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# A new ophiostomatoid genus from *Protea* infructescences

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***Abstract:*** In recent years, two unusual ophiostomatoid fungi, *Ceratocystiopsis proteae* and *Ophiostoma capense,* have been described from infructescences of *Protea* spp. They are unique in having *Knoxdaviesia* anamorphs and differ from each other in ascospore morphology. Both species are sensitive to cycloheximide, typical of *Ceratocystis* s. s. In this study, RFLP analyses were done on the rRNA operon regions of the type species of *Ceratocystis, Ophiostoma* and *Ceratocystiopsis* as well as *C. proteae* and *O. capense* using the restriction enzymes *Eco* RI, *Hae* II, *Pst* I, *Sal* I, *Sma* I and *Xba* I. By constructing restriction maps, phylogenetic relationships between these species were determined. From the data, it was evident that *C. proteae* and *O. capense* are phylogenetically unrelated to both *Ceratocystis* and *Ophiostoma,* but showed a closer affinity to *Ceratocystis* than to *Ophiostoma.* Based on the RFLP analyses, cycloheximide sensitivity, cell saccharides, and anamorph morphology, a new genus, *Gondwanamyces,* is established to accommodate these two unusual fungi.

***Key Words:*** Ceratocystiopsis, Ceratocystis, Gondwanamyces, Ophiostoma, RFLP

## INTRODUCTION

The central genus, *Ceratocystis* Ellis & Halst. sensu lato, has been subdivided into the genera, *Ceratocystis* Ellis & Halsted sensu stricto, *Ophiostoma* H. & P. Sydow and *Ceratocystiopsis* Upadhyay & W.B. Kendr. Species in *Ceratocystis* s. s. are characterized by having *Chalara* (Corda) Rabenh. anamorphs in which conidia develop through ring-wall building (Minter et al., 1983), are sensitive to cycloheximide and lack cellulose and rhamnose in their cell walls (de Hoog and Scheffer, 1984). In contrast, species in *Ophiostoma* have anamorphs in the genera *Sporothrix* Hektoen & C.F. Perkins, *Graphium* Corda, *Leptographium* Lagerb. & Melin and *Hyalorhinocladiella* Upadhyay & W.B. Kendr. (Mouton et al., 1994). In all the latter genera, conidia are produced by apical wall building (Minter et al., 1982; Mouton et al., 1994). These species possess rhamnose and cellulose in their cell walls (Smith et al., 1967) and are tolerant to high concentrations of cycloheximide (Harrington, 1981). Species in *Ceratocystiopsis* differ from the other genera by their falcate ascospores (Upadhyay and Kendrick, 1975). By analyzing ribosomal DNA, the validity of these chemical and morphological characteristics have been confirmed by various authors (Hausner et al., 1992a; 1992b; 1993; Spataphora and Blackwell, 1994).

Two new ophiostomatoid species, *Ceratocystiopsis proteae* M.J. Wingf., P.S. Van Wyk & Marasas (Wingfield et al., 1988) and *Ophiostoma capense* M.J. Wingf. & P.S. Van Wyk (Wingfield and Van Wyk, 1993) have been described from various *Protea* species in the western Cape Province of South Africa. *Ceratocystiopsis proteae* and *O. capense* are unlike species in *Ophiostoma* and *Ceratocystiopsis* in that they do not contain rhamnose in their cell walls (Marais and Wingfield, unpublished), are sensitive to cycloheximide (Wingfield et al., 1988; Wingfield and Van Wyk, 1993), are unable to utilize rhamnose (Marais and Wingfield, 1991), and have *Knoxdaviesia* anamorphs (Wingfield et al., 1988; Wingfield and Van Wyk, 1993). With the exception of the anamorph, these characteristics are also those of *Ceratocystis* s. s.

