Discovery of Fungus–Mite Mutualism in a Unique Niche

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ABSTRACT The floral heads (infructescences) of South African *Protea* L. represent a most unusual niche for fungi of the economically important genus *Ophiostoma* Syd. and P. Syd. emend. Z.W. de Beer et al. Current consensus holds that most members of *Ophiostoma* are vectored by tree-infesting bark beetles. However, it has recently been suggested that mites, phoretic on these bark beetles, may play a central role in the dispersal of *Ophiostoma*. No bark beetles are known from *Protea*. Therefore, identifying the vectors of *Ophiostoma* in *Protea* infructescences would independently evaluate the role of various arthropods in the dispersal of *Ophiostoma*. Infructescence-colonizing arthropods were tested for the presence of *Ophiostoma* DNA using polymerase chain reaction (PCR) and for reproductive propagules by isolation on agar plates. PCR tests revealed that few insects carried *Ophiostoma* DNA. In contrast, various mites (*Proctolaelaps vandenbergi* Ryke, two species of *Tarsonemus* Canestrini and Fonzago, and one *Trichouropoda* Berlese species) frequently carried *Ophiostoma* propagules. DNA sequence comparisons for 28S ribosomal DNA confirmed the presence of *O. splendens* G. J. Marais and M. J. Wingf., *O. palmiculminatum* Roets et al., and *O. phasma* Roets et al. on these mites. Two apparently undescribed species of *Ophiostoma* were also identified. Light and scanning electron microscopy revealed specialized structures in *Trichouropoda* and one *Tarsonemus* sp. that frequently contained *Ophiostoma* spores. The *Trichouropoda* sp. was able to complete its life cycle on a diet consisting solely of its identified phoretic *Ophiostoma* spp. This study provides compelling evidence that mites are the primary vectors of infructescence-associated *Ophiostoma* spp. in South Africa.

KEY WORDS *Ophiostoma*, symbiosis, *Protea*, mycangia, sporothecae

The Cape Floristic Region is located at the southwestern tip of Africa and is internationally recognized for its exceptional richness in flowering plants (Goldblatt and Manning 2000). It displays very high levels of gamma diversity, which correlates well with the unusually high levels of local endemism (Linder 2003). The most striking component of the Cape Floristic Region is the unique Fynbos Biome, which includes the majority of ~9,000 constituent vascular plant species. Landscapes within the Fynbos are often dominated by members of the endemic African genus *Protea* L. (proteas) (Linder 2003).

Proteas produce large, colorful floral heads (inflorescences), and numerous species are economically important in generating revenue from ecotourism, horticulture, and the dried-flower industries (Crous et al. 2004). A number of species also represent pivotal members of the ecosystems in which they occur. The seeds of several species are retained within compact structures known as infructescences that serve as above-ground seed storage structures, usually releasing seeds after fire (Bond 1985).

Infructescences of *Protea* spp. can be viewed as miniature ecosystems (Zwölfer 1979), in which many fungal species are known to thrive (Marais and Wingfield 1994, Lee et al. 2005). One of the most unusual contemporary discoveries related to *Protea* has been the detection of so-called ophiostomatoid fungi in the infructescences of serotinous *Protea* spp. (Wingfield et al. 1988). This fungal group includes diverse genera (*Gondwanamyces* G. J. Marais and M. J. Wingf., *Ceratocystis* Ellis and Halst., *Ophiostoma* Syd. and P. Syd. emend. Z.W. de Beer et al., and their asexual states) that are grouped together based on their morphology rather than common descent. The ophiostomatoid fungi are adapted to arthropod dispersal, typically by having sticky spores carried on stalked fruiting structures (Malloch and Blackwell 1992, 1993). These fungi are best known as associates of insects such as bark beetles (Coleoptera: Scolytinae) that construct galleries in the bark/cambium interface of trees (Upadhyay 1981, Harrington 1987, Wingfield et al. 1993, Jacobs and Wingfield 2001) or picnic beetles (Coleoptera: Nitidulidae) that colonize wounds on trees (Gibbs and French 1980, Juzwik 2001). These fungi also include some of the world's most serious tree pathogens such as the causal agents of Dutch elm

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disease [*Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier] and *Ceratocystis fagacearum* (Bretz) J. Hunt, the causal agent of Oak wilt (Sinclair et al. 1987, Brasier 1991). Their dominant presence in *Protea* infructescences (Marais and Wingfield 2001, Roets et al. 2005) has thus been considered curious and inexplicable.

Five species of *Ophiostoma* have been described from the infructescences of *Protea* spp. (Marais and Wingfield 1994, 1997, 2001, Roets et al. 2006a). *O. splendens* G. J. Marais and M. J. Wingf., *O. protearum* G. J. Marais and M. J. Wingf., and *O. palmiculminatum* Roets et al. are each thought to be confined to a specific *Protea* sp., whereas *O. africanum* G. J. Marais and M. J. Wingf. and *O. phasma* Roets et al. have been isolated from different *Protea* spp. (Marais and Wingfield 1997, 2001, Roets et al. 2005, 2006a). It is unknown whether the varying host specificity of the *Ophiostoma* spp. relates to host factors, the environment, or to the mechanisms of dispersal of these fungi.

It is unknown how *Ophiostoma* spp. move from one *Protea* infructescence to another. The fungi appear in the infructescences relatively soon after flowering when the infructescences close (Roets et al. 2005). Although *Ophiostoma* spp. that occur elsewhere are known to be vectored by many different types of insects, conifer-infesting bark beetles are the most common vectors (Francke-Grosmann 1967, Upadhyay 1981, Wingfield et al. 1993, Kirisits 2004). It is thus reasonable to assume that the *Ophiostoma* spp. found in *Protea* infructescences would also have insect vectors and their morphological characteristics are consistent with this view.

Mites, particularly those carried by bark beetles, are also known to act as vectors of *Ophiostoma* spp., carrying them between various coniferous trees (Bridges and Moser 1983, Moser 1985, Moser et al. 1995). The association between mites and the fungi that they vector can be highly specialized (Klepzig et al. 2001a, 1b, Klepzig and Six 2004), and some are known to have evolved specialized spore-carrying structures (sporothecae) that contain spores of ophiostomatoid fungi (Bridges and Moser 1983, Moser 1985, Moser et al. 1995). The association between these mites and their phoretic fungi may thus be mutualistic (Klepzig et al. 2001b).

