Molecular Phylogenetics and Evolution 51 (2009) 111–119

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Fungal radiation in the Cape Floristic Region: An analysis based on *Gondwanamyces* and *Ophiostoma*

F. Roets [a,b,*], M.J. Wingfield [b], P.W. Crous [c], L.L. Dreyer [a]

[a] Department of Botany and Zoology, Stellenbosch University, Stellenbosch 7600, South Africa
[b] Forestry and Agricultural Biotechnology Institute (FABI), Department of Microbiology, University of Pretoria, Pretoria 0002, South Africa
[c] Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht 3584, The Netherlands

ARTICLE INFO

Article history:
Received 4 January 2008
Revised 21 May 2008
Accepted 30 May 2008
Available online 7 June 2008

Keywords:
Ophiostoma
Gondwanamyces
Molecular phylogeny
Speciation
ITS
β-Tubulin
Cape Floristic Region
Protea

ABSTRACT

The Cape Floristic Region (CFR) displays high levels of plant diversity and endemism, and has received focused botanical systematic attention. In contrast, fungal diversity patterns and co-evolutionary processes in this region have barely been investigated. Here we reconstruct molecular phylogenies using the ITS and β-tubulin gene regions of the ophiostomatoid fungi *Gondwanamyces* and *Ophiostoma* associated with southern African *Protea* species. Results indicate that they evolved in close association with *Protea*. In contrast to *Protea*, *Ophiostoma* species migrated to the CFR from tropical and subtropical Africa, where they underwent subsequent radiation. In both *Gondwanamyces* and *Ophiostoma* vector arthropods probably facilitated long-distance migration and shorter-distance dispersal. Although ecological parameters shaped most associations between ophiostomatoid fungi and *Protea*, there is congruence between fungal–host-associations and the systematic classification of *Protea*. These results confirm that the entire biotic environment must be considered in order to understand diversity and evolution in the CFR as a whole.

1. Introduction

The southern tip of Africa displays a flora of such diversity that this region has been identified as one of the few global biodiversity hotspots (Myers et al., 2000). It includes both the Cape Floristic Region (CFR) and the Succulent Karoo Region (Van Wyk and Smith, 2001), each characterized by a specialized flora rich in endemics. Born et al. (2006) proposed an expansion of the CFR to include the entire winter-rainfall region as the Greater CFR, which resulted in an increased level of total endemism.

In comparison to plants, fungi have been grossly under-studied worldwide. Only about 7% of an estimated 1.5 million fungal species, are thought to be formally described (Hawksworth, 2004). Conservatively estimated, there are ca. 200,000 fungal species associated with South African plants (Crous et al., 2006). Although studies are certainly incomplete, the CFR fungi have been more intensively studied than those in any other floristic region in South Africa (Crous et al., 2006). The high levels of plant endemism in the Greater CFR predict equally high numbers of unique fungal species in this region. Studies on the fungi associated with various CFR plants (Crous et al., 2004; Lee et al., 2004, 2005) found between three to ten unique species per plant species. As the total number of endemic CFR plant species is estimated at around 6000 (Goldblatt and Manning, 2000), up to 42,000 unique fungal species may thus be expected in the CFR alone. It is thus surprising that only 780 endemic fungal species have been described from all of South Africa to date (Crous et al., 2006). Clearly, more studies should focus on documenting fungal diversity in South Africa and particularly in the Greater CFR.

Plant taxa of the Greater CFR have received substantial phylogenetic attention (e.g. Klak et al., 2003; Linder, 2003, 2005; Verboom et al., 2004; Galley and Linder, 2006; Mummenhoff et al., 2005). Patterns and some of the processes underlying the plant radiations in the region are now beginning to emerge (Johnson, 1996; Verboom et al., 2004; Bakker et al., 2005; Van der Niet et al., 2006). Numerous ecological parameters have been shown to influence plant speciation in the Greater CFR, including abiotic factors such as edaphic diversity, landscape topography, alternation between dry summers and wet winters, fire regimes and the relatively stable climatic history (Linder, 2003 and references therein). Studies of biological interactions as potential drivers of radiation have mostly been limited to assessments of the role of pollinators in driving plant speciation in the CFR (Johnson, 1996; Johnson et al., 1998; Manning and Goldblatt, 2005). While substantial new knowledge is emerging regarding the evolution of plant taxa in

* Corresponding author. Address: Department of Botany and Zoology, Natural Science Building, Stellenbosch University, Marriman Street, Stellenbosch 7600, South Africa. Fax: +27 021 808 2408.
E-mail address: fr@sun.ac.za (F. Roets).

1055-7903/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2008.05.041

the CFR, virtually no attention has been paid to the radiation of fungi (Mitchell and Gibson, 2006). Given their close association with plants, fungi would clearly provide an interesting group for study and comparison.

Of all the fungi studied in the CFR, those associated with Proteaceae are the best known (Crous et al., 2004, 2006). This is largely because these plants are commercially cultivated, resulting in a need to survey the associated pathogenic fungal species (Crous et al., 2004; Knox-Davies, 1975, 1981; Knox-Davies et al., 1986, 1987; von Broembsen, 1989; van Wyk et al., 1975; Swart, 1999). A detailed recent inventory of some saprophytes on *Protea* (Lee et al., unpublished) is purely taxonomic and does not provide a phylogenetic foundation for understanding patterns of evolution and radiation.

Proteaceae is the seventh largest vascular plant family in the CFR (Rourke, 1998; Goldblatt and Manning, 2000; Linder, 2003). Within the CFR, Proteaceae often form the dominant elements in the landscape, both in terms of physical plant size and numbers. Various attempts have been made to resolve the taxonomy and systematics of southern African Proteaceae (Rebelo, 1995; Rourke, 1998; Barker et al., 2002). Rebelo (1995) provided a taxonomic classification of all the southern African taxa. More recently Barraclough and Reeves (2005) reconstructed a DNA sequence-based phylogeny of *Protea* and concluded that current diversity in this genus resulted from the coexistence of species that diversified over a long period of time. This implies that any organisms that are dependent on *Protea* may have had a very long co-evolutionary history with this genus.

Recently an unusual group of non-pathogenic fungi, the ophiostomatoid fungi (Wingfield et al., 1993), was discovered within *Protea* infructescences (Marais and Wingfield, 1994, 2001). They are opportunists/saprophytes confined to the infructescences of various *Protea* species and appear to have developed a symbiotic relationship with insects and mites that associate with these plants (Roets et al., 2007). This group contains two distantly related genera, *Gondwanamyces* and *Ophiostoma*, both of which are adapted to dispersal via arthropods. Species of *Gondwanamyces* are confined to *Protea* species within the CFR, while species of *Ophiostoma* have also been described from *Protea* in other parts of South Africa. *Protea*-associated *Ophiostoma* do not form a monophyletic unit, suggesting multiple invasions of this unique niche (Roets et al., 2006a, in press-a). Speciation has subsequently occurred in some of these lineages, and probably entailed host-switching, vector-switching and geographic isolation.

The *Protea*-associated ophiostomatoid fungi are widespread on *Protea* species but are clearly *Protea*-specific. These interesting patterns of association provide an outstanding example on which to base a first attempt at studying fungal evolutionary patterns in the CFR. *Protea* has been phylogenetically well-studied, thus allowing for inter-organismal comparison. For these reasons, the *Protea*-associated ophiostomatoid fungi provide a unique opportunity to consider co-evolution within the CFR. In this study we reconstruct phylogenies for the ophiostomatoid fungi *Gondwanamyces* and *Ophiostoma* associated with southern African *Protea* species. We compare the resultant patterns of evolution to those known for *Protea*, and interpret the observed evolutionary and association patterns in terms of the general biology and ecology of these two fungal lineages.

