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Two new *Ophiostoma* species from *Protea caffra* in Zambia

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Key words

β-tubulin
ITS
Ophiostoma
phylogeny
Protea
taxonomy

Abstract The genus *Ophiostoma* (*Ophiostomatales*) has a global distribution and species are best known for their association with bark beetles (*Curculionidae*: *Scolytinae*) on conifers. An unusual assemblage of these fungi is closely associated with the African endemic plant genus *Protea* (*Proteaceae*). *Protea*-associated *Ophiostoma* species are ecologically atypical as they colonise the fruiting structures of various serotinous *Protea* species. Seven species have been described from this niche in South Africa. It has been speculated that novel species may be present in other African countries where these host plants also occur. This view was corroborated by recent collections of two unknown species from *Protea caffra* trees in Zambia. In the present study we evaluate the species delineation of these isolates using morphological comparisons with other *Protea*-associated species, differential growth studies and analyses of DNA sequence data for the β-tubulin and internal transcribed spacer (ITS1, 5.8S, ITS2) regions. As a result, the species *O. protea-sedis* sp. nov., and *O. zambiensis* sp. nov. are described here as new. This study brings the number of *Protea*-associated *Ophiostoma* species to nine and highlights the need for more inclusive surveys, including additional African countries and hosts, to elucidate species diversity in this uncharacteristic niche.

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INTRODUCTION

The *Proteaceae* is a prominent Southern Hemisphere flowering plant family that includes c. 1 400 species (Rebelo 1995, Barker et al. 2007). It comprises five sub-families with the *Proteoideae* predominant in southern Africa (Weston & Barker 2006). The genus *Protea* is one of the principle members of the *Proteoideae* and has its origin in the Cape Floristic Region (CFR) at the southernmost tip of Africa (Reeves 2001, Barraclough & Reeves 2005). The genus has subsequently expanded its range from the CFR, migrated along the African escarpment and up into the tropics (Reeves 2001, Barraclough & Reeves 2005). Today the genus includes more than a hundred species, 80 % of which are confined to the CFR (Rourke 1998). The remainder is associated with various non-CFR vegetation types, ranging from tropical forests in west-central and eastern Africa, to savannah in the north-eastern parts of South Africa (Rourke 1998).

Protea species vary from low-growing shrubs (e.g. *P. acaulos*) to trees of up to 10 m tall (e.g. *P. caffra*) (Rebelo 1995). Their flowers are grouped in large inflorescences that form compact seed-storage structures after pollination. In serotinous species, these structures remain on the plants for at least one year and provide a moist, sheltered environment in which numerous arthropods and saprophytic fungi thrive (Coetzee & Giliomee 1985, Lee et al. 2003, 2005, Roets et al. 2006b). The ophiostomatoid fungi, including members of *Gondwanamyces* (*Microascales*) and *Ophiostoma* (*Ophiostomatales*), dominate fungal communities within such *Protea* infructescences (Roets et al. 2005).

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Ophiostomatoid fungi are morphologically adapted to arthropod spore dispersal, producing sticky spores at the tips of conidiophores or of usually elongated ascomatal necks (Münch 1907, 1908, Francke-Grosmann 1967, Upadhyay 1981, Malloch & Blackwell 1993). The vectors of *Ophiostoma* include bark beetles and ambrosia beetles (*Curculionidae*: *Scolytinae*), longhorn beetles (*Cerambycidae*) and mites (*Acari*) (Barras & Perry 1975, Upadhyay 1981, Bridges & Moser 1983, 1986, Moser 1997, Jacobs & Wingfield 2001, Roets et al. 2007). The genus is best known as an associate of bark-beetles within larval galleries constructed in the phloem and wood of coniferous trees (Kirisits 2004). The interactions between these fungi and their vectors can be mutualistic (Francke-Grosmann 1967, Beaver 1989, Berryman 1989, Six & Paine 1998, Ayres et al. 2000, Jacobs & Wingfield 2001, Klepzig et al. 2001a, b).

Recent studies on the dispersal of *Protea*-associated *Ophiostoma* have shown that mites act as primary vectors of various species, while beetles play an intermediary role by carrying the mites between infructescences (Roets et al. 2006c, 2007, 2009b). Some of these mites feed exclusively on *Ophiostoma*, confirming that the association between these mites and the fungi they carry is also mutualistic (Roets et al. 2007). These ecological studies have led to the discovery of two new *Ophiostoma* species, both of which were first isolated from mites (Roets et al. 2008). Results further highlighted the need for extended surveys, including a greater number of host species and additional localities, to elucidate the total number of species associated with this *Protea*-infructescence niche.

Seven species of *Ophiostoma* have been described from *Protea*. These include *Ophiostoma africanum*, *O. gemellus*, *O. palmiculminatum*, *O. phasma*, *O. protearum*, *O. splendens* and the anamorphic *Sporothrix variecibatus* (Marais & Wingfield 1994, 1997, 2001, Roets et al. 2006a, 2008). All *Protea*-associated *Ophiostoma* species are known only from South Africa.

With the aim of gaining a more comprehensive understanding of the association between *Ophiostoma* and *Protea*, Roets et al. (2009b) conducted surveys including areas and hosts not

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previously considered. Collections from Zambia resulted in the isolation of two putatively novel *Ophiostoma* species from *Protea caffra*. Infructescence-associated *Ophiostoma* species are morphologically very similar. These fungi do not regularly produce sexual structures in culture making species delineation using mating studies difficult. Consequently, DNA sequence comparisons provide an important tool to confirm their identity. In the present study we evaluate the validity of the proposed new taxa (Roets et al. 2009b) based on morphological and DNA sequence-based comparisons with known *Protea*-associated *Ophiostoma* species.

MATERIALS AND METHODS

Isolates

Isolates of the putative new taxa (*Ophiostoma* sp. nov. 1 and *Ophiostoma* sp. nov. 3 (Roets et al. 2009b) were collected in Nchila, Zambia during October 2006. Three additional isolates of each of these species, not included in the study of Roets et al. (2009b) were included in the present study (Table 1). Isolates were maintained in Petri dishes containing 2 % malt extract agar (MEA, Biolab, Midrand, South Africa) at 4 °C. Representative cultures of the taxa considered in this study have been deposited in both the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Herbarium specimens were deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM). Nomenclatural novelties and descriptions were deposited in MycoBank (www.MycoBank.org; Crous et al. 2004).

Morphology and growth in culture

Isolates of *Ophiostoma* sp. nov. 1 and *Ophiostoma* sp. nov. 2 (Table 1) were grown in the dark for 5 d at 25 °C on 2 % MEA (Biolab, Midrand, South Africa). Ascomata collected from colonized infructescences and conidiogenous cells formed in culture were mounted on microscope slides in lactophenol (Stephens 1974). Specimens were studied using a Nikon Eclipse E600 light microscope (Nikon Corporation, Tokyo, Japan) with differential interference contrast. Fifty measurements of each taxonomically informative structure were made and, where appropriate, means (± standard deviation) calculated. Photographic images were captured using a Nikon DXM1200 digital camera (Nikon Corporation, Tokyo, Japan).

