H. werneckii in optimal (17% NaCl), hyposaline (0% NaCl) and hypersaline (25% NaCl) media without (white symbols) and with (black symbols) 50 μM lovastatin.

lipids from cellular proteins as a consequence of its the inhibitory effect on production of endogenous mevalonate. However, this was most evident in salt-free medium, where lovastatin treatment had less effect on growth.

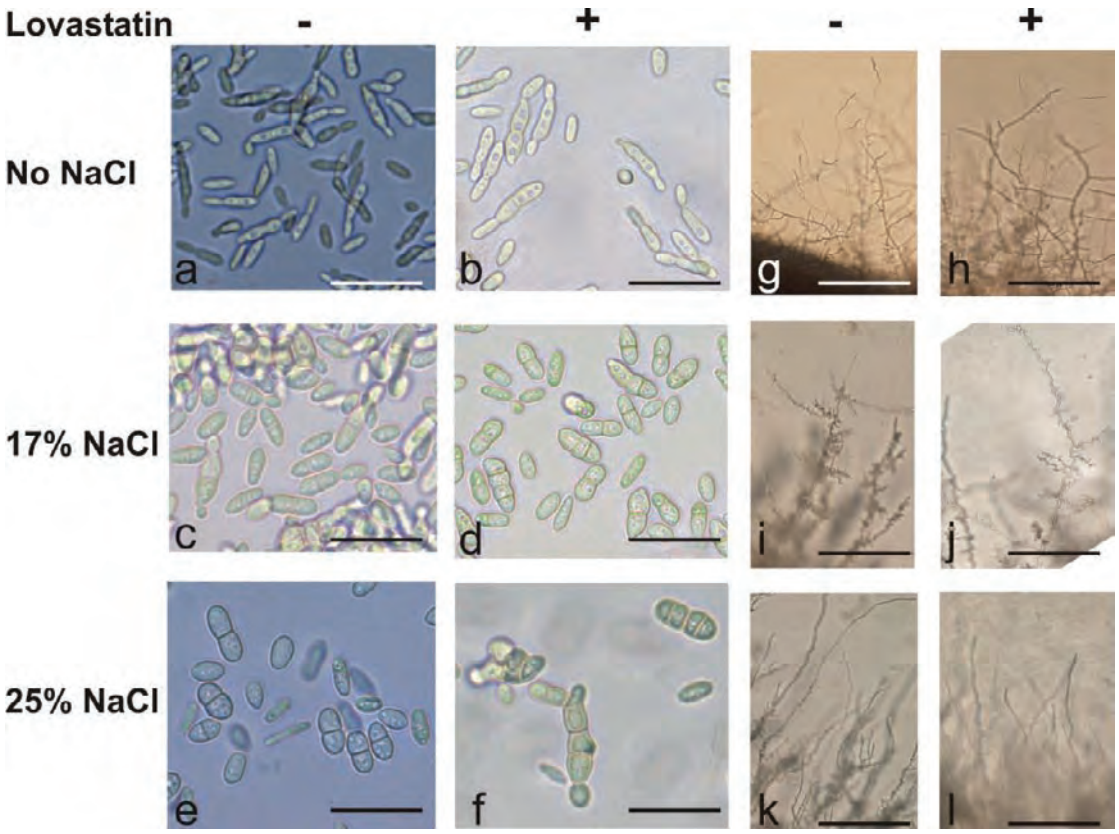

Fig. 3. Morphological changes in cells and hyphae growth caused by salt and lovastatin in *H. werneckii.* Cells were grown in media or agar plates with the indicated salt concentrations without or with lovastatin. Morphology was investigated using bright field microscopy under 40x and 10x magnification for cells and hyphae, respectively. Panels a-f, bar = 60 µm; panels g-l, bar = 240 µm.

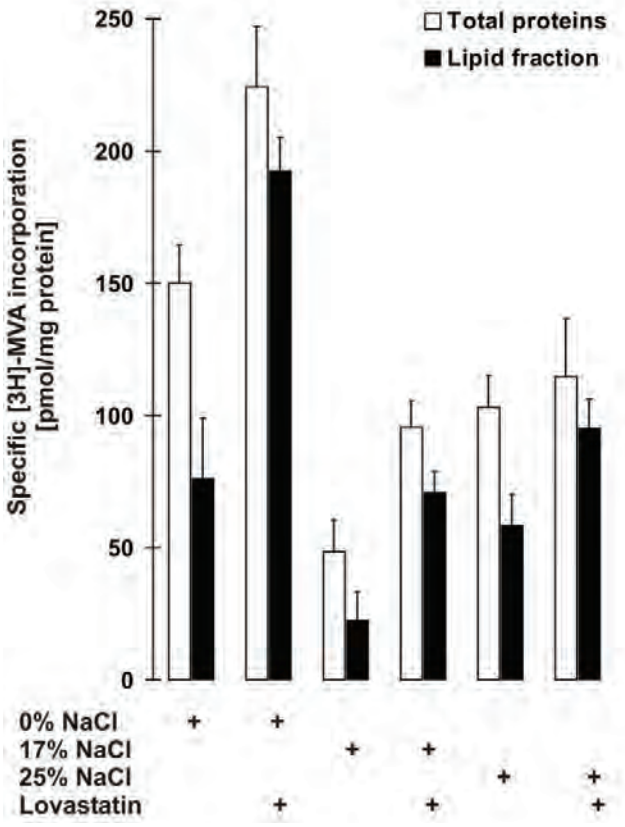

Fig. 4. Incorporation of [3H]-mevalonate-derived lipid moiety into cellular proteins in *H. werneckii*. After metabolic labelling of cellular proteins with [3H]-mevalonate, scintillation counting was carried out on total protein before delipidation (white bars) and then on the lipid fraction after protein delipidation (black bars). The data are presented as pmol incorporated radioactively labelled mevalonate per mg protein.

DISCUSSION

There have been numerous studies on stress responses of various salt-sensitive unicellular eukaryotes to saline stress. The present study of halophilic fungi represents adaptive metabolism in high-saline media rather than a stress response, due to the evolutionarily acquired halotolerance of these species. Our previous studies on ecophysiological characteristics of these fungal species have shown that *H. werneckii*, *W. ichthyophaga*, *E. amstelodami*, *P. triangularis* and *T. salinum* are halophiles, while *A. pullulans* is instead a moderately halotolerant species (Blomberg 2000, Gunde-Cimerman 2000, Butinar *et al*. 2005, Butinar *et al*. 2005, Zalar *et al*. 2005). Observing the changes in cellular HMGR activity we can conclude that the different HMGR activities in cells grown under different environmental salinities were the consequence of different amounts of cellular HMGR protein. The similar HMGR activity profiles in the moderately halotolerant *A. pullulans* and salt-sensitive *S. cerevisiae*, which lack the U-shaped HMGR profile, indicate that both have their growth optima in salt-free medium. This correlates well with their non-halophilic characters. Alternatively, the U-shaped HMGR profiles obtained in halophilic species corresponded well to previously proposed optimal salinities. These all showed the lowest HMGR activity at the optimal growth salinity and high HMGR activity in both salt-free medium, as hyposaline conditions for these species, and under extremely high salt concentrations, defined as hypersaline conditions, proposing the HMGR level and activity as a sensor of non-optimal growth salinity. Despite frequent reports of *D. hansenii* (*Saccharomycetales*) as a prime fungal halophile, we have shown here that according to the HMGR profile, its optimal salinity is lower than those of the halophilic fungi used in comparison. This fits well with the ecology of these species, since *D. hansenii* is most

often isolated from sea water with only 3 % NaCl, while the other halophilic fungi were from the hypersaline waters of solar salterns, where salt concentrations can reach saturation levels.

Very little is known about the regulation of HMGR in extremophilic eukaryotes. To date, some reports have demonstrated that expression and/or activity of HMGR is regulated in response to non-optimal salinity, i.e. in the halophilic archaeon *Haloferax volcanii*, where authors demonstrated the HMGR at the level of protein ampunt and activity was conspicuously increased during growth at high salinity (Bidle *et al*. 2007). In our previous reports on halophilic black yeast, we also showed a similar response in halophilic yeast *Hortaea werneckii* (Petrovič *et al.* 1999). In our ongoing investigations into proteins linked to halophily in *H. werneckii* (Vaupotič *et al*. 2007), HMGR is one of the so-called "salt-responsive" enzymes, where proten levels and/or enzyme activities increase under both hyposaline and hypersaline conditions. We have shown that at optimal growth salinity, HMGR undergoes ubiquitination and proteasomal degradation (Vaupotič & Plemenitaš 2007). Here, we provide additional evidence that salinity-dependent regulation of HMGR activity in *H. werneckii* is linked to its halotolerant character (Figs 2, 3), as inhibition of HMGR by lovastatin resulted in a considerably more salt-sensitive phenotype. Also, the morphological changes with both salt and lovastatin clearly indicate a defect in cell proliferation (Fig. 3). These data demonstrate further that HMGR activity is required for the halotolerant character of *H. werneckii*. Based on the data in the present study, we can speculate that this could similarly be true for other halophilic fungi in this study, where similar HMGR enzyme activities and protein profiles were seen (Fig. 1).

As we have shown previously, the sterol composition in *H. werneckii* did not change significantly at different salinities (Mejanelle *et al.* 2001, Turk *et al.* 2004) as we could expect according to HMGR activity fluctuations. Therefore, we sought evidence for changes in other mevalonate-derived, but pre-squalene isoprenoid intermediates, to reflect the metabolic effects of the HMGR activity profile in *H. werneckii*. Derivatives of pre-squalen isoprenoids are important regulatory determinants of prenylated proteins implicated in cell-cycle progression and cell proliferation, since their attachement to proteins directes them to cellular mebranes (Brown & Goldstein 1980, Siperstein 1984). Using [^{3}H]-mevalonate and the highly selective HMGR inhibitor lovastatin in metabolic labelling experiments, we have clearly demonstrated that modification of protein by the mevalonate-derived lipid moiety not only reflects the profile of HMGR activity, but also the responses to HMGR inhibition (Fig. 4). Combining the growth inhibitory effect of lovastatin treatment in salt containing media with the HMGR activity-dependent protein prenylation profile, we might conclude that lovastatin-mediated reduction in halotolerance was not merely the result of a non-specific inhibitory effect on overall cellular function, but rather reflects a protein-prenylation-specific event. Based on this data we can conclude, that in salterns-inhabiting fungi the regulation of HMGR activity by environmental salinity reflects more distinctively at the level of the metabolic flux through the pre-squalene part of the mevalonate pathway, rather than at the level of post-squalene regulation of sterol content. The higher activity of HMGR in hyposaline and hypersaline media could be connected with specific metabolic demands, when increased flux through the mevalonate pathway may be needed for prenylation and the subsequent membrane localisation of specific proteins that are not essential for growth in an optimal environment. In evolutionary terms, the maintenance of high levels of HMGR in hyposaline and hypersaline environments may also reflect physiological adaptation of halophilic fungi to metabolic demands under extreme conditions.

In conclusion, the key metabolic enzyme, HMGR, has been studied in a previously non-characterised group of halophilic fungi. The present study documents that both HMGR activity and protein levels in halophilic fungi depend on environmental salinity. In the extremely halotolerant *H. werneckii*, the biological consequence of HMGR regulation relates to posttranslational modification of proteins by prenylation. Therefore our findings provide a new insight for understanding the regulation of the mevalonate pathway as a response to changes in environmental NaCl concentrations. We propose the HMGR enzyme as an important biochemical signature of halophily in the Fungal kingdom .

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Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: a molecular perspective at a glance

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Abstract: Halophilic adaptations have been studied almost exclusively on prokaryotic microorganisms. Discovery of the black yeast *Hortaea werneckii* as the dominant fungal species in hypersaline waters enabled the introduction of a new model organism to study the mechanisms of salt tolerance in eukaryotes. Its strategies of cellular osmotic adaptations on the physiological and molecular level revealed novel, intricate mechanisms to combat fluctuating salinity. *H. werneckii is* an extremely halotolerant eukaryotic microorganism and thus a promising source of transgenes for osmotolerance improvement of industrially important yeasts, as well as in crops.

Key words: Compatible solutes, differential gene expression, Hal2, halophile, HOG signaling pathway, *Hortaea werneckii*, hypersaline water, ions, melanin.

INTRODUCTION

Water is of vital importance to all organisms. In an aqueous environment of high salt concentration, loss of internal water is a consequence of osmosis (Yancey 2005). Investigations have shown that most strategies of cellular osmotic adaptations are conserved from bacteria to man (Klipp *et al*. 2005). Salt sensitive *Saccharomyces cerevisiae* is a well-studied model system for studies of osmotic adaptation (Blomberg 2000, Hohmann 2002, Mager & Siderius 2002, Klipp *et al*. 2005). While 0.5 M NaCl represents a concentration that is already toxic for *S. cerevisiae*, the same concentration of NaCl is close to growth optimum of another model organism, halotolerant yeast *Debaryomyces hansenii* (Prista *et al*. 1997). Black yeast *Hortaea werneckii* can grow, albeit extremely slowly, in a nearly saturated salt solution (5.2 M NaCl), and completely without salt, with a broad growth optimum from 1.0 – 3.0 M NaCl (Gunde-Cimerman *et al*. 2000). As few extremely salt tolerant eukaryotic microorganisms are known, black yeast in general and *H. werneckii* in particular represent a group of highly appropriate microorganisms for studying the mechanisms of salt tolerance in eukaryotes (Petrovic *et al*. 2002). Since the first isolation of *H. werneckii* from hypersaline water in 1997, we have studied various aspects of its adaptation to saline environment. It has previously been shown that *H. werneckii* has distinct mechanisms of adaptation to high-salinity environments that were neither observed neither in salt-sensitive nor in moderately salt-tolerant fungi (Plemenitaš & Gunde-Cimerman 2005). The most relevant differences studied to date are in plasma membrane composition and properties (Turk *et al*. 2004, 2007), osmolyte composition and accumulation of ions (Petrovic *et al*. 2002, Kogej *et al*. 2005, 2006), melanisation of cell wall (Kogej *et al*. 2004, 2006), differences in HOG signaling pathway (Turk & Plemenitaš 2002), and differential gene expression (Petrovic *et al*. 2002, Vaupotič & Plemenitaš 2007).

ECOLOGY OF *HORTEA WERNECKII*

Hypersaline environments worldwide are dominated by halophilic prokaryotes (Oren 2002). Nevertheless, some rare representatives of *Eukarya* have also adapted to extreme conditions prevailing in man-made salterns and salt lakes. Besides the brine shrimp *Artemia salina,* the alga *Dunaliella,* and some species of protozoa, a surprising diversity of fungi are well adapted to these extreme conditions (Gunde-Cimerman *et al*. 2005).

The dominant group of fungi in hypersaline waters of the salterns are black yeasts (de Hoog 1977) or meristematic ascomycetes (Sterflinger *et al*. 1999) from the order *Dothideales*. *Hortea werneckii* is the dominant black yeast species in hypersaline waters at salinities above 3.0 M NaCl (Gunde-Cimerman *et al*. 2000). Morphology of *H. werneckii* is characteristically polymorphic (de Hoog *et al*. 1993, Wollenzien *et al*. 1995, Sterflinger *et al*. 1999, Zalar *et al*. 1999), hence it has received many designations in the past (Plemenitaš & Gunde-Cimerman 2005). Its molecular differentiation is based on the sequencing of the ITS rDNA region and RFLP markers from SSU rDNA and ITS rDNA regions (de Hoog *et al*. 1999).

Hortea werneckii was primarily known as the etiological pathogen of human dermatosis called *tinea nigra*, a superficial infection of the human hand, strictly limited to the salty, greasy *stratum corneum* of the skin (de Hoog & Gerrits van den Ende 1992, Göttlich *et al*. 1995). It was also known as a contaminant of salty food (Mok *et al*. 1981, Todaro *et al*. 1983) and other low-water-activity substrates such as arid inorganic and organic surfaces (Wollenzien *et al*. 1995), seawater (Iwatsu & Udagawa 1988) and beach soil (de Hoog & Guého 1998). Two successive yr of investigations of potential mycobiota in evaporite ponds of solar salterns along the Slovenian Adriatic coast revealed that the primary environmental ecological niche of *H. werneckii* is hypersaline water (Gunde-Cimerman *et al*. 2000, Butinar *et al*. 2005). *Hortea werneckii* was found within the

entire environmental salinity range (0.5 – 5.2 M NaCl), with three prominently expressed seasonal peaks, which correlated primarily with high environmental nitrogen values. At 3 – 4.5 M NaCl, at the highest peak in August, *H. werneckii* represented 85 – 90 % of all isolated fungi, whereas it was detected only occasionally when NaCl concentrations were below 1.0 M. Although it was later also identified in hypersaline waters of eight other salterns on three continents (Gunde-Cimerman *et al*. 2000, Butinar *et al*. 2005, Cantrell *et al*. 2006), it has never been isolated from oligotrophic hypersaline waters nor from athalasso-haline waters of salt lakes and only rarely from hypersaline waters with elevated temperatures (Gunde-Cimerman *et al*. 2005). Its complex polymorphic life cycle enables *H. werneckii* to colonise other ecological microniches in the salterns besides brine, such as the surface and interior of wood submerged in brine (Zalar *et al*. 2005), thick bacterial biofilms on the surface of hypersaline waters, the soil in dry evaporite ponds and the saltern microbial mats (Butinar *et al*. 2005, Cantrell *et al*. 2006).

COMPATIBLE SOLUTE STRATEGY IN THE CELLS OF *H. WERNECKII*

Cells living in natural saline systems must maintain lower water potential than their surroundings to survive and proliferate. Osmotic strategy employed by most eukaryotic microorganisms inhabiting hypersaline environments is based on the cytoplasmic accumulation of "compatible solutes" – low-molecular-weight organic compounds (Oren 1999) and on maintaining the intracellular concentrations of sodium ions bellow the toxic level for the cells. Mechanisms of salt tolerance have been studied in salt-sensitive *S. cerevisiae* (Blomberg 2000) and in a few halotolerant fungi such as *Debaryomyces hansenii, Candida versatilis*, *Rhodotorula mucilaginosa* and *Pichia guillermondii* (Andre *et al*. 1988, Almagro *et al*. 2000, Silva-Graca & Lucas 2003, Prista *et al*. 2005, Ramos 1999, 2005). Although in *D. hansenii* osmotic adjustments of the major intracellular cations occurs in response to osmotic stress (Blomberg & Adler 1992, Ramos 2005), data from the other investigated fungi show that the maintenance of positive turgor pressure at high salinity is mainly due to an increased production and accumulation of glycerol as a major compatible solute (Pfyffer *et al*. 1986, Blomberg & Adler 1992).

Initial physiological studies in *H. werneckii* showed that, in contrast to *D. hansenii,* it keeps very low intracellular potassium and sodium levels even when grown in the presence of 4.5 M NaCl. Interestingly, in *H. werneckii* the amounts of K^+ and Na^+ were the lowest in the cells grown at 3.0 M NaCl. At this salinity of the medium *H. werneckii* still grows well, but most probably this salinity represents a turning point, shown in restricted colony size, slower growth rate and characteristic changes of physiological behaviour (Plemenitaš & Gunde-Cimerman 2005, Kogej *et al.* 2007). Our primary studies showed that glycerol is the most important compatible solute in *H. werneckii* (Petrovic *et al*. 2002), although these authors indicates the possible presence of other compatible solute(s). Further studies have indeed revealed that *H. werneckii,* when grown in hypersaline media, also accumulates a mixture of organic compounds besides glycerol, including the polyols such as erythritol, arabitol and mannitol. They varied in amounts both with the salinity of the growth medium and with the growth phase of the fungal culture (Table 1). However, the total amount of polyols correlated well with increasing salinity mostly for the account of glycerol and during all growth phases (Kogej *et al*. 2007).

When the growth-phase dependence of compatible solutes in *H. werneckii* grown at extremely high salt concentrations was followed, it appeared that glycerol accumulated predominantly during the exponential growth phase and diminished steeply during the stationary phase. On the other hand, the amount of erythritol increased gradually during the exponential growth phase and reached its highest level during the stationary phase. The amounts

Table 1. Compatible solutes in *H. werneckii*. Intracellular amounts of polyols and mycosporine-glutaminol-glucoside (myc-gln-glc) in *H. werneckii* grown at various salinities and measured A. in the logarithmic growth phase; B. in the stationary phase (data from Kogej *et al*. 2007). The values are in mmol per g dry weight.

A.						
	without NaCl	**0.86 M NaCl**	**1.71 M NaCl**	**2.91 M NaCl**	**3.42 M NaCl**	**4.28 M NaCl**
Glycerol	0.244	1.259	2.294	2.458	2.823	2.941
Erythrytol	0.026	0.104	0.314	0.309	0.252	0.275
Arabitol	0.315	0.165	0.043	0	0	0
Mannitol	0.249	0.155	0.018	0	0	0
Myc-gln-glc	0.011	0.003	0.008	0.004	0.003	0.003
B.						
	without NaCl	**0.86 M NaCl**	**1.71 M NaCl**	**2.91 M NaCl**	**3.42 M NaCl**	**4.28 M NaCl**
Glycerol	0.021	0.102	0.021	1.243	1.225	0.929
Erythrytol	0.016	0.420	0.597	0.728	0.557	0.544
Arabitol	0.128	0.067	0.004	0	0	0
Mannitol	0.443	0.087	0	0	0	0.37
Myc-gln-glc	0.060	0.159	0.146	0.036	0.024	0.019

of other compatible solutes remained low, thus the total amount of polyols decreased during the stationary phase. In the stationary growth phase, *H. werneckii* also accumulated different amounts of two different mycosporines in addition to polyols. Mycosporines, substances with an aminocyclohexenone unit bound to an amino acid or amino alcohol group, were initially known as morphogenetic factors during fungal sporulation and as UV-protecting compounds (Bandaranayake 1998). The hypothesis that in certain microorganisms the mycosporines or mycosporine-like amino acids might play a role as complementary compatible solutes (Oren & Gunde-Cimerman 2007) was lately confirmed for *H. werneckii* with identification of mycosporine-glutaminol-glucoside in produced during the stationary growth phase. This mycosporine accumulated steeply from up to 1.0 M NaCl, and was decreasing at higher NaCl concentrations (Kogej *et al*. 2006). This pattern corresponded with the growth curve of *H. werneckii*. Given their lower content in the cells (Table 1B), they probably do not have as significant a role in osmoadaptation as polyols, but they still contribute to the internal osmotic potential.

CELL-WALL MELANISATION REDUCES GLYCEROL LOSS IN *H. WERNECKII*

Cell walls of black yeasts are melanised. *Hortea werneckii* synthesises a 1,8-dihydroxynaphthalene-(DHN)-melanin under saline and non-saline growth conditions (Kogej *et al*. 2004, 2006). The ultrastructure of melanised cells was compared to the ones grown in the presence of the melanisation inhibitor tricyclazole (Andersson *et al*. 1996). In melanised *H. werneckii* cells, melanin was observed as electron-dense granules in or on the electron-translucent cell walls, whereas the cells with blocked melanin biosynthesis either had no electron-dense granules or these were smaller and lighter in colour. In cells grown without NaCl, melanin granules were deposited in the outer layer of the cell wall forming a thin layer of melanin with separate larger granules. When grown at optimal salinity, *H. werneckii* formed a dense shield-like layer of melanin granules on the outer side of the cell wall. At higher salinities the melanin granules were larger and scarce, and they did not form a continuous layer. In conclusion, *H. werneckii* is highly melanised at low salinities close to the growth optimum, whereas melanisation is reduced at higher salinities (Kogej *et al*. 2007).

We hypothesised that melanin might have a role in the osmoadaptation of *H. werneckii*. A physiological response of *H. werneckii* to the elevated concentrations of NaCl is hyperaccumulation of glycerol in the cells. Compared to other uncharged polar molecules, glycerol has a high permeability coefficient for passage through the lipid bilayers due to its small molecular mass. Therefore, eukaryotic cells using glycerol as a compatible solute combat this either by accumulation of the lost glycerol by transport systems (Oren 1999), which is energetically costly, or by a special membrane structure (high sterol content or reduced membrane fluidity (Oren 1999). For example, in the halophilic alga *Dunaliella*, the lowered membrane permeability for glycerol is correlated with its high sterol content (Sheffer *et al*. 1986, Oren 1999).

Although in *H. werneckii* the ergosterol as the principal sterol together with 23 other types of sterols (Turk *et al*. 2004) constitute the most distinct lipid fraction of cell membranes (Mejanelle *et al*. 2001), the total sterol content remains mainly unchanged with increased salinity. In addition, the plasma membrane of *H. werneckii* is significantly more fluid over a wide range of salinities in comparison with the membranes of the salt-sensitive and halotolerant fungi (Turk *et al*. 2004, 2007). *Hortea werneckii* can thus grow at very high salinities, which require high intracellular amount of glycerol, but at the same time it maintains a very fluid membrane and constant sterol content. It seems that instead of modifying its membrane structure, *H. werneckii* uses a modification of the cell-wall structure to reduce glycerol leakage from the cells. The cell-wall melanisation namely minimises glycerol loss from the cells: as melanin granules form a continuous layer in the outer part of the cell wall, they create a mechanical permeability barrier for glycerol by reducing the size of pores in the cell wall (Jacobson & Ikeda 2005), and thus improving glycerol retention. At optimal salinities *H. werneckii* probably maintains a balance between energetically cheap production of glycerol, which partially leaks out of the cells and therefore needs to be recovered, and by energetically more costly synthesis of other compatible solutes, which escape less easily from the cells and are therefore retained more efficiently. Melanised cell walls reduce the energy needs of *H. werneckii* by retaining the glycerol in the cells. At higher salinities, where melanisation is diminished, higher energy demands of *H. werneckii* are reflected in reduced growth rates and biomass yield at salinity above 3.0 M NaCl (Kogej, unpubl. data). Perhaps the higher proportion of polymorphic cells observed at the increased salinity is another mechanism for reducing glycerol leakage when melanisation is diminished.

As mentioned above, *H. werneckii* maintains a highly fluid membrane also at increased salinities: it decreases C16:0 and increases *cis*-C18:2$^{\Delta 9,12}$ fatty-acyl residues of the membrane lipids (Turk *et al*. 2004), a phenomenon, which is otherwise observed in cells, subjected to low temperatures. A molecular mechanism contributing to such an adaptation mode is partly enabled by the salinity-regulated expression of genes involved in fatty-acid modification. In *S. cerevisiae*, such a response has been observed for genes encoding a Δ^9-desaturase (*OLE1*) and two long-chain fatty-acid elongases (*ELO2*, *ELO3*) (Causton *et al.* 2001). Recently, multiple copies of genes encoding desaturases and elongases were identified in the genome of *H. werneckii*. Their expression pattern, which was determined at different salinities and osmotic stresses, suggests that desaturases and elongases play an important role particularly after sudden (acute) changes in environmental salinity (Gostinčar, unpubl. data). Gene duplication observed in desaturases, elongases and many other genes in *H. werneckii* (see below) has already been accepted as a general mechanism of adaptation to various stresses also in other organisms. In *S. cerevisiae*, for example, most of the duplicated genes are membrane transporters and genes involved in stress response (Kondrashov *et al*. 2002).
By modifying the cell-wall structure instead of lowering the membrane fluidity, *H. werneckii* can maintain high membrane fluidity even at high salinities, which might be one of the factors enabling its growth at decreased water availability.

SENSING THE INCREASED OSMOLARITY - THE HOG SIGNAL TRANSDUCTION PATHWAY IN *H. WERNECKII*

Multiple signaling pathways allow organisms to respond to different extracellular stimuli and to adjust their cellular machinery to changes in the environment. The sensing of changes in environmental osmolarity is vital for cell survival. In *S. cerevisiae*, the pathway for the sensing of osmolarity changes is known as the high-osmolarity

glycerol (HOG) signaling pathway, and is one of the best understood mitogen-activated protein kinase (MAPK) cascades. Upon osmotic stress, the osmosensors Sho1 and Sln1 stimulate this pathway by two distinct mechanisms, converging the signal at the MAPK kinase Pbs2, which phosphorylates its downstream MAP kinase Hog1, a key MAP kinase of the pathway (Hohmann 2002, O'Rourke *et al*. 2002, Westfall *et al*. 2004). Phosphorylated Hog1 controls the transcription of a family of osmoresponsive genes (Tamas *et al*. 2000, Yale & Bohnert 2001, Proft *et al*. 2006).

Hortea werneckii's ability to adapt to a wide range of salinities indicates the presence of an efficient system that can both sense and respond to these changes. The existence of a signaling pathway similar to the *S. cerevisiae* HOG pathway was demonstrated by identification of putative sensor proteins HwSho1 and histidine kinase-like osmosensor HwHhk7, together with two MAP kinases: MAPKK HwPbs2 and the final MAPK HwHog1 (Lenassi *et al*. 2007, Turk & Plemenitaš 2002). We found that the genome of *H. werneckii* contains one copy of the *S. cerevisiae* homologue gene for the osmosensor Sho1, *HwSHO1.* When compared to other known Sho1 proteins, HwSho1 shows a distinct membrane topology with inverted orientation, suggesting different localisation of HwSho1. To obtain better insight into the role of the HwSho1, the protein was expressed in *S. cerevisiae sho1* mutant strain. We demonstrated that the HwSho1 protein can rescue the osmosensitivity of the *S. cerevisiae sho1* mutant, despite its much lower binding affinity to the scaffold protein Pbs2, when compared to the binding affinity of *S. cerevisiae* Sho1 to Pbs2. It appears that the affinity of binding between HwSho1 and Pbs2 depends not only on the SH3 domain at the C-terminus of HwSho1, but also on the amino-acid sequence surrounding the domain. We also assessed the salt-dependent gene expression and found that the expression of *HwSHO1* is only weakly salt-responsive. We proposed that a preferred role of HwSho1 is in general cellular processes rather than in quick responses to the changes in osmolarity (Lenassi, unpubl. data).

The genome of *H. werneckii* contains two copies of histidine kinase genes with the putative role in osmosensing (Lenassi & Plemenitaš 2007). As many of the *H. werneckii* genes that have so far been associated with adaptation to high osmolarity are present in two copies in the genome (Plemenitaš & Gunde-Cimerman 2005), perhaps the histidine kinase duplication could be beneficial for *H. werneckii* living in environments with fluctuations in salt concentration. A comparison of the translated nucleotide sequence of the product from *H. werneckii* with the protein database revealed a high homology with the histidine kinase ChHhk17 from *Cochliobolus heterostrophus*. ChHhk17 and the related BfHhk17 of *Botryotinia fuckeliana* are members of the group 7 of fungal histidine kinases. The isolated genes from *H. werneckii* were therefore named *HwHHK7A* and *HwHHK7B*. An inspection of the relative positions of all fungal histidine kinase groups on a phylogenetic tree (Catlett *et al*. 2003) shows that histidine kinase Sln1 from *S. cerevisiae* and HK7 group position close together, indicating late separation from a common ancestor. The most obvious difference between the Sln1 and HK7 group, however, is the intracellular localisation of the proteins. While histidine kinases of the Sln1 group are membrane bound, histidine kinases from HK7 group are soluble, cytosolic proteins. Since the secondary structure of some histidine kinases are known, we could predict the secondary structures of the described domains with a high degree of certainty. We confirmed that HwHhk7A and HwHhk7B isoforms have all the regions necessary to function as eukaryotic hybrid-type histidine kinases (Wolanin *et al*. 2002). No transmembrane domain could be predicted in the HwHhk7 proteins from *H. werneckii*, which distinguished them from the *S. cerevisiae* Sln1 protein with two transmembrane domains.

Transcription of *HwHHK7A* gene was not very responsive to the changes in NaCl concentration. In contrast, the expression of *HwHHK7B* gene was highly salt-responsive, with higher levels of expression through the whole range of salinities when compared to *HwHHK7A* gene expression. Salt-dependent expression pattern of *HwHHK7* indicated the existence of two types of responses, an early response to hyposaline and a late response to hypersaline stress (Lenassi & Plemenitaš 2007). Our data suggest that the high induction of *HwHHK7B* gene expression as an early response to hyposaline stress could be the result of the specialised role of this histidine kinase in response to conditions of modest osmolarity, as has already been demonstrated for the Sln1 (O'Rourke & Herskowitz 2004). These results lead us to speculate that the role of isoform HwHhk7B in the adaptation of *H. werneckii* is mostly in sensing and adapting to the sudden changes of salinity, which are very common in this organism's natural habitat.

The role of Sln1 in the HOG pathway is generally well studied and well evidenced (Hohmann 2002). By contrast, none of the HK7 group protein members has a known function. Interestingly, all other fungal species but *H. werneckii*, which code for HK group 7, are known as plant or human pathogens (Furukawa *et al*. 2005, Nemecek *et al*. 2006). The lifestyle of some plant pathogens has similarities with life in a high osmolarity environment, as they must also be able to adapt to fluctuating osmolarity when invading the victim organism (Han & Prade 2002). As controlling the osmotic response on the cellular level is of great importance to the pathogenicity of fungi, other HK7 group members could also have a role in osmosensing, as it was predicted for HwHhk7B in *H. werneckii.* The absence of hybrid histidine kinases from animals makes these proteins prominent antimicrobial targets (Santos & Shiozaki 2001), thus group 7 of HKs could present novel sites for the development of fungal inhibitors.

Both osmosensors, Sho1 and Sln1 proteins in *S. cerevisiae* transmit the signals to the downstream MAP kinase cascade of the HOG signal transduction pathway (Hohmann 2002). In *H. werneckii*, we found homologues of two MAP kinases: HwPbs2 and HwHog1 (Turk & Plemenitaš 2002). In *S. cerevisiae*, Pbs2 functions both as a MAPK kinase and as a scaffold protein, which recruits multiple proteins involved in the activation of the HOG pathway. Upon activation, Pbs2 then phosphorylates the target kinase Hog1 (Hohmann 2002). In *H. werneckii,* we found two gene copies of *HwPBS2* that are transcribed and translated into three different isoforms: HwPbs2A, HwPbs2B1 and HwPbs2B2. The expression of *HwPBS2A* and *HwPBS2B2* isoforms was increased 4-fold in the cells adapted to 4.5 M NaCl, whereas the expression of *HwPBS2B1* was not salt-responsive. As suggested with RNA polymerase II-chromatin immunoprecipitation (RNAPol-ChIP) experiments and promoter analysis, the higher steady-state concentration of *HwPBS2A* transcript in respect to *HwPBS2B2* is the consequence of the activation of *HwPBS2A* gene transcription. The expression profiles of *HwPBS2* genes suggested the putative role of HwPbs2A and HwPbs2B2 in response to quick adaptation to severe hyperosmotic shock, whereas the role of HwPbs2B1 is in response to moderate stress adaptation (Lenassi, unpubl. data). In contrast to *S. cerevisiae*, we showed that HwPbs2 proteins are not only localised to the cytosol, but they also bind to the plasma membrane at higher salinities (Turk & Plemenitaš 2002). The HwPbs2 complemented the defect of the *S. cerevisiae pbs2* mutant strain only weakly. This could be explained by the absence of the appropriate binding partners for the HwPbs2 isoforms in *S. cerevisiae* and may indicate the existence of specialised roles of multiple isoforms in the HOG signaling pathway of *H. werneckii.* This explanation could be supported by our finding that HwPbs2

isoforms have a conserved kinase domain, but a very diverse scaffold binding part.

Moving downstream through the cascade, we have also identified the *S. cerevisiae* homologue of the key MAP kinase in *H. werneckii* - HwHog1 (Turk & Plemenitaš 2002). As in *S. cerevisiae*, the genome of *H. werneckii* contains only one copy of the *HOG1* gene. The *HwHOG1* open reading frame encodes a protein of 359 amino-acid residues with a predicted molecular weight of 46 kDa and with all of the conserved regions that are specific for the MAPKs, such as the common docking (CD) domain at the C-terminal end, a TGY phosphorylation motif at amino-acid residues 171–173, and an Asp in the active site. The 3-dimensional model of the full-length HwHog1 protein revealed an overall structural homology with other known MAPKs (Turk & Plemenitaš 2002, Lenassi *et al*. 2007). Although the HwHog1 protein shows high homology to the *S. cerevisiae* Hog1, important differences in both activation and localisation of the phosphorylated and non-phosphorylated forms of HwHog1 have been observed. An *in vitro* kinase assay demonstrated that in contrast to *S. cerevisiae*, where Hog1 is activated even at very low salt concentrations, HwHog1 is fully active only at extremely high salt concentrations (Turk & Plemenitaš 2002). *HwHOG1* successfully complemented the *S. cerevisiae hog1* phenotype at increased osmolarity, caused by 1.0 M NaCl, 1.0 M KCl, or 1.5 M sorbitol. We demonstrated not only that the cells expressing HwHog1 have restored tolerance to sodium and potassium ions and to sorbitol, but also that the osmotolerance was restored only in the presence of the MAPKK Pbs2 (Lenassi *et al*. 2007).

The HOG pathway has classically been considered as specific to osmotic stress. Recent studies have suggested that Hog1 can also be activated in response to heat shock, cold stress, oxidative stress, and UV injury (Gacto *et al*. 2003, Panadero *et al*. 2006). To test the response of HwHog1 to these alternative stresses, we analysed the growth ability of *S. cerevisiae* wild-type, *hog1* and *pbs2* strains expressing the HwHog1, after exposure to UV, high pH, H_2O_2, and low or high temperatures. We found that the activation of HwHog1 is less efficient in response to UV stress than in wild-type *S. cerevisiae* (Lenassi *et al*. 2007). However, when both yeasts were exposed to UV irradiation, *H. werneckii* was much more resistant to UV than *S. cerevisiae* (Turk, unpublished). As melanin is a well-known UV protectant, we can speculate that it is responsible for high viability in melanised *H. werneckii*, and therefore, we can also conclude that the activation of the HOG signaling pathway might not be involved in the UV stress response in *H. werneckii*. In contrast, the HOG signaling pathway is important for the oxidative stress in *H. werneckii cells*. *S. cerevisiae* cells expressing HwHog1 are much more resistant to H_2O_2 than wild-type cells. Furthermore, this phenotype depends on the presence of the MAPKK Pbs2. The ability of *H. werneckii* to combat oxidative stress has recently been addressed again, using hydrogen peroxide as the reactive oxygen species (ROS)-generating compound. Exposure to H_2O_2 resulted in a decrease in *H. werneckii* viability at extremely high salt concentrations, suggesting that the level of ROS degradation and resistance determine the upper limits of the salt tolerance of *H. werneckii* (Petrovic 2006). HwHog1 also appears to mediate the response to high-temperature, but not low-temperature stresses. Amongst all tested stresses, only the heat-shock response is independent of the Pbs2 protein (Lenassi *et al*. 2007). These data suggest that heat-shock signals that activate HwHog1 are transmitted via a pathway distinct from the classical HOG pathway, in which this MAPK and the scaffold protein Pbs2 have crucial roles. High temperature is stressful for *H. werneckii*, as has been shown by ecological studies. So far only a few strains of *H. werneckii* with optimal growth at 32°C were isolated, while the majority typically prefers lower environmental temperatures (Cantrell *et al*. 2006). Activation of HwHog1 could be of general importance in regulating the transcription of the gene set that is involved in combating high-temperature stress. In contrast, *H. werneckii* seems to be more adapted to lower temperatures and therefore HwHog1 is not activated upon low-temperature exposure. Likewise, the exposure of cells to elevated pH turned out not to be connected to HOG pathway activation (Lenassi *et al*. 2007).

RESPONDING TO INCREASED OSMOLARITY BY DIFFERENTIAL GENE EXPRESSION

When an organism is subjected to extreme environmental conditions for extended periods of time, physiological and metabolic changes lead to adaptive responses and tolerance that depend on the response mechanisms available to the system. Previous studies on *S. cerevisiae* have suggested a critical role of differential protein expression to counteract changes in environmental salinity (Norbeck & Blomberg 1997, Li *et al*. 2003, Liska *et al*. 2004). In contrast to *S. cerevisiae*, *H. werneckii* is well adapted to fluctuations in NaCl concentrations. Differentially expressed genes in *H. werneckii* cells grown at different salinities therefore represent the transcriptional response of the adapted cells rather than their stress response. By applying a suppression subtractive hybridisation (SSH) technique coupled with a mirror orientation selection (MOS) method, we identified a set of 95 osmoresponsive genes as differentially expressed in *H. werneckii* adapted to moderately saline environment of 3 M NaCl or extremely saline environment of 4.5 M NaCl. Among them, more than half were functionally related to general metabolism and energy production. Thirteen unclassified genes with no orthologues in other species, which we called *SOL* genes, represented a specific transcriptional response unique to *H. werneckii* (Vaupotič & Plemenitaš 2007). The transcriptional induction or repression of approximately 500 genes in *S. cerevisiae* that are strongly responsive to salt stress was highly or fully dependent on the MAPK Hog1, indicating that the Hog1-mediated signaling pathway plays a key role in global gene regulation under saline stress conditions (Posas *et al*. 2000, O'Rourke & Herskowitz 2004). We approached the study of a possible interaction of endogenous HwHog1 with the chromatin regions of identified up-regulated genes in optimal salinity- or hypersaline-adapted *H. werneckii* cells by a chromatin immunoprecipitation (ChIP) assay. Lacking the information about promoter regions for the identified differentially-expressed genes in *H. werneckii*, a ChIP-coding region PCR amplification was performed (Vaupotič & Plemenitaš 2007). Recently, it has been shown that the activated Hog1 in *S. cerevisiae* is associated with elongating RNA polymerase II and is therefore recruited to the entire coding region of osmoinducible genes (Proft *et al*. 2006). HwHog1 cross-linked with the coding region of 36 of the differentially expressed genes. For 34 up-regulated genes, the interaction with HwHog1 was stronger in cells adapted to 4.5 M NaCl, whereas for 2 down-regulated genes the HwHog1-ChIP signal was stronger in cells adapted to 3 M NaCl, showing not only the transcriptional induction but also the transcriptional repression by HwHog1 (Vaupotič & Plemenitaš 2007). Genome-wide expression profiling studies using wild-type and *hog1* mutant *S. cerevisiae* cells were performed to comparatively identify genes whose up-regulation of expression was dependent on Hog1 (Yale & Bohnert

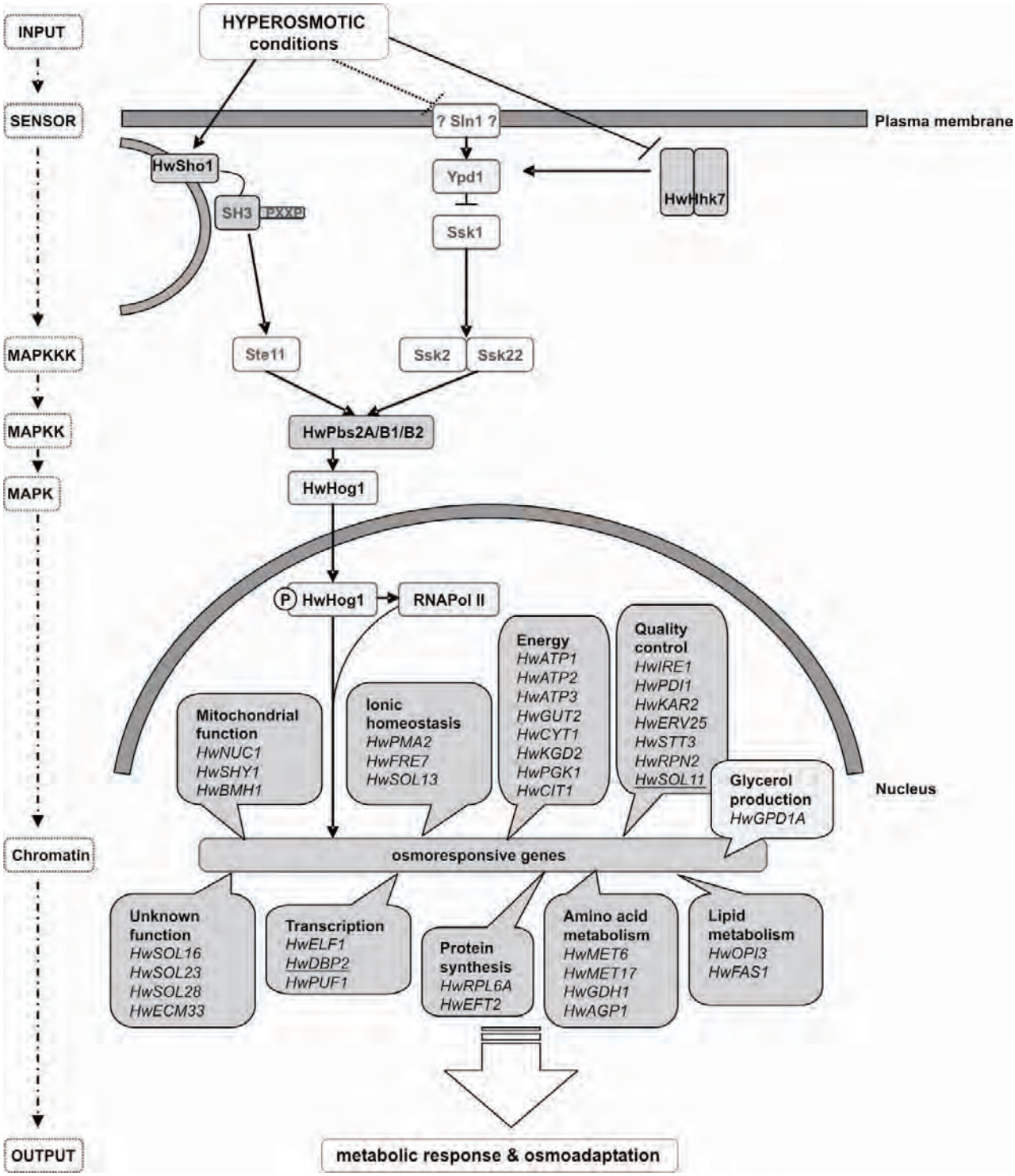

Fig. 1. The model of HOG signaling pathway response during the long-term hypersaline adaptation in the extremely halophilic *H. werneckii*.
Hyperosmotic conditions (4.5 M NaCl) activate the plasma membrane localised osmosensor of the pathway. However, unlike in *S. cerevisiae*, HwSho1 is most likely localised on an inner cell membrane. The Sln1-Ypd1-Ssk1 phosphorelay is much more complex, with an input from at least one more histidine kinase (HwHhk7) and with a questionable role of Sln1 homologue. The signals from both pathways converge at the level of Pbs2 MAPKK homologues (HwPbs2A, HwPbs2B1, and HwPbs2B2). HwPbs2 isoforms putatively activate the HwHog1, a key MAP kinase of the pathway. Upon phosphorylation and translocation into the nucleus, the phosphorylated HwHog1 associates with the chromatin of osmoresponsive genes and thereby promotes (or represses; underlined genes) the transcription, either by recruitment and/or activation of transcriptional factors or by direct association with the RNA polymerase II (RNAPol II), or both. The protein products of HwHog1-interracting osmoresponsive genes belonging to indicated functional groups contribute to the crucial metabolic changes required for successful adaptation to the severe osmotic environment. Although *H. werneckii* has roughly retained the structure of the HOG pathway, it has also developed many distinctive features. The identified components of the *H. werneckii* HOG pathway are shown in dark grey, the evolutionary highly conserved components are shown in light grey, the known components of the *S. cerevisiae* HOG pathway are colorless. HwHog1 responsive genes are: *HwAGP1*, amino acid permease; *HwATP1*, ATPase alpha-subunit; *HwATP2*, ATPase beta-subunit; *HwATP3*, ATPase gamma-subunit; *HwBMH1*, 14-3-3 protein; *HwCIT1*, citrate synthase; *HwCYT1*, cytochrome c1; *HwDBP2*, RNA helicase; *HwECM33*, extracellular matrix protein 33; *HwEFT2*, translation elongation factor 2 (eEF-2); *HwELF1*, transcription elongation factor; *HwERV25*, p24 component of the COPII-coated vesicles; *HwFAS1*, fatty-acid synthase acyl-carrier protein; *HwFRE7*, ferric-chelate reductase 7; *HwGDH1*, glutamate dehydrogenase; *HwGPD1A*, glycerol-3-phosphate dehydrogenase A; *HwGUT2*, FAD-dependent glycerol-3-phosphate dehydrogenase; *HwIRE1*, protein kinase/endoribonuclease; *HwKAR2*, endoplasmic reticulum luminal chaperone; *HwKGD2*, dihydrolipoamide succinyltransferase; *HwMET17*, cystein synthase, *HwMET6*, methionine synthase; *HwNUC1*, mitochondrial nuclease; *HwOPI3*, unsaturated phospholipid methyltransferase; *HwPDI1*, protein disulphide isomerase; *HwPGK1*, 3-phosphoglycerate kinase; *HwPMA2*, plasma membrane proton-exporting ATPase; *HwPUF1*, pumilio-family RNA-binding domain protein; *HwRPL6A*, 60S ribosomal protein 6A; *HwRPN2*, 26S proteasome regulatory subunit; *HwSHY1*, mitochondrial inner membrane protein chaperone; *HwSTT3*, oligosaccharyltransferase catalytic subunit; *SOL11*, mannose-P-dolichol utilization defect 1 protein; *SOL13*, opsin 1; *SOL16*, senescence-associated protein; *SOL23*, hyperosmolarity-induced mRNA 23; *SOL28*, hyperosmolarity-induced mRNA 28.

2001, O'Rourke & Herskowitz 2004, Proft *et al*. 2006). Only the *UGP1* orthologue was also induced in *H. werneckii* cells adapted to 4.5 M NaCl and in cells exposed to a sudden change in salinity. However, in contrast to *S. cerevisiae*, upregulation of *HwUGP1* turned out to be independent of HwHog1 (Vaupotič & Plemenitaš 2007). Other HwHog1-ChIP positive genes in *H. werneckii* were reported for the first time in connection with MAPK Hog1 by our study, reflecting the complexity of HOG signaling pathway. The relative distribution of HwHog1-dependent genes was approximately equivalent among functional categories, except for transcription, cellular transport, signal transduction mechanism, and cell fate functional categories, where the HwHog1-ChIP positive genes represented more than 70 % fraction of tested genes. Only 2 of 10 tested genes with unknown function (*SOL23* and *SOL28*) were HwHog1-ChIP positive.

It has been previously shown that during the HOG response, the nuclear retention and chromatin association of Hog1 in *S. cerevisiae* depends on the co-localisation with general transcription machinery components (Alepuz *et al*. 2001, Alepuz *et al*. 2003). A sequential HwHog1-ChIP analysis (SeqChIP) using primers specific for the genes identified as HwHog1-positive was performed after the primary RNAPol-ChIP in *H. werneckii* (Vaupotič & Plemenitaš 2007). The co-localisation of HwHog1 and RNA polymerase II existed in 17 out of 36 HwHog1-ChiP positive differentially expressed genes. Co-occupation of HwHog1 and RNA polymerase II on target genes resulted in an increased PCR signal in SeqChIP with the accompanying increased level of corresponding transcript in RT-PCR analyses. These observations indicate a stimulating role for HwHog1 and RNA polymerase II co-localisation on the efficiency of transcription of indicated genes in high-salt adapted *H. werneckii* and reflect HwHog1-RNAPolII-chromatin interactions, relevant for the extremely hypersaline conditions, which have so far not been studied in salt-sensitive organisms. Based on our results and in comparison with *S. cerevisiae*, we built the model of HOG signaling pathway in *H. werneckii*, which is shown in Fig.1.

CONCLUSIONS

Black yeast *H. werneckii* is so far the most studied extremely halotolerant eukaryotic model organism. According to our data, *H. werneckii* can be classified as a sodium extruder with an intricate compatible solute strategy, as a response to elevated NaCl concentrations. The main compatible solute of *H. werneckii* is glycerol, which is complemented by erythritol and partially by mycosporine-glutaminol-glucoside in the stationary-phase cells. At low salinities, *H. werneckii* accumulates a mixture of glycerol, erythritol, arabitol and mannitol, whereas glycerol and erythritol prevail at high salinities. At optimal growth salinities, the melanised cell wall helps in retaining high concentrations of glycerol in the cells of *H. werneckii,* despite the highly fluid membrane. The novelty of osmoadaptation of the halophilic fungus *H. werneckii*, probably contributing to its growth at a wide salinity range, is an effective combination of the accumulation of known compatible solutes polyols and of melanised cell walls for improved osmolyte retention.

Our studies confirmed the important role of the HOG signaling pathway in the osmoadaptation and in the stress response of *H. werneckii*. This pathway is activated not only in response to hyperosmotic stress, but also to oxidative and heat stress, both typical for solar salterns. At high salt concentrations, the induction of a completely different set of osmoresponsive genes was observed in *H. werneckii* when compared to salt-sensitive *S. cerevisiae*. Most of these are novel in terms of their interaction with the major transcriptional regulator HwHog1, the mitogen-activated protein kinase of the HOG signaling pathway. Moreover, in *H. werneckii*, HwHog1 mediates not only the early phase of the osmotic induction of many osmo-responsive genes, but it also supports a high RNA-polymerase II-dependent elongation rate of target genes in long-term-adapted cells growing at extremely high salinities. Our studies revealed distinct molecular mechanisms in sensing and responding to changes in environmental osmolarity in *H. werneckii* when compared to the conventional model yeasts, such as salt-sensitive *S. cerevisiae* and moderately halotolerant *D. hansenii*. Differences in protein structure, different intracellular localisation of the components, which are involved in signal transduction, and multiple gene copies, are crucial for these adaptations.

Since salt stress is an increasing threat to agriculture in many productive areas of the world, it is important to bridge the gap between salt toxicity in plants and knowledge of molecular mechanisms of adaptation in extremely halotolerant model eukaryotic cells. Our studies showed that *H. werneckii* is also a promising source of salt tolerant transgenes for agriculture. We identified and characterised two novel isoforms of 3'-phosphoadenosine-5'-phosphatases or Hal2-like proteins from *H. werneckii.* Overexpression of both isoenzymes, HwHal2A and HwHal2B from a low copy number vector in *S. cerevisiae* remarkably increased its halotolerance (Vaupotič *et al*. 2007).

Taken together, an interplaying array of adaptational mechanisms at different levels make *H. werneckii* a very versatile halophile, which is able to grow at a broader salinity range than most known microorganisms. Our findings contribute an important advance in understanding the molecular mechanisms underlying the adaptive response of *H. werneckii*, an increasingly useful model organism for studying the mechanisms of salt tolerance in eukaryotic cells.

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Tinea nigra by *Hortaea werneckii*, a report of 22 cases from Mexico

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Abstract: Tinea nigra is a superficial mycosis caused by *Hortaea werneckii*. It is an infrequent asymptomatic infection that affects human palms and soles, and is mostly observed in tropical countries. We evaluate retrospectively twenty-two confirmed cases of tinea nigra from a total of eleven yr (1997–2007) and discuss the epidemiology, clinical features and treatment of this disease. In twelve cases, adults were involved, in 10, children. In nineteen cases the disorder was located on palms of hands and in three on soles of feet. In all cases, the obtained isolates were morphologically identified as *Hortaea werneckii* and the identification of ten isolates was retrospectively confirmed with the help of sequences of the internal transcribed spacer regions of the ribosomal DNA. The patients received topical treatment with Whitfield ointment, ketoconazole, bifonazole, or terbinafine. Treatment with keratolytic agents and topical antifungals was effective.

Key words: *Hortaea werneckii*, keratolysis, melanized fungi, superficial mycosis, tinea nigra, tinea palmaris.

INTRODUCTION

Tinea nigra is a superficial mycosis caused by the melanized, yeast-like fungus *Hortaea werneckii* (Horta) Nishimura & Miyaji, formerly incorrectly classified in genera such as *Cladosporium, Cryptococcus, Exophiala* and *Phaeoannelomyces* (McGinnis *et al.* 1985, de Hoog *et al*. 2000). It is an infrequent, asymptomatic infection, limited to tropical and subtropical countries. Most typically, it affects palms of hands but is occasionally found on other parts of the body. Symptoms include hyperchromic plaques, in which the fungus may or may not live in commensalism with other organisms (Hughes *et al*. 1993, Bonifaz 2001, Gupta *et al.* 2003). The disorder has longtime been regarded as an infection and therefore *Hortaea werneckii* was classified as a BioSafety Level 2 organism (Anon 2004). However, de Hoog & Gerrits van den Ende (1992) and Göttlich *et al*. (1992) noted that tinea nigra is subclinical, only dead keratin cells on the skin being colonized. No keratinolysis can be observed and the adhesion to human hands is to be explained by the hydrophobic character of the yeast cells. It has been proven that the natural habitat of the fungus comprises hypersaline environments due to its halophilic behaviour (Zalar *et al*. 1999, Plemenitaš *et al*. 2008). The present article is a retrospective report of cases of tinea nigra, its epidemiological, clinical, and therapeutic features, as well as a review of the disorder.

MATERIAL AND METHODS

This is an eleven-yr retrospective study (1997−2007) of confirmed clinical cases of tinea nigra (Table 1). Each patient underwent clinical examinations and laboratory tests such as direct KOH (20 %) analysis and culturing on Sabouraud glucose agar without or with antibiotics (Mycobiotic, Difco Co), incubated at 28 °C for 8−30 ds. Each of the strains was identified using macro- and microscopical features. The identification of ten strains was verified with sequences of the internal transcriber spacer regions (ITS) of the rDNA. Methods for DNA extraction and sequencing were those of Badali *et al*. (2008). Sequences were compared using a black yeast molecular database maintained at the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

After diagnosis, the patients received Whitfield ointment topical treatment (salicylic acid 3 %, benzoic acid 2 %) twice daily for 15 ds, or one of the following antifungals as creams: ketoconazole 2 % (Nizoral), bifonazole 1 % (Mycospor) and terbinafine 1 % (Lamisil). Subsequently, follow-up of patients was performed for 1−2 mos after application of the last dose. Cure was defined as the absence of clinical signs and negative culturing (Table 2).

RESULTS

Twenty-two confirmed cases of tinea nigra were reviewed and patient data recorded (Table 1). The ITS region of ten strains was identical with the ITS of the ex-type strain of *Hortaea werneckii*, CBS 107.67. Table 1 shows the strain numbers and the main demographical data of the patients. Twelve out of 22 patients were adults and the remaining were adolescents and children. Mean overall age was 20.7 yr; mean age of the adult patients was 27.3 yr, and 11.2 yr for the adolescents/children. All adults were farmers. Most of the adolescents/children were in school-age. Most patients originated from rural areas in tropical and humid regions characterized by abundant subtropical and tropical vegetation. Several of them reported to have close contact with plants and grasses. Eleven cases originated from coastal zones where patients may have been in contact with substrata of high salinity: the majority of them lived near the sea, salt-marsh or river estuaria. The remaining 11 patients came from metropolitan zones and did not provide any specific information (Fig. 1). The

Table 1. Demographic data of 22 patients with tinea nigra.

Case nr	Age (yr)	Gender	Disease duration (mos)	Geographical zone	Occupation	Predisposing factor	Disease location	Direct exam	Culture	CBS Number
1	23	M	2	MA	Farmer	–	Palm	+	*H. werneckii*	–
2	9	M	1	MA	Junior	–	Palm	+	*H. werneckii*	–
3	25	F	2	LC Tampico	Home	Hyperhydrosis, running on beach	Sole	+	*H. werneckii*	CBS 123043
4	18	M	4	MA	Student	Hyperhydrosis	Palm	+	*H. werneckii*	CBS 123041
5	40	F	3	MA	Home	Hyperhydrosis	Palms (bilateral)	+	*H. werneckii*	–
6	9	M	2	LR Tabasco	Junior	–	Palm	+	*H. werneckii*	–
7	11	M	2	LR Tabasco	Junior	–	Palm	+	*H. werneckii*	–
8	20	M	8	MA	Farmer	–	Palm	+	*H. werneckii*	CBS 123046
9	15	F	2	MA	Home	Hyperhydrosis	Palm	+	*H. werneckii*	CBS 122348
10	2	F	1	LC Tapachula Chis	None	–	Palm	+	*H. werneckii*	–
11	28	M	2	MA	Home	–	Palm	-	*H. werneckii*	CBS 122344
12	25	F	8	MA	Farmer	Hyperhydrosis	Palm	+	*H. werneckii*	CBS 123044
13	16	M	3	LC, Tampico	Junior	Hyperhydrosis	Foot (interdigital)	+	*H. werneckii*	CBS 123045
14	8	F	1	MA	Junior	–	Palm	+	*H. werneckii*	–
15	20	M	1.5	MA	Student	Hyperhydrosis	Palm	+	*H. werneckii*	
16	28	F	2	LC. Cabo San Lucas BC	Farmer & worker	Saltpan worker	Palm	+	*H. werneckii*	–
16	28	F	2	LC. Cabo San Lucas BC	Farmer & worker	Saltpan worker	Palm	+	*H. werneckii*	–
17	12	M	1	LC, LR Veracruz	Junior	–	Palm	+	*H. werneckii*	CBS 123042
18	15	F	3	LC, LR Veracruz	Junior	–	Palm	+	*H. werneckii*	–
19	12	F	2	MA	Home	–	Palm	+	*H. werneckii*	–
20	61	M	18	LC Tampico	Farmer and fisher	–	Palm	+	*H. werneckii*	CBS 122342
21	30	F	2	LC, LR Salinacruz Oax	Junior	Hyperhydrosis, running on beach	Sole	+	*H. werneckii*	CBS 122340
22	28	M	6	LR Acapulco	Student	Hyperhydrosis	Palm	+	*H. werneckii*	–

MA = Metropolitan area; LC = living near coast; LR = living near river.

Table 2. Summary of treatment of tinea nigra.

Topical	No. of cases	No. of applications per d	Mean treatment duration	Cases / Response
Whitfield's ointment	11	2	18 ds	10/ Cure 1 /Relapse*
Ketoconazole	4	2	15 ds	4 /Cure
Bifonazole	4	1	12 ds	4/Cure
Terbinafine	2	1	15 ds	2 /Cure
None	2	–	–	2 / Spontaneous cure

*Treated later with bifonazole.

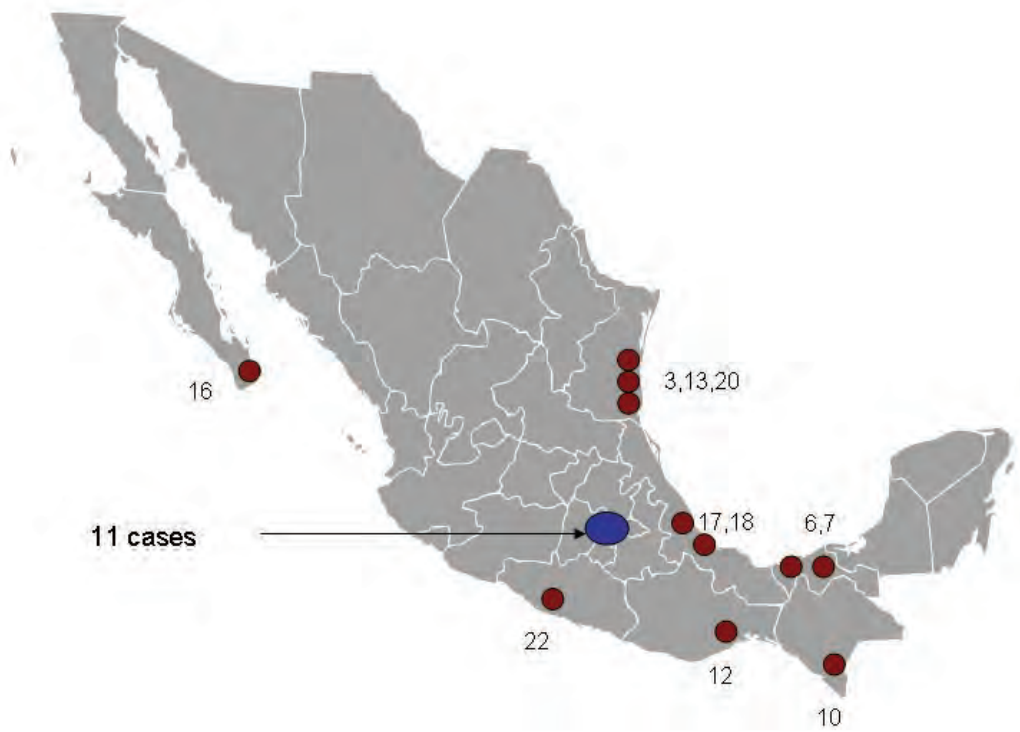

Fig. 1. Map showing the distribution of cases analysed.

majority of patients were without apparent specific predisposing factors for fungal infection. Palm hyperhydrosis was present in 6/22 cases; one case reported professional activities in a saltpan. Two patients used to run barefoot along the beach. The clinical location of tinea nigra was the palms in most cases; in 17 out of 18 cases the infection was unilateral and one was bilateral (Figs 2A–C). Of the cases located on the feet, two occurred on the sole (Fig. 2D) and one was located in three interdigital spaces (Fig. 2E). These three patients reported sole hyperhydrosis; two of them used to walk barefoot along the beach.

Direct examinations were positive in 21/22 cases (95.4 %; Figs 2F–H). All patients had a positive culture that was preliminarily identified as *Hortaea werneckii*; active growth was evident on average after 5.5 ds (Figs 2–4). Biopsies were taken in two patients (due to confusion with nevi); however, perivascular infiltrates were reported in both cases and short fungal filaments could be found on the stratum corneum.

In view of generating a retrospective report, various types of treatment were applied (Table 2). Eleven cases were treated with Whitfield ointment. Treatment failed in one case, which is why ketoconazole cream 2 % was applied successively. Using the antifungals ketoconazole 2 %, bifonazole 1 %, and terbinafine 1 %, all patients achieved clinical and cure within 12–18 ds, with a mean total treatment period of 15 ds. Two patients were not treated with any medication due to uncertain initial diagnosis, but presented spontaneous cure was achieved within about two mos.

DISCUSSION

Hortaea werneckii is best known from (sub)tropical climates and lives in environments with reduced water activity such as sea water, natural or man-made saltpans (Zalar *et al.* 1999), occasionally it can also be present in house dust (Uezato *et al.* 2006). It is a halophilic species, having the capacity to support high salt concentrations (3–30 % NaCl). Its prevalence reaches a peak in highly saline water of crystallization ponds (Gunde-Cimerman *et al.* 2000). Therefore this microorganism has been used as a model to study these conditions of extremotolerance, for example, its role of oxidative stress, osmotic adaptation and melanization (Petrovič 2006, Kogej *et al*. 2007).

The melanized, polymorphic and yeast-like fungus *Hortaea werneckii* may be difficult to recognize by morphological characters. Its relatively restricted, black primary cultures, 1 µm wide annellated zones and one-septate conidia facilitate its specific identification. ITS sequences show limited variation, and the species is clearly distinct from other, closely related taxa (Zalar *et al*. 1999). No teleomorph of this fungus has been found. It is known to be phylogenetically affiliated to the order *Capnodiales* (Crous *et al.* 2007a).

In some regions of Venezuela, *Stenella araguata* Syd. has been reported as a causative agent of tinea nigra (Perez *et al.* 2005). This species is known from only two occasions: the original specimen caused leaf spots on vegetation (*Pithecellobium lanceolatum, Mimosaceae*) and its taxonomic status is now unclear. Another isolate came from a case of tinea nigra (Crous *et al*. 2007b). The ITS of the latter, CBS 105.75, was sequenced (EU019250) and mentioned twice under invalid name *"Catenulostroma castellanii"* (Crous *et al.* 2007a,b). Based on ITS rDNA data, the strain CBS 105.75 is clearly different from *H. werneckii* (G.S. de Hoog, unpublished data), but judging from rDNA large subunit data (Crous *et al*. 2007a) the species is a close relative and might represent a second agent of tinea nigra. As long as its identity with the type of *S. araguata* is pending, it is difficult to attribute a taxonomic name to this fungus.

Tinea nigra is an uncommon discolouration of the skin. Most reports originate from tropical, humid climate zones. Cases from Latin America have been reported from Panama, Colombia, Venezuela, Brazil and Mexico (Chang & Arenas 1983, Durán *et al.* 1983, Severo *et al*. 1994, Pegas *et al*. 2003, Perez *et al.* 2005), while Asian reports came from India, Sri Lanka, Myanmar

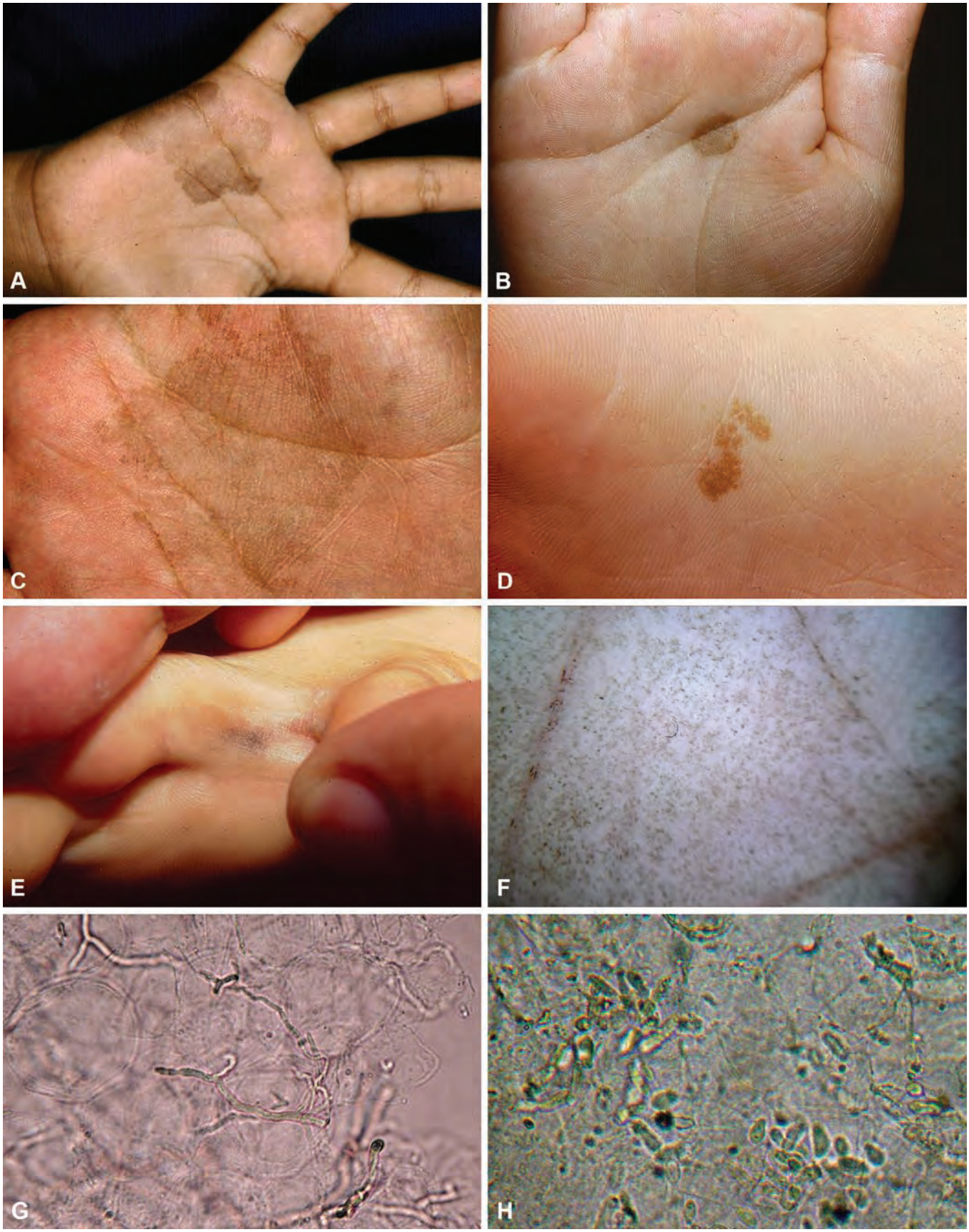

Fig. 2. A. Extensive tinea nigra palmaris (case 1; Table 1); B. Localized tinea nigra (case 9, CBS 122348; Table 1); C. Close-up of a pigmented macula (case 20, CBS 122342; Table 1); D. Tinea nigra plantaris, panoramic; E. Tinea nigra interdigital (case 13, CBS 123045; Table 1); F. Tinea nigra under dermatoscopy, multiple pigmented lesions; G. Direct examination of pigmented and septate hyphae (KOH, 40×); H. Direct examination of short filaments and multiple blastoconidia (KOH, 40×) (case 20, CBS 122342, Table 1).

and Polynesia (Uezato *et al.* 2006). The disorder is rare in Europe (Hughes *et al*. 1993, Reid 1998) and the United States (Burke 1993, Shanon *et al.* 1999, Tseng *et al.* 1999). Predisposing factors associated with the condition are hyperhydrosis (Severo *et al.* 1994, Bonifaz 2001, Padilla *et al.* 2002) and presence in coastal see areas or hypersaline environments, where the causative agent may be picked up from the natural habitat (de Hoog & Gerrits van den Ende 1992).

Our 22 cases present a large series and, unlike other published reports (for example, Severo *et al.* 1994, Perez *et al.* 2005), cover a long follow-up period. Our cases are clinically and demographically similar to the ones reported from other continents (Uezato *et al.* 2006). Tinea nigra is considered a rare disease. According to statistics in our hospital in Mexico City, D.F., it accounts for 0.085 % of all mycoses. The frequency of the disorder may be higher, but due to its asymptomatic nature and the possibility of spontaneous cure, patients seldom reach the doctor's office. This is the case only when patients worry because of confusion with other pigmented skin diseases, such as melanoma.

The disorder has no preference for age categories (Table 1), cases equally occurring in adults and children, and no gender differences are observed (Durán *et al*. 1993, Pegas *et al*. 2003). Course of the infection according to our data was 1−18 mos, with a mean incubation time of 3.8 mos. According to the patient's information, we think that the developing lesions become visible within 15–30 ds. The lesions are invariably flat, not elevated and without inflamed margins; they consist of pigmented, mostly brownish, irregular and asymptomatic macules and with well-defined borders, and are covered by fine scaling. Some patients report that throughout the day a change in colour of the macules can be observed: early in the morning macules can appear in more intense pigmentations but they fade as the day goes by. This is probably due to the fact that the fungus is cleared from the hands as a result of daily activities. Only one patient in our study, presented with erythema, also reported moderate itching as was noted in other studies (Hughes *et al.* 1993, Perez *et al*. 2005).

Fig. 3. Culture of *Hortaea werneckii* on SGA. Two phases: yeast-like phase with moist colonies, and filamentous colony (case 11, CBS 122344; Table 1).

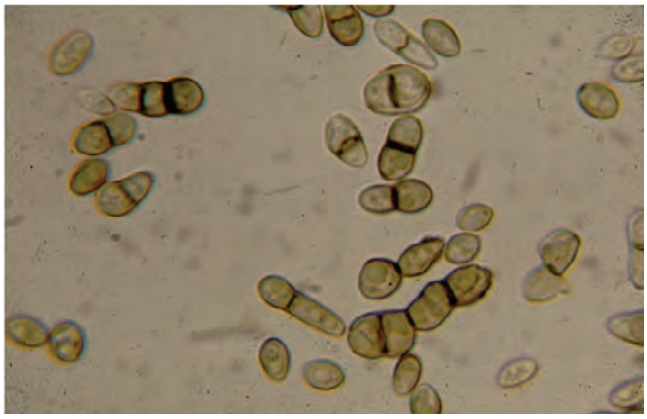

Fig. 4. Multiple blastoconidia with central septa (KOH, 40×). (case 20, CBS 122342; Table 1).

Tinea nigra has a major clinical relevance because it can be mistaken for various types of nevi. In some reports it even was misidentified as melanomas. Also some of our cases, seen at external dermatology services, were initially diagnosed as various types of nevi (Hall & Perry 1998, Tseng *et al.* 1999). With this incorrect diagnosis, two of the cases underwent biopsies, which lead to the observation of fungal elements in the stratum corneum with a discrete perivascular infiltrate. Differential diagnosis is essential, due to the extremely different prognosis of the various conditions. The tinea nigra macule denotes the superficial growth of the fungus, strictly limited to the stratum corneum. This may be revealed by dermatoscopy, which is a technique allowing the observation of fungal elements with certainty, particularly when flanked with microbiological tests (Smith *et al*. 2001; Fig. 2F).

