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ORIGINAL ARTICLE

Diversity of fungal endophytes from the medicinal plant *Dendropanax arboreus* in a protected area of Mexico

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Abstract With the aim of studying the biodiversity and the biotechnological potential of endophytic fungi associated with the medicinal plant *Dendropanax arboreus*, 45 fungal isolates were recovered from ten plants grown at the "El Cielo" Biosphere Reserve, Mexico. Based on the sequence analysis of internal transcribed spacer (ITS) regions and the observation of morphological traits, the isolates were grouped into 28 genotypes corresponding to 14 genera with a predominance of *Fusarium*, *Phomopsis*, *Alternaria*, and *Colletotrichum* species. Enzymatic activity assays revealed numerous isolates as having xylanase (66.6 %), cellulase (57.1 %), pectinase (51.2 %), and amylase (20.9 %) activities. Only the isolate *Paecilomyces* sp. HER3-5 exhibited chitinase and chitosanase activities, and only the unidentified isolate HET1-5 had phosphate solubilization capacity. Isolates of five fungal genera had antimicrobial activity against at least one among the *Staphylococcus aureus*, *Candida albicans*, and *Candida glabrata* target strains. To the best of our knowledge, this is the first study on the endophytic fungi of *D. arboreus*, and provides evidence that: (1) endophytes commonly produce enzymes associated with the colonization process (xylanases, cellulases, and pectinases), while enzymes associated with pathogenic infection (amylases) or phosphate solubilization were relatively rare; (2) isolates of the genera *Corynespora*, *Endomelanconiopsis*, and *Thozetella* are potential sources of novel antimicrobial compounds; and (3) distinctive endophytic fungal communities occur in different plant tissues (the root, trunk, and leaf), but this was less evident in the sampling sites (elevation).

Keywords Angelica tree · Antimicrobial · Diversity · ITS · Microbiota · Phylogeny

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Introduction

Endophytes are microorganisms (often bacteria or fungi) living within plant tissues, at least for part of their life cycles, without causing visible symptoms of disease. Endophytes usually exert profound effects on plant ecology, fitness, and evolution (Brundett 2006) through several different mechanisms, including induction of resistance to pathogens or heavy metals, improvement of nutrient supplies (e.g., by nitrogen-fixing or phosphate solubilization), enhancement of plant growth by producing phytohormones, and synthesis of compounds with biological activity, such as antibiotics, agrochemicals, and immunosuppressants (Ryan et al. 2008; Rodríguez et al. 2009; Aly et al. 2011).

Dendropanax arboreus (L.) Decne. & Planch, common name Angelica tree, belongs to the family Araliaceae. It is an evergreen canopy tree (14–25 m in height), distributed from Mexico to South America at elevations of up to 1500 m.a.s.l. (Figueroa-Esquivel et al. 2010). It is a traditional medicinal plant used frequently in Mexico and Latin America

for the treatment of fever, snakebites, and intestinal parasites (Bourdy et al. 2000). Falcarindiol, a compound having protective effects against certain types of cancer in animal models, has been identified in extracts of this tree (Setzer et al. 2000). Other compounds found in this plant with structures related to falcarindiol may be of potential use as antineoplastic agents. The traditional medicinal uses of this plant make it an attractive potential resource in the search for endophytic fungi able to produce novel bioactive compounds, as some previous studies have already revealed (Lv et al. 2010; Aly et al. 2011).

Since endophytes have significant potential as sources of novel plant-growth promoters and novel bioactive compounds, and given that no study has yet reported on the endophytes of *D. arboreus*, we characterized 45 endophytic fungi recovered from this plant. The objectives of this study were to: (1) evaluate the diversity of endophytic fungi associated with different tissues of plants grown at two distinct altitudes; (2) study the activity of enzymes related to the colonization of fungi in plants, such as cellulase, xylanase, pectinase, chitinase, and chitosanase; and (3) study the phosphate solubilizing capacity and the antimicrobial activity of the endophytic fungi.

Materials and methods

Sampling of plant tissues

The Biosphere Reserve "El Cielo" is located in the Northeast part of Mexico, near the town of Gomez Farias in the Tamaulipas state, and comprises 2400 km^2 between 22°55′–23°30′N and 99°02′–99°30′W at elevations ranging from 200 to 2200 m.a.s.l. "El Cielo" has unique biodiversity in flora and fauna due to the existence of four different ecosystems within the reserve: tropical rainforest, cloud mountain forest, pine-oak forest, and desert scrub. The two sampling sites were located within the tropical rainforest region at 500 m.a.s.l. (site 1) and 1000 m.a.s.l. (site 2), respectively, and separated by 5 km along a small road near the Gomez Farías village. Eighty samples per plant tissue (entire leaves, pieces of trunk bark, and of root bark; 240 samples in total) were collected in September 2009 from randomly selected healthy plants (five plants/site) without visible symptoms of necrosis, chlorosis, or parasites. All the plants had stem diameters >20 cm at breast height. The samples were stored in plastic bags kept on ice, transported to our laboratory within 24 h, and stored at 4 °C for 1 or 2 days before their use for fungal isolation. This sampling strategy was used in view of the relatively low genetic variation among populations of this tree in Mexico, and because 91.5 % of that genetic variation is attributable to individual plant differences within populations (Figueroa-Esquivel et al. 2010).

Isolation of fungi

Endophytic fungi were isolated according to Photita et al. (2001). Briefly, leaves and portions of the trunk bark and root bark, which had been previously peeled to eliminate the rough outer layer, were sliced into 2×2 cm pieces. Subsequently, tissue pieces were immersed for 30 s in 96 % ethanol (v/v), then for 6 min in sodium hypochlorite solution (1 % w/v, 1:5 dilution of Clorox regular bleach), and washed six times with sterile water. To test the sterilization efficiency, 0.1 mL of the final washing water was placed in Petri dishes containing potato dextrose agar (PDA) medium [4 g potato infusion, 20 g dextrose, 15 g agar, 1 L distilled water, (pH 5.5)]. Eight pieces of each tissue from each plant were placed evenly in Petri dishes (four pieces/plate) containing PDA medium supplemented with streptomycin (50 mg L^{-1}) and incubated at 28 °C for 5–10 days. The plates were checked daily until mycelia were observed. To purify the fungal isolates, small agar plugs were sliced from the leading edge of each colony, transferred to new PDA plates, and further incubated under the same conditions. This procedure was repeated as necessary, until isolates were morphologically clean. The pure isolates were stored on PDA slants at 4 °C and kept as living vouchers in our laboratory. The fungal isolation rate (IR) was determined as the ratio of the number of isolates recovered from the plant tissue to the total number of pieces laid in Petri dishes (80) of the tissue.

