Characterization of Botryosphaeriaceae from plantation-grown *Eucalyptus* species in South China

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Abstract

The Botryosphaeriaceae is a species-rich family that includes pathogens of a wide variety of trees, including *Eucalyptus* species. Symptoms typical of infection by the Botryosphaeriaceae have recently been observed in *Eucalyptus* plantations in South China. The aim of this study was to identify the Botryosphaeriaceae associated with these symptoms. Isolates were collected from branch cankers and senescent twigs of different *Eucalyptus* spp. All isolates resembling Botryosphaeriaceae were separated into groups based on conidial morphology. Initial identifications were made using PCR-RFLP fingerprinting, by digesting the ITS region of the rDNA operon with the restriction enzymes *Cfo*I and *Ksp*I. Furthermore, to distinguish isolates in the *Neofusicoccum parvum*/*N. ribis* complex, a locus (BotF15) previously shown to define these species, was amplified and restricted with *Cfo*I. Selected isolates were then identified using comparisons of DNA sequence data for the ITS rDNA and translation elongation factor 1-alpha (TEF-1α) gene regions. Based on anamorph morphology and DNA sequence comparisons, five species were identified: *Lasiodiplodia pseudotheobromae*, *L. theobromae*, *Neofusicoccum parvum*, *N. ribis sensu lato* and one undescribed taxon, for which the name *Fusicoccum fabicercianum* sp. nov. is provided. Isolates of all species gave rise to lesions on the stems of an *E. grandis* clone in a glasshouse inoculation trial and on the stems of five *Eucalyptus* genotypes inoculated in the field, where *L. pseudotheobromae* and *L. theobromae* were most pathogenic. The five *Eucalyptus* genotypes differed in their susceptibility to the Botryosphaeriaceae species suggesting that breeding and selection offers opportunity for disease avoidance in the future.

Introduction

The Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) is a family of fungi that have a cosmopolitan distribution and occur on a wide range of monocotyledonous, dicotyledonous and gymnosperm hosts (Barr, 1987; Crous *et al*., 2006; Slippers & Wingfield, 2007). These fungi are generally regarded as weak, opportunistic pathogens that cause disease symptoms on plants exposed to stressful environmental conditions such as those associated with drought, frost, hail and damage caused by other pathogens and pests (Smith *et al*., 1994; Slippers & Wingfield, 2007). It has also been shown that the Botryosphaeriaceae occur in symptomless tissues as endophytes and latent pathogens on a variety of trees, including *Eucalyptus* spp. (Smith *et al*., 1996; Mohali *et al*., 2007;

Pavlic *et al*., 2007; Slippers & Wingfield, 2007; Slippers *et al*., 2009). These fungi can cause different symptoms on *Eucalyptus*, but are typically associated with cankers and die-back followed by kino exudation and in severe cases tree death (Smith *et al*., 1996; Slippers & Wingfield, 2007).

Species of the Botryosphaeriaceae are considered to be a significant threat to the production and sustainability of plantations of *Eucalyptus* spp., where they are grown as non-native crops (Smith *et al*., 1994, 2001; Slippers *et al*., 2004b, 2009; Mohali *et al*., 2009; Rodas *et al*., 2009). At least 23 species of Botryosphaeriaceae have been associated with *Eucalyptus* spp. in commercially grown plantations worldwide (Slippers *et al*., 2009). Some of these species, such as *Botryosphaeria dothidea* and *Neofusicoccum ribis* were the most commonly reported species from *Eucalyptus* spp. in the past (Slippers *et al*., 2009). However, the application of DNA-based molecular tools for identification of species in the Botryosphaeriaceae has shown that these two species are rare on *Eucalyptus* spp. Furthermore, a large number of new or cryptic sister species have been identified on *Eucalyptus* spp., mostly in the genera *Neofusicoccum*, *Pseudofusicoccum* and *Lasiodiplodia* (Slippers *et al*., 2009).

Eucalyptus plantations are of increasing importance in China where they provide the country with structural timber and pulp products such as paper. Approximately 2.6 million hectares of *Eucalyptus* plantations have been established in China to meet the needs of the country, especially for the rapidly growing pulp industry (Xie, 2006; Iglesias-Trabad & Wilstermann, 2008). *Eucalyptus* trees are thus important in China, both for the development of the economy and protection of native ecosystems that have been strictly protected from logging since the beginning of the 21st century.

Similar to the situation in other countries (Wingfield *et al*., 2008), *Eucalyptus* plantations in China are threatened by various pests and diseases (Zhou *et al*., 2008). However, limited research has been conducted on *Eucalyptus* diseases in this country. A recent survey of *Eucalyptus* plantations in South China reported a number of fungal pathogens, including species within the Botryosphaeriaceae (Zhou *et al*., 2008). The aims of the current study were to identify the Botryosphaeriaceae occurring on *Eucalyptus* spp. in the FuJian, GuangXi and HaiNan Provinces in South China, using PCR-RFLP fingerprinting analysis, DNA sequence comparisons and morphology of the anamorph stages. Furthermore, the pathogenicity of these fungi was tested on *Eucalyptus* genotypes in glasshouse and field trials.

Materials and methods

Isolates

Isolates used in this study were collected from different *Eucalyptus* clones/species in plantations in FuJian, GuangXi and HaiNan during the period of 2006 and 2007. Isolations were made from diseased branches, and from pycnidia formed on senescing branches following the protocols described by Pavlic *et al*. (2007, 2008). Cultures were maintained on 2% malt extract agar (MEA) (20 g malt extract, 15 g agar, 1 L water; Biolab) at 25°C under near-fluorescent light and stored at 5°C. To induce sporulation of cultures, isolates were transferred to 2% water agar (WA) (20 g agar, 1 L water; Biolab) with sterilized pine needles placed on the agar surface and incubated at 25°C for 10–14 days until fruiting structures appeared on the surface of the pine needles.

Conidial masses from fruiting structures were spread on the surface of WA in sterile drops of water. Single germinating conidia were isolated after 4–12 h, and transferred to clean 2% MEA plates. All the single-spore cultures are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa and the China Eucalypt Research Centre (CERC), Chinese Academy of Forestry (CAF), China.

DNA extraction and PCR amplification

For DNA extraction, single-conidial cultures were grown on 2% MEA for 7–10 days at 25°C in the dark. Mycelium used for DNA extraction was scraped directly from the medium using a sterile scalpel, and transferred to 1.5 mL Eppendorf tubes. DNA was extracted following a modified DNA extraction method (Raeder & Broda, 1985; Smith *et al*., 2001). The DNA pellets were re-suspended in 50 µL sterile SABAX water. RNAse (1 mg mL^{-1}) was added to DNA suspensions and incubated overnight at 37°C for RNA degradation. DNA was separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light.

The internal transcribed spacer (ITS) regions, ITS1 and ITS2, and the 5.8S gene of the ribosomal DNA (rDNA) operon, were amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*., 1990). Part of the translation elongation factor 1-alpha (TEF-1α) gene was amplified using the primers EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) (Carbone *et al*., 1999). The PCR reactions were performed following the PCR protocol described in Slippers *et al*. (2004a). In the case of isolates that were difficult to amplify using primers EF1-728F and EF1-986R, the primers EF1F (5′-TGCGGTGGTATCGACAAGCGT-3′) and EF2R (5′-AGCATGTTGTCGCCGTTGAAG-3′) (Jacobs *et al*., 2004) were used. The PCR amplicons were viewed on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. The fragment sizes were estimated against a standard 100 bp molecular weight marker (Roche Molecular Biochemicals). The PCR products were cleaned using 6% Sephadex G-50 columns (Sigma-Aldrich).

PCR-RFLP analysis

A PCR-RFLP fingerprinting technique was applied to identify groups among the collected isolates and to select representative isolates for sequencing. The ITS rDNA locus was amplified from all the isolates using primers ITS1 and ITS4 and amplicons were digested with the restriction enzymes (RE) *Cfo*I and *Ksp*I (Roche Diagnostics). Isolates identified as representing the *Neofusicoccum parvum*/*N. ribis* complex were further separated by PCR-RFLP analysis of the amplicons obtained using primers BotF15 and BotF16 (Slippers *et al*., 2004b) and digested with *Cfo*I. The RFLP reaction mixtures consisted of 20 µL PCR products, 0.30 µL RE and 2.5 µL matching enzyme buffers. The reaction mixtures were incubated for 18 h at 37°C. The resulting restriction fragments were separated on 2% agarose gels, stained with ethidium bromide and visualized under UV light. A standard 100 bp molecular marker was used to estimate the fragment sizes. The banding patterns were compared with those previously published (Slippers *et al*., 2004b).

DNA sequencing and analysis

Representative isolates from all groups identified based on PCR-RFLP analysis were sequenced (Table 1). To determine their identities, ITS and TEF-1α sequences of known Botryosphaeriaceae species were obtained from GenBank and included in the analysis (Table 1). The PCR products were sequenced in both directions using the same primers that were used for PCR reactions, and were purified using 6% Sephadex G-50 columns. Sequence reactions were run on an ABI PRISM 3100™ autosequencer (Perkin-Elmer Applied Biosystems).

