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Chapter Five
Identification of the Causal Agent of Botryosphaeria Stem Canker in Ethiopian *Eucalyptus* plantations

ABSTRACT

Plantations of exotic *Eucalyptus* spp. constitute more than 30% of Ethiopia's plantation forests, providing fuel and construction timber to the country. Species such as *E. camaldulensis, E. saligna, E. grandis, E. citriodora* and *E. globulus* are the most commonly planted. During a disease survey of *Eucalyptus* spp. in 2000 and 2001, Botryosphaeria stem canker was observed in most plantations. Characteristic symptoms included tip die-back, coppice failure and stem cankers characterised by kino exudation. The aim of this study was to identify the species responsible for Botryosphaeria stem canker in Ethiopia. Culture and conidial morphology, as well as DNA-based identification involving RFLP's and sequencing of the ITS regions of the ribosomal DNA gene and the elongation factor 1-alpha (EF1-α) gene, were used to identify isolates. Pathogenicity studies were conducted in the greenhouse and under field conditions. Results showed that *B. parva* is responsible for Botryosphaeria stem canker of *Eucalyptus* spp. in Ethiopia. This is the first report of the fungus from the country. The results of greenhouse and field inoculation studies showed that the Ethiopian isolates are highly virulent. Careful site species selection and breeding trials are needed to reduce the impact of this disease in Ethiopia.

INTRODUCTION

In Ethiopia, the planting of *Eucalyptus* species commenced with the introduction of *E. globulus* Labil. in the late 1890's (Pohjonen & Pukkala 1990, Persson 1995). *Eucalyptus* spp., including *E. camaldulensis* Dehnh., *E. saligna* Sm., *E. grandis* Hill ex Maid and *E. citriodora* Hook are also commonly planted today. Together, they constitute the major proportion of the plantation resource, which covered an estimated 100 000 ha in 1990 (Pohjonen & Pukkala 1990, Persson 1995). The wood from these plantations is used as a source of fuel, construction timber and for the production of poles and posts.

Fungi in the genus *Botryosphaeria* are associated with diseases on a wide range of hosts. On *Eucalyptus* spp., these fungi are known as saprophytes, opportunistic pathogens and endophytes (Davison & Tay 1983, Barnard *et al.* 1987, Shearer, Tippett & Bartle 1987, Smith, Kemp & Wingfield 1994, Smith, Wingfield & Petrini 1996). Damage due to *Botryosphaeria* spp. is more pronounced when plants are under stress caused by drought, frost, water logging and insect damage (Wene & Schoenewesis 1980, Swart, Wingfield & Knox-Davies 1987, Pusey 1989, Old *et al.* 1990). Recently, it has been recognised that *Botryosphaeria* spp. also exist as symptomless endophytes in *Eucalyptus* spp. For example *B. dothidea* (Moug.) Ces. & De. Not. (anamorph = *F. aesculi* Corda) has been reported as an endophyte in *E. nitens* (Deane Et Maid.) Maid. in England (Fisher, Petrini & Sutton 1993) and in *E. grandis, E. camaldulensis, E. nitens* and *E. smithii* R. T. Baker in South Africa (Smith *et al.* 1996). When trees or tree parts are affected by stress, these fungi become active and can cause die-back.

The taxonomy of *Botryosphaeria* spp. is complicated and has been the subject of considerable debate (Sivanesan 1984, Butin 1993, Jacobs & Rehner 1998). This is because many of the species are almost impossible to distinguish from each other in culture. Furthermore, sexual structures of different *Botryosphaeria* spp. on infected tissues are morphologically similar and often indistinguishable. In the past, there has been a tendency to describe new species for collections from different hosts and this

has caused substantial confusion. Names such as *B. dothidea*, *B. ribis* Gressenb. & Dugg., (anamorph = *Fuscicoccum ribis* Gressenb. & Dugg.) and *B. berengeriana* De Not. have been used interchangeably (Slippers *et al.* 2003). In recent years, molecular techniques, particularly DNA sequencing have been used to clarify questions pertaining to *Botryosphaeria* taxonomy (Jacobs & Rehner 1998, Zhou & Stanosz 2001, Slippers *et al.* 2003). These data are showing that names used in past descriptions of diseases, must be treated with some circumspection.

Botryosphaeria ribis has been found associated with *Eucalyptus* spp. in different countries. In Florida, *B. ribis* has been associated with seed capsule abortion and twig die-back of *E. camaldulensis*, where it subsequently resulted in the abandonment of commercial seed production (Webb 1983). Infection by *B. ribis* has also been found associated with basal cankers and coppice failure of *E. grandis* in Florida (Barnard *et al.* 1987). In Australia, *B. ribis* is associated with twig, branch and stem cankers of *E. marginata* Donn. ex Sm. (Davison & Tay 1983). This fungus was also responsible for the death of *E. radiata* Sieb. ex DC. in species selection trials in Western Australia (Shearer *et al.* 1987).

In Africa, Botryosphaeria die-back and canker, caused by *B. dothidea, B. rhodina* (Cooke) Von Arx (anamorph = *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl.) and *B. eucalyptorum* Crous, H. Smith et M. J. Wingf. (anamorph = *Fusicoccum eucalyptorum* Crous, H. Smith et M. J. Wingf.) has been reported from several countries including South Africa, Republic of Congo and Uganda (Smith *et al.* 1994, 2001, Roux *et al.* 2000, 2001). In South Africa, wide-spread twig die-back and stem cankers were observed on *E. grandis, E. nitens* and *E. smithii*, clones of *E. grandis*, hybrids of *E. grandis* with *E. camaldulensis* as well as with *E. urophylla* S. T. Blake. *B. dothidea* and *B. eucalyptorum* were identified from symptomatic trees (Smith *et al.* 1994, 2001) and are considered to be the most common cause of *Eucalyptus* disease in South Africa. In the Republic of Congo, *B. rhodina* was found associated with root disease on *E. grandis* (Roux *et al.* 2000). Similarly, *B. rhodina* was associated with stem cankers on *Eucalyptus* spp. in the Republic of Congo and Uganda (Roux *et al.* 2000, 2001).

A recent disease survey conducted in *Eucalyptus* plantations of Ethiopia has shown that symptoms, typical of Botryosphaeria canker and die-back are present on several *Eucalyptus* spp. (Alemu, Roux & Wingfield 2003). The aim of this study is to identify the *Botryosphaeria* spp. associated with stem canker of *Eucalyptus* species in Ethiopia. To achieve this, morphological characterization, PCR-RFLP analysis and DNA sequencing were used.

MATERIALS AND METHODS

Symptoms, sample collection and fungal isolation

In 2000 and 2001, disease surveys were conducted in plantations of *Eucalyptus* spp. in Southern and South Western Ethiopia, from Munessa Shashemen, Wondo Genet, Menagesha and Jima (Figure 1). Different symptoms were noted on several *Eucalyptus* spp. Segments of symptomatic plant parts were incubated in moist chambers for 2-3 days to enhance development of fruiting structures. These were then transferred to MEA (2% Biolab Malt Extract and 1.5% Biolab Agar) and incubated at 25°C. Isolation from symptomatic tissue was also made directly onto MEA. Isolations were also made onto MEA from fruiting structures occurring on *Eucalyptus* twigs collected from the forest floor. All isolates have been deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Botryosphaeria isolates were inoculated on sterilised pine needles placed on the surface of water agar (2% Biolab Agar) in Petri dishes for 2-3 weeks at 25 °C to induce sporulation. Conidial masses from fruiting structures were spread on the surface of MEA in a sterile drop of water. Germinating single conidia were isolated after 12-16 hr.

Morphological characterisation

Isolates were initially characterized based on culture morphology on the MEA. Conidia from each of these cultures were mounted in lactophenol and examined using a Zeiss Axioskop light microscope. Widths and lengths of ten conidia were measured for each isolate and their length:width ratios were calculated based on the mean length and width of each isolate (Table 1).

DNA extraction

Total genomic DNA was extracted from selected Ethiopian *Botryosphaeria* isolates (Table 2). These isolates were selected to represent different morphological groups. Mycelium used for DNA extraction was scraped directly from MEA plates covered with mycelium using a sterile scalpel and placed into 1.5 µl Eppendorf tubes. The DNA extraction method of Raeder and Broda (1985), with slight modifications, was used to extract DNA. Extraction buffer, 200 µl (100 mM Tris-HCl, pH 8; 50 mM EDTA; 500 mM NaCl; 5 g CTAB) was added to each Eppendorf tube and the mycelium ground into small pieces with a sterile toothpick. When the mycelium was thoroughly broken, a further 800 µl of extraction buffer was added and incubated in a 60 °C water bath for one hour. Thereafter, 500 µl phenol and 300 µl chloroform was added and mixed. The cell debris was precipitated by centrifugation (12000 g, for 60 min) at 4 °C. The upper aqueous layer was transferred into new tubes and a further phenol:chlorophorm extraction was carried out by adding 200 µl phenol and 200 µl chloroform. This mixture was centrifuged at 12000 g for 5 min at 4 °C. The upper aqueous layer was again transferred into clean tubes. Following this, 400 µl chloroform was added to remove the excess phenol. When a clear interface was obtained, 0.1 volume of 3M NaAc (pH 5.5) and two volumes of absolute ethanol were added and the mixture centrifuged for 30 min at 4 °C to precipitate the nucleic acids. The ethanol was discarded and the precipitated nucleic acids were washed by the addition of 500 µl ethanol (70%) and centrifuged for 5 min at 4 °C. The ethanol was removed and the DNA pellets vacuum dried to remove excess ethanol. The DNA pellet was resuspended in 50 µl sterilised water. RNase A (1mg/ml) was added to the

DNA solution to remove the contaminating RNA and incubated over night at 37 °C in a water bath. A 1% agarose gel, stained with ethidium bromide was run and the DNA visualised under UV light.

PCR amplification

The internal transcribed spacer regions and 5.8S gene of the ribosomal RNA operon and the elongation factor gene (EF1-α) of the *Botryosphaeria* isolates used in this study were amplified using the Polymerase Chain Reaction (PCR). To amplify the ITS rDNA regions, primers ITS 1 (5' TCC GTA GGT GAA CCT GCG G '3) and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC '3) (White *et al.* 1990) were used. For the EF1-α gene, forward primer EF1-728F (5'-CAT CGA GAA GTT CGA GAA GG-3') and reverse primer EFI-986R (5'-TAC TTG AAG GAA CCC TTA CC-3') was used (Carbone, Anderson & Kohn 1999).

The PCR reaction mixture contained DNA polymerase (*Taq*, 2.5U/µl, Roche), 0.2 mM DNTP's, 10x Buffer, with 1 mM $MgCl_2$ supplied by the manufacturer, 25 mM $MgCl_2$ and 0.75 mM of each primer and approximately 1µl of DNA. Sterile water (37 µl) was added to give a total reaction volume of 50 µl. Denaturation was performed at 96 °C for 1 min. This was followed by 35 cycles of primer annealing at 55 °C for 30 s, chain elongation at 72 °C for 1 min and denaturation at 92 °C for 1 min. Final chain elongation was carried out at 72 °C for 5 min.

Restriction Fragment Length Polymorphisms (RFLP)

The ITS amplicons of the Ethiopian *Botryosphaeria* isolates used in this study were digested with *Cfo* I restriction endonuclease to determine the RFLP profile of the isolates (Jacobs 2002, Slippers *et al.* 2002). The RFLP reaction mix contained 20 µl DNA template, 0.5 µl enzyme and 2.5 µl enzyme buffer. The mixture was digested at 37 °C in a water bath for 6 hr. The RFLP fragments were separated on a 3% agarose gel stained with ethidium bromide and visualised under UV light. A standard 100 bp molecular marker was used to estimate the fragment sizes. These banding patterns were compared with those published by Jacobs (2002) and Slippers *et al.* (2002).

DNA sequencing

PCR products were purified using the High Pure PCR Product Purification Kit (QUIAGEN, GmbH, Hilden, Germany) and sequenced in both directions. The Big Dye Cycle Sequencing kit with Amplitaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, UK), was used following the manufacturer's protocols, on an ABI PRISM^{TM} 377 DNA Autosequencer (Perkin-Elmer). Primers ITS 1 and ITS 4 were used for sequencing the ITS rDNA regions, and primers EF1-728F and EF1-986R were used to sequence the EF1-α gene.

Sequence analysis

The possible identity of the Ethiopian isolates was considered by comparing their ITS sequences with those in the GenBank database [National Centre for Biotechnology Information (NCBI) US National Institute of Health Bethesda (http:/www.ncbi.nlm.nih.gov/BLAST)]. The Ethiopian *Botryosphaeria* sequences were aligned against *Botryosphaeria* sequences obtained from GenBank and from Slippers *et al.* (2003). Alignment of both ITS and EF 1-α gene sequences was done manually using PAUP version 4.0b (Swofford 1998). Gaps were treated as missing data. The sequences were analysed using parsimony, with trees generated by heuristic searches with simple addition and Tree Bisection Reconstruction (TBR) branch swapping. In the phylogenetic analysis *Guignardia philoprina* (Ellis) Viala & Ravaz was used as outgroup. Confidence intervals were determined using DNA BOOTSTRAP analysis (Bootstrap confidence intervals on DNA parsimony) (1000 replicates) (Felsenstein 1993). A partition homogeneity test (Farris *et al.* 1995, Huelsenbeck, Bull & Cunningham 1996) was used to consider whether the data sets for the ITS and EF1-α sequences could be combined.

Pathogenicity tests

Greenhouse inoculation studies were conducted on an 18-month-old *E. grandis* clone (ZG14). The trees were maintained in a greenhouse for one week to allow them to

acclimatise to the greenhouse environment. Thirteen *Botryosphaeria* isolates from Ethiopia were used in the greenhouse inoculation trial (Table 3).

Isolates used for inoculation tests were grown on MEA for ten days. A cork borer with a diameter of 9 mm was used to remove the bark and expose the wood for inoculation. Mycelial plugs of equal size were placed into each wound with the mycelial surface facing the xylem. After inoculation, the wounds were covered with Parafilm (Pechiney Plastic Packaging, Chicago) to prevent contamination and desiccation of the inoculum. Each isolate was inoculated on ten trees. Ten trees were also inoculated with sterile MEA to serve as a control.

After six weeks, disease development was evaluated by measuring the lesion lengths on inoculated trees. These measurements were subjected to statistical analysis (One-way ANOVA) using Statistica for Windows (StatSoft. Inc. 1995) to determine whether the lesions associated with the various *Botryosphaeria* isolates differed statistically from each other.

For the field inoculation trials, three representative isolates (CMW11059, CMW11065 and CMW11073) were selected based on the results of the greenhouse inoculation trial and these are representative of the range of lesion sizes (Table 3). Isolates were inoculated onto two-year-old coppice stems of *E. citriodora* trees in a plantation at Wondo Genet. Each isolate was inoculated into 20 trees and 20 trees were inoculated with sterile MEA as a control. A 9 mm cork borer was used to remove the bark and the same protocol used in the greenhouse trial was used. All inoculation wounds were covered with masking tape to prevent desiccation. Lesion development was evaluated after 8 weeks. A one-way analysis of variance ($P<0.0001$) was carried out to determine statistically, the differences in lesion development. Comparison of means was made using Dunnett's t Test method available in Statistica for Windows (StatSoft. Inc. 1995).

RESULTS

Symptoms, sample collection and fungal isolation

Symptoms of Botryosphaeria canker were commonly found in *Eucalyptus* plantations at Munessa Shashemene, Wondo Genet, Jima and Menagesha. Disease symptoms were found on different *Eucalyptus* species including *E. globulus, E. saligna, E. grandis* and *E. citriodora*. Symptoms of Botryosphaeria stem canker were observed on both coppice stems and first generation stands and on trees of all ages (Figure 2a-d). Bark cracking, production of copious amounts of kino (Figure 2a, 2d), stem discoloration and malformation, tip die-back and death (Figure 2b), as well as the occurrence of kino pockets in the xylem were the most common symptoms observed. When the bark was removed from symptomatic trees, well-developed kino pockets (Figure 2d) were visible in the cambium and xylem. Of all *Eucalyptus* spp., *E. citriodora* plantations at Wondo Genet and Jima/Belete were the most severely damaged by this stem canker disease. Large basal cankers (Figure 2a), as well as two to three layers of black kino rings (Figure 2c) were commonly found on *E. citriodora* trees, indicating different seasons of infection. Isolates of *Botryosphaeria* associated with these stem cankers were easily collected from all samples.

Morphological characterisation

The Ethiopian *Botryosphaeria* isolates grown on MEA showed some variation in colony growth and morphology. Some of the isolates had fluffy light brown aerial mycelium, whereas others had flat colony growth with little aerial mycelium (Figure 3d, 3e). Considerable variation was observed between the conidial lengths of *Botryosphaeria* isolates obtained from Ethiopia (Table 1). The lengths of the individual conidia for all isolates ranged from 12.5 µm to 27.5 µm and the average conidial length for different isolates ranged from 15.25 µm to 24.25 µm. The widths of the conidia showed limited variation and ranged from 5 µm to 7.5 µm. The conidia were grouped into three categories, namely (a) Those with long, narrow conidia, (b) those with long, wide conidia and (c) those with short conidia (Figure 3a-c). No teleomorph structures were observed for isolates examined in this study.

PCR amplification

Amplification of the ITS regions and 5.8S gene yielded a PCR product with a fragment length of approximately 500 base pairs (bp). For the EF1-α gene, fragments of approximately 300 base pairs were obtained.

Restriction Fragment Length Polymorphisms (RFLP)

All of the *Botryosphaeria* isolates from Ethiopia produced the same banding pattern (Figure 4) when the ITS PCR products were cut with *Cfo* I. This suggested that these isolates might represent the same species, even though they displayed substantial morphological variation. Comparison of the RFLP pattern for the Ethiopian isolates with banding patterns described for *Botryosphaeria* spp. (Jacobs 2002, Slippers *et al.* 2002) showed that the Ethiopian isolates had a banding pattern similar to that of *B. parva* (Figure 4).

DNA sequencing and analysis

When compared with sequences in GenBank, the ITS sequences of the Ethiopian *Botryosphaeria* isolates, most closely matched those of *B. ribis*. Alignment of these sequences with sequences of *B. ribis* and with representative sequences of other *Botryosphaeria* spp. (Slippers *et al.* 2003) yielded a total of 518 characters. Analysis of the data set for the EF 1-α sequences produced a total of 343 characters.

The partition homogeneity test revealed that the ITS and EF1-α data sets could be combined. The phylogenetic tree generated for the combined sequences of the ITS and EF 1-α produced five clades (Figure 5). Based on this tree, the Ethiopian *Botryosphaeria* isolates resided within the *B. parva* group. Other clades were similar to those defined by Slippers *et al.* (2003) and included *B. ribis* (Clade II), *B. eucalyptorum* (Clade III), *B. lutea* (clade IV) and *B. dothidea* (clade V). All clades were supported with bootstrap values of 100%. This phylogenetic tree was generated

based on a total of 855 characters, where 254 variable characters were parsimony uninformative and 194 characters were parsimony informative. The phylogenetic tree generated from the combined sequences had a CI value of 0.928 and RI value 0.905.

Pathogenicity tests

All Ethiopian *Botryosphaeria* isolates used in the greenhouse inoculation trial produced lesions on the *E. grandis* clone (Figure 6a, b). The mean lesion lengths produced ranged from 24.9 mm and 91.8 mm (Table 3). Isolate CMW11073, produced the largest lesions while the smallest average lesions were associated with isolate CMW11065. No lesions developed on seedlings inoculated with the sterile MEA. Statistical analysis showed that the lesions produced by the majority of isolates were significantly different from the control ($P<0.0001$) (Table 3). An R-square value of 0.47 was recorded for the data obtained in the greenhouse trial. Isolates CMW11073, CMW10095, CMW11066, CMW11064, CMW11063, CMW11069, CMW11059, CMW10094, CMW11067 and CMW11068 produced lesions that were significantly different from the control. The average lesion lengths associated with isolates CMW11071, CMW11070 and CMW11065 (Table 3, Figure 7) were not statistically different from the controls.

The three isolates used in the field inoculation trial produced lesions ranging in average length between 63 mm and 255.1 mm. The largest lesion was recorded for isolate CMW11073 (average = 255.1mm) and the smallest lesion (average = 63.35) was that associated with CMW11065 (Table 4). Some trees inoculated as controls also developed lesions. However, the controls were statistically different ($P=0.0001$) to those where *Botryosphaeria* isolates were used for inoculation (Table 4, Figure 6c-e). An R-square value of 0.71 was recorded for the data obtained from the field study. The results of the field inoculation trial also showed that the lesions associated with isolates CMW11073 and CMW11059 were statistically different to those of the control. CMW11065 produced lesions that did not vary significantly from the control (Table 4, Figure 8). The field and greenhouse trials, therefore, gave similar results.

