Ophiostoma protearum sp.nov. associated with *Protea caffra* infructescences

G.J. Marais and M.J. Wingfield

Abstract: A new ophiostomatoid fungus, *Ophiostoma protearum*, and its *Sporothrix* anamorph, is described based on isolations from infructescences of *Protea caffra*. The species is compared with ophiostomatoid fungi previously described from *Protea* infructescences in the Western Cape Province of South Africa. Based on differences in their anamorphs, there appear to be two evolutionary lines among these fungi in this niche.

Key words: *Ceratocystis*, *Ceratocystiopsis*, *Ophiostoma protearum*, *Sporothrix*, *Knoxdaviesia*, biogeography.

Résumé : Les auteurs décrivent un nouveau champignon ophiostomatoïde, l'*Ophiostoma protearum*, ainsi que le *Sporothrix*, son anamorphe, à partir d'isolements pratiqués sur les infructescences du *Protea caffra*. Ils comparent l'espèce avec des champignons ophiostomatoïdes déjà décrits à partir d'infructescences récoltées dans la province occidentale du Cap, en Afrique du sud. Sur la base des différences au niveau des anamorphes, il semble qu'ils constituent deux lignées évolutives distinctes dans cet habitat.

Mots clés : *Ceratocystis*, *Ceratocystiopsis*, *Ophiostoma protearum*, *Sporothrix*, *Knoxdaviesia*, biogéographie.
[Traduit par la rédaction]

Introduction

The *Ceratocystis* Ellis & Halst. sensu lato complex includes the genera *Ceratocystis* sensu stricto, *Ophiostoma* H. & P. Sydow, and *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. *Ceratocystis* s.s. can be separated from *Ophiostoma* and *Ceratocystiopsis* by the presence of *Chalara* anamorphs and ring wall building conidium development in the former and anamorphs with apical wall building development in the latter genera (Minter et al. 1982; 1983; de Hoog and Scheffer 1984). *Ceratocystiopsis* is similar to *Ophiostoma* but has been separated from the latter genus because these species have falcate ascospores (Upadhyay and Kendrick 1975), although recent molecular evidence (Hausner et al. 1993) suggests that most of these species should reside in *Ophiostoma*. Species in *Ceratocystis* lack rhamnose and cellulose in their cell walls and are sensitive to cycloheximide (de Hoog and Scheffer 1984; Samuels 1993). In contrast, *Ophiostoma* and *Ceratocystiopsis* contain rhamnose and cellulose and are tolerant to cycloheximide.

Two ophiostomatoid fungi, *Ceratocystiopsis proteae* M.J. Wingf., Van Wyk & Marasas (1988) and *Ophiostoma capense* M.J. Wingf. & Van Wyk (1993), have been described from *Protea* infructescences in the Western Cape Province of South Africa. These fungi have anamorphs in *Knoxdaviesia* M.J. Wingf., Van Wyk & Marasas, which do not occur in other species of *Ceratocystis* s.l. A third ophiostomatoid fungus, *Ophiostoma splendens* Marais & M.J. Wingf. (1994) with a *Sporothrix* anamorph, has also recently been described from this unusual niche.

Until the present study, only infructescences of *Protea* spp. occurring in the Western Cape Province were examined for ophiostomatoid fungi. The discovery of *O. splendens* led us to hypothesize that additional ophiostomatoid species might occur in other parts of the country. In this study, we collected infructescences of *Protea caffra*, a species with a wide distribution in South Africa outside the Western Cape Province (Fig. 1), in an effort to discover additional ophiostomatoid fungi.

Materials and methods

Infructescences of *Protea caffra* were collected in the Ugie area (Eastern Cape Province), Drakensberg Mountains (Kwazulu-Natal Province) and Pretoria (Gauteng Province of South Africa) (Fig. 1). A single ophiostomatoid species was present in most infructescences, producing abundant perithecia as well as a *Sporothrix* anamorph. Ascospore masses at the tips of ascocarps were removed using a sterile needle with a small piece of agar at the tip. These were then inoculated onto 2% malt extract agar (MEA) (20 g Difco malt extract, 20 g Difco Bacto Agar per 1000 mL water) and incubated at 20°C in the dark.

