South African Journal of Botany 2004, 70(2): 241–248
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SOUTH AFRICAN JOURNAL OF BOTANY
ISSN 0254–6299

Identification of the causal agent of Botryosphaeria stem canker in Ethiopian *Eucalyptus* plantations

Alemu Gezahgne, J Roux*, B Slippers and MJ Wingfield

Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa
** Corresponding author, e-mail: jolanda.roux@fabi.up.ac.za*

Received 7 July 2003, accepted in revised form 20 October 2003

Plantations of exotic *Eucalyptus* make up more than 30% of Ethiopia's plantations, providing fuel and construction timber to the country. Species such as *E. camaldulensis, E. saligna, E. grandis, E. citriodora* and *E. globulus* are most commonly planted. During a survey of *Eucalyptus* diseases in 2000 and 2001, Botryosphaeria stem canker was observed in most plantations. The disease 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 Restriction Fragment Length Polymorphisms (RFLPs) and sequencing of the Internal Transcribed Spacer regions (ITS) of the ribosomal RNA 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 *Botryosphaeria parva* is responsible for Botryosphaeria stem canker of *Eucalyptus* in Ethiopia. This is the first report of the fungus from this country. Greenhouse and field inoculation studies showed that the Ethiopian isolates are highly virulent. Careful site species selection and breeding trials are thus needed to reduce the impact of this disease in Ethiopia.

Introduction

Fungi in the genus *Botryosphaeria* are associated with diseases on a wide range of hosts. On *Eucalyptus* spp., these fungi are known as saprophytes and opportunistic pathogens (Davison and Tay 1983, Barnard *et al*. 1987, Shearer *et al*. 1987, Smith *et al.* 1994). Damage due to *Botryosphaeria* spp. is more pronounced when plants are under stress caused by drought, frost, water logging and insect damage (Wene and Schoeneweiss 1980, 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.:Fr.) Ces. & De. Not. (anamorph = *F. aesculi* Corda) has been reported as an endophyte in *E. nitens* (Deane Et Maid.) Maid. in England (Fisher *et al.* 1993) and in *E. grandis* Hill ex Maid., *E. camaldulensis* Dhen., *E. nitens* and *E. smithii* R. T. Baker in South Africa (Smith *et al.* 1996a). When trees or tree parts are affected by stress, these fungi can cause disease and death.

Botryosphaeria ribis Grossenb. & Duggar has commonly been reported associated with *Eucalyptus* diseases 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) and in Australia, *B. ribis* is associated with twig, branch and stem cankers on *E. marginata* Donn.: Sm. (Davison and Tay 1983). This fungus was also responsible for the death of *E. radiata* Sieb.: DC. in species selection trials in Western Australia (Shearer *et al.* 1987).

In Africa, Botryosphaeria die-back and canker, caused by *B. parva* (reported as *B. dothidea*), *B. rhodina* (Cooke) Von Arx and *B. eucalyptorum* Crous, H. Smith et M. J. Wingf. has been recorded in several countries including South Africa, Republic of Congo and Uganda (Smith *et al*. 1994, 2001, Roux *et al*. 2000, 2001, Slippers *et al*. 2004). In South Africa, wide-spread twig die-back and stem cankers caused by *B. dothidea* and *B. eucalyptorum* 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 (Smith *et al.* 1994, 2001). 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).

In Ethiopia, *Eucalyptus* plantations cover approximately 100 000ha, providing wood for fuel, construction timber and

for the production of poles and posts (Pohjonen and Pukkala 1990). 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 *et al*. 2003). The aim of this study was to identify the *Botryosphaeria* spp. associated with stem canker of *Eucalyptus* spp. in this country. To achieve this, characterisation of isolates based on morphology, PCR–RFLP analysis and DNA sequence data was used. Pathogenicity of Ethiopian isolates was, furthermore, assessed under greenhouse and field conditions.

