Chapter 3

Botryosphaeriaceous fungi as endophytes on *Terminalia* species in Cameroon

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ABSTRACT

In Cameroon, native *Terminalia* spp. represent an important component of the forestry industry, but limited information is available regarding the fungal pathogens that affect them. The Botryosphaeriaceae are endophytic fungi and latent pathogens that can result in wood stain, cankers, die-back and death of trees, particularly when trees are under stress. The aim of this is study was, therefore, to identify and characterize the Botryosphaeriaceae occurring as endophytes on *Terminalia* spp. in Cameroon as part of a larger project to identify potential pathogens of these trees in the country. Samples were collected from three *Terminalia* spp. in the Central, Southern and Eastern Regions and the resultant Botryosphaeriaceae were identified using morphology and DNA sequence comparisons for the ITS and *tef 1-α* gene regions. Furthermore, inoculation trials were conducted to consider the relative pathogenicity of the isolates collected. The majority of isolates (88 %) represented species of *Lasiodiplodia,* including *L. pseudotheobromae, L. theobromae* and *L. parva.* The remaining isolates were identified as *Endomelanconiopsis endophytica.* Pathogenicity trials on young *T. mantaly* and *T. catappa* trees revealed that *L. pseudotheobromae* was the most pathogenic species followed by *L. theobromae*.

1. INTRODUCTION

The forestry sector in Cameroon plays an important role in the national economy of the country. Timber is the second most exported product, after petroleum, with wood-based exports generating revenue of US $210 million in 2001 (Anonymous 2005a). The total forest area in Cameroon is estimated to represent ~12.8 million ha of natural forests and about 17000 ha of planted forests (Anonymous 2005b), made up of a variety of native trees such as *Terminalia* spp.

Species of *Terminalia* currently found in forest plantations in Cameroon include *T. ivorensis* and *T. superba*. These tree species have a well acknowledged commercial value with a total volume of exported logs representing 10 % of the national round wood production (Laird 1999). Besides their high commercial value, *Terminalia* spp. are commonly used in agriculture to establish a "taungya" agri-sylvicultural system in which they provide shade or improve soil fertility for crops (Norgrove and Hauser 2002). Furthermore, species such as *T. ivorensis* are important components for traditional medicine (Kamtchouing *et al.* 2006). Additional to native *Terminalia* spp., non-native species such as *T. mantaly* and *T. catappa* are frequently encountered as ornamentals in urban areas in Cameroon. The socio-economical importance of *Terminalia* spp. in Cameroon, coupled with their fast growth account for their extensive exploitation in national regeneration programs.

Among the potential threats to forest tree species are fungal pathogens belonging to the family Botryosphaeriaceae. Species in the Botryosphaeriaceae have a worldwide occurrence, causing a wide range of diseases, predominantly die-back, canker and blue stain, on numerous hosts, including trees (Brown and Britton 1986; Denman *et al.* 1999; 2000; Desprez-Laustaud *et al.* 2006). This group of fungi commonly exist as endophytes in healthy plant tissues (Smith *et al.* 1996; Swart *et al.* 2000; Slippers and Wingfield 2007). Disease symptoms typically appear only after stress caused by abiotic and biotic disturbances (Schoeneweiss 1981; Slippers and Wingfield, 2007). Their occurrence as endophytes makes them especially important in international trade, as they may be spread undetected from one area to another, causing potentially serious damage to hosts that might have no co-evolved resistance (Slippers and Wingfield 2007).

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Species of Botryosphaeriaceae contribute directly or indirectly to economic and environmental losses, although the impact of their diseases is difficult to assess in forestry. In South African pine plantations, for instance, up to 55 % loss of production have been

recorded after hail damage and die-back due to *Diplodia pinea* Fries (Zwolinski et al. 1990). In the United States of America (U.S.A.) several tree diseases associated with non-aggressive pathogens belonging to the Botryosphaeriaceae caused extensive mortality of Aspen during the 1930s (Schoeneweiss 1981). Moreover, other reports appear in literature recognizing severe decline of *Quercus* spp. due to species in the Botryosphaeriaceae in 1980 in the Mediterranean Basin (Sanchez et al. 2003).

In Africa, species of *Terminalia* occur in environments ranging from evergreen, primary and secondary forests to open woodlands or wooded savannahs (Carr 1994; Dale and Greenway 1961; Keay 1989; Lebrun and Stork 1991). Although these trees have tended to display natural resistance to pests and diseases (Groulez and Wood 1985; Lamb and Ntima 1971), their wide ecological distribution exposes them to highly variable climatic conditions, environmental stress and other negative factors such as human activities and diverse pests and diseases. These factors may play an important role in predisposing *Terminalia* spp. in Africa to infection by species of the Botryosphaeriaceae (Jurskis 2005).

The aim of this study was to identify species in the Botryosphaeriaceae that occur on *Terminalia* trees in Cameroon. This information will be valuable in the management of the health of these trees, because they are a key group of pathogens that generally affect forest trees, and especially given projections of changing weather patterns, that will negatively affect these trees. Identification were done using a combination of morphological and DNA sequence data of the ITS and *tef 1-α* gene regions. Furthermore, inoculation trials using species of the Botryosphaeriaceae from *Terminalia* spp. were conducted to determine their relative pathogenicity.

2. MATERIALS AND METHODS

2.1. Sample collection and fungal isolation

Plant material was collected in 2007 and 2008 from two species of native and one non-native *Terminalia* in Cameroon. The tree species sampled were the non-native *T. mantaly* and native *T. ivorensis* and *T. superba.* Four sites, located in three regions, were chosen for sampling (Table 1). Depending on the availability of trees at each location, at least 15 trees per species were randomly chosen for sampling without considering either their size or age. In each area,

samples from healthy twigs or bark were collected and placed in paper bags and transferred to the laboratory where they were processed within a few days.

For each sample, two pieces of twig or bark (1 cm in length) were split longitudinally. Samples were surface disinfected by sequential soaking in 70 % ethanol (1min), undiluted bleach (3.5 % sodium hypochlorite for 1 min), 70 % ethanol (1 min), rinsed in sterile water and allowed to dry under sterile conditions. Three disinfected pieces were placed onto 2 % malt extract agar (MEA) (2 % malt extract, 1.5 % agar; Biolab, Midrand, Johannesburg, S.A.) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri plates were sealed with Parafilm (Pechiney Plastic Packaging, Chicago, USA) and incubated at 20 °C under continuous near-UV light for one week. Single hyphal tips growing from the plant tissues were transferred to new Petri plates containing MEA. After two weeks of incubation under near UV-light, cultures resembling species of the Botryosphaeriaceae (fast growth, mycelium white originally, turning dark greening-grey or greyish within few days) were selected and transferred to new Petri dishes containing MEA.

2.2. Morphology and cultural characteristics

To encourage formation of fruiting structures, isolates were inoculated onto sterile pine needles on 1.5 % water agar (WA) (Biolab, S.A.) as described previously (Slippers et al. 2004). The plates were incubated at 25 °C under near UV-light for 4-6 weeks. Microscope slides of conidia from pycnidia formed on the pine needles were prepared in lactic acid for morphological observations. Conidial dimensions were taken from digital images using a HRc Axiocam digital camera and accompanying Axiovision 3.1 (Carl Zeiss Ltd., München, Germany) microscope. For each isolate, fifteen measurements of both conidial length and width were made. Colony appearance of cultures growing on 2 % MEA at 25 °C under near UV-light for two weeks was described and colours of the colonies were recorded. Cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.3. DNA extraction, PCR reactions and DNA sequencing

Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae were the same as those described in Begoude et al. (2009),

using two gene regions. The entire Internal Transcribed Spacer region (ITS) of the nrDNA, including the 5.8S operon, was amplified by PCR (polymerase chain reaction), for all isolates collected, using the primers ITS1 and ITS4 (White et al. 1990). A part of the Translation Elongation Factor-1α (*tef 1-α*) gene was amplified, for selected isolates, using the primers EF1F and EF1R (Jacobs et al. 2004).

2.4. Sequence Analyses

Sequences of the Botryosphaeriaceae generated in this study were edited using MEGA version 4 (Tamura et al. 2007). For the phylogenetic analyses, DNA sequences from this study, together with those retrieved from published sequences in GenBank (http://www.ncbi.nlm.gov) were aligned online using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh et al. 2005). The aligned sequences were transferred to PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 2001) where a final manual alignment was made.

A phylogenetic analysis was run for separately each of the ITS and *tef 1-α* data sets, followed by combined analyses of these data sets for core isolates. A partition homogeneity test (Farris et al. 1995) was conducted in PAUP version 4.0b10 (Swofford 2001) to assess the possibility of combining the ITS and *tef 1-α* data sets. In all analyses, gaps were treated as fifth character and characters were unordered and of equal weight. The phylogenetic analyses for the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition in 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithm, and random taxon addition of sequences for the construction of maximum parsimonious trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. *Guignardia mangiferae* A.J. Roy was used as the outgroup in the analyses of ITS and *tef 1-α* data sets. The support for branches of the most parsimonious trees was assessed using a 1000 bootstrap replicates (Felsenstein 1985). Other measures considered were the tree length (TL), consistency index (CI), rescaled consistency index (RC), and retention index (RI) (Hillis and Huelsenbeck 1992).

Bayesian phylogenetic analyses using Markov Chain Monte Carlo (MCMC) were performed in MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001) for all three data sets described above. Version 2.2 of MrModeltest (Nylander 2004) was used to select the model that best fits each

of the partitions. The Likelihood settings from best-fit models, SYM+I+G and HKY+G, were selected based on the Akaike Information Criteria (AIC) for ITS and *tef 1-α* respectively. Bayesian analyses were performed for one million generations, with four independent chains, and sampled every 100th tree. The first 1000 trees were graphically identified as the burn-in and deleted when constructing consensus trees and calculating posterior probabilities. A total of 9001 trees were imported into MEGA version 4 to construct a 50% majority-rule consensus tree.

2.5. Pathogenicity

Plants of native species of *Terminalia* are rare and could not be obtained. Pathogenicity tests were consequently carried out on one-year-old non-native *T. mantaly* and *T. catappa* trees grown in the Yaoundé Urban Council nursery, Cameroon. These trials were conducted between October and December 2008. This period falls at the end of the rain season and the beginning of the dry season, with average day and night temperatures of 26 °C. The trees were maintained under shade in 15 cm diameter plastic bags and watered daily. At the time of inoculation, the stem diameters were approximately 10 mm and the trees varied from 15-30 cm in height. For inoculations, 14 isolates of the Botryosphaeriaceae, representing all the species identified in the study, were grown on 2 % MEA for 10 days prior to inoculation.

To inoculate trees, wounds were made on the stems, half way between the soil level and the first branch by removing the outer bark with a 5 mm diameter cork-borer. A 5 mm-diameter plug of each isolate was placed into each wound, with the mycelium facing the cambium, and wrapped with a strip of Parafilm to prevent desiccation and cross contamination of the wounds and inoculum. The trees were divided into four separate blocks and within each block, six trees arranged in a completely randomized design, were used for inoculation with each isolate. The entire trial was repeated once. For the controls, sterile MEA plugs were used in place of the fungal cultures.

After six weeks, the lengths of the lesions produced in the cambium, including the inoculation point, were measured to obtain an indication of the virulence of the isolates tested. Furthermore, a small piece of necrotic tissue was cut from the edges of all lesions and placed on MEA for isolations to show that the inoculated fungus was associated with the lesions. As no significant differences were noticed between results obtained for the two inoculations (P >

0.05), the data for all isolates were pooled in a single dataset for analyses. Variations in the extent of the lesions were assessed through a one-way analysis of variance (ANOVA) using SAS (SAS systems, version 8.2; SAS Institute).

3. RESULTS

3.1. Isolates and morphology

A total of 115 trees were sampled at four localities. These included 35 *T. ivorensis* trees, 50 *T. superba* and 30 *T. mantaly* trees. Isolates of Botryosphaeriaceae were obtained from 55 of the 110 trees sampled. In total, 43 isolates were obtained from 37 *T. ivorensis* trees, 20 isolates from seven *T. superba* trees and 27 isolates from 11 *T. mantaly* trees. No sign of disease, caused by fungi in the Botryosphaeriaceae, was observed on any trees at the time of collection. It was thus assumed that all isolates were from healthy trees.

The isolates obtained were assigned to two groups based on colony and conidial morphology. The majority of isolates collected (82 isolates) produced aerial mycelium which was white at first, turning dark grey-green or grey after four to five days at 25 °C under near UV-light. These isolates produced thick-walled, hyaline conidia that turned dark with age (Figure 1). The conidia were aseptate when young, becoming uniseptate with age. Conidia were ovoid in shape and some developed longitudinal striations as they aged. These isolates were identified as belonging to species of *Lasiodiplodia* based on their conidial morphology. The second group of isolates (eight isolates) were characterized by dark grey or green to black mycelium, producing small dark brown conidia (Figure 1) and resembling species of *Endomelanconiopsis* (Rojas et al. 2008).

No sexual fruiting structures were produced on pine needles by any of the isolates from *Terminalia* spp. in Cameroon. The Botryosphaeriaceae occurring on *Terminalia* spp. in Cameroon were compared to similar species described in previous studies (Table 3). Isolates from the *Lasiodiplodia* group were found at all the localities sampled and from all three host species. Isolates residing in the second group were found only in three locations (Belabo, Nkoemvone and Mbalmayo) and only on *T. ivorensis* and *T. superba*.

3.2. DNA extraction and PCR amplification

A total of 55 isolates, each originating from a single *Terminalia* tree, were selected for sequencing of their ITS and 5.8S rDNA regions to obtain a broad indication of their identities and to select isolates for the data sets used in the final analyses. These were comprised of 51 isolates from the morphological group resembling *Lasiodiplodia* and four from the group resembling *Endomelanconiopsis.* Based on results of the ITS sequences, fourteen isolates were selected for sequencing of the *tef 1-α* gene region and considered in the final analyses. PCR fragments for the ITS and 5.8S gene regions were ~ 580 bp in size, and ~ 710 bp for the *tef 1-α* gene region, The *tef 1-α* sequences were larger than those retrieved from GenBank, which spanned 244 to 500 bp, and only the corresponding regions were used in the phylogenetic analyses.

3.3. Phylogenetic analyses

A BLAST search against the GenBank database, using ITS sequences obtained from *Terminalia* spp. in Cameroon, showed that isolates resembling species of *Lasiodiplodia* were most closely related to *L. theobromae* (Pat.) Griff. & Maubl. and *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous. Isolates from the second group, with small dark brown conidia, were identified as *Endomelanconiopsis endophytica* Rojas & Samuels.

ITS phylogeny. The ITS dataset comprised 91 sequences of which 55 were for isolates from *Terminalia* spp. and 36 sequences were retrieved from GenBank. After alignment, the ITS sequence data set consisted of 575 characters of which 313 were constant, 112 were parsimony uninformative and 150 were parsimony informative. The MP analyses generated 100 trees with identical topologies with respect to the major clades (TL = 563, CI = 0.627, RI = 0.868, RC = 0.544).

Four main clades including isolates from *Terminalia* spp. in Cameroon emerged from the MP analyses of the ITS gene region. These clades represented the two genera *Lasiodiplodia* [Bootstrap support (BS) = 100 % and Bayesian posterior probabilities (BPP) = 0.99] and *Endomelanconiopsis* (BS = 100 % and BPP = 1) (Figure 2). In the *Endomelanconiopsis* group, except for two isolates where very little divergence (two to three base pairs) was observed, sequences from *Terminalia* spp. in Cameroon were identical to *E. endophytica* and

clustered with isolates from South America. The *Lasiodiplodia* group included most of the isolates obtained in this study and it was subdivided into three sub-clades with no significant Bootstrap support. The first sub-clade (20 isolates) consisted of isolates grouping with *L. theobromae*. Except for two isolates, no sequence variation was detected between isolates in this clade. The second sub-clade (25 isolates) accommodated isolates clustering with *L. pseudotheobromae*. Small sequence variations were observed in a few isolates of this group. The third group, consisting of five isolates from Cameroon was not clearly resolved and clustered close to *L. mahajangana* Begoude, Jol. Roux, Slippers. and *L. parva* A.J.L. Phillips, A. Alves & Crous. No statistical support was observed for any of these sub-clades. For this reason, representative isolates from the *Endomelaconiopsis* clade and the three sub-clades in the *Lasiodiplodia* group were selected for *tef 1-α* gene region sequencing.

Combined ITS and tef 1-α analyses. The partition homogeneity test indicated congruence between the ITS and *tef 1-α* partitions (P = 0.355) suggesting that the data sets could be combined. The combined dataset consisted of 48 isolates with 887 characters of which, 377 were constant, 146 were parsimony uninformative and 364 were parsimony informative. Gaps were treated as a fifth character. After heuristic searches, 42 most parsimonious trees were obtained (TL = 1068; CI = 0.738, RI = 0.914, RC = 0.674; TreeBase Accession No. SN4630) and one of them was chosen for representation (Figure 3). All 42 trees displayed the same topology with regard to the identified clades. The topology of the tree generated from the combined analyses with MP, as well as with the 50 % majority rule consensus tree from the trees obtained through Bayesian analysis, was congruent with the trees obtained with the individual analyses of ITS and *tef 1-α*, identifying the same clades.