2. Materials and methods

2.1. Isolates

Isolates of *Gondwanamyces* and *Ophiostoma* species were obtained from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Additional isolates of these genera were made from the infructescences of *Protea* species collected from numerous sites in South Africa and Zambia between February 2005 and August 2007. At least 20 infructescences of each *Protea* sp. encountered at each site were collected and screened for the presence of ophiostomatoid fungi. These collections resulted in the assessment of nearly all South African *Protea* species and, for the majority of species, from across their entire distribution ranges. Ascospores were germinated on 2% malt extract agar (MEA; Biolab, Midrand, South Africa) and pure cultures were maintained on petri dishes containing MEA at 4 °C. Representative cultures of all new isolates (Table 1) have been deposited in CMW.

2.2. DNA isolation, amplification and sequencing

Genomic DNA from fungal mycelium of Gondwanamyces and Ophiostoma isolates was extracted using a Sigma GenElute™ plant genomic DNA miniprep kit (Sigma-Aldrich Chemie CMBH, Steinheim, Germany) following the manufacturer's instructions. The following primers were used for amplification: ITS1–F (White et al., 1990) and ITS4 (White et al., 1990) for the internal transcribed spacers and 5.8S rDNA regions (ITS); T10 (O' Donnell and Cigelnik, 1977), T1 (O' Donnell and Cigelnik, 1977) and Bt2b (Glass and Donaldson, 1995) for the β-tubulin gene region. Fifty microlitre PCR reaction volumes for the rDNA amplifications consisted of: 32.5 μL ddH_2O, 1 μL DNA, 5 μL (10×) reaction buffer (Super-Therm, JMR Holdings, USA), 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, USA). DNA fragments were amplified using a Gene Amp®, PCR System 2700 thermal cycler (Applied Biosystems, Foster City, USA). PCR reaction conditions were: an initial denaturation step of 2 min at 95 °C, followed by 35 cycles of: 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 1 min elongation at 72 °C. The PCR process terminated with a final elongation step of 8 min at 72 °C.

Reaction mixtures to amplify part of the β-tubulin gene region were identical to ribosomal DNA, except that 1.5 μL DNA, 32 μL of ddH_2O and primers T10 or T1 (depending on isolate) were used in conjunction with Bt2b (Glass and Donaldson, 1995). The amplification protocol for β-tubulin was as follows: initial denaturation for 4 min at 95 °C, 35 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1.5 min, elongation at 72 °C for 1 min, and a termination step of 7 min at 72 °C.

All amplified PCR products were cleaned using the Wizard® SV gel and PCR clean up system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The purified fragments were sequenced using the PCR primers and the Big Dye™ Terminator v. 3.0 cycle sequencing premix kit (Applied Biosystems, Foster City, CA, USA). The fragments were analyzed on an ABI PRISIM™ 3100 Genetic Analyzer (Applied Biosystems).

2.3. Phylogenetic analyses

Sequence data, additional to those generated specifically for this study, were obtained from previous studies (Roets et al., 2007, in press-a) from the NCBI's GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and compared to sequences of closely related *Ophiostoma* species (De Beer et al., 2003; Aghayeva et al., 2004, 2005). Sequences were aligned using Sequence Alignment Editor v2.0a11 (Rambaut, 2002). Compatibility of the ITS and the β-tubulin data sets was scrutinised with a SH test (Shimodaira and Hasegawa, 1999), before combining them into a single data set.

For maximum parsimony analysis, one thousand random stepwise addition heuristic searches were performed using the software package PAUP* (Phylogenetic Analysis Using Parsimony)

Table 1
GenBank Accession Numbers for isolates used in phylogenetic analysis

Species	Isolate number	Host	Country	GenBank Accession	
				ITS	β-tubulin
Ceratocystis fimbriata	CMW7765	*Eucalyptus* sp.	South Africa	DQ520635	
Custingophora olivacea	CBS335.68	Compost	Germany	AM267269	
Gondwanamyces capensis	**CMW1040**	***Protea neriifolia***	**South Africa**	EU660447	
G. capensis	**CMW927**	***Protea lepidocarpodendron***	**South Africa**	EU660437	
G. capensis	**CMW922**	***Protea lepidocarpodendron***	**South Africa**	EU660438	
G. capensis	**CMW19962**	***Protea laurifolia***	**South Africa**	EU660445	
G. capensis	**CMW1038**	***Protea magnifica***	**South Africa**	EU660446	
G. capensis	**CMW997**	***Protea longifolia***	**South Africa**	EU660439	
G. capensis	**CMW1147**	***Protea lepidocarpodendron***	**South Africa**	EU660440	
G. capensis	**CMW978**	***Protea neriifolia***	**South Africa**	EU660443	
G. capensis	**CMW1150**	***Protea magnifica***	**South Africa**	EU660444	
G. capensis	**CMW974**	***Protea coronata***	**South Africa**	EU660442	
G. capensis	**CMW1145**	***Protea coronata***	**South Africa**	EU660441	
G. proteae	CBS 486.88	*P. repens*	South Africa	AY372072	
G. proteae	**CMW3757**	***P. repens***	**South Africa**	EU660435	
G. proteae	**CMW1042**	***P. repens***	**South Africa**	EU660436	
G. proteae	**CMW1043**	***P. repens***	**South Africa**	EU660434	
Gondwanamyces sp. nov 1	CCF 3569	Scolytinae beetle from *Cecropia* sp.	Costa Rica	AM267268	
Gondwanamyces sp. nov 2	CCF 3568	Scolytinae beetle from *Cecropia* sp.	Costa Rica	AM267266	
Gondwanamyces sp. nov 2	CCF 3565	Scolytinae beetle from *Cecropia* sp.	Costa Rica	AM267267	
Ophiostoma abietinum	CMW 1468	*Dendroctonus ponderosa*	Canada	AF484457	AY280468
O. abietinum	CMW 110	*Pinus echinata*	USA	AF280488	AY280470
O. africanum	CMW 1104	*Protea caffra*	South Africa	DQ316200	DQ316162
O. africanum	CMW 1822	*Protea dracomontana*	South Africa	DQ316179	DQ316159
O. aurorae	CMW 19362	*Pinus eliottii*	South Africa	DQ396796	DQ393800
O. aurorae	CMW 19363	*Pinus eliottii*	South Africa	DQ396797	DQ393801
O. dentifundum	CMW 13017	*Quercus* sp.	Poland	AY495435	AY495446
O. dentifundum	CMW 13016	*Quercus* sp.	Hungary	AY495434	AY495445
O. fusiforme	CMW 9968	*Populus nigra*	Azerbaijan	AY280481	AY280461
O. fusiforme	CMW 10565	*Larix decidua*	Austria	AY280484	AY280465
O. gemellus	CMW 23056	*Protea caffra*	South Africa	DQ821557	DQ821551
O. gemellus	CMW 23055	*Protea caffra*	South Africa	DQ821558	DQ821552
O. lunatum	CMW 10564	*Larix decidua*	Austria	AY280486	AY280467
O. lunatum	CMW 10563	*Carpinus betulus*	Austria	AY280458	AY280465
O. nigrocarpum	CMW 651	*Pseudotsuga menziesii*	USA	AY280490	AY280480
O. nigrocarpum	CMW 560	*Abies* sp.	USA	AY280489	AY280479
O. phasma	CMW 20676	*Protea laurifolia*	South Africa	DQ316219	DQ316181
O. phasma	CMW 20698	*Protea laurifolia*	South Africa	DQ316222	DQ316184
O. palmiculminatum	CMW 20677	*Protea repens*	South Africa	DQ316191	DQ821543
O. palmiculminatum	CMW 23049	mite from *P. repens*	South Africa	DQ821563	DQ821550
O. protearum	CMW 1107	*Protea caffra*	South Africa	DQ316201	DQ316163
O. protearum	CMW 1103	*Protea caffra*	South Africa	DQ316203	DQ316165
O. splendens	CMW 872	*Protea repens*	South Africa	DQ316215	DQ296071
O. splendens	CMW 20675	*Protea repens*	South Africa	DQ316205	DQ316167
***Ophiostoma* sp. nov. 1**	**CMW 28600**	***Protea caffra***	**Zambia**	EU660448	EU660463
***Ophiostoma* sp. nov. 1**	**CMW 28601**	***Protea caffra***	**Zambia**	EU660449	EU660464
***Ophiostoma* sp. nov. 2**	**CMW 28602**	***Protea caffra***	**South Africa**	EU660458	EU660468
***Ophiostoma* sp. nov. 2**	**CMW 28603**	***Protea caffra***	**South Africa**	EU660459	EU660469
***Ophiostoma* sp. nov. 3**	**CMW 28604**	***Protea caffra***	**Zambia**	EU660453	EU660473
***Ophiostoma* sp. nov. 3**	**CMW 28605**	***Protea caffra***	**Zambia**	EU660454	EU660474
O. stenoceras	CMW 11192	Sapwood	New Zealand	AY280492	AY280474
O. stenoceras	CMW 2344	*Eucalyptus smithii*	South Africa	AY280491	AY280472
Sporothrix inflata	CBS 239.68	Soil	Germany	AY495426	AY495445
S. inflata	CBS 841.73	Wood	Chile	AY495431	AY495442
S. schenckii	CMW 7612	Human	South Africa	AY280494	AY280476
S. schenckii	CMW 7614	Human	South Africa	AY280495	AY280477
S. variecibatus	CMW 23051	Mite from *Protea repens*	South Africa	DQ821568	DQ821539
S. variecibatus	CMW 23060	*Protea longifolia*	South Africa	DQ821569	DQ821573