Morphological and molecular identification of the fungi

Distinctive morphotypes were grouped according to phenotypic characteristics on PDA. Microscopic examination of conidia was performed in microcultures grown in PDA at 28 °C for 14 days. When possible, isolates were identified according to published taxonomy systems. For example, the system of Seifert for the genera in the Class Hyphomycetes, which relies on the examination of colony morphology, diffusible pigments, conidia production, and microscopic observations of microcultures (Seifert et al. 2011; more references for other genera are listed in Supplementary Table S1). Conidia production was observed microscopically with lactophenol cotton blue staining (Seifert et al. 2011).

Total genomic DNA was extracted from 14-day-old fungal cultures according to Allers and Litchen (2000), as modified by Rodriguez Tovar et al. (2005). The DNA was dissolved in sterile water and stored at -20 °C. The internal transcribed spacer region (ITS rDNA) containing the ITS1, 5.8S, and ITS2 regions was PCR-amplified using the primers ITS1 (Fw 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (Rv 5′-TCC TCC GCT TAT TGA TAT GC-3′) (Gardes and Bruns 1993). The reaction mixture (50 μL) contained 50 ng genomic DNA, 20 pmol of each primer, 1× *Taq* buffer, 3 mM

$MgCl_2$, 0.2 mM dNTP, and 1 U *Taq* polymerase (Thermo Scientific, Waltham, MA). The PCR reactions comprised a DNA denaturation step at 94 °C for 10 min, followed by 35 cycles of 1 min at each temperature: 94 °C, 54 °C, and 72 °C, and a final extension step at 72 °C for 7 min. The amplified products were examined by electrophoresis in 1.0 % (w/v) agarose gel in 0.5× TBE buffer (Green and Sambrook 2012) and purified using a PCR DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) according to manufacturer's instructions. The amplicons were bidirectionally sequenced with an ABI 3100 Analyzer (Applied Biosystems, Foster City, CA) in the UBIPRO platform FES-IZTACALA of the National Autonomous University of Mexico. All the sequences acquired in this study have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers JQ716993 through JQ717331 and KF668285.

The ITS rDNA sequence data was used in phylogenetic reconstruction to estimate the taxonomic placement and for genotype designation of the recovered isolates. BLASTn searches of ITS rDNA sequence data in the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to guide taxon sampling for the phylogenetic analysis. All sequences were edited manually with BIOEDIT (Hall 1999) and aligned using CLUSTALX v1.7 software (Thompson et al. 1997). The phylogenetic tree was constructed by the maximum-likelihood method using PhyML software (http://www.atgc-montpellier.fr/phyml; Guindon and Gascuel 2003). The best model for sequence evolution was selected with jModeltest 3.06 software (Posada 2008) based on Akaike information criterion. The model of Tamura Nei (TrN) + I (invariant proportion) + Gamma (G) was selected. The parameters were α=0.6610, A=0.2274, C=0.2838, G=0.2381, and T=0.2507 for the gamma distribution. The statistical validation at each node was determined by 500 bootstrap replicates. *Saccharomyces cerevisiae* was used as the outgroup for tree rooting.

Definition of ITS genotypes and diversity estimation

Sequencher 4.1 software (Gene Codes, Ann Arbor, MI) was used to delimit groups at 90 %, 95 %, 97 %, and 99 % ITS rDNA sequence similarity, with an expectation of at least 40 % sequence overlap for global delimitation. Genotypes defined at 97 % ITS similarity were considered as species, based upon comparisons between phylogenetic relationship, and identity was assigned on the basis of morphological traits (Socca-Chafre et al. 2011). Species accumulation curves, bootstrap estimates of total richness, and the Fisher's, Shannon, and Simpson's diversity indices were inferred with EstimateS v8.2 (Colwell 2005).

In vitro antimicrobial activity

Five mycelial plugs (0.5×0.5 cm) were taken from agar culture incubated at 28 °C for 7 days on PDA and inoculated into a 1 L Erlenmeyer flask containing 200 mL Czapek malt broth (15 g malt extract, 30 g sucrose, 1 g KH_2PO_4, 2 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 0.010 g $FeSO_4$, and distilled water to 1 L total volume). Following incubation at 28 °C with agitation (120 rpm) for 3 weeks, the flask content was filtered and the mycelium-free supernatant was extracted three times (30 min each time with agitation at 120 rpm) with 200 mL dichloromethane to obtain an organic phase (culture extract). The mycelia were frozen in liquid nitrogen, crushed to obtain a white powder, and extracted three times with 200 mL dichloromethane/methanol (1:1) mixture (Calcul et al. 2013). The supernatant and the mycelial extracts were separately concentrated under vacuum at 45 °C to obtain a powder, which was weighed and dissolved in dimethylsulfoxide (DMSO) at a final concentration of 100 mg mL^{-1}. The solutions were stored at −20 °C until assayed.

Aliquots (50 μL) of the concentrated extracts (100 mg mL^{-1}) were used to evaluate the antimicrobial activity by the Kirby-Bauer method (Bauer et al. 1966). The target strains were the Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 35218, the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212, and the opportunistic pathogen yeasts *Candida glabrata* CBS138 and *Candida albicans* ATCC 10231. Aliquots of 20 μL penicillin (50 μg mL^{-1}) or amphotericin B (50 μg mL^{-1}) were used as positive controls for the antimicrobial activity test against the target bacteria and fungi, respectively, while 50 μL of the dissolvent (DMSO) was used as a blank control for the same tests.