Materials and Methods

Isolation

Disease surveys were conducted during 2000 and 2001 in plantations of *Eucalyptus* spp. in southern and south western Ethiopia, around Munessa Shashemene, Wondo Genet, Menagesha and Jima. Segments of symptomatic plant parts were incubated in moist chambers for 2–3 days to induce 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 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.

Morphological characterisation

Botryosphaeria isolates were inoculated onto 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 sterile drops of water. Single, germinating conidia were isolated after 12–16h and transferred to clean 2% MEA plates.

Isolates were initially characterised based on culture morphology on 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.

DNA extraction

Total genomic DNA was extracted from selected Ethiopian *Botryosphaeria* isolates. 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 in 1.5µl Eppendorf tubes. The DNA was extracted using the method described by Smith *et al*. (2001), which was modified from that of Raeder and Broda (1985). The DNA pellet was resuspended in 50µl sterilised water. RNase A (1mg ml^{-1}) was added to the DNA solution to remove the contaminating RNA and incubated overnight 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 (ITS) and 5.8S gene of the ribosomal RNA operon, between the 18S and 28S genes, and the elongation factor (EF1-α) gene of the selected *Botryosphaeria* isolates were amplified using the Polymerase Chain Reaction (PCR). To amplify the ITS rDNA regions, primers ITS 1 and ITS 4 (White *et al*. 1990) were used. For the EF1-α gene, forward primer EF1-728F and reverse primer EFI-986R (Carbone *et al.* 1999) were used. The PCR reaction mixtures and the PCR cycles were the same as those published by Slippers *et al.* (2004).

Restriction Fragment Length Polymorphisms (*RFLP*)

The ITS amplicons of 10 Ethiopian *Botryosphaeria* isolates were digested with the restriction endonuclease *Cfo* I to determine their RFLP profiles (Jacobs 2002, Slippers *et al*. 2002). The RFLP reaction mix contained 20µl DNA template, 0.5µl enzyme and 2.5µl enzyme buffer. The mixture was digested at 37°C in a water bath for 6hr. The RFLP fragments were separated on a 3% agarose gel stained with ethidium bromide and visualised under UV light. A standard 100bp 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 and analyses

PCR products were purified using the High Pure PCR Product Purification Kit (QIAGEN, 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™ 377 DNA Autosequencer (Perkin-Elmer). The same primers were used for sequencing as for the original PCR.

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*. (2004). Alignment of both ITS and EF 1-α gene sequences was done manually using PAUP version 4.0b (Swofford 1998). 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 (1 000 replicates) (Felsenstein 1993). Slippers *et al*. (2004) showed that these data sets can be combined for *Botryosphaeria* species. Hence the sequences of ITS rDNA and EF1-α genes were combined and analysed as described by Slippers *et al.* (2004).

Pathogenicity tests

Greenhouse inoculation studies were conducted on an 18-month-old *E. grandis* clone (ZG14). Thirteen 10-day-old *Botryosphaeria* isolates grown on MEA were used in the inoculation trial (Table 3).

A cork borer (9mm diameter) was used to remove the bark and expose the cambium for inoculation. Mycelial plugs of equal size were placed into each wound with the mycelium surface facing the xylem. After inoculation, the wounds were covered with Parafilm (Pechiney Plastic Packaging, Chicago) to prevent contamination and desiccation of the wound and the inoculum. Each isolate was inoculated onto ten trees. Ten trees were inoculated with sterile MEA to serve as controls. 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 or from the controls.

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

Results

Isolations

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. Bark cracking, production of copious amounts of kino, stem discoloration and malformation, tip die-back and death, 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 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. Large basal cankers, as well as two to three layers of black kino rings 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 19 Ethiopian *Botryosphaeria* isolates grown on MEA showed some variation in colony growth and morphology (Table 1). Some of the isolates had fluffy light brown aerial mycelium, whereas others had flat colony growth with little aerial mycelium. Considerable variation was observed in the conidial lengths of the isolates (Table 1). The lengths of the individual conidia for all isolates ranged from 12.5µm to 27.5µm and the average conidial length for different isolates ranged from 15.3µm to 24.3µm. The widths of the conidia showed limited variation and ranged from 5µm to 7.5µm. 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. No teleomorph structures were observed for isolates examined in this study.

