Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infructescences in South Africa

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Five ophiostomatoid taxa have been found associated with the infructescences of *Protea* species, an ancient group of flowering plants endemic to South Africa. Two of these fungi are characterized by their unusual *Knoxdavesia* anamorphs and have been placed in *Gondwanamyces*. The three remaining species have *Sporothrix* anamorphs and have accordingly been accommodated in *Ophiostoma*. The phylogenetic relationships between the fungi associated with *Protea* spp. and other ophiostomatoid fungi in *Ceratocystis* and *Ophiostoma* are unknown. Large subunit ribosomal RNA sequence data was obtained for the fungi associated with *Protea* infructescences as well as for the type species of *Ceratocystis* and *Ophiostoma*. Both groups of ophiostomatoid fungi were phylogenetically distinct from either *Ceratocystis* or *Ophiostoma*, despite sharing morphological and physiological characters with these genera. The species of *Ophiostoma* associated with *Protea* infructescences group within the Ophiostomatales while species of *Gondwanamyces* group within the Microascales. Furthermore, the *Ophiostoma* spp. from *Protea* should reside in a separate genus and are a fascinating example of convergent evolution towards insect dispersal.

Ceratocystis sensu lato includes *Ceratocystis sensu stricto*, *Ceratocystiopsis* and *Ophiostoma*. Species of *Ceratocystis* are very different from species of *Ceratocystiopsis* and *Ophiostoma* based on morphological, physiological and molecular data (Rosinski & Campana, 1964; Smith, Patik & Rosinski, 1967; Weijman & De Hoog, 1975; Harrington, 1981; De Hoog & Scheffer, 1984; Hausner & Reid, 1993). The distinction between *Ceratocystiopsis* and *Ophiostoma* is less clear, with species in these genera having various characters in common (De Hoog & Scheffer, 1984; Hausner, Reid & Klassen, 1993; Wingfield, 1993).

Species of *Ceratocystis s.s.*, are characterized by having *Chalara* anamorphs with enteroblastic conidiogenesis (De Hoog & Scheffer, 1984). In contrast, species of *Ophiostoma* have *Sporothrix*, *Hyalorhinocladiella*, *Leptographium* and *Graphium* Corda anamorphs, with holoblastic conidiogenesis. *Ceratocystiopsis* includes one species with a *Chalara* anamorph while others have anamorphs with holoblastic conidiogenesis, similar to many species of *Ophiostoma*. The distinction between *Ophiostoma* and *Ceratocystiopsis* is based solely on ascospore morphology (Upadhyay & Kendrik, 1975; Upadhyay, 1981) and is probably not justified (Harrington, 1981; Hausner *et al*., 1993; Wingfield, 1993).

Ceratocystiopsis protea M. J. Wingf., P. S. van Wyk & Marasas (Wingfield, Van Wyk & Marasas, 1988) and *Ophiostoma capense* M. J. Wingf. & P. S. van Wyk (Wingfield & Van Wyk, 1993) were described from infructescences of *Protea* spp. in South Africa. They have unusual *Knoxdavesia* anamorphs that are unique for *Ceratocystis s.l.* *C. proteae* and *O. capense* are closely related, differing only in ascospore sheath morphology (Wingfield, 1993) and both are distantly related to other species in *Ceratocystis s.l.* and have, accordingly, been transferred to *Gondwanamyces* (Marais & Wingfield, 1994; Marais, 1996; Marais *et al*., 1998).

