Chapter 1

Botryosphaeriaceae occurring on native *Syzygium cordatum* in South Africa and their potential threat to *Eucalyptus*

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ABSTRACT

Eight species of the Botryosphaeriaceae (canker and dieback pathogens) were identified on native *S. cordatum* in South Africa, based on anamorph morphology, ITS rDNA sequence data and PCR-RFLP analysis. The species identified were *Neofusicoccum parvum, N. ribis, N. luteum, N. australe, N. mangiferae*, *Botryosphaeria dothidea, Lasiodiplodia gonubiensis* and *L. theobromae*. Their pathogenicity on *S. cordatum* seedlings and a *Eucalyptus grandis* × *camaldulensis* clone was determined in glasshouse inoculation trials. Isolates of all identified species, except one of *N. mangiferae* were more pathogenic on the *Eucalyptus* clone than on *S. cordatum*. Some of the species that cross-infected these hosts, such as *N. ribis, N. parvum* and *L. theobromae*, were amongst the most pathogenic on the *Eucalyptus* clone, while *B. dothidea* and *L. gonubiensis* were the least pathogenic. Results of this study illustrate that species of the Botryosphaeriaceae from native hosts could pose a threat to introduced *Eucalyptus* spp., and *vice versa.*

INTRODUCTION

The Botryosphaeriaceae (Dothideales) is comprised of fungal species that have a wide geographic distribution and extensive host range, including *Eucalyptus* spp. (Myrtaceae) (von Arx and Müller 1954, Crous et al 2006). These fungi are latent and opportunistic pathogens that occur as endophytes in symptomless plant tissues and they can cause rapid disease development when plants are exposed to unsuitable environmental conditions such as drought, freezing, hot or cold winds, hail wounds or damage caused by insects or other pathogens (Fisher et al 1993, Smith et al 1996). Species of the Botryosphaeriaceae cause a wide variety of symptoms on all parts of *Eucalyptus* trees and on trees of all ages, but are mostly associated with cankers and dieback followed by extensive production of kino, a dark-red tree sap, and in severe cases mortality of trees (Smith et al 1994, 1996, Old and Davison 2000).

The Myrtaceae is a predominantly southern hemisphere angiosperm family that accommodates more than 3000 species, largely distributed in the tropical and temperate regions of Australasia, as well as Central and South America (Johnson and Briggs 1981). Species of the Myrtaceae also form an integral part of the southern African indigenous flora (Palgrave 1977). In this context, the most widespread myrtaceous tree in South Africa is *Syzygium cordatum* Hochst. (Palgrave 1977). *Eucalyptus* species, native Australasian Myrtaceae, are the most widely grown trees in commercial forestry plantations, particularly in the tropics and southern hemisphere, including South Africa.

Movement of pathogens between native and introduced hosts has been recognised as a significant threat to plant communities (Slippers et al 2005b). Because of the potential threat of native pathogens to non-native *Eucalyptus* plantations, various recent studies have considered fungal pathogens on native hosts in areas where *Eucalyptus* spp. are intensively planted (Wingfield et al 2003, Burgess et al 2006). These studies showed that pathogens that can cause severe diseases on *Eucalyptus* spp. also occur on native plants and thus pose a threat to *Eucalyptus* spp. Where plantations of non-native *Eucalyptus* spp. are established amongst closely related native myrtaceous trees, pathogens could cross-infect either the native or introduced host group and cause serious diseases (Burgess and Wingfield 2001). For example, the rust fungus *Puccinia psidii* G. Winter, which occurs on a variety of native Myrtaceae in South America, has become one of the main pathogens on exotic *Eucalyptus* spp. in that area (Coutinho et al 1998).

In South Africa, species of the Botryosphaeriaceae are amongst the most important canker pathogens in plantations of non-native *Eucalyptus* spp., causing twig dieback, branch and stem cankers and mortality of diseased trees (Smith et al 1994). These fungi have also been reported recently as endophytes from native South African trees closely related to *Eucalyptus*, such as *S. cordatum* and *Heteropyxis natalensis* (Smith et al 2001). The *Eucalyptus* plantations mostly occur in the eastern part of the country where *S. cordatum* is widely distributed (Palgrave 1977, Anonymous 2002, FIG. 1). Thus, Botryosphaeriaceae that occur on this native tree could pose a threat to exotic *Eucalyptus* and *vice versa*. However, there have not been any detailed studies on Botryosphaeriaceae on native hosts closely related to *Eucalyptus* in South Africa. Because of the economic importance of *Eucalyptus* plantations, as well as the need to protect native flora, identification and characterization of Botryosphaeriaceae from *S. cordatum* is of great concern.

Recent studies combined morphological characteristics and DNA sequence data to distinguish and identify species within the Botryosphaeriaceae (Denman et al 2000, Zhou and Stanosz 2001, Crous et al 2006). Molecular approaches most commonly used to study Botryosphaeriaceae are comparisons of sequence data from internal transcribed spacer (ITS) gene region of the rDNA operon (Denman et al 2000, Zhou and Stanosz 2001). However, some closely related or cryptic species of the Botryosphaeriaceae have been difficult to distinguish based on single gene genealogies. Comparisons of sequence data for multiple genes or gene regions were thus used to discriminate between these species (Slippers et al 2004a, c). Furthermore, identification of large numbers of species has been facilitated by PCR restriction fragment length polymorphism (RFLP) techniques (Slippers et al 2004b).

The aims of this study were to identify Botryosphaeriaceae occurring on native *S. cordatum* in South Africa, based on ITS rDNA sequence data, PCR-RFLP analysis and anamorph morphology. Isolates belonging to the Botryosphaeriaceae on *S. cordatum* and *Eucalyptus* were also compared, with special attention given to overlaps and the potential for cross infection. The pathogenicity of the Botryosphaeriaceae isolates from *S. cordatum* was furthermore, tested on both a *Eucalyptus* clone and *S. cordatum* in glasshouse trials.

MATERIALS AND METHODS

Isolates

Isolates used in this study were collected in surveys of Botryosphaeriaceae on native *S. cordatum* in different geographical regions of South Africa, in 2001 and 2002 (TABLE I, FIG.

1). The 148 isolates that were collected from 11 *S. cordatum* sites during these surveys form the basis of this study. Between 5 and 45 trees were sampled from each site. From each tree, isolations were made from dying twigs and symptomless, visually healthy twigs and leaf tissues. Leaves and twig portions (5 cm in length) were washed in running tap water and surface sterilized by placing them sequentially for 1 min in 96 % ethanol, undiluted bleach (3.5–5 % available chlorine) and 70 % ethanol, then rinsed in sterile water. Treated twig portions were halved and pieces from the pith tissue (2 mm²) and segments of the leaves (3 mm²) were placed on 2 % malt extract agar (MEA; 2 % malt extract, 1.5 % agar; Biolab, S.A.) in Petri dishes. Following incubation for 2 weeks at 20 °C under continuous near-fluorescent light and colonies resembling Botryosphaeriaceae with grey-coloured, fluffy aerial mycelium, were selected. These colonies were transferred to 2 % MEA at 25 °C and stored at 5 °C. All isolates have been maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative isolates were deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extraction and ITS rDNA amplification

Single-conidial cultures from 21 isolates were grown on MEA for 7 days at 25 °C in the dark. Template DNA was obtained from the mycelium using the modified phenol-chloroform DNA extraction method described in Smith et al (2001). DNA was separated by electrophoresis on 1.5 % agarose gels, stained with ethidium bromide and visualized under ultraviolet light. DNA concentrations were estimated against λ standard size markers.

The internal transcribed spacer (ITS) regions ITS1 and ITS2, and the intermediate 5.8S gene of the ribosomal RNA (rRNA), were amplified using the primer pair ITS1 and ITS4 (White et al 1990). The PCR reactions were performed using the PCR protocol of Slippers et al (2004b). PCR products were separated in a 1.5 % agarose gel, stained with ethidium bromide and visualized under UV light. Sizes of PCR products were estimated against a 100 bp molecular weight marker XIV (Roche Diagnostics). The PCR products were purified using High Pure PCR Product Purification Kit (Roche Diagnostics).

DNA sequencing and analysis

Based on conidial morphology, the isolates of Botryosphaeriaceae from *S. cordatum* in South Africa were tentatively separated into eight groups. ITS rDNA sequences were

determined for representative samples from all morphological groups (TABLE I). To determine the identity and phylogenetic relationship of these isolates, ITS sequences of known species of the Botryosphaeriaceae were obtained from GenBank and included in the analyses (TABLE I). The purified PCR products were sequenced using the same primers that were used for the PCR reactions. The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) was used for sequencing reactions, as specified by the manufacturers. Sequence reactions were run on an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer).

Nucleotide sequences were analyzed using SEQUENCE NAVIGATOR version 1.0.1. (Perkin-Elmer Applied BioSystems, Inc.) software and alignments were made online using MAFFT version 5.667 (http://timpani.genome.ad.jp/~mafft/server/) (Katoh et al 2002). Gaps were treated as fifth character and all characters were unordered and of equal weight. Phylogenetic analyses of aligned sequences were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999). Most parsimonious trees were found using the heuristic search function with 1000 random addition replicates and the tree bisection and reconstruction (TBR) selected as branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Branch support was determined using 1000 bootstrap replicates (Felsenstein 1985). The trees were rooted using the GenBank sequences of *Guignardia philoprina* and *Mycosphaerella africana*. The sequence alignments and phylogenetic tree have been deposited in TreeBASE as S1412, M2541.

PCR-RFLP analyses

PCR-RFLP fingerprinting techniques were applied to confirm the identity of isolates that were not sequenced and to identify the isolates that could not be separated based on ITS rDNA sequences. Amplicons obtained using primer pairs ITS1 and ITS4, or *BotF15* (5′ CTGACTTGTGACGCCGGCTC) and *BotF16* (5′ CAACCTGCTCAGCAAGCGAC) (Slippers et al 2004a) were digested with the restriction endonuclease *Cfo*I. The RFLP reaction mixture consisted of 10 µL PCR products, 0.3 µL *Cfo*I and 2.5 µl matching enzyme buffer (Roche Diagnostics). The reaction mixture was incubated at 37 °C overnight. Restriction fragments were separated on 1.5 % agarose gel as described for PCR products. The results were compared with those of Slippers (2003).

Morphology and cultural characteristics

Fungal isolates were grown on 2 % water agar (WA; Biolab) with sterilized pine needles placed onto the medium, at 25 °C under near-UV light, to induce sporulation. Conidia that were released from pycnidia on the pine needles were mounted in lactophenol on glass slides and examined microscopically. Ten conidia of each isolate were measured. Measurements and digital photographs were taken using a light microscope, an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd.). Colony morphology and colour were determined from cultures grown on 2 % MEA at 25 °C under near-UV light. Colony colors (upper surface and reverse) were compared to those in the color charts of Rayner (1970).

Pathogenicity

Fifteen isolates, representing eight species of Botryosphaeriaceae isolated from native *S. cordatum* in South Africa, were used in this study (TABLE I). One isolate of *Botryosphaeria dothidea* and two isolates for each of the other seven species were randomly selected for inoculations. The isolates were grown on 2 % MEA at 25 °C under continuous near-fluorescent light for 7 days prior to inoculation.

Two-year-old trees of an *E. grandis* × *camaldulensis* clone (GC-540) and 1-year-old saplings of *S. cordatum* were selected for the pathogenicity trials under glasshouse conditions. Saplings of *S. cordatum* were raised from seeds taken from a single tree grown in Kwambonambi (KwaZulu Natal province) area. Trees and saplings selected for inoculations were grown in pots outside, and maintained in the glasshouse for acclimatization for 3 weeks prior to inoculation. Trees were inoculated during the spring-summer season (September 2003–February 2004). The glasshouse was subjected to natural day/night conditions and a constant temperature of approximately 25 °C. Each of the isolates representing the different species was inoculated into the stems of 10 trees of each host species. Ten trees were also inoculated with sterile MEA plugs to serve as controls. The 160 inoculated trees, 10 for each fungal species and 10 as a control, were arranged in a randomised block design. The entire trial was repeated once under the same conditions, giving a total of 320 trees inoculated for each host species.

For inoculations, wounds were made on the stems of trees using a 6-mm-diameter (*Eucalyptus* clone) or a 4-mm-diameter (*S. cordatum*) cork borer to remove the bark and expose the cambium. Wounds were made between two nodes on the stems of trees approximately 250 mm (*Eucalyptus*) or 150 mm (*S. cordatum*) above soil level. Plugs of

mycelium were taken from 7-day-old cultures grown on MEA using the same size cork borer, and were placed into the wounds with mycelium surface facing the cambium. Inoculated wounds were sealed with a laboratory film (Parafilm M, Pechiney Plastic Packaging) to prevent desiccation and contamination. Lesion lengths (mm) were measured 6 weeks after inoculation. The fungi were re-isolated by cutting small pieces of wood from the edges of lesions and plating them on 2 % MEA at 25 °C. Re-isolations were made from two randomly selected trees per isolate and tree species and from all trees inoculated as controls.

Pathogenicity for all isolates inoculated on *Eucalyptus* clone and *S. cordatum* was determined based on the length of lesions (mm) that developed after 6 weeks. There was no significant difference between the two repeats of the pathogenicity trials and the data were therefore combined to represent one data set for the analyses. Statistical analyses of the data were performed using SAS statistical software (version 8, SAS Institute). The 95 % confidence limits were determined for all means based on full model analysis of variance (ANOVA). Differences between means were, therefore, considered significant at the $P \leq 0.05$ level.

RESULTS

DNA sequence analyses

DNA fragments of approximately 600 bp were amplified. The ITS dataset consisted of 53 ingroup sequences, with *G. philoprina* and *M. africana* as outgroup taxa (TABLE I). After alignment, the ITS dataset consisted of 593 characters; 432 uninformative characters were excluded, and 161 parsimony informative characters were used in the analysis. The parsimony analysis (using heuristic searches) produced 276 most-parsimonious trees of 414 steps (consistency index (CI) = 0.702, retention index (RI) = 0.915), one of which was chosen for presentation (FIG. 2).

The isolates considered in the phylogenetic analyses formed 12 clades, designated as groups I to XII (FIG. 2). These groups were resolved in two major clades that corresponded to species of Botryosphaeriaceae with *Fusicoccum*-like or *Diplodia*-like anamorphs. The *Fusicoccum* clade comprised six groups that represented: *Neofusicoccum parvum* and *N. ribis* (group I), *Neofusicoccum mangiferae* (group II), *N. eucalyptorum* (group III), *N. australe* (group IV), *N. luteum* (group V) and *B. dothidea* (group VI). Groups VII and VIII represented species with *Lasiodiplodia* anamorphs: *Lasiodiplodia theobromae* (group VII) and *L. gonubiensis* (group VIII). These two groups (VII and VIII) formed a distinct subclade

(supported by 100 % bootstrap value) within the *Diplodia* clade. The other major subclade within the *Diplodia* clade contained four groups corresponding to: *D. mutila* (group IX), *D. corticola* (group X), *Diplodia seriata* (group XI) and *Diplodia pinea* (= *Sphaeropsis sapinea*) (group XII) (FIG. 2).

All the isolates obtained from *S. cordatum* in this study resided in seven groups (FIG. 2) as follows: *N. parvum* and *N. ribis* (group I), *N. mangiferae* (group II), *N. australe* (group IV), *N. luteum* (group V), *B. dothidea* (group VI), *L. theobromae* (group VII) and *L. gonubiensis* (group VIII).

PCR-RFLP analyses

Isolates that were not identified from DNA sequence comparisons were subjected to ITS PCR-RFLP analyses. Digests of the PCR products, obtained using primers ITS1 and ITS4, with the RE *Cfo*I produced two distinctive banding patterns. These profiles matched those of *N. parvum* / *N. ribis* (99 isolates) and *N. luteum* / *N. australe* (5 isolates) as shown by Slippers et al (2004b). To further distinguish isolates of *N. parvum* from those of *N. ribis,* amplicons obtained using primers *BotF15* and *BotF16* were digested using the same restriction endonuclease (RE). The two banding patterns obtained matched those of *N. parvum* (42 isolates) and *N. ribis* (57 isolates) as described by Slippers (2003). However, *N. luteum* and *N. australe* could not be separated using this technique.

Morphology and cultural characteristics

All 148 isolates of the Botryosphaeriaceae from *S. cordatum* produced anamorph structures on pine needles on WA within 2–3 weeks. No teleomorph (sexual) structures were observed. Based on conidial morphology, isolates were separated into eight groups. Five of these groups corresponded to Botryosphaeriaceae with *Neofusicoccum* anamorphs (FIG. 3a–f), one with a *Fusicoccum* anamorph (FIG. 3g) and two with *Lasiodiplodia* (*Diplodia*-like) anamorphs (FIG. 4a, b).

Representative samples from the groups emerging from morphological comparisons were identified based on ITS rDNA sequence comparison. As described earlier, isolates of *N. parvum* and *N. ribis* were separated based on PCR-RFLP analyses. Further morphological examination of isolates, identified based on DNA data, provided support for their identity.

Cultures of *N. parvum* were initially white with fluffy, aerial mycelium, becoming pale olivaceous grey from the middle of colony after 3–4 days; columns of the mycelium

formed in the middle of colony reaching the lid; margins were regular; reverse sides of the colonies were olivaceous grey. Conidia were hyaline, smooth, aseptate and fusiform to ellipsoid (average of 420 conidia: 18.2 × 5.5 µm, l/w 3.3) (FIG. 3a). The 42 isolates were identified as *N. parvum*.

Colonies of *N. ribis* were initially white, becoming pale olivaceous grey from the middle of colony, with thick aerial mycelium reaching the lids of Petri dishes; margins were regular; reverse sides of the colonies were olivaceous grey. Conidia were hyaline, unicellular, aseptate, fusiform, apices tapered (average of 570 conidia: 21 × 5.5 µm, l/w 3.8) (FIG. 3b). The 57 isolates were identified as *N. ribis*.

The culture of the single *B. dothidea* isolate identified in this study produced greenish olivaceous appressed mycelium, its margins regular and the reverse sides of the colonies olivaceous grey to iron grey. Conidiomata were readily formed in the middle of colony after 3–4 for days of incubation. Conidia were hyaline, smooth with granular contents, aseptate, narrowly fusiform (average of 10 conidia: 27.8 × 5.4 µm, l/w 5.1) (FIG. 3g).

Isolates of *N. mangiferae* produced pale olivaceous grey appressed mycelium, slightly fluffy on the edges of colonies, with sinuate margins and the reverse sides of colonies were olivaceous. Conidiomata were readily formed in the middle of coloniesa after 3–4 days and covered the entire surface of the colonies within 7–10 days. Conidia were hyaline, fusiform (average of 300 conidia: 14.2 × 6.3 µm, l/w 2.25) (FIG. 3f). The 30 isolates were identified as *N. mangiferae*.

Cultures of *N. luteum* were initially white, becoming pale olivaceous grey from the middle of colonies within 3–4 days, with suppressed mycelium, moderately fluffy in the middle and with regular margins. A yellow pigment was noticeable after 3–5 days of incubation and was seen as amber yellow on the reverse side of Petri dishes; after 5–7 days colonies become olivaceous buff to olivaceous gray. Conidiomata were readily formed from the middle of colonies within 3–4 days and covered the whole surface of colonies within 7–10 days. Conidia were hyaline, fusiform to ellipsoid, sometimes irregularly fusiform, smooth with granular contents, unicellular, forming one or two septa before germination (average of 40 conidia: 18.9 × 6.3 µm, l/w 3.0) (FIG. 3d, e). The four isolates were identified as *N. luteum*.

Cultures of *N. australe* were very similar in morphology to those of *N. luteum*, but the yellow pigment produced in young cultures was brighter and a honey yellow colour when viewed from the bottom of the Petri dishes. Conidiomata readily formed at the middle

of colonies within 3–4 days and covered the colony surfaces within 7–10 days. Conidia were hyaline, fusiform, apices rounded, aseptate, rarely uniseptate (average of 70 conidia: 20.5 × 5.7 µm, l/w 3.6) (FIG. 3c). These conidia are slightly longer and narrower on average than *N. luteum,* which also reflected in higher l/w ratio. The seven isolates were identified as *N. australe*.

Isolates of *L. theobromae* produced initially white to smoke grey fluffy aerial mycelium, becoming pale olivaceous grey within 5–6 days with regular margins; the reverse sides of the cultures were olivaceous grey to iron, becoming dark slate blue after 7–10 days. Conidia were hyaline, aseptate, ellipsoid to ovoid, thick-walled with granular contents (average of 50 conidia: 27 × 14.7 µm, l/w 1.85) (FIG. 4b). Dark, septate conidia typical for this species were not observed in this study. The five isolates were identified as *L. theobromae*.

Isolates of *L. gonubiensis* were similar in culture morphology to those of *L. theobromae.* Conidia of *L. gonubiensis* were initially hyaline, unicellular, ellipsoid to obovoid, thick-walled with granular contents, rounded at apex and occasionally truncate at base. Aging conidia became cinnamon to sepia with longitudinal striations, forming one to three septa (average of 20 conidia: 33.9 × 18.9 µm, l/w 1.8) (FIG. 4a). The two isolates were identified as *L. gonubiensis*.

KEY TO SPECIES OF BOTRYOSPHAERIACEAE AND THEIR ANAMORPHS FROM *SYZYGIUM CORDATUM* IN SOUTH AFRICA

1. Fusoid to ellipsoid, thin-walled, *Fusicoccum*-like conidia **2**
1. Ovoid, thick-walled, *Diplodia*- or *Lasiodiplodia*-like conidia **3**
2. Colonies on MEA producing yellow pigment in young cultures **4**
2. Colonies on MEA not producing yellow pigment in cultures **5**
3. Conidia on average <30 µm long; aging conidia become dark brown with longitudinal striations and uniseptated as reported by Punithalingam (1976) ***Lasiodiplodia theobromae***
3. Conidia on average >30 µm long; aging conidia become cinnamon to sepia with longitudinal striations and 1–3 septata ***L. gonubiensis***
4. Colonies producing amber yellow pigment noticeable between 3–5 days after incubation; conidia on average <20 µm long ***Neofusicoccum luteum***
4. Colonies producing honey yellow pigment noticeable between 3–5 days after incubation; conidia on average >20 µm long ***N. australe***

5. Conidia on average >25 µm long, narrowly fusiform	***Botryosphaeria dothidea***
5. Conidia on average <25 µm long	**6**
6. Conidia on average <15 µm long, l/w 2–2.5	***N. mangiferae***
6. Conidia on average ≥15um long, l/w 3–5	**7**
7. Conidia 15–27 × 4–7 µm, aseptate, fusiform, apices tapered	***N. ribis***
7. Conidia 13–25 × 3.5–6 µm, aseptate, fusiform to ellipsoid	***N. parvum***

Pathogenicity

All Botryosphaeriaceae isolates tested for pathogenicity on the *E. grandis* × *camaldulensis* clone (GC-540) produced lesions within six weeks. Small lesions were found on trees inoculated with sterile MEA plugs as controls. The fungi re-isolated from the lesions that developed on trees were the same as those used for inoculations. The original Botryosphaeriaceae species were re-isolated from all trees chosen for re-isolations. No Botryosphaeriaceae were re-isolated from the controls.

Statistical analyses showed that the mean lesion length for the majority of isolates used in the trial differed significantly from that of the controls (FIG. 5a). The longest lesions were produced by isolates of *L. theobromae*, while the size of lesions produced by *B. dothidea* and *L. gonubiensis* were not significantly different to those of the controls (FIG. 5a). The mean lesion lengths for different strains of the same Botryosphaeriaceae species were not significantly different from one another, except for the isolates of *L. theobromae*. Thus *L. theobromae* isolate CMW14116 was significantly more pathogenic than isolate CMW14114 (FIG. 5a).

All Botryosphaeriaceae isolates inoculated on *S. cordatum* saplings produced lesions within six weeks. However, the mean lesion lengths produced by majority of the isolates were not significantly different from those of the controls (FIG. 5b). Some trees inoculated as controls also developed small lesions, but no Botryosphaeriaceae could be re-isolated from these lesions, which appeared to represent wound reactions. The fungi re-isolated from the lesions on trees inoculated with fungal mycelium were the same as those used for inoculations. The longest lesions were produced by one isolate of *N. mangiferae* (CMW14034) and the mean lesion length obtained for this isolate was significantly greater than that of the other isolate (CMW14102) of the same species (FIG. 5b). However, there were no statistically significant differences between the lesion lengths for the different isolates of the other species of the Botryosphaeriaceae (FIG. 5b). The mean lengths of lesions

produced by one isolate of *N. ribis* (CMW13992) and one isolate of *L. theobromae* (CMW14116) were also significantly different from that of the control (FIG. 5b). All the other isolates inoculated onto *S. cordatum* saplings produced lesions that were not significantly different from those of the controls (FIG. 5b).

Isolates of all the Botryosphaeriaceae used in this study, except those of *Neofusicoccum mangiferuae,* were more pathogenic on *Eucalyptus* clone than on *S. cordatum*. Analyses of variance showed that the interactions between mean lesion length produced by the species of Botryosphaeriaceae on *Eucalyptus* clone and those on *S. cordatum* were statistically significant ($P \leq 0.001$).

DISCUSSION

Eight species of the Botryosphaeriaceae were identified on native *Syzygium cordatum* in South Africa in this study. They were *N. ribis, N. parvum, N. luteum, N. australe, N. mangiferae, B. dothidea*, *L. theobromae* and *L. gonubiensis*. The isolates were identified based on ITS rDNA sequence data, PCR-RFLP analysis and anamorph morphology. With exception of *B. dothidea* and *L. gonubiensis,* this is the first report of all of these species of Botryosphaeriaceae on native *S. cordatum*. All eight species had the ability to infect and cause lesions on the stems of a *Eucalyptus grandis* × *camaldulensis* clone and *S. cordatum* in glasshouse trials. Although lesions produced by most of isolates on *S. cordatum* saplings were not significantly different from those on the controls, the pathogens could be re-isolated from these lesions. In the case of some species, such as *N. ribis, L. theobromae* and *F. mangiferae*, one isolate did not produce lesions that differed from those of the control, while the other isolate did. From these data, and knowledge of the fungi on other hosts, we conclude that this group of fungi could be regarded as potential pathogens of *Syzygium*. However, apart from the isolates of *B. dothidea* and *L. gonubiensis,* all the other Botryosphaeriaceae produced lesions on the *Eucalyptus* clone that were significantly different from those of the controls. They should be considered as potential threats to plantation-grown *Eucalyptus* spp. in South Africa.

Neofusicoccum ribis was the dominant species collected from native *S. cordatum* in South Africa. This fungus represented 38 % of all isolates obtained in this study and it was found in most of the areas surveyed. This abundant and wide distribution on a native host might indicate that this species is native to this region. *Neofusicoccum ribis* has been reported from *Eucalyptus* (Myrtaceae) in its native range in Australia and on non-native

Eucalyptus spp. in plantations (Old and Davison 2000), but has not been identified on *Eucalyptus* spp. in South Africa (Slippers et al 2004a). These identifications should, however, be interpreted with caution, as the distinction between *N. parvum* and *N. ribis* had not been recognised at the time of these studies (Slippers et al 2004a). Furthermore, *N. ribis* as identified in this study (using RFLPs) was also interpreted as representing the *N. ribis sensu lato* group rather than strictly conspecific populations with the type isolates of this species, as identified by Slippers (2003). Further analyses using sequence data for additional gene regions and other variable markers will be required to more clearly characterise populations and potential cryptic species in this group. *Neofusicoccum ribis* was one of the most pathogenic species of the Botryosphaeriaceae on the *Eucalyptus* clone in this study. This fungus should thus be considered as a potentially important pathogen of *Eucalyptus* spp. in South Africa.

Isolates of *N. parvum* represented 28 % of the total number of isolates obtained in this study. Recent studies showed that *N. parvum* is an important and widely distributed pathogen of non-native *Eucalyptus* plantations in South Africa (Slippers et al 2004b). The wide distribution of *N. parvum* on non-native and native Myrtaceae in South Africa raises intriguing questions, such as whether these populations are native or introduced and how they might be interacting with each other. The movement of this pathogen between these important host groups represents a potential threat for both groups and should be further investigated. Isolates of *N. parvum* used in this study also developed only slightly smaller lesions than those of closely related *N. ribis*, illustrating its potential threat to *Eucalyptus* plantations in South Africa.

Only one isolate obtained from *S. cordatum* was identified as *B. dothidea* (anamorph *Fusicoccum aesculi*). This species has been one of the most commonly reported members of the Botryosphaeriaceae from a wide variety of hosts, including *Eucalyptus* spp. (von Arx and Muller 1954, Smith et al 2001). While *B. dothidea* was considered to be an important canker pathogen of *Eucalyptus* spp. in South Africa (Smith et al 1994), some of these isolates that were the most pathogenic (Smith et al 2001) were re-identified as *N. parvum* (Slippers et al 2004b). *Botryosphaeria dothidea* was seldom encountered on *Eucalyptus* spp. in other studies on this host (Slippers et al 2004b) and results of the present study suggest that it is probably not an important pathogen of this tree.

High numbers of isolates from *S. cordatum* were identified as *N. mangiferae*. This species is best known as a pathogen of mango (*Mangifera indica*) worldwide, particularly in Australia (Johnson et al 1992). *Neofusicoccum mangiferae* was earlier reported under

different names from mango in South Africa (Darvas 1991). Interestingly, however, a recent comprehensive study of Botryosphaeriaceae from mango plantations in South Africa, using a combination of DNA-based techniques and morphological data, did not report this species (Jacobs 2002). The fact that this fungus is highly pathogenic on *S. cordatum* might imply that it has been introduced into South Africa on other woody plants. Studies focused on the origin of *N. mangiferae* are likely to yield intriguing results, relevant to commercial forestry and to the protection of natural biodiversity in South Africa.

Neofusicoccum luteum and phylogenetically closely related *N. australe* were identified on *S. cordatum* in this study, but have not been recorded on *Eucalyptus* spp. in South Africa. *Neofusicoccum australe* is a recently described species (Slippers et al 2004c) and the present study is the first to consider the pathogenicity of this fungus on *Eucalyptus. Neofusicoccum luteum* was highly pathogenic to *Eucalyptus* clone and its occurrence on the related *S. cordatum* in South Africa is of concern. *Neofusicoccum luteum* and *N. australe* were not the most commonly encountered species of the Botryosphaeriaceae on *S. cordatum,* but their presence alone provides sufficient evidence that they are well established in the country.

Two *Lasiodiplodia* species were identified in this study. *Lasiodiplodia theobromae* was isolated from *S. cordatum* in subtropical areas of South Africa. This fungus is an opportunistic pathogen with an extremely wide host range, including more than 500 host plants, mostly in tropical and sub-tropical regions (Punithalingam 1976), and has previously been isolated from exotic *Acacia, Eucalyptus* and *Pinus* spp. in South Africa (Crous et al 2000, Burgess et al 2003). *Lasiodiplodia theobromae* was the most pathogenic species to the *Eucalyptus* clone in this study. Although the two isolates of *L. theobromae* displayed different levels of pathogenicity, both were highly pathogenic. *Lasiodiplodia theobromae* might be considered a potentially important pathogen of *Eucalyptus* in South Africa and studies to consider its pathogenicity to different species and hybrid clones would be warranted. Another *Lasiodiplodia* species isolated from *S. cordatum* has recently been described as *L. gonubiensis* (Pavlic et al 2004) and was isolated from a geographical region with a moderate climate where *L. theobromae* was absent. *Lasiodiplodia gonubiensis* appears to be very mildly pathogenic to the *Eucalyptus* clone, even though it is most closely related to the highly pathogenic *L. theobromae*.

The results of this study have provided an interesting insight into the diversity of Botryosphaeriaceae occuring on native *S. cordatum* in South Africa. Some of these fungi appear to be potentially important pathogens of *Eucalyptus* spp. and future surveys should

recognize this fact. Clearly, additional studies such as the one presented here, considering the pathogenicity of these fungi, will be needed to better understand their importance. This study emphasises the threat of cross-infecting species of the Botryosphaeriaceae, to both native and introduced Myrtaceae. In a recent study, Burgess et al (2006) showed that there is no restriction to the movement of *N. australe* between native and planted eucalypts in Western Australia. Population studies on other species of the *Botryosphaeriaceae* are, therefore, planned to provide further insight into their movement between native and cultivated hosts in South Africa.

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TABLE I. Isolates considered in the phylogenetic study and pathogenicity trials

Culture no.[a,b,c]	Other no.[a]	Identity	Host	Location[d]	Isolator	GenBank ITS
CMW7772		*Neofusicoccum ribis*	*Ribis* sp.	New York	B Slippers, G Hudler	AY236935
CMW7054	CBS121.26	*N. ribis* (chromagena)	*R. rubrum*	New York	NE Stevens	AF241177
CMW14011		*N. ribis*	*Syzygium cordatum*	SA, Sodwana Bay	D Pavlic	DQ316072
CMW14012		*N. ribis*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316073
CMW13990		*N. ribis*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316074
CMW13991	CBS118822	*N. ribis*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316075
CMW14016		*N. ribis*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316079
CMW14031[c]		*N. ribis*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316076
CMW14025		*N. ribis*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316080
CMW13992[c]		*N. ribis*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	
CMW9081	ICMP8003	*Neofusicoccum parvum*	*Populus nigra*	New Zealand	GJ Samuels	AY236943
CMW9078	ICMP7925	*N. parvum*	*Actinidia deliciosa*	New Zealand	SR Pennycook	AY236940
CMW994	ATCC58189	*N. parvum*	*Malus sylvestris*	New Zealand	GJ Samuels	AF243395
CMW9071		*N. parvum*	*Ribes* sp.	Australia	MJ Wingfield	AY236938
CMW10122		*N. parvum*	*Eucalyptus grandis*	SA, Mpumalanga	H Smith	AF283681
CMW14030[c]		*N. parvum*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316077
CMW14029	CBS118832	*N. parvum*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316078
CMW14097[c]		*N. parvum*	*S. cordatum*	SA, Port St Johns	D Pavlic	
CMW7801	BRIP23396	*Neofusicoccum mangiferae*	*Mangifera indica*	Australia	GI Johnson	AY615187
CMW7024	BRIP24101	*N. mangiferae*	*M. indica*	Australia	GI Johnson	AY615185
CMW13998	CBS118821	*N. mangiferae*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316081
CMW14005		*N. mangiferae*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316082
CMW14102[c]		*N. mangiferae*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316083
CMW14034[c]		*N. mangiferae*	*S. cordatum*	SA, Kwambonambi	D Pavlic	
CMW9072		*Neofusicoccum australe*	*Acacia* sp.	Australia, Melbourne	J Roux, D Guest	AY339260
CMW6837		*N. australe*	*Acacia* sp.	Australia, Batemans Bay	MJ Wingfield	AY339262
CMW1110		*N. australe*	*Widdringtonia nodiflora*	SA, Cape province	WJ Swart	AY615166
CMW1112		*N. australe*	*W. nodiflora*	SA, Cape province	WJ Swart	AY615167
CMW3386		*N. australe*	*Wollemia nobilis*	Australia, Queensland	M Ivory	AY615165
CMW14074		*N. australe*	*S. cordatum*	SA, East London	D Pavlic	DQ316089
CMW13986	CBS 118839	*N. australe*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316085
CMW13987[c]		*N. australe*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316086
CMW14013[c]		*N. australe*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316087
CMW9076	ICMP7818	*Neofusicoccum luteum*	*Malus domestica*	New Zealand	SR Pennycook	AY236946
CMW992	KJ93.52	*N. luteum*	*Actinidia deliciosa*	New Zealand	GJ Samuels	AF027745
CMW10309	CAP002	*N. luteum*	*Vitis vinifera*	Portugal	AJL Phillips	AY339258
CMW14071[c]	CBS118842	*N. luteum*	*S. cordatum*	SA, East London	D Pavlic	DQ316088
CMW14073[c]		*N. luteum*	*S. cordatum*	SA, East London	D Pavlic	DQ316090
CMW10125		*Neofusicoccum eucalyptorum*	*E. grandis*	SA, Mpumalanga	H Smith	AF283686

TABLE I. Continued

Culture no.[a,b,c]	Other no.[a]	Identity	Host	Location[d]	Isolator	GenBank ITS
CMW11705		*N. eucalyptorum*	*E. nitens*	South Africa	B Slippers	AY339248
CMW9075	ICMP8019	*Botryosphaeria dothidea*	*P. nigra*	New Zealand	GJ Samuels	AY236950
CMW8000		*B. dothidea*	*Prunus* sp.	Switzerland, Crocifisso	B Slippers	AY236949
CMW14009[c]	CBS118831	*B. dothidea*	*S. cordatum*	SA, Sodwana Bay	D Pavlic	DQ316084
CMW10130		*Lasiodiplodia theobromae*	*Vitex donniana*	Uganda	J Roux	AY236951
CMW9074		*L. theobromae*	*Pinus* sp.	Mexico	TI Burgess	AY236952
CMW14114[c]	CBS118843	*L. theobromae*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316091
CMW14116[c]		*L. theobromae*	*S. cordatum*	SA, Kwambonambi	D Pavlic	DQ316092
CMW14077[c]	CBS115812	*Lasiodiplodia gonubiensis*	*S. cordatum*	SA, Eastern Cape	D Pavlic	AY639595
CMW14078[c]	CBS116355	*L. gonubiensis*	*S. cordatum*	SA, Eastern Cape	D Pavlic	AY639594
CMW7774		*Diplodia seriata*	*Ribes* sp.	USA, New York	B Slippers, G Hudler	AY236953
	KJ93.56	*D. seriata*	Hardwood shrub	USA, New York	GJ Samuels	AF027759
CMW7060	CBS431	*Diplodia mutila*	*Fraxinus excelsior*	Netherlands	HA van der Aa	AY236955
	ZS94-6	*D. mutila*	*Malus pumila*	New Zealand	N Tisserat	AF243407
	CBS112545	*Diplodia corticola*	*Quercus ilex*	Spain	MA Sanchez, A Trapero	AY259089
	CBS112551	*D. corticola*	*Quercus suber*	Portugal	A Alves	AY259101
	KJ94.07	*Diplodia pinea*	*Pinus resinosa*	USA, Wisconsin	DR Smith	AF027758
CMW3025		*Mycosphaerella africana*	*Eucalyptus viminalis*	SA, Stellenbosch	PW Crous	AF 283690
CMW7063	CBS447.68	*Guignardia philoprina*	*Taxus baccata*	Netherlands	HA van der Aa	AF312014

[a] Culture collections: CMW = Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; KJ = Jacobs and Rehner (1998); ATCC = American Type Culture Collection, Manassas, Virginia, USA; BRIP = Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; CAP = Culture collection of AJL Phillips, Lisbon, Portugal; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; ZS = Zhou and Stanosz (2001).

[b] Isolates sequenced in this study are given in bold.

[c] Isolates used in pathogenicity trials.

[d] SA = South Africa.

FIG. 1. A map of South Africa indicating the area of natural distribution of *Syzygium cordatum* (left) and sites from where isolates of the Botryosphaeriaceae identified in this study were obtained (stars, right).

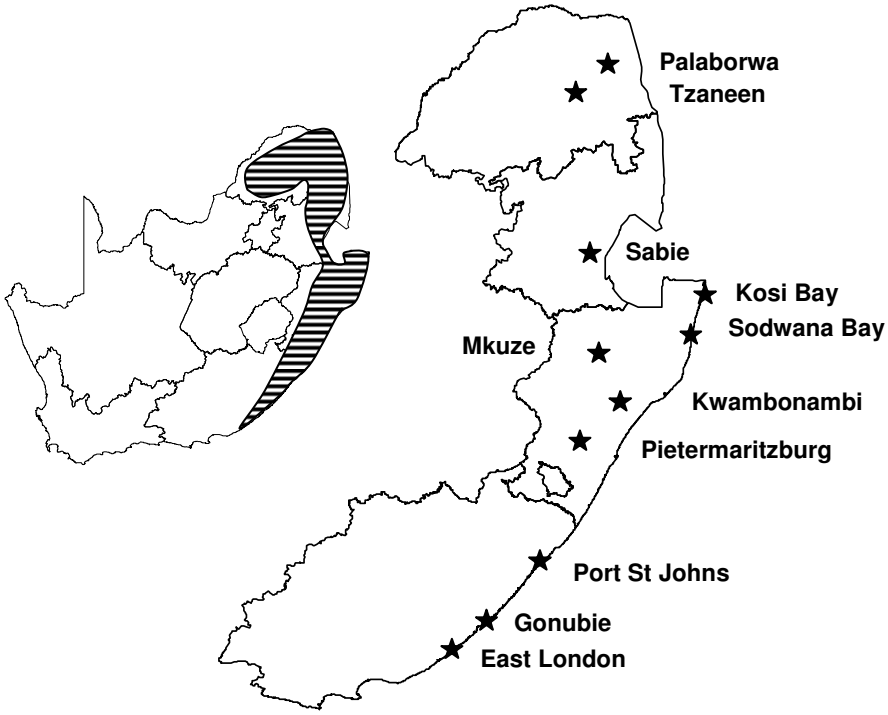

Palaborwa
Tzaneen
Sabie
Kosi Bay
Sodwana Bay
Mkuze
Kwambonambi
Pietermaritzburg
Port St Johns
Gonubie
East London

FIG. 2. One of 276 most parsimonious trees obtained from heuristic searches of the ITS1, 5.8S and ITS2 rDNA sequence data (tree length = 414 steps, CI = 0.702, RI = 0.915). Branch lengths, proportional to the number of steps, are indicated above the internodes, and bootstrap values (1000 replicates) below the internodes. The tree is rooted to the outgroup taxa *Guignardia philoprina* and *Mycosphaerella africana*. Isolates sequenced in this study are presented in bold.

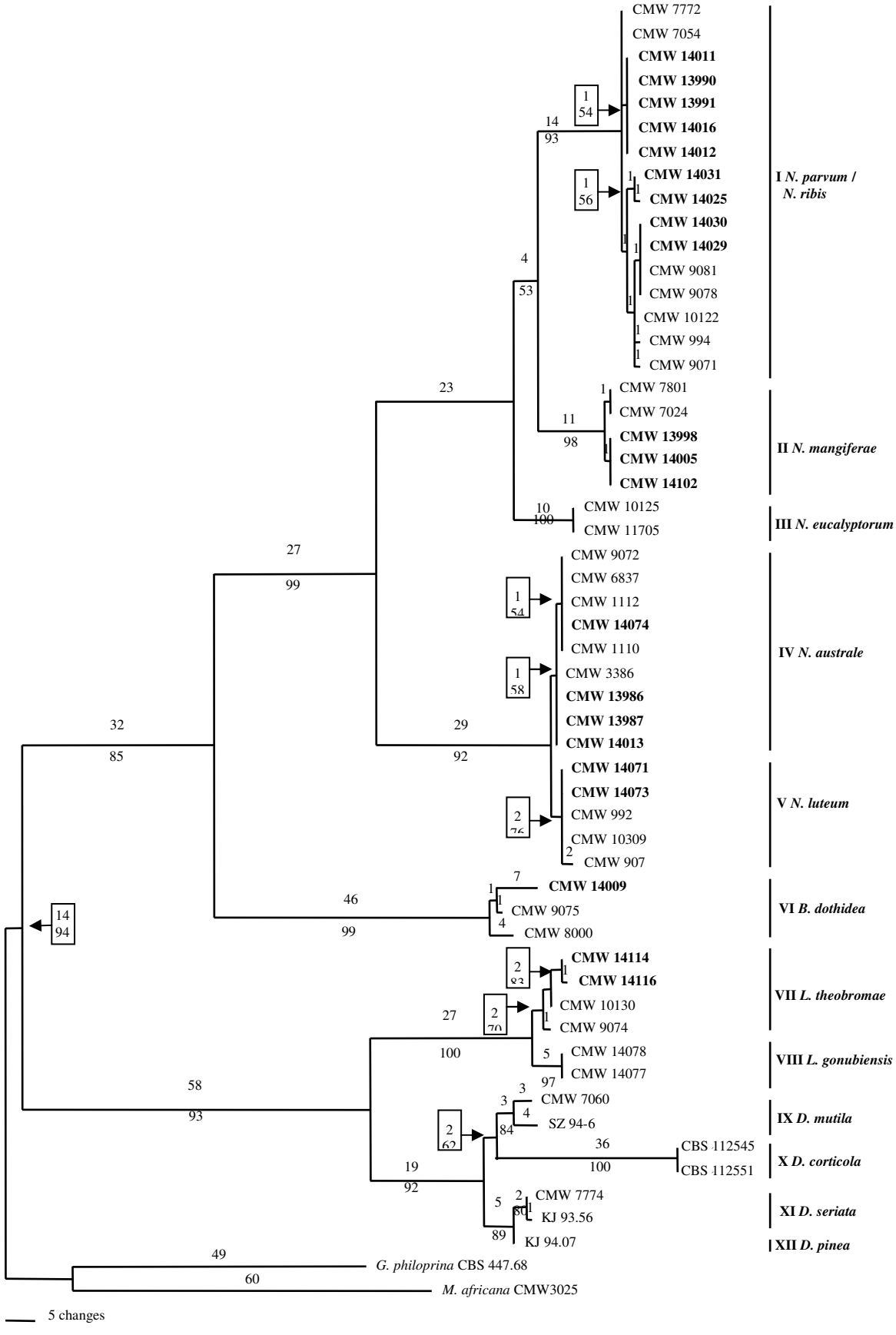

CMW 7772
CMW 7054
CMW 14011
CMW 13990
CMW 13991
CMW 14016
CMW 14012
CMW 14031
CMW 14025
CMW 14030
CMW 14029
CMW 9081
CMW 9078
CMW 10122
CMW 994
CMW 9071
I N. parvum / N. ribis
CMW 7801
CMW 7024
CMW 13998
CMW 14005
CMW 14102
II N. mangiferae
CMW 10125
CMW 11705
III N. eucalyptorum
CMW 9072
CMW 6837
CMW 1112
CMW 14074
CMW 1110
CMW 3386
CMW 13986
CMW 13987
CMW 14013
IV N. australe
CMW 14071
CMW 14073
CMW 992
CMW 10309
CMW 907
V N. luteum
CMW 14009
CMW 9075
CMW 8000
VI B. dothidea
CMW 14114
CMW 14116
CMW 10130
CMW 9074
CMW 14078
CMW 14077
VII L. theobromae
VIII L. gonubiensis
CMW 7060
SZ 94-6
IX D. mutila
CBS 112545
CBS 112551
X D. corticola
CMW 7774
KJ 93.56
KJ 94.07
XI D. seriata
XII D. pinea
G. philoprina CBS 447.68
M. africana CMW3025
5 changes

FIG. 3. Light micrographs of conidia of six Botryosphaeriaceae species with *Fusicoccum*-like anamorphs. a. *N. parvum*. b. *N. ribis*. c. Aseptate and one-septate conidia of *N. australe*. d, e. Aseptate and germinating one- and two-septate conidia of *N. luteum*. f. *N. mangiferae*. g. *B. dothidea*. Bars = 10 µm.

