FUNGAL ECOLOGY 4 (2011) 94–102

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Linking *ex planta* fungi with their endophytic stages: *Perisporiopsis*, a common leaf litter and soil fungus, is a frequent endophyte of *Hevea* spp. and other plants

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ARTICLE INFO

Article history:
Received 3 May 2010
Revision received 21 August 2010
Accepted 10 September 2010
Available online 12 November 2010
Corresponding editor: Kevin Hyde

Keywords:
Endophyte
Environmental sampling
Functional ecology
Fungal biodiversity
Fungi barcode
Host specificity
Loculoascomycetes
Species identification

ABSTRACT

It has been hypothesized that endophytic fungi originate from local environmental sources, and endophyte identity and composition can be predicted based on surrounding *ex planta* fungi. As part of a pilot study to characterize endophytes and *ex planta* fungi (e.g. saprotrophs) of wild *Hevea* trees, endophytes were isolated with almost identical ITS sequences, but these were unidentifiable using GenBank database. Along with the endophytes, *ex planta* fungi on *Hevea* leaves were also collected at the sampling sites. The objective of this study was to determine if selected unknown *Hevea* endophytes could be linked with the *ex planta* fungi using DNA sequences. Methods included BLAST searches, phylogenetic analysis, and morphological characterizations of the endophytes and saprotrophs. Results show that the BLAST-unidentifiable *Hevea* endophytes belong in the fungal genus *Perisporiopsis* (Parodiopsidaceae, Dothideomycetes, Ascomycota). In addition, this *Hevea* endophyte is at least 98 % identical to >20 other sequences from GenBank, demonstrating that *Perisporiopsis* is a common endophyte from various plants, and a leaf litter and soil fungus. Connecting a DNA sequence with a name that has biological meaning enables microbial (i.e. fungal) biologists to make more accurate inferences about the functional ecology of a particular species in a community or ecosystem.

Introduction

Endophytes are microorganisms (e.g. fungi and bacteria) that live for all or part of their life cycle inside the tissues of aboveground plant parts without causing any visible sign of infection (Hyde & Soytong 2008). Of all the plants (and their organs) sampled until now, most have a high diversity of culturable and non-culturable endophytic fungi (Carroll 1988; Evans *et al.* 2003; Berg *et al.* 2005; Mahesh *et al.* 2005; Summerbell 2005a, b; Crozier *et al.* 2006; Samuels *et al.* 2006; Arnold *et al.* 2007; Baird *et al.* 2007; Gond *et al.* 2007; Verma *et al.* 2007). Studies have shown that individual plants may harbor several dozens of endophytic fungal species (Lodge *et al.* 1996; Arnold *et al.* 2000; Frohlich *et al.* 2000; Gazis & Chaverri 2010). This high diversity of endophytic fungi, in addition to the non-sporulating characteristic of many of them, poses a challenge for their identification.

It has been hypothesized that endophytes have local environmental sources, and endophyte identity and composition can be predicted based on surrounding *ex planta* fungi (Saikkonen *et al.* 1998; Ghimire & Hyde 2004; Schulz & Boyle 2005; Promputtha *et al.* 2007). This hypothesis is centered on the mode of transmission of endophytes, which can be either vertical (*via* host seeds) or horizontal (*via* fungal propagules,

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1754-5048/$ – see front matter
doi:10.1016/j.funeco.2010.09.003

such as ascospores or conidia) (Clay 1993; Saikkonen *et al.* 1998; Schulz & Boyle 2005). Endophytes of woody plants are thought to be transmitted horizontally due to their generalized absence from tree seeds (Espinosa-Garcia & Langenheim 1990; Faeth & Hammon 1997; Arnold *et al.* 2000; Boyle *et al.* 2001). Thus, infection by horizontally transmitted endophytes depends on the availability and viability of fungal propagules in the surrounding environment (Schulz & Boyle 2005). In addition, the life cycle of some fungi may include an endophytic stage, and thus, for example, some endophytes may become saprotrophs at host senescence or pathogens when the host becomes susceptible (Petrini 1986; Saikkonen *et al.* 1998; Schulz & Boyle 2005; Hyde *et al.* 2007; Promputtha *et al.* 2007; Duong *et al.* 2008; Oses *et al.* 2008; Parfitt *et al.* 2010). Studies suggest that saprotrophic fungi having an endophytic stage may have an advantage over those that are exclusively saprotrophic because when the plant dies they are the first or "pioneer" colonizers of the decaying plant material (Müller *et al.* 2001; Oses *et al.* 2008; Parfitt *et al.* 2010).

