A PCR-based method to detect species of *Gondwanamyces* and *Ophiostoma* on surfaces of insects colonizing *Protea* flowers

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

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

Résumé : Les cimes florales de membres économiquement importants du genre *Protea* L. mûrissent sous forme d'infructescences voyantes et de longue durée, lesquelles sont souvent colonisées, en Afrique du Sud, par des espèces fongiques ophiostomatoïdes de *Gondwanamyces* G.J. Marais & M.J. Wingf et d'*Ophiostoma* Syd. & P. Syd. On pense que ces champignons sont transportés d'une infructescence à l'autre par des insectes. Afin de développer des techniques qui pourraient permettre la détection des champignons ophiostomatoïdes sur les insectes, les auteurs ont développé les amorces GPR1 et OSP1, basées sur des séquences singulières de l'ADN ribosomal 28S de *Gondwanamyces* et d'*Ophiostoma*, provenant de *Protea*. Une polymérisation en chaîne multiplex de ces amorces, combinée avec l'amorce universelle LR6, produit des fragments longs de 885 et 637 pb. On a obtenu une amplification positive à partir d'aussi peu que 30 et 45 pg d'ADN génomique, pour les *Gondwanamyces* et *Ophiostoma*, respectivement, et on a pu amplifier des fragments de longueurs identiques à partir d'insectes artificiellement inoculés avec ces champignons. Aucune autre espèce fongique testée n'a montré d'amplification avec le GPR1 ou les OSP1 et LR6. En utilisant ces amorces, on a pu confirmer que deux espèces d'insectes (*Genuchus hottentottus* Fabricius et *Oxycarenus maculates* Stal.), récoltées sur des infructescences de *Protea repens* L., constituent des vecteurs du *Gondwanamyces proteae* (M.J. Wingfield et al.) G.J. Marais & M.J. Wingfield et de l'*Ophiostoma splendens* G.J. Marais & Wingfield, respectivement. La méthode développée dans cette étude constitue un système rapide de détection, qui peut être utilisé pour comprendre la relation entre les insectes et les champignons ophiostomatoïdes associés aux fleurs des espèces sud-africaines de *Protea*.

Mots clés : insectes vecteurs de champignons, fynbos, infructescenses, champignons.

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Introduction

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

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

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

The ophiostomatoid fungi from members of the genus *Protea* in South Africa differ in host specificity. *Gondwanamyces capensis* and *O. splendens*, for example, occur in the infructescences of various members of the genus *Protea*, while *O. africanum* and *O. protearum* are each known from only a single host plant (Wingfield et al. 1988; Wingfield and Van Wyk 1993; Marais and Wingfield 1994, 1997, 2001). The basis of this specificity is unknown and could include chemical and (or) morphological characteristics of their host plants. Although it is suspected that insects are involved in the transport of these fungi, no vectors have been identified from these infructescences. The ecological role that these fungi play within the *Protea* infructescences also remains to be determined.

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

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

Taxa	USEC collection No.*	No. of insects tested
Argyroploce Hübner (Tortricidae)	68	3
Blattidae	26	1
Braconidae	52	1
Capys alphaeus Cramer (Lycaenidae)	66	1
Chrysomelidae	17	1
Crematogaster Lund (Formicidae)	15	15
Curculionidae	48	2
Diptera	5	7
Euderes lineicollis Wiedemann (Curculionidae)	33	2
Formicidae	23	8
G. hottentottus (Scarabaeidae)	70	8 (1†)
Gyponyx Gorham (Cleridae)	55	3
Histeridae	32	5
Miridae	20	3
Nitidulidae	25	8
O. maculates (Lygaeidae)	7	18 (1‡)
Pentatomidae	24	1
Psocoptera (sp. 1)	31	22
Psocoptera (sp. 2)	12	1
Psocoptera (sp. 3)	13	10
Sphenoptera Solier (Buprestidae)	49	2
Staphylinidae	35	18
Tinea L. (Tineidae)	67	3

*Reference collection in the Department of Entomology and Nematology, University of Stellenbosch, South Africa.

†Individual showing positive amplification results for *G. proteae* DNA.

