

**ECOLOGY AND SYSTEMATICS OF SOUTH AFRICAN *PROTEA*-  
ASSOCIATED *OPHIOSTOMA* SPECIES**

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## DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or part been submitted at any university for a degree.

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F. Roets

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Date

*"Life did not take over the globe by combat, but by networking"*

(Margulis and Sagan 1986)

## SUMMARY

The well-known, and often phytopathogenic, ophiostomatoid fungi are represented in South Africa by the two phylogenetically distantly related genera *Ophiostoma* (Ophiostomatales) and *Gondwanamyces* (Microascales). They are commonly associated with the fruiting structures (infructescences) of serotinous members of the African endemic plant genus *Protea*. The species *O. splendens*, *O. africanum*, *O. protearum*, *G. proteae* and *G. capensis* have been collected from various *Protea* spp. in South Africa where, like other ophiostomatoid fungi, they are thought to be transported by arthropod vectors.

The present study set out to identify the vector organisms of *Protea*-associated members of mainly *Ophiostoma* species, using both molecular and direct isolation methods. A polymerase chain reaction (PCR) and taxon specific primers for the two *Protea*-associated ophiostomatoid genera were developed. Implementation of these newly developed methods revealed the presence of *Ophiostoma* and *Gondwanamyces* DNA on three insect species. They included a beetle (*Genuchus hottentottus*), a bug (*Oxycarenus maculatus*) and a psocopteran species. It was, however, curious that the frequency of these insects that tested positive for ophiostomatoid DNA was very low, despite the fact that ophiostomatoid fungi are known to colonise more than 50% of *Protea* infructescences. Subsequent direct isolation methods revealed the presence of reproductive propagules of *Ophiostoma* spp. on four *Protea*-associated mite species (*Oodinychus* sp., two *Tarsonemus* spp. and *Proctolaelaps vandenbergi*). These mites are numerous within *Protea* infructescences and *Ophiostoma* spp. were isolated from a high frequency of these individuals. The *Oodinychus* sp. mite was found to vector most of the *Protea*-associated *Ophiostoma* species. It was thus postulated that the mites (in particular the *Oodinychus* sp.) act as primary vectors of the *Protea*-associated *Ophiostoma* species. The association between *Oodinychus* mites collected from *P. repens* and *O. splendens* proved to be mutualistic. Mites feeding on this fungus showed significantly higher population growth than mites feeding on any of the other fungal species tested.

The short- and long-distance dispersal methods of these mites were also investigated. Firstly the ability of mites to move from drying infructescences to moist and sheltered

areas such as provided by intact infructescences on the same plant was investigated experimentally. Significantly more mites were found to actively disperse from drying infructescences to artificially manufactured infructescences containing moistened filter paper shreds than to artificially manufactured infructescences containing dry filter paper shreds. The frequent fires associated with the habitat of these mites would, however, require movement over larger areas than what would be possible through self-dispersal. Dispersal of mites via air currents was thus investigated using sticky traps, but no *Ophiostoma*-vectoring mites were captured in this way. Self-dispersal aided by air currents could thus be ruled out, and our investigations shifted to vectored dispersal. Numerous insects emerging from *Ophiostoma*-containing *P. repens* and *P. neriifolia* infructescences were collected using specially designed emergence cages. Scanning electron microscopy and stereo-microscopy revealed that all three *Ophiostoma*-vectoring mite genera were phoretic on the beetle *G. hottentottus*. *Tarsonemus* spp. and *P. vanderbergi* were also phoretic on the beetles *Trichostetha fascicularis* and *T. capensis* associated with *P. repens* and *P. neriifolia* flowers. Mites collected from the surface of these beetles were found to vector reproductive propagules of various *Ophiostoma* spp. This thus seems to be the only method of long-distance dispersal of these mites and subsequently also the *Protea*-associated *Ophiostoma* species.

Molecular phylogenetic reconstruction based on large subunit, ITS and beta-tubulin DNA sequence data suggests a polyphyletic origin for the *Protea*-associated members of *Ophiostoma*, which proposes multiple invasions of this unusual niche by these fungi. These studies also revealed the presence of four new species of *Ophiostoma* associated with *Protea* spp. The new species *O. palmiculminatum*, *O. phasma*, *O. gemellus* and *Sporothrix variecibatus* were thus described. *Ophiostoma palmiculminatum* is associated with *P. repens* infructescences and the *Oodinychus* mites collected from them. *Ophiostoma phasma* was collected from various *Protea* and mite species. *Ophiostoma gemellus* and *Sporothrix variecibatus* were initially only isolated from mites, but have subsequently also been isolated from *Protea* spp.

The present study clarifies many aspects pertaining to the phylogeny and ecology of the interesting members of *Ophiostoma* associated with *Protea* hosts. As such this

study will form the platform for further studies on the co-evolution of these insect / mite / fungi / plant associations.

## OPSOMMING

Die bekende, en dikwels fitopatogene, ophiostomatoïde fungi is in Suid Afrika verteenwoordig deur die twee filogeneties verlangs-verwante genera *Ophiostoma* (Ophiostomatales) en *Gondwanamyces* (Microascales). Hulle word algemeen geassosieer met die vrugstrukture (saadkoppe) van die saadhoudende lede van die Afrika-endemiese plant genus *Protea*. Die spesies *O. splendens*, *O. africanum*, *O. protearum*, *G. proteae* en *G. capensis* is op verskeie *Protea* spp. in Suid Afrika versamel, waar hulle, soos ander ophiostomatoïde fungi, waarskynlik deur geleedpotige diere-vektore vervoer word.

Die huidige studie het ten doel gehad om die vektor-organismes van die Suid Afrikaanse lede van hoofsaaklik *Ophiostoma* spesies te identifiseer deur die gebruik van beide molekulêre en direkte isolasie metodes. 'n Polimerase ketting-reaksie (PKR) en takson-spesifieke voorvoeders vir die *Protea*-geassosieerde ophiostomatoïde genera is ontwikkel. Implimentasie van hierdie nuut-ontwikkelde metodes het die aanwesigheid van *Ophiostoma* en *Gondwanamyces* DNS op drie insek spesies aangedui. Hulle sluit die kewer (*Genuchus hottentottus*), 'n besie (*Oxycarenus maculatus*) en 'n boekluis spesie in. Die frekwensie van hierdie insekte wat positief getoets het vir ophiostomatoïde DNS was egter baie laag, ten spyte daarvan dat ophiostomatoïde fungi bekend is om meer as 50% van *Protea* saadkoppe te koloniseer. Latere direkte isolasie het die aanwesigheid van reprodktiewe eenhede van *Ophiostoma* spesies op vier *Protea*-geassosieerde myt spesies (*Oodinychus* sp., twee *Tarsonemus* spp. en *Proctolaelaps vandenbergi*) aangetoon. Hierdie myt spesies is vollop binne meeste *Protea* vrugkoppe, en *Ophiostoma* spp. is vanaf 'n hoë frekwensie van hierdie individue geïsoleer. Daar is gevind dat die *Oodinychus* sp. myt meeste van die *Protea*-geassosieerde *Ophiostoma* spesies vektor. Dit is dus gepostuleer dat die myte (en spesifiek die *Oodinychus* sp.) as primêre vektor van die *Protea*-geassosieerde *Ophiostoma* spesies optree. Daar is gevind dat die assosiasie tussen *Oodinychus* myte vanaf *P. repens* en *O. splendens* mutualisties is. Myte wat op hierdie fungus voed het beduidend hoër populasie groei getoon as myte wat op enige van die ander fungus spesies wat getoets is gevoed het.

Die kort- en langafstand verspreidingsmetodes van hierdie myte is ook ondersoek. Eerstens is die vermoë van myte om te beweeg vanaf uitdrogende saadkoppe na klam, beskutte areas soortgelyk aan dié verskaf deur heel saadkoppe van dieselfde plant eksperimenteel ondersoek. Beduidend meer myte het aktief versprei vanaf die uitdrogende saadkoppe na die kunsmatig geproduseerde saadkoppe wat klam filtreerpapier repe bevat het as na die kunsmatige saadkoppe met droë filtreerpapier repe. Die gereelde vure wat met die habitat van hierdie myte geassosieer word sou egter beweging oor groter areas verg as wat deur self-verspreiding moontlik sou wees. Verspreiding van myte via lugstrome is dus ondersoek deur gebruik te maak van gomlokvalle, maar geen *Ophiostoma*-draende myte is op hierdie wyse gevang nie. Self-verspreiding met behulp van lugstrome kon dus uitgesluit word, en die ondersoek het verskuif na verspreiding deur vektore. Die groot hoeveelheid insekte wat verskyn het vanuit *Ophiostoma*-draende *P. repens* en *P. neriifolia* saadkoppe is versamel in spesiaal ontwerpte uitkruip-hokke. Skandeerelektron-mikroskopie en stereo-mikroskopie het aangetoon dat al drie *Ophiostoma*-draende myt genera foreties is op die kewer *G. hottentottus*. *Tarsonemus* spp. en *P. vandenbergi* is ook foreties op die kewers *Trichostetha fascicularis* en *T. capensis* wat met *P. repens* en *P. neriifolia* blomme geassosieer was. Daar is gevind dat myte wat van die oppervlak van hierdie kewers versamel is reprodusiewe eenhede van verskeie *Ophiostoma* spp. dra. Hierdie is dus skynbaar die enigste metode van langafstand-verspreiding van hierdie myte en dus ook die *Protea*-geassosieerde *Ophiostoma* spesies.

Molekulêr-filogenetiese rekonstruksie gebaseer op groot subeenheid, ITS en beta-tubulien DNS basisvolgorde data stel 'n polifiletiese oorsprong vir die *Protea*-geassosieerde lede van *Ophiostoma* voor, wat dus suggereer dat hierdie ongewone nis meermale deur hierdie fungie betree is. Hierdie studies het ook die aanwesigheid van vier nuwe *Protea*-geassosieerde *Ophiostoma* spesies aangedui. Die nuwe spesies *O. palmiculminatum*, *O. phasma*, *O. gemellus* en *Sporothrix variecibatus* is dus beskryf. *Ophiostoma palmiculminatum* is geassosieer met *P. repens* vrugkoppe en *Oodinychus* myte wat uit hulle versamel is. *Ophiostoma phasma* is vanaf verskeie *Protea* en myt spesies versamel. *Ophiostoma gemellus* en *Sporothrix variecibatus* is aanvanklik slegs vanaf myte geïsoleer, maar is later ook vanaf *Protea* spesies, geïsoleer.



Die huidige studie verduidelik verskeie aspekte met betrekking tot die filogenie en ekologie van die interessante lede van *Ophiostoma* wat met *Protea* gashere geassosieer is. As sulks sal hierdie studie die basis vorm vir verdere studies op die ko-evolusie van hierdie insek / myt / fungi / plant assosiasies.

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## Chapter 1: Introduction

The species-rich Fungal Kingdom is thought to be at least 900 million years old (Blackwell 2000, Berbee and Taylor 2001, Heckman *et al.* 2001) and some approximations estimate that there are over 1.5 million extant species (Hawksworth 2001). It is estimated that only about 5–7 percent of these have been described yet (Hawksworth 1991, 2004, Crous *et al.* 2006). Fungi are encountered in virtually every aerobic environment and have evidently been highly successful colonists. Since the successful dissemination of fungal spores is critical to the survival of a species, they have evolved many specialised abiotic (anemophily and hydrophily) and biotic (vectored) dispersal mechanisms (Ingold 1953, Kendrick 1999).

Clearly one cannot deal with all of the many different dispersal modes of fungi in extensive detail within the restrictive bounds of a thesis. Therefore, each of these broad topics will only be briefly introduced in general terms. Thereafter, I will focus the review on vectored dispersal of fungal propagules by arthropods, because this is most germane to the thesis topic. In many instances these interactions have lead to interesting symbiotic arthropod-fungus relationships, some of which I will briefly discuss. Greater focus is directed towards the ophiostomatoid fungi and their arthropod associations. The last part of this review introduces the ophiostomatoid fungi associated with *Protea* infructescences and outlines the objectives of the studies presented in this thesis.

### 1. ANEMOPHILOUS DISPERSAL

Spores of most fungi are dispersed *via* air currents and a cubic meter of air may contain up to 200 000 fungal spores (Gregory 1952, 1961). Air dispersal of fungal propagules can be achieved in various ways. Some ascomycetes (e.g. *Pseudoplectania*) and basidiomycetes (e.g. *Sphaerobolus*) forcibly eject spores into the air from their fruiting bodies to ensure dispersal over long distances (Walker 1927, Buller 1933, Ingold 1972). The asexual spores (conidia) of hyphomycetes are commonly borne in loosely arranged clusters or chains (e.g.

*Penicillium*) that are easily dislodged and dispersed by air currents (Zoberi 1961). Spores dispersed by air are usually dry and can travel over thousands of kilometres, often becoming a major cause of human allergies (Feinberg 1946, Nagarajan and Singh 1990).

Although anemophily is an effective means of dispersal, as supported by the number of fungal species that employ this strategy, fungi that rely on anemophilous dispersal and that are confined to specialised substrata, face certain limitations. The most obvious would be that only a small proportion of the numerous spores produced would reach suitable uncolonised sites. The vast majority of spores produced by these species are likely to perish without ever reaching suitable substrates. Focused spore dispersal towards resources that are limited in space and / or time (e.g. fresh dung) would probably give species that utilise this dispersal method a competitive advantage over species that rely solely on anemophily. Low numbers of spores need to be produced by the former group to reach new sites and greater resources can thus be directed towards other physiological processes.

## 2. HYDROPHILOUS DISPERSAL

The Chytridiomycota, Hyphochytriomycota and Oomycota are aquatic taxa with spores that are often equipped with flagella (known as zoospores) (e.g. *Pythium* and *Phytophthora*) (Matthews 1931, Middleton 1943, Carlile 1983). The small size of these spores, however, makes it unlikely that they will be dispersed far from the parental structure without the additional aid of water currents or animal involvement (Duniway 1976, Ingold 1979). In order to utilise water currents for dispersal some hyphomycetes have evolved spores that can float on the surface of water, and thus be dispersed to new substrates (Ingold 1979). Others have spores that are dispersed underneath the surface of the water (Ingold 1966, 1979). Due to the limitations posed by hydrophilous dispersal as sole dispersal mechanism (how to reach water bodies beyond those currently colonised), many species also need to be dispersed via other means. In order to overcome this problem most fungi that form specialised water-dispersed spores usually also produce spores that can be wind dispersed (Ingold 1971).



### 3. VECTORED DISPERSAL

Fungi that prefer specialised substrata (e.g. dung) or those that need to colonise areas that are not generally accessible to air or water-borne spores (e.g. the wood of living trees) need more specialised means of dispersal. Fungal species utilising such specific substrata are usually dispersed by other organisms (e.g. arthropods) that exploit these same niches (Talbot 1952, Malloch and Blackwell 1993). These so-called vector organisms are usually well-equipped (i.e. are very mobile and may have specialised olfactory abilities) to seek and colonise these often widely dispersed niches (Talbot 1952, Ingold 1971, Malloch and Blackwell 1993).

Vectored dispersal can be defined as: “*dispersal by an organism, which consciously or unconsciously aids in the dispersal of another*” (Kendrick 1999). Following this definition, almost all fungal species, even those adapted to other forms of dispersal, may be vector dispersed. A more exclusive definition may thus require the addition of an adaptation(s) to the transported organism to promote dispersal via vectors. Almost all fungal groups (ascomycetes, basidiomycetes, zygomycetes, hyphomycetes) include species that are adapted to animal dispersal.

Among vector-dispersed fungi, the mammal-vectored species are among the best known examples. Fungi dependent on mammals for dispersal include basidiomycetes (e.g. *Rhizopogon* spp.) and ascomycetes (e.g. *Tuber* spp.). They produce appealing, strong odours (Fogel and Trappe 1978) that attract diverse animal species (Maser *et al.* 1978, Viro and Sulkava 1985, Malajczuk *et al.* 1987, Launchbaugh and Umess 1992). The mammals feed on the fungal fruiting structures (Fogel and Trappe 1978, Claridge and May 1994), and disperse the fungal spores through their droppings (Trappe and Maser 1976, Claridge and May 1994).

The dispersal of fungal reproductive propagules by mammals is most notable in the above-mentioned macro-fungi, as many of these species are also sought-after delicacies for human consumption such as truffles in the genus *Tuber*. Smaller fungal species, such as those in the hyphomycetes and the majority of ascomycetes, which rely on substrates not generally accessed by mammals (e.g. the wood of dying trees), also need smaller vector organisms for dissemination of their propagules.

## VECTORED DISPERSAL BY ARTHROPODS

Arthropods play a dominant role in vectored dispersal of fungal spores (Talbot 1952) and many fungal groups that rely on arthropod vectors have evolved similar morphological traits to enhance this mode of dispersal (Chain 1972, Pirozynski and Hawksworth 1988, Malloch and Blackwell 1993). Amongst others, these morphological adaptations often include the production of spores in sticky droplets rather than the dry spores that are usually carried by air currents (Malloch and Blackwell 1993, Cassar and Blackwell 1996). Groups of fungi with such adaptations that employ arthropod vectors include the hyphomycetes, basidiomycetes, ascomycetes, zygomycetes and the non-fungal group, the myxomycetes (Ingold 1953, Kendrick 1999, Stephenson and Stempen 1994). Given that the first three fungal groups include the highest diversity of species adapted towards this mode of dispersal, the arthropod-mediated dispersal of these groups are further discussed below.

### 3A. ARTHROPOD VECTORED DISPERSAL IN THE HYPHOMYCETES

In the hyphomycetes, spores are commonly produced in sticky droplets that can adhere to the surface of small arthropods (Ingold 1971, Carmichael *et al.* 1980). Any arthropod that comes into contact with these spores can potentially act as vector of the fungal species. Examples of fungal genera that make use of this mode of dispersal include *Acremonium*, *Fusarium* and *Gliomastix*. Hyphomycetous genera such as *Gliocladium*, *Graphium*, *Leptographium*, *Pesotum* and *Stilbella* produce spores in sticky drops at the tips of long stalks (conidiophores). These elongated structures present the fungal propagules in such a way that they can easily come into contact with fairly large insects that climb over the substrate (Upadhyay 1981, Seifert 1985, Wingfield *et al.* 1993, Jacobs and Wingfield 2001). Some hyphomycetes that produce only dry spores may utilise insects in addition to air currents for the dissemination of their spores. Such species are known to produce synnemata (e.g. members of the genus *Penicillium*), which dust insects with fungal spores on contact (Abbott 2000).

### 3B. ARTHROPOD VECTORED DISPERSAL IN THE BASIDIOMYCETES

Fruiting bodies of some basidiomycetous species produce spores in viscous layers or masses. A prominent example is the stinkhorns (*Aseroë*, *Mutinus* and *Phallus*) that fabricate a strong putrid odour and produce spores that are surrounded by a sugary slime coating (Stoffalano *et al.* 1989). Flies are attracted to these fruiting bodies and they ingest the spores, while some spores also adhere to the surface of the flies. Fungal spores are vectored to novel sites, where the insects excrete them without any apparent adverse effect or damage to the spores (Stoffalano *et al.* 1989). Other basidiomycetes (e.g. *Cryptopus volvatus*) are vectored by mycophagous beetles (Borden and McClaren 1970, Castello *et al.* 1976). The spores collect on the inner surface of a sheath formed by the fungus and are dispersed to new substrata by the fungus feeding beetles.

Certain heterothallic (with more than one mating type) rust fungi have evolved elaborate ways in which to ensure vectored-dissemination of their propagules. Such adaptations could affect both the fungus itself and the substrate on which it occurs. For example, in the rust fungal genera *Uromyces* and *Puccinia*, the fungus induces its host plant to produce a pseudo-flower (a modification of leaves that resemble a flower), while also inhibiting the production of normal flowers by the host plant (Roy 1993). This pseudoflower attracts potential insect ‘pollinators’, which then act as fungal spore carriers (Craigie 1931, 1972, Roy 1993, Pfunder and Roy 2000). The fungi produce sugary nectar in which they present their gametes (Buller 1950, Roy 1994) and floral-like fragrances to attract insect vectors (Raguso and Roy 1998). The insect visits facilitate the completion of the sexual stage of the life cycle of the fungus.

The fungus *Microbotryum violaceum* (= *Ustilago violacea*) is a pathogen that causes anther-smut disease in almost 100 species of Caryophyllaceae (Thrall and Antonovics 1993). The fungus induces the production of anther-like structures that contain fungal spores instead of pollen. The fungus also destroys the ovary in the flowers, thereby sterilising the plant (Baker 1947, Uchida *et al.* 2003). The fungal spores are transmitted from diseased plants to healthy plants by visiting pollinators (Baker 1947).

### 3C. ARTHROPOD VECTORED DISPERSAL IN THE ASCOMYCETES

Almost half of the known fungal species belong to the Ascomycota, which contain more than 32 000 described species (Hawksworth *et al.* 1995). The ascomycetes are also diverse in terms of the number of fungal species adapted to spore dispersal by arthropods and the mechanisms through which this is achieved. These mechanisms represent evolutionary adaptations of distantly related fungal taxa towards dispersal by taxonomically and biologically very diverse arthropods. Mechanisms of arthropod-mediated dispersal include not only the dispersal of single spores (usually sticky), but also dispersal of whole ascomata between hosts. The interactions between some ascomycetes and arthropod vectors led to such close associations that the fungi are now considered obligate external commensalists of their hosts. A few of these arthropod-mediated dispersal mechanisms are highlighted below.

#### 3C (1) *Dispersal of whole-ascomata*

Diverse families such as Arthrodermataceae, Gymnoascaceae, Myxotrichaceae and Onygenaceae have all developed distinctive hooked, curved or barbed appendages on the peridia (Currah 1985, von Arx *et al.* 1986). These appendages attach to the hairs of insects (Grief and Currah 2003), and spores are released when the insects move about on the substrate (Currah 1985, von Arx *et al.* 1986) or during their grooming activities (Grief and Currah 2003). The adaptation of these fungi to the morphology and behaviour of their vector insects was the driving force behind the evolution of these structures in unrelated groups of cleistothecial ascomycetes (Grief and Currah 2003).

#### 3C (2) *Ascospores with sticky holdfasts*

Another fairly well-studied arthropod-fungal spore dispersal system involves the dispersal of *Pyxidiophora* and its *Thaxteriola* and *Acariniola* anamorphs (Blackwell *et al.* 1986, 1988, Blackwell and Malloch 1989). In this system, mites are responsible for carrying the fungal spores from one habitat to the next. In order to achieve this, the mites climb onto bark or dung beetles, which transport the spore-bearing mites between suitable habitats (Blackwell *et al.* 1986). The ascospores of *Pyxidiophora* spp. have evolved a special holdfast at their one end, which enables them to adhere to the mites that carry them. While

attached to the mite, the ascospores differentiate into complex thalli that also produce conidia in the form of phialoconidia (Blackwell and Malloch 1989). These phialoconidia are responsible for the inoculation of new substrates. In addition to mites, many other insects from diverse orders can also vector these fungi directly (Blackwell *et al.* 1986).

### 3C (3) *Arthropod Ectoparasites*

Most species of the ascomycete class Laboulbeniomycetes have arthropod-dependant life histories and are obligate external commensalists of arthropods (Tavares 1985, Weir and Blackwell 2001). Most have no detrimental effect on the life of their hosts (Benjamin 1971, Tavares 1985, De Kesel 1996). The fungus attaches to the integument of the host and absorbs nutrients through the cuticular pores or by active penetration of the integument (Tavares 1985, De Kesel 1996). The fungus has no free-living stages and its entire life cycle is spent on the host, with only sexual reproduction taking place (De Kesel 1996). Dispersal of the fungus occurs when uninfected insects come into contact with infected hosts, or when transferred from one host to the next by contaminated phoretic mites (Seeman and Nahrung 1999).

### 3C (4) *Sticky spore drops*

Ophiostomatoid fungi (Wingfield *et al.* 1993) usually have ascospores in viscous droplets present on long perithecial necks (Malloch and Blackwell 1993, Cassar and Blackwell 1996). The group includes diverse taxa such as members of the Ophiostomatales (eg. *Ophiostoma*) and Microascales (e.g. *Ceratocystis*) (Upadhyay 1981, Wingfield *et al.* 1993). Interestingly, in some ascomycetes, both the teleomorphs and their anamorphs (e.g. *Graphium*, *Knoxdaviesia*, *Leptographium* and *Pesotum*) may be adapted to insect spore dispersal by producing spores in sticky droplets (Ingold 1971, Carmichael *et al.* 1980, Malloch and Blackwell 1993, Wingfield *et al.* 1993). Convergent evolution has probably resulted in many unrelated ascomycete genera exhibiting similar morphological adaptations to facilitate arthropod dispersal of their spores (Münch 1907, 1908, Francke-Grosmann 1967, Whitney 1982, Beaver 1989, Malloch and Blackwell 1993, Cassar and Blackwell 1996).

#### 4. ARTHROPOD-FUNGUS SYMBIOSIS, ESPECIALLY IN THE OPHIOSTOMATOID FUNGI

Adaptations of fungi to arthropod dispersal may have induced symbiotic interactions. Symbiosis can be defined as “*the acquisition and maintenance of one or more organisms by another that results in novel structures and (or) metabolism*” (Klepzig and Six 2004). Mutualism is a form of symbiosis where the different species both benefit from association with one another. Several insect groups are known to form mutualistic relationships with fungi including certain ants (e.g. *Atta*, *Acromyrex*) (Wheeler 1907, Fisher *et al.* 1994), termites (e.g. *Termes*) (Korb and Aanen 2003), Coleoptera (beetles) (Francke-Grosmann 1967, Norris 1979, Beaver 1989, Berryman 1989), Homoptera (bugs) (Couch 1931), Hymenoptera (bees, ants and wasps, notably *Sirex* sp.) (Talbot 1977, Slippers 1998, Slippers *et al.* 2003) and Diptera (flies) (Graham 1966, Harrington 1987, Kluth *et al.* 2002). The former three groups also represent the only currently known fungus-farming insects, and their associations are reportedly ancient, dating back at least 40–60 million years (Mueller and Gerardo 2002).

##### 4A. SYMBIOSIS BETWEEN FUNGI AND BARK AND AMBROSIA BEETLES

The association between the wood boring bark and ambrosia beetles (e.g. *Dendroctonus*, *Ips* and *Xyleborus*), and their fungal symbionts, mainly ophiostomatoid fungi (*Ceratocystiopsis*, *Ceratocystis*, *Ophiostoma* and their anamorphs), have been studied in some detail (Barras and Perry 1975, Upadhyay 1981, Whitney 1982, Price *et al.* 1992, Wingfield *et al.* 1993, Cassar and Blackwell 1996, Paine *et al.* 1997, Klepzig *et al.* 2001a, 2001b, Klepzig and Six 2004). Harrington (2005) and Kirisits (2004) have provided recent reviews of the topic. These ancient (60 – 80 million years old) (Farrell *et al.* 2001) interactions have received focussed attention mainly because of the phytopathogenic nature of some of the fungal species in these systems, and their ability to kill mature trees in association with their beetle vectors (Thatcher *et al.* 1980, Drooz 1985, Brasier 1988, 1991, Price *et al.* 1992, Wingfield *et al.* 1993, Paine *et al.* 1997).

#### 4B. MECHANISMS FOR TRANSPORT

Many bark and ambrosia beetles (Curculionidae: Scolytinae) have special spore-carrying structures called mycangia (Batra 1963, Farris and Funk 1965, Farris 1969, Livingston and Berryman 1972, Beaver 1986, Furniss *et al.* 1987, Lévieux *et al.* 1991, Six 2003a), and the shared presence of these structures on the insects suggests a long co-evolutionary history between beetles and their associated fungi (Six and Paine 1999, Six 2003b). According to the definition of Six (2003a), a mycangium is any structure that consistently functions to transport specific fungi, regardless of the fine detail of the structure. She also defined three different types of mycangia on the basis of their morphological structure, namely pit mycangia, sac mycangia and setal brush mycangia (Six 2003a). This classification system is convenient, as it is independent of the fine detail (presence or absence of gland cells) within the structures (Six 2003a, Klepzig and Six 2004).

Sexual (ascospores) and asexual spores (conidia) of most mutualistic fungal species, including the ophiostomatoid fungi, are either carried within mycangia (Whitney and Farris 1970, Barras and Perry 1971, Furniss *et al.* 1990, Moser *et al.* 1995, Six 2003b, Six and Bentz 2003) or passively adhere to the cuticle of the insect (Whitney and Farris 1970, Whitney 1971, Furniss *et al.* 1990, Harrington 1993, Malloch and Blackwell 1993, Paine and Hanlon 1994). It has also been reported that ophiostomatoid spores may be carried in the gut and frass of adult beetles (Leach *et al.* 1934, Francke-Grosmann 1967, Whitney 1982, Furniss *et al.* 1990, 1995, Paine *et al.* 1997, Kopper *et al.* 2004). The insects carry the spores to new substrates and thus ensure the survival of the fungal species (Price *et al.* 1992, Paine *et al.* 1997, Wingfield *et al.* 1993, Klepzig and Six 2004, Klepzig *et al.* 2001b). Thus, many of the beetle-associated fungi are almost exclusively dependent on their vectors for transportation between host trees (Francke-Grosmann 1967, Dowding 1969, Upadhyay 1981).

#### 4C. FACTORS DRIVING SYMBIOSIS

*"Life did not take over the globe by combat, but by networking"* (Margulis and Sagan 1986). The association between organisms is extremely important in their evolution. The ophiostomatoid fungi and their vectors provide an ideal opportunity for the investigation of organisms co-evolving as a result of close association. In some instances the association

between these organisms is thought to be mutualistic as both the fungi and their vectors benefit from association with one another.

#### 4C (1) BENEFIT TO FUNGI

In addition to transportation, beetles may also help to protect fungal spores in transit against desiccation and UV light (Klepzig and Six 2004). Secretions by glandular cells within certain mycangia may selectively benefit the fungal species they include during transportation, while such secretions may negatively affect, and thus reduce the numbers, of other fungi (Barras and Perry 1971). A few ophiostomatoid species are even known to reproduce within these mycangia and force their spores from the openings as the structure is filled by the growing fungi (Barras 1975).

Another benefit to the fungi is that in some cases, as in the case of ambrosia beetles, fungal growth is encouraged by the ability of the beetles to actively care for their fungal ‘gardens’. This ability of the beetle also seems to protect the symbiotic fungi from antagonistic or unwanted fungal species (Francke-Grosman 1967, Beaver 1989). This results in a dominance of the mycangial fungi within the beetle galleries on which both the adults and larvae feed (Francke-Grosman 1967, Beaver 1989). Other ophiostomatoid fungi may improve spore production when growing on the frass of bark beetles that vector them (Goldhammer *et al.* 1989). In both of these cases the beetles may enhance the ability of their fungal associates to survive and reproduce.

Interactions among different fungi in the bark beetle system may depend on external factors (Klepzig *et al.* 1991, Bronstein 1994a, 1994b, Callaway and Walker 1997, Haberkern *et al.* 2002, Kopper *et al.* 2004). Klepzig *et al.* (2004) and Klepzig and Six (2004) have, for example, demonstrated that the results of competition experiments between various bark beetle-associated fungi (e.g. *Ceratocystiopsis ranaculosus* and *Ophiostoma minus*) differ in relation to different water potentials. Thus changes in water potential (e.g. when a tree dies) are likely to affect the ability of the fungi to compete with one another (Webb and Franklin 1978, Klepzig and Six 2004).



#### 4C (2) BENEFIT TO THE INSECTS

Beetles benefit from this mutualism in that the fungal symbionts may supplement beetle nutrition (Baker and Norris 1968, Barras 1973, Furniss and Carolin 1977, Strongman 1982, Fox *et al.* 1993, Coppedge *et al.* 1995, Six and Paine 1998, Eckhardt *et al.* 2004) by concentrating nitrogen (Ayres *et al.* 2000) and by providing sterols for hormone synthesis and egg production (Clayton 1964, Svoboda *et al.* 1978, Strongman 1982, Coppedge *et al.* 1995, Morales-Ramos *et al.* 2000). This is important, since many of these wood-boring beetles feed in nutritionally poor substrates such as xylem and bark (Franke-Grosman 1967, Wood 1982, Beaver 1989).

Apart from the above-mentioned direct benefits to the bark beetles resulting from associations with fungi, some indirect benefits have also been identified. Most notable is the association between certain bark beetles (e.g. *Scolytus* spp.) and *Ophiostoma novo-ulmi*, the causal agent of Dutch elm disease (Webber and Brasier 1984, Brasier 1991). The beetles are regarded as secondary colonisers of elm trees (*Ulmus* spp.), only colonising stressed trees (Postner 1974). The beetles vector the fungi from diseased to healthy trees where they feed on the bark of twigs in the crown of healthy trees (Webber and Brasier 1984). The fungus can kill infected trees and subsequently provide more suitable breeding sites for the beetles (Postner 1974, Webber and Brasier 1984).

Most xylem-feeding bark beetles (or ambrosia beetles) are totally dependant on their associated fungi, and it has been shown that it is possible to rear the beetles on a diet of the fungal symbionts only (Franke-Grosman 1967, Norris 1979, Beaver 1989). In this case the association between the bark beetles and their fungal symbionts can be described as being mutualistic. The beetles actively care for their fungal 'gardens' and protect them from 'weed' fungi (Franke-Grosman 1967, Beaver 1989), and in return the fungi provide the exclusive source of food to the adult beetles and larvae (Franke-Grosman 1967, Norris 1979, Beaver 1989, Berryman 1989).

Phloem is much richer in nutrients than xylem, and most phloem-feeding bark beetles probably feed mainly on the phloem of their host trees (Kirisits 2004). It is thus unlikely that phloem-feeding bark beetles are obligate fungal feeders (Franke-Grosman 1967, Whitney 1982). It has, for example, been shown that the beetle *Dendroctonus frontalis*

construct greater numbers of galleries and lay more eggs (and at faster rates) than beetles not associated with their mycangial fungi (Goldhammer *et al.* 1990). The beetle, therefore, retained the ability to reproduce without its symbiotic fungus. The production of nutrients by the fungi in this system may thus only supplement the diet of the beetles and result in beetles being more fit for flight, gallery construction, mating etc. This boost of nutrients supplied by the fungi may then lessen the amount of phloem tissue needed to complete their development and allow for shorter galleries in the wood (Harrington 2005). This will result in reduced competition between the bark beetles and other wood boring beetles (Harrington 2005).

Reasons for the symbiotic association between phloeophagous bark beetles and their associated fungi could also, in some instances, reside with the protection of the beetle galleries from invasion by detrimental fungi. It has been shown that the mycangial fungi of *Dendroctonus frontalis* protect the developing beetle larvae from antagonistic fungal species (e.g. *O. minus*) when competing for resources (Klepzig and Wilkens 1997, Klepzig 1998). This may suggest a mutualistic association between *Ophiostoma minus* and *D. frontalis*.

#### 4C (3) SECONDARY VECTORSHIP BY MITES

The complexity of the interactions between ophiostomatoid fungi and bark beetles cannot be fully understood before the impact that other organisms have on this system is evaluated. In this regard the importance of mites as potential vectors of ophiostomatoid fungi should not be underestimated (Moser *et al.* 1989, Lévieux *et al.* 1989, Klepzig *et al.* 2001a, 2001b, Klepzig and Six 2004). Over 90 species of mites are, for instance, associated with the southern pine beetle *Dendroctonus frontalis*, 14 of which are phoretic on the beetle (Moser and Roton 1971, Moser 1976a). Many of these phoretic mites are fungivorous, and may thus also carry fungal propagules (Moser and Roton 1971, Moser *et al.* 1971, 1974, Moser and Bridges 1986, Lévieux *et al.* 1989, Moser *et al.* 2005).

Amongst the contingent of phoretic mites on *D. frontalis*, species of the genus *Tarsonemus* (*T. ips*, *T. krantzii* and *T. fusarii*) are of special interest. They are not injurious to the beetle while in transit (Moser and Roton 1971, Smiley and Moser 1974, Moser 1976b, Bridges and Moser 1983, Moser and Bridges 1986), but may impact the beetles indirectly by

transporting additional fungal spores (Lombardero *et al.* 2000, Lombardero *et al.* 2003). The *Tarsonemus* mites possess specialised spore-carrying structures (sporothecae) that have been shown to frequently contain spores of the ophiostomatoid fungi (e.g. *Ophiostoma minus* and *Ceratocystis ranaculosus*) (Bridges and Moser 1983, Moser 1985, Moser *et al.* 1995). These mites have positive population growth rates when feeding on *O. minus* and *C. ranaculosus*, suggesting a mutualistic association between the mites and their phoretic fungi (Lombardero *et al.* 2000).

How mites affect the survival and reproduction of the bark beetles that carry them is largely unknown. Nonetheless the life cycles of all three organisms are interwoven and may even be mutually dependent (Bridges and Moser 1983, Klepzig and Six 2004). Mites influence the population dynamics of *D. frontalis* by vectoring *O. minus*, a fungus that limits the success of the beetle mycangial fungi, and consequently lower the success of the beetles (Lombardero *et al.* 2000, Klepzig *et al.* 2001a, 2001b, Lombardero *et al.* 2003). Thus, these associations are very complex and include a communalism (mites and beetles), two mutualisms (mites-fungi and mycangial fungi-beetles) and competition (mite fungi vs. beetle mycangial fungi) (Lombardero *et al.* 2003).

The presence of sporothecae is not exclusively restricted to mites associated with bark and ambrosia beetles alone. *Imparipes haeseleri* and *I. apicola* (Acari: Scutacaridae) also carry fungal spores in their atrium genitale and are phoretic on wasps and wild bees (Ebermann and Hall 2003). Mites in the families Trochometridiidae and Siteroptiidae reportedly also bear specialised spore-carrying structures (Suski 1973, Lindquist 1985). The mycophagous *Siteroptes avenae* carries conidia of *Fusarium poae* in elongated internal sporothecae and together they cause the wheat disease Glume Sot (Kemp *et al.* 1996). The largely unexplored role mites play in vectoring different fungal species may thus extend to many different environments.

## 5. OPHIOSTOMATOID FUNGI ASSOCIATED WITH *PROTEA* IN SOUTH AFRICA

There are an estimated 9 000 vascular plant species (*ca.* 44 % of the southern African flora) in the Cape Floristic Region (CFR) of South Africa (Arnold and De Wet 1993, Cowling and Hilton-Taylor 1997, Goldblatt 1978, Goldblatt and Manning 2000). The flora, including the Proteaceae, is world-renowned for its remarkable species richness, and the high levels of endemism that typify it (Goldblatt 1978, Takhtajan 1986, Cowling *et al.* 1992, Linder 2003). The majority of CFR species are found in the Fynbos biome (Rutherford and Westfall 1986), with *ca.* 69 % of these species endemic to the CFR (Goldblatt and Manning 2000). This area also supports five of the 12 plant families endemic to southern Africa (Goldblatt and Manning 2000).

The Proteaceae (including the genus *Protea*) is a family with a world-wide distribution and contains about 1400 species in more than 60 genera (Rebelo 1995). The vast majority of Proteaceae species are confined to the Southern Hemisphere. It is the seventh largest vascular plant family in the CFR, with about 96.7% of its African members confined to this region (Goldblatt and Manning 2000). The 340 species in the CFR are grouped into 13 Cape-centred genera, of which ten are endemic to the region (Rourke 1998, Linder 2003).

The approximately 90 species of *Protea* are found in South Africa are not only of considerable economic importance (eco-tourism, horticulture and the dried-flower industry), but are also considered as keystone members of the CFR (Anon. 1999, Goldblatt and Manning 2000). Within the CFR, they often form the dominant elements in the landscape, both in terms of physical size and in numbers. *Protea* is considered to be ancient (36 million years old) with the species diversity in the CFR ascribed to the coexistence of species that diversified over a long period of time, rather than a recent and rapid radiation of this lineage (Reeves 2001). This implies that any organisms that are dependent on a *Protea* sp. could potentially have had a very long co-evolutionary history with this genus.

Flowers of *Protea* spp. are borne in fairly large and often colourful inflorescences. After flowering, the seeds of many species are retained on the plant (serotiny) within conspicuous, mostly tightly closed infructescences. The seeds can remain in this canopy-stored seed bank for more than five years, and are only released after fire or when the water supply between the infructescence and the rest of the plant is severed (usually when fire

kills the parent plant) (Bond 1985). Boring insects may also be significant in this regard, facilitating the premature release of seeds when feeding in infructescence bases and on the seeds contained within them.

The infructescences of *Protea* species can be considered as miniature ecosystems (Zwölfer 1979) that house different food chains and trophic levels. They contain a multitude of heterotrophic fungal species (Marais and Wingfield 1994, Lee *et al.* 2005) that represent one of the basal trophic levels. Within the infructescences they are fed upon by small arthropods, which in turn serve as nutrition for predatory arthropods and other animals. As the fungi form the basal trophic level within this unique niche and are potentially pathogenic to their hosts, these fungi merit closer study. Although many fungal species are associated with *Protea* species (Crous *et al.* 2000, Swart *et al.* 2000, Taylor and Crous 2000, Crous *et al.* 2004), the apparently non-pathogenic fungi associated with these plants have received only very limited attention (Marais and Wingfield 1994, Lee *et al.* 2005).

The so-called ophiostomatoid fungi include species in well-known genera such as *Ophiostoma* and *Ceratocystis* and their anamorphs. These fungi are commonly treated collectively, since they are morphologically similar in having ascospores produced in slimy masses at the apices of typically long-necked ascomata (Wingfield *et al.* 1993). Over 100 species of ophiostomatoid fungi are known (Seifert *et al.* 1993), and their taxonomy has been problematic since the first description of the genera *Ceratocystis* and *Ophiostoma*. Currently it is accepted that the two genera are distantly related with *Ophiostoma* residing in the Ophiostomatales, while *Ceratocystis* is accommodated in the Microascales (Haussner *et al.* 1992, Paulin-Mahady *et al.* 2002, Haussner *et al.* 1993a, 1993b, Spatafora and Blackwell 1994). The shared morphology between these genera is probably the result of directed evolution towards arthropod-vectored dispersal rather than phylogenetic affinity.

Five ophiostomatoid species have been described from the infructescences of serotinous South African *Protea* species (Wingfield *et al.* 1988, Marais and Wingfield 1994, 1997, 2001, Wingfield and Van Wyk 1993). They reside in the genera *Ophiostoma* (*Sporothrix* anamorph) and *Gondwanamyces* (*Knoxdaviesia* anamorph), two genera that are morphologically very similar. Studies based on large subunit nuclear-encoded ribosomal DNA sequence data have, however, shown that these genera are only distantly related, with species of *Gondwanamyces* closely related to *Ceratocystis* species in the Microascales

(Wingfield *et al.* 1999). The similarity in morphology between *Ophiostoma* and *Gondwanamyces* may thus also relate to similarities in their ecology and not to a shared common ancestry.

It is interesting to note that the ophiostomatoid fungal associates in the bark beetle system (notably *Ophiostoma* and *Ceratocystis* spp.) and those of the *Protea* system (*Ophiostoma* and *Gondwanamyces* spp.) are phylogenetically similar. This probably suggests a common origin of the two systems. The commonality between the two systems cannot be explained by the relations between the plant hosts as *Protea* spp. are distantly related to the conifers on which the bark beetle system is usually based (Bowe *et al.* 2000, APG II 2003).

Bark beetles and their associated fungi have switched hosts from coniferous ancestors to angiosperms several times over their evolutionary history (Farrell *et al.* 2001). The maintenance of similar systems between the bark beetle and *Protea* systems may thus relate to similarities in vectors for these fungi. In the bark beetle system, a specific bark beetle species can vector both ophiostomatoid fungal genera (see Kirisits 2004). No bark beetles are, however, associated with the infructescences of *Protea* spp. (Myburg *et al.* 1973, 1974, Myburg and Rust 1975a, 1975b, Coetzee and Giliomee 1987a, 1987b, Coetzee 1989, Roets *et al.* 2006) and information on the vectors of the *Protea*-associated ophiostomatoid fungi will greatly improve our understanding of the apparent similarities between the two systems.

Three species of *Ophiostoma* are known from *Protea* infructescences. One of these, *O. splendens*, has been recorded from the Western Cape Province (Marais and Wingfield 1994). This species colonises the infructescences of many different *Protea* hosts (Marais and Wingfield 1994, Roets *et al.* 2005). The other two *Ophiostoma* species, *O. africanum* and *O. protearum*, are more host-specific and are confined to *P. caffra* and *P. gagedi*, respectively (Marais and Wingfield 1997, 2001). These two *Protea* species occur naturally in the northern parts of South Africa and extend into neighbouring African countries (Rebello 1995). Although it has not yet been confirmed, it is suspected that the *Ophiostoma* spp. associated with these two *Protea* species are also present on plants from the rest of Africa (Marais 1996). With such a large geographical distribution, and such a wealth of possible *Protea* host species, it is reasonable to assume that many more *Ophiostoma* species await discovery in this niche.

In contrast to *Ophiostoma* spp., *Gondwanamyces* species are confined to species of *Protea* occurring in the Cape region of South Africa. Similar to *O. splendens*, *G. capensis* colonises many different Cape *Protea* hosts in the southwestern Cape (Wingfield and Van Wyk 1993). *Gondwanamyces proteae* on the other hand, is host-specific and colonises only the widespread *P. repens* (Wingfield *et al.* 1988). *Ophiostoma splendens* and *Gondwanamyces* species are known to co-inhabit the same infructescence (Marais 1996) and even sporulate concurrently (pers. observ.).

As outlined above, *Ceratocystis* and *Ophiostoma* species are usually associated with insect vectors. Due to morphological similarities between these two genera and *Gondwanamyces*, it is very likely that *Gondwanamyces* spores are also vector dispersed. All three of these taxa develop fairly long-necked ascomata in their sexual stage. Ascospores are produced within the ascomatal bases, pushed through the necks and collect at the tip in sticky masses. Here insects can readily come into contact with these spores and transport them to new substrates. A large number of arthropods are known to colonise *Protea* infructescences (Myburg *et al.* 1973, 1974, Myburg and Rust 1975a, 1975b, Coetzee and Giliomee 1987a, 1987b, Coetzee 1989, Roets *et al.* 2006). Many of these are thought to be monophagous and exclusively associated with *Protea* species. While any of these may act as vector of the ophiostomatoid fungi, no comprehensive attempt has been made to identify the specific arthropods involved in vectoring *Ophiostoma* and *Gondwanamyces* spp. occurring in *Protea* infructescences. As ophiostomatoid fungi sporulate only within infructescences, and not within inflorescences, Marais (1996) suggested that borers are the most likely vectors of ophiostomatoid fungi. Roets (2002) subsequently used molecular techniques to identify six insects as putative vector organisms, but his identification techniques were preliminary and required refinement.

Interestingly, ophiostomatoid fungi are often found to be the dominant fungal species within a colonised infructescence (Marais and Wingfield 2001, Roets *et al.* 2005), where they are thought to grow saprophytically (Marais 1996). They inhabit the infructescences from an early age (Roets *et al.* 2005), and colonise the styles and other floral structures, including the fruits and inner bracts in acute infestations (Marais 1996, Roets *et al.* 2005). The dominance of these fungi within *Protea* infructescences may be ascribed to the ability of the ophiostomatoid fungi to out-compete other fungal species also present within this

niche. The presence of ophiostomatoid fungi may thus be beneficial to the plant, as they may enhance seed-survival by limiting the growth of seed-destroying fungal species. It is plausible that there is a constructive symbiotic relationship between fungus and plant. Further studies focussed on the effect of these fungi on other fungal species and on *Protea* seed production are required.