The growth of isolates of the two putative new *Ophiostoma* species was compared to that of closely related species. Based on results of previous molecular analyses (Roets et al. 2009b) three isolates of each of *O. africanum*, *O. gemellus*, *O. palmiculminatum*, *O. protearum* and *O. splendens* were thus included. For these initial studies, conidia were removed from actively growing 1 wk old cultures using a sterile needle with a small piece of agar at the tip and transferred to the centre of fresh dishes containing 20 mL MEA. Plates were incubated at 25 °C for 8 d in the dark, after which mean (± standard deviation) colony diameters (three measurements per isolate) were determined.

Species that are morphologically closely related to *Ophiostoma* sp. nov. 1 and *Ophiostoma* sp. nov. 2 from *P. cafra* in Zambia, respectively, and that had similar growth rates at 25 °C were used in subsequent growth studies to test for significant differences in growth response at different temperatures. For these studies three isolates each of *Ophiostoma* sp. nov. 1, *O. gemellus*, *O. palmiculminatum*, *O. splendens* and *Ophiostoma* sp. nov. 2 were included. Mycelium-covered agar discs (5 mm diam) were excised from the edges of actively growing 1 wk old cultures and transferred to the centers of fresh MEA dishes. Plates were incubated in the dark at temperatures ranging from 5–35 °C at 5 °C intervals. Colony diameters were measured after 2 d and again after an additional 8 d of growth. The mean diameter of additional growth (three measurements per isolate) and the mean growth diameter (± standard deviation) for each isolate were calculated. A factorial one-way analysis of variance (ANOVA) with Sigma-restricted parameterization was used to analyze the data using Statistica 8 (Statsoft Corp., Tulsa, Oklahoma). Significant differences between growth rates of these fungal species are reported when statistically different at the 99 % level ($P \leq 0.001$).

DNA isolation, amplification and sequencing

Genomic DNA was extracted from fungal mycelium using a Sigma GenElute™ plant genomic DNA miniprep kit (Sigma-Aldrich Chemie CMBH, Steinheim, Germany) following the manufacturer's instructions. The primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White et al. 1990) were used for amplification of the internal transcribed spacers and 5.8S rDNA regions (ITS). For amplification of the β-tubulin gene region the primers T1 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) were used. PCR reaction mixtures and amplification conditions followed those of Roets et al. (2006a, 2009b). PCR products were purified using the Wizard® SV gel and PCR clean up system (Promega, Madison, Wisconsin, USA) following the manufacturer's instructions. The amplicons were sequenced using the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

ITS and β-tubulin DNA sequences for four isolates, two of each of the two putative new species from *P. caffra* in Zambia, generated by Roets et al. (2009b) were included in the present study. DNA sequence data for all other *Ophiostoma* spp. included in phylogenetic reconstructions were obtained from NCBI GenBank (www.ncbi.nlm.nih.gov/), and accession numbers are presented in the phylogenetic trees. Sequences were aligned using the L-INS-I strategy in the online version of MAFFT v6 (Katoh & Toh 2008).

The ITS sequence dataset included several *Ophiostoma* and *Sporothrix* species to illustrate the phylogenetic positions of the species from *Protea* in relation to other species and species groups in the *Ophiostomatales*. Three *Grosmannia* species were designated as outgroup taxa in the ITS analyses.

Sequence data of the β-tubulin gene were used to distinguish between closely related species and were analyzed in two datasets. The first dataset included several *Ophiostoma* and *Sporothrix* species from the *S. schenckii*–*O. stenoceras* complex, and all available β-tubulin sequences of species associated with *Protea*. The region included in the analyses spanned β-tubulin exons 4, 5 and 6, as well as intron 5. In all the species included in this dataset, intron 4 was absent. In the second β-tubulin dataset, an extended fragment was analyzed, consisting of exons 3–6, as well as introns 3 and 5. Data of the longer fragment are available for fewer species, thus fewer species were included in the analyses.

All three datasets were subjected to maximum parsimony (MP), Bayesian inference (BI) and maximum likelihood (ML) analyses. The protocols followed for each analysis are described below. Isolate numbers for all species from *Protea* (including those from previous studies) are given in the phylogenetic trees. GenBank accession numbers and all other information regarding the *Protea* isolates are presented in Table 1. GenBank accession numbers of all non-*Protea* species of *Ophiostoma* are presented in the phylogenetic trees.

Table 1 Origin, hosts and GenBank accession numbers for fungal isolates associated with *Protea* species in Zambia and South Africa, used in phylogenetic analyses. Isolate and accession numbers of sequences obtained in this study are printed in **bold** type.