Nucleotide sequences were analysed using mega4 software (Tamura *et al*., 2007). Sequence alignments were conducted online using mafft version 5.667 (Katoh *et al*., 2002) with the iterative refinement method (FFT-NS-i settings) and adjusted manually. Gaps were treated as a fifth character and all characters were unordered and of equal weight. A partition homogeneity test (PHT) was used to determine the congruence of the ITS and TEF-1α datasets (Farris *et al*., 1995;

Huelsenbeck *et al*., 1996). After an outcome indicating congruence between the datasets, phylogenetic analyses of the combined datasets were done in paup (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2002). Most parsimonious trees were found using the heuristic search function with 1000 random addition replicates and tree bisection and reconstruction (TBR) as branch swapping algorithm. Maxtrees were unlimited, branches of zero length were collapsed, and all most parsimonious trees were saved. A bootstrap analysis (1000 replicates) was done to determine the confidence levels of the tree-branching points (Felsenstein, 1985). The sequence of *Guignardia philoprina* from GenBank was used as the outgroup taxon (Table 1).

Table 1. Isolates considered in the phylogenetic study and pathogenicity tests.

Isolate No.[a,b]	Other No.[a]	Identity	Host	Location	Collector	GenBank ITS	GenBank TEF 1-α
CBS 119047		*Botryosphaeria corticis*	*Vaccinium corymbosum*	New Jersey, USA	P.V. Oudemans	DQ299245	EU017539
ATCC 22927		*B. corticis*	*Vaccinium* sp.	North Carolina, USA	R.D. Milholland	DQ299247	EF614951
CMW 7780		*Botryosphaeria dothidea*	*Fraxinus excelsior*	Molinizza, Switzerland	B. Slippers	AY236947	AY236896
CMW 8000		*B. dothidea*	*Prunus* sp.	Crodifeso, Switzerland	B. Slippers	AY236949	AY236898
CMW 13425	CBS 117445	*Botryosphaeria mamane*	*Acacia mangium*	Portuguesa state, Venezuela	S. Mohali	EF118046	GU134939
CMW 13429	CBS 117446	*B. mamane*	*Eucalyptus* hybrid	Cojedes state, Venezuela	S. Mohali	EF118048	GU134940
CMW 22674	CBS 124934	*Fusicoccum stromiferi*	*Pterocarpus angolensis*	South Africa	J. Mehl & J. Roux	FJ888473	FJ888486
CMW 22682	CBS 124935	*F. stromiferi*	*P. angolensis*	South Africa	J. Mehl & J. Roux	FJ888476	FJ888487
CMW 26167	CBS 122069	*Fusicoccum ramosum*	*E. camaldulensis*	Bell Gorge, Australia	T. Burgess	EU144065	EU144070
CMW 24703[a,b]	CBS 127187	*Fusicoccum fabioanianum*	*E. grandis* hybrid	GuangXi, China	M.J. Wingfield & X.D. Zhou	HQ332195	HQ332211
CMW 27091[c]		*F. fabioanianum*	*E. urophylla* × *E. tereticornis* clone	Hainan, China	M.J. Wingfield & X.D. Zhou	HQ332196	HQ332212
CMW 27094[a,b]	CBS 127193	*F. fabioanianum*	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332197	HQ332213
CMW 27106[c]		*F. fabioanianum*	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332199	HQ332215
CMW 27108		*F. fabioanianum*	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332200	HQ332216
CMW 27121[c]	CBS 127194	*F. fabioanianum*	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332198	HQ332214
CMW 7063		*Guignardia philoprina*	*Terminalia bacata*	Netherlands	H.A. van der As	AY236956	AY236905
CMW 27801		*Lasiodiplodia mahajangana*	*T. catappa*	Madagascar	J. Roux	FJ900895	FJ900641
CMW 27820		*L. mahajangana*	*T. catappa*	Madagascar	J. Roux	FJ900897	FJ900643
CBS 35659		*Lasiodiplodia parva*	*Theobroma cacao*	Sri Lanka	A. Riggenbach	EF622082	EF622052
CBS 49478		*L. parva*	Cassava field soil	Colombia	O. Rangel	EF622084	EF622054
STE-U 5808		*Lasiodiplodia plurivora*	*Prunus salicina*	South Africa	U. Damm	EF445362	EF445395
STE-U 4583		*L. plurivora*	*Vitis vinifera*	South Africa	F. Halleen	AY343482	EF445396
CMW 24699[a,b]		*Lasiodiplodia pseudotheobromae*	*Eucalyptus* sp.	GuangXi, China	M.J. Wingfield & X.D. Zhou	HQ332191	HQ332207
CMW 24700[a,b]		*L. pseudotheobromae*	*Eucalyptus* sp.	GuangXi, China	M.J. Wingfield & X.D. Zhou	HQ332192	HQ332208
CBS 116459		*L. pseudotheobromae*	*Gmelina arborea*	Costa Rica	J. Carranza-Velasquez	EF622077	EF622057
CBS 30479		*L. pseudotheobromae*	*Rosa* sp.	Netherlands	Ns	EF622079	EF622051
CMW 24701[a,b]		*Lasiodiplodia theobromae*	*Eucalyptus* sp.	GuangXi, China	M.J. Wingfield & X.D. Zhou	HQ332193	HQ332209
CMW 24702[a,b]		*L. theobromae*	*Eucalyptus* sp.	GuangXi, China	M.J. Wingfield & X.D. Zhou	HQ332194	HQ332210
CMW 18420	BOT 979	*L. theobromae*	*Casuarina cunninghamii*	Uganda	J. Roux	DQ103554	DQ103564
CMW 9074		*L. theobromae*	*Pinus* sp.	Mexico	T. Burgess	AY236952	AY236901
CBS 16496		*L. theobromae*	Fruit stung coral reef coast	New Guinea	Ns	AY640255	AY640258
CBS 111530		*L. theobromae*	Ns	Ns	Ns	EF622074	EF622054
CMW 13511	WAC 12539	*Lasiodiplodia venezuelensis*	*A. mangium*	Acarigua, Venezuela	S. Mohali	DQ103547	DQ103568
WAC 12540		*L. venezuelensis*	*A. mangium*	Venezuela	S. Mohali	DQ103547	DQ103568
CMW 28315		*Neofusicoccum batangarum*	*Terminalia catappa*	Cameroon	D. Begoude & J. Roux	FJ900606	FJ900652
CMW 28369		*N. batangarum*	*T. catappa*	Cameroon	D. Begoude & J. Roux	FJ900607	FJ900653
CMW 13992	CBS 123634	*Neofusicoccum cordaticola*	*Syzygium cordatum*	Sodwana Bay, South Africa	D. Pavlic	EU821898	EU821868
CMW 14151	CBS 123637	*N. cordaticola*	*S. cordatum*	Sabi, South Africa	D. Pavlic	EU821922	EU821892
CMW 10125	CBS 115791	*Neofusicoccum eucalyptorum*	*E. grandis*	Mpumalanga, South Africa	H. Smith	AF283686	AY236891
CMW 11705		*N. eucalyptorum*	*E. nitens*	South Africa	B. Slippers	AY339248	AY339264
CMW 14023	CBS 123639	*Neofusicoccum kwambonambiense*	*S. cordatum*	Kwambonambi, South Africa	D. Pavlic	EU821900	EU821870
CMW 14123	CBS 123643	*N. kwambonambiense*	*S. cordatum*	Ricards Bay, South Africa	D. Pavlic	EU821894	EU821864
CMW 9079	ICMP 7933	*Neofusicoccum parvum*	*Actinidia deliciosa*	New Zealand	S.R. Pennycook	AY236941	AY236886
CMW 9081	ICMP 8003	*N. parvum*	*Populus nigra*	New Zealand	G.J. Samuels	AY236943	AY236888
CMW 994	ATCC 58189	*N. parvum*	*Malus sylvestris*	New Zealand	G.J. Samuels	AF243395	AY236883
CMW 24704[c]		*N. parvum*	*E. grandis* hybrid	GuangXi, China	M.J. Wingfield & X.D. Zhou	HQ332201	HQ332217
CMW 27110[c]		*N. parvum*	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332202	HQ332218
CMW 27111		*N. parvum*	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332203	HQ332219
CMW 27125[a,b]		*N. parvum*	*E. urophylla* × *E. grandis* clone	Fujian, China	M.J. Wingfield	HQ332204	HQ332220
CMW 27135[c]		*N. parvum*	*E. dunnii*	Fujian, China	M.J. Wingfield	HQ332205	HQ332221
CMW 7772		*Neofusicoccum ribis*	*Ribes* sp.	New York, USA	B. Slippers & G. Hudler	AY236935	AY236877
CMW 7773		*N. ribis*	*Ribes* sp.	New York, USA	B. Slippers & G. Hudler	AY236936	AY236878
CMW 27119[a,b]		*N. ribis* s.l.	*Eucalyptus* sp.	Fujian, China	M.J. Wingfield	HQ332206	HQ332222
CMW 14058	CBS 123645	*N. umdonicola*	*S. cordatum*	Kosi Bay, South Africa	D. Pavlic	EU821904	EU821874
CMW 14127	CBS 123648	*N. umdonicola*	*S. cordatum*	Kwambonambi, South Africa	D. Pavlic	EU821926	EU821896

Ns: Not available.
[a]Designation of isolates and culture collections: CMW: Tree Protection Co-operative Program, Forestry and Agricultural Biotechnology Institute, University of Pretoria; ATCC: American Type Culture Collection; CBS: Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; ICMP: International Collection of Microorganisms from Plants, Auckland, New Zealand; WAC: Department of Agriculture, Western Australia Plant Pathogen Collection, Perth, Australia; STE-U: University of Stellenbosch, South Africa.
[b]Isolates sequenced in this study are given in bold.
[c]Isolates used in glasshouse pathogenicity trials.
[d]Isolates used in field pathogenicity trials in China.