The palms of hands constitute the major location of the disease, which explains the often-used name "tinea nigra palmaris". Most cases are unilateral but also bilateral infections can be observed (Tseng *et al.* 1999), probably resulting from autoinoculation (Fig. 2A). In our study, in 19/22 cases the disorders were located on the palms and 3 on the soles. Surprisingly, one of the sole infections was located in the interdigital spaces (Fig. 2E) and had a discrete pigmentation (case 13, strain CBS 123045; see Table 1), which led to suspect dermatosis neglecta caused by the chronic deposition of dirt or filth (Ruiz-Maldonado *et al.* 1999). We are unaware of any case of a tinea nigra located in the interdigital spaces. Recently a similar condition caused by chaetothyrialean black yeast-like fungi was reported (Badali *et al*. 2008), differing by the fact that the etiologic fungus concerned, *Cladophialophora saturnica* showed invasive behaviour, while *Hortaea werneckii* is strictly commensal. The two cases located on the plantar region were also informative, as they concerned patients with the habit of running barefoot along the seaside, an environment with high salinity and probably the natural niche of the fungus. Both cases had a clinical history of hyperhydrosis probably resulting in saline plantar cutaneous conditions and adhesion of the fungus (Fig. 2C; Göttlich *et al*. 1995). It is important to comment on case 16 (Table 1), an irregular worker in saltpans, that is considered the natural enviroment of *H. werneckii*. It is probable that more saltpan-workers carry the disorder but due to the asymptomatic nature of the disease do not seek medical help (Gunde-Cimerman *et al.* 2000).

Recently Ng *et al*. (2005) reported the isolation of *H. werneckii* from the serum and a splenic abscess of a human patient. This is an exceptional case, in which the fungus behaved as an opportunistic pathogen and was the causative agent of a systemic phaeohyphomycosis.

Given the taxonomic uncertainties mentioned above, confirmation of identification with microbiological and molecular tests is compulsory. Direct KOH examinations provide us with quick information and demonstrate the short, tortuous, thick, light brown hyphae, which may occasionally be darkened, and sometimes present short filaments and yeast-like cells. The pigmentation of hyphae unambiguously distinguishes tinea nigra from various other types of dermatophytoses or skin infections (Hughes *et al.* 1993, Gupta *et al.* 2003). The use of chlorazol black (Feuilhade de Chauvin 2005) as a clearing solution is not recommended because

this reagent stains fungal elements black, which interferes with distinction from dermatophyte hyphae. In our study nearly all cases were diagnosed by direct examination and were confirmed by culture. Colonies of *Hortaea werneckii* grow on a standard media within 5–8 ds. They are initially black with a creamy appearance and later become filamentous. This morphological transition is characteristic for *H. werneckii*, but is also known in *Exophiala* (*Chaetothyriales*). Filamentous isolates may be mistaken for some chaetothyrialean fungi or for *Cladosporium* spp. Conidia appear as pigmented yeast cells with a dark central septum, the outer wall later becoming thick-walled, heavily pigmented. Conidia finally germinate with hyphae resulting in yeast-like colonies that gradually change over into filaments to complete the anamorph life cycle.

Molecular diagnostics of *H. werneckii* were developed by Uijthof *et al*. (1994) using PCR-fingerprinting techniques and by Zalar *et al*. (1999) using sequencing of rDNA ITS. Uezato *et al.* (1989) applied molecular diagnostics in clinical practice. Abliz *et al.* (2003) developed specific primers on the basis of ITS data and validated the primers by a comparison with 42 other melanized fungal species, including chaetothyrialean agents of cutaneous and subcutaneous disease.

The treatment of tinea nigra is very simple and effective. Most cases resolve with only keratinolytic agents like urea, salicylic acid and Whitfield ointment, applied once or twice a day (Sayegh-Carreno *et al.* 1989, Bonifaz 2001). Most of the cases in our report were managed with Whitfield ointment. Mean treatment duration (Table 2) was approximately 15 ds; two cases presented spontaneous cure. Most topical antifungals are also effective. Good treatment results have been reported using miconazole (Marks *et al.* 1980), ketoconazole (Chang & Arenas 1983, Burke 1993), bifonazole (Meisel 1984), terbinafine (Shanon *et al.* 1999) and ciclopirox olamine (Rossen & Lingappan. 2006). There is even a report of oral itraconazole therapy (Gupta *et al.* 1997), which is not recommended for this commensal fungus. Most cases in our study resolved with topical therapy within two weeks.

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Black fungi in lichens from seasonally arid habitats

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Abstract: We present a phylogenetic study of black fungi in lichens, primarily focusing on saxicolous samples from seasonally arid habitats in Armenia, but also with examples from other sites. Culturable strains of lichen-associated black fungi were obtained by isolation from surface-washed lichen material. Determination is based on ITS rDNA sequence data and comparison with published sequences from other sources. The genera *Capnobotryella*, *Cladophialophora*, *Coniosporium*, *Mycosphaerella*, and *Rhinocladiella* were found in different lichen species, which showed no pathogenic symptoms. A clade of predominantly lichen-associated strains is present only in *Rhinocladiella*, whereas samples of the remaining genera were grouped more clearly in clades with species from other sources. The ecology of most-closely related strains indicates that *Capnobotryella* and *Coniosporium*, and perhaps also *Rhinocladiella* strains opportunistically colonise lichens. In contrast, high sequence divergence in strains assigned to *Mycosphaerella* could indicate the presence of several lichen-specific species with unknown range of hosts or habitats, which are distantly related to plant-inhabitants. Similar applies to *Cladophialophora* strains, where the closest relatives of the strains from lichens are serious human pathogens.

Key words: Aridity, black fungi, *Chaetothyriomycetidae, Dothideomycetidae*, phylogeny, symbioses.

INTRODUCTION

"Black fungi" is a practical term to group heterogeneous lineages of *Chaetothyriomycetidae* and *Dothideomycetidae* with melanised cell walls. It comprises fungi with diverse ecology and different growth styles, such as black yeasts or meristematic black fungi. In black yeasts, daughter cells form yeast-like multilateral or polar budding. The term meristematic was originally introduced for black fungi by de Hoog and Hermanides-Nijhof (1977) and describes non-disintegrating phenotypes which form aggregates of thick-walled, melanised cells. Meristematic growth is infrequent in the fungal kingdom, yet it was for example found in several fungi that parasitise lichens (Diederich 1990).

Black fungi can occur in extreme environments and under poor nutrient conditions, where they often grow meristematically (Sterflinger *et al.* 1999). In the last yr dematiaceous fungi with meristematic and yeast-like growth patterns, turned out to be, together with lichens and cyanobacteria, among the most successful inhabitants of marble, limestone, granite, and other rock types in arid and semi-arid environments (Sterflinger & Krumbein 1997, Wollenzien *et al.* 1997, Sterflinger 1998, Ruibal *et al.* 2005). They have been found in hot deserts of Arizona (U.S.A.) (Staley *et al.* 1982; Palmer *et al.* 1987), in cold Antarctic deserts (Nienow & Friedmann 1993, Selbmann *et al.* 2005), in Mediterranean countries as *e.g.* Italy, Greece, Turkey (Gorbushina *et al.* 2005, Ruibal *et al.* 2005, Sert & Sterflinger 2005), on stone monuments in Austria (Sterflinger & Prillinger 2001), and on granites of the Ivory Coast (Büdel *et al.* 2000). Sterflinger & Krumbein (1995) hypothesised that the ability to grow meristematically provides the colonies an optimal surface/volume ratio for enhanced stress tolerance. In particular they resist elevated temperatures, low water availability (Wollenzien *et al.* 1995), UV radiation (Urzì *et al.* 1995), high salt concentration (Zalar *et al.* 1999) or combinations of these factors and further stresses (Selbmann *et al.* 2005, Scott *et al.* 2007).

Due to the high selective pressure exerted by these stresses on the microbial community, black fungi are rarely found in complex microbial populations, rather they occur solitary or in communities with similarly stress resistant organisms such as lichens (Onofri *et al.* 2007a) and cyanobacteria (Sterflinger 2006). This is perfectly shown on rock surfaces where black fungi can form communities with epi- and endolithic lichens, cyanobacteria, chemoorganotrophic bacteria and fungi (Nienow & Friedmann 1993).

In the course of current lichenological explorations of Armenia, the first author frequently found lichen thalli with obscure discolourations. Microscopic study showed that they were caused by fungal colonisation, although not leading to sexual structures or distinctive anamorphs on lichen thalli as characteristic of most lichenicolous fungi (Lawrey & Diederich 2003). Rather, the hyphae grew isolated or as sclerotial aggregates on the thallus surface. Further diagnostic characters of the dematiaceous hyphae were missing. The phylogenetic relationships of lichen-dwelling hyphomycetes are generally unclear, due to the lack of sequence data. Despite some of the described taxa seem to be specific to their host, it is not known if other lichenicolous black fungi can colonise a wider range of hosts with similar habitat ecology.

MATERIALS AND METHODS

Sampling

Lichen material originates from Armenia. The samples were collected at different altitudes (up to *ca.* 2800 m), and on different substrates (basalt, calcareous and siliceous rocks, and few also bark) representing comparatively dry habitats (precipitation mostly between 400–600 mm/yr^{-1}). Additionally, we used samples from the mountain and the Mediterranean belts of Austria, Italy and Spain

Table 1. List of investigated specimens with collecting sites, herbarium numbers, GenBank accessions of the ITS sequences of the black fungal strains.

Lichen species	Origin	Black fungi isolated				
		***Capnobotryella* sp.**	***Cladophialophora* sp.**	***Coniosporium* sp.**	***Mycosphaerella* sp.**	***Rhinocladiella* sp.**
Caloplaca erodens Tretiach, Pinna & Grube	Austria, Styria, Mt. Hochlantsch, c. 1620 m a.s.l., on limestone, 2005, *Muggia* (TSB 36936).	FJ265744				
C. erythrocarpa (Pers.) Zwackh	Armenia, Syunik, Goris, c. 1550 m a.s.l., on rock, 2006, *Harutyunyan* (GZU 23-1006).		FJ265749			
C. gomerana J. Steiner	Spain, Canary Islands, Tenerife, Punta Roja, 15 m a.s.l., on basalt, 2005, *Muggia* (TSB 36862).					FJ265765
C. holocarpa (Ach.) A.E. Wade	Armenia, Kotayk, Jrvej, c. 1300-1400 m a.s.l., on *Prunus* sp., 2005, *Harutyunyan* (GZU 36-105).	FJ265745				
C. saxicola (Hoffm.) Nordin	Armenia, Kotayk, Garni gorge, c. 1180 m a.s.l., on basalt, 2006, *Harutyunyan & Mayrhofer* (GZU 05-1000).	FJ265742		FJ265756	FJ265760	FJ265766
Dermatocarpon miniatum (L.) W. Mann	Austria, Styria, Grazer Bergland, Kaschlsteig, 800 m a.s.l., on limestone, 2005, *Muggia & Hafellner* (TSB 36921).					FJ265762
Fulgensia fulgida (Nyl.) Szatala	Italy, Sardegna, Nuoro, Mt. Albo Massiv, 1000 m a.s.l., on limestone, 2006, *Muggia* (TSB 37496).		FJ265750			
Protoparmeliopsis muralis (Schreb.) M. Choisy	Armenia, Kotayk, Garni gorge, c. 1180 m a.s.l., on basalt, 2006, *Harutyunyan & Mayrhofer* (GZU 05-1001).			FJ265754		FJ265770
"	Armenia, Kotayk, Geghardavank, c. 1600 m a.s.l., on *Rhamnus catarthica*, 2005, *Harutyunyan* (GZU 38-183).		FJ265752			
"	Armenia, Kotayk, Tsaghkadzor, c. 1750 m a.s.l., on rock, 2005, *Harutyunyan* (GZU 34-206).	FJ265743			FJ265757	FJ265768
"	Armenia, Kotayk, Geghard, c. 1875 m a.s.l., on basalt, 2006, *Harutyunyan & Mayrhofer* (GZU 08-1002).		FJ265747			
"	Armenia, Vayots Dzor, Jermuk, c. 2050 m a.s.l., on basalt, 2006, *Harutyunyan & Mayrhofer* (GZU 12-1005).		FJ265748			FJ265764
"	Armenia, Shirak, Mt. Aragats, c. 2465 m a.s.l., on basalt, 2006, *Harutyunyan & Mayrhofer* (GZU 18-1004).					FJ265771
"	Armenia, Kotayk, Mt. Taghenyac, c. 2820 m a.s.l., on basalt, 2006, *Harutyunyan & Mayrhofer* (GZU 02-1003).					FJ265767
Lecidella stigmatea (Ach.) Hertel & Leuckert	Austria, Styria, Röthelstein, 1260 m a.s.l., on limestone, 2006, *Muggia & Hafellner* (TSB 37332).					FJ265772
Physcia dimidiata (Arnold) Nyl.	Armenia, Kotayk, Garni gorge, c. 1180 m a.s.l., on *Salix* sp., 2006, *Harutyunyan & Mayrhofer* (GZU 05-289).		FJ265751			
Physconia americana Esslinger	Armenia, Syunik province, Goris, c. 1550 m a.s.l., on *Quercus* sp., 2006, *Harutyunyan* (GZU 23-727).		FJ265753			
Tephromela atra (Huds.) Hafellner	Italy, Toscana, Mt. Labbro, 1150 m a.s.l., on silicate, 2004, *Tretiach* (TSB 37086).				FJ265759	
Xanthoria elegans (Link) Th.Fr.	Armenia, Kotayk, Tsaghkadzor, c. 1750 m a.s.l., on rock, 2005, *Harutyunyan* (GZU 34-205).		FJ265746		FJ265758	FJ265763

to explore a wider geographic range. The selected lichen species, origins, and corresponding fungal strains isolated from the lichen thalli are listed in Table 1. Both lichen material without symptoms and with black discolourations on the thalli were selected for the isolations.

Optical analysis

Lichens were analysed for visible colonisation with black fungi using a stereomicroscope. Hand-made transversal sections (using Gillette razor-blades) were examined in water with a Zeiss Axioscope compound microscope (Zeiss, Vienna).

Isolation of fungal strains in culture

We used young areoles or lobes, respectively, of freshly collected lichen material. The isolations were performed following the "lichen tissue culture method" as described by Yamamoto *et al.* (2002), with some modifications as applied in Stocker-Wörgötter (2002). To remove attached debris and substrate, small pieces of lichen thalli were cleaned mechanically using a forceps. Additionally the pieces were washed in water and in diluted Tween 80 to eliminate other organisms loosely attached to the thallus surface (Bubrick & Galun 1986). Fragments were homogenised using a mortar and pestle. The suspensions, containing small pieces of cortex, medulla and algal layer, were filtered through two sieves of 500 and 150 µm mesh size, respectively. Single fragments of about 150 µm in size were picked up with sterile bamboo sticks under a dissecting microscope and transferred on slanted agar in test tubes.

The fungal cultures were grown on MY, TM (Ahmadjian 1967), and LBM (Lilly & Barnett 1951) media for 4–5 mo at 15–20 °C, with a cycle of 14 h of light and 10 h of dark. As soon as the mycelia reached *ca*. 0.5 cm in diam, a small part was sub-cultured and another was used for DNA isolation. Subcultures were prepared on up to five small Petri dishes having either the same original agar media or different media. The fungus was transferred on fresh media for subcultures after 3–4 mo.

Molecular analysis: DNA-isolation, PCR-amplification and sequencing

DNA isolation was performed on a part of the mycelium grown in the slant tubes, following Cubero *et al*. (1999). Identity of the cultured strains were checked with sequences of the ITS regions, amplified with the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). PCR reactions were prepared for a 30 µL final volume containing 4.05 µL double-distilled water, 3 µL 10 x *Taq* polymerase reaction buffer (10 mM Tris pH 8.3), 1.8 µL $MgCl_2$ (25 mM), 3 µL of 2.5 mM dNTPs, 0.15 µL *Taq* DNA polymerase, 1.5 µL for each of the 10 µM primers. PCR amplification were performed under the following conditions: an initial heating step of 2 min at 94 °C, 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 53 °C, and 2 min of extension at 72 °C, and one final extension step of 7 min at 72 °C, after which the samples were kept at 4 °C. PCR products were cleaned using Qiaquick spin columns (Qiagen, Vienna, Austria). Both complementary strands were sequenced with the BigDye Cycle Sequencing Ready Reaction Kit (Applera, Vienna, Austria) according to the manufacturer's instructions, and sequences were run on an ABI310 automated sequencer (Applera, Vienna, Austria).

Alignment and phylogenetic analysis

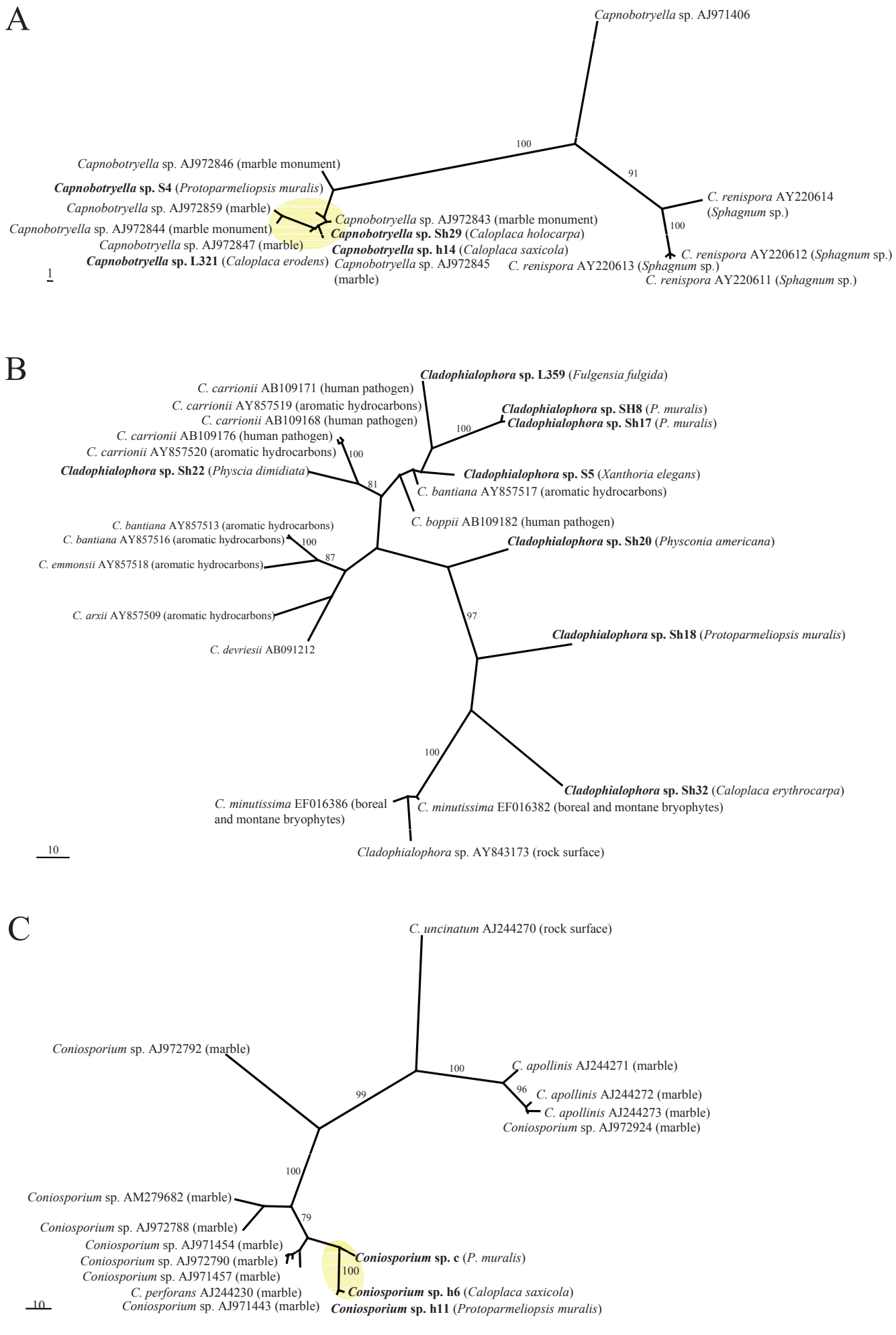
Sequences of the isolated fungal strains were subjected to BLAST search (Altschul *et al.* 1990, http://www.ncbi.nlm.nih.gov/BLAST/), and were assigned to genera that appeared as first matches in the GenBank. Sequences that matched closer to our data were selected from GenBank and included in the analyses.

All fungal strains corresponding to the same genus were grouped together and for each of them a small alignment was produced automatically with ClustalW (Thompson *et al*. 1994) as implemented in BioEdit v. 5.0.6 (Hall 1999, http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) and then manually adjusted. Small phylogenetic analyses were carried out to outline the genetic similarity of the isolated strains with the closest matches resulted from the BLAST search, The maximum parsimony (MP) algorithm was used in PAUP v. 4.0b10 (Swofford 2002). A heuristic search using 100 random addition replicates was conducted with tree-bisection-reconnection (TBR), branch swapping, and MulTree options. Bootstrappping was performed on 1000 pseudoreplicates (Felsenstein 1985). Ambiguously aligned positions were excluded and no root was selected. The phylogenetic trees were drawn using the program TreeView (Page 1996).

RESULTS

Several lichen species used in the present study (*Caloplaca erythrocarpa*, *C. saxicola*, some samples of *Protoparmeliopsis muralis*, *Physconia americana* and *Xanthoria elegans*) showed visual signs of fungal colonisation in the form of minute black hyphae or dots. Attempts to determine the colonising species using available literature that includes keys to several hyphomycetous genera occurring on lichens (*e.g.* Hawksworth 1979, Clauzade *et al.* 1989, etc.) did not give clear results. Other samples, such as *C. holocarpa*, some samples of *P. muralis* and *Physcia dimidiata* were free of any visual contaminants.

We obtained 70 black fungal cultures from 20 different thalli belonging to 14 lichen species, representing crustose, foliose or fruticose growth (Table 1). On the same media, black fungi grew slowly but clearly faster than lichen mycobionts (*ca.* 1 cm diameter black fungal colony is reached after about 3 mos, whereas most of the lichen mycobionts show only initial development of compact mycelium after this period). Sequencing of the complete ITS regions from cultures, from which DNA was successfully extracted, revealed 31 distinct strains. According to results of the BLAST searches, the obtained strains were classified as representatives of the subclasses *Chaetothyriomycetidae* and *Dothideomycetidae*. In particular, they belong to five genera: *Capnobotryella*, *Cladophialophora*, *Coniosporium*, *Mycosphaerella* and *Rhinocladiella*. Molecular data of 16 cultures were not subjected to phylogenetic analyses because we so far obtained incomplete sequences from these strains. According to BLASTn searches these latter were most closely assigned to uncultured soil fungi (isolated from *Protoparmeliopsis muralis*, *Teloschistes contortuplicatus* and *Xanthoria. elegans*), melanised limestone ascomycetes (from *Caloplaca saxicola*, *C. holocarpa*, *Fulgensia fulgida*, *Leptogium corniculatum* and *P. muralis*), *Capronia* and *Exophiala* (representing *Chaetothyriomycetidae* on *Physconia americana, P. muralis* and *X. elegans*). In addition to black fungal groups we also found a species showing sequence similarity with *Nectria* (representing *Hypocreales*) in *Physcia scopulorum*.


A
Capnobotryella sp. AJ971406
100
91
Capnobotryella sp. AJ972846 (marble monument)
Capnobotryella sp. S4 (Protoparmeliopsis muralis)
Capnobotryella sp. AJ972859 (marble)
Capnobotryella sp. AJ972844 (marble monument)
Capnobotryella sp. AJ972847 (marble)
Capnobotryella sp. L321 (Caloplaca erodens)
Capnobotryella sp. AJ972843 (marble monument)
Capnobotryella sp. Sh29 (Caloplaca holocarpa)
Capnobotryella sp. h14 (Caloplaca saxicola)
Capnobotryella sp. AJ972845 (marble)
C. renispora AY220614 (Sphagnum sp.)
100
C. renispora AY220612 (Sphagnum sp.)
C. renispora AY220613 (Sphagnum sp.)
C. renispora AY220611 (Sphagnum sp.)
1
B
Cladophialophora sp. L359 (Fulgensia fulgida)
C. carrionii AB109171 (human pathogen)
C. carrionii AY857519 (aromatic hydrocarbons)
C. carrionii AB109168 (human pathogen)
C. carrionii AB109176 (human pathogen)
C. carrionii AY857520 (aromatic hydrocarbons)
Cladophialophora sp. Sh22 (Physcia dimidiata)
Cladophialophora sp. SH8 (P. muralis)
Cladophialophora sp. Sh17 (P. muralis)
100
100
81
Cladophialophora sp. S5 (Xanthoria elegans)
C. bantiana AY857517 (aromatic hydrocarbons)
C. boppii AB109182 (human pathogen)
C. bantiana AY857513 (aromatic hydrocarbons)
C. bantiana AY857516 (aromatic hydrocarbons)
C. emmonsii AY857518 (aromatic hydrocarbons)
100
87
Cladophialophora sp. Sh20 (Physconia americana)
C. arxii AY857509 (aromatic hydrocarbons)
C. devriesii AB091212
97
Cladophialophora sp. Sh18 (Protoparmeliopsis muralis)
100
Cladophialophora sp. Sh32 (Caloplaca erythrocarpa)
C. minutissima EF016386 (boreal and montane bryophytes)
C. minutissima EF016382 (boreal and montane bryophytes)
Cladophialophora sp. AY843173 (rock surface)
10
C
C. uncinatum AJ244270 (rock surface)
Coniosporium sp. AJ972792 (marble)
100
C. apollinis AJ244271 (marble)
96
C. apollinis AJ244272 (marble)
C. apollinis AJ244273 (marble)
Coniosporium sp. AJ972924 (marble)
99
100
Coniosporium sp. AM279682 (marble)
79
Coniosporium sp. AJ972788 (marble)
Coniosporium sp. AJ971454 (marble)
Coniosporium sp. AJ972790 (marble)
Coniosporium sp. AJ971457 (marble)
C. perforans AJ244230 (marble)
Coniosporium sp. AJ971443 (marble)
Coniosporium sp. c (P. muralis)
100
Coniosporium sp. h6 (Caloplaca saxicola)
Coniosporium sp. h11 (Protoparmeliopsis muralis)
10

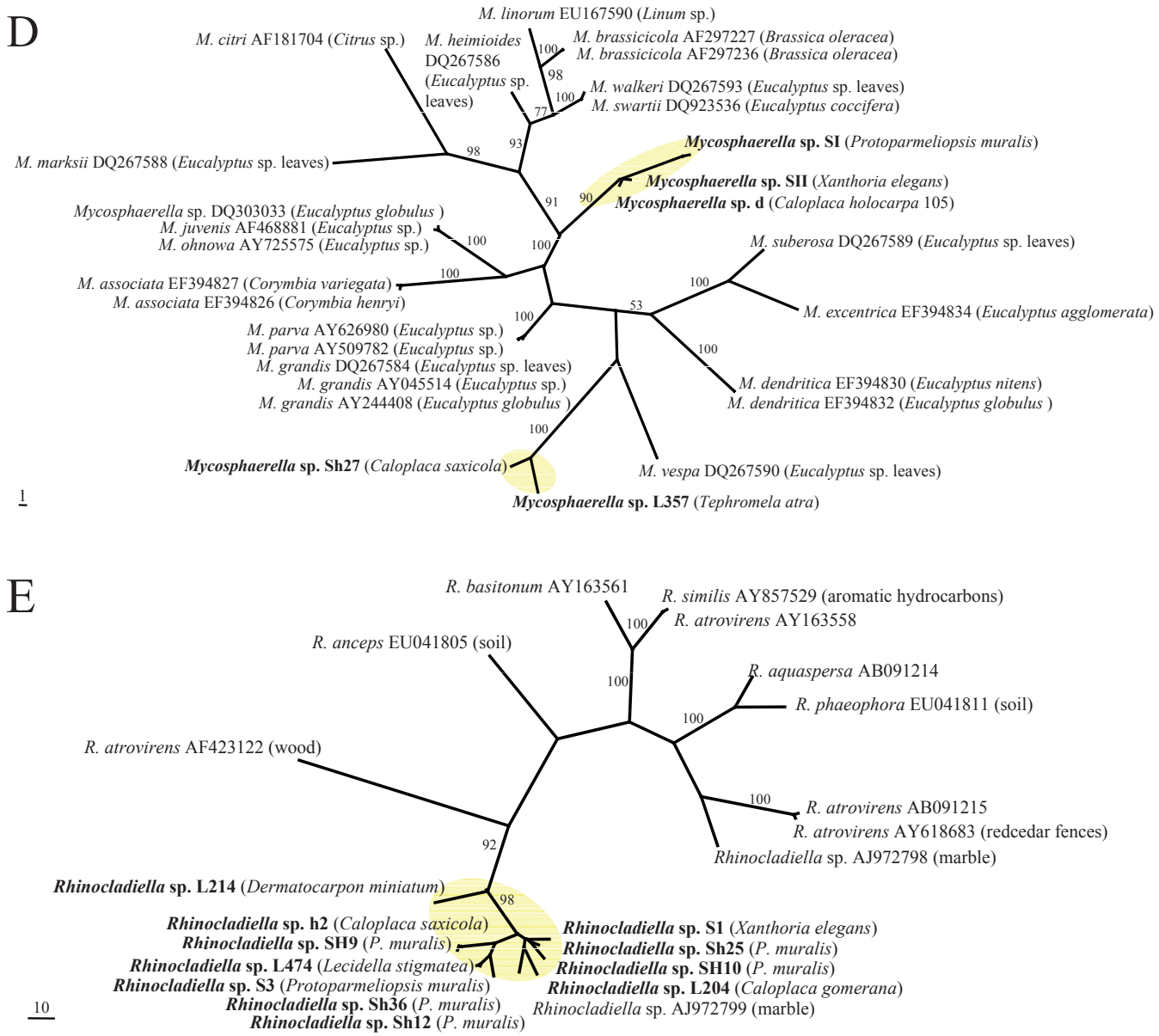


Fig 1. MP phylogenetic hypotheses based on ITS sequences of the black fungal genera *Capnobotryella* (a), *Cladophialophora* (b), *Coniosporium* (c), *Mycosphaerella* (d), and *Rhinocladiella* (e). Sequences obtained in this study are shown in bold, the isolation sources are written in parentheses, and yellow ellipses highlight the clades they form. Trees are unrooted, bootstrap cut-offs higher then 75% are reported.

Strains belonging to the genera *Cladophialophora* (8) and *Rhinocladiella* (11) were found more frequently in lichen thalli than *Capnobotryella* (4), *Coniosporium* (3), and *Mycosphaerella* (5). *Cladophialophora* and *Rhinocladiella* did not show any preference to elevation and were isolated from six lichen species at different altitudes. Not supporting a species-specific occurrence, the same fungal strains representing *Coniosporium* were isolated from co-occurring *Caloplaca saxicola* and *Protoparmeliopsis muralis* in Armenia (h11 and h6). Fungal strains identified as *Capnobotryella*, *Cladophialophora*, and *Rhinocladiella* did not show any specificity for the lichen growth habit. All of them were isolated from crustose, foliose and fruticose lichens, respectively. Fungal strains assigned to *Coniosporium* and *Mycosphaerella* were so far isolated only from crustose lichens. Among all the five genera only *Cladophialophora* was isolated also from the two epiphytic lichens included, *i.e. Physcia dimidiata* and *Physconia americana*. These strains represented distinct lineages.

In the phylogenetic analyses (Fig. 1A–E), all fungal strains identified as *Rhinocladiella*, are grouped in a single clade with the exception of one rock-inhabiting strain from Turkey (AJ972799). Except for the latter, these strains are genetically rather similar although they were isolated from different lichen species. In the remaining genera, by contrast, sequences from lichens are genetically rather heterogeneous and are scattered among strains coming from other different sources. Two lineages of lichen-colonisers were so far found in *Mycosphaerella* and more lineages colonising lichens are present in *Cladophialophora*. Several distinct strains were also found in *Capnobotryella*.

Multiple strains of black fungi were isolated from the same thallus of four crustose lichen species (*Caloplaca holocarpa*, *C. saxicola*, *Protoparmeliopsis muralis* and *Xanthoria elegans*) (Table 1). The most diverse of these was *C. saxicola*, yielding four isolates representing the genera *Capnobotryella*, *Coniosporium*, *Mycosphaerella* and *Rhinocladiella*. *P. muralis* contained *Capnobotryella*, *Mycosphaerella* and *Rhinocladiella*, and *X. elegans* hosted *Cladophialophora*, *Mycosphaerella* and *Rhinocladiella* sp. occurring simultaneously. The thallus of *C. holocarpa* was a substrate for *Capnobotryella* and *Mycosphaerella*. Two separate

thalli of *P. muralis* hosted *Coniosporium* with *Rhinocladiella* and *Cladophialophora* with *Rhinocladiella* respectively. Only one culturable fungal strain from the above-mentioned genera of black fungi was present in the lichen samples of *Caloplaca erodens*, *Dermatocarpon miniatum*, *Fulgensia fulgida*, *Physcia dimidiata*, *Physconia americana*, and *Tephromela atra* according to the sequencing results with several isolates retrieved from these lichens (Table 1).

DISCUSSION

High diversity of life-styles characterises the *Chaetothyriomycetidae* and *Dothideomycetidae* (Ruibal *et al.* 2008). In these two subclasses, genera can comprise animal and human pathogens, endophytes or epiphytes of living plants and fungi (de Hoog 1994, Geiser *et al.* 2006, Schoch *et al.* 2006). On the other hand, phylogenetic studies indicate a rather scattered distribution of lichenised fungi in *Dothideomycetidae* (Del Prado *et al.* 2006, Muggia *et al.* 2007), whereas in *Chaetothyriomycetidae*, lichenised forms belong to the large monophyletic orders *Pyrenulales* and *Verrucariales* (Geiser *et al.* 2006). Repeated loss of lichenisation and lichenicolous habit occurred in *Verrucariales* (Navarro-Rosinés *et al.* 2007). However, black fungi detected by us do not belong to any of the lineages of lichenised fungi in *Chaetothyriomycetidae* or *Dothideomycetidae*. The BLAST analysis revealed indeed high sequence similarity primarily with the genera *Capnobotryella*, *Cladophialophora*, *Coniosporium*, *Mycosphaerella*, and *Rhinocladiella.* Within these genera genetic similarities were found with undetermined fungal strains isolated directly from rock surfaces, or with plant and human pathogenic species.

Lichens can host a wide range of associated fungi with rather varied ecologies, specificities, and biological behaviours (Lawrey & Diederich 2003). Some fast-growing lichenicolous species (*e.g. Athelia*, *Marchandiomyces*) with often low host specificity can rapidly eradicate lichen vegetation, whereas many others grow slowly without expressing any or showing only local pathogenic symptoms on their specific hosts. Pathogenic and commensalic interactions with their hosts appear to be corners of an ecological continuum, yet, known lichenicolous fungi have clear affinity to lichens as hosts and are not found without their hosts.

In this publication we find indirect evidence that some lineages of black fungi can opportunistically grow on lichens. Generally lichen-colonising forms did not form monophyletic groups, although in *Rhinocladiella* (Arnanlou *et al.* 2007), only one published marble-colonising strain was found among a genetically homogeneous group of lichen inhabitants. This pattern might nevertheless be incomplete because only few strains from rock are published so far. We suspect that *Rhinocladiella* is only a facultative lichen coloniser (Fig. 2), also because the same strains can be found in different co-occurring lichen species. More evidence for this hypothesis is found in *Coniosporium* and *Capnobotryella*, where lichen-inhabiting strains are scattered among groups of rock-inhabitants. This clearly contrasts with the ecological relationships found in *Mycosphaerella* and *Cladophialophora*. *Mycosphaerella* strains from lichens form two clades which are related to plant-associated fungi. Sequence divergence suggests that the lichen associates could represent distinct species with so far undetermined host specificity. The lichenicolous genus *Stigmidium* is phenotypically recognised in the mycological literature by the similar phenotypic features as the phytopathogenic *Mycosphaerella* (*Mycophaerella* Johanson being a younger name than *Stigmidium* Trevis.). The understanding of relationships between these two genera will clarify whether some our *Mycosphaerella*-like strains could in fact represent *Stigmidium* species. If this is the case, it will be interesting to assess whether they represent new species or individuals growing cryptically in a suboptimal host. *Stigmidium* species are regarded as highly specialised for their lichen host species but they are only recognised by their fertile structures. *Cladophialophora* isolates from lichens are represented in several distinct lineages. Some of them are related to the human pathogens *C. bantiana*, *C. boppi* and *C. carrionii*, while others are related to the moss-inhabitant *C. minutissima* (and a strain isolated from rocks).

Microscopic observations of the interactions between black fungi and their host lichens are difficult. Some black fungi, including the ones found by us, are well pigmented only at the surface of the thalli. Their hyphal walls can lose their pigmentation when they stretch downwards through lichen cortex plectenchyma into the thallus, and then become unapparent. At present we cannot assess how extensively hyphae can invade the thallus and how they interact with the lichen symbionts. Only *Intralichen* (Hawksworth & Cole 2002) species form mycelia that also visibly extend deep into the thallus. Clear mycoparasitic or algal-parasitic behaviour is not evident and the hyphae of *Intralichen* grow well between fungal plectenchyma, which might be evidence for an affinity to the fungal partner in lichens. However, this is perhaps not a general feature of lichen-colonising black fungi. A direct involvement of black fungi in fungal-algal interactions was earlier described for *Coniosporium aeroalgicolum*, which seems to establish a balanced stage of algal parasitism (Turian 1977). Also, co-culture of various rock-inhabiting microcolonial fungi with lichen algae can develop into lichenoid structures after 2–12 mo, with the fungi contacting algae by haustoria- and appressoria-like structures (Gorbushina *et al.* 2005). Finally, Brunauer *et al.* (2007) showed that a lichen-associated black fungus (with unclear position at the basis of *Chaetothyriomycetidae*) discovered on *Lecanora rupicola* forms lichenoid structures with a range of coccal green algae *in vitro*.

As some *Cladophialophora* strains are involved in degradation of aromatic hydrocarbons (Prenafeta-Boldú *et al.* 2006), it might be possible that black fungi can take benefits from the numerous aromatic polyketide secondary metabolites found in lichens, but there is so far no clear evidence for this from our observations. Parts of the lichens colonised by black fungi are not necessarily bleaching or devoid of secondary compounds. Moreover, aromatic hydrocarbons can also originate from other sources. Turian (1975) noticed that *Coniosporium aeroalgicolum* was tolerant to high levels of air pollution, and was abundant at urban places where hydrocarbons originate from traffic exhausts. High incidence of black fungi is also noticed on roadside trees in Armenia (de Hoog, unpubl. data).

In extremely hostile places on Earth, meristematic fungi are frequently associated with lichens (Onofri *et al.* 2007b), where they can directly or indirectly benefit from primary production of the algae at very low temperatures and limiting water conditions. Lichens from increasingly arid habitats are often more pronouncedly colonised by dark pigmented fungi, not only at the edges of the thallus areoles and squamules, but also on the more central surfaces. The isolation of black fungi from several visually uninfected thalli suggests that black fungi are ubiquitous in lichens and wait for chances to grow out, *e.g.* when parts of the host become senescent (Aptroot & Alstrup 1999). Microscopic studies also confirm the frequent presence of dark-walled hyphal fragments in otherwise healthy parts of lichens. We hypothesise that black fungi readily colonise most, if not all,

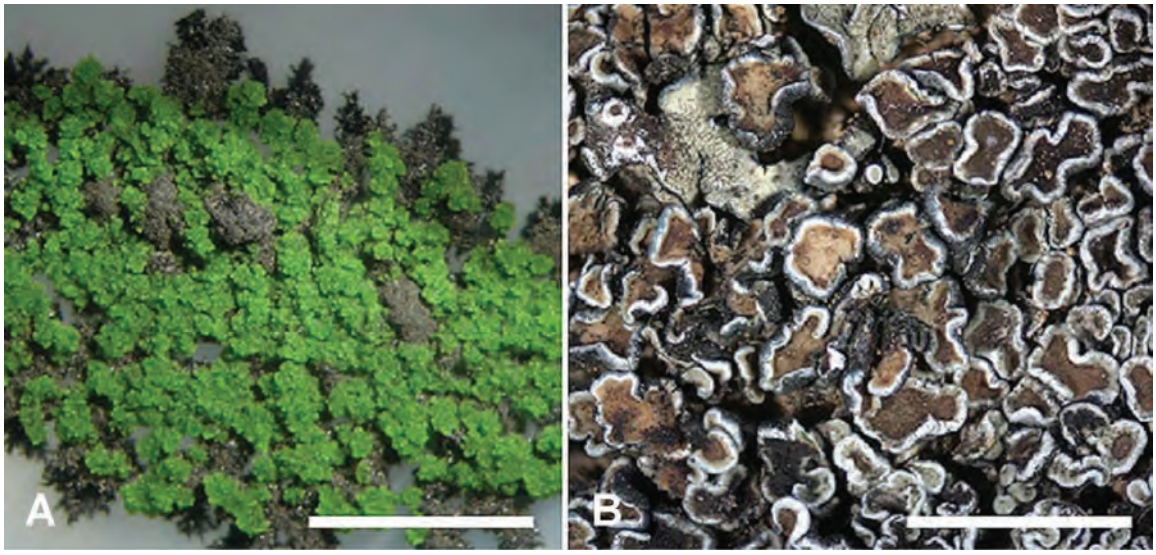


Fig 2. A. Co-culture on LB medium of *Rhinocladiella* sp. and *Trebouxia* sp. both isolated from *Lecanora muralis*, Bar = 1 mm; B. Thallus of *Lecanora muralis* showing symptoms of black fungal infection, Bar = 1 mm.

lichen thalli from dry sites. Moreover, we have shown here that several distinct strains can be present on a single thallus at the same time. Preliminary results using single strand conformation polymorphisms (SSCP, L. Muggia, prelim. data), indicate that up to 8 fungi can be present in a single lichen thallus (incl. the mycobiont). It is possible that we might have been able to isolate additional black fungal strains from the same samples with specific media for xerophilic fungi. However, black discolourations, especially at the thallus or areole margins do not always indicate the presence of black fungi. They may rather represent thallospores or prothalline edges formed by the lichen itself. These dark coloured mitospores can be found in a various crustose lichens, and are particularly common in arid habitats (Poelt & Obermayer 1990).

We have no evidence that associated black fungi overgrow the host when it is in a wealthy ecological state, but they may become more prominent when the lichen hosts are somewhat affected by sustained aridity. We observed that the upper cortex becomes more brittle in arid habitats than found in samples of the same species collected from more humid situations. Water dropped on such brittle surfaces is usually taken up very quickly. The thalli also do not keep the water for extended time in a gelatinous intercellular matrix, which is often better developed in the samples from more humid locations (unpubl. observation). Some of the lichen-associated meristematic black fungi may be rock-inhabitants, which appear as opportunists on lichens under certain circumstances. We observed more commonly meristematic forms on the brittle surfaces, whereas gelatinous surfaces might more often host filamentous forms of hyphomycetes. Adjacent lichens, which slightly differ in their cortical structures, can contain black fungi that were different in appearance and abundance, but further studies are required to see whether these represent different co-inhabiting species or just represent growth modifications of the lichen-colonisers. We also observed that the algal layers beneath the colonised parts of the host lichens can look rather wealthy, indicating that the algae seem to proliferate under a cover of black fungi rather than being negatively affected. It is still unclear whether black fungi could influence hydration of the lichens or even dissipate excessive sunlight to protect the algae.

However, when black fungal hyphae become abundant on the surface, the thallus structure is severely impaired. Reinfection of *Lecanora rupicola* with a concentrated inoculum of a black fungus isolated from the same lichen actually led to necrotic symptoms (Brunauer *et al.* 2007). Moreover, there are also examples for negative interactions and rather aggressive dematiaceous hyphomycetes on lichens. Black fungi apparently interact in various ways with lichens and they likely have different degrees of specificity. Future studies will show which black fungi are facultative opportunists on lichens, and which ones represent obligate and specialised lichen inhabitants. Such studies will also elucidate relationships with described lichenicolous fungi. Growing on their hosts, some of our fungi have at least some similarities with poorly understood lichenicolous species assigned to, *e.g.*, the genera *Taeniolella* or *Torula*. Some of these described species are regarded as highly host-specific (*e.g.* Etayo & Calatayud 2005). This may perhaps be questioned, if future sequencing data may reveal that growth and behaviour of these fungi can be modified in other potential lichen hosts. Future studies may also resolve the relationships with the fertile lichenicolous genus *Lichenostigma* and poorly known species assigned to the lichenised genus *Lichenothelia*, which are capable of meristematic growth.

ACKNOWLEDGEMENTS

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Cellular responses of microcolonial rock fungi to long-term desiccation and subsequent rehydration

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Abstract: Melanised rock-inhabiting fungi are astonishingly resistant to environmental stresses. Also known as micro-colonial fungi (MCF), they are ubiquitous and even colonise bare rocks in deserts. To survive in nutrient poor and extremely stressful conditions, MCF have reduced morphogenetic complexity to a minimum, and rely on a broad spectrum of stress protection mechanisms. Although visual signs of carotenoid presence are masked by heavily melanised black cell-walls, we were able to isolate and characterise a variety of carotenoids (ß-carotene, ζ-carotene, phytoene, torularhodin and torulene) in the rock-inhabiting, relatively fast-growing strain A95. The desiccation/rehydration stress response was used to measure the ability of A95 to adapt to slow or fast changes in external conditions. Revival of MCF after prolonged desiccation and rehydration was documented by biochemical (analyses of lipids and protective pigments), cultivation, and microscopic methods. Survival of MCF is enhanced when desiccation is rapid and mycostasis is instant rather than following prolonged periods of low metabolic activity.

Key words: Anhydrobiosis, carotenoids, lipids, mycostasis, protective pigments, stress responses.

INTRODUCTION

Micro-colonial fungi (MCF) are the only inhabitants of varnished rock surfaces in arid regions (Staley *et al*. 1982) as well as ubiquitous settlers on sub-aerial rock surfaces in other climatic zones (Gorbushina *et al*. 1993, Urzí *et al*. 1995, Wollenzien *et al.* 1995, Sterflinger & Prillinger 2001, Ruibal 2004, Selbmann *et al*. 2005, Gorbushina 2007). Rock-inhabiting ascomycetes form a peculiar ecological group with simple morphology that exhibits a remarkable tolerance to stress (Palmer *et al.* 1987, Sterflinger & Krumbein 1995). Often stress resistance in micro-organisms is strongly correlated with an easy to simulate desiccation challenge, and here we chose desiccation / rehydration stress to investigate the capability of rock inhabiting MCF to adapt to slow or fast changes in external conditions.

Different pro- and eukaryotic organisms are able to withstand almost complete desiccation (Billi & Potts 2002). To test whether MCF are capable of surviving the removal of all but 0.1 g water / g dry weight (a condition that occurs during matric stress as well as through travel in simulated space), we took a representative strain of rock-inhabiting fungi (*Sarcinomyces petricola* strain A95) and measured its ability to revive. A matric stress (physical removal of water by desiccation in air) characteristic of the natural habitat of these fungi was applied for eight wks followed by sudden rehydration. Biochemical and ultra-structural changes in strain A95 were followed by analysing lipid- and pigment-composition as well as by microscopy.

MATERIAL AND METHODS

Strain

The black microcolonial fungus *S. petricola* strain A95 (= CBS 123872) was isolated from a marble rock surface near the Philopappos monument on Musaios Hill, Athens (Greece). This relatively fast growing strain belongs to the *Chaetothyriales* (Gueidan *et al*., 2008) and is maintained in the Geomicrobiology culture collection at the University of Oldenburg (ICBM, Oldenburg University, Germany).

Media, growth and desiccation conditions

Inocula were taken from two-wk-old pre-cultures grown on 2 % malt−extract agar (MEA) and suspended in physiological saline using a homogeniser (Ultra-Turrax T25, IKA Labortechnik, Staufen, Germany). Sterile nitrocellulose filters (Sartorius 0.22 µm, 25 mm diam) laid on MEA were inoculated with 50 µL of this suspension and fungal colonies allowed to develop in the sub-aerial environment (Fig. 1A). After eight wks of growth, the supporting filters were transferred to a desiccator. Two types of desiccation were employed: (i) fast removal of free water (to imitate environmental conditions on rock surfaces), and; (ii) slow removal of water. Phosphorus pentoxide was used as the desiccant in both cases, but for fast desiccation the nitrocellulose filters were placed in dry Petri dishes while for slow desiccation the filters were placed in Petri dishes containing a layer of an agar medium. After one wk all colonies (both desiccation types) had dried down to a constant weight (water content less then 0.1 g water / dry weight). Eight wks later, the colonies were sub-sampled for lipid and pigment analysis, as well as for ultra-structural studies by light- and transmission-

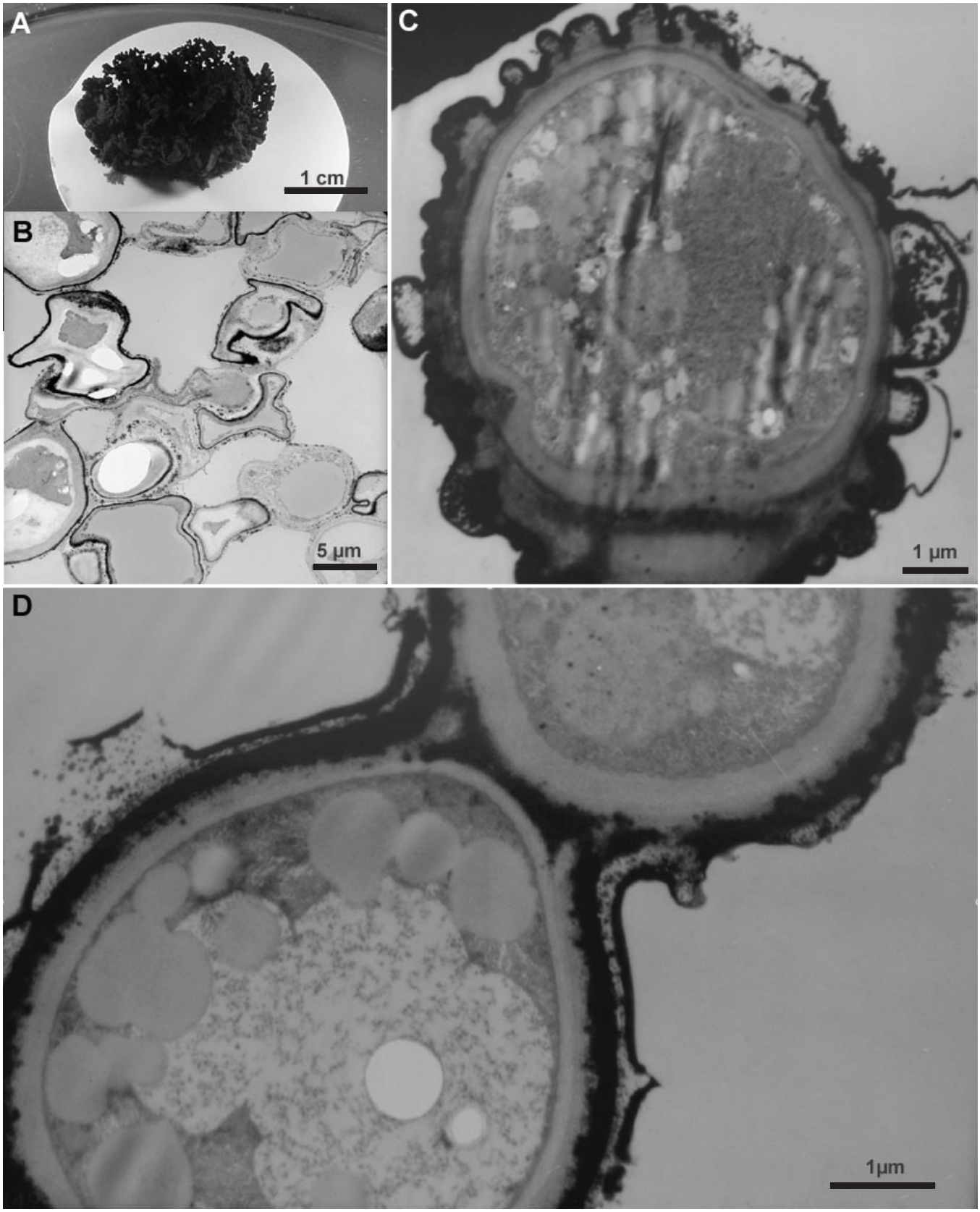

Fig. 1. Colonies of rock-inhabiting strain A95 were grown on nitrocellulose filters (A), and subjected to fast and slow desiccation (8 wk in a desiccator containing P_2O_5 with and without underlying agar) and analysed microscopically. A. experimental setup, well-developed single colony just before treatment; B. TEM micrograph of a colony sectioned after 8 wk of desiccation (overview showing collapsed dehydrated cells filled with coalescent lipid inclusions). Differences were not observed between fast and slow desiccated colonies; C, D. TEM micrographs of restored cells just 24 h after rehydration.

electron microscopy (TEM).

Determination of dry weight and rate of water loss

A separate set of colonies was used to determine the rate of water loss. After transfer to a desiccator, colonies (fast- and slow-desiccated) were removed on consequent days and weighed until constant weight was achieved. These experiments were carried out in triplicate.

Cultivation studies

Discs (~ 5 mm diameter) were cut out of desiccated colonies and placed on MEA for revival studies. These experiments were carried out in triplicate.

TEM and SEM studies

Colonies were fixed in 4 % (v/v) glutaraldehyde in 0.1 M sodium-potassium-phosphate buffer (pH 7.2) for 2 h at room temperature and post-fixed in 2 % (w/v) OsO_4 overnight. An ethanol solutions series (v/v) of 30 % for 30 min, twice 50 % for 30 min, 70 % for 30 min, overnight at 80 %, 1 h at 90 % 1 h and absolute ethanol 30 min was used for dehydration. Spurr resin was used for embedding, sections were cut with an ultra-microtome. Uranyl acetate and lead citrate were used to enhance contrast (Reynolds 1963). Cryo-SEM (Hitachi S-320M, Tokyo, Japan equipped with an Oxford CT 1500 Cryostation, Oxford Instruments, U.K.) was used to examine the colonies in their native status with undisturbed extracellular matrix.

Lipid analysis

Colonies were homogenised and extracted at room temperature with chloroform / methanol (1:2) (Bligh & Dyer 1959). Lipids were fractionated on a silica-gel column eluted with chloroform, acetone, and methanol (Kates 1972). High-performance thin-layer chromatography (HPTLC) was conducted on pre-coated silica gel 60 plates (Merck, Darmstadt, Germany). Phospholipids were analysed by two-dimensional HPTLC according to the method of Vaskovsky & Terekhova (1979) using chloroform / methanol / toluene / 28 % ammonia (65:30:10:6) and chloroform / methanol / toluene / acetone / acetic acid / water (70:30:10:5:4:1) in the first and the second dimensions, respectively. Neutral lipids were separated by one-dimensional HPTLC. Toluene / hexane / formic acid (140:60:1) and hexane / diethyl ether / formic acid (60:40:1) mixtures were used sequentially as the mobile phases. The lipid spots were visualized by spraying with 5 % sulphuric acid in methanol. The contents of the individual classes of phospholipids and neutral lipids were determined by estimating phosphorus (Vaskovsky *et al.* 1975) and carbon (Kabara & Chen 1976), respectively. Four fungal replicates were prepared and each was sampled three times. Fatty acids were extracted using a method recommended for the Sherlock Microbial Identification System (MIDI Inc., Newark, Delaware, U.S.A.) which involves saponification of cellular lipds in hot NaOH / methanol, methylation of fatty acids with hot HCl / methanol, and extraction with hexane – methyl –*tert*-butyl ether. The methylated fatty acids were analysed by gas chromatography (GC 6890 Agilent Technologies, Santa Clara, CA, U.S.A.) and identified in comparison with bacterial acid methyl esters mix (Sigma-Aldrich 47080-U, St. Louis, MO, U.S.A.).

Carotenoid analysis

Methanolic extracts were separated by HPLC using a mixture of acetonitrile / tetrahydrofuran / water (5:3:1, v/v/v) at a flow rate of 1 mL / min using a C18 column (Nucleosil 100 RP 18 5 µm; 4,8 x 250 mm; Varian, Palo Alto, Ca, U.S.A.) and a diode-array detector. Carotenoids were identified by comparisons of retention times and spectral characteristics to those of pure compounds and literature data. Quantitative spectrophotometric analyses were performed on methanolic extracts that were re-extracted with hexane. Phytoene concentrations were calculated by using its specific extinction coefficient $E^{1\,\%}_{1cm}$= 1100 (Foppen 1971). Carotenoids that absorb visible radiation (red carotenoids) were quantified by using $E^{1\,\%}_{1cm}$= 3240 for torulene (Foppen 1971).

RESULTS

Water loss

In colonies subjected to fast desiccation (FD), constant weight (corresponding to the complete loss of free water) was reached in 2 to 3 h. In contrast, colonies left to dry on agar (slow desiccation – SD), more than 80 h was necessary to achieve the same result. As a consequence, fungal metabolic activities were rapidly terminated in the first treatment (FD), but only slowly declined in the latter (SD). After 7 d, all colonies were completely desiccated and this status was maintained for 8 wks under both FD and SD conditions.

Morphology

Colonies of control samples consisted of mostly grossly deformed and highly-stressed cells that contained only a limited number of lipid globules. Nevertheless, the intracellular membranes were preserved and the cytoplasm remained granular. An overwhelming majority of desiccated cells had lost turgor, contained an increased number of lipid globules (Fig. 1B). Nuclei and intracellular membranous structures were not always visible, the cytoplasm was not evident and mostly replaced by lipid inclusions (abundant coalescing lipid droplets as shown in Fig. 1B). Although the shape of the cells was restored in rehydrated colonies, intracellular structures were not always re-formed and only some cells showed fully functional granular cytoplasm and intracellular membranes (Fig. 1C,D). Budding cells were observed in rehydrated colonies (Fig. 2). Nevertheless, lipid inclusions were the most obvious feature of dehydrated cells subjected to FD and SD.

Colonies of A95 were examined by cryo-SEM to reveal extra-cellular slime (Fig. 3). Following rehydration, external layers of the cell walls were remarkably swollen (Figs 1C,D), showing that the extra-cellular matrix of A95 was capable of rapidly absorbing large amounts of water.

Lipid composition

The lipid fraction comprised of monoacylglycerols, 1,2- and 1,3,-diacylglycerols, triacylglycerols, phosphotidincholine, phosphatidlethanolamine, phosphatidylinositole, phosphatidic acids and sterols (both sterol esters and free fatty acids). Fatty acids were mostly unsaturated C16:0, and C18:0 although some C18:2 were present. These lipids play strikingly different physiological roles including in membranes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), sterols (S)] and as reserves or neutral lipids [diacyl- and triacyl-glycerols, sterol ethers (SE) and free fatty acids (FA)].

Lipid contents varied significantly between samples that were subjected to fast- or slow-desiccation (cf. Figs 4, 5). Colonies that were desiccated slowly lost the major part of their sterols during drying (Fig. 4A), whereas those that dried rapidly maintained a stable level of membrane lipids (Fig. 4B). The different rehydration regimes only magnified these differences: major membrane lipids like phosphatidylcholine and phosphatidylethanolamine

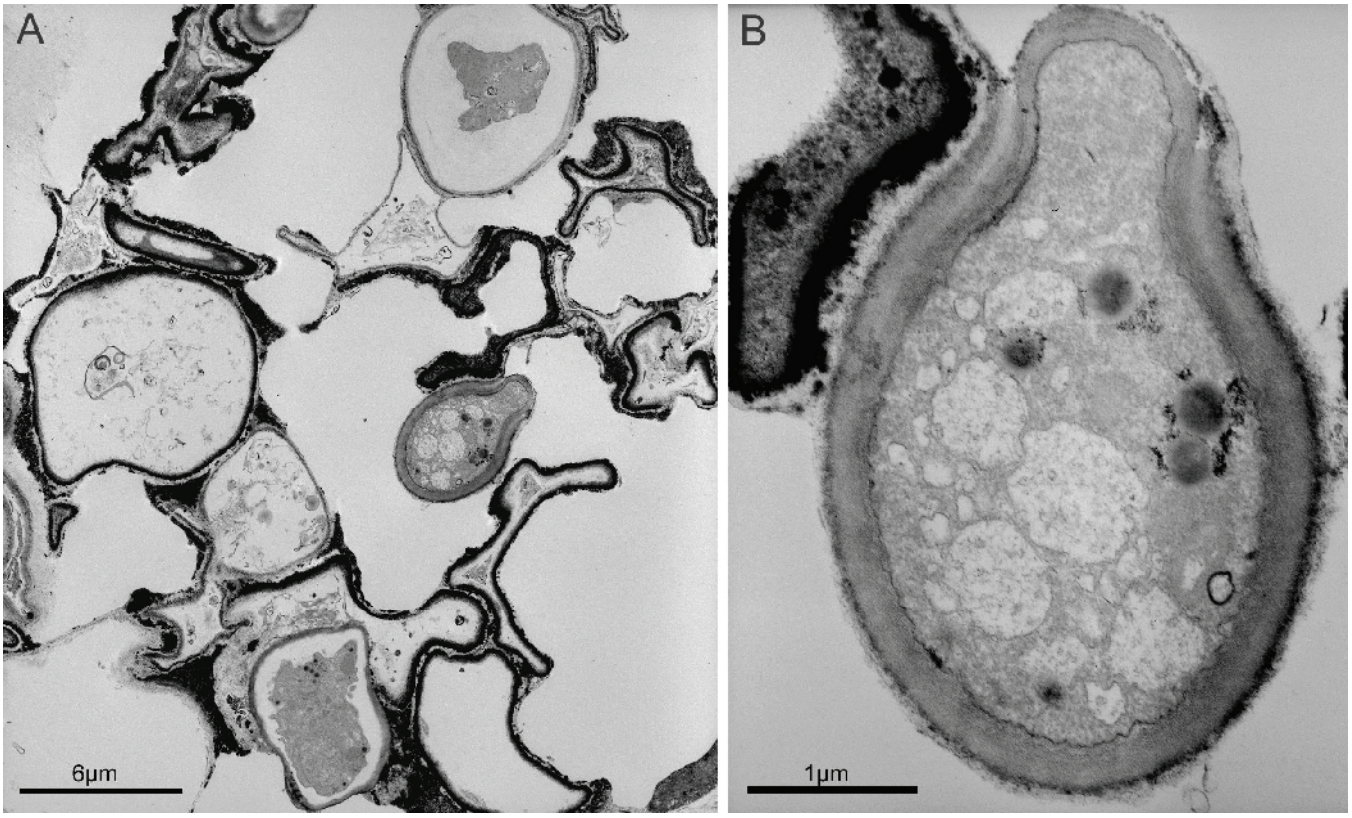

Fig. 2. TEM micrographs of microcolonial strain A95 subjected to 8 wks of fast desiccation and rehydrated by the addition of water. A. TEM micrograph of a colony showing one surviving cell among many deformed and seemingly inactive ones. B. close-up of a budding survivor cell.

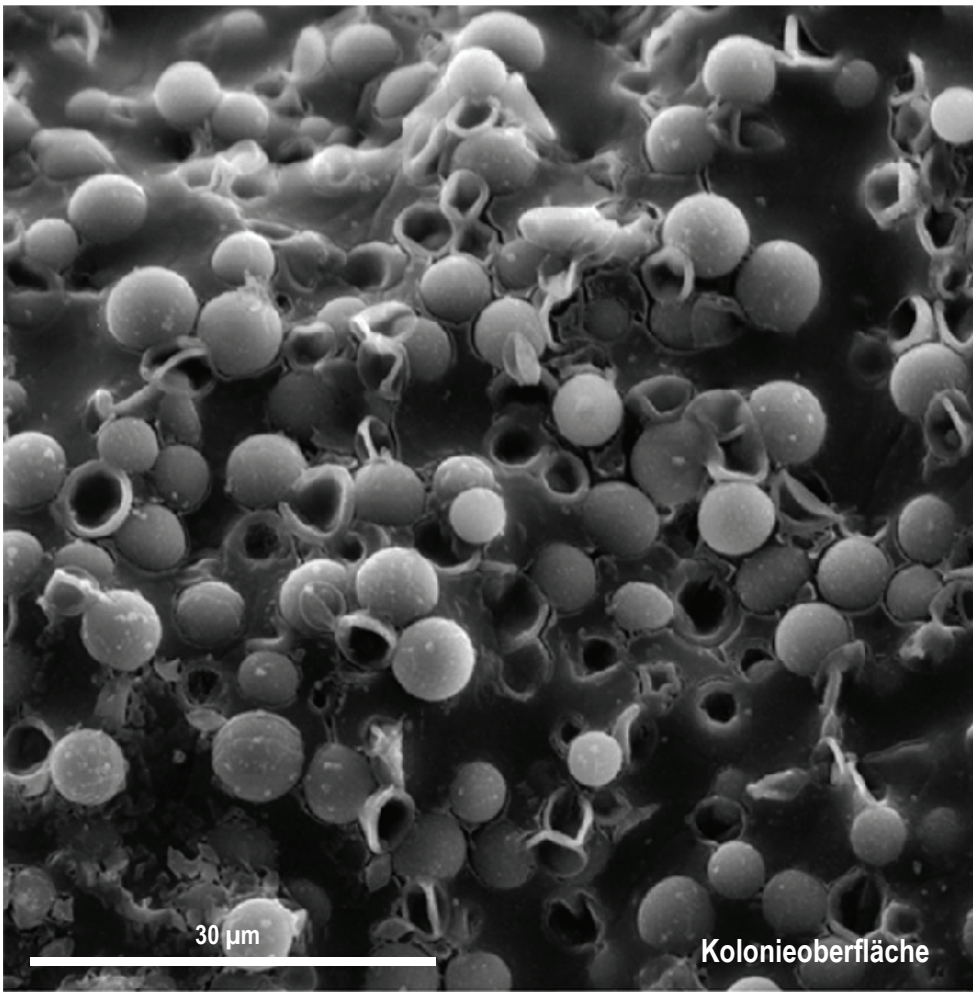

Fig. 3. Cryo-SEM micrograph of microcolonial strain A95 showing abundant colony slime.

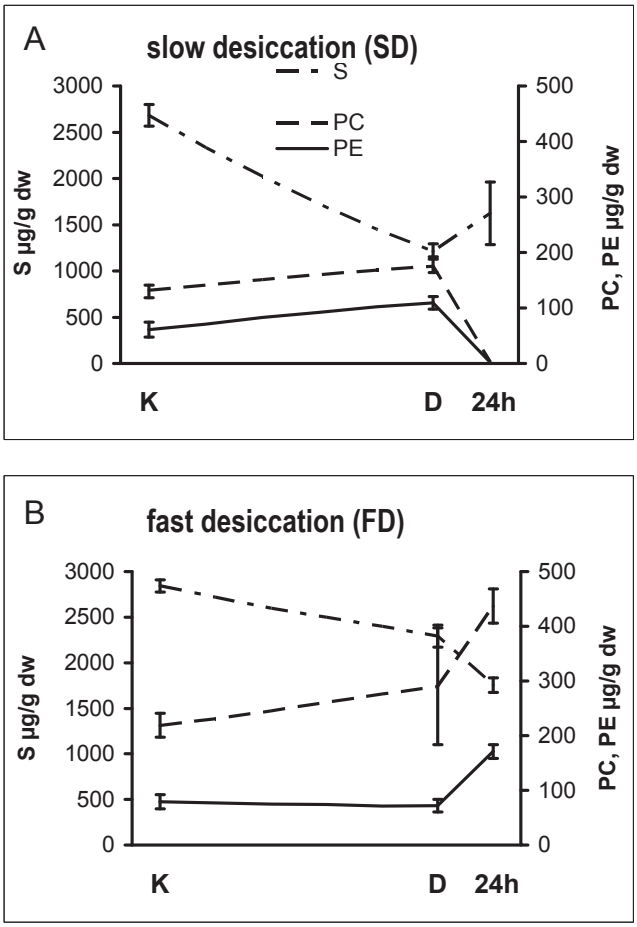

Fig. 4. Membrane lipids in colonies of microcolonial strain A95 subjected to slow (A) and fast (B) desiccation. The time axis is not to scale, as the distance between K and D equals 8 wks of desiccation, while 24 h is 24 hs after water was added to the desiccated colonies. K = control (stationary phase colony, before being transferred to a desiccator), D = after 8 wk of desiccation; 24 h = re-hydrated colony 24 h later. S = sterols, PC = phosphatidylcholine, PE = phosphatidylethanolamine.

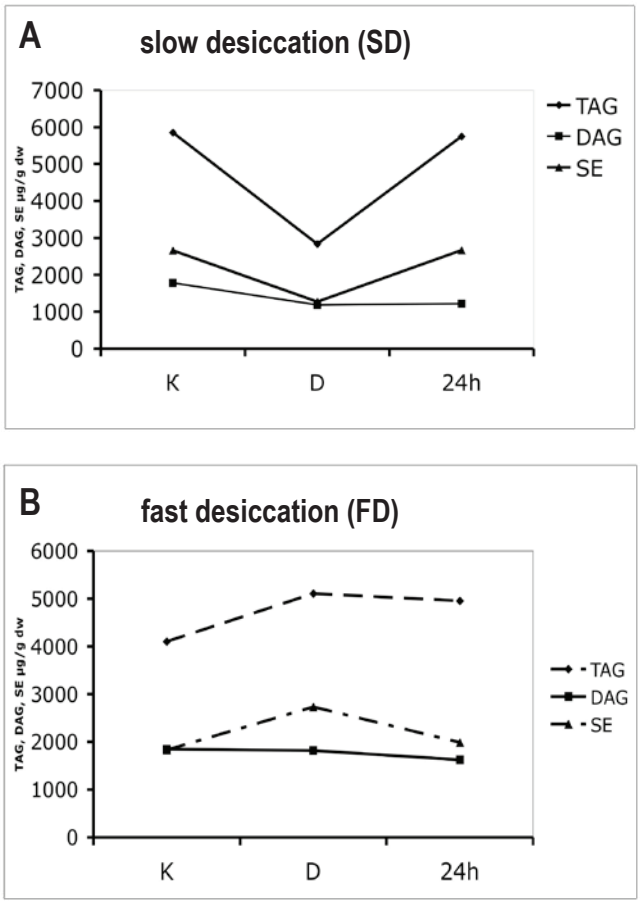

Fig. 5. Neutral (storage) lipids in colonies o microcolonial strain A95 subjected to slow (A) and fast (B) desiccation. The time axis is not to scale - the distance between K and D equals 8 wk of desiccation, while 24 h represents 24 hs after re-hydration. K = control, D = after 8 wk of desiccation; 24 h = re-hydrated colony after 24 h. TAG = triacylglycerol; DAG = diacylglycerol, SE = sterol esters.

were practically absent in SD colonies (Fig. 4A, 24 h), while FD colonies showed a steady increase in both phosphatidylcholine and phosphatidylethanolamine levels (Fig. 4B).

Desiccated cells contained high amounts of neutral lipids like triacylglycerol (TAG) and diacylglycerol (DAG). Storage lipids also displayed significantly diverse dynamics between slow- and fast-dehydrated mycelia. Neutral lipids decreased during the dehydration when the process was slow (Fig. 5A), while a slight increase occurred when desiccation was fast. TAG levels of SD cells were reduced by 50 % as compared to controls (Fig 5A), while FD cells contained 1,4-fold higher amounts of TAG (Fig. 5B). After rehydration, the constant levels of neutral lipids like triacylglycerols and sterol ethers suggest a stable metabolic state that was not significantly influenced by desiccation stress (Fig. 5B). The proportion of rapidly and slowly oxidised lipids in cell membranes and intracellular inclusions was checked in A95 following the desiccation challenge. The proportion of unsaturated fatty acids was always relatively high. In control cells, 81 % of all fatty acids were unsaturated. After 8 wk of anhydrobiosis the ratio of unsaturated fatty acids in SD and FD cells equalled respectively 80 and 67 % of the total fatty acids. In desiccated cells after 24 h of rehydration the values reached 86 % in SD cells, while rehydrated FD cells had equal proportions of saturated and unsaturated fatty acids (50:50 %).

As shown by biochemical methods, intra-cellular structures in fast desiccated cells were largely preserved and their biochemical status (synthesis of carotenoids and phospholipids) supported these observations (Figs 4B, 5B, 6B). In contrast, SD colonies (Figs 4A, 5A, 6A), were less viable although some cells in a colony were still capable of re-growth. Decreased levels of triglycerols and sterol esters suggested lower levels of storage lipids in SD cells. Only trace amounts of phospholipids were found following rehydration, confirming the complete loss of membrane structure in SD colonies. Under TEM, these cells looked like vials filled with lipid droplets, confirming the decay of intracellular membrane structures.

Pigment analysis

As expected (Gorbushina *et al*. 1993, Butler & Day 1997) melanin was obvious under TEM (Figs 1, 2) and no further characterisation of this pigment group was carried out. Pigments found in A95 absorbed radiation in UV- and visible-wavelengths. Both red carotenoids and colourless UV-absorbing carotenoids as well as mycosporines were present. Five different carotenoid pigments were identified in A95: ß-carotene, ζ-carotene, phytoene, torulene and torularhodin. Mycosporines included mycosporine-glutaminol, mycosporine-glutaminol-glucoside, mycosporine-glutamicol, and mycosporine-glutamicol-glucoside (Volkmann & Gorbushina 2006). Carotenoid contents also varied during desiccation / rehydration stress (Fig. 6). Levels of phytoene, a colourless precursor of carotenoid synthesis were particularly sensitive to stresses, but the amounts of red carotenoids decreased under SD (Fig. 6A) or remained unchanged in conditions of FD (Fig. 6B).

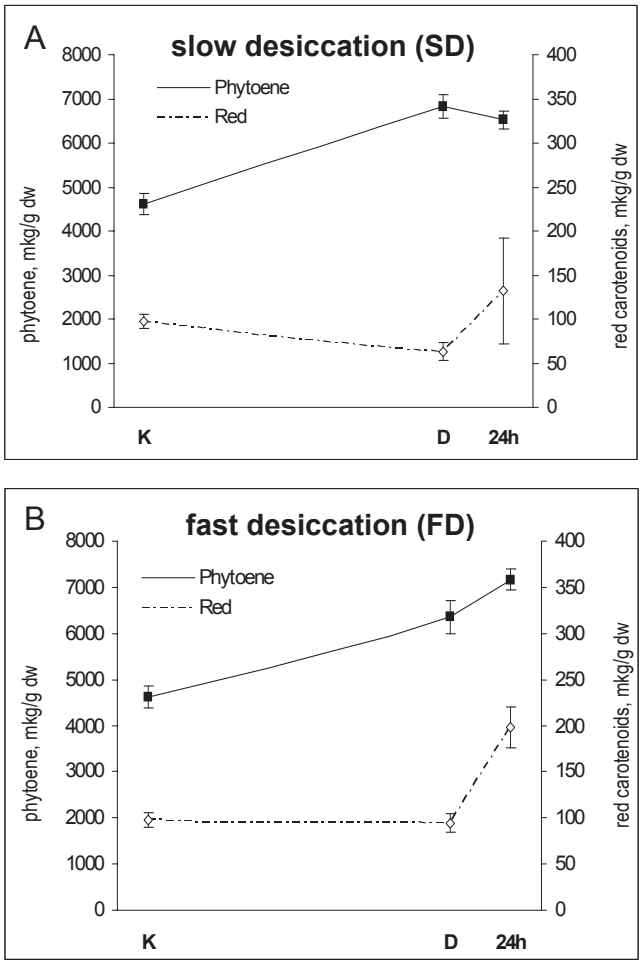

Fig. 6. Pigment composition in colonies of microcolonial strain A95 subjected to slow (A) and fast (B) desiccation. For further details see Figs 3 and 4.

DISCUSSION

Cultivation, biochemical- and ultra-structural analyses have shown that MCF strain A95 is capable of surviving desiccation / rehydration stress and sudden changes in the availability of water. Desiccated cells maintained their typical MCF spore-like ultra-structure (Sterflinger 1998, Gorbushina 2003, Gorbushina *et al.* 2003), and all colonies were able to re-grow after long-term desiccation, as well as following the rehydration challenge. Large inocula (discs of 5 mm) aided survival as parts of colonies always contained viable cells that gave rise to new colonies. Even when viable cells were few, some survivor cells in the middle of a colony were protected by the surrounding cells (Fig. 2) and were abundant enough to permit re-growth.

The decrease in the proportion of unsaturated fatty acids in both SD and FD colonies with and without dehydration indicates significant stress. Unsaturated fatty acids in membranes of A95 remained high (50 to 80 %) in all cases. Rehydrated SD colonies managed to keep the ratio of unsaturated fatty acids at the level of unstressed (unchallenged) controls, while in FD cells this parameter decreased to slightly more than 50 %. This value is, however, still high and shows an intrinsic ability of A95 to adapt to desiccation / rehydration stress.

Desiccated A95 cells contained high amounts of neutral lipids that served as a reserve for the synthesis of phospholipid-membrane following stress relief. Rapidly desiccated cells largely retained their normal physiological status. After 8 wk of matric stress and one day of rehydration, phospholipid synthesis was restored to levels that permitted correct functioning of membranes (Fig. 4B). TEM showed that budding cells were indeed present after 24 h of rehydration, confirming that the colonies remained viable (Fig. 2).