Species	Isolate no.		Host	Origin	Collector	GenBank no.	
	CBS	CMW				ITS	β-tubulin
Ophiostoma africanum	116374	1822	*Protea dracomontana*	KZ-Natal, South Africa	M.J. Wingfield	DQ316197	DQ316159
	116376	1812	*Protea dracomontana*	KZ-Natal, South Africa	M.J. Wingfield	DQ316198	DQ316160
	116566	1104	*P. caffra*	Gauteng, South Africa	Unknown	DQ316200	DQ316162
	116571	823	*P. gaguedi*	Unknown	M.J. Wingfield	DQ316199	DQ296073
O. gemellus	121957	23054	*P. caffra*	Gauteng, South Africa	F. Roets	DQ821557	DQ821551
	121958	23056	*P. caffra*	Gauteng, South Africa	F. Roets	DQ821558	DQ821552
	121959	23057	*Tarsonemus* sp. from *P. caffra*	Gauteng, South Africa	F. Roets	DQ821560	DQ821554
		23055	*P. caffra*	Gauteng, South Africa	F. Roets	DQ821559	DQ821553
		23058	*Tarsonemus* sp. from *P. caffra*	Gauteng, South Africa	F. Roets	DQ821561	DQ821555
		23059	*Tarsonemus* sp. from *P. caffra*	Gauteng, South Africa	F. Roets	DQ821562	DQ821556
O. palmiculminatum	119590	20677	*P. repens*	Western Cape, South Africa	F. Roets	DQ316191	DQ821543
	119591	20693	*P. repens*	Western Cape, South Africa	F. Roets	DQ316192	DQ821544
	119592	20694	*P. repens*	Western Cape, South Africa	F. Roets		DQ821546
	119593	20697	*P. repens*	Western Cape, South Africa	F. Roets		DQ821547
		20695	*P. repens*	Western Cape, South Africa	F. Roets		DQ821545
		20696	*P. repens*	Western Cape, South Africa	F. Roets		DQ821548
		23049	*Trichouropoda* sp. from *P. repens*	Western Cape, South Africa	F. Roets	DQ821563	DQ821550
O. phasma	119721	20676	*P. laurifolia*	Western Cape, South Africa	F. Roets	DQ316219	DQ316181
		20683	*P. laurifolia*	Western Cape, South Africa	F. Roets	DQ316227	DQ316189
		20684	*P. laurifolia*	Western Cape, South Africa	F. Roets	DQ316218	DQ316180
		20692	*P. laurifolia*	Western Cape, South Africa	F. Roets	DQ316228	DQ316190
		20698	*P. laurifolia*	Western Cape, South Africa	F. Roets	DQ316222	DQ316184
		20699	*P. laurifolia*	Western Cape, South Africa	F. Roets	DQ316220	DQ316182
O. protearum	116567	1103	*P. caffra*	Gauteng, South Africa	M.J. Wingfield	DQ316203	DQ316165
	116568	1102	*P. caffra*	Gauteng, South Africa	M.J. Wingfield	DQ316202	DQ296072
	116654	1107	*P. caffra*	Gauteng, South Africa	M.J. Wingfield	DQ316201	DQ316163
***Ophiostoma* sp. nov. 2 =**	**124909**	**28600**	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660448**	**EU660463**
(*O. protea-sedis* sp. nov.)	**124910**	**28601**	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660449**	**EU660464**
	124911	**29074**	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660450**	**EU660465**
		29075	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660451**	**EU660466**
		29076	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660452**	**EU660467**
O. splendens	116377	873	*P. repens*	Unknown	M.J. Wingfield	DQ316214	DQ316176
	116378		Unknown	Unknown	Unknown	DQ316208	DQ316170
	116379	896	*P. repens*	Unknown	M.J. Wingfield	DQ316207	DQ316169
	116569	872	*P. repens*	Unknown	M.J. Wingfield	DQ316215	DQ296071
		2753	*P. neriifolia*	Western Cape, South Africa	G.J. Marais	DQ316206	DQ316168
		20674	*P. repens*	Western Cape, South Africa	F. Roets	DQ316204	DQ316166
		20675	*P. repens*	Western Cape, South Africa	F. Roets	DQ316205	DQ316167
		20678	*P. repens*	Western Cape, South Africa	F. Roets	DQ316210	DQ316172
		20679	*P. repens*	Western Cape, South Africa	F. Roets	DQ316212	DQ316174
		20680	*P. repens*	Western Cape, South Africa	F. Roets	DQ316211	DQ316173
		20685	*P. repens*	Western Cape, South Africa	F. Roets	DQ316213	DQ316175
		20691	*P. repens*	Western Cape, South Africa	F. Roets	DQ316209	DQ316171
***Ophiostoma* sp. nov. 1 =**	**124912**	**28604**	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660453**	**EU660473**
(*O. zambiensis* sp. nov.)	**124913**	**28605**	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660454**	**EU660474**
	124914	**29077**	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660455**	**EU660475**
		29078	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660456**	**EU660476**
		29079	***P. caffra***	**Nchila, Zambia**	**F. Roets**	**EU660457**	**EU660477**
Sporothrix variecibatus	121960	23060	*Protea longifolia*	Western Cape, South Africa	F. Roets	DQ821569	DQ821573
	121961	23051	*Trichouropoda* sp. from *Protea repens*	Western Cape, South Africa	F. Roets	DQ821568	DQ821539
	121962	2543	*Eucalyptus* leaf litter	Western Cape, South Africa	P.W. Crous	DQ821567	DQ821572

Maximum parsimony

Five thousand random stepwise addition heuristic searches were performed using the software package PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford 2003) with tree-bisection-reconnection (TBR) branch swapping active. All characters were unordered and of equal weight and alignment gaps were treated as a fifth character state. Ten trees were saved per replicate and branches of zero length were collapsed. Confidence levels were estimated by performing 1 000 bootstrap replicates (Felsenstein 1985) with fast-stepwise addition.

Bayesian inference

Bayesian inference, based on a Markov Chain Monte Carlo (MCMC) approach, was performed in the software package MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). Evolutionary models were determined using MrModeltest v2.2 (Nylander 2004) based on the Akaike Information Criterion (AIC). Base equilibrium frequencies, instantaneous substitution rates and among-site rate variation were estimated independently on shared topologies. Two independent Markov chains were initiated from a random starting tree where after runs of 5 000 000 generations (sample frequency of 100) were performed. Burnin values were determined using Tracer v1.4 (http://tree.bio.ed.ac.uk/software/tracer/), and all sampled trees lower than the burnin values were discarded. The remaining trees were pooled into a 50 % majority rule consensus tree.

Maximum likelihood

Maximum likelihood (ML) analyses were conducted in the online version of PhyML v3.0 (Guindon & Gascuel 2003). The best fit substitution models were determined by AIC in Modeltest v3.7 (Posada & Crandall 1998). Confidence values were estimated using bootstrap analysis (1 000 replicates).

RESULTS

Morphology and growth in culture

Morphological comparisons revealed close similarities between *O. africanum*, *O. protearum*, *O. splendens* and *Ophiostoma* sp. nov. 1 (hereafter *O. zambiensis*). *Ophiostoma protearum* differs from the other species in that it has hyphal ornamentation on the ascomatal bases. *Ophiostoma protearum* and *O. africanum* have ostiolar hyphae, while these structures are not found in *O. splendens* or *O. zambiensis* from *P. caffra* in Zambia. Morphological distinction between *O. splendens* and *O. zambiensis* is not unambiguous, as most characters overlap in their dimensions. Ascospores of *O. zambiensis* are on average slightly shorter and wider (4 × 2 µm) than those of *O. splendens* (5.7 × 1.1 µm).

Based on morphology, *Ophiostoma* sp. nov. 2 (hereafter *O. protea-sedis*) is closely related to *O. gemellus* and *O. palmiculminatum*. The size-ranges of most morphological characters overlap between these three species. All three species form fairly large ascomata with long necks that have ostiolar hyphae at the tips. These ostiolar hyphae vary slightly in size between the three species. The ostiolar hyphae of *O. gemellus* are 32–42 µm (35 ± 3) long, while those of *O. palmiculminatum* are 10–25 µm (16 ± 5) long. The ostiolar hyphae of *O. protea-sedis* are intermediate (25–27 µm (25 ± 1)) in length between *O. gemellus* and *O. palmiculminatum*. Another morphological character that aids delineation between these species is the presence of hyphal ornamentation on the ascomatal bases. *Ophiostoma gemellus* lacks hyphal ornamentation on the ascomatal base, while *O. palmiculminatum* usually has very sparse hyphae originating from these structures. *Ophiostoma protea-sedis*, in contrast, usually has very dense hyphal ornamentation on the ascomatal bases. In addition to these morphological differences, conidia of *O. gemellus* are usually strongly curved, while those of *O. palmiculminatum* and *O. protea-sedis* are clavate. Conidia of *O. protea-sedis* and *O. palmiculminatum* can be distinguished by their respective dimensions. Those of *O. protea-sedis* are generally slightly wider than those of *O. palmiculminatum*.