*Ophiostoma capense* differs from *C. proteae* by having allantoid ascospores, as apposed to the falcate ascospores of *C. proteae.* Ascospore morphology was the primary reason for placing the two species in different genera (Wingfield et al., 1988; Wingfield and Van Wyk, 1993). It has recently been noted that the ascospore sheath in *C. proteae* might represent the remains of the perithecial cavity and probably is not a true sheath such as those found in other ophiostomatoid fungi (Van Wyk and Wingfield, 1991; Van Wyk et al., 1991; Wingfield and Van Wyk, 1993). Wingfield (1993) emphasised the fact that *C. proteae* is unlike other ophiostomatoid species and, together with *O. capense,* would be most acceptable in *Ophios-*

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TABLE I. List of cultures from which DNA was isolated

| Fungus | Isolate numbers* | Collector/Origin |
|---|---|---|
| *Ophiostoma capense* | CMW 1147 | M. J. Wingfield, Cape of Good Hope Nature Reserve, South Africa |
| *Ceratocystiopsis proteae* | CMW 986 | M. J. Wingfield, Jonkerskoek, South Africa |
| *Ceratocystis fimbriata* | CMW 2219 | C. Grosclaude, St. Maurice, France |
| *Ceratocystiopsis minuta* | CMW 1018, CBS 393.77 | R. W. Davidson, Colorado, USA |
| *Ophiostoma piliferum* | CMW 1591, IFO 6702 | K. Aoshima, Japan |

[a] CBS = Centraal Bureau voor Schimmelcultures; CMW = Culture collection of M. J. Wingfield, Univ. Free State, Bloemfontein, South Africa; IFO = Institute for Fermentation, Osaka.

*toma*. In contrast and based on the analysis of partial rDNA sequences, Hausner et al. (1993) found that *C. proteae* could have been derived from the same lineage as *Ceratocystis* s. s.

Other than *Ceratocystiopsis proteae* and *Ophiostoma capense,* two additional ophiostomatoid fungi have also been isolated from the infructescences of *Protea* spp. These are *O. splendens* Marais & M. J. Wingf. from various *Protea* spp. in Western Cape Province of South Africa (Marais and Wingfield, 1994) and *Ophiostoma protearum* Marais & M. J. Wingf. from *Protea caffra* Meisn. in the Drakensberg mountains (Marais and Wingfield, 1997). These species have *Sporothrix* anamorphs, which is typical of species of *Ophiostoma*. They are also tolerant to cycloheximide and have rhamnose in their cell walls (Marais and Wingfield, unpublished data). Although *C. proteae* and *O. capense* share the same ecological niche as the other species of *Ophiostoma,* it is clear that there are two distinct groups of ophiostomatoid fungi associated with infructescences of *Protea* spp.

*Ceratocystiopsis proteae* and *O. capense* are closely related and, based on current evidence, should clearly reside in the same genus. Substantial evidence also exists to suggest that they represent a taxon distinct from *Ceratocystis* s. s., *Ophiostoma* and *Ceratocystiopsis*. The aim of this study was to compare *C. proteae* and *O. capense* further with the type species of these three genera by using RFLP analysis of the rRNA operon regions and to consider a more acceptable generic placement for these fungi.

## MATERIALS AND METHODS

*Culturing procedures.*—Cultures of *Ceratocystiopsis proteae* (CMW 986), *Ophiostoma capense* (CMW 1147), *Ceratocystis fimbriata* (CMW 2219), *Ophiostoma piliferum* (CMW 1591, IFO 6702) and *Ceratocystiopsis minuta* (CMW 1018, CBS 393.77) were used and are maintained in and available from the culture collection of M.J. Wingfield, University of the Free State, Bloemfontein, South Africa (TABLE I). Cellophane disks covering the surface of Petri dishes containing MEA (20 g Difco malt extract, 20 g Difco Bacto agar per L water) were inoculated with each of the test fungi and incubated at 25 C until the surface was covered with mycelium. The cellophane disks were then removed from the medium, lyophilized and stored at −20 C.

*DNA extraction.*—Genomic DNA was isolated from cultures using the method of Chirgwin et al. (1979), modified by Viljoen et al. (1993). Approximately a quarter of the cell material from one cellophane disk was placed in 1.5-mL Eppendorf tubes together with 100 μL of 4 M GT buffer (4 M guanidinium thiocyanate, 24 mM trisodium citrate). The cell material was homogenized with an Eppendorf pestle and additional GT was added to this mixture to a total volume of 1.5 mL. After incubation on ice for 15 min, cellular debris were removed by centrifugation for 10 min at 14 000 rpm. Nucleic acid was precipitated from the supernatant with 0.1 vol. 3 M sodium acetate and 0.6 vol. isopropanol. This precipitate was pelleted by centrifugation for 20 min at 14 000 rpm. The nucleic acid pellet was washed with 70% ethanol and suspended in 500 μL sterile distilled H2O.