DISCUSSION

Results of this study and a prior survey in 2000/2001 have shown that Botryosphaeria canker is the most common disease of *Eucalyptus* in Ethiopia. This disease affects all the major *Eucalyptus* spp. including *E. globulus, E. grandis, E. saligna* and *E. citriodora* (Alemu *et al.* 2003). The results of the current study have, furthermore, shown that *B. parva* is the major cause of Botryosphaeria canker in Ethiopian *Eucalyptus* plantations. This is the first report of this fungus from Ethiopia.

Ethiopian *Botryosphaeria* isolates used in this study showed some variation in colony growth, as well as in conidial length and shape. Based on culture morphology two groups could be distinguished. When the morphology of the conidia was considered, three morphological groups emerged. The morphological variation detected in this study, was however, not consistent with the results of the DNA-based comparison, which showed that the Ethiopian *Botryosphaeria* isolates represent a single species. Results of this study support the view (Jacobs & Rehner 1998, Smith & Stanosz 2001, Slippers *et al.* 2003) that morphological characteristics are insufficient to identify many *Botryosphaeria* spp. with confidence. They also provide additional evidence to suggest that names used for *Botryosphaeria* spp. in the past, are questionable.

Analysis of the banding patterns of the RFLP of ITS rDNA PCR product has been successfully used to distinguish between *Botryosphaeria* spp. obtained from different hosts (Jacobs 2002, Slippers *et al.* 2002). In this study the RFLP analysis showed that all Ethiopian isolates might represent a single species. It was, however, not useful in determining a species name for the fungus because *B. ribis* and *B. parva* have the same banding pattern (Slippers *et al.* 2002).

Ethiopian *Botryosphaeria* isolates had identical ITS sequences, which were sufficient only to determine that the isolates represented either *B. ribis* or *B. parva*. Inability to distinguish between these two species based on ITS sequences has been reported previously by Smith & Stanosz (2001) and Zhou & Stanosz (2001). However, the combination of the ITS rDNA and EF1-α sequence data was useful to separate *B.*

ribis and *B. parva* and showed that Ethiopian isolates belong to *B. parva*. These combined sequences were also used by Slippers *et al.* (2003) who showed that *B. ribis* and *B. parva* represent two distinct species. It is interesting that only one species of *Botryosphaeria* is associated with die-back in Ethiopia, while three species, *B. parva, B. dothidea* and *B. eucalyptorum* are associated with die-back on this host in South Africa (Smith *et al.* 1994, Smith *et al.* 2001, Slippers *et al.* 2003).

Botryosphaeria parva was first recorded in 1985 as a new species from Kiwifruit in New Zealand (Pennycook & Samuels 1985). There has, however, been considerable controversy surrounding its taxonomic status. It has, for example, been suggested that *B. parva* represents a synonym of *B. ribis* (Smith & Stanosz 2001, Zhou & Stanosz 2001). More recent studies have, however, shown that *B. ribis* and *B. parva* are distinct (Zhou, Smith & Stanosz 2001, Slippers *et al.* 2003). *Botryosphaeria parva* was previously most frequently found associated with fruit trees (Pennycook & Samuels 1985) and little information is available on the importance of this species in *Eucalyptus* plantations. Recently, Slippers *et al.* (2003) showed that *B. parva* is dominant in plantations of *Eucalyptus* spp. in South Africa. The results of the current study also showed that this fungus is important in *Eucalyptus* plantations of Ethiopia.

Greenhouse and field inoculation trials revealed that most *Botryosphaeria* isolates obtained from *Eucalyptus* in Ethiopia are pathogenic to *E. grandis* (clone ZG 14) and to *E. citriodora*. The *B. parva* isolates used in this study showed variation in pathogenicity both in the greenhouse and field study. Development of lesions on some trees inoculated as controls might have been due to contamination at the time of inoculation, wound stress or entophytic infections. The variations in virulence of the three isolates were concordant between greenhouse and field inoculation studies. These findings are similar to those of Jacobs (2000) who showed that *B. parva* is pathogenic to Mango, but isolates varied substantially in pathogenicity.

Botryosphaeria spp. have long been recognised as stress related opportunistic pathogens (Schoeneweiss 1981, Pusey 1989). A contemporary view is that they are latent pathogens that commonly occur in leaf and branch tissues of healthy woody

plants, and cause disease when trees are stressed (Fisher *et al.* 1993, Smith *et al.* 1996). In this respect, they are very similar to fungi such as *Sphaeropsis sapinea* (Fr.:Fr.) Dyko & Sutton (Syn=Diplodia pinea (Desm.) Kickx), which biologically and phylogenetically is a typical species of *Botryosphaeria* (Smith *et al.* 1996). The latter fungus is commonly found in healthy pine tissue but causes serious damage under conditions of stress such as after hail damage (Zwolinski, Swart & Wingfield 1990). Hence, *B. parva* must be considered an important pathogen in Ethiopia, where it almost certainly resides in healthy trees, but causes die-back and death of trees under stress conditions.

Plantations in Ethiopia are commonly developed on marginal sites where moisture stress is a limiting factor for tree growth. This could favour disease development associated with *B. parva*. Moreover, the association of Botryosphaeria canker with *Eucalyptus* coppice stands is of great concern, because regenerating *Eucalyptus* species by coppicing is widely practiced in Ethiopia, particularly by small scale tree growers. This practice evidently stresses trees, facilitating infection by *B. parva*. In the future, efforts will need to be made to match species and genotypes to sites and thus to minimise the impact of this opportunistic pathogen.

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Table 1. Conidial sizes of *Botryosphaeria* isolates from *Eucalyptus* in Ethiopia

Isolate	Origin	Host	Range and Average Length (µm)	Range and Average Width (µm)	Length: Width (ratio)
CMW10088	Wondo Genet	*Eucalyptus* sp.	(15) 18.3 (22.5)	(5.25) 5 (5.5)	3.65
CMW10089	Wondo Genet	*Eucalyptus* sp.	(20) 24.3 (27.5)	(7) 7.5 (7.75)	3.23
CMW10092	Menagesha	*E. globulus*	(15) 17.3 (20)	(5) 5.8 (7.5)	3.17
CMW10093	Wondo Genet	*E. saligna*	(12.5) 15 (17.5)	(4.75) 5 (5.5)	3
CMW10094	Wondo Genet	*E. saligna*	(17.5) 19 (20)	(5) 5 (5.25)	3.8
CMW10095	Wondo Genet	*E. grandis*	(15) 15.3 (17.5)	(5) 5 (5.5)	3.05
CMW10096	Wondo Genet	*Eucalyptus* sp.	(15) 16.3 (17.25)	(5) 5 (5.25)	3.25
CMW11059	Jima/Belete	*E. citriodora*	(15) 17.5 (20)	(5) 5 (5.5)	3.5
CMW11060	Jima/Belete	*E. citriodora*	(17.5) 18.3 (20)	(4.75) 5 (5.5)	3.17
CMW11061	Jima/Belete	*E. citriodora*	(15) 17.8 (22.5)	(5) 5.3 (7.5)	3.38
CMW11062	Jima/ Belete	*E. citriodora*	(17.5) 19.5 (22.5)	(7) 7.5 (7.5)	2.6
CMW11063	Jima/Belete	*E. citriodora*	(15) 16.5 (17.5)	(4.75 5 (5.5)	3.3
CMW11064	Jima/Belete	*E. citriodora*	(17.5) 21.8 (25)	(5) 5 (5.5)	4.35
CMW11066	Jima/Belete	*E. citriodora*	(17.5) 20.8 (25)	(5) 5.3 (7.5)	3.95
CMW11068	Munessa	*E. globulus*	(15) 18.5 (22.5)	(5) 5 (5.5)	3.7
CMW11069	Menagesha	*E. globulus*	(15) 16.8 (20)	(5) 5.8 (7.5)	2.91
CMW11070	Menagesha	*E. globulus*	(17.5) 17.5 (20)	(5.25) 5 (5.5)	3.55
CMW11071	Menagesha	*E. globulus*	(17.5) 18.5 (20)	(5) 6.5 (7.5)	2.85
CMW11072	Menagesha	*E. globulus*	(17.5) 19.8 (22.5)	(5) 5 (5.5)	3.95

Each mean values and ranges are based on measurements from 10 conidia.

CMW numbers are culture collection numbers of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

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Table 2. Isolates used in the DNA sequence analysis.

Culture No.	Identity	Host	Origin	Collector	Accession No.	
					ITS	EF 1-α
CMW7780	*B. dothidea*	*Fraxinus excelsior*	Switzerland	B. Slippers	AY236947	AY236896
CMW8000	*B. dothidea*	*Prunus* sp.	Switzerland	B. Slippers	AY236949	AY236898
CMW10125	*B. eucalyptorum*	*E. grandis*	S. Africa	H. Smith	AF283686	AY236891
CMW10126	*B. eucalyptorum*	*E. grandis*	S. Africa	H. Smith	AF283687	AY236892
CMW992/3	*F. luteum*	*Actinidia deliciosa*	New Zealand	G. J. Smuels	AF027745	AY236894
CMW9076	*B. lutea*	*Malus X domestica*	New Zealand	S. R .Pennycook	AY236946	AY236893
CMW7772	*B. ribis*	*Ribis* sp.	New York	B. Slippers/ G. Hudler	AY236935	AY236877
CMW7773	*B. ribis*	*Ribis* sp.	New York	B. Slippers/ G. Hudler	AY236936	AY236878
CMW9071	*B. parva*	*Ribis* sp.	Australia	M. J. Wingfield	AY236938	AY236880
CMW994	*B. parva*	*Malus sylvestris*	New Zealand	G. J Samuels	AY243395	AY236883
CMW9077	*B. parva*	*Actinidia deliciosa*	New Zealand	S. R. Pennycook	AY236939	AY236884
CMW10122	*B. parva*	*E. grandis*	S. Africa	H. Smith	AF283681	AY236882
CMW11060	*Botryosphaeria* sp.	*E. citriodora*	Ethiopia	Alemu Gezahgne & J. Roux	AY210474	AY210480
CMW11062	,,	,,	,,	,,	AY210475	AY210481
CMW11064	,,	,,	,,	,,	AY210476	AY210482
CMW10089	,,	*E. globulus*	,,	,,	AY210477	AY210483
CMW10095	,,	*E. grandis*	,,	,,	AYS20478	AY210485
CMW10094	,,	*E. saligna*	,,	,,	AY210479	AY210484
CMW7063	*Guignardia philoprina*	*Taxus baccata*	Netherlands	H. A. van der Aa	AY236979	AY236905

Isolates from Ethiopia were sequenced in this study. All other sequences are from the study of Slippers *et al.* (2003).
CMW numbers are culture collection numbers of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Table 3. Mean lesion lengths and confidence limits for greenhouse inoculations using *Botryosphaeria* isolates from Ethiopia.

Isolate	Mean Lesion length (mm)	95% Confidence limits
CMW11059	54.2^{bcd}	38.35-70.05
CMW11063	66.0^{abc}	49.29-72.71
CMW11064	71.7^{ab}	55.85-87.55
CMW11065	24.9^{de}	9.05-40.75
CMW11066	72.8^{ab}	56.95-88.65
CMW11067	48.1^{bcd}	32.25-63.95
CMW11068	43.5^{bcd}	27.65-59-35
CMW11069	60.8^{abc}	44.95-76.65
CMW11070	34.9^{cde}	19.05-50.75
CMW11071	39.6^{cde}	23.75-55.45
CMW11073	91.8^{a}	75.95-107.65
CMW10095	83.2^{ab}	67.35-99.05
CMW10094	49.5^{bcd}	33.65-65.35
Control	10.9^{e}	4.95-26.75

CMW numbers are culture collection numbers of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Means are averages of 10 measurements.

Means with the same letter are not significantly different from each other at $P<0.05$ significance level.

Table 4. Mean lesion lengths and confidence limits for trees inoculated with *Botryosphaeria* sp. on *E. citriodora* in the field.

Isolates	Mean Lesion length (mm)	95% Confidence limits
CMW11059	226.8[a]	197.95-255.65
CMW11065	63.35[b]	34.50-92.20
CMW11073	255.1[a]	226.25-283.95
Control	29.35[b]	0.50-58.20

CMW numbers are culture collection numbers of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Means are averages of 20 measurements.

Means with the same letter are not significantly different from each other at $P<0.05$ significance level.

Figure 1. Map of Ethiopia showing the plantation areas where samples were collected.

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Figure 2. Symptoms of Botryosphareia stem canker in Ethiopia. (a) Basal canker, (b) Wilting and die-back, (c) Black kino rings, (d) Xylem discoloration.

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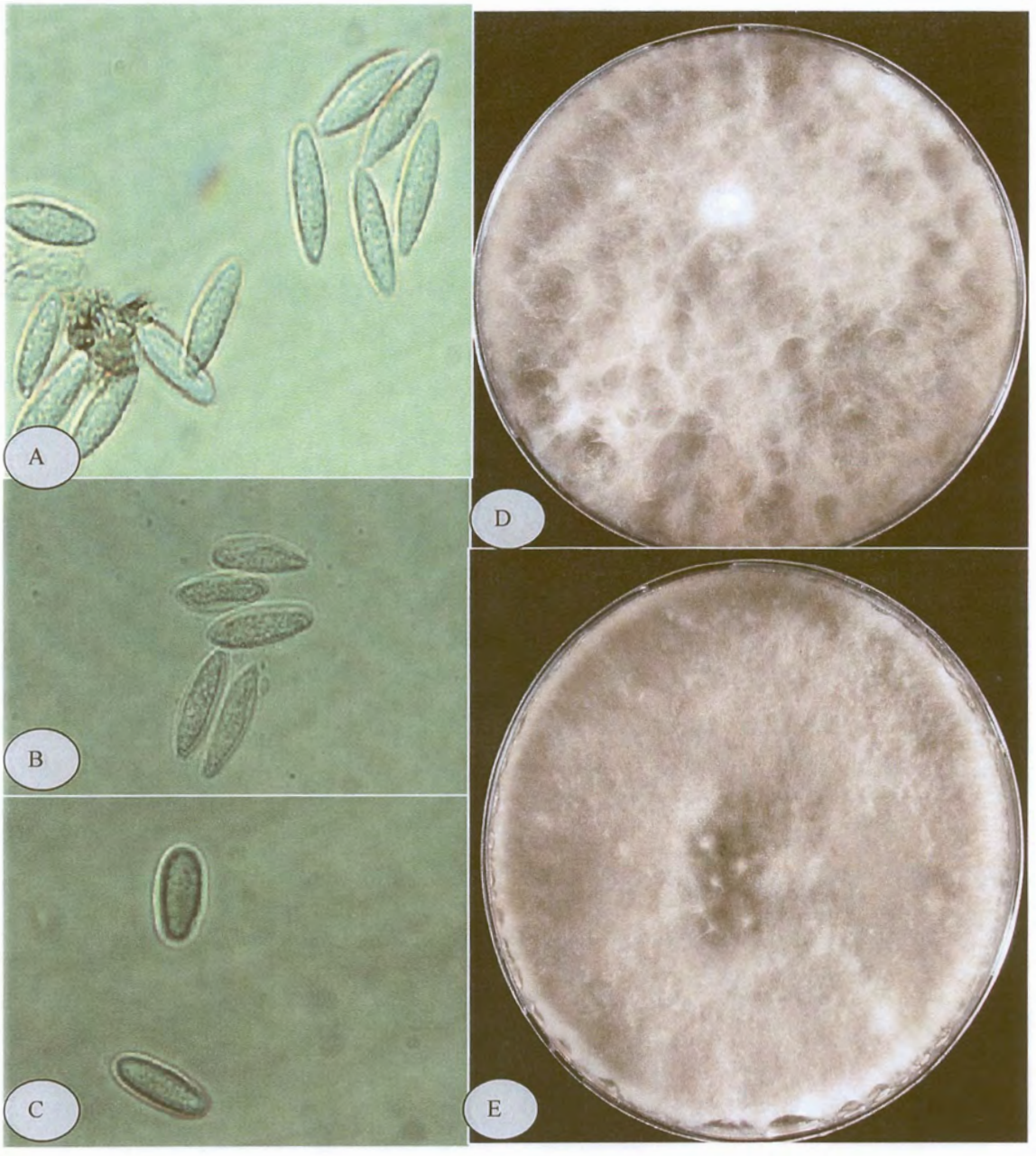

Figure 3. Culture and conidial morphology for *B. parva*, (A) Long, narrow and cylindrical conidia, (B) Long, wide and cylindrical conidia, (C) short, and wide conidia, (D) fluffy light brown aerial mycelial growth and (E) flat light brown mycelial growth.

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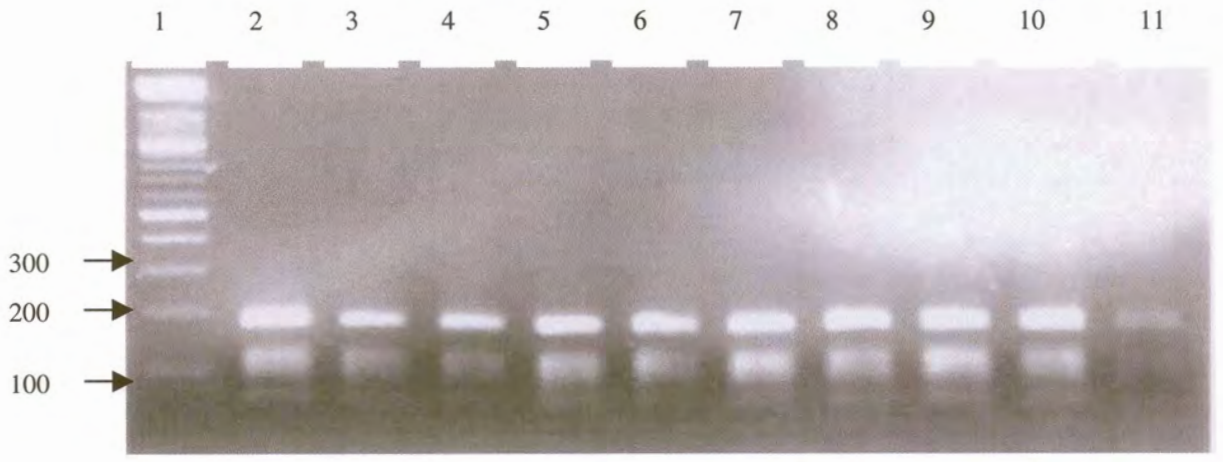

Figure 4. Restriction fragment patterns of *Botryosphaeria* isolates from Ethiopia after electrophoresis on a 3% agarose gel stained with ethidium bromide. Lane 1=100 base pair Molecular Weight Marker, Lane 2-11 representative isolates CMW11060, CMW11062, CMW11063, CMW11064, CMW10089, CMW10090, CMW10091, CMW10094 and CMW10095 respectively.