By using the Markov Chain Monte Carlo (MCMC) method, Bayesian analyses were performed to ascertain the topology of the trees obtained with paup. These were conducted on the combined datasets of ITS and TEF-1α sequences as used in the parsimony analysis. First MrModeltest v.2.3 (Nylander, 2004) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with MrBayes v. 3.1 (Ronquist & Huelsenbeck, 2003). GTR+G was chosen as best-fitting model for the ITS and TEF-1α combined dataset. Two independent runs of MCMC with four chains were run for 1 000 000 generations. Trees were sampled every 100th generation for a total of 10 000 trees. The first 1000 trees were discarded as the burn-in phase of

each analysis, well after the likelihood values converted to stationary, leaving 9000 trees from which the consensus trees and posterior probabilities were calculated. The sequence of *Guignardia philoprina* from GenBank was used as the outgroup taxon (Table 1).

Morphology

Single-spore cultures of each species identified using DNA sequence data were transferred to WA media with sterilized pine needles placed on the agar surface and incubated at 25°C to induce sporulation. Released conidia from the pycnidia formed on pine needles were mounted in 85% lactic acid on glass slides and examined microscopically. Digital images were taken using a light microscope, a HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd.). Widths and lengths of 20 conidia were measured for each taxon and average (mean), standard deviation (std. dev), minimum (min) and maximum (max) measurements presented as [(min-) (average−std. dev.)-(average + std. dev.)(-max)], and their length: width ratios were calculated. The morphology of fungal colonies and the growth rates of cultures were conducted following the method described in Begoude *et al*. (2010b).

Pathogenicity tests

Glasshouse trial

Fourteen isolates, representing different species of the Botryosphaeriaceae identified based on PCR-RFLP fingerprinting, anamorph morphology and DNA sequence comparisons were used in a glasshouse pathogenicity trial (Table 1). These isolates were randomly selected to represent different locations and hosts. Six-day-old isolates grown on 2% MEA at 25°C were used for inoculations.

Trees of a *Eucalyptus grandis* clone (TAG-5) were selected for pathogenicity tests under glasshouse conditions. The trees were approximately 2 m tall and had diameters of approximately 10 mm. Before conducting the inoculations, the trees were allowed to acclimatize to the glasshouse conditions of 25°C and 14 h daylight, with 10 h darkness, for 1 month. Each of the 14 selected isolates was inoculated into the stems of 10 trees. Ten trees were also inoculated with sterile MEA plugs to serve as controls. The total of 150 inoculated trees was randomly arranged in a glasshouse.

For inoculations, wounds were made on the stems of the plants using a 6 mm diameter cork borer to remove the bark and expose the cambium. Wounds were made on the stems of the trees, approximately 300 mm above soil level. Plugs of mycelium were taken from 10-day-old cultures grown on MEA using the same size cork borer, and were placed into the wounds with the mycelium facing the cambium. Inoculated wounds were sealed with laboratory film (Parafilm M, Pechiney Plastic Packaging) to prevent desiccation and contamination.

After 6 weeks, the bark of the inoculated plants was removed and internal lesion lengths (mm) on the cambium were measured. The inoculated fungi were re-isolated by cutting small pieces of wood from the edges of lesions and plating them on 2% MEA at 25°C. Re-isolations were made from four randomly selected trees per isolate and from all trees inoculated as controls. Results were analysed in sas (Version 8) using proc glm (General Linear Model) (SAS Institute, 1999). Analysis of variance (anova) was used to determine the effects of fungal strain on lesion length. Prior to anova, homogeneity of variance across treatments was verified. To test the significance of the comparison means, Fisher's protected test was used and F values with $P < 0.05$ were considered significant.

Field inoculation trial

The eight most aggressive isolates identified in the glasshouse trial, belonging to five different species of Botryosphaeriaceae, were selected for field inoculations in China (Table 1). The field inoculations were conducted in an experimental *Eucalyptus* plantation in the ZhanJiang area of South China. Five *Eucalyptus* genotypes (1-year-old, 40–70 mm diameter), commonly grown in commercial plantations in South China were used in the field trial. These included an *E. grandis* clone (CEPT-1), an *E. grandis* × *E. tereticornis* clone (CEPT-2), an *E. pellita* genotype (CEPT-3), an *E. urophylla* × *E. grandis* clone (CEPT-4) and an *E. urophylla* clone (CEPT-5). Each of the eight isolates was inoculated into eight trees for every *Eucalyptus* genotype. Eight trees of each *Eucalyptus* genotype were also inoculated with sterile MEA plugs to serve as controls.

Inoculations were made on the stems of trees 50–100 cm above the soil level. A 9-mm-diameter sterile metal cork borer was used to remove the bark and to expose the cambial layer. Discs of mycelium of the same size were taken from 7-day-old cultures and placed into the exposed wounds with the mycelium facing the cambium. The inoculated wounds were sealed with Parafilm to protect them from desiccation and contamination. The inoculations were conducted in October–November, 2008. Internal lesion lengths in the cambium were measured after 5 weeks. Results from the field trial were analysed in sas (Version 8) using proc glm (SAS Institute Inc., 1999) and in a similar manner to those for the glasshouse inoculation test.

Results

Isolates

Forty-eight isolates of Botryosphaeriaceae were collected from 14 trees in three provinces during collection trips in 2006 and 2007 (Table 1). Six of these isolates were collected from a single *E. grandis* hybrid tree and two trees of an unknown *Eucalyptus* clone at two sites in GuangXi Province in November of 2006. Three isolates were each collected from three different trees of an *E. urophylla* × *E. tereticornis* clone at a site in the HaiNan Province in June of 2007. Thirty-nine isolates were collected from four different sites in FuJian Province in September of 2007, nine isolates were collected from a single *E. urophylla* × *E. grandis* clone at one site, four isolates from two trees of an *E. dunnii* clone at another site, and 26 isolates were collected from five trees of an unknown *Eucalyptus* sp. at two different sites. Nine isolates from the GuangXi and HaiNan Provinces were collected from diseased twigs, while the 39 isolates from FuJian Province were collected from senescing twigs on different trees (Table 1).

PCR-RFLP analysis

The 48 isolates of Botryosphaeriaceae from South China were separated into four groups based on PCR-RFLP profiles. After digestion of ITS rDNA amplicons with *Cfo*I, the 48 isolates were separated into three groups (Fig. 1a–b). These profiles were compared with those published by Slippers *et al*. (2004b). Four isolates, identified as *Lasiodiplodia* spp. based on morphology, produced the same profile (Group 1) (Fig. 1a) and 17 isolates in Group 2 represented members of the *N. parvum*/*N. ribis* complex (Fig. 1a–b). Profiles of 27 isolates in Group 3 matched those of the *B. dothidea* complex or *N. luteum*/*N. australe* (Fig. 1a–b). The ITS amplicons of these isolates were therefore also digested with *Ksp*I and identified as *B. dothidea* complex (Fig. 1c).

The 17 isolates residing in the *N. parvum*/*N. ribis* complex (Group 2) produced two profiles after digestion of BotF15 and BotF16 amplicons with *Cfo*I (Fig. 1d). These profiles matched those of *N. parvum* (16 isolates) and *N. ribis s. l.* (one isolate) as described by Slippers *et al*. (2004b).