Enzymatic activities and phosphate solubilization

The assays for amylase, pectinase, chitinase, and chitosanase activities were performed in Castañeda medium: 0.156 g KH_2PO_4, 0.093 g Na_2CO_3, 0.093 g NaCl, 0.062 g $MgSO_4 \cdot 7H_20$, 4.5 g Noble agar in 250 mL distilled water. The substrate concentrations (w/v) were 2 % starch, 1 % pectin, 10 % chitin, and 5 % chitosan. The cellulase and xylanase activities were tested on Congo Red Agar: 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 2 g gelatine, 18 g Noble agar, 0.02 g Congo red, and 1.8 g cellulose or xilan in 1 L distilled water. The solubilization of inorganic phosphate was determined on Pikovskaya medium: 10 g $Ca_3(PO_4)_2$, 5 g $(NH_4)_2SO_4$, 0.5 g KCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 20 g Noble agar in 1 L distilled water. A PDA plug (2×2 mm) containing 1-week-old mycelia of fungal isolate was used to inoculate the plates containing medium corresponding to each assay, and then plates were incubated at 28 °C for 3–5 days.

Amylase activity was determined by the presence of a clear zone around a colony after immersing the fungal colonies in iodine solution (1 %, w/v). Positive pectinase activity was recognized by the presence of a clear ring around a colony following immersion in cethyl trimetyl ammonium bromide solution (5 %, w/v). A positive degradation of chitin, chitosan, cellulose, and xylan, as well as phosphate solubilization, was identified by the presence of a clear or semitransparent ring around a given colony.

Statistical analysis

Principal component analysis (PCA) was performed with the Software ade4 (Dray and Dufour 2007) in R ® (Development Core Team 2015; https://www.r-project.org/) to assess the correspondence among the genotypes (28 levels), plant tissues (three levels: leaf, trunk, and root), and sampling sites (two levels: 500 m.a.s.l. and 1000 m.a.s.l.) as the variables. A multivariate analysis (MANOVA) was made to evaluate the distinctive fungal communities among the plant tissues and between the sampling sites. MANOVA was performed using distance matrices and the software 'vegan' (Oksanen et al. 2015) in R.

Results

Isolation and morphological identification of fungi

Overall, 45 endophytic fungal isolates (Table 1) were recovered from 240 pieces of plant tissues. Among these fungi, 13 were from leaves (IR=0.162), seven from trunk bark (IR= 0.087), and 25 from root bark (IR=0.312). Sampling site 1 contributed 23 isolates, including 3 from leaves, 7 from stem bark, and 13 from root bark, whereas site 2 provided 22 isolates, of which 10 were from leaves and 12 were from root bark. No fungus was recovered from trunk bark at site 2.

Microscopic observation revealed that 15 isolates were able to form asexual spores (conidia; Fig. 1), whereas the complementary 30 isolates were mycelia sterilia. Based on morphological characteristics, the 15 conidia-producing isolates were identified as members of the genera *Colletotrichum* (2), *Alternaria* (3), *Corynespora* (1), *Fusarium* (7), *Paecilomyces*, and *Stemphylium* (Table 1). Microscopic observations showed 27 hyaline and 18 pigmented mycelia. The morphological features of the 15 conidia-producing isolates are shown in Supplementary Table S1.

Molecular identification and diversity of fungi

PCR amplification returned the expected amplicon size (600–900 bp) corresponding to the ITS rDNA for 42 isolates. Since identical sequences were found between the isolates HER3-4 and HER3-4P, and between HEH4-1 and HEH7-1, only 40 ITS sequences were deposited in the GenBank database. Due to unknown reasons, amplification failed in three isolates (HETI-5, HER8-1, and HER8-5). In the phylogenetic tree, the 42 isolates were identified into 28 genotypes with 97 % sequence similarity (Fig. 2, Table 1). Upon comparison with the ITS sequences extracted from the GenBank database (90 named and 14 unnamed), all of the isolates were identified as *Ascomycetes* belonging to 14 genera in nine orders: *Botryosphaeriales*, *Capnodiales*, *Chaetosphaeriales*, *Diaporthales*, *Eurotiales*, *Glomerellales*, *Hypocreales*, *Magnaporthales*, and *Pleosporales* (Fig. 2, Table 1). *Phomopsis* (16 isolates) and *Fusarium* (seven isolates) were the most abundant groups, while one to three isolates presented in each of the other 12 genera.

The definitions of 28 genotypes in our endophytic fungi captured 76 % of the fungal species in *D. arboreus* (species accumulation curve available as Supplementary Fig. S1), suggesting that more fungal species may be recovered from *D. arboreus* with further isolation efforts. The species richness of 39.43, together with the diversity indices of Shannon (3.19), Simpson (26.29) and Fisher (46.49), also indicated the existence of diverse endophytic fungi in this tree.

Enzymatic and antimicrobial activities

Table 2 shows the enzymatic activities found in the fungi recovered from *D. arboreus*. A significant number of the isolates showed activity by xylanase (66.6 %), cellulase (57.1 %), pectinase (51.2 %), and amylase (20.9 %). Only the isolate *Paecilomyces* sp. HER3-5 showed chitinase and chitosanase activities, and only the unnamed strain HET1-5 was able to solubilize inorganic phosphate. *Stemphylium* sp. HER7-1 and two unidentified isolates, HER8-1, HER8-5, were not included in Table 2, since they grew too poorly in the medium (Czapek malt broth) for enzymatic assays and did not present antimicrobial activity.

Antimicrobial activity against at least one of the target strains, *S. aureus* ATCC 25923, *C. albicans* ATCC 10231, and *C. glabrata* CBS138, was found in culture extracts of nine isolates belonging to *Phomopsis*, *Paraconiothyrium*, *Corynespora*, *Endomelanconiopsis*, *Thozetella*, and a *Dothideomycetes* isolate (Table 2). None of the culture extracts inhibited *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, or *E. faecalis* ATCC 29212. In addition, none of the extracts obtained from mycelia inhibited the growth of any of the target microorganisms.