As part of a pilot study to characterize endophytes and *ex planta* fungi (e.g. saprotrophs) of wild *Hevea* spp. trees (including *Hevea brasiliensis*, the source of natural rubber), several endophyte samples with almost identical ITS sequences were isolated. A BLAST search showed the *Hevea* endophyte sequences had 98 % identity with 26 sequences in GenBank, most of them unidentified endophytes, soil fungi, and leaf litter fungi. When sampling for endophytes, some saprotrophs were also collected on decaying *Hevea* leaves. The objective of this study was to determine if it was possible to link the unknown *Hevea* endophytes with the *ex planta* fungi using DNA sequences. By identifying and naming the *ex planta* stage of the fungal endophyte, it will be possible to identify and name the unknown *Hevea* endophytes.

Materials and methods

Endophyte isolation

Living leaves and sapwood (cambium and vascular phloem) of *ca.* 50 wild *H. brasiliensis* and *Hevea guianensis* (Euphorbiaceae) trees were sampled in various sites in the Peruvian Amazon, i.e. Iquitos (Dpto. Loreto, Prov. Maynas), Tambopata (Dpto. Madre de Dios, Prov. Tambopata), Los Amigos (Dpto. Madre de Dios, Prov. Manú), and Iberia (Dpto. Madre de Dios, Prov. Tahuamanu). To isolate endophytes from leaves, three healthy leaflets from different parts of each tree were collected. In the field station, five segments from each leaflet were surface-sterilized through sequential immersion in 2 % sodium hypochlorite (bleach), 70 % ethanol, and sterilized water (Arnold *et al.* 2001, 2003). Each segment was placed in a 3 cm Petri plate containing BBL™ cornmeal-dextrose-agar plus 2 % dextrose (CMD) supplemented with a 0.2 % antibiotic solution (Sigma–Aldrich neomycin–streptomycin–penicillin). Endophytes from the living sapwood tissue were isolated using methods modified from Evans *et al.* (2003) and Kowalski & Kehr (1996). Three slivers of *ca.* 3 × 6 cm of dead bark were shaved from each tree in the field. Five pieces of *ca.* 5 mm of sapwood were cut from the exposed sapwood and transferred to 3 cm Petri plates containing CMD plus antibiotics. Petri plates were incubated for several days and then transported to the U.S. for analyses and purification. Once cultures were purified and transferred to clean plates, they were incubated at 25 °C with alternating 12 hr darkness/12 hr fluorescent light. The pure cultures were then preserved in cryovials with 20 % glycerol at −80 °C at University of Maryland. The unknown endophytes with almost identical ITS sequences were isolated from the sapwood in Tambopata (PP 75, PP 114), and from leaves in Iquitos (IQ 232) and Iberia (IB 145b).

Saprotroph collection

Selected sexual fruit bodies (ascomata) on leaves were collected from two sites, i.e. Los Amigos and Tambopata, with specimen numbers P.C. 987 and P.C. 811, respectively. (Not all ascomata found were collected or processed; future studies will include an extensive collection of saprotrophs.) Fungal fruit bodies on decaying *Hevea* spp. leaves were collected near the base of *Hevea* trees and placed in brown paper bags. Specimens were air dried and placed in glassine bags within plastic Ziploc™ bags with silica gel for culturing in the United States. Although sexual spore (ascospore) germination was attempted by isolating them onto CMD supplemented with antibiotics (Sigma–Aldrich; streptomycin–neomycin–penicillin) (Chaverri & Samuels 2003), these did not germinate after 2 weeks. Specimens were preserved and labelled for accession in the U.S. National Fungus Collection (P.C. 987 = BPI 880186; P.C. 811 = BPI 880185).

