Antonie van Leeuwenhoek **71:** 281–288, 1997.

Sarcinomyces petricola, a new microcolonial fungus from marble in the Mediterranean basin

U. Wollenzien[1], G.S. de Hoog[2], W. Krumbein[1] & J.M.J. Uijthof[2]
[1]*Geomikrobiologie, ICBM, Carl von Ossietzky-Universität, P.O. Box 2503, D–26111 Oldenburg, Germany;*
[2]*Centraalbureau voor Schimmelcultures, P.O. Box 273, NL–3740 AG Baarn, The Netherlands*

Received 26 January 1996; accepted 18 July 1996

Key words: black yeasts, microcolonial fungi, taxonomy, microbial damage, stone monuments, marble

Abstract

A new meristematic black yeast species, *Sarcinomyces petricola,* is described. All strains known to date were isolated from sun-exposed marble in the Mediterranean basin. On the basis of PCR-ribotyping and nutritional physiology, the species is classified as an anamorph member of the ascomycete family Herpotrichiellaceae (Chaetothyriales).

Introduction

Microbial activity of fungi and other microorganisms is one of the causes of the degradation of architectural monuments and works of art, in addition to physical and chemical factors (Wollenzien et al. 1993; Urzì et al. 1995). Melanized fungi with restricted, meristematic growth (microcolonial fungi, MCF; Staley et al. 1984) are particularly significant (Diakumaku et al. 1995). Such species are able to endure extreme environmental conditions and to propagate by slow endoconidiation rather than by germination (Wollenzien et al. 1995). Some species appear to be epiphytic fungi which are also found on other substrates like conifer needles and leathery plant leaves (Butin et al. 1995).

During a prolonged investigation of fungi inhabiting marble and calcarenite statues and monuments, especially in the Mediterranean basin, a black yeast-like fungus was repeatedly isolated and proven to be a new species. It is described below.

Material and methods

Isolation

Samples were taken from a statue in the Messina Museum (Italy; Figure 1), from the Dionysos Theatre near the Acropolis (Greece) and from an obelisque at Corfu (Greece). Isolation in Messina was carried out directly from the statue with a sterile needle using the selective medium DRBC (containing dichlorane, rose bengal and chloramphenicol; King et al. 1979) and occasionally 2% malt and diluted Czapek agars. Isolates from specimens from the Dionysos theatre were obtained in the laboratory by the same method one week after sampling, while the Corfu material was sampled by using a spiral plater system (Jarvis 1977).

Collection data of the strains: CBS 726.95 (= UW H13), from Carrara (?) marble, outdoor angel statue in Messina museum garden, Italy; CBS 600.93 (= UW D2b), from penthelic marble, Dionysos Theatre, Acropolis, Athens, Greece; CBS 725.95 (= UW P2), from calcarenite obelisque, Corfu, Greece.

Morphology

Stock cultures were maintained on 2% malt agar at room temperature. Slides were made in lactophenol with or without cotton blue, in water or in Melzer's reagent.

Physiology

Growth and fermentative abilities were tested in duplicate in liquid medium, using the method described by Van der Walt & Yarrow (1984) and adapted according to de Hoog et al. (1995b) with prolonged incuba-

Figure 1. Part of Carrara marble statue at Messina, exposed to outdoor conditions since 1908, with initial patination and macropitting caused by *Sarcinomyces petricola*, CBS 726.95. Spots are visible to the naked eye (macro 1:4).

tion. For assimilation, cells were grown in test tubes at 25 °C and aerated by shaking in nearly horizontal position at 50 rpm. Urease activity was tested on Christensen's agar. Salt tolerance was tested by comparison of biomass production at three salt concentrations (2.5, 5 and 10%, w/v) in shaken liquid medium. Cycloheximide tolerance was tested both in liquid medium at 0.01, 0.05 and 0.1% and on commercial Mycosel agar (0.4%; Becton Dickinson, Cockeysville, U.S.A.). DNAse activity was tested using commercial DNAse agar (Difco, Detroit, U.S.A.). Acid production was scored as dissolution of $CaCO_3$ powder suspended in agar.

Molecular biology

DNA isolation. Two ml of cell suspension was centrifuged in an Eppendorf centrifuge at 14,000 rpm and the cells were washed twice with sterile water. The pellets (approximately 100 μl) were stored at – 70 °C.