Correspondence analysis

The correspondence analysis (Fig. 3) represented 58.5 % of the variance of the data (see Supplementary Table S2 for details). Considering all of the isolates, the distribution of

Table 1 Endophytic fungi isolated from different tissues of *Dendropanax arboreus* and their relevant information

Isolate no.[a]	Phylogenetic relative (name, sequence accession number)	Identity (%)	Morphological identification	Genotype[b]	Identification
Pleosporales					
HEH6-1, HEH6-I, HEH9-0	*Alternaria alternara*, GU594744	90-92	*Alternaria* spp.	E/H	*Alternaria* spp.
HET1-3	*Corynespora citricola*, FJ852594	89	*Corynespora* sp.	AB	*Corynespora* sp.
HET1-1	*Paraconiothryrium brasilense*, EU295634	89	*Montacnulaceae*	AA	*Paraconiothryrium* sp.
HET1-2	Pleosporales, GQ923982	94	Not identified	V	"*Paraconiothryrium*" sp. I
HEH4-1, HEH7-1	*Pyrenochaeta cava*, AY8553248	90/90	Not identified	G	*Pyrenochaeta* sp.
HER7-1	*Stemphylium vesicarium*, GU065719	93	*Sthemphylium* sp.	Y	*Stemphylium* sp.
Diaporthales					
HET4-1, HER0-1	*Diaporthe helianthi*, AY746005	96	Not identified	D	*Phomopsis* sp. I
HER8-2	*Diaporthe helianthi*, AY746005	90	Not identified	M	*Phomopsis* sp. II
HEH10-1	*Phomopsis* sp. CML 1935,	96	Not identified	I	*Phomopsis* sp. III
HER1-1, HER4-2, HER4-3, HER3-3, HER4-4, HER5-1, HER6-3	Fungal endophyte (AF373049)	95-97	Not identified	A	"*Phomopsis*" sp. IV
HER9-1	Endophytic Diaporthales AF373049	92	Not identified	U	"*Phomopsis*" sp. V
HEH3-II, HEH6-II, HET2-3	*Phomopsis* sp. EU715618/ EU715618	96	Not identified	B	*Phomopsis* sp. VI
HER8-4	*Phomopsi*s sp. EU715618	94	Not identified	S	*Phomopsis* sp. VII
Hypocreales					
HER2-1	*Fusarium oxysporum* HQ691412	94	*Fusarium* sp.	O	*Fusarium* sp. I
HER2-2, HER3-1, HER6-1	*Fusarium oxysporum* HQ691412	98	*Fusarium* sp.	C	*F. oxysporum*
HER6-2	*Fusarium solan*I GU355666	93	*Fusarium* sp.	R	*Fusarium* sp. II
HER9-4	*Fusarium solani* GU355666	92	*Fusarium* sp.	N	*Fusarium* sp. III
HER8-3	*Fusarium* sp. AY729073	92	Not identified	T	*Fusarium* sp. IV
Glomerellales					
HEH5-1	*Colletotrichum gloesporoides* HM222947	97	*Colletotrichum* sp.	K	*Colletotrichum gloesporoides*
HEH5-II	*Colletotrichum gloesporoides* HM222947	90	*Colletotrichum* sp.	J	*Colletotrichum* sp.
HET2-2	*Glomerella septospora* GU935911	97	Not identified	W	*Glomerella septospora*
Capnodiales HER1-1-A	Uncultured Dothideomycetes JF519601	95	Not identified	Z	Dothideomycetes
Magnaporthales HEH3-1	*Mycoleptodiscus indicus* GU980698	89	Not identified	F	*Mycoleptodiscus* sp.
Botryosphaeriales					
HER4-1	*Endomelanconiopsis microspora* EU683655	96	Not identified	Q	*Endomelanconiopsis* sp.
HEH8-10	*Guignardia mangiferae* GU060440	97	Not identified	L	*Guignardia* sp.
Chaetosphaeriales					
HER3-4, HER3-4P	*Thozetella havanensis* EF029184	97	Not identified	X	*Thozetella havanensis*
Eurotiales HER3-5	*Paecilomyces lilacinus* EU553303	96	*Paecilomyces* sp.	P	*Paecilomyces* sp.
Unidentified					
HET1-5, HER8-1, HER8-5	Not amplified		Not identified	Mycelia	Unknown

[a] The first two letters "HE" mean endophytic fungi; the third letter "H, T and R" represent the tissue of origin: leaf, trunk and root, respectively

[b] Genotype was defined based upon the threshold 97 % of the internal transcribed spacer (ITS) sequence similarity

endophytic fungi showed significant correlation with the origin of plant tissues ($P<0.01$), but not to the elevation of the sampling site (Supplementary Table S3). The fungal isolates were clearly divided into three groups (Fig. 3). Group I covered 14 genotypes associated with leaves and trunk bark at site 1, as well as with leaves at site 2. This group included *Paraconiothyrium* sp. (AA), *Corynespora* sp. (AB), *Colletotrichum* sp. (W), *Phomopsis* sp. (D), the *Pleosporales* isolate (V), *Phomopsis* sp. (B), *Colletotrichum* spp. (J, K), *Mycoleptodiscus* (F), *Alternaria* sp. (E), *Pyrenochaeta* sp. (G), *Phomopsis* sp. (H), *Diaporthe* sp. (I), and *Guignardia* sp. (L). Group II comprised six genotypes recovered from root