Morphological characterization of the saprotrophs and endophytes

For morphological characterization of the sexual state (teleomorph), the macro- and micro-morphology of the sexual fruit bodies (ascomata) and spores (ascospores) were observed and described. To observe internal and microscopic characteristics, the ascomata were rehydrated briefly in KOH, then supported by Tissue-Tek O.C.T. Compound 4583 (Miles Inc., Elkhart, Indiana, U.S.A.), and sectioned at a thickness of *ca.* 15 μm with a freezing microtome. Characteristics of the ascospores were observed by rehydrating the ascomata in 3 % potassium hydroxide (KOH), removing part of the center with a fine glass needle, and placing it on a glass slide. Measurements of continuous characters such as length and width were made using Scion Image software beta version 4.0.2 (Scion Corporation, Frederick, Maryland, U.S.A.). Continuous measurements were based on at least 30 measured units. Morphological observations of the colonies and asexual state (anamorph) of the endophytes in culture were based on isolates grown on Difco™ potato-dextrose-agar (PDA) for 2 weeks at 25 °C with alternating 12 hr/12 hr fluorescent light/darkness. Color terminology is from Rayner (1970).

DNA extraction, PCR and sequencing

Cultures of the endophytes (PP 75, PP 114, IQ 232, IB 145b) were grown in 6 cm diam Petri dishes containing Difco™ potato-dextrose broth. Plates were incubated at 25 °C for *ca.* 1 week. DNA was extracted from the mycelial mat harvested from the surface of the broth using the PowerPlant™ DNA Isolation Kit

(MO BIO Laboratories, Inc., Carlsbad, California, U.S.A.). DNA was also extracted from the ascomata of the saprotroph (P.C. 811) by removing the centrum contents of several ascomata with a fine glass needle and placing them in the bead-beating microtubes of the PowerPlant™ DNA isolation kit.

Partial large subunit (LSU) and complete internal transcribed spacers 1 and 2 sequences including 5.8S (ITS) of the nuclear ribosomal DNA were used in the analyses. The primers used for LSU were LROR and LR5 (Vilgalys & Hester 1990); and for ITS were ITS 5 and ITS 4 (White *et al.* 1990). Each 50 µl PCR reaction consisted of 25 µl GoTaq® Green Master Mix (Promega Corporation, Madison, Wisconsin, U.S.A.), 2.5 µl 10 mM ITS 5, 2.5 µl 10 mM LR5, 1 µl of the DNA template, and 19 µl of sterile RNAase-free water. PCR reactions were run in an Eppendorf Mastercycler *ep* using the following parameters: (1) 94 °C for 5 min, (2) 35 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min, and (3) 72 °C for 5 min. PCR products were cleaned using ExoSAP-IT® (USB Corporation, Cleveland, Ohio, U.S.A.) following the manufacturers instructions. Clean PCR products were sequenced using ITS 5, ITS 4, LROR, and LR5 primers at the DNA Sequencing Facility (Center for Agricultural Biotechnology, University of Maryland, College Park, Maryland, U.S.A.). Sequences were assembled and edited with Sequencher 4.9 (Gene Codes, Madison, Wisconsin, U.S.A.). Sequences have been deposited in GenBank (Table 1).

Phylogenetic analysis

Ninety-six ITS and 18 LSU sequences were analyzed, including Leptosphaeriaceae and Phaeosphaeriaceae sequences from GenBank (accession numbers are indicated in Figs 1 and 2). Several taxa in the Pleosporaceae and Botryosphaeriaceae were used as outgroups for the ITS and LSU trees, respectively. Sequences were aligned with MAFFT 5 (Katoh *et al.* 2005) using the E-INS-i strategy. The alignment was improved by hand with Seaview 2.4 (Galtier *et al.* 1996) and MESQUITE 2.5 (Maddison & Maddison 2009). Gaps (insertions/deletions) were treated as missing data. Neighbor-Joining (NJ) and Maximum Likelihood (ML) were performed with all sequences. jMODELTEST (Rannala & Yang 1996; Posada & Buckley 2004; Posada 2008) was used to select the models of nucleotide substitution for the ML analysis. Once the likelihood scores were calculated, the models were selected according to the Akaike Information Criterion (AIC), and corrected for smaller samples (AICc). According to jMODELTEST, the general time reversible model with gamma distribution (GTR+G) best fit the sequence data for both LSU and ITS regions.

