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RESEARCH ARTICLE

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Elucidating the *Ramularia eucalypti* species complex

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Key words

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plant pathogen
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Abstract The genus *Ramularia* includes numerous phytopathogenic species, several of which are economically important. *Ramularia eucalypti* is currently the only species of this genus known to infect *Eucalyptus* by causing severe leaf-spotting symptoms on this host. However, several isolates identified as *R. eucalypti* based on morphology and on nrDNA sequence data of the ITS region have recently been isolated from other plant hosts, from environmental samples and also from human clinical specimens. Identification of closely related species based on morphology is often difficult and the ITS region has previously been shown to be unreliable for species level identification in several genera. In this study we aimed to resolve this species-complex by applying a polyphasic approach involving morphology, multi-gene phylogeny and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Six partial genes (ITS, ACT, TEF1-α, HIS3, GAPDH and RPB2) were amplified and sequenced for a total of 44 isolates representing *R. eucalypti* s.lat. and closely related species. A multi-gene Bayesian phylogenetic analysis and parsimony analysis were performed, and both the resulting trees showed significant support for separation of seven species in *R. eucalypti* s.lat., including two previously described (*R. eucalypti* and *R. miae*), four novel species here described (*R. haroldporteri*, *R. glennii*, *R. mali* and *R. plurivora*) and one undescribed *Ramularia* species (sterile). Additionally, *Mycosphaerella nyssicola* is newly combined in *Ramularia* as *R. nyssicola*. Main mass spectra (MSPs) of several *R. eucalypti* s.lat. strains were generated using MALDI-TOF MS and were compared through a Principal Component Analysis (PCA) dendogram. The PCA dendrogram supported three clades containing *R. plurivora*, *R. glenni*/*R. mali* and *R. eucalypti*/*R. miae*. Although the dendrogram separation of species differed from the phylogenetic analysis, the clinically relevant strains were successfully identified by MALDI-TOF MS.

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INTRODUCTION

Ramularia (Unger 1833) is a species-rich genus in the order *Capnodiales* that includes more than 1 000 legitimate species names (www.MycoBank.org, acc. Apr. 2014). The genus has been monographed by Braun (1995, 1998), who defined *Ramularia* species as hyphomycetes with hyaline conidiophores and conidia with distinct, thickened, darkened and refractive conidial scars and hila. The sexual morph of *Ramularia* species belongs to *Mycosphaerella* (*Mycosphaerellaceae*) but the number of experimentally proven links is small and some species may be true asexual holomorphs (Sivanesan 1984, Braun 1995, Verkley et al. 2004, Crous et al. 2009b, Koike et al. 2011). Currently *Ramularia* species are accepted as being host-specific, though some exceptions are likely to emerge (Braun 1998). Most species are phytopathogenic and associated with leaf spots, necrosis or chlorosis, but some species can be saprobic or even hyperparasitic. Foliar diseases occur mostly under conditions of high air humidity and low temperatures and result indirectly in crop loss due to defoliation. The most harmful pathogens in this genus are *R. collo-cygni*, *R. beticola* and *R. grevilleana* that cause severe economic losses in barley, sugarbeet and strawberry crops, respectively.

Ramularia eucalypti is a recently described species that was isolated from mature *Corymbia grandifolia* leaves collected in Italy that exhibited severe leaf spotting symptoms (Crous et al. 2007). It is currently the only species of the genus known to infect *Eucalyptus* and *Corymbia*, since *R. pitereka* and aggregate species have been reassigned to *Quambalaria* (*Quambalariaceae*) (de Beer et al. 2006). Over the past few years several isolates have been collected and identified as *R. eucalypti* based on morphology and on sequence data of the ITS region of the nrDNA operon which has recently been adopted as the universal DNA barcode for fungi (Schoch et al. 2012). However, in several genera of phytopathogenic fungi, ITS phylogenies have often failed to separate closely related species, and a better resolution could only be achieved by using protein-coding loci (Lombard et al. 2010, Cabral et al. 2012, Crous et al. 2013, Groenewald et al. 2013, Quaedvlieg et al. 2013, Woudenberg et al. 2013).

In Italy, *R. eucalypti* has been reported as an emerging problem on pome fruit in cold storage where it causes lenticel rot in healthy fruits of apple (*Malus domestica* cv. Ambrosia) and pear (*Pyrus communis* cv. Conference) (Giordani et al. 2012). Investigations into the epidemiology showed that apple trees in the orchards had leaf spots caused by *R. eucalypti*, and symptomless fruits harvested from infected plants exhibited disease symptoms during the subsequent four months of cold storage (Gianetti et al. 2012).

In the Netherlands, *R. eucalypti* has been isolated not only from different plant hosts but has also been obtained from clinical specimens in different hospitals. This is the first time a *Ramularia* species is associated with human infection and little is known of its epidemiology. However, this is not the first time a plant pathogenic fungus has been reported to be able

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Table 1 Collection details and GenBank accession numbers of isolates included in this study.

Species	Accession number(s)[1,2]	Host/isolation source	Country	Collector	GenBank Accession numbers[3]					
					ITS	ACT	TEF1-α	GAPDH	RPB2	HIS3
Ramularia agrimoniae	CPC 11653	*Agrimonia pilosa*	South Korea	H.-D. Shin	KJ504784	KJ504448	KJ504699	KJ504567	KJ504655	KJ504611
Ramularia calcea	CBS 101612	*Symphytum* sp.	Germany	G. Arnold	KJ504785	KJ504449	KJ504700	KJ504568	KJ504656	KJ504612
Ramularia collo-cygni	CBS 101181	*Hordeum vulgare*	Germany	E. Sachs	KJ504786	KJ504450	KJ504701	KJ504569	KJ504657	KJ504613
Ramularia decipiens	CBS 114300	*Rumex aquaticus*	Sweden	E. Gunnerbeck	KJ504787	KJ504451	KJ504702	KJ504570	KJ504658	KJ504614
Ramularia eucalypti	CBS 155,82	*Puccinia* sp. on *Carex acutiformis*	Netherlands	W. Gams & O. Constantinescu	KJ504789	KJ504453	KJ504704	KJ504572	KJ504660	KJ504616
	CBS 356,69	*Malus sylvestris*	Netherlands	–	KJ504790	KJ504454	KJ504705	KJ504573	KJ504661	KJ504617
	CBS 101045	*Geranium pusillum*	Netherlands	H.A. van der Aa	KJ504791	KJ504455	KJ504706	KJ504574	KJ504662	KJ504618
	CBS 120726 T, CPC 13043	*Corymbia grandifolia*	Italy	W. Gams	KJ504792	KJ504456	KJ504707	KJ504575	KJ504663	KJ504619
	CBS 120728, CPC 13304	*Eucalyptus* sp.	Australia	P.W. Crous	KJ504793	KJ504457	KJ504708	KJ504576	KJ504664	KJ504620
	CPC 13044	*Corymbia grandifolia*	Italy	W. Gams	KJ504794	KJ504458	KJ504709	KJ504577	KJ504665	KJ504621
	CPC 13045	*Corymbia grandifolia*	Italy	W. Gams	KJ504795	KJ504459	KJ504710	KJ504578	KJ504666	KJ504622
	CPC 16804	*Pinus wallichiana*	Netherlands	W. Quaedvlieg	KJ504796	KJ504460	KJ504711	KJ504579	KJ504667	KJ504623
	CPC 19187	*Phragmites* sp.	Netherlands	P.W. Crous	KJ504797	KJ504461	KJ504712	KJ504580	KJ504668	KJ504624
	CPC 19188	*Phragmites* sp.	Netherlands	P.W. Crous	KJ504798	KJ504462	KJ504713	KJ504581	KJ504669	KJ504625
Ramularia glechomatis	CBS 108979	*Glechoma hederacea*	Netherlands	G. Verkley	KJ504799	KJ504463	KJ504714	KJ504582	KJ504670	KJ504626
Ramularia glennii	CBS 120727, CPC 13046	*Corymbia grandifolia*	Italy	W. Gams	KJ504767	KJ504431	KJ504682	–	KJ504638	KJ504594
	CBS 122989, CPC 15195	Human skin	Netherlands	–	KJ504768	KJ504432	KJ504683	KJ504551	KJ504639	KJ504595
	CBS 129441 T	Human lungs	Netherlands	–	KJ504769	KJ504433	KJ504684	KJ504552	KJ504640	KJ504596
	CPC 13047	*Corymbia grandifolia*	Italy	W. Gams	KJ504770	KJ504434	KJ504685	KJ504553	KJ504641	KJ504597
	CPC 13048	*Corymbia grandifolia*	Italy	W. Gams	KJ504771	KJ504435	KJ504686	KJ504554	KJ504642	KJ504598
	CPC 16560	*Eucalyptus camaldulensis*	Iraq	A. Saadoon	KJ504772	KJ504436	KJ504687	KJ504555	KJ504643	KJ504599
	CPC 16561	*Eucalyptus camaldulensis*	Iraq	A. Saadoon	KJ504773	KJ504437	KJ504688	KJ504556	KJ504644	KJ504600
	CPC 16565	*Eucalyptus camaldulensis*	Iraq	A. Saadoon	KJ504774	KJ504438	KJ504689	KJ504557	KJ504645	KJ504601
	CPC 18468	Rubber of refrigerator	USA: Athens	A.E. Glenn	KJ504775	KJ504439	KJ504690	KJ504558	KJ504646	KJ504602
	CPC 18469	Rubber of refrigerator	USA: Athens	A.E. Glenn	KJ504776	KJ504440	KJ504691	KJ504559	KJ504647	KJ504603
	CPC 18470	Rubber of refrigerator	USA: Athens	A.E. Glenn	KJ504777	KJ504441	KJ504692	KJ504560	KJ504648	KJ504604
Ramularia haroldporteri	CBS 137272 T, CPC 16296	Unidentified bulb plant	South Africa	P.W. Crous	KJ504766	KJ504430	KJ504681	–	KJ504637	KJ504593
Ramularia major	CPC 12543	*Petasites japonicus*	South Korea	H.-D. Shin	KJ504800	KJ504464	KJ504715	KJ504583	KJ504671	KJ504627
Ramularia mali	CBS 129581 T	Apple in storage	Italy	–	KJ504778	KJ504442	KJ504693	KJ504561	KJ504649	KJ504605
Ramularia miae	CBS 120121 T, CPC 12736	*Wachendorfia thyrsiflora*	South Africa	M.K. Crous & P.W. Crous	KJ504801	KJ504465	KJ504716	KJ504584	KJ504672	KJ504628
	CPC 12737	*Wachendorfia thyrsiflora*	South Africa	M.K. Crous & P.W. Crous	KJ504802	KJ504466	KJ504717	KJ504585	KJ504673	KJ504629
	CPC 12738	*Wachendorfia thyrsiflora*	South Africa	M.K. Crous & P.W. Crous	KJ504803	KJ504467	KJ504718	KJ504586	KJ504674	KJ504630
	CPC 19835	*Gazania rigens* var. *uniflora*	South Africa	P.W. Crous	KJ504804	KJ504468	KJ504719	KJ504587	KJ504675	KJ504631
	CPC 19770	*Leonotis leonurus*	South Africa	P.W. Crous	KJ504805	KJ504469	KJ504720	KJ504588	KJ504676	KJ504632
Ramularia nyssicola	CBS 127665 ET	*Nyssa ogeche* × *sylvatica* hybrid	USA: Maryland	R. Olsen	KJ504765	KJ504429	KJ504680	–	KJ504636	KJ504592
Ramularia plurivora	CBS 118693, CPC 12206	Human skin	Netherlands	–	KJ504779	KJ504443	KJ504694	KJ504562	KJ504650	KJ504606
	CBS 118743 T, CPC 12207	Human bone marrow	Netherlands	–	KJ504780	KJ504444	KJ504695	KJ504563	KJ504651	KJ504607
	CPC 11517	*Coleosporium plectanthri* on *Plectranthus excisus*	South Korea	H.-D. Shin	KJ504781	KJ504445	KJ504696	KJ504564	KJ504652	KJ504608
	CPC 16123	Melon in storage	Netherlands	–	KJ504782	KJ504446	KJ504697	KJ504565	KJ504653	KJ504609
	CPC 16124	Melon in storage	Netherlands	–	KJ504783	KJ504447	KJ504698	KJ504566	KJ504654	KJ504610
Ramularia pratensis	CBS 136.23	–	–	A. Weber	KJ504806	KJ504470	KJ504721	KJ504589	KJ504677	KJ504633
Ramularia sp.	CBS 114568	*Epilobium hirsutum*	Sweden	E. Gunnerbeck	KJ504788	KJ504452	KJ504703	KJ504571	KJ504659	KJ504615
Ramularia tovarae	CBS 113305	*Persicaria filiformis*	South Korea	H.-D. Shin	KJ504807	KJ504471	KJ504722	KJ504590	KJ504678	KJ504634
Ramularia vizellae	CBS 130601 T, CPC 18283	*Vizella interrupta*	South Africa	P.W. Crous	KJ504808	KJ504472	KJ504723	KJ504591	KJ504679	KJ504635