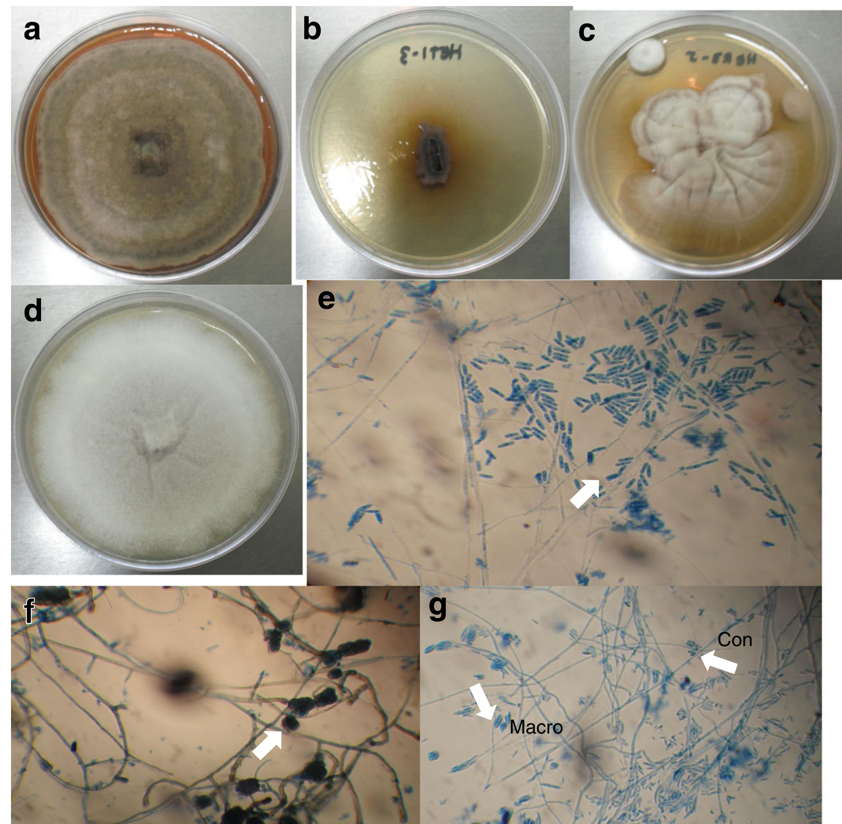

Fig. 1a–g Colony morphology and conidia formed in microculture. **a–d** Colony morphology of *Alternaria* sp. (HEH9-0), *Corynespora* sp. (HETI-3), *Paecilomyces* sp. (HER3-5), and *Fusarium oxysporum* HER2-2. **e–g** Spore morphology of *Colletotrichum* sp. (HEH5-II), *Stemphylium* sp. (HER7-1), and *Fusarium oxysporum* HER3-1. Conidia (Con) and macroconidia (Macro) are marked, respectively

bark at site 2, and included *Fusarium* spp. (M, N, R, T, and U) and *Phomopsis* sp. (S). Group III contained eight genotypes, including *Phomopsis* sp. (A), *Fusarium* spp. © and O), *Paecilomyces* (P), *Endomelanconiopsis* sp. (Q), *Thozetella* (X), *Stemphylium* sp. (Y), and *Dothideomycetes* (Z), which were all associated with root bark at site 1.

Discussion

Diversity of endophytic fungi

Based on morphological traits, ITS rDNA genotypes, and ITS rDNA phylogeny, we studied the taxonomic placement, richness, and diversity of fungal endophytes associated with *D. arboreus* at "El Cielo" Biosphere Reserve. All 45 recovered fungi were *Ascomycetes* belonging to 14 genera, and three unclassified isolates (Table 1, Fig. 2). The predominance of *Ascomycota* found in this study is typical of endophytic mycota (Rubini et al. 2005), which might imply that this fungal phylum has co-evolved with plants (Lane et al. 2000). The species richness and coverage value (about 76 %; Supplementary Fig. S1) attained in this study suggested that this tree harbors a diverse fungal microbiota, similar to those reported previously for other plants (Lv et al. 2010; Larran et al. 2002; Park et al. 2012).

Among the endophytic fungi associated with *D. arboreus*, *Phomopsis*, *Colletotrichum*, *Fusarium*, *Alternaria*, and *Stemphylium* have been described as endophytes and/or plant pathogens (Larran et al. 2002; Arnold and Lutzoni 2007; Lv et al. 2010). Of the abundant genera found in *D. arboreus*, *Phomopsis* (16 isolates), *Fusarium* (7 isolates), *Alternaria*, and *Colletotrichum* were also the predominant endophytic groups found in *Panax ginseng* (Park et al. 2012), another member of the family Araliaceae. However, these dominant endophytic groups were different from those found in wheat (Larran et al. 2002) and wild rubber trees (Gazis and Chaverri 2010). Therefore, some specificity may exist between *D. arboreus* trees and their endophytic fungi. Additionally, fungi, such as *Guignardia* (Rodrigues et al. 2004) and *Thozetella* (Reyes-Estebanez et al. 2011), may be potential biocontrol agents.

Endophytic features and potential importance of the endophytic fungi

In general, xylanase, cellulose, and pectinase are cell-wall degrading enzymes related to the virulence of phytopathogens (Knogge 1996). The occurrence of pectinase, cellulase, and xylanase in both the pathogenic (*Pyrenochaeta*, *Fusarium*, *Colletotrichum*, and *Mycoleptodiscus*) and the non-pathogenic fungi (*Thozetella* strains HER3-4 and HER3-4P) in the present study (Table 2) may imply that these enzymes