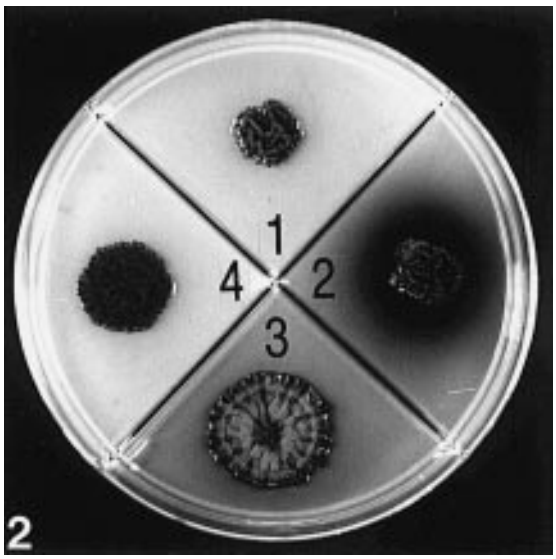


Figure 2. Colonies of *Sarcinomyces petricola*, CBS 600.93 after 2 months incubation, (1) Czapek agar; (2) Sabouraud's glucose agar; (3) 2% malt extract agar; (4) potato dextrose agar.

Total DNA was extracted by using a miniprep protocol. The pellet was dissolved in 0.5 ml of TES buffer (0.1 mM Tris-HCl, pH 8.0; 10 mM EDTA; 2% SDS) and approximately 400 μl of glass beads (diam 0.45–0.50 mm) were added. After homogenization, the mixture was vortexed for at least 10 min. Proteinase K was added and the mixture was incubated for 30 min at 55–60 °C. After centrifugation, the supernatant was transferred to a new tube. Subsequently the procedure of Möller et al. (1992) was followed, starting with the addition of CTAB in high salt concentration. The resulting DNA pellet was dissolved in water and the DNA concentration was determined spectrophotometrically.

DNA amplification. 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 $MgCl_2$, 0.01% gelatin, 200 μM of each deoxynucleotide triphosphate, 50 pmol of each primer, 10–100 ng nuclear DNA and 0.25 U of Taq DNA polymerase (Super Taq; Sphaero Q, Leiden, The Netherlands). Primers NS1, NS24, ITS1 and ITS4 (White et al. 1990) were employed. Forty amplification cycles were performed: 94 °C, 1 min; 48 °C, 2 min; 74 °C, 3 min (with initial and terminal delay of 1 min) in a Biomed thermocycler (type 60).

Restriction analysis. The amplicons were digested with restriction enzymes (1 U) for at least 2 h. The fol-

lowing enzymes were used: HinfI, HaeIII, RsaI, NdeII, HhaI, DdeI, TaqI and HpaII. Digests were subjected to electrophoresis on 2% agarose gels (TAE buffer) containing ethidium bromide. Resulting banding patterns were characterized with letters, identical patterns bearing the same letter.

Sarcinomyces petricola *Wollenzien & de Hoog, sp. nov. – Figures 1–11*

Coloniae nigrae, primum madidae, butyraceae, deinde siccae, firmae, maturae radiatim plicatae; lentissime crescentes, post 2 menses ad 10 mm (in agaro Czapekii) vel 20 mm diam. (in agaro maltoso); in agaro Sabouraudii saepe pigmentum obscure olivaceobrunneum diffundens. Cellulae gemmantes praedominant, late ellipsoideae vel globosae, ad quinas cellulas secundarias distantes proferentes. Cellulae juvenes 4.5—5.5 μm diam., paulatim inflatae et uno vel compluribus septis obliquis divisae, demum ad 13 μm diam. et usque ad 7 cellulas continentes. Hyphae in coloniis vetustis praesentes; cellulis circa 5 μm latis et ad 10 μm longis; gradatim dilatatae et septis transversalibus, longitudinalibus vel obliquis divisae, deinde in singulas cellulas dilabentes. Hyphae juvenes dilute brunneae, gradatim fuscescentes et atrobrunneae. Cellulae maximae crassitunicatae, demum pariete exteriore diffracto verrucis obscuris coopertae.

Proprietates assimilativae et fermentativae et tolerantiae in Tabula 1 enumeratae.

Holotypus: CBS 726.95, isolatus e marmore in sculptura angeli, Messinae in Italia, exsiccatus et vivus in Collectione CBS, Baarn, praeservatur.