All the isolates collected in this study grouped with previously described species of *Lasiodiplodia* and *Endomelanconiopsis*, strongly supported with Bootstrap and Bayesian posterior probability values (Figure 3). Similar to results obtained for the ITS gene region, isolates from Cameroon could be identified as *L. theobromae* (BS = 100 %, BPP = 1), *L. pseudotheobromae* (BS = 100 %; BPP = 1). The third group of *Lasiodiplodia* isolates clustered with *L. parva* (BS = 97 %; BPP = 1), but one base pair difference in the *tef 1-α* sequences was noticed among isolates in this later group. The fourth group of isolates consisted of *E. endophytica* from *Terminalia* spp. in Cameroon which formed a well supported clade (BS = 100 % and BPP = 1) with sequences from authentic isolates of this species from GenBank (Figure 3).

Isolates of Botryosphaeriaceae found on *Terminalia* spp. that were phylogenetically related to *L. parva* based on ITS and *tef 1-α* sequence comparisons, mostly conformed to previous morphological description of *L. parva*. However, important differences in conidial sizes were observed for isolates from Cameroon (Table 3), raising the question as to whether they represent a different and unique species. DNA sequence data for the ITS and *tef 1-α* gene regions, however, did not support the description of a discrete species for these isolates. Further sequences from additional gene regions (*β*-tubulin, LSU and SSU) not reported in this paper were found to be identical with those of original species of *L. parva* and, therefore, suggested that all these isolates represent the same species.

3.4. Pathogenicity

Pathogenicity trials conducted on *T. mantaly* using isolates of the Botryosphaeriaceae collected in this study revealed visible lesions within six weeks after inoculation (Figure 4). Trees inoculated with sterile MEA also produced small lesions that represented only wound reactions as no Botryosphaeriaceae could be isolated from them. All the isolates of Botryosphaeriaceae were successfully re-isolated from the lesions emerging from inoculations. Analysis of variance showed that the mean lengths of lesions produced by all of the isolates on *T. mantaly* differed significantly ($P < 0.0001$) from the controls (Figure 4). *L. pseudotheobromae* produced the longest lesions followed by *L. theobromae, L. parva* and *E. endophytica*.

On *T. catappa* trees, all isolates collected from *Terminalia* trees in Cameroon produced lesions significantly longer than those of the control inoculations (Figure 5). Similar to the situation on *T. mantaly*, control inoculations showed only small lesions. However, re-isolations did not yield any Botryosphaeriaceae from the controls, whereas the original Botryosphaeriaceae were re-isolated from all the trees inoculated with fungal cultures. Analysis of variance indicated that lesion lengths produced on the cambium by all the isolates were significantly different ($P < 0.0001$) to those associated with the controls (Figure 5). Isolates representing *L. pseudotheobromae* were most virulent and produced longer lesions than *L. theobromae* and *L. parva*. *E. endophytica* produced substantially smaller lesions than either *L. pseudotheobromae* or *L. theobromae*.

A positive correlation (R^2 = 77 %) was found between inoculations on *T. mantaly* and *T. catappa.* On both tree species, *L. pseudotheobromae* was most virulent. In general, the lesions observed in *T. catappa* were longer than those of *T. mantaly* for *L. pseudotheobromae* and *L. theobromae* isolates. In contrast, the lengths of lesions produced by isolates of *L. parva* and *E. endophytica* on *T. catappa* were smaller than those observed on *T. mantaly.* However, this difference in susceptibility between *T. catappa* and *T. mantaly* was not statistically significant.

4. DISCUSSION

This study represents the first attempt to identify the Botryosphaeriaceae on native *Terminalia* trees in Africa. Four species of the Botryosphaeriaceae were collected from *T. ivorensis* and *T. superba*, and three species were found on samples from the non-native *T. mantaly*. A combination of morphological characteristics and DNA sequence comparisons was used to identify these species as *L. theobromae, L. pseudotheobromae, L. parva* and *E. endophytica.* These fungi are reported on these hosts for the first time. Except for *E. endophytica,* which was isolated only from *T. superba* and *T. ivorensis*, *L. pseudotheobromae, L. theobromae* and *L. parva* were collected from all the tree species sampled in this study.

The majority of isolates obtained in this study represented species of *Lasiodiplodia* of which isolates were identified as *L. theobromae, L. pseudotheobromae* and *L. parva* based on sequence data of the ITS and *tef 1-α* gene regions. Until recently, most *Lasiodiplodia* spp. from tropical trees were treated as *L. theobromae* (Punithalingam 1976). However, application of DNA sequence comparisons for the ITS and *tef 1-α* gene regions has resulted in the description of 10 new *Lasiodiplodia* spp. (Pavlic et al. 2004; Burgess et al. 2006; Damm et al. 2007; Alves et al. 2008; Begoude et al. 2009; Pavlic et al. 2008). These species share similar morphological characteristics, such as slowly maturing conidia with thick walls that turn dark with age and develop longitudinal striations.

Lasiodiplodia theobromae has a wide geographic distribution and it has been found on more than 500 forest and agricultural plant species in tropical and subtropical areas (Punithalingam 1980). It is well known as an endophyte on healthy tropical trees (Suryanarayanan et al. 2002; Begoude et al. 2009). Furthermore, *L. theobromae* can act as a latent pathogen causing disease symptoms after onset of conditions unfavourable for the host (Schoeneweiss 1981;

Mullen et al. 1991; Slippers and Wingfield 2007). *L. theobromae* has previously been reported as an endophyte in the inner bark and twigs of healthy *T. arjuna* (Tejesvi et al. 2005), leaves of *T. tomentosa* and *T. bellerica* (Suryanarayanan et al. 2002) and the twigs and bark of healthy *T. catappa* (Begoude et al. 2009) in the tropics. On *Terminalia* spp., *L. theobromae* has mostly been recorded as the causal agent of blue stain of logs, soon after felling (Groulez and Wood 1985; Lamb and Ntima 1971; Apetorgbor et al. 2004). However, in Cameroon, *L. theobromae* is best known as the cause of die-back of cacao (*Theobromae cacao*) (Mbenoun et al. 2008). In the current study, *L. theobromae* was the second most abundant species identified on *Terminalia* spp. All isolates collected were from healthy trees, but pathogenicity trials on young *T. catappa* and *T. mantaly* showed that it is highly pathogenic to these trees. Pathogenicity tests on *T. ivorensis* and *T. superba* should, however, be conducted to determine whether it can cause disease on these important native trees.

Lasiodiplodia pseudotheobromae was the most commonly collected species of Botryosphaeriaceae, collected from all the species of *Terminalia* sampled in this study. This fungus was originally described from *Rosa* sp. in the Netherlands, *Gmelina arborea* and *Acacia mangium* in Costa Rica, *Coffea* sp. in Democratic Republic of Congo and *Citrus aurantium* in Suriname (Alves et al. 2008). In a recent study investigating the Botryosphaeriaceae on *T. catappa* in Cameroon, South Africa and Madagascar (Begoude et al. 2009), *L. pseudotheobromae* was also the most abundant species found in all the sampled areas. The information generated in the current study, which is supported by a previous one on *T. catappa*, suggests that *L. pseudotheobromae* has a worldwide distribution. In pathogenicity trials *L. pseudotheobromae* was found to be the most virulent species tested. This was also the case in a study of *T. catappa* (Begoude et al. 2009). *L. pseudotheobromae* is, therefore, the most likely species of Botryosphaeriaceae to cause health problems on *Terminalia* trees in Africa where they are subjected to stressful conditions.

Lasiodiplodia parva was only recently described and was previously treated as *L. theobromae*, together with *L. pseudotheobromae* (Alves et al. 2008). Isolates collected from *Terminalia* spp. in this study, however, differed in their conidial sizes from descriptions for the type specimen. The conidia of isolates from Cameroon were larger than those previously described for *L. parva*. DNA sequence data for both ITS and *tef 1-α, β*-tubulin, LSU and SSU, however, confirmed that isolates from Cameroon represent *L. parva*, despite minor differences for two nucleotides in ITS sequences and a single nucleotide in *tef 1-α* sequences.

Our results thus show that some isolates of *L. parva* can produce conidia as large as those produced by other closely related species, such as *L. pseudotheobromae* and *L. theobromae*. This emphasizes the importance of considering multiple criteria for species identification when treating species of the Botryosphaeriaceae.

Prior to this study, *L. parva* was known only to occur in agricultural field soil and crops in Latin America (Alves et al. 2008). Although *L. parva* was the least abundant *Lasiodiplodia* sp. isolated from *Terminalia* spp., its occurrence on these trees in Cameroon has substantially broadened its host range and geographic distribution. Previously, the only plant host from which *L. parva* was known was *Theobroma cacao* in Colombia (Alves et al. 2008) and no information concerning its pathogenicity to this tree is available. In the current study, assessment of its pathogenicity on *T. mantaly* and *T. catappa* trees showed that *L. parva* consistently produced lesions on both hosts. However, in comparison to *L. theobromae* and *L. pseudotheobromae*, *L. parva* was only mildly pathogenic, suggesting that this fungus is unlikely to emerge as an important pathogen on these trees.

Endomelanconiopsis endophytica is a recently described species found as an endophyte in leaves of *T. cacao* and associated native woody hosts in the same environment (Rojas et al. 2008). Isolates of *E. endophytica* found in the present study were shown to group with the South American isolates of the fungus. The Cameroonian isolates were obtained from *T. ivorensis* and *T. superba*. These tree species are commonly used in cacao farms to establish a "taungya" agri-sylvicultural system in which they provide shade or improve soil fertility (Norgrove and Hauser 2002). It would not, therefore, be surprising to obtain further isolates of this fungus on hosts such as cocoa trees in Cameroon. The collection of *E. endophytica* from native *Terminalia* spp. in Cameroon adds to previous records of the fungus from plants in South America (Rojas et al. 2008). Even though very few isolates representing *E. endophytica* were found in this study, its presence on tropical species of *Terminalia* is particularly interesting as this could indicate a possible tropical origin of the fungus.

Two distinct genera of Botryosphaeriaceae, *Lasiodiplodia* and *Endomelanconiopsis,* were found associated with species of *Terminalia* in Cameroon. Although little information related to the ecology of the genus *Endomelanconiopsis* is available, both *Lasiodiplodia* and *Endomelanconiopsis* appear to be tropical species. Apart from *E. endophytica,* which was isolated only from *T. superba* and *T. ivorensis*, no evidence of host specialization was

observed for species of *Lasiodiplodia* identified in this study. This is characteristic of many species of Botryosphaeriaceae (Slippers and Wingfield, 2007) and contributes to their potential to cause diseases on trees. Although this study focussed exclusively on healthy tree tissue, the common occurrence of generalist species such as *L. theobromae* and *L. pseudotheobromae* which are reputed virulent pathogens on a wide range of hosts (Punithalingam 1980; Slippers and Wingfield 2007; Begoude et al. 2009) suggests that they could be pathogens if unfavourable conditions for the host occur.

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Table 1. Locations and characteristics of sites from where *Terminalia* trees were sampled.

Site		**GPS coordinates**	**Tree species sampled**
Region	Locality		
Central	Mbalmayo	N3 26.034 E11 29.344	*T. superba, T. ivorensis*
South	Nkoemvone	N2 49.045 E11 07.577	*T. superba*
	Kribi	N2 58.064 E9 54.904	*T. mantaly*
Eastern	Belabo	N4 57.376 E13 19.433	*T. superba, T. ivorensis, T. mantaly*

Table 2. Botryosphaeriaceae used in phylogenetic analyses in this study.

Species	Culture number	Origin	Host	Collectors	Genbank Accession No.				
					ITS	*tef* 1-α	*β-tub*	LSU	SSU
Botryosphaeria dothidea	CMW7999	Switzerland	*Ostrya* sp.	B. Slippers	AY236948	AY236897			
	CMW8000	Switzerland	*Prunus* sp.	B. Slippers	AY236949	AY236898			
Diplodia mutila	CBS112553	Portugal	*Vitis vinifera*	A.J.L. Phillips	AY259093				
	CBS230.30	USA	*P. dactylifera*	L.L. Huilllier	DQ458886				
Diplodia. seriata	CMW7774	USA	*Ribes* sp.	B. Slippers/G.Hudler	EF445343	EF445382			
	CMW7775	USA	*Ribes* sp	B. Slippers/G.Hudler	EF445344	EF445383			
Endomelanconiopsis endophytica	**CMW28618**	Cameroon	*Terminalia ivorensis*	D. Begoude	GQ469966	GQ469906			
	CMW28551	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469967	GQ469907			
	CMW28552	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469968	GQ569908			
	CMW28563	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469965				
	CBS120397	Panama	*Theobroma cacao*	E. Rojas/L.Mejia/Z. Maynard	EU633656	EU683637			
	CBS122546	Panama	*T. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683661	EU683642			
	CBS122550	Panama	*T. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683664	EU683645			
Endomelanconiopsis microspora	CBS353.97	Panama	Soil	E. Rojas/L.Mejia/Z. Maynard	EU683655	EU683636			
Guignardia mangiferae	1095	Panama	*T. cacao*	E. Rojas/L.Mejia/Z. Maynard	EU683671	EU683652			
Lasiodiplodia crassispora	WAC12533	Venezuela	*Eucalyptusurophylla*	S. Mohali	DQ103552	DQ10355			
	WAC12534	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103550	DQ103557			
	WAC12535	Australia	*S. album*	T.I. Burgess/B. Dell	DQ103551	DQ103558			

Lasiodiplodia gonubiensis	CBS115812	South Africa	*S. cordatum*	D. Pavlic	DQ458892	DQ458877			
	CBS116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567			
Lasiodiplodia mahajangana	CMW27801	Madagascar	*T. catappa*	J. Roux	FJ900595	FJ900641			
	CMW27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642			
	CMW27820	Madagascar	*T. catappa*	J. Roux	FJ900597	FJ900643			
Lasiodiplodia margaritacea	CMW26162	Australia	*A. gibbosa*	D. Pavlic	EU144050	EU144065			
	CMW26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066			
Lasiodiplodia parva	CBS356.59	Sri Lanka	*T. cacao*	A. Riggenbach	EF622082	EF622062			
	CBS494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064			
	CMW28333	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469961	GQ469903	GQ469892	GQ469909	GQ469912
	CMW28309	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469962	GQ469904	GQ469894	GQ469911	GQ469914
	CMW28292	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469963	GQ469905	GQ469893	GQ469910	GQ469913
	CMW28295	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469964				
	CMW28628	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469960				
Lasiodiplodia plurivora	STEU-5803	South Africa	*P. salicina*	U.Damm	EF445362	EF445395			
	STEU-4583	South Africa	*V. vinifera*	F.Halleen	AY343482	EF445396			
Lasiodiplodia pseudotheobromae	**CMW28297**	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469937	GQ469899			
	CMW28300	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469939	GQ469900			
	CMW28574	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469947	GQ469901			
	CMW28624	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469956	GQ469902			
	CMW28328	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469935				
	CMW28330	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469936				
	CMW28299	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469938				
	CMW28301	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469940				
	CMW28332	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469941				
	CMW28566	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469942				
	CMW28314	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469943				
	CMW28568	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469944				
	CMW28569	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469945				
	CMW28633	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469946				
	CMW28632	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469948				

	CMW28310	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469949	
	CMW28557	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469950	
	CMW28558	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469951	
	CMW28313	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469952	
	CMW28560	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469953	
	CMW28561	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469954	
	CMW28562	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469955	
	CMW28298	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469957	
	CMW28627	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469958	
	CMW28622	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469959	
	CBS116459	Costa Rica	*Gmelinea arborea*	J.Carranza/Velásquez	EF622077	EF622057
	CBS447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060
Lasiodiplodia rubropupurea	WAC12535	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103553	DQ103571
	WAC12536	Australia	*E. grandis*	T.I. Burgess/G.Pegg	DQ103554	DQ103572
Lasiodiplodia theobromae	**CMW28550**	Cameroon	*T. mantaly*	D. Begoude/ J.Roux	GQ469921	GQ469895
	CMW28570	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469923	GQ469896
	CMW26571	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469924	GQ469897
	CMW27311	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469932	GQ469898
	CMW28326	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469915	
	CMW28327	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469916	
	CMW28329	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469918	
	CMW28547	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469919	
	CMW28548	Cameroon	*T. mantaly*	D. Begoude/J. Roux	GQ469920	
	CMW28573	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469925	
	CMW28575	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469926	
	CMW28308	Cameroon	*T. superba*	D. Begoude/J. Roux	GQ469927	
	CMW28312	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469928	
	CMW28554	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469929	
	CMW28555	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469930	
	CMW28556	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469931	
	CMW28625	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469933	
	CMW28626	Cameroon	*T. ivorensis*	D. Begoude/J. Roux	GQ469934	
	CMW9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054
	CBS164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258

Lasiodiplodia venezuelensis	WAC12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568
	WAC12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569
Neofusicoccum parvum	CMW9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888
	CMW9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885
Neofusicoccum ribis	CMW7772	USA	*Ribes* sp.	B. Slippers/G.Hudler	AY236935	AY236877
	CMW7773	USA	*Ribes* sp.	B. Slippers/G Hudler	AY236936	AY236878

Table 3. Conidial dimensions of the Botryosphaeriaceae from *Terminalia* spp. and comparison with those reported in previous studies.