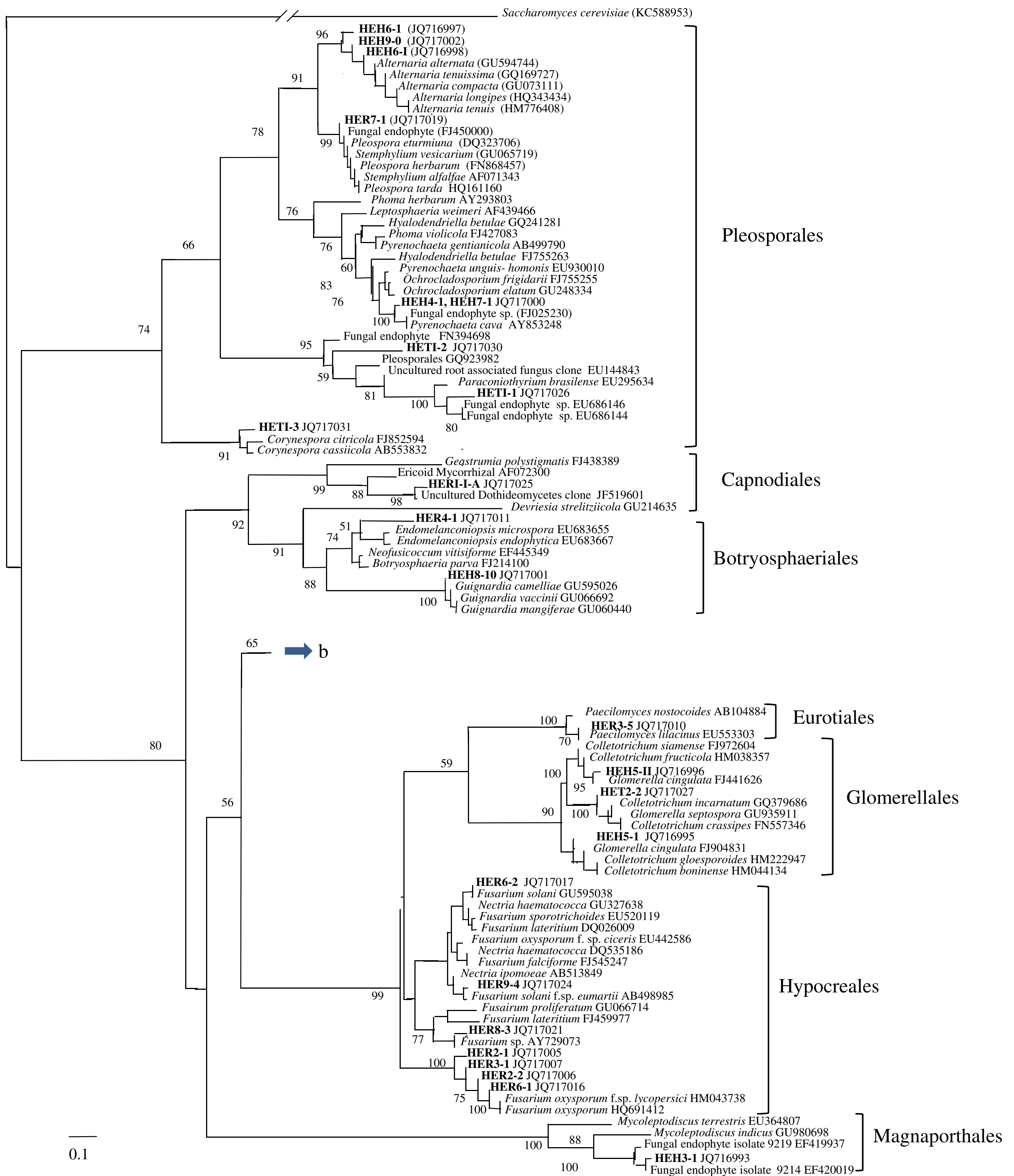

Fig. 2 Maximum-likelihood phylogenetic tree based on internal transcribed spacer (ITS) rDNA data for 42 sequenced *Ascomycetes* (−InL=8778.9459). *Branches* indicate bootstrap support above 70 % (BS>70 %). *Bar* 1 % nucleotide substitutions

are related not only to pathogenicity, but also to endophytic colonization. According to Schulz and Boyle (2005), endophytes secrete cell-wall-degrading enzymes in order to penetrate and colonize plant cells, but without causing infection symptoms, such as induction of callus or papillae in plants. Nevertheless, these enzymes may also help endophytic

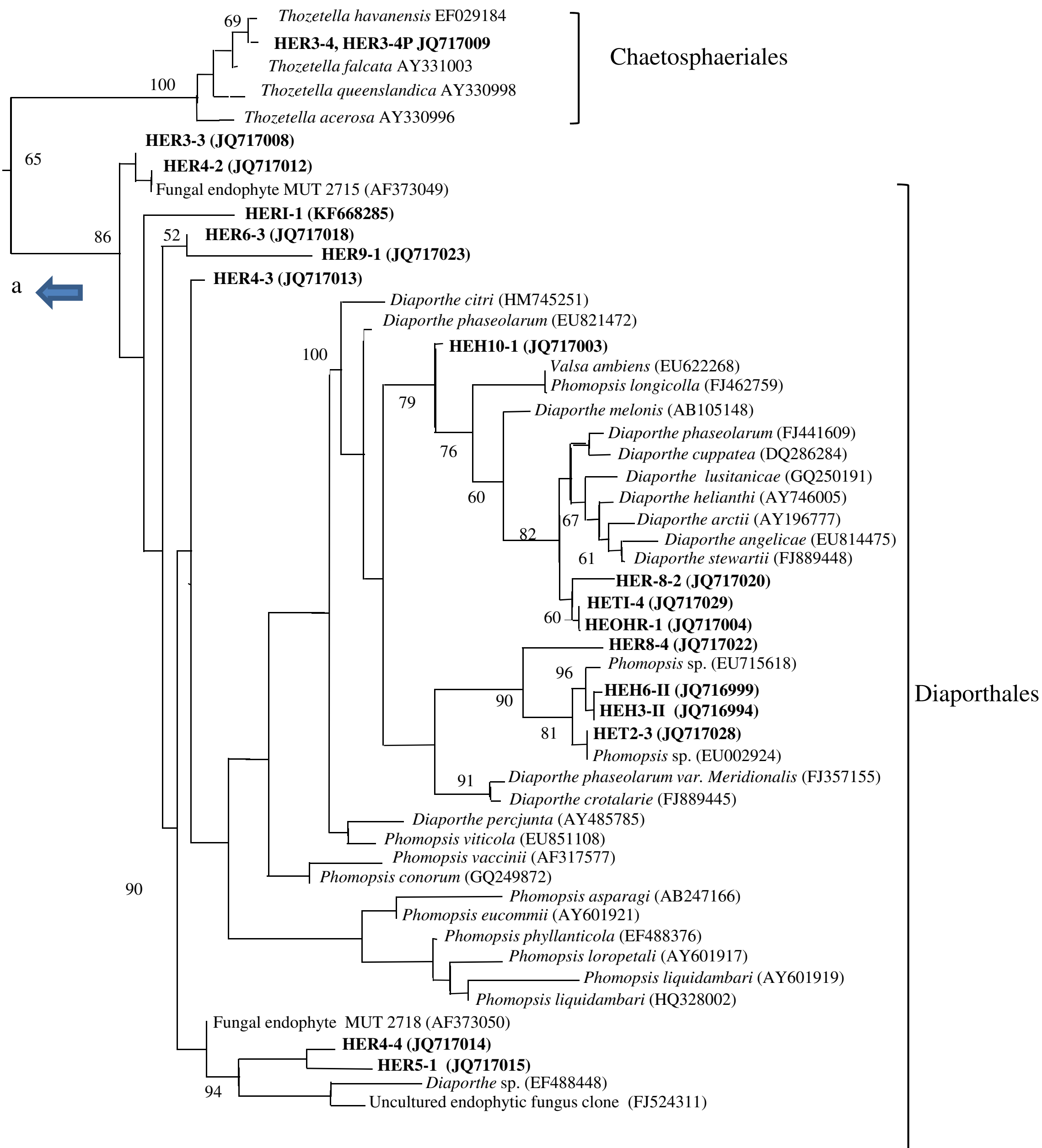

Fig. 2 continued.

fungi become saprophytes when the host plant dies (Promputtha et al. 2007). Amylase activity is associated with starch degradation at the plant senescence stage, whereas phosphate solubilization activity acts as a plant growth promoter (Kuklinsky-Sobral et al. 2004; Alpinia et al. 2012). Since only nine endophytes showed amylase activity and only one isolate (HET1-5) showed phosphate solubilization activity, these activities may not be the determinants of the fungal endophytes of *D. arboreus*.

