# Botryosphaeriaceae associated with die-back of *Schizolobium parahyba* trees in South Africa and Ecuador

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Summary

Die-back of *Schizolobium parahyba* var. *amazonicum* is a serious problem in plantations of these trees in Ecuador. Similar symptoms have also been observed on trees of this species in various parts of South Africa. The most common fungi isolated from disease symptoms on *S. parahyba* var. *amazonicum* in both locations were species of the Botryosphaeriaceae. The aim of this study was to identify these fungi from both Ecuador and South Africa, and to test their pathogenicity in greenhouse and field trials. Isolates obtained were grouped based on culture morphology and identified using comparisons of DNA sequence data for the Internal Transcribed Spacer (ITS) and Translation Elongation Factor 1α (TEF-1α) gene regions. The β-tubulin-2 (BT2) locus was also sequenced for some isolates where identification was difficult. Three greenhouse trials were conducted in South Africa along with a field trial in Ecuador. *Neofusicoccum parvum* was obtained from trees in both areas and was the dominant taxon in South Africa. *Lasiodiplodia theobromae* was the dominant taxon in Ecuador, probably due to the subtropical climate in the area. Isolates of *N. vitifusiforme* (from South Africa only), *N. umdonicola*, and *L. pseudotheobromae* (from Ecuador

only) were also obtained. All isolates used in the pathogenicity trials produced lesions on inoculated plants, suggesting that the Botryosphaeriaceae contribute to the die-back of *S. parahyba* trees. While the disease is clearly not caused by a single species of the Botryosphaeriaceae in either region, *N. parvum* has been introduced into at least one of the regions. This species has a broad host range and could have been introduced on other hosts.

## 1. Introduction

The Botryosphaeriaceae (Ascomycota) include well-known endophytes and opportunistic pathogens of woody plants. These fungi infect via natural openings (WEAVER 1979; MICHAILIDES 1991; MICHAILIDES and MORGAN 1993; KIM et al. 1999) or wounds (MICHAILIDES 1991; AROCA et al. 2006; WHITELAW-WECKERT et al. 2006). They remain latent and persist endophytically within plant tissue, until stress arises (SMITH et al. 1994, 1996; STANOSZ et al. 1997; FLOWERS et al. 2001). Stresses reported, in relation to diseases caused by the Botryosphaeriaceae, include drought (PAOLETTI et al. 2001) and/or extreme cold or heat (RAYACHHETRY et al. 1996). Disease symptoms caused by the Botryosphaeriaceae include blights, cankers and die-back of plant parts or the death of entire trees (SLIPPERS and WINGFIELD 2007).

*Schizolobium parahyba* (Vell.) S. F. Blake var. *amazonicum* (Ducke) Barneby is a tree species native to South America, occurring in Ecuador and the Amazon Basin (DUCKE 1949). The species is locally known in South America as pachaco, guanacastle, guapuruvu, Brazilian fern tree or the feather duster tree. Although cultivated as an ornamental globally, the species is also prized for its light-colored veneer and plywood, and is used in furniture and paper production (ABRAF 2012). Along with these economic incentives, *S. parahyba* var. *amazonicum* grows rapidly, facilitating an important ecological role in reforestation (SILVA et al. 2011). In 1950, germplasm of *S. parahyba*

var. *amazonicum*, originating from Costa Rica, was introduced to Ecuador by the Instituto Nacional de Investigaciones Agrícolas y Pecuarias (INIAP) (CANCHIGNIA-MARTINEZ et al. 2007) enabling the subsequent establishment of plantations of the species in that country in 1982.

In 1987, *S. parahyba* var. *amazonicum* trees in plantations in Ecuador began to suffer from a serious die-back disease (GELDENHUIS et al. 2004). The first symptoms of this disease began at the branch tips and die-back progressed down the stems, resulting in epicormic shoot production, leaf loss, discoloration and rot of the pith and surrounding wood, and eventually tree death. Many diseased trees also had machete wounds resulting from the clearing of undergrowth by foresters (GELDENHUIS 2005). Isolations from wounds resulted in the discovery of putative pathogens such as *Ceratocystis fimbriata sensu lato* (*s.l.*) and non-pathogenic ophiostomatoid fungi such as *C. moniliformis*, *Graphium penicillioides*, *Ophiostoma quercus*, *Pesotum* sp. and *Thielaviopsis basicola* (GELDENHUIS et al. 2004; GELDENHUIS 2005; VAN WYK et al. 2011).

Apart from die-back in Ecuador, mortalities of *S. parahyba* var. *amazonicum* trees have also been reported in Brazil. In Ilha Grande, Rio de Janeiro, variable rainfall from the weather events El Niño and La Niña (first reduced then increased) and increased humidity from La Niña were thought to contribute to the development of disease and death of trees of varying ages (CALLADO and GUIMARÃES 2010). In another unrelated report, plantation trees in Dom Eliseu County, Para State, began to suffer from cankers and rotting. These symptoms were linked to infections by *Lasiodiplodia theobromae*, a member of the Botryosphaeriaceae (TREMACOLDI et al. 2009).

Die-back has recently been reported amongst ornamental *S. parahyba* var. *amazonicum* trees in Pretoria, South Africa (Fig 1), and it has continued to develop in Ecuador. Isolations from trees in

