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).

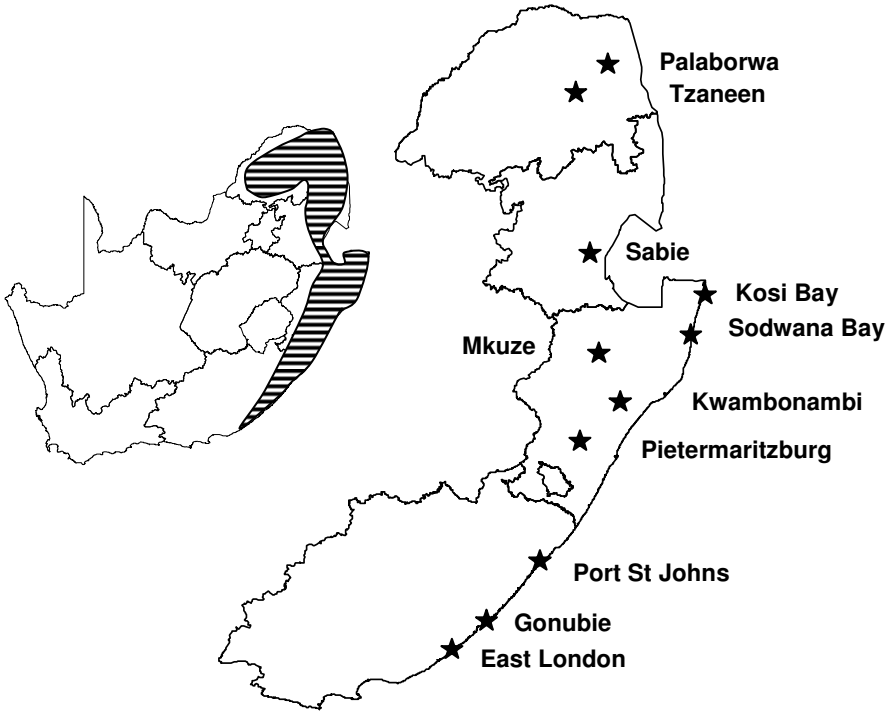

Palaborwa
Tzaneen
Sabie
Kosi Bay
Sodwana Bay
Mkuze
Kwambonambi
Pietermaritzburg
Port St Johns
Gonubie
East London

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.

