Chapter 2

Botryosphaeriaceae, including two new species, identified from a *Eucalyptus* arboretum in Pretoria, South Africa

Abstract

Disease symptoms that are typical for the Botryosphaeriaceae were observed in a small *Eucalyptus* arboretum in Pretoria, which is maintained to provide feed for Koala bears at the Pretoria Zoo. The aim of this study was to identify and characterize species of Botryosphaeriaceae from the 20 *Eucalyptus* species maintained in this arboretum and to determine their pathogenicity. Isolations were made from diseased and asymptomatic twigs and leaves. All isolates resembling species of Botryosphaeriaceae were induced to sporulate in culture and were subsequently characterized based on anamorph morphology. DNA sequence data for the ITS rDNA, translation elongation factor 1-α (EF- 1 α) and β-tubulin region of selected isolates were compared with known species to establish their identity. A previously developed PCR-RFLP technique was used to separate isolates of *Neofusicoccum parvum* and *N. ribis*. Isolates that did not sporulate in culture were also distinguished using a PCR-RFLP fingerprinting technique. Results revealed five species of the Botryosphaeriaceae including *B. dothidea, N. parvum*, *N. eucalypti* comb. nov. and two new species described here as *Neofusicoccum ursorum* sp. nov. and *N. crypto-australe* sp. nov. While there was no indication of host specificity, all five species were pathogenic on *Eucalyptus*.

Introduction

The Botryosphaeriaceae are among the most common fungi that cause diseases of various commercially grown trees. These fungi have been reported on branches and leaves of gymnosperms and angiosperms, culms of monocotyledons, herbaceous stalks or leaves of dicotyledons, worldwide (von Arx 1987). The various Botryosphaeriaceae are associated with disease symptoms such as cankers and dieback, leaf lesions and tip blight, fruit rots and even death of the trees (Shearer *et al*. 1987; Crous *et al*. 1989; Smith *et al*. 1994). Species of Botryosphaeriaceae can also exist in plant tissue as endophytes or latent pathogens. These fungi typically do not cause any visible symptoms until the onset of stressful environmental conditions such as drought, hail and frost damage, hot and cold winds and damage caused by hail and frost (Pusey 1989; Old *et al*. 1990).

The taxonomy of the Botryosphaeriaceae has been confused in the past. Identification was commonly achieved based on morphological characteristics or the host on which species were found (e.g. von Arx and Müller 1954; Shoemaker 1964; Pennycook and Samuels 1985). The many overlapping morphological characteristics among different species and the fact that some morphological features change with age lead to a substantially misleading taxonomy for these fungi. Recent studies, combining molecular and morphological characters, have led to an extensive revision of the taxonomy of the Botryosphaeriaceae (Denman *et al*. 2000; Slippers *et al*. 2004*a*; Crous *et al*. 2006). For example, *B*. *dothidea* (Moug.: Fr.) Ces. & De Notaris, previously considered one of the most important canker and dieback causing fungi on *Eucalyptus* in South Africa, was shown to represent *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers and A.J.L. Phillips and *N*. *eucalyptorum* (Crous, H. Smith. & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (Slippers *et al*. 2004*b*).

Eucalyptus have been planted as exotics in many parts of the world, including South Africa. It has been previously suggested that the introduction of these trees can also introduce pathogens into new areas via planting stock or seed (Wingfield *et al*. 2001). Evidence of the presence of pathogenic fungi such as species of Botryosphaeriaceae on seeds has been documented on *Eucalyptus* and other hosts (Webb 1983; Cilliers *et al*. 1993; Lupo *et al*. 2001; Gure *et al*. 2005). Due to their seed-borne nature and existence in

asymptomatic plant material, species of Botryosphaeriaceae can be expected to be easily moved unnoticed into new areas together with *Eucalyptus* (Slippers and Wingfield 2007).

Botryosphaeriaceae have a wide host range with the ability to move between native and introduced hosts (Slippers *et al*. 2005; Slippers and Wingfield 2007). For example, no restrictions of the gene flow of canker pathogen *N. australe* (Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips were detected between non-native *Eucalyptus* globulus plantations and native eucalypt forests in Western Australia (Burgess *et al*. 2006). As another example, all species of Botryosphaeriaceae found on the native *Syzygium cordatum* in South Africa, were found to be more pathogenic on *Eucalyptus*, with a number of these species overlapping in occurrence between the two hosts (Pavlic *et al*. 2007). Consequently, *Eucalyptus* can be expected to acquire new species of Botryosphaeriaceae from the surrounding trees in a new area, and to donate species to native plant communities.

An arboretum of 20 different *Eucalyptus* spp. has been established in Pretoria, South Africa. This resource was established to provide feed for Koala bears housed at the Pretoria Zoo. As part of an overall disease evaluation, a survey was conducted to identify and characterize species of Botryosphaeriaceae on these trees and to compare them to species found on naturally regenerated *Eucalyptus* trees surrounding the arboretum.

Materials and methods

Isolates

Eighty four isolates were obtained from 20 *Eucalyptus* spp. growing in an arboretum in Pretoria, South Africa, as well as from surrounding wild *Eucalyptus* trees (Table 1). The arboretum consisted of 12 blocks (20 rows in a block), with each row having 11 trees of each *Eucalyptus* sp. Three trees (tree 1, 5 and 10 of each row) of each species were sampled from three of the blocks (block 1, 6 and 7). Twenty five *Eucalyptus* trees surrounding the arboretum were sampled. Isolations were made using the protocol described by Pavlic *et al*. (2004). All cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphology and cultural characteristics

Isolates were induced to sporulate on sterilized pine needles placed on 2 % water agar (WA) (Agar; Biolab, South Africa) and incubated at 25 °C under near-UV light. Pycnidia formed on pine needles after two to three weeks of incubation. A mass of conidia oozing from the pycnidia were mounted in 85 % lactic acid on microscopic slides and examined using a light microscope. Images were captured using an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd., Münhen, Germany). Conidia (20–50) and 50 conidiogenous cells were measured for each isolate. Colony morphology and colour were noted for cultures grown on 2 % malt extract agar (MEA) (Biolab, South Africa) at 25 °C and culture colours were defined by comparison to the colour charts of Rayner (1970). Growth studies were conducted for selected isolates representing each species of Botryosphaeriaceae at temperatures ranging from 10–35 °C in the dark. Each culture was grown in duplicate and subsequently growth was measured perpendicularly. Culture growth at different temperatures was measured daily until 90 mm plates had been covered by mycelium.

DNA extraction and PCR amplification

Single conidial cultures were grown on 2 % MEA at 25 °C for 7 days. The mycelium was scraped directly from the medium and transferred to Eppendorf tubes (1.5 mL) and 300 µL of an extraction buffer (200 mM Tris-HCl pH 8.5, 150 mM NaCl, 25 mM EDTA, 0.5 % SDS) was added. A modified phenol:chloroform method for DNA extraction was followed (Raeder and Broda 1985, Slippers *et al*. 2004*a*). The resulting DNA pellets were re-suspended in 30 µL sterile SABAX water. RNAse (1mg/ µL) was added to DNA suspensions and left overnight at the room temperature for RNA degradation. DNA electrophoresis was performed on a 1.5 % agarose gel, stained with ethidium bromide. Bands were visualised under ultra-violet light. DNA concentration was estimated against a λ standard size marker.

The ITS region was amplified using primers ITS 1 and ITS 4 (White *et al*. 1990) and part of the β-tubulin gene region was amplified using the primers Bt2a and Bt2b (Glass and Donaldson 1995). A portion of the EF-1α gene region, was amplified with primer set EF-AF (5´ CAT CGA GAA GTT CGA GAA 3´) and EF-BR (5´ CRA TGG

TGA TAC CRC GCT C 3´) (Sakalidis 2004). The reaction mixture contained 2.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Almeda, California), 10 × PCR buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM dNTPs and 10 mM of each primer. The reaction mixture was made up to the final volume of 25 µL with sterile water. The following PCR program was used: denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing temperature at 55 °C for 45s, elongation at 72 °C for 1½ min and a final elongation step at 72 °C for 5 min. The PCR amplicons were viewed on a 1 % agarose gel, stained with ethidium bromide under UV-light. To estimate the band sizes, a 100 bp marker was used.

PCR-RFLP analysis

Two groups of isolates could be separated based on conidial morphology. Variations observed in the largest group indicated that there can be more than one species. A PCR-RFLP technique developed by Slippers (2003) was used to distinguish the species identified in this study based on morphology. ITS rDNA amplicon digestion with the restriction enzymes (RE) *Hha*I and *Ksp*I could separate three species from isolates identified using morphology. Isolates identified to belong to the *Neofusicoccum parvum* / *N. ribis* complex based on this ITS rDNA PCR-RFLP technique, were further identified by PCR-RFLP analysis of the amplicon obtained using primers *Bot 15* and *Bot 16* (Slippers *et al*. 2004*b*) and RE *Hha*I. The PCR-RFLP reaction mixture for all the above reactions consisted of 20 µL PCR product, 0.3 µL RE *Hha*I or *Ksp*I and 2.5 µL of the matching enzyme buffer (Fermentas, South Africa). The reaction mixture was incubated at 37 °C overnight. Digested fragments were separated on a 3 % agarose gel ran at a low voltage (60V) for 1 hour.

DNA sequencing and analysis

Representative isolates from each group (twenty six in total) including those that could not be identified based on morphology and PCR-RFLP technique, were sequenced using the primers that were used for the PCR amplification. The ITS sequences were compared to those of known Botryosphaeriaceae obtained from GenBank, with a particular focus on those previously isolates from *Eucalyptus* (Table 1). Two sequences of

Lasiodiplodia theobromae (Pat. Griffon and Maubl.) were used as outgroup. Five isolates that grouped separately from known species were further sequenced using EF-1α and β-tubulin primers in order to confirm their identity. Sequencing of the purified products was carried out by using ABI PRISM 3100^{TM} automated DNA sequencer (Perkin-Elmer). Nucleotide sequences were analysed and edited using SEQUENCE NAVIGATOR version 1.0.1. (Perkin- Elmer Applied Bio-Systems, Foster City, CA) software. Online software, MAFFT version 5.667 (http://timpani.genome.ad.jp/~mafft/ server/), was used for original alignments (Katoh *et al*. 2002), after which manual adjustments were made. Phylogenetic analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b8 (Swofford 1999) with gaps treated as the fifth character and all characters assumed to be unordered and of equal weight. The heuristic search function was used to obtain the maximum parsimony tree and random stepwise addition and tree bisection and reconstruction (TBR) were chosen as the branch swapping algorithm. A thousand (1000) bootstrap replicates were used as a measure of branch support (Felsenstein 1985).