PCR amplification

Amplification of the ITS regions and 5.8S gene yielded PCR products 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 (Table 2) produced the same banding pattern when the ITS PCR products were cut with *Cfo* I. This suggested that they 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 identical to that of *B. parva* Pennycook & Samuels and *B. ribis*.

DNA sequencing and analysis

When compared with sequences in GenBank, the ITS sequences of the Ethiopian *Botryosphaeria* isolates (Table 2) 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*. 2004) yielded a total of 833 characters where 182 variable characters were parsimony uninformative and 219 characters were parsimony informative for the combined ITS and EF 1-α data sets. Sequence analysis using parsimony generated 26 phylogenetic trees (CI = 0.928 and RI = 0.905) and these trees showed only minor variation in the arrangements of samples within clades. The consensus phylogenetic tree generated for the combined sequences produced five clades (Figure 1). 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*. (2004) and included *B. ribis* (clade II), *B. eucalyptorum* (clade III), *B. lutea* (clade IV) and *B. dothidea* (clade V). All clades were supported by bootstrap values of 100% (Figure 1).

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

Isolate[a]	Origin	Host	Range and Average Length (µm)[b]	Range and Average Width (µm)	Length:Width (ratio)
CMW10088	Wondo Genet	*Eucalyptus* sp.	(15.0) 18.3 (22.5)	(5.2) 5.0 (5.5)	3.65
CMW10089	Wondo Genet	*Eucalyptus* sp.	(20.0) 24.3 (27.5)	(7.0) 7.5 (7.7)	3.23
CMW10092	Menagesha	*E. globulus*	(15.0) 17.3 (20.0)	(5.0) 5.8 (7.5)	3.17
CMW10093	Wondo Genet	*E. saligna*	(12.5) 15.0 (17.5)	(4.7) 5.0 (5.5)	3.00
CMW10094	Wondo Genet	*E. saligna*	(17.5) 19.0 (20.0)	(5.0) 5.0 (5.2)	3.80
CMW10095	Wondo Genet	*E. grandis*	(15.0) 15.3 (17.5)	(5.0) 5.0 (5.5)	3.05
CMW10096	Wondo Genet	*Eucalyptus* sp.	(15.0) 16.3 (17.2)	(5.0) 5.0 (5.2)	3.25
CMW11059	Jima/Belete	*E. citriodora*	(15.0) 17.5 (20.0)	(5.0) 5.0 (5.5)	3.50
CMW11060	Jima/Belete	*E. citriodora*	(17.5) 18.3 (20.0)	(4.7) 5.0 (5.5)	3.17
CMW11061	Jima/Belete	*E. citriodora*	(15.0) 17.8 (22.5)	(5.0) 5.3 (7.5)	3.38
CMW11062	Jima/Belete	*E. citriodora*	(17.5) 19.5 (22.5)	(7.0) 7.5 (7.5)	2.60
CMW11063	Jima/Belete	*E. citriodora*	(15.0) 16.5 (17.5)	(4.7) 5.0 (5.5)	3.30
CMW11064	Jima/Belete	*E. citriodora*	(17.5) 21.8 (25.0)	(5.0) 5.0 (5.5)	4.35
CMW11066	Jima/Belete	*E. citriodora*	(17.5) 20.8 (25.0)	(5.0) 5.3 (7.5)	3.95
CMW11068	Munessa	*E. globulus*	(15.0) 18.5 (22.5)	(5.0) 5.0 (5.5)	3.70
CMW11069	Menagesha	*E. globulus*	(15.0) 16.8 (20.0)	(5.0) 5.8 (7.5)	2.91
CMW11070	Menagesha	*E. globulus*	(17.5) 17.5 (20.0)	(5.2) 5.0 (5.5)	3.55
CMW11071	Menagesha	*E. globulus*	(17.5) 18.5 (20.0)	(5.0) 6.5 (7.5)	2.85
CMW11072	Menagesha	*E. globulus*	(17.5) 19.8 (22.5)	(5.0) 5.0 (5.5)	3.95