In contrast, slow desiccation (complete water loss over a period of several days, which kept metabolism active for approx. 80 h) followed by rehydration, drastically reduced phospholipid contents (Fig. 4A), and resulted in complete degradation of membrane systems. This could have been caused by the exhaustion of internal lipid resources as TAG levels of SD cells were reduced by 50 % compared to control values (Fig. 5A).

Typical fungal carotenoids include molecules with 13 conjugated double bonds that are important antioxidants (*e.g.* torulene and torularhodin), which help to stabilise membranes under unfavourable conditions All carotenoids found in A95 have been previously observed in different asco- and basidiomycetous yeasts and thus are typical fungal pigments (Davoli & Weber 2002, Weber & Davoli 2002, Davoli *et al.* 2004). As A95 was grown in the dark, the carotenoids described here belong to the natural metabolites of MCF. Constitutive levels of carotenoids in a majority of MCF strains (data not shown) suggest a readiness to counteract stress. Fungal carotenoids are reported to occur predominantly in cytoplasmic lipid bodies, the endoplasmatic reticulum, cell walls and EPS layers (Rikkinen 1995). Carotenoids are abundant in lipid inclusions of fungi, but are also major components of the cell wall and the cell membrane (Rikkinen 1995).

Phytoene is generally accepted as a precursor of less-saturated C_{40} carotenoids, which are synthesised from phytoene through a series of desaturation reactions (Simpson 1972). All types of environmental stresses promote accumulation of this colourless precursor (Fig. 6). The amount of red carotenoids either remained unchanged under conditions of fast desiccation or UV-radiation or was reduced when the cells were slowly desiccated. Fast desiccation stimulated both phytoene and red carotenoid synthesis (Fig. 6B) while preventing degradation of protective pigments.

During slow desiccation, colonies are forced to subsist with ever lower levels of available water and as a result retain synthetic activities until their internal resources (especially reserve lipids) are exhausted. In contrast, fast drying forces vegetative MCF to shift rapidly to a dormant state in which levels of reserve storage compounds, protective carotenoid precursors and constitutive antioxidants like melanins and mycosporines are maintained.

In many rock surface environments, access to sufficient sources of energy, nutrients and water rarely coincides. For this reason, micro-organisms that dwell on and in rocks need to be able to maintain biomass during extended periods of dormancy. We have shown here that MCF are capable of fast recovery after prolonged desiccation, proving that the cellular machinery remains in a state of suspended animation. This immediate revival from an anhydrobiotic state clearly demonstrates the ability of MCF to recover from water deficits that might be lethal to many prokaryotes.

The most important findings of this study are: A the fact that MCF responses to stresses are unspecific and thus can be employed against various environmental challenges (in this sense, tolerance to desiccation is perhaps part of a broader range of adaptations to other stresses; Mattimore & Battista 1996), and B that rapid as opposed to slow desiccation best preserves the viability of MCF. This preference reflects an inherent capacity of MCF to respond to drastic changes in the environmental conditions typical of subaerial rock surfaces (Gorbushina 2003). Enhanced survival of MCF when subjected to rapid changes in the environment provides strong experimental support for the poikilotolerance hypothesis proposed for rock inhabiting organisms (Gorbushina & Krumbein 2000).

CONCLUSIONS

During desiccation-induced mycostasis (dormancy), MCF employ a broad range of unspecific stress response mechanisms including:

- cell wall re-encrustation with irradiation-protective and antioxidant melanins (Gorbushina *et al.* 1993, 2003);
- layers of EPS containing lipids, polysaccharides, and proteins act as an additional irradiation filter and may have properties similar to those described for glass-forming polymers (Hill *et al*. 1997);
- presence of diverse carotenoids that are known to stabilise cell membranes;
- presence of intracellular mycosporines (typical of fungal survival structures such as spores, fruit bodies and sclerotia) that act as UV-filters, antioxidants and minor osmolytes (Gorbushina *et al.* 2003, Volkmann *et al*. 2003, Kogej *et al*. 2006, Kogej *et al*. 2007), and;
- as witnessed by a high proportion of unsaturated fatty acids and normal levels of membrane lipids (phosphatidylcholine, phosphatidylethanolamine), membranes in dormant desiccated MCF are maintained in a stable physiological state.

ACKNOWLEDGEMENTS

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Resistance of Antarctic black fungi and cryptoendolithic communities to simulated space and Martian conditions

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Abstract: Dried colonies of the Antarctic rock-inhabiting meristematic fungi *Cryomyces antarcticus* CCFEE 515, CCFEE 534 and *C. minteri* CCFEE 5187, as well as fragments of rocks colonized by the Antarctic cryptoendolithic community, were exposed to a set of ground-based experiment verification tests (EVTs) at the German Aerospace Center (DLR, Köln, Germany). These were carried out to test the tolerance of these organisms in view of their possible exposure to space conditions outside of the International Space Station (ISS). Tests included single or combined simulated space and Martian conditions. Responses were analysed both by cultural and microscopic methods. Thereby, colony formation capacities were measured and the cellular viability was assessed using live/dead dyes FUN 1 and SYTOX Green. The results clearly suggest a general good resistance of all the samples investigated. *C. minteri* CCFEE 5187, *C. antarcticus* CCFEE 515 and colonized rocks were selected as suitable candidates to withstand space flight and long-term permanence in space on the ISS in the framework of the LIchens and Fungi Experiments (LIFE programme, European Space Agency).

Key words: Astrobiology, cryptoendolithic community, fungi, ground-based experiments, lithopanspermia, panspermia, space conditions, stress resistance, viability.

INTRODUCTION

Astrobiology raises the fascinating question about the possible existence of life forms on other planets, survival in space conditions and possible interplanetary transfers. This emerging field of research encounters evident challenges in carrying out *in situ* experiments. Most of the information on the behaviour of organism under extraterrestrial conditions has been obtained by space simulation experiments performed on Earth (Nicholson *et al*. 2000, Rettberg *et al.* 2004, de Vera *et al.* 2003, 2004). Earth is our only reference for studying the possibility of life on other planetary bodies: extreme terrestrial environments host specifically adapted organisms, among which anhydrobionts are generally considered the best models for exobiological studies (Finster *et al.* 2007). Extreme environments include the cold and dry polar regions, permafrost soils, deep sea, alkaline and hypersaline habitats, arid regions, or acidic sites (González-Toril *et al.* 2003, McKay *et al.* 2003, Gunde-Cimerman *et al.* 2005, Gilichinsky *et al.* 2007, Onofri *et al.* 2007a). Organisms able to thrive in extreme environments are generally defined as 'extremophiles'. The existence of extremophiles has led to speculations about the survival of organisms during interplanetary transfer and that life could be present even on other planets of the Solar system, where water might be present *e.g.* under the icy surfaces of some of Jupiter's moons or in the underground caverns of Mars (Miller 2005, Mustard *et al*. 2008).

Antarctica is a continent where a combination of dry, cold, and oligotrophic extremes exists, and huge fluxes of dangerous radiations such as UV radiation can be present as well. These conditions become harsher in the ice-free McMurdo Dry Valleys area in continental Antarctica where cryptoendolithic microbial communities are almost the only life-form possible. Black meristematic microfungi of these communities were already suggested as eukaryotic models for the biological exploration of Mars (Onofri *et al.* 2004). The McMurdo Dry Valleys area, also known as Ross Desert, is the largest ice-free area in Antarctica. It is located within the Transantarctic Mountains in the Southern part of Victoria Land. Winter air temperature fluctuates between –20 and –50 °C (occasionally lower), rising to mean daily values of about –15 °C in the summer, up to 15 °C or higher values at ground surfaces. Wide and repeated thermal fluctuations are a stress factor, more than the minimum values reached. Dryness is also extreme: water is mainly supplied by snow (less than 100 mm water equivalent / yr), that mostly sublimes without visibly wetting the ground or it is blown away (Nienow & Friedmann 1993). High evaporation leads to high salt concentration on rock surfaces and a poor nutrient soil is occasionally present because of the scarcity of organic matter. Finally, UV radiation is high, mainly in the springtime, as a consequence of stratospheric ozone depletion during this period. As for it's oxygenic atmosphere no place on Earth is truly comparable to what is present on Mars, the McMurdo Dry Valleys could be called the closest terrestrial analogue. Therefore, they are one of the best investigated areas as a model environment for astrobiological studies since the 1970ies (Horowitz *et al.* 1972, Wynn-Williams & Edwards 2000, Onofri *et al.* 2004).

Friedmann and co-workers (Friedmann & Ocampo-Friedmann 1976, Friedmann 1982, Nienow & Friedmann 1993) discovered various microbial communities in the Ross Desert. The communities live sheltered under the rock surface, where they find a more favorable nanoclimate in the rock pore-spaces. Among these, the "lichen dominated cryptoendolithic community" is the most studied and known (Friedmann 1982). Under a reddish superficial crust, eukaryotic and prokaryotic autotrophic and heterotrophic microorganisms form a clearly stratified community. Antarctic black non-lichenised rock fungi, showing meristematic growth, are recurrent components of this community and common

Table 1. Test parameters (EVT-E1 and EVT-E2).

EVT	Parameters	Duration/exposure		No. samples
E1				
	Vacuum 10^{-5} Pa	1 h	1.3×10^{-5} Pa	3
		1 wk	2.3×10^{-6} Pa	3
	Temperature oscillation 50 cycles –20 °C to +20 °C, 1 atm air	2 wk		3
	UV-C irradiation monochromatic 254 nm, 1 atm air, 71.4 μW/cm²	14 s	10 Jm^{-2}	3
		2 min 20 s	100 Jm^{-2}	3
		23 min 20 s	1000 Jm^{-2}	3
	UV irradiation polychromatic 200–400 nm, 1 atm air	3 s (SOL2000)	1.44 kJm^{-2}	3
		52 min (SOL2000)	1.5×10^{3} kJm^{-2}	3
		87 h (SOL2000)	1.5×10^{5} kJm^{-2}	3
	total number of samples EVT-E1			27
E2				
	Vacuum 10^{-5} Pa (dark)	22 d		3
	Vacuum 10^{-5} Pa + UV irradiation polychromatic (200–400 nm)	22 d 244.5 h (SOL1000)	1.5×10^{5} kJm^{-2}	3
	Mars atmosphere 600 Pa (dark)	21 d		3
	Simulated CO_2 Mars atmosphere 600 Pa + UV irradiation polychromatic (200–400 nm)	21 d 18 min (SOL2000) + 10 d 3 h 40 min 48 s (SOL1000)	1.5×10^{5} kJm^{-2}	3
	total number of samples EVT-E2			12
Control				
	Room temperature, dark, 1 atm air	2 mo		3

inhabitants of the black zone immediately below the lichen crust. Their biodiversity has been studied only recently. Sampling is still limited, yet some new genera were discovered in these habitats, viz. *Friedmanniomyces*, *Cryomyces, Recurvomyces* and *Elasticomyces* (Onofri *et al.* 1999, 2004, Selbmann *et al.* 2005, 2008).

The results from freeze-thaw experiments, after UV exposures and osmotic stress tolerance of two isolates of *C. antarcticus* (CCFEE 515 and CCFEE 534 = CBS 116301), and one isolate of *C. minteri* (CCFEE 5187 = CBS 116902), revealed an unusual ability to survive under these pressures (Onofri *et al.* 2007b).

The opportunity to expose these isolates to space conditions by the "EXPOSE-E" facility of the European Space Agency (ESA) on the EuTEF platform (part of the European Columbus Laboratory) outside of the International Space Station (ISS) inspired ground-based experiments that will be described in this contribution. The experiments have been carried out at the German Aerospace Center (DLR, Köln, Germany), where we tested single and combined simulated space and Martian conditions. Furthermore, we extended the experiments to the entire Antarctic crytptoendolithic community as well, also to test the potential protective role of the rock substratum.

We considered this as a preliminary and essential step before launching Antarctic black fungi and cryptoendolithic communities to outer space and for exposure on the ISS in the framework of the Lichens and Fungi Experiments (LIFE, European Space Agency).

MATERIALS AND METHODS

Biological material

The biological material used for the Experiment Verification Tests (EVTs) consisted of two isolates of *C. antarcticus* (CCFEE 515 and CCFEE 534), one isolate of *C. minteri* (CCFEE 5187), and colonised rock fragments.

Cryomyces antarcticus CCFEE 515 was isolated by R. Ocampo-Friedmann from sandstone collected at Linnaeus Terrace (Southern Victoria Land) by H. Vishniac, in the Antarctic expedition 1980-81; *C. antarcticus* CCFEE 534 was isolated by R. Ocampo-Friedmann from weathered rock collected at Linnaeus Terrace by E.I. Friedmann, during the Antarctic expedition 1981–82; *C. minteri* CCFEE 5187 was isolated by S. Pagano from weathered rocks collected by S. Onofri at Battleship Promontory (Southern Victoria Land) on Dec 28, 1996. For the EVTs, dehydrated fungal colonies were prepared as follows: cell suspensions were spread on MEA (malt extract agar: malt extract, powdered 30 g/L; peptone 5 g/L; agar 15 g/L; Applichem, GmbH) medium (5 mm thick) in Petri dishes and, once grown, maintained at 15 °C for 1 yr. Agar disks (12 mm diameter) containing 1–3 colonies each were then drilled and used for tests.

The colonised sandstone sample, with a well developed and stratified colonisation, was collected by L. Zucconi at Battleship

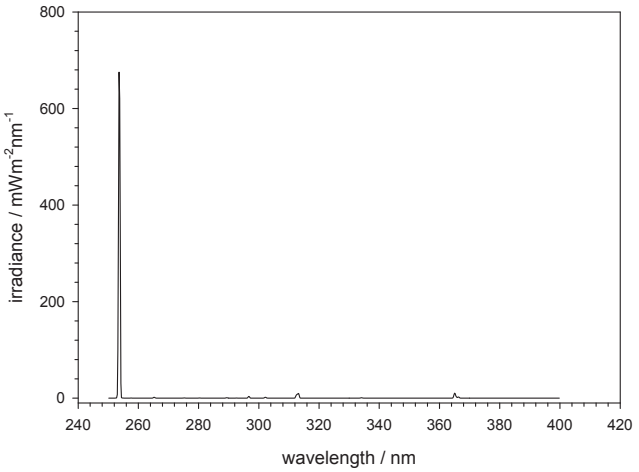

Fig. 1. Spectral irradiance of the monochromatic UV source, a Hg-low-pressure lamp, mainly emitting at 254 nm (data provided by DLR).

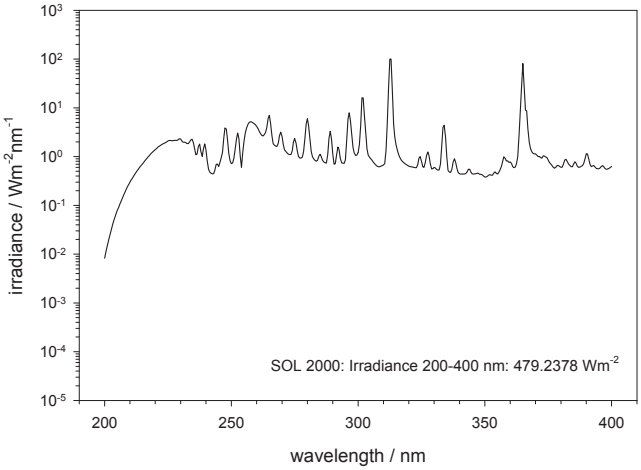

Fig. 2. Spectrum of the solar simulator SOL2000 in the range of the polychromatic UV, 200–400 nm (data provided by DLR).

Promontory (76°54'37.6"S 160°55'27.5"E), Southern Victoria Land, on Jan. 24, 2004. Colonised rock fragments (11 mm wide, maximum 6 mm thick) were obtained by hitting the rock lengthwise, and then dehydrated at room temperature.

Tests facilities and exposure conditions

Two sets of ground-based Experiment Verification Tests for EXPOSE-E (EVT-E1 and EVT-E2) were performed, using the Planetary and Space Simulation facilities (PSI) at the Institute of Aerospace Medicine (German Aerospace Center, DLR, Köln, Germany). Twenty-seven samples of each isolate and colonised rock for the EVT-E1 (9 tests) and 12 for the EVT-E2 (4 tests) were prepared, plus 3 controls each. The aim of EVT-E1 was to test the response of rock fungi and cryptoendolithic communities to exposure to the following space conditions: vacuum, temperature fluctuations (–20 / +20 °C), laboratory standard monochromatic UV-C radiation and high polychromatic UV radiation (Table 1).

The aim of EVT-E2 was to test the responses of rock fungi and cryptoendolithic community to simulated space vacuum, simulated CO_2 Martian atmosphere and pressure, simulated space vacuum combined with polychromatic UV radiation, and simulated CO_2 Martian atmosphere combined with polychromatic UV radiation (Table 1). All tests were performed in triplicate.

Vacuum (E1 and E2)

The pressure was set at the value of 10^{-5} Pa as expected to prevail during the space flight. Samples were accommodated in a vacuum facility, called PSI 6, and exposed to vacuum for 1 h and 1 wk for E1, and 22 d for E2, after reaching 10^{-5} Pa (monitored by a Pirani cold cathode inserted into the vacuum chamber).

Temperature fluctuation (E1)

Temperature fluctuations (–20 / +20 °C) are expected during the space flight. 50 cycles were therefore performed inside a facility, called PSI 2, within 2 wk, programming 2 h heating, 2.5 h cooling, and maintenance at –20 °C and +20 °C respectively for 1 h. The temperature was monitored with a sensor attached to the inner side of a sample carrier.

UV Radiation conditions and fluences (E1 and E2)

E1. The monochromatic UV-C at 254 nm was obtained using a Hg-low-pressure lamp, the spectral irradiance was measured using a Bentham spectroradiometer (Fig. 1) giving a total irradiance of 0.7 W/m^2; in addition the irradiance was controlled before and after each exposure with a calibrated UVX-meter at the sample site. Samples were arranged within the homogeneously irradiated area. Due to the low infrared (IR) output of this lamp, no cooling was required. The exposure was performed additively, by covering the samples that received their assigned UV-C fluence of 10, 100 and 1000 Jm^{-2}, with UV opaque filters.

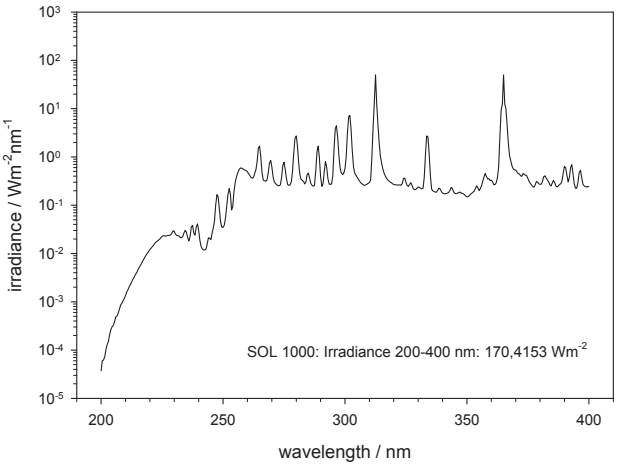

Fig. 3. Spectrum of the solar simulator SOL1000 in the range of the polychromatic UV, 200–400 nm (data provided by DLR).

The polychromatic UV irradiance was obtained using the solar simulators SOL2000 and SOL1000 (the latter only used in EVT-E2). Figs 2–3 show only the polychromatic UV irradiation spectra in the range 200–400 nm. The samples were accommodated on a cold plate inside the homogeneously irradiated area inside the facility PSI 2 (Fig. 4), and kept below 30 °C during the whole irradiation, by cooling the cold plate to 10 °C. Temperature was monitored during the irradiation by a sensor attached to the sample carrier (Fig. 5). The fluences for irradiation with polychromatic UV in EVT-E1 were selected to simulate the final dose of irradiation (1.5×10^5 kJm^{-2}) and the attenuated fluences beneath 1 % and 0.01 % neutral density filters, expected on the ISS during the mission in this wavelength range. Similar fluences were reached with the solar simulator SOL2000 after 87 h irradiation for 1.5×10^5 kJm^{-2}, 52 min for 1.5×10^3 kJm^{-2} and 3 s for 1.5 kJm^{-2} (Table 1).

Fig. 4. Planetary and Space Simulation facility PSI 2: irradiation with solar simulator SOL2000.

E2. A combination of polychromatic UV irradiation and vacuum or simulated Martian atmosphere was applied (Table 1). For the polychromatic UV irradiance in vacuum (10^{-5} Pa) as well as in a simulated CO_2 Martian atmosphere (composed of argon 1.56 %, oxygen 0.16 %, nitrogen 2.72 %, carbon dioxide 95.56 %; pressure of 600 Pa), the samples were accommodated on the cold plate kept at 10 °C inside the homogeneous irradiated area inside the facility PSI 2. Temperature was monitored during the irradiation by a sensor attached to the sample carrier. The IR radiation of the solar simulators leads to heating the irradiated samples in the low pressure and hence low-convection environment. Because the SOL2000 caused a high heating of the samples, for more prolonged irradiation times the weaker SOL1000 solar simulator was used (Table 1).

The measured and integrated irradiance of the SOL2000 for the polychromatic UV range 200–400 nm was 479.2 W/m^2, and 170.4 W/m^2 for the SOL1000, at the sample site. The selected final dose of 1.5 × 10^5 kJm^{-2} (full polychromatic irradiation) was reached after a total irradiation time of 18 min with SOL2000 plus 243 h with SOL1000 in simulated CO_2 Martian atmosphere, and after a total irradiation time of 244.5 h with SOL1000 irradiation in simulated space vacuum.

Analyses of responses to tested parameters

Responses to test parameters were analysed both by cultural and staining methods. Viability of isolates of *C. antarcticus* (CCFEE 515 and CCFEE 534) and of *C. minteri* (CCFEE 5187) after EVTs by cultural methods was evaluated as number of formed colony. Tested colonies were collected, preliminarily treated in a rotator (9 g/min) with 1 mL of a sterile solution of Tween 20 (0.35 %) for 15 min to remove eventual external contaminants, and then washed with 1 mL of sterile physiological solution (4 times), for 15 min, to remove Tween 20. Fungal suspensions were obtained by crumbling colonies with a sterile needle, followed by serial dilutions up to a final concentration of about 10^4 cells per mL of inoculum in physiological solution (0.9 % NaCl). Cultural tests were performed by spreading 0.15 mL of standardised inocula on MEA (Malt Extract Agar, Applichem Gmbh), with the addition of chloramphenicol (100 ppm) to prevent bacterial growth. Cultures were then incubated at 15 °C. The number of growing microcolonies was recorded after 1 and 2 mo of incubation. Data are reported as percentage of colonies compared to the untreated control. All tests were performed in triplicate.

Viability of samples from rocks was demonstrated by directly spreading small fragments of rocks on 5 different cultural media: MEA (Malt Extract Agar; Applichem Gmbh) and DRBC (Oxoid) media (Dichloran Rose-Bengal Chloramphenicol agar: peptone 5 g/L; dextrose 10 g/L; biacid potassium phosphate 1 g/L; magnesium sulphate 0.5 g/L; dichloran 0.002 g/L; rose-bengal 0.025 g/L; agar 15 g/L) for fungal growth (filamentous and black fungi respectively); TM medium (*Trebouxia* Medium: Bold's Basal Medium 970 mL; proteose peptone 2.5 g/L; glucose 5 g/L; agar 15 g/L) for algal growth; TY and BG11 media (Trypton Yeast Medium: trypton 5 g/L; yeast extract 3 g/L; $CaCl_2$ anhydrous 0.4 g/L; agar 17 g/L. Blue Green Algae Medium: $NaNO_3$ 1.5 g/L; K_2HPO_4 0.04 g/L; $MgSO_4 \times 7H_2O$ 0.075 g/L; $CaCl_2 \times 2H_2O$ 0.036 g/L; citric acid 0.006 g/L; ammonium ferric citrate 0.006 g/L; EDTA 0.001 g/L; metal traces 1 mL/L: H_3BO_3 2.86 g/L, $MnCl_2 \times 4H_2O$ 1.81 g/L, $ZnSO_4 \times 7H_2O$ 0.222 g/L, $NaMoO_4 \times 2H_2O$ 0.39 g/L, $CuSO_4 \times 5H_2O$ 0.079 g/L, $Co(NO_3)_2 \times 6H_2O$ 49.4 mg/L; agar 10 g/L) for bacterial and cyanobacterial growth, respectively. The occurrence / absence of growing colonies was simply recorded after 3 mo of incubation in the dark for fungi and bacteria, and in the light for algae and cyanobacteria.

Viability was also evaluated by staining methods using two dyes, the LIVE / DEAD dye FUN 1 (30 µM) and SYTOX Green (10 µM) in PBS (Dulbecco's Phosphate Buffered Saline: NaCl 8 g/L; KCl 0.2 g/L; Na_2HPO_4 anhydrous 1.15 g/L; KH_2PO_4 0.259 g/L). This enabled us to detect viable and metabolically active cells as well as non-viable or damaged cells. Staining methods were applied only to *Cryomyces minteri* isolate.

Each tested colony was rehydrated with a PBS solution for 1 h, included in a polyethylene glycol mixture (Killik, Bio-Optica) inside a cryostat chamber (Leica, CM1510 S) at –20 °C for 10 min, cut into sections about 25 µm thick by a microtome, and coloured on microscope slides. The best dye penetration for FUN 1 and SYTOX Green was obtained with an incubation of 1–3 h and 1.5 h respectively, under dark conditions at room temperature.

A fluorescence microscope Axioskop 2 plus (Zeiss), provided with long-pass filters (488 nm with emission ≥ 530 nm for fluorescein isothiocyanate, 546 nm with emission ≥ 580 nm for the rhodamine), was used to analyse the viability of the *C. minteri* samples after exposure to test parameters. Red (viable and metabolically active) and green (viable but not metabolically active) emitting images of stained samples were separately acquired by a mounted Axiocam

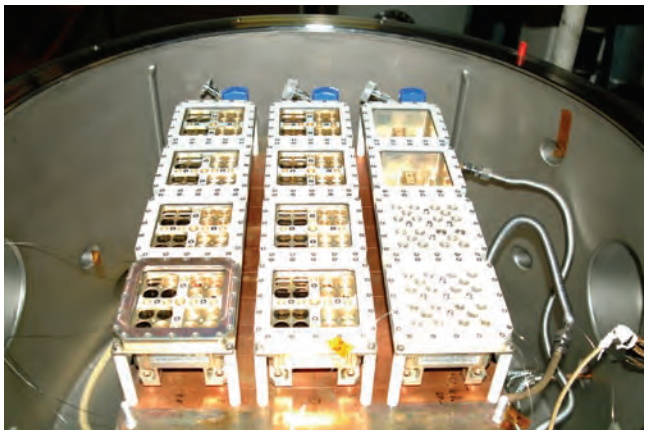

Fig. 5. Sample carrier, composed of three trays, 16 wells each, in PSI 2.

using AxioVision v. 4 software. The combined image obtained by overlapping both images allows to distinguish clearly the viability state. The SYTOX Green dye (S-7020, Molecular Probes) stains the DNA of non-viable cells green when excited at 450–500 nm, and using an appropriate filter (504 nm with emission ≥ 524 nm). The imagines were imported in Adobe Photoshop whereas the quantitative analyses were performed using KS300 software and expressed as percentage of live / dead cells.

Responses to high temperatures

Thermal model calculations by ESA estimated that during the space flight the temperature may reach high values (up to 90 °C without any coverage). Therefore we tested the resistance of *C. antarcticus* CCFEE 515 and CCFEE 534 and *C. minteri* CCFEE 5187 at 60 °C (selected as limit value for closing automatically the EXPOSE sample carrier lids) and of *C. antarcticus* CCFEE 515 and *C. minteri* CCFEE 5187, previously selected for flight, also at 80 and 90 °C. Six-mo-old dried colonies of all the isolates were incubated at 60 °C for 15 and 60 min (3 replicates for each test) and six-mo old dried colonies of *C. antarcticus* CCFEE 515 and *C. minteri* CCFEE 5187 were incubated for 60 min at 80 and 90 °C (5 replicates). All colonies were subsequently maintained overnight in a desiccator, to allow a slow cooling to room temperature. Survival was tested by cultural methods, as described above, as number of colonies growing after two mo of incubation on MEA at 15 °C, for the tests at 60 °C, and as presence / absence of growing colonies for tests at 80 and 90 °C. Viability after exposure at 80 and 90 °C was also evaluated in *C. minteri* by staining methods.

RESULTS

Fungal isolates

Figure 6 shows cell viability of fungal isolates after Experiment Verification Tests E1 and E2, analysed by cultural methods.

Vacuum

The viability of dried isolates after 1 h, 1 wk (EVT-E1) and 22 d (EVT-E2) of exposure to simulated space vacuum is reported as percentage variations, compared to the untreated controls, in the number of colonies grown after 2 mo of incubation on MEA at 15 °C. Colony formation of all isolates was negatively affected by the vacuum treatment, more markedly in *C. antarcticus* than in *C. minteri*. *Cryomyces antarcticus* CCFEE 534 is more markedly affected, with a complete loss of growth ability already after 1 wk of exposure, whereas isolate CCFEE 515 maintains the ability to grow also after 22 d of exposure.

Freeze and thawing cycles

Results of the viability of dried isolates after 50 repeated freeze and thawing cycles, recorded as above and compared to the untreated controls, showed different responses: *C. antarcticus* CCFEE 515 was the most negatively affected with a strong reduction of growth ability, *C. minteri* CCFEE 5187 showed a 75 % reduction, while *C. antarcticus* CCFEE 534 seemed to be almost unaffected.

UV radiation at different spectral ranges

With respect to the capability of dried fungal isolates to form colonies after increasing monochromatic or polychromatic radiation doses, respectively, *C. antarcticus* isolates were both negatively affected by the higher doses of polychromatic UV, whereas the species showed diversified response to the monochromatic irradiation (Fig. 6). CCFEE 515 remained practically unaffected, and CCFEE 534 was totally inhibited after the highest exposure only. *Cryomyces minteri* CCFEE 5187 showed a good survival after both radiation types and doses, with a certain decrease only at the highest doses.

UV radiation plus vacuum or Martian atmosphere and pressure

Figure 6 shows the results of exposure of dried samples to polychromatic UV spectrum in space-simulating vacuum as well as in simulated Martian atmosphere compared to vacuum and Martian atmosphere exposure, respectively. Growth obtained with both isolates of *C. antarcticus* was scarce, with a complete inhibition of isolate CCFEE 534 and a substantial reduction in viability in CCFEE 515. This response was consistent with negative results obtained after vacuum and 1.5×10^5 kJm^{-2} polychromatic irradiation. Propagules of *C. minteri* CCFFEE 5187 showed a higher survival than both *C. antarcticus* strains after exposure either to vacuum or Martian CO_2, and a reduction, but not complete inhibition, at combined UV radiation and Martian atmosphere. A total absence in viability apparently appears in the combined test of vacuum and maximum UV (200−400 nm) dose of 1.5×10^5 kJm^{-2}, but this result was disproved by the staining techniques.

The LIVE / DEAD dye FUN1 (F-7030, Molecular Probes) passively penetrates the cytoplasm and stains cells with a yellowish green to green fluorescence: the intravacuolar enzymatic activity of viable and metabolically active cells is indicated by the appearance of compact cylindrical intravacuolar structures (CIVS) with an orange to red fluorescence (when a λ_{ex} between 470 and 590 nm is used) and a concomitant reduction of the green to yellow cytoplasmatic fluorescence.

The staining procedure failed with the isolates of *C. antarcticus* because the thickness of cell wall prevented the penetration of the dyes, while the same procedure was successful with *C. minteri*. Good viability percentages obtained in all EVTs with the LIVE / DEAD dye FUN 1 (Fig. 7) confirmed the positive responses obtained by the cultural methods and the high resistance of *C. minteri* to simulated space and Martian conditions in both EVTs, while combined vacuum and maximum UV (200−400 nm) exposure gave ambiguous results when the responses were tested with cultural or staining approach. In fact, even though *C. minteri* was unable to grow after this treatment, cell viability in a 25 µm section of a colony was suggested by the red and green fluorescence shown in Fig. 8. Data (not shown) obtained by the SYTOX Green dye, confirmed results reported in Fig. 7.

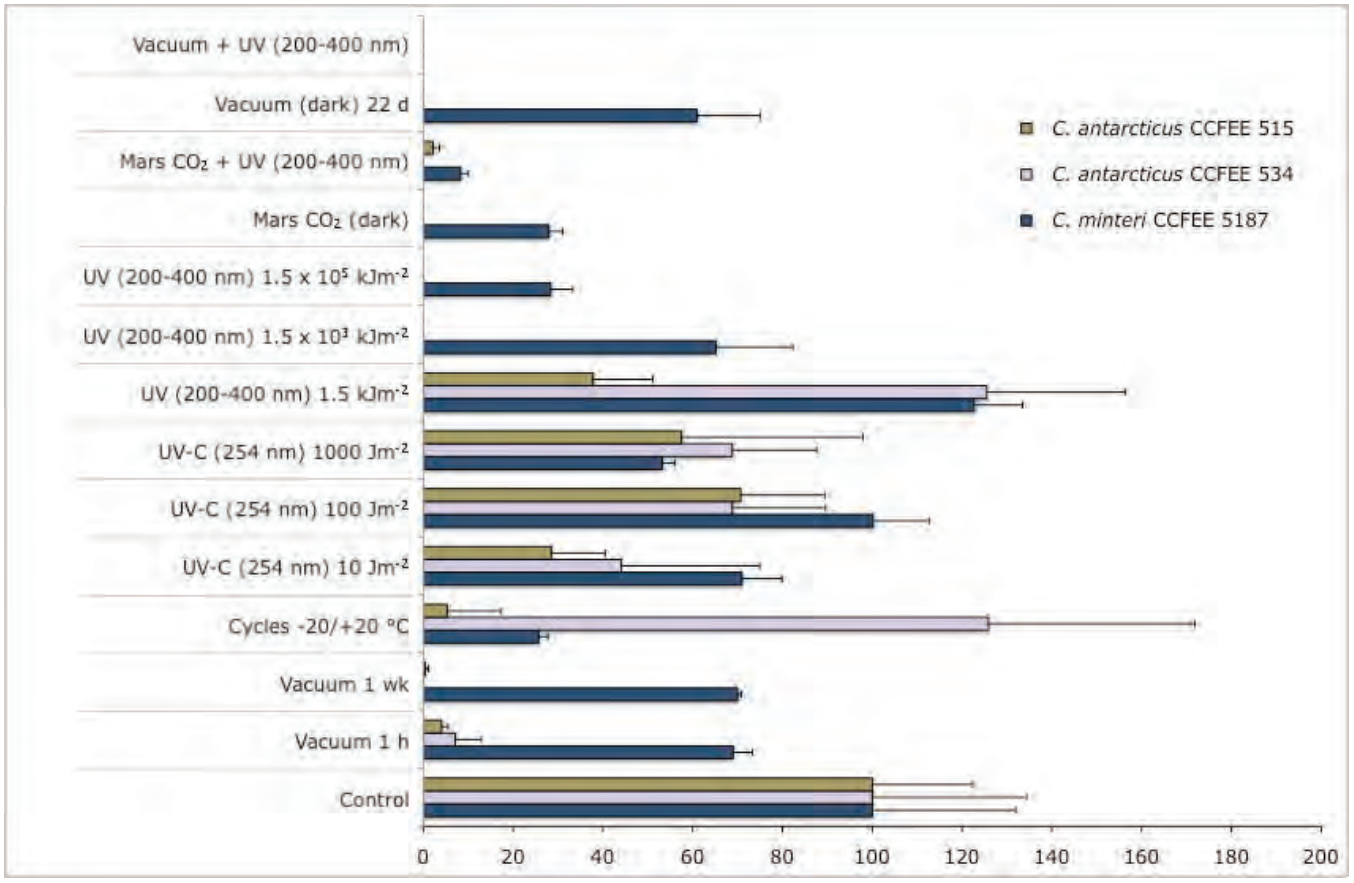

Fig. 6. Percentage variations, compared to untreated control, in the number of colonies of *C. antarcticus* CCFEE 534 and CCFEE 515 and *C. minteri* CCFEE 5187 after exposure to single or combined simulated space and Mars conditions (EVT-E1 and E2), evaluated by cultural methods.

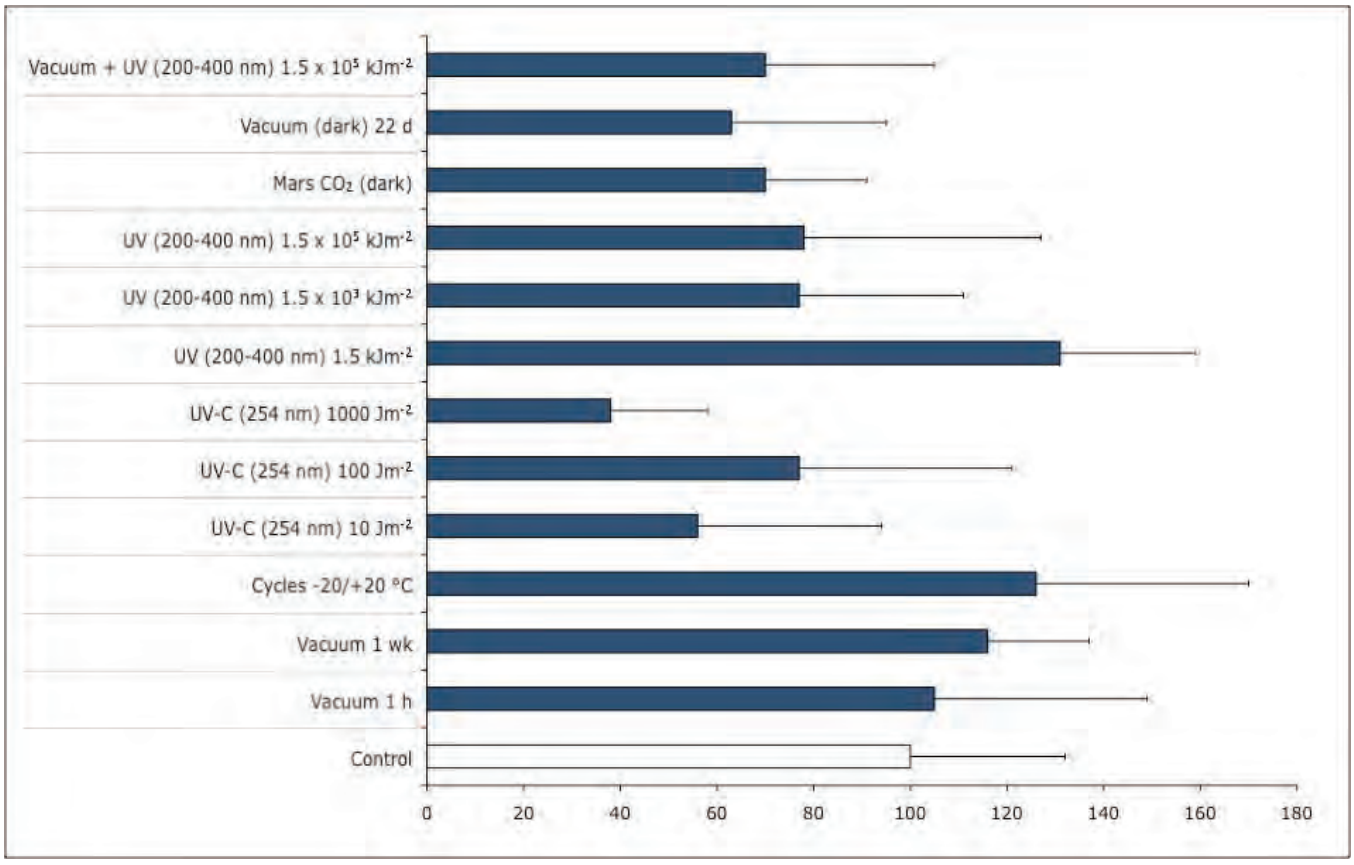

Fig. 7. Percentage variations, compared to untreated control, of *C. minteri* CCFEE 5187 cell viability after experiment verification tests E1 and E2, evaluated *in vivo* by LIVE / DEAD dye FUN 1.

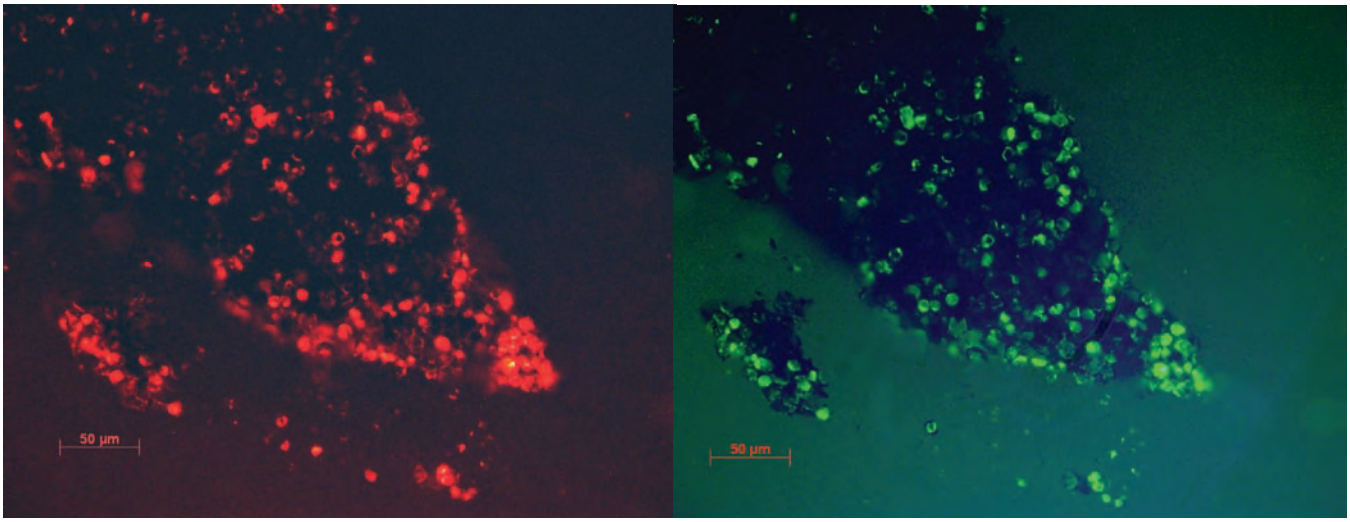

Fig. 8. Red and green fluorescence of viable and metabolic active cells in 25 µm sections of a *C. minteri* colony treated with LIVE / DEAD dye FUN 1, after combined exposure to vacuum and maximum polychromatic UV 200–400 nm dose (bar = 50 µm).

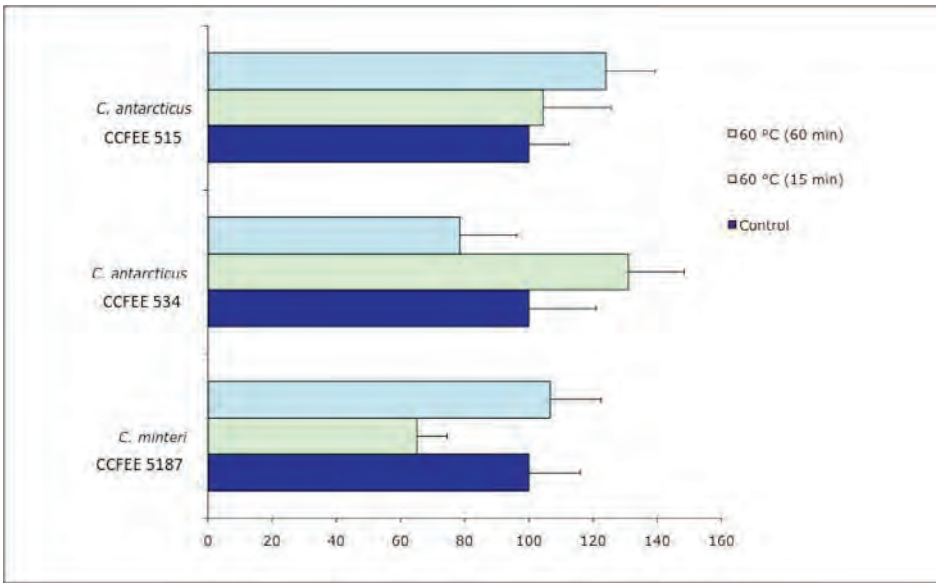

Fig. 9. Percentage variations, compared to controls, in the number of colonies of *C. antarcticus* CCFEE 534 and CCFEE 515 and *C. minteri* CCFEE 5187 after incubation at 60 °C for 15 and 60 min.

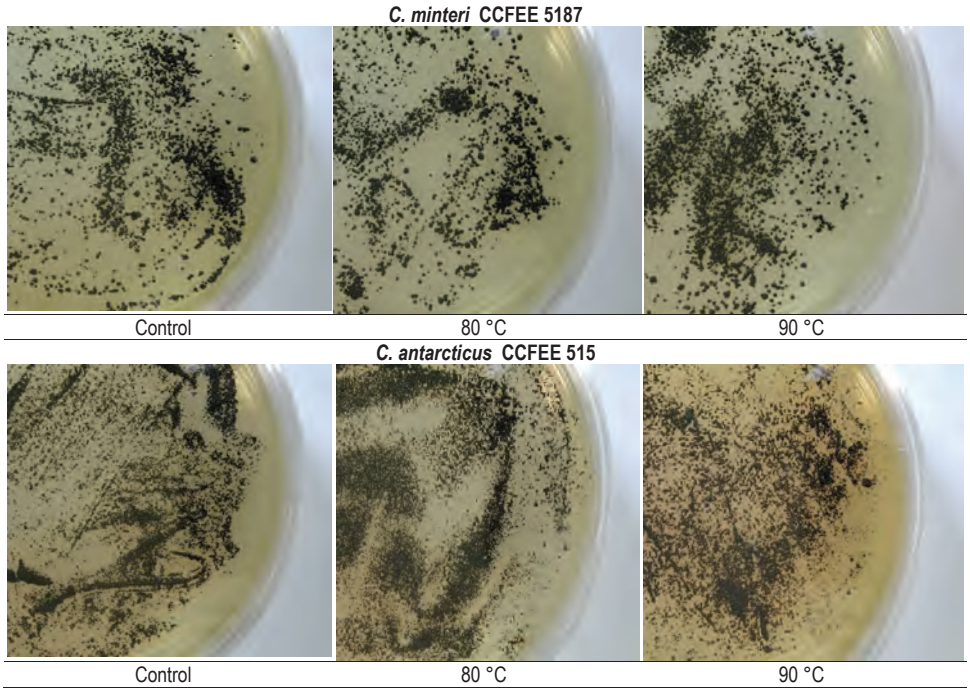

Fig. 10. Colonies on MEA of *C. minteri* CCFEE 5187 and *C. antarcticus* CCFEE 515 after incubation at 80 °C (center) and 90 °C (right) for 60 min, compared to the controls (left).

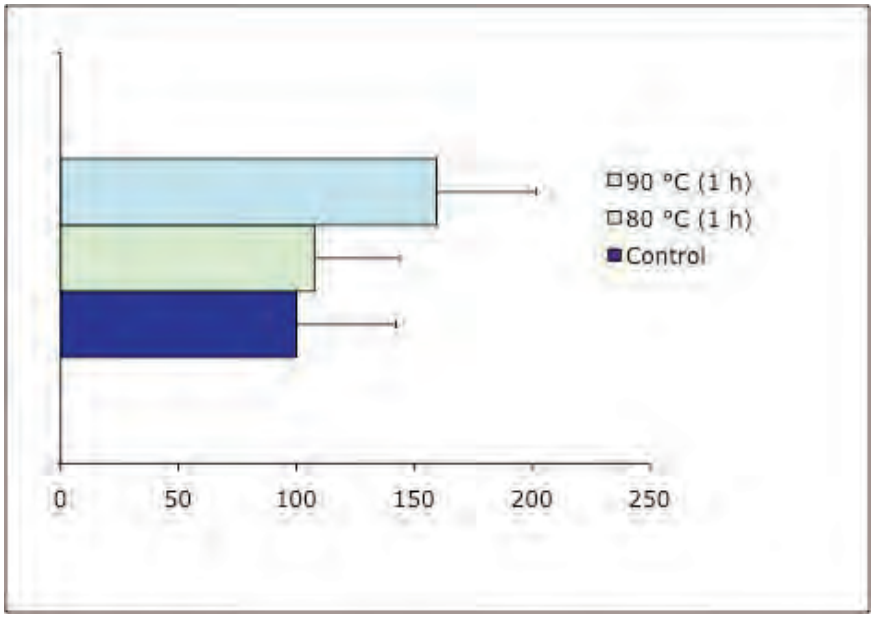

Fig. 11. Percentage of cell viability of *C. minteri* CCFEE 5187 after incubation at 80 and 90 °C for 60 min, compared to the control, evaluated by using FUN 1 *in vivo*.

Table 2. Presence (+) / absence (-) of colonies of fungi (filamentous fungi and black yeast fungi), algae, and bacteria (cyanobacteria included) from rock fragments after EVTs.

TEST			Colony formation		
EVT-E1			**Algae**	**Fungi**	**Bacteria**
Vacuum	10^{-5} Pa	1 h	-	-	+
		1 wk	+	-	+
Freeze and thawing cycles		2 wk	+	+	+
UV (254 nm), 1 atm					
Fluence:	10 Jm^{-2}		+	+	-
	100 Jm^{-2}		+	+	+
	1000 Jm^{-2}		+	+	+
UV (200–400 nm), 1 atm					
Fluence:	1.5 kJ m^{-2}		-	+	-
	1.5 × 10^{3} kJ m^{-2}		-	-	-
	1.5 × 10^{5} kJ m^{-2}		-	-	-
EVT-E2					
Vacuum	10^{-5} Pa	22 d	+	+	-
Vacuum + UV (200–400 nm)					
Fluence:	1.5 × 10^{5} kJ m^{-2}		-	-	-
Control					
Room temperature, dark, 1 atm, air		2 mo	+	+	+

High temperatures

Effects of incubation at 60 °C for 15 and 60 min, and of 80 and 90 °C for 1 h respectively, on the viability of the tested isolates, was evaluated by counting the number of colonies (Fig. 9) and the presence / absence of grown colonies (Fig. 10). A good resistance of all strains was found at 60 °C for both 15 min and 60 min, with low variations compared to the controls. Both tested isolates of *C. minteri* CCFEE 5187 and *C. antarcticus* CCFEE 515 showed an unexpected high viability of growing colonies after exposure to 80 and 90 °C, already after one mo incubation (Fig. 10). Viability of C. *minteri* was confirmed by LIVE / DEAD dye FUN1 staining (Fig. 11).

Table 3. Summarisation of responses of the tested isolates to the EVTs, reporded as survival (+) or non-survival (-).

		C. antarcticus CCFEE 515	*C. antarcticus* CCFEE 534	*C. minteri* CCFEE 5187
EVT-E1	Vacuum 10^{-5} Pa/1h	+	+	+
	Vacuum 10^{-5} Pa/1 wk	+	-	+
	50 cycles –20/+20 °C/2 wk	+	+	+
	UV-C (254 nm) 10 Jm^{-2}	+	+	+
	UV-C (254 nm) 100 Jm^{-2}	+	+	+
	UV-C (254 nm) 1000 Jm^{-2}	+	+	+
	UV (200–400 nm) 1.5 kJm^{-2}	+	+	+
	UV (200–400 nm) 1.5 × 10^3 kJm^{-2}	-	-	+
	UV (200–400 nm) 1.5 × 10^5 kJm^{-2}	-	-	+
EVT-E2	Vacuum/22 d (dark)	+	-	+
	Vacuum + UV (200–400 nm) 1.5 × 10^5 kJm^{-2}	-	-	+(*)
	Mars CO_2/21 d (dark)	+	-	+
	Mars CO_2 + UV (200–400 nm) 1.5 × 10^5 kJm^{-2}	+	-	+
Control	Room temperature, dark, 1 atm air	+	+	+

(*) Result obtained only by staining techniques.

Cryptoendolithic community

Table 2 reports the presence or absence of colonies grown from rock fragment samples after EVTs. Eleven verification tests E1 and E2 were carried out, and colony appearance was recorded in 8 of them. Negative results are those concerning polychromatic irradiation at higher doses and vacuum plus polychromatic UV-irradiation at the lower dose. Some samples treated with simulated Martian atmosphere with or without combined polychromatic UV radiation have been lost and EVT-E2 data for these experiments are missing.

DISCUSSION

This is the first report on resistance of fungal isolates and cryptoendolithic communities from terrestrial extreme environments, to simulated space and Martian conditions, which were applied individually or in different combinations. Selected fungal isolates were previously demonstrated to survive some extreme terrestrial factors, such as repeated freezing and thawing cycles, high salt concentrations and UV-B irradiation (Onofri *et al.* 2007b). This study demonstrates high resistance of all isolates to simulated space or Martian conditions, despite wide standard deviations. Thirteen verification tests were carried out in both EVT-E1 and E2. Reporting results as survival (+) or non-survival (-) (Table 3), 13 positive responses were recorded for *C. minteri* CCFEE 5187, 10 for *C. antarcticus* CCFEE 515 and 6 for *C. antarcticus* CCFEE 534. Moreover, results obtained by staining methods in *C. minteri* (Fig. 7) showed no significant reductions of living cells both in control and treated cultures, with the exception of the UV-C treatment (254 nm, 1000 Jm^{-2}). The higher resistance of *C. minteri* CCFEE 5187 to both EVTs and the good survival shown to heat shocks suggested that this isolate might be selected as a good candidate to withstand space flight and long-term permanence in space. Under the most selective combined condition of vacuum and maximum dose of polychromatic UV used, this strain was unable to grow. Its positive response in staining techniques but absence of growth might be due to the transition to a state of "viable but non-culturable cells" (VBNC), as described for bacteria (Weichart 1999). Apparently cells can maintain their integrity and viability but nevertheless may lose reproductive ability. Growth of *C. minteri* may escape detection because of the transition to a specific survival state characterised by deceleration of vital activity (DVA) (Feofilova 2003). Because *C. antarcticus* cells were not stainable, we did not have the opportunity to verify the occurrence of viable but not culturable cells after exposure to stressing conditions. Particularly surprising were the results concerning single and combined irradiation of *C. minteri* at the maximum UV polychromatic dose, since it corresponds to the irradiation expected during the whole planned space exposure of 1.5 yr without attenuation, *i.e.* without neutral density filter.

Cryomyces antarcticus CCFEE 515, which gave better results compared with CCFEE 514 of the same species, was also selected to investigate short and long term resistance to space conditions on the ISS.

The high resistance to space conditions shown by the three isolates tested could be ascribed to the peculiar morpho- and physiological features of black meristematic fungi. These microorganisms produce slowly expanding, cauliflower-like colonies, barely differentiated structures, and thick and heavily pigmented cell walls (Selbmann *et al.* 2005, Onofri *et al.* 2007a). These characteristics convey to high tolerance to extreme terrestrial environments and, by coincidence, spacial conditions. Melanin, for instance, is a biological macromolecule, ubiquitous in nature, mainly known for its protective role against UV and ionising radiation, extreme temperatures, and desiccation (Sterflinger 2005). The high tolerance to the UV-B exposure of single cells of the tested fungi has been reported recently (Onofri *et al.* 2007b). The thickness of the colony itself may represent an additional protection for the cells in the inner layers.

The high resistance of tested isolates to temperatures up to 90 °C is in agreement with literature on survival of dehydrated colonies of other meristematic fungi subjected to high temperatures (Sterflinger & Krumbein 1995, Sterflinger *et al.* 1999, Sterflinger

2005). The ability to enter a cryptobiotic state under poikilohydric conditions could be aided by the presence of abundant extracellular polymeric substances (EPS) in many species. EPS production may be abundant (Selbmann *et al*. 2005) and may appear as a gelatinous matrix in lichen thalli (de Vera *et al*. 2004) which may serve as a water reservoir to survive long dry periods (de los Ríos *et al.* 2004, 2005). Not surprisingly, many meristematic black fungi are commonly recorded from Mediterranean areas and hot deserts, where substrate surface temperatures can reach very high values. The ability to survive long-term desiccation makes these isolates pre-adapted to the extreme conditions of space, since high-vacuum conditions produce an extreme dehydrating effect.

Recent studies also show that lichens, as well as their isolated photobionts and mycobionts, cope with the extreme conditions of outer space in ground-based experiments (de Vera *et al.* 2003, 2004). *Xanthoria elegans* was able to photosynthesise under simulated Martian conditions with light in visible wave-lengths and in the presence of water (de Vera *et al.* 2007). Finally, samples of the lichens *Rhizocarpon geographicum* and *X. elegans* survived 16 d of exposure to space in the BIOPAN-5 facility of the European Space Agency located on the outer shell of the Earth-orbiting FOTON-M2 Russian satellite (Sancho *et al.* 2007).

By means of the NASA Space Shuttle Atlantis flight launched on Feb 7, 2008, these fungi are now exposed to actual space in the EXPOSE facility (created by Kaiser-Threde - DE) on the outside platform EuTEF of the International Space Station, orbiting round the Earth at a height of ~300 km, where space conditions include pressures of 10^{-5} Pa, temperatures ranges between –20 and +20 °C, and full solar (including UV-A, UV-B, and UV-C) and cosmic radiation. These conditions are normally prohibitive for life.

Lithopanspermia postulates the feasibility of interplanetary transfer of living material, protected against extraterrestrial solar UV and possibly heat within asteroids, comets and meteorites (Nicholson *et al*. 2000). Our knowledge on the limits of life has largely expanded in the last decades. The discovery of extremophiles, the high survival of *Bacillus subtilis* spores over six yr in space (Horneck *et al*. 1994), the survival of lichens after ground-based experiments (de Vera *et al.* 2003, 2004), as well as in space for 2 wk during the Biopan experiments (Sancho *et al.* 2007), and our results with the Experiment Verification Tests on meristematic fungi and cryptoendolithic communities give additional support to the idea of lithopanspermia. Considering that 1 yr is the minimum flight time estimated for Martian meteorites landing on Earth (Mileikowsky *et al.* 2000), the eventual survival after 1.5 yr permanence in space planned in the LIFE experiment represents a further contribution in the scenario of interplanetary transfer of life.

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A rock-inhabiting ancestor for mutualistic and pathogen-rich fungal lineages

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Abstract: Rock surfaces are unique terrestrial habitats in which rapid changes in the intensity of radiation, temperature, water supply and nutrient availability challenge the survival of microbes. A specialised, but diverse group of free-living, melanised fungi are amongst the persistent settlers of bare rocks. Multigene phylogenetic analyses were used to study relationships of ascomycetes from a variety of substrates, with a dataset including a broad sampling of rock dwellers from different geographical locations. Rock-inhabiting fungi appear particularly diverse in the early diverging lineages of the orders *Chaetothyriales* and *Verrucariales*. Although these orders share a most recent common ancestor, their lifestyles are strikingly different. *Verrucariales* are mostly lichen-forming fungi, while *Chaetothyriales*, by contrast, are best known as opportunistic pathogens of vertebrates (*e.g. Cladophialophora bantiana* and *Exophiala dermatitidis*, both agents of fatal brain infections) and saprophytes. The rock-dwelling habit is shown here to be key to the evolution of these two ecologically disparate orders. The most recent common ancestor of *Verrucariales* and *Chaetothyriales* is reconstructed as a non-lichenised rock-inhabitant. Ancestral state reconstructions suggest *Verrucariales* as one of the independent ascomycetes group where lichenisation has evolved on a hostile rock surface that might have favored this shift to a symbiotic lifestyle. Rock-inhabiting fungi are also ancestral to opportunistic pathogens, as they are found in the early diverging lineages of *Chaetothyriales*. In *Chaetothyriales* and *Verrucariales*, specific morphological and physiological traits (here referred to as extremotolerance) evolved in response to stresses in extreme conditions prevailing on rock surfaces. These factors facilitated colonisation of various substrates including the brains of vertebrates by opportunistic fungal pathogens, as well as helped establishment of a stable lichen symbiosis.

Key words: Evolution of rock-dwelling habit, evolution of lichenisation, multigene phylogeny, ancestral state reconstruction, *Verrucariales* and *Chaetothyriales* (*Chaetothyriomycetidae*, *Eurotiomycetes*).

INTRODUCTION

During the evolutionary history of the Fungi, associations with various substrates have evolved, together with a wide range of nutritional modes and numerous symbiotic interactions. As a consequence, fungi are often referred to as "ecological opportunists", in order to emphasise their ecological diversity (Thompson 1994, Gargas *et al.* 1995). The evolution of ecological traits in fungi has been the focus of several studies, in particular for symbiotic fungi like lichens and mycorrhiza (Gargas *et al.* 1995, Hibbett *et al.* 2000, Lutzoni *et al.* 2001, Grube *et al.* 2004, Schmitt *et al.* 2005). Recent improvement in higher-level classification of Fungi has been reached by large-scale multigene analyses (Lutzoni *et al.* 2004, James *et al.* 2006, Schoch *et al.* in press). These studies have clarified phylogenetic relationships within and between main groups of fungi, and some clades were delimited with an increased certainty. Several of these newly defined groups comprise puzzling assemblages of ecologically diverse taxa, for which we need to develop a deeper understanding of the evolution of their lifestyles.

Verrucariales and *Chaetothyriales*, two closely related ascomycete orders from the class *Eurotiomycetes*, are a good example of one of these evolutionary puzzles. Strongly supported as sister groups in many studies (Lutzoni *et al.* 2001, 2004, Lumbsch *et al.* 2005), these two orders are extremely disparate ecologically. *Verrucariales* are mostly lichenised, *i.e.*, forming stable symbiotic associations with one or two photosynthetic partners (mostly from the green algae), and have a preference for mineral substrates. *Chaetothyriales*, in the other hand, was originally best known for its animal and human opportunistic pathogens (Winka *et al.* 1998), often called black yeasts in reference to their melanisation and growth form. This order of mostly anamorphic species also includes teleomorphs that occur as saprophytes on decaying wood and mushrooms (Untereiner *et al.* 1995, Untereiner 1997, 2000). Previous studies of the evolution of lichenisation have shown that the ancestor of these two orders was likely lichenised, and that opportunistic pathogens and saprophytes within *Chaetothyriales* probably derived from this ancestor by a loss of lichenisation (Lutzoni *et al.* 2001, James *et al.* 2006). Since two additional non-lichenised orders, *Coryneliales* and *Mycocaliciales*, have been affirmatively placed within this class (Geiser *et al.* 2006, Spatafora *et al.* 2006), our comprehension of the phylogenetic relationships and hence of the class *Eurotiomycetes* was drastically changed. Association with different habitats and substrates has gained an important role while the evolution of the *Chaetothyriales* and a broader selection of fungi from bare rock surfaces came into consideration.

This peculiar guild of ascomycetes, which inhabit bare rock surfaces, has consistently been overlooked when considering the evolution of ecological traits in fungi. First discovered in extreme environments, in the hot deserts and in Antarctica (Krumbein & Jens 1981, Friedmann 1982, Staley *et al.* 1982, Henssen 1987, Danin 1993), these fungi were shown to persistently colonise rock surfaces under more temperate climates (Urzi *et al.* 1995, Sterflinger & Prillinger 2001). These rock-inhabitants are particularly diverse in semiarid and arid habitats, where they thrive largely

Table 1. Support values for the phylogeny of the *Pezizomycotina* dataset (PP and ML bootstrap) and for the ancestral state reconstruction of lichenisation. The state "non lichenised" was coded as 0, and the state "lichenised" as 1. For the Bayesian reconstructions (MCMC), the PPs of each state are indicated [P(0) and P(1)]. For the ML reconstructions on the Bayesian tree sample (ML), the number of trees supporting each state [Nt(0) and Nt(1)] or ambiguous [Nt(a)] are indicated, as well as the mean of the probabilities for each state across the 5000 trees [Pm(0) and Pm(1)]. Node numbers in this table refer to node numbers in Figure 1. Bold numbers indicate PP greater than 95 %. Results with AddNode option: [1] P(0)=1.00 for node 5; [2] P(0)=0.98 for node 7; [3] P(0)=0.83 for node 10; [4] P(1)=1.00 for node 12. Results using RAxML BS replicates: [5] Pm(0)=0.81 for node 13; [6] Pm(0)=0.89 for node 17; [7] Pm(0)=0.68 for node 18; [8] Pm(0)=0.78 for node 21.

				Ancestral state reconstruction of lichenisation (0=non lichenised, 1=lichenised)						
				MCMC		**ML**				
Nodes	**Taxonomical groups**	**PP (%)**	**ML BS (%)**	P(0)	P(1)	Nt(0)	Nt(a)	Nt(1)	$P_m(0)$	$P_m(1)$
1	*Pezizomycotina*	**100**	88	**0.98**	0.02	5000	0	0	**1.00**	0.00
2	*Pezizomycotina* without *Pezizomycetes*	**100**	100	0.91	0.09	4983	17	0	**0.99**	0.01
3	-	**100**	84	0.71	0.29	270	4730	0	0.88	0.12
4	*Leotiomycetes* + *Sordariomycetes*	**100**	99	**0.99**	0.01	5000	0	0	**1.00**	0.00
5	*Leotiomycetes* *	65.38	67	**1.00**[1]	0.00	5000	0	0	**1.00**	0.00
6	*Sordariomycetes*	**100**	100	**0.99**	0.01	5000	0	0	**1.00**	0.00
7	*Pezizomycetes* *	**99.66**	74	**1.00**[2]	0.02	5000	0	0	**0.99**	0.01
8	*Arthoniomycetes*	**100**	100	0.01	**0.99**	0	0	5000	0.00	**1.00**
9	*Trypetheliaceae*	**100**	100	0.00	**1.00**	0	0	5000	0.00	**1.00**
10	*Dothideomycetes* *	50.34	<50	0.58[3]	0.42	14	4986	0	0.63	0.37
11	*Dothideomycetes* without *Trypetheliaceae*	**100**	91	**1.00**	0.00	5000	0	0	**1.00**	0.00
12	*Lichinomycetes* + *Lecanoromycetes* *	**99.68**	<50	0.00	**1.00**[4]	0	14	4986	0.00	**1.00**
13	*Eurotiomycetes*	**100**	88	**0.98**	0.02	5000	0	0	**1.00**[5]	0.00
14	*Mycocaliciales*	**100**	100	**1.00**	0.00	5000	0	0	**1.00**	0.00
15	*Eurotiomycetidae*	**100**	100	**0.99**	0.01	5000	0	0	**1.00**	0.01
16	*Eurotiales*/*Onygenales*	**100**	100	**1.00**	0.00	5000	0	0	**1.00**	0.00
17	*Eurotiomycetidae* + *Chaetothyriomycetidae*	**100**	99	0.91	0.09	4969	31	0	**0.98**[6]	0.03
18	*Chaetothyriomycetidae*	**100**	100	0.55	0.45	0	5000	0	0.64[7]	0.36
19	*Pyrenulales*	**100**	100	0.05	**0.95**	0	555	4445	0.04	**0.96**
20	*Chaetothyriales*	**100**	100	**0.99**	0.01	5000	0	0	**1.00**	0.00
21	*Chaetothyriales* + *Verrucariales*	**100**	100	0.93	0.07	4985	15	0	**0.98**[8]	0.02
22	Rock Fungi/*Verrucariales*	**100**	75	0.59	0.41	0	5000	0	0.65	0.35
23	*Verrucariales*	**100**	100	0.00	**1.00**	0	0	5000	0.00	**1.00**

due to the absence of competition, but also their extraordinary extremotolerance. A number of specific and universally present morphological and physiological characters enable them to tolerate surprisingly wide ranges of temperature, irradiation and osmotic stress (Palmer *et al.* 1990, Sterflinger 1998, Ruibal 2004, Gorbushina 2007). Melanisation protects the cells against UV and solar radiation, but also extremes of temperature and desiccation. The production of internal asexual spores and their typical isodiametrical (meristematic) growth form keep the volume-surface ratio optimal and, therefore, enable these fungi to survive extreme drought (Wollenzien *et al.* 1995). Finally, their ability to rely exclusively on sparse, airborne, low molecular weight nutrients (oligotrophism), contributes to the amazing survival capabilities that extremotolerance confers to rock-inhabiting fungi in these hostile habitats. The remarkable survival abilities of these fungi along with a capacity to penetrate minerals make this guild an attractive study object in microbial ecophysiology and applied research, such as biodeterioration of monuments and exobiology (Gorbushina *et al.* 1993, Diakumaku *et al.* 1995, Wollenzien *et al.* 1997, Gorbushina *et al.* 2002, Gorbushina 2003).

The first molecular phylogenetic studies to include some of these rock fungi showed that they belonged to two main groups of ascomycetes, the *Dothideomycetes* and the *Chaetothyriales* (Sterflinger *et al.* 1997, 1999, Ruibal 2004). These early studies did not allow a precise phylogenetic placement of most rock-inhabiting fungi as they either included many strains but a fast evolving phylogenetic marker (Ruibal 2004, Sert *et al.* 2007), or a slowly evolving marker but limited taxon sampling (Sterflinger *et al.* 1997, 1999). A recent detailed study of a broad sampling of rock-inhabiting fungi from Central Spain and Mallorca (Ruibal *et al.* 2005, 2008) revealed a large number of undiscovered rock-inhabiting strains and allowed us to infer the phylogenetic relationships of their Chaetothyrialean members in respect to other members of this order using a multigene analysis. By incorporating these unusual fungi within a phylogenetic study, we were able to obtain a better representation of the ecological diversity of this group. Taking into account recent contributions to fungal molecular phylogenetics and newly-discovered lineages of rock-inhabiting fungi, two main questions were addressed: (i) is the ancestor of the two orders *Chaetothyriales* and *Verrucariales* still reconstructed

as lichenised, as in previous studies, and (ii) is the rock-dwelling habit an ancestral trait to both the lichenised *Verrucariales* and the pathogen-rich *Chaetothyriales*. To answer these questions, we explore the evolution of the lichenisation and the rock-dwelling habit using ancestral state reconstruction methods. Our results are discussed within the broader framework of the origin of lichenisation and pathogenicity in ascomycetes.

MATERIAL AND METHODS

Taxon sampling

Two different datasets were used. The evolution of lichenisation, which was shown in previous studies to be highly dependent on the relationships inferred between classes of ascomycetes (Lutzoni *et al.* 2001, James *et al.* 2006), was analysed with a dataset representative of almost all main groups of this phylum, except for some early diverging lineages. This dataset included a total of 92 accessions (*Pezizomycotina* dataset; Appendix 1 in Supplementary Information), sampled in order to represent the phylogenetic and ecological (lichenised vs. non-lichenised) diversity of this phylum. The study of the evolution of the rock-inhabiting habit was limited to our group of interest, the class *Eurotiomycetes*, and included a total of 183 accessions (*Eurotiomycetes* dataset; Appendix 2 in Supplementary Information). This dataset includes 25 isolates of rock-inhabiting fungi, most of which selected from a larger pool of strains (Ruibal 2004, Ruibal *et al.* 2008), to represent all groups separated by a 95 % ITS similarity criterion.

Molecular data

The *Pezizomycotina* dataset included three phylogenetic markers, the small and large subunits of the nuclear ribosomal RNA gene (nucSSU and nucLSU) and the largest subunit of the RNA polymerase II (*RPB*1). For this dataset, the gene sequences of 75 taxa were obtained from GenBank. The *Eurotiomycetes* dataset included the same three loci, and one additional phylogenetic marker, the small subunit of the mitochondrial ribosomal RNA gene (mtSSU). In total, this study generated 68 sequences of nucLSU, 64 of nucSSU, 129 of mtSSU and 61 of *RPB*1, most of them for taxa within the *Eurotiomycetes* (Appendices 1 and 2). The sequences of nucLSU, nucSSU and *RPB*1 were generated using protocols described in Gueidan *et al.* (2007), and sequences of mtSSU were obtained following the protocol published in Zoller *et al.* (1999).

Alignments and phylogenetic analyses

Sequence editing, alignment and congruence assessment were done as described in Gueidan *et al.* (2007). Phylogenetic relationships and confidence were inferred using a Bayesian approach. Additional support values were estimated using maximum likelihood (ML) bootstrap. For the Bayesian approach, the Akaike Information Criterion, as implemented in Modeltest 3.7 (Posada & Crandall 1998), was used to estimate models of molecular evolution. For both datasets, a GTR+I+G model was used for the different partitions (nucLSU, nucSSU, mtSSU, *RPB*1 first, second and third codon positions), except for the 3rd codon position of *RPB*1 region D-G, which was not concatenated with region A-D in the *Eurotiomycetes* dataset, and was subjected to a HKY+I+G model. For each dataset, two independent analyses of two parallel runs and four chains were carried out for 5,000,000 generations using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), and trees were sampled every 500 generations. A burn-in sample of 5000 trees was discarded from the first run. The remaining 5000 trees were used to estimate branch lengths and posterior probabilities (PPs) with the sumt command in MrBayes. The program RAxML-VI-HPC (Stamatakis *et al.* 2005) was used for the ML bootstrap analysis with 1000 replicates and a GTRMIX model of molecular evolution applied to the partitions previously defined.

Ancestral state reconstructions

A recent study showed that estimates of ancestral states may vary depending on the reconstruction methods applied (Ekman *et al.* 2008). For this reason, two methods were used (comparisons in Tables 1 and 2): a Bayesian approach using BayesMultiState (Pagel *et al.* 2004) and an ML approach applied to a sample of 5 000 Bayesian trees using MultiState (Pagel *et al.* 2004). Ancestral state reconstructions of lichenisation were carried out on 23 nodes of the *Pezizomycotina* dataset (Fig. 1, Table 1), and the rock-dwelling habit on 28 nodes of the *Eurotiomycetes* dataset (Fig. 2, Table 2). All nodes were present in all 5 000 Bayesian trees, except for nodes 5, 7, 10, and 12 of the *Pezizomycotina* dataset (Table 1). The program uses a continuous-time Markov model of trait evolution, allowing unequal rates of losses and gains (Lewis 2002). For the Bayesian approach, a uniform prior (0, 100) was chosen, and the analysis was allowed to run for 5 000 000 iterations, with a sample period of 100 and with the option AddMRCA. Output files from BayesMultiState were analysed using Tracer v.1.2.1 (Rambaut & Drummond), with a Burn-in of 500 000 iterations, and output files from MultiState were analysed with Excel v. 11.3.3 (Microsoft).

RESULTS

Ancestral state reconstructions

Ancestral state reconstructions gave similar results with maximum likelihood (ML) and Bayesian methods (MCMC), except for some nodes supported in more than 95 % of the Bayesian trees with ML (nodes 2 and 17), but with only moderate supports with MCMC (90 % < posterior probability [PP] < 95 %).

Analyses were done using the option AddMRCA, which allows the reconstruction to be carried out on all trees, even if they do not have the node of interest, by finding, for these particular trees, a node that contains at least all the species otherwise present in the node of interest. For the four nodes that were not present in all Bayesian trees (nodes 5, 7, 10 and 12 of the *Pezizomycotina* dataset), the option AddNode, restricting the reconstructions to the trees having the node of interest, was also used for comparison (see legend of Table 1). For these four nodes, whether all trees were used (option AddMRCA) or only the trees with the node of interest (option AddNode), did not have a major effect on most of the reconstructions, at the exception of node 3, for which the posterior probability to be non-lichenised increased from 0.58 to 0.83 when using the option AddNode.