Species	**Conidial size (µm)**		**Source of data**
	This study	**Previous studies**	
L. pseudotheobromae	(20.5-)23.5-27.5(-31.5) x (10.5-)12.0-14.0(-16.5)	(22.5-)23.5-32(-33) x (13.3-)14-18(-20)	Alves *et al.* 2008.
L. theobromae	(17.5-)21.5-27.5(-31.0) x (10.5-)12.0-14.0(-16.5)	(19-)21-31(-32.5) x (12-)13-15.5(-18.5)	Alves *et al.* 2008.
L. parva	(24.5-)26.5-29.5(-33.5) x (11.0-)12-14.5(-17.5)	(15.5-)16-23.5(-24.5) x (10-)10.5-13(-14.5)	Alves *et al.* 2008
E. endophytica	(5.5-)6.0-7.5(-8) x (3.0-)3.5-4.0(-4.5)	(4.7-)5.5-7.5(-10.0) x (3.0-)3.5-4.5(-6.2)	Rojas *et al.* 2008

Figure 1. Conidial morphology of species of the Botryosphaeriaceae from *Terminalia* spp. (a) young hyaline thick-walled conidia of *Lasiodiplodia theobromae,* (b) *L. pseudotheobromae,* (c) *L. parva* (d) dark brown conidia of *E. endophytica.* Bars: a, b, c, d = 10 µm.

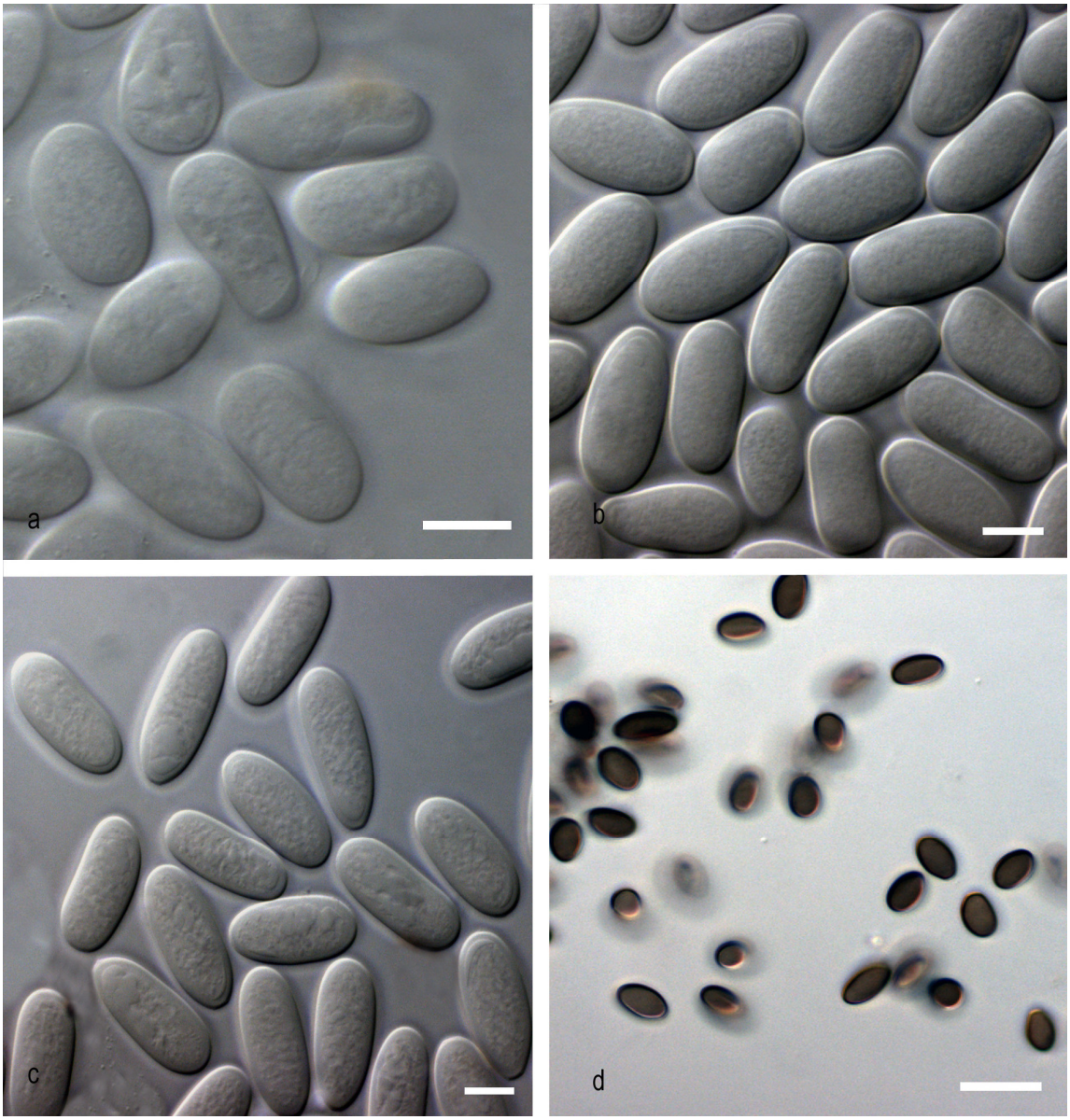

a
b
c
d

Figure 2. One of the most parsimonious trees obtained from analyses of the ITS sequence data of the Botryosphaeriaceae from *Terminalia* spp. Bootstrap support (%) followed by Posterior probabilities from 1000 replications are given on the branches (BS/ PP). Isolates marked in bold represent those obtained from *Terminalia* spp. in Cameroon. Isolates marked in grey were selected for *tef 1-α* sequencing.

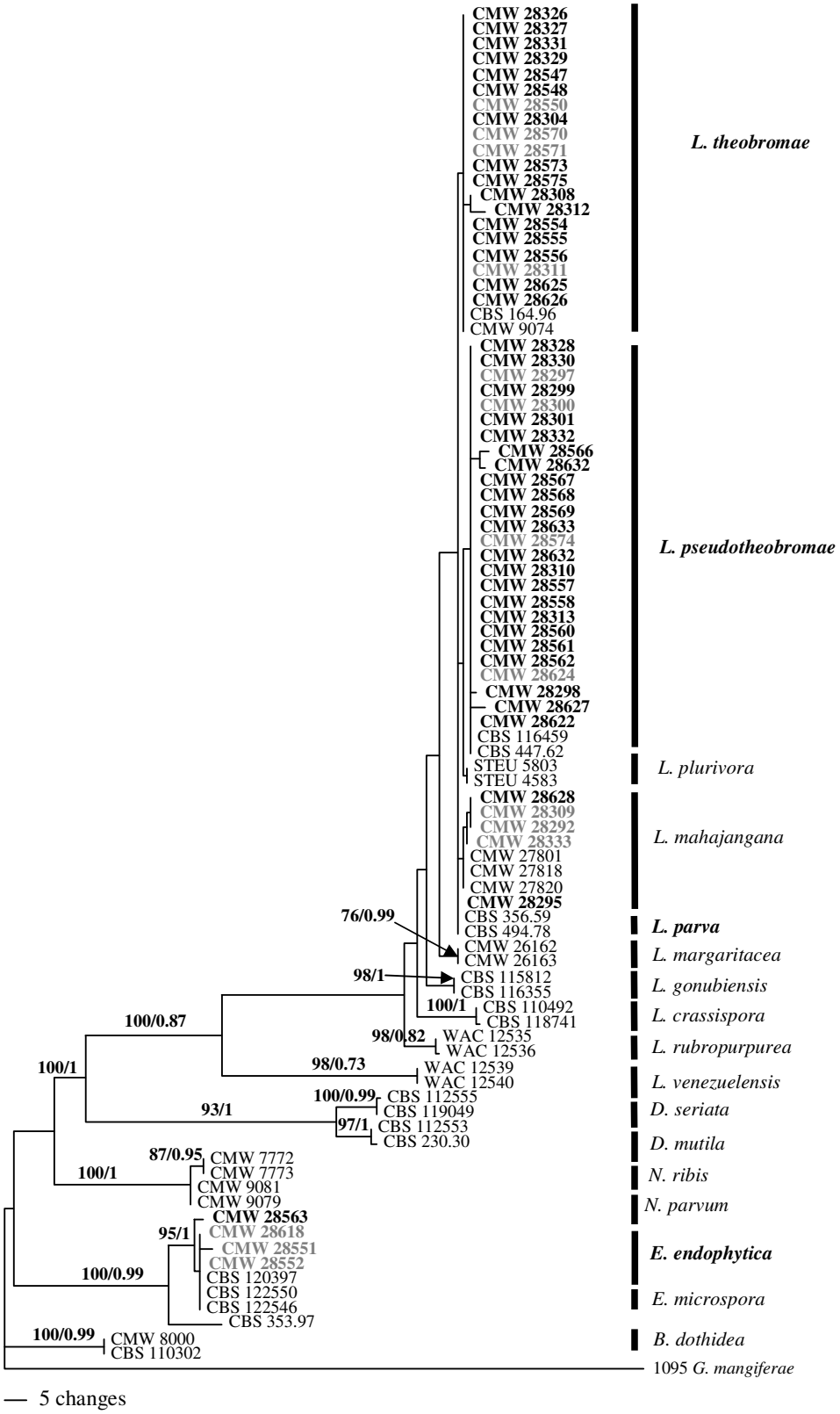

CMW 28326
CMW 28327
CMW 28331
CMW 28329
CMW 28547
CMW 28548
CMW 28550
CMW 28304
CMW 28570
CMW 28571
CMW 28573
CMW 28575
CMW 28308
CMW 28312
CMW 28554
CMW 28555
CMW 28556
CMW 28311
CMW 28625
CMW 28626
CBS 164.96
CMW 9074
L. theobromae
CMW 28328
CMW 28330
CMW 28297
CMW 28299
CMW 28300
CMW 28301
CMW 28332
CMW 28566
CMW 28632
CMW 28567
CMW 28568
CMW 28569
CMW 28633
CMW 28574
CMW 28632
CMW 28310
CMW 28557
CMW 28558
CMW 28313
CMW 28560
CMW 28561
CMW 28562
CMW 28624
CMW 28298
CMW 28627
CMW 28622
CBS 116459
CBS 447.62
L. pseudotheobromae
STEU 5803
STEU 4583
L. plurivora
CMW 28628
CMW 28309
CMW 28292
CMW 28333
CMW 27801
CMW 27818
CMW 27820
L. mahajangana
CMW 28295
CBS 356.59
CBS 494.78
L. parva
CMW 26162
CMW 26163
L. margaritacea
CBS 115812
CBS 116355
L. gonubiensis
CBS 110492
CBS 118741
L. crassispora
WAC 12535
WAC 12536
L. rubropurpurea
WAC 12539
WAC 12540
L. venezuelensis
CBS 112555
CBS 119049
D. seriata
CBS 112553
CBS 230.30
D. mutila
CMW 7772
CMW 7773
CMW 9081
CMW 9079
N. ribis
N. parvum
CMW 28563
CMW 28618
CMW 28551
CMW 28552
CBS 120397
CBS 122550
CBS 122546
CBS 353.97
E. endophytica
E. microspora
CMW 8000
CBS 110302
B. dothidea
1095 G. mangiferae
76/0.99
98/1
100/1
100/0.87
98/0.82
98/0.73
100/1
100/0.99
93/1
97/1
87/0.95
100/1
95/1
100/0.99
100/0.99
— 5 changes

Figure 3. One of the most parsimonious trees obtained from analyses of the combined ITS and *tef 1-α* sequence data of the Botryosphaeriaceae from *Terminalia* spp. Bootstrap support (%) followed by Posterior probabilities from 1000 replications are given on the branches (BS/ PP). Isolates marked in bold represent those obtained from *Terminalia* spp. in Cameroon.

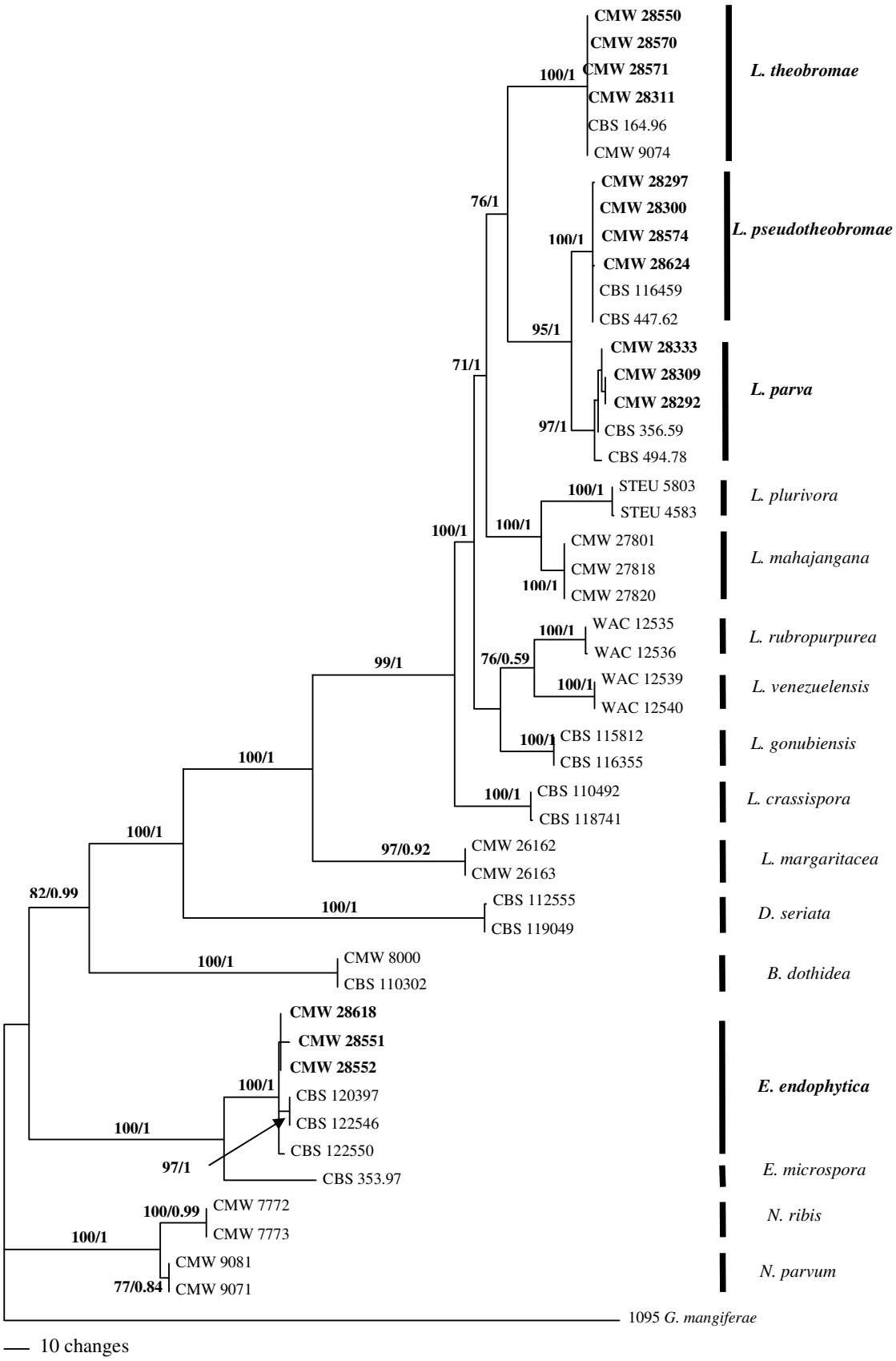

CMW 28550
CMW 28570
CMW 28571
CMW 28311
CBS 164.96
CMW 9074
L. theobromae
CMW 28297
CMW 28300
CMW 28574
CMW 28624
CBS 116459
CBS 447.62
L. pseudotheobromae
CMW 28333
CMW 28309
CMW 28292
CBS 356.59
CBS 494.78
L. parva
STEU 5803
STEU 4583
L. plurivora
CMW 27801
CMW 27818
CMW 27820
L. mahajangana
WAC 12535
WAC 12536
L. rubropurpurea
WAC 12539
WAC 12540
L. venezuelensis
CBS 115812
CBS 116355
L. gonubiensis
CBS 110492
CBS 118741
L. crassispora
CMW 26162
CMW 26163
L. margaritacea
CBS 112555
CBS 119049
D. seriata
CMW 8000
CBS 110302
B. dothidea
CMW 28618
CMW 28551
CMW 28552
CBS 120397
CBS 122546
CBS 122550
E. endophytica
CBS 353.97
E. microspora
CMW 7772
CMW 7773
N. ribis
CMW 9081
CMW 9071
N. parvum
1095 G. mangiferae
10 changes

Figure 4. Mean lesion lengths (mm) on cambium for each Botryosphaeriaceae isolate six weeks after inoculation on *T. mantaly* ($P < 0.0001$). *L. pseudotheobromae* (LPs), *L. theobromae* (LT), *L. parva* (LP), *E. endophytica* (EE), Control.

