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 Southern Africa. Phylogenetic relationships between Ophiostoma spp. from Protea infructescences were studied using DNA sequence data from the β-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: β–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 & Hillary-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 (Rebelo 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 infructescences. After a bud stage that can last for several months (Fig. 1B), the infructescences will open to reveal the often brightly coloured involucral bracts that attract many insect and bird pollinators (Fig. 1C–G). After pollination, the involucral 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 (Rebelo 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 africanaum G.J. Marais & M.J. Wingf. is reportedly specific to its P. gaguëdi 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. nerifolia, 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 Ophiostomatidae. 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 β–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. cynaroides. G. Inflorescence of P. repens showing visiting pollinators (Apis mellifera capensis, Hymenoptera: Apidae). H. Infructescences (ca. 4-mo-old) of P. repens. I. Same, opened to show tightly packed florets and undamaged involucral receptacle. J. Same, showing damage by insect larvae boring into involucral 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 Centaalbureau 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 (± 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 (± 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 LRS 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 µL consisting of: 32.5 µL ddH2O, 1 µL DNA, 5 µL (10×) reaction buffer (Super-Therm, JMR Holdings, U.S.A.), 5 µL MgCl2, 5 µLdNTP (10 mM of each nucleotide), 0.5 µL (10 mM) of each primer and 0.5 µL Super-Therm Taq polymerase (JMR Holdings, U.S.A.). DNA fragments were amplified using a GeneAmp®, 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 β-tubulin gene region were the same as for ribosomal DNA, except that 1.5 µL DNA, 32 µL of ddH2O and primers T10 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995) were used. The amplification protocol for β-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 PRISIM™ 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 β-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       | CBS Isolate no. | CMW Herbarium no. | Host                | Geographical origin       | Collector            | GenBank accession no. | LSU     | ITS     | β-tubulin |
|------------------------|-----------------|--------------------|---------------------|---------------------------|-----------------------|-----------------------|---------|---------|-----------|
| *Ophiostoma africanum* | CBS 116571       | CMW 823            | *Protea gagedi*     | Unknown                   | M.J. Wingfield        | AF221015              | DQ316199| DQ296073|           |
|                        | CBS 116374       | CMW 1822           | *P. dracomontana*   | Cathedral Peak, KZ-Natal  | M.J. Wingfield        | DQ316197              | DQ316159|         |           |
|                        | CBS 116376       | CMW 1812           | *P. dracomontana*   | Cathedral Peak, KZ-Natal  | M.J. Wingfield        | DQ316198              | DQ316160|         |           |
|                        | CBS 116566       | CMW 1104           | *P. caffra*         | Irene, Gauteng            | Unknown               | DQ316147              | DQ316200| DQ316162|           |
| *O. palmiculminatum*   | CBS 119590       | CMW 20677          | PREM58942H          | *P. repens*               | JS Marais Park, SW Cape | F. Roets             | DQ316143| DQ316153|           |
|                        | CBS 119591       | CMW 20693          | PREM58949P          | *P. repens*               | JS Marais Park, SW Cape | F. Roets             | DQ316192| DQ316154|           |
|                        | CBS 119592       | CMW 20694          | PREM58950P          | *P. repens*               | JS Marais Park, SW Cape | F. Roets             | DQ316193| DQ316155|           |
|                        | CMW 20696        |                    | *P. repens*         | JS Marais Park, SW Cape   | F. Roets             | DQ316194             | DQ316156|         |           |
|                        | CBS 119593       | CMW 20697          | PREM58951P          | *P. repens*               | JS Marais Park, SW Cape | F. Roets             | DQ316195| DQ316157|           |