Chitinases and chitosanases play important roles as biocontrol agents against phytophagous nematodes and pathogenic fungi, and as bioactive compounds for plant production (Govinda Rajulu et al. 2011). The detection of these two enzymes in *Paecilomyces* sp. HER3-5 may be related to the entomopathogenic activities recognized in some species in this genus (Castillo Lopez et al. 2014).

Many novel compounds with distinctive biological activities have been isolated from endophytic fungi (Aly et al. 2011). Since many antibiotics commonly possess other useful biological activities, the search for this activity has been used as a primary screen in several prospecting studies with endophytes (Lv et al. 2010; Aly et al. 2011). In the present study, the detection of antimicrobial activities in the culture extracts of eight isolates (Table 2) determined the potential of searching *D. arboreus* endophytes for bioactive compounds. Some of the species found in this study have been isolated

Table 2 Enzymatic, phosphate solubilization and antimicrobial activities of endophytic fungi. *DMSO* Dimethylsulfoxide

Isolate no.	Enzyme activity of[b]				P solubilization	Antimicrobial activity to[c]		
	Pectinases	Celullases	Xylanases	Amylases		*Staphylococcus aureus*	*Candida albicans*	*Candida glabrata*
Alternaria spp. HEH6-1, HEH9-0	-	+	+	-	-	-	-	-
HEH6-I	-	-	-	-	-	-	-	-
Paraconiothryrium spp. HET1-1, HET1-2[a]	-	+	+	-	-	+	-	-
Pyrenochaeta spp. HEH4-1, HEH7-1	+	+	+	+	-	-	-	-
Corynespora sp. HET1-3[a]	-	-	+	+	-	-	-	++
Phomopsis spp. HER4-2	-	-	-	-	-	-	-	-
HER3-3, HET1-4, HER4-4[a], HER5-1	+	-	-	-	-	+	+	++
HER1-1, HER9-1[a]	-	+	-	-	-	-	+	-
HER4-3, HEH10-1, HEH0-1	-	-	+	-	-	-	-	-
HER8-2	NG	+	+	-	-	-	-	-
HER8-4	-	+	+	-	-	-	-	-
HEH3-II, HEH6-II[a], HET2-3	+	-	+	-	-	-	-	++
HER6-3[a]	NG	NG	NG	NG	NG	+	++	-
Fusarium spp. HER6-1	NG	+	+	-	-	-	-	-
HER6-2, HER9-4, HER2-1	+	+	+	+	-	-	-	-
HER8-3	NG	+	+	-	-	-	-	-
HER3-1	+	+	+	-	-	-	-	-
HER2-2	+	NG	NG	-	-	-	-	-
Glomerella septospora HET2-2	+	-	-	-	-	-	-	-
Colletotrichum spp. HEH5-1, HEH5-II	+	+	+	+	-	-	-	-
Paecilomyces sp. HER3-5	NG	+	+	-	-	-	-	-
Guignardia sp. HEH8-10	-	+	-	+	-	-	-	-
Endomelanconiopsis sp. HER4-1[a]	-	-	+	-	-	+	+	-
Thozetella spp. HER3-4[a], HER3-4P	+	+	+	-	-	-	+	-
Mycoleptodiscus sp. HEH3-1	+	+	+	-	-	-	-	-
Diothidomycetes HER1-1-A[a]	+	-	-	-	-	-	-	+
Unidentified HET1-5	-	-	-	-	+	-	-	-
Control positive (Penicillin)						++	-	-
Control positive (Amphotericin B)						-	++	++
Control negative (DMSO)						-	-	-
Origin of tissue	L, T, R	L, T, R	L, T, R	L, T, R	T	T, R	R	T, R
Positive isolates /tested isolates	20/39	24/42	28/42	9/43	1/43			
%	51.2	57.1	66.6	20.9	2.3			

[a] Strains presenting antimicrobial activity

[b] +, presence of activity; −, absence of activity; NG, no growth; *Paecilomyces* sp. HER3-5 was the only isolate with activities of chitinases and chitosanases

[c] Strains used in analysis: *S. aureus* ATCC 25923, *C. glabrata* CBS138 and *C. albicans* ATCC 10231; no inhibition was observed against *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212. -, growth not inhibited; +, growth inhibited; ++, growth strongly inhibited

from other plants and recorded as producers of antimicrobial compounds, including *Phomopsis* (Silva et al. 2006). *Corynespora* (Zhao et al. 2015), and *Paraconiothyrium* (Paul et al. 2007). Regarding the isolates of *Endomelanconiopsis*, *Thozetella*, and *Diothideomycetes* (HER1-1-A), no reports on antimicrobial activity were found,

