DIVERSITY AND ECOLOGY OF OPHIOSTOMATOID FUNGI AND ARTHROPODS ASSOCIATED WITH PROTEACEAE INFRUCTESCENCES

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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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SUMMARY

Five new species of ophiostomatoid fungi, colonising the infructescences of serotinous *Protea* species, have recently been discovered in South Africa. Prior to this, ophostomatoid fungi were thought to be restricted to the Northern Hemisphere. The discovery of these five species thus extensively expanded the known geographical range of these fungi, now also to include the Southern Hemisphere. Since this discovery, few studies have focused on the interesting ecology of this group, which is uniquely adapted for spore dispersal by arthropods. Studies focussed on ophiostomatoid fungi provide an unique opportunity to study inter-organism interactions between fungi, their host plants and the arthropods responsible for their spore dispersal. Very few similar studies have been undertaken, particularly in the Fynbos Biome, to which most of our economically important *Protea* species are confined. The current study provides insight into some ecological aspects of these complex interactions.

The seasonal distribution of the ophiostomatoid fungi associated with the infructescences of members of the Proteaceae was investigated. Definite seasonal patterns were observed, with peak fungal colonisation occurring during the wetter winter months. While determining the host specificity of these fungi, a new *Protea* host for *Ophiostoma splendens* was identified, and a new species of *Rhyncomeliola* was discovered. All ophiostomatoid species native to Fynbos in the Stellenbosch region are exclusively associated with the infructescences of species in the plant genus *Protea*.

By employing a multiplex Polymerase Chain Reaction (PCR), a set of group specific primers was developed for the identification of South African *Ophiostoma* and *Gondwanamyces* fungal DNA. This newly developed method was used to scan arthropods collected from the infructescences of *P. repens* for the presence of *O. splendens* and *G. proteae* spores. By using these group-specific primers,

three insects possibly responsible for long range dispersal of fungal spores were identified. Three further insect species, possibly contributing to the short-range dispersal of ophiostomatoid spores, were also implicated through this method.

All arthropods associated with the fruiting structures of selected members of the Proteaceae were also investigated. A total of 62 pseudospecies (*ca.* 7500 individuals), belonging to 45 different arthropod families, were collected. Proteaceae species with larger fruiting structures housed more arthropod species and higher numbers of individuals than Proteaceae taxa with smaller fruiting structures. Some plant species housed similar arthropod communities, while others housed unique suites of arthropods. Seasonal patterns in arthropod numbers were observed, and it was found that, in most instances, arthropod numbers peaked during the autumn and winter months.

Twenty-five fungal taxa were isolated from various Proteaceae arthropods, many of which are genera known to include Proteaceae pathogenic species. Ophiostomatoid fungi have a saprophytic relationship with their Protea hosts, and may deter some of these potentially harmful fungi from colonising the infrutescences. Some fungi showed a high degree of specificity towards potential vectors, while others were found on a diverse range of arthropods.

This study highlights the existence of complex inter-organismal interactions within the Fynbos Biome, a study area where the interactions between plants, fungi and insects have been grossly neglected. The few studies that have been conducted in this field have mostly focussed on a limited number of organisms, and no wide-scale attempts, such as presented here, have been published. It is important to obtain a holistic view in any ecological study that focuses on interactions between different suites of organisms. Ultimately this will aid in the development of better conservation strategies. This study thus provides a much-needed start in studies on multi-organismal interactions in the Fynbos Biome.

OPSOMMING

Vyf nuwe ophiostoma-agtige fungus spesies is onlangs in die vrug-liggame van Suid Afrikaanse *Protea* plante ontdek. Voor hierdie ontdekking, was die algemene opvatting dat ophiostoma-agtige fungi beperk is tot die Noordelike Halfrond. Die ontdekking van hierdie vyf spesies het dus die bekende geografiese verspreiding van hierdie tipe fungi vergroot om nou ook die Suidelike Halfrond in te sluit. Sederdien het min studies gefokus op die interesante ekologie van hierdie groep, wat aangepas is vir spoorverspreiding deur geleedpotiges. Studies wat fokus op die ophiostoma-agtige fungi voorsien 'n unieke geleentheid om die inter-organismiese-interaksies tussen fungi, hul gasheer plante en die geleedpotiges wat hul spore versprei, te bestudeer. Baie min soortgelyke studies is al voorheen in die Fynbos Bioom, waar die meeste van ons ekonomies-belangrike *Protea* spesies voorkom, onderneem. Die huidige studie verleen meer inligting oor sommige ekologiese aspekte van hierdie komplekse interaksies.

Seisoenale verspreidingspatrone van ophiostoma-agtige fungi geassosieer met die vrug-liggame van lede van die Proteaceae, is ondersoek. Daar is defnitiewe seisoenale patrone in die aanwesigheid van fungi gevind, met 'n piek in fungi kolonisasie-getalle gedurende die vogtiger wintermaande. 'n Nuwe *Protea* gasheer vir *Ophiostoma splendens* en 'n nuwe fungus spesie (*Rhyncomeliola* sp.), is ontdek. Alle Fynbos ophiostoma-agtige spesies in die Stellenbosch area, is beperk tot *Protea* spesies.

'n Multipleks Polimerase Ketting Reaksie is ontwikkel om die spesifieke DNS van ophiostoma-agtige fungi te herken. Hierdie metode is gebruik om te toets vir die aanwesigheid van spore van hierdie fungi op die liggame van geleedpotiges (geassosieerd met *P. repens*). Hierdeur is drie insekte wat waarskynlik verantwoordelik is vir die langafstand verspreiding van spore geidentifiseer. Drie

verdere insekte wat moontlik verantwoordelik is vir die kortafstand verspreiding van ophiostoma-agtige fungi is ook aangewys.

Alle geleedpotiges geassosieer met die vrugstrukture van geselekteerde lede van die Proteaceae is ondersoek. 'n Totaal van 62 pseudo-spesies (omtrent 7500 individue), wat tot 45 families behoort, is versamel. Proteaceae spesies met groter vrugstrukture het meer geleedpotige spesies en individue gehuisves as taksa met kleiner vrugstrukture. Sommige plant spesies het soortgelyke geleedpotige gemeenskappe gehuisves, terwyl unieke groepe geleedpotiges in ander plante voorgekom het. Alhoewel die geleedpotiges seisonale variasies in getalle geopenbaar het, het die meeste groepe 'n piek in getalle getoon gedurende die herfs- en wintermaande.

Vyf-en-twintig fungi taksa is geïsoleer vanaf verskeie Proteaceae geleedpotiges. Sommige van hierdie is genera wat Proteaceae patogeniese spesies insluit. Sommige fungi het 'n hoë graad van spesifisiteit teenoor potensiële vektore getoon, terwyl ander weer algemeen op verskeie geleedpotige spesies gevind is.

Hierdie studie dui op die bestaan van komplekse inter-organismiese-interaksies in die Fynbos Bioom, in 'n area waar die studie van interaksies tussen plante, fungi en insekte nog baie min aandag geniet het. Die beperkte aantal studies wat tot dusver in hierdie veld onderneem is, fokus gewoonlik slegs op 'n paar organismes. Geen grootskaalse studies, soos die een hier voorgelê, is al gepubliseer nie. In enige ekologiese studie wat fokus op interaksies tussen verskeie organismes, is dit belangrik om 'n holistiese siening te probeer verkry. Uiteindelik sal dit bydra tot die ontwikkeling van beter bewarings-strategië. Hierdie studie verleen dus 'n noodsaaklike begin tot studies op multi-organismiese-interaksies.

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INTRODUCTION

South Africa has the richest temperate flora in the world, including *ca.* 24 500 plant taxa (more than 20 000 species). This constitutes almost 10% of the known flowering plants of the world (Arnold & De Wet 1993). An estimated number of *ca.* 9 000 vascular plant species, representing *ca.* 44% of the southern African flora, are found in the Cape Floristic Region (CFR) alone (Arnold & De Wet 1993; Cowling & Hilton-Taylor 1997; Goldblatt & Manning 2000). The majority of CFR species are found in the Fynbos Biome (Rutherford & Westfall 1986), with 69% of these species endemic to the CFR. This area also supports five of the 12 southern African endemic plant families (Goldblatt & Manning 2000).

The main reasons for the tremendous species radiation and diversification in the south-western Cape are believed to be linked to the physical heterogeneity of this region. The region displays heterogeneous precipitation patterns, a variety of soil types including sandstone, granite, limestone and alluvial sands, numerous mountain ranges and a very high frequency of fire. Different combinations of these variables produce a wide range of different microhabitats (Goldblatt & Manning 2000). Although plant diversity has been well studied, limited attention has been given to other biotic elements, which may also influence plant species diversity. Very few studies have focused on aspects such as pollination, herbivory and interspecies competition of the Cape Flora (Rourke & Wiens 1977; Bond & Slingsby 1983; Coetzee & Giliomee 1985; Coetzee 1989; Wright 1990; Vlok 1995; Midgley *et al.*1998).

Many authors believe that the Fynbos vegetation has a faunal and fungal diversity that not only matches that of plants, but may even far exceed it (Coetzee & Giliomee 1987a, b; Coetzee 1989; Visser 1992; Swart *et al.* 2000; Taylor & Crous 2000). The Cape Flora is marked by a very high level of endemism. Species endemism levels of 90% and higher have been reported for many plant families (Oliver *et al.* 1983; Goldblatt & Manning 2000). Despite the lack of published data, a number of endemic insects and fungi are also

known from the CFR (Coetzee & Giliomee 1987a, b; Coetzee 1989; Wright 1990; Visser 1992; Crous *et al.* 2000a; Taylor *et al.* 2001). Many of these are dependent on specific Fynbos plant species for their development and survival. This further highlights the need for integrated biological studies to investigate relationships and patterns of co-evolution between different interactive biological groups within this world-renown floristic region.

Proteaceae

The Proteaceae, along with the families Restionaceae and Ericaceae, form a major component of Fynbos vegetation (Cowling & Richardson 1995). The Proteaceae is the seventh largest family of vascular plants in the CFR, with about 96.7% of its members confined to this area (Goldblatt & Manning 2000). The family is of major economic importance to South Africa in terms of the flower export industries and eco-tourism. Unfortunately it is also considered to be one of the most threatened plant families in South Africa, since many included species are in danger of becoming extinct. More than 140 of 400 southern African Proteaceae species are listed in the Red Data Book for rare and endangered plants (Hilton-Taylor 1996). There is thus an urgent need for a carefully managed conservation programme for this taxon.

The family contains about 1400 species world-wide and is divided into more than 60 genera (Rebelo 1995). The vast majority of Proteaceae species are found in the Southern Hemisphere, with the majority of genera occurring in South Africa and Australia. Two subfamilies are recognised in the family, the Proteoideae and the Grevilleoideae. Members of the Proteoideae occur mainly in southern Africa, with a few representatives in Australia and New Zealand. The Grevilleoideae is found predominantly in Australia, with only one species present in South Africa (Rebelo 1995), a few in South America and a few on the south-western Pacific islands. According to Rourke (1998) the African Proteaceae (including Madagascar) is represented by 16 genera and about 400 species, while Australia includes over 800 species in 45 genera (Rebelo 1995). Central and South America house about 90 species, while the

islands east of New Guinea contain about 80 species, and New Caledonia has some 45 species. Madagascar, Southeast Asia, New Guinea and New Zealand include only a few species each (Rebelo 1995). The south-western Cape alone hosts more than 330 species grouped into 13 Cape-centred genera. Ten of these genera are endemic to the region (Rourke 1998).

South African Proteaceae genera include: *Aulax* Berg., *Brabejum* L., *Diastella* Salisb., *Faurea* Harv., *Grevillea* R. Br. ex Knight, *Hakea* Schard., *Leucadendron* R. Br., *Leucospesmum* R. Br., *Macadamia* F. Müll., *Mimetes* Salisb., *Orothamnus* Pappe ex Hook., *Paranomus* Salisb., *Protea* L., *Serruria* Salisb., *Sorocephalus* R. Br., *Spatella* Salisb. and *Vexatorella* Rourke (Rebelo 1995). *Grevillea*, *Hakea* and *Macadamia* were introduced, and have subsequently become naturalised within this region.

Only four of the 13 southern African genera are also represented outside the CFR, namely *Faurea*, *Leucadendron*, *Leucospermum* and *Protea* (Rebelo 1995). More than two thirds of the *Protea* species are, however, confined to the southern tip of Africa, and more than 130 species are found in the Caledon region alone (Oliver *et al.* 1983).

The Proteaceae is considered to be an ancient family (Carpenter *et al.* 1994; Johnson 1998); in fact, some authors believe that it is one of the oldest angiosperm families on earth, dating back to at least 140 million years (Rebelo 1995). The basal position of the Proteaceae in the angiosperms has long been suspected (Chase *et al.* 1993; APG 1998) and modern classification systems seem to confirm this placing (Stevens 2002). Along with the orders Buxales and Trochodendrales, the Proteales (including Nelubonaceae, Plantaceae and Proteaceae) appear as an unresolved basal polytomy in the eudicots. This position thus excludes them from the more recently derived eudicots clade. The estimated age of the genus *Protea* is about 36 million years (Reeves 2001). Reeves (2001) also concluded that the diversity of the *Protea* species in the CFR may be due to high coexistence of species that diversified over a long period of time, rather than a recent and rapid radiation of this lineage. This implies that any organisms that are

dependent on members of the Proteaceae could potentially have a very long co-evolutionary history with this family.

Ophiostomatoid fungi

Many pathogenic fungal species are known to be associated with the Proteaceae (Crous *et al.* 2000b; Swart *et al.* 2000; Taylor & Crous 2000; Table 1). Limited attention has, however, been focused on the apparently non-pathogenic fungi associated with these plants (Marais & Wingfield 1994; Taylor *et al.* 2001).

Since the fairly recent discovery of five new species of ophiostomatoid fungi within the infructescences of serotinous *Protea* species (Wingfield & Van Wyk 1993; Marais & Wingfield 1994; Marais 1996), there has been renewed interest in this ecologically diverse fungal group.

As noted by Marais (1996), ophiostomatoid fungi were previously believed only to be associated with plants from the Northern Hemisphere. The discovery of related fungi restricted to indigenous South African plants has confirmed that these fungi are also naturally represented in the Southern Hemisphere. Wingfield & Van Wyk (1993) noted that most members of the Ophiostomataceae in the Northern Hemisphere are associated with primitive and drought tolerant conifers. Interestingly, the members of this group of fungi from the Southern Hemisphere are also associated with primitive, drought tolerant members of the Proteaceae (Wingfield & Van Wyk 1993). Although the Proteaceae is an Angiosperm and thus considered more derived than the Gymnosperm host plants, it is interesting that the Proteaceae is a very basal eudicot family.

Over 100 species of ophiostomatoid fungi are currently known (Seifert *et al.* 1993), but their taxonomy has proven to be problematic

Table 1 A list of some Proteaceae disease-causing fungi, with an indication of the site of infection on their hosts (J.E. Taylor unpublished).

Leaves	Flowers	Stems	Roots
Alternaria alternata	*Botrytis cinerea*	*Botryosphaeria dothidea*	*Armillaria luteobubalina*
Ascochyta sp.	*Drechslera biseptata*	*Botryosphaeria proteae*	*Cylindrocladium* sp.
Batcheloromyces leucadendri	*Drechslera dermatiodea*	*Botrytis cinerea*	*Cylindrocarpon* sp.
Batcheloromyces leucospermi	*Elsinoë* sp.	*Botrytis* sp.	*Curvularia* sp.
Botryosphaeria proteae		*Colletotrichum* sp.	*Fusarium* sp.
Bipolaris sp.		*Chondrostereum purpureum*	*Graphium* sp.
Calonectria colhounii var. *colhounii*		*Drechslera biseptata*	*Macrophomina phaesolina*
Cercostigmina protearum		*Drechslera dermatiodea*	*Pestalotia* sp.
Cercostigmina protearum var. *leucadendri*		*Elsinoë* sp.	*Phytophthora cinnamomi*
Clasterosporium proteae		*Lasiodiplodia theobromae*	*Phytophthora nicotianae*
Cirsosia		*Pestalotia* sp.	*Pythium vexans*
Coleroa senniana		*Phoma sorghina*	*Rhizoctonia solani*
Coniothyrium leucospermi		*Phoma* sp.	*Rosellinia* sp.
Coniothyrium nitidae		*Phomopsis* sp.	*Stemphylium* sp.
Coniothyrium proeteae		*Schizophyllum commune*	*Verticillium dahliae*
Didymosporium congestum		*Sclerotinia* sp.	
Elsinoë sp.			
Helicosingula leucadendri			
Leptosphaeria leucadendri			
Leptosphaeria protearum			
Lophiostoma fuckelii			
Mycosphaerella bellula			
Mycosphaerella jonkershoekensis			
Mycosphaerella sp.			
Passalora protearum			
Pestalotiopsis sp.			

(Marais 1996). The three main genera included in the ophiostomatoid fungi are: *Ceratocystis s.s.* Ellis & Halst., *Ophiostoma* H. Syd. & P. Syd. and *Ceratocystiopsis* H.P. Upadhyay & W.B. Kend. Nine anamorphic genera are recognised within the ophiostomatoid fungi (Wingfield *et al.* 1988; Nag Raj & Kendrick 1993). A new genus, *Gondwanamyces* Marais & M.J. Wingf., was proposed to accommodate the phylogenetically distinct (based on morphological and physiological characters) species with *Knoxdaviesia* M.J. Wingf. *et al.* anamorphs found in the infructescences of *Protea* species (Marais *et al.* 1998; Wingfield *et al.* 1999). It was also suggested that the species of *Ophiostoma* found on *Protea* species represent an unique lineage in the Ophiostomales and that these species should reside in a new genus (Wingfield *et al.* 1999). Marais (1996) presented an excellent account of the taxonomic history of *Ceratocystis* (*senso lato*), while Upadhyay (1981, 1993) presented thorough reviews on the taxonomy of ophiostomatoid fungi.

Five species of ophostomatoid fungi are currently known from the infructescences of serotinous *Protea* species in South Africa. These include *Ophiostoma protearum* Marais & M.J. Wingf., *O. splendens* Marais & M.J. Wingf., *O. africanum* Marais & M.J. Wingf. (which have *Sporothrix* Hektoen & Perkins anamorphs), and two from genus *Gondwanamyces*, which have *Knoxdaviesia* anamorphs. These are *G. capensis* (M.J. Wingf. & P.S. Van Wyk) Marais & Wingf. and *G. proteae* (M.J. Wingf., P.S. Van Wyk & W.F.C. Marasas) Marais & Wingf.

These species show varying degrees of host specificity and it is not known to what extent the fungi occur on other *Protea* species in the rest of Africa. Known host species for *O. splendens* include *P. lepidocarpodendron* L., *P. longifolia* Andrews, *P. neriifolia* R. Br. *and P. repens* L. (Marais & Wingfield 1994). Host species for *G. capensis* include *P. burchelii* Stapf, *P. coronata* Lam., *P. lepidocarpodendron., P. longifolia, P. magnifica* Link and *P. neriifolia* (Wingfield & Van Wyk 1993; Marais & Wingfield 1994). Each of the remaining three ophiostomatoid species are restricted to only one species of *Protea*: *G. proteae* is found on *P. repens* (Wingfield *et al.* 1988), *O. africanum* is found on *P. gaguedi* J.F. Gmel. (Marais 1996), and *O. protearum* is only found on *P.*

caffra Meisn. (Marais & Wingfield 1997). More fungal species are likely to be discovered when more *Protea* species are included in the study.

Although the ecology of these fungi is quite varied, most Northern Hemisphere members are associated with plant stems and roots. The ophiostomatoid fungi found on *Protea* species are unique in that they occupy a very different niche, namely the infructescences of some of the serotinous species.

Members of ophiostomatoid fungi are known to include devastating plant pathogens such as the well-known Dutch elm disease, caused by *Ophiostoma ulmi* (Buis.) Nannf. (Braiser 1988), and oak wilt, caused by *Ceratocystes fagacearum* (Bretz) Hunt (Sinclair *et al.* 1987; Table 2). At present it is unknown whether the *Protea* fungi are pathogenic to their hosts. A pilot study suggested a non-pathogenic association, as the fungi only appear to infect dead floral parts (Roets 2000).

South African ophiostomatoid fungi have a similar anatomy and morphology to the species native to the Northern Hemisphere. These fungi have a flask-shaped base and an elongated neck. Spores collect at the tip of this neck in a sticky mass, where insects can readily come into contact with it. This morphology suggests insect dissemination of their spores. The vectors of many Northern Hemisphere species have been identified and include Diptera, Coleoptera (e.g. Curcilionidae & Scolytidae) and a few other orders (Davidson & Robinson-Jeffrey 1965; Davidson *et al.* 1967; Davidson 1978; Dowding 1984; Harrington 1993; Kile 1993; Malloch & Blackwell 1993; Nag Raj & Kendric 1993). It is, however, still unclear which specific insects act as vectors for the different South African ophiostomatoid fungi. These fungi have thus far only been found in insect-infested flower heads (Wingfield *et al.* 1988; pers. observ.), and it is feasible that one or more of these insects transport the fungal spores.

As mentioned before, these fungi vary in their degree of host specificity. This aspect is thought to be linked either to specific insect vectors that visit only

certain *Protea* species or to differences in the chemical profiles of the host plants (Wingfield & Van Wyk 1993). Identifying the insect vectors of these fungi on *Protea* hosts will greatly expand our current knowledge and understanding of the ecology of the ophiostomatoid fungi and their plant hosts.