Pathogenicity

Thirteen isolates representing five species of the Botryosphaeriaceae that were identified in this study were used in pathogenicity tests in the greenhouse (Table 1). Two or three of the fastest growing isolates (on MEA) per species were selected and grown on MEA at 25 ^{o}C under continuous near-fluorescent light for seven days before inoculations.

Two-year-old trees of an *Eucalyptus grandis* clone (ZG-14) were maintained in the green house for approximately three weeks prior to inoculations for acclimatization. The greenhouse was exposed to natural day and night light cycles at a constant temperature of about 25 ^{o}C. Ten trees were inoculated with each of the 13 isolates selected. As a control, 30 trees were inoculated with sterile MEA plugs. Wounds were made on the stems of trees using 8 mm diameter cork borer approximately 250 mm above the soil level. Mycelial plugs were prepared from seven-day-old cultures, with the same size cork borer. Mycelial plugs were placed into wounds with the mycelium facing the exposed cambium and sealed with laboratory film (Parafilm “M”, Pechiney Plastic Packaging, Chicago, U.S.A) to prevent drying out and contamination. Lesion lengths were measured six weeks after inoculation. Re-isolation of the fungi from resulting lesions was done by cutting small

pieces of the wood from the edge of lesions and plating it on 2 % MEA at 25 ^{o}C. The entire trial was repeated once to verify the pathogenicity of all isolates under the same conditions.

Lesion lengths that developed six weeks after inoculation were used as a measure of the pathogenicity. SAS® version 8.2 (2001) was used for statistical analysis of the data produced. The data were analyzed separately for each of the two trials and because there were no significant difference between them, the data were combined. P-values generated after the statistical analysis of the combined dataset were used as a measure of determining the significant differences amongst lesions produced.

Results

Morphology and cultural characteristics

Forty four of the 84 isolates produced anamorph structures on WA amended with pine needles. No teleomorph structures were observed. Two groups were obtained based on length to width (L/W) ratio of conidial morphology (Table 2). Isolates representing the first group with the L/W ratio of 2.8–4.0 could not be distinguished to species level based on conidial and cultural morphology, therefore PCR-RFLP technique and sequencing was used. In that group, few isolates were distinguished from others based on yellow pigmentation in culture. The second group could be easily distinguished from other species based on a higher L/W ratio. All isolates produced conidia typical for the *Neofusicoccum* species, and two groups were identified to species level. The morphology of the other three groups could not be linked to species with confidence and are described in detail later under the taxonomy section, following molecular identification.

PCR-RFLP analysis

Three profiles (restriction length polymorphism fingerprints) were observed after digesting ITS rDNA PCR product with RE *Hha*I (Fig. 4) and were matched with those specified by Slippers (2003). Based on this comparison, forty isolates represented *Neofusicoccum parvum / N. ribis* (profile A), 28 isolates represented either *D. eucalypti* or one of the two undescribed *Neofusicoccum* species identified using sequencing (profile B) and 10 isolates represented *Botryosphaeria dothidea* (profile C). The ITS rDNA

amplicons of all isolates were then digested with (RE) *Ksp*I and three profiles were observed (Fig. 5). *Neofusicoccum* sp. 1 (clade VII) (profile B) could be separated from the rest following this digestion with (RE) *Ksp*I. All the remaining isolates had the same profile (profile A) and their identity were confirmed with DNA sequence data (*D. eucalypti* and *Neofusicoccum* sp. 2).

All forty isolates that were identified as belonging to the *N. parvum* / *N. ribis* complex produced the same profile following *Hha*I digestion of the *Bot 15* and *Bot 16* primers amplicons (Fig. 6). This profile matched that of *N. parvum* as described by Slippers (2003). With the use of these PCR-RFLP profiles and DNA sequence data, the number of isolates per species obtained from both *Eucalyptus* in the arboretum, as well as in the surrounding *Eucalyptus* spp. could be confirmed for all 84 isolates (Table 4).

DNA sequencing and analysis

Sequences of approximately 550 bp (ITS rDNA), 300 bp (EF-1α) and 390 bp (β-tubulin) were obtained. Aligned ITS dataset consists of 60 in-group sequences of which 26 represent isolates obtained in this study and 34 sequences of known species of Botryosphaeriaceae that occur on *Eucalyptus*, obtained from GenBank (Table 1). The primary analysis produced 112 most parsimonious (MP) trees of 154 steps (consistency index (CI) = 0.747, retention index (RI) = 0.952) and one of them is presented (Fig. 1).

Twelve terminal clades were observed for all isolates considered in the phylogenetic analyses of ITS sequence data (Fig.1). Most isolates obtained in this study grouped with other species of *Neofusicoccum* and some isolates grouped with *Botryosphaeria*. The *Neofusicoccum* clade consisted of *N. ribis* (Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade I), *N. parvum* (clade II), *N. macroclavatum* (T. Burgess, Barber & G.E Hardy) T. Burgess, Barber and G.E Hardy (clade III), *D. eucalypti* (G. Winter) B. Sutton (clade IV), *N. vitifusiforme* (van Niekerk & Crous) Crous, Slippers and A.J.L Phillips (clade V), an unknown species (*Neofusicoccum* sp. 1) closely related to *Dichomera eucalypti* (clade VI), an unknown species (*Neofusicoccum* sp. 2) closely related to *N. australe* (clade VII), *N. australe* (Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade VIII), *N. luteum* (Pennycook & Samuels) Crous, Slippers and A.J.L. Phillips (clade IX), *N. eucalypticola*

(Slippers, Crous & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade X), *N. eucalyptorum* (Crous, H. Smith. ter & M.J. Wingfield) Crous, Slippers and A.J.L Phillips (clade XI). The other major clade represents *B. dothidea* (clade XII). All isolates obtained in this study grouped into five different clades, namely clades II (*N. parvum*), IV (*D. eucalypti*), VI (*Neofusicoccum* sp. 1) and VII (*Neofusicoccum* sp. 2), XII (*B. dothidea*) (Fig. 1).

A subset of isolates were analysed using EF-1α data to strengthen support for the branches observed using ITS sequences and to test congruence between the trees. EF-1α aligned sequences contained 42 taxa and 360 characters of which 123 characters were parsimony informative. The data for the EF-1α gene region produced 100 MP trees of 390 steps (consistency index (CI) = 0.831, retention index (RI) = 0.957). A partition homogeneity test produced a P-value = 0.1, showing that the ITS rDNA and EF-1α sequence data are combinable (Cunningham 1997). The ITS rDNA and EF-1α data sets were, therefore, combined and the aligned dataset consists of 40 in-group sequences, of which fourteen sequences represent isolates from this study and the rest were obtained from GenBank (Table 1). Of the 882 characters in the combined dataset, 200 characters were parsimony informative. Combined data for the ITS rDNA and EF-1α gene regions produced 88 MP trees of 369 steps (consistency index (CI) = 0.805, retention index (RI) = 0.953), of which one is presented (Fig. 2). Twelve terminal clades were observed (Fig. 2) and they were consistent with those observed in the tree based on ITS rDNA sequence data.

In the ITS rDNA sequence dataset, Clade VII included an isolate previously isolated from *Wollemia nobilis* and three from *Syzygium cordatum*. This clade was very closely related to clade VIII (*N. australe*) and was, therefore, also characterized using sequence data from third gene region namely β-tubulin. The three datasets contained 525 (ITS rDNA), 274 (EF-1α) and 388 (β-tubulin) characters, of which 3, 7 and 3 characters in each datasets respectively, were indicated in the analysis. All the variable characters in all three gene regions were parsimony informative. One MP tree was obtained for ITS rDNA, (Tree length = 3, consistency index (CI) = 1.00, retention index (RI) = 1.00); three for EF-1α (Tree length = 8, CI = 0.875, RI = 0.971) and one for β-tubulin (Tree length = 3, CI = 1.00, RI = 1.00) (Fig. 3). Fixed, unique polymorphism were identified in all three gene

regions, which produced congruent trees from the individual loci that separated clade VII (*Neofusicoccum* sp. 2) and clade VIII (*N. australe*), supporting the view that they are distinct species (Fig. 3; Table 3).

Taxonomy

Two previously undescribed species of the Botryosphaeriaceae were identified in this study. They reside in *Neofusicoccum* and were distinguished from known *Neofusicoccum* species based on anamorph morphology, DNA sequence data and PCR-RFLP analysis. These species are described below. Furthermore, *Dichomera eucalypti* was shown in this and previous studies to belong to *Neofusicoccum*, and a new combination is therefore proposed in this genus.

Neofusicoccum ursorum. *Maleme*, *Pavlic*, *Slippers sp. nov. Mycobank MB 512478* (Fig. 7)

Etymology: Name refers to the Koala 'bears', that feed on *Eucalyptus* spp. that were sampled in this study

Teleomorph: Unknown

Conidiomata: pycnidia solitaria subimmersa papillata, pilis hyphalibus tecta, nigra, usque ad 645 µm diametro. *Conidia* hyalina laevia granulis tenuibus, non septata, fusiformia vel ellipsoidea, (20.8–)22–26(–28.4) × (5.6–)6.5–8(–8.2) µm. *Cellulae conidiogenae* hyalinae holoblasticae cylindricae vel subcylindricae (8.8–)10–14(–15.5) × (2.0–)2.5–3(–3.3) µm.