Other than *Gondwanamyces proteae* (M. J. Wingf., P. S. van Wyk & Marasas) Marais & M. J. Wingf. and *G. capense* (M. J. Wingf. & P. S. van Wyk) Marais & M. J. Wingf., three other ophiostomatoid fungi have also been described from infructescences of *Protea* spp., *O. splendens* Marais & M. J. Wingf., *O. protearum* Marais & M. J. Wingf. and *O. africanum* Marais & M. J. Wingf. (Marais & Wingfield, 1994; Marais, 1996; Marais *et al*., 1998). These have *Sporothrix* anamorphs typical of *Ophiostoma*. Most species of *Ophiostoma* are associated with bark beetles that primarily infest conifers (Perry, 1991). The occurrence of *Ophiostoma* spp. outside this niche, and particularly restricted to *Protea* infructescences is, therefore, enigmatic and it seems unlikely that they would be related to other *Ophiostoma* spp. Furthermore, the ophiostomatoid fungi isolated from the *Protea* infructescences appear to be unique to South Africa. Their association with *Protea* spp. raises important questions regarding the evolution and phylogenetic relatedness of these fungi to other ophiostomatoid genera. For example, are they related to other ophiostomatoid fungi or have they evolved separately? The use of ribosomal RNA sequence data has proven extremely

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useful for inferring fungal phylogenies (e.g. Bruns, White & Taylor, 1991; Hillis & Dixon, 1991). In this study, we used parsimony analyses of the DNA sequences of a PCR generated fragment for the Large subunit (LSU) ribosomal RNA gene to determine relationships between the fungi associated with *Protea* spp., and for the type species of *Ceratocystis* and *Ophiostoma*.

MATERIALS AND METHODS

Isolates of species associated with *Protea* infructescences and the type species of other ophiostomatoid genera, *Ceratocystis fimbriata* Ellis & Halst. and *Ophiostoma piliferum* (Fr.) Syd. & P. Syd., are maintained in the culture collection of the Tree Pathology Co-operative Programme, University of Pretoria, Pretoria, South Africa and various other internationally recognized culture collections (Table 1). Cultures were grown in 200 ml malt extract (20 g l^{-1} Biolab malt extract) in shake flasks at 20 °C, for 2 wk. Mycelium was harvested by centrifugation for 10 min at 10000 rpm, washed twice with sterile distilled water, lyophilized and stored at −20°.

Total nucleic acids were extracted using the method of Viljoen *et al.* (1993). Lyophilized mycelium was homogenized in liquid nitrogen using a mortar and pestle. The homogenate was resuspended in 20 ml buffer (0·01 M Tris-HCl pH 8, 5 mM EDTA pH 8 and 0·5% SDS). Proteinase K (Boehringer Mannheim Randburg, South Africa), (50 μg ml^{-1}) was added and the suspension incubated for 12 h at 37°. CTAB, 1% (w/v) (Sigma H5882) (Sigma-Aldrich Company Ltd, Dorset, U.K.) and 1 M NaCl final concentration was added and the suspension incubated at 55° for 1 h. Chloroform-isoamyl alcohol (24:1 v/v) extractions were performed to remove proteins. The nucleic acids were precipitated from the aqueous phase by adding 2·25 vol. of ethanol. The precipitate was collected by centrifugation at 10000 rpm for 30 min, washed with 70% ethanol and resuspended in sterile dH_2O.

The Expand High Fidelity PCR System (Boehringer Mannheim) was used to generate PCR fragments for sequencing. Primer pair LS1 (Gutell & Fox, 1988) and LR6 (Vilgalys & Hester, 1990) were used to amplify a DNA fragment from the LSU rRNA gene in all the isolates studied. PCR fragments were treated with proteinase K and purified using the PCR Wizard Magic Preps (Promega). Forward and reverse sequence reactions were performed using primers, LS1, LR3, LR3R, LR5, LR5R, LR6 (Vilgalys & Hester, 1990).

Table 1. List of fungi studied.

Isolate	Origin	Collector
CMW2219 *C. fimbriata*	France	C. Grosclaude
CMW1591 *O. piliferum*	Japan	K. Aoshima
CMW3936 *G. protea*	South Africa	C. D. Viljoen
CMW997 *G. capense*	South Africa	M. J. Wingfield
CMW823 *O. africanum*	South Africa	M. J. Wingfield
CMW1102 *O. protearum*	South Africa	M. J. Wingfield
CNW872 *O. splendens*	South Africa	M. J. Wingfield

[a] CMW – the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa.