Table 2 A summary of some ophiostomatoid fungi, the diseases they cause, their host plants and geographic distribution. *

Fungus	Host	Disease	Distribution
Ophiostoma minus	Pines	Canker stain	N. America
Ophiostoma montia	Pines	Canker stain	N. America
Ophiostoma polonica	Spruce	Canker stain	Europe
Ophiostoma ulmi	Elms	Dutch elm disease	Widespread
Ophiostoma sp.	Oak	Oak decline	Central Europe
Sporothrix schenkii	Humans	Sporotrichosus	Cosmopolitan
Ceratocystis coerulescens	Sugar maple	Sapstreak	U.S.A.
Ceratocystis fagacearum	Red oak	Oak wilt	U.S.A., Europe
Ceratocystis fimbriata	Coffee	Trunk rot	S. America
	Fig	Canker	Japan
	Mango	Wilt	Brazil
	Poplar	Canker	N. America, Europe
	Rubber	Mouldy rot	Brazil
	Sweet potato	Black rot	E. Asia
	Sycamore	Canker	Europe
Ceratocystis laricicola	Larch	Canker stain	Europe
Ceratocystis paradoxa	Citrus	Soft rot	India
	Coconut	Stem bleeding	Asia
	Pineapple	Pineapple disease	Tropical
	Sugar cane	Pineapple disease	Tropical
Leptographium procerum	Pines	Root disease	N. America, Europe
Leptographium wageneri	Conifers	Black stain	N. America
Leptographium serpens	Pines		Europe, S. Africa

* Adapted from Wingfield *et al.* (1993).

Insects and vectors

Insects found on proteas (members of the Proteaceae) fulfil many functions, from herbivory and seed predation to pollination and protection (e.g. ants and other predators could deter herbivorous insects from causing damage to the plant). Insects are known to influence the evolution of pollination mechanisms and anti-herbivore defence systems in plants (Bond & Slingsby 1983). It is therefore also possible that insects could have played a significant role in the speciation and radiation of proteas.

Many insects have been found associated with the infructescences of members of the Proteaceae (Guess 1968; Myburg *et al.* 1973, 1974; Myburg & Rust 1975a, b; Coetzee & Giliomee 1987a, b; Coetzee 1989; Wright 1990; Visser 1992). Most of these have been found to inhabit the infructescences of serotinous members of the genus *Protea*. It is possible that one or more of them could act as the vector(s) of the ophiostomatoid fungi present on proteas.

Related fungi from the Northern Hemisphere are known to be dispersed by insects belonging to a wide range of different families, predominantly beetles (Coleoptera) (Davidson & Robinson-Jeffrey 1965; Davidson *et al.* 1967; Davidson 1978; Dowding 1984; Harrington 1993; Kile 1993; Malloch & Blackwell 1993; Nag Raj & Kendric 1993). Insects belonging to some of these families have also been collected from Southern Hemisphere *Protea* species. Since these fungi are only found within older infructescences, pollinators (such as bees) are ruled out as possible vectors, as they will only visit nectar and pollen containing inflorescences. Marais (1996) suggested that borers are the most likely vectors of ophiostomatoid fungi. Many other insects have, however, also been isolated from closed *Protea* flower heads. Mites and nematodes, which are commonly found within *Protea* flower heads, are carried from one flower to another by other insects and may also act as vectors of these fungi.

There is a symbiotic relationship between the vector-insects and some Northern Hemisphere ophiostomatoid species. Once the insects invade the plants, some construct galleries, and in the process create favourable conditions for ophiostomatoid fungal growth (Harrington & Cobb 1988). The benefit to the insects in this relationship is unknown. In some cases the symbiotic relationship between certain bark beetles and pathogenic *Ophiostoma* species was found to be mutualistic. The fungi cause wounds around the infected areas where beetles have invaded, which can ultimately even lead to the death of the host. This, in turn, creates more suitable environments for the insects to breed in (Berryman 1972). Several reviews on the association between *Ophiostoma* species and different insects have been published (Graham 1967; Dowding 1984).

Ophiostomatoid fungi are often found to be the dominant fungal species encountered within infected infructescences. They are also the most common fungi found within this niche (Marais & Wingfield 2001). It seems possible that they might manage to out-compete other seed-destructive fungal species also present within this niche. Should this prove to be the case, ophiostomatoid fungi may well be an asset to the plant, as they may then favourably influence seed production. It is thus possible that there is a constructive symbiotic relationship between fungus and plant.

PCR detection of fungi

The polymerase chain reaction (PCR) assay developed in 1983 (Mullis & Faloona 1987; Mullis 1990), is an approach that allows for the rapid amplification of small amounts of specific DNA fragments. It allows the synthesising of millions of copies of a specific DNA target fragment, theoretically from as little as a single DNA molecule (Li *et al.* 1988; Erlich *et al.* 1991). In many instances PCR methods have proven to be useful in the detection and identification of fungi from a range of complex environments (Steffan & Atlas 1991; Kim *et al.* 1999; Edel *et al.* 2000; Groenewald *et al.* 2000; Hamelin *et al.* 2000; Hirsch *et al.* 2000; Schmidt & Moreth 2000; Vaino

& Hantula 2000; Ganley & Bradshaw 2001; Lee *et al.* 2001; Mazzaglia *et al.* 2001; Zhou *et al.* 2001).

The process firstly involves the extraction of DNA, whereafter specific oligomer primers are added in a buffered solution that contains deoxynucleotides (dNTP's), magnesium ions and a thermally stable DNA synthesising enzyme (*Taq* DNA polymerase). By manipulating the temperature of the solution many copies of the DNA fragment flanked by the two primers can be synthesised exponentially. When the synthesised DNA is placed in a gel matrix over which a current is applied, the negatively charged DNA fragments will migrate towards the positive pole. The size of the fragment determines the speed at which they will travel through the gel. Smaller fragments will travel much faster than larger ones. When the intercalating DNA stain ethidium bromide is added to the gel and the gel is studied under UV light, the DNA can be seen as fluorescent bands on the darker gel background. The size of the DNA fragments forming the fluorescent bands can be determined by comparing the distances of these bands from the loading pits, and comparing these with size markers (DNA fragments of known sizes). These are run simultaneously with the amplified DNA fragments. If the sizes of the amplified fragments correspond with the expected fragment sizes for the specific target fungal DNA and the primers are specific for the target organisms, it can be concluded that DNA of the target species was present. This can be validated by sequencing (determining the sequence of the nucleotides) the DNA fragments.

When two or more species need to be detected (i.e. to amplify several DNA segments simultaneously) it is possible to perform multiplex PCRs, where you have different primer pairs mixed in the same medium for each of the species tested. Primer pairs are chosen such that they produce fragments of different sizes for each of the species.

Ribosomal genes are often used in molecular studies, since their high copy number increases PCR sensitivity (Edel *et al.* 2000). They also display areas with high base pair conservation and areas with low base pair conservation.

This is especially useful in phylogenetic studies, and lends itself both to the design of universal and of species- or group-specific primers.

Nuclear rDNA consists of different sized subunits: 5.8S, 18S and 28S. The internal transcribed spacers (ITS) separate these units. The ITS units are mostly used in the design of species-specific primers, as they are not transcribed and have more sequence variation than transcribed regions. It is also possible to design species-specific primers using transcribed areas of DNA, provided there are some areas that show enough sequence variation. Publications on the methodology of PCR systems abound in literature (Erlich 1989; Innis *et al.* 1990; Steffan & Atlas 1991; Venigalla 1994). Developing a PCR-based assay to detect ophiostomatoid fungal DNA on insects is critical in the search for the vector(s) of these fungi.

Objectives of this study

The main objective of the present study is to find the specific vector organism(s) of the ophiostomatoid fungi present within the infructescences of serotinous South African *Protea* species (Chapter 2). In order to achieve this, an ophiostomatoid fungal specific DNA extraction protocol had to be developed, tested and refined (Chapter 1). In addition, a number of sub-objectives were also identified. The first of these was to examine fungal seasonality and related ecological aspects (Chapter 3), while also scanning for new fungal and host species.

A second sub-objective was to assess the seasonally and host specificity of the arthropods found within the infructescences of different Proteaceae species (Chapter 4). In this part of the study the presence and specificity of other, non-ophiostomatoid, fungal species present on the surface of some of the captured arthropod species was also investigated.

Results of the present study will contribute to a more thorough understanding of the biological interactions at play within the Fynbos Biome. It will also provide methods for the faster detection of insect vectors.

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CHAPTER 1

A PCR-BASED METHOD FOR THE DETECTION OF *OPHIOSTOMA* AND *GONDWANAMYCES* ON INSECTS COLLECTED FROM THE INFRUCTESCENCES OF SOUTH AFRICAN *PROTEA* SPECIES

Abstract

The oligonucleotide primers, OSP1 and GPR1, were synthesised from the large subunit (LSU) ribosomal DNA for the genera *Ophiostoma* and *Gondwanamyces* found in infructescences of *Protea* species in South Africa. Multiplex Polymerase chain reaction (PCR) of the primer pairs OSP1 and GPR1 combined with universal primer LR6 yielded fragment lengths of about 900- and 600-bp, respectively. PCR detection of these fragments was achieved from as little as 45 pg and 30 pg of fungal genomic DNA for *Ophiostoma* and *Gondwanamyces* respectively. The same fragment lengths were amplified from inoculated insects. Fungal DNA from no other fungal species tested showed any amplification with the designed primers. These primers can now be used in an attempt to detect the vectors of ophiostomatoid fungi that are associated with South African *Protea* species. This, in turn, will aid in ecological assessments of the insect-plant-fungal interactions present in the Fynbos Biome.

Introduction

In recent years, a number of novel ophiostomatoid fungi were isolated from serotinous *Protea* L. species (Wingfield *et al.* 1988; Wingfield & Van Wyk 1993; Marais & Wingfield 1994, 1997, 2001). These ascomycetes include three species belonging to the genus *Ophiostoma* H. Syd. & P. Syd. (*Ophiostoma protearum* Marais & Wingf., *O. splendens* Marais & Wingf. and *O. africanum* Marais & Wingf.) and two species belonging to the genus *Gondwanamyces* Marais & Wingf. (*G. capensis* (M.J. Wingf. & P.S. Van Wyk) Marais & Wingf. and *G. proteae* (M.J. Wingf., P.S. Van Wyk & Marasas) Marais & Wingf.). It was recently suggested that the species of *Ophiostoma* isolated from *Protea* species should reside in a new genus, but these suggested changes have not yet been formally published (Wingfield *et al.* 1999). *Protea*-ophiostomatoid fungi differ with regard to their host specificity. *G. capensis* and *O. splendens,* for example, have a wide spectrum of *Protea* hosts, whereas *O. africanum* and *O. protearum* are confined to only one *Protea* species each. It is presently still unknown whether this specificity is related to differences between the *Protea* species (chemical and/or physical), or whether it is due to different vectors associated with each of the fungal species. The role that these fungi play within the *Protea* infructescences also remains to be determined.

Closely related fungi from the Northern Hemisphere (e.g. *O. ulmi* (Buis.) Nannf. – the fungus responsible for Dutch elm disease) are known to be insect vectored (Berrymin 1972; Dowding 1984; Braiser 1988; Malloch & Blackwell 1993). The morphology of the *Protea* fungi are very similar to the Northern Hemisphere ophiostomatoid species (Marais 1996). Spores are produced in a perithecial sac, pushed through a narrow perithecial neck and collect at the tip in a sticky mass. In this position, the spores can adhere to an insect that comes into contact with it and can thus be dispersed. The same system seems to apply to *Protea*-ophiostomatoid fungi, suggesting insect dispersal. Many insects are known to be associated with *Protea* species (Coetzee & Giliomee 1985, 1987a, b; Coetzee 1989; Wright 1990; Visser 1992), but the specific vectors of these fungi are still unknown.

In a pilot study, arthropods collected from *Protea* infructescences were crushed and plated on 2 % malt agar plates (MEA; Biolab, Midrand, South Africa), containing Sigma Streptomycin sulphate (0.04 g/l), in an attempt to identify the arthropod vector responsible for the dispersal of the fungi (Roets 2000). However, no ophiostomatoid fungi were recovered using this method. Since it is known that ophiostomatoid fungi can grow on this medium, it was concluded that either the fungi were not present on the arthropods, or that other fungi out-competed the slower-growing ophiostomatoid fungi. As it was highly unlikely that not a single one of the arthropods tested was carrying ophiostomatoid fungal spores, the second option seemed more plausible. This led to the approach followed in the present paper, where molecular techniques are tested as an alternative method to detect ophiostomatoid fungi on arthropods.

The polymerase chain reaction (PCR) assay is an approach that allows for the rapid detection of even small amounts of target DNA. This technique significantly improves the possibility of detecting target DNA sequences in heterogeneous DNA mixtures. In many instances PCR methods have proven to be useful in the identification and detection of fungi, even in very complex environments (Johnston & Aust 1994; Kim *et al.* 1999; Groenewald *et al.* 2000; Lee *et al.* 2001). When two or more fungal species need to be detected simultaneously, it is possible to perform multiplex PCR's (Chamberlain *et al.* 1988), where different primer pairs testing for different fungal species are present in one mixture. Primer pairs are chosen such that they produce different sized fragments for the respective fungi.

Ribosomal regions (rDNA) are often used in molecular studies, because their high copy numbers increase PCR sensitivity. They also display areas with high as well as areas with low base pair conservation. This is useful in phylogenetic studies and in the design of universal primers, species-specific primers or group-specific primers.

Nuclear rDNA consists of subunits of different sizes: 5.8S, 18S and 28S. The internal transcribed spacers (ITS) separate these units. ITS regions are

mostly used to design species-specific primers, as they are not transcribed and have more sequence variation than transcribed regions. It is also possible to design species specific primers using transcribed areas of DNA, provided they include areas that show enough sequence variation.

The present study sets out to test the feasibility of using group-specific PCR primers for the detection of ophiostomatoid fungi on or in insects. This method will hopefully help to identify vectors that carry ophiostomatoid fungi in the Southern Hemisphere. As all other species of ophiostomatoid fungi are naturally restricted to the Northern Hemisphere, it will be interesting to determine whether the vectors of the Southern Hemisphere fungi are related to vectors in the Northern Hemisphere. Ultimately this study will lead to a better understanding of the inter-organismal interactions taking place within the Fynbos Biome.

Materials and methods

Fungal isolates.

Cultures of *O. splendens (*CMW872), *O. protearum* (CMW1102), *O. africanum* (CMW823), *G. proteae* (CMW3936) and *G. capense* (CMW 997) were obtained from the culture collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Further cultures of *O. splendens* and *G. capense* were obtained from infected *P. repens* infructescences collected from Jonkershoek Nature Reserve, Stellenbosch (S: 33º 59.555' E: 18º 58.287'). Fungal spore masses of these fungi were collected directly from the tips of the perithecial necks by removing them with a small piece of 2 % malt extract agar (MEA, Biolab, Midrand, South Africa) at the tip of a dissecting needle, and transferring them onto agar plates. Isolates of other fungi screened in this study (Table 1) were obtained from the culture collection at the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa (STE-U).

Table 1 Fungal isolates used to test primer specificity and their culture reference numbers

Fungal species	Ref. no.
Alternaria sp.	SL 20
Aspergillus sp.	SL 3
Beauvaria sp.	SL 48
Chaetomium sp.	SL 13
Cladosporium sp.	SL 34
Dicyma sp.	SLE 10
Epicoccum sp.	SL 9
Fusarium sp. 1	SLE 16
Fusarium sp. 2	SLE 20
Fusarium sp. 3	SLE 4
Clonostachys sp.	SL 35
Clonostachys sp.	SL 23
Melanospora sp.	SL 15
Nigrospora sp.	SLE 17
Penicillium sp.	SL 101
Pestalotia sp.	SL 47
Sarcostroma sp.	SL 82
Sordaria sp.	SLE 80
Trichoderma sp.	SL 6
Unidentified Hyphomycete 1	SL 49
Unidentified Hyphomycete 2	SL 85
Unidentified Coelomycete	SL 83

These isolates were specifically chosen, as they were previously isolated from insects collected from Proteaceae infructescences (Roets 2000). All isolates were grown on 2 % MEA plates at 24 °C for 2-4 weeks.

DNA extraction from fungal mycelia for specificity testing of the primer sets

A modified DNA extraction protocol developed by Lee & Taylor (1990) was used to isolate Fungal DNA from mycelia. Fungal mycelia were collected from the surface of the agar plates by scraping them off with a scalpel. They were placed in Eppendorf tubes containing 1 ml SDS (pH 8) extraction buffer. This suspension was boiled for 3 min, and then placed on ice for a further 10 min. Tubes were incubated at 65 ºC in a water tub for 1.5 h, where after 600 µl chloroform : isoamyl alcohol (24:1 v / v) and 400 µl phenol was added. Extraction was then performed for 10 min by shaking the tubes at room temperature to remove proteins. The resulting suspensions were centrifuged at 14 000 rpm for 15 min, after which the supernatant was pipetted into a new tube. A second chloroform : isoamyl alcohol (24:1) extraction was done and again the supernatant was transferred to new tubes. After boiling the RNAase A (20 mg/ml stock solution) for 5 min, 10 µl was added to each tube and the tubes were then incubated at 37 ºC for 2 h. Then, 5 µl 20 % SDS buffer and 10 µl Proteinase K (20 mg/ml stock solution) was added to this mixture, and it was again incubated at 37 ºC overnight. The next morning a third chloroform : isoamyl alcohol (24:1) extraction was performed, after which 10 µl 3M NaOAc and 600 µl isopropanol were added to precipitate the nucleic acids. This mixture was left in the freezer for 1 h. The tubes containing the mixture were then centrifuged for 10 min at 14 000 rpm to collect the precipitate and the supernatant was decanted. The pellets were washed by adding 1 ml 70 % Ethanol and draining it off. The pellets were dried in an oven at 65 ºC for 1 h. The DNA was then resuspended in 100 µl ddH_2O and stored at 4 ºC until further use.

Design of species- and genus-specific primers

A total of 20 partial 18s rDNA sequences were obtained for a variety of fungal species. Sequence data for the five *Protea* ophiomatoid species, four Northern Hemisphere ophiostomatoid species and 11 fungal species included in genera previously isolated from insects collected from the infructescences of *Protea* species (Roets 2000), were obtained from GenBank (Table 2). DNA

sequences were aligned using the DNA and Protein Sequence alignment (DAPSA) programme developed by Harley (1998). The 18s rDNA region was chosen for this study, since a comparison of sequence data from this area showed sufficient base pair variation to develop group-specific primers. After comparing nucleotide sequences of the various fungal species, two DNA regions were identified as being group-specific for South African *Ophiostoma* species and genus-specific for *Gondwanamyces*. The two primers OSP1 (5' – GACGCCTAGCCTCTACAA –3') for *Ophiostoma* species and GPRI (5' – CCAGCATCGGTTTGTTA –3') for *Gondwanamyces* species were subsequently designed. The universal right binding primer (LR6 - 5' – CGCCAGTTCTGCTTACC –3'), designed by Vilgalys & Hester (1990), was used in combination with these two. The primers OSP1 and GPR1 were designed to amplify areas within the large subunit (LSU) ribosomal RNA gene (Appendix 1). The new primers were synthesised at the DNA Synthesis Laboratory, Department of Biochemistry, University of Cape Town, South Africa. Primers were designed to produce DNA fragments of different lengths so that the different ophiostomatoid taxa could be identified based on fragment length. The expected DNA fragment length amplified by the combination of primers OSP1 and LR6 is about 900 bp, while the fragment amplified by the primer pair GPR1 and LR6 is about 600 bp long. Expected melting temperatures for the individual primers were: OSP1 = 64 ºC, GPR1 = 63 ºC and LR6 = 64 ºC. After their development, the primers were tested for their specificity in the amplification of target 18S rDNA fragments of South African ophiostomatoid fungi.

Table 2 Sequence data of the 18S rDNA area for 20 fungal species representing 11 genera, obtained from GenBank.

Fungal species	Accession no.
Botrytis pori	AT226077
Ceratocystes adiposa	AF222481
Ceratocystes fimbriata	U47820
Cladosporium sp.	U26886
Fusarium aculatum	U34544
Gondwanamyces capensis	AF221012
Gondwanamyces proteae	AF221011
Leptographium lundgergii	AF155664
Melanospora zamiae	U17405
Mucor hiemalis f. hiemalis	AF113468
Ophiostoma africanum	AF221015
Ophiostoma protearum	AF221014
Ophiostoma splendens	AF221013
Ophiostoma piliferum	U47837
Penicillium chrysogenum	AF034557
Penicillium sp. 1	U26865
Penicillium sp. 2	U26851
Penicillium turbatim	AF034454
Penicillium namyslowskii	AB000487
Sordaria fimicola	AF132330

Testing the specificity of the newly developed primers OSP1 and GPR1

The specificity of the newly developed primers was tested using a number of fungi isolated from insects collected from Proteaceae species (Table 1). Prior to this, the optimal fungal DNA concentration for PCR's had to be established. This was done by testing different fungal DNA concentrations (no further dilution, 10x- and 50x-dilutions of the isolated DNA) for success of

amplification with universal primers ITS1 and ITS4 (White *et al.* 1990). These PCR's were performed in a Gene Amp®, PCR System 2 700 thermal cycler (Applied Biosystems, Foster City, U.S.A.). Thermal cycling conditions started with an initial denaturation of isolated DNA stage, performed at 96 °C for 5 min. This was followed by 30 cycles of (1) further denaturation at 94 °C for 30 s, (2) an annealing stage at 50 °C for 30 s and (3) an elongation stage of 1 min, 30 s at 72 °C. Before the termination of the PCR, a final elongation stage was performed at 72 °C for 7 min.

PCR mixtures (25 μl) in each tube contained 5 μl of the extracted fungal genomic DNA for the tested dilution, 8 mM $MgCl_2$ (Bioline, Londen), 1X NH_4 reaction buffer (Bioline, Londen), 0.25 mM of each of the four dNTP's, 10 pmol of each of the primers and 0.626 units of Biotaq (Bioline, London).

Once established, the fungal DNA dilutions that yielded the best amplification results with primer pair ITS1 and ITS4 were used in all further testing of the newly developed primers. (The actual fungal DNA concentrations at which amplification was optimal were not determined).