Initial cultures on MEA produced no perithecia, but the *Sporothrix* anamorph was present and easily isolated. One shredded infructescence of *Protea repens* was autoclaved at 121°C for 15 min in a medium containing 20 g Difco Bacto Agar per 1000 mL water. The fungus was then inoculated on Petri dishes containing 20 mL of the medium. Fully developed fertile perithecia developed after 1 month on the surface of the *Protea* flowers.

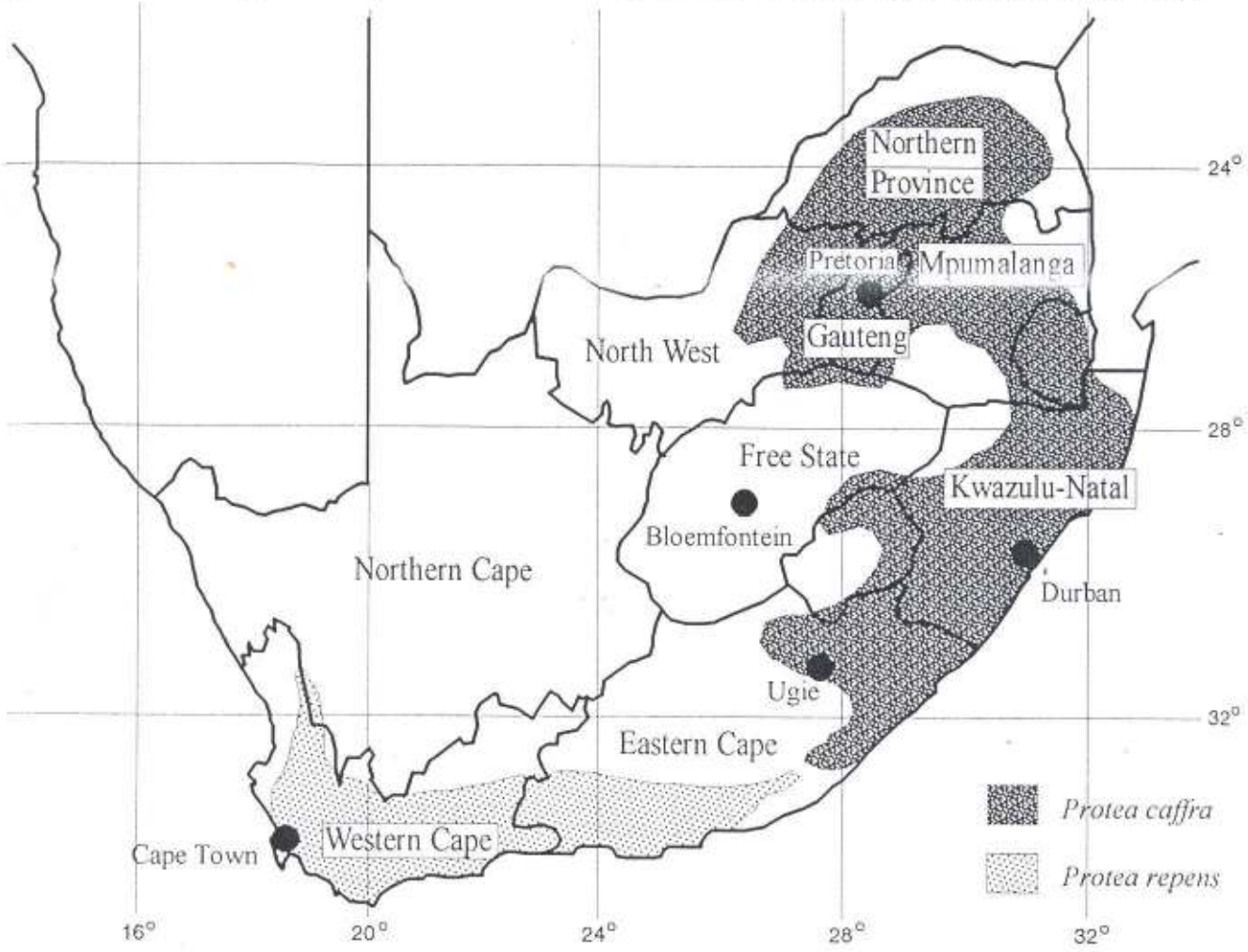
Single ascospore isolates were obtained by using surface sterilized perithecia from which the necks were removed. The oozed spore masses were suspended in heavy water (dH_2O) and plated out on MEA by using the dilution plate method. After 24 h, single germination tubes of 30 single spores were removed from the plates with a sterile needle and cultivated on MEA. An isolate was randomly chosen as the main isolate and plated out together with the remaining 29 isolates, by placing 4 mm diameter disks of 1-week-

