
CHAPTER 2

MULTIPLE GENE GENEALOGIES AND MICROSATELLITE MARKERS REFLECT RELATIONSHIPS BETWEEN MORPHOTYPES OF *SPHAEROPSIS SAPINEA* AND DISTINGUISH A NEW SPECIES OF *DIPLODIA*

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ABSTRACT

Sphaeropsis sapinea is an opportunistic pathogen causing serious damage to conifers, predisposed 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 both analyses, 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*.

INTRODUCTION

Sphaeropsis sapinea (Fr.) Dyko & Sutton (=*Diplodia pinea* (Desm.) Kickx.) 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). *Sphaeropsis sapinea* causes extensive losses in commercial plantation forestry, especially where susceptible *Pinus* spp. are intensively propagated (Zwolinski *et al*. 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 *et al*. 1986; Palmer *et al*. 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* has been described as being intermediate between the A and B morphotypes (Hausner *et al*. 1999), but subsequent studies based on SSR markers (Burgess *et al*. 2001a) showed that this fungus represents the anamorph state of *Botryosphaeria obtusa* (Schw.) Shoemaker.

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, ISSR (inter simple or short sequence repeats) fingerprinting and SSR (simple sequence repeats) markers have also been used to provide increased resolution to the differentiation between these morphotypes (Burgess *et al*. 2001a; Zhou *et al*. 2001). 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 combined with highly polymorphic microsatellite loci (Geiser *et al*. 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 1α [EF-1α], 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 *S. sapinea* isolates (Table 1) from the United States, Australia, Mexico, California and Indonesia were used in this study. These isolates represented all three morphotypes described for *S. sapinea*. Four isolates of the closely-related species *B. obtusa*, were included for comparison and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (*B. rhodina* (Cooke) Arx) 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 ºC. All the single conidial cultures were transferred to 2 % MEA slants in McCartney bottles and stored at 4 ºC. 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, Netherlands and the National Collection of Fungi (PREM), Pretoria, South Africa.

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 °C. 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/µl 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 for 14 isolates (Table 1). The 25 µl reaction mixture consisted of 2.5 µl Expand PCR buffer (2 mM Tris-HCl, pH 7.5; 1.5 mM $MgCl_2$; 10 mM KCl), 100 µM of each dNTP, 300 nM of each primer, 2 ng template and 0.25 U Expand High^{TM} Fidelity *Taq* polymerase (Roche Biochemicals). The following temperature profile was followed: 2 min at 94 °C, 10 cycles of 30 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C, the last three temperature intervals were repeated for another 30 cycles with a 5 s increase per cycle for the elongation step at 72 °C.

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 Prism^{TM} 377 DNA sequencer (Applied Biosystems, Warrington WA1 4SR, UK). 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 (Smithsonian Institution, 1993). A partition homogeneity test was also performed to test whether the protein-coding and microsatellite genealogies could be combined. 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 length was zero. Bootstrap values were determined after 1000 replications and only groups with frequencies >50 % were retained. Distances were determined using "neigbour-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 for *S. sapinea* and *B. obtusa*, while only protein-coding gene regions could be amplified for *B. rhodina* isolates. Sequences generated from the amplification products ranged from 170 – 565 bp in length. Introns occurring in the partial gene sequences of Bt2 of β-tubulin, EF-1α, 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 were observed for them. Isolates of the B morphotype encompassed a high degree of genetic diversity and groupings according to geographical origin were observed. Based on the protein-coding gene

genealogy, the B morphotype isolates from the United States (CMW189, CMW5870, CMW8228, CMW4898, CMW4900) grouped separately from the single European B morphotype isolate (CMW8753). The microsatellite genealogy could, furthermore, differentiate the B morphotype isolates from the 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 anam. 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 25º, et superficiem medii in 8 diebus velabant. Mycelium atratum septatum ad agarum appressum. *Conidiomata* in foliis pinorum pycnidialia, mycelio obtecta. Pycnidia atro-vinaceo-brunnea in foliis pinorum vel in agaro immersa, 150 Φm diametro. *Cellulae conidiogenae* holoblasticae, proliferatione percurrenti limitata, ut videtur annellationibus paucis, 10 Φm diametro. *Conidia* clavata vel truncata, 1–3 septata, parietibus crassis, scrobiculatis, atrovinacea vel atrobrunnea, 39.4 H 14.1 Φm.