Conidiomata: pycnidia (produced *in vitro* on pine needles on water agar within 14 days) solitary, semi-immersed, papillate, covered by hyphal hairs, black, up to 645 µm diam. *Conidia:* hyaline, smooth with contents having fine granular appearence, aseptate, fusiform to ellipsoid (20.8–)22–26(–28.4) × (5.6–)6.5–8(–8.2) µm (ave. of 50 conidia 24 × 7.1 µm, l/w 3.4). *Conidiogenous cells:* hyaline, holoblastic, cylindrical to subcylindrical (8.8–)10–14(–15.5) × (2.0–)2.5–3(–3.3) µm (ave. of 50 conidiogenous cells 12.1 × 2.8 µm). *Cultural characteristics:* colonies initially white with fluffy aerial mycelium changing after 3-4 days to pale olivaceous grey from the middle of the colony (both sides), margins regular. Optimum temperature for growth 30 ^{o}C, colonies grown on MEA covering a 90 mm diam plate after 7 days of incubation in the dark.

Habitat: Endophytic in healthy leaves of *Eucalyptus* spp.

Known distribution: Pretoria, South Africa

Material examined: South Africa, Pretoria, Gauteng province, from branches and leaves of living *Eucalyptus* trees, May 2005, H.M. Maleme (Holotype PREM 59815) (Culture CMW 24480); Gauteng province, *Eucalyptus* trees, H.M. Maleme (Paratype PREM 59816) (Culture CMW 23790).

Neofusicoccum crypto-australe. *Maleme, Pavlic, Slippers sp. nov. Mycobank MB 512477* (Fig. 8)

Etymology: Referring to cryptic species closely related to *N. australe*

Teleomorph: Unknown

Conidiomata: pycnidia solitaria subimmersa papillata, pilis hyphalibus tecta, nigra usque ad 575 µm diametro. *Conidia* hyalina laevia contentis granularibus, non septata, fusiformia apicibus rotundatis (21.0–)18–20.5(–26.4) × (4.9–)5–6(–6.2) µm, cum maturitate brunnescentia et semel vel bis septata. Cellulae conidiogenae hyalinae holoblasticae cylindricae vel subcylindricae (11–)11.5–12.5(–12.9) × (2.0–)2.2–2.7(–2.9) µm.

Conidiomata: pycnidia (produced *in vitro* on pine needles on water agar within 14 days) solitary, semi-immersed, papillate, covered by hyphal hairs, black, up to 575 µm diam. *Conidia:* hyaline, smooth with granular contents, aseptate, fusiform, apices rounded (18–)20.5–21.0(–26.4) × (4.9–)5–6(–6.2) µm (ave. of 50 conidia 19 × 5.5 µm, l/w 3.5), becoming brown and one or two-septate with maturation. *Conidiogenous cells:* hyaline, holoblastic, cylindrical to subcylindrical (11–)11.5–12.5(–12.9) × (2.0–)2.2–2.7(–2.9) µm (ave. of 50 conidiogenous cells 11.9 × 2.4 µm). *Cultural characteristics:* colonies initially white with fluffy aerial mycelium, changing to straw yellow after 3 days of incubation. After 4-7 days the colour changed to pale olivaceous grey from the middle of the colony from the top and reverse, margins regular. Optimum temperature for growth at 25 ^{o}C, covering a 90 mm diam MEA plate after 3 days of incubation in the dark.

Habitat: Endophytic in healthy leaves of *Eucalyptus* spp.

Known distribution: Pretoria, South Africa

Material examined: South Africa, Pretoria, Gauteng province from branches and leaves of living *Eucalyptus* trees, May 2005, H.M. Maleme (Holotype PREM 59817) (Culture

CMW 23785); Gauteng province, *Eucalyptus* trees, H.M. Maleme (Paratype PREM 59818) (Culture CMW 20738); Gauteng province, *Eucalyptus* trees, H.M. Maleme (PREM 60063) (Culture CMW 23787); Gauteng province, *Eucalyptus* trees, H.M. Maleme (PREM 60064) (Culture CMW 23784); Gauteng province, *Eucalyptus* trees, H.M. Maleme (PREM 60065) (Culture CMW 23786).

Neofusicoccum eucalypti. *(Winter) Maleme*, *Pavlic*, *Slippers comb. nov. Mycobank MB 512501* (Fig. 9)

Basionym: Camarosporium eucalypti G. Winter (1886).

≡*D. eucalypti* B. Sutton, *Mycol. Pap.* **138**: 182 (1975).

≡*Camarosporellum eucalypti* (G. Winter) Tassi, *Bull*, *Lab*, *ort. Bot.* Siena **5**: 62 (1902).

=*Coryneum viminale* Cooke and Massee, Grevillea **20**: 36 (1891).

Conidiomata: pycnidia (produced *in vitro* on pine needles on water agar within 14 days) solitary, semi-immersed, papillate, covered by hyphal hairs, black, up to 625 µm diam. *Conidia:* hyaline, smooth with granular contents, aseptate, fusiform, apices rounded (16.8–)17.7–19.7(–21.6) × (4.1–)4.9–5.9(–6.3) µm (ave. of 50 conidia 18.7 × 5.4 µm, l/w 3.5). *Conidiogenous cells:* hyaline, holoblastic, cylindrical to subcylindrical (9.8–)11–15(–15.5) × (3.0–)3.5–4(–3.3) µm (ave. of 50 conidiogenous cells 13.1 × 3.8 µm). *Cultural characteristics:* colonies initially white with fluffy aerial mycelium changing after 3-4 days to pale olivaceous grey from the middle of the colony (both sides), margins regular. Optimum temperature for growth 30 ^{o}C, colonies grown on MEA covering a 90 mm diam plate after 7 days of incubation in the dark.

Notes: *Dichomera eucalypti* was formally described by Sutton (1975), based on *Camarosporium eucalypti* (G. Winter) and including *Coryneum viminale* Cke and Massee as synomym. This description was based on similarity of the conidial morphology. These species are known to form irregular, globose to pyriform conidia that are brown and muriform with transverse, longitudinal and oblique septa. Barber *et al.* (2005) confirmed these observations by examining type material. Barber *et al.* (2005) also designated an

epitype specimen and ex-epitype isolate for this species. Sequence data of this ex-epitype isolate is linked here to isolates obtained from *Eucalyptus* in our study.

'*Dichomera*' *eucalypti* groups within the *Neofusicoccum* clade in this and other recently published studies (Barber *et al*. 2005; Burgess *et al*. 2005; Crous *et al*. 2006). *Neofusicoccum* is represented by species with *Fusicoccum*-like conidia sometimes having *Dichomera*-like synanamorphs (Crous *et al*. 2006). *Fusicoccum*-like conidia were observed in this study as opposed to muriform, globose conidia observed in the previous studies. Based on these morphological observations and the phylogenetic grouping of our isolates with isolates from Barber *et al*. (2005) (Fig. 1, clade IV), which were morphologically linked to the epitype, we transfer this taxon to *Neofusicoccum*.

Pathogenicity

All isolates inoculated in this study on the *Eucalyptus* clone ZG-14 produced lesions after six weeks that were significantly different to those of the controls (R-square = 0.58, Coefficient variable = 39.7, Root MES = 16.2), confirming their pathogenicity on this host (Fig. 10). The isolates could be recovered by re-isolations from the lesions. Some lesions were observed on trees used as controls, but no Botryosphaeriaceae were re-isolated from these lesions.

Isolates could be separated into two virulence classes (high and low) based on the mean lesion lengths produced. Virulence variation was observed amongst isolates within species, such that individual isolates of a species could group into either the higher or lower virulence category. The more virulent group of isolates is typified by one isolate of *N. eucalypti* (CMW 24571) and two isolates of *N. crypto-australe* (CMW 23785 and CMW 23786). The lesions produced by these isolates were significantly longer than those of all other isolates used for inoculations. The less virulent group represented by isolates from each species with the variation providing a continuous range (Fig. 10). On average, *B. dothidea* isolates were the least virulent and *N. crypto-australe* and *N. parvum* the most virulent (Fig. 10).

Discussion

Five species of the Botryosphaeriaceae were identified in this study, including *Botryosphaeria dothidea, Neofusicoccum parvum*, *N. eucalypti*, and the newly described species, *N. ursorum* and *N. crypto-australe.* These species were found in a small plot on 20 *Eucalyptus* spp. planted in a Pretoria arboretum and a few surrounding *Eucalyptus* trees. No host specificity was evident amongst these Botryosphaeriaceae and *Eucalyptus* spp. from which they were collected. Furthermore, three of these species of Botryosphaeriaceae are recorded on *Eucalyptus* in South Africa for the first time, which is interesting given the numerous previous studies considering these fungi on this host in the country.

Neofusicoccum parvum was the most commonly isolated species from *Eucalyptus* in this study. Subsequent to its first description from kiwifruit in New Zealand in 1985 (Pennycook and Samuels 1985), this species has been described from a wide variety of hosts in various parts of the world (Phillips *et al*. 2002; Gure *et al*. 2005; Pavlic *et al*. 2007). On *Eucalyptus,* it is commonly reported as the cause of canker and dieback, in countries including Uganda, Chile, Ethiopia, South Africa, Australia and Venezuela (Nakabonge 2002; Ahumada 2003; Gezahgne *et al*. 2004; Slippers *et al*. 2004*b*; Barber *et al*. 2005; Mohali *et al*. 2006). Its common occurrence on *Eucalyptus* in this study is thus not surprising. *Neofusicoccum parvum* is also known from native Myrtaceae in South Africa including *Heteropyxis natalensis* and *Syzygium cordatum* (Smith *et al*. 2001; Slippers *et al*. 2004*b*; Pavlic *et al*. 2007). Individual *N. parvum* isolates were pathogenic, but not the most virulent in this study. On average they were at least as virulent as any other species.