Received April 5, 1996.

G.J. Marais[1] and M.J. Wingfield. Department of Microbiology and Biochemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein, South Africa.

[1] Author to whom all correspondence should be addressed at Foodtek CSIR, P.O. Box 395, Pretoria 1000, South Africa. e-mail: gmarais@csir.co.za

Fig. 1. Distribution of *P. caffra* and *P. repens* with which different groups of ophiostomatoid fungi are associated (Rourke 1980).



old cultures together on *P. repens* flower medium, 0.5 cm from each other.

Growth rate of the ophiostomatoid fungus from *P. caffra* was determined by inoculating Petri dishes containing 2% MEA with mycelial plugs from 1-week-old cultures. Plates (three per temperature) were then incubated at temperatures ranging from 5 to 30°C at 5°C intervals. Colony diameters were measured after 8 days and averages calculated. Cycloheximide tolerance was tested by inoculating six plates containing different concentrations (0, 0.05, 0.1, 0.5, and 2.5 g per 1000 mL MEA) of cycloheximide. Colony diameters were measured after 8 days at 25°C and average growth at each concentration was calculated.

Specimens were prepared for scanning electron microscopy (SEM) by fixing fungal material on agar blocks in 3% glutaraldehyde and 1% osmium tetroxide in a sodium phosphate buffer (pH 7). The material was dehydrated in a graded acetone series and then critical point dried and coated with gold palladium. Examination of material was with a JSM 6400 scanning electron microscope.