[1] CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands; CPC: Culture collection of P.W. Crous, housed at CBS.
[2] T: ex-type strain; ET: ex-epitype strain
[3] ITS: Internal transcribed spacers 1 and 2 together with 5.8S nrDNA; ACT: actin; TEF1-α: translation elongation factor 1-alpha; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; RPB2: RNA polymerase II second largest subunit; HIS3: histone H3.

to infect human hosts (Mostert et al. 2006, van Baarlen et al. 2007, Phillips et al. 2013). The number of infections caused by filamentous fungi previously considered of low clinical relevance has increased in the past few years, especially among immunocompromised patients (Cassagne et al. 2011, Lu et al. 2013). Mycoses caused by hyaline, septate fungal hyphae fall under the medical term hyalohyphomycosis and some of the pathogens involved have been demonstrated to be resistant to certain antifungals (Tortorano et al. 2014). Therefore, a fast and accurate diagnosis is critical for patient management in order to determine appropriate treatment. The identification of microbial species is usually based on microscopy and biochemical methods that are time consuming and require high expertise. The DNA sequencing approach gives more reliable and faster results but still remains laborious. Recently, a technique known as Matrix-Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) has been revolutionising the clinical diagnostics field. This technique allows the identification of microorganisms by analysing their unique protein peak pattern and comparing it with a database of reference main mass spectra (MSPs). The peaks present in the MSP represent mostly ribosomal proteins but structural proteins, cold-shock proteins and others may also be detected. MALDI-TOF MS is a simple, fast and accurate procedure that has been validated in numerous laboratories for the identification of yeasts (Goyer et al. 2012, Kolecka et al. 2013) and bacteria (Seng et al. 2009) from clinical samples. Recently, an effort has been made towards the validation of standardised procedures for routine mould identification (Cassagne et al. 2011, Lau et al. 2013) and dermatophytes (L'Ollivier et al. 2013) from clinical samples and all reports showed that MALDI-TOF MS had a good discrimination power for species separation, effectively decreased the time of identification and improved its accuracy. Furthermore, MALDI-TOF MS has also been used in studies were it was used as a complementary tool for taxonomical discriminatory purposes: Degenkolb et al. (2008) used MALDI-TOF MS in a polyphasic approach to support the description of *Trichoderma brevicompactum* as a novel species and Brun et al. (2013) tested this technique to discriminate closely related species of *Alternaria*.

In this study we aimed to:

i. resolve the *R. eucalypti* species-complex by applying a polyphasic approach involving morphology, multi-gene phylogeny and MALDI-TOF mass spectrometry; and
ii. build an in-house library of MSPs of *R. eucalypti* s.lat. strains in order to evaluate the taxonomic resolution power of this technique for the identification of the set of clinical isolates within this species complex.

MATERIALS AND METHODS

Fungal strains

The 44 isolates used in this study are maintained in the culture collection of the CBS-KNAW Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands, and the working collection of Pedro Crous (CPC), housed at CBS (Table 1).

DNA extraction, amplification and sequencing

The fungal strains (Table 1) were grown on Malt Extract Agar (MEA), for 7 d at room temperature (20 °C). The mycelium was harvested with a sterile scalpel and the genomic DNA was isolated using the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA, USA) following the manufacturers' protocols. Ten partial nuclear genes were initially targeted for PCR amplification and sequencing, namely, 28S nrRNA gene (LSU), internal transcribed spacer regions and intervening 5.8S nrRNA gene (ITS) of the nrDNA operon, actin (ACT), translation elongation factor 1-α (TEF1-α), histone H3 (HIS3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNA polymerase II second largest subunit (RPB2), calmodulin (CAL), β-tubulin (bTUB) and chitin synthase I (CHS-1). The primers employed are listed in Table 2. The PCR amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR mixtures consisted of 1 µL genomic DNA, 1× GoTaq® Flexi buffer (Promega, Madison, WI, USA), 2 µM $MgCl_2$, 40 µM of each dNTP, 0.2 µM of each primer and 0.5 Unit GoTaq® Flexi DNA polymerase (Promega) in a total volume of 12.5 µL. The PCR mixtures for HIS3, GAPDH, RPB2, CAL, bTUB and CHS-1 contained 2 µL

Table 2 Details of primers used and/or developed for this study for the PCR amplification and sequencing of the different genes.

Gene	Primer Name	Sequence 5'→3'	Annealing temperature (°C)	Orientation	Reference
ACT	ACT-512F	ATG TGC AAG GCC GGT TTC GC	55	Forward	Carbone & Kohn (1999)
	ACT-783 R	TAC GAG TCC TTC TGG CCC AT	55	Reverse	Carbone & Kohn (1999)
	ACT-2Rd	ARR TCR CGD CCR GCC ATG TC	55	Reverse	Groenewald et al. (2013)
bTUB	T1	AAC ATG CGT GAG ATT GTA AGT	52	Forward	O'Donnell & Cigelnik (1997)
	β-Sandy-R	GCR CGN GGV ACR TAC TTG TT	52	Reverse	Stukenbrock et al. (2012)
	Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC	52	Forward	Glass & Donaldson (1995)
	Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	52	Reverse	Glass & Donaldson (1995)
CAL	CAL-228F	GAG TTC AAG GAG GCC TTC TCC C	58	Forward	Carbone & Kohn (1999)
	CAL-737R	CAT CTT TCT GGC CAT CAT GG	58	Reverse	Carbone & Kohn (1999)
	Cal2Rd	TGR TCN GCC TCD CGG ATC ATC TC	58	Reverse	Groenewald et al. (2013)
CHS-1	CHS-79F	TGG GGC AAG GAT GCT TGG AAG AAG	52	Forward	Carbone & Kohn (1999)
	CHS-354R	TGG AAG AAC CAT CTG TGA GAG TTG	52	Reverse	Carbone & Kohn (1999)
GAPDH	gpd1	CAA CGG CTT CGG TCG CAT TG	55	Forward	Berbee et al. (1999)
	gpd2	GCC AAG CAG TTG GTT GTG C	55	Reverse	Berbee et al. (1999)
HIS3	CylH3F	AGG TCC ACT GGT GGC AAG	52	Forward	Crous et al. (2004b)
	CylH3R	AGC TGG ATG TCC TTG GAC TG	52	Reverse	Crous et al. (2004b)
ITS	V9G	TTA CGT CCC TGC CCT TTG TA	52	Forward	de Hoog & Gerrits van den Ende (1998)
	ITS4	TCC TCC GCT TAT TGA TAT GC	52	Reverse	White et al. (1990)
LSU	LSU1Fd	GRA TCA GGT AGG RAT ACC CG	52	Forward	Crous et al. (2009a)
	LR5	TCC TGA GGG AAA CTT CG	52	Reverse	Vilgalys & Hester (1990)
RPB2	RPB2-f5f	GAY GAY MGW GAT CAY TTY GG	60→58→54	Forward	Liu et al. (1999)
	RPB2-7cR	CCC ATR GCT TGY TTR CCC AT	60→58→54	Reverse	Liu et al. (1999)
	Rpb2-F1	GGTGTCAGTCARGTGYTGAA	60→58→54	Forward	This study
	Rpb2-R1	TCC TCN GGV GTC ATG ATR ATC AT	60→58→54	Reverse	This study
Tef1-α	EF-728F	CAT CGA GAA GTT CGA GAA GG	54	Forward	Carbone & Kohn (1999)
	EF-2	GGA RGT ACC AGT SAT CAT GTT	54	Reverse	O'Donnell et al. (1998)
	TEF-1R	CTT GAT GAA ATC ACG GTG ACC	54	Reverse	This study

genomic DNA. The PCR conditions were: initial denaturation (94 °C, 3 min); 35 cycles amplification (94 °C, 30 s; annealing (Table 2), 30 s; 72 °C, 45 s) and final extension (72 °C, 5 min). For GAPDH and HIS3, 40 amplification cycles were used. To obtain the partial RPB2, a touchdown PCR protocol was used: initial denaturation (94 °C, 3 min), five amplification cycles (94 °C, 45 s; 60 °C, 45 s; 72 °C, 2 min), five amplification cycles (94 °C, 45 s; 58 °C, 45 s; 72 °C, 2 min), 30 amplification cycles (94 °C, 45 s; 54 °C, 45 s; 72 °C,2 min) and a final extension (72 °C, 8 min). The resulting fragments were sequenced in both directions using the PCR primers and a BigDye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems Life Technologies, Carlsbad, CA, USA). DNA sequencing amplicons were purified through Sephadex G-50 Superfine columns (Sigma-Aldrich, St. Louis, MO) in MultiScreen HV plates (Millipore, Billerica, MA). Purified sequence reactions were analysed on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, Carlsbad, CA, USA). The DNA sequences generated were analysed and consensus sequences were computed using the BioNumerics v. 4.61 software package (Applied Maths, St-Martens-Latem, Belgium).

Phylogenetic analyses

Ramularia nyssicola (CBS 127665) has recently been revised and separated from *R. endophylla* (= *Mycosphaerella punctiformis*) (Minnis et al. 2011). *Ramularia nyssicola* is basal in the genus *Ramularia* (Videira, unpubl. data) and was therefore considered as an adequate outgroup for the *R. eucalypti* species complex. The generated sequences for each gene were aligned with MAFFT v. 6.864b (http://mafft.cbrc.jp/alignment/server/index.html) according to the gene characteristics. The alignments were manually checked and improved where necessary using MEGA v. 5 (Tamura et al. 2011) and were concatenated with Mesquite v. 2.75 (Maddison & Maddison 2011). In order to check the stability of each species clade a neighbour-joining analysis using the HKY85 substitution model was applied to each gene partition individually using PAUP v. 4.0b10 (Swofford 2003) (data not shown). Alignment gaps were treated as missing data and all characters were unordered and of equal weight. When ties were encountered they were randomly broken. The robustness of the obtained trees was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993).

Parsimony and Bayesian analyses were used to estimate phylogenetic relationships for the aligned combined dataset. Parsimony analyses were conducted with PAUP v. 4.0b10 (Swofford 2003). Alignment gaps were treated as fifth base and all characters were unordered and of equal weight. The robustness of the obtained trees was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993).