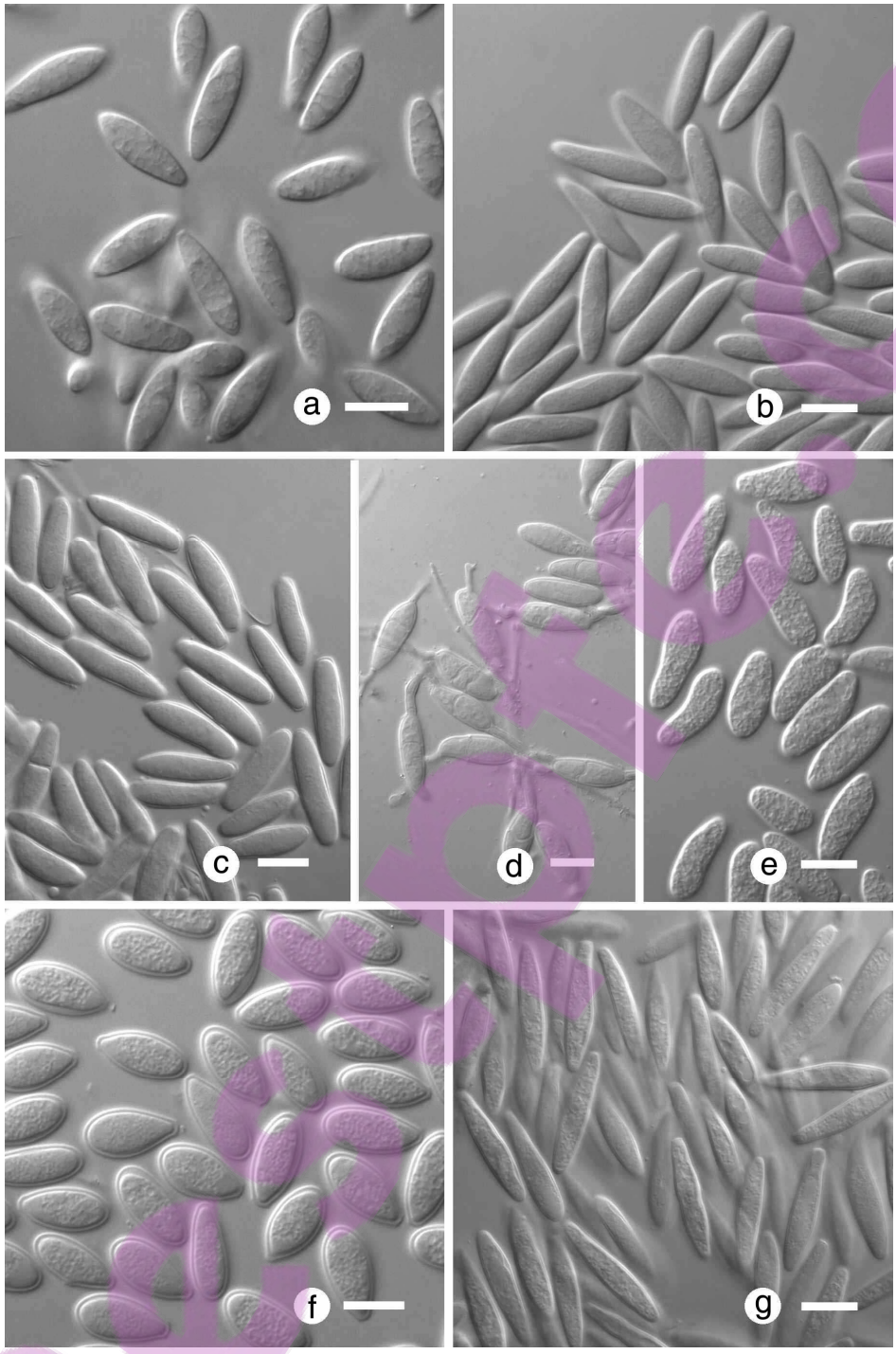


FIG. 4. Light micrographs of conidia of two Botryosphaeriaceae species with *Lasiodiplodia* anamorphs. a. *Lasiodiplodia gonubiensis*. b. *Lasiodiplodia theobromae*. Bars = 10 µm.

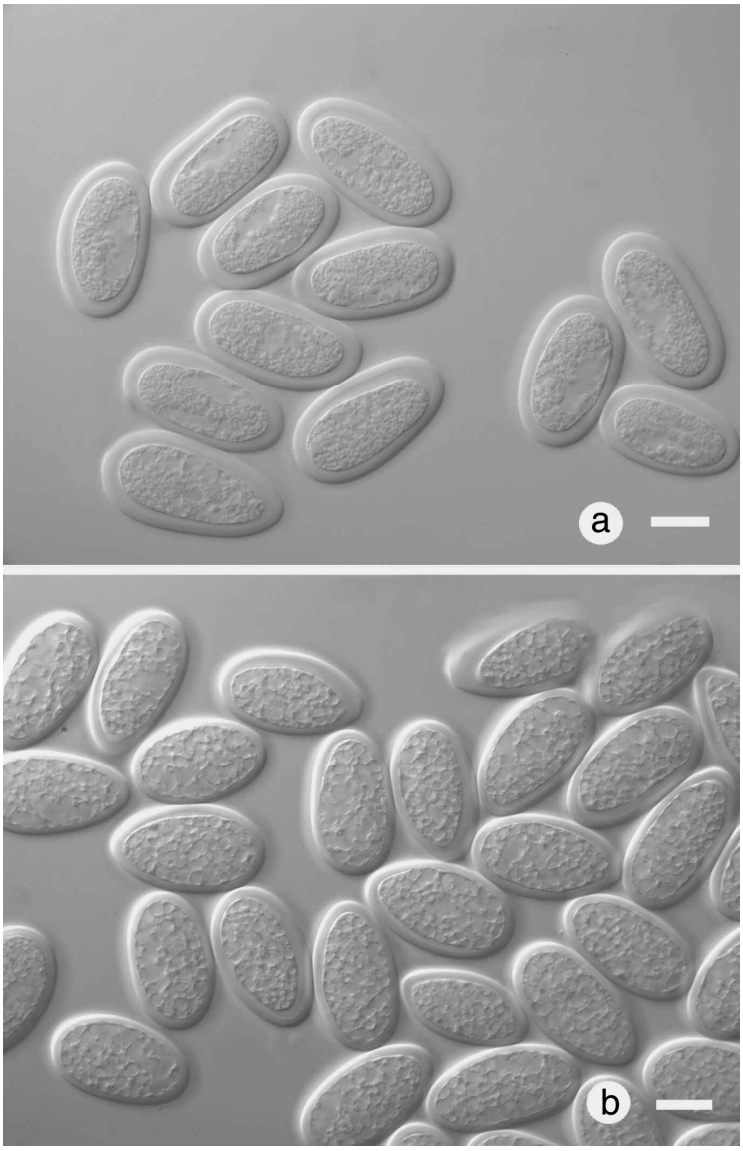
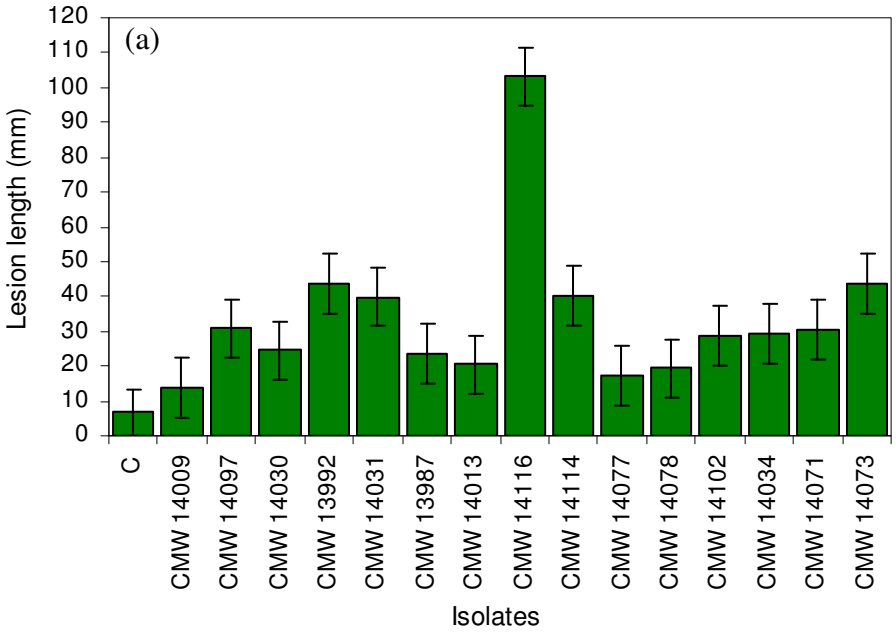
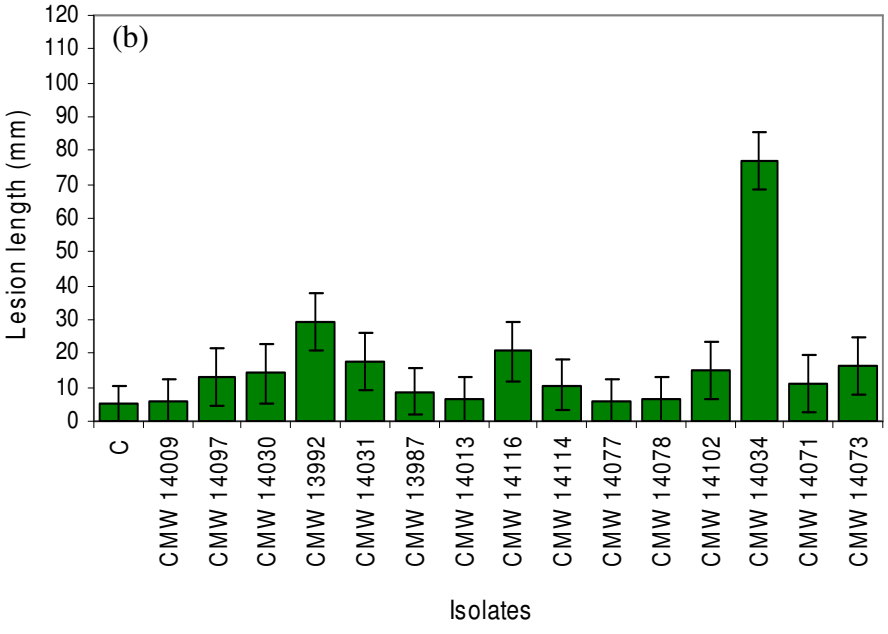


FIG. 5. Mean lesion lengths (mm) obtained for each isolate of different species of the Botryosphaeriaceae six weeks after inoculations on (a) *E. grandis* × *camaldulensis* clone (GC-540) and (b) on *S. cordatum*. Bars represent 95 % confidence limits for each isolate. C = Control. *B. dothidea* (CMW14009), *N. parvum* (CMW14097, 14030), *N. ribis* (CMW13992, 14031), *N. australe* (CMW13987, 14013), *L. theobromae* (CMW14116, 14114), *L. gonubiensis* (CMW14077, 140780), *N. mangiferae* (CMW14102, 14034), *N. luteum* (CMW14071, 14073).


(a)
Lesion length (mm)
120
110
100
90
80
70
60
50
40
30
20
10
0
C
CMW 14009
CMW 14097
CMW 14030
CMW 13992
CMW 14031
CMW 13987
CMW 14013
CMW 14116
CMW 14114
CMW 14077
CMW 14078
CMW 14102
CMW 14034
CMW 14071
CMW 14073
Isolates


(b)
Lesion length (mm)
120
110
100
90
80
70
60
50
40
30
20
10
0
C
CMW 14009
CMW 14097
CMW 14030
CMW 13992
CMW 14031
CMW 13987
CMW 14013
CMW 14116
CMW 14114
CMW 14077
CMW 14078
CMW 14102
CMW 14034
CMW 14071
CMW 14073
Isolates

Chapter 2

Multiple gene genealogies and phenotypic data reveal cryptic species of the Botryosphaeriaceae: A case study on the *Neofusicoccum parvum / N. ribis* complex

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ABSTRACT

Neofusicoccum parvum and *N. ribis* (Botryosphaeriaceae, Ascomycetes) are closely related, plant pathogenic fungi with a worldwide distribution on a wide range of woody hosts. Species boundaries in the *N. parvum* / *N. ribis* complex have eluded definition, despite the application of various tools for characterization. In this study, we test the hypothesis that only one species exists amongst isolates from the *N. parvum* / *N. ribis* complex, identified from *Syzygium cordatum* trees across their native distribution in South Africa. Genealogical concordance phylogenetic species recognition (GCPSR) was applied based on concordance of genealogies obtained from DNA sequence data for five nuclear loci. These data showed that the single species hypothesis must be rejected. Rather, all analyses support the existence of three previously unrecognised, cryptic species within the *N. parvum* / *N. ribis* complex from *S. cordatum*, in addition to *N. parvum* and *N. ribis*. The three lineages reflecting these cryptic taxa are sympatric across their geographical range, indicating barriers to gene flow other than geographic isolation. Phenotypic characters failed to detect all the species uncovered by the GCPSR. Sequence data of the Internal Transcribed Spacer (ITS) of the ribosomal DNA locus, which is thought to be useful for barcoding in fungi, did not distinguish all the species with confidence. RNA polymerase II subunit (RPB2) was the most informative to distinguish all the species *a posteriori* to the application of GCPSR. The results reflect the critical importance of using multiple gene genealogies and adequate sampling to identify cryptic species and to characterise the true diversity within the Botryosphaeriaceae.

INTRODUCTION

Most fungal species are identified solely based on phenotypic characters. However, morphological features used to define species might not be noticeable until well after genetic separation has occurred (Taylor et al 2006). The rapidly increasing number of taxonomic studies utilizing DNA sequence comparisons is revealing increasing numbers of cryptic fungal species and species complexes, previously identified as single morphospecies (Taylor et al 2000, Bickford et al 2006). This is especially true where the genealogical concordance phylogenetic species recognition (GCPSR), a form of phylogenetic species concept (PSC), has been applied (Taylor et al 2000). The GCPSR is based on concordance of multiple gene genealogies and has been used to study cryptic speciation in important human and plant pathogenic fungal complexes, such as *Fusarium graminearum* and *Gibberella fujikuroi* (O'Donnell et al 2000a, b, Steenkamp et al 2002), *Aspergillus flavus* and *A. fumigatus* (Geiser et al 1998, Pringle et al 2005), *Coccidioides immitis* (Koufopanou et al 1997) and others. These studies have revealed numerous previously unidentified, cryptic species.

Since molecular data have been incorporated in species separation and identification of the Botryosphaeriaceae, new sibling species have been recognized within morphologically described taxa. In some cases multiple gene sequence data, using the GCPSR (although not always explicitly stating it as such), needed to be combined with phenotypic characters to identify closely related species. For example, the GCPSR was effectively used to detect *Diplodia scrobiculata* as a sister species of *D. pinea* (de Wet et al 2003). *Neofusicoccum eucalypticola* and *N. australe,* were also identified using the GCPSR as sister species of *N. eucalyptorum* and *N. luteum*, respectively (Slippers et al 2004c, d). The cryptic species recognized in these studies were overlooked or uncertain when using morphology or single-locus sequence data alone (Denman et al 2000, Smith et al 2001, Zhou and Stanosz 2001, Pavlic et al 2007).

Neofusicoccum parvum and *N. ribis* are closely related species that belong to the Botryosphaeriaceae (Ascomycetes, Botryosphaeriales) (Crous et al 2006). *Neofusicoccum ribis* was originally described from *Ribes* spp. in New York, USA as "*Botryosphaeria*" *ribis* (Grossenbacher and Duggar 1911), while *Neofusicoccum parvum* was described from Kiwifruit and a *Populus* sp. in New Zealand as "*Botryosphaeria*" *parva* (Pennycook and Samuels 1985). Both of these species were subsequently identified as pathogens on numerous woody hosts worldwide (Punithalingam and Holliday 1973, Slippers et al 2004a, Mohali et al 2007, Pavlic et al 2007). These fungi are known to have both sexual (teleomorph) and asexual (anamorph) stages in their life cycle, but they are most commonly

encountered as anamorphs. Sexual reproduction in these species is still unexplored and little is known regarding their mating strategy. *Neofusicoccum parvum* and *N. ribis* overlap in the morphological characteristics of their teleomorphs and anamorphs that were used for their original descriptions, making all subsequent identifications difficult and unreliable (Grossenbacher and Duggar 1911, Pennycook and Samuels 1985). The uncertainty regarding their identification was seemingly resolved when *N. parvum* and *N. ribis* were characterised based on multiple gene phylogenies combined with phenotypic characters (Slippers et al 2004b). However, this study was based on a few ex-type and other isolates related to the types of each species. In subsequent phylogenetic analyses, where more isolates were included from larger numbers of hosts and locations, the distinction between these species became less clear (Farr et al 2005, Slippers et al 2005, Pavlic et al 2007). It thus appears to be inadequate to rely only on ex-type specimens of *N. parvum* and *N. ribis* to represent populations across the distribution of these species.

The difficulty in distinguishing *N. parvum* and *N. ribis* is illustrated by conflicting results in two related studies aimed at resolving their identity using multiple approaches. Slippers (2003) characterised a large number of isolates from different hosts and geographical regions using simple sequence repeat (SSR) markers and multiple gene DNA sequence data. In that study, these species were recognised as distinct and *sensu stricto* and *sensu lato* groups were identified for each. The *sensu lato* groups of *N. parvum* and *N. ribis* could be separated using a PCR-RFLP diagnostic tool, but not the further subdivisions of *sensu stricto* groups (Slippers 2003). In a similar study on populations of these species obtained from variety of hosts around the world, and using multiple gene DNA sequence data, SSR marker data, phenotypic characters and AFLP analysis, it was concluded that these two species could not be distinguished from each other (Sakalidis 2004). The separation of the type species was viewed as the end of a genetic continuum of populations. Both studies, however, suffered from sampling deficiencies, where some populations were undersampled, originating from different continents, and from both native and non-native hosts, where opportunities for mating were difficult to judge.

A recent study of Botryosphaeriaceae on *S. cordatum* across its native range in South Africa gave rise to a large number of isolates in the *N. parvum* / *N. ribis* complex (Pavlic et al 2007). Initial data indicated significant variation in conidial morphology and ITS rDNA sequences amongst these isolates, but without supporting a clear distinction of species. In this study we test the hypothesis that these isolates represent one species. For this purpose, we use GCPSR with multiple genes DNA sequence data for five nuclear loci. In addition, we

compare results obtained from the multilocus genealogies with a single locus approach in order to identify the most suitable loci for future recognition of cryptic species in this complex. Variation in conidial morphology, the traditional tool used to distinguish species in this group, was also compared to the GCPSR results to determine their value in species delineation.

MATERIALS AND METHODS

Fungal isolates

The 30 isolates used in this study were selected from a larger collection of 103 isolates collected during the course of a survey of the Botryosphaeriaceae on native *S. cordatum* in different geographical locations of South Africa (TABLE I). All the isolates were identified as *N. parvum* or *N. ribis sensu lato* based on PCR-RFLP analysis (Pavlic et al 2007). The 30 isolates were selected to represent the diversity observed previously in conidial morphology and ITS rDNA sequence data (Pavlic 2004, Pavlic et al 2007), as well as to represent the geographical area and different trees from which they were collected. Three isolates of each of *N. parvum* and *N. ribis* that included the ex-type specimen and two specimens linked to the ex-type were used for comparison (TABLE I). The single-conidial cultures were prepared as reported previously (Pavlic et al 2007), to ensure that only haploid genotypes were characterized for each representative culture. The collection of single-conidial strains used in this study is maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphometric analysis

In a previous study, all 103 isolates were induced to sporulate in culture and conidia were measured and characterized using light microscopy (Pavlic, 2004, Pavlic et al 2007). The lengths and widths of ten conidia were measured for each isolate and the data were analysed in this study. Averages of ten conidial measurements per isolate were calculated and used in the analyses.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the single-conidial cultures following the modified phenol-chloroform DNA extraction method outlined in Smith et al (2001). Five different gene regions were selected for characterization, including the internal transcribed spacer

(ITS) regions 1 and 2 and the 5.8S gene of the ribosomal RNA (rRNA) (White et al 1990), the portion of gene encoding translation elongation factor 1 alfa (EF-1α) (Sakalidis 2004), Bt2 regions of the β-tubulin gene (Glass and Donaldson 1995), a portion of RNA polymerase II subunit (RPB2) (Sakalidis 2004) and locus *BotF15*, an unknown locus containing microsatellite repeats (Slippers et al 2004a). The primer sequences, their respective annealing temperatures and expected product size are presented in TABLE II. The selected regions were amplified using the polymerase chain reaction (PCR) from genomic DNA. The amplifications were performed using an Eppendorf Mastercycler PERSONAL (Perkin-Elmer, Germany) and the following protocol: 94 ºC for 2 min initial denaturation; 40 cycles of 94 ºC for 30 s, 55 or 62 ºC for 30 s, 72 ºC for 1 min; and 72 ºC for 7 min final extension. PCR products were cleaned using the High Pure PCR Product Purification kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Both strands were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Warrington, U.K.), as specified by the manufacturer. Sequence reactions were run on an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, U.K.).

The nucleotide sequences for both strands were examined with SEQUENCE NAVIGATOR version 1.0.1. (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) software and alignments were done online using MAFFT version 5.667 (http://timpani.genome.ad.jp/~mafft/server/) (Katoh et al 2002). Aligned sequences for each gene region were analysed in DnaSP v. 4.00.6 (Rozas et al 2003) for nucleotide polymorphisms.

Phylogenetic analyses

To determine whether analyses of combined sequences can be conducted, statistical congruence was tested using a partition homogeneity test (PHT) (Farris et al 1995, Huelsenbeck et al 1996). The PHT was performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000) using 1000 replicates and the heuristic standard search options.

Maximum-parsimony (MP) genealogies, for single genes and all five genes combined, were constructed in PAUP version 4.0b10 (Swofford 2000), using the heuristic search function with 1000 random addition replicates and tree bisection and reconstruction (TBR) selected as branch swapping algorithm. Gaps were treated as fifth characters and all characters were unordered and of equal weight. Insertions/deletions (indels), irrespective of

their size were each treated as one evolutionary event and weighted as one base substitution. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. To estimate branch support, maximum parsimony bootstrap values were determined using 1000 bootstrap replicates (Felsenstein 1985).

Bayesian analyses were performed using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003) for single gene data and for the combined data set of all five genes. The best-fitting evolutionary models were estimated for each gene region and for the combined data using MrModeltest v. 2.2 software (Nylander 2004). The Markov Chain Monte Carlo (MCMC) chains were initialised from a random tree and were run for 1000000 generations and trees were saved every 100 generations, counting 10000 trees. Burn in was set to 100000 generations. To determine the confidence of the tree topologies, values of Bayesian posterior probabilities (BPPs) (Rannala and Yang 1996) were estimated using MrBayes (Ronquist and Huelsenbeck 2003).

RESULTS

Morphometric analysis

Conidial lengths and widths varied significantly among the isolates. This variation was continuous and did not support any clear distinction of groups. Isolates used for DNA sequence comparisons were selected to represent the full range of conidial sizes and are indicated on the graph reflecting these data (FIG. 1).

DNA sequencing

The sequences obtained in this study have been deposited in GenBank with accession numbers as follows: ITS1, 5.8S, and ITS2 (EU821898-EU821927), EF-1α (EU821868-EU821897), β-tubulin (Bt-2a/b) (EU821838-EU821867), *BotF15* (EU821802-EU821837) and RPB2 (EU821928-EU821963). The sequence alignments and phylogenetic trees have been deposited in TreeBASE as SN3948. Polymorphic nucleotide positions observed in the five sequenced DNA regions are presented in TABLE IV.

Phylogenetic analyses

The phylogenies obtained from sequence data of the gene regions were first determined separately. MrModeltest v. 2.2 predicted appropriate evolutionary models for Bayesian analyses for each of the datasets as follows: K80 model for ITS, HKY model (Hasegawa et

al 1985) with a proportion of invariable sites (*I*) for β-tubulin, GTR model (Rodriguez et al 1990) for RPB2 and HKY model for the *BotF15* and EF-1α datasets. The topologies of trees representing all the gene regions were identical in the maximum-parsimony and Bayesian consensus analyses. Therefore, only unrooted maximum-parsimony trees are presented, with the parsimony bootstrap values and the posterior probabilities shown for well-supported branches (FIG. 2). Statistical data for individual trees are summarised in TABLE III. Five distinct groups were consistently observed, of which two correspond to *N. parvum* and *N. ribis*, while the other three groups represent distinct lineages referred to as R1, R2 and R3. The isolates from *S. cordatum* considered in this study grouped within the *N. parvum* clade (n = 14), and clades R1 (n = 5), R2 (n = 6) and R3 (n = 5).

The R3 and *N. ribis* groups were the most closely related. The three isolates of *N. ribis* (one of which is the ex-type isolate) formed a separate clade in four of the gene regions analysed, while the fifth locus (*BotF15*) contained no polymorphisms between *N. ribis* and R3 (FIG. 2). Bootstrap support and BPPs were generally low for the *N. ribis* clade except in the EF-1α dataset (FIG. 2), but each of the four gene regions contained unique fixed polymorphisms (FIG. 2, TABLE IV). Groups R1 and R2 were strongly supported in four of the five gene genealogies, except the EF-1α dataset, which had only one unique, fixed polymorphism distinguishing R1 and R2 (FIG. 2, TABLE IV). The *Neofusicoccum parvum* clade was recognised in four gene genealogies, with the exception of the β-tubulin dataset in which unique fixed polymorphisms were not identified for the *N. parvum* group (FIG. 2, TABLE IV). The phylogenies constructed based on RPB2 sequences showed the best resolution and highest support for the groups (FIG. 2), followed by the ITS rDNA sequences based genealogy.

Subsequent to individual analyses, the datasets were also analysed collectively. The partition homogeneity test for all the datasets combined indicated that there was no significant conflict among the datasets ($P \geq 0.05$) (Cunningham 1997). MrModeltest v2.2 predicted HKY model with a proportion of invariable sites (*I*) as the most appropriate evolutionary model for Bayesian analyses. Two most parsimonious trees of the same overall topology were obtained for the combined dataset (FIG. 3, TABLE III). In the phylogenetic reconstruction from this combined dataset, the same partitions observed in the individual gene genealogies were recognised. All of these were also strongly supported with bootstrap values close to or equal to100 % and posterior probabilities above 0.95 (FIG. 3).

DISCUSSION

Application of the GCPSR in this study led us to reject the hypothesis that a single variable species in the *N. parvum* / *N. ribis* complex occurs on native *S. cordatum* trees in South Africa. Analysis of five DNA sequence loci showed congruent phylogenies supporting five lineages and indicating a lack of recombination between loci amongst the lineages. The high number of shared single nucleotide polymorphisms (SNPs) and short branches in the phylogenetic trees suggest recent speciation events within the *N. parvum / N. ribis* complex. Nevertheless, the unique SNPs fixed for each of the five lineages, which were linked across all five gene regions, support their treatment as distinct species. What was previously referred to as the *N. parvum* / *N. ribis* clade, therefore, represents a species complex that contains at least five cryptic species, of which three are recognised here for the first time and designated as *Neofusicoccum* sp. R1, R2 and R3. Results of this study reflect the critical importance of using multiple gene genealogies and GCPSR to identify cryptic species and to characterise the true diversity within the Botryosphaeriaceae.

Neofusicoccum parvum and the three new phylogenetic species occur sympatrically across the native geographical range of *S. cordatum*. In addition, more than one species was identified from the same tree, apparently occupying the same niche. This raises the question as to how the genetic barriers that separate the taxa would have evolved. One hypothesis is that these species previously occurred in allopatry, or on different hosts and that they have expanded their geographical or host ranges. Alternatively, genetic barriers might have evolved in sympatry in response to ecological forces not currently known to us. Le Gac et al (2007), based on studies of *Microbotryum violaceum*, and Le Gac and Giraud (2008), after an extensive analysis of published data for various fungi, concluded that such genetic barriers frequently exist among Ascomycetes, to which the Botryosphaeriaceae also belong. This is even when they occur in sympatry and despite the absence of, or only weak, pre-zygotic mating barriers. The genetic barriers appear to be mostly post-zygotic in these fungi, and Le Gac and Giraud (2008) speculated that this is strongly influenced by some 'phylogeny-dependent' life history traits. *In vitro* mating with isolates of the Botryosphaeriaceae has not previously been achieved, making a test of these hypotheses difficult. This should be the focus of future studies if the process of evolution in the group is to be more completely understood.

The focus of this study was specifically to consider members of the *N. parvum* / *N. ribis* complex from a single native tree species occurring in a clearly defined geographical area. Previous studies on these species have considered limited numbers of isolates obtained

from various hosts, including native and non-native trees, and from different geographical regions of the world (Slippers 2003, Sakalidis 2004). In these studies *N. parvum* and *N. ribis* were either recognised as *sensu lato* groups with high levels of inter-specific and intra-specific variation (Slippers 2003) or treated as a single species (Sakalidis 2004). It is likely that the under representation of certain populations in those studies failed to reveal the concordant phylogenies between sequence data sets from different loci. Slippers (2003) recommended that species of Botryosphaeriaceae should be analyzed separately for each host and geographical area of origin due to the possibility for under-sampled, native species occurring sympatrically. The recognition of four cryptic phylogenetic species, occurring sympatrically on native *S. cordatum* supports this view.

None of our isolates from *S. cordatum* were found to represent *Neofusicoccum ribis*. This species has thus far only been confirmed from *Ribes* sp. in the USA using multiple gene phylogenies (Slippers et al 2004b). Although *N. ribis* has been reported from the other hosts and regions (Cunnington et al 2007, Mohali et al 2007) those isolates were characterized only based on the ITS sequences and their identity needs to be reconsidered. Phylogenetic species R3 is recognised in this study as the most closely related taxon to *N. ribis*. Differentiation between these two species was consistent across four gene regions with six fixed unique SNPs that distinguish them. Similarly, a recent study on Southern Hemisphere conifers based on multiple gene genealogies identified three isolates from native coniferous trees in Australia that were also more closely related to *N. ribis* than *N. parvum* (Slippers et al 2005). Four unique fixed SNPs across three gene regions distinguish these three isolates from *N. ribis*. Based on sequence comparison (data not shown) none of those isolates represent any of the phylogenetic species recognised in the present study. The number of cryptic species recognized in the *N. parvum* / *N. ribis* complex in the present study may thus increase in future when isolates from other hosts and areas are considered. It is especially important to better characterize the diversity in *N. ribis*, for which only three isolates has been confirmed thus far.

Evaluation of the single gene genealogies showed that the RPB2 gene region contains the highest number of parsimony informative characters. The RPB2 single-locus phylogeny consequently also provided the highest support for the clades or phylogenetic species. The RPB2 phylogeny was most congruent with ITS sequences and these two datasets combined were the most appropriate for delimitation of phylogenetic species in this study. The RPB2, encoding the second largest RNA polymerase subunit, with its single copy in Ascomycetes and relatively slow evolutionary rate (Liu 1999), has proven useful for

phylogenetic resolution of the Ascomycetes at different taxonomic levels (Liu 1999, Schoch et al 2006, Hofstetter et al 2007, Tang et al 2007). However, it has not been used extensively in the studies of the Botryosphaeriaceae at the species level. DNA sequence based characterisation of these fungi has most commonly been based on the ITS rDNA sequences combined with EF-1α (Luque at al 2005, Phillips et al 2005, Burgess et al 2006). Based on the data presented here, we propose that RPB2 and ITS sequences be used in combination for delimitation of species in the *N. parvum* / *N. ribis* complex in the future. Furthermore, we recommend that its utility for identification of other species of Botryosphaeriaceae should also be assessed.

The ITS rDNA sequence data has been most commonly used for DNA sequence based identification of fungi (Hajibabaei et al 2007). This locus has also been proposed as the DNA barcoding region for fungi (Nilsson et al 2006, www.allfungi.org/its-barcode.php). ITS rDNA sequence data, however, need to be used in combination with other data to delimit cryptic species. The support for the subclades obtained in phylogenetic analyses of ITS sequence data in this study was very low, leaving uncertainty as to their interpretation. Similar results have been obtained in other studies of fungi based on multiple gene genealogies, where ITS data did not provide sufficient resolution for separation of closely related species or varieties. Examples are found in *Neurospora* and *Gelasinospora* (Dettman et al 2001), the human pathogenic fungus *Cryptococcus neoformans* (Xu et al 2000) and many others. As have been discussed in previous studies (Will and Rubinoff 2004, Trewick 2007), the attempt to sort the complex task of species identification based on DNA sequences of one gene region is unlikely, especially when closely related species are considered. After the basis of the variation had been clarified using GCPSR in this study, SNPs could, however, be identified in ITS rDNA regions that would be useful for identification of cryptic species in the *N.* parvum / *N. ribis* complex.

Significant variation in conidial morphology was observed for isolates within the *N. parvum* / *N. ribis* complex from *S. cordatum*. Conidial measurements and the conidial morphology of many of the isolates differed from those in the original descriptions of *N. parvum* and *N. ribis*, suggesting that additional species could exist in this complex. This conidial morphological variation represented a continuum for the phylogenetic species recognised here using multiple gene genealogies and GCPSR. This indicates that genetically isolated species do not necessarily show divergence in character states such as conidial morphology, which is consistent for many other fungi that have been considered in a similar manner (Taylor et al 2000, Chaverri et al 2003, Dettman et al 2003, O'Donnell at al 2004).

In these studies, morphospecies were also recognised as species complexes comprising of a number of phylogenetic species when analysed using GCPSR. *A priori* selection of isolates to represent the full spectrum of the conidial variation (together with ITS sequences and geographic variation), however, proved to be useful in our study to sample representatives of different cryptic species. Observed morphological differences should thus not be underestimated for initial selection of isolates from a larger collection prior to molecular identification. This, together with molecular and ecological data, as well as adequate sampling, should be considered in combination when selecting isolates to test hypotheses regarding cryptic species in the Botryosphaeriaceae.

The common occurrence of *N. parvum sensu stricto* throughout the native distribution of *S. cordatum*, and the intraspecific genetic variation observed, suggests that this is a native fungal species. However, to address hypotheses relating to the origin of species in the *N. parvum* / *N. ribis* complex, population and phylogeographic studies are needed. The delimitation of species boundaries and diagnostic tools tested in this study provide a foundation for such further studies. Significant DNA sequence variation observed amongst *N. parvum* isolates raises questions about population differentiation or even speciation in this group. Sequence data or other more variable molecular tools, such as microsatellite markers, and extended collections is necessary to clarify the origin and distribution of this observed variability within *N. parvum*. Since the *N. parvu* / *N. ribis* species complex includes some of the most aggressive members of the Botryosphaeriaceae (Burgess et al 2005, Pavlic et al 2007), identification of variation in phenotypic characters such as pathogenicity and virulence for the newly recognized species must also be a key area for research in future.

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TABLE I. Isolates analysed in this study

Culture no. [1, 2, 3]	Other no. [1]	Identity	Geographic origin	Host
CMW13992	CBS123634	*Neofusicoccum* sp. R1	South Africa, Sodwana Bay	*Syzygium cordatum*
CMW14056	CBS123635	*Neofusicoccum* sp. R1	South Africa , Kosi Bay	*S. cordatum*
CMW14054	CBS123636	*Neofusicoccum* sp. R1	South Africa , Mkuze	*S. cordatum*
CMW14124	CBS123638	*Neofusicoccum* sp. R1	South Africa, Richards Bay	*S. cordatum*
CMW14151	CBS123637	*Neofusicoccum* sp. R1	South Africa , Sabie	*S. cordatum*
CMW14023	CBS123639	*Neofusicoccum* sp. R2	South Africa, Kwambonambi	*S. cordatum*
CMW14025	CBS123640	*Neofusicoccum* sp. R2	South Africa, Kwambonambi	*S. cordatum*
CMW14140	CBS123641	*Neofusicoccum* sp. R2	South Africa, Tzaneen	*S. cordatum*
CMW14155	CBS123642	*Neofusicoccum* sp. R2	South Africa, Sabie	*S. cordatum*
CMW14123	CBS123643	*Neofusicoccum* sp. R2	South Africa, Richards Bay	*S. cordatum*
CMW14106	CBS123644	*Neofusicoccum* sp. R3	South Africa, Sodwana Bay	*S. cordatum*
CMW14058	CBS123645	*Neofusicoccum* sp. R3	South Africa, Kosi Bay	*S. cordatum*
CMW14060	CBS123646	*Neofusicoccum* sp. R3	South Africa, Kosi Bay	*S. cordatum*
CMW14079	CBS123647	*Neofusicoccum* sp. R3	South Africa, Gonubie	*S. cordatum*
CMW14096		*Neofusicoccum* sp. R3	South Africa, Port St Johns	*S. cordatum*
CMW14127	CBS123648	*Neofusicoccum* sp. R3	South Africa, Kwambonambi	*S. cordatum*
CMW14029		*Neofusicoccum parvum*	South Africa, Kwambonambi	*S. cordatum*
CMW14082		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14085	CBS123649	*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14087		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14088		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14089		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14094		*N. parvum*	South Africa, Pietermaritzburg	*S. cordatum*
CMW14097	CBS123650	*N. parvum*	South Africa, Port St Johns	*S. cordatum*
CMW14080	CBS123651	*N. parvum*	South Africa, Gonubie	*S. cordatum*
CMW14129		*N. parvum*	South Africa, Tzaneen	*S. cordatum*
CMW14135		*N. parvum*	South Africa, Tzaneen	*S. cordatum*
CMW14141		*N. parvum*	South Africa, Tzaneen	*S. cordatum*
CMW14143	CBS123652	*N. parvum*	South Africa, Palaborwa	*S. cordatum*
CMW27901		*N. parvum*	South Africa, Pretoria	*S. cordatum*
CMW9079	ICMP7933	*N. parvum*	New Zealand	*Actinidia deliciosa*
CMW9080	ICMP8002	*N. parvum*	New Zealand	*Populus nigra*
CMW9081	ICMP8003	*N. parvum*	New Zealand	*P. nigra*
CMW7772		*Neofusicoccum ribis*	USA. New York	*Ribes* sp.
CMW7773		*N. ribis*	USA, New York	*Ribes* sp.
CMW7054	CBS121.26	*N. ribis*	USA, New York	*Ribes rubrum*

[1]Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria South Africa; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand.

[2] Isolates in bold are ex-types.

[3] All isolates other than CMW 9079, CMW 9080, CMW 9081, CMW 7772, CMW 7773, and CMW 7054 were collected by D. Pavlic.

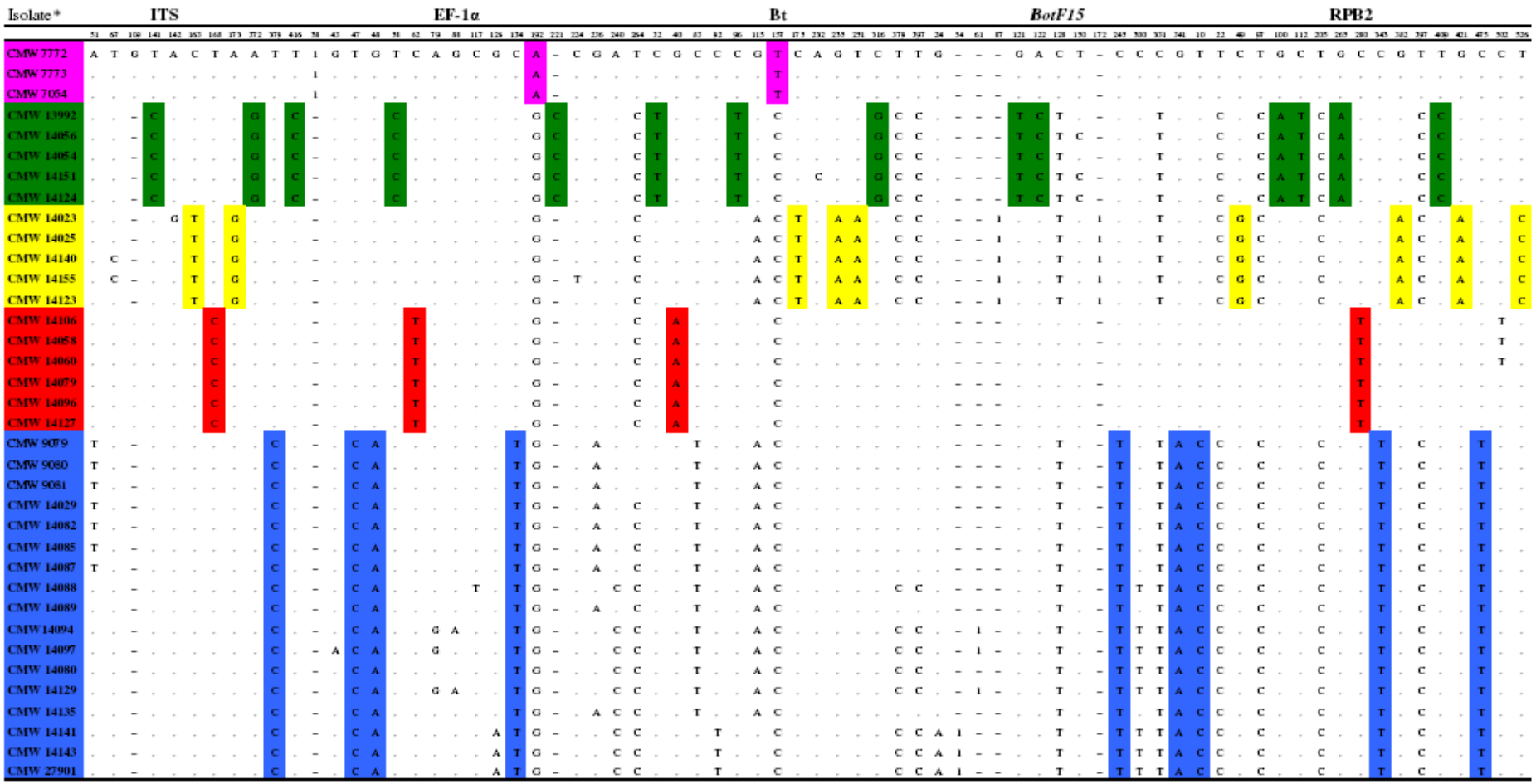
TABLE II. Primer sets used to amplify the five loci analysed in this study

Region	Oligos	Oligo Sequences	Amplicon size (bp)	AT (ºC)	Reference
ITS	ITS1	5′ TCCGTAGGTGAACCTGCGG	600	55	(White et al 1990)
	ITS4	5′ TCCTCCGCTTATTGATATGC			
EF-1α	EF-AF	5′ CATCGAGAAGTTCGAGAAGG	310	55	(Sakalidis 2004)
	EF-BR	5′ CRATGGTGATACCRCGCTC			
β-tubulin	Bt2a	5′ GGTAACCAAATCGGTGCTGCTTTC	450	55	(Glass and Donaldson 1995)
	Bt2b	5′ ACCCTCAGTGTAGTGACCCTTGGC			
RPB2	RPB2bot6F	5′ GGTAGCGACGTCACTCCC	500	55	(Sakalidis 2004)
	RPB2bot7R	5′ GGATGGATCTCGCAATGCG			
BotF15	Bot15	5′ CTGACTTGTGACGCCGGCTC	350	62	(Slippers et al 2004a)
	Bot16	5′ CAACCTGCTCAGCAAGCGAC			

TABLE III. Information on the sequence dataset and maximum parsimony (MP) trees for each locus and all five loci combined

	Locus					
	ITS	**EF-1α**	**β-tubulin**	***BotF15***	**RPB2**	**Combined all**
Total no. of alignable characters	499	286	420	376	565	2146
No. of excluded characters	0	13	0	38	0	51
Total no. of variable characters	11	17	14	13	17	72
No. of informative characters	10	14	13	13	17	67
No. of most parsimonious trees	1	1	6	1	1	2
Tree length	10	15	15	13	17	72
Consistency index (CI)	1	0.933	0.867	1	1	0.931
Retention index (RI)	1	0.989	0.979	1	1	0.989

TABLE IV. Single nucleotide polymorphisams (SNPs) from the sequence data of ITS rDNA, EF-1α, β-tubulin, *BotF15* and RPB2 loci



* Isolates in bold are obtained from *S. cordaeum*. *Neofusicoccum ribis* = pink; *Neofusicoccum* sp. R1 = green; *Neofusicoccum* sp. R2 = yellow; *Neofusicoccum* sp. R3 = red; *N. parvum* = blue. (1, –) = Insertions /deletions (indels) irrespective of their size.