Isolates from which sequences were generated in this study are indicated in bold type face.

v4.0b10 (Swofford, 2003) with alignment gaps treated as a fifth character state and all characters were unordered and of equal weight. Tree Bisection-Reconnection (TBR) was chosen as the branch-swapping algorithm, branches of zero length were collapsed and 10 trees were saved per replicate. Internal node support was assessed using the bootstrap procedure (Felsenstein, 1985), with one thousand replicates of simple taxon addition. Other measures calculated included tree length, consistency index and retention index (TL, CI and RI). The resulting trees were printed with TreeView v1.6.6 (Page, 1996).

Data were also analyzed using Bayesian inference based on a Markov Chain Monte Carlo (MCMC) approach in the software package MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003). Evolutionary models were determined using the MrModeltest 2.2 software package (Nylander, 2004) based on AIC criteria. Models chosen included: GTR + I + G (shape parameter using four rate categories) for the analysis of the *Ophiostoma* data set and GTR + G (shape parameter using four rate categories) for the *Gondwanamyces* data set. Two independent Markov chains were initiated from a random starting tree. Runs of 1,000,000 generations with a sample

frequency of 50 were implemented. Burnin trees (first 20,000 generations) were discarded and the remaining trees from both runs were pooled into a 50% majority rule consensus tree.

2.4. Ancestral character state reconstructions

Ancestral character state reconstructions were based on the tree with the highest posterior probability score generated in Bayesian analysis. These reconstructions were analysed using the Mesquite system for phylogenetic computing (Maddison and Maddison, 2006) using categorical character coding. *Ophiostoma* host-association data were coded as three discreet states: species associated with *Protea* in the CFR, species associated with *Protea* in tropical/subtropical Africa and species not associated with *Protea* (largely from conifers in the Northern Hemisphere). Ancestral character state reconstructions were performed using both parsimony and likelihood reconstruction methods. Likelihood reconstructions were based on the Mk1 model (Markov k-state one parameter) that corresponds to Lewis's (2001) Mk model.

3. Results

3.1. Isolates

Compared to the study by Roets et al. (in press-a), the present study provides supplementary ITS and β-tubulin sequence data for six additional *Ophiostoma* isolates (Table 1). These additional isolates represent three newly collected and currently undescribed *Ophiostoma* species (two isolates per species), one from the Gauteng Province of South Africa (*Ophiostoma* sp. nov. 2) and two from Zambia (*Ophiostoma* sp. nov. 1 and *Ophiostoma* sp. nov. 3). In addition, ITS sequences of 14 *Gondwanamyces* isolates from collections throughout the CFR were generated in this study (Table 1).

3.2. Gondwanamyces

The ITS data set for *Gondwanamyces* contained 19 taxa (including outgroup) and 677 characters. Of these, 111 were parsimony-informative, 202 were variable and parsimony-uninformative, and 364 were constant. The parsimony analysis yielded 100 most parsimonious trees, one of which is shown in Fig. 1.

Strong support for the monophyly of CFR isolates of *Gondwanamyces* (parsimony BS = 100, Bayesian PP = 1) were obtained. This Cape clade resolved as sister to Central American *Gondwanamyces* species, again with strong support (BS = 100, PP = 1). The isolates from *Protea* resolved in two distinct, well-supported clades. The first contains all the isolates from *P. repens* and represents isolates of *G. proteae*. The second CFR clade contains isolates from various other *Protea* species and represents *G. capensis*. The genus *Gondwanamyces* resolves with strong support as sister to *Custingophora olivacea*, a fungus known only from a single collection from compost in Germany (Stolk and Hennebert, 1968).

Within the CFR, *Gondwanamyces* species appear to be highly specific to *Protea* (Marais, 1996; Marais and Wingfield, 1997, 2001) and are commonly found in their infructescences. Despite extensive sampling, these fungi have not been found in any other niche (Roets, 2002, 2006). *Gondwanamyces proteae* appears to be specific to *P. repens* across all localities over the entire distribution range of this plant, even when it grows sympatrically with other *Protea* species that harbor *G. capensis*. In contrast, *G. capensis*, is not specific to a single host, and was isolated from various *Protea* species from across the CFR, including *P. burchellii* (Stellenbosch Mountain), *P. coronata* (Nuweberg and Jonkershoek),