Table 2. List of pyrenomycete taxa and the GenBank loci or source of DNA sequence.

	GenBank locus/source
Aphysiostroma stercorarium Barrasa, A. T. Martínez & G. Moreno	U47820
Ceratocystis fimbriata	U47822
C. virescens (R. W. Davidson) C. Moreau	U47824
Cercophora septentrionalis N. Lundq.	U47823
Chaetomium globosum Kunze	U47825
Claviceps paspali F. Stevens & J. G. Hall	U47826
Cylindrocladium floridanum (Sobers) C. P. Seym.	U17408
C. scoparium Morgan	U17409
Daldinia concentrica (Bolton) Ces & De Not.	U47828
Diaporthe phaseolorum (Cooke & Ellis) Sacc.	U47830
Glomerella cingulata Stoneman) Spauld. & H. Schrenk	U48428
Hirsutella thompsonii F. E. Fisher	U47831
Hypocrea schweinitzii (Fr.) Sacc.	U47833
Microascus trigonosporus C. W. Emmons & B. O. Dodge	U47835
Neocosmospora vasinfecta E. F. Sm.	U47836
Ophiostoma piliferum	U47837
Petriella setifera (J. C. Schmidt) Curzi	U48421
Xylaria curta Fr.	U47840
X. hypoxylon (Fr.) Grev.	U47841

Sequences were aligned manually and analysed using PAUP version 3.1.1 (Swofford, 1993). Bootstrapping (1000 replicates) was performed on sequence data to determine tree confidence intervals. The sequence data of species of *Gondwanamyces* and *Ophiostoma* associated with *Protea* infructescences were also compared with those available from GenBank for other pyrenomycete taxa (Table 2).

RESULTS

Approximately 1100 bp of DNA sequence from the LSU ribosomal RNA gene was aligned for the different taxa used in this study (Fig. 1). Of the 236 variable sites, there were 103 phylogenetically informative sites with parsimony methods (Swofford, 1993). The branch and bound option of PAUP was used to find the most parsimonious tree. One most parsimonious tree was generated (Fig. 2). Bootstrap analysis (1000 replicates) was performed to determine branch confidence intervals.

The tree branching separated taxa into four groups. *Ceratocystis fimbriata* and *Ophiostoma piliferum*, the type species of their respective genera, did not group within either of the two clades. Species of *Gondwanamyces* clustered together while *O. africanum*, *O. protearum* and *O. splendens*, also from *Protea* spp., formed a separate clade (Fig. 2).

Phylogenetic analysis of pyrenomycete sequences for a wide range of genera produced one most parsimonious tree through a process of 10 replicates of a heuristic search utilizing the random sequence addition option in PAUP (Fig. 3). The pyrenomycetes clustered together according to ordinal affiliation, Microascales, Polystigmatales, Ophiostomatales, Xylariales, Diaporthiales, Sordariales, Clavicipitales and Hypocreales (Spatafora & Blackwell, 1993). Species of *Gondwanamyces* clustered within the Microascales with a 100% confidence interval. Species of *Ophiostoma* associated with

