Two new Fusicoccum species from Acacia and Eucalyptus in Venezuela, based on morphology and DNA sequence data

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ABSTRACT

Botryosphaeria spp. are common endophytes of woody plants, and they also include some serious pathogens of Eucalyptus and Acacia species. Numerous anamorphs have been associated with Botryosphaeria, of which the species Fusicoccum are amongst the most common. Here, we characterize two new Fusicoccum species, isolated from Eucalyptus and Acacia trees in Venezuela, based on morphological features in culture and comparisons of DNA sequence data. The two taxa named Fusicoccum andinum and F. stromaticum spp. nov, reside in two well-supported clades (BS values = 100 %) based on a combined data set of the ITS of the rDNA operon and translation elongation factor 1-α (EF1-α) gene sequences. The conidia of F. andinum are unusually large amongst Botryosphaeria anamorphs, and peripherally resemble those of B. mamane and B. melanops. F. stromaticum is characterized by large conidiomata in cultures, growth at 35 °C and slightly thickened conidial walls, which is different to most other Fusicoccum spp. No teleomorphs were observed for these fungi, but DNA sequence data show that they are anamorphs of Botryosphaeria.

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Introduction

The genus Botryosphaeria is cosmopolitan and its species occur on a wide range of monocotyledonous, dicotyledonous, and gymnosperm hosts. Botryosphaeria spp. infect the stems, branches and twigs, leaves of many woody plants, and they have also been found in the stems of grasses and thalli of lichens (Barr 1987). These fungi include opportunistic pathogens that give rise to symptoms such as such shoot blights, stem cankers, fruit rots, die-back, and gummosis (von Arx 1987).

The taxonomy of Botryosphaeria has been confused for many years. This is mainly due to the similar morphology of the teleomorphs (Jacobs & Rehner 1998; Slippers et al. 2004a). Host association has been used to assign names to species, but this has led to confusion because some species are host specific, whilst others are generalists (Jacobs & Rehner 1998; Crous & Palm 1999; Smith et al. 2001; Smith & Stanosz 2001; Slippers et al. 2004a, d).

The anamorphs of Botryosphaeria species are generally encountered in culture and on diseased plant parts. For this reason, identification of Botryosphaeria spp. has commonly been based on conidial morphology of the anamorphs (Jacobs & Rehner 1998; Smith & Stanosz 2001; Smith et al. 2001; Phillips et al. 2002; Slippers et al. 2004a, d).

Conidial characters considered to be useful for the taxonomic delimitation of Botryosphaeria anamorphs are size, colour, septation, wall thickness and texture, as well as the presence of microconidia and mode of conidiogenesis (Sutton...
lates have also been deposited in CBS (Utrecht). University of Pretoria, South Africa, and representative isolates from the Forestry and Agricultural Biotechnology Institute (FABI), used in this study are maintained in the collection (CMW) of cool-white fluorescent light to induce sporulation. All isolates were incubated for 3–6 wk at 25°C on water agar (WA; 2 % Biolab agar, Midrand, South Africa) with sterilized pine needles placed on the agar surface and these were incubated for 1 min. Small tissue pieces (4–5 mm) were cut from the plant tissue and placed on 2 % malt extract agar (MEA; DIFCO, Becton Dickinson, MD) and incubated at 25°C.

For isolations, plant tissues were surface disinfested with 70 % ethanol for 30 s and thereafter rinsed in sterile water for 1 min. Small tissue pieces (4–5 mm) were cut from the plant tissue and placed on 2 % malt extract agar (MEA; DIFCO, Becton Dickinson, MD) and incubated at 25°C. Cultures resembling Botryosphaeria spp. were transferred to water agar (WA; 2 % Biolab agar, Midrand, South Africa) with sterilized pine needles placed on the agar surface and these were incubated for 3–6 wk at 25°C under a combination of near-UV and C14 light. The sizes of the PCR amplicons were estimated using DNA molecular weight marker XIV (100 bp ladder) (Roche BioSystems, Foster City, California).

**Sequence analysis**

In all 27 isolates were used in the phylogenetic analysis (Table 1). BLAST searches were done to determine whether any related sequences are present in GenBank, but none were found that were more closely related to the test isolates than those chosen for comparison here. The trees were rooted to sequence data of an isolate of a Bionectria sp., which was included as an outgroup taxon in the analysis of 30 ingroup taxa.