FIG. 1. The averages of the lengths and widths of ten conidia measured for each of 103 isolates representing *Neofusicoccum parvum* / *N. ribis* complex from *Syzygium cordatum*. The thirty isolates used for DNA sequence comparisons in this study were selected to represent the full range of conidial sizes and are indicated on the graph as unfilled squares.

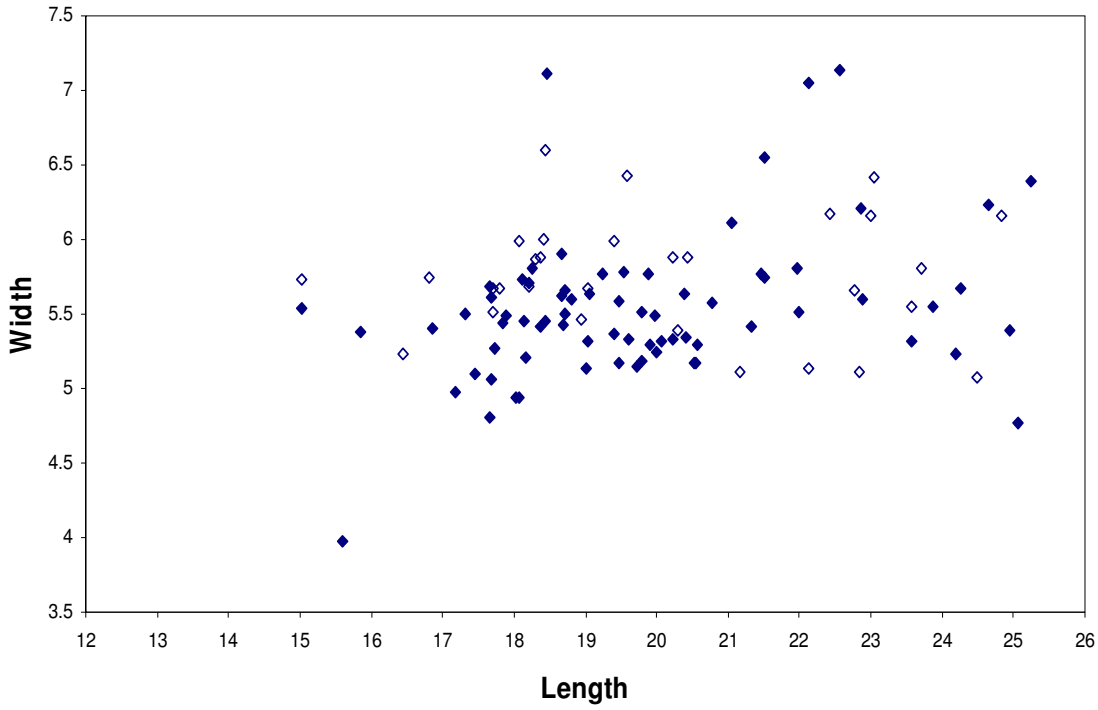
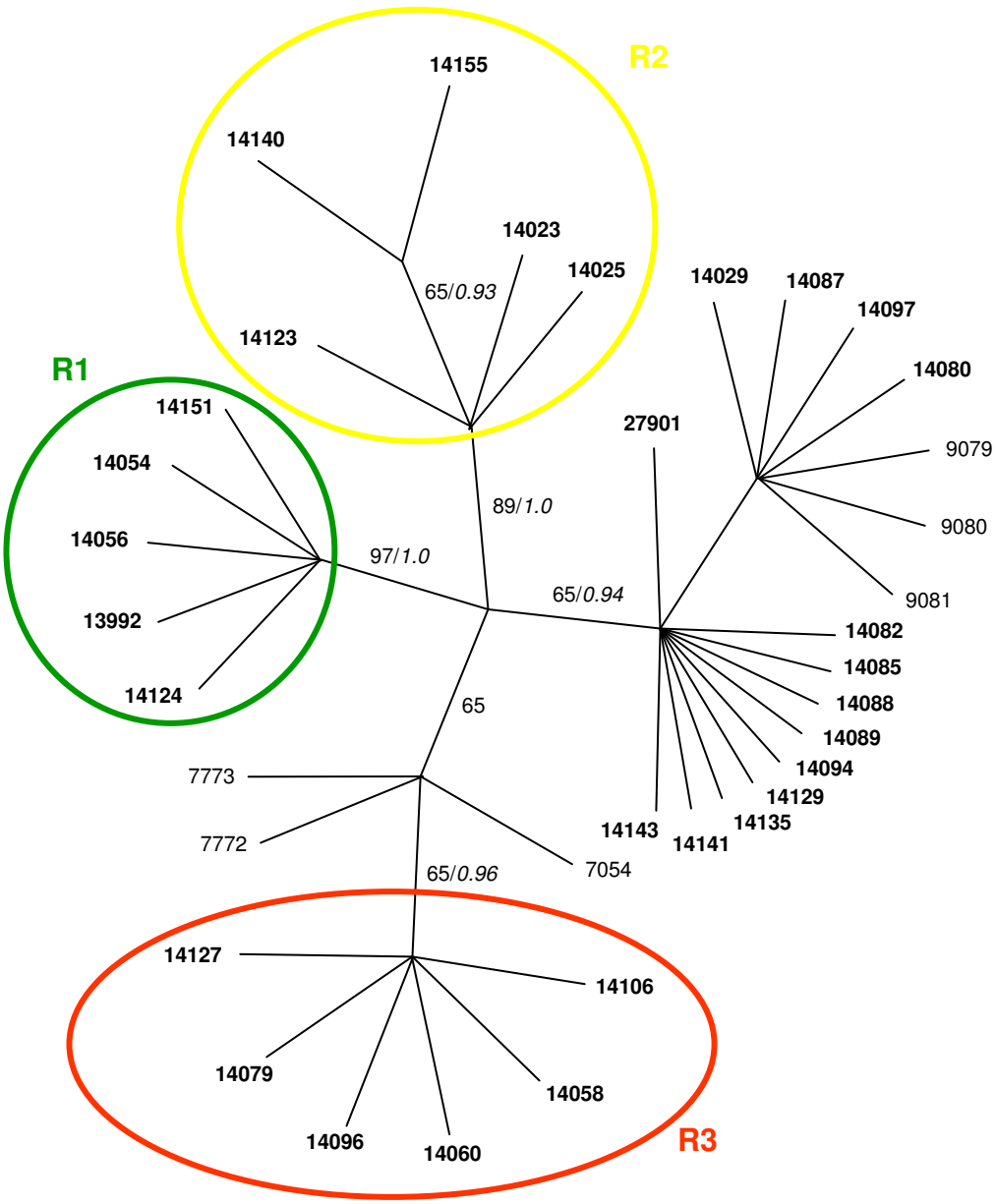
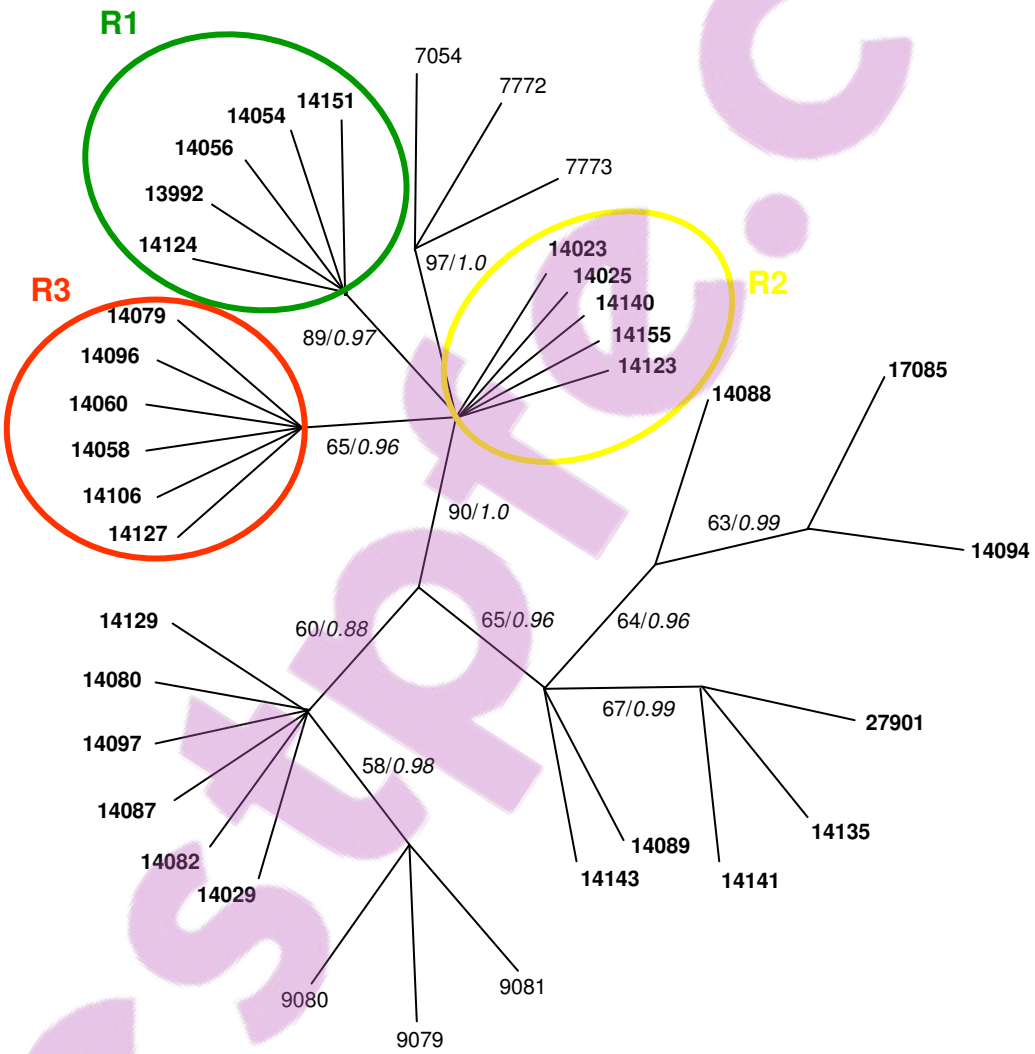

Width
Length
7.5
7
6.5
6
5.5
5
4.5
4
3.5
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26

FIG. 2. Unrooted maximum-parsimony trees resulting from the separate analysis of the sequence data of the ITS (a), EF-1α (b), Bt2 regions of the β-tubulin gene (c), locus *BotF15* (d) and RPB2 (e). Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates of the *Neofusicoccum* spp. obtained from *S. cordatum* are indicated in bold.

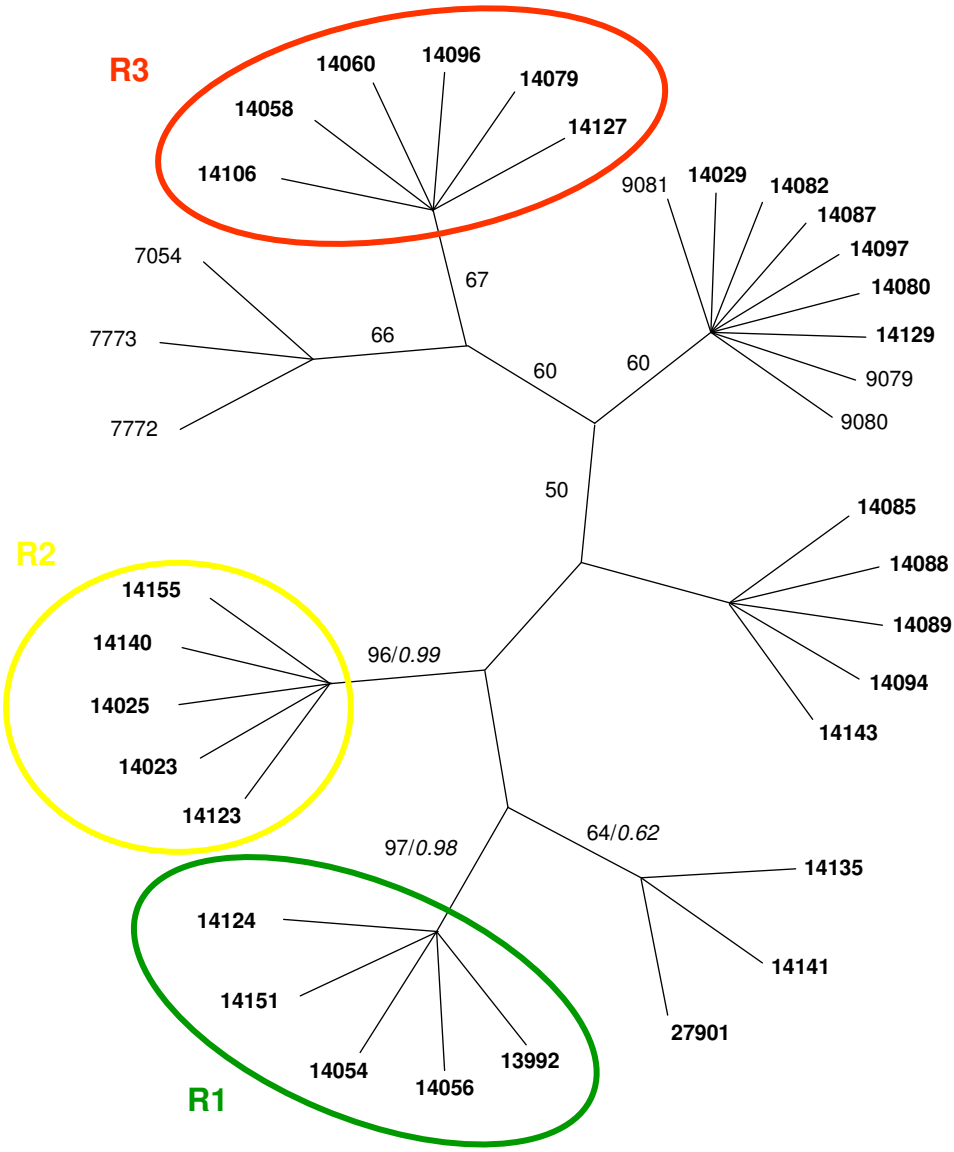
(a)


R2
14155
14140
14023
14025
65/0.93
14123
R1
14151
14054
14056
13992
14124
97/1.0
89/1.0
65/0.94
65
14029
14087
14097
14080
9079
9080
9081
27901
14082
14085
14088
14089
14094
14129
14135
14143
14141
7773
7772
7054
65/0.96
14127
14106
14079
14058
14096
14060
R3

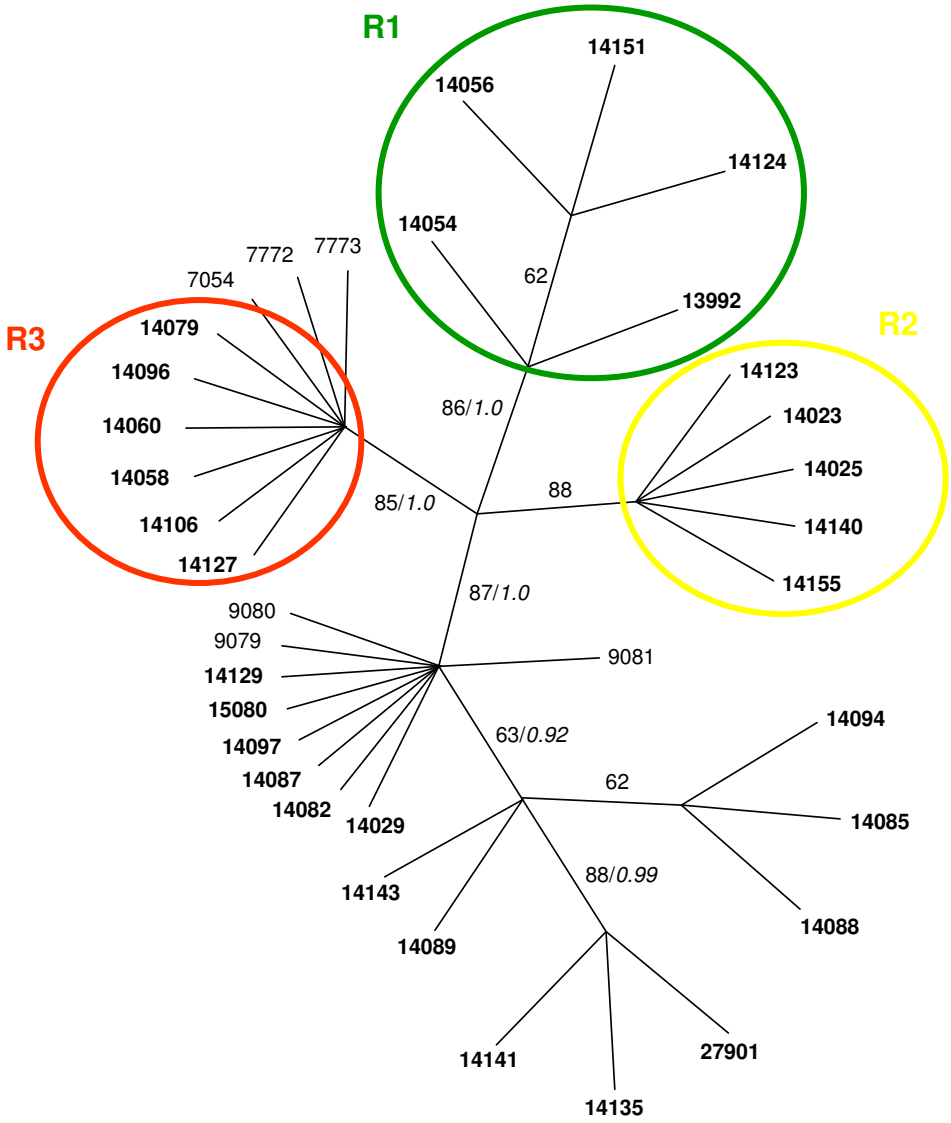
(b)



(c)


R3
14060
14096
14079
14058
14127
14106
9081
14029
14082
14087
14097
14080
14129
9079
9080
7054
67
7773
66
60
60
7772
50
14085
14088
14089
14094
14143
R2
14155
14140
96/0.99
14025
14023
14123
97/0.98
64/0.62
14135
14124
14141
14151
27901
14054
14056
13992
R1

(d)



(e)

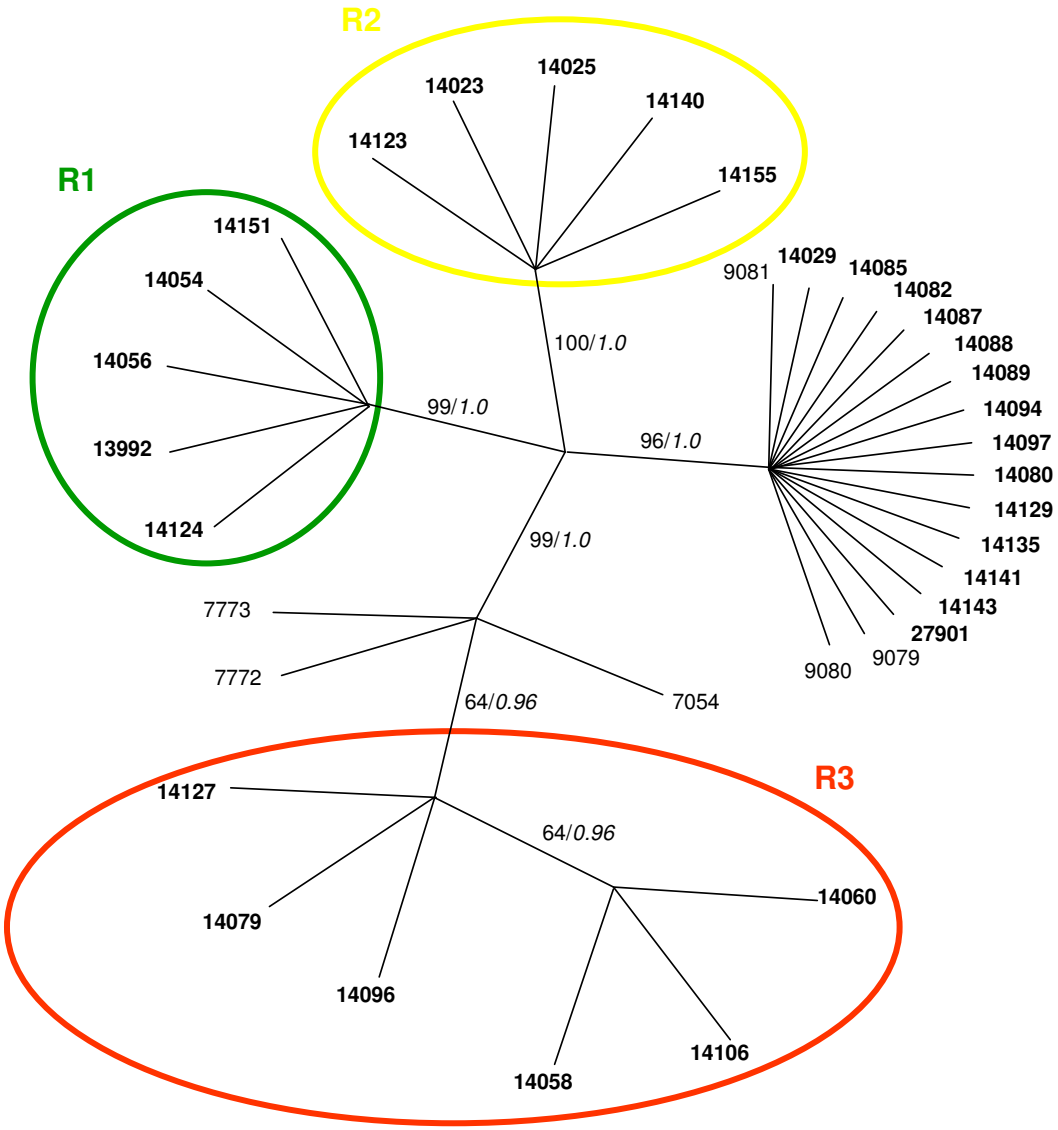
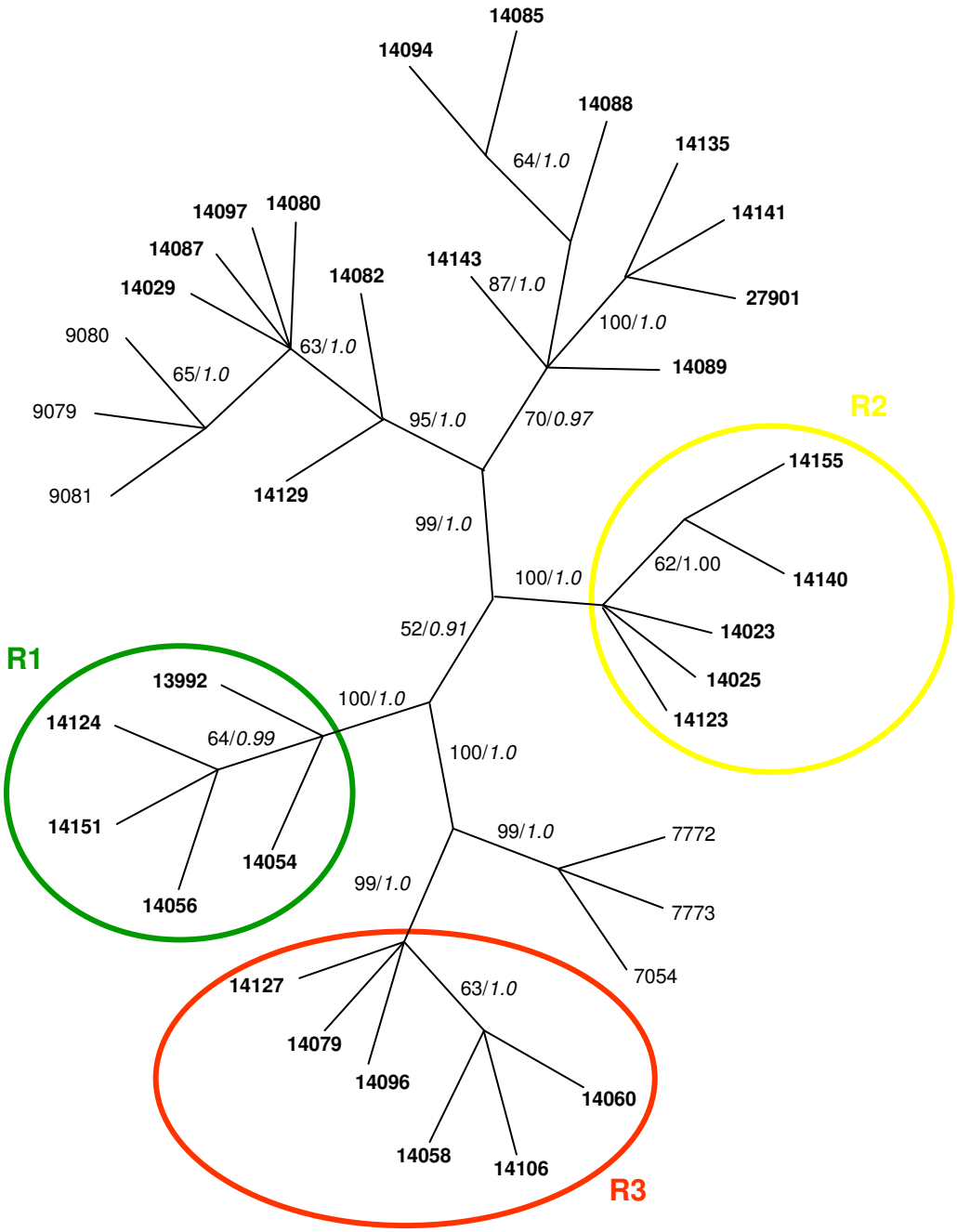

R2
14025
14023
14140
14123
14155
R1
14151
14054
14056
13992
14124
100/1.0
99/1.0
96/1.0
99/1.0
9081
14029
14085
14082
14087
14088
14089
14094
14097
14080
14129
14135
14141
14143
27901
9079
9080
7773
7772
7054
64/0.96
R3
14127
64/0.96
14060
14079
14096
14106
14058

FIG. 3. One of two unrooted maximum-parsimony trees resulting from the analysis of the combined sequence data. Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates of the *Neofusicoccum* spp. obtained from *S. cordatum* are indicated in bold.


14085
14094
14088
14135
64/1.0
14141
14097
14080
14087
14143
14082
87/1.0
14029
27901
100/1.0
9080
63/1.0
14089
65/1.0
9079
95/1.0
70/0.97
R2
14155
9081
14129
99/1.0
62/1.00
14140
100/1.0
52/0.91
14023
R1
14025
13992
100/1.0
14123
14124
64/0.99
100/1.0
14151
99/1.0
7772
14054
99/1.0
7773
14056
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14127
63/1.0
14079
14096
14060
14058
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R3

Chapter 3

Molecular and phenotypic characterisation of three phylogenetic species discovered within the *Neofusicoccum parvum / N. ribis* complex

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ABSTRACT

Neofusicoccum parvum and *N. ribis* are closely related species whose identities have often been confused. These fungal plant pathogens were recently identified as the most abundant species of Botryosphaeriaceae (Ascomycetes) isolated from native *Syzygium cordatum* trees in South Africa. In another study using multiple gene genealogies from five nuclear loci, three undescribed cryptic phylogenetic species, as well as *N. parvum,* were identified among thirty of these isolates. The aim of this study was to clarify the identity of the remaining isolates in the *N. parvum* / *N. ribis* complex from *S. cordatum* in South Africa, to describe newly identified cryptic species and to test their pathogenicity. Based on the RNA polymerase II subunit (RPB2) sequence comparisons the isolates were identified as *N. parvum* or one of three previously recognized phylogenetic species that are described here as *N. cordaticola, N. kwambonambiense* and *N. umdonicola*. These species cannot be separated *a priori* based on morphological characteristics, although *a posteriori* analysis of variance showed that the differences in conidial length and width between the species were statistically significant. The isolates of the newly described species as well as *N. parvum* and *N. ribis* were tested for pathogenicity on *S. cordatum* under greenhouse conditions. Isolates representing the three new species were significantly more aggressive than *N. parvum* and *N. ribis*, with *N. kwambonambiense* being the most aggressive. This study resolved long-standing questions of identity of species within *N. parvum* / *N. ribis* complex and lays a foundation for further studies on this group of pathogens.

INTRODUCTION

The phylogenetic species concept (PSC) (Taylor et al 2000) and genealogical concordance phylogenetic species recognition (GCPSR) have been increasingly applied in studies of species boundaries in both human and plant pathogenic fungi (e.g. Koufopanou et al 1997, Geiser et al 1998, O'Donnell et al 2000a, b, Steenkamp et al 2002, O'Donnell et al 2004, Pringle et al 2005). In these studies, using GCPSR based on concordance of multiple gene sequence genealogies, numerous cryptic species and species complexes were revealed in fungal taxa previously identified as one morphospecies. GCPSR was also used with good results in the detection of cryptic species within Botryosphaeriaceae, e.g. *Diplodia scrobiculata* as a sister species of *D. pinea* (de Wet et al 2003) and *Neofusicoccum eucalypticola* and *N. australe* as sister species of *N. eucalyptorum* and *N. luteum* respectively (Slippers et al 2004b, c). The cryptic species recognized in these studies could not have been acknowledged based on morphology or single-locus data alone, methods commonly used for identification of Botryosphaeriaceae (e.g. Jacobs and Rehner 1998, Denman et al 2000, Smith et al 2001, Zhou and Stanosz 2001, Pavlic et al 2004).

Neofusicoccum parvum and *N. ribis* are closely related cryptic species within the recently described genus *Neofusicoccum* (Botryosphaeriaceae, Ascomycetes) (Slippers et al 2004a, Crous et al 2006). Although known to develop teleomorph (sexual) structures, these fungi are commonly encountered in their anamorph (asexual) stage (Pennycook and Samuels 1985, Slippers et al 2004a, Pavlic et al 2007). The cosmopolitan distribution, sympatric occurrence on native and non-native hosts, as well as plasticity and overlap in the morphological characteristics of both their teleomorphs and anamorphs, make these species difficult to distinguish based upon morphological, ecological and geographical criteria. Consequently, these plant pathogens have often been mistaken for each other. These species could also not be separated with confidence based on ITS sequence data alone, the method most commonly used in molecular identification and phylogenetic analyses of the Botryosphaeriaceae (Smith et al 2001, Zhou and Stanosz 2001, Slippers et al 2005, Pavlic et al 2007).

Nucleotide sequence data from multiple genes were used to distinguish the identity of the type specimens of *N. parvum* and *N. ribis* (Slippers et al 2004a). However, when more isolates were included in subsequent analyses, many clustered intermediate to the type, but did not clearly cluster with either of these species (Ahumada 2002, Slippers 2003, Slippers et al 2005, Rodas et al 2009). These isolates have been referred to as the *N.* parvum / *N. ribis*

complex. Isolates that belong to the *N. parvum* / N. *ribis* complex could be separated into two groups using a PCR-RFLP fingerprinting technique. They were then referred to as *N. parvum sensu lato* and *N. ribis sensu lato* (Slippers 2003). It was not clear in those studies, however, whether these groups comprise more than one cryptic species or represent inter-specific variation.

Neofusicoccum parvum sensu lato and *N. ribis sensu lato* were recently identified as the most abundant species of Botryosphaeriaceae isolated from native *Syzygium cordatum* (Myrtaceae) in South Africa (Pavlic et al 2007). In a subsequent study using multiple gene genealogies of five nuclear loci, three undescribed cryptic phylogenetic species, as well as *N. parvum,* were identified among these isolates (Pavlic et al 2009). None of the isolates were identified as *N. ribis*. In this study, we characterise a larger collection of these isolates using genotypic data and combine this with phenotypic characteristics such as conidial morphology and pathogenicity to describe the taxa. Consequently, three new phylogenetically recognised cryptic species within the *Neofusicoccum parvum* / *N. ribis* species complex are described here as *N. cordaticola* sp. nov. , *N. umdonicola* sp. nov. and *N. kwambonambiense* sp. nov.

MATERIALS AND METHODS

Isolates

The 103 isolates used in this study were collected during the survey of the Botryosphaeriaceae on native *S. cordatum* in South Africa from 2001 to 2003 (TABLE I). The collection spanned the north to south natural distribution of *S. cordatum* in South Africa, from Tzaneen in the Northern Province to Gonubie in the Eastern Cape Province. Isolations were made from dying twigs and asymptomatic, visually healthy twigs and leaves, as described in Pavlic et al (2007). Isolations were also made from visually healthy fruits. Fruits were washed in running tap water and surface disinfected by spraying them with 70 % ethanol and left dried on filter paper. The disinfected fruits were halved and pieces from the fruit pulp (2 mm^2) were placed on 2 % malt extract agar (MEA) and incubated and maintained as described in Pavlic et al (2007). All cultures used in this study have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and representative isolates have been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA sequence comparisons

Thirty isolates from *S. cordatum* were selected and identified in a previous study (Pavlic et al 2009) as *N. parvum* or one of the undescribed phylogenetic species termed as *Neofusicoccum* sp. R1, R2 and R3. This distinction was based on multiple gene genealogies of DNA sequence data for five nuclear loci, including the internal transcribed spacer rDNA (ITS1, 5.8S, and ITS2), partial translation elongation factor 1α (EF-1α), β-tubulin-2 (βt-2a/b), a portion of the RNA polymerase II subunit (RPB2) and locus *BotF15* (an unknown locus containing a simple sequence repeat), and the results were compared with a single gene sequence data. The RPB2 region was found to contain the most informative characters considering fixed single nucleotide polymorphisms (SNPs) in each species. Following the same protocol as Pavlic et al (2009), a portion of the RNA polymerase II subunit (RPB2) was sequenced for the remaining 73 isolates. The type specimens and two specimens related to the types of *N. parvum* and *N. ribis* were included for comparison. The nucleotide sequences from one strand were examined with SEQUENCE NAVIGATOR version 1.0.1. software (Perkin-Elmer Applied BioSystems, Inc., Foster City, California) and alignments were prepared online using MAFFT version 5.667 (http://timpani.genome.ad.jp/~mafft/server/) (Katoh et al 2002) to compare it to the data from Pavlic et al (2009).

Phylogenetic analyses

A maximum-parsimony (MP) tree was constructed in PAUP version 4.0b10 (Swofford 2000) using the heuristic search function, with 1000 random addition replicates and tree bisection and reconstruction (TBR) selected as branch swapping algorithm. Gaps were treated as fifth characters and all characters were unordered and of equal weight. Branches of zero length were collapsed and all multiple equally parsimonious trees were saved. To estimate branch support, maximum parsimony bootstrap values were determined using 1000 bootstrap replicates (Felsenstein 1985).

Bayesian analyses were performed using MrBayes v. 3.0b4 (Ronquist and Huelsenbeck 2003) and the best-fitting evolutionary model was estimated using MrModeltest v. 2.2 software (Nylander 2004). The Markov Chain Monte Carlo (MCMC) chains were initialised from a random tree and were run for two million generations and trees were saved every hundred generations, counting twenty thousand trees. Burn-in was set at one thousand generations, leaving just over thirty eight thousand (38002) trees from which

the consensus tree was calculated. To determine the confidence of the tree topologies, values of Bayesian posterior probabilities (BPPs) (Rannala and Yang 1996) were estimated using MrBayes (Ronquist and Huelsenbeck 2003).

Morphological characteristics

In an earlier study, the 103 isolates (TABLE I) were induced to sporulate in culture as described in Pavlic et al (2007). Conidia were mounted in lactophenol on microscope slides and inspected by light microscopy. Ten measurements of conidial lengths and widths were taken for each isolate and the ranges and averages, as well as length and width ratio were calculated. Measurements were made and digital photographs taken with a HRc Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss Ltd., Munich, Germany). SAS® version 8.2 undmc vm/cms statistical software was used to analyse variability in conidial lengths and widths between the isolates. Single conidial cultures grown on 2 % malt extract agar (MEA) at 25 ºC under continuous near fluorescent light were used to characterise culture morphology as described previously (Pavlic et al 2007).

Pathogenicity

A total of twenty isolates representing the three new *Neofusicoccum* species and *N. parvum* identified from *S. cordatum*, as well as type specimens of *N. parvum* and *N. ribis* (TABLE I) were selected for pathogenicity trials under greenhouse conditions. Isolates obtained from *S. cordatum* were randomly selected for inoculations and all isolates were grown on 2 % MEA at 25 ºC under continuous near fluorescent light for seven days prior to inoculation.

Twenty-month old *S. cordatum* saplings were grown in the pots in an open plant nursery and moved into the greenhouse for acclimatization four weeks prior to inoculations. The greenhouse temperature was constant (25 ºC) and regular day/night conditions were kept. Trees were inoculated during the Spring–Summer season (October–November 2007). Each isolate was inoculated into stems of ten trees and ten trees were inoculated with sterile MEA plugs as a control. The inoculations were carried out following the procedure described by Pavlic et al (2007). The inoculated trees were arranged in a randomized block design. The trial was repeated under the same conditions.

Tree diameter at the inoculation height and the length of the lesion developed six weeks after inoculations were measured. SAS® version 8.2 undmc vm/cms statistical software was used to analyse variability in lesion lengths between the isolates. We modelled

lesion length as a linear function of greenhouse, fungal species, and isolates nested within the species, interaction of greenhouses and fungal species, and interaction of greenhouses and isolates nested within the species. This model was repeated using tree diameter as the co-variable. The 95 % confidence limits were determined for all means based on full model analysis of variance (ANOVA). Differences between means were considered significant at the $P \leq 0.05$ level.

RESULTS

Phylogenetic analyses

The sequence alignment consisted of 550 characters of which 16 were parsimony informative and were included in the analyses. The parsimony analyses resulted in one most parsimonious tree (CI = 1.0, RI = 1.0) (FIG. 1). MrModeltest v2.2 predicted K80 as an appropriate evolutionary model for Bayesian analyses. The topologies of the trees were identical in the maximum-parsimony and Bayesian consensus analyses. Therefore, only the consensus tree derived from Bayesian analyses is presented, with the parsimony bootstrap values and the posterior probabilities shown at the branches (FIG. 1). The sequences of *N. ribis* were used as an out-group. In-group taxa formed four distinct clades of which one corresponded to *N. parvum*, while the other three clades represent distinct lineages referred to as R1, R2 and R3. The isolates from *S. cordatum* considered in this study grouped within the *N. parvum* clade (n = 43), and clades R1 (n = 15), R2 (n = 14) and R3 (n = 31).

The sequences obtained in this study have been deposited in GenBank (TABLE I). The sequence alignment and phylogenetic trees have been deposited in TreeBASE as SN4175.

Morphological characteristics

No differences were observed in cultural morphology among the isolates of the different *Neofusicoccum* species analysed in this study. Cultures were initially white with fluffy aerial mycelium, turning pale olivaceous grey from the middle of colony after 3–4 days. They formed thick aerial mycelium, occasionally with columns of the mycelium in the middle of colony reaching the lid. The margins were regular with the reverse sides of the colonies olivaceous grey to black.

Conidial dimensions (lengths and widths) of isolates that belong to the *N. parvum* / N. *ribis* complex from *S. cordatum* are highly variable and overlap among newly recognised species (FIG. 2). As such, these characteristics cannot be used for morphological species

recognition *a priori*. However, *a posteriori* analysis of variance showed that the differences in conidial length and width among phylogenetically recognised species in the *N. parvum* / *N. ribis* complex were statistically significant ($P \leq 0.001$). Therefore, conidial measurements are included in the description of newly recognised phylogenetic species. On average, conidia of *Neofusicoccum* sp. R1 and R2 are longer than those of *Neofusicoccum* sp. 3, and with rounded apices. Conidia of *Neofusicoccum* sp. R1 are on average longer and narrower with a higher length to width ratio than those of *Neofusicoccum* sp. R2, which are shorter and wider with lower length to width ratio. *Neofusicoccum* sp. R3 differ from the *Neofusicoccum* sp. R1 and R2 by conidia that are on average shorter with tapered apices, but they overlap in shape and size with those of *N. parvum* identified in this study, as well as *N. parvum* and *N. ribis* described in previous studies (Slippers et al 2004a). Although the conidia of different ages (2–6 weeks) were examined, as well as after discharge from pycnidia and until germination, no septate conidia were observed for any of newly recognised species or *N. parvum*.

Pathogenicity

All isolates induced lesions on stems of *S. cordatum* saplings within six weeks demonstrating potential pathogenicity of all species. The respective *Neofusicoccum* species that were re-isolated from the edge of the lesions on the inoculated trees were the same as those used for inoculations. Small lesions developed on some trees inoculated with a sterile MEA plugs as controls. No species of Botryosphaeriaceae were re-isolated from controls. Therefore the lesions associated with the controls are considered as reaction of trees to inoculation wounds.

Analyses of variance showed that the interactions between mean lesion lengths produced in two trials were statistically significant ($P \leq 0.05$) and therefore data from these trials could not be combined. Data for both trials are presented on the same graph (FIG. 3). Statistical analyses showed that there was no correlation between tree diameter and lesion length. With exception of two *N. parvum* isolates (CMW14143, CMW9079), one isolate of *N. ribis* (CMW7054) and another of *Neofusicoccum* sp. R1 (CMW14151), all the other isolates in trial one produced lesions significantly different from the controls (FIG. 3). Lesions produced by four isolates of *N. parvum* (CMW14080, CMW14143, CMW9079, CMW9080), one of *N. ribis* (CMW7054) and another of *Neofusicoccum* sp. R2 (CMW14140) in the second trial were not significantly different from the control (FIG. 3).

All the other isolates in the second trial produced lesions significantly different from the controls (FIG. 3). Intra-specific variation in mean lesion length was observed for all four species obtained from *S. cordatum* and at the 95 % significance level for some of isolates in both trials (FIG. 3). Mean lesion lengths produced by some of the isolates (CMW14097, 14140, 14155, 14058, 14106) differed significantly between two trials (FIG. 3). In such cases, significantly smaller lesions were observed on the trees that were in better conditions.

TAXONOMY

Based on combined sequence data of five gene regions, four phylogenetic groups were recognised within the *N. parvum* / *N. ribis* species complex from native *S. cordatum* in South Africa. Three of these groups are closely related, but clearly separated from *N. parvum* and *N. ribis* and are recognised as three undescribed phylogenetic species. These species can only be consistently diagnosed based on genotypic characters. The three new phylogenetic species are therefore described here as follows:

Neofusicoccum cordaticola MB512498 Pavlic, Slippers, M.J. Wingfield, sp. nov.
= *Neofusicoccum* sp. R1 *sensu* Pavlic et al Mol Phylogenet Evol 51: 259–268 (2009)

N. cordaticola speciebis aliis in complexo specierum *N. parvi* / N. *ribis* similis; conidia *N. cordaticola* hyalina unicellularia anguste fusiformia vel ovalia apicibus rotundatis 18–28 × 4.5–7 µm. *N. cordaticola* a speciebus aliis locis 5 nuclearibus differt: ITS1, 5.8S, et ITS2 sitibus 141 (C), 372 (G), et 416 (C); loco 'translation elongation factor (1α)' dicto sitis 58 (C) et 221 (C); loco 'β-tubulin-2' dicto sitis 32 (T), 96 (T) et 316 (G); loco *BotF15* sitis 121 (T) et 122 (C); et loco 'RNA polymerase II subunit' dicto sitis 100 (A), 112 (T), 265 (A) et 409 (C).