Cultures (Fig 4) pale mouse grey(15""d) to mouse grey (15"") viewed from the top of the Petri dish, dark mouse grey (15""k) to fuscous black (13""m) viewed from the bottom of the Petri dish, colonies with sinuate edges; optimal growth at 25°C, covering the medium surface in eight days. *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 diameter, single, papillate ostiole. *Conidiogenous cells* (Figs 6-7) discrete, dark, smooth, 10 µm in diameter, holoblastic with limited percurrent proliferation seen as small numbers of annellations. *Conidia* (Figs 8-9) clavate to truncate, dark mouse grey (15""k), (37.4)39.4(41.3) µm (12.8)14.1(15.5) µm, 1-3 septa, thick, pitted walls (Wang *et al*. 1986; Wang *et al*. 1985).

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

Distribution: USA: Wisconsin, Minnesota, California; Mexico; Europe: France, Italy.

Holotype: **USA:** Wisconsin: Jackson County, *Pinus banksiana*. 1987, M.A. Palmer, (CMW189) in Herb. PREM57461.

Paratypes: **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/CBS117836); all in Herb. PREM57462, PREM57463, PREM57464, PREM57465, PREM57466.

DISCUSSION

Using multiple gene genealogies constructed from six protein-coding gene regions and six microsatellite-rich loci, we have been able to provide robust evidence showing that the B morphotype isolates of *S. sapinea* represent a distinct species. We have thus provided the name *D. scrobiculata* to 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 has enabled us to infer reliable and consistent phylogenetic relationships between the morphotypes of *S. sapinea*. We found that isolates of the A and C morphotypes are much more closely related to each other, than they are to *D. scrobiculata*. *Diplodia scrobiculata* isolates were equally distant from those of the A and C

morphotypes of *S. sapinea*, as they were from isolates of *B. 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 difficult to distinguish based on their teleomorph morphology, but they can more easily be divided into two groups using anamorph characteristics. These represent a group with dark-spored conidia, best treated in the genus *Diplodia* and a group with predominantly hyaline conidia residing in *Fusicoccum* (Denman *et al*. 2000). *Sphaeropsis sapinea* closely resembles the *Diplodia*-anamorphs of *Botryosphaeria* spp. (Denman *et al*. 2000; Jacobs & Rehner 1998) and was segregated from *Diplodia* based primarily on characteristics of conidial development (Sutton 1980). Phylogenetic data derived from this study provide substantial additional evidence to justify reverting to the name *Diplodia pinea* and in future, we recommend doing so.

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 the A morphotype isolates representing *S. sapinea sensu stricto* are overwhelmingly clonal. *Diplodia 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 unpublished). *Diplodia 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.

Sphaeropsis 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 (Burgess & Wingfield 2001; Swart *et al*. 1991). 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). *Diplodia scrobiculata* has a much more restricted distribution. The fungus was initially known only on *Pinus banksiana* and *P. 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 *et al*. 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 description of *D. scrobiculata*. This is also supported by the results of other molecular genetic comparisons (Burgess *et al*. 2001a; Zhou *et al*. 2001), 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.