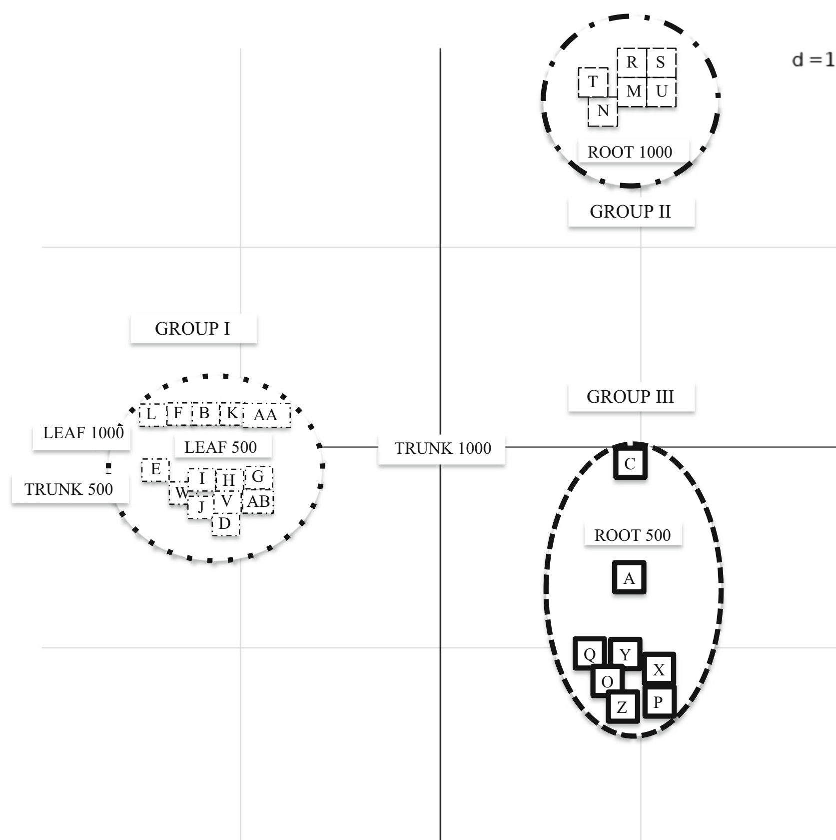

Fig. 3 Correspondence analysis showing the distribution of genotypes for different tissues and elevations (sites). Three groups were formed: group I included fungi from the aerial parts sampled at the two sites, group II covered fungi from root tissues sampled at site 2, and group III was composed of fungi from root tissues sampled at site 1

making these particularly attractive to future investigation for novel antimicrobial molecules.

These results showed that a high proportion (17.8 %) of the *D. arboreus* endophytic fungi possessed antifungal activity. Additionally, the specific activity against certain target microbes may be advantageous for potential uses (such as in human patients). For example, *Corynespora* sp. HET1-3 and *Phomopsis* sp. HEH6-II strongly inhibited *C. glabrata*, but did not affect *C. albicans* and bacteria, while *Phomopsis* sp. HER6-3 only inhibited *C. albicans*, but not *C. glabrata*. For these reasons, the mycobiota of this plant may be a valuable resource in the search for novel antifungal drugs. Currently, fungal infections are common diseases. Although there have been some drugs capable of treating fungal infections, new agents, including those targeting yeast infections, are still needed.

The existence of high proportions of antifungal activities in the endophytic fungi might explain the medicinal applications of *D. arboreus* (Bourdy et al. 2000), since both *S. aureus* and *Candida* are common infectious pathogens that are inhibited by many endophytic fungi from this tree (Table 2). The absence of chitinase and chitosanase activities in the antifungal isolates implies that the anti-yeast activities of these fungi should be based on the synthesis of other compounds (Vandeputte et al. 2012). The absence of antimicrobial activity, but presence of activities for all of the four other tested enzymes in the isolates *Pyrenochaeta* spp. HEH4-1 and HEH7-1, *Fusarium* spp. HER6-2, HER9-4, and HER2-1, as well as *Colletotrichum* spp. HEH5-1 and HEH5-II (Table 2) also suggested that the antimicrobial activities and the tested enzyme activities are not related to each other, which is not surprising, given that many antimicrobial compounds are products of secondary metabolism.

Distribution and specificity of endophytic fungi in plant tissues/sampling sites

The identity of fungal isolates recovered from leaves, trunk bark, and root bark of *D. arboreus* was distinctive (Table 1, Fig. 3), similar to earlier studies (Petrini and Fisher 1986). All of the isolates of *Alternaria*, *Colletotrichum*, *Corynespora*, *Glomerella*, *Guignardia*, *Mycoleptodiscus*, *Paraconiothyrium*, *Pyrenochaeta*, and two of *Phomopsis* covering the isolates HEH3-II, HEH6-II, HET2-3, and HEH10-1, were recovered from the leaves and/or trunks of *D. arboreus*. These fungi are also common to the aerial tissues of other plants (Larran et al. 2002; Lumyong et al. 2009; Lin et al. 2011; Han et al. 2012; Ortega et al. 2013). The isolates of *Dothideomycetes* HER1-1-A, *Endomelanconiopsis*, *Fusarium*, *Paecilomyces*, *Stemphylium*, and *Thozetella*, as well as some of *Phomopsis*, were only recovered from the root bark of *D. arboreus*. These fungi were also reported as root

endophytes in other plants (Paulus and Hyde 2004; Paul et al. 2007; Baral et al. 2011; Fu et al. 2011; Khan et al. 2012). *Endomelanconiopsis* is a phytopathogen described previously (Rojas et al. 2008), but the present work is the first record of its classification as a root endophyte. Our results in combination with previously cited reports suggest that: (1) the endophytic fungal community in leaves is clearly different from that found in roots, but the trunk can be a transit habitat between leaves and roots for some fungi; and (2) some genera identified in this study are widespread endophytes in other plant types.

Further studies are needed to understand the effects of other environmental factors on the incidence of certain endophytic fungi of *D. arboreus*. Notably, the correspondence analysis indicated that the root endophytes form two groups according to the sampling site (Fig. 3), which may be related to variable environmental conditions, such as soil characteristics (Photita et al. 2001).

Conclusions

Forty-five endophytic fungi isolated from *D. arboreus* were identified as *Ascomycota* belonging to 14 different genera. In general, the abundance of fungi was superior in root bark relative to stem bark, and was lowest in the leaves. The correspondence analyses showed clear specificity of some fungal genotypes (species) with the plant tissues and sampling sites (elevation). Many endophytes of *D. arboreus* produced cell-wall-degrading enzymes. Antifungal and antibacterial activity was found in culture extracts of *Corynespora*, *Endomelanconiopsis*, and *Thozetella* strains, three fungi whose metabolites have been little studied or have not been studied at all. These fungi may be valuable resources for searching novel compounds.

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