GARLI version 0.96 (Zwickl 2006) was used for the ML and bootstrap analyses. To search for the best tree in GARLI, the starting tree was obtained by random stepwise-addition, the number of independent searches was set to 10, and the number of generations was set to 20 000. For the bootstrap analysis (BP), the starting tree was obtained randomly, was replicated 1 000 times, and the consensus of the resulting bootstrap trees was calculated in PAUP* version 4.0a109 (Swofford 2002). PAUP* was used for the NJ analysis and the tree search was done with the LogDet/Paralinear distance option. NJ bootstrap analysis was also done in PAUP* (1 000 replicates).

A BLAST search or query in the nucleotide database using the "megablast" option was done with the unknown endophytic isolates. To define if the unknown endophyte belonged to the same "phylogenetic species" or Operational Taxonomic Unit (OTU) as sequences in GenBank, a 98 % similarity threshold was used (Nilsson *et al.* 2008). The resulting sequences from the BLAST search were downloaded and compared to those produced in the present study. Only sequences with *ca.* ≥95 % query coverage were used in the analyses.

Results

Saprotroph and endophyte collections

Approximately 248, 190, 246 and 175 endophytic strains were collected from Iberia, Iquitos, Los Amigos and Tambopata, respectively. These endophytic strains represent an estimated 76, 50, 39 and 58 OTUs (or putative species), respectively, based on a 98 % ITS-sequence similarity threshold (Nilsson *et al.* 2008). The identification of these isolates is still in progress. The most abundant genera were *Clonostachys*, *Colletotrichum*, *Fusarium*, *Lasiodiplodia*, *Pestalotiopsis*, *Phomopsis*, *Trichoderma* and *Xylaria* (results not shown, but see Gazis & Chaverri 2010 for results from Tambopata). The endophytes selected for this study were not abundant, but were found at three sites: Tambopata (PP 75, PP 114), Iquitos (IQ 232) and Iberia (IB 145b). Saprotrophs mainly from Dothideomycetes and Hypocreales (Sordariomycetes) were collected only in Tambopata and Los Amigos; their identification is still in progress. The leaf saprotroph used in the present study (P.C. 811 and P.C. 987) was selected because its sequences matched those of the above-mentioned endophytes (see following sections).

Identification of the unknown endophyte using a BLAST search

One of the sequences of the unknown endophytes (IB 145b) was used in the BLAST search. The query resulted in *ca.* 25 sequences with ≥98 % similarity or "maximum identity" (Table 1), indicating that all of these sequences may represent one OTU. Out of these resulting sequences, the majority were unidentified soil, leaf litter or endophytic fungi. Isolate IB 145b was 99.5 % identical to a *Perisporiopsis* (P.C. 811, accession number FJ884129), which was collected from decaying *Hevea* leaves as part of this study. One of the sequences was labelled as *Dokmaia monthadangii* (98.3 % identical) and others were identified as "*Leptosphaeria*" sp. In the ITS tree, all of the sequences with ≥98 % similarity clustered with the unknown endophytes in Clade A (Fig 1). Other close BLAST matches identified to species were *Phoma leveillei* FJ571477 (97.8 % identical), *Pyrenochaeta nobilis* EU930011 (86.4 % identity) and *Ochrocladosporium elatum* GU248334 (85.4 % identity), all members of the *Dothideomycetes*.

Phylogenetic analysis

Alignment of ITS and LSU sequences resulted in 680 and 905 base pairs (bp), respectively, including gaps. An ambiguously aligned region of 125 characters in the ITS alignment was

Table 1 – Results of BLAST search, using ITS sequence of IB 145b as the query, and sorted by % identity. Only % identities >95 % are included. GenBank accession numbers for the unknown endophytes are included