The aim of this study was to determine how *Ophiostoma* spp. move from one *Protea* infructescence to another and particularly to consider whether insects or mites might be involved in this process. We question whether the host specificity of *Ophiostoma* spp. associated with *Protea* spp. can be explained by the vector relationships of the fungi. Furthermore, we consider whether there are mutualistic relationships between specific *Ophiostoma* spp. and their vectors, such as is found in the bark beetle systems.

Materials and Methods

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

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

Emergence cages were maintained at room temperature in the laboratory. They were inspected every 2–3 d over a 40-d period, and all emerging arthropod individuals were collected and classified into morphospecies. Use of the emergence cages ensured simultaneous collection of arthropods as they emerged from numerous infructescences, and presumably after they would have acquired spores from fungi in the infructescences. After 40 d, the infructescences were opened, and all remaining arthropods were extracted using fine tweezers and a dissecting needle. The surfaces of larger arthropods were cleared of debris and/or smaller phoretic arthropods using a fine camelhair brush and dissecting needle. All arthropods were stored at −20°C until further analysis.

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

Vector Identification Using Polymerase Chain Reaction. A newly developed polymerase chain reaction (PCR) protocol (Roets et al. 2006c) was used to test a subset of infructescence-associated arthropods collected from the emergence cages for the presence of *Ophiostoma* DNA. The subset included individuals ($n \leq 30$) of each arthropod species collected per *Protea* sp. (Table 1). All individuals of *Genuchus hot-*

Table 1. Total no. of arthropods collected from the infructescences of the four *Protea* spp. ($n = 70$ for each species) and tested for the presence of *Ophiostoma* DNA using PCR techniques

Arthropod taxa	Ref. no.	*Protea* species			
		P. repens	*P. longifolia*	*P. neriifolia*	*P. laurifolia*
Insects					
Argyroploce sp. Hbner (Tortricidae)	68	7	1	1	3
Blattidae	26		1	2	
Braconidae	52	2		1	
Bruchidae	51				1
Capys alphaeus Cramer (Lycaenidae)	66	2	1		1
Carabidae	29				4
Chrysomelidae	17		17	2	
Crematogaster sp. Lund (Formicidae)	15	10	21		
Curculionidae	48	4	1	1	1
Dermaptera	42				1
Diptera	5	12		4	2
Euderes lineicolis Wiedemann (Curculionidae)	33	9	1		1
Formicidae (sp. 1)	23	4			
Formicidae (sp. 2)	56	9			79
Genuchus hottentottus (F) (Scarabaeidae)	70	28 (2) **39**		15	1
Gyponyx sp. Gorham (Cleridae)	55	1			3
Histeridae	32	7	1	1	5
Hopliini (Scarabaeidae)	47		1	4	3
Miridae	20	1			1
Nitidulidae	25	30	18	8	7
Oxycarenus maculates Stal. (Lygaeidae)	7	51 (2) **75**	37	19	46
Pentatomidae	24	2		1	
Psocoptera (sp. 1)	31	12, **50**	9	35	1
Psocoptera (sp. 2)	12	4, **50**	1	1	3
Psocoptera (sp. 3)	13	66 (1) **108**		8	1
Sphenoptera Solier sp. (Buprestidae)	49	2	4	1	11
Staphylinidae	35	1			4
Thysanoptera	34		2	5	1
Tinea sp. L. (Tineidae)	67	4		1	1
Spiders					
Clubionidae	59	1			
Spider (sp. 1)	64			4	
Spider (sp. 2)	63	1			
Spider (sp. 3)	60	2			1
Mites					
Ameroseius proteaea Ryke (Ameroseiidae)	M1	18, **50**	**3**	1, **29**	**50**
Trichouropoda sp. Berlese (Uropodidae)	M2	23, **50**	4	6, **24**	13
Lorryia sp. Oudemans (Tydeidae)	M3	1, **42**		1	3, **1**
Tenuelamellarea hispanica Subias & Itor. (Lamellareidae)	M4	3, **33**			1
Humerobates setosus Behan-Pelletier & Mahunka (Humerobatidae)	M5	2, **50**	1	**2**	1, **5**
Bdellodes sp. Oudemans (Bedellidae)	M6	1, **50**	**50**	1, **50**	**50**
Proctolaelaps vandenbergi Ryke (Ascidae)	M7	14, **50**	6	19, **50**	1, **28**
Zygoribatula setosa Evans (Oribatulidae)	M8	2, **9**			

Numbers in parentheses indicate the no. of individuals verified to be positive for *Ophiostoma* DNA. Numbers in bold indicate the no. of additional arthropod individuals collected and tested for the presence of *Ophiostoma* spp. reproductive propagules by plating techniques.

tenntottus (F.) (Scarabaeidae) and *Oxycarenus maculates* Stal. (Lygaeidae) were tested, because these two taxa had previously been noted as putative vectors (Roets et al. 2006c). Individuals used for the PCR procedures were macerated in Eppendorf tubes, after which the total genomic DNA was extracted (Lee and Taylor 1990).

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

Vector Identification by Direct Plating of Arthropods. All individuals ($n \leq 50$) of the small (<1 mm long) arthropod species and the species that yielded positive PCR results were crushed, vortexed in 2–10 ml ddH_2O (depending on the size of the arthropod), and plated (1 ml of suspension per plate) on petri dishes containing 2% malt extract agar (MEA; Biolab, Midrand, South Africa), streptomycin sulfate (0.04 g/liter), and cycloheximide (0.05 g/liter), which is selective for *Ophiostoma* spp. This plating technique made it possible to verify putative vectors for the *Protea*-associated *Ophiostoma* spp., and it also provided an indication of the number of reproductive propagules carried per individual insect. Spore num-

Table 2. Isolates, frequency (*F*), and mean no.colony-forming units (CFUs) of *Ophiostoma* spp. isolated from *Ophiostoma* spore-carrying mites collected from *Protea* infructescences from various localities