Neofusicoccum crypto-australe is described here as a cryptic sister species to *N. australe*. This species has previously been isolated from *Wollemia nobilis*, a native conifer in eastern Australia (Slippers *et al*. 2005) and on native *Syzygium cordatum* trees in South Africa (Pavlic *et al*. 2007). In those studies, it was not provided with a formal description due to the small number of isolates available. The occurrence of *N. crypto-australe* on two different native hosts in Australia and South Africa and now on non-native *Eucalyptus* in South Africa, makes it difficult to suggest a possible origin for the fungus. In this study, five isolates were obtained from *Eucalyptus* spp. in the arboretum and the surrounding

wild *Eucalyptus* trees. Its existence on *Eucalyptus* spp. could be explained by the movement of species of Botryosphaeriaceae from different continents through plant material, possibly from Australia where *Eucalyptus* is native. Alternatively, it could have jumped hosts from native *Syzygium cordatum* to introduced *Eucalyptus*, or *visa versa* in South Africa since both hosts were shown to share similar pathogens in the work done by Pavlic *et al*. (2007). Its wide host and geographic range, as well as the high level of virulence revealed in this study, makes it a potential threat to both native and non-native hosts in South Africa and Australia.

Neofusicoccum ursorum was represented by two isolates in this study. Both isolates were obtained from *Eucalyptus* trees surrounding the arboretum. *Neofusicoccum ursorum* has, to the best of our knowledge also never been isolated from any other area or host. It is thus possible that this species is native to the region and could be more common on other native trees surrounding the arboretum. A situation similar to this has previously been observed (Pavlic *et al*. 2007; Slippers and Wingfield 2007). *Neofusicoccum ursorum* was pathogenic, but only mildly virulent when compared to other species studied here.

Morphology alone is typically insufficient when identifying closely related fungal species and this approach tends to underestimate the true diversity of fungi on a host or in an area. This is also true for the identification of cryptic species of the Botryosphaeriaceae such as *N. crypto-australe* and *N. ursorum* discovered in this study. Molecular tools coupled with morphology have, however, been very successfully applied in recent years to overcome this problem in the Botryosphaeriaceae (Slippers and Wingfield 2007). *Neofusicoccum ursorum* is for example virtually indistinguishable from *N. parvum* and *N. eucalypti* in this study based on morphology, but clearly distinct from them based on phylogenetic analysis of ITS rDNA sequence data.

Some cryptic species may be very difficult to distinguish with confidence when considering only one gene region and, therefore, the application of multiple gene regions has been promoted for identification of the Botryosphaeriaceae (De Wet *et al*. 2003; Slippers *et al*. 2004*a*, *b*, *c*; Alves *et al*. 2008). In this study, *N. crypto-australe*, the sister species to *N. australe*, could have gone unidentified if only one gene region had been analysed. An analysis of concordance between three gene regions, ITS rDNA, EF-1α and β-tubulin, were needed to confidently separate these two species. Such concordance

between gene regions can only emerge in the absence of recombination between the groups.

'*Dichomera*' *eucalypti* (= *Camarasporellum eucalypti*) was described from *Eucalyptus* spp. in Australia (Sutton 1975) as producing globose, subglobose, obvoid, obpyriform, muriform or somewhat fusiform with septate conidia. This was confirmed by Barber *et al*. (2005) who designated an epitype specimen (and ex-type culture) for '*D. eucalypti*'. The isolates obtained in the present study were identical to these ex-type cultures in ITS rDNA and Ef-1α sequence data, but did not show the morphological characteristics described above. Instead, they produced *Fusicoccum*-like hyaline, aseptate, fusiform to ellipsoid conidia. This observation, together with the consistent grouping with other species of *Neofusicoccum*, validates its description here as *N. eucalypti*. Some other *Neofusicoccum* species (e.g. *N. parvum*, *N. australe*, see Barber *et al*. (2005) are also known to produce both *Fusicoccum*-like as well as *Dichomera*-like conidia, but is interesting that some isolates produce one of these very distinct conidial forms and not the other. The genetic basis of this unusual characteristic deserves further study.

Neofusicoccum eucalypti (= '*D. eucalypti*') is well known from woody tissues, foliage and bark samples of *Eucalyptus* spp. in Australia (Sutton 1975; Barber *et al*. 2005; Burgess *et al*. 2005). In this study, two isolates of this species were identified as endophytes from asymptomatic leaves in the *Eucalyptus* arboretum and one from the surrounding *Eucalyptus* spp. This is the first report of this fungus on *Eucalyptus* in South Africa. Its occurrence on non-native *Eucalyptus* in South Africa might have been anticipated due to its common association with *Eucalyptus* in Australia and the fact that the trees sampled in this study were generated from seed introduced from that country. Although the isolates of *N. eucalypti* varied significantly in virulence, it is noteworthy that one of the isolates was the second most virulent. The presence of *N. eucalypti* in South Africa, albeit at low levels currently, poses a potential threat to this host and its presence should be monitored.

Five isolates of *Botryosphaeria dothidea* were obtained in this study. This pathogen has been documented on many hosts worldwide, including *Eucalyptus* (Farr *et al*. 1989; Fisher *et al*. 1993; Smith *et al*. 1996). Recent studies have, however, indicated that this pathogen is not common on *Eucalyptus* and other related hosts (Slippers *et al*.

2004*b*; Pavlic *et al*. 2007). *Botryosphaeria dothidea* was found to be only mildly virulent in this study, which is consistent with recent studies that have considered its pathogenicity on species of Myrtaceae in South Africa, Venezuela and Colombia (Rodas 2003; Mohali 2005; Pavlic *et al*. 2007). Therefore, the previous assumption that *B*. *dothidea* is an important canker and dieback pathogen on *Eucalyptus*, appears to be incorrect.

Fifteen species of Botryosphaeriaceae have been identified in the past five years on *Eucalyptus*, applying both morphological characters and DNA sequence data (Slippers *et al.* 2007). Some are thought to be host specific and/or with a local distribution, while others have a broad host range and are more widely distributed. For example, *Lasiodiplodia theobromae* has been reported to occur on more that 500 host species (Punithalingam 1976). On *Eucalyptus* it has been documented in countries such as South Africa, Venezuela, Congo and Uganda (Roux *et al*. 2000, 2001; Burgess *et al*. 2003; Mohali *et al*. 2007). Species of the Botryosphaeriaceae, such as *B*. *mamane*, *N*. *stromaticum* and *N*. *andinum*, have, however, only been identified on *Eucalyptus* in Venezuela (Mohali *et*. *al*. 2006). The current study adds to this global picture of a combination of a few common generalists and some rare, apparently specific, species of the Botryosphaeriaceae that infect *Eucalyptus* at any given site. Future studies should also focus on native hosts surrounding plots, such as the one studied here, to better understand host and geographic distribution of certain species. Furthermore, it would be valuable to study the distribution of these Botryosphaeriaceae at a finer spatial scale on the trees in this plot, in order to better understand their biology and interaction.