Neofusicoccum cordaticola is morphologically similar to other species in the *N. parvum* / N. *ribis* species complex. Conidia of *N. cordaticola* are hyaline, unicellular, narrowly fusiform to oval, apices rounded 18–28 × 4.5–7 µm (av. of 150 conidia 23.3 × 5.3 µm, l/w 4.3). *N. cordaticola* differs from other species in the *N. parvum / N. ribis* complex by uniquely fixed nucleotides in five nuclear loci: internal transcribed spacer rDNA (ITS1, 5.8S, and ITS2) position 141 (C), 372 (G), and 416 (C); translation elongation factor (1α) positions 58 (C), and 221 (C); β-tubulin-2 position 32 (T), 96 (T), and 316 (G); locus *BotF15* position 121 (T), and 122 (C); RNA polymerase II subunit positions 100 (A), 112 (T), 265 (A), and 409 (C).

Teleomorph. Not known.

Etymology. Refers to the host *Syzygium cordatum* from which isolates were collected, (*[in]cola* = an inhabitant).

Habitat: Symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum*.

Known distribution: South Africa.

HOLOTYPE. SOUTH AFRICA. KWAZULU NATAL PROVINCE: Sodwana bay, on *Syzygium cordatum,* Mar 2002, D. Pavlic, (PREM 60066, a dry culture ex CMW 13992 on pine needles; ex-type culture CMW 13992 = CBS 123634).

Additional specimens examined. See TABLE I.

Neofusicoccum kwambonambiense MB512499 Pavlic, Slippers, M.J. Wingfield, sp. nov. = *Neofusicoccum* sp. R2 *sensu* Pavlic et al Mol Phylogenet Evol 51: 259–268 (2009)

N. kwambonambiense speciebis aliis in complexo specierum *N. parvi* / *N. ribis* similis; conidia *N. kwambonambiense* hyalina unicellularia fusiformia vel ellipsoidia apicibus rotundatis 16–28 × 5–8 µm. *N. kwambonambiense* a speciebus aliis locis 4 nuclearibus differt: ITS1, 5.8S, et ITS2 sitibus 163 (T) et 173 (G); loco 'β-tubulin-2' dicto sitis 175 (T), 235 (A), et 251 (A); loco *BotF15* sitis 87 et 172; loco 'RNA polymerase II subunit' dicto sitis 49 (G), 382 (A), 421 (A), et 526 (C).

Neofusicoccum kwambonambiense is morphologically similar to other related species in the *N. parvum / N. ribis* species complex. Conidia of *N. kwambonambiense* are hyaline, unicellular, fusiform to ellipsoid, apices rounded 16–28 × 5–8 µm (av. of 140 conidia 22.3 × 6.3 µm, l/w 3.6). *N. kwambonambiense* differs from other species in the *N. parvum / N. ribis* complex by uniquely fixed nucleotides in four nuclear loci: internal transcribed spacer rDNA (ITS1, 5.8S, and ITS2) position 163 (T), and 173 (G); β-tubulin-2 position 175 (T), 235 (A), and 251 (A); locus *BotF15* position 87, and 172; RNA polymerase II subunit positions 49 (G), 382 (A), 421 (A), and 526 (C).

Teleomorph. Not known.

Etymology. Refers to the town Kwambonambi, South Africa from where the type isolate was collected.

Habitat: Symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum*.

Known distribution: South Africa.

HOLOTYPE. SOUTH AFRICA. KWAZULU NATAL PROVINCE: Kwambonambi, on *Syzygium cordatum,* Mar 2002, D. Pavlic, (PREM 60067, a dry culture ex CMW 14023 on pine needles; ex-type culture CMW 14023 = CBS 123639).

Additional specimens examined. See TABLE I.

Neofusicoccum umdonicola MB512500 Pavlic, Slippers, M.J. Wingfield, sp. nov.
= *Neofusicoccum* sp. R3 *sensu* Pavlic et al Mol Phylogenet Evol 51: 259–268 (2009)

N. umdonicola speciebis aliis in complexo specierum *N. parvi / N. ribis* similis; conidia *N. umdonicola* hyalina unicellularia fusiformia vel ovalia apicibus angustatis 15–23.5 × 4.5–6.5 µm. *N. umdonicola* a speciebus aliis locis 4 nuclearibus differt: ITS1, 5.8S, et ITS2) situ 168 (C); loco 'translation elongation factor (1α)' dicto situ 62 (T); loco 'β-tubulin-2'dicto situ 40 (A); loco 'RNA polymerase II subunit' dicto situ 280 (T).

Neofusicoccum umdonicola is morphologically similar to other related species in the *N. parvum / N. ribis* species complex. Conidia of *N. umdonicola* are hyaline, unicellular, fusiform to oval, apices tapered 15–23.5 × 4.5–6.5 µm (av. of 310 conidia 19.4 × 5.5 µm, l/w 3.5). *N. umdonicola* differs from other species in the *N. parvum / N. ribis* complex by uniquely fixed nucleotides in four nuclear loci: internal transcribed spacer rDNA (ITS1, 5.8S, and ITS2) position 168 (C); translation elongation factor (1-α) positions 62 (T); β-tubulin-2 position 40 (A); RNA polymerase II subunit position 280 (T).

Teleomorph. Not known.

Etymology. Refers to common Zulu and also KZN-english name, Umdoni for the *Syzygium cordatum*, the host from which isolates were obtained, (*[in]cola* = an inhabitant).

Habitat: Symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum*.

Known distribution: South Africa.

HOLOTYPE. SOUTH AFRICA. KWAZULU NATAL PROVINCE: Kosi bay, on *Syzygium cordatum,* Mar 2002, D. Pavlic, (PREM 60068, a dry culture ex CMW 14058 on pine needles; ex-type culture CMW 14058 = CBS 123645).

Additional specimens examined. See TABLE I.

DISCUSSION

In this study we described three phylogenetic species within the *N. parvum / N. ribis* species complex from native *S. cordatum* in South Africa, namely *Neofusicoccum cordaticola, N.*

kwambonambiense and *N. umdonicola*. These species were recognized previously (Pavlic et al 2009) using the genealogical concordance phylogenetic species recognition (GCPSR) as a form of phylogenetic species concept (PSC) (Taylor et al 2000), based on DNA sequence data for five nuclear loci. The phylogenetic species are characterized primarily by fixed single nucleotide polymorphisms (SNPs) (O'Donell et al 2004, Grünig et al 2008) that were identified for each of three species described in this study. Although many cryptic, phylogenetic species have been recently recognized in the fungal kingdom, there are very few descriptions of these species. This is the first description of phylogenetic species in the Botryosphaeriaceae using sequence data as defining characters.

Neofusicoccum umdonicola is the sister species to *N. ribis*. Despite the fact that these two species can be distinguished using DNA sequence data from multiple loci, these two species cannot be separated from each other, or from *N. parvum* using conidial morphology observed in this study. Slippers et al (2004a) used septation of conidia to distinguish *N. parvum* and *N. ribis*, but such septa were not observed in this study for any of the newly described species or *N. parvum*. Since conidial septation is not a constant character in these *Neofusicoccum* spp. it cannot be used as a reliable feature in separation and identification of these species.

The pathogenicity trials showed that *N. umdonicola* is the most aggressive to *S. cordatum* of all five species tested in this study. There is no significant difference in pathogenicity between *N. cordaticola* and *N. kwambonambiense* to *S. cordatum*, but they both appear to be significantly more aggressive to this host then *N. parvum* and *N. ribis.* Barring *N. ribis*, all of these species were isolated from *S. cordatum* growing in close association with commercially grown *Eucalyptus* plantations in South Africa. In an earlier study isolates of *N. parvum*, *N. cordaticola* and *N. kawambonambiense* (the latter two species were then identified as *N. ribis sensu lato* (Pavlic et al 2007)) were recognized as more aggressive to *Eucalyptus* than to *S. cordatum* in greenhouse trials. In the field pathogenicity trials on different *Eucalyptus* clones grown commercially in Venezuela (Mohali et al 2009) and Colombia (Rodas et al 2009), isolates identified as '*N. ribis*' were shown to be highly aggressive to *Eucalyptus*. It is possible that some of these isolates represent cryptic species in the *N. parvum / N. ribis* complex. The trials conducted on different *Eucalyptus* clones in Venezuela showed that *N. parvum* was significantly more aggressive than *N. ribis* (Mohali et al 2009). Clearly, most of the members of the *N. parvum / N. ribis* complex have potential to become important pathogens to native and commercially grown Myrtaceae.

All three new species grow endophytically on different parts of *S. cordatum* tree. These include symptomless twigs, leaves and fruits. More than one species were commonly found within a single tree and even within one leaf or one fruit. Species of Botryosphaeriaceae are known as endophytes that grow within different plant tissues without exhibiting any disease symptoms (Smith et al 1996, Pavlic et al 2004, Slippers and Wingfield 2007) and were also identified as seed-born, for example *N. parvum* in *Podocarpus falcatus* and *Prunus africana* seeds (Gure et al 2005). As endophytes they can be easily moved into new regions and pose an equally serious threat to native and cultivated plants alike (Burgess and Wingfield 2002, Slippers and Wingfield 2007). Occurrence of more than one species within a small piece of plant tissue or in one fruit of *S. cordatum* implies that more than one species can be easily introduced into a new area with this plant material. This is important, given that these new species are more aggressive than known species *N. parvum* and *N. ribis* on *Syzygium*.

The correct identification of plant pathogenic fungi is of utmost importance for quarantine and control measures. A PCR-RFLP fingerprinting technique was developed to distinguish *sensu lato* groups of *N. parvum* and *N. ribis* (Slippers 2003). Recently, Alves et al (2007) designed MSP-PCR (microsatellite-primed polymerase chain reaction) and rep-PCR (repetitive-sequence-based polymerase chain reaction) fingerprinting methodologies for rapid identification of species of Botryosphaeriaceae, including closely related species such as *N. parvum* and *N. ribis*, or *N. luteum* and *N. australe*. Such PCR based methodologies are quick and reliable for the identification of large numbers of isolates and development of such methods for the identification of new *Neofusicoccum* species should be considered in future studies. The isolates recognized in previous studies (Slippers 2003, Slippers et al 2005, Mohali et al 2009, Rodas et al 2009) as *N. ribis sensu lato* group, based on PCR-RFLP profiles, should be re-evaluated since these groups can comprise cryptic species, such as those described in this study. As it was shown in Pavlic et al. (2009), the RPB2 sequences are the most valuable for delimitation of these cryptic species and should be used in further identification and re-evaluation of species in the *N. parvum* / *N. ribis* complex.

In many studies on Botryosphaeriaceae, preliminary groupings of isolates have been based on cultural and conidial morphology (e.g. Slippers et al 2004a, Burgess et al 2005, Pavlic et al 2007, 2008). In those studies, groups identified based on morphological characters were usually found congruent with those recognized based on DNA sequence data and *vice versa*. Interestingly, in our earlier study on Botryosphaeriaceae from *S. cordatum* in South Africa, differences in conidial morphology were used to select isolates from *N.*

parvum / *N. ribis* group for further ITS rDNA sequencing (Pavlic et al 2007). Groups recognized based on differences in conidial morphology were consistent with groupings observed within *N. parvum* / *N. ribis* clade based on ITS sequences. These observations initiated further study on this group of isolates and recognition of cryptic species based on multiple gene genealogies (Pavlic et al 2009). Despite its use in selection of isolates for further study, the variation amongst the larger group of isolates was continuous and overlapping between what was later identified as distinct species. The morphological characters alone were thus insufficient for confident identification of all isolates representing the species in the *N. parvum* / *N. ribis* complex.

The use of molecular tools and specifically DNA sequence data allowed us to detect and discriminate numerous new species. Without these powerful tools, closely related or cryptic species and species complexes would stay unrecognised. However, morphological and other phenotypic characteristics such as pathogenicity cannot be underestimated, because differences in these characteristics may indicate presence of cryptic species and present valuable data in their delimitation, as it is shown in this study. Thus, an integrated approach should be imperative in species delineation and identification of Botryosphaeriaceae as it was suggested in the other studies (Dayrat 2005, Roe and Sperling 2007).

The species described in this study are only recognised from native *Syzygium cordatum*. These species were not recognised during intensive studies on related or other non-native hosts grown in close proximity (Jacobs 2002, Slippers et al 2004b). This indicates that more studies should be focus on identification of fungal species on native trees. They are clearly a source of fungal diversity, which could serve as a source of inoculum onto economically important crops. Furthermore, such studies on fungi on native trees will give us an opportunity to extend our knowledge about the natural history, ecology and biogeography of fungal biodiversity that is at present poorly understood.

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TABLE I. Isolates considered in this study

Culture no. [1, 2, 3, 4]	Other no. [1]	Identity	Geographic origin [5]	Host	Substratum	GenBank [6] RPB2
CMW13992[a]	CBS123634	*Neofusicoccum cordaticola*	SA, Sodwana Bay	*Syzygium cordatum*	twig	*EU821928*
CMW14035[c]		*N. cordaticola*	SA, Kwambonambi	*S. cordatum*	twig	FJ389275
CMW14041		*N. cordaticola*	SA, Kwambonambi	*S. cordatum*	twig	FJ389277
CMW14042		*N. cordaticola*	SA, Kwambonambi	*S. cordatum*	twig	FJ389276
CMW14056[d]	CBS123635	*N. cordaticola*	SA, Kosi Bay	*S. cordatum*	twig	*EU821933*
CMW14054	CBS123636	*N. cordaticola*	SA, Mkuze	*S. cordatum*	twig	*EU821936*
CMW14144		*N. cordaticola*	SA, Sabie	*S. cordatum*	twig	FJ389269
CMW14145		*N. cordaticola*	SA, Sabie	*S. cordatum*	leaf	FJ389271
CMW14147		*N. cordaticola*	SA, Sabie	*S. cordatum*	leaf	FJ389270
CMW14148		*N. cordaticola*	SA, Sabie	*S. cordatum*	leaf	FJ389274
CMW14149		*N. cordaticola*	SA, Sabie	*S. cordatum*	leaf	FJ389268
CMW14150		*N. cordaticola*	SA, Sabie	*S. cordatum*	leaf	FJ389273
CMW14151	CBS123637	*N. cordaticola*	SA, Sabie	*S. cordatum*	twig	*EU821952*
CMW14152		*N. cordaticola*	SA, Sabie	*S. cordatum*	twig	FJ389272
CMW14124[h]	CBS123638	*N. cordaticola*	SA, Richards Bay	*S. cordatum*	fruit	*EU821955*
CMW14023	CBS123639	*Neofusicoccum kwambonambiense*	SA, Kwambonambi	*S. cordatum*	twig	*EU821930*
CMW14025[b]	CBS123640	*N. kwambonambiense*	SA, Kwambonambi	*S. cordatum*	twig	*EU821931*
CMW14031		*N. kwambonambiense*	SA, Kwambonambi	*S. cordatum*	twig	FJ389280
CMW14046		*N. kwambonambiense*	SA, Kwambonambi	*S. cordatum*	twig	FJ389282
CMW14136		*N. kwambonambiense*	SA, Tzaneen	*S. cordatum*	twig	FJ389286
CMW14140[g]	CBS123641	*N. kwambonambiense*	SA, Tzaneen	*S. cordatum*	twig	*EU821949*
CMW14153		*N. kwambonambiense*	SA, Sabie	*S. cordatum*	twig	FJ389285
CMW14154		*N. kwambonambiense*	SA, Sabie	*S. cordatum*	twig	FJ389283
CMW14155	CBS123645	*N. kwambonambiense*	SA, Sabie	*S. cordatum*	fruit	*EU821953*
CMW14156		*N. kwambonambiense*	SA, Sabie	*S. cordatum*	fruit	FJ389284
CMW14119		*N. kwambonambiense*	SA, Richards Bay	*S. cordatum*	fruit	FJ389279
CMW14120		*N. kwambonambiense*	SA, Richards Bay	*S. cordatum*	fruit	FJ389248
CMW14121		*N. kwambonambiense*	SA, Richards Bay	*S. cordatum*	fruit	FJ389281
CMW14123[h]	CBS123643	*N. kwambonambiense*	SA, Richards Bay	*S. cordatum*	fruit	*EU821954*
CMW13990[a]		*Neofusicoccum umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389310
CMW13991		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389293
CMW13993		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389306
CMW13994		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389300
CMW13995		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389298
CMW13997		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389289
CMW14006		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389295
CMW14007		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389303
CMW14106	CBS123644	*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	leaf	*EU821929*
CMW14008		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	leaf	FJ389287
CMW14010		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389304
CMW14012		*N. umdonicola*	SA, Sodwana Bay	*S. cordatum*	twig	FJ389290
CMW14016		*N. umdonicola*	SA, Kwambonambi	*S. cordatum*	twig	FJ389297
CMW14028		*N. umdonicola*	SA, Kwambonambi	*S. cordatum*	twig	FJ389294
CMW14055[d]		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389305
CMW14057		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389301
CMW14058	CBS123645	*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	*EU821934*
CMW14098		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389288

TABLE I. Continued

Culture no. [1, 2, 3, 4]	Other no. [1]	Identity	Geographic origin [5]	Host	Substratum	GenBank [6] RPB2
CMW14099		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389307
CMW14059		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389291
CMW14060	CBS123646	*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	*EU821935*
CMW14100		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389299
CMW14101		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389311
CMW14068		*N. umdonicola*	SA, Kosi Bay	*S. cordatum*	twig	FJ389309
CMW14047		*N. umdonicola*	SA, Mkuze	*S. cordatum*	twig	FJ389308
CMW14051		*N. umdonicola*	SA, Mkuze	*S. cordatum*	twig	FJ389292
CMW14096[e]		*N. umdonicola*	SA, Port St Johns	*S. cordatum*	leaf	*EU821943*
CMW14079[f]	CBS123647	*N. umdonicola*	SA, Gonubie	*S. cordatum*	leaf	*EU821945*
CMW14127	CBS123648	*N. umdonicola*	SA, Kwambonambi	*S. cordatum*	fruit	*EU821956*
CMW14125		*N. umdonicola*	SA, Kwambonambi	*S. cordatum*	fruit	FJ389296
CMW14126		*N. umdonicola*	SA, Kwambonambi	*S. cordatum*	fruit	FJ389302
CMW14018		*Neofusicoccum parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389333
CMW14019		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389317
CMW14021		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389321
CMW14022		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389322
CMW14024		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389320
CMW14027[b]		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389339
CMW14029		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	*EU821932*
CMW14030		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389319
CMW14032[c]		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389332
CMW14036		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389318
CMW14038		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389335
CMW14039		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389316
CMW14040		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389334
CMW14045		*N. parvum*	SA, Kwambonambi	*S. cordatum*	twig	FJ389314
CMW14081		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	FJ389338
CMW14082		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	*EU821937*
CMW14085	CBS123649	*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	leaf	*EU821938*
CMW14086		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	leaf	FJ389312
CMW14087		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	*EU821939*
CMW14088		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	*EU821940*
CMW14089		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	leaf	*EU821941*
CMW14090		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	FJ389336
CMW14091		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	leaf	FJ389337
CMW14092		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	FJ389315
CMW14093		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	FJ389323
CMW14094		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	*EU821942*
CMW14095		*N. parvum*	SA, Pietermaritzburg	*S. cordatum*	twig	FJ389329
CMW14097[e]	CBS123650	*N. parvum*	SA, Port St. Johns	*S. cordatum*	leaf	*EU821944*
CMW14080[f]	CBS123651	*N. parvum*	SA, Gonubie	*S. cordatum*	leaf	*EU821946*
CMW14112		*N. parvum*	SA, Tokai, Cape Town	*S. cordatum*	leaf	FJ389326
CMW14128		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389313
CMW14129		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	*EU821947*
CMW14130		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389327
CMW14133		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389330
CMW14134		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389328

TABLE I. Continued

Culture no. [1, 2, 3, 4]	Other no. [1]	Identity	Geographic origin [5]	Host	Substratum	GenBank [6] RPB2
CMW14135		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	*EU821948*
CMW14137		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389324
CMW14138		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389325
CMW14139		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	FJ389340
CMW14141[g]		*N. parvum*	SA, Tzaneen	*S. cordatum*	twig	*EU821950*
CMW14142		*N. parvum*	SA, Palaborwa	*S. cordatum*	twig	FJ389331
CMW14143	CBS123652	*N. parvum*	SA, Palaborwa	*S. cordatum*	twig	*EU821951*
CMW27901		*N. parvum*	SA, Pretoria	*S. cordatum*	twig	*EU821957*
CMW9079	ICMP7933	*N. parvum*	New Zealand	*Actinidia deliciosa*		*EU821961*
CMW9080	ICMP8002	*N. parvum*	New Zealand	*Populus nigra*		*EU821962*
CMW9081	ICMP8003	*N. parvum*	New Zealand	*Populus nigra*		*EU821963*
CMW7772		*Neofusicoccum ribis*	USA, New York	*Ribis* sp.		*EU821958*
CMW7773		*N. ribis*	USA, New York	*Ribis* sp.		*EU821959*
CMW7054	CBS121.26	*N. ribis*	USA, New York	*Ribis rubrum*		*EU821960*

[1] Abbreviations of culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures Utrecht, The Netherlands; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand.

[2] Isolates used in pathogenicity trials are given in bold.

[3] All isolates other than CMW9079, CMW9080, CMW9081, CMW7772, CMW7773, and CMW7054 were collected by D. Pavlic.

[4] Isolates of different *Neofusicoccum* spp. collected from a single tree or from one leaf, twig or fruit are marked with the same latter.

[5] SA = South Africa.

[6] Sequence numbers in italics were obtained from the GenBank public database. All others were obtained in this study.

FIG. 1. Consensus phylogram of 38002 trees resulting from Bayesian analyses of the RNA polymerase II subunit (RPB2) sequence data of the *Neofusicoccum* species in the *N. parvum* / *N. ribis* complex. The tree is rooted to sequences of *Neofusicoccum ribis*. Bootstrap values of maximum parsimony analyses are indicated above the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics).

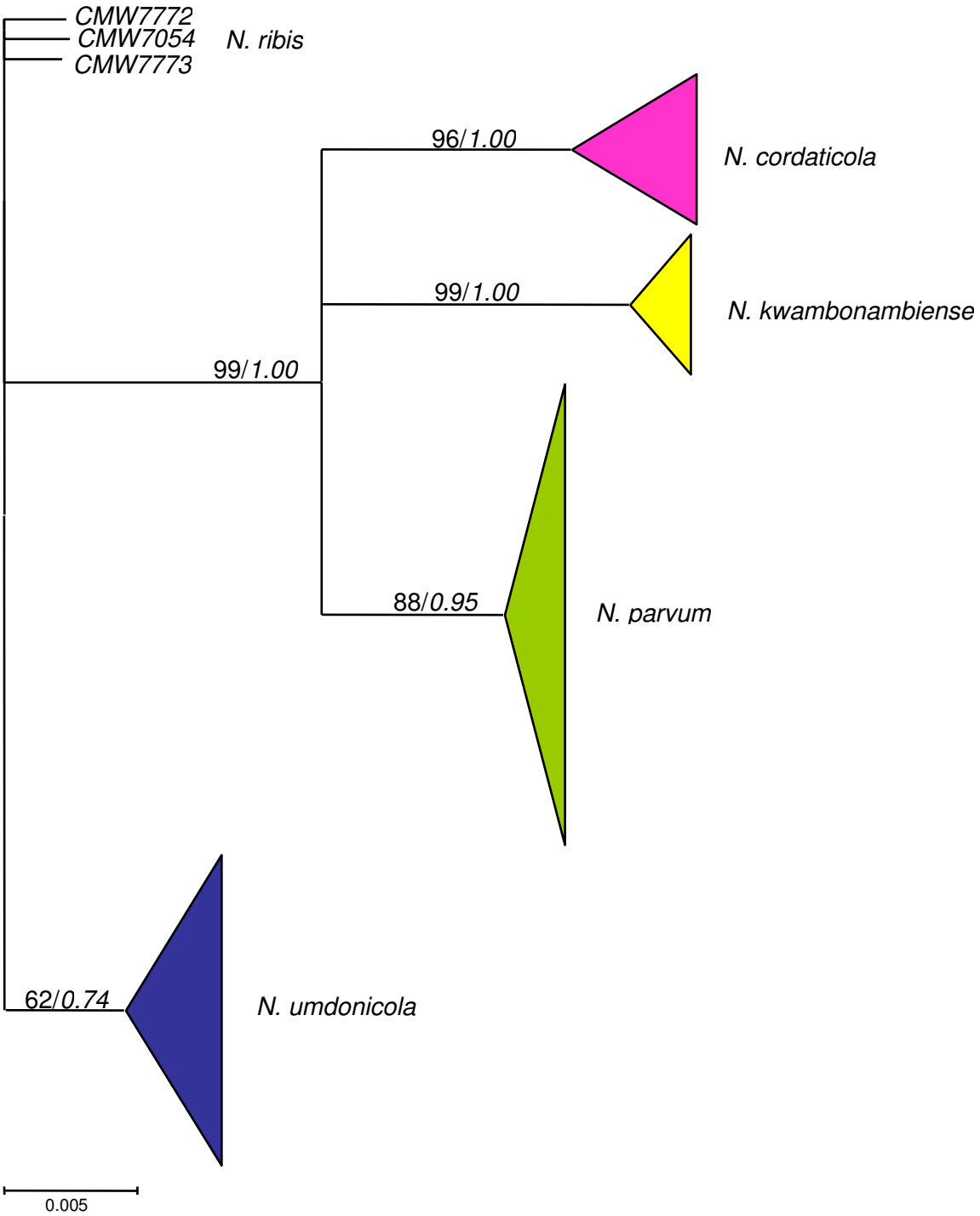

CMW7772
CMW7054
CMW7773
N. ribis
96/1.00
N. cordaticola
99/1.00
N. kwambonambiense
99/1.00
88/0.95
N. parvum
62/0.74
N. umdonicola
0.005

FIG. 2. The averages of the lengths and widths of ten conidia measured for each of 103 isolates representing *Neofusicoccum parvum* / *N. ribis* complex from *Syzygium cordatum*.

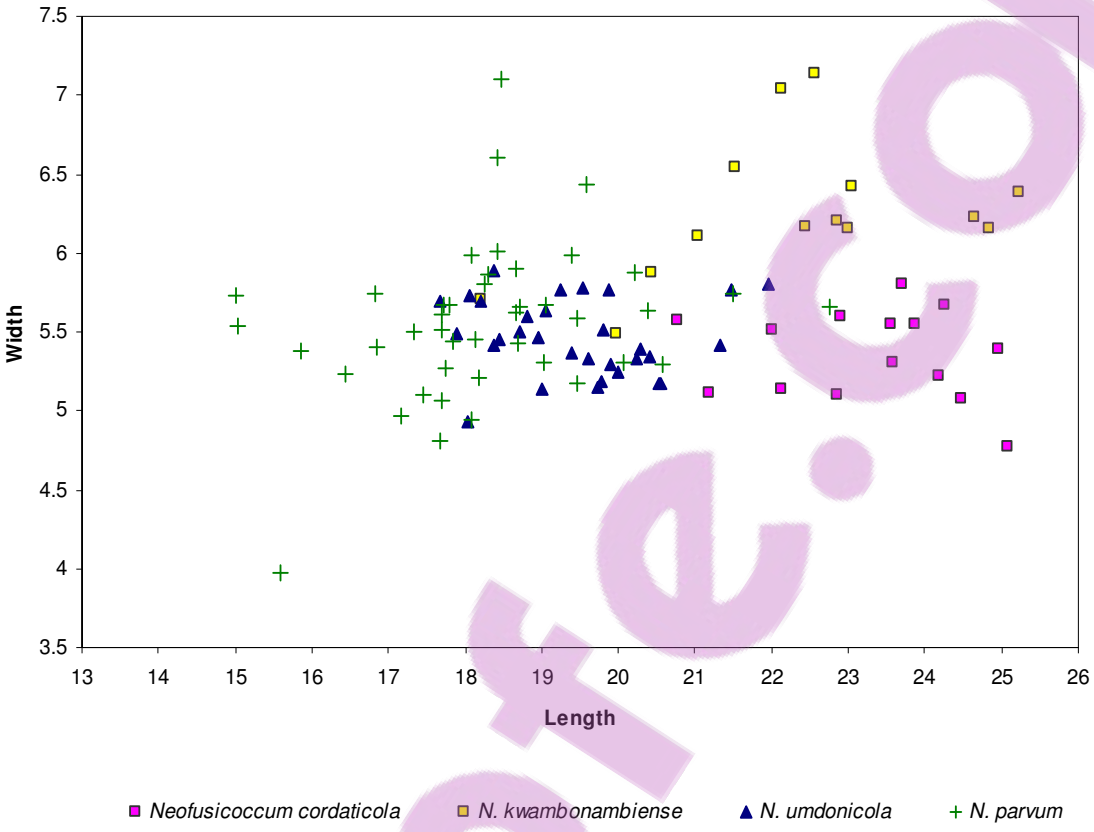
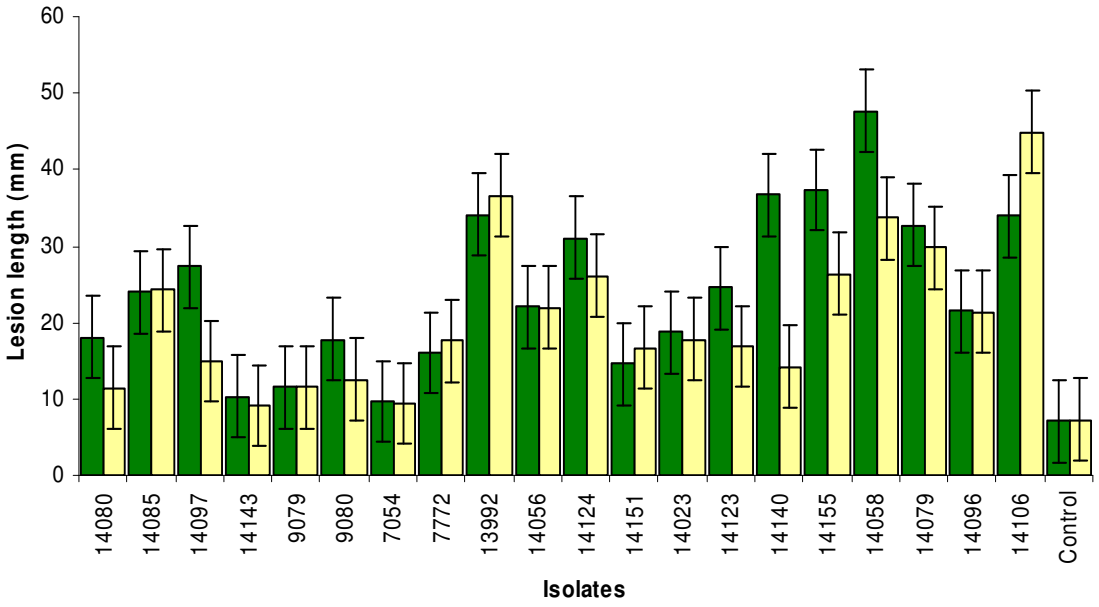

7.5
7
6.5
6
5.5
5
4.5
4
3.5
Width
13
14
15
16
17
18
19
20
21
22
23
24
25
26
Length
Neofusicoccum cordaticola
N. kwambonambiense
N. umdonicola
N. parvum

FIG. 3. Mean lesion lengths (mm) obtained for each isolate of different species of the *Neofusicoccum* six weeks after inoculations on *Syzygium cordatum*. Bars represent 95 % confidence limits for each isolate. *N. parvum* (CMW14097, 14080, 14085, 14143, 9079, 9080); *N. ribis* (CMW7772, 7054); *N. cordaticola* (CMW13992, 14056, 14151, 14124), *N. kwambonambiense* (CMW14023, 14140, 14155, 14123); *N. umdonicola* (CMW14106, 14058, 14079, 14096); C = Control.



Chapter 4

Cryptic diversity and distribution of species in the *Neofusicoccum parvum / N. ribis* complex as revealed by microsatellite markers

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ABSTRACT

Delineation of cryptic species by molecular identification tools is drastically changing our view of fungal species diversity and distribution. For example, the *Neofusicoccum parvum* / *N. ribis* species complex (Botryosphaeriaceae, Ascomycetes) was thought to consist of two sister species, but genealogical concordance species recognition has led to the recent delineation of five cryptic sibling species in this complex. Their cryptic nature and the small number of isolates available in previous studies has, however, led to questions regarding the distinction, diversity and distribution of *N. cordaticola*, *N. kwambonambiense*, *N. umdonicola* and *N. parvum* on native *Syzygium cordatum* trees. Microsatellite markers were thus used to investigate inter- and intra-species genetic diversity and structure amongst 114 isolates in this complex from across the distribution of *S. cordatum* in South Africa. The microsatellite data support the fact that four distinct species exist sympatrically on this host. The distribution of these species on *S. cordatum* shows very clear structure across the *S. cordatum* distribution, with *N. parvum* isolates being dominant and most abundant in areas influenced by humans, and absent in isolated natural stands of these trees. *Neofusicoccum parvum* populations from *S. cordatum* in disturbed environments were also structured, with those from trees growing alongside stands of non-native *Eucalyptus* less genetically diverse than trees planted in urban environments. These results suggest a strong influence of human activity on the composition of *Neofusicoccum* species on *S. cordatum* and that cross infections between native and non-native plants are important in structuring the diversity of these fungi.

INTRODUCTION

The availability and improvement of molecular tools have led to the identification of numerous cryptic species in the fungal kingdom. The ability to efficiently identify such cryptic species has consequently substantially changed our understanding of species diversity and distribution (Taylor et al 2006, Bickford et al 2007). This is also true for many plant pathogenic fungi affecting economically important crops. These fungi might, however, have the ability to also infect surrounding native vegetation. To fully understand their ecological role, diversity and distribution these pathogens must not be viewed separately from those occurring on plants in the surrounding natural ecosystems.

The *Neofusicoccum parvum* / *N. ribis* species complex (Botryosphaeriaceae, Ascomycetes) (Crous et al 2006) was thought to include two sister species that are fungal plant pathogens on a variety of woody hosts, most frequently reported from the Southern Hemisphere (Slippers and Wingfield 2007, de Wet et al 2008). These fungi have been recorded on cultivated, economically important non-native fruit and forestry trees (Slippers et al 2004b, 2007, Mohali et al 2007) and also on trees in native ecosystems (Slippers et al 2005, Burgess et al 2005, Pavlic et al 2007). They are also commonly isolated as endophytes that reside in asymptomatic plant tissues of numerous woody hosts (Slippers and Wingfield 2007). This makes them ideal candidates for undetected, long distance dispersal by humans, together with plant germplasm traded for agriculture and forestry.

Species that belong to the *Neofusicoccum parvum* / *N. ribis* complex were the most abundant species of Botryosphaeriaceae isolated from the native tree, *Syzygium cordatum* (Myrtaceae) across its native range in South Africa (Pavlic et al 2007). In that study, *N. parvum sensu lato* and *N. ribis s. l.* were identified based on ITS sequence comparison and PCR-RFLP profiles. In more recent studies, three undescribed species from this host were discovered using a Genealogical Concordance Phylogenetic Species Recognition (GCPSR) approach based on multiple locus sequence data (Pavlic et al 2009a). These were described as the phylogenetic species *N. cordaticola, N. kwambonambiense* and *N. umdonicola* (Pavlic et al 2009b). These three cryptic species are known only from *S. cordatum* in South Africa, while *N. parvum* has been found on many different hosts in the country, including native *S. cordatum* (Pavlic et al 2007), closely related to *Eucalyptus* in non-native plantations (Slippers *et al*. 2004b) and on the unrelated mango (Jacobs 2002).

A limited number of isolates was considered in GCPSR of *N. cordaticola, N. kwambonambiense, N. umdonicola* and *N. parvum* on *S. cordatum* by Pavlic et al (2009a),

which leaves a number of questions regarding the distribution and interaction of these species unanswered. The small number of fixed single nucleotide polymorphisms (SNPs) in sequenced loci in that study also demands further evidence to support distinction of the phylogenetic species. Considering the sub-clades observed in the *N. parvum* clade in combined multi-gene genealogies, the question as to whether other cryptic species also exist in this group has been raised. In order to clarify these questions, verification using an additional molecular tool is required.

Simple Sequence Repeat (SSR) or microsatellite markers are frequently applied in population genetic studies on a variety of fungal species. However, microsatellite markers have also been utilised to provide an additional molecular tool to be used in the delineation of closely related human pathogenic fungi, which had initially been recognised as phylogenetic species based on GCPSR (e.g. Fisher et al 2000, Taylor and Fisher 2003, Matute et al 2006). In the case of the Botryosphaeriaceae, Burgess et al (2001) distinguished morphotypes of *D. sapinea* with microsatellite markers. Two of these morphotypes were later shown to represent the distinct species, *Diplodia pinea* and *D. scrobiculata*, by de Wet et al (2003) using GCPSR. In these studies, the microsatellite markers were useful to type strains and species, in support of GCPSR, because they can easily be applied to large numbers of isolates, they are reproducible and they often reveal more diversity than sequence analyses alone.

Microsatellite markers have previously been developed for Botryosphaeriaceae with *Fusicoccum*-like anamorphs, which include *Neofusicoccum* species (Slippers et al 2004a). In this study, we use these markers to: (i) test the GCPSR based hypothesis of Pavlic et al (2009a) regarding the coexistence of four cryptic species, *N. parvum*, *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, in the *N. parvum* / *N. ribis* complex on *S. cordatum* in South Africa, (ii) analyse their inter- and intra-species genetic structure and diversity and (iii) map their geographical distribution on *S. cordatum* trees in natural stands and in undisturbed sites or areas disturbed by human activity such as trees growing along non-native *Eucalyptus* plantations or those planted as ornamentals in urban areas.

MATERIALS AND METHODS

Fungal isolates

The isolates used in this study were collected during a survey of the Botryosphaeriaceae on native *Syzygium cordatum* in different geographical locations of South Africa, between

February 2001 and March 2003 (TABLE I, II, FIG. 1). *Syzygium cordatum* trees do not grow in persistent forests, but rather in patches or as solitary trees and sampling areas were defined accordingly. Of the 114 isolates used in this study, 81 were collected from natural stands of *S. cordatum* that are isolated in natural reserves or are growing alongside *Eucalyptus* plantations (8 sites) and 33 isolates were collected from planted *S. cordatum* in urban areas (4 sites) (TABLE I, II, FIG. 1). Between 1 and 45 trees were sampled from each site. From each tree, isolations were made from dying twigs and, visually healthy twig and leaf tissues as described by Pavlic et al (2007). All isolates were identified in previous studies to belong to the *N. parvum / N. ribis* species complex including *N. parvum* (48 isolates), *N. cordaticola* (17), *N. kwambonambiense* (14) and *N. umdonicola* (35), based on DNA sequence data of at least one locus and PCR-RFLP analysis (Pavlic et al 2007, 2009a, b). All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and representative isolates have been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA extraction, microsatellite-PCR amplification and genotyping

The haploid, single conidial cultures were grown on 2 % malt extract agar (MEA) (20 g malt extract, 15 g agar; Biolab, Midrand, Johannesburg, S.A. and 1000 mL deionised water) for 7 days at 25 °C in the dark. DNA was extracted from the mycelium following the modified phenol-chloroform DNA extraction method described in Pavlic et al (2007). DNA was separated by electrophoresis on 1.5 % agarose gels, stained with ethidium bromide and visualized under ultraviolet light. DNA concentrations were estimated against λ standard size marker. Seven loci that contained microsatellite sequences were amplified for all isolates, using fluorescently-labeled primer pairs designed for species of Botryosphaeriaceae with *Fusicoccum*-like anamorphs (Slippers et al 2004a). PCR reactions were performed using an Eppendorf Mastercycler PERSONAL (Perkin-Elmer, Germany) and the following protocol: 94 °C for 2 min initial denaturation; 40 cycles of 94 °C for 30 s, 55 or 62 °C for 30 s, 72 °C for 1 min; and 72 °C for 7 min final extension. PCR products were separated in 2 % agarose gels stained with ethidium bromide and visualized under UV light. Sizes of PCR products were estimated by comparison with a 100 bp molecular weight marker (Fermentas Life Sciences). Allele sizes of labeled microsatellite-PCR products were determined on an ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer, Warrington, U.K.) and

compared against a GENESCAN−500 LIZ (Perkin-Elmer Applied Biosystems, Warrington, U.K.) internal size standard. Because of the overlap of fragment sizes for some of the amplicons, two separate gels were run for each sample. Allele sizes were analyzed with GENESCAN 3.7 and GENOTYPER 3 software (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Microsatellite analyses

The Bayesian clustering algorithm in the program STRUCTURE version 2.2 (Pritchard et al 2000, Falush et al 2003) was used to determine whether isolates in the *N. parvum* / *N. ribis* complex could be subdivided into *K* genetically distinct groups. STRUCTURE assumes a model in which there are *K* populations (where *K* may be unknown), each of which is characterized by a set of allele frequencies at each locus. Individuals in the sample are probabilistically assigned to a single population, or jointly to two or more populations if their genotypes indicate that they are admixed. To determine the most likely number of genetically distinct groups or clusters (*K*) in the sample, 20 independent runs of $K = 1-10$ were carried out at 100 000 Markov chain Monte Carlo (MCMC) repetitions following a burn-in of 20 000 iterations. The program was run assuming no admixture among the populations and additional parameters assumed were: different values of F_{ST} for different subpopulations, prior mean of F_{ST} 0.01, standard deviation (SD) of F_{ST} 0.05 and constant lambda value at 1. The most probable number of clusters was taken using the highest mean log-likelihood of *K*. The analyses were run with a clone-corrected data set where only one of each of the genotypes was included in the analyses.

The program POPGENE version 1.31 (Yeh et al 1999) was used to calculate allele frequencies at each microsatellite locus and to estimate genetic diversity across all loci for each of four populations representing four species identified in the *N. parvum* / *N. ribis* complex from *S. cordatum*. For each population, the observed number of alleles (n_a), number of unique alleles, number and percentage of polymorphic loci (P) and mean genetic diversity across all loci (H), which was calculated as $H = 1-\Sigma x_k^2$, where x_k is the frequency of the k^{th} allele (Nei 1973), were evaluated.

Three geographically defined populations of *N. parvum* collected in South Africa from *S. cordatum* in Kwambonambi (KWM), Pietermaritzburg (PTM) and Tzaneen (TZ) were further analysed. For each isolate, a data matrix of multistate characters was composed by assigning a different letter to each allele at each of 7 loci (e.g. ABDCEFB), and the total

number of multilocus genotypes across the dataset was determined. Each genotype was assigned a unique number and genotypic diversity (G) was calculated using the equation $G = n / n - 1 (1 - \Sigma p_i^2)$ where p_i is the observed frequency of the i^{th} genotype and n is the number of individuals sampled in the population (Stoddart and Taylor 1998). To compare genotypic diversity (G) between populations, the maximum percentage of genotypic diversity was obtained using the equation $\hat{G} = G/N \times 100$, where N is the population size (Chen et al 1994). Isolates with the same genotype were considered to be clones and only one representative of each genotype was included in the analyses.

The analysis of molecular variance (AMOVA) was carried out using software GENALEX version 6.1 (Peakall and Smouse 2006). We examine the partitioning of genetic variation among and within the three geographically defined (Kwambonambi, Pietermaritzburg and Tzaneen) populations of *N. parvum*. Analysis was performed on clone-corrected datasets, where only one representative of each genotype was included in the analysis, to prevent over-representation of alleles in frequently occurring clones.

RESULTS

Genetic structure and diversity

Six clusters were identified using STRUCTURE analyses with no prior population knowledge assumed. The identified clusters supported the species distinctions recognized by Pavlic et al (2009a, b) based on multiple gene sequence genealogies, and they distinguished added subdivision in *N. parvum*. Isolates of each of the three species, *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, were grouped in three different clusters (1–3), showing no further substructure within populations of these species. The three remaining clusters (4–6) contained only *N. parvum* isolates (FIG. 2) representing subdivision of this species into three sub-populations.

For the entire dataset of 114 isolates that belong to *N. parvum* / *N. ribis* complex from native *S. cordatum,* a total of 61 alleles were observed across seven loci examined (TABLE III). There were 26 alleles detected amongst the *N. cordaticola* individuals, 16 alleles amongst the *N. kwambonambiense* individuals, 15 alleles amongst the *N. umdonicola* individuals and 38 alleles in the *N. parvum* populations (TABLE III). Only one allele was shared among all four species. Private alleles were identified in each of these groups that represent the species. Twenty private alleles were detected in *N. parvum*, eight in *N. cordaticola,* four in *N. kwambonambiense*, and two in *N. umdonicola* (TABLE III). The mean

total gene diversity across all isolates was the highest in *N. parvum* (H = 0.57). Moderate gene diversity was observed in *N. cordaticola* (H = 0.44) and low gene diversity in *N. kwambonambiense* (H = 0.27) and *N. umdonicola* populations (H = 0.21) (TABLE III).

