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Preliminary studies on *Botryosphaeria* species from Southern Hemisphere conifers in Australasia and South Africa

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Abstract. *Wollemia nobilis* is an ancient coniferous tree species that was recently discovered in eastern Australia. This tree is highly threatened due to its limited distribution. No genetic variation has been detected within the wild populations of ∼100 adult plants. A recent study has revealed that a species of *Botryosphaeria* is highly pathogenic to *W. nobilis*. The aim of the present study was to identify this fungus, as well as *Botryosphaeria* isolates of unknown identity from other Southern Hemisphere coniferous hosts, *Araucaria* from New Zealand and *Widdringtonia* from South Africa. To facilitate their identification, sequence data for the ITS rDNA, as well as the β-tubulin and translation elongation factor 1-α genes were combined to determine the phylogenetic relationship of these isolates with those of known *Botryosphaeria* spp. Isolates from *W. nobilis* included two *Botryosphaeria* spp. The first is closely related to *B. ribis*, but also shares some unique sequence polymorphisms with *B. parva*. One isolate grouped with *B. australis*, but also varied slightly from this taxon in the gene regions analysed. Additional isolates will be needed to determine whether these sequence variations represent speciation events or merely variation within populations of *B. ribis* and *B. australis*. In addition to this, *B. parva* was identified from *Araucaria* in New Zealand, and *B. australis* was found on *Widdringtonia* trees in South Africa. All three reports of these fungi are new records for their various hosts and could represent important pathogens of these trees.

Additional keywords: canker, die-back, *Fusicoccum*, gene genealogies, pathogen, phylogeny, conidia, new record.

Introduction

The Araucariaceae is an ancient conifer family that occurs mainly in the Southern Hemisphere, and includes the genera *Araucaria*, *Agathis* and *Wollemia* (Gilmore and Hill 1997). Some genera and species from this group are widely grown as ornamentals. Others occur only in small populations and areas, and are threatened by loss of genetic diversity and habitat.

Wollemia nobilis is a unique member of the family Araucariaceae that was discovered for the first time in Australia less than 10 years ago (Jones *et al*. 1995). This monotypic genus is an ancient plant relic that is linked to fossils dating back to the Mesozoic era (Gilmore and Hill 1997; Hill 1997). The total population of adult plants of *W. nobilis* includes ∼100 individuals that occur as several groups that grow a few km apart in a gorge in the Wollemi National Park, north-west of Sydney (Offord *et al*. 1999; Peakall *et al*. 2003). Population studies using allozymes, AFLP and SSR markers have not detected any genetic variation among the adult individuals (Peakall *et al*. 2003).

The small population size, proximity of the trees to each other and lack of genetic variation make *W. nobilis* vulnerable to diseases (Offord 1996; Bullock *et al*. 2000; Peakall *et al*. 2003). Surveys to determine the level of fungal diversity associated with *W. nobilis* have identified more than 50 fungal taxa from these plants, including potential pathogens such as a *Botryosphaeria* sp. and a *Fusarium* sp. (Summerell, www.rbgsyd.gov.au/wollemi/research). A subsequent pathogenicity study revealed that *Phytophthora cinnamomi*

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(which was not found on the site) and a *Botryosphaeria* sp. were highly pathogenic to *W. nobilis*, killing plants in the glasshouse within 4 weeks (Bullock *et al.* 2000). *P. cinnamomi* is a serious pathogen of exotic and indigenous plants in Australia, while *Botryosphaeria* spp. are endophytes and stress related pathogens of various woody hosts in this region (Slippers 2003; Slippers *et al.* 2004*b*, 2004*c*; Shearer and Smith 2000).

Seedlings and cuttings have been produced in an effort to conserve *W. nobilis* (Offord 1996; Hogbin *et al.* 2000). This material is used for two purposes. Firstly, to discourage poaching and secondly, the *ex situ* population is kept in several different localities to reduce the risk of diseases. The genetic diversity of this planting stock is as small as that of the wild population and diseases in the nursery, such as those already experienced due to *Botryosphaeria* die-back, thus threaten this planting stock. However, there is also the concern that distributing these plants could result in the spread of unique pathogens that may have evolved on Wollemi pine.

Apart from the Araucariaceae, other unique conifers are found in the Southern Hemisphere including the African cypresses (*Widdringtonia*: Cupressaceae). The four species in this genus have a limited distribution in southern Africa and are all considered rare to highly endangered (Pauw and Linder 1997). Similar to *W. nobilis*, efforts are being made to produce sufficient numbers of these plants in nurseries to discourage poaching and replenish natural populations (Mustart and Bond 1995). These nursery plants and those in natural stands are also at risk of pathogens. However, in a preliminary study no significant diseases were found in natural stands and plantations of adult *W. cedarbergensis* plants (Wingfield *et al.* 1988).

Botryosphaeria spp. are notoriously difficult to identify and the taxonomy of this group of fungi has been confused for many years (Denman *et al.* 2000). In a suite of recent studies, species have been successfully defined using a combination of morphological (mostly of the anamorph) and DNA-based data (Smith *et al.* 2001; Phillips *et al.* 2002; Denman *et al.* 2003). In some cases, combined gene genealogies using sequences of ITS rDNA, β-tubulin and translation elongation factor 1α (EF-1α) regions have been necessary to distinguish closely related species (Slippers *et al.* 2004*a*, 2004*b*). The aim of this study was to identify botryosphaeriaceous fungi that have been isolated from *W. nobilis* as well as some other conifers in the Southern Hemisphere.

Methods

Isolates and morphological characterisation

Eight *Botryosphaeria* isolates from Southern Hemisphere conifers were used in this study (Table 1). Three isolates were from die-back symptoms on *W. nobilis* plants in nurseries in Sydney, NSW and Queensland, Australia. Two isolates were obtained from *Araucaria heterophylla* (Norfolk Island Pine) in Auckland, New Zealand and one isolate was from *A. cunninghamii* (Hoop Pine) in Queensland. A further two isolates were obtained from diseased *Widdringtonia nodiflora* (Mountain Cypress) plants from the Cape Province, South Africa.

Isolates were maintained on malt and yeast-extract agar (MYA) (2% malt extract, 0.2% yeast extract and 2% agar; Biolab, Johannesburg, South Africa) at 25°C in the dark or under near-UV light. Isolates are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Molecular phylogenetic characterisation

The eight isolates from Southern Hemisphere conifers that formed the basis of this study were compared with other *Botryosphaeria* spp. that are known from there and from several other areas, or from similar hosts in other parts of the world. These comparisons were made based on phylogenetic reconstruction using sequence data of the 16S (small subunit) rRNA gene, the first internal transcribed spacer (ITS1), the complete 5.8S rRNA gene, the second ITS (ITS2) and the 5′ end of the 26S (large subunit) rRNA gene, a region of the β-tubulin gene and a part of the EF 1-α gene.

A phenol : chloroform DNA extraction technique was used to isolate the genomic DNA, as described in Raeder and Broda (1985) and Smith *et al.* (2001). PCR reaction mixtures, PCR conditions and visualisation of amplicons were as described in a previous study by Slippers *et al.* (2004*a*). The amplicons of all three DNA regions were also cleaned and sequenced as described in Slippers *et al.* (2004*a*). Sequences for fungi other than those produced in this study were obtained from GenBank or from Slippers *et al.* (2004*a*) (Table 1).

Sequence data were analysed using Sequence Navigator version 1.0.1 (Perkin Elmer Applied Biosystems, Foster City, CA) and manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were unordered and of equal weight. Maximum parsimonious trees were determined using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999), using heuristic searches with stepwise (random) addition and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved.

After partition homogeneity tests (Farris *et al.* 1994; Huelsenbeck *et al.* 1996), the sequence datasets of all three gene regions were combined and treated as one. Retention and consistency indices, a g1-value (Hillis and Huelsenbeck 1992) and bootstrap support (1000 replicates) (Felsenstein 1985) for branches were also determined in PAUP. Decay analysis of the branch nodes was determined using Autodecay (Eriksson 1998). Phylogenetic hypotheses were also tested using distance analyses with the Neighbour-Joining algorithm and either an uncorrected p-factor or the HKY85 parameter in PAUP.

Morphological characterisation

Isolates from *W. nobilis* were induced to sporulate by transferring them onto sterilised pine needles or *Populus* sticks placed on the surface of 2% water agar (WA) (Biolab) in Petri dishes and incubating these at 25°C under near-UV light. Fruiting structures were sectioned by hand and mounted in clear lactophenol. Morphological observations and photographs were made using an Axiocam digital camera (Carl Zeiss, Germany).

Results

Phylogenetic relationships

PCR amplicons of ~600, 450 and 300 base pairs were amplified and sequenced for the ITS rDNA, β-tubulin

Table 1. Isolates compared in the phylogenetic study

Culture no.[A]	Other no.	Identity[B]	Host	Location	Collector	GenBank		
						β-tubulin	EF1-α	ITS rDNA
CMW7772		*Botryosphaeria ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236906	AY236877	AY236925
CMW7773		*B. ribis*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236907	AY236878	AY236936
CMW7054	CBS121	*B. ribis*	*Ribes* sp.	New York, USA	N.E. Stevens	AY236908	AY236879	AF241177
CMW3386		*Botryosphaeria* sp.	*Wollemia nobilis*	Queensland, Australia	M. Ivory	AY615149	AY615157	AY615165
CMW3388		*Botryosphaeria* sp.	*Araucaria cunninghamii*	Queensland, Australia	M. Ivory	AY615146	AY615154	AY615162
CMW3389		*Botryosphaeria* sp.	*W. nobilis*	Queensland, Australia	M. Ivory	AY615147	AY615155	AY615163
CMW9070		*Botryosphaeria* sp.	*W. nobilis*	Sydney, Australia	B. Summerell	AY615148	AY615156	AY615164
CMW9078	ICMP7925	*B. parva*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY236914	AY236885	AY236940
CMW9081	ICMP8003	*B. parva*	*Populus nigra*	New Zealand	G.J. Samuels	AY236917	AY236888	AY236943
CMW10120		*B. parva*	*A. heterophylla*	Auckland, New Zealand	M.J. Wingfield	AY615144	AY615152	AY615160
CMW10121		*B. parva*	*A. heterophylla*	Auckland, New Zealand	M.J. Wingfield	AY615145	AY615153	AY615161
CMW9072		*B. australis*	*Acacia* sp.	Australia	D. Guest/J. Roux	AY339252	AY339268	AY339260
CMW1110		*B. australis*	*Widdringtonia nodiflora*	Cape Province, S Africa	W.J. Swart	AY615150	AY615158	AY615166
CMW1112		*B. australis*	*W. nodiflora*	Cape Province, S Africa	W.J. Swart	AY615151	AY615159	AY615167
CMW9075	ICMP8019	*B. dothidea*	*Pinus nigra*	New Zealand	G.J. Samuels	AY236928	AY236899	AY236950
CMW8000		*B. dothidea*	*Prunus sp.*	Crocifisso, Switzerland	B. Slippers	AY236955	AY236955	AY236949
CMW10309	CAP002	*B. lutea*	*Vitis vinifera*	Portugal	A.J.L. Phillips	AY339250	AY339266	AY339258
CMW992	KJ93.52	*B. lutea*	*A. deliciosa*	New Zealand	G.J. Samuels	AY236923	AY236894	AF027745
CMW10125		*B. eucalyptorum*	*Eucalyptus grandis*	Mpumalanga, S Africa	H. Smith	AY236920	AY236891	AF283686
CMW11705		*B. eucalyptorum*	*E. nitens*	S Africa	B. Slippers	AY339256	AY339264	AY339248
CMW7774		*B. obtusa*	*Ribes* sp.	New York, USA	B. Slippers/G. Hudler	AY236931	AY236902	AY236953
CMW7060	CBS431	*B. stevensii*	*Fraxinus excelsior*	Netherlands	H.A. van der Aa	AY236933	AY236904	AY236955
CMW9074		*B. rhodina*	*Pinus* sp.	Mexico	T. Burgess	AY236930	AY236901	AY236952

[A]Abbreviations for culture collections and isolates: CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; CMW = Tree Pathology Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ICMP = International Collection of Micro-organisms from Plants, Auckland, New Zealand; KJ = Jacobs and Rehner (1998).
[B]Identities as determined in this study.

and EF1-α regions, respectively (Table 1, GenBank AY615144–AY615167). A partition homogeneity test showed that the sequences of these three regions were significantly concordant (P value = 0.36). The data of the three regions were subsequently treated as a single larger dataset. The total dataset contained 1324 characters after alignment. Twelve characters that were repetitive in isolates of *B. ribis* were excluded from the EF1-α region. The 327 parsimony informative characters contained significant phylogenetic signal compared with random sampling ($P < 0.01$; g1 = −1.09) (Hillis and Huelsenbeck 1992). Three most parsimonious trees were retained after heuristic searches (543 steps, CI = 0.838, RI = 0.918) (Fig. 1).

Two isolates from *W. nobilis* (CMW3389, CMW9070) and one isolate from *A. cunninghamii* (CMW3388) grouped most closely to *B. ribis*, but formed a strongly supported separate branch (Clade II, d2/93% bootstrap) (Fig. 1). Analysis of the polymorphic sites that separate the closely related clades I–III (*B. ribis*, *Botryosphaeria* sp. and *B. parva*) revealed that Clade II had four unique polymorphisms,

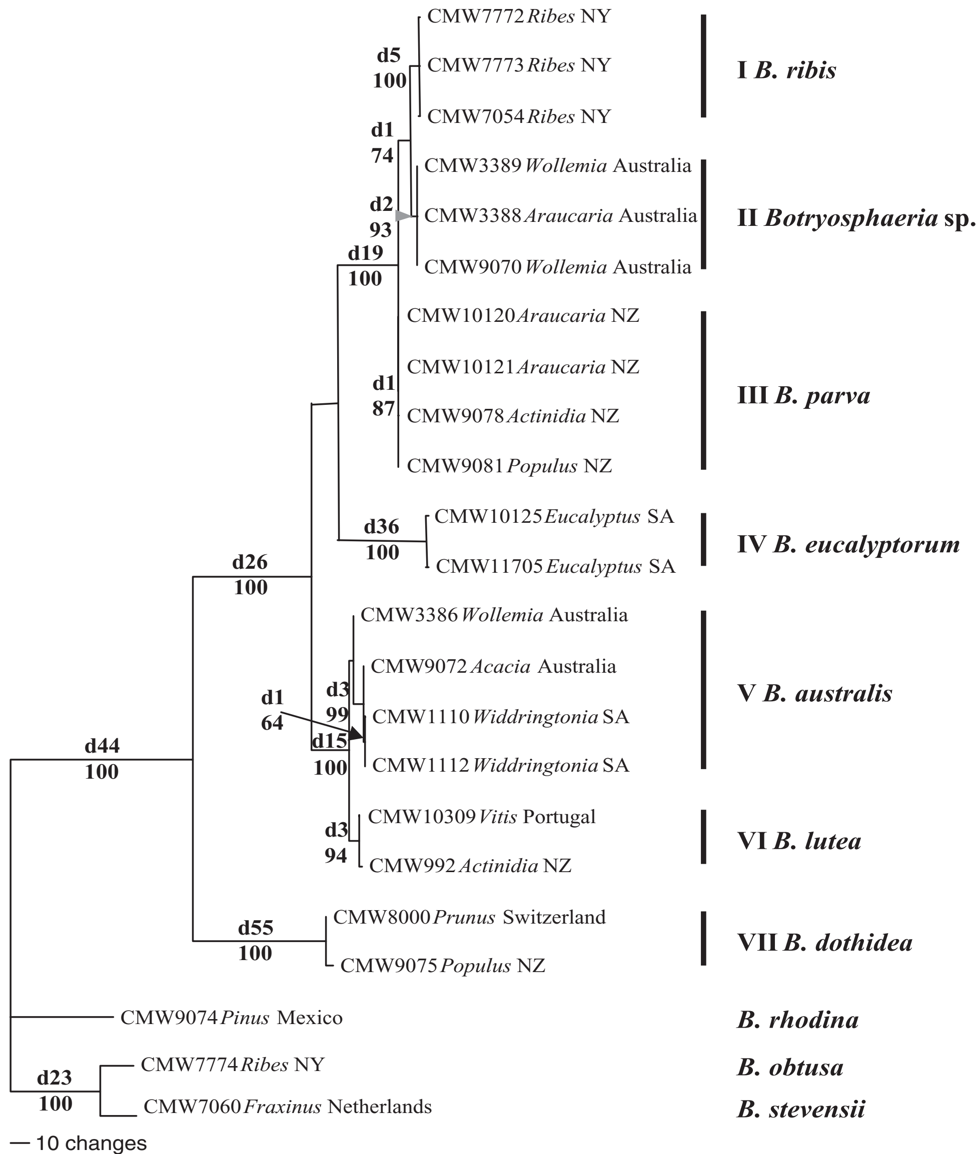

Fig. 1. One of the most parsimonious trees obtained through heuristic searches of the combined datasets of ITS rDNA, β-tubulin and elongation factor 1-α. Branch supports are indicated as decay values above and bootstrap values below the branches. The tree is rooted to *Botryosphaeria rhodina*, *B. obtusa* and *B. stevensii*. These species all have *Diplodia*-like anamorphs, unlike the ingroup taxa that have *Fusicoccum*-like anamorphs. The host genera and geographic origins (SA = South Africa; NY = New York, USA; NZ = New Zealand) of each isolate are indicated.

four shared polymorphisms with *B. parva* and eight shared polymorphisms with *B. ribis* (Table 2).

Two isolates from *Araucaria* from New Zealand (CMW10120, CMW10121) grouped with isolates of *B. parva* (Fig. 1, Clade III). Two isolates from *Widdringtonia* (CMW1110, CMW1112) grouped with *B. australis* (Fig. 1, Clade V). One isolate from *W. nobilis* (CMW3386) grouped most closely with *B. australis*, but also distinctly separate from them (Fig. 1). Given that only one isolate was available in this case, further phylogenetic analysis could not be made.

Morphological characterisation

Isolates from *W. nobilis* sporulated on pine needles and *Populus* sticks after 2–3 weeks incubation. Pycnidia were globose, mostly solitary, with apical ostioles, occasionally with small conical necks, superficial on the needles and 100–300 µm in diameter. Conidia of isolates residing in Clade II were hyaline, fusiform to ellipsoid and (17–)18–19(–20) × 5–6 µm (Fig. 2). Conidia of isolate CMW3386 from *W. nobilis* were similar in shape to those in Clade II, but were slightly longer, (19–)21–22(–25) × 5–6 µm (Fig. 3).

Discussion

At least four *Botryosphaeria* spp. were identified from the small collection of isolates associated with the Southern Hemisphere coniferous hosts, *Wollemia*, *Araucaria* and *Widdringtonia*. Two taxa were identified as *B. parva* and *B. australis*. The identities of the two other taxa remain uncertain, but they are most closely related to *B. ribis* and *B. australis*, respectively. All these species have *Fusicoccum* anamorphs and all are new records for the hosts on which they were collected.

Based on multiple gene sequence comparisons, three isolates (Clade II) from Southern Hemisphere coniferous hosts were most closely related to *B. ribis*. This is the first DNA-based evidence to show that isolates more similar to *B. ribis* than to *B. parva* occur in Australia. *B. ribis* has previously been reported from Australia on *Eucalyptus* and this record was based on morphological characters (Davison and Tay 1983; Shearer *et al*. 1987; Old *et al*. 1990). It is, however, likely that these reports refer to the morphologically similar *B. parva* (Slippers *et al*. 2004*a*). Currently, *B. ribis* and *B. parva* can be separated only with certainty by using DNA sequence data (Slippers *et al*. 2004*a*). Based on these data, *B. ribis* has thus far been identified only from *Ribes* sp. in the USA. In contrast, *B. parva* has a wide host range and has been reported from native and exotic hosts in Australia and elsewhere in the Southern Hemisphere (Slippers *et al*. 2004*a*, 2004*c*).

The DNA sequences for isolates grouping in Clade II were not identical to those of *B. ribis*. Isolates residing in this clade share eight polymorphisms with *B. ribis* and four with *B. parva*. They also have four alleles that are different from those of *B. ribis* and *B. parva*. All four of these unique alleles are also present in the outgroup taxa with *Diplodia* anamorphs and are, thus, either plesiomorphic characters or homoplasies. These results suggest that Clade II isolates represent an older lineage that share a common ancestor with both *B. ribis* and *B. parva*, but have begun to accumulate unique alleles due to geographic or host separation. Alternatively, these isolates in Clade II might represent remnants of past genetic exchange between populations of *B. ribis* and *B. parva* before these lineages were reproductively separated (Davis and Nixon 1992). However, the limited sample size and absence of other distinguishing

Table 2. Polymorphic nucleotides (or alleles) from sequence data of the ITS rDNA, β-tubulin and EF-1α, that show the relationship between the unidentified *Botryosphaeria* sp. in Clade II of the phylogenetic analysis, and isolates of *B. ribis* and *B. parva*. Polymorphisms that are unique to the unidentified *Botryosphaeria* sp. are in bold type. All other polymorphisms that are shared between the isolates of the unidentified *Botryosphaeria* sp. and either *B. ribis* or *B. parva* are highlighted

Culture no.	β-tubulin					ITS				EF1-α						
	96	129	188	419	437	513	585	797	864	1081	1082	1138	1145	1190	1216	1251	1309
B. ribis																	
CMW7772	C	G	T	T	T	A	G	T	T	T	G	1	1	C	G	A	G
CMW7773	C	G	T	T	T	A	G	T	T	T	G	1	1	C	G	A	G
CMW7045	C	G	T	T	T	A	G	T	T	T	G	1	1	C	G	A	G
Botryosphaeria sp.																	
CMW3388	C	G	C	**C**	**C**	A	G	**C**	T	T	G	0	0	T	**A**	G	G
CMW3389	C	G	C	**C**	**C**	A	G	**C**	T	T	G	0	0	T	**A**	G	G
CMW9070	C	G	C	**C**	**C**	A	G	**C**	T	T	G	0	0	T	**A**	G	G
B. parva																	
CMW9078	T	A	C	T	T	T	–	T	C	C	A	0	0	T	G	G	A
CMW9081	T	A	C	T	T	T	–	T	C	C	A	0	0	T	G	G	A
CMW10120	T	A	C	T	T	T	–	T	C	C	A	0	0	T	G	G	A
CMW10121	T	A	C	T	T	T	–	T	C	C	A	0	0	T	G	G	A

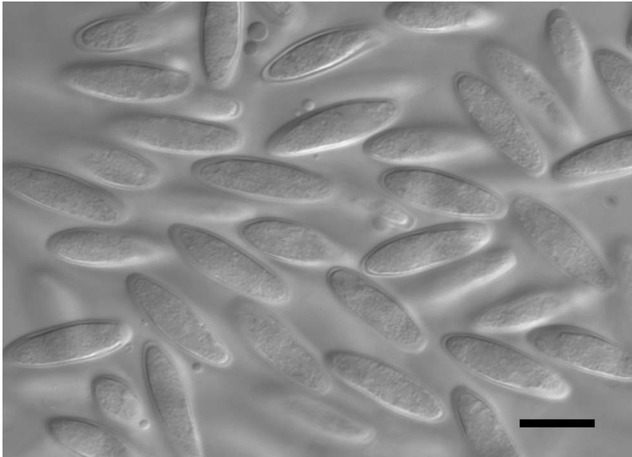

Fig. 2. Conidia of *Botryosphaeria* spp. produced in culture on pine needles and water agar. This figure corresponds to isolates in clade II in the phylogenetic study. Bars = 10 µm.

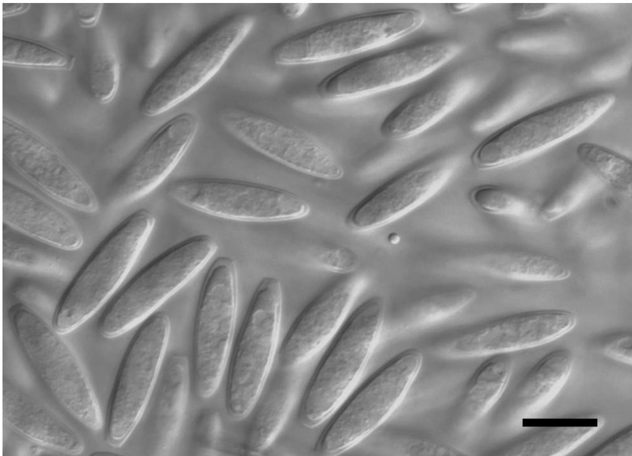

Fig. 3. Conidia of *Botryosphaeria* spp. produced in culture on pine needles and water agar. This figure corresponds to isolates in clade V in the phylogenetic study. Bars = 10 µm.

characters for Clade II isolates make it impossible to test whether these isolates represent a distinct species.

Isolates that grouped in Clade II, and that remain unidentified, were from diseased *W. nobilis* nursery plants. Symptoms of *Botryosphaeria* die-back now occur on *Wollemia* plants in nurseries in Sydney and Queensland where these plants are propagated (Summerell, unpublished data). The origin of the pathogen cannot be determined from the limited isolates used here. However, it is possible that they originated from native stands of Wollemi Pine and were brought into nurseries on cuttings, seeds or other plant material. *Botryosphaeria* spp. are often endophytic and are apparently commonly moved around the world in this way (Smith *et al*. 1996; Burgess and Wingfield 2002; Slippers 2003; Slippers *et al*. 2004*a*). The pathogen might also have originated from other plants such as the related native Hoop Pine which is common in these nurseries, or from other hosts. Both hypotheses relating to the origin of this pathogen are of concern for the conservation of Southern Hemisphere coniferous species. They would imply that a pathogen from *Wollemia* could threaten other coniferous species in the nurseries or gardens where *Wollemia* trees are grown, and ultimately plants beyond these areas, or *vice versa*. *Wollemia* could also be susceptible to *Botryosphaeria* spp. present in other countries.

B. parva was identified from an *Araucaria* sp. from New Zealand. The fungus is well known from this region where it was initially described by Pennycook and Samuels (1985) on the exotic plants such as *Malus domestica*, *Populus nigra* and *Actinidia deliciosa*. It has subsequently been shown that this species has a wide distribution and host range in the Southern Hemisphere (Slippers 2003; Slippers *et al*. 2004*a*, 2004*c*). *B. parva* is known to be a virulent pathogen when plants are under stress (reported as *B. dothidea* in Smith *et al*. 2001) and its presence on Araucariaceae would be worth further study.

Conidial morphology of *B. parva* and isolates of the species residing in Clade II from *Araucaria* and *W. nobilis* resemble *Hendersonula agathi*, both in size and in shape. Young (1948) described *H. agathi* as the pathogen responsible for a leaf and twig disease of *Agathis* spp. in nurseries in Queensland, Australia. Sutton and Dyko (1989) considered *H. toruloidea* and *H. agathi* to be synonymous with a new taxon, *Natrassia mangiferae* Sutton & Dyko. In Slippers *et al*. (2005) this last named taxon is described as *Fusicoccum mangiferum* Slippers, Johnson & M.J. Wingf., based on DNA and morphological similarities with other *Fusicoccum* species. The synonymy of *H. agathi* and *F. mangiferum* was, however, rejected in this study, because the conidia of the former species are more similar to those of *B. ribis* and *B. parva* in size and appearance (Slippers *et al*. 2005). It is thus possible that the *B. parva* and Clade II isolates, reported here from coniferous hosts that are also related to *Agathis*, are conspecific with *H. agathi*. A more extensive sample, including isolates from *Agathis*, is required to test this hypothesis.

B. australis was identified from *W. nodiflora* from South Africa in this study. This fungus was first described from *Acacia* and *Sequoiadendron* trees in Australia, but also occurs on other native and exotic hosts (*Banksia* and *Eucalyptus*) in Australia and elsewhere (Slippers 2003; Slippers *et al*. 2004*b*). It is not clear whether the current finding of this fungus represents that of a native pathogen on a South African native host, or whether *B. australis* has been introduced into South Africa. In both cases, this fungus poses a risk to this rare and threatened genus of tree, especially where plants are produced in nurseries to replenish depleted natural populations (Mustart and Bond 1995). As discussed earlier, *Botryosphaeria* spp. are known to cause nursery diseases on other coniferous hosts in the Southern Hemisphere

(Young 1948; Bullock *et al*. 2000). Wingfield *et al*. (1988) did not find *Botryosphaeria* spp. associated with *Widdringtonia* in an earlier survey, but warned of the potential dangers of introducing pathogens into natural populations when these plants are propagated in nurseries.

One isolate from *W. nobilis* grouped sister to the *B. australis* clade. Conidia of this isolate were morphologically similar to those of *B. australis*, although they were slightly shorter on average than those of the latter taxon (Slippers *et al*. 2004*b*). *B. australis* conidia are, on average, 24–25 µm long, unlike those of the isolate from *Wollemia* that had conidia that were 21–22 µm long. It is, however, not possible to judge the value of these measurements based on a single isolate. This isolate might represent a unique species or simply a variant of *B. australis*, but more isolates will need to be studied to test these hypotheses. Nevertheless, this second species of *Botryosphaeria* on *Wollemia* could be important when considering conservation strategies for this tree.

The number of samples of *Botryosphaeria* spp. from *Wollemia*, *Araucaria* and *Widdringtonia* in this study was small. Results should thus be seen as providing a preliminary indication of the identity and variation of *Botryosphaeria* spp. that could potentially affect Southern Hemisphere coniferous hosts. Although *Botryosphaeria* spp. are mostly stress related pathogens, the potential impact of these pathogens is well illustrated by the pathogenicity trials of Bullock *et al*. (2000). We hope that this investigation will encourage further collections of isolates and studies to determine the extent of variation among, and within, these *Botryosphaeria* spp. The accurate identification of these fungi, their origin and distribution, as well as their pathogenicity to the coniferous and other hosts in the Southern Hemisphere, will clearly be important in managing their potential impact.

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References

Bullock S, Summerell BA, Gunn LV (2000) Pathogens of the Wollemi Pine, *Wollemia nobilis*. *Australasian Plant Pathology* **29**, 211–214.

Burgess T, Wingfield MJ (2002) Quarantine is important in restricting the spread of exotic seed-borne tree pathogens in the Southern Hemisphere. *International Forestry Review* **4**, 56–65.

Davis JI, Nixon KC (1992) Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology* **41**, 421–435.

Davison EM, Tay CS (1983) Twig, branch and upper trunk cankers of *Eucalyptus marginata*. *Plant Disease* **67**, 1285–1287.

Denman S, Crous PW, Groenewald JZ, Slippers B, Wingfield BD, Wingfield MJ (2003) Circumscription of *Botryosphaeria* species associated with Proteaceae on morphology and DNA sequence data. *Mycologia* **95**, 294–307.

Denman S, Crous PW, Taylor JE, Kang JC, Pascoe I, Wingfield MJ (2000) An overview of the taxonomic history of *Botryosphaeria* and a re-evaluation of its anamorphs based on morphology and ITS rDNA phylogeny. *Studies in Mycology* **45**, 129–140.

Eriksson T (1998) Autodecay ver. 4.0 (program distributed by the author). (Department of Botany, Stockholm University: Stockholm)

Farris JS, Kallersjo M, Kluge AG, Bult C (1994) Testing significance of incongruence. *Cladistics* **10**, 315–319. doi: 10.1006/clad.1994.1021

Felsenstein J (1985) Confidence intervals on phylogenetics: an approach using bootstrap. *Evolution; International Journal of Organic Evolution* **39**, 783–791.

Gilmore S, Hill KD (1997) Relationships of the Wollemi pine (*Wollemia nobilis*) and a molecular phylogeny of the Araucariaceae. *Telopea* **7**, 275–291.

Hill KD (1997) The Wollemi Pine – a recently discovered Australian genus of Araucariaceae. *Botany* **84**, 202–203 [abstract].

Hillis DM, Huelsenbeck JP (1992) Signal, noise, and reliability in molecular phylogenetic analyses. *The Journal of Heredity* **83**, 189–195.

Hogbin PM, Peakall R, Sydes MA (2000) Achieving practical outcomes from genetic studies of rare Australian plants. *Australian Journal of Botany* **48**, 375–382. doi: 10.1071/BT98080

Huelsenbeck JP, Bull JJ, Cunningham CW (1996) Combining data in phylogenetic analysis. *Trends in Ecology & Evolution* **11**, 152–158. doi: 10.1016/0169-5347(96)10006-9

Jacobs KA, Rehner SA (1998) Comparison of cultural and morphological characters and ITS sequences in anamorphs of *Botryosphaeria* and related taxa. *Mycologia* **90**, 601–610.

Jones WG, Hill KD, Allen JM (1995) *Wollemia nobilis*, a new living Australian genus and species in the Araucariaceae. *Telopea* **6**, 173–176.

Mustart P, Bond W (1995) The decline of the Clanwilliam cedar — natural or man made? *Africa — Environment & Wildlife* **2**, 80–81.

Offord CA (1996) Conserving the Wollemi pine: an integrated approach. *Danthonia* **5**, 12–14.

Offord CA, Porter CL, Meagher PF, Errington G (1999) Sexual reproduction and early plant growth of the Wollemi pine (*Wollemia nobilis*), a rare and threatened Australian conifer. *Annals of Botany* **84**, 1–9. doi: 10.1006/anbo.1999.0882

Old KM, Gibbs R, Craig I, Myers BJ, Yaun ZQ (1990) Effect of drought and defoliation on the susceptibility of *Eucalyptus* to cankers caused by *Endothia gyrosa* and *Botryosphaeria ribis*. *Australian Journal of Botany* **38**, 571–581.

Pauw CA, Linder HP (1997) Tropical African cedars (*Widdringtonia*, Cuppressaceae): systematics, ecology and conservation status. *Botanical Journal of the Linnean Society* **123**, 297–319. doi: 0.1006/bojl.1996.0091

Peakall R, Ebert D, Scott LJ, Meagher PF, Offord CA (2003) Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). *Molecular Ecology* **12**, 2331–2343. doi: 10.1046/j.1365-294X.2003.01926.x

Pennycook SR, Samuels GJ (1985) *Botryosphaeria* and *Fusicoccum* species associated with ripe fruit rot of *Actinidia deliciosa* (Kiwifruit) in New Zealand. *Mycotaxon* **24**, 445–458.

Phillips AJL, Fonseca F, Povoa V, Castilho R, Nolasco G (2002) A reassessment of the anamorphic fungus *Fusicoccum luteum* and description of its teleomorph *Botryosphaeria lutea* sp. nov. *Sydowia* **54**, 59–77.

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology* **1**, 17–20.

Shearer BL, Smith IW (2000) Diseases of eucalypts caused by *Phytophthora* and *Pythium*. In 'Diseases and pathogens of *Eucalyptus*'. (Eds PJ Keane, GA Kile, FD Podger, BN Brown) (CSIRO Publishing: Melbourne, Australia)

Shearer BL, Tippett JT, Bartle JR (1987) *Botryosphaeria ribis* infection associated with death of *Eucalyptus radiata* in species selection trials. *Plant Disease* **71**, 140–145.

Slippers B (2003) The taxonomy, phylogeny and ecology of Botryosphaeriaceous fungi occurring on various woody hosts. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Slippers B, Crous PW, Denman S, Coutinho TA, Wingfield BD, Wingfield MJ (2004*a*) Combined multiple gene genealogies and phenotypic characters differentiate several species previously identified as *Botryosphaeria dothidea. Mycologia* **96**, 83–101.

Slippers B, Fourie G, Crous PW, Coutinho TA, Wingfield BD, Wingfield MJ (2004*b*) Multiple gene sequences delimit *Botryosphaeria australis* sp. nov. from *B. lutea. Mycologia* **96**, 1028–1039.

Slippers B, Fourie G, Crous PW, Coutinho TA, Wingfield BD, Carnegie A, Wingfield MJ (2004*c*) Speciation and distribution of *Botryosphaeria* spp. on native and introduced Eucalyptus trees. *Studies in Mycology* **50**, 343–358.

Slippers B, Johnston GI, Crous PW, Coutinho TA, Wingfield BD, Wingfield MJ (2005) Phylogenetic and morphological re-evaluation of the *Botryosphaeria* species causing diseases of *Mangifera indica* in Australia. *Mycologia* **97**, 102–113.

Smith H, Crous PW, Wingfield MJ, Coutinho TA, Wingfield BD (2001) *Botryosphaeria eucalyptorum* sp. nov., a new species in the *B. dothidea*-complex on *Eucalyptus* in South Africa. *Mycologia* **93**, 277–284.

Smith H, Wingfield MJ, Crous PW, Coutinho TA (1996) *Sphaeropsis sapinea* and *Botryosphaeria dothidea* endophytic in *Pinus* spp. and *Eucalyptus* spp. in South Africa. *South African Journal of Botany* **62**, 86–88.

Sutton BC, Dyko BJ (1989) Revision of *Hendersonula. Mycological Research* **93**, 466–488.

Swofford DL (1999) 'PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4'. (Sinauer Associates: Sunderland, Massachusetts)

Wingfield MJ, Von Broembsen SL, Manders PT (1988) A preliminary assessment of the threat of diseases and pests to *Widdringtonia cedarbergensis. SA Forestry Journal* **147**, 32–34.

Young HE (1948) *Hendersonula agathi* n. sp., the cause of leaf cast of Kauri Pine in Queensland. *Queensland Journal of Agricultural Science* **5**, 9–12.

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