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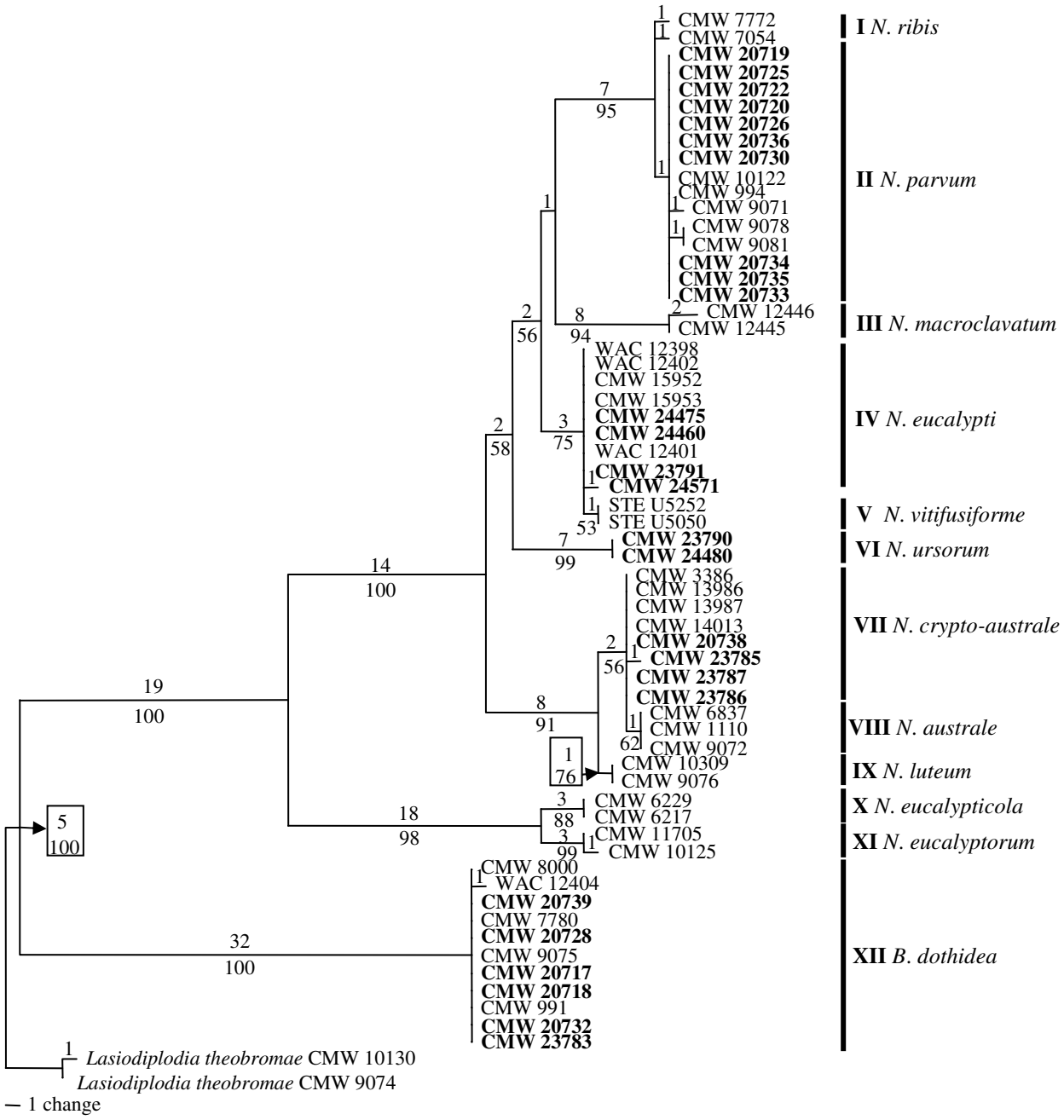
Fig. 1. One of the 112 most parsimonious trees obtained from sequence data of the ITS rDNA locus (tree length = 154 steps, CI = 0.747, RI = 0.952). Bootstrap values based on 1000 bootstrap replicates are shown below the branches and number of steps above the branches. Isolates sequenced in this study are in bold. The tree is rooted to *Lasiodiplodia theobromae*.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CMW 20728
CMW 9075
CMW 20717
CMW 20718
CMW 991
CMW 20732
CMW 23783
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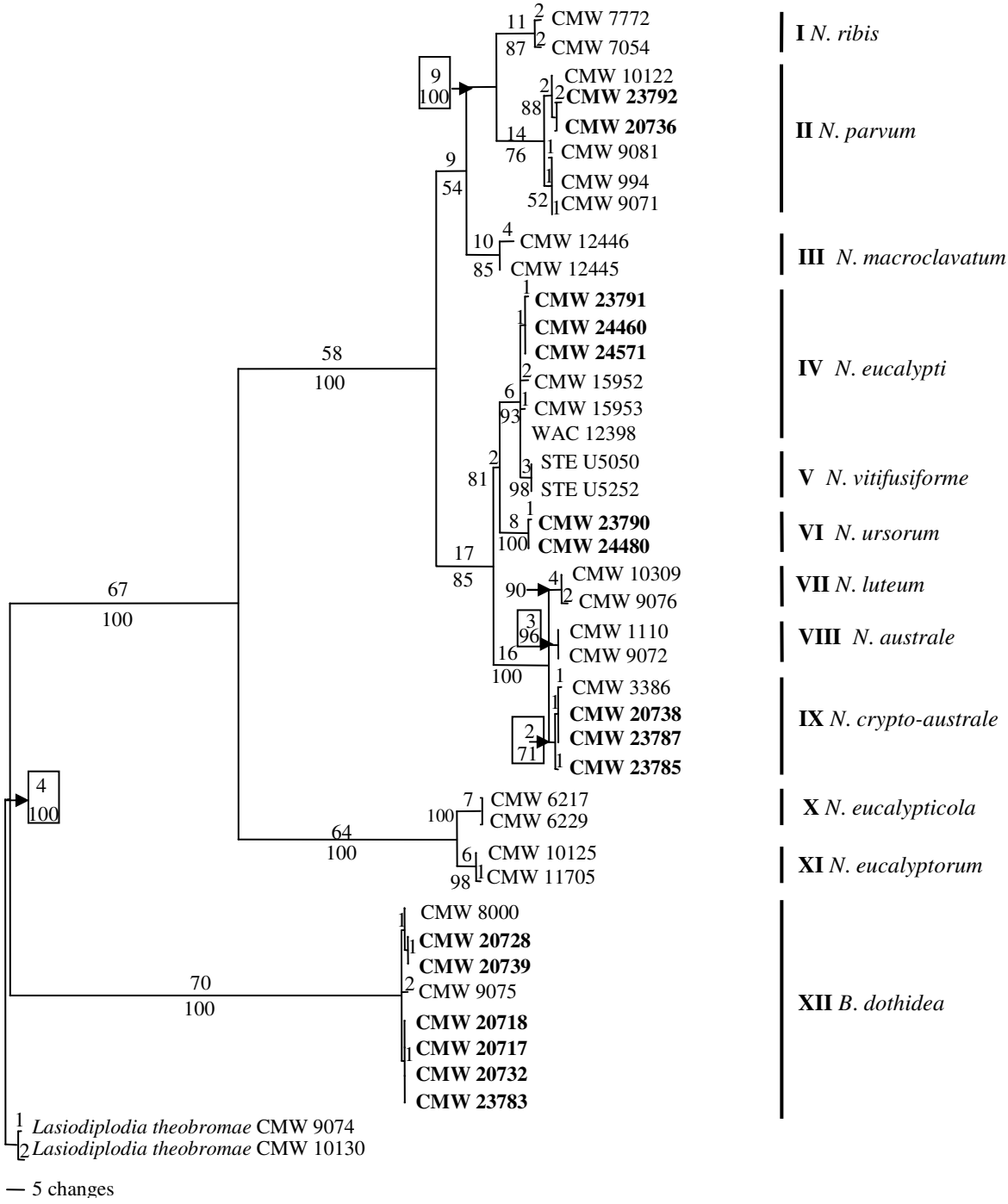
Lasiodiplodia theobromae CMW 9074
1 change
I N. ribis
II N. parvum
III N. macroclavatum
IV N. eucalypti
V N. vitifusiforme
VI N. ursorum
VII N. crypto-australe
VIII N. australe
IX N. luteum
X N. eucalypticola
XI N. eucalyptorum
XII B. dothidea

Fig. 2. One of the 88 most parsimonious trees obtained from the combined sequence datasets of the ITS rDNA and EF-1α loci (tree length = 369 steps, CI = 0.805, RI = 0.953). Bootstrap values based on 1000 bootstrap replicates are shown below the branches and number of steps above the branches. Isolates sequenced in this study are in bold. The tree is rooted to *Lasiodiplodia theobromae*.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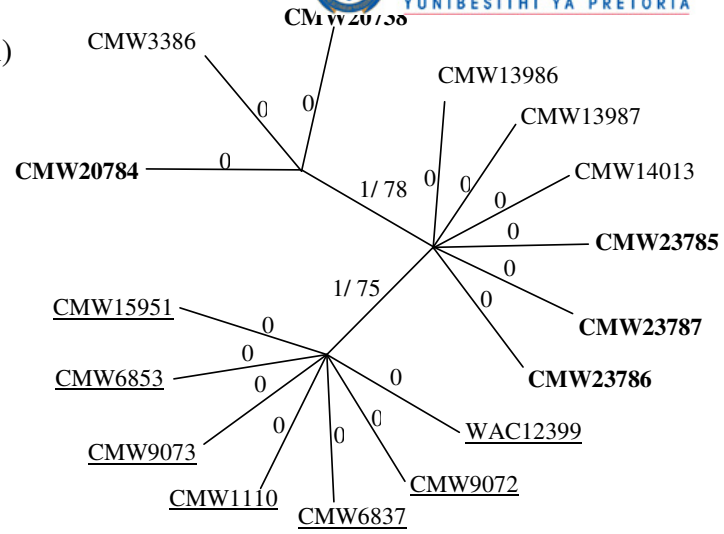
— 5 changes
I N. ribis
II N. parvum
III N. macroclavatum
IV N. eucalypti
V N. vitifusiforme
VI N. ursorum
VII N. luteum
VIII N. australe
IX N. crypto-australe
X N. eucalypticola
XI N. eucalyptorum
XII B. dothidea

Fig. 3. Unrooted cladograms obtained from DNA sequence comparison of three gene regions, (*A*) ITS rDNA, (*B*) EF-1α, and (*C*) β-tubulin. The trees represent isolates belonging to *N. australe* (underlined) and *N. crypto-australe* which group congruently in analyses of the three loci. Isolates sequenced in this study are in bold. The first numbers represent branch lengths and the second numbers bootstrap values (1000 replicates).

(A)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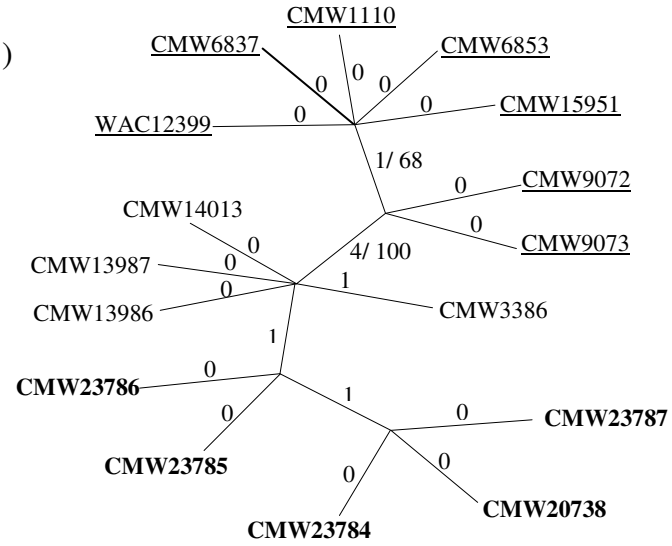
(B)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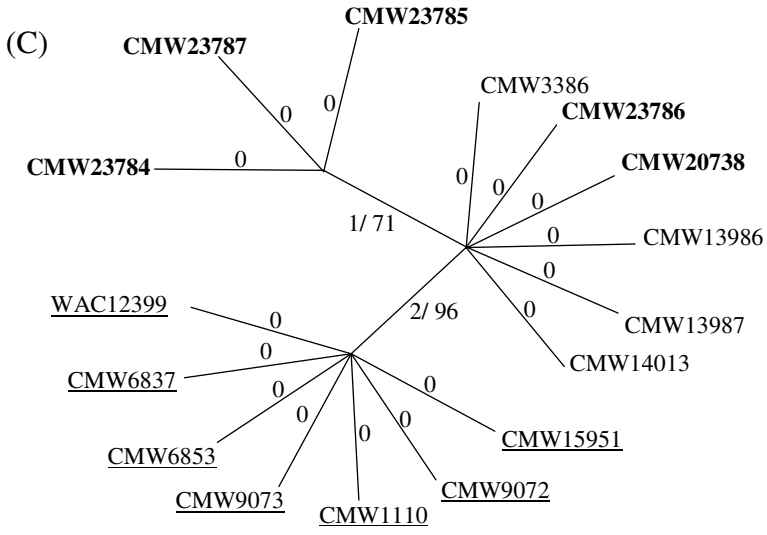
(C)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CMW6853
CMW9072
CMW9073
CMW1110

Fig. 4. Restriction fragment length polymorphism maps of five species of Botryosphaeriaceae from *Eucalyptus* after digestion of the ITS rDNA PCR products with the (RE) *Hha*I. *N. parvum* / *N. ribis* (*A*), *N. eucalypti*; *N. ursorum*; *N. crypto-australe* (*B*) and *B. dothidea* (*C*). The total length of each fragment is given in brackets and the fragment lengths are given below each line.

