

# Multi-gene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences

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**Abstract:** *Ophiostoma* represents a genus of fungi that are mostly arthropod-dispersed and have a wide global distribution. The best known of these fungi are carried by scolytine bark beetles that infest trees, but an interesting guild of *Ophiostoma* spp. occurs in the infructescences of *Protea* spp. native to South Africa. Phylogenetic relationships between *Ophiostoma* spp. from *Protea* infructescences were studied using DNA sequence data from the  $\beta$ -tubulin, 5.8S ITS (including the flanking internal transcribed spacers 1 and 2) and the large subunit DNA regions. Two new species, *O. phasma* sp. nov. and *O. palmiculminatum* sp. nov. are described and compared with other *Ophiostoma* spp. occurring in the same niche. Results of this study have raised the number of *Ophiostoma* species from the infructescences of serotinous *Protea* spp. in South Africa to five. Molecular data also suggest that adaptation to the *Protea* infructescence niche by *Ophiostoma* spp. has occurred independently more than once.

**Taxonomic novelties:** *Ophiostoma phasma* Roets, Z.W. de Beer & M.J. Wingf. sp. nov., *Ophiostoma palmiculminatum* Roets, Z.W. de Beer & M.J. Wingf. sp. nov.

**Key words:**  $\beta$ -tubulin, ITS, LSU, *Ophiostoma*, phylogeny, *Protea*.

## INTRODUCTION

The southern tip of Africa is recognised for its floral diversity, accommodating the world's smallest floral kingdom that is commonly referred to as the Fynbos. The Fynbos Biome is a major constituent of the Cape Floristic Region (CFR) in which approximately 9000 vascular plant species (ca. 44 % of the southern African flora) are found (Arnold & De Wet 1993, Cowling & Hilton-Taylor 1997, Goldblatt & Manning 2000). Amongst these plants, the CFR also includes approximately 330 species of *Proteaceae* in 14 genera, 10 of which are endemic to the region (Rebello 1995, Rourke 1998). Members of the *Proteaceae*, including the genus *Protea* (proteas), commonly dominate plant communities of the Fynbos Biome (Fig. 1A) (Cowling & Richardson 1995). The *Proteaceae* are not only ecologically significant, but provide the basis for the South African protea cut-flower industry that generates an annual income of more than US \$ 10 million (Anon. 1999, Crous *et al.* 2004).

Florets of *Protea* spp. are arranged in inflorescences. After a bud stage that can last for several months (Fig. 1B), the inflorescences will open to reveal the often brightly coloured involucre bracts that attract many insect and bird pollinators (Fig. 1C–G). After pollination, the involucre bracts close, forcing the florets together in compact infructescences (Fig. 1H–J). The infructescence may persist on the plants for several years, and act as an above-ground seed bank (Bond 1985) that opens to release seeds after a fire (Rebello 1995). During this time, the infructescences are colonised by many different arthropods (Myburg

*et al.* 1973, 1974, Myburg & Rust 1975a, b, Coetzee & Giliomee 1985, 1987a, b, Coetzee 1989, Wright 1990, Visser 1992, Roets *et al.* 2006a) and micro-fungi (Marais & Wingfield 1994, Lee *et al.* 2003, 2005), some of which are specific to their *Protea* hosts.

Three species of *Ophiostoma* Syd. & P. Syd. have been described from *Protea* infructescences in South Africa, showing varying degrees of host specificity. *Ophiostoma africanum* G.J. Marais & M.J. Wingf. is reportedly specific to its *P. gagedi* host (Marais & Wingfield 2001), while *O. protearum* G.J. Marais & M.J. Wingf. is confined to the infructescences of *P. caffra* (Marais & Wingfield 1997). *Ophiostoma splendens* G.J. Marais & M.J. Wingf., in contrast, has been reported from *P. repens*, *P. neriifolia*, *P. laurifolia*, *P. lepidocarpodendron*, and *P. longifolia* (Marais & Wingfield 1994, Roets *et al.* 2005). All three species are characterised by *Sporothrix* Hekt. & C.F. Perkins anamorphs, tolerance to high levels of the antibiotic cycloheximide and contain rhamnose in their cell walls (Marais *et al.* 1998).

Wingfield *et al.* (1999) suggested that the *Ophiostoma* spp. from proteas possibly reside in a discrete genus of the *Ophiostomatales*. This observation was based on the marked differences between these species and *O. piliferum* (Fr.) Syd. & P. Syd., the type species of *Ophiostoma*. A recent study (Zipfel *et al.* 2006) has, however, confirmed that the *Protea*-associated species reside in the *O. stenoceras* (Robak) Nannf. clade of *Ophiostoma*.

The present study aimed to determine the phylogenetic relationships of the three known *Protea*-associated *Ophiostoma* spp., using ribosomal ITS and partial  $\beta$ -tubulin gene sequences. We also reconsidered



**Fig. 1.** Growth habit and flower phenology of *Protea* species. A. Natural Fynbos landscape dominated by *Protea repens*. B. Flower-bud stage of *P. cynaroides*. C. Flowering stage of *P. repens*. D. Flowering stage of *P. eximia*. E. Inflorescence of *P. scolymocephala*. F. Inflorescence of *P. repens* showing visiting pollinators (*Apis mellifera capensis*, Hymenoptera: Apidae). G. Inflorescence of *P. repens* (ca. 4-month-old) showing tightly packed florets and undamaged involucrel receptacle. H. Infructescences of *P. repens*. I. Same, opened to show tightly packed florets and undamaged involucrel receptacle. J. Same, showing damage by insect larvae boring into involucrel receptacle.

the phylogenetic position of these species at the generic level using ribosomal large subunit (LSU) data. The study included *Ophiostoma* spp. described from proteas in previous studies, as well as new isolates collected from *Protea* spp. from a wider geographical range than that considered previously.

## MATERIALS AND METHODS

### Isolates

Infructescences of various *Protea* spp. were collected from different sites in South Africa between Feb. 2003 and Jun. 2005, and examined for the presence of *Ophiostoma* spp. Ascospores were removed from the apices of ascomatal necks with a small piece of agar attached to the tip of a dissecting needle and transferred to 2 % malt extract agar (MEA; Biolab, Midrand, South Africa) amended with 0.05 g/L cycloheximide (Harrington 1981). Once purified, all cultures were maintained on Petri dishes containing MEA at 4 °C. Representative cultures of all species (Table 1) have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Herbarium specimens of both the teleomorph and anamorph states of the new species have been deposited in the National Fungus Collection (PREM), Pretoria, South Africa (Table 1).

### Microscopy

Perithecia of *Ophiostoma* spp. collected from within the *Protea* infructescences, and conidiophores and conidia of the *Sporothrix* anamorphs formed in culture, were mounted on microscope slides in clear lactophenol. Specimens were studied using a Nikon SMZ800 dissecting microscope and a Nikon Eclipse E600 light microscope with differential interference contrast (DIC). Photos were taken with a Nikon DXM1200 digital camera mounted on the microscopes. Measurements (25) of each taxonomically useful structure were made and means ( $\pm$  standard deviation) calculated.

### Growth in culture

The growth of the unidentified species was determined by transferring a 5 mm diam piece of mycelium-covered agar from the edges of actively growing 1-wk-old cultures to the centre of fresh Petri dishes containing 20 mL MEA. Plates were incubated at a range of temperatures between 5–35 °C with 5 °C intervals. Three replicate plates were used for each temperature interval and colony diameters (two per plate) were determined after 2 d and again after 10 d of growth in the dark. The mean difference between growth diameter at 2 and 10 d was determined ( $\pm$  standard deviation) for each species.