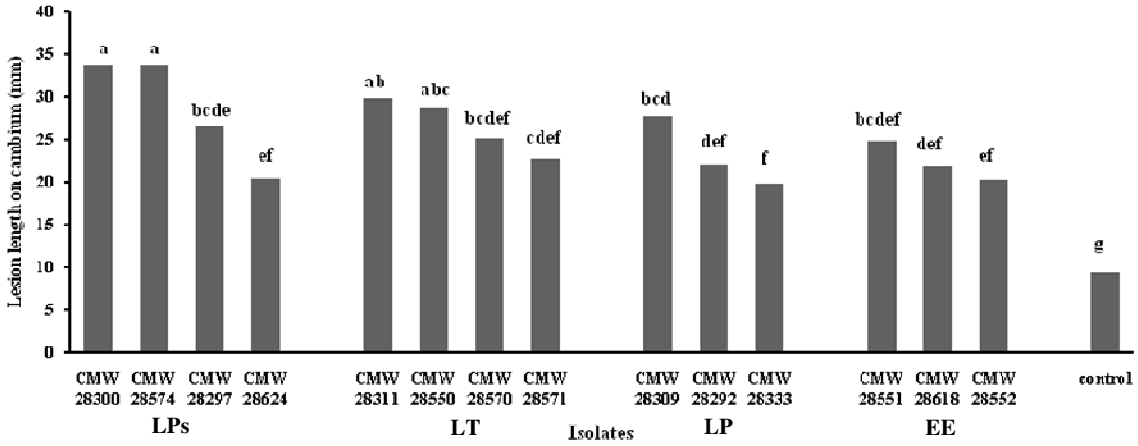
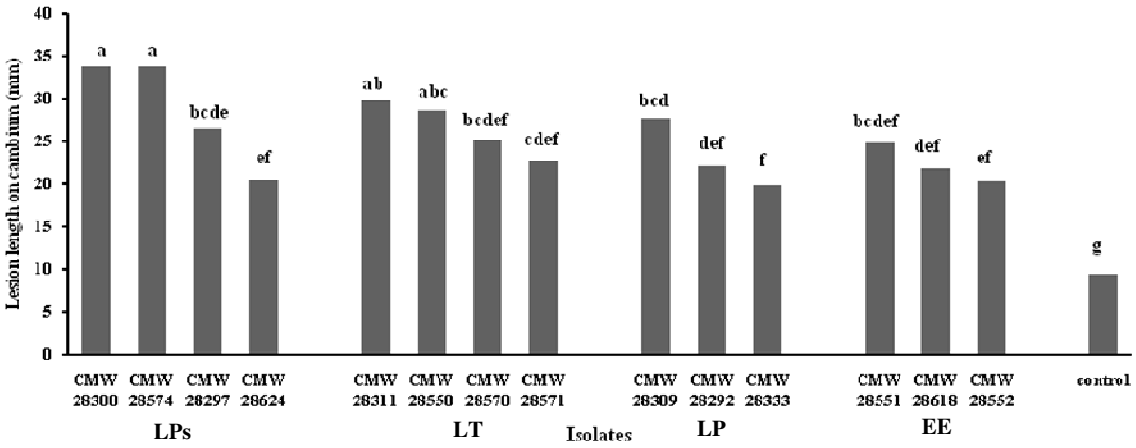

Lesion length on cambium (mm)
40
35
30
25
20
15
10
5
0
a
a
bcde
ef
ab
abc
bcdef
cdef
bcd
def
f
bcdef
def
ef
g
CMW 28300
CMW 28574
CMW 28297
CMW 28624
CMW 28311
CMW 28550
CMW 28570
CMW 28571
CMW 28309
CMW 28292
CMW 28333
CMW 28551
CMW 28618
CMW 28552
control
LPs
LT
LP
EE
Isolates

Figure 5. Mean lesion lengths (mm) on cambium for each Botryosphaeriaceae isolate six weeks after inoculation on *T. catappa* ($P < 0.0001$). *L. pseudotheobromae* (LPs), *L. theobromae* (LT), *L. parva* (LP), *E. endophytica* (EE), Control.


Lesion length on cambium (mm)
40
35
30
25
20
15
10
5
0
a
a
bcde
ef
ab
abc
bcdef
cdef
bcd
def
f
bcdef
def
ef
g
CMW 28300
CMW 28574
CMW 28297
CMW 28624
CMW 28311
CMW 28550
CMW 28570
CMW 28571
CMW 28309
CMW 28292
CMW 28333
CMW 28551
CMW 28618
CMW 28552
control
LPs
LT
Isolates
LP
EE

Chapter 4

Phenotypic and molecular characterization of the *Botryosphaeriaceae* associated with native *Terminalia* spp. in Southern Africa

ABSTRACT

As part of a broad investigation considering fungal host jumps between native and non-native trees in Africa, this study considers the diversity of Botryosphaeriaceae from healthy native *Terminalia* spp. in Southern Africa. A combination of morphological characteristics, DNA sequence data for the ITS, *tef 1-α* and *β-tub* gene regions were used to identify species. From a total of 135 isolates obtained from 232 *T. sericea* and *T. sambesiaca* trees sampled in Southern Africa, nine species were identified. These included seven known species, *Lasiodiplodia crassispora, L. pseudotheobromae, Diplodia alatafructa, Pseudofusicoccum olivaceum, Neofusicoccum parvum, N. kwambonambiense* and *N. vitifusiforme,* as well as two taxa described here as *L. cryptotheobromae* sp. nov. and *N. terminaliae* sp. nov. Other than *L. pseudotheobromae* and *N. parvum,* all are recorded on *Terminalia* spp. for the first time. Inoculation trials on branches of healthy *T. sericea* trees showed that all species are capable of causing lesions on these trees. *N. vitifusiforme, D. alatafructa* and *P. olivaceum* were mildly pathogenic and *L. pseudotheobromae* was the most virulent.

1. INTRODUCTION

The flora of Southern Africa is remarkably diverse with ~1700 indigenous tree species belonging to more than 90 families, including the Combretaceae (Palmer and Pitman 1972; Pooley 1999). The Combretaceae includes 20 genera and about 475 species (Thiombiano *et al.* 2006). Of these about 200 belong to the genus *Terminalia,* making it the second largest genus in the family (McGaw *et al.* 2001). At least 13 indigenous species of *Terminalia* are found in Southern Africa, of which the most abundant are *T. sericea* Burch. ex DC., *T. prunoïdes* Lawson. and *T. sambesiaca* Engl. & Diels (Coastes-Palgrave 1988). In Southern Africa, species of *Terminalia* are small shrubs to medium sized trees, found in open woodlands and wooded savannahs (Dale and Greenway 1961; Carr 1994). Indigenous species of *Terminalia* provide various benefits such as food for animals, improved soil fertility, control of soil erosion, wood for various social activities, fodder and shelter for livestock, human and veterinary medicines (Schmidt *et al*. 2002; Smith *et al.* 2004; Singh *et al*. 2002; Masoko *et al*. 2005; Katjiua and Ward 2006). A number of *Terminalia* spp. are also grown in plantations for commercial purposes, especially in Central and Eastern Africa (Groulez and Wood 1985; Lamb and Ntima 1971).

In Southern Africa, very few indigenous tree species are suitable for timber and pulp production (Immelman *et al.* 1973). For this reason non-native tree species have been introduced in many countries to meet the domestic needs for wood and simultaneously to relieve pressure on native forests (Immelman *et al.* 1973). Consequently, increasing land areas are being afforested and plantations of non-native tree species are reshaping the natural vegetation. Strips of native trees, such as *Terminalia* spp., thus occur adjacent to, and intermixed, with trees such as *Eucalyptus* or *Pinus* spp., introduced for commercial forestry. Many introduced, commercially propagated trees are related to native woody plants and there is growing evidence that pathogens are able to move between them (Wingfield 2003; Slippers *et al.* 2005; Slippers and Wingfield 2007). Knowledge of potentially pathogenic fungi on native trees contributes to assessments of the vulnerability of both native forests and plantations of non-native trees.

Diseases affecting forest tree species are of increasing concern to countries in the Southern African region. Both native pathogens that have undergone host shifts (Slippers *et al.* 2005)

from indigenous trees as well as non-native pathogens that have been accidentally introduced with germplasm of non-indigenous tree species are responsible for these diseases (Wingfield 2003; Wingfield *et al.* 2008). As forest plantations commonly adjoin native communities of trees, this close association can constitute a risk as both plant communities can act as sources of pests and pathogens (Slippers *et al.* 2005; Strauss 2001). For example, *Phytophthora cinnamomi* Rands., an important pathogen of *Eucalyptus* spp. in Australia, has been introduced into South Africa, where it seriously affects native Proteaceae and *Ocotea bullata* (Birch) Baill trees (Von Broembsen & Kruger 1985; Linde *et al.* 1999). In contrast, *Ceratocystis albifundus* M. J. Wingf., De Beer & M. J. Morris, a native African fungus (Barnes *et al.* 2005; Roux *et al.* 2007), has spread from native tree species to plantation grown Australian *Acacia mearnsii* De Wild., resulting in disease and death of these economically important trees (Roux *et al*. 2007).

The Botryosphaeriaceae represents a cosmopolitan group of fungi with a wide host range. Members of this family include important canker and dieback pathogens of numerous tree species (Denman *et al.* 1999; 2000; Slippers and Wingfield 2007). These fungi have the ability to live in healthy plant organs as symptomless endophytes making their detection difficult (Smith *et al.* 1996; Swart *et al.* 2000). This facilitates their ability to spread into new environments where they can infect new hosts. A number of recent studies have shown the occurrence of species of Botryosphaeriaceae on both native and introduced hosts in Southern Africa. *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips. has, for example, been found commonly on non-native *Eucalyptus* spp. and *T. catappa* L. trees, and on native *Syzygium cordatum* Hochst (Slippers *et al.* 2004; Pavlic *et al.* 2007; Begoude *et al.* 2009a). The common occurrence and wide host range of *N. parvum* on native trees in South Africa has led to the suggestion that this fungus is native in southern Africa. This hypothesis is further supported by the fact that it causes disease on non-native *Eucalyptus* spp. and *T. catappa*, but not on native *S. cordatum* (Slippers *et al.* 2004; Pavlic *et al.* 2007; Begoude *et al.* 2009a;). In contrast, *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips. a native pathogen of *Eucalyptus* spp. in Australia has been found on both non-native *Eucalyptus* spp. and native *S. cordatum* in South Africa (Slippers *et al.* 2004; Pavlic *et al.* 2007; Maleme 2008).

Fungi belonging to the Botryosphaeriaceae constitute interesting model organisms to study the movement of fungal pathogens between native and introduced hosts. Not only can they be spread unnoticed, but increasing knowledge on the taxonomy and ecology of these fungi makes them ideal to study broader scale aspects of fungal movement and ecology. Following this view, the aim of the present study was to investigate the diversity of the Botryosphaeriaceae associated with native *Terminalia* spp. in Southern Africa. Isolates obtained in the study were characterized based on morphology, DNA sequence data and their pathogenicity to a *Terminalia* sp.

2. MATERIALS AND METHODS

2.1. Sample collection and fungal isolation

Plant material was collected in 2007 from native *T. sericea* and *T. sambesiaca* in Namibia, Botswana, South Africa and Tanzania (Figure 1). In each area, five to 20 trees were randomly chosen for sampling without considering either their size or age. A single healthy branch was collected from each tree, placed in a paper bag and transported to the laboratory for processing.

From each branch, two one centimetre long segments, were cut and split vertically. Samples were surface sterilized by immersing them in 96 % ethanol for 1 min, followed by 1 min in undiluted 3.5 % sodium hypochlorite and 1 min in 70 % ethanol, before rinsing in sterile distilled water and allowing them to dry under sterile conditions. The four disinfected branch pieces from each tree were plated on 2 % malt extract agar (MEA) (2 % malt extract, 1.5 % agar; Biolab, Midrand, Johannesburg, S.A.) supplemented with 1 mg ml^{-1} streptomycin (Sigma, St Louis, MO, USA) to suppress bacterial growth. The Petri dishes were sealed with Parafilm and incubated at 20 °C under continuous near-Ultra Violet (UV) light. One week later, filamentous fungi growing from the plant tissue and resembling the Botryosphaeriaceae were transferred to new Petri dishes containing fresh MEA.

All cultures used in this study were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. Representatives of all species were also deposited with the Centraalbureau voor

Schimmelcultures (CBS, Utrecht, Netherlands). Herbarium materials for previously undescribed species were deposited at the National Fungal Collection (PREM), Pretoria, South Africa.

2.2. Morphology and culture characteristics

Fungal isolates were grown in Petri plates containing 1.5 % water agar (Biolab, S.A.) overlaid with three pine needles that had been autoclaved twice, and incubated at 25 °C under near UV-light for two to six weeks to induce the formation of fruiting bodies. Morphological features of the resultant fruiting bodies were observed using a HRc Axiocam and accompanying Axiovision 3.1 camera (Carl Zeiss Ltd., München, Germany) and measurements of all relevant characters were made. These measurements are presented in descriptions as the extremes in brackets and the ranges calculated as the mean of the overall measurements plus or minus the standard deviation.

The morphology of fungal colonies growing on 2 % MEA at 25 °C under near UV-light for two weeks was described and colony colours (upper and reverse surfaces) of the isolates were recorded using the colour notations of Rayner (1970). Growth rates of cultures on 2 % MEA in the dark was determined at 5 °C temperature intervals from 10 to 35 °C. For growth rates, evaluations of five plates were used for each isolate at each temperature. Two measurements, perpendicular to each other, were made after three days for each plate resulting in 10 measurements for each isolate at each temperature. The experiment was repeated once.

2.3. DNA extraction, PCR reactions and DNA sequencing

Procedures and protocols for genomic DNA extraction and sequencing of representative isolates of the Botryosphaeriaceae were as described in Begoude *et al.* (2009a) using three gene regions. The entire Internal Transcribed Spacer region (ITS) of the nrDNA, including the 5.8S operon was amplified by PCR (polymerase chain reaction) using the primers ITS1 and ITS4 (White *et al*. 1990). A portion of the Translation Elongation Factor-1α (*tef 1-α*) gene was amplified using the primers EF1F and EF1R (Jacobs *et al*. 2004) and part of the β-tubulin (*β-tub*) gene region was amplified with primers Bt2a and Bt2b (Glass and Donaldson, 1995).

2.4. DNA Sequence Analyses

Sequences of the Botryosphaeriaceae generated in this study were edited using MEGA version 4 (Tamura *et al.* 2007). For the phylogenetic analyses, DNA sequences from this study, together with those retrieved from published sequences in GenBank (http://www.ncbi.nlm.gov) were aligned online using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) version 6 (Katoh *et al.* 2005). The aligned sequences were transferred to PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford 1998) where a final manual alignment was made.

A single gene phylogenetic analysis was done for the dataset from each locus, followed by a combined analysis of ITS and *tef 1-α* sequence data. Additional analyses were also done with combined data from the ITS, *tef 1-α* and *β-tub* for a cryptic species in the *Lasiodiplodia* clade. In the analyses, gaps were treated as fifth character and all characters were unordered and of equal weight. The phylogenetic analyses for all the datasets were performed using the maximum parsimony (MP) option, with trees generated by heuristic searches with random stepwise addition of 1000 replicates, tree bisection and reconnection (TBR) as branch swapping algorithms, and random taxon addition sequences for the construction of maximum parsimony trees. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. MAXTREES was set to auto-increase in all analyses. *Guignardia mangiferae* A.J. Roy was used as outgroup in analyses of ITS and *tef 1-α* data sets. The support for branches of the most parsimonious trees was assessed with a 1000 bootstrap replications (Felsenstein 1985). Other measures considered were the tree length (TL), consistency index (CI), rescaled consistency index (RC), and the retention index (RI) (Hillis and Huelsenbeck 1992). A partition homogeneity test (Farris *et al.* 1995) was conducted in PAUP to assess the possibility of combining the ITS and *tef 1- α* data sets in analyses of all the isolates. Incongruence Length Difference (Farris *et al.* 1995) was used in combined analyses for *Lasiodiplodia* isolates.

Both ITS and *tef 1-α* data sets were subjected to Bayesian phylogenetic analyses employing Markov Chain Monte Carlo (MCMC) implemented in MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001). MrModeltest version 2.2 (Nylander 2004) was used to estimate separately the model that best fit each of the partitions. The Likelihood settings from best-fit models

[(GTR+I+G) and (HKY+G)] were selected based on the Akaike Information Criteria (AIC) for the ITS and *tef 1- α* data sets, respectively. In the additional analyses, aiming to clarify the relationship among species close to *L. theobromae,* the K80 model was selected for the ITS data set whilst HKY was chosen for the *tef 1- α* and *β-tub* data sets, respectively. Bayesian analyses were performed for one million generations, with four independent chains, and sampled every 100th tree. The first 1000 trees were graphically identified as the burn-in and deleted when constructing consensus trees and calculation of posterior probabilities. A total of 9001 trees were imported into MEGA version 4 to construct a 50 % majority-rule consensus tree.

2.5. Pathogenicity

Pathogenicity tests were performed on branches of healthy seven to 12-year-old field grown *T. sericea* trees with branch diameters ranging from 10-20 mm. For inoculations, two isolates per species of the Botryosphaeriaceae identified in the study (Table 1) were grown on 2 % MEA for 10 days prior to inoculation. For each isolate, as well as the control, 10 trees were used. The method described by Begoude *et al.* (2009a) was used to inoculate branches and obtain an indication of the pathogenicity of the isolates tested. Data were analysed using the statistical analyses software programme SAS (SAS systems, version 8.2; SAS Institute).