After determining the ability to amplify the extracted fungal DNA (as described above) with the primers ITS1 and ITS4, we determined optimal amplification conditions for the new primers OSP1 and GPR1 used in conjunction with LR6, respectively. Different annealing temperatures (50 °C, 60 °C and a program in which the annealing temperature varied), primer concentrations (4 pmol/μl, 0.4 pmol/μl and 0.2 pmol/μl) and $MgCl_2$ concentrations (0.5 mM, 1.5 mM, 2.5 mM, 3.5 mM 4.5 mM, 5 mM and 8 mM) were tested. Optimal primer concentrations were 0.4 pmol/μl and optimal $MgCl_2$ concentration was 8 mM. Concentrations of all the other constituents of the PCR mixture were the same as those used for the amplification of the fungal DNA with the ITS primers. The programme with the different annealing temperatures yielded the best amplification results. These thermal cycling conditions were: an initial DNA denaturating step at 94 °C for 2 min followed by 13 cycles of (1) denaturation at 94 °C for 30 s, (2) annealing at 65 °C for 30 s and (3) elongation of DNA fragments at 72 °C for 1 min. This was again followed with 23 cycles with the

same allocated times for the different stages, denaturation temperature and DNA extension temperature as for the previous 13 cycles. The annealing temperature used in these cycles was 56 °C rather than 65 °C. Before the termination of the PCR process a final extension phase at 72 °C for 5 min was performed.

After the optimisation of the PCR process, both the two left primers and the right primer were combined into a multiplex reaction and the specificity of the developed primers were tested again.

For these PCR reactions the PCR mixtures (25 µl) in each tube of the multiplex reaction again contained 5 µl of the extracted fungal genomic DNA for the tested dilution, 8 mM $MgCl_2$ (Bioline, London), 1X NH_4 reaction buffer (Bioline, London) and 0.25 mM of each of the four dNTP's. However, the primer concentrations were different in this case, with 0.002 pmol/µl of each of the primers OSP1 and GPR1 and 0.4 pmol/µl of the right binding primer LR6, being added to the mixture. As before 0.625 units of Biotaq (Bioline, London) enzyme were added. The same thermal cycling conditions used during the separate testing of the developed primers, were also used for the multiplex reactions.

The next step was to establish the minimum amount of ophiostomatoid fungal DNA needed in the reaction mixture to show positive amplification with primers OSP1, GPR1 and LR6. This was done in order to determine the sensitivity of the developed primers in the multiplex reaction.

All reactions were analysed by separating 10 µl of the PCR products and 3 µl loading buffer on a 1.5 % agarose gel (Promega corporation, Madison, U.S.A.) in Tris-acetate-EDTA buffer (TAE) containing ethidium bromide. PCR products were viewed under an UV transilluminator.

This newly developed multiplex PCR was then tested for its application and specificity in detecting South African ophiostomatoid fungal spores on arthropods.

Detection of Ophiostoma and Gondwanamyces on arthropods

A range of different insects and other arthropods were collected from Helderrand farm, Lynedoch, Stellenbosch (Table 3). As these insects were not collected from *Protea* species, and no proteas are known to grow in this area, it was assumed that the control organisms were not carrying ophiostomatoid fungal spores. This arthropod reference collection is preserved in the insect collection (USEC) of the department of Entomology and Nematology, University of Stellenbosch, Stellenbosch, South Africa. The arthropods were killed by exposing them to -20 ºC in a freezer for 24 h. Some of them were then inoculated (Table 3) by bringing them into contact with ophiostomatoid fungal spores present at the tips of the perethecial necks of *O. splendens* and *G. proteae*, while others were not inoculated and thus served as negative controls. The same DNA extraction protocol used for the extraction of fungal DNA from fungal mycelia (with some minor adjustments) was followed to extract DNA from the surface of the arthropods. The first adjustment entailed that arthropods were first vortexed for 1 min prior to further DNA isolation, in an attempt to loosen fungal spores from their surfaces. Extracted DNA was suspended in 15 µl rather than in 100 µl ddH_2O, since low quantities of DNA were obtained from the surfaces of the arthropods.

The newly developed multiplex reaction conditions were then used to amplify the target fungal DNA fragments isolated from the surface of the inoculated arthropods. Again the reactions were analysed by separating 10 µl of the PCR products and 3 µl loading buffer on a 1.5 % agarose (Promega corporation, Madison, U.S.A.) gel in TAE containing ethidium bromide and viewed under an UV transilluminator.

An adjusted fast detection method developed by Kim *et al.* (1999) was also tested for the extraction of fungal DNA from the arthropods. This method extracts DNA by heating the sample in a microwave (700 W) for 5 min after which cooled extraction buffer (SDS) is added (100 µl at 4 ºC). Tubes containing samples are then vortexed for 1 min. The supernatant was used

Table 3 Insects belonging to 10 different families, used for the detection of ophiostomatoid with primers OSP1 and GPR1 in conjunction with LR6 using the variable annealing temperature PCR programme.

Insect taxa	Ref. no.	No. of individuals tested	Amplification (yes/no)
Insects not inoculated (- controls)			
Crematogaster sp. (Formicidae)	1	2	No
Curculionidae sp. 1	2	1	No
Pyrochorridae sp. 1	3	3	No
Chironomidae sp. 1	4	2	No
Noctuidae sp. 1	5	2	No
Gryllidae sp. 1	6	1	No
Dermaptera sp. 1	7	1	No
Blattidae sp. 1	8	1	No
Lygaeidae sp. 1	9	2	No
Musca domestica (Muscidae)	10	2	No
Insects inoculated (+ controls)			
Crematogaster sp. (Formicidae)	1	4	Yes
Curculionidae sp. 1	2	1	Yes
Pyrochorridae sp. 1	3	2	Yes
Chironomidae sp. 1	4	1	Yes
Noctuidae sp. 1	5	2	Yes
Gryllidae sp. 1	6	1	Yes
Dermaptera sp. 1	7	1	Yes
Blattidae sp. 1	8	2	Yes
Lygaeidae sp. 1	9	1	Yes
Musca domestica (Muscidae)	10	5	Yes

directly for PCR's, using the above mentioned multiplex reaction mixture, without performing any additional extraction steps.

Results and discussion

Amplification of isolated fungal DNA with primer pair ITS1 and ITS4 was achieved for all the fungal isolates tested (Table 1), including all the South African ophiostomatoid fungal isolates. This confirmed the presence of the extracted fungal DNA and the ability to amplify these DNA fragments. As mentioned previously, three different concentrations (no dilution, 10X- and 50X-dilutions) of extracted DNA was tested for its ability to amplify with primer pair ITS1 and ITS4. These results are summarised in Table 4.

The same concentrations that yielded positive amplification results with the primer pair ITS1 and ITS4 were used for testing the specificity of PCR's with the new left-binding primers OSP1 and GPR1 (separately) and the right-binding primer LR6. Positive amplification was obtained for all five the tested ophiostomatoid species with the designed primers. As expected the fragment length for the *Ophiostoma* species was approximately 900 bp (with primer pair OSP1 and LR6) and about 600 bp for both *Gondwanamyces* species (with primer pair GPR1 and LR6). Although all fungal isolates were tested with primers OSP1, GPR1 and LR6, a positive amplification was obtained with *Ophiostoma* and *Gondwanamyces* species only (Fig. 1). After the specificity of the newly developed primers for the amplification of fungal DNA of South African ophiostomatoid species was established, the PCR protocol had to be optimised by testing different annealing temperatures, primer concentrations and $MgCl_2$ concentrations.

Testing of the different annealing temperatures revealed that the programme with the varying annealing temperatures yielded the best results. This programme starts off with a high annealing temperature (65 °C), which allows for highly specific annealing of the primers to the fungal genomic DNA. After a few repetitions at this highly specific high annealing temperature the

Table 4 Best amplification results for different dilutions of DNA extracted from fungal mycelia for testing the presence and amplify ability of this DNA with primer pair ITS1 and ITS4 (50 °C annealing temperature programme).

Fungal taxa	Ref. No.	Amplification result (indicated by a + sign)		
		No dilution	10X dilution	50X dilution
Alternaria sp.	SL 20			+
Aspergillus sp.	SL 3			+
Beauvaria sp.	SL 48		+	
Chaetomium sp.	SL 13		+	
Cladosporium sp.	SL 34			+
Dicyma sp.	SLE 10			+
Epicoccum sp.	SL 9			+
Fusarium sp. 1	SLE 16		+	
Fusarium sp. 2	SLE 20			+
Fusarium sp. 3	SLE 4			+
Clonostachys sp. 1	SL 35		+	
Clonostachys sp. 2	SL 23			+
Melanospora sp.	SL 15		+	
Nigrospora sp.	SLE 17			+
Penicillium sp.	SL 101			+
Pestalotia sp.	SL 47		+	
Sarcostroma sp.	SL 82			+
Sordaria sp.	SLE 80			+
Trichoderma sp.	SL 6		+	
Unidentified Hyphomycete 1	SL 49	+		
Unidentified Hyphomycete 2	SL 85		+	
Unidentified Coelomycete	SL 83		+	

annealing temperature drops to 56 °C, which facilitates less specific binding to the DNA. As this protocol results in an increase in the number of specific DNA fragments, these are more likely to be used as subsequent annealing templates, and are thus more likely to be copied.

The design of a multiplex reaction, which can test for both South African genera simultaneously, was crucial, as the same arthropod could serve as the vector for both genera at the same time. Two species of ophiostomatoid fungi are, for example, are known to co-occur on *Protea repens*, namely *O. splendens* and *G. proteae*. They are known to infect the same flower head at the same time (Marais 1996), and any arthropod can potentially come into contact with both these fungal species during a single visit. Beside these biological advantages, reducing arthropod scans to a single run testing for both fungi simultaneously also brought down costs significantly.

Specificity testing of the combined designed primer pairs in the multiplex reactions revealed that this method caused no loss in specificity. The expected fragment sizes were obtained for all the ophiostomatoid genomic DNA, while no amplification was detected when template DNA from any other fungal species (Table 1) was used (Fig. 1). Amplification was achieved from a minimum of 45 pg of genomic DNA for *Ophiostoma* and 30 pg genomic DNA for *Gondwanamyces*. After the development of the multiplex PCR method, this protocol was tested for its application in the detection of South African ophiostomatoid fungal spores on inoculated insects.

Ophiostomatoid fungal DNA could be detected on all inoculated arthropods (Table 3), while none of the control arthropod specimens showed amplification with the designed primers. However, all positive amplification results obtained from *Protea* arthropods should be sequenced and compared to the known sequences of the ophiostomatoid fungi in order to verify the presence of these species.

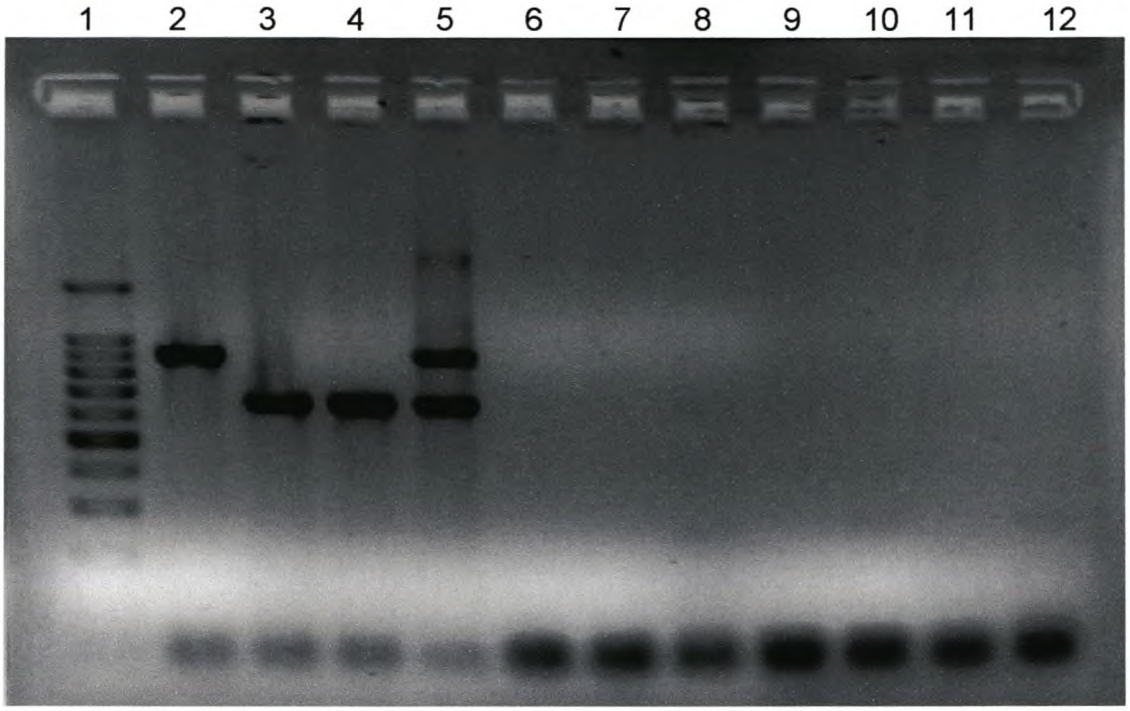

Fig. 1 1, 5 % Agarose (Promega corporation, Madison, U.S.A.) gel in Tris-acetate-EDTA buffer (TAE) containing ethidium bromide and viewed under an UV transilluminator, showing the specificity of developed primers (OSP1 and GPR1) using the newly developed multiplex reaction protocol. Lane 1, DNA size marker (100 bp ladder) which show banding at 1 500 bp, 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp 400 bp and 300 bp lengths of DNA; Lane 2, *O. splendens* DNA; Lane 3, *G. proteae* DNA; Lane 4, *G. capensis*; Lane 5, combined *Ophiostoma* and *Gondwanamyces* DNA; 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.

The adjusted rapid detection method developed by Kim *et al.* (1999) failed to produce fungal DNA that could be amplified. It is possible that proteins and other cellular chemicals present in insects interacted with the extracted DNA, making amplification using this method impossible.

The multiplex PCR method developed here enables the rapid and accurate identification of DNA of the Southern Hemisphere *Ophiostoma* and

Gondwanamyces fungal genera on arthropod vectors. These methods are therefore ideally suited for the identification of the natural arthropod vector organism(s) of these two fungal groups. As this method of detection is group specific, the same method and primers (OSP1 and GPR1) can be used to detect the insect vectors of all the ophiostomatoid fungi known to occur naturally on South African *Protea* species. The technique thus has the potential to help us understand the broader ecological processes occurring in the Cape Fynbos and in our economically important family Proteaceae. This method may also find broader application in the identification of pathogen vectors on other crop plants.

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Appendix 1 Aligned South African ophiostomatoid fungal 18s rDNA sequence data obtained from GenBank shown here in the 5' to 3' direction. Areas in grey indicate binding areas for the newly developed primers OSP1 and GPR1 and the universal right binding primer LR6. Dashes represent gaps in the aligned DNA sequences, while dots represent nucleotides that correspond to those of *O. splendens*. (Osplend = *O. splendens*, Oafrican = *O. africanum*, Oprotea = *O. protearum*, Gproteae = *G. proteae* and Gcapen = *G. capensis*).

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Osplend     ---------- ---------T CAATAAGCGG AGGAAAAGAA ACCAACAGGG ATTGCCCC-A
Gproteae    ---------- ---------. .......... .......... .......... .......AT.
Oafrican    ---------- ---------. .......... .......... .......... ........-.
Gcapen      ---------- ---------. .......... .......... .......... .......AT.
Oprotea     ---------- ---------. .......... .......... .......... ........-.

Osplend     GTAACGGCGA GTGAAGCG-C AACAGCTCAA ATTTGAAATC TGGCCCCCC- ----GGCCCG
Gproteae    .......... .C..C..--A .......... .......... ...T.---TT C-TG......
Oafrican    .......... ........-. .......... .......... ....TA.ATT CAGT..T...
Gcapen      .......... .C..C.---. .......... .......... .........- ----......
Oprotea     .......... ........-. .......... .......... ....TA.ATT CAGT..T...

Osplend     AGTTGTAATT TGGAGAGGAT GGTTTTGGCG CGGCGCC-GT CCGAGTTCCT T-GGAACAGG
Gproteae    .......... ..C....... .C..C....A A..T...-T. .........C .-.....G..
Oafrican    .......... ..T....... .......... .......-.. .......... .-........
Gcapen      .......... .......... .C..C....A A..T...-T. .........C .-.....G..
Oprotea     .......... ..T....... .......... .......-.. .......... .-........

                                                  OSP 1 Binding Area

Osplend     ACGCCACAGA GGGTGAGAGC CCCGTACGGA ---CGGACGC CTAGCCTCTA CAAAGCTCC-
Gproteae    ......T... .......... .........T ---T....A. .G....GT.G TGT.......-
Oafrican    .......... .......... .......... ---.......... .......... .......-
Gcapen      ......T... .......... .........T ---T....A. .G....GT.G TGT.......-
Oprotea     .......... .......... .......... ---.......... .......... .......-

Osplend     ------TTCG ACGAGTCGAG TAGTTTGGGA ATGCTGCTCT AAA-TGGGAG GTAAATTTCT
Gproteae    ------...A .......... .......... .......... ...-...... ...T..C...
Oafrican    ------.... .......... .......... .......... ...-...... ..........
Gcapen      ------...A .......... .......... .......... ...-...... ...T..C...
Oprotea     ------.... .......... .......... .......... ...-...... ..........

Osplend     TCTAAAGCTA AATAC--GC- -AGAGA-CGA TAGCGCACAA GTAGAGTGAT CGAAAGATGA
Gproteae    .......... .....--..- -.....-... .......... ....GA.... ..........
Oafrican    .......... .....--..- -.....-... .......... .......... ..........
Gcapen      .......... .....--..- -.....-... .......... .......... ..........
Oprotea     .......... .....--..- -.....-... .......... .......... ..........
```

Appendix 1 (cont.)

```
Osplend    AAAGCACTTT GAAAAGAGGG TTAAA--GCT ACGTGAAATT GTTGAAAGGG AAGCGCCTGT
Gproteae   .......... ........A. .....CA..- .......... .......... ......T...
Oafrican   .......... .......... .....--... .......... .......... ..........
Gcapen     .......... ........A. .....CA..- .......... .......... ......T...
Oprotea    .......... .......... .....--... .......... .......... ..........

Osplend    GACCAGACTT GCGCCCCCG- TGGACCA-CC G-CGTTCTC- GCCGGTGC-- ACT---CCGC
Gproteae   .......... .T....--.G ..A.TT.G.T .GT......- A..A..CG-- ...TTG..--
Oafrican   .......... .........- .......-.. .-........- ........-- ...---....
Gcapen     .........- .T....--.G ..A.TT.G.T .GT......- A..A..CG-- ...TTG..--
Oprotea    .......... .........- .......-.. .CG-.....- ........-- ...---....
```

GPR 1 Binding Area

```
Osplend    GGGCGCAGG- CAGCATCGGT TC-TCCCAGG GGGATAAAGG CCGCGGGAAC GTAGCTC-T-
Gproteae   -...T....C .......... .T-GTTATT. ....G...AA .GAT.....A ..G....-.C
Oafrican   .........C .......... ..-...T... ....C....A .......... .......-.-
Gcapen     -...T....C .......... .T-GTTATT. ....G...AA .GAT.....T ..G....TCT
Oprotea    .........C .......... ..-...T... ....C....A .......... .......-.-

Osplend    TC-------G GGAGTGTTAT AGCCCGCGGT GGCATGCCCC TGGGGG-GAC CGAGGACCGC
Gproteae   ..-------. .......... .......C.. ATA..A...T .T..-CA... ..........
Oafrican   ..-------. .......... .......... .......... ....-.G... ..........
Gcapen     C--------. .......... .......C.. ATA..A...T .T..-CA... ..........
Oprotea    ..-------. .......... .......... .......... ......-... ..........

Osplend    GCTT------ ---CGGCAA- ---------- ---------- ---------- ----------
Gproteae   ..A.------ ---.T...T- ---------- ---------- ---------- ----------
Oafrican   ....------ ---......- ---------- ---------- ---------- ----------
Gcapen     ..A.------ ---.T...T- ---------- ---------- ---------- ----------
Oprotea    ....C----- ---...-..- ---------- ---------- ---------- ----------

Osplend    -------GGA TGCT-G-CG- TAA-TGGTCA CAGGACGCCC GT-CTTGAAA CACGGACCAA
Gproteae   -------... ....-.-..- ...-...... .......... ..-....... ..........
Oafrican   -------... ....-.-..- ...-...... .......... ..-....... ..........
Gcapen     -------... ....-.-..- -..-...... .......... ..-....... ..........
Oprotea    -------... ....-.-..- ...-...... .......... ..-....... ..........

Osplend    GGAGT-CAAC ACTTGGG-CG AGTGTATGGG TGCCAAACGC CA-CGCGCAA ATGAAAGTAA
Gproteae   .....-.... CT.AT.TG.A .....T.... ..TT....C. .TA....-G. ..---C..G.
Oafrican   .....T.... .......-.. .......... .......... ..-....... ..........
Gcapen     .....-.... CT.AT.TG.A .....T.... ..TT....C. .TA....-G. ..T..C..G.
Oprotea    .....T.... .......-.. .......... .......... ..G....... ..........

Osplend    ATCGCAGGTG AGAGC--TTC -GG-CGCATC ATCGACCGAT CCTGATGTCT TCGGATGGAT
Gproteae   .-..T..... .....--... -..G...... .......... T.......TC ..........
Oafrican   .......... .....--... -..-...... .......... .......... ..........
Gcapen     .-..T..... .....--... -..G...... .......... T.......TC ..........
Oprotea    .......... .....--... -..-...... .......... .......... ..........
```

Appendix 1 (cont.)

```
Osplend     TTGAGTAAGA GCCTTACCGC TTGGACCCGA AAGATGGTGA ACTATGCCTG AATAGGGTGA
Gproteae    .......... ..ACACAG.G .......... ....A..... .......T.. T.........
Oafrican    .......... .......... .......... .......... .......... ..........
Gcapen      .......... ..ACACAG.G .......... ....A..... .......T.. T.........
Oprotea     .......... .......... .......... .......... .......... ..........