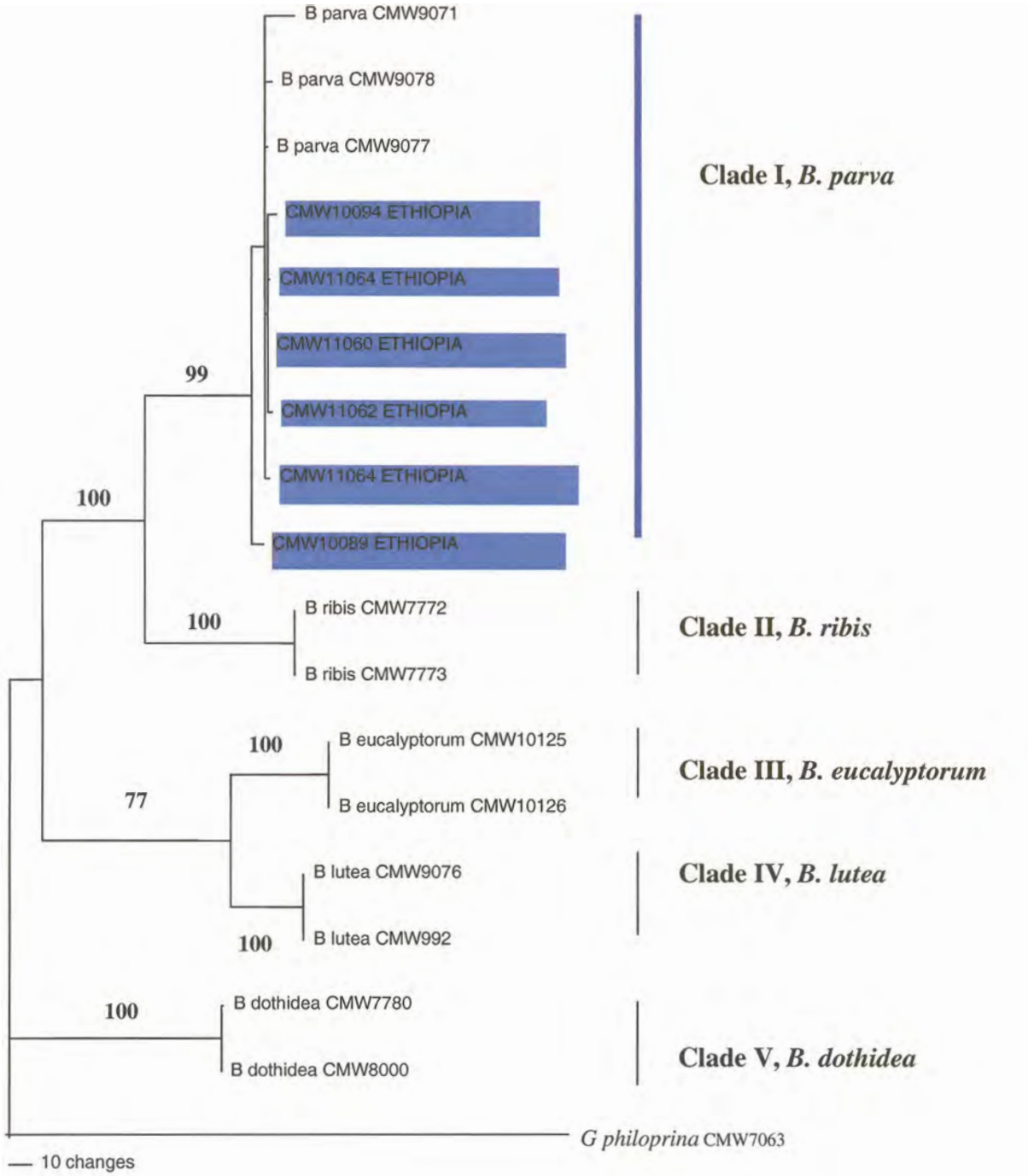

Figure 5. Phylogenetic tree for combined ITS rDNA and EF1-α sequences. (CI=0.928, RI=0.905). Bootstrap values are shown on the branches.

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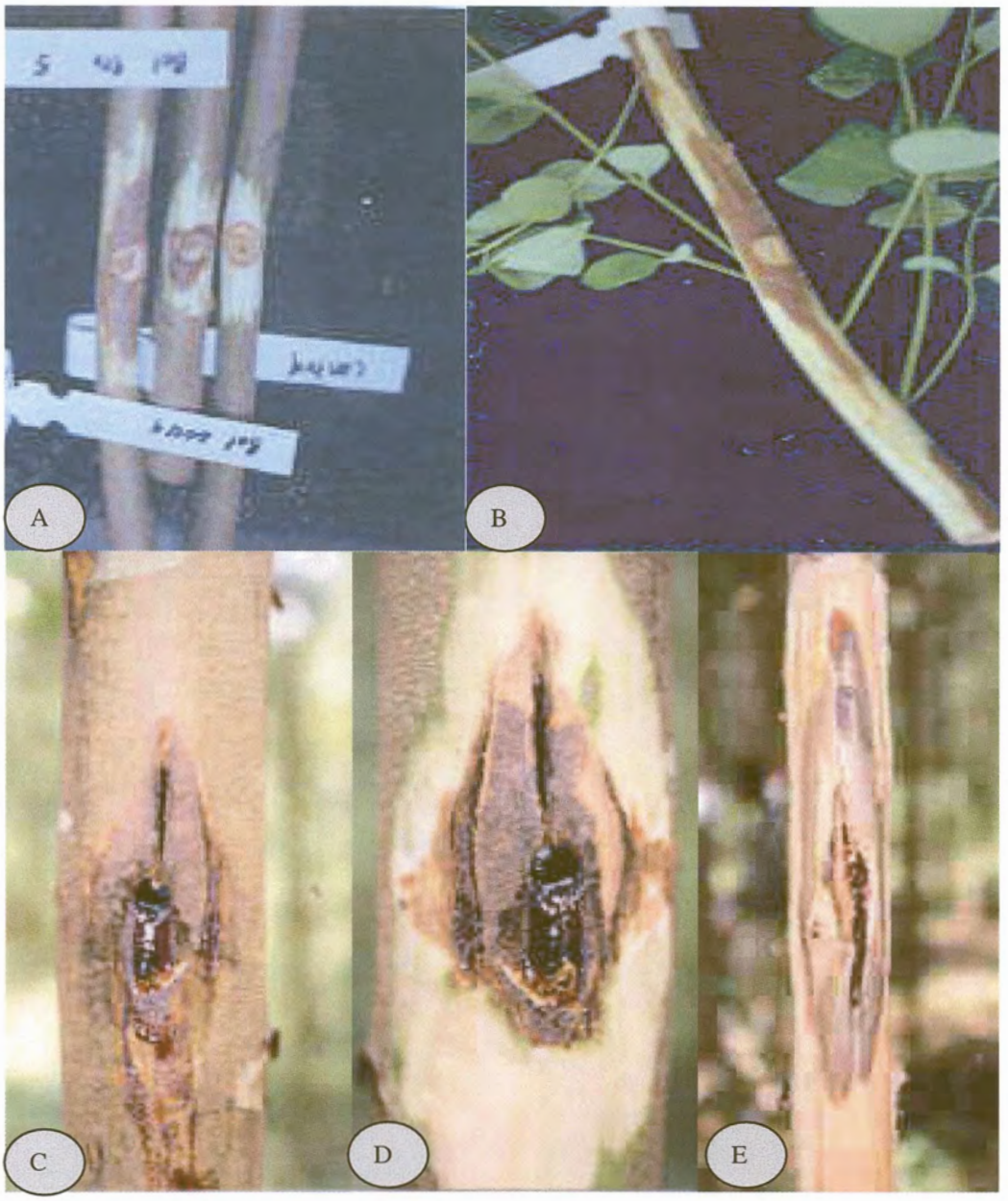

Figure 6. Results of pathogenecity tests. (A) Lesions from the greenhouse inoculation trial. (B) Development of epicormic shoots on *E. grandis* clone. (C, D, E) Lesion development in the field inoculation trial.

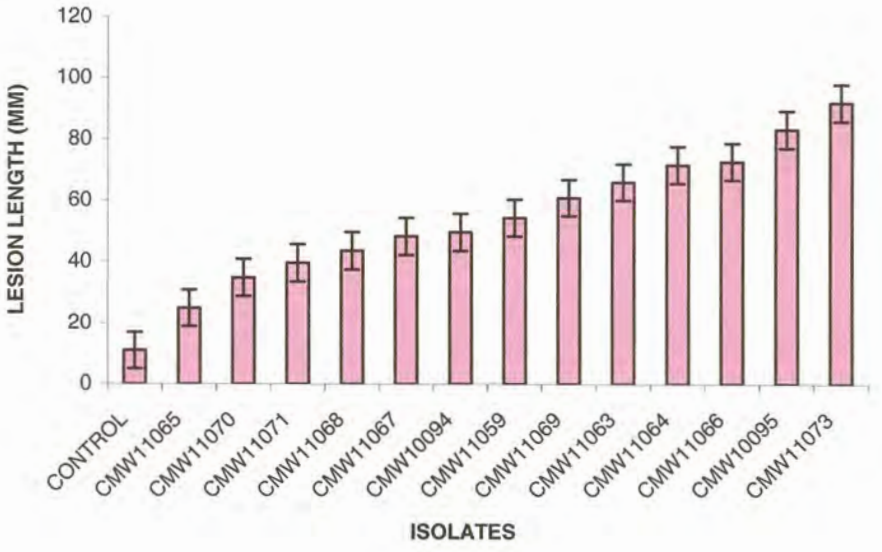

Figure 7. Lesion lengths and confidence limits for *Botryosphaeria* isolates and controls used in the greenhouse inoculation study (P=0.05).

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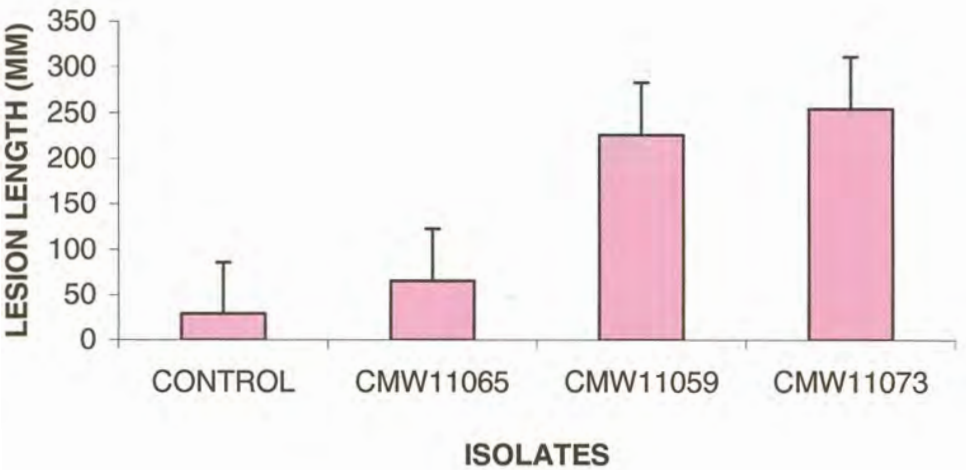

Figure 8. Lesion lengths and confidence limits for *Botryosphaeria* isolates and controls used in the field inoculation trial (P=0.05).

Figure 9. Aligned sequences of the ITS and Elongation Factor (1-alpha) genes of *Botryosphaeria* isolates. (-) = Gaps, (.) = homologous nucleotides (N) = Unknown bases

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                                10        20        30        40        50        60        70
B_parva_CMW9071                 GAAGTTCGAG AAGGTAAGAA -AG-TTTTTC C-TTCCGCTG CACGCGC--T GGGTGCCAGG TGCTGGGT--
B_PARVA_CMW9078                 ....G.---- -----..... -..-..... .-........ .......--. .......... ........--
B_PARVA_CMW9077                 ....G.---- -----..... -..T....-. .-........ .......--. .......... ........--
B_DOTHIDEA_CMW7780              ....G.---- -----...C. C.CA....-. TG.G..--.. ....-----. ---------. ........--
B_DOTHIDEA_CMW8000              ....G.---- -----...C. C.CA....-. TG.G..--.. ....-----. ---------. ........--
CMW10094ETHIOPIA                ....G.---- -----..... -..T....-. .-........ .......--. .......... ........--
CMW10089ETHIOPIA                ....G.---- -----..... -..T....-. .-........ .......--. .......... ........--
CMW11064ETHIOPIA                ....G.---- -----..... -..T....-. .-........ .......--. .......... ........--
CMW11060ETHIOPIA                ....G.---- -----..... -..T....-. .-........ .......--. .......... ........--
CMW11062ETHIOPIA                ....G----- -----..... -..T....-. .-........ .......--. .......... ........--
CMW10095ETHIOPIA                ....G----- -----..... -..T....-. .-........ .......--. .......... ........--
B_eucalyptorum_CMW10125         .......... .......... -.AT....-. .-........ .....TTCC. ......---- ---.....--
B_eucalyptorum_CMW10126         .......... .......... -.AT....-. .-........ .....TTCC. ......---- ---.....--
B_lutea_CMW9076                 .......... .......... G.-T....-. -G........ .C.....GA. ..-...---- ---....A--
B_lutea_CMW992                  .......... .......... G.-T....-. -G........ .C.....GA. ..-...---- ---....A--
B_RIBIS_CMW7772                 ....G.---- -----..... -..T....-. .-........ .......--. ......TG.. ........GC
B_RIBIS_CMW7773                 ....G.---- -----..... -..T....-. .-........ .......--. ......TG.. ........GC
G_PHILOPRINA_CMW7063            ....G.---- -----..... C..------. .--------- ---------- ---------- ----------
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```
                                  80         90         100        110        120        130        140
B_parva_CMW9071          ---------- --TCCCGCAC TCAATTTGCC TTATC--GCT TCGGTGAGGG GCA-TTT--T GGTGGTGGGG
B_PARVA_CMW9078          ---------- --......... .......... .....--... .......... ...-...--. ..........
B_PARVA_CMW9077          ---------- --......... .......... .....--... .......... ...-...--. ..........
B_DOTHIDEA_CMW7780       ---------- --...T..G. CGG....... .....-A-.. CT........ ...A...C-. --.........
B_DOTHIDEA_CMW8000       ---------- --...T..G. CG........ .....-A-.. CT........ ...A...C-. --.........
CMW10094ETHIOPIA         ---------- --.........  ........T. .....--... ....A..... ...-...--. ..........
CMW10089ETHIOPIA         ---------- --......... .......... .....--... .......... ...-...--. ..........
CMW11064ETHIOPIA         ---------- --......... .......... .....--... .......... ...-...--. ..........
CMW11060ETHIOPIA         ---------- --......... .......... .....--... .......... ...-...--. ..........
CMW11062ETHIOPIA         ---------- --......... .......... .....--... .......... ...-...--. ..........
CMW10095ETHIOPIA         ---------- --......... .......... .....--... .......... ...-...--. ..........
B_eucalyptorum_CMW10125  ---------- --......... .......... .....--... ..A....... ...-...--. .....---..
B_eucalyptorum_CMW10126  ---------- --......... .......... .....--... ..A....... ...-...--. .....---..
B_lutea_CMW9076          ---------- --.G....... .........T. .....--... .......... ...-...--. ..........
B_lutea_CMW992           ---------- --.G....... .........T. .....--... .......... ...-...--. ..........
B_RIBIS_CMW7772          TGGGTGCTGG GT........ .......... .....--... .......... ...-...--. ..........
B_RIBIS_CMW7773          TGGGTGCTGG GT........ .......... .....--... .......... ...-...--. ..........
G_PHILOPRINA_CMW7063     ---------- --------.. ..-C....-- A..C.CA.-A ..-...C..C .---.C.CGC A-.CTCACA-
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```
                                150         160         170         180         190         200         210
B_parva_CMW9071                 T-TGGCCCGC GCTAAGCCTC GTTTGGGCT- CGGCAAAATG TCCGCATC-- TGGTTTTTTT GCGACCGGCG
B_PARVA_CMW9078                 .-........ .......... .........- .......... ........-- .......... ..........
B_PARVA_CMW9077                 .-........ .......... .........- .......... ........-- .......... ..........
B_DOTHIDEA_CMW7780              -C........ .......... ......T..T .........C ........-- ...A...... .T........
B_DOTHIDEA_CMW8000              -C........ .......... ......T..T .........C ........-- ...A...... .T........
CMW10094ETHIOPIA                .-........ .......... ?........- .......... ........-- .......... ..........
CMW10089ETHIOPIA                .-........ .......... .........- .......... ........-- .......... ..........
CMW11064ETHIOPIA                .-........ ......T... .........- .......... ........-- .......... ..........
CMW11060ETHIOPIA                .-........ .......... .........- .......... ........-- .......... ..........
CMW11062ETHIOPIA                .-........ .......... .........- .......... ........-- .......... ..........
CMW10095ETHIOPIA                .-........ .......... .........- .......... ........-- .......... ..........
B_eucalyptorum_CMW10125         .C-....... .....T...G .....T...T .........C ....T...-- .........- ..........
B_eucalyptorum_CMW10126         .C-....... .....T...G .....T...T .........C ....T...-- .........- ..........
B_lutea_CMW9076                 .C-....... .......... .....T...T .........C .........- .........- ..........
B_lutea_CMW992                  .C-....... .......... .....T...T .........C .........- .........- ..........
B_RIBIS_CMW7772                 .-........ .......... ...C.....- .......... ........-- .......... ..........
B_RIBIS_CMW7773                 .-........ .......... ...C.....- .......... ........-- .......... ..........
G_PHILOPRINA_CMW7063            CC....ATT. TG.GCC...? -..ACCC..C .TCA.....- --------.AA --T....... .T.G..CTTT
```

```
                                220        230        240        250        260        270        280
B_parva_CMW9071                 TGCGAC-CGA AGCG--CGCC CCTCGCCAGA ------CACG CCAC------ --GCATGTGC G-----ACCA
B_PARVA_CMW9078                 ......-... ....--.... .......... ------.... ....------ --......... .-----....
B_PARVA_CMW9077                 ......-... ....--.... .......... ------.... ....------ --......... .-----....
B_DOTHIDEA_CMW7780              ......-... C...AA.A.. ....A...-. CGCTTC..-. ....TCACGT TC.TC.A...  .-----....
B_DOTHIDEA_CMW8000              ......-... C...AA.A.. ....A...-. CGCTTC..-. ....TCACGT TC.TC.A...  .-----....
CMW10094ETHIOPIA                ......-... ....--.... .......... ------.... ....------ --......... .-----....
CMW10089ETHIOPIA                ......-... ....--.... .......... ------.... ....------ --......... .-----....
CMW11064ETHIOPIA                ......-... ....--.... .......... ------.... ....------ --......... .-----....
CMW11060ETHIOPIA                ......-... ....--.... .......... ------.... ....------ --......... .-----....
CMW11062ETHIOPIA                ......-... ....--.... .......... ------.... ....------ --......... .-----....
CMW10095ETHIOPIA                ......-... ....--.... .......... ------.... ....------ --......... .-----....
B_eucalyptorum_CMW10125         ......-... .AT.--.... ........-- ---T-G..-- ---------- -------... .--CGG-...
B_eucalyptorum_CMW10126         ......-... .AT.--.... ........-- ---T-G..-- ---------- -------... .--CGG-...
B_lutea_CMW9076                 ......-... C...--.A.. .......... --CTCG..-. ....AC---- ------.... .ATCGG----
B_lutea_CMW992                  ......-... C...--.A.. .......... --CTCG..-. ....AC---- ------.... .ATCGG----
B_RIBIS_CMW7772                 ......-... ....--.A.. .......... ------.... ....------ --......... .-----....
B_RIBIS_CMW7773                 ......-... ....--.A.. .......... ------.... ....------ --......... .-----....
G_PHILOPRINA_CMW7063            .TA.TGGG.C C----A.AA. ..C-...... GTTCTCG.TA ---------- --....C.CA A---GG.---
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                                290        300        310        320        330        340        350
B_parva_CMW9071          -GACGCTAAC -----AGCCA TCCC---AGG AAGCCACCGA GTTGATTCGA GCTCCGGC-T CGACTCTCCC
B_PARVA_CMW9078          -......... -----..... ....---... .......... .......... ........-. ..........
B_PARVA_CMW9077          -......... -----..... ....---... .......... .......... ........-. ..........
B_DOTHIDEA_CMW7780       T-.T...... CA-----..G C.A.AAC... .......... .........G ........C- ...TC-CT..
B_DOTHIDEA_CMW8000       T-.T...... CA-----..G C.A.AAC... .......... .........G ........C- ...TC-CT..
CMW10094ETHIOPIA         -......... -----..... ....---... .....NNNNN NNNNNNNNNN NNNNNNNNNN NNNN......
CMW10089ETHIOPIA         -......... -----G...C ....---... .......... .......... ........-. ..........
CMW11064ETHIOPIA         -......... -----..... ....---... .......... .......... ........-. ..........
CMW11060ETHIOPIA         -......... -----..... ....---... .......... .......... ........-. ..........
CMW11062ETHIOPIA         -......... -----..... ....---... .......... .......... ........-. ..........
CMW10095ETHIOPIA         -......... -----..... ....---... .......... .......... ........-. ..........
B_eucalyptorum_CMW10125  -........T -----GC... ....---... .......... .....C.... ........-. ..........
B_eucalyptorum_CMW10126  -........T -----GC... ....---... .......... .....C.... ........-. ..........
B_lutea_CMW9076          --........ -----GA..G ..T.---... .......... .......... ........-. ..........
B_lutea_CMW992           --........ -----GA..G ..T.---... .......... .......... ........-. ..........
B_RIBIS_CMW7772          -......... -----G.... ....---... .......... .......... ........-. ..........
B_RIBIS_CMW7773          -......... -----G.... ....---... .......... .......... ........-. ..........
G_PHILOPRINA_CMW7063     --.G..--.. GCGCTGA.AG ....---.AA .TAGG.AT.. .A.C..G.C- -..T...GG. A...-.....
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                                360        370        380        390        400        410        420
B_parva_CMW9071          ACCCAATGTG TACC-TACCT CTGTTGCTTT GGCGGGCCGC GGTCCTCCGC -ACCGG-CGC CC-TTC-GGG
B_PARVA_CMW9078          ....T..... ....-..... .......... .......... .......... -.....-... ..-...-...
B_PARVA_CMW9077          .......... ....-..... .......... .......... .......... -.....-... ..-...-...
B_DOTHIDEA_CMW7780       CA..CT.TGT GTA.C..... .......... .......... .......... GG....C.C. ..TCC.C...
B_DOTHIDEA_CMW8000       CA..CT.TGT GTA.C..... .......... .......... .......... GG....C.C. ..TCC.C...
CMW10094ETHIOPIA         ....T..... ....-..... .......... .......... .......... -.....-... ..-...-...
CMW10089ETHIOPIA         .......... ....-..... .......... .......... .......... -.....-... ..-...-...
CMW11064ETHIOPIA         ....T..... ....-..... .......... .......... .......... -.....-... ..-...-...
CMW11060ETHIOPIA         ....T..... ....-..... .......... .......... .......... -.....-... ..-...-...
CMW11062ETHIOPIA         ....T..... ....-..... .......... .......... .......... -.....-... ..-...-...
CMW10095ETHIOPIA         .......... ....-..... .....T.... .......... .......... -.....-... ..-...-...
B_eucalyptorum_CMW10125  ....T..... ....-..... .......... .......... .......... -.....-.T. ..T..--...
B_eucalyptorum_CMW10126  ....T..... ....-..... .......... .......... .......... -.....-.T. ..T..--...
B_lutea_CMW9076          ....C..... ....-..... .......... .......... .......... -....AC.C. .G-...-...
B_lutea_CMW992           ....C..... ....-..... .......... .......... .......... -....AC.C. .G-...-...
B_RIBIS_CMW7772          .......... ....-..... .......... .......... .......... -.....-... ..-...G...
B_RIBIS_CMW7773          .......... ....-..... .......... .......... .......... -.....-... ..-...G...
G_PHILOPRINA_CMW7063     ....TT...T ...AA..... T......... .....C..-. .-..--G.AA G..-AAC..G .--.C.-..-
```