| *O. phasma*            | CMW 20699        |                    | *P. laurifolia*     | Giftberg top, SW Cape     | F. Roets             | DQ316222              | DQ316184|         |           |
|                        | CMW 20699        |                    | *P. laurifolia*     | Giftberg top, SW Cape     | F. Roets             | DQ316220              | DQ316182|         |           |
|                        | CBS 119722       | CMW 20681          | PREM58943P          | *P. neriifolia*           | Jonkershoek, SW Cape  | F. Roets             | DQ316216| DQ316178|           |
|                        | CBS 119589       | CMW 20682          | PREM58944P          | *P. neriifolia*           | Jonkershoek, SW Cape  | F. Roets             | DQ316217| DQ316179|           |
|                        | CMW 20683        |                    | *P. laurifolia*     | Bainskloof, SW Cape       | F. Roets             | DQ316227              | DQ316189|         |           |
|                        | CMW 20684        |                    | *P. laurifolia*     | Bainskloof, SW Cape       | F. Roets             | DQ316218              | DQ316180|         |           |
|                        | CBS 119721       | CMW 20676          | PREM58941H          | *P. laurifolia*           | JS Marais Park, SW Cape | F. Roets             | DQ316151| DQ316181|           |
|                        | CMW 20686        |                    | *P. laurifolia*     | Bainskloof, SW Cape       | F. Roets             | DQ316223              | DQ316185|         |           |
|                        | CMW 20687        |                    | *P. laurifolia*     | Bainskloof, SW Cape       | F. Roets             | DQ316221              | DQ316183|         |           |
|                        | CMW 20688        |                    | *P. laurifolia*     | Bainskloof, SW Cape       | F. Roets             | DQ316224              | DQ316186|         |           |
|                        | CBS 119588       | CMW 20689          | PREM58946P          | *P. laurifolia*           | Bainskloof, SW Cape  | F. Roets             | DQ316225| DQ316187|           |
|                        | CMW 20690        |                    | *P. laurifolia*     | Bainskloof, SW Cape       | F. Roets             | DQ316226              | DQ316188|         |           |
|                        | CMW 20692        | PREM58948          | *P. neriifolia*     | Jonkershoek, SW Cape      | F. Roets             | DQ316228              | DQ316190|         |           |
| *O. protearum*         | CBS 116654       | CMW 1107           | *P. caffra*         | Irene, Gauteng            | M.J. Wingfield        | DQ316145              | DQ316163|         |           |
|                        | CBS 116567       | CMW 1103           | *P. caffra*         | Irene, Gauteng            | M.J. Wingfield        | DQ316203              | DQ316165|         |           |
|                        | CBS 116568       | CMW 1102           | *P. caffra*         | Irene, Gauteng            | M.J. Wingfield        | AF221014              | DQ316202| DQ296072|           |
| *O. splendens*         | CMW 20679        |                    | *P. repens*         | JS Marais Park, SW Cape   | F. Roets             | DQ316150              | DQ316174|         |           |
|                        | CMW 20680        |                    | *P. repens*         | JS Marais Park, SW Cape   | F. Roets             | DQ316121              | DQ316173|         |           |
|                        | CMW 20685        |                    | *P. repens*         | JS Marais Park, SW Cape   | F. Roets             | DQ316123              | DQ316175|         |           |
|                        | CMW 20691        |                    | *P. repens*         | JS Marais Park, SW Cape   | F. Roets             | DQ316209              | DQ316171|         |           |
|                        | CMW 20678        |                    | *P. repens*         | JS Marais Park, SW Cape   | F. Roets             | DQ316210              | DQ316172|         |           |
|                        | CMW 20674        |                    | *P. repens*         | George, SW Cape           | F. Roets             | DQ316204              | DQ316166|         |           |
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 β-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 involucral 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 dendogram 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 %.](image-url)
β-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 β-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 β-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 β-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