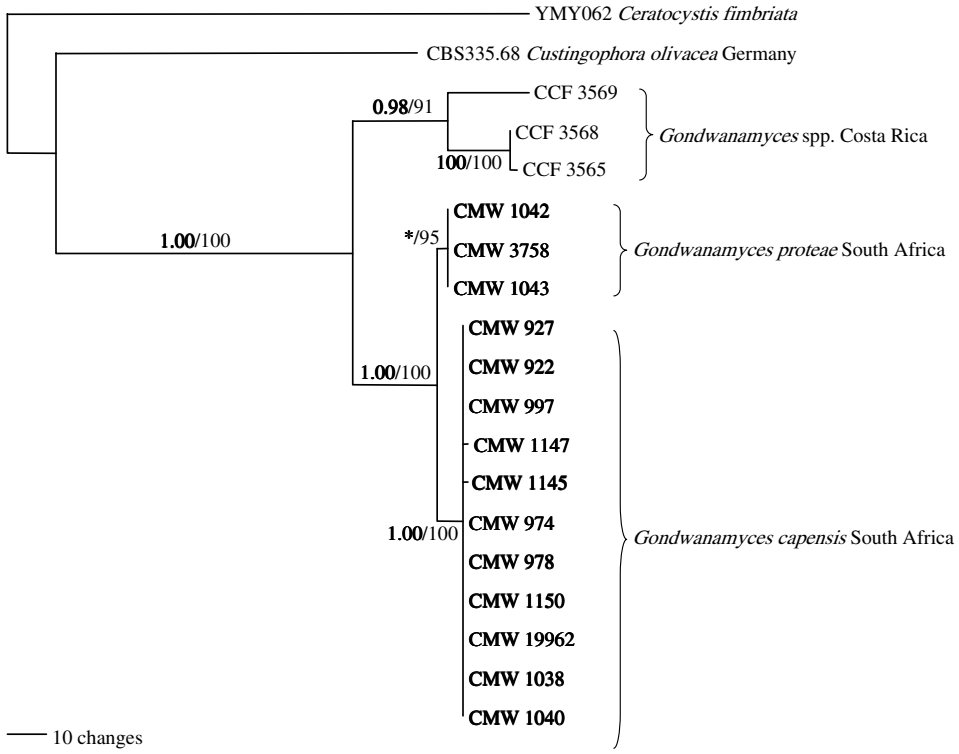

Fig. 1. One of 100 most parsimonious trees (TL = 420 steps, CI = 0.9281, RI = 0.8908) obtained from a parsimony analysis using ITS sequence data of *Gondwanamyces* and its closest relatives. The bar indicates 10 changes. The numbers at the nodes indicated in bold type face represent posterior probabilities obtained through Bayesian inference (values below 0.95 replaced by asterisk). Numbers in normal type face indicate parsimony bootstrap values based on 1000 resampling replicates. The topology of the chosen tree corresponds to the strict consensus tree. The accession numbers of isolates from *Protea* are printed in bold type face. *Ceratocystis fimbriata* was included as the outgroup taxon.

P. laurifolia (Stellenbosch Mountain), *P. lepidocarpodendron* (Stellenbosch and Cape of Good Hope Nature Reserve), *P. longifolia* (Hermanus), *P. lorifolia* (Riviersonderend Range), *P. magnifica* (Groenland Range), *P. magnifica* (Hermanus), *P. neriifolia* (Jonkershoek) and *P. neriifolia* (Montaque Pass). *Gondwanamyces capensis* was also never isolated from *P. repens*, despite its various *Protea*-associates often growing sympatrically with *P. repens*.

All *Gondwanamyces* isolates were obtained from infructescences of serotinous *Protea* species, and the genus seems not to be associated with non-serotinous *Protea* species (Roets, 2006) or with *Protea* leaf litter (Lee et al., 2004). The non-serotinous *Protea* species evaluated in this study included: *P. angolensis*, *P. laetans*, *P. parvula*, *P.* roupelliae and *P. simplex*. *Gondwanamyces* species were also not found in the infructescences of many serotinous *Protea* species screened for their presence, including *P. acaulos, P. amplexicaulis, P. angustata*, *P. aspera, P. aurea*, *P. canaliculata*, *P. compacta, P. cordata, P. cynaroides*, *P. eximia, P. grandiceps*, *P. humiflora*, *P. nana, P. nitida*, *P. scabra*, *P. scolymocepela*, *P. speciosa*, *P. stokoei*, *P. subvestita*, *P. susannae*, *P. roupelliae* and *P. witzenbergiana*.

3.3. Ophiostoma

The manually adjusted alignment of sequences for species of *Ophiostoma* (combined ITS and β-tubulin data set) contained 38 taxa (including outgroup) and 1674 characters; of these 552 were parsimony-informative, 11 were variable and parsimony-uninformative and 1111 were constant. The ITS and β-tubulin data sets were combined in spite of the outcome of the SH test ($P < 0.05$), as the observed differences between these were most likely the result of ambiguous alignment due to the variability of the β-tubulin intron areas. Species of interest were placed congruently in all analyses. Parsimony analysis yielded one most parsimonious tree (Fig. 2).

Similar to results of Roets et al. (2006a, in press-a), *Ophiostoma* species associated with *Protea* resolved into four separate clades in this study. Two of these included only a single *Protea*-associated species (*O. phasma* and *S. variecibatus*). The other two clades both included more than one *Ophiostoma* species associated with *Protea*. The first of these (*Protea*-host Clade 1, Fig. 2) was well-supported (BS = 100, PP = 1) and includes *Ophiostoma* sp. nov. 1 from Zambia as sister to *O. gemellus* from Gauteng (SA) and *O. palmiculminatum* from the CFR. The second clade (*Protea*-host Clade 2, Fig. 2) included *O. protearum* and *O. africanum* (both from Gauteng, SA) as sister to *O. splendens* from the CFR. These three species are sister to an undescribed *Ophiostoma* sp. (*Ophiostoma* sp. nov. 3) from Zambia. An undescribed *Ophiostoma* sp. (*Ophiostoma* sp. nov. 2) from Gauteng (SA) resolved as sister species to all of the above. All branches were well-supported.

Ophiostoma phasma was isolated from a wide range of associated *Protea* species, comprised mostly of the "bearded sugarbushes" (Rebelo, 1995), including *P. laurifolia, P. lepidocarpodendron, P. longifolia* and *P. neriifolia*. *Protea longifolia* is the only recorded host that forms part of Rebelo's (1995) "spoon bract" group. *Sporothrix variecibatus* has been collected from the Western Cape Province only, and is known from *P. longifolia*, *P. repens* and a *Eucalyptus* species (Roets et al., in press-a).

In *Protea*-host Clade 1, the undescribed species collected from Zambia (*Ophiostoma* sp. nov. 1) was found in *P. caffra*, which is also a host to *O. gemellus*. *Ophiostoma palmiculminatum* was restricted to *P. repens* infructescences. In *Protea*-host Clade 2 *P. caffra* was the host of *Ophiostoma* sp. nov. 2, *Ophiostoma* sp. nov. 3 and *O. protearum*. *Ophiostoma africanum* was isolated from a fairly wide range of *Protea* species, including *P. caffra*, *P. dracomontana* and *P. gaguedi*. *Ophiostoma splendens* was also associated with many *Protea* species including: *P. burchellii, P. coronata, P. laurifolia*, *P. lepidocarpodendron, P. longifolia*, *P. lorifolia, P. neriifolia* and *P. repens*. This list corresponds closely with the host range of *Gondwanamyces capensis*, and may indicate similar ecological barriers affecting both fungi.

Numerous other *Protea* species from South Africa and Zambia were inspected for the presence of *Ophiostoma* species, and were found to be free of these fungi. As with *Gondwanamyces*, all *Protea*-associated *Ophiostoma* species remain known only from serotinous *Protea* species. In addition to the above-mentioned *Gondwanamyces*-free *Protea* species, the following serotinous species were screened and found to be free of *Ophiostoma*: *P. comptonii, P. curvata*, *P. rubropilosa* and *P. subvestita.*

3.4. Ancestral character state reconstructions

Ancestral character state reconstructions are reported for the basal nodes of *Protea*-host clade 1 and 2 only (Fig. 2), as these nodes represent the only known radiations of *Ophiostoma* species on *Protea* hosts. Parsimony based ancestral character state reconstructions resolved both ancestral nodes as being *Ophiostoma* species associated with *Protea* from tropical and subtropical Africa. Likelihood methods also provided good support for this hypothesis, with support at the basal nodes of *Protea*-host clade 1 and *Protea*-host clade 2 being 0.864 and 0.974 (proportion of total likelihood), respectively. These values were greater than two log likelihood units higher than other reconstructions.

4. Discussion

In this study we attempted to understand some of the patterns of evolution and radiation of fungi present in the CFR. For this purpose we have chosen a group of fungi that includes two distinct phylogenetic lineages for which DNA sequence data are available. We know that the ophiostomatoid fungi treated in this study are native to the area, so that patterns of host association can be used to reflect patterns of radiation in species of *Protea*.