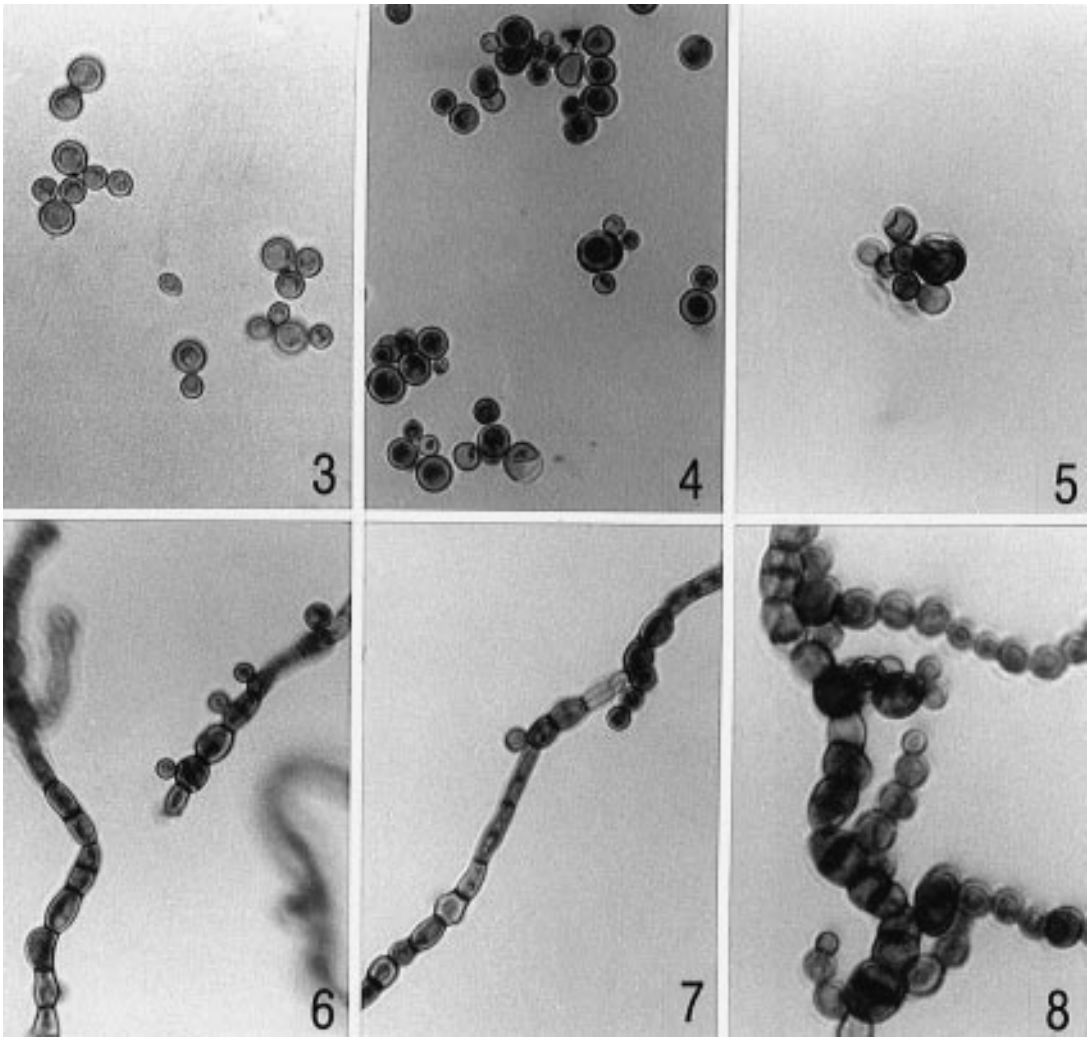
Colonies black, initially moist, buttery and glossy at the centre, later becoming dry, hard and finally with some grey, velvety aerial mycelium at least at the centre; mature colonies radially folded, centrally mostly cerebriform with age. Margin sharp, finely lobed. Colonies attaining up to 10 (Czapek) to 20 (2% malt) mm diameter in 2 months; on Sabouraud's glucose agar frequently a dark olivaceous brown pigment diffuses into the medium. Yeast cells preponderant, broadly ellipsoidal to spherical, producing up to five, widely spaced, spherical daughter cells by multilateral budding. Young budding cells 4.5–5.5 μm diam, gradually inflating isodiametrically and becoming subdivided by one or several oblique septa, finally containing up to 7 cells about 13 μm diam. Hyphae present in old cultures, with limited expansion growth; cells about 5 μm wide, up to 10 μm long, gradually widening and developing transverse, longitudinal and oblique septa, finally falling apart into individual cells. Hyphae pale brown at the apex, gradually turning dark to blackish brown. Cells thick-walled, finally bursting open whereby patches of outer cell wall remain as local, darker warts. Spherical hyphal clumps resembling sterile fruit bodies were occasionally observed.