Mite species	*n*	Host	Locality	Fungal species	*F* (%)	CFUs
Trichouropoda sp.	50	*P. repens*	J. S. Marais Park	*O. splendens*	3 (6)	1–8 (4.33)
Trichouropoda sp.	50	*P. repens*	J. S. Marais Park	*O. palmiculminatum*	4 (8)	1–8 (5.50)
Trichouropoda sp.	50	*P. repens*	J. S. Marais Park	*Sporothrix* sp. 1	1 (2)	1
Trichouropoda sp.	24	*P. neriifolia*	Jonkershoek	*O. phasma*	1 (4.17)	19
Trichouropoda sp.	11	*P. repens*	George	*O. splendens*	3 (27.27)	1–2 (1.33)
P. vandenbergi	50	*P. neriifolia*	Jonkershoek	*O. phasma*	1 (2)	1
P. vandenbergi	50	*P. repens*	J. S. Marais Park	—	0	0
P. vandenbergi	28	*P. laurifolia*	Piekenierskloof	—	0	0
Tarsonemus cf. sp. A	50	*P. laurifolia*	Piekenierskloof	*O. phasma*	2 (4)	9–51 (30.00)
Tarsonemus cf. sp. A	50	*P. repens*	J. S. Marais Park	—	0	0
Tarsonemus cf. sp. B	19	*P. caffra*	Walter Sisulu Garden	*Sporothrix* sp. 2	3 (15.79)	1–9 (5.67)

bers were based on numbers of *Ophiostoma* colony-forming units growing from each arthropod individual. The mean number of colony-forming units was calculated for each putative *Ophiostoma* spp. isolated from each arthropod species (Table 2).

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

Vector Identification by Light and Scanning Electron Microscopy. The position of fungal spores on arthropod exoskeletons was studied using a Leo 1430VP scanning electron microscope (SEM). Individuals ($n = 50$ per arthropod species) of the suspected primary vectors of the *Ophiostoma* spp. were collected from *P. neriifolia* and *P. repens* infructescences from the Jonkershoek Nature Reserve and J. S. Marais Park, respectively. These arthropod species were also examined using light microscopy ($n = 50$ per species). In addition, representatives of *Genuchus hottentottus* ($n = 15$) and *Oxycarenus maculates* ($n = 33$) were studied by SEM, because these two species had been recognized as potential vectors in a previous study (Roets et al. 2006c).

For the SEM studies, the arthropods were frozen (−20°C) and dried (3 d at 50°C) and mounted onto stubs using double-sided carbon tape. They were sputter coated with gold-palladium using standard methods. SEM scans made it possible to locate spores on the surfaces of the arthropods. We focused specifically on detecting ascospores, because of the problems associated with the identification of fungal taxa based on the asexual conidia. Ascospores were presumed to belong to *Ophiostoma* when they had an allantoid shape, were between 5 and 7 μm long, and tended to stick together. These characteristics are typical of the *Ophiostoma* spp. found in *Protea* infructescences (Marais and Wingfield 2001, Roets et al. 2006a). Arthropods were collected only from *Protea* infructescences that were heavily infected with *Ophiostoma* spp.

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

DNA Extraction and Amplification of Fungal Isolates. Genomic DNA was extracted from isolates using a Sigma GenElute plant genomic DNA miniprep kit (Sigma-Aldrich Chemie, Steinheim, Germany) following the manufacturer's instructions. For amplification and sequencing of the nuclear large subunit (LSU) 28S rDNA region, the primers LROR and LR5 (White et al. 1990) were used. PCR reaction volumes (50 μl) contained 32.5 μl ddH_2O, 1 μl DNA, 5 μl (10×) reaction buffer (Super-Therm; JMR Holdings), 5 μl $MgCl_2$, 5 μl dNTP (10 mM of each nucleotide), 0.5 μl (10 mM) of each primer, and 0.5 μl Super-Therm *Taq* polymerase (JMR Holdings). PCR runs were performed on a Gene Amp, PCR System 2 700 thermal cycler (Applied Biosystems). PCR reaction conditions included an initial denaturation step of 2 min at 95°C followed by 35 cycles of 30 s denaturation at 95°C, 30 s annealing at 55°C, and 1 min elongation at 72°C. A final elongation step of 8 min at 72°C was performed before the PCR process was terminated. Purification and sequencing of PCR products followed the methods outlined above.

Sequence data obtained in this study were compared with sequences of both *Protea*-associated and non–*Protea*-associated *Ophiostoma* spp. obtained from GenBank (Table 3) using the software package Clustal X (1.81). These included the large subunit sequences of the ex-type cultures of all *Ophiostoma* spp. described from *Protea* infructescences.

Protea-Associated *Ophiostoma* spp. as Food Source for Vector Arthropods. The most common arthropod identified as a vector of *Ophiostoma* spp. spores was a species of mite collected from the infructescences of *P. repens* (~5 mo old) in the J.S. Marais Park. To test the ability of this mite to feed and reproduce on a diet of *Protea*-associated *Ophiostoma* species only, mites

Table 3. GenBank accession numbers for fungal isolates used for comparisons of 28S large subunit DNA sequence data