4.1. Origin of Protea-associated Ophiostoma in the CFR

Phylogenetic reconstruction of *Protea*-associated *Ophiostoma* suggests that species from *Protea* species north of the CFR are ancestral. This would mean that the *Ophiostoma* species in the CFR are derived from those associated with northern *Protea* species This result is in direct contrast to the phylogeny of the genus *Protea* (Barraclough and Reeves, 2005), where it is suggested that CFR taxa are ancestral and that the northern *Protea* species are derived from Cape species. If this south to north phylogeographic history for *Protea* (Barraclough and Reeves, 2005) is correct (and in order to explain the diversity of the *Protea*-associated *Ophiostoma* species in the CFR), an *Ophiostoma* species would have had to undergo a host jump from a northern *Protea* species to a CFR *Protea* sp. and subsequently migrated back down to the CFR. Although we know of no northern and CFR *Protea* species that are host to the same *Ophiostoma* species and that exist sympatrically, there are *Protea* species such as *P. repens* in the CFR and *P. caffra* in the northern parts of South Africa that have very wide distributions. Thus, these northern and southern hosts may have grown in close enough proximity to one another during past cooler climatic conditions that a host jump would have been possible.

The ecology of *Protea*-associated *Ophiostoma* species could also have supported a host jump between northern and CFR *Protea* species. Recent research has shown that these *Ophiostoma* species are vectored by mites (Roets et al., 2007) phoretic on beetles (Roets, 2006; Roets et al., in press-b). These secondary vector beetles could, at least occasionally, have crossed geographic barriers between host species, as they are able to fly over extended distances. Indirect evidence for this is found in the presence of the beetle

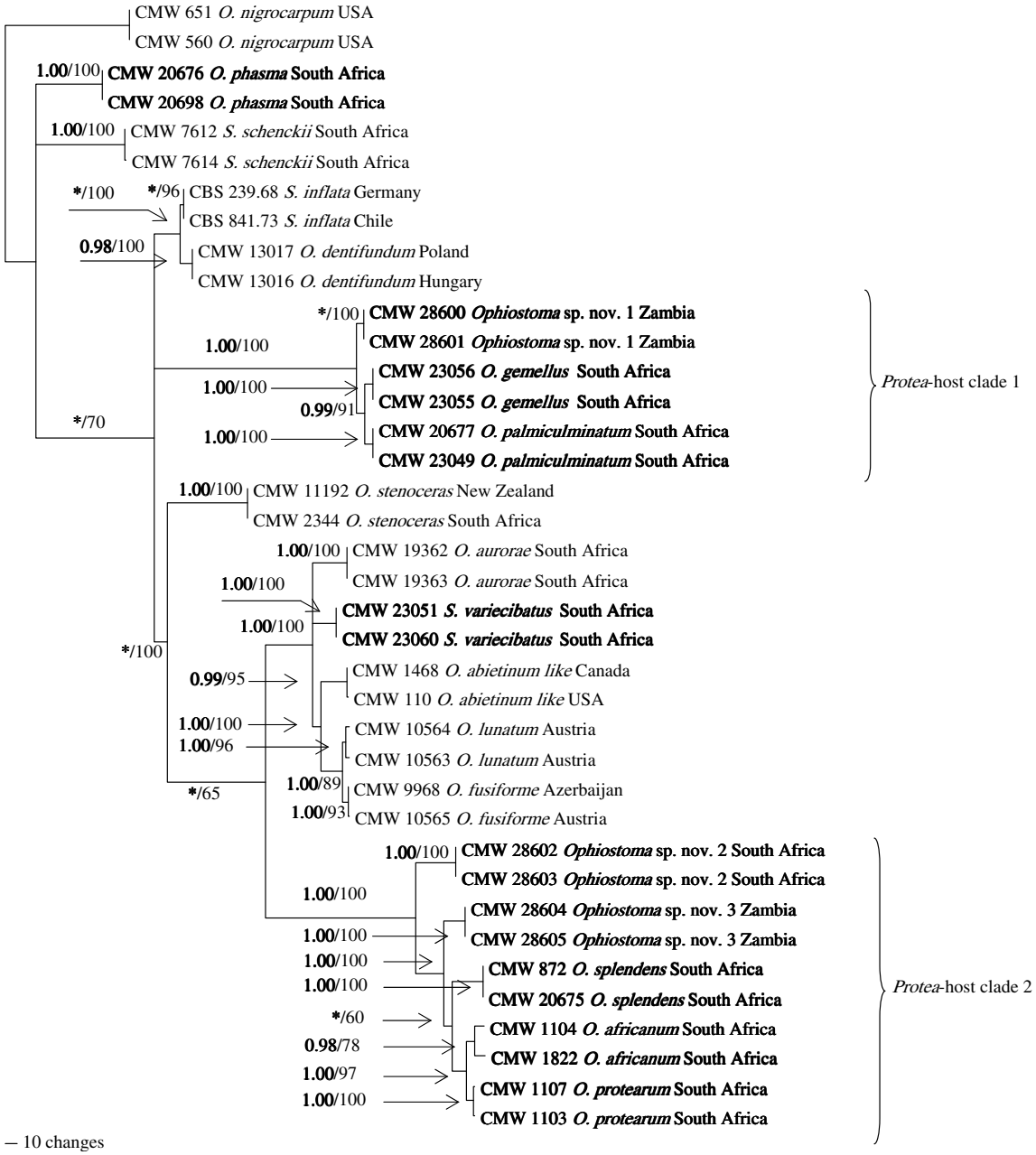

Fig. 2. The most parsimonious tree (TL = 941 steps, CI = 0.7928, RI = 0.9264) obtained from a parsimony analysis using ITS and β-tubulin sequence data of members of the genus *Ophiostoma*. The bar indicates 10 changes. The numbers at the nodes indicated in bold type face represent posterior probabilities obtained through Bayesian inference (values below 0.95 replaced by asterisk). Numbers in normal type face indicate parsimony bootstrap values based on 1000 resampling replicates. The accession numbers of isolates from *Protea* are printed in bold type face. *Ophiostoma nigrocarpum* was used as the outgroup taxon because it has previously been shown to be appropriate for this purpose (Zipfel et al., 2006; Roets et al., 2006a, in press-a).

Trichostetha fascicularis in both the Western Cape Province and Kwa-Zulu Natal (Holm and Marais, 1992). This beetle is exclusively associated with *Protea* species (Holm and Marais, 1992) and it carries *Ophiostoma*-vectoring mites in the Western Cape Province (Roets, 2006; Roets et al. in press-b). Also, mite communities (and thus probably the primary *Ophiostoma* vectors) in the infructescences of the CFR *Protea* species and the northern *Protea* species are similar (N. Theron, unpublished). It is thus plausible that *T. fascicularis* or a similar secondary vector may have facilitated the transport of mites and their fungal associates across geographic barriers.

The Proteaceae are estimated to have evolved in the mid-Cretaceous, between 130 and 100 Mya, shortly after the onset of the Gondwanan break-up (Barker et al., 2007; Sauquet et al., this issue). West Gondwana (including Africa and America) is estimated to have separated from east Gondwana (including Australia, Antarctica, Madagascar and India) around 180–150 Mya (Scotese et al., 1988), while the southern Indian Ocean opened between 120 and 100 Mya (Scotese, 1997). The disjunct distributions observed in present day Proteaceae is shown to have resulted from both vicariance and trans-oceanic dispersal events (Barker et al., 2007; Sauquet et al., this issue). The lineage to which the genus *Protea* belongs may have been in Africa since the Late Cretaceous (Sauquet et al., this issue). The specific association between ophiostomatoid fungi and their proteaceous hosts thus suggests that these fungi may also have an early evolutionary origin.