Assimilative and fermentative abilities and results of tolerance tests are listed in Table 1. Restriction patterns of amplicons NS1-NS24 are summarized in Table 2 and representative patterns generated with ITS1-ITS4 are displayed in Figure 12.

Holotype: CBS 726.95, dried culture preserved at Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; type culture: CBS 726.95 (= UW H13), isolated from Carrara marble, angel statue in Messina museum garden, Italy.

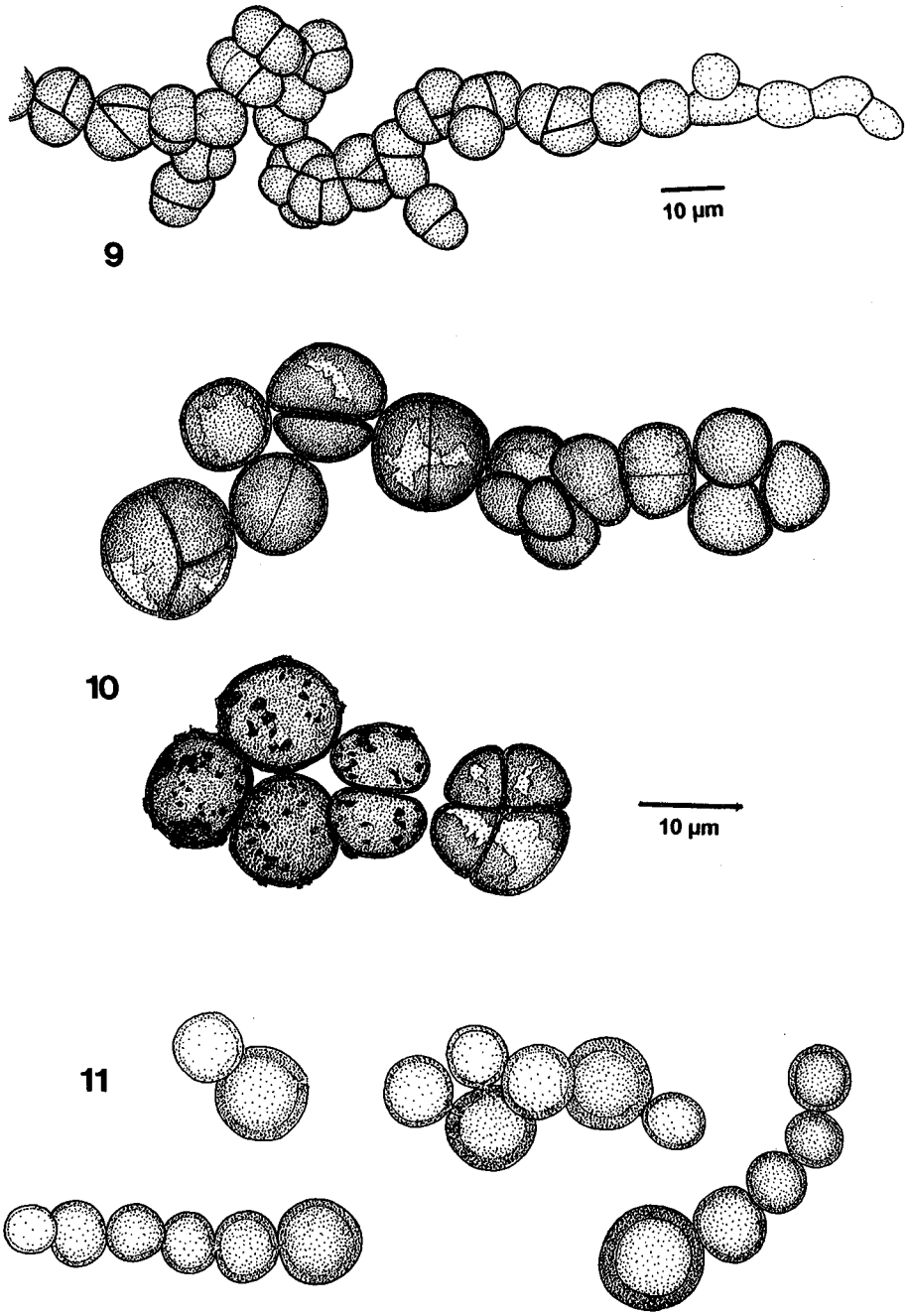
Discussion

The identity of *Sarcinomyces petricola* as a new species was established by comparison of restriction fragment length polymorphisms with a panel of meristematic black yeasts. This panel included the type strains of the genera *Phaeococcomyces, Phaeotheca* [including the recently described *P. triangularis* de Hoog & Beguin (1996)], *Botryomyces, Phaeosclera* and representative strains of *Sarcinomyces* and *Trimmatostroma*. *Aureobasidium pullulans* (De Bary) Arnaud and *Exophiala jeanselmei* (Langeron) McGinnis & Padhye were added as reference strains. The lengths of small subunit ribosomal DNA amplicons in this panel was found to differ, being either 1800 or 2100 bp (Table 2), probably due to the occurrence of introns (Uijthof et al. 1995, Haase et al. 1995). The three strains of *S. petricola*, with 1800 bp, lacked introns. The spectrum of SSU restriction patterns of *S. petricola* obtained differed consistently from that of the remaining species, including the morphologically most similar species, *S. phaeomuriformis* (Table 2). Little similarity was observed with *Aureobasidium pullulans* and related meristematic fungi of supposed dothideaceous relationship (de Hoog et al. 1996b). Only patterns obtained after HaeIII digestion of the SSU rDNA were identical to those of *Trimmatostroma abietis* Butin & Pehl and some *Phaeotheca* and *Phaeosclera* species (Table 2). Digestion of the amplicon containing the two internal transcribed spacer regions (amplicon ITS1-ITS4) yielded unique patterns with eight enzymes tested (Figure 12). No intraspecific variability was noted.