Bayesian and ML ancestral state reconstructions on Bayesian trees allow taking into account phylogenetic uncertainties (Pagel *et al.* 2004). However, because the Bayesian approach as implemented in MrBayes was shown to sometime overestimate posterior probabilities on short internodes (Alfaro *et al.* 2003),

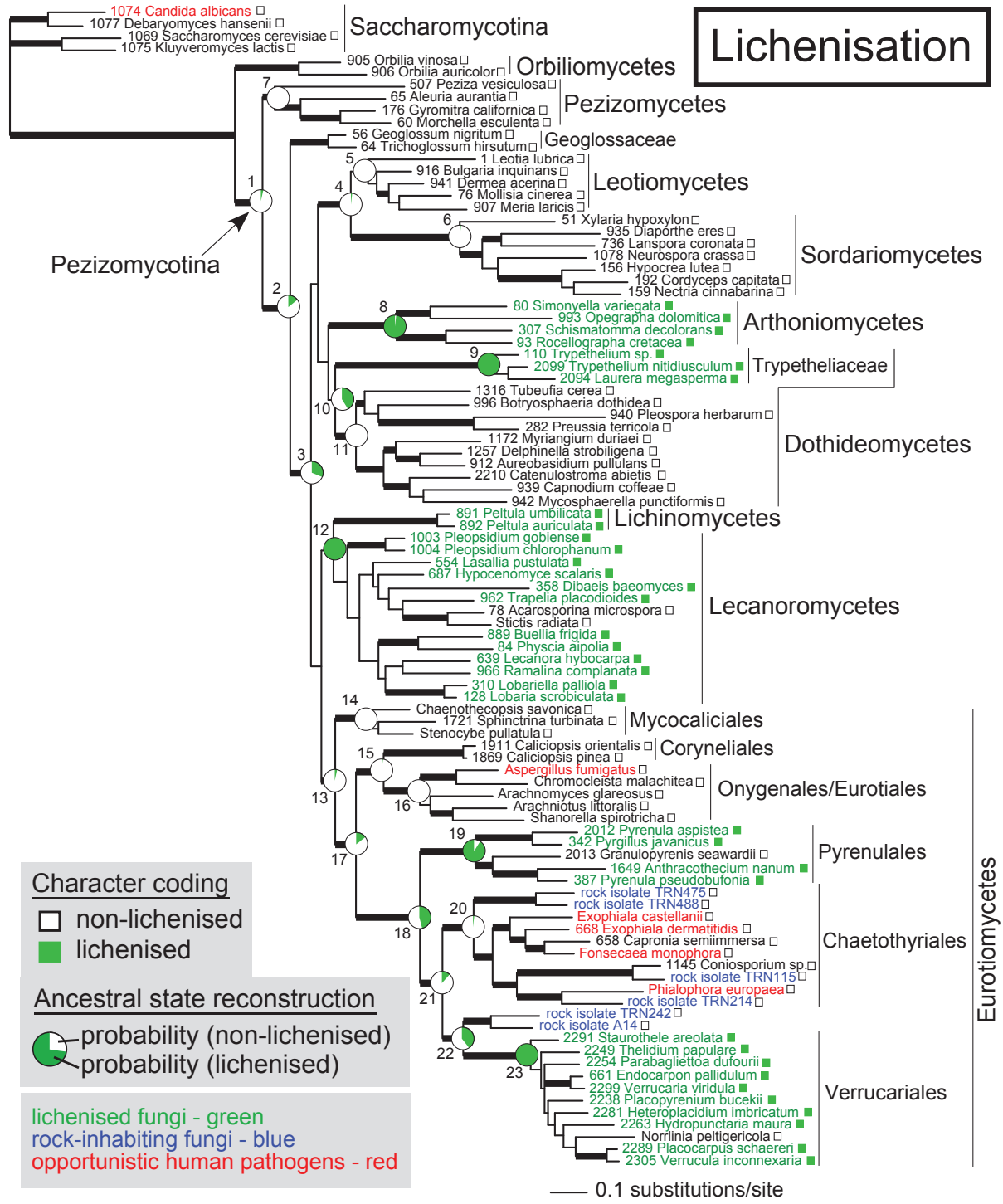

Fig. 1. Three-locus phylogeny of the *Pezizomycotina* obtained from a Bayesian MCMC analysis depicting ancestral state reconstruction of lichenisation. Thick branches represent nodes supported by PP ≥ 95 % and ML bootstrap ≥ 70 % (see Table 1 for numerical values). Boxes after each name indicate the state for each extant taxon (white box = non-lichenised, green box = lichenised). Posterior probabilities for each of the two states are represented in pie charts at each reconstructed node (numbered from 1 to 23). Additional information for these 23 nodes is provided in Table 1.

phylogenetic uncertainty linked to nodes with 100 % PP but low or moderate bootstrap (BS) values is not accounted for in our analyses. Therefore, the effect of the underestimation of the phylogenetic uncertainty linked to PPs was further explored for four nodes of interest of the *Pezizomycotina* dataset (nodes 13, 17, 18, and 21). Ancestral state reconstructions were carried out on these four nodes using a ML approach on 500 trees obtained with branch lengths from bootstrap replicates in RAxML (see legend of Table 2). The results show that the probability for the state "non-lichenised" for nodes 13, 17 and 21 (all superior to 98 % when using the Bayesian tree sample) gave only moderate support when using the bootstrap tree sample (PPs ranging from 78 to 89 %).

Evolution of lichenisation

A first phylogenetic analysis was conducted on a dataset including representatives from almost all main groups of ascomycetes (Fig. 1). The topology inferred is similar to those recovered in two recent

Table 2. Support values for the phylogeny of the *Eurotiomycetes* dataset (PP and ML bootstrap) and for the ancestral state reconstruction of the rock-dwelling habit (PP). The state "non rock-inhabitant" was coded as 0, and the state "rock-inhabitant" as 1. For the Bayesian reconstructions (MCMC), PPs of each state are indicated [P(0) and P(1)]. For the ML reconstructions on the Bayesian tree sample (ML), the number of trees supporting each state [Nt(0) and Nt(1)] or ambiguous [Nt(a)] are indicated, as well as the mean of the probabilities for each state across the 5000 trees [Pm(0) and Pm(1)]. Node numbers in this table refer to node number in Figure 1. **Bold** numbers indicate PP greater than 95 %.

				Ancestral state reconstruction of the rock dwelling habit (0=non rock-inhabitant, 1=rock-inhabitant)						
				MCMC		ML				
Nodes	Taxonomic groups	PP (%)	ML BS (%)	P(0)	P(1)	Nt(0)	Nt(a)	Nt(1)	$P_m(0)$	$P_m(1)$
1	*Eurotiomycetes*	**100**	100	0.80	0.20	0	5000	0	0.83	0.17
2	*Eurotiomycetidae*	**100**	98	0.80	0.20	0	5000	0	0.83	0.17
3	*Eurotiales*/*Onygenales*/*Arachnomycetales*	**100**	100	**0.95**	0.05	4765	235	0	**0.96**	0.04
4	*Onygenales*	**100**	80	0.94	0.06	4973	24	0	**0.97**	0.03
5	*Eurotiales*	**100**	100	**0.99**	0.01	5000	0	0	**1.00**	0.00
6	*Eurotiomycetidae*/*Chaetothyriomycetidae*	**100**	100	0.59	0.41	0	5000	0	0.59	0.42
7	*Chaetothyriomycetidae*	**100**	100	0.22	0.78	0	5000	0	0.18	0.82
8	*Pyrenulales*	**100**	100	0.85	0.15	0	5000	0	0.88	0.12
9	*Chaetothyriales*	**100**	100	0.14	0.86	0	4774	226	0.08	0.92
10	-	**100**	93	0.27	0.73	0	5000	0	0.21	0.79
11	-	**100**	100	0.05	**0.95**	0	4	4996	0.03	**0.97**
12	*Coniosporium*/*Sarcinomyces*	**100**	100	0.03	**0.97**	0	0	5000	0.02	**0.98**
13	-	**100**	81	0.58	0.42	0	5000	0	0.58	0.42
14	-	**100**	100	0.92	0.09	717	4283	0	0.94	0.07
15	*Cladophialophora*/*Fonsecaea*	**100**	78	0.88	0.12	0	5000	0	0.90	0.10
16	-	**100**	94	0.34	0.66	0	5000	0	0.30	0.70
17	-	**100**	86	**0.98**	0.03	5000	0	0	**0.99**	0.01
18	*Verrucariales*/*Chaetothyriales*	**100**	100	0.05	**0.95**	0	0	5000	0.02	**0.98**
19	*Verrucariales*	**100**	100	0.01	**0.99**	0	0	5000	0.00	**1.00**
20	*Staurothele*/*Catapyrenium*/*Placidiopsis*	**100**	96	0.00	**1.00**	0	0	5000	0.00	**1.00**
21	*Polyblastia*/*Thelidium*/*Verrucaria*	**100**	99	0.00	**1.00**	0	0	5000	0.00	**1.00**
22	*Placopyrenium*/*Verruculopsis*	**100**	97	0.92	0.08	578	4422	0	0.94	0.08
23	*Endocarpon*/*Neocatapyrenium*	**100**	95	0.00	**1.00**	0	0	5000	0.00	**1.00**
24	*Hydropunctaria*	**100**	99	0.02	**0.99**	0	0	5000	0.01	**0.99**
25	*Verrucula*/*Placocarpus*/*Norrlinia*	**100**	82	0.53	0.47	0	5000	0	0.53	0.47
26	*Placidium*/*Heteroplacidium*	**100**	100	0.84	0.16	0	5000	0	0.87	0.13
27	*Bagliettoa*/*Parabagliettoa*	**100**	88	0.00	**1.00**	0	0	5000	0.00	**1.00**
28	*Mycocaliciales*	**100**	100	0.93	0.07	1725	3265	0	**0.95**	0.06

large-scale phylogenetic analyses (James *et al.* 2006, Spatafora *et al.* 2006), except for the placement of the *Geoglossaceae*, which is here strongly supported as an early diverging lineage in the *Pezizomycotina*, the largest subphylum of ascomycetes (see also Wang *et al.* 2006, Schoch *et al.* in press). Lichenisation is shown to have evolved independently three to five times in the *Pezizomycotina*: once in the lineage including Lecanoromycetes and Lichinomycetes (node 12; PP_{MCMC}= 1.00), once or twice in the lineage including Pyrenulales (node 19; PP_{MCMC}= 0.95) and *Verrucariales* (node 23; PP_{MCMC}= 1.00), and once or twice in the lineage including Arthoniomycetes (node 8; PP_{MCMC}= 0.99) and *Dothideomycetes* (node 9; PP_{MCMC}= 1.00 for Trypetheliaceae). In contrast to previous studies (Lutzoni *et al.* 2001, James *et al.* 2006), the ancestor of *Eurotiomycetes* is reconstructed as non-lichenised (node 13; PP_{MCMC}= 0.98), as well as the ancestor of both *Verrucariales* and *Chaetothyriales*, although with lower support (node 21; PP_{MCMC}= 0.93).

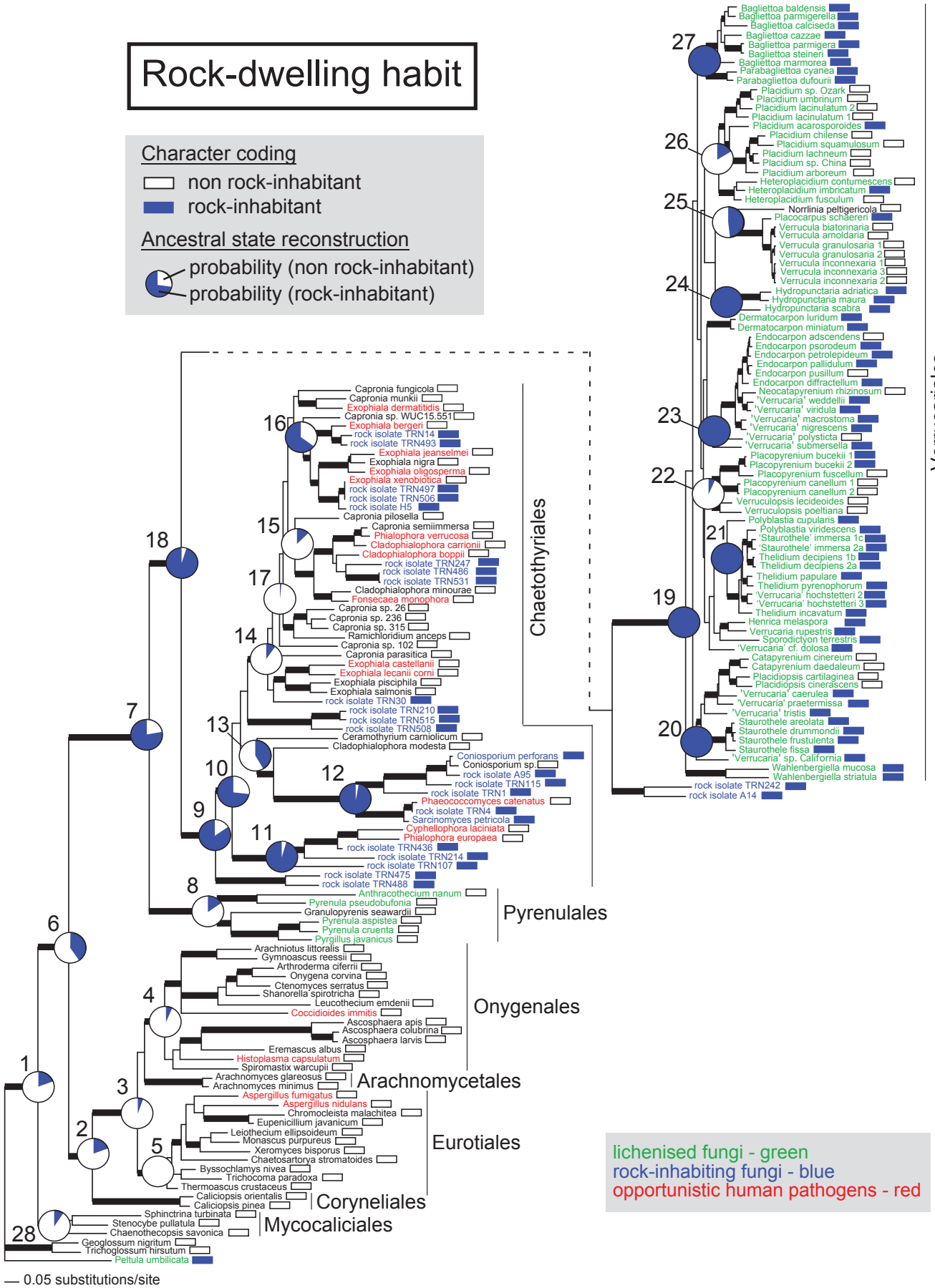

Fig. 2. Four-locus phylogeny of the *Eurotiomycetes* obtained from a Bayesian MCMC analysis depicting ancestral state reconstruction of the rock-dwelling habit. Thick branches represent nodes supported by PP ≥ 95 % and ML bootstrap ≥ 70 % (see Table 2 for numerical values). Boxes after each name indicate the state for each extant taxon (white box = non rock substrate, blue box = rock substrate). Posterior probabilities for each of the two states are represented in pie charts at each reconstructed node (numbered from 1 to 28). Additional information for these 28 nodes is provided in Table 2.

Evolution of the rock-dwelling habit

A second phylogenetic analysis was conducted on a dataset including representatives from all orders within *Eurotiomycetes* (Fig. 2). Relationships between orders were strongly supported and congruent with previous analyses (Lutzoni *et al.* 2004, Geiser *et al.* 2006). Most of the rock isolates are shown to belong to the *Chaetothyriales*, except for two strains (rock isolates TRN242 and A14), which are here resolved (but not supported) as sister to the lichen order *Verrucariales*. Within the *Chaetothyriales*, some rock-isolates are closely related to previously described species (*e.g.*, rock isolate TRN4 and *Phaeococcomyces catenatus*), and are nested within a large group including most of the saprophytes and opportunistic pathogens. Other strains consist in early diverging lineages, some of which are novel (*e.g.*, lineage including rock isolates TRN475 and TRN488). When reconstructing the rock-dwelling habit, the ancestor of the monophyletic group including *Verrucariales* and *Chaetothyriales* (node 18; PP_{MCMC}= 0.95), as well as the ancestor of the *Verrucariales* (node 19; PP_{MCMC}= 0.99), are supported as inhabiting rock surfaces. The ancestor of the *Chaetothyriales* is also reconstructed as rock inhabitant, although with lower support (node 9; PP_{MCMC}= 0.86).

DISCUSSION

During the evolutionary history of the Fungi, many transitions in lifestyles and habitats have occurred, giving rise to an amazing ecological diversity. In order to better understand their evolution, ancestral state reconstructions methods have been used to characterise these ecological shifts (Hibbett *et al.* 2000, Lutzoni *et al.* 2001). However, the discovery of new fungal lineages and the recent contributions to fungal molecular phylogenetics have greatly changed our perception of the evolution of some group of fungi, as, for example, the two ascomycetes orders *Verrucariales* and *Chaetothyriales*. In our study, the inclusion of the newly discovered rock-inhabiting fungi and other groups recently shown to belong to the *Eurotiomycetes* is reshaping our understanding of the evolution of this group. Reconstructions prove the key-role of primordial habitats like rock surfaces by suggesting that the ancestor of both lichenised *Verrucariales* and pathogen-rich *Chaetothyriales* was most likely non-lichenised and dwelled on rock surfaces. Similar to present-day rock-inhabiting fungi, this ancestor most probably possessed characters universally effective against different stresses (temperature, desiccation, pH, radiation), such as melanisation, and meristematic growth. Our results further imply that existing views on the evolutionary history of lichenisation within ascomycetes should be revised. Rock surfaces are proposed as the ancestral habitat for the lichenised *Verrucariales*. The results also support the hypothesis that, in *Chaetothyriales*, factors involved in pathogenicity evolved from characters enabling survival to physical stresses and life in extreme environments.

Evolution of lichenisation

Previous studies have suggested that the lichen symbiosis has evolved multiple times in fungi (Gargas *et al.* 1995, Lutzoni *et al.* 2001). In ascomycetes, where the great majority of lichen species belong, the evolution of lichenisation was shown to be limited to only one-three origins, and three-four losses (Lutzoni *et al.* 2001). These results supported the hypothesis that lichenisation results from complex and specialised interactions between symbiotic partners, unlikely to have evolved frequently and independently in different lineages, but more conceivably to have been lost multiple times. Our present work, which aimed at reassessing these previous results based on a taxon sampling more representative of the ecological diversity of these fungi (lichenised versus non-lichenised), suggests a higher number of origins of lichenisation in the ascomycetes (three-five) than previously estimated (Lutzoni *et al.* 2001, James *et al.* 2006). In contrast to Lutzoni *et al.* (2001), the ancestor of the Eurotiales and Onygenales, two orders comprising medically and commercially important fungi (such as the genera *Aspergillus* and *Penicillium*), is here supported as being non-lichenised. The addition of non-lichenised lineages previously overlooked (*Mycocaliciales*, *Coryneliales* and the rock-inhabiting fungi) seems to account for most of the differences observed in ancestral state reconstructions (Lutzoni *et al.* 2001, James *et al.* 2006 and this study). A recent work (Schoch *et al.* in press), including the most exhaustive taxon and gene sampling currently available for ascomycetes (434 taxa and 6 genes), also suggests a higher number of lichenisation events (four-seven), supporting that, lichen symbiosis, although resulting from complex interactions, might have evolved independently many more times than previously inferred.

Rock surfaces as a cradle to lichen symbiosis

Based on our reconstructions, the *Verrucariales* likely resulted from an independent lichenisation event, and the ancestor of *Verrucariales* and *Chaetothyriales* inhabited rock surfaces. This rock-dwelling ancestor most likely relied on sparse nutrients provided by the winds or rainfalls. It is possible that, where unicellular free-living algae could survive on rock surfaces (mainly the northerly outcrops, which are less exposed to the sun), ancestral lineages of rock-inhabiting fungi were able to evolve symbiotic relationships with photosynthetic microorganisms in order to ensure a more constant source of carbohydrates. Recent studies have shown that, when cultured *in vitro* together with pure isolates of lichen algal symbionts, some rock-inhabiting fungi and one melanised lichen-colonising fungus could develop structures allowing a cellular contact with the algal cells (Gorbushina *et al.* 2005, Brunauer *et al.* 2007). This ability to develop symbiotic interactions with unicellular free-living algae might have allowed some rock-inhabiting fungal lineages to evolve lichenisation, such as the *Verrucariales*. Strikingly, preliminary data suggest that rock-inhabiting fungi also constitute early diverging lineages in Arthoniomycetes, another independently derived lichen lineage nested within a primarily non-lichenised group (data not shown).

From extremotolerance to pathogenicity

The order *Chaetothyriales* is particularly well known for its animal and human opportunistic pathogens (de Hoog *et al.* 2000). They infect their hosts either by inhalation, ingestion or traumatic inoculation, provoking a variety of illnesses, ranging from skin to fatal brain infections, both in immunocompromised and immunocompetent patients. Non-pathogenic asexual strains of *Chaetothyriales* can be isolated from the environment using selective culture methods (Prenafeta-Boldú *et al.* 2006), and closely related sexual saprophytes are found as secondary decomposers on decaying wood and mushrooms (Haase *et al.* 1999, Untereiner & Naveau 1999). Primary habitats of opportunistic pathogens and factors involved in pathogenicity are still not entirely

understood (Prenafeta-Boldú *et al.* 2006). Factors conferring invasive capability include the production of melanin and the meristematic growth (Schnitzler *et al.* 1999, Feng *et al.* 2001). It was previously suggested that these factors might have primarily evolved in response to extreme environments (de Hoog 1993, Haase *et al.* 1999, Prenafeta-Boldú *et al.* 2006). This hypothesis is now supported by our results, as they show that lineages diverging early in the evolution of the *Chaetothyriales* comprise a particularly high diversity in rock-inhabiting fungi, and that the ancestor of this order is a rock-inhabitant. Therefore, specific traits evolved by a rock-inhabiting ancestor for life in extreme habitats and potentially shared by all descendants within this lineage, can explain the numerous independent shifts to pathogenicity that have occurred in this group of fungi. Because clinical strains have to survive and multiply at body temperature, relying only on nutrients provided by the hosts, tolerance to high temperatures and oligotrophism might also be prerequisites to the evolution of animal and human pathogens (Prenafeta-Boldú *et al.* 2006).

This new insight in the evolution of the *Eurotiomycetes* accentuates the role that traits enabling survival in extreme conditions might have had on lifestyle transitions often observed in this class. Other opportunistic fungal pathogens in *Eurotiomycetes* have also been shown to primarily colonise extreme habitats, such as *Coccidioides immitis*, the agent of the respiratory disease known as valley fever, which occurs in desert regions in the Americas (Fisher *et al.* 2001). Unfortunately, as for many other environmental opportunistic fungal pathogens, limited knowledge on primary niches, ecological preferences and factors involved in pathogenicity, is available for the *Eurotiomycetes*. Because opportunistic fungal pathogens have become an important concern in Public Health, further work on their ecology and evolution is of highest importance.

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Evolution of *CDC42*, a putative virulence factor triggering meristematic growth in black yeasts

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Abstract: The cell division cycle gene (*CDC42*) controlling cellular polarization was studied in members of *Chaetothyriales*. Based on ribosomal genes, ancestral members of the order exhibit meristematic growth in view of their colonization of inert surfaces such as rock, whereas in derived members of the order the gene is a putative virulence factor involved in expression of the muriform cell, the invasive phase in human chromoblastomycosis. Specific primers were developed to amplify a portion of the gene of 32 members of the order with known position according to ribosomal phylogeny. Phylogeny of *CDC42* proved to be very different. In all members of *Chaetohyriales* the protein sequence is highly conserved. In most species, distributed all over the phylogenetic tree, introns and 3rd codon positions are also invariant. However, a number of species had paralogues with considerable deviation in non-coding exon positions, and synchronous variation in introns, although non-synonomous variation had remained very limited. In some strains both orthologues and paralogues were present. It is concluded that *CDC42* does not show any orthologous evolution, and that its paralogues haves the same function but are structurally relaxed. The variation or absence thereof could not be linked to ecological changes, from rock-inhabiting to pathogenic life style. It is concluded that eventual pathogenicity in *Chaetothyriales* is not expressed at the DNA level in *CDC42* evolution.

Key words: Cell Division Cycle *CDC42*, *Chaetothyriales*, chromoblastomycosis, muriform cell, paralogue evolution, phylogeny, virulence factors.

INTRODUCTION

Human infection by agents of chromoblastomycosis is accompanied by dramatic morphogenetic changes of fungal cells in tissue. The fungi concerned have the ability of morphogenetic switching from polarized filamentous growth to isodiametric expansion, leading to large spherical cells (Szaniszlo *et al.* 1983). Subdivision of the cells gives rise to muriform cells (Matsumoto *et al.* 1993), the invasive phase of the fungi concerned and triggering hyperphasia characteristic for the disease. Chromoblastomycosis is exclusively known to be caused by members of the ascomycete order *Chaetothyriales* (Haase *et al.* 1999, Badali *et al.* 2008): primarily by *Cladophialophora* and *Fonsecaea* species and occasionally by species of *Exophiala, Phialophora* or *Rhinocladiella*. The order comprises a large number of pathogens and opportunists on warm- and cold-blooded vertebrates (de Hoog *et al.* 2000), and hence is likely to have ancestral virulence factors.

The order *Chaetothyriales* is remarkable in the fungal Kingdom, for two reasons. First, a large number of the infections are observed in individuals without known immune disorder. Of the about 77 species confirmed to belong to the order by sequence data (Barr 1990, Gueidan *et al.* 2008), about 33 have been encountered as etiologic agents of infections in vertebrates (de Hoog *et al.* 2000, Zeng *et al.* 2006, Badali *et al.* 2008). This high percentage of species with an infective potential is only matched by the order *Onygenales* containing the dermatophytes and classical systemic fungi. Second, the diversity in clinical pictures caused by members of the *Chaetothyriales* is bewildering. Species of *Onygenales* are very consistent in their pathology, displaying a similar clinical course by members causing cutaneous or systemic infections, whereas those of *Chaetothyriales* encompass a wide diversity of diseases, which are nevertheless more or less characteristic within a single species (de Hoog *et al.* 2000). This pathogenic potential is particularly observed in the more derived parts of the order, comprising the ascomycete family *Herpotrichiellaceae* (Untereiner 2000). Recently it was established that members of this same group show 'dual ecology', i.e., they also possess the uncommon ability to assimilate monoaromatic pollutants (Prenafeta-Boldú *et al.* 2006).

A second recurrent trend in the *Chaetothyriales* is extremotolerance, i.e. growth on exposed surfaces, having a competitive advantage at high temperature and dryness (Sterflinger *et al.* 1998, Ruibal 2004). Phylogenetic trees published by Lutzoni *et al.* (2001) and Gueidan *et al.* (2008) indicate that some deep branches among the pathogenic black yeasts have a shared evolution with rock-inhabiting fungi. A meristematic growth form, morphologically similar to the muriform cells described above, may be expressed under adverse environmental conditions of nutrient depletion, high temperature and dryness. This suggests a functional change in the course of evolution, from an ancestral rock-inhabiting lifestyle to a derived strategy in which ultimately pathogenicity to vertebrate hosts enhances the fitness of species.

Polarized *vs.* isodiametric growth in fungi is regulated by the Rho-related GTPase Cdc42p (Cdc = Cell Division Cycle), reviewed by Johnson (1999). Cdc42p is essential for the reorganization of the actin cytoskeleton during the shift from polarized to isodiametric growth. Upon activation, Cdc42p is recruited to the plasma membrane to initiate actin nucleation. Localization and activity of Cdc42p are mediated by guanine-nucleotide exchange factors (GEFs). In *S. cerevisiae*, the only GEF for Cdc42p is Cdc24p,

Table 1. Strains used in this study

Name	CBS no.	Status	Ortho-/para-	Other reference	Source	Origin
Exophiala dermatitidis	CBS 525.76	T	+/+	ATCC 34100; NIH 8656	Human sputum	Japan
	CBS 292.49		+/-	DH 15696	Faeces	U.S.A.
	CBS 207.35	T	+/+	ATCC 28869; UAMH 3967	Man, facial chromoblastomycosis	Japan; Osaka University
Exophiala heteromorpha	CBS 232.33	T	-/+	MUCL 9894; NCMH 17	Wood pulp	Sweden
Capronia mansonii	CBS 101.67	T	+/-	ATCC 18659; IMI 134456	*Populus tremula*	Sweden
Capronia munkii	CBS 615.96	T	+/-	DH 16078	*Populus tremuloides*, wood	Canada, Alberta; south of Hinton
Capronia epimyces	CBS 606.96		+/+	DH 16065	*Nectria,* fruit bodies, on *Pinus* wood	Canada; Ontario
Rhinocladiella mackenziei	CBS 650.93	T	+/-	MUCL 40057	Human, cerebral phaeohyphomycosis	Saudi Arabia
Capronia acutiseta	CBS 618.96	T	-/+	ATCC 56428;ATCC 76482	*Dacrydium cupressinum*, wood	New Zealand; Saltwater State, Westland County
Capronia parasitica	CBS 123.88		+/-	DH 15347	*Hypoxylon cohaerens* var. *microsporum*, on *Quercus* sp.	France; Bois de Lourdes
Rhinocladiella anceps	CBS 181.65	NT	+/-	ATCC 18655; IMI 134453; MUCL 8233	Soil under *Thuja plicata*	Canada, Ontario; Campbellville
Capronia villosa	CBS 616.96		+/-	ATCC 56206	Decorticated wood	New Zealand
Fonsecaea monophora	CBS 269.37		+/-	DH 12659	Human, chromoblastomycosis	South America
Phialophora verrucosa	CBS 286.47		-/+	ATCC 9541; MUCL 9768	Mycetoma hand, human	Brazil
Phialophora americana	CBS 840.69		-/+	MUCL 15537	Decaying timber	Finland; Helsinki
Cladophialophora carrionii	CBS 260.83		-/+	ATCC 44535	Human, chromoblastomycosis	Venezuela, Falcon State, Uganda
Cladophialophora boppii	CBS 126.86	T	+/+	DH 15357	Human, skin lesion, on limb	Brazil
Exophiala castellanii	CBS 158.58	NT	+/-	ATCC 18657; IFM 4702; MUCL 10097	Human	Sri Lanka
Exophiala nigra	CBS 546.82		+/-	DH 15993	Soil under ice	Russia
Exophiala bergeri	CBS 353.52	T	+/-	DH 15792	Human, chromoblastomycosis	Canada
Exophiala spinifera	CBS 899.68	T	+/-	ATCC 18218; IHM 1767; NCMH 152	Human, nasal granuloma	U.S.A.
Exophiala jeanselmei	CBS 507.90	T	+/-	ATCC 34123; CBS 664.76; IHM 283; NCMH 123	Human	Uruguay
Exophiala oligosperma	CBS 725.88	T	+/-	DH 16212	Human, tumour of sphenoidal cavity	Germany; Würzburg
Phialophora europaea	CBS 129.96	T	-/+	DH 10389	Human, chromoblastomycosis of toe	Germany; Giessen
Fonsecaea pedrosoi	CBS 271.37	NT	+/-	ATCC 18658;IMI 134458	Human, chromoblastomycosis	Argentina
Coniosporium perforans	CBS 885.95	T	+/-	DH 16308	Marble	Greece
Phaeoannellomyces elegans	CBS 122.95		+/-	DH 15343; NCMH 1286	Human, skin infection of toe nail	Canada; Toronto
Exophiala pisciphila	CBS 661.76		+/-	DH 16145	*Heterodera schachtii* egg from cyst, recovered from soil	Germany; Elsdorf
Phialophora reptans	CBS113.85	T	+/-	DH 5543	Food	Sweden
Exophiala mesophila	CBS 402.95	T	+/-	DH 15838	Silicone, shower cabinet,	Germany, Hamburg
Cladophialophora modesta	CBS 985.96	T	+/-	NCMH 108; UAMH 4004	Human, brain	U.S.A.; Chapel Hill
Exophiala salmonis	CBS 157.67	T	+/-		Brain, Salmo clakii	Canada; Galgary

controlled by cell cycle proteins (Cdc28p) and additional proteins (Cla4p and Bemp1; Bose *et al.* 2001). The eleven current members of the CDC42 family display between 75 and 100 % amino acid identity and are functionally as well as structurally homologous. In filamentous fungi another member of this family is present, RacAp. This is a well studied GTP-binding protein in mammalian cells and it has been shown that RacAp is required for the formation of lamellipodia in fibroblast cells. Recently, RacAp was found in basidiomycetes (Gorfer *et al.* 2001) as well as in ascomycetes (Hurtado *et al.* 2000, Boyce *et al.* 2001, Virag *et al.* 2007), but RacAp orthologus are absent in *S. cerevisiae* and have not been reported to be present in *Chaetothyriales*.Analysis of transformants overexpressing a dominant active allele of RacAp(*racAG12V*) displays unpolarized growth in *Aspergillus niger*, resembling the muriform cells, the pathogenic tissue-phase characteristic for chromoblastomycosis (A.F.J. Ram, unpublished data). This form in the agents of chromoblastomycosis, and even the agent of phaeohyphomycosis, *Exophiala dermatitidis*, is easily expressed in culture by a low pH of the growth medium and conditions of calcium limitation at more neutral pH (Mendoza *et al.* 1993, Karuppayil & Szaniszlo 1993, Szaniszlo *et al.* 1993, Badali *et al.* 2008). The Cdc42 proteins act as molecular switches by responding to exogenous and/or endogenous signals and relaying those signals to activate downstream components of a biological pathway. Ye & Szaniszlo (2000) confirmed that Cdc42p plays a unique regulatory role in the morphogenesis of the black yeast *E.dermatitidis* during its phenotype transition from yeast to isodiametric cells and muriform cells in vitro.. They also found that the derived Cdc42 protein is highly conserved member of the Cdc42 subfamily. These results suggest that the *CDC42* gene products seem to play an important role in the regulation of stress-induced fungal cellular morphogenesis. One of the aspects of this therefore is its implication in human chromoblastomycosis.

When the functional change of Cdc42p, from extremotolerance to pathogenicity, concerns an evolutionary adaptation, we might expect the transition to be reflected at the DNA level. Comparing *CDC42* DNA gene sequences with the phylogenetic scaffold based on ribosomal genes as the gold standard, might thus provide additional insights about whether structural and functional changes in the gene concern adaptive changes in ecological strategies. In the present paper, tools are developed for the detection *of the CDC42 gene* in *Chaetothyriales* in view of a phylogenetic study of the *CDC42* gene, in parallel to genes with known phylogenetic content.

MATERIAL AND METHODS

Strains and culture conditions

The isolates studied are listed in Table 1. A total of 32 members of *Chaetothyriales* including basal lineages were obtained from the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre and grown on Malt Extract Agar (MEA) at 25 °C.

DNA extraction

Approximately 1 cm^2 mycelium of 30-d-old cultures was transferred to a 2 mL Eppendorf tube containing 300 µL TES-buffer (Tris 1.2 % w/v, Na-EDTA 0.38% w/v, SDS 2 % w/v, pH 8.0) and about 80 mg of a silica mixture (Silica gel H, Merck 7736, Darmstadt, Germany / Kieselguhr Celite 545, Machery, Düren, Germany, 2 : 1, w/w). Cells were disrupted mechanically in a tight-fitting sterile pestle for approximately 1 min. Subsequently 200 µL TES-buffer was added, the mixture was vortexed, 10 µL proteinase K was added and incubated for 10 min at 65 °C. After addition of 140 µL of 5 M NaCl and 1/10 vol CTAB 10 % (cetyltrimethylammoniumbromide) solution, the material was incubated for 30 min at 65 °C. Subsequently 700 µL SEVAG (24 : 1, chloroform : isoamylalcohol) was added to the solution and shortly mixed by shaking, incubated for 30 min on ice water and centrifuged for 10 min at 1125 × g. The supernatant was transferred to a new tube with 225 µL 5 M NH_4-acetate, incubated on ice water for 30 min. and centrifuged again for 10 min at 1125 × g. The supernatant was transferred to another Eppendorf tube with 0.55 vol isopropanol mixed carefully by flipping and spin for 5 min at 1125 × g. Subsequently, the pellet was washed with ice cold 70 % ethanol. After drying at room temperature it was re-suspended in 48.5 µL TE buffer (Tris 0.12 % w/v, a-EDTA 0.04 % w/v) plus 1.5 µL RNAse 20 U/mL and incubated for 15–30 min at 37 °C. DNAs were purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) as recommended by the manufacturer.

CDC42 primer design

Primers specific for *CDC42* amplifications were selected using a complete alignment of the amino acid sequences of species listed in Table 2. Two highly conserved areas were detected for *CDC42* that were absent from the Rac and Rho gene families. Degenerated forward (*CDC42*-F1, *CDC42*-F2) and reverse (*CDC42*-R1, *CDC42*-R2) primers were designed matching the target regions. In addition, primers (Table 4) were synthesized in the same positions but that were specific for the published sequence of *Exophiala dermatitidis*, CBS 525.76 (NIH 8656 = AF162788) as control (Ye & Szaniszlo 2000). The resulting specific primers *CDC42*-F1s, *CDC42*-F2s, *CDC42*-R1s and *CDC42*-R2s (Table 4) were subsequently tested with the aim to establish amplification conditions. Different combinations of specific primers were then tested for the 32 strains listed in Table 1.

Table 2. *CDC42* reference sequences taken from GenBank.

Species	Gene	GenBank no. protein	GenBank no. DNA
Exophiala dermatitidis	*CDC42*	AAD46909	AF162788
Emericella nidulans	*CDC42*	AAF24514	AF217199
Penicillium marneffei	*CDC42*	AAK56917	AF330694
	RAC	AAN77094	
Aspergillus niger	*RAC*	AAT09022	
Aspergillus nidulans	*RHO*	XP-663344	

Amplification of *CDC42* orthologue

Approximately 100 ng of genomic DNA was used as a template in semi-nested PCR with primer pair *CDC42*-F1s-R2s and followed by a second PCR using the amplicon of the first PCR with primer *CDC42*-F1s-R1s. Both PCR were performed in 25 µL PCR-mix consisting of GoTaq green master mix 7 µL (Promega, Leiden), MQ 13 µL, DMSO 1 µL, primers 1 µL each, DNA 2 µL, using a Biosystems 2720 thermal cycler with an initial cycle of 1 min at 98

Table 3. Primer sequences for PCR amplification and sequencing of LSU and SSU.

Gene	PCR primers	Sequencing primers	References
LSU rDNA	LRORa, LR7b	LRoR LR3R, LR5, LR7b	a Rehner & Samuels (1994)
			b Vilgalys & Hester (1990)
SSU rDNA	NS1a, NS24b	BF83, Oli1, Oli9, BF951, BF963, BF1438, Oli3, BF1419 c	c White *et al.* (1990)
			b Gargas & Taylor (1992)
			c de Hoog *et al*. (2005)

Table 4. Degenerate and specific primers designed in this study.

Degenerate primer:		
Gene:	Amino acid sequence:	Nucleotide sequence:
CDC42-F1:	M V V A T I	5'-ATG GTI GTI GCI ACI ATH
CDC42-F2:	I G D E PYT	5'-ATH GGI GAY GAR CCI TAY AC
CDC42-R1:	R M A K EL G	5'-CCI ARY ICY TTI GCC ATI CK
CDC42-R2:	Y K L K D VF	5'-RAA IAC RTC YTT IAR YTT RTA
Specific primers for *CDC42* orthologue:		
CDC42-F1s: 5'-ATG GTT GTC GCA ACG ATC		
CDC42- F2s: 5'-GGA TTA CGA CCG GCT TCG		
CDC42-R1s: 5'-CCA ACT CCT TGG CCA TTC		
CDC42-R2s: 5'-AAA GAC GTC TTT GAG TTT GTA		
Primer combination		
CDC42 F1s--R2s 698 bp	*CDC42* F1s--R1s 639 bp	*CDC42* F2s--R2s 372 bp	*CDC42* F2s--R1s 315 bp
Six specific backward primers for *CDC42* paralogue:		
CDC42-F1s:	5'-ATG GTT GTC GCA ACG ATC	
CDC42- R1d:	5'-GGA CTT GTG GGT CGT CA	
CDC42-R2d:	5'-TCA GCG ACG GAT GGG T	
CDC42-R3d (CBS 232.33):	5'-GTT CCA ACA ATC AGA CA	
CDC42-R4d (CBS 606.96):	5'-TCC CAA CAA TCA GAC AT	
CDC42-R5d (CBS 616.96):	5'-CTT GCG GGT AGT CAC GAA	
CDC42-R6d (CBS 618.96):	5'-AAC AAT CAA ACA AGG CAC T	

°C, subsequently 30 cycles of 30 s at 98 °C, 30 s at 54 °C, and 1 min at 72 °C, and a final extension of 7 min at 72 °C.

Specific primers for *CDC42* paralogue

Backward primers were designed (Table 4) specific for each deviating motif using *CDC42*-F1s as forward primer. With both PCR amplifications, approximately 100 ng of genomic DNA was used as a template in first PCR with the forward primer combined with the backward primer in six separate reactions, and nested with the same primer pair. PCR was performed in a 25 µL PCR-mix consisting of GoTaq green master mix 7 µL (Promega, Leiden), MQ 13 µL, DMSO 1 µL, primers 1 µL each, DNA 2 µL using a Biosystems 2720 thermal cycler with an initial cycle of 1 min at 98 °C. Program was as follows: 30 cycles of 30 s at 98 °C, 30 s at 54 °C and 1 min at 72 °C, and a final extension of 7 min at 72 °C. The sond PCRs used amplicons from the first PCR and a touch-down program with an initial cycle of 1 min at 95 °C, subsequently 10 cycles of 30 s at 95 °C, 30 s at 62 °C/-1°C/cycle and 1 min at 72 °C, followed by 20 cycles of 30 s at 95 °C, 30 s at 52 °C, and 1 min at 72 °C, and a final extension of 7 min at 72 °C in sond PCR.

Cloning of the *CDC42* paralogue

CDC42 paralogues were cloned using a cloning kit (pGEM-T vector; Promega, Madison, WI, U.S.A.), according to the manufacturer's instructions. We picked up one white colony to do direct PCR with primer M13 fw [5´-GTA AAA CGA CGG CCA GT-3´], M13 rv [5´-GGA AAC AGC TAT GAC CAT G-3]. PCR was performed in a 25 µL PCR-mix consisting of PCR buffer 10x 2.5 µL, MQ 15 µL, dNTP mix (1 mM) 2.5 µL, *Taq* polymerase (1 U/ µL) 1 µL, primers 1 µL each and one white colony. amplifications were with a Biosystems 2720 thermal cycler using an initial cycle of 3 min at 94 °C, 28 subsequent cycles of 1 min at 93 °C, 1 min at 52 °C, and 2 min at 72 °C, and a final extension of 3 min at 72 °C. The resulting *CDC42* paralogue sequences were compared to the previously obtained orthologous *CDC42* gene sequences using BIONUMERICS software v. 4.61 (Applied Maths, Kortrijk, Belgium)

SSU and LSU amplification and sequencing

Primers (http://www.biology.duke.edu/fungi/mycolab/primers.htm) shown in Table 3 were used to amplify part of the nuclear rDNA operon spanning the 3' end of the 18S r RNA gene (SSU), then

first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and 5' end of the 28S rRNA gene (LSU). The PCR conditions followed the methods of Crous *et al.* (2006b). PCR amplifications were performed as follows: 95 °C for 1 min, followed by 30 cycles consisting of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Reaction products were then purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data were adjusted using the SeqMan of Lasergene software (DNAStar Inc., Madison, Wisconsin, U.S.A.).

Alignment and phylogenetic reconstruction

Phylogenetic analyses were carried out on data with a taxon sampling representative of the order *Chaetothyriales* and including the three genes nucLSU, nucSSU and *CDC42*. This dataset was used in order to assess the phylogenetic evolution of *CDC42* gene on diverse species within *Chaetothyriales*. SSU, LSU and *CDC42* data sets were analyzed separately. Alignments were adjusted manually. Ambiguous regions were excluded from the alignments. Concatenated sequences of LSU and SSU were submitted to the Cipres Portal v.1.14 (http://www.phylo.org/portal/Home.do) using the RAxML web server. Maximum likelihood searches for the best-scoring tree were made after the bootstrap estimate proportion of invariable sites automatically determined the number of bootstrapping runs: if checked, RAxML will automatically determine the point at which enough bootstrapping replicates have been produced (Stamatakis *et al.* 2008). *CDC42* data were analyzed in the same way. Bootstrap values equal to or greater than 80 % were considered significant (Hillis & Bull 1993).

RESULTS

Evaluation of designed primers for *CDC42*

The degenerate primers designed proved to be insufficiently specific to amplify the *CDC42* gene. Four oligonucleotide primer sets, i.e. *CDC42*-F1s-R2s, *CDC42*-F1s-R1s, *CDC42*-F2s-R2s and *CDC42*-F2s-R1s were synthesized and tested for amplification with genomic DNA from 32 selected strains in the CBS reference collection representing the order *Chaetothyriales*. Primer pairs *CDC42*-F2s-R2s and *CDC42*-F2s-R1s provided poor results and were excluded from the analysis. Primer sets *CDC42*-F1s-R2s yielded a PCR product of 690 bp, whereas primer pair *CDC42*-F1s-R1s produced an amplicon of 630 bp in length, spanning two introns. The primer sets were found to be largely specific for their target groups. Semi Nested PCRs were successfully employed, with the first PCR giving only very weak bands but clear bands being visible with second PCR. With the second amplification the ratio of concentration of inhibitors *vs.* template DNA allowed amplification of the product. In general it was difficult to amplify the desired *CDC42* gene fragments from strains of *Chaetothyriales,* because of the following: (1) PCRs had frequently to be repeated using the first amplicon, because the product from the first PCR was mostly not visible on the gel; (2) Successful PCRs were only obtained with GoTaq and DMSO, not with BioTaq; (3) Although the PCR programs were optimized for amplification of *Chaetothyriales*, some strains had to be done with touch-down programs in order to avoid generation of unspecific bands; (4) Frequent heavy backgrounds in electropherograms suggested contaminated PCR products. BLAST searches in GenBank showed that the sequences determined in this study deviated maximally 7 % from the published *CDC42* sequence of CBS 525.76, *E. dermatitidis* (AF162788) (Ye & Szaniszlo 2000).

Phylogenetic analysis

A phylogenetic tree was constructed for 32 members of *Chaetothyriales* using concatenated SSU and LSU ribosomal genes, with *Conisporium perforans* as an outgroup. This species was shown previously to be basal to the *Herpotrichiellaceae* by Badali *et al.* (2008). Four of the five clades recognized previously (e.g., Haase *et al.* 1999) were separated with high bootstrap support (Fig. 2): *Exophiala dermatitidis* clade (1), *Fonsecaea pedrosoi* clade (2) and *Exophiala spinifera* clade (3), while Haase's clade 5 is now known to represent the ancestral group (lineage 2) close to *Ceramothyrium* (Badali *et al.* 2008) and members of this clade 4 were not included in the present analysis.

DNA sequences of the partial of *CDC42* were strictly identical for 25 strains (Fig 1). In these strains also the intron and the 3rd codon positions of the exons were identical. The strains were distributed randomly over the ribosomal tree (Fig. 2). No difference was detected between *Coniosporium perforans* CBS 885.96, selected as the ribosomal outgroup, and the most derived groups containing e.g. *Exophiala oligosperma* CBS 725.88. In a number of strains significant deviations were found (Fig. 1), which were limited to the third codon positions and to the introns (Table 5). The strains clustered in four ribosomal clades (1, 2, 3, 5; Fig. 2). Nearest neighbours in the *CDC42* trees were found to belong to the same ribosomal clades.

In order to verify the different motifs obtained, specific primers were developed for each sequence type of 32 strains. Amplification of

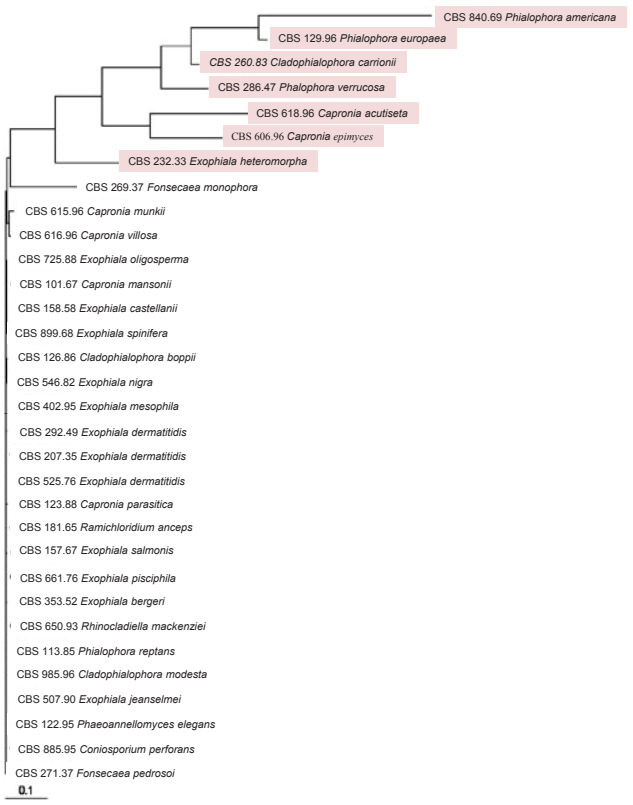

Fig. 1. Initial tree constructed for 32 members of *Chaetothyriales* based on partial *CDC42* sequences.

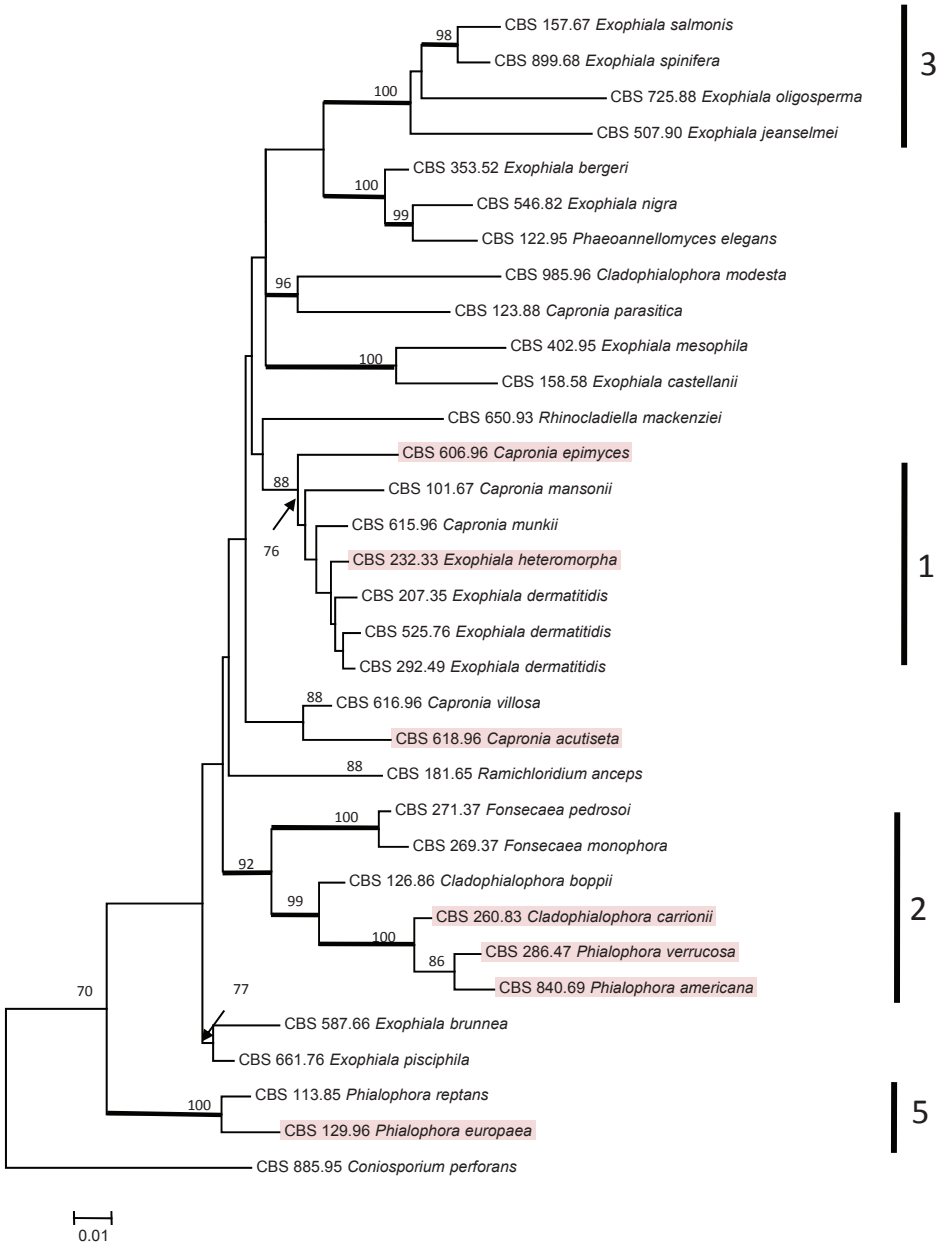

Fig. 2. Tree constructed for 32 members of *Chaetothyriales* obtained from a ML analysis of two combined loci (SSU and LSU) using RAxML. Bootstrap support values were estimated based on 500 replicates and are shown above the branches (thick branch for values ≥ 90 %). The tree was rooted using *Coniosporium perforans*, CBS 885.95.

the invariant type was relatively straightforward: the same sequence was obtained consistently. However, specific amplifications of deviating types were mostly unsuccessful. Strains CBS 232.33 and CBS 618.96 consistently provided a deviating sequence. Two strains of *Exophiala dermatitidis* (CBS 525.76 and CBS 207.37), *Capronia epimyces* (CBS 606.96) and *Cladophialophora boppii* (CBS 126.86) revealed two deviating sequences, one identical to the core sequence of 25 strains, another clearly deviating in intron and 3rd codon sequences. Cloning results proved that the deviating genotypes were common in many strains. In some strains the invariant type could not be amplified (Table 1)

DISCUSSION

CDC42 is an essential GTPase that is ubiquitously expressed in eukaryotes, where it participates in the regulation of the cytoskeleton and a wide range of cellular processes, including cytokinesis, gene expression, cell cycle progression, apoptosis, and tumorogenesis. It transduces signals to the actin cytoskeleton to initiate and maintain polarized growth and to promote mitogen-activated protein morphogenesis. In filamentous fungi the *CDC42* gene product is involved in the transition of hyphae to isodiametrically growing cells. This transition becomes apparent in the formation of meristematic cells under conditions of environmental stress, such as with growth on rock, but also concerns muriform cells, the invasive form in human chromoblastomycosis.

In the evolution of *Chaetothyriales*, we witness a functional change from a rock-inhabiting life style prevalent in *Coniosporium* and relatives (Fig. 2; Sterflinger *et al.* 1998) to an increased ability to infect humans and other vertebrates, e.g. in cases of chromoblastomycosis. Both life styles are characterized by isodiametric growth at least during part of the life cycle. Thus a major ecological and functional transition has taken place, the same characteristics of isodiametric expansion becoming applied in an entirely different setting. Exposure on rock is an ancestral condition in *Coniosporium* (Lutzoni *et al.* 2001, Gueidan *et al.* 2008), while the pathogenic role in agents of chromoblastomycosis

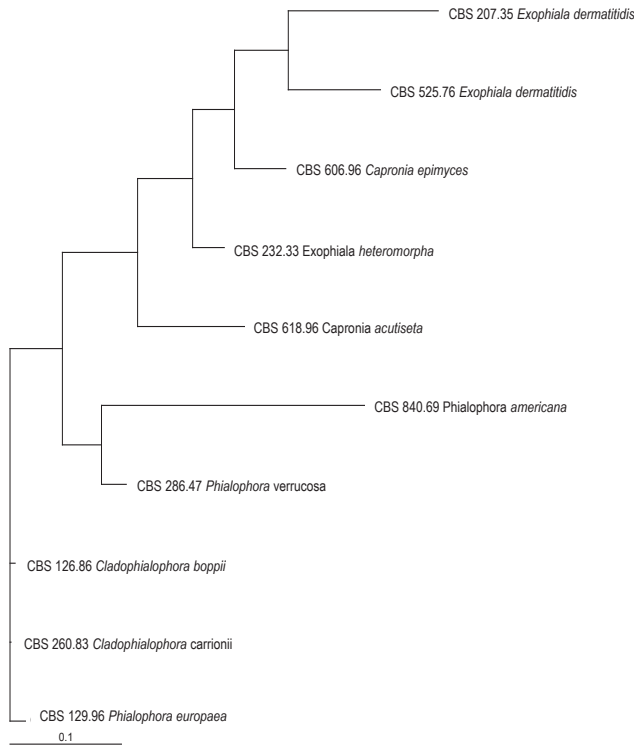

Fig. 3. Tree constructed for 10 strains based on the partial exon of the *CDC42* paralogue.

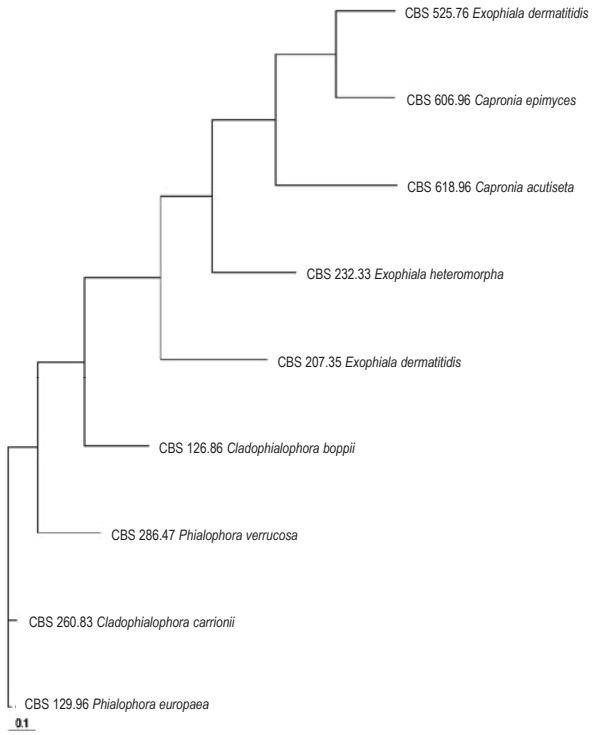

Fig. 4. Tree constructed for 9 strains based on the sequenced intron of the *CDC42* paralogue.

in *Cladophialophora, Fonsecaea* and *Phialophora* is a derived condition (Badali *et al.* 2008). In recent publications the ribosomal tree based on SSU and LSU exhibits a basal lineage that has become individualized more clearly and referred to as the separate family *Chaetothyriaceae* within the *Chaetothyriales* (Badali *et al.* 2008). It contains primarily species from rock, but also those occasionally colonizing human skin or causing mild cutaneous infections (Li *et al.* 2008). The majority of species reported from human chromoblastomycosis (*Phialophora verrucosa* and *Cladophialophora carrionii*) are united in a separate clade which can be attributed to the family *Herpotrichiellaceae* (Untereiner 2000). An eventual directional evolution of CDC should reflect both ecologies including rock-inhabiting and pathogenic life styles.

Our research was initiated with the development of primer sets to detect *CDC42* in *Chaetothyriales.* We constructed four primer sets from protein coding regions in which the PCR product would span at least one intron. Four degenerate primer sets were tested for their ability to amplify *CDC42* in members of *Chaetothyriales.* Specific primer sets were subsequently synthesized. Only two sets worked well such that they amplified PCR products matching the published *CDC42* sequence of CBS 525.76, *Exophiala dermatitidis* (AF162788) and that were clearly different from genes that encode functionally related proteins such as Rac and Rho. Further amplification in related species was done with these primer sets (*CDC42*-F1s-R2s and *CDC42*-F1s-R1s).

The majority of the strains, irrespective of their phylogenetic position based on rDNA, were invariable in *CDC42*. Part of the strains, however, showed considerable deviation in non-coding positions and introns (Table 5). Since the relationship with the ribosomal tree of the combined dataset was not directly obvious, we were uncertain whether the deviating sequences were homologous with *CDC42*, despite consistent highest scores in GenBank. For most *CDC42* amplifications, PCRs were difficult and had to be repeated several times due to frequent heavy background in the electropherogram suggesting contaminated PCR products. In order to verify the different motifs obtained, we developed specific primers for each sequence type. PCR efficiency and sequencing was particularly difficult in the deviating sequences, even when specific backward primers were used. Amplification of the invariant type was relatively straightforward: the same sequence was obtained consistently, with CBS 618.96, CBS 232.33 as the only exceptions (Table 1). However, specific amplifications of deviating haplotypes were mostly unsuccessful. In two strains the deviating sequence was obtained in addition to the invariant type. This strongly suggests that the deviating type was a paralogue resulting from gene duplication. When the fragments were cloned, ortho- and paralogues were obtained repeatedly, the orthologue remaining absent from CBS 232.33.

The *CDC42* orthologue evolution shows an unexpected topology. The analyzed exons showed very limited non-synonymous change throughout the entire phylogenetic history of *Chaetothyriales*, even between such remote species as *Coniosporium perforans* and *Exophiala dermatitidis*. A very strong constraint is observed on the gene, the exon having remained identical and the protein structure having remained unchanged. Remarkably, also intron and 3rd codon sequences had remained identical (Fig. 1). The gene was subject of strong functional and structural constraints.

In main traits the evolution of the *CDC42* paralogue was comparable to that of the scaffold of the multilocus phylogeny of the *Chaetothyriales* (Badali *et al.* 2008). SSU, LSU and *CDC42* were analysed using the same set of strains (Figs 1–4). SSU and LSU gene evolution is likely to reflect the phylogenetic history of *Chaetothyriales*. The paralogue showed considerable evolution in intron and 3rd codon positions, the tertiary structure being relaxed, allowing considerable mutation. The translated protein sequence, however, showed only limited non-synonymous change (Table 5 Figs 3, 4). Non-synonymous change is observed in *Phialophora* and *Cladophialophora* agents of chromoblastomycosis, but not in *Fonsecaea* even though all three cause the same disease. Hence these mutations cannot be linked to selection of an adapted

Table 5. Number of mutations in 89 codons of partial *CDC42* coding region of ten strains which had paralogues. Amino acid changes from orthologue are listed

Accession No.	Species name	Total codon	1st base	2nd base	3rd base	Amino acid change
CBS 618.96	*Capronia acutiseta*	89	2	1	38	CAA → TCA / Q → S
CBS 232.33	*Exophiala heteromorpha*	89	1	-	33	-
CBS 260.83	*Cladophialophora carrionii*	89	2	1	39	CAA → TCG / Q → S
CBS 126.86	*Cladophialophora boppii*	89	2	1	39	CAA → TCG / Q → S
CBS 286.47	*Phialophora verrucosa*	89	3	1	46	CAA → TCC / Q → S
CBS 606.96	*Capronia epimyces*	89	-	-	35	-
CBS 207.35	*Exophiala dermatitidis*	89	2	-	33	-
CBS 525.76	*Exophiala dermatitidis*	89	1	-	23	-
CBS 616.96	*Capronia villosa*	89	2	1	5	GAC → TAC / D → Y
CBS 615.96	*Capronia munkii*	89	-	-	5	-

Q glutamin; S serin; D asparaginic acid; Y tyrosin

genotype.

We hypothesise that both types are expressed with identical proteins, with gene duplication as an ancient event, and that subsequent structural evolution has taken place in the paralogue without loss of function. In the majority of the species investigated only one of the types was detected, suggesting that ortho- or paralogues may be lost. When non-synonymous *vs.* synonymous nucleotide divergence is high between species, functional divergence is assumed to be high, due to positive selection and/or relaxed selective constraint (e.g. Friedman & Hughes 2004). When this ratio is low, the functional properties of the gene products involved are thought to be conserved, because selective constraint is high and there is little or no positive selection (e.g. Sehgal & Lovette 2003). In *CDC42*, both ortho- and paralogues show a very low ratio, indicating high functional and structural selective constraint.

In summary, we hypothesise that members of *Chaetothyriales* may have two duplicate *CDC42* genes, producing exactly the same protein, but having a conserved *versus* relaxed tertiary structure, which involves 3rd codon positions as well as introns. The evolution that takes place in the paralogue follows the main traits of evolution seen in ribosomal genes, but the genes are considerably more variable and difficult to align. The possibility is not excluded that in the course of evolution one of the duplicate genes – either ortho- or paralogue – was lost in individual species. In that case *CDC42* would be a poor phylogenetic marker, as trees will consist of non-homologous genes. From the fact that both orthogue and paralogue have a considerable constraint at the protein level, it can be concluded that any eventual change of function of *CDC42* in the course of evolution of *Chaetothyriales* (using *CDC42* for rock-inhabiting or pathogenic life styles, respectively) is not reflected at the DNA level. The limited number of non-synonymous changes in the paralogue do not coincide with evolution in species causing chromoblastomycosis, and thus do not suggest any positive selection of a functional change from rock-inhabiting life styles to pathogenicity. Any role of *CDC42* as a virulence factor has thus not been proven.

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Coniosporium epidermidis sp. nov., a new species from human skin

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Abstract: *Coniosporium epidermidis* sp. nov. is described from a superficial skin lesion with blackish discolouration in an 80-yr-old Chinese patient. The species produces dark, thick-walled, inflated, reluctantly liberating arthroconidia without longitudinal septa. Sequences of the ribosomal operon, as well as of the translation elongation factor 1-α support its novelty. The species is found in a lineage basal to the order *Chaetothyriales*, amidst relatives from rock, but also species repeatedly isolated from human skin and nails and eventually causing mild cutaneous infections. *Coniosporium epidermidis* is consistently found on humans, either asymptomatic or symptomatic. The species indicates a change of life style towards human pathogenicity, which is a recurrent type of ecology in derived *Chaetothyriales*. Superficial and cutaneous infection by melanized fungi is a new category in dermatology.

Key words: Black yeasts, *Coniosporium*, superficial mycosis, taxonomy.
Taxonomic novelties: *Coniosporium epidermidis* D.M. Li, de Hoog, Saunte & X.R. Chen, sp. nov.

INTRODUCTION

In recent years the clinical significance of melanized fungi involved in cutaneous infections has been underlined (Badali *et al.* 2008a). Several of the species concerned, although causing relatively mild infections, are regularly encountered in dermatological specimens, but usually discarded as purported contaminants. Some species, such as *Phialophora europaea* de Hoog *et al.* and *Cyphellophora laciniata* de Vries, however, are recurrently observed on humans, and their environmental niches thus far have remained unknown (de Hoog *et al.* 2000). We here report on a species that originated from the skin of an 80-yr-old male patient who manifested with a 3 yrs history of black bilateral maculae on his feet, with scales, maceration, and fissures. The infection was caused by a *Coniosporium*-like fungus that could not be identified with any of the known species and is therefore introduced here as a new taxon.

The genus *Coniosporum* is considered to comprise environmental fungi forming black spots or patches on plant leaves, bamboo surface, rotten wood, and recently particularly on rock surfaces (Hyde *et al*. 2002, De Leo *et al.* 1999, Sterflinger *et al*. 1997, 2001). Species have black, velvety colonies on the natural substrate, and are characterized microscopically by thick-walled, heavily pigmented arthroconidia with subsequent meristematic development. This report concerns the first human infection caused by a *Coniosporium* species. *Coniosporium* is not among the recognized human pathogens in dermatology. Several melanized fungi have been reported cause mild cutaneous infections, e.g. *Cyphellophora laciniata* de Vries (1962), *Phialophora europaea* de Hoog *et al*. (2000b), and *Cladophialophora saturnica* Badali *et al*. (2009). Such fungi are encountered fairly regularly in samples from human skin and nail (de Hoog *et al*. 2000a). A new dermatological category may be concerned, which will be introduced in this paper.

MATERIALS AND METHODS

Isolation

Clinical specimens were scraped with a scalpel from superficially sterilized blackish skin lesions. A skin biopsy was performed on the black lesion and histological specimens were stained with hematoxylin-eosin. Samples of skin flakes were plated on Sabouraud's glucose agar (SGA) with chloramphenicol and incubated at 27 °C. Strain T22 (= CBS 120353) was isolated from specimens of the first visit of the patient. Another isolate was recovered one year later from the same patient and turned out to be identical by sequence data. Studied strains of the same species included for comparison were isolates encountered during analysis of routine dermatological specimens from Denmark, and an isolate from ant garbage from Brazil. Related strains studied are listed in Table 1.

Morphology

Strains were transferred to malt extract agar (MEA), potato dextrose agar (PDA), cormeal agar (CMA), oatmeal agar (OA) and Czapek agar (CZA) and incubated at 25 °C and 37 °C for at least 4 wk under alternate near-ultraviolet light for growth rate determination and phenetic description of colonies. For study of microscopic morphology strains were point-inoculated on PDA. Blocks of agar of approximately 1 × 1 cm were excised aseptically on sterile microscope slides. Blocks were inoculated, covered with sterile cover slips and incubated in moist chambers for 14 d at 27 °C. Structure and branching pattern of conidiophores were observed at magnifications ×100, ×200 and ×400 in intact slide cultures under the microscope without removing the cover slips from the agar blocks. For higher magnifications, cover slips were removed and mounted in lactic acid with aniline blue.

Sequencing

Approximately 0.1 g of fungal material was transferred to a 2-mL Eppendorf tube containing a 2:1 (w/w) mixture of silica gel and Celite (silica gel H, Merck 7736/Kieselguhr, Celite 545, Machery, Merck, Amsterdam, The Netherlands); DNA was extracted according to methods described previously (Li *et al*. 2008). Amplifications were done with primers ITS1 and ITS4 (for rDNA Internal Transcribed Spacer ITS), NS1, BF83, OLI1, BF963, BF1438 and NS24 (for rDNA Small Subunit nucSSU), D1/D2 (for rDNA Large SubUnit nucLSU), and EF1-728F and EF1-986R (for Translation Elongation Factor 1-α *EF*1α). PCR was performed in 50 µL volumes of a reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 0.01 % gelatin, 200 mM of each deoxynucleotide triphosphate, 25 pmol of each primer, 10−100 ng rDNA, and 0.5 U Taq DNA polymerase (Bioline, GC Biotech, Alphen a/d Rijn, The Netherlands), as follows: 95 °C for 4 min, followed by 35 cycles consisting of 94 °C for 45 s, 52 °C for 30 s, and 72 °C for 2 min. Amplicons were cleaned with GFX columns (GE Healthcare, Sweden). Sequence PCR was performed as follows: 95 °C for one min, followed by 30 cycles consisting of 95 °C for 10 s, 50 °C for five s, and 60 °C for two min. DNA was purified with Sephadex G-50 Superfine. Purified amplicons were then sequenced on both strands using the same primers described above. BigDye terminator cycle sequencing Ready Reaction kits (Perkin Elmer Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) were used according to the manufacturer's instructions and DNA was sequenced using a DYE-ET terminator.

Sequence analysis and taxonomy

Sequences were compared in GenBank and using a research database available at the Centraalbureau voor Schimmelcultures Biodiversity Centre (CBS), Utrecht, The Netherlands. Alignment

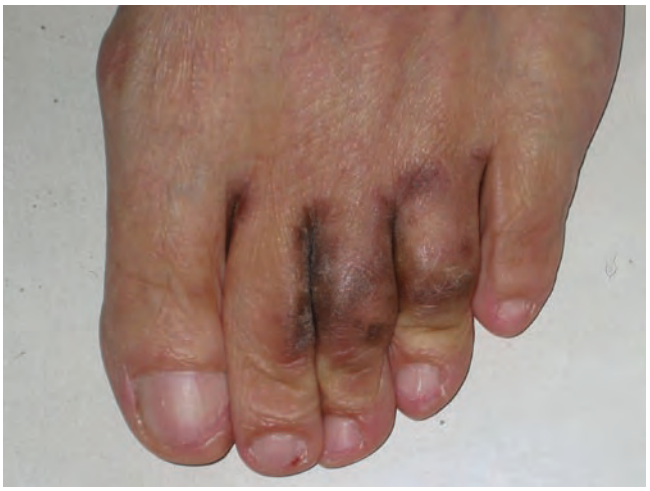

Fig. 1. Blackish discoloured skin of toes and toe webs with scaling.

was done in a database using BioNumerics software v. 4.61 (Applied Maths, Kortrijk, Belgium). SSU sequences were aligned with the ARB beta-package (v. 22-08-2003) developed by Ludwig *et al.* (2004). A distance tree of *Coniosporium epidermidis* and allied black fungi based on the completed ITS 1-2 domain including the 5.8S rDNA gene were reconstructed using neighbor-joining algorithm with Kimura 2 correction with 100 bootstrap replications in Treefinder.

RESULTS

Mycology

A skin biopsy performed on the black lesion (Fig. 1) and histological specimens stained with hematoxylin-eosin (Fig. 2) showed hyperkeratosis and acanthosis. Numerous hyphae and swollen cells were observed in the stratum corneum (Fig. 2). Pigmented hyphae and loose cells were displayed in the entire layers of epidermis, predominated among the low layers. Cells also penetrated the basal membrane to the dermis. Direct examination of skin scrapings with KOH was positive for pigmented arthroconidia and dark-walled hyphae. The disease was considered to be an infection.

At primary isolation, isolate CBS 120353 grew slowly with 8 mm/wk. Colonies on SGA were convex with papillate surface. Obverse and reverse were black, while colonies on MEA, OA, CMA, CZA and PDA were velvety with dark brown to olive obverse. Growth was stimulated under near-UV light; 37 °C was tolerated. Subcultures initially were cream-coloured, smooth and turned black within a wk; this phenomenon disappeared after several transfers. Hyphae were septate, olivaceous-black, forming reluctantly disarticulating arthroconidia with transverse but without longitudinal septa (Fig. 3). Cells were pigmented, thick-walled, and maturated meristematically, the mother cell wall frequently rupturing in an irregular fashion. With time, monilioid conidia (Fig. 3h, i) were predominant and occasional chlamydospores occurred.

Molecular data

1743 bp of the rDNA SSU gene were sequenced of strain CBS 120353 (data not shown). Phylogenetic analysis of aligned sequences revealed close relationship with species in *Cladophialophora, Exophiala, Phialophora, Rhinocladiella, Fonsecaea* and *Capronia*, which all are members of the order *Chaetothyriales*. However,

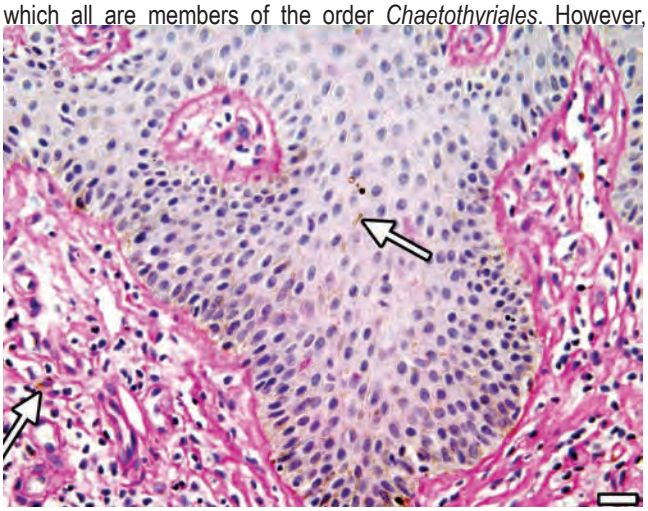

Fig. 2. Skin biopsy stained with HAE; some fungal elements are visible (arrows). Size bar = 5µm.

nearest neighbours were *Coniosporium perforans* Sterflinger (CBS 665.80), and *C. apollinis* Sterflinger (CBS 352.97). Of the LSU domain, 616 bp were sequenced. Nearest neighbour at 97 % similarity was a species published by Crous *et al.* (2007) in a tree as '*Exophiala* sp. 3', CPC 12173 = EU035422.

Length of ITS domain of CBS 120353 was 541 bp. ITS rDNA sequences compared in a dedicated black yeast data base maintained at CBS and containing about 11,000 entries revealed no

Table 1. Isolation data of examined strains.

Name	Accession no.	Country	Source	GenBank
Antarctic black fungus	CCFEE 5323	Antarctic	Thallus of *Lecanora* sp.	FJ392866
	CCFEE 5314	Antarctic	Thallus of *Xanthoria elegans*	FJ392865
	CCFEE 5324	Antarctic	Thallus of *Acarospora flavocordia*	FJ392867
Coniosporium apollinis	CBS 100213	Greece	Rock	AJ244271
	CBS 100218	Greece	Marble	AJ244273
	CBS 109867	Greece	Marble	
	CBS 100216	Spain	Rock	AJ244272
	CBS 109860	Spain	Rock	
	CBS 109865	Italy	Rock	
Coniosporium epidermidis	CBS 123233	Denmark	Hand, female	
	dH 17028	Denmark	Axilla, male	
	CBS 123466	China	Nail with onychomycosis	
	CBS 120353 (T)	China	Skin infection	EU730589
	CBS 120388	Denmark	Toenail, female	
	CBS 123279	Denmark	Toenail, female	
	dH 17006	Denmark	Toenail, female	
	CBS 123261	Denmark	Toenail, male	
	dH 17086	Denmark	Toenail, male	
Coniosporium perforans	dH 17016	Denmark	Axilla, female	
	CBS 109861	Italy	Marble	
	dH 16682	Denmark	Nail, male	
	CBS 885.95 (T)	Greece	Marble	
Coniosporium species	CBS119726	Italy	Stone monument	
	dH 14071	Australia	Cattle	
	dH 16979	France	Chronic nasal oedema	
	dH 14084	Italy	Marble monument	
	dH 14085	Italy	Rock monument	
	CBS 109864	Italy	Rock	
	CBS 109866	Italy	Rock	
	CBS 665.80	Italy	Rock	
Cryptoendolithic fungus	CCFEE 457	Antarctic	University Valley	
Exophiala placitae	CPC 13707	Australia	*Eucalyptus placita*	EU040215.1
Exophiala species	CPC 12172	Canada	*Prunus* sp.	
	CPC 12173	Canada	*Prunus* sp.	
	CPC 12171	Canada	*Prunus* sp.	EU035420
Meristematic fungus	CBS119729	Italy	Stone	
Phaeococcomyces catenatus	Det M175	Netherlands	Nail	
	CBS 650.76 (T)	Switzerland	Air	AF050277
	dH 11392		Unknown	
	dH 14721	Austria	Bloodplasma	
	TRN4	Spain	Rock surface	AY843222
Phialophora europaea	CBS 656.82	France	Nail	FJ489612
	dH 12320	Germany	Nail	
	CBS 101466 (T)	Netherlands	Skin scales	
Sarcinomyces petricola	CBS 726.96 (T)	Guinea	Dung of cow	FJ489613
	CBS 600.93	Greece	Pentelic marble	
	CDC 2008006858	USA	Scalp rash, male	
Uncultured ascomycete	AM901753	Finland	Indoor dust	AM901753

Abbreviations used: CBS = CBS Fungal Biodiversity Centre, Utrecht, The Netherlands; CCFEE = Culture Collection of Fungi from Extreme Environments, Viterbo, Italy; CDC = Centers for Disease Control and Prevention, Atlanta, U.S.A.; CPC = culture collection of Pedro Crous, housed at CBS; dH = G.S. de Hoog working collection; TRN = Tino Ruibal working collection.
T = ex-type culture.