4.2. Origin of Protea-associated Gondwanamyces in the CFR

The genus *Gondwanamyces* was first described from the CFR where it was discovered in infructescences of *P. repens* (Wingfield et al., 1988). Later, a second species was discovered in this niche (Wingfield and Van Wyk, 1993). They were considered unusual fungi associated with an early divergent group of plants. Recently two additional species have been discovered in Costa Rica from *Scolytodes* bark beetles associated with *Cecropia* trees (Hulcr et al., 2007). This discovery adds credence to the African–South American link and to the view (Wingfield et al., 1999) that this is a southern Hemisphere fungal group. It further supports an early origin for *Gondwanamyces*, with a maximum age estimate between 180 and 150 Mya (Scotese et al., 1988). The two CFR *Gondwanamyces* species resolve as sister to the two South American species. There are no obvious relationships between *Cecropia* and *Protea* and although *Protea* has no bark beetle associations (Myburg and Rust, 1975; Coetzee and Giliomee, 1987; Roets et al., 2006b) it is likely that mites are the vectors of these fungi, as is true for *Ophiostoma* species in *Protea* infructescences (Roets et al., 2007) as well as in pine-infesting bark beetle galleries (Moser, 1985; Moser et al., 1995; Klepzig et al., 2001).

4.3. Links between conifer-associated and Protea-associated ophiostomatoid communities

Phylogenetically, *Gondwanamyces* is related to a large group of ophiostomatoid fungi in the genus *Ceratocystis sensu lato* and these fungi are distantly related to the *Ophiostoma* species residing in different orders of the fungi (Assembling the Fungal Tree Of Life (AFTOL), http://aftol.org/). Similar to the co-occurrence of *Gondwanamyces* and *Ophiostoma* on *Protea*, some *Ophiostoma* species associated with bark beetles on pines in the Northern Hemisphere also occur in close association with *Ceratocystis* species (Harrington and Wingfield, 1998). It is unknown whether the *Ceratocystis* species that occur sympatrically with *Ophiostoma* on conifers are more or less primitive than those species that occur in other niches. Nevertheless, this relationship is comparable to that of the *Ophiostoma* and *Gondwanamyces* species that co-occur in *Protea* infructescences and it is likely that they would have analogous evolutionary histories.

Recent large-scale phylogenetic studies on *Ophiostoma* (Zipfel et al., 2006; Roets et al., 2006a, b, in press-a) suggest that *Protea*-associated species evolved from largely conifer-associated, bark beetle-dispersed Northern Hemisphere ancestors (e.g. *O. nigrocarpum* (R.W. Davidson) de Hoog) (Farrell et al., 2001). The association between *Ophiostoma*, bark beetles and conifers (e.g. *Pinus*) is estimated to be at least 85 million years old (Bright and Stock, 1982; Berbee and Taylor, 1995; Sequeira and Farrell, 2001; Farrell et al., 2001). As the Proteaceae has an early origin, it would thus have been possible for the Proteaceae-*Ophiostoma* association to have established at around the same time as the establishment of the conifer-*Ophiostoma* association, just prior to the onset of the Gondwanan breakup (140–180 Mya, McLoughlin, 2001) at a time when the respective host plants may have been in close association (Sanderson et al., 2004). As in *Gondwanamyces*, we suspect that mites rather than bark beetles facilitated the host jumping of *Ophiostoma* between conifers and Proteaceae. This is corroborated by recent studies confirming the involvement of closely related mite species (*Tarsonemus* species) as specialised vectors of *Ophiostoma* species in both the conifer and *Protea* systems (Moser, 1985; Moser et al., 1995; Klepzig et al., 2001; Roets et al., 2007).

4.4. Patterns of host association

Phylogenetic analyses in this study support previous suggestions that the ophiostomatoid fungi in *Protea* infructescences are restricted to serotinous species (Roets et al., 2005, Roets et al., 2006a,b, in press-a). These fungi are vectored by mites that live in the infructescences and tend to require a relatively moist, protected environment typical of such infructescence structures (Roets et al., 2007). The absence of *Ophiostoma* species from other serotinous *Protea* species may be ascribed to various ecological factors. *Protea rubropilosa*, for example, is the only serotinous, and thus ecologically suitable, member of the mostly non-serotinous "mountain sugarbush" group (Rebelo, 1995) that might have housed ophiostomatoid fungi. The absence of *Ophiostoma* here may be due to ecological barriers such as host chemistry. The absence of ophiostomatoid fungi from many other serotinous *Protea* species screened in this study may be attributed to the open morphology of their infructescences, e.g. *P. nitida* and species in the monophyletic "rodent sugarbush" clade (Barraclough and Reeves, 2005; Rebelo, 1995), the "dwarf tufted sugarbush", and the well-supported clade of "western ground proteas" (Barraclough and Reeves, 2005; Rebelo, 1995). These open infructescences do not retain moisture for extended periods, which would prevent the establishment of the slow-growing ophiostomatoid species. The absence of ophiostomatoid fungi on the serotinous *P. scolymocepela*, *P. canaliculata*, *P. nana* and *P. witzenbergiana* corresponds with their phylogenetic placement with other non-host *Protea* species.

Even though the *Protea* hosts of ophiostomatoid fungi are morphologically similar, they do not form well-defined monophyletic groups (Barraclough and Reeves 2005). For example, the hosts of *G. capensis* and *O. splendens* namely *P. burchellii, P. laurifolia, P. lepidocarpodendron* and *P. lorifolia* all resolve in a single clade, but the other host group housing these fungi (*P. coronata*, *P. longifolia* and *P. neriifolia*) resolve in a different clade (Barraclough and Reeves, 2005). The presence of *P. burchellii* in the first host clade is unusual, as it is the only non-"bearded sugarbush" representative (Rebelo, 1995), and was taxonomically traditionally grouped with the "spoon bract" species *P. compacta*, *P. eximia*, *P. roupelliae* and *P. susannae* (Rebelo, 1995) that never hosts ophiostomatoid fungi. Both the phylogenetic data for *Protea* and the ophiostomatoid fungal association data thus suggest that *P. burchellii* may be a member of the "bearded sugarbush" *Protea* group rather than the "spoon bract sugarbushes".

An unusual aspect of the ophiostomatoid fungi occurring in the infructescences of *Protea* is that one fungal species may be restricted to a single plant species (e.g. *G. proteae* and *O. palmiculminatum*) over a wide geographic range within the CFR. In contrast, other species (e.g. *G. capensis* and *O. phasma*) have wide ranges of *Protea* hosts, some restricted to limited geographical areas. In the CFR, both *Ophiostoma* and *Gondwanamyces* species with limited host ranges tend to be specific to *P. repens* (e.g. *O. palmiculminatum* and *G. proteae*), whereas species associated with other *Protea* species (e.g. *G. capensis* and *O. phasma*) tend to have wide host ranges. The high level of specificity found for the ophiostomatoid fungi that occur in *P. repens* infructescences is particularly intriguing, as *Protea repens* is phylogenetically distantly related to all other host *Protea* species (Barraclough and Reeves, 2005) of the other ophiostomatoid fungi. It is also ecologically different from other host *Protea* species in having infructescences that are more tightly closed and that might result in niches with higher levels of moisture retention. *Protea repens* also harbors very different mite communities to most other *Protea* species (N. Theron, unpublished) and we believe that this is linked to the very different and specific ophiostomatoid fungi found associated with this plant.

The differences in ophiostomatoid fungal communities in *P. repens* and other serotinous *Protea* species could be the result of their having different mite vectors. However, two ophiostomatoid fungi (*O. splendens* and *S. variecibatus*) occur in the infructescences of *P. repens* as well as in a range of other *Protea* species. This may imply that certain *Ophiostoma* and *Gondwanam-*

yces species have specific mite vectors, while others are more promiscuous and associate with a wider range of vectors. *Sporothrix variecibatus* is thought to be indigenous to the CFR (Roets et al., in press-a) where it was isolated from *P. longifolia* and *P. repens*. This species has, however, also been isolated from a *Eucalyptus* sp. (Roets et al., in press-a), suggesting that native fungi on native CFR plant taxa may undergo host shifts (Slippers et al., 2005). Given the apparent ease of past and present host jump events (between unrelated host plants) observed in the genera *Gondwanamyces* and *Ophiostoma*, it appears as if association between these fungi and their vector insects may be more important than the association between these genera and their host plants in determining the distribution of these fungi. Thus, the apparent host specificity observed in CFR ophiostomatoid species probably has more to do with the specificity (and ecology) of the vectors than with the specificity of the fungus.

