

Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*

Juanita de WET¹, Treena BURGESS^{2†}, Bernard SLIPPERS¹, Oliver PREISIG³, Brenda D. WINGFIELD² and Michael J. WINGFIELD^{3*}

¹Department of Microbiology and Plant Pathology, Forestry Agricultural Biotechnology Institute (FABI), Tree Pathology Co-operative Programme (TPCP), University of Pretoria, South Africa.

²Department of Genetics, Forestry Agricultural Biotechnology Institute (FABI), Tree Pathology Co-operative Programme (TPCP), University of Pretoria, South Africa.

³Forestry Agricultural Biotechnology Institute (FABI), Tree Pathology Co-operative Programme (TPCP), University of Pretoria, South Africa.

E-mail: mike.wingfield@fabi.up.ac.za

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Sphaeropsis sapinea is an opportunistic pathogen causing serious damage to conifers, pre-disposed by adverse environmental conditions or mechanical damage. Three different morphological forms of the fungus have been described and are commonly referred to as the A, B and C morphotypes. Isolates of the different morphotypes have also been separated based on differences in pathogenicity and molecular characteristics. These differences, however, overlap and have not been considered sufficiently robust to justify the description of separate taxa. The aim of this study was to consider relationships between isolates representing different *S. sapinea* morphotypes, using multiple gene genealogies inferred from partial sequences of six protein-coding genes and six microsatellite loci. Genealogies generated for the protein-coding genes and microsatellite loci were not congruent but both consistently grouped isolates representing the A and C morphotypes in separate but closely related clades. In contrast, isolates of the B morphotype grouped together in a clade that was equally different to the A and C morphotypes as it was to the clade encompassing isolates of *Botryosphaeria obtusa*. These results provide strong evidence to show that the B morphotype isolates are distantly related to *S. sapinea* and represent a discrete taxon, which we describe here as *Diplodia scrobiculata* sp. nov.

INTRODUCTION

Sphaeropsis sapinea (syn. *Diplodia pinea*) is a latent, opportunistic pathogen of conifers occurring world-wide (Eldridge 1961, Swart & Wingfield 1991). It can have devastating effects on trees when it is associated with stress-inducing factors such as drought, hail, adverse temperatures or mechanical wounding (Purnell 1957, Chou 1987). *S. sapinea* causes extensive losses in commercial plantation forestry, especially where susceptible *Pinus* spp. are intensively propagated (Zwolinski, Swart & Wingfield 1990).

Three distinct morphotypes (A, B and C) have been described for *S. sapinea*. The A morphotype is characterised by fluffy mycelium and smooth conidial walls, while the B morphotype has mycelium appressed to the surface of the agar and pitted conidial walls (Wang *et al.* 1985, Wang, Blanchette & Palmer 1986, Palmer,

Stewart & Wingfield 1987). C morphotype isolates have fluffy mycelium and smooth conidial walls similar to the A morphotype, but the conidia are significantly longer in the C morphotype (de Wet *et al.* 2000). Isolates of the C morphotype are also considerably more pathogenic than those of the A morphotype (de Wet *et al.* 2002). An I morphotype of *S. sapinea* was described as being intermediate between the A and B morphotypes (Hausner *et al.* 1999), but subsequent studies based on SSR markers (Burgess, Wingfield & Wingfield 2001a) showed that this fungus represents the anamorph state of *Botryosphaeria obtusa*.

The authenticity of the morphotypes of *S. sapinea* has been confirmed using DNA-based techniques, such as randomly amplified polymorphic DNA (RAPDs) (Smith & Stanosz 1995, de Wet *et al.* 2000), restriction fragment length polymorphisms (RFLPs) (Hausner *et al.* 1999) and DNA sequences of the rRNA operon (de Wet *et al.* 2000). More recently, inter simple or short sequence repeat (ISSR) fingerprinting and simple sequence repeat (SSR) markers have also been used to

* Corresponding author.

† Present address: School of Biological Sciences and Biotechnology, Murdoch University, Perth, Western Australia.

Table 1. Isolates used in this study.

Isolates ^a	Type ^b	Origin	Host	Collector	Other collection numbers ^c	Reference
CMW8225	A	Australia	<i>P. radiata</i>	T. Burgess		
CMW190	A	USA	<i>P. resinosa</i>	M. A. Palmer		
CMW4885	C	Indonesia	<i>P. patula</i>	M. J. Wingfield		
CMW4876	C	Indonesia	<i>P. patula</i>	M. J. Wingfield		
CMW5870 (PREM57463)	B	CA, USA	<i>P. radiata</i>	T. Gordon		
CMW8228 (PREM57464)	B	CA, USA	<i>P. radiata</i>	T. Gordon		
CMW4898 (PREM57465)	B	Mexico	<i>P. greggii</i>	M. J. Wingfield		
CMW4900 (PREM57466)	B	Mexico	<i>P. greggii</i>	M. J. Wingfield		
CMW189 (PREM57461)	B	USA	<i>P. banksiana</i>	M. A. Palmer	124	Palmer <i>et al.</i> (1987)
CMW4334 (PREM57462)	B	USA	<i>P. resinosa</i>	G. R. Stanosz	474	Blodgett & Stanosz (1999)
^CMW8753	B	Italy	<i>Pinus. sp.</i>	L. Sparapano	97-73	Stanosz <i>et al.</i> (1999)
CMW8230	<i>B. obtusa</i>	Canada	<i>Picea glauca</i>	J. Reid	920729	Hausner <i>et al.</i> (1999)
CMW8231	<i>B. obtusa</i>	Canada	<i>P. banksiana</i>	J. Reid	810704	Hausner <i>et al.</i> (1999)
CMW8232	<i>B. obtusa</i>	South Africa	<i>Malus domestica</i>	W. A. Smit		
*CMW8233	<i>B. obtusa</i>	South Africa	<i>M. domestica</i>	W. A. Smit		
^CMW4891	<i>Lasiodiplodia theobromae</i>	Indonesia	<i>P. patula</i>	M. J. Wingfield		

^a Isolates marked (*) were included only in the microsatellite genealogy and those marked (^) were included only in the protein-coding gene genealogy.

^b Morphotype designation is based on descriptions provided by Palmer *et al.* (1987) and de Wet *et al.* (2000).

^c Isolation numbers used in previous studies for which references are provided in the last column.

provide increased resolution to the differentiation between these morphotypes (Zhou, Smith & Stanosz 2001, Burgess *et al.* 2001a). These techniques alone, however, are not always informative when comparing closely related species or elements of the same species. This weakness can be resolved by using genealogies inferred from multiple protein-coding genes (Taylor *et al.* 2000) combined with highly polymorphic microsatellite loci (Geiser, Pitt & Taylor 1998, Fisher *et al.* 2000, Koufopanou *et al.* 2001, Steenkamp *et al.* 2002). In this study, our aim was to construct multiple gene genealogies from partial sequences of six protein-coding genes (Bt2 of β -tubulin, chitin synthase (CHS), elongation factor 1 α (EF-1 α), actin (ACT), calmodulin (CAL) and glutaraldehyde-6-phosphate (GPD)), and six microsatellite loci (SS5, SS7, SS8, SS9, SS10 and SS11) to elucidate the phylogenetic relationships between isolates of *S. sapinea* representing the different morphotypes.

MATERIALS AND METHODS

Fungal isolates

Eleven *Sphaeropsis sapinea* isolates (Table 1) from the USA, Australia, Mexico, California, Italy, and Indonesia were used in this study. These isolates represented all three morphotypes described for *S. sapinea*. Four isolates of the closely-related *Botryosphaeria obtusa*, were included for comparison, and *Lasiodiplodia theobromae* (syn. *B. rhodina*) was used as an outgroup taxon

(Table 1). The *S. sapinea* isolate from south-western Australia was obtained by direct isolation from the pith tissue of *P. radiata* cones, and those from Mexico from *P. greggii* cones. The Indonesian and Californian isolates were obtained from pycnidia on *P. patula* or *P. radiata* shoots, with die-back symptoms. Single conidial cultures were generated for all the isolates and cultured on 2% Malt Extract Agar (MEA) (2% m/v Biolab malt extract; 2% m/v Biolab agar) in Petri dishes at 25 °C. All the single conidial cultures were transferred to 2% MEA slants in McCartney bottles and stored at 4 °. All isolates are maintained in the Culture Collection of the Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representative isolates have also been deposited in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, and the National Collection of Fungi (PREM), Pretoria.

DNA extractions

The single conidial isolates (Table 1) were grown in liquid ME medium in 1.5 ml Eppendorf tubes for one week at 25 °. After centrifugation, the mycelium pellet was freeze dried and DNA was extracted using the technique described by Raeder & Broda (1985). The DNA concentrations of the samples were determined against a standard molecular marker and diluted to 5 ng μ l⁻¹ for further studies.

Amplification of partial protein-coding genes and microsatellite loci

The Bt2 regions of the β -tubulin gene (Glass & Donaldson 1995), parts of five other protein-coding genes (Carbone & Kohn 1999) and six microsatellite loci (Burgess *et al.* 2001a) were amplified from 14 isolates (Table 1). The 25 μ l reaction mixture consisted of 2.5 μ l Expand PCR buffer (2 mM Tris-HCl, pH 7.5; 1.5 mM MgCl₂; 10 mM KCl), 100 μ M of each dNTP, 300 nM of each primer, 2 ng DNA template and 0.25 U Expand HighTM Fidelity *Taq* polymerase (Roche Biochemicals). The following temperature profile was followed: 2 min at 94 °, 10 cycles of 30 s at 94 °, 45 s at 60 ° and 1 min at 72 °, the last three temperature intervals were repeated for another 30 cycles with a 5 s increase per cycle for the elongation step at 72 °.

Sequencing

PCR products were visualised on a 1% agarose gel containing ethidium bromide using UV illumination. The PCR products were purified using the Roche High Pure PCR product purification kit (Roche Diagnostics). Both DNA strands were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI PrismTM 377 DNA sequencer (Applied Biosystems, Warrington). All the reactions were done using protocols recommended by the manufacturers. Sequence data for all the isolates (Table 1) were processed using Sequence Navigator version 1.0.1 (Perkin Elmer) and manually aligned.

Phylogenetic analyses

Parsimony and distance analyses were performed on the individual data sets, as well as the combined data sets after partition homogeneity tests were performed on the individual protein-coding gene and microsatellite sequences using PAUP (Swofford 2002). A partition homogeneity test was also performed to test whether the protein-coding and microsatellite genealogies could be combined (Farris *et al.* 1994, Huelsenbeck, Bull & Cunningham 1996). In all cases, parsimony analyses were based on a strict heuristic search with a tree-bisection reconnection (TBR) branch swapping algorithm, stepwise addition and collapse of branches if maximum lengths was zero. Bootstrap values were determined after 10³ replications and only groups with frequencies > 50% were retained. Distances were determined using 'neighbour-joining' with an uncorrected 'p' parameter.

RESULTS

Amplification and sequencing of protein-coding genes and microsatellite loci

Portions of six protein-coding genes and six microsatellite loci were successfully amplified from *Sphaeropsis*

sapinea and *Botryosphaeria obtusa* isolates, while only protein-coding gene regions could be amplified from *B. rhodina* isolates. Sequences generated from the amplification products ranged from 170 to 565 bp in length. Introns occurring in the partial gene sequences of Bt2 of β -tubulin, EF-1 α , ACT, CAL and GPD and the sequences flanking the microsatellites were included in the phylogenetic analyses.

Phylogenetic analyses

Neighbour-joining distance phylograms were generated for each of the six protein-coding genes with bootstrap values (Fig. 1). The partition homogeneity test showed that no significant conflict exists between the phylogenies of the individual protein-coding genes ($P=0.01$). The individual sequences were consequently combined into one data set containing 2272 characters, of which 62 variable characters were parsimony informative, 238 were parsimony uninformative and the remainder were constant.

Neighbour-joining distance phylograms were also generated for each of the six microsatellite loci (Fig. 2). The partition homogeneity test on these data also showed that no significant conflict exists between the individual microsatellite phylogenies ($P=0.01$). They were thus combined into one data set containing 1783 characters, of which 146 variable characters were parsimony informative, 263 were parsimony uninformative and the remainder were constant.

The partition homogeneity test showed that significant conflict exists between the combined microsatellite and the combined protein-coding gene phylogenies ($P=0.26$) and that they could not be combined. Three distinct clades with bootstrap values higher than 50% emerged from the combined neighbour-joining distance phylogram, generated from the protein-coding gene sequences, as well as the microsatellite sequences (Fig. 3). One clade included all the A and C morphotype isolates of *S. sapinea*. These isolates were closely related but clearly distinguishable from each other. A second clade contained all of the B morphotype isolates. A third clade contained *B. obtusa* isolates together with isolates (CMW8230 and CMW8231), previously described as the I morphotype of *S. sapinea* (Hausner *et al.* 1999) and now known to represent *B. obtusa* (Burgess *et al.* 2001a). The clade containing the B morphotype isolates was equally distant from the clade encompassing the A and C morphotype isolates as it was from that including isolates of *B. obtusa*.

High levels of sequence similarity were observed for *S. sapinea* isolates representing the A and C morphotypes and no correlation to geographical distribution was observed for them. Isolates of the B morphotype encompassed a high degree of genetic diversity. Although a more thorough study with more isolates from a wider geographical distribution needs to be done, groupings of isolates according to geographical origin were starting to emerge. Based on the combined

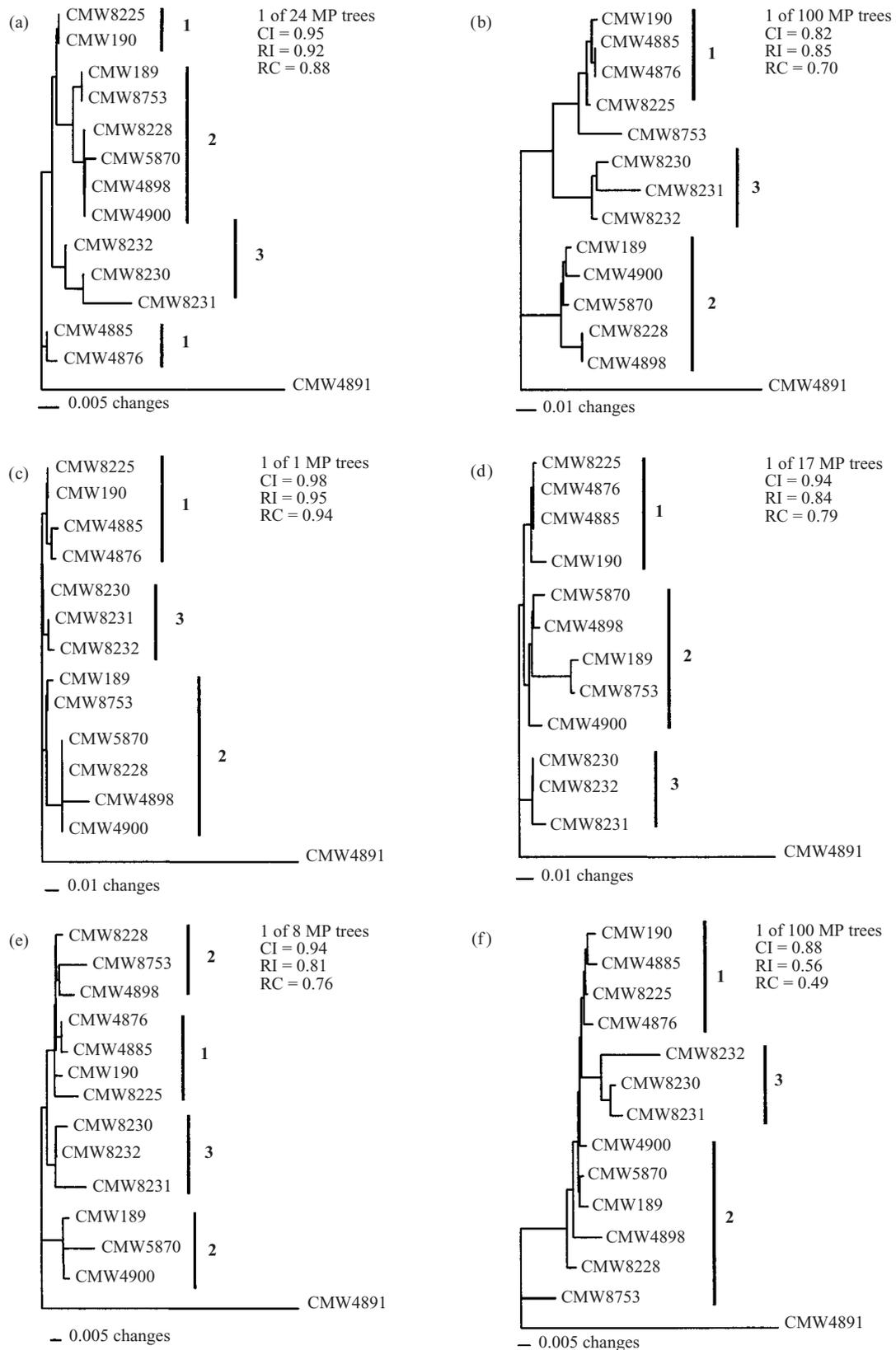


Fig. 1. Phenograms constructed for partial sequences of six protein coding genes, (a) Bt2 of the β -tubulin gene, (b) ACT, (c) EF-1 α , (d) CAL, (e) CHS, (f) GPD, using neighbour-joining distance analysis with an uncorrected ‘*p*’ parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 10^3 replications and only groups with frequencies > 50% were retained. Clade 1, *Sphaeropsis sapinea* (A and C morphotypes); Clade 2, *Diplodia scrobiculata*; and Clade 3, *Botryosphaeria obtusa*. MP, most parsimonious; CI, consistency index; RI, retention index; and RC, reconstructed consistency index.

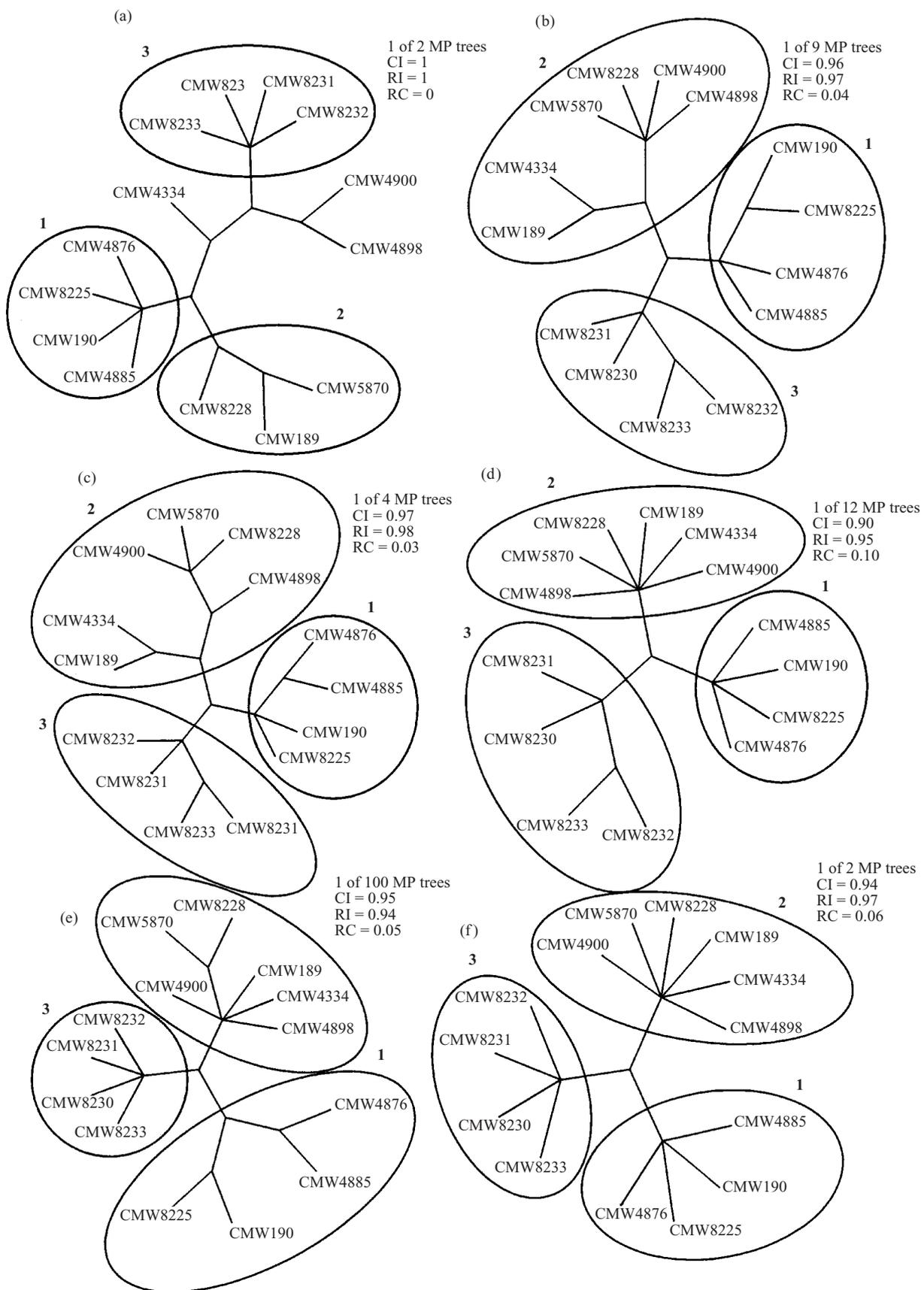


Fig. 2. Phenograms constructed for sequence data of six SSR loci, (a) SS5, (b) SS7, (c) SS8, (d) SS9, (e) SS10, (f) SS11, using neighbour-joining distance analysis with an uncorrected 'p' parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 10⁸ replications and only groups with frequencies > 50% were retained. Clade 1, *Sphaeropsis sapinea* (A and C morphotypes); Clade 2, *Diplodia scrobiculata*; and Clade 3, *Botryosphaeria obtusa*. MP, most parsimonious; CI, consistency index; RI, retention index; and RC, reconstructed consistency index.

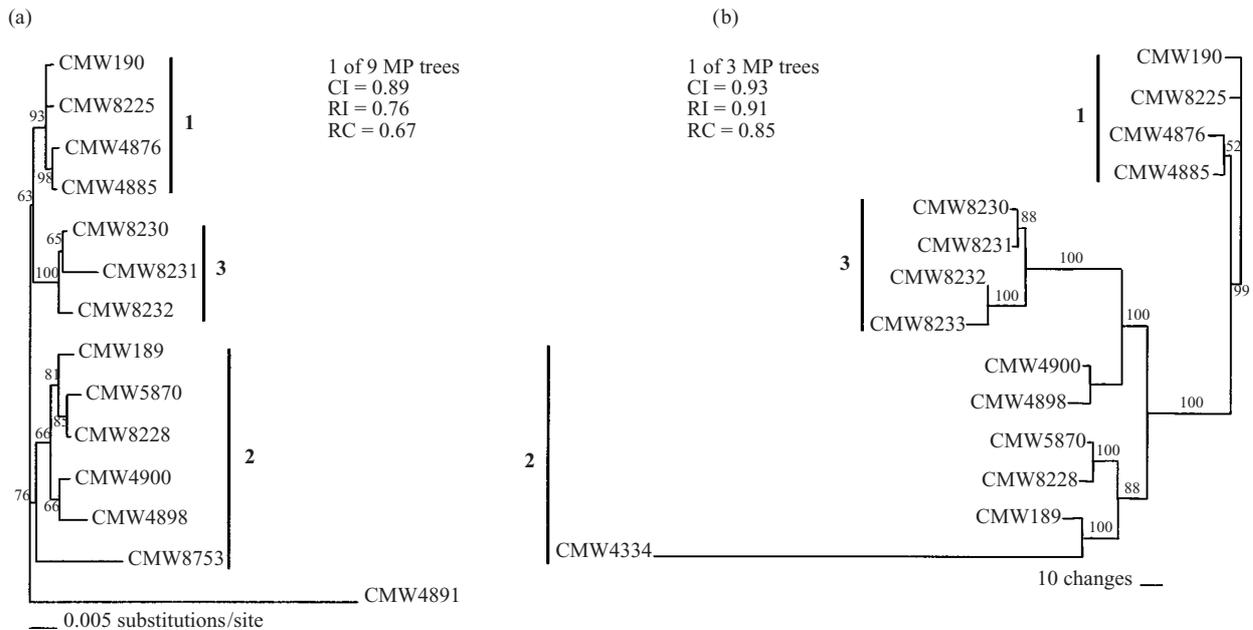


Fig. 3. (a) Phenogram constructed for combined sequences of the six protein-coding genes, (b) phenogram constructed for combined sequence data of six SSR loci using neighbour-joining distance analysis with an uncorrected 'p' parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 10^3 replications and only groups with frequencies $> 50\%$ were retained. Clade 1, *Sphaeropsis sapinea* (A and C morphotypes); Clade 2, *Diplodia scrobiculata*; and Clade 3, *Botryosphaeria obtusa*. MP, most parsimonious; CI, consistency index; RI, retention index; and RC, reconstructed consistency index.

protein-coding gene genealogy (Fig. 3), the B morphotype isolates from the universal United States (Central, West and Mexico) (CMW189, CMW5870, CMW8228, CMW4898, CMW4900) grouped separately from the single European B morphotype isolate (CMW8753). Furthermore, the combined microsatellite genealogy could differentiate the B morphotype isolates from the universal United States into three sub-clades, one from the Central US (CMW189, CMW4334), one from California (CMW5870, CMW8228) and one from Mexico (CMW4898, CMW4900).

TAXONOMY

The results of the phylogenetic comparisons presented in this study provide robust evidence to justify treating isolates of the B morphotype of *S. sapinea* as a discrete taxon. We, therefore, provide the following description for the fungus:

Diplodia scrobiculata J. de Wet, B. Slippers & M. J. Wingfield, *sp. nov.* (Figs 4–10)

Etym.: Latin, *scrobiculata* = minutely pitted, in reference to the texture of the conidial walls.

Culturae colonias supra submurinas vel murinas, infra atromurinas, marginibus sinuatis faciunt. Coloniae creverunt optime ad $25\text{ }^{\circ}\text{C}$, et superficiem medii in 8 diebus velabant. Mycelium atratum septatum ad agarum appressum. *Conidiomata* in foliis pinorum pycnidialia, mycelio oblecta. Pycnidia atro-vinaceo-brunnea in foliis pinorum vel in agaro immersa, $150\text{ }\mu\text{m}$ diam *Cellulae conidiogenae* holoblasticae,

proliferatione percurrenti limitata, ut videtur annellationibus paucis, $10\text{ }\mu\text{m}$ diam *Conidia* clavata vel truncata, 1–3 septata, parietibus crassis, scrobiculatis, atrovinea vel atrobrunnea, $39.5 \times 14\text{ }\mu\text{m}$.

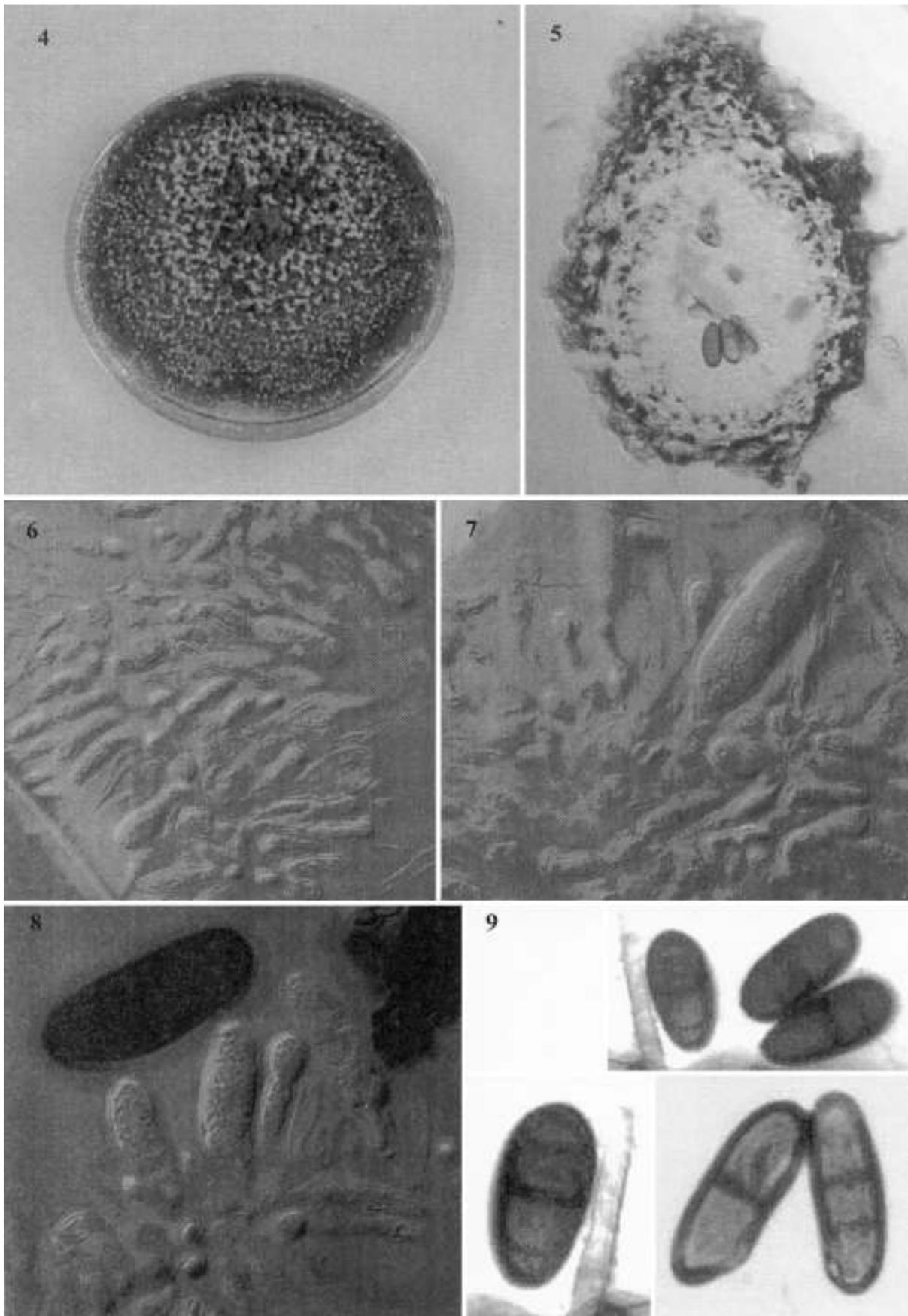
Typus: USA: Wisconsin: Jackson County, *Pinus banksiana*. 1987, M. A. Palmer CMW189 (PREM 57461 – holotypus).

Cultures (Fig. 4) pale mouse grey ($15^{\text{''''}}\text{d}$) to mouse grey ($15^{\text{''''}}$) viewed from the top of the Petri dish, dark mouse grey ($15^{\text{''''}}\text{k}$) to fuscous black ($13^{\text{''''}}\text{m}$) viewed from the bottom of the Petri dish, colonies with sinuate edges; optimal growth at 25 ° , covering the medium surface (9 cm Petri dishes) in 8 d. *Mycelium* dark, septate, appressed to the agar surface. *Conidiomata* (Fig. 5) pycnidial, covered in mycelium, dark, immersed in pine needles or in the agar, (100–)150 (–250) μm diam, single, papillate ostiole. *Conidiogenous cells* (Figs 6–7) discrete, dark, smooth, $10\text{ }\mu\text{m}$ in diameter, holoblastic with limited percurrent proliferation seen as small numbers of annellations. *Conidia* (Figs 8–9) clavate to truncate, dark mouse grey ($15^{\text{''''}}\text{k}$), (37.5–)39.5 (–41.5) μm (13–)14 (–15.5) μm , 1–3 septa, thick, pitted walls (Wang *et al.* 1985, Wang *et al.* 1986).

Substratum: Needles of *Pinus banksiana*, *P. resinosa*, and *P. greggii*.

Distribution: USA (Wisconsin, Minnesota, California), Mexico, and Europe (France, Italy).

Other specimens examined: USA: Minnesota: Wadena County, *Pinus resinosa*, 1987, G. R. Stanosz CMW4334. California: *Pinus radiata*, 2000, T. Gordon CMW5870, CMW8228. Mexico: *Pinus greggii*, 1998, M. J. Wingfield CMW4898, CMW4900. [All in PREM57462, PREM57463, PREM57464, PREM57465, PREM57466.]



Figs 4–9. *Diplodia scrobiculata* (holotype). **Fig. 4.** Colony characteristics on malt extract agar. **Fig. 5.** Section through pycnidium with conidia. **Figs 6–8.** Conidiophores with conidiogenous cells. **Fig. 9.** Conidia with up to three septa.

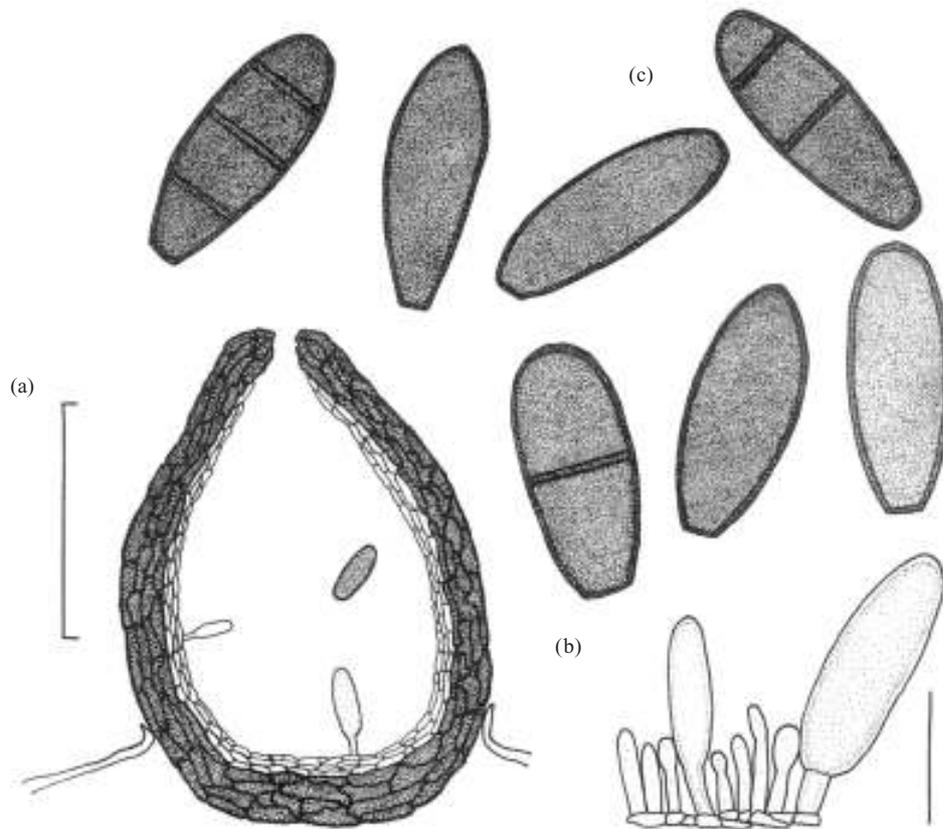


Fig. 10. *Diplodia scrobiculata* (holotype). (a) Pycnidium; (b) conidiogenous cells; and (c) conidia. Bars (a) = 100 μm ; (b)–(c) = 20 μm .

DISCUSSION

Multiple gene genealogies constructed from six protein-coding gene regions and six microsatellite-rich loci provide robust evidence that the B morphotype isolates of *Sphaeropsis sapinea* represent a distinct species. We have thus provided the name *Diplodia scrobiculata* for this fungus. Our results also reinforce those of Zhou *et al.* (2001) using dominant ISSR markers, and Burgess *et al.* (2001a) using co-dominant SSR markers, suggesting the A and B morphotypes of *S. sapinea* represent distinct taxa.

The construction of multiple gene genealogies, supported by distinct morphological differences, has enabled us to infer reliable and consistent phylogenetic relationships between the morphotypes of *S. sapinea s. lat.* We found that isolates of the A and C morphotypes are much more closely related to each other than they are to *D. scrobiculata*. *D. scrobiculata* isolates were equally distant from those of the A and C morphotypes of *S. sapinea* as they were from isolates of *Botryosphaeria obtusa*. Phylogenetic relationships inferred from these gene genealogies corroborate results obtained using SSR markers, based on sizes (Burgess *et al.* 2001a). Therefore, in this case SSR markers alone would have been adequate to infer species level relationships, even though initial empirical studies have suggested otherwise (Fisher *et al.* 2000).

Botryosphaeria spp. are very similar based on their teleomorph characteristics but their anamorph characteristics are very diverse and reside in a number of anamorphs genera. Recent morphological and molecular data suggested that these anamorphs can be divided into two main groups (Jacobs & Rehner 1988, Crous & Palm 1999, Denman *et al.* 2000, Zhou & Stanosz 2001). The first group have ellipsoid, thick-walled conidia that septate regularly and darken with age, and are characterised by the genus *Diplodia*. The second group have fusoid, thin-walled conidia that septate and darken less often with age, and are characterised by the genus *Fusicoccum*. The new species described here is concurrent with *Diplodia* group described by the above authors, and is therefore described in this genus. Based on the above evidence, we agree with the argument of Denman *et al.* (2000), that percurrent proliferation and time of septation of *S. sapinea*, that is considered characteristic, is not sufficient to separate it from the genus *Diplodia*. We, therefore, suggest reverting to the older name *D. pinea* for this taxon.

No sexual state is known for any form of *S. sapinea*, although, together with *D. scrobiculata*, molecular evidence (Burgess *et al.* 2001a) shows that it clearly represents an anamorph of *Botryosphaeria*. Burgess, Wingfield & Wingfield (2001b), have also shown that the A morphotype isolates representing *S. sapinea sensu stricto* are overwhelmingly clonal. *D. scrobiculata*

isolates occasionally produce spermatia-like spores (Palmer *et al.* 1987), suggesting the presence of a sexual state in this fungus. Recent studies using SSR markers have shown a considerably higher degree of genetic diversity amongst isolates of *D. scrobiculata* than those of the A and C morphotypes of *S. sapinea* (Burgess, unpubl.). *D. scrobiculata* could represent a recently derived lineage of *Botryosphaeria*, which has only recently lost its ability to reproduce sexually. Alternatively, sexual reproduction in this fungus may possibly be suppressed by unfavourable conditions such as those in culture and sexual structures may yet be found in nature. In contrast, we believe the A and C morphotypes of *S. sapinea* represent ancient lineages that have stabilised over time and have acquired a virtually clonal existence.

S. sapinea, as reflected by the A and C morphotypes of this fungus, appears to be native to and widely distributed across the natural range of *Pinus* species. The two morphotypes that represent this species differ in their distribution, host specificity and virulence. The A morphotype is common and has a wide distribution in Southern hemisphere countries including South Africa, Australia and New Zealand, where it was probably introduced together with pine seed imports (Swart *et al.* 1991, Burgess & Wingfield 2001). The C morphotype of *S. sapinea* has, thus far, been found only on *Pinus* spp. in Indonesia and isolates are significantly more virulent than those of the A morphotype (de Wet *et al.* 2002). *D. scrobiculata* has a much more restricted distribution. The fungus was initially known only on *Pinus banksiana* and *Pinus resinosa* in the north central United States (Wang *et al.* 1985, Palmer *et al.* 1987), but has recently been reported from other conifers in Europe (Stanosz, Swart & Smith 1999). There is no conclusive evidence to show that it has been introduced into pine-growing areas of the southern hemisphere.

The wide array of phylogenetic comparisons presented in this study provide robust evidence to support the recognition of *D. scrobiculata* (formerly *S. sapinea* B morphotype) as a distinct species. This is also supported by the results of other molecular genetic comparisons (Zhou *et al.* 2001, Burgess *et al.* 2001a), as well as useful morphological and ecological data previously published (Wang *et al.* 1985, Palmer *et al.* 1987, de Wet *et al.* 2002). Isolates of *D. scrobiculata* are characterized by dark, septate mycelium appressed to the surface of the agar. This is consistently different to *S. sapinea* isolates that have fluffy, aerial mycelium. Conidia of *D. scrobiculata* are dark brown with thick, pitted walls and 1–3 septa (Wang *et al.* 1985, Wang *et al.* 1986, Palmer *et al.* 1987). Conidiogenous cells are holoblastic with annelidic proliferations and based on this characteristic, *D. scrobiculata* and *S. sapinea* are apparently indistinguishable.

S. sapinea was one of the earliest fungi to be recognised as a common inhabitant of *Pinus* spp. (Fisher 1912). It is also one of the best-known pathogens of *Pinus* spp. grown as exotics in the tropics and southern

hemisphere (Burgess & Wingfield 2001). Thus, the discovery of taxonomically and ecologically meaningful differences in isolates of *S. sapinea* in the north central United States in the late 1980's, was relatively recent. During the past 15 years, substantial evidence has accumulated to show that these differences reflect both inter- and intraspecies variation. While the description of *D. scrobiculata* represents an important step in this process, the fungus is probably not of particular relevance in terms of pathology. *D. scrobiculata* is known to be a very weak pathogen (Palmer *et al.* 1987) and it is probably best recognised as a relatively harmless endophyte. This is in contrast to the A and C morphotypes of *S. sapinea* that are important pathogens whose movement should be carefully managed.

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Corresponding Editor: S. J. Assinder