Cultures of *O. africanum*, *O. gemellus*, *O. palmiculminatum*, *O. protearum*, *O. splendens* and the two putative new *Ophiostoma* spp. from *P. caffra* in Zambia showed differences in their growth rates. Mean colony diameters (± standard deviation) for *O. africanum*, *O. protearum*, *O. splendens* and *O. zambiensis* were 25.8 mm (± 1.1), 25.1 mm (± 0.3), 30.2 mm (± 0.4) and 29.7 mm (± 0.7), respectively, after 8 d in the dark at 25 °C. Mean colony diameters (± standard deviation) for *O. gemellus*, *O. palmiculminatum* and *O. protea-sedis* were 25.2 mm (± 0.6), 23.3 mm (± 0.4) and 21.1 mm (± 1.2), respectively, after 8 d in the dark at 25 °C.

Further growth tests (at different temperatures) focused on comparisons between *O. splendens* and *O. zambiensis* and comparisons between *O. gemellus*, *O. palmiculminatum* and *O. protea-sedis* as these species are morphologically closely related to the Zambian isolates and had similar growth rates at 25 °C. Cultures of the two *Ophiostoma* species from *P. caffra* in Zambia grew optimally on MEA at 30 °C and 25 °C, respectively (Fig. 1). *Ophiostoma zambiensis* grew significantly slower than *O. splendens* at the tested temperatures ($F = 52.99$; $P \leq 0.001$). *Ophiostoma splendens* and *O. zambiensis* also reacted differently towards different temperatures ($F = 16604$, $P \leq 0.001$). *Ophiostoma protea-sedis* grew significantly slower than *O. gemellus* ($F = 18.72$, $P \leq 0.001$) and *O. palmiculminatum* ($F = 15.52$, $P \leq 0.001$) when growth rates of these species at different temperatures are compared (Fig. 1). In addition, *O. protea-sedis* and *O. gemellus* reacted significantly different to the different temperatures ($F = 12149$, $P \leq 0.001$). *Ophiostoma protea-sedis* and *O. palmiculminatum* differed significantly in their reaction to different growth temperatures ($F = 18417$, $P \leq 0.001$).

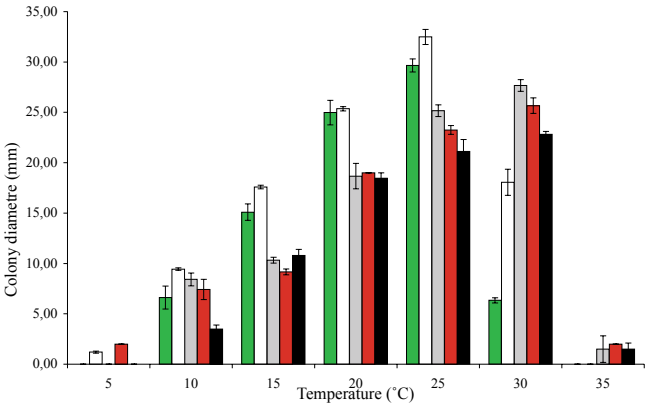

Fig. 1 Mean growth on MEA (three isolates per tested species, ± standard deviation) of *O. splendens* (white bars), *O. zambiensis* (green bars), *O. gemellus* (grey bars), *O. palmiculminatum* (red bars), and *O. protea-sedis* (black bars) at a range of temperatures after 8 d in the dark.

DNA isolation, amplification and sequencing

Amplified fragments obtained using the primers ITS1-F and ITS4 were 547 bp for *O. protea-sedis* and 541 bp long for *O. zambiensis*. Amplification of extracted genomic DNA with the primers T1 and Bt2b resulted in fragment lengths of 999 bp for *O. protea-sedis* and 879 bp for *O. zambiensis*.