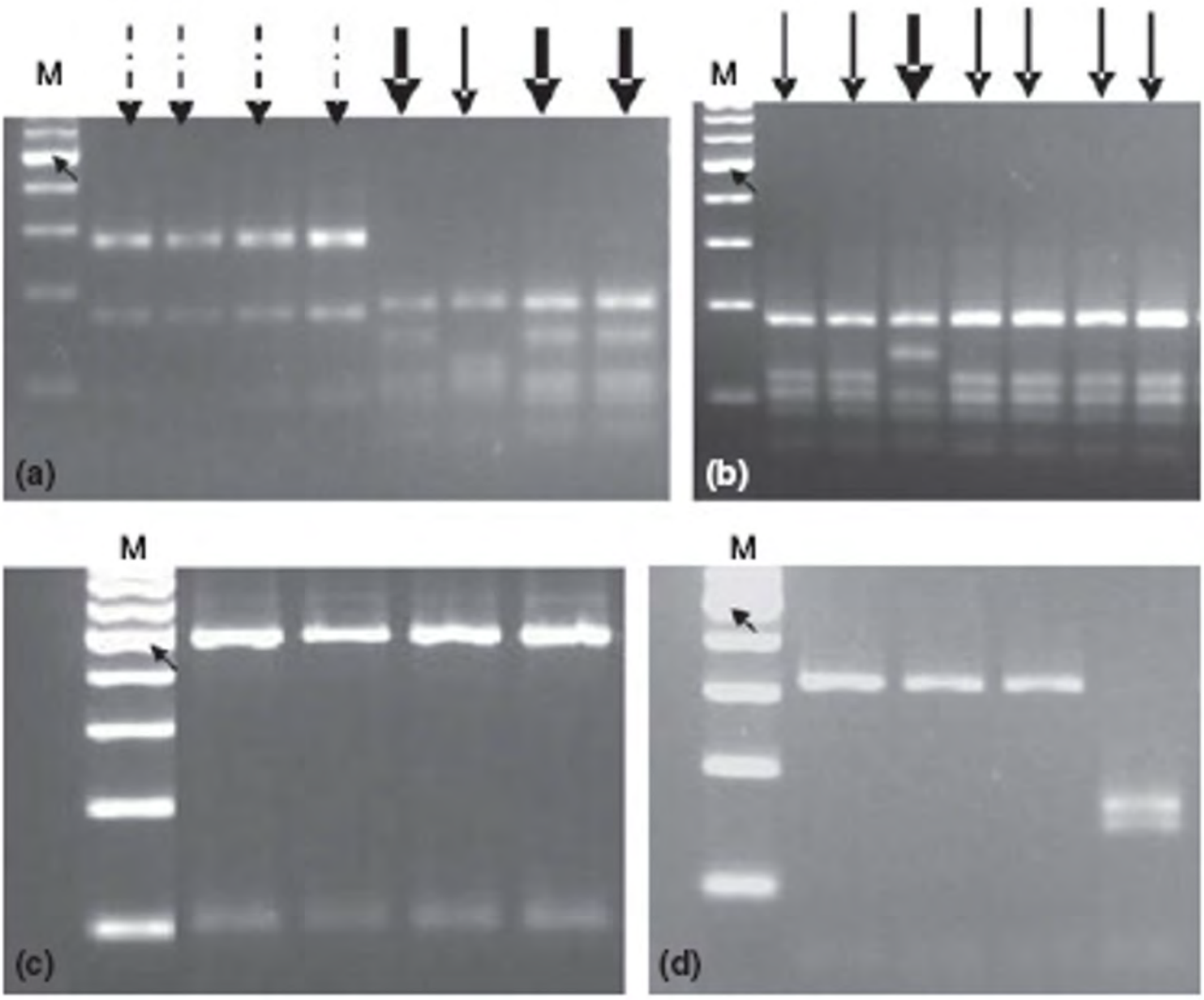

Figure 1. Agarose gel showing *Cfo*I restriction fragments of the ITS and DNA locus BotF15 PCR, and *Ksp*I restriction fragments of the ITS PCR products of different clades of Botryosphaeriaceae from China. (a–b) All the isolates are distinguished into three groups by using restriction enzyme *Cfo*I to digest ITS PCR products, (a) Group 1 (dash arrow): *Lasiodiplodia pseudotheobromae* and *L. theobromae*, (a–b) Group 2 (bold arrow): *Neofusicoccum parvum*/*N. ribis* species complex; Group 3 (thin arrow): species within *Botryosphaeria dothidea* complex; (c) ITS PCR products of isolates of *B. dothidea* complex digested by restriction enzyme *Ksp*I; (d) DNA locus BotF15 PCR products of isolates of *N. parvum*/*N. ribis* species complex digested by restriction enzyme *Cfo*I, one isolate is in group of *N. ribis s. l.*, and other isolates in *N. parvum*. The oblique arrow indicates the 500 bp marker, and lane 'M' contains a 100 bp size marker.

Phylogenetic analysis

The partition homogeneity test comparing the ITS and TEF-1α datasets gave a PHT value of P = 0.297, indicating that these two datasets were congruent and could be combined in the phylogenetic analysis. The aligned sequences of the combined datasets were submitted to TreeBASE (11035; http://www.treebase.org), and consisted of 863 characters of which 481 were constant and 382 were parsimony informative. Heuristic searches resulted in 20 most parsimonious trees. A consensus tree (50% majority rule) (tree length = 784 steps; CI = 0.802; RI = 0.969; RC =0.777) was computed (Fig. 2; TreeBASE 11035).

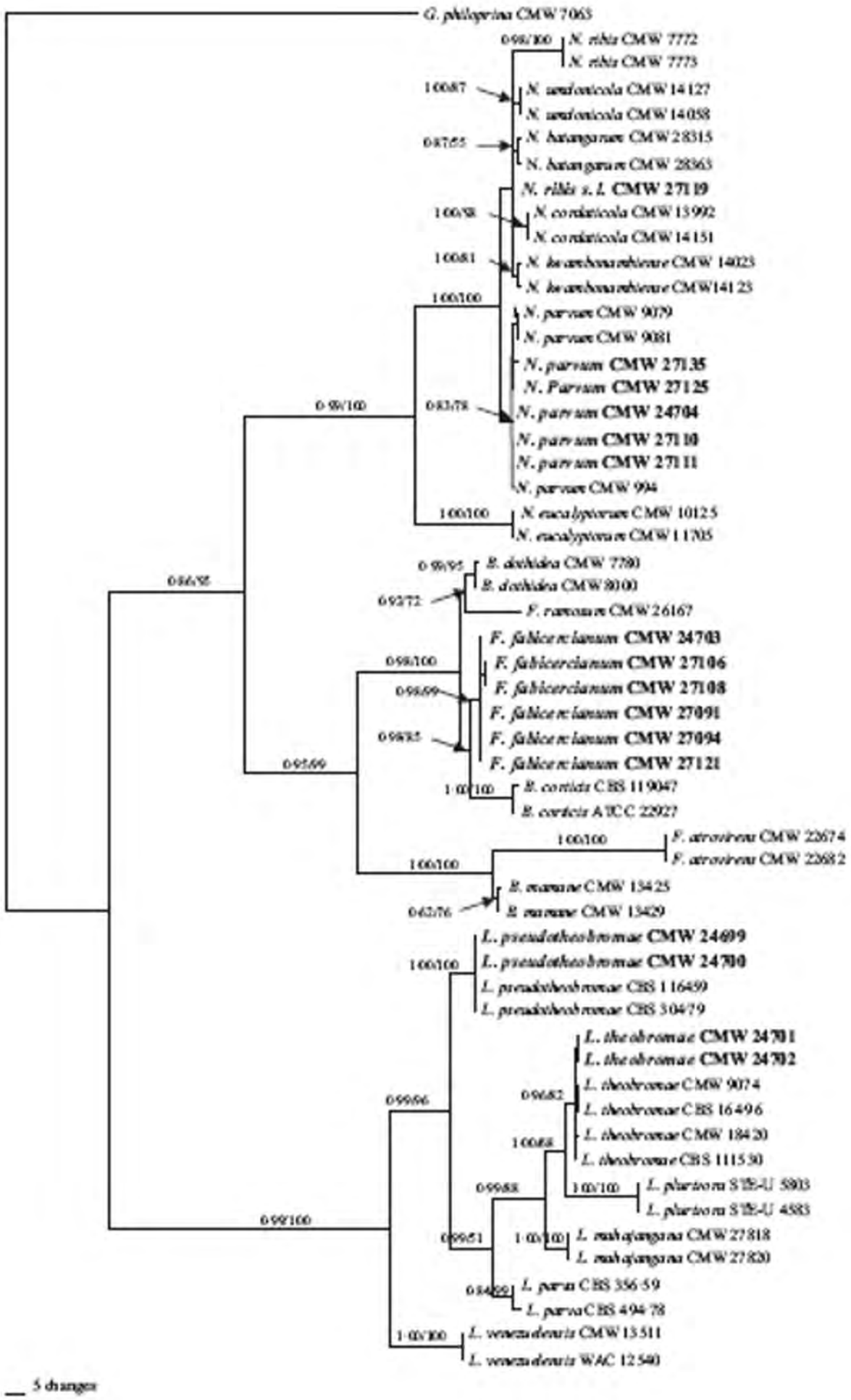
G. philoprina CMW 7065
0.98/100
N. ribis CMW 7772
N. ribis CMW 7773
1.00/87
N. umtiensicola CMW 14127
N. umtiensicola CMW 14058
0.87/65
N. hatungarum CMW 28315
N. hatungarum CMW 28363
N. ribis s. l. CMW 27119
1.00/98
N. conidiicola CMW 13992
N. conidiicola CMW 14151
1.00/81
N. kwambonambiense CMW 14023
N. kwambonambiense CMW14125
1.00/100
N. parvum CMW 9079
N. parvum CMW 9081
N. parvum CMW 27135
N. Parvum CMW 27125
0.99/100
0.83/78
N. parvum CMW 24704
N. parvum CMW 27110
N. parvum CMW 27111
N. parvum CMW 904
1.00/100
N. eucalyptorum CMW 10125
N. eucalyptorum CMW 11705
0.99/95
B. dothidea CMW 7780
B. dothidea CMW 8000
0.86/92
0.92/72
F. ramosum CMW 26167
F. fabicerciamum CMW 24703
F. fabicerciamum CMW 27106
0.98/100
F. fabicerciamum CMW 27108
0.98/69
F. fabicerciamum CMW 27091
F. fabicerciamum CMW 27094
0.98/85
F. fabicerciamum CMW 27121
0.97/69
1.00/100
B. cortecis CBS 119047
B. cortecis ATCC 22927
1.00/100
F. atrovirens CMW 22674
F. atrovirens CMW 22682
1.00/100
B. eucrasiae CMW 13425
0.62/76
B. eucrasiae CMW 13429
L. pseudotheobromae CMW 24699
1.00/100
L. pseudotheobromae CMW 24700
L. pseudotheobromae CBS 116459
L. pseudotheobromae CBS 130479
L. theobromae CMW 24701
L. theobromae CMW 24702
0.99/96
0.96/82
L. theobromae CMW 9074
L. theobromae CBS 116496
1.00/88
L. theobromae CMW 18420
L. theobromae CBS 111530
0.99/88
1.00/100
L. plurivora STE-U 5903
L. plurivora STE-U 4583
0.99/100
0.99/51
1.00/100
L. mahajangana CMW 27818
L. mahajangana CMW 27820
0.84/99
L. parva CBS 356.59
L. parva CBS 494.78
1.00/100
L. venezuelensis CMW 13511
L. venezuelensis WAC 12540
5 changes