PCR amplicons were purified and sequenced as described in Slippers et al. (2004a), except that products were run on an ABI PRISM 3100 automated sequencer (Perkin-Elmer Applied BioSystems, Foster City, California, USA). Sequence data were analysed using Sequence Navigator version 1.0.1 (Perkin-Elmer Applied BioSystems, Foster City, California, USA) and manually aligned by inserting gaps. Gaps were treated as a fifth character and all characters were given equal weight. Phylogenetic analyses were done using PAUP version 4.0b8 (Swofford 1999). Heuristic searches were done using random stepwise addition tree bisection and reconstruction (TBR) as branch swapping algorithm for the construction of maximum parsimonious trees. One thousand BS replicates (Felsenstein 1985) were run to determine the confidence intervals of branching points on the shortest tree. Branches with a length of zero were collapsed and all multiple equally parsimonious trees were saved. Levels of homoplasy (retention and consistency indices) (Hillis & Huelsenbeck 1992) were determined.

**Materials and methods**

**Fungal isolation**

A survey was conducted during 2003 in plantations of Eucalyptus urophylla, an unidentified Eucalyptus sp., a Eucalyptus hybrid, and Acacia mangium. Isolations were made from twigs, stems and branches displaying symptoms of blue stain, dieback and from dead trees. Single conidial isolates were obtained after cultures were induced to sporulate on water agar to which sterile pine needles had been added.

For isolations, plant tissues were surface disinfested with 70 % ethanol for 30 s and thereafter rinsed in sterile water for 1 min. Small tissue pieces (4–5 mm) were cut from the plant tissue and placed on 2 % malt extract agar (MEA; DIFCO, Becton Dickinson, MD) and incubated at 25°C. Cultures resembling Botryosphaeria spp. were transferred to water agar (WA; 2 % Biolab agar, Midrand, South Africa) with sterilized pine needles placed on the agar surface and these were incubated for 3–6 wk at 25°C under a combination of near-UV and cool-white fluorescent light to induce sporulation. All isolates used in this study are maintained in the collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa, and representative isolates have also been deposited in CBS (Utrecht).

**DNA isolation and amplification**

DNA was extracted from isolates of unknown identity (Table 1) using the technique described by Slippers et al. (2004a). The quantification of nucleic acids was made using a spectrophotometer (Eppendorf, Hamburg) with a ratio of absorbance at 260 nm and 280 nm.

The DNA extraction was used as template to amplify part of the nuclear rDNA operon in PCR reactions using the primers ITS1 and ITS4 (White et al. 1990). The amplified fragments included the 3′ end of the small subunit (SSU) rDNA gene, ITS1 region, the complete 5.8 S rRNA gene, the ITS2 region and the 5′ end of the LSU rRNA gene. A part of the EF1-α was amplified using the primers EF1-728F and EF1-986R (Carbone et al. 1999). The PCR reaction mixtures and conditions were described in Slippers et al. (2004a), except that the PCR annealing temperatures varied between 52–60°C for EF1-α region.

Extracted DNA (20–25 ng μl−1) was used as template in the reactions. PCR amplicons were separated on 1.5 % (w/v) agarose gels, stained with ethidium bromide and visualized under UV-light. The sizes of the PCR amplicons were estimated using DNA molecular weight marker XIV (100 bp ladder) (Roche Molecular Biochemicals, Alameda, California).

In all 27 isolates were used in the phylogenetic analysis (Table 1). BLAST searches were done to determine whether any related sequences are present in GenBank, but none were found that were more closely related to the test isolates than those chosen for comparison here. The trees were rooted to sequence data of an isolate of a Bionectria sp., which was included as an outgroup taxon in the analysis of 30 ingroup taxa.

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**Morphological characterization**

Conidial morphology was studied using a light microscope with an Axiocam digital camera and software to analyse photographs (Carl Zeiss, Jena). Sections through some of the pycnidia and stomatal structures were made with a Leica CM100 cryostat (Leica, Wetzlar). Length, breadth, shape and colour of the conidia were recorded after mounting in clear lactophenol. At least 50 conidia of each isolate of two different Fusarium spp. were measured.