The remaining contaminating proteins were removed by extracting twice with a mixture consisting of equal volumes of phenol, chloroform and nucleic acid suspension. The nucleic acids were again precipitated from the aqueous phase, washed and resuspended in sterile distilled H2O. The concentration of the purified DNA was assessed by agarose gel electrophoresis.

*Ribotyping.*—Restriction enzymes used for cleaving the DNA included *Eco* RI, *Hae* II, *Pst* I, *Sal* I, *Sma* I and *Xba* I (10 units enzyme/μg DNA). All enzymes were supplied by Boehringer Mannheim, GMBH, Biochemica, Germany. Restriction of the DNA was done according to manufacturer's specifications. Single and double digests were done.

Restriction digests were resolved on 0.85% agarose gels (molecular biological grade, FMC) by means of electrophoresis. The DNA was stained with ethidium bromide and detected using UV-light. The total ex-

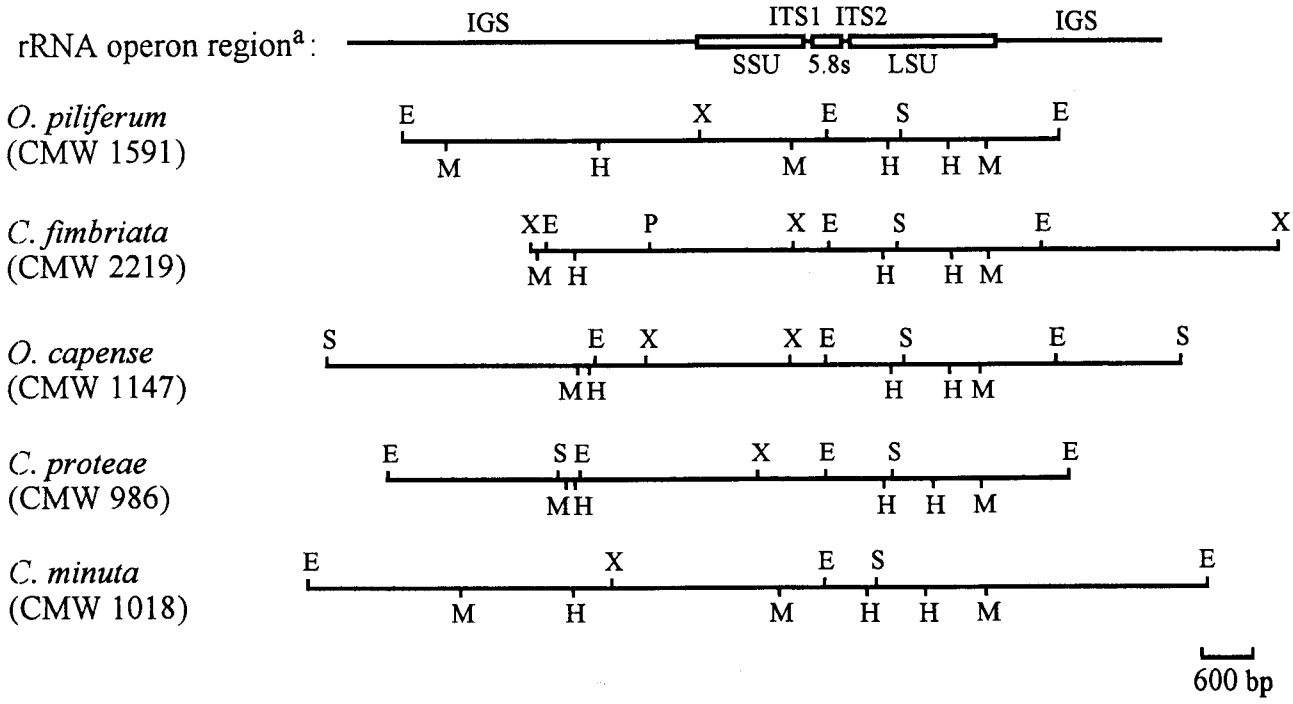


FIG. 1. Restriction maps of the species of the major genera in *Ceratocystis* s. l. as well as species associated with *Protea* infructescences. E= *Eco* RI; H= *Hae* II; S= *Sal* I; M= *Sma* I; X= *Xba* I; P= *Pst* I. [a]A schematic representation of the rRNA operon from the 5′ end with flanking IGS regions (intergenic spacer), SSU (small subunit rRNA gene), ITS1 (first internal transcribed spacer), 5.8S rRNA gene, ITS2 (second internal transcribed spacer) and the LSU (large subunit rRNA gene) is included for purposes of comparison.

posure time of the DNA to UV-light was 30 sec. in order to depurinate the DNA, after which it was denatured with a solution containing 0.5 M NaOH and 1.5 M NaCl and neutralized with a solution containing 1 M Tris HCl buffer (pH 8) and 1.5 M NaCl. Denatured DNA was transferred to a Hybond N nylon membrane (Amersham International plc, Amersham, UK) using Southern blotting.

PCR fragments spanning the rRNA operon of *C. proteae* were used as probes to detect the digested rRNA operon fragments. The probes were PCR generated using primers NS1 and NS8 for the small subunit rRNA gene, ITS1 to ITS4 (White et al., 1990) which amplifies a region including the ITS (internal transcribed spacer) regions and the 5.8S rRNA gene and 5.8SR to LR6 (Garber et al., 1988) in the large subunit rRNA gene. The same gene order as found in *Saccharomyces cerevisiae* was assumed. This was confirmed with results using the PCR probes from different rRNA operon regions. The PCR probes were labeled and detected with digoxigenin (Nonradioactive DIG labeling and detection kit, Boehringer Mannheim, GMBH, Biochemica, Germany). Fragment sizes were calculated based on the molecular weight markers of lambda DNA cut with *Eco* RI and *Hind* III and restriction maps were compiled and aligned manually.