Results

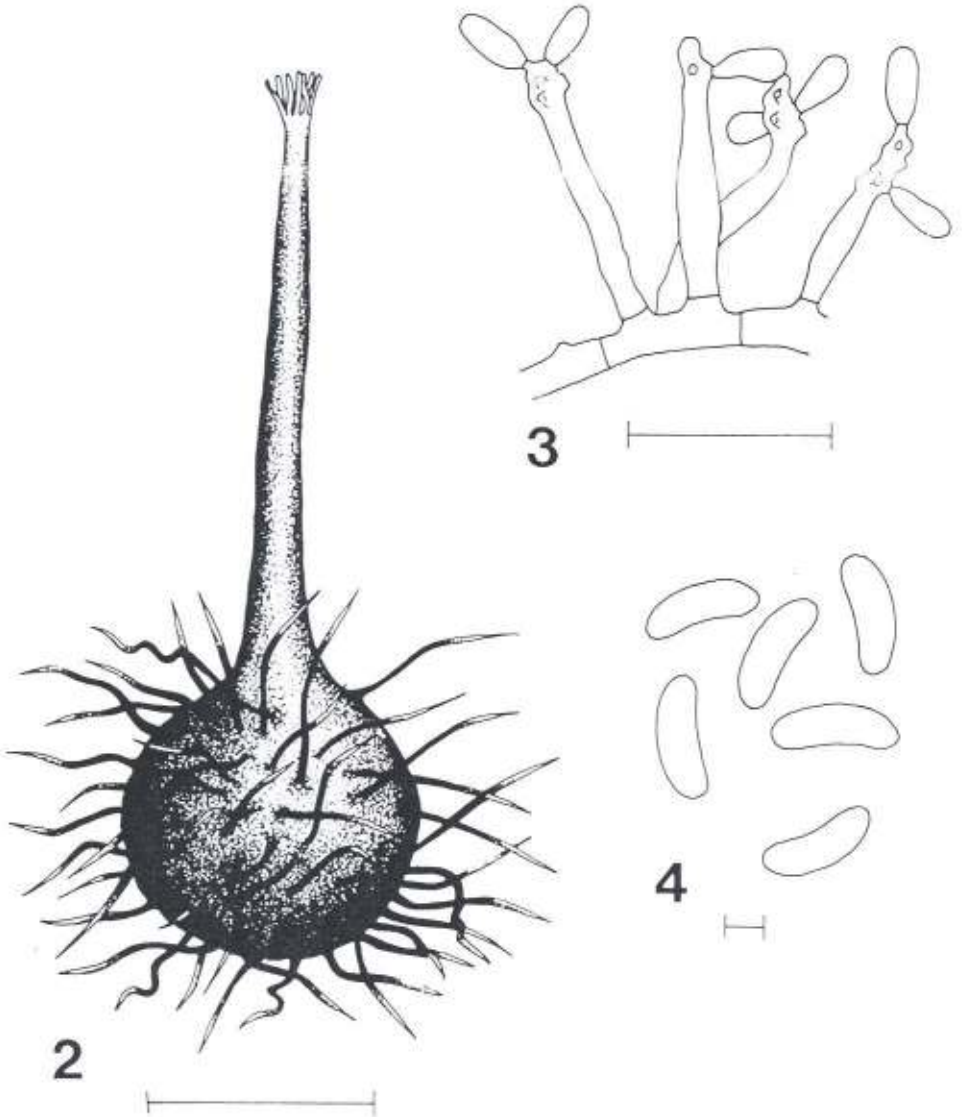
A single ophiostomatoid fungus occurred in the infructescences of *P. caffra* in all three provinces where material was collected. The fungus has an obvious *Sporothrix* anamorph and is best placed in *Ophiostoma*. This species of *Ophiostoma* is distinct from all others associated with *Protea* infructescences and can also be distinguished from other species in *Ceratocystis* s.l. Therefore, we consider it to represent a new taxon and provide the following descriptions for the teleomorph and anamorph states.

Ophiostoma protearum Marais & M.J. Wingf. sp.nov. Perithecia genita in hospitis textu vel in agari patellis continentibus flores *Protea repens* minutim dissectos. Perithecia superficialia in mycelio; bases anthracinae, globosae, hyphali ornatione, 93–167 ($\bar{x}$ = 130) μm diametro; colla anthracina, glabro-tunicata, 217–267 ($\bar{x}$ = 245) μm longa cum matura sunt, 19–31 ($\bar{x}$ = 26) μm lata ad basim, 6–18 ($\bar{x}$ = 9) μm lata ad apicem, digredientibus hyphis ostiolaribus 14–19 ($\bar{x}$ = 17) μm longis. Asci evanescentes. Ascosporae unicellulares, hyalinae, lunatae, vaginis absentibus, 3–4 ($\bar{x}$ = 3.3) × 1–1.5 μm. HOLOTYPUS: PREM 51886.

Perithecia produced on host tissue or on agar plates containing shredded *Protea repens* flowers (Figs. 2, 5). Perithecia superficial on the mycelium; bases black, globose, hyphal ornamentation, 93–167 ($\bar{x}$ = 130) μm in diameter; necks black, smooth walled, 217–267 ($\bar{x}$ = 245) μm long, 19–31 ($\bar{x}$ = 26) μm wide at the base, 6–18 ($\bar{x}$ = 9) μm wide at the apex, ostiolar hyphae 14–19 ($\bar{x}$ = 17) μm long (Fig. 6). Asci evanescent. Ascospores one-celled, hyaline, lunate, sheaths absent, 3–4 ($\bar{x}$ = 3.3) × 1–1.5 μm (Figs. 4, 7).