Figure 2. One of the 20 most parsimonious trees obtained from Maximum Parsimony analysis of the combined ITS and TEF-1α sequence data of the representative taxa of the Botryosphaeriaceae. Posterior probabilities followed by bootstrap support (%) from 1000 replications are given on the branches (PP/BS). Isolates marked in bold represent those obtained from *Eucalyptus* trees in China.

In the Bayesian analysis, the position of the genera in relation to each other was different, but within each genus the topology was similar to the parsimony tree (TreeBASE 11035). Three clades were identified, each corresponding to a separate genus and each supported with high Bayesian posterior probabilities (PP) and bootstrap support (BS) (Fig. 2, PP = 0.99, BS = 100%; PP = 0.95, BS = 99%; PP = 0.99, BS = 100%, respectively). These were Clade 1 (*Neofusicoccum*), Clade 2 (*Botryosphaeria*) and Clade 3 (*Lasiodiplodia*). Within the *Neofusicoccum* clade, a single Chinese isolate was phylogenetically distant from other species within this clade, while other Chinese isolates in this clade resided with *N. parvum*. Within the *Botryosphaeria* clade, the Chinese isolates were found to be distinct from the known species in the genus by congruent distinction in both the datasets, and high Bayesian posterior probability and high bootstrap value (PP = 0.98, BS = 99%), suggesting that they represent an undescribed species. Within the Lasiodiplodia clade, the isolates in this study resided in clades identified as *L. theobromae* and *L. pseudotheobromae*, respectively (Fig. 2).

Morphology and taxonomy

All 48 isolates of the Botryosphaeriaceae from *Eucalyptus* plantations in South China produced anamorph fruiting structures on pine needles on WA media within 2–3 weeks. Teleomorph structures were not observed. All isolates were separated into two main groups based on conidial morphology. Forty-four isolates produced hyaline, *Fusicoccum*-like conidia, and four isolates produced dark, septate and striate conidia typical for *Lasiodiplodia* spp. Based on PCR-RFLP fingerprinting, anamorph morphology and DNA sequence comparisons, five species were identified. The conidial morphology of the four species and taxonomy of the undescribed species are described below.

Two isolates from one tree of an unknown *Eucalyptus* sp. in GuangXi Province were identified as *L. pseudotheobromae*. Conidia of *L. pseudotheobromae* were ellipsoidal, apices and bases rounded, widest in their middle to upper two-thirds, thick-walled, initially hyaline and aseptate, becoming one-septate and dark walled, (26.5-) 27-29(-30.5) × (13-)14.5-16(-17) µm (average of 40 conidia 28.1 × 15.2 µm, l/w 1.8) (Fig. 3a).

Two isolates from a single unknown *Eucalyptus* tree in GuangXi Province were identified as *L. theobromae*. Conidia of *L. theobromae* were ellipsoid or oblong, broadly rounded at their apices, tapering to truncate bases, widest in their middle to upper one-quarter sections, initially hyaline and aseptate, becoming one-septate and dark brown, (22.5-)23.5-26(-27) × (12-)13-14.5(-16) µm (average of 40 conidia 24.7 × 13.9 µm, l/w 1.8) (Fig. 3b).

Conidia of isolates identified as *N. parvum* and *N. ribis s. l.* were fusiform to ellipsoidal, aseptate, hyaline, smooth with granular contents. Conidia of *N. parvum* (Fig. 3c) were (16.5-)17-20(-23.0) × (5-)5.5-6.5(-7) µm (average of 100 conidia 18.7 × 6.0 µm, l/w 3.1) and *N. ribis s. l.* (Fig. 3d) were (19-)19.5-21(-22.5) × (5.5–6.5) µm, (average of 20 conidia 20.6 × 6.0 µm, l/w 3.5). Sixteen isolates were identified as *N. parvum*. One isolate was from a single *E. grandis* hybrid tree in GuangXi Province, 15 isolates from FuJian Province, including four isolates from two trees of an unknown Eucalyptus sp., seven isolates from a single *E. urophylla* × *E. grandis* tree and another four isolates from two *E. dunnii* trees. One isolate, from a single unknown *Eucalyptus* tree in FuJian Province was identified as *N. ribis s. l.*

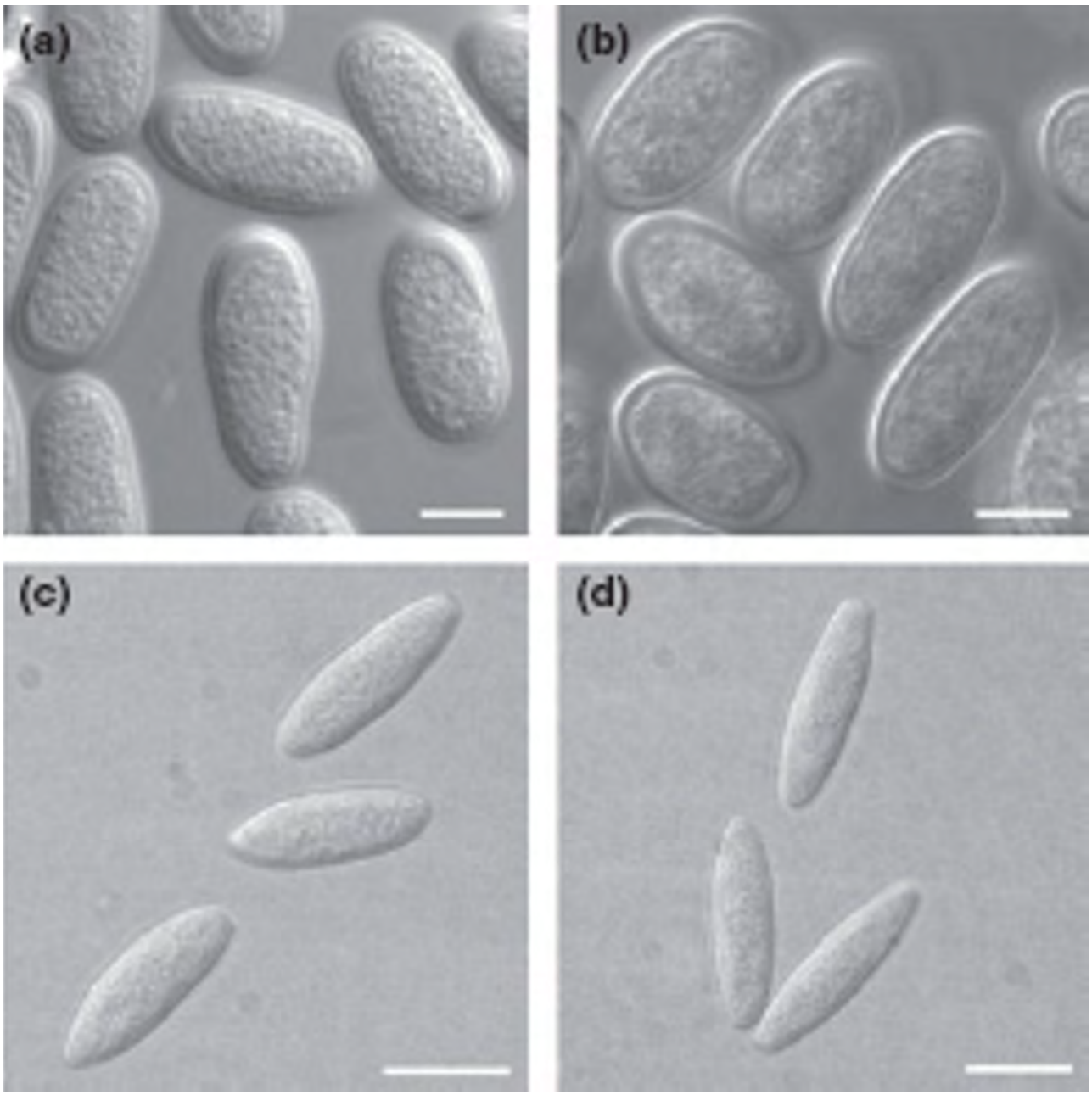

Figure 3. Conidia of *Lasiodiplodia*, *Fusicoccum* and *Neofusicoccum* species isolated from Chinese *Eucalyptus* plantations. (a) *Lasiodiplodia pseudotheobromae*; (b) *L. theobromae*; (c) *Neofusicoccum parvum*; (d) N. ribis s. l. Bars = 10 μm.