Accession number	Description	Substrate	% identity
HM031459	*Perisporiopsis lateritia* IB 145b[a]	Leaf endophyte, *Hevea brasiliensis*	–
FJ884129	*Perisporiopsis lateritia* P.C. 811[a]	Decaying leaf of *Hevea brasiliensis*	99.52
FJ884130	*Perisporiopsis lateritia* PP 114[a]	Sapwood endophyte, *Hevea brasiliensis*	>99
FJ890410	*Perisporiopsis lateritia* PP 75[a]	Sapwood endophyte, *Hevea brasiliensis*	>99
HM031458	*Perisporiopsis lateritia* IQ 232[a]	Leaf endophyte, *Hevea brasiliensis*	>99
FJ450030	Fungal endophyte strain 1168	Endophyte, *Dendrobium* sp.	99.34
AF502815	Leaf litter ascomycete strain its301	Leaf litter	99.17
FJ612653	Fungal sp. ARIZ B151	*Cecropia insignis*	99.17
AF502886	Leaf litter ascomycete strain its404	Leaf litter	99.16
EU686970	Fungal endophyte isolate 1872	Endophyte, *Oplismenus hirtellus*	99.15
FN386301	"*Leptosphaeria*" sp. 2809	Endophyte, *Holcus lanatus*	99.15
FN394721	"*Leptosphaeria*" sp. 3813	Endophyte, *Holcus lanatus*	99.15
AM262434	"*Leptosphaeria*" sp. *SS-1551*	Endophyte, *Dactylis glomerata*	99.11
AF502612	Leaf litter ascomycete strain its017	Leaf litter	99.05
EU003010	Uncultured ascomycete clone 12h	Soil?	98.96
EU076968	Soil fungal sp. 4-M-1	Soil	98.96
EU076941	Soil fungal sp. 4-M-3	Soil	98.95
AF502626	Leaf litter ascomycete strain its039	Leaf litter	98.79
GU056020	Uncultured Pezizomycotina isolate 58	Spore trap	98.68
AY336132	"*Leptosphaeria*" sp. L413	Endophyte, unidentified plant	98.55
FJ439576	Fungal sp. GMG_C2	Soil	98.54
FJ439577	Fungal sp. GMG_C3	Soil	98.54
FJ439594	Fungal sp. GMG_ppb9	Soil	98.54
FJ439595	Fungal sp. GMG_ppb12	Soil	98.54
AM924151	"*Leptosphaeria*" sp. 4009	Endophyte, *Elymus farctus*	98.48
FJ450026	Fungal endophyte strain 937	Endophyte, *Dendrobium* sp.	98.46
FJ235878	Uncultured fungus isolate F-RISA	Soil	98.34
DQ780454	*Dokmaia monthadangii*	Leaf litter	98.33
AY243059	Ascomycete sp. A75	Endophyte(?), *Ammophila arenaria*	98.17
AJ879672	Uncultured Leptosphaeriaceae	Endophyte of root tips	98.08
FJ571477	*Phoma leveillei* strain OY13807	Marine sponge	97.77
DQ092530	Leptosphaeriaceae HKC16[b]	Marine sponge	97.44
AF502852	Leaf litter ascomycete strain its348	Leaf litter	97.15
DQ342361	Leptosphaeriaceae GF014[b]	Endophyte(?), *Porphyra yezoensis*	97.08
GQ220340	Fungal sp. ZH S52-6-1	Endophyte, *Oryza granulata*	96.92
FJ752615	Fungal sp. JIA4-7-1	Endophyte, *Oryza granulata*	96.72
DQ420974	Uncultured soil fungus clone 68a28[b]	Soil	95.26

a Sequences produced for the present study. All other sequences were obtained from GenBank.
b Not included in Fig 1.

excluded from the analysis. Maximum Likelihood and Neighbor-Joining analyses showed that the unknown endophytes (isolates IB 145b, IQ 232, PP 75, PP 114) clustered with a specimen found in surrounding decaying *Hevea* leaves (P.C. 811 from Tambopata) identified as *Perisporiopsis* (Fig 1: ITS tree, clade A). These specimens also cluster with the additional sequences obtained from GenBank that had ≥98 % similarity in the BLAST search (Fig 1, Clade A). Phylogenetic analyses of the ITS and LSU regions also showed that the unidentified endophytes and saprotrophs of *Hevea* are taxonomically related to species in the fungal families Leptosphaeriaceae and Phaeosphaeriaceae (Dothideomycetes, Ascomycota) (Figs 1 and 2). However, the endophytes and saprotrophs did not seem to cluster within any of those families.