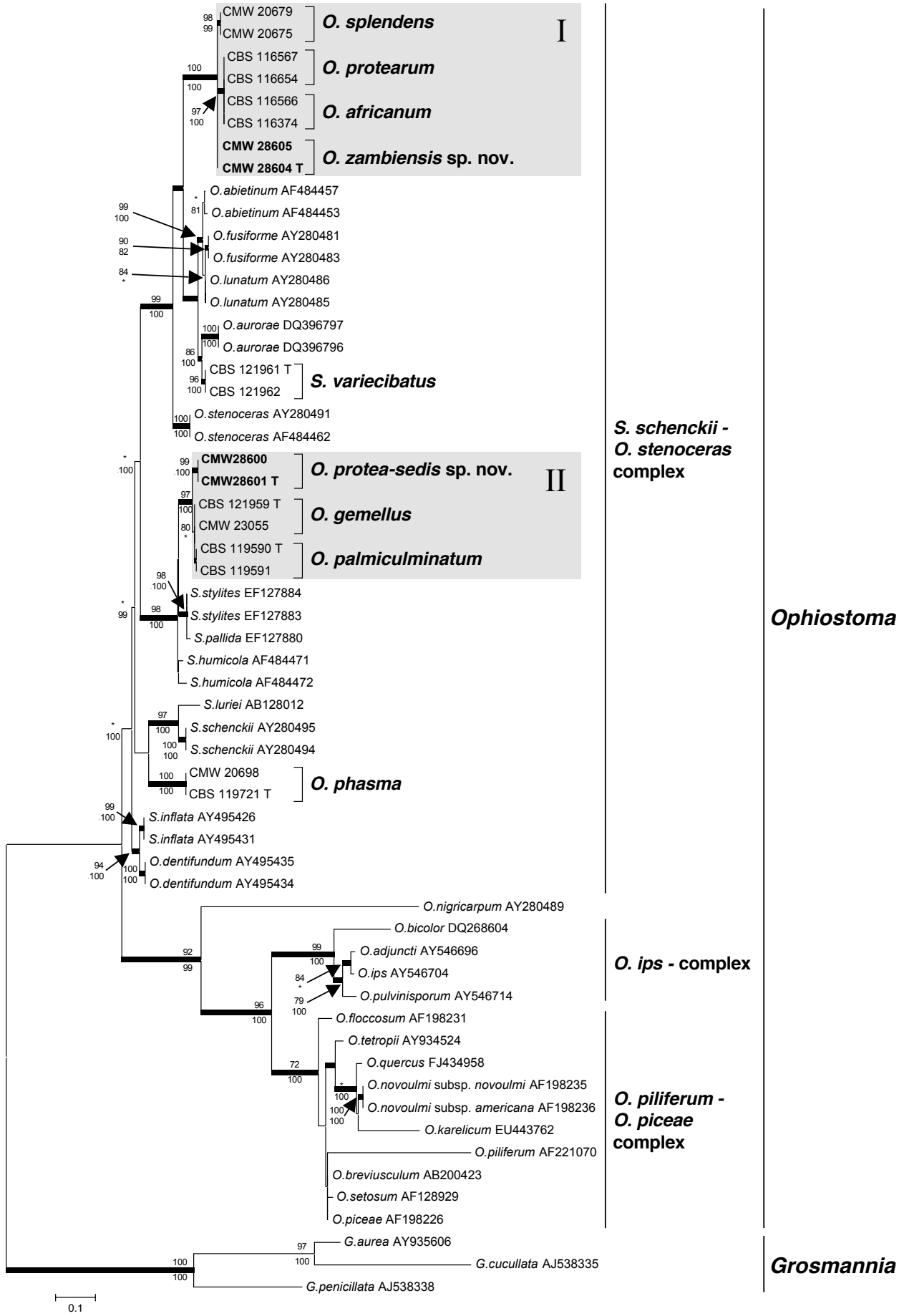

Fig. 2 Phylogram obtained from maximum likelihood (ML) analysis of the ribosomal ITS data. Isolate numbers of sequences obtained in the present study are printed in **bold** type. Isolates from *Protea* are indicated by square brackets and their species names printed in **bold** type. Bootstrap support values (> 70 %) for ML and MP are respectively presented above and below branching points. Branches supported by posterior probabilities > 90 % obtained with Bayesian inference, are printed in **bold**. Trees were rooted against the three species of *Grosmannia*.

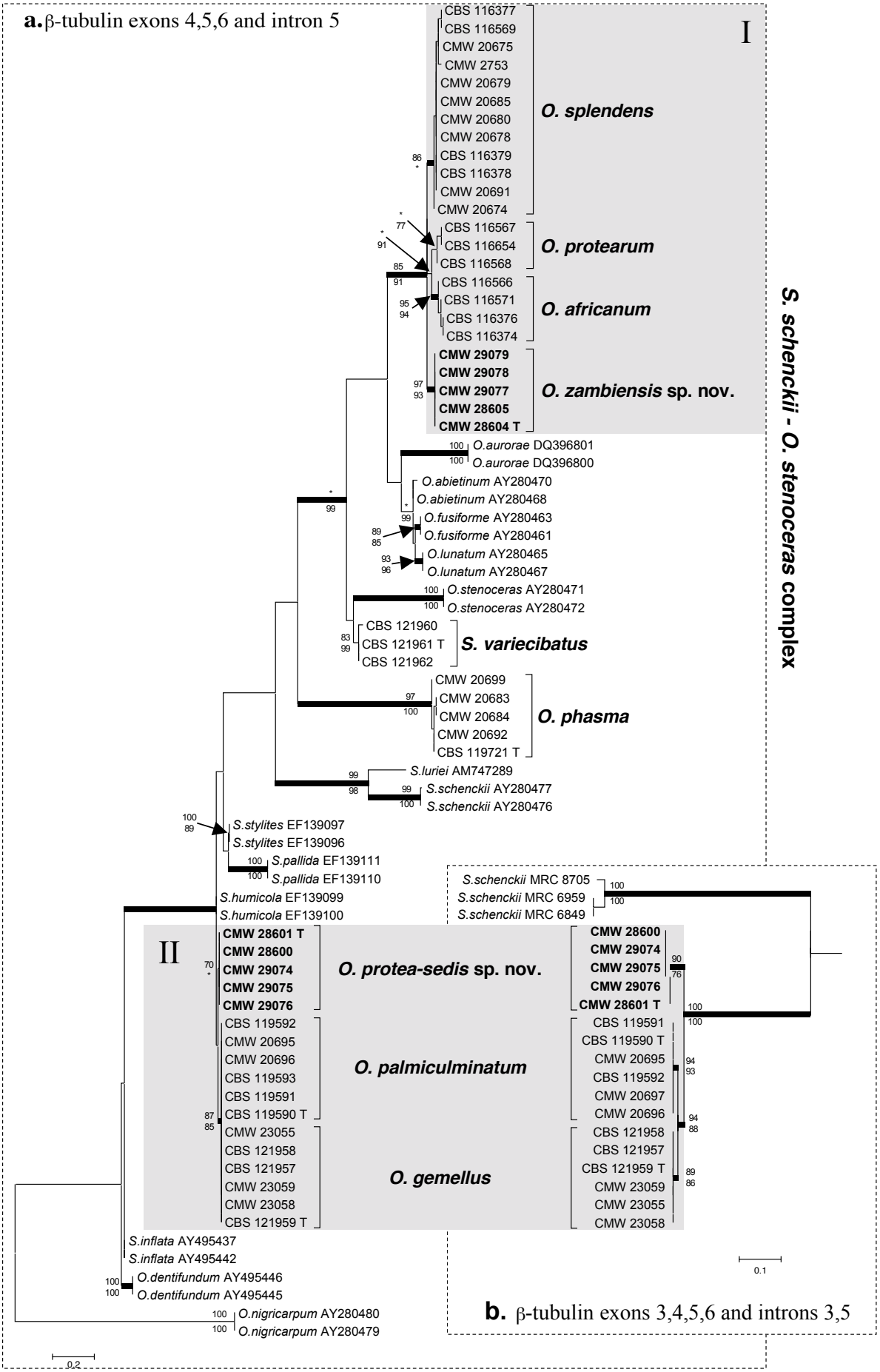

Fig. 3 Phylograms obtained from maximum likelihood (ML) analysis of the shorter (a) and extended (b) β-tubulin gene regions. Isolate numbers of sequences obtained in the present study are printed in **bold** type. Isolates from *Protea* are indicated by square brackets and their species names printed in **bold** type. Bootstrap support values (> 70 %) for ML and MP are respectively presented above and below branching points. Branches supported by posterior probabilities > 90 % obtained with Bayesian inference, are printed in **bold**. Two isolates of *Ophiostoma nigricarpum* was used as outgroup in (a), while the tree obtained in (b) was midpoint-rooted.

Table 2 Statistics resulting from the different phylogenetic analyses.