Figures 3–8. *Sarcinomyces petricola*, CBS 726.95 on MEA2% agar. (3,4) Yeast cells with multilateral budding (3: x 720; 4, 5: x 640); (5) cell subdivided by 4 septa, with some small cells (x 640); (6,7) hyphal formation in 2-month-old culture (x 640); (8) hyphae with meristematic development (x 720).

Meristematic black yeasts are notoriously hard to classify because of their morphological plasticity. Depending on environmental conditions, a single strain may change its morphology drastically, which would justify classification in different anamorph genera (Figueras et al. 1995). *Sarcinomyces petricola* shows a conversion from yeast-like cells with multilateral budding (matching the anamorph genus *Phaeococcomyces* de Hoog) to meristematic growth (matching the anamorph genus *Sarcinomyces* Lindner), eventually leading to endogenous reproduction (matching the anamorph genus *Phaeotheca* Sigler et al.), while cells may also arise after meristematic swelling of hyphal cells (matching the anamorph genus *Phaeosclera* Sigler et al.). Attribution of the fungus to one of these genera on the basis of morphology alone therefore remains arbitrary. De Hoog et al. (1996b) presented PCR-ribotyping and physiological data indicating that most meristematic fungi possibly are anamorph members of Dothideaceae, while only very few are are related to the Herpotrichiellaceae. Anamorph genera should reflect natural relationships. However, since the affinity of a few meristematic genera could not be established (de Hoog et al. 1997) due to the presence of introns in 18S rDNA of type strains, the meristematic genera could not yet be sorted out adequately. We



Figures 9–11. (9) *Sarcinomyces petricola,* CBS 600.93, hyphae of 2-week-old colony on OA, gradually developing into meristematic conidia. (10) *Sarcinomyces petricola,* CBS 600.93, 6-week-old culture on OA. Meristematic conidia in final stage of development, with bursting of outer cell wall layer which finally remains as flakes on the inner cell wall. (11) *Sarcinomyces petricola,* CBS 600.93, 8-week-old culture on MEA. Budding cells in final stage of development.

therefore prefer to classify our fungus in the oldest anamorph genus of meristematic fungi, *Sarcinomyces*.