Fungal species	Isolate no.		Host	Geographical origin	Collector	GenBank accession no.
	CBS	CMW				
Ceratocystiopsis manitobensis		13792	*Pinus resinosa*	Canada	J. Reid	DQ294358
C. minima	128.86	162	*Pinus banksiana*	USA	M. J. Wingfield	DQ294361
C. minuta		4586	*Ips cembrae*	Scotland	T. Kirisits	DQ294360
C. minuta-bicolor	393.77	1018	*Ips* sp. from *Pinus* sp.	USA	R. W. Davidson	DQ294359
C. ranaculosa		13940	*Pinus echinata*	USA	F. Hains	DQ294357
C. rollhanseniana	118669	13791	*Pinus sylvestris*	Norway	J. Reid	DQ294362
Grosmannia galeiformis	115711	5290	*Pinus sylvestris*	Scotland	T. Kirisits	DQ294383
G. grandifoliae		703	*Fagus grandifolia*	USA	R. W. Davidson	DQ294399
G. penicillata	116008	2644	*Picea abies*	Norway	H. Solheim	DQ294384
G. piceiperda	366.75	660	*Piceae abies*	Finland	A. M. Hallaksel	DQ294392
G. robusta		2805	unknown	Unknown	T. Hinds	DQ294398
G. serpens	67.76	290	unknown	Italy	Gambagi	DQ294394
G. wageneri		491	*Pinus jeffreyi*	Unknown	T. Harrington	DQ294396
Leptographium lundbergii	352.29	217	unknown	Unknown	M. Lagerberg	DQ294388
Ophiostoma africanum	116571	823	*Protea gaguedi*	Unknown	M. J. Wingfield	AF221015
	116566	1104	*Protea caffra*	South Africa	Unknown	DQ316147
O. ainoae	118672	1903	*Picea abies*	Norway	O. Olsen	DQ294368
O. araucariae	114.68	671	*Araucaria* sp.	Chile	H. Butin	DQ294373
O. canum	118668	5023	*Tomicus minor*	Austria	T. Kirisits	DQ294372
O. carpenteri	118670	13793	*Trypodendron lineatum*	USA	SE Carpenter	DQ294363
O. distortum	397.77	467	*Picea engelmannii*	USA	R. W. Davidson	DQ294369
O. flexuosum	208.83	907	*Picea abies*	Norway	H. Solheim	DQ294370
O. floccosum		1713	*Pinus ponderosa*	USA	C. Bertagnole	DQ294367
O. fusiforme	112912	9968	*Populus nigra*	Azerbaijan	D. N. Aghayeva	DQ294354
O. ips	137.36	7075	*Ips integer*	USA	C. T. Rumbold	DQ294381
	CBS	CMW				
O. lunatum	112928	10564	*Larix decidua*	Austria	T. Kirisits	DQ294355
O. montium	151.78	13221	*Pinus ponderosa*	USA	R. W. Davidson	DQ294379
O. multiannulatum	357.77	2567	*Pinus* sp.	USA	Unknown	DQ294366
O. nigrocarpum	638.66	651	*Pseudotsuga menziesii*	USA	R. W. Davidson	DQ294356
O. novo-ulmi		10573	*Picea abies*	Austria	Neumuller	DQ294375
O. palmiculminatum		20677	*Protea repens*	South Africa	F. Roets	DQ316143
		20694	*Protea repens*	South Africa	F. Roets	DQ316144
		23048	*Trichouropoda* **sp. from *Protea repens***	**South Africa**	**F. Roets**	**DQ821527**
		23049	*Trichouropoda* **sp. from *Protea repens***	**South Africa**	**F. Roets**	**DQ821525**
		23052	*Trichouropoda* **sp. from *Protea repens***	**South Africa**	**F. Roets**	**DQ821526**
		23053	*Trichouropoda* **sp. from *Protea repens***	**South Africa**	**F. Roets**	**DQ821524**
O. piceae		8093	*Tetropium* sp.	Canada	K. Harrison	DQ294371
O. piliferum	12932	7879	*Pinus sylvestris*	Unknown	H. Diddens	DQ294377
O. phasma		20698	*Protea laurifolia*	South Africa	F. Roets	DQ316152
		20676	*Protea laurifolia*	South Africa	F. Roets	DQ316151
		26	*P. vandenbergi* **from *P. neriifolia***	**South Africa**	**F. Roets**	**DQ821535**
O. pluriannulatum	118684	75	unknown	Unknown	R. W. Davidson	DQ294365
O. protearum	116654	1107	*Protea caffra*	South Africa	M. J. Wingfield	DQ316145
	116568	1102	*Protea caffra*	South Africa	M. J. Wingfield	AF221014
O. pulvinisporum	118673	9022	*Pinus pseudostrobus*	Mexico	X. Zhou	DQ294380
O. quercus	118713	3110	*Juglans cinerea*	USA	M. J. Wingfield	DQ294376
O. splendens		20679	*Protea repens*	South Africa	F. Roets	DQ316150
		23050	*Trichouropoda* **sp. from *Protea repens***	**South Africa**	**F. Roets**	**DQ821534**
	116569	872	*Protea repens*	Unknown	M. J. Wingfield	AF221013
O. stenoceras	237.32	3202	*Pinus* sp.	Norway	H. Robak	DQ294350
O. subannulatum	118667	518	*Pinus ponderosa*	Unknown	W. Livingston	DQ294364
O. ulmi		1462	*Ulmus procera*	USA	C. Brasier	DQ294374
Sporothrix inflata	239.68	12527	soil	Germany	W. Gams	DQ294351
S. schenckii	117842	7614	human	South Africa	H. Vismer	DQ294352
S. schenckii-like		7617	soil	South Africa	H. Vismer	DQ836010
Sporothrix **sp. 1**		**23057**	*Tarsonemus* **sp. from *Protea caffra***	**South Africa**	**F. Roets**	**DQ821531**
		23058	*Tarsonemus* **sp. from *Protea caffra***	**South Africa**	**F. Roets**	**DQ821532**
		23059	*Tarsonemus* **sp. from *Protea caffra***	**South Africa**	**F. Roets**	**DQ821533**
Sporothrix **sp. 2**		**23051**	*Trichouropoda* **sp. from *Protea repens***	**South Africa**	**F. Roets**	**DQ821537**

Isolates obtained from mites in this study are indicated in bold.

were transferred to petri dishes containing 1-wk-old cultures of *O. splendens* growing on MEA plates. The first generation progeny of these individuals that had been caught in the wild were used in all subsequent experiments. All experiments were carried out on MEA plates kept at 25°C in the dark.

The population growth rate of the mite species was tested on a diet of *O. palmiculminatum, O. phasma, O. splendens,* and eight nonophiostomatoid fungal species isolated from species of *Protea* available from the culture collection of Stellenbosch University, Stellenbosch, South Africa. These included representatives of the genera *Cladosporium* Link (STU5664), *Conoplea* Pers. (STU5660), *Dactylaria* Sacc. (STU5657), *Gliocladium* Corda (STU5661), *Monodictys* S. Hughes (STU5656), *Penicillium* Link (SL646), *Phaeoisaria* Höhn. (STU5659), and *Pithomyces* Berk. and Broome (STU5662). Mature mite individuals ($n = 10$) were placed on 1-wk-old cultures of the 11 fungal species. As a control, mites were placed on petri dishes containing only MEA. The experiment was replicated three times. After 40 d, the number of individuals in each colony was determined. Differences in mite population size between the various fungal species were compared statistically using a *t*-test (Statistica 7; StafSoft, Tulsa, OK). Significant differences are reported when $P \leq 0.05$.

Results

Arthropod Collection. Forty-one arthropod morpho-species (811 individuals) were collected from the different *Ophiostoma*-colonized *Protea* infructescences using the emergence cages (Table 1). *P. repens* infructescences contained the greatest number of arthropod individuals (341) and also had the greatest diversity of taxa (33). *P. neriifolia* (richness = 24, abundance = 142), *P. laurifolia* (richness = 29, abundance = 201), and *P. longifolia* (richness = 20, abundance = 180) showed lower arthropod richness and abundance levels than *P. repens,* but their richness and abundance levels were comparable with each other. Most arthropods were found to be associated with more than one *Protea* sp.