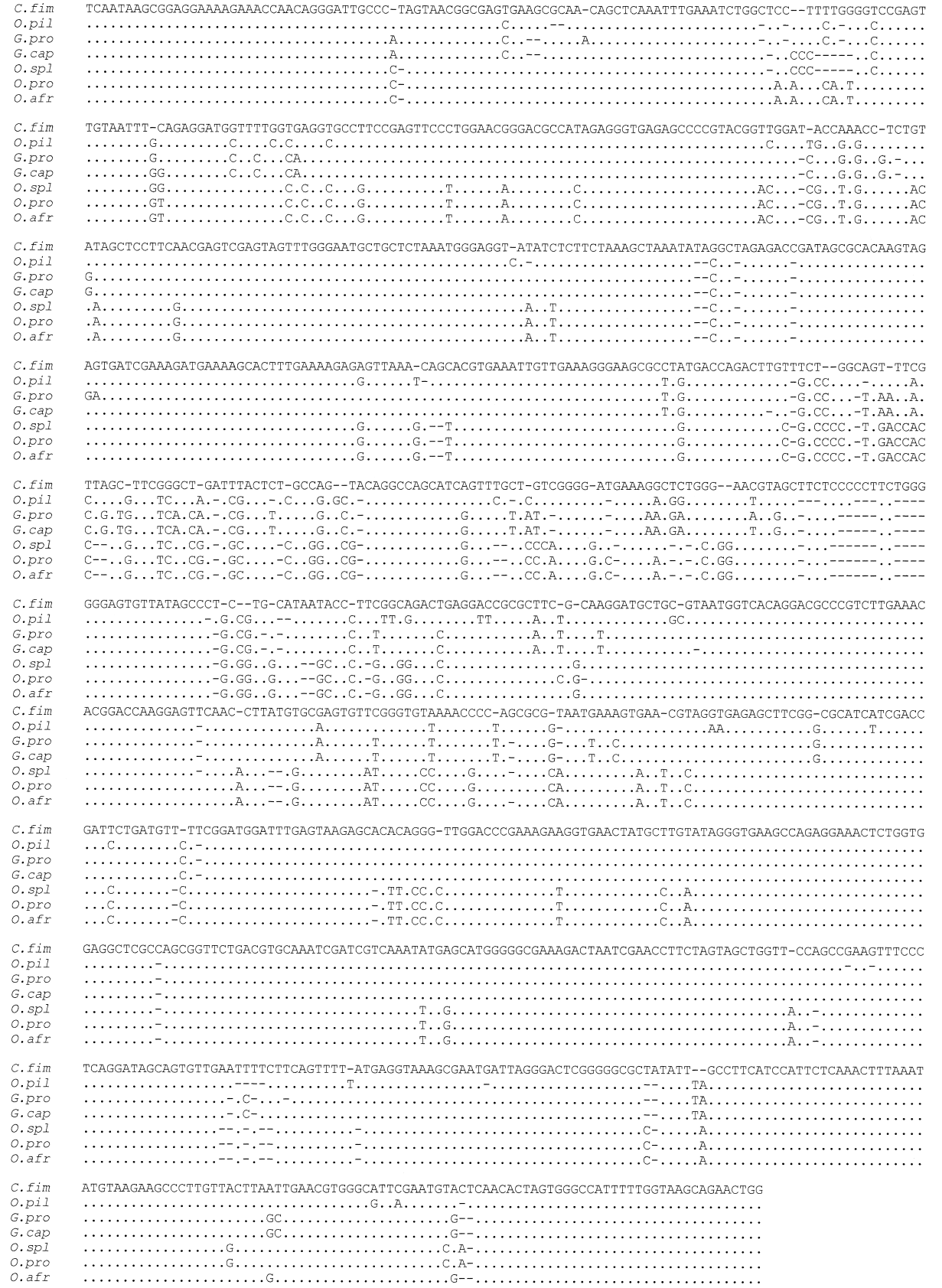

Fig. 1. Approximately 1100 bp of aligned sequence from the LSU ribosomal RNA gene. A dash indicates a gap in the sequence. A dot indicates a base homologous and identical to the corresponding base in *C. fimbriata* sequence.

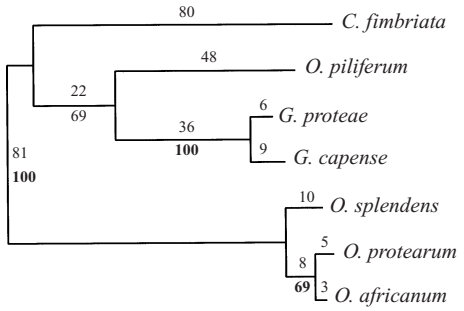

Fig. 2. Phylogenetic tree produced using PAUP branch and bound option with midpoint rooting. Branch lengths are indicated above the branches on the tree. Bootstrap values (percentages of 1000 bootstrap replicates) are indicated in bold below the branches of the tree.