Sarcinomyces petricola has an 18S ribotype similar to that of the majority of *Exophiala* species (Uijthof & de Hoog 1995) and is therefore believed to be

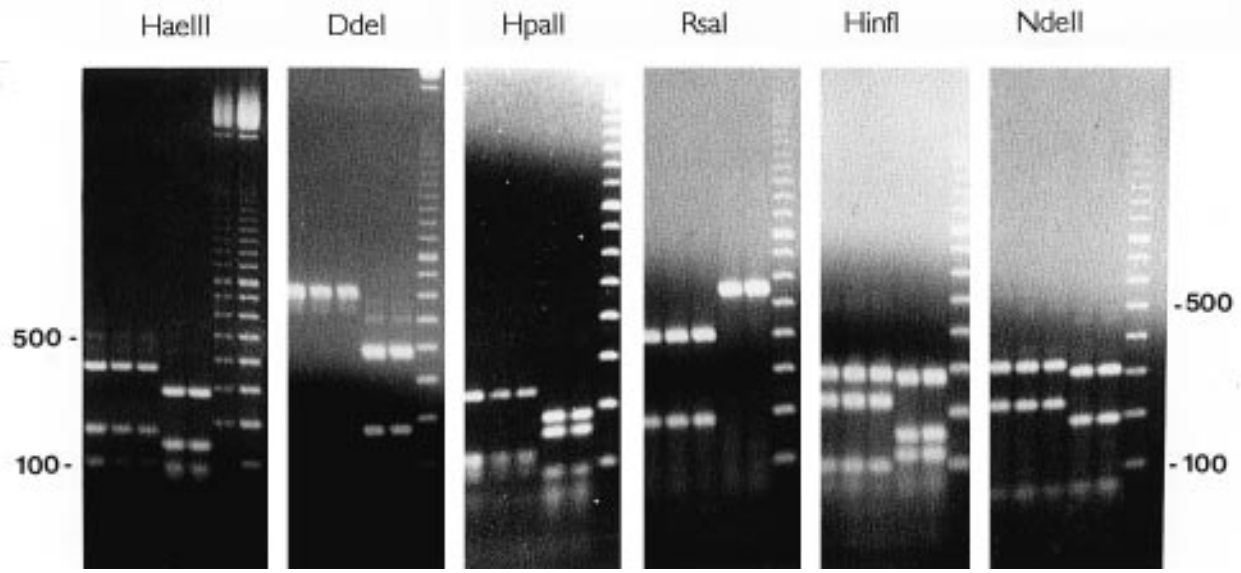


Figure 12. Digestion patterns of amplicon ITS1-ITS4 from *Sarcinomyces petricola*, CBS 600.93, 725.95, 726.95 (lanes 1–3, in every panel) and *Phaeotheca triangularis*, CBS 469.90, 471.90 (lanes 4 and 5 in every panel), digested with HaeIII, DdeI, HpaII, RsaI, HinfI and NdeII.

an anamorph member of the Herpotrichiellaceae. Its lack of extracellular DNAse corroborates this supposition. It is morphologically similar to yeast-like synanamorphs of *Exophiala* Carmichael and to *Phaeococcomyces*. However, in *Exophiala* a clear difference in shape is always seen between young budding cells and mature cells after subsequent swelling (de Hoog et al. 1994), while in *S. petricola* only conidial sizes are different, all budding cells being spherical. Physiologically the species differs from the generic type species of *Phaeococcomyces*, *P. nigricans* (Rich & Stern) de Hoog, in many physiological parameters (compare de Hoog et al. 1995a).

Sarcinomyces phaeomuriformis Matsumoto et al. is morphologically similar to *S. petricola*, and is also of herpotrichiellaceous relationship. PCR-Ribotyping of amplicon ITS1- ITS4 of that species yields patterns identical to those of *Exophiala dermatitidis* (Kano) de Hoog (Uijthof et al. 1996). These patterns differ from those of *S. petricola* for all restriction enzymes used in this study (Uijthof & De Hoog 1995; J.M.J. Uijthof, unpublished results). In addition, the species are physiologically different in growth reactions to D-ribose, L-rhamnose, lactose, inulin, nitrate, nitrite and growth at 40 °C (de Hoog et al. 1997).

The meristematic genera *Botryomyces*, *Phaeosclera* and *Phaeotheca* have hitherto been defined on the basis of morphological criteria. *Botryomyces* is well delimited from the other genera by cells being initially subhyaline rather than melanized. The type species of *Phaeosclera* and *Phaeotheca* are clearly different from *S. petricola* in their physiological profiles (de Hoog et al. 1997).