Tolerance of the unidentified species to cycloheximide was tested by transferring a 5 mm diam piece of agar containing fungal mycelia and conidia to MEA plates containing varying concentrations of cycloheximide (0,

0.05, 0.1, 0.5, 1.0 and 2.5 g/L). The colony diameter of three replicate plates per tested concentration was calculated as described for the study of growth at different temperatures after incubation at 25 °C in the dark for 10 d.

### DNA extraction, amplification and sequencing

Mycelium was collected for DNA extraction by scraping the surface of the agar plates with a sterile scalpel. Genomic DNA from fungal mycelium was extracted using a Sigma GenElute™ plant genomic DNA miniprep kit (Sigma-Aldrich Chemie CMBH, Steinheim, Germany) according to the manufacturer's instructions.

The following primers were used for amplification: LR0R and LR5 for nuclear LSU rDNA (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>), ITS1–F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) for the ITS and 5.8S regions. PCR reaction volumes for the rDNA amplifications were 50  $\mu$ L consisting of: 32.5  $\mu$ L ddH<sub>2</sub>O, 1  $\mu$ L DNA, 5  $\mu$ L (10 $\times$ ) reaction buffer (Super-Therm, JMR Holdings, U.S.A.), 5  $\mu$ L MgCl<sub>2</sub>, 5  $\mu$ L dNTP (10 mM of each nucleotide), 0.5  $\mu$ L (10 mM) of each primer and 0.5  $\mu$ L Super-Therm Taq polymerase (JMR Holdings, U.S.A.). DNA fragments were amplified using a Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). PCR reaction conditions were: an initial denaturation step of 2 min at 95 °C, followed by 35 cycles of: 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 1 min elongation at 72 °C. The PCR process terminated with a final elongation step of 8 min at 72 °C.

Reaction mixtures to amplify part of the  $\beta$ -tubulin gene region were the same as for ribosomal DNA, except that 1.5  $\mu$ L DNA, 32  $\mu$ L of ddH<sub>2</sub>O and primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) were used. The amplification protocol for  $\beta$ -tubulin was as follows: initial denaturation for 4 min at 95 °C, 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1.5 min, elongation at 72 °C for 1 min, and a termination step of 7 min at 72 °C.

All amplified PCR products were cleaned using the Wizard® SV gel and PCR clean up system (Promega, Madison, Wisconsin, U.S.A.) according to the manufacturer's instructions. The purified fragments were sequenced using the PCR primers and the Big Dye™ Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.). The fragments were analysed on an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems).

### Analysis of sequence data

LSU sequences obtained in this study (Table 1) were compared to sequences of species of *Ophiostoma* and related genera from the study of Zipfel *et al.* (2006). ITS and partial  $\beta$ -tubulin sequences from the present study (Table 1) were compared with sequences of closely related *Ophiostoma* spp. from previous studies (De Beer *et al.* 2003, Aghayeva *et al.* 2004, 2005). Sequences were aligned using Clustal X v. 1.81.

Table 1. Fungal isolates and herbarium specimens obtained from *Protea* spp. and used in this study.

Species identity	Isolate no.		Herbarium	Host	Geographical origin	Collector	GenBank accession no.		
	CBS	CMW					LSU	ITS	$\beta$ -tubulin
<i>Ophiostoma africanum</i>	CBS 116571	CMW 823		<i>Protea gagedi</i>	Unknown	M.J. Wingfield	AF221015	DQ316199	DQ296073
	CBS 116374	CMW 1822		<i>P. dracomontana</i>	Cathedral Peak, KZ-Natal	M.J. Wingfield		DQ316197	DQ316159
	CBS 116376	CMW 1812		<i>P. dracomontana</i>	Cathedral Peak, KZ-Natal	M.J. Wingfield		DQ316198	DQ316160
	CBS 116566	CMW 1104		<i>P. caffra</i>	Irene, Gauteng	Unknown	DQ316147	DQ316200	DQ316162
	CBS 119590	CMW 20677	PREM58942 <sup>H</sup>	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316143	DQ316191	DQ316153
	CBS 119591	CMW 20693	PREM58949 <sup>P</sup>	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316192	DQ316154
	CBS 119592	CMW 20694	PREM58950 <sup>P</sup>	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316144	DQ316193	DQ316155
		CMW 20695		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316194	DQ316156
		CMW 20696		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316195	DQ316157
		CMW 20697	PREM58951 <sup>P</sup>	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316152	DQ316196	DQ316158
		CMW 20698		<i>P. laurifolia</i>	Gifberg top, SW Cape	F. Roets		DQ316222	DQ316184
		CMW 20699		<i>P. laurifolia</i>	Gifberg top, SW Cape	F. Roets		DQ316220	DQ316182
<i>O. phasma</i>	CBS 119722	CMW 20681	PREM58943 <sup>P</sup>	<i>P. nerifolia</i>	Jonkershoek, SW Cape	F. Roets		DQ316216	DQ316178
	CBS 119589	CMW 20682	PREM58944 <sup>P</sup>	<i>P. nerifolia</i>	Jonkershoek, SW Cape	F. Roets		DQ316217	DQ316179
		CMW 20683	PREM58945 <sup>P</sup>	<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316227	DQ316189
		CMW 20684		<i>P. laurifolia</i>	Piekenierskloof, SW Cape	F. Roets		DQ316218	DQ316180
	CBS 119721	CMW 20676	PREM58941 <sup>H</sup>	<i>P. laurifolia</i>	JS Marais Park, SW Cape	F. Roets	DQ316151	DQ316219	DQ316181
		CMW 20686		<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316223	DQ316185
		CMW 20687		<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316221	DQ316183
		CMW 20688		<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316224	DQ316186
	CBS 119588	CMW 20689	PREM58946 <sup>P</sup>	<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316225	DQ316187
		CMW 20690	PREM58947 <sup>P</sup>	<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316226	DQ316188
		CMW 20692	PREM58948	<i>P. nerifolia</i>	Jonkershoek, SW Cape	F. Roets		DQ316228	DQ316190
	<i>O. protearum</i>	CBS 116654	CMW 1107		<i>P. caffra</i>	Irene, Gauteng	M.J. Wingfield	DQ316145	DQ316201
CBS 116567		CMW 1103		<i>P. caffra</i>	Irene, Gauteng	M.J. Wingfield		DQ316203	DQ316165
CBS 116568		CMW 1102		<i>P. caffra</i>	Irene, Gauteng	M.J. Wingfield	AF221014	DQ316202	DQ296072
<i>O. splendens</i>		CMW 20679		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316150	DQ316212	DQ316174
		CMW 20680		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316211	DQ316173
		CMW 20685		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316213	DQ316175
		CMW 20691		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316209	DQ316171
		CMW 20678		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316210	DQ316172
	CMW 20674		<i>P. repens</i>	George, SW Cape	F. Roets		DQ316204	DQ316166	

Table 1. (Continued).