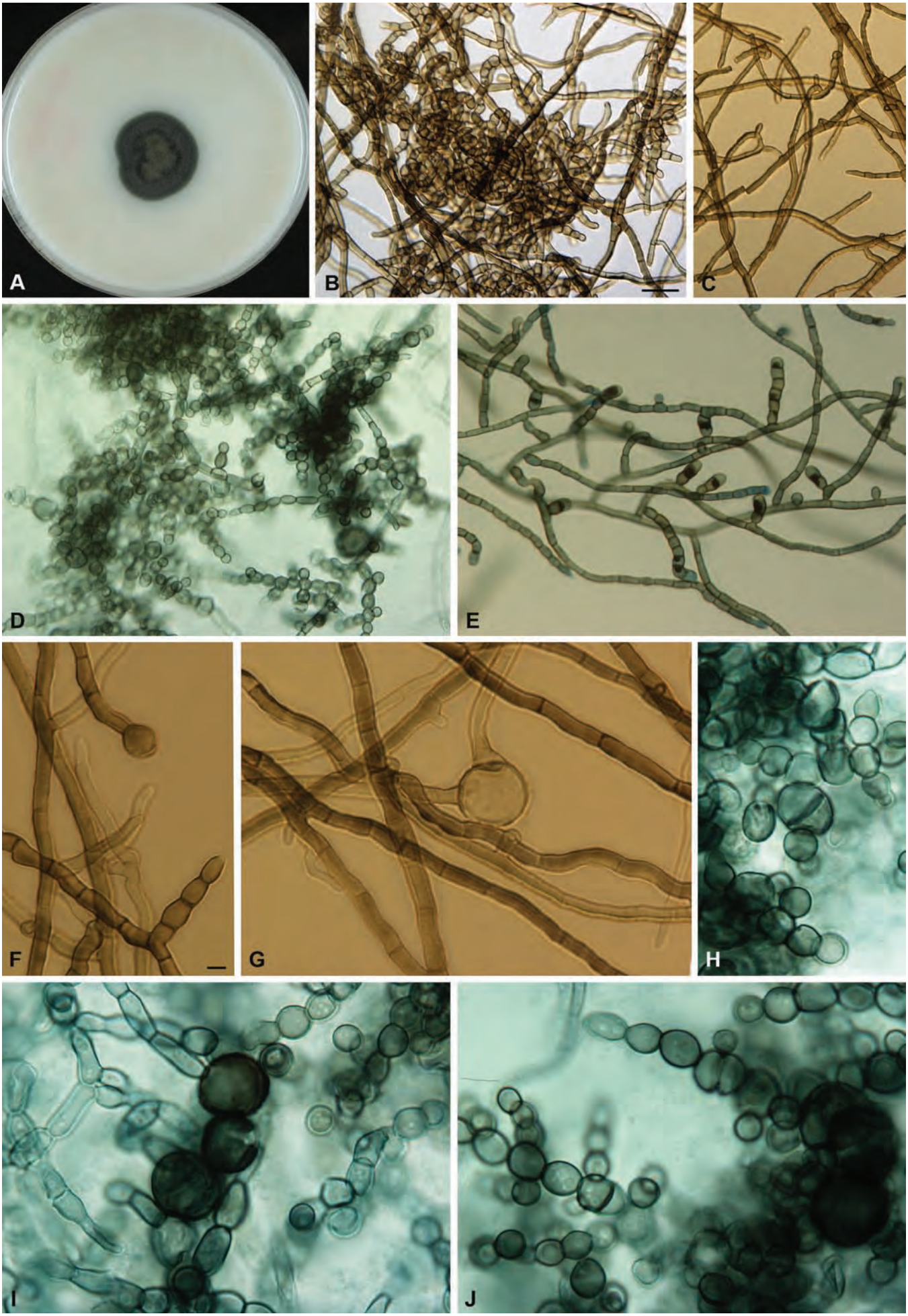

Fig. 3. *Coniosporium epidermidis*, CBS 120353. A. Colony on OA (3 wks). B, C. Elongated and monilioid hyphae, anthroconidia; D, E. Conidial chains; F–J. Mature conidial chains and large conidia with transverse septa. Scale bars 5 µm (B–E); 1 µm (F–J).

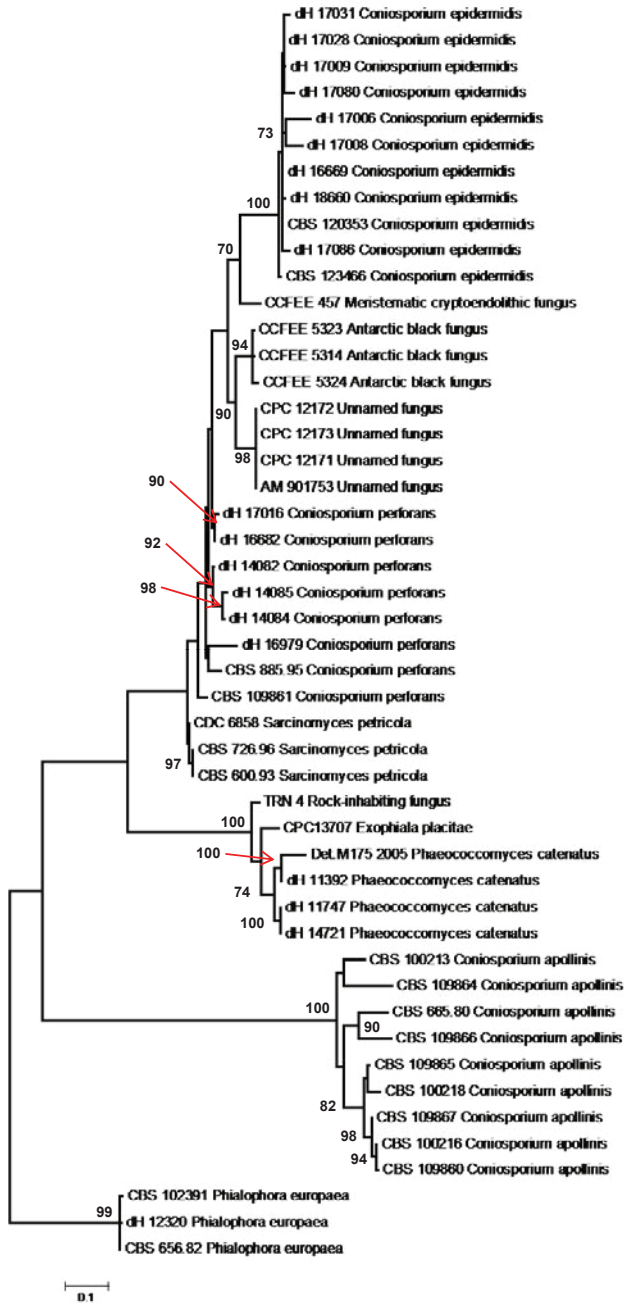

Fig. 4. Phylogenetic tree of *Coniosporium epidermidis* and allied black fungi based on the completed ITS 1−2 domain including the 5.8S rDNA gene, generated with the TREEFINDER package using the Neighbor-joining algorithm and Kimura correction. The tree was subjected to 100 bootstrap replications; *Phialophora europaea*, CBS 656.82 was selected as outgroup.

close match with any known species. Alignment was only partially confident. Among the nearest neighbours were the known rock-inhabiting species *Coniosporium perforans* and *C. apollinis*, as well as a number of undescribed rock-inhabiting species prevalently from the Mediterranean and from the Antarctic (Table 1). '*Exophiala* sp. 3' of Crous *et al.* (2007) was also close; the ITS of this species proved to be identical to a hyperparasitic '*Coniosporium* sp.', AM901753 published by Harutyunyan *et al.* (2008). In addition, strains from human skin and nail samples were involved (Table 1). Identity was found with a group of strains from dermatological skin and nail samples from Denmark, as well as with an environmental sample from Brazil; all these strains either were morphologically identical to CBS 120353, or consisted of sterile melanized hyphae. These strains were therefore regarded to represent a hitherto undescribed taxon, which is introduced below.

Coniosporium epidermidis D.M. Li, de Hoog, Saunte & X.R. Chen, **sp. nov**. – MycoBank MB512506, Figs 3, 5.

Coloniae primum fuscae, effusae, deinde elevatae, velutinae vel cottoneae, griseo-olivaceae; reversum olivaceo-nigrum. Coloniae in agaroso maltoso, agaro PDA, vel agaro farina avenae confecto (OSD) dicto 25 °C 10−15 mm diam post 28 dies. Mycelium immersum vel superficiale, ex hyphis ramosis, septatis compositum, olivaceo-nigrum vel brunneum. Conidiophora vix distinguenda, ramose vel simplicia, terminalia vel intercalaria. Conidia singula, primaria in apice conidiophori, ellipsoidea vel subglobosa, levia, 2-3 µm diam. Mycelium torulosum praesens. Teleomorphosis ignota.

Holotype: dried culture in CBS herbarium (CBS-H-20167); ex-type strain CBS 120353 = T22, isolated from nigramacula, superficial infection of the feet of a 80-yr-old male patient, China, D.M. Li. Additional strains listed in Table 1.

The following description is of CBS 120353 on PDA after 28 ds incubation at 25 °C.

Colonies effuse, becoming raised, attaining 10-15 mm diam, velvety to fluffy, black, blackish-brown to greyish olivaceous; reverse olivaceous-black. Mycelium superficial, regularly and densely septate, profusely branched at nearly right angles, olivaceous-black or dark brown, smooth-, or occasionally rough-walled. Cells gradually swelling at maturation up to 3-8 µm diam; cell walls very thick at maturity. Conidia formed by liberation of arthric cells, swelling to become ellipsoidal or nearly spherical, smooth-walled, mostly 2-3 µm diam, up to 8 µm wide, then frequently the mother cell wall remaining visible on the daughter cell. Truly muriform cells absent. Teleomorph unknown. Cardinal temperatures: optimum 27 °C, maximum 37 °C.

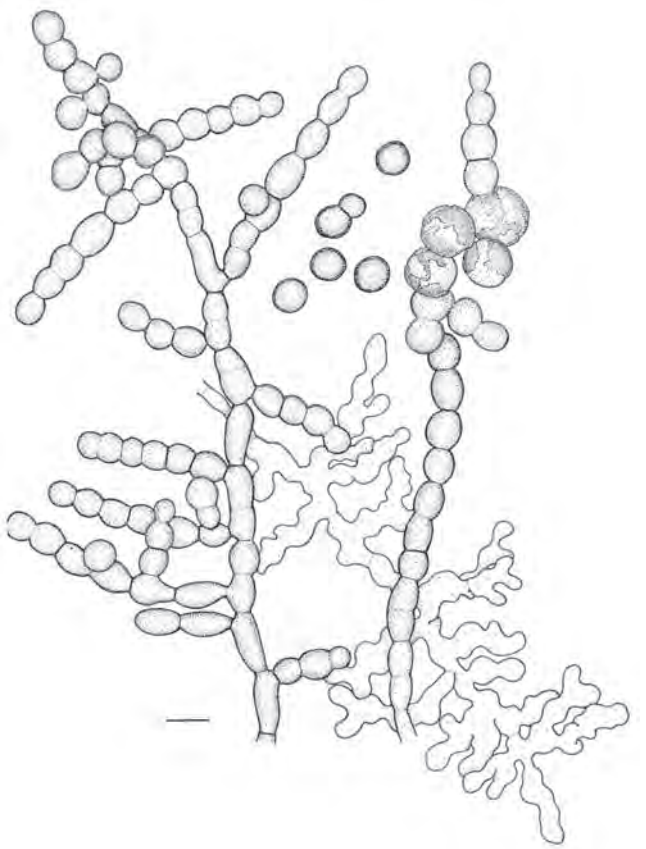

Fig. 5. Microscopic morphology of *Coniosporium epidermidis*, CBS 120353; slide culture on SGA, 28 d.

DISCUSSION

Coniosporium classically includes black, slow-growing anamorphs, most of which are plant colonizers (Hyde *et al.* 2002). Species are characterized by pigmented anthroconidia, and tend to develop meristematically into chains (Ellis 1971). The mother cell wall may eventually rupture during development, leading to irregular cell wall ornamentation (Ellis 1976).

Coniosporium epidermidis was isolated from a tinea nigra-like skin infection on the foot. Tinea nigra, characterized by brown to black superficial macules, is a strictly asymptomatic colonization of dead epidermis (Schwartz 2004, Bonifaz *et al*. 2008) caused by a halophilic member of the order *Capnodiales*, *Hortaea werneckii* (Zalar *et al*. 1999). In contrast, pathological slides of *C. epidermidis* clearly showed that the fungus grew into all layers of the epidermis, and some cells penetrated down to the basal membrane reaching the superficial dermis. Thus the present species causes a real disease process, as was also observed with the tinea nigra-like infection caused by *Cladophialophora saturnica* Badali *et al*. (2008a), another member of *Chaetothyriales*. The optimal growth temperature of *C. epidermidis* at 27 °C (maximum 37 °C) is comparable to that of true pathogens of the skin, the dermatophytes, ranging between 25 and 35 °C (Weitzman & Summerbell 1999). We thus conclude that ordinal relationships predict opportunistic potential. In the course of the present study *Coniosporium epidermidis* was repeatedly isolated from routine dermatological samples in Denmark. It is supposed that the fungus may be common in cutaneous samples, but is generally discarded as a contaminant. We therefore recommend to pay more attention to melanized fungi occurring on skin and nails, and to establish their precise role in pathology.

With SSU rDNA, the black fungus recovered from affected human skin appeared to be an undescribed species, located within a group of species causing mild cutaneous infections, such as *Phialophora europaea* and *Cyphellophora laciniata*. The group was basal to the order *Chaetothyriales,* an order having *Capronia* teleomorphs and being notorious for containing numerous opportunists (Badali *et al*. 2008b). In the corresponding ITS tree (Fig. 4) the group showed considerable diversification among species. The similarity of *C. epidermidis* to the nearest described species, *Coniosporium perforans,* was less than 91 %. This species is, however, a colonizer of rock and monuments in the Mediterranean Basin (Sterflinger *et al*. 1997), as is the case in the majority of taxa in this group. The rock-inhabiting species were attributed to the genus *Coniosporium*, although the generic type species, *C. olivaceum* Link (Ellis 1971), has as yet not been redefined according to modern standards.

Sterflinger *et al.* (1997) supposed that the *Coniosporium*-clade of *Chaetothyriales* was entirely rock-associated. However, with recent additions to this group we notice that it contains several undescribed taxonomic species from human skin and nail samples. In addition, many of the species described as having a rock-inhabiting life-style, such as *Sarcinomyces petricola* Wollenzien & de Hoog, *Coniosporium perforans* and *Phaeococcomyces catenatus* (de Hoog & Hermanides-Nijhof) de Hoog, can also be found on human skin (Table 1). A similar dual ecology was found earlier in the unrelated meristematic fungus *Catenulostroma abietis* (Butin & Pehl) Crous *et al.* (Butin *et al.* 1996). We suppose that these predominantly meristematic skin colonizers are taken up from the environment where they live as oligotrophs on rock, leathery plant leaves and other relatively inert surfaces. They are likely to display a similar oligotrophic character on human skin, and thus probably behave as commensals rather than pathogens. Nevertheless occasional mild infections may occur, such as the ones observed in *Cladophialophora saturnica* (Badali *et al*. 2008a) and the present fungus.

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Environmental isolation of black yeast-like fungi involved in human infection

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Abstract: The present study focuses on potential agents of chromoblastomycosis and other endemic diseases in the state of Paraná, Southern Brazil. Using a highly selective protocol for chaetothyrialean black yeasts and relatives, environmental samples from the living area of symptomatic patients were analysed. Additional strains were isolated from creosote-treated wood and hydrocarbon-polluted environments, as such polluted sites have been supposed to enhance black yeast prevalence. Isolates showed morphologies compatible with the traditional etiological agents of chromoblastomycosis, *e.g. Fonsecaea pedrosoi* and *Phialophora verrucosa*, and of agents of subcutaneous or systemic infections like *Cladophialophora bantiana* and *Exophiala jeanselmei*. Some agents of mild disease were indeed encountered. However, molecular analysis proved that most environmental strains differed from known etiologic agents of pronounced disease syndromes: they belonged to the same order, but mostly were undescribed species. Agents of chromoblastomycosis and systemic disease thus far are prevalent on the human host. The hydrocarbon-polluted environments yielded yet another spectrum of chaetothyrialean fungi. These observations are of great relevance because they allow us to distinguish between categories of opportunists, indicating possible differences in pathogenicity and virulence.

Key words: Black yeasts, *Chaetothyriales*, chromoblastomycosis, enrichment, environmental isolation, opportunists, phaeohyphomycosis, virulence.

INTRODUCTION

Knowledge of natural ecology and evolution is essential for a better understanding of pathogenicity and opportunism. Members of different fungal orders and families tend to be differentially involved in human mycoses. Among melanised fungi, for example, etiologies of members of *Dothideaceae* and *Herpotrichiellaceae* show basic differences (de Hoog 1993, 1997). Of these families, only species belonging to the *Herpotrichiellaceae* (black yeasts and relatives) are associated with recurrent, clearly defined disease entities such as chromoblastomycosis and neurotropic dissemination in immunocompetent individuals. In contrast, members of *Dothideaceae* show coincidental opportunism, whereby the infection is largely dependent on the portal of entry and the immune status of the host.

Among the diseases caused by chaetothyrialean fungi (teleomorph family *Herpotrichiellaceae*), chromoblastomycosis and other traumatic skin disorders are the most frequent (Attili *et al.* 1998, Zeng *et al.* 2007). Although the agents are supposed to originate from the environment, their isolation from nature is difficult. This is probably due to their oligotrophic nature, low competitive ability, and in general insufficient data on their natural habitat. Several selective techniques have been developed enabling recovery of these fungi (de Hoog *et al.* 2005; Dixon *et al.* 1980, Prenafeta-Boldú *et al.* 2006, Satow *et al*. 2008, Zhao *et al*. 2008, Sudhadham *et al.* 2008). These investigations indicated that opportunism of these fungi must be explained from the perspective of unexpected environments such as rock, creosote-treated wood, hydrocarbon-polluted soil, and hyperparasitism of fungi and lichens (Sterflinger *et al*. 1999, Wang & Zabel 1997, Lutzoni *et al.* 2001).

In the present study we tried to find recover chaetothyrialean fungi from the natural environment in the State of Paraná, Southern Brazil, where chromoblastomycosis and phaeohyphomycosis are frequent in endemic areas. In addition, human-made substrates like creosote-treated wood and hydrocarbon-polluted soil were sampled. Strains morphologically similar to etiological agents of chromoblastomycosis, such as *Fonsecaea pedrosoi* and *Phialophora verrucosa*, and to agents of subcutaneous and systemic infections, such as *Exophiala jeanselmei* and *Cladophialophora bantiana*, were selected. The aim of this investigation was to clarify whether these fungi were identical to known etiologic agents of disease. Isolates were compared with clinical reference strains on the basis morphological, physiological and molecular parameters.

MATERIALS AND METHODS

Study area and strains

Samples were obtained from 28 localities belonging to three different geographical regions in the State of Paraná, Southern Brazil (Fig. 1). Locations were chosen on the basis of known records of chromoblastomycosis in the "Hospital de Clínicas" of the Paraná Federal University (HC-UFPR). The climate in the region is subtropical, with relatively regular rainfall throughout the

Fig. 1. Sampling locations in the State of Paraná, S.P., Brazil.

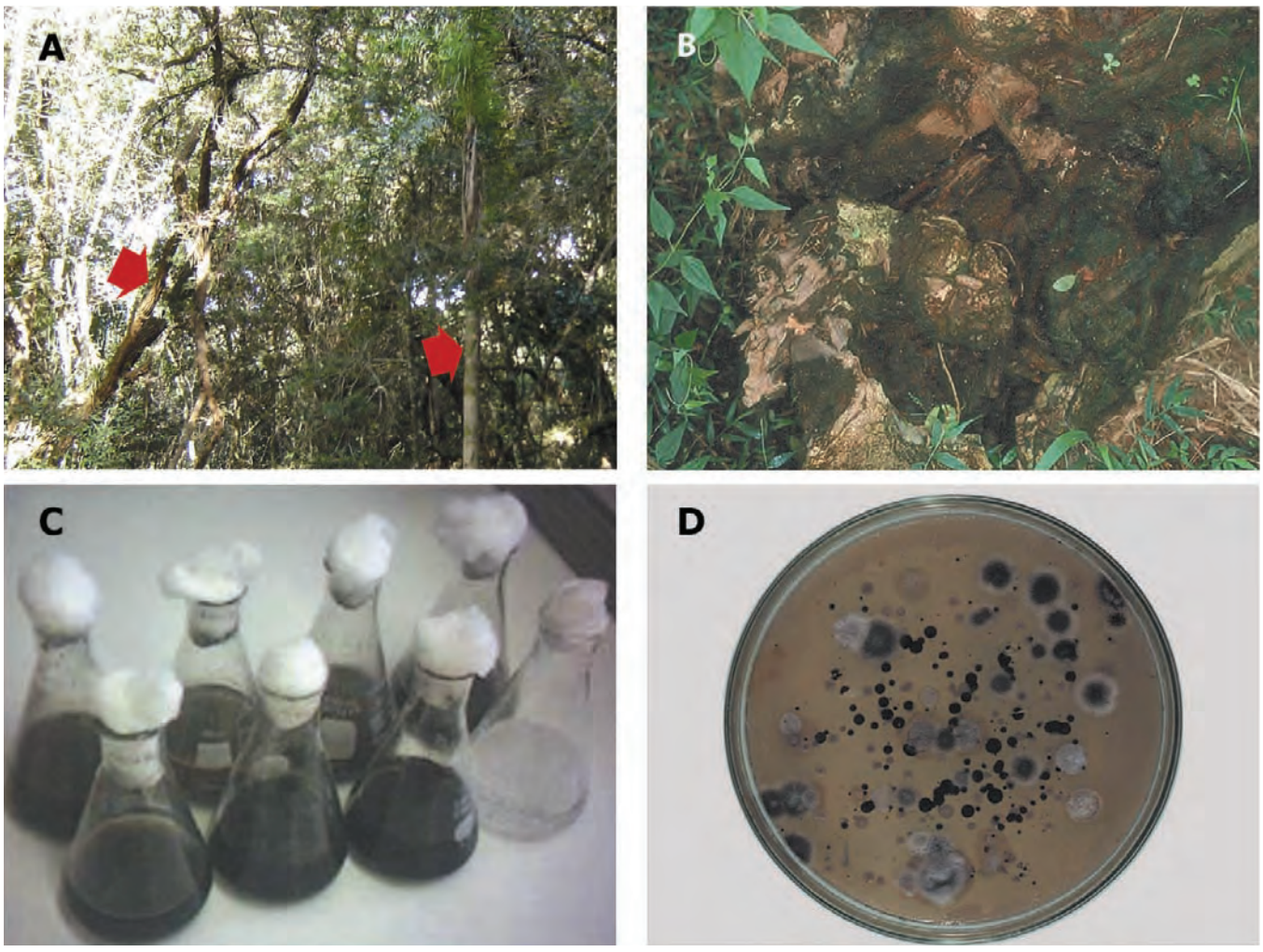

Fig. 2. A. Sampling location in the area of "FE" series of samples in the first plateau at Colombo (Paraná) with native species (arrows) dominated by cambara tree (*Gochnathia polymorpha*) and stem palm (*Syagrus romanzoffiana*); B. Sampling location of FE5P4 of *Fonsecaea monophora*, from decaying cambara wood; C. Sample incubated at room temperature in sterile saline solution, containing antibiotics added with sterile mineral oil after vigorous shaking; D. Black yeast colonies on Mycosel medium.

yr (average 12500 mm/yr). The isolates obtained were compared to clinical isolates from the hospital of the Universidade Federal do Paraná, as well as to reference strains from the Centraalbureau voor Schimmelcultures (CBS, The Netherlands; Table 1). Samples from *Eucalyptus* wood in the "Navarro de Andrade" forest and creosote-treated railway ties near Rio Claro, and from hydrocarbon-polluted soil from the oil refinery of Paulínia, Paulínia state of São Paulo, Brazil, were also investigated (Table 1).

Fungal isolation

In each location, fragments of litter and decaying wood showing the presence of black spots, as well as soil samples, were randomly collected. Approximately 20 g from each sample were processed for fungal isolation, with 10 replicates per sample. Each sample was incubated at room temperature for 30 min in 100 mL sterile saline solution containing 200 U penicillin, 200 µg/L streptomycin, 200 µg/L chloramphenicol and 500 µg/L cycloheximide (Fig. 2C). After the initial incubation, 20 mL of sterile mineral oil was added to the solution, followed by vigorous 5 min shaking and the flasks were left to settle for 20 min. The oil-water interphase was carefully collected, inoculated onto Mycosel agar (Difco) and incubated for 4 wks at 36 ºC (Dixon & Shadomy 1980, Iwatsu *et al.* 1981). The grown dark colonies were then isolated and stored on Mycosel agar (Fig. 2D).

Morphology

Preliminary identification was carried out based on macro- and microscopic features of the colonies after slide culturing on Sabouraud's dextrose agar at room temperature (de Hoog *et al.* 2000a). In addition, vacuum-dried samples were mounted on carbon tape and sputtered with gold for 180 s for SEM. Observations were done in a Zeiss DSM 940 A microscope, operated at 5 kV.

Nutritional physiology

Some isolates with cultural and morphological similarity to known agents of disease were selected for physiological testing. Growth and fermentative abilities were tested in duplicate, negative controls were added. The fungi were incubated at 28 and 36 °C on the following culture media: Mycosel, Potato Dextrose Agar (PDA), Minimal Medium (MM), Complete Medium (CM), and Malt Extract Agar (MEA). Assimilation and fermentation tests were carried out in liquid medium according to de Hoog *et al*. (1995). Halotolerance was tested in a liquid medium at 2.5, 5 and 10 % (w/v) NaCl and $MgCl_2$. Cycloheximide tolerance was determined in liquid medium at 0.01, 0.05 and 0.1 % (w/v).

DNA extraction

About 1 cm^2 mycelium of 20 to 30-d-old cultures was transferred to a 2 mL Eppendorf tube containing 300 µL CTAB (cetyltrimethylammonium bromide) buffer [CTAB 2% (w/v), NaCl 1.4 M, Tris-HCl 100 mM, pH 8.0; EDTA 20 mM, b-mercaptoethanol 0.2 % (v/v)] and about 80 mg of a silica mixture (silica gel H, Merck 7736, Darmstadt, Germany / Kieselguhr Celite 545, Machery, Düren, Germany, 2:1, w/w). Cells were disrupted manually with a sterile pestle for approximately 5 min. Subsequently 200 µL CTAB buffer was added, the mixture was vortexed and incubated for 10 min at 65 °C. After addition of 500 µL chloroform, the solution was mixed and centrifuged for 5 min at 20 500 g and the supernatant transferred to a new tube with 2 vols of ice cold 96 % ethanol. DNA was allowed to precipitate for 30 min at −20°C and then centrifuged again for 5 min at 20 500 g. Subsequently the pellet was washed with cold 70 % ethanol. After drying at room temperature it was resuspended in 97.5 µL TE-buffer plus 2.5 µL RNAse 20 $U.mL^{-1}$ and incubated for 5 min at 37 °C, before storage at −20 °C (Gerrits van den Ende & de Hoog 1999).

Sequencing

rDNA Internal Transcribed Spacer (ITS) was amplified using primers V9G and LS266 (Gerrits van den Ende & de Hoog 1999) and sequenced with ITS1 and ITS4 (White *et al*. 1990). Amplicons were cleaned with GFX PCR DNA purification kit (GE Healthcare, U.K.). Sequencing was performed on an ABI 3730XL automatic sequencer. Sequences were edited using the Seqman package (DNAStar, Madison, U.S.A.) and aligned using BioNumerics v.4.61 (Applied Maths, Kortrijk, Belgium). Sequences were compared in a research data database of black fungi maintained at CBS, validated by ex-type strains of all known species.

RESULTS

Eighty-one isolates from a total of 540 showed morphologies compatible with the traditional etiological agents of chromoblastomycosis and phaeohyphomycosis. Twenty -six strains were selected and processed for taxonomic studies and listed in Table 1 with additional strains from hydrocarbon-polluted soil and wood (natural and creosote-treated).

Isolate FE9 was morphologically very similar to *Cladophialophora bantiana*. Physiological testing demonstrated ability to assimilate ethanol, lactose and citrate, but it was unable to grow at 40 °C (Table 2). Sequence data proved identity to *C. immunda* (Table 1). Strain F10PLB was physiologically similar to FE9 of *C. immunda* (Table 2), which was confirmed by molecular data (Table 1). F10PLA showed physiological characteristics close to the FE9, differing only by growth in the presence of creatine and creatinine (Table 2); also this strain was identified by ITS sequence data as *C. immunda*. The isolate FP4IIB was capable of growing with 0.1 % cycloheximide, showed reduced growth in the presence of ethanol and had a maximum growth temperature of 37 °C (Table 2). It presented ellipsoidal to fusiform conidia originating from denticles, consistent with *Cladophialophora devriesii*. However, molecular data identified the strain as *C. saturnica* (Table 1). FP4IIA, phenetically identified as *Cladophialophora* sp. and physiologically similar to FP4IIB was identified as *C. saturnica* by ITS sequencing (Table 1). FE1IIA and F11PLA had fusiform conidia in chains. FE1IIA was unable to assimilate galacticol, developed poorly in the presence of D-glucoronate, but was able to grow in a medium with ethanol; F11PLA assimilated glucoronate having a weak development in the presence of ethanol (Table 2). With ITS sequencing two undescribed *Cladophialophora* species appeared to be concerned (Table 1).

Strain FE5P4 was isolated from decaying cambara wood (Fig. 2B) in an area of native species (Fig. 2A) dominated by cambara trees (*Gochnathia polymorpha*) and stem palm (*Syagrus romanzoffiana*) near Colombo city (Fig. 1). This isolate was morphologically identified as *Fonsecaea pedrosoi*. Physiologically it differed from *F. pedrosoi* by assimilation of L-sorbose, melibiose,

Table 1. Isolates from clinical and environmental sources in southern Brazil.

Morphological ID	Final ITS ID	CBS	dH	Vicente / Attili	Origin	Source
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*		18223	Fp28III	Marmeleiro	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*		18331	Queiros	São Paulo	Subcutaneous, compromised
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*	102244	11608	Fp37	Ipora	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea pedrosoi*	102245	11610	Fp36I	Ampere	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea monophora*	102248	11613	Fp82	Piraquara	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea monophora*	102246	11611	Fp65	Campo Largo	Chromoblastomycosis
Fonsecaea pedrosoi	*Fonsecaea monophora*	102243	11607	Fp31I	Ibituva	Chromoblastomycosis
Cladophialophora bantiana[1]	*Cladophialophora immunda*	102227	11588	FE9 = 9EMB	Colombo	Stem palm (*Syagrum roman zoffianum*)
Cladophialophora bantiana	*Cladophialophora* sp. 3	102231	11592	FE1IIA	Colombo	Rotten *Gochnatia polymorpha* stem
Cladophialophora devriesii[1]	*Cladophialophora saturnica*[4]	102230	11591	FP4IIB	Piraquara	Plant litter
Cladophialophora sp.	*Cladophialophora saturnica*	102228	11589	FP4IIA	Piraquara	Rotten wood
Cladophialophora sp.	*Fonsecaea monophora*[5]	102229	11590	FP8D = 8DPIRA	Piraquara	Plant litter
Cladophialophora sp.	*Cladophialophora* sp. 2	102236	11600	F11PLA	Telêmaco Borba	Plant litter
Cladophialophora sp.	*Cladophialophora immunda*	102237	11601	F10PLA	Telêmaco Borba	Plant litter
Exophiala lecanii-corni[2]	*Exophiala xenobiotica*[4]	102232	11594	FE4IIB	Colombo	Rotten wood
Exophiala jeanselmei[2]	*Exophiala bergeri*[4]	102241	11605	F14PL	Cianorte	Soil under coffee tree
Exophiala sp.	*Capronia semi-immersa*	102233	11595	FE6IIB	Colombo	Rotten *Araucaria* trunk
Fonsecaea pedrosoi[3]	*Fonsecaea* sp. 3	102223	11583	FCL2	Castro	Rotten root
Fonsecaea pedrosoi	*Fonsecaea* sp. 1	102224	11584	F9PRA	Terra Roxa	*Grevillea robusta* wood
Fonsecaea pedrosoi	*Fonsecaea monophora*[5]	102225	11585	FE5P4	Colombo	Rotten wood (*Gochnatia polymorpha*)
Fonsecaea pedrosoi	*Fonsecaea* sp. 1	102254	11619	FE5P6	Colombo	Rotten wood
Fonsecaea pedrosoi	*Fonsecaea* sp. 2	102226	11587	FE5II	Colombo	Rotten *Araucaria* trunk
Phialophora verrucosa[3]	*Phialophora* sp. 1	102234	11596	FE3	Colombo	*Lantana camara* rhizosphere
Rhinocladiella sp.	*Rhinocladiella* sp. 1	102235	11597	F9PR	Terra Roxa	*Grevillea robusta* wood
Rhinocladiella sp.	*Cladophialophora immunda*	102249	11614	F10PLB	Sarandi	Rotten *Cinnamomum* trunk
Rhinocladiella sp.	*Cladophialophora chaetospira*	102250	11615	F3PLB	Sertanópolis	Plant litter
Rhinocladiella sp.	*Exophiala xenobiotica*[4]	102251	11616	F3PLC	Sertanópolis	Plant litter
Rhinocladiella sp.	*Fonsecaea* sp. 3	102239	11603	FE1IIB	Colombo	Rotten *Lantana camara* stem
Rhinocladiella sp.	*Fonsecaea* sp. 3	102252	11617	FE10IIB	Colombo	Plant litter
Rhinocladiella sp.	*Rhinocladiella* sp. 1		11618	FE10IIB1	Colombo	Plant litter
Rhinocladiella sp.	*Rhinocladiella* sp. 1	102240	11604	F9PRC	Terra Roxa	*Podocarpos lambertii* branch
Rhinocladiella sp.	*Exophiala xenobiotica*[4]	102255	11621	F20PR3	Jacutinga	Soil
Rhinocladiella sp.	*Fonsecaea monophora*[5]	102238	11602	F1PLE	Rio Tibagi	Soil
Exophiala sp.	*Exophiala bergeri*[4]	122844	18627	D0009	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122843	18629	D0020	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122842	18636	D0035	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122841	18643	D0201	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala bergeri*[4]	122840	18654	D0213	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122839	18635	D0029b	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122838	18646	D0204a	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122837	18651	D0210	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122836	18648	D0206	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122835	18652	D0211	Rio Clarov	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122834	18653	D0212	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis*[6]	122833	18656	D0215	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala dermatitidis* (mel⁻ mut)	122830	18650	D0209	Rio Claro	Railway tie treated with creosote 16 yr ago
Exophiala sp.	*Exophiala xenobiotica*[4]	122832	18647	D0205	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala xenobiotica*[4]	122910	18638	D0044	Rio Claro	*Eucalyptus* wood
Exophiala sp.	*Exophiala xenobiotica*[4]	122831	18655	D0214	Rio Claro	*Eucalyptus* wood

Table 1. (Continued).

Morphological ID	Final ITS ID	CBS	dH	Vicente / Attili	Origin	Source
Exophiala sp.	*Exophiala xenobiotica*[4]	122829	18631	D0023	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala xenobiotica*[4]	122846	18632	D0024	Rio Claro	Railway tie treated with creosote 15 yr ago
Exophiala sp.	*Exophiala xenobiotica*[4]	122828	18637	D0037	Rio Claro	Railway tie treated with creosote 15 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122826	18639	D0045	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122824	18640	D0047	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122822	18641	D0060	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122825	18642	D0063	Rio Claro	Railway tie treated with creosote 20 yr ago
Black fungus sp.	*Veronaea botryosa*[4]	122823	18628	D0017	Rio Claro	Railway tie treated with creosote 20 yr ago
Aureobasidium sp.	*Aureobasidium pullulans*[4]	122827	18657	D0216	Paulinia	Polluted soil, Replan Co.

Abbreviations used: CBS = Centraalbureau voor Schimmelcultures; dH = G.S. de Hoog working collection.

Known agent of: [1]systemic and disseminated disease; [2]mycetoma; [3]chromoblastomycosis; [4]mild cutaneous disease; [6]systemic disease and pulmonary colonization

[5]Known opportunistic agent (including chromoblastomycosis).

ribitol, xylitol, myo-inositol, glucono-δ-lactone, D- and L-lactate, succinate, nitrite, urease and tolerance to 5% NaCl (Table 2). This physiological profile was similar to that of clinical strains FP65 and FP82 (Table 2) originating from symptomatic patients of the same geographic region (first plateau, Fig. 1). With ITS sequencing FE5P4 was identified as *Fonsecaea monophora.* Environmental isolate FP8D morphologically was cladophialophora-like but was identified as *F. monophora* based on molecular data. It had physiological similarity with clinical strain FP82 of *F. monophora* (Table 2) and was isolated from the same location where the patient, a carrier of chromoblastomycosis, had acquired his infection (Piraquara city, Fig. 1). All strains grew at 37 °C but not at 40 ºC, similar to known *Fonsecaea* species (de Hoog *et al*. 2004). Isolate F1PLE was recovered from soil, located on the second plateau (Fig. 1). It showed similar morphology to *Rhinocladiella* but through molecular data it was identified as *F. monophora* (Table 1). Strains FE5P6, FE5II and FCL2 strains appeared to represent undescribed species of the genus *Fonsecaea* (Table 1).

In the same region isolate (FE3) was recovered which was morphologically identified as *Phialophora verrucosa* on the basis of pronounced funnel-shaped collarettes from which the conidia were released. The isolate did not assimilate glucose, ribose and inulin but was capable of L-lysine assimilation (Table 2), a result that is consistent with the physiological characteristics of the *P. verrucosa* reference strain (de Hoog *et al.* 1999). With molecular ID a hitherto undescribed *Phialophora* species was found (Table 1).

Strain FE6IIB, morphologically identified as *Exophiala* species (Table 1) physiologically differed from reference strains of *Exophiala* (de Hoog *et al*. 2000a) by positive responses to lactose, L-arabinose, myo-inositol, D-gluconate and DL-lactate, by being able to assimilate D-ribose, and also presenting weak assimilation of glucoronate. By molecular identification was identified as the anamorph of Capronia semi-immersa. Isolate F14PL was preponderantly yeast-like and was provisionally identified as *Exophiala jeanselmei*, but ITS sequence data suggested *E. bergeri* (Table 1). Isolate FE4IIB showed morphological similarity to *E. lecanii-corni*, but differed from reference strains (de Hoog *et al*. 2000a) by positive assimilation of lactose, L-arabinose, myo-inositol, D-glucuronate and DL-lactate and by being able to assimilate D-ribose and D-glucoronate. Molecular data suggested identity with *E. xenobiotica*.

Isolates F20PR3 and F3PLC, morphologically having the appearance of *Rhinocladiella* species were identified as *Exophiala xenobiotica* by ITS data. These strains were unable to ferment glucose, to assimilate methanol, to grow at 40 ºC and were citrate negative. None of the strains analysed produced extracellular DNAse. Rhinocladiella-like strains F9PR and F9PRC were physiologically similar, differing only in assimilation of glycerol and L-lysine (Table 2). Using molecular data, they were identified as an undescribed *Rhinocladiella* species (Table 1). Strains FE10IIB and FE10IIB1 were initially thought to be Rhinocladiella- or fonsecaea-like species. FE10IIB1 did not assimilate inulin and was physiologically similar to *Rhinocladiella atrovirens* (CBS 264.49 and CBS 380.59). No close molecular match was found for either of these strains (Table 1).

DISCUSSION

Chaetothyrialean black yeasts and relatives are interesting microorganisms from ecological as well as clinical points of view. The recurrent and consistent infections cause by many representatives of the order indicates a possible adaptation of the fungi to the human host. In the environment they occupy specific micro-habitats, probably due to their low competitive ability towards co-occurring microorganisms. Their oligotrophism (Satow *et al*. 2008) enables them to thrive and maintain at low density in adverse substrates where common saprobes are absent (de Hoog 1993, 1997). An eventual potential as an environmental pathogen may involve a composite life cycle of the fungi concerned. However, the invasive potential is polyphyletic and differs significantly between species (Badali *et al.* 2008). Recurrent, consistently identifiable diseases are caused by relatively few species, which may be morphologically very similar to environmental counterparts which in many cases seem to be undescribed (Table 1). Therefore a reliable taxonomic system is mandatory to obtain better understanding of the link between clinical disease and environmental ecology.

The state of Paraná in southern Brazil is an endemic region for chromoblastomycosis. *Fonsecaea pedrosoi* is supposed to be responsible for more than 95% of the clinical cases, mainly infecting agricultural laborers (Queiroz-Telles 1997). This species is now known to comprise two cryptic entities, causing the same disease but seemingly differing in virulence (de Hoog *et al*. 2004). Out of five clinical strains tested from Paraná, two appeared to be

Table 2. Physiological test results of Brazilian isolates.

	FE3 *Phialophora* sp. 1	FE9 *C. immunda*	F10PLA *C. immunda*	FE1IIA *Clad.* sp. 3	FP4IIB *C. saturnica*	FP4IIA *C. saturnica*	F10PLB *C. immunda*	F11PLA *Clad.* sp. 2	F14PL *E. bergeri*	FE4IIB *E. xenobiotica*	FE6IIB *Cap. semiimmersa*	F20PR3 *E. xenobiotica*	F3PLC *E. xenobiotica*	F9PR *Rhin.* sp. 1	F9PRC *Rhin.* sp. 1	FE10IIB1 *Rhin.* sp. 1	Fp82 *F. monophora*	Fp65 *F. monophora*	FE5P4 *F. monophora*	FP8D *F. monophora*	F1PLE *F. monophora*	F9PRA *Fonsecaea* sp. 1	FE10IIB *Fonsecaea* sp. 3	FE1IIB *Fonsecaea* sp. 3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	+	w	+	w	+	+	+	+	+	w	+	-	w	+	w	w	w	+	+	w	w/+	w	w	w
D-Glucosamine	w/+	w	w	+	w	w	w	w	w/+	+	w	+	+	w	w/-	-	w	+	w	w	w	w/-	+	+
D-Ribose	-	+	+	+	+	+	+	+	+	+	w	+	+	w	-	w/-	w/-	w/-	w/-	w/-	-	-	+	+
D-Xylose	+	+	+	+	+	+	+	w	+	+	+	+	+	+	w/+	+	w/+	+	+	w/+	w/+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+	+	+	+	w/+	+	+	+
D-Arabinose	+	w	+	+	w	w	+	w	+	w/+	w	+	+	w	-	-	-	+	w	-	w/-	-	+	+
L-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+	w/+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	w/+	+	+	w/+	w/+	+	+	+
α,α-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl-α-D-Glucoside	w	+	+	-	+	+	+	+	-	-	+	w	-	+	+	+	w/-	+	w	w/-	w	+	-	-
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	w/+	+	w	w/+	w/+	+	+
Salicin	+	+	+	+	+	+	+	+	w/-	+	+	+	+	+	+	+	+	+	+	+	+	w	+	+
Arbutin	+	+	+	+	+	+	+	+	w/+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+
Melibiose	-	+	+	+	w	w	+	+	-	+	+	+	+	+	w/+	+	+	+	+	+	w/+	w/+	+	+
Lactose	-	+	+	-	+	+	+	+	w/-	-	-	-	w	+	+	+	w/-	+	w	w/-	w/-	w/+	-	-
Raffinose	+	+	+	+	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Melezitose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	w	-	-	-	-	-	w/-	w	w	w	-	-	-	-	-	-	-	-	-	w	w
Sol. starch	w	w	w	w/+	-	-	w	-	-	w/-	-	w/+	w/+	-	w/-	w/-	w/-	w/-	-	w/-	w/-	w/-	w/+	w/+
Glycerol	+	+	+	+	+	+	+	w	+	+	w	+	+	w	+	+	w	+	w	w	w	+	+	+
meso-Erythritol	+	+	+	+	+	+	+	+	w/-	+	+	+	+	+	+	+	w/-	+	w	w/-	w	+	+	+
Ribitol	+	w	+	+	+	+	+	+	+	+	+	+	+	w	w	w/+	w/-	+	w	w/-	w	w/-	+	+
Xylitol	-	+	+	+	+	+	+	+	+	+	w	+	+	+	+	+	-	+	w	-	w/-	+	+	+
L-Arabinitol	w	+	+	w/+	w	w	+	+	+	+	w	w/+	w/+	w	w	w/+	w/+	+	+	w/+	+	w/+	w/+	w/+
D-Glucitol	+	+	+	+	w	w	+	+	+	+	+	+	+	+	w	w/+	w/+	+	+	w/+	w/+	w/+	+	+
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/+	+	+	+
Galactitol	w	+	+	-	w	w	+	w	-	-	w	-	-	w	+	+	w/-	+	w	w/-	w	+	-	-
myo-Inositol	+	+	+	+	+	+	+	+	+	+	w	+	+	-	-	+	-	w/+	+	-	w	-	+	+
Glucono-d-Lactone	+	+	+	+	+	+	+	w	w/+	w/+	w	+	+	+	w	w	w	+	+	w	w	w	+	+
D-Gluconate	+	w	w	w	+	+	w	w	-	w	+	w	-	w	w	w	w	+	+	w	w	w/-	w	w
D-Glucuronate	+	w	w	w	+	+	w	+	w	w	w	w	w	w	w	w	w	+	+	w	w/-	w/-	w	w
D-Galacturonate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w/-	w/-	w	w/-	w	w/+	+	+
DL-Lactate	+	+	+	+	w	w	+	w	+	+	w	+	+	w	+	+	w	-	w	w	w/+	w	+	+
Succinate	+	w	+	+	+	+	+	+	w	w/+	w	+	+	+	w	w	w	w/+	w	w	w	w	+	+
Citrate	+	w	w	-	w/-	w/-	w	+	-	-	-	-	-	w	w/-	w/-	w	w	w	w	w	w/-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	w/+	w/+	+	+	w	w	+	W	+	w/+	w	+	+	w	+	w/+	w/+	+	w	w/+	w/+	+	+	+
Nitrate	+	+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. (Continued).

	FE3 P*hialophora* sp. 1	FE9 *C. immunda*	F10PLA *C. immunda*	FE1IIA *Clad.* sp. 3	FP4IIB *C. saturnica*	FP4IIA *C. saturnica*	F10PLB *C. immunda*	F11PLA *Clad.* sp. 2	F14PL *E. bergeri*	FE4IIB *E. xenobiotica*	FE6IIB *Cap. semiimmersa*	F20PR3 *E. xenobiotica*	F3PLC *E. xenobiotica*	F9PR *Rhin.* sp. 1	F9PRC *Rhin.* sp. 1	FE10IIB1 *Rhin.* sp. 1	Fp82 *F. monophora*	Fp65 *F. monophora*	FE5P4 *F. monophora*	FP8D *F. monophora*	F1PLE *F. monophora*	F9PRA *Fonsecaea* sp. 1	FE10IIB *Fonsecaea* sp. 3	FE1IIB *Fonsecaea* sp. 3
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Nitrite	+	w/-	+	+	w	w	+	w	+	+	+	+	+	w	+	w	+	+	w	+	+	w/+	+	+
Ethylamine	+	+	w/+	w/+	w	w	w/+	+	w/+	+	w	+	w/+	w	w/+	w/+	w	w	w	w/+	w	w/+	w/+	w/+
L-Lysine	+	w	w/+	w	-	-	w/+	w	w/-	w/+	-	-	w	-	w/+	w/+	+	+	w	+	w/+	w/+	w	w
Cadaverine	+	+	+	+	+	+	+	w	+	+	+	-	+	+	+	+	+	+	+	w/+	+	+	+	+
Creatine	+	+	w	+	-	-	w/+	w	-	+	w	+	+	w	+	w	w/+	w/+	w	+	w/+	w/+	+	+
Creatinine	+	+	w	+	-	-	w/+	w/-	-	+	w	+	+	w	+	-	w/+	w/+	w	w/+	w	w	+	+
2.5 % MgCl2	+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.0 % MgCl2	+	+	w/+	+	w/+	w/+	w/+	w/+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	+
10 % MgCl2	w	+	w/+	+	w/+	w/+	w/+	w/+	+	+	+	+	+	w/+	w/+	+	w/+	+	+	w/+	+	w/+	+	+
2.5 % NaCl	+	+	+	+	+	+	+	w/+	+	+	+	+	+	+	+	+	w/+	+	+	w/+	+	+	+	+
5.0 % NaCl	w	+	w	+	w/+	w/+	W	w/+	+	+	+	+	+	+	w/+	+	-	+	+	-	+	+	+	+
10 % NaCl	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.1 % Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.05 % Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.01 % Cycloheximide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	-	-	+	-	-	-	-	-	-	-	+	+	w	w/+	+	+	-	+	+	+	-	+	+
30 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37 ºC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40 ºC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acid production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mycosel	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatine	-	-	-	-	w	w	-	w	-	-	w	-	-	w	-	-	-	-	w	-	w	-	-	-
DNAse	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutin	?	?	w/+	w/-	w/+	w/+	w/+	w/+	-	w/+	-	w/-	-	-	-	-	-	-	-	-	-	-	w/-	w/-

Abbreviations used: + = growth; w = weak growth; - = no growth; ? = ambiguous or unknown.

C. = *Cladophialophora; Cap* = *Capronia; E* = *Exophiala; Phial* = *Phialophora; Rhin* = *Rhinocladiella*. 1*Phialophora* sp.1; 2-8 *Cladophialophora* species; 9-13 *Exophiala* species; 14 -16 *Rhinocladiella.* sp; 17-18 *Clinical strain*: (m*) Fonsecaea monophora* and (p) *F. pedrosoi*; 19−21 (m) *Fonsecaea monophora*; 22-24 (sp) *Fonsecaea* sp. (molecular identifications partly according to Najafzadeh *et al.* 2008)

F. monophora (Table 1). Our extensive environmental sampling in 56 locations in the state of Paraná showed that *Fonsecaea pedrosoi* was not isolated from nature, but instead we repeatedly encountered *F. monophora*. The natural source and route of infection of *F. pedrosoi* therefore still remains a mystery.

Several chaetothyrialean opportunists were isolated which are known to be associated with mild disorders, such as the cutaneous species *Cladophialophora saturnica* (Badali *et al.* 2008) and *Exophiala xenobiotica* (de Hoog *et al.* 2006). None of the systemic pathogens, such as *Cladophialophora bantiana*, were found. Several species listed in Table 1 concern hitherto undescribed, apparently saprobic representatives of the order *Chaetothyriales* that have never been reported as agents of human or animal disorders. The discrepancy of molecular identification and morphological and physiological results that were validated by analysis of ex-type strains of chaetothyrialean fungi (de Hoog *et al.* 1995) indicated that a vast number of saprobic species still awaits discovery and description.

The hydrocarbon-polluted environments yielded another spectrum of chaetothyrialean fungi. *Exophiala dermatitidis* is a fairly common opportunist, occasionally causing fatal, systemic disease. *Exophiala bergeri, E. xenobiotica, E. angulospora* and *Veronaea botryosa* are exceptional and/or low-virulent opportunists. *Exophiala bergeri* has thus far rarely been reported as an agent of disease, but was abundantly isolated when monoaromatic hydrocarbons were used for enrichment. The presence of aromatic compounds in

the sample increases colony density and diversity of black yeasts. The ecological and physiological patterns of species concerned suggests an evolutionary connection between the ability to develop on alkylbenzenes and the ability to cause diseases in humans and animals (Prenafeta-Boldú *et al.* 2006).

The present study was an attempt to verify whether infections caused by *Fonsecaea pedrosoi* and other agents of human mycosis are likely to be initiated by traumatic inoculation of environmental strains, and, more in general, to find the source of infection of invasive black yeasts-like fungi. Our results showed that this link is complex: environmental strains cannot always be linked directly to clinical cases. This is illustrated above by the genus *Fonsecaea*, known from two clinically relevant species. Mostly *F. monophora* or unknown *Fonsecaea* species were isolated. The apparently more virulent species *F. pedrosoi* is likely to require special, hitherto unknown parameters for isolation, such as the use of an animal bait (Dixon *et al*. 1980, Gezuele *et al*. 1972). Thus far it only has been encountered on the human patient, always causing chromoblastomycosis when the host is immunocompetent. In contrast, *F. monophora* can be isolated from the environment without an animal bait, and is a less specific opportunist (Surash *et al.* 2006). In general, pathogenicity and virulence of chaetothyrialean black yeasts may differ between closely related species. The group can be divided in three ecological groups, as follows. (1) Saprobes not known from vertebrate disorders, such as the majority of undescribed strains reported in Table 1; (2) Low-virulent opportunists that can directly be isolated from the environment, such as *F. monophora*, and (3) Highly specific pathogens that cannot be isolated from the environment directly but require a living mammal bait, resp. a human host. This suggests that isolation efficiencies differing between species reflect different pathogenic tendencies in pathogenic adaptation of the species.

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The neurotropic black yeast *Exophiala dermatitidis* has a possible origin in the tropical rain forest

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Abstract: The black yeast *Exophiala dermatitidis* is known as a rare etiologic agent of neurotropic infections in humans, occurring particularly in East and Southeast Asia. In search of its natural habitat, a large sampling was undertaken in temperate as well as in tropical climates. Sampling sites were selected on the basis of the origins of previously isolated strains, and on the basis of physiological properties of the species, which also determined a selective isolation protocol. The species was absent from outdoor environments in the temperate climate, but present at low abundance in comparable habitats in the tropics. Positive outdoor sites particularly included faeces of frugivorous birds and bats, in urban as well as in natural areas. Tropical fruits were found *E. dermatitidis* positive at low incidence. Of the human-made environments sampled, railway ties contaminated by human faeces and oily debris in the tropics were massively positive, while the known abundance of the fungus in steam baths was confirmed. On the basis of the species' oligotrophy, thermotolerance, acidotolerance, moderate osmotolerance, melanization and capsular yeast cells a natural life cycle in association with frugivorous animals in foci in the tropical rain forest, involving passage of living cells through the intestinal tract was hypothesized. The human-dominated environment may have become contaminated by ingestion of wild berries carrying fungal propagules

Key words: Black yeasts, *Exophiala dermatitidis*, frugivorous animals, human faeces, intestinal colonization, neurotropism.

INTRODUCTION

The black yeast *Exophiala dermatitidis* is an uncommon etiologic agent of fatal infections of the central nervous system in otherwise healthy, mainly adolescent patients in East Asia (Hiruma *et al*. 1993, Matsumoto *et al*. 1993, Chang *et al*. 2000). The species shows neurotropism in animal experiments (Dixon *et al*. 1989, 1992). In the U.S.A., cases have been reported where inoculation of patients with medical fluids containing contaminated water led to nosocomial common-source outbreaks with sometimes fatal neurological implications (Woollons *et al*. 1996, Engemann *et al*. 2002). More frequently than brain infection, asymptomatic colonization is observed in protected body sites, e.g. in the mucus of lungs in 2-8 % of patients with cystic fibrosis (CF) (Haase *et al.* 1991, Horré *et al.* 2003), in the intestinal tract in 0.3 % of the European population (de Hoog *et al.* 2005) and occasionally in the wax of human external ear canals (Kerkmann *et al.* 1999, G. Haase unpublished data). The fungus is a constitutive producer of melanin (Langfelder *et al.* 2003), is consistently able to grow at temperatures above 37 °C (Padhye *et al*. 1978) and produces extracellular polysaccharide capsules (Yurlova *et al.* 2002), which all are regarded to be virulence factors.

The route of infection is still a mystery. The species is known to occur in the environment, but is not among the commonly encountered saprobes. It is practically absent from dead plant material or soil, and has never been reported from outdoor air (Matos *et al*. 2002). The somewhat odd spectrum of main sources of isolation of strains presently available in culture collections (fruit surfaces, steam baths, faeces, and human tissue) suggests that a hitherto unknown, quite specific natural niche must be concerned.

Particularly the occurrence in steam rooms of public bathing facilities is consistent and with high colony counts (Nishimura *et al.* 1987, Matos *et al.* 2002). The artificial environment of the steam bath apparently provides a novel environmental opportunity for this fungus. The transition from the hitherto unknown natural niche to the human-dominated environment may be accompanied by selection and/or adaptation to the new habitat, facilitated by the stress protection provided by melanin. Given the nature of the fungus as an opportunistic agent of potentially fatal infections in humans, this process may have clinically relevant consequences. The present article documents a possible natural habitat of the fungus and on processes taking place during transition from nature to the domestic environment.

MATERIAL AND METHODS

Samples

Varying numbers of replicates each containing 0.1−0.5 g of fresh and dry faeces of wild or semi-wild birds, chickens, bats, flying foxes, jackdaws, rats and elephants were collected from different localities in the Netherlands and/or in Thailand. (Fig. 1) Forty-four bathing facilities, toilets, hot springs and railway ties (Tables 4−9) were sampled, either by using sterile cotton swabs, or by collecting 0.5−1.0 g (wet weight) bottom soil or aliquots of 10 L were filtered over a 0.8 µm pore size, sterile membrane cellulose acetate filter (Göttingen, Germany). In addition, 731 intestinal samples (~1 g)

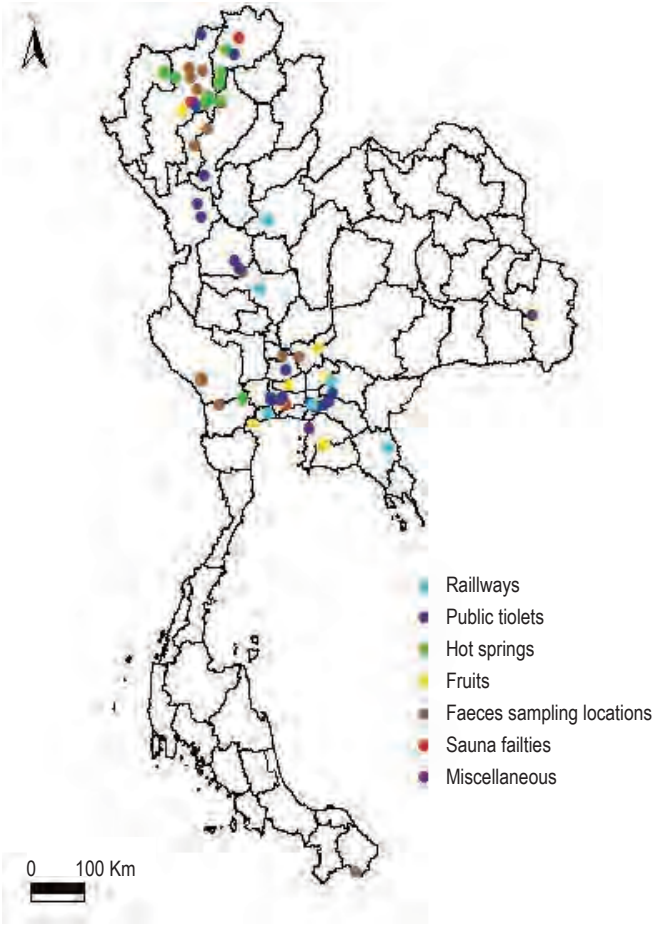

Fig 1. Map of Thailand showing the sampling sites.

from small zoo animals from the Netherlands and autopsied at the Veterinary Faculty of the University of Utrecht, The Netherlands were included. Also large numbers of wild fruits and berries from the Netherlands and Thailand were analyzed (Table 3). Solid specimens were incubated in 5.5 mL Raulin's solution (Booth 1971) in test tubes for 2-3 d at 25 ° C in nearly horizontal position and shaken at 10 r.p.m. Subsequently 0.5 mL was transferred using a glass Drigalski spatula to Erythritol Chloramphenicol Agar (ECA) (de Hoog & Haase 1993) and Sabouraud's Glucose Agar (SGA) (de Hoog *et al.* 2000) – both media containing the same concentration of chloramphenicol – and incubated for up to 40 d at 40 °C. Small, blackish-brown colonies were transferred to new growth media on PDA using a loop, or were separated from contaminating yeasts and aspergilli by repeatedly washing agar blocks with black yeast cells in 0.1 % Tween 80 in sterile water followed by dispersing the visually clean blocks over fresh agar plates.

Validation of isolation procedure

Three strains were used to test the efficiency of recovery by the above method: CBS 207.35 (genotype A, capsular), CBS 116014 (genotype B, capsular) and CBS 109143 (genotype B, non-capsular). Strains were pre-cultured in Potato Dextrose Broth (PDB) for 3 d at 30 °C. Suspensions were adjusted to 10^2 cells/mL and recovery verified by plating on ECA and SGA at 25 °C and 40 °C. *Aureobasidium pullulans,* CBS 584.75 was used as positive control at 25 °C. Suspensions in Raulin's solution with a final concentration of 10^6 cells/mL were incubated for 3 d under conditions specified above and the recovery rate was counted on ECA, SGA and Potato Dextrose Agar (PDA) at 25 °C and 40 °C.

Identification of accompanying biota

White yeasts were identified physiologically by testing fermentation and carbon and nitrogen assimilation. C-assimilative capabilities were tested using API-ID 32 C strips (bioMérieux, Marcy-l'Étoile, France). Single colonies were grown at 25 °C for 3 d as a maximum. Suspensions were made and the densities were scored by McFarland turbidity standard point 2. Strips were inoculated according to specifications provided by the manufacturer and incubated at 25 °C. For nitrogen assimilation the substrates nitrate, ethylamine, L-lysine, cadaverine, D-glucosamine, HCl and tryptophane were used. Peptone was used as positive control. Suspensions were applied in N-auxanograms in culture plates. After the medium was cooled and solidified, a small amount of each N-source was put on the medium. For fermentation, D-glucose, D-galactose, maltose, sucrose, lactose and raffinose were tested. Sugars solutions were 2 %, except for raffinose which 4 % solution was used. These solutions were sterilized in tubes with Durham inserts and suspensions of McFarland turbidity standard point 2 were added. The identification software BIOLOMICS was used to score combined physiological results. Morphology was investigated to confirm the identifications (Kurtzman & Fell 1998).

Diagnostics

Strains were recognised as black yeasts were provisionally identified at the species level by colony appearance, morphology, and temperature tolerance. Species-identification and ITS-genotype attribution (Matos *et al.* 2002) was done on the basis of ITS rDNA, either by restriction length polymorphism (Sudhadham *et al*. 2009a) or by sequencing ITS1 and 2 (Sudhadham *et al*. 2009b) using standard methodology (de Hoog & Gerrits van den Ende 1998). The ITS-genotypes were recognized on the polymorphic sites listed in Table 2. A small number of isolates was genotype-attributed by similarity of patterns of amplified fragment length polymorphism (AFLP) to strains of which to ITS-genotype was known.

RESULTS

Recovery efficiency

The recovery rate of *Exophiala dermatitidis* suspensions (10^6 cells/mL) was not significantly altered by three days of incubation in Raulin's solution when strain CBS 116014 (genotype B) was used. In contrast, cell counts increased with a factor 4.3 in CBS 207.35 (genotype A), while those of the non-capsular strain CBS 109143 (genotype B) was reduced to about 9.5 % (Table 1). *Aureobasidium pullulans* showed an 83.3 % reduction in colony-forming units (CFU) at 25 °C and did not grow at 40 °C. Average recovery rate in 0.1−0.5 g (wet weight) of faeces was between 0−3 colonies per culture plate, which corresponded to 0−3 CFU per gram original material for genotype B and 0−0.69 for genotype A, judging from isolation efficiency described above.

The isolation protocol applied to animal faeces and to fruits and berries collected in temperate and tropical climates proved to be highly selective, judging from the very few ubiquitous saprobes that were recovered. The rarity of *E. dermatitidis* was proven by selective isolation from a large diversity of environments in temperate (The Netherlands) and tropical (Thailand) climates, supplementing data

of Matos *et al*. (2002) which involved leaves, fruits and berries, animal faeces and soil in a temperate climate.

Genotyping

Nuclear rDNA ITS sequencing of 86 reference strains confirmed the existence of two major genotypes differing in three positions in ITS1 (Table 2). Genotype A was more common than B (A:B = 57:29). Two out of nine animal faeces samples from Thailand belonged to genotype A, while seven were B. Two sets of samples from bird guano in Khao Khaew Zoo (where occasional mechanic cleaning is carried out) and flying fox faeces at a temple complex in Thailand contained genotypes A and B, of which the latter was isolated more frequently.

Isolation in temperate climate

Fruits and berries

Two areas in The Netherlands were chosen for isolation from berries, namely Boswachterij Noordwijk, in a dune area near the Northsea coast near Leiden, and a lane planted with shrubs in a rural area in Maartensdijk in the central part of the country. In Noordwijk, samples were taken in Autumn, when berries were predomantly eaten by migratory frugivorous birds such as *Turdus pilaris* (fieldfare). Twenty-one samples were taken from berries of *Rosa pimpinellifolia*, 345 samples from *Hippophae rhamnoides* and 187 samples from *Ligustrum vulgare* (Table 3). The shrubs near Maartensdijk were predominantly frequented by sedentary birds such as *Corvus monedula* (jackdaw) and *Sturnus vulgaris* (European starling). Thirty samples were taken from *Crataegus monogyna*, 92 samples from *Viburnum opulus*, 61 samples from *Ilex aquifolium*, 22 samples from *Rosa canina*, 20 samples *Rosa rubiginosa*, 36 samples *Prunus spinosa*, 19 samples *Ligustrum vulgare* and 51 samples from *Taxus baccata***.** With our isolation protocol plates mostly remained blank, or white yeasts were encountered. No black yeast was isolated.

Faeces of omnivorous birds

Faeces mixed with soil under *Thuja* conifers harbouring a large combined roosting site of *Corvus monedula* (jackdaw) and *Sturnus vulgaris* (European starling) in a park near Hilversum, The Netherlands, was sampled, as well as a roosting site of jackdaw alone. All samples were negative for *E. dermatitidis* (Table 4).

Intestinal contents from sectioned zoo animals

A total of 731 samplings from dead animals originating from different zoos in the Netherlands, were received at the Veterinary Faculty at Utrecht for autopsy. In addition to routine analysis, the contents of the intestines with visible disorders such as discoloration or halfway digested food was subjected to selective isolation for *E. dermatitidis*. Results are available from 16 reptiles, 406 birds, 183 mammals, 8 fishes, 3 turtles, 2 amphibians, 5 lizards and 11 snakes; these data will not be included in this article, but are available as attachment at www.cbs.knaw.nl. Culture plates mostly remained blank. White yeasts were common in the intestinal tract of frugivorous animals. A single strain of *E. dermatitidis* (genotype A) was obtained from a bonobo monkey (*Pan paniscus*) with diarrhoea in the Apeldoorn Zoo, The Netherlands. Bonobo's are omnivorous with a marked preference of fruit.

Isolation in tropical climate

Fruits and berries

On grapes (*Vitis vinifera*) numerous white yeasts were isolated, which were not identified down to the species level. The only black fungus obtained was a *Cladosporium* species. On green papaya fruits (*Carica papaya*) mainly white yeasts occurred. Few filamentous fungi were obtained, mainly biverticillate *Penicillium* species, among which was *Eupenicillium cinnamopurpureum* (anamorph: *P. phaeniceum*). Three types of fruit were found positive on isolation for *E. dermatitidis*: papaya, pineapple (*Ananas comosus*) and mango (*Mangifera indica*). In papaya and pineapple, genotypes A and B were found, while in mango only genotype B was encountered. Mango fruits further contained white yeasts, a *Rhizopus* species, a recurrent *Aspergillus* species, a biverticillate *Penicillium* and *Talaromyces intermedius* (dH 13728). On lemon fruits (*Citrus* sp.) white yeasts were common, and a single colony of a white filamentous fungus was obtained. On fruits of tamarind (*Tamarindus indica*), Queen's flower (*Lagerstroemia calyculata*) and *Ficus lacor*, only *Aspergillus* species were obtained. In contrast, fruits of *Ficus annulata* contained much more white yeasts in addition to *Aspergillus* species; three times a hitherto undescribed species of *Munkovalsaria* species was isolated. On fruits of yellow santol (*Sandoronicum indicum*) the main filamentous fungi acquired were *Aspergillus* species, and infrequently white yeasts were encountered. Fruits of rambutan (*Nephelium lappaceum*), pomelo (*Citrus maxima***)** and mangrove palm (*Nypa fruticans*) contained many white yeasts only. Rose apple fruits (*Syzygium jambos*) contained biverticillate *Penicillium* species (Table 3).

Faeces of frugivorous birds

Samples were taken from faeces of a number of frugivorous birds from two zoos in Thailand. Bird species are described as follows. Columbiformes: *Ducula bicolor* (pied imperial-pigeon); Coraciiformes: *Aceros undulatus* (wreathed hornbill)*, Anorrhinus galeritus* (bushy-crested hornbill)*, Anorrhinus tickelli* (rusty-cheeked hornbill)*, Anthracoceros albirostris* (oriental pied-hornbill)*, Anthracoceros malayanus* (black hornbill)*, Berenicornis comatus* (white-crowned hornbill), *Buceros bicornis* (great hornbill)*, Buceros hydrocorax* (rufous hornbill)*, Buceros rhinoceros* (rhinoceros hornbill), *Rhinoplax vigil* (helmeted hornbill); Passeriformes: *Acridotheres tristis* (common myna)*, Sturnus burmannicus* (vinous-breasted starling)*, Pycnonotus finlaysoni* (stripe-throated bulbul); and Piciformes*: Megalaima virens* (great barbet). White yeasts were predominant on isolation media. Some of these were selected to be identified by ID32C and they invariably turned out to be *Candida tropicalis,* while a single strain was identified as *Trichosporon loubieri. Exophiala dermatitidis* (genotype B) was isolated from fresh faeces of *Acridotheres tristis* (common myna). In a feeding area for birds by fruits of papaya (*Carica papaya*), banana (kluai namwa; *Musa* 'ABB'), two isolates of *Exophiala dermatitids*, genotype B were found (dH 13132, dH 13134) and genotype A (dH 13135) (Fig. 2 D,E). Frugivorous bird faeces were also analyzed in the national park HalaBala Wildlife Sanctuary, established in 1996. Samples were taken in the Hala portion of the Sanctuary, approximately 22 km west from the Malaysian border, comprising a mix of forest broadly classified as tropical lowland evergreen forest, and small-scale agricultural land. The area is particularly famous for harbouring nine species of hornbill birds being one of richest areas for these birds in Southeast Asia (Fig H). *Exophiala dermatitidis* (dH 13148 genotype B) was found from the faeces of *Buceros rhinoceros* (rhinoceros hornbill) (Table 4).

Table 1. Efficiency of live recovery of *E. dermatitidis* after Raulin's incubation, verified on ECA, PDA and SGA at 25 °C and 40 °C.

	Pre-Raulin:	**Post-Raulin:**
CBS 207.35 (genotype A, capsular)	10^6	0.43 x 10^7
CBS 109143 (genotype B, non-capsular)	10^6	0.95 x 10^5
dH 13133 (genotype B, capsular)	10^6	0.83 x 10^6

Table 2. Genotypes within *E. dermatitidis* based on polymorphisms in rDNA Internal Transcribed Spacer 1.

	ITS1 (210)			**ITS2 (220)**
	162	**184**	**196**	**-**
Genotype A:	T	-	A	-
Genotype B:	C	T	C	-
Genotype C: deletion 74-99	T	-	A	-

Table 3. Overview of fruit and berry sampling locations in Thailand and The Netherlands*.

No.	**Plant**		**Province/ country**	**No. of samples**	***Exophiala dermatitidis***	
	Common name	**Scientific name**			**Genotype A**	**Genotype B**
1	Grape	*Vitis vinifera*	Nakornratchasima; Thailand	41	-	-
2	Yellow papaya	*Carica papaya*	Bangkok; Thailand	25	-	-
	Green papaya	*Carica papaya*		17	-	-
	Papaya with antrachnose disease	*Carica papaya*		30	-	-
	Lemon	*Citrus aurantifolia*		40	-	-
3	Bo-tree fruit	*Ficus religiosa*	Chachoengsao; Thailand	54	-	-
	Tamarind	*Tamarindus indica*		5	-	-
	Queen's flower	*Lagerstroemia calyculata*		1	-	-
	Liap	*Ficus lacor*		6	-	-
4	Papaya	*Carica papaya*	Rayong; Thailand	6	-	-
	Rose apple	*Syzygium jambos*		15	-	-
	Yellow sentol	*Sandoricum indicum*		9	-	-
	Breadfruit	*Artocarpus altilis*		12	-	-
	Jackfruit	*Artocarpus heterophyllus*		15	-	-
	Pineapple	*Ananas comosus*		20	3	1
6	Mango	*Mangifera indica*	Bangkok; Thailand	6	-	5
7	Lychee	*Litchi chinensis*		2	-	-
8	-	*Ficus annulata*		59	-	-
9	Mangrove palm	*Nypa fruticans*	Samutsakorn; Thailand	40	-	-
10	Autumn sampling from berries eaten by mainly migratory birds, e.g.	*Rosa pimpinellifolia*	Leiden; The Netherlands	21	-	-
	Turdus pilaris (fieldfare)	*Hippophae rhamnoides*		345	-	-
		Ligustrum vulgare		187	-	-
11	Autumn sampling from berries eaten by mainly resident birds , e.g.	*Crataegus monogyna*	Leiden; The Netherlands	30	-	-
		Viburnum opulus		92	-	-
	Corvus monedula (jackdaw),	*Ilex aquifolium*		61	-	-
	Sturnus vulgaris (European starling)	*Rosa canina*		22	-	-
		Rosa rubiginosa		20	-	-
		Prunus spinosa		36	-	-
		Ligustrum vulgare		19	-	-
		Taxas baccata		51	-	-

**Exact localities available upon request.*

Table 4. Overview of animal faeces sampling in Thailand*.

No.	Animal's faeces	Location	Province/ country	No. of samples	*Exophiala dermatitidis*	
					Genotype A	Genotype B
1	Rats	University	Bangkok; Thailand	4	-	-
2	Elephants	Domesticated elephant	Lum Pang; Thailand	27	-	-
3	Corn-eating animals	Chicken farm	Lum Poon; Thailand	58	-	-
4	Frugivorous birds	Dusit zoo	Bangkok; Thailand	20	-	-
5	Granivorous birds	Dusit zoo	Bangkok; Thailand	15	-	-
6	Frugivorous birds	Khao Khaew open zoo	Chonburi; Thailand	49	1	1
7	Granivorous birds	Khao Khaew open zoo	Chonburi; Thailand	105	-	-
8	Five different species of hornbill	Hala-Bala wildlife sanctuary	Narathiwat; Thailand	96	-	2
9	*Pteropus scapulatus* (flying fox): fresh, dry and dry faeces mixed with soil	Temple	Ayutthaya; Thailand	112	-	-
10	*Pteropus scapulatus* (flying fox): faeces mixed with soil	Temple	Ayutthaya; Thailand	62	-	-
11	*Pteropus scapulatus* (flying fox)	Temple	Saraburi; Thailand	23	-	-
12	*Pteropus scapulatus* (flying fox): fresh, dry and dry faeces mixed with soil	Temple	Chachoengsao; Thailand	88	1	2
13	Insectivorous bat: mix of old and fresh faeces	Temple	Chiang Mai; Thailand	14	-	-
14	Insectivorous bat: fresh and old faeces and soil	Cave near temple	Chiang Mai; Thailand	28	-	-
15	Insectivorous bat: mix of old and fresh faeces	Temple	Chiang Mai; Thailand	44	-	-
16	Insectivorous bat: old faeces mixed with soil	Temple	Chiang Mai; Thailand	5	-	-
17	Insectivorous bat: mix of old and fresh faeces	Temple	Chiang Mai; Thailand	10	-	-
18	Insectivorous bat: mix of old and fresh faeces	Bat-inhabited newly-built house	Lumpoon; Thailand	10	-	-
19	Insectivorous bat: fresh faeces	Temple	Nakornsawan; Thailand	20	-	-
20	Insectivorous bat: mix of old and fresh faeces	Temple	Kanchanaburi; Thailand	36	-	-
21	Insectivorous bat: mix of old and fresh faeces	Temple	Ratchaburi; Thailand	10	-	-
22	Omnivorous birds	Park	Hilversum, The Netherlands	60	-	-
23	Frugivorous bats	Blijdorp zoo artificial cave	Rotterdam, The Netherlands	110	-	-

*Exact localities available upon request.