Genotypic diversity of *N. parvum*

Twenty-three genotypes were observed among 48 isolates of *N. parvum* from *S. cordatum* in South Africa. The three geographically defined populations of *N. parvum* encompassed a total of 41 isolates represented by 22 genotypes, four of which were from the Kwambonambi (KWM) population and nine in each of the Pietermaritzburg (PTM) and the Tzaneen (TZ) populations (TABLE IV). The maximum genotypic diversity ($\hat{G}$) of the *N. parvum* populations was moderate to high for populations PTM (62.8 %) and TZ (77.5 %) and low in the KWM population (36 %) (FIG. 3, TABLE IV). The low diversity in the KWM population was due to the predominance of a single genotype (S14), which accounted for 66 % of the isolates collected in Kwambonambi area (10 of 15 isolates). Genotype S14 was the only genotype shared between populations of *N. parvum* from Kwambonambi and Pietermaritzburg and three genotypes (S9, S11 and S12) were shared between the PTM and TZ populations. There were no genotypes shared between KWM and TZ populations. From the AMOVA analysis that was applied to the *N. parvum* dataset, the highest fraction of variability (96 %) was within populations and only 4 % among the geographic populations.

Distribution of *Neofusicoccum* spp. on *S. cordatum*

The occurrence of the four *Neofusicoccum* species on *S. cordatum* varied significantly for the twelve collection sites (FIG. 4). In ten of the twelve areas, one of the species was dominant (FIG. 4). *Neofusicoccum parvum* was the only species identified in Pietermaritzburg and it was also the dominant species in Tzaneen and Kwambonambi. In Tzaneen, *N. kwambonambiense* was found together with *N. parvum*. Kwambonambi was the only area where all four species co-exist. *Neofusicoccum cordaticola* was the dominant species in the Sabie area, and it was found together with *N. kwambonambiense*. The same composition as that in the Sabie area was found in Richards Bay, but with *N. kwambonambiense* being the dominant species. Two areas close to the Indian Ocean, Kosi Bay and Sodwana Bay had the same species compositions, with *N. umdonicola* dominant and found together with *N. cordaticola* (FIG. 4). Overall *N. parvum* was the dominant *Neofusicoccum* species on *S. cordatum* in South Africa making up 42 % of all isolates.

DISCUSSION

Application of microsatellite markers clearly supported the earlier discovery (Pavlic et al 2009a) that four sister species, *N. parvum*, *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, co-exist within the *N. parvum* / *N. ribis* complex on native *S. cordatum* trees in South Africa. Intriguingly, this study also showed that there is a strong correlation between the distribution of the cryptic species in the *N. parvum* / *N. ribis* complex on *S. cordatum* and human disturbance. Thus, at sites disturbed by or in close contact with human activities, *N. parvum*, a generalist and serious pathogen with a wide geographic distribution (Slippers and Wingfield 2007, de Wet et al 2008), was dominant. This is in contrast to the three other cryptic species in the *N. parvum* / *N. ribis* complex that were dominant in natural, undisturbed stands of *S. cordatum*.

Pavlic et al (2009a) discovered four sympatric cryptic species in the *N. parvum* / N. *ribis* complex, using GCPSR based on DNA sequence data for five nuclear loci. An important outcome of the present work was the fact that the microsatellite data were in concordance with those previous findings. Private alleles were observed in each of the four *Neofusicoccum* spp., which supported their distinction in STRUCTURE. The concordant phylogenies indicated by the private microsatellite alleles, together with the fixed SNPs observed in the five sequenced loci (Pavlic et al 2009a), provide evidence for the absence of recombination amongst these alleles between the groups. These fungi not only occur sympatrically at a larger spatial scale, but in some cases inhabit the same tree or even a single leaf (Pavlic at al 2009b). A sexual state is also known in the *N. parvum* / *N. ribis* complex and the absence of recombination is, therefore, interpreted as being due to reproductive isolation over long period (Fisher et al 2000, Taylor et al 2000).

These four species identified here and by Pavlic et al (2009) also shared alleles at all loci examined. Most of the identical alleles were shared between two species and only one was shared among all four species. The occurrence of shared alleles has also been recognized in other closely related species such as in the human pathogens *Coccidioides immitis* and *C. posadasii* (Fisher et al 2002). However, the congruent phylogenies obtained using microsatellite and gene sequence data for the latter species suggest that the shared alleles between the two taxa were a result of mutational convergence or ancestral shared origin of the alleles, and not due to interbreeding (Fisher et al 2000, 2002, Taylor et al 2000). This is consistent with the interpretation of the results in the present study. The microsatellite

markers used in this study are evidently useful as a part of an integrative approach in studies on speciation and delimitation of cryptic species in the Botryosphaeriaceae.

This study on the *N. parvum* / *N. ribis* complex was focused on the single species *S. cordatum* and in a single country. More species diversity is to be expected in this group on other hosts and in other areas. For example, isolates of an undescribed *Neofusicoccum* sp., was recently identified within *N. parvum* / *N. ribis* complex from the ancient *Wollemia nobilis* and *Araucaria cunninghamii* growing in Australia and New Zealand (Slippers et al 2005). More work is needed to confirm that this is a distinct species, but patterns of variation indicated that this is most likely the case. To fully understand patterns of diversity in these fungi additional studies on native species in additional areas will be needed.

The existence of numerous isolates with identical multilocus haplotypes, in all the different species and in particular in different populations of *N. parvum*, suggests that either asexual reproduction or a homothallic sexual cycle plays an important role in structuring these populations (Coppin et al 1997, Turgeon 1998) Although *Neofusicoccum* species produce both teleomorph and anamorph structures in their life cycle, these species are most commonly encountered in the asexual state, which might suggest that asexual reproduction is the main cause of the identical haplotypes. Sexual structures are known for *N. parvum,* but they have not been identified for *N. cordaticola, N. kwambonambiense* and *N. umdonicola*. There have been no studies considering whether they are exclusively outcrossing or alternatively whether they can also reproduce homothallically and since mode of reproduction plays an important role in population structure and diversity, future studies should interrogate this question. The small population sizes did not allow us to further analyse these questions for these species.

Isolates of *N. parvum* were assigned to three sub-populations by the STRUCTURE analysis, based on allele frequencies across the loci, with very low or no admixture among the groups. This indicates that *N. parvum* population associated with native *S. cordatum* in South Africa is a mixture of at least tree independent sources, with different ancestral origins. The origin of the three groups is, however, unclear, because they could not be assigned to specific geographic regions or hosts in this study, Genetic variation identified by microsatellites and subdivision of *N. parvum* isolates into three sub-populations was consistent with significant sequence variation observed in a previous study among *N. parvum* isolates based on multiple gene sequence data (Pavlic et al 2009a). However, there was no concordance between phylogenies from multiple gene genealogies and GCPSR to further separate these groups into species. Furthermore, in this study the highest fraction of

genetic variability (96 %) was within the populations and only 4 % among the populations of *N. parvum*, which is expected for populations of the same species (Linde et al 2002, Grünig et al 2006).

Distribution of the four species in the *N. parvum* / *N. ribis* complex across *S. cordatum* displayed clear differences on trees in environments affected by human activity or those in isolated natural stands. In most areas studied, at least two *Neofusicoccum* species coexisted on *S. cordatum*, with one of the species typically dominant. For example, *N. cordaticola* was the dominant species in the Sabie area of the Mpumalanga Province. In contrast, this species was present only in low numbers in the KwaZulu Natal Province, co-existing either with *N. kwambonambiense* or *N. umdonicola*, and it was absent from any other area. The Sabie area is at high altitude and has a climate very different to other areas sampled and with a very different floral composition surrounding the collection site (Mucina and Rutherford 2006). In contrast, *N. umdonicola* was the dominant species in the three isolated collection sites in the National Reserves in northern KwaZulu Natal including Sodwana Bay, Kosi Bay and Makuze, which are at low altitude with a subtropical climate and surrounded by undisturbed native flora. The dominance of one species in a particular niche might be related to local adaptation of each species to defined environmental conditions.

Neofusicoccum parvum is a known generalist that infests various woody hosts around the world (Pennycook and Samuels 1985, Slippers and Wingfield 2007, de Wet et al 2008). This was also the dominant species in the *N. parvum* / *N. ribis* complex isolated from *S. cordatum* in the present study. With the exception of two isolates of *N. kwambonambiense*, only *N. parvum* was ever found on planted *S. cordatum*. In contrast, *N. parvum* was not found in the isolated natural stands of *S. cordatum*. This effect was despite the close geographical proximity and climatic similarity of regions where other *Neofusicoccum* spp. are common. The results suggest that *N. parvum* is not native to the region on *S. cordatum* and that this species is spreading from non-native hosts to native *S. cordatum*, rather than *vice versa*.

The genotypic diversity among the isolates of *N. parvum* differed between the population from *S. cordatum* from natural stands along *Eucalyptus* plantations in the Kwambonambi area and two populations from planted *S. cordatum* in urban areas Pietermaritzburg and Tzaneen. A low level of genotypic diversity and dominance of one multilocus genotype was observed in the *N. parvum* population from the Kwambonambi area. This population derives from naturally grown *S. cordatum* that remained amongst non-

native *Eucalyptus* plantations, also residing in the Myrtaceae that dominate this area. *Neofusicoccum parvum* is the most common species of Botryosphaeriaceae found in non-native *Eucalyptus* plantations in South Africa (Slippers et al 2004b, Maleme 2008). Interestingly, it is rare on *Eucalyptus* in its native range in Australia and likely originates from another host and geographic origin (Slippers et al 2004b, Burgess et al 2005). Some of the genotypes identified in the Kwambonambi population from *S. cordatum* were identical with *N. parvum* genotypes identified on *Eucalyptus* in this area (authors, unpublished). This finding strongly supports the hypothesis that this pathogen spreads between these two hosts and that proximity to non-native *Eucalyptus* shaped the population structure of *N. parvum* on *S. cordatum* in this area.

In contrast to the Kwambonambi population, two populations from planted *S. cordatum* trees in Pietermaritzburg and Tzaneen, exhibit high levels of genotypic diversity with some genotypes overlapping between them, despite the fact that these two sites are more than 600 kilometers apart. *Neofusicoccum parvum* isolates with different genotypes, but with the same ancestral origin (as defined by STRUCTURE), were also found on planted *S. cordatum* in other areas distant from each other such as Pretoria and the Tokai. The *S. cordatum* trees in urban environments are surrounded by various known host of *N. parvum*, such as *Eucalyptus*, *Vitis vinifera* and *Mangifera indica* (Jacobs 2002, van Niekerk et al 2004, Slippers et al 2004b) and many ornamental plants that could harbor this species. Thus, multiple introductions through human activities and movement between hosts would be common in these areas and could have influenced the genetic diversity and population structure of *N. parvum* on the planted *S. cordatum*. Such movement of pathogens, in particular generalists such as *N. parvum*, could result in a population continuum over a large area, where gene flow among isolates might serve to maintain similar populations even at distant locations (McDonalds and Linde 2002).

Introduction of plants into non-native areas, changes in land use and intense forestation are some of the human activities that directly influence plant pathogen movement as well as interactions with their hosts. The patterns of distribution of *N. parvum, N. cordaticola, N. kwambonambiense* and *N. umdonicola* on *S. cordatum* in different areas, from isolated natural stands to environments where they have been affected by human activity provide vivid examples of this influence. This study illustrates the importance of considering surrounding native tree communities in studies that seek to understand fungal tree pathogens of importance to forestry and agriculture, and *vice versa*. Our results also

provide a foundation for future studies to characterize the biology and temporal changes of members of *N. parvum* complex in native and human disturbed environments.

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TABLE I. Isolates analysed in this study

Culture no. [1]	Other no. [2]	Identity	Geographic origin [3, 4]
CMW14056	CBS123635	*Neofusicoccum cordaticola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14035		*N. cordaticola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14041, 14042		*N. cordaticola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14054	CBS123636	*N. cordaticola*	SA, KwaZulu Natal Province, Mkuzi
CMW14122		*N. cordaticola*	SA, KwaZulu Natal Province, Richards Bay
CMW14124	CBS123638	*N. cordaticola*	SA, KwaZulu Natal Province, Richards Bay
CMW14144-14150		*N. cordaticola*	SA, Mpumalanga Province, Sabie
CMW14151	CBS123637	*N. cordaticola*	SA, Mpumalanga Province, Sabie
CMW14152		*N. cordaticola*	SA, Mpumalanga Province, Sabie
CMW13992	CBS123634	*N. cordaticola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14023	CBS123639	*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14046		*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14025	CBS123640	*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14031		*N. kwambonambiense*	SA, KwaZulu Natal Province, Kwambonambi
CMW14119-14121		*N. kwambonambiense*	SA, KwaZulu Natal Province, Richards Bay
CMW14123	CBS123643	*N. kwambonambiense*	SA, KwaZulu Natal Province, Richards Bay
CMW14153, 14154		*N. kwambonambiense*	SA, Mpumalanga Province, Sabie
CMW14155	CBS123645	*N. kwambonambiense*	SA, Mpumalanga Province, Sabie
CMW14156		*N. kwambonambiense*	SA, Mpumalanga Province, Sabie
CMW14140	CBS123641	*N. kwambonambiense*	SA, Northern Province, Tzaneen
CMW14136		*N. kwambonambiense*	SA, Northern Province, Tzaneen
CMW14079	CBS123647	*N. umdonicola*	SA, Eastern Cape Province, Gonubie
CMW14055		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14057		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14058	CBS123645	*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14098, 14099		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14059		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14060	CBS123646	*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14100, 14101		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14061, 14062		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14068		*N. umdonicola*	SA, KwaZulu Natal Province, Kosi Bay
CMW14028		*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14016		*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14125, 14126		*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14127	CBS123648	*N. umdonicola*	SA, KwaZulu Natal Province, Kwambonambi
CMW14047		*N. umdonicola*	SA, KwaZulu Natal Province, Mkuze
CMW14051		*N. umdonicola*	SA, KwaZulu Natal Province, Mkuze
CMW13993-13997		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14007		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14106	CBS123644	*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14008		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14010		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW13990, 13991		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14006		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14011, 14012		*N. umdonicola*	SA, KwaZulu Natal Province, Sodwana Bay
CMW14096		*N. umdonicola*	SA, KwaZulu Natal Province, Port St Johns
CMW14080	CBS123651	*Neofusicoccum parvum*	SA, Eastern Cape Province, Gonubie
CMW14018-14022		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14024		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14027		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14032		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14036-14040		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14045		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14030		*N. parvum*	SA, KwaZulu Natal Province, Kwambonambi
CMW14081, 14082		*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW14084		*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW14085	CBS123649	*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW14086-14095		*N. parvum*	SA, KwaZulu Natal Province, Pietrmaritzburg
CMW27901		*N. parvum*	SA, Gauteng Province, Pretoria

TABLE I. Continued

Culture no. [1]	Other no. [2]	Identity	Geographic origin [3, 4]
CMW29125		*N. parvum*	SA, Gauteng Province, Pretoria
CMW14097	CBS123650	*N. parvum*	SA, Eastern Cape Province, Port St Johns
CMW14110-14112		*N. parvum*	SA, Western Cape Province, Tokai
CMW14128		*N. parvum*	SA, Northern Province, Tzaneen
CMW14137-14139		*N. parvum*	SA, Northern Province, Tzaneen
CMW14141, 14142		*N. parvum*	SA, Northern Province, Tzaneen
CMW14143	CBS123652	*N. parvum*	SA, Northern Province, Tzaneen
CMW14129, 14130		*N. parvum*	SA, Northern Province, Tzaneen
CMW14133-14135		*N. parvum*	SA, Northern Province, Tzaneen

[1, 2] Abbreviations of culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa; CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands.

[3] SA = South Africa.

[4] All isolates were collected from *S. cordatum* by D. Pavlic.

TABLE II. Distribution of isolates of *Neofusicoccum* spp. collected from *S. cordatum* in South Africa

Collection sites [1, 2]	***N. cordaticola***	***N. kwambonambiense***	***N. umdonicola***	***N. parvum*** [3]
Tzaneen (1) **P**	-	2	-	**12**
Pretoria (2) **P**	-	-	-	2
Sabie (3) **N**	9	4	-	-
Kosi Bay (4) **N**	1	-	12	-
Mkuze (5) **N**	1	-	2	-
Sodwana Bay (6) **N**	1	-	14	-
Kwambonambi (7) **N**	3	4	5	**15**
Richards Bay (8) **N**	2	4	-	-
Pietermaritzburg (9) **P**	-	-	-	**14**
Port St Johns (10) **N**	-	-	1	1
Gonubie (11) **N**	-	-	1	1
Tokai (12) **P**	-	-	-	3
Total	17	14	35	48

[1] Numbers in brackets indicate collections sites as marked on the South African map (FIG. 1).

[2] N = Isolates were collected from *S. cordatum* trees on natural stands; P = Isolates collected from the planted trees in urban areas.

[3] Numbers in bold indicate isolates considered in population analyses.

TABLE III. Allele size (bp) and frequency at 7 loci in four *Neofusicoccum* spp. collected from *Syzygium cordatum* in South Africa

Locus	*Allele*[1]	*N. cordaticola*	*N. kwambonambiense*	*N. umdonicola*	*N. parvum*
***BotF*11**	420	-	**1.000**	-	-
	427	-	-	-	**0.104**
	428	-	-	-	**0.854**
	431	0.471	-	0.400	-
	432	-	-	0.543	0.021
	433	-	-	**0.057**	-
	435	**0.471**	-	-	-
	null	0.058	-	-	0.021
***BotF*15**	365	-	-	-	**0.187**
	374	-	-	-	**0.021**
	377	1.000	-	1.000	0.646
	378	-	-	-	**0.021**
	389	-	-	-	**0.083**
	390	-	-	-	**0.042**
	395	-	**0.857**	-	-
	396	-	**0.143**	-	-
***BotF*17**	229	-	1.000	0.971	-
	233	0.117	-	-	0.104
	234	**0.882**	-	-	-
	244	-	-	-	**0.104**
	246	-	-	-	**0.083**
	247	-	-	-	**0.021**
	249	-	-	0.029	0.479
	256	-	-	-	**0.104**
	259	-	-	-	**0.104**
***BotF*21**	203	-	0.071	0.200	-
	204	0.176	0.143	0.743	0.062
	206	-	-	-	**0.021**
	207	0.118	-	-	0.187
	208	0.118	0.143	-	0.104
	209	0.059	0.071	-	0.125
	219	0.294	0.429	-	0.500
	229	-	0.071	0.057	-
	234	**0.176**	-	-	-
	null	0.059	0.071	-	-
***BotF*23**	422	-	-	0.914	0.562
	423	-	-	-	**0.021**
	424	-	-	-	**0.042**
	425	-	-	-	**0.333**
	426	0.059	0.286	-	0.042
	427	0.059	0.714	-	-
	428	0.882	-	0.057	-
	null	-	-	**0.029**	-
***BotF*35**	222	-	-	-	**0.083**
	225	0.059	-	-	0.480
	238	-	-	0.800	0.125
	239	-	-	-	**0.083**
	244	0.529	-	-	0.187
	245	-	-	0.200	0.021
	247	**0.294**	-	-	-
	253	0.059	0.429	-	-
	255	-	**0.571**	-	-
	265	-	-	-	**0.021**
	null	**0.059**	-	-	-
***BotF*37**	303	**0.412**	-	-	-
	306	**0.412**	-	-	-
	312	-	-	1.000	0.542
	313	0.059	-	-	0.417
	314	-	-	-	**0.042**
	320	0.059	1.000	-	-
	null	**0.059**	-	-	-

TABLE III. Continued

N	**17**	**14**	**35**	**48**
n_a	**26**	**16**	**15**	**38**
No. of private alleles	**8**	**4**	**2**	**20**
Polymorphic loci	**6**	**4**	**5**	**7**
P	**85.71**	**57.14**	**71.43**	**100**
H	**0.438**	**0.271**	**0.212**	**0.572**

N = number of isolates

n_a = observed number of alleles

P = percentage of polymorphic loci

H = mean gene diversity

[1] Alleles in bold are unique for each of species

TABLE IV. *Neofusicoccum parvum* genotypes as estimated from multilocus profiles generated from the 7 microsatellite loci; Genotypes were distributed among populations collected in from *S. cordatum* in three different areas in South Africa; Kwambonambi (KWM), Pietermaritzburg (PTM) and Tzaneen (TZ)

Genotype[1]	KWM	PTM	TZ
S1			1
S2			1
S3	1		
S4		1	
S5			2
S6			
S7			1
S8			3
S9		1	1
S10		1	
S11		1	1
S12		1	1
S13	3		
S14	10	5	
S15			
S16		1	
S17		1	
S18			
S19		1	
S20			
S21			
S22		1	
S23	1		
N	**15**	**14**	**12**
N(g)	**4**	**9**	**9**
G	**0.54**	**0.88**	**0.93**
Ĝ (%)	**36**	**62.8**	**77.5**

N = number of isolates

N (g) = number of genotypes

G = Genotypic diversity (Stoddart and Taylor 1988)

Ĝ = percent maximum diversity

[1] Genotypes in bold overlap between populations

FIG. 1. Map of South Africa showing sites where the isolates were collected. Tzaneen (1U), Pretoria (2U), Sabie (3N), Kosi Bay (4NR), Mkuze (5NR), Sodwana Bay (6NR), Kwambonambi (7E), Richards Bay (8N), Pietermaritzburg (9U), Port St. Johns (10N), Gonubie (11N), Tokai (12U). U = Urban area; N = Natural stand; NR = Nature Reserve; E = Naturally regenerated *S. cordatum* amongst *Eucalyptus* stands. The timber plantations areas are highlighted in red. Source: Forestry South Africa.

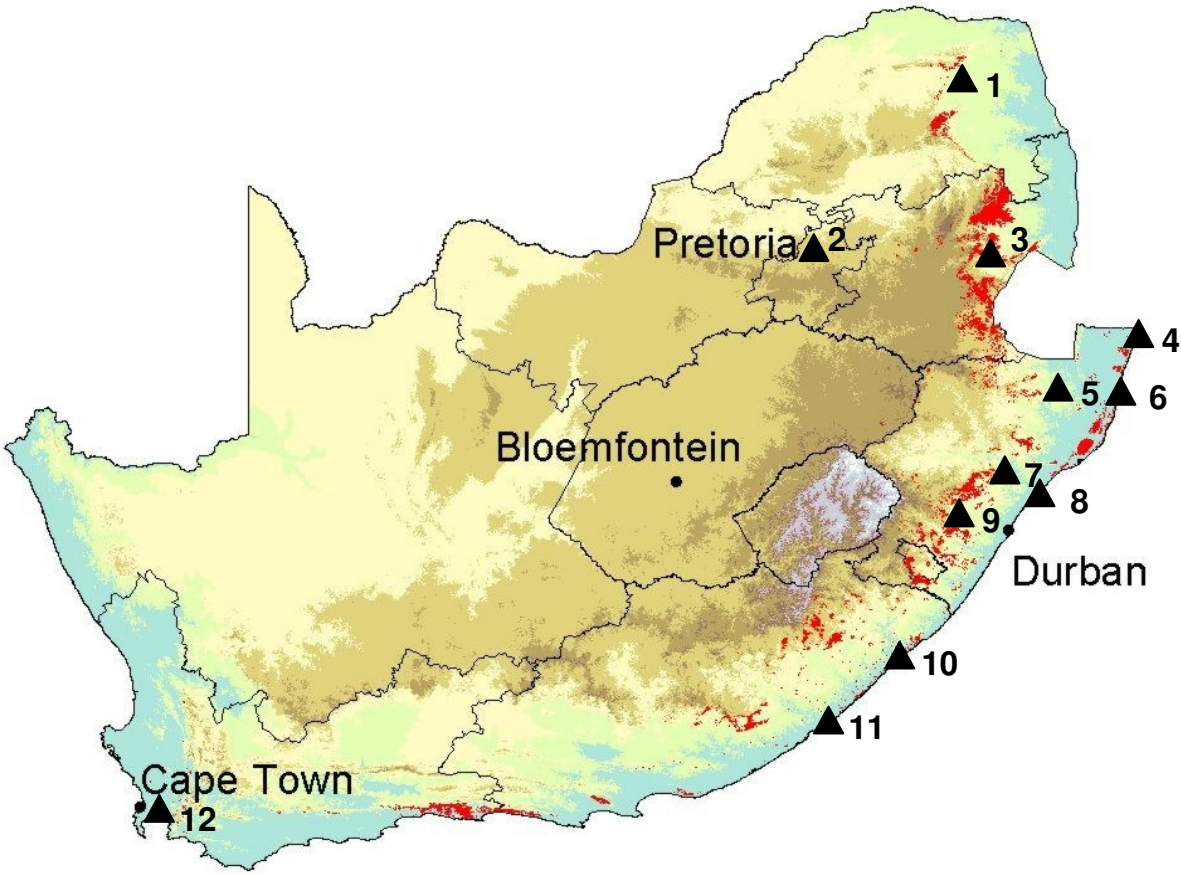

1
Pretoria
2
3
4
5
6
Bloemfontein
7
8
9
Durban
10
11
Cape Town
12

FIG. 2. The clustering outcome from STRUCTURE analyses of the clone-corrected dataset of all isolates at $K = 6$. Each color represents one cluster. Labels underneath the outcome (1–4) correspond to clusters related to each of four *Neofusicoccum* species, *N. cordaticola* (1), *N. kwambonambiense* (2), *N. umdonicola* (3) and isolates of *N. parvum* (4). Note that isolates of *N. parvum* are distributed in three clusters indicated by green, yellow and blue.

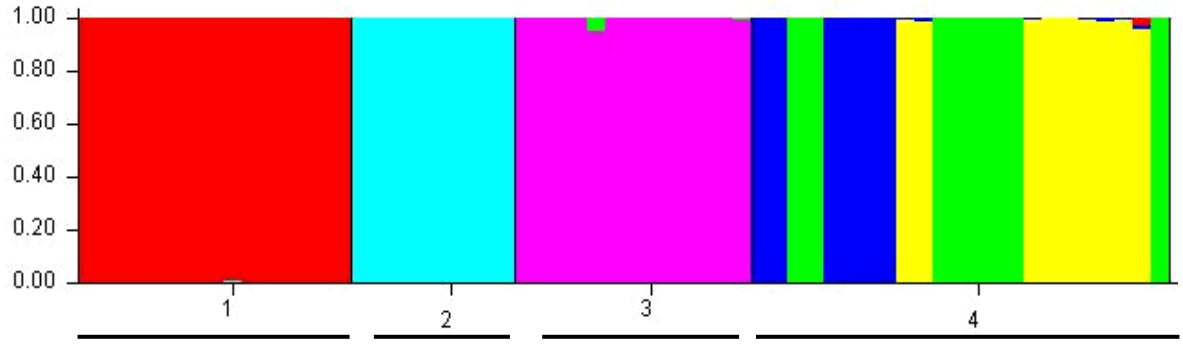
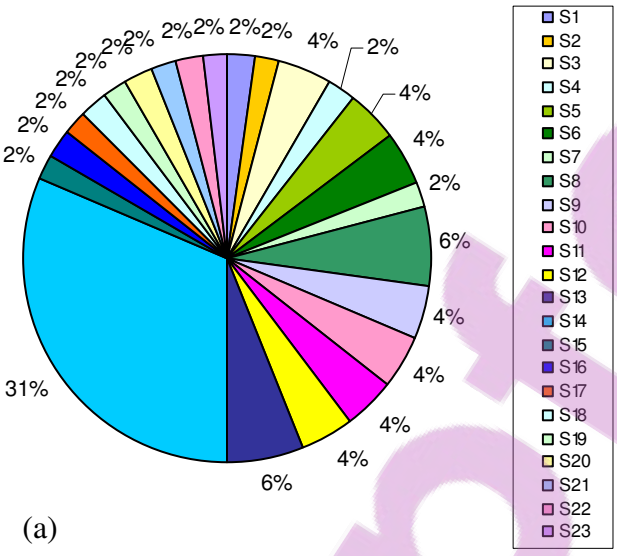
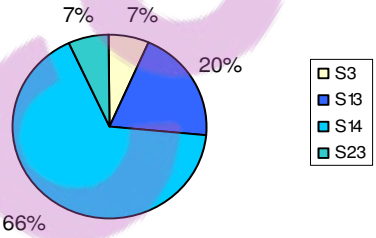

1.00
0.80
0.60
0.40
0.20
0.00
1
2
3
4

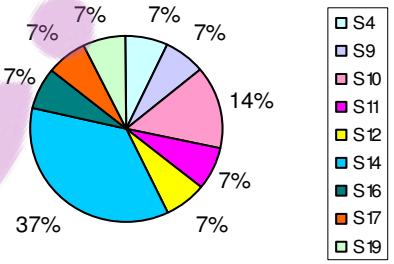
FIG. 3. Pie charts representing genotypic diversity of the *N. parvum* populations from *S. cordatum.* All South African isolates (a), the population collected from naturally regenerated trees growing amongst *Eucalyptus* plantations in the Kwambonambi (KWM) area (b), and the populations collected from planted trees in the towns of Pietermaritzburg (PTM) (c) and Tzaneen (TZ) (d). Different multilocus genotypes are indicated as S1-S23.



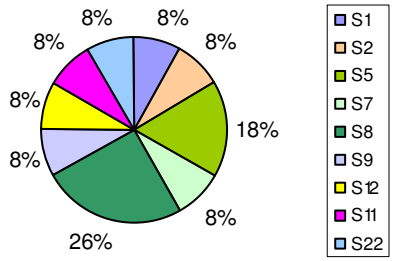
(a)



(b)

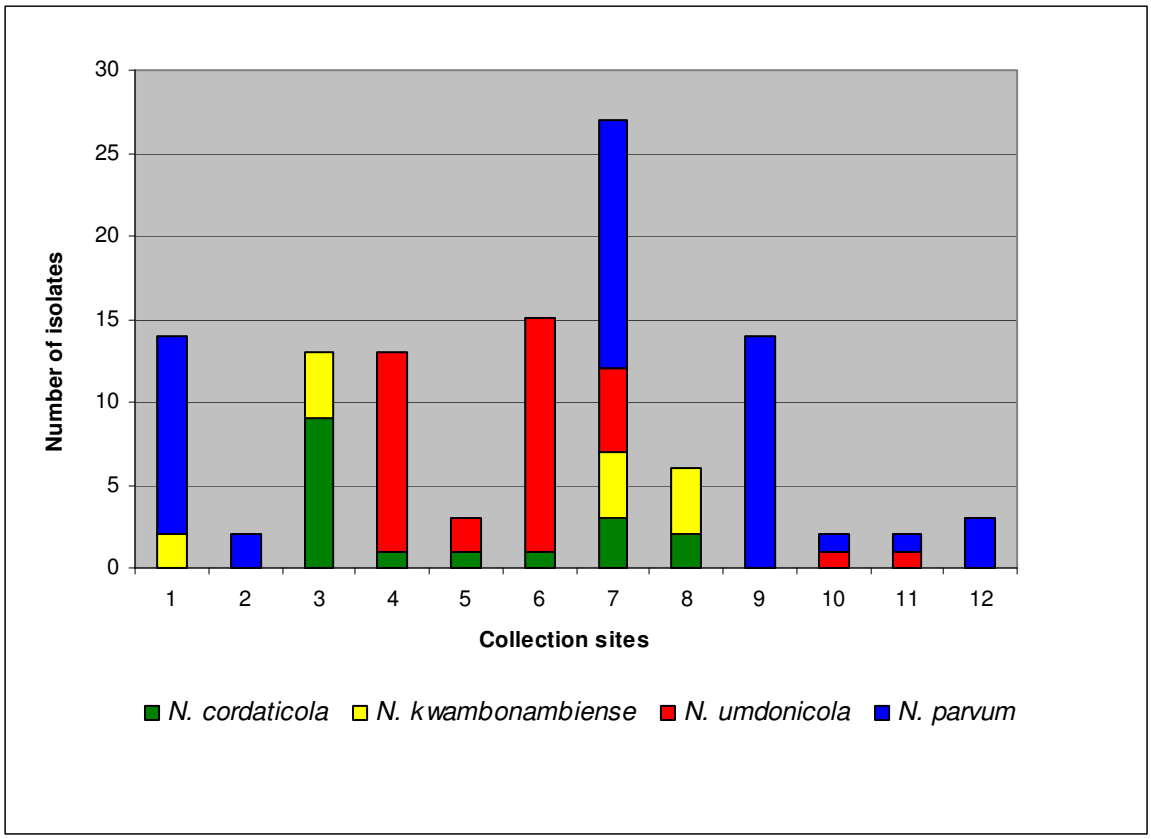


(c)



(d)

FIG. 4. Bars representing the distribution of four species from the *Neofusicoccum parvum* / *N. ribis* complex on *Syzygium cordatum* in the twelve collection sites. Tzaneen (1U), Pretoria (2U), Sabie (3N), Kosi Bay (4NR), Mkuze (5NR), Sodwana Bay (6NR), Kwambonambi (7E), Richards Bay (8N), Pietermaritzburg (9U), Port St Johns (10N), Gonubie (11N), Tokai (12U). U = Urban area; N = Natural stand; NR = Nature Reserve; E = Naturally regenerated *S. cordatum* amongst *Eucalyptus* stands.



Chapter 5

Seven new species of the Botryosphaeriaceae from baobab and other native trees in Western Australia

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ABSTRACT

In this study, seven new species of the Botryosphaeriaceae are described from baobab (*Adansonia gibbosa*) and surrounding endemic tree species growing in the Kimberley region of northwestern Australia. Members of the Botryosphaeriaceae were predominant endophytes isolated from apparently healthy sapwood and bark of endemic trees; others were isolated from dying branches. Phylogenetic analyses of ITS and EF-1α sequence data revealed seven new species: *Dothiorella longicollis*, *Fusicoccum ramosum Lasiodiplodia margaritacea*, *Neoscytalidium novaehollandiae*, *Pseudofusicoccum adansoniae*, *P. ardesiacum* and *P. kimberleyense*.

INTRODUCTION

Only eight species of baobabs (*Adansonia* spp.) are known. *Adansonia gibbosa* is the only baobab species endemic to Australia and is restricted to the northwestern part of the country (Crisp et al 2004). *Adansonia digitata* has a wide natural distribution throughout tropical parts of Africa and the six other species are found on Madagascar (Bowman 1997, Baum et al 1998). A recent biogeographical study on baobabs presented the intriguing view that the distribution of these unusual trees between Africa and Australia occurred after the division of Gondwana (Baum et al 1998). The same study revealed that *A. gibbosa* in Australia is more closely related to *A. digitata* from Africa than it is to species from Madagascar.

In this first study of fungi associated with *A. gibbosa* and surrounding endemic tree species in northwestern Australia, members of the Botryosphaeriaceae were found as non-sporulating endophytes in apparently healthy sapwood and bark of branches collected from all tree species sampled; they were also found sporulating and releasing conidia on dying branches of baobabs. Numerous studies have combined phenotype with DNA sequence analyses in defining genera and species in the Botryosphaeriaceae (Jacobs and Rehner 1998, Denman et al 2000, Zhou and Stanosz 2001, Slippers et al 2004, Phillips et al 2005). Crous et al (2006) summarised this work and represented several lineages in the Botryosphaeriaceae that were identified with generic names based on large sub-unit (LSU) sequence data, including *Botryosphaeria, Dothidotthia*, *Macrophomina*, *Neofusicoccum*, *Neoscytalidium*, *Pseudofusicoccum, Saccharata* and *Guignardia*. The identity and generic placement of the numerous species included in *Diplodia* and *Lasiodiplodia* were unclear in the study of Crous et al (2006), but they are clearly separated in ITS and EF-1α phylogenies (Burgess et al 2005, Phillips et al 2005, Damm et al 2007, Alves et al 2008).

In this study, we describe seven new species of Botryosphaeriaceae associated with *A. gibbosa* and other native trees in the northwestern Australia. The new taxa are characterised and described based on ITS and EF-1α sequence data combined with anamorph morphology.

MATERIALS AND METHODS

Isolates

Isolates used in this study were collected from *A. gibbosa* and surrounding native tree in northwestern Australia in June and July of 2006 (TABLE I). Asymptomatic and dying twigs of *A. gibbosa* were collected from 26 locations approximately 20 km apart along the Gibb

River Road. At three locations asymptomatic twigs were also collected from eight other tree species. The other tree species were different at the three locations, but included: *Acacia synchronica*, *Crotalaria medicaginea*, *Eucalyptus camaldulensis*, an unidentified *Eucalyptus* sp., *Ficus opposita*, *Grevillia agrifolia*, *Lysiphyllum cunninghamii* and a *Terminalia* sp. (TABLE I). Isolations were made from visually healthy sapwood and bark collected from branches following Burgess et al (2006b). Collections were also made from pycnidia formed on dying branches. When pycnidia were found on dying branches, masses of conidia were directly transferred to 2 % malt extract agar (MEA) (Biolab, S.A.). Single-conidial cultures of all isolates used in this study are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, and the Murdoch University Culture Collection (MUCC). A representative set of isolates has also been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands.

DNA sequence comparisons

DNA was extracted from fungal mycelium from 7 d old single-conidial cultures as described by Burgess et al (2005). DNA was purified using the Ultrabind ® DNA purification kit following the instructions given by the manufacturer (MO BIO Laboratories). Two gene regions were used for phylogenetic analyses. The internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) operon was amplified for all isolates using primers ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al 1990). For selected isolates, a part of the elongation factor 1α (EF-1α) gene was amplified using primers EF1-728F and EF1-986R (Carbon and Kohn 1999). The PCR reaction mixture, PCR conditions and visualization were as described by Pavlic et al (2004) except that 0.5 U of Taq polymerase (Biotech International, Needville, Texas) was used. PCR products were cleaned with the Ultrabind ® DNA purification kit and sequenced with the BigDye terminator cycle sequencing kit (PE Applied Biosystems) in both directions, using the same primers used for the PCR reactions. Products were separated with an ABI 3730 48 capillary sequencer (Applied Biosystems, Foster City, California). Data were collected with ABI data collection software.

Sequence data for isolates of the unknown species were deposited in GenBank (TABLE I). Sequences of known species were obtained from GenBank, and the isolate code, identity and accession numbers for sequence data used are given in TreeBASE (http://www.treebase.org/treebase/index.html, accession number SN3768). Parsimony

analysis was performed on individual datasets (individual trees are not illustrated) and on the combined data set after partition homogeneity tests (PHT) was performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2000) to determine whether sequence data from the ITS and EF-1α gene regions were statistically congruent (Farris et al 1995, Huelsenbeck et al 1996). Non-informative characters were removed prior to analysis and characters were unweighted and unordered. The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed and all multiple equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (Hillis and Huelsenbeck 1992). Branch and branch node supports were determined using 1000 bootstrap replicates (Felsenstein 1985). The tree is rooted to a *Guignardia* sp.

Bayesian analysis was conducted on the same individual and combined dataset as that used in the parsimony analysis. First, MrModeltest v. 2.5 (Nylander, 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v. 3.1 (Ronquist and Huelsenbeck, 2003) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 1 000 000 generations. Trees were saved each 1000 generation, resulting in 10 001 trees. Burn-in was set at 50 001 generations (i.e. 51 trees), well after the likelihood values converged to stationery, leaving 9950 trees from which the consensus trees and posterior probabilities were calculated.

Morphological characteristics

To induce sporulation, cultures were inoculated onto sterilized pine needles and/or eucalypt twigs placed on the surface of 2 % water agar (WA) (Biolab, S.A.) and incubated at 25 ºC under near-UV light. To obtain single-conidial cultures, releasing conidia from pycnidia formed on pine needles and/or eucalypt twigs, were transferred on WA and spread on the medium surface by sterilised streaking loop. Plates were incubated at 25 ºC under near-UV light for approximately 12 h and single germinating conidia were transferred on the MEA, using sterilised needle, and incubated under the same conditions. A single pycnidium was placed in a drop of lactoglycerol on a microscope slide and cut in pieces using a sterile

medical needle before adding the cover slip. Fifty released conidia and 30 of pycnidia, conidiogenous cells and paraphyses, were measured for each isolate, and the ranges and averages were computed. Measurements and digital images were made using an HRc Axiocam digital camera and accompanying Axiovision 3.1 software (Carl Zeiss Ltd., Munich, Germany). Drawings were prepared with a drawing tube and finalized using the method described by Barber and Keane (2007). Colony morphology and color were determined from cultures grown on MEA at 25 ºC in the dark. Colony colors (upper surface and reverse) were determined by comparison to the color charts of Rayner (1970).

Growth rates at temperatures ranging from 5 to 35 ºC, at 5 ºC intervals were determined from cultures grown in the dark. To determine growth rate, mycelial plugs, 6 mm diam, were taken from the actively growing edges of 7 d old single-conidial cultures and transferred to the centers of MEA in 90 mm diam Petri dishes. Three replicate plates were used for each isolate at each temperature. Two perpendicular measurements were taken of the colony diameter daily until the mycelium of the fastest growing isolates had covered the plates.

RESULTS

DNA sequence comparisons

The partition homogeneity test comparing the ITS and EF-1α data sets was significant (P = 0.007) indicating that the individual data sets were not congruent and produced trees with differing topology. These differences were not due to the relationships among species in a genus, but rather the relationship of the genera to each other. Thus, when the data were combined, support for the placement of species within a genus was high, but the support for the deeper branches, indicating relationships between genera, was low. Similar discrepancies were found when comparing the phylogeny obtained from parsimony and Bayesian analyses. Thus, the analyses for the individual ITS and EF-1α datasets are available from TreeBASE (SN3768), while the results emerging from the combined dataset are presented here.

The combined dataset consisted of 996 characters of which 546 were parsimony informative. The data set contained significant phylogenetic signal compared to 1000 random trees (P < 0.01, g1 = -0.43). Heuristic searches resulted in 2 most parsimonious trees of 1566 steps (CI = 0.60, RI = 0.90) (FIG. 1, TreeBASE SN3768). In the Bayesian analysis, the positions of the genera in relation to each other differed, but within each genus, the topology was similar to the parsimony tree (TreeBASE SN3768). Eight clades were

identified, each corresponding to a separate genus and each supported with bootstrap values of 100 % and Bayesian probabilities of 1.00. These were Clade 1 (*Lasiodiplodia*), Clade 2 (*Diplodia*), Clade 3 (*Dothiorella*), Clade 4 (*Neofusicoccum)*, Clade 5 (*Botryosphaeria*), Clade 6 (*Macrophomina)*, Clade 7 (*Neoscytalidium*) and Clade 8 (*Pseudofusicoccum*). Isolates obtained in this study resided in Clades 1, 3, 5, 7 and 8.

Within the *Lasiodiplodia* clade, two isolates were found to be distinct from the known species in this genus (FIG. 1). Three isolates, two from *Lysiphyllum cunninghamii* and one from a *Terminalia* sp., formed a well-supported lineage in the *Dothidotthia/Dothiorella* clade (FIG. 1). Within the *Botryosphaeria* clade, a single isolate from *Eucalyptus camaldulensis* was phylogenetically distinct from the two previously sequenced (for ITS and EF-1α) species, *B. dothidea* and *B. corticis* (FIG. 1). Four isolates obtained in the present study from *Acacia synchronica*, *Adansonia gibbosa*, *Crotalaria medicaginea* and *Grevillia agrifolia* formed a separate sub-clade within the *Neoscytalidium* clade (FIG. 1). Although support for this sub-clade was low, these isolates produce *Dichomera*-like synanamorphs that distinguish them from known *Neoscytalidium* species and are described in this study as a new *Neoscytalidium* species. *Pseudofusicoccum* is currently monotypic for *P. stromaticum*. In this study, three new species were found that phylogenetically reside in this genus (FIG. 1).

Morphology

With exception of one isolate of *Pseudofusicoccum ardesiacum* (CMW26160), all the isolates of the Botryosphaeriaceae obtained from *A. gibbosa* and other native trees in northwestern Australia produced pycnidia on the pine needles and eucalyptus twigs on WA within two to three weeks. No ascomata were observed. Based on culture and conidial morphology, isolates were separated into seven species: three in *Pseudofusicoccum* and one species each in *Dothiorella, Fusicoccum*, *Lasiodiplodia* and *Neoscytalidium*. These species are described as follows.

TAXONOMY

Pseudofusicoccum adansoniae Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512048 FIGS. 2, 3.

Pycnidia subimmersa solitaria globosa papillata castanea, mycelio tecta, usque ad 500 µm diametro. Cellulae conidiogenae holoblasticae laeves cylindricae hyalinae, conidio

primo holoblastico, posteriora enteroblastica. Conidia mediocriter 22.5 × 5.2 µm, 4.3 plo longiora quam latiora, hyalinae, parietibus tenuibus, viscida strato persistenti muci tecta, laeves contentu tenue granulari, raro subflexa vel irregularia, apicibus rotundatis, unicellularia, ante germinationem 1–2 septa formantia.