Fusicoccum fabicercianum S.F. Chen, D. Pavlic, M.J. Wingf. & X.D. Zhou, sp. nov., MycoBank No. MB519065

Etymology: Name refers to the co-operation between FABI (Forestry and Agricultural Biotechnology Institute, South Africa) and CERC (China Eucalypt Research Centre, China), that led to the discovery of this new species (Fig. 4).

Conidiomata superficialia solitaria vel aggregata atrobrunnea globosa, hyphis vel mycelio tecta. *Conidiophorae* absunt. *Cellulae conidiogenae* cylindricae vel lageniformes, hyalinae laeves parietibus tenuibus holoblasticae conidium unicum in apice formantes. *Paraphyses* absunt. *Conidia* hyalina parietibus tenuibus laevia contento granuloso, unicellularia non septata, fusiformia medio vel in tertia parte summa latissima, apice acuta, basi truncata fimbriis minutis marginalibus, mediocriter 22.0 × 5.8 μm, 3.8–plo longiora quam latiora; unum vel duo septa ante germinationem formantes.

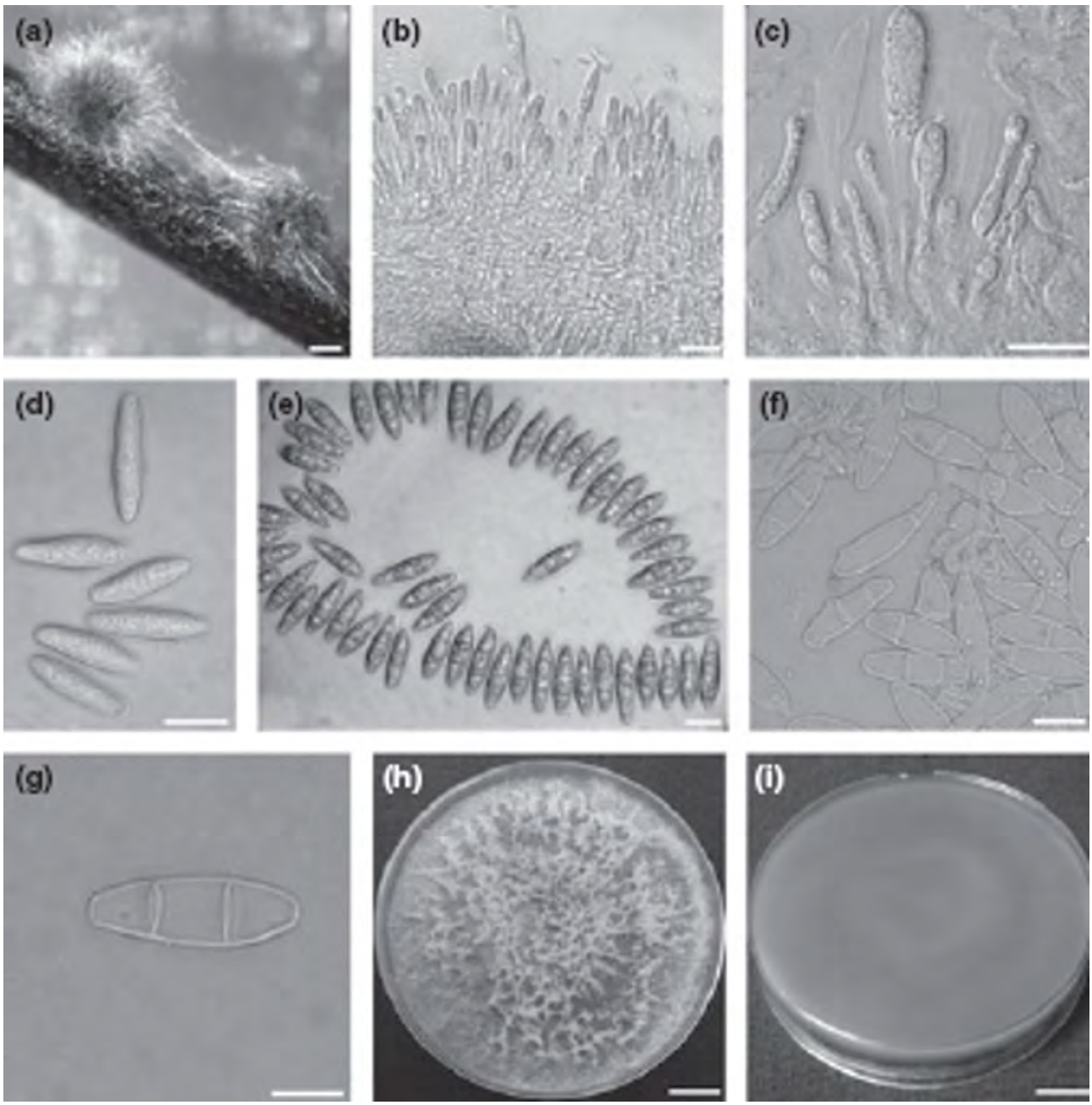

Figure 4. *Fusicoccum fabicercianum* CMW 27094 = CBS 127193 (culture ex-epitype). (a) Conidiomata formed in culture on pine needle; (b) immature, developing conidia produced by conidiogenous cells; (c) conidiogenous cells and developing conidia; (d) smooth, aseptate, fusiform mature conidia; (e) mature conidia with granular contents; (f) germinating conidia with one or two septa; (g) a typical germinating conidium with two septums; (h) living culture after growing 10 days on MEA (front); (i) living culture after growing 10 days on MEA (reverse). Bars: a = 100 μm; b–g = 10 μm; h–i = 10 cm.

Conidiomata developing in culture on pine needles after 10 days and producing conidia after 14 days, superficial, solitary to aggregated, dark brown, globose, covered with hyphae/mycelium, diameter (245-)346-470(-525) μm (average of 50 conidiomata 408 μm); wall composed of three layers: an outer thick-walled dark to light brown textura angulari; a middle layer of thin-walled light brown cells; and an inner layer of thin-walled hyaline cells (Fig. 4a). *Conidiophores* absent. *Conidiogenous cells* (Fig. 4b–c) cylindrical to lageniform, hyaline, smooth, thin-walled, holoblastic producing a single conidium at the tip, rarely proliferating at same level giving rise to periclinal thickenings, (6.5-)10.5-13.5(-16.0) × (2.0-)2.5-3.5(-4.5) μm (average of 50 conidiogenous cells 12.0 × 3.0 μm). *Paraphyses* absent. *Conidia* (Fig. 4d–g) hyaline, thin-walled, smooth (Fig. 4d) with

granular contents (Fig. 4e); unicellular, aseptate, fusiform, widest in the middle to upper third, apex acute, base truncate with a minute marginal frill, (16.7-)19.6-24.4(-26.1) × (4.5-)5.2-6.4(-7.5) µm (average of 100 conidia 22.0 × 5.8 µm, l/w = 3.8); forming one or two septa before germination (Fig. 4f–g).

Culture characteristics: Colony mycelium fluffy, initially white turning smoke grey (21""f) from the middle of colonies within 4–6 days, with an appressed mycelial mat, sparse to moderately dense. Cottony aerial mycelium toward the edge of colony, becoming pale olivaceous grey (21""d) to olivaceous grey (21""i), and greenish black (33""k) (reverse) within 12–16 days (Fig. 4h–i). Optimal temperature for growth 25(−30)ºC, colony covering the 90 mm diameter Petri dish after 5 days in the dark.

Teleomorph: Not observed, but expected to be *Botryosphaeria*-like based on phylogenetic inference.

Habitat: Branches and twigs of *Eucalyptus* species and hybrids.

Hosts and distributions: *Eucalyptus urophylla* ×*E. grandis* and a *Eucalyptus* sp. in FuJian Province; *E. urophylla* × *E. tereticornis* in HaiNan Province; *Eucalyptus* grandis hybrid in GuangXi Province; China.

Specimens examined: CHINA. FuJian Province, from senescing twigs of an unknown *Eucalyptus* sp. Aug., 2007, M.J. Wingfield, Herb. PREM 60449, holotype of *F. fabicercianum* sp. nov., culture ex-type CMW 27094 = CBS 127193; FuJian Province, from senescing twigs of unknown *Eucalyptus* sp. Aug., 2007, M.J. Wingfield, Herb. PREM 60450, culture CMW 27121 = CBS 127194; GuangXi Province, from senescing twigs of *Eucalyptus grandis* hybrid, Nov., 2006, M.J. Wingfield & X.D. Zhou, culture CMW 24703 = CBS 127187; HaiNan Province, from diseased living twigs of *E. urophylla* × *E. tereticorni*; June, 2007, M.J. Wingfield & X.D. Zhou, culture CMW 27091.