```
                                430        440        450        460        470        480        490
B_parva_CMW9071          GGGCTGGCCA GCGCCCGCCA GAGGACCAT- AAAACTCCAG TCAGTGAAC- TTCGCAGTCT GAAAAACAAG
B_PARVA_CMW9078          .......... .......... .........- .......... .........- .......... ..........
B_PARVA_CMW9077          .......... .......... .........- .......... .........- .......... ..........
B_DOTHIDEA_CMW7780       ...-...... .......... .........C ...-...... .....A...G A.-....... .........-T
B_DOTHIDEA_CMW8000       ...-...... .......... .........C ...-...... .....A...G A.-....... .........-T
CMW10094ETHIOPIA         .......... .......... .........- .......... .........- .......... ..........
CMW10089ETHIOPIA         .......... .......... .........- .......... .........- .......... ..........
CMW11064ETHIOPIA         .......... .......... .........- .......... .........- .......... ..........
CMW11060ETHIOPIA         .......... .......... .........- .......... .........- .......... ..........
CMW11062ETHIOPIA         ..-....... .......... .........- .......... .........- .......... ..........
CMW10095ETHIOPIA         .......... .......... .........- .......... .........- .......... ..........
B_eucalyptorum_CMW10125  ..-....... ...T...... .........C .......... .....A...G ..-....... ..........
B_eucalyptorum_CMW10126  ..-....... ...T...... .........C .......... .....A...G ..-....... ..........
B_lutea_CMW9076          ....C..... .......... .........C .......... .....A...G -......... ..G.......
B_lutea_CMW992           ....C..... .......... .........C .......... .....A...G -......... ..G.......
B_RIBIS_CMW7772          .......... .......... .........- .......... .........- .......... ..........
B_RIBIS_CMW7773          .......... .......... .........- .......... .........- .......... ..........
G_PHILOPRINA_CMW7063     ---....T.. ...G...... .......--.C .......ATA ----.T.TTA ..-.TC.... ..GT.-.T.T
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                                500        510        520        530        540        550        560
B_parva_CMW9071          TTAATAAACT AAAACTTTCA ACAACGGATC TCTTGGTTCT GGCATCGATG AAGAACGCAG CGAAATGCGA
B_PARVA_CMW9078          .......... .......... .......... .......... .......... .......... ..........
B_PARVA_CMW9077          .......... .......... .......... .......... .......... .......... ..........
B_DOTHIDEA_CMW7780       .......... .......... .......... .......... .......... .......... ..........
B_DOTHIDEA_CMW8000       .......... .......... .......... .......... .......... .......... ..........
CMW10094ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW10089ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW11064ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW11060ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW11062ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW10095ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125  .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10126  .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW9076          .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW992           .......... .......... .......... .......... .......... .......... ..........
B_RIBIS_CMW7772          .......... .......... .......... .......... .......... .......... ..........
B_RIBIS_CMW7773          .......... .......... .......... .......... .......... .......... ..........
G_PHILOPRINA_CMW7063     A.....G-T. .......... .......... .......... .......... .......... ..........
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                          570        580        590        600        610        620        630
B_parva_CMW9071      TAAGTAATGT GAATTGCAGA ATTCAGTGAA TCATCGAATC TTTGAACGCA CATTGCGCCC CTTGGTATTC
B_PARVA_CMW9078      .......... .......... .......... .......... .......... .......... ..........
B_PARVA_CMW9077      .......... .......... .......... .......... .......... .......... ..........
B_DOTHIDEA_CMW7780   .......... .......... .......... .......... .......... .......... T.........
B_DOTHIDEA_CMW8000   .......... .......... .......... .......... .......... .......... T.........
CMW10094ETHIOPIA     .......... .......... .......... .......... .......... .......... ..........
CMW10089ETHIOPIA     .......... .......... .......... .......... .......... .......... ..........
CMW11064ETHIOPIA     .......... .......... .......... .......... .......... .......... ..........
CMW11060ETHIOPIA     .......... .......... .......... .......... .......... .......... ..........
CMW11062ETHIOPIA     .......... .......... .......... .......... .......... .......... ..........
CMW10095ETHIOPIA     .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125 .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10126 .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW9076      .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW992       .......... .......... .......... .......... .......... .......... ..........
B_RIBIS_CMW7772      .......... .......... .......... .......... .......... .......... ..........
B_RIBIS_CMW7773      .......... .......... .......... .......... .......... .......... ..........
G_PHILOPRINA_CMW7063 .......... .......... .......... .......... .......... .......... .C........
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```
                                640        650        660        670        680        690        700
B_parva_CMW9071          CGAGGGGCAT GCCTGTTCGA GCGTCATTTC AACCCTCAAG CTCTGCTTGG TATTGGGCCC CGTCCTCCAC
B_PARVA_CMW9078          .......... .......... .......... .......... .......... .......... ..........
B_PARVA_CMW9077          .......... .......... .......... .......... .......... .......... ..........
B_DOTHIDEA_CMW7780       ...A...... .......... ........A. .......... .......... ........A. ......T--T
B_DOTHIDEA_CMW8000       ...A...... .......... ........A. .......... .......... ........A. ......T--T
CMW10094ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW10089ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW11064ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW11060ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW11062ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
CMW10095ETHIOPIA         .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125  .......... .......... .......... .......... ..T....... .......... ......-.T-
B_eucalyptorum_CMW10126  .......... .......... .......... .......... ..T....... .......... ......-.T-
B_lutea_CMW9076          .......... .......... .......... .......... .......... ........T. ......-.T-
B_lutea_CMW992           .......... .......... .......... .......... .......... ........T. ......-.T-
B_RIBIS_CMW7772          .......... .......... .......... .......... .......... ........T. ..........
B_RIBIS_CMW7773          .......... .......... .......... .......... .......... ........T. ..........
G_PHILOPRINA_CMW7063     ..G....... .......... ........A. .......... .......... ........-- -...AC..C.
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```
                          710        720        730        740        750        760        770
B_parva_CMW9071       GGACGC---G CCTCAAAGAC CTCGGCGGTG GCGTCTTGCC TCAAGCGTAG TAGAA--AAC ACCTCGCTTT
B_PARVA_CMW9078       .......---. ...T...... .......... .......... .......... .....--... ..........
B_PARVA_CMW9077       .......---. .......... .......... .......... .......... .....--... ..........
B_DOTHIDEA_CMW7780    .CGG..-GC. .......... .......... .......... .......... .....CAT.. .T.......C
B_DOTHIDEA_CMW8000    .CGG..-GC. .......... .......... .......... .......... .....CAT.. .T.......C
CMW10094ETHIOPIA      .......---. ...T...... .......... .......... .......... .....--... ..........
CMW10089ETHIOPIA      .......---. ...T...... .......... .......... .......... .....--... ..........
CMW11064ETHIOPIA      .......---. ...T...... .......... .......... .......... .....--... ..........
CMW11060ETHIOPIA      .......---. ...T...... .......... .......... .......... .....--... ..........
CMW11062ETHIOPIA      .......---. ...T...... .......... .......... .......... .....--... ..........
CMW10095ETHIOPIA      .......---. ...T...... .......... .......... .......... .....--... ..........
B_eucalyptorum_CMW10125 -.TG.ACGC. .......... .......... .......... .......... .....--.T. ..........
B_eucalyptorum_CMW10126 -.TG.ACGC. .......... .......... .......... .......... .....--.T. ..........
B_lutea_CMW9076       -.TG.ACGC. ....G..... .......... .......... .......... .....-A.-. ..........
B_lutea_CMW992        -.TG.ACGC. ....G..... .......... .......... .......... .....-A.-. ..........
B_RIBIS_CMW7772       .......---. ...T...... .......... .......... .......... .....--... ..........
B_RIBIS_CMW7773       .......---. ...T...... .......... .......... .......... .....--... ..........
G_PHILOPRINA_CMW7063  ..GT..---. ...T...AT. AGT....... C.....G..T .......... .--..--T.. TT........
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                          780        790        800        810        820        830 833
B_parva_CMW9071      GGAGCGCACG GCGTCGCCCG CCGGACGAAC CTTT-GAATT ATTTCTCAAG GTTGACCTCG GAT
B_PARVA_CMW9078      .......... .......... .......... ....-..... .......... .......... ...
B_PARVA_CMW9077      .......... .......... .......... ....-..... .......... .......... ...
B_DOTHIDEA_CMW7780   ........G. .......... .......... ...CT...C. -......... .......... ...
B_DOTHIDEA_CMW8000   ........G. .......... .......... ...CT...C. -......... .......... ...
CMW10094ETHIOPIA     .......... .......... .......... ....-..... .......... .......... ...
CMW10089ETHIOPIA     .......... .......... .......... ....-..... .......... .......... ...
CMW11064ETHIOPIA     .......... .......... .......... ....-..... .......... .......... ...
CMW11060ETHIOPIA     .......... .......... .......... ....-..... .......... .......... ...
CMW11062ETHIOPIA     .......... .......... .......... ....-..... .......... .......... ...
CMW10095ETHIOPIA     .......... .......... .......... ....-..... .......... .......... ...
B_eucalyptorum_CMW10125 ........T. .......... .......... ....-..... -......... .......... ...
B_eucalyptorum_CMW10126 ........T. .......... .......... ....-..... -......... .......... ...
B_lutea_CMW9076      .......... .......... .......... ....-..... -......... .......... ...
B_lutea_CMW992       .......... .......... .......... ....-..... -......... .......... ...
B_RIBIS_CMW7772      .......... .......... .......... ....-..... .......... .......... ...
B_RIBIS_CMW7773      .......... .......... .......... ....-..... .......... .......... ...
G_PHILOPRINA_CMW7063 ....TC.GG. CGAG..T..T G.CA.--... .CCC--.--. ....T.T... .......... ...
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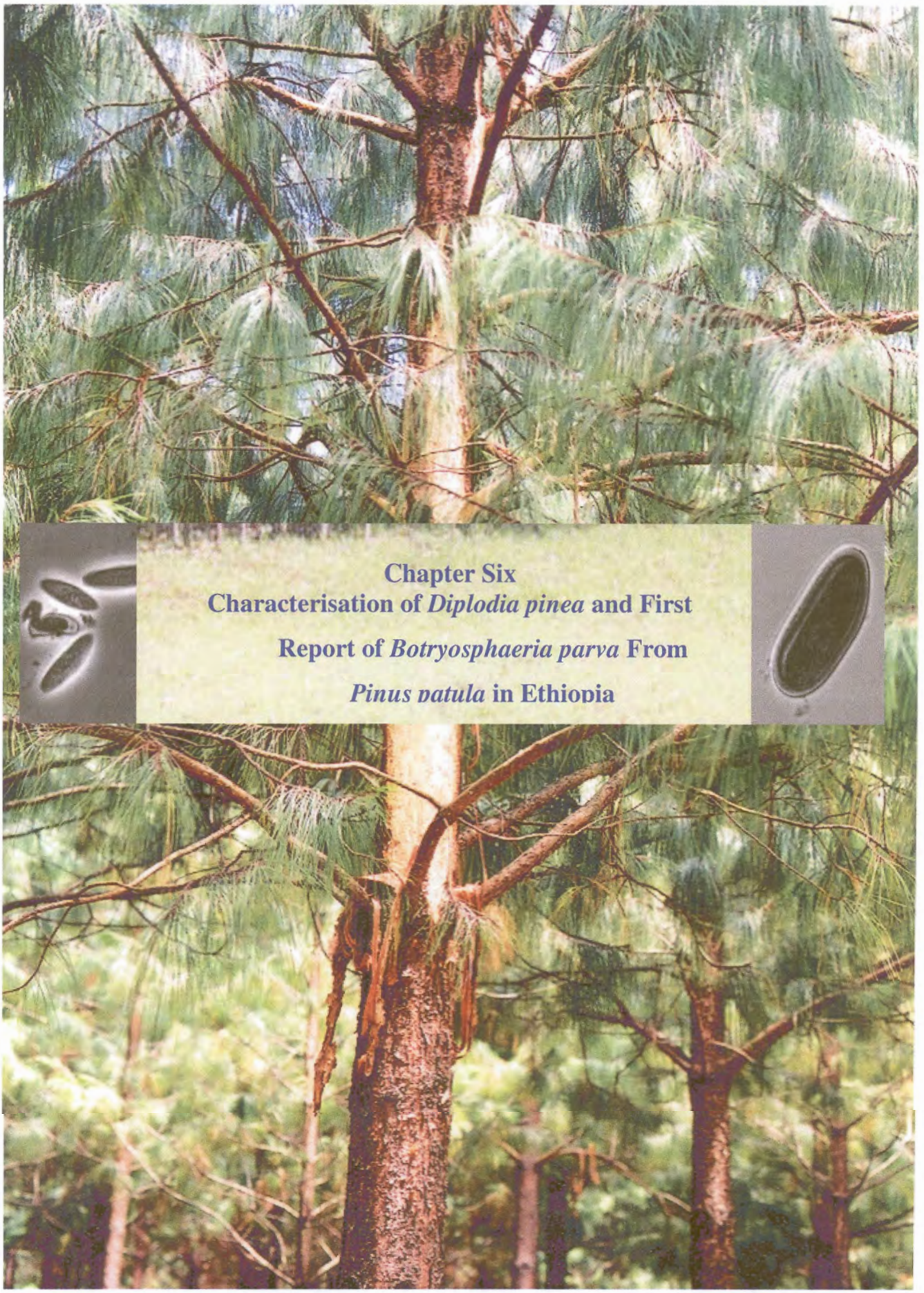

Chapter Six
Characterisation of *Diplodia pinea* and First Report of *Botryosphaeria parva* From *Pinus patula* in Ethiopia

ABSTRACT

Pinus patula is extensively used in Ethiopian reafforestation. In recent disease surveys a dark grey fungus resembling *Diplodia* sp. was isolated from cones of *P. patula* in Ethiopia. *Diplodia pinea* has a cosmopolitan distribution on *Pinus* spp. and is an important pathogen on exotic *Pinus* spp. in the tropics and Southern Hemisphere. *D. pinea* is a stress related opportunistic pathogen that can result in branch and shoot die-back, root disease and blue stain of the sapwood. *D. pinea,* including the two morphotypes (A and C), and *D. scrobiculata* are commonly associated with *Pinus* spp. The aim of this study was to characterise dark grey fungal isolates obtained from the cones of *P. patula* in Ethiopia. These were compared based on morphology and DNA based techniques. Morphological comparisons showed that the fungi isolated from pine cones represent a species of *Fusicoccum* as well as *D. pinea*. DNA sequences for the ITS, 5.8S and β-tubulin gene regions confirmed the identification of *D. pinea* and showed that the fungus with the *Fusicoccum* anamorph is closet to *Botryosphaeria parva* or *B. ribis*. Analysis of SSR sequences showed that the *D. pinea* isolates from Ethiopia represent the A morphotype of the fungus, which is widely distributed in countries where pines are grown as exotics. Pathogenicity tests showed that isolates of both *Fusicoccum* sp. and *D. pinea* were able to cause lesions but the *D. pinea* isolates were most pathogenic.

INTRODUCTION

In Ethiopia, *P. patula* Schiede & Deppe and *P. radiata* D. Don. have been used extensively for afforestation, having been introduced into the country more than 100 years ago. *P. patula* is still planted in Ethiopia whereas planting of *P. radiata* has been abandoned probably due to Dothistroma needle blight. Plantations of *Pinus* spp. cover approximately 30 000 ha, of which *P. patula* constitutes the major proportion (Anonymous 1994). Information available on the disease situation in these *P. patula* plantations is very limited. In a recent disease survey conducted in Ethiopia, a fungus resembling *Diplodia pinea* (Desm.) Kick (= *Sphaeropsis sapinea* (Fr.:Fr.) Dykco & Sutton) was isolated from cones of *P. patula* (Alemu, Roux & Wingfield 2003).

Diplodia pinea has a world-wide distribution and an extensive host range mainly among the conifers (Birch 1937, Eldridge 1961, Gibson 1979). This fungus is known from *Pinus* spp., wherever they are planted (Currie & Toes 1978, Gibson 1979) and it is known as an opportunistic pathogen (Marks & Minko 1969, Swart, Knox-Davies & Wingfield 1985). Stress, associated with environmental conditions and mechanical damage, predispose trees to disease caused by *D. pinea.* The fungus exists in pine cones and stems as an endophyte (Smith *et al.* 1996), where it lives in the plant without necessarily showing disease symptoms. Expression of symptoms commences when the trees are under stress (Swart *et al.* 1985, Smith *et al.* 1996, Stanosz *et al.* 1997), such as that caused by hail damage, drought or frost (Marks & Minko 1969, Bega *et al.* 1978, Swart *et al.* 1985, Swart & Wingfield 1991).

Diplodia pinea is associated with various disease symptoms (Puntinhalingam & Waterson 1970, Swart *et al.* 1985). These include shoot blight, shoot die-back, stem canker (Gilmour 1964, Marks & Minko 1969, Swart *et al.* 1985, Stanosz *et al.* 1997), root diseases (Wingfield & Knox-Davies 1980) and staining of wood (Da Costa 1955, Eldridge 1961). In South Africa, for example, *D. pinea* was found associated with seedling root rot (Wingfield & Knox-Davies 1980) and associated with hail damage (Swart *et al.* 1985). For this reason, *P. radiata* is not planted in summer rainfall areas in South Africa (Swart, Wingfield & Knox-Davies 1987, Swart & Wingfield 1991).

In the past, four morphotypes (A, B, C and I) have been described for *D. pinea* (Wang *et al.* 1985, Palmer, Swart & Wingfield 1987, De Wet *et al.* 2002, Hausner *et al.* 1999). Burgess, Wingfield & Wingfield (2001) showed that the I morphotype represents the anamorph of *Botryosphaeria obtusa* (Schw.) Shoemaker. In a recent study, De Wet *et al.* (2003) showed that the A and C morphotypes, once encompassed in *S. sapinea*, are closely related to each other and treated them in *D. pinea.* They also showed that the B morphotype of *S. sapinea* represent a new *Diplodia* sp. recently described as *D. scrobiculata* J. de Wet, B. Slippers & M. J. Wingfield (De Wet *et al.* 2003).

All of the abovementioned fungi, broadly treated as *D. pinea* are known to be pathogenic to a wide range of *Pinus* spp. (Wang *et al.* 1985, Palmer *et al.* 1987, De Wet *et al.* 2002). The C morphotype of *D. pinea* is more pathogenic than the A morphotype or *D. scrobiculata* (De Wet *et al.* 2002). The A morphotype is known to occur on a wide range of conifers world-wide (Morelet & Chandelier 1993, Smith & Stanosz 1995, Hausner *et al.* 1999, De Wet *et al.* 2000). *D. scrobiculata* is best known from the North Central United States and is only mildly pathogenic (Wang *et al.* 1985, Smith & Stanosz 1995, Stanosz, Swart & Smith 1999, De Wet *et al.* 2000, Burgess *et al.* 2001). The C morphotype has been reported only from Indonesia (De Wet *et al.* 2002).