## 6. OBJECTIVES OF THIS STUDY

A modern approach to evolutionary biology promotes the use of integrated biological studies to assess holistic relationships and patterns of co-evolution between different biological groups. The *Protea* ophiostomatoid fungi present an ideal case study, in which inter-organism interactions between the ophiostomatoid fungi, their vector organisms, the *Protea* plant hosts, and other fungi present within the infructescences, can be considered together. The assessment of these interactions forms the main objective of the studies that are presented in this thesis. Results are presented in manuscript format. The formatting for accepted papers may vary slightly according to the preferred editorial style of the journal involved.



## THESIS CHAPTERS WITH A BRIEF STATEMENT OF OBJECTIVES

### CHAPTER 2. A PCR-based method to detect species of *Gondwanamyces* and *Ophiostoma* from the surfaces of insects colonising *Protea* flowers

The polymerase chain reaction (PCR) developed by Mullis (1990) and Mullis and Faloona (1987), allows for the amplification of small amounts of specific DNA fragments. This approach has been used successfully for the identification of fungal DNA fragments (for specific fungal groups) from a range of complex environments (e.g. Hwan Kim *et al.* 1999, Edel *et al.* 2000, Groenewald *et al.* 2000, Hamelin *et al.* 2000, Hirsch *et al.* 2000, Ganley and Bradshaw 2001, Lee *et al.* 2001, Mazzaglia *et al.* 2001). Chapter 2 deals with the development of a PCR-based method to detect *Ophiostoma* and *Gondwanamyces* from insects that colonise *Protea* flowers. The manuscript prepared from this chapter has been accepted for publication in *Canadian Journal of Botany* (Paper co-authored by Michael J. Wingfield, Léanne L. Dreyer, Pedro W. Crous, and Dirk U. Bellstedt).

### CHAPTER 3. Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences

Most biological studies require a thorough understanding of the phylogenetic relationships between the experimental organisms. This chapter thus focuses on the delimitation and identification of *Ophiostoma* species present in various *Protea* species using sequence-based phylogenetic reconstruction. This chapter clarifies the number of *Ophiostoma* species involved in this association based on sequence data obtained from the Large Subunit, ITS and Beta-tubulin DNA regions of *Ophiostoma* spp. collected from various *Protea* spp. infructescences. The manuscript prepared from this chapter has been accepted for publication in *Studies in Mycology* (Paper co-authored by Wilhelm Z. De Beer, Léanne L. Dreyer, Renate Zipfel, Pedro W. Crous and Michael J. Wingfield).

#### CHAPTER 4. Discovery of fungus-mite-mutualism within a unique niche of the Cape Floral Kingdom

This chapter sets out to identify the specific vector organisms of *Ophiostoma* spp. associated with *Protea* infructescences. It also aims to identify ophiostomatoid fungus spores from arthropods through visual detection by light and scanning electron microscopy and by direct isolation using plating techniques. Possible mutualistic interactions between *Ophiostoma* spp. and their vector organism(s) are investigated.

#### CHAPTER 5. *Ophiostoma gemellus* prov. nom. and *Sporothrix variecibatus* prov. nom. (Ophiostomatales) from mites infesting *Protea* infructescences in South Africa

Investigations on the specific species of *Ophiostoma* isolated from arthropods in Chapter 4 revealed the presence of two possible undescribed species. In this chapter we assess the taxonomy of these isolates in conjunction with additional isolates collected from *Protea* infructescences. For this study, data from the ITS and beta-tubulin gene fragments, morphological and physiological data are considered in order to identify the two unknown species. The species are given provisional names, hence the name of the taxa are followed by prov. nom.

#### CHAPTER 6. Hyperphoretic dispersal of the *Protea*-associated fungi, *Ophiostoma phasma* and *O. splendens* by mites

The aim of this chapter was to examine various dispersal methods of the *Protea*-associated *Ophiostoma* species and their vector organisms between infructescences. It also sets out to reconstruct the life cycle of these fungi.

#### CHAPTER 7. The taxonomy and ecology of ophiostomatoid fungi associated with *Protea* infructescences: a review of current knowledge

This chapter summarizes the main conclusions reached in each of the different chapters, and uses these as a basis to draw conclusions for the inclusive broad study. Suggestions for future research are also provided.

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## Chapter 2: A PCR-based method to detect species of *Gondwanamyces* and *Ophiostoma* from the surfaces of insects colonising *Protea* flowers

### Abstract

Flower heads of economically important members of the genus *Protea* mature into conspicuous, often long-lived infructescences, which in South Africa are commonly colonised by species of the ophiostomatoid fungi, *Gondwanamyces* and *Ophiostoma*. It is suspected that these fungi are transported between infructescences by insects. In order to develop techniques that would enable detection of ophiostomatoid fungi on insects, primers GPR1 and OSP1 were designed based on unique 28S ribosomal DNA sequences of *Gondwanamyces* and *Ophiostoma* from *Protea*. Multiplex polymerase chain reaction of these primers, combined with universal primer LR6, yielded fragment lengths of 885- and 637-bp. Positive amplification was achieved from as little as 30 pg and 45 pg of fungal genomic DNA for *Gondwanamyces* and *Ophiostoma*, respectively and fragments of identical lengths were amplified from insects artificially inoculated with these fungi. No other tested fungal species showed amplification with GPR1 or OSP1 and LR6. Using these primers two insect species (*Genuchus hottentottus* and *Oxycarenus maculatus*) collected from *Protea repens* infructescences were confirmed as carriers of *Gondwanamyces proteae* and *Ophiostoma splendens* respectively. The method developed in this study represents a rapid detection system that can be used to understand the relationship between insects and ophiostomatoid fungi found associated with flowers of South African species of *Protea*.

*Key words:* insect-vectored fungi, fynbos, infructescence, ophiostomatoid fungi, Proteaceae

## Introduction

In 1999 the South African Proteaceae industry generated an annual income of more than US \$ 30 million, 30 % of which can be attributed to cut-flower sales of members of the genus *Protea* L. (Anon. 1999, Crous *et al.* 2004). This genus is of considerable economic importance to South Africa, and phytosanitary problems caused by arthropod and fungal damage and colonisation to these plants pose a serious threat to the South African export market.

The ophiostomatoid fungi include species of *Ceratocystis* Ellis & Halst., *Gondwanamyces* G.J. Marais & M.J. Wingf. and *Ophiostoma* Syd. & P. Syd. These ascomycetes produce ascospores in slimy masses at the apices of typically long-necked ascomata (Wingfield *et al.* 1993), a feature interpreted as an adaptation for insect dispersal (Upadhyay 1981, Malloch and Blackwell 1993, Cassar and Blackwell 1996). For example, most species of *Ophiostoma* and their closest relatives are dispersed by scolytine bark beetles, especially those that infest conifers (Upadhyay 1981, Dowding 1984, Wingfield *et al.* 1993, Paine *et al.* 1997, Klepzig *et al.* 2001, Klepzig and Six 2004).

A number of ophiostomatoid fungi occur on the floral parts within infructescences of serotinous members of *Protea* (Wingfield *et al.* 1988, Wingfield and Van Wyk 1993, Marais and Wingfield 1994, 1997, 2001). These ascomycetes include two species of *Gondwanamyces* (*G. capensis* (M.J. Wingf. *et al.*) G.J. Marais & M.J. Wingf. and *G. proteae* (M.J. Wingf. *et al.*) G.J. Marais & M.J. Wingf. and three species of *Ophiostoma* (*O. africanum* G.J. Marais & M.J. Wingf., *O. protearum* G.J. Marais & M.J. Wingf. and *O. splendens* G.J. Marais & M.J. Wingf.), representing two distinct phylogenetic lineages.

The ophiostomatoid fungi from members of the genus *Protea* in South Africa differ in host specificity. *Gondwanamyces capensis* and *O. splendens*, for example, occur in the infructescences of various members of the genus *Protea*, while *O. africanum* and *O.*

*protearum* are each known from only a single host plant (Wingfield *et al.* 1988, Wingfield and Van Wyk 1993, Marais and Wingfield 1994, 1997, 2001). The basis of this specificity is unknown and could include chemical and / or morphological characteristics of their host plants. Although it is suspected that insects are involved in the transport of these fungi, no vectors have been identified from these infructescences. The ecological role that these fungi play within the *Protea* infructescences also remains to be determined.

Species of *Ophiostoma* found in *Protea* infructescences are similar morphologically and closely related phylogenetically to Northern Hemisphere taxa, known to be vectored by insects (Upadhyay 1981, Dowding 1984, Malloch and Blackwell 1993, Wingfield *et al.* 1993, Paine *et al.* 1997, Klepzig *et al.* 2001, Klepzig and Six 2004). Infructescences of *Protea* are colonised by a number of economically important insects that may play a role in the dispersal of ophiostomatoid fungi (Coetzee and Giliomee 1985, 1987a, 1987b, Coetzee 1989, Wright 1990, Visser 1992). However, the identification of vectors of *Gondwanamyces* and *Ophiostoma* employing conventional methods has proven challenging. These fungi grow very slowly in pure culture and are typically overgrown by faster growing fungal contaminants found on the insects and floral parts (Roets 2000). DNA-based detection of ophiostomatoid fungi from the surface of insects may be a more effective alternative to these conventional methods (Schweigkofler *et al.* 2005). The objectives of the present study were to develop sensitive DNA-based technique to detect species of *Gondwanamyces* and *Ophiostoma* on insect surfaces and to test the hypothesis that insects colonising the infructescences of *Protea* are capable of vectoring ophiostomatoid fungi.

## **Materials and methods**

### **Fungal isolates**

All isolates were grown on 2 % MEA plates at 24 °C in the dark for 2–4 wks. Cultures of *G. capense* (CMW 997, CBS pending), *G. proteae* (CMW 3936, CBS 486.88), *O. africanum* (CMW823, CBS 116571), *O. protearum* (CMW1102, CBS 116568) and *O.*

*splendens* (CMW872, CBS 116379) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representative cultures of all species are available from the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Additional fungi isolated from insects collected from the infructescences of *Protea* that were used in this study are housed in the culture collection at the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa (STE-U). These include unidentified representatives of the following genera: *Alternaria* Nees (SL 20), *Aspergillus* Link (SL 3), *Botrytis* P. Micheli ex Pers. (SL 48), *Chaetomium* Kunze (SL 13), *Cladosporium* Link (SL 34), *Clonostachys* Corda (SL 35, -23), *Dicyma* Boulanger (SLE 10), *Epicoccum* Link (SL 9), *Fusarium* Link (SL 16, -20, -4), *Melanospora* Corda (SL 15), *Nigrospora* Zimm. (SLE 17), *Penicillium* Link (SL 101), *Pestalotia* De Not. (SL 47), *Sarcostroma* Cooke (SL 82), *Sordaria* Ces. & De Not. (SL 80), *Trichoderma* Pers. (SL 6), two unidentified hyphomycetes (SL 49, -85) and an unidentified coelomycete (SL 83). Additional cultures of *G. capense* and *O. splendens* were collected from *P. repens* L. infructescences in the Jonkershoek Nature Reserve, Stellenbosch, South Africa (S: 33° 59.555' E: 18° 58.287'). Ascospore masses were lifted directly from the tips of the ascomatal necks with a small piece of MEA placed at the tip of a dissecting needle, and transferred to agar plates.

### **Design of taxon-specific primers**

Partial 28S rDNA sequences were obtained from GenBank for five species of ophiostomatoid fungi isolated from *Protea* (*G. capense* AF221012, *G. proteae* AF221011, *O. africanum* AF221015, *O. protearum* AF221014, *O. splendens* AF221013), two species of *Ceratocystis* (*Ceratocystes adiposa* (E.J. Butler) C. Moreau AF222481, *C. fimbriata* Ellis & Halst. AF222484), *Leptographium lundbergii* Lagerb. & Melin AF155664, and *Ophiostoma piliferum* (Fr.) Syd. & P. Syd. U47837. Sequences of the following 11 species of fungi, representing genera commonly isolated from *Protea* infructescence inhabiting insects (Roets 2000) were also included: *Botrytis tulipae* (Lib.) Lind AJ226078, *Cladosporium* Link U26886, *Fusarium acumilnatum* Ellis & Everh. U34544, *Melanospora zamiae* Corda U17405, *Mucor hiemalis* f. *hiemalis* Wehmer

AF113468, *Penicillium* Link sp. 1 U26865, *Penicillium* sp. 2 U26851, *P. chrysogenum* Thom AF034857, *P. namyslowskii* K. M. Zalesky AB000487, *P. turbatim* Westling AF034454, and *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. AF132330. DNA sequences were aligned using DAPSA (Harley 1988).

Based on the comparison of 28S rDNA sequences, we identified two regions within this gene as genus-specific for *Gondwanamyces* and group-specific for members of *Ophiostoma* from *Protea* hosts. The forward primers GPR1 (5'–CCAGCATCGGTTTGTTA –3') and OSP1 (5' –GACGCCTAGCCTCTACAA –3') were designed for species of *Gondwanamyces* and *Ophiostoma*, respectively. The universal reverse primer LR6 (Vilgalys and Hester 1990) was used in combination with these two primers to yield fragments 637 bp (GPR1 and LR6) and 885 bp (OSP1 and LR6) in length. Expected primer melting temperatures were GPR1 = 63 °C, LR6 = 64 °C and OSP1 = 64 °C. PCR products were sequenced to confirm their identities.

### **Primer-specificity tests**

The specificity of GPR1 and OSP1 were tested using the fungal cultures listed above and amplification conditions were optimised for the primer pair combinations GPR1 - LR6 and OSP1 - LR6 before they were combined in a multiplex reaction. DNA was isolated from fungal mycelia following the protocol of Lee and Taylor (1990). PCR mixtures (25 µL) in each tube contained 5 µL of the extracted fungal genomic DNA, 8 mM MgCl<sub>2</sub> (Bioline, London), 1× NH<sub>4</sub> reaction buffer (Bioline, London), 0.25 mM of each of the four dNTP's, 0.4 pmol.µL<sup>-1</sup> of each of the primers and 0.626 units of Biotaq (Bioline, London).

The best amplification results were obtained using a touchdown PCR program with an initial denaturation at 94 °C for 2 min, 13 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 1 min followed by 23 cycles for which the annealing temperature was lowered to 56 °C and the final extension was increased to 5 min. Amplification was achieved using GPR1, LR6 and OSP1 in a multiplex reaction employing a minimum of 30 pg genomic DNA for species of

*Gondwanamyces* and 45 pg of genomic DNA for species of *Ophiostoma*. The specificity of the developed primers was retested using the concentrations of the constituents of the PCR mixtures and thermal cycling conditions outlined above, but 0.2 pmol. $\mu\text{L}^{-1}$  rather than 0.4 pmol. $\mu\text{L}^{-1}$  of GPR1 and OSP1.

Controls were included to verify the presence of amplifiable amounts of target DNA for all the fungi tested. These reactions contained the universal primers LROR (White *et al.* 1990) and LR6 with the remaining PCR constituents as described above. PCR conditions were: a 2 min denaturation at 95 °C followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min with a final elongation 72 °C for 8 min. All PCR's were performed using a Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). Reactions were analysed by separating 10  $\mu\text{L}$  of the PCR products and 3  $\mu\text{L}$  loading buffer on a 1.5 % agarose gel (Promega corporation, Madison, U.S.A.) in TAE containing ethidium bromide prior to viewing under an UV transilluminator.

#### **Detection of *Gondwanamyces* and *Ophiostoma* on inoculated insects**

A subset of 10 insects (one individual per family) was inoculated by bringing each individual insect into contact with the ascospores oozing from the tip of one ascomatal neck of *G. proteae* (n = 10). A second subset of 10 insects (one individual per family) was inoculated in the same manner with the ascospores of *O. splendens* (n = 10). The ascomata of both fungi were produced *in vivo* within the infructescences of *P. repens* collected from the J.S. Marais Park, Stellenbosch, South Africa. The remaining uninoculated insects (n = 12) served as negative controls.

Insects were placed in Eppendorf tubes containing 1 mL SDS extraction buffer (pH 8) and vortexed for 1 min to loosen the fungal spores. The insects were then removed and the suspension was centrifuged for 5 minutes (13,800 X g). DNA was extracted following Lee and Taylor (1990) and resuspended in 15  $\mu\text{L}$  ddH<sub>2</sub>O.

The method of Kim *et al.* (1999) was also tested for the extraction of fungal DNA from 10 artificially inoculated insects (n = 5 for both *G. proteae* and *O. splendens*). DNA was extracted by heating the sample insect in a microwave (700 W) for 5 min, after which cooled extraction buffer (SDS) was added (100 µL at 4 °C). Tubes containing samples were vortexed for 1 min and the supernatant was used as template for multiplex PCR amplification.

### **Screening of insects collected from *P. repens* infructescences**

Twenty-three insect morphospecies (145 individuals) were removed from 20 of the closed infructescences of *P. repens* collected from the Jan S. Marais Park, Stellenbosch, South Africa (Table 1). Infructescences were opened under sterile conditions and the collected insects were frozen at -20 °C. DNA was extracted and the target fragments were amplified as described previously. Amplified DNA fragments were analysed on a 3100 ABI automated sequencer.

## **Results**

### **Primer specificity tests**

Amplification products were obtained for all of the fungi tested using the universal primers LR0R and LR6 and the standard PCR protocol (results shown for eight fungal taxa in Fig. 1a). Amplification products were obtained using GPR1 and OSP1 for species of *Gondwanamyces* and *Ophiostoma*, but not for any of the other fungi. Combining the primers in a multiplex reaction resulted in no loss of specificity and fragments of the expected sizes were obtained for the ophiostomatoid fungi examined (Fig. 1b).

### **Detection of *Gondwanamyces* and *Ophiostoma* on inoculated insects**

DNA of *Gondwanamyces* and *Ophiostoma* could be detected on all insects that had been touch-inoculated with ascospores of these fungi. In contrast, none of the uninoculated arthropod specimens showed amplification with the designed primers. We were unable to amplify fungal DNA following the method of Kim *et al.* (1999).



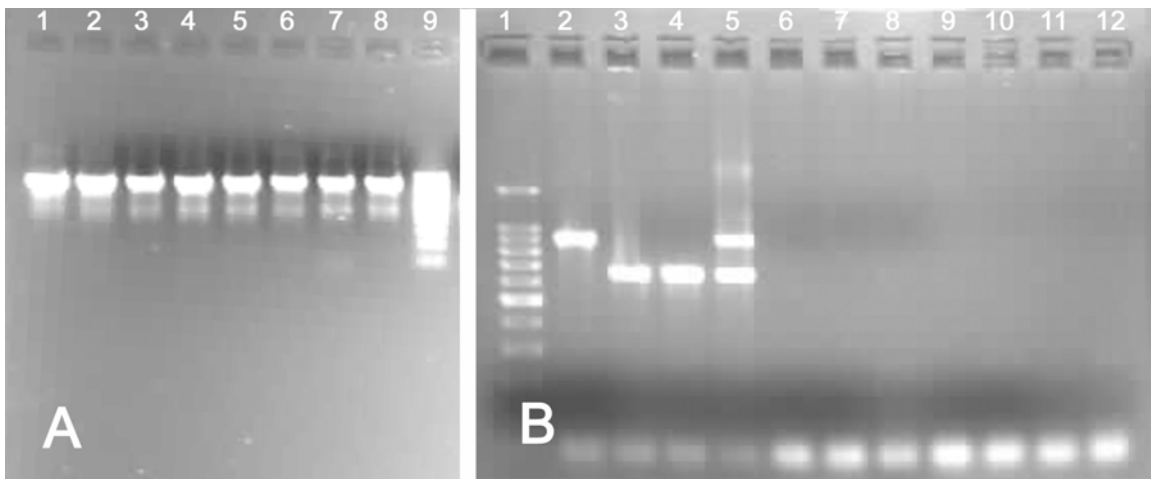
**Table 1.** Arthropods collected from *Protea repens* infructescences from the Jan S. Marais Park, Stellenbosch, South Africa (Jan. 2002 – Nov. 2002) and tested for the presence of ophiostomatoid fungi using PCR protocols.

Species	USEC Coll. Nr.*	Number of insects tested
<i>Argyroploce</i> Hübner (Tortricidae)	68	3
Blattidae	26	1
Braconidae	52	1
<i>Capys alphaeus</i> Cramer (Lycaenidae)	66	1
Chrysomelidae	17	1
<i>Crematogaster</i> Lund (Formicidae)	15	15
Curculionidae	48	2
Diptera	5	7
<i>Euderus lineicollis</i> Wiedemann (Curculionidae)	33	2
Formicidae	23	8
<i>G. hottentottus</i> (Scarabaeidae)	70	8 (1†)
<i>Gyponyx</i> Gorham (Cleridae)	55	3
Histeridae	32	5
Miridae	20	3
Nitidulidae	25	8
<i>O. maculates</i> (Lygaeidae)	7	18 (1‡)
Pentatomidae	24	1
Psocoptera (sp. 1)	31	22
Psocoptera (sp. 2)	12	1
Psocoptera (sp. 3)	13	10
<i>Sphenoptera</i> Solier (Buprestidae)	49	2
Staphylinidae	35	18
<i>Tinea</i> L. (Tineidae)	67	3

**Note:** \*Reference collection in the Department of Entomology and Nematology, University of Stellenbosch, South Africa. †Individual showing positive amplification results for *G. proteae* DNA, ‡Individual showing positive amplification results for *O. splendens* DNA

### Screening of insects collected from *P. repens* infructescences

Testing of insects collected from *P. repens* (Table 1) using the newly developed multiplex PCR method revealed the presence of *G. proteae* on *Genuchus hottentottus* Fabricius (Cetoniidae, Coleoptera) and *O. splendens* on *Oxycarenus maculatus* Stal. (Lygaeidae, Heteroptera). Sequencing confirmed that these fragments were identical to corresponding GenBank sequences for *G. proteae* (AF221011) and *O. splendens* (AF221013).



**Fig. 1.** A: Agarose gel showing amplification of partial 28S rDNA with universal primers (LROR and LR6) for selected taxa. Lane 1, *Cladosporium* sp.; Lane 2, *Nigrospora* sp.; Lane 3, *Clonostachys* sp.; Lane 4, *Aspergillus* sp.; Lane 5, *Sarcostroma* sp.; Lane 6, *Epicoccum* sp.; Lane 7, *O. splendens*; Lane 8, *Trichoderma* sp.; Lane 9, DNA size marker (100 bp ladder). B: Agarose gel showing the specificity of developed primers (OSP1 and GPR1) using the developed multiplex reaction protocol. Lane 1, DNA size marker; Lane 2, *O. splendens* DNA; Lane 3, *G. proteae* DNA; Lane 4, *G. capensis*; Lane 5, DNA representing both *G. proteae* and *O. splendens*; Lane 6, no DNA control; Lane 7, *Cladosporium* sp.; Lane 8, *Nigrospora* sp.; Lane 9, *Clonostachys* sp.; Lane 10, *Aspergillus* sp.; Lane 11, *Sarcostroma* sp.; Lane 12, *Epicoccum* sp.

## Discussion

In this study we developed taxon-specific primer sets that permit the rapid and accurate detection of DNA of *Gondwanamyces* and *Ophiostoma* from the surfaces of insects associated with species of *Protea*. The multiplex PCR protocol proved to be highly specific for the two fungal taxa, and enabled successful identification of fungal DNA from both artificially and naturally inoculated insects from the infructescences of *Protea*. The method also proved to be very sensitive, as we were able to detect low quantities of the target fungal DNA. Application of this protocol led to the identification of the two putative vector insects for *G. proteae* and *O. splendens* from *P. repens*. This PCR-based method can now be used for further intensive investigations of the biology of members of *Gondwanamyces* and *Ophiostoma* associated with South African species of *Protea*.

Schweigkofler *et al.* (2005) developed a PCR test and were able to detect target ophiostomatoid fungi on 37 % of the bark beetles collected. Although a previous study (Roets *et al.* 2005) indicated that up to 60 % of *P. repens* infructescences can be colonised by ophiostomatoid fungi, only two of the 145 individuals screened yielded amplification products using the primers designed in this study. Tests of the PCR protocol revealed that amplification was possible for the load of spores transferred to insects from contact with a single sporulating ascoma. As ophiostomatoid fungi form the dominant fungal component within *Protea* infructescences (Marais 1996, Roets *et al.* 2005) and insects departing from infructescences colonised by these fungi likely come into contact with numerous sporulating ascomata, it can be assumed that detectable amounts of DNA should be present on the putative vectors.

The low retrieval of ophiostomatoid fungi from insects may be associated with the synchronisation of fungal sporulation and insect emergence. In this study, the insects were physically extracted from the infructescences, possibly prior to their having come into contact with the fungi. The lifecycles of these organisms may be synchronised in such a way that the ascospores are deposited onto the insects just prior to their departure from the infructescences. This has been reported in some bark beetle-associated fungi

(Bridges 1983). The fungi may also be transported endozootically by the vector insects. We will employ macerated insects emerging naturally from *Protea* infructescences in future investigations of this system.

Both putative vector insects identified in this study are known to be associated with various members of the Proteaceae, mainly in the genus *Protea*, and they have not been reported from other plant families (Coetzee and Giliomee 1985, 1987a, 1987b, Coetzee 1989, Wright 1990, Visser 1992). Similarly, species of *Gondwanamyces* and *Ophiostoma* found in *Protea* infructescences are known only from the genus *Protea* (Wingfield *et al.* 1988, Wingfield and Van Wyk 1993; Marais and Wingfield 1994, 1997, 2001). This suggests that there may be a symbiotic relationship between the plants, vector insects and / or associated ophiostomatoid fungi. A more comprehensive study, including large numbers of arthropods collected from all species of *Protea* known to be colonised by ophiostomatoid fungi, can now be undertaken to elucidate the interactions among these organisms.

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## Chapter 3: Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences

### Abstract

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

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

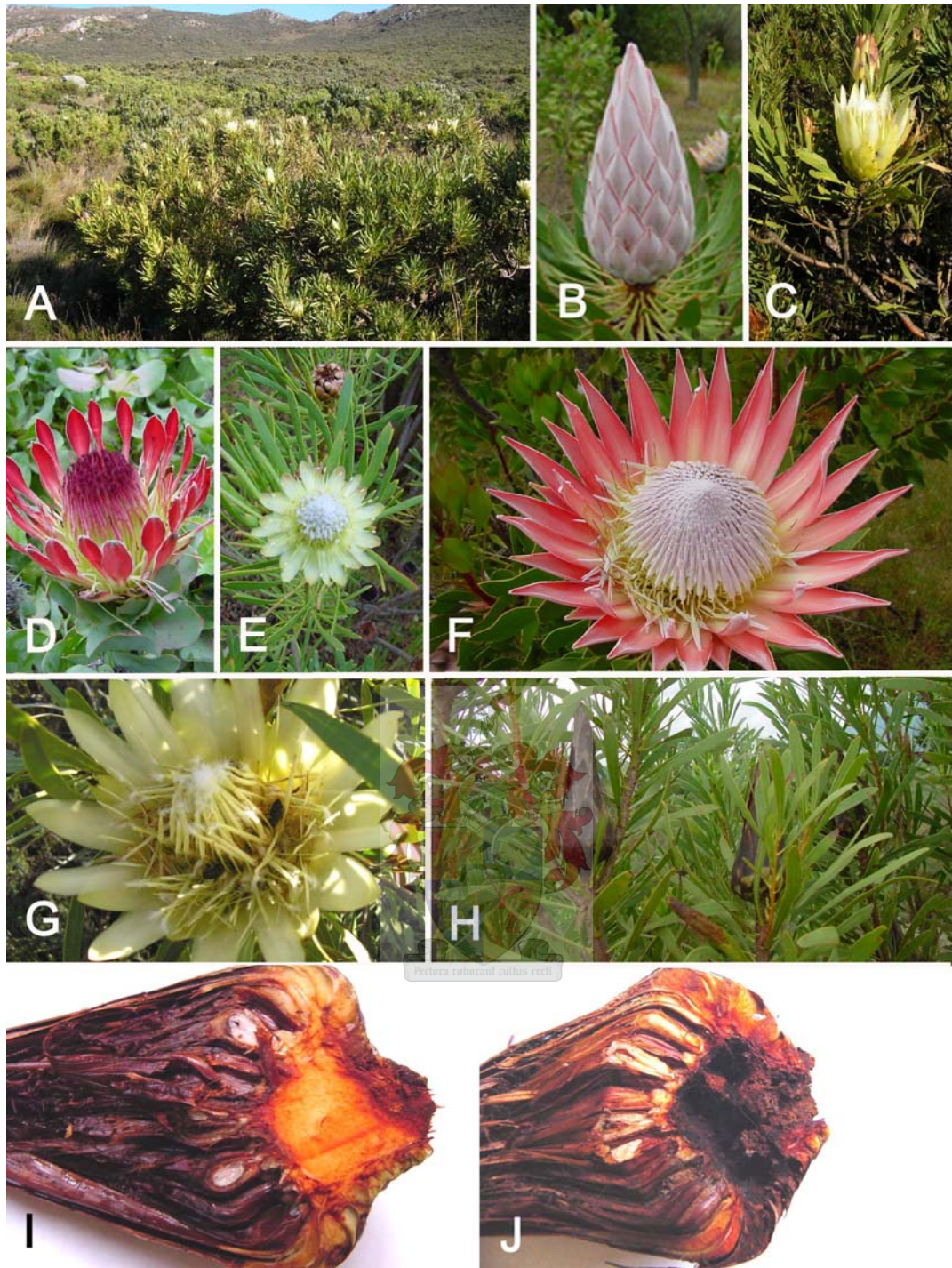
*Key words:*  $\beta$ -tubulin, ITS, LSU, *Ophiostoma*, phylogeny, *Protea*.

## Introduction

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

Florets of *Protea* spp. are arranged in inflorescences. After a bud stage that can last for several months (Fig. 1B), the inflorescences will open to reveal the often brightly coloured involucre bracts that attract many insect and bird pollinators (Fig. 1C–G). After pollination, the involucre bracts close, forcing the florets together in compact infructescences (Fig. 1H–J). The infructescence may persist on the plants for several years, and act as an above-ground seed bank (Bond 1985) that opens to release seeds after a fire event (Rebelo 1995). During this time, the infructescences are colonised by many different arthropods (Myburg *et al.* 1973, 1974, Myburg and Rust 1975*a*, 1975*b*, Coetzee and Giliomee 1985, 1987*a*, 1987*b*, Coetzee 1989, Wright 1990, Visser 1992, Roets *et al.* 2006*a*) and micro-fungi (Marais and Wingfield 1994, Lee *et al.* 2003, 2005), some of which are specific to their *Protea* hosts.

Three species of *Ophiostoma* Syd. & P. Syd. have been described from *Protea* infructescences in South Africa showing varying degrees of host specificity. *Ophiostoma africanum* G.J. Marais & M.J. Wingf. is reportedly specific to its *P. gagedi* J.F. Gmel. host (Marais and Wingfield 2001), while *O. protearum* G.J. Marais & M.J. Wingf. is confined to the infructescences of *P. caffra* Meisn. (Marais and Wingfield 1997). *Ophiostoma splendens* G.J. Marais & M.J. Wingf., in contrast, has been reported from *P. repens* L., *P. neriifolia* R. Br., *P. laurifolia* Tunb., *P. lepidocarpodendron* L., and *P. longifolia* Andrews (Marais and Wingfield 1994, Roets *et al.* 2005). All three species are characterised by *Sporothrix* Hekt. &

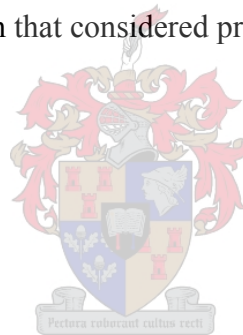


**Fig. 1.** Growth habit and flower phenology of *Protea* species. A. Natural Fynbos landscape dominated by *Protea repens*. B. Flower-bud stage of *P. cynaroides*. C. Flowering stage of *P. repens*. D. Flowering stage of *P. eximia*. E. Inflorescence of *P. scolymocephala*. F. Inflorescence of *P. cynaroides*. 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 involucre receptacle. J. Same, showing damage by insect larvae boring into involucre receptacle.

C.F. Perkins anamorphs, tolerance to high levels of the antibiotic cycloheximide, and contain rhamnose in their cell walls (Marais *et al.* 1998).

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

The present study aimed to determine the phylogenetic relationships of the three known *Protea*-associated *Ophiostoma* spp., using ribosomal ITS and partial  $\beta$ -tubulin gene sequences. We also reconsidered 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 February 2003 and June 2005, and examined for the presence of *Ophiostoma* spp. Ascospores were removed from the apices of ascomatal necks with a small piece of agar attached to the tip of a dissecting needle and transferred to 2 % malt extract agar (MEA, Biolab, Midrand, South Africa) amended with 0.05 g/L cycloheximide (Harrington 1981). Once purified, all cultures were maintained on Petri dishes containing MEA at 4 °C. Representative cultures of all species (Table 1) have been deposited in the culture collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Herbarium specimens of both the teleomorph and anamorph states of the new species have been deposited in the National Fungus Collection (PREM), Pretoria, South Africa (Table 1).

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

Species identity	Isolate no.		Herbarium	Host	Geographical origin	Collector	GenBank accession no.		
	CBS	CMW					LSU	ITS	$\beta$ -tubulin
<i>Ophiostoma africanum</i>	CBS 116571	CMW 823		<i>Protea gaguedi</i>	Unknown	M.J. Wingfield	AF221015	DQ316199	DQ296073
	CBS 116374	CMW 1822		<i>P. dracomontana</i>	Cathedral Peak, KZ-Natal	M.J. Wingfield		DQ316197	DQ316159
	CBS 116376	CMW 1812		<i>P. dracomontana</i>	Cathedral Peak, KZ-Natal	M.J. Wingfield		DQ316198	DQ316160
	CBS 116566	CMW 1104		<i>P. caffra</i>	Irene, Gauteng	Unknown	DQ316147	DQ316200	DQ316162
<i>O. palmiculminatum</i>	CBS 119590	CMW 20677	<sup>H</sup> PREM58942	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316143	DQ316191	DQ316153
	CBS 119591	CMW 20693	<sup>P</sup> PREM58949	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316192	DQ316154
	CBS 119592	CMW 20694	<sup>P</sup> PREM58950	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316144	DQ316193	DQ316155
		CMW 20695		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316194	DQ316156
<i>O. phasma</i>		CMW 20696		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316195	DQ316157
	CBS 119593	CMW 20697	<sup>P</sup> PREM58951	<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316152	DQ316196	DQ316158
		CMW 20698		<i>P. laurifolia</i>	Giftberg top, SW Cape	F. Roets		DQ316222	DQ316184
		CMW 20699		<i>P. laurifolia</i>	Giftberg top, SW Cape	F. Roets		DQ316220	DQ316182
	CBS 119722	CMW 20681	<sup>P</sup> PREM58943	<i>P. neriifolia</i>	Jonkershoek, SW Cape	F. Roets		DQ316216	DQ316178
	CBS 119589	CMW 20682	<sup>P</sup> PREM58944	<i>P. neriifolia</i>	Jonkershoek, SW Cape	F. Roets		DQ316217	DQ316179
		CMW 20683	PREM58945	<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316227	DQ316189
		CMW 20684		<i>P. laurifolia</i>	Piekenierskloof, SW Cape	F. Roets		DQ316218	DQ316180
	CBS 119721	CMW 20676	<sup>H</sup> PREM58941	<i>P. laurifolia</i>	JS Marais Park, SW Cape	F. Roets	DQ316151	DQ316219	DQ316181
		CMW 20686		<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316223	DQ316185
	CMW 20687		<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316221	DQ316183	
	CMW 20688		<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316224	DQ316186	
CBS 119588	CMW 20689	<sup>P</sup> PREM58946	<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316225	DQ316187	
	CMW 20690	PREM58947	<i>P. laurifolia</i>	Bainskloof, SW Cape	F. Roets		DQ316226	DQ316188	

**Table 1. Continued.**

<i>O. phasma</i>		CMW 20692	PREM58948	<i>P. neriifolia</i>	Jonkershoek, SW Cape	F. Roets		DQ316228	DQ316190
<i>O. protearum</i>	CBS 116654	CMW 1107		<i>P. caffra</i>	Irene, Gauteng	M.J. Wingfield	DQ316145	DQ316201	DQ316163
	CBS 116567	CMW 1103		<i>P. caffra</i>	Irene, Gauteng	M.J. Wingfield		DQ316203	DQ316165
	CBS 116568	CMW 1102		<i>P. caffra</i>	Irene, Gauteng	M.J. Wingfield	AF221014	DQ316202	DQ296072
<i>O. splendens</i>		CMW 20679		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets	DQ316150	DQ316212	DQ316174
		CMW 20680		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316211	DQ316173
		CMW 20685		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316213	DQ316175
		CMW 20691		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316209	DQ316171
		CMW 20678		<i>P. repens</i>	JS Marais Park, SW Cape	F. Roets		DQ316210	DQ316172
		CMW 20674		<i>P. repens</i>	George, SW Cape	F. Roets		DQ316204	DQ316166
		CMW 20675		<i>P. repens</i>	George, SW Cape	F. Roets		DQ316205	DQ316167
	CBS 116379	CMW 896		<i>P. repens</i>	Unknown	M.J. Wingfield		DQ316207	DQ316169
	CBS 116569	CMW 872		<i>P. repens</i>	Unknown	M.J. Wingfield	AF221013	DQ316215	DQ296071
	CBS 116377	CMW 873		<i>P. repens</i>	Unknown	M.J. Wingfield		DQ316214	DQ316176
		CMW 2753		<i>P. neriifolia</i>	Sir Lowrey's Pass, SW Cape	G. Marais		DQ316206	DQ316168
	CBS 116378			Unknown	Unknown	Unknown		DQ316208	DQ316170

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

## **Microscopy**

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

## **Growth in culture**

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

Tolerance of the unidentified species to cycloheximide was tested by transferring a 5 mm diameter piece of agar containing fungal mycelia and conidia to MEA plates containing varying concentrations of cycloheximide (0, 0.05, 0.1, 0.5, 1.0 and 2.5 g/L). The colony diameter of three replicate plates per tested concentration was calculated as described for the study of growth at different temperatures after incubation at 25 °C in the dark for 10 d.

## **DNA extraction, amplification and sequencing**

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

The following primers were used for amplification: LR0R and LR5 for nuclear LSU rDNA (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>), ITS1–F (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990) for the ITS and 5.8S regions. PCR reaction volumes for the

rDNA amplifications were 50  $\mu$ L consisting of: 32.5  $\mu$ L ddH<sub>2</sub>O, 1  $\mu$ L DNA, 5  $\mu$ L (10 $\times$ ) reaction buffer (Super-Therm, JMR Holdings, U.S.A.), 5  $\mu$ L MgCl<sub>2</sub>, 5  $\mu$ L dNTP (10 mM of each nucleotide), 0.5  $\mu$ L (10 mM) of each primer, and 0.5  $\mu$ L Super-Therm Taq polymerase (JMR Holdings, U.S.A.). DNA fragments were amplified using a Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). PCR reaction conditions were: an initial denaturation step of 2 min at 95 °C, followed by 35 cycles of: 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 1 min elongation at 72 °C. The PCR process terminated with a final elongation step of 8 min at 72 °C.

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

All amplified PCR products were cleaned using the Wizard® SV gel and PCR clean up system (Promega, Madison, Wisconsin, U.S.A.) according to the manufacturer's instructions. The purified fragments were sequenced using the PCR primers and the Big Dye™ Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.). The fragments were analysed on an ABI 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  $\beta$ -tubulin sequences from the present study (Table 1) were compared with sequences of closely related *Ophiostoma* spp. from previous studies (De Beer *et al.* 2003, Aghayeva *et al.* 2004, 2005). Sequences were aligned using Clustal X v. 1.81.

*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 and Crandall 1998). Selected evolutionary models were: GTR+I+G (proportion invariable sites 0.6899 and rates for variable sites following a gamma distribution with shape parameter of 1.0185) for LSU, TrN+I+G (proportion invariable sites 0.4213 and rates for variable sites following a gamma distribution with shape parameter of 0.6253) for ITS, and HKY+G (rates for variable sites following a gamma distribution with shape parameter of 0.1783) for  $\beta$ -tubulin. Trees were constructed using the neighbour-joining tree-building algorithm (Saitou and 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 and 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 7 isolates of *O. splendens* that came from the same host, *P. repens*. Some old isolates of *O. africanum* from *P. dracomontana* Beard and *P. caffra* were newly identified.

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

## Growth in culture

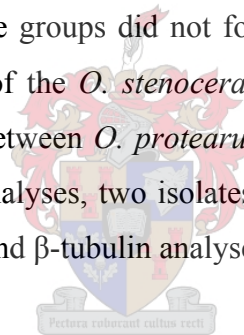
Isolates of both the unknown *Ophiostoma* species showed optimum growth at 30 °C. Mean colony diameter for the species collected from *P. repens* was 26 mm ( $\pm 1$ ), while the species from *P. neriifolia* and *P. laurifolia* had a colony diameter of 18 mm ( $\pm 1$ ) at this temperature after 8 d 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 ( $\pm 1$ ) 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 ( $\pm 1$ ) on 0.05 g/L to 12 mm ( $\pm 1$ ) on 2.5 g/L cycloheximide.

## Phylogenetic analysis

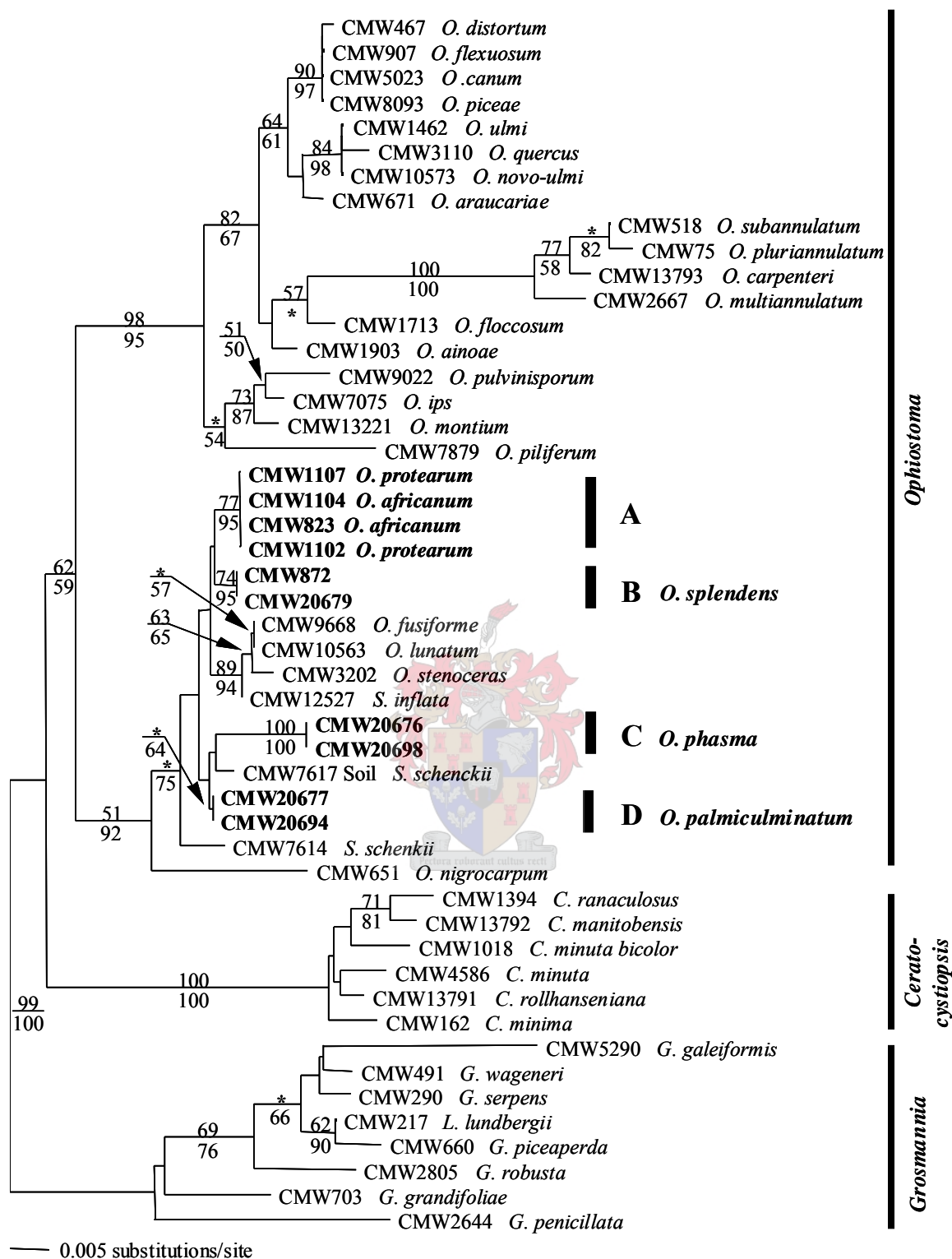
Alignment of the amplified products with Clustal X resulted in data sets of 709 characters for LSU, 531 characters for ITS, and 307 characters for part of the  $\beta$ -tubulin gene. Placement of isolates in the resulting trees based on phylogenetic analyses for each gene region was similar. For all three gene regions, the trees presented (Figs 2–4) were obtained from neighbour-joining analyses.

For the LSU region there were 98 parsimony-informative characters, 611 parsimony-uninformative characters, and 581 constant characters. For the ITS region there were 98 parsimony-informative characters, 433 parsimony-uninformative characters, and 389 constant characters. For the  $\beta$ -tubulin region there were 112 parsimony-informative characters, 195 parsimony-uninformative characters, and 194 constant characters. Analysis using the parsimony algorithm yielded 38, 9990 and 9530 equally most parsimonious trees of 291, 234 and 268 steps long for the LSU, ITS and  $\beta$ -tubulin data sets respectively. The Consistency Indices were 0.765, 0.533 and 0.705, while the Retention Indices were 0.957, 0.856 and 0.940 for the ITS, LSU and  $\beta$ -tubulin regions, respectively. Apart from group C (PP 1.0), PP values obtained for LSU were not statistically significant for the groups of interest and were omitted (Fig. 2).