3. RESULTS

3.1. Isolation, morphology and culture characteristics

In total, 135 isolates of the Botryosphaeriaceae were obtained from 232 trees sampled in Southern Africa. A total of 82 isolates were obtained from 134 *T. sericea* trees in the Moloto, Makhado, Musina, Pongola and Sudwala Caves areas in South Africa, 26 isolates from 58 *T. sericea* trees in Namibia from Katima Mulilo and Bagani, three isolates from 30 *T. sericea* trees in the Nata area in Botswana and 24 isolates from 10 *T. sambesiaca* trees sampled in the Ifakara area of Tanzania.

All isolates from *Terminalia* spp. derived from single conidia could be grouped into two categories based on conidial morphology. The isolates in the first category produced hyaline, elongated, thin-walled, *Fusicoccum*-like conidia. Isolates in this group represented two

different morphological forms. One group of isolates (Group 1) produced hyaline, bacilliform, aseptate and thin-walled conidia, and the other (Group 2) had hyaline, elongate, thin-walled, fusoid conidia. The second category contained isolates with pigmented, broad, thick-walled, *Diplodia*-like conidia. This category could also be sub-divided into two groups. Several isolates were characterized by thick-walled conidia; hyaline when immature and turning brown with age, aseptate and one-septate, sometimes exhibiting longitudinal striations (Group 3). A small set of three isolates (Group 4), differed from Group 3 isolates in that they had dark, thick-walled conidia. All the isolates produced only anamorph structures on pine needles in culture. Conidial dimensions of species of the Botryosphaeriaceae occurring on *Terminalia* spp. in Southern Africa were compared with those of similar species described in previous studies (Table 1).

All of the groups of isolates had similar culture morphology. Isolates on MEA grew fast, covering the surfaces of the Petri dishes within five days. The aerial mycelium was initially white, turning dark greenish or greyish after four to five days at 25 °C under near UV-light. It was not possible to identify the isolates to species level based on conidial or culture morphology.

3.2. DNA extraction and PCR amplification

A total of 47 isolates were selected for ITS sequencing to represent the four groups identified based on conidial morphology, hosts and areas sampled. These comprised 27 from Groups 1 and 2, and 20 from Groups 3 and 4. After ITS characterization, 27 isolates were selected for *tef 1- α* sequencing and three isolates for *β-tub* sequencing. DNA extraction and PCR was conducted successfully for all gene regions selected. PCR fragments for the ITS were ~ 580 bp, for *tef 1- α* they were 710 bp and for the *β-tub* gene region they were 440 bp.

3.3. Phylogenetic analyses

Combined ITS and tef 1- α analyses. The partition homogeneity test showed no significant conflict between the ITS and *tef 1-α* partitions (P = 0.103) suggesting that the data sets could be combined. The combined dataset consisted of 72 isolates with 900 characters of which, 388 characters were constant, 146 characters were parsimony uninformative and 366 characters were parsimony informative. Gaps were treated as a fifth character. After heuristic

searches, 42 most parsimonious trees were obtained (Tree length (TL) = 1053; Consistency index (CI) = 0.752, Retention index (RI) = 0.963, Rescaled consistency index (RC) = 0.724 TreeBase Accession No. SN4634) and one of these was chosen for presentation (Figure 2). All 42 trees displayed the same overall topology, with slightly different arrangements of isolates within the terminal clades. The topology of the tree generated from the combined analyses with MP, as well as with the 50 % majority rule consensus tree, was congruent with the trees obtained with the individual analyses of *tef 1-α* and ITS (data not shown).

Isolates from *Terminalia* spp. grouped with seven previously described species corresponding to four genera, namely *Neofusicoccum, Lasiodiplodia, Pseudofusicoccum* and *Diplodia*. All these clades were strongly supported with Bootstrap and Bayesian posterior probability values (Figure 2).

The *Neofusicoccum* clade included isolates obtained from *Terminalia* spp. in South Africa only and represented four sub-clades (Figure 2). Three of these sub-clades represented the previously described species *N. parvum, N. kwambonambiense* Pavlic, Slippers, & M.J. Wingf. and *N. vitifusiforme* (Niekerk & Crous) Crous, Slippers & A.J.L. Phillips. The isolates residing in the fourth clade (BS = 87 % and BPP = 0.99), did not group with any known *Neofusicoccum* sp., suggesting that it represented an undescribed species. This clade clustered close to *N. ursorum* Maleme, Pavlic, Slippers. with three and two fixed nucleotide differences in their ITS and *tef 1- α* sequences, respectively (Table 3). No sequence variation was observed between isolates from *Terminalia* spp. and those from GenBank in any group other than in the *N. parvum* cluster, where a single fixed nucleotide difference was seen in the ITS sequence.

Three main clades within *Lasiodiplodia* accommodated isolates from *Terminalia* spp. These were *L. mahajangana* Begoude, Jol. Roux, Slippers., *L. pseudotheobromae* A.J.L. Phillips, A. Alves & Crous and *L. crassispora* Burgess, Barber. No sequence variation was observed in the ITS region for isolates from *Terminalia* spp. and isolates from GenBank for any of these groups. However, based on *tef 1-α* sequence data, and despite the high statistical values supporting the group containing isolates of *L. pseudotheobromae,* a sub-structure made up of two isolates was observed in the clade. This had two base pairs separating these isolates from other isolates of *L. pseudotheobromae*. Another clade (BS = 78 % and BPP = 0.93)

accommodating an apparently undescribed species, nested alone between *L. mahajangana* and *L. theobromae.* Ten and six nucleotide modifications respectively were observed between isolates from *Terminalia* spp. and representatives of *L. mahajangana* and *L. theobromae* (Table 4).

The remaining isolates from *Terminalia* spp. formed single clades with *D. alatafructa* J.W.M. Mehl & B. Slippers (BS = 100 % and BPP = 0.96) and *P. olivaceum* J.W.M. Mehl & B. Slippers (BS = 99 % and BPP = 0.97), respectively. The isolates included in the *Diplodia* clade originated from South Africa only, whereas those included in the *Pseudofusicoccum* clade originated from South Africa and Namibia. Pairwise sequence comparisons revealed no divergence in the ITS sequence and minor divergence in the *tef 1-α* sequence (one to two nucleotides) between isolates.

Additional analyses: To resolve uncertainties in the relationships between the seven taxa in the clade containing *L. mahajangana, L. theobromae* and isolates from *Terminalia* spp. (CMW26709, CMW26699, CMW26710) (Figure 2), individual and collective analyses were conducted using part of the ITS, *tef 1- α* and *β-tub* gene regions (Table 4). The tree topologies obtained from MP analyses were identical to those obtained in Bayesian analyses for each partition separately, as well as in combination. While no differences were observed in ITS sequences between isolates from *Terminalia* spp. and those representing *L. mahajangana* (Table 4) (Figure 3a), the separation of the apparently undescribed isolates in this clade was consistently observed in individual partitions of both *tef 1- α* and *β-tub* (Figure 3b, 3c). These regions contained two unique fixed polymorphisms each. Furthermore, a better resolution in the relationship of these isolates was provided after combining the data from each partition into one phylogenetic analysis.

The Incongruence Length Difference (Farris *et al.* 1995) calculated for all the data, related to all the isolates included in the clade (I = 0), indicated that the gene phylogenies were congruent (BS = 89 %; BPP = 0.90) for the consensus tree obtained from both MP and Bayesian analyses (Figure 3d) of the combined data set. These analyses clearly showed that isolates from *Terminalia* spp. consistently formed a sub-clade distinct from *L. theobromae* and *L. mahajangana,* confirming previous observations from the *tef 1-α* gene region.

3.4. Taxonomy

DNA sequence data for the ITS, *tef 1-α* and *β-tub* gene regions distinguished two previously undescribed species of Botryosphaeriaceae from amongst the isolates collected from *T. sericea* and *T. sambesiaca* in this study. The morphology of these isolates was studied and this confirmed that they represent previously undescribed species, for which we provide the following descriptions:

Lasiodiplodia cryptotheobromae Begoude, Jol. Roux, Slippers, sp. nov. MB515130 FIGURE 4.

Etymology: the name refers to the fact that this is a cryptic species closely related to *L. theobromae*.

Conidiomata pycnidialia usque ad 420.0 µm diametro, in foliis pini in MEA in 14 diebus facta, nigra solitaria mycelio tecta subimmersa conica. *Paraphyses* inter cellulas conidiogenas, cylindricae hyalinae. *Cellulae conidiogenae* holoblasticae discretae hyalinae cylindricae percurrente proliferantes ita incrassationem periclinalem formantes. *Conidia* primo non septata, hyalina ellipsoidea vel ovoidea, parietibus crassis (< 2.5 µm), contentis granularibus, liberata semel septata, colorata, dum matura strias verticales visas, 24.86 x 14.02 µm.

Conidiomata: pycnidial (up to 420.0 µm diam.), produced on sterilized pine needles on MEA within 14 days, black, solitary and covered by mycelium, semi-immersed, conical. *Paraphyses*: formed among conidiogenous cell, cylindrical, hyaline, 1-2 septate (38.5-) 49.0-73.5 (-66.0) x (2.0-) 2.5-3.0 (-3.5) µm, (average 50 paraphyses 61.29 x 2.61 µm), rounded at the tips, unbranched. *Conidiophores*: reduced to conidiogenous cells. *Conidiogenous cells*: holoblastic, discrete, hyaline, cylindrical, proliferating percurrently to form a periclinal thickening (14.0-) 15.5-23.0 (-28.0) x (2.5-) 3.0-4.5 (-5.0) µm (average 50 conidiogenous cells 19.04 x 3.76 µm). *Conidia*: initially aseptate, hyaline, ellipsoid to ovoid, thick-walled (< 2.5 µm), granular content, becoming one-septate and pigmented after release, vertical striations observed at maturity, (17.0-) 22.5-27.5 (-29.0) x (10.5-) 13.0-15.0 (-16.0) µm (average 50 conidia 24.86 x 14.02 µm, l/w 1.7). *Culture characteristics*: white fluffy and

abundant aerial mycelium, becoming pale olivaceous grey (23’’’’’f) after 4 days, with the reverse sides of the colonies olivaceous grey (23’’’’’b). *Optimum temperature for growth* 30 °C, with a growth rate of 30 mm/day on MEA in the dark, no growth observed at 10 °C.
Teleomorph: not observed
Host: *Terminalia sambesiaca, Terminalia sericea.*
Distribution: Ifakara, Tanzania; Bagani, Namibia.
Specimen examined: TANZANIA, IFAKARA: isolated from healthy branches of *Terminalia sambesiaca*, April 2007, collected by J. Roux, Holotype (PREM 60319) a dried culture of isolate CMW26699 = CBS125262 on pine needles; ex-Holotype (PREM 60315) representing dried culture of isolate CMW26710 = CBS125267.
Additional specimens: TANZANIA, IFAKARA: isolated from healthy branches of *Terminalia sambesiaca*, April 2007, collected by J. Roux, Paratype (PREM 60318) representing a dried culture of isolate CMW26709 = CBS125266.

Neofusicoccum terminaliae Begoude, Jol. Roux, Slippers, sp. nov. MB515131
FIGURE 5.

Etymology: The name refers to the host from which the type specimen was isolated.

Conidiomata pycnidialia in foliis pini in 14 diebus facta, solitaria vel aggregata mycelio tecta primo immersa, matura ¾ erumpentia, papillata nigra usque ad 2100.0 µm longa 1300.0 µm diametro. *Paraphyses* abundantes cylindricae hyalinae non septatae. *Conidiophorae* ad cellulas conidiogenas reductae. *Cellulae conidiogenae* holoblasticae hyalinae percurrente proliferantes interdum incrassationem periclinalem formantes, laeves conidium unicum facientes. *Conidia* non vel ad ter septata, hyalina laevia fusiformia parietibus tenuibus apice rotundata 20.02 x 6.84 µm.

Conidiomata: pycnidial produced on sterilized pine needles within 14 days, solitary to aggregate and covered by mycelium, initially embedded, ¾ erumpant through the pine needles at maturity, papillate, black, up to 2100.0 µm in length and 1300.0 µm diam. *Paraphyses*: abundant, cylindrical, hyaline, aseptate (27.0-) 32.5-52.5 (-64.0) x (1.5-) 2.0-2.5 (-3.0) µm, (average 50 paraphyses 42.61 x 2.37 µm). *Conidiophores*: reduced to conidiogenous cells. *Conidiogenous cells*: holoblastic, hyaline, cylindrical, proliferating

percurrently, sometimes forming a periclinal thickening, smooth producing a single conidium, (11.5-) 15.5-24.0 (-30.0) x (1.5-) 2.0-2.5 (-3.0) µm (average of 50 conidiogenous cells (19.6 x 2.4 µm). *Conidia*: aseptate to 3-septate, hyaline, smooth, fusiform, thin-walled, round at apex (14.5-) 18.0-22.5 (-25.0) x (3.5-) 6.0-7.5 (-8.5) µm (average 50 conidia 20.02 x 6.84 µm, l/w 2.9). *Culture characteristics*: colonies initially white, becoming olivaceous grey (21''''b) from the centre after seven days. Aerial mycelium dense, fluffy, edge smooth to crenalate. *Optimum temperature for growth* 25 °C, with a growth rate of 22.5 mm/day on MEA in the dark, little growth observed at 10 °C.

Teleomorph: not observed

Host: *Terminalia sericea*

Distribution: Moloto, Gauteng Province, South Africa.

Specimen examined: SOUTH AFRICA, MOLOTO: isolated from healthy branches of *Terminalia sericea*, Jan 2007, collected by D. Begoude and J. Roux, Holotype (PREM 60316), a dried culture of isolate CMW26683 = CBS125264 on pine needles; ex-Holotype (PREM 60314), representing dried culture of isolate CMW26679; ex-paratype culture CMW26679 = CBS125263.

Additional specimens: SOUTH AFRICA, MOLOTO: isolated from healthy branches of *Terminalia sericea*, Feb 2007, collected by D. Begoude and J. Roux. ex-paratype culture CMW26687; CMW26685 = CBS125265.

3.5. Pathogenicity

All inoculations with isolates of Botryosphaeriaceae collected in this study resulted in visible lesions on the bark and cambium of *T. sericea* trees after six weeks. Analysis of variance showed that there were significant differences in the level of pathogenicity between species ($P < 0.0001$). *L. pseudotheobromae, N. parvum, L. cryptotheobromae*, *L. crassispora* and *N. terminaliae* produced the longest lesions in both the bark and cambium, whereas *N. vitifusiforme, D. alatafructa* and *P. olivaceum* produced the smallest lesions (Figure 6). Considerable variation in levels of pathogenicity was also observed among isolates of the same species. There was a positive correlation (R^2 = 89 %) between lesions produced on the bark and those in the cambium. Isolations from lesions on the inoculated trees resulted in recovery of the inoculated fungi, confirming that they were the cause of the lesions.

4. DISCUSSION

This study represents the first attempt to characterize species of the Botryosphaeriaceae on native *Terminalia* spp. in Southern Africa. Nine species, corresponding to four genera, were identified from *T. sericea* and *T. sambesiaca*. Seven of these, *L. crassispora, L. pseudotheobromae, D. alatafructa, P. olivaceum, N. parvum, N. kwambonambiense* and *N. vitifusiforme,* represent previously described species and two, *L. cryptotheobromae* and *N. terminaliae,* were described as new. *L. pseudotheobromae* and *N. parvum* have previously been reported from *Terminalia* spp. (Begoude *et al*. 2009a,b) and the remaining species are recorded on *Terminalia* spp. for the first time.

Three species of *Lasiodiplodia* were identified in this study. These are *L. pseudotheobromae*, *L. crassispora* and the newly described *L. cryptotheobromae*. The taxonomy of species in *Lasiodiplodia* has undergone considerable change in recent years. The best known species, *L. theobromae,* was originally described as *Botryodiplodia theobromae* Pat. from cacao fruit in Ecuador (Patouillard and Lagerheim 1892). Later, Griffon and Maublanc (1909) renamed this species in *Lasiodiplodia* (Goos *et al*. 1961). Subsequently, all collections of fungi in the group with dark conidia, becoming striated with age, were treated as *L. theobromae*. This resulted in *L. theobromae* representing a pleomorphic, plurivorous group of fungi with a global geographic distribution and a very wide host range (Punithalingam 1976). The advent of DNA sequence comparisons to define species boundaries has shown that *L. theobromae* represents a relatively large group of distinct species. To date, 10 species, previously grouped with *L. theobromae*, have been described using DNA sequence comparisons (Pavlic *et al.* 2004; Burgess *et al.* 2006; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Begoude *et al.* 2009a). In the current study, it was thus necessary to use DNA sequence data for multiple gene regions to distinguish *Lasiodiplodia* spp. collected from *Terminalia* spp.