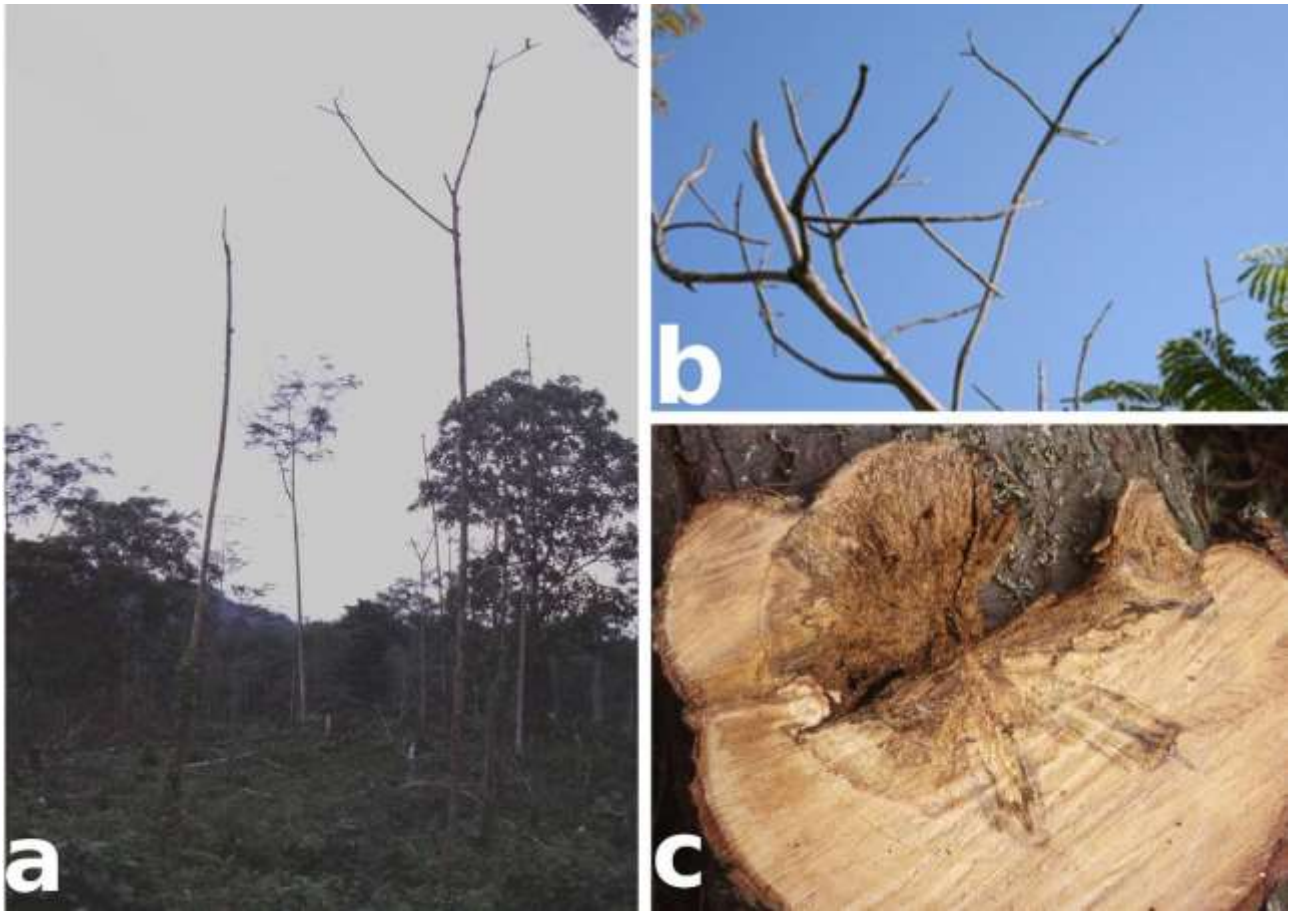


Fig 1. Disease symptoms of *Schizolobium parahyba* var. *amazonicum* trees in Ecuador and South Africa. a) Trees suffering from die-back and death in Ecuador. b) Branch die-back of trees in South Africa. c) Canker caused by species of the Botryosphaeriaceae from a diseased tree in Ecuador.

both areas yielded isolates of the Botryosphaeriaceae (HINZE et al. 2005). The aim of this study was to identify the isolates of the Botryosphaeriaceae collected from diseased *S. parahyba* var. *amazonicum* trees in Ecuador and South Africa and to test their pathogenicity in greenhouse and field trials. In this way, we considered whether the disease is caused by a specific pathogen, or generalist pathogens in this group that occur in each region.

## 2. Materials and Methods

### 2.1. Isolations

South African *S. parahyba* var. *amazonicum* trees were sampled from April-June 2005. Isolations were made from asymptomatic twigs and leaves, as well as dying branches following the method of PAVLIC et al. (2004) on malt extract agar (1.5% malt extract, 2% agar) (Biolab, Midrand, South Africa) and incubated at 25 ºC for 7 days. Trees in Ecuador were sampled in April 1997, January 1998, December 2000, October 2001 and December 2005. Isolations were made from the edges of visible lesions on branches and stems of trees displaying die-back. Resultant cultures from both regions were purified and isolates resembling species of the Botryosphaeriaceae were retained for further study.

Isolates were transferred to 2 % water agar (Biolab, South Africa) overlaid with sterilized pine needles (SMITH et al. 1996) or *S. parahyba* branch tissue. Sporulation was induced by incubating plates on a lab bench under artifical fluorescent light. Single conidial or single hyphal tip cultures were produced as outlined by MEHL et al. (2011). Cultures were then grouped based on culture morphology. All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria, Pretoria, South Africa.

### 2.2. DNA extraction and PCR amplifications

Three to four South African isolates of each culture morphological group were selected to compare DNA sequence data. All isolates from Ecuador were identified based on DNA sequence comparisons for the ITS locus. DNA extractions were done following the methods of MEHL et al. (2011).

The ITS rDNA locus (including the ITS1, 5.8S and ITS2 regions), the translation elongation factor 1α (TEF-1α) locus, and the β-tubulin-2 (BT2) locus were selected for DNA sequence comparisons and phylogenetic analyses. PCR mixtures for amplification of the ITS, BT2 and TEF-1α loci of South African isolates consisted of 5 µl 5× MyTaq Reaction Buffer (Bioline GmbH, Luckenwalde, Germany), 0.5 U MyTaq DNA Polymerase, 0.2 mM each primer and 10-50 ng template DNA. Sterile Sabax water (Adcock Ingram, Johannesburg, South Africa) was added to adjust the mixes to a volume of 25 µl. Primers ITS1 and ITS4 (WHITE et al. 1990) were used to amplify the ITS locus, BT2A and BT2B for the β-tubulin-2 locus (GLASS and DONALDSON 1995), while primer sets EF1F and EF2R (JACOBS et al. 2004) and EF688F and EF1251R (ALVES et al. 2008) were used to amplify the TEF-1α locus. Cycling conditions consisted of an initial denaturation step of 94 ºC for 2 minutes followed by 30 cycles of 94 ºC for 30 s, 54 ºC for 45 s and 72 ºC for 90 s then a final extension step of 72 ºC for 5 minutes.

PCR products were stained with Gel-Red (Biotium, Hayward, US) and viewed on 2% agarose gels run on a TAE buffer system (MANIATIS et al. 1982) under ultraviolet light. Product sizes were estimated using a Lambda DNA/*Eco*RI + *Hin*dIII marker 3 (Fermentas Life Sciences, Pittsburgh, PA, USA).

### 2.3. DNA sequencing and phylogenetic analysis

PCR product purification and sequencing were done as outlined by MEHL et al. (2011). Sequences were visually checked and edited using MEGA5 (TAMURA et al. 2011). Additional sequences required for phylogenetic analyses were obtained from GenBank. Sequence datasets were aligned using MAFFT 6 (http://mafft.cbrc.jp/alignment/server/) (KATOH and TOH 2008) using the L-INS-i algorithm. Phylogenetic analyses, both maximum parsimony (MP) and maximum likelihood (ML),

were performed on both the individual sequence datasets and the combined datasets. MP analyses were done using PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (SWOFFORD 2002) with the heuristic search option of 100 random addition search replications and tree-bisection-reconnection (TBR) selected, and MAXTREES limited to 1000. Uninformative flanking regions were excluded prior to analyses and gaps were treated as a fifth character. Partition homogeneity tests (PHTs) of 1000 replications were done to test for congruence between the datasets. All resulting equally parsimonious trees were saved. Measures such as tree length (TL), consistency index (CI), retention index (RI), and rescaled consistency index (RC) (HILLIS and HUELSENBECK 1992) were recorded.

The best nucleotide substitution model for each dataset was determined using jModelTest 0.1.1 (POSADA 2008) with the corrected Akaike information criterion (AIC) (SIGIURA 1978) selected. ML analyses were done using PhyML v3.0.1 (GUINDON et al. 2010) and with the respective parameters of the model selected for each dataset. Bootstrap analyses were done to determine the robustness of trees obtained from both MP and ML analyses. Trees were rooted to two isolates of *Botryosphaeria dothidea* (SLIPPERS et al. 2004a) as the outgroup taxon.

Some isolates from Ecuador grouped within the *Neofusicoccum parvum-ribis* complex based on sequence data for the ITS locus, but their taxonomic position remained unresolved after including sequence data for the TEF-1α locus. Thus the BT2 locus of these isolates was also amplified and sequenced.