Very little information is available regarding diseases affecting *P. patula* in Ethiopia. In a recent survey, Alemu *et al.* (2002), identified a fungus similar to *Armillaria fuscipes* Petch as a cause of mortality in certain plantation of *Pinus patula* in Western and South Western Ethiopia. Alemu *et al.* (2003), also reported the presence of *D. pinea* in cones of *P. patula* in Ethiopia, but these authors did not identify the morphotype of the *Diplodia* isolates. The aim of this study was to identify and characterise these isolates. This was based both on morphological characteristics and DNA based techniques. Pathogenicity of the isolates was also considered in greenhouse trials.

MATERIALS AND METHODS

Fungal isolations and morphological characterisation

During a disease survey conducted in 2000, cones were randomly collected from the forest floor in a *P. patula* plantation at Munessa Shashemene and isolations were made from them. The cones were opened and ~3 mm sections were taken from the pith of the cones. These were surface-sterilised with ethanol (100%), washed with sterile water, plated onto 2% MEA (20 g Biolab Malt Extract, 15 g Biolab Agar) and incubated at 25 °C. Isolates with dark grey mycelium, typical of *D. pinea* were selected and transferred to MEA in Petri dishes. Pure cultures were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute, University of Pretoria.

Isolates were transferred to water agar (WA) (15 g Biolab Agar, 1L H_2O) with sterilised pine needles laid on the surface and incubated for three weeks to promote production of fruiting structures. Single conidial isolates were made from the resultant fruiting structures. This was achieved by spreading spore masses on MEA in a drop of sterile water and incubating plates for 24 hr at 25 °C. After 24 hr, single germinating spores were picked and transferred to MEA and incubated at 25 °C.

Conidia were mounted in lactophenol and examined using a Zeiss Axioskop light microscope. Ten spores were selected randomly from each isolate and the lengths and widths of the conidia were measured (Table 1).

DNA extraction

Total genomic DNA was extracted from isolates selected to represent the different morphological groups (Table 2). Mycelium for DNA extraction was obtained by scraping the surface of the agar plates with a sterile scalpel and placing mycelial mats into 1.5 µl Eppendorf tubes. The mycelium was freeze dried and ground to a fine powder in liquid nitrogen using a pestle and mortar. The method of Raeder and Broda (1985) was used to extract DNA from the mycelium.

The DNA pellets were vacuum dried to remove excess ethanol and re-suspended in 50 µl sterilised water. RNase A (1mg/ml) (Roche Diagnostics, South Africa) was added to the DNA solution to remove the contaminating RNA and incubated at 37 °C in a water bath over night. The concentration of the DNA in the samples was detected by comparison with a standard on a 1% agarose gel, stained with ethidium bromide and visualised under UV light.

PCR amplification

The internal transcribed spacer (ITS) regions of the ribosomal RNA operon and the 5.8S gene were amplified using the polymerase chain reaction (PCR). PCR was conducted using primers ITS 1 (5' TCC GTA GGT GAA CCT GCG G '3) and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC '3) (White *et al.* 1990). The PCR reaction mixture contained DNA polymerase (*Taq*, 2.5U/µl, Roche), 2.5 mM dNTP's, 10x PCR Buffer (10 mM Tris-HCl, HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.15 mM of each primer, 1 µl of DNA and 37 µl sterilised water to make up a final volume of 50 µl. Denaturation was performed at 96 °C for 1 min. This was followed by 35 cycles of primer annealing at 55 °C for 30 s, chain elongation at 72 °C for 1 min and denaturation at 92 °C for 1 min. Final chain elongation was carried out at 72 °C for 5 min.

The β-tubulin gene was partially amplified using the forward primer Bt2a (5' GGT AAC CAA ATC GGT GCT GCT TTC 3') and the reverse primer Bt2b (5' ACC CTC AGT GTA G TG ACC CTT GGC 3') (Glass & Donaldson 1995). The PCR reaction mix included DNA polymerase (*Taq*, 2.5U/µl), 2.5 mM dNTP's, 10x Buffer, 25 mM $MgCl_2$ supplied by the manufacturer, 0.15 mM of each primer, 1 µl of DNA and 37 µl sterilized water. PCR reaction conditions involved an initial denaturation at 94 °C for 1 min, which was followed by 40 cycles at 94 °C for 1 min, primer annealing at 51 °C for 30 s, chain elongation at 72 °C for 1 min and an additional chain elongation step at 72 °C for an additional minute.

Morphotype determination

Three sets of unlabelled Simple Sequence Repeat (SSR) markers (Burgers *et al.* 2001) were used in PCR reactions to determine the morphotype of the *D. pinea* isolates. The sets of the SSR primers used included SS7 (forward primer TB23 5' GAC AGA CAT CTA GGC CCT GC 3' and reverse primer TB24 5' GAT CAG TCG GTC GAG ACG AG 3'), SS9 (forward primer TB37 5' CAG CGG TTT CAT TGA AAT GCC 3') and reverse primer TB38 5' GAC TTG TCT CCT ACC GAT TCC 3') as well as SS10 (forward primer TB41 5' GCC AAC CCT AAT GCT TCC ATG 3' and reverse primer TB42 5' CAG CGG CGA TTG CGG TAT GG 3') (Burgess *et al.* 2001). The PCR reactions and conditions used were similar to those described by Burgess *et al.* (2001). All PCR products were detected on 1% agarose gels stained with ethidium bromide and visualised under UV illumination.

DNA sequencing

All PCR products were purified using the High Pure PCR Product Purification Kit (QUIAGEN, GmbH, Hilden, Germany). The PCR products were sequenced in both directions using the Big Dye Cycle Sequencing kit with Amplitaq® DNA Polymerase, FS (Perkin-Elmer, Warrington, UK), according to the manufacturer's protocol, on an ABI PRISM^{TM} 3100 DNA Autosequencer (Perkin-Elmer). Primers ITS 1 and ITS 4 were used for sequencing the ITS regions whereas the β-tubulin genes were sequenced using primers Bt2a and Bt2b. For the morphotype determination the SSR primers mentioned above were used.

Sequence analysis

The identity of the isolated fungi was determined by comparing the ITS rDNA sequences of the Ethiopian isolates against sequences obtained from GenBank [National Centre for Biotechnology Information (NCBI), US National Institute of Health Bethesda (http:/www.ncbi.nlm.gov/BLAST)]. Thereafter, the ITS rDNA and β-tubulin gene sequences of the isolates were combined and aligned manually using

PAUP 4.0b (Swofford 1998) against the sequence data set of *D. pinea* and *Botryoshpaeria* spp. obtained from De Wet *et al.* (2000) and Slippers *et al.* (2003) (Table 2). Gaps were inserted manually and were treated as missing data. The sequences were analysed using parsimony, with trees generated by heuristic searches with simple addition and Tree Bisection Reconstruction (TBR) branch swapping. Confidence intervals were determined using DNA BOOTSTRAP analysis (Bootstrap confidence intervals on DNA parsimony) (1000 replicates) (Felsenstein 1993). *Guignardia philoprina* (Ellis) Viala & Ravaz as well as *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. were used as outgroup taxa in the phylogenetic analysis.

SSR sequences of the Ethiopian isolates were aligned against each other and with representative sequences of the three morphotypes of *D. pinea* obtained from De Wet *et al.* (2003). The sequences were analysed using parsimony, with trees generated by heuristic searches with simple addition and Tree Bisection Reconstruction (TBR) branch swapping. A phylogenetic tree showing the relationships of the isolates was obtained using the mid point rooting option.

Pathogenicity trials

Greenhouse inoculation trials using isolates CMW10717, 11240, 11246, 11250, 11252 and 11253 were conducted to evaluate the pathogenicity of the fungi in question. The greenhouse pathogenicity tests were conducted on 2-year-old *P. taeda* seedlings. Prior to inoculation, the trees were kept in the greenhouse for ten days to allow them to acclimatise to the environment. Six isolates were selected to represent the two conidial forms emerging from morphological and DNA sequence results.

Isolates used in the inoculation trials were grown on MEA for ten days. A 9 mm cork borer was used to wound the trees and expose the cambium. Mycelial plugs of equal size were placed in the wounds with mycelium facing the exposed cambium. Each isolate was inoculated onto 20 trees. Plugs of sterile MEA, were also inoculated onto 20 trees, to serve as controls. The inoculated wounds were covered with Parafilm (Pechiney Plastic Packageing, Chicago, USA) to prevent desiccation.

After six weeks, lesion lengths were measured to evaluate disease development on inoculated plants. One-way analysis of variance (ANOVA) using Statistica for Windows (StatSoft. Inc. 1995) was carried out to evaluate statistical differences between treatments. Mean variation was compared using Dunnett's T -test available in Statsitica for Windows (StatSoft. Inc. 1995).

RESULTS

Fungal isolations and morphological characterisation

Several dark coloured isolates were obtained from the pine cones collected from *P. patula* plantations at Munessa Shashemene. Cultures showed grey to black mycelial growth on MEA. These cultures also had a fluffy mycelial growth covering the whole surface of the Petri dish. No distinct variation was detected in mycelial growth of the isolated fungi. In total, twenty isolates, each from a different cone were retained for further study.

Of the 20 isolates inoculated onto water agar, only 15 produced fruiting structures on the pine needles and conidial morphology was determined for these. Evaluation of conidial morphology showed that two different fungi were present. One group had conidia similar to those of *Fusicoccum* spp. (Figure 1a) and the other group had conidia similar to those of *D. pinea* (Figure 1b). The walls of the conidia in the latter group were smooth and the conidia were aseptate. The lengths of the conidia that resembled *D. pinea* varied between 34 µm and 35 µm (Table 1). Their widths ranged from 16 µm to 17 µm. The length of the conidia that resembled *Fusicoccum* spp. ranged from 17 µm to 19 µm and their average widths ranged from 5.2 µm to 5.7 µm (Table 1). No sexual structures were found. Four of the 15 isolates examined had conidia similar to *D. pinea* whereas 11 of the isolates had conidia similar to *Fusicoccum* sp.

PCR amplification and *DNA sequence comparisons*

A fragment size of approximately 500 base pairs (bp) was obtained when the ITS rDNA of *D. pinea* and the *Fusicoccum* isolates were amplified with primers ITS1 and

ITS4. Partial amplification of the β-tubulin gene of the *D. pinea* and *Fusicoccum* isolates with primers Bt2a and Bt2b produced a fragment size of approximately 400 bp.

The ITS sequence data of the *D. pinea* isolates were compared with sequences in Genbank. Sequences of *Diplodia* isolates showed that they were closely related to *D. pinea*. Similarly, when sequences of the *Fusicoccum* isolates were compared, they showed a high degree of homology to sequences of *Botryosphaeria ribis* Grossenb. & Dugg. (anamorph =*Fusicoccum ribis* Grossenb. & Dugg.) and *B. parva* (Pennycook & Samuels).

The ITS rDNA and β-tubulin sequences of the isolates used in this study were combined and aligned against each other and against sequence data obtained from De Wet *et al.* (2002) and Slippers *et al.* (2003). After alignment a total of 1029 characters was obtained (Figure 6). Of these 486 characters were constant whereas 278 characters were variable and parsimony uninformative, while 265 characters were parsimony informative. Phylogenetic analysis using parsimony produced 9 trees. The topologies of these trees were the same with only minor variation in arrangements within the groups. The phylogenetic tree (CI=0.886, RI=0.886) (Figure 2) showed that sequences of the Ethiopian isolates grouped together with *D. pinea* isolates with a 100% bootstrap value and the Ethiopian *Fusicoccum* isolates resided in a clade containing *B. parva* and *B. ribis* with 97% bootstrap value. The Ethiopian *Fusicoccum* isolates, however, formed their own subgroup of which the exact identity is unclear (Figure 2). This clade was supported by a bootstrap value of 100%.

Morphotype determination

To determine the morphotype of the *D. pinea* isolates a further sequence analysis was conducted using three SSR markers. Alignment of the combined SSR sequences of the *D. pinea* isolates with sequences obtained from De Wet *et al.* (2003) produced a total of 1051 base pairs (Figure 7). Sequence analysis using mid point rooting produced a single tree. This phylogenetic tree showed that the *D. pinea* isolates from Ethiopia group together with the 'A' morphotype of *D. pinea* (Figure 3).

Pathogenicity trials

All isolates tested in the two inoculation studies produced lesions on the two-year-old *P. taeda* seedlings (Figure 3). The mean lesion lengths produced by these isolates in the first inoculation trial was in a range of 30 mm to 56.8mm (Table 3) and in the second inoculation trial the lesion lengths produced were between 29.75 mm and 95.93 mm (Table 4). The *Diplodia* isolates produced the largest lesions compared to the *Fusicoccum* isolates. Both these fungi developed lesions that were significantly different (P>0.0001) compared to the lesions of the controls (Table 3, Figure 5). Analysis of variance showed statistically significant differences, in pathogenicity for the *D. pinea* and *Fusicoccum* sp., *D. pinea isolates* were more pathogenic than the *Fusicoccum* sp.

DISCUSSION

Pinus patula is the only *Pinus* sp. currently planted in Ethiopia and is of great importance to the country. During a survey conducted in *P. patula* plantations in Ethiopia, *D. pinea* was isolated from pine cones (Alemu *et al.* 2003). The results of the present study showed that two fungal species are associated with *P. patula* cones in Ethiopia. Based on morphological characteristics and sequence analysis, they were identified as a *Fusicoccum* sp. and *D. pinea*. This is the first report defining the morphotype of *D. pinea* and the presence of a *Botryosphaeria* sp. from *Pinus* spp. in Ethiopia. Our results, although preliminary, suggests that in contrast to other countries, the *Botryosphaeria* sp. is more commonly associated with *P. patula* cones than *D. pinea*.

Results of DNA based comparisons confirmed identifications based on morphology. Use of sequence analysis of the ITS and β-tubulin sequence, however, could not assist in determining the morphotype of *D. pinea* in Ethiopia. Further sequence analysis using SSR markers, however, showed that the *D. pinea* obtained from cones of *P. patula* from plantations at Munessa Shashemene in Ethiopia, belong to the A morphotype. De Wet *et al.* (2003) used SSR sequences to determine the relationships

of the morphotypes of *Diplodia*. The A morphotype has been frequently found associated with seed and seed chaff (Anderson, Belcher & Miller 1984), indicating the endophytic nature of the fungus. The A morphotype is also the most widely distributed morphotype of *D. pinea* (Wang *et al.*, 1985, De wet *et al.* 2000). Its presence in Ethiopia is, therefore, not surprising.

Identification of the *Fusicoccum* isolates to species level, was not possible using only conidial morphology. Evaluation of the ITS and β-tubulin sequences, however, showed that the *Fusicoccum* sp. associated with *P. patula* in Ethiopia is closely related to *B. ribis* and *B. parva*. Several *Fusicoccum* spp. are known anamorphs of *Botryosphaeria* spp. (Sutton 1980, Pennycook & Samuels 1985). *Botryosphaeria* spp. are also known as opportunistic wound and stress related pathogens and as symptomless endophytes on several hosts (Smith *et al.* 1996). It has been shown that *Botryosphaeria* spp. cause die-back and cankers on a wide range of woody plants including *Eucalyptus* spp. (Smith, Kemp & Wingfield 1994). In Hawaii, *B. dothidea* has been found associated with wilting and dying *P. taeda* and *P. elliottii* Engelm. (Hodges 1983). The importance of the *Fusicoccum* sp. in pine plantations of Ethiopia, however, needs further investigation.

The results of the greenhouse inoculation studies showed that both *D. pinea* and the *Fusicoccum* sp. are pathogenic to *P. taeda*. The *D. pinea* isolates, however, produced larger lesions than those of the *Fusicoccum* sp. The A morphotype of *D. pinea* has been shown to be highly pathogenic to several *Pinus* spp. (Wang *et al.* 1985, Palmer *et al.* 1987). In Swaziland symptoms similar to those of *D. pinea* were observed on *P. elliottii* Englem and *P. taeda* (Wingfield & Knox-Davies 1980) suggesting that *P. taeda* is susceptible to *D. pinea* infection. This was confirmed in our study where *P. taeda* was used as a substitute for *P. patula*, due to the lack of available trees.

The occurrence of *D. pinea* in Ethiopian *P. patula* plantations could have a serious impact on the management, utilisation and future development of *P. patula*. It has been shown that *D. pinea* was introduced into several countries, apparently with seeds (Burgess *et al.* 2001, Smith *et al.* 1996). It is therefore, essential to manage future introductions of pine seed into Ethiopia to minimize the risks of introduction of the

other morphotypes of *D. pinea*. For example, the C morphotype of this fungus is considerably more pathogenic than isolates of the A morphotype (De Wet *et al.* 2002). Multiple introductions increase the clonal diversity and risk of disease from this pathogen. Species site matching and selection for disease resistance have to be considered to minimise severe damage from *D. pinea*. The importance of the *Fusicoccum* sp. in *P. patula* plantation also needs further investigation.

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Table 1. Average conidial lengths of fungal isolates obtained from *Pinus patula* cones.

Isolate No.	Species	Average Conidial Length (µm)	Average Conidial Width (µm)
CMW11249	*Diplodia pinea*	34.6	15.6
CMW11250	„	33.6	16.5
CMW11252	„	33.2	16.1
CMW10717	„	34.3	15.8
CMW11240	*Fusicoccum sp.*	17.4	5.5
CMW11241	„	18.8	5.7
CMW11242	„	17.1	5.5
CMW11243	„	17.5	5.3
CMW11244	„	18.2	5.7
CMW11245	„	17.2	5.2
CMW11246	„	16.8	5.7
CMW11247	„	17.2	5.5
CMW11248	„	18.5	5.2
CMW11251	„	18.9	5.5
CMW11253	„	18.3	5.7

CMW numbers are those of the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

Values of conidial length and width are averages of 10 measurements.

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Tables 2. Isolates used in the DNA sequence analyses

Culture Nr.	Identity	Morhotype	Host	Origin	Collector
CMW189	*Diplodia pinea*	B	*Pinus resinosa*	United States	M. A. Palmer
CMW190	,,	A	*P. banksiana*	United States	M. A. Palmer
CMW4876	,,	C	*P .patula*	Indonesia	M. J. Wingfield
CMW11250[a]	,,	A	*P. patula*	Ethiopia	Alemu Gezahgne & Jolanda Roux
CMW11246[a]	,,	A	*P. patula*	Ethiopia	Alemu Gezahgne & Jolanda Roux
CMW10717[a]	,,	A	*P. patula*	Ethiopia	Alemu Gezahgne & Jolanda Roux
CMW4891	*Lasiodiplodia theobromae*	-		South Africa	W. A. Smith
CMW7780	*B. dothidea*	-	*Fraxinus excelsior*	Switzerland	B. Slippers
CMW8000	*B. dothidea*	-	*Prunus* sp.	Switzerland	B. Slippers
CMW10125	*B. eucalyptorum*	-	*E. grandis*	S. Africa	H. Smith
CMW10126	*B. eucalyptorum*	-	*E. grandis*	S. Africa	H. Smith
CMW992/3	*F. luteum*	-	*Actinidia deliciosa*	New Zealand	G.J. Smuels
CMW9076	*B. lutea*	-	*Malus X domestica*	New Zealand	S.R .Pennycook
CMW7772	*B. ribis*	-	*Ribis* sp.	New York	B. Slippers/ G. Hudler
CMW7773	*B. ribis*	-	*Ribis* sp.	New York	B. Slippers/ G. Hudler
CMW9071	*B. parva*	-	*Ribis* sp.	Australia	M.J. Wingfield
CMW994	*B. parva*	-	*Malus sylvestris*	New Zealand	G.J Samuels
CMW9077	*B. parva*	-	*Actinidia deliciosa*	New Zealand	S.R. Pennycook
CMW9071	*B. parva*	-	*Ribis* sp.	Australia	M.J. Wingfield
CMW10122	*B. parva*	-	*E. grandis*	S. Africa	H. Smith
CMW11246[a]	*B. parva*	-	*Pinus patula*	Ethiopia	Alemu Gezahgne & Jolanda Roux
CMW10717[a]	*B. parva*	-	*Pinus patula*	Ethiopia	Alemu Gezahgne & Jolanda Roux
CMW7060	*B. stevensii*	-	*Fraxinus ecelsior*	Netherlands	H. A. van der Aa
CMW7774	*B. obtusa*	-	*Ripes* spp.	New York, USA	B. Slippers/G. Hudler
CMW7775	*B. obtusa*	-	*Ripes* spp.	New York, USA	B. Slippers/G. Hudler
CMW10130	*B. rhodina*	-	*Vitex donniana*	Uganda	J. Roux
CMW9074	*B. rhodina*	-	*Pinus* sp.	Mexico	T. Burgess
CMW7063	*Guignardia phyiloprina*	-	*Taxus baccata*	Netherlands	H.A. van der Aa

[a]/ Sequences of the isolates from Ethiopia were obtained in this study. All other sequences are those from the studies of Slippers *et al.* (2003) and De Wet *et al.* 2000, 2003.