A data matrix was established based on the scoring of the absence and presence of restriction sites. These were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1991). The branch and bound option was used to find the most parsimonious tree using the midpoint rooting option. One thousand bootstrap replicates were performed to determine the bootstrap values for the respective branching points. All bootstrap values above 70% are indicated on the tree.

## RESULTS

Lengths of the rRNA operon regions ranged from approximately 7000 bp in *O. piliferum* to 9700 bp in *C. minuta*. Certain restriction sites were highly conserved and present in all five species. This facilitated the alignment process and comparison of the restriction maps (FIG. 1).

All *Hae* II sites, one *Sma* I, one *Sal* I and one *Eco* RI site were conserved in all species. Although the occurrence of these sites are uniform for all the species, slight differences in the distances between species were observed (smaller than 20 bp). These differences were considered insignificant due to the lack of resolution when determining fragment sizes.

Apart from those previously mentioned, two other

| | S | E | E | M | X | E | H | M | E | S | X | P | X | X | M | E | H | S | H | M | E | S | E | X |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *O. capense* | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| *C. proteae* | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| *C. minuta* | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 |
| *C. fimbriata* | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 |
| *O. piliferum* | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |

FIG. 2. Binary codes based on the restriction sites of the rRNA operon region of the type species of *Ceratocystis, Ophiostoma, Ceratocystiopsis* and the two ophiostomatoid species from *Protea* spp. A data matrix was established based on the scoring of the absence and presence of restriction sites. 1 = present, 0 = absent.

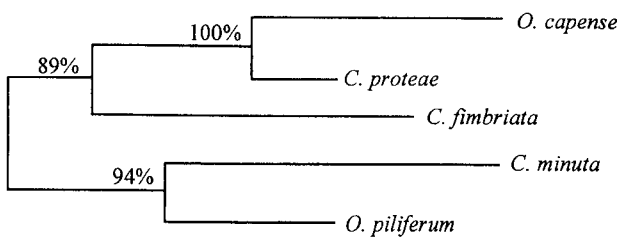


FIG. 3. PAUP cladistic analysis of the rRNA operon region of the type species of *Ceratocystis, Ophiostoma, Ceratocystiopsis* and the two species from *Protea* infructescences gave only one phylogenetic tree. The tree is rooted to midpoint. The percentages represent the bootstrap values for the respective branching points of one thousand bootstrap replicates.