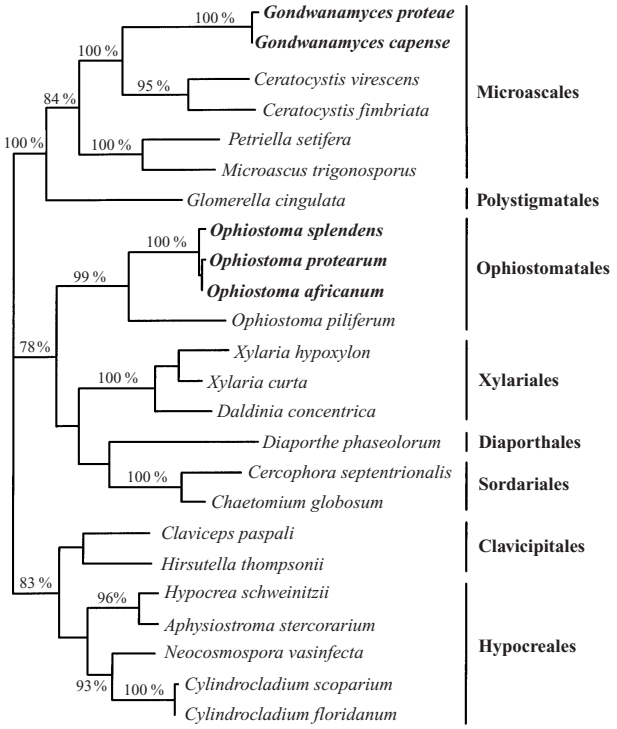

Fig. 3. The most parsimonious phylogenetic tree generated by 10 replicates of an heuristic search option of PAUP 3.1.1. The percentages indicated on the tree are frequencies with which branching occurred in 1000 bootstrap replications.

Protea infructescences grouped within the Ophiostomatales (Fig. 3). The Ophiostomatales formed a group with the Xylariales, Diaporthiales and the Sordariales with 78 % support. The tree consistency (CI), homoplasy (HI) and retention (RI) indices were 0·538, 0·462 and 0·705, respectively.

DISCUSSION

The ophiostomatoid fungi associated with *Protea* infructescences are an unusual and intriguing group. The fact that two very distinct groups of these fungi occur in this niche is an additional source of interest. Morphologically, these two groups are very different from each other, with one very closely resembling *Ophiostoma*. Our results clearly show that the ophiostomatoid fungi associated with the Proteaceae are distinct from both *Ceratocystis fimbriata* and *Ophiostoma piliferum*, the type species for the genera (Fig. 2). The similarity in morphological and physiological characters (Marais, 1996; Marais *et al*., 1998) amongst the fungi associated with *Protea* is clearly a feature of convergent evolution, mediated through insect association and not their phylogeny.

Gondwanamyces proteae and *G. capense* in the Microascales are phylogenetically very closely related, despite differences in ascospore sheath morphology. Similarly *Ophiostoma africanum*, *O. protearum* and *O. splendens* clustered closely together in the Ophiostomatales (Fig. 2). These fungi, therefore, represent two very distinct lineages, which appear to have independently evolved association with *Protea*.

Gondwanamyces species are relatively sensitive to the antibiotic cycloheximide and contain no rhamnose in their cell walls (Marais, 1996), which makes them very similar to *Ceratocystis* spp. It is, therefore, not surprising that *Gondwanamyces* was originally thought to be more closely related to *Ceratocystis* than to *Ophiostoma* (Wingfield *et al*., 1988). Comparison of sequence data has confirmed *Gondwanamyces* as a genus allied to *Ceratocystis* within the Microascales.