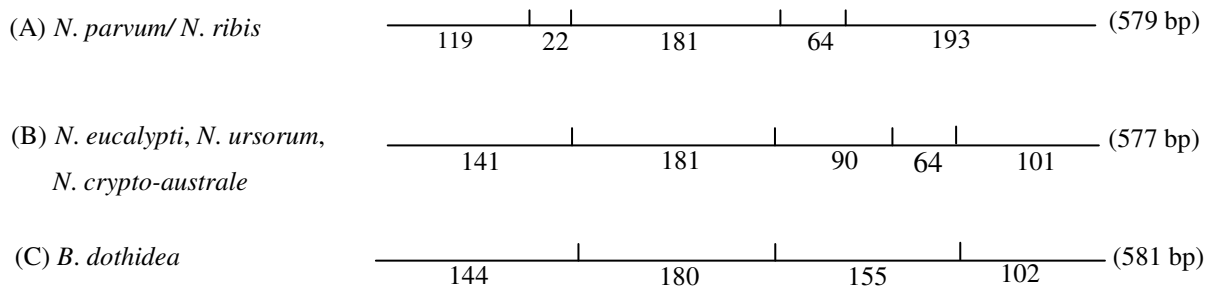
(A) N. parvum/ N. ribis
119
22
181
64
193
(579 bp)
(B) N. eucalypti, N. ursorum,
N. crypto-australe
141
181
90
64
101
(577 bp)
(C) B. dothidea
144
180
155
102
(581 bp)

Fig. 5. Restriction fragment length polymorphism maps of five species of Botryosphaeriaceae obtained from digestion of the ITS rDNA PCR products with (RE) *Ksp*I. *N. parvum*, *N. ursorum*, *N. eucalypti* (*A*), *B. dothidea* (*B*), *N. crypto-australe* (*C*). The total length of each fragment is given in brackets and the fragment lengths are given below each line.

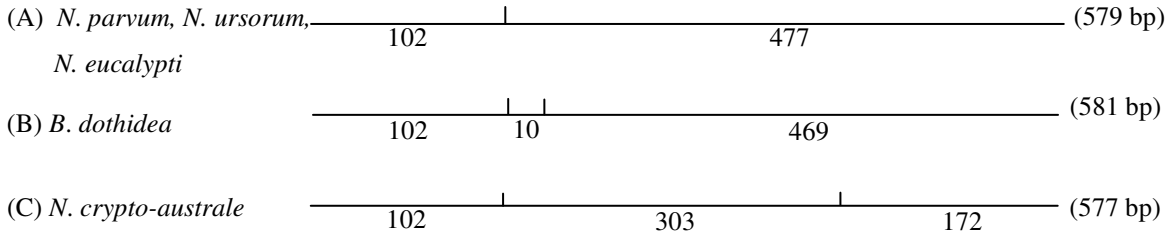
(A) N. parvum, N. ursorum, N. eucalypti
102
477
(579 bp)
(B) B. dothidea
102
10
469
(581 bp)
(C) N. crypto-australe
102
303
172
(577 bp)

Fig. 6. Restriction fragment length polymorphism maps of *N. ribis* (*A*) and *N. parvum* (*B*). PCR amplicons were amplified with primers *Bot 15* and *Bot 16* and digested with (RE) *Hha*I. The total length of each fragment is given in brackets and the fragment lengths are given below each line.

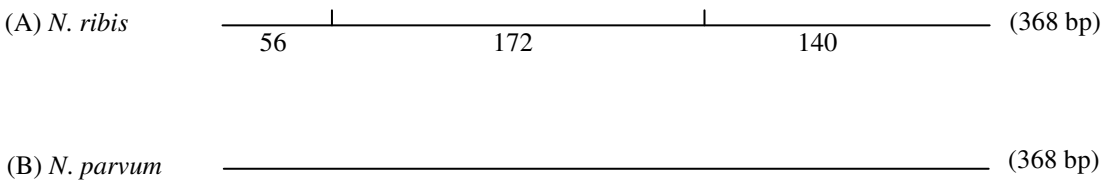
(A) N. ribis
56
172
140
(368 bp)
(B) N. parvum
(368 bp)

Fig. 7. Light micrographs of *Neofusicoccum ursorum*: (*A*) Pycnidia, (*B*,*C*) Conidiogenous cells, (*D*) Mature conidia, (*E*,*F*) Germinating conidia. Scale bar (*A*) = 500 µm, Scale bars (*B*-*F*) = 10 µm.

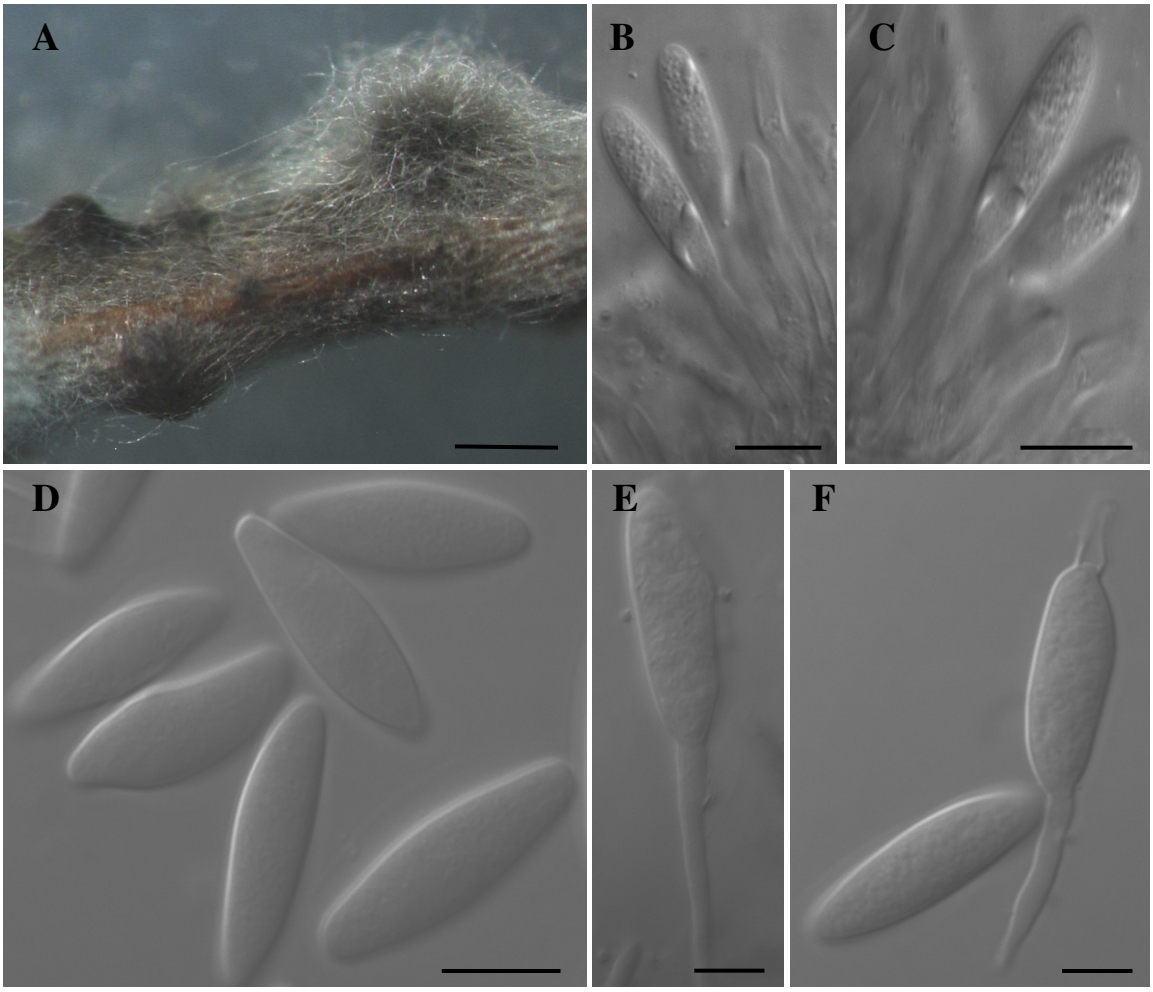
A
B
C
D
E
F

Fig. 8. Light micrographs of *Neofusicoccum crypto-australe*: (*A*) Pycnidium, (*B*) Conidiogenous cells, (*C*) 2-septate conidium, (*D*) One and two-septate germinating conidia, (*E*) aseptate conidia, (*F*) One and two-septate dark conidia. Scale bars (*A*) = 500 µm, Scale bar (*B-F*) = 10 µm.