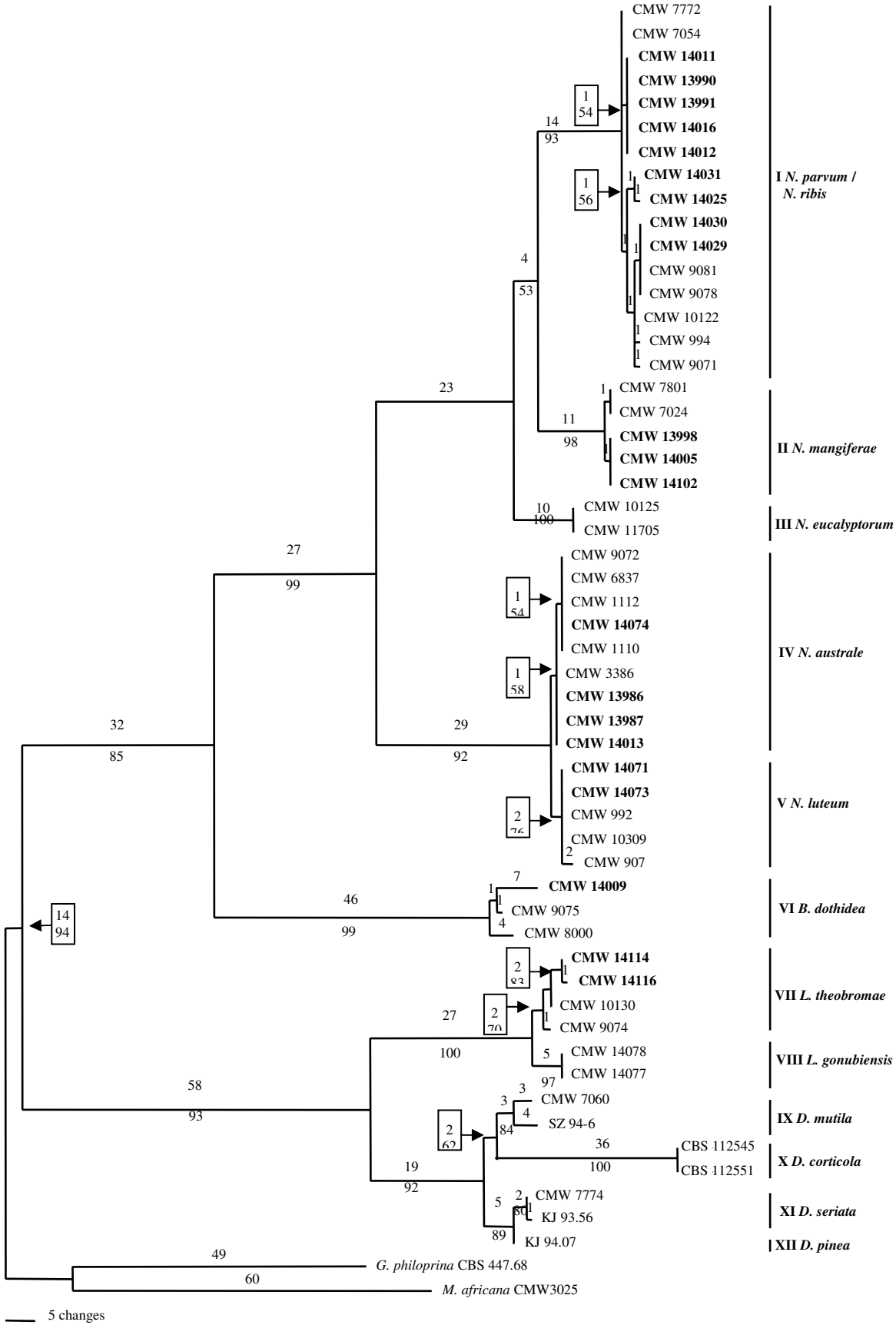

CMW 7772
CMW 7054
CMW 14011
CMW 13990
CMW 13991
CMW 14016
CMW 14012
CMW 14031
CMW 14025
CMW 14030
CMW 14029
CMW 9081
CMW 9078
CMW 10122
CMW 994
CMW 9071
I N. parvum / N. ribis
CMW 7801
CMW 7024
CMW 13998
CMW 14005
CMW 14102
II N. mangiferae
CMW 10125
CMW 11705
III N. eucalyptorum
CMW 9072
CMW 6837
CMW 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.