Species identity	Isolate no.		Herbarium	Host	Geographical origin	Collector	GenBank accession no.		
	CBS	CMW					LSU	ITS	$\beta$ -tubulin
<i>O. splendens</i>		CMW 20675		<i>P. repens</i>	George, SW Cape	F. Roets		DQ316205	DQ316167
	CBS 116379	CMW 896		<i>P. repens</i>	Unknown	M.J. Wingfield		DQ316207	DQ316169
	CBS 116569	CMW 872		<i>P. repens</i>	Unknown	M.J. Wingfield	AF221013	DQ316215	DQ296071
	CBS 116377	CMW 873		<i>P. repens</i>	Unknown	M.J. Wingfield		DQ316214	DQ316176
		CMW 2753		<i>P. nerifolia</i>	Sir Lowrey's Pass, SW Cape	G. Marais		DQ316206	DQ316168
	CBS 116378		Unknown	Unknown	Unknown		DQ316208	DQ316170	

<sup>H</sup>Holotype.<sup>P</sup>Paratype.

**Maximum parsimony:** One thousand random stepwise addition heuristic searches were performed using the software package PAUP v. 4.0 beta 10 (Swofford 2000) with Tree Bisection-Reconnection (TBR) on and 10 trees saved per replicate. Internal node support was assessed using the bootstrap algorithm (Felsenstein 1985), with 1000 replicates of simple taxon addition.

**Neighbour-joining:** Relationships between taxa were determined using distance analysis in PAUP. Evolutionary models for the respective data sets were determined based on AIC (Akaike Information Criteria) using the Modeltest 3.06 (Posada & Crandall 1998). Selected evolutionary models were: GTR+I+G (proportion invariable sites 0.6899 and rates for variable sites following a gamma distribution with shape parameter of 1.0185) for LSU, TrN+I+G (proportion invariable sites 0.4213 and rates for variable sites following a gamma distribution with shape parameter of 0.6253) for ITS, and HKY+G (rates for variable sites following a gamma distribution with shape parameter of 0.1783) for  $\beta$ -tubulin. Trees were constructed using the neighbour-joining tree-building algorithm (Saitou & Nei 1987) and statistical support was determined by 1000 NJ bootstrap replicates.

**Bayesian inference:** Data were analysed using Bayesian inference based on a Markov chain Monte Carlo (MCMC) approach in the software package MrBayes v. 3.1.1 (Ronquist & Huelsenbeck 2003). The most parameter-rich model available in MrBayes, GTR+I+G (shape parameter using 4 rate categories) was used for the analysis. All parameters were inferred from the data. Two independent Markov chains were initiated from a random starting tree. Runs of 1 million generations with a sample frequency of 50 were implemented. Burn-in trees (first 20000 generations) were discarded and the remaining trees from both runs were pooled into a 50 % majority rule consensus tree.

## RESULTS

### Isolates

A total of 38 isolates obtained from proteas were included in this study, with 12 isolates from five *Protea* spp. derived from previous collections by Wingfield and Marais (Table 1). The remaining 26 isolates were obtained from three *Protea* spp. in surveys that formed part of this study.

### Microscopy

Among all isolates studied, five groups could be distinguished based on morphology. Three of these groups included isolates of the three *Ophiostoma* spp. previously described from *Protea* infructescences. No recent isolates were added to this group, except for seven isolates of *O. splendens* that came from the same host, *P. repens*. Some old isolates of *O. africanum* from *P. dracomontana* and *P. caffra* were newly identified.

The remaining isolates collected resided in two clear morphological groups that did not resemble any of the three *Ophiostoma* species described from proteas, or any other *Ophiostoma* species. Isolates in the one group were commonly collected on the styles of *P. neriifolia* and *P. laurifolia*. The fungus often occurred sympatrically with *Gondwanamyces capensis* (M.J. Wingf. & P.S. van Wyk) G.J. Marais & M.J. Wingf. Isolates representing the second morphological group were found only in the insect-damaged involucre receptacles of *Protea repens* (Fig. 1J).

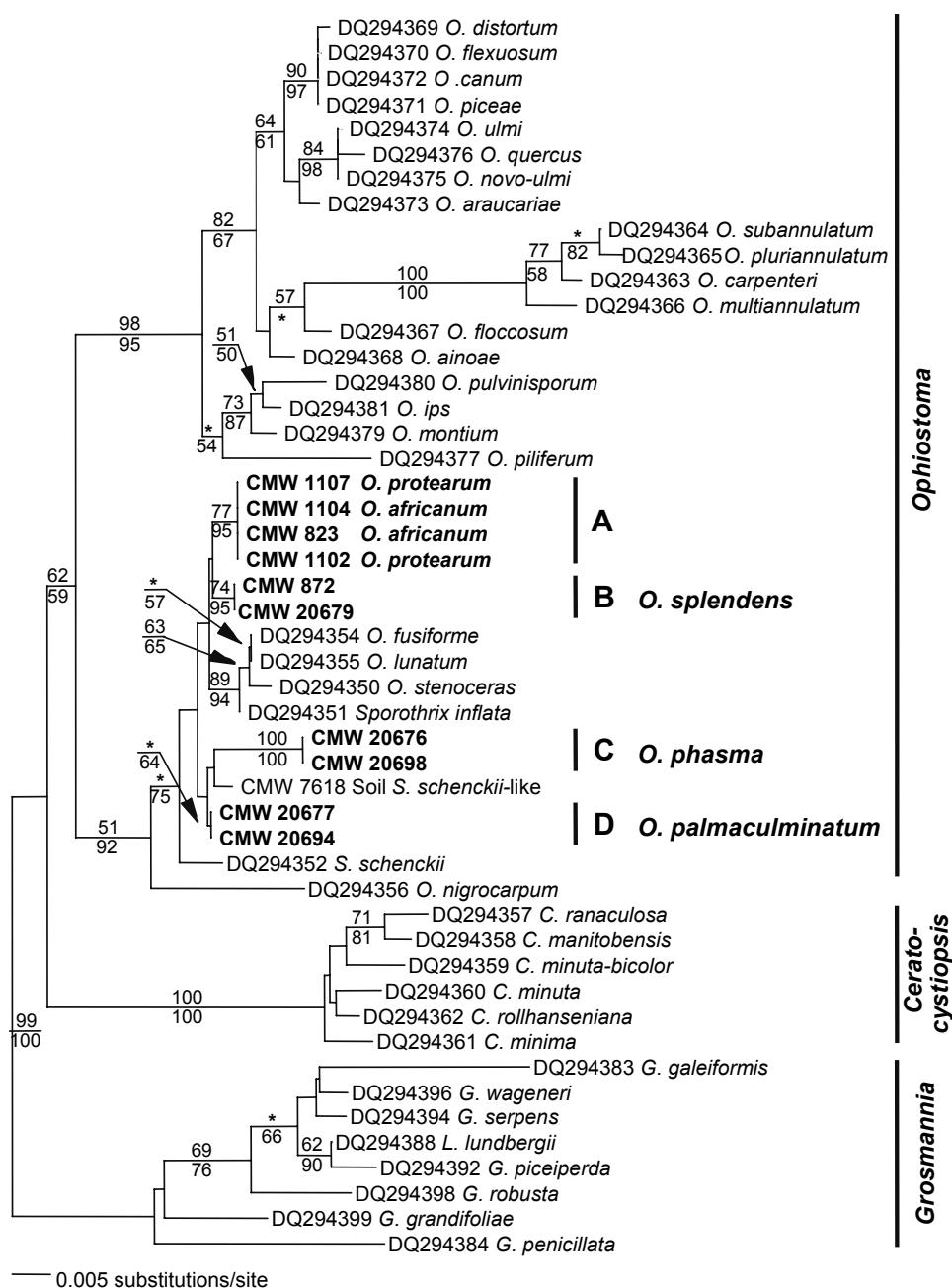
**Growth in culture**

Isolates of both the unknown *Ophiostoma* species showed optimum growth at 30 °C. Mean colony diameter for the species collected from *P. repens* was

26 mm (± 1), while the species from *P. neriifolia* and *P. laurifolia* had a colony diameter of 18 mm (± 1) at this temperature after 8 d in the dark. Both of the unknown *Ophiostoma* species were tolerant to cycloheximide and were able to grow on all tested concentrations of this antibiotic. Mean colony diameter for the species collected from *P. repens* declined from 27 mm (± 1) on 0.05 g/L to 17 mm on 2.5 g/L cycloheximide. Mean colony diameter for the species from *P. neriifolia* and *P. laurifolia* declined from 20 mm (± 1) on 0.05 g/L to 12 mm (± 1) on 2.5 g/L cycloheximide.