Numerous fungi can be isolated from stone (Braams 1992) and are able to grow and reproduce during the wet season (Wollenzien et al. 1995). However, only very few species with extremely slow growth are able to survive on unprotected stone during summer. Among these are the MCF which are also known from extreme environments such as desert rock (Staley et al. 1984). *Sarcinomyces petricola* is the first species which has not been found on substrates other than stone, and thus might be the first unlichenized fungus with its natural ecological niche on stone. Its exact role in biodegradation still has to be established.

Acknowledgements

Collection and isolation of samples was partially supported by a Vigoni travel programme and a CNR contribution to a bilateral project Italy / Germany 1992–1994. We further acknowledge financial support of the EU through grant Nr EV5V-CT94–0569. We are particularly indebted to Dr Clara Urzì for her open hospitality at the Istituto di Microbiologia in Messina. Prof. T. Moropolou of the NTU at Athens is acknowledged for sampling at Corfu, the Acropolis Committee for

Table 1. Physiological profile of *Sarcinomyces petricola*

	600.93	725.95	726.95		600.93	725.95	726.95
D-Glucose	+	+	+	keto-2-Gluconate	w	-	w
D-Galactose	+	+	+	D-Gluconate	+	+	+
L-Sorbose	+	+	+	D-Glucuronate	+	+	+
D-Glucosamine	-	-	-	D-Galacturonate	+	w	+
D-Ribose	+	+	+	DL-Lactate	-	-	-
D-Xylose	+	+	+	Succinate	+	w	+
L-Arabinose	+	+	+	Citrate	-	-	-
D-Arabinose	+	+	+	Methanol	-	-	-
L-Rhamnose	-	-	w	Ethanol	+	+	+
Sucrose	+	+	+	Nitrate	+	+	+
Maltose	+	+	+	Nitrate	+	+	+
α-α-Trehalose	+	+	+	Ethylamine	+	+	+
methyl-α-D-Glucoside	w	-	+	L-Lysine	+	+	+
Cellobiose	+	+	+	Cadaverine	+	+	+
Salicin	w	-	w	Creatine	-	+	w
Arbutin	-	-	-	Creatinine	+	+	+
Melibiose	+	+	+	5% $MgCl_2$	+	+	+
Lactose	-	-	-	10% $MgCl_2$	+	+	+
Raffinose	+	+	+	5% NaC1	+	+	+
Melezitose	+	+	+	10% NaC1	w	+	-
Inulin	+	w	+	0.01% Cycloheximide	-	-	-
Sol. starch	w	w	w	0.1% Cycloheximide	-	-	-
Glycerol	+	+	+	Mycosel	-	-	-
meso-Erythritol	+	+	+	Urease	+	+	+
Ribitol	+	+	+				
Xylitol	+	+	+	30C	+	+	+
L-Arabinitol	+	+	+	37C	-	-	-
D-Glucitol	+	+	+	Fermentation	-	-	-
D-Mannitol	+	+	+	Gelatin	w	w	w
Galacitol	+	+	+	DNAse	-	-	-
myo-Inositol	+	w	+	Acid production	-	-	-
Glucono-δ-lactone	+	w	+				

Table 2. Meristematic black yeasts, representative restriction patterns of amplicons NS1–NS24

Species	CBS No.	SSU size	Restriction enzymes				
			HinfI	HaeIII	RsaI	DdeI	TaqI
Aureobasidium pullulans	755.96	1800	A	A	A	A	A
Trimmatostroma abietis	459.93	1800	A	B	B	A	A
Phaeotheca fissurella	520.89	1800	A	B	B	A	A
Phaeotheca triangularis	469.90	1800	A	B	-	A	A
Phaeosclera dematioides	157.81	1800	-	B	C	A	A
Botryomyces caespitosus	177.80	2100	B	C	D	B	B
Sarcinomyces crustaceus	156.89	2100	C	D	E	C	C
Sarcinomyces phaeomuriformis	131.88	3000	D	E	F	D	-
Sarcinomyces petricola	600.93	1800	E	B	G	E	D
Sarcinomyces petricola	725.95	1800	E	B	G	E	D
Sarcinomyces petricola	726.95	1800	E	B	G	E	D
Exophiala jeanselmei	507.90	1800	E	F	G	F	D

sampling at the Acropolis monuments, and Dr F. Campagna, Director of the Messina Museum, for sampling at Messina.

References

Braams J (1992) Ecological studies on the fungal microflora inhabiting historical sandstone monuments. Ph. D. Thesis, Oldenburg, 104 pp.