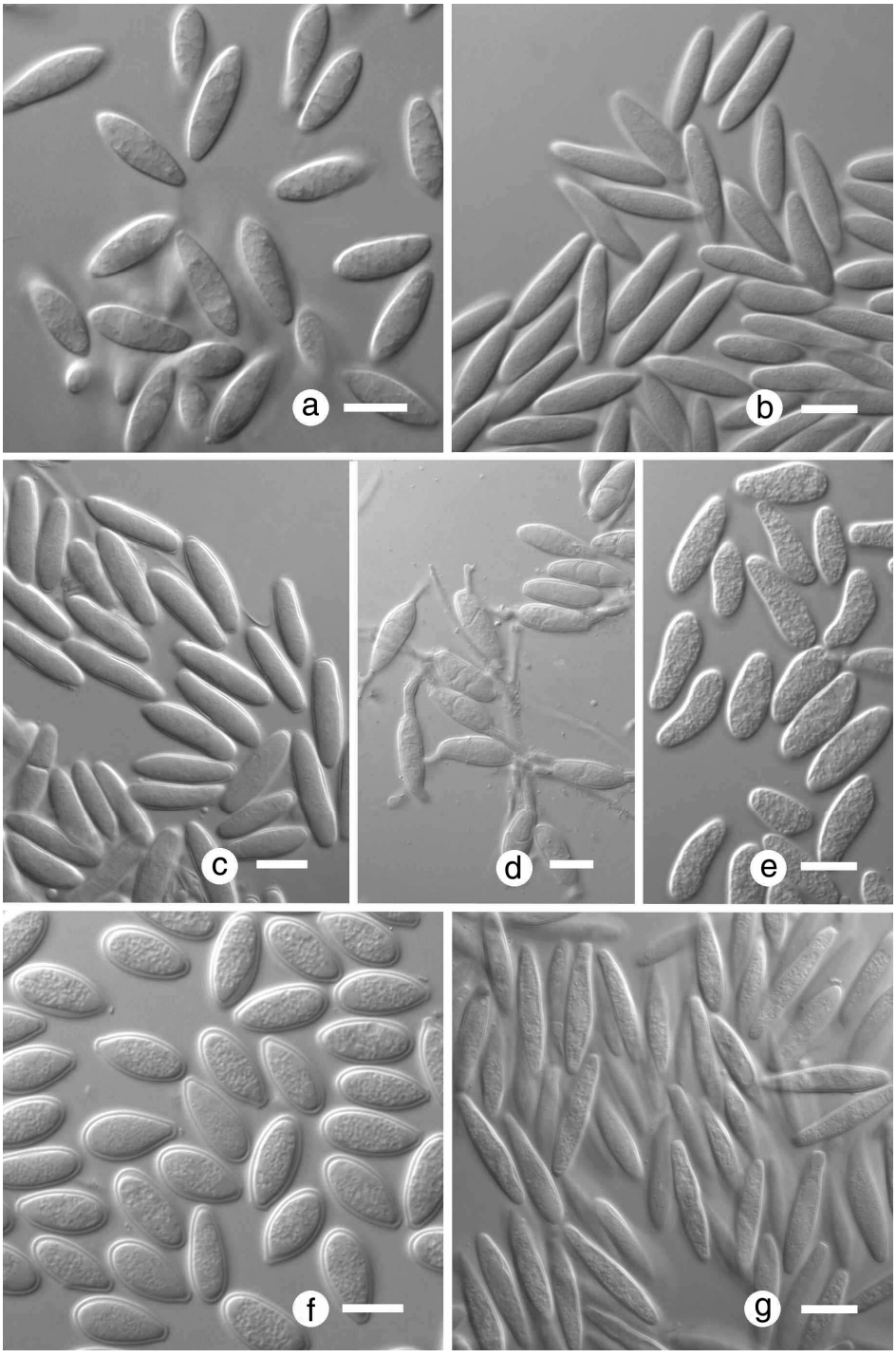

a
b
c
d
e
f
g

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

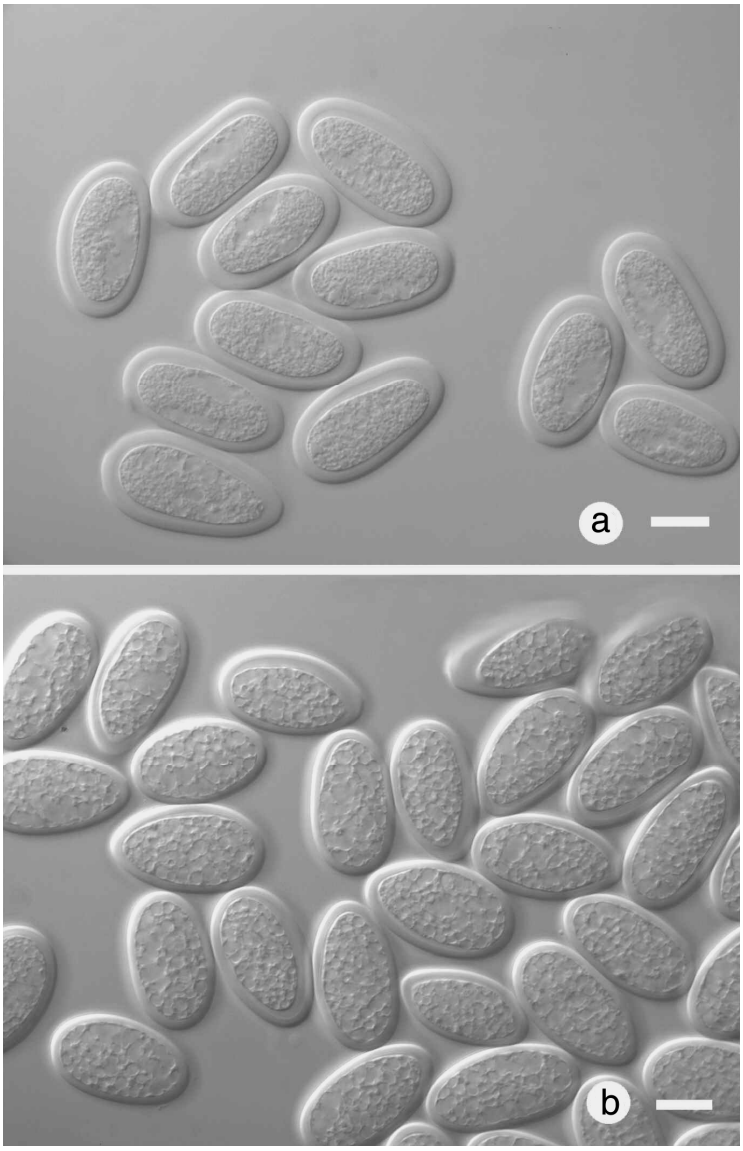