Dataset	β-tubulin		Maximum Parsimony						Maximum Likelihood			MrBayes	
	exons	introns	PIC[a]	No. of trees	Tree length	CI[b]	RI[c]	HI[d]	Subst.[e] model	Pinvar[f]	Gamma[g]	Subst.[e] model	Burn-in
ITS	–	–	382	96	1432	0.624	0.864	0.376	GTR+I+G	0.3504	1.4792	GTR+I+G	500
β-tubulin	4–6	5	116	84	455	0.642	0.936	0.358	GTR+I+G	0.5396	1.7180	GTR+I+G	100
β-tubulin	3–6	3, 5	303	1	517	0.899	0.973	0.101	HKY+I	0.5246	–	HKY+I	100

[a] PIC = number of parsimony informative characters; [b] CI = consistency index; [c] RI = retention index; [d] HI = homoplasy index;
[e] Subst.model = best fit substitution model; [f] Pinvar = proportion of invariable sites; [g] Gamma = Gamma distribution shape parameter.

Phylogenetic analyses

Parameters used and statistical values obtained from the various analyses of the ITS, β-tubulin and extended β-tubulin datasets, are presented in Table 2. The three aligned datasets were respectively 725, 269 and 600 base pairs in length. Phylograms obtained with ML for the three datasets are presented in Fig. 2, 3a, b.

Trees generated by analyses of the ITS dataset (Fig. 2) revealed that the two putative new taxa from Zambia grouped in two distinct clades (hereafter referred to as Group I and Group II) in the *S. schenckii*–*O. stenoceras* complex of the genus *Ophiostoma*. Group I included *O. africanum*, *O. protearum* *O. splendens* and the new species, *O. zambiensis*. The two isolates of *O. zambiensis* did not form a clade with strong statistical support, and ITS sequences also failed to distinguish between *O. africanum* and *O. protearum* as has been reported previously (Roets et al. 2006a). Group II isolates included *O. gemellus*, *O. palmiculminatum* and the second putative new species from Zambia, *O. protea-sedis*. The two isolates of *O. protea-sedis* had good statistical support, but this was not the case for *O. gemellus* or *O. palmiculminatum* (Roets et al. 2008).

The trees obtained from the β-tubulin dataset that included exons 4–6, and intron 5 (Fig. 3a), showed the same two clades (Groups I and II) containing the species from *Protea*. The four species in Group I, including *O. zambiensis*, grouped separately with good statistical support. These included *O. africanum*, *O. protearum*, (both from the Gauteng Province) and *O. splendens* from the Western Cape Province. In Group II, *O. protea-sedis* had some level of support from ML analyses, separating it from *O. gemellus* and *O. palmiculminatum*, but this was not convincing. Neither could the latter two species be separated based on this region, as was shown previously (Roets et al. 2007). This lack of resolution necessitated analyses of an extended region of the β-tubulin gene used previously to distinguish between *O. gemellus* and *O. palmiculminatum* (Roets et al. 2007). This analysis also incorporated exon 3 and intron 3 of the β-tubulin gene (Fig. 3b). Apart from *S. schenckii* and the species in Group II, intron 3 is absent from all the other *Protea*-associated species. Analyses of this extended region distinguished clearly, with strong support (Fig. 3b), between *O. gemellus* from the Gauteng Province, *O. palmiculminatum* from the Western Cape Province of South Africa, and *O. protea-sedis* from Zambia.

Taxonomy

Both the analyses of phylogenetic data in Roets et al. (2009b) and in the present study provided strong support for the hypothesis that the *Ophiostoma* isolates from *P. caffra* infructescences in Zambia represent two undescribed species. They are described here as follows:

Ophiostoma zambiensis Roets, M.J. Wingf. & Z.W. de Beer, *sp. nov.* — MycoBank MB513284; Fig. 4a–h

Asomata superficialia, basi globosa, atro, 90–120 µm diam, nonnumquam paucis hyphis circumdata, collo atro, 135–165 × 17–27 µm, sursum ad 10–18 µm angustato, nonnumquam paucis hyphis ostiolaribus. Asci evanescentes. Ascosporae allantoideae, unicellulares, hyalinae, vagina gelatinosa carentes, 4 × 2 µm, aggregatae incoloratae. Anamorphe *Sporothrix* sp., conidiis clavatis 3.5–5 × 2 µm.

Etymology. The epithet *zambiensis* refers to Zambia, the country from which this species was collected.

Ascomata superficial on host tissue. *Ascomatal bases* globose, dark, 90–120 µm (101 ± 13) diam, without hyphal ornamentation. *Ascomatal necks* black, 135–165 µm (155 ± 12) long, 17–27 µm (23 ± 5) wide at the base, 10–18 µm (13 ± 4) wide at the apex. *Ostiolar hyphae* absent. *Asci* evanescent. *Ascospores* allantoid, 1-celled, hyaline, sheaths absent, 4 µm long, 2 µm wide, collecting in a hyaline gelatinous droplet at the apex of the neck.

Culture of the Sporothrix anamorph on MEA 29.7 mm (± 0.7) diam after 8 d at 25 °C in the dark, cream coloured, effuse, circular with an entire edge, surface smooth. Growth reduced at temperatures below and above the optimum of 25 °C. *Hyphae* superficial on 2 % MEA plates. Sporulation profuse on MEA. *Conidiogenous cells* 3–12 µm long, 2–3 µm wide, arising directly from hyphae and from 2–43 µm long aerial conidiophores, proliferating sympodially, hyaline, becoming denticulate. *Denticles* 0.5–1.5 µm (1 ± 0.5) long, usually in an apical crown of 3–10, scattered, solitary or in nodes. *Conidia* holoblastic, hyaline, 1-celled, clavate, smooth, thin-walled, 3.5–5 µm (4 ± 0.5) long and 2 µm wide. Conidia formed singly, but aggregate to form slimy masses.

Specimens examined. Zambia, Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, holotype Herb. PREM 60350, culture ex-type CMW 28604 = CBS 124912; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, paratype Herb. PREM 60351, culture ex-type CMW 28605 = CBS 124913; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, paratype Herb. PREM 60352, culture ex-type CMW 29077 = CBS 124914; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, paratype Herb. PREM 60353, culture ex-type CMW 29079; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, culture CMW 29078.

Notes — Based on morphological characteristics, *O. zambiensis* is closely related to *O. africanum*, *O. protearum* and *O. splendens*. *Ophiostoma protearum* can be distinguished from *O. splendens*, *O. zambiensis* and *O. africanum* by the presence of hyphal ornamentation at the ascomatal base, structures absent for all the other species. The only morphological character that distinguishes *O. zambiensis* from *O. africanum* is the presence of short ostiolar hyphae in the latter species, while these structures are absent in *O. zambiensis*. Except for slightly smaller and wider conidia in *O. zambiensis*, no other consistent morphological characters distinguish this species from *O. splendens*. The ITS and β-tubulin gene nucleotide sequences of these four species differ markedly.

Ophiostoma protea-sedis Roets, M.J. Wingf. & Z.W. de Beer, *sp. nov.* — MycoBank MB513285; Fig. 4i–p

Asomata superficialia, basi globosa, atro, 130–300 µm diam, hyphis circumdata 40–70 µm, collo atro, 540–850 × 30–60 µm, sursum ad 11–13 µm angustato, 8–12 hyphis ostiolaribus rectis vel curvatis, hyalinis vel subhyalinis, 25–27 µm longis palmam fingentibus ornato. Asci evanescentes.