MrModeltest v. 2.2 (Nylander 2004) was used to determine the best nucleotide substitution model settings for each data partition in order to perform a model-optimised Bayesian phylogenetic reconstruction using MrBayes v. 3.2.0 (Ronquist & Huelsenbeck 2003). The heating chain was set to 0.15 and the Markov Chain Monte Carlo (MCMC) analysis of four chains was started in parallel from a random tree topology and lasted until the average standard deviation of split frequencies reached a value of 0.01. Burn-in was set to 25 % after which the likelihood values were stationary. Trees were saved each 100 generations and the resulting phylogenetic tree was printed with Geneious v. 5.5.4 (Drummond et al. 2011). All new sequences generated in this study were deposited in NCBIs GenBank nucleotide database (www.ncbi.nlm.nih.gov) and the accession numbers of the sequences used for the phylogenetic analyses are detailed in Table 1. The alignment and phylogenetic tree were deposited in TreeBASE (www.TreeBASE.org).

MALDI-TOF MS

Sample preparation

The cultures were prepared according to the method used in the protocol for the construction of the Filamentous fungi v. 1 Library (Bruker Daltonics, Germany) with a few modifications. Falcon tubes (15 mL) containing 7 mL of Saboraud dextrose broth (Difco, REF 238230) were inoculated with the isolates and incubated at 21 °C for 48–72 h on a tube rotator SB2 (Stuart). The tubes were centrifuged (1 min, 3 000 rpm) and 1.5 mL of the sediment was collected into 1.5 mL Eppendorf tubes. These were centrifuged (3 min, 14 000 rpm), the supernatant was removed and 1 mL of sterile Milli-Q water was added to the pellet followed by vortexing. This washing step was performed twice. The supernatant was removed and 1.2 mL of 70 % ethanol was added. The samples were stored up to 5 d at room temperature. The crude protein content was extracted using the Formic Acid/Ethanol sample preparation method (Bruker Daltonics, Germany) with a few modifications. The samples were centrifuged (3 min, 14 000 rpm), the supernatant was removed and the pellets were air-dried in a laminar flow cabinet for 30 min. The pellets were incubated for 10–20 min in 20–40 µL of 70 % formic acid (FA) (Sigma-Aldrich, Zwijndrecht, The Netherlands), followed by 10–20 min in 20–40 µL of 100 % acetonitrile (ACN) (Fluka) and were then centrifuged (2 min, 14 000 rpm). The supernatant, now containing the protein crude extract, was immediately used to generate mass spectra.

In-house library and identification

The in-house library of *Ramularia* comprises 22 reference MSPs, of which 21 were created from strains of *R. eucalypti* s.lat. and one from a strain of *R. vizellae*. The reference MSPs were generated with a MALDI Biotyper 3.0 Microflex LT (Bruker Daltonics, Germany) mass spectrometer. For each strain, 1 µL of protein crude extract was deposited on eight spots of a polished steel target plate (Bruker Daltonics, Germany), air-dried and covered with 1 µL of alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Kolecka et al. 2013). Twenty-four spectra were acquired per isolate using FlexControl v. 2.4 (Bruker Daltonics, Germany). A minimum of 20 high quality spectra were selected with Flex analysis v. 3.3 (Bruker Daltonics, Germany) to create the respective reference MSP entry to be stored in the in-house library. Comparison of the MSPs was performed by Principal Component Analysis (PCA) (Shao et al. 2012) resulting on a distance score-oriented dendrogram (Fig. 1). The library was challenged with the identification of a set of four clinical isolates. The identification of each isolate was performed in duplicate using MALDI Biotyper 3.0 RTC application (Bruker Daltonics, Germany) with the standardised parameters recommended by the manufacturer for routine diagnostics in hospitals (Kolecka et al. 2013). In the automatic identification runs the clinical isolates were compared with reference MSPs selected simultaneously from the BDAL Bruker database (5627 MSPs), the Bruker Filamentous fungi v. 1 Library (365 MSPs) and the *Ramularia* in-house library (24 MSPs). Identification results were scored as log-values and, according to the manufacturer, classified as follows: secure genus and species identification (> 2.0), secure genus identification (1.7–2.0) and no reliable identification (< 1.7).

Taxonomy

The 33 isolates belonging to *R. eucalypti* s.lat. were inoculated on Synthetic Nutrient-poor Agar (SNA) (Crous et al. 2009c) and incubated at 21 °C for 7 d. Morphology of the strain CBS 118743 was also observed and described at 33 °C, because it showed morphological dimorphism at different temperatures. Observations of the conidiogenous structures were performed using a

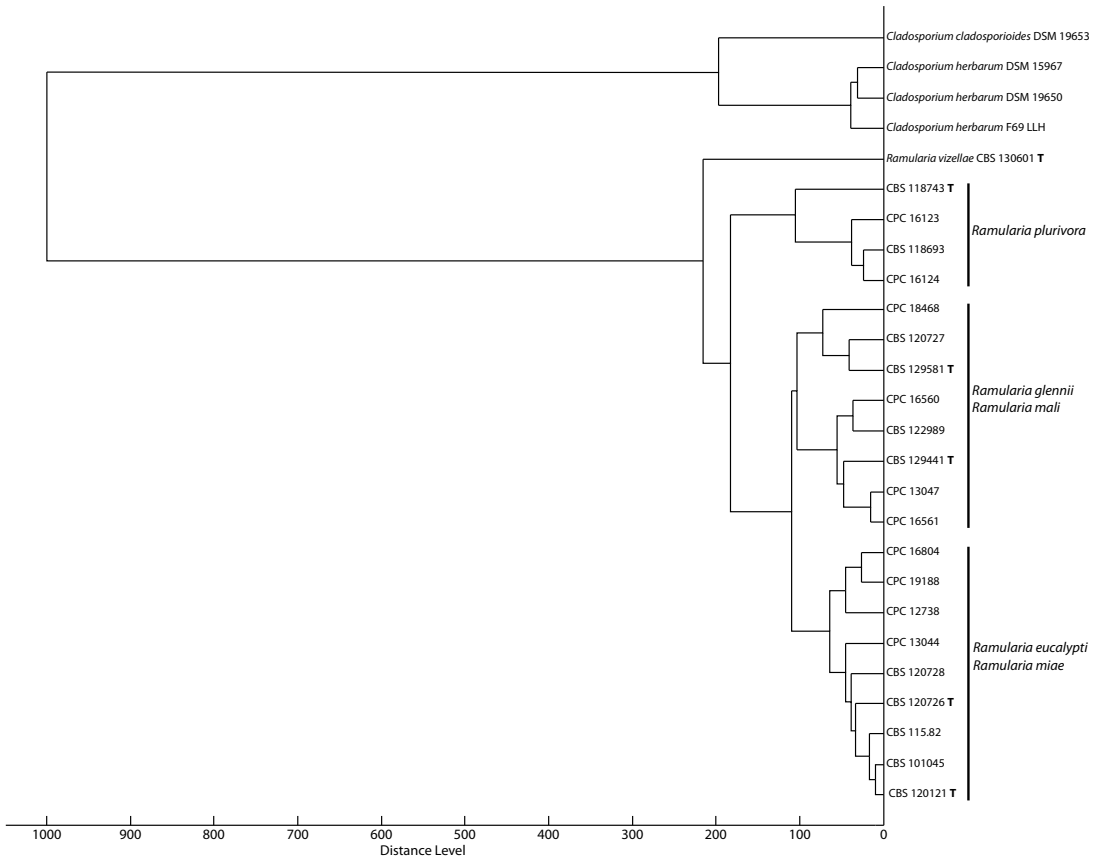

Fig. 1 PCA dendrogram based on the measured MSPs.

Nikon Eclipse 80i light microscope with differential interference contrast (DIC) illumination (Fig. 4–9). Slides were prepared using the inclined coverslip method (Kawato & Shinobu 1959, Nugenta et al. 2006) and also with transparent adhesive tape (Titan Ultra Clear Tape, Conglom Inc., Toronto, Canada) (Bensch et al. 2012). Lactic acid (clear) was used as mounting medium for the measurements and Lactophenol cotton blue was used in some preparations to improve the contrast of the naturally hyaline structures. The terminology of morphological structures followed those used for description of *Ramularia* species by Crous et al. (2011). The recorded measurements represent the minimum value followed by the 95 % confidence interval of 30 individual measurements and the maximum value for both length and width. For colony macro-morphology the isolates were inoculated on Potato Dextrose Agar (PDA), Oatmeal Agar (OA) and Malt Extract Agar (MEA) (Crous et al. 2009c), and incubated in the dark at 25 °C. After 14 d, the colony diameter was measured and the colony colour was described according to the mycological colour charts of Rayner (1970). Additionally, for each species, representative strains were selected to be

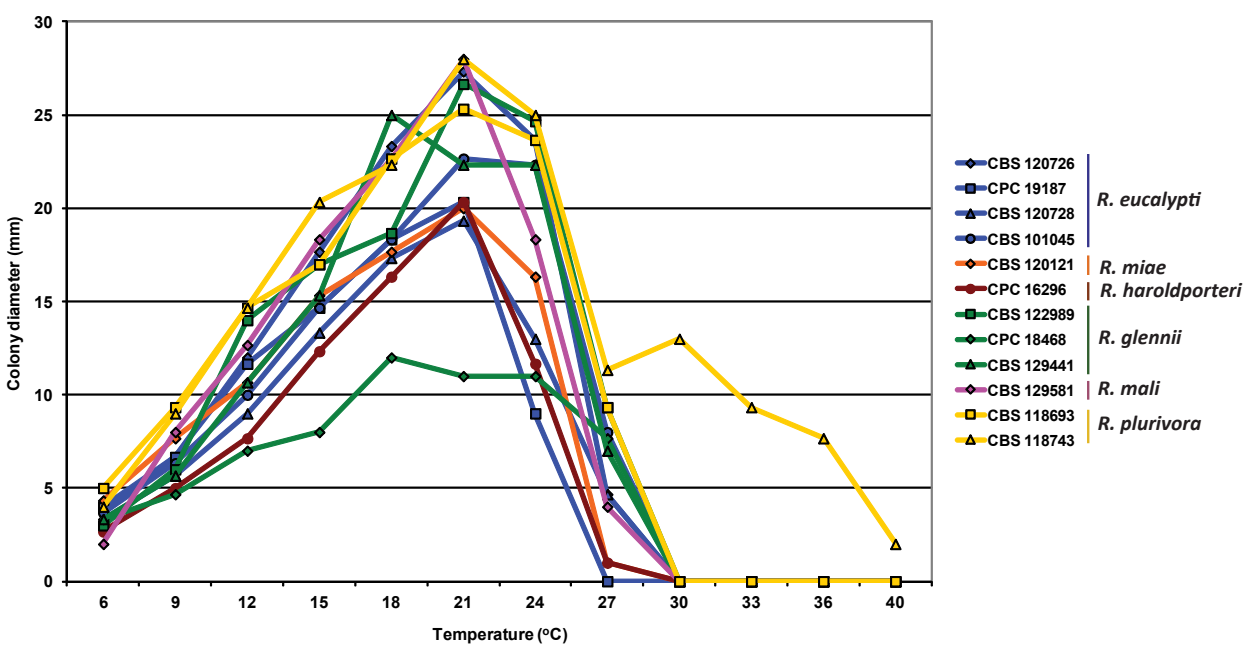

Fig. 2 Growth measurements of colony diameters (mm) of representative isolates from each clade (Fig. 3) taken from 6–36 °C, with 3 °C intervals, and also at 40 °C. Lines with the same colour represent strains from the same clade. Different strains within each clade are represented with different symbols. Colony diameters differed with less than 2 mm between replicates and are therefore not supplied with error bars.