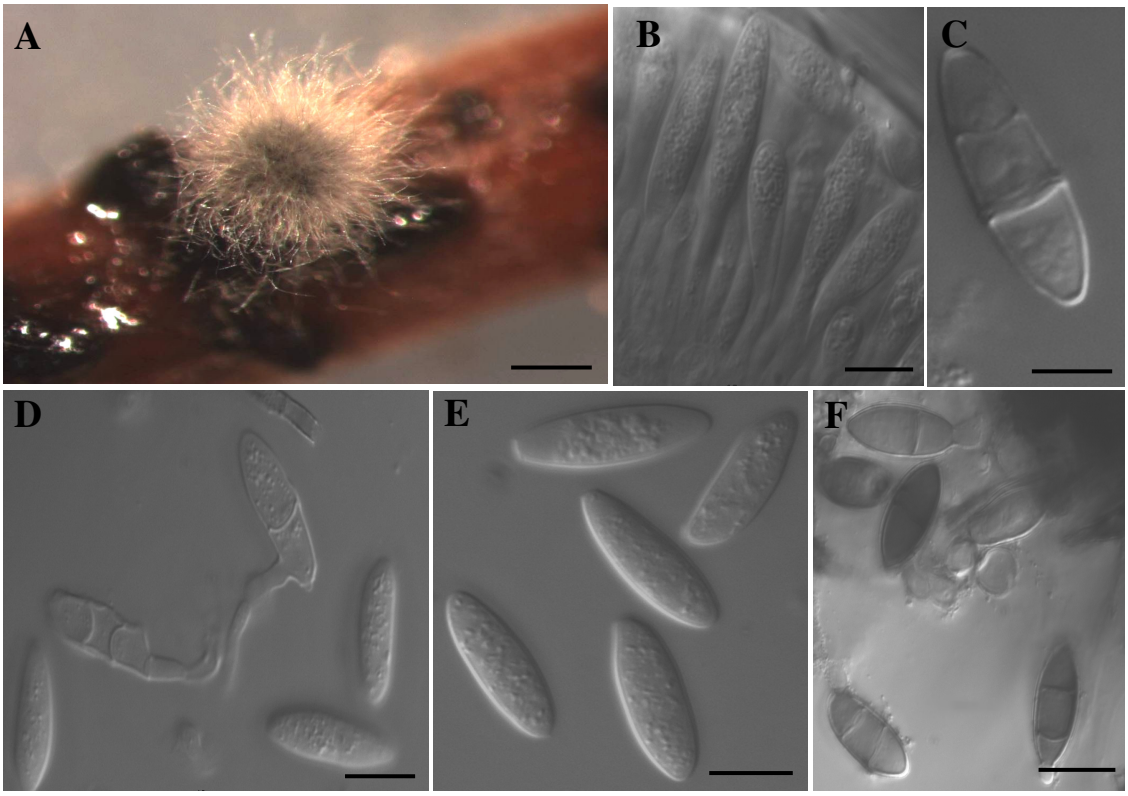
A
B
C
D
E
F

Fig. 9. Light micrograph of *N. eucalypti* conidia. Scale bar = 10 µm.

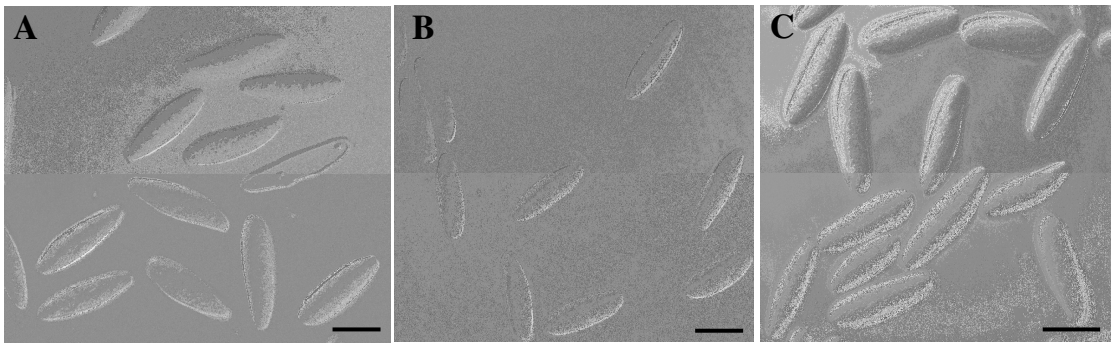
A
B
C

Fig. 10. Mean lesion lengths (mm) for each isolate of five species of Botryosphaeriaceae after inoculation on a *Eucalyptus grandis* clone (ZG-14), including *Botryosphaeria dothidea*, *Neofusicoccum eucalypti*, *Neofusicoccum crypto-australe*, *Neofusicoccum parvum*, *Neofusicoccum ursorum*. Control inoculations were done with MEA agar. Bars indicate the 95 % confidence limit for each isolate.

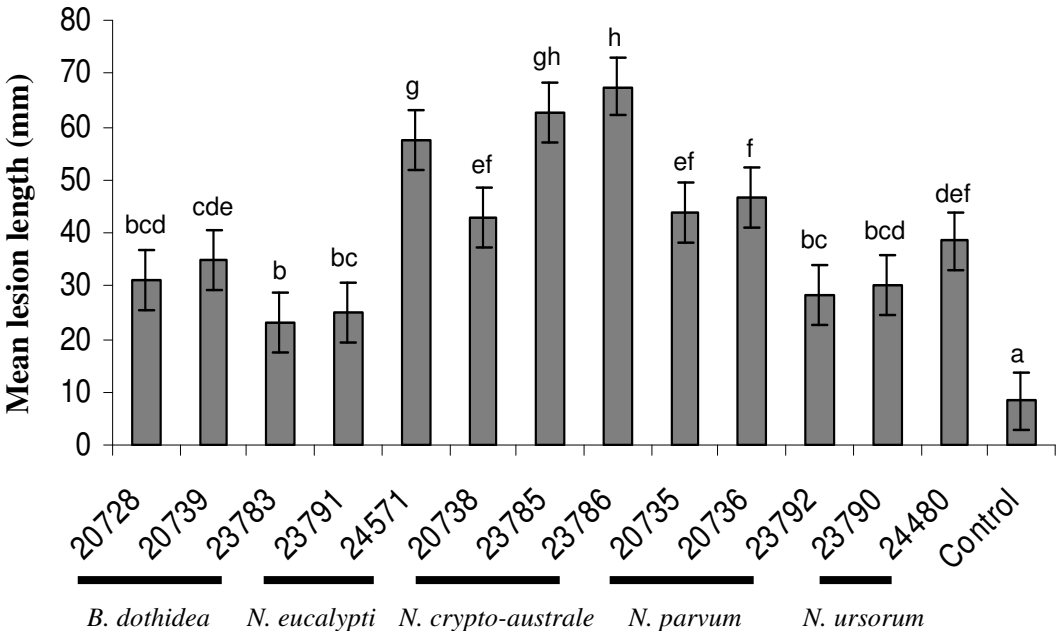
Mean lesion length (mm)
80
70
60
50
40
30
20
10
0
bcd
cde
b
bc
g
ef
gh
h
ef
f
bc
bcd
def
a
20728
20739
23783
23791
24571
20738
23785
23786
20735
20736
23792
23790
24480
Control
B. dothidea
N. eucalypti
N. crypto-australe
N. parvum
N. ursorum

Table 1. Isolates representing species of the Botryosphaeriaceae considered in a phylogenetic study and pathogenicity test

Culture no.[A,B]	Other no.[A]	Identity	Host	Location	Isolator	Genbank		
						ITS	EF	Bt
CMW7772		*Neofusicoccum ribis*	*Ribes* sp.	New York	B. Slippers & G. Hudler	AY236935	AY236877	AY236906
CMW7054	CBS 121.26	*N. ribis*	*R. rubrum*	New York	N. E. Stevens	AF241177	AY236879	AY236908
CMW10122		*Neofusicoccum parvum*	*Eucalyptus grandis*	Mpumalanga Province, S. Africa	H. Smith	AF283681	AF283681	AF283681
CMW994	ATCC58189	*N. parvum*	*Malus Sylvestris*	New Zealand	G. J. Samuels	AF243395	AY236883	AY236912
CMW9071		*N. parvum*	*Ribes* sp.	Australia	M. J. Wingfield	AY236938	AY236880	AY236909
CMW9081	ICMP8003	*N. parvum*	*Populus nigra*	New Zealand	G. J. Samuels	AY236943	AY236888	AY236917
CMW9078	ICMP7925	*N. parvum*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236940	AY236885	AY236914
CMW23792[C]		*N. parvum*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme			
CMW20736[C]		*N. parvum*	*E. robusta*	Pretoria, S. Africa	H. M. Maleme			
CMW20727		*N. parvum*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme			
CMW20719		*N. parvum*	*E. ovata*	Pretoria, S. Africa	H. M. Maleme			
CMW20724		*N. parvum*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW20722		*N. parvum*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme			
CMW20720		*N. parvum*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW20726		*N. parvum*	*E. robusta*	Pretoria, S. Africa	H. M. Maleme			
CMW20735[C]		*N. parvum*	*E. nicholii*	Pretoria, S. Africa	H. M. Maleme			
CMW20725		*N. parvum*	*E. scorparia*	Pretoria, S. Africa	H. M. Maleme			
CMW20730		*N. parvum*	*E. tereticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW20733		*N. parvum*	*E. tereticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW20734		*N. parvum*	*E. tereticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW3386		*Neofusicoccum crypto-australe*	*Wollemia nobilis*	Queensland, Australia	M. Ivory	AY615165	AY615157	AY615179
CMW13986		*N. crypto-australe*	*Syzygium cordatum*	Sodwana bay, South Africa	D. Pavlic	AY615166		
CMW13987		*N. crypto-australe*	*S. cordatum*	Sodwana bay, South Africa	D. Pavlic	AY615167		
CMW14013		*N. crypto-australe*	*S. cordatum*	Sodwana bay, South Africa	D. Pavlic	AY615168		
CMW20738[C]		*N. crypto-australe*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme			
CMW23787		*N. crypto-australe*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme			