The growth of selected isolates was determined by placing mycelial discs (5 mm diam) at the centres of MEA plates, with
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<th>Other</th>
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a Culture collections and isolates abbreviations: CMW, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria; CBS, Centraalbureau voor Schimmelcultures, Utrecht; ICMP, International Collection of Microorganisms from Plants, Auckland, NZ; BRIP, Plant Pathology Herbarium, Department of Primary Industries, QL; KJ, Jacobs and Rehner (1998); isolates CMW 7999 and CMW 7772 are ex-type isolates.

b Identities determined in this study.
three replicate plates for each of three isolates for each of the two morphologically different Fusicoccum spp. Plates were incubated at temperatures ranging from 15–40 ºC at 5 ºC intervals. Two diameter measurements were taken perpendicular to each other after 4 d for each colony, and averages computed. Colony colours were determined using the colour charts of Rayner (1970).

Results

Phylogenetic analyses

The partition homogeneity test indicated that the ITS-rDNA (547 characters) and EF1-α (340 characters) sequence partitions were congruent and that the data sets could be combined (P = 0.440). This resulted in a final data set of 887 characters after alignment of which 284 characters were constant, 165 variable characters were parsimony uninformative and 426 were parsimony informative. Heuristic search analysis in PAUP of the sequence data resulted in one tree (CI = 0.753; RI = 0.857; HI = 0.247).

The isolates in the tree obtained from the combined data sets resided in 13 principal clades (I–XIII) (Fig 1). Isolates in clades I–X all have hyaline and thin-walled conidia and are thus Fusicoccum-like anamorphs. In contrast, isolates residing in clades XI–XIII all have pigmented and thick-walled conidia which can be referred to as Diplodia-like anamorphs (Denman et al. 2000; Slippers et al. 2004a). Isolates from Venezuela resided in clade I and clade IX were distinct from all other clades that included known Botryosphaeria spp. (Fig 1). The Fusicoccum sp. from Venezuela residing in clade I and B. dothidea (Slippers et al. 2004a) in clade X were each strongly supported (100 % BS) and distinct from other Botryosphaeria spp. with Fusicoccum-like anamorphs. The Fusicoccum sp. from Venezuela residing in clade IX (100 % BS support) was closely related to B. parva and B. ribis (clade V, II, III), B. eucalyptorum (clade IV), B. eucalypticola (clade V), B. australis (clade VI), B. lutea (clade VII) and F. mangiferum (clade VIII) (Phillips et al. 2002; Slippers et al. 2004b, c, d). B. obtusa, B. stevensii and B. rhodina (clades XI, XII, XIII), all with Diplodia-like anamorphs, formed a well-defined group (Alves et al. 2004; Punithalingam 1976).

Morphological characters

The two unknown Fusicoccum spp. from Acacia and Eucalyptus in Venezuela and residing in distinct clades in the phylogenetic trees, produced conidiomata on sterilized pine needles at 25 ºC on WA after 3 wk.

The colonies of Fusicoccum sp. isolated from Eucalyptus and Acacia in Portuguesa and Cojedes state (Table 1), grew rapidly at 30 ºC, but produced little or no growth at extremes of 15 ºC and 40 ºC. This fungus produced few, but large conidiomata, on MEA. The conidia were hyaline, aseptate, bacilliform, and had thin to slightly thickened walls (Fig 2).

Colonies of the Fusicoccum sp. isolated from Eucalyptus spp. growing on the mountains in Merida state (Table 1), grew at 15 ºC with an optimum growth temperature of 20–30 ºC. Abundant pycnidia were produced on MEA at 25 ºC (Fig 3A–B). Conidia were clavate to slightly navicular and large when compared with other Fusicoccum anamorphs (Table 2, Fig 3E–H, see Taxonomy).

Taxonomy

Based on conidial morphology, cultural characteristics and DNA sequence phylogeny we conclude that the two Fusicoccum spp. from Eucalyptus and Acacia in Venezuela represent undescribed taxa. We thus provide the following descriptions of them here.

Fusicoccum stromaticum Mohali, Slippers & M. J. Wingf., sp. nov.

Etym.: The name refers to the very large conidiomata on MEA at 25 ºC.