SPECIMENS EXAMINED: On flowers within infructescences

Figs. 2–4. *Ophiostoma protearum*. Fig. 2. Perithecium with a moderately long neck, divergent ostiolar hyphae at the apex and hyphal ornamentation at the base. Scale bar = 100 μm. Fig. 3. *Sporothrix* anamorph with denticulate conidiogenous cells and smooth walled clavate, hyaline conidia. Scale bar = 10 μm. Fig. 4. One-celled, lunate ascospores without sheaths. Scale bar = 1 μm.



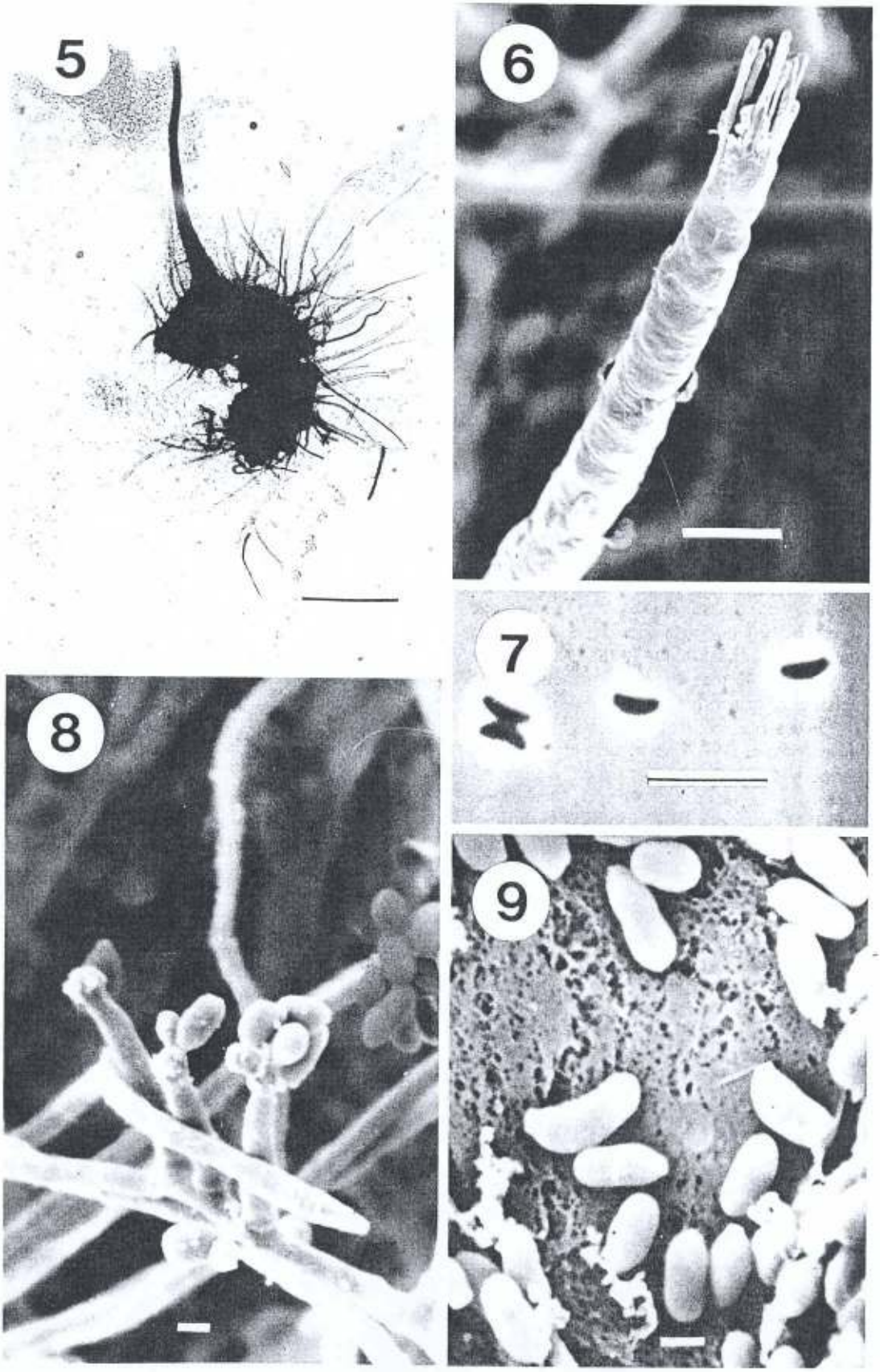
of *P. caffra* infested with insects, Drakensberg Mountains, Kwazulu-Natal, South Africa, 6 July 1992, G.J. Marais, HOLOTYPE: PREM 51886, PARATYPES: PREM 51888 on insect-infested *P. caffra* flowers, Ugie, Eastern Cape Province, 31 January 1992, G.J. Marais; PREM 51890 on insect-infested *P. caffra* flowers, Pretoria, Gauteng, 29 September 1987, M.J. Wingfield.