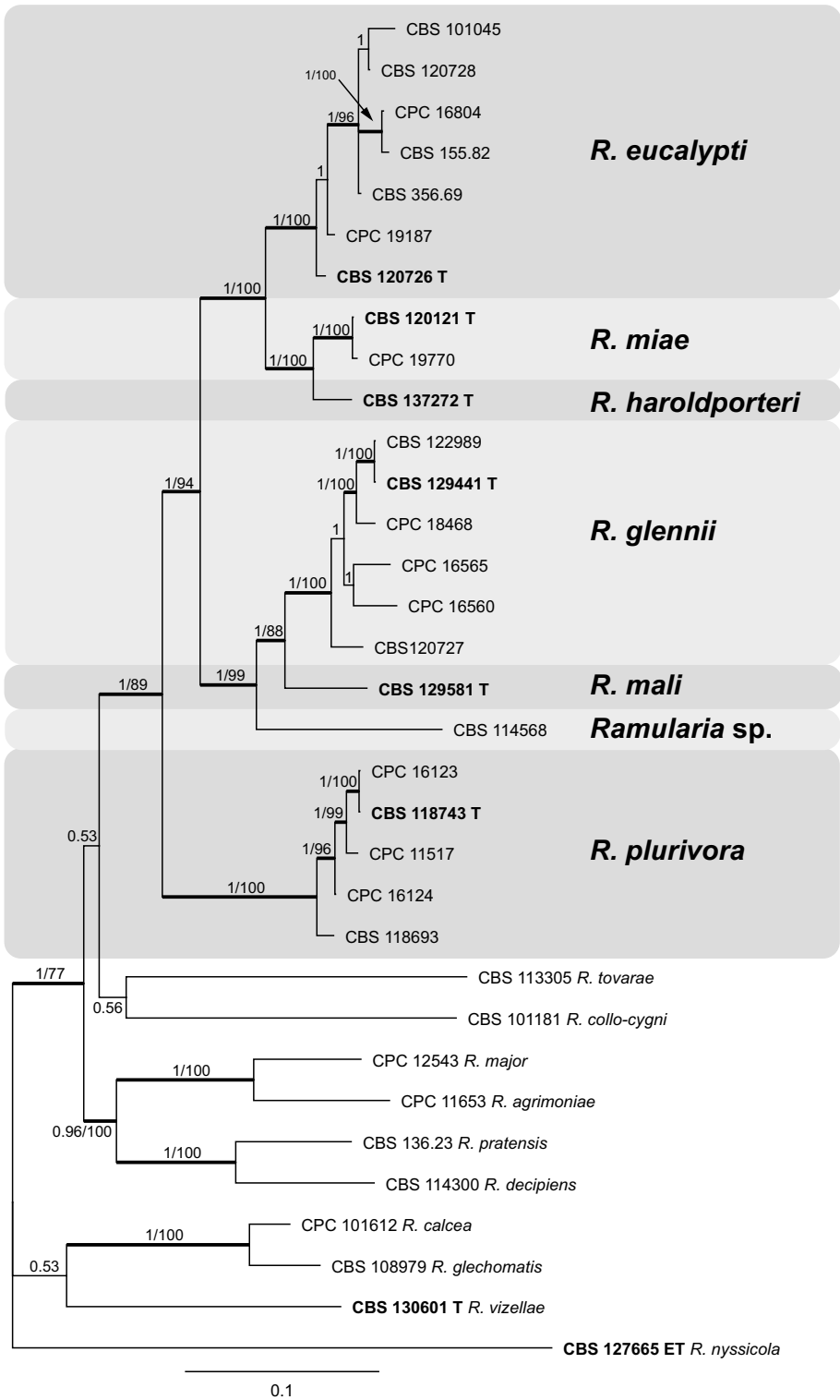

Fig. 3 Phylogenetic tree resulting from a Bayesian analysis of the combined 6-gene sequence alignment. Both Bayesian posterior probabilities (left number) and parsimony bootstrap support values > 75 % (right number) are indicated at the nodes, and the scale bar represents the expected number of changes per site. Branches in a thicker stroke represent the branches present in the strict consensus parsimony tree. Species clades in the *R. eucalypti* complex are indicated in coloured blocks and species names in black text. Ex-type strains are in **bold** and indicated with the letter T while ex-epitype strains are indicated with ET. The tree was rooted to *R. nyssicola* (CBS 127665).

included in a growth study. The isolates were inoculated onto MEA plates in triplicate, and placed in a serial incubator, in the dark, at temperatures ranging from 6–36 °C, with 3 °C intervals, and also at 40 °C. Measurements of colony diameters were taken after 14 d (Fig. 2). Nomenclatural data was deposited in MycoBank (Crous et al. 2004a).

RESULTS

DNA amplification and phylogenetic analysis

New primers for the GAPDH, TEF-1α and RPB2 loci were designed based on a larger dataset of *Ramularia* and other cercosporoid genera (Videira, unpubl. data) that proved to be effective for species within the genus *Ramularia.* These primers were used when no amplification was obtained with the standard primers (Table 2).

In the phylogenetic analysis six of the 10 screened loci were used, namely ITS, RPB2, GAPDH, ACT, TEF-1α and HIS3. The LSU sequences obtained were nearly identical to one another and did not provide useful information to resolve the species-complex and were therefore not included in the subsequent phylogenetic analyses. The amplification of CAL and CHS-1 was not successful for all the isolates and the inclusion of missing data in the alignment would negatively influence the posterior probability and bootstrap support values. The amplification of bTUB often generated multiple PCR products and was only successful for a reduced number of isolates. Although these sequences were excluded from the phylogenetic analyses, they have been deposited in GenBank under accession numbers KJ504473–KJ504495 (TUB), KJ504496–KJ504529 (CAL), KJ504530–KJ504550 (CHS) and KJ504724–KJ504764 (LSU).

Neighbour-joining analysis using the HKY85 substitution model was applied to each data partition in order to check the stability and robustness of each species clade (data not shown). The ITS locus did not differentiate species well, supporting only *R. eucalypti*, *R. miae*, *R. plurivora* and *Ramularia* sp., while most of the isolates formed a basal polytomy. The tree based on the ACT gene had a better resolution by additionally segregating strains of *R. glennii* and *R. haroldporteri*. The HIS3 phylogeny resolved seven species but with very low bootstrap support values. The individual trees based on the RPB2, GAPDH and TEF1-α loci all supported seven species with high bootstrap support. These genes also suggested a split of *R. glennii* in two clades but with a low support value and with internal subclades that were not supported either by the geographical origin or by the morphological characteristics of the isolates.

The concatenated alignment contained 33 strains, including the outgroup sequence (*R. nyssicola*). A representative strain was selected from strains representing the same substrate and country and which shared identical sequences for all loci (Table 1). The final alignment contained a total of 2 651 characters divided in 6 partitions containing 665 (RPB2), 486 (ITS), 551 (GAPDH), 358 (HIS3), 177 (ACT), 389 (TEF-1α) characters, respectively. From the total alignment, 40 characters were excluded from the phylogenetic analysis: 25 characters were artificially introduced as spacers to separate the genes and 15 characters in the GAPDH locus (alignment positions 1216–1230, see TreeBASE) represented a longer sequence in the outgroup compared to the ingroup sequences.

The results of the MrModelTest analyses indicated that the ITS partition had fixed (equal) base frequencies, whereas all the other partitions had dirichlet base frequencies. The optimised models for this dataset were K80+I+G for ITS and GTR+I+G for all the other partitions.

The Bayesian analysis generated 1 702 trees from which 424 trees were discarded (25 % burnin). The 50 % majority rule consensus tree (Fig. 3) and posterior probabilities (left numbers) were calculated from the remaining 1 278 trees. The alignment contained a total of 933 unique site patterns: 255 (RPB2), 74 (ITS), 210 (GAPDH), 81 (HIS3), 99 (ACT), 214 (TEF-1α).

The parsimony analysis generated the maximum limit of 1 000 equally most parsimonious trees and the bootstrap support values (right numbers) higher than 75 % are displayed (Fig. 3). The gaps in the alignment were treated as fifth base and from the analysed characters 1 670 were constant, 234 were variable and parsimony-uninformative and 707 were parsimony-informative. A parsimony consensus tree was calculated from the equally most parsimonious trees and the branches present in the strict consensus tree are mapped with a thicker stroke on the Bayesian tree (Fig. 3).

Phylogenetic trees based on the combined dataset and generated with both parsimony and Bayesian analyses (Fig. 3) separated strains into seven well-supported species within this complex: *R. eucalypti*, *R. glennii*, *R. haroldporteri*, *R. mali*, *R. miae*, *R. plurivora* and *Ramularia* sp. *Ramularia eucalypti* is no longer the only species of the genus to be found on *Eucalyptus* with the addition of the newly described *R. glennii*. The clinical isolates do not cluster in the same clade as *R. eucalypti*, and are here described as *R. glennii* and *R. plurivora*. The species causing the apple and pear fruit damage in storage is a new species as well (*R. mali*) considering both the branch length and the posterior probability value separating it from the closest species (*R. glennii*). The clades of *R. eucalypti*, *R. glennii* and *R. plurivora* show some interspecific variability within the evaluated genes, but not strong enough to support further division into additional species.

MALDI-TOF MS

A total of 22 strains from *Ramularia* were used to create the *Ramularia* in-house library. Twenty-one strains belonged to *R. eucalypti* s.lat. and one strain of *R. vizellae* was used as a reference species outside the complex while still within the same genus. It was not always possible to obtain good quality MSPs for all strains (e.g. *R. haroldporteri* and *Ramularia* sp.) as the crude protein extraction performed with the current protocol was problematic for a few strains. For the PCA dendrogram 26 MSPs were used in total, including the 22 MSPs from the *Ramularia* in-house library and four *Cladosporium* strains from the Bruker Filamentous fungi v. 1 Library that were used as an outgroup (Fig. 1). The distance level presented on the dendrogram is a relative measure of the differences among the MSP peak patterns and three clades can be observed: *R. plurivora*, *R. glennii*/*R. mali* and *R. eucalypti*/*R. miae*. The PCA dendogram topology shows a broadly similar topology to the DNA phylogeny but it is unable to separate the species *R. glennii* from *R. mali* and *R. eucalypti* from *R. miae*, which are closely related. The MALDI-TOF MS identification results of the four clinical isolates confirmed their identity as *R. plurivora* and *R. glenni*, respectively, as secure genus and species identification was attained with log-score > 2.0. The identification results showed that the top ten identification hits per tested spot per isolate were matching only with MSPs of *Ramularia* species.

Taxonomy

The multigene analysis resulted in seven well-supported species. Four new species are described, two are redescribed on different cultural media, and one new combination is proposed. Culture growth curves were not consistent among isolates within the same phylogenetic clade (Fig. 2). Lines representing isolates within the same clade are depicted with the same colour, but with different symbols. The optimal growth temperature for the majority of the isolates was 21 °C and only two isolates, CBS 18468 and CBS 129441, grew better at 18 °C. The isolates within the *R. eucalypti* clade (blue) reached diameters between 18 and 24 mm while isolates of *R. glennii* reached 18, 22 and 26 mm, respectively. The isolate CBS 118743 from the *R. plurivora* clade, isolated from human bone marrow in the Netherlands, presented morphological dimorphism (Fig. 9). The mycelium was filamentous until 27 °C, while from 30 °C upwards, the morphology switched into an arthroconidial yeast form that was even able to grow at 40 °C. None of the other isolates within this clade displayed morphological dimorphism and were unable to grow from 30 °C onwards.