Lasiodiplodia cryptotheobromae is described here based primarily on evidence from DNA sequence comparisons. *L. cryptotheobromae* is phylogenetically most closely related to *L. theobromae* and *L. mahajangana*. Although ITS sequences for both *L. cryptotheobromae* and *L. mahajangana* were identical, considerable sequence variation was observed across *tef 1-α* and *β-tub* gene regions, providing support for their distinction as distinct species. Eighteen fixed, unique, single nucleotide polymorphisms (SNPs) out of 23 informative characters

across the three gene regions differentiated these species. Morphologically, conidial measurements of *L. crytotheobromae* overlapped with those of *L. theobromae*, but they were larger than those of *L. mahajangana*. Moreover, septate paraphyses were observed in *L. cryptotheobromae,* similar to *L. theobromae*. It is thus not possible to distinguish *L. cryptotheobromae* from *L. theobromae* based only on morphology.

Isolates of *L. cryptotheobromae* were found as endophytes in twigs of healthy *T. sericea* and *T. sambesiaca* collected in Namibia and Tanzania. However, pathogenicity tests on branches of *T. sericea* trees showed that this fungus was among the most pathogenic species. This suggests that it could be a pathogen of these trees, for example if it were introduced into an area where it does not occur naturally, or when the trees are under stress. This is consistent with the fact that a number of species in the Botryosphaeriaceae are known as virulent pathogens of trees under environmental stress (Schoeneweiss 1981; Mullen *et al.* 1991; Smith *et al.* 1994; Ahimera *et al.* 2003).

Four isolates of *L. crassispora* were found on healthy twigs of *T. sericea* and *T. sambesiaca* collected in South Africa, Botswana and Tanzania. This fungus was first reported from *Santalum album* and *Eucalyptus urophylla* in Australia and Venezuela (Burgess *et al.* 2006). Its range has remained restricted to these hosts and localities until recently, when it was found on *Pterocarpus angolensis*. in South Africa (Mehl *et al*. 2009). Results of the present study have, therefore, substantially increased the host range and geographic distribution of *L. crassispora*. Conidial dimensions of isolates from *Terminalia* spp. were very similar to those of the type isolate. Judging from the length of lesions produced on branches of *T. sericea* trees, *L. crassispora* could be considered as a potential pathogen of these trees.

Lasiodiplodia pseudotheobromae was commonly collected from healthy twigs of *T. sericea* and *T. sambesiaca* in Namibia and Tanzania. Previously, it has been reported from *T. catappa* in Madagascar, South Africa and Cameroon (Begoude *et al.* 2009a), *T. ivorensis, T. superba* and *T. mantaly* in Cameroon (Begoude *et al.* 2009b) as well as on various genera of woody plants in other localities (Alves *et al.* 2008). Isolates from Southern Africa displayed slight divergences in *tef 1-α* sequences and clustered as a sub-clade within the larger *L. pseudotheobromae* clade. However, these minor differences were considered to

represent normal variation within the species, similar to that described previously for *Diplodia scrobiculata* J. de Wet, B. Slippers & M.J. Wingfield (Lazzizera *et al*. 2008a). Pathogenicity trials showed that *L. pseudotheobromae* was consistently the most virulent species among those Botryosphaeriaceae tested in this study. This is similar to results of other studies, such as those on *P. angolensis* (Mehl *et al*. 2009) and *T. catappa* and *T. mantaly* (Begoude *et al.* 2009a,b), where *L. pseudotheobromae* was found to be highly virulent. Even though these results stemmed from artificial inoculations, the consistently high levels of pathogenicity of *L. pseudotheobromae* on different hosts and in different locations add to the view that this is an important pathogen of trees.

Neofusicoccum species represented the most abundant and the most diverse group of fungi identified from *Terminalia* spp. in Southern Africa. Four species were collected from *T. sericea* and *T. sambesiaca*. These included the previously undescribed *N. terminaliae*, which was found from asymptomatic *T. sericea*. Based on the phylogeny inferred from MP analyses of individual and combined ITS and *tef 1-α* gene regions, isolates representing *N. terminaliae* consistently resided in a single and well supported clade. The morphological features of this species are consistent with those described for other members of the genus, such as fusoid, hyaline, elongate and thin-walled conidia (Crous *et al.* 2006). The closest relative of *N. terminaliae* is the newly described species *N. ursorum* (Maleme 2008) with which three and two nucleotide differences in the ITS and *tef 1-α* sequences were respectively observed. Morphologically, *N. terminaliae* can be distinguished from *N. ursorum* by its wider pycnidia, the presence of paraphyses and up to 3-septate conidia. Similar to many members of the Botryosphaeriaceae, the presence of *N. terminaliae* on healthy tissue confirmed its endophytic nature. However, *N. terminaliae* produced lesions on branches of *T. sericea* in pathogenicity trials showing that it has the capacity to cause disease.

Two species were identified in this study in what is known as the *N. parvum / N. ribis* complex. This complex, which originally consisted of two species, has recently been amended based on multiple gene genealogies, and now includes six species (Begoude *et al.* 2009a; Pavlic *et al.* 2009a,b). The most commonly collected species from *Terminalia* spp. in this study was *N. parvum*, which accounted for 21 % of the total number of isolates. *N. parvum* was, however, collected only from healthy twigs of *T. sericea* in South Africa and not

from any of the other areas surveyed. *N. parvum* has been found abundantly on non-native *Eucalyptus* trees and on native *S. cordatum* in previous studies conducted in South Africa (Slippers *et al.* 2004; Pavlic *et al.* 2007). Very recently, the fungus was also reported from non-native *T. catappa* in South Africa (Begoude *et al.* 2009a). The high levels of occurrence on both introduced and native hosts in South Africa has led to suggestions that *N. parvum* might be native to the country (Pavlic *et al.* 2007; 2009b; Begoude *et al.* 2009a) and the results of this study add credence to this view. In this study *N. parvum* was able to produce lesions in pathogenicity trials. The pathogenicity of *N. parvum* has previously been recognized on native *S. cordatum* and non-native *T. catappa* and *Eucalyptus* trees in Southern Africa (Slippers *et al.* 2004; Pavlic *et al.* 2007; Begoude *et al.* 2009a). This remarkable association of *N. parvum* with native and non-native hosts in South Africa supports evidence for movement of the pathogen between native and non-native tree hosts in this area (Strauss 2001; Slippers *et al.* 2005). Further investigations on populations of *N. parvum* from native and non-native hosts are needed to clarify the question of origin of this fungus.

A small number of isolates from *T. sericea* in South Africa were identified as *N. kwambonambiense.* This fungus was one of the first phylogenetic species described in the Botryosphaeriaceae using sequence differences as the most important defining characteristics (Pavlic *et al.* 2009b). In that study, it was sister to *N. ribis* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips that was found on native *S. cordatum* in South Africa. In the present study, isolates representing *N. kwambonambiense* were isolated as endophytes from native *T. sericea* in South Africa and this represents the second record of this fungus on a native South African tree species. Its presence in this niche suggests that the fungus is most likely native to South Africa.

Neofusicoccum vitifusiforme, collected in this study from a native tree, was first described from South Africa when it was discovered on *Vitis vinifera* (van Niekerk *et al.* 2004). Interestingly, it has not previously been found on a forest tree host and other reports of the fungus are from *Prunus salicina* Lindell. and *P. persica* trees in South Africa (Damm *et al.* 2007) and on olive drupes in Italy (Lazzizera *et al.* 2008b). In their study, Lazzizera *et al.* (2008b) found that isolates of *Botryosphaeria* studied by Barber *et al.* (2005) clustered with ex-type cultures of *N. vitifusiforme* and subsequently concluded that *Dichomera*

eucalypti is the synanamorph of *N. vitifusiforme.* At the time of its identification, *N. vitifusiforme* was considered as a weak pathogen restricted to *V. vinifera* (van Niekerk *et al.* 2004). Our consideration of its pathogenicity on branches of *T. sericea* trees also showed that it is not a pathogen.

Three isolates found in this study clustered with each of *D. alatafructa* and *P. olivaceum.* Both fungi were previously isolated from native *P. angolensis* trees in South Africa, *P. olivaceum* from asymptomatic branches and *D. alatafructa* from branch lesions on this host (Mehl *et al.* 2009). Of particular interest is the fact that some of the isolates obtained from *T. sericea* in South Africa were found at the same location as the reference strains. The fact that *D. alatafructa* and *P. olivaceum* occurred on two native hosts in the same area suggests that these species are most likely native to South Africa. They do not appear to be pathogenic and are currently of little concern in terms of the health of *T. sericea* in South Africa.

The inoculation trials conducted on branches of healthy *T. sericea* trees in this study tested the ability of the mycelium of the Botryosphaeriaceae to infect living plant tissue. In terms of pathogenicity, the species tested fell into one of two groups. These accommodated the potentially pathogenic species that produced distinct lesions on the branches. These were, in order of decreasing virulence, *L. pseudotheobromae, N. parvum, L. cryptotheobromae*, *L. crassispora* and *N. terminaliae*. The second group included species with low levels of pathogenicity, including *N. vitifusiforme, D. alatafructa* and *P. olivaceum.* There was considerable variation in the levels of pathogenicity for different isolates of some species. For example, in the case of *L. pseudotheobromae* and *L. crassispora*, isolates CMW26702 and CMW26688 produced lesions that were three times as large as those produced by the other isolates of these species. Similar variation in pathogenicity amongst isolates of the same species has previously been reported for *N. australe* and *Diplodia seriata* on grapevine (Larignon *et al.* 2001; van Niekerk *et al.* 2004; Taylor *et al.* 2005) and for *B. dothidea* on apple fruit (Latorre and Toledo 1984; Parker and Sutton 1993). This suggests that pathogenicity data for the Botryosphaeriaceae must be interpreted with some circumspection. In addition, this group of fungi are known to be associated with conditions of stress (Smith *et al.* 1994; Ahimera *et al.* 2003) and the impact of the environment of the trees being studied should also be considered.

A large number of taxonomic lineages of the Botryosphaeriaceae were found on two species of native *Terminalia* in Southern Africa. This number was as high as previous surveys of the Botryosphaeriaceae on other native hosts in subtropical and temperate areas in Southern Africa and Australia (Pavlic *et al.* 2008; Mehl *et al.* 2009; Taylor *et al.* 2009; van der Walt 2008).The high diversity of species found on native trees supports the views of Taylor *et al.* (2009) that there is a need to extend knowledge regarding fungi on native trees. Furthermore, some of these fungi could be important pathogens of economically important crops with the native trees serving as a source of inoculum (Pavlic *et al.* 2009b). It is, therefore, important to promote studies in natural ecosystems to better understand the diversity of these environments.

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Table 1. Conidial measurements of species of the Botryosphaeriaceae isolated from *Terminalia* spp. in Southern Africa and comparison with those reported in previous studies.

	Conidial size (µm)		Source of data
Species	This study	Previous studies	
N. parvum	(15.5-)16.5-20.5(-21.5) x (4.5-)6.0-7.5(-8.0)	(12-)15-19(-24) x 4-6	Slippers *et al.* 2004
N. kwambonambiense	(16.0-)20.5-21.0(-22.0) x (5.0-)4.5-7.5	16-28 x 5-8	Pavlic *et al.* 2009
N. terminaliae	(14.5-)18.0-22.5(-25.0) x (3.5-)6.0-7.5(-8.5)		This study
N. vitifusiforme	(14.5-)18.0-21.0(-24.5) x (4.0-)4.5-8.5	(18-)19-21(-22) x (4.5-)5.5-6.5(-8)	van Niekerk *et al.* 2004
P. olivaceum	(20.0-)22.0-25.5(-27.5) x (5.0-)5.5-7.0(-8.0)	(17.9-)19.9-25.7(-30.4) x (5.9-)6.3-7.7(-8.9)	Mehl *et al.* 2009
L. crassispora	(19.5-)23.0-28.5(31.5) x (15.0-)16.0-18.5(-20.5)	27-30(-33) x 14-17	Burgess *et al.* 2006
L. cryptotheobromae	(17.0-)22.5-27.5(-29.0) x (10.5-)13.0-15.0(-16.0)		This study
L. pseudotheobromae	(21.5-)24.5-27.0(30.5) x (12.0-)13.5-15.5(-16.5)	(22.5-)23.5-31(-32.5) x (12-) 13-15.5(-18.5)	Alves *et al.* 2008
D. alatafructa	(20.0-)22.0-25.5(-27.5) x (5.0-)5.5-7.0(-8.0)	(22.4-)24.6-29.2(-32.9) x (9.3-)11-13.8(-15.8)	Mehl *et al.* 2009

Table 2. Botryosphaeriaceae used for phylogenetic analyses in this study.

Species	Culture number	Origin	Host	Collectors	Genbank Accession No.		
					ITS	*tef* 1-α	*β-tub*
Diplodia alatafructa	CMW 22627	South Africa	*Pterocarpus angolenssis*	J. Mehl/J. Roux	FJ888460	FJ888444	
	CMW 22721	South Africa	*P. angolensis*	J. Mehl/J. Roux	FJ888478	FJ888446	
	CMW 26854	South Africa	*Terminalia sericea*	D. Begoude	GQ471835	GQ471793	
	CMW 26840	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471834	GQ471795	
	CMW 26838	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471833	GQ471794	
D. seriata	CMW 7774	USA	*Ribes* sp.	B. Slippers/G. Hudler	EF445343	EF445382	
	CMW 7775	USA	*Ribes* sp.	B. Slippers/G. Hudler	EF445344	EF445383	
Guignardia mangiferae	1095	Panama	*Theobroma cacao*	E. Rojas/L. Mejia/Z. Maynard	EU683671	EU683652	
Lasiodiplodia crassispora	CBS 118741	Venezuela	*Eucalyptus urophylla*	S. Mohali	DQ103552	DQ103556	
	CBS 110492	Australia	*Santalum album*	T.I. Burgess/B. Dell	DQ103551	DQ103558	
	CMW 26688	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471822	GQ471775	
	CMW 26698	Botswana	*T. sericea*	J. Roux	GQ471823	GQ471776	
L. cryptotheobromae	**CMW 26699**	Tanzania	*Terminalia sambesiaca*	J. Roux	GQ471812	GQ471778	GQ471773
	CMW 26709	Tanzania	*T. sambesiaca*	J. Roux	GQ471814	GQ471777	GQ471772
	CMW 26710	Tanzania	*T. sambesiaca*	J. Roux	GQ471813	GQ471779	GQ471774
L. gonubiensis	CBS 115812	South Africa	*Syzygium cordatum*	D. Pavlic	DQ458892	DQ458877	
	CBS 116355	South Africa	*S. cordatum*	D. Pavlic	AY639594	DQ103567	
L. mahajangana	CMW 27801	Madagascar	*T. catappa*	J. Roux	FJ900595	FJ900641	FJ900630
	CMW 27818	Madagascar	*T. catappa*	J. Roux	FJ900596	FJ900642	FJ900631
L. margaritacea	CMW 26162	Australia	*Adansonia gibbosa*	D. Pavlic	EU144050	EU144065	
	CMW 26163	Australia	*A. gibbosa*	D. Pavlic	EU144051	EU144066	

L. parva	CBS 356.59	Sri Lanka	*T. cacao*	A. Riggenbach	EF622082	EF622062	
	CBS 494.78	Colombia	Cassava-field soil	O. Rangel	EF622084	EF622064	
L. plurivora	STEU-5803	South Africa	*Prunus salicina*	U. Damm	EF445362	EF445395	
	STEU-4583	South Africa	*Vitis vinifera*	F. Halleen	AY343482	EF445396	
L. pseudotheobromae	**CMW 26702**	Tanzania	*T. sambesiaca*	J. Roux	GQ471830	GQ471796	
	CMW 26724	Namibia	*T. sericea*	J. Roux	GQ471832	GQ471797	
	CMW 26695	Namibia	*T. sericea*	J. Roux	GQ471831	GQ471798	
	CBS 116459	Costa Rica	*Gmelina arborea*	J. Carranza/Velásquez	EF622077	EF622057	
	CBS 447.62	Suriname	*Citrus aurantium*	C. Smulders	EF622081	EF622060	
L. rubropupurea	WAC 12535	Australia	*E. grandis*	T.I. Burgess/G. Pegg	DQ103553	DQ103571	
	WAC 12536	Australia	*E. grandis*	T.I. Burgess/G. Pegg	DQ103554	DQ103572	
L. theobromae	CMW 9074	Mexico	*Pinus* sp.	T. Burgess	EF622074	EF622054	AY236930
	CBS 164.96	New Guinea	Fruit along coral reef coast	Unknown	AY640255	AY640258	EU673110
L. venezuelensis	WAC 12539	Venezuela	*Acacia mangium*	S. Mohali	DQ103547	DQ103568	
	WAC 12540	Venezuela	*A. mangium*	S. Mohali	DQ103548	DQ103569	
Neofusicoccum batangarum	CMW 28315	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900606	FJ900652	
	CMW 28363	Cameroon	*T. catappa*	D. Begoude/ J. Roux	FJ900607	FJ900653	
N. cordaticola	CMW 13992	South Africa	*S. cordatum*	D. Pavlic	EU821898	EU821868	
	CMW 14056	South Africa	*S. cordatum*	D. Pavlic	EU821903	EU821873	
N. kwambonambiense	CMW 14023	South Africa	*S. cordatum*	D. Pavlic	EU821900	EU821870	
	CMW 14025	South Africa	*S. cordatum*	D. Pavlic	EU821901	EU821871	
	CMW 26850	South Africa	*T. sericea*	D. Begoude	GQ471846	GQ471799	
	CMW 26856	South Africa	*T. sericea*	D. Begoude	GQ471843	GQ471800	
	CMW 26865	South Africa	*T. sericea*	D. Begoude	GQ471845	GQ471801	
N. parvum	CMW 9081	New Zealand	*P. nigra*	G.J. Samuels	AY236943	AY236888	
	CMW 9079	New Zealand	*A. deliciosa*	S.R. Pennicook	AY236940	AY236885	
	CMW 26690	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471815	GQ471787	
	CMW 26851	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471817	GQ471788	