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

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

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

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

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

Vector Identification by Light and Scanning Electron Microscopy. No ascospores of *Ophiostoma* spp. were observed on the surfaces of any of the insect (*G. hottentottus* or *O. maculates*) individuals using SEM. SEM also failed to disclose the presence of any *Ophiostoma* ascospores from wild-caught *P. vandenbergi* mites, whereas spores of several undetermined fungal

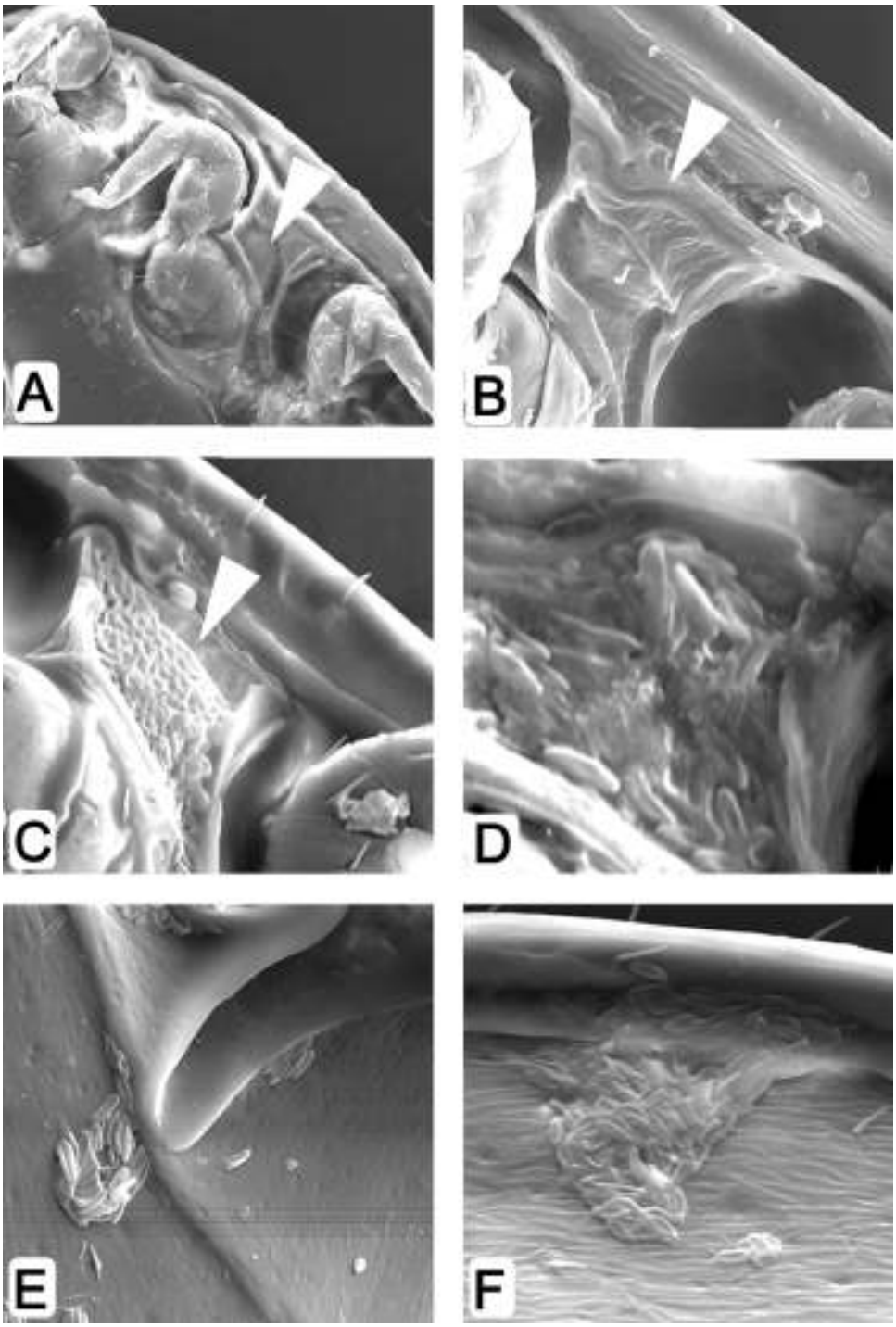

Fig. 1. Scanning electron micrographs of unidentified conidia and ascospores of *Ophiostoma* spp. from the surface of *Trichouropoda* sp. individuals. (A) Ventral view of a mite showing the depression between the legs where spores were commonly observed (arrow). (B) Close-up view of the same structure.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