Conidiomata magna in superficie MEA, multilocularia, eucormatica, cum hyphis tecta; loculus omnino inclusus sine ostiolis. Cellulae conidiogenae hyalinae, holoblasticae, cylindraceae, conidium unicum apicale effereentes, primo holoblastice, dein enteroblastice. Conidia hyalina, parietibus tenuibus vel subincarcassatis, non septata, granularia, bacilliformia, apice basique obtusa rotundata vel obtusa, (19–)20–23–(24) × (4–)5–6 μm.


Cultures fluffy, greenish olivaceous (23 “b”) (surface) and olivaceous (21”k”) (reverse) after 15 d on MEA at 25 ºC (Fig 2A). Colonies reaching 70–75 mm diam on MEA after 4 d in the dark at 25 ºC. Cardinal temperatures for growth; min. 15 ºC (little or no growth), max. 40 ºC (no growth), optimum 30–35 ºC. Conidiomata large, superficial on MEA (Fig 2A–B), multilocular, eucormastic, covered with hyphae; locule totally embedded without ostioles, locule walls consisting of a dark brown texture angularis, becoming thinner and hyaline towards the conidiogenous region (Fig 2C). Conidiogenous cells hyaline, holoblastic, smooth, cylindrical, producing a single apical conidium, the first conidium produced holoblastically and subsequent conidia produced enteroblastically (Fig 2D), (10–)11–15–(17) × (1.5–)2–3 μm (average of 50 conidiogenous cells 13 × 2.5 μm, 1b 5.33). Conidia hyaline, thin to slightly thickened walled, aseptate, granular, bacilliform, straight to slightly curved, apex and base both bluntly rounded or just blunt (Fig 2E), (19–)20–23–(24) × (4–)5–6 μm (average of 50 conidia 21.5 × 5.5 μm, 1b 4.01).

Teleomorph. Not observed, but expected to be a Botryosphaeria sp. based on phylogenetic analyses.


Fusicoccum andinum Mohali, Slippers & M. J. Wingf., sp. nov.

Etym.: Refers to the region where the species was isolated. Cordillera of Los Andes.

Pycnidia superficiales, copiosae in superficie MEA ad 25 ºC facta, solitaria vel botryosa, stromaticata, globosa, pariete pycnidii et textura angularis brunnea. Cellulae conidiogenae hyalinae, holoblasticae,

Typus: Venezuela: Merida State: Merida; Mucuchies (3140 m), Cordillera of Los Andes, on branches of Eucalyptus sp., Feb. 2003, S. Mohali (PREM 58238 - holotypus; cultura viva CBS 117453).

Cultures fluffy and flat becoming pale olivaceous grey (21""""d) (surface) and olivaceous buff (21""""d) (reverse) 15 d after inoculation on MEA at 25 °C (Fig 3A), producing columns of mycelium reaching the Petri dish lid after 30 d at 25 °C. Colonies reaching 80 mm diam on MEA after 4 d in the dark at 25 °C. Cardinal temperatures for growth were min. 15 °C.
reaching an average 24 mm diam), max. 35 °C (no growth), optimum 20–30 °C. Pycnidia superficial, produced abundantly on MEA surface at 25 °C (Fig 3A), oozing conidia after 30 d at 25 °C on MEA (Fig 3B), solitary or botryose on the colonies, stromatic, globose (Fig 3C–E), (331–)374–597(–740) μm (average of 50 pycnidia 486 × 448 μm, l:b 1.08); pycnidial wall, composed of brown textura angularis, 6–8 cell layers thick. Conidiogenous cells hyaline, holoblastic, smooth, cylindrical, producing a single apical conidium, the first conidium holoblastic and subsequent conidia enteroblastic (Fig 3E), (8–)11–17(–23) (1.5–)2–2.5 (–3) μm (average of 50 conidiogenous cells 14 × 2 μm, l:b 6.62). Conidia hyaline, granular, clavate to slightly navicular, apex obtuse and base truncate, 0–1 septa (Fig 3F–H), (19–)23–31(–40) × (4–)5–6 (–8) μm (average of 50 conidia 27 × 5.5 μm, l:b 4.84).

Teleomorph. Not observed, but expected to be a Botryosphaeria sp. based on phylogenetic analyses.