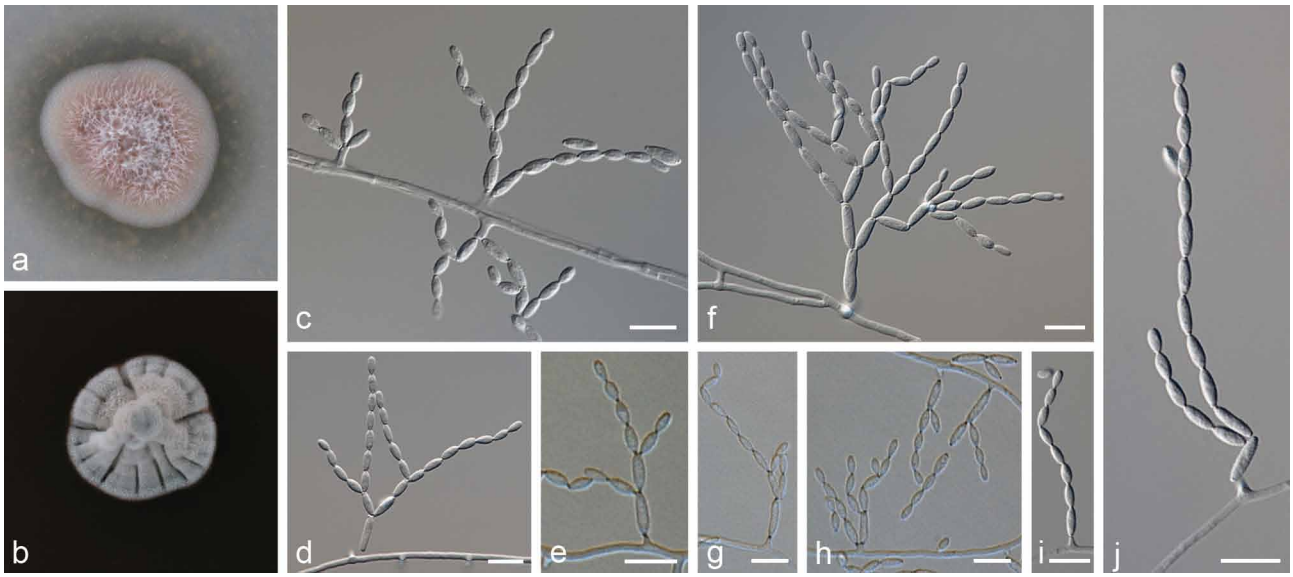

Fig. 4 *Ramularia eucalypti* (CBS 120726). a. Culture on OA; b. culture on MEA; c–j. hypha, conidiophores and conidia. — Scale bars = 10 µm.

Ramularia eucalypti Crous, Fung. Diversity 26: 174. 2007 — MycoBank MB501270; Fig. 4

Mycelium consisting of septate, branched, smooth to finely verruculose, hyaline, 1–1.5 µm diam hyphae. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline, smooth to finely verruculose, terminal and lateral, (6–)11–13(–20) × 1(–2) µm, with 1–3 apical loci almost flat or short cylindrical; *scars* thickened, darkened, refractive, 0.5–1 µm diam. *Ramoconidia* hyaline, smooth to finely verruculose, subcylindrical to fusiform, aseptate, (5–)7–8(–11) × (1.5–)2(–3) µm. *Intercalary conidia* hyaline, smooth to finely verruculose, aseptate, fusiform to oval, (4–)5.5–6(–9) × (1.5–)2(–2.5) µm, in branched chains (–11). *Terminal conidia* hyaline, smooth to finely verruculose, aseptate, obovoid, (3–)3.5–4(–6) × (1–)1.5–2 µm; *hila* thickened, darkened, refractive, 0.5–1 µm diam.

Culture characteristics — On MEA surface folded, mostly dirty white but with pale greenish grey tones, radially striated with lobate, concave, feathery margin, with fluffy aerial mycelium, reverse isabeline with iron-grey patches and with small buff margin, reaching 22 mm after 2 wk at 25 °C. On OA surface with sparse fluffy aerial mycelium in the centre, rosy-buff with greenish grey patch, low convex, forming a 5 mm ring of media discoloration, reaching 20 mm after 2 wk at 25 °C. On PDA colony flat, radially striated with entire edge, mostly flat aerial mycelium, greenish grey with dirty white thin margin, reverse olivaceous-grey with dirty white margin, reaching 20 mm after 2 wk at 25 °C.

Specimens examined. Australia, Queensland, Cairns, Kuranda, Karoomba River Walk, on leaves of *Eucalyptus* sp., 19 Aug. 2006, *P.W. Crous & J. Stone*, CPC 13304 = CBS 120728. – Italy, Norcia, on *Corymbia grandifolia*, 10 May 2006, *W. Gams* (holotype CBS H- 19832, ex-type cultures CPC 13043 = CBS 120726, CPC 13044, CPC 13045). – The Netherlands, Gelderland, Wageningen, on *Phragmites* sp., 19 Feb. 2011, *P.W. Crous*, CPC 19187, CPC 19188; Noord-Holland, Kortenhoef, Kortenhoefse Plassen, associated with *Puccinia* sp. on *Carex acutiformis*, Jan. 1982, *W. Gams & O. Constantinescu*, CBS 155.82; Anloo, Pinetum Anloo, on *Pinus wallichiana*, 8 June 2009, *W. Quaedvlieg*, CPC 16804; unknown location, on *Malus sylvestris* (cv. Golden Delicious), Mar. 1969, *Van der Scheer*, CBS 356.69; Baarn, on *Geranium pusillum*, May 1998, *H.A. van der Aa*, CBS 101045.

Notes — Currently, *R. eucalypti* is the only confirmed member of *Ramularia* known from *Eucalyptus* since *R. pitereka* and similar species were allocated to *Quambalaria*. The specimens examined show that this is a plurivorous species, able to colonise very different hosts like *Eucalyptus* (*Myrtaceae*), *Pinus* (*Pinaceae*) and *Phragmites* (*Poaceae*). Among the examined strains, CBS 356.69 sporulated sparsely and never formed conidial chains longer than two conidia, probably due to the fact that this is an old culture (from 1969), and strain CBS 101045 produced long chains with up to 13 intercalary conidia.

Ramularia glennii Videira & Crous, *sp. nov.* — MycoBank MB808138; Fig. 5

Etymology. Named after the collector of one of the isolates, Anthony E. Glenn, a plant pathologist from the Agricultural Research Service of the United States Department of Agriculture (ARS/USDA), who found it growing in the rubber of the refrigerator where he usually stored the samples related to his *Fusarium* research.

Mycelium consisting of septate, branched, smooth, hyaline, 1–1.5 µm diam hyphae. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* smooth, hyaline, terminal and lateral, (5–)13–16(–25) × 1(–2) µm, sympodial proliferation with 1–3 apical loci almost flat or protuberant, cylindrical; *scars* thickened, darkened, refractive, 0.5–1 µm diam. *Ramoconidia* hyaline, smooth to finely verruculose, subcylindrical to clavate or oval, 0–1-septate, hyaline, (6–)9–11(–15) × (2–)3(–4) µm. *Intercalary conidia* hyaline, smooth to finely verruculose, aseptate, fusiform or oval, (5–)6.5–8(–12) × (2–)2.5(–3) µm, in branched chains of up to 7. *Terminal conidia*, hyaline, smooth to finely verruculose, aseptate, obovoid, (3–)5–5.5(–8) × (1.5–)2(–3) µm; *hila* thickened, darkened, refractive, 0.5–1 µm diam.

Culture characteristics — On MEA surface folded, radially striated and sinking into the media, vinaceous-buff, undulate feathery and concave margin, reverse ochreous, reaches 27 mm after 2 wk at 25 °C. On OA surface folded and slightly depressed, rosy-buff, margin undulate and with flat mycelium while fluffy aerial mycelium covers the centre, 5 mm halo around the colony, reaches 22 mm after 2 wk at 25 °C. On PDA surface mostly flat, white, pale mouse-grey in the centre, undulate margins, reverse olivaceous-grey in the centre and buff towards the margin, reaches 24 mm after 2 wk at 25 °C.

Specimens examined. Iraq, Al-Kora, Basrah, on leaves of *Eucalyptus camaldulensis*, 1 Mar. 2009, *A. Saadoon*, CPC 16560, CPC 16561, CPC 16565. – Italy, Viterbo, on leaves of *Corymbia grandifolia*, 1 Apr. 2006, *W. Gams*, CPC 13047 = CBS 120727, CPC 13048. – The Netherlands, Rotterdam Maasstad Ziekenhuis (Clara), on human bronchial alveolar lavage, 2011, unknown collector (holotype CBS H-21617, type culture CBS 129441); Rotterdam Maasstad Ziekenhuis (Clara), on human skin tissue, 2008, unknown collector, CBS 122989. – USA, Athens, on rubber of refrigerator, Sept. 2010, *A. Glenn*, CPC 18468, CPC 18469, CPC 18470.

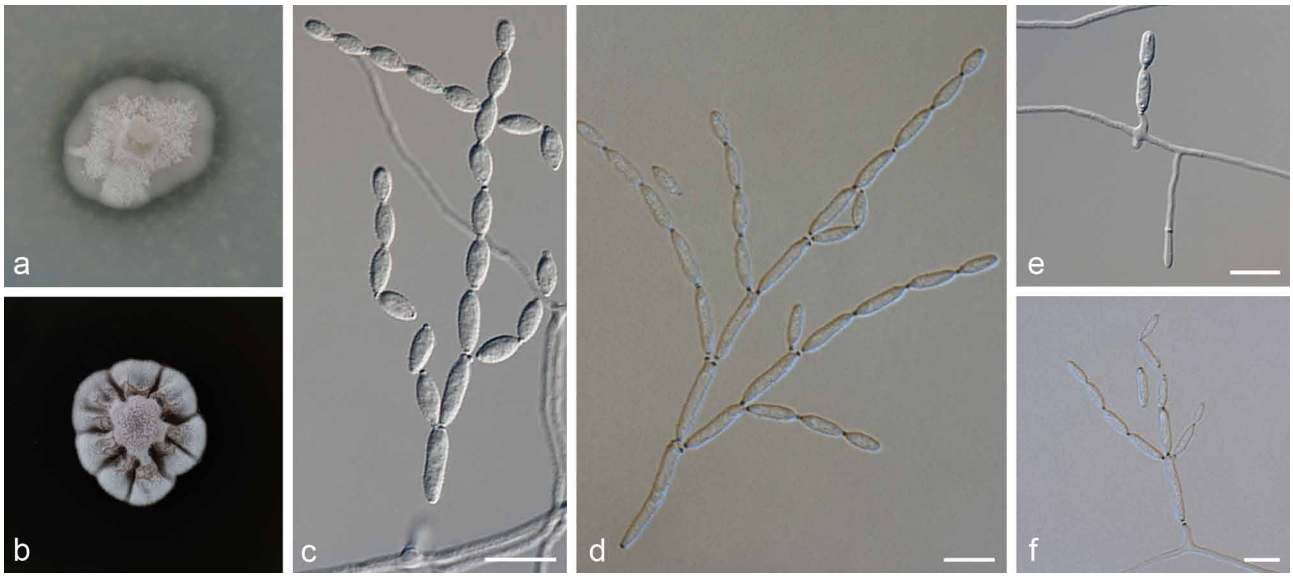

Fig. 5 *Ramularia glennii* (CBS 129441). a. Culture on OA; b. culture on MEA; c–f. hypha, conidiophores and conidia. — Scale bars = 10 µm.