*Eco* RI sites were also present in all five species studied. The first *Eco* RI site indicated on the restriction map (FIG. 1) was the same in *Ceratocystis fimbriata, C. proteae* and *O. capense. Ophiostoma piliferum* and *C. minuta,* however, did not have this site. The third *Eco* RI site indicated was the same in all species other than *C. minuta.*

All species were found to have only one *Sal* I site. *Ophiostoma capense* and *Cop. proteae,* however, each had an extra site which occurred at different points on the respective restriction maps.

*Sma* I digestions demonstrated a single conserved site in all species. Another *Sma* I site was found to be similar in *Ceratocystis fimbriata, O. capense* and *C. proteae.* In contrast, *O. piliferum* and *C. minuta* shared two *Sma* I cutting sites.

Digestions with *Xba* I also showed that *Ceratocystis fimbriata, O. capense* and *C. proteae* had the same cutting sites. *Ophiostoma capense* also shared a similar *Xba* I site with *O. piliferum* and *C. minuta. Ceratocystis fimbriata* had an additional *Xba* I site as well as a *Pst* I site not found in any of the other species studied.

The presence or absence of restriction sites was used to obtain a binary code (FIG. 2) for each species which was subjected to the extensive branch and bound option of PAUP. This resulted in a single unrooted tree (FIG. 3). Here, *Ceratocystiopsis proteae* and *O. capense* clustered together and closest to *Ceratocystis fimbriata. Ceratocystiopsis minuta* and *O. piliferum* were distantly removed from the other three species and showed a closer affinity to each other.

Bootstrap analysis showed that *O. capense* and *C. proteae* grouped together with a 100% bootstrap value. These two species grouped with *Ceratocystis fimbriata* with a bootstrap value of 89%. *Ceratocystiopsis minuta* and *O. piliferum* grouped together with a 94% bootstrap value and clustered apart from the other three species under consideration.

These results provided additional evidence that *C. proteae* and *O. capense* are more closely related to the type species of *Ceratocystis* (*Ceratocystis fimbriata*) than to *Ceratocystiopsis* (*C. minuta*) and *Ophiostoma* (*O. piliferum*). They also confirm results of a previous study by Hausner et al. (1993) in which *C. proteae* was considered to be phylogenetically allied to *Ceratocystis* s. s.

The unusual ecological niche in which these fungi occur (Marais and Wingfield, 1994), cycloheximide sensitivity (Wingfield et al., 1988; Wingfield and Van Wyk, 1993), anamorph states and molecular evidence (this study; Hausner et al., 1993) lead us to propose the following new genus for *C. proteae* and *O. capense*:

**Gondwanamyces** Marais & M.J. Wingf., gen. nov.

Perithecia nigra, globosa vel subglobosa; colla comparate longa, attenuata apicem versus angustata; hyphae ostiolares praesentes; asci evanescentes, hyalini; ascosporae hyalinae, aseptatae, cum vel sine vagina. Conditio conidica *Knoxdaviesia,* formans phialoconidia holoblastice apicalibus parietibus struendis in obovoideis cellis conidiogenis. Eveniens a infructescentiis specierum variarum *Protea* in Occidentali Provincia Capitis Bonae Spei Africae Australis.

Perithecia black, globose to subglobose; necks relatively long, tapered toward the apex; ostiolar hyphae short and divergent; asci evanescent, hyaline; ascospores hyaline, aseptate, with or without a sheath. Conidial state *Knoxdaviesia,* forming phialoconidia holoblastically through apical wall building on obovoid conidiogenous cells. Occurring in the infructescences of various species of *Protea* in the Western Cape Province of South Africa.
Species typica:

**Gondwanamyces proteae** (M.J. Wingf., P.S. Van Wyk & Marasas) Marais & M.J. Wingf., comb. nov.

≡ *Ceratocystiopsis proteae* M.J. Wingf., P.S. Van Wyk & Marasas, Mycologia 80: 23–30, 1988.