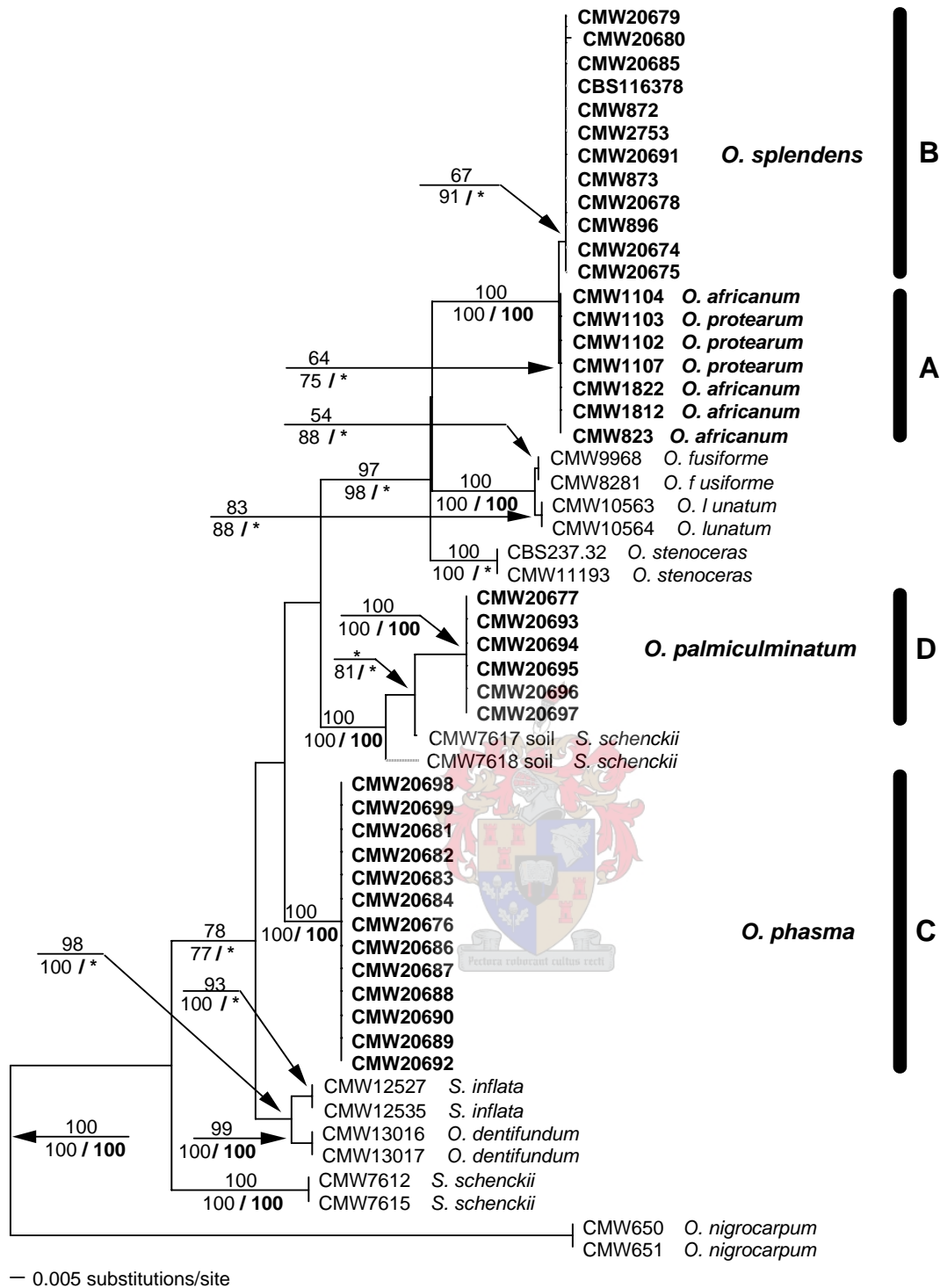
Trees obtained using different analyses of the LSU data resembled each other, and only the neighbour-joining tree (Fig. 2) is presented. The five taxa from proteas formed four distinct, well-supported groups (A–D). These groups did not form a monophyletic lineage, but were distributed among various species 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). Based on these analyses, two isolates of *O. nigrocarpum* were selected as outgroup for the more focused ITS and  $\beta$ -tubulin analyses.



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



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



**Fig. 3.** Distance dendrogram obtained with the TrN+I+G parameter model ( $G = 0.6253$ ) on the 5.8S (including the flanking internal transcribed spacers 1 and 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).



## Taxonomy

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

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

*Anamorph: Sporothrix* sp.

*Etymology:* The epithet *phasma* (*phasma* = ghost) refers to the small and inconspicuous perithecia growing within a cryptic habitat.

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

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

*Colonies* on malt extract agar 22 µm (± 1) mm diam in 8 d at 25 °C in the dark, white to cream-coloured, effuse, circular with an entire edge, surface smooth becoming mucoid, with a distinctive soapy odour, hyphae semi-immersed (Fig. 5D). Growth reduced at temperatures below and above the optimum of 30 °C. Sporulation profuse on MEA. *Conidiogenous cells* arising directly from hyphae on the surface of the agar and from aerial conidiophores,

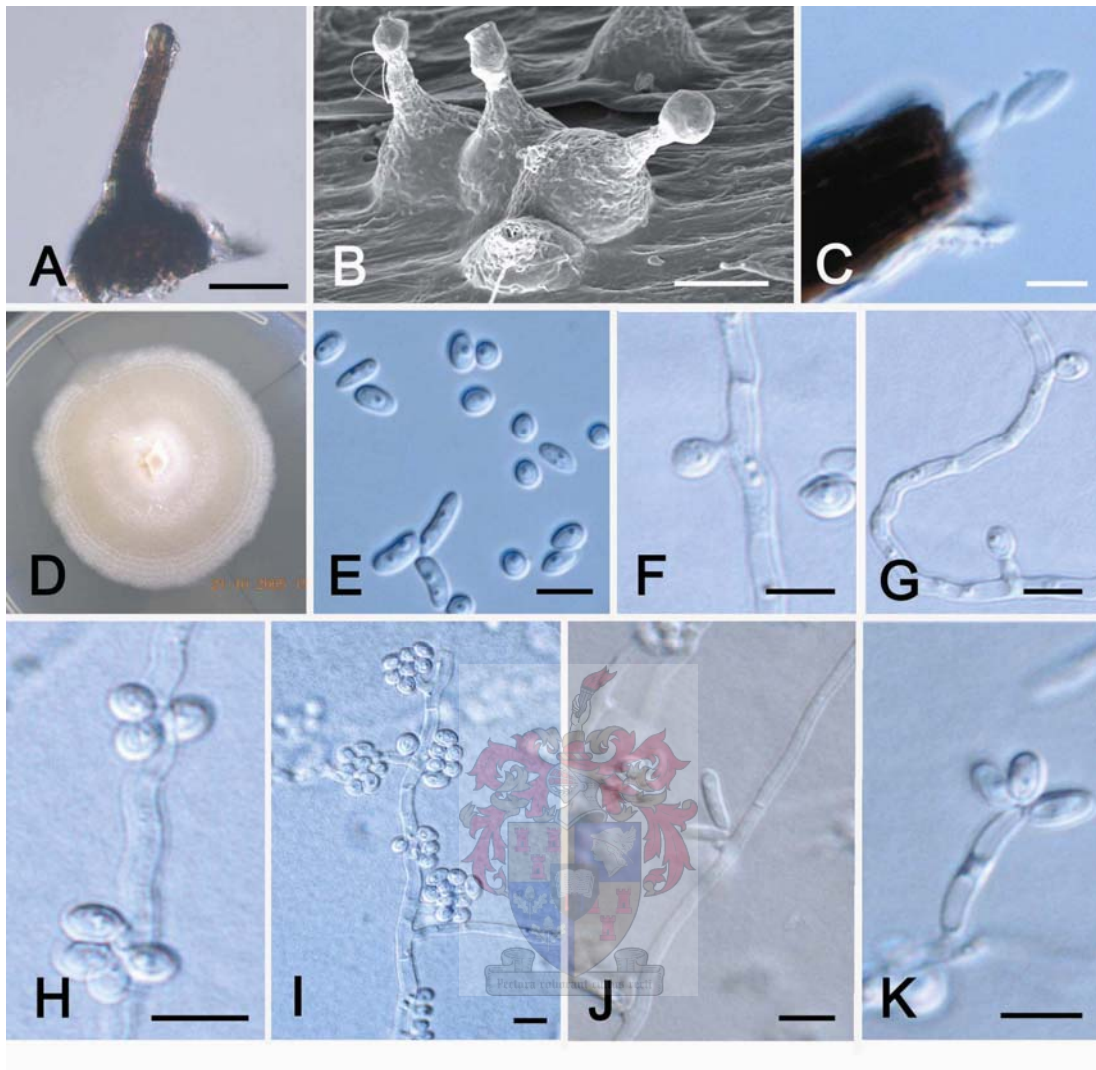
proliferating sympodially, hyaline (Fig. 5F–K). *Conidia* holoblastic and hyaline and of two forms, one ellipsoidal to clavate, smooth, thin-walled, 5–8 x 2–3 µm (Fig. 5E) and the other globose to obovate, smooth, thin-walled, 3–5 x 2–3 µm (Fig. 5E). Conidia forming singly, but aggregating into slimy masses, often also produced directly on hyphae (5H–I).

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

*Distribution*: South Africa, Western Cape Province.

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





**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  $\mu\text{m}$ ; C = 5  $\mu\text{m}$ ; E–K = 3  $\mu\text{m}$ .

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

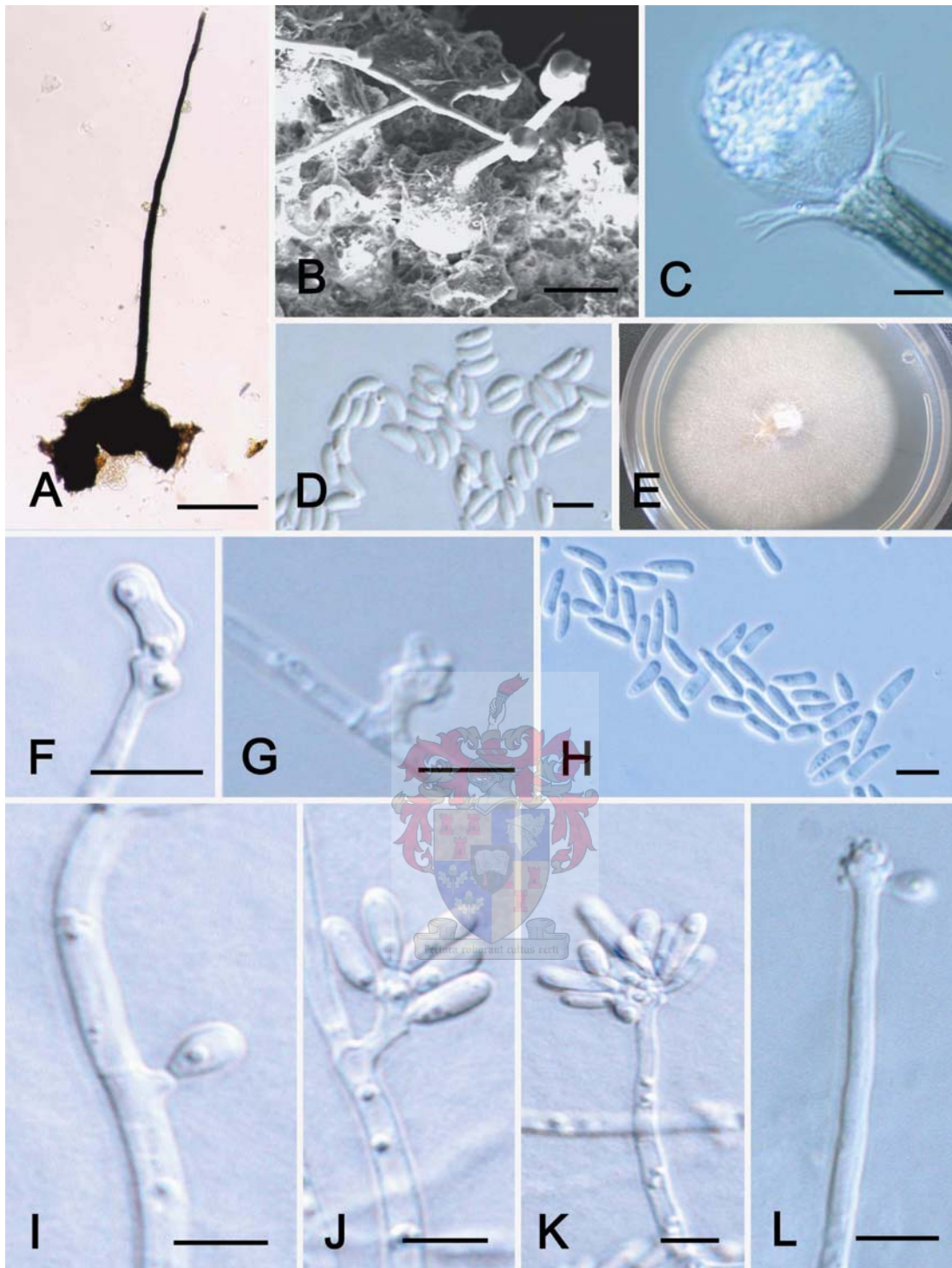
*Anamorph: Sporothrix* sp.

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

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

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

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



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

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

*Distribution:* South Africa, Western Cape Province.

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

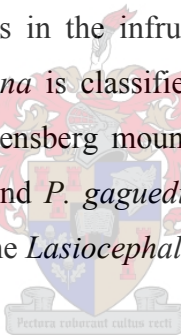
## Discussion

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

The *Ophiostoma* spp. found in *Protea* infructescences look morphologically very similar and in this respect, analyses of DNA sequence data enhance our ability to recognise distinct taxa. Thus, two new *Ophiostoma* spp. are recognised 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 morphologically distinct from each other and from the other three species, although these differences would have been difficult to define in the absence of DNA

sequence comparisons. Results of this study also represent the first report of *O. africanum* from *Protea dracomontana* and *P. caffra*.

Analyses of LSU and ITS sequence data was insufficient to distinguish between *O. africanum* and *O. protearum*. This shows that the two species are very closely related. Analyses of the more variable  $\beta$ -tubulin gene regions, however, support the notion that the two species represent distinct taxa as defined by Marais and Wingfield (2001) based on morphological characteristics. 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 and Wingfield 1997, 2001) that is classified in the section *Leiocephalae* and occurs in the eastern and northern provinces of South Africa (Rebelo 1995). *Ophiostoma africanum* was previously thought to be specific to *P. gagedi* (Marais and Wingfield 2001), but sequence data from the present study show that it also occurs in the infructescences of *P. dracomontana* and *P. caffra*. Like *P. caffra*, *P. dracomontana* is classified in the section *Leiocephalae*, and the latter species is restricted to the Drakensberg mountain range. This area overlaps with the distribution ranges of both *P. caffra* and *P. gagedi*, although *P. gagedi* is classified in a different section of the genus *Protea*, the *Lasiocephalae* (Rebelo 1995).

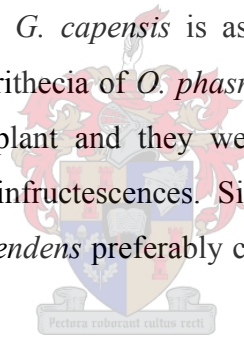


Phylogenetic analyses of DNA sequences of three gene regions investigated in this study suggest that *O. splendens* is closely related to *O. africanum* and *O. protearum*. *Ophiostoma splendens* has been recorded from *P. repens*, *P. neriifolia*, *P. lepidocarpodendron* and *P. longifolia* in the Western Cape Province (Marais and 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 and Wingfield 1994, Roets *et al.* 2006a). We did not isolate *O. splendens* from any *Protea* species other than *P. repens*. Other than the single isolate of *O. splendens* from *P. neriifolia*, the fungus appears to be confined to *P. repens*, which resides in the section *Melliferae*. The explanation for the close phylogenetic relationship between *O. splendens* and its northern

counterparts, *O. protearum* and *O. africanum*, will probably only be revealed once a robust phylogeny for the genus *Protea* becomes available.

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

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



*Ophiostoma palmiculminatum* is the only species of *Ophiostoma* or *Gondwanamyces* that has been collected from the tunnels of insects found within the involucre receptacles of *P. repens*. These tunnels are made by either coleopteran or lepidopteran larvae (Coetzee and Giliomee 1987b). The 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 characteristics represent adaptations for arthropod-vectored dispersal (Malloch and 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 and 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 and Paine 1998, 1999, Klepzig *et al.* 2001a, 2001b, Six 2003a, 2003b, Six and Bentz 2003, Klepzig and 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 techniques 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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## Chapter 4: Discovery of fungus-mite-mutualism within a unique niche of the Cape Floral Kingdom

### Abstract

The floral heads (infructescences) of *Protea* species in South Africa represent one of the most unusual niches in which fungi belonging to genus *Ophiostoma* have been found. This fungal group, well-known for its plant pathogenic members, is morphologically adapted for insect dispersal. Although most species appear to be vectored by tree-infesting bark beetles, how the *Protea*-associated *Ophiostoma* species are dispersed, is not yet known. In order to identify the primary vectors of these species, infructescences-colonising arthropods were collected and tested for the presence of *Ophiostoma* DNA using PCR techniques. Putatively identified vectors were subsequently screened for the presence of fungal spores using agar plate isolations. Small arthropods were tested using both techniques irrespective of the outcome of PCR tests, as their potentially low spore loads might have been insufficient to ensure PCR amplification. PCR tests revealed the presence of *Ophiostoma* DNA on three insect species only, but no isolates of *Ophiostoma* were retrieved from these insects in the subsequent plating studies. However, mites collected from various infructescences were found to carry *Ophiostoma* propagules. These mite species included *Proctolaelaps vandenbergi*, two species of *Tarsonemus* and one *Oodinychus* species. DNA sequences of 28S ribosomal DNA confirmed the presence of *O. splendens*, *O. palmiculminatum* and *O. phasma* on these mites. Light and scanning electron microscopy revealed specialised structures in the *Oodinychus* and one *Tarsonemus* species; in the case of *Oodinychus*, these frequently contained spores of *Ophiostoma*. The *Oodinychus* species was able to complete its life cycle on a diet consisting of only its phoretic *Ophiostoma* species: *O. palmiculminatum*, *O. phasma* and *O. splendens*. The population growth of this mite was significantly higher when fed these fungal species than when it was presented with a diet of various other fungi. Results of this study provide compelling evidence that mites are the primary vectors of infructescence-associated *Ophiostoma* in South Africa. There also appears to be a close mutualistic association between these fungal species and the *Oodinychus* sp. Morphological observations and DNA-based

phylogenetic reconstruction of the genus also revealed the presence of two apparently undescribed species of *Ophiostoma* on these mites.

*Key words:* *Ophiostoma*, symbiosis, *Protea*, phoresy, mycangia, sporothecae

## **Introduction**

More than 90 species of the remarkable plant genus *Protea* L. (proteas) are found in South Africa (Rebello 1995). Members of this endemic African genus produce large, colourful floral heads (inflorescences) and numerous species are economically important in generating revenue from eco-tourism, horticulture and the dried-flower industries (Anon. 1999, Crous *et al.* 2004). Some species also represent pivotal members of the ecosystems in which they occur. Landscapes within the unique Fynbos Biome, in particular, are often dominated by these attractive plants (Cowling and Richardson 1995). The Fynbos Biome forms a major component of the Cape Floristic Region (CFR), which is located at the southwestern tip of Africa and is internationally recognised for its exceptional richness in flowering plants (Goldblatt and Manning 2000). It displays very high levels of gamma diversity, which correlates well with the unusually high levels of local endemism (Goldblatt and Manning 2000, Linder 2003). While CFR plant taxa (including *Protea* species) have been extensively surveyed, very little is known about the biological associations between these plants and other organisms.

The brightly colored inflorescences of most *Protea* spp. are pollinated by animals including insects, birds and rodents (Rebello 1995). The seeds of many species are retained within compact structures known as infructescences. In serotinous *Protea* spp., these infructescences serve as above-ground seed-storage structures, releasing seeds only after fire or when the water supply between the infructescences and the rest of the plant is severed (Bond 1985, Cowling and Richardson 1995).

Infructescences of *Protea* spp. can be viewed as miniature ecosystems (Zwölfer 1979) in which many fungal species are known to thrive (Marais and Wingfield 1994, Lee *et al.* 2005). One of the most unusual contemporary discoveries related to *Protea* was the detection of so-called ophiostomatoid fungi in the infructescences of serotinous *Protea* spp. (Wingfield *et al.* 1988). These fungi are best-known as associates of insects such as bark beetles (Coleoptera:

Scolytinae) that make galleries in the bark / cambium interface of trees, or picnic beetles (Coleoptera: Nitidulidae) that colonise wounds on trees (Upadhyay 1981, Harrington 1987, Wingfield *et al.* 1993, Jacobs and Wingfield 2001, Jacobs *et al.* 2003). Ophiostomatoid fungi are morphologically adapted to be dispersed by arthropods, typically by having sticky spores carried on stalked fruiting structures (Malloch and Blackwell 1992, 1993. Cassar and Blackwell 1996). These fungi also include some of the world's most serious tree pathogens such as the causal agents of Dutch elm disease (*Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier) and *Ceratocystis fagacearum* (Bretz) J. Hunt, the causal agent of Oak wilt (Sinclair *et al.* 1987, Brasier 1991). Their dominant presence in *Protea* infructescences has thus been considered curious and inexplicable.

The ophiostomatoid fungi, including the genera *Gondwanamyces* G.J. Marais & M.J. Wingf. and *Ophiostoma* Syd. & P. Syd. emend. Z.W. de Beer *et al.*, typically form the dominant fungal component within the infructescences of *Protea* spp. Their fruiting structures regularly colonise more than 50% of the older infructescences in a population (Roets *et al.* 2005). These two fungal genera are phylogenetically distantly related (Hausner *et al.* 1992, 1993a, 1993b, Spatafora and Blackwell 1994, Marais *et al.* 1998, Wingfield *et al.* 1999). The *Protea*-specific genus *Gondwanamyces* resides in the Microascales and is related to the well-known pathogen genus *Ceratocystis* Ellis & Halst., while the cosmopolitan genus *Ophiostoma* belongs to the Ophiostomatales (Wingfield *et al.* 1999, Zipfel *et al.* 2006).

Five species of *Ophiostoma* have been described from the infructescences of *Protea* spp. These are *O. splendens* G.J. Marais & M.J. Wingf. (Marais and Wingfield 1994), *O. protearum* G.J. Marais & M.J. Wingf. (Marais and Wingfield 1997), *O. africanum* G.J. Marais & M.J. Wingf. (Marais and Wingfield 2001) and the recently described *O. phasma* Roets *et al.* and *O. palmiculminatum* Roets *et al.* (Roets *et al.* 2006a, Chapter 3). All of these species have *Sporothrix* Hekt. & C.F. Perkins asexual states. *Ophiostoma splendens*, *O. protearum* and *O. palmiculminatum* are each thought to be confined to a specific *Protea* species, while *O. africanum* and *O. phasma* have been isolated from different *Protea* species (Marais and Wingfield 1997, Marais and Wingfield 2001, Roets *et al.* 2005, 2006a, Chapter 3).

The mode of dispersal of *Ophiostoma* species from the infructescences of one *Protea* plant to another is unknown. The fungi appear in the infructescences relatively soon after flowering



when the infructescences close (Roets *et al.* 2005). Although *Ophiostoma* spp. that occur elsewhere are known to be vectored by many different insects, bark beetles are the most common vectors (Barras and Perry 1975, Upadhyay 1981, Price *et al.* 1992, Wingfield *et al.* 1993, Paine *et al.* 1997, Klepzig *et al.* 2001a, 2001b, Kirisits 2004, Klepzig and Six 2004, Harrington 2005). It is thus reasonable to assume that the *Ophiostoma* spp. found in *Protea* infructescences would also have insect vectors. Insects are common in the closed infructescences of *Protea* species (Coetzee and Giliomee 1985, 1987a, 1987b, Roets *et al.* 2006b) providing numerous candidate vectors for these fungi. Like other *Ophiostoma* spp., those in *Protea* infructescences have elongated necks bearing sticky spores that could easily be transported from one infructescence to another by insects that occupy this niche.

Mites and particularly those carried by bark beetles, are also known to act as vectors of some ophiostomatoid fungi, including *Ophiostoma* spp. (Moser and Roton 1971, Smiley and Moser 1974, Moser 1976, Bridges and Moser 1983, Moser 1985, Moser and Bridges 1986, Moser *et al.* 1995), and hence could also play a role in the dispersal of the *Protea*-associated members of *Ophiostoma*. The association between mites and the fungi they vector can be highly specialised (Klepzig *et al.* 2001a, 2001b, Klepzig and Six 2004). Some mite species have evolved specialised spore-carrying structures (sporothecae) that have been shown to contain spores of ophiostomatoid fungi (Bridges and Moser 1983, Moser 1985, Moser *et al.* 1995). The association between these mites and their phoretic fungi may be mutualistic (Klepzig *et al.* 2001b).

The aim of this study was to identify the possible vectors of the *Ophiostoma* spp. found in *Protea* infructescences. We question whether the host specificity of *Protea* species and *Ophiostoma* species associated with them might be explained by the vector relationships of the fungi. Furthermore, we consider whether there are mutualistic relationships between specific *Ophiostoma* spp. and their vectors.

## Materials and methods

### *Arthropod collection*

A total of 280 3-mo to 1-y-old *Ophiostoma*-colonised infructescences representing four *Protea* species (n = 70) were collected from different sites in the Western Cape Province, South Africa, between January 2003 and August 2005. *Protea* species included: *P. repens* L. from the Jan S. Marais Park, Stellenbosch, *P. neriifolia* R. Br. from the Jonkershoek Forestry Reserve, Stellenbosch, *P. longifolia* Andrews from the Kogelberg Nature Reserve, Betties Bay and *P. laurifolia* Thunb. from Piekenierskloof Pass, Citrusdal.

Infructescences were placed in specially designed emergence cages from which arthropods were collected. Emergence cages were made up of two large plastic containers (64 cm long x 39 cm wide x 20 cm deep) stacked on top of one another. A total of 28 holes (3.5 cm diam) were drilled into the base of the upper container through which PVC piping (10 cm in length, 3.5 cm diam) was secured. The lower container was filled with water and the stalks of infructescences were pushed through the piping such that the bases of the infructescences blocked the aperture at the top of the pipe. The stalks of the infructescences extend through the pipes into the lower container where they were kept immersed in water. The upper container was then covered with fine gauze.

Emergence cages were maintained at room temperature in the laboratory. They were inspected every two to three days over a 40 d period, and all emerging arthropod individuals were collected and classified into morpho-species. Using the emergence cages ensured simultaneous collection of arthropods as they emerged from numerous infructescences, and presumably after they would have acquired spores from fungi in the infructescences. After 40 d, the infructescences were opened and all remaining arthropods were extracted using fine tweezers and a dissecting needle. The surfaces of larger arthropods were cleared of debris and / or smaller phoretic arthropods using a fine camel-hair brush and dissecting needle. All arthropods were stored at -20 °C until further analysis.

Additional arthropod individuals were collected directly from *Ophiostoma*-colonised infructescences at the natural collection sites mentioned above as well as at two additional sites. Infructescences of *Protea caffra* Meisn. were obtained from the Walter Sisulu National

Botanic Garden, Gauteng Province, while infructescences of *Protea repens* were collected from an additional site in George, Western Cape Province. The infructescences were opened and arthropods were extracted as described above. All arthropod individuals collected directly from infructescences, were cleared of debris and stored at 4 °C until further analysis. Voucher specimens of all the morpho-species collected are maintained in the insect collection (USEC), Department of Conservation Ecology and Entomology, University of Stellenbosch, Stellenbosch, South Africa.

#### *Vector identification using polymerase chain reaction (PCR)*

A newly developed PCR protocol (Roets *et al.* 2006c, Chapter 2) was used to test a subset of infructescence-associated arthropods collected from the emergence cages for the presence of *Ophiostoma* DNA. The subset included individuals ( $n \leq 30$ ) of each arthropod species collected per *Protea* species (Table 1). All individuals of *Genuchus hottentottus* (F) (Scarabaeidae) and *Oxycarenus maculatus* Stal. (Lygaeidae) were tested, as these two insect taxa had previously been noted as putative vectors (Roets *et al.* 2006c, Chapter 2). Individuals used for the PCR procedures were macerated in Eppendorf tubes, after which the total genomic DNA was extracted (Lee and Taylor 1990).

Expected product length after amplification of *Ophiostoma* DNA with the primers OSP1 (Roets *et al.* 2006c, Chapter 2) and LR6 (Vilgalys and Hester 1990) was *ca.* 900 bp. PCR products of the appropriate length were cleaned using the Wizard® SV gel and PCR clean-up system (Promega, Madison, Wisconsin, U.S.A.). The fragments were sequenced using the PCR primers and the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.) with an ABI PRISIM™ 3100 Genetic Analyzer (Applied Biosystems) to verify positive amplification results.

#### *Vector identification by direct plating of arthropods*

All individuals ( $n \leq 50$ ) of the small (less than 1 mm long) arthropod species and the species that yielded positive PCR results, were crushed, vortexed in 2 – 10 ml ddH<sub>2</sub>O (depending on the size of the arthropod) and plated (1 ml of suspension per plate) on Petri dishes containing 2 % malt extract agar (MEA, Biolab, Midrand, South Africa), streptomycin sulphate (0.04 g/L) and cycloheximide (0.05 g/L), which is selective for *Ophiostoma* spp. (Harrington 1981).

This plating technique made it possible to verify putative vectors for the *Protea*-associated *Ophiostoma* spp., and also provided an indication of the number of reproductive propagules carried per individual insect. Spore numbers were based on numbers of *Ophiostoma* colony-forming units (CFU's) growing from each arthropod individual. The mean number of CFU's was calculated for each putative *Ophiostoma* spp. isolated from each arthropod species (Table 2).

### *Isolates*

Colony- and microscopic fungal characteristics were used to determine the number of putative *Ophiostoma* spp. (as *Sporothrix* anamorphic states) isolated from arthropods. In all cases, where suspected *Ophiostoma* spp. were present on plates containing crushed individual arthropods, the colonies were found to represent a single species. One *Ophiostoma* sp. colony per arthropod individual was chosen at random and purified as representative of that fungal species. Representative cultures of all species were deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 3).

### *Vector identification by light- and scanning electron microscopy*

The part of the arthropods exoskeleton on which the fungal spores was carried was considered using a Leo 1430VP Scanning Electron Microscope (SEM). Individuals (n = 50 per arthropod species) of the suspected primary vectors of the *Ophiostoma* spp. were collected from *P. neriifolia* and *P. repens* infructescences from the Jonkershoek Nature Reserve and J. S. Marais Park, respectively. These arthropod species were also examined using light microscopy (n = 50 per species). In addition, representatives of *Genuchus hottentottus* (n = 15) and *Oxycarenus maculatus* (n = 33) were also studied by SEM, since these two species had been recognised as potential vectors in a previous study (Roets *et al.* 2006c, Chapter 2).

For the SEM studies, the arthropods were frozen (-20 °C) and then dried (3 d at 50 °C) and mounted onto stubs using double-sided carbon tape. They were sputter coated with gold-palladium using standard methods. SEM scans made it possible to locate spores on the surfaces of the arthropods. We focussed specifically on detecting ascospores, because of the

problems with the identification of fungal taxa based on the asexual conidia. Ascospores were presumed to belong to *Ophiostoma* species when they had an allantoid shape, were between 5 and 7  $\mu\text{m}$  in size and tended to stick together. These characteristics are typical of the *Ophiostoma* spp. found in *Protea* infructescences (Marais and Wingfield 2001, Roets *et al.* 2006a, Chapter 3). Arthropods were collected only from *Protea* infructescences that were heavily infected with *Ophiostoma* spp. We could then conclude that ascospores conforming to the above-mentioned criteria represented *Ophiostoma* spp.

In addition to the SEM studies, smaller arthropod specimens such as mites were mounted on microscope slides in lactophenol containing cotton blue (Stephens 1974). Mounts were heated over an open flame for 10 s and left overnight. Mounted arthropods were studied with the aid of a Nikon Eclipse E600 light microscope with differential interference contrast (DIC). Photographic images were captured using a Nikon DXM1200 digital camera.

#### *DNA extraction, amplification and sequencing of fungal isolates*

Genomic DNA was extracted from isolates using a Sigma GenElute™ plant genomic DNA miniprep kit (Sigma-Aldrich Chemie CMBH, Steinheim, Germany) following the manufacturer's instructions. For amplification and sequencing of the nuclear large subunit (LSU) 28S rDNA region, the primers LROR and LR5 (White *et al.* 1990) were used. PCR reaction volumes (50  $\mu\text{L}$ ) contained 32.5  $\mu\text{L}$  ddH<sub>2</sub>O, 1  $\mu\text{L}$  DNA, 5  $\mu\text{L}$  (10X) reaction buffer (Super-Therm JMR Holdings, U.S.A.), 5  $\mu\text{L}$  MgCl<sub>2</sub>, 5  $\mu\text{L}$  dNTP (10 mM of each nucleotide), 0.5  $\mu\text{L}$  (10 mM) of each primer and 0.5  $\mu\text{L}$  Super-Therm Taq polymerase (JMR Holdings, U.S.A.). PCR runs were performed on a Gene Amp®, PCR System 2 700 thermal cycler (Applied Biosystems, Foster City, U.S.A.), and PCR reaction conditions included an initial denaturation step of 2 min at 95 °C followed by 35 cycles of: 30 sec denaturation at 95 °C, 30 sec annealing at 55 °C and 1 min elongation at 72 °C. A final elongation step of 8 min at 72 °C was performed before the PCR process was terminated. Purification and sequencing of PCR products followed the methods outlined above.

### *Phylogenetic analyses*

Sequence data obtained in this study were compared to sequences of both *Protea*-associated and non-*Protea*-associated *Ophiostoma* species obtained from GenBank (Table 3). These included the large subunit sequences of the ex-type cultures of all *Ophiostoma* spp. described from *Protea* infructescences. Sequence data were aligned using the software package Clustal X (1.81). The aligned data set consisted of 706 characters that were treated as unweighted. Numbers of parsimony informative, parsimony uninformative and constant characters for the data set was 98, 29, and 579, respectively. A heuristic search in PAUP, v.4.0 beta 10 (Swofford 2000) was performed with tree-bisection-reconnection (TBR) branch swapping active. Starting trees were obtained through step-wise addition and resulting trees were combined into a consensus tree. One tree was saved per replicate to facilitate optimal searching of tree space. A total of 1000 bootstrap replicates (Felsenstein 1985) were performed with the fast-stepwise addition option active in order to estimate confidence levels.

Distance analysis was performed using the neighbour-joining algorithm (Saitou and Nei 1987) in PAUP. The evolutionary model GTR+I+G (proportion of invariable sites at 0.7012 and the rates for variable sites following a gamma distribution with shape parameter of 1.0849) was selected using Modeltest 3.06 based on Akaike Information Criteria (Posada and Crandall 1998). Statistical support for nodes obtained by distance analysis was determined by 1000 bootstrap replicates using the TBR algorithm.

Bayesian analysis was performed using the GTR+I+G (shape parameter with 4 rate categories) model and the Markov Chain Monte Carlo approach in the software package MrBayes v.3.1.1 (Ronquist and Huelsenbeck 2003). All parameters were inferred from the data. Two independent Markov chains of 1000000 generations each (sample frequency of 50) were initiated from a random starting tree. The first 20000 generations were discarded as burnin and the remaining trees were pooled into a 50 % majority rule consensus tree.

## *Protea-associated Ophiostoma spp. as food source for vector arthropods*

The most common arthropod identified as a vector of *Ophiostoma* spp. spores was a species of mite collected from the infructescences of *P. repens* (ca. 5-month-old) in the J.S. Marais Park. To test their ability to feed and reproduce on a diet of *Protea*-associated *Ophiostoma* species only, these mites were transferred to Petri dishes containing 1-week-old cultures of *O. splendens* growing on MEA plates. The first generation progeny of these individuals that had been caught in the wild were used in all subsequent experiments. All experiments were carried out on MEA plates that were kept at 25 °C in the dark.

The population growth rate of the mite species was tested on a diet of *O. palmiculminatum*, *O. phasma*, *O. splendens* and eight non-ophiostomatoid fungal species isolated from species of *Protea* available from the culture collection of Stellenbosch University, Stellenbosch, South Africa. These included representatives of the genera *Cladosporium* Link (STU pending), *Conoplea* Pers. (STU5660), *Dactylaria* Sacc. (STU5657), *Gliocladium* Corda (STU5661), *Monodictys* S. Hughes (STU5656), *Penicillium* Link (STU pending), *Phaeoisaria* Höhn. (STU5659) and *Pithomyces* Berk. & Broome (STU5662). Mature mite individuals (n = 10) were placed on 1-week-old cultures of the 11 fungal species. As a control, mites were placed on Petri dishes containing only MEA. The experiment was replicated three times. After 40 d, we determined the number of individuals for each colony. Differences in mite population sizes between the various fungal species were compared statistically by performing a t-test (Statistica 7, Statsoft corporation, Tulsa, U.S.A.). Significant differences are reported when  $P \leq 0.05$ .

## **Results**

### *Arthropod collection*

Forty-one arthropod morpho-species (811 individuals) were collected from the different *Ophiostoma*-colonised *Protea* infructescences using the emergence cages (Table 1). *Protea repens* infructescences contained the greatest number of arthropod individuals (341) and also had the greatest diversity of taxa (33). *Protea neriifolia* (richness = 24, abundance = 142), *P. laurifolia* (richness = 29, abundance = 201) and *P. longifolia* (richness = 20, abundance = 180) showed lower arthropod richness and abundance levels than *P. repens*, but their richness and

abundance levels were comparable with each other. Most arthropods were found to be associated with more than one *Protea* spp.

#### *Vector identification using PCR*

Using PCR, 21 individuals (six arthropod morpho-species) yielded amplified fragments of the appropriate length to represent species of *Ophiostoma*. Sequencing of these products, however, showed that only three insect species (five individuals) carried DNA of *Ophiostoma* species (Table 1). Two individuals each of the putative vector arthropods *Genuchus hottentottus* (Scarabeidae: Coleoptera) and *Oxycarenus maculatus* (Lygaeidae: Hemiptera) and one individual of a Psocopteran (sp. 3) were found to carry *Ophiostoma* DNA (Table 1). Although the PCR method used was not limited to amplifying *Ophiostoma* DNA, it allowed for the rapid identification of putative vectors from large numbers of arthropod individuals.

#### *Direct isolation from arthropods*

Based on the presence of *Ophiostoma* spp. on these insects, additional specimens of *G. hottentottus*, *O. maculatus* and the Psocopteran (sp. 3) were collected from *P. repens* in the J.S. Marais Park, Stellenbosch (Table 1). Isolation from *G. hottentottus* and *O. maculatus* on selective medium for species of *Ophiostoma* failed to yield evidence of *Ophiostoma* spp. Plates were dominated by yeasts. Although contamination was less problematic than with the other insects, this technique also failed to produce colonies of *Ophiostoma* spp. from the additionally collected individuals of this Psocopteran sp. Likewise, no *Ophiostoma* spp. were isolated from the other Psocopteran species tested (Table 1).

In contrast to isolation from insects, isolations from four mite morpho-species collected from the different *Protea* species sampled (Table 2) commonly yielded cultures of *Ophiostoma* spp. The mites included *Proctolaelaps vandenbergi* Ryke, two members of the genus *Tarsonemus* Canestrini & Fonzago and a species of the genus *Oodinychus* Berlese. None of the numerous individuals of any other mite species tested (Table 1) gave rise to cultures of *Ophiostoma* spp. About 14 % of all *Oodinychus* sp. individuals (n = 85), 2 % of all the individuals of *Tarsonemus cf. sp. A* (n = 100), 15.8 % of *Tarsonemus cf. sp. B* (n=19) and 0.8 % of *Proctolaelaps vandenbergi* (n = 128) gave rise to cultures of *Ophiostoma* spp. (Table 2).



**Table 1.** Total number of arthropods collected from the infructescences of the four *Protea* spp. (n = 70 for each species) and tested for the presence of *Ophiostoma* DNA using PCR techniques. Numbers in brackets indicate the number of individuals verified to be positive for *Ophiostoma* DNA. Numbers in bold indicate the number of additional arthropod individuals collected and tested for the presence of *Ophiostoma* spp. reproductive propagules by plating techniques.

Arthropod taxa	Ref. nr.	<i>Protea</i> species				
		<i>P. repens</i>	<i>P. longifolia</i>	<i>P. neriifolia</i>	<i>P. laurifolia</i>	
<b>INSECTS</b>						
<i>Argyroploce</i> sp. Hübner (Tortricidae)	68	7	1	1	3	
Blattidae	26		1	2		
Braconidae	52	2		1		
Bruchidae	51				1	
<i>Capys alphaeus</i> Cramer (Lycaenidae)	66	2	1		1	
Carabidae	29				4	
Chrysomelidae	17		17	2		
<i>Crematogaster</i> sp. Lund (Formicidae)	15	10	21			
Curculionidae	48	4	1	1	1	
Dermaptera	42				1	
Diptera	5	12		4	2	
<i>Euderus lineicollis</i> Wiedemann (Curculionidae)	33	9	1		1	
Formicidae (sp. 1)	23	4				
Formicidae (sp. 2)	56	9			79	
<i>Genuchus hottentottus</i> (F) (Scarabaeidae)	70	28 (2)	<b>39</b>	15	1	
<i>Gyponyx</i> sp. Gorham (Cleridae)	55	1			3	
Histeridae	32	7	1	1	5	
Hopliini (Scarabaeidae)	47		1	4	3	
Miridae	20	1			1	
Nitidulidae	25	30	18	8	7	
<i>Oxycarenus maculatus</i> Stal. (Lygaeidae)	7	51 (2)	<b>75</b>	37	19	46
Pentatomidae	24	2		1		
Psocoptera (sp. 1)	31	12, <b>50</b>	9	35	1	
Psocoptera (sp. 2)	12	4, <b>50</b>	1	1	3	
Psocoptera (sp. 3)	13	66 (1)	<b>108</b>	8	1	
<i>Sphenoptera</i> Solier sp. (Buprestidae)	49	2	4	1	11	
Staphylinidae	35	1			4	
Thysanoptera	34		2	5	1	
<i>Tinea</i> sp. L. (Tineidae)	67	4		1	1	
<b>SPIDERS</b>						
Clubionidae	59	1				
Spider (sp. 1)	64			4		
Spider (sp. 2)	63	1				
Spider (sp. 3)	60	2			1	

**Table 1.** Continued.

<b>MITES</b>					
<i>Ameroseius proteaea</i> Ryke (Ameroseiidae)	M1	18, <b>50</b>	<b>3</b>	1, <b>29</b>	<b>50</b>
<i>Oodinychus</i> sp. Berlese (Uropodidae)	M2	23, <b>50</b>	4	6, <b>24</b>	13
<i>Lorryia</i> sp. Oudemans (Tydeidae)	M3	1, <b>42</b>		1	3, <b>1</b>
<i>Tenuelamellarea hispanica</i> Subias & Itor. (Lamellareidae)	M4	3, <b>33</b>			1
<i>Humerobates setosus</i> Behan-Pelletier & Mahunka (Humerobatidae)	M5	2, <b>50</b>	1	<b>2</b>	1, <b>5</b>
<i>Bdellodes</i> sp. Oudemans (Bedellidae)	M6	1, <b>50</b>	<b>50</b>	1, <b>50</b>	<b>50</b>
<i>Proctolaelaps vandenbergi</i> Ryke (Ascidae)	M7	14, <b>50</b>	6	19, <b>50</b>	1, <b>28</b>
<i>Zygoribatula setosa</i> Evans (Oribatulidae)	M8	2, <b>9</b>			

**Table 2.** Isolates, frequency (F) and mean number of colony forming units (CFU's) of *Ophiostoma* spp. isolated from *Ophiostoma*-spore carrying mites collected from *Protea* infructescences from various localities.

<b>Mite species</b>	<b>n</b>	<b>Host</b>	<b>Locality</b>	<b>Fungal species</b>	<b>F (%)</b>	<b>CFU's</b>
<i>Oodinychus</i> sp.	50	<i>P. repens</i>	Jan S. Marais Park	<i>O. splendens</i>	3 (6)	1–8 (4.33)
<i>Oodinychus</i> sp.	50	<i>P. repens</i>	Jan S. Marais Park	<i>O. palmiculminatum</i>	4 (8)	1–8 (5.50)
<i>Oodinychus</i> sp.	50	<i>P. repens</i>	Jan S. Marais Park	<i>Sporothrix</i> sp. 1	1 (2)	1
<i>Oodinychus</i> sp.	24	<i>P. neriifolia</i>	Jonkershoek	<i>O. phasma</i>	1 (4.17)	19
<i>Oodinychus</i> sp.	11	<i>P. repens</i>	George	<i>O. splendens</i>	3 (27.27)	1–2 (1.33)
<i>P. vandenbergi</i>	50	<i>P. neriifolia</i>	Jonkershoek	<i>O. phasma</i>	1 (2)	1
<i>P. vandenbergi</i>	50	<i>P. repens</i>	Jan S. Marais Park	–	0	0
<i>P. vandenbergi</i>	28	<i>P. laurifolia</i>	Piekenierskloof pass	–	0	0
<i>Tarsonemus</i> cf. sp. A	50	<i>P. laurifolia</i>	Piekenierskloof pass	<i>O. phasma</i>	2 (4)	9–51 (30.00)
<i>Tarsonemus</i> cf. sp. A	50	<i>P. repens</i>	Jan S. Marais Park	–	0	0
<i>Tarsonemus</i> cf. sp. B	19	<i>P. caffra</i>	Walter Sisulu N.B.G.	<i>Sporothrix</i> sp. 2	3 (15.79)	1–9 (5.67)

*Tarsonemus cf. sp. A*, *P. vanderbergi* and the *Oodinychus sp.* were commonly collected from larval galleries of boring insects, especially that of *G. hottentottus* in *P. repens* infructescences. These galleries were generally located in the fruit-bearing bases of the infructescences. In many instances, these three mites were found sympatrically in *G. hottentottus* larval galleries, and they were present at the time when the larvae were still feeding. However, none of the three mite species were restricted to insect galleries, and they were also collected from all other internal parts of *Protea* infructescences throughout the collection period. Individuals of *Tarsonemus cf. sp. B* were collected from between the styles and other dead floral parts within *P. caffra* infructescences.

### *Isolates*

Eighteen isolates of putative *Ophiostoma* spp. were obtained from mites that were collected from the *Protea* spp. considered (Table 2). These isolates were divided into five groups based on culture and morphological characteristics. Three of the isolate groups were similar to those of *O. splendens*, *O. palmiculminatum* and *O. phasma*, respectively. Isolates representing the remaining two groups did not resemble any of the known *Ophiostoma* species associated with *Protea*. They were provisionally identified as *Sporothrix sp. 1* and *Sporothrix sp. 2* (Table 2). The single isolate of *Sporothrix sp. 1* was collected from an *Oodinychus sp.* associated with *P. repens*, while three isolates of *Sporothrix sp. 2* were collected from *Tarsonemus cf. sp. B* associated with *P. caffra*.