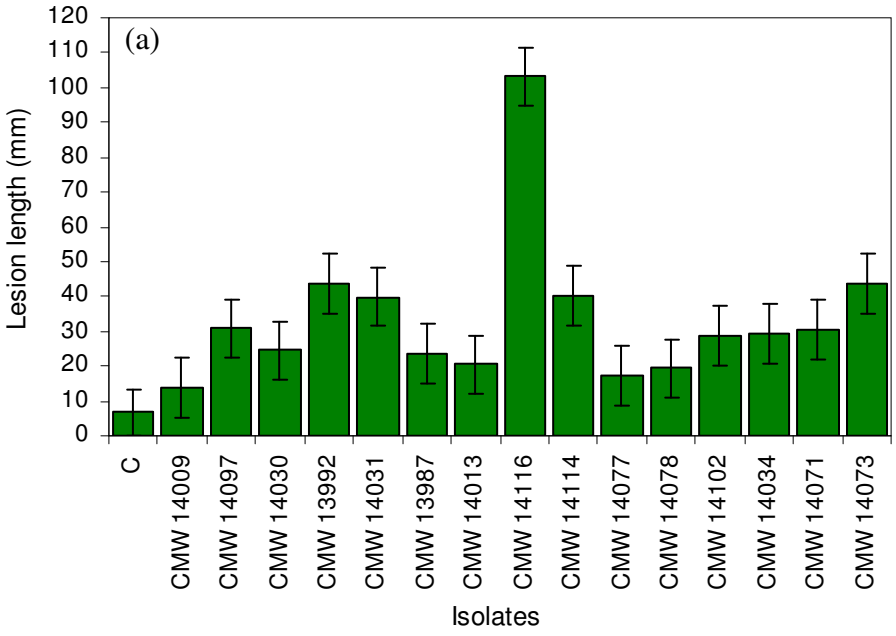
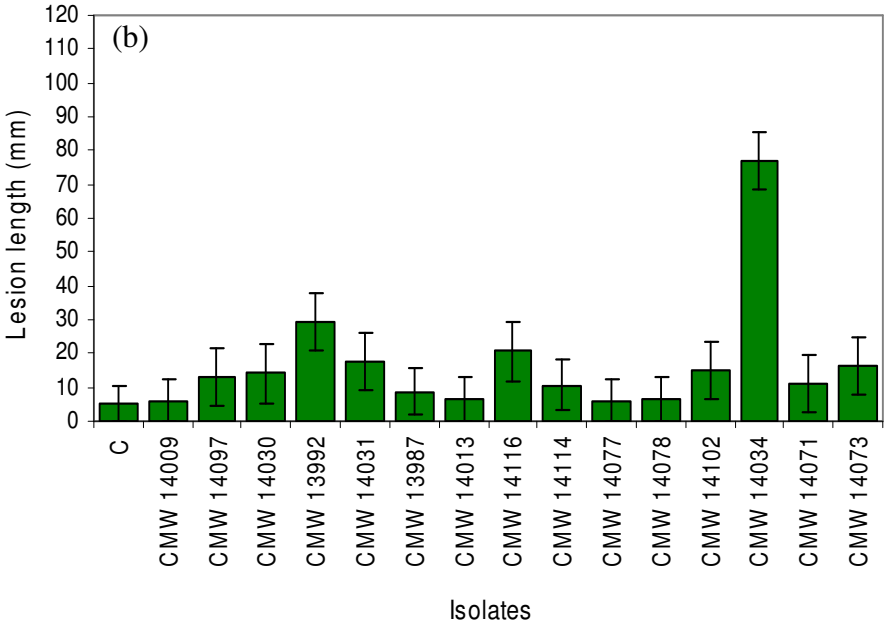

a
b

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