**Phylogenetic analysis**

Alignment of the amplified products with Clustal X resulted in data sets of 709 characters for LSU, 531 characters for ITS, and 307 characters for part of the



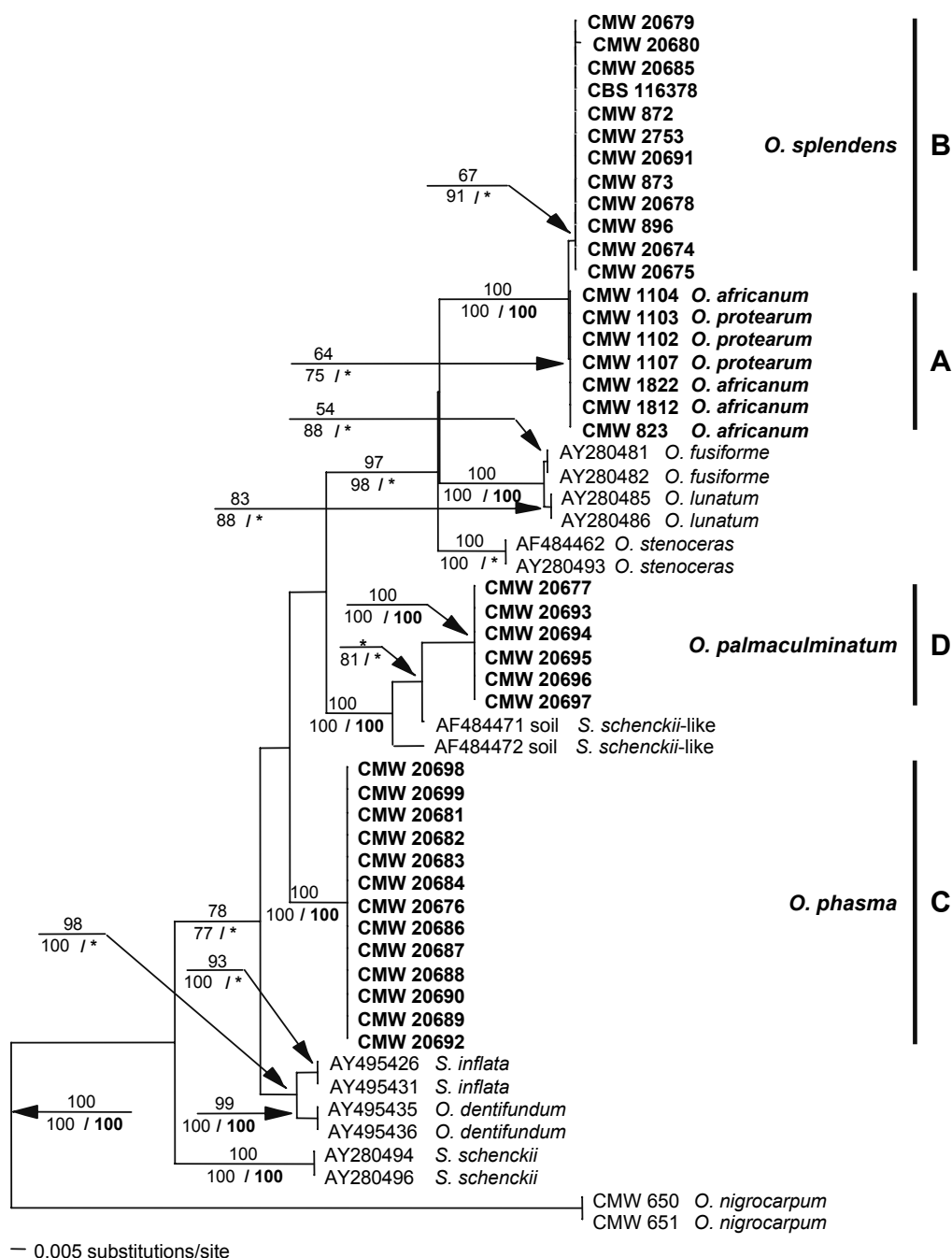
**Fig. 2.** Distance dendrogram obtained with the GTR+I+G parameter model (G = 1.0185) for the partial 28S rDNA data set. Values above nodes indicate parsimony-based bootstrap values (1000 replicates). Values below nodes indicate bootstrap values (1000 replicates) obtained from neighbour-joining analysis. \* = value lower than 50 %.

$\beta$ -tubulin gene. Placement of isolates in the resulting trees based on phylogenetic analyses for each gene region was similar. For all three gene regions, the trees presented (Figs 2–4) were obtained from neighbour-joining analyses.

For the LSU region there were 98 parsimony-informative characters, 611 parsimony-uninformative characters, and 581 constant characters. For the ITS region there were 98 parsimony-informative characters, 433 parsimony-uninformative characters, and 389 constant characters. For the  $\beta$ -tubulin region there were 112 parsimony-informative characters, 195 parsimony-uninformative characters, and 194 constant characters.

Analysis using the parsimony algorithm yielded 38, 9990 and 9530 equally most parsimonious trees of 291, 234 and 268 steps long for the LSU, ITS and  $\beta$ -tubulin data sets respectively. The Consistency Indices were 0.765, 0.533 and 0.705, while the Retention Indices were 0.957, 0.856 and 0.940 for the ITS, LSU and  $\beta$ -tubulin regions, respectively. Apart from group C [(Fig. 2), (PP 1.0)], PP values obtained for LSU were not statistically significant for the groups of interest and were omitted.