Sporothrix protearum Marais & Wingf. sp.nov.
Coloniae in MEA 2–2.5 ($\bar{x}$ = 2.3) cm diametro post 8 dies ad 25°C, albae vel cremeae. Incrementum deminutum ad temperaturas supra 25°C vel infra 30°C. Sporulatio profusa in 2% MEA. Cellas conidiogenas hyalina, septata, 1.5–2.5 ($\bar{x}$ = 1.9) μm crassa et 6–16 ($\bar{x}$ = 10.8) μm longa, prolificantur sympodice cum conidia formantur, denticulescentes; denticulae 0.5–1 ($\bar{x}$ = 0.9) μm longae. Conidia quoque nata sunt in huphis ipsis. Conidia holoblastica, hyalina, unicellularia, clavata, glabra, tenuitunicata, 3.5–4.5 ($\bar{x}$ = 3.9) × 1–2 ($\bar{x}$ = 1.5) μm, formata singulatim, aggregascentia in massis mucosis. HOLOTYPUS: PREM 51887.

Colonies on MEA 2–2.5 ($\bar{x}$ = 2.3) cm in diameter after 8 days at 25°C, white to creamy white. Growth reduced at temperatures below 25°C and above 30°C. Sporulation profuse on 2% MEA. Conidiogenous cells arising directly from the surface of the agar or from aerial mycelium, hyaline, septate, 1.5–2.5 ($\bar{x}$ = 1.9) μm thick and 6–16 ($\bar{x}$ = 10.8) μm long, proliferating sympodially, becoming denticulate; denticles 0.5–1 ($\bar{x}$ = 0.9) μm long (Figs. 3, 8). Conidia also produced directly on hyphae. Conidia holoblastic, hyaline, one-celled, clavate, sometimes curved, smooth, thin walled, 3.5–4.5 ($\bar{x}$ = 3.9) × 1–2 ($\bar{x}$ = 1.5) μm formed singly, becoming aggregated in slimy masses (Fig. 9).

SPECIMENS EXAMINED: From perithecia on flowers in insect infested *P. caffra* infructescences, Drakensberg Mountains, Kwazulu-Natal, South Africa, 6 July 1992,

Figs. 5–9. *Ophiostoma protearum*. Fig. 5. Perithecium. Scale bar = 100 μm. Fig. 6. Apex of perithecium neck showing the presence of ostiolar hyphae. Scale bar = 10 μm. Fig. 7. Lunate ascospores without sheaths. Scale bar = 10 μm. Fig 8. Conidia produced on denticulate conidiogenous cells. Scale bar = 1 μm. Fig. 9. Clavate conidia with distinct points of attachment Scale bar = 1 μm.



G.J. Marais, HOLOTYPE: PREM 51887, PARATYPES: PREM 51889 on insect infested *P. caffra* flowers, Ugie, Eastern Cape Province, 31 January 1992, G.J. Marais; PREM 51891 on insect-infested *P. caffra* flowers, Pretoria, Gauteng, 29 September 1987, M.J. Wingfield.