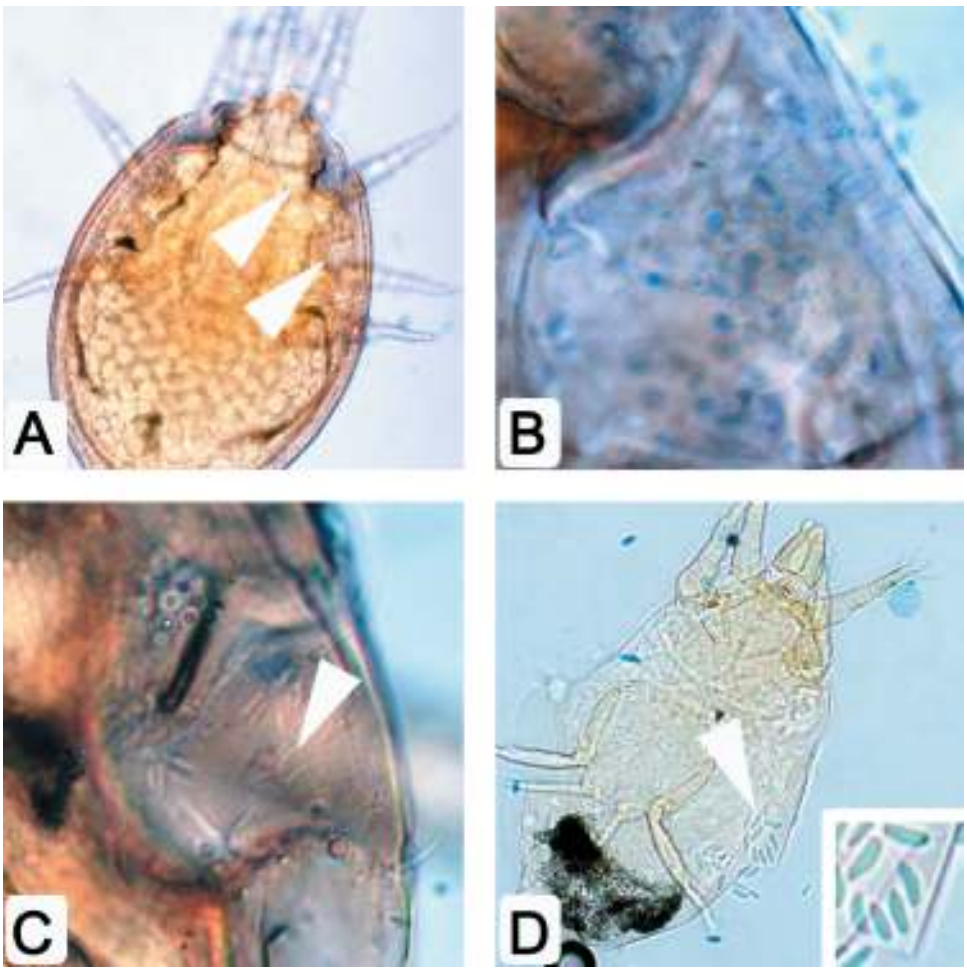

Fig. 2. Light microscope micrographs depicting *Ophiostoma* sp. ascospores from *Trichouropoda* sp. and unidentified fungal conidia from *Tarsonemus cf.* sp. A individuals. (A) *Trichouropoda* sp. mite showing areas where ascospores accumulate (arrow). (B) Close-up of depression filled with unknown conidia.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

species were commonly observed. In contrast, SEM of wild-caught *Trichouropoda* sp. mites revealed the presence of *Ophiostoma* ascospores within the grooves and depressions associated with the legs (Fig. 1A–F) of 3 of the 50 individuals tested. In one instance, *Ophiostoma* ascospores were also observed on the upper surface of a mite (Fig. 1F). Light micrographs confirmed these observations (Fig. 2A–C). Spores of many other unidentified fungal species were also observed on these mites.

Conidia (asexual spores) analogous to those of *Sporothrix* sp. 1 were observed underneath flap-like structures of the integument formed by tergite one in two individuals of *Tarsonemus cf.* sp. A, using light microscopy (Fig. 2D). It is likely that *Ophiostoma* ascospores would be carried in a similar fashion. Because of a lack of material, no *Tarsonemus cf.* sp. B individuals were studied using SEM or light microscopy.

***Ophiostoma* spp. Isolated from Mites.** Amplified fragments obtained using the primers LROR and LR5 were ~700 bp long. Sequences from all putative *Ophiostoma* spp. isolated from mites were used in DNA comparisons (Tables 2 and 3). These comparisons confirmed that *O. palmiculminatum*, *O. splendens*, and *O. phasma* (Table 2) were collected from *Tarsonemus cf.* sp. A, *P. vandenbergi*, and the *Trichouropoda* sp. The single isolate of *Sporothrix* sp. 1 from the *Trichouropoda* sp. was distinct from any of the *Ophiostoma* spp. known from *Protea* infructescences.

No differences were found in comparisons between large subunit data of *O. palmiculminatum* and the three isolates from *Tarsonemus cf.* sp. B collected from *P. caffra.* Isolates representing *O. palmiculminatum* and those of *Sporothrix* sp. 2 were, however, distinct based on morphological comparisons. Conidia of *O. palmiculminatum* are clavate in shape (Roets et al.

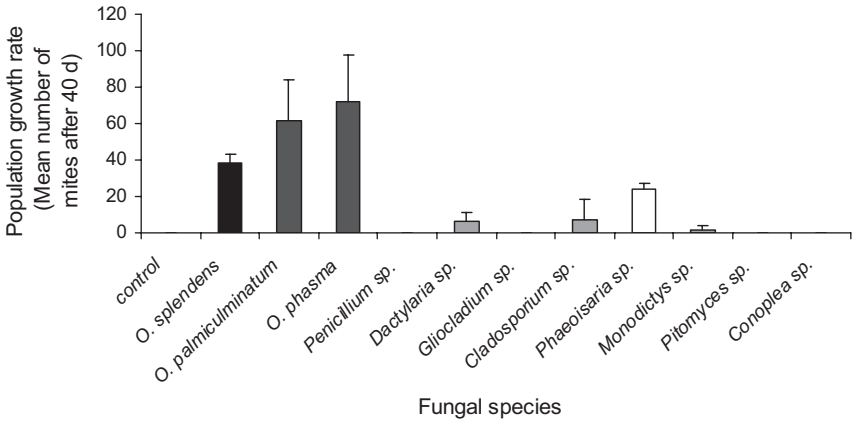

Fig. 3. Mean population size (±SD) after 40 d for *Trichouropoda* sp. mites feeding on various fungal species associated with members of the genus *Protea*. Different colored bars indicate significant differences between the population sizes ($P \leq 0.05$) on the various fungal species tested.

2006a), whereas c-shaped conidia were formed by isolates of *Sporothrix* sp. 2. These three isolates probably represent another undescribed species of *Ophiostoma* closely related to *O. palmiculminatum*.

***Ophiostoma* spp. as a Food Source for *Trichouropoda* sp.** Results of this study showed that mites of the genus *Trichouropoda* are the main vectors of the spores of various *Ophiostoma* spp. (Table 2). They were consequently used in studies to test their ability to feed on *Ophiostoma* sp. This mite is fairly large (~400–500 μm), which facilitated easy handling of individuals. Individuals that were caught in the wild and placed on colonies of *O. splendens* reproduced regularly. Their progeny failed to reproduce on the control plates or when exposed to a potential diet of *Penicillium*, *Gliocladium*, *Conoplea*, or *Pithomyces* spp. (Fig. 3). Compared with the control, a significant increase in population size of this mite species was observed when it was fed on colonies of *O. palmiculminatum* ($t = 4.8634$, $P = 0.0398$), *O. phasma* ($t = 4.7244$, $P = 0.0420$), *O. splendens* ($t = 14.8523$, $P = 0.0045$), and the species of *Phaeoisaria* ($t = 12.0000$, $P = 0.0069$). The population growth of the *Trichouropoda* sp. on the remaining fungal species tested was not significant compared with the control (Fig. 3). Mites feeding on the species of *Phaeoisaria* had significantly smaller population sizes after 40 d than mites feeding on *O. palmiculminatum* ($t = 2.9343$, $P = 0.0426$), *O. phasma* ($t = 3.1153$, $P = 0.0357$), and *O. splendens* ($t = 4.4675$, $P = 0.0111$). This mite species had significantly larger population sizes after 40 d on *O. palmiculminatum* and *O. phasma* compared with when feeding on *O. splendens*.