Butin H, Pehl L, De Hoog GS & Wollenzien U (1995) *Trimmatostroma abietis* sp. nov. (hyphomycetes) and related species. Antonie van Leeuwenhoek 69: 203–209

Diakumaku E, Gorbushina AA, Krumbein WE, Panina L & Soukharjevski S (1995) Black fungi in marble and limestones – an aesthetical, chemical and physical problem for the conservation of monuments. Sci. Total Environm. 167: 295–304

Figueras MJ, De Hoog GS, Takeo K & Guarro J (1995) Stationary phase development of *Trimmatostroma abietis*. Antonie van Leeuwenhoek 69: 217–222

Haase G, Sonntag L, Van de Peer Y, Uijthof JMJ, Podbielski A & Melzer-Krick B (1995) Phylogenetic analysis of ten black yeast species using nuclear small subunit rRNA gene sequences. Antonie van Leeuwenhoek 68: 19–33

Hoog GS de, Beguin H & Batenburg-van der Vegte WH (1997) *Phaeotheca triangularis*, a new meristematic black yeast from a humidifier. Antonie van Leeuwenhoek 71: 289–295

Hoog GS de, Gerrits van den Ende AHG, Uijthof JMJ, Boos C & Gené J (1997) Taxonomy of meristematic black yeast-like fungi (in prep.)

Hoog GS de, Gerrits van den Ende AHG, Uijthof JMJ & Untereiner WA (1995a) Nutritional physiology of type isolates of currently accepted species of *Exophiala* and *Phaeococcomyces*. Antonie van Leeuwenhoek 68: 43–49

Hoog GS de, Guého E, Masclaux F, Gerrits van den Ende AHG, Kwon-Chung KJ & McGinnis MR (1995b) Nutritional physiology and taxonomy of human-pathogenic *Cladosporium-Xylohypha* species. J. Med. Vet. Mycol. 33: 339–347

Hoog GS de, Takeo K, Yoshida S, Göttlich E, Nishimura K & Miyaji M (1994) Pleoanamorphic life cycle of *Exophiala (Wangiella) dermatitidis*. Antonie van Leeuwenhoek 65: 143–153

Jarvis R (1977) Evaluation of the Spiral Plate Maker for the enumeration of microorganisms in foods. J. Appl. Bacteriol. 43: 149–157

King AD, Hocking AD & Pitt JI (1979) Dichlorane rose bengal medium for enumeration and isolation of moulds from foods. Appl. Environm. Microbiol. 43: 149–157

Möller EM, Bahnweg G, Sandermann H & Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissue. Nucl. Acids Res. 20: 6115–6116

Staley JT, Palmer F & Adams JB (1984) Microcolonial fungi: common inhabitants on desert rocks? Science 215: 1093–1095

Uijthof JMJ, Van Belkum A, De Hoog GS & Haase G (1996) *Exophiala dermatitidis* and *Sarcinomyces phaeomuriformis:* physiology, ITS1-sequencing and the development of a molecular probe. J. Med. Vet. Mycol. (in press)

Uijthof JMJ & De Hoog GS (1995) PCR-Ribotyping of type isolates of currently accepted *Exophiala* and *Phaeococcomyces* species. Antonie van Leeuwenhoek 68: 35–42

Urzì C, Krumbein WE, Lyalikova N, Petushkova J, Wollenzien U & Zagari M (1994) Microbiological investigations of marbles exposed to natural and anthropogenic influences in northern and southern climates. In Fassina V, Ott H & Zezza F (eds): Stone and monuments pp. 297–304, Proc. 3rd Int Symp. Cons. Monum. Medit. Basin, Venice

Walt JP van der & Yarrow D (1984) Methods for the isolation, maintenance, classification and identification of yeasts. In: Kreger-van Rij NJW (Ed) The Yeasts, a Taxonomic Study. 4th ed. (pp 45–104) Elsevier, Amsterdam

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfland DH, Sninsky JJ, White TH (Eds) PCR Protocols (pp 315–322) Academic Press, San Diego, California

Wollenzien U, De Hoog GS, Krumbein WE & Urzì C (1995) On the isolation of microcolonial fungi occurring on and in marble and other calcareous rocks. Sci. Total Environm. 167: 287–294

Wollenzien U, Urzì C & Krumbein WE (1993) Report on the microbial and biochemical analyses of some Carrara marble statues of the old Nord- and Südfriedhof München. Proc. 3rd Workshop Euromarble, Gothenburg pp 47–61