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Diversity, phylogeny and pathogenicity of Botryosphaeriaceae on non-native *Eucalyptus* grown in an urban environment: A case study

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ABSTRACT

The Botryosphaeriaceae are opportunistic pathogens mostly of woody plants, including *Eucalyptus*. These fungi can cause cankers and die-back diseases on non-native *Eucalyptus* trees in South African plantations. Botryosphaeriaceae were isolated from diseased and asymptomatic twigs and leaves from 20 *Eucalyptus* spp. grown in a Pretoria, South Africa arboretum and its surroundings. The isolates were initially grouped based on conidial morphology and Internal Transcribed Spacer (ITS) rDNA PCR-RFLP profiles. They were further identified using DNA sequence data for the ITS rDNA and translation elongation factor 1-α (TEF-1α) gene regions and tested for pathogenicity. Five species were identified including *Botryosphaeria dothidea* and four *Neofusicoccum* species namely *Neofusicoccum parvum*; *N. cryptoaustrale* and *N. ursorum* that were recently described from plant tissues collected as a part of the current study; and *Neofusicoccum eucalypti* (Winter) Maleme, Pavlic & Slippers comb. nov. The latter species is recorded for the first time on *Eucalyptus* in South Africa. Most of the identified species were collected from the leaves of 17 different *Eucalyptus* spp. *Neofusicoccum parvum* was most commonly isolated (72% of all isolates) followed by *B. dothidea* species complex (17%). With exception of *N. parvum* which was isolated from majority of *Eucalyptus* spp. the other species were isolated from limited number of *Eucalyptus* species indicating host-preferences. All the isolated Botryosphaeriaceae species produced lesions on inoculated *Eucalyptus grandis* plants that were significantly larger than those associated with the controls.

1. Introduction

The Botryosphaeriaceae (Botryosphaeriales, Dothideomycetes) are among the most common fungi associated with diseases of trees and shrubs in both native and non-native environments worldwide (Slippers and Wingfield, 2007; Slippers et al., 2009). These fungi are typically associated with symptoms such as branch and stem cankers, die-back as well as leaf and tip blights. Species of Botryosphaeriaceae commonly exist in asymptomatic plant tissues as endophytes or latent pathogens, causing disease symptoms at the onset of stressful environmental conditions (Slippers and Wingfield, 2007; Mehl et al., 2013). Their cryptic nature as endophytes combined with increasing occurrences of extreme weather conditions due to climate change makes these fungi threatening to economically and environmentally important woody plants globally (Desprez-Loustau et al., 2006).

The taxonomy of the Botryosphaeriaceae has been confused in the past. Identification was commonly achieved based on morphological characteristics or the host plants on which species were found. The many overlapping morphological characteristics among different species of the Botryosphaeriaceae and the fact that some morphological features change with age has also resulted in a substantially misleading taxonomy for these fungi. Recent taxonomic studies, combining morphological characters and multigene phylogenies, have led to extensive revisions of the taxonomy of the Botryosphaeriaceae (Crous et al., 2006; Liu et al., 2012; Phillips et al., 2013; Slippers et al., 2013). Based on these analyses, the identities of Botryosphaeriaceae species known from culture has been revised, and they have been placed in 17 genera currently recognised in this family (Phillips et al., 2013; Slippers et al., 2013).

Eucalyptus trees have been planted as non-natives in many parts of the world, including South Africa. It has been previously suggested that the global movement of these trees has also resulted in the introduction of pathogens into new areas via planting stock or seed (Wingfield et al., 2001, 2015). In this regard, species of Botryosphaeriaceae have been

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found on the seeds of *Eucalyptus* and other tree species (Lupo et al., 2001; Gure et al., 2005). Their association with seeds and their presence in asymptomatic plant tissues provides evidence that species of Botryosphaeriaceae can be expected to be easily moved unnoticed into new areas together with *Eucalyptus* (Slippers and Wingfield, 2007; Slippers et al., 2009).

Species of Botryosphaeriaceae have wide host ranges and they can move between native and introduced tree species (Slippers and Wingfield, 2007; Sakalidis et al., 2011). For example, no restrictions to gene flow between non-native *Eucalyptus globulus* plantations and native eucalypt forests in Western Australia could be found in the canker pathogen *Neofusicoccum australe* (Burgess et al., 2006). Similarly, all species of Botryosphaeriaceae identified from the native *Syzygium cordatum* in South Africa, were found to be more pathogenic on *Eucalyptus*, with a several of these species overlapping in occurrence between the two hosts (Pavlic et al., 2007). Consequently, *Eucalyptus* can be expected to acquire new species of Botryosphaeriaceae from the surrounding trees in a new area, and to provide a source of species to native plant communities.

An arboretum of 20 different *Eucalyptus* spp. has been established in Pretoria, South Africa, in 2001, to provide a food-source for Koala Bears at the nearby National Zoological Gardens of South Africa (www.nzg.co.za). Canker and die-back symptoms were observed on these trees and an attempt was made to identify and characterize species of Botryosphaeriaceae on these trees, as well as on apparently healthy *E. camaldulensis* trees growing near the arboretum. This was achieved using (ITS) rDNA PCR-RFLP profiles, DNA sequence data for the ITS rDNA and translation elongation factor 1-α (TEF-1α) gene regions of cultures isolated from these trees. Inoculations were also conducted to consider the pathogenicity of the identified species.