	CMW 26844	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471819	GQ471789
N. ribis	CMW 7772	USA	*Ribes* sp.	B. Slippers/G. Hudler	AY236935	AY236877
	CMW 7773	USA	*Ribes* sp.	B. Slippers/G. Hudler	AY236936	AY236878
N. terminaliae	**CMW 26679**	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471802	GQ471780
	CMW 26687	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471803	GQ471781
	CMW 26683	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471804	GQ471782
	CMW 26685	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471805	GQ471783
N. umdonicola	CMW 14106	South Africa	*S. cordatum*	D. Pavlic	EU821899	EU821869
	CMW 14058	South Africa	*S. cordatum*	D. Pavlic	EU821904	EU821874
N. ursorum	CMW 23790	South Africa	*Eucalyptus* sp.	H. M. Maleme	FJ752745	FJ752708
	CMW 24480	South Africa	*Eucalyptus* sp.	H. M. Maleme	FJ752746	FJ752709
N. vitifusiforme	STE-U 5252	South Africa	*V. vinifera*	J. M. van Niekerk	AY343383	AY343343
	STE-U 5820	South Africa	*Prunus salicina*	U. Damm	EF445347	EF445389
	CMW 26689	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471838	GQ471790
	CMW 26676	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471841	GQ471791
	CMW 26686	South Africa	*T. sericea*	D. Begoude/ J. Roux	GQ471840	GQ471792
Pseudofusicoccum olivaceum	CMW 20881	South Africa	*P. angolensis*	J. Roux	FJ888459	FJ888437
	CMW 22637	South Africa	*P. angolensis*	J. Mehl/ J. Roux	FJ888462	FJ888438
	CMW 26673	South Africa	*T. sericea*	D. Begoude	GQ471806	GQ471784
	CMW 26824	South Africa	*T. sericea*	J. Roux	GQ471807	GQ471785
	CMW 26836	South Africa	*T. sericea*	J. Roux	GQ471808	GQ471786
P. stromaticum	CBS 117448	Venezuela	*Eucalyptus hybrid*	S. Mohali	AY693974	AY693975
	CBS 117449	Venezuela	*Eucalyptus hybrid*	S. Mohali	DQ436935	DQ436936

Table 3. Polymorphic nucleotides from sequence data of the ITS and *tef* 1-α gene regions for isolates representing two closely related *Neofusicoccum* spp.

Identity	**Culture number**	**ITS**			***tef* 1-α**	
		103	**132**	**141**	**61**	**78**
N. ursorum	CMW23790	T	G	T	T	T
	CMW24480	T	G	T	T	T
N. terminaliae	CMW26679	C	A	C	C	G
	CMW26687	C	A	C	C	G
	CMW26683	C	A	C	C	G
	CMW26685	C	A	C	C	G

Table 4. Polymorphic nucleotides from sequence data of the ITS, *tef* 1-α and *β-tub* gene regions for isolates of three *Lasiodiplodia* spp. Unique, fixed polymorphisms in each species are shaded.

Identity	**Culture number**	**ITS**		***tef* 1-α**								***β-tub***						
		46	**98**	**36**	**44**	**59**	**60**	**68**	**82**	**99**	**261**	**85**	**124**	**143**	**157**	**177**	**189**	**244**
L. theobromae	CBS164.96	C	C	C	A	C	1	A	C	C	G	C	C	G	T	T	A	C
	CMW9074	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. mahajangana	CMW27801	T	T	G	T	-	0	G	T	T	.	T	T	C	C	C	G	A
	CMW27818	T	T	G	T	-	0	G	T	T	.	T	T	C	C	C	G	A
L. cryptotheobromae	CMW26709	T	T	.	T	T	.	G	T	T	A	T	.	C	C	C	G	.
	CMW26699	T	T	.	T	T	.	G	T	T	A	T	.	C	C	C	G	.
	CMW26710	T	T	.	T	T	.	G	T	T	A	T	.	C	C	C	G	.

Table 5. Sequence characteristics and phylogenetic information for ITS, *tef 1-α* and *β-tub* and combined data sets of *Lasiodiplodia* spp.

Data set	Sequence range	No. of variable sites	No. of informative sites	No. of most parsimonious trees	Tree length	Consistency index	Retention index
ITS	461	4	2	1	4	1	1
***tef* 1-α**	270	14	14	1	15	1	1
β-tub	422	7	7	1	7	1	1
Combined data	1153	25	23	1	26	1	1

Figure 1. Collection sites and distribution of species of the Botryosphaeriaceae collected from *Terminalia* spp. in Southern Africa

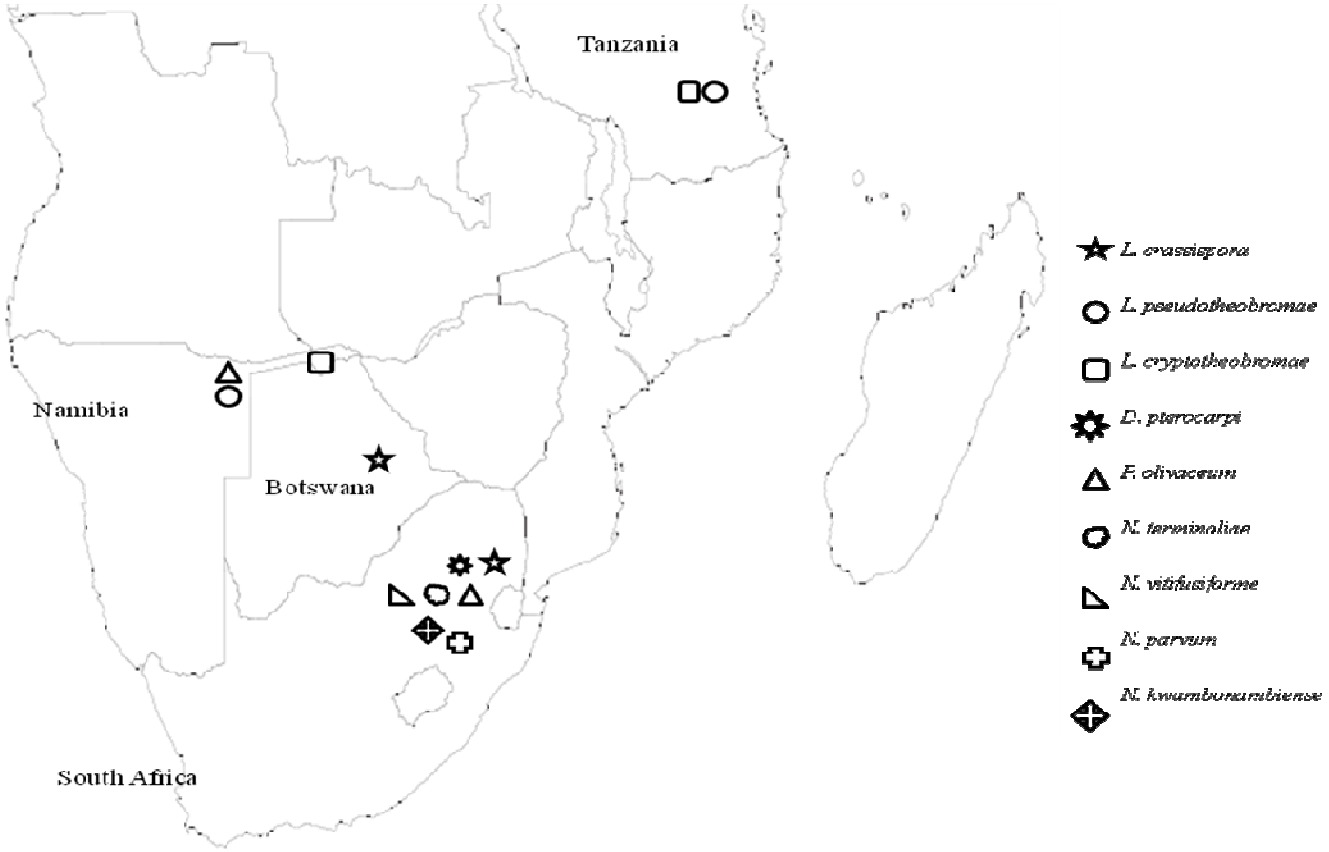

Tanzania
Namibia
Botswana
South Africa
L. ovassiepora
L. pseudotheobromae
L. cryptotheobromae
D. pterocarpi
F. olivaceum
N. terminalina
N. vitifusiforme
N. parvum
N. kwambonambiense

Figure 2. One of the most parsimonious trees obtained from MP analyses of the combined ITS and *tef* 1-α sequence data of the Botryosphaeriaceae from *Terminalia* spp. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branches (PP/BS). Isolates marked in bold represent those obtained from *Terminalia* spp.

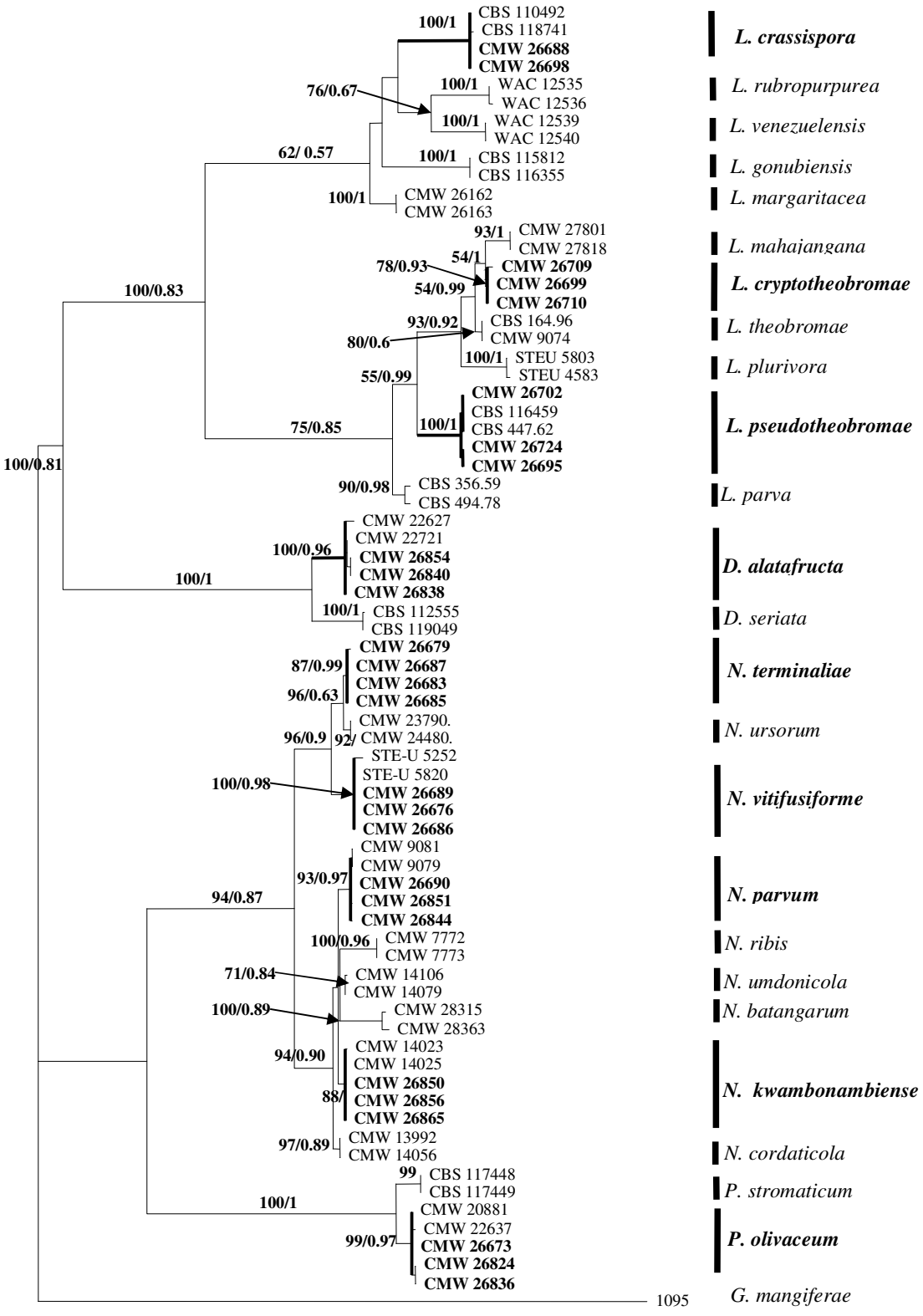
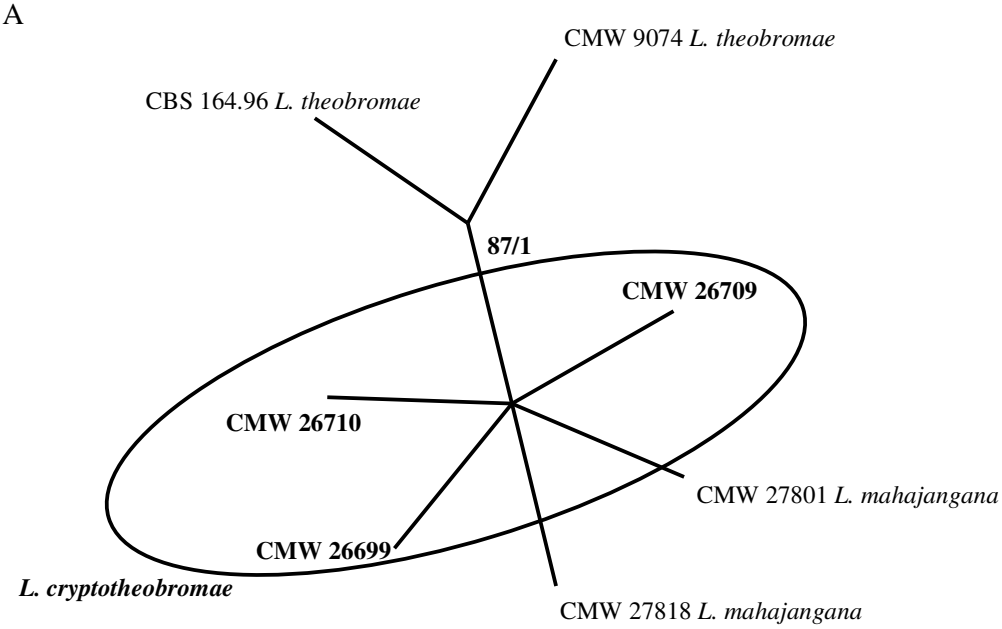
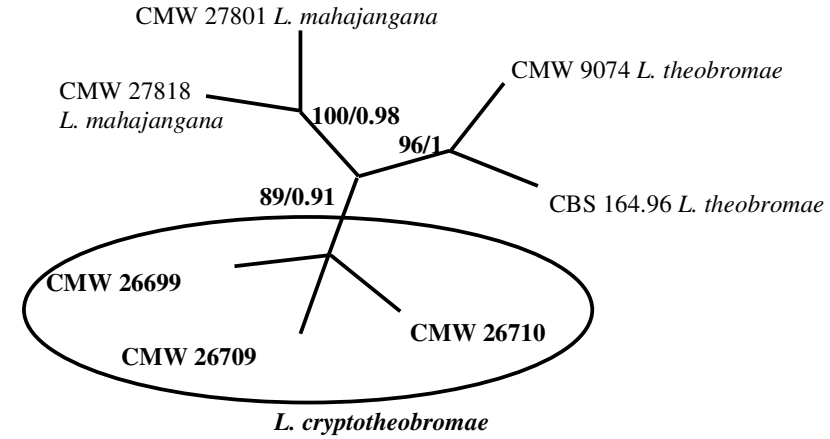

100/1
CBS 110492
CBS 118741
CMW 26688
CMW 26698
L. crassispora
76/0.67
100/1
WAC 12535
WAC 12536
L. rubropurpurea
100/1
WAC 12539
WAC 12540
L. venezuelensis
62/ 0.57
100/1
CBS 115812
CBS 116355
L. gonubiensis
100/1
CMW 26162
CMW 26163
L. margaritacea
93/1
CMW 27801
CMW 27818
L. mahajangana
54/1
78/0.93
CMW 26709
CMW 26699
CMW 26710
L. cryptotheobromae
100/0.83
54/0.99
93/0.92
CBS 164.96
CMW 9074
L. theobromae
80/0.6
100/1
STEU 5803
STEU 4583
L. plurivora
55/0.99
CMW 26702
CBS 116459
CBS 447.62
CMW 26724
CMW 26695
L. pseudotheobromae
75/0.85
100/1
100/0.81
90/0.98
CBS 356.59
CBS 494.78
L. parva
CMW 22627
CMW 22721
100/0.96
CMW 26854
CMW 26840
CMW 26838
D. alatafructa
100/1
100/1
CBS 112555
CBS 119049
D. seriata
CMW 26679
87/0.99
CMW 26687
CMW 26683
96/0.63
CMW 26685
N. terminaliae
CMW 23790.
96/0.9
92/
CMW 24480.
N. ursorum
STE-U 5252
100/0.98
STE-U 5820
CMW 26689
CMW 26676
CMW 26686
N. vitifusiforme
CMW 9081
CMW 9079
93/0.97
CMW 26690
CMW 26851
CMW 26844
N. parvum
94/0.87
100/0.96
CMW 7772
CMW 7773
N. ribis
71/0.84
CMW 14106
CMW 14079
N. umdonicola
100/0.89
CMW 28315
CMW 28363
N. batangarum
CMW 14023
94/0.90
CMW 14025
CMW 26850
88/
CMW 26856
CMW 26865
N. kwambonambiense
97/0.89
CMW 13992
CMW 14056
N. cordaticola
99
CBS 117448
CBS 117449
P. stromaticum
100/1
CMW 20881
CMW 22637
99/0.97
CMW 26673
CMW 26824
CMW 26836
P. olivaceum
1095
G. mangiferae
– 5 changes

Figure 3. Most-parsimonious unrooted trees inferred from independent analyses of each data set (A=ITS; B=*tef 1-α*; C=*β-tub*; D= combination of sequences of the three loci) of the isolates representing *L. theobromae, L. mahajangana* and *L. cryptotheobromae*. Posterior probabilities followed by Bootstrap support (%) from 1000 replications are given on the branches (PP/BS).