### *Vector identification by light- and scanning electron microscopy*

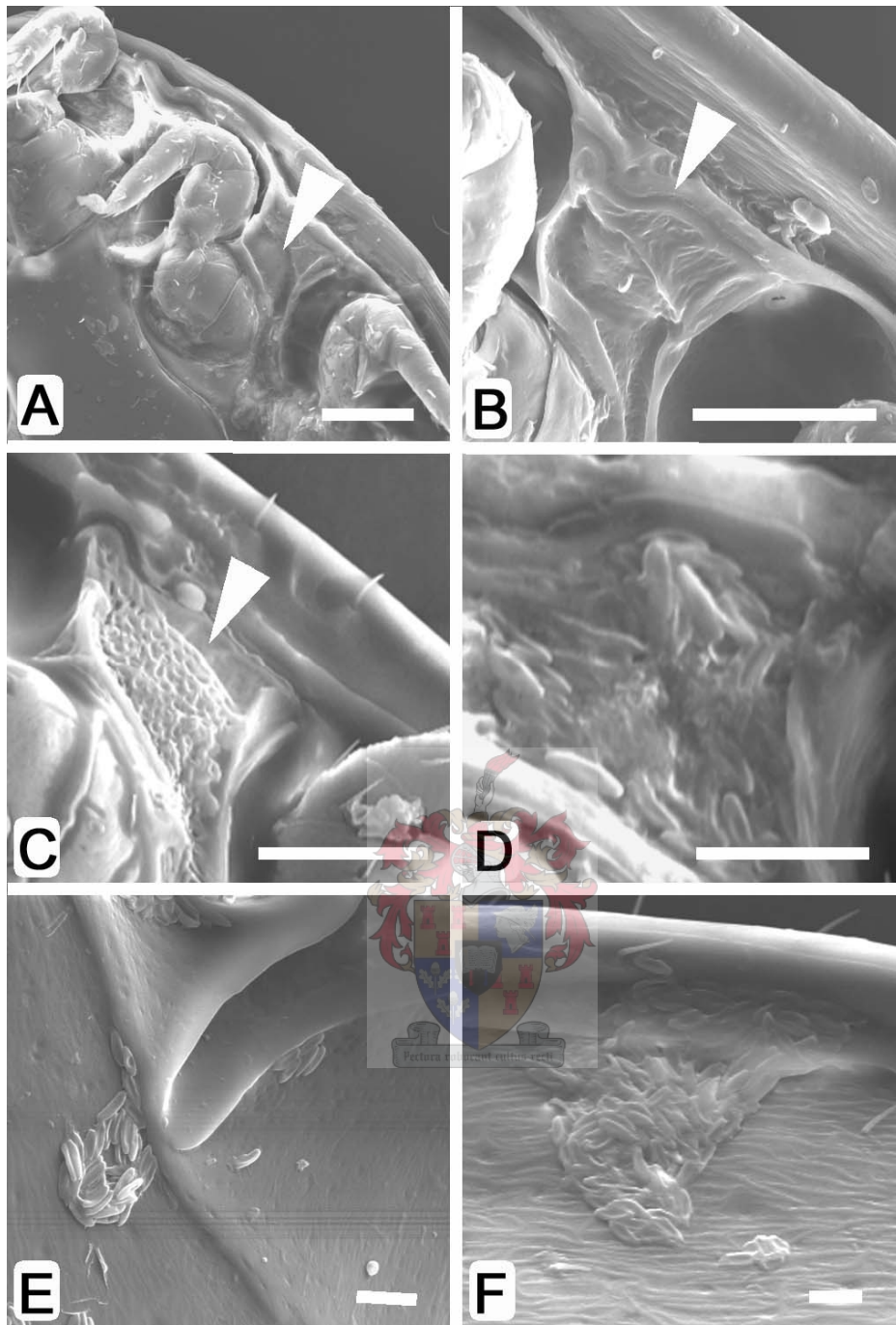
No ascospores of *Ophiostoma* spp. were observed on the surface of any *G. hottentottus* or *O. maculates* individuals using SEM. SEM also failed to disclose the presence of any *Ophiostoma* ascospores from wild-caught *P. vanderbergi*, while spores of several undetermined fungal species were commonly observed. In contrast, SEM of wild-caught *Oodinychus sp.* mites revealed the presence of *Ophiostoma* ascospores within the grooves and depressions associated with the legs (Fig. 1A–F) of 3 of the 50 individuals tested. In one instance *Ophiostoma* ascospores were also observed on the upper surface of a mite (Fig. 1F). Light micrographs confirmed these observations (Fig. 2A–C). Again, spores of many other unidentified fungal species were also observed on these mites.

Conidia of an unknown fungal species were observed underneath flap-like structures of the integument formed by tergite 1 in two individuals of *Tarsonemus cf. sp. A* using light microscopy (Fig. 2D). It is likely that *Ophiostoma* ascospores will be carried in a similar fashion. Due to a lack of material, no *Tarsonemus cf. sp. B* individuals were studied with the SEM or light microscope.

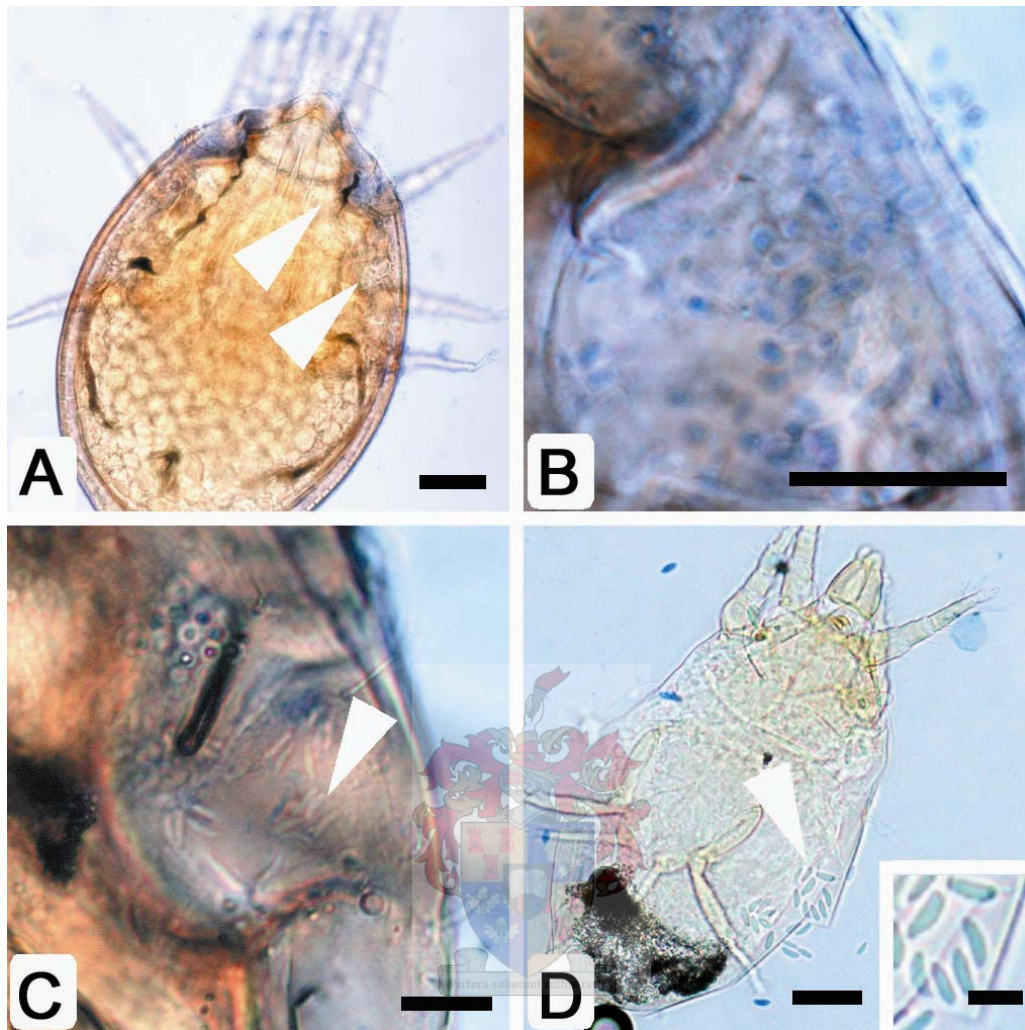
*Phylogenetic relations of Ophiostoma spp. isolated from mites.*

Amplified fragments obtained using the primers LROR and LR5 were *ca.* 700 bp long. Sequences from all putative *Ophiostoma* species isolated from mites were used in DNA comparisons (Tables 2 and 3). Analysis using the parsimony algorithm yielded 67 equally most parsimonious trees of 287 steps long. The Consistency Index (CI) was 0.4321, while the Retention Index (RI) was 0.8378. Phylogenetic reconstruction of the genus based on LSU sequences indicated that all isolates from mites represented *Ophiostoma* spp, even though sexual structures were not observed for most of the isolates (Fig. 3). These analyses confirmed that *O. palmiculminatum*, *O. splendens* and *O. phasma* (Fig. 3, Table 2) were collected from *Tarsonemus cf. sp. A*, *P. vandenbergi* and the *Oodinychus* sp. The phylogenetic reconstruction also revealed that the single isolate of *Sporothrix* sp.1 (CBS nr. pending) from the *Oodinychus* sp. mite, resided in a clade distinct from any of the *Ophiostoma* spp. known from *Protea* infructescences (Fig. 3).

No differences were found in comparisons between large subunit data of *O. palmiculminatum* and the three isolates from *Tarsonemus cf. sp. B* collected from *P. caffra* (Fig. 3). Isolates representing *O. palmiculminatum* and those of *Sporothrix* sp. 2 were, however, distinct based on morphological comparisons. Conidia of *O. palmiculminatum* are clavate in shape (Roets *et al.* 2006a, Chapter 3), whereas c-shaped conidia were formed by isolates of *Sporothrix* sp. 2. These three isolates thus probably represent another undescribed species of *Ophiostoma* closely related to *O. palmiculminatum*.



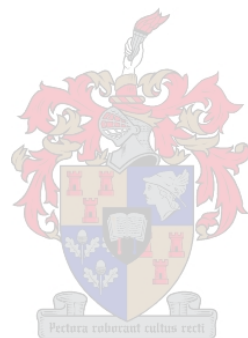
**Fig. 1** Scanning electron micrographs of unidentified conidia and ascospores of *Ophiostoma* spp. from the surface of *Oodinychus* sp. individuals. A. Ventral view of mite showing the depression between the legs where spores were commonly observed (arrow). B. Close-up view of the same structure. C. Depression filled with unidentified conidia (arrow) of a wild *Oodinychus* sp. mite from *P. repens*. D. Same, with depression filled with *Ophiostoma* sp. ascospores. E. *Ophiostoma* sp. ascospores from the depressions at the base of the hind legs of a wild *Oodinychus* sp. mite from *P. repens*. F. *Ophiostoma* sp. ascospores from the dorsal surface of a wild *Oodinychus* sp. mite from *P. repens*. Scale bars: A–C = 20  $\mu\text{m}$ , D–F = 10  $\mu\text{m}$ .



**Fig. 2** Light microscope micrographs depicting *Ophiostoma* sp. ascospores from *Oodinychus* sp. and unidentified fungal conidia from *Tarsonemus* cf. sp. A individuals. A. *Oodinychus* sp. mite showing areas where ascospores accumulate (arrow). B. Close-up of depression filled with unknown conidia. C. Same, filled with *Ophiostoma* sp. ascospores (arrow) from an *Oodinychus* sp. mite collected from *P. repens*. D. Image of *Tarsonemus* cf. sp. A showing fungal conidia (arrow) underneath flap-like structures formed by tergite 1. Insert to D. enlargement of the conidia contained within the structure. Scale bars: A = 30  $\mu$ m, B = 25  $\mu$ m, C = 10  $\mu$ m, D = 15  $\mu$ m, Insert = 7  $\mu$ m.

**Table 3.** GenBank accession numbers for fungal isolates used in the phylogenetic analysis.

Fungal species	Isolate no.		Host	Geographical origin	Collector	GenBank accession no.
	CBS	CMW				
<i>Ceratocystiopsis manitobensis</i>		13792	<i>Pinus resinosa</i>	Canada	J. Reid	DQ294358
<i>C. minima</i>	128.86	162	<i>Pinus banksiana</i>	USA	M.J. Wingfield	DQ294361
<i>C. minuta</i>		4586	<i>Ips cembrae</i>	Scotland	T. Kirisits	DQ294360
<i>C. minuta-bicolor</i>	393.77	1018	<i>Ips</i> sp. from <i>Pinus</i> sp.	USA	R.W. Davidson	DQ294359
<i>C. ranaculosa</i>		13940	<i>Pinus echinata</i>	USA	F. Hains	DQ294357
<i>C. rollhanseniana</i>	118669	13791	<i>Pinus sylvestris</i>	Norway	J. Reid	DQ294362
<i>Grosmannia galeiformis</i>	115711	5290	<i>Pinus sylvestris</i>	Scotland	T. Kirisits	DQ294383
<i>G. grandifoliae</i>		703	<i>Fagus grandifolia</i>	USA	R.W. Davidson	DQ294399
<i>G. penicillata</i>	116008	2644	<i>Picea abies</i>	Norway	H. Solheim	DQ294384
<i>G. piceiperda</i>	366.75	660	<i>Piceae abies</i>	Finland	A.M. Hallaksela	DQ294392
<i>G. robusta</i>		2805	unknown	unknown	T. Hinds	DQ294398
<i>G. serpens</i>	67.76	290	unknown	Italy	Gambagi	DQ294394
<i>G. wagneri</i>		491	<i>Pinus jeffreyi</i>	unknown	T. Harrington	DQ294396
<i>Leptographium lundbergii</i>	352.29	217	unknown	unknown	M. Lagerberg	DQ294388
<i>Ophiostoma africanum</i>	116571	823	<i>Protea gagedi</i>	Unknown	M.J. Wingfield	AF221015
	116566	1104	<i>Protea caffra</i>	Irene, Gauteng	Unknown	DQ316147
<i>O. ainoae</i>	118672	1903	<i>Picea abies</i>	Norway	O. Olsen	DQ294368
<i>O. araucariae</i>	114.68	671	<i>Araucaria</i> sp.	Chile	H. Butin	DQ294373
<i>O. canum</i>	118668	5023	<i>Tomicus minor</i>	Austria	T. Kirisits	DQ294372
<i>O. carpenteri</i>	118670	13793	<i>Trypodendron lineatum</i>	USA	S.E. Carpenter	DQ294363
<i>O. distortum</i>	397.77	467	<i>Picea engelmannii</i>	USA	R.W. Davidson	DQ294369
<i>O. flexuosum</i>	208.83	907	<i>Picea abies</i>	Norway	H. Solheim	DQ294370
<i>O. floccosum</i>		1713	<i>Pinus ponderosa</i>	USA	C. Bertagnole	DQ294367
<i>O. fusiforme</i>	112912	9968	<i>Populus nigra</i>	Azerbaijan	D.N. Aghayeva	DQ294354
<i>O. ips</i>	137.36	7075	<i>Ips integer</i>	USA	C.T. Rumbold	DQ294381
<i>O. lunatum</i>	112928	10564	<i>Larix decidua</i>	Austria	T. Kirisits	DQ294355
<i>O. montium</i>	151.78	13221	<i>Pinus ponderosa</i>	USA	R.W. Davidson	DQ294379
<i>O. multiannulatum</i>	357.77	2567	<i>Pinus</i> sp.	USA	Unknown	DQ294366
<i>O. nigrocarpum</i>	638.66	651	<i>Pseudotsuga menziesii</i>	USA	R.W. Davidson	DQ294356

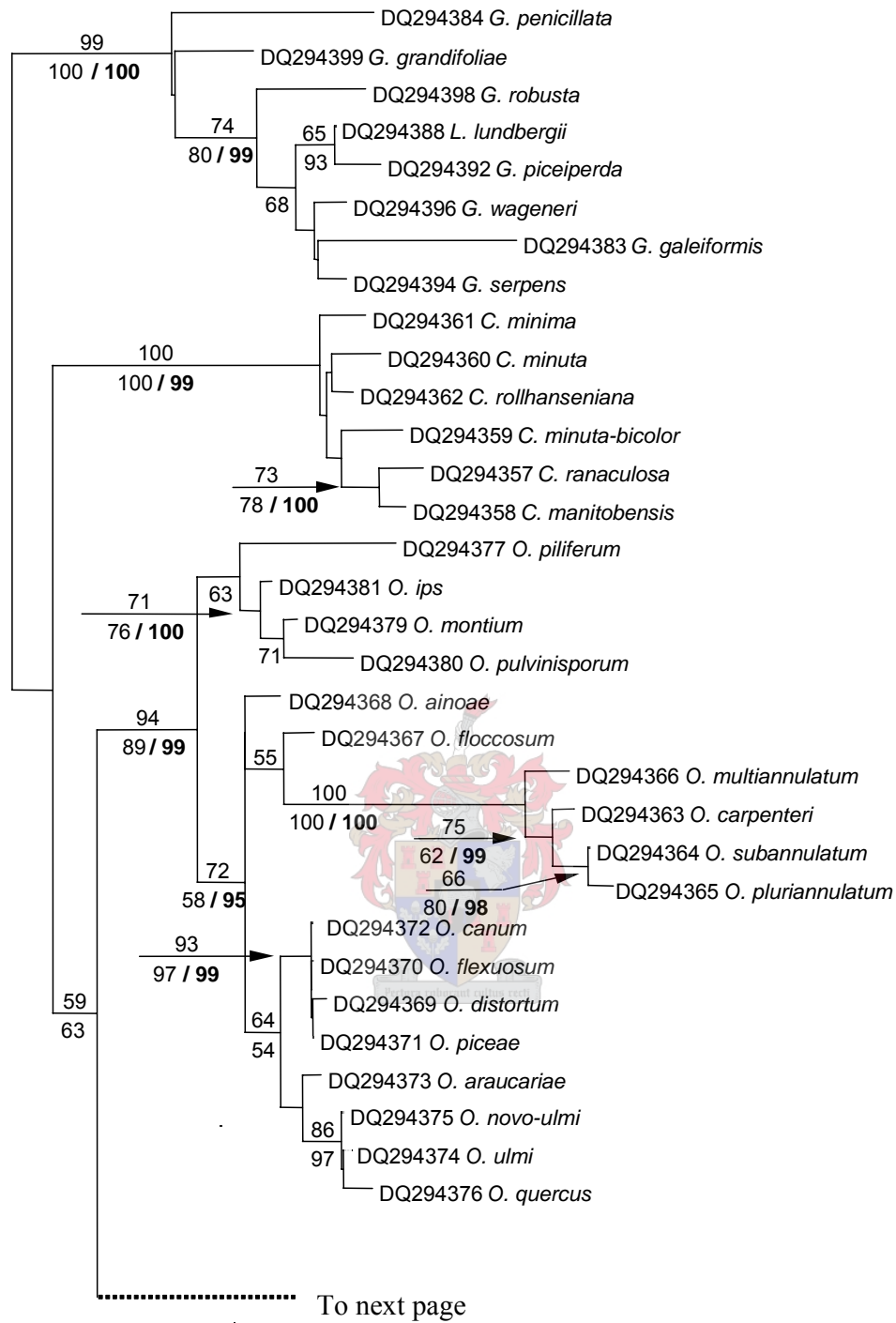


**Table 3.** Continued.

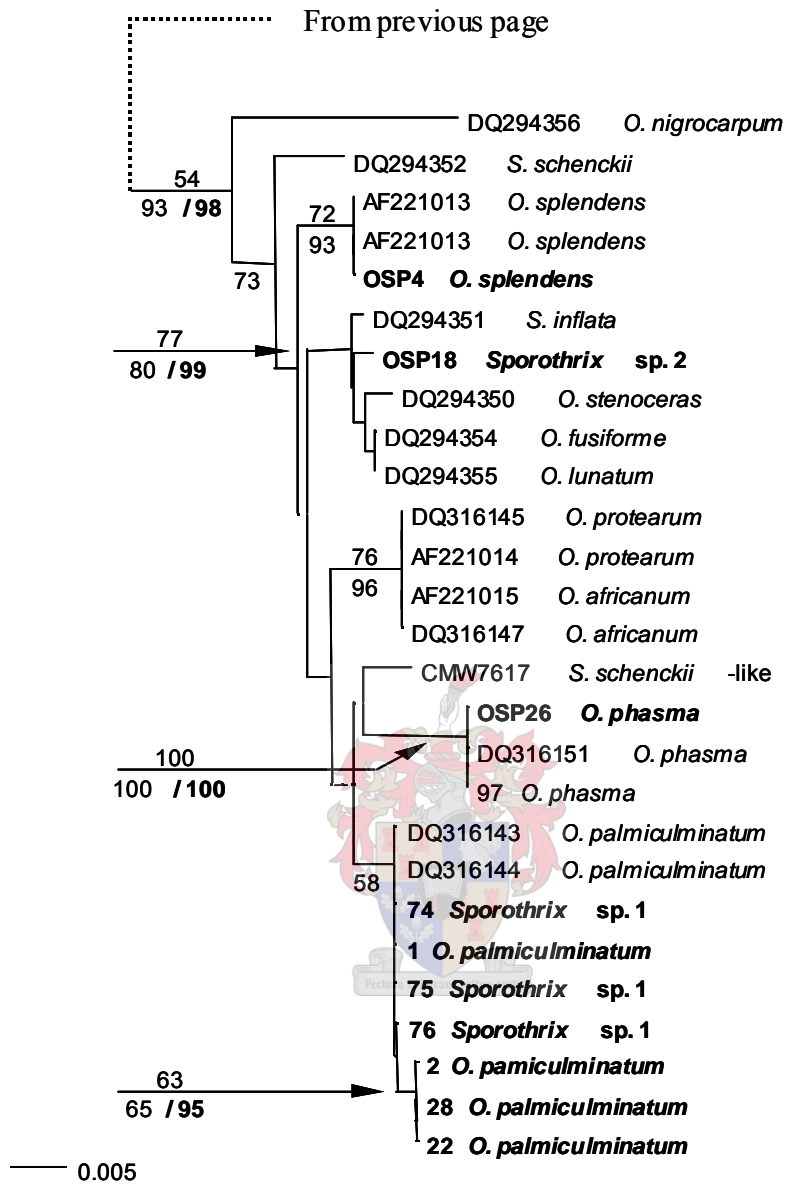
<i>O. novo-ulmi</i>		10573	<i>Picea abies</i>		Austria	Neumuller	DQ294375
<i>O. palmiculminatum</i>		20677	<i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ316143
		20694	<i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ316144
		23048	<i>Oodinychus</i> sp. from <i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ821527
		23049	<i>Oodinychus</i> sp. from <i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ821525
		23052	<i>Oodinychus</i> sp. from <i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ821526
		23053	<i>Oodinychus</i> sp. from <i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ821524
<i>O. piceae</i>		8093	<i>Tetropium</i> sp.		Canada	K. Harrison	DQ294371
<i>O. piliferum</i>	12932	7879	<i>Pinus sylvestris</i>		unknown	H. Diddens	DQ294377
<i>O. phasma</i>		20698	<i>Protea laurifolia</i>		Giftberg top, SW Cape	F. Roets	DQ316152
		20676	<i>Protea laurifolia</i>		JS Marais Park, SW Cape	F. Roets	DQ316151
		26	<i>Proctolaelaps vandenbergi</i> from <i>P. neriifolia</i>		Jonkershoek, SW Cape	F. Roets	DQ821535
<i>O. pluriannulatum</i>	118684	75	unknown		unknown	R.W. Davidson	DQ294365
<i>O. protearum</i>	116654	1107	<i>Protea caffra</i>		Irene, Gauteng	M.J. Wingfield	DQ316145
	116568	1102	<i>Protea caffra</i>		Irene, Gauteng	M.J. Wingfield	AF221014
<i>O. pulvinisporum</i>	118673	9022	<i>Pinus pseudostrabus</i>		Mexico	X. Zhou	DQ294380
<i>O. quercus</i>	118713	3110	<i>Juglans cinerea</i>		USA	M.J. Wingfield	DQ294376
<i>O. splendens</i>		20679	<i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ316150
		23050	<i>Oodinychus</i> sp. from <i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ821534
	116569	872	<i>Protea repens</i>		Unknown	M.J. Wingfield	AF221013
<i>O. stenoceras</i>	237.32	3202	<i>Pinus</i> sp.		Norway	H. Robak	DQ294350
<i>O. subannulatum</i>	118667	518	<i>Pinus ponderosa</i>		unknown	W. Livingston	DQ294364
<i>O. ulmi</i>		1462	<i>Ulmus procera</i>		USA	C. Brasier	DQ294374
<i>Sporothrix inflata</i>	239.68	12527	soil		Germany	W. Gams	DQ294351
<i>S. schenckii</i>	117842	7614	human		South Africa	H. Vismer	DQ294352
<i>S. schenckii</i> -like		7617	soil		South Africa	H. Vismer	DQ836010
<i>Sporothrix</i> sp. 1		23057	<i>Tarsonemus</i> sp. from <i>Protea caffra</i>		W. Sisulu Garden, Gauteng	F. Roets	DQ821531
		23058	<i>Tarsonemus</i> sp. from <i>Protea caffra</i>		W. Sisulu Garden, Gauteng	F. Roets	DQ821532
		23059	<i>Tarsonemus</i> sp. from <i>Protea caffra</i>		W. Sisulu Garden, Gauteng	F. Roets	DQ821533
<i>Sporothrix</i> sp. 2		23051	<i>Oodinychus</i> sp. from <i>Protea repens</i>		JS Marais Park, SW Cape	F. Roets	DQ821537







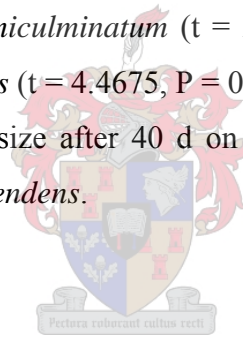
**Fig. 3.** Neighbour-joining tree derived from the 28S rDNA data set. Values above nodes indicate parsimony-based bootstrap values obtained by 1000 replicates. Values below nodes indicate bootstrap values (1000 replicates) obtained from neighbour-joining analysis. Values in bold typeface below nodes represent posterior probabilities (%) obtained through Bayesian inference.

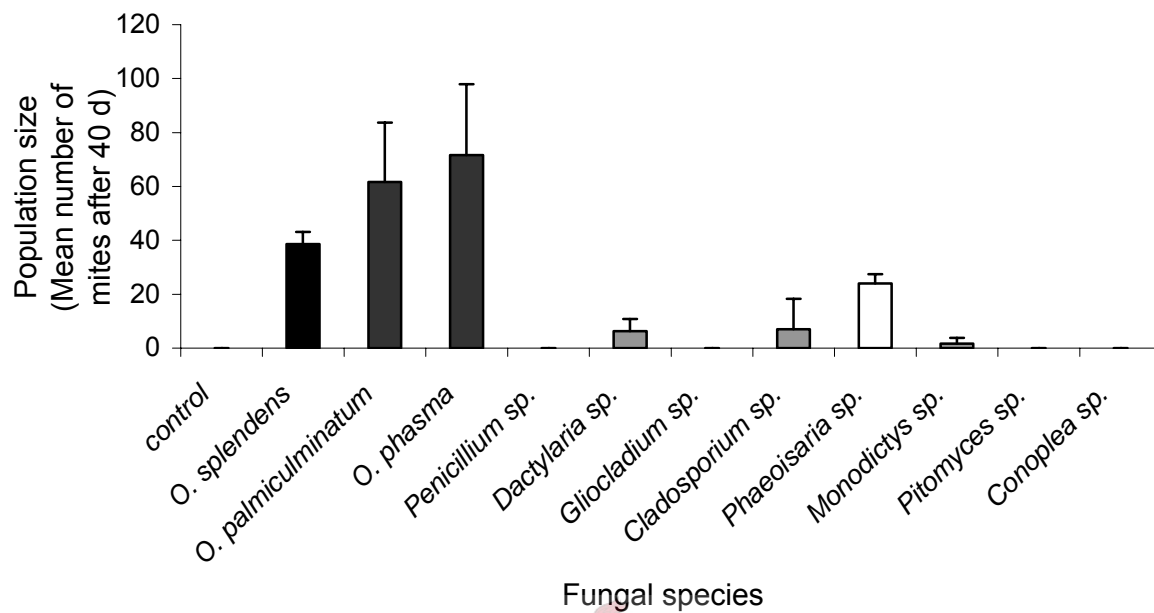


**Fig. 3.** Continued. Neighbour-joining tree derived from the 28S rDNA data set. Values above nodes indicate parsimony-based bootstrap values obtained by 1000 replicates. Values below nodes indicate bootstrap values (1000 replicates) obtained from neighbour-joining analysis. Values in bold typeface below nodes represent posterior probabilities (%) obtained through Bayesian inference.

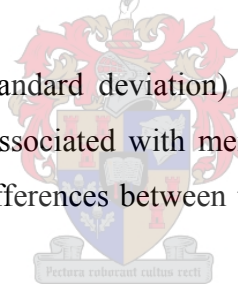
*Ophiostoma* spp. as food source for *Oodinychus* sp.

Mites belonging to the genus *Oodinychus* are clearly the main vectors of the spores of various *Ophiostoma* spp. (Table 2). They were consequently used in studies to test their ability to feed on *Ophiostoma* species. This mite is also fairly large (ca. 400 – 500 µm), which facilitated handling of individuals. Individuals caught in the wild and placed on colonies of *O. splendens* reproduced regularly. Their progeny failed to reproduce on the control plates or when exposed to a potential diet of *Penicillium*, *Gliocladium*, *Conoplea* or *Pithomyces* spp. (Fig. 4). Compared to the control, a significant increase in population size for this mite species was observed when it was fed on colonies of *O. palmiculminatum* ( $t = 4.8634$ ,  $P = 0.0398$ ), *O. phasma* ( $t = 4.7244$ ,  $P = 0.0420$ ), *O. splendens* ( $t = 14.8523$ ,  $P = 0.0045$ ) and the species of *Phaeoisaria* ( $t = 12.0000$ ,  $P = 0.0069$ ). The population growth for the *Oodinychus* sp. on the remaining fungal species tested were not significant when compared to the control (Fig. 4). Mites feeding on the species of *Phaeoisaria* had significantly smaller population sizes after 40 d than when feeding on *O. palmiculminatum* ( $t = 2.9343$ ,  $P = 0.0426$ ), *O. phasma* ( $t = 3.1153$ ,  $P = 0.0357$ ) and *O. splendens* ( $t = 4.4675$ ,  $P = 0.0111$ ), respectively. This mite species had significantly larger population size after 40 d on *O. palmiculminatum* and *O. phasma* compared to when feeding on *O. splendens*.





**Fig. 4.** Mean population size (+ standard deviation) after 40 d for *Oodinychus* sp. mites feeding on various fungal species associated with members of the genus *Protea*. Different coloured bars indicate significant differences between the population sizes ( $P \leq 0.05$ ) on the various fungal species tested.



## Discussion

The infructescences of *Protea* spp. represent one of the most intriguing habitats in which *Ophiostoma* spp. have ever been found. The results of the present study provide the first conclusive evidence for vectors of the *Ophiostoma* spp. found in this *Protea* niche. Given that insects and mites vector other species of these fungi from different habitats, it was reasonable to hypothesise that the same might be true of the *Protea*-associated species. Discovery of mites as vectors of the *Protea*-associated *Ophiostoma* spp. is, however, important and it provides a framework for future studies on these unusual species of *Ophiostoma*.

Of the ten mite species tested for the presence of *Ophiostoma* spp., only four (*Proctolaelaps vandenbergi*, two *Tarsonemus* spp. and an *Oodinychus* sp.) tested positive. This was interesting, as numerous of the mite species that did not have an association with the *Ophiostoma* spp. are similar in size and habit to those that displayed this association. These results suggest a specific relationship between mites, at least in the case of the two *Tarsonemus* spp. and the *Oodinychus* sp., and *Protea*-associated *Ophiostoma* spp.

The mite species most closely associated with the *Ophiostoma* spp. from *Protea* was the *Oodinychus* sp. The relationship between this mite and *Ophiostoma* spp. was determined through direct isolations and *via* SEM in which ascospores could be seen in specialised structures. In addition, the *Oodinychus* sp. had the highest frequency of individuals carrying species of *Ophiostoma* and was found to carry spores of four of the five *Ophiostoma* spp. isolated in this study. The *Oodinychus* sp. may thus play a principle role in carrying various *Protea*-associated *Ophiostoma* spp. within the *Protea* ecosystem. The non-specificity of the *Oodinychus* sp. mites towards species of *Ophiostoma* is demonstrated by the ability of these mites to reproduce on all tested species with more or less equal success. In contrast to the *Oodinychus*-*Ophiostoma* association, the *Tarsonemus* spp. appeared to have a more specific association with particular species of *Ophiostoma*. Although the data from this study are insufficient to fully understand vector patterns, specific associations between certain mite species and their phoretic *Ophiostoma* spp. may help to explain the co-existence of a large number of *Ophiostoma* species within a restricted niche such as *Protea* infructescences.

Of the 29 insect and four arachnid species examined, only three different insects (*G. hottentottus*, *O. maculates* and Psocoptera sp. 3) carried DNA of *Ophiostoma* spp. Compared to most other infructescence-inhabiting arthropods, *G. hottentottus* and *O. maculates* are fairly large insects and may easily come into contact with sporulating perithecia of *Ophiostoma* spp. as they move within infructescences. The low success rate in attempts to isolate *Ophiostoma* spp. directly from these insects was probably due to the extensive contamination by yeasts.

*Oxycareus maculates* and *G. hottentottus* occur in infructescences in very low numbers (Myburg *et al.* 1973, 1974, Myburg and Rust 1975*a*, 1975*b*, Coetzee and Giliomee 1985, 1987*a*, 1987*b*). This was also true in the infructescences investigated in the present study. In contrast, up to 70 % of infructescences of *Protea* are known to be dominated by *Ophiostoma* spp. (Roets *et al.* 2005). This suggests that *O. maculates* and *G. hottentottus* may not be

important vectors of *Ophisotoma* spp. We, therefore, believe that the presence of the *Ophiostoma* spp. on these insects was accidental and not related to a specific vector/ fungus relationship. The same appears to also be true for the Psocopteran specimens that were found to occasionally carry *Ophiostoma* DNA.

Light- and scanning electron microscopy revealed the deposition of *Ophiostoma* ascospores within grooves and depressions surrounding the legs on the lower surface of the *Oodinychus* sp. mite. The legs of the mites can be retracted within these grooves, mainly when they adopt a defensive posture (pers. observ.). In this position the tibia and tarsi are in close proximity to the depressions that frequently contain the fungal spores. From here, the spores could easily attach to the legs of the mites and thus be transferred to the substrate. If the terminology of Six (2003) is followed, these spore-containing structures may be regarded as pit mycangia as they commonly contained *Ophiostoma* ascospores, lack setae and are not deeply invaginated structures. Mycangia (or sporothecae) bearing fungal spores have been described in the mites *Imparipes* Berlese (Ebermann and Hall 2003), *Siteroptes* Amerling (Suski 1973), *Tarsonemus* (Moser 1985) and *Trochometridium* Cross (Lindquist 1985). To the best of our knowledge, this is the first report of the presence of mycangia in the mite genus *Oodinychus*.

*Tarsonemus* cf. sp. A was found to carry conidia of unknown origin in flap-like structures formed by tergite 1. We suspect that these areas also serve as specialised spore-bearing structures for *Ophiostoma* spp. In contrast to *Oodinychus* and *Tarsonemus* spp., no specialised spore-carrying structures were observed on *P. vandenbergii* mites, which may suggest that they are only loosely associated with *Ophiostoma* spp. Interestingly, some *Tarsonemus* spp. associated with conifers in the northern hemisphere have similar structures to those found in the *Protea*-associated *Tarsonemus* sp. and have been shown to frequently contain spores of ophiostomatoid fungi, including *Ophiostoma* spp. (Bridges and Moser 1983, Moser 1985, Moser *et al.* 1995, Klepzig *et al.* 2001a, 2001b). The *Ophiostoma*-*Tarsonemus* associations in these systems are thought to be mutualistic as the mites are able to feed on the fungi they vector (Klepzig *et al.* 2001b). Similarly, the *Ophiostoma*-*Tarsonemus* associations in *Protea* may also be mutualistic. Thus, a relationship between mites and *Ophiostoma* spp. in *Protea* infructescences is not unusual, but it does provide many intriguing questions regarding the movement of the fungi from one infructescence to another. The analogy with the bark beetle/ mite/ *Ophiostoma* ecosystem would be that the mite vectors of the *Protea*-*Ophiostoma* spp. would move from one infructescence to another phoretically on insects.

Dispersal of mites between plants may occur by wind, self-dispersal (climbing between branches) or phoresy. Many bark beetle-associates of *Ophiostoma* spp. carry large numbers of phoretic mites and these might be more important vectors of the fungi than the insects themselves (Klepzig *et al.* 2001a, 2001b). Known phoretic genera include *Oodinychus*, *Proctolaelaps* and *Tarsonemus* (Lindquist 1969, Moser and Roton 1971, Smiley and Moser 1974, Moser 1976, Bridges and Moser 1983, Moser and Bridges 1986, Blackwell *et al.* 1986, 1988). It is thus possible that the vector mites reported here are phoretic on larger insects.

Five *Ophiostoma* spp. were isolated from four species of mites encountered in this study. Morphological characteristics and phylogenetic relationships confirmed the presence of *O. splendens*, *O. phasmae* and *O. palmiculminatum* on these mites. Three of the unknown isolates were obtained from *Tarsonemus cf.* sp. B collected from *P. caffra* from the Gauteng Province. They represented a species closely related to *O. palmiculminatum* that is associated with *P. repens* from the Western Cape Province (Roets *et al.* 2006a, Chapter 3). Based on comparative morphology between these isolates and the disjunct distribution of their host species, these two groups probably represent distinct species. The other unidentified isolate grouped close to *O. stenoceras* (Robak) Melin & Nannf., *O. fusiforme* Aghayeva & M.J. Wingf., *O. lunatum* Aghayeva & M.J. Wingf. and *S. inflata* de Hoog (Aghayeva *et al.* 2004, 2005). This undescribed species represents another case for the non-monophyly (Roets *et al.* 2006a, Chapter 3) of the *Protea*-associated *Ophiostoma* species. As mentioned by Roets *et al.* (2006a, Chapter 3) these results probably suggest multiple colonisation of the *Protea*-infructescence niche by species of *Ophiostoma*. These provisional findings need to be verified by further in-depth molecular phylogenetic studies and comparative morphology.

The ability of *Oodinychus* sp. to feed and multiply on a diet of *Protea*-associated *Ophiostoma* spp. alone suggests a mutualistic association between these mites and their phoretic fungi. In this symbiosis the fungi benefit, since they are vectored to uncolonised substrates. The mites on the other hand, would benefit by receiving nourishment from the fungi. Similar associations may exist between the other *Protea*-associated *Ophiostoma* species and the other mites vectoring their propagules. Future studies must thus focus on clarifying these intricate *Protea* / *Ophiostoma* / mite interactions.

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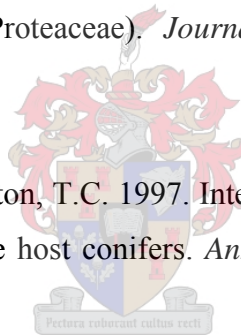
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Chapter 5: *Ophiostoma gemellus* prov. nom. and *Sporothrix variecibatus* prov. nom. (Ophiostomatales) from mites infesting *Protea* infructescences in South Africa

**Abstract**

*Ophiostoma* (Ophiostomatales) represents a large genus of fungi that are mainly associated with bark beetles (Curculionidae: Scolytinae) that infest conifers in the Northern Hemisphere. Few species are known as natives from the Southern Hemisphere, and the five known species that consistently occur in the infructescences of *Protea* spp. in South Africa are ecologically rather unusual. Very little is known about the vectors of *Ophiostoma* spp. from *Protea* infructescences, and recent studies have considered the possible role of insects and mites in the distribution of these exceptional fungi. In this study, we describe a new species of *Ophiostoma* and a new *Sporothrix* species with affinities to *Ophiostoma*, both initially isolated from mites associated with *Protea* spp. They are described as *Ophiostoma gemellus* prov. nom. and *Sporothrix variecibatus* prov. nom. based on their morphology, and comparisons of DNA sequence data of the  $\beta$ -tubulin and internal transcribed spacer (ITS1, 5.8S, ITS2) regions. DNA sequences of *S. variecibatus* were identical to those of a *Sporothrix* isolate obtained from *Eucalyptus* leaf litter in the same area in which *S. variecibatus* occurs in *Protea* infructescences. Results of this study suggest that mites might be vectors of *Ophiostoma* spp. that colonise *Protea* infructescences, and emphasise the fact that DNA sequence comparisons are likely to reveal more cryptic species of *Ophiostoma* in this unusual niche.

*Taxonomic novelties:* *Ophiostoma gemellus* Roets, Z.W. de Beer & P.W. Crous, prov. nom., *Sporothrix variecibatus* Roets, Z.W. de Beer & P.W. Crous, prov. nom.

*Key words:*  $\beta$ -tubulin, ITS, *Ophiostoma*, phylogeny, *Protea*, vector, mite



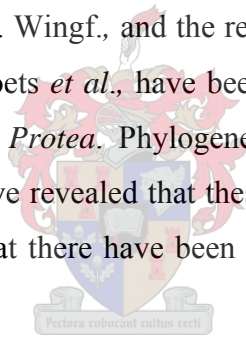
## Introduction

*Ophiostoma sensu lato* Syd. & P. Syd. is a species-rich (ca. 140 species) ascomycete genus that includes many ecologically important taxa (Upadhyay 1981, Whitney 1982, Wingfield *et al.* 1993, Jacobs and Wingfield 2001, Sinclair and Lyon 2005). Recent DNA-based phylogenetic reconstructions identified three well-supported monophyletic lineages in *Ophiostoma* that are tightly linked to morphological features such as the anamorph state or ascospore morphology (Zipfel *et al.* 2006). Thus, species with *Leptographium* Lagerb. & Melin anamorphs have been accommodated in the re-instated teleomorph genus *Grosmannia* Goid. emend. Z.W. de Beer *et al.* Likewise, *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. emend. Z.W. de Beer *et al.* has been re-instated for species with short ascomatal necks, falcate ascospores, *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendr. anamorphs, and that are sensitive to the antibiotic cycloheximide. Species with *Sporothrix* Hekt. & C.F. Perkins anamorphs and / or synnematus *Pesotum* J.L. Crane & Schokn. emend. G. Okada & Seifert anamorphs have been retained in *Ophiostoma* Syd. & P. Syd. emend. Z.W. de Beer *et al.* Although this group has substantial sub-structure and will most likely resolve into a number of distinct monophyletic lineages with the addition of species and DNA loci, it is treated as *Ophiostoma sensu stricto* in the present study.

Species of *Ophiostoma s.str.* typically produce ascospores in short-lived asci within flask-shaped ascomata. The ascospores are borne in gloeoid droplets at the tips of extended ascomatal necks. These characters represent adaptations to arthropod-mediated dispersal of their reproductive propagules (Münch 1907, 1908, Francke-Grosmann 1967, Upadhyay 1981, Whitney 1982, Harrington 1987, Beaver 1989, Malloch and Blackwell 1993, Cassar and Blackwell 1996). The vectors of *Ophiostoma* spp. include diverse arthropod taxa such as bark beetles (Curculionidae: Scolytinae), longhorn beetles (Cerambycidae) and mites (Acari) (Barras and Perry 1975, Upadhyay 1981, Bridges and Moser 1983, 1986, Lévieux *et al.* 1989, Moser *et al.* 1989, Moser 1997, Price *et al.* 1992, Wingfield *et al.* 1993, Jacobs and Wingfield 2001). The fungi are usually associated with galleries constructed in the phloem and wood of mainly, but not exclusively, coniferous trees by the larvae of the vector beetles (Kirisits 2004). Apparent co-evolution between the fungi and their vectors has resulted in close associations that, at least in some instances, have been shown to be mutualistic (Francke-Grosmann 1967, Norris 1979, Whitney 1982, Beaver 1989, Berryman 1989, Jacobs and Wingfield 2001, Klepzig *et al.* 2001a, 2001b).

Most species of *Ophiostoma* are known from the Northern Hemisphere. Where species have been recorded from Southern Hemisphere substrates, they are commonly associated with introduced insects or their origin is unknown (De Beer *et al.* 1995, 1999, Zhou *et al.* 2004, 2006). One of the most unusual and intriguing assemblages of seemingly native *Ophiostoma* spp. occurs in the infructescences (fruiting structures) of the uniquely African genus *Protea* L. (Proteaceae), which has its centre of diversity in the Cape Floristic Region (Marais and Wingfield 1994, Rebelo 1995, Linder 2003). Five species of *Ophiostoma* have been described from *Protea* spp. in South Africa (Marais and Wingfield 1994, 1997, 2001, Roets *et al.* 2006a, Chapter 3). Interestingly, these fungi usually form the dominant fungal component within this protected environment (Roets *et al.* 2005). Unlike various conifer-associated species, the *Ophiostoma* species associated with *Protea* are not pathogenic to their hosts and have an ecological function that has yet to be defined (Roets *et al.* 2005, 2006a, Chapter 3).

*Ophiostoma africanum* G.J. Marais & M.J. Wingf., *O. protearum* G.J. Marais & M.J. Wingf. and *O. splendens* G.J. Marais & M.J. Wingf., and the recently described *O. palmiculminatum* F. Roets *et al.* and *O. phasma* F. Roets *et al.*, have been isolated only from members of the economically important host genus *Protea*. Phylogenetic analyses of DNA sequences for *Ophiostoma* species from *Protea* have revealed that these fungi are paraphyletic (Roets *et al.* 2006a, Chapter 3). This suggests that there have been multiple invasions of this specialised niche.



Very little is known about the vectors of the *Protea*-associated *Ophiostoma* species. Their morphology does, however, suggest that insects or other small animals carry their spores between infructescences. In a preliminary attempt to find the vectors of the *Protea*-associated *Ophiostoma* spp., Roets *et al.* (2006b, Chapter 4) identified the mites *Proctolaelaps vandenbergi* Ryke, two *Tarsonemus* Canestrini & Fonzago species and an *Oodinychus* Berlese species as the primary vectors of *O. palmiculminatum*, *O. phasma* and *O. splendens*. In that study, they also isolated two unidentified species of *Sporothrix* from mites, one from an *Oodinychus* sp., and the other from a *Tarsonemus* sp. Based on DNA sequence comparisons (Zipfel *et al.* 2006, Roets *et al.* 2006b, Chapter 4), both of these unidentified anamorph taxa could also be assigned to the teleomorph genus *Ophiostoma*. One of these species later produced teleomorph structures in culture. The aim of the present study was to identify the unknown *Ophiostoma* sp. and *Sporothrix* sp. based on morphological and

physiological features, as well as comparisons of DNA sequences of the  $\beta$ -tubulin and 5.8S rDNA (including the internal transcribed spacers 1 and 2) gene regions.

## Materials and Methods

### *Isolates*

Cultures used in this study included three isolates of the unknown *Ophiostoma* sp. and one isolate of the unknown *Sporothrix* sp. collected from mites by Roets *et al.* (2006b, Chapter 4) (Table 1). Additional isolates of both these fungi were collected from *P. caffra* Meisn. (*Ophiostoma* sp.) and *P. longifolia* Andrews (*Sporothrix* sp.) (Table 1). An isolate from the leaf-litter of a *Eucalyptus* L'Her. sp. (CMW2543), previously shown to be related to but distinct from *O. stenoceras* (Robak) Melin & Nannf. (de Beer *et al.* 2003), was also included, as it was morphologically similar to the unknown *Sporothrix* sp. from *P. longifolia* and from mites.

For morphological and physiological comparisons, representative isolates including the ex-type culture of *O. palmiculminatum* were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands (Table 1). All isolates were maintained in Petri dishes containing 2 % malt extract agar (MEA, Biolab, Midrand, South Africa) at 4 °C. Representative cultures of the new taxa treated in this study have been deposited in both the CBS, and the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Herbarium specimens of the anamorph and teleomorph structures of the unknown *Ophiostoma* sp. and anamorph structures of the unknown *Sporothrix* sp. were deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM) (Table 1). DNA sequence data used for phylogenetic reconstructions of all other *Ophiostoma* species and isolates included in this study were obtained from GenBank (Table 1).

**Table 1.** GenBank accession numbers for ITS and  $\beta$ -tubulin nucleotide data for fungal isolates used in phylogenetic analysis.