Sphaeropsis 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. *Diplodia 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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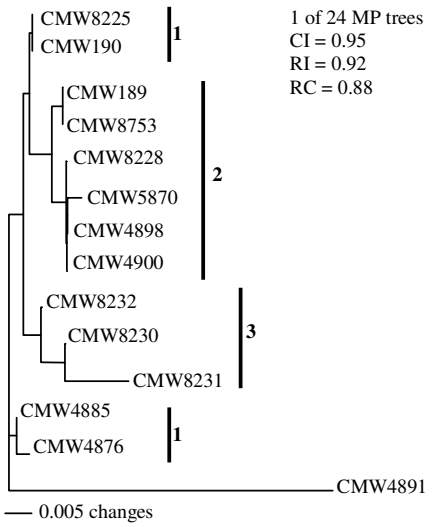
Table 1. Isolates used in this study.

Isolates[a/]	Type/Species[b/]	Origin	Host	Collector	Other collections[c/]	References
CMW8225	A	Australia	*Pinus radiata*	T. Burgess		
CMW190	A	United States	*P. resinosa*	M.A. Palmer		
CMW4885	C	Indonesia	*P. patula*	M.J. Wingfield		
CMW4876	C	″	″	″		
CMW5870	B	California	*P. radiata*	T. Gordon		
CMW8228	B	″	″	″		
CMW4898	B	Mexico	*P. greggii*	M.J. Wingfield		
CMW4900	B	″	″	″		
CMW189	B	United States	*P. banksiana*	M.A. Palmer	124	Palmer *et al*. 1987
*CMW4334	B	″	*P. resinosa*	G.R. Stanosz	474	Blodgett & Stanosz 1999
^CMW8753	B	Italy	*Pinus. sp.*	L. Sparapano	97-73	Stanosz *et al*. 1999
CMW8230	*B. obtusa*	Canada	*Picea glauca*	J. Reid	920729	Hausner *et al*. 1999
CMW8231	″	″	*P. banksiana*	″	810704	″
CMW8232	″	South Africa	*Malus domestica*	W.A. Smit		
*CMW8233	″	″	″	″		
^CMW4891	*Lasiodiplodia theobromae*	Indonesia	*P. patula*	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 for *D. pinea* is based on morphotype descriptions provided by Palmer *et al*. 1987; De Wet *et al*. 2000. [c/]Isolation numbers used in previous studies for which references are provided in the last column.

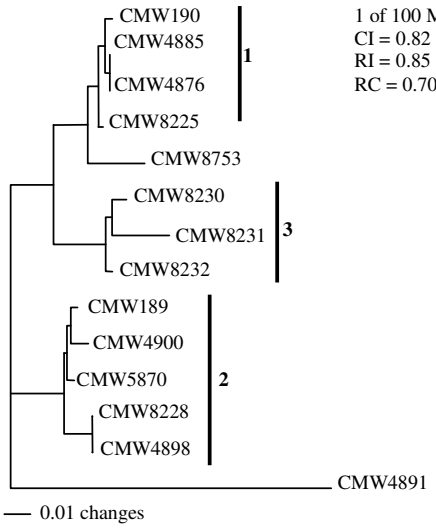
Figure 1. Phenograms constructed for the partial sequences of six protein coding genes (a) Bt2 of the β-tubulin gene; (b) ACT; (c) EF-1α; (d) CAL; (e) CHS; (f) GPD using neighbour-joining distance analysis with an uncorrected "p" parameter and parsimony based on a strict heuristic search to determine bootstrap values. Bootstrap values were determined after 1000 replications and only groups with frequencies >50% were retained. Clade 1 = *Sphaeropsis sapinea* (A and C morphotypes), Clade 2 = *Diplodia scrobiculata*, Clade 3 = *Botryosphaeria obtusa*. MP = most parsimonious, CI = consistency index, RI = retension index, RC = reconstructed consistency index.