[a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI)
[b] Mean and range values are based on measurements from 10 conidia

Table 2: Isolates used in the DNA sequence analysis

Culture No.[a]	Identity	Host	Origin	Collector	GenBank Accession No. ITS	GenBank Accession No. 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*	South Africa	H Smith	AF283686	AY236891
CMW10126	*B. eucalyptorum*	*E. grandis*	South Africa	H Smith	AF283687	AY236892
CMW992	*F. luteum*	*Actinidia deliciosa*	New Zealand	GJ Samuels	AF027745	AY236894
CMW9076	*B. lutea*	*Malus X domestica*	New Zealand	SR Pennycook	AY236946	AY236893
CMW7772	*B. ribis*	*Ribes* sp.	New York	B Slippers/G Hudler	AY236935	AY236877
CMW7773	*B. ribis*	*Ribes* sp.	New York	B Slippers/G Hudler	AY236936	AY236878
CMW9071	*B. parva*	*Ribes* sp.	Australia	MJ Wingfield	AY236938	AY236880
CMW994	*B. parva*	*Malus sylvestris*	New Zealand	GJ Samuels	AY243395	AY236883
CMW9077	*B. parva*	*Actinidia deliciosa*	New Zealand	SR Pennycook	AY236939	AY236884
CMW10122	*B. parva*	*E. grandis*	South Africa	H Smith	AF283681	AY236882
CMW11060[b]	*Botryosphaeria* sp.	*E. citriodora*	Ethiopia	Alemu Gezahgne and J Roux	AY210474	AY210480
CMW11062[b]	*Botryosphaeria* sp.	*E. citriodora*	Ethiopia	Alemu Gezahgne and J Roux	AY210475	AY210481
CMW11064[b]	*Botryosphaeria* sp.	*E. citriodora*	Ethiopia	Alemu Gezahgne and J Roux	AY210476	AY210482
CMW10089[b]	*Botryosphaeria* sp.	*E. globulus*	Ethiopia	Alemu Gezahgne and J Roux	AY210477	AY210483
CMW10095[b]	*Botryosphaeria* sp.	*E. grandis*	Ethiopia	Alemu Gezahgne and J Roux	AYS20478	AY210485
CMW10094[b]	*Botryosphaeria* sp.	*E. saligna*	Ethiopia	Alemu Gezahgne and J Roux	AY210479	AY210484
CMW7063	*Guignardia philoprina*	*Taxus baccata*	Netherlands	HA van der Aa	AY236979	AY236905

[a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI)
[b] Isolates from Ethiopia were sequenced in this study. All other sequences are from the study of Slippers *et al*. (2004)

Pathogenicity tests

All Ethiopian *Botryosphaeria* isolates used in the greenhouse inoculation trial produced lesions on the *E. grandis* clone. The mean lesion lengths produced ranged from 24.9mm to 91.8mm (Table 3). Isolate CMW11073 produced the longest and CMW11065 the shortest average lesion lengths. No lesions developed on plants inoculated with the sterile MEA as controls. 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 all produced lesions that were significantly different from the

control. In contrast, the average lesion lengths associated with isolates CMW11071, CMW11070 and CMW11065 (Table 3) were not statistically different from the controls.

The three isolates (CMW11059, CMW11065 and CMW11073) used in the field inoculation trial produced lesions ranging in average length between 63mm and 255mm. The longest lesions were recorded for isolate CMW11073 (average = 255mm) and the shortest lesions (average = 63.4) were those 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). An r-square value of 0.71 was recorded for the data obtained from the field study. The results of this inoculation trial also showed that the lesions associated with isolates CMW11073 and CMW11059 were statistically different to those of the control. Isolate CMW11065 produced lesions that did not vary significantly from the control (Table 4). The field and greenhouse trials, therefore, gave similar results.