Osplend     AGCCAGAGGA AACTCTGGTG GAGGCTCGCA GCGGTTCTGA CGTGCAAATC GATCGTCAAA
Gproteae    .......... .......... .......... .......... .......... ..........
Oafrican    .......... .......... .......... .......... .......... ..........
Gcapen      .......... .......... .......... .......... .......... ..........
Oprotea     .......... .......... .......... .......... .......... ..........

Osplend     TTTGGGCATG GGGGCGAAAG ACTAATCGAA CCTTCTAGTA GCTGGTTACC GCCGAAGTTT
Gproteae    .A..A..... .......... .......... .......... .......C.A ..........
Oafrican    .......... .......... .......... .......... .......... ..........
Gcapen      .A..A..... .......... .......... .......... .......C.A ..........
Oprotea     .......... .......... .......... .......... .......... ..........

Osplend     CCCTCAGGAT AGCAGTGTTG TTTT---CAG TTTT-TGAGG TAAAGCGAAT GATTAGGGAC
Gproteae    .......... .......... A.C.CT-... ....A..... .......... ..........
Oafrican    .......... .......... ....---... ....-..... .......... ..........
Gcapen      .......... .......... A.C.CTT... ....A..... .......... ..........
Oprotea     .......... .......... ....---... ....-..... .......... ..........

Osplend     TCGGGGGCGC CTATT-AGCC TTCATCCATT CTCAAACTTT AAATATGTAA GAAGCCCTTG
Gproteae    .......... -....T.... .......... .......... .......... ..........
Oafrican    .......... .....-.... .......... .......... .......... ..........
Gcapen      .......... -....T.... .......... .......... .......... ..........
Oprotea     .......... .....-.... .......... .......... .......... ..........
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LR 6-

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Osplend     TTGCTTAATT GAACGTGGGC ATTCGAATG- -CAACAACAC TAGTGGGCCA TTTTTGGTAA
Gproteae    ..A....GC. .......... .........T G...---... .......... .....-----
Oafrican    ..A....G.. .......... .........T G...---... .......... .....-----
Gcapen      ..A....GC. .......... .........T G...---... .......... .....-----
Oprotea     .......... .......... .........- -......... .......... .....-----
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Binding Area