Species identity	Isolate no.		Host	Geographical origin	Collector	GenBank accession no.		
	PREM	CBS				CMW	ITS	B-tubulin
<i>Ophiostoma</i> sp. (Unknown) [= <i>O. gemellus</i> prov. nom.]			23054	<i>Protea caffra</i>	W. Sisulu Park, South Africa	F. Roets	DQ821557	DQ821551
			23056	<i>Protea caffra</i>	W. Sisulu Park, South Africa	F. Roets	DQ821558	DQ821552
			23055	<i>Protea caffra</i>	W. Sisulu Park, South Africa	F. Roets	DQ821559	DQ821553
			23057	<i>Tarsonemus</i> sp. from <i>Protea caffra</i>	W. Sisulu Park, South Africa	F. Roets	DQ821560	DQ821554
			23058	<i>Tarsonemus</i> sp. from <i>Protea caffra</i>	W. Sisulu Park, South Africa	F. Roets	DQ821561	DQ821555
			23059	<i>Tarsonemus</i> sp. from <i>Protea caffra</i>	W. Sisulu Park, South Africa	F. Roets	DQ821562	DQ821556
<i>O. abietinum</i>			1468	<i>Dendroctonus ponderosa</i>	Canada	Y. Hiratsuka	AF484457	AY280468
			110	<i>Pinus echinata</i>	USA	F. Hinds	AF280488	AY280470
			109	<i>Pinus echinata</i>	USA	F. Hinds	AF280487	AY280469
<i>O. africanum</i>	116566		1104	<i>Protea caffra</i>	Irene, South Africa	Unknown	DQ316200	DQ316162
	116374		1822	<i>Protea dracomontana</i>	KZ-Natal, South Africa	M.J. Wingfield	DQ316179	DQ316159
<i>O. aurorae</i>			19362	<i>Pinus eliottii</i>	South Africa	Unknown	DQ396796	DQ393800
			19363	<i>Pinus eliottii</i>	South Africa	Unknown	DQ396797	DQ393801
<i>O. dentifundum</i>	115856		13017	<i>Quercus</i> sp.	Poland	T. Kowalski	AY495435	AY495446
	115790		13016	<i>Quercus</i> sp.	Hungary	C. Delatour	AY495434	AY495445
<i>O. fusiforme</i>	112912		9968	<i>Populus nigra</i>	Azerbaijan	D.N. Aghayeva	AY280481	AY280461
	112926		10565	<i>Larix decidua</i>	Austria	T. Kirisits	AY280484	AY280465
<i>O. lunatum</i>	112928		10564	<i>Larix decidua</i>	Austria	T. Kirisits	AY280486	AY280467
	112927		10563	<i>Carpinus betulus</i>	Austria	T. Kirisits	AY280458	AY280465
	638.66		651	<i>Pseudotsuga menziesii</i>	USA	R.W. Davidson	AY280490	AY280480
<i>O. nigrocarpum</i>	637.66		560	<i>Abies</i> sp.	USA	R.W. Davidson	AY280489	AY280479
			20677	<i>Protea repens</i>	J. S. Marais Park, South Africa	F. Roets	DQ316191	DQ821543
<i>O. palmiculminatum</i>			23049	<i>Oodinychus</i> sp. from <i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets	DQ821563	DQ821550
			23052	<i>Oodinychus</i> sp. from <i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets	DQ821566	DQ821574
			20693	<i>Protea repens</i>	J. S. Marais Park, South Africa	F. Roets	DQ316192	DQ821544
			23048	<i>Oodinychus</i> sp. from <i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets	DQ821565	DQ821549
			20694	<i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets		DQ821546
			20695	<i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets		DQ821545
			20696	<i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets		DQ821548
			20697	<i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets		DQ821547



**Table 1. (Continued)**

<i>O. palmiculminatum</i>		23053	<i>Oodinychus</i> sp. from <i>P. repens</i>	J. S. Marais Park, South Africa	F. Roets	DQ821564	DQ821575
<i>O. phasma</i>		20676	<i>Protea laurifolia</i>	J. S. Marais Park, South Africa	F. Roets	DQ316219	DQ316181
		20698	<i>Protea laurifolia</i>	Giftberg top, South Africa	F. Roets	DQ316222	DQ316184
		20684	<i>Protea laurifolia</i>	Citrusdal, South Africa	F. Roets		DQ821540
		20676	<i>Protea laurifolia</i>	J. S. Marais Park, South Africa	F. Roets		DQ821541
<i>O. protearum</i>	116654	1107	<i>Protea caffra</i>	Irene, South Africa	M.J. Wingfield	DQ316201	DQ316163
	116567	1103	<i>Protea caffra</i>	Irene, South Africa	M.J. Wingfield	DQ316203	DQ316165
<i>O. splendens</i>	116569	872	<i>Protea repens</i>	unknown	M.J. Wingfield	DQ316215	DQ296071
		20675	<i>Protea repens</i>	George, South Africa	F. Roets	DQ316205	DQ316167
<i>O. stenoceras</i>		11192	sapwood	New Zealand	R. Farrell	AY280492	AY280474
		2344	<i>Eucalyptus smithii</i>	South Africa	G.H.J. Kemp	AY280491	AY280472
	237.32	3202	<i>Pinus</i> sp.	Norway	H. Robak	AF484462	AY280471
<i>Sporothrix</i> sp. (unknonwn)		2543	<i>Eucalyptus</i> sp.	Stellenbosch, South Africa	P.W. Crous	DQ821567	DQ821572
[= <i>S. variecibatus</i> prov. nom.]		23051	<i>Oodinychus</i> sp. from <i>Protea repens</i>	Stellenbosch, South Africa	F. Roets	DQ821568	DQ821539
		23060	<i>Protea longifolia</i>	Kleinmond, South Africa	F. Roets	DQ821569	DQ821573
<i>S. inflata</i>	239.68	12572	soil	Germany	W. Gams	AY495426	AY495445
	841.73		wood	Chile	J. Grinbergs	AY495431	AY495442
<i>S. schenckii</i>	117440	7612	Human	South Africa	H. Vismer	AY280494	AY280476
	117842	7614	Human	South Africa	H. Vismer	AY280495	AY280477
		7615	Human	South Africa	H. Vismer	AY280496	AY280478



### *Morphology and growth in culture*

Isolates of the unknown *Ophiostoma* sp. and *Sporothrix* sp. were grown in the dark for 8 days at 25 °C on MEA (Biolab, Midrand, South Africa). Ascospores and conidiogenous cells that formed in culture were mounted onto microscope slides in lactophenol (Stephens 1974). Specimens were studied using a Nikon Eclipse E600 light microscope with differential interference contrast. Photographic images were captured using a Nikon DXM1200 digital camera. Measurements (25) of each taxonomically informative structure were made in all of the investigated cultures and means ( $\pm$  standard deviation) calculated.

Mycelium-covered agar disks (5 mm diam) were excised from actively growing 1-w-old cultures of three different isolates of each of *O. palmiculminatum*, the unknown *Ophiostoma* species and the unknown *Sporothrix* species. These discs were transferred to the centres of fresh dishes containing 20 mL 2 % MEA. The plates were then incubated at temperatures ranging from 5–35 °C with 5 °C intervals for 2 days in the dark, after which colony diameters were determined. The procedure was repeated after an additional 8 days of growth in the dark. Both the mean diameter of additional growth (two measurements per replicate) and the mean growth diameter ( $\pm$  standard deviation) for each test species (three replicates) were calculated. Tolerance of these species to varying concentrations of cycloheximide (0.05, 0.1, 0.5, 1.0 and 2.5 g/L) was determined as described by Roets *et al.* (2006a, Chapter 3) after 10 days of growth in the dark at 25 °C.

The growth rates of the unknown *Ophiostoma* and *Sporothrix* species on different concentrations of cycloheximide and varying temperatures were statistically compared to those of *O. palmiculminatum*. This was done in order to differentiate between these two species, which were found to be morphologically similar. A one-way analysis of variance (ANOVA) was used to analyse the data in the Statistica 7 (Statsoft Corporation, Tulsa, U.S.A.) software package with Sigma-restricted parameterisation. Significant differences between the growth rates of these fungal species are reported when  $P \leq 0.05$ .

### *DNA isolation, amplification and sequencing*

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 primers ITS1–F (Gardes and Bruns 1993) and ITS4 (White *et al.* 1990) were used to amplify the ITS and 5.8S regions, while the primers T10 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) were used to amplify the partial  $\beta$ -tubulin DNA regions.

Due to similarities in the DNA sequences of *O. palmiculminatum* and the unknown *Ophiostoma* species,  $\beta$ -tubulin gene fragments from selected isolates of these two species and *O. phasma*, *O. splendens*, as well as the unknown *Sporothrix* sp. were also amplified with the primers T1 (O'Donnell and Cigelnik 1997) and Bt2b. This was done in order to obtain longer fragments of this gene region for comparisons. The extended  $\beta$ -tubulin data set included the introns 2, 3 and 5 (amplified using primers T1 and Bt2b), while only intron 5 was amplified with the primer set T10 and Bt2b. PCR reaction mixtures and conditions for amplification of all gene regions followed the methods described by Roets *et al.* (2006a, Chapter 3).

All amplified PCR products were cleaned using the Wizard® SV gel and PCR clean-up system (Promega, Madison, Wisconsin, U.S.A.) following the manufacturer's instructions. The purified fragments were sequenced using the respective PCR primers and the Big Dye™ Terminator v3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, U.S.A.). The fragments were analysed on an ABI PRISIM™ 3100 Genetic Analyser (Applied Biosystems).

### *Phylogenetic analyses*

The sequence data obtained in the laboratory were compared with sequence data acquired from GenBank for all of the known *Protea*-associated and various non-*Protea*-associated *Ophiostoma* species (Table 1). *Ophiostoma nigrocarpum* (R.W. Davidson) de Hoog was chosen as outgroup based on results of previous studies (Zipfel *et al.* 2006; Roets *et al.* 2006a, 2006b, Chapters 3 and 4). Sequences were aligned using the Clustal X (1.81) software package. Compatibility of the ITS and the  $\beta$ -tubulin (non-extended) data sets was tested with a SH test (Shimodaira and Hasegawa 1999) before combining them into a single data set.

**Maximum parsimony.** The most parsimonious trees were obtained by performing 1000 random stepwise addition heuristic searches (1 tree saved per replicate) with the Tree Bisection-Reconnection (TBR) algorithm in the Phylogenetic Analysis Using Parsimony (PAUP) v4.0 b10 (Swofford 2000) software package. Confidence intervals for nodes were assessed using the bootstrap algorithm (Felsenstein 1985) with 1000 replicates of simple taxon addition.

**Neighbour-joining (NJ).** Akaike information criteria were applied to determine evolutionary models for distance analysis using Modeltest 3.06 (Posada and Crandall 1998). The selected evolutionary model for the combined data set was: GTR + I + G (proportion invariable sites 0.4598 and rates for variable sites following a gamma distribution with shape parameter of 0.5207). For distance analysis of the extended  $\beta$ -tubulin data set the selected evolutionary model was HKY + I (proportion invariable sites 0.5326). Trees were again constructed with PAUP, using the neighbour-joining tree-building algorithm (Saitou and Nei 1987). Statistical support for nodes was determined by 1000 NJ bootstrap replicates.

**Bayesian inference.** Data were analysed using Bayesian inference in the software package MrBayes v3.1.1 based on a Markov chain Monte Carlo (MCMC) approach (Ronquist and Huelsenbeck 2003). The general time-reversal model of DNA substitution (Tavare 1986) with rate variation (four rate classes) and invariant sites was selected for these analyses. Parameter values were treated as unknown with uniform priors using the default values. Two independent Markov chains were initiated from a random starting tree and allowed to run for 1000000 generations. The Markov chains were sampled at intervals of 50 to obtain 20000 sample points for the respective chains. The Bayesian analyses were carried out multiple times in order to test for uniformity. The first 1000 burn-in trees were discarded and the remaining trees from both runs were pooled into a 50 % majority rule consensus tree.



## Results

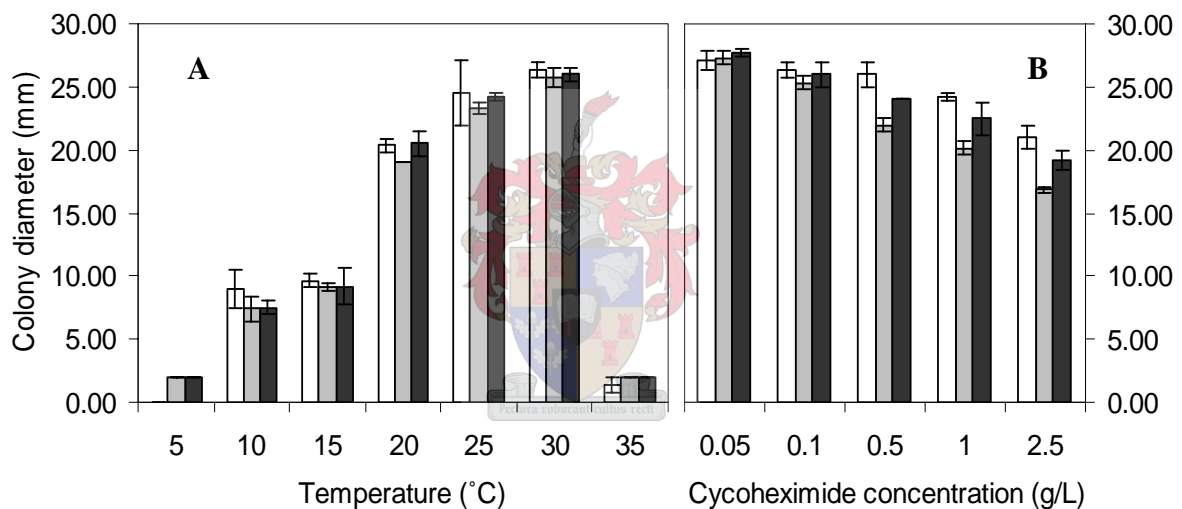
### *Morphology and growth in culture*

Roets *et al.* (2006b, Chapter 4) showed that isolates obtained from mites collected from *Protea* infructescences could be divided into five morphological groups. Three of these groups are consistent with descriptions of the anamorphs of *O. palmiculminatum*, *O. phasma* and *O. splendens*, respectively. The remaining two groups represented isolates of the unknown *Ophiostoma* and unknown *Sporothrix* spp. treated in this study. Differences in morphology between the anamorphs of *O. palmiculminatum* and the unknown *Ophiostoma* sp. were only slight, and mostly related to the size of the taxonomically informative structures. For instance, the length of the denticles of the unknown *Ophiostoma* sp. (ca. 2  $\mu\text{m}$ ) is usually twice the length of those of *O. palmiculminatum* (ca. 1  $\mu\text{m}$ ). The most reliable distinction is, however, the presence of clavate conidia in *O. palmiculminatum* while the unknown *Ophiostoma* sp. also produces c-shaped conidia. These differences were consistent between isolates representing the two species. The unknown *Sporothrix* sp. was morphologically different to the *Sporothrix* states of all known *Protea*-associated *Ophiostoma* species.

After their initial isolation from mites, two isolates of the unknown *Ophiostoma* sp. (CBS numbers pending) formed mature ascomata on the MEA after 3 mo of growth at 25 °C. Teleomorph structures of this species could thus be included in the morphological assessments. Subsequent sub-cultures using ascospore masses failed to produce mature ascomata. Comparisons of the morphology of the unknown *Sporothrix* sp. and other *Ophiostoma* spp. were based on anamorph structures only, as no teleomorph structures of this taxon were found.

Cultures of *O. palmiculminatum*, the unknown *Ophiostoma* sp. and the unknown *Sporothrix* sp. grew optimally at 30 °C (Fig. 1A). The mean colony diameter of the *Ophiostoma* sp. was 26.3 mm ( $\pm 0.6$ ), while the unknown *Sporothrix* sp. had a colony diameter of 26 mm ( $\pm 0.5$ ) at this temperature after 8 days of growth in the dark. Under these conditions the mean colony diameter at the optimum growth temperature for *O. palmiculminatum* was 25.7 mm ( $\pm 0.8$ ). All species were tolerant to relatively high levels of the antibiotic cycloheximide in the growth media. The mean colony diameter of the unknown *Ophiostoma* sp. declined from 27.2

mm ( $\pm 0.8$ ) on 0.05 g/L to 21 mm ( $\pm 0.9$ ) on 2.5 g/L cycloheximide after 10 days (Fig.1B). The mean colony diameter of the *Sporothrix* sp. declined from 27.7 mm ( $\pm 0.3$ ) on 0.05 g/L to 19.2 mm ( $\pm 0.8$ ) on 2.5 g/L cycloheximide after 10 days (Fig. 1B). Mean colony diameter for *O. palmiculminatum* declined from 27 mm ( $\pm 1$ ) on 0.05 g/L to 17 mm on 2.5 g/L cycloheximide after 10 days (Fig. 1B).



**Fig. 1.** Comparison between the mean growth on MEA (three isolates per tested species,  $\pm$  standard deviation) of *O. palmiculminatum* (grey bars), the unknown *Ophiostoma* species (white bars) and the unknown *Sporothrix* species (black bars) at A: different temperatures after 8 days of growth in the dark and B: on different concentrations of cycloheximide at 25 °C after 10 days of growth in the dark.

The difference in growth between the unknown *Ophiostoma* sp. and *O. palmiculminatum* on the different cycloheximide concentrations (Fig. 1B) was highly significant ( $F = 124.16$ ,  $P = 0.000000$ ). In addition, the two taxa also reacted significantly different to changes in cycloheximide concentration ( $F = 15.23$ ,  $P = 0.000007$ ). *Ophiostoma palmiculminatum* was more sensitive to this antibiotic than the isolates of the unknown *Ophiostoma* sp. Comparisons of growth between the unknown *Ophiostoma* sp. and *O. palmiculminatum* at different temperatures revealed no significant differences. Both had similar growth at the different temperature intervals, with peaks at 30 °C, whereafter a rapid decline was observed to 35 °C (Fig. 1A).

#### *DNA isolation, amplification and sequencing*

Amplification of extracted genomic DNA with the primers ITS1–F and ITS4 resulted in fragments of *ca.* 550–600 bp in length. DNA fragments of *ca.* 500–560 bp lengths were amplified using the primers T10 and Bt2b. Substantially longer fragments (*ca.* 700–800 bp) were obtained when amplifying the extracted genomic DNA with the primer pairs T1 and Bt2b.



#### *Phylogenetic analyses*

Alignment of the amplified sequence fragments resulted in data sets of 603, 273 and 545 characters for the ITS,  $\beta$ -tubulin and extended  $\beta$ -tubulin respectively. Numbers of potentially parsimony informative, parsimony uninformative and constant characters were: 171, 0 and 432 for ITS; 108, 1 and 164 for  $\beta$ -tubulin; and 147, 34, and 364 for the extended  $\beta$ -tubulin data sets.

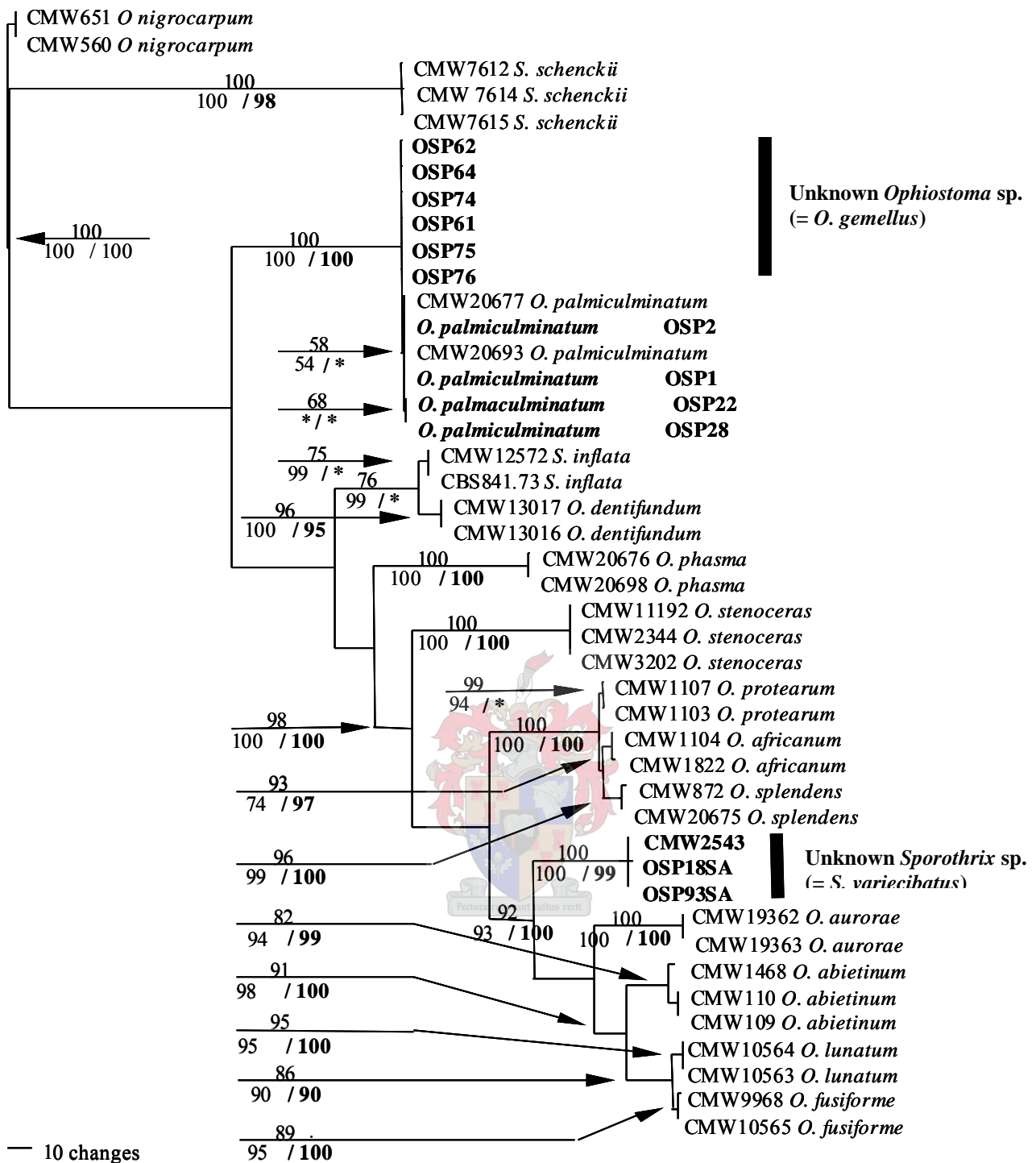
The ITS and  $\beta$ -tubulin data sets (excluding the extended  $\beta$ -tubulin data) were combined regardless of the outcome of the SH test ( $P < 0.05$ ), as the observed differences between these were most likely the result of ambiguous alignment due to the variability of the  $\beta$ -tubulin intron areas of the various species. Placement of isolates of the various species of interest in this study in the trees resulting from phylogenetic analysis of the data sets for each separate gene region, was similar. Combining the data sets did not affect the grouping of terminal nodes of interest compared to the phylogenetic reconstructions using the separate data sets.

After alignment, the combined data set for the ITS and  $\beta$ -tubulin gene regions consisted of 876 characters. Numbers of potentially parsimony informative, parsimony uninformative and constant characters for the combined data set were: 279, 1, and 596, respectively. Parsimony analysis of these data resulted in 70 equally most parsimonious trees of 573 length and had a CI = 0.716 and RI = 0.923.

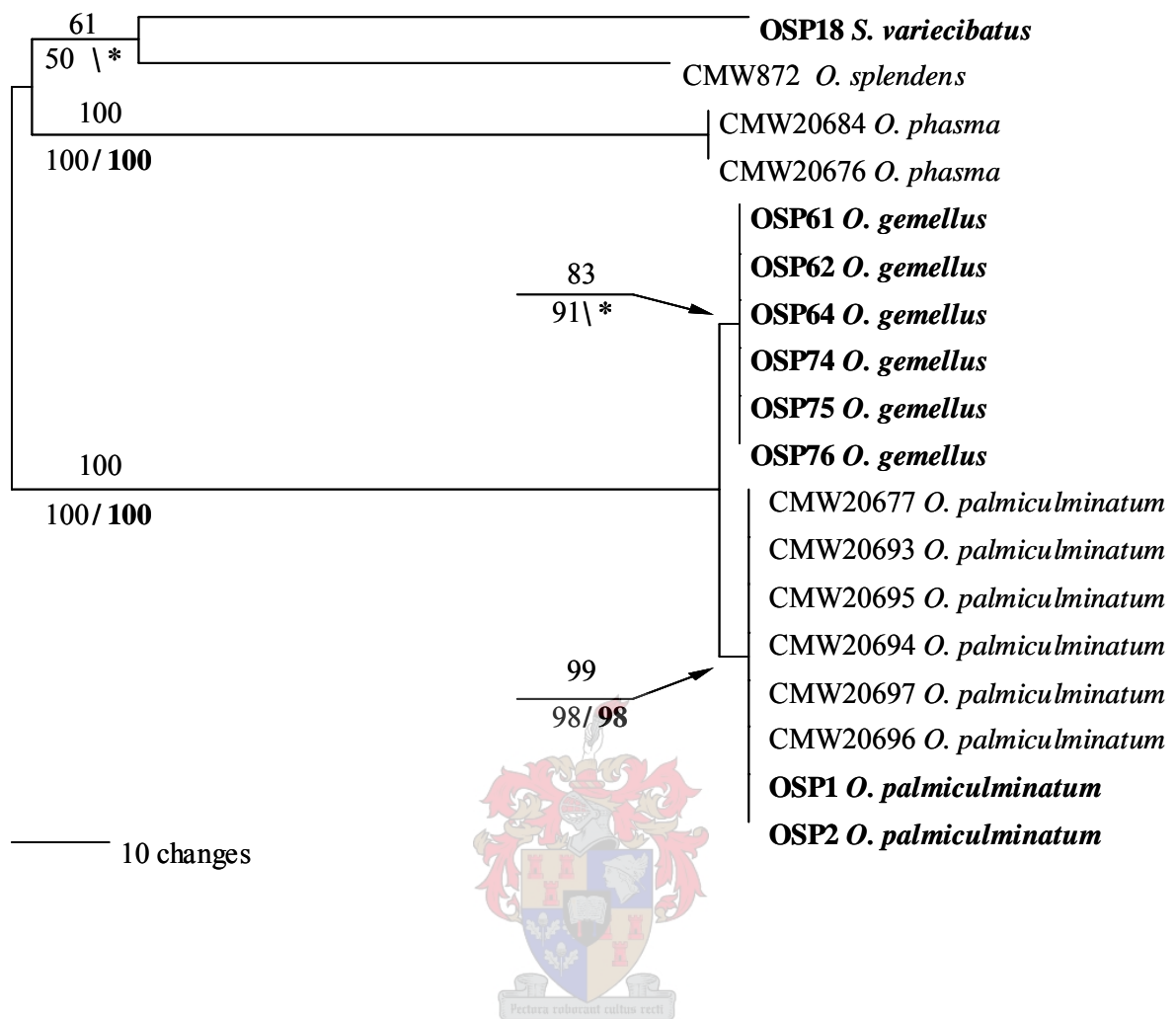
Isolates of the unknown *Sporothrix* sp. grouped with the isolate (CMW2543) from *Eucalyptus* with strong support obtained by all three phylogenetic node support algorithms (Fig. 2). They formed a strongly supported monophyletic clade sister to *Ophiostoma abietinum* Marm. & Butin, *O. aurorae* X.D. Zhou & M.J. Wingf., *O. fusiforme* Aghayeva & M.J. Wingf. and *O. lunatum* Aghayeva & M.J. Wingf., deeply embedded within the phylogenetic reconstruction of the genus.

Analysis of the combined ITS and  $\beta$ -tubulin gene regions accentuated a close relationship between the unknown *Ophiostoma* sp. and *O. palmiculminatum* as they were separated by weak support using the three phylogenetic support algorithms (Fig. 2). Isolates of these two taxa grouped together into one well-supported clade, suggesting a very close affinity between them. The phylogenetic difference between *O. palmiculminatum* and the unknown *Ophiostoma* sp. is better demonstrated by analyses of the sequence data for the extended  $\beta$ -tubulin gene region of these taxa. This data set included eight isolates of *O. palmiculminatum*, two of *O. phasma*, one of *O. splendens*, six of the unknown *Ophiostoma* sp. and one of the unknown *Sporothrix* sp. Parsimony analysis of this data set resulted in 1 most parsimonious tree of 244 length and had a CI = 0.955 and RI = 0.961 (Fig. 3).

Strong support values were attained for the divergence between the isolates representing *O. palmiculminatum* and the unknown *Ophiostoma* sp. when the data from the extended  $\beta$ -tubulin gene region was analysed using both neighbour-joining and parsimony bootstrap support algorithms (Fig. 3). The isolates representing these lineages were found to diverge in terms of 5 base pair positions (Table 2). These differences were also consistent for the two lineages (Table 2). Strong Bayesian support values (posterior probabilities) were obtained for the monophyly of *O. palmiculminatum*, *O. phasma*, the clustering of *O. palmiculminatum* and the unknown *Ophiostoma* sp. (Fig. 3).



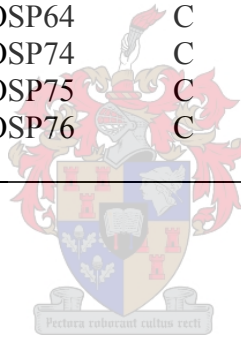
**Fig. 2.** One of 70 equally parsimonious trees obtained for the combined ITS and  $\beta$ -tubulin data set. Values above nodes indicate bootstrap values (1000 replicates) of neighbour-joining analysis obtained with the GTR+I+G parameter model ( $G = 0.5207$ ). Values below nodes indicate parsimony-based bootstrap values (1000 replicates). Values in bold typeface represent confidence values (posterior probabilities as percentage) obtained through Bayesian inference. \* = value below 50 (= value below 95 % for Bayesian analysis)



**Fig. 3.** The most parsimonious tree obtained for the extended  $\beta$ -tubulin data set (including exons 2–5, partial exon 6 and introns 2, 3 and 5). Values above nodes indicate bootstrap values (1000 replicates) of neighbour-joining analysis obtained with the HKY + I parameter model. Values below nodes indicate parsimony-based bootstrap values (1000 replicates). Values in bold typeface represent confidence values (posterior probabilities as percentage) obtained through Bayesian inference. \* = value below 95 %

**Table 2.** Differences in rDNA and  $\beta$ -tubulin base pair sequence data for *O. palmiculminatum* and the unknown *Ophiostoma* sp.

Species	Isolate	rDNA	$\beta$ -tubulin				
		ITS1	Intron 2			Intron 3	
		Position →	188	59	74	115	31
<i>O. palmiculminatum</i>	CMW20677	T	G	C	A	A	C
	CMW20693	T	G	C	A	A	C
	CMW20695	T	G	C	A	A	C
	CMW20694	T	G	C	A	A	C
	CMW20697	T	G	C	A	A	C
	CMW20696	T	G	C	A	A	C
	OSP1	T	G	C	A	A	C
	OSP2	T	G	C	A	A	C
Unknown <i>Ophiostoma</i> sp. ( <i>O. gemellus</i> prov. nom.)	OSP61	C	A	A	C	C	A
	OSP62	C	A	A	C	C	A
	OSP64	C	A	A	C	C	A
	OSP74	C	A	A	C	C	A
	OSP75	C	A	A	C	C	A
	OSP76	C	A	A	C	C	A



## Taxonomy

From the morphological comparisons and growth study data obtained, it was clear that the two mite-associated *Ophiostoma* spp. with *Sporothrix* anamorphs from *Protea* infructescences were different to any *Ophiostoma* previously described from this niche. These fungi could also be distinguished from previously described *Ophiostoma* spp. based on DNA comparisons. They are, therefore, newly described as follows:

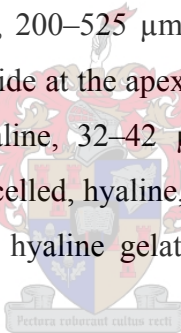
*O. gemellus* Roets, Z.W. de Beer & P.W. Crous., **prov. nom.** MycoBank MB (pending). Fig. 4.

*Anamorph: Sporothrix* sp.

*Etymology:* The epithet *gemellus* (*gemellus* = twin) refers to the close resemblance of the ascomata to its sister species *O. palmiculminatum*.

*Ophostomati palmiculminato simile, sed basi ascomatum latiore (70–270 µm), collo ascomatum brevior et crassior (200–525 x 12–18 µm), hyphis ostiolaribus longioribus (32–42 µm) et conidiis curvatis differens.*

*Ascomata* superficial on 2 % MEA plates after 2 mo of growth at room temperature. *Ascomatal bases* globose, dark, 70–270 µm (176 ± 75) diam, without hyphal ornamentation. *Ascomatal necks* dark brown to black, 200–525 µm (430 ± 101) long, 40–50 µm (46 ± 4) wide at the base, 12–18 µm (15 ± 2) wide at the apex. 10–13 *ostiolar hyphae* usually present, somewhat curved, hyaline to subhyaline, 32–42 µm (35 ± 3) long (Fig. 4A–C). *Asci* evanescent. *Ascospores* allantoid, one-celled, hyaline, sheaths absent, 3–5 µm (5 ± 1) long, 1–2 µm wide (Fig. 4C) collecting in a hyaline gelatinous droplet at the apex of the neck, remaining uncoloured when dry.



*Culture of the Sporothrix* anamorph on MEA 24.5 µm (± 2.64) mm diam after 8 d at 25 °C in the dark, white to cream coloured, effuse, circular with an entire edge, surface smooth. Growth reduced at temperatures below and above the optimum of 30 °C. *Hyphae* superficial on 2 % MEA plates (Fig. 4D). Sporulation profuse on MEA. *Conidiogenous cells* 3–44 µm long, 1.5–2.5 µm wide, arising directly from hyphae and from 5–45 µm long aerial conidiophores, proliferating sympodially, hyaline (Fig. 4F–K) becoming denticulate. *Denticles* 0.5–2.5 µm (2 ± 0.5) long, usually in an apical crown of 5–12, sometimes in an extended zone 4–8 µm long, scattered, solitary or in nodes. *Conidia* holoblastic, hyaline, one-celled, clavate to strongly curved, smooth, thin-walled, 3–7 µm (5 ± 2) long and 2–3.5 µm (3) wide (Fig. 4E). Conidia formed singly, but aggregate to form slimy masses.

*Notes:* Based on morphological characteristics, *O. gemellus* is closely related to *O. palmiculminatum*. The following nucleotide characters are diagnostic (presented as the gene

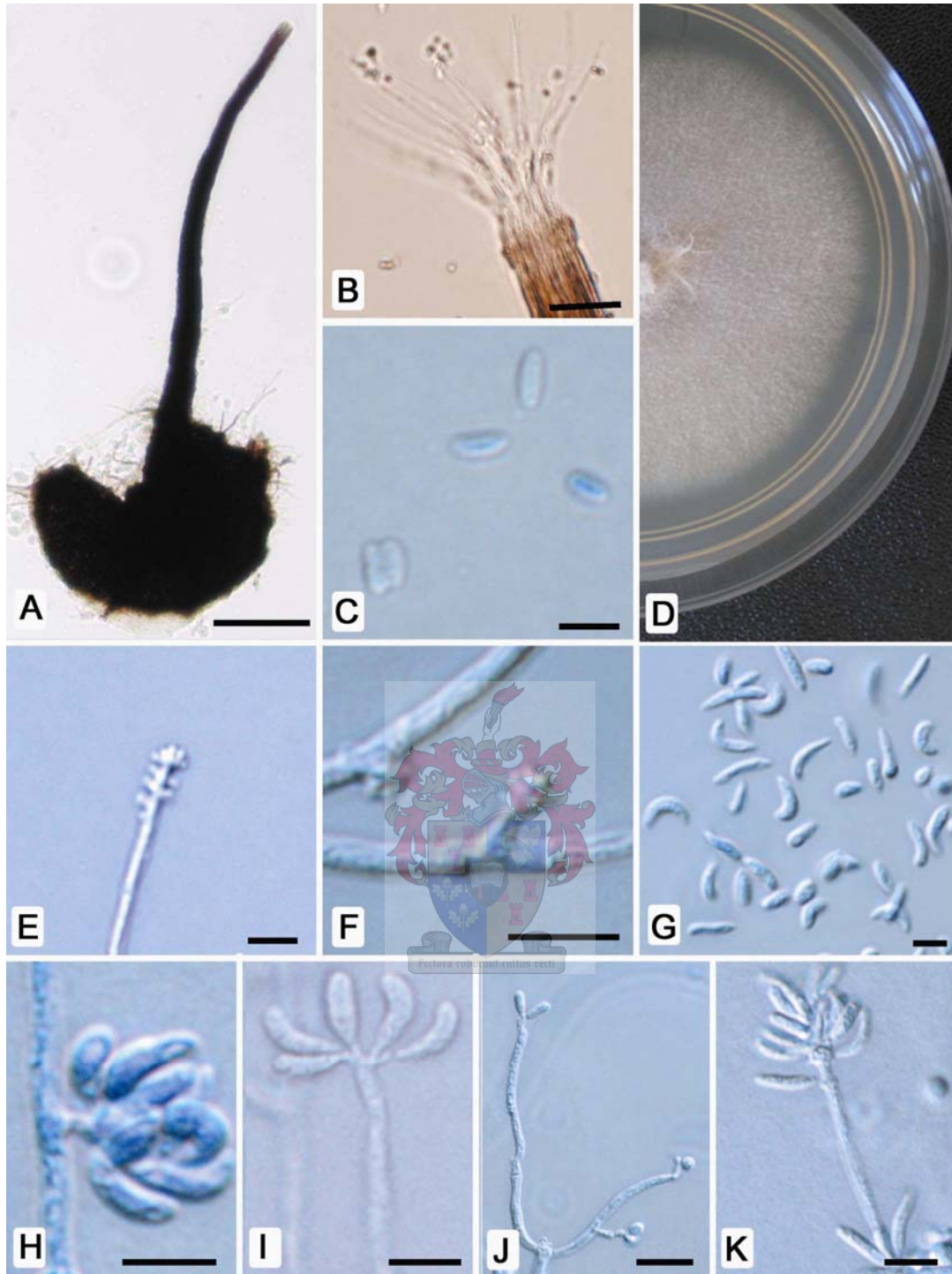


followed by the nucleotide position in the gene in brackets) of *O. gemellus* as compared to *O. palmiculminatum* (Table 2): Internal transcribed spacer 1 of the nuclear encoded rDNA position 188 (C in stead of T);  $\beta$ -tubulin gene intron 2 positions 59 (G in stead of A), 74 (A in stead of C) and 115 (C in stead of A);  $\beta$ -tubulin gene inton 3 positions 31 (C in stead of A) and 34 (A instead of C).

Specimens examined: **South Africa**, Gauteng Province, Walter Sisulu National Botanical Garden, from the mite *Tarsonemus* sp. from within the infructescences of *P. caffra*, April 2005, F. Roets, **holotype** PREM (pending), culture ex-type 74, CMW (pending) = CBS (pending); Gauteng Province, Walter Sisulu National Botanical Garden, from the mite *Tarsonemus* sp. from within the infructescences of *P. caffra*, April 2005, F. Roets, **paratype** PREM (pending), culture ex-paratype 75, CMW (pending) = CBS (pending); Gauteng Province, Walter Sisulu National Botanical Garden, from the mite *Tarsonemus* sp. from within the infructescences of *P. caffra*, April 2005, F. Roets, **paratype** PREM (pending), culture ex-paratype 76, CMW (pending) = CBS (pending); Gauteng Province, Walter Sisulu National Botanical Garden, within *P. caffra* infructescences, May 2004, F. Roets, **paratype** PREM (pending), culture ex-paratype 62, CMW (pending) = CBS (pending); Gauteng Province, Walter Sisulu National Botanical Garden, within *P. caffra* infructescences, May 2004, F. Roets, culture 63 CMW (pending); Gauteng Province, Walter Sisulu National Botanical Garden, within *P. caffra* infructescences, May 2004, F. Roets, culture 64 CMW (pending).

*Substrate*: Isolated from the infructescences of *P. caffra* and from *Tarsonemus* sp. mites associated with infructescences of this species.

*Distribution*: South Africa, Gauteng Province.



**Fig. 4.** Micrographs of *Ophiostoma gemellus* prov. nom. A. Ascoma produced on the surface of MEA agar after 3 mo of growth at 24 °C. B. Ostiolar hyphae. C. Ascospores. D. Two-week-old colony of the *Sporothrix* anamorph on MEA. E. Conidiogenous cell on long conidiophore. F. Conidiogenous cells arising directly from hyphae. G. Conidia. H–K. Conidiogenous cells arising from hyphae and conidiophores of varying lengths. Scale bars A = 100  $\mu$ m, B = 10  $\mu$ m; C–I = 5  $\mu$ m; J = 10  $\mu$ m; K = 5  $\mu$ m.

*Sporothrix variecibatus* Roets, Z.W. de Beer & P.W. Crous, **prov. nom.** MycoBank MB (pending). Fig. 5.

*Teleomorph*: not observed, phylogenetically *Ophiostoma*.

*Etymology*: The epithet *variecibatus* (*varie* = diverse, *cibatus* = food) refers to the taxonomically diverse host range from which isolates of this species were collected.

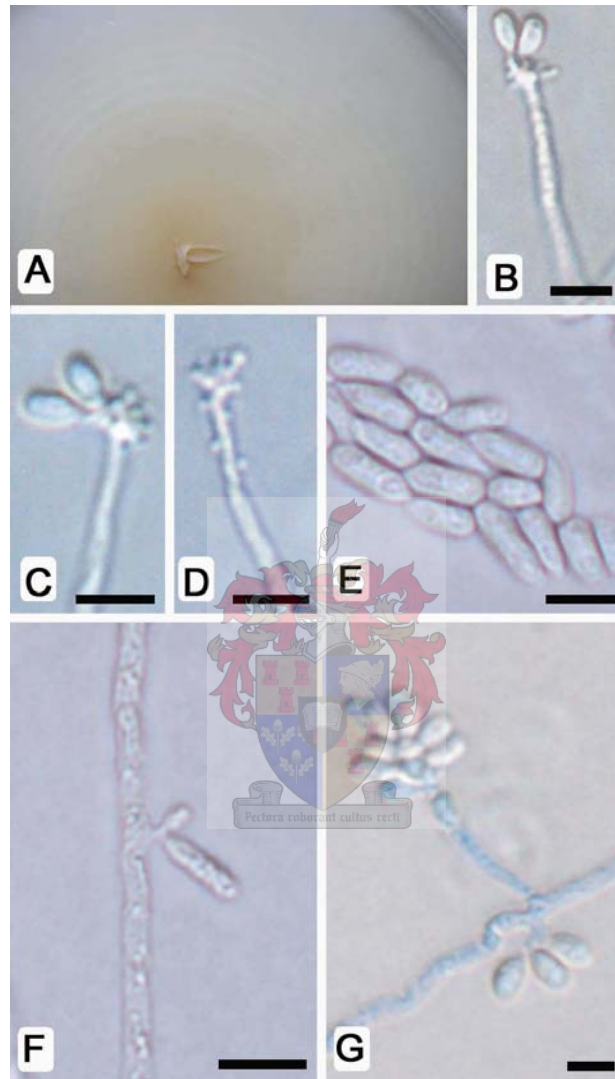
*Anamorphe* *Ophiomatis aurorae similis, sed conidiis minoribus, 3–7 x 2–3 µm, differens.*

*Ascomata* not observed. Cultures of the *Sporothrix* sp. on MEA 24.17 mm ( $\pm$  0.29) diam after 8 d at 25 °C in the dark, white to cream coloured, effuse, circular with an entire edge, surface smooth. Growth reduced at temperatures below and above the optimum of 30 °C. *Hyphae* superficial on 2 % MEA plates (Fig. 5A). Sporulation profuse on MEA. *Conidiogenous cells* 5–20 µm long, 1.5–2 µm wide, arising directly from hyphae or from short (19 µm  $\pm$  6) aerial conidiophores, proliferating sympodially, hyaline (Fig. 5B–E) becoming denticulate. *Denticles* 1–2 µm (1.5  $\pm$  1) long, usually in an apical crown of 9–16, sometimes in an extended zone 5–10 µm long. *Conidia* holoblastic, hyaline, one-celled, clavate, smooth, thin-walled, 3–7 µm (6  $\pm$  2) long and 2–3 µm (2) wide (Fig. 5F). *Conidia* forming singly, aggregating to form slimy masses.



*Specimens examined*: **South Africa**, Western Cape Province, Stellenbosch, Jan S. Marais Park, from *Oodinychys* sp. mite associated with *P. repens*, Jul. 2004, F. Roets, **holotype** PREM (pending), culture ex-holotype 18, CMW (pending) = CBS (pending); Kleinmond district, within the infructescences of *P. longifolia*, Jul. 2004, F. Roets, **paratype** PREM (pending), culture ex-paratype 93 CMW (pending) = CBS (pending); Stellenbosch district, from the leaf-litter of *Eucalyptus* sp., Apr. 1993, P.W. Crous, **paratype** PREM (pending), culture ex-paratype CMW2543 = CBS (pending).

*Distribution*: South Africa, Western Cape Province.



**Fig. 5.** Micrographs of *Sporothrix variecibatus* prov. nom. A. Two-week-old colony on MEA B–D. Conidiogenous cells. E. Conidia. F–G. Conidia arising directly from hyphae and conidiophores of various lengths. Scale bars: 5  $\mu$ m

## Discussion

Results of this study led to the discovery of two new ophiostomatoid species associated with the infructescences of *Protea* spp. in South Africa. *Ophiostoma gemellus* is known from both the teleomorph and anamorph states. In contrast, *Sporothrix variecibatus* is recognised as a new species of *Ophiostoma* based on its phylogenetic placement in this genus, but in the absence of a teleomorph. Description of these two new species brings to seven the species of *Ophiostoma* known from *Protea* hosts in South Africa. These fungi are typically restricted to the infructescences of serotinous members of the host genus and occur widespread throughout South Africa. The newly described *O. gemellus* and *S. variecibatus* were previously known only from mites collected from *Protea* plants (Roets *et al.* 2006, Chapter 3). In this study, they were also collected from the infructescences of *Protea* species from which the mites had been collected.

From both the DNA sequence comparisons and morphological characters, it is clear that *O. gemellus* is closely related to its sister species *O. palmiculminatum*. This is indicated by the low support values obtained for the separation of these taxa using parsimony, Bayesian, and neighbour-joining analyses of the combined ITS and  $\beta$ -tubulin data set. Phylogenetically the species were, however, separated by analyses of the extended  $\beta$ -tubulin data set. High support values were obtained when analysing this data using both the parsimony and neighbour-joining algorithms. Low support for the monophyly of *O. gemellus* using Bayesian inference was likely due to the long branches leading to the *O. palmiculminatum* - *O. gemellus* clade and the outgroups. Morphological distinctions between the anamorphs of *O. palmiculminatum* and *O. gemellus* include the conidial shape with the conidia of *O. palmiculminatum* being clavate, while *O. gemellus* usually also formed c-shaped conidia in culture. Morphologically the teleomorphs also differ slightly, most notably in the length of their ostiolar hyphae. The ostiolar hyphae of *O. gemellus* are about twice as long as those of *O. palmiculminatum*. Physiologically the two species differ markedly in their responses to different cycloheximide concentrations. The most obvious distinction between these two species is, however, their completely different host species. *Ophiostoma gemellus* is only known from *P. caffra*, while *O. palmiculminatum* is specific to *P. repens*.

*Sporothrix variecibatus* appears to be related to *O. stenoceras* that has been reported globally from wood and soil (De Beer *et al.* 2003), to hardwood infecting species such as *O. fusiforme*

and *O. lunatum* (Aghayeva *et al.* 2005), and to the conifer bark-beetle associates *O. abietinum* and *O. aurorae* (Zhou *et al.* 2006). Our data suggest that, the closest relative of *S. variecibatus* is *O. aurorae*, which was recently described from bark beetles infesting *Pinus* spp. in the Mpumalanga province of South Africa (Zhou *et al.* 2006). Morphologically the *Sporothrix* anamorph of *O. aurorae* and *S. variecibatus* are very similar. They closely resemble other species in the *O. stenoceras*-complex (De Beer *et al.* 2003), and in the absence of a teleomorph, these species are morphologically virtually indistinguishable. These two species can be distinguished from other species in the complex by their swollen clavate conidia, with those of *O. aurorae* being slightly larger than conidia produced by *S. variecibatus*. Comparisons of ITS and partial  $\beta$ -tubulin sequence data also showed that *O. aurorae* and *S. variecibatus* are distinct species and that they differ from other similar species in the *O. stenoceras*-complex for which data was available.