Notes: *Fusicoccum fabicercianum* sp. nov. is morphologically similar to *Botryosphaeria corticis*, but can be distinguished from that species by having smaller conidiogenous cells and smaller conidia. *Botryosphaeria corticis*: conidiogenous cells 12.5–17.5 × 2.5–4.5 µm, conidia (20.5-)23.5-32.5(-34.5) × (5-)5.5-7(-7.5) µm, av. = 28.9 × 6.4 µm, l/w = 4.5 (Phillips *et al*., 2006). Compared to the conidial morphology of other species of *Fusicoccum*, the conidia of *F. fabicercianum* sp. nov. are larger than those of *F. ramosum* (13.4 × 5.7 µm in culture) (Pavlic *et al*., 2008), similar to *B. dothidea* (24.7 × 4.9 µm in culture, 19.6 × 4.8 µm on a natural *Prunus* sp.) (Slippers *et al*., 2004a), smaller than *F. atrovirens* (33.5 × 8.5 µm in culture) (Mehl *et al*., 2010) and *B. mamane* (33.5 × 6.1 µm in culture) (Mohali *et al*., 2007). *Fusicoccum fabicercianum* sp. nov. is also characterized by one or two septa that develop in the conidia before germination, which is not uncommon amongst species of *Botryosphaeria* (Slippers *et al*., 2004a).

Twenty-seven isolates were identified as *F. fabicercianum* sp. nov. in this study. One isolate was from a single unknown *E. grandis* hybrid tree in GuangXi Province, three isolates were from three different trees of an *E. urophylla* × *E. tereticornis* clone in the HaiNan Province, 21 isolates were from four trees of an unknown *Eucalyptus* sp. and an additional two isolates from a single *E. urophylla* × *E. grandis* tree were identified from the FuJian Province.

Pathogenicity tests

Glasshouse trial

All isolates of the Botryosphaeriaceae inoculated on the *Eucalyptus* clone TAG-5 produced lesions within 6 weeks, while only small lesions were produced in the controls (Fig. 5). The comparison of means showed that the lesions produced by the isolates of Botryosphaeriaceae were significantly longer than those of the controls ($P < 0.0001$) (Fig. 5). The inoculated fungi were successfully re-isolated from the lesions. Although small lesions were produced on the controls, no Botryosphaeriaceae were re-isolated from those trees.

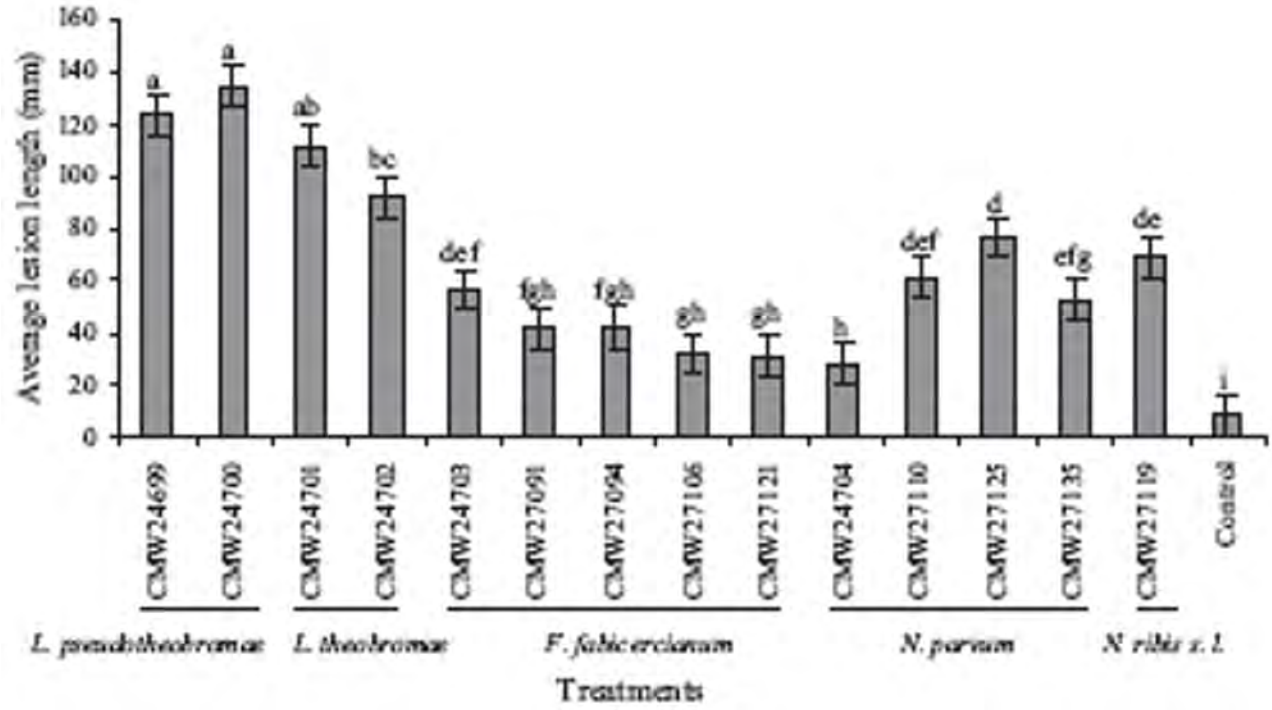

Figure 5. Column chart indicating the mean lesion lengths (mm) resulting from inoculation trials with *L. pseudotheobromae*, *L. theobromae*, *Fusicoccum fabicercianum*, *Neofusicoccum parvum* and *N. ribis s. l.* onto *E. grandis* clone (TAG-5) under glasshouse conditions. Bars represent 95% confidence limits for each treatment. Different letters above the bars indicate treatments that were significantly different ($P = 0.05$).

The mean comparison tests showed that lesions produced by isolates of *L. pseudotheobromae* and *L. theobromae* were significantly longer than those induced by isolates of *F. fabicercianum* sp. nov., *N. parvum* and *N. ribis s. l.* ($P < 0.05$) (Fig. 5). Isolates of *L. pseudotheobromae* were more aggressive than those of *L. theobromae* (Fig. 5). The two most aggressive isolates of each species of *L. pseudotheobromae*, *L. theobromae* and *F. fabicercianum* sp. nov., and one most aggressive isolate of *N. parvum* and *N. ribis s. l.* were selected for field inoculations.

Field trial

All isolates used in the field trial produced lesions on the *Eucalyptus* genotypes. Small lesions were also produced for the control inoculations but they were significantly shorter ($P < 0.05$) (Fig. 6). anovas showed significant isolate × genotype interaction ($P < 0.0001$), indicating that not all the Botryosphaeriaceae isolates reacted in the same manner to all the tested Eucalyptus genotypes, and this was supported by the mean comparison test results. For example, lesions produced by *L. pseudotheobromae* on *Eucalyptus* genotypes CEPT-1, CEPT-2 and CEPT-4 were significantly

longer than those on CEPT-3 and CEPT-5 ($P < 0.05$) (Fig 6), while no significant differences in lesion length were found for the five *Eucalyptus* genotypes inoculated with isolates of *F. fabicercianum* sp. nov. ($P > 0.05$) (Fig. 6). Similar to the glasshouse inoculations, *L. pseudotheobromae* was found to be the most aggressive pathogen.

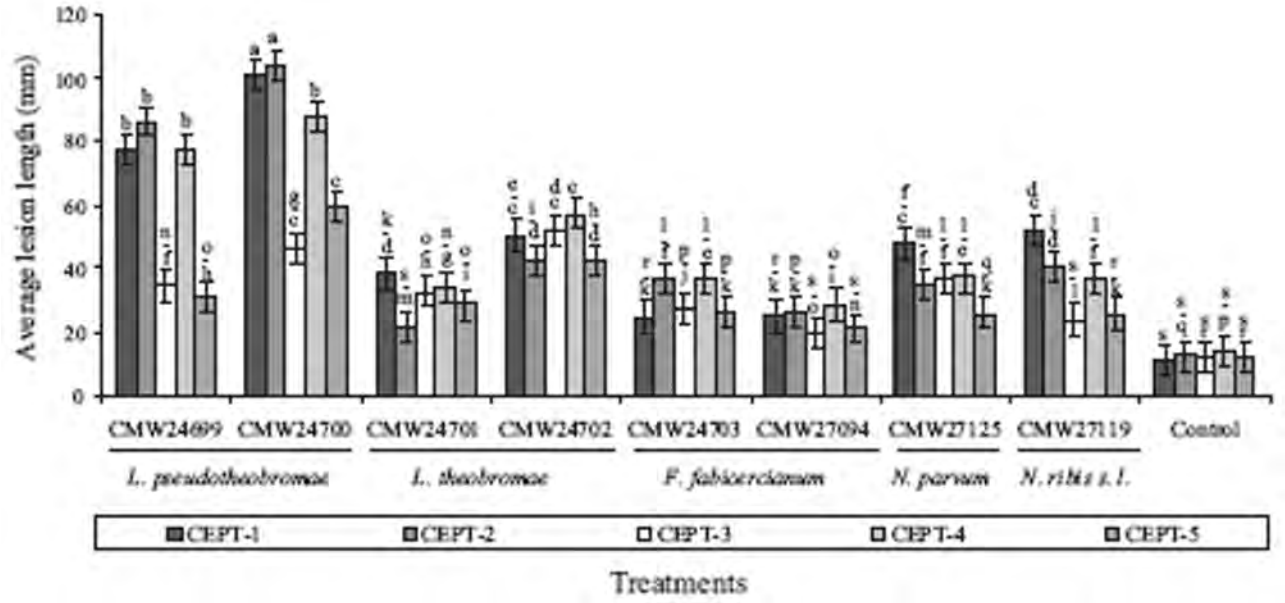

Figure 6. Column chart indicating the mean lesion lengths (mm) resulting from inoculation trials with *Lasiodiplodia pseudotheobromae*, *L. theobromae*, *Fusicoccum fabicercianum*, *Neofusicoccum parvum* and *N. ribis s. l.* onto five *Eucalyptus* genotypes in Chinese plantations. Bars represent 95% confidence limits for each treatment. Different letters above the bars indicate treatments that were significantly different ($P = 0.05$).