Discussion

The infructescences of *Protea* spp. represent one of the most intriguing habitats in which *Ophiostoma* spp. have ever been found. The fact that nothing is known regarding their mode of transmission represents a substantial void in our understanding of a group of ecologically important fungi. Results of this study provided the first conclusive discovery of vectors for the *Ophiostoma* spp. found in this *Protea* niche. Given that insects and mites vector other species of *Ophiostoma* from different habitats, it was reasonable to hypothesize that the same might be true of the *Protea*-associated species. Discovery of mites as vectors of the *Protea*-associated *Ophiostoma* spp. is, however, important, and it provides a framework for future studies on these unusual species of *Ophiostoma*.

Of the 10 mite species tested for the presence of *Ophiostoma* spp., only 4 (*Proctolaelaps vandenbergi*, 2 *Tarsonemus* spp., and a *Trichouropoda* sp.) tested positive. This was interesting, as many of the tested mite species have no known association with *Ophiostoma* spp. despite similarities in size and fungivorous habit to those that display this association. These results suggest a specific relationship between mites, at least in the case of the two *Tarsonemus* spp., the *Trichouropoda* sp., and *Protea*-associated *Ophiostoma* spp.

Trichouropoda sp. was the mite species most closely associated with the *Ophiostoma* spp. that live on *Protea* hosts. The relationship between this mite and *Ophiostoma* spp. was determined through direct isolations and through SEM, which revealed the presence of ascospores carried in specialized structures. In addition, the *Trichouropoda* sp. had the highest frequency of individuals carrying species of *Ophiostoma*, and it was found to carry spores of four of the five *Ophiostoma* spp. isolated in this study. The *Trichouropoda* sp. may thus play a principal role in carrying various *Protea*-associated *Ophiostoma* spp. within the *Protea* ecosystem. The nonspecificity of the *Trichouropoda* sp. mites toward species of *Ophiostoma* is shown by the ability of these mites to reproduce on a diet of all tested *Ophiostoma* spp. with more or less equal success. In contrast to the *Trichouropoda–Ophiostoma* association, the *Tarsonemus* spp. appeared to have a more specific association with particular species of *Ophiostoma*. Although the data from this study are insufficient to fully understand vector patterns, spe-

cific associations between certain mite species and their phoretic *Ophiostoma* spp. may help to explain the co-existence of a large number of *Ophiostoma* spp. within a restricted niche such as *Protea* infructescences.

Of the 29 insect and 4 arachnid species examined, only three different insects (*G. hottentottus*, *O. maculates*, and Psocoptera sp. 3) carried DNA of *Ophiostoma* spp. Compared with most other infructescence-inhabiting arthropods, *G. hottentottus* and *O. maculates* are fairly large insects and may easily come into contact with sporulating perithecia of *Ophiostoma* spp. as they move within infructescences. The low success rate in attempts to isolate *Ophiostoma* spp. directly from these insects was probably because of the extensive contamination by yeasts. *O. maculates* and *G. hottentottus* are known to occur in infructescences in very low numbers (Coetzee and Giliomee 1987a, b, Roets et al. 2006b). This was also true in the infructescences investigated in this study. In contrast, up to 70% of infructescences of *Protea* are known to be dominated by *Ophiostoma* spp. (Roets et al. 2005). This suggests that *O. maculates* and *G. hottentottus* are probably not important vectors of *Ophisotoma* spp. We, therefore, believe that the presence of the *Ophiostoma* spp. on these insects was accidental and not related to a specific vector/fungus relationship. The same seems to be true for the Psocopteran specimens that were found to occasionally carry *Ophiostoma* DNA.

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

Tarsonemus cf. sp. A was found to carry conidia, probably those of the *Ophiostoma* asexual state identified as *Sporothrix* sp. 1, in flap-like structures formed by tergite 1. We suspect that these areas also serve as specialized spore-bearing structures for *Ophiostoma* spp. No specialized spore-carrying structures were observed on *P. vandenbergii* mites, which may suggest that they are only loosely associated with *Ophiostoma* spp. Interestingly, some *Tarsonemus* spp. associated with conifers in the northern hemisphere have similar structures to those found in the *Protea*-associated *Tarsonemus* sp. and have been shown to frequently contain spores of ophiostomatoid fungi, including *Ophiostoma* spp. (Bridges and Moser 1983, Moser 1985, Moser et al. 1995, Klepzig et al. 2001a, b). The *Ophiostoma–Tarsonemus* associations in these systems are thought to be mutualistic, because the mites are able to feed on the fungi they vector (Klepzig et al. 2001b). Similarly, the *Ophiostoma-Tarsonemus* associations in *Protea* may also be mutualistic. Thus, a relationship between mites and *Ophiostoma* spp. in *Protea* infructescences is not unusual, but it does provide many intriguing questions regarding the evolution and ecological role of mites in these systems.

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

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

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References Cited

Bond, W. J. 1985. Canopy-stored seed reserves (serotiny) in Cape Proteaceae. S. Afr. J. Bot. 51: 181–186.

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

Bridges, J. R., and J. C. Moser. 1983. Role of two phoretic mites in transmission of bluestain fungus *Ceratocystis minor.* Ecol. Entomol. 8: 9–12.

Coetzee, J. H., and J. H. Giliomee. 1987a. Seed predation and survival in the infructescences of *Protea repens* (Proteaceae). S. Afr. J. Bot. 53: 61–64.

Coetzee, J. H., and J. H. Giliomee. 1987b. Borers and other inhabitants of the inflorescences and infructescences of *Protea repens* in the western Cape. Phytophylactica 19: 1–6.

Crous, P. W., S. Denman, J. E. Taylor, L. Swart, and E. Palm. 2004. Cultivation and diseases of Proteaceae: *Leucadendron, Leucospermum* and *Protea.* Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Ebermann, E., and M. Hall. 2003. First record of sporothecae within the mite family Scutacaridae (Acari: Tarsonemina). Zool. Anz. 242: 367–375.

Francke-Grosmann, H. 1967. Ectosymbiosis in wood-inhabiting insects, pp. 171–180. *In* S. M. Henry (ed.), Symbiosis, vol. 2. Academic, New York.

Gibbs, J. N., and D. W. French. 1980. The transmission of oak wilt. USDA, Washington, DC.

Goldblatt, P., and J. Manning. 2000. Cape plants: a conspectus of the Cape Flora of South Africa, Strelitzia 9. National Botanical Institute of South Africa, Pretoria, South Africa.