## 2.4. Pathogenicity tests

### 2.4.1. Inoculations in South Africa

Three inoculation trials were undertaken with South African isolates on *S. parahyba* trees. For all three trials, inoculations were done using the same method as described in MEHL et al. (2011), except that wounds were sealed with cotton wool, a piece of aluminum foil and parafilm to maintain humidity and to prevent dessication and contamination. While this is the standard method for determining pathogenicity, it is harsh in that it involves placing a large amount of inoculum supplemented by nutrient rich media onto an open wound. Nevertheless, the aim was to compare the aggressiveness between species recovered and amongst isolates, although done under artificial conditions. In all three trials, inoculations were performed in a greenhouse with natural day/night conditions and a constant temperature of 25 ºC. Trees were maintained for six weeks post-inoculation after which lesion lengths were measured. Re-isolations were done from four trees per isolate per trial to verify that the lesion formed was caused by the inoculated fungus.

The first trial in September 2005 included 50 one-year-old trees. A 3 mm cork borer was used to wound the trees and the wounds were inoculated with 4 isolates of the Botryosphaeriaceae species obtained from South African trees. In total, 10 trees were inoculated with each isolate and an equal number were inoculated with sterile agar discs to serve as controls.

The second and third trials were done in November and December 2011. In both tests, 35 one metre tall (6-year-old) trees were wounded using a 7 mm cork borer and the wounds were inoculated with 2 different isolates of the 2 species obtained from South African trees. A set of trees were also wounded and the wounds inoculated with a sterile agar disc that acted as a control, so that 7 trees

were wounded for inoculation with an isolate or a control.

### 2.4.2. Inoculations in Ecuador

A single inoculation trial was done in 2001 on 3-year-old plantation trees in Rio Pitzara near Las Golondrinas (Pichincha province). 15 trees were each wounded using a 10 mm cork borer and the wounds inoculated with 4 of the isolates obtained from trees in Ecuador (total 60 trees). A set of 12 trees were also wounded and the wounds inoculated with a sterile agar disc that served as controls.

### 2.4.3. Statistical analyses

Statistical analysis of data from the pathogenicity trials was done in R, using the R Commander package (FOX 2005; R DEVELOPMENT CORE TEAM 2011). Cork borer diameters were subtracted from the data prior to analyses. Outliers were identified using boxplots and log transformed. A Shapiro-Wilk test was done on all samples to test for normality. Data for the same isolate between trials were tested using a t-test to determine whether the data could be combined. One-way analysis of variance (ANOVA) tests were done on data within trials. *Post hoc* analysis was done using Fisher's least significant differences (LSD) test to evaluate whether significant differences occurred amongst treatments. Means were considered significantly different at $P = 0.05$.

## 3. Results

### 3.1. Isolations and species identifications

A total of 28 isolates were collected from multiple South African *S. parahyba* var. *amazonicum* trees, of which 20 originated from trees in Pretoria and 8 from trees in Nelspruit. South African isolates could be placed in two morphological groups. Cultures of the first group were creamy yellow in colour while cultures from the second group had white mycelium. Isolates of the first

group were collected from trees in both Pretoria and Nelspruit while isolates of the second group were collected only from trees in Pretoria. In Ecuador, 65 isolates were collected from the various diseased trees.

Isolates from *S. parahyba* var. *amazonicum* grouped in two genera of the Botryosphaeriaceae; specifically *Lasiodiplodia* Ellis & Everh. and *Neofusicoccum* Crous, Slippers & Phillips. Datasets for each locus as well as the combined loci were analyzed for each genus separately. Sequence data generated for this study were deposited in GenBank (Table 1). Alignments and phylogenetic trees emerging from analyses undertaken on the individual ITS, BT2 and TEF-1α datasets, as well as the combined datasets for these loci were deposited in TreeBase (http://www.treebase.org/treebase-web/home.html) under accession number S14951 (http://purl.org/phylo/treebase/phylows/study/TB2:S14951).

For the *Lasiodiplodia* analyses, the ITS dataset consisted of 525 characters (51 parsimony informative, 472 constant, 2 parsimony uninformative), and yielded 22 most parsimonious trees (TL=68, CI=0.7941, RI=0.9119, RC=0.7242). The model selected for ML analysis was TPM1 (gamma=0.011). The TEF-1α dataset consisted of 283 characters (136 parsimony informative, 141 constant, 6 parsimony uninformative), and yielded 43 most parsimonious trees (TL=234, CI=0.7607, RI=0.9026, RC=0.6866). The model selected for ML analysis was HKY (ti/tv=1.3838, gamma=0.584). The combined analysis consisted of 808 characters (187 parsimony informative, 613 constant, 8 parsimony uninformative), and yielded 5 most parsimonious trees (TL=312, CI=0.7436, RI=0.8910, RC=0.6625) (Fig 2). The model TPM2uf (gamma=0.21) was selected for ML analysis. The PHT value was 0.002.

Table 1. Isolates used for the phylogenetic analysis. Ex-type cultures are indicated in boldface. Sequence data from GenBank are italicized.

| **Identity** | **Culture number** | **Other numbers** | **Host** | **Location** | **Collector(s)** | **GenBank accession number** | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | **ITS** | **BT2** | **EF-1α** |
| *Botryosphaeria dothidea* | CMW7780 | BOT1636 | *Fraxinus excelsior* | Molinizza, Ticino, Switzerland | B. Slippers | *AY236947* | *AY236925* | *AY236896* |
| *B. dothidea* | CMW8000 | CBS115476 | *Prunus* sp. | Crocifisso, Ticino, Switzerland | B. Slippers | *AY236949* | *AY236927* | *AY236898* |
| *Dichomera versiformis* | **WAC12403** | VIC3, PD295 | *E. camaldulensis* | Victoria, Australia | P. Barber | *GU251222* | *GU251882* | *GU251354* |
| *Guignardia* sp. | MUCC684 | | *Agonis flexuosa* | Yalgorup, W.A. | T. Burgess | *EU675682* | | *EU686573* |
| *Guignardia* sp. | MUCC685 | | *Ag. flexuosa* | Yalgorup, W.A. | T. Burgess | *EU675681* | | *EU686572* |
| *Lasiodiplodia citricola* | CBS124706 | IRAN1521C | *Citrus* sp. | Chaboksar, Sari, Iran | A. Shekari | *GU945353* | | *GU945339* |
| *L. citricola* | **CBS124707** | IRAN1522C | *Citrus* sp. | Chaboksar, Sari, Iran | J. Abdollahzadeh/ A. Javadi | *GU945354* | | *GU945340* |
| *L. crassispora* | CMW13488 | | *E. urophylla* | Venezuela | S. Mohali | *DQ103552* | | *DQ103559* |
| *L. crassispora* | CMW14688 | WAC12534 | *Santalum album* | Ord River, Kununurra, W.A. | T. Burgess | *DQ103551* | | *DQ103558* |
| *L. crassispora* | **CMW14691** | WAC12533 | *San. album* | Ord River, Kununurra, W.A. | T. Burgess | *DQ103550* | | *DQ103557* |