Table 3. Lesion lengths and confidence limits for trees inoculated with *D. pinea* and *Fusicoccum* sp. obtained from *Pinus patula* cones in Ethiopia.

Isolates	Species	Trial 1		Trial 2	
		Mean Lesion Length (mm)[1]	95% Confidence limits[1]	Mean Lesion Length (mm)[2]	95% Confidence limits[2]
CMW11250	*Diplodia pinea*	56.80[a]	48.819-64.781	38.45[bc]	30.873-46.026
CMW10717	,,	54.30[a]	46.319-62.281	51.3[b]	43.732-58.876
CMW11252	,,	48.65[ab]	40.669-56.631	95.93[a]	87.185-104-681
CMW11246	*Fusicoccum* sp.	38.85[b]	30.869-46.831	52.30[b]	44.723-59.876
CMW11240	,,	37.90[b]	29.918-45.881	37.40[bc]	29.823-44.976
CMW11253	,,	30.00[bc]	22.019-37.981	29.75[c]	22.173-37.326
CONTROL	—	14.35[c]	6.369-22.331	11.95[d]	4.373-19.526

Each value is the average of 20 measurements.

Means followed by the same letters are not significantly different from each other at P=<0.05 significance level.

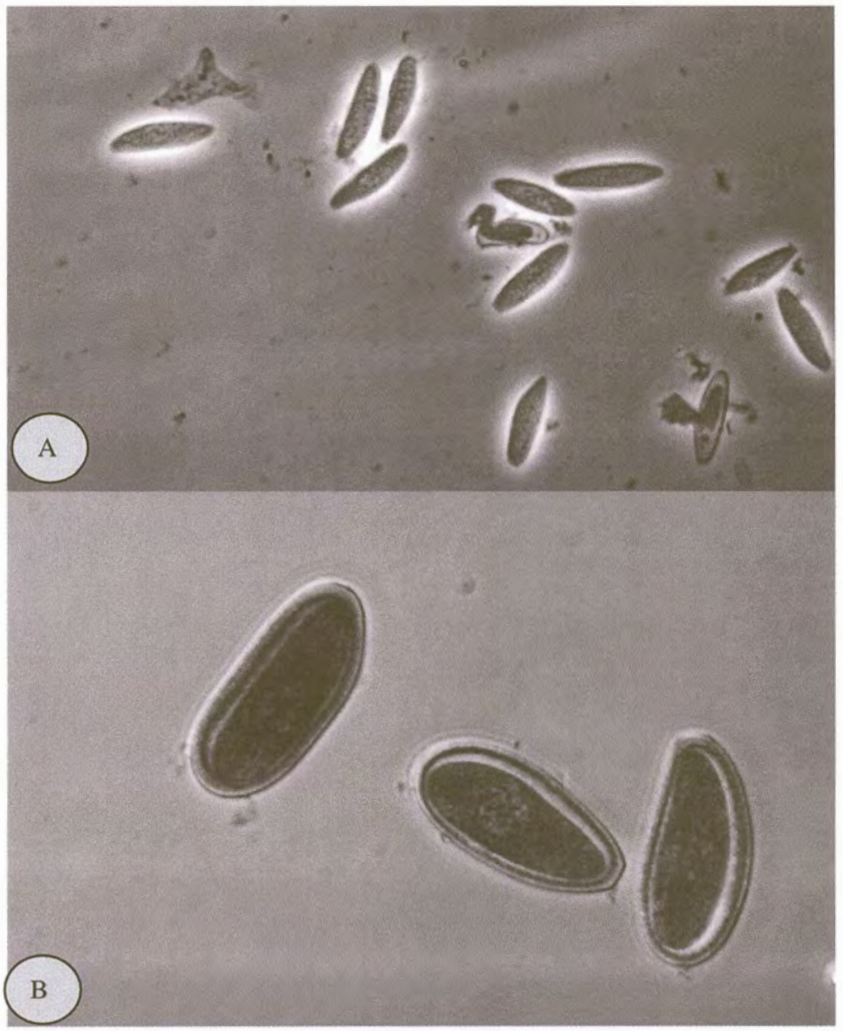

Figure 1. Condial morphology for fungal isolates from *P. patula* cones in Ethiopia. (a) *Fusicoccum* sp. (b) *D. pinea.*

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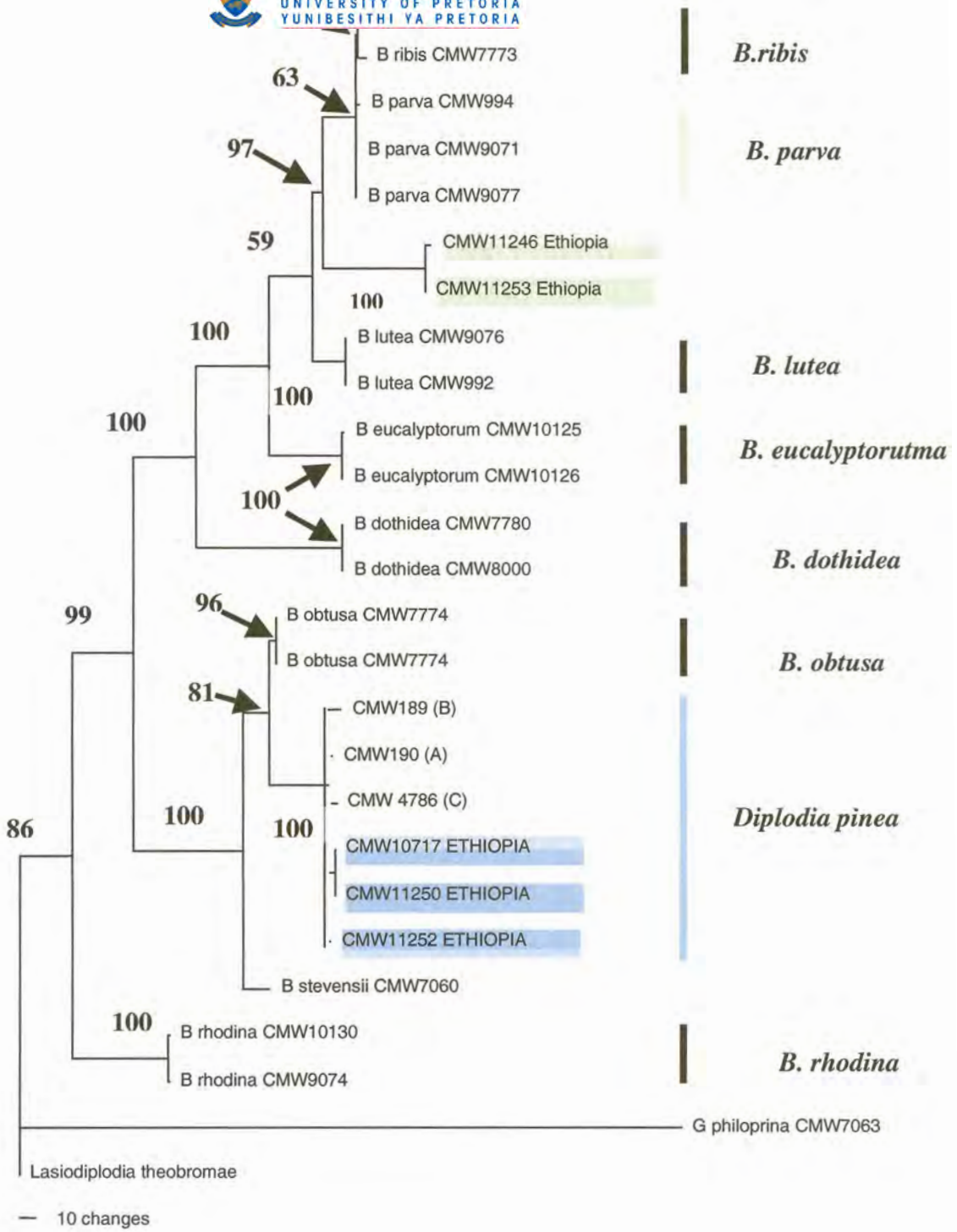

Figure 2. Phylogenetic tree of the combined sequences of the ITS rDNA and β-tubulin gene of *Diplodia* and *Fusicoccum* sp. Bootstrap values are shown at each branch.

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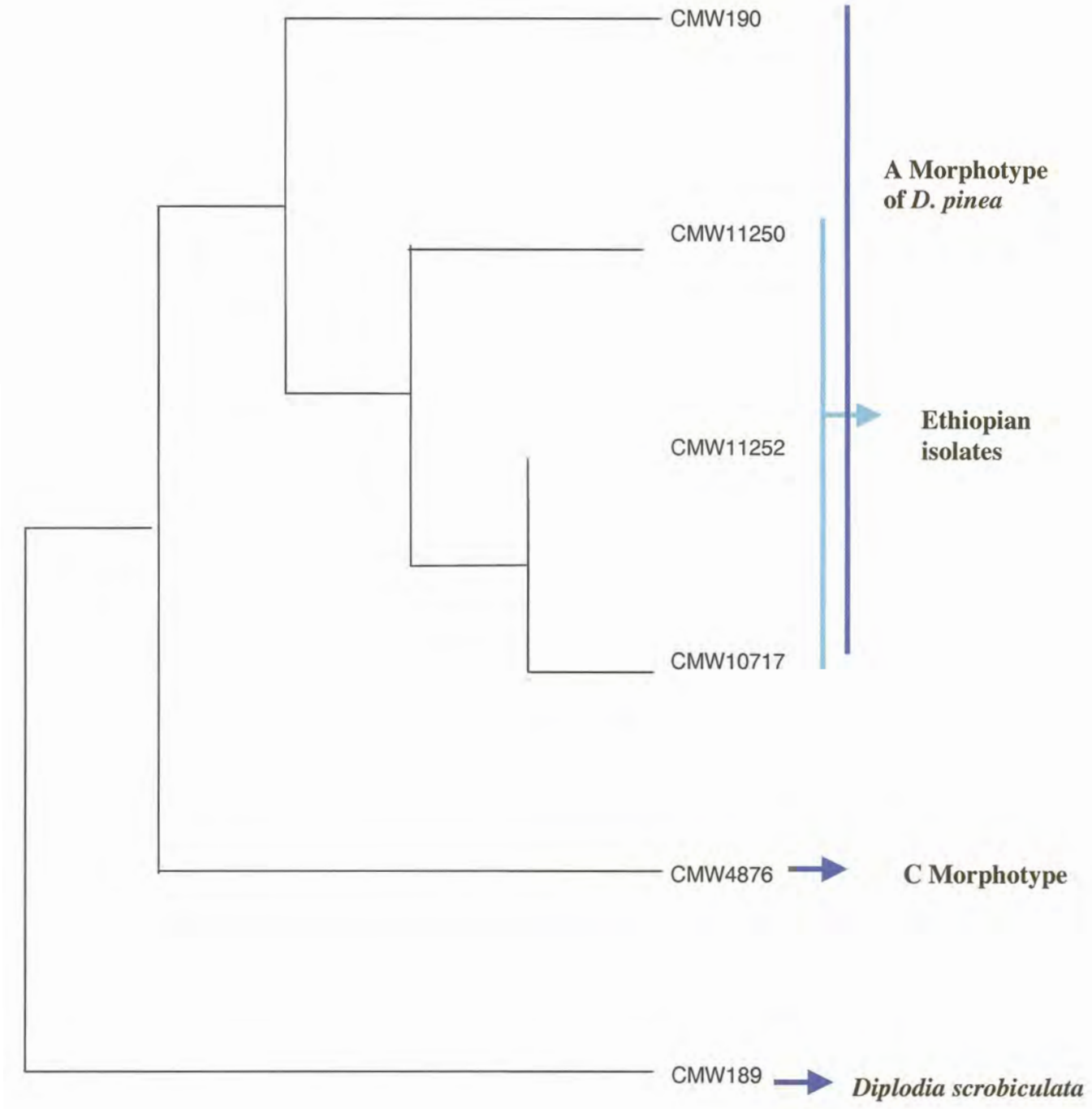

Figure 3. Phylogram of sequence data generated using SSR markers separating the A and C morphotypes of *Diplodia pinea*.

Figure 4. Lesion development on *Pinus tadea* in the greenhouse inoculation trial (a) Control, (b) Lesion from *Fusicoccum* isolate, and (c) Lesion from *D. pinea*.

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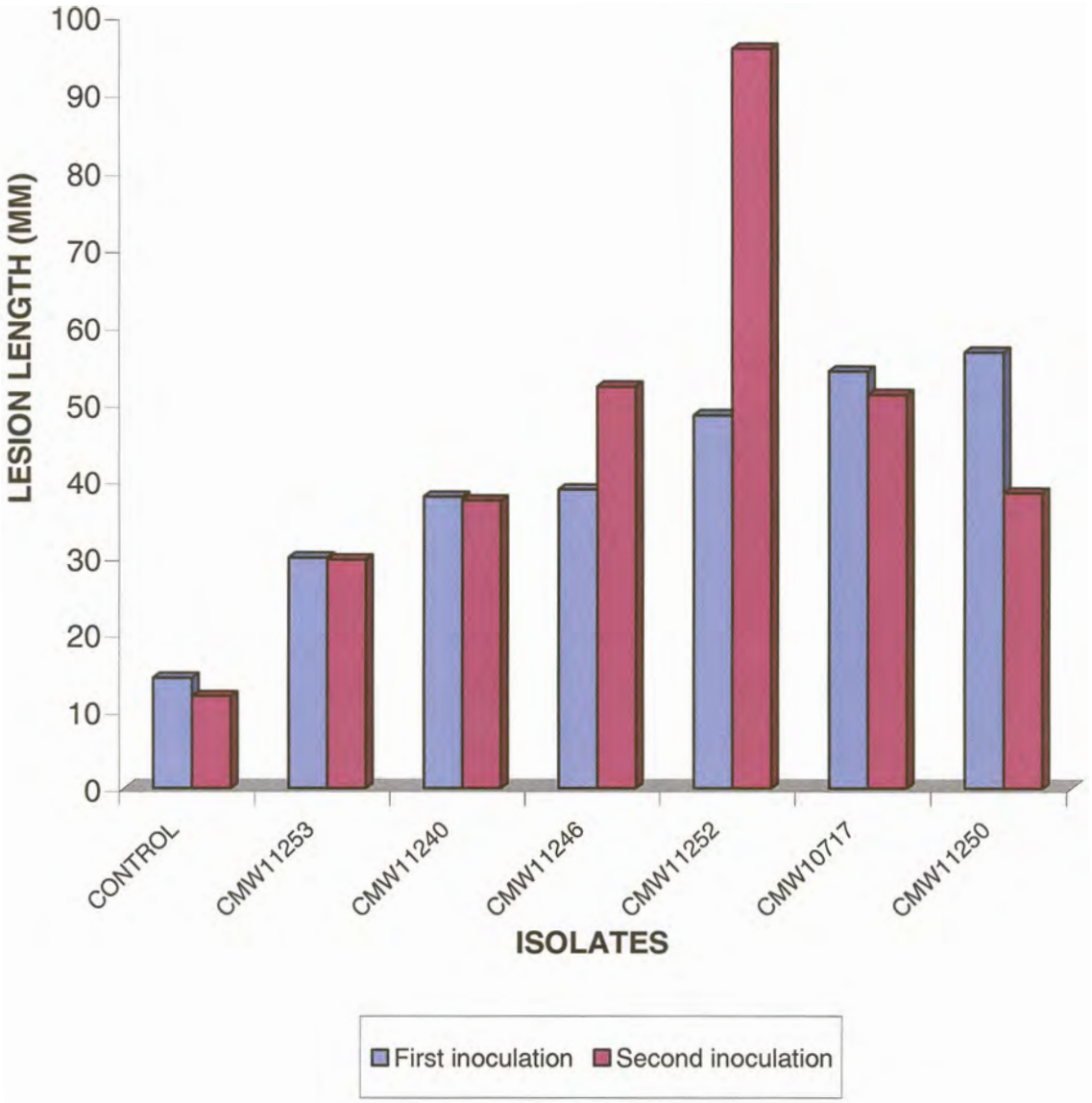

Figure 5. Mean lesion lengths of *Diplodia pinea* and *Fusicoccum* sp. in greenhouse inoculation trials. Isolates CMW11240, 11246 and 11253 represent *Fusicoccum* sp. whereas CMW10717, 11250 and 11252 represent *Diplodia pinea* isolates.

Figure 6. Alignment of combined sequences of The ITS rDNA and β-tubulin genes of *Fusicoccum* sp. and *D. pinea*. (-)= gaps, (.)= Homologous nucleotides, (N)= Unknown bases.