Ophiostoma protearum was relatively tolerant to cycloheximide in culture. Mean colony diameter declined from 2.2 cm in the absence of cycloheximide to 1.3 cm on plates containing 2.5 g cycloheximide per 1000 mL MEA.

Discussion

Ophiostoma splendens and *O. protearum* are the only two *Ophiostoma* species with *Sporothrix* anamorphs associated with *Protea* infructescences. These two species can be distinguished from each other based on a number of morphological characteristics. *Ophiostoma splendens* has perithecial necks 143–217 μm long, ostiolar hyphae are absent, ornamentation is absent on the ascomatal bases, and ascospores are 5.5–6 μm long (Marais and Wingfield 1994). In contrast, *O. protearum* has perithecial necks that are 217–265 μm long, ostiolar hyphae are present, hyphal ornamentation is present on the ascocarp bases, and ascospores are an average of 3–4 μm long. Both species have anamorphs in *Sporothrix*, but conidium size differs between them.

The morphological characteristics of *O. protearum* resemble those of *Ophiostoma ulmi* (Buism.) Nannf. and *Ophiostoma novo-ulmi* Brasier (1991). The perithecium neck lengths fall in the same range, all have ostiolar hyphae and ascospores are similar in shape. However, *O. ulmi* and *O. novo-ulmi* have *Graphium* Corda as well as *Sporothrix* anamorphs, ornamentation of the bases in the latter species are bristly as opposed to the long hyphal ornamentation of *O. protearum*, and the ascospores are longer (4.5–6 μm) than those of *O. protearum*. Moreover, these fungi cause Dutch elm disease (Brasier 1991), a niche very different from that associated with *O. protearum*.

Ceratocystis tenella Davidson (1979) and *Ophiostoma narcissi* Limber (1950) have neck lengths similar to those of *O. protearum*. However, both these species differ from *O. protearum* in the absence of hyphal ornamentation on the bases of the ascocarps. *Ceratocystis tenella* has lunate ascospores such as those of *O. protearum*, but these differ in size for the two species. *Ophiostoma narcissi* is different in having ellipsoid to ovoid ascospores. The neck lengths of *Ceratocystis denticulata* Davidson (1979), *Ceratocystis angusticollis* Wright & Griffin (Upadhyay 1981), and *Ophiostoma valdivianum* (Butin) Rulamort (Butin and Aquilar 1984) are similar to those of *O. protearum*. *Ceratocystis denticulata*, however, has allantoid ascospores and ascospores in *C. angusticollis* are reniform. Both species lack ostiolar hyphae as well as hyphal ornamentation on the bases of ascocarps. *Ophiostoma valdivianum* is described as having cylindrical ascospores but the illustration given in Butin and Aquilar (1984) shows lunate ascospores. This fungus, however, differs from *O. protearum* in the presence of a *Leptographium* anamorph and the absence of ornamentation at the bases of perithecia.

Ophiostoma protearum is the fourth ophiostomatoid fungus to be associated with *Protea* infructescences. Two of the species, *Ceratocystiopsis proteae* and *Ophiostoma capense*, have *Knoxdaviesia* anamorphs and are morphologically very similar to each other, differing only in ascospore shape. These species are also sensitive to cycloheximide (Wingfield and Van Wyk 1993; Wingfield et al. 1988), which is characteristic of species of *Ceratocystis* but not species of *Ophiostoma* and *Ceratocystiopsis*. This has led to confusion regarding their generic placement (Wingfield 1993). In contrast, *O. protearum* and *O. splendens* have *Sporothrix* anamorphs, are relatively tolerant to cycloheximide, and are considered to be closely related and similar to species of *Ophiostoma* occurring in the Northern Hemisphere.