*Sporothrix variecibatus* represents the first known case of an *Ophiostoma* species associated with *Protea* that has been isolated from material of an unrelated host, in this case the leaf litter of an exotic *Eucalyptus* sp. Known *Protea* hosts include *P. repens* from the J. S. Marais Park in Stellenbosch and *P. longifolia* from a site in the Kleinmond district. *Eucalyptus* and *P. repens* plants were found growing together close to the site where *S. variecibatus* was isolated from *Eucalyptus* in the J. S. Marais Park. At this stage the data are insufficient to draw clear conclusions on whether *S. variecibatus* shifted from native *Protea* spp. hosts to the *Eucalyptus* litter environment or *vice versa*. As *S. variecibatus* was also isolated from an *Oodinychus* mite, we believe that this mite could have facilitated the movement of the fungus from *Protea* to the *Eucalyptus* leaf litter.

*Ophiostoma palmiculminatum* and *O. gemellus* were also isolated from mites associated with the infructescences of their respective hosts. Although *O. palmiculminatum* and *O. gemellus* represent sister species, the respective mite species associated with them are very distantly related. *Ophiostoma gemellus* was isolated from a *Tarsonemus* sp. (Tarsonemidae), while *O. palmiculminatum* was isolated from an *Oodinychus* sp. (Uropodidae). *Oodinychus* mites have not been observed from the infructescences of *P. caffra*, but are common within the infructescences of *P. repens* (pers. observ.). In contrast, *Tarsonemus* sp. has been recorded from *P. repens* infructescences (Roets *et al.* 2006, Chapter 4), but no isolates of *O. palmiculminatum* were collected from *Tarsonemus* sp. mites in that study. The speciation

event that resulted in the separation of *O. gemellus* and *O. palmiculminatum* may thus have been driven both by differences in host species and a switch in vectors.

Geographically the distribution of the respective hosts of the sister species *O. gemellus* (*P. caffra*) and *O. palmiculminatum* (*P. repens*) are completely disjunct, with *P. caffra* occurring in the northern and eastern parts South Africa, while *P. repens* is confined to the Western Cape Province of South Africa. Although not in the same monophyletic lineage as *O. gemellus* and *O. palmiculminatum*, the sister species *O. protearum* and *O. africanum* (from *P. caffra* and *P. gaguidi*, respectively) show the same pattern of north-south disjunction with their closest relative *O. splendens*. The *Protea* hosts of *O. protearum* and *O. africanum* are restricted to north-eastern parts of South Africa, while their sister species *O. splendens* only occurs on *Protea* species in the south-western regions of South Africa. The northern and southern *Protea*-rich areas of South Africa are separated by the dry central Karoo region that is effectively devoid of *Protea* spp. A repeated pattern of speciation in the *Protea*-associated members of *Ophiostoma* thus appears to have been driven by a combination of geographical separation and accompanying host switches.

The *Ophiostoma* species associated with *Protea* infructescences are morphologically very similar. The morphological uniformity has rendered molecular phylogenetic analysis essential for the identification and taxonomic placement of these fungi. More cryptic species are likely to be discovered as isolates from a wider geographical range and a wider host range are included. The close morphological similarity between the various *Protea*-associated *Ophiostoma* species may be ascribed to their shared arthropod-vectored mode of spore dispersal, which suggests that they have been subjected to similar ecological and evolutionary pressures. This is supported by the recent confirmation that at least four of the *Protea*-associated *Ophiostoma* species are strongly associated with mite species present on their host plants (Roets *et al.* 2006b, Chapter 4). Future studies will focus on defining the vectors of the remaining *Protea*-associated *Ophiostoma* species and clarifying the number of species involved in these multi-organism interactions.

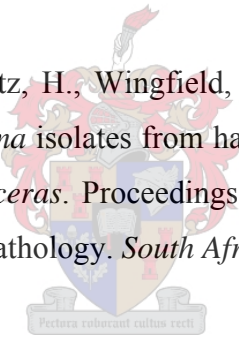
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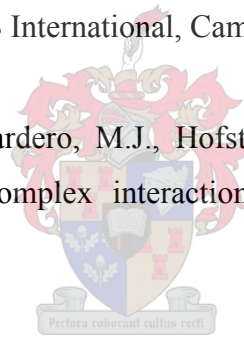
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## Chapter 6: Hyperphoretic dispersal of the *Protea*-associated fungi, *Ophiostoma phasma* and *O. splendens* by mites

### Abstract

Ophiostomatoid fungi are well-known for their association with arthropods, and many are economically important pathogens or agents of timber degradation. A unique and curious assemblage of these fungi (including members of the genera *Gondwanamyces* and *Ophiostoma*) occurs in the floral heads (infructescences) of *Protea* spp. in South Africa. The ecology of these fungi is understudied and it has only recently been discovered that members of *Ophiostoma* are vectored by mites (Acarina) associated with *Protea* infructescences. Two *Tarsonemus* spp., *Proctolaelaps vanderbergi* and an *Oodinychus* sp. are recognised as vectors of *Ophiostoma* spores, although it is not known how the mites move from one plant to another. In this study we consider the mode of dispersal of these three mite species. The different modes of dispersal assessed included: 1. Self-dispersal by testing the movement of mites between infructescences and ‘artificial infructescences’ that provided various means of protection (shelter and / or moisture). 2. Anemophilous dispersal was studied using sticky traps. 3. Dispersal via insect vectors was scrutinised by inspecting both arthropods emerging from laboratory-kept infructescences and wild-caught *Protea*-flower visiting insects for the presence of phoretic mites. Results indicated that mites self-disperse from infructescences to moist, sheltered ‘artificial infructescences’ under desiccating conditions. Mites carrying *Ophiostoma* spp. were not collected on sticky traps, which rules out wind-dispersal as a mode of transport. Long distance dispersal was restricted to vectored dispersal via the three beetle species *Genuchus hottentottus*, *Trichostetha fascicularis* and *T. capensis*. *Ophiostoma phasma* and *O. splendens* were isolated from mites phoretic on *G. hottentottus*. The hyperphoretic dispersal of *O. splendens* and *O. phasma* was very effective, as their hosts were successfully colonised during the first flowering season 3–4 years after fire.

*Key words:* fungal transmission, ophiostomatoid fungi, phoresy, vector

## Introduction

Ophiostomatoid fungi (Wingfield *et al.* 1993) are best-known as associates of bark beetles that infest trees, especially conifers. These fungi include some of the most important pathogens of trees and many impart sapstain to lumber, which results in substantial reduction in the profitability of timber industries worldwide (Sinclair *et al.* 1987, Brasier 1988, Webber and Gibbs 1989, Wingfield *et al.* 1993, Jacobs and Wingfield 2001). One of the most unusual assemblages of these fungi occur in the flower heads (infructescences) of *Protea* L. spp. that grow in the unique Fynbos Biome in the Western Cape province of South Africa (Cowling and Richardson 1995, Goldblatt and Manning 2000).

Ophiostomatoid fungi provide a very good example of fungi that have evolved novel structures to promote dispersal by vectors such as insects and other small animals (Upadhyay 1981, Bridges and Moser 1983, 1986, Harrington 1987, Wingfield *et al.* 1993, Jacobs and Wingfield 2001, Jacobs *et al.* 2003, Kirisits 2004, Harrington 2005). The group includes species in well-known and phylogenetically distantly related genera, *Ophiostoma* Syd. & P. Syd. emend. Z.W. de Beer *et al.* and *Ceratocystis* Ellis & Halst. These fungi have co-evolved to live in association with insects and mites and most are characterised by spores produced in sticky masses, usually at the apices of elongated ascomata or conidiophores (Upadhyay 1981, Harrington 1987). This morphological architecture promotes dispersal via vector arthropods to which sticky spores typically attach (Münch 1907, 1908, Francke-Grosmann 1967, Whitney 1982, Beaver 1989, Malloch and Blackwell 1993, Cassar and Blackwell 1996).

The interactions between scolytine bark beetles and their phoretic fungal partners, including *Ophiostoma* spp., have been relatively well studied, although many questions still surround various aspects of the relationships (Malloch and Blackwell 1993, Paine *et al.* 1997, Kirisits 2004, Harrington 2005). The relationship between some of these bark beetles and their phoretic fungi is believed to be mutualistic (Francke-Grosmann 1967, Norris 1979, Beaver 1989, Berryman 1989, Bridges 1985, Jacobs and Wingfield 2001). Thus, beetles associated with these fungi in their galleries are reproductively more fit than beetles that exclude the fungi from their diet (Six and Paine 1998, Eckhardt *et al.* 2004).

In addition to bark beetles, mites are suspected to play a significant role in the dispersal of some ophiostomatoid fungi (Bridges and Moser 1983, Moser 1985, Moser and Bridges 1986, Lombardero *et al.* 2000, Klepzig *et al.* 2001a, 2001b, Lombardero *et al.* 2003, Roets *et al.* 2006c, Chapter 4). The relationship between certain *Ophiostoma* spp. and specific mite species may also be mutualistic (Bridges and Moser 1983, Moser 1985, Moser *et al.* 1995). Many of the mite species are phoretic on bark beetles (e.g. *Tarsonemus* Canestrini & Fonzago spp.) (Moser and Roton 1971, Moser *et al.* 1974, Moser 1976, Bridges and Moser 1983, Klepzig *et al.* 2001a, 2001b). In these interactions the fungus is hyperphoretically dispersed, with the mites acting as primary vectors, while the beetles play a secondary role. For example, it has been demonstrated that *Ophiostoma minus* (Hedgc.) H. & P. Sydow, a fungus frequently vectored by mites, limits the reproductive success of the bark beetle *Dendroctonus frontalis* Zimmermann (Bridges 1985, Lombardero *et al.* 2000, Klepzig *et al.* 2001a, 2001b, Lombardero *et al.* 2003). The interactions in this three-way symbiosis are thus extremely complex.

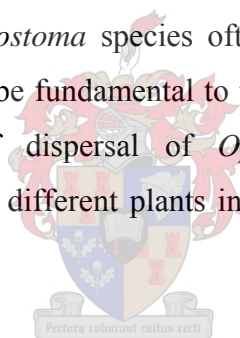
Another reasonably well-documented insect-fungus-mite interaction is found in the spore dispersal of *Pyxidiophora* Bref. & Tav. sp. and its *Thaxteriola* Speg. and *Acariniola* Maj. & Wiśn. anamorphs by more than 35 mite species (Blackwell *et al.* 1986a, 1986b, Blackwell and Malloch 1989, 1990). In this system, beetles are responsible for carrying fungus-vectored mites from one habitat to the next (Blackwell *et al.* 1986b). The coprophilous fungus, *Stylopaga anomala* Wood. is also dispersed from one dung pad to another by mites of dung beetles (Blackwell and Malloch 1991). Spore dispersal by mites thus appears to play a pivotal role in the survival and evolution of several unrelated fungal taxa. At present the above-mentioned systems are known to be naturally confined to the Northern Hemisphere. Similar interactions may, however, exist naturally in the Southern Hemisphere, and *Ophiostoma* species associated with indigenous *Protea* (Proteaceae) hosts in South Africa could provide a fascinating case study.

Species of *Protea* often dominate plant communities in the Fynbos biome of the Cape Floristic Kingdom and thus constitute an important ecological component of the Cape Flora (Cowling and Richardson 1995, Linder 2003). They are also of considerable economic value to South Africa, as revenue from *Protea* product exports generate



over U.S. \$10 million annually (Anon. 1999, Crous *et al.* 2004). This figure excludes the revenue generated through eco-tourism and horticulture. A thorough biological understanding of *Protea*-associated organisms that could cause phytosanitary problems is thus of substantial biological and economic importance.

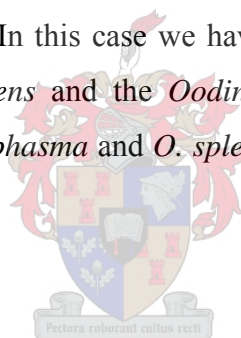
The conspicuous inflorescences of *Protea* spp. are visited by various animal pollinators (e.g. insects, birds and rodents) (Rebelo 1995). After pollination the inflorescences mature into tightly-packed fruiting structures (infructescences). The infructescences of serotinous members of the genus *Protea* only open to release their seeds once the water supply to the infructescence is severed (i.e. when the plant dies after fire or when insects bore into the bases of the infructescences) (Bond 1985). These structures may thus persist on the plant for several years, during which time they are occupied by various arthropod and fungal species (Coetzee and Giliomee 1985, 1987a, 1987b, Marais and Wingfield 1994, Lee *et al.* 2003, 2005, Roets *et al.* 2006d). *Protea*-associated *Ophiostoma* species often dominate fungal communities within infructescences and may be fundamental to the ecology of these plants (Roets *et al.* 2005). The means of dispersal of *Ophiostoma* populations between infructescences of the same and different plants in the community is, however, still poorly understood.



Seven species of *Ophiostoma* have been described from *Protea* spp. hosts in South Africa (Wingfield and Van Wyk 1993, Marais and Wingfield 1994, 1997, 2001, Roets *et al.* 2006a, 2006b, Chapters 3 and 5). Most of these (*O. splendens* G.J. Marais & M.J. Wingf., *O. phasma* Roets *et al.*, *O. palmiculminatum* Roets *et al.*, *Sporothrix variecibatus* Roets *et al.* and *O. gemellus* Roets *et al.*) have been shown to be phoretic on four mite species (Roets *et al.* 2006c, Chapter 4). The remaining two (*O. protearum* G.J. Marais & M.J. Wingf. and *O. africanum* G.J. Marais & M.J. Wingf.) are suspected to be transported in a similar way. At least two of the *Ophiostoma*-vectoring mite species that have been identified (one of the *Tarsonemus* spp. and a species of *Oodinychus* Berlese) possess seemingly specialised spore-carrying structures that, in the case of the *Oodinychus* sp., frequently contain spores of *Ophiostoma* species (Roets *et al.* 2006c, Chapter 4). The *Oodinychus* sp. is thought to be the main vector of *Protea*-associated *Ophiostoma* species. It is, for example, able to feed and reproduce on a diet consisting exclusively of these fungi (Roets *et al.*

2006c, Chapter 4). The interaction between this *Oodinychus* sp. and *Ophiostoma* spp. is, therefore, likely to be mutualistic.

It is not known how the *Protea*-associated *Ophiostoma* vectoring mites are dispersed, but amongst others, representatives of the genera *Tarsonemus*, *Proctolaelaps* Berlese and *Oodinychus* from the Northern Hemisphere have been implicated in phoresy on other insects (Bridges and Moser 1983, Lindquist 1969, Moser and Roton 1971, Blackwell *et al.* 1986a, 1988). It is thus plausible that the *Protea*-associated representatives of these genera have similar means of transport between hosts. Other potential means of transport include anemophilous dispersal and / or self-dispersal (climbing between the branches of the host plant). The present study sets out to determine the specific means of dispersal of the *Protea*-associated *Ophiostoma*-vectoring mites. We also consider the timing and effectiveness of colonisation of the hosts by *O. splendens* and *O. phasma*, as well as the transfer of fungal spores from the mites onto the host substratum. In this case we have focussed on the apparent close relationship between *O. splendens* and the *Oodinychus* sp. Ultimately we aim to reconstruct the life cycles of *O. phasma* and *O. splendens* and to compare them to the conifer-based systems.



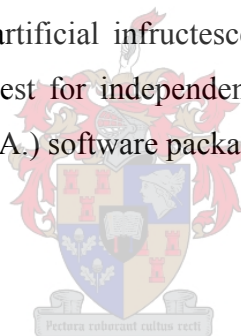
## Materials and methods

### *Self-dispersal of mites and fungi*

The movement of mites between infructescences on the same plant was investigated by capturing mites that moved from drying infructescences to artificially constructed infructescence-like containers. We tested for the preferential movement of mites, if any, towards moist and sheltered areas such as those provided by intact moist infructescences. The manufactured ‘infructescences’ (n = 52) consisted of small glass containers (30 ml wide-neck bottles) filled with shredded filter paper. The filter paper in half of these bottles (n = 26) was used dry, while the other half was slightly moistened with 3 ml dH<sub>2</sub>O. Half of the bottles (n = 13) from each of the two treatments were then placed within larger containers covered with black plastic bags to block out light, while the rest were left uncovered.

Fifty-two *P. repens* L. shoots (ca. 60 cm long) that contained a single ca. 4-month-old infructescence colonised both by *O. splendens* and one or more of the mites *Tarsonemus* sp. A, *P. vandenbergi* and / or *Oodinychus* sp. were collected from the Jonkershoek Forestry Reserve, Stellenbosch, South Africa in December 2005. We selected morphologically similar shoots in which the infructescences were situated a third of the way down from the tip of the main branch. After the side branches and leaves had been removed from the main branches, the shoots were placed in empty plastic containers to maintain them in an upright position. Due to the unavailability of water, the infructescence bases dried out rapidly, which caused the involucre bracts to open and release the enclosed seeds. The absence of water below the opening infructescences also allowed free upward or downward movement by the mites. The apices of the branches were covered with the upturned glass containers described above. Mites were collected from these containers at six-day intervals over a period of one month and stored at 4 °C. Comparisons were made between the total numbers of mites accumulating within the artificial infructescences of the different treatments. Data were analysed using a T-test for independent samples within the Statistica 7 (Statsoft corporation, Tulsa, U.S.A.) software package.

### *Vectored dispersal*



To test whether mites were phoretic on other arthropods, 56 *Ophiostoma*-colonised *P. repens* and *P. neriifolia* R. Br. infructescences (3 – 12 months old) were collected from the Jonkershoek Forestry Reserve between May 2004 and July 2005. These were placed in the specially designed emergence cages as described by Roets *et al.* (2006c, Chapter 4), and all arthropods that emerged from them over a 3-mo period were collected. In addition, various larger insects ( $\geq 5$  mm) were randomly collected from the open flower heads of these two plant species during August 2005. All the collected arthropods were classified into morpho-species and inspected for the presence of phoretic mites using a Nikon SMZ800 dissecting microscope. Photos were taken with a Nikon DXM1200 digital camera. When present, individual mites were removed with a fine dissecting needle and stored at 4 °C. A few arthropod specimens were also studied with a Leo 1430 VP7 scanning electron microscope (SEM). For these studies, the arthropods were frozen at -20 °C overnight and then

dried for 3 d at 50 °C. Specimens were mounted onto stubs using double-sided carbon tape, sputter coated with gold-palladium and studied using standard SEM methods.

#### *Dispersal by wind*

We confirmed the presence of *Tarsonemus* sp. A, *Oodinychus* sp. and *P. vandenbergi* Ryke within *P. repens* and *P. neriifolia* infructescences in the Jonkershoek Forestry Reserve during November 2005. We then proceeded to collect any wind-borne arthropods with the aid of 30 sticky traps (15 x 15 cm, Bayer, Stellenbosch, South Africa). Traps were randomly suspended at various heights (62 – 141 cm) and directions between plants in a *Protea* population consisting mainly of *P. repens* and *P. neriifolia*. Traps were retrieved after five days and inspected for the presence of the target mites using a dissecting microscope.

#### *Phoretic mites and hyperphoretic Ophiostoma spp.*

All mites collected were identified to morpho-species, crushed, mixed with 1 ml ddH<sub>2</sub>O and plated onto 2 % malt extract agar plates (MEA, Biolab, Midrand, South Africa). The medium was amended with the antibiotics streptomycin sulphate (0.04 g/L) and cycloheximide (0.05 g/L) to restrict the growth of fungal contaminants and bacteria (Harrington 1981). Plates were periodically inspected for the presence of *Sporothrix* Hekt. & C.F. Perkins anamorphic states of the *Protea*-associated *Ophiostoma* species, all of which were identified using morphological characters. Voucher specimens of all arthropods collected are housed in the insect collection (USEC), Department of Conservation Ecology and Entomology, University of Stellenbosch, Stellenbosch, South Africa.

#### *Timing of colonisation*

The floral development of both *P. repens* and *P. neriifolia* was studied, and flowering was divided into the following six flowering stages: 1. Young bud stage (*ca.* 3 months before the inflorescence opens). 2. Late bud stage (just prior to the opening of the inflorescence). 3. Early flowering stage (30–50 % of individual flowers within the inflorescences were open). 4. Late flowering stage (> 70 % of flowers within the

inflorescence were open). 5. After flowering (all of the flowers had opened and the involucre bracts started to close). 6. One month after flowering. A total of 20 flower heads per flowering stage of each of these two species were then covered with fine gauze to exclude insect visits to flower heads at different stages of floral maturity. This was done in order to determine the time when *Ophiostoma* spp. first appear in the infructescences. Study sites included the Jonkershoek Forestry Reserve, Franschoek Pass, Franschoek and the Riviersonderend mountains, Riviersonderend. The stems beneath the infructescences (*ca.* 10 cm) were smeared with oil to prevent mites and other small arthropods from migrating up the stems to the infructescences after they had been bagged. This experiment was repeated during the main *P. repens* and *P. neriifolia* flowering season in May to August 2003, 2004 and 2005. All infructescences were inspected for the presence of *Ophiostoma* ascomata and their anamorphs two to three months after flowering (Roets *et al.* 2005).

A univariate test of significance (ANOVA) was performed on the data within the Statistica version 7 (Statsoft corporation, Tulsa, U.S.A.) software package with Sigma-restricted parameterisation. A significance of  $P = 0.05$  was used as minimum value for reports of significance.

#### *Inoculation of uncolonised material with Ophiostoma spp. by Oodinychus sp.*

Mites identified as *Oodinychus* sp. were collected from *O. splendens*-colonised *P. repens* infructescences from the Jan S. Marais Park, Stellenbosch. They were placed in 40 ml specimen vials (20 individuals per vial) containing double autoclaved *P. repens* floral parts (collected from flower heads at flowering stage 4). The experiment was replicated 20 times, and included two additional negative controls containing only autoclaved floral parts. The vials were kept at 24 °C in the dark for three months. The floral parts were then removed from the vials, cleared of any mites and agitated using a vortex mixer in 10 ml ddH<sub>2</sub>O under sterile conditions in order to loosen fungal spores deposited or produced on the plant material. The suspension was transferred to MEA plates (1 ml/ plate) that had been amended with streptomycin sulphate (0.04 g/L) and cycloheximide (0.05 g/L). Plates were regularly inspected for the presence of *Sporothrix* colonies. In a separate experiment, eighty *Oodinychus* sp. mites collected from *O. splendens*-colonised *P. repens* infructescences were allowed to move freely

on MEA plates (one mite per plate) in an effort to isolate *Ophiostoma* spp. directly from the mites.

### *Efficiency of dispersal*

The efficiency of dispersal of *Ophiostoma* spp. to uncolonised sites was assessed by determining the percentage of *Protea* plants containing *Ophiostoma* spp. in areas with natural Fynbos vegetation of different ages. Reseeding *Protea* species such as *P. repens* and *P. neriifolia* usually flower for the first time three years after germination (le Maitre and Midgley 1992). After flowering, the main stem forks to form two branches, with the infructescence situated in the fork. This pattern of branching and flowering is repeated in all subsequent flowering seasons, so that it is possible to roughly estimate the age (time after most recent fire) of proteaceous vegetation.

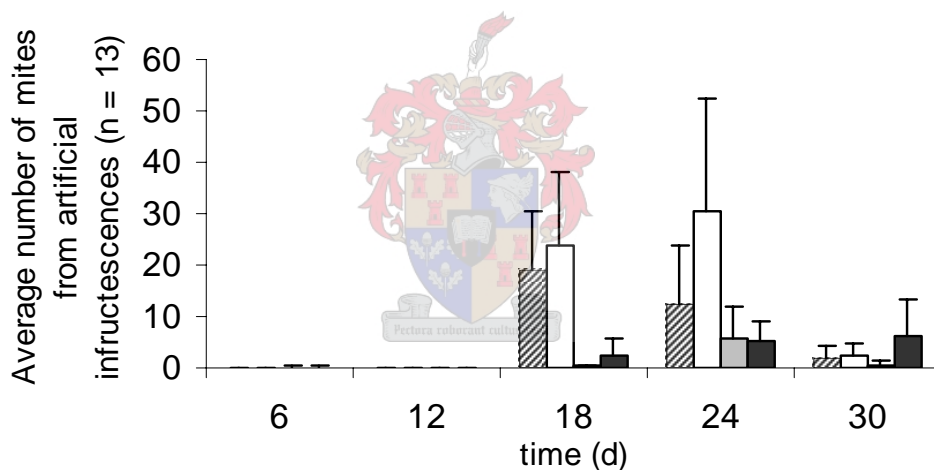
Three sites containing populations of *P. repens* and *P. neriifolia* at various ages (between 3–4, 9–11 and 14–17 years, respectively) were selected in the Jonkershoek Forestry Reserve during November 2005. The ages of the sites were estimated by counting the branching nodes, as outlined above. Plants were chosen at random (n = 10 per *Protea* species per site) and all the infructescences of a chosen plant were inspected with a hand lens for the presence of ascomata of *Ophiostoma* sp. When no ascomata were found, infructescences (n ≈ 10) were collected and inspected with a dissecting microscope for the presence of the anamorphic states of these fungi. Plants were counted as positive for colonisation by *Ophiostoma* spp. if any of the collected infructescences contained ascomata or anamorphs of these fungi.

## **Results**

### *Self-dispersal*

A total of 779 mites were collected from the false ‘infructescences’ throughout the duration of the experiment. Most of these belonged to one of the three species known to carry spores of *Ophiostoma* spp. These included *Tarsonemus* sp. A (n = 688), *P. vanderbergi* (n = 19) and *Oodinychus* sp. (n = 54). Only two mites, both individuals of *P. vanderbergi*, were observed within the bottles prior to the opening of the

infructescences *ca.* 2 weeks after the infructescences were picked. A significant increase in mite numbers was, however, observed at the time when the infructescences started to open ( $t = 4.20$ ,  $P = 0.000$ ) (Fig. 1). A significant decrease in mite numbers was observed from day 24 to day 30 ( $t = 2.94$ ,  $P = 0.004$ ) (Fig. 1). Furthermore, significantly more mites were collected from containers with moist filter paper than those without moisture ( $t = 2.42$ ,  $P = 0.02$  for open and  $t = 2.47$ ,  $P = 0.02$  for closed containers). No significant difference was found between the numbers of mites that were collected in the open versus closed containers, except when the closed containers contained moistened filter paper ( $t = 2.94$ ,  $P = 0.01$ ). A small number of mites were still accumulating in the containers when the experiment was terminated.



**Fig. 1.** Average number of mites (+ standard deviation) collected from artificial infructescences over a 30 day period: Bars with diagonal stripes = mites collected from uncovered artificial infructescences containing moist filter paper shreds, White bars = mites collected from covered artificial infructescences containing moist filter paper shreds, Grey bars = mites collected from uncovered artificial infructescences containing dry filter paper shreds, Black bars = mites collected from covered artificial infructescences containing dry filter paper shreds.

## Vectored dispersal

Thirteen insect morpho-species (168 individuals) were collected from the emergence cages (Table 1). These belonged to various families, all of which had been reported on these *Protea* species in previous studies (Roets *et al.* 2006c, 2006e, Chapters 2 and 3). Mites were, however, only observed on the surface of *G. hottentottus* F. Coleoptera: Scarabaeidae individuals that emerged from these infructescences. Although four species of mite were collected from the surface of this beetle (Table 2), only three species were commonly observed. They were *Tarsonemus* sp. A, *P. vandenbergi* and the *Oodinychus* sp. (Fig. 2 A–D). The fourth mite species on this beetle was represented by only two hypopi (a special type of deutonymph stage) of a *Caloglyphus* Berlese sp. In one case all four mite species were found to co-occur on the same *G. hottentottus* individual.

**Table 1.** Insects that were collected from the infructescences of *P. repens* and *P. neriifolia* during May 2004 to Jul. 2006 (n = 56 for each *Protea* species) in emergence cages and searched for the presence of phoretic mites. Arthropods indicated in bold were found to vector mites. Reference numbers indicate collection number of reference specimen housed in insect collection (USEC), Department of Conservation Ecology and Entomology, University of Stellenbosch, Stellenbosch, South Africa

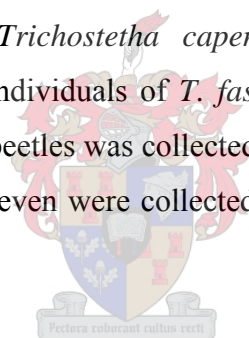
Insect taxa	Ref. nr.	<i>P. repens</i>	<i>P. neriifolia</i>
<i>Argyroploce</i> Hübner sp. (Tortricidae)	68	2	1
Braconidae	52	1	1
<i>Crematogaster</i> Lund sp. (Formicidae)	15	35	12
Diptera	5	5	0
<i>Euderes lineicolis</i> Wiedemann (Curculionidae)	33	2	0
<b><i>Genuchus hottentottus</i> (F) (Scarabaeidae)</b>	70	<b>9</b>	<b>2</b>
<i>Gyponyx</i> Gorham sp. (Cleridae)	55	3	0
Miridae	20	4	0
<i>Oxycarenum maculatum</i> Stal. (Lygaeidae)	7	18	24
Pentatomidae	24	0	1
Psocoptera (sp. 3)	13	15	29
<i>Sphenoptera</i> Solier sp. (Buprestidae)	49	2	0
Thysanoptera	34	1	1



The number of mites carried by an individual *G. hottentottus* beetle varied considerably. Most beetles carried individuals of *Tarsonemus* sp. A and *Oodinychys* sp. When present, *Tarsonemus* sp. A was particularly numerous, with up to 108 individuals collected from a single beetle. This same beetle also carried 3 individuals of the *Oodinychus* sp. and one individual of *P. vandenbergi*.

Mites were observed on the ventral side of the beetles only (Fig 2 A–D). Here they were usually found in groups associated with the front legs and head of the beetle. A smaller number of mites were also observed from the bases of the middle pair of legs. The *Tarsonemus* sp. A appeared to be gregarious, as numerous individuals usually occupied the space between the head and the front legs of the beetle.

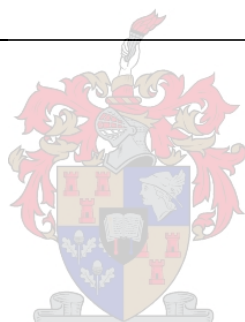
Various insects were collected in the field from the flower heads of *P. neriifolia* and *P. repens*, including bees, flies and beetles. Mites were, however, observed from two beetle species only, namely *Trichostetha capensis* L. and *T. fascicularis* L. Scarabeidae: Cetoniinae. Four individuals of *T. fascicularis* were collected from *P. repens*, while only one of these beetles was collected from *P. neriifolia*. Of the nine *T. capensis* individuals collected, seven were collected from *P. neriifolia* and two from *P. repens*.



Individuals of the mite *P. vandenbergi* were common (usually > 100 individuals) on all five *Trichostetha capensis* and nine *T. fascicularis* individuals. They were located on the hairy ventral surface of the beetles (Fig. 2 E). Individuals of *Tarsonemus* sp. A were also observed on all *T. fascicularis* and four of the *T. capensis* individuals (Table 2). Instead of being ventrally carried as *P. vandenbergi* individuals, the *Tarsonemus* sp. A was more common on the upper surface of the beetle within the mesoscutellum groove in numbers exceeding 50 (Fig. 2 F). *Tarsonemus* sp. A were also occasionally observed underneath the elythera of both beetle species.

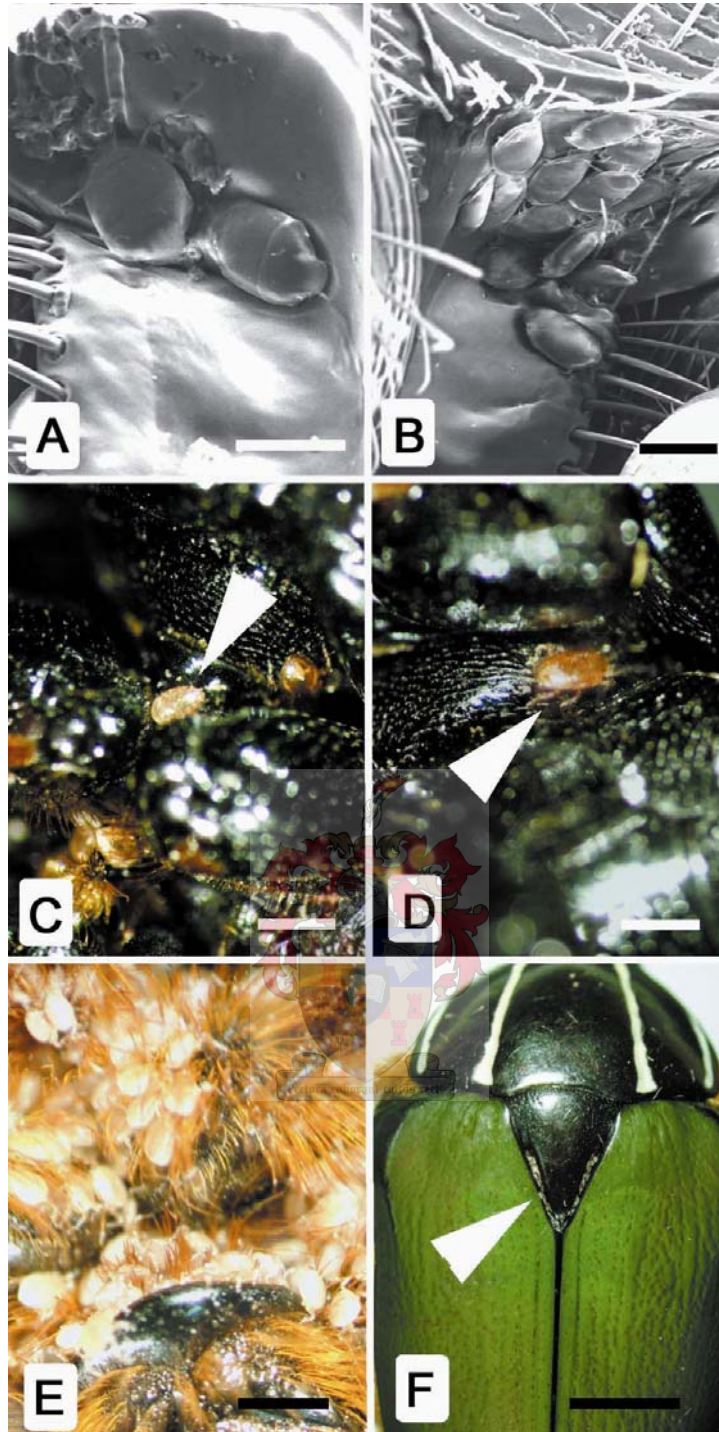
**Table 2.** Average number ( $\pm$  standard deviation) of phoretic mites collected from the surface of three beetles (*Genuchus hottentottus*, *Trichostetha capensis* L. and *T. fascicularis* L.) associated with *Protea repens* and *P. neriifolia* in the Jonkershoek Forestry Reserve, Stellenbosch, South Africa during May 2004 to Aug. 2005.

Mite taxa	<i>Protea neriifolia</i>		
	<i>G. hottentottus</i>	<i>T. capensis</i>	<i>T. fascicularis</i>
<i>Tarsonemus</i> sp. A	7 (9.90)	9.29 (24.57)	0
<i>Oodinychus</i> sp.	0	0	0
<i>Proctolaelaps vanderbergi</i>	0	135.86 (131.68)	39
<i>Caloglyphus</i> sp.	0	0	0
	<i>Protea repens</i>		
<i>Tarsonemus</i> sp. A	31.22 (39.28)	15.5 (6.36)	89 (106.24)
<i>Oodinychus</i> sp.	4.67 (6.48)	0	0
<i>Proctolaelaps vanderbergi</i>	1.67 (3.20)	80 (72.13)	321 (264.92)
<i>Caloglyphus</i> sp.	0.22 (0.44)	0	0



#### *Dispersal by wind*

The arthropods that were caught in the sticky traps consisted mainly of coleopterans (beetles,  $n = 249$ ), dipterans (flies,  $n = 165$ ) and hymenopterans (bees and wasps,  $n = 185$ ). Small numbers of thysanopterans (thrips,  $n = 33$ ), spiders ( $n = 4$ ) and hemipterans (bugs,  $n = 42$ ) were also collected using this technique. No individuals of *Oodinychus*, *P. vanderbergi*, or *Tarsonemus* sp. A were collected. The only wind-borne mites that were caught in the sticky traps were two individuals belonging to the genus *Microtydeus* Thor.



**Fig. 2.** Mites phoretic on *Protea*-associated beetles. A and B. Scanning electron micrographs of *Tarsonemus* sp. on ventral surface of *Genuchus hottentottus*. C. Light micrograph of *Oodinychus* sp. on ventral surface of *G. hottentottus*. D. Light micrograph of *Proctolaelaps vanderbergi* on ventral surface of *G. hottentottus*. E. Light micrograph of *P. vanderbergi* on ventral side of *Trichostetha fascicularis*. F. Light micrograph of *Tarsonemus* sp. in mesoscutellar groove of *T. fascicularis*. Scale bars, A, B = 100  $\mu$ m, C, D = 400  $\mu$ m, E = 800  $\mu$ m, F = 5 mm.

*Phoretic mites and hyperphoretic Ophiostoma spp.*

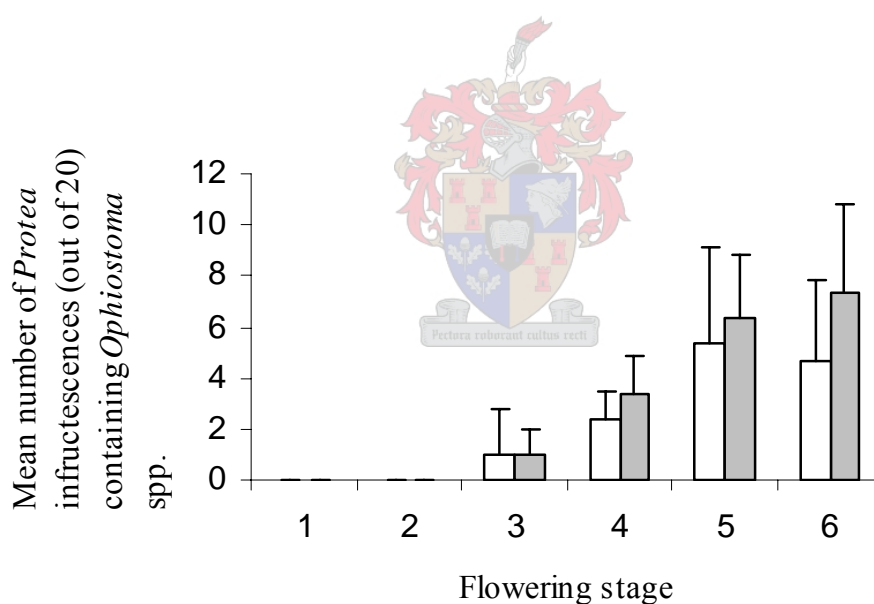
Many different fungal species were isolated from mites collected using the various methods. A total of ten isolates of *Ophiostoma* spp. were obtained from mites (Table 3). *O. splendens* was isolated nine times; twice from *Oodinychus* sp. collected from the artificial "infructescences", twice from *Tarsonemus* sp. A collected on the surface of a *G. hottentottus* individual that emerged from a *P. repens* infructescence, and five times from *Oodinychus* sp. also from emerging *G. hottentottus* individuals from *P. repens* infructescences. The single isolate of *O. phasma* was obtained from a *Tarsonemus* sp. A that was carried on a *G. hottentottus* individual that emerged from *P. neriifolia* infructescences. Humidity problems were encountered during the storage of *T. capensis* and *T. fascicularis* specimens that were collected from the flower heads of the two *Protea* species. Mites stuck to the sides of the collection vials containing the beetles due to moisture released by respiration of the insects. It was, therefore, not possible to isolate fungi from these mites.

**Table 3.** Isolates of *Ophiostoma* spp. obtained from mites phoretic on *Genuchus hottentottus* and collected in false 'infructescences'. Collections were made from *Protea neriifolia* and *P. repens* from the Jonkershoek Forestry Reserve, Stellenbosch, South Africa. The CMW number refers to the reference number in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

Fungal species	CMW	Phoretic mite / false infructescence	Host plant
<i>Ophiostoma phasma</i>	23061	<i>Tarsonemus</i> sp. A	<i>P. neriifolia</i>
<i>O. splendens</i>	Osp101	<i>Oodinychus</i> sp.	<i>P. repens</i>
<i>O. splendens</i>	23062	<i>Oodinychus</i> sp.	<i>P. repens</i>
<i>O. splendens</i>	23063	<i>Oodinychus</i> sp.	<i>P. repens</i>
<i>O. splendens</i>	OSP104	<i>Oodinychus</i> sp.	<i>P. repens</i>
<i>O. splendens</i>	23064	<i>Oodinychus</i> sp.	<i>P. repens</i>
<i>O. splendens</i>	OSP106	<i>Tarsonemus</i> sp. A	<i>P. repens</i>
<i>O. splendens</i>	23065	<i>Tarsonemus</i> sp. A	<i>P. repens</i>
<i>O. splendens</i>	23066	false infructescence	<i>P. repens</i>
<i>O. splendens</i>	23067	false infructescence	<i>P. repens</i>

### Timing of colonisation

Ascomata of *Ophiostoma* spp. and their anamorphs were never observed from *P. neriifolia* and *P. repens* inflorescences that were covered with the gauze bags at the first two bud stages (Fig. 3). This confirmed that the exclusion method followed in this study was effective in preventing arthropods carrying spores of *Ophiostoma* spp. to come into contact with the flower heads. There was a significant increase in colonisation numbers of these fungi with an increase in inflorescence age for both *P. neriifolia* ( $F = 3.5$ ,  $P = 0.0350$ ) and *P. repens* ( $F = 8.5$ ,  $P = 0.001$ ) with five degrees of freedom (Fig. 3). No significant increase in colonisation numbers of *Ophiostoma* spp. was observed between flowering stages 5 (late flowering stage) and 6 (1 month after flowering).



**Fig. 3.** Mean number of inflorescences (+ standard deviation,  $n = 20$ ) containing *Ophiostoma* spp. at various flowering stages. White bars = *P. neriifolia*, Grey bars = *P. repens*.

### *Inoculation of uncolonised material with Ophiostoma spp. by Oodinychus sp. mites*

Various fungal species were isolated from the autoclaved floral parts artificially colonised by the *Oodinychus* mites, while no fungi were isolated from the negative controls. Colonies of *O. splendens* (in the *Sporothrix* anamorphic state) were observed from suspensions made from four of the vials containing floral parts colonised by the *Oodinychus* sp. No colonies of *Ophiostoma* spp. were initiated from mites placed directly onto agar plates.

### *Efficiency of dispersal*

Colonies of *O. splendens* and *O. phasma* were observed in the infructescences of *P. repens* and *P. neriifolia* formed after their first flowering season following a fire (ca. 3–4 years old). Seventy percent of *P. repens* plants and 50 % of *P. neriifolia* plants contained colonies of *O. splendens* and *O. phasma*, respectively. When these colonisation numbers are compared to colonisation numbers in the two older vegetation sites, it is apparent that there is a slight increase in colonisation numbers with an increase in plant population age. *O. splendens* was found to colonise 80 % and 70 % of plants at the 9–11 and 14–16 year old populations, respectively, while the colonisation numbers for *O. phasma* were 70 % at both of the older vegetation sites.

## **Discussion**

The present study reports the first known case of hyperphoretic dispersal of *Ophiostoma* spp. associated with hosts native to the Southern Hemisphere. The three mite species, *Oodinychus* sp., *P. vanderbergi* and *Tarsonemus* sp. A, that have been shown to vector *Ophiostoma* spp. (Roets *et al.* 2006c, Chapter 4) were found to be phoretic on the beetles *G. hottentotus*, *T. fascicularis* and *T. capensis*. In turn, mites phoretic on *G. hottentotus* were shown to vector *O. splendens* and *O. phasma*. *Ophiostoma splendens* was also isolated from *Tarsonemus* sp. A for the first time in this study.

We failed to trap any *Tarsonemus* sp. A, *P. vanderbergi* and *Oodinychus* sp. individuals in the sticky traps designed to collect wind-borne arthropods. This

suggests that wind plays a minor role in the dispersal of mites (and the fungal spores they carry), and that the three beetles identified in this study are crucial to the long-distance dispersal of the mites and fungi. Although the frequency of phoretic mites carrying spores of *Ophiostoma* species was low, beetles did carry numerous mite individuals. The number of mites vectored by the beetles varied greatly, but mite loads upwards of 200 individuals were common on both species of *Trichostetha*. *Genuchus hottentottus* beetles generally carry fewer mites, although one individual was found carrying 112 mite individuals. A limited number of beetles would thus still be able to transport many *Ophiostoma*-carrying mite individuals to a new host plant. This explains why successful *Ophiostoma* spore transfer to new substrates appears to be highly effective, despite the relatively low percentage of spore-carrying mites observed on the beetles.

Colonisation of *P. repens* and *P. neriifolia* infructescences by *O. splendens* and *O. phasma* mostly took place during the late flowering and post-flowering stages. This coincides with the peak in activity for the secondary *Ophiostoma*-vectoring arthropods (*G. hottentottus*, *T. fascicularis* and *T. capensis*) as these feed on nectar and pollen of *Protea* species (Coetzee and Giliomee 1985). The main *Protea* flowering period in the Jonkershoek Forestry Reserve (May – August, pers. observ.) also coincides with a peak in numbers of *Ophiostoma* within *Protea* infructescences (Roets *et al.* 2005). Although not tested, the primary vectors of these fungi (mites) are also expected to be more active and / or more numerous during the flowering season of the host plants. These observations suggest that the timing of *Ophiostoma* spore deposition on the host plant coincides closely with periods of activity of both the primary (mites) and secondary (beetles) *Ophiostoma* vectors. It remains to be confirmed whether the mites also disperse *Ophiostoma* fungal propagules outside of the *Protea* flowering season. We suspect this to be the case, as some fungal species (e.g. *Ophiostoma gemellus*) were isolated from *Tarsonemus* sp. mites associated with *P. caffra* Meisn. infructescences collected outside the flowering season of the host plants (Roets *et al.* 2006c, Chapter 4).

The beetle-mediated phoretic dispersal of mites and hyperphoretic dispersal of *Ophiostoma* species is evidently very effective, as the first *P. repens* and *P. neriifolia* infructescences that formed after the first flowering season (3 – 4 years after a fire)

were already colonised by *O. splendens* and *O. phasma* respectively. The natural fire cycle ranges between 5 to 50 years (Van Wilgen 1981, 1987) and reseeded *Protea* species will take at least three years to mature and commence flowering (le Maitre and Midgley 1992). It is thus essential that the mites and the fungi that they vector are able to move over large distances to ensure successful recolonisation of regenerating post-fire *Protea* populations.