Discussion

This study represents the most comprehensive consideration of species of Botryosphaeriaceae from *Eucalyptus* spp. in China to date. Using comparisons of ITS rDNA and TEF-1α sequence data combined with PCR-RFLP analysis and morphology of the anamorph structures, it was possible to identify five species from a relatively small collection of isolates. They included *L. pseudotheobromae*, *L. theobromae*, *N. parvum*, *N. ribis sensu lato* and *F. fabicercianum* sp. nov. Except for *N. parvum* (Slippers *et al*., 2009), all species are reported for the first time from *Eucalyptus* trees in China.

The distribution of the species collected in this study varied among the regions. *Fusicoccum fabicercianum* was found on diseased *Eucalyptus* trees in the GuangXi and HaiNan Provinces, and also on dying *Eucalyptus* twigs in FuJian Province. *Lasiodiplodia pseudotheobromae* and *L. theobromae* were identified on a diseased *Eucalyptus* sp. in GuangXi Province. *Neofusicoccum parvum* was found on diseased *Eucalyptus* trees in GuangXi Province and dying twigs in FuJian Province. *Neofusicoccum ribis s. l.* was collected from senescing twigs on a *Eucalyptus* sp. in FuJian Province. *Fusicoccum fabicercianum* sp. nov., *N. parvum* and *N. ribis s. l.* were isolated from senescing twigs on the same tree in FuJian Province, indicating that different species of Botryosphaeriaceae share the same ecological environment in China, which is similar to that found in other studies (Slippers & Wingfield, 2007). The relatively limited distribution of *L. pseudotheobromae* and *L. theobromae* is most likely related to a collecting bias as these fungi are known from numerous countries, especially from tropical regions (Punithalingam, 1980; Mohali *et al*., 2005, 2007; Pavlic *et al*., 2007; Alves *et al*., 2008; Begoude *et al*., 2010a).

Fusicoccum fabicercianum resides in the *Botryosphaeria* (anamorph *Fusicoccum*) clade of the Botryosphaeriaceae and pathogenicity tests indicate that it is a weak pathogen. Although the species was most commonly isolated in samples taken in three provinces of South China, it was less pathogenic than the other species collected. Phylogenetically, *F. fabicercianum* is closely related to *Botryosphaeria corticis*, *B. dothidea* and *F. ramosum*. Interestingly, the results further showed that *Botryosphaeria* spp. with *Fusicoccum* anamorphs clustered in two clades supported by high statistical values. One clade includes *B. corticis*, *B. dothidea*, *F. fabicercianum* and *F. ramosum*, while *B. mamane* and *F. atrovirens* reside in the other clade (Fig. 2). Previous studies have shown that there are conidial differences amongst the species residing in the two clades, for example the conidia of species in the former clade are shorter (average: <30 µm) than those of *B. mamane* and *F. atrovirens* (average: more than 30 µm) (Slippers *et al*., 2004a; Phillips *et al*., 2006; Mohali *et al*., 2007; Pavlic *et al*., 2008; Mehl *et al*., 2010). More representative species and further work is needed to better understand the relevance of this divergence, and whether these groups validate distinct generic descriptions.

Neofusicoccum parvum is a well known pathogen of *Eucalyptus* spp. (Slippers *et al*., 2004b; Burgess *et al*., 2005; Pavlic *et al*., 2007; Slippers & Wingfield, 2007; Mohali *et al*., 2009; Rodas *et al*., 2009). The pathogen has been identified from *Eucalyptus* trees in Australia (Slippers *et al*., 2004b), Africa (Gezahgne *et al*., 2004; Slippers *et al*., 2004b), South America (Mohali *et al*., 2007) and Southeast Asia (Slippers *et al*., 2009). Results of this study also showed that *N. parvum* was amongst the most pathogenic species except *Lasiodiplodia* spp., supporting the view that it is an important pathogen of *Eucalyptus* spp. (Slippers *et al*., 2004b; Slippers & Wingfield, 2007).

The taxonomy of *N. ribis* and closely related species has been confused for many years. Isolates of *N. ribis* were initially separated from its sister species *N. parvum*, based on concordance between sequence data of multiple gene regions and PCR-RFLP analysis and were treated as *N. ribis* s. l. (Slippers *et al*., 2004b). Subsequently, *N. ribis* has been reported from *Eucalyptus* in Australia (Barber *et al*., 2005), Colombia (Rodas *et al*., 2009) and Venezuela (Mohali *et al*., 2007). Recently, four species, *N. batangarum*, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* were identified in the *N. ribis s. l.* complex based on multiple gene genealogies (Pavlic *et al*., 2009a,b; Begoude *et al*., 2010b). Thus, isolates previously identified as *N. ribis* based on PCR-RFLP analysis could represent any of a number of cryptic species. The isolate included in this study grouped closely, but separately from *N. batangarum*, *N. cordaticola* and *N. umdonicola* in the phylogenetic analysis of the ITS rDNA and TEF-1α sequences. Thus, it could represent another cryptic species in the *N. parvum*/N. ribis complex. Additional isolates and DNA sequence data for a greater number of gene regions will be required to resolve its identity. Pathogenicity tests in this study showed that the isolate has the ability to produce lesions on *Eucalyptus* trees, which were generally longer than those produced by *F. fabicercianum*.

Two *Lasiodiplodia* species, *L. pseudotheobromae* and *L. theobromae*, were identified in this study. The latter species is a widely distributed plant pathogen that has most commonly been reported from the tropics and subtropics (Punithalingam, 1980). It has been associated with more than 500 host plants including *Eucalyptus* (Punithalingam, 1976; Roux *et al*., 2000, 2001; Burgess *et al*., 2006; Mohali *et al*., 2007). This pathogen has been reported from *Eucalyptus* trees in Africa (Roux *et al*., 2000, 2001), Australia (Burgess *et al*., 2006) and South America (Mohali *et al*., 2007), and is considered the most aggressive species of Botryosphaeriaceae on these trees (Pavlic *et al*., 2007; Mohali *et al*., 2009). In the current study, *L. theobromae* was second only to *L. pseudotheobromae* in terms of its pathogenicity, which further supports the view that *L. theobromae* is one of the most damaging species of Botryosphaeriaceae on *Eucalyptus* (Slippers & Wingfield, 2007).

Lasiodiplodia pseudotheobromae is a sister species to *L. theobromae* and was recently described from species of *Acacia*, *Citrus*, *Coffea*, *Gmelina* and *Rosa* (Alves *et al*., 2008). It has also been

identified on *Eucalyptus* spp. in eastern Australia (Mohali *et al*., 2005; Alves *et al*., 2008; Slippers *et al*., 2009) and Venezuela (Mohali *et al*., 2005; Alves *et al*., 2008; Slippers *et al*., 2009). In this study, isolates of this species were the most aggressive of all the Botryosphaeriaceae tested. Previous inoculations on *Terminalia* trees also showed that *L. pseudotheobromae* is the most pathogenic among all the tested species of Botryosphaeriaceae on those trees (Begoude *et al*., 2010a).

Overall results of this study suggest that it is important to consider the impact of genotype by isolate and environment interaction when conducting artificial screening trials. For example, in the field pathogenicity trial on *Eucalyptus* genotypes CEPT-3, CEPT-4 and CEPT-5, lesions produced by isolate CMW 24702 of *L. theobromae* were significantly longer than those of *N. parvum* and the controls. In contrast, lesions produced by *L. theobromae* on *Eucalyptus* clones in Venezuela were significantly shorter than those of *N. parvum*, while the lesions produced by *L. theobromae* and the controls were not significantly different (Mohali *et al*., 2009). It is thus important to select the appropriate isolates for screening trials and to consider the involvement of isolate by tree genotype interaction, which has also been shown to be important for the *Eucalyptus* stem pathogen *Chrysoporthe austroafricana* (Van Heerden *et al*., 2005; Gryzenhout *et al*., 2009).

Species of Botryosphaeriaceae are considered to be a significant threat to the production and sustainability of *Eucalyptus* plantations. These fungi can infect native and introduced or cultivated hosts and appear to have been moved easily between different countries and continents (Slippers & Wingfield, 2007; Slippers *et al*., 2009). The results of this study provide a foundation for future work aimed at managing diseases caused by the Botryosphaeriaceae in *Eucalyptus* plantations in China.

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