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|---|---|---|---|---|---|---|---|
| *L. gilanensis* | **CBS124704** | IRAN1523C | Unknown | Gilan, Iran | J. Abdollahzadeh/ A. Javadi | *GU945351* | *GU945342* |
| *L. gilanensis* | CBS124705 | IRAN1501C | Unknown | Gilan, Iran | J. Abdollahzadeh/ A. Javadi | *GU945352* | *GU945341* |
| *L. gonubiensis* | CMW14077 | CBS115812 | *Syzygium cordatum* | Gonubie, Eastern Cape, S. Africa[2] | D. Pavlic | *AY639595* | *DQ103566* |
| *L. gonubiensis* | CMW14078 | CBS116355 | *Syz. cordatum* | Gonubie, Eastern Cape, S. Africa | D. Pavlic | *AY639594* | *DQ103567* |
| *L. hormozganensis* | CBS124708 | IRAN1498C | *Mangifera indica* | Hormozgan, Iran | J. Abdollahzadeh/ A. Javadi | *GU945356* | *GU945344* |
| *L. hormozganensis* | **CBS124709** | IRAN1500C | *M. indica* | Hormozgan, Iran | J. Abdollahzadeh/ A. Javadi | *GU945355* | *GU945343* |
| *L. iraniensis* | **CBS124710** | IRAN1520C | *Salvadora persica* | Hormozgan, Iran | J. Abdollahzadeh/ A. Javadi | *GU945348* | *GU945336* |
| *L. iraniensis* | CBS124711 | IRAN1502C | *Juglans* sp. | Golestan, Iran | A. Javadi | *GU945347* | *GU945335* |
| *L. mahajangana* | CMW27801 | CBS124925 | *Terminalia catappa* | Mahajanga, Madagascar | J. Roux | *FJ900595* | *FJ900641* |
| *L. mahajangana* | CMW27818 | CBS124926 | *T. catappa* | Mahajanga, Madagascar | J. Roux | *FJ900596* | *FJ900642* |
| *L. mahajangana* | **CMW27820** | CBS124927 | *T. catappa* | Mahajanga, Madagascar | J. Roux | *FJ900597* | *FJ900643* |
| *L. margaritacea* | **CMW26162** | CBS122519 | *Adansonia gibbosa* | Tunnel Creek Gorge, W.A. | T. Burgess | *EU144050* | *EU144065* |

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|---|---|---|---|---|---|---|---|
| *L. margaritacea* | CMW26163 | CBS122065 | *Ad. gibbosa* | Tunnel Creek Gorge, W.A. | T. Burgess | *EU144051* | *EU144066* |
| *L. parva* | CBS356.59 | ETH2977 | *Theobroma cacao* | Agalawatta, Sri Lanka | A. Riggenbach | *EF622082* | *EF622062* |
| *L. parva* | **CBS456.78** | | Cassava-field soil | Dep. Meta, Vilavicencio, Colombia | O. Rangel | *EF622083* | *EF622063* |
| *L. parva* | CBS494.78 | | Cassava-field soil | Dep. Meta, Vilavicencio, Colombia | O. Rangel | *EF622084* | *EF622064* |
| *L. plurivora* | **CBS120832** | STE-U5803 | *Pr. salicina* | Stellenbosch, S. Africa | U. Damm | *EF445362* | *EF445395* |
| *L. plurivora* | CBS121103 | STE-U4583 | *Vitis vinifera* | S. Africa | F. Halleen | *AY343482* | *EF445396* |
| *L. pseudotheobromae* | CMW22933 | 46 | *Schizolobium parahyba* | Buenos Aires, Esmeraldas, Ecuador | L. Lombard | KF886704 | KF886727 |
| *L. pseudotheobromae* | CMW22937 | 103 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | L. Lombard | KF886705 | KF886728 |
| *L. pseudotheobromae* | CMW22945 | 127 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | L. Lombard | KF886706 | KF886729 |
| *L. pseudotheobromae* | CBS447.62 | | *Citrus aurantium* | Suriname | C. Smulders | *EF622081* | *EF622060* |
| *L. pseudotheobromae* | **CBS116459** | KAS2 | *Gmelina arborea* | San Carlos, Costa Rica | J. Carranza-Velásquez | *EF622077* | *EF622057* |
| *L. rubropurpurea* | **CMW14700** | WAC12535 | *E. grandis* | Tully, Queensland | T. Burgess/G. Pegg | *DQ103553* | *DQ103571* |

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|---|---|---|---|---|---|---|---|---|
| *L. rubropurpurea* | CMW15207 | WAC12536 | *E. grandis* | Tully, Queensland | T. Burgess/G. Pegg | *DQ103554* | | *DQ103572* |
| *L. theobromae* | CMW4695 | BOT531 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | M. J. Wingfield | KF886707 | | KF886730 |
| *L. theobromae* | CMW9271 | BOT2490, 26 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | M. J. Wingfield | KF886708 | | KF886731 |
| *L. theobromae* | CMW22924 | 3 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | L. Lombard | KF886709 | | KF886732 |
| *L. theobromae* | CMW9074 | | *Pinus* sp. | Mexico | T. Burgess | *AY236952* | | *AY236901* |
| *L. theobromae* | CBS164.96 | | Fruit on coral reef coast | Papua New Guinea | A. Aptroot | *AY640255* | | *AY640258* |
| *L. venezuelensis* | **CMW13511** | WAC12539 | *Acacia mangium* | Acarigua, Venezuela | S. Mohali | *DQ103547* | | *DQ103568* |
| *L. venezuelensis* | CMW13512 | WAC12540 | *Ac. mangium* | Acarigua, Venezuela | S. Mohali | *DQ103548* | | *DQ103569* |
| *Neofusicoccum andinum* | CMW13446 | CBS117452 | *Eucalyptus* sp. | Mountain Range, Mérida state, Venezuela | S. Mohali | *DQ306263* | | *DQ306264* |
| *N. andinum* | **CMW13455** | CBS117453, PD252 | *Eucalyptus* sp. | Mountain Range, Mérida state, Venezuela | S. Mohali | *AY693976* | *GU251815* | *AY693977* |
| *N. arbuti* | **CBS116131** | AR4014, BPI863597, PD281 | *Arbutus menziesii* | Washington, U.S.A. | M. Elliott | *AY819270* | *GU251811* | *GU251283* |
| *N. arbuti* | CBS117089 | AR4100, BPI863937, UW22 | *Ar. menziesii* | Sonoma, California, U.S.A. | M. Elliott | *GU251154* | *AY820313* | *GU251286* |