(a)



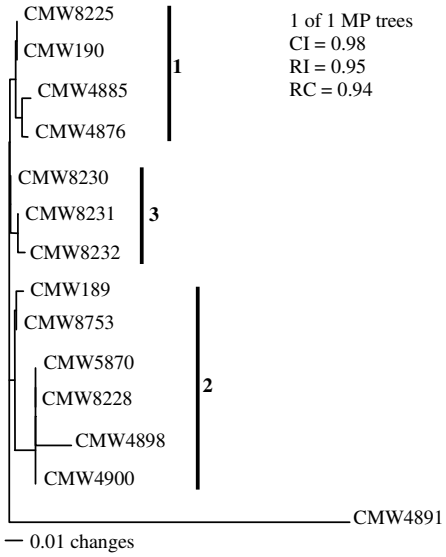
1 of 24 MP trees
CI = 0.95
RI = 0.92
RC = 0.88

(b)



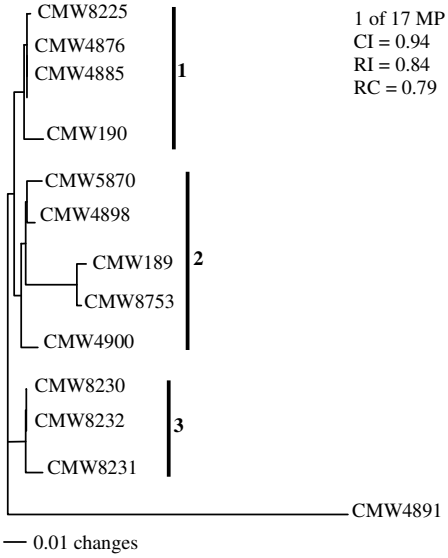
1 of 100 MP trees
CI = 0.82
RI = 0.85
RC = 0.70

(c)



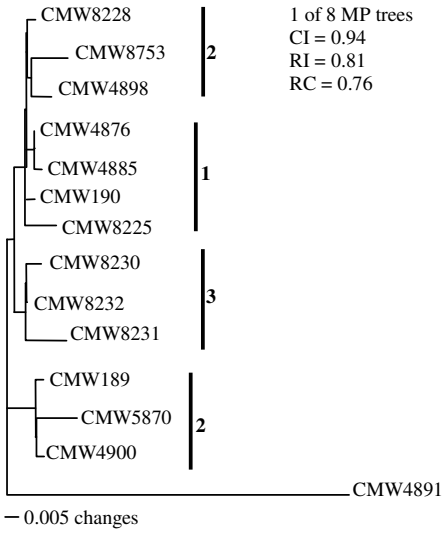
1 of 1 MP trees
CI = 0.98
RI = 0.95
RC = 0.94

(d)



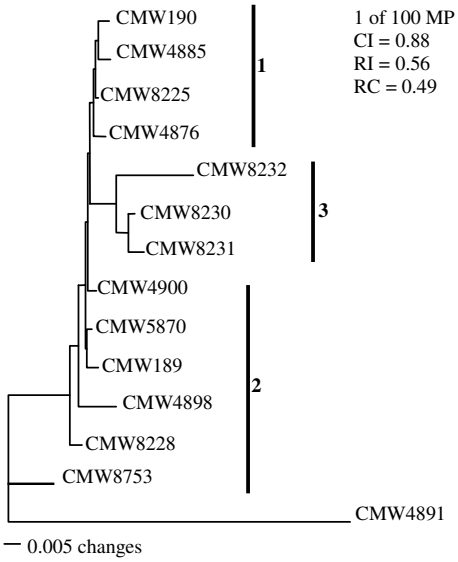
1 of 17 MP trees
CI = 0.94
RI = 0.84
RC = 0.79

(e)



1 of 8 MP trees
CI = 0.94
RI = 0.81
RC = 0.76

(f)



1 of 100 MP trees
CI = 0.88
RI = 0.56
RC = 0.49

Figure 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 1000 replications and only groups with frequencies >50% were retained. Clade 1 = *Sphaeropsis sapinea* (A and C morphotypes), Clade 2 = *Diplodia scrobiculata*, Clade 3 = *Botryosphaeria obtusa*. MP = most parsimonious, CI = consistency index, RI = retension index, RC = reconstructed consistency index.