Table 1. Continued

CMW23784		*N. crypto-australe*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW23785[C]		*N. crypto-australe*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW23786[C]		*N. crypto-australe*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW9072		*N. australe*	*Acacia* sp.	Melbourne, Australia	J. Roux & D. Guest	AY339260	AY339268	AY339252
CMW1110		*N. australe*	*Widdringtonia nodiflora*	Cape Province, S. Africa	W. J. Swart	AY615166	AY615158	AY615150
CMW9073		*N. australe*	*Acacia* sp.	Melbourne, Australia	J. Roux & D. Guest	AY339261	AY615269	AY339253
CMW6837		*N. australe*	*Acacia* sp.	Australia	M. J. Wingfield	AY339262	AY339270	AY339254
CMW6853		*N. australe*	*Sequiadendron giganteum*	Australia	B. Slippers, T. Burgess/K.-	AY339263	AY339271	AY339255
CMW15951		*N. australe*	*E. diversicolor*	Western Australia	T. Burgess/K.- L. Goei	DQ093201	DQ093225	DQ093212
WAC12399		*N. australe*	*E. diversicolor*	Western Australia	T. Burgess/K.- L. Goei	AY744374	DQ093222	DQ093209
CMW10309	CAP 002	*Neofusicoccum luteum*	*Vitis vinifera*	Portugal	A. J. L. Phillips	AY339258	AY339266	AY339250
CMW9076	ICMP 7818	*N. luteum*	*Malus domestica*	New Zealand	S. R. Pennycook	AY236946	AY236893	AY615126
CMW6217	CBS 115766	*Neofusicoccum eucalypticola*	*E. rossi*	Australia	B. Slippers	AY615143	AY615135	AY615127
CMW6229		*N. eucalypticola*	*E. rossi*	Australia	B. Slippers	AY615142	AY615134	AY615126
CMW10125	CBS 115791	*Neofusicoccum eucalyptorum*	*E. grandis*	Mpumalanga Province, S. Africa	H. Smith	AF283686	AY236891	AY236920
CMW11705		*N. eucalyptorum*	*E. nitens*	South Africa	B. Slippers	AY339248	AY339264	AY339256
CMW15948	WAC 12445	*Neofusicoccum macroclavatum*	*E. globulus*	Western Australia	T. Burgess	DQ093197	DQ093218	DQ093207
CMW15949	WAC 12446	*N. macroclavatum*	*E. globulus*	Western Australia	T. Burgess	DQ093198	DQ093219	DQ093208
CMW23790[C]		*Neofusicoccum ursorum*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW24480[C]		*N. ursorum*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
CMW15952		*Neofusicoccum eucalypti*	*E. diversicolor*	Australia	T. Burgess	DQ093194	DQ093215	DQ093204
CMW15953		*N. eucalypti*	*E. diversicolor*	Australia	T. Burgess	DQ093195	DQ093216	DQ093205
CMW24571		*N. eucalypti*	*E. paniculata*	Pretoria, S. Africa	H. M. Maleme			
CMW23791[C]		*N. eucalypti*	*Eucalyptus* sp.	Pretoria, S. Africa	H. M. Maleme			
WAC12401		*N. eucalypti*	*E. pauciflora*	Victoria, Australia	P. J. Keane	AY744371		
WAC12402		*N. eucalypti*	*E. camaldulensis*	Victoria, Australia	G. Whyte	AY744372		
WAC12398		*N. eucalypti*	*E. diversicolor*	Western Australia	T. Burgess/K.- L. Goei	AY744373	DQ093214	DQ093203
CMW24460		*N. eucalypti*	*E. pilularis*	Pretoria, S. Africa	H. M. Maleme			
CBS110887	STE-U 5252	*Neofusicoccum vitifusiforme*	*Vitis vinifera*	South Africa	J. M. van Niekerk	AY343383	AY343343	
CBS110880	STE-U 5050	*N. vitifusiforme*	*V. vinifera*	South Africa	J. M. van Niekerk	AY343382	AY343344	
CMW8000		*Botryosphaeria dothidea*	*Prunus* sp.	Crocifisso, Switzerland	B. Slippers	AY236949	AY236898	AY236927

Table 1. Continued

CMW9075	ICMP 8019	*B. dothidea*	*P. nigra*	New Zealand	G. J. Samuels	AY236950	AY236899	AY236928
CMW991		*B. dothidea*	*P. nigra*	New Zealand	B. Slippers	AY236947	AY236896	AY236925
CMW20739[C]		*B. dothidea*	*E. microcorys*	Pretoria, S. Africa	H. M. Maleme			
CMW20728		*B. dothidea*	*E. saligna*	Pretoria, S. Africa	H. M. Maleme			
CMW20718		*B. dothidea*	*E. teresticornis*	Pretoria, S. Africa	H. M. Maleme			
CMW20717		*B. dothidea*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme			
CMW20732		*B. dothidea*	*E. citriodora*	Pretoria, S. Africa	H. M. Maleme			
CMW23783		*B. dothidea*	*E. dorrigoensis*	Pretoria, S. Africa	H. M. Maleme			
CMW10130		*L. theobromae*	*Vitex doniana*	Uganda	J. Roux	AY236951	AY236900	AY236929
CMW9074		*L. theobromae*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236901	AY236930

[A]Culture collections: CMW = Tree Protection Co-operative Programme, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ATCC = American Type Culture Collection, Fairfax, VA, USA; BRIP = Plant Pathology Herbarium, Department of Primary industries, Queensland, Australia, CAP = Culture collection of A. J. L. Phillips, Lisbon, Portugal; CBS = Centraalbureau voor isolates Schimmelcultures, Utrecht, Netherlands; ICMP = International Collection of Microorganisms from Plant, Auckland, New Zealand.
[B]Isolates sequenced in this study are given in bold.
[C]Isolates used in the pathogenicity tests.

Table 2. Conidial measurements of five species of the Botryosphaeriaceae identified in this study

Identity	CMW no.[A]	Conidial measurement (µm)[B]		
		Length	Width	L/W
Neofusicoccum parvum	20719	(16.4) 18.7 (22.3)	(5.3) 6.8 (7.9)	2.8
	20725	(16.6) 18.5 (22.6)	(5.6) 6.1 (6.8)	3.1
	20724	(13.0) 16.0 (17.6)	(4.9) 5.3 (5.8)	3
	20722	(16.5) 18.7 (22.2)	(4.7) 4.8 (5.7)	3.9
	20720	(18.8) 18.5 (23.2)	(6.0) 5.1 (5.2)	3.7
	20726	(15.5) 17.7 (21.7)	(6.3) 5.8 (5.3)	3.3
	20736	(17.1) 18.6 (21.7)	(4.0) 4.8 (5.7)	3.9
	20730	(15.5) 18.7 (21.2)	(4.1) 5.8 (5.4)	3.3
	20734	(14.3) 16.5 (17.8)	(4.0) 5.4 (5.5)	3.3
	20735	(17.8) 18.8 (20.2)	(6.0) 5.4 (4.7)	3.5
	20733	(18.2) 18.9 (19.8)	(4.9) 5.4 (6.2)	3.5
	20723	(15.7) 16.9 (18.5)	(4.9) 5.1 (5.8)	3.3
	23792	(15.3) 17.1 (18.8)	(4.1) 5.4 (6.4)	3.2
	20727	(17.4) 18.6 (21.3)	(5.3) 6.4 (7.4)	3
Neofusicoccum eucalypti	23791	(20.1) 21.8 (23.6)	(5.1) 6.1 (7.4)	3.6
	24571	(14.9) 18.6 (18.7)	(4.8) 4.7 (5.8)	3.6
Neofusicoccum crypto-australe	20738	(17.8) 21.0 (22.2)	(5.2) 6.0 (6.0)	3.5
	23787	(16.2) 18.8 (20.7)	(4.8) 5.6 (6.4)	3.4
	23786	(19.2) 19.8 (22.3)	(4.9) 5.5 (6.5)	3.6
	23785	(18.2) 19.7 (21.6)	(4.9) 5.5 (6.2)	3.6
Neofusicoccum ursorum	23790	(20.8) 23.9 (28.4)	(5.6) 7.1 (8.2)	3.4
	24480	(21.0) 24.0 (26.4)	(5.7) 6.9 (8.4)	3.5
Botryosphaeria dothidea	20728	(21.6) 25.6 (28.4)	(5.7) 6.0 (6.7)	4.3
	20739	(22.6) 25.8 (26.6)	(3.8) 5.7 (5.8)	4.5
	20718	(22.8) 26.1 (30.1)	(4.6) 5.3 (5.9)	4.9
	20717	(20.4) 25.8 (30.6)	(4.4) 5.7 (7.4)	4.5
	23783	(22.2) 23.8 (29.6)	(5.1) 5.7 (6.3)	4.2

[A]CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[B]Measurements in brackets are actual ranges. Values outside brackets are averages of 30 conidia.

Table 3. Fixed polymorphic nucleotides from the sequence data of ITS rDNA, EF-1α and β-tubulin between isolates of *N. australe* and *N. crypto-australe*

Culture no.[A, B]	ITS	EF- 1α			β- tubulin		Identity
	463	55	240	248	44	58	
CMW9072	T	T	T	C	C	T	
CMW6837	-	-	-	-	-	-	
CMW1110	-	-	-	-	-	-	
CMW9073	-	-	-	-	-	-	*Neofusicoccum australe*
CMW6853	-	-	-	-	-	-	
CMW15951	-	-	-	-	-	-	
WAC12399	-	-	-	-	-	-	
CMW13986	C	C	C	T	T	C	
CMW14013	C	C	C	T	T	C	
CMW13987	C	C	C	T	T	C	
CMW 3386	C	C	C	T	T	C	
CMW 23787	**C**	**C**	**C**	**T**	**T**	**C**	*N. crypto-australe*
CMW 23786	**C**	**C**	**C**	**T**	**T**	**C**	
CMW 23785	**C**	**C**	**C**	**T**	**T**	**C**	
CMW 20738	**C**	**C**	**C**	**T**	**T**	**C**	
CMW 23784	**C**	**C**	**C**	**T**	**T**	**C**	

[A] Culture Collections: CMW= Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; [B]WAC= Department of Agriculture Western Australia Plant Pathogen Collection, Perth, Australia; KJ = Jacobs and Rehner (1998).
Isolates sequenced in this study are in bold.

Table 4. Distribution of five Botryosphaeriaceae isolated from 20 different *Eucalyptus* spp. in the Pretoria arboretum and from surrounding *Eucalyptus* trees

Species of Botryosphaeriaceae	*Eucalyptus* spp. surrounding the arboretum	*E. camaldulensis*	*E. nicholii*	*E. microcorys*	*E. punctata*	*E. terreticorris*	*E. dorrrigoensis*	*E. propinqua*	*E. citriodora*	*E. saligna*	*E.obligua*	*E. coniocalyx*	*E. ovata*	*E. sideroxlon*	*E. pilularis*	*E. maculata*	*E. botryoides*	*E. paniculata*	*E. scorparia*	*E. uiminalis*	*E. robusta*	Total
N. parvum	13	-	5	3	-	9	6	2	2	5	-	-	4	3	-	1	4	5	2	-	2	66
N. eucalypti	1	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	1	-	-	1	5
N. ursorum	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
N. crypto-australe	1	-	-	-	1	1	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	5
B. dothidea	4	2	-	2	-	2	2	-	2	-	-	-	-	-	-	2	-	-	-	-	-	16