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|---|---|---|---|---|---|---|---|---|
| *N. australe* | CBS112872 | STE-U4425 | *V. vinifera* | Stellenbosch, Western Cape, S. Africa | F. Halleen | *AY343388* | | *AY343347* |
| *N. australe* | CBS112877 | STE-U4415 | *V. vinifera* | Stellenbosch, Western Cape, S. Africa | F. Halleen | *AY343385* | | *AY343346* |
| *N. batangarum* | CMW28315 | CBS124922 | *T. catappa* | Kribi, Cameroon | D. Begoude/J. Roux | *FJ900606* | *FJ900633* | *FJ900652* |
| *N. batangarum* | CMW28320 | CBS124923 | *T. catappa* | Kribi, Cameroon | D. Begoude/J. Roux | *FJ900608* | *FJ900635* | *FJ900654* |
| *N. cordaticola* | **CMW13992** | CBS123634 | *Syz. cordatum* | Sodwana Bay, S. Africa | D. Pavlic | *EU821898* | *EU821838* | *EU821868* |
| *N. cordaticola* | CMW14056 | CBS123635 | *Syz. cordatum* | Kosi Bay, S. Africa | D. Pavlic | *EU821903* | *EU821843* | *EU821873* |
| *N. eucalypticola* | CMW6217 | CBS115766 | *E. rossii* | Tidbinbilla, N.S.W., Australia | M. J. Wingfield | *AY615143* | *AY615127* | *AY615135* |
| *N. eucalypticola* | **CMW6539** | CBS115679 | *E. grandis* | Orbost, Victoria, Australia | M. J. Wingfield | *AY615141* | *AY615125* | *AY615133* |
| *N. eucalyptorum* | CMW6233 | CBS15768 | *E. nitens* | Canberra, N.S.W., Australia | M. J. Wingfield | *AY615138* | *AY615122* | *AY615130* |
| *N. eucalyptorum* | CMW10125 | CBS115791 | *E. grandis* | Mpumalanga, S. Africa | H. Smith | *AF283686* | *AY236920* | *AY236891* |
| *N. kwambonambiense* | **CMW14023** | CBS123639 | *Syz. cordatum* | Kwambonambi, S. Africa | D. Pavlic | *EU821900* | *EU821840* | *EU821870* |
| *N. kwambonambiense* | CMW14123 | CBS123643 | *Syz. cordatum* | Kwambonambi, S. Africa | D. Pavlic | *EU821924* | *EU821864* | *EU821894* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *N. luteum* | CMW9076 | BOT2482 | *Malus* × *domestica* | Kemeu, New Zealand | S. Pennycook | *AY236946* | *AY236922* | *AY236893* |
| *N. luteum* | **CBS110299** | | *V. vinifera* | Quinta do Marquês, Oeiras, Portugal | A. Phillips | *AY259091* | *DQ458848* | *AY573217* |
| *N. macroclavatum* | **CMW15955** | CBS118223, WAC12444 | *E. globulus* | Denmark, W.A. | T. Burgess | *DQ093196* | *DQ093206* | *DQ093217* |
| *N. macroclavatum* | CMW15948 | WAC12445 | *E. globulus* | Denmark, W.A. | T. Burgess | *DQ093197* | *DQ093208* | *DQ093218* |
| *N. mediterraneum* | CBS121558 | PD311 | *Olea europaea* | Lepre, Scorrano, Italy | C. Lazzizera | *GU799463* | *GU251835* | *GU799462* |
| *N. mediterraneum* | **CBS121718** | CPC13137, PD312 | *Eucalyptus* sp. | Rhodes, Greece | P. Crous, M. J. Wingfield, A. Phillips | *GU251176* | *GU251836* | *GU251308* |
| *N. nonquaesitum* | **CBS126655** | PD484 | *Umbellularia californica* | St. Helena, Napa, California, U.S.A. | F. Trouillas | *GU251163* | *GU251823* | *GU251295* |
| *N. nonquaesitum* | PD301 | B62–07 | *Vaccinum corymbosum* cv. Elliot | Río Negro, Osorno, X Region, Chile | E. Briceño, J. Espinoza, B. Latorre | *GU251164* | *GU251824* | *GU251296* |
| *N. parvum* | CMW8313 | CM6 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | N. Geldenhuis | KF886710 | KF886719 | KF886733 |
| *N. parvum* | CMW18662 | B9 | *Sch. parahyba* var. *amazonicum* | Pretoria, Gauteng, S. Africa | B. Hinze | KF886711 | KF886720 | KF886734 |
| *N. parvum* | CMW18671 | B18 | *Sch. parahyba* var. *amazonicum* | Pretoria, Gauteng, S. Africa | B. Hinze | KF886712 | KF886721 | KF886735 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *N. parvum* | CMW19379 | BT02A | *Sch. parahyba* var. *amazonicum* | Nelspruit, Mpumalanga, S. Africa | B. Hinze | KF886713 | | KF886736 |
| *N. parvum* | CMW19813 | BT03E | *Sch. parahyba* var. *amazonicum* | Nelspruit, Mpumalanga, S. Africa | B. Hinze | KF886714 | KF886722 | KF886737 |
| *N. parvum* | **CMW9081** | ICMP8003, ATCC58191 | *Populus nigra* | TePuke/BP, New Zealand | G. Samuels | *AY236943* | *AY236917* | *AY236888* |
| *N. parvum* | CBS110301 | CAP074 | *V. vinifera* | Palmella, Portugal | A. Phillips | *AY259098* | *EU673095* | *AY573221* |
| *N. pennatisporum* | **MUCC510** | WAC13153 | *Allocasuarina fraseriana* | Yalgorup, W.A. | K. Taylor | *EF591925* | *EF591959* | *EF591976* |
| *N. protearum* | MUCC497 | | *Santalum acuminatum* | Yalgorup, W.A. | K. Taylor | *EF591912* | *EF591948* | *EF591965* |
| *N. ribis* | CMW7772 | CBS115475 | *Ribes* sp. | New York, U.S.A. | B. Slippers/G Hudler | *AY236935* | *AY236906* | *AY236877* |
| *N. ribis* | CMW7773 | | *Ribes* sp. | New York, U.S.A. | B. Slippers/G Hudler | *AY236936* | *AY236907* | *AY236878* |
| *N. umdonicola* | CMW4692 | BOT528 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | M. J. Wingfield | KF886715 | KF886723 | KF886738 |
| *N. umdonicola* | CMW8314 | CM9 | *Sch. parahyba* var. *amazonicum* | Buenos Aires, Esmeraldas, Ecuador | N. Geldenhuis | KF886716 | KF886724 | KF886739 |
| *N. umdonicola* | **CMW14058** | CBS123645 | *Syz. cordatum* | Kosi Bay, S. Africa | D. Pavlic | *EU821904* | *EU821844* | *EU821874* |
| *N. umdonicola* | CMW14106 | CBS123644 | *Syz. cordatum* | Sodwana Bay, S. Africa | D. Pavlic | *EU821905* | *EU821839* | *EU821875* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *N. viticlavatum* | **CBS112878** | STE-U5044 | *V. vinifera* | Stellenbosch, Western Cape, S. Africa | F. Halleen | *AY343381* | | *AY343342* |
| *N. viticlavatum* | CBS112977 | STE-U5041 | *V. vinifera* | Stellenbosch, Western Cape, S. Africa | F. Halleen | *AY343380* | | *AY343341* |
| *N. vitifusiforme* | CMW18655 | B2 | *Sch. parahyba* var. *amazonicum* | Pretoria, Gauteng, S. Africa | B. Hinze | KF886717 | KF886725 | KF886740 |
| *N. vitifusiforme* | CMW18666 | B13 | *Sch. parahyba* var. *amazonicum* | Pretoria, Gauteng, S. Africa | B. Hinze | KF886718 | KF886726 | KF886741 |
| *N. vitifusiforme* | CBS110880 | STE-U5050 | *V. vinifera* | Stellenbosch, Western Cape, S. Africa | J. van Niekerk | *AY343382* | | *AY343344* |
| *N. vitifusiforme* | **CBS110887** | STE-U5252 | *V. vinifera* | Stellenbosch, Western Cape, S. Africa | J. van Niekerk | *AY343383* | | *AY343343* |