Other combinations:

**Gondwanamyces capensis** (M.J. Wingf. & P.S. Van Wyk) Marais & M.J. Wingf., comb. nov.

≡ *Ophiostoma capense* M.J. Wingf. & P.S. Van Wyk, Mycological Research 97: 709–716, 1993.

## DISCUSSION

Results of this study show that *G. proteae* and *G. capensis* are closely related to each other but distinct from other genera of ophiostomatoid fungi such as *Ceratocystis* s. s., *Ophiostoma* and *Ceratocystiopsis*. These results also confirm those of Hausner et al. (1993) who suggested that *C. proteae* is more closely related to *Ceratocystis* s. s. than to *Ophiostoma*. Although a relatedness to *Ceratocystis* is indicated, these genera are clearly distinct in many characteristics. The most conspicuous of these distinguishing characteristics are the presence of *Knoxdaviesia* anamorphs in *Gondwanamyces* as opposed to *Chalara* in *Ceratocystis* s. s.

Spatafora and Blackwell (1994) have recently, and on the basis of partial sequences of the ssrDNA, confirmed previous suggestions that *Ceratocystis* s. s. and *Ophiostoma* are phylogenetically distantly related with the former genus best being accommodated in the Microascales and the latter in the Ophiostomatales. The relatedness of *Gondwanamyces* and *Ceratocystis* s. s. as indicated in this study and that of Hausner et al. (1993), therefore, suggests that the new genus could be a member of the Microascales.

The two species of *Gondwanamyces* are very similar to each other but could be distinguished from each other based on RFLP analysis used in this study. The differences that did occur were in the IGS region. These differences are greater than would be expected within a single species. Furthermore, they can also be distinguished from each other based on distinct ascospore morphology and minor differences in anamorph characteristics (Wingfield and Van Wyk, 1993). *Gondwanamyces proteae* is also restricted to infructescences of *Protea repens* whereas *G. capensis* occurs in the infructescences of a wide range of *Protea* spp. This is despite the fact that *P. repens* often grows alongside other *Protea* spp. infected with *G. capensis* (Wingfield and Van Wyk, 1993). The two species are also heterothalic (Marais and Wingfield, unpublished data).

Ascospore morphology has been a major characteristic used to separate genera of ophiostomatoid fungi as well as sections within these genera (Griffin, 1968; Olchowecki and Reid, 1974; Upadhyay, 1981). It has, however, recently been shown that this characteristic is not phylogenetically conserved within any specific group of ophiostomatoid fungi (Hausner et al., 1992b; 1993; Wingfield et al., 1994). The similarity between *G. capensis* and *G. proteae*, despite their differences in ascospore morphology, provides further support for the notion.

Based on the analyses of partial rDNA sequences from both the small and large subunit genes, Hausner et al. (1993) have chosen to reduce *Ceratocystiopsis* to synonymy with *Ophiostoma*. In doing so, they transferred the majority of *Ceratocystiopsis* spp. to *Ophiostoma* but failed to provide genera to accommodate *C. proteae* and *C. falcata* (Wright and Cain) Upadhyay. Results of this study have provided a generic residence for *C. proteae* but *C. falcata* must, for the time being remain in *Ceratocystiopsis*. The fact that the latter genus was reduced to synonymy with *Ophiostoma* complicates this matter and we can thus not accept that synonymy. Whether most *Ceratocystiopsis* spp., transferred to *Ophiostoma* by Hausner et al. (1993), are now correctly placed, remains uncertain.

Results of this study, and those derived from previous studies (Wingfield et al., 1988; Wingfield and Van Wyk, 1993; Hausner et al., 1993) have confirmed that *G. proteae* and *G. capensis* are unusual and that they almost certainly represent a distinct phylogenetic lineage. These unique and unusual fungi remain poorly understood and, given their dominant nature in *Protea* infructescences, deserve further study. One of the most fascinating and intriguing questions that has yet to be resolved relates to their vectors. Whether they have specific vectors such as most *Ophiostoma* spp. or whether they have a broad range of insect associates such as in the case of most species of *Ceratocystis* s. s. will add considerably to our knowledge, not only of their taxonomy but also their ecology and importance.

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