Based on the results of this study, we are able to propose a life history for *O. splendens* and *O. phasma* (Fig. 4). This is divided into four phases as follows:

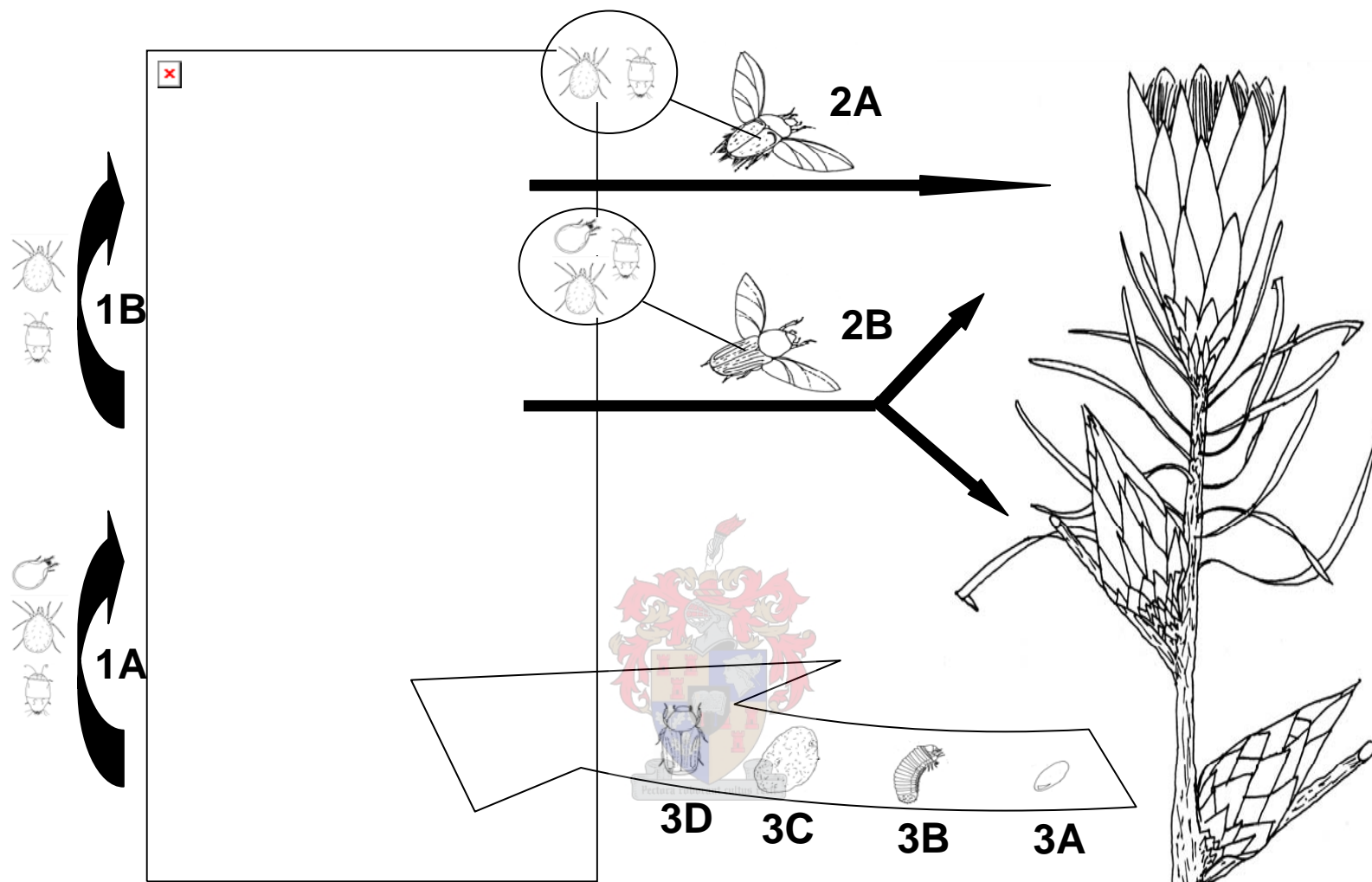
**Spore acquisition by mites** - Sporulating ascomata of *O. splendens* and *O. phasma* are present in *P. repens* and *P. neriifolia* infructescences within three to four months after flowering (Roets *et al.* 2005). These fungi persist in this niche for several years, and display sporulation peaks during the cooler and wetter winter and autumn months (Roets *et al.* 2005). Many species of mites (including *Tarsonemus* sp. A, *P. vanderbergi* and the *Oodinychus* sp.) and insects (including *G. hottentottus*) are also present within the infructescences throughout the year (Coetzee and Giliomee 1985, 1987a, 1987b, Roets *et al.* 2006d). The mites acquire spores of these fungi while they feed. Some mite species, notably *Oodinychus* sp., also feed directly on certain *Ophiostoma* species, which would certainly aid spore acquisition. Roets *et al.* (2006c, Chapter 4) showed that the spores of *Ophiostoma* spp. may be carried in specialised spore-carrying structures (e.g. *Tarsonemus* spp. and the *Oodinychus* sp.). We believe spore deposition onto the mites, or acquisition by the mites, occurs mainly during the peak fungal growing season, which coincides with the peak of the *Protea* flowering season (Roets *et al.* 2005, 2006c, Chapter 4), but this needs further investigation.

**Short distance dispersal** - As prevailing weather conditions become warmer and drier towards the end of the *Protea* flowering season, many of the infructescences that have been damaged by boring insects during the cool, wet winter months will open to release their seeds (Bond 1985). We have shown that mites migrate along the main stem of the plant, from the open, desiccating infructescences to closed artificial infructescences that provide a sheltered, moist environment analogous to the environment within intact infructescences and / or inflorescences. During this migration, the mites carry spores of various fungal species, including those of the



*Ophiostoma* spp. *In vitro* experiments showed that the mites would be able to inoculate uncolonised plant material with *Ophiostoma* species, at least in the case of *O. splendens* vectored by *Oodinychus* sp. mites. Mites are thus able to spread *Ophiostoma* species from colonised infructescences to uncolonised habitats by simply moving between the branches (Fig. 4. 1A and 1B). The limited size of the mites and their need for moist, sheltered habitats would probably restrict their movements, and should limit this mainly to migrations between infructescences of the same or neighbouring plants.

**Long distance dispersal** - *Tarsonemus* sp. A and *P. vanderbergi* were collected from *T. fascicularis* and *T. capensis* adults. These two mites must thus move both between infructescences and between infructescences and open flower heads (inflorescences) of the same plant (Fig. 1B) as the beetles are only associated with the inflorescences of *Protea* spp. (Holm and Marais 1992). A move to inflorescence would bring them into contact with the two beetle species. The *Oodinychus* sp. was not collected from these two beetles, and it is thought to primarily be transported by *G. hottentottus* that feeds within infructescences when still immature (Coetzee and Giliomee 1978b). *P. vanderbergi* and the *Oodinychus* sp. were often observed to display phoretic activity (they move towards any moving object in their vicinity) at exactly the same time when *G. hottentottus* adults were observed emerging from the infructescences of *P. repens* and *P. neriifolia*. At this stage, both of the plant species also carried older flowering stage flower heads to which *Genuchus hottentottus*, *T. fascicularis* and *T. capensis* are attracted in search of nectar and pollen. We propose that, due to the phoretic activity of the mites, they crawl onto the young adult *G. hottentottus* (Fig. 4. 2B) beetles as they emerge from the infructescences or onto *Protea*-flower visiting *Trichostetha* spp. (Fig. 4. 2A), and are carried between flower heads by the beetles in their quest for food. The beetles are strong fliers and are capable of covering vast distances in search of food. As the mites disembark from their vectors on arrival at the flower head, they colonise the new substrate and in so doing also transfer *Ophiostoma* spp. fungal spores.



**Fig. 4.** Schematic drawing of the proposed life history and dispersal of *O. phasma* and *O. splendens* on *Protea* spp. (not to scale). Mites acquire spores when moving and feeding within *Ophiostoma* spp. colonised infructescences. 1A. From top to bottom: *Oodinychus* sp., *P. vanderbergi* and *Tarsonemus* sp. move between infructescences by climbing among the branches. 1B. *P. vanderbergi* and *Tarsonemus* sp. also move from infructescences to open flowers. 2A. *P. vanderbergi* and *Tarsonemus* sp. hitching a ride on *Trichostetha* spp. to open flowers as the beetles search for *Protea* spp. pollen and nectar. 2B. *Oodinychus* sp., *P. vanderbergi* and *Tarsonemus* sp. hitching a ride on *G. hottentottus* to open flowers as the beetles search for *Protea* spp. pollen and nectar or to infructescences where the beetles lay their eggs. 3A–D. Development of *G. hottentottus* from egg to adult where-upon the adults will emerge during the next *Protea* flowering season to complete the cycle.

***Transfer of fungal spores to new substrate*** – Our results suggest that the transfer of *Ophiostoma* spores from mites may be an active process. If the transfer of fungal spores from these mites were passive, we would expect mites to relocate spores of these fungi to any surface within the infructescences. We were unable to initiate *O. splendens* colonies (collected from infructescences colonised by sporulating *O. splendens*) from numerous individual *Oodinychus* sp. placed directly on agar plates. Colonies of this fungus were, however, isolated from autoclaved host material colonised by these mites. Due to their sticky nature, it is improbable that the fungal spores are attracted to specific surfaces. We can thus infer that spore transfer by the mites is done actively, although the method in which this is regulated is not understood.

During the *Protea* non-flowering season the eggs of *G. hottentottus* (Fig. 4. 3A) laid during the flowering season develop into c-shaped larvae (Fig. 4. 3B) that bore into the seeds and involucrel receptacle whilst feeding (Coetzee and Giliomee 1987b). These will form pupae within ovoid structures constructed from frass and plant debris by the larvae (Fig. 4. 3C). Mature beetles will emerge the following *Protea* flowering season and leave the infructescences in search of nectar and pollen (Fig. 4. 3D).

The dispersal and life history of *O. splendens* and *O. phasma* differ from that of most of the conifer-associated *Ophiostoma* species. The *Protea*-associated species appear to be primarily dispersed by mites, with beetles playing a secondary role. This phenomenon however, has recently been confirmed for some conifer-based *Ophiostoma* spp. and may be more significant than generally accepted (Klepzig *et al.* 2001a, 2001b). *G. hottentottus* have been implicated in carrying DNA (probably as spores) of *O. splendens* in previous studies and may be linked to the fungus (Roets *et al.* 2006c, 2006e, Chapters 2 and 3). The low numbers of individuals found to carry DNA and the low number of infructescences found to contain individuals of these beetles do, however, complicate an explanation of the high observed colonisation numbers of these fungi (Roets *et al.* 2005). The abundance of mites within almost all infructescences and on most *G. hottentottus*, *T. fascicularis* and *T. capensis* individuals supports the notion that these mites represent the primary vectors of *Protea*-associated *Ophiostoma* species.

Interesting parallels can be drawn between the conifer-associated and *Protea*-associated *Ophiostoma* spp. In both systems, the host plants are drought tolerant woody plants of ancient origin (Bowe *et al.* 2000, Reeves 2001). Likewise, the beetles involved in both systems are borers (excluding *Trichostetha* sp.) that are strongly associated with and dependant on their host plants. Various mites belonging to the genus *Tarsonemus* have been implicated as associates of the *Ophiostoma* fungal species on both conifers and *Protea* spp. Finally, *Tarsonemus* spp. associated with *Ophiostoma* spp. on both conifers and *Protea* spp. possess sporothecae formed by tergite 1 (Moser 1985, Roets *et al.* 2006c, Chapter 4). These parallels do not only suggest similar origins of the two systems, but also predict that they have been maintained over a very long period of time, which may even predate the Gondwanan break-up 140 m.y.a. (Goldblatt and Manning 2000, Farrell *et al.* 2001). This long-term continuation of these similar systems is probably the result of a close association between at least mites in the genus *Tarsonemus* and species of *Ophiostoma*. The co-evolution between *Tarsonemus* sp. and species of *Ophiostoma* should prove to be an interesting field for future study.

In addition to *O. phasma* and *O. splendens*, the *Protea*-associated *O. palmiculminatum*, *O. gemellus* and *S. varicibatus* have all been isolated from mites in a previous study (Roets *et al.* 2006c, Chapter 4). In the present study we only report on the isolation of *O. phasma* and *O. splendens* from mites phoretic on beetles, but we predict that the remaining species of *Protea*-associated *Ophiostoma* are also hyperphoretically dispersed by mites. Future studies should focus on reconstructing the life histories and dispersal of the remaining *Protea*-associated *Ophiostoma* species in order to draw comparisons between the life histories and co-evolution of *Protea* associated and conifer associated *Ophiostoma* species and their vectors.

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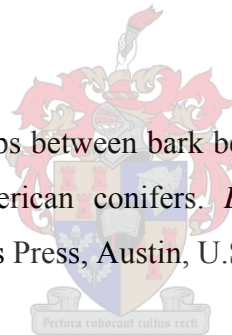


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## Chapter 7: The taxonomy and ecology of ophiostomatoid fungi associated with *Protea* infructescences: a review of current knowledge

### Introduction

The term ophiostomatoid fungi was coined at a time when there was considerable confusion between the insect-associated fungal genera *Ceratocystis* Ellis & Halst. and *Ophiostoma* Syd. & P. Syd. emend. Z.W. de Beer *et al.* (Wingfield *et al.* 1993). Broadly, the term refers to a group of fungi that have assumed similar morphology through convergent evolution and include well-known arthropod-associated genera such as *Ophiostoma*, *Ceratocystis* and their anamorphs. Despite the morphological similarities of their ascomata (sexual states), molecular evidence strongly suggest that species belonging to these genera are distantly related, with *Ophiostoma* belonging to the Ophiostomatales, while *Ceratocystis* resides in the Microascales (Hausner *et al.* 1992, 1993a, 1993b, Spatafora and Blackwell 1994). The shared morphology between these genera probably relates to ecological convergence (i.e. dispersal biology) rather than phylogenetic affinity. The group is characterised by having globose ascomatal bases that usually taper towards long ostiolate beaks bearing spores carried in sticky masses. This morphology promotes spore dispersal via arthropods (Münch 1907, 1908, Francke-Grosmann 1967, Whitney 1982, Beaver 1989, Malloch and Blackwell 1993, Cassar and Blackwell 1996).

The ophiostomatoid fungi have a global distribution, but are most prevalent in the Northern Hemisphere where they are typically associated with the galleries of bark-beetles (Coleoptera: Scolytinae) that are particularly well-known on conifers (Francke-Grosmann 1967, Upadhyay 1981, Whitney 1982, Christiansen *et al.* 1987, Wingfield *et al.* 1993, Paine *et al.* 1997, Kirisits 2004). The group includes important and devastating plant pathogens such as *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier (the causes of Dutch elm disease) and *Ceratocystis fagacearum* (Bretz) J. Hunt (the causal agent of Oak wilt) (Webber and Brasier 1984, Sinclair *et al.* 1987, Brasier 1988,

Webber and Gibbs 1989, Brasier 1991). Many non-pathogenic species also colonise trees and timber causing discoloration of the wood that result in large monetary losses (Münch 1907, Upadhyay 1981, Whitney 1982, Sinclair *et al.* 1987, Seifert 1993, Jacobs and Wingfield 2001).

### **Taxonomy of the ophiostomatoid fungi**

Although the economic importance of ophiostomatoid fungi has been well established, their taxonomy has proven to be problematic (Parker 1957, Wright and Cain 1961, Olchowecki and Reid 1973, De Hoog 1974, Weijman and De Hoog 1975, Upadhyay 1981, De Hoog and Scheffer 1984, Harrington 1987). Current consensus is that species sensitive to the antibiotic cycloheximide, and with *Thielaviopsis* Went anamorphs reside in *Ceratocystis* (Microascales) (Hausner *et al.* 1993a, Spatafora and Blackwell 1994, Paulin-Mahady *et al.* 2002), while species tolerant to cycloheximide, containing rhamnose in their cell walls, and with *Sporothrix* Hekt. & C.F. Perkins, *Hyalorhinocladia* H.P. Upadhyay & W.B. Kendr., *Leptographium* Lagerb. & Melin, or *Pesotum* J.L. Crane & Schokn. emend. G. Okada & Seifert anamorphs, reside in *Ophiostoma* (Ophiostomatales) (Hausner *et al.* 1993b, Spatafora and Blackwell 1994). Zipfel *et al.* (2006) recently reconsidered the relationship between various *Ophiostoma* spp. and their anamorphs. In their study, a multi-marker DNA sequence-based phylogeny of the genus was reconstructed. In it three well-supported monophyletic lineages were identified, each supported by the morphology of the anamorph states of the constituent species. The three lineages were subsequently ascribed to three different genera. Species with *Leptographium* anamorphs were assigned to *Grosmannia* Goid. emend. Z.W. de Beer *et al.*, species with *Hyalorhinocladia* H.P. Upadhyay & W.B. Kendr. anamorphs, short-necked ascomata and falcate ascospores were included in *Ceratocystiopsis* H.P. Upadhyay & W.B. Kendr. emend. Z.W. de Beer *et al.* and species with either *Sporothrix* or *Pesotum* J.L. Crane & Schokn. anamorphs, having a range of ascomatal and ascospore forms were retained in *Ophiostoma s. s.* (Zipfel *et al.* 2006). The latter group most likely represents a number of distinct taxa, but current sampling is insufficient to resolve this further.

Recent research on the taxonomy and ecology of the *Protea* L.-associated ophiostomatoid fungi has made it possible to compare this system with the better-known conifer-associated system. The aim of the current review is to consider all the available literature pertaining to the taxonomy and ecology of the *Protea*-associated ophiostomatoid fungi and in so-doing to provide a basis for future studies on this interesting system.

### ***Protea* spp. and their associated organisms**

Members of the specious, African endemic genus *Protea* (Proteaceae – commonly referred to as proteas) are of considerable economic importance to South Africa (Anon. 1999, Crous *et al.* 2004). Several species of *Protea* are also dominant members of landscapes within the unique Fynbos Biome of the Cape floral kingdom and play a critical role in the functioning of this vegetation (Cowling 1992). The fynbos flower export market contributes the bulk of the revenue generated by these plants (Anon. 1999), and this financial potential has also contributed to an increase in the number of studies relating to the commercial production of proteas. Amongst others, this research resulted in a number of studies focused on the interactions with both fungal and faunal associates of the Proteaceae (Myburg *et al.* 1973, 1974, Myburg and Rust 1975*a*, 1975*b*, Coetzee and Giliomee 1985, 1987*a*, 1987*b*, Taylor and Crous 2000, Swart *et al.* 2000, Crous *et al.* 2004). The priority of this research has been the assessments of potential detrimental organisms on these plants, while for example the saprobic fungal associates of *Protea* species have received less attention (Marais and Wingfield 1994, Lee *et al.* 2003, 2005). This is unfortunate, as saprobic fungi represent a large component of the total *Protea*-associated fungal biodiversity, and it merits more focused attention.

The large flower heads of *Protea* species mature into often long-lived, conspicuous fruiting structures (infructescences), which may be retained on plants for a number of years in the form of canopy-stored seed reserves (Bond 1985). In South Africa, several species of *Protea* store their seeds in these serotinous structures (Rebelo 1995, Rourke 1998) that open to release seeds only once the water supply between them and the rest of the plant is severed. Seed-release is usually triggered by fire (Bond 1985), but insects

boring in the infructescence base may also disrupt the water supply and thus trigger the opening of the infructescences (Cowling 1992). Intact *Protea*-infructescences are colonised by many species of arthropods (Myburg *et al.* 1973, 1974, Myburg and Rust 1975a, 1975b, Coetzee and Giliomee 1985, 1987a, 1987b, Roets *et al.* 2006e) and micro-fungi (Marais and Wingfield 1994, Lee *et al.* 2003, 2005). Intriguingly, the ophiostomatoid fungi are, however, the most prominent fungal constituent within these structures (Roets *et al.* 2005) and may thus play a vital role in *Protea* ecology.

### **Taxonomic history and phylogenetic affinities of the *Protea*-associated ophiostomatoid fungi**

Ophiostomatoid fungi were discovered in *Protea*-infructescences about 20 years ago (Wingfield *et al.* 1988). This was considered a most unusual niche in which to find the fungi, the first species of which was discovered on *P. repens* L. The fungus was placed in the genus *Ceratocystiopsis* (*C. proteae* M.J. Wingf. *et al.*), based on the presence of ascospores that have long falcate sheaths. The new genus *Knoxdaviesia* Wingf. *et al.* was described to accommodate the unique anamorph of this species. Although morphology provided a reasonable accommodation for *C. proteae*, some uncertainty was felt regarding its generic placement. This was because the fungus is sensitive to the antibiotic cycloheximide (Wingfield *et al.* 1988) like the species in *Ceratocystis*, but unlike *Ophiostoma* spp. (Harrington 1981, Hausner *et al.* 1993a, 1993b, Spatafora and Blackwell 1994, Paulin-Mahady *et al.* 2002). The anamorph of *C. proteae* is also very unlike the *Thielaviopsis* anamorphs of *Ceratocystis* and produces conidia through a process of apical wall building (Minter *et al.* 1983), a character shared with species in the genus *Ophiostoma* (Wingfield *et al.* 1988, Wingfield and Van Wyk 1993). These characteristics and the fact that *C. proteae* had been found in a most unusual niche provided a firm indication that the fungus deserved more careful study.

Collections following the discovery of *C. proteae* revealed the presence of a similar species from other *Protea* host species, which also has a *Knoxdaviesia* anamorph. Although this species was morphologically related to *C. proteae*, it has allantoid, non-



sheathed ascospores and it was, therefore, assigned to *Ophiostoma* as *Ophiostoma capense* G.J. Wingf. & P.S. van Wyk (Wingfield and Van Wyk 1993). This discovery of this second species of ophiostomatoid fungi in *Protea* infructescences raised interesting questions regarding the phylogenetic placement of these two species within the ophiostomatoid fungi.

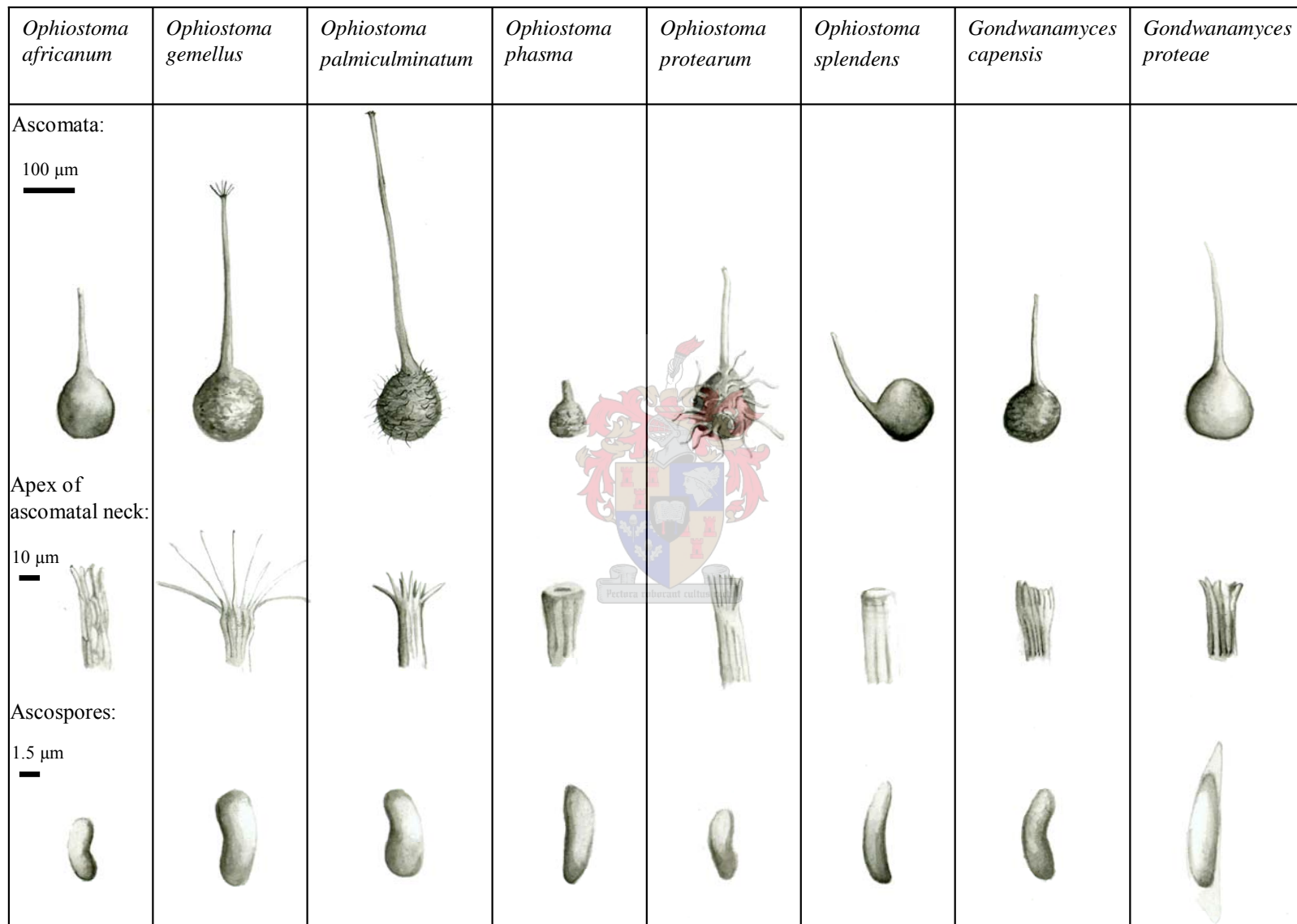
Surveys of *Protea* infructescences, subsequent to the discovery of *C. proteae* and *O. capense* led to the description of an additional ophiostomatoid species from this unusual habitat (Marais and Wingfield 1994). This species (*O. splendens* G.J. Marais & M.J. Wingf.) was more typical of the genus *Ophiostoma* than were the species with *Knoxdaviesia* anamorphs. It has a distinct *Sporothrix* anamorph, is tolerant to high concentrations of cycloheximide in culture and contains rhamnose in its cell walls (Marais and Wingfield 1994).

More extensive surveys (including *Protea* species from the northern parts of South Africa) revealed the presence of two additional ophiostomatoid fungi from *Protea* infructescences. These are also typical of the genus *Ophiostoma* as they have *Sporothrix* anamorphs that are tolerant to high concentrations of cycloheximide (Marais and Wingfield 1997, 2001). The species *O. africanum* G.J. Marais & M.J. Wingf. and *O. protearum* G.J. Marais & M.J. Wingf. were consequently described (Marais and Wingfield 1997, 2001). Thus, two very distinct forms of ophiostomatoid fungi became well-known from *Protea* infructescences in South Africa.

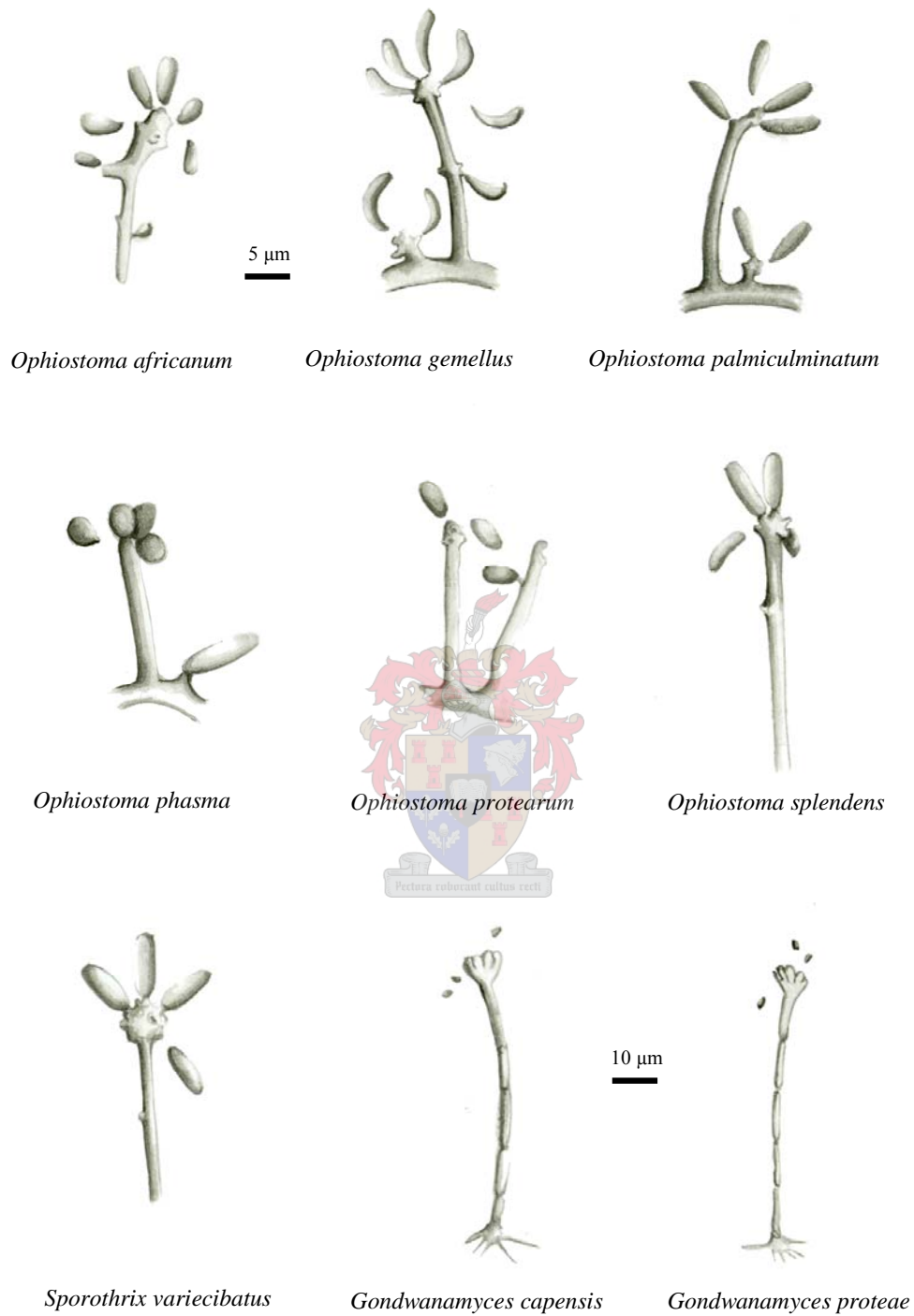
There was clearly a deep interest in understanding more regarding the phylogenetic relationships of the two morphological groups of fungi from *Protea* infructescences. Thus, as DNA sequence-based comparisons became available for phylogenetic studies, the ophiostomatoid fungal species from *Protea* spp. provided excellent material for study. Early analyses of the *Protea*-associated ophiostomatoid fungi using RFLP analysis on the operon regions of the rRNA subsequently revealed that species with *Knoxdaviesia* anamorphic states are closely related to each other and also to *Ceratocystis* (Marais *et al.* 1998). In contrast, species with *Sporothrix* anamorphs grouped with *O. piliferum* (Fr.)

Syd. & P. Syd., the type species of *Ophiostoma* (Marais *et al.* 1998, Wingfield *et al.* 1999). Species with *Knoxdaviesia* anamorphic states were thus recognised as unique to the Fynbos biome and they were thus provided with the new teleomorph genus *Gondwanamyces* G.J. Marais & M.J. Wingf. as *G. capensis* (M.J. Wingf. *et al.*) G.J. Marais & M.J. Wingf. and *G. proteae* (M.J. Wingf. *et al.*) G.J. Marais & M.J. Wingf. Like *Ceratocystis*, species of *Gondwanamyces* have close affinities with species in the Microascales (Wingfield *et al.* 1999). Based on results of their study, Wingfield *et al.* (1999) suggested that the *Ophiostoma* species associated with *Protea* infructescences might also reside in a separate genus. There was, however, insufficient evidence to make this distinction. More recent studies based on multiple gene genealogies have also failed to resolve a clear monophyletic grouping for the *Protea*-associated *Ophiostoma* spp. (Zipfel *et al.* 2006, Roets *et al.* 2006a, 2006b, Chapters 3 and 5).

Advances in molecular phylogenetic techniques made it possible to identify cryptic species that had previously been overlooked due to morphological similarities to known species. This led to the discovery of four additional species of *Protea*-associated ophiostomatoid fungi from the infructescences of various *Protea* spp. (Roets *et al.* 2006a, 2006b, Chapters 3 and 5). In a phylogenetic reconstruction of *Ophiostoma* s. l. (based on sequence data from the large subunit, ITS and beta-tubulin gene regions) these grouped within the genus *Ophiostoma* s. s. (Zipfel *et al.* 2006, Roets *et al.* 2006a, 2006b, Chapters 3 and 5). The species *O. palmiculminatum* F. Roets *et al.*, *O. phasma* F. Roets *et al.* and *Sporothrix variecibatus* F. Roets *et al.* were thus described from *Protea* spp. native to the fynbos, while *O. gemellus* F. Roets *et al.* was described from a species of *Protea* occurring in the northern parts of the country only (Roets *et al.* 2006a, 2006b, Chapters 3 and 5). The South African *Protea* infructescence niche thus currently accommodates nine species of ophiostomatoid fungi residing in two genera. These are *G. capensis*, *G. proteae*, *O. africanum*, *O. gemellus*, *S. variecibatus* (phylogenetically *Ophiostoma*), *O. palmiculminatum*, *O. phasma*, *O. protearum* and *O. splendens* (Fig. 1A). All *Protea*-associated *Ophiostoma* species have *Sporothrix* anamorphs, while *Gondwanamyces* species have *Knoxdaviesia* anamorphic states (Fig. 1B).



**Fig. 1A.** Teleomorph characteristics of eight of the ophiostomatoid fungi associated with *Protea* infructescences.

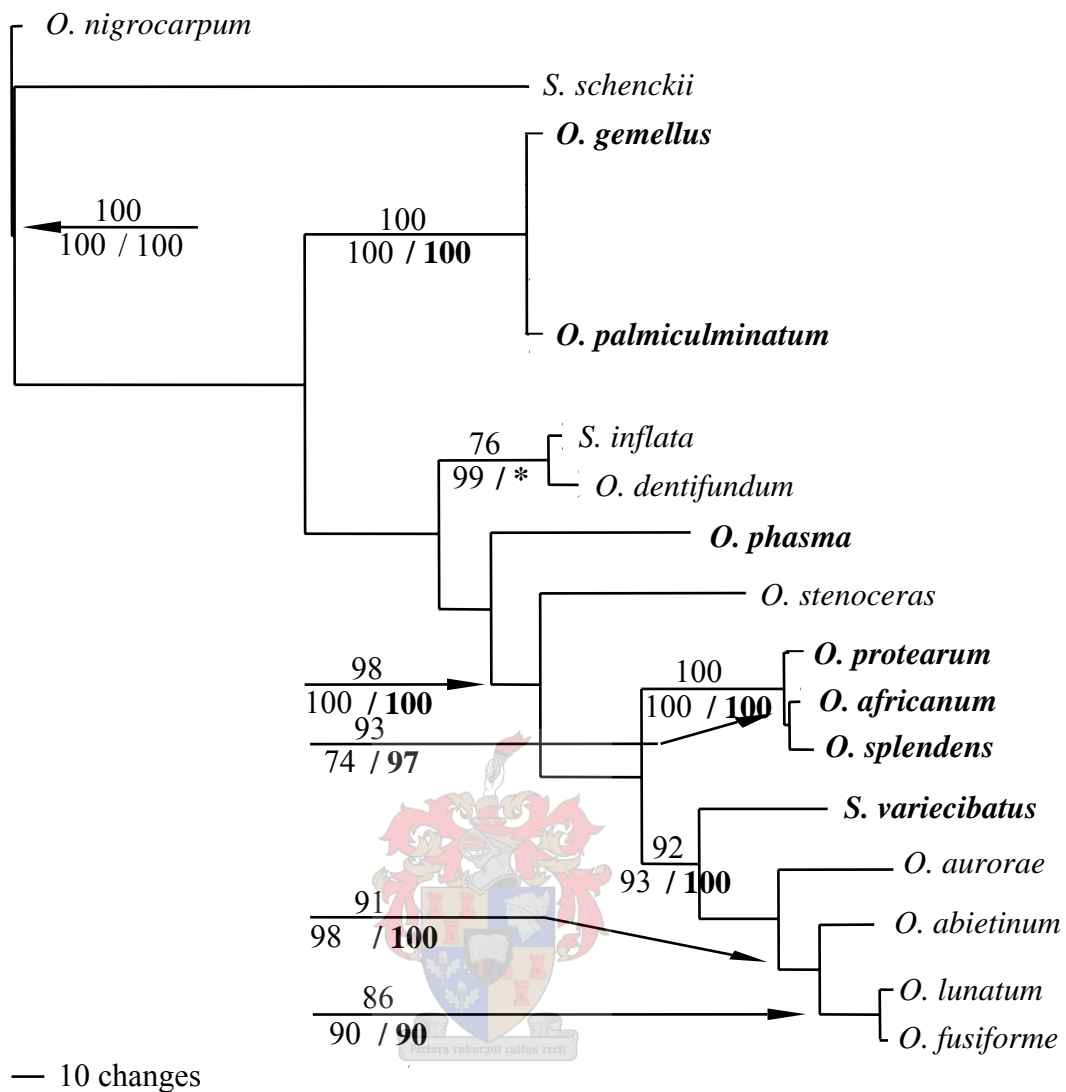


**Fig. 1B.** Anamorph characteristics of nine ophiostomatoid fungi associated with *Protea* infructescences.

*Gondwanamyces* species are confined to South African *Protea* species and form a discrete monophyletic unit closely related to *Ceratocystis* in the Microascales (Marais *et al.* 1998, Wingfield *et al.* 1999). Interestingly, recent molecular phylogenetic reconstruction of the large subunit, ITS and beta-tubulin DNA regions has revealed that the *Ophiostoma* species from *Protea*, in contrast, do not form a monophyletic unit (Fig. 2, adopted from Roets *et al.* 2006a, Chapter 5). *Ophiostoma splendens*, *O. africanum* and *O. protearum* form a well supported monophyletic lineage, with *O. protearum* and *O. africanum* resolving as closely related sister species. The grouping of *O. phasma* varies according to the marker used, but is always separate from the other *Protea*-associated *Ophiostoma* spp. (Roets *et al.* 2006a, 2006b, Chapters 3 and 5). *O. palmiculminatum* and *O. gemellus* reside in a well-supported monophyletic lineage also separate from the other species (Roets *et al.* 2006a, 2006b, Chapters 3 and 5). This lineage (*O. palmiculminatum* and *O. gemellus*) seems to be closely related to soil-borne South African isolates of *Sporothrix schenkii*. Thus, the *Ophiostoma* species associated with *Protea* are polyphyletic and they probably emerged more than once. The fourth clade that contains representatives of *Protea*-associated *Ophiostoma* species includes *S. variecibatus* (Fig. 2) that group with isolates of *O. aurorae* Zhou & M.J. Wingf., *O. abietinum* Marm. & Butin, *O. lunatum* Aghayeva & M.J. Wingf. and *O. fusiforme* Aghayeva & M.J. Wingf.



The polyphyletic origin of the *Protea*-associated *Ophiostoma* species suggests that the *Protea*-infructescence niche was independently invaded by *Ophiostoma* species more than once. The initial colonisation events were probably followed by subsequent speciation events in some lineages (e.g. the *O. splendens* / *O. protearum* / *O. africanum* clade, Fig.2). Possible driving forces of these speciation events may have included host differences and geographical isolation, which will be discussed in more detail below.



**Fig. 2.** One of 70 equally parsimonious trees obtained for analysis of combined ITS and  $\beta$ -tubulin DNA sequence data sets (adopted from Roets *et al.* 2006a, Chapter 5). Values above nodes indicate bootstrap values (1000 replicates) obtained from neighbour-joining analysis obtained with the GTR+I+G parameter model ( $G = 0.5207$ ). Values below nodes indicate parsimony-based bootstrap values (1000 replicates). Values in bold typeface represent confidence values (posterior probabilities as percentage) obtained through Bayesian inference. Species associated with *Protea* spp. are indicated in bold typeface. \* = value below 95 %.

The *Ophiostoma* phylogeny suggests that species associated with *Protea* probably evolved from largely conifer-associated, bark beetle dispersed Northern Hemisphere ancestors (e.g. *O. nigrocarpum* (R.W. Davidson) de Hoog, Fig. 2) (see Farrell *et al.* 2001). This is curious, as bark beetles are not known to be associated with *Protea* species (Myburg *et al.* 1973, 1974, Myburg and Rust 1975a, 1975b, Coetzee and Giliomee 1985, 1987a, 1987b, Roets *et al.* 2006e), and phylogenetically the angiospermous Proteaceae are very distantly related to the conifers (Bowe *et al.* 2000, APG II 2003). In addition, all attempts to find species of *Ophiostoma* from native South African conifers (*Widringtonia* Endl. and *Podocarpus* L'Her. ex Pers. spp.) have failed (Roets/ Wingfield unpublished data). These studies did, however, reveal the presence of unidentified bark beetles species from these Southern Hemisphere conifers. It is possible that more focused studies may yet reveal the presence ophiostomatoid fungi associated with these plants.

The association between the ophiostomatoid fungi (as members of the pyrenomycetes) and conifers is thought to date back to *ca.* 200 million years ago (Berbee and Taylor 2001, Farrell *et al.* 2001). The genus *Ophiostoma* is estimated to be at least 85 million years old (Farrell *et al.* 2001) and the association between *Ophiostoma* and bark beetles is thought to have started at around the same time (Bright and Stock 1982, Berbee and Taylor 1995, Sequeira and Farrell 2001). Bark beetles have switched hosts from coniferous ancestors to angiosperms several times over their evolutionary history, and each switch was accompanied by subsequent species diversification (Farrell *et al.* 2001). The Proteaceae is considered to be one of the oldest living eudicot families on earth (APG II 2003), and the oldest fossilised members of the family have been dated to 108 million years ago (Linder 2003). It would thus have been possible for the Proteaceae-*Ophiostoma* association to have established at around the same time as the establishment of the conifer-*Ophiostoma* association just prior the onset of the Gondwanan breakup (140–180 mya, Cowling 1992, McLoughlin 2001) at a time when the respective host plants may have been in close association (Sanderson *et al.* 2004). The age of the genus *Protea* has been established as between 10 to 36 million years old (Reeves 2001). It is thus likely that organisms associated with members of the Proteaceae have had a very long co-evolutionary history with the family, which has resulted in the species diversification and specific relationships observed today.

## Host relations and geographical distribution of the *Protea*-associated ophiostomatoid fungi

Numerous serotinous South African *Protea* species (Rebelo 1995, Rourke 1998) have been extensively tested for the presence of ophiostomatoid fungi, but only a few of them have been found to accommodate members of *Gondwanamyces* and / or *Ophiostoma*. All of the reported hosts have large infructescences with compactly arranged floral parts, which suggest that infructescence morphology may influence the colonisation potential. Infructescence morphology may influence moisture availability such that small or open-structured infructescences will tend to dry out more rapidly. This, in turn, will influence ophiostomatoid fungal growth negatively, as illustrated by the comparative infructescence-morphology of *P. compacta* R. Br. and *P. neriifolia* R. Br. The two species are phylogenetically closely related (Reeves 2001), their infructescences are of similar sizes and their distribution ranges overlap (Rebelo 1995). Their infructescence structures are, however, quite different, with *P. neriifolia* producing compact structures, while the floral parts are loosely arranged within infructescences of *P. compacta* (despite the misleading species epithet). The presence of three species of ophiostomatoid fungi has been confirmed in *P. neriifolia* infructescences, while repeated surveys of the infructescences of *P. compacta* have failed to reveal any fungal association. Similarly, *Protea repens*, the species with the most compact infructescences in the genus, also houses the largest number (four) of ophiostomatoid fungal species. Interestingly, the infructescences of *P. repens* also house the richest *Protea*-associated arthropod fauna (Roets *et al.* 2006e).

The most commonly encountered fungal species within the infructescences of *P. repens* is *Gondwanamyces proteae*. It is confined to this host (Wingfield *et al.* 1988, Marais and Wingfield 1994, Roets *et al.* 2005) and has a restricted geographical range. This fungus has most commonly been collected from *P. repens* infructescences from the cooler and wetter parts of the range of its host plant (Fig. 3) and occurs from Table Mountain in the west, along the southern coast up to around Port Elizabeth in the east. Several attempts to isolate *G. proteae* from infructescences collected from the drier northern parts of the *P.*



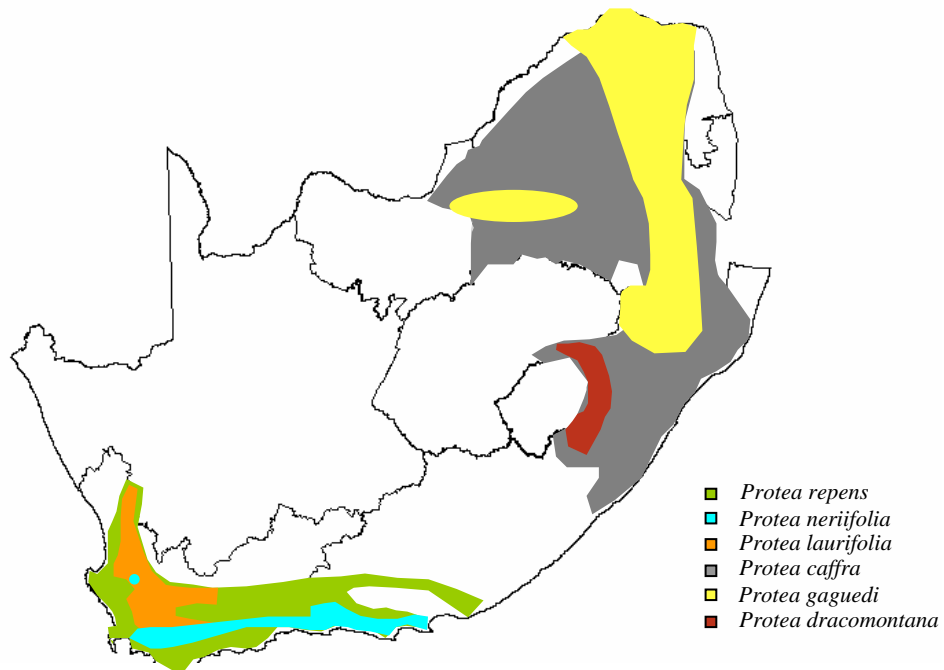
*repens* distribution range (Fig. 3) have failed. Similarly, attempts to isolate *G. capensis*, a species with a much wider host range (including *P. neriifolia*, *P. laurifolia* Thunb., *P. longifolia* Andrews, *P. burchellii* Stapf, *P. coronata* Lam., *P. magnifica* Link, *P. lepidocarpodendron* (L.) L.) from these areas have also failed (Roets, unpublished data). The geographical location of the host plants, coupled with the availability of moisture within infructescences, thus appears to limit the natural distribution of *Gondwanamyces* species. Unlike *Ophiostoma*, *Gondwanamyces* species have never been reported from *Protea* hosts in the northeastern parts of South Africa (Fig. 3), but seem to be confined to the floristically unique Fynbos biome of the Western Cape Province in South Africa.

Similar to the case with *Gondwanamyces*, *Ophiostoma* species associated with *Protea* show varying degrees of host specificity. Thus *O. palmiculminatum* is similar to *Gondwanamyces proteae* in having been found only in *P. repens* (Roets *et al.* 2006b, Chapter 3), while other species (e.g. *O. splendens*) have been collected from various *Protea* hosts including *P. neriifolia*, *P. laurifolia*, *P. longifolia*, *P. burchellii*, *P. repens* and *P. lepidocarpodendron* (Marais and Wingfield 1994, Roets *et al.* 2005). These *Protea* species are closely related (except for *P. repens*) (Rebelo 1995, Rourke 1998, Reeves 2001) and it is thus not surprising that they host similar fungal communities. The host range of *O. splendens* does, however, still need to be confirmed as surveys conducted by Roets *et al.* (2006b, Chapter 3) failed to reveal the presence of this species on any non-*P. repens* hosts other than *P. neriifolia*.