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Osplend     GCAGAACTGG CG-------- ---------- ---------- ---------- ----------
Gproteae    .......... CG-------- ---------- ---------- ---------- ----------
Oafrican    .......... CG-------- ---------- ---------- ---------- ----------
Gcapen      .......... CG-------- ---------- ---------- ---------- ----------
Oprotea     .......... CG-------- ---------- ---------- ---------- ----------
```

CHAPTER 2

POSSIBLE VECTOR INSECTS OF THE OPHIOSTOMATOID FUNGI ASSOCIATED WITH *PROTEA REPENS*

Abstract

Over 35 arthropod species (375 individuals) belonging to 32 families were collected from the infructescences of *P. repens* between Jan. 2001 and Jul. 2002. These were tested for the presence of ophiostomatoid fungal DNA on their surfaces using a newly developed multiplex Polymerase Chain Reaction (PCR). This assay was developed for the specific amplification of 18s rDNA fragments of *Ophiostoma* and *Gondwanamyces* species, both of which are associated with *Protea* species only. Six possible vector organisms are identified; three of which could act as the main organisms responsible for the dispersal of ophiostomatoid spores between *P. repens* infructescences. They are *Genuchus hottentottus* (Scarabaeidae), *Oxycarenus maculates* (Lygaeidae) and *Sphenoptera* sp. (Buprestidae). Further studies entailing the sequencing of the amplified PCR products, are needed to confirm the identity of the specific organisms responsible for the dispersal of *Ophiostoma* sp. and *Gondwanamyces* sp. spores between *P. repens* infructescences.

Introduction

The Cape Floristic Region (CFR) is of significant economic importance to South Africa, as it is a major attractant for the thriving local eco-tourism industry. The most famous aspect of this unique region is its great floral diversity. It is estimated that the CFR contains well over 9 000 vascular plant species (Goldblatt & Manning 2000), many of which produce flowers and other organs that are exported (e.g. leaves used as fillers in flower arrangements).

The Proteaceae is one of the most important plant families in this region. Members of the Proteaceae are often keystone members in the Fynbos biome, and are usually the structural dominant members of the vegetation (Cowling & Richardson 1995). The Proteaceae is extremely diverse, with at least ten endemic genera present in the south-western Cape alone (Rourke 1998). The family contains many species of which flowers are commercially grown for the export and dried flower industries. Fungi that directly or indirectly influence Proteaceae population dynamics could, therefore, lead to substantial commercial and ecological losses.

Since the discovery of five species of ophiostomatoid fungi colonising the infructescences of some serotinous *Protea* L. species from South Africa (Wingfield & Van Wyk 1993; Marais & Wingfield 1994; Marais 1996), there has been renewed interest in this biologically interesting group of fungi. This discovery was the first record of the presence of native Southern Hemisphere ophiostomatoid fungi. South African ophiostomatoid fungi are grouped into two genera, namely *Ophiostoma* H. Syd. & P. Syd. (*Ophiostoma protearum* Marais & Wingf., *O. splendens* Marais & Wingf. and *O. africanum* Marais & Wingf.) and the *Gondwanamyces* Marais & Wingf. (*G. capensis* (M.J. Wingf., & P.S. Van Wyk) Marais & Wingf. and *G. proteae* (M.J. Wingf., P.S. Van Wyk & W.F.C. Marasas) Marais & Wingf. It is believed that the *Protea* ophiostomatoid fungi are non-

pathogenically associated with their hosts, as the fungi only colonise dead floral parts (Marais 1996, Roets 2000; Chapter 3).

The dispersal of ophiostomatoid fungi is biologically interesting, as insects are known to act as vectors for many of the fungi in this group (Marais 1996). Most ophiostomatoid fungi are morphologically adapted for insect spore dispersal. They have flask shaped perithecial bases and long perithecial necks. Spores collect at the tip of the necks in sticky masses. Here passing insects can come into close contact with these spores, and can readily disperse them from one host plant to the next (Marais 1996). The morphology of the *Protea* fungi is similar, displaying both a flask shaped base and a long neck. This suggests that their spores are also insect or arthropod dispersed (Marais 1996). The Southern Hemisphere ophiostomatoid fungi are found within the closed infructescences of some *Protea* species. It would therefore also be difficult to disperse spores by means of any other vector (wind or water) than arthropods. The vectors for many of the Northern Hemisphere ophiostomatoid fungi have already been identified (Davidson & Robinson-Jeffrey 1965; Davidson *et al.* 1967; Davidson 1978; Dowding 1984; Harrington & Cobb 1988; Harrington 1993; Kile 1993; Malloch & Blackwell 1993; Nag Raj & Kendric 1993). Insects responsible for dispersing ophiostomatoid species of the Northern Hemisphere are mostly species of bark beetles and weevils. Many vector insects, belonging to a wide range of other families and orders, have also been identified. Insects belonging to some of the same vector families (e.g. Curculionidae) and orders (e.g. Coleoptera) were also collected from Southern Hemisphere *Protea* species (Guess 1968; Myburg *et al.* 1973, 1974; Myburg & Rust 1975a, b; Coetzee & Giliomee 1987a, b; Coetzee 1989; Wright 1990; Visser 1992; Chapter 4). It is very likely that one or more of these insects act as vector for the South African ophiostomatoid species.

Protea ophiostomatoid fungi have thus far only been found in insect-infested flower heads (Wingfield *et al.* 1988; pers. observ.). It is, however, still unclear which specific insects act as vectors for these fungi. The main aim of the present

study was to detect possible vector arthropods for ophiostomatoid species associated with *Protea repens* L. Finding the insect vectors of these fungi on *Protea* species will greatly expand our current knowledge and understanding of the biology and ecology of ophiostomatoid fungi.

Materials and Methods

Insect collection

Infructescences of *Protea repens* were periodically collected between Jan. 2001 and Jul. 2002 (See Table 1) from Stellenbosch Mountain (S: 33º 56.743' E: 18º 52.716') and Jonkershoek Nature Reserve (S: 33º 59.555' E: 18º 58.287'). They were then placed in a fridge (4 °C) to reduce the activity of the arthropods contained within them. Hereafter, arthropods were extracted from the infructescences by cutting them open with pruning sheers and then shaking the animals out. Boring insects were extracted with the aid of tweezers. All arthropod collections were done under sterile conditions. The individual arthropods were sorted into pseudospecies, and all individuals of a given pseudospecies were placed together in a specimen vial. The animals were then stored at –20 °C until further use.

Screening the arthropods for the presence of ophiostomatoid fungal DNA

The DNA extraction method developed in Chapter 1 was used to extract DNA from fungal spores found on the surface of the animals. All arthropods that were tested are summarised in Table 1. In many instances more than one individual of a particular pseudospecies were used in a single extraction (Table 1) in order to increase the initial concentration of extracted fungal DNA. Three different concentrations of each extraction (No further dilution, 10X dilution and 50X dilution) were tested for its success of amplification with the multiplex PCR

detection method. This method was developed (Chapter 1) especially for the detection of fungal 18s rDNA of *Ophiostoma* and *Gondwanamyces* species only. Amplified DNA was separated on a 1,5 % Agarose (Promega corporation, Madison, U.S.A.) gel in TAE buffer containing ethidium bromide and visualised under UV light.

Results and Discussion

A total of 39 arthropod species (375 individuals) belonging to 32 different arthropod families were isolated from *P. repens* infructescences (Table 1). Despite the fact that over 300 reactions were performed, positive amplification of DNA extracted from the spores found on the surface of these arthropods was only achieved for very few of the reactions (Table 1). Positive scores were obtained for the following arthropod pseudospecies: Archaeognatha, Blattidae, *Crematogaster* sp., *Oxycarenus maculates* Stal. and *Spenoptera* sp. (Table 1). It was, unfortunately not possible to verify the positive results by sequencing of the PCR products obtained, due to problems with the purification of the PCR products from the gel matrix. However, if we take the high specificity of the PCR primer pairs used in the multiplex reaction into account (Chapter 1), we can predict with a high degree of certainty that these positive amplifications represent amplification of *Ophiostoma* and *Gondwanamyces* DNA only. PCR amplification of fragments with lengths of ca. 600 and 900 bp respectively is very likely to represent positive indications of the presence of *G. proteae* and *O. splendens*, as these are the only two ophiostomatoid species known to occur on *P. repens*.

Two arthropod species showed positive amplification of a 600 bp fragment length, while four arthropod species showed positive amplification of a 900 bp fragment. One insect species (*Crematogaster* sp.) tested positive for both 600 bp and 900 bp lengths (Table 1). It is evident that, if these positive results do indeed represent amplification of ophiostomatoid fungal DNA, these fungi do not seem to

be vector specific. It appears as if they rely on many arthropods to disperse spores from one fruiting structure to the next. Many Northern Hemisphere ophiostomatoid fungi are also known to rely on more than one insect for spore dispersal (Harrington 1988). However, it is possible that the *Protea* fungi are usually dispersed by a certain vector species, as was found to be the case for some Northern Hemisphere ophiostomatoid species (Alexander *et al.* 1988; Harrington 1988). Indirect confirmation of this is the fact that the arthropods responsible for the dispersal of these fungi are probably highly mobile (i.e. able to fly). This is necessary because, in many instances, these fungi need to be dispersed over great distances. In one case, the distance between an isolated ophiostomatoid colonised plant and the nearest next *P. repens* population was *ca.* 400-500 metres. If this is the case, then members of the Blattidae (Cockroaches), *Crematogaster* sp. (Ant) and Archaeognatha (Fish-moth like insects) could not be the usual vectors, even though they showed positive amplification with the developed primers, as they are unable to fly. The vector species should also be fairly common, as the colonisation numbers for these fungi can be very high (Chapter 3). Both *O. maculates* and *Spenoptera* sp. are common on *P. repens* (Chapter 4) and are highly mobile (both are winged). They are thus more likely to act as a vector of *O. splendens* and *G. proteae* between different *P. repens* plants. The other arthropods that showed positive amplification may also disperse fungal spores, but they will most likely only be able to disperse fungal spores over short distances (e.g. between different infructescences on the same plant or neighboring plants).

It was curious that, when ophiostomatoid fungi were found within the infructescences of *P. repens* collected from Stellenbosch Mountain during Jul. 2002, 16 out of 18 fungal-colonised infructescences studied showed distinct signs of damage done by the borer *Genuchus hottentottus* (F). Damage done by the larvae of this insect is very distinctive, as they destroy most of the seeds in the infructescences they infest (Coetzee & Giliomee 1987b; pers. observ.). The larvae are also easily distinguished from all other larval species found within an

infructescence. Additional factors suggesting *G. hottentottus* as another potential vector are the fairly common occurrence of this insect (Chapter 4) and the ability of adults to fly. They visit inflorescences of *Protea* species to feed on pollen and nectar (Coetzee & Giliomee 1987b) and could in the process transfer fungi from a fungal-colonised infructescence (from which the emerged as adults) to an uncolonised inflorescence. The dispersed fungal spores can start to grow after the inflorescences close and conditions become more suitable for fungal growth. Very few adult *G. hottentottus* specimens were encountered during this study, as only insects enclosed within infructescences were collected. Here the insects are usually found only in their larval or pupae stages. Future studies that aim to identify the specific vector organisms for *Protea* ophiostomatoid fungi, should thus also include flower visitors.

When *G. hottentottus* bores into an infructescence, it leaves moist plant material (trichomes and other particles from the flowers) mixed with excreta (Coetzee & Giliomee 1987b). This could enhance fungal growth and indicate a possible symbiotic effect between the insect and the fungus. It is thought that the *Protea* ophiostomatoid fungi deter the growth of other fungal species in infected infructescences, because, when these fungi are found inhabiting *Protea* infructescences, very few other fungal species are present (M.J. Wingfield, pers. comm.; pers. observ.). If this is the case, then there could be a mutualistic relationship between *G. hottentottus* and the fungus. The insect could disperse the fungi and make conditions more favourable for fungal growth, while the fungi deters the growth of other fungal species that may attack the larvae or pupae of *G. hottentottus.*

Table 1 Multiplex PCR amplification results for DNA extracted from the surface of arthropods collected from the infructescences of *P. repens* (Jan. 2001 to Jul. 2002). Positive results (amplification of 18s rDNA fragments by using the varying annealing temperature PCR programme) are indicated by the length of the resultant PCR amplification products (ca. 600 bp and 900 bp for *G. proteae* and *O. splendens* respectively), while negative results are indicated by a dot. Reference arthropod specimens are housed in the insect collection of the Department Entomology and Nematology (USEC), University of Stellenbosch, Stellenbosch, South Africa. See the end of Table for an explanation of symbols.

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Aradidae	Jan. (S)		2	.	.	.
Aradidae	Apr. (J)		1	.	.	.
Aradidae	Jan. (S)		2	.	.	.
Aradidae	Feb. (S)		1	.	.	.
Aradidae	Jun. (S)		2	.	.	.
Archaeognatha	Apr. (S)		4	.	.	.
Archaeognatha	Mar. (S)		13	.	.	.
Archaeognatha	Jan. (S)		1	.	.	.
Archaeognatha	Jan. (S)		1	.	.	.
Archaeognatha	May (S)		2	.	.	.
Archaeognatha	May (S)		1	600 bp	.	.
Archaeognatha	May (S)		6	.	.	.
Archaeognatha	Jul. (S)		1	.	.	.
Arthropleona (Collembola)	Mar. (S)	9	1	.	.	.
Blattidae	Feb. (S)	26	1	.	.	.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Blattidae	May (S)	26	1	900 bp	.	.
Blattidae	May (S)	26	2	900 bp	.	.
Braconidae	Apr. (S)		4	.	.	.
Bruchidae	Feb. (J)		1	.	.	.
Carabidae (larvae)	Jan. (S)		1	.	.	.
Carabidae (larvae)	Mar. (S)		1	.	.	.
Carabidae (larvae)	May (S)		1	.	.	.
Chironomidae	Jul (S)		1	.	.	.
Chrysomelidae (sp. 1)	Feb. (J)	18	1	.	.	.
Chrysomelidae (sp. 1)	Mar. (S)	18	1	.	.	.
Chrysomelidae (sp. 1)	Mar. (S)	18	1	.	.	.
Chrysomelidae (sp. 1)	Apr. (S)	18	1	.	.	.
Chrysomelidae (sp. 2)	May (S)	17	2	.	.	.
Clubionidae (spider sp. 1)	Jan. (S)		1	.	.	.
Coccinellidae	May (S)		1	.	.	.
Crematogaster sp. (Formicidae)	Jan. (S)	15	9	.	.	.
Crematogaster sp. (Formicidae)	May (S)	15	30	.	.	.
Crematogaster sp. (Formicidae)	May (S)	15	6	.	.	.
Crematogaster sp. (Formicidae)	May (S)	15	5	900 bp + 600 bp	600 bp	.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Crematogaster sp. (Formicidae)	May (S)	15	8	.	.	.
Crematogaster sp. (Formicidae)	Jul. (S)	15	15	.	.	.
Crematogaster sp. (Formicidae)	Jul. (S)	15	5	.	.	.
Cryptophagidae	Jul. (S)		2	.	.	.
Curculionidae (sp. 2)	May (S)		1	.	.	.
Diptera (larvae)	Apr. (J)		6	.	.	.
Euderes lineicolis (Curculionidae)	Apr. (S)	33	1	.	.	.
Euderes lineicollis (Curculionidae) (larvae)	Jan. (S)	28	1	.	.	.
Formicidae (sp. 1)	May (S)		3	.	.	.
Formicidae (sp. 2)	Apr. (S)	23	32	.	.	.
Formicidae (sp. 2)	May (S)	23	4	.	.	.
Formicidae (sp. 2)	Jul. (S)	23	2	.	.	.
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	May (S)	14	1	.	.	.
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	May (S)	14	1	.		.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	May (S)	14	1	.		.
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	Jul. (S)	14	1	.		.
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	Jul. (S)	14	1	.		.
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	Jul. (S)	14	1	.		.
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	Jul. (S)	14	1	.		.
Genuchus hottentottus (F) (Scarabaeidae) (adult)	Apr. (S)	14	1	.	.	.
Histeridae	Jan. (S)	32	2	.	.	.
Hymenoptera (pupae)	Feb. (S)	38	1	.	.	.
Lygaeidae (sp. 1)	Jul. (S)	26	1	.	.	.
Membracidae	May (S)		1	.	.	.
Mite sp. 1	Jan. (S)		4	.	.	.
Mite sp. 2	Jul. (S)		3	.	.	.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Oxycarenus maculates Stal. (Lygaeidae)	Mar. (S)	7	10	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Apr. (S)	7	7	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Jan. (S)	7	3	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Jan. (J)	7	14	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Mar. (J)	7	21	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Jan. (S)	7	7	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Mar. (S)	7	3	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Jan. (S)	7	9	900 bp	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Mar. (S)	7	1	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Jun. (S)	7	1	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	May (S)	7	12	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	May (S)	7	1	.	.	.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Oxycarenus maculates Stal. (Lygaeidae)	May (S)	7	6	.	.	.
Oxycarenus maculates Stal. (Lygaeidae)	Jul (S)	7	2	.	.	.
Pentatomidae	Jan. (S)	24	1	.	.	.
Pentatomidae	Mar. (S)	24	1	.	.	.
Pentatomidae	Jul. (S)	24	1	.	.	.
Psocoptera	Mar. (S)	13	2	.	.	.
Psocoptera	Jan. (S)	13	3	.	.	.
Psocoptera	Jul. (S)	13	23	.	.	.
Reduviidae	Mar. (J)		1	.	.	.
Scutelleridae	May (S)		1	.	.	.
Solifugidae	Mar. (S)		1	.	.	.
Solifugidae	Jan. (S)		1	.	.	.
Sphenoptera sp. (Buprestidae) (mature)	May (S)	30	1	900 bp	900 bp	.
Sphenoptera sp. (Buprestidae) (pupae)	Jan. (S)		1	.	.	.
Sphenoptera sp. (Buprestidae) (pupae)	Mar. (S)		1	.	.	.
Sphenoptera sp. (Buprestidae) (pupae)	Mar. (J)		1	.	.	.
Sphenoptera sp. 1 (Buprestidae) (larvae)	Mar. (S)	37	2	.	.	.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Sphenoptera sp. 1 (Buprestidae) (larvae)	Jan. (J)	37	2	.	.	.
Sphenoptera sp. 1 (Buprestidae) (larvae)	Jun. (s)	37	2	.	.	.
Sphenoptera sp. 1 (Buprestidae) (larvae)	May (s)	37	1	.	.	.
Sphenoptera sp (Buprestidae) (adult)	Apr. (S)		3	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	Mar. (S)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	Feb. (S)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	Feb. (J)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	Feb. (S)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	Jun. (S)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	May (S)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	May (S)	30	1	.	.	.
Sphenoptera sp. 2 (Buprestidae) (larvae)	Jul. (S)	30	1	.	.	.

Table 1 (cont.)

Arthropod pseudospecies	Collection date and site	Ref. No.	Number of individuals used per extraction	Amplification result		
				No dilution	10X dilution	50X dilution
Sphenoptera sp. 2 (Buprestidae) (larvae)	Jul. (S)	30	1	.	.	.
Spider sp. 1	Jan. (S)		1	.	.	.
Spider sp. 2	Feb. (S)		1	.	.	.
Spider sp. 2	Mar. (S)		1	.	.	.
Spider sp. 2	May (S)		1	.	.	.
Spider sp. 2	Jul. (S)		1	.	.	.
Spider sp. 3	Jan. (S)		1	.	.	.
Spider sp. 3	Mar. (J)		1	.	.	.
Spider sp. 3	May (S)		1	.	.	.
Spider sp. 4	Feb. (S)		1	.	.	.
Thysanoptera	Mar. (S)	34	1	.	.	.

(J) = Jonkershoek, (S) = Stellenbosch Mountain

Symbiotic relationships have been found between some Northern Hemisphere ophiostomatoid species and their vector-insects. The insects attack the plants, constructing galleries, and in the process make conditions favourable for ophiostomatoid fungal growth (Harrington & Cobb 1988). In some cases, the symbiotic relationship between bark beetles and pathogenic *Ophiostoma* species have been found to be mutualistic. The fungi infect areas where beetles have invaded, which sometimes can lead to the death of the host. This creates more suitable environments for the insects to breed (Berryman 1972). Several reports

on the association between *Ophiostoma* species and insects have been published (Graham 1967; Dowding 1984).

The positive amplification results achieved for the insects listed in Table 1 might also be due to the presence of other fungal species not included in tests for primer specificity (Chapter 1). It should thus be evident that, in order to make final decisions about the vectors for *O. splendens* and *G. proteae* on *P. repens*, verifiable amplifications (verified with DNA sequencing) must be obtained. It must also be remembered that the presence of fungal spores alone cannot be used as proof that a certain organism is the vector for these fungi. To prove this Koch's postulates must be satisfied (Hansen *et al.* 1988). These postulates specify that insects (1) must constantly be collected from fungi-colonised infructescences, (2) insects must visit uncolonised infructescesces under conditions suitable for fungal transmission, (3) fungal spores must be present on insects in the field and (4) the insects must transmit the fungus in the field and under laboratory conditions.