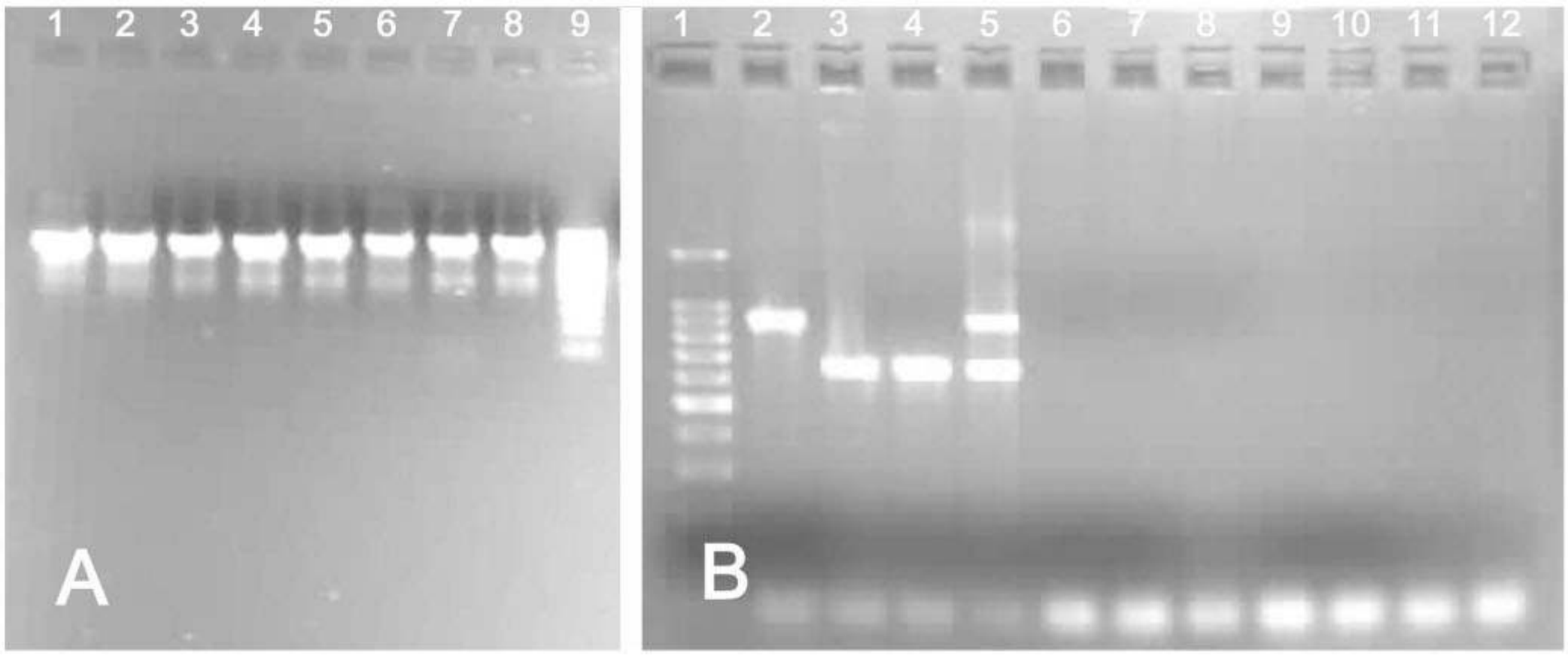
‡Individual showing positive amplification results for *O. splendens* DNA.

Materials and methods

Fungal isolates

All isolates were grown on 2% MEA plates at 24 °C in the dark for 2–4 weeks. Cultures of *G. capense* (CMW 997, CBS 120015), *G. proteae* (CMW 3936, CBS 486.88), *O. africanum* (CMW823, CBS 116571), *O. protearum* (CMW1102, CBS 116568), and *O. splendens* (CMW872, CBS 116379) were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

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



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

Design of taxon-specific primers

Partial 28S rDNA sequences were obtained from GenBank for five species of ophiostomatoid fungi isolated from *Protea* (*G. capense* accession No. AF221012, *G. proteae* accession No. AF221011, *O. africanum* accession No. AF221015, *O. protearum* accession No. AF221014, *O. splendens* accession No. AF221013), two species of *Ceratocystis* (*Ceratocystes adiposa* (E.J. Butler) C. Moreau accession No. AF222481, *C. fimbriata* Ellis & Halst. accession No. AF222484), *Leptographium lundbergii* Lagerb. & Melin accession No. AF155664, and *Ophiostoma piliferum* (Fr.) Syd. & P. Syd. accession No. U47837. Sequences of the following 11 species of fungi, representing genera commonly isolated from *Protea* infructescence inhabiting insects (Roets 2000) were also included: *Botrytis tulipae* (Lib.) Lind accession No. AJ226078, *Cladosporium* Link accession No. U26886, *Fusarium acumilnatum* Ellis & Everh. accession No. U34544, *Melanospora zamiae* Corda accession No. U17405, *Mucor hiemalis* f. *hiemalis* Wehmer AF113468, *Penicillium* Link sp. 1 accession No. U26865, *Penicillium* sp. 2 accession No. U26851, *Penicillium chrysogenum* Thom accession No. AF034857, *Penicillium namyslowskii* K. M. Zalessky accession No. AB000487, *Penicillium turbatim* Westling accession No. AF034454, and *Sordaria fimicola* (Roberge ex Desm.) Ces. & De Not. accession No. AF132330. DNA sequences were aligned using DAPSA (Harley 1988).