Harrington, T. C. 1987. New combinations in *Ophiostoma* of *Ceratocystis* species with *Leptographium* anamorphs. Mycotaxon 28: 39–43.

Jacobs, K., and M. J. Wingfield. 2001. *Leptographium* species: tree pathogens, insect associates and agents of bluestain. APS Press, St Paul, MN.

Juzwik, J. 2001. Overland transmission of *Ceratocystis fagacearum:* extending our understanding, pp. 83–92. In C. L. Ash (ed.), Shade tree: wilt diseases. APS Press, St Paul, MN.

Kirisits, T. 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi, pp. 1–55. In F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire, and H. Evans (eds.), Bark and wood boring insects in living trees in Europe, a synthesis. Kluwer Academic, Dordrecht, The Netherlands.

Klepzig, K. D., and D. L. Six. 2004. Bark beetle-fungal symbiosis: context dependency in complex associations. Symbiosis 37: 189–205.

Klepzig, K. D., J. C. Moser, F. J. Lombardero, M. P. Ayres, R. W. Hofstetter, and C. J. Walkinshaw. 2001a. Mutualism and antagonism: ecological interactions among bark beetles, mites and fungi, pp. 237–267. In M. J. Jeger and N. J. Spence (eds.), Biotic interactions in plant-pathogen associations. CAB International, New York.

Klepzig, K. D., J. C. Moser, F. J. Lombardero, R. W. Hofstetter, and M. P. Ayres. 2001b. Symbiosis and competition: complex interactions among beetles fungi and mites. Symbiosis 30: 83–96.

Lee, S. B., and J. W. Taylor. 1990. Isolation of DNA from fungal mycelia and single spores, pp. 282–287. In M. A. Innis, D. H. Gelfand, J. Shinsky, and T. J. White (eds.), PCR protocols: a guide to methods and applications. Academic, New York.

Lee, S., F. Roets, and P. W. Crous. 2005. Biodiversity of saprobic microfungi associated with the infructescences of *Protea* species in South Africa. Fungal Divers. 19: 69–78.

Linder, H. P. 2003. The radiation of the Cape flora of Southern Africa. Biol. Rev. 78: 597–638.

Lindquist, E. 1969. New species of *Tarsonemus* (Acarina: Tarsonemidae) associated with bark beetles. Can. Entomol. 101: 1291–1314.

Lindquist, E. 1985. Discovery of sporothecae in adult female *Trochometridium* Cross with notes on analogous structures in *Siteroptes* Amerling (Acari: Heterostigmata). Exp. Appl. Acarol. 1: 73–85.

Malloch, D., and M. Blackwell. 1992. Dispersal of fungal diasporas, pp. 195–260. In G. C. Caroll and D. T. Wicklow (eds.), The fungal community: its organization and role in the ecosystem, 2nd ed. APS Press, St Paul, MN.

Malloch, D., and M. Blackwell. 1993. Dispersal biology of the ophiostomatoid fungi, pp. 195–206. In M. J. Wingfield, K. A. Seifert, and J. F. Webber (eds.), *Ceratocystis* and *Ophiostoma:* taxonomy, ecology and pathogenicity. APS Press, St Paul, MN.

Marais, G. J., and M. J. Wingfield. 1994. Fungi associated with infructescences of *Protea* species in South Africa including a new species of *Ophiostoma.* Mycol. Res. 98: 396–374.

Marais, G. J., and M. J. Wingfield. 1997. *Ophiostoma proteae-rum* sp. nov. associated with *Protea caffra* infructescences. Can. J. Botany 75: 362–367.

Marais, G. J., and M. J. Wingfield. 2001. *Ophiostoma africanum* sp. nov. and a key to ophiostomatoid species from *Protea* infructescences. Mycol. Res. 105: 240–246.

Moser, J. C. 1985. Use of sporothecae by phoretic *Tarsonemus* mites to transport ascospores of coniferous bluestain fungi. Trans. Br. Mycol. Soc. 84: 750–753.

Moser, J. C., and L. M. Roton. 1971. Mites associated with southern pine bark beetles in Allen Parish Lousiana. Can. Entomol. 103: 1775–1798.

Moser, J. C., T. J. Perry, J. R. Bridges, and H. F. Yin. 1995. Ascospore dispersal of *Ceratocystiopsis ranaculosus,* a mycangial fungus of the southern pine beetle. Mycologia 87: 84–86.

Roets, F., P. W. Crous, and L. L. Dreyer. 2005. Seasonal trends in colonization of *Protea* infructescences by *Gondwanamyces* and *Ophiostoma* spp. South Afr. J. Bot. 71: 307–311.

Roets, F., Z. W. de Beer, L. L. Dreyer, R. Zipfel, P. W. Crous, and M. J. Wingfield. 2006a. Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. Stud. Mycol. 55: 199–212.

Roets, F., L. L. Dreyer, H. G. Geertsema, and P. W. Crous. 2006b. Arthropod communities in *Proteaceae* infructescences: seasonal variation and the influence of infructescence phenology. Afr. Entomol. 14: 257–265.

Roets, F., M. J. Wingfield, L. L. Dreyer, P. W. Crous, and D. U. Bellstedt. 2006c. A PCR-based method to detect species of *Gondwanamyces* and *Ophiostoma* from the surfaces of insects colonizing *Protea* flowers. Can. J. Botany 84: 989–994.

Sinclair, W. A., H. Lyon, and W. T. Johnson. 1987. Diseases of trees and shrubs. Cornell University Press, Ithaca, NY.

Six, D. L. 2003. Bark beetle-fungal symbiosis, pp. 97–114. *In* K. Bourtzis and T. Miller (eds.), Insect symbiosis. CRC, Boca Raton, FL.

Suski, Z. W. 1973. A revision of *Siteroptes cerealium* (Kirchner) complex (Acarina: Heterostigmata: Pyemitidae). Ann. Zool. 30: 509–535.

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

Vilgalys, R., and M. Hester. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J. Bacteriol. 172: 4238–4246.

White, T. J., T. D. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.), PCR protocols: a guide to methods and applications. Academic, New York.

Wingfield, M. J., K. A. Seifert, and J. F. Weber. 1993. *Ceratocystis* and *Ophiostoma:* taxonomy, ecology, and pathogenicity. APS Press, St Paul, MN.

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

Zwölfer, H. 1979. Strategies and counterstrategies in insect population systems competing for space and food in flower heads and plant galls. Forts Zool. 25: 331–353.

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