[1]W.A. - Western Australia

[2]S. Africa – South Africa

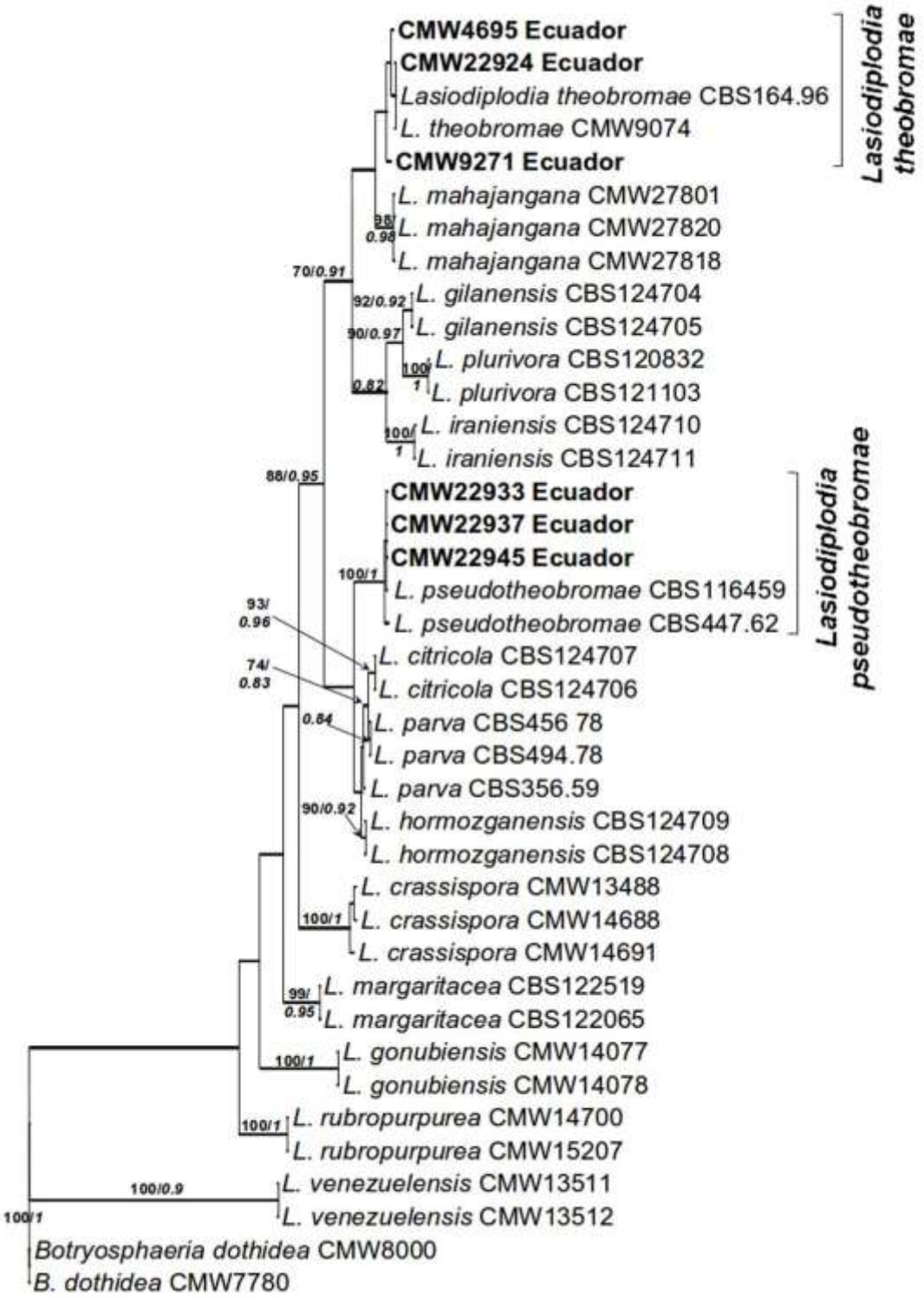


Fig 2. One of 5 most parsimonious trees of 312 steps obtained from the analysis of the combined ITS and TEF-1α datasets for isolates grouping within the *Lasiodiplodia* genus. Bootstrap values (≥ 70 %) resulting from MP analysis (non-italicized) and ML analysis (italicized) are indicated above the branches. Isolates obtained from *S. parahyba* var. *amazonicum* trees indicated in boldface. The trees is rooted with two isolates of *Botryosphaeria dothidea*.

For the *Neofusicoccum* analyses, the ITS dataset consisted of 491 characters (65 parsimony informative, 408 constant, 18 parsimony uninformative), and yielded 1000 most parsimonious trees (TL=126, CI=0.6111, RI=0.8533, RC=0.5215). The model selected for ML analysis was TIM1 (gamma=0.118). The TEF-1α dataset consisted of 277 characters (88 parsimony informative, 181 constant, 8 parsimony uninformative), and yielded 1000 most parsimonious trees (TL=145, CI=0.7793, RI=0.9266, RC=0.7221). The model selected for ML analysis was K80 (ti/tv=2.3854, gamma=0.531). The BT2 dataset consisted of 430 characters (61 parsimony informative, 350 constant, 19 parsimony uninformative), and yielded 28 most parsimonious trees (TL=92, CI=0.75, RI=0.9119, RC=0.6839). The model selected for ML analysis was HKY (ti/tv=3.5071, gamma=0.175). The combined analysis consisted of 1198 characters (206 parsimony informative, 943 constant, 49 parsimony uninformative), and yielded 1000 most parsimonious trees (TL=372, CI=0.6586, RI=0.8483, RC=0.5587) (Fig 3). The model TrN (gamma=0.184) was selected for ML analysis. The PHT value was 0.001.

The topologies of the trees resulting from the MP and ML analyses undertaken on the individual and combined loci were similar for both genera. However, clades representing individual species were not clearly defined and collapsed under one locus, but were evident when the other loci analyzed were visually examined.

Based on culture morphologies and sequence data, five species could be distinguished. Isolates of *N. parvum* originated from Ecuador (n = 4) and from both regions sampled in South Africa; Pretoria, Gauteng (n = 15) and Nelspruit, Mpumalanga (n = 8). Isolates of *N. vitifusiforme* were obtained only from trees in Pretoria, Gauteng (n = 5). Isolates of *L. theobromae* (n = 56), *L. pseudotheobromae* (n = 3) and *N. umdonicola* (n = 2) were isolated from trees in Ecuador only.