Faeces of frugivorous bats

On leaves and branches of boh tree (*Ficus religiosa*) contaminated with faeces of Lyle's flying foxes (*Pteropus lylei*) we repeatedly encountered *Exophiala dermatitidis.* Sequencing showed that three of these were genotype A and B. Soil samples at from a temple in Chachoengsao were mixed with faeces of flying foxes provided white yeasts, among which were *Candida tropicalis* and *C. guillermondii.* Two colonies of an *Aspergillus* species were found and two isolates of *Penicillium* which were sequenced and proved to be *P. islandicum* and *P. pupurogenum* (Tables 4, 9) (Fig. 1 A–C).

Faeces of granivorous birds

Faeces samples were collected from two zoos in Thailand and involved the bird species Galliformes: *Pavo cristatus* (Indian peafowl); Psittaciformes: *Cacatua ducorpsii* (Ducorps' cockatoo), *Cacatua goffini* (Goffin's cockatoo), *Cacatua moluccensis* (Moluccan cockatoo), *Cacatua sulphurea* (yellow-crested cockatoo), *Cacatua tenuirostris* (long-billed corella), *Calyptorhynchus magnificus* (red-tailed black cockatoo), *Electus auratus* (eclectus parrot), *Probosciger aterrimus* (palm cockatoo), *Psittacula alexandri* (red-breasted parakeet), *Psittacula eupatria* (Alexandrine parakeet), *Psittacus erithacus* (African grey parrot), and *Psittrichas fulgidus* (Pesquet's parrot). Very few white yeasts were encountered, but filamentous fungi were relatively common. No *Exophiala* was isolated (Table 4).

Faeces of insectivorous bats

Thirteen mostly limestone caves were chosen for sampling of insectivorous bat faeces from different geographical regions in Thailand. Most of them were located in montane rain forest, about 1 000–1 900 meters above sea level. Others were touristic places surrounded by agricultural land, or were part of a temple complex. Numerous filamentous fungi were obtained (Table 4), most of these being rapidly growing *Aspergillus* species, biverticillate penicillia and zygomycetes. No black yeasts were detected (Table 4) (Fig. 2 F, G).

Public toilets

Public toilets of gas stations can be found along many highways in Thailand. They differ only slightly with the company with respect to building structure, hygienic level and intensity of warding. Most of them are pedestal squat toilets, which means that there is no

Table 5. Overview of public toilet sampling locations in Thailand*.

No.	Public toilets	Source	Province / country	No. of samples	*Exophiala dermatitidis*	
					Genotype A	Genotype B
1	Toilet in gas station	Wall , squat toilet, tap zink	Ayutthaya; Thailand	6	-	-
2	Toilet in gas station	Wall , squat toilet, tap zink	Nakhonsawan; Thailand	6	-	-
3	Toilet in gas station	Wall , squat toilet, tap zink	Nakhonsawan; Thailand	6	-	-
4	Toilet in gas station	Wall , squat toilet, tap zink	Tak; Thailand	6	-	-
5	Toilet in gas station	Wall , squat toilet, tap zink	Tak; Thailand	6	-	-
6	Toilet in gas station	Wall , squat toilet, tap zink	Tak; Thailand	6	-	-
7	Toilet in gas station	Wall , squat toilet, tap zink	Lumpoon; Thailand	6	-	-
8	toilet in hotspring	Wall , squat toilet, tap zink	Chiangmei; Thailand	6	-	-
9	toilet in hotspring	Floor in bath room, wall in toilet room	Chiangrai; Thailand	6	-	-
10	Toilet in gas station	Floor , squat toilet, tap zink	Bangkok; Thailand	6	-	-
11	Toilet in gas station	Floor , squat toilet, tap zink	Chachoengsao; Thailand	6	-	-
12	Toilet in gas station	Floor , squat toilet, tap zink	Chachoengsao; Thailand	6	-	-
13	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
14	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
15	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
16	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
17	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
18	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
19	Toilet in gas station	Squat toilet, tap zink	Nakhon Pathom; Thailand	5	-	-
20	Toilet in gas station	Squat toilet, tap zink	Chachoengsao; Thailand	5	-	-
21	Toilet in gas station	Squat toilet, tap zink	Chachoengsao; Thailand	6	-	-
22	Toilet in gas station	Squat toilet, tap zink	Prachinburi; Thailand	6	-	-
23	Toilet in gas station	Squat toilet, tap zink	Prachinburi; Thailand	6	-	-
24	Toilet in gas station	Squat toilet, tap zink	Chachoengsao; Thailand	6	-	-

*Exact localities available upon request.

Table 6. Overview of sauna facility sampling locations in Thailand*.

No.	Sauna facilitites	Source	Province / country	Number of samples	*Exophiala dermatitidis*	
					Genotype A	Genotype B
1	Steam bath	Floor, wall, seat	Bangkok; Thailand	12	-	10
2	Steam bath	Floor, wall, seat	Chiangrai; Thailand	20	8	53
3	Dry sauna	Floor, wall, seat	Bangkok; Thailand	12	-	-
4	Dry sauna	Floor, wall, seat	Bangkok; Thailand	12	-	-
5	Dry sauna	Floor, wall, seat	Bangkok; Thailand	12	-	-
6	Dry sauna	Floor, wall, seat	Bangkok; Thailand	55	-	-
7	Dry sauna	Floor, wall, seat	Bangkok; Thailand	30	-	-
8	Dry sauna	Floor, wall, seat	Lampang; Thailand	12	-	-
9	Dry sauna	Floor, wall, seat	Narathiwat; Thailand	13	-	-
10	Dry sauna	Floor, wall, seat	Chiangmai; Thailand	3	-	-
11	Dry sauna	Floor, wall, seat	Chiangmai; Thailand	12	-	-

*Exact localities available upon request.

flushing system installed to clean the toilet after use. Instead, water is collected from a tap into a bucket placed close to the squat toilet. Due to this situation, most of the time floors in the toilets were wet, and the hygienic level was low. Samples were taken at different points, such as the floor in the toilet room, the wall, as well as swabs taken from the bowl above the water level. The occurrence of fungi which grew after incubation of swabs in Raulin's solution and incubation on ECA at 40 °C was similar in all toilets: we found *Aspergillus* species and some white yeasts, but no *Exophiala* (Table 5) (Fig. 2 J, K).

Sauna facilities

The saunas in Thailand that were chosen for this experiment were dry sauna with continuous heating. The room was located inside the building complex with other steam baths and Gyms for sport entertainment. Inside the sauna room, seats and walls were made of wood. The steam rooms were also included in this experiment; these were mostly located on the same floor as the sauna room. The walls and floors were made of tiles while the seats were made from polyvinyl chloride. Black yeasts appeared abundantly in the

Table 7. Overview of railway sampling locations in Thailand*.

No.	Railw tiess	Source	Province/ country	Number of samples	*Exophiala dermatitidis*	
					Genotype A	Genotype B
1	Railway station	Stone on railway	Samut Sakhon; Thailand	30	-	-
2	Railway station	Stone on railway	Chachoengsao; Thailand	10	-	-
3	Railway station	Wood stained with petroleum oil	Prachinburi; Thailand	11	108	5
4	Railway station	Stone stained with petroleum oil	Srakaew; Thailand	10	31	-
5	Railway station	Stone stained with petroleum oil	Nakornsawan; Thailand	39	176	29
6	Railway station	Stone stained with petroleum oil	Pitsanulok; Thailand	40	59	-

*Exact localities available upon request.

Table 8. Overview of hot spring water sampling locations in Thailand*.

No.	Water sampling	Source	Province / country	Number of samples	*Exophiala dermatitidis*	
					Genotype A	Genotype B
1	Water from hot srping	Natural hot spring	Chiang Mai; Thailand	20 liters	-	1
2	Water from hot srping	Natural hot spring	Chiang Mai; Thailand	20 liters	-	-
3	Water from hot srping	Natural hot spring	Chiang Mai; Thailand	10 liters	-	-
4	Water from hot srping	Natural hot spring	Chiang Mai; Thailand	30 liters	-	-
5	Water from hot srping	Natural hot spring	Chiangrai; Thailand	10 liters	-	-
6	Water from hot srping	Natural hot spring	Chiangrai; Thailand	10 liters	-	-
7	Water from hot srping	Natural hot spring	Chiangrai; Thailand	10 liters	-	-
8	Water from hot srping	Natural hot spring	Chiangrai; Thailand	30 liters	-	-
9	Water from hot srping	Natural hot spring	Mae Hong son; Thailand	10 liters	-	-
10	Water from hot srping	Natural hot spring	Mae Hong son; Thailand	10 liters	-	-
11	Water from hot srping	Natural hot spring	Lampang; Thailand	10 liters	-	-
12	Water factory	Raw material pond	Ubonratchathani; Thailand	25 liters	-	-
13	Water from pond in the zoo	Dusit zoo	Bangkok; Thailand	1 liter	-	-
14	Water from pond in the zoo	Khao Khaew open zoo	Chonburi; Thailand	3 liters	-	-

*Exact localities available upon request.

Table 9. Overview of hot spring soil sampling locations in Thailand*.

No.	Soil	Source	Province / country	Amount	*Exophiala dermatitidis*	
					Genotype A	Genotype B
1	Soil from hot spring	Natural hot spring	Chiang Mai; Thailand	40 g	-	-
2	Soil from hot spring	Natural hot spring	Chiang Mai; Thailand	40 g	-	-
3	Soil from hot spring	Natural hot spring	Chiang Mai; Thailand	40 g	-	-
4	Soil from hot spring	Natural hot spring	Chiang Mai; Thailand	40 g	-	-
5	Soil from hot spring	Natural hot spring	Chiang Rai; Thailand	40 g	-	-
6	Soil from hot spring	Natural hot spring	Chiang Rai; Thailand	40 g	-	-
7	Soil from hot spring	Natural hot spring	Chiang Rai; Thailand	40 g	-	-
8	Soil from hot spring	Natural hot spring	Chiang Rai; Thailand	40 g	-	-
9	Soil from hot spring	Natural hot spring	Mae Hong son; Thailand	40 g	-	-
10	Soil from hot spring	Natural hot spring	Mae Hong son; Thailand	40 g	-	-
11	Soil from hot spring	Natural hot spring	Lampang; Thailand	40 g	-	-
12	Soil from spring in zoo	Dusit zoo	Bangkok; Thailand	40 g	-	-
13	Soil from spring in zoo	Khao Khaew open zoo	Chonburi; Thailand	130 g	1	2

*Exact localities available upon request.

Fig 2. A. Temple complex at Chachaosao province, Thailand, with colony of flying foxes positive for *E. dermatitidis*; B. Roosting flying foxes; C. Faeces of flying foxes; D. Cage in the zoo of Chonburi province, Thailand, with indoor feeding area (E) positive for two genotypes of E. dermatitidis (dH 13132, dH 13133, dH 13134); F. Mountain at Nakornsawan province, Thailand, with numerous caves harboring insectivorous bats and surrounded by agricultural land; G. Collecting bat faeces in cave at Chiengmai province, Thailand, with bat faeces (insert); H: Hala-Bala Wildlife Sanctuary, Narathiwat province, Thailand, with hornbill nesting sites positive for dH 13183; I. Public hot sping in Chiangmai province, Thailand, positive for dH 13145; J. Public toilet in Chiangmai province, Thailand; K. Squat toilet in public toilet; L. Railway ties in Prachinburi province, Thailand, with collection sites (a) outside ties contaminated with petroleum oil, and (b) between ties (arrows).

isolation step. Only *Exophiala dermatitidis* could be found in these samples, no other fungi were encountered. Sequencing results showed that *E. dermatitidis* could be assigned to both genotypes A and B (Table 6).

Railway ties

Six creosote-treated oak railways ties in Thailand were chosen for this experiment. One of these was located at a fresh market, where heavy contamination and regular cleaning lead to nutritional enrichment, and yielded negative results. The remaining ties showed a line of blackish debris, probably a mixture of faeces and machine oil. These samples contained an enormous amount of *Exophiala dermatitidis* presenting both genotypes from all locations. Srakhaew railway ties yielded 31 isolates of *Exophiala dermatitidis* genotype A, Prachinburi railway ties had 108 genotype A and 5 genotype B, Nakornsawan railways had 176 genotype A, 29 genotype B and 2 genotype C, Pitsanulok railway yielded 61 genotypes A and 1 genotype C (Table 7) (Fig. 2 L).

Hot springs

In ten litre-samples of water from eleven hot springs, a few biverticillate *Penicillium* species were found: dH 13743 = *P. mineoluteum,* dH 13790 = *P. pinophilum* and dH 13744 = *P. funiculosum.* No white yeasts were detected, but one sample yielded a colony of *Exophiala dermatitidis*, genotype A. In the soil samples from the same hot springs, after incubation in Raulin's solution, analyzed with a dilution series and plated on ECA, few filamentous fungi were observed, but there was no evidence of black yeasts (Table 8; Fig. 2 I).

DISCUSSION

From our data, subjecting about 3 000 samples over a 3 yr period of collecting with a selective protocol, it has become apparent that *Exophiala dermatitidis* is not a ubiquitous fungus. Being rare in the environment, the baseline of occurrence is low, with small numbers of strains in all environment samples except for steam baths and creosote-treated and petroleum oil-polluted railway ties. Given its probable low competitive ability as an oligotroph (Satow *et al.* 2008), it may even be infrequent in its natural habitat. This makes it methodically difficult to detect this habitat, which should be indicated by small relative increase in frequency. The detection of even a few samples, contrasted with environments that are consistently negative, may therefore be significant. Matos *et al*. (2003) noted that in preferred habitats the fungus is in an active metabolic state, and grows on isolation plates within three day. Outside its natural habitat it is in a survival stationary state and may require wks before visible growth occurs.

During the present research we selected environments which are likely to be positive. Samples taken on the basis of strains already available in the reference collection of CBS, and on the basis of physiology. The species had previously recurrently been isolated from human sputum, particularly from CF patients, from deep infections, particularly from brain, from stool, from bathing facilities, from fruits and berries, and from creosote-treated wood (http://www.cbs.knaw.nl). This remarkably discontinuous spectrum of sources of isolation of *E. dermatitidis* suggests that the organism might be adapted to a particular, hitherto undiscovered habitat, rather than being a saprobe on dead plant material, as is frequently suggested in the literature (Gold *et al*. 1994). Based on its physiology, we hypothesized a niche containing a number of key elements. First, it must be dynamic, with simultaneously or consecutively occurring phases differing in environmental conditions, since the organism itself is polymorphic, exhibiting yeast-like, filamentous and meristematic phases (de Hoog *et al*. 1994). Second, the phases are likely to be nutritionally diverse, as is concluded from the fungus' consistent occurrence as an epiphyte on low-nitrogenous substrates such as fruit surfaces – promoted by its moderate osmotolerance – and bath tiles (Mayr 1999) combined with equally consistent occurrence in faeces (de Hoog *et al.* 2005). Third, the latter environment, combined with a consistent tolerance of *E. dermatitidis* of 40 °C (Padhye *et al*. 1978) and of very low pH values (de Hoog *et al.* 1994) led to the supposition of passage of the digestive tract of warm-blooded animals (G.S. de Hoog unpublished data). Fourth, the organism shows strong adhesion to artificial surfaces (Mayr 1999), probably promoted by production of sticky extracellular polysaccharides (Yurlova *et al*. 2002). An occurrence on wild fruits and berries that are subsequently ingested by frugivorous animals and dispersed via their faeces thus seems to provide a possible connection of the divergent sources of isolation. The hypothesis led to the successful development of a selective protocol used in this study, which involved an acidic enrichment step (Booth 1971) followed by a high temperature step on a nutritionally specific medium (de Hoog & Haase 1993). The protocol enabled the isolation of minute quantities of the fungus. Massive isolation studies further underlined that *E.dermatitidis* is a rare species in most outdoor environments. We believe our recovery data broadly reflect the actual presence of *E. dermatitidis* in the environment, for two reasons. (1) Environments known to harbor the species were indeed found to be positive at rates comparable to those published earlier (Matos *et al.* 2002). (2) Recently Zhao *et al.* (2008) applied a new, *Chaetothyriales*-specific isolation method based on toluene-enrichment at ambient temperature to the same shrubs near Maartensdijk and indeed found numerous mesophilic *Exophiala* species, but never the thermophilic species *E. dermatitidis*.

The recovery rate was tested experimentally and on average found to reflect the number of cells present prior to incubation in acid. However, slight differences were noted among the three strains analyzed. The representative of ITS-genotype A (CBS 207.35) was stimulated with a factor 4.3 by incubation in Raulin's solution, whereas genotype B (CBS 1160124) remained practically unaltered. The non-capsular strain (CBS 109143) was inhibited tenfold, but since such strains are extremely rare in the natural environment (Matos *et al*. 2002, Yurlova *et al*. 2002) we believe that this more vulnerable phenotype has little effect on the recovery rate of the species.

We analyzed a large diversity of substrates using a highly selective protocol, with accent on substrates bearing similarity to origins of reference strains (Table 2). Despite that, isolation was mostly unsuccessful outdoors in the temperate climate of The Netherlands (Table 3). Only a single strain from a berry of *Sorbus aucuparia* had been found (CBS 109142, genotype B) by Matos *et al*. (2002). The negative samples included berries commonly eaten by migratory and sedentary birds such as *Turdus pilaris, Corvus monedula* and *Sturnus vulgaris,* as well as faeces from several of these birds. From these extended environmental studies including 944 samples it may be concluded that *E. dermatitidis* does not occur naturally in temperate climates.

In contrast, the fungus was confirmed to reside consistently and abundantly from known foci in several artificial, indoor environments, such as steam rooms (Matos *et al*. 2002), in Slovenia, Austria, Thailand as well as in The Netherlands. Several of the negative

berry-sampling locations were only a few kilometers away from steam baths that proved to be highly positive. The species was recovered at high frequency (about 1 000 CFU.cm^{-2}) from thirteen bathing facilities. Steam baths (and not the adjacent sauna's) of public bathing facilities represent an artificial environment with conditions thought to be similar to parts of the natural habitat of *E. dermatitidis*, where high (body) temperature and epiphytie adhesion to (fruit) surfaces play a significant role.

In tropical Thailand, most fruits and berries were also negative (Table 3), although the species was encountered a few times on mango and pineapple. Positive samples were more regularly derived at low frequencies from animal faeces (Table 4). The consistent presence of *E. dermatitidis* in bat faeces and bird guano analyzed is demonstrated by the sample from guano-littered soil in the Khao Khaew Zoo in Chonburi, Thailand, and from flying fox faeces at the temple complex in Chachongsao, Thailand, where both genotypes A and B were recovered (Table 4), despite the overall environmental scarcity of *E. dermatitidis* (Fig. 2 A−C). Recovery rates were low, with a maximum of three colonies per culture plate. The isolation method used reflects the real frequency of genotype B in the original samples (Table 2), which means that positive samples contain maximally 3 CFU per gram faeces. Positive samples were obtained from birds as well as mammals such as flying foxes.

Sampling of autopsied zoo animals was almost always negative for black yeasts. The great majority of these animals fed on corn and seeds, and also yielded very few white yeasts or filamentous fungi. The single black yeast-positive animal intestinal sample (www.cbs.knaw.nl) was a bonobo monkey, which is a largely frugivorous animal. A common factor linking this sample with positive samples elsewhere in the study is the diet of the animals: *E. dermatitidis* was almost exclusively found in animals that fed partially or entirely on wild fruits and berries. Herbivores have a large caecum, where digestion of food is enhanced by fermentation aided by a resident bacterial flora and white yeasts. Frugivorous animals, as those that feed on honey and nectar, may have problematic yeast overgrowth due to a high sugar content in the intestinal tract. *Exophiala dermatitidis* has a slight preference for osmotic environments. In clinical practice this was noted with its occurrence in the lungs of patients with cystic fibrosis, a disease characterized by an elevated salt content of tissues (de Hoog & Haase 1993).

De Hoog *et al*. (2005) found that *E. dermatitidis* occurs at a low incidence in the intestinal tract of humans. This matches with the abundant presence of *E. dermatitidis* on railway ties in Thailand, which are heavily contaminated by faeces (Fig. 2 L). Similar samples were taken in The Netherlands (data not shown), and these were positive for black yeasts other than *E. dermatitidis*. This situation is comparable with our isolation data from berries, which were negative in temperate but positive in tropical climates. Apparently the environmental temperature plays a significant role in the life cycle of *E. dermatitidis*. Public toilets in Thailand (Fig. 2 J, K) were, somewhat against expectations, also negative. This may be explained by competition of other, rapidly growing saprobes in this environment, such as white yeasts and *Aspergillus* species .

Exophiala dermatitidis, similar to other *Exophiala* species, is an oligotroph, as shown *in vitro* by Satow *et al*. (2008) on the basis of the ability of growth utilizing inoculum cells only. The property may be useful for growth on fruit surfaces, but is particularly expressed, in combination with thermotolerance, in abundant replication on the smooth surface of tiles and plastics of steam bath walls. The fungus was detected in large numbers in nearly all bathing facilities investigated located in temperate as well as in tropical climates. The species was also occasionally encountered in natural hot springs, which may be somewhat more difficult to colonize for the fungus due to their relative richness in nutrients. A single strain of *E. dermatitidis* was found in one of the hot springs but its presence might be explained by local people using the spring to clean and boil bamboo shoots after harvest from the forest. The sugary shoots may have been contaminated by *E. dermatitidis* and the fungus may have survived for a short period without significant colonization. A second sampling one year later without human activities was negative (Fig. 2 I).

Combining thermotolerance of the species with the knowledge that *E. dermatitidis* tolerates very acidic conditions (de Hoog *et al*. 1994), it is hypothesised that the fungus is able to pass through the intestinal tract of warm-blooded animals. About 80 % of positive hosts had diarrhoea at the moment of isolation of *E. dermatitidis,* a condition also encountered in positive bonobo. This suggests that the fungus may be present at a higher frequency but can only be isolated when the host has diarrhoea. De Hoog *et al.* (2005) reported isolation of the fungus at 3-wk-intervals from the faeces of a single hospitalized patient with diarrhoea, which indicates that maintenance in the intestines is possible. As a route of infection, translocation from the intestines may thus be supposed. For pulmonary cases an inhalative route would be more logical, but the apparent absence of *E. dermatitidis* from air remains in conflict with this explanation. Other similar phenomenon which support this hypothesis was the strain which was successful isolated from the bonobo from the zoo in the Netherlands. At that time , the sample was taken from the bonobo with the presence of diarrhoea (G. Dorrestein, personal information). Unfortunately, further sampling could not be continued due to the fact that the monkey had returned to the mother.

Reis & Mok (1979) and Muotoe-Okafor & Gugnani (1993) repeatedly found the species in internal organs of tropical frugivorous bats (*Phyllostomus discolor, Sturnira lilium* and *Eudolon helvum*) in American as well as African tropical rain forests. Representative isolates were verified to be *E. dermatitidis* genotype B (Table 4). Despite isolating the fungus from bat organs, Mok (1980) failed to isolate it from roosting sites. This may have been due to the use of inadequate isolation procedures. Reis & Mok (1979) also reported the species from two insectivorous bats (*Myotis albescens* and *M. molossus*), but the identity of these strains could not be verified.

Since in the reference set genotype A is about twice as common as genotype B and genotype A shows a higher recovery rate with the used isolation method (Table 2), our technique would expect to yield an A : B ratio of 10.4 : 1 would be expected in the fruit-eating animal faeces from Thailand. However, these samples showed a ratio A : B = 2 : 7, which would mean that the frequency of genotype B in tropical fruit-eating animal faeces samples deviates with a factor 36.4 from the average of all other sources of isolation.

This model study aims to prove the supposition that aspects of human behaviour i.e. the creation of environment that are extreme from a fungal perspective, by being hot, poor in nutrients, or poisonous, lead to the emergence of new, potentially virulent genotypes. The fungus under study causes a potentially fatal brain disease in otherwise healthy humans; this clinical picture is known in eastern Asia only (Horré & de Hoog 1999). Understanding the origin and course of this evolution may eventually lead to the development of measures which canalize speciation processes into a direction which is less harmful to humanity. The human community creates opportunities for adaptation and the emergence of pathogenic host races. Artificial, human-made environments may stimulate evolution and generate pathogenic genotypes which otherwise would not

have evolved. The source of contamination of a potentially harmful microorganism and its routes on infection and transmission will be studied, potentially leading to protocols for hygiene and prevention. This may be particularly significant in bathing facilities connected to hospitals, where susceptible populations of patients with cystic fibrosis or, in Asia, with immunosuppression are warded. Though the disease under study is extremely rare, our approach can be viewed as a model study for understanding emergence of new microbial pathogens in general and their translocation from the tropical rain forest to the human environment.

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Selective factors involved in oil flotation isolation of black yeasts from the environment

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Abstract: The oil flotation isolation technique has been successfully applied to recover chaetothyrialean black yeasts and relatives from the environment. The selective mechanisms playing a role in isolation are unknown. The fungi concerned are supposed to occupy specialized microniches in nature, taking advantage of (1) oligotrophism. Mineral oil as a main selective agent may be based on (2) hydrophobicity or on (3) assimilation. All three hypotheses are tested in this paper. Results show that cell wall hydrophobicity is unlikely to be a selective factor. Incubation under poor nutrient conditions provides competitive advantage for black yeasts, especially for *Exophiala* strains, which are subsequently enriched by mineral oil which enhances growth in this group of fungi. Incubation under mineral media and mineral oil can be used as selective factor.

Key words: Black yeasts, oil flotation, polluted soil, selective isolation.

INTRODUCTION

Black yeasts belonging to the *Chaetothyriales* are infrequently isolated from the environment. Recent studies have shown, however, that if selective methods are applied, these fungi may be encountered in a wide diversity of environments (Badali *et al.,* 2008). Special attention has been paid to environments rich in hydrocarbons, because there are indications that these compounds can be used as substrates by black yeast and filamentous relatives (Prenafeta-Boldú *et al*. 2006); the fungi may have a significant potential for bioremediation. Particularly difficult is isolation of black yeasts from natural sources, classical techniques usually revealing only a limited number of strains (Iwatsu *et al.* 1981, Marques *et al.* 2006, Vicente *et al.* 2001). This is supposed to be due to the abundance of rapidly growing saprobes in the same samples. This is one of the reasons that knowledge on the distribution of this group of fungi is still incomplete (Marques *et al*. 2006).

The oil flotation technique has been reported as an effective method for isolation of chaetothyrialean black yeasts (Dixon *et al.* 1980, Gezuele *et al*. 1972, Iwatsu *et al.* 1981, Richard-Yegres *et al.* 1987, Vicente *et al.* 2001, Marques *et al.* 2006). This technique applies mineral oil in the procedure. The black yeast and relatives are able to assimilate monoaromatic hydrocarbons and are promoted in environments rich in these compounds (Prenafeta-Boldú *et al.* 2002, Sterflinger & Prillinger 2001, Woertz *et al.* 2001), so mineral oil – a complex mixture of petrol hydrocarbons – could act as an enrichment factor favoring their isolation. Another hypothesis is that the cells of black yeast and relatives are hydrophobic and remained on the interphase solution-oil which inoculum is subsequently plated. The other hypothesis, not related to the mineral oil, is that black yeasts could continue grow in poor nutrient media, like the solution of this technique, due to their oligotrophic metabolism. So the aim of this article is to verify which selective factor is determinant for the isolation of black yeast and relatives in this technique. Such approach is of great relevance because it raises useful information for the improvement of black yeast isolation methods and consequently the better understanding of the ecology of this group.

MATERIAL AND METHODS

Sampling area

The soil samples were collected in a landfarming area of Paulínia Oil Refinery (REPLAN) in São Paulo state, Brazil (-22.726213 latitude, -47.135259 longitude). This site receives large amounts of waste petrol hydrocarbons. Samples were collected from different regions of the landfarming cell at 0–10 cm depth using sterile lab tools, they were placed in plastic bags and maintained at 4 ºC until use. In the laboratory the samples were homogenised and processed within a period of 2–15 d.

Fungal isolation

The oil flotation technique was based in previous studies (Iwatsu *et al.* 1981, Marques *et al.* 2006, Vicente *et al.* 2001). Twenty g soil sample was added to a sterile Erlenmeyer flask (250 mL) with 100 mL saline containing 200 U/mL penicillin; 200 µg/mL chloramphenicol; 200 µg/mL streptomycin and 500 µg/mL cycloheximide. The solution was homogenised and incubated for 30 min at 20–22 ºC. Subsequently 20 mL mineral oil was added, followed by vigorous vortexing for 5 min. Flasks were allowed to settle for 20 min. Aliquots from the oil/saline interphase were plated

Table 1. Composition of the media in different culture conditions of the assimilation test (volumes in µL).

	Culture Condition				
Component	**A**	**B**	**C**	**D**	**E**
Cell suspension (1.0 × 10^6 cells/mL)	40	40	40	40	40
Basal growth media	360	280	280	---	---
Mineral oil	---	80	---	360	---
n-Hexadecane	---	---	80	---	360
Total	400	400	400	400	400

on Mycosel agar and incubated at 28 °C until dark, slow-growing colonies appeared (about 4 wk). Colonies were purified by plating and transferred to slants with 2 % malt extract agar (MEA) incubated at 28 °C for 2 wk and then, they were maintained at 4 °C.

Morphology

Macroscopic morphology was observed by growing isolates on MEA at 28 ºC. Slide cultures were prepared with strains grown on MEA at 28 ºC and mounted in lactophenol cotton blue.

Molecular identification

About 1 cm^2 mycelium of 20 to 30-d-old cultures was transferred to a 2 mL Eppendorf tube containing 400 µL TEx buffer (pH 9.0) and about 80 mg of glass beads (Sigma G9143). Samples were homogenised for 1 min in MoBio vortex and subsequently 120 µL SDS 10 % and 10 µL Proteinase K (Merck 124568) were added, the mixture was vortex and incubated in water bath for 30 min at 55 °C. The samples were vortex again for 3 min on MoBio vortex. Then, 120 µL 5 M NaCl and 0.1 vol CTAB (hexadecyltrimethylammoniumbromide, Sigma H-5882) 10 % was added and the tubes were incubated in a water bath for 1 h at 55 ºC. Subsequently, samples were submitted to agitation in MoBio vortex for 3 min. One vol of SEVAG was added and it was carefully mixed by hand, inverting the flasks 50 times. Then, the solution was centrifuged at 20 400 g, 4 ºC for 5 min and the supernatant was transferred to a new tube. 225 µL 5 M NH_4-acetate was added and mixed carefully by inverting. After 30 min incubation on ice water samples were centrifuged for 5 min at 4 ºC at 20 400 g. Supernatant was transferred to a new Eppendorf tube and 0.55 vol isopropanol was added and mixed carefully. Samples were incubated at –20 ºC for 1 h and centrifuged for 5 min at 20 400 g. The supernatant was decanted and the pellet was washed with cold 70 % EtOH. After drying at room temperature it was resuspended in 100 µL TE-buffer and incubated for 5 min at 37 °C prior to storage at –20 °C. rDNA Internal Transcribed Spacer (ITS) was amplified using primers V9G and LS266 and sequenced with ITS1 and ITS4. Amplicons were cleaned with GFX PCR DNA and gel band purification kit (GE Healthcare, U.K.). Sequencing was performed on an ABI 3730XL automatic sequencer. Sequences were edited using the SEQMAN package (DNAStar Inc., Madison, United States of America) and aligned using BIONUMERICS v. 4.61 (Applied Maths, Kortrijk, Belgium).

Preparation of cell suspensions

Stock cultures were transferred to 5 MEA slants and incubated for 10–15 d at 28 °C. Five millilitres physiological salt solution was added to the grown culture and vortexed for 1 min. Aliquots were filtered and cell densities were measured using a Neubauer's counting chamber. Cell concentration was adjusted by adding physiological salt solution, mineral medium or inoculum.

Hydrophobicity

Six isolates and three reference strains from CBS collection were tested. The test was an adaptation of methodology of Göttlich *et al*. (1995). One millilitres of n-hexadecane or mineral oil was added to a 20 mL glass flask with rubber cap containing 5 mL of cell suspension and submitted to vigorous vortexing for 30 s. The flask was allowed to settle for 2 min for the complete separation of the phases. A small volume of the aqueous phase was removed with a 1 mL syringe and the cell density in the aqueous phase was established by visual counting using a Neubauer's counter chamber. The procedure was repeated at least five times for each culture condition and the results were expressed as the percentage of cells remained in the aqueous phase. Values above 50 % were considered to indicate hydrophily, whereas with values below 50 % the strain was considered to be hydrophobic.

Assimilation

Mineral oil and n-hexadecane assimilation tests were performed with a spectophotometer Bioscreen C (Labsystems, Helsinki, Finland). Yeast nitrogen base (Difco) was used as basal growth medium. Mineral oil and n-hexadecane were filter-sterilised. Five different culture conditions were established: Test A: only mineral medium (Yeast Base Nitrogen 0.65 % YNB); B: YNB + 20 % mineral oil; C: YNB +20 % n-hexadecane; D: YNB + 80 % mineral oil; E: YNB + 80 % n-hexadecane. The volumes of the reagents are summarised in Table 1. Each culture condition was done with five replications. Initial densities of cell suspensions were set to 1.0 x 10^6 cells/mL. Control tests without inocula were done to measure the blank (absorbance of the media) in order to compare the readings of the different culture conditions. The Bioscreen was set to maintain a temperature of 28 °C for 7 d with continuous shaking and absorbance reading at 540 nm every 2 h. Data were registered automatically. Growth curves were done with values resulted of the absorbance readings discounted of the blank, so the values shown in the graphics refer only to the growth of the strain in different culture conditions.

Oligotrophism

The same procedure of the assimilation test was used, but basal growth media was replaced by physiological salt solution.

Table 2. Molecular identification of melanised isolates from oil-polluted soil.

Accession no.	Identification based on ITS
dH 18460	*Cladophialophora minourae*
dH 18466 / CBS 122275	*Cladophialophora minourae*
dH 18463	*Cladophialophora immunda*
dH 18465 / CBS 122257	*Cladophialophora immunda*
dH 18468 / CBS 122253	*Cladophialophora immunda*
dH 18469	*Cladophialophora immunda*
dH 18471 / CBS 122255	*Cladophialophora immunda*
dH 18473 / CBS 122636	*Cladophialophora immunda*
dH 18474	*Cladophialophora immunda*
dH 18476	*Cladophialophora immunda*
dH 18477	*Cladophialophora immunda*
dH 18478	*Cladophialophora immunda*
dH 18462	*Cladosporium halotolerans*
dH 18458 / CBS 122258	*Exophiala xenobiotica*
dH 18459	*Exophiala xenobiotica*
dH 18461	*Exophiala xenobiotica*
dH 18464	*Exophiala xenobiotica*
dH 18467	*Exophiala xenobiotica*
dH 18470	*Exophiala xenobiotica*
dH 18472	*Exophiala xenobiotica*
dH 18475	*Exophiala xenobiotica*

RESULTS

Isolation

A total of 107 strains suspected to belong to chaetothyrialean black yeast and relatives were isolated from three landfarming soil samples. 20 of them were identified based on molecular techniques and one appeared to be a *Cladosporium* species (*Capnodiales*). The remaining strains belonged to at least three different species (Table 2). *Exophiala xenobiotica* was the preponderant species, while also *Cladophialophora minourae* was isolated. Eleven *Cladophialophora* strains did not match with any known species, neither in GenBank nor in a research database containing about 7,000 black yeast sequences maintained at CBS. Their nearest, undescribed neighbours in the latter database all originated from environments rich in hydrocarbons or had been isolated using alkylbenzene enrichment method.

Tested chaetothyrialean black yeast-like fungi (*Cladophialophora* and *Exophiala*) differentially responded to our hydrophobicity test (Table 3). The n-hexadecane test, applied previously to fungi by Göttlich *et al*. (1995) was validated using the same control strains, viz. hydrophilic strain *Rhodotorula graminis* (CBS 2826) and hydrophobic strain *Penicillium chrysogenum* (CBS 776.95). The strains responded as expected, viz. with very few *versus* many cells remaining in the oil phase. Isolated black yeast-like strains responded differentially to culture condition: *Cladophialophora*-type strains were hydrophobic, whereas *Exophiala*-type strains (strains of *Exophiala xenobiotica* CBS 122258 and CBS 118157) proved to be hydrophilic.

In the strains from genus *Cladophialophora*, high hydrophobicity values were observed and they were higher with n-hexadecane except strain CBS 122255.

Table 3. Identification, CBS numbers and cell density results of hydrophobicity tests.

			Cell density		
			Initial		
Species	**Accession no.**	**Culture Condition**	**(1.00×10^7)**	**Aqueous phase**	**Oil phase**
Penicillium chrysogenum	CBS 776.95	Mineral oil	100 %	2.40 %	97.60 %
		n-Hexadecane	100 %	2.81 %	94.75 %
Rhodotorula graminis	CBS 2826	Mineral oil	100 %	79.91 %	20.81 %
		n-Hexadecane	100 %	85.98 %	14.01 %
Cladophialophora minourae	CBS 122275 = dH 18466	Mineral oil	100 %	5.25 %	94.75 %
		n-Hexadecane	100 %	3.30 %	96.70 %
Cladophialophora immunda	CBS 122253 = dH 18468	Mineral oil	100 %	31.91 %	68.08 %
		n-Hexadecane	100 %	17.17 %	82.82 %
	CBS 122257 = dH 18465	Mineral oil	100 %	16.93 %	83.07 %
		n-Hexadecane	100 %	10.22 %	89.78 %
	CBS 122255 = dH 18471	Mineral oil	100 %	25.80 %	74.20 %
		n-Hexadecane	100 %	36.92 %	63.08 %
	CBS 122636 = dH 18473	Mineral oil	100 %	47.27 %	52.73 %
		n-Hexadecane	100 %	32.40 %	67.60 %
Exophiala xenobiotica	CBS 118157	Mineral oil	100 %	55.20 %	44.80 %
		n-Hexadecane	100 %	56.70 %	43.30 %
	CBS 122258 = dH 18458	Mineral oil	100 %	67.70 %	32.30 %
		n-Hexadecane	100 %	83.75 %	16.25 %

Assimilation of mineral oil and n-hexadecane

The readings of 80 % hydrocarbon concentration were not considered because at this percentage an interference on the absorbance occurred. Growth curves of the strains in culture conditions A, B and C (values with blank discounted) are shown (Figs 1–3).

Strains not belonging to the black yeast group: *Rhodotorula graminis* (CBS 2826) and *Penicillium chrysogenum* (CBS 776.95) showed minimum growth in culture conditions A, B and C (Figs 1–3). Similar growth was observed for the black yeast strain CBS 122275. Ability to use hydrocarbons as sole source of carbon and energy seemed to be absent for these strains.

Cladophialophora sp. CBS 122255 showed growth in all culture conditions, however its growth was higher in culture condition A, without hydrocarbon (Fig. 1). Better growth with hydrocarbon as sole source of carbon and energy was observed in strains

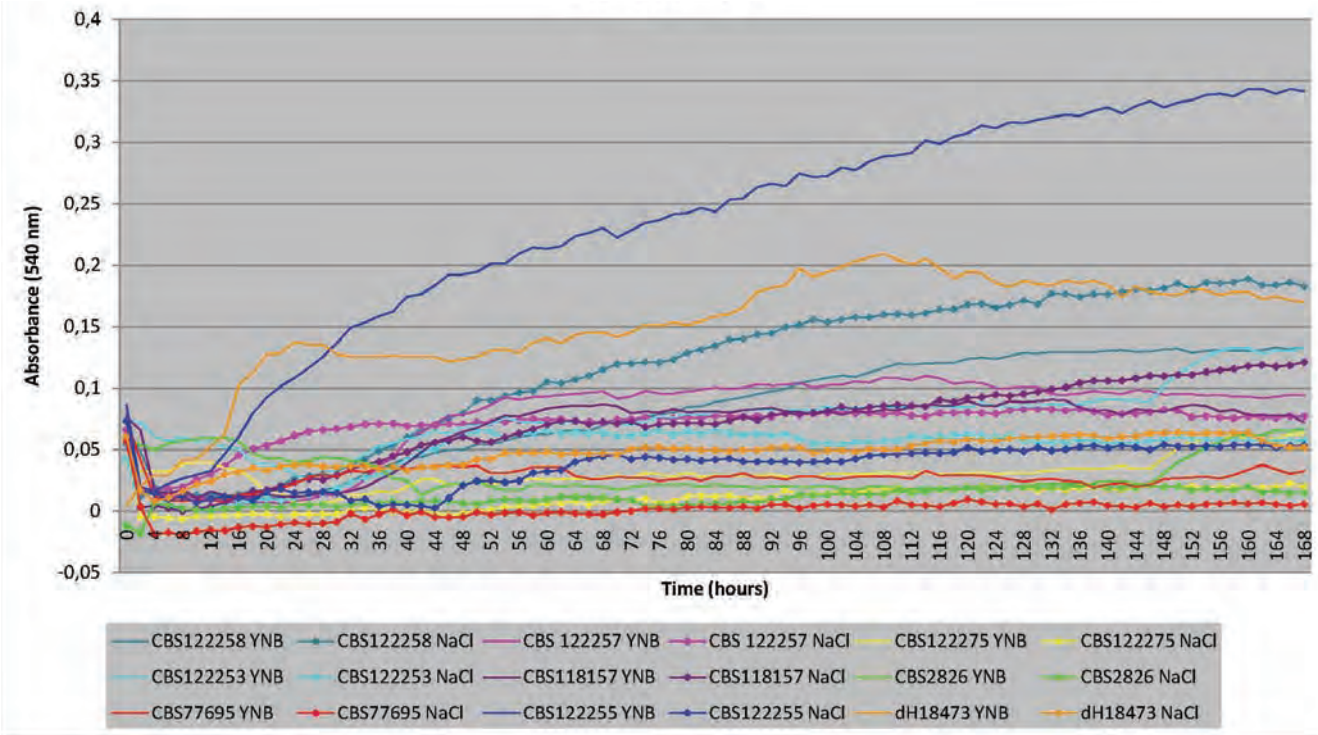

Fig. 1. Culture condition A: growth curves of studied strains in mineral medium, YNB (continuous line), or in physiological salt solution, NaCl (line and dots).

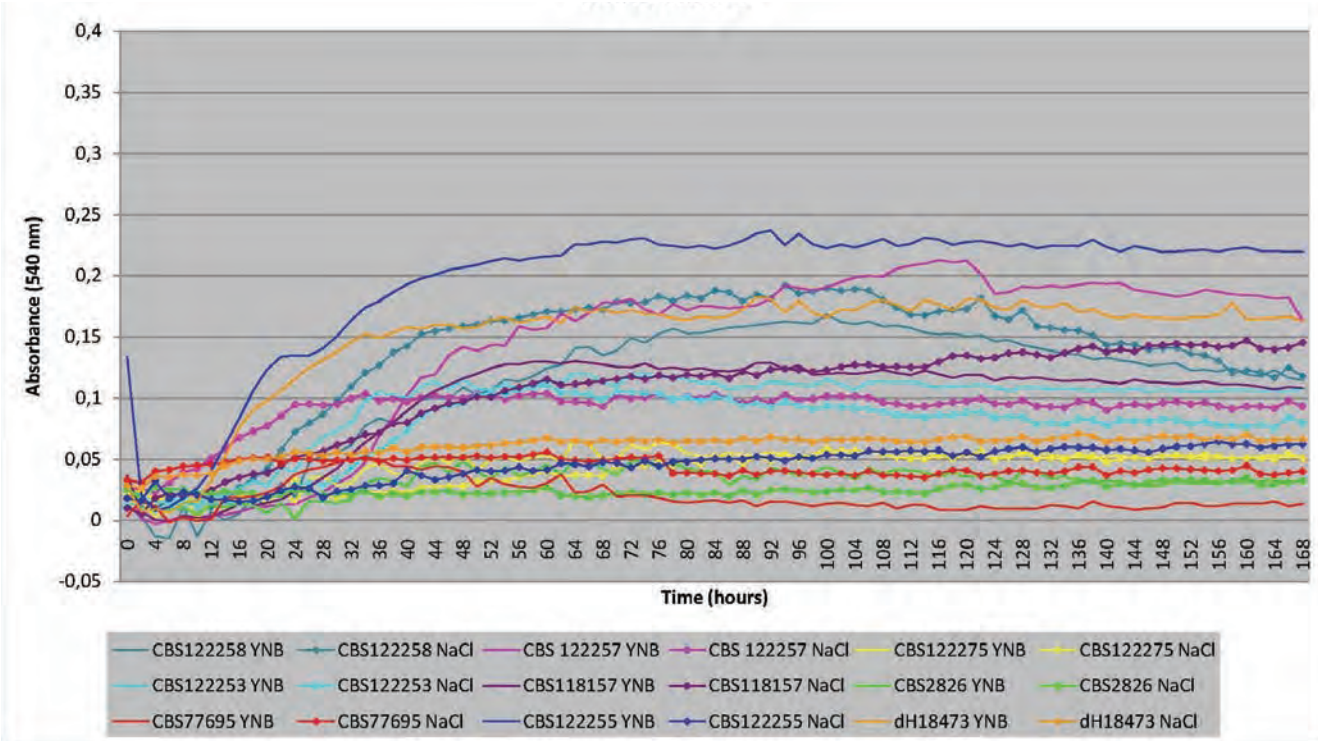

Fig. 2. Culture condition B: growth curves of studied strains in mineral medium (YNB) + 20 % mineral oil (continuous line), and in physiological salt solution (NaCl) + 20 % mineral oil (line and dots).

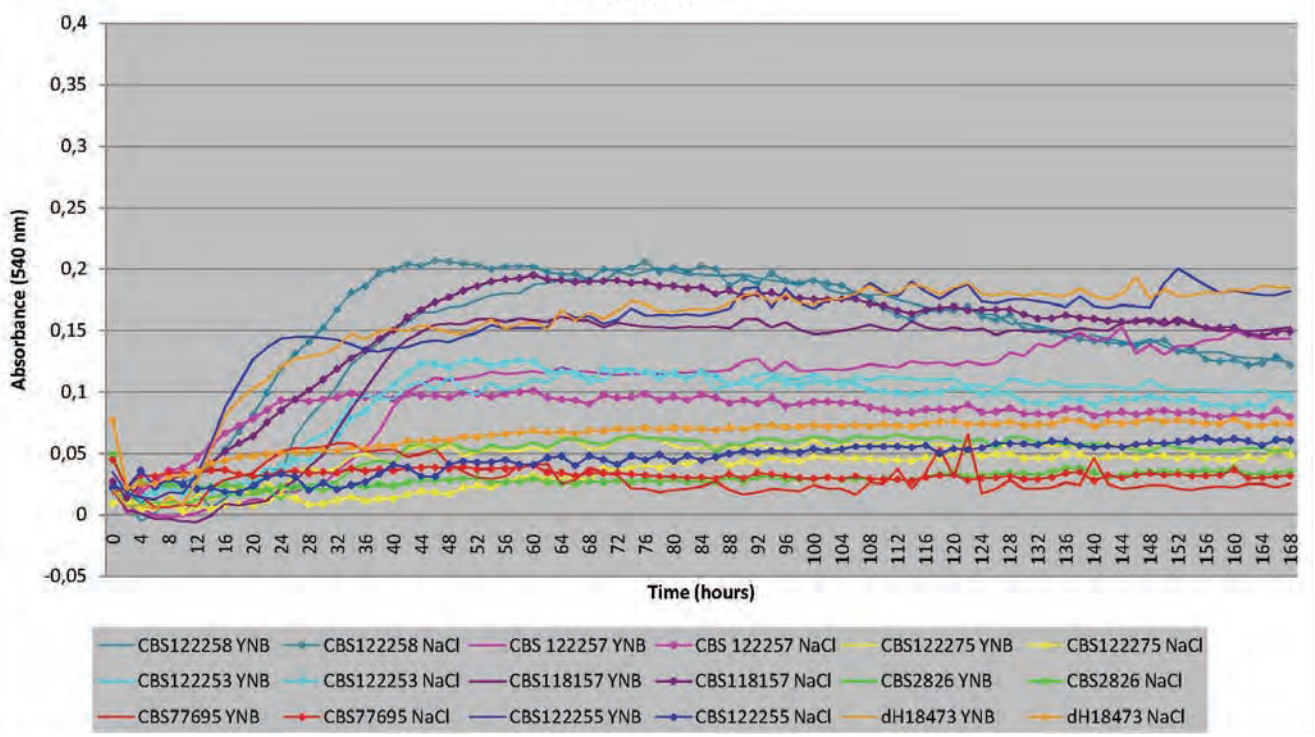

Fig. 3. Culture condition C: growth curves of studied strains in mineral medium (YNB) + 20 % n-hexadecane (continuous line), and in physiological salt solution (NaCl) + 20 % n-hexadecane (line and dots).

CBS 122253, CBS 122257, CBS 122258 and CBS 118157. The difference between the growth rates in culture conditions B and C were small: strain CBS 122253 and CBS 122258 showed the same rates for both culture conditions; higher growth rates with mineral oil was observed for strain CBS 122257; and strain CBS 118157 seemed to be stimulated by adding n-hexadecane (Figs 2–3). Strain dH 18473 had similar growth in culture conditions A, B and C (Figs 1–3).

Oligotrophism

When growth was tested in physiological salt solution, several black yeasts and relatives (CBS 122258; CBS 122257; CBS 122253; CBS 118157) were able to grow for few ds. However, growth rates were lower than in assimilation tests as presumed due to the poor concentration of nutrients. Stationary phase was mostly reached after 36−68 h. *Cladophialophora minourae* (CBS 122275) and *Cladophialophora* sp. (dH 18473) showed the same pattern as reference strains (*Rhodotorula graminis* and *Penicillium chrysogenum*): they all did not have significant growth in NaCl (Figs 1–3). Surprisingly some growth curves in mineral oil and n-hexadecane 20 % from oligotrophism tests were higher than observed in assimilation tests. Strain CBS 122258 and CBS 118157 (both *Exophiala xenobiotica* strains) continued growth at a minimal level. Addition of 20 % mineral oil slightly stimulated growth of black yeast-like fungi in that stationary phase was reached somewhat earlier (Fig. 2). Addition of 20 % n-hexadecane stimulated growth in *Exophiala* strains that performed best in previous tests; clear exponential and stationary phases were distinguishable (Fig. 3). Strains CBS 122255 and dH 18473 also did not show significant growth in NaCl. Strains CBS 122257 and CBS 122253 showed ability to grow in media poor in nutrients; growth levels were not so high as for *Exophiala* strains but were higher than in *Cladophialophora* sp.

DISCUSSION

Previous studies on black yeasts isolation using the oil flotation technique reported that soil was poor in black yeast (Iwatsu *et al.,* 1981; Marques *et al*., 2006; Vicente *et al.,* 2001), but in the present article using polluted soil, a large number of isolates was obtained. In these researches few black yeast strains per sample were recovered: Iwatsu *et al.* (1981) isolated 83 dematiaceous strains from 177 samples; Vicente (2000) obtained 81 strains from 540 samples and Marques *et al.* (2006) 9 isolates from 68 samples. In this study 107 strains were isolated from 3 samples. Hence it may be concluded that hydrocarbons present in the soil might favor the growth of the black yeasts and function as an enrichment factor, as supposed earlier (Prenafeta-Boldú *et al.* 2002, 2006, Sterflinger & Prillinger 2001) or inhibited growth of competing species. Judging from the number of strains recovered, and the number of strains for which no match was obtained with any known species, land farming soil was considered an interesting substrate for recovering this group of fungi. Studies on the ecology, phylogeny and bioremediation seem to be promising. Previous studies in this area obtained strains able to degrade aromatic hydrocarbons such as fungi from genera *Fusarium*, *Penicillium*, *Trichoderma*, *Aspergillus* (Kataoka 2001, Satow 2005) and some fungi with black colonies, however, molecular identification was not done to confirm if they belonged to black yeast-like fungi (Conceição *et al.* 2005).

The recovery of *Exophiala xenobiotica* from land farming soil confirms that particularly those members of *Chaetothyriales* showing a preference for habitats rich in monoaromatic hydrocarbons and alkanes are recovered (de Hoog *et al.* 2006, Sterflinger & Prillinger 2001, Woertz *et al.* 2001). Given their potential as opportunists or human pathogens (*e.g.*, Prenafeta-Boldú *et al*. 2006, Zeng *et al.* 2007) the results of this study may contribute for the biosafety rules established for the employees of the refinery. On the other hand, black yeasts and relatives could be also developed as agents for bioremediation for industrial purposes, such as treatment of volatile

pollutants in bioreactors (Woertz *et al.* 2001) and other types of bioremediation in soil and water.

Cladophialophora strains were also isolated from land farming soil; its natural niche is not well known yet, but Prenafeta-Boldú *et al.* (2002, 2004, 2005) observed the ability of strains from this genus to degrade aromatic compounds and to survive in habitats with high concentrations of volatile hydrocarbons.

In the present study, besides the fact that only one fifth of isolated strains was identified by molecular techniques, it was shown that they belonged to only three genera. Prevalence of these fungi seems to be due to the niche, rich in hydrocarbons which could select the strains able to survive under toxic conditions, and also the absence of plant matter that could inhibit the growth of fungi associated to this substrate. *Fonsecaea*, *Phialophora*, *Rhinocladiella* and *Veronaea* species were observed in previous isolations (Iwatsu *et al.*1981, Marques *et al.* 2006, Vicente 2000) from diverse sources from nature, using the same technique, indicating that the method can recover different strains from the black yeast group and the diversity depends on the conditions of the habitat.

The application of the method on landfarming soil samples revealed that cell hydrophobicity is not the main selective factor of the method, because *Exophiala xenobiotica* (hydrophilic) as well as *Cladophialophora* species (hydrophobic) were repeatedly recovered from soil samples. Considering the aim of this technique, this result supports its efficiency because it succeeds in recovering both types of black yeast-like strains observed in the hydrophobicity test. However, the method seems not to be adequate for quantitative studies due to the different rates of hydrophobicity shown by black yeast strains. Strains with higher cell hydrophobicty tend to be more prevalent in the interphase than the hydrophilic ones.

Melanised fungi of the order *Chaetothyriales* are highly polymorphic. Closely related members may be morphologically very different, and even a single strain may exhibit various types of morphology. For that reason a practical, ecological classification of anamorphs has been proposed: *Cladophialophora* for catenate anamorphs which are mostly hydrophobic, and *Exophiala* for annellidic anamorphs producing slimy, mostly hydrophilic conidia and budding cells. In this paper we have proven that *Cladophialophora* cells are indeed strongly hydrophobic, and *Exophiala* cells are hydrophilic, underlining the differential ecological roles of these morphotypes. The presence of yeast cells does not *a priori* indicate hydrophilic character, as yeast cells of *Horteae werneckii* have 94–98 % hydrophobicity suggesting distribution by rain splash (Göttlich *et al.* 1995). These authors also showed that *Exophiala* species are differentially hydrophilic: *Exophiala dermatitidis* has a hydrophobicity of 63 % and *Exophiala jeanselmei* of 37 %.

Assimilation test revealed that most black yeast strains were able to grow in media with mineral oil or n-hexadecane as sole source of carbon and energy, so incubation in medium with hydrocarbon may improve the selection of this group of fungi, as enrichment factor or inhibiting other fungal species with faster growth rate. Growth differences between the culture conditions B and C are probably due to the composition of the reagents: n-hexadecane has a defined chemical composition ($C_{16}H_{34}$) and a short, simple molecular chain; mineral oil is a complex mixture of long chain hydrocarbons, where degradation is more difficult.

As mentioned above strain CBS 122275 has low ability to use hydrocarbons as sole carbon source, but high cell hydrophobicity what may indicate that the selective factor, in this case, could be the hydrophobicity instead of hydrocarbon assimilation or oligotrophism.

Mineral oil and n-hexadecane serve to be an enrichment factor for members of both genera, *Exophiala* and *Cladophialophora*, enhancing growth of many black yeast-like but not of competing saprobes. The addition of a hydrocarbon source for a short period (2–3 d) in media lacking adequate nutrients might just be sufficient to be used as a selective factor for this group of fungi. Extended periods of incubation under these conditions seem to further promote the isolation of members of *Chaetothyriales*, as several tend to continue growth at a very low level (Fig. 1). Since they are able to show some growth with or without these compounds and with or without additional N-source, they can be regarded as being truly oligotrophic. In this study, the primary advantage of the isolation of *Cladophialophora* hydrophobic strains above the *Exophiala* hydrophilic ones could be compensated by a short incubation under oligotrophic conditions and mineral oil enrichment. *Exophiala* strains showed faster growth than most *Cladophialophora* strains under these conditions, so after some ds of incubation, they could have more chance to be recovered.

Incubation of the sample for 2 d in mineral medium and 20 % mineral oil (culture condition B) seems to enrich the growth of most of black yeast strains and could be used to increase the chance to isolate them. In the present study it was not possible to clearly identify the main selective factor of mineral oil in black yeast isolation. The strains of this fungal group are very diverse concerning their ecology, physiology and phylogenetic aspects so it is presumed that their cell surface hydrophobic characteristics, hydrocarbon assimilation and oligotrophism should be distinct, as demonstrated with the tests performed.

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Characterisation of the substrate specificity of the nitrile hydrolyzing system of the acidotolerant black yeast *Exophiala oligosperma* R1

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Abstract: The 'black yeast' *Exophiala oligosperma* R1 can utilise various organic nitriles under acidic conditions as nitrogen sources. The induction of a phenylacetonitrile converting activity was optimised by growing the strain in the presence of different nitriles and /or complex or inorganic nitrogen sources. The highest nitrile hydrolysing activity was observed with cells grown with 2-cyanopyridine and $NaNO_3$. The cells metabolised the inducer and grew with 2-cyanopyridine as sole source of nitrogen. Cell extracts converted various (substituted) benzonitriles and phenylacetonitriles. They usually converted the isomers carrying a substituent in the *meta*-position with higher relative activities than the corresponding *para*- or *ortho*-substituted isomers. Aliphatic substrates such as acrylonitrile and 2-hydroxy-3-butenenitrile were also hydrolysed. The highest specific activity was detected with 4-cyanopyridine. Most nitriles were almost exclusively converted to the corresponding acids and no or only low amounts of the corresponding amides were formed. The cells hydrolysed amides only with extremely low activities. It was therefore concluded that the cells harboured a nitrilase activity. The specific activities of whole cells and cell extracts were compared for different nitriles and evidence obtained for limitation in the substrate-uptake by whole cells. The conversion of 2-hydroxy-3-butenenitrile to 2-hydroxy-3-butenoic acid at pH 4 demonstrated the unique ability of cells of *E. oligosperma* R1 to hydrolyse aliphatic α-hydroxynitriles under acidic conditions. The organism could grow with phenylacetonitrile as sole source of carbon, energy and nitrogen. The degradation of phenylacetonitrile presumably proceeds via phenylacetic acid, 2-hydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid (homogentisate), maleylacetoacetate and fumarylacetoacetate.

Key words: Acidotolerance, biotransformation, black yeasts, *Exophiala*, homogentisate pathway, induction, nitrilase.

INTRODUCTION

Organic nitriles (R-CN) are widely used in organic chemistry as intermediates for the synthesis of various compounds such as carboxylic acids and amides (Banerjee *et al*. 2002). Since several yr there is an increasing interest in identifying and using nitrile converting biocatalysts in order to replace the traditional chemical synthetic reactions for the production of acids and amides which require rather harsh acidic or alkaline conditions (Singh *et al*. 2006). Furthermore, several examples have been described that demonstrate that nitrile-converting biocatalysts allow chemo-, regio-, or enantioselective reactions that are difficult to achieve by purely chemical reactions (Bunch 1998a, Martínková & Křen 2001, Schulze 2002, Brady *et al*. 2004). These investigations resulted in the isolation of several nitriles converting Gram-positive and Gram-negative bacteria (often *Rhodococcus* or *Pseudomonas* strains) and some yeasts and fungi (Banerjee *et al*. 2002, Bunch 1998b, Kaplan *et al*. 2006a, b, Kaul *et al.* 2004, Kiziak *et al*. 2005, Rezende *et al*. 1999). These organisms converted nitriles either to the acids by using nitrilases or to the amides by using nitrile hydratases.

An interesting application for nitrile hydrolysing organisms or enzymes is the conversion of α-hydroxynitriles, because (chiral) α-hydroxycarboxylic acids and amides are interesting products for the chemical industry (Gröger 2001). Unfortunately, the biotransformation of α-hydroxynitriles in aqueous systems is hampered by the low stability of the substrates under neutral conditions. In contrast, these substrates are generally more stable under acidic conditions (Rustler *et al*. 2007). However, most of the known nitrilases are unstable at acidic pH values (Banerjee *et al.* 2002). Therefore, we are trying to find whole cell catalysts which are able to catalyse these biotransformations under acidic conditions. We have recently isolated after an enrichment at pH 4 with phenylacetonitrile as sole source of nitrogen an acidotolerant black yeast which was subsequently identified as a new strain of *Exophiala oligosperma* (Rustler & Stolz 2007). This strain (R1) was the first 'black yeast' for which the ability to convert organic nitriles was described and it was found that the organism could grow at pH 4 with phenylacetonitrile as sole source of carbon, nitrogen and energy. Although this nitrile converting system showed some biotechnological potential because of the unique taxonomic position of the producing organism and the acid-resistance of the process, the observed activities were not sufficient for a detailed analysis of the reaction and any possible application. Therefore, in the present communication we further optimised the induction conditions for the production of the nitrile converting activity and subsequently analysed the responsible enzymatic activity.

MATERIALS AND METHODS

Microorganisms and culture conditions

Exophiala oligosperma R1 [deposited at the Centraalbureau voor Schimmelcultures in Utrecht (The Netherlands) as strain CBS 120260] was routinely grown in Na-citrate-phosphate mineral media consisting of 20 % (*v/v*) 0.5 M Na-citrate-phosphate buffer (pH 4) and the nitrogen-free mineral medium described previously (Rustler & Stolz 2007) plus 0.2 % (*w/v*) casamino acids and 20 mM

glucose as sources of nitrogen, carbon and energy. The cultures were usually grown for 72–120 h in 3 L Erlenmeyer flasks with baffles at 30 °C on a rotary shaker (100 rpm).

Growth measurements

The growth of the strain was monitored spectrophotometrically by measuring the optical density at 600 nm (OD_{600nm}) with a Cary 100 Bio spectrophotometer (Varian Inc., Mulgrave, Australia) or by using a Klett photometer (Klett Manufactoring Co. Inc., Brooklyn, NY). An OD_{600nm} of 1 corresponded to about 75 Klett units and 0.25 mg of cell dry weight per mL of culture.

Preparation of cell extracts

The cells were harvested at the beginning of the stationary growth phase by centrifugation (11,000 g, 15 min, 4 °C) and washed twice in 0.1 M Na-phosphate buffer (pH 6.5). The cells were then resuspended in 0.1 M Tris/HCl buffer (pH 7) to an OD_{600nm} of about 300 and disintegrated by using a French Press (Aminco, Silver Springs, Md., U.S.A.). The cells were disintegrated seven times at 4 °C by using the miniature pressure cell (Aminco, Silver Springs, Md., U.S.A.) with a cell pressure of about 16 000 psi. The samples were centrifuged (6 000 g, 10 min, 4 °C) and the liquid phase was finally clarified by ultracentrifugation (100 000 g, 60 min, 4 °C). The supernatant was immediately frozen in liquid nitrogen and stored at –70 °C. The protein content of the cell extract was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzyme assays

One unit of enzyme activity was defined as the amount of enzyme that converted 1 µmol of substrate per minute.

For the determination of nitrile hydrolysis with resting cells, the cells were harvested at the beginning of the stationary phase by centrifugation (11 000 g, 15 min, 4 °C), washed twice in 0.1 M Na-citrate-phosphate buffer (pH 4) and finally resuspended in 0.1 M Na-citrate-phosphate buffer (pH 4). The standard assay usually contained 1.5–2 mL 0.1 M Na-citrate-phosphate buffer (pH 4), 1–10 mM substrate and cells corresponding to an OD_{600nm} of 2–15. The reactions were started by the addition of the substrates and performed in Eppendorf cups incubated in a thermoshaker at 30 °C and 1 400 rpm (Thermomixer Comfort, Eppendorf AG, Hamburg, Germany). The stock solutions (1 M) of the nitriles (or amides) were routinely prepared in methanol (or a 1:1 mixture of methanol and water for some amides). After different time intervals, samples (50–100 µL each) were taken and the reactions were stopped by adding 1 M HCl (5–10 µL). The cells were removed by centrifugation (21 000 *g*, 10 min, 4 °C) and the supernatants finally analysed by using high pressure liquid chromatography (HPLC).

The nitrile hydrolysing activity of cell extracts was determined in reaction mixtures (0.75–1 mL) containing 0.1 M Tris/HCl (pH 7), 0.15–0.2 mg/mL of protein and 1–5 mM of the substrate. The reactions were then performed as described for the resting cell experiments. The reactions were usually terminated by the addition of 10 % (*v/v*) 1 M HCl and the precipitated proteins were removed by centrifugation (21 000 g, 10 min, 4 °C). The concentrations of the substrates and products in the supernatants were determined by HPLC.

The activities were usually calculated from the turn-over of the substrate and correlated to the dry weight of the cells or the amount of protein applied.

Comparison of the conversion of different nitriles by resting cells and cell extracts

Exophiala oligosperma R1 was cultivated in 3 L Erlenmeyer flasks with baffles in 1 L of the Na-citrate-phosphate mineral medium (pH 4) containing glucose (20 mM), $NaNO_3$ (4 mM), and 2-cyanopyridine (15 mM). The cultures were incubated at 30 °C on a rotary shaker (100 rpm). At the end of the exponential growth phase (after 72 h of cultivation; $OD_{600nm} \approx 3.5$) the cells were harvested by centrifugation (11 000 g, 15 min, 4 °C) and washed in 0.1 M Na-phosphate buffer (pH 6). An aliquot of the cells was immediately frozen in liquid nitrogen and stored at –70 °C. The other aliquot was resuspended in 0.1 M Tris/HCl (pH 7) and used for the preparation of cell extracts as described above. For the experiments cells and cell extracts were thawed and resuspended or diluted, respectively, in 0.1 M Tris/HCl (pH 7). The reaction mixtures finally contained in 0.1 M Tris/HCl (pH 7) resting cells with an OD_{600nm} of 2 or a protein concentration of 0.2 mg/mL in case of the cell extracts.

Analytical methods

The concentrations of the nitriles and their corresponding amides and acids were analysed by HPLC (ChemStation LC3D, Autosampler G1329A, Thermostat 1330B, Diode Array Detector G1315B, Quat-HPLC pump G1311A; Agilent Technologies, Santa Barbara, CA). The individual compounds were usually detected spectrophotometrically at 210 nm.

A reversed-phase column [125 by 4 mm (internal diam); Trentec, Gerlingen, Germany] filled with 5-µm-diameter particles of Lichrospher-RP8 endcapped (E. Merck AG, Darmstadt, Germany) was used in most experiments for separation of individual compounds. The conversion of cyanopyridines, acrylonitrile and 2-hydroxy-3-butenenitrile was analysed by using a column [size 250 by 4 mm (internal diameter) Trentec, Gerlingen, Germany] filled with 5 µm particles of Nucleosil-100 C18 (Macherey & Nagel, Düren, Germany). The columns were incubated at 21 °C in a column heater /chiller (Jones Chromatography Model 7956, Alltech Associates Inc., Hesperia, CA) and the samples were cooled at 4 °C.

The solvent systems for the analysis of the turn-over experiments with benzonitrile, 2-, 3-, and 4-tolunitrile, 2-, 3-, and 4-chlorobenzonitrile, 2-, 3-, and 4-hydroxybenzonitrile, phenylacetonitrile, 2-phenylpropionitrile and 2-, 3-, and 4-chlorophenylacetonitrile contained 30–50 % (v/v) acetonitrile and 0.3 % H_3PO_4 (v/v) in H_2O as mobile phases. The conversion of mandelonitrile was analysed by using a solvent system which consisted of 40 % (v/v) methanol, 0.3 % (v/v) H_3PO_4 and water.

Acrylonitrile, 2-hydroxy-3-butenenitrile and their potential products were analysed by using a solvent system consisting of 0.5 % (v/v) acetonitrile, 0.1 % (v/v) H_3PO_4 and 99.4 % (v/v) H_2O. The detection of these aliphatic substances was performed at 195 nm.

2-, 3-, and 4-cyanopyridine were analysed by using a solvent system which contained 20 % (v/v) acetonitrile and 0.3 % (v/v) H_3PO_4 in water by using a Lichrospher-RP8 column. The products formed from 3- and 4-cyanopyridine were detected by using a solvent system consisting of 10 % (v/v) acetonitrile plus 90 % (v/v) 30 mM Na-PO_4-buffer (pH 7) and 5 mM of the ion-pair reagent TBAHS (tetrabutylammoniumhydrogensulfate) and by using a

Nucleosil-100 C18 column as the stationary phase (Dazzi *et al*. 2001).

Products formed from 2-cyanopyridine were analysed with a Nucleosil-100 C18 column using a solvent system composed of 99 % (v/v) 30 mM Tris/HCl (pH 9.0) plus 1 % (v/v) acetonitrile and 5 mM TBAHS. The detection of substances which were separated in solvent systems with TBAHS was performed at 265 nm. The average flow rate was 1 mL/min.

Synthesis of 2-hydroxy-3-butenenitrile

Into a 250 mL round bottom flask 0.09 mol (5 g) acrolein, 0.1 mol (9.9 g) trimethylsilyl cyanide and 0.01 mol (3.19 g) zinc iodide were added, followed by addition of 200 mL dichloromethane. The reaction was stirred overnight at RT. Then, the solvent was evaporated under reduced pressure, giving the silyl protected cyanohydrin. The latter was hydrolyzed at 40 °C to 2-hydroxy-3-butenenitrile by reacting it with 150 mL 3 M HCl. The reaction was completed after 2 h. The pure product (dark brown liquid) was isolated from the reaction mixture by triple extraction with diethyl ether (3 x 150 mL), drying on $MgSO_4$ and concentrating the product using a rotary evaporator (Gassman and Talley 1978). Proton NMR analysis confirmed the product formation. ^{1}H NMR (300 MHz, $CDCl_3$): δ= 5.9 (ddd, 2H, *J'*=16.2 *J'*=11.1, *J'*=6.3 Hz), 5.6 (d, 3 H, *J*=17.1), 5.4 (d, 3 H,*J* =10.2), 5.0 (d, 1 H, *J* =5.1).

Synthesis of 2-hydroxy-3-butenoic amide

Into a 10 mL glass reactor, 12 mmol (1 g) 2-hydroxy-3-butenenitrile and 3.2 mL 37 % HCl were added. The reaction was shaken for 3.5 h at 10 °C until the cyanohydrin was completely converted into the corresponding 2-hydroxy-3-butenoic amide. The latter product was extracted to ethyl acetate, dried on Na_2SO_4 and the resulting clear solution was concentrated using a rotary evaporator (van Langen *et al*. 2004). The formation of the product was confirmed by proton NMR. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.9 (bs, $CONH_2$), 6.2 (ddd, 2H, *J'*=16.8, *J'*=11.4, *J'*=6.6 Hz), 5.5 (d, 3 H, *J*=16.5), 5.3 (d, 3 H, *J*=10.5), 4.7 (d, 1H, *J*=5.7 Hz).

Synthesis of 2-hydroxy-3-butenoic acid

Into a 10 mL round bottom flask, 6 mmol (0.5 g) 2-hydroxy-3-butenoic amide and 3.0 mL 37 % HCl were added. The reaction was refluxed at 125 °C for 1.5 h. 2-hydroxy-3-butenoic acid was extracted to ethyl acetate, dried on Na_2SO_4 and the product was concentrated *in vacuo* (van Langen *et al*. 2004). The formation of the product was confirmed by proton NMR. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.0 (ddd, 2H, *J'*=15.4, *J'*=11.4, *J'*=6.6 Hz), 5.5 (d, 3H, *J*=17.4), 5.3 (d, 3H,*J*=10.5,), 4.8 (m,1H).

Chemicals

Trimethylsilyl cyanide and zinc iodide (98+ %) were obtained from Acros Organics BVBA (Geel, Belgium). All other chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) or E. Merck AG (Darmstadt, Germany). All the chemicals were used as supplied, without further purification.

RESULTS

Induction of the nitriles converting activity after addition of different possible inducers

There are several examples found in the literature which demonstrate that the addition of certain nitriles may have pronounced inducing effects on nitriles converting enzymes. Thus, the addition of ε-caprolactam or isovaleronitrile resulted in *Rhodococcus rhodochrous* J1 and *R. rhodochrous* K22 in the formation of relatively large amounts of the respective nitrilases which corresponded to 20–30 % of the total soluble protein (Nagasawa *et al*. 1990, Kobayashi *et al.* 1991). Recently, 2-cyanopyridine and valeronitrile have been described as especially potent inducers of the nitrilase from *Aspergillus niger* K10 (Kaplan *et al.* 2006b). Therefore, the influence of these and structural similar compounds was assayed on the induction of the nitrile converting activity of *E. oligosperma* R1. The strain was grown in Na-citrate-phosphate medium (pH 4) with glucose (20 mM), casamino acids (0.2 % w/v) and different potential inducers: phenylacetonitrile, 2-, 3-, and 4-chlorophenylacetonitrile, indole-3-acetonitrile, 4-hydroxyphenylacetonitrile, 2-, 3-, and 4-cyanopyridine, isovaleronitrile, or benzonitrile (2 mM each). Furthermore, also ε-caprolactam (45 mM) was analysed.

Exophiala oligosperma R1 did not grow in the presence of the indicated concentrations of 2-, 3-, and 4-chlorophenylacetonitrile and indole-3-acetonitrile. In contrast, no inhibitory effects of the other compounds were observed on growth. The cells were harvested at the beginning of the stationary growth phase by centrifugation (11,000 *g*, 15 min, 4 °C), washed, and resupended in 0.1 M Na-citrate-phosphate buffer (pH 4) to an OD_{600nm} of 11. Finally, phenylacetonitrile (2 mM) was added and the conversion of the nitrile analysed by HPLC.

The experiments demonstrated that the addition of 2-cyanopyridine, ε-caprolactam, and isovaleronitrile increased the nitrile converting activity compared to the constitutive level 4.4-, 2.9-, and 2-fold, respectively. In contrast, the previously used inducer phenylacetonitrile resulted only in a 1.5-fold increase in enzyme activity. Also the other tested putative inducers only resulted in a less than 1.5-fold increase in the nitrile converting activity.

Influence of different nitrogen sources on induction and optimisation of the inducer concentrations

In the following experiment the influence of different nitrogen sources on the induction of the nitriles converting activity was assayed. The cells were grown in Na-citrate-phosphate medium (pH 4) with glucose (20 mM) plus casamino acids (0.2 % *w/v*), $NaNO_3$ (3 mM) or $(NH_4)_2SO_4$ (1.5 mM) with or without phenylacetonitrile (2 mM), 2-cyanopyridine (2 mM), ε-caprolactam (45 mM), or a mixture of 2-cyanopyridine plus isovaleronitrile (2 mM each). In these experiments the highest activities were obtained in a growth medium containing $(NH_4)_2SO_4$ plus ε-caprolactam (45 mM), followed by a growth medium which contained $NaNO_3$ plus 2-cyanopyridine.