Pycnidia semi-immersed, solitary, globose, papillate, chestnut, covered by hyphal hairs, up to 500 µm diam. *Conidiogenous cells* holoblastic, smooth, cylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (9–) 10–15 (–16) × (1.5–) 2–3 (–3.5) µm (av. 12.7 × 2.4 µm). *Conidia* ellipsoid, occasionally slightly bent or irregularly shaped, (19–) 21–24 (–26) × (3.5–) 4.5–6 (–6.5) µm (av. 22.5 × 5.2 µm, l/w 4.3), apices rounded, smooth with fine granular content, hyaline, thin-walled, covered with a persistent mucus layer, unicellular, forming 1 or 2 septa prior to germination. *Cultural characteristics*. Colonies initially white with moderately dense, appressed mycelial mat. Submerged mycelium, turning grey olivaceous (21’’’’b) to olivaceous black (27’’’’m) from the middle of colony after 3–5 d and becoming dark slate-blue (39’’’’k) with age. Aerial mycelium slightly fluffy, becoming dense, cottony with age, sometimes remaining white to smoke grey (21’’’’f), usually turning pale olivaceous grey (21’’’’’d) within 7 d and becoming olivaceous grey (21’’’’’i) to iron grey 23’’’’’k) with age. Colonies slightly irregular, occasionally radially striated with lobate edges and/or forming concentric, irregular circles. Conidiomata readily formed from the middle of colony within 7–10 d, covering the entire surface of the colony and immersed in the medium (seen as a round black structures on the reverse side of Petri dishes) 14 d after incubation. Optimum growth temperature 30 ºC, covering the 90 mm diam Petri dish after 4 d in the dark.

Teleomorph. Not known.

Etymology. Refers to the host from which the type specimen was isolated.

Habitat. Dying branches of *Adansonia gibbosa* and asymptomatic branches of *Acacia synchronica*, *Eucalyptus* sp. and *Ficus opposita*.

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Derby (17°21’03.150S, 123°40’07.578E), on *Adansonia gibbosa*, Jul 2006, T.I. Burgess (PREM 59841, a dry culture ex CMW 26147 on pine needles; ex-type culture CMW 26147 = CBS 122055).

Additional specimens examined. See TABLE I.

Pseudofusicoccum kimberleyense Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512049 FIGS. 4, 5.

Pycnidia subimmersa solitaria globosa papillata castanea, mycelio tecta, usque ad 500 µm diametro. Cellulae conidiogenae holoblasticae laeves cylindricae vel subcylindricae hyalinae, conidio primo holoblastico, posteriora enteroblastica. Conidia mediocriter 30.7 × 7.4 µm, 4.1 plo longiora quam latiora, hyalinae, parietibus tenuibus, viscida strato persistenti muci tecta, laeves contentu tenue granulari, ellipsoidea, recta vel subfalcata, apicibus rotundatis, unicellularia, ante germinationem 1–4 septa formantia.

Pycnidia semi immersed, solitary, globose, papillate, chestnut, covered by hyphal hairs, up to 500 µm diam. *Conidiogenous cells* holoblastic, smooth, cylindrical to subcylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (7–) 8.5–11 (–14) × (2.5–) 3–3.5 (–4) µm (av. 9.8 × 3.3 µm). *Conidia* ellipsoid, straight or slightly curved, (24–) 28–33 (–34) × (6.5–) 7–8 (–8.5) µm (av. 30.7 × 7.4 µm, l/w 4.1), apices rounded, smooth with fine granular content, hyaline, thin-walled, covered with a persistent mucus layer, unicellular, forming 1–4 septa prior to germination. *Cultural characteristics*. Colonies initially white, hyphae forming a moderately dense, appressed mycelial mat. Submerged mycelium citrine (21k) to grey olivaceous (21’’’’b) from the middle of colony after 3–5 d, becoming olivaceous black (27’’’’m) to black with age. Aerial mycelium slightly fluffy, becoming dense, cottony with age, smoke grey (21’’’’f) to pale olivaceous grey (21’’’’’d). Colonies slightly irregular with sinuate edges. Optimum growth temperature 30 ºC, covering the 90 mm diam Petri dish after 4 d in the dark.

Teleomorph. Not known.

Etymology. Refers to Kimberley region, Western Australia where the substratum was collected from which the fungus was isolated.

Habitat. Dying branches of *Adansonia gibbosa* and asymptomatic branches of *Acacia synchronica*, *Eucalyptus* sp. and *Ficus opposita*.

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Tunnel Creek National Park (17°54’33.342S, 125°17’01.686E), on *Acacia synchronica,* Jul 2006, T.I. Burgess (PREM 59842, a dry culture on pine needles ex CMW 26156; ex-type culture CMW 26156 = CBS 122058).

Additional specimens examined. See TABLE I.

Pseudofusicoccum ardesiacum Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512051 FIGS. 6, 7.

Pycnidia subimmersa solitaria globosa papillata castanea, mycelio tecta, usque ad 510 µm diametro. Cellulae conidiogenae holoblasticae laeves cylindricae vel subcylindricae hyalinae, conidio primo holoblastico, posteriora enteroblastica. Conidia mediocriter 25 × 7.5 µm, 3.3 plo longiora quam latiora, hyalinae, parietibus tenuibus, viscida strato persistenti muci tecta, laeves contentu tenue granulari, ellipsoidea vel vergata, recta vel subflea, apicibus rotundatis, unicellularia, ante germinationem 1–3 septa formantia.

Pycnidia semi-immersed, solitary, globose, papillate, chestnut, covered by hyphal hairs, up to 510 µm diam. *Conidiogenous cells* holoblastic, smooth, cylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (6–) 7.5–10 (–11) × (2.7–) 3–4 (–4.3) µm (av. 8.6 × 3.5 µm). *Conidia* ellipsoid to rod-shape, straight or slightly bent, (17.5–) 21–29 (–32) × (6.3–) 7–8 (–9) µm (av. 25 × 7.5 µm, l/w 3.3), apices rounded, smooth with fine granular content hyaline, thin-walled, covered with a persistent mucus layer, unicellular, forming 1–3 septa prior to germination. *Cultural characteristics*. Colonies initially white with sparse to moderately dense appressed mycelial mat. Submerged mycelium dark violet (59m) to dark blue (47m) (middle of the colony) and smoke grey (21’’’’f) to grey olivaceous (21’’’’b) towards edges within 3–5 days, becoming violaceous grey (59’’’’i) to slate blue (47’’’k) with age. Aerial mycelium slightly fluffy, becoming dense, cottony with age, turning smoke grey (21’’’’f) to pale purplish grey (71’’’’’d) in the middle of colony and smoke grey (21’’’’f) to grey olivaceous (21’’’’b) towards edges after 5–7 days, becoming lavender grey (45’’’’’f) with age; occasional columns of aerial mycelium in the middle of colony, reaching the lid. Colonies slightly irregular with sinuate edges. Conidiomata readily formed and immersed in aerial mycelia on the entire colony surface within 7–10 days. Optimum growth temperature 30 ºC, covering the 90 mm diam Petri dish after 4 d in the dark.

Teleomorph. Not known.

Etymology. Refers to the slate blue-violet pigment found in cultures.

Habitat. Dying branches of *Adansonia gibbosa* and asymptomatic branches of *Eucalyptus* sp.

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Mt Hardman, Great Northern Highway (17°16’05.952S, 123°45’26.930E), on *Adansonia gibbosa*, Jul 2006, T.I. Burgess

(PREM 59843, a dry culture ex CMW 26159 on pine needles; ex-type culture CMW 26159 = CBS 122062).

Additional specimens examined. See TABLE I.

Dothiorella longicollis Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512053 FIGS. 8, 9.

Pycnidia subimmersa plerumque solitaria, basi globosa usque ad 550 µm diametro, collis longis, interdum ramosis, usque ad 1.5 mm longis, e substrato orientia. Cellulae conidiogenae holoblasticae cylindricae vel subcylindricae hyalinae, conidio primo holoblastico, posteriora enteroblastica. Conidia mediocriter 20.4 × 8.7µm, 2.3 plo longiora quam latiora, primo hyalina unicellularia, dum etiam ad cellulas conidiogenas affixa cinnamomeo- vel sepiaceo-brunnescentia, uniseptata, ovalia vel ovoidea apice rotundata basi truncata.

Pycnidia semi-immersed, mostly solitary, with globose base (up to 550 µm diam) and long neck (sometimes branching), up to 1.5 mm long, arising from the substrate. *Conidiogenous cells* holoblastic, cylindrical to subcylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (5–) 6–8 (–10) × (2.5–) 3–4 (–4.5) µm (av. 7.3 × 3.4 µm). *Conidia* oval to ovoid, (17–) 19–22 (–23) × (7–) 8–9.5 (–10.5) µm (av. 20.4 × 8.7 µm, l/w 2.3), apices rounded and truncate base, initially hyaline, unicellular, becoming cinnamon (13'') to sepia (13''k) and one-septate while still attached to conidiogenous cells. *Cultural characteristics*. Colonies initially white to olivaceous-buff (21'''d), becoming greenish-olivaceous (23''') to citrine (21k) from the middle of colonies within 7 d, iron grey (23''''') (surface) and black (beneath) with age, with suppressed, moderately fluffy mycelium, edges smooth appearing sinuate as the colony darkens with age. Conidiomata readily formed from the middle of colony within 7–10 days, covering the entire surface of the colony and immersed in the medium (seen as round black structures on the reverse side of Petri dishes) 14 days after incubation. Optimum growth temperature 25 ºC, covering the 90 mm diam Petri dish after 4 d in the dark.

Teleomorph. Not known.

Etymology. Refers to the fact that the pycnidia have long necks.

Habitat. Asymptomatic branches of *Lysiphyllum cunninghamii* (Caesalpiniaceae) and *Terminalia* sp. (Combretaceae).

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Tunnel Creek National Park (17°54'33.342S, 125°17'01.686E), on *Lysiphyllum cunninghamii*, Jul 2006, T.I. Burgess

(PREM 59845, a dry culture ex CMW 26166 on pine needles; ex-type culture CMW 26166 = CBS 122068).

Additional specimens examined. See TABLE I.

Note. Cultures transferred onto WA with pine needles formed numerous pycnidia on the surface and immersed in the medium. *Dothiorella longicollis* conforms well to morphological concept of the genus proposed by Phillips et al (2005).

Lasiodiplodia margaritacea Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512052 FIG. 10, 11.

Pycnidia subimmersa solitaria globosa papillata nigra mycelio tecta, usque ad 520 µm diametro. Paraphyses cylindricae, 1–2-septatae, hyalinae. Cellulae conidiogenae holoblasticae, cylindricae vel subcylindricae, hyalinae, conidio primo holoblastico, posteriora enteroblastica. Conidia mediocriter 15.3 × 11.4µm, 1.3 plo longiora quam latiora primo unicellularia, hyalina globosa subglobosa vel obovoidea, parietibus crassis, contentu granuloso, cinnamomeo- vel sepiaceo-brunnescentia, cum maturitate 1-septata longitudinaliter striata.

Pycnidia semi-immersed, solitary, globose, papillate, black, covered by hyphal hairs, up to 520 µm diam. *Paraphyses* cylindrical, 1–2 septate, hyaline, (19–) 28–46 (–54) × (1.5–) 2–2.5 (–3) µm (av. 37.1 × 2.2 µm), formed among conidiogenous cells. *Conidiogenous cells* holoblastic, cylindrical to subcylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (6–) 10–11 (–19.5) × (2–) 3–4 (–4.5) µm (av. 10.3 × 3.3 µm). *Conidia* globose to subglobose to obovoid, (12–) 14–17 (–19) × (10–) 11–12 (–12.5) µm (av. 15.3 × 11.4 µm, l/w 1.3), with granular content, thick-walled (1–2 µm), initially unicellular, hyaline, becoming cinnamon (13'') to sepia (13''k), forming one septum and longitudinal striations with maturation. *Cultural characteristics*. Colonies initially white to smoke grey (21''''f) with woolly aerial mycelium, becoming pale olivaceous grey (21'''''d) within 5–7 d, olivaceous grey (21'''''i) to iron grey (23'''''k) with age, margins regular. Submerged mycelium dense, reverse grey olivaceous (21''''b) to olivaceous black (27''''m) after 7 d, becoming black with age. Optimum growth temperature 30 ºC, covering the 90 mm diam Petri dish after 3 d in the dark.

Teleomorph. Not known.

Etymology. The name refers to the conidia that have a pearl-like appearance.

Habitat. Asymptomatic branches of *Adansonia gibbosa*.

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Tunnel Creek Gorge (17°36'22.884S, 125°108'46.056E), on *Adansonia gibbosa*, Jul 2006, T.I. Burgess (PREM 59844, a dry culture ex CMW 26162 on pine needles; ex-type culture CMW 26162 = CBS 122519).

Additional specimens examined. See TABLE I.

Notes. Isolates of *L. margaritacea* clustered with other *Lasiodiplodia* species with high bootstrap support (100 %). The septate conidia with striations that darken with age, as well as paraphyses, are typical of the genus (Punithalingham 1976, Pavlic et al 2004, Burgess et al 2006a). However, the smaller, subglobose conidia clearly distinguish this species from previously described species (Punithalingham 1976, Pavlic et al 2004, Burgess et al 2006a, Damm et al 2007, Alves et al 2008).

Fusicoccum ramosum Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512054 FIGS. 12, 13.

Pycnidia subimmersa solitaria globosa papillata castanea, mycelio tecta, usque ad 510 µm diametro, interdum collis ad 1.7 mm longis, e substrato orientia. Cellulae conidiogenae holoblasticae, cylindricae vel subcylindricae, hyalinae, conidio primo holoblastico, posteriora enteroblastica. Conidiophorae laeves cylindricae septatae usque ad 2 µm latae 50 µm longae, simplices vel ramosae. Conidia mediocriter 13.4 × 5.7 µm, 2.3 plo longiora quam latiora, hyalinae, parietibus tenuibus vel subcrassis, laeves contentu tenue granulari, fusiformia ellipsoidea vel ovalia apicibus rotundatis basibus truncatis, unicellularia vel 1-septata.

Pycnidia semi-immersed, solitary, globose, papillate, chestnut, covered by hyphal hairs, up to 510 µm diam, sometimes with a neck to 1.7 mm long, arising from the substrate. *Conidiogenous cells* smooth, cylindrical to subcylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (6–) 7.5–10 (–11) × (2–) 2–3 (–3.5) µm (av. 8.7 × 2.5 µm). *Conidiophores* smooth, cylindrical, septate, up to 2 µm wide and 50 µm long, simple or branching. *Conidia* fusiform to ellipsoid to oval, (11–) 12–15 (–16) × (4.7–) 5–6 (–7) µm (av. 13.4 × 5.7 µm, l/w 2.3), apices rounded or round at apex and truncate at base, smooth with fine granular contents, hyaline, wall thin to slightly thickened, unicellular or 1 septate. *Cultural characteristics*. Colonies initially white turning grey olivaceous (21''''b) from the middle of colonies within 5–7 days, with appressed mycelial mat and white moderately dense, cottony aerial mycelium towards the edge of the colony, becoming smoke grey (21''''f) to olivaceous grey (21'''''i) (surface) and iron grey

(23’’’’’k) (beneath) within 10–14 days. Optimum growth temperature 25 ºC, covering the 90 mm diam Petri dish after 4 d in the dark.

Teleomorph. Not known.

Etymology. Name refers to the branched conidiophores of this species.

Habitat. Asymptomatic branches of *Eucalyptus camaldulensis*.

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Bell Gorge (17°00’58.584S, 125°13’47.866E), on *Eucalyptus camaldulensis*, Jul 2006, T.I. Burgess (PREM 59846, a dry culture ex CMW 26167 on pine needles; ex-type culture CMW 26167 = CBS 12206).

Notes. The only known culture of “Botryosphaeria”, anamorph *Fusicoccum ramosum*, is distinguished from other species in the genus by its long, simple or branching conidiophores. Its conidia develop a single septum before germinating, as is typical of *Botryosphaeria* (Slippers et al 2004). It did not produce a *Dichomera* synanamorph, which is reported for some isolates of the type species *Botryosphaeria dothidea* (Barber et al 2005). The conidia of *Fusicoccum ramosum* are significantly shorter then those of known species in this genus.

Neoscytalidium novaehollandiae Pavlic, Burgess, M.J. Wingfield, sp. nov. MB512103 FIGS. 14, 15.

Pycnidia ad dimidium immersa vel superficiales, solitaria vel in stromata multilocularia, nigra, cum basim globosa, diametrus usque ad 300 µm, collis usque ad 600 µm longis. Cellulae conidiogenae holoblasticae, cylindricae vel subcylindricae, hyalinae, conidio primo holoblastico, posteriora enteroblastica. Conidia (1) mediocriter 11.5 × 4.4 µm, 2.6 plo longiora quam latiora, apices rotundati, primo hyalina, evadentes cinnamomeo- vel sepiaceo-brunnescentia cum maturitate, sive ellipsoidea vel ovoidea et 0–1–2-septata cum maturitate; (2) mediocriter 10.6 × 6.9 µm, 1.5 plo longiora quam latiora, primo hyaline, evadentes cinnamomeo- vel sepiaceo-brunnescentia cum maturitate, sive in forma variabilia, irregularia, globosa, subglobosa vel obpyriformia, cum septis muriformibus, Arthroconidia catenulata in mycelio aerio, mediocriter 6.5 × 4 µm, 1.6 plo longiora quam latiora, pulveriformia, disarticulantia, cylindrica, oblonga, obtusa vel doliiformia, crasse tunicata, primo hyalina et unicellularia, cinnamomeo- vel sepiaceo-brunnescentia et 0–1-septata cum maturitate.

Pycnidia semi-immersed or superficial, solitary or in multilocular stromata, black, with globose base, up to 300 µm diam and long neck, up to 600 µm long. *Conidiogenous*

cells holoblastic, cylindrical to subcylindrical, hyaline, the first conidium produced holoblastically and subsequent conidia enteroblastically, (6–) 7–10 (–11) × (2–) 2–3 (–4) µm (av. 8.6 × 2.5 µm). *Conidia* of two distinct types: (1) ellipsoidal to oval, (8–) 10.5–12.5 (–14) × (3–) 4–5 (–5) µm (av. 11.5 × 4.4 µm, l/w 2.6), apices rounded, initially hyaline, unicellular, becoming cinnamon (13'') to sepia (13''k), and 0–1-septate or 2-septate with darker central cell; (2) variable in shape, globose, subglobose to obpyriform with muriform septa, (8–) 8.5–12.5 (–15.5) × (5–) 5.5–7.5 (–8) µm (av. 10.6 × 6.9 µm, l/w 1.5), initially hyaline becoming cinnamon (13'') to sepia (13''k). Aerial mycelium forms chains of arthroconidia, (5–) 5.5–7.5 (–9.5) × (3–) 3.5–4.5 (–5) µm (av. 6.5 × 4 µm, l/w 1.6), unicellular, powdery to the touch, disarticulating, cylindrical, oblong to obtuse to doliiform, thick-walled, initially hyaline becoming cinnamon (13'') to sepia (13''k) and 0–1-septate. *Cultural characteristics*. Colonies initially white to olivaceous-buff (21'''d), becoming greenish-olivaceous (23''') to citrine (21k) from the middle of colonies within 7 d, and black (surface and beneath) with age, with suppressed, moderately fluffy mycelium, edges smooth. Optimum growth temperature 35 C, covering the 90 mm diam Petri dish after 3 d in the dark.

Teleomorph. Not known.

Etymology. Name refers to original Dutch name for Western Australia, where the substratum was collected from which the fungus was isolated.

Habitat. Asymptomatic branches (sapwood) of *Acacia synchronica*, *Adansonia gibbosa, Crotalaria medicaginea* and *Grevillia agrifolia*.

Known distribution. Western Australia.

HOLOTYPE. AUSTRALIA. WESTERN AUSTRALIA: Bell Gorge (17°00'58.584S, 125°13'47.866E), on *Crotalaria medicaginea*, Jul 2006, T.I. Burgess (PREM 60069, a dry culture ex CMW 26170 on pine needles; ex-type culture CMW 26170 = CBS 122071).

Additional specimens examined. See TABLE I.

Note: Isolates of *Neoscytalidium novaehollandiae* are similar in morphological characteristics to those of the type species *N. dimidiatum* (Punithalingam and Waterston 1970, Crous et al 2006). However, isolates obtained in this study produce muriform, *Dichomera*-like conidia that distinguish this species from known *Neoscytalidium* spp.

KEY TO *PSEUDOFUSICOCCUM* SPECIES

1. Blue-violet pigment in cultures visible after 3–5 days; conidia averaging 25 µm long, l/w 3.3, aseptate, forming 1–3 septa prior to germination ***P. ardesiacum***

1. Blue-violet pigment absent in cultures	2
2. Conidia on average >30 µm long	***P. kimberleyense***
2. Conidia on average <25 µm long	3
3. Conidia aseptate, l/w 4	***P. stromaticum***
3. Conidia aseptate, forming 1or 2 septa prior to germination, l/w 4.3	***P. adansoniae***

DISCUSSION

Seven new species of Botryosphaeriaceae were isolated from endemic trees in Western Australia. Combined ITS and EF-1α sequence data distributed these isolates among the genera *Botryosphaeria, Dothiorella, Lasiodiplodia*, *Neoscytalidium* and *Pseudofusicoccum*. Teleomorphs were not observed for any of the species identified in this study.

Three of the seven new fungi are species of *Pseudofusicoccum*, a genus previously monotypic for *P. stromaticum* (Crous et al 2006, Mohali et al 2006). *Pseudofusicoccum* is separated from *Fusicoccum* by the presence of persistent mucous sheaths surrounding the conidia (Crous et al 2006). *Pseudofusicoccum stromaticum* was described on non-native *Acacia* and *Eucalyptus* spp. in Venezuela (Mohali et al 2006). Strains of *P. adansoniae* and *P. kimberleyense* described in this study were obtained from four unrelated hosts (*Acacia* sp., *Eucalyptus* sp., *Ficus* sp. and *A. gibbosa*) residing in four families all native to Western Australia. Isolates of *P. ardesiacum* were obtained from two of these native hosts, *Eucalyptus* and *A. gibbosa.* The fact that all *Pseudofusiccum* spp. occured on native hosts in a relatively undisturbed area of Australia or in the case of *P. stromaticum* on Australian plants suggests that the species are most likely native to Australia.

Isolates of *P. adansoniae* came from different hosts but were morphologically uniform. This is in contrast to isolates of *P. kimberleyense,* which displayed differences in conidial morphology, and variation in DNA sequences in both gene regions analysed. These variations could indicate that *P. kimberleyense* is comprised of more than one species. *Pseudofusicoccum ardesiacum* was easily distinguished from other species in the genus by its smaller conidia and the distinct slate blue-violet pigment that it produces in culture.

Two species with dark conidia were identified in this study. Based on phylogenetic analyses and phenotype, they have been placed in *Lasiodiplodia* and *Dothiorella*. *Lasiodiplodia margaritacea* was identified only from dying branches of *A. gibbosa*. High numbers of dead and dying baobabs (*A. digitata*) have been reported in Southern Africa, particularly in Zimbabwe (Anonymous 1991, Piearce et al 1994). The symptoms identified

on the trees in Zimbabwe were originally reported as "sooty bark disease" caused by species of sooty mould fungi (Calvert 1989, Anonymous 1991). However, Piearce et al (1994) reported that the "sooty" baobabs were dying due to drought, related to climatic change, rather than being caused by fungal pathogens. A recent study on diseases of baobabs in South Africa, showing symptoms of die-back and death of branches followed by sap exudation, revealed that *Lasiodiplodia theobromae* was the most abundant fungus present (Roux 2002). This fungus is a well-known latent, stress-associated pathogen on more then 500 hosts world-wide (Punithalingham 1976), and as such, could also be involved in the decline of baobab trees in African countries (Roux 2002). Since *Lasiodiplodia margaritacea* was found only on *A. gibbosa* that shows die-back symptoms, this fungus could be pathogenic to this host.

Dothiorella longicollis is another species with dark conidia described in this study. This species is morphologically similar to the other species with *Dothiorella* anamorphs, *D. iberica, D. sarmentorum* and *D. viticola* (Luque et al 2005, Phillips et al 2005). Except for the pycnidia with long necks, which are distinct feature of *D. longicollis*, other morphological characteristics such as conidial shape and size, overlap among these species and cannot be used to separate them with confidence. However, their distinction is well supported in the ITS and EF-1α phylogenies. *Dothiorella longicollis* occured as an endophyte in asymptomatic branches of two unrelated hosts, *Lysiphyllum cunninghamii* (Caesalpiniaceae) and a *Terminalia* sp. (Combretaceae) endemic to Western Australia and nothing is known regarding its ecology.

A number of isolates obtained from asymptomatic branches on different hosts, including *Acacia*, *Adansonia, Crotalaria* and *Grevillia*, were identified as *Neoscytalidium novaehollandiae*. *Neoscytalidium*, with *N. dimidiatum* as a type, accommodates species with *Scytalidium*-like synanamorphs (Crous et al 2006). These are characterized by conidia held in arthric chains in the aerial mycelium. In addition to arthroconidia, the cultures produce *Fusicoccum*-like conidia in pycnidia. Isolates of *N. novaehollandiae* identified in this study produce a *Dichomera*-like synanamorph, which is not known for other species in this genus. *Dichomera*-like synanamorphs were recently described for *Botryosphaeria dothidea*, *Neofusicoccum parvum, N. ribis* and *N. australe* (Barber et al 2005), however this is the first time that *Dichomera*-like synanamorph is identified for *Neoscytalidium*. *Neoscytalidium dimidiatum* has been isolated from different substrates including plant tissues, soil, human skin and nails, and is known as plant pathogen (Punithalingam and Waterston 1970, Crous et

al 2006). The isolates examined in this study were collected as endophytes from plant tissues. This is the first report of *Neoscytalidim* sp. on *A. gibbosa.*

Fusicoccum ramosum was isolated as endophyte from asymptomatic twigs of *Eucalyptus camaldulensis*. Numerous species of Botrosphaeraceae with '*Fusicoccum*' anamorphs identified from *Eucalyptus* have now been placed in a new genus *Neofusicoccum* (Crous et al 2006). *Neofusicoccum* spp. are the most common endophytes and latent pathogens of *Eucalyptus* (Burgess et al 2006b, Slippers and Wingfield and 2007), however no *Neofusicoccum* spp. were isolated from *Euclyptus* in this study.

Species of Botryosphaeriaceae are well-known as endophytes and latent, opportunistic canker and die-back pathogens on numerous woody hosts worldwide (von Arx 1987, Slippers and Wingfield 2007, de Wet et al 2008). However, this is the first detailed study to consider these fungi on *Adansonia gibbosa*, and also other endemic trees in Western Australia, including *Acacia synchronica*, *Crotalaria medicaginea*, *Eucalyptus camaldulensis*, *Eucalyptus* sp., *Ficus opposita*, *Grevillia agrifolia*, *Lysiphyllum cunninghamii* and *Terminalia* sp. The seven new species emerging from this study, of which five were recorded on *A. gibbosa,* reflects a lack of knowledge regarding the fungi on *A. gibbosa* and of the Botryosphaeriaceae on native plants in this region. The role of these fungi in the ecology of the trees from which they were collected will be considered in future studies.

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TABLE I. Isolates included in the phylogenetic study

Culture no.[1]	Other no. [1]	Identity	Host	Location [2]	Genbank no. [3]	
					ITS	EF-1α
CMW26145	MUCC520, CBS122053	*Pseudofusicoccum adansoniae*	*Acacia synchronica*	WA, Tunnel Creek NP	EF585525	EF585569
CMW26148	MUCC521, CBS122056	*P. adansoniae*	*Ficus opposita*	WA, Tunnel Creek NP	EF585524	EF295489
CMW26147	MUCC522, CBS122055	*P. adansoniae*	*Adansonia gibbosa*	WA, Derby	EF585523	EF585571
CMW26146	MUCC533, CBS122054	*P. adansoniae*	*Eucalyptus* sp.	WA, Tunnel Creek NP	EF585532	EF585570
CMW26161	CBS122061	*P. kimberleyense*	*F. opposita*	WA, Tunnel Creek NP	EU144059	EU144074
CMW26156	CBS122058	*P. kimberleyense*	*Ac. synchronica*	WA, Tunnel Creek NP	EU144057	EU144072
CMW26157	CBS122059	*P. kimberleyense*	*Eucalyptus* sp.	WA, Tunnel Creek NP	EU144056	EU144071
CMW26158	CBS122060	*P. kimberleyense*	*Ad. gibbosa*	WA, Tunnel Creek NP	EU144058	EU144073
CMW26155	CBS122063	*P. ardesiacum*	*Ad. gibbosa*	WA, Derby	EU144061	EU144076
CMW26159	CBS122062	*P. ardesiacum*	*Ad. gibbosa*	WA, Mt Hardman, Great North Hwy	EU144060	EU144075
CMW26160	CBS122064	*P. ardesiacum*	*Eucalyptus* sp.	WA, Tunnel Creek NP	EU144062	EU144077
CMW13434		*P. stromaticum*	*Eucalyptus* hybrid	Venezuela, Cojedes state	*AY693974*	AY693975
CMW13435		*P. stromaticum*	*Eucalyptus* hybrid	Venezuela, Cojedes state	*DQ436935*	*DQ436936*
CMW26162	CBS122519	*Lasiodiplodia margaritacea*	*Ad. gibbosa*	WA, Tunnel Creek Gorge	EU144050	EU144065
CMW26163	CBS122065	*L. margaritacea*	*Ad. gibbosa*	WA, Tunnel Creek Gorge	EU144051	EU144066
CMW10130		*L. theobromae*	*Vitex donniana*	Uganda	*AY236951*	*AY236900*
CMW9074		*L. theobromae*	*Pinus* sp.	Mexico	*AY236952*	*AY236901*
CMW14077	CBS115812	*L. gonubiensis*	*Syzygium cordatum*	South Africa, Gonubie	*AY639595*	*DQ103566*
WAC12538		*L. rubropurpurea*	*E. grandis*	Queensland, Tully	*DQ103556*	*DQ103574*
CMW13511	WAC12539	*L. venezuelensis*	*Ac. mangium*	Venezuela, Acarigua	*DQ103547*	*DQ103568*
CMW14691	WAC12533	*L. crassispora*	*Santalum album*	Western Australia, Kununurra	*DQ103550*	*DQ103557*
	STE-U5803, CBS120832	*L. plurivora*	*Prunus salicina*	South Africa, Stellenbosch	*EF445362*	*EF445395*
	CBS304.79	*L. pseudotheobromae*	*Rosa* sp.	Netherlands	*EF622079*	*EF622061*
	CBS447.62	*L. pseudotheobromae*	*Citrus aurantium*	Suriname	*EF622081*	*EF622060*
	CBS495.78	*L. parva*	*Cassava*-field soil	Colombia	*EF622085*	*EF622065*
	CBS494.78	*L. parva*	*Cassava*-field soil	Colombia	*EF622084*	*EF622064*
ZS94-6		*Diplodia mutila*	*Malus pumila*	New Zealand	*AF243407*	*AY236904*
CMW7774		*Di. seriata*	*Ribes* sp.	U.S.A., New York	*AY236953*	*AY236902*
KJ94-07		*Di. pinea*	*Pinus resinosa*	U.S.A., Wisconsin	*AF027758*	*AY624251*
CMW26164	CBS122066	*Dothiorella longicollis*	*Terminalia* sp.	WA, Bell Gorge	EU144052	EU144067

TABLE I. Continued

Culture no.[1]	Other no. [1]	Identity	Host	Location [2]	Genbank no. [3]	
					ITS	EF-1α
CMW26165	CBS122067	*Do. longicollis*	*Lysiphyllum cunninghamii*	WA, Tunnel Creek NP	EU144053	EU144068
CMW26166	CBS122068	*Do. longicollis*	*L. cunninghamii*	WA, Tunnel Creek NP	EU144054	EU144069
CBS117008		*Do. viticola*	*Vitis vinifera*	Spain, Sant Sadurní d'Anoia	*AY905557*	AY905560
CBS117010		*Do. viticola*	*V. vinifera*	Spain, Sant Esteve Sesrovires	*AY905558*	AY905561
CBS115041		*Do. iberica*	*Quercus ilex*	Spain, Aragon	*AY573202*	*AY573222*
CBS115035		*Do.iberica*	*Q. ilex*	Spain, Aragon	*AY573213*	*AY573228*
CBS115038		*Do.sarmentorum*	*M. pumila*	Netherlands, Delft	*AY573206*	*AY573223*
IMI63581		*Do.sarmentorum*	*Ulmus* sp.	England, Warwickshire	*AY573212*	*AY573235*
CMW26167	CBS122069	*Fusicoccum ramosum*	*E. camaldulensis*	WA, Bell Gorge	EU144055	EU144070
CMW991	ATCC58188	*B. dothidea*	*Prunus nigra*	New Zealand	*AF241175*	*AY236895*
CMW8000		*B. dothidea*	*Prunus* sp.	Switzerland, Crocifisso	*AY236949*	AY236898
ATCC22928		*B. corticis*	*Vaccinium* sp.	U.S.A., North Carolina	*DQ299248*	EF614932
ATCC22927		*B. corticis*	*Vaccinium* sp.	U.S.A., North Carolina	*DQ299247*	EF614931
MUCC531		*Macrophomina phaseolina*	*Sesbania formosa*	Western Australia, Kununurra	*EF585505*	*EF585560*
MUCC532		*M. phaseolina*	*S. formosa*	Western Australia, Kununurra	*EF585506*	*EF585561*
CMW6837		*Neofusicoccum australe*	*Acacia* sp.	New South West, Batermans Bay	*AY339262*	*AY339270*
CMW7054	CBS121.26	*N. ribis*	*Ribis rubrum*	U.S.A., New York	*AF236908*	*AY236879*
CMW9081	ICMP8003	*N. parvum*	*Populus nigra*	New Zealand	*AY236943*	*AY236888*
CMW26171	MUCC534, CBS122072	*Neoscytalidium novaehollandiae*	*Ad. gibbosa*	WA, Gibb River Rd, 50 km E of Derby	EF585535	EF585581
CMW26168	MUCC535, CBS122610	*N. novaehollandiae*	*Ac. synchronica*	WA, Gibb River Rd, near Meda	EF585536	EF585578
CMW26169	MUCC536, CBS122070	*N. novaehollandiae*	*Grevillia agrifolia*	WA, Gibb River Rd, near Meda	EF585539	EF585579
CMW26170	MUCC537, CBS122071	*N. novaehollandiae*	*Crotalaria medicaginea*	WA, Bell Gorge	EF585540	EF585580
CBS499.66		*Neoscytalidium dimidiatum*	*Mangifera indica*	Mali	*AY819727*	EU144063
CBS204.33		*N. dimidiatum*	*Prunus* sp.	Egypt	*AY819728*	EU144064
	MUCC684	*Guignardia* sp.	*Agonis flexuosa*	Western Australia, Yalgorup	*EU675682*	EU686573
	MUCC685	*Guignardia* sp.	*Ag. flexuosa*	Western Australia, Yalgorup	*EU675681*	EU686572

[1] Abbreviations of isolates and culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, The Netherlands; CMW = Forestry and Agricultural Biotechnology Institute, University of Pretoria South Africa; MUCC = Murdoch University Culture Collection, Perth, Australia; KJ = Jacobs and Rehner (1998); ATCC = American Type Culture Collection, Manassas, VA, U.S.A; ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand; IMI = CABI Bioscience, Egham, U.K; ZS = Zhou and Stanosz (2001); WAC = Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia.

[2] WA=Western Australia, NP=National Park.

[3] Sequence numbers in italics were obtained from the GenBank public database. All others were obtained in this study.

FIG. 1. One of 2 most parsimonious trees of 1566 steps resulting from the analysis of the combined ITS–EF-1α sequence data. Bootstrap values of the branch nodes are given in italics and the posterior probabilities resulting from Bayesian analysis are indicated in bold. Isolates from this study are in bold. Tree is rooted to a *Guignardia* sp. The strongly supported clades that represent different genera within the Botryosphaeriacea according to Crous et al. (2006) are indicated by circles at the nodes.

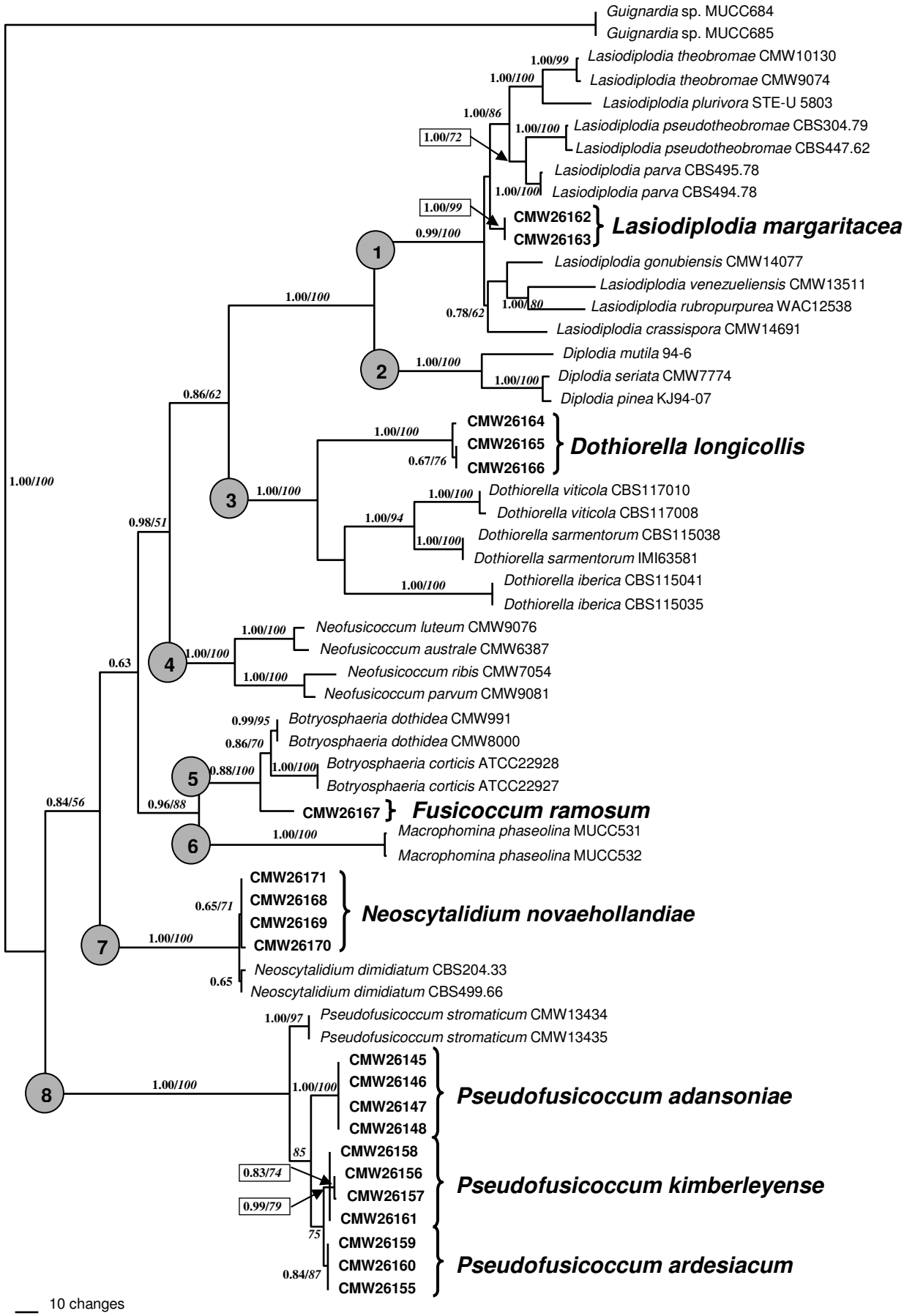

Guignardia sp. MUCC684
Guignardia sp. MUCC685
1.00/99
Lasiodiplodia theobromae CMW10130
1.00/100
Lasiodiplodia theobromae CMW9074
Lasiodiplodia plurivora STE-U 5803
1.00/86
1.00/100
Lasiodiplodia pseudotheobromae CBS304.79
1.00/72
Lasiodiplodia pseudotheobromae CBS447.62
Lasiodiplodia parva CBS495.78
1.00/100
Lasiodiplodia parva CBS494.78
1.00/99
CMW26162
CMW26163
Lasiodiplodia margaritacea
0.99/100
1
Lasiodiplodia gonubiensis CMW14077
Lasiodiplodia venezueliensis CMW13511
1.00/100
1.00/80
Lasiodiplodia rubropurpurea WAC12538
0.78/62
Lasiodiplodia crassispora CMW14691
2
1.00/100
Diplodia mutila 94-6
1.00/100
Diplodia seriata CMW7774
Diplodia pinea KJ94-07
0.86/62
1.00/100
CMW26164
CMW26165
CMW26166
Dothiorella longicollis
0.67/76
1.00/100
3
1.00/100
1.00/100
Dothiorella viticola CBS117010
Dothiorella viticola CBS117008
0.98/51
1.00/94
1.00/100
Dothiorella sarmentorum CBS115038
Dothiorella sarmentorum IMI63581
1.00/100
Dothiorella iberica CBS115041
Dothiorella iberica CBS115035
1.00/100
Neofusicoccum luteum CMW9076
0.63
4
1.00/100
Neofusicoccum australe CMW6387
1.00/100
Neofusicoccum ribis CMW7054
Neofusicoccum parvum CMW9081
0.99/95
Botryosphaeria dothidea CMW991
0.86/70
Botryosphaeria dothidea CMW8000
0.88/100
1.00/100
Botryosphaeria corticis ATCC22928
5
Botryosphaeria corticis ATCC22927
0.84/56
0.96/88
CMW26167
Fusicoccum ramosum
6
1.00/100
Macrophomina phaseolina MUCC531
Macrophomina phaseolina MUCC532
CMW26171
CMW26168
CMW26169
CMW26170
Neoscytalidium novaehollandiae
0.65/71
7
1.00/100
Neoscytalidium dimidiatum CBS204.33
0.65
Neoscytalidium dimidiatum CBS499.66
1.00/97
Pseudofusicoccum stromaticum CMW13434
Pseudofusicoccum stromaticum CMW13435
CMW26145
CMW26146
CMW26147
CMW26148
Pseudofusicoccum adansoniae
1.00/100
8
1.00/100
85
CMW26158
0.83/74
CMW26156
CMW26157
Pseudofusicoccum kimberleyense
0.99/79
CMW26161
75
CMW26159
CMW26160
CMW26155
Pseudofusicoccum ardesiacum
0.84/87
10 changes

FIG. 2. *Pseudofusicoccum adansoniae*. a. Conidiogenous cells (CBS122056). b. Conidia (CBS122054, CBS122055, CBS122056). c. Germinating conidia (CBS122056). Bar = 10 µm.

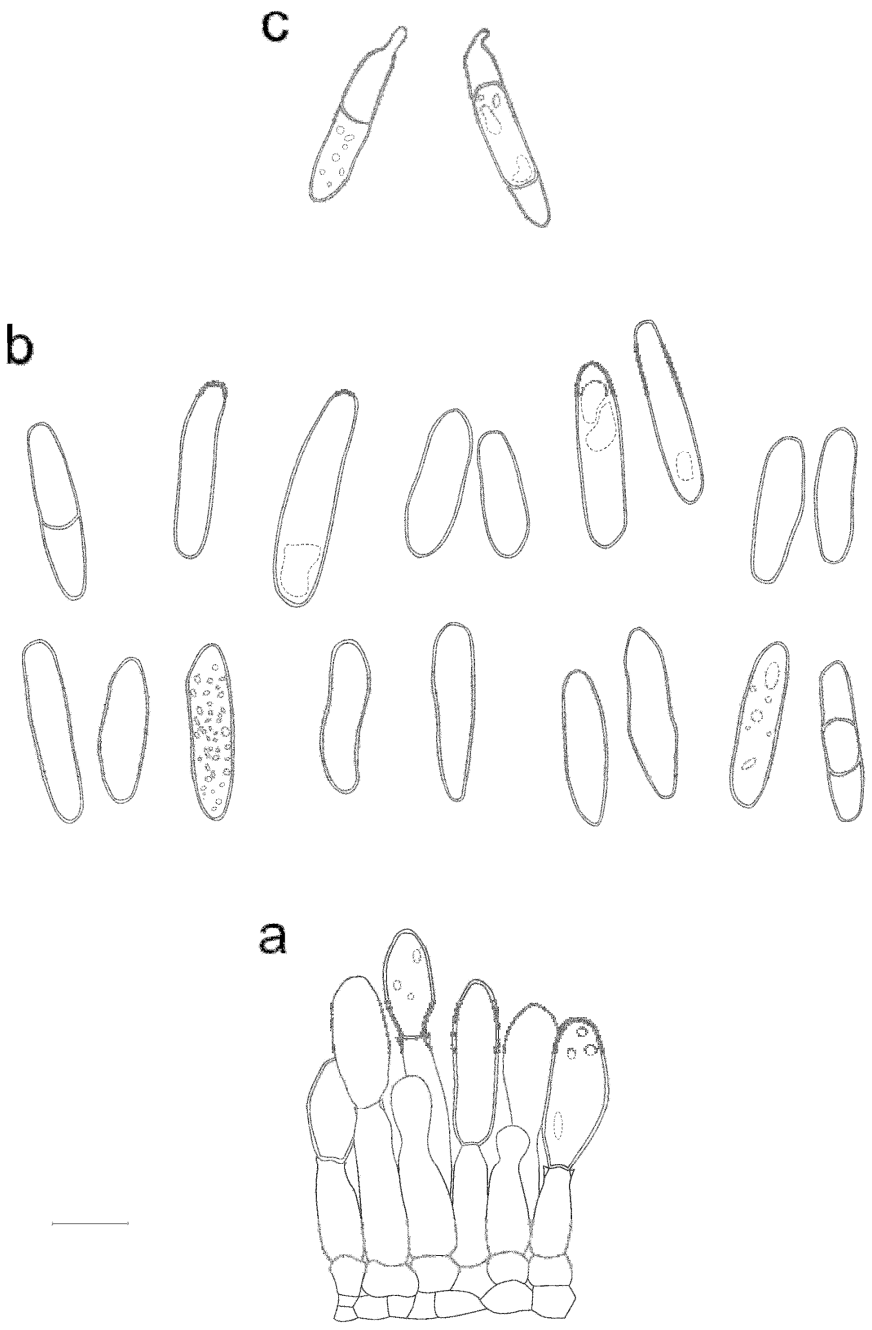

c
b
a

FIG. 3. *Pseudofusicoccum adansoniae*. a. Pycnidia formed in culture on pine needles (CBS122054). b. Aseptate conidia (CBS122055). c, d. Conidiogenous cells (CBS122053). Scale bars: a = 500 µm, b–f = 10 µm.