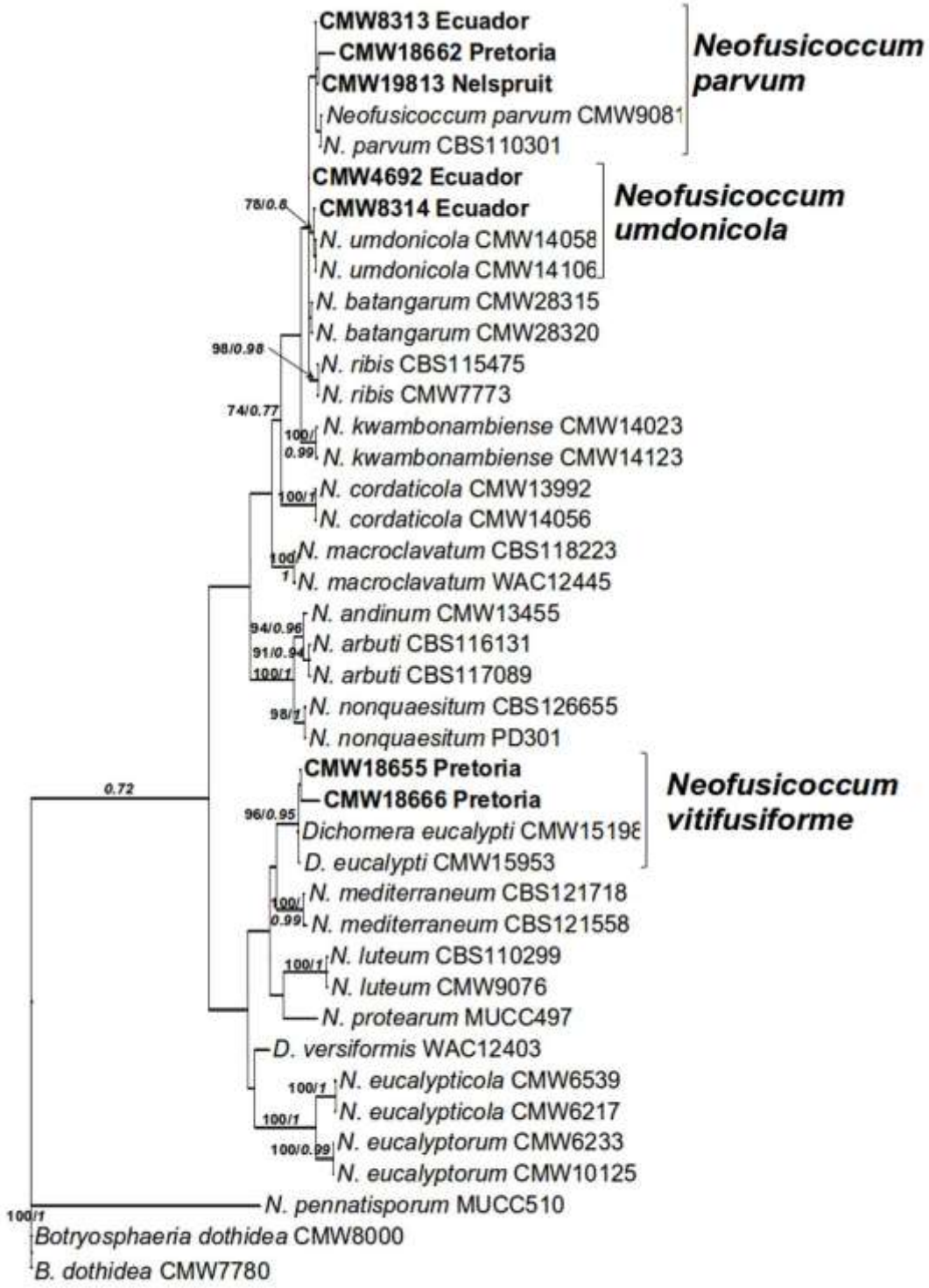


Fig 3. One of 1000 most parsimonious trees of 372 steps obtained from the analysis of the combined ITS, BT2 and TEF-1α datasets for isolates grouping within the *Neofusicoccum* genus. Bootstrap values (≥ 70 %) resulting from MP analysis (non-italicized) and ML analysis (italicized) are indicated above the branches. Isolates obtained from *S. parahyba* var. *amazonicum* trees indicated in boldface. The trees is rooted with two isolates of *Botryosphaeria dothidea*.


Fig 4. Lesions resulting from the pathogenicity trials conducted in South Africa and Ecuador. a) Lesions produced on 7-year old trees in the third trial conducted in South Africa. From left to right: Control inoculation, CMW18655, CMW18659 (both *N. vitifusiforme*), CMW18666 and CMW19380 (both *N. parvum*). b) Control inoculation on an 8-year old tree in Ecuador. c) Lesion produced by CMW4695 (*L. theobromae*) on an 8-year old tree in Ecuador.

Table 2. Summary of mean lesion lengths (mm) ($\alpha = 0.05$) and associated standard errors, measured after six weeks, for pathogenicity trials conducted in South Africa and Ecuador. Means are significantly different at P = 0.05 and were determined using Fisher's least significant differences test.

| Region | Trial | Isolate | Identity | Mean ± SE |
|---|---|---|---|---|
| South Africa | 1 | Control | | 17.398 ± 2.465 a |
| | | CMW18653 | *Neofusicoccum vitifusiforme* | 28.985 ± 3.494 ab |
| | | CMW18660 | *N. parvum* | 54.597 ± 6.068 c |
| | | CMW18662 | *N. parvum* | 30.988 ± 3.609 b |
| | | CMW18666 | *N. parvum* | 40.078 ± 4.559 b |
| | 2 | Control | | 2.491 ± 0.525 a |
| | | CMW18655 | *N. vitifusiforme* | 6.217 ± 1.339 ab |
| | | CMW18659 | *N. vitifusiforme* | 7.461 ± 1.469 b |
| | | CMW18666 | *N. parvum* | 5.187 ± 1.008 ab |
| | | CMW19380 | *N. parvum* | 39.245 ± 2.567 c |
| | 3 | Control | | 3.063 ± 0.187 a |
| | | CMW18655 | *N. vitifusiforme* | 6.596 ±1.180 ab |
| | | CMW18659 | *N. vitifusiforme* | 10.444 ± 1.645 b |
| | | CMW18666 | *N. parvum* | 8.637 ± 1.009 b |
| | | CMW19380 | *N. parvum* | 24.416 ± 2.842 c |
| Ecuador | 1 | Control | | 18.000 ± 1.030 a |
| | | CMW4695 | *L. theobromae* | 40.167 ± 3.628 c |
| | | CMW4697 | *L. theobromae* | 36.900 ± 1.955 bc |
| | | CMW8313 | *N. parvum* | 41.250 ± 3.080 c |
| | | CMW8314 | *N. umdonicola* | 30.700 ± 1.262 b |

### 3.2. Pathogenicity tests

All stem inoculations resulted in lesions (Fig 4). Data generated fitted a normal distribution based on the results from the Shapiro-Wilk test (data not shown). All isolates were pathogenic to *S. parahyba* trees (Table 2).

Comparisons of isolate data between the South African inoculation trials showed that none of the data could be combined, probably due to the different age classes of trees inoculated. Each trial was, therefore, analyzed separately. For the first trial conducted in South Africa, the three isolates of *N. parvum* produced lesions that differed significantly in length from the control inoculations ($F_{1, 48}$ = 22.67, $P$ = 1.81 × $10^{-5}$). For the second and third South African trials, one isolate of *N. parvum* (CMW19380) and one isolate of *N. vitifusiforme* (CMW18659) produced significantly different lesion lengths relative to the control inoculations (second trial: $F_{4, 29}$ = 92.86, $P$ = 4.25 × $10^{-16}$, third trial: $F_{4, 26}$ = 19.82, $P$ = 1.37 × $10^{-7}$) (Table 2). Re-isolations done from inoculated stems resulted in the isolation of the species inoculated in all but two cases. It seems possible in these cases that the isolate inoculated was outcompeted, most likely by secondary saprophytes. No isolates of the Botryosphaeriaceae were obtained from the control inoculations.

For the trial conducted in Ecuador, the lesion lengths of isolates of *N. parvum*, *N. umdonicola* and *L. theobromae* were similar (Table 2). Isolates of *L. pseudotheobromae* were not included in the inoculation trial because they were morphologically indistinguishable from other isolates of *L. theobromae* and the trial preceded the recognition of *L. pseudotheobromae* as a distinct species. There was no opportunity to repeat this trial with additional isolates.