Identification using morphology

The specimens of saprotrophs on decaying leaves of *H. brasiliensis* and *H. guianensis*, P.C. 811 and P.C. 987, respectively, were identified as *Perisporiopsis* (Ascomycota, Dothideomycetes, Parodiopsidaceae) by examination and characterization of sexual and asexual stages on the natural substratum, and by the use of taxonomic literature. Specimens P.C. 811 and P.C. 987 had abundant sexual and asexual fruiting structures on the underside of the decaying leaves. The characteristics of the sexual and asexual stages did not match any of the previously described species of *Perisporiopsis* (Sivanesan 1984). Therefore, this species is considered as new and is being formally described as *Perisporiopsis lateritia* (Chaverri & Gazis 2010).

The colony appearance matched that of other members in the Dothideomycetes and the microconidial anamorph that of *Perisporiopsis* (Sivanesan 1984). The cultures of the unknown endophytes on PDA were discrete, somewhat cottony or wooly, slow growing, reaching *ca.* 30 mm after 2 weeks, the mycelium at first colourless with tinges of pale grayish-brown, and with a grayish sepia pigmentation of the reverse of the colony. The cultures sporulated after 2 weeks, forming few

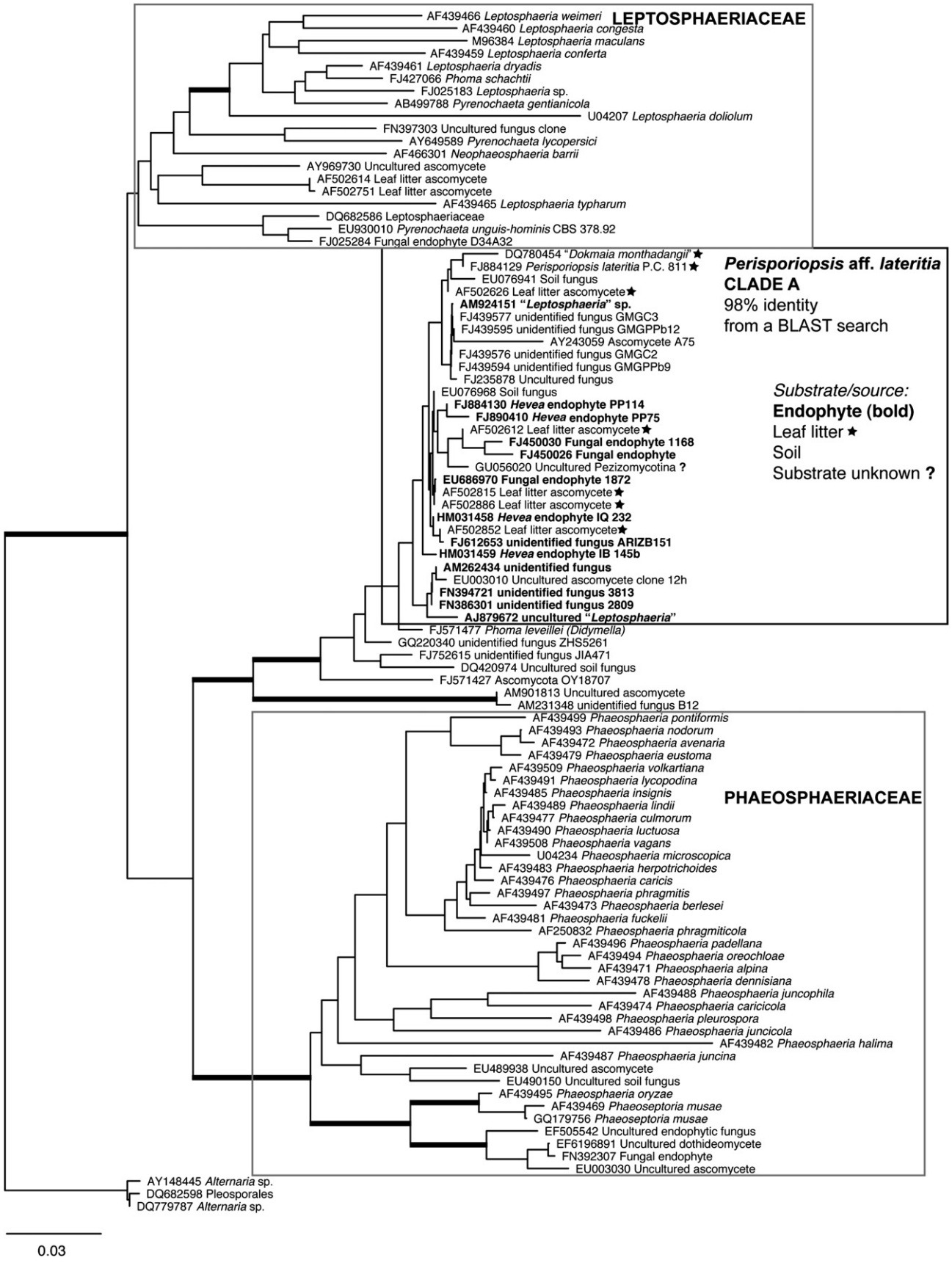

Fig 1 – ITS nrDNA Neighbor-Joining tree. Bootstrap support ≥70 % in NJ and ML analyses indicated by the thicker branches. Log likelihood (Ln) of the ML tree = −11 194.0284. Clade A includes resulting sequences from a BLAST search. Outgroup taxa: *Pleosporales* and *Alternaria* sp.