Notes — The specimens examined were collected from a wide range of substrates worldwide. The multigene phylogeny showed some internal structure that was insufficient to confidently split this group in more than one species. Morphologically, all the strains were similar but strain CBS 129441 had slightly longer ramoconidia than the rest and the isolate CPC 18468 showed an optimal growth rate at 18 °C instead of 21 °C (Fig. 2), which may reflect some intraspecific variation.

Ramularia haroldporteri Videira & Crous, *sp. nov.* — MycoBank MB808136; Fig. 6

Etymology. Named after Harold Porter, who bequeathed the land in Leopard's Kloof (Gorge in Afrikaans) to the National Botanical Gardens of South Africa, who in turn named this garden in his honour.

Mycelium consisting of septate, branched, smooth to finely verruculose, hyaline, 1–1.5 µm diam hyphae. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline, smooth to finely verruculose, terminal and lateral, (7–)10–13(–19) × 1(–2) µm, sympodial proliferation with 1–3 apical loci almost flat or short cylindrical; *scars* thickened, darkened, refractive, 0.5–1 µm diam. *Ramoconidia* subcylindrical, oval or ellipsoid, aseptate, hyaline, smooth to finely verruculose, (5–)8–9(–13) × (1.5–)2 µm. *Intercalary conidia* hyaline, smooth to finely verruculose, aseptate, oval or ellipsoid, (4–)5–6(–8) × (1.5–)2(–2.5) µm, in branched chains of up to 8. *Terminal conidia*, hyaline, smooth to finely verruculose, aseptate, obovoid, (2.5–)3–4(–4.5) × (1.5–)2(–2.5) µm; *hila* thickened, darkened, refractive, 0.5–1 µm diam.

Culture characteristics — On MEA surface convex, strongly folded, smoke-grey, with undulate and concave margin, flat aerial mycelium, reverse greyish sepia, reaches 18 mm after 2 wk at 25 °C. On OA folded with undulate margins, smoke-grey, flat aerial mycelium, reaches 15 mm after 2 wk at 25 °C. On PDA surface folded with undulate margins, smoke-grey, flat aerial mycelium, reverse olivaceous-grey, reaches 15 mm after 2 wk at 25 °C.

Specimen examined. South Africa, Western Cape Province, Betties Bay, Harold Porter Botanical Garden, on leaves of unidentified bulb plant, 14 Jan. 2009, *P.W. Crous* (holotype CBS H-21616, ex-type culture CPC 16296 = CBS 137272).

Notes — *Ramularia haroldporteri* differs from *R. miae* by producing significantly shorter ramoconidia, intercalary and terminal conidia and by not producing exudate droplets on top of the mycelium. In the individual gene phylogenetic trees, all genes except the ITS separates *R. haroldporteri* from *R. miae*.

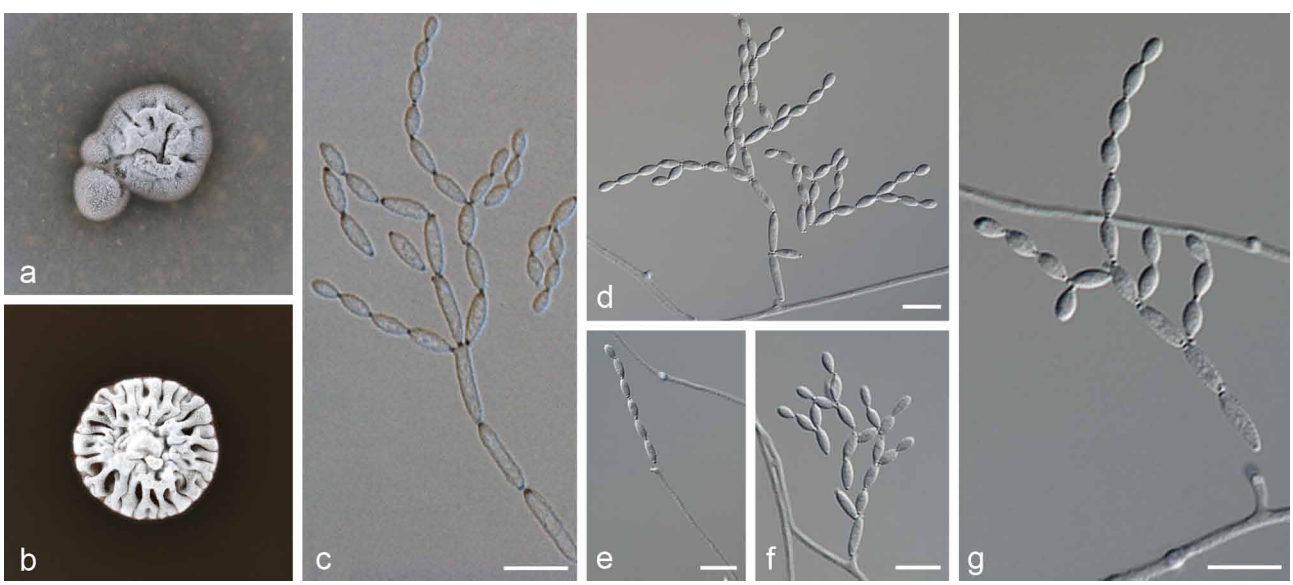

Fig. 6 *Ramularia haroldporteri* (CBS 137272). a. Culture on OA; b. culture on MEA; c–g. hypha, conidiophores and conidia. — Scale bars = 10 µm.

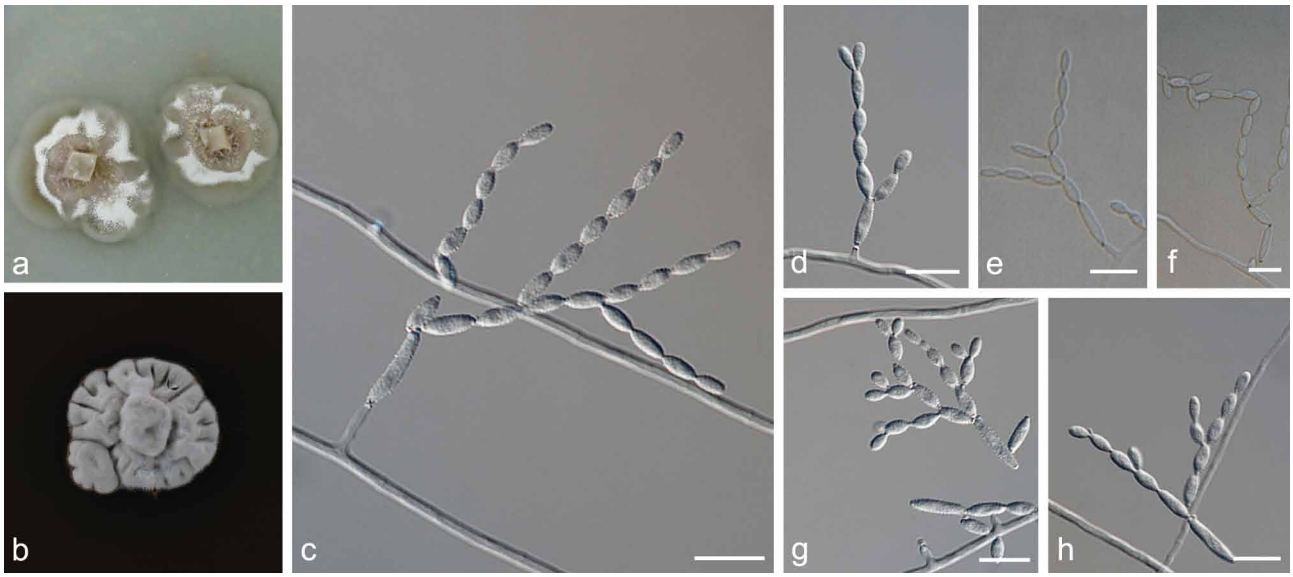

Fig. 7 *Ramularia mali* (CBS 129581). a. Culture on OA; b. culture on MEA; c–h. hypha, conidiophores and conidia. — Scale bars = 10 µm.

Ramularia mali Videira & Crous, *sp. nov*. — MycoBank MB808135; Fig. 7

Etymology. Named after its occurrence on apple (*Malus*).

Mycelium consisting of septate, branched, smooth, hyaline, (1–)1.5(–2) µm diam hyphae. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* finely verruculose, hyaline, terminal and lateral, (6.5–)11–13.5(–18) × (1–)1.5(–2) µm, sympodial proliferation with 1–2 apical loci flattened or protuberant cylindrical; *scars* thickened, darkened, refractive, 0.5–1 µm diam. *Ramoconidia* subcylindrical to clavate or fusoid, 0(–1)-septate, hyaline, finely verruculose, (5–)7–9(–16) × 2(–3) µm, with 1–2(–3) apical loci. *Intercalary conidia* hyaline, finely verruculose, aseptate, fusoid or ovoid, 5–6(–8) × 2(–3) µm, in branched chains of up to 6. *Terminal conidia* hyaline, finely verruculose, aseptate, obovoid, (3–)4–4.5(–6) × (1–)1.5–2(–2.5) µm; *hila* thickened, darkened, refractive, 0.5–1 µm diam.

Culture characteristics — On MEA surface folded, undulate margin, white greyish, feathery and concave margin, reverse iron-grey with greyish sepia margin, reaches 21 mm after 2 wk at 25 °C. On OA surface flat, smooth, entire edge, buff, 3 mm halo around the colony, reaches 18 mm after 2 wk at 25 °C. On PDA surface low convex, white greyish, flat aerial mycelium, slightly undulate margin, reverse iron-grey with rosy-buff patch, reaches 25 mm after 2 wk at 25 °C.

Specimen examined. Italy, Piemont, on *Malus domestica* fruit in cold storage, May 2011, unknown collector (holotype CBS H-21618, culture ex-type CBS 129581).

Notes — This species, previously identified as *R. eucalypti*, is an emerging problem causing a post-harvest disorder in healthy pome fruits in cold storage, namely apple cv. Ambrosia and pear cv. Conference (Giordani et al. 2012). An epidemiological study reports that symptomless fruits harvested from trees showing leaf spot symptoms caused by this pathogen, developed the lenticel rot disease during the subsequent months of cold storage (Gianetti et al. 2012). *Ramularia mali* differs from *R. glennii* by forming shorter conidiogenous cells, shorter and thinner ramoconidia and shorter intercalary and terminal conidia.

Ramularia miae Crous, Fungal Planet 3. 2006. — MycoBank MB501004; Fig. 8

Mycelium consisting of septate, branched, smooth to finely verruculose, hyaline, 0.5–1 µm diam hyphae. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* hyaline, smooth to finely verruculose, terminal and lateral, (5.5–)9–12(–24) × 1 µm, sympodial proliferation with 1–2 apical loci almost flat or short cylindrical; *scars* thickened, darkened, refractive, 0.5–1 µm diam. *Ramoconidia* hyaline, smooth to finely verruculose, subcylindrical to clavate or fusiform, 0–1-septate, (6–)9–10(–16) × (1.5–)2 µm. *Intercalary conidia* hyaline, smooth to finely verruculose, aseptate, subcylindrical to oval, (5.5–)7–8.5(–12.5) × (1.5–)2(–3) µm, in branched chains of up to 7. *Terminal conidia*, hyaline, smooth to finely verruculose, aseptate, obovoid, (4–)5–6(–9) × (1.5–)2(–3) µm; *hila* thickened, darkened, refractive, 0.5–1 µm diam.

Culture characteristics — On MEA surface convex, folded, dirty-white to pale olivaceous-grey, with lobate margin, short fluffy aerial mycelium, reverse iron-grey with small buff margin, reaches 15 mm after 2 wk at 25 °C, produces small droplets of slimy exudate. On OA surface flat or slightly folded with undulate margins, pale olivaceous-grey mycelium, 5 mm halo in the media, producing several droplets of colourless slimy exudates, reaches 15 mm after 2 wk at 25 °C. On PDA surface folded with lobate margins, olivaceous-grey with white-grey patch, producing large droplets of colourless slimy exudates, reaches 15 mm after 2 wk at 25 °C.