## 4. Discussion

Different species of the Botryosphaeriaceae are associated with die-back of *S. parahyba* var. *amazonicum* trees in Ecuador and two regions of South Africa. Four species were isolated from diseased trees in Ecuador, namely *N. parvum*, *N. umdonicola*, *L. theobromae* and *L. pseudotheobromae*. In South Africa, only *N. parvum* and *N. vitifusiforme* were isolated, with *N. vitifusiforme* found only in Pretoria. Prior to this study, only an unknown species of *Physalospora* Niessl. (VIÉGAS 1944; HANLIN 1992) and *L. theobromae* had been associated with diseased *S. parahyba* var. *amazonicum* trees in Brazil (TREMACOLDI et al. 2009). It is possible that the *Physalospora* sp. identified by VIÉGAS (1944) is the same fungus as *L. theobromae* as the taxonomy of these fungi was confused for a considerable period of time (ALVES et al. 2008; PHILLIPS et al. 2013).

*Neofusicoccum parvum* was isolated from *S. parahyba* var. *amazonicum* trees at all three sites and is reported for the first time from Ecuador. The species was the dominant taxon associated with trees in South Africa, comprising 82.1 % of the isolates. In contrast, it comprised only 6.2 % of the isolates from Ecuador. In South Africa, *N. parvum* has been reported from diseased plantation forestry trees (*Eucalyptus* spp.), grapevines (*Vitis vinifera*), native *Heteropyxis natalensis* and *Syzygium cordatum*, and non-native *Sequoia gigantea*, *Terminalia catappa* and *Tibouchina urvilleana* (SLIPPERS et al. 2004a, b; VAN NIEKERK et al. 2004; PAVLIC et al. 2007; BEGOUDE et al. 2010; HEATH et al. 2011). The species has a broad distribution in the country, having been reported from six of the nine provinces. Furthermore, it has a high level of genetic variation within South Africa, strengthening the argument that it might be native to this country (PAVLIC et al. 2009b, SAKALIDIS et al. 2013). The discovery of this pathogen on another ornamental, non-native tree species extends the known host range of this pathogen in South Africa,

and supports the hypothesis that it is native to the country.

*Neofusicoccum umdonicola*, a cryptic species closely related to *N. parvum*, was isolated only from trees in Ecuador, albeit at a low frequency (3.1 %). This species was recently recognized as a distinct species in the *N. parvum-ribis* species complex based on congruence between several gene phylogenies (PAVLIC et al. 2009a, b). *Neofusicoccum umdonicola* had previously only been reported from *Syz. cordatum* trees along the east coast of South Africa (from Kosi Bay down to Gonubie) and from Panama (close to Ecuador), from ungerminated seed (PAVLIC et al. 2009a, b; SAKALIDIS et al. 2013). The species seemingly occupies subtropical areas.

*Neofusicoccum vitifusiforme* was isolated only from *S. parahyba* var. *amazonicum* trees in Pretoria, Gauteng. This fungus was first discovered on grapevines in the Western Cape Province of South Africa (VAN NIEKERK et al. 2004). It has since been reported from plum and peach trees in South Africa (DAMM et al. 2007), rotten olive drupes in Italy (LAZZIZERA et al. 2008) and from blueberry seedlings in China (KONG et al. 2010). Interestingly, it is clear that *N. vitifusiforme* is associated with plants under cultivation, and has not been reported from any native trees in these areas. Plum, peach, olive and blueberry trees are all cultivated for their fruit, and *S. parahyba* var. *amazonicum* trees are cultivated as ornamentals. However, this might be due to less intensive sampling efforts on native trees compared to agricultural or horticultural trees.

The discovery of *L. theobromae* associated with *S. parahyba* var. *amazonicum* trees in Ecuador was not surprising as the fungus was originally described from *Theobroma cacao* in the same country (PATOUILLARD and DE LAGERHEIM 1892). It occurs in tropical and subtropical regions globally (ALVES et al. 2008) and is dominant in Ecuador, comprising 86.2 % of the isolates. Its

absence amongst isolates from trees in Nelspruit, South Africa was surprising, as this is a subtropical area, and it has previously been reported from pines (*Pinus* spp.) and kiaat (*Pterocarpus angolensis*) trees in the province (MOHALI et al. 2005; MEHL et al. 2011). A larger sample size might well have revealed it at a lower frequency in this region.

*Lasiodiplodia pseudotheobromae* was isolated from *S. parahyba* var. *amazonicum* trees in Ecuador, but at a low frequency (4.6 %). Its occurrence in Ecuador is documented here for the first time, although it has been reported from Suriname, Uruguay and Costa Rica in South and Central America (ALVES et al. 2008; PÉREZ et al. 2010). Initially described from five hosts in four countries, the fungus is a sibling species of *L. theobromae* (ALVES et al. 2008). There are minor morphological differences between both species, specifically regarding conidial size and shape (ALVES et al. 2008), necessitating sequence data to establish the identity of cultures. It is possible that many disease reports linked to *L. theobromae* from past literature actually concern infections by *L. pseudotheobromae*, or that some isolates of the latter species occur amongst the former, as shown in this study. In other areas such as Cameroon, China, Iran, Madagascar, and South Africa, both species occur together in relative abundance (ABDOLLAHZADEH et al. 2010; BEGOUDE et al. 2010, 2011; CHEN et al. 2011; MEHL et al. 2011).

Data from the pathogenicity trials showed that all isolates inoculated into *S. parahyba* var. *amazonicum* trees produced lesions. Lesion lengths differed significantly from control inoculations, irrespective of the age of trees inoculated. Differences in aggressiveness amongst isolates of the same species were noted. In the case of isolates of *N. parvum* in the South African trials, these differences were sometimes significant. This was not surprising as large differences in the aggressiveness of isolates of the same species have been noted before amongst the

Botryosphaeriaceae (PAVLIC et al. 2007; STANOSZ et al. 2007; MOHALI et al. 2009; MEHL et al. 2011). Results of these and other pathogenicity trials conducted in Ecuador (M.J. Wingfield, unpublished data) suggest that these fungi can have significant effects on the health of *S. parahyba*, regardless of the age at which trees are infected.

Results of this study have shown that the assemblages of the Botryosphaeriaceae associated with diseased trees in Ecuador and South Africa partially overlap. This overlap is solely due to the occurrence of *N. parvum* in both countries. Although *N. parvum* is the dominant taxon associated with *S. parahyba* var. *amazonicum* trees in the areas sampled in South Africa, it is eclipsed in Ecuador by *L. theobromae*, probably because of the subtropical climate in that area. Both restrictive climate and competitive exclusion by a single dominant species have previously been noted as potential explanations for why particular species of the Botryosphaeriaceae occur in or are absent from a particular region (SLIPPERS and WINGFIELD 2007; SAKALIDIS et al. 2013).

All the species isolated from *S. parahyba* var. *amazonicum* trees in this study occur on multiple continents and it is likely that some have been introduced into the areas sampled. This is evidenced by the low isolation frequencies of *L. pseudotheobromae*, *N. parvum* and *N. umdonicola* in Ecuador and the occurrence of *N. vitifusiforme* in Pretoria. Potential introductions of these fungi may have occurred as a result of introducing infected germplasm into either Ecuador or South Africa, either that of *S. parahyba* var. *amazonicum* or of other host species that were established close to these trees. The ability of the Botryosphaeriaceae to remain quiescent within infected germplasm has already resulted in unintented introductions of these fungi into novel areas (SLIPPERS and WINGFIELD 2007; SAKALIDIS et al. 2013). Quarantine measures that restrict or limit the importation of this material would undoubtedly reduce the incursions of both new species and of

genotypes of already established species, and would facilitate better management and control of diseases produced by the Botryosphaeriaceae.

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