![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).](http://example.com/dendrogram.png)
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 β-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 β-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.

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**Fig. 4.** Distance dendogram obtained with the HKY+G parameter model (G = 0.1783) on the partial β-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 ostiolares absentes. Asci evanescentes. Ascospores allantoidae, unicellulares, hyalinae, vagina gelatinosa carentes, 4–6 x 2 μm, aggregatae electrinae. Anamorpha *Sporothrix* sp., conidii ellipsoides 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). Ascii 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,

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![Micrographs of Ophiostoma phasma](image)

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, Steellenbosch, Jan S. Marais Park, on Protea laurifolia, Jun. 2005, F. Roets, holotype PREM 58941, culture ex-type CMW 20676 = CBS 119721; Steellenbosch, 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; Steellenbosch, Jonkershoek NR, on P. neriifolia, Jul. 2004, F. Roets, paratype PREM 58944, culture ex-paratype CMW 20682 = CBS 119589; Giftberg top, on P. laurifolia, Jun. 2005, F. Roets, culture CMW 20698; Giftberg 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; Pienkierskloof 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: Spororthix sp.

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

Asomata superficialia, basi globosa, atra, 80–195 μm diam, nonnumquam pauciars hyphis circumdata, collo atro, 360–760 x 20–35 μm, sursum ad 10–15 μm angustato, 8–12 hyphis ostiolariibus rectis vel curvatis, hyalinis vel subhyalinis, 10–25 μm longis palmis fingeintibus ornato. Asci evanescentes. Ascospores allantoidae, uniloculare, hyalinae, vagina gelatino-sa carentes, 3.5–5.5 x 2.0–2.5 μm, aggregatae incoloratae. Anamorphe Spororthix 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 ostior hyphae, straight or slightly curved, hyaline to sub-hyaline, 10–25 (16 ± 5) μm long (Fig. 6C). Asci evanescent. Ascospores allantoid, asceptate, 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 (Fig. 6F–G). Denticles 0.5–2 μm (1 ± 0.5) long (Fig. 6G). Conidia holoblastic, hyaline, asceptate, 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, Steellenbosch, Jan S. Marais Park, on Protea laurifolia, Jun. 2005, F. Roets, holotype PREM 58942, culture ex-type CMW 20677 = CBS 119590; Steellenbosch, Jonkershoek NR, on P. neriifolia, May 2004, F. Roets, paratype PREM 58949, culture ex-paratype CMW 20693 = CBS 119591; Steellenbosch, Jon S. Marais Park, on P. repens, Jun. 2005, F. Roets, paratype PREM 58950, culture ex-paratype CMW 20697 = CBS 119593; Steellenbosch, Jon S. Marais Park, on P. repens, Jun. 2005, F. Roets, paratype PREM 58951, culture ex-paratype CMW 20694 = CBS 119592; Steellenbosch, Jon S. Marais Park, on P. repens, Jun. 2005, F. Roets, paratype PREM 58952, culture ex-paratype CMW 20695 = CBS 119594; Steellenbosch, Jon S. Marais Park, on P. repens, Jun. 2005, F. Roets, culture CMW 20695; Steellenbosch, 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. afericanum 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 µm; C = 10 µm; D = 5 µm; F–G = 3 µm; H = 5 µm; I–L = 3 µm.
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. africanaum* from Protea dracomontana and *P. caffra*.

Analyses of LSU and ITS sequence data was insufficient to distinguish between *O. africanaum* and *O. protearum*. This shows that the two species are very closely related. Analyses of the more variable β-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 (Rebelo 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 (Rebelo 1995). *Ophiostoma africanaum* was previously thought to be specific to *P. gaguedi* (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. gaguedi*, although *P. gaguedi* is classified in a different section of the genus *Protea*, the *Lasiocephalae* (Rebelo 1995).

Phylogenetic analyses of DNA sequences of three gene regions investigated in this study suggest that *O. splendens* is closely related to *O. africanaum* 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 *Melilferae*. The explanation for the close phylogenetic relationship between *O. splendens* and its northern counterparts, *O. protearum* and *O. africanaum*, 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. africanaum* 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 involucral receptacles of *P. repens*. These tunnels are either made by coleopteran or lepidopteran larvae (Coetzee & Giliomee 1987b). The involucral 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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