2. Materials and methods

2.1. Isolates

Isolations were made from 20 *Eucalyptus* spp. in the Pretoria Zoological Garden (www.nzg.co.za) arboretum in Pretoria, South Africa (Fig. 1a), as well as from surrounding eucalypt trees planted as ornamentals in the area (Table 1). The arboretum consisted of 12 blocks, each of them having 20 rows (each row representing one *Eucalyptus* species) of 11 trees. Three trees (Tree 1, 5 and 10 of each row) were sampled from three of the blocks (block 1, 6 and 7), thus having in total 9 trees sampled per each *Eucalyptus* sp. In addition, twenty-five *Eucalyptus camaldulensis* trees surrounding the arboretum were sampled. Twig die-back on terminal leader shoots (Fig. 1a) and main stem cankers, identified as cracks in the bark exuding kino (Fig. 1b, c), were observed on approximately 10% of these trees. Cankers were spread widely on the trunks of some trees that appeared reddish in colour due to the extensive production of kino, indicating variation in susceptibility between *Eucalyptus* spp. (Fig. 1b). The trees were sampled during March and April 2005. Isolations were made from diseased and asymptomatic (visually healthy) twigs, and from asymptomatic leaves collected from 205 trees, using the protocol described by Pavlic et al. (2004). All the resulting cultures are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

2.2. Morphological characteristics

Isolates were induced to sporulate on sterilized pine needles (Smith et al., 2001) placed on 2% water agar (WA) (Agar; Biolab, South Africa) and incubated at 25 °C under near-UV light. Pycnidia formed on pine needles after two to three weeks of incubation. Masses of conidia oozing from the pycnidia were mounted in 85% lactic acid on microscopic slides and examined using a light microscope. Images were captured using an HRc Axiocam digital camera and accompanying software (Carl Zeiss Ltd., Munich, Germany). Conidia (20–50) and 50 conidiogenous cells were measured for each isolate. Colony morphology and colour were noted for cultures grown on 2% malt extract agar (MEA) (Biolab, South Africa) at 25 °C and culture colours were defined using the colour charts of Rayner (1970). Growth studies were conducted for selected isolates at temperatures ranging from 10 to 35 °C at 5 °C intervals in the dark. Two dishes were prepared for each isolate and two measurements of colony diameter perpendicular to each other were made daily until growth reached the edges of the 90 mm plates.

2.3. DNA extraction and PCR amplification

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

The ITS region was amplified using primers ITS 1 and ITS 4 (White et al., 1990) and a portion of the TEF-1α gene region, was amplified with primer set EF-AF and EF-BR (Sakalidis et al., 2011). The reaction mixture contained 2.5 units of *Taq* polymerase (Roche Molecular Biochemicals, Almeda, California), 10× PCR buffer with $MgCl_2$ (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 0.2 mM dNTPs and 10 mM of each primer. The reaction mixture was made up to the final volume of 25 µl with sterile water. The following PCR program was used: denaturation at 94 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing temperature at 55 °C for 45s, elongation at 72 °C for 1½ min and a final elongation step at 72 °C for 5 min. The PCR amplicons were viewed on a 1% agarose gel, stained with ethidium bromide under UV-light. To estimate the band sizes, a 100 bp marker XIV (Roche Molecular Biochemicals, Almeda, California) was used.

2.4. PCR-RFLP analysis

A PCR-RFLP technique was used on the ITS amplicons to group all the isolates resembling Botryosphaeriaceae based on culture morphology. ITS rDNA amplicons were digested with the restriction enzymes (RE) *Hha*I that recognises the same sequences as *Cfo*I that had been previously used to distinguish species of the Botryosphaeriaceae (Slippers et al., 2007). The PCR-RFLP reaction mixture for each reaction consisted of 20 µl PCR product, 0.3 µl RE *Hha*I and 2.5 µl of the matching enzyme buffer (Fermentas, South Africa). The reaction mixture was incubated at 37 °C overnight. Digested fragments were separated on a 3% agarose gel run at a low voltage (60 V) for 1 h.

2.5. DNA sequencing and phylogenetic analysis

Representative isolates from each of three groups identified based on PCR-RFLP analyses were sequenced using the primers that were used for the PCR amplification. The sequences were compared to those of known Botryosphaeriaceae obtained from GenBank, with a focus on those previously isolated from *Eucalyptus* (Table 1). Sequencing of the purified products was carried out by using ABI PRISM 3100™ automated DNA sequencer (Perkin-Elmer). Nucleotide sequences were analysed and edited using SEQUENCE NAVIGATOR version 1.0.1. (Perkin- Elmer Applied Bio-Systems, Foster City, CA) software. Online software, MAFFT version 7 under E-INS-i algorithm was used for alignments (Katoh and Standley, 2013). Maximum likelihood analyses using 10,000 rapid bootstrap inferences (command f–a) under the