It is unlikely that the positive amplification results obtained (Table 1) are due to the amplification of DNA from the insects themselves. If this was the case, then all the individuals of that particular species should, theoretically, also show positive amplification using this method. This was not found to be the case (Table 1). Further studies are presently being undertaken to determine the specific vector organisms for the South African ophiostomatoid species.

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CHAPTER 3

SEASONAL TRENDS IN OPHIOSTOMATOID FUNGAL COLONISATION OF *PROTEA* INFRUCTESCENCES

Abstract

Seasonal colonisation numbers of ophiostomatoid fungi in the fruiting structures of serotinous Proteaceae species was investigated. New fungal and host species were determined, and their ability to colonise plant tissues investigated. Although fruiting structures of a wide range of Proteaceae were included in this study, ophiostomatoid species were exclusively collected from the infructescences of serotinous species of *Protea*. A definite seasonal pattern was observed, with fungal infection numbers peaking during the wetter, winter months. Competition seems to occur when two ophiostomatoid species co-inhabit the same *Protea* host. *P. laurifolia* was found to be a new host for *Ophiostoma splendens* and *Gondwanamyces capensis*, while a new species of *Rhynchomeliola* was collected from the infructescences of *P. laurifolia* and *P. burchellii*. This fungal species was found to share the same ecological niche and dispersal mechanism as the ophiostomatoid fungi. Sectioning of flower parts showed the ophiostomatoid fungi to probably be non-pathogenic to *Protea* species, as no perithecia were detected penetrating plant tissue, and these fungi were found to be prolific only on the dead floral parts. These results highlight the presence of a unique group of fungi that are adapted for insect dispersal, that exist within *Protea* infructescences. Their vectors and specific ecological function, however, remain to be determined.

Introduction

The south-western Cape is world renowned for it's floral diversity. This area includes the Fynbos Biome, which contains most of the *ca.* 9 000 plant species (Goldblatt & Manning 2000) found within the Cape Floristic Region (CFR). The Fynbos contains three dominant plant families: the Ericaceae, Restionaceae and the Proteaceae (Cowling & Richardson 1995), of which the Proteaceae is often the dominant and keystone members.

The Proteaceae is diverse in terms of species numbers and in the range of morphological forms that exist within the family. The south-western Cape alone hosts more than 330 species in 14 genera (Rebelo 1995). In total 13 of these genera are Cape-centred with 10 endemic to the area (Rourke 1998). The Proteaceae is the seventh largest family of vascular plants in the CFR, with about 96.7% of its members confined to this area (Goldblatt & Manning 2000). Proteas are of considerable economic importance, especially in the fields of eco-tourism, horticulture and the dried-flower industry. Proteaceae fungal diseases could cause monetary losses amounting to millions of rands.

Several fungi have been discovered that are closely associated with the Proteaceae (Crous *et al.* 2000a, b; Swart *et al.* 2000; Taylor & Crous 2000). Many of these cause fungal infections, including diseases of the leaves, stems, roots and seedlings (Crous *et al.* 2000a). Very limited attention has, however, been focused on the apparently non-pathogenic fungi associated with these plants (Marais & Wingfield 1994; Taylor *et al.* 2001). Although apparently not damaging to plants, they may also cause phytosanitary problems.

Five ophiostomatoid fungal species are currently known to inhabit the infructescences of some serotinous *Protea* L. species, where they are thought to grow saprophytically (Marais 1996). These ophiostomatoid fungi are known to be associated with the infructescences of *Protea* species from South Africa only

(Marais 1996) and are grouped into two genera: Three species of *Ophiostoma* H. Syd. & P. Syd., *O. protearum* Marais & Wingf., *O. splendens* Marais & Wingf. and *O. africanum* Marais & Wingf. (*Sporothrix* Hektoen & Perkins anamorphs), and two species of *Gondwanamyces* Marais & Wingf., *G. capensis* (M.J. Wingf., & P.S. Van Wyk) Marais & Wingf. and *G. proteae* (M.J. Wingf., P.S. Van Wyk & Marasas) Marais & Wingf. *Knoxdaviesia* Wingf. *et al.* anamorphs occur on these infructescences.

Closely related fungi from the Northern Hemisphere are insect-vectored (Davidson & Robinson-Jeffrey 1965; Davidson *et al.* 1967; Davidson 1978; Dowding 1984), and cause important tree diseases such as Dutch elm disease (caused by *O. ulmi* (Buis.) Nannf.) (Braiser 1988) and oak wilt (caused by *Ceratocystes fagacearum* (Bretz) Hunt) (Sinclair *et al.* 1987). They can also assist aggressive bark beetles in overwhelming host tree resistance (Christeansen & Solheim 1990) indicating the existence of a possible mutualistic relationship between fungus and its vector insect.

The morphology of these fungi is indicative of insect spore dispersal. They have flask shaped perithecial bases and long necks. Spores collect at the tip of the neck in a sticky mass, where insects can readily come into contact with it. The morphology of the *Protea* ophiostomatoid fungi is no different, which suggests insect dispersal of their spores. Convergent evolution has resulted in the *Protea* fungi resembling those from the Northern Hemisphere (Wingfield *et al.* 1999). Based on a rDNA fungal phylogeny, however, they are quite distinct (Marais *et. al.* 1998). These fungi have thus far only been found in insect-infested *Protea* flower heads (Wingfield *et al.* 1988), and it is possible that one or more of these insects transport the fungal spores.

Insects associated with *Protea* infructescences belong to a wide range of families (Coetzee & Giliomee 1987a, b; Coetzee 1989; Wright 1990; Visser 1992) and

probably fulfil a range of ecological functions. The specific vectors of ophiostomatoid fungi on *Protea* spp. are still being investigated.

The present study sets out to examine the seasonality of ophiostomatoid fungi within *Protea* infructescences, while also scanning for new fungal and host species. The nature and extent of ophiostomatoid colonisation are also investigated. Results will help in the search for the vector of the South African ophiostomatoid fungi, while clarifying the number of fungi involved and elucidating their host ranges. This study will also contribute to better understanding of the ecological processes operational within the Fynbos Biome, which will help to determine better conservation strategies.

Materials and Methods

Seasonality and host specificity

Three-month to two-year-old infructescences and other fruiting structures of 11 Proteaceae species were collected from various sites in the Stellenbosch region, South Africa (Table 1). Ten fruiting structures of each of the species were collected on a monthly basis from Feb. 2000 to Jan. 2002, and searched (light microscope examination) for the presence of ophiostomatoid perithecia. Species were selected according to the availability of their infructescences in the Stellenbosch region.

A range of other Proteaceae taxa present at the above-mentioned sites and also in the Betties Bay – Kleinmond area and Cape Point, were spot-checked at various times of the year for the presence of ophiostomatoid fungi in their fruiting structures. These include a number of other *Protea* species (*P. compacta* R. Br.,

Table 1 Collection sites of Proteaceae infructescences and other fruiting structures in the Stellenbosch region.

	Population site	Grid reference
Leucadendron rubrum Burm. f.	Jonkershoek	S: 33° 58, 591' E: 18° 56, 817'
Leucadendron salignum Bergius	Jonkershoek	S: 33° 59, 210' E: 18° 57, 361'
Protea acaulos (L.)	Jonkershoek	S: 33° 59, 210' E: 18° 57, 361'
Protea burchellii Stapf.	Stellenbosch mountain	S: 33° 56, 743' E: 18° 52, 711'
Protea cynaroides L.	Jan S. Marais Park	S: 33° 55, 984' E: 18° 52, 375'
Protea eximia Fourc.	Jan S. Marais Park	S: 33° 55, 984' E: 18° 52, 375'
Protea laurifolia Thunb.	Stellenbosch mountain	S: 33° 56, 743' E: 18° 52, 716'
Protea neriifolia R. Br.	Jonkershoek	S: 33° 59, 555' E: 18° 58, 287'
Protea nitida Mill.	Jonkershoek	S: 33° 59, 210' E: 18° 57, 361'
Protea repens L.	Jonkershoek	S: 33° 59, 555' E: 18° 58, 287'
Serruria fasciflora Salisb. ex Knight	Jonkershoek	S: 33° 58, 591' E: 18° 56, 817'

P. lepidocarpodendron (L.) L., *P. longifolia* Andrews, *P. magnifica* Link, *P. grandiceps* Tratt., *P. scabra* R. Br. & *P. speciosa* (L.) L.), a number of *Leucospermum* R. Br. species (*L. conocarpadendron* (L.) H. Beuk, *L. oleifolium* (P.J. Bergius) R. Br. & *L. cordifolium* (Salisb. ex Knight) Fourc.), two *Leucadendron* R. Br. species (*L. laureolium* (Lam.) Fourc. & *L. xanthoconus* (Knutze) K. Schum.), *Diastella thymelaeoides* (P.J. Bergius) Rourke, *Mimetes cuculatos* (L.) R. Br., *Aulax umbellata* (Thunb.) R. Br. and *Spatella curvifolia* Salisb. ex Knight. These plants were, however, not included in the monthly monitoring due to their limited numbers of fruiting structures. This scanning, however, helped in clarifying the host range of South African ophiostomatoid species.

Fungal cultures

Fungal cultures were obtained by removing ascospores from the apex of perithecial necks with a sterile needle with a small piece of agar at the tip. These

were plated out onto Petri dishes containing 2 % malt extract agar (MEA; Biolab, Midrand, South Africa) and Sigma Streptomycin sulphate (0.04 g/l). The isolates were then incubated at 21 °C in the dark for 7-9 d prior to fungal identification.

Determining tissue colonisation

Using standard freezing microtome techniques, 6 μm thick sections were made through infected floral parts of *P. laurifolia* using a Leica CM 1100 microtome (Leica instruments GmgH, Germany) and Tung tissue freezing medium ™ (Leica instruments GmgH, Germany). Light microscopy was used to study the infection sites, and to scan for fungal hyphae penetrating green- (living), healthy- (dead without obvious ophiostomatoid infection) and senescent- (dead with ophiostomatoid colonisation) plant tissues. This facilitated an assessment of the ability of the ophiostomatoid fungi to colonise the host *Protea* species.

Results

Host specificity

Many of the *Protea* species that were surveyed were colonised by ophiostomatoid fungi (Table 2). Perithecia of ophiostomatoid fungi were exclusively found in infructescences of *Protea* sp.

No ophiostomatoid fungal perithecia were found in flower heads less than 5-mo-old, while infructescences as old as 5 years still showed colonisation by ophiostomatoid fungi (*G. proteae* on *P. repens*, collected at Stellenbosch mountain).

Table 2 Ophiostomatoid fungal colonisation of Proteaceous species with an indication of the type of association between fungus and host plant (# = specific association, 1 = general association, 0 = no association found)

Proteaceae species	Fungal species		
	O. splendens	*G. capensis*	*G. proteae*
A. umbellata	0	0	0
D. thymelaeoides	0	0	0
Leuca. laureolium	0	0	0
Leuca. rubrum	0	0	0
Leuca. salignum	0	0	0
Leuca. xanthoconus	0	0	0
Leuco. conocarpadendron	0	0	0
Leuco. cordifolium	0	0	0
Leuco. oleifolium	0	0	0
M. cuculatos	0	0	0
P. acaulos	0	0	0
P. burchellii	0	1	0
P. compacta	0	0	0
P. cynaroides	0	0	0
P. eximia	0	0	0
P. grandiceps	0	0	0
P. laurifolia	1	1	0
P. lepidocarpodendron	1	1	0
P. longifolia	1	1	0
P. magnifica	0	1	0
P. neriifolia	1	0	0
P. nitida	0	0	#
P. repens	0	0	0
P. scabra	0	0	0
P. speciosa	0	0	0
S. curvifolia	0	0	0

A new ascomycete was isolated from *P. laurifolia* and *P. burchellii*. This new species is morphologically very similar to the ophiostomatoid fungi, and seems to share the same dispersal mechanism (Fig. 1). It was found within the same ecological niche as the ophiostomatoid fungi, growing on the individual flowers within infected infructescences. This species is currently being described as a new species of *Rhynchomeliola* Speg. (Lee *et al.* unpublished data).

Host tissue colonisation

In an anatomical study of the infection sites, no fungal perithecia were found penetrating the host tissue (Fig. 2). Furthermore, no perithecia of these fungi were found to colonise green or healthy plant tissues either. Fungi were confined to the dead pollen presenters and other dead floral parts of the individual flowers within the host infructescences. In highly colonised infructescences, some perithecia were found growing on the inside of the outer protective bracts of infructescences. No perithecia were found on the fruits of any of the infected infructescences.

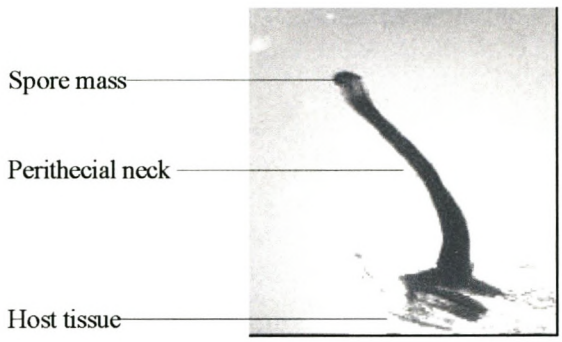

Fig. 1 *Rhynchomeliola* sp. on the pollen presenter of *P. laurifolia*.

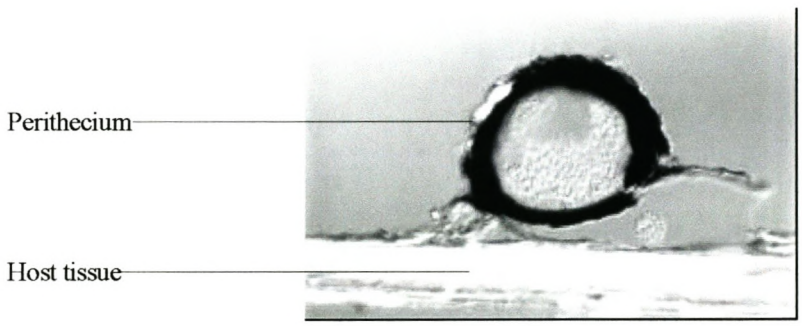

Fig. 2 Cross section (6 µm) through the perithecial base of *O. splendens* on the pollen presenter of *P. laurifolia*.

Seasonality

The infection numbers of ophiostomatoid fungi within *Protea* infructescences display distinct seasonality (Fig. 3). There is a marked increase in the number of infected *Protea* infructescences during the wetter, winter months with infection numbers in some instances being as high as 80 % (*G. capensis* on *P. laurifolia*).

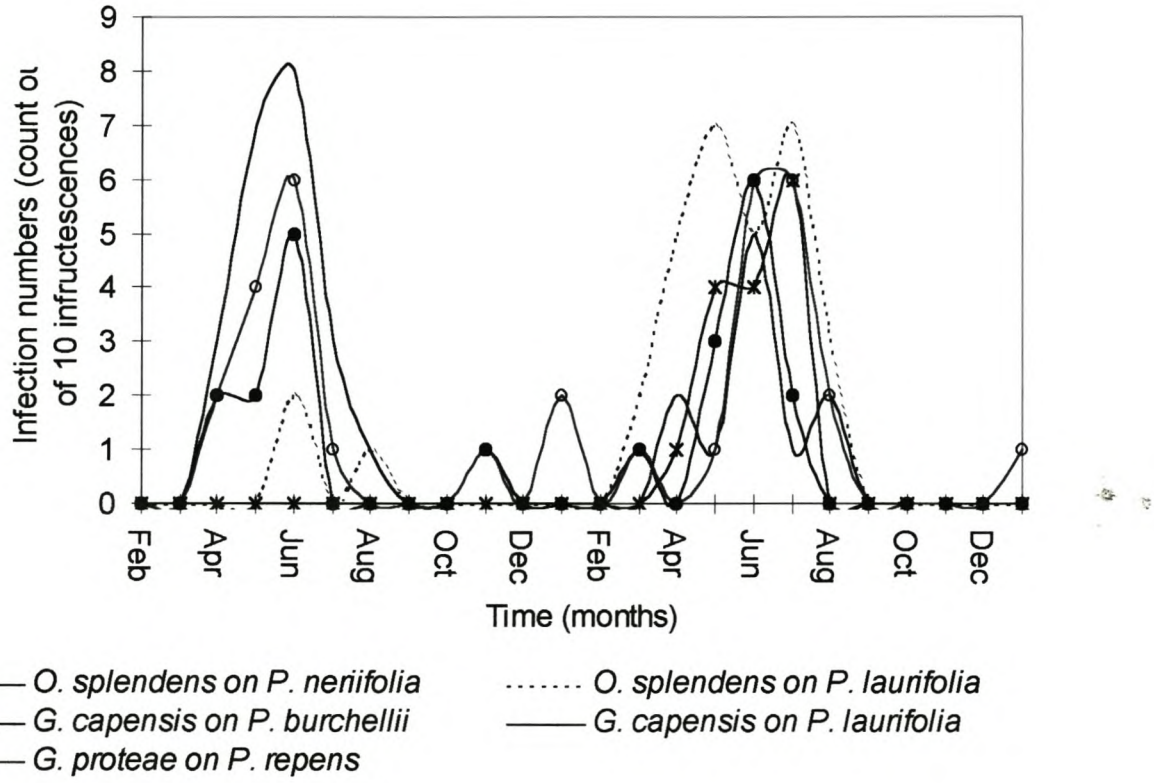

Fig. 3 Ophiostomatoid fungal colonisation of *Protea* infructescences over time (Feb. 2000 - Jan 2001) in the Stellenbosch region.

Interestingly, it was noted that when the infection numbers of *G. capensis* on *P. laurifolia* was high, the corresponding infection numbers of *O. splendens* was low during the first year of collection. During the second year the opposite was true during the months of May and August. In June of the second year, there was a decrease in the numbers of *O. splendens*, with a peak in the infection numbers of *G. capensis*. It seems as if these two fungal species are competing against each other in *P. laurifolia* infructescences. This is very likely, since these two species share exactly the same ecological niche.

Discussion

Ophiostomatoid fungi seem to colonise the infructescences of serotinous *Protea* species only, as no fungi were found on the fruiting structures of any of the other Proteaceae genera. It is also evident that not all *Protea* species are colonised by these fungi. In the south-western Cape, ophiostomatoid fungi seem to be confined to *Protea* species belonging to the 'true' sugarbush, the bearded sugarbush, and the spoon-bract sugarbush groups (Rebelo 1995). The precise factors that control host specificity is still unknown, but may include microclimatic conditions or differences in the chemical structure of the different *Protea* species. Different vectors for each of the fungal species may also play a role.

Two species of ophiostomatoid fungi were found to be prolific on *P. laurifolia.* They are *O. splendens* and *G. capensis*, and in both cases it represents a new host record for these fungi. Known host species for *O. splendens* now include *Protea laurifolia, P. lepidocarpodendron, P. longifolia, P. neriifolia and P. repens* (Marais & Wingfield 1994). Host species for *G. capensis* include *Protea laurifolia, P. burchellii, P. coronata* Lam., *P. lepidocarpodendron, P. longifolia, P. magnifica* and *P. neriifolia* (Wingfield & Van Wyk 1993; Marais & Wingfield 1994). Each of the remaining three ophiostomatoid species are restricted to one species of *Protea* only: *G. proteae* is exclusively found on *P. repens* (Wingfield *et al.* 1988),

O. africanum is exclusively found on *P. gaguedi* J.F. Gmel. (Marais 1996), and *O. protearum* is exclusively found on *P. caffra* Meisn. (Marais & Wingfield 1997). More ophiostomatoid species are likely to be discovered as more *Protea* species are studied. Apart from the Proteaceae, many other Fynbos plants also store their fruit in serotinous structures. These species should also be surveyed for the presence of ophiostomatoid fungi, in order to obtain a more detailed overview of the host specificity of these fungi.

The age of the youngest infructescence found to be colonised by ophiostomatoid fungi (5-mo-old) suggests that any potential vectors must at least be active after flowering to ensure fungal dispersal. Pollinators, such as bees, can thus be excluded as possible vectors of these fungi as, at this age, the flower heads of the *Protea* species are already closed (Marais 1996). However, not all flower visitors can be excluded as vectors, as some insect borers (e.g. *Genuchus hottentottus* F.) complete their immature stages in closed infructescences of various ages (Coetzee & Giliomee 1987a), after which they emerge as adults and feed within the inflorescences of *Protea* species. When these adults depart they could potentially come into contact with ophiostomatoid spores within the infructescences and carry them to uncolonised inflorescences of *Protea* species. The teliomorphic form of the fungi will then only be detectable at a later stage, as it may initially only reproduce asexually (anamorphically). The anamorphic form of the fungi is far less visible to the naked eye than the teliomorphic form, and was not surveyed in the present study. Thus, it is possible that the anamorphic form of ophiostomatoid fungi are present within *Protea* infructescences of a much younger age than the teliomorphic form of the fungus. Further studies are needed to elucidate relationships between South African ophiostomatoid fungi and their insect vectors. This is currently being investigated.

The discovery of a fungal species, which share the same ecological niche and dispersal mechanism as ophiostomatoid fungi (Fig. 1), is of great taxonomic and ecological interest. Similar species have been isolated from *Grevillia*

(Proteaceae) in Australia and also from plants in South America (Müller & Von Arx 1962). With the discovery of members of the same genus in South Africa, it seems as if this fungal group might be of Gondwanan origin. It is also known that the Proteaceae is a very old family of flowering plants (Chase *et al.* 1993; APG 1998; Reeves 2001). This could indicate that the relationship between this fungal group and members of the Proteaceae could date back to a time prior to Gondwanan-breakup (*ca.* 140 million years ago). It is unknown whether ophiostomatoid fungi also occur on Proteaceae species in Australia and South America, but if they do, it would confirm that the association between ophiostomatoid species and their Proteaceae hosts is very old as well.

No ophiostomatoid fungi were found growing on the fruits of any of the colonised *Protea* infructescences and no perithecia was found penetrating the host tissue (Fig. 2), indicating that the fungi may not have a direct influence on seed viability. They were also only found on dead and senescent floral parts, which suggests that these fungi have a saprophytic, rather than a parasitic relationship with their plant hosts. The fungi may, however, influence the composition of the insect taxa that infest the infructescences and may so indirectly influence seed production, viability and/or dispersal. It also deserves special mention that, when these fungi are found growing within *Protea* infructescences, no other fungi are found to co-inhabit the infructescences in significant numbers, while these were prolific in non-ophiostomatoid infected infructescences (pers. observ.). This may be the result of the ophiostomatoid fungi out-competing other fungi once they become established within the flower heads. This competitiveness may protect the *Protea* seeds from destruction by other, more destructive, fungal species (Marais 1996). Further research is needed to elucidate these fungal interactions and their impact on *Protea* seed viability.

As previously mentioned, the occurrence of ophiostomatoid fungi within the *Protea* infructescences display distinct seasonal patterns (Fig. 3). The peak in fungal numbers during autumn and winter indicates that sufficient moisture and

maybe also low temperatures could promote fungal growth. To what extent this pattern of seasonality in fungal numbers is repeated in the Fynbos at large, is still unknown. It is, however, likely that other areas, with different moisture regimes, will show different fungal growth patterns. Personal observations indicated differences in the seasonal distributions of ophiostomatoid fungi in *P. repens* infructescences collected from Cape Point and Stellenbosch. The fungi were found to be prolific in the Cape Point infructescences collected in February. Figure 3 indicates that ophiostomatoid fungi infected very few infructescences during the same period at the Stellenbosch collection sites. Other environmental parameters must thus clearly also influence the abundance of ophiostomatoid fungi within the *Protea* infructescences. Further studies, at different study sites and including more environmental parameters, need to be undertaken to expand our understanding of the ecological parameters controlling the growth of these fungi on *Protea* species.