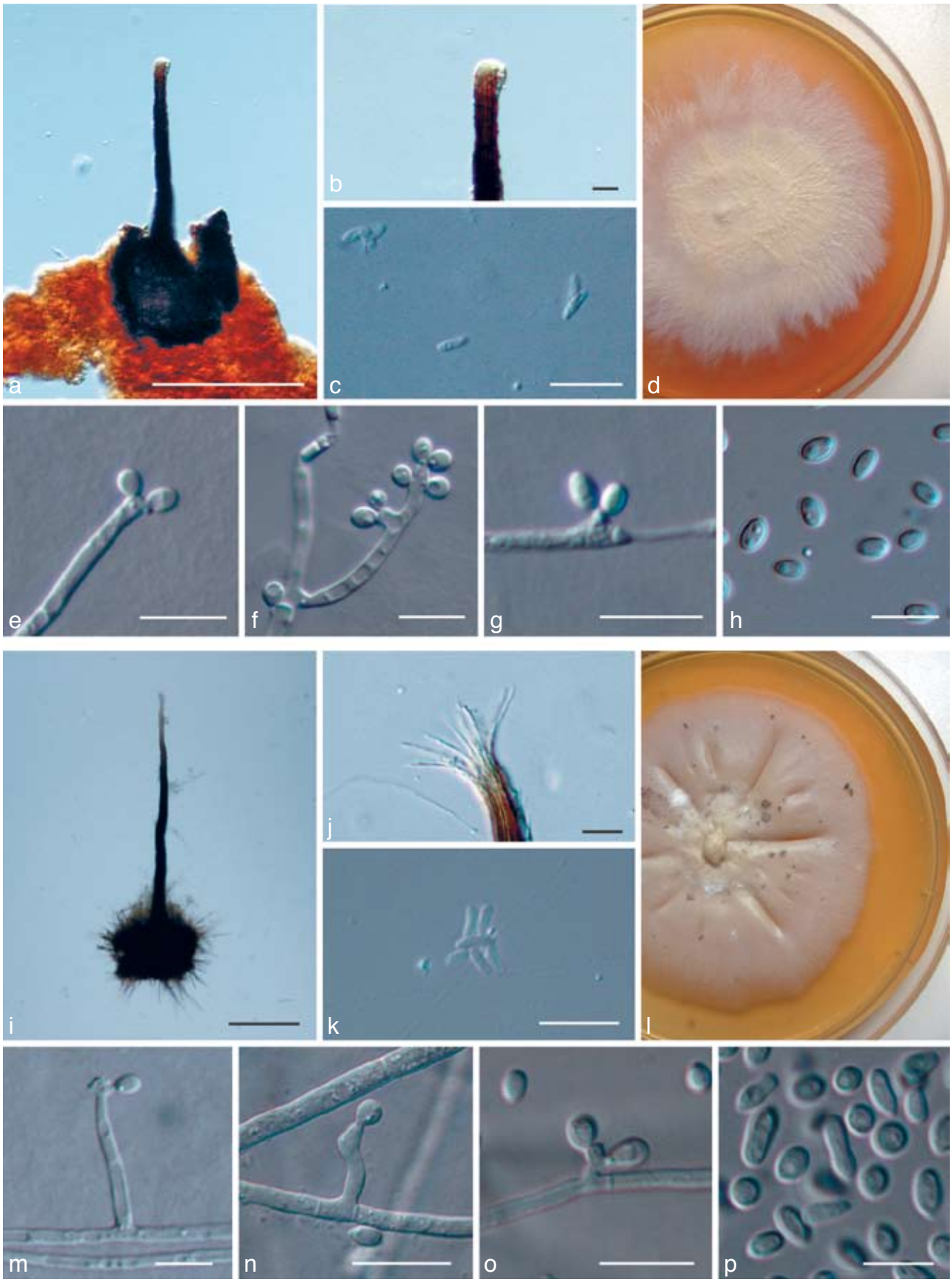

Fig. 4 *Ophiostoma* from Zambian *P. caffra*. a–h: *O. zambiensis*. a. Perithecium; b. perithecial tip; c. ascospores; d. 3 wk old colony on MEA; e–h. conidiogenous cells, conidiophores and conidia. — i–p: *O. protea-sedis*. i. Perithecium; j. perithecial tip; k. ascospores; l. 3 wk old colony on MEA; m–p. conidiogenous cells, conidiophores and conidia. — Scale bars: a, i = 100 µm; all others = 10 µm.

Ascosporae allantoideae, unicellulares, hyalinae, vagina gelatinosa carentes, 4–5 × 1–2 µm, aggregatae incoloratae. Anamorphe *Sporothrix* sp., conidiis clavatis 3.5–7 × 2–3.5 µm.

Etymology. The epithet *protea-sedis* (*protea* = host genus, *sedis* = home) refers to the close association between this species and its plant host.

Ascomata superficial on host tissue. *Ascomatal bases* globose, black, 130–300 µm (229 ± 66) diam, usually with dense hyphal ornamentation, hyphae 40–70 µm long. *Ascomatal necks* black, 540–850 µm (677 ± 95) long, 30–60 µm (41 ± 11) wide at the base, 11–13 µm (12 ± 1) wide at the apex. 8–12 *Ostiolar hyphae* usually present, divergent, somewhat curved, hyaline to subhyaline, 25–27 µm (25 ± 1) long. *Asci* evanescent. *Ascospores* allantoid, 1-celled, hyaline, sheaths absent, 4–5 µm (4 ± 1) long, 1–2 µm wide collecting in a hyaline gelatinous droplet at the apex of the neck.

Culture of the Sporothrix anamorph on MEA 21.1 mm (± 1.2) diam after 8 d at 25 °C in the dark, cream coloured, effuse, circular with an entire edge, surface smooth. Growth reduced at temperatures below and above the optimum of 30 °C. *Hyphae* superficial on 2 % MEA plates. Sporulation profuse on MEA. *Conidiogenous cells* 1–42 µm long, 1–2 µm wide, arising directly from hyphae and from 5–63 µm long aerial conidiophores, proliferating sympodially, hyaline becoming denticulate. *Denticles* 0.5–1 µm long, usually in an apical crown of 7–14, scattered, solitary or in nodes. *Conidia* holoblastic, hyaline, 1-celled, clavate, smooth, thin-walled, 3.5–7 µm (4 ± 1) long and 2–3.5 µm (3) wide. Conidia formed singly, but aggregate to form slimy masses.