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

Primer-specificity tests

The specificity of GPR1 and OSP1 were tested using the fungal cultures listed above and amplification conditions were optimized for the primer pair combinations GPR1–LR6 and OSP1–LR6 before they were combined in a multiplex reaction. DNA was isolated from fungal mycelia following the protocol of Lee and Taylor (1990). PCR mixtures (25 μL) in each tube contained 5 μL of the extracted fungal genomic DNA, 8 mmol·L^{-1} $MgCl_2$ (Bioline, London), 1× NH_4 reaction buffer (Bioline, London), 0.25 mmol·L^{-1} of each of the four dNTPs, 0.4 pmol·μL^{-1} of each of the primers and 0.626 units of Biotaq (Bioline, London).

The best amplification results were obtained using a touchdown PCR program with an initial denaturation at 94 °C for 2 min, 13 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 1 min, followed by 23 cycles for which the annealing temperature was lowered to 56 °C and the final extension was increased to 5 min. Amplification was achieved using GPR1, LR6, and OSP1 in a multiplex reaction employing a minimum of 30 pg genomic DNA for species of *Gondwanamyces* and 45 pg of genomic DNA for species of *Ophiostoma*. The specificity of the developed primers was retested using the concentrations of the constituents of the PCR mixtures and thermal cycling conditions outlined above, but with 0.2 pmol·μL^{-1} rather than 0.4 pmol·μL^{-1} of GPR1 and OSP1.

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

Detection of *Gondwanamycs* and *Ophiostoma* on inoculated insects

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

Insects were placed in Eppendorf tubes containing 1 mL SDS extraction buffer (pH 8) and vortexed for 1 min to loosen the fungal spores. The insects were then removed and the suspension was centrifuged for 5 min (13 800g). DNA was extracted following Lee and Taylor (1990) and resuspended in 15 μL double-distilled H_2O.

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

Screening of insects collected from *P. repens* infructescences

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

Results

Primer specificity tests

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

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

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

Screening of insects collected from *P. repens* infructescences

Testing of insects collected from *P. repens* (Table 1) using the newly developed multiplex PCR method revealed the presence of *G. proteae* on *Genuchus hottentottus* Fabricius (Cetoniidae, Coleoptera) and *O. splendens* on *Oxycarenus maculates* Stal. (Lygaeidae, Heteroptera). Sequencing confirmed that these fragments were identical to corresponding GenBank sequences for *G. proteae* (Accession No. AF221011) and *O. splendens* (Accession No. AF221013).

Discussion

In this study we developed taxon-specific primer sets that permit the rapid and accurate detection of DNA of *Gondwanamyces* and *Ophiostoma* from the surfaces of insects asso-

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

Schweigkofler et al. (2005) developed a PCR test and were able to detect target ophiostomatoid fungi on 37% of the bark beetles collected. Although a previous study (Roets et al. 2005) indicated that up to 60% of *P. repens* infructescences can be colonized by ophiostomatoid fungi, only 2 of the 145 individuals screened yielded amplification products using the primers designed in this study. Tests of the PCR protocol revealed that amplification was possible for the load of spores transferred to insects from contact with a single sporulating ascoma. As ophiostomatoid fungi form the dominant fungal component within *Protea* infructescences (Roets et al. 2005; Marais 1996) and insects departing from infructescences colonized by these fungi likely come into contact with numerous sporulating ascomata, it can be assumed that detectable amounts of DNA should be present on the putative vectors.

The low retrieval of ophiostomatoid fungi from insects may be associated with the synchronization of fungal sporulation and insect emergence. In this study, the insects were physically extracted from the infructescences, possibly prior to their having come into contact with the fungi. The life-cycles of these organisms may be synchronized in such a way that the ascospores are deposited onto the insects just prior to their departure from the infructescences. This has been reported in some bark beetle-associated fungi (Bridges 1983). The fungi may also be transported endozootically by the vector insects. We will employ macerated insects emerging naturally from *Protea* infructescences in future investigations of this system.

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

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