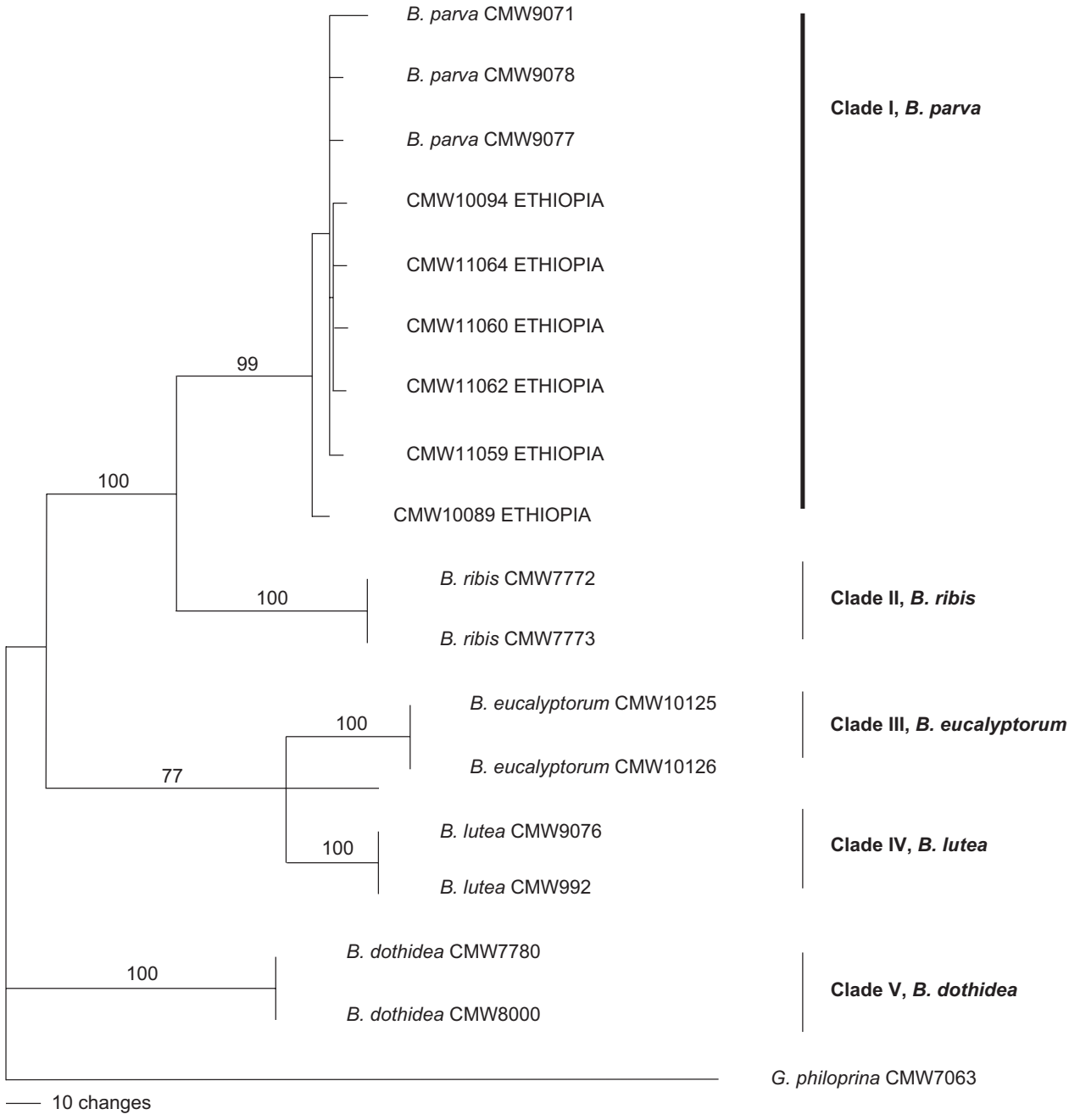

Figure 1: One of the 26 most parsimonious phylogenetic trees obtained from heuristic search of a combined ITS rDNA and EF1-α sequence data sets (CI = 0.928, RI = 0.905). Bootstrap values are shown above the branches