Specimens examined. Zambia, Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, holotype Herb. PREM 60354, culture ex-type CMW 28601= CBS 124910; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, paratype Herb. PREM 60355, culture ex-type CMW 29074 = CBS 124911; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, paratype Herb. PREM 60357, culture ex-type CMW 28600 = CBS 124909; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, paratype Herb. PREM 60356, culture ex-type CMW 29076; Nchila, from the infructescences of *Protea caffra*, Oct. 2006, *F. Roets*, culture CMW 29075.

Notes — Based on morphological characteristics, *O. protea-sedis* is closely related to *O. palmiculminatum* and *O. gemellus*. The only reliable means to distinguish between these species is by comparison of their respective ITS and β-tubulin gene nucleotide sequences.

DISCUSSION

This study confirms an earlier contention by Roets et al. (2009b) that isolates of *Ophiostoma* collected from *P. caffra* infructescences in Zambia represent two new taxa. *Ophiostoma protea-sedis* and *O. zambiensis* represent the first *Protea*-associated members of the genus described from a country other than South Africa. These species were recognised based on morphological characteristics as well as comparisons of DNA sequence data and comparisons of growth in culture with closely related species. With the description of *O. protea-sedis* and *O. zambiensis*, nine *Ophiostoma* species are now known to occur in the unusual *Protea*-infructescences niche.

Most species of *Ophiostoma* from *Protea* infructescences are morphologically very similar and the size ranges for some characters overlap. *Ophiostoma phasma* and the anamorphic *S. variecibatus* are morphologically well-defined and can readily be distinguished from other *Protea*-associated species based on morphology alone. These species are also phylogenetically well-separated from other *Protea*-associated species. The remaining species reside in two well-defined groups based on morphology, physiology and phylogenetic inference. The first of these groups (Group I) comprises species that form slightly smaller ascomatal bases and have much shorter necks (135–167 µm) than species in Group II. These species (*O. africanum*, *O. protearum*, *O. splendens* and *O. zambiensis*) grow optimally at 25 °C. Species in the second group (*Protea*-host Group II), produce fairly large ascomatal bases and very long necks (up to 850 µm as for *O. protea-sedis*). All species in this group (*O. gemellus*, *O. palmiculminatum* and *O. protea-sedis*) grow optimally at 30 °C, rather than 25 °C. Differentiating species residing in these two groups based on morphology alone is tenuous. The only reliable method to readily distinguish between them is by comparison of DNA sequence data.

The two distinct groups of *Ophiostoma* species residing in *Protea* infructescences have varied host plant preferences. *Ophiostoma gemellus* and *O. protea-sedis* are confined to *P. caffra*, while *O. palmiculminatum* is restricted to the infructescences of *P. repens* (Roets et al. 2009b). *Protea caffra* also hosts *O. protearum* and *O. zambiensis*, while *O. africanum* is associated with various different *Protea* species (*P. caffra*, *P. dracomontana* and *P. gaguedi*). *Ophiostoma splendens* is also associated with many different *Protea* species (*P. burchellii*, *P. coronata*, *P. laurifolia*, *P. lepidocarpodendron*, *P. longifolia*, *P. lorifolia*, *P. neriifolia* and *P. repens*) (Roets et al. 2009b). Based on these host preferences and the distribution of the hosts, Roets et al. (2009b) concluded that the *Protea*-associated *Ophiostoma* species probably originated in tropical Africa and migrated southwards to the tip of Africa. This migration of *Ophiostoma* into the CFR was probably facilitated by vector arthropods (Roets et al. 2009b). This contrasts with the hypothesised origin of the genus *Protea* in the Cape region of South Africa and its subsequent migration into the tropics following the Great Rift Valley (Barraclough & Reeves 2005).

The specific evolutionary origin of *Protea*-associated *Ophiostoma* species remains unclear. The closest sister species of *Protea*-host Group I includes *O. abietinum*, *O. aurorae*, *O. fusiforme*, *O. lunatum*, *O. stenoceras* and *S. variecibatus* (de Beer et al. 2003, Roets et al. 2008). These species have been isolated from a large diversity of niches that include wood, leaves, soil, bark beetles and even human tissues. The sister species of *Protea*-host Group II includes *S. humicola*, *S. pallida*, and *S. stylites*. These species have only been isolated from soil and from the bases of planted wooden utility poles (de Meyer et al. 2008). Interestingly, the latter two species have been reported only from South Africa (de Meyer et al. 2008). A soil-linked origin for *Ophiostoma* species in *Protea*-host Group II seems plausible. It is also possible that species currently known only from *Protea* infructescences may eventually also be isolated from soil samples in the habitats where the hosts occur. The proposed evolutionary migratory pathway for *Ophiostoma* on *Protea* hosts (Roets et al. 2009b) may be a reflection of an underlying soil-dictated pattern and not because of movement on the *Protea* host species alone. Studies focused on intensive soil sampling from a range of different *Ophiostoma* host-*Protea* localities are required to corroborate this hypothesis.

Various *Protea*-associated *Ophiostoma* species are primarily dispersed by mites, while beetles play an intermediary role and carry the mites between infructescences (Roets et al. 2007, 2009a). It has also been shown that these mites may have a mutualistic association with their fungal partners, as they can feed and reproduce on a diet consisting solely of *Ophiostoma* (Roets et al. 2007). It is not known whether mites also disperse *O. protea-sedis* and *O. zambiensis* in Zambia, but this seems likely, because the closely related *O. gemellus*, *O. palmiculminatum* and *O. splendens* have confirmed relationships with mites (Roets et al. 2007, 2009a). Identifying the mites and also the secondary dispersal agents of these fungi (beetles) will lead to a more comprehensive understanding of the co-evolution between these organisms.

The dispersal of soil-inhabiting *Ophiostoma* species has not yet been investigated, but a large diversity of soil-borne mites may well be involved. Should this be verified, mites may well have facilitated a jump from a soil niche to the *Protea* infructescence niche. Such a jump could have occurred either early in the evolutionary history of the fungi with subsequent speciation on the host genus (Roets et al. 2009b) or, if these species are also detected from soil samples, contentious current movement between soil and infructescences is likely. This could then provide an alternative explanation of how the isolated *Protea* infructescence niche could be re-populated with *Ophiostoma* species in the first flowering season after fire, other than by long-distance dispersal facilitated by insects (Roets et al. 2009a).

Future studies should focus on clarifying natural host ranges and number of *Ophiostoma* species found in the unusual *Protea*-infructescence niche. It is likely that more cryptic species remain to be discovered when new hosts and hosts from a wider geographical range are evaluated. Future studies should also include the evaluation of soil and other niches for the presence of these fungi in order to elucidate the evolutionary origin of *Protea*-associated *Ophiostoma*. Investigations into the ecology of these fungi, focussed on the interactions between specific fungi, their mite and beetle vectors and their influence on *Protea* ecology will undoubtedly be a fruitful field for future study.

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