*Ophiostoma palmiculminatum* has been collected only from an isolated population of *P. repens* consisting of less than 1000 individual plants (J. S. Marais Park, Stellenbosch, Roets *et al.* 2006b, Chapter 3). Similarly, its sister species *O. gemellus* has been collected from a small population of *P. caffra* in the Gauteng Province only (W. Sisulu Botanical Garden, Roets *et al.* 2006a, Chapter 5). These two species may, however, have wider distributions, as their host species have amongst the widest distribution ranges of all South African *Protea* species (Fig. 3). Further surveys in the ranges of these two fungi are thus required.

*Ophiostoma gemellus*, *O. protearum* and *O. africanum* are associated with *Protea* species restricted to the north- and eastern parts of South Africa (Marais and Wingfield 1997, 2001) (Fig. 3). *Ophiostoma protearum* is confined to *P. caffra* Meisn., while *O. africanum* has been isolated from *P. gagedi* J.F. Gmel., *P. dracumontana* Beard and *P. caffra* (Marais and Wingfield 1997, 2001, Roets *et al.* 2006b, Chapter 3). The distribution range of *P. caffra* overlaps with that of *P. gagedi* and *P. dracumontana* (Fig. 3) within South Africa, but the ranges of the former two *Protea* species are also known to extend north of the South African borders (Rebelo 1995). It is very likely that *O. protearum* and *O. africanum* follow these distribution patterns.

*Sporothrix variecibatus* is associated with the infructescences of two *Protea* species (*P. repens* and *P. longifolia*) and with a non-native *Eucalyptus* L'Her. sp. (Roets *et al.* 2006a, Chapter 5). The isolates of this species from *P. repens* and the *Eucalyptus* sp. were obtained from host populations that occur close together (Stellenbosch region, Roets *et al.* 2006a, Chapter 5), while the isolate from *P. longifolia* were obtained from a natural host population in the Kleinmond district, *ca.* 100 km from the Stellenbosch site. No *Eucalyptus* spp. were observed growing in close proximity to the *P. longifolia* population site (Roets *et al.* 2006a, Chapter 5). It may thus be concluded that this species jumped hosts from native *Protea* species to the exotic *Eucalyptus* sp. in the Stellenbosch region. More thorough surveys are needed to clarify these preliminary observations.

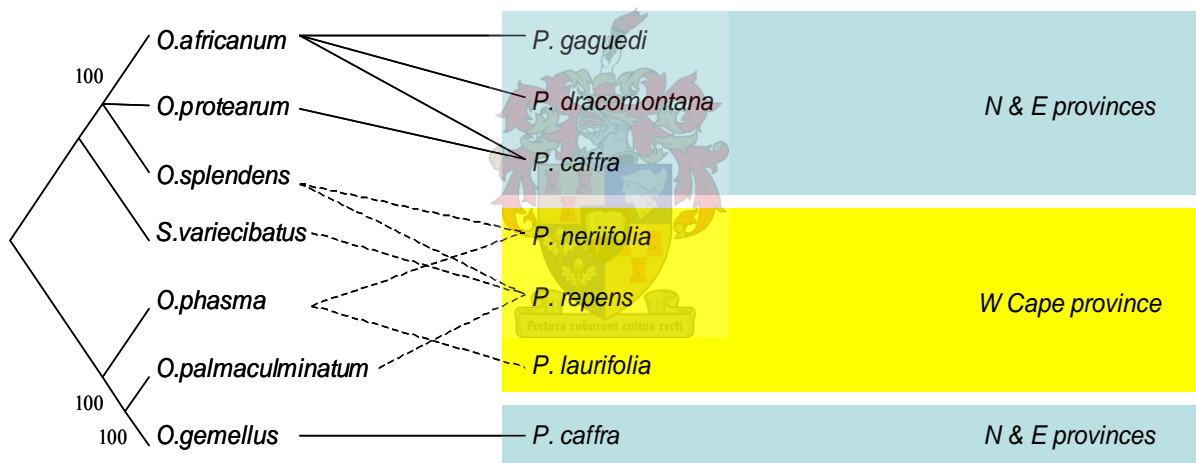


**Fig. 3.** Distribution of some South African *Protea* spp. (after Rebelo 1995) that have confirmed relationships with ophiostomatoid fungi.



A relatively extensive diversity of ophiostomatoid fungi has now been isolated from serotinous *Protea* species since the first discovery of these fungi approximately 20 years ago. There are, however, some serotinous *Protea* species that have not been inspected for these intriguing fungi. When the distributions of the *Protea* hosts are plotted on the *Ophiostoma* phylogeny (Fig. 4), it is evident that the geographical isolation of host plants resulted in speciation in at least two of the *Protea*-associated *Ophiostoma* lineages (*O. palmiculminatum*/*O. gemellus* lineage and *O. splendens*/*O. protearum*/*O. africanum* lineage). Geographical distribution patterns of host species thus appear to have markedly

influenced *Ophiostoma* species richness. Future surveys should, therefore, focus on both unexplored hosts and collections from known hosts over a wider and more representative geographical range. Studying the fungal communities in the infructescences of *Protea* species from tropical Africa would be particularly interesting and would most likely provide further interesting discoveries. We are quite confident that additional *Protea*-associated ophiostomatoid species await discovery and that these will be ecologically and biogeographically interesting.



**Fig. 4.** Diagram showing confirmed relationships between the *Protea*-associated *Ophiostoma* spp. and their hosts. Different colours indicate different regions within South Africa where the host plants are found. The phylogramme was obtained by 1000 bootstrap replicates of the neighbour-joining algorithm in PAUP v. 4.0 beta 10 (Swofford 2000) using partial ITS data of the ex-type cultures of the represented species where possible.

**Key to the species of ophiostomatoid fungi associated with *Protea* infructescences based on teleomorph structures (modified from Marais and Wingfield (2001)).**

- |    |   |   |
|----|---|---|
| 1  | Ascoma neck shorter than 70 $\mu\text{m}$<br>Ascoma neck longer than 70 $\mu\text{m}$                     | <i>Ophiostoma phasma</i><br>2                                   |
| 2  | Ornamented ascomatal base<br>Ornamentation of ascomatal base lacking                                      | 3<br>9  |
| 3  | Ostiolar hyphae longer than 10 $\mu\text{m}$<br>Ostiolar hyphae shorter than 10 $\mu\text{m}$ or lacking  | 4<br>6  |
| 4  | Associated with <i>Protea repens</i><br>Associated with <i>Protea</i> species other than <i>P. repens</i> | <i>Ophiostoma palmiculminatum</i><br>5                          |
| 5  | Ostiolar hyphae longer than 20 $\mu\text{m}$<br>Ostiolar hyphae shorter than 20 $\mu\text{m}$             | <i>Ophiostoma gemellus</i><br><i>Ophiostoma protearum</i>       |
| 6  | Ascospores alantoid<br>Ascospores falcate   | 7<br><i>Gondwanamyces proteae</i>                               |
| 7  | Ascomatal neck shorter than 200 $\mu\text{m}$<br>Ascomatal neck longer than 200 $\mu\text{m}$             | <i>Gondwanamyces capensis</i><br>8                              |
| 8  | Associated with <i>Protea repens</i><br>Associated with <i>Protea caffra</i>                              | <i>Ophiostoma palmiculminatum</i><br><i>Ophiostoma gemellus</i> |
| 9  | Ascospores longer than 5.5 $\mu\text{m}$<br>Ascospores shorter than 5.5 $\mu\text{m}$                     | <i>Ophiostoma splendens</i><br>10                               |
| 10 | Ostiolar hyphae shorter than 20 $\mu\text{m}$<br>Ostiolar hyphae longer than 20 $\mu\text{m}$             | <i>Ophiostoma africanum</i><br>9                                |
| 9  | Associated with <i>Protea repens</i><br>Associated with <i>Protea caffra</i>                              | <i>Ophiostoma palmiculminatum</i><br><i>Ophiostoma gemellus</i> |



## Ecology of ophiostomatoid fungi associated with *Protea* infructescences

From an ecological perspective, the infructescences of *Protea* species represent an unusual habitat for ophiostomatoid fungi to colonise. Similarly, from a phylogenetic perspective the association of these fungi with the Proteaceae is unique, as the original plant hosts of the ophiostomatoid fungi are likely to have been conifers, a plant group phylogenetically very distantly related to Proteaceae (Bowe *et al.* 2000, APG II 2003). It may be argued that a host jump between these two very different host plants would have required large physiological adaptation, as the chemical composition of the plants would probably differ markedly (Jörg *et al.* 1998). Successful host jumping would, however, have brought about tremendous competitive advantages over the ancestral species, as interspecies competition would be minimised.

The unique nature of the *Protea*-infructescences habitat is illustrated by the clear differences between the fungal species that colonise them and the fungi associated with the rest of the plant (Wingfield *et al.* 1994, Lee *et al.* 2003, Crous *et al.* 2004). The micro-ecological differences between these microhabitats can be used to explain the different fungal compositions on these different plant parts. The infructescences form a closed, moist environment, dissimilar to any other plant part, in which many fungal species can prevail. The infructescences also provide a suitable substrate (dead floral parts) for the growth of saprobic fungal species.

In addition to advantages related to protection and substrate availability, the infructescences provide the fungal species that colonise them protection against the fires that frequent the Cape Floristic region. Although the fungi within the infructescences will survive fires, they would need to be dispersed soon thereafter, since fire promotes the opening of infructescences to release the enclosed seeds (Bond 1985). Given that it takes at least 3 years after a fire before a subsequent generation of *Protea* spp. produce new infructescences (le Maitre and Midgley 1992), this dispersal dilemma becomes even more evident.

The same conditions that promote fungal growth within *Protea* infructescences are also believed to promote the colonisation of these structures by numerous species of arthropods. In this sense, the infructescences may be considered as mini-ecosystems with different trophic levels (Zwölfer 1979). The fungi form one of the basal levels of the within-infructescence ecosystem. Fungal feeding arthropods such as mites and psocopterans constitute a subsequent level, while their predators represent the top trophic level. The rich diversity of arthropods within infructescences also ensures that there are many potential vectors available for long distance dissemination of fungal reproductive propagules.

### *Spore dispersal*

As is the case for other wood inhabiting fungi, the sheltered nature of *Protea* infructescences present the fungal species that colonise them with a dispersal dilemma. It is necessary to assure dispersal of reproductive propagules from an environment where dispersal via air currents or water-splash is ineffective. To overcome this problem the ophiostomatoid fungi have evidently evolved mechanisms (morphological adaptations including long necks and sticky spore drops) to promote vectored dispersal of their spores (Malloch and Blackwell 1993, Cassar and Blackwell 1996). In this regard arthropods and bark beetles in particular, play a central role in the dissemination of ophiostomatoid fungal spores (Barras and Perry 1975, Upadhyay 1981, Price *et al.* 1992, Wingfield *et al.* 1993, Cassar and Blackwell 1996, Paine *et al.*, 1997, Klepzig *et al.* 2001b, Klepzig and Six 2004). These arthropods may even form mutualistic associations with the fungi they disperse (Francke-Grosmann 1967, Norris 1979, Whitney 1982, Beaver 1989, Berryman 1989, Jacobs and Wingfield 2001). Other known vector beetles include members of the Cerambycidae, Curculionidae and Nitidulidae (Upadhyay 1981, Harrington 1987, Wingfield *et al.* 1993, Jacobs and Wingfield 2001, Jacobs *et al.* 2003).

Apart from beetles, mites have also been shown to be critical in the dissemination of spores of ophiostomatoid fungi (Bridges and Moser 1983, 1986, Lévieux *et al.* 1989, Moser *et al.* 1989, Moser 1997, Klepzig *et al.* 2001a, 2001b). The association of

*Tarsonemus* Canestrini & Fonzago (Tarsonemidae) spp. with bark beetles and ophiostomatoid fungi, in particular, has received much attention (Moser and Roton 1971, Smiley and Moser 1974, Moser 1976b, Bridges and Moser 1983, Moser and Bridges 1986). These mites possess specialised spore-carrying structures (sporothecae) that have been shown to contain spores of ophiostomatoid fungi (Moser 1985, Moser *et al.* 1995). The association between the mites and ophiostomatoid fungi may also be mutualistic, as the mites can reproduce on a diet consisting solely of *Ophiostoma* spp. (Klepzig *et al.* 2001b).

Like fungal spores, mites have been shown to disperse phoretically via insect vectors. In one example, about 14 species of mites, including amongst others the ophiostomatoid-vectoring *Tarsonemus* spp., have been shown to be phoretic on *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae) (Moser and Roton 1971, Moser 1976a). This hyperphoretic dispersal of fungal propagules via phoretic mites is not unique to the bark beetle-associated fungi, but has also been reported in various other systems. For example, the dispersal of *Pyxidiophora* Bref. & Tav. and its *Thaxteriola* Speg. and *Acariniola* Maj. & Wiśn. anamorphs are achieved by mites phoretic on among others, bark beetles (Blackwell *et al.* 1986, 1988, Blackwell and Malloch 1989). Mites in the family Scutacaridae that are phoretic on wasps and wild bees have also been shown to carry fungal spores in specialised structures (Ebermann and Hall 2003). Other mite families bearing similar structures include the Trochometridiidae and Siteroptiidae (Suski 1973, Lindquist 1985, Kemp *et al.* 1996). The role of mites in the dissemination of fungal spores is clearly relatively unexplored, extending to various environments and it may be a common phenomenon.

The discovery of ophiostomatoid fungi within *Protea* infructescences sparked intense interest in their mode of dispersal. The possible role of arthropods in the dissemination of these spores and how this system would compare to the conifer-based system is of particular interest. One of the problems relating to understanding the possible role of arthropods in the dispersal of these fungi is that they are relatively slow-growing and arthropods are covered with fast-growing mold spores as well as bacteria. Thus, direct



isolations from the insects without appropriate selective media would most likely not be feasible. With this in mind, Roets *et al.* (2006f, Chapter 2) developed taxon-specific primers for the detection of small quantities of DNA from *Protea* associated *Ophiostoma* and *Gondwanamyces* species. These methods led to the discovery of three putative vector insects (*Genuchus hottentottus* F.: Coleoptera, *Oxycarenus maculatus* Stal: Lygaeidae and a psocopteran species). Although this discovery was very promising, the low frequency of ophiostomatoid fungal DNA detected on these arthropods, despite the observed high colonisation rates of these fungi, was difficult to interpret or explain (Roets *et al.* 2005, 2006c, 2006f, Chapters 2 and 4).

Subsequent studies focused on the dispersal of the *Ophiostoma* spp. associated with *Protea* infructescences (Roets *et al.* 2006c, Chapter 4) and led to the identification of mites (members of the genera *Tarsonemus*, *Proctolaelaps* Berlese and *Oodinychus* Berlese) as potential spore vectors. Spores of ophiostomatoid fungi were identified on the mites both through direct isolation and visual detection with the aid of a scanning electron microscope. Based on the abundance of mites within *Protea* infructescences and the high frequency of individuals carrying *Ophiostoma* spores, it was concluded that mites play a primary role in the dispersal of *Ophiostoma* spores (Roets *et al.* 2006c, Chapter 4). The most prominent mite, *Oodinychus* sp., was then also shown to be able to transfer *O. splendens* from *Ophiostoma* colonised infructescences to sterilised plant material. Interestingly, these mites did not transfer any *Ophiostoma* spores to Petri dishes containing only malt extract agar. The mites may thus be involved in the transfer of fungal spores from one specific substrate to the next, suggesting high levels of specificity within the system. The transfer of fungal spores to uncolonised material by the mites *Tarsonemus* sp. and *P. vandenbergi* Ryke has not yet been investigated.

Studies on the dispersal mechanisms of the *Ophiostoma*-vectoring mites led to the discovery of phoretic behavior in all three mite genera that have been found associated with these fungi. The main mite vectors of *Ophiostoma* spp. in *Protea* infructescences include *Tarsonemus*, *Proctolaelaps* and *Oodinychus*. These were found to be phoretic on the beetles *G. hottentottus* F., *Trichostetha capensis* L. and *T. fascicularis* L. (Coleoptera:

Scarabaedae: Cetoniinae) (Roets *et al.* 2006d, Chapter 6). As the mites do not disperse via air currents (Roets *et al.* 2006d, Chapter 6), it was deduced that beetle-mediated hyperphoretic dispersal provides the only means of long-distance dispersal for the spores of these fungal species. Long-distance dispersal is especially important for the *Protea* associated *Ophiostoma* species, as the host plants grow within fire prone habitats (van Wilgen 1981, 1987). Interestingly, like species of *Tarsonemus*, mite species in the genera *Proctolaelaps* and *Oodinychus* have also been found to be phoretic on the bark beetle *Dendroctonus frontalis* (Kinn 1976).

Vector dispersal is evidently extremely effective in the case of the *Protea*-associated ophiostomatoid fungi, as these fungi have been observed within infructescences of *Protea* plants after the first flowering season after a fire (Roets *et al.* 2006d, Chapter 6). Short-distance dispersal of the mites that have been found to carry the fungi is apparently achieved by migration between infructescences on the same or neighbouring plants (Roets *et al.* 2006d, Chapter 6).

#### *Life cycle of O. splendens and O. phasma*

The increased understanding of the biology of *Ophiostoma* spp. in *Protea* infructescences has made it possible to establish a tentative life cycle of *O. splendens* and *O. phasma* associated with *Protea* infructescences (Fig. 4). Mites acquire spores when moving within fungal colonised infructescences. Roets *et al.* (2006d, Chapter 6) demonstrated that the mites will disperse from desiccating infructescences to more moist and sheltered areas as provided by intact infructescences and inflorescences on the same or surrounding *Protea* plants. In the field, desiccating conditions will be experienced during the warmer and rain-free spring and summer months. This will cause infructescences that experienced extensive insect damage during the winter to open and release their seeds. Mites probably acquire most fungal spores during ophiostomatoid fungal sporulation peaks that occur during winter (Roets *et al.* 2005). Short distance dispersal of these fungi probably occurs when mites flee these desiccating conditions and carry the spores to undamaged infructescences. (Fig. 4, stage 1). The *Tarsonemus* spp. and *P. vanderbergi*

are thought not only to move between infructescences, but also from infructescences to flower heads (inflorescences) because of their phoretic association with adult *Trichostetha* spp. beetles (Roets *et al.* 2006d, Chapter 6) (Fig. 4, stage 2). These beetles are exclusively associated with the flowers of *Protea* spp. (Holm and Marais 1992). Mites belonging to the *Oodinychus* sp. are thought to be restricted to infructescences, and never to move to inflorescences, as no individuals have ever been encountered within an inflorescence (unpublished data).

Stage three in the proposed life cycle of *O. splendens* and *O. phasma* (Fig. 4) is initiated during the flowering season of the *Protea* spp. The peak in flowering time for the ophiostomatoid-colonised *Protea* species coincide with the peak in fungal colonisation times during winter and early spring (Roets *et al.* 2005). In addition, the three *Ophiostoma*-vectoring mite species have only been collected from beetles (*G. hottentottus*, *T. fascicularis* and *T. capensis*) that feed on nectar and pollen as adults (Holm and Marais 1992, Roets *et al.* 2006d, Chapter 6). The main fungal sporulation times and activity of the secondary vectors thus also coincide. The mites (primary vectors) show phoretic readiness during this time (Roets *et al.* 2006d, Chapter 6), but it is not known whether they are more abundant during this time.

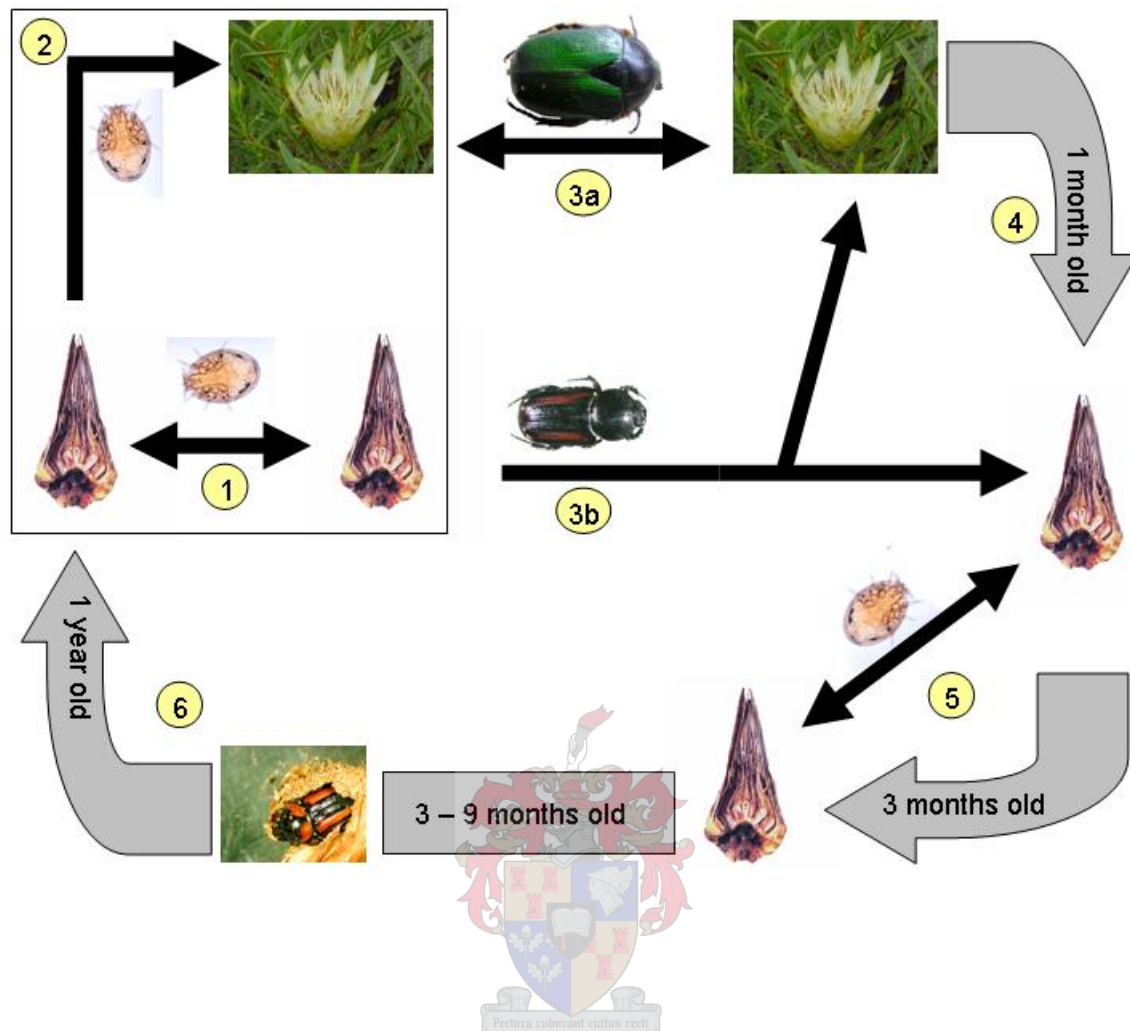


Mites differ in their associations with the various vectors. The *Oodinychus* sp. is phoretic only on *G. hottentottus*, while the other mite species have been collected from all three beetle species (Roets *et al.* 2006d, Chapter 6). This may largely be ascribed to the apparent inability of the *Oodinychus* sp. to move from infructescences to flower heads where they would encounter beetles belonging to the genus *Trichostetha*. Mites phoretic on *Trichostetha* sp. would be dispersed only between inflorescences, as these beetles are not associated with the infructescences of *Protea* spp. (Coetzee and Giliomee 1985, 1987a, 1987b) (Fig. 4, stage 3a). Mites that are phoretic on *G. hottentottus*, in contrast, may be carried to either inflorescences, where the beetles feed on nectar and pollen, or to young infructescences within which they lay their eggs (Coetzee and Giliomee 1987b) (Fig. 4, stage 3b).

Ophiostomatoid fungi become prominent within infructescences that are *ca.* 2–3 months old (Roets *et al.* 2005) (Fig. 4, stages 4 and 5). This developmental time coincides with the time needed for the *in vitro* formation of fully developed fertile ascomata after *O. splendens* inoculation on autoclaved flower parts (Marais and Wingfield 1994). At this stage mites can again acquire and transport spores between infructescences and inflorescences, both through short-distance self-dispersal and via longer-distance beetle-aided dispersal. This cycle (Fig 4, stags 1-5) could then be repeated throughout the flowering period of the *Protea* hosts.

Dispersal of spores of *Ophiostoma* spp. during the non-flowering stages of the host plants is probably restricted to short distance dispersal by mites. Mites have been shown to carry spores of *Ophiostoma* species during these non-flowering periods (Roets *et al.* 2006c, 2006d, Chapters 4 and 6), a time when the beetles are inactive (Coetzee and Giliomee 1985, 1987a, 1987b). During these stages, *G. hottentottus* larvae feed on the styles and seed within infructescences (Coetzee and Giliomee 1987b). Interestingly, *Oodinychus* sp., *Tarsonemus* spp. and *P. vanderbergi* are concentrated in areas where the beetle larvae feed actively. At maturity the larvae construct ovoid chambers from plant debris in which they pupate (Coetzee and Giliomee 1987b, Fig. 4, stage 6). The adult beetles emerge from the infructescences at the onset of the next flowering season and carry the phoretic *Ophiostoma*-vectoring mites to uncolonised sites. As the beetles emerge, they too would come into contact with sporulating ascomata and may thus also be involved in the dispersal of *Protea*-associated *Ophiostoma* species.

Although not yet adequately studied, we suspect that *Gondwanamyces* spp. share the vectored-mode of dispersal with *Protea*-associated *Ophiostoma* species. *Ophiostoma* and *Gondwanamyces* species sporulate simultaneously and share similar colonisation numbers within *Protea* infructescences (Roets *et al.* 2005). *Gondwanamyces* species colonise the more loosely arranged upper areas of the styles of *Protea* spp. (Marais 1996), suggesting associations with larger arthropods such as *G. hottentottus* (Roets *et al.* 2006d, Chapter 6).



**Fig. 4.** Proposed life cycle of *O. splendens* and *O. phasma* on *Protea* spp. Mites acquire spores when moving and feeding within *Ophiostoma* spp. colonised infructescences. 1. *Oodiniychus* sp., *P. vanderbergi* and *Tarsonemus* sp. migrating between infructescences. 2. *P. vanderbergi* and *Tarsonemus* sp. migrating from infructescences to open flowers. 3a. *P. vanderbergi* and *Tarsonemus* sp. hitching a ride on *Trichostetha* spp. to open flowers. 3b. *Oodiniychus* sp., *P. vanderbergi* and *Tarsonemus* sp. hitching a ride on *G. hottentottus* to open flowers or infructescences. 5. Mites migrating between infructescences throughout the year. Grey arrows. Development of *G. hottentottus* to adulthood over *ca.* 1 year, to emerge again during the next *Protea* flowering season.

### *Fungus-Protea associations*

The association between ophiostomatoid fungi and their *Protea* host species are thought to be non-destructive. The host would thus provide the fungi with a suitable habitat, while the fungi appear not to affect the host adversely. Except for *O. palmiculminatum*, all species colonise and are confined to dead floral substrates within the infructescences. *O. palmiculminatum* has been collected from supposedly living basal parts of the infructescences of *P. repens* (Roets *et al.* 2006b, Chapter 3). This species is still considered to be saprophytic, as in all instances the infructescence bases from which it has been isolated had been hollowed and damaged by boring insects, such that they appear to comprise of dead tissue only.

As saprobes, the ophiostomatoid fungi probably only have an indirect effect on *Protea* ecology. Ophiostomatoid fungi are the dominant fungi found in the *Protea* infructescences. This led Marais (1996) to suggest that they exclude other fungal species from colonising infructescences, which suggests that they may be beneficial to their *Protea* hosts. In contrast, infructescences without ophiostomatoid fungi contain a large concentration of many different fungal species (Lee *et al.* 2005), some of which (e.g. *Penicillium* spp.) may be detrimental to the host, possibly affecting the viability of seeds or the sustainability of the infructescences until a time when seed might best be dispersed.

### *Fungus-Mite associations*

*Protea* infructescences provide the *Ophiostoma*-vectoring mites with a moist and sheltered habitat in which to flourish. Nothing is known regarding the effect of the mites on their *Protea* hosts, but as they are probably non-phytophagous the mites are not considered to be damaging (Coetzee *et al.* 1986). *Proctolaelaps vandenbergi* is thought to feed on *Protea* pollen and nectar (Coetzee *et al.* 1986), but is also often present in the infructescences at times when nectar and pollen is absent. This mite must, therefore, be able to supplement its diet with other sources such as detritus and / or fungi. Similar to *P. vandenbergi*, the *Tarsonemus* spp. and *Oodinychus* sp. may feed on various substrates.

The association between the *Ophiostoma*-vectoring mites and the fungi they vector reveals strong symbiotic relationships between these organismal groups. The obvious benefit to the fungi is that they are dispersed to uncolonised sites that are unreachable by other means. The benefit to the mites is less obvious, and has been studied in detail in the association between *O. splendens*, *O. palmiculminatum*, *O. phasma* and the *Oodinychus* sp. mites only. The population growth of this mite was found to be significantly higher when feeding on these fungal species, than when feeding on other fungal species common to *Protea* infructescences (Roets *et al.* 2006c, Chapter 4). The association between this mite and *O. splendens* is therefore assumed to be mutualistic. *Oodinychus* mites may, in addition, also feed on detritus and / or other fungal species, as they have been observed within infructescences that are apparently devoid of *Ophiostoma* species (Roets, pers. observ.). The extent to which *Oodinychus* sp. mites depend on the fungi they vector is thus still unknown.

As is the case for the conifer-associated *Tarsonemus* spp., the interaction between *Tarsonemus* spp. and their associated *Protea-Ophiostoma* fungi may also be mutualistic. The possibility of strong mutualistic interactions between *Tarsonemus*, the *Oodinychus* sp. and *Protea* associated *Ophiostoma* spp. are supported by the presence of specialised spore-carrying structures on both of these mite species (Roets *et al.* 2006c, Chapter 4). In the case of *Oodinychus* sp., these structures commonly contain spores of *Ophiostoma* spp. (Roets *et al.* 2006c, Chapter 4). The conidia observed within the sporothecae of the *Tarsonemus* sp. have not yet been identified, but it is highly likely that *Ophiostoma* species will be carried within these structures (Roets *et al.* 2006c, Chapter 4). This is substantiated by the morphological similarities of these structures in the conifer- and *Protea*-associated *Tarsonemus* spp. (Moser 1985) and the frequent isolation of *Protea*-associated *Ophiostoma* spp. from these mites (Roets *et al.* 2006c, 2006d, Chapters 4 and 6). The interpretation of these associations as mutualistic is substantiated by the maintenance of such similar processes in both the conifer- and *Protea-Ophiostoma* systems over extended evolutionary times.

The evolution of sporothecae indicates a long and specific association between the mites and fungi. Interestingly, the conifer-associated *Ophiostoma minus* (Hedgc.) Syd. & P. Syd. is now considered to be primarily dispersed by *Tarsonemus* spp. mites and not the bark beetles in the galleries of which these fungi thrive (Lombardero *et al.* 2000, Klepzig *et al.* 2001a, 2001b, Lombardero *et al.* 2003). The absence of bark beetles (or their close relatives) from *Protea* also suggests that mites play a primary role in the dispersal of the *Protea*-associated *Ophiostoma* species and that they may have been involved in the initial transfer of *Ophiostoma* spores between conifers and members of the Proteaceae. Additional studies on the interactions between mites and various *Ophiostoma* species are, however, still needed to corroborate this.

#### *Beetle-Protea associations*

The interactions between the mite-vectoring beetles (*G. hottentottus*, *T. capensis* and *T. fascicularis*) and the different *Protea* spp. vary. *Trichostetha* species are important in the pollination of various *Protea* species. (Mybyrg *et al.* 1973). Like the *Trichostetha* spp., adult *G. hottentottus* beetles feed on nectar and pollen of various *Protea* spp. and in the process act as their pollinators. Adult *G. hottentottus* individuals are thus beneficial to their hosts. The larvae of *Trichostetha* spp. are thought to be associates in ant or termite nests and thus not damaging to proteas (Holm and Marais 1992), while the larval *G. hottentottus* injure their *Protea* hosts. They bore into the infructescences and mainly feed on the enclosed seeds (Coetzee and Giliomee 1987b). These boring activities may trigger premature seed release due to the damage to the involucrel receptacles of the infructescences (Bond 1985, Cowling 1992).

#### *Fungus-Beetle associations*

Other than acting as secondary fungal vectors, *Trichostetha* spp. are unlikely to have further association with the *Protea-Ophiostoma* species, as they never come into direct contact with the spore-bearing structures of the fungi in the infructescences. Conversely, *G. hottentottus* beetles may be more closely linked to these fungi. Their larvae are commonly found feeding within ophiostomatoid fungus-colonised infructescences, and



mature ascomata of these fungi can be found on their faeces (pers. observ.). The beetles and the fungi are thus found in close proximity with one another and would undoubtedly either directly or indirectly influence one another. In addition, DNA of both *Ophiostoma* spp. and *Gondwanamyces* spp. has been isolated from these beetles (Roets *et al.* 2006f, 2006c, Chapters 2 and 4). Studies on the interaction between *G. hottentottus* and the ophiostomatoid fungi have unfortunately thus far been severely hampered by extensive yeast contamination (Roets *et al.* 2006c, Chapter 4).

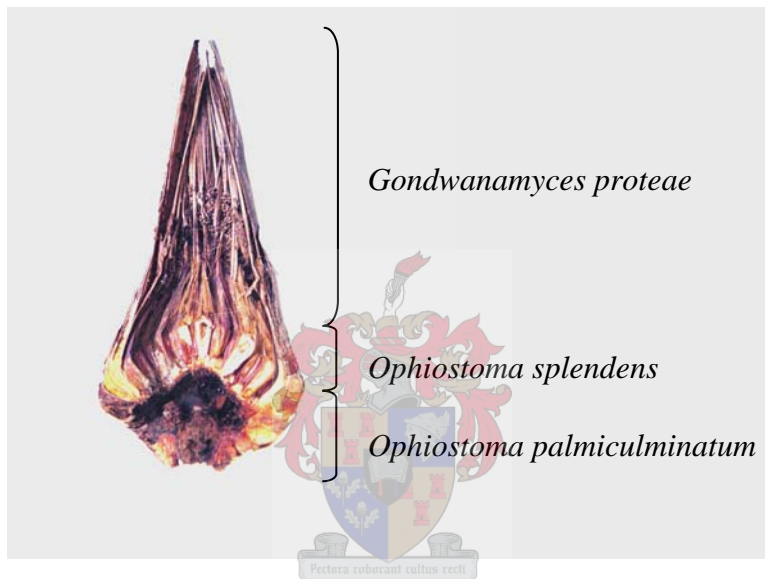
### *Mite-Beetle associations*

As far as is known the mites, *P. vanderbergi*, *Tarsonemus* spp. and *Oodinychus* sp. do not injure the beetles that carry them. They merely use the beetles for transport from one host to the next. The extent of the association between these organisms is thus unlikely to be more than a commensalism.

### *Competition*

*Protea* infructescences harbour a wealth of fungal and arthropod species (Myburg *et al.* 1973, 1974, Myburg and Rust 1975a, 1975b, Coetzee and Giliomee 1985, 1987a, 1987b, Marais and Wingfield 1994, Lee *et al.* 2003, 2005). Most of these effectively compete for the same resources (space and food) within this limited niche. Succession of species may be an important way in which to avoid competition, but very few succession studies have been undertaken on the ophiostomatoid fungi (Bramble and Holst 1940, Käärrik 1975, Solheim 1992a, 1992b). For these species to co-exist, one would expect the presence of small-scale niche differentiation within infructescences. Spatial separation of ophiostomatoid fungi growing and sporulating simultaneously (Roets, pers observ.) may present one such an example. Such separation is especially evident between the ophiostomatoid fungi associated with *P. repens*. Figure 6 depicts a cross section of a mature infructescence (*ca.* 1-y-old) of *P. repens* after colonisation by insect borers and ophiostomatoid fungi. *Gondwanamyces proteae* is often restricted to the regions of the styles, while *O. splendens* almost exclusively resides at the base of the styles and on the

*Protea* seed coats. *O. palmiculminatum* has been collected only from the insect- damaged involucrel receptacle of *P. repens*, and thus occurs even lower down in the same infructescence. These very similar species may thus co-exist successfully within such a limited space due to niche differentiation.

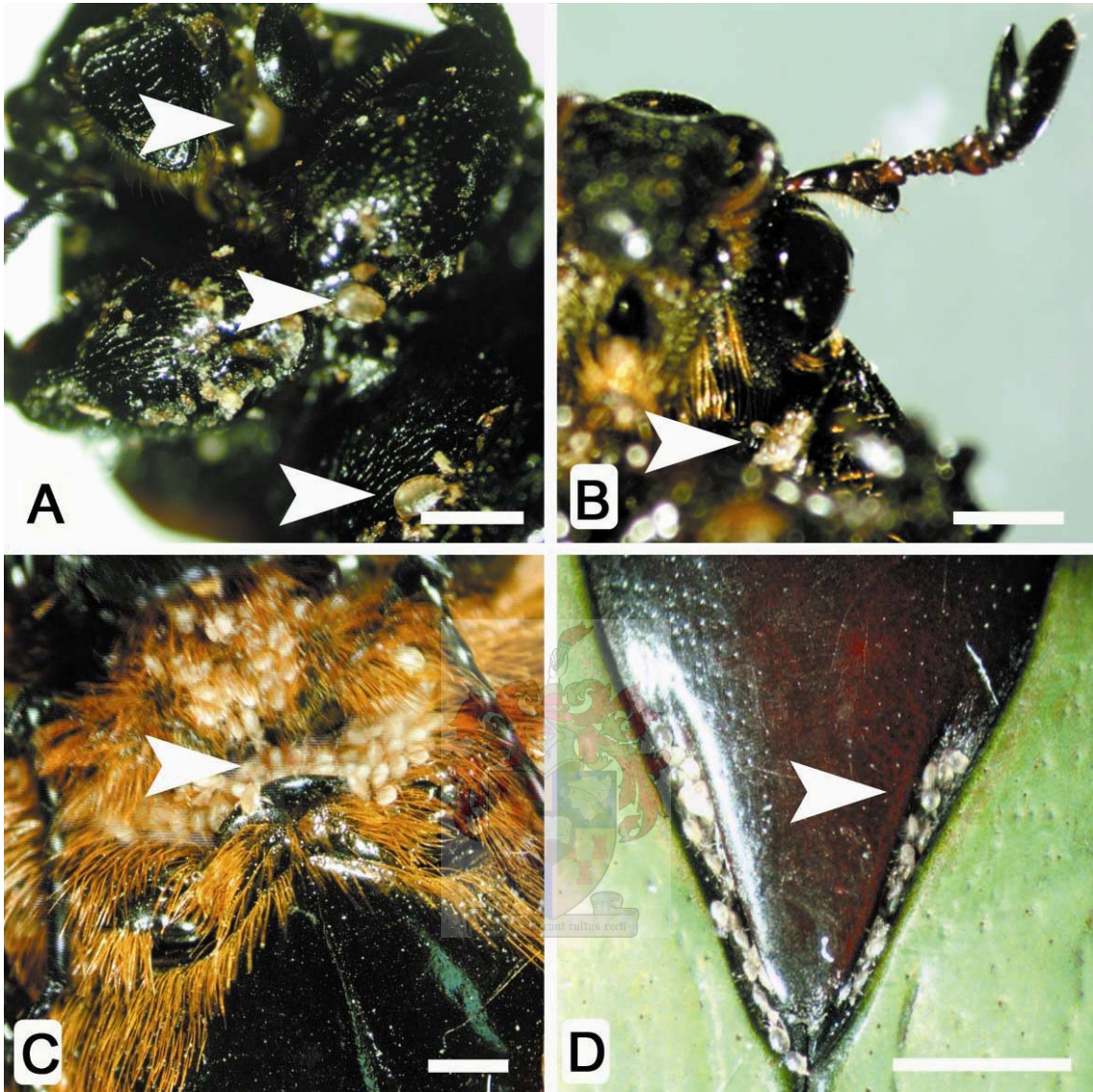


**Fig. 5.** Cross section of *Protea repens* infructescence (ca. 1-year old) showing colonisation zones of three *Protea*-associated ophiostomatoid fungi.

The different niches that the various ophiostomatoid fungi colonise appear to correlate with the morphology of the respective fungal species. The basal portion of a *P. repens* infructescence, in which the seeds are located, is more compact than the upper portions in which the styles are more loosely arranged (Fig. 5). Correspondingly, the size and length of the ascomata necks of fungi occupying the upper regions (*G. proteae*) is generally much larger than of fungi occupying the lower regions (*O. splendens*) within these

infructescences. In addition, the necks of *G. proteae* are usually straight (rarely slightly curved), while the necks of *O. splendens* are usually distorted. This distortion is required, as the spaces within which this fungus grows are inordinately small to accommodate extended necks. The ascomatal necks of *O. palmiculminatum*, in contrast, are exceptionally long when compared to other *Protea*-associated ophiostomatoid fungi (Roets *et al.* 2006b, Chapter 3, Fig. 1). This correlates well with the niche occupied by this species, which is comprised of large cavities in the involucral receptacles created by boring insects (Fig. 5).

Niche differentiation is not restricted to the areas within infructescences that are colonised by different ophiostomatoid fungi (Fig. 5), but it is also evident on the surface of the beetles onto which the different mites attach when vectored. On *G. hottentottus*, mites mostly occur on the ventral side of the beetle, while *Oodinychus* sp. and to a lesser extent *P. vanderbergi* cling to the area surrounding the base of the anterior legs of the beetles (Roets *et al.* 2006e, Chapter 6). These two species are also sometimes found in the cavity between the head and prosternum of the beetle (Fig. 6A). When *Tarsonemus* spp. are present (usually in numbers exceeding 30 individuals) they always occupy the head-prosternum cavity and exclude other mites from these areas (Fig. 6B). On *Trichostetha* spp., *P. vanderbergi* individuals are found clinging to the hairs on the ventral sides of the beetles (Fig. 6C). When present *Tarsonemus* spp. are almost without exception found within the groove formed by the scutellum and elytra or underneath the elytra (Fig. 6D). This distribution is similar to that observed for *Tarsonemus krantzi* individuals that congregate either near the cupped area where the elytra attach to the body or underneath the elytra of the bark beetle *Dendroctonus frontalis* (Moser 1976b). Small-scale niche differentiation probably reduces competition for space on the limited surface area of the beetles.



**Fig. 6.** Mites phoretic on *G. hottentottus* and *T. fascicularis*. A. *Oodinychus* sp. mites on the ventral side of *G. hottentottus*. B. *Tarsonemus* sp. mites occupying the head-prosternum cavity on the ventral side of *G. hottentottus*. C. *P. vandenbergi* mites on the hairy ventral side of *T. fascicularis*. D. *Tarsonemus* sp. mites within the groove formed by the scutellum and elytra of *T. fascicularis*.

Differential host species dependence would undoubtedly also aid the avoidance of competition between the various ophiostomatoid fungi from *Protea* infructescences. As has been noted above, some ophiostomatoid fungi associated with *Protea* infructescences have very clear and specific host ranges. The specificity of the *Ophiostoma*-vectoring mites to different *Protea* spp. is currently still unknown, but unpublished data (Roets in prep.) suggest that the *Tarsonemus* sp. found on Cape *Protea* species and *P. vanderbergi* are especially common on bearded proteas such as *P. neriifolia* and *P. laurifolia*, with few individuals observed on *P. repens*. In contrast, the *Oodinychus* sp. is very abundant within *P. repens* infructescences, but absent from the infructescences of most other *Protea* species. The *Tarsonemus* sp. from the northern and eastern parts of South Africa (sp. B) was collected from *P. caffra* only (Roets *et al.* 2006c, Chapter 4). There are clearly patterns of occurrence of these mites on *Protea* spp. and studies focussed on the acarifauna of the different *Protea* spp. are thus needed to show possible co-evolutionary processes.

Differentiation between vector organisms appears to be of lesser importance to the avoidance of competition. The *Oodinychus* sp. mite, for example, has been shown to vector *O. splendens*, *O. palmiculminatum*, *S. variecibatus* and *O. phasma* (Roets *et al.* 2006c, Chapter 4). *Tarsonemus* sp. A (Roets *et al.* 2006c, Chapter 4) vectors *O. phasma*, *Tarsonemus* sp. B. (Roets *et al.* 2006c, Chapter 4) vectors *O. gemellus* and *P. vanderbergi* vectors *O. phasma* (Roets *et al.* 2006c, Chapter 4). More data are clearly needed before the possible co-evolution of these fungi and their vectors can be fully understood.

## Future research

Future studies on the intriguing relationship between the ophiostomatoid fungi and *Protea* spp. should focus on unraveling the patterns and potential mechanisms of co-evolution between all three organism groups. Thus far, studies on the ecology and species diversity of the *Protea*-associated ophiostomatoid fungi have been largely focused on the genus *Ophiostoma* (Roets *et al.* 2006a, 2006b, 2006c, 2006d, Chapters 3–6). As *Gondwanamyces* species are morphologically similar to species of *Ophiostoma*, we assume that these genera share associations with arthropods as vectors. Ecological studies on *Gondwanamyces* have been hampered by problems with the isolation of these slow-growing fungi from among many other fungal species on the arthropods that carry them.

The interactions between different fungal species within infructescences will also be an interesting topic for future research. As mentioned, it has been observed that when ophiostomatoid fungi are absent within an infructescence, other fungal species appear to thrive and *vice versa*. It is thus plausible that the ophiostomatoid fungi are able to exclude other fungal species from this niche. *In vitro* and field studies on the specific interactions between these fungi and other organisms are needed if we wish to fully comprehend the effect of ophiostomatoid fungi on *Protea* population dynamics.

Many other fungal species present within *Protea* sp. infructescences are also suspected to rely on arthropod spore dispersal, as other spore dispersal options are limited within this niche. The morphological characteristics of many of the fungal species in this niche accordingly suggest vectored spore dissemination. These include species such as *Rhyncostoma proteae* S. Lee & P.W. Crous (Lee *et al.* 2003), *Sordaria* Ces. & De Not. sp., *Phaeacremonium* W. Gams *et al.* sp. and various synnematosus spp. (Lee *et al.* 2005, Roets unpublished data). Infructescences of *Protea* spp. thus provide a unique opportunity to study multi-organism interactions within a relatively restricted ecological niche.

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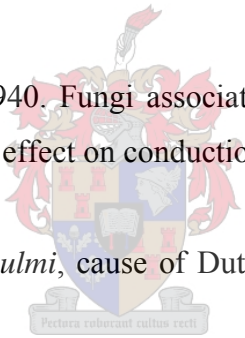
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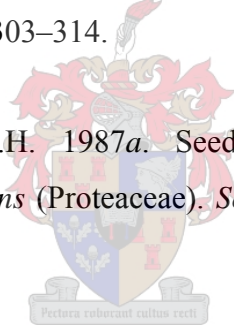
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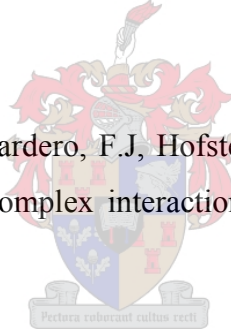
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