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

Isolate[a]	Mean lesion length (mm)[b]	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

[a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI)
[b] Means are averages of 10 measurements and those followed by 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 parva* on *E. citriodora* in the field

Isolates[a]	Mean lesion length (mm)[b]	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

[a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI)
[b] Means are averages of 20 measurements and those followed by the same letter are not significantly different from each other at $P < 0.05$ significance level

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* grown in Ethiopia (Alemu *et al*. 2003). The results 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 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 support the view (Jacobs and Rehner 1998, Smith and Stanosz 2001, Slippers *et al*. 2004) that morphological characteristics are insufficient to identify many *Botryosphaeria* spp. with confidence.

Analysis of RFLP banding patterns of the ITS region has been successfully used to distinguish between *Botryosphaeria* spp. obtained from different hosts (Jacobs 2002, Slippers *et al*. 2002). In this study, the RFLP analyses showed that all Ethiopian isolates belong to the *B. ribis* and *B. parva* complex. This technique 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 they represented either *B. ribis* or *B. parva*. Inability to distinguish between these two species based on ITS sequences has been reported previously by Smith and Stanosz (2001) and Zhou and Stanosz (2001). However, the combination of the ITS rDNA and EF1-α sequence data made it possible 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*. (2004) who confirmed that *B. ribis* and *B. parva* represent distinct species. 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 and Stanosz 2001, Zhou and Stanosz 2001). More recent studies have, however, shown that *B. ribis* and *B. parva* are distinct (Zhou *et al.* 2001, Slippers e*t al*. 2004).

It is interesting that only one species of *Botryosphaeria* is associated with die-back of *Eucalyptus* spp. in Ethiopia, while two species, *B. parva* and *B. eucalyptorum* are associated with die-back on this host in South Africa (Slippers *et al*. 2004). *Botryosphaeria parva* was first recorded in 1985

as a new species from kiwifruit in New Zealand and the fungus was previously most frequently found associated with fruit trees (Pennycook and Samuels 1985). Little information is available on the importance of this species in *Eucalyptus* plantations. Recently, Slippers *et al*. (2004) showed that *B. parva* is dominant in plantations of *Eucalyptus* spp. in South Africa. The results of the current study also show that this fungus is important in *Eucalyptus* plantations of Ethiopia.

Greenhouse and field inoculation trials revealed that most *Botryosphaeria* isolates obtained from *Eucalyptus* spp. 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 studies. Development of lesions on some trees inoculated as controls might have been due to contamination at the time of inoculation, wound stress or endophytic infections. The variability in virulence of the three isolates was consistent in greenhouse and field inoculation studies. These findings are similar to those of Jacobs (2002) who showed that *B. parva* is pathogenic to mango, but isolates varied substantially in pathogenicity.

Botryosphaeria parva must be considered an important pathogen of *Eucalyptus* spp. in Ethiopia, where it causes die-back and death of trees under stress conditions. *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*. 1996a, 1996b).

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.

Acknowledgments — The Agricultural Research and Training Project (ARTP) of the Ethiopian Agricultural Research Organisation (EARO) are thanked for financing this study. We acknowledge the Forestry Research Centre of Ethiopia and its staff for facilitating the fieldwork. The collaboration and support of Wondo Genet Forestry College, Munesa Shashemene Forest Development and Marketing Enterprise as well as the Jima Zonal Agricultural Bureau of Ormoia is highly appreciated.

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Edited by PD Hare