Additional specimens examined: VENEZUELA, Merida State: Merida, Mucuchies (3140 m) Cordillera of Los Andes, on branches of Eucalyptus sp., Feb. 2003, S. Mohali (PREM 58518, 58519, 58520, 58521, 58522, 58523, 58524, 58525, 58526, 58527, 58528, 58529, 58530, 58531, 58532).

Discussion

Two new Fusicoccum spp. collected in Venezuela have been characterized here, based on both morphology and their unique DNA sequences. One of these, F. andinum, was isolated exclusively from Eucalyptus spp. at high altitude sites, whereas F. stromaticum was from both Eucalyptus spp. and Acacia spp. at lower altitude sites in Venezuela. To the best of our knowledge, these are the first two species of Fusicoccum to be described from Venezuela.

F. andinum was collected from Eucalyptus spp. growing in the Cordillera Los Andes mountains of Venezuela at an altitude of approx. 3000 m. The daily mean temperature of this region is 10 °C, and the extreme environmental conditions most probably explain the low optimum growth temperature of F. andinum in culture. F. andinum grew at 15 °C, had an optimum at 20–30 °C and showed no growth above 35 °C. This is a low optimum temperature for growth when compared with other Botryosphaeria species such as B. dothidea, B. parva, B. ribis, B. mamane, B. corticola, B. lutea, B. eucalyptorum, B. eucalyptica, B. australis and B. protearum (Morgan-Jones & White 1987; Gardner 1997; Denman et al. 1999; Smith et al. 2001; Alves et al. 2004; Slippers et al. 2004a, c, d).

F. andinum was isolated from old Eucalyptus trees, and mainly from asymptomatic branches, without causing apparent damage to trees. We thus assume that the fungus is an endophyte and that it is not pathogenic. This would be consistent with many Botryosphaeria spp. that are known to reside as endophytes in asymptomatic or healthy plant tissues on a non-native host (Fisher et al. 1993; Smith et al. 1996).
different areas or under different environmental conditions, such endophytic species have, however, been considered important pathogens (Fisher et al. 1993; Smith et al. 2001).

Isolates of F. andinum formed a well-defined group based on analyses of sequence data. They are also morphologically distinct. The conidia of this fungus are large when compared with those of other Fusicoccom species. Two other Botryosphaeria spp. with comparatively large conidia are B. mamane (Gardner 1997) and B. melanops (Shear & Davidson 1936), although these are larger than those of F. andinum. Other than the relatively large conidia found in F. andinum, this species can also be distinguished by clavate to slightly navicular conidia. These are different to those of F. andinum, with and without symptoms. These trees were growing in the Portuguesa and Cojedes states 150–200 m. The annual medium temperatures of these regions ranges between 26–30 °C, and this is also consistent with the fungus having a relatively high optimum temperature for growth in culture of 30–35 °C, compared with many other Botryosphaeria spp. (Pennycook & Samuels 1985; Morgan-Jones & White 1987; Gardner 1997; Denman et al. 1999; Smith et al. 2001; Alves et al. 2004).

Isolates of F. stromaticum resided in a well-defined group with strong BS support. This confirmed that the fungus represents the anamorph of an undescribed Botryosphaeria sp. Furthermore, there were three conspicuous morphological characteristics that distinguish this fungus from other Fusicocom spp. F. stromaticum has unusually large conidiomata, it grows at 35 °C, and the conidia have slightly thickened walls.

F. stromaticum was isolated from asymptomatic, as well as dead and dying branches and stems of Eucalyptus spp. and A. mangium trees. The presence of the fungus on asymptomatic tissue suggests that it is an endophyte. In this regard, it is similar to F. andinum described here. Whether F. stromaticum is pathogenic is unknown, as it may have simply been present on the dying tissue as a saprophyte, without necessarily being the cause of the symptoms observed. A. mangium and Eucalyptus spp. are important plantation trees in Venezuela, and pathogenicity tests with this fungus should be conducted to determine its relative importance in tree health.

Isolates of F. andinum and F. stromaticum originated from trees that are not native to Venezuela. These fungi have not been found elsewhere in the world, despite the extensive surveys that have been conducted on Acacia and Eucalyptus spp. (Keane et al. 2000; Slippers et al. 2004b, c), suggesting that these newly described species might be native to Venezuela. However, extensive surveys of native woody plants would be necessary to establish this.

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