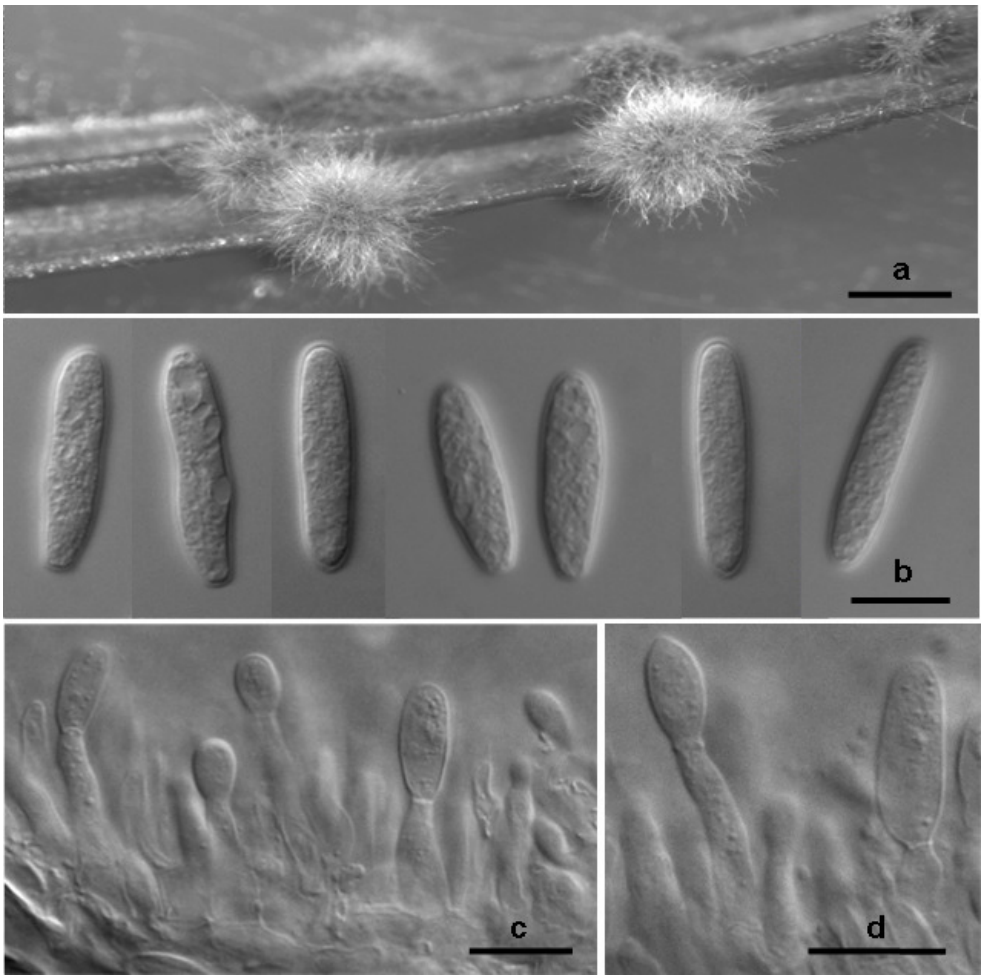

a
b
c
d

FIG. 4. *Pseudofusicoccum kimberleyense*. a. Conidiogenous cells (CBS122059). b. Aseptate conidia (CBS122058, CBS122060, CBS122061). c. 1–4 septate conidia (CBS122059, CBS122060, CBS122061). Bar = 10 µm.

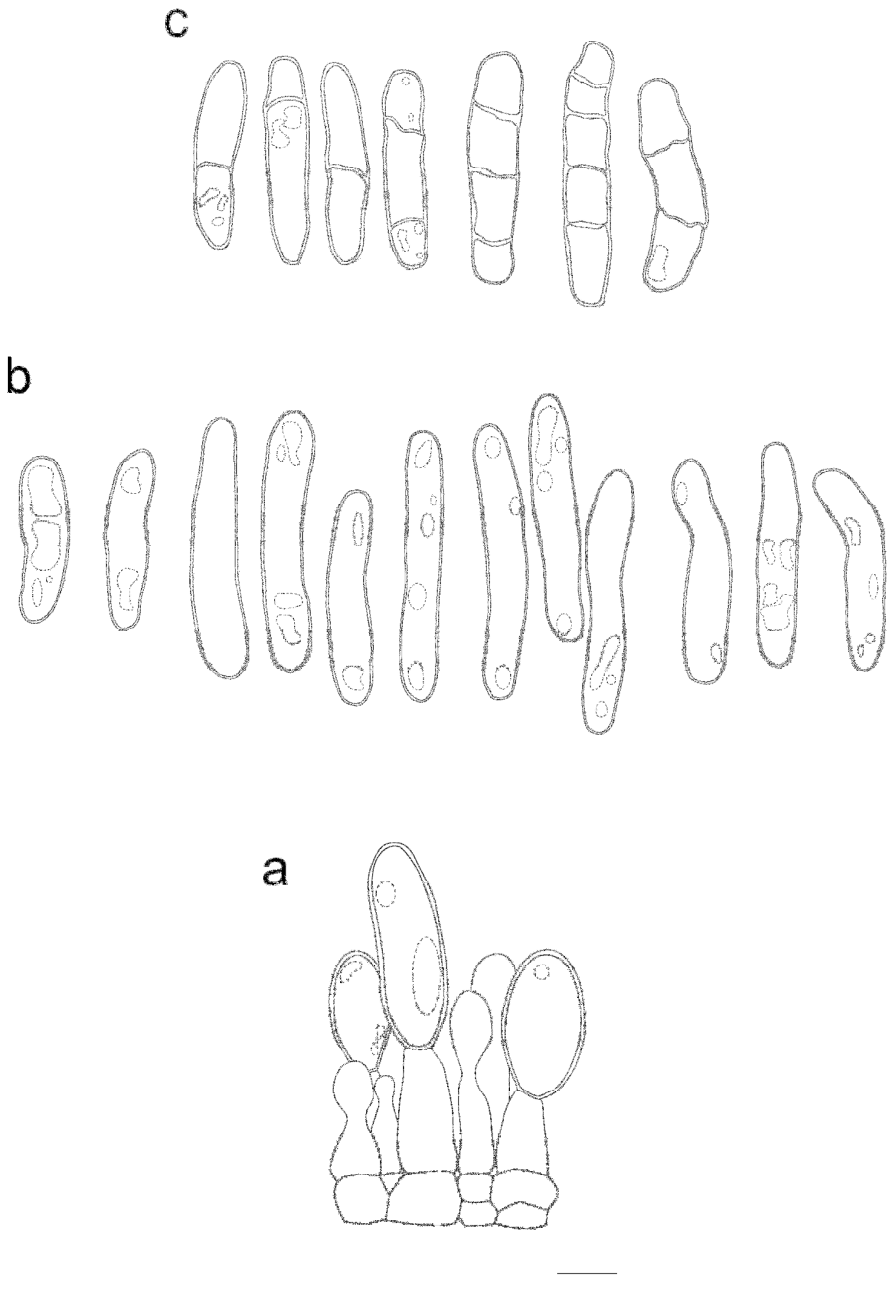
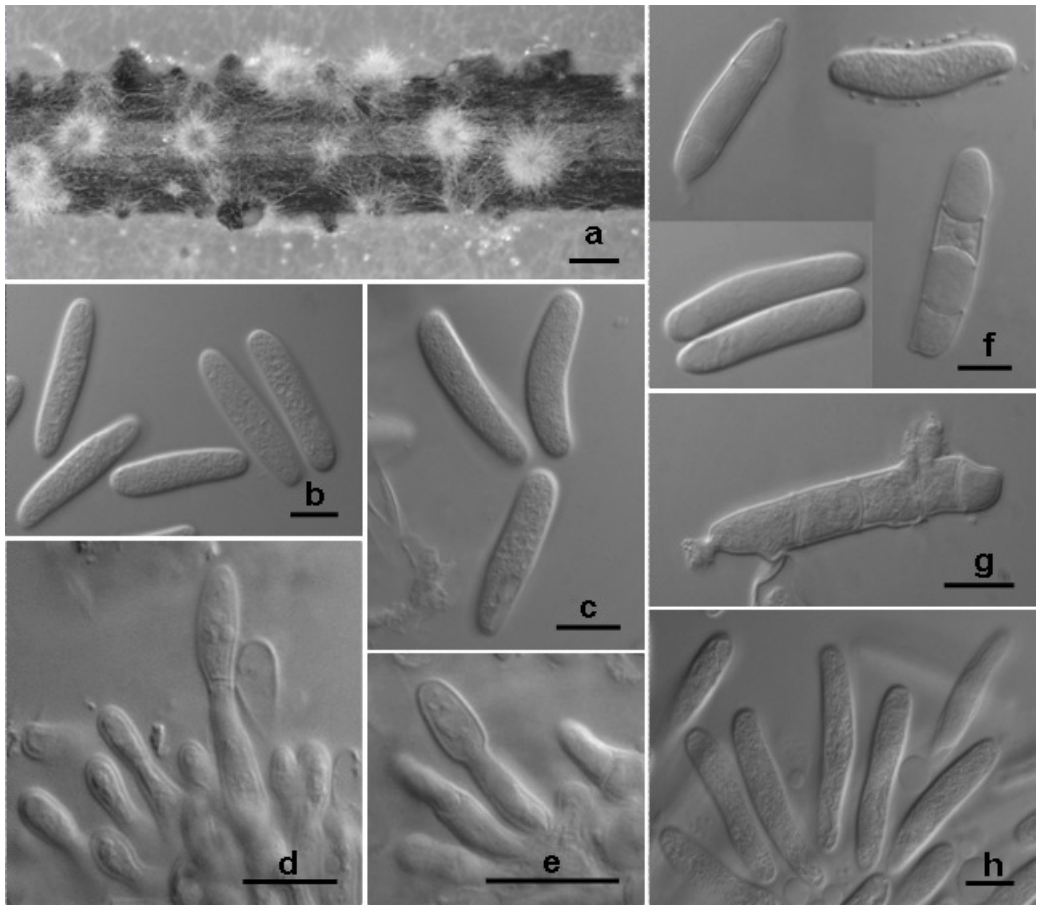

c
b
a

FIG. 5. *Pseudofusicoccum kimberleyense*. a. Pycnidia formed in culture on pine needles (CBS122058). b, c. Aseptate conidia (CBS122058). d, e. Conidiogenous cells (CBS122058). f, g. Aseptate and 2–4 septate conidia (CBS122060). h. Aseptate conidia (CBS122061). Scale bars: a = 500 µm, b–h = 10 µm.


a
b
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e
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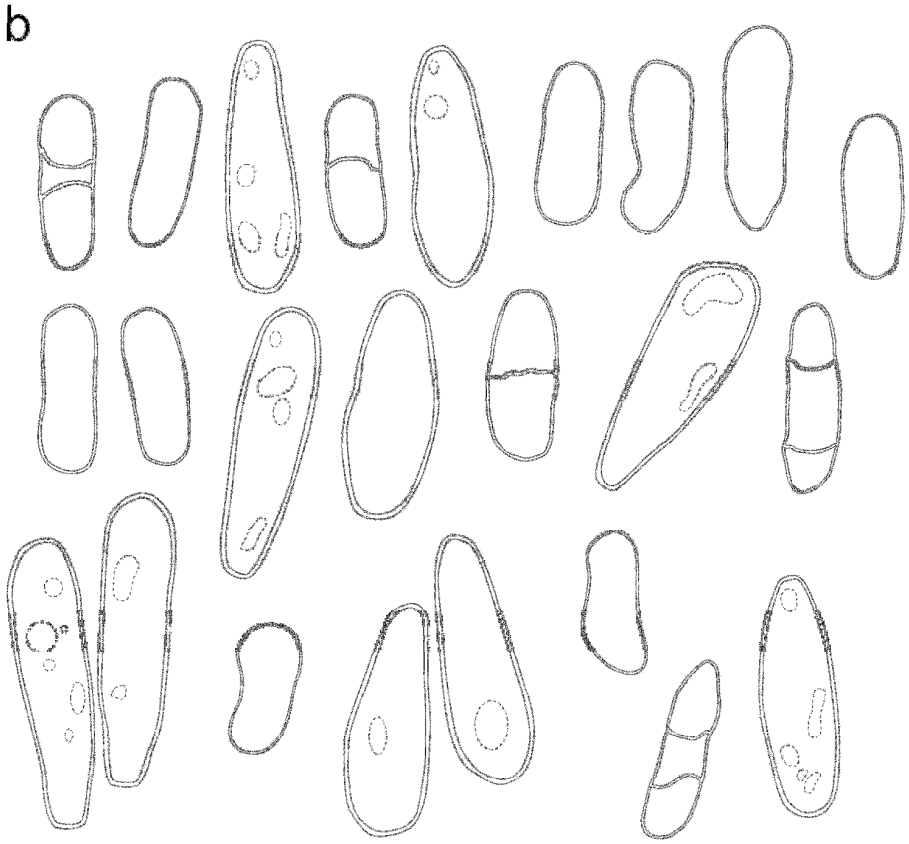
g
h

FIG. 6. *Pseudofusicoccum ardesiacum*. a. Conidiogenous cells (CBS122062). b. Conidia (CBS122062, CBS122063). Bar = 10 µm.

b



a

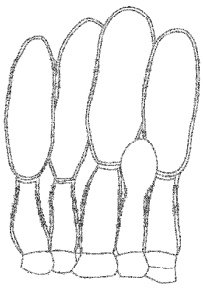
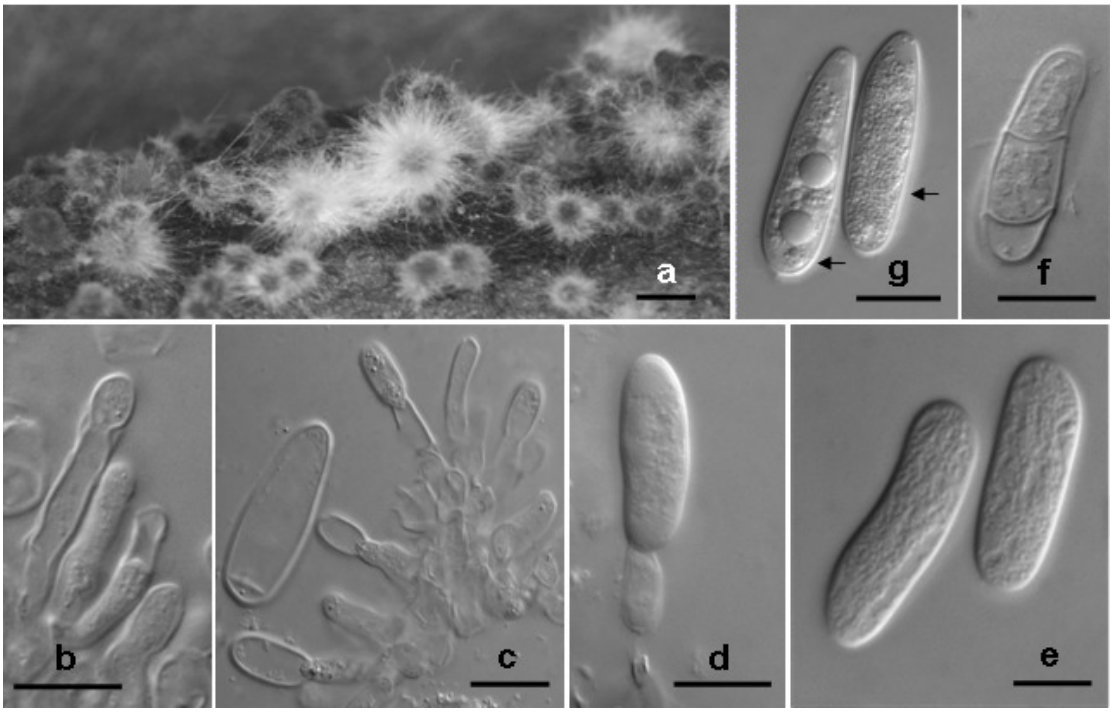


FIG. 7. *Pseudofusicoccum ardesiacum*. a. Pycnidia formed in culture on eucalypt twig (CBS122063). b, c. Conidiogenous cells (CBS122063). d. Conidium attached to conidiogenous cell (CBS122062). e. Aseptate conidia (CBS122062). f. Two-septate conidium (CBS122062). g. Aseptate conidia covered with mucus layer (indicated by arrow) (CBS122063). Scale bars: a = 500 µm, b–g = 10 µm.


a
g
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e

FIG. 8. *Dothiorella longicollis*. a. Conidiogenous cells (CBS122067, CBS122068). b. Conidia (CBS122067, CBS122068). Bar = 10 µm.

b

a

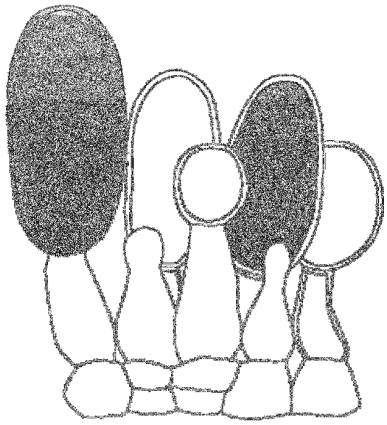


FIG. 9. *Dothiorella longicollis*. a. Pycnidia formed in culture on water agar (CBS122068). b. Pycnidium formed in culture releasing dark one-septate conidia (CBS122068). c. Cross section through a pycnidium showing outer layers of dark brown cells and inner layers of hyaline cells with conidiogeneous cells arising from the pycnidial wall (CBS122067). d. Dark one-septate conidia (CBS122067). Scale bars: a = 500 µm, b–d = 10 µm.

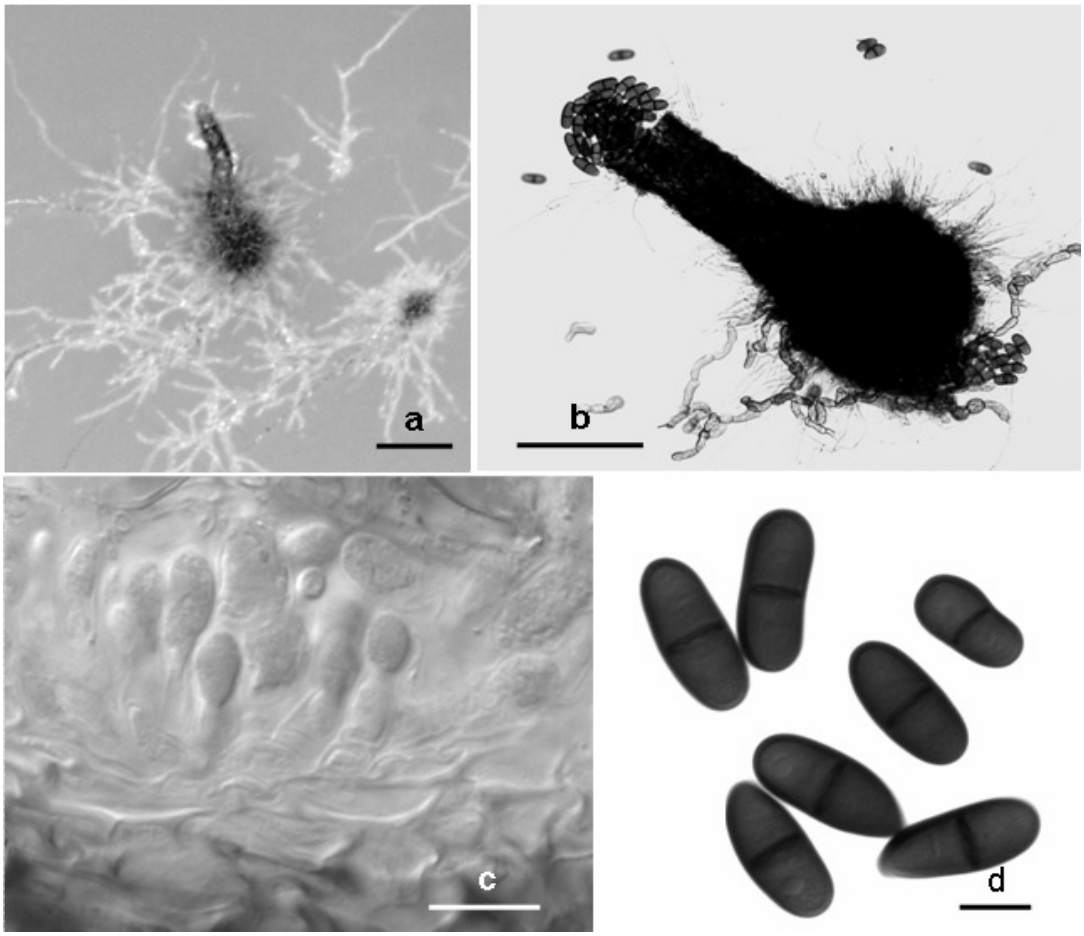

a
b
c
d

FIG. 10. *Lasiodiplodia margaritacea*. a. Conidiogenous cells and paraphyses. b. Immature conidia. c. Mature conidia. (a–c CBS122519). Bar = 10 µm.

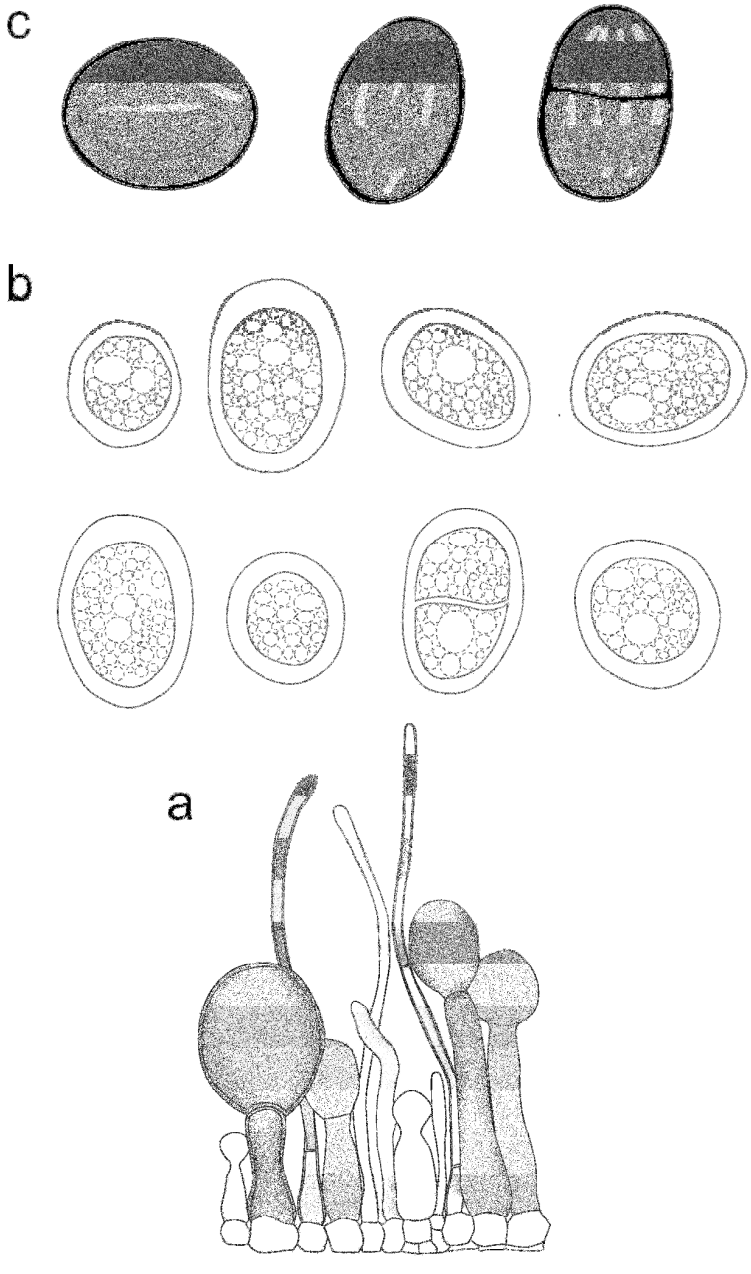

c
b
a

FIG. 11. *Lasiodiplodia margaritacea*. a. Pycnidia emerging through the eucalypt bark in culture. b. Conidium attached to conidiogenous cell. c. Germinating conidium. d. Paraphyses. e. Conidia in various stages of development, including young, hyaline, aseptate conidia, unicellular conidia with developing pigmentation with and without septation, mature striate conidia. (a–e CBS122519). Scale bars: a = 500 µm, b–h = 10 µm.

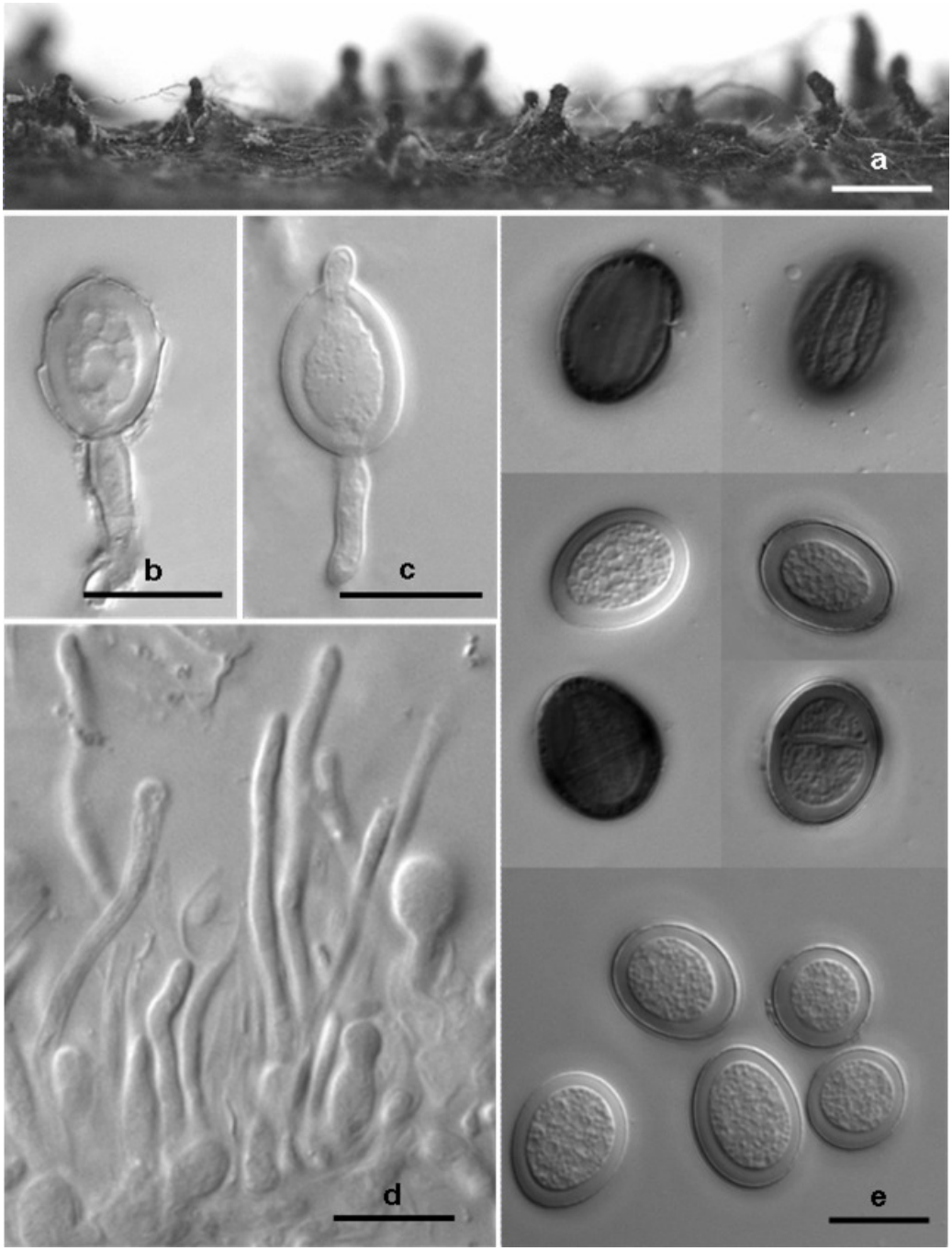

a
b
c
d
e

FIG. 12. *Fusicoccum ramosum*. a. Conidiogenous cells and conidiophores. b, Branching conidiophores, c. Conidia. (a–c CBS122069). Bar = 10 µm.

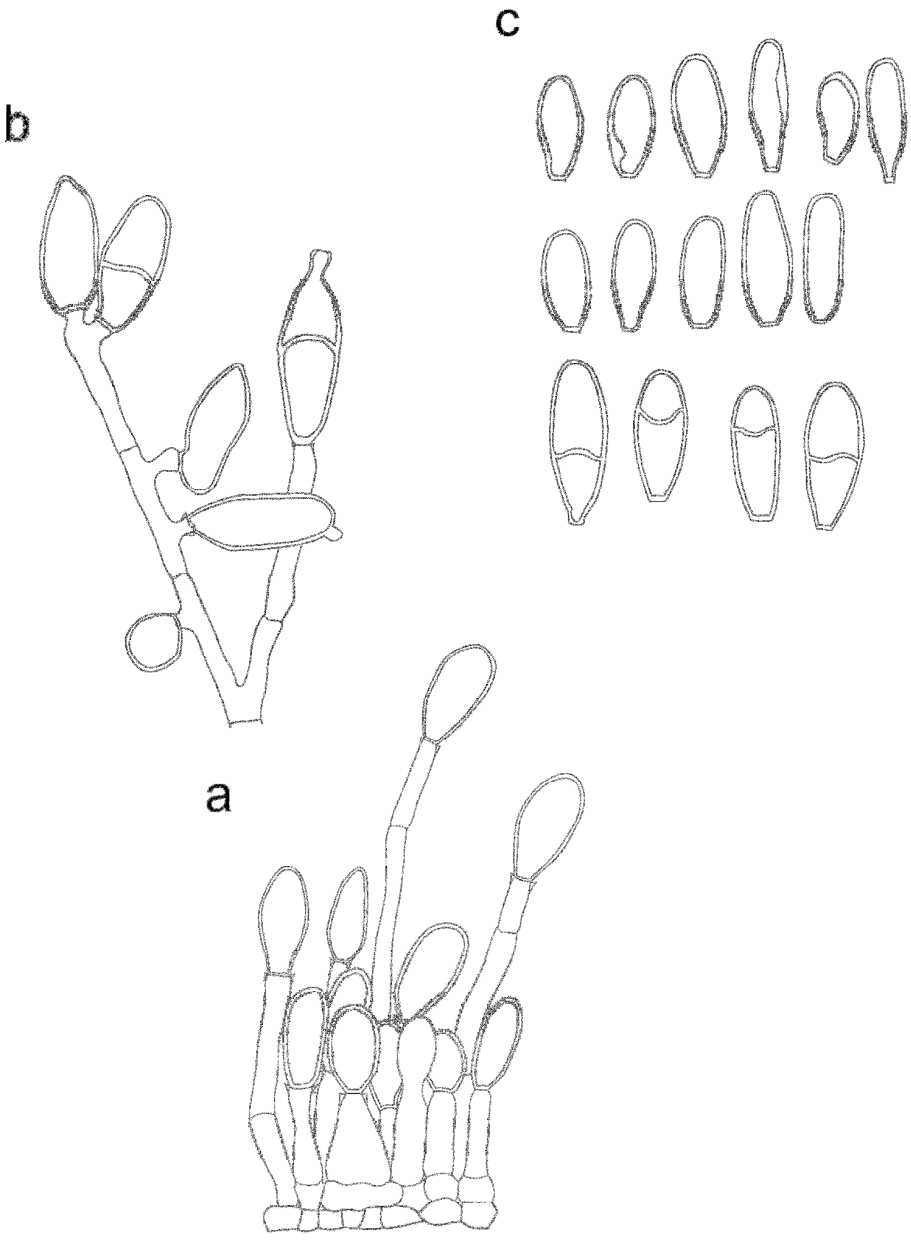
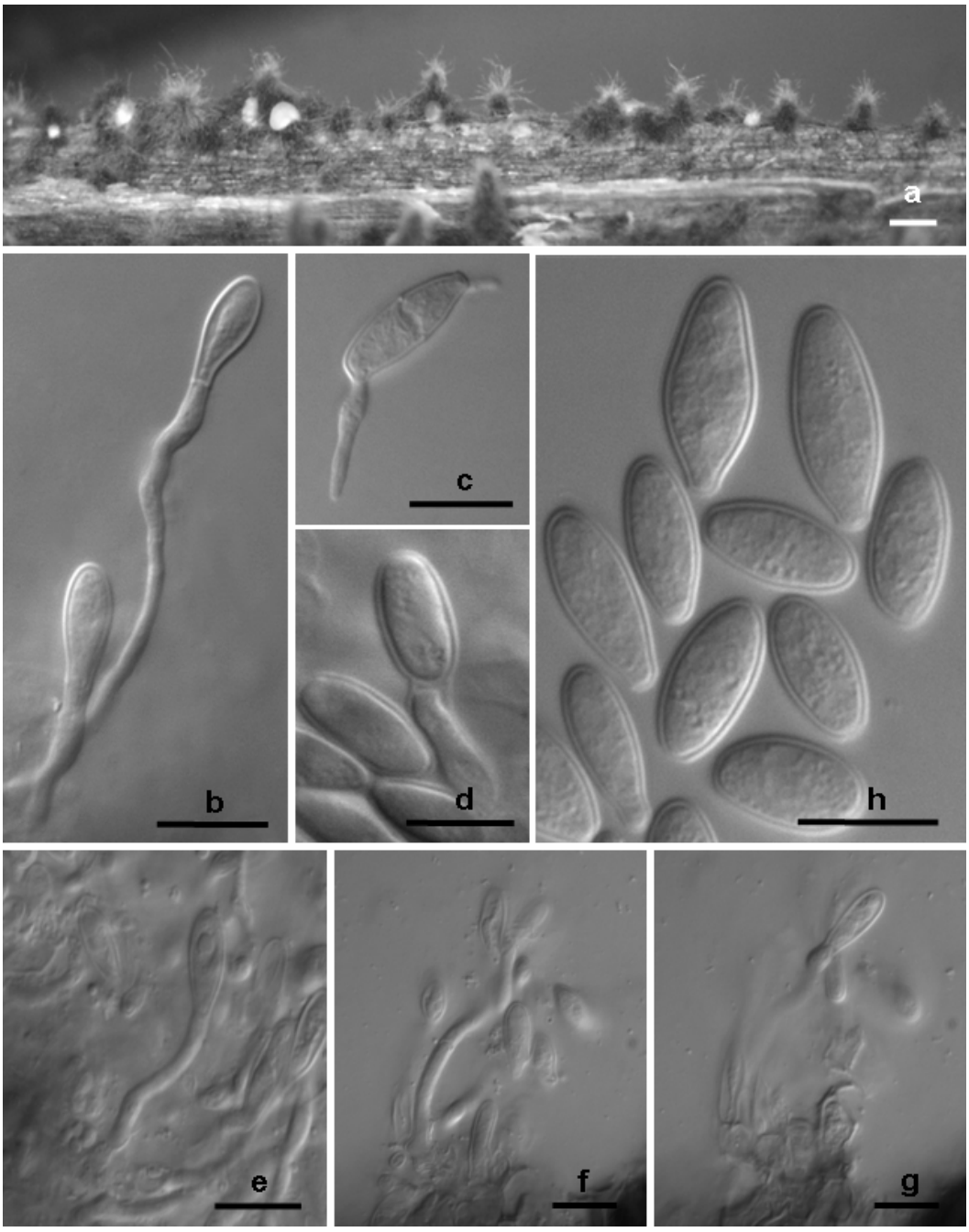

c
b
a

FIG. 13. *Fusicoccum ramosum*. a. Pycnidia emerging through the eucalypt bark in culture releasing white masses of conidia. b. Conidiophores with attached conidia c. Germinating one-septate conidium. d. Conidium attached to conidiogenous cell. e. Conidiophores arising from the pycnidial wall. f–g Conidiophore with attached conidium, at two different focuses. h. Aseptate conidia. (a–h CBS122069). Scale bars: a = 500 µm, b–g = 10 µm.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d
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g

FIG. 14. *Neoscytalidium novaehollandiae*. a. Conidiogenous cells (CBS122610). b. Conidia. c. Muriform conidia. d. Chains of arthroconidia. b–d (CBS122071). Bar = 10 µm.

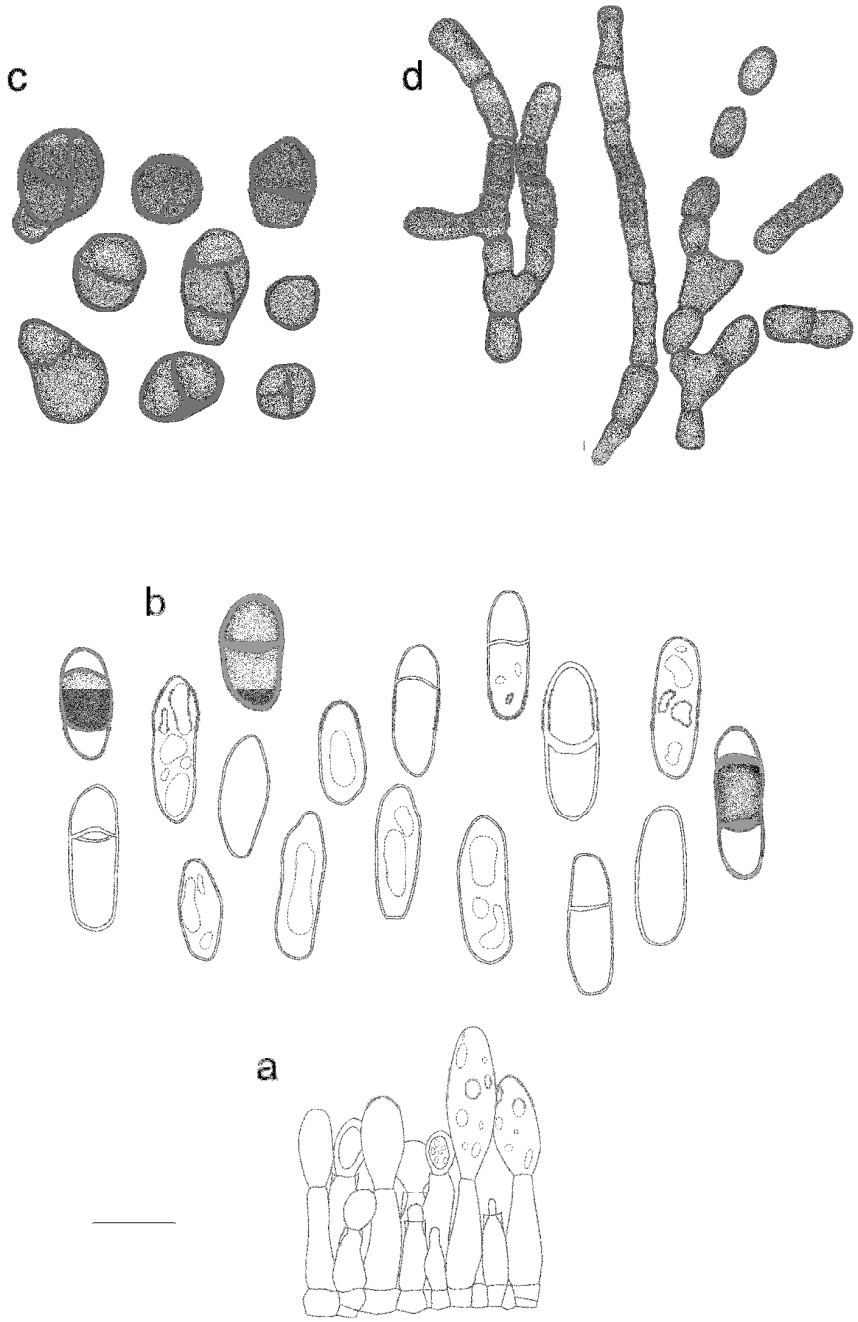
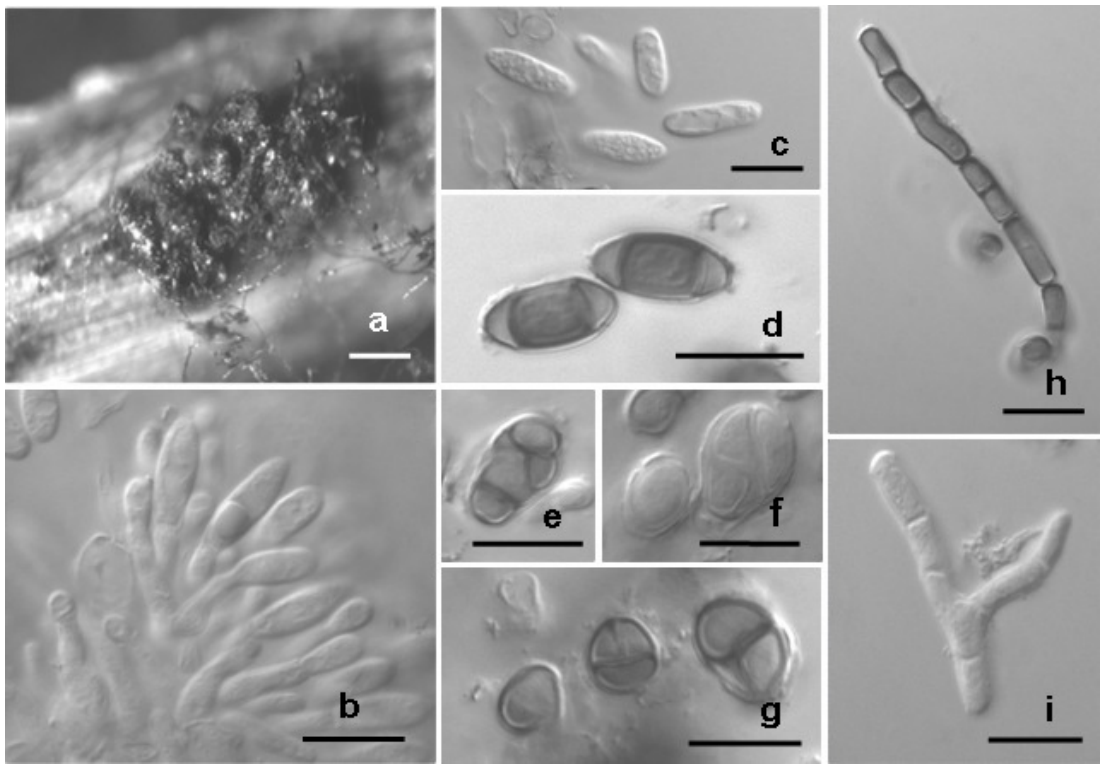

c
d
b
a

FIG. 15. *Neoscytalidium novaehollandiae*. a. Pycnidia emerging from a pine needle in culture. b. Conidiogenous cells (CBS122610). c. Hyaline aseptate conidia. d. Two-septate dark conidia. e–g. Muriform conidia. h, i. Chains of arthroconidia. (a, c–h CBS122071). Bars: a = 500 µm, b–h = 10 µm.


a
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Chapter 6

Molecular phylogenetics in the recognition of fungal species, with a particular focus on the Botryosphaeriaceae

ABSTRACT

DNA-based molecular techniques and molecular phylogenetics in species delineation has revolutionised the taxonomy of fungi. Along with deployment of the phylogenetic species concept and genealogical concordance phylogenetic species recognition (GCPSR), cryptic species and species complexes have been revealed where one taxonomic entity was previously known. The *Gibberella fujikuroi* species complex provides one of best examples of fungal plant pathogens, where numerous cryptic phylogenetic species were recognized in one morphospecies. Likewise, the genus *Botryosphaeria* has been radically revised during the past decade based on molecular evidence and a number of new genera and species have been introduced for taxa that previously resided in this genus. This diverse and cosmopolitan group of fungi includes serious plant pathogens as well as some medically important species. In this review, the molecular approaches that are currently applied to delineate fungal species, in particular in the Botryosphaeriaceae, are considered and their implications for the taxonomy of the Botryosphaeriaceae are discussed.