In the previous experiments fixed concentrations of the inducers (2 mM of the nitriles or 45 mM ε-caprolactam) were used. In the following experiments the concentrations of the previously identified best inducers (ε-caprolactam and 2-cyanopyridine) were varied (2–45 mM). The cells were grown in 300 mL Klett-flasks with baffles in 50 mL of a medium which contained Na-citrate-phosphate buffer (pH 4), nitrogen-free mineral medium, 20 mM glucose. Furthermore,

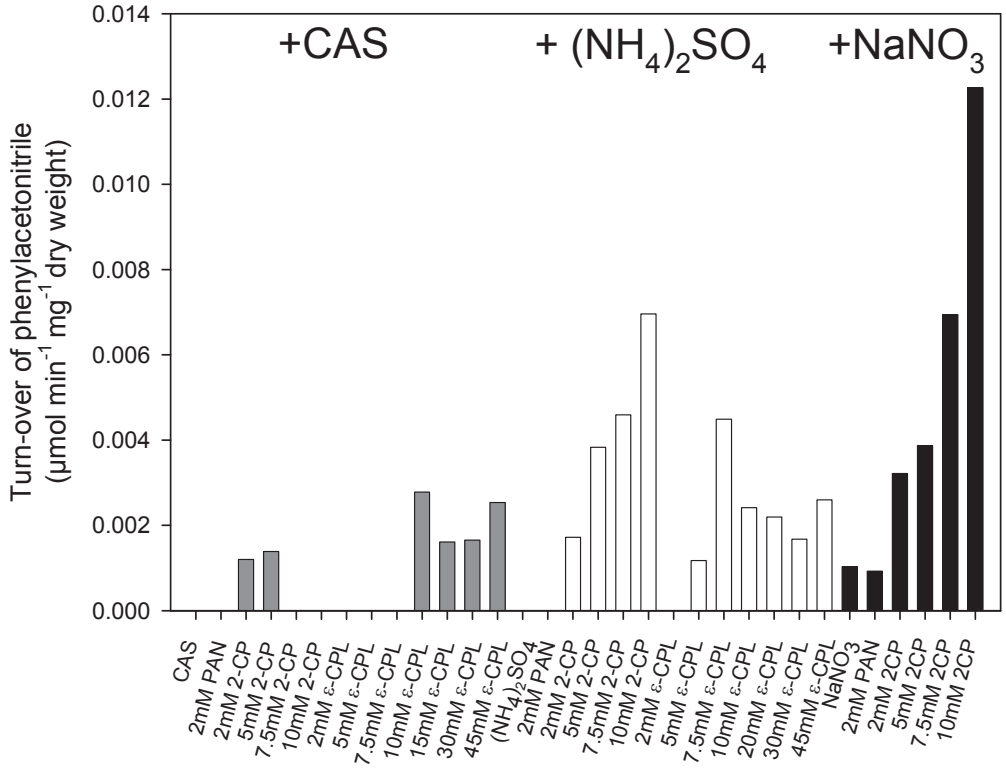

Fig. 1. Nitrile hydrolyzing activity of resting cells of *E. oligosperma* R1 after growth in the presence of different nitrogen sources and different concentrations of ε-caprolactam or 2-cyanopyridine. The cells were cultivated as described in the text in media containing Na-citrate buffer (pH 4), glucose (20 mM) and 0.2 % (*w/v*) casamino acids (CAS; ■), 4 mM $NaNO_3$ (■), or 2 mM $(NH_4)_2SO_4$ (□). The cells were harvested by centrifugation (11 000 g, 15 min, 4 °C), washed in 0.1 M Na-citrate-phosphate buffer (pH 4), and finally resuspended in 0.1 M Na-citrate-phosphate buffer (pH 4) to an OD_{600nm} of approximately 15. The conversion of phenylacetonitrile (2 mM) by the resting cells was analysed using a thermoshaker (30 °C, 1 400 rpm) as described in the materials and methods section.

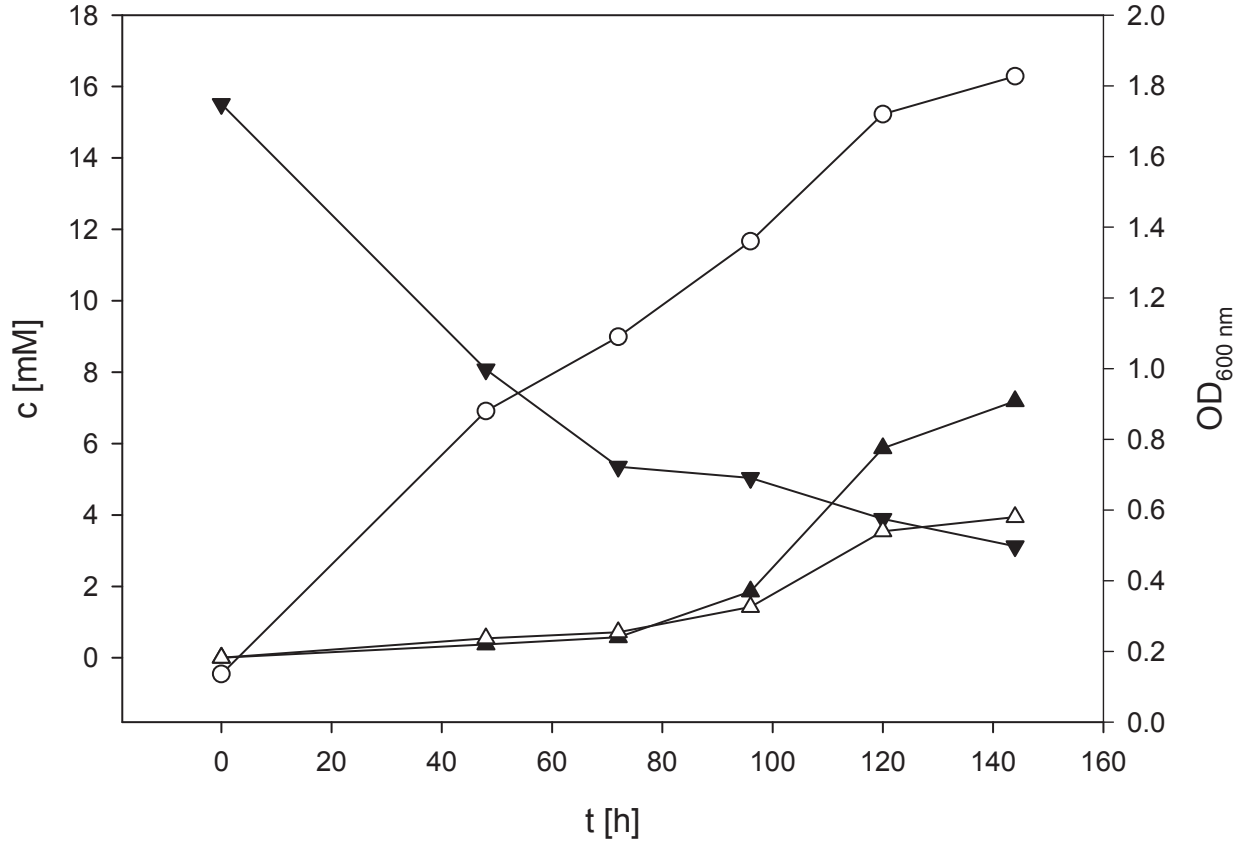

Fig. 2. Turn-over of 2-cyanopyridine by growing cells of *E. oligosperma* R1**.** The cells were grown in Na-citrate-phosphate medium (pH 4) with glucose (20 mM), $NaNO_3$ (4 mM), and 2-cyanopyridine (15 mM). The cells were incubated in Erlenmeyer flasks on a rotary shaker (100 rpm) at 30 °C. The growth of the culture was monitored by measuring the optical density at 600 nm (O). After different time intervals samples were taken and the reactions terminated by adding 10 % (*v/v*) 1 M HCl. Cells were removed by centrifugation (21 000 g, 10 min, 4 °C) and the concentration of 2-cyanopyridine (▼), picolinic acid (▲), and picolinic amide (Δ) in the supernatants determined by HPLC.

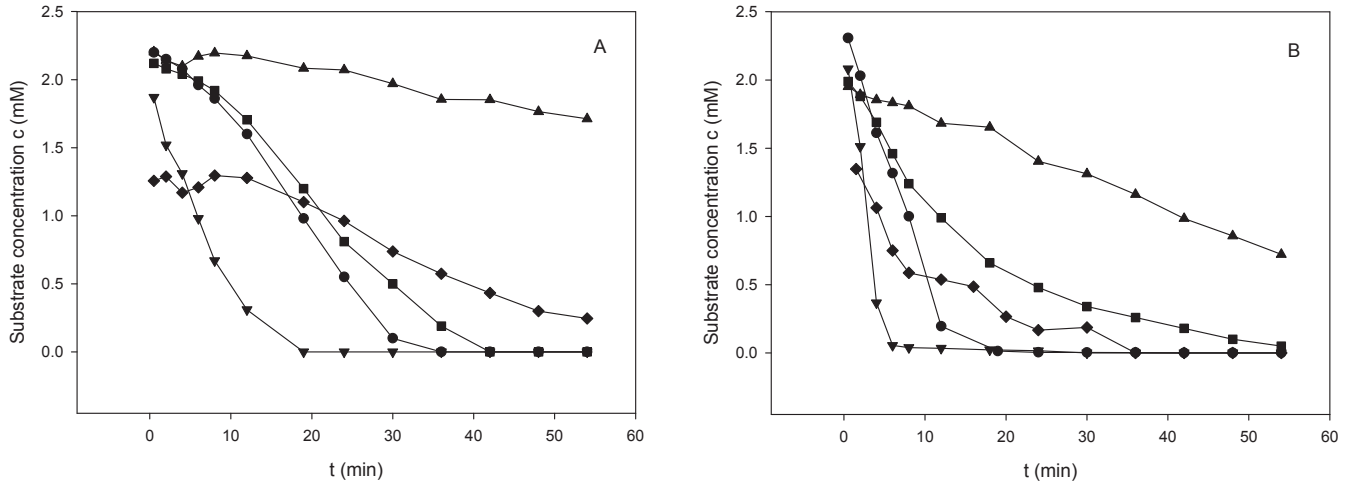

Fig. 3. Turn-over of different nitriles by whole cells (A) and cell extracts (B) of *E. oligosperma* R1**.** The resting cells and cell extracts were prepared as described in the materials and methods section. The reaction mixtures (750 µL each) were incubated in 1.5 mL cups in a thermoshaker (1 400 rpm, 30 °C). The reactions were started by the addition of 4-cyanopyridine (▼), 3-cyanoypridine (●), benzonitrile (■), phenylacetonitrile (▲) (2 mM each), or acrylonitrile (♦; 1.5 mM). After different time intervals samples were taken and the reactions terminated by adding 20 % (*v/v*) 1 M HCl. The samples were clarified by centrifugation (21 000 g, 10 min, 4 °C), and the supernatants analysed by HPLC (see material and methods).

casamino acids (0.2 % *w/v*), $(NH_4)_2SO_4$ (2 mM), or $NaNO_3$ (4 mM) were offered as nitrogen sources. The cells were harvested at the end of the exponential growth phase (after 96 h with casamino acids or after 112 h with inorganic nitrogen sources). The cells reached in the presence of the casamino acids significantly higher final cell densities than after growth with the inorganic nitrogen sources (OD_{600nm} of about 6.5 or 3.5, respectively). In both systems (inorganic or organic nitrogen sources) the addition of ε-caprolactam and 2-cyanopyridine did not result in significant decreases in the finally reached optically densities. The results demonstrated that with ε-caprolactam the highest nitrile hydrolysing activities were found with 7.5 mM ε-caprolactam and $(NH_4)_2SO_4$. In these experiments 2-cyanopyridine was a better inducer than ε-caprolactam. Almost twice as much activity could be detected in samples that had been grown with $NaNO_3$ and 10 mM 2-cyanopyridine (Fig. 1).

Conversion of 2-cyanopyridine by the cells

The analysis of further induction experiments with 2-cyanopyridine demonstrated that the cells metabolised the inducer. Thus, in the presence of 4 mM $NaNO_3$ an (almost) complete disappearance of 2-CP (10–15 mM) was observed within 96–144 h. The analysis of these reactions by HPLC [solvent system: 1 % (*v/v*) acetonitrile and 99 % (*v/v*) 30 mM Tris/HCl, pH 9.0, plus 5 mM TBAHS, see materials and methods] demonstrated the turn-over of 2-CP (R_t= 19.4 min) and the formation of two metabolites. These metabolites were according to their retention times and *in situ* spectra identified as picolinic acid (pyridine-2-carboxylic acid) (R_t= 11.2 min) and picolinic amide (2-pyridinecarboxamide) (R_t= 10.5 min) (Fig. 2). The strain also grew in the absence of $NaNO_3$ with 10–15 mM 2-CP as sole source of nitrogen and also under these conditions almost completely converted the nitrile with similar specific activities. Further, resting cell experiments with cells grown with 2-cyanopyridine (12.5 mM) in the absence or presence of $NaNO_3$ (4 mM) did not show any significant differences in the ability to convert phenylacetonitrile.

Identification of better substrates for the nitrile converting enzyme

The optimisation of the induction conditions described above resulted in an approximately 20-fold increase of the nitrile-converting activity compared to the initially found values (for cells grown with phenylacetonitrile and casamino acids) (Rustler & Stolz 2007). Nevertheless, even these 'optimised' cells (grown in Na-citrate-phosphate medium with 4 mM $NaNO_3$ and 15 mM 2-cyanopyridine) demonstrated with phenylacetonitrile as substrate only activities of about 0.021 U per mg of dry weight. It was therefore tested if nitriles which had been previously identified as good substrates for fungal nitrilases (Kaplan *et al*. 2006a, Goldlust & Bohak 1989) were converted with higher specific activities. Therefore, whole resting cells were incubated with benzonitrile, 3-, and 4-cyanopyridine (2 mM each) and acrylonitrile (1.5 mM) (Fig. 3A).

The experiments demonstrated that the resting cells converted 3- and 4-cyanopyridine about 7 and 13-times faster, respectively, than phenylacetonitrile (Table 1). The resting cells converted 4-cyanopyridine after induction with 2-cyanopyridine with specific activities of 0.27 U/mg of dry weight. This represented an approximately 270-fold increase compared to the specific activity previously reported for the conversion of phenylacetonitrile by cells grown with casamino acids plus phenylacetonitrile (Rustler & Stolz 2007). The experiments further showed that benzonitrile and acrylonitrile were also converted with higher activities than phenylacetonitrile.

Comparison of the nitrile hydrolysing activity of whole cells and cell extracts

In the following experiments it was tested if the uptake of the substrates could be a limiting factor for the conversion of nitriles by *E. oligosperma* R1. Therefore, cell extracts were prepared and the conversion of benzonitrile, phenylacetonitrile, 3-, and 4-cyanopyridine and acrylonitrile compared between resting cells and cell extracts (Fig. 3). The experiments demonstrated that the cell extracts exhibited significant activities for the transformation of the cyanopyridines and specific activities of cell extracts for

Table 1. Conversion of different nitriles by whole cells and cell extracts of *E. oligosperma* R1.

	Activity of whole cells		Activity of cell extracts	
Compound	**U/mg of dry weight**	**Relative activity**	**U/mg of total protein**	**Relative Activity**
Benzonitrile	0.115	100	0.48	100
Phenylacetonitrile	0.021	18	0.11	22
3-Cyanopyridine	0.14	124	1	208
4-Cyanopyridine	0.27	232	2.46	512
Acrylonitrile	0.058	50	0.58	121

The specific activities were calculated from the experiments shown in Fig. 3 based on the decrease of the nitrile concentrations and related to the dry weight of the cells or the protein concentration in the experiments with cell extracts, respectively.

the conversion of 4-cyanopyridine up to 2.5 U/mg of protein were calculated (Table 1). In the literature generally protein contents of 39–56 % (expressed as % of dry weight) have been described for different yeast species (Verduyn 1991). An average of 50 % protein content (related to the dry weight) was used for the comparison of the turn-over-rates of cell extracts and resting cells. Thus, it was calculated that the cell extracts converted 3-, and 4-cyanopyridine as well as acrylonitrile with up to 5-times higher specific activities than the resting cells. This suggested that at least in the case of rapidly converted substrates the uptake of the nitriles has a limiting effect on the *in vivo* metabolism of organic nitriles by the yeast cells. Furthermore, also the comparison of the relative activities of the whole cells and the cell extracts with different nitriles suggested a significant influence of the cell membrane on the nitrile metabolism because the whole cells converted the cyanopyridines and acrylonitrile in comparison to benzonitrile with decreased relative activities (Table 1). This was especially evident for the substrates benzonitrile and acrylonitrile, because crude extracts converted acrylonitrile faster than benzonitrile, while this was the opposite in the whole cell system.

Conversion of different nitriles by cell extracts

The substrate specificity of the nitrile hydrolysing activity was determined with cell extracts and compared to other fungal nitrile converting enzymes (Table 2). These experiments demonstrated that cell extracts from *E. oligosperma* R1 converted in addition to benzonitrile and the cyanopyridines various methyl-, chloro-, and hydroxy-substituted benzonitriles and phenylacetonitriles. The cell extracts converted in general all isomers of a given substituted benzonitrile or phenylacetonitrile. Nevertheless, it became evident that in most cases the *meta*-substituted isomers were converted with the highest relative activities. In most cases the *meta*-substituted benzonitriles were even faster converted than benzonitrile. In contrast, the *ortho*-substituted isomers were generally converted with the lowest relative activities.

Phenylacetonitriles which carried a larger substituent at the α-position than a hydrogen atom (such as mandelonitrile and 2-phenylpropionitrile) were converted with significantly reduced conversion rates. The aliphatic substrate acrylonitrile was converted with high relative activities (121 % compared to benzonitrile).

The conversion of the nitriles resulted in almost all experiments (with the exceptions of 2-chlorobenzonitrile, 4-cyanopyridine, and acrylonitrile as substrates) in the formation of one clearly prominent product which represented (according to its signal intensity during the HPLC analysis) more than 95 % of the products formed (Table 2). These products were identified by the comparison with authentic standards by their retention times and *in situ* UV/VIS spectra as the corresponding acids. In the cases of 4-cyanopyridine and acrylonitrile two products were formed in a ratio of about 9:1. The conversion of 2-chlorobenzonitrile resulted in two signals in a ratio of 7:3. However, also for these substrates it was shown by using authentic standards that the prominent products were the corresponding acids. The minor products were identified according to their retention times as the corresponding amides.

The conversion of 2-cyanopyridine and 2-hydroxybenzonitrile did not result in the formation of detectable amounts of products. However, in the case of 2-cyanopyridine the conversion of the substrate to picolinic acid and picolinic amide had already been observed in the growth experiment described before (see Fig. 2). The cell extracts were also incubated with several amides, which would be intermediately formed if the cells would harbour a nitrile hydratase. The experiments showed that picolinic amide and nicotinic amide were not converted and isonicotinic amide only with extremely low activities to the corresponding acid. This indicated only a rudimental amidase activity and gave strong evidence that the nitriles were indeed converted by a nitrilase activity.

Conversion of 2-hydroxy-3-butenenitrile by whole cells of *E. oligosperma* R1

It was previously shown that resting cells of *E. oligosperma* R1 were able to convert nitriles at pH values ≥1.5 (Rustler & Stolz 2007). This could allow the conversion of α-hydroxynitriles, which are unstable at neutral pH-values but stabilised under acidic conditions by resting cells of *E. oligosperma* R1. The conversion of acrylonitrile by whole cells and cell extracts demonstrated the general ability of *E. oligosperma* R1 to convert aliphatic nitriles with high relative reaction rates. Therefore, 2-hydroxy-3-butenenitrile was synthesised as substrate (see material and method section) and incubated in a reaction mixture containing 0.1 M Na-citrate-phosphate buffer (pH 4) and resting cells of *E. oligosperma* R1. The analysis of the reaction mixture demonstrated a disappearance of almost 50 % of the initial amount of 2-hydroxy-3-butenenitrile within the first 80 min of the reaction and the formation of one product (Fig. 4). In contrast, no significant decrease of 2-hydroxy-3-butenenitrile was observed in a control experiment without cells. Only traces of acroleine (2-propenal, acrylaldehyde) which would be formed by the chemical decomposition of 2-hydroxy-3-butenenitrile were detected in the reaction mixtures with and without cells. Thus, it was concluded that 2-hydroxy-3-butenenitrile was almost completely stabilised by the acidic reaction buffer and that the resting cells indeed converted the nitrile. The product formed from 2-hydroxy-3-butenenitrile by the cells was identified according to its retention time (R_t=3.45 min) and UV/VIS spectrum in comparison with a chemically synthesised authentic standard as 2-hydroxy-3-butenoic acid.

Table 2. Comparison of the relative nitrile hydrolysing activities of cell extracts from *Exophiala oligosperma* R1, *Fusarium solani* O1, *Penicillium multicolor* CCF 2244 and the purified nitrilase from *Aspergillus niger* K1.

Substrate	*E. oligosperma* R1		*P. multicolor* CCF 2244[1] (whole cells)	*F. solani* O1[2] (purified enzyme)	*A. niger* K1[3] (purified enzyme)
	Relative activity (%)	Formation of amide (% of total products)	Relative activity (%)	Relative activity (%)	Relative activity (%)
Benzonitrile	100	nd	100	100	100
2-Tolunitrile	≤5	nd	nd	nd_m	nd
3-Tolunitrile	154	nd	11.3	33	5.5
4-Tolunitrile	16	nd	nd	16	3.4
2-Hydroxybenzonitrile	≤5	nd	nd	nd_m	nd
3-Hydroxybenzonitrile	140	nd	0.8	80	5.8
4-Hydroxybenzonitrile	10	nd	nd	3	nd
2-Chlorobenzonitrile	<10	29	nd	nd_m	nd
3-Chlorobenzonitrile	100	<5	14.3	87	41
4-Chlorobenzonitrile	49	nd	2.8	40	29.8
2-Cyanopyridine	<10	nd	40	nd_m	14.2
Picolinic amide	nd	-	nd	nd_m	-
3-Cyanopyridine	208	nd	15	28	32.4
Nicotinic amide	nd	-	18	nd_m	-
4-Cyanopyridine	566	<10	72	130	410.7
Isonicotinic amide	<5	-	nd	nd_m	-
Phenylacetonitrile	19	nd	2.3	nd_m	10.8
2-Chlorophenylacetonitrile	11	nd	nd_m	nd_m	nd_m
3-Chlorophenylacetonitrile	35	nd	nd_m	nd_m	nd_m
4-Chlorophenylacetonitrile	22	nd	nd_m	nd_m	nd_m
Mandelonitrile	<5	Traces	nd_m	nd_m	nd_m
2-Phenylpropionitrile	<10	nd	nd	nd	1
Acrylonitrile	121	<10	nd_m	nd_m	nd_m

Cell extracts of *E. oligosperma* R1 were produced as described in the materials and methods section. The reaction mixtures (750 µL each) contained 0.1 M Tris/HCl (pH 7) and 0.2 mg/mL of protein and were incubated in 1.5 mL cups in a thermoshaker (30 °C, 1 400 rpm). The reactions were started by the addition of the respective substrates (2 mM each). At different time intervals samples were taken, and the reactions terminated by the addition of 20 % (*v/v*) 1 M HCl. The reaction mixtures were centrifuged (21 000 g, 10 min, 4 °C) and the supernatants analysed by HPLC according to the procedures described in the material and method section. The reaction rates were calculated from the turn-over of the substrates. The activitiy of benzonitrile was taken as 100 % (0.48 U/mg of protein).
[1,2,] The data for the *Penicillium,* and the *Aspergillus* strains were adapted from Kaplan *et al.* (2006a, c) and [3] those for the *Fusarium* strain from Vejvoda *et al.* 2008, nd: not detected; nd_m: not determined

Degradation of aromatic compounds by *E. oligosperma* R1

It was previously demonstrated that *E. oligosperma* R1 could grow with phenylacetonitrile as sole source of carbon, energy, and nitrogen and that resting cells converted phenylacetonitrile via phenylacetate to 2-hydroxyphenylacetate, which finally also disappeared from the culture supernatants. This suggested that *E. oligosperma* R1 could further metabolise 2-hydroxyphenylacetate and finally cleave the aromatic ring (Rustler & Stolz 2007). Therefore, *E. oligosperma* R1 was grown with phenylacetonitrile as sole source of carbon and energy, the cells harvested by centrifugation and resting cells ($OD_{546nm} \cong 200$) incubated with 2 mM 2-hydroxyphenylacetate and different inhibitors of Fe(II)-ions containing ring-fission dioxygenases (ortho-phenanthroline, 8-hydroxyquinoline, or 2,2´-bipyridyl, 1 mM each). The reactions were analysed by HPLC (40 % methanol, 0.3 % H_3PO_4 in water, flow rate 0.6 mL/min) and it was found that the cells converted 2-hydroxyphenylacetate (R_t= 5.0 min) in the presence of all three inhibitors to a product which was identified in comparison with an authentic standard according to its retention time (R_t= 2.8 min) and *in situ* spectrum (λ_{max}= 222 nm, 292 nm) as 2,5-dihydroxyphenylacetate (homogentisate). In order to analyse the further metabolism of homogentisate, cell extracts were prepared from cells of *E. oligosperma* R1 grown with phenylacetonitrile as sole source of carbon and energy. The cell extracts were incubated in 25 mM Tris/HCl (pH 7.4) with 0.1 mM homogentisate and the reactions analysed spectrophotometrically using overlay-spectra. This demonstrated that the cell extracts rather slowly converted homogentisate causing a batho- and hyperchromic shift. Homogentisate-1,2-dioxygenases are known to contain ferrous iron ions in their catalytical center (Adachi *et al*. 1966). Therefore, the cell extracts were incubated for 30 min with 2 mM $Fe(NH_4)_2(SO_4)_2$ prior to the enzyme assays. This resulted in a pronounced increase in the enzyme activity and it could be demonstrated that homogentisate was converted to a product with an absorption maximum at λ_{max}= 317 nm. This suggested that homogentisate was converted by a homogentisate-1,2-dioxygenase to maleylacetoacetate (Knox & Edwards 1955). The

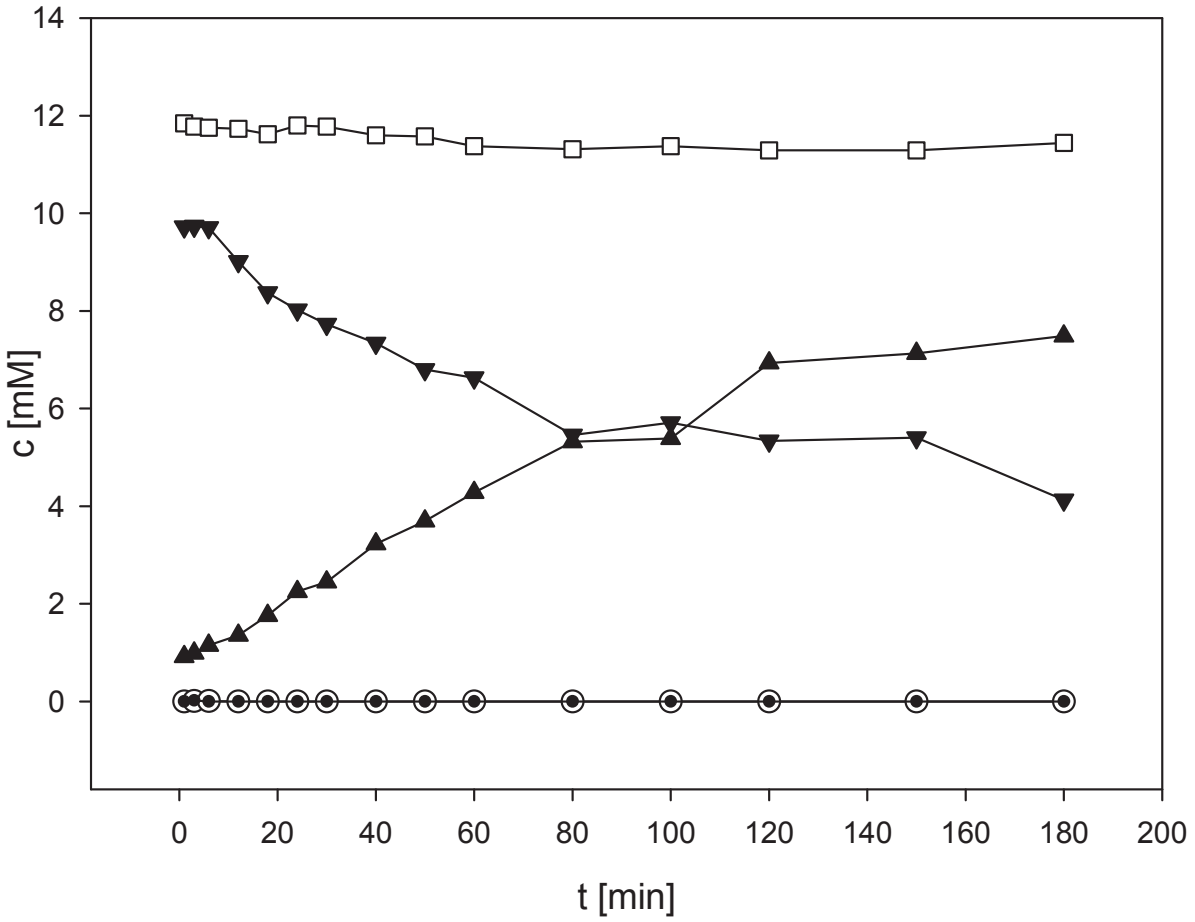

Fig. 4. Turn-over of 2-hydroxy-3-butenenitrile by resting cells of *E. oligosperma* R1 at pH 4. The resting cells were prepared as described in Fig. 3. The reaction mixture contained 0.1 M Na-citrate-phosphate-buffer (pH 4) and resting cells corresponding to an OD_{600nm} of 100. The control experiment contained 2 mL of the 0.1 M Na-citrate-phosphate-buffer (pH 4) without cells. The reaction mixtures were incubated in Eppendorf cups (2 mL at 30 °C in a thermoshaker (1 400 rpm). The reactions were started by the addition of 2-hydroxy-3-butenenitrile (10 mM). After different time intervals, samples (100 µL each) were taken, and the reactions terminated by the addition of 10 % (*v/v*) 1 M HCl. Cells were removed by centrifugation (21 000 g, 10 min, 4 °C) and the supernatants analysed by HPLC as described in the material and method section. The concentrations of 2-hydroxy-3-butenenitrile (▼), acroleine (●), and 2-hydroxy-3-butenoic acid (▲) in the experiment with cells as well as the concentrations of 2-hydroxy-3-butenenitrile (□) and acroleine (○) in the control experiment were calculated based on their signal intensities during the HPLC analysis.

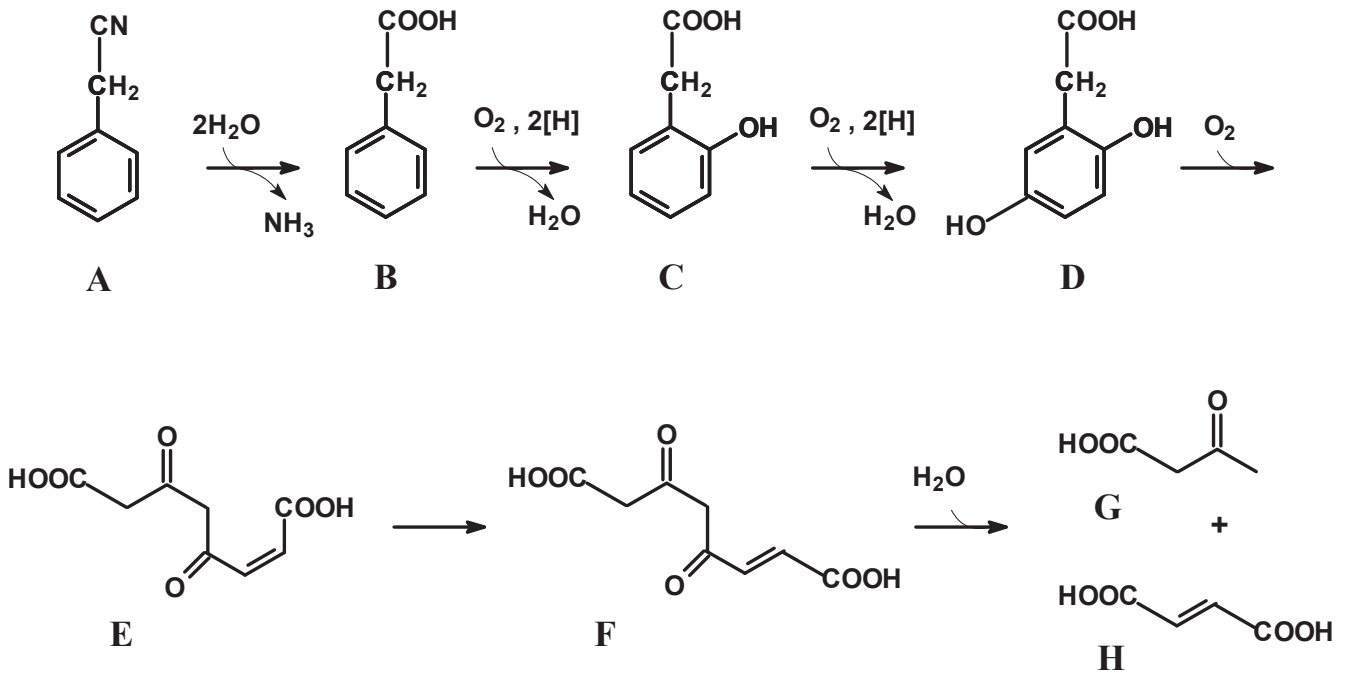

Fig. 5. Proposed pathway for the degradation of phenylacetonitrile by *E. oligosperma* R1. Key to compounds: A. phenylacetonitrile, B. phenylacetic acid, C. 2-hydroxyphenylacetic acid, D. 2,5-dihydroxyphenylacetic acid (homogentisic acid), E. maleylacetoacetic acid, F. fumarylacetoacetic acid, G. acetoacetic acid, H. fumaric acid.

specific activity was calculated from the known molar extinction coefficient of maleylacetoacetate (ε_{330nm}= 14 $mM^{-1}cm^{-1}$; Adachi *et al*. 1966) as 0.11 U mg^{-1} of protein. The reaction terminated when the initial concentration of homogentisate was converted and the calculated almost stoichiometric amounts of maleylacetoacetate were formed. The maleylacetoacetate was stable in the cuvettes for more than an hour. In most organisms maleylacetoacetate is converted by glutathione-dependent maleylacetoacetate isomerases (Fernánandez-Canón & Penalva 1998). Therefore, glutathione (2 mM) was added to the cuvettes and an immediate decrease in the absorbance at λ_{max}= 317 nm observed. This suggested that homogentisate is metabolised by *E. oligosperma* R1 via maleylacetoacetate and fumarylacetoacetate to fumarate and acetoacetate (Fig. 5). Thus, it appears that homogentisate is an important ring-fission substrate in "black yeasts", because it has already been suggested that *E. jeanselmei* (nowadays *Phialophora sessilis*) and *E. lecanii-corni* degrade compounds such as styrene and ethylbenzene via homogentisate (Cox *et al*. 1996; Gunsch *et al*. 2005; Prenafeta-Boldú *et al*. 2006).

DISCUSSION

The main aim of the present study was to obtain sufficient amounts of active biomass in order to allow a comparison of the nitrile converting system of *E. oligosperma* R1 with other (fungal) nitrile hydrolysing systems. The initial induction experiments demonstrated that the highest nitrile hydrolysing activities were achieved after growth of *E. oligosperma* R1 in the presence of 2-cyanopyridine. This compound had already been previously identified as a powerful inducer for the nitrile hydrolysing systems of different filamentous fungi such as *A. niger* K10, *Fusarium oxysporum* CCF1414, *F. oxysporum* CCF 483, *F. solani* O1, and *P. multicolor* CCF 244 (Kaplan *et al*. 2006b). 2-Cyanopyridine did not only serve as inducer of the nitrile converting activity of *E. oligosperma* R1 but was also metabolised and utilised as sole source of nitrogen as has been previously reported for *F. solani* O1 (Kaplan *et al*. 2006b).

The experiments with whole cells and cell extracts demonstrated that *E. oligosperma* R1 converted nitriles primarily to the corresponding acids and that the organism formed only from very few substrates low amounts of the corresponding amides. In addition, the cell extracts exhibited only a rudimentary amidase activity. Thus, it can be concluded that the nitriles were converted by a nitrilase. It therefore appears that most fungi convert organic nitriles using nitrilases and that nitrile hydratases are not common among fungi.

The extracts prepared from cells of *E. oligosperma* which had been grown in the presence of 2-cyanopyridine converted all available isomers of methyl-, hydroxy-, or chloro-substituted benzonitriles and phenylacetonitriles as well as phenylpropionitrile, 2-, 3- and 4-cyanopyridine. These cell extracts exhibited for these substrates compared to benzonitrile as substrate significantly higher relative activities than cell extracts or purified nitrilase fractions from *F. solani* O1, *P. multicolor* CCF 2244, or *A. niger* K10 (see Table 2). This was especially evident for the *meta*-substituted benzonitriles, because cell extracts of *E. oligosperma* R1 in general converted these substrates with higher specific activities than benzonitrile. In contrast, the opposite was reported for cell extracts from *F. solani* O1, *P. multicolor* CCF 2244, and the purified nitrilase of *A. niger* K10 (Kaplan *et al*. 2006a, c). The enzyme from *E. oligosperma* R1 also converted different *ortho*- substituted benzonitriles (*e.g.* 2-tolunitrile, 2-hydroxy- and 2-chlorobenzonitrile) which, probably due to sterical hindrances, could not be converted by the other strains (Kaplan *et al.* 2006a, c). In addition to the (substituted) benzonitrile(s) and phenylacetonitrile(s), the cell extracts from *E. oligosperma* R1 also converted aliphatic substrates such as acrylonitrile and 2-hydroxy-3-butenenitrile. The turn-over of acrylonitrile had not been analysed in the studies by Kaplan *et al*. (2006a, c) but had been previously described for another fungal nitrilase from *F. oxysporum* f. *sp melonis* (Goldlust & Bohak 1989). However, the enzyme of *F. oxysporum* f. *sp melonis* converted acrylonitrile (in comparison to benzonitrile as substrate) with significantly lower relative activities. Thus it can be concluded that the nitrile converting activity from *E. oligosperma* R1 appears to accept a slightly wider range of substrates than other fungal nitrilases.

The formation of amides as by-products of nitrilase catalyzed reactions had already been observed with various nitrilases from bacteria, plants, and fungi, *e.g.* from *Rhodococcus* ATCC 39484, *Pseudomonas fluorescens* EBC191, *Arabidopsis thaliana*, and *F. oxysporum* f. *sp. melonis*, and *A. niger* K10 (Goldlust & Bohak 1989, Stevenson *et al*. 1992, Effenberger & Osswald 2001, Osswald *et al*. 2002, Šnajdrová *et al.* 2004**,** Kiziak *et al*. 2005, Mateo *et al*. 2006, Fernandes *et al.* 2006, Kaplan *et al*. 2006b, c, Rustler *et al.* 2007). It was proposed that the formation of these by-products is based on an atypical cleavage of the tetrahedral intermediate formed during the reaction which might be triggered by electron withdrawing effects of different substituents (Fernandes e*t al*. 2006). The nitrilase activity from *E. oligosperma* R1 produced the largest relative amounts of amides during the induction experiments from 2-cyanopyridine (Fig. 2; ratio acid: amide about 7:4) and during the experiments with cell extracts (Table 2) from 2-chlorobenzonitrile (ratio acid: amide about 7:3). Furthermore some amides (< 10 molar % of total products) were also formed during the turn-over of 4-cyanopyridine and 3-chlorobenzonitrile. These results follow the emerging trend for the relationship between substrate structure and the degree of amide formation for fungal nitrilases. Thus, also *P. multicolor* CCF 2244 and the purified nitrilase from *A. niger* K10 produced relatively large amounts of amides from chlorobenzonitriles and cyanopyridines (Kaplan *et al*. 2006b, c). From the available data it appears that the nitrilase from *E. oligosperma* R1 is regarding to its tendency for amide formation situated somehow intermediate between the enzymes from *F. solani* O1 (less amide formation) and those from *P. multicolor* CCF 2244 and *A. niger* K10 (stronger tendency for amide formation).

In conclusion it might be summarised that the recent work in the group of L. Martinková (Kaplan *et al.* 2006a,b,c; Vejvoda *et al.* 2008) about the nitrile converting systems of filamentous fungi together with our study about the black yeast *E. oligosperma* R1 (Rustler & Stolz, 2007, this manuscript) suggest that evolutionary rather different fungi synthesise nitrilases which clearly resemble each other regarding their induction system and substrate specificity. These fungal systems might be of some relevance because of the high specific activities that can be obtained under optimal induction conditions and also the high specific activities of the purified enzymes with their preferred substrates. In addition, it was shown in the present study that the acid tolerance of fungi allows their utilisation as whole cell catalysts for the conversion of nitriles that are stabilised under acidic conditions. This observation might significantly enhance the importance of fungal nitrilases for biotransformation reactions.

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Biodiversity of the genus *Cladophialophora*

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Abstract: *Cladophialophora* is a genus of black yeast-like fungi comprising a number of clinically highly significant species in addition to environmental taxa. The genus has previously been characterized by branched chains of ellipsoidal to fusiform conidia. However, this character was shown to have evolved several times independently in the order *Chaetothyriales*. On the basis of a multigene phylogeny (nucLSU, nucSSU, *RPB*1), most of the species of *Cladophialophora* (including its generic type *C. carrionii*) belong to a monophyletic group comprising two main clades (*carrionii*- and *bantiana*-clades). The genus includes species causing chromoblastomycosis and other skin infections, as well as disseminated and cerebral infections, often in immunocompetent individuals. In the present study, multilocus phylogenetic analyses were combined to a morphological study to characterize phenetically similar *Cladophialophora* strains. Sequences of the ITS region, partial Translation Elongation Factor 1-α and β-Tubulin genes were analysed for a set of 48 strains. Four novel species were discovered, originating from soft drinks, alkylbenzene-polluted soil, and infected patients. Membership of the both *carrionii* and *bantiana* clades might be indicative of potential virulence to humans.

Key words: Biodiversity, bioremediation, *Cladophialophora*, chromoblastomycosis, disseminated infection, MLST, mycetoma.
Taxonomic novelties: *Cladophialophora samoënsis* Badali, de Hoog & Padhye, sp. nov., *Cladophialophora subtilis* Badali & de Hoog, sp. nov., *Cladophialophora mycetomatis* Badali, de Hoog & Bonifaz, sp. nov., *Cladophialophora immunda* Badali, Satow, Prenafeta-Boldú, Padhye & de Hoog, sp. nov.

INTRODUCTION

Cladophialophora is a genus of black yeast-like fungi which are remarkably frequently encountered in human infections, ranging from mild cutaneous lesions to fatal encephalitis. The genus is morphologically characterized by one-celled, ellipsoidal to fusiform, dry conidia arising through blastic, acropetal conidiogenesis, and arranged in branched chains. The chains are usually coherent and conidial scars are nearly unpigmented (Borelli 1980, Ho *et al.* 1999, de Hoog *et al.* 2000). The genus was initially erected to accommodate species exhibiting *Phialophora*-like conidiogenous cells in addition to conidial chains (Borelli 1980) but thus far this feature is limited to the species *C. carrionii*. Teleomorphs have not been found, but judging from SSU rDNA phylogeny data, these are predicted to belong to the ascomycete genus *Capronia*, a member of the order *Chaetothyriales* (Haase *et al.* 1999).

The type species of *Cladophialophora*, *C. carrionii*, is an agent of chromoblastomycosis, a cutaneous and subcutaneous disease histologically characterized by muriform cells in skin tissue. Muriform cells represent the invasive form of fungi causing chromoblastomycosis (Mendoza *et al.* 1993). Infections are supposed to originate by traumatic implantation of fungal elements into the skin and are chronic, slowly progressive and localised. Tissue proliferation usually occurs around the area of inoculation, producing crusted, verrucose, wart-like lesions. The genus has been expanded to encompass several other clinically significant species, including the neurotropic fungi *C. bantiana* and *C. modesta* causing brain infections (Horré & de Hoog 1999), *C. devriesii* and *C. arxii* causing disseminated disease (de Hoog *et al.* 2000) and *C. boppii, C. emmonsii* and *C. saturnica* causing cutaneous infections (de Hoog *et al.* 2007, Badali *et al*. 2008).

Based on molecular data, anamorphs morphologically similar to *Cladophialophora* have been found in other groups of ascomycetes, particularly in the *Dothideales* / *Capnodiales* (*e.g.*, *Pseudocladosporium*, *Fusicladium*; Crous *et al.* 2007). Distinction between chaetothyrialean and dothidealean / capnodialean anamorphs is now also supported by their teleomorphs. Braun & Feiler (1995) reclassified the dothidealean species *Venturia hanliniana* (formerly *Capronia hanliniana*) as the teleomorph of *Fusicladium brevicatenatum* (formerly *Cladophialophora brevicatenata*). Further distinction between *Chaetothyriales* and *Dothideales / Capnodiales* lies in their ecology, with recurrent human opportunists being restricted to the *Chaetothyriales*. Braun (1998), summarizing numerous statements in earlier literature, separated *Cladophialophora* with *Capronia* teleomorphs (*Herpotrichiellaceae, Chaetothyriales* mostly as opportunistic or pathogens), from the predominantly saprobic or plant associated isolates in the *Dothideomycetes*.

Some species attributed to *Cladophialophora* may be found in association with living plants. De Hoog *et al.* (2007) reported a cactus endophyte, *Cladophialophora yegresii*, as the nearest neighbour of *C. carrionii*, which is a major agent of human chromoblastomycosis. The latter fungus was believed to grow on debris of tannin-rich cactus spines, which were also supposed to be the vehicle of introduction into the human body. Crous *et al*. (2007) described several host-specific plant pathogens associated with the *Chaetothyriales*. *Cladophialophora hostae* caused spots on living leaves of *Hosta plantaginea*, *C. proteae* was a pathogen of *Protea*

Table 1. Isolation data of examined strains.

Name	CBS	Status	Other reference	GenBank ITS, *TUB*, *EF1α*	Source	Origin
Cladophialophora carrionii	CBS 114392		UNEFM 82267 = dH 13261	EU137267, EU137150, EU137211	Chromoblastomycosis; leg; female,	Venezuela, Falcon State
	CBS 114393		UNEFM 9801 = dH 13262	EU137268, EU137151, EU137212	Chromoblastomycosis; hand; male	Venezuela, Falcon State
	CBS 114396		UNEFM 2001/1 = dH 13265	EU137269, EU137152, EU137213	Chromoblastomycosis; arm; male	Venezuela, Falcon State
	CBS 114398		UNEFM 2003/1 = dH 13267	EU137271, EU137154, EU137215	Chromoblastomycosis; arm; female	Venezuela, Falcon State
	CBS 260.83		CDC B-1352 = FMC 282 = ATCC 44535	EU137292, EU137175, EU137234	Skin lesion in human	Venezuela, Falcon State
	CBS 160.54	LT	CDC A-835 =(ex-LT of *C. carrionii)* = ATCC 16264	EU137266, EU137201, EU137210	Chromoblastomycosis, human	Venezuela, Falcon State
Cladophialophora yegresii	CBS 114406		UNEFM SgSR1; dH 13275	EU137323, EU137208, EU137263	*Stenocereus griseus* asymptomatic plant	Venezuela, Falcon State
	CBS 114405		UNEFM SgSr3; dH 13276	EU137322, EU137209, EU137262	*Stenocereus griseus* asymptomatic plant	Venezuela, Falcon State
	CBS 114407		UNEFM SgSR1; dH 13274	EU137324, –, U137264	*Stenocereus griseus* asymptomatic plant	Venezuela, Falcon State
Cladophialophora emmonsii	CBS 640.96		CDC B-3634; NCMH 2248; 4991	EU103995, –, U140584	Sub-cutaneous lesion, cat	–
	CBS 979.96		CDC B-3875; NCMH 2247	EU103996, –, U140583	Sub-cutaneous lesion right forearm, human	U.S.A., Virginia
Cladophialophora boppii	CBS 126.86		FMC 292; dH 15357	EU103997, –, U140596	Skin lesion, on limb, male	Brazil
	CBS 110029		det M-41/2001 56893; dH 12362	EU103998, –, U140597	Scales of face, male	Netherlands, Dordrecht
Cladophialophora bantiana	CBS 173.52	T	CBS 100433	EU103989, –, U140585	Brain abscess, male	U.S.A.
	CBS 444.96		–	EU103994, –, U140591	Disseminated infection, dog	South Africa, Pretoria, Onderstepoort
	CBS 678.79		CDC B-3658; NCMH 2249; NIH B-3839	EU103992, –, U140592	Skin lesion, cat	U.S.A., Bethesda
	CBS 648.96		UAMH 3830	EU103993, –, U140587	Liver, dog	Barbados
Cladophialophora saturnica	CBS 109628		dH 12333; IHM 1727	EU103983, –, U140601	Dead tree	Uruguay, Isla Grande del Queguay
	CBS 109630		dH 12335; IHM 1733	FJ385270, –, –	Trunk, cut tree	Uruguay, Isla Grande del Queguay
	CBS 118724	T	157D; dH 12939	EU103984, – , EU140602	Interdigital toe lesion, child	Brazil, Paraná, Curitiba
	CBS 102230		dH 11591; 4IIBPIRA	AY857508, – , EU140600	Litter, vegetable cover/soil	Brazil, Paraná, Curitiba
	CBS 114326		ATCC 200384	AY857507, –, EU140603	Toluene biofilter	Netherlands, Wageningen
Cladophialophora devriesii	CBS 147.84	T	ATCC 56280; CDC 82-030890	EU103985, –, EU140595	Disseminated infection, male	U.S.A., Grand Cayman Island
	CBS 118720		ISO 13F	FJ385275, –, –	Litter, vegetable cover/soil	Brazil, Paraná, Curitiba
Cladophialophora arxii	CBS 306.94	T		EU103986, – , EU140593	Tracheal abscess, male	Germany
Cladophialophora minourae	CBS 987.96		IFM 4701; UAMH 5022	EU103988, – , EU140599	Rotting wood	Japan, Yachimata, Chiba
	CBS 556.83		ATCC 52853; IMI 298056	AY251087, – , EU140598	Decaying wood	Japan, Shiroi

Table 1. (Continued).

Name	CBS	Status	Other reference	GenBank ITS, *TUB*, *EF1α*	Source	Origin
Cladophialophora immunda	CBS 110551		dH15250	FJ385274, EU137207, EU137261	Gasolin-station soil	Netherlands, Apeldoorn
	CBS 109797		dH 11474	FJ385271, EU137206, EU137260	Biofilter inoculated with soil	Germany, Kaiserslautern
	CBS 834.96	T	CDCB-5680; de H.10680	EU137318, EU137203, EU137257	Sub-cutaneous phaeohyphomycosis, male	U.S.A., Georgia, Atlanta
	CBS 102227		dH 11588	FJ385269, –, EU137259	Syagrum romanzoffianum, stem	Brazil, Paraná, Colombo
	CBS 102237		dH 11601	FJ385272, EU137205, EU137285	Decaying cover vegetable	Brazil, Paraná, Sarandi
Cladophialophora samoënsis	CBS 259.83	T	CDC B-3253; dH 15637	EU137291, EU137174, EU137233	Chromoblastomycosis skin lesion, male	U.S.A., Samoa
Cladophialophora subtilis	CBS 122642	T	dH 14614	FJ385273, –, –	Ice tea	Netherlands, Utrecht
Cladophialophora mycetomatis	CBS 454.82		dH 15898	EU137293, EU137176, EU137235	Culture contaminant	Netherlands
	CBS 122637	T	dH 18909	FJ385276, –, –	Eumycetoma, male	Mexico, Jicaltepec
Fonsecaea monophora	CBS 289.93	T	dH 15691	AY366925, EU938554, –	Lymphnode, aspiration-biopsy	Netherlands (zoo)
	CBS 102238		dH 11602, 1PLE	AY366927, EU938546, –	Soil	Brazil
	CBS 102248		dH 11613	AY366926, EU938550, –	Chromoblastomycosis, male	Brazil
Fonsecaea pedrosoi	CBS 271.37	T	ATCC 18658; IMI 134458; dH 15659	AY366914, EU938559, –	Chromoblastomycosis, male	South America
	CBS 272.37		dH 15661	AY366917, –, –	Chromoblastomycosis, male	–
Cladophialophora australiensis	CBS 112793		CPC 1377	EU035402, –, –	Sports drink	Australia
Cladophialophora chaetospira	CBS 491.70		–	EU035405, –, –	Roots of *Picea abies*	Denmark
Cladophialophora potulenturum	CBS 112222		CPC 1376; FRR 4946	EU035409, –, –	Sports drink	Australia
	CBS 114772		CPC 1375; FRR 4947	EU035410, –, –	Sports drink	Australia

Abbrevations used: ATCC = American Type Culture Collection, Manassas, U.S.A.; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DH = G.S. de Hoog private collection; IFM = Research Institute for Pathogenic Fungi, Chiba, Japan; IHM = Laboratory of Mycology, Faculty of Medicine, Montevideo Institute of Epidemiology and Hygiene, Montevideo, Uruguay; IMI = International Mycological Institute, London, U.K.; IWW = Rheinisch Westfählisches Institut für Wasserforschung, Mülheim an der Ruhr, Germany; GHP = G. Haase private collection; MUCL = Mycotheque de l'Université de Louvain, Louvain-la-Neuve, Belgium; NCMH = North Carolina Memorial Hospital, Chapel Hill, U.S.A.; RKI = Robert Koch Institute, Berlin, Germany; UAMH = Microfungus Herbarium and Collection, Edmonton, Canada; UTHSC = Fungus Testing Laboratory, Department of Pathology, University of Texas Health Science Center at San Antonio, U.S.A.; UTMB = Medical Mycology Research Center, Galveston, U.S.A.; UNEFM = Universidade Nacional Experimental Francisco de Miranda, Coro, Falcon, Venezuela. *T = ex-type culture; LT = ex-lectotype culture;*

cynaroides, and *C. scillae* caused leaf spots on *Scilla peruviana*. Finally, Davey & Currah (2007) described *Cladophialophora minutissima* from mosses collected at boreal and montane sites in central Alberta, Canada. Within the *Chaetothyriales*, most of these plant-associated species are found at relatively large phylogenetic distance from the main clade of *Cladophialophora* comprising most of the opportunistic species.

The core of the genus *Cladophialophora* does comprise a number of environmental saprobes. *Cladophialophora minourae* and *C. chaetospira* occur in plant litter. Badali *et al.* (2008) described *C. saturnica* from plant debris in the environment, but the species was also found causing an interdigital infection in a Brazilian child with HIV infection. The species *C. australiensis* and *C. potulentorum* were found in soft drinks (Crous *et al*. 2007). If environmental species are able to provoke opportunistic infections, the question arises whether members of *Chaetothyriales* isolated from food products might imply a health risk. Understanding of the phylogeny and ecology of *Cladophialophora* is therefore essential. Many review articles incorrectly mention that black yeast-like fungi are commonly found on decomposing plant debris and in soil. In fact, Chaetothyrialean members are difficult to isolate from the environment as they seem to have quite specific, hitherto undiscovered ecological niches (Satow *et al.* 2008, Vicente *et al.* 2008). For their isolation, selective methods are required, *e.g.* by the use of high temperatures (Sudhadham *et al.* 2008), a mouse vector (Gezuele *et al.* 1972, Dixon *et al.* 1980), alkyl benzenes (Prenafeta-Boldú *et al.* 2006) or isolation via mineral oil (Satow *et al.* 2008, Vicente *et al.* 2001, 2008). An association with assimilation of toxic monoaromatic compounds has been hypothesised. Black yeasts and their filamentous relatives in the *Chaetothyriales* are potent degraders of monoaromatic compounds and tend to accumulate in industrial biofilters (Cox *et al*. 1997, Prenafeta-Boldú *et al.* 2001, de Hoog *et al.* 2006). This might be a clue to dissecting their dual behavior as rare environmental oligotrophs as well as invaders of human tissue containing aromatic neurotransmitters.

The present paper combines ecological information with phylogenetic and taxonomic data, and interprets them in the light of potential health hazards of seemingly saprobic species that may occur in food products. We applied multilocus sequence analysis and phenetic characterization to distinguish novel *Cladophialophora* species from various sources.

Table. 2. Primer sequences for PCR amplification and sequencing.

Gene	PCR primers	Sequencing primers	References
ITS rDNA	V9G[a], LS266[b]	ITS1[c], ITS4[c]	[a] de Hoog *et al*. (1998) [b] Masclaux *et al.* (1995) [c] White *et al*. (1990)
TUB	Bt2a, Bt2b	Bt2a, Bt2b	Glass & Donaldson (1995)
EF1-α	EF1-728F, EF1-986R	EF1-728F, EF1-986R	Carbone & Kohn (1999)
SSU rDNA	NS1[a], NS24[b]	(BF83, Oli1, Oli9, BF951, BF963, BF1438, Oli3, BF1419)[c]	[a] White *et al*. (1990) [b] Gargas & Taylor (1992) [c] de Hoog *et al.* (2005)

MATERIALS AND METHODS

Fungal strains

Strains used in this study were obtained from the Centraalbureau voor Schimmelcultures (Table 1). Stock cultures were maintained on slants of 2 % malt-extract agar (MEA, Difco) and oatmeal agar (OA, Difco) and incubated at 24 °C for two weeks (Gams *et al.* 1998). All cultures in this study are maintained in the culture collection of CBS (Utrecht, The Netherlands) and taxonomic information for new species was deposited in MycoBank (www.MycoBank.org).

DNA extraction

The fungal mycelia were grown on 2 % (MEA) plates for 2 wks at 24 °C (Gams *et al.* 1998). A sterile blade was used to scrape off the mycelium from the surface of the plate. DNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA 92010, U.S.A.) according to the manufacturer's instructions. DNA extracts were stored at −20 °C prior to use.

Amplification and sequencing

Four genes were amplified: the internal transcribed spacer region (ITS), the translation elongation factor 1 alpha (*EF1-α*), the partial beta tubulin gene (*TUB*), and the small subunit of the nuclear ribosomal RNA gene (nucSSU). The primers used for amplification and sequencing are shown in Table 2. PCR reactions were performed on a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA) in 50 µL volumes containing 25 ng of template DNA, 5 µL reaction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M KCl, 15 mM $MgCl_2$, 0.1 % gelatine, 1 % Triton X-100), 0.2 mM of each dNTP and 2.0 U Taq DNA polymerase (ITK Diagnostics, Leiden, The Netherlands). Amplification of ITS and nucSSU was performed with cycles of 2 min at 94 °C for primary denaturation, followed by 35 cycles at 94 °C (45 s), 52 °C (30 s) and 72 °C (120 s), with a final 7 min extension step at 72 °C. Annealing temperatures used to amplify EF1α and *TUB* genes were 55 and 58 °C, respectively. Amplicons were purified using GFX PCR DNA and gel band purification kit (GE Healthcare, Ltd., Buckinghamshire U.K.). Sequencing was performed as follows: 95 °C for 1 min, followed by 30 cycles consisting of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 2 min. Reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and sequencing was done on an ABI 3730XL automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data obtained in this study were adjusted using the SeqMan of Lasergene software (DNAStar Inc., Madison, Wisconsin, U.S.A.).

Alignment and phylogenetic reconstruction

Phylogenetic analyses were carried out on three different datasets. The first dataset included a taxon sampling representative of the order *Chaetothyriales* (94 taxa) and the three genes nucLSU, nucSSU and *RPB*1. This dataset was used in order to assess the phylogenetic placement of diverse species of *Cladophialophora* within the *Chaetothyriales*. For this first analysis, sequences of nucSSU obtained from investigated strains of *Cladophialophora* were added to an existing dataset representing the *Chaetothyriales* (Gueidan *et al.* 2008). Alignments were done manually for each gene

using MacClade 4.08 (Maddison & Maddison 2003) with the help of amino acid sequences for protein coding loci. Ambiguous regions and introns were excluded from the alignments. The program RAxML-VI-HPC v.7.0.0 (Stamatakis *et al.* 2008), as implemented on the Cipres portal v.1.10, was used for the tree search and the bootstrap analysis (GTRMIX model of molecular evolution and 500 bootstrap replicates). Bootstrap values equal or greater than 70 % were considered significant (Hillis & Bull 1993).

The second and third datasets focused on two main monophyletic clades nested within the core group of *Cladophialophora* (Clade I and Clade II, Fig. 1). They included three genes, ITS, EF1α and *TUB*. The goal of these two analyses was to assess the delimitation of species of *Cladophialophora*. The second dataset (Clade I or *carrionii*-clade) comprised 15 taxa, and the third dataset (Clade II or *bantiana*-clade) 33 taxa. Phylogenetic reconstructions and bootstrap values were first obtained for each locus separately using RAxML (as described above). The congruence between loci was assessed using a 70 % reciprocal bootstrap criterion (Mason-Gamer & Kellogg 1996). The loci were then combined and analysed using RAxML (as described above). The phylogenetic trees were edited using Tree View v.1.6.6.

Morphological identification and cultural characterisation

Strains were cultured on 2 % MEA and OA and incubated at 24 °C in the dark for two wks (Gams *et al.* 1998). Identification was based primarily on macroscopic and microscopic morphology. Microscopical observations were based on slide culture techniques using potato dextrose agar (PDA) or OA because these media readily induce sporulation and suppress growth of aerial hyphae (de Hoog *et al.* 2000). Mounts of two-wk-old slide cultures were made in lactic acid or lactophenol cotton blue and light micrographs were taken using Nikon Eclipse 80*i* microscope with a Nikon digital sight DS-Fi1 camera.

Physiology

Cardinal growth temperatures of strains were determined on 2 % MEA (Difco) (Crous *et al*. 1996). Plates were incubated in the dark for two wks at temperatures of 6–36 °C at intervals of 3 °C; in addition, growth at 40 °C was recorded. Experiments consisted of three simultaneous replicates for each isolate; the entire procedure was repeated once.

Muriform cells

All strains were tested for the production of muriform cells, *i.e.*, the meristematic tissue form of agents of human chromoblastomycosis. Strains were incubated at 25 and 37 °C for one wk in a defined medium with low pH containing 0.1 mM/L calcium chloride. The basal medium was prepared by adding the following components to 1 L deionized distilled water: 30 g glucose, 3 g $NaNO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.265 g NH_4Cl, 0.003 g thiamin and 1 mM $CaCl_2$; the pH was adjusted to 2.5 with HCl for all experiments (Mendoza *et al.* 1993).

RESULTS

Phylogeny

Chaetothyriales dataset

Fig. 1 shows that, in the order *Chaetothyriales*, species of *Cladophialophora* belong to at least four different lineages: one lineage corresponding to the family *Herpotrichiellaceae* (lineage 1, Fig. 1), and three basal lineages including mostly rock-inhabiting strains (lineages 2, 3 and 4, Fig. 1). Most of the human-opportunistic species (including the most virulent ones) belong to two well-supported clades within the *Herpotrichiellaceae*. Clade I (*carrionii*-clade, Fig. 1, 2) includes the pathogenic *Cladophialophora* species *C. carrionii* and *C. boppii*, as well as two pathogenic species of *Phialophora* (*P. verrucosa* and *P. americana*). Clade II (*bantiana*-clade, Fig. 1, 3) includes the pathogenic species *C. bantiana*, *C. arxii*, *C. immunda*, *C. devriesii, C. saturnica, C. emmonsii,* and *C. mycetomatis*, as well as the pathogenic genus *Fonsecaea* (*F. pedrosoi* and *F. monophora*). All the plant associated species of *Cladophialophora* belong to lineages 2, 3 and 4. Lineage 2 includes rock-inhabiting strains and the opportunistic pathogen *C. modesta*. Lineage 3 includes rock-inhabiting strains, the two opportunistic pathogens *P. europaea,* and *C. laciniata*, and three plant associated species of *Cladophialophora* (*C. proteae, C. hostae,* and *C. scillae*). Lineage 4 includes exclusively rock-inhabiting strains and plant leaving strains (C. *minutissima, C. humicola,* and *C. sylvestris*).

Carrionii-clade dataset

Phylogenetic reconstructions of Clade I (*carrionii*-clade; Fig. 2) were first carried out for each gene separately (ITS: 542 characters, *EF1-α*: 117 characters, *TUB*: 402 characters). Topological conflicts were detected for all genes within both *C. carrionii* and *C. yegresii*. This incongruence involving only below species-level relationships, and therefore – in agreement with the genealogical recognition species concept (Taylor *et al.* 2000) –, the conflicts were ignored and the three loci were combined. In this combined analysis, *C. boppii*, an agent of cutaneous infection, was taken as an out-group for clade I (Fig. 2). The combined analysis shows that both the pathogenic species *C. carrionii* (represented by 6 strains) and the environmental species *C. yegresii* (represented by 3 strains) are monophyletic and well supported (100 % bootstrap, Fig. 2). The two species of *Phialophora* (*P. verrucosa* and *P. americana*) are sister taxa (100 % bootstrap, Fig. 2), and are nested among species of *Cladophialophora*. Two newly investigated strains (CBS 122642, isolated from soft drink; CBS 259.83, isolated from a patient with chromoblastomycosis) are shown to be phylogenetically distinct from other members of Clade I, and are described below as new species (*C. samoënsis* and *C. subtilis*).

Bantiana-clade dataset

Phylogenetic reconstructions of Clade II (*bantiana*-clade, Fig. 3) were first carried out for each gene separately (ITS: 495 characters, *EF1-α*: 133 characters, and *TUB*: 355 characters). As topological incongruence was detected only within the species *C. saturnica* (relationships obtained with ITS differed from relationships obtained with EF1- α or *TUB*), the loci were combined. The resulting tree was rooted using *C. mycetomatis*, a species newly described here. A phylogenetic analysis at larger scale (Fig. 1) shows that this taxon is phylogenetically distinct from other species of *Cladophialophora*, and sister to all the other members of the *bantiana*-clade. The three species *C. immunda*, *C. devriesii* and *C. saturnica* all form

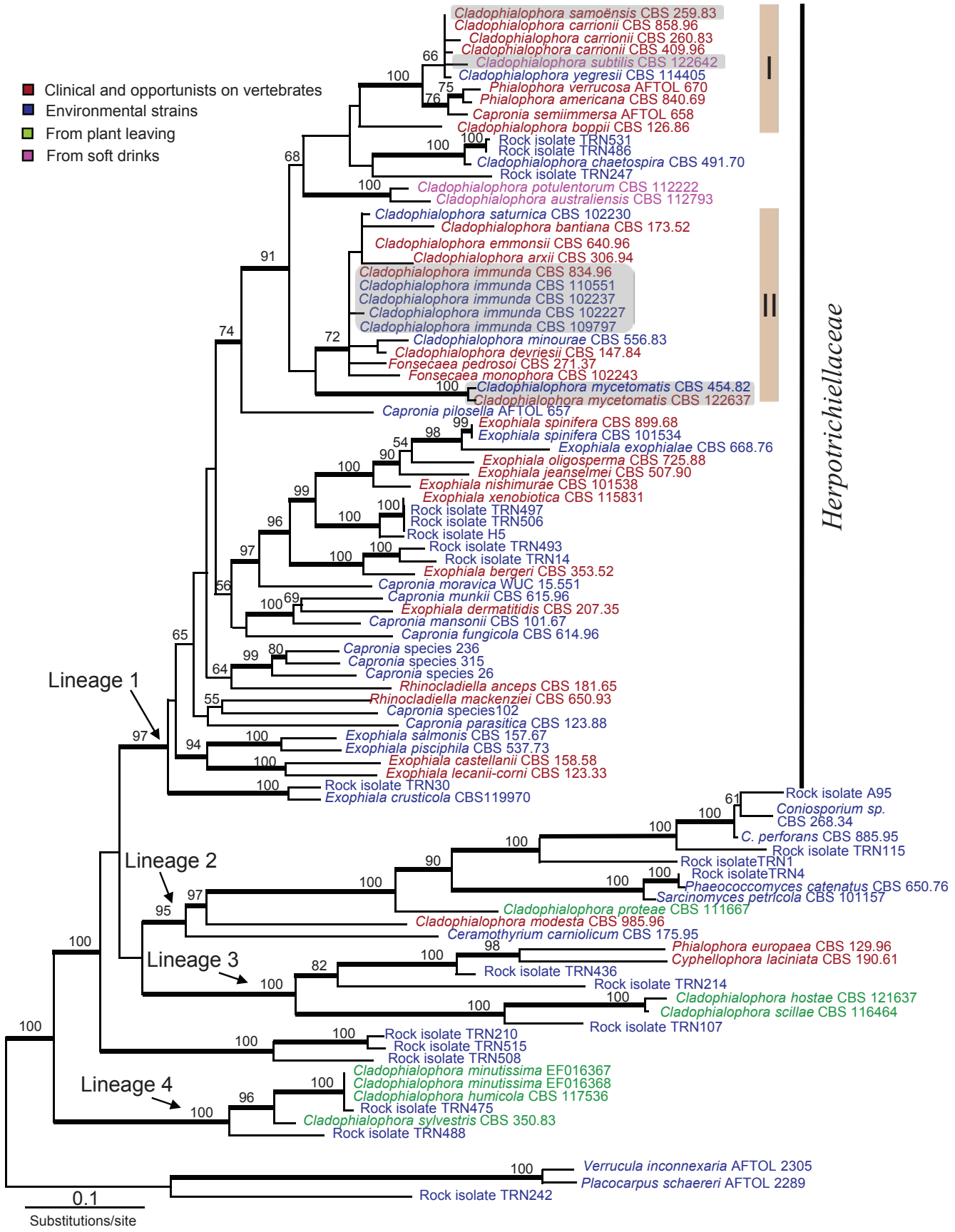

Fig.1. Phylogeny obtained from a ML analysis of three combined loci (SSU, LSU and *RPB1*) using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches (thick branch for values ≥ 70 %). The tree was rooted using *Verrucula inconnexaria*, *Placocarpus schaereri* and the rock isolate TRN242. New species are highlighted with grey boxes.

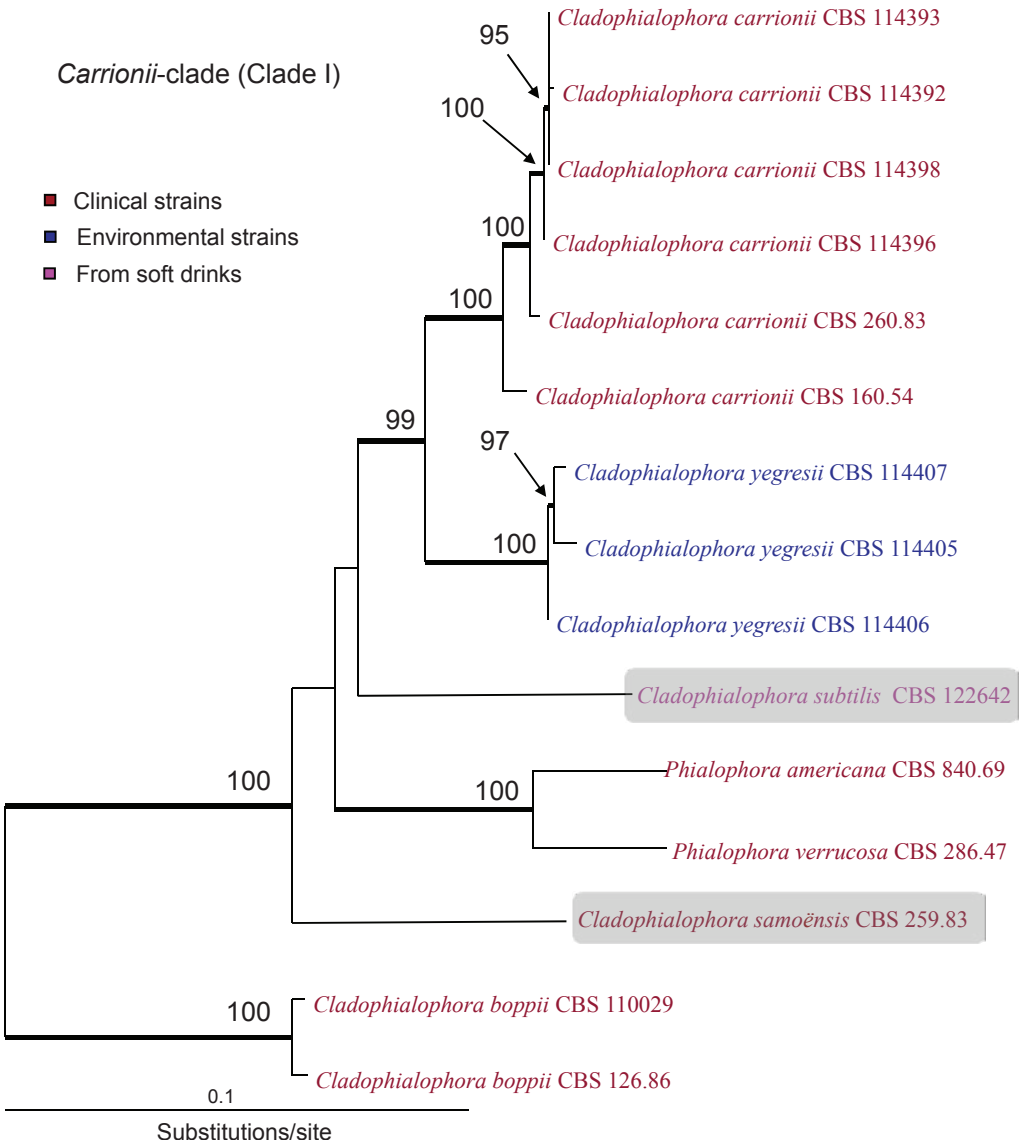

Fig. 2. Phylogeny of Clade I obtained from a ML analysis of three combined loci (ITS rDNA, *EF1-α* and *TUB*) using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches (thick branch for values ≥ 70 %). New species are highlighted using with grey boxes. *Cladophialophora boppii* (CBS 126.86 and CBS 110029) were taken as outgroup.

monophyletic clades including both clinical and environmental strains. For *C. immunda*, strains from different geographical areas and ecological preferences all cluster together. The environmental species *C. minourae* is sister to the pathogenic species *C. arxii*. The truly pathogenic species *C. bantiana* is sister to *C. emmonsii*, although with no support. Finally, the genus *Fonsecaea* forms a well-supported monophyletic group nested within this clade of *Cladophialophora*.

Physiology

The cardinal growth temperature test showed that all cultures obtained in this study had their optimal development at 27-30 °C, with growth abilities ranging between 9-37 °C. No growth was observed at 40 °C. For *C. samoënsis*, *C. immunda* and *C. mycetomatis,* the optimum growth temperature on MEA and PDA was 27 °C, with minimum and maximum of 15 and 37 °C, respectively. For all the other species, growth temperatures were identical except for the minimum temperature, which was 12 °C in *C. subtilis*. However, neither plant associated species nor strains isolated from sport drink nor apple juice (*C. australiensis* and *C. potulentorum*) had the ability to grow at 37 and 40 ° C (Fig. 4). Growth characteristics were studied at low pH after addition of 0.1 mM $CaCl_2$ to the basal medium, inducing conversion of hyphae of *Cladophialophora* species into muriform cells when incubated at 25−37 °C (Table 3). *Cladophialophora subtilis* developed extensive mycelia and produced muriform cells at 25 °C after one wk incubation (Fig. 5). Hyphae were generally attached to these muriform cells in either terminal or intercalary positions (Fig. 5). However, no muriform cells were observed under the same conditions at 25 °C in other species (*C. immunda, C. mycetomatis* and *C. samoënsis*). Hyphae of *C. subtilis, C. immunda* and *C. samoënsis* converted to large numbers of muriform cells when incubated in the same conditions at 37 °C. Moreover, muriform cells were not observed for plant-associated species of *Cladophialophora* and for *C. mycetomatis* and *C. yegresii* neither at 25 nor at 37 °C (Table 3).

Taxonomy of *Cladophialophora*

Cladophialophora carrionii (Trejos) de Hoog, Kwon-Chung & McGinnis, J. Med. Vet. Mycol. 33: 345 (1995).