The possible competitiveness of *G. capensis* and *O. splendens* within *P. laurifolia* infructescences is very interesting. It is known that these two species of ophiostomatoid fungi can co-inhabit the same infructescence at the same time (pers. observ.). It is not known to what extent these fungal species compete within the infructescences of other *Protea* species, but they also co-inhabit infructescences of other *Protea* species. It is possible that when these two fungi co-inhabit the same infructescence at the same time, the stronger competitor will out-compete the less competitive species. The presence of both species in the same infructescece may, in this case, only be temporary. It is also possible that these two species could co-inhabit the same infructescence without much competition taking place. If this is the case, some other factor must affect the co-occurrence, such as spatial separation within the same infructescence. Wingfield (pers. comm.) mentioned that *Ophiostoma* and *Gondwanamyces* inhabit the pollen presenters at different heights within the same infructescence, thus effectively spatially separating the two genera.

Observations have shown that, while the fungal numbers in the field declined, samples taken in June and kept at room temperature under dry conditions in the laboratory, did not display any decline in fungal numbers. This could be due to the absence of fungi-feeding Psocoptera living within the flower heads under laboratory conditions. In the field, these insects could be responsible for the decline in fungal numbers by devouring perithecia after the completion of the fungal life cycle. Psocoptera are common in field-infructescences, but very few living specimens were found within the laboratory kept samples.

Results of this study indicate that the South African ophiostomatoid fungi are restricted to *Protea* species and are most likely non-pathogenically associated with these plants. They show definite seasonal growth patterns. A new fungal species was discovered, which share the same ecological niche as the ophiostomatoid fungi. It also seems to share the same dispersal mechanism as its ophiostomatoid counterparts.

These results explain some of the ecological aspects pertaining to the ophiostomatoid fungi found within the infructescences of *Protea* species. In a broader sense it also contributes to a better understanding of the ecological processes active in the Fynbos Biome. Further studies are urgently needed to assess the impact these fungi may have on other fungal species and insect communities within the infructescences, as this may be of great economic and ecological importance.

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CHAPTER 4

PROTEACEAE-INFRUCTESCENCES, ARTHROPOD COMMUNITIES AND THEIR FUNGAL INHABITANTS: AN UNIQUE ECOSYSTEM

Abstract

Arthropods associated with the fruiting structures of nine species of Proteaceae were collected on a monthly basis over a two-year period. A total of 62 pseudospecies (over 7500 individuals), belonging to 45 different arthropod families were collected. Plant species with larger infructescences housed the most insect species and the highest numbers of individuals. *Protea laurifolia*, *P. repens*, *P. nitida*, *P. burchellii* and *P. neriifolia* harboured very similar arthropod species. Seasonal patterns were found in the insects associated with Proteaceae infructescences, with abundance levels in most plant species peaking during the wetter autumn and winter months. Twenty-five fungal taxa were isolated from the various insects inhabiting the infructescences. Many of the isolated fungal genera are known to include species pathogenic to Proteaceae. Some fungi showed a high degree of specificity towards potential vectors, while others were found on a diverse range of insects. These results suggest that some of these insects could play a significant role in the dispersal of plant pathogenic fungi between their hosts. Further studies are needed to elucidate these insect-plant-fungal interactions within the Cape Fynbos.

Introduction

The Cape Fynbos is considered one of the floristically most diverse regions in the world, containing well over 9 000 vascular plant species (Goldblatt & Manning 2000). This area houses almost 10% of the flowering plants of the world (Arnold & De Wet 1993).

The African Proteaceae is represented by 16 genera, 13 of which are centred within the Cape Floristic Kingdom (CFK). Ten of these genera are endemic to the Western Cape Province (Rourke 1998). The family is of major ecological importance, as it forms one of the dominant and defining families within this unique Floral Kingdom (Bond & Goldblatt 1984). Some members of the family are considered keystone species, and are essential for the continued functioning of plant and animal communities in the Fynbos. The Proteaceae is also of significant economic importance to South Africa. Many Proteaceae species are utilised in the horticultural and flower export industries, and it is also a major contributor to the thriving local eco-tourism industry.

Many insects have been found inhabiting the infructescences of Proteaceae (Guess 1968; Myburgh *et al.* 1973; Myburgh & Rust 1975; Wright 1990; Visser 1992). Very limited attention has, however, been paid to their general ecology, and to the role they play within the *Protea* infructescences (Coetzee & Giliomee, 1985, 1987a, b; Coetzee 1989; Hattingh & Giliomee, 1989; Wright 1990; Visser 1992). Insects associated with these plants include members that are seed destroyers (Coetzee & Giliomee 1987b), influencing the numbers of viable seeds kept within the canopy-stored seed bank. Not only are viable seeds needed to ensure the long-term survival of the species (Bond 1985), but they are also essential to maintain the functionality of the Fynbos Biome (Bond & Breytenbach 1985). It is thus possible that insects could have a direct effect on the distribution and abundance of the Proteaceae in South Africa (Coetzee 1989).

Zwölfer (1979) considers the fruiting structures of flowering plants as "miniature ecosystems", which house different food chains and trophic levels.

The present study sets out to address some of the more general issues pertaining to the ecology of the insects and other arthropods associated with Proteaceae-infructescences. Firstly, what is the role of the different arthropods within the infructescences, secondly, does the composition of the arthropod fauna within the infructescences change over time due to seasonal climatic variations, and thirdly, how do the arthropod communities compare between different plant species? Answers to these questions are of both ecological and economic importance (Myburgh *et al.* 1973; Myburgh & Rust 1975; Coetzee 1986). Results of this study should also to some extent indicate whether the diversity of Fynbos arthropod fauna matches the floral diversity of this biome.

Studies on Fynbos invertebrate ecology have been limited to only a few arthropod (Donnelly 1983; Schlettwein 1984) and plant species (Coetzee & Giliomee, 1985, 1987a, b; Coetzee 1989; Wright 1990; Visser 1992). A comprehensive study, including a range of different Proteaceae species and all the arthropods associated with their infructescences is still lacking. The present study sets out to provide the first step towards such an overview.

Many fungi are known to be associated with Proteaceae species (Crous *et al.* 2000a, b; Swart *et al.* 2000; Taylor & Crous 2000; Taylor *et al.* 2001). Some of these cause fungal infections, including diseases of the leaves, stems, roots and seedlings (Marasas *et al.* 1975; Gorter 1976; Von Broemsen & Brits 1986; Knox-Davies *et al.* 1987; Crous *et al.* 2000a). They may cause major financial losses, and yet their means of transmission between plants is still poorly investigated and understood.

Some insects are known to transmit fungal diseases between plants (Dowding 1984), and may act as chief vectors of some of the Proteaceae-fungi. A further aim of this study was thus to investigate the fungal genera carried by insects associated with Proteaceae infructescences, to identify possible vectors for some Proteaceae pathogens.

The presence of saprobic fungi within infructescences may also lead to seed losses. They cause rotting of infructescence tissues, which may in turn lead to the degradation of viable seeds. It was therefore decided to include these fungi in the present study as well.

Materials and Methods

Study sites

Ten infructescences (6-24-mo-old) of nine Proteaceae species were collected on a monthly basis from two sites in the Stellenbosch region of the Western Cape Province, South Africa (Table 1). Proteaceae species were selected according to their availability in the demarcated areas. Sites included the Jonkershoek Forestry Reserve and Stellenbosch Mountain. Proteaceae infructescences were collected over a two-year period (Feb. 2000 – Jan. 2002) and inspected for the presence of insects and other arthropods.

Table 1 Collection sites of Proteaceae infructescences.

	Population site	Grid Reference
Leucadendron rubrum Burm. f.	Jonkershoek	S: 33° 58, 591' E: 18° 56, 817'
Leucadendron salignum Bergius	Jonkershoek	S: 33° 59, 210' E: 18° 57, 361'
Protea acaulos (L.)	Jonkershoek	S: 33° 59, 210' E: 18° 57, 361'
Protea burchellii Stapf.	Stellenbosch Mountain	S: 33° 56, 743' E: 18° 52, 711'
Protea laurifolia Thunb.	Stellenbosch Mountain	S: 33° 56, 743' E: 18° 52, 716'
Protea neriifolia R. Br.	Jonkershoek	S: 33° 59, 555' E: 18° 58, 287'
Protea nitida Mill.	Jonkershoek	S: 33° 59, 210' E: 18° 57, 361'
Protea repens L.	Jonkershoek	S: 33° 59, 555' E: 18° 58, 287'
Serruria fasciflora Salisb. ex Knight	Jonkershoek	S: 33° 58, 591' E: 18° 56, 817'

Arthropod collection

Flower heads were cut open using pruning sheers. All arthropods contained within the flower heads were collected, placed in separate vials and frozen (-20 ºC). The arthropods were identified to the level of pseudospecies (hereafter referred to as species) groups. These were then classified into feeding classes (guilds). The following eight guilds were identified: (1) sucking (sap feeding) insects, (2) detritivorous insects (plant non-detrimental feeders), (3) nectar and pollen feeders, (4) ants, (5) predators and parasitoids, (6) boring insects, (7) foliage feeding insects (phytophytic chewing insects) and (8) fungal feeding insects. Allocation of arthropods to the various guilds was based on personal observations and similar allocations done by Coetzee (1989) and Wright (1990). Voucher specimens are housed in the insect collection (USEC), Department of Entomology and Nematology, University of Stellenbosch, Stellenbosch, South Africa.

Similarity indices

Sørensen's coefficient of similarity (Southwood 1978) was used to determine the degree of similarity of the arthropod species (C_s = 2j/(a +b)) and individuals (C_n = 2jN/(aN + bN)) between the different Proteaceae species. [j = number of arthropod species in common between two plants, a and b respectively = total number of arthropod species present per plant, aN and bN respectively = total number of individuals on each plant, and jN = the sum of the smaller values (individuals count) for the species collected from both plants].

Fungal isolates

Insects collected in the first seven months (Feb. 2000 – Aug. 2000) were crushed under sterile conditions and transferred to Petri dishes containing 2% malt extract agar (MEA, Biolab, Midrand, South Africa) and Sigma Streptomicin sulphate (0.04 g/l). These were incubated at 21ºC in the dark for 7-9 d, whereafter all fungi that grew from these were identified to genus level

(where possible). Voucher cultures are housed in the Department of Plant Pathology, University of Stellenbosch, Stellenbosch, South Africa (STE-U).

Results and Discussion

Guild composition

A total of 62 insect and other arthropod species (7 617 individuals), representing *ca.* 45 families, were collected from the infructescences of the nine Proteaceae species over the entire collection period (Table 2). Figure 1 shows the total numbers of arthropod species (and guild composition) associated with the infructescences of these nine Proteaceae species. The highest numbers of arthropod species were collected from *Protea laurifolia* (44 species), *P. nitida* (43 species) and *P. neriifolia* (41 species). The lowest numbers of arthropod species were collected from the fruiting bodies of *Protea acaulos* (14 species), *Serruria fasciflora* (13 species), *Leucadendron salignum* (11 species), and *L. rubrum* (9 species), while *P. repens* (37 species) and *P. burchellii* (27 species) housed intermediate numbers of arthropod species. The same trend can be seen in the number of arthropod individuals occupying infructescences of the different Proteaceae species (Fig. 2). The size of the proteaceous fruiting structures correlates well with the number of individuals. Large infructescences, such as those of *Protea laurifolia* (40-60 mm across), *P. nitida* (80-160 mm across), *P. repens* (70-90 mm across), *P. burchellii* (50-70 mm across) and *P. neriifolia* (60-80 mm across), house more insect individuals (and thus possibly species) than smaller infructescences such as those of *Leucadendron salignum* (10-14 mm across), *L. rubrum* (35 mm across), *Serruria fasciflora* (15-50 mm across) and *P. acaulos* (30-50 mm across) (The size of the different infructescences, according to Rebelo (1995), is given in brackets). Other factors that may influence the numbers of species associated with the different plant species are plant size, plant architecture and plant distribution range (Lawton 1978, 1983). These factors have not been considered in this study.

Arthropods showed varying specificity towards their plant hosts (Table 2). Some arthropods (e.g. the Aradidae species) have a very narrow host range and were found on only one (or a few) of the proteaceous species. Other arthropods appear to have a very wide host range (e.g. *Oxycarenus maculates* Stal. and Psocoptera sp. 1) and were found in the infructescences of most of the plant species included in this study. The extent to which these arthropods occupy other Fynbos plant species is unknown, but it is possible that some are monophagous, being associated with only one or a few related plant species.

The predator and parasite guild contained the most arthropod species (20) followed by the phytophagous sucker guild (11), the phytophagous chewer guild (8) and the borer feeding guild (8) (Table 2). The lowest number of species was found in the pollen and nectar-feeding guild (2). As was found by Coetzee (1989), there is a major similarity in the proportions of the number of arthropod species (gamma-diversity) belonging to the different guilds between the different Proteaceae species (Fig. 1). This was especially true for the predators and parasites, borers and sucking guilds. It is interesting to note that, although Coetzee (1989) focused on the insects collected from the foliage of *Protea* species, similar results were obtained in the present study of arthropod communities within the Proteaceae infructescences.

As mentioned above, the predator and parasitoids constituted most of the arthropod species in all of the plant species. Coetzee (1989) found that phytophagous insects (including chewers, suckers and borers) constituted most of the species in most of the Proteaceae species (*P. repens*, *P. cynaroides* (L.) L. and *Leucospermum cordifolium* (Salisb. ex Knight) Fourc. This was confirmed by Wright (1990), who compared the guild structures of arthropods collected from the foliage of *P. magnifica* Link and *P. laurifolia*. Results of the present study show the same trend for insects associated with the infructescences of most of the plant species studied (*P. laurifolia*, *P. nitida, P. repens, P. burchellii, P. acaulos* and *P. neriifolia*). Coetzee (1989) found that most epigaic species collected on *P. neriifolia* and *Leucadendron laureolium* (Lam.) Fourc. were grouped into the predator and parasitoid guild.

This contrasts to what we found in our study of *P. neriifolia* infructescences. Predator and parasitoid guilds were found to be the most species diverse in *Serruria fasciflora* and in the two *Leucadendron* species included in our study.

Table 2 Checklist of insects and other arthropods collected from Proteaceae infructescences between Feb. 2000 and Jan. 2002. The animals are grouped into eight different feeding guilds. Reference numbers indicate arthropod pseudospecies reference organisms housed in the Department of Entomology and Nematology, University of Stellenbosch, Stellenbosch, South Africa.

Arthropod taxa and feeding guilds	Ref. number	Proteaceae species								
		P. acaulos	*L. salignum*	*P. repens*	*P. nitida*	*L. rubrum*	*P. burchellii*	*S. fasciflora*	*P. neriifolia*	*P. laurifolia*
PHYTOPHYTIC SUCKERS										
Aradidae										1
Fulgoridae					1				3	1
Miridae				5	4		1			
Lygaeidae (sp. 1)	26								3	1
Oxycarenus maculates Stal. (Lygaeidae)	7	2	2	253	252	9	148		149	254
Oligonychus coffeae (Mite)	36				13		4		744	250
Mite (sp. 1)				1	1		1		6	1
Pseudococcidae	37	4			3				3	
Pentatomidae	24			19	10					
Scutelleridae				3				1		
Thysanoptera	34				4		2		3	6
DERTRITIVORES										
Archaeognatha							97			109
Arthropleona (Collembola)	9	8		4	12		17	1	5	16
Blattidae	26	2					5	5	2	2

Table 2 (cont.)

Arthropod taxa and feeding guilds	Ref. number	Proteaceae species								
		P. acaulos	*L. salignum*	*P. repens*	*P. nitida*	*L. rubrum*	*P. burchellii*	*S. fasciflora*	*P. neriifolia*	*P. laurifolia*
DERTRITIVORES (cont.)										
Symphypleona (Collembola)	21		1		16		3			9
Dermaptera								1	2	2
PREDATORS AND PARASITOIDS										
Carabidae	29	2			2		1	1		3
Carabidae (larvae)				4	5		1			1
Chrysopidae			3						2	
Gyponyx sp. (Cleridae)				4			2			1
Clubionidae			1	1	1			1	2	1
Coccinellidae									2	
Coleoptera (larvae)				3					3	
Histeridae	32			2	1	8			1	4
Braconidae				7	6				8	1
Hymenoptera (pupae)	38			6	2		1			2
Latrodectus geometricus	11			1				1		
Mite (sp. 2)		4		5	25	9	4	3	2	8
Pseudoscorpionidea	16				4		4		1	

Table 2 (cont.)

Arthropod taxa and feeding guilds	Ref. number	Proteaceae species								
		P. acaulos	*L. salignum*	*P. repens*	*P. nitida*	*L. rubrum*	*P. burchellii*	*S. fasciflora*	*P. neriifolia*	*P. laurifolia*
PREDATORS AND PARASITOIDS (cont.)										
Spider (sp. 1)						1				
Spider (sp. 2)			1	1				1		1
Spider (sp. 3)										2
Spider (sp. 4)					8				1	1
Spider (sp. 5)					12		1	1	1	11
Staphylinidae	35			10	27				6	7
Mantidae					1	1			1	1
Salticidae				17	34				28	15
ANTS										
Crematogaster sp. (Formicidae)	15			27	19	2	96		5	331
Formicidae (sp. 1)		69	2	16	78					79
Formicidae (sp. 2)	4			48			1		74	1
Formicidae (sp. 3)	23			20	27					
Sima sp. (Formicidae)				2						

Table 2 (cont.)

Arthropod taxa and feeding guilds	Ref. number	Proteaceae species								
		P. acaulos	*L. salignum*	*P. repens*	*P. nitida*	*L. rubrum*	*P. burchellii*	*S. fasciflora*	*P. neriifolia*	*P. laurifolia*
BORERS										
Argyroploce sp. (Tortricidae) (larvae)	6			13	7		6		8	10
Bruchidae					4			1		4
Sphenoptera sp. (Buprestidae) (larvae sp. 1)	37			17	14		8		11	11
Sphenoptera sp. (Buprestidae) (larvae sp. 2)	30			15	14		8		33	20
Sphenoptera sp. (Buprestidae)									2	5
Sphenoptera sp. (Buprestidae) (pupae)				5			3		1	1
Capys alphaeus (Lycaenidae) (larvae)	10			3	4		4			
Euderes lineicollis (Curculionidae) (larvae)	28	1	1	6	4	2	2		5	26
Genuchus hottentottus (F) (Scarabaeidae) (larvae)	14			30	12				1	11
Tinea sp. (Tineidae) (larvae)		3			5				8	5
PHYTOPHYTIC CHEWERS										
Chrysomelidae (sp. 1)	18			15					36	22
Chrysomelidae (sp. 2)					18					
Chrysomelidae (sp. 3)	17	5			3		2			

Table 2 (cont.)

Arthropod taxa and feeding guilds	Ref. number	Proteaceae species								
		P. acaulos	*L. salignum*	*P. repens*	*P. nitida*	*L. rubrum*	*P. burchellii*	*S. fasciflora*	*P. neriifolia*	*P. laurifolia*
PHYTOPHYTIC CHEWERS (cont.)										
Euderes lineicolis (Curculionidae)	33			7	11				3	3
Curculionidae (sp. 2)				3		1			2	1
Curculionidae (sp. 3)	3			8	6					
Elateridae	2				2				1	
Nitidulidae	25			239	28		137		20	20
FUNGAL FEEDERS										
Psocoptera (sp. 1)	31	45	16	370	855	1	188	31	86	498
Psocoptera (sp. 2)	13	22	7	32	26		9		56	55
Psocoptera (sp. 3)	12	29		10	66		31	2	18	32
POLLEN AND NECTAR FEEDERS										
Diptera	5			1	3				6	3
Hopliini (Rutelinae: Scarabaeidae)									11	

Visser (1992) studied the guild composition of the insects associated with *P. nitida* infructescences collected at three different sites. His results were similar to what we found for *P. nitida*. Table 3 supplies a comparison between the results of the present study and those of Visser (1992).

Table 3 Insect and other arthropod species (%) in *P. nitida* belonging to the different feeding guilds. Results of Visser (1992) are compared to those of the present study. Visser (1992) did not include arachnids in this specific study.

	Visser (1992) (n = 51)	Present study (n = 43)
Detritivores	22%	32%
Chewers (including borers)	29%	30%
Suckers	20%	19%
Predators and parasites	18%	30%
Ants	12%	7%

As was found by Coetzee (1989), the guild composition based on individual level (Fig. 2) varied greatly from one proteaceous species to another. Most individuals present in the infructescences of *P. neriifolia*, *P. laurifolia*, *P. burchellii*, *P. repens* and *L. rubrum* belong to the phytophytic (especially sucking) guilds. *L. salignum*, *P. nitida* and *Serruria fasciflora* housed more individuals belonging to the fungal feeding guild, while *P. acaulos* housed more ants than individuals of any other guild.

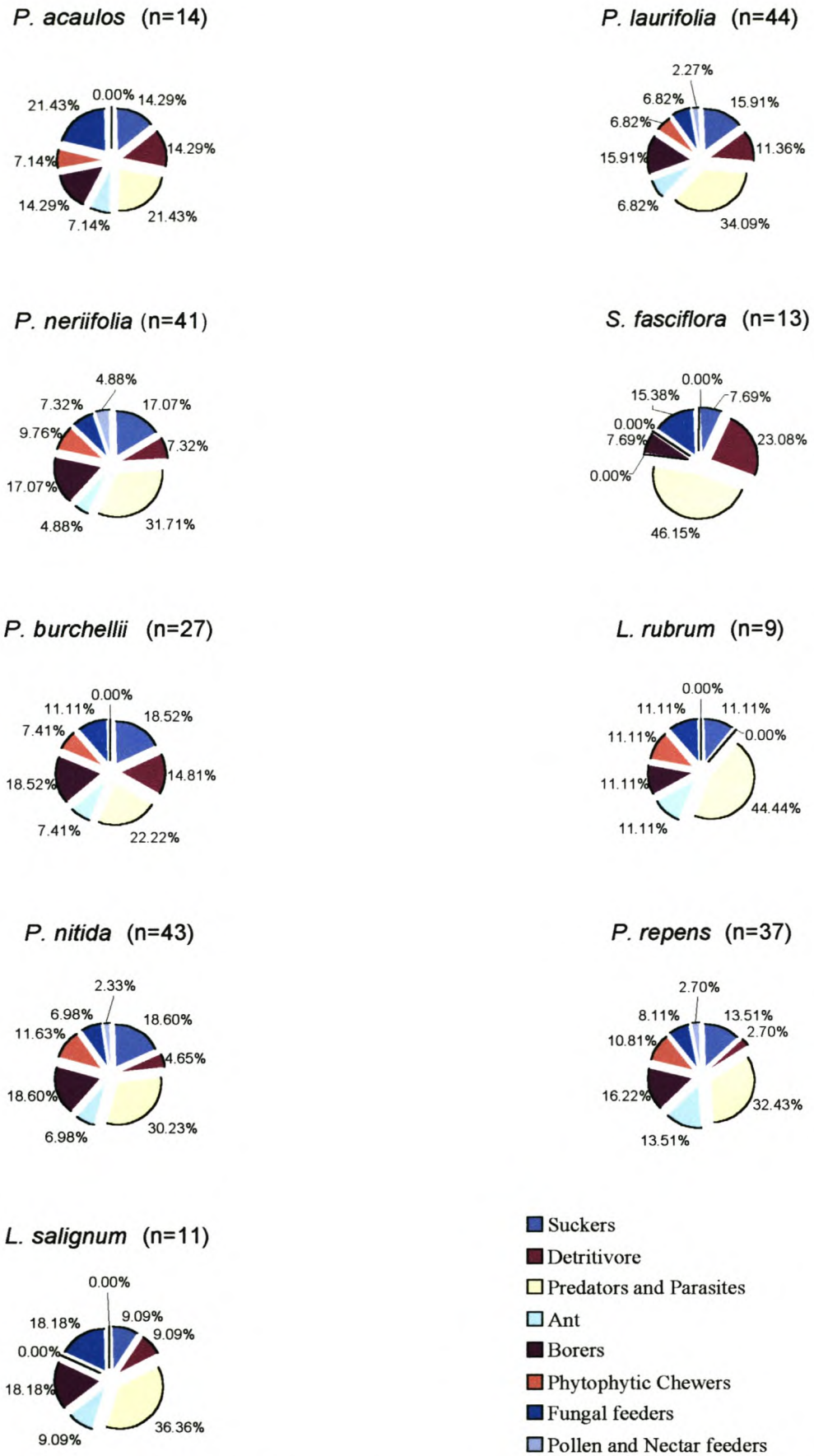

Fig. 1 Guild structure of the arthropod fauna of the different Proteaceae species expressed as a percentage of the total number of extracted arthropod species per guild. (n: total number of arthropod species collected from 240 infructescences)

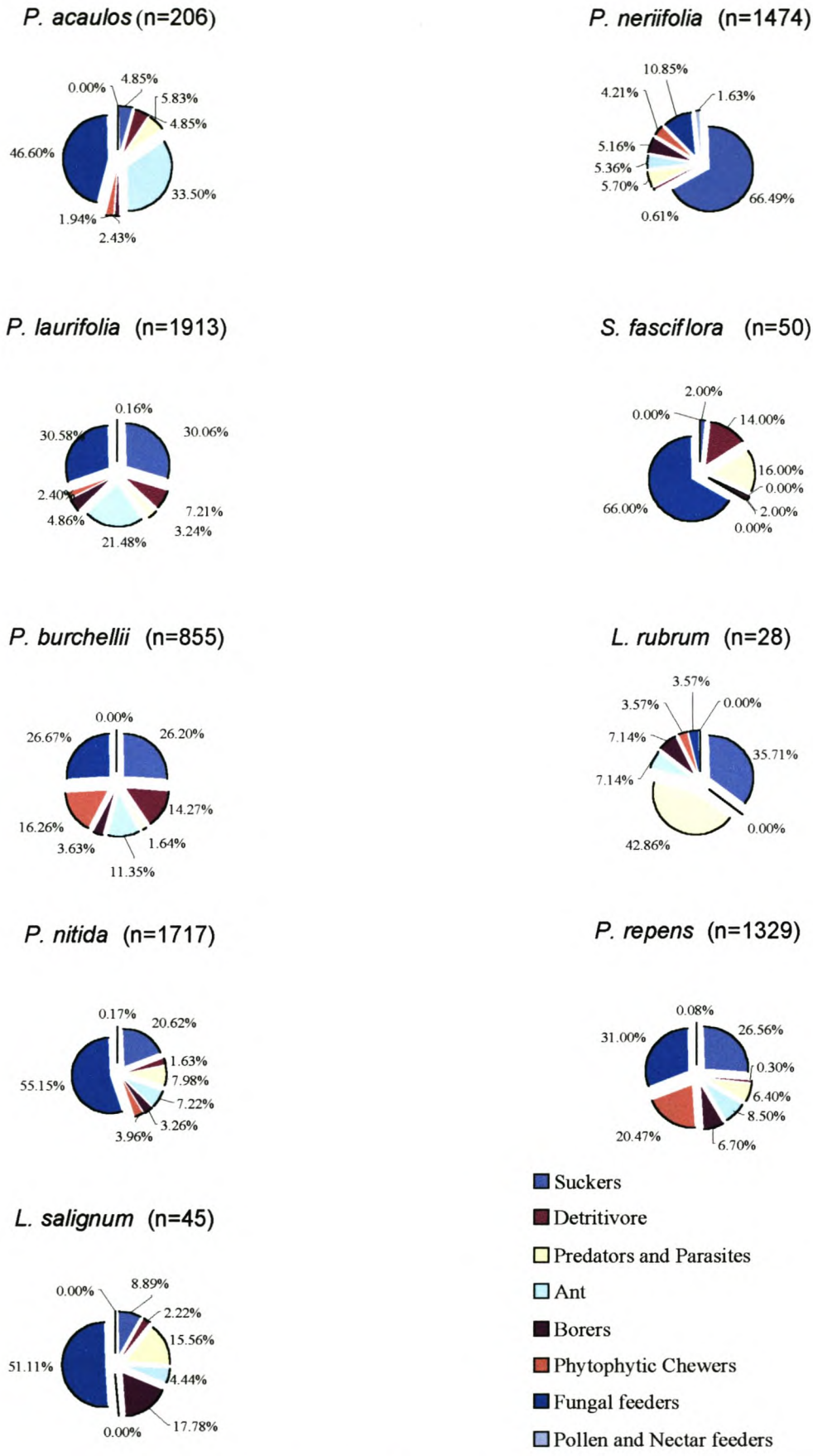

Fig. 2 Guild structure of the arthropod fauna of the different Proteaceae species expressed as a percentage of the total number of extracted arthropod individuals per guild. (n: total number of arthropod Individuals collected from 240 infructescences)