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                                  10         20         30         40         50         60         70
B_ribis_CMW7772                   NNNNNACCAA A-TCGGTGCT GCTTTCTGGT TTGTTGCCAA AACACTCCCG CTCCCGCGCC CCC--GCTGA
B_ribis_CMW7773                   NNNNN..... .......... .......... .......... .......... .......... ..........
B_parva_CMW994                    NNNNN..... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071                   NNNNNNNNNN N......... .......... .......... .......... .......... ..........
B_parva_CMW9077                   NNNNNNNNNN .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125           NNNNN..... .......... .......... .......... .......T.. ....T..... ..........
B_eucalyptorum_CMW10126           NNNNNNNNNN .......... .......... .......... .......T.. ....T..... ..........
B_lutea_CMW9076                   NNNNN..... .......... .......... .......... ......G... .......... ..........
B_lutea_CMW992                    NNNNN..... .......... .......... .......... ......G... .......... ..........
B_dothidea_CMW7780                NNNNN..... .......... .......... .......... .....--... .......... .........A.
B_dothidea_CMW8000                NNNNN..... .......... .......... .......... .....--... .......... .........A.
B_rhodina_CMW10130                NNNNN..... .......... .......... .......... ........T. ....T..... ...C......
B_rhodina_CMW9074                 NNNNN..... .......... .......... .......... ........T. ....T..... ...C......
B_obtusa_CMW7774                  NNNNN..... .......... .......... .......... .......... ..G....... ...C......
B_obtusa_CMW7774                  NNNNN..... .......... .......... .......... .......... ..G....... ...C......
B_stevensii_CMW7060               NNNNN..... .......... .......... .......... .......... .AG....... ...CC.....
CMW11246_Ethiopia                 NNNNN..... .......... .......... .......... .......... .......... ..........
CMW11253_Ethiopia                 NNNNN..... .......... .......... .......... .......... .......... ..........
CMW189 (B)                        TGGTA..... .A........ .......... .......... .......... ..G....... ...C......
CMW190 (A)                        TGGTA..... .......... .......... .......... .......... ..G....... ...C......
CMW4786(C)                        TGGTA..... .......... .......... .......... ........T. ..G....... ...C......
CMW10717_ETHIOPIA                 TGGTA..... .......... .......... .......... .......... ..G....... ...C......
CMW11252_ETHIOPIA                 TGGTA..... .......... .......... .......... .......... ..G....... ...C......
CMW11250_ETHIOPIA                 TGGTA..... .......... .......... .......... .......... ..G....... ...C......
LasioBt2                          NNNNN..... .......... .......... .......... ........T. ..-....... ...C......
G_philoprina_CMW7063              NNNNN..... .......... .........- ---------- ---------- ---------- ---..------
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                                  80         90         100        110        120        130        140
B_ribis_CMW7772          CGCGAATCGA CACCACAGGC AGACCATTTC CGGCGAGCAC GGCCTGGACG GCTCTGGCGT GTGAGTCTGC
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... T......... .......... .......... ..A.......
B_parva_CMW9071          .......... .......... .......... T......... .......... .......... ..A.......
B_parva_CMW9077          .......... .......... .......... T......... .......... .......... ..A.......
B_eucalyptorum_CMW10125  .......... .....T.... .......... T..T..A... .......... .......... ..A.......
B_eucalyptorum_CMW10126  .......... .......... .......... T..T..A... .......... .......... ..A.......
B_lutea_CMW9076          .......... ....G..... .......... T......... .......... .......... ..A.......
B_lutea_CMW992           .......... ....G..... .......... T......... .......... .......... ..A.......
B_dothidea_CMW7780       .......... .......... .......C.. .......... .......... .......... ..A.......
B_dothidea_CMW8000       .......... .......... .......C.. .......... .......... .......... ..A.......
B_rhcdina_CMW10130       ..-...G... .....T.... .......C.. .......... ........T. ....C..T.. ..A...G...
B_rhcdina_CMW9074        ..-...G... .....T.... .......C.. .......... ........T. ....C..T.. ..A...G...
B_obtusa_CMW7774         ...C...... .......... ....T..C.. T......... .......... ....C..... ..A...T...
B_obtusa_CMW7774         ...C...... .......... ....T..C.. T......... .......... ....C..... ..A...T...
B_stevensii_CMW7060      .C.C...... ...-...... .......C.. T......... .......... .......... ..A...G...
CMW11246_Ethiopia        .......... .......... .......... T......... .......... .......A.. ..A.......
CMW11253_Ethiopia        .......... .......... .......... T......... .......... .......... ..A.......
CMW189 (B)               ...C...... .......... ....T..C.. T......... ........T. ....C..... ..A...T...
CMW190 (A)               ...C...... .......... ....T..C.. T......... .......... ....C..... ..A...T...
CMW4786 (C)              ...C...... ..-....... ....T..C.. T......... .......... ....C..T.. ..A...T...
CMW10717_ETHIOPIA        ...C...G.. .......... ....T..C.. T......... .......... ....C..... ..A...T...
CMW11252_ETHIOPIA        ...C...... .......... ....T..C.. T......... .......... ....C..... ..A...T...
CMW11250_ETHIOPIA        ...C...G.. .......... ....T..C.. T......... .......... ....C..... ..A...T...
LasioBt2                 ..G-..G... .....T.... .......C.. .......... ........T. ....C..T.. ..A...G...
G_philoprina_CMW7063     ---------- -------... .......C.. T......... .....C...A ..AA...T.. C.ACA---..
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                                150        160        170        180        190        200        210
B_ribis_CMW7772          GCCGTTTC-- -CCGCGC--- --GAA--TGG CAATGGCTGA CCC-GTAGCA GC------TA CAATGGCACC
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... .......... .....C.... .......... ..........
B_parva_CMW9071          .......... .......... .......... .......... .....C.... .......... ..........
B_parva_CMW9077          .......... .......... .......... .......... .....C.... .......... ..........
B_eucalyptorum_CMW10125  .......... .....T.... .......... .......... .....C.A.. .......... ..........
B_eucalyptorum_CMW10126  .......... .....T.... .......... .......... .....C.A.. .......... ..........
B_lutea_CMW9076          ........TT .--........ .......... .......... .....C.... .......... ..........
B_lutea_CMW992           ........TT .--........ .......... .......... .....C.... .......... ..........
B_dothidea_CMW7780       AT.-A...TC A--...-TGG GA...CA.-- .....A..-. AA.T...... .......... ..........
B_dothidea_CMW8000       AT.-A...TC A--...-TGG GA...CA.-- .....A..-. AA.T...... .......... ..........
B_rhodina_CMW10130       ...-..CTCC G.....-... ..---CA... ....C..... .-.T...... .......... .........T
B_rhodina_CMW9074        ...-..CTCC G.....-... ..---CA... ....C..... .-.T...... .......... .........T
B_obtusa_CMW7774         ..T..C.TT. G......TC. ..---....- ....C..... ...TTG-... .......... ..........
B_obtusa_CMW7774         ..T..C.TT. G......TC. ..---....- ....C..... ...TTG-... .......... ..........
B_stevensii_CMW7060      ..T..C.TT. G......TG. ..---....- ....C..... .T.TCG-... .......... ..........
CMW11246_Ethiopia        ....--.TTC .......... .......... .......--- ---T.ACC.- ..AGCAGC.. .........A
CMW11253_Ethiopia        ....--.TTC .......... .......... .......--- ---T.ACC.- ..AGCAGC.. ..........
CMW189 (B)               ..T..C.TT. G......... ..---TC.-. ....C..... ...TT.-... .......... ..........
CMW190 (A)               ..T..C.TT. G......... ..---TC.-. ....C..... ...TTG-... .......... ..........
CMW4786 C)               ..T..C.TT. G......... ..---TC.-. ....C..... ...TTG-... .......... ..........
CMW10717_ETHIOPIA        ..T..C.TT. G......... ..---TC.-. ....C..... ...TTG-... .......... ..........
CMW11252_ETHIOPIA        ..T..C.TT. G......... ..---TC.-. ....C..... ...TTG-... .......... ..........
CMW11250_ETHIOPIA        ..T..C.TT. G......... ..---TC.-. ....C..... ...TTG-... .......... ..........
LasioBt2                 ...-..CT.. .....CGCGC AT---..-.. ....C..... ..-.TG--T. ..AGC..... .........T
G_philoprina_CMW7063     --------.. .------... ..---..--- ---------- ---.------ --.......-- ----..T...
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                                220        230        240        250        260        270        280
B_ribis_CMW7772                 TCCGACCTGC AGCTCGAGCG CATGAACGTC TACTTCAACG AGGTACTCTC TC-ACTAATT GCACAAACAC
B_ribis_CMW7773                 .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994                  .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071                 .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077                 .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125         ........C. .......... .......... .......... .......... .........C A.........
B_eucalyptorum_CMW10126         ........C. .......... .......... .......... .......... .........C A.........
B_lutea_CMW9076                 ........C. .......... .......... .......... .......... .........C ....G.....
B_lutea_CMW992                  ........C. .......... .......... .......... .......... .........C ....G.....
B_dothidea_CMW7780              ..G.....T. .......... .......... ..T....... .......... .......... AG........
B_dothidea_CMW8000              ..G.....T. .......... .......... ..T....... .......... .......... AG........
B_rhodina_CMW10130              ..G.....C. .A..G..... .......... .......... .......... ..C.-..... AG........
B_rhodina_CMW9074               ..G.....C. .A..G..... .......... .......... .......... ..C.-..... AG........
B_obtusa_CMW7774                ........C. ....G..... .......... .......... .......... .-.....G.. AG........
B_obtusa_CMW7774                ........C. ....G..... .......... .......... .......... .-.....G.. AG........
B_stevensii_CMW7060             ........C. .A..G..... .......... .......... .......... .TG-...... AG........
CMW11246_Ethiopia               .......... .......... .........N .......... .......... .-C....... ..........
CMW11253_Ethiopia               .......... .......... .......... .......... .......... .-C....... ..........
CMW189  (B)                     ........C. ....G..... .......... .......... .......... .-.....G.. AG........
CMW190  (A)                     ........C. ....G..... .......... .......... .......... .-.....G.. AG........
CMW4786 (C)                     ........C. ....G..... .......... .......... .......... .-.....G.. AG........
CMW10717_ETHIOPIA               ........C. ....G..... .......... .......... .......... .-.....G.. AG........
CMW11252_ETHIOPIA               ........C. ....G..... .......... .......... .......... .-.....G.. AG........
CMW11250_ETHIOPIA               ........C. ....G..... .......... .......... .......... .-.....G.. AG........
LasioBt2                        ..G.....C. .A..G..... .......... .......... .......... ..C.-..... AG........
G_philoprina_CMW7063            .....G..C. .......... .......... .......... .....-.G-- ..C.GACCGA ..TTC.CATA
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                                290        300        310        320        330        340        350
B_ribis_CMW7772          GTAAAGTATG GCAATCTTCT GAACG----- -CGCAGCAGG CGTC---C-- AACAACAAGT ACGTTCCTCG
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077          .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125  A......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10126  A......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW9076          .......... .......... .......... .......... .......-G. .......... ..........
B_lutea_CMW992           .......... .......... .......... .......... .......-G. .......... ..........
B_dothidea_CMW7780       .......... .......... .......... .......... .......... .......... ..........
B_dothidea_CMW8000       .......... .......... .......... .......... .......... .......... ..........
B_rhodina_CMW10130       .......... .......... .......... .......... .......... .......... ..........
B_rhodina_CMW9074        .......... .......... .......... .......... .......... .......... ..........
B_obtusa_CMW7774         .......... .......... .......... .......... .A........ .....T.... ..........
B_obtusa_CMW7774         .......... .......... .......... .......... .A........ .....T.... ..........
B_stevensii_CMW7060      .......... .......... .......... .......... .T........ .......... ..........
CMW11246_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW11253_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW189  (A)              .C........ .......... .......... .......... .-.T....G. .....T.... ..........
CMW190  (B)              .......... .......... .......... .......... .-.T....G. .....T.... ..........
CMW4786 (C)              .......... .......... .......... .......... .-.T....G. .....T.... ..........
CMW10717_ETHIOPIA        .......... .......... .......... .......... .-.T....G. .....T.... ..........
CMW11252_ETHIOPIA        .......... .......... .......... .......... .-.T....G. .....T.... ..........
CMW11250_ETHIOPIA        .......... .......... .......... .......... .-.T....G. .....T.... ..........
LasioBt2                 .......... .......... .......... .......... .......... .......... ..........
G_philoprina_CMW7063     T.CTG..GAT TTTCATC.TC TG..-CGAGA TTTGG.T.TA G.C.TCCGGC .....---.. .T........
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                                360        370        380        390        400        410        420
B_ribis_CMW7772          TGCCGTCCTC GTCGACCTCG AGCCCGGCAC CATGGATGCC GTCCGCGCCG GCCCCTTCGG CCAGCTCTTC
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077          .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125  .......... ..T....... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10126  .......... ..T....... .......... .......... .......... .......... ..........
B_lutea_CMW9076          .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW992           .......... .......... .......... .......... .......... .......... ..........
B_dothidea_CMW7780       .......... .......... .......... G......... .......... .......... ......T...
B_dothidea_CMW8000       .......... .......... .......... G......... .......... .......... ......T...
B_rhodina_CMW10130       ...T...... .......... .......... .......... .......... .......... ..........
B_rhodina_CMW9074        ...T...... .......... .......... .......... .......... .......... ..........
B_obtusa_CMW7774         ...T...... ..T....... .......... .......... .......... .......... ..........
B_obtusa_CMW7774         ...T...... ..T....... .......... .......... .......... .......... ..........
B_stevensii_CMW7060      ...T...... ..T....... .......T.. .......... .....T.... .......... ..........
CMW11246_Ethiopia        .......... .......... .......... ........T. .......... .......... ..........
CMW11253_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW189  (A)              ...T...... ..T....... .......... .......... .......... .......... ..........
CMW190  (B)              ...T...... ..T....... .......... .......... .......... .......... ..........
CMW4786 (C)              ...T...... ..T....... .......... .......... .......... .......... ..........
CMW10717_ETHIOPIA        ...T...... ..T....... .......... .......... .......... .......NNN NNNNNNNNNN
CMW11252_ETHIOPIA        ...T...... ..T....... .......... .......... .......... .......... ..........
CMW11250_ETHIOPIA        ...T...... ..T....... .......... .......... .......... .......... ..........
LasioBt2                 ...T...... .......... .......... .......... .......... .......... ..........
G_philoprina_CMW7063     C..T...... .....T..T. .......T.. .......... .....T..T. .A..T..... ..........
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                                430        440        450        460        470        480        490
B_ribis_CMW7772                 CGCCCTGACA ACTTCGTCTT CGGTCAGTCT GGCGCCGGTA ACAACTGGGA AGGATCATTA CCGAGTTGAT
B_ribis_CMW7773                 .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994                  .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071                 .......... .......... .......NNN NNNNNNNNNN NNNNNNN... .......... ..........
B_parva_CMW9077                 .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125         .....C.... ....T..... ...C...... ..T....... ....T..... .......... .........C
B_eucalyptorum_CMW10126         .....C.... ....T..... ...C...... ..T....... ....T..... .......... .........C
B_lutea_CMW9076                 .....C.... ....T..... ...C...... ..T....... .......... .......... ..........
B_lutea_CMW992                  .....C.... ....T..... ...C...... ..T....... .......... .......... ..........
B_dothidea_CMW7780              .....C.... .......... .........C ..T....... .......... .......... ..........
B_dothidea_CMW8000              .....C.... .......... .........C ..T....... .......... .......... ..........
B_rhodina_CMW10130              .....C.... .......... ...C...... ..T....... .......... .......... .......--.
B_rhodina_CMW9074               .....C.... .......... ...C...... ..T....... .......... .......... .......--.
B_obtusa_CMW7774                ..T..C.... .......T.. ...C...... ..T....... .......... ..N....... .......--C
B_obtusa_CMW7774                ..T..C.... .......T.. ...C...... ..T....... .......... .......... .......--C
B_stevensii_CMW7060             ..T..C.... .......T.. ...C...... ..T....... .......... .......... .......--C
CMW11246_Ethiopia               .......... .......... .......... .......... .......... .......... ..........
CMW11253_Ethiopia               .......... .......... .......... .......... .......... .......... ..........
CMW189  (A)                     ..T..C.... .......T.. ...C...... ..T.....NN NNNNNNNNNN NNNNN..... .......--C
CMW190  (B)                     ..T..C.... .......T.. ...C...... ..T.....NN NNNNNNNNNN NNNNN..... .......--C
CMW4786 (C)                     ..T..C.... .......T.. ...C...... ..T.....NN NNNNNNNNNN NNNNN..... .......--C
CMW10717_ETHIOPIA               NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNN..... .......--C
CMW11252_ETHIOPIA               ..T..C.... .......T.. ...C...... ..T.....NN NNNNNNNNNN NNNNN..... .......--C
CMW11250_ETHIOPIA               ..T..C.... .......T.. ...C...... ..T.....NN NNNNNNNNNN NNNNN..... .......--C
LasioBt2                        .....C.... .......... ...C...... ..T.....NN NNNNNNNNNN NNNNN..... .......---
G_philoprina_CMW7063            .....C.... .......T.. .........C ..T..T..C. .......... .......... .......---
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                                500        510        520        530        540        550        560
B_ribis_CMW7772                 TCGAGCTCCG -GCTC-GAC- TC--TC--CC ACCCAA--TG TGTACCTACC ----TCTGTT GCTTTGGCGG
B_ribis_CMW7773                 .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994                  .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071                 .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077                 .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125         .......... .......... .......... ....T..... .......... .......... ..........
B_eucalyptorum_CMW10126         .......... .......... .......... ....T..... .......... .......... ..........
B_lutea_CMW9076                 .......... .......... .......... ....C..... .......... .......... ..........
B_lutea_CMW992                  .......... .......... .......... ....C..... .......... .......... ..........
B_dothidea_CMW7780              ...G...... ...C....-. ...C...... ....--TT.. .......... .......... ..........
B_dothidea_CMW8000              ...G...... ...C....-. ...C...... ....--TT.. .......... .......... ..........
B_rhodina_CMW10130              ...G...T.. .......... .....N.... ....--TT.. ..A..G.... .......... ..........
B_rhodina_CMW9074               .......... .......... .......... ....--TT.. ..A..G.... .......... ..........
B_obtusa_CMW7774                ...G...T.. ........A. .......... ....--TT.. ..A..A.... .......... ..........
B_obtusa_CMW7774                ...G...T.. ........A. .......... ....--TT.. ..A..A.... .......... ..........
B_stevensii_CMW7060             .......T.. ........A. .......... ....--TT.. ..A..A.... .......... ..........
CMW11246_Ethiopia               .......... .......... .......... .......... ..----.... TACC...... ..........
CMW11253_Ethiopia               .......... .......... .......... .......... ..----.... TACC...... ..........
CMW189 (B)                      ...G...T.. ........A. --....TC.. ....--TT.. ..A..A.... TCTG.TGC.. ----......
CMW190 (A)                      ...G...T.. ........A. --....TC.. ....--TT.. ..A..A.... TCTG.TGC.. ----......
CMW4786(C)                      ...G...T.. ........A. --....TC.. ....--TT.. ..A..A.... TCTG.TGC.. ----......
CMW10717_ETHIOPIA               ...G...T.. ........A. --....TC.. ....--TT.. ..A..A.... TCTG.TG-.. ---C......
CMW11252_ETHIOPIA               ...G...T.. ........A. --....TC.. ....--TT.. ..A..A.... TCTG.TGC.. ----......
CMW11250_ETHIOPIA               ...G...T.. ........A. --....TC.. ....--TT.. ..A..A.... TCTG.TG-.. ---C......
LasioBt2                        ------.T.. A....C.G.T CGAC..TC.. ....--TT.. ..A..G.... TCTG.TGC.. ---.G.CG.C
G_philoprina_CMW7063            ---------- .--.ACA..T C-..--CCAA .....-.... ..A..A.... TA...TGT.G CT.CG.CG..
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                                570        580        590        600        610        620        630
B_ribis_CMW7772                 GCCGCGGTCC T--CCGC-AC CGG-CGCCC- TT--CG-GGG GGGCTGGCCA GCGC-----C CGCCAGAGGA
B_ribis_CMW7773                 .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994                  .......... .......... .......... .......A.. ..-....... .......... ..........
B_parva_CMW9071                 .......... .......... .......... .......... ..-....... .......... ..........
B_parva_CMW9077                 .......... .......... .......... .......... ..-....... .......... ..........
B_eucalyptorum_CMW10125         .......... .......... .....T...T ....-..... .--....... ...T...... ..........
B_eucalyptorum_CMW10126         .......... .......... .....T...T ....-..... .--....... ...T...... ..........
B_lutea_CMW9076                 .......... .......... ..AC.C..G. .......... ..-.C..... .......... ..........
B_lutea_CMW992                  .......... .......... ..AC.C..G. .......... ..-.C..... .......... ..........
B_dothidea_CMW7780              .......... .......GG. ...C.C.... .CCC...... ..--...... .......... ..........
B_dothidea_CMW8000              .......... .......GG. ...C.C.... .CCC...... ..--...... .......... ..........
B_rhodina_CMW10130              -.-----..- -..---.NG- ---.-----. --..--.--- ---------- ----...... .....A....
B_rhodina_CMW9074               -.-----..- -..---.GG- ---.-----. --..--.--- ---------- ----...... .....A....
B_obtusa_CMW7774                -.-----..T .TG....G.- -..AG..... .-....C... CC--CCC..- ....GCTTT. ..........
B_obtusa_CMW7774                -.-----..T .TG....G.- -..AG..... .-....C... CC--CCC..- ....GCTTT. ..........
B_stevensii_CMW7060             -.-----..T ..G....GT- -..AG..... .-...AAAAA .C--CCC..C .T..GCT.T. ..........
CMW11246_Ethiopia               .......... .......... ...C-....T .-....G... .C--...... .......... ..........
CMW11253_Ethiopia               .......... .......... ...C-....T .-....G... .C--...... .......... ..........
CMW189 (B)                      C------..T .TG....G.- -..AG....T --....C... CCC.CCA.GC ......TTT. ..........
CMW190 (A)                      C------..T .TG....G.- -..AG....T --....C... CCC.CC-.GC ......TTT. ..........
CMW4786(C)                      C------..T .TG....G.- -..AG....T --....C... CCC.CC-.GC ......TTT. ..........
CMW10717_ETHIOPIA               C------..T .TG....G.- -..AG....T --....C... CCC.CC-.GC ......TTT. ..........
CMW11252_ETHIOPIA               C------..T .TG....G.- -..AG....T --....C... CCC.CC-.GC ......TTT. ..........
CMW11250_ETHIOPIA               C------..T .TG....G.- -..AG....T --....C... CCC.CC-.GC ......TTT. ..........
LasioBt2                        T...------ -.G....C.A -..G-----. --..--.--- ---------- ----.....- ----------
G_philoprina_CMW7063            ------A.T- -.G..C.G-- -..CGC.--T CGTGT.CCCC ..ATCA.G.G C---...... ....TAG.A.
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                                640        650        660        670        680        690        700
B_ribis_CMW7772          CCAT-AAAAC TCCAGTCAGT GAAC-TTCGC AGTCTGAAAA AC-AAGTTAA TAAACTAAAA -CTTTCAACA
B_ribis_CMW7773          ..........  ..........  ..........  ..........  ..........  ..........  ..........
B_parva_CMW994           ..........  ..........  ..........  ..........  ..........  ..........  ..........
B_parva_CMW9071          ..........  ..........  ..........  ..........  ..........  ..........  ..........
B_parva_CMW9077          ..........  ..........  ..........  ..........  ..........  ..........  ..........
B_eucalyptorum_CMW10125  ...-C.....  ..........  A...G..-..  ..........  ..........  ..........  ..........
B_eucalyptorum_CMW10126  ...-C.....  ..........  A...G..-..  ..........  ..........  ..........  ..........
B_lutea_CMW9076          ...-C.....  ..........  A...G-....  .......G..  ..........  ..........  ..........
B_lutea_CMW992           ...-C.....  ..........  A...G-....  .......G..  ..........  ..........  ..........
B_dothidea_CMW7780       ....C...-.  ..........  A...GA.-..  ..........  ....T-....  ..........  ..........
B_dothidea_CMW8000       ....C...-.  ..........  A...GA.-..  ..........  ....T-....  ..........  ..........
B_rhodina_CMW10130       ..T.C...-.  ..........  A...GCA-.A  C......T..  ..........  ..........  ..........
B_rhodina_CMW9074        ..T.C...-.  ..........  A...GCA-.A  C......T..  ..........  ..........  ..........
B_obtusa_CMW7774         ..T.C...-.  ..........  A...G-...A  C......T..  ..........  ..........  ..........
B_obtusa_CMW7774         ..T.C...-.  ..........  A...G-...A  C......T..  ..........  ..........  ..........
B_stevensii_CMW7060      ..T.C...-.  ..........  A...G-...A  C..........  ..........  ..........  ..........
CMW11246_Ethiopia        ..........  ..........  ..........  ..........  ..........  ..........  ..........
CMW11253_Ethiopia        ..........  ..........  ..........  ..........  ..........  ..........  ..........
CMW189 (B)               ..T.C..-T.  ..........  A...G.CGA-  C......T..  ..T.......  ...G......  ..........
CMW190 (A)               ..T.C...-.  ..........  A...G.CGA-  C......T..  ..........  ..........  ..........
CMW4786(C)               ..T.C...-.  ..........  A...G.CGA-  C......T..  ..........  ..........  ..........
CMW10717_ETHIOPIA        ..T.C...-.  ..........  A...G.CGA-  C......T..  ..........  ..........  ..........
CMW11252_ETHIOPIA        ..T.C...-.  ..........  A...G.CGA-  C......T..  ..........  ..........  ..........
CMW11250_ETHIOPIA        ..T.C...-.  ..........  A...G.CGA-  C......T..  ..........  ..........  ..........
LasioBt2                 ..T.C...-.  ..........  A.-.G-C-AG  C......T..  ..........  ..........  ..........
G_philoprina_CMW7063     A.T..-..-.  ..TT..TTTA  TTTTG-GAAT  CT.....GT.  GTTTTTAC..  AT..A.....  A.........
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                                710        720        730        740        750        760        770
B_ribis_CMW7772          ACGGATCTCT TGGTTCTGGC ATCGATGAAG AACGCAGCGA AATGCGATAA GTAATGTGAA TTGCAGAATT
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077          .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125  .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10126  .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW9076          .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW992           .......... .......... .......... .......... .......... .......... ..........
B_dothidea_CMW7780       .......... .......... .......... .......... .......... .......... ..........
B_dothidea_CMW8000       .......... .......... .......... .......... .......... .......... ..........
B_rhodina_CMW10130       .......... .......... .......... .......... .......... .......... ..........
B_rhodina_CMW9074        .......... .......... .......... .......... .......... .......... ..........
B_obtusa_CMW7774         .......... .......... .......... .......... .......... .......... ..........
B_obtusa_CMW7774         .......... .......... .......... .......... .......... .......... ..........
B_stevensii_CMW7060      .......... .......... .......... .......... .......... .......... ..........
CMW11246_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW11253_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW189  (B)              .......... .......... .......... .......... .......... .......... ..........
CMW190  (A)              .......... .......... .......... .......... .......... .......... ..........
CMW4786 (C)              .......... .......... .......... .......... .......... .......... ..........
CMW10717_ETHIOPIA        .......... .......... .......... .......... .......... .......... ..........
CMW11252_ETHIOPIA        .......... .......... .......... .......... .......... .......... ..........
CMW11250_ETHIOPIA        .......... .......... .......... .......... .......... .......... ..........
LasioBt2                 .......... .......... .......... .......... .......... .......... ..........
G_philoprina_CMW7063     .......... .......... .......... .......... .......... .......... ..........
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                                780        790        800        810        820        830        840
B_ribis_CMW7772          CAGTGAATCA TCGAATCTTT GAACGCACAT TGCGCCCCTT GGTATTCCGA GGGG-CATGC CTGTTCGAGC
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077          .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125  .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10126  .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW9076          .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW992           .......... .......... .......... .......... .......... .......... ..........
B_dothidea_CMW7780       .......... .......... .......... .......T.. .......... A......... ..........
B_dothidea_CMW8000       .......... .......... .......... .......T.. .......... A......... ..........
B_rhodina_CMW10130       .......... .......... .......... .......... .........G .......... ..........
B_rhodina_CMW9074        .......... .......... .......... .......... .........G .......... ..........
B_obtusa_CMW7774         .......... .......... .......... ........C. ..C......G .......... ..........
B_obtusa_CMW7774         .......... .......... .......... ........C. ..C......G .......... ..........
B_stevensii_CMW7060      .......... .......... .......... .......... ..C....... .......... ..........
CMW11246_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW11253_Ethiopia        .......... .......... .......... .......... .......... .......... ..........
CMW189  (B)              .......... .......... .......... .......... ..C....... .......... ..........
CMW190  (A)              .......... .......... ....C..... .......... ..C....... .......... ..........
CMW4786 (C)              .......... .......... ....C..... .......... ..C....... .......... ..........
CMW10717_ETHIOPIA        .......... .......... .......... .......... ..C....... .......... ..........
CMW11252_ETHIOPIA        .......... .......... .......... .......... ..C....... .......... ..........
CMW11250_ETHIOPIA        .......... .......... .......... .......... ..C....... .......... ..........
LasioBt2                 .......... .......... .......... .......... .........- ....G..... ..........
G_philoprina_CMW7063     .......... .......... .......... .......GCC A......T.- .C..G..... ....CT....
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                                850        860        870        880        890        900        910
B_ribis_CMW7772          GTCATTTCAA CCCTCAAGCT CT----GCTT GGTATTGGGC TCCGTCCTCC A----CGGAC GCGCCTTAAA
B_ribis_CMW7773          .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994           .......... .......... .......... .......... C......... .......... ..........
B_parva_CMW9071          .......... .......... .......... .......... C......... .......... ......C...
B_parva_CMW9077          .......... .......... .......... .......... C......... .......... ......C...
B_eucalyptorum_CMW10125  .......... .......... T......... .......... C.........- -..TGT.... ......C...
B_eucalyptorum_CMW10126  .......... .......... T......... .......... C.........- -..TGT.... ......C...
B_lutea_CMW9076          .......... .......... .......... .......... ..........- -..TGT.... ......CG..
B_lutea_CMW992           .......... .......... .......... .......... ..........- -..TGT.... ......CG..
B_dothidea_CMW7780       ......A... .......... .......... .......... A.......T- -..TG...G. ......C...
B_dothidea_CMW8000       ......A... .......... .......... .......... A.......T- -..TG...G. ......C...
B_rhodina_CMW10130       ......A... .......... .......... ..A....... A.........A -.CTG..... ......C...
B_rhodina_CMW9074        ......A... .......... .......... ..A....... A.........A -.CTG..... ......C...
B_obtusa_CMW7774         ......A... .......... .......... .......... G.........- -TCTG..... ..........
B_obtusa_CMW7774         ......A... .......... .......... .......... G.........- -TCTG..... ..........
B_stevensii_CMW7060      ......A... .......... .......... .......... GA........- -TCTG..... ......C...
CMW11246_Ethiopia        .......... .......... .......... .......... C......... .......... ..........
CMW11253_Ethiopia        .......... .......... .......... .......... C......... .......... ..........
CMW189  (B)              ......A... .......... .......... .......... G.........- -TCTG..... ..........
CMW190  (A)              ......A... .......... .......... .......... G.........- -TCTG..... ..........
CMW4786 (C)              ......A... .......... .......... .......... G.........- -TCTG..... ..........
CMW10717_ETHIOPIA        ......A... .......... .......... .......... G.........- -TCTG..... ..........
CMW11252_ETHIOPIA        ......A... .......... .......... .......... G.........- -TCTG..... ..........
CMW11250_ETHIOPIA        ......A... .......... .......... .......... G.........- -TCTG..... ..........
LasioBt2                 ......A... .......... .......... ..A....... A.........A -.CTG..... ..........
G_philoprina_CMW7063     .......... ......T..C .CTAGG..G. ...G.....G AT..G..AAA GCCCG..AGG .ACGGCCGGC
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                          920        930        940        950        960        970        980
B_ribis_CMW7772           GACCTCGGCG GTGGC-GTCT TGCC-TCAAG CGTAGTAGAA AA--CACCTC GCTTTGGAGC GCACGGCGTC
B_ribis_CMW7773           .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW994            .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9071           .......... .......... .......... .......... .......... .......... ..........
B_parva_CMW9077           .......... .......... .......... .......... .......... .......... ..........
B_eucalyptorum_CMW10125   .......... .......... .......... .......... -.T....... .......... ...T......
B_eucalyptorum_CMW10126   .......... .......... .......... .......... -.T....... .......... ...T......
B_lutea_CMW9076           .......... .......... .......... .......... .......... .......... ..........
B_lutea_CMW992            .......... .......... .......... .......... .......... .......... ..........
B_dothidea_CMW7780        .......... .......... .......... .......... C.TA..T... ....C..... ...G......
B_dothidea_CMW8000        .......... .......... .......... .......... C.TA..T... ....C..... ...G......
B_rhodina_CMW10130        .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
B_rhodina_CMW9074         .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
B_obtusa_CMW7774          .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
B_obtusa_CMW7774          .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
B_stevensii_CMW7060       .......... .....T..TC A...C..... ....A..... --TA...... .......... .GTT......
CMW11246_Ethiopia         .......... .....GTCT. GC.-...... .......... .-.A...... .......... .......C..
CMW11253_Ethiopia         .......... .....GTCT. GC.-...... .......... .-.A...... .......... ..........
CMW189  (B)               .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
CMW190  (A)               .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
CMW4786 (c)               .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
CMW10717_ETHIOPIA         .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
CMW11252_ETHIOPIA         .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
CMW11250_ETHIOPIA         .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
LasioBt2                  .......... .....T..TC A...C..... .......... --TA...... .......... .GTT......
G_philoprina_CMW7063      -C...AAATC TA.TGGCGG- AC..G..GT. GCCTCCTCTG CGAAGTAG.G ATA..CCGCA T.GGA.A.CG
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                                990       1000       1010       1020       1029
B_ribis_CMW7772                 GCCCGCCGGA CGAACCTT-T GAATTATTT- CTCAAGGTTG ACCTCGGAT
B_ribis_CMW7773                 .......... .......... .......... .......... .........
B_parva_CMW994                  .......... .......... .......... .......... .........
B_parva_CMW9071                 .......... .......... .......... .......... .........
B_parva_CMW9077                 .......... .......... .......... .......... .........
B_eucalyptorum_CMW10125         .......... .......... .....-.... .......... .........
B_eucalyptorum_CMW10126         .......... .......... .....-.... .......... .........
B_lutea_CMW9076                 .......... .......... .....-.... .......... .........
B_lutea_CMW992                  .......... .......... .....-.... .......... .........
B_dothidea_CMW7780              .......... .........C. ...C.-.... .......... .........
B_dothidea_CMW8000              .......... .........C. ...C.-.... .......... .........
B_rhodina_CMW10130              .......... .........C. ...C.-.... .......... .........
B_rhodina_CMW9074               .......... .........C. ...C.-.... .......... .........
B_obtusa_CMW7774                .......... .........C. ...C.-.... .......... .........
B_obtusa_CMW7774                .......... .........C. ...C.-.... .......... .........
B_stevensii_CMW7060             .......... .........C. ...C.-.... .......... .........
CMW11246_Ethiopia               .......... .......... .......... .......... .........
CMW11253_Ethiopia               .......... .......... .......... .......... .........
CMW189  (B)                     .......... .........C. ...C.-.... .......NNN NNNNNNNNN
CMW190  (A)                     .......... .........C. ...C.-.... .......NNN NNNNNNNNN
CMW4786 (C)                     .......... .........C. ...C.-.... .......NNN NNNNNNNNN
CMW10717_ETHIOPIA               .......... .........C. ...C.-.... .......NNN NNNNNNNNN
CMW11252_ETHIOPIA               .......... .........C. ...C.-.... .......NNN NNNNNNNNN
CMW11250_ETHIOPIA               .......... .........C. ...C.-.... .......NNN NNNNNNNNN
LasioBt2                        .......... .........C. ...C.-.... .......NNN NNNNNNNNN
G_philoprina_CMW7063            A.GA...CCT GCCGTTAAAC CCCCA.C..T .-........ .....A...
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Figure 7. Alignment of combined SSR sequences of *D. pinea* sequenced with SS7, SS9 and SS10 markers. (-)= gaps, (.)= Homologous nucleotides, (N)= Unknown bases.