Specimens examined. South Africa, Western Cape Province, Betties Bay, Harold Porter Botanical Garden, on *Wachendorfia thyrsiflora*, Jan. 2006, *P.W. Crous & M.K. Crous* (holotype CBS H-19763, ex-type cultures CBS 120121 = CPC 12736, CPC 12737, CPC 12738); Western Cape Province, Kirstenbosch Botanical Garden, on *Gazania rigens* var. *uniflora*, 9 Aug. 2011, *P.W. Crous*, CPC 19835; Kirstenbosch Botanical Garden, on *Leonotis leonurus*, 30 July 2011, *P.W. Crous*, CPC 19770.

Notes — Morphologically, *R. miae* differs from *R. eucalypti* by having shorter conidiogenous cells and ramoconidia and longer intercalary and terminal conidia. *Ramularia miae* was first observed causing black leaf spots on *Wachendorfia thyrsiflora*, a tall evergreen geophyte with bright red roots that belongs to the Bloodwort family (*Haemodoraceae*). This host is native to South Africa and *R. miae* is likely to occur wherever it is cultivated. In addition, the specimens examined were isolated from two new hosts native to South Africa: *Gazania rigens* var.

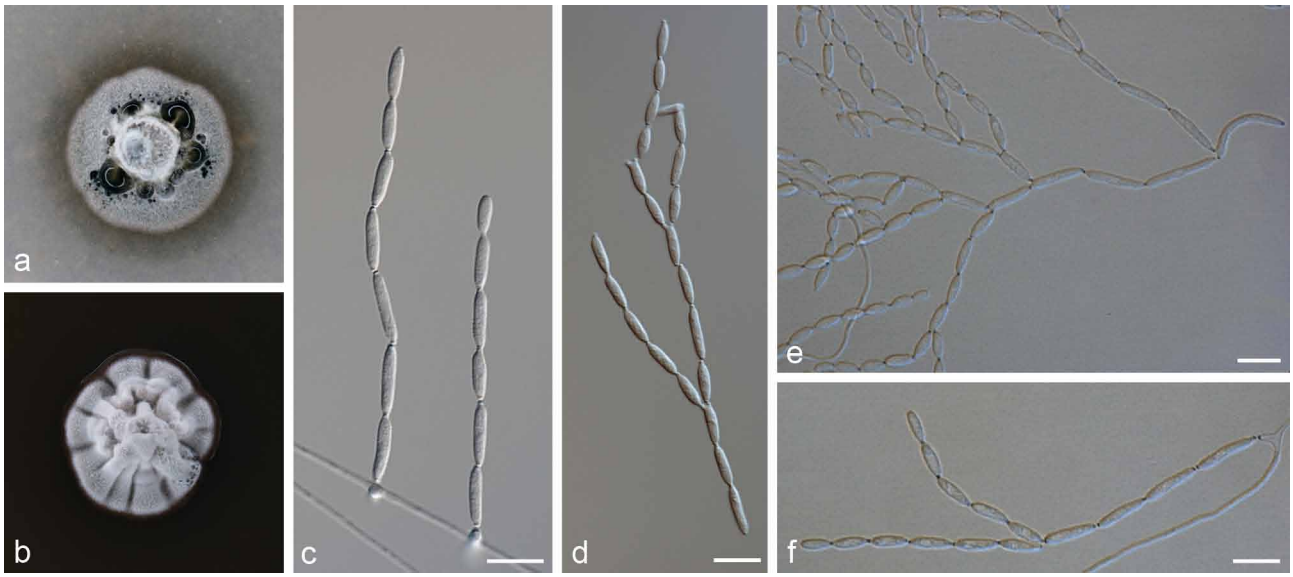

Fig. 8 *Ramularia miae* (CBS 120726). a. Culture on OA; b. culture on MEA; c–f. hypha, conidiophores and conidia. — Scale bars = 10 µm.

uniflora (*Asteraceae*), a flowering plant that is cultivated as an ornamental worldwide, and *Leonotis leonurus* (*Lamiaceae*), a broadleaf evergreen shrub that is known for its medicinal and slightly psychoactive properties, suggesting a plurivorous *Ramularia* species.

Ramularia nyssicola (Cooke) Videira & Crous, *comb. nov.* — MycoBank MB809667

Basionym. *Sphaerella nyssicola* Cooke as '*nyssaecola*'. Hedwigia 17: 40. 1878.

≡ *Mycosphaerella nyssicola* (Cooke) F.A. Wolf as '*nyssaecola*'. Mycologia 32: 333. 1940.

Specimen examined. USA, Maryland, Prince George's County, Glen Dale, on fallen overwintered leaves of *Nyssa ogeche* × *sylvatica* hybrid, June 2009, *R. Olsen*, ex-epitype culture CBS 127665.

Notes — *Mycosphaerella nyssicola* has been recently epitypified from overwintered leaves of *Nyssa sylvatica* trees freshly collected in Maryland, USA (Minnis et al. 2011). *Nyssa sylvatica* or black gum trees (*Cornaceae*) are cultivated as ornamental plants and *M. nyssicola* causes leaf spots that reduce their aesthetic appeal and cause early defoliation. The ITS and LSU sequences supported *M. nyssicola* as a distinct species from *R. endophylla* (= *M. punctiformis*), even though they were almost indistinguishable morphologically (Aptroot 2006). Minnis et al. (2011) did not propose a new combination in *Ramularia* at the time because they did not observe the asexual *Ramularia* morph, and the name *M. nyssicola* correctly adhered to the ICBN Art. 59.1. However, the previous Art. 59 has been deleted from the new International Code of Nomenclature for Algae, Fungi and Plants (ICN) and, since January 2013, both asexual and sexual morph names have equal status. We propose a new combination in *Ramularia* because the name *Ramularia* (Unger 1833) predates *Mycosphaerella* (Johanson 1884), and species of *Mycosphaerella* s.str. have been shown to be confined to taxa with *Ramularia* asexual morphs (Crous 2009a), which is also supported by the DNA data generated in this study. Furthermore, the genus *Ramularia* has recently been monographed (Braun 1995, 1998), while *Mycosphaerella* (Aptroot 2006) contains an assemblage of more than 40 different genera (Crous 2009b).

Ramularia plurivora Videira & Crous, *sp. nov.* — MycoBank MB808132; Fig. 9

Etymology. Named after its wide host range.

Mycelium consisting of septate, branched, smooth, hyaline, (0.5–)1–1.5 µm diam hyphae. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* smooth, hyaline, terminal and lateral, (6–)10–13(–17) × (0.5–)1(–2) µm, sympodial proliferation with 1–3 apical loci flattened or protuberant cylindrical; *scars* thickened, darkened, refractive, 0.5–1 µm diam. *Ramoconidia* subcylindrical to ellipsoid, 0–1-septate, hyaline, smooth to finely verruculose, (6–)9–11(–18) × (1.5–)2 µm. *Intercalary conidia* hyaline, smooth, aseptate, ellipsoid, smooth to finely verruculose, (6–)7.5–8(–10.5) × (1.5–)2 µm, in branched chains (–7). Terminal conidia hyaline, smooth to finely verruculose, aseptate, ellipsoid, (4–)5–6(–9) × (1–)1.5–2 µm; hila thickened, darkened, refractive, 0.5–1 µm diam. On MEA, *Arthroconidia* smooth, bacilliform, oblong with apices rounded or truncate, 0–3-septate, slightly constricted at the septa, 1-septate, (3.5–)4.5–5(–7) × (1–)1.5–2 µm, 2-septate, (6–)8–9(–12) × 1.5–2 µm, 3-septate, (8–)10–11(–13.5) × (1.5–)2(–2.5) µm.

Culture characteristics — On MEA surface dirty white with a greenish grey tinge, folded, radially striated with undulate margins, reverse fuscous black with a buff margin, reaches 25 mm after 2 wk at 25 °C. On OA surface dirty white to light greenish grey, smooth, with entire edge, central area sporulating profusely and outer ring sparse in mycelium, reaches 35 mm after 2 wk at 25 °C. On PDA colonies have a dirty white and greenish grey aspect, low convex, undulate margins, central area sporulating profusely and outer ring sparse in mycelium, reaches 25–35 mm after 2 wk at 25 °C.

Specimens examined. Korea, on *Coleosporium plectanthri* on *Plectranthus excisus*, 2004, *H.D. Shin*, CPC 11517. – The Netherlands, Den Haag, Laboratory of Medical Microbiology, Hospital Leyenburg, from human bone marrow, 2005, holotype CBS H-21619, ex-type culture CBS 118743 = CPC 12207; Hilversum, Central Biological and Serological Laboratory, on human skin from neck, 20 May 2005, CBS 118693 = CPC 12206; on melon in storage, 1 Jan. 2008, *J.H. Houbraken*, CPC 16123, CPC 16124.

Notes — The strain CBS 118743 presented temperature-induced morphological dimorphism being filamentous until 27 °C and an arthroconidial yeast form from 30 °C up to 40 °C. This temperature-induced dimorphism may be related with the ability to cause disease. Isolates CPC 16123, CPC 11517, CBS 118693 were not able to grow at 40 °C. However, after a week at

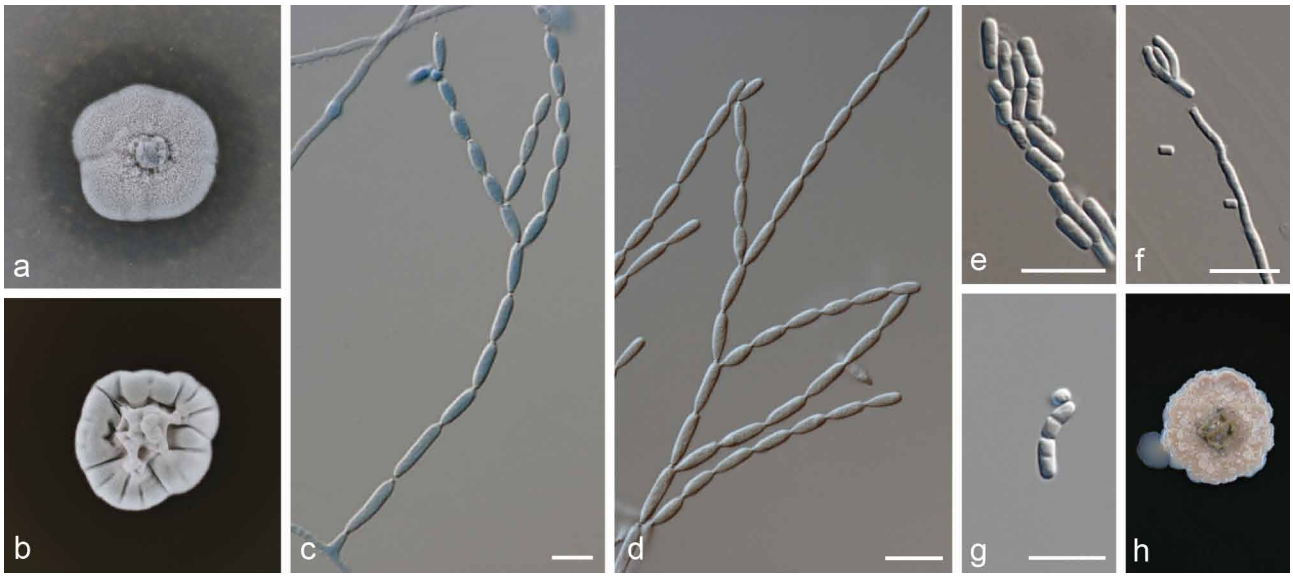

Fig. 9 *Ramularia plurivora* (CBS 118743). a. Culture on OA; b. culture on MEA; c, d. hypha, conidiophores and conidia; e–g. arthroconidia formed at 33 °C; h. culture on MEA at 33 °C. — Scale bars = 10 µm.