Similarity of arthropod species composition and arthropod individual composition between the different Proteaceae species.

Results of the application of Sørensen's similarity coefficient on the different arthropod species isolated from the infructescences of the nine Proteaceae species are presented in Fig. 3. It indicates the similarity of the arthropod species composition between the different plant species.

High values are obtained when the arthropod species from *P. laurifolia*, *P. repens*, *P. nitida*, *P. burchellii* and *P. neriifolia* are compared using Sørensen's similarity coefficient (Fig. 3). This indicates that there is great overlap between the arthropod species found on these five plant species. This is especially true for the arthropods shared between *P. nitida* and *P. laurifolia* (C_s = 0.752) and between *P. neriifolia* and *P. laurifolia* (C_s = 0.774). What makes this even more significant is that infructescences of each of these species were collected from different sites (Table 1). These closely related plant species are very similar in habit and in the size and structure of their infructescences (Rebelo, 1995). This could perhaps explain the high similarity in arthropod species diversity between the infructescences of these plants.

Low C_s values for the other plant species show that these plants contain many faunal species within their infructescences that are restricted to certain Proteaceae species. These arthropod species could be either monophagous (utilizing only one or a few plant species) or tourists, using the infructescences merely for shelter. Tourists are important to consider in any insect-plant association study, as they form part of the feeding chain within the Proteaceae.

Results of the application of Sørensen's similarity coefficient when the numbers of the different arthropod individuals, isolated from the infructescences of the nine Proteaceae species, are taken into account are presented in Fig. 4. This gives an indication of the similarity of the arthropod species composition and,

more specifically, the similarity in their numbers, between the different plant species.

The low C_n values obtained for most of the plants studied (Fig. 4) indicate that there are marked differences in the number of arthropod individuals housed within the infructescences of the different plant species. The highest C_n value was obtained when comparing arthropod individuals found on *P. nitida* with those found on *P. laurifolia* (C_n = 0.620). This means that these plants not only share the same arthropod species, but they also share them at similar levels of abundance.

A comparison between *P. neriifolia* and *P. laurifolia* yielded a low C_n value (<0.1), despite the fact that they are closely related plants (Rebelo 1995). The reason for this is unclear, but it may be caused by differences in the microclimatic conditions between the two sites from where the plants were sampled. There is also a difference in the age of the veld (time after the most recent fire) between these two sites, which may have an influence on the numbers of the arthropods associated with the Proteaceae infructescences. This may well prove to be the case as Donnely (1983) and Schlettwein (1984) have found differences in insect communities of Fynbos study sites under different fire regimes.

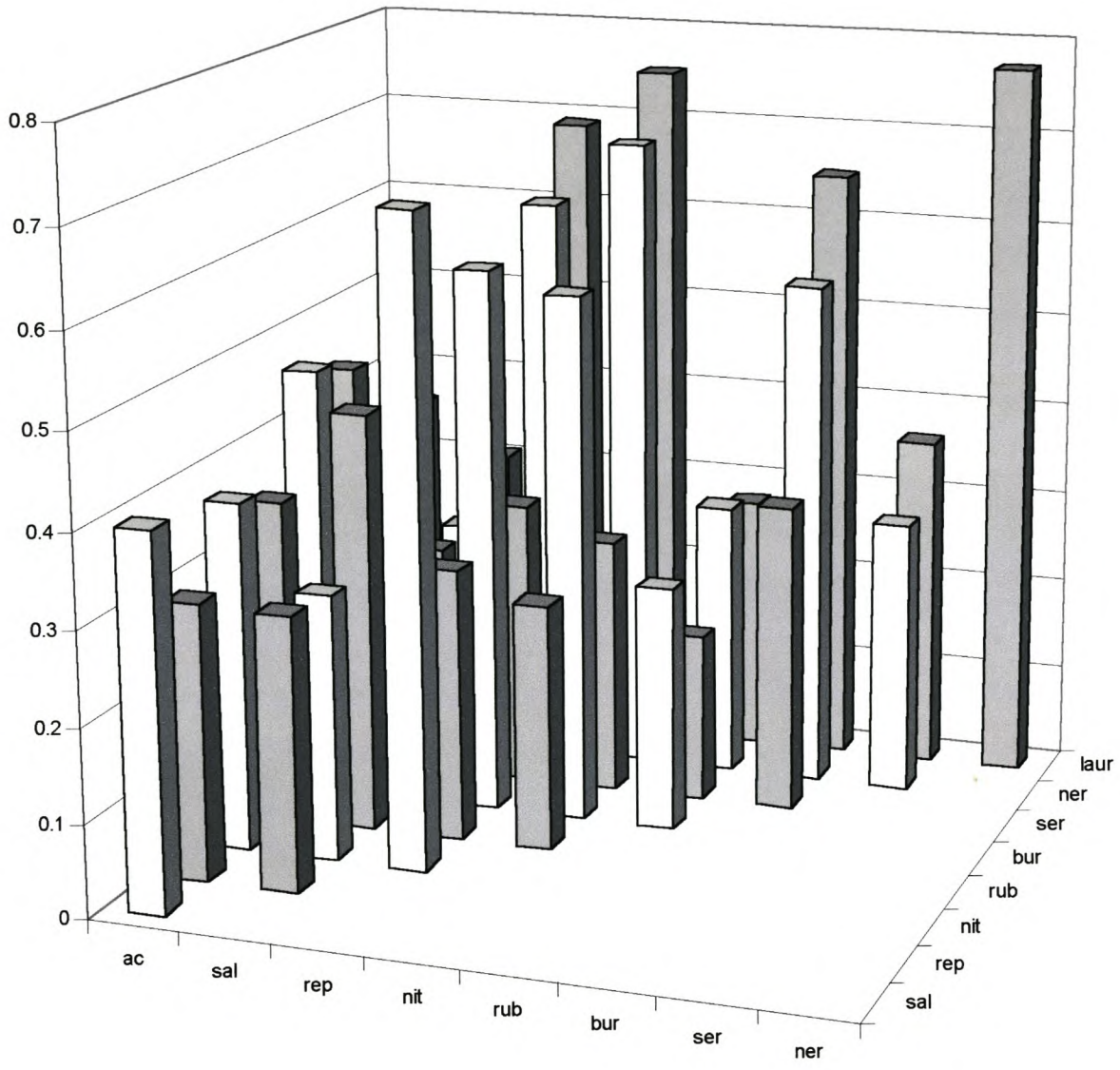

Fig. 3 Sørenson's coefficients of similarity for the arthropod species associated with the Proteaceae species (C_s). ac = *P. acaulos*, sal = *L. salignum*, rep = *P. repens*, nit = *P. nitida*, rub = *L. rubrum*, bur = *P. burchellii*, ser = *Serruria fasciflora*, ner = *P. neriifolia*, laur = *P. laurifolia*.

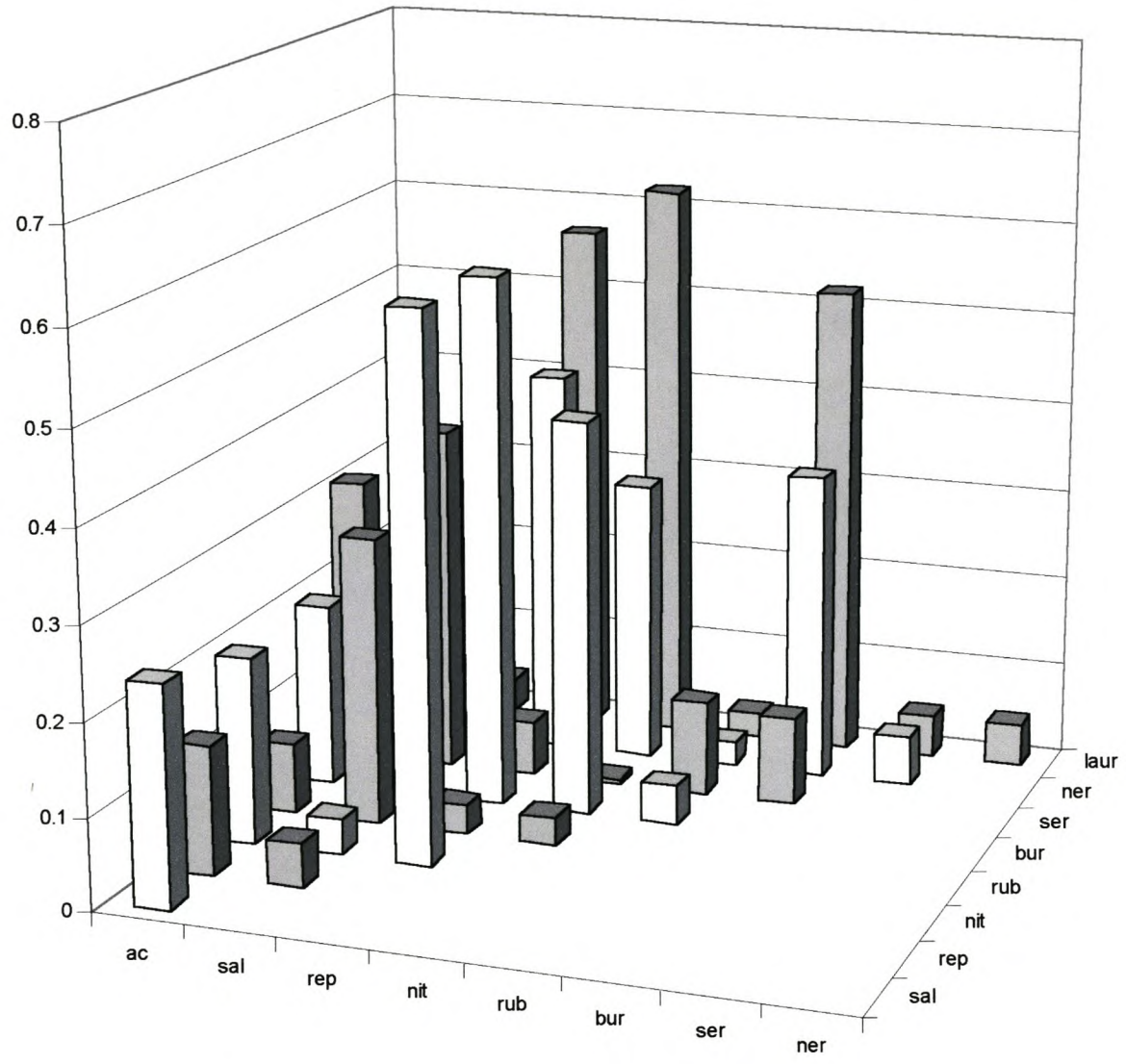

Fig. 4 Sørenson's coefficients of similarity for the number of arthropod individuals associated with the Proteaceae species (C_n). ac = *P. acaulos*, sal = *L. salignum*, rep = *P. repens*, nit = *P. nitida*, rub = *L. rubrum*, bur = *P. burchellii*, ser = *Serruria fasciflora*, ner = *P. neriifolia*, laur = *P. laurifolia*.

Seasonal distribution of the arthropods associated with the different Proteaceae species

The arthropod fauna occupying the Proteaceae infructescences displays a distinct seasonal pattern (Fig. 5, 6). There is a marked increase in the abundance of arthropod individuals during the wetter autumn and winter months. Coetzee (1989) found similar results when comparing seasonal arthropod patterns of five Proteaceae species. However, he included insects collected from flowers, which were more abundant in the winter months. The present study largely excludes flower visitors, as it focuses on infructescences only. When flower visitors are excluded from the study of Coetzee (1998), there is very little difference between the number of arthropods collected from Proteaceae foliage during winter and those collected during summer. In contrast, Wright (1990) found that the arthropods collected from the foliage of *P. magnifica* and *P. laurifolia* showed seasonal variations, which appeared to be strongly correlated with winter and summer conditions. In the present study, definite seasonal distribution patterns were found for arthropods collected from Proteaceae infructescences. This also seems to correlate well with the known rainfall patterns for the Stellenbosch region, with more precipitation during the wetter autumn and winter months.

When comparing the seasonal distributions of the total number of arthropods collected for each of the *Protea* species (excluding *P. acaulos*) (Fig. 6), it is apparent that the plant species harboured peak numbers of arthropods during different seasons. The other Proteaceae genera studied were excluded from this comparison due to the low numbers of arthropod individuals found within their fruiting structures. In *P. neriifolia*, *P. nitida* and *P. repens*, arthropod numbers peak during autumn, while *P. laurifolia* and *P. burchellii* house slightly more arthropod individuals during winter. Visser (1992) also found that there is an increase in insect numbers within the infructescences of *P. nitida* during autumn. It is unknown whether there is a general increase in arthropod abundance during the wetter autumn and winter seasons in the Fynbos as a whole, or whether this phenomenon is restricted to the Proteaceae.

Guild seasonality (total number of individuals per guild collected from all the Proteaceae species) was observed for many of the feeding guilds (Fig. 5). These include the ant, phytophytic chewers and the fungal feeding guilds, all of which display peak numbers during the wetter winter months. Fungal feeders probably also peak during this time, since it coincides with fungal growth peaks (pers. observ.).

Of special interest is the peak in the number of ants found within the infructescences during the colder autumn and winter months. Coetzee (1989) found a decrease in ants during winter and spring on the foliage and inflorescences of the Proteaceae species he studied. Visser (1992) and Wright (1990) obtained similar results for epigaic insects. In contrast, as in the present study, Visser (1992) found an increase in ant numbers in the infructescences of *P. nitida* during summer and autumn (Fig. 6). Donnelly (1983) found an increase in ant numbers during summer for ants collected on other Fynbos species.

A possible explanation for increased overall ant numbers during autumn and winter is that ants may use the infructescences as shelter against cold and rain during this period. They inhabit the bases of infructescences that were previously hollowed out by borers, and only depart when conditions become more suitable for foraging. This is supported by personal observations that some ants (e.g. *Crematogaster* sp. and *Sima* sp.) actually build their nests within the hollow base of the infructescences, and thus do not use these sites only as foraging grounds.

Not all of the *Protea* species, however, showed peak ant numbers during the same time (Fig. 6). Peak ant numbers in autumn and winter was observed for *P. laurifolia*, *P. neriifolia* and *P. repens*. *P. nitida* showed a peak in ant numbers during summer and autumn, while *P. burchellii* showed a peak in ant numbers during spring. Most of the arthropod guilds showed dissimilar temporal distribution patterns when comparing different *Protea* species.

It is clear that, in order to study the plant-insect interactions within the Fynbos, one should include as many study organisms as possible in order to avoid making erroneous assumptions about the ecology of the biome as a whole.

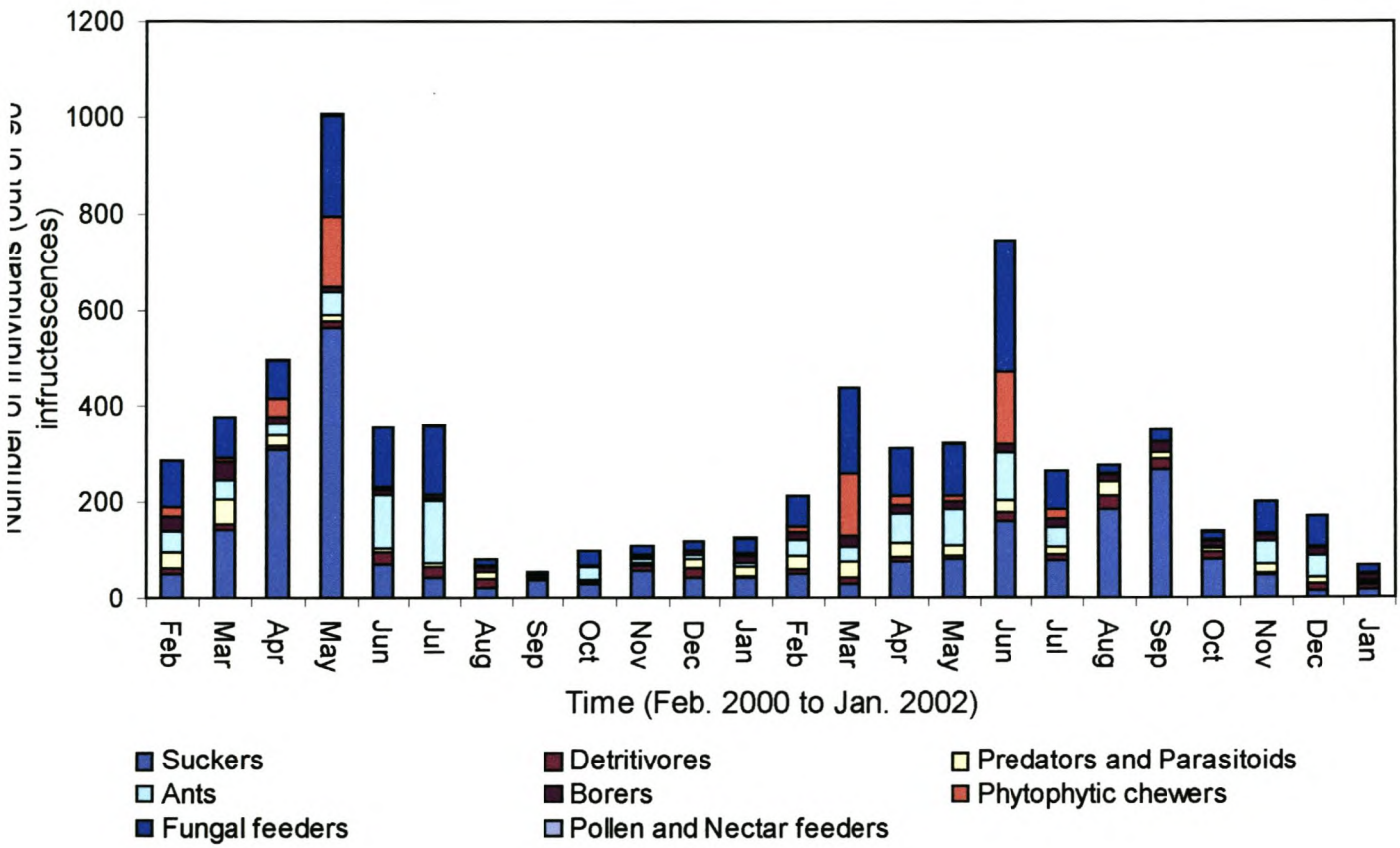

Fig. 5 Seasonal distribution of the feeding guilds on all the proteaceous species over the entire collection period from Feb. 2000 to Jan. 2002.

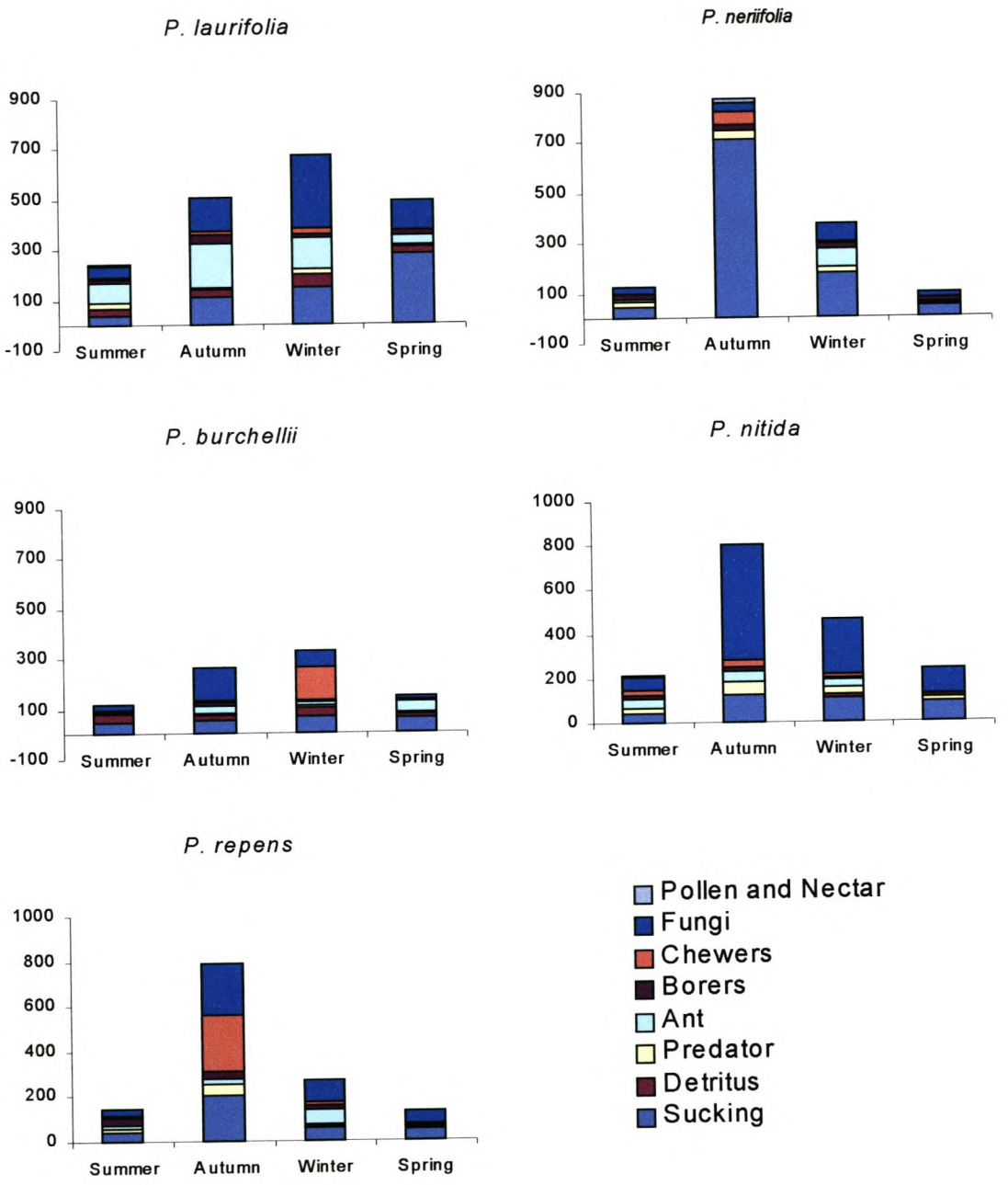

Fig. 6 Seasonal abundance of arthropod guilds (total number of arthropod individuals collected over the two year period) for five *Protea* species.

Fungal associations

Twenty-five fungal taxa were isolated from the various insect species inhabiting Proteaceae infructescences (Table 4). Many of these fungal genera are known to have representatives that cause Proteaceae plant diseases (Marasas *et al.* 1975; Gorter 1976; Von Broemsen & Brits 1986; Knox-Davies *et al.* 1987; Crous *et al.* 2000a, b; Swart *et al.* 2000; Taylor & Crous 2000; Taylor *et al.* 2001). It is possible that these insects play a significant role in the dispersal of the pathogenic fungi between plants.

Some of the fungi displayed specificity towards certain vector insects, while others were found to probably have more generalist associations (Table 4). *Gliomastix* sp., for example, was only isolated from juvenile specimens of *Euderes lineicolis* (Curcilionidae), while other fungi (e.g. *Alternaria* sp. and *Penicillium* sp.) were isolated from most of the insects tested. Additionally, some insects carried fungal spores of a wide variety of fungal species, while others carried spores of only a few fungal species (Table 4).

It should be noted that not all of the fungal species associated with the Proteaceae arthropods were isolated. Certain fungal species did not grow on MEA medium while some species of fungi deterred the growth of other fungal species by outcompeting the less competitive species. The number of different fungal species carried by these insects should thus be much higher, especially if we take into account that there is a conservative estimate of *ca.* 1,5 million fungal species on earth (Hawksworth 1991, 2001). The number of fungi specific to insects alone is estimated to be *ca.* 50 000 (Rossman 1994).

No ophiostomatoid fungi were isolated from the surface of any arthropod included in this study, but it is likely that insects also disperse these (Chapters 1, 2, 3). Other fungal taxa also not isolated in this study were members of the Trichomycetes, a group of fungi occurring in the hindguts of invertebrates and Laboulbeniales, a group of fungi found on the exoskeleton of beetles and flies (Hawksworth & Rossman 1997). These fungal groups represent a major component of the fungi found on insects, yet none were isolated using this

technique. It should thus be evident that the isolation process used in this study is not optimal for detecting all fungal species associated with the arthropods. It does, however, supply a basis for the determination of at least some fungal species found on insects associated with Proteaceae. A range of refinements in terms of methods of isolation and suitable growth media could expand upon the results presented here.

The low C_s values obtained in the present study indicate that many insects collected from Proteaceae infructescences are possibly monophagous. This suggests that some Fynbos plants could act as speciation islands, giving rise to new arthropod species adapted to associations with only one or a few plant species. This, in turn, may indicate that Fynbos, known for its floral diversity, could have an arthropod diversity that not only matches, but could well exceed, the floral diversity. It is also likely that the fungal diversity associated with Fynbos plants could far exceed the number of plant species, as calculated fungi:plant ratios are often very high (Hawksworth 2001). Along with studies such as those by Coetzee (1989), Wright (1990), Visser (1992) and Marais (1996), the present study provides a basis for further studies on arthropod, fungi and plant interactions within the Fynbos Biome.

The arthropods associated with the infructescences of the different plant species displayed distinct seasonal patterns. These results can potentially impact on aspects of the flower export industry. By knowing when certain arthropods occur on these plants, preventative measures can be taken at the correct time, before the presence of insects could pose a threat to the export industry.

Once we know which insects are the vectors of pathogenic fungi, this knowledge can be used to prevent agricultural crop losses. If the numbers of vector insects can be controlled, it will also reduce plant infection by pathogenic fungi.

Table 4 A checklist of fungi isolated from different insects associated with Proteaceae species. See Table 2 for arthropod reference numbers.

Arthropod taxa	Fungal taxa and reference numbers (SL number)																							
	Acremonium	Actinomycetes	*Alternaria* (20)	*Aspergillus* (3)	*Beauveria* (48)	*Chaetomium* (13)	*Botrytis*	*Cladosporium* (34)	*Dicyma* (10)	*Epicoccum* (10)	*Fusarium* (4)	*Clonostachys* (35)	*Gliomastix*	*Mucor* (85)	*Melanospora* (15)	*Nigrospora* (17)	Myxomycetes	*Penicillium*	*Pestalotia* (47)	*Rhizopus*	*Sclerotium*	*Trichoderma* (6)	*Trichothecium*	Yeast	Zygomycetes (49)
Agyropluce sp. (larvae)			*	*			*	*										*	*						*
Aradidae			*			*		*		*					*			*						*	*
Archaeognatha			*	*	*		*	*		*	*	*						*	*				*		
Arthropleona			*				*	*			*							*							*
Blattidae											*			*						*					
Bruchidae					*		*	*							*			*							
Sphenoptera sp. 1 (larvae)								*			*							*						*	
Sphenoptera sp. 2 (laarvae)	*	*		*			*	*			*							*						*	*
Sphenoptera sp. (pupae)				*							*							*	*						
Capys alphaeus (larvae)																		*							*
Carabidae				*			*	*										*	*						
Crematogaster sp.			*	*			*	*		*	*					*		*						*	*
Chrysomelidae sp. 1			*		*	*	*			*								*						*	*
Chrysomelidae sp. 2			*	*			*	*			*							*			*			*	
Chrysomelidae sp. 3			*	*				*			*				*			*						*	

Table 4 (cont.)

Arthropod taxa	Fungal taxa and reference numbers (SL number)																							
	Acremonium	Actinomycetes	*Altemaria* (20)	*Aspergillus* (3)	*Beauveria* (48)	*Chaetomium* (13)	*Botrytis*	*Cladosporium* (34)	*Dicyma* (10)	*Epicoccum* (10)	*Fusarium* (4)	*Clonostachys* (35)	*Gliomastix*	*Mucor* (85)	*Melanospora* (15)	*Nigrospora* (17)	Myxomycetes	*Penicillium*	*Pestalotia* (47)	*Rhizopus*	*Sclerotium*	*Trichoderma* (6)	*Trichothecium*	Yeast	Zygomycetes (49)
Chrysomelidae sp. 4			*					*										*							
Chrysomelidae sp. 5		*																*							
Cleridae							*	*			*							*						*	
Euderes lineicolis				*			*	*		*								*							
Curculionidae sp. 1																									
Curculionidae sp. 2			*				*	*										*	*						
E. lineicolis (larvae)											*		*					*						*	
Curculionidae sp. 3			*					*			*							*						*	
Dermaptera				*														*	*						
Diptera			*					*										*						*	
Formicidae sp. 2								*			*				*			*						*	
Formicidae sp. 3		*	*	*			*	*		*	*							*						*	*
G. hottentottus (F) (larvae)			*	*		*	*	*									*	*						*	*
Histeridae			*	*			*	*			*							*				*		*	
Braconidae			*	*				*										*							
O. maculatus		*	*	*	*		*	*		*	*	*			*			*				*		*	*
Fulgoridae											*							*							
Pseudococcidae			*					*										*							
Psocoptera sp. 1	*		*	*	*		*	*		*	*							*						*	

Table 4 (cont.)

Arthropod taxa	Fungal taxa and reference numbers (SL number)										
	Zygomycetes (49)	*									
	Yeast			*		*	*	*			
	Trichothecium										
	Trichoderma (6)										
	Sclerotium										
	Rhizopus										
	Pestalotia (47)	*	*								
	Penicillium	*	*	*	*	*	*	*	*		
	Myxomycetes										
	Nigrospora (17)			*							
	Melanospora (15)										
	Mucor (85)										
	Gliomastix										
	Clonostachys (35)										
	Fusarium (4)	*	*	*	*	*		*			
	Epicoccum (10)			*				*			
	Dicyma (10)						*				
	Cladosporium (34)	*	*	*	*	*		*	*		
	Botrytis	*	*	*				*			
	Chaetomium (13)										
	Beauveria (48)					*					
	Aspergillus (3)			*							
	Alternaria (20)	*	*	*	*		*	*	*		
	Actinomycetes	*									
	Acremonium										
Psocoptera sp. 2											
Psocoptera sp. 3											
Scutelleridae											
Sima sp.											
Staphylinidae											
Mantidae											
Thysanoptera											
Tinea sp. (larvae)											

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CONCLUSIONS

This study has indicated that the ophiostomatoid fungi and arthropod communities associated with the fruiting structures of Proteaceae represent an unique ecosystem, with its own feeding chains and ecological interactions. Some plant species were shown to be unique in the specific arthropods and fungal species contained within their infructescences. It is known that the arthropod communities have an influence on seed bank dynamics, as many insects destroy seeds while others may protect seeds from destruction (parasitic wasps, for instance will destroy certain boring insects). What is not so evident is that some fungal taxa (e.g. ophiostomatoid fungi) associated with infructescences of *Protea* species may also influence these seed bank dynamics indirectly by deterring the growth of more harmful fungal species. It is evident that many more studies on these interactions should be undertaken to highlight all the different factors that influence the seed bank dynamics of these economically important proteas.

The development and use of a PCR system that can detect the organisms that act as vectors of fungal species, already have a significant impact on agricultural practices. These techniques are presently being expanded to develop similar protocols for use in identifying pathogen vectors on crops, where other methods currently still fail to do so. Once the vector organisms for pathogenic fungi are known, it will be possible to significantly reduce crop losses, by employing more integrated control strategies.

In this study, six insects possibly responsible for the dispersal of *O. splendens* and *G. proteae* spores have been identified using a set of specifically designed PCR primers. Further studies are, however, needed to determine the specific insect(s) responsible for the dispersal of these fungi. Once this information becomes available, a comparison can be drawn between the vectors for the Southern Hemisphere fungi and those of the Northern Hemisphere

ophiostomatoid fungi. This should provide further insight into the evolutionary history of this biologically interesting group of fungi.

Seasonality of the different organisms associated with the infructescences of *Protea* species has been observed. Both arthropods and ophiostomatoid fungi showed peak numbers during the wetter autumn and winter months. Fungal numbers probably peak during this time due to the prevailing more moist conditions. The overlap in high arthropod numbers during this time suggests that more animals are available then for the dispersal of fungal spores. Results on the seasonal distribution of arthropods can potentially impact on aspects of the commercial cultivation of proteas. By knowing when certain arthropods occur on plants, preventative measures can be taken in time, before the animals could pose a threat to this industry.

It has been suggested that there may be mutualistic relationships between the ophiostomatoid fungi and *Protea* species, and between the insect vectors and the fungi. The plants provide a niche for the fungi to thrive in, while the fungi may prevent other, more detrimental fungi, from growing in the specific *Protea* infructescence. The insect *G. hottentottus* may also aid ophiostomatoid fungal growth by providing moisture and substrate inside the infructescences, while the fungi in this case may in turn deter other fungal species from attacking the developing larvae and pupae. If the latter suggestion proves to be correct, then ophiostomatoid fungi may well impact negatively on the plants by protecting an organism that is ultimately responsible for seed losses. It should be evident that seed bank dynamics of economically important proteas appear to be a complicated process, with many influencing factors. The dynamics of the process need to be understood if informed decisions are to be made on conservation strategies of the Fynbos Biome.

The identification of the arthropods collected during this study proved to be one of the major problems encountered. It became acutely apparent that more

taxonomic and systematic studies focused on Fynbos invertebrates are critically important, as very limited published data is available. This seriously hinders correct identification of the diversity of invertebrates present within the Fynbos.

Future research should concentrate on determining the specific vectors for all of the Southern Hemisphere ophiostomatoid species. Once this is known, the factors influencing the host specificity of these fungi should become clearer. More studies are also required on the arthropods associated with different Proteaceae fruiting structures, as this impacts directly on the economic viability of the cut-flower industry of South Africa.