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                    10         20         30         40         50         60         70
CMW190A         GACAAGACAT CTAGGCCCTG CCGGTCCCG- TCCCCGTCTC CAGGCTCACA TGGAAACAAA -CTGTACAGG
CMW11250        ....-..... .......... .........- .......... .......... .......... -.........
CMW11252        ....-..... .......... .........- .......... .......... .......... -.........
CMW10717        ....-..... .......... .........- .......... .......... .......... -.........
CMW4786         NNNNNNNNN  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
CMW189B         ....-..... T......... ....C...CA .......T.. .G..G..... A......... A...C..T..

                    80         90         100        110        120        130        140
CMW190A         CAAAAGCAGT GAAGTCGTAA GGCCCGGACC CTA-GAAGAG GCGCTTTCCT CTCCACGGAG TAACCACCGG
CMW11250        .......... .......... .......... ...-...... .......... .......... ..........
CMW11252        .......... .......... .......... ...-...... .......... .......... ..........
CMW10717        .......... .......... .......... ...-...... .......... .......... ..........
CMW4786         NNNN...... .......... .......... ...-...... .......... .......... ..........
CMW189B         GG...A.... .....T.... .......... ..-GA..... .......G.. T......... C.........

                    150        160        170        180        190        200        210
CMW190A         CTCGGCTACG CTAGAAAGCA AATTCCCCGA TCTTAGTGGC ATTTTTTCTT TTGCATCATT CCCGGGCCTC
CMW11250        .........C .......... .......... .......... .......... .......... ..........
CMW11252        .......... .......... .......... .......... .......... .......... ..........
CMW10717        .......... .......... .......... .......... .......... .......... ..........
CMW4786         .......... .......... ...C...... .......... .......... .......... ..........
CMW189B         G....G.... G..A...... ...C...... .......... .......... ........A. ..........
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                220        230        240        250        260        270        280

CMW190A         TTTGGAAATT GCTTTTTTTT -----GATTT TGATTTT--- CTTCTTTTCC TCCTCCTCCT CC--------
CMW11250        .......... .......... -----..... .......--- .......... .......... ..--------
CMW11252        .......... .......... -----..... .......--- .......... .......... ..--------
CMW10717        .......... .......... -----..... .......--- .......... .......... ..--------
CMW4786         .......... .......... T-----..... .......--- .......... .......... ..TCC-----
CMW189B         ........A. .G........ TTTTG.T... .......TCT .......... .......... ..TCCTCCTC


                290        300        310        320        330        340        350

CMW190A         -TCTCTTTCT CAACACGAGG CTCACCAATC ACGATGACGA CGACGACGCC GCTGAGAATG AGCGGAAAAT
CMW11250        -.......... .......... .......... .......... .......... .......... ..........
CMW11252        -.......... .......... .......... .......... .......... .......... ..........
CMW10717        -.......... .......... .......... .......... .......... .......... ..........
CMW4786         -.......... .......... .......... .......... .......... .......... ..........
CMW189B         C.......... .......... .......... .......... .......... ......T... .........C


                360        370        380        390        400        410        420

CMW190A         TATCCGAGAA TCATTCCAC- TTCACCGNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNCA GCGGTTTCAT
CMW11250        .......... .........C .......GAT GGGCCCCTCG TCTCGACCCG ACTGATCANN NN....C...
CMW11252        .......... .........C .......GAT GGGCCCCTCG TCTCGACCCG ACTGATCANN NN....C...
CMW10717        .......... .........C .......GAT GGGCCCCTCG TCTCGACCCG ACTGATCANN NN....C...
CMW4786         .......... .........C .......GAT GGGCCCCNNN NNNNNNNNNN NNNNNNNNNN N.........
CMW189B         .......... ...C...G.C .......GAN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN N.........
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                    430        440        450        460        470        480        490

CMW190A         TGAAATGCCA TCTTCAGTAT CTTGGATATC TTTTTTTTTT TTTTTGATGA GTGCGCGCGC ACACTGCGTT
CMW11250        .A...A...C .T...CAG.. T.....A..T .......... .......... .......... ..........
CMW11252        GA...A...C .T...CAG.. T.....A..T .......... .......... .......... ..........
CMW10717        .A...A...C .T...CAG.. T.....A..T .......... .......... .......... ..........
CMW4786         .......... .......... .......... .......... ..----.... .......... ..........
CMW189B         .......... .G........ .......... ......---- -----....C .......... ....C.T...


                    500        510        520        530        540        550        560

CMW190A         GAGTGAGGAC GGTGTGCTGG TGGCGG---T GATGTATGTG TGTTGTTGGT GGTG---TGG GTAGTGTGTG
CMW11250        .......... .......... ......---. .......... .......... ....---... ..........
CMW11252        .......... .......... ......---. .......... .......... ....---... ..........
CMW10717        .......... .......... ......---. .......... .......... ....---... ..........
CMW4786         .......... .......... ......---. .......... .......... ....---... ..........
CMW189B         .......... ...A...... ...T..TGG. ....----.. .......... ....TGG... ..--------


                    570        580        590        600        610        620        630

CMW190A         GATGGAGTGG ATGGAGGAAG GGGTCCGGGA GTGTTGGTTG TTGTATCTGC TCTTCGGGCG AGAGAGAGTC
CMW11250        .......... .......... ..A....... .......... ..CN-..... .AGNGAN... ..........
CMW11252        .......... .......... ..A....... .......... ..CN-..... .AGNGAN... ..........
CMW10717        .......... .......... ..A....... .......... CGACTG.... .AGNGAN... ..........
CMW4786         .......... .......... .......... .......... .......... .......... ..........
CMW189B         ---..T.... .......... .......... .......... .......... .......... ..........
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                640        650        660        670        680        690        700
CMW190A         CAAGGAAGAA G-GAAG-TGG GAATCGGTAG GAGACAAGTC GCCAACCCTA ATGCTTCCAT AGAAACCAAT
CMW11250        .......... .AA.NNNNNN NNNNNNNNNN NNNNNNNNNN .......... .......... ......G...
CMW11252        .......... .AA.NNNNNN NNNNNNNNNN NNNNNNNNNN .......... .......... ..........
CMW10717        .......... .AA.NNNNNN NNNNNNNNNN NNNNNNNNNN .......... .......... ..........
CMW4786         .......... ANNNNNNNNN NNNNNNNNNN NNNNNNNNNN .......... .......... C.........
CMW189B         ...A...... AG.NNNNNNN NNNNNNNNNN NNNNNNNNNN .G........ .......... C.........

                710        720        730        740        750        760        770
CMW190A         TGACGGCGGA AAGACAAAGG AGCTTACACC GCAGCACCAT TCCCTCCCAC AATCCCTGGT CACAAGACAC
CMW11250        A......... .......... .......... .......... .......... .......... ..........
CMW11252        .......... .......... .......... .......... .......... .......... ..........
CMW10717        .......... .......... .......... .......... .......... .......... ..........
CMW4786         C......... .......... .......... -......... .......... .........C ..........
CMW189B         C......... ...T...... .........A .......... .A......G. .........C ..........

                780        790        800        810        820        830        840
CMW190A         ATACAGACAC ACACACACAC ACACACACAC ACCCAACACA CACATACAAC CTCTCCAACT CACCACCACG
CMW11250        .......... .......... .......... .......... .......... .......... ..........
CMW11252        .......... .......... .......... .......... .......... .......... ..........
CMW10717        .......... .......... .......... .......... .......... .......... ..........
CMW4786         .......T.. .......... .......... ..---..... .........T .......... ..........
CMW189B         .......... .....----- ---------- ---------- ----....G. .......... ..........
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                    850        860        870        880        890        900        910
CMW190A       GCGCCTTCAA CGCCCCGATC TGTTCCCTCG GACCACCCAG CAGCAGCATG AACTCCCGCG CACCGTCACT
CMW11250      .......... .......... .......... .......... .......T.. .......... .........T.
CMW11252      .......... .......... .......... .......... .......T.. .......... .....G....
CMW10717      .......... .......... .......... .......... .......T.. .......... .....G....
CMW4786       .......... .......... .......... .......... .......... .......... ..........
CMW189B       .......... T......... .......... .......... .......... .G........ ..........

                    920        930        940        950        960        970        980
CMW190A       AACCTCCCTT CCTTCATCGA CTCCTGGCGC TTCCACCGCC GCCGAAGTGG CAGAACCCTC CAGACCGCAA
CMW11250      .......... ........A. T...G..G.T .......... ...A.G.G.. ..A....... ..T....G.G
CMW11252      .......... .A.....NNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
CMW10717      .......... .A..G..NNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
CMW4786       .......... .......... .......... .......... .......... .......... ..........
CMW189B       .......... .......... .......... .......... ..NNNNNNNN NNNNNNNNNN NNNNNNNNNN

                   989
CMW190A       TCGCGGTTG
CMW11250      ....NNNNN
CMW11252      NNNNNNNNN
CMW10717      NNNNNNNNN
CMW4786       ....C.C..
CMW189B       NNNNNNNNN
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