Trees obtained using different analyses of the LSU data resembled each other, and only the neighbour-joining tree (Fig. 2) is presented. The five taxa from

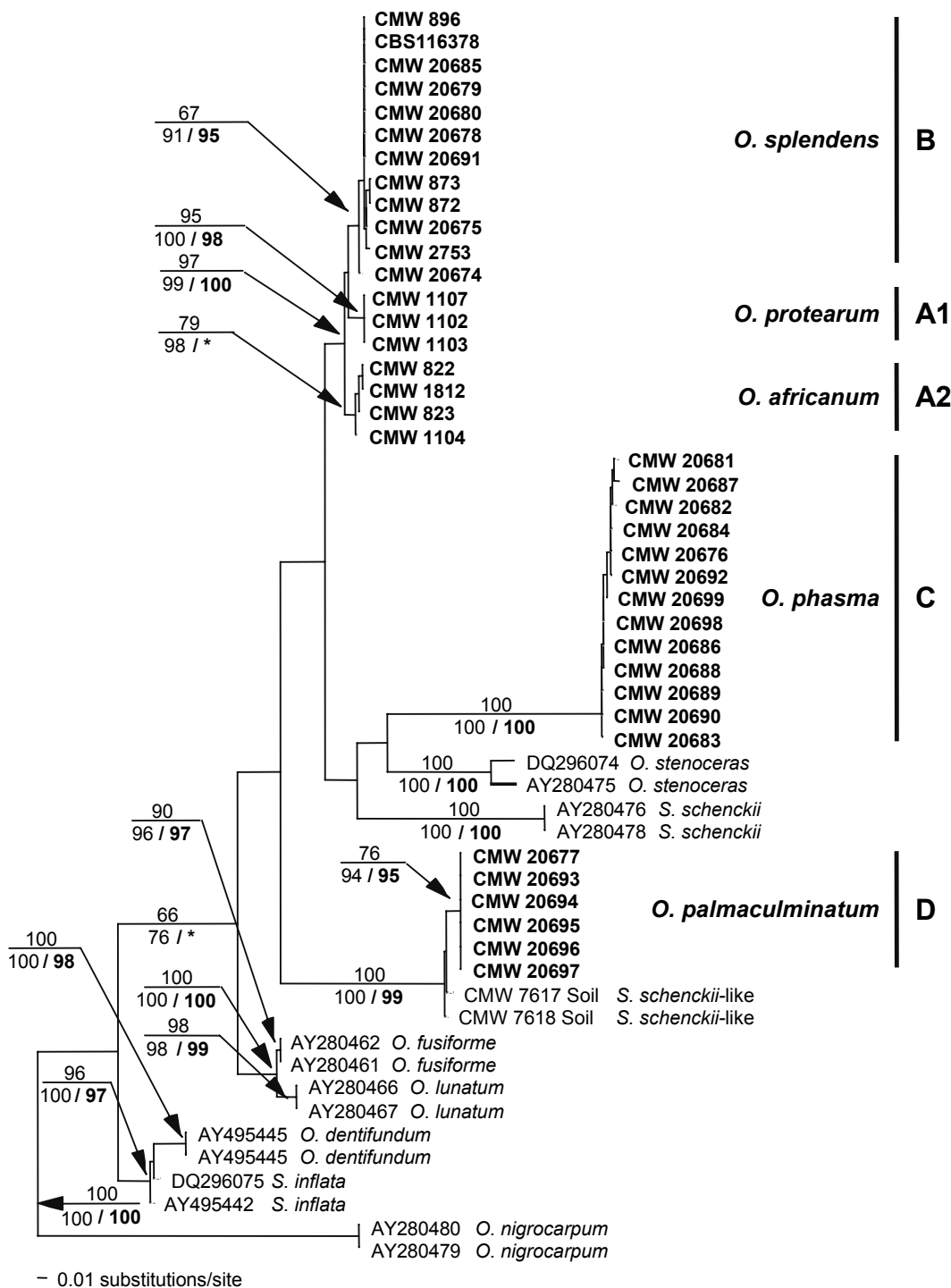


**Fig. 3.** Distance dendrogram obtained with the TrN+I+G parameter model ( $G = 0.6253$ ) on the 5.8S (including the flanking internal transcribed spacers 1 & 2) data set. Values above nodes indicate bootstrap values (1000 replicates) obtained by parsimony-based methods. Non-bold typeface values below nodes indicate bootstrap values (1000 replicates) obtained from NJ/UPGMA analysis. Values in bold typeface represent confidence values (posterior probabilities as percentage) obtained through Bayesian inference. \* = value lower than 50 % (= value lower than 95 % for Bayesian analysis).

proteas formed four distinct, well-supported groups (A–D). These groups did not form a monophyletic lineage, but were distributed among various species of the *O. stenoceras* complex in the genus *Ophiostoma*. The LSU data did not distinguish between *O. protearum* and *O. africanum*, which formed a single group [(A), (Fig. 2)]. Based on these analyses, two isolates of *O. nigrocarpum* were selected as outgroup for the more focused ITS and  $\beta$ -tubulin analyses.

Analyses of the ITS data (Fig. 3) confirmed the topology of the LSU tree. The protea isolates formed

four well-supported groups (A–D), with isolates of *O. protearum* and *O. africanum* grouping together (group A) similar to the outcome of the LSU sequence comparisons. The topology of the tree arising from analyses of part of the  $\beta$ -tubulin gene region (Fig. 4) differed from both the LSU and ITS trees (Figs 2–3). Groups B–D remained well-resolved with strong bootstrap support, but group A was sub-divided into two distinct, well-supported sub-groups, representing *O. protearum* (group A1) and *O. africanum* (group A2), respectively.



**Fig. 4.** Distance dendrogram obtained with the HKY+G parameter model (G = 0.1783) on the partial  $\beta$ -tubulin data set. Values above nodes indicate bootstrap values (1000 replicates) obtained by parsimony-based methods. Non-bold typeface values below nodes indicate bootstrap values (1000 replicates) obtained from NJ analyses. Values in bold typeface represent confidence values (posterior probabilities as percentage) obtained through Bayesian inference. \* = value lower than 50 % (= value lower than 95 % for Bayesian analysis).



## TAXONOMY

Phylogenetic and morphological differences distinguished two groups of *Ophiostoma* isolates from each other as well as from the three *Ophiostoma* species previously described from the infructescences of *Protea* spp. Isolates in these groups were also distinct from other closely related *Ophiostoma* spp. The fungi residing in these two morphologically and phylogenetically distinct groups are described as new species as follows:

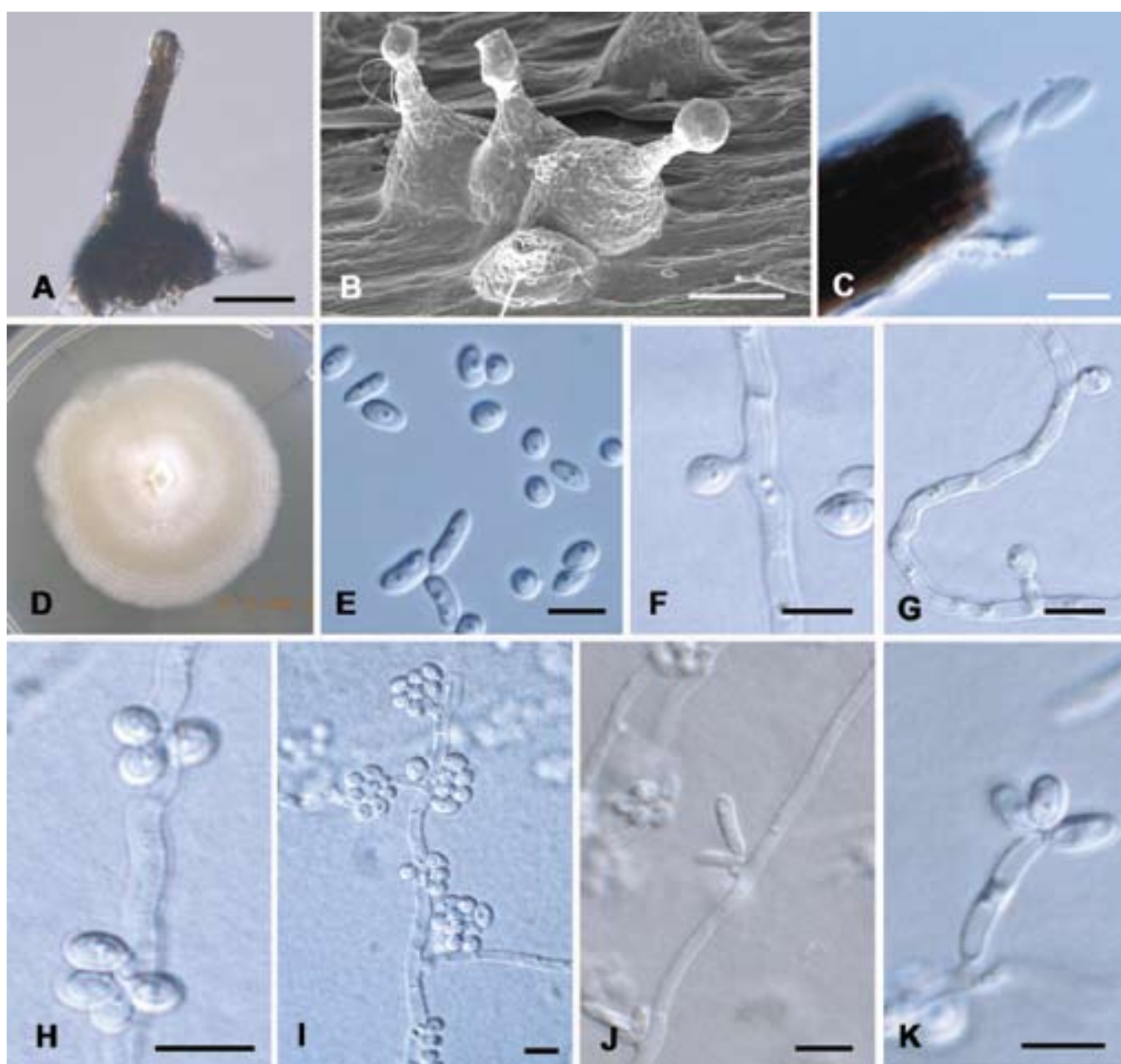
***Ophiostoma phasma*** Roets, Z.W. de Beer & M.J. Wingf., **sp. nov.** MycoBank MB500684. Fig. 5.  
Anamorph: *Sporothrix* sp.

**Etymology:** The epithet *phasma* (*phasma* = ghost)

refers to the small and inconspicuous perithecia growing within a cryptic habitat.

Ascomata superficialia, basi depressa globosa, atra, nuda, 35–70 µm diam, collo atro, 20–60 x 15–25 µm, sursum ad 10–15 µm angustato, hyphae ostiolaris absentes. Asci evanescentes. Ascospores allantoideae, unicellulares, hyalinae, vagina gelatinosa carentes, 4–6 x 2 µm, aggregatae electrinae. Anamorphe *Sporothrix* sp., conidiis ellipsoideis vel clavatis, 5–8 x 2–3 µm.

*Ascomata* superficial on the host substrate, bases depressed–globose, wider at base, black without hyphal ornamentation, 35–70 (51 ± 8) µm diam; necks black, 20–60 (42 ± 10) µm long, 15–25 (19 ± 3) µm wide at the base, 10–15 (11 ± 2) µm wide at the apex, ostiolar hyphae absent (Fig. 5A–C). *Asci* evanescent. *Ascospores* allantoid, aseptate, hyaline, sheaths absent, 4–6 (5 ± 1) µm, 2 µm (Fig. 5C), accumulating in a hyaline gelatinous droplet at the apex of the neck,



**Fig. 5.** Micrographs of *Ophiostoma phasma*. A. Perithecium removed from the style of *Protea neriifolia*. B. Electronmicrograph of sporulating perithecia on *P. laurifolia* host tissue. C. Ascospores at the tip of perithecial neck. D. Two-week-old colony of the *Sporothrix* anamorph on MEA. E. Conidia. F–K. Conidia arising directly from hyphae and short conidiophores. Scale bars A, B = 30 µm; C = 5 µm; E–K = 3 µm.

becoming amber-coloured when dry.

**Colonies** on malt extract agar 22 µm (± 1) mm diam in 8 d at 25 °C in the dark, white to cream-coloured, effuse, circular with an entire edge, surface smooth becoming mucoid, with a distinctive soapy odour, hyphae semi-immersed (Fig. 5D). Growth reduced at temperatures below and above the optimum of 30 °C. Sporulation profuse on MEA. *Conidiogenous cells* arising directly from hyphae on the surface of the agar and from aerial conidiophores, proliferating sympodially, hyaline (Fig. 5F–K). *Conidia* holoblastic and hyaline and of two forms, one ellipsoidal to clavate, smooth, thin-walled, 5–8 x 2–3 µm (Fig. 5E) and the other globose to obovate, smooth, thin-walled, 3–5 x 2–3 µm (Fig. 5E). Conidia forming singly, but aggregating into slimy masses, often also produced directly on hyphae (5H–I).

**Substrate:** Confined to the dead styles and petals of florets within serotinous infructescences of *Protea* spp.

**Distribution:** South Africa, Western Cape Province.

**Specimens examined:** **South Africa**, Western Cape Province, Stellenbosch, Jan S. Marais Park, on *Protea laurifolia*, Jun. 2005, F. Roets, **holotype** PREM 58941, culture ex-type CMW 20676 = CBS 119721; Stellenbosch, Jonkershoek NR, on *P. neriifolia*, May 2004, F. Roets, **paratype** PREM 58943, culture ex-paratype CMW 20681 = CBS 119722; Bainskloof Pass, on *P. laurifolia*, Aug. 2004, F. Roets, **paratype** PREM 58946, culture ex-paratype CMW 20689 = CBS 119588; Stellenbosch, Jonkershoek NR, on *P. neriifolia*, Jul. 2004, F. Roets, **paratype** PREM 58944, culture ex-paratype CMW 20682 = CBS 119589; Giffberg top, on *P. laurifolia*, Jun. 2005, F. Roets, culture CMW 20698; Giffberg top, on *P. laurifolia*, Jun. 2005, F. Roets, culture CMW 20699; Bainskloof Pass, on *P. laurifolia*, Aug. 2004, F. Roets, PREM 58945, culture CMW 20683; Piekenierskloof Pass, Aug. 2004, on *P. laurifolia*, F. Roets, culture CMW 20684; Jonkershoek NR, Aug. 2004, on *P. neriifolia*, F. Roets, PREM 58948, culture CMW 20692; Bainskloof Pass, Sep. 2004, on *P. laurifolia*, F. Roets, PREM 58947, culture CMW 20690.

***Ophiostoma palmiculminatum*** Roets, Z.W. de Beer & M.J. Wingf., **sp. nov.** MycoBank MB500685. Fig. 6.

**Anamorph:** *Sporothrix* sp.

**Etymology:** The epithet *palmiculminatum* (*palma* = palm; *culmen* = peak) refers to the palm-like hyphal ornamentation of the ostiolar tip.

Ascomata superficialia, basi globosa, atra, 80–195 µm diam, nonnumquam paucis hyphis circumdata, collo atro, 360–760 x 20–35 µm, sursum ad 10–15 µm angustato, 8–12 hyphis ostiolaribus rectis vel curvatis, hyalinis vel subhyalinis, 10–25 µm longis palmam fingentibus ornato. Asci evanescentes. Ascospores allantoidae, unicellulares, hyalinae, vagina gelatinosa carentes, 3.5–5.5 x 2.0–2.5 µm, aggregatae incoloratae. Anamorphe *Sporothrix* sp., conidii clavatis 3–11 x 1.5–2.5 µm.