40 °C, when transferred back to 21 °C, they were able to grow, meaning they were able to survive at 40 °C for that period of time.

***Ramularia* sp.**

Culture characteristics — On MEA surface convex, folded, white with very few and small droplets of pale luteous exudates, margin undulate and feathery, reverse umber with ochreous margin, reaching 18 mm after 2 wk at 25 °C. On OA surface convex, white and feathery, margin undulate and without aerial mycelium, 2 mm hazel ring around the colony, reaching 20 mm after 2 wk at 25 °C. On PDA surface convex, white, margin slightly undulate and feathery, reverse dark mouse grey with pale luteus margin, reaching 18 mm after 2 wk at 25 °C; culture sterile.

Specimen examined. Sweden, Uppland, Knovsta, isolated from *Epilobium hirsutum* L., 22 Sept. 1989, *E. Gunnerbeck*, CBS 114568.

Notes — This strain was previously identified as *R. epilobiana*. The type specimen of *R. epilobiana* was described from *Epilobium hirsutum* in France, and no ex-type culture is available. The culture CBS 114568 was sterile and we were unable to compare its morphology with that of the type description. However, it is very doubtful that it represents the true *R. epilobiana* since all species of the *R. eucalypti* complex have catenate, narrow conidia, and *R. epilobiana* is characterised by having broadly ellipsoid-ovoid conidia that are formed singly. The DNA sequences obtained from this strain differ significantly from the sequences of the closest strain CBS 129581. Therefore, we rename it as '*Ramularia* sp.' and retain it as a potential new species pending the collection of fresh material from the same host and country.

DISCUSSION

Eucalyptus is one of the most important commercially afforested genera cultivated to meet the increasing global demand for wood and paper pulp. Over the years, more than 50 species of the family *Mycosphaerellaceae* have been described causing diseases on *Eucalyptus* trees (Quaedvlieg et al. 2014). However, since the introduction of molecular techniques, many well-established plant pathogens have been revealed to represent species complexes (Crous & Groenewald 2005, Groenewald et al. 2005, Damm et al. 2012a, b, Weir et al. 2012). The pathogen *R. eucalypti* has certainly proved to be no exception.

Using a polyphasic approach involving morphology, multi-gene phylogeny and MALDI-TOF MS, a total of seven species were accepted within the complex: *R. eucalypti*, *R. glennii*, *R. haroldporteri*, *R. mali*, *R. miae*, *R. plurivora* and one undescribed *Ramularia* species. Species discrimination was mostly based on the multigene phylogeny since it clearly separated them into stable and strongly supported monophyletic clades while the morphological features and the MALDI-TOF MS PCA dendrogram did not consistently discriminate all species.

Within the clades of *R. eucalypti* and *R. glennii*, some phylogenetic structure was observed that was not resolved consistently in all gene trees (data not shown) and, in accordance with the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept, the transition from concordance to conflict determined the limit of these species (Taylor et al. 2000). The isolates within these clades have been collected worldwide and the phylogenetic structure observed suggests that the isolates studied may represent populations in the process of divergence. It has been shown that *Mycosphaerella* populations can be carried within asymptomatic *Eucalyptus* trees transported and planted across the world and, given time, they have genetically diverged sometimes to the point of being recognised as distinct species (Crous & Groenewald 2005).

The ITS barcode was not sufficient to achieve species level identification, just like previously reported among other cercosporoid genera, e.g. *Cercospora* (Groenewald et al. 2013) and *Pseudocercospora* (Crous et al. 2013). The need to use secondary barcodes to achieve species identification has been highlighted in several studies in recent years (e.g. Fitzpatrick et al. 2006, Aguileta et al. 2008, Quaedvlieg et al. 2012). Secondary barcodes are usually protein-coding genes since their intron sequences introduce more variability that is valuable for species discrimination. From the five protein-coding genes that were used in this study, any of the partial genes TEF1-α, RPB2 or GAPDH could be used as a secondary barcode since they all delineate the seven recognised species. However, further studies are necessary to determine which of these loci would be more adequate to discriminate species within the genus *Ramularia*.

Cultural morphological traits have been used in the past for species discrimination within species complexes in other genera,

e.g. *Cercospora apii* s.lat. (Groenewald et al. 2005). The species in this study (Fig. 2), however, showed few morphological or cultural features that could be consistently and reliably used to identify them. All the strains used in this study were cultures that were deposited in the CBS or CPC fungal collections and no fresh material was collected. Therefore, any features that may exclusively develop in association with the original host or substrate have not been examined.

Although the genus *Ramularia* is currently accepted as a host-specific genus this assumption has not been tested experimentally. In the present study, *R. haroldporteri* and *R. mali* have been isolated from a single host while *R. eucalypti*, *R. glennii*, *R. miae* and *R. plurivora* were isolated from multiple hosts, suggesting that both host-specific and plurivorous species may occur in this genus, even within the same species complex. Some species of the *Mycosphaerellaceae* are known to have the ability of colonising different hosts in order to disperse further in an attempt to find the host to which they are truly pathogenic (Crous & Groenewald 2005). This ability makes it more difficult to determine whether they act as true pathogens, are opportunistic and take advantage of an already debilitated host, or if they are simply saprophytes.

The pathogen responsible for causing lenticel rot in fruits of apple (*Malus malus* cv. Ambrosia) and pear (*Pyrus communis* cv. Conference) in the Piemont Province in Italy (Gianetti et al. 2012, Giordani et al. 2012) is here newly described as *R. mali*. The apple tree orchards in Piemont are an important crop that in 2011 produced 140 000 t of fruit. Healthy apple fruits (*Malus domestica* cv. Ambrosia) collected from trees with leaf spots caused by *R. mali* in the orchards, exhibited disease symptoms during the subsequent months of cold storage (Gianetti et al. 2012). Artificial inoculations of healthy apple (*Malus domestica* cv. Ambrosia) with *R. mali* also caused the development of symptoms indicating that this is a true pathogen (Giordani et al. 2012). It is thought that the fungus was already present in the country and that the gradual abandonment of the use of broad-spectrum fungicides in the fruit sector allowed the emergence of this pathogen that had passed unnoticed until now. In 2013, in the Trentino Alto-Adige province in Italy, apples from a different cultivar (*Malus domestica* cv. Golden Delicious), also developed the lenticel rot in cold storage and the disease affected 50–60 % of the crop. In the same year and province, *Malus domestica* cv. Braeburn and *Malus domestica* cv. Rosy Glow were also affected. Molecular analysis of these isolates were identical to those of *R. eucalypti* (Crous et al. 2007) (100 % ITS, 99–100 % LSU) deposited on GenBank. However, artificial inoculation of these isolates on ripe fruits of *Malus domestica* cv. Golden Delicious did not result in de development of disease symptoms (Lindner 2013). No isolates from this province were available in the present study. Since the ITS barcode is not sufficient for species identification, the mentioned pathogen can be *R. eucalypti*, *R. mali*, or a different species. If it is *R. mali*, it may be a mere opportunist on *Malus domestica* cv. Golden Delicious and only truly pathogenic to the Ambrosia cultivar. Information on the biology and behaviour of *R. mali* is still lacking and no preventive measures to control this fungus from spreading have been taken.

The newly described species *R. glennii* and *R. plurivora* include strains that were obtained not only from plants but also from human clinical specimens. This is the first time species of the genus *Ramularia* are reported in association with a human host and little is known about their pathogenicity. Some pathogens are able to infect hosts from different kingdoms (van Baarlen et al. 2007) and other plant pathogens have been reported capable of infecting humans (Mostert et al. 2006, Phillips et al. 2013). The fact that only a limited number of isolates was obtained and no previous report is known about *Ramularia* species infecting patients support the hypothesis that this is an opportunistic fungus. However, if potential host species are immunocompromised, opportunistic pathogens may turn into aggressive pathogens (van Baarlen et al. 2007). Furthermore, *R. plurivora* (strain CBS 118743) displayed morphological dimorphism (Fig. 9) and was able to grow at 40 °C (Fig. 2). These characteristics are similar to, for example, *Talaromyces marneffei* (syn. *Penicillium marneffei*, *Eurotiomycetes*) (Vanittanakom et al. 2006, Houbraken & Samson 2011), a human pathogen known to cause lethal systemic infections in immunocompromised patients. Therefore, further studies are needed to appraise the pathogenicity of *R. plurivora* in order to determine if measures for its rapid identification, containment and treatment should be taken.

MALDI-TOF MS has become a powerful tool in the clinical microbiology workflow for the identification of bacteria and yeasts (Bader 2013, Lau et al. 2013). The use of MALDI-TOF MS for routine filamentous fungal identification from clinical samples has only recently been standardised and validated for several species (Cassagne et al. 2011, Lau et al. 2013, L'Ollivier et al. 2013). Filamentous fungi present some challenges when compared to yeast and bacteria. They have thicker cell walls that make the protein extraction more difficult, the presence of cell wall pigments inhibit the ionisation process and spore-based protein extractions result in a low variability of mass spectra peaks (Bader 2013). The use of Sabouraud broth as culture media has been shown to inhibit pigmentation and spore production in most species thus improving the quality of the spectra. Furthermore, filamentous fungi have complex phylogenetic relationships that make their species boundaries more difficult to define. The need to use secondary barcodes to resolve species complexes also challenges the MALDI-TOF MS to perform identifications almost at the level of intraspecies subtyping (Degenkolb et al. 2008, Cassagne et al. 2011, Welker & Moore 2011, Bader 2013, Brun et al. 2013).

The species in this complex are very closely related and the PCA dendrogram topology (Fig. 1) individualised only three clades containing *R. plurivora*, *R. glennii* / *R. mali* and *R. eucalypti* / *R. miae*. The dendrogram represents the relative similarity of the peak patterns and is based on a scoring algorithm that is influenced not only by the available number of MSPs that are representative of each species, but also by the intensity of the peaks. The first parameter can be improved by creating more MSPs from different strains of the same species. However, the second parameter can only be improved by preparing all the samples on the same day, using the same amount of protein and using the same settings on the machine, which is virtually impossible when building a large database. Furthermore, the protocol for the crude protein extraction recommended by the manufacturer still needs to be optimised, since it did not work for all strains in this study.

Nevertheless, the use of MALDI-TOF MS as an identification tool has still proven to be reliable not only in previous studies but also in this one. When the in-house *Ramularia* library was challenged with the identification of the clinical isolates of *R. glenni* and *R. plurivora*, a secure genus and species identification log-score (> 2.0) was attained.

In conclusion, the *R. eucalypti* species complex has been resolved with the circumscription of the *R. eucalypti* s.str. and the description of four new species. *Ramularia eucalypti* and *R. glennii* are the only species of this genus described so far from the economically important *Eucalyptus* hosts. *Ramularia mali* is an important pathogen of apple cv. Ambrosia and may become a serious pathogen on other apple cultivars. For the first time, two *Ramularia* species, *R. glennii* and *R. plurivora*

have been reported from clinical specimens and *R. plurivora* has the potential of becoming an important human pathogen. The identification of the clinical isolates with MALDI-TOF MS was successful and their MSPs should be added to the commercially available Bruker database of MSPs (BDAL). This would promote a fast and accurate identification of these species in clinical laboratories and would contribute to further investigate the epidemiological relationship with the human host.

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