≡ *Cladosporium carrionii* Trejos, *Revista de Biologia Tropicale*, Valparaiso 2:

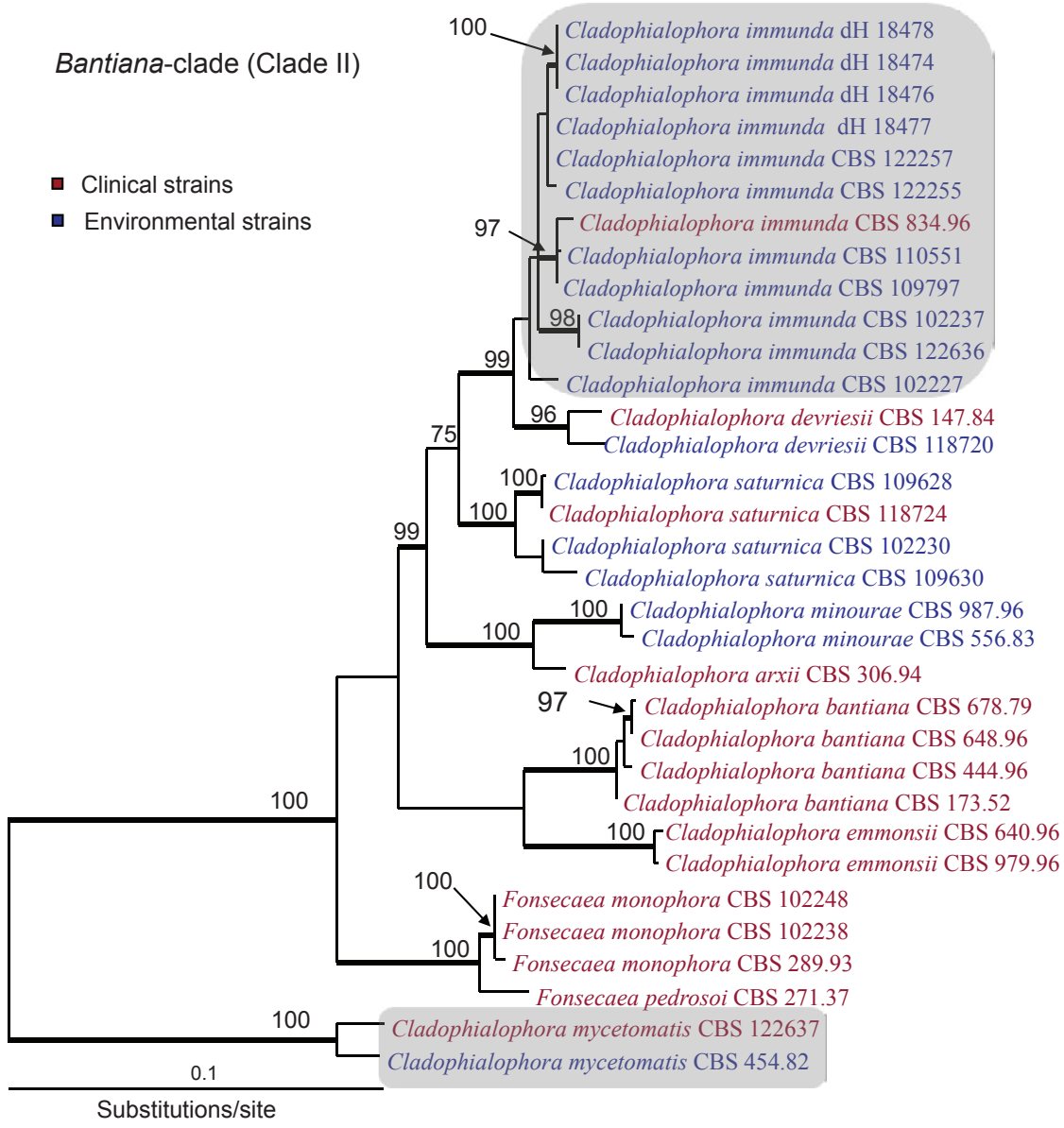

Fig. 3. Phylogeny of Clade II obtained from a ML analysis of three combined loci (ITS rDNA, *EF1-α* and *TUB*) using RAxML. Bootstrap support values were estimated based on 500 replicates, and are shown above the branches (thick branch for values ≥ 70 %). New species are highlighted using with grey boxes. The tree was rooted with two strains of *Cladophialophora mycetomatis* (CBS 454.82 and CBS 122637).

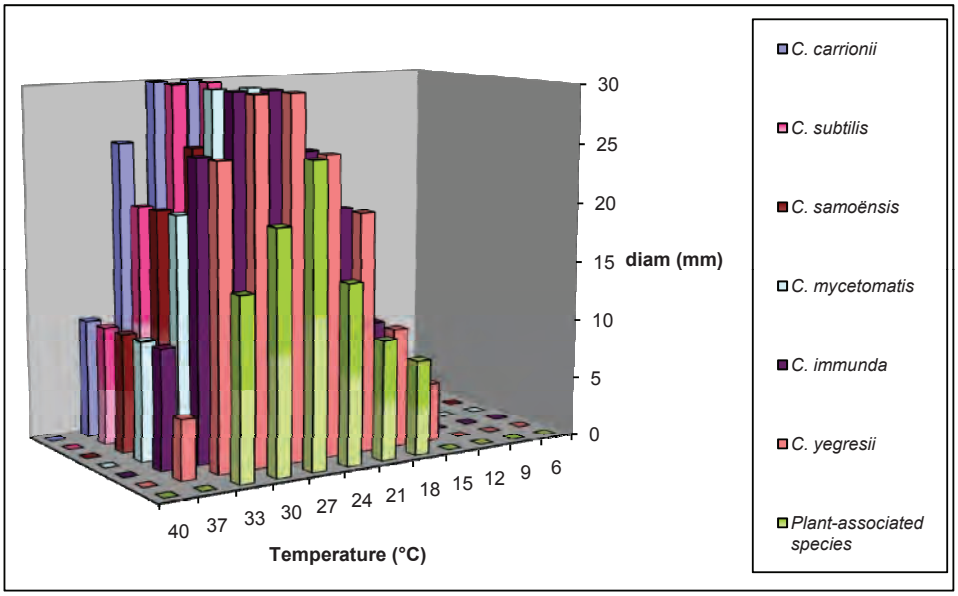

Fig. 4. Colony diameters of novel *Cladophialophora* species at different temperatures ranging from 6 to 40 °C, measured after two wks on 2 % MEA.

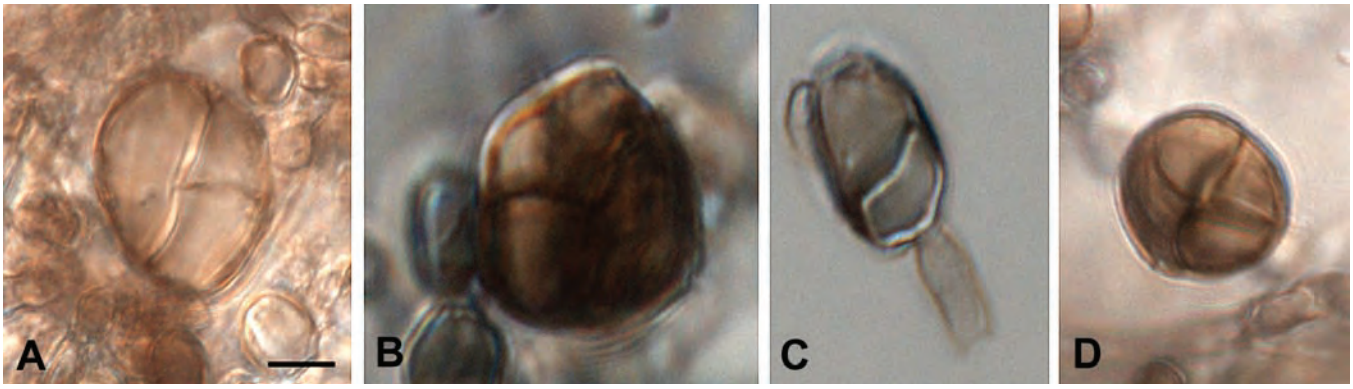

Fig. 5. Morphology of muriform cells in three species of *Cladophialophora*. A–B. *Cladophialophora subtilis*. C. *Cladophialophora immunda*. D. *Cladophialophora carrionii*. Scale bar = 10 µm.

Table. 3. Effect of calcium and temperature on production of muriform cells under the acidic conditions in basal medium for 13 species of *Cladophialophora*.

***Cladophialophora* species**	**Muriform Cells at 25 °C, 0.1 mM Ca^{2+} and pH 2.5**	**Muriform Cells at 37 °C, 0.1 mM Ca^{2+} and pH 2.5**
C. carrionii	+	++
C. samoënsis	–	+
C. subtilis	+	+++
C. mycetomatis	–	–
C. immunda	–	+
C. australiensis	–	–
C. potulentorum	–	–
C. yegresii	–	–
C. sylvestris	–	–
C. humicola	–	–
C. hostae	–	–
C. proteae	–	–
C. scillae	–	–

No production of muriform cells is indicated by –, low production of small muriform cells by +, moderate production of large muriform cells by ++, and large production of large muriform cells by +++.

106 (1954)
= *Cladophialophora ajelloi* Borelli, *Proceedings of the 5th International Conference on Mycoses*: 335 (1980)

Type: Trejos 27 (CBS H-18465, lectotype designated here; CBS 160.54 = ATCC 16264 = CDC A-835 = MUCL 40053, ex-type).

Trejos (1954) introduced *Cladophialophora carrionii* but he did not indicate a holotype. For this reason, the isolate Trejos 27 = Emmons 8619 = CBS 160.54, the first strain mentioned by Trejos (1954), is selected here as **lectotype** for *C. carrionii*. The ex-type strain of *Cladophialophora ajelloi*, CBS 260.83, proved to be indistinguishable from *C. carrionii* based on both morphology and molecular data. This former species was also known to be able to produce phialides in addition to catenate conidia (Honbo *et al*. 1984). *Cladophialophora ajelloi* is here proposed as a taxonomic synonym of *C. carrionii*.

Cladophialophora samoënsis Badali, de Hoog & Padhye, **sp. nov**. MycoBank MB511809. Fig. 6–7.

Etymology: Named after Samoa, the Pacific Island where the species was encountered in a human patient.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies, olivaceo-virides vel griseae, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, 2–3 µm latae. Conidiophora semi-macronemata, septata, lateralia vel terminalia; stipites et ramoconidia denticulata. Conidia holoblastica, dilute brunnea, late fusiformia, unicellularia, levia, catenas longas ramosas cohaerentes formantia, cicatricibus dilute brunneis, 3–4 × 2–3 µm. Synanamorphe not visa. Chlamydosporae absentes. Teleomorphe ignota.

Description based on CBS 259.83 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies growing moderately slowly, reaching up to 30 mm diam, olivaceous-green to grey, with a thin, dark, well-defined margin, dry, velvety, darker after 4 wks; reverse olivaceous-black. Cardinal temperatures: minimum 15 °C, optimum 27−30 °C, maximum 37 °C. No growth at 40 °C.

Microscopy: Budding cells absent. Hyphae smooth, hyaline to pale brown, branched, 2−3 µm wide, locally forming hyphal

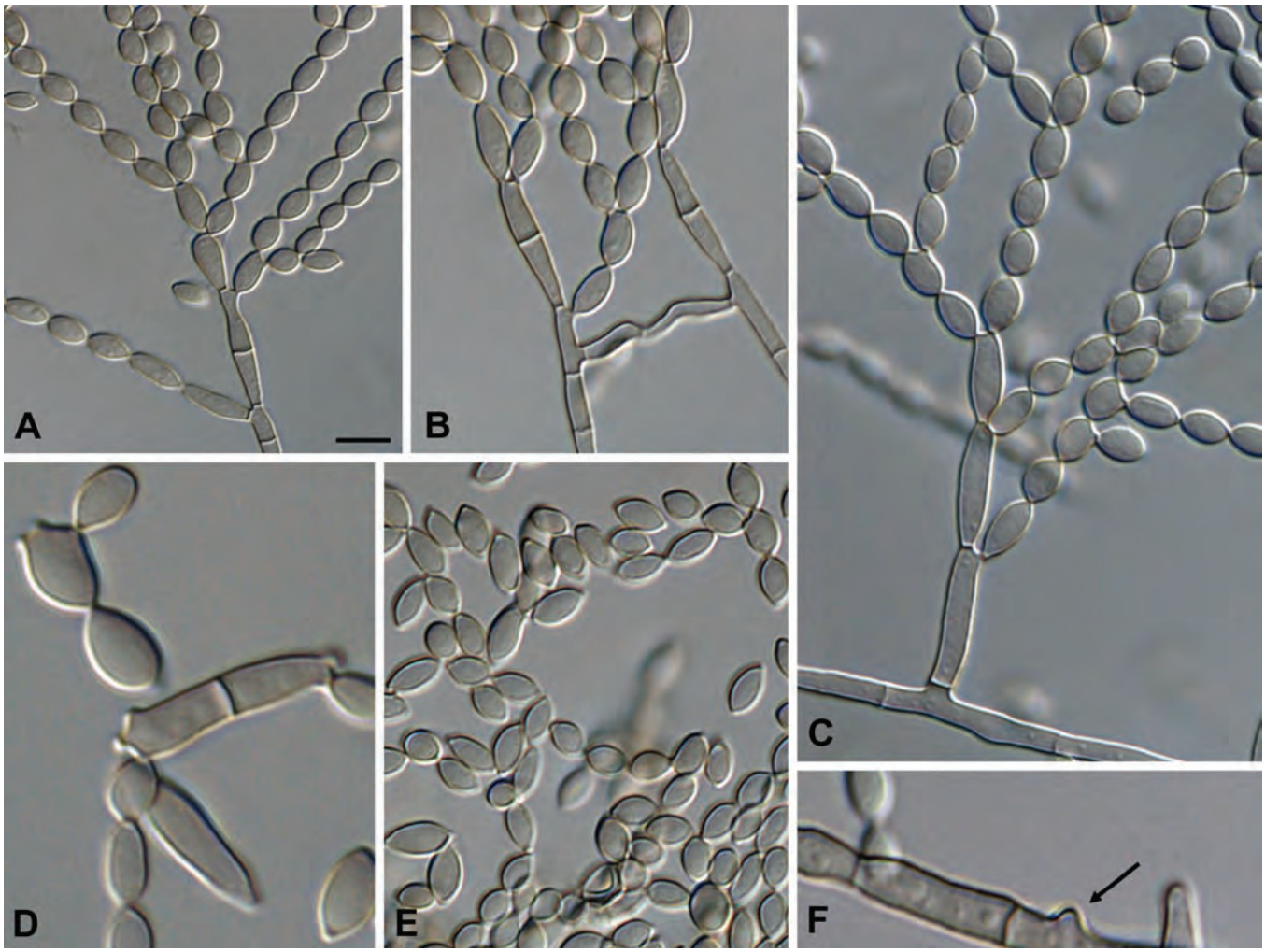

Fig. 6. *Cladophialophora samoënsis* (CBS 259.83). A. Conidiophore. B–E. Conidial chains with ramoconidia and conidia. F. Conidiogenous loci (arrows). Scale bar = 10 µm.

strands and coils. Conidiophores semi-macronematous, septate, lateral or terminal, with denticles on the stipe and on 0–1-septate ramoconidia. Conidia holoblastic pigmented, broadly fusiform, one-celled, pale brown, smooth-walled, forming long, cohering, branched acropetal chains of conidia; conidial scars pale. Conidia 3–4 × 2–3 µm. Synanamorph not seen. Chlamydospores absent. Teleomorph unknown.

Specimen examined: **U.S.A.**, Samoa (Pacific), isolated from human patient with chromoblastomycosis, November 1979 (CBS H-20113, **holotypus**; CDC B-3253 = CBS 259.83, ex-type).

Notes: This strain (CBS 259.83, from Samoa) was previously identified as *C. ajelloi* (Goh *et al*. 1982). In our multigene analysis, it clustered within the *carrionii* complex (Clade I; Fig. 2), but proved to be consistently different from all described species.

Case Report: According to the description by Goh *et al.* (1982), a healthy, 43-yr-old male patient had a 5 × 3 cm erythematous, scaling lesion on his arm which was first observed about 3 yr earlier. The patient did not remember the circumstances under which the infection had been acquired. Muriform cells were revealed in superficial dermis and stratum corneum after skin scrapings. Histological examination showed segments of well-differentiated stratified squamous epithelium with moderate keratin production and underlying coarsely fibrillar dermal connective tissue. In this tissue, dense aggregates of chronic inflammatory cells, including lymphocytes, plasma cells and multinucleated foreign bodies (giant cells), were observed. Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained sections characterized dark brown, thick-walled, multiseptate muriform cells, measuring 6−12 µm in diameter, and dividing by fission. The histopathological observations led to the diagnosis of chromoblastomycosis, and the strain was identified as *C. ajelloi* (Goh *et al.* 1982). The taxon name *C. ajelloi* is not available for this taxon, as it was shown here to be a synonym of *C. carrionii*. Hence *Cladophialophora samoënsis* is described as a novel agent of chromoblastomycosis. Morphological observation showed branched chains of holoblastic conidia identical to those of *Cladophialophora carrionii*.

Cladophialophora subtilis Badali & de Hoog, **sp. nov**. MycoBank MB511842. Figs 8, 11.

Etymology: Named after thin-walled, conidial structures.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies, velutinae, olivaceo-virides vel griseae, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, 2−3 µm latae. Conidiophora micronemata, septata, lateralia vel terminalia; stipites et ramoconidia denticulata. Conidia holoblastica, dilute brunnea, late fusiformia, unicellularia, levia, catenas longas ramosas cohaerentes formantia, cicatricibus dilute brunneis, 5−6 × 2−3 µm. Synanamorphe not visa. Chlamydosporae absentes. Teleomorphe ignota.

Description based on CBS 122642 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies growing slowly, reaching 30 mm

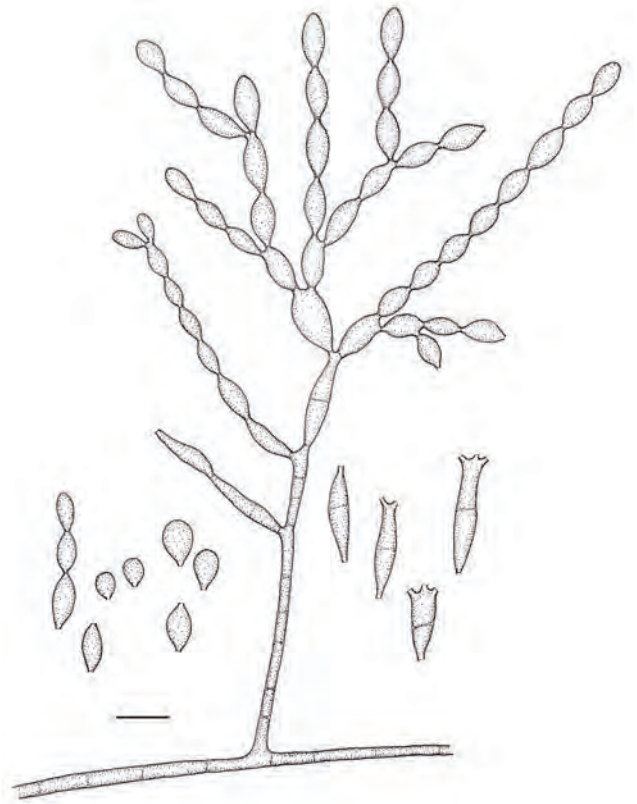

Fig. 7. Microscopic morphology of *C. samoënsis* (CBS 259.83). Branched conidial chains with ramoconidia and conidia. Conidiophores septate, lateral or terminal, with denticles on the stipe and on 0–1-septate ramoconidia. Scale bar = 10 µm.

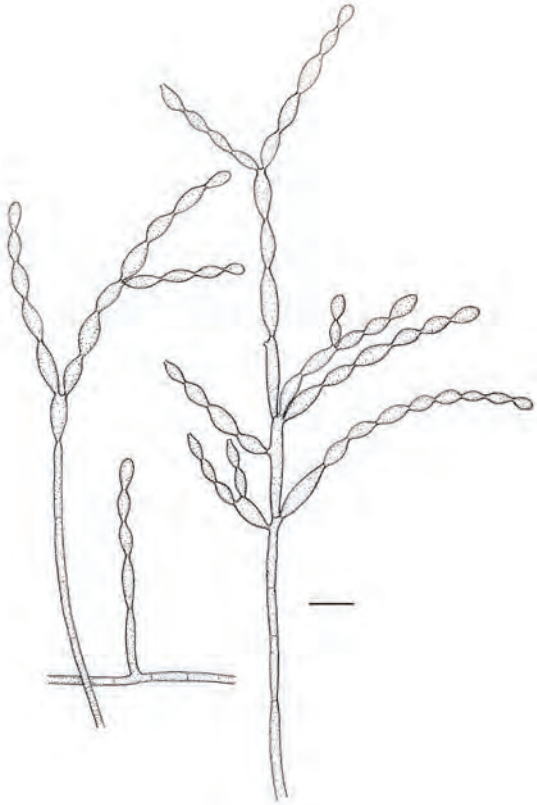

Fig. 8. Microscopic morphology of *C. subtilis* (CBS 122642). Fertile hyphae septate, ascending to erect. Conidiophores apically branched, cylindrical to sub-cylindrical Branched conidial chains with ramoconidia and conidia. Scale bar = 10 µm.

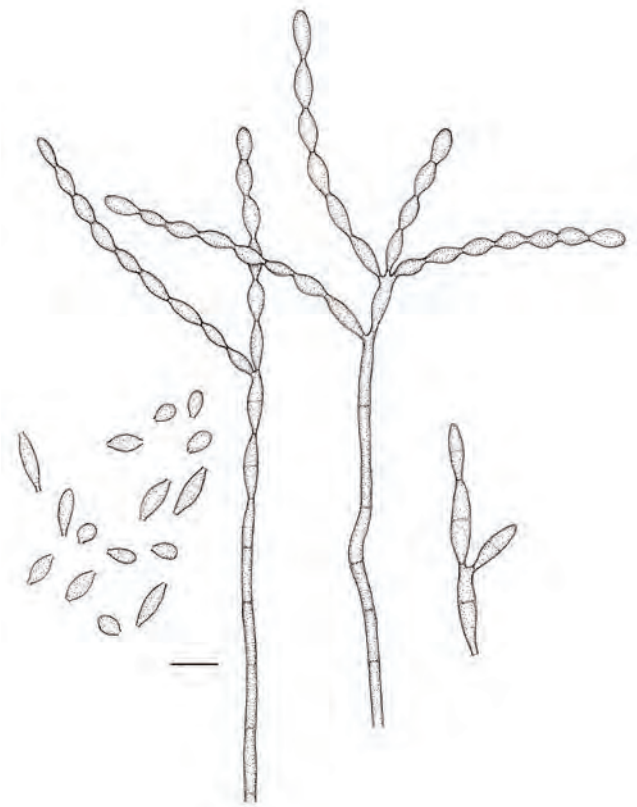

Fig. 9. Microscopic morphology of *C. mycetomatis* (CBS 122637 and CBS 454.82). Septate hyphae creeping, ascending to sub-erect. Conidiophores solitary, micronematous, cylindrical, apically branched. Conidia holoblastic, fusiform produced in long chains. Scale bar = 10 µm.

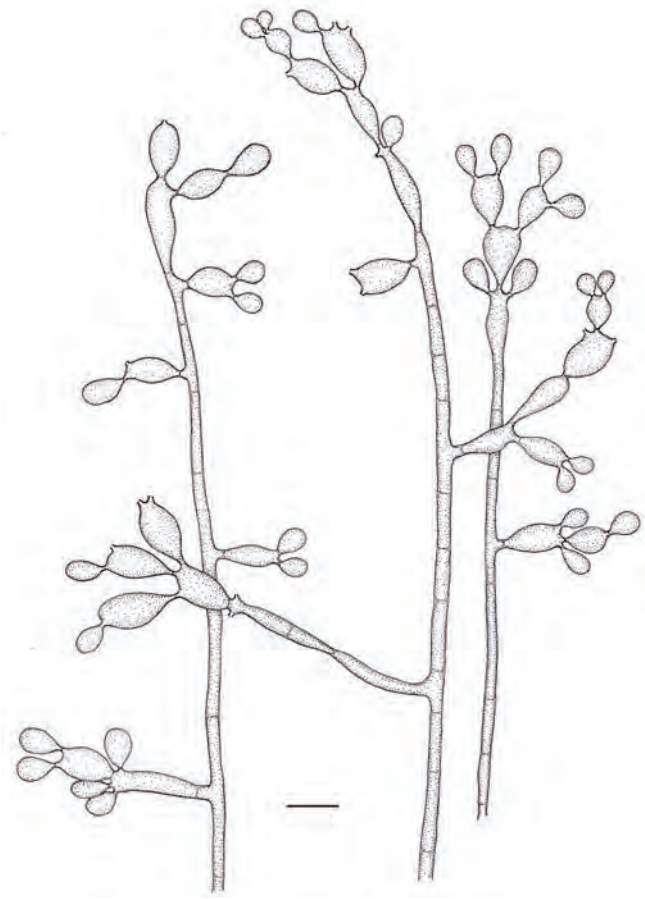

Fig. 10. Microscopic morphology of *C. immunda* (CBS 834.96, CBS 109797, CBS 110551, CBS 102227, CBS 102237). Hyphae branched, septate, straight, ascending to erect, Hyphae giving rise to conidiophores. Lemon-shaped to pyriform to guttuliform, narrowed towards one or both ends, coherent or deciduous. Scale bar = 10 µm.

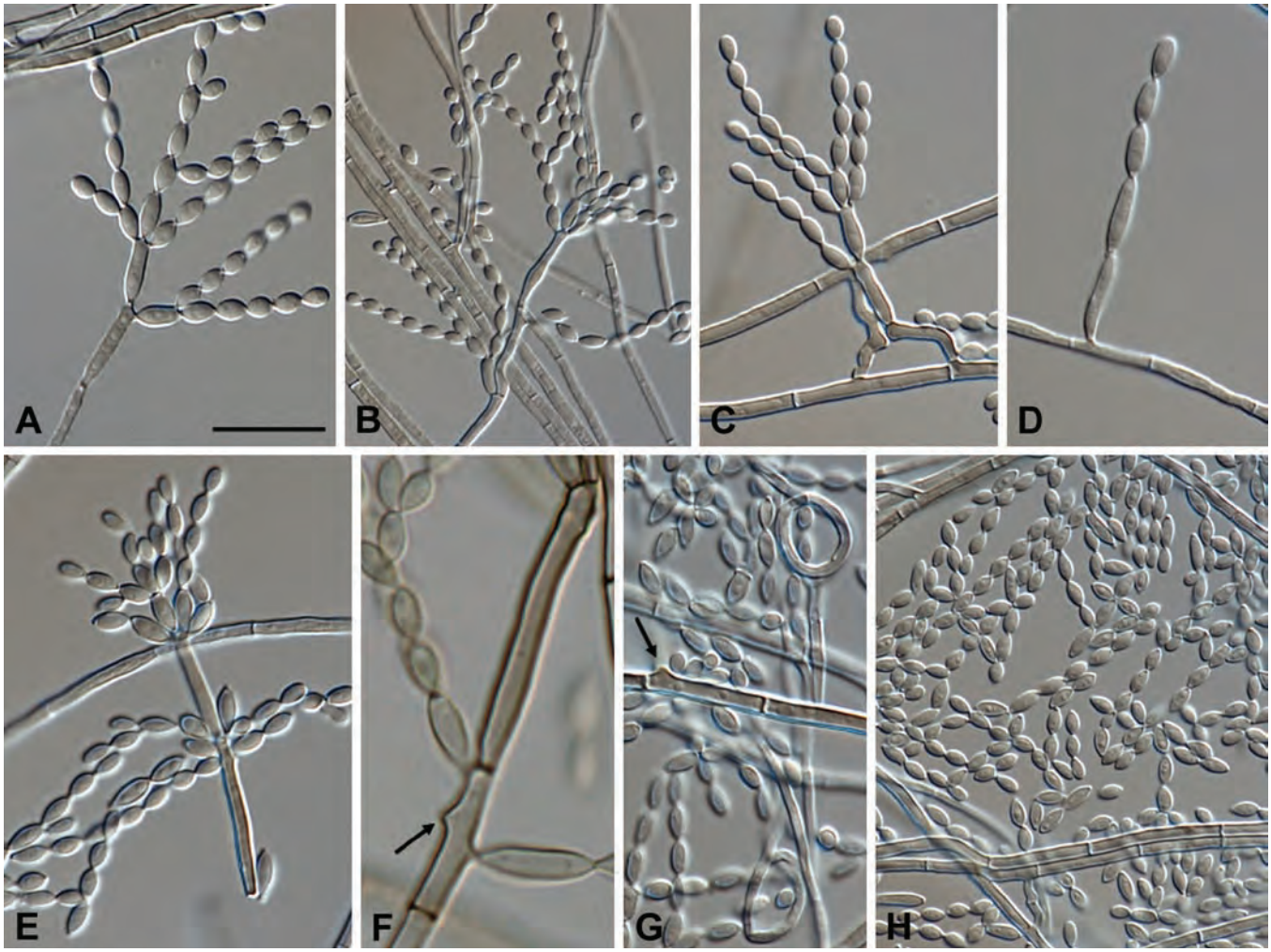

Fig. 11. *Cladophialophora subtilis* (CBS 122642). A–D. Conidiophores with branched conidial chains and ramoconidia. E. Sympodial conidiogenesis. F–G. Conidiogenous loci (arrows). H. Conidia. Scale bars = 10 µm.

diam (10 mm at 37 °C on PDA). Colonies velvety, olivaceous-black, with a wide well defined margin darker than the colony centre, and a compact suede-like to downy surface; reverse olivaceous-black. Cardinal temperatures: minimum 12 °C, optimum 27−30 °C, maximum 37 °C. No growth at 40 °C.

Microscopy: Fertile hyphae septate, ascending to erect, smooth, thin-walled, hyaline to pale olivaceous, guttulate, branched, 2−3 µm wide, forming hyphal strands and hyphal coils. Conidiophores either micronematous, erect, sub-cylindrical, often reduced to a conidiogenous cell, or semi-macronematous, with stalks 60−80 µm long, guttulate, hyaline to pale olivaceous, cylindrical to sub-cylindrical, apically branched, 2.5−3 µm wide. Conidiogenous cells pale brown, slightly darker than conidia, sub-cylindrical to fusiform, with pigmented scars, smooth-walled, proliferating sympodially with 1−3, denticle-like extensions and guttulate. Ramoconidia present. Conidia one-celled, produced in long coherent chains, subhyaline to hyaline, smooth, thin-walled, guttulate; conidia and ramoconidia ellipsoidal to ovoid, 5−6 × 2−3 µm, non-septate. Chlamydospores absent. Teleomorph unknown.

Specimen examined: **The Netherlands**, Utrecht, isolated from ice tea, December 2004, (CBS H-20114, **holotypus**; CBS 122642 = dH 14614, ex-type).

Notes: The species is morphologically similar to *C. carrionii*, an agent of chromoblastomycosis. However, *C. subtilis* has distinct erect conidiophores which arise at right angles from fertile hyphae; conidiogenous cells are pale brown, slightly darker than conidia, sub-cylindrical to fusiform, with pigmented scars, proliferating sympodially with 1−3, denticle-like extensions.

The species is known from a single strain originating from commercial ice tea. Several *Cladophialophora* species have been isolated from sugared drinks (*C. potulentorum, C. australiensis*), while pathogenic *Exophiala* species also have a preference for sugar-rich surfaces of fruits (Sudhadham *et al.* 2008). The association of Chaetothyrialean anamorphs with drinks is thus not surprising. The group is also associated with human disorders (de Hoog *et al*. 2000, Levin *et al*. 2004). Our species has the ability to grow at 37 °C and produces muriform cells when incubated at 25 and 37 °C at low pH (pH = 2.5). Further studies are required to evaluate its pathogenic ability.

Cladophialophora mycetomatis Badali, de Hoog & Bonifaz, **sp. nov**. MycoBank MB511843. Figs 9, 12.

Etymology: Named after the clinical picture mycetoma caused by one of the strains.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies, velutinae, olivaceo-griseae, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, 2.5–3 µm latae. Conidiophora solitaria micronemata, dilute brunnea, cylindrica, sursum ramosa, 3.0–3.5 µm lata. Cellulae conidiogenae dilute brunneae, leves, sympodialiter proliferentes. Ramoconidia 0–(1)-septata, cylindrica vel fusiformia, 2.5–4 × 2.5–3 µm. Conidia holoblastica, dilute brunnea, late fusiformia , unicellularia, levia in catenis longis ramosis cohaerentia, cicatricibus dilute brunneis, 2.5−3 × 2−3. Synanamorphe not visa. Chlamydosporae absentes. Teleomorphe ignota.

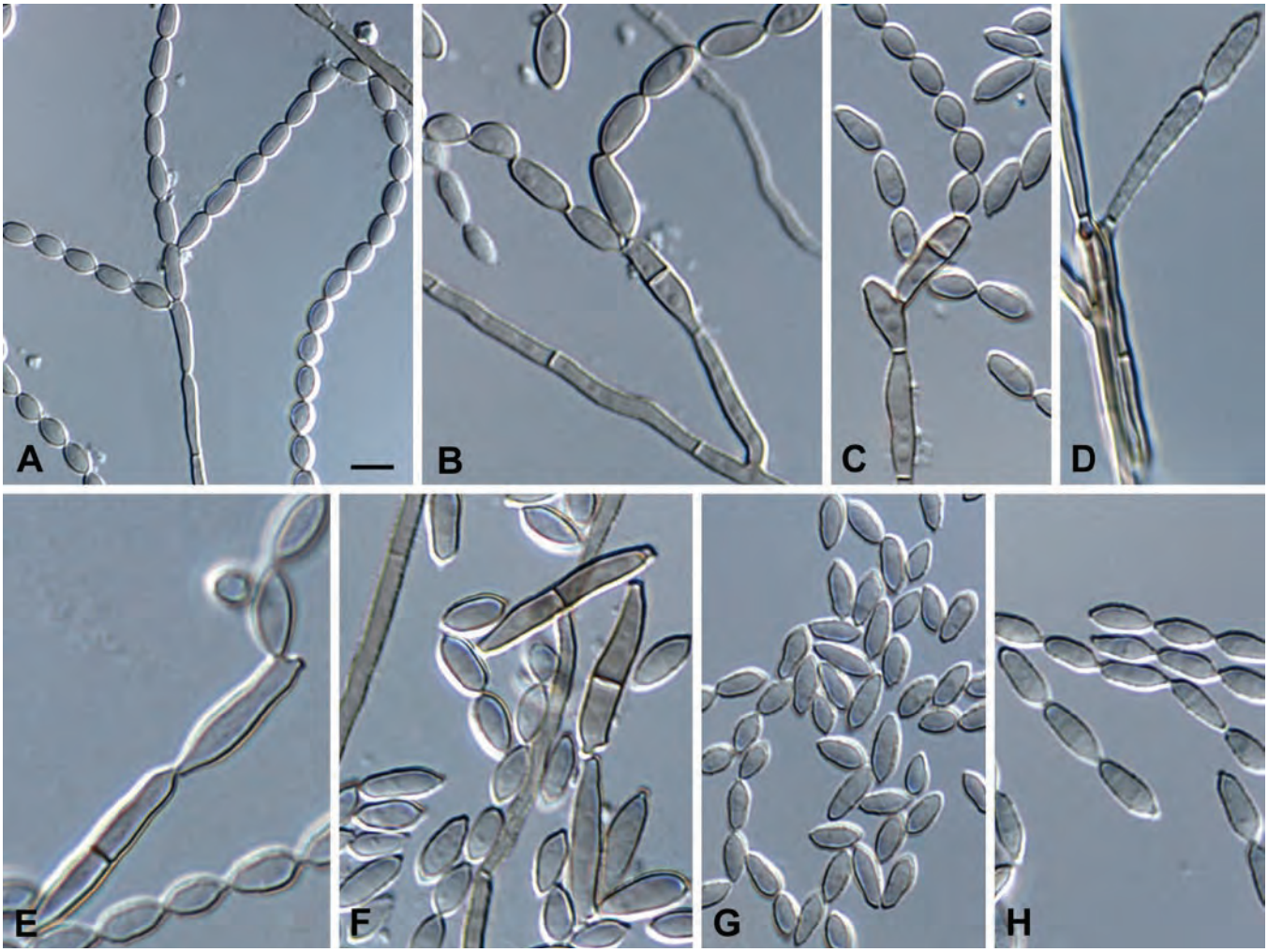

Fig. 12. *Cladophialophora mycetomatis* (CBS 122637 and CBS 454.82). A–B. Conidiophores and conidial chains with ramoconidia and conidia. C–D. Cylindrical, septate conidiophores. G. Ellipsoidal to fusiform conidia. Scale bars = 10 µm.

Description based on CBS 122637 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies moderately expanding, reaching up to 30 mm diam, olivaceous-grey, velvety; reverse olivaceous-black. Cardinal temperatures: minimum 15 °C, optimum 27−30 °C, maximum 37 °C [maximum growth temperature 33 °C; CBS 454.82].

Microscopy: Budding cells absent. Septate hyphae creeping, ascending to sub-erect, smooth-walled, hyaline to pale olivaceous, guttulate, branched, 2.5−3.0 µm wide. Conidiophores solitary, micronematous, pale brown, cylindrical, apically branched, 3.0−3.5 µm wide. Conidiogenous cells pale brown, smooth-walled, sympodially proliferating. Ramoconidia cylindrical to fusiform, 2.5−4.0 × 2.5−3.0 µm. Conidia holoblastic, fusiform produced in long chains; subhyaline to pale olivaceous, guttulate. Chlamydospores absent. Teleomorph unknown.

Specimen examined: **Mexico**, Jicaltepec, isolated from human patient with mycetoma, 2006 (CBS H-20116, **holotypus**; CBS 122637, ex-type).

It is remarkable that an environmental strain of *C. mycetomatis* (CBS 454.82) was isolated by W. Gams as a culture contaminant in the strain of *Scytalidium lignicola* Pesante (CBS 204.71) from the Netherlands. It was morphologically very similar to *C. carrionii* but was methyl-α-D-glucoside and melibiose negative and assimilated D-glucosamine and galactitol (de Hoog *et al.* 1995).

Case Report: A 49-yr-old male farmer mainly growing corn and resident of Jicaltepec, in the semi-arid zone Pinotepa Nacional Oaxaca, approximately 450 km south of Mexico City presented with a dermatosis localised to the left leg at the dorsum of the foot, affecting the third toe (Fig. 13A). The lesion consisted of a tumorous area, with deformation, and nodules with draining sinuses releasing thread-like material including black granules. The dermatosis had begun one and a half yr before, after a trauma with a thorn of cactaceous plant called nopal (*Opuntia* sp.). These led to progressive swelling of the region and occasional pain (Fig. 13A). Initial treatment included penicillin and sulfametoxazole-trimetroprim. The presumptive clinical diagnosis was that of mycetoma. Direct examination with KOH (10 %) showed some black granules approximately 500 µm in size. The granules were composed of branched, septate, dematiaceous hyphae (Fig. 13B). Clinical specimens cultured on Sabouraud Glucose Agar (SGA) with or without antibiotics (Mycosel) resulted in the growth of a dematiaceous fungus morphologically identified as *Cladophialophora* sp. Once the diagnosis of eumycetoma had been made, laboratory tests consisted of a complete blood count, blood chemistry and liver function tests; all of these were within normal limits. Foot radiographs (lateral and PA) showed no involvement of bones. Treatment with itraconazole, 200 mg/d was instituted, with significant clinical improvement at 8 mos. Liver and hematological function tests were monitored throughout the treatment period at

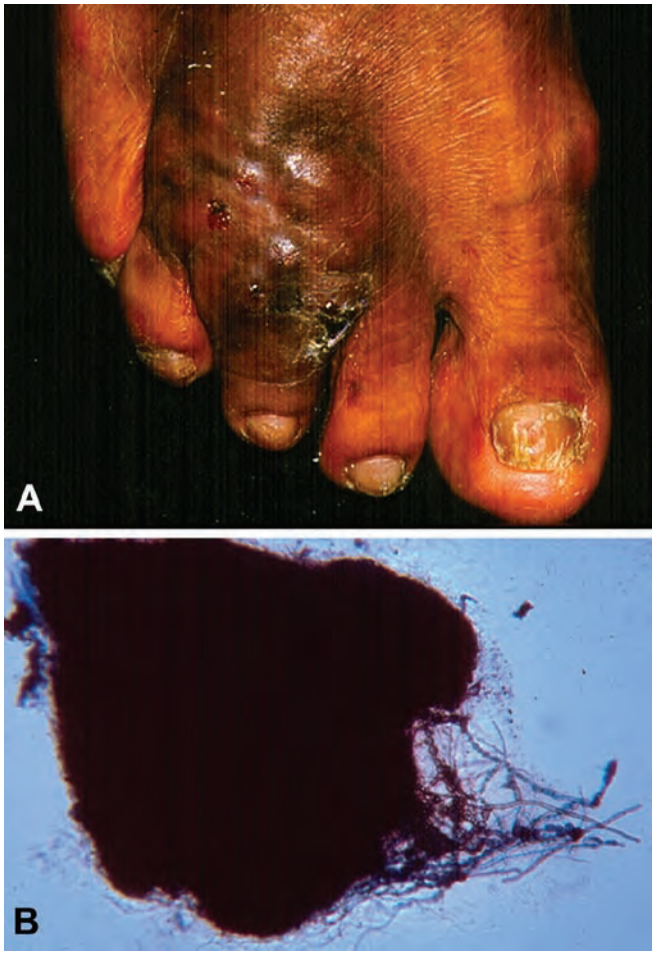

Fig.13. Clinical manifestations of Eumycetoma caused by *Cladophialophora mycetomatis*. A. Deformed tumorous area of the foot, with nodules, draining sinuses and discharging purulent fluid. B. Branched black granule, approximately 500 µm in size (in 10 % KOH), with septate hyphae.

4-mo intervals, with no alterations.

The province where the patient lived in the South of Mexico is a very poor region in a semi-arid area climate zone. The majority of inhabitants are farmers growing corn and other vegetables. Cactaceous plants are very common. The trauma with a thorn of a nopal cactus and the micromorphology of the fungus reminded of *Cladophialophora carrionii*, which is abundant under similar climatic conditions in Venezuela (de Hoog *et al.* 2007), but according to molecular data the fungus proved to be clearly separate. In addition to *Cladophialophora*, *Exophiala* species (particularly *E. jeanselmei*) are also known to cause mycetoma (de Hoog *et al.* 2003). Otherwise, subcutaneous infections by members of *Chaetothyriales* mostly lead to chromoblastomycosis-like infections which are characterised by muriform cells rather than granules, and do not lead to necrosis and draining.

Cladophialophora immunda Badali, Satow, Prenafeta-Boldú & de Hoog, **sp. nov**. MycoBank MB511844. Figs 10, 14.

Etymology: Named after its preference for polluted environments.

Coloniae fere lente crescentes, ad 30 mm diam post 14 dies 27–30 °C, velutinae, olivaceo-griseae vel olivaceo-virides, reversum olivaceo-nigrum. Cellulae gemmantes absentes. Hyphae leves, hyalinae vel pallide brunneae, adscendentes vel erectae, 2.5–4 µm latae, saepe circiter aggregatae. Conidiophora micronemata, septata. Conidia holoblastica, dilute brunnea, late fusiformia, unicellularia, levia, catenas cohaerentes formantia vel dilabentia, cicatricibus dilute brunneis, 3.0−4.5×2.5−4.0 µm. Synanamorphe not visa. Chlamydosporae vulgo absentes, Teleomorphe ignota.

Description based on CBS 834.96 at 27 °C on MEA after 2 wks in darkness.

Cultural characteristics: Colonies growing moderately slowly, attaining up to 30 mm diam at 27−30 °C and 10 mm [5 mm in CBS 102237; 20 mm in CBS 110551] at 37 °C. Colonies dark olivaceous-grey [greyish green to olivaceous-green in CBS 102237] with a thin, dark, well-defined margin [wide grey margin in CBS 109797], spreading, downy, velvety; reverse olivaceous-black. Cardinal temperatures: minimum 15 °C, optimum 27−30 °C, maximum 37 °C. No growth at 40 °C.

Microscopy: Budding cells absent. septate hyphae , straight, ascending to erect, smooth, thin-walled, hyaline to pale brown, branched, 2−3 µm wide, frequently forming hyphal strands and coils [no hyphal coils in CBS 102237, CBS 110551]. Hyphae giving rise to conidiophores which are pale brown, erect, mostly straight, branched or unbranched, long, sub-cylindrical and cylindrical to fusiform [fusiform to ellipsoidal in CBS 110551, CBS 102227], 2.5−4 µm wide [with T-shaped foot cell in CBS 102227], with up to 6−8 septa. Conidiogenous cells branched, conspicuously denticulate, smooth-walled, guttulate, with pigmented scars. Conidia one-celled, acropetal, catenulate, sub-hyaline to pale brown [olivaceous brown in CBS 102227, CBS 102237], smooth [slightly verrucose in CBS 109797, CBS 102227], thin-walled, lemon-shaped to pyriform to guttuliform, narrowed towards one or both ends, with pale pigmented scars. Conidia 3.0−4.5 × 2.5−4.0 µm [3−4 × 2−3 µm in CBS 102227], coherent or deciduous. Phialidic synanamorph not seen. Chlamydospores absent [present in CBS 109797]. Teleomorph unknown.

Specimens examined: **U.S.A.**, Georgia, Atlanta, isolated from a sub-cutaneous ulcer on a 68-yr-old female treated with long-term immunosuppressive therapy, (CBS H-20115, **holotypus**; CDC B-6580 = CBS 834.96, ex-type). **Brazil,** Paraná, Colombo, isolated from *Syagrum romanzoffianum* stem, CBS 102227. **Brazil,** Paraná, isolated from decaying cover vegetable, CBS 102237. **Germany,** Kaiserslautern, isolated from biofilter inoculated with soil, CBS 109797. **The Netherlands**, isolated from hydrocarbon-polluted soil, CBS 110551 (Prenafeta-Boldú *et al.* 2006). **Brazil** isolated from hydrocarbon-polluted soil, CBS 122636, CBS 122255, CBS 122257, dH 18477, dH 18476, dH 18476, dH 18478 (Satow *et al.* 2008).

Case Report: According to the description by Padhye *et al*. (1999), a 68 yr-old-female who underwent long-term immunosuppressive therapy in view of a history of recurrent sinusitis, pneumonia, genital herpes, hysterectomy, chronic hypergammaglobulinemia, low grade lymphoma, Sjogren's disease, rheumatois arthritis. She had not any history of predisposing factor such as truma. The pretibial lesion was non-responsive to cephalexin or ofloxacin. Due to the clinical manifestation of the lesion, a biopsy was performed which consisted of dermis and subcutaneous tissue. Biopsy tissue section was stained by PAS (Fig. 15A) and Gomori's methanamine-silver (GMS, Fig. 15B) stains, showing septate hyphae, moniliform hyphae of different lengths, and thick-walled cells. The melanized fungal elements were within intense infiltrates of neutrophils and necrotizing granulomas with many giant cells. The histopathological observations led to the diagnosis of a subcutaneous phaeohyphomycosis infection. The strain was identified as *Cladophialophora* species (Padhye *et al*. 1999) resembling *C. devriesii* and *C. arxii*. It formed dry conidia in branched acropetal chains inserted on pronounced denticles, fusiform to lemon-shaped conidia, and being involved in a human infection. Unlike *C. arxii*,

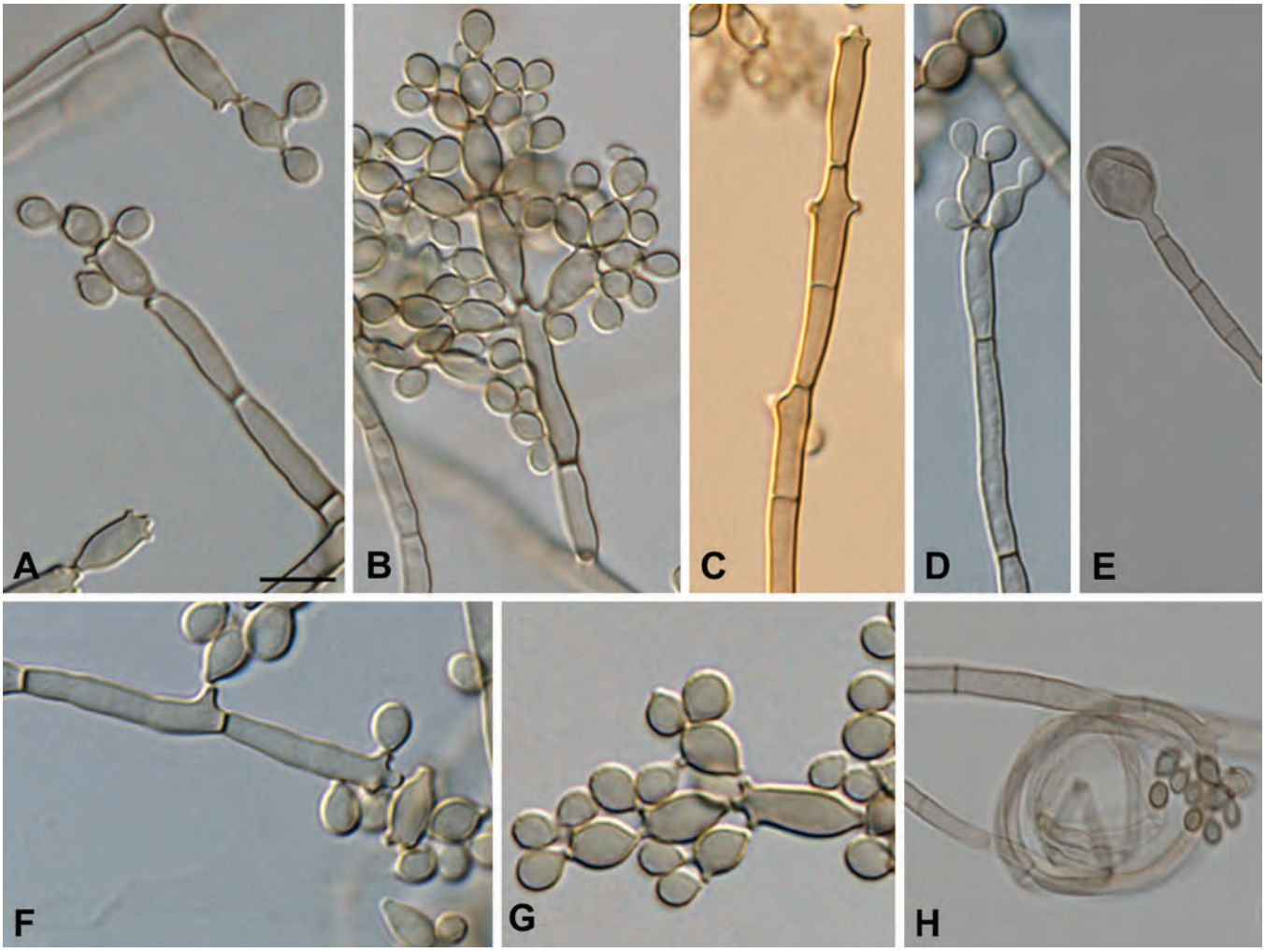

Fig. 14. *Cladophialophora immunda* (CBS 834.96, CBS 109797, CBS 110551, CBS 102227, CBS 102237). A–D. Conidiophores and conidial apparatus with T-shaped foot cell and cylindrical, septate, denticulate conidiogenesis. E. Chlamydospores. F–G. Thin-walled, lemon-shaped to pyriform to guttuliform conidia. H. Hyphal coil. Scale bar = 10 µm.

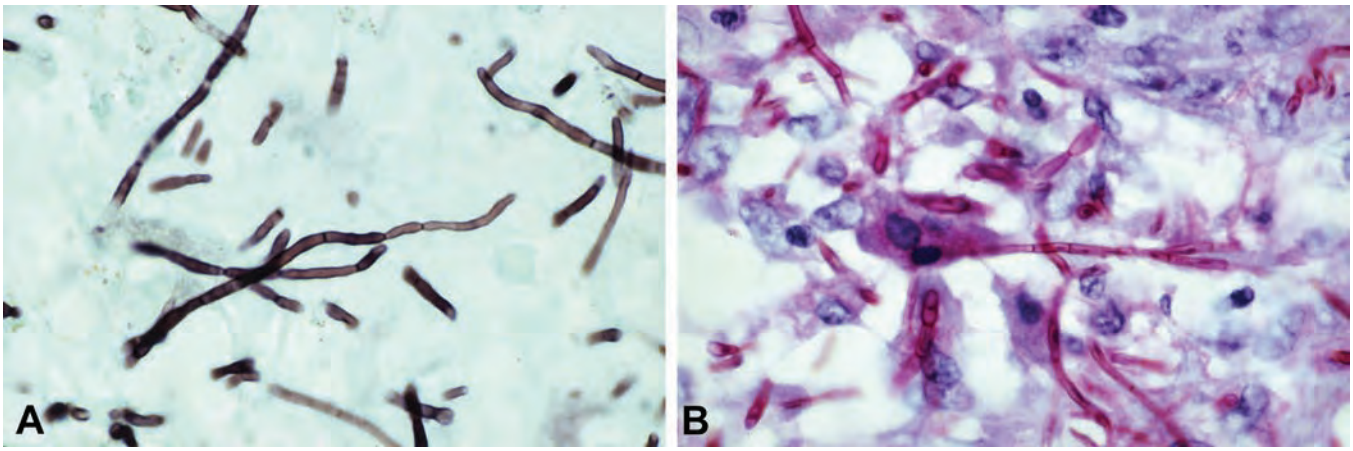

Fig. 15. *Cladophialophora immunda* (CBS 834.96). A. Gomori Methenamine-silver and B. Periodic acid-Schiff stained sections revealed septate hyphae, moniliform hyphae of different lengths, and thick-walled cells. (GMS & PAS × 360)

the species did not grow above 37 °C. We identified the strain with several environmental isolates by multilocus sequencing (Figs 1, 3 and Table 1). For the environmental strains, a striking association with aromatic hydrocarbons was observed: eight out of twelve strains of *C. immunda* originated from hydrocarbon-polluted soils. An association with assimilation of toxic monoaromatic compounds and infective potentials have been hypothesised (Prenafeta-Boldú *et al*. 2006). Black yeasts and their filamentous relatives in the ascomycete order *Chaetothyriales* are potent degraders of monoaromatic compounds and eventually tend to accumulate in industrial biofilters (Cox *et al*. 1997, Prenafeta-Boldú *et al*. 2001, de Hoog *et al*. 2006).

DISCUSSION

Cladophialophora-like anamorphs are polyphyletic

The genus *Cladophialophora* is morphologically characterized by poorly or profusely branched chains of dry, rather strongly coherent, moderately melanized conidia. Our results show that this *Cladophialophora* anamorphic type is convergent, as the genus *Cladophialophora* is polyphyletic. In our combined phylogeny of the *Chaetothyriales* (Fig. 1), the plant-associated species (*C. hostae, C. scillae, C. proteae, C. sylvestris, C. minutissima,* and *C. humicola*) appeared to be closer to *Ceramothyrium carniolicum* (a putative member of the family of *Chaetothyriaceae*), and only distantly related to most remaining *Cladophialophora* species. It is interesting to note that *Cladophialophora* species that are consistently associated with pathology to humans mostly belong to the *Herpotrichiellaceae*, a family particularly diverse in human opportunists (*e.g.*, *Exophiala*, *Fonsecaea*, *Rhinocladiella*, and *Veronaea*). The lineages to which the plant-associated species of *Cladophialophora* (lineages 2, 3 and 4) belong contain comparatively few human opportunists, all of them causing only mild cutaneous infections (*e.g.*, *Phialophora europaea*, and *Cyphellophora laciniata*), or traumatic infection (*Cladophialophora modesta*).

Similar Cladophialophora-like morphologies have also been observed in several unrelated environmental fungi, particularly in *Cladosporium*, *Pseudocladosporium* and *Devriesia* (Braun *et al*. 2003, Crous *et al.* 2007, Seifert *et al.* 2004). These genera are assigned to different families within the *Dothideales* and the *Capnodiales*, two ascomycete orders for which species are only exceptionally encountered in a clinical setting. Moreover, the conidial scars have a pale pigmentation in *Cladophialophora*, which is in contrast to members of the saprobic genera *Cladosporium*, *Devriesia* and *Pseudocladosporium*, where pronounced darker conidial scars are present. Furthermore, conidiophores in most *Cladophialophora* species are poorly differentiated, while those of *Cladosporium* species are usually erect and significantly darker than the rest of the mycelium. Finally, conidial chains of *Cladophialophora* species are coherent, while those of *Cladosporium* detach very easily.

Evolution of pathogenicity in *Cladophialophora*

Melanin pigments are common to all Chaetothyriales, although little is known concerning the pathogenic mechanisms by which these fungi cause disease, particularly in immunocompetent individuals. However, the production of melanin was shown to be involved in pathogenicity. Melanins are pigments of high molecular weight formed by oxidative polymerization of phenolic compounds and usually are dark brown or black; in case of fungal pathogens melanin appears to function in virulence by protecting fungal cells against fungicidal oxidants, by impairing the development of cell-mediated responses, interfere with complement activation and reduce the susceptibility of pigmented cells to antifungal agents. In the environment, they protect organisms against environmental factors (Butler *et al.* 1998, Jacobson 2000).

Another putative virulence factor is thermotolerance. According to de Hoog *et al.* (2000), species of *Cladophialophora* show a differential maximum growth at temperatures more or less coinciding with clinical predilections, species causing systemic infections being able to grow at 40 °C. The agents of chromoblastomycosis have a growth maximum at 37 °C, while a mildly cutaneous species, such as *C. boppii,* is not able to grow at this temperature (de Hoog *et al.* 2000). In the present study, all studied strains of *Cladophialophora* had an optimum growth around 27 °C, and were still able to grow at 37 °C, but not at 40 °C (Fig. 4). This observation agrees with the prevalent nature of these species as environmental saprobes, and their potential to cause superficial infections in humans, similarly to other opportunistic species. Tolerance of human body temperature is an essential requirement for pathogenicity, but this trait may have incidentally been acquired via adaptation to warm environmental habitants, such as hot surfaces in semiarid climates.

Early experiments involving the inoculation of *Cladophialophora* in several species of cold-blooded animals have shown the abundant production of characteristic muriform cells *in vivo* (Trejos 1953). For *C. yegresii* and *C. carrionii*, the muriform propagules are also present in cactus spines (de Hoog *et al.* 2007); in this plant host, they can be regarded as an extremotolerant survival phase, and are likely to play an essential role in the natural life cycle of these organisms. The capacity of some *Herpotrichiellaceae* to grow in a meristematic form both in human hosts and in extreme environmental conditions supports the suggestion that the muriform cells may indeed be a main virulence factor in the development of the disease, representing an adaptation to the conditions prevailing in host tissues. The conversion of hyphae to muriform cells can be induced *in vitro* under acidic conditions (pH = 2.5) and low concentration of calcium. In the present study, we observed different morphogenetic responses between environmental and clinical species of *Cladophialophora*. *C. subtilis* isolated from ice tea, under the above circumstances formed structures resembling muriform cells at 27 °C which became larger when incubated at 37 °C. The pathogenic *C. samoënsis* and *C. immunda* also produced muriform cells at 37 °C. Muriform cells were not observed, neither on the plant-associated species (plant leaving) of *Cladophialophora*, nor in the *C. mycetomatis* isolated from both environment and a patient.

CONCLUSIONS

Some members of *Herpotrichiellaceae* grow as ordinary filamentous moulds in pure culture, but can also be induced to form strongly melanized, isodiametrically expanding meristematic cells *in vitro*. This type of cells, dividing by sclerotic fission, is characteristically found in chromoblastomycosis. However, they can also be observed when these fungi grow in natural niches, in particular the ones characterized as extreme (*e.g.*, *F. pedrosoi* within dried cactus thorns; Sterflinger *et al.* 1999) and Vicente *et al*. (2008) have shown that the hydrocarbon-polluted environments yielded yet another spectrum of chaetothyrialean fungi. A generalized suite of adaptations to extreme environments, including melanin production and meristematic growth, as well as thermotolerance, is suggested to contribute to pathogenic potential (Gueidan *et al*. 2008). Many of the natural and artificial habitats that are associated with growth of *Herpotrichiellaceae*, such as decaying tree bark and creosoted poles and ties, are likely to favor fungi that, in addition to being able to break down the aromatic compounds occurring in these substrata, have a generalized set of adaptations to conditions that at least occasionally become highly stressful (extreme temperatures, pH, low availability of nutrients and growth factors, etc.). Many of these adaptations may incidentally predispose fungi towards human opportunistic pathogenesis.

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Studies in Mycology 60: Neotropical *Hypocrella* (anamorph *Aschersonia*), *Moelleriella*, and *Samuelsia*

Priscila Chaverri, Miao Liu and Kathie T. Hodge

The present taxonomic revision deals with Neotropical species of three entomopathogenic genera that were once included in *Hypocrella s. l.*: *Hypocrella s. str.* (anamorph *Aschersonia*), *Moelleriella* (anamorph aschersonia-like), and *Samuelsia* gen. nov (anamorph aschersonia-like). Species of *Hypocrella*, *Moelleriella*, and *Samuelsia* are pathogens of scale insects (Coccidae and Lecaniidae, Homoptera) and whiteflies (Aleyrodidae, Homoptera) and are common in tropical regions. Phylogenetic analyses of DNA sequences from nuclear ribosomal large subunit (28S), translation elongation factor 1-α (TEF 1-α), and RNA polymerase II subunit 1 (RPB1) and analyses of multiple morphological characters demonstrate that the three segregated genera can be distinguished by the disarticulation of the ascospores and shape and size of conidia. *Moelleriella* has filiform multi-septate ascospores that disarticulate at the septa within the ascus and aschersonia-like anamorphs with fusoid conidia. *Hypocrella s. str.* has filiform to long-fusiform ascospores that do not disarticulate and *Aschersonia s. str.* anamorphs with fusoid conidia. The new genus proposed here, *Samuelsia*, has filiform to long-fusiform ascospores that do not disarticulate and aschersonia-like anamorphs with small allantoid conidia. In addition, the present study presents and discusses the evolution of species, morphology, and ecology in *Hypocrella*, *Moelleriella*, and *Samuelsia* based on multigene phylogenetic analyses.

66 pp., illustrated with colour pictures (A4 format), paperback, 2008. € 40

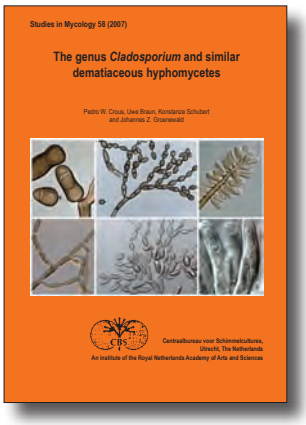
Studies in Mycology 59: *Aspergillus* systematics in the genomic era

editors Robert A. Samson and János Varga

Since Pier Antonio Micheli described Aspergillus is his Nova Plantarum Genera in 1729 the genus attracted an immense interest. Many species were found as spoilage agents, or responsible for human and animal diseases. On the other hand Aspergilli were also found as beneficial micro organisms in the fermentation of Asian food and beverages. With the discovery of aflatoxins, the interest and research of the Aspergilli increased even more. In the present days Aspergillus research has grown to such a level, that it could be stated that Aspergilli might be the most studied fungi.

This issue comprises 14 papers and is a summary of presentations and discussions of the international workshop entitled "*Aspergillus* systematics in the genomics era" with a multidisciplinary audience held in Utrecht, The Netherlands (12-14 April, 2007). The papers discuss topics such as the current species concept; what can comparative genomics tell us about species concepts in *Aspergillus*; sexual and vegetative compatibility genes in the aspergilli; secondary metabolite (including mycotoxins) profiling, growth profiles and other tools for species recognition; identification in the clinical setting; *Aspergillus* strain typing in the genomics era and the biodiversity of *Aspergillus* species in some important agricultural products. Nomenclatural considerations in naming species of *Aspergillus* and its teleomorphs were discussed in a separate paper, while the recommendations of an international panel are included. Additionally, the polyphasic methods applied recently on aspergilli resulted in four monographs included in this issue: *Aspergillus* sections *Candidi, Clavati, Fumigati* and *Usti*. Diagnostic tools developed for the identification of the economically extremely important but taxonomically problematic black aspergilli (*Aspergillus* section *Nigri*) are also covered in a separate paper. This issue contains many colour illustrations, particularly in the four monographs.

206 pp., fully illustrated with colour pictures (A4 format), paperback, 2007. € 65



Studies in Mycology 58: The genus *Cladosporium* and similar dematiaceous hyphomycetes

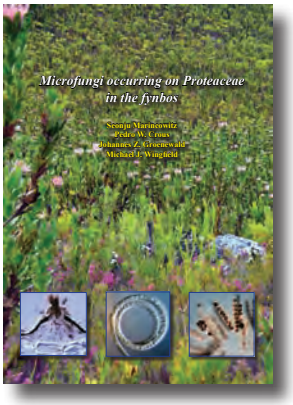
Pedro W. Crous, Uwe Braun, Konstanze Schubert and Johannes Z. Groenewald

Species of *Cladosporium* are common and widespread, and interact with humans in every phase of life, from producing allergens in the indoor environment, to causing fruit decay and plant disease, or being associated with human mycoses. Although *Cladosporium* is one of the largest and most heterogeneous genera of hyphomycetes, only a mere fraction of these species are known from culture, and few have been characterised based on molecular data. The present volume consists of nine research papers, and introduces 71 new combinations, 53 new species, 15 genera, and one family. Specific conditions and media are recommended to study *Cladosporium* and allied genera, while the genus is circumscribed, and separated from morphologically similar taxa with which it has been confused in the past. Generic issues related to the anamorph genera and associated *Dothideomycete* teleomorph genera (*Davidiella*, *Mycosphaerella*, *Venturia*) are addressed, and multi-allelic data sets provided to facilitate species recognition. The volume is richly illustrated, with more than 100 colour plates, numerous line drawings, and scanning electron micrographs.

253 pp., illustrated with colour pictures (A4 format), paperback, 2007. € 65

CBS Biodiversity Series

No. 7: Microfungi occurring on *Proteaceae* in the fynbos

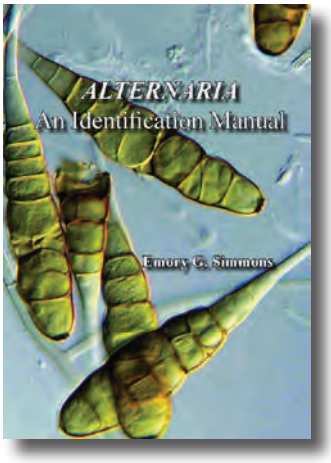


Seonju Marincowitz, Pedro W. Crous, Johannes Z. Groenewald and Michael J. Wingfield

The fynbos is a shrubland characterised by a fire-prone ecosystem and Mediterranean climate. Although it is extremely rich in plant species, and has a high degree of floral endemism, very little is known regarding the fungi in this unique and fascinating environment. The present study investigated the saprobic microfungi that colonise and utilise leaf and twig litter and senescent flowerheads of *Proteaceae*. A total of 29 species and sub-species belonging to four genera of *Proteaceae* were sampled from 12 sites in the Western Cape Province of South Africa spanning a period of two yr (2000–2001). An attempt was made to culture all fungi encountered, and where successful, the ITS and partial 28S nrDNA, and in some cases the translation elongation factor 1-α or the β-tubulin gene regions were sequenced. A total of 62 bags of litter yielded 316 individuals, consisting of 141 fungal species residing in 103 genera and 43 families. Of these, 59 species, including eight species that had been previously published, represented novel taxa. Thirty-eight species reflected new records for South Africa, and 48 species were new reports on *Proteaceae*. Two new genera and one new combination were also introduced. Seventy-three species were represented by teleomorphs and 68 species by anamorphs, which were made up of 30 hyphomycetes and 38 coelomycetes. Eighty percent of the species occurred on only one type of substrate. The fungal community found on twig litter had the highest species richness, while flowerhead-styles yielded the highest percentage (100 %) of unique species. These results showed that the species richness for the fynbos Mycota was moderately high with every 2.2 collections representing a different fungal species. The percentage of new fungal taxa (43 % of the total species) was exceptionally high, and most of these probably host-specific. More than 80 % of the fungi collected in this study had hard and closed fruiting structures, indicating an adaptation to the constraints of the harsh fynbos environment. Other than providing a foundation for further studies, this investigation highlights a disturbing paucity of knowledge regarding the fynbos Mycota in one of the world's most threatened and unusual floral Kingdoms.

166 pp., illustrated with 93 colour pictures and 6 B&W pictures (A4 format). Hardbound, 2008. € 50

CBS Biodiversity Series



No. 6: *Alternaria* An Identification Manual

Emory G. Simmons

This book will fill a very large void in the scientific literature and it is quite certain that the volume will become the standard reference for those needing to have critical access to *Alternaria* literature and taxonomic information. There are many scientists, both research and regulatory, who are in desperate need of resources like this book to facilitate routine identification. More than 1 100 published names are associated with taxa that must be considered in the *Alternaria* context. Of these, 276 species with validly published names are maintained here as currently identifiable; these are keyed in the main text of the volume. An additional 16 named taxa, although requiring expanded information and comparison, also are accepted. A few species that have been associated with the genus for yr but which now are considered anomolous in the genus have been removed to other genera. Chapters of species and genus characterisations are followed by a comprehensive list of all the nearly 1 200 names involved historically with *Alternaria* taxonomy in the period 1796-2007. Each name is listed with its source, type, and an opinion on its validity and taxonomic disposition. A host index to all accepted species is followed by a comprehensive list of literature cited and a general index. Within the context of the manual, 88 names are assigned to newly described species and genera and to taxa whose epithets appear in new combinations.

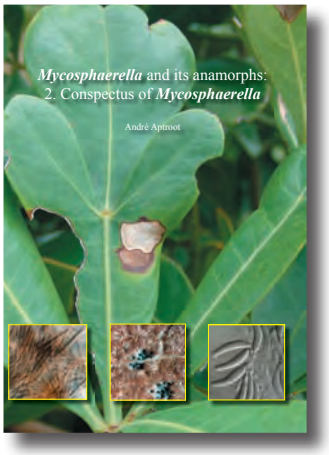
775 pp., with more than 288 line drawings (A4 format). Hardbound, 2007. € 170

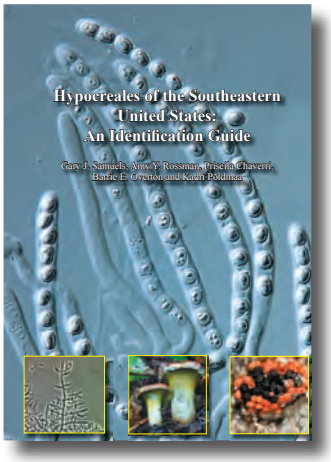
No. 5: *Mycosphaerella* and its anamorphs: 2. Conspectus of *Mycosphaerella*

André Aptroot

A revision of the species described in *Mycosphaerella* and *Sphaerella* is presented, together with observations on the types of most species or their disposition. The genus *Stigmidium* is expanded to encompass fungicolous species and internal parasites of algae, and includes the genus *Mycophycias*.

173 pp., 115 plates (A4 format), paperback with spiral binding, 2005. € 50





No. 4: Hypocreales of the Southeastern United States: An Identification Guide

Gary J. Samuels, Amy Y. Rossman, Priscila Chaverri, Barrie E. Overton and Kadri Põldmaa

An illustrated guide is presented to the members of the ascomycete order Hypocreales that are known to occur in the southeastern states of the United States, including North and South Carolina, Tennessee and Georgia. Species were selected mainly based on records in the United States National Fungus Collections (BPI). These states include or surround the Great Smoky Mountains National Park. Species of the Hypocreales are among the most numerous, and certainly most conspicuous, of the microfungi. The order also includes some of the most economically important fungi. This guide is intended for individuals who are participating in All Taxa Biological Diversity studies of the Great Smoky Mountains National Park as well as other interested professionals and amateurs. Short descriptions and colour illustrations of one-hundred and one species and two varieties in twenty genera are provided. Keys to genera and species are included. The new combination Neonectria ditissima is proposed.

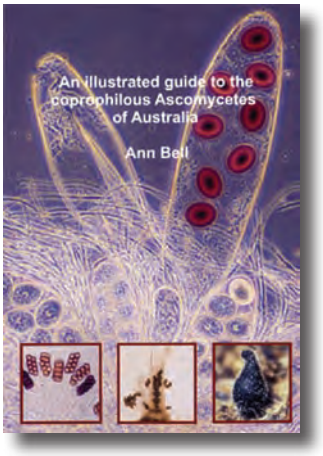
145 pp., over 120 colour pictures (A4 format), paperback with spiral binding, 2006. € 70

No. 3: An illustrated guide to the coprophilous Ascomycetes of Australia

Ann Bell

Descriptions, keys and illustrations (many in colour). Ann Bell's observations of her own collections and some 2 000 microscope slides and assorted notebooks on Australian coprophilous fungi made by the late Major Harry Dade during his retirement yr in Victoria.

173 pp., 115 plates (A4 format), paperback with spiral binding, 2005. € 55



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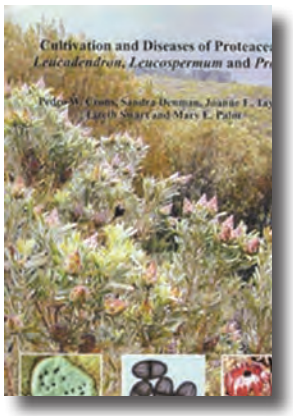


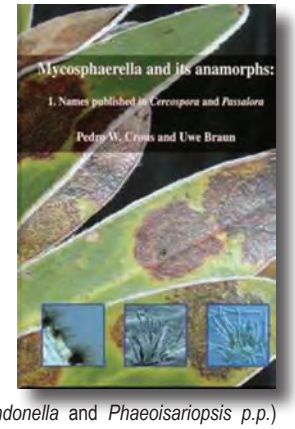
No. 2: Cultivation and Diseases of *Proteaceae*: *Leucadendron, Leucospermum* and *Protea*

Pedro W. Crous, Sandra Denman, Joanne E. Taylor, Lizeth Swart and Mary E. Palm

The Proteaceae represent one of the Southern Hemisphere"s most prominent flowering plant families, the cultivation of which forms the basis of a thriving export industry. Diseases cause a loss in yield and also limit the export of these flowers due to strict phytosanitary regulations. In this publication the fungi that cause leaf, stem and root diseases on Leucadendron, Leucospermum and Protea are treated. Data are provided pertaining to the taxonomy, identification, host range, distribution, pathogenicity and control of these pathogens. Taxonomic descriptions and illustrations are provided and keys are included. Desease symptoms are illustrated with colour photographs.

510 pp., (17 x 25 cm), paperback, 2004. € 55

No. 1: *Mycosphaerella* and its anamorphs: 1. Names published in *Cercospora* and *Passalora*

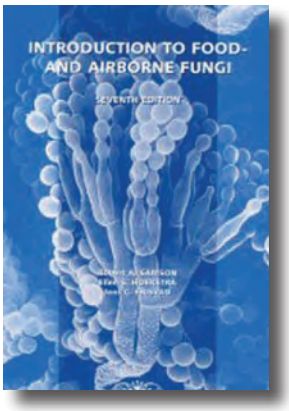


Pedro W. Crous and Uwe Braun

This book contains a compilation of more than 3000 names that have been published or proposed in *Cercospora*, of which 659 are presently recognised in this genus, with a further 281 being referred to *C. apii s.lat*. Approximately 550 names of *Passalora* emend. (incl. *Mycovellosiella*, *Phaeoramularia*, *Tandonella* and *Phaeoisariopsis p.p.*) are treated in a second list. In total 5720 names are treated. 553 taxonomic novelties are proposed.

571 pp., 31 figures (17 x 25 cm), hard cover, 2003. € 80

Selection of other CBS publications



Introduction to food- and airborne fungi

Robert A. Samson, Ellen S. Hoekstra and Jens C. Frisvad

Seventh edition with updated taxonomy and addition of some important species. The keys to the taxa were improved. The taxonomy and the nomenclature of *Fusarium* is revised and the number of *Penicillium* species has been increased because they are frequently encountered on food and indoor environments. The identification of *Penicillium* based on morphological characters remains difficult and therefore synoptic keys and tables are added to assist with the identification.

389 pp., 120 plates (A4 format), paperback, 2004. € 55

Identification of Common *Aspergillus* Species

Maren A. Klich

Descriptions and identification keys to 45 common *Aspergillus* species with their teleomorphs (*Emericella*, *Eurotium*, *Neosartorya* and *Sclerocleista*). Each species is illustrated with a one page plate and three plates showing the most common colony colours.

116 pp., 45 black & white and 3 colour plates (Letter format), paperback, 2002. € 45

A revision of the species described in *Phyllosticta*

Huub A. van der Aa and Simon Vanev

2936 taxa are enumerated, based on the original literature and on examination of numerous herbarium (mostly type) specimens and isolates. 203 names belong to the genus *Phyllosticta s.str.*, and are classified in 143 accepted species. For seven of them new combinations are made and for six new names are proposed. The great majority, 2733 taxa, were redisposed to a number of other genera. A complete list of these novelties, as included in the book"s abstract, can also be consulted on the web-site of CBS.

510 pp. (17 x 25 cm), paperback, 2002. € 55

Atlas of Clinical Fungi

G.S. de Hoog, J. Guarro, J. Gené and M.J. Figueras (eds)

The second fully revised and greatly expanded edition of the Atlas of Clinical Fungi appeared in 2000. The modest and very competitive price of this standard work has certainly contributed to the popularity of the first edition. In recent yr the application of molecular biology has become within reach for many routine laboratories. The new Atlas will provide ample molecular data for the majority of clinically relevant fungi. It will set a standard for innovative techniques in medical mycology. In addition, antifungal susceptibility data will be given for most species, which will provide essential knowledge for the clinician in view of adequate therapy.

1126 pp., fully illustrated with line drawings and black & white photo plates (A4 format), hard cover, 2004. € 140.

Interactive CD-ROM v. of the Atlas € 65, Book plus CD-ROM € 180

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