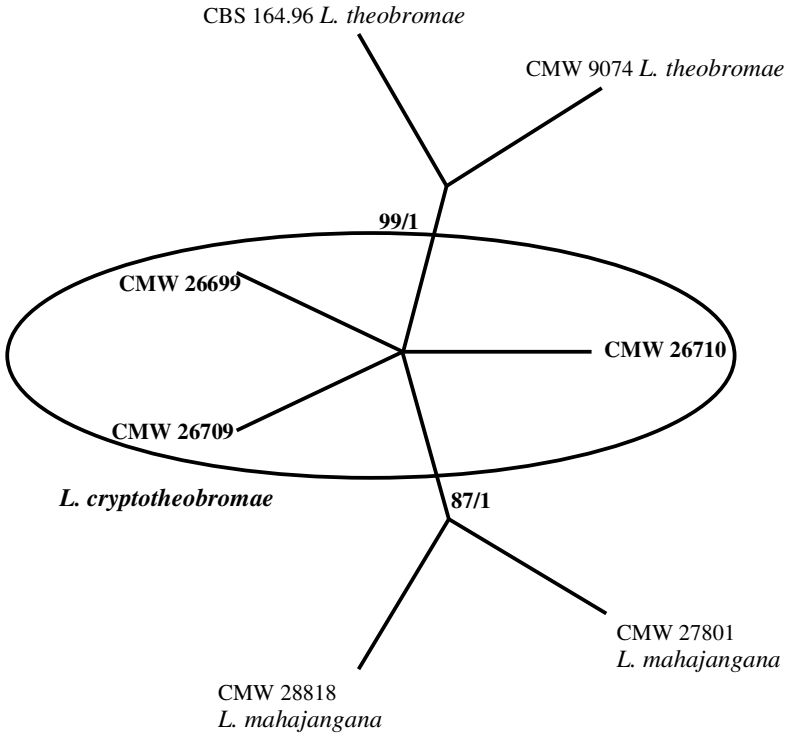
A


CMW 9074 L. theobromae
CBS 164.96 L. theobromae
87/1
CMW 26709
CMW 26710
CMW 27801 L. mahajangana
CMW 26699
L. cryptotheobromae
CMW 27818 L. mahajangana

B


CMW 27801 L. mahajangana
CMW 9074 L. theobromae
CMW 27818
L. mahajangana
100/0.98
96/1
89/0.91
CBS 164.96 L. theobromae
CMW 26699
CMW 26710
CMW 26709
L. cryptotheobromae

C


CBS 164.96 L. theobromae
CMW 9074 L. theobromae
99/1
CMW 26699
CMW 26710
CMW 26709
L. cryptotheobromae
87/1
CMW 27801
L. mahajangana
CMW 28818
L. mahajangana

D

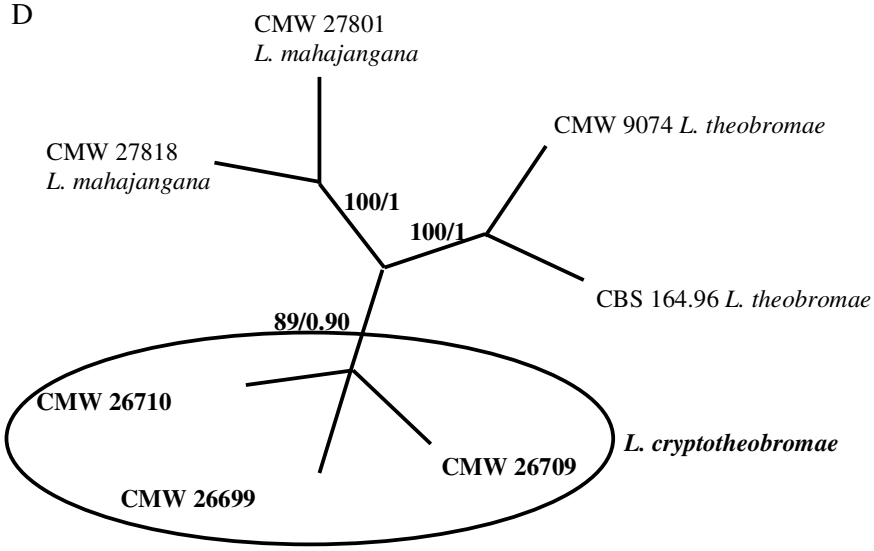

CMW 27801
L. mahajangana
CMW 9074 L. theobromae
CMW 27818
L. mahajangana
100/1
100/1
CBS 164.96 L. theobromae
89/0.90
CMW 26710
CMW 26709
L. cryptotheobromae
CMW 26699

Figure 4. *Lasiodiplodia cryptotheobromae.* (a) Pycnidium formed on pine needle in culture, (b, c) conidiogenous cells with developing conidia, (d) paraphyses, (e) young and mature conidia and (f) mature conidium showing striations. Bars: a = 200 µm; b, c, d, e, f = 10 µm.

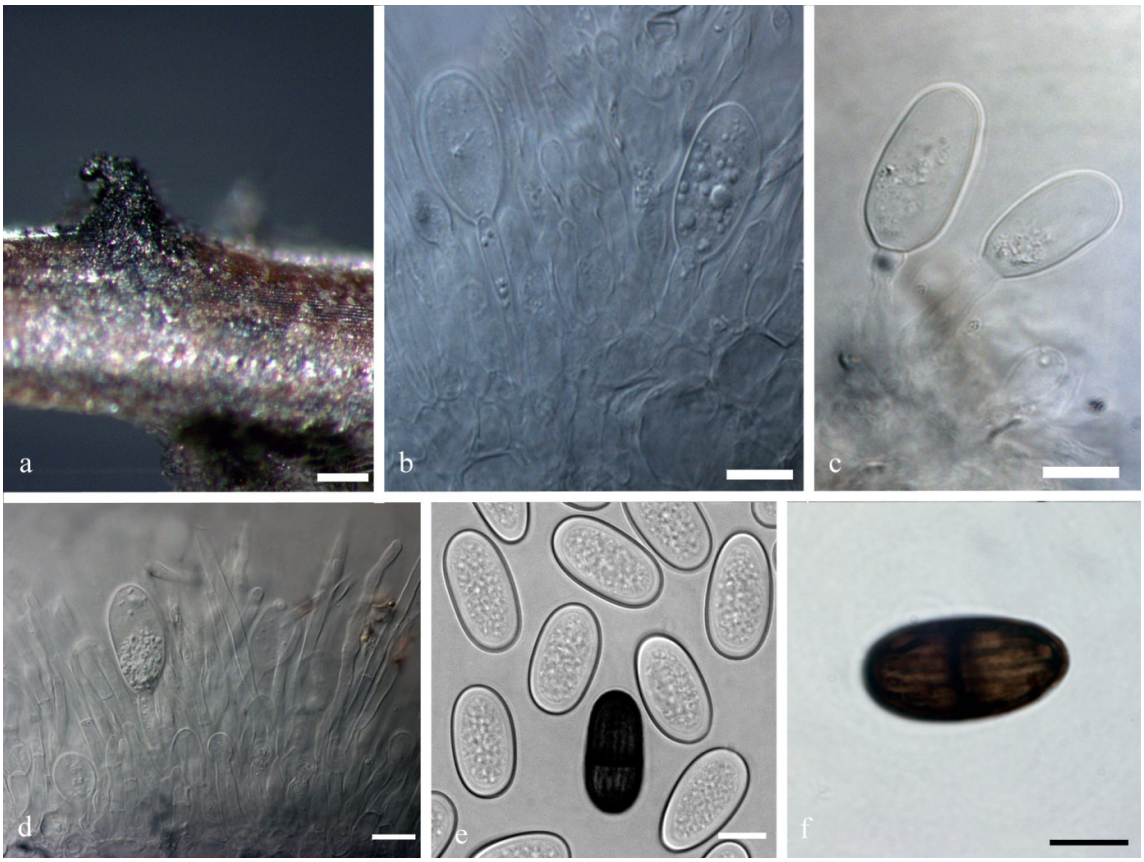

a
b
c
d
e
f

Figure 5. *Neofusicoccum terminaliae.* (a) Pycnidium formed on pine needles in culture, (b, c) conidiogenous cells with developing conidia, (d) paraphyses, (e) aseptate conidia and (f) 3-septate conidium. Bars: a = 200 µm; b, c, d, e, f = 10 µm.

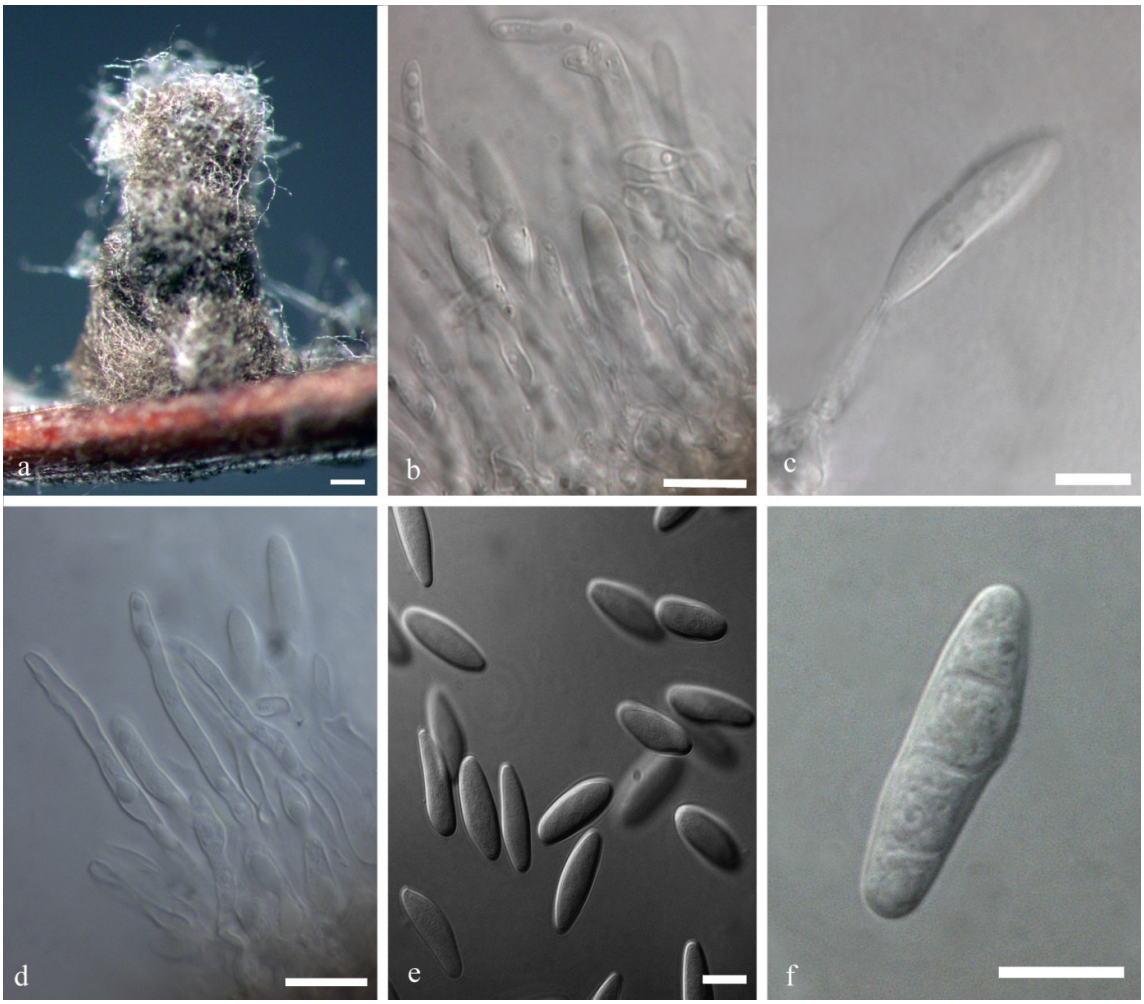
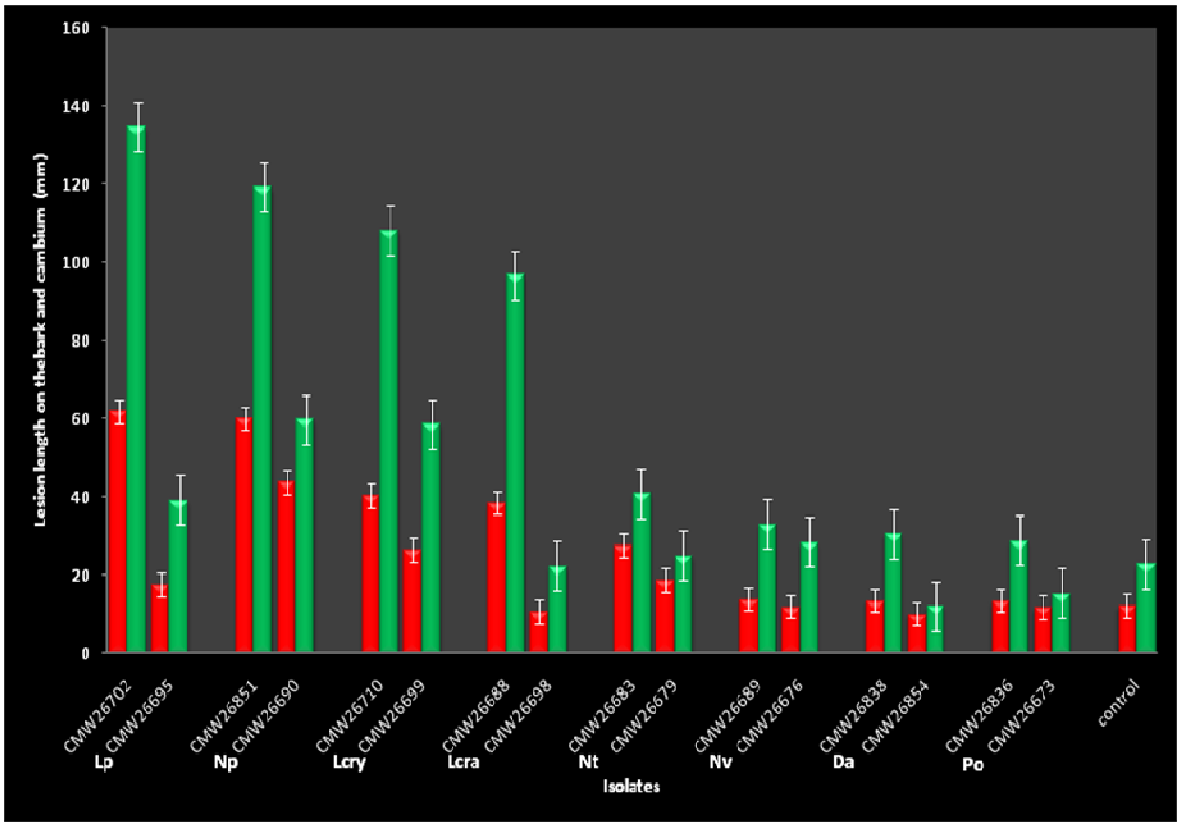

a
b
c
d
e
f

Figure 6. Mean lesion lengths (mm) on bark (red) and cambium (green) for each Botryosphaeriaceae isolate six weeks after inoculation on branches of *T. sericea* ($P < 0.0001$). *L. pseudotheobromae* (Lp), *N. parvum* (Np), *L. cryptotheobromae* (Lcry), *L. crassipora* (Lcra), *N. terminaliae* (Nt), *N. vitifusiforme* (Nv), *D. alatafructa* (Da), *P. olivaceum* (Po) and the control.


Lesion length on the bark and cambium (mm)
160
140
120
100
80
60
40
20
0
CMW26702
CMW26695
CMW26851
CMW26690
CMW26710
CMW26699
CMW26688
CMW26698
CMW26683
CMW26679
CMW26689
CMW26676
CMW26838
CMW26854
CMW26836
CMW26673
control
Lp
Np
Lcry
Lcra
Nt
Nv
Da
Po
Isolates