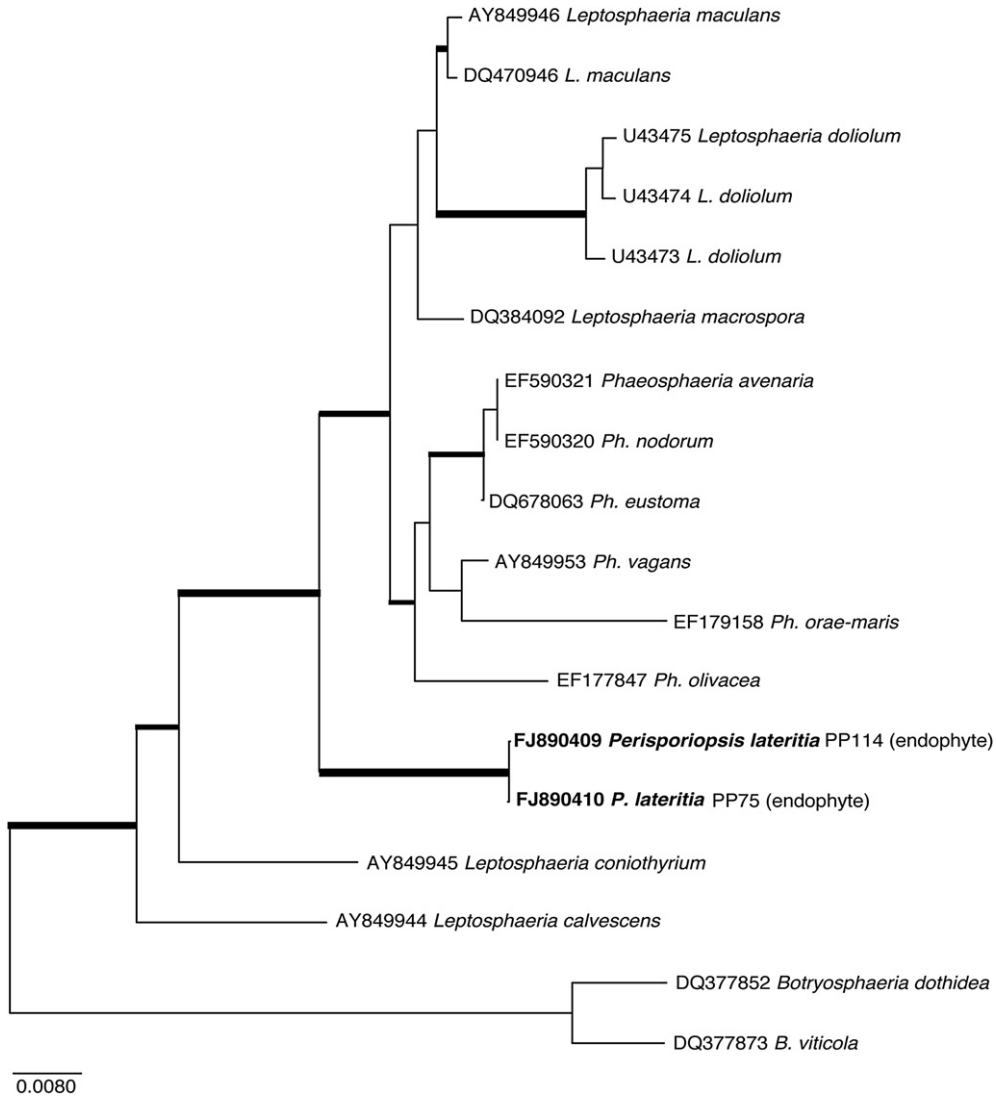

Fig 2 – LSU (28S) nrDNA Neighbor-Joining tree. Bootstrap support ≥70 % in NJ and ML analyses indicated by the thicker branches. Log likelihood (Ln) of the ML tree = −2 540.7520. Endophytes of *Hevea* spp. are in bold. Outgroup taxa: *Botryosphaeria* spp.

brown conidiophores and colourless unicellular spores near the source of inoculum.

The original description (Promputtha *et al.* 2002) and holotype (PDD 74980) of *D. monthadangii*, a leaf litter fungus that had 98 % sequence identity with the *Hevea* endophytes, did not match the morphology of either the *Hevea* endophytes or saprotrophs. The morphology of the asexual fruit bodies on PDA described for *D. monthadangii* also did not match that of the *Hevea* endophytes on PDA. In addition, none of the asexual stages of Leptosphaeriaceae, Parodiopsidaceae, Phaeosphaeriaceae, or other related families in the Dothideomycetes reported in the literature have morphology similar to *Dokmaia*.

Discussion

Based on the results of the DNA sequence analyses (BLAST and phylogenies) and morphological comparisons of endophytes and saprotrophs of *Hevea*, it is concluded that the BLAST-unidentifiable endophytes (IB 145b, IQ 232, PP 75, PP 114) are *P. lateritia* (P.C. 811). All other species of *Perisporiopsis* are also described as being hypophyllous (growing on the underside of decaying leaves) in tropical regions (Sivanesan 1984). According to Sivanesan (1984) *Perisporiopsis* is taxonomically related to the Leptosphaeriaceae in the Dothideomycetes and our phylogenetic analyses confirm this relationship (Figs 