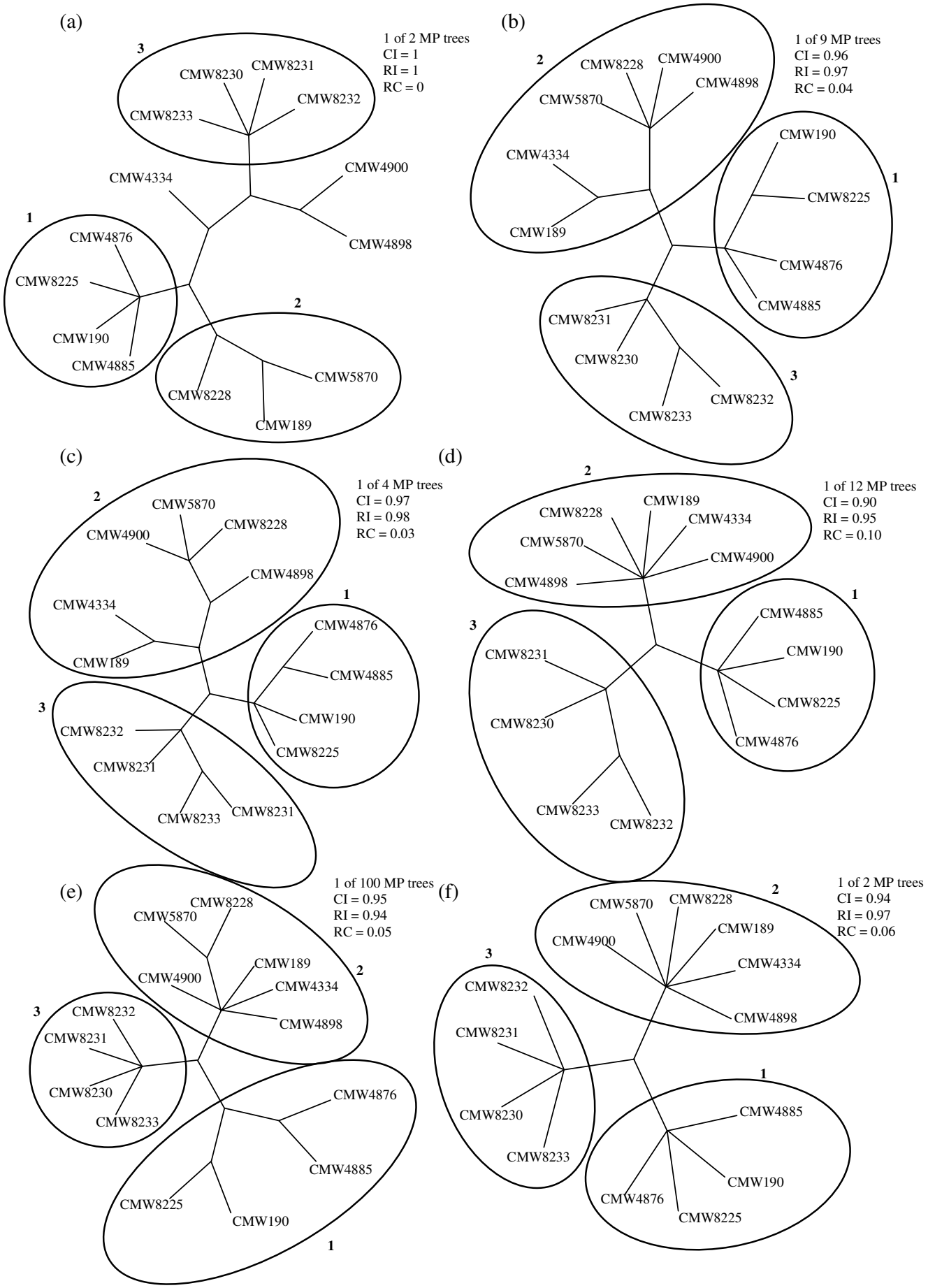
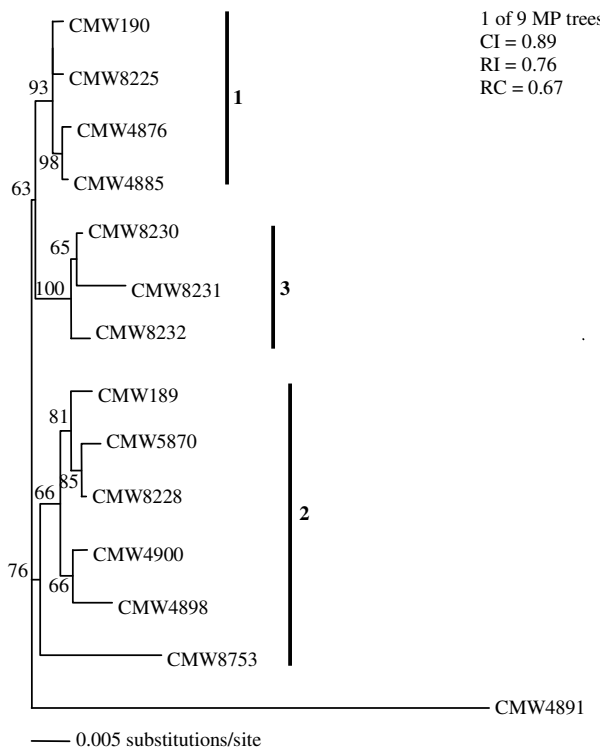
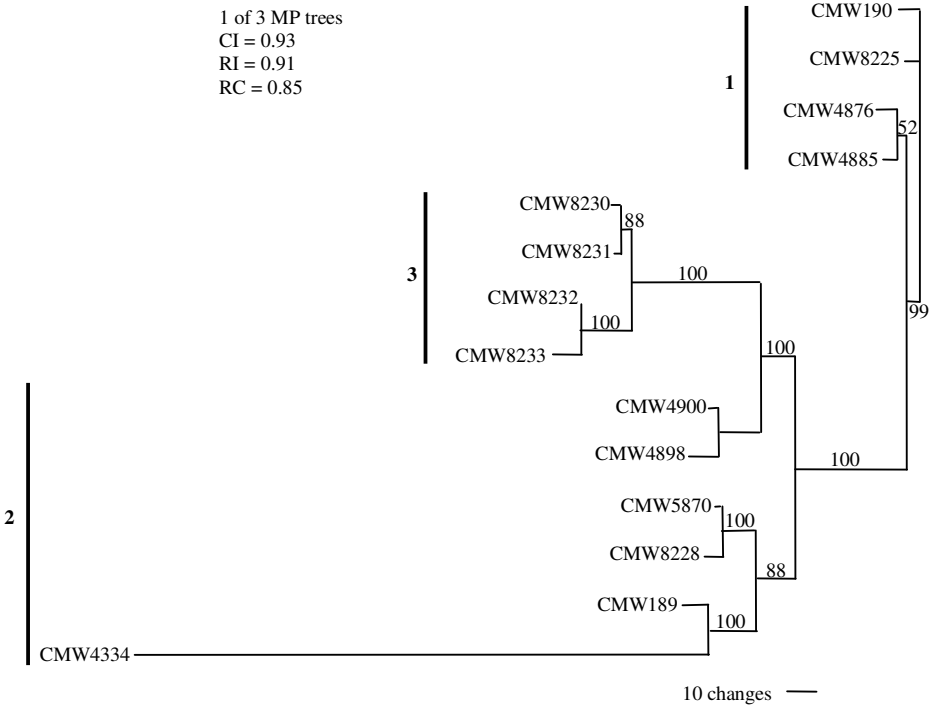

(a)
1 of 2 MP trees
CI = 1
RI = 1
RC = 0
3
CMW8230
CMW8231
CMW8232
CMW8233
CMW4900
CMW4334
CMW4898
1
CMW4876
CMW8225
CMW190
CMW4885
2
CMW5870
CMW8228
CMW189
(b)
1 of 9 MP trees
CI = 0.96
RI = 0.97
RC = 0.04
2
CMW8228
CMW4900
CMW4898
CMW5870
CMW4334
CMW189
CMW190
1
CMW8225
CMW4876
CMW4885
CMW8231
CMW8230
3
CMW8232
CMW8233
(c)
1 of 4 MP trees
CI = 0.97
RI = 0.98
RC = 0.03
2
CMW5870
CMW8228
CMW4900
CMW4898
CMW4334
CMW189
1
CMW4876
CMW4885
CMW190
CMW8225
3
CMW8232
CMW8231
CMW8233
CMW8231
(d)
1 of 12 MP trees
CI = 0.90
RI = 0.95
RC = 0.10
2
CMW8228
CMW189
CMW4334
CMW5870
CMW4900
CMW4898
1
CMW4885
CMW190
CMW8225
CMW4876
3
CMW8231
CMW8230
CMW8233
CMW8232
(e)
1 of 100 MP trees
CI = 0.95
RI = 0.94
RC = 0.05
CMW8228
CMW5870
CMW189
CMW4900
CMW4334
CMW4898
2
3
CMW8232
CMW8231
CMW8230
CMW8233
CMW4876
CMW4885
CMW8225
CMW190
1
(f)
1 of 2 MP trees
CI = 0.94
RI = 0.97
RC = 0.06
CMW5870
CMW8228
2
CMW189
CMW4900
CMW4334
CMW4898
3
CMW8232
CMW8231
CMW8230
CMW8233
1
CMW4885
CMW190
CMW4876
CMW8225

Figure 3. (a) Phenogram constructed for the combined sequences of the six protein-coding genes (b) phenogram constructed for the 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 1000 replications and only groups with frequencies >50% were retained. Clade 1 = *Sphaeropsis sapinea* (A and C morphotypes), Clade 2 = *Diplodia scrobiculata*, Clade 3 = *Botryosphaeria obtusa*. MP = most parsimonious, CI = consistency index, RI = retension index, RC = reconstructed consistency index.

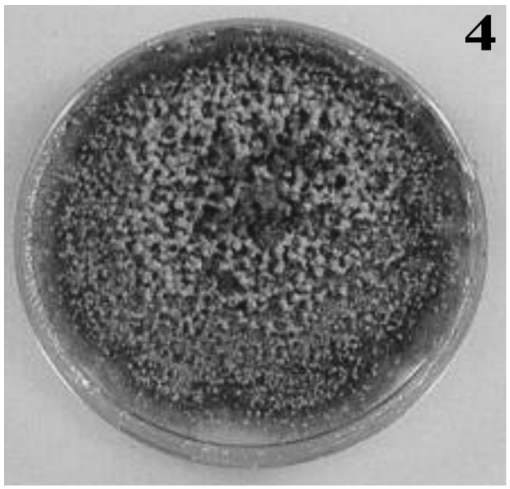
(a)


1 of 9 MP trees
CI = 0.89
RI = 0.76
RC = 0.67
CMW190
CMW8225
CMW4876
CMW4885
CMW8230
CMW8231
CMW8232
CMW189
CMW5870
CMW8228
CMW4900
CMW4898
CMW8753
CMW4891
1
2
3
93
98
63
65
100
81
85
66
76
66
0.005 substitutions/site

(b)


1 of 3 MP trees
CI = 0.93
RI = 0.91
RC = 0.85
CMW190
CMW8225
CMW4876
CMW4885
CMW8230
CMW8231
CMW8232
CMW8233
CMW4900
CMW4898
CMW5870
CMW8228
CMW189
CMW4334
1
2
3
52
88
100
100
99
100
100
88
100
10 changes

Figures 4-9. *Diplodia scrobiculata*. **Fig. 4.** Colony characteristics on malt extract agar. **Fig. 5.** Section through pycnidium with conidia. **Fig. 6-8.** Conidiophores with conidiogenous cells. **Fig. 9.** Conidia with up to three septa, scale bar = 10 µm.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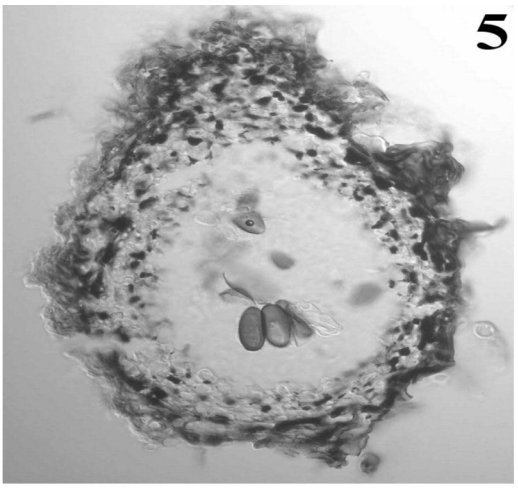
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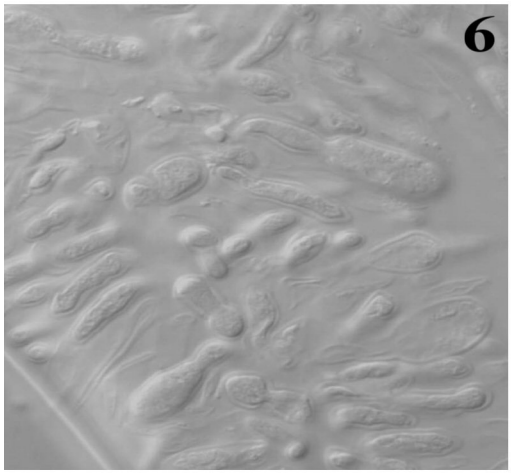
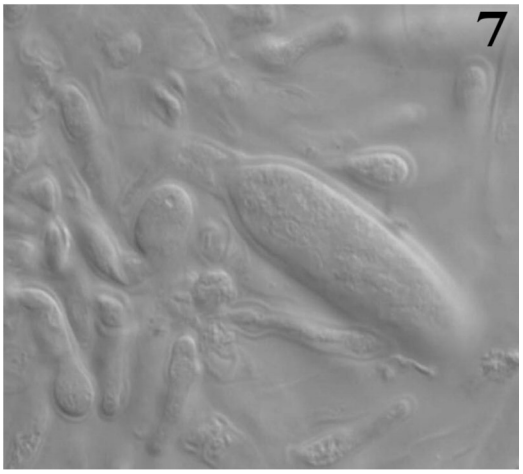

6


7


8

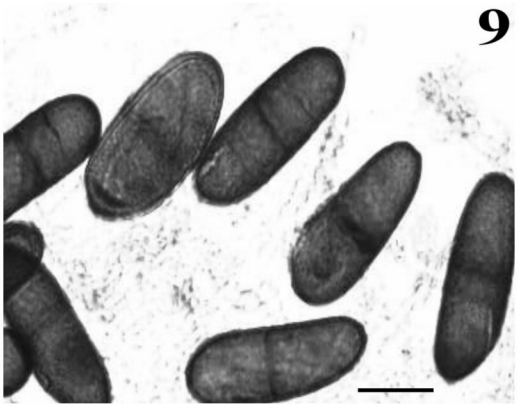

9

Figure 10. Illustration of (a) pycnidium, scale bar = 100 μm, (b) conidiogenous cells and (c) conidia, scale bar = 20 μm.