**Ascomata** superficial on the host substrate, also produced on agar plates after 2 mo of growth at 25 °C in the dark. Bases globose, black, 80–195 (146 ± 33) µm diam, occasionally with sparse hyphal ornamentation; necks black, 360–760 (569 ± 114) µm long, 20–35 (28 ± 5) µm wide at the base, 10–15 (12 ± 2.5) µm wide at the apex (Fig. 6A–B). 8–12 ostiolar hyphae, straight or slightly curved, hyaline to sub-hyaline, 10–25 (16 ± 5) µm long (Fig. 6C). **Asci** evanescent. **Ascospores** allantoid, aseptate, hyaline, sheaths absent, 3.5–5.5 x

2–2.5 µm (Fig. 6D), collecting in a hyaline gelatinous droplet at the apex of the neck (Fig. 6C), remaining uncoloured when dry.

**Colonies** on MEA reaching 23 mm diam in 8 d at 25 °C in the dark, white to cream-coloured, circular, effuse, with an entire edge and somewhat rough surface, not producing an odour (Fig. 6E). Growth reduced at temperatures below and above the optimum of 30 °C. Sporulation profuse on MEA. *Conidiogenous cells* arising directly from hyphae on the surface of the agar and from aerial conidiophores, proliferating sympodially, hyaline, becoming denticulate (Fig. 6F–G). Denticles 0.5–2 µm (1 ± 0.5) long (Fig. 6G). *Conidia* holoblastic, hyaline, aseptate, clavate, smooth, thin-walled, 3–11 x 1.5–2.5 µm (Fig. 6H). Conidia forming singly, but aggregating in slimy masses, also produced directly on hyphae (Fig. 6I–J).

**Substrate:** Confined to the insect-damaged involucral receptacles of *Protea repens* infructescences.

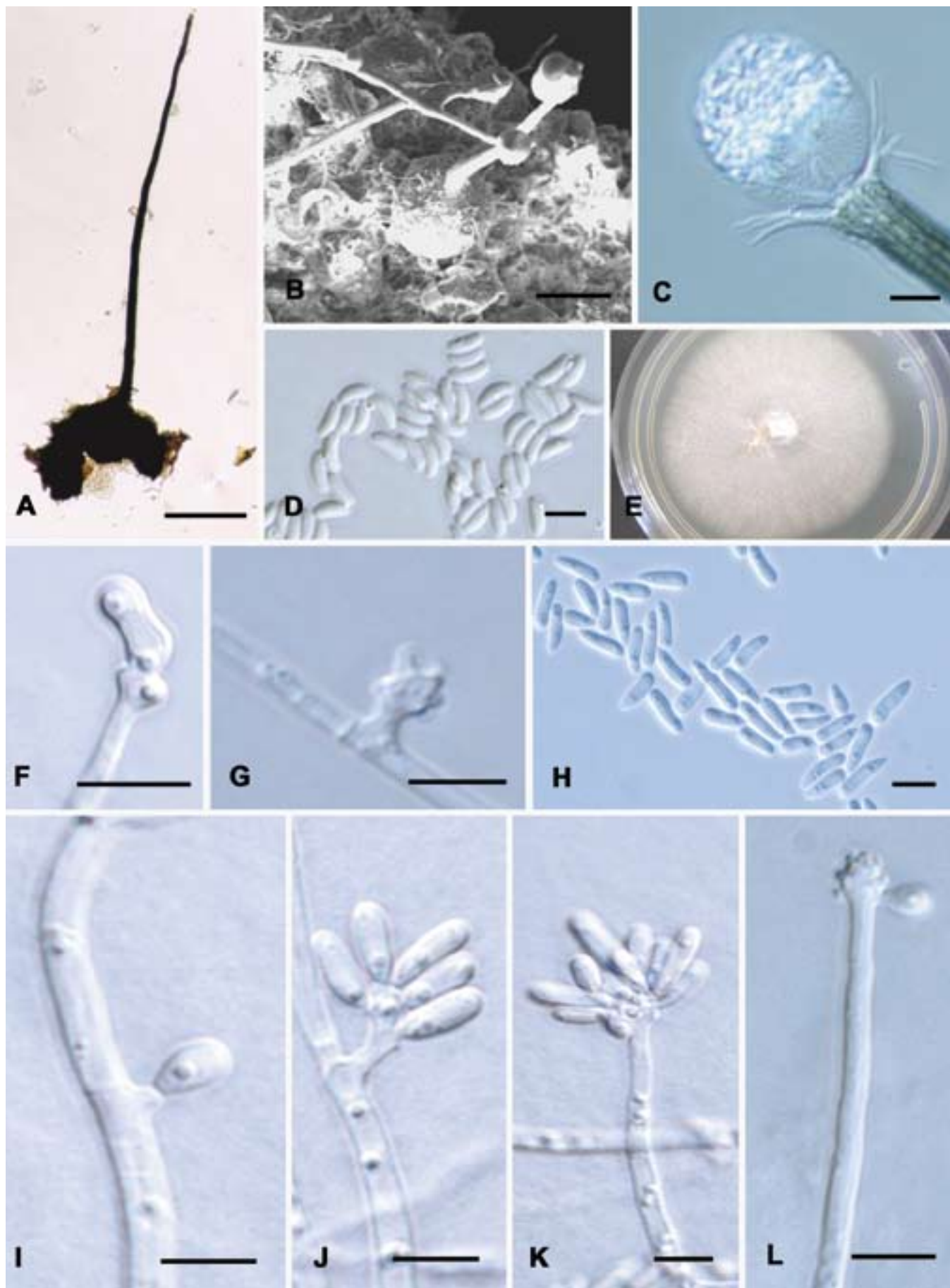
**Distribution:** South Africa, Western Cape Province.

**Specimens examined:** **South Africa**, Western Cape Province, Stellenbosch, Jan S. Marais Park, on *P. repens*, Jun. 2005, F. Roets, **holotype** PREM 58942, culture ex-type CMW 20677 = CBS 119590; Stellenbosch, Jan S. Marais Park, on *P. repens*, Jun. 2005, F. Roets, **paratype** PREM 58949, culture ex-paratype CMW 20693 = CBS 119591; Stellenbosch, Jan S. Marais Park, on *P. repens*, Jun. 2005, F. Roets, **paratype** PREM 58950, culture ex-paratype CMW 20694 = CBS 119592; Stellenbosch, Jan S. Marais Park, on *P. repens*, Jun. 2005, F. Roets, **paratype** PREM 58951, culture ex-paratype CMW 20697 = CBS 119593; Stellenbosch, Jan S. Marais Park, on *P. repens*, Jun. 2005, F. Roets, culture CMW 20695; Stellenbosch, Jan S. Marais Park, on *P. repens*, Jun. 2005, F. Roets, culture CMW 20696.

## DISCUSSION

The infructescences of *Protea* spp. in southern Africa represent a unique and unusual habitat for *Ophiostoma* spp. Their ecology is poorly understood and knowledge of their relatedness to other species of *Ophiostoma* is only just emerging. Phylogenetic analyses of DNA sequence data added substantially to our understanding of the placement of these fungi amongst their close relatives. We have been able to show that *Ophiostoma splendens*, *O. africanum* and *O. protearum*, previously described from *Protea* infructescences, represent well-defined species of *Ophiostoma sensu* Zipfel *et al.* (2006). These three species form a monophyletic lineage within the *O. stenoceras*-complex.