This study provides an initial attempt at understanding evolutionary processes that have shaped a group of fungi associated with a definitive CFR plant genus. The *Protea*-associated ophiostomatoid fungi have clearly evolved in close association with their hosts, although their co-habitation of this niche may have occurred separately. Results indicate that *Protea*-associated *Ophiostoma* species migrated from tropical and subtropical Africa to the CFR and the directionality of this migration contrasts with that proposed for their hosts. This emphasizes that understanding evolutionary processes that have shaped the phenomenal diversity of the CFR must include contributions from the entire biotic environment. In this regard, the fungi and certainly the mites provide outstanding models for further study.

Acknowledgments

We thank the National Research Foundation and the NRF/DST Centre of Excellence in Tree Health Biotechnology (CTHB), the Leverhulme Trust and the South African Big Genera Group for financial support. The Western Cape Nature Conservation Board is acknowledged for permission to work on conserved land. We extend our gratitude to K.C. Oberlander for assistance with phylogenetic analyses and two anonymous reviewers for valuable suggestions made for the improvement of our manuscript.

References

Aghayeva, D.N., Wingfield, M.J., De Beer, Z.W., Kirisits, T., 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. Mycologia 96, 866–878.

Aghayeva, D.N., Wingfield, M.J., Kirisits, T., Wingfield, B.D., 2005. *Ophiostoma dentifundum* sp. nov. from oak in Europe, characterized using molecular phylogenetic data and morphology. Mycol. Res. 109, 1127–1136.

Bakker, F.T., Culham, A., Marais, E.M., Gibby, M., 2005. Nested radiation in Cape *Pelargonium*. In: Bakker, F.T., Chatrou, L.W., Gravendeel, B., Pesler, P.B. (Eds.), Plant Species-level Systematics: New Perspectives on Patterns and Process. Koeltz, Königstein, pp. 75–100.

Barker, N.P., Weston, P.H., Rourke, J.P., Reeves, G., 2002. The relationships of the southern African Proteaceae as elucidated by internal transcribed spacer (ITS) DNA sequence data. Kew Bull. 57, 867–883.

Barker, N.P., Weston, P.H., Rutschmann, F., Sauquet, H., 2007. Molecular dating of the 'Gondwanan' plant family Proteaceae is only partially congruent with the timing of the break-up of Gondwana. J. Biogeogr. 34, 2012–2027.

Barraclough, T.G., Reeves, G., 2005. The causes of speciation in flowering plant lineages: species-level DNA trees in the African genus *Protea*. In: Bakker, F.T., Chatrou, L.W., Gravendeel, B., Pesler, P.B. (Eds.), Plant Species-level Systematics: New Perspectives on Patterns and Process. Koeltz, Königstein, pp. 31–36.

Berbee, M.L., Taylor, J.W., 1995. From 18S ribosomal sequence data to morphology among the fungi. Can. J. Bot. 73, S677–S683.

Born, J., Linder, H.P., Desmet, P., 2006. The greater cape floristic region. J. Biogeogr. 34, 147–162.

Bright, D.E., Stock, M.W., 1982. Taxonomy and geographic variation. In: Mitton, J.B., Sturgeon, K.B. (Eds.), Bark Beetles in North American Conifers: A System for the Study of Evolutionary Biology. University of Texas Press, Austin, pp. 46–73.

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

Crous, P.W., Denman, S., Taylor, J.E., Swart, L., Palm, E., 2004. Cultivation and diseases of Proteaceae: *Leucadendron*, *Leucospermum* and *Protea*. CBS Biodiversity Series 2. Centraalbureau voor Schimmelcultures, Utrecht.

Crous, P.W., Rong, I.H., Wood, A., Lee, S., Glen, H., Botha, W., Slippers, B., de Beer, Z.W., Wingfield, M.J., Hawksworth, D.L., 2006. How many species of fungi are there at the tip of Africa? Stud. Mycol. 55, 13–33.

De Beer, Z.W., Harrington, T.C., Vismer, H.F., Wingfield, B.D., Wingfield, M.J., 2003. Phylogeny of the *Ophiostoma stenoceras–Sporothrix schenckii* complex. Mycologia 95, 434–441.

Farrell, B.D., Sequeira, A.S., O'Meara, B.C., Normark, B.B., Chung, J.H., Jordal, B.H., 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). Evolution 55, 2011–2027.

Felsenstein, J., 1985. Confidence limits on phylogenetics: an approach using the bootstrap. Evolution 39, 783–791.

Galley, C., Linder, H.P., 2006. Geographical affinities of the Cape Flora, South Africa. J. Biogeogr. 33, 236–250.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous Ascomycetes. Appl. Environ. Microbiol. 61, 1323–1330.

Goldblatt, P., Manning, J., 2000. Cape Plants: A Conspectus of the Cape Flora of South Africa. National Botanical Institute, Pretoria, South Africa.

Harrington, T.C., Wingfield, M.J., 1998. The *Ceratocystis* species on conifers. Can. J. Bot. 76, 1446–1457.

Hawksworth, D.L., 2004. Fungal diversity and its implications for genetic resource collections. Stud. Mycol. 50, 9–18.

Holm, E., Marais, E., 1992. Fruit Chafers of Southern Africa. Sigma Press, Pretoria, South Africa.

Hulcr, J., Kolarik, M., Kirkendall, L.R., 2007. A new record of fungus-beetle symbiosis in *Scolytodes* bark beetles (Scolytinae, Curculionidae, Coleoptera). Symbiosis 43, 151–159.

Johnson, S.D., 1996. Pollination, adaptation and speciation models in the Cape Flora of South Africa. Taxon 45, 59–66.

Johnson, S.D., Linder, H.P., Steiner, K.E., 1998. Phylogeny and radiation of pollination systems in *Disa* (Orchidaceae). Am. J. Bot. 85, 402–411.

Klak, C., Reeves, G., Hedderson, T., 2003. Unmatched tempo of evolution in southern African semi-desert ice plants. Nature 427, 63–65.

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

Knox-Davies, P.S., 1975. Decline disease of silver trees and other indigenous species. Veld and Flora 61, 20–21.

Knox-Davies, P.S., 1981. Comments on fungus diseases of plants indigenous to the south western Cape. Veld and Flora 67, 88–91.

Knox-Davies, P.S., van Wyk, P.S., Marasas, W.F.O., 1986. Diseases of proteas and their control in the South–western Cape. Acta Hort. 185, 189–200.

Knox-Davies, P.S., van Wyk, P.S., Marasas, W.F.O., 1987. Diseases of Protea, *Leucospermum* and *Leucadendron* recorded in South Africa. Phytophylactica 19, 327–337.

Lee, S., Mel'nik, V., Taylor, J.E., Crous, P.W., 2004. Diversity of saprobic hyphomycetes on Proteaceae and Restionaceae from South Africa. Fungal Divers. 17, 91–114.

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

Lewis, P.O., 2001. A likelihood approach to estimating phylogeny from discrete morphological character data. Syst. Biol. 50, 913–925.

Linder, H.P., 2003. The radiation of the Cape Flora, southern Africa. Biol. Rev. 78, 597–638.

Linder, H.P., 2005. Evolution of diversity: the Cape Flora. Trends Plant Sci. 10, 536–541.