Species of *Gondwanamyces* produce conidia through a process of apical wall building, similar to species of *Ophiostoma* and other ophiostomatoid fungi associated with *Protea*. Conidium ontogeny is generally considered an important morphological character used to distinguish the ophiostomatoid species of *Ceratocystis* and *Ophiostoma* (De Hoog & Scheffer, 1984; Mouton, Wingfield & Van Wyk, 1994). The occurrence, therefore, of holoblastic conidium development in species with *Knoxdavesia* anamorphs is unusual and enigmatic. The presence of this type of conidium development could indicate that this lineage may be derived from an *Ophiostoma*-like ancestor. Alternatively this characteristic could be the result of convergent evolution.

Species of *Ophiostoma* associated with *Protea* infructescences are typical *Ophiostoma* species. In contrast to *Gondwanamyces*, they are tolerant of cycloheximide and have rhamnose in their cell walls (Marais, 1996; Marais *et al*., 1998). Furthermore, they have *Sporothrix* anamorphs, which are also typical of *Ophiostoma*. These fungi, however, represent a unique lineage, divergent to the type species of *Ophiostoma*. We suggest, therefore, that the *Ophiostoma* spp. from *Protea* should reside in a separate genus within the Ophiostomatales. A more complete study of this group of fungi would be necessary to further clarify this distinction.

Ceratocystis fimbriata and *Ophiostoma piliferum* are both pyrenomycetes and have similar perithecia with passively discharged, wet spore masses, adapted to insect dispersal. Nevertheless, they have diverged to the point where they are now considered relatively distantly related (Berbee & Taylor, 1992*a*, *b*; Hausner *et al*., 1993). In comparing sequence data from the LSU rRNA gene, *C. fimbriata* and *O. piliferum* have 21 point mutations in common. In comparison, the number of shared point mutations amongst species with *Knoxdavesia* anamorphs, *C. fimbriata* and *O. piliferum*, is eight and 24, respectively. Amongst all three groups, including *C. fimbriata*, *O. piliferum* and species with *Knoxdavesia* anamorphs, a total of 77 point mutations are common. This implies that species with *Knoxdavesia* anamorphs share more point mutations with *C.*

fimbriata and *O. piliferum* together, than with these two species separately. It is possible that these changes are the result of coincidental mutations giving rise to these similarities. Another possible explanation for this rather surprising phenomenon is, however, that the lineage giving rise to species with *Knoxdavesia* anamorphs (*G. proteae* and *G. capense*) is a result of a hybridization event between the ancestors of *Ceratocystis* and *Ophiostoma*. It would be necessary to examine other gene sequences to support or refute this suggestion.

The origin of the ophiostomatoid fungi associated with the Proteaceae is an intriguing question. It is interesting that insect association is a common character shared by many of these fungi (Wingfield *et al*., 1988; Perry, 1991; Marais & Wingfield, 1994). The evolution of species of *Ophiostoma* and their vectors, mainly bark beetles, is thought to have begun approximately 140 million years ago (Bright & Stock, 1982; Mitten & Sturgeon, 1982; Berbee & Taylor, 1995). The Proteaceae, although one of the oldest living families of flowering plants dating back 95 million years, only gave rise to present day *Protea* spp. approximately 5 million years ago (Cowling & Richardson, 1995), when we suggest that the lineages of ophiostomatoid fungi associated with the Proteaceae originated.

The species in each of the two distinct groups of ophiostomatoid fungi associated with *Protea* infructescences are very closely related. This would suggest that the speciation of these two groups of fungi has occurred recently. This is supported by the fact that *Protea* spp. are considered to be neo-endemic, having given rise to many species over a relatively short period of time (Cowling, Holmes & Rebelo, 1992; Cowling & Richardson, 1995).

The results of this study clearly show that the unusual groups of ophiostomatoid fungi associated with *Protea* infructescences have evolved independently in *Ceratocystis sensu lato*, despite sharing many morphological and physiological characters. It would also appear that the two distinct lineages are present within the currently recognized *Ophiostoma*.

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