The *Ophiostoma* spp. found in *Protea* infructescences look morphologically similar and in this respect, analyses of DNA sequence data enhance our ability to recognise distinct taxa. Thus, two new *Ophiostoma* spp. are recognized that had probably been overlooked during the period when the first of these fungi were discovered and described. The two new species, *O. phasma* and *O. palmiculminatum*, can easily be distinguished from each other and from the other three *Ophiostoma* spp. occurring in *Protea* infructescences based on DNA sequence comparisons. They are also



**Fig. 6.** Micrographs of *Ophiostoma palmiculminatum*. A. Perithecium. B. Electronmicrograph of sporulating perithecia in tunnels in the base of *P. repens* infructescence created by insect borers. Short basal hyphae can be seen. C. Close-up of perithecial tip showing ostiolar hyphae and ascospores in a sticky mass. D. Ascospores. E. Habit of the *Sporothrix* anamorph on MEA after 2 wk of growth. F, G. Conidiogenous cells showing denticles. H. Conidia. I–J. Conidiogenous cells arising directly from hyphae. K–L. Conidiophores of varying lengths. Scale bars A–B = 100  $\mu$ m; C = 10  $\mu$ m; D = 5  $\mu$ m; F–G = 3  $\mu$ m; H = 5  $\mu$ m; I–L = 3  $\mu$ m.

morphologically distinct from each other and from the other three species, although these differences would have been difficult to define in the absence of DNA sequence comparisons. Results of this study also represent the first report of *O. africanum* from *Protea dracomontana* and *P. caffra*.

Analyses of LSU and ITS sequence data was insufficient to distinguish between *O. africanum* and *O. protearum*. This shows that the two species are very closely related. Analyses of the more variable  $\beta$ -tubulin gene regions, however, support the notion that the two species represent distinct taxa as defined by Marais & Wingfield (2001) based on morphological characters. The close phylogenetic relationship of these species indicates that they share a common ancestor. These affinities may be explained by the fact that they occur in the infructescences of closely related *Protea* spp. that have overlapping geographical distribution ranges (Rebello 1995). *Ophiostoma protearum* appears to be specific to *P. caffra* (Marais & Wingfield 1997, 2001) that is classified in the section *Leiocephalae* and occurs in the eastern and northern provinces of South Africa (Rebello 1995). *Ophiostoma africanum* was previously thought to be specific to *P. gagedi* (Marais & Wingfield 2001), but sequence data from the present study show that it also occurs in the infructescences of *P. dracomontana* and *P. caffra*. Like *P. caffra*, *P. dracomontana* is classified in the section *Leiocephalae*, and the latter species is restricted to the Drakensberg mountain range. This area overlaps with the distribution ranges of both *P. caffra* and *P. gagedi*, although *P. gagedi* is classified in a different section of the genus *Protea*, the *Lasiocephalae* (Rebello 1995).

Phylogenetic analyses of DNA sequences of three gene regions investigated in this study suggest that *O. splendens* is closely related to *O. africanum* and *O. protearum*. *Ophiostoma splendens* has been recorded from *P. repens*, *P. neriifolia*, *P. lepidocarpodendron* and *P. longifolia* in the Western Cape Province (Marais & Wingfield 1994). However, morphological data arising from this study (results not shown) show that all *O. splendens* isolates from non-*P. repens* hosts from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), were misidentified and belong in *Gondwanamyces*. The only exception was one isolate (CMW 2753) collected from *P. neriifolia*. It is suspected that in most of these cases, *O. splendens* was confused with *G. capensis* due to superficial similarities in the teleomorph structures of these species (Marais & Wingfield 1994, Roets *et al.* 2005). We did not isolate *O. splendens* from any *Protea* species other than *P. repens*. Other than the single isolate of *O. splendens* from *P. neriifolia*, the fungus appears to be confined to *P. repens*, which resides in the section *Melliferae*. The explanation for the close phylogenetic relationship between *O. splendens* and its northern counterparts, *O. protearum* and *O. africanum*, will probably only be revealed once a robust phylogeny for the genus *Protea* becomes available.

*Ophiostoma phasma* was isolated from *P. neriifolia* and *P. laurifolia*. Perithecia with features closely resembling those of *O. phasma* were also observed

in the infructescences of *P. lepidocarpodendron* and *P. longifolia*. However, we were not able to isolate *Ophiostoma* spp. from these *Protea* spp. because the perithecia were old and the ascospores appeared not to be viable. Although we were unable to identify the species definitively, we believe that the perithecia in *P. lepidocarpodendron* and *P. longifolia* represent *O. phasma*. It thus appears as if this species is associated with a number of different *Protea* spp. belonging to different sections.

The seemingly wide host range of *O. phasma* in comparison to the restricted host range of *O. splendens* mirrors the situation in *Gondwanamyces*. *Gondwanamyces proteae* is exclusively associated with *P. repens*, whereas *G. capensis* is associated with numerous *Protea* spp. (Wingfield & Van Wyk 1993). Perithecia of *O. phasma* appear to be confined to the styles and petals of florets of the host plant and they were never observed in insect tunnels commonly found in the bases of infructescences. Similar to *O. phasma*, the species *O. protearum*, *O. africanum* and *O. splendens* preferably colonise the styles and petals of florets of their host plants.

*Ophiostoma palmiculminatum* is the only species of *Ophiostoma* and *Gondwanamyces* that has been collected from the tunnels of insects found within the involucre receptacles of *P. repens*. These tunnels are either made by coleopteran or lepidopteran larvae (Coetzee & Giliomee 1987b). The involucre receptacles consist of living tissue, contrasting with the substratum in the *Protea* infructescences. The ability of *O. palmiculminatum* to exclusively exploit this substrate probably results in reduced competition between this species, *O. splendens* and *Gondwanamyces proteae* that can colonise the same infructescence simultaneously (pers. observ.). Whether *O. palmiculminatum* is pathogenic to its host remains to be determined.

*Ophiostoma* spp. produce ascospores in evanescent asci within the bases of their ascomata. The spores are exuded through the necks and carried in sticky masses on the apices of the necks. These morphological characters represent adaptations for arthropod-vectored dispersal (Malloch & Blackwell 1990). In the Northern Hemisphere scolytine bark beetles infesting conifers are the most common vectors of *Ophiostoma* spp. (Wingfield *et al.* 1993, Paine *et al.* 1997, Klepzig & Six 2004). The interactions between the beetles and the fungi may, in some cases, lead to the death of the host plant (Wingfield *et al.* 1993, Paine *et al.* 1997). As a result, many studies have focused on unravelling the complexity of these associations (Six & Paine 1998, 1999, Klepzig *et al.* 2001a, b, Six 2003a, b, Six & Bentz 2003, Klepzig & Six 2004). Based on similarities in morphology, the *Ophiostoma* spp. on proteas appear to share this mode of vectored spore dispersal, and may thus also be involved in mutualistic associations with arthropods. The nature of these multi-organism interactions is currently being investigated.

The large number of insects representing diverse habits complicates these studies and it has been necessary to develop specialised DNA-based tech-

niques to study the vector relationships of *Ophiostoma* spp. from *Proteaceae* (Roets *et al.* 2006b). Preliminary observations have shown that insects are involved, at least occasionally, in transporting spores of *Ophiostoma* spp., and we expect that the discovery of new species of *Ophiostoma* will enhance our understanding of these fungi and the invertebrates that transport them from one *Protea* infructescence to another.

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