1.0. Introduction

Defining a "species" is fundamental to studies on speciation, understanding of this process and its underlying mechanisms. It is also essential for practical reasons such as disease control and in the application of quarantine regulations. At least 25 different species concepts have been used to define species in the past (Coyne and Orr 2004). These species concepts are classified as theoretical, such as the Evolutionary Species Concept (ESC) (Mayden 1997), or operational of which the more commonly accepted include the Morphological Species Concept (MSC), the Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC) (Taylor et al 2000). Operational species concepts classify practical criteria that can be used to delineate species (Mayden 1997, Berlocher 1998, de Queiroz 2007). Taylor et al (2000) introduced the term "species recognition" for the operational approaches e.g. Morphological Species Recognition (MSR), Biological Species Recognition (BSR) and Phylogenetic Species Recognition (PSR), in order to distinguish them from theoretical concepts and to emphasize their use in species delimitation, particularly in fungal species diagnoses.

Changes in operational species concepts and the use of PSC and PSR that have been conceptualised in last few decades (Berlocher 1998), have all been influenced by the development of new molecular tools and their availability for species recognition. The most revolutionary change to have arisen is the direct analyses of DNA sequences that became broadly applied in species delimitation in the late 1980's, with the discovery of the Polymerase Chain Reaction (PCR) (Berlocher 1998). Since then, the number of studies on cryptic speciation has increased dramatically in all fields of biology and for all taxonomic groups of living organisms (Bickford et al 2006). One of the important outcomes of the application of molecular based diagnoses has been the recognition that many previously described taxa incorporate cryptic species, which traditionally applied phenotypic characters have failed to reveal.

The application of DNA-based molecular techniques and molecular phylogenetics in species delineation has revolutionised the taxonomy of fungi. Apart from its influence on higher classification, increasing numbers of studies based on DNA sequence variation and application of PSR reveal an escalating number of cryptic species and species complexes in fungal Kingdom (Taylor et al 2000). Based on the outcomes of these studies, it is expected that most of fungal species described based on morphology, comprise more than one closely related cryptic or sibling species, or species complexes. A lack of distinguishing morphological characters, difficulties to induce sporulation in culture, failure of isolates to

mate under laboratory conditions or the lack of living cultures are the main reasons why these species remained cryptic. The increasing number of recognised cryptic fungal species has also necessitated a new approach to the description of these species, and a need to move towards what is referred to as phylogenetic taxonomy.

The rising numbers of species distinguished based on molecular approaches, and cryptic species in fungi in general, is mirrored in the recognition of species and the resulting taxonomy of the Botryosphaeriaceae. Since 1998, when the DNA sequence data were first applied to distinguish species in this family, at least twenty cryptic species have been identified in species of this group, previously defined based on morphology. Numerous others are currently being described. Recently, three cryptic species were described in this family using DNA sequence data and single nucleotide polymorphisms (SNPs) as defining characters for the first time (Pavlic et al 2009b). In this review we consider these developments specifically in the Botryosphaeriaceae, which in many ways provides a leading example that can equally be applied to other fungal groups.

2.0. The historical development of *Botryosphaeria* taxonomy

The Botryosphaeriaceae (Botryosphaeriales, Ascomycota) is referred to here in the strict sense as referring to taxa that were described in the genus *Botryosphaeria*, or anamorphs of *Botryosphaeria*, before 2006, following the classification system of von Arx (von Arx and Müller 1954). This group comprises more than 2000 species (http://www.indexfungorum.com) that are commonly known as endophytes and latent, stress-associated, opportunistic plant pathogens with cosmopolitan distributions on a variety of angiosperms and gymnosperms (Denman et al 2000, Slippers and Wingfield 2007, de Wet et al 2008). Some of the Botryosphaeriaceae are also medically important fungi that may cause diseases in humans (Tan et al 2008, Woo et al 2008).

The taxonomic history and identification of species of *Botryosphaeria sensu* von Arx (von Arx and Müller 1954) can be split in two periods, which are related to pre- and post-the application of the DNA sequence data. The first period started in 1863 when Cesati and de Notaris established *Botryosphaeria*, with 12 species, including *B. dothidea*, which was later identified as the lectotype of the genus (Barr 1972). Until a decade ago, the taxonomy of this group of fungi was based exclusively on morphology, and this period is characterised by morphological species recognition (MSR) (Taylor et al 2000).

Morphological species recognition in *Botryosphaeria* has been complex in the past for a number of reasons. In the initial stages, morphological species identification was

usually combined with a single host-one species approach, which led to the description of new species based on host association (Cesati and De Notaris 1863, Saccardo 1877, 1882, Grossenbacher and Duggar 1911, Putterill 1919). Many of the early-described species were, however, synonymised in a major revision of the genus by von Arx and Müller (1954) based almost exclusively on teleomorph morphology. The occurrence of more than one species on the same host and simultaneous existence of anamorph and teleomorph structures further complicated species identification. Connections between *Botryosphaeria* species and their anamorphs have also not always been available. For example, at the time when *B. dothidea* was described, its anamorph, *Fusicoccum aesculi* Corda, was known, but there were no connections made between these taxa (Pennycook and Samuels 1985, Crous and Palm 1999, Slippers et al 2004b). Identification of species based exclusively on morphological characters either of their anamorphs or teleomorphs is unreliable given that these phenotypic characters overlap between species and in many cases are not sufficiently informative for species delimitation. Denman et al (2000) provided a detailed overview of the taxonomic history of *Botryosphaeria* during this early taxonomic period.

The use of DNA sequence comparisons for the identification and classification of Botryosphaeriaceae was initiated by the study of Jacobs and Rehner (1998). These authors attempted to define species in *Botryosphaeria* and associated anamorphic fungi, combining morphological characters with nuclear rDNA ITS sequence analyses. In this revision, several anamorph genera were linked to *Botryosphaeria* providing the foundation for further taxonomic studies. A subsequent ITS based phylogenetic re-evaluation of *Botryosphaeria* combined with anamorph morphology, by Denman et al (2000), elucidated two main groups for the *Botryosphaeria* anamorphs. These corresponded to species with hyaline, *Fusicoccum*-like conidia and those with dark *Diplodia*-like conidia. Thus, anamorphs of *Botryosphaeria* that were related to 18 different genera were suggested to be synonymised with either *Fusicoccum* or *Diplodia*. The use of the ITS rDNA sequence data for species identification in *Botryosphaeria sensu lato* has subsequently been widely applied (e.g. Zhou and Stanosz 2001, Alves et al 2004, Barber et al 2005, Gure et al 2005, Phillips et al 2006, Pavlic et al 2007).

A major revision of the taxa included in *Botryosphaeria* followed after the analysis of LSU rDNA sequences data by Crous et al (2006). In that study, species of the Botryosphaeriaceae were assigned to at least 10 lineages, which were related to different genera recognised by anamorph morphology. *Botryosphaeria* was reduced to the two species *B. dothidea* and *B. corticis*, and the remaining taxa were accommodated in "*Botryosphaeria*"

quercuum, *Dothidotthia, Guignardia, Neofusicoccum, Neosyitalidium, Macrophomina, Pseudofusicoccum* and *Saccharata,* while the phylogenetic status of *Diplodia* and *Lasiodiplodia* remained unresolved. Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the Botryosphaeriaceae based on a combined phylogeny of five loci (SSU, ITS, LSU, EF-1α and β-tubulin), Phillips et al (2008) recognised *Diplodia* and *Lasiodiplodia* as separate genera, described new dark-spored genera such as *Barriopsis* and *Spencermartinsia*, and re-instated genera *Neodeightonia, Phaeobotryon, Phaeobotryosphaeria* that were synonymised under *Botryosphaeria* by von Arx and Müller (1954). Furthermore, the genus *Dothidotthia* described by Crous et al (2006) was renamed as *Dothiorella*, while *Dothidotthia* species, previously placed in the Botryosphaeriaceae, were shown to belong to the newly established family Dothidotthiaceae (Pleosporales). Recently, two additional anamorph genera, *Aplosporella* (Damm et al 2008) and *Endomelanconiopsis* (Rojas et al 2008), were described in the Botryosphaeriaceae. All of these studies confirm the significance of molecular phylogenetics not only for species level identification but also as an important tool used to resolve the phylogenetic and taxonomic status in higher-level taxa in the Botryosphaeriaceae.

3.0. Phylogenetic species recognition in the Botryosphaeriaceae

In recent years, a number of new or cryptic species have been recognised in the Botryosphaeriaceae (de Wet et al 2003, Slippers et al 2004b, c, d, Burgess et al 2005, Alves et al 2008, Maleme 2008, Phillips et al 2008, Pavlic 2009a, b). Although phenotypic characters were considered in all of these studies, data obtained using molecular markers and DNA sequences, together with the phylogenetic species concept, were used as a foundation on which to base the identification and delimitation of species.

Single locus approach

The Internal Transcribed Spacer (ITS) region of the rDNA operon has been most commonly used for DNA sequence-based identification of fungi (Hajibabaei et al 2007, Nilsson et al 2008). The first DNA-based study on the Botryosphaeriaceae included the sequence data for the ITS region in combination with conidial characters, culture morphology and growth rate to analyse anamorphs of *Botryosphaeria* and related taxa (Jacobs and Rehner 1998). That study indicated that there was not always consensus between morphospecies and phylogenetic clades. For example, strains of *B. dothidea* (anamorph *Fusicoccum aesculi*)

resided in two ITS clades, one of which also included *B. ribis* strains (Jacobs and Rehner 1998). During the course of the decade following that study, numerous studies were conducted in which ITS sequences were used to re-evaluate the relationships amongst known species in this group as well as to confirm the identity and to describe new species (e.g. Denman et al 2000, Smith et al 2001, Zhou and Stanosz 2001, Denman et al 2003, Alves et al 2004, Pavlic et al 2004, 2007, Barber et al 2005, Gure et al 2005, Phillips 2007, Slippers et al 2007).

Comparisons of ITS sequences alone have not always been sufficient to clarify species boundaries in the Botryosphaeriaceae. For example, where isolates of *N. parvum* and *N. ribis* grouped in the same clade in ITS-based phylogenies, they were treated as a species complex or referred to as a *N. parvum* / *N. ribis* clade (Farr et al 2005, Slippers et al 2005, Pavlic et al 2007). In this case, data from ITS sequences were insufficient to either separate these two species or to determine whether other cryptic species existed within this complex. Such observations suggested strongly that there was a need for the inclusion of additional gene sequences or other molecular tools in order to clarify genetic variation observed.

An example of the strengths and limitations of ITS rDNA sequence data can be found in the studies of Pavlic et al (2004, 2007) on Botryosphaeriaceae on native *Syzygium cordatum* trees in South Africa. Prior to these studies, it was thought that *B. dothidea* occurs on this host (Smith et al 2001), but it was later shown that the isolates from *S. cordatum* represented *N. parvum* (Slippers et al 2004b). ITS rDNA sequence data, combined with anamorph morphology and PCR-RFLP analyses of the same region, later revealed that eight species occur on this host, of which *L. gonubiensis* was described as new (Pavlic et al 2004, 2007). Although the ITS phylogeny was sufficient to discriminate *L. gonubiensis* in these studies, this region alone could not separate the two closely related species *N. parvum* and *N. ribis*. Isolates within the *N. parvum* / *N. ribis* complex exhibited much variation in conidial morphology and ITS sequences. However, support for the sub-clades obtained in phylogenetic analyses of ITS sequence data was very low, leaving uncertainty as to their interpretation.

Multiple locus approach

The limitations of using single locus sequence data, especially for closely related sister species where ITS rDNA do not provide sufficient resolution, has led to sequences for more than one locus being used to delimit species in recent years. Examples can be found in studies on *Neurospora* and *Gelasinospora* (Dettman et al 2001, 2003), the human pathogenic

fungus *Cryptococcus neoformans* (Xu et al 2000), and other important human and plant pathogenic fungal complexes, such as *Fusarium graminearum* and *Gibberella fujikuroi* (O'Donnell et al 2000a, b, Steenkamp et al 2002, O'Donnell et al 2004), *Trichoderma harzianum* / *Hypocrea lixii* complex (Chaverri et al 2003), *Aspergillus flavus* and *A. fumigatus* (Geiser et al 1998, Pringle et al 2005), *Coccidioides immitis* (Koufopanou et al 1997) and many others. In all of these studies, various previously unidentified, cryptic phylogenetic species were revealed.

Genealogical concordance phylogenetic species recognition (GCPSR) was applied to the gene genealogies of multiple loci in the studies described above, in order to identify cryptic species. The GCPSR is a form of PSR that has most commonly been applied to study members of the fungal Kingdom (Taylor et al 2000). By relying on concordance of more than one gene genealogy, this method eliminates the limits of application of phylogenetic analyses of single genes (Taylor et al 2000).

GCPSR based on multi-locus sequences was first applied in a study on Botryosphaeriaceae by de Wet et al (2003). In that study, partial sequences of six protein-coding genes and six microsatellite loci, were used to elucidate phylogenetic relationships amongst isolates of *Diplodia pinea* (= *Sphaeropsis sapinea*) representing the A, B and C morphotypes previously described for this fungus. Although these morphotypes were described based on differences in pathogenicity, morphological and molecular characters, it was not clear whether they represent different taxa, because some characters overlapped between them. Application of GCPSR provided evidence that the B morphotype isolates were genetically distinct from *D. pinea* and this morphotype was consequently recognised as a new species described as *D. scrobiculata* (de Wet et al 2003). This was the first species in the Botryosphaeriaceae identified by the explicit application of GCPSR.

The application of GCPSR has been used to resolve long-standing uncertainty regarding the existence of cryptic species in the *N. parvum* / *N. ribis* complex. *Neofusicoccum parvum* and *N. ribis* were described as separate taxonomic entities based on morphological features (Grossenbacher and Duggar, 1911, Pennycook and Samuels, 1985). Although combined sequences for three gene regions separated these species (Slippers et al 2004b), they could not be delineated in many other studies, even where multiple gene sequences were used. This raised the question as to whether cryptic species were present in the complex. In the study of Pavlic et al (2009a), using sequences from five loci and GCPSR, three cryptic species were identified in the *N. parvum* / *N. ribis* complex from

Syzygium cordatum in South Africa. These species were described as *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* (Pavlic et al 2009b).

Increased numbers of cryptic species have been detected in different genera of the Botryosphaeriaceae using multiple gene phylogenies generated using ITS rDNA, EF-1α and β-tubulin sequence data. Phylogenetic analyses distinguished *N. eucalypticola* from *N. eucalyptorum* (Slippers et al 2004c) and *N. australe* as a sister species to *N. luteum* (Slippers et al 2004d). The same method was recently used to separate of *N. crypto-australe prov. nom.* as an additional sister species in the *N. luteum* / *N. australe* complex (Maleme 2008). Another example of species delineation using multiple gene sequences can be found in the morphologically similar species *B. dothidea* and *N. ribis* (= *B. ribis*) that were thought to represent a species complex. In a study by Zhou and Stanosz (2001), the ITS phylogeny supported separation of these two species that could not clearly be distinguished in the study of Jacobs and Rehner (1998). Using combined multiple gene sequences of the ITS rDNA, EF-1α and β-tubulin gene regions, along with phenotypic characters, Slippers et al (2004a) clarified the identity of *B. dothidea* and *N. ribis,* as well as *N. parvum* (= *B. parva*). In all of these studies, genetic variation observed within the clades in the phylogenetic analyses based on ITS sequences alone, gave a clear indication of new species, although their identity could only be clarified using multiple gene sequences.

Based on combined ITS and EF-1α sequences, a number of new species have been recently been recognised in *Diplodia, Lasiodiplodia* and *Dothiorella* (Luque et al 2005, Phillips et al 2005, Burgess et al 2006a, Damm et al 2007, Lazzizera et al 2008). For example, this approach was used to identify *Diplodia cupressi,* previously known as *D. pinea* f. sp. *cupressi*, as a distinct species (Alves et al 2006). It was also used to identify cryptic species, *L. pseudotheobromae* and *L. parva,* among a collection of isolates previously identified as *L. theobromae* (Alves et al 2008). Although not always explicitly applied as the phylogenetic species concept or phylogenetic species recognition, but rather as combined phylogenies used to clarify the identity of unresolved taxa based on single gene phylogeny, these studies represented the first steps towards PSR.

Microsatellite marker data

Single Sequence Repeat (SSR) or microsatellites are short repeat sequences found throughout the genomes of Eukarya that are commonly used as co-dominant markers in various typing studies. Amplified loci that contain SSR repeats can be analysed for variation in sequence data or for fragment size variation that depends on the number of repeats

contained in the microsatellite region (Squirrell et al 2003). This method can be used in combination with multilocus gene sequences as a form of multilocus species typing (MLST), typically used in studies of bacterial diversity (Taylor and Fisher 2003). Such microsatellite markers were, for example, used in the diagnosis of the phylogenetically recognised human fungal pathogens *Coccidioides posadassi* as well as in cryptic species in *Paracoccidioides brasiliensis* (Fisher et al 2002, Matute et al 2006). These studies showed that microsatellite loci could be used as molecular markers to characterise and type strains, as well as to assign strains to the described species. They could thus provide a simple and reliable means for the identification of genetically recognised cryptic species.

Microsatellites have been used for typing of populations and cryptic species in the Botryosphaeriaceae, especially in the pine pathogen *D. pinea* and related species (Burgess et al 2002, de Wet et al 2003). Microsatellite markers designed for this fungus clearly distinguished the three morphotypes of *D. pinea* (de Wet et al 2000, Burgess et al 2001, de Wet et al 2002). The sequences of the microsatellite regions were also used in combination with sequences from introns of six functional genes to analyse the relationship between morphotypes of *D. pinea*, and to distinguish *D. scrobiculata* amongst them (as discussed above) (de Wet et al 2003). Comparison of the multiple gene genealogies in the latter paper with those from the sequenced microsatellite loci confirmed that the sequences of microsatellite markers alone would be adequate for species recognition.

Microsatellite markers have been developed for *N. parvum*, but these also amplify corresponding loci in a few other Botryosphaeriaceae with *Fusicoccum* and *Neofusicoccum* anamorphs (Slippers et al 2004a). They have further been used in a population study on *N. australe* to show gene flow between native forests and plantations of *Eucalyptus globulus* in Western Australia (Burgess et al 2006b). These microsatellite markers have also been useful to delineate cryptic species in the *N. parvum* / *N. ribis* complex, and appropriate for analyses of inter- and intra-specific variation and population structure of sister species *N. cordaticola*, *N. kwambonambiense, N. umdonicola* and *N. parvum* (Pavlic et al 2009c).

Other molecular tools

The application of multiple gene genealogies and SSR markers is critical for the identification of cryptic species in the Botryosphaeriaceae. However, these methods can be time consuming and expensive and there is a need for accurate and rapid screening protocols following the initial delineation of the species. One approach that can be used is to find characteristic SNP or SSR alleles that characterize a species. Such data have been used to

develop PCR-RFLP fingerprinting techniques to distinguish species in the Botryosphaeriaceae. For example, the *sensu lato* groups of *N. parvum* and *N. ribis* could be distinguished based on *Cfo*I digestion of an SSR locus (*BotF15*) (Slippers 2003). Similarly, Alves et al (2005) used amplified ribosomal DNA restriction analyses (ARDRA) to differentiate isolates of twelve Botryosphaeriaceae species. Recently, Alves et al (2007) designed MSP-PCR (microsatellite-primed polymerase chain reaction) and rep-PCR (repetitive-sequence-based polymerase chain reaction) fingerprinting methodologies for the rapid identification of Botryosphaeriaceae species, including closely related species such as *N. parvum* and *N. ribis*, or *N. luteum* and *N. australe*. All of these techniques provide rapid and simple methods that can be readily used in species identification in the Botryosphaeriaceae. Thus, further application of such tools should be considered for newly identified phylogenetic species.

4.0. Towards phylogenetic systematics in the Botryosphaeriaceae

The majority of the more than 70000 described fungal species (Hawksworth et al 2004) have been defined based on morphological or other phenotypic characters, also referred to as MSR. However, speciation is not always correlated with morphological change (Taylor et al 2000). Comparisons of MSR and PSR have, therefore, not surprisingly shown that PSR performs the best, because changes in gene sequences occur and can be diagnosed before changes have occurred in mating behavior or morphology (Taylor et al 2000). Biological Species Recognition (BSR), which is commonly used in other fungi such as, for example, in the *Gibberella fujikuroi* complex (Leslie 1995, Kvas et al 2009), has not been applied to the Botryosphaeriaceae, because they do not produce sexual structures in culture. The BSR is, therefore, not be considered further here. Thus, with the application of PSR, numerous species have been identified that were previously morphologically or biologically cryptic, due to the lack of taxonomically informative phenotypic characters or incomplete reproductive isolation amongst the species. This reality is driving a need for changes to the way that the taxonomy of fungi is approached, and this is especially true for the Botryosphaeriaceae.

Due to their plasticity, inconsistency and overlapping nature, morphological features have been insufficient to distinguish closely related or sister species of the Botryosphaeriaceae with confidence. However, in many studies on the Botryosphaeriaceae, preliminary groupings of isolates have been based on cultural and conidial morphology (e.g. Slippers et al 2004a, Burgess et al 2005, Pavlic et al 2007, 2008). In these studies, groups

identified based on morphological characters were usually found congruent with those recognized based on DNA sequence data and *vice versa*. Although some morphological characters commonly used in the identification of Botryosphaeriaceae, such as conidial and ascospore shape, size, septation, wall thickness and color, as well as culture morphology and pigmentation, have provided strong indication of potentially cryptic species, further confirmation using other less subjective tools has typically been required.

Culture morphology has been useful to distinguish between some species in the Botryosphaeriaceae. For example, *N. luteum* was distinguished from related species by a yellow pigment formed in young cultures (Pennycook and Samuels 1985). However, some of isolates included in a study by Slippers et al (2004d), that were originally thought to represent this species based on conidial and culture morphology, exhibited slight differences in pigmentation from the original strains of *N. luteum*. However, differences in ITS rDNA sequence data amongst *N. luteum* isolates in that and other studies (Smith and Stanosz 2001, Denman et al 2003) were inordinately small to make conclusive decisions regarding potential cryptic species. It was only after fixed alleles across multiple gene regions indicated a genetic barrier between the groups representing the different cultural morphologies that *N. australe* could be described as distinct (Slippers et al 2004d). Culture morphology has also been useful in separation of other species in the Botryosphaeriaceae, but in many cases molecular support remained necessary to confirm species boundaries (de Wet et al 2003, Burgess et al 2005, Pavlic et al 2008).

Conidial morphology has been most extensively used in species identification in the Botryosphaeriaceae. It has been shown that variation in conidial morphology can indicate species diversity, but could not *a priori* confirm species differences. For example, isolates that resided in the *N. parvum* / *N. ribis* complex from *S. cordatum* in South Africa exhibited high levels of variation in conidial measurements and morphology, which differed from those in the original descriptions of *N. parvum* and *N. ribis*. This suggested that additional species could exist in this complex (Pavlic et al 2007). In a follow-up study (Pavlic et al 2009a), the selection of isolates for DNA sequencing based on variation in conidial mophology proved to be useful to sample representatives of different cryptic species, which were then recognised as *Neofusicoccum* spp. R1, R2 and R3 in that paper. When additional isolates were identified using molecular tools and included in morphometric analyses, a continuum in conidial variation was observed for these phylogenetically recognised species (Pavlic et al 2009b). Thus, while morphological differences can be used for initial selection

of isolates from a larger collection prior to molecular identification, species can not be identified based on this character alone.

The discovery of morphologically cryptic species using molecular tools makes it technically impossible to describe them taxonomically, because the International Code of Botanical Nomenclature requires morphologically distinct characters. This has led to the description of species using molecular characters (DNA sequence data), although this is not strictly allowed by the Code. The phylogenetic species are characterized primarily by fixed single nucleotide polymorphisms (SNPs) (O'Donnell et al 2004, Grünig et al 2008, Pavlic et al 2009a, b). Although many cryptic, phylogenetic species have been recently recognized in the fungal Kingdom, there are very few descriptions of these species. Some examples include the description of human pathogen *Coccidioides posadasii* (Fisher et al 2000), nine phylogenetically distinct species within *Fusarium graminearum* clade (O'Donnell et al 2004) and six cryptic species of the *Phialocephala fortinii* s.l.-*Acephala applanata* species complex (Grünig et al 2008). Fixed nucleotide characters, given by gene and nucleotide position were used in diagnoses of these species. Without the descriptions, these phylogenetically recognised species would remain cryptic. Increasing numbers of phylogenetic species that cannot be diagnosed based on morphological characters, provide a strong case for changes to the regulations regarding descriptions of fungal species.

The first phylogenetically recognized species in the Botryosphaeriaceae were recently described using sequence data and SNPs as defining characters (Pavlic et al 2009b). Three new species were thus recognized in the *N. parvum* / *N. ribis* complex as *Neofusicoccum* spp. R1, R2 and R3 using multiple gene genealogies and GCPSR (Pavlic et al 2009a) (Fig. 1) and described as *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, respectively (Pavlic et al 2009b). The conidial sizes of these species lie in a continuum, and can not be used to distinguish the species *a priori* the application of DNA sequence data (Fig. 2). Analyses of conidial measurements following molecular identification, however, revealed statistically significant differences between the average conidial dimensions for *N. parvum*, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* (Pavlic et al 2009b). Even with this information, their correct diagnosis can only be achieved by SNPs (Pavlic et al 2009b). Phenotypic characters can identify many cryptic species once revealed using molecular tools, however due to small differences in morphological features, these species must be described using molecular characters.

5.0. Utility of one name per fungal species

The International Code of Botanical Nomenclature (ICBN) requires two separate names for the anamorph and teleomorph states of fungi (Article 59) (McNeill et al 2005). Where both are known, the teleomorph name takes preference. The growing numbers of fungal species that have been identified based on DNA sequence data or using other molecular tools are, however, intensifying the need to use one name per fungal species. This is because new species can be linked to either teleomorph or anamorph genera based on DNA sequence data, irrespective of what state of the fungus was collected or could be induced in culture. Furthermore, many morphologically defined teleomorph genera are being shown to include numerous anamorph genera, and some anamorph genera are clearly polyphyletic (Crous et al 2006, Phillips et al 2008). Application of molecular tools allows us to define relationships of asexually producing fungi and establishes anamorph-teleomorph connections based on molecular phylogeny, even when the teleomorph state is unknown. Consequently the holomorph concept that provides one name per fungal species that reflects the phylogeny of taxa (Rossman and Samuels 2005) should be more widely accepted by the mycological community. Although a proposal for a single scientific name for fungi was recently made (Rossman and Samuels 2005), it appears that the transition towards the application of one name for a fungal taxon will not be an easy task.

A one species-one name approach to fungal taxonomy would aid the taxonomy of the Botryosphaeriaceae, since the sexual state is less commonly found in the nature than the anamorph and it is also rarely induced in culture. This problem was highlighted in a recent study when phylogenetic lineages in the Botryosphaeriaceae (all previously linked to the teleomorph *Botryosphaeria*) were characterized as distinct genera based on anamorph differences (Crous et al 2006). Not all these genera could be linked to teleomorph taxa, and many of the genera recognised by Crous et al (2006) are, therefore, known only by their anamorph names. An example is found in the well-known pathogen of fruit and fruit trees, '*Botryosphaeria' obtusa*. Following the taxonomic changes this name was no longer valid and only the anamorph name, *Diplodia seriata*, can be used (Phillips et al 2007). Similarly, *Lasiodiplodia theobromae* now represents '*Botryosphaeria' rhodina*, *Diplodia mutila* represents '*Botryosphaeria' stevensii*, *Neofusicoccum parvum* represents '*Botryosphaeria' parva* and many more (Crous et al 2006). These changes reflect the evolutionary divergence amongst the groups much more accurately than was true in previous taxonomic treatments. They also facilitate thinking and communication regarding the evolutionary history of the groups (de Wet et al 2008).

6.0. DNA-barcoding

The DNA Barcode of Life Initiative aims to provide unique DNA sequences, the 'barcode,' for the identification of all biological species (http://barcoding.si.edu/). The ITS rDNA locus is currently the preferred region to serve as the universal tool in identification of fungal species (Nilsson et al 2006, 2008; www.allfungi.org/its-barcode.php). Although ITS rDNA sequences have been broadly used for fungal DNA based identification (Hajibabaei et al 2007, Nilsson et al 2008), it has been argued above that this region alone is not sufficient to distinguish closely related or cryptic species of the Botryosphaeriaceae (e.g. Smith et al 2001, Slippers et al 2004b, Farr et al 2005, Pavlic et al 2007). This is also true for many species in other groups of fungi (Bruns and Shefferson 2004, Bischoff et al 2006, Kvas et al 2009). However, no other region currently provides a more suitable basis for barcoding the Botryosphaeriaceae in terms of ease of amplification and distinguishing power. Despite its shortcomings, the ITS rDNA thus remains the most suitable region to serve as an effective barcoding locus.

The fact that the majority of fungal species are described based on morphology alone, presents a significant challenge to DNA barcoding efforts. This challenge is also substantial in the Botryosphaeriaceae. Although more than 2000 species are known in the Botryosphaeriaceae (www. indexfungorum.org), a limited number are represented by sequence data in GenBank or even cultures. The application of DNA sequence based characterisation without consulting previous descriptions based on phenotypic characters can thus lead to the description of species that have already been described, but for which sequence data are not available. Furthermore, not all the sequences in GenBank are linked to type specimens and might not represent the taxa they are labelled with. In general, as many as 27 % of ITS rDNA fungal sequences deposited in GenBank have been found to be from wrongly identified specimens and cultures (Nilsson et al 2006). Much work thus remains to be done, before a reliable database will exist and upon which DNA based taxonomy and DNA barcoding systems can rely.

7.0. Consequences of phylogenetic species recognition in the Botryosphaeriaceae

Similarly to other fungal groups (see Le Gac et al 2007), the application of molecular tools has contributed substantially to our understanding of host relationships in the Botryosphaeriaceae. Some species previously thought to be generalists have been shown to represent complexes of species and cryptic species, which are specialists on one host or on a

few related hosts. For example, *B. dothidea* was considered to be a widely distributed species on a variety of native and cultivated hosts. Species previously treated under *B. dothidea* are now known to vary from specialists such as *N. eucalyptorum* on *Eucalyptus* and *N. protearum* on Proteaceae, to generalists such as *N. parvum* that has been associated with a variety of hosts worldwide (Slippers and Wingfield 2007, de Wet et al 2008). It is clear that incorrect species identifications underestimate the diversity of fungal communities on host plants and they also obscure the specificity of many species.

Accurate species identification is important for understanding patterns in the distribution of the Botryosphaeriaceae and to implement suitable quarantine measures to reduce the probability of their spread to new environments. Species of the Botryosphaeriaceae are known as endophytes that can easily be moved unnoticed into new areas (Burgess and Wingfield 2002, Slippers and Wingfield 2007). Once introduced into new areas, they are likely to infect new hosts. For example, a recent population study on the plant pathogen *L. theobromae* revealed high gene flow between populations from three different hosts in Venezuela, *Pinus caribaea*, *Eucalyptus urophylla* and *Acacia mangium,* and indicated that there was no host specificity for isolates of this fungus (Mohali et al 2005). Movement of these species between native and non-native hosts and their introduction into new areas could pose a serious threat to agricultural crops, trees in plantations and native flora.

Recently, eight species of the Botryosphaeriaceae were identified from native *S. cordatum* in South Africa (Pavlic et al 2007). These species were also shown to be able to infect *Eucalyptus* and were more virulent on this host than on *S. cordatum*, at significantly different levels (Pavlic et al 2007). Isolates treated as *N. ribis*-like in the study of Pavlic et al (2007), were later shown to represent three cryptic species that were significantly more virulent than *N. parvum* and *N. ribis* to *S. cordatum* in greenhouse trials (Pavlic et al 2009b). Isolates identified as '*N. ribis*' were also highly pathogenic to different *Eucalyptus* clones grown commercially in Venezuela (Mohali et al 2009) and Colombia (Rodas et al 2009), but the identity of these isolates remains to be confirmed. Other examples include the *Diplodia pinea* morphotypes and *D. scrobiculata* that differed in virulence to *Pinus* (de Wet et al 2000, 2003). Inoculation trials on different host plants, such as *Eucalyptus* and grapevines identified *B. dothidea* as least virulent, while *N. parvum* was amongst the most virulent Botryosphaeriaceae tested by van Niekerk et al (2004) and Pavlic et al (2007). Two closely related species in *Lasiodiplodia, L. theobromae* and *L. gonubiensis,* differ significantly in their virulence, with *L. theobromae* being more virulent (Pavlic et al 2007). Since isolates of

cryptic species differ in virulence, their correct identification is of enormous importance for control purposes and management strategies. Application of the phylogenetic species concept will allow us to recognise morphologically and ecologically cryptic species in under-explored environments, such as natural stands of different species of plants. This has been particularly evident in the Botryosphaeriaceae where applications of DNA based tools in species identification have revealed substantial unknown diversity in recent years (Pavlic et al 2004, Slippers et al 2005, Pavlic et al 2008, van der Walt 2008, Taylor et al 2009).

In two extensive studies recently conducted on more than thirty native tree species, including *Adansonia digitata* (baobab), *Acacia* spp. and *Eucalyptus gomphocephala,* eleven new species of Botryosphaeriaceae were described (Pavlic et al 2008, Taylor et al 2009). An additional twelve new species were recognised from native *Acacia* spp. in Southern Africa (van der Walt 2008). Discovery of many new fungal species on the plants in native environments indicates that plants in natural stands are under explored and will most likely harbour numerous new species. These findings underpin the necessity of having a holistic view of fungal communities on native and planted trees in order to record and conserve their true diversity.

Molecular tools have proven useful in the identification of medically important species in the Botryosphaeriaceae (Tan et al 2008, Woo et al 2008). The fungi identified in these studies included *L. theobromae, Macrophomina phaseolina* and *Neoscytalidium dimidiatum* (= *Scytalidium dimidiatum*). Interestingly, all of these species are also well-known plant pathogens (Punithalingam 1976, Crous et al 2006, Avilés et al 2008). It is thought that in all of the cases, humans were infected through environmental exposure and through contact with contaminated plant material and soil (Tan et al 2008). What triggers these species to infect and cause diseases in humans will need further clarification. However, identification of these clinical isolates based on morphology was difficult. For example, one of the isolates was initially thought to represent, *Pseudallescheria boydii*, based on colony morphology, and then later identified as *L. theobromae*, of which identity was also uncertain since the isolate failed to produce fruiting structures. The ITS rDNA sequence comparisons, however, determined the isolate as *Macrophomina phaseolina* (Tan et al 2008). This is another example that highlights the necessity of using molecular tools in the correct identification of species in Botryosphaeriaceae, which can be particularly difficult in non-sporulating isolates.

8.0. Conclusions

Phylogenetic inference based on DNA sequence data has had an enormous impact on the taxonomy of the Botryosphaeriaceae. At the species level, a phylogenetic approach has revealed that a number of previously well defined taxa encompass cryptic species that had previously been overlooked. The result has been the description of numerous new species, many of which can hardly be distinguished from their sister species based on morphology. DNA sequences have also been used in the re-evaluation of *Botryosphaeria sensu lato* and its placement in higher orders of fungal classification. Thus, new genera have been recognised and their phylogenetic relationships, anomorph-teleomorph connections and placement in the family have been clarified.

Although ITS rDNA sequence comparisons were useful at the early stages of DNA based identification of the Botryosphaeriaceae, and fungi in general, sequences for additional loci often revealed cryptic species that could not be delineated based on ITS rDNA sequences alone. Through these studies GCPSR, as a form of PSR based on concordance of multiple gene genealogies, has emerged as the most powerful tool in species recognition. It is expected that through the application of this approach, new species and species complexes will be discovered. Although it is debatable whether ITS rDNA region will be most suitable for DNA barcoding in fungi, molecular phylogenetics will provide the most important basis for species identification as well as for molecular systematics.

The application of molecular tools other than single or multiple locus sequence data, such as microsatellite markers, and a polyphasic approach will lead to more detailed insights into inter- and intra-species diversity for the Botryosphaeriace. This will improve our knowledge of evolution of fungal species and understanding of processes that drive speciation. It will further advance and clarify criteria for species delineation and assist in the identification of species boundaries among closely related species and species complexes. It is, however, apparent that this is a process that is far from complete and many new species of agricultural or medical importance have yet to be discovered.

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FIG. 1. One of two unrooted maximum-parsimony trees resulting from the analysis of the combined sequence data of five loci, including ITS rDNA, EF-1α, RPB2, the Bt2 region of the β-tubulin gene and *BotF15*, shows distinct clades for *N. parvum, N. ribis, N. cordaticola, N. kwambonambiense* and *N. umdonicola*. The combined sequence data analysis, and in particular also the linked divergence indicated by the individual gene genealogies (data not shown), indicate species barriers that was not evident by considering morphological characters alone. Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates obtained from *S. cordatum* are indicated in bold. Ex-type isolates and isolates linked morphologically and geographically to the types of *N. parvum* and *N. ribis* are underlined. Isolate numbers are those of the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

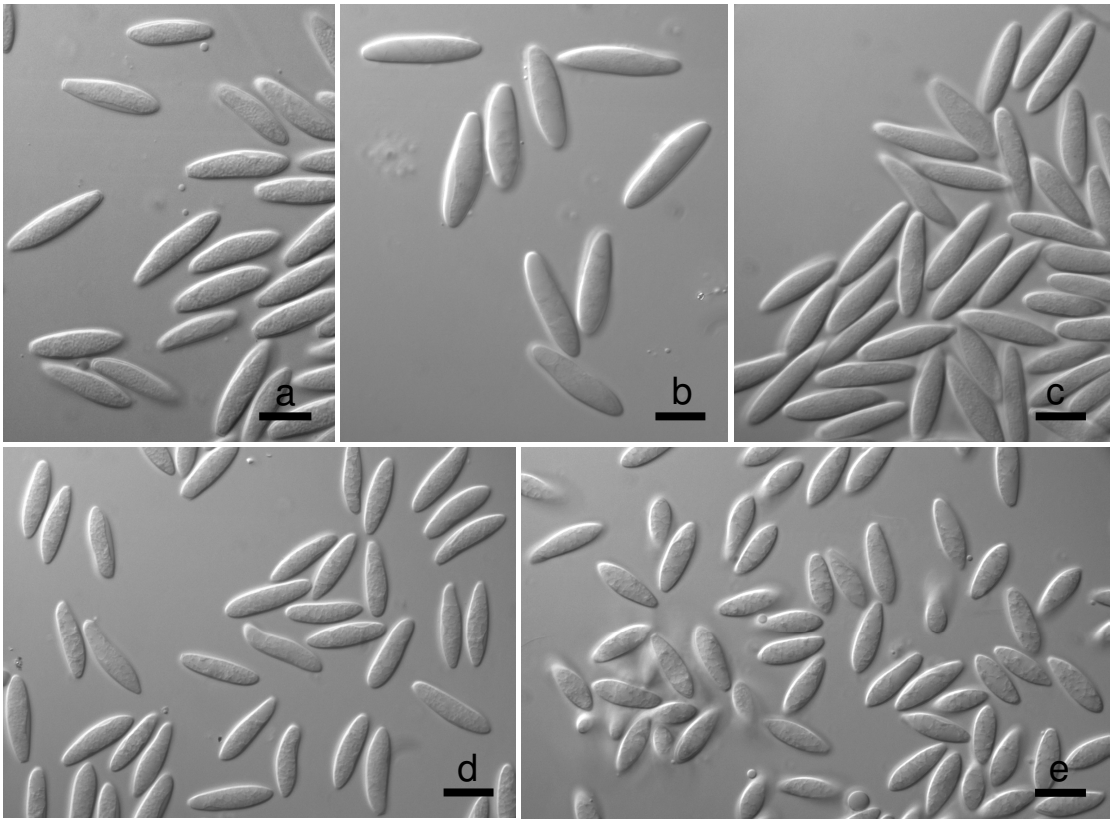
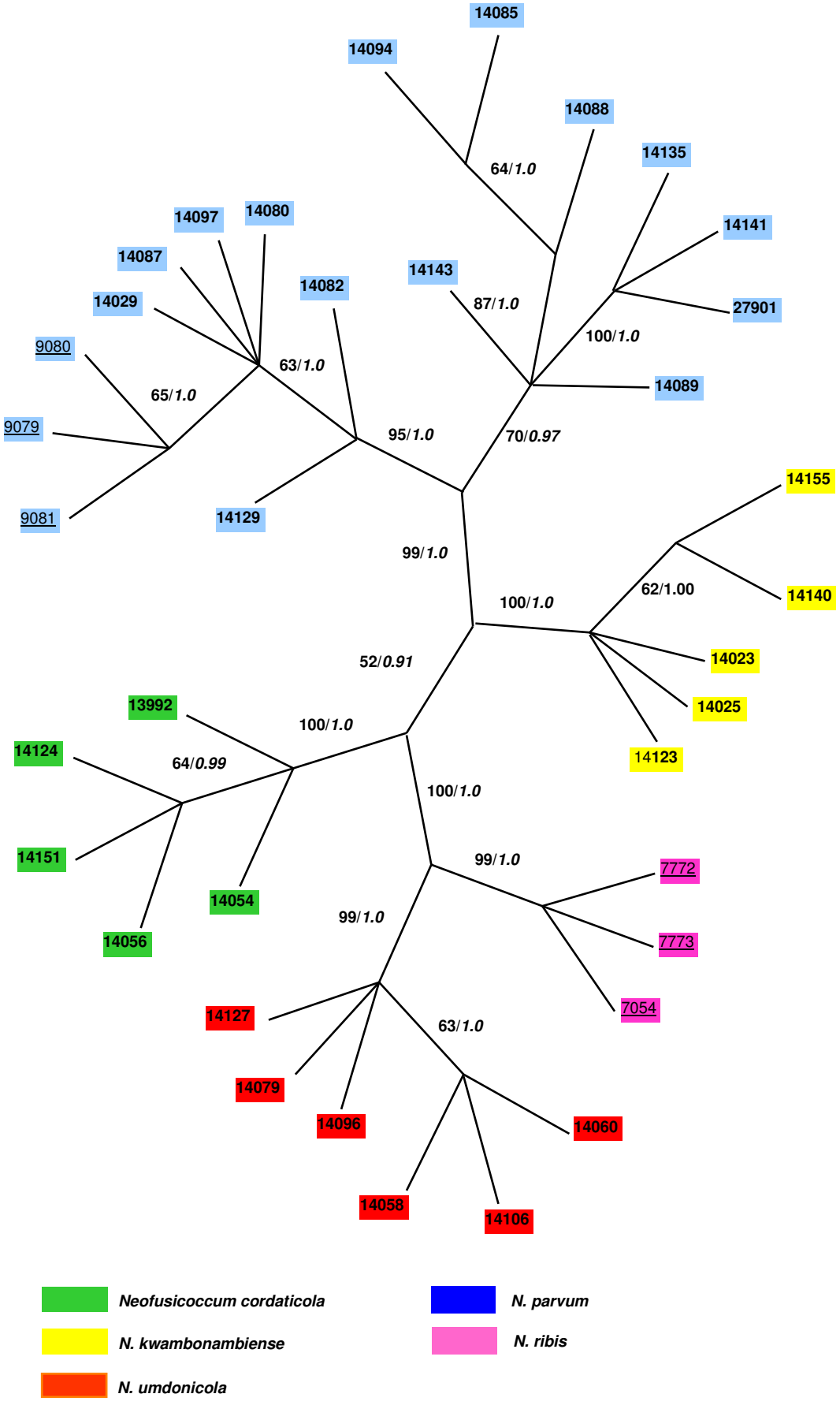

a
b
c
d
e

FIG. 2. Conidia of four cryptic species in the *N. parvum* / *N. ribis* complex recognised as *N. umdonicola* (a), *N. kwambonambiense* (b), *N. cordaticola* (c) and *N. parvum* (d, e) using GCPSR of five sequenced loci. These species cannot be distinguished from each other with certainty based on conidial morphology alone, which was commonly used in the past for this purpose.


14085
14094
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14135
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14143
14082
14029
87/1.0
27901
100/1.0
9080
63/1.0
14089
65/1.0
9079
95/1.0
70/0.97
14155
9081
14129
99/1.0
62/1.00
14140
100/1.0
52/0.91
14023
13992
14025
100/1.0
14123
14124
64/0.99
100/1.0
14151
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Neofusicoccum cordaticola
N. parvum
N. kwambonambiense
N. ribis
N. umdonicola