Maddison, W.P., Maddison, D.R., 2006. Mesquite: a modular system for evolutionary analysis. Version 1.12 Avaliable from: http://mesquiteproject.org.

Manning, J., Goldblatt, P., 2005. Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. Int. J. Plant Sci. 166, 459–474.

Marais, G.J., 1996. Fungi associated with the infructescences of *Protea* species with special reference to the Ophiostomatales. PhD Thesis, University of Pretoria, Pretoria, SA.

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

Marais, G.J., Wingfield, M.J., 1997. *Ophiostoma protearum* sp. nov. associated with *Protea caffra* infructescences. Can. J. Bot. 75, 362–367.

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

McLoughlin, S., 2001. The breakup history of Gondwana and its impact on pre-Cenozoic floristic provincialism. Aust. J. Bot. 49, 271–300.

Mitchell, D.T., Gibson, B.R., 2006. Ericoid mycorrhizal association: ability to adapt to a broad range of habitats. Mycologist 20, 2–9.

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

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

Mummenhoff, K., Al-Shehbaz, I., Bakker, F.T., Linder, H.P., Mühlhausen, A., 2005. Phylogeny, morphological evolution and speciation of endemic Brassicaceae genera in the Cape Flora of southern Africa. Ann. Mo. Bot. Gard. 92, 400–424.

Myburg, A.C., Rust, D.J., 1975. A survey of pests of the Proteaceae in the western and southern Cape Province. J. Entomol. Soc. S. Afr. 38, 55–60.
Myers, N., Mittelmeier, R.A., Mittelmeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858.
Nylander, J.A.A., 2004. MrModeltest v2, Program distributed by the author, Evolutionary Biology Centre, Uppsala University, The Netherlands.
O' Donnell, K., Cigelnik, E., 1977. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are onorthologous. Mol. Phylogenet. Evol. 7, 103–116.
Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.
Rambaut, A., 2002. Sequence Alignment Editor. Version 2.0. Department of Zoology, University of Oxford, Oxford.
Rebelo, T., 1995. Proteas of South Africa. Fernwood press, Vlaeberg, South Africa.
Roets, F., 2002. Diversity and ecology of ophiostomatoid fungi and arthropods associated with Proteaceae infructescences. M.Sc. Thesis, Department of Botany and Zoology, Stellenbosch University, South Africa.
Roets, F., Crous, P.W., Dreyer, L.L., 2005. Seasonal trends in colonization of *Protea* infructescences by *Gondwanamyces* and *Ophiostoma* spp. S. Afr. J. Bot. 71, 307–311.
Roets, F., 2006. Ecology and systematics of South African *Protea*-associated *Ophiostoma* species. Ph.D. Thesis, Department of Botany and Zoology, Stellenbosch University, South Africa.
Roets, F., De Beer, Z.W., Dreyer, L.L., Zipfel, R., Crous, P.W., Wingfield, M.J., 2006a. Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. Stud. Mycol. 55, 199–212.
Roets, F., Dreyer, L.L., Geertsema, H.G., Crous, P.W., 2006b. Arthropod communities in *Proteaceae* infructescences: seasonal variation and the influence of infructescence phenology. Afr. Entomol. 14, 257–265.
Roets, F., Wingfield, M.J., Crous, P.W., Dreyer, L.L., 2007. Discovery of fungus-mite-mutualism in a unique niche. Environ. Entomol. 36, 1226–1237.
Roets, F., De Beer, Z.W., Wingfield, M.J., Crous, P.W., Dreyer, L.L., in press-a. *Ophiostoma gemellus* and *Sporothrix variecibatus* from mites infesting *Protea* infructescences in South Africa. Mycologia.
Roets, F., Dreyer, L.L., Crous, P.W., Wingfield, M.J., in press-b. Mite-mediated hyperphoretic dispersal of *Ophiostoma* spp. from the infructescences of South African *Protea* spp. Environ. Entomol.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Rourke, J.P., 1998. A review of the systematics and phylogeny of the African Proteaceae. Aust. Syst. Bot. 11, 267–285.
Sanderson, M.J., Thorne, J.L., Wikström, N., Bremer, K., 2004. Molecular evidence on plant divergence times. Am. J. Bot. 91, 1656–1665.
Sauquet, H., Weston, P.H., Barker, N.P., Anderson, C.L., Cantrill, D.J., Savolainen, V., this issue. The age and origins of floral biodiversity in the Cape: contrasting patterns in the protea family (Proteaceae). Mol. Phylogenet. Evol.
Scotese, C.R., Gahagan, L.M., Larson, R.L., 1988. Plate tectonic reconstructions of the Cretaceous and Cenozoic ocean basins. Tectonophysics 155, 27–48.
Scotese, C.R., 1997. Paleogeographic atlas, PALEOMAP progress report 90-0497. Department of Geology, University of Texas at Arlington, Arlington, TX.
Sequeira, A.S., Farrell, B.D., 2001. Evolutionary origins of Gondwanan interaction: how old are *Araucaria* beetle herbivores? Biol. J. Lin. Soc. 74, 459–474.
Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.
Slippers, B., Stenlid, J., Wingfield, M.J., 2005. Emerging pathogens: fungal host jumps following anthropogenic introduction. Trends Ecol. Evol. 20, 420–421.
Stolk, A.C., Hennebert, G.L., 1968. New species of *Thysanophora* and *Custingophora* gen. nov. Persoonia 5, 189–199.
Swart, L., 1999. Pathogens associated with diseases of *Protea*, *Leucospermum* and *Leucadendron* spp. MSc Thesis, Department of Plant Pathology, University of Stellenbosch, Stellenbosch.
Swofford, D.L., 2003. PAUP*: Phylogenetic Analyses using Parsimony (* and other methods), Version 4.0b10. Sinauer, Sunderland.
Van der Niet, T., Johnson, S.D., Linder, H.P., 2006. Macroevolutionary data suggest a role for reinforcement in pollination system shifts. Evolution 60, 1596–1601.
Van Wyk, A.E., Smith, G.F., 2001. Regions of Floristic Endemism. Umdaus Press, Pretoria.
Van Wyk, P.S., Marasas, W.F.O., Knox-Davies, P.S., 1975. Ascomycetous leaf pathogens of *Protea*, *Leucadendron* and *Leucospermum* in South Africa. Phytophylactica 7, 91–94.
Verboom, G.A., Linder, H.P., Stock, W.D., 2004. Testing the adaptive nature of radiations: growth form and life history divergence in the African grass genus *Ehrharta* (Poaceae: Ehrhartoideae). Am. J. Bot. 91, 1364–1370.
Von Broembsen, S.L., 1989. Handbook of Diseases of Cut-Flower Proteas. International Protea Association, Victoria.
White, T.J., Bruns, T.D., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: a Guide to Methods and Applications. New York Academic Press, New York, pp. 315–322.
Wingfield, M.J., Seifert, K.A., Webber, J.F., 1993. *Ceratocystis* and *Ophiostoma*: Taxonomy, Ecology and Pathogenicity. American Phytopathological Society Press, St. Paul, Minnesota, USA.
Wingfield, M.J., Van Wyk, P.S., 1993. A new species of *Ophiostoma* from *Protea* infructescences in South Africa. Mycol. Res. 97, 709–716.
Wingfield, M.J., Van Wyk, P.S., Marasas, W.F.C., 1988. *Ceratacystiopsis proteae* sp. nov. with a new anamorph genus. Mycologia 80, 23–30.
Wingfield, B.D., Viljoen, C.D., Wingfield, M.J., 1999. Phylogenetic relationships of ophiostomatoid fungi associated with *Protea* infructescences in South Africa. Mycol. Res. 103, 1616–1620.
Zipfel, R.D., De Beer, Z.W., Jacobs, K., Wingfield, B.D., Wingfield, M.J., 2006. Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud. Mycol. 55, 75–97.