Ophiostoma protearum is consistently found among populations of *P. caffra* that are geographically widespread across Southern Africa (Fig. 1). In contrast, *O. splendens*, which is most similar to *O. protearum*, is apparently restricted to the Western Cape Province but has a wide host range amongst species of the Proteaceae in that area (Marais and Wingfield 1994). The basis of this host specificity remains unknown but is thought to be associated with specific insect vectors, as yet unidentified, that visit flowers.

The discovery of *O. protearum* adds further credence to the supposition of Marais and Wingfield (1994) that two distinct lineages of ophiostomatoid fungi have evolved in association with *Protea* spp. in Southern Africa. Those species with *Knoxdaviesia* anamorphs appear to be unique and unrelated to other ophiostomatoid fungi (Hausner et al. 1993; Wingfield 1993). *Ophiostoma protearum* and *O. splendens* are, at least based on morphology, more similar to *Ophiostoma* spp. If this is indeed so, the question of this origin is an intriguing one and the possibility of a common ancestor for these and species found in the Northern Hemisphere seems probable. We are currently in the process of comparing *Ophiostoma* spp. from Proteaceae with other *Ophiostoma* spp. on the basis of their DNA and hope that this will improve our understanding of these fascinating fungi.

Acknowledgement

The authors gratefully acknowledge the financial support of the Foundation for Research Development.

References

Brasier, C.M. 1991. *Ophiostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. Mycopathologia, **115**: 151–161.

Butin, H., and Aquilar, A.M. 1984. Bluestain fungi on *Nothofagus* from Chile: including two new species of *Ceratocystis* Ellis & Halst. Phytopathology, **109**: 80–89.

Davidson R.W. 1979. A *Ceratocystis* associated with an ambrosia beetle in *Dendroctonus*-killed pines. Mycologia, **71**: 1085–1089.

de Hoog, G.S., and Scheffer, R.J. 1984. *Ceratocystis* versus *Ophiostoma*. A reappraisal. Mycologia, **76**: 292–299.

Hausner, G., Reid, J., and Klassen, G.R. 1993. *Ceratocystiopsis*: a reappraisal based on molecular criteria. Mycol. Res. **97**: 625–633.

Limber, D.P. 1950. *Ophiostoma* on *Narcissus* bulbs. Phytopathology, **40**: 493–496.

Marais, G.J., and Wingfield, M.J. 1994. Fungi associated with infructescences of *Protea* species in South Africa including a new species of *Ophiostoma*. Mycol. Res. **98**: 369–374.

Minter, D.W., Kirk, P.M., and Sutton, B.C. 1982. Holoblastic phialides. Trans. Brit. Mycol. Soc. **79**: 75–93.

Minter, D.W., Kirk, P.M., and Sutton, B.C. 1983. Thalic phialides. Trans. Brit. Mycol. Soc. **80**: 39–66.

Rourke, J.P. 1980. The Proteaceae of South Africa. Purnell, Cape Town, South Africa.

Samuels, G.J. 1993. The case for distinguishing *Ceratocystis* and *Ophiostoma*. *In Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. *Edited by* M.J. Wingfield, K.A. Seifert, and J.F. Webber. American Phytopathological Society Press, St. Paul, Minn. pp. 14–19.

Upadhyay, H.P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens, Ga. 176 pp.

Upadhyay, H.P., and Kendrick, W.B. 1975. Prodromus for a revision of *Ceratocystis* (Microascales, Ascomycetes) and conidial states. Mycologia, **67**: 799–805.

Wingfield, M.J. 1993. Problems in delineating the genus *Ceratocystiopsis*. *In Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. *Edited by* M.J. Wingfield, K.A. Seifert, and J.F. Webber. APS Press, St. Paul, Minn. pp. 21–26.

Wingfield, M.J., and Van Wyk, P.S. 1993. A new species of *Ophiostoma* from *Protea* infructescences in South Africa. Mycol. Res. **97**: 709–716.

Wingfield, M.J., Van Wyk, P.S., and Marasas, W.F.O. 1988. *Ceratocystiopsis proteae* sp. nov., with a new anamorph genus. Mycologia, **80**: 23–30.