1 and 2). The identification of the sequence with GenBank accession number DQ780454 as *D. monthadangii* is doubtful. First, the authors who described *D. monthadangii* mention its taxonomic relationship to *Phaeoisaria* (Promputtha *et al.* 2002), a genus that belongs in the Sordariomycetes, not in the Dothideomycetes. Second, Promputtha *et al.* (2002) reported the formation of asexual fruiting structures (synnemata) and spores in culture; none of the endophytic cultures studied in the present paper formed synnemata in culture. Therefore, the more likely name for these *Hevea* endophytes is *Perisporiopsis*.

Little is known about the ecology of *Perisporiopsis*. This genus includes 19 hypophyllous species, all of them from tropical

regions (Sivanesan 1984). In the present study, two endophytic isolates originated from the sapwood of *Hevea*, yet most other endophytic isolates, including those sequences from GenBank (Table 1), show a substratum preference for leaves (Schulz & Boyle 2005; Arnold 2007; Hyde & Soytong 2008; Tao *et al.* 2008; Gazis & Chaverri 2010). The results of the present study also suggest that for part of its life cycle this fungus lives in the soil, possibly "waiting" for the preferred host or substratum (Arnold 2007; Hyde & Soytong 2008; Tao *et al.* 2008; De Errasti *et al.* 2010). The darkly pigmented mycelium and asexual spores may allow these fungi to lay dormant in the soil (Rotem & Aust 1991; Robinson 2001).

Species in the genus *Perisporiopsis* appear to be plant host specific, at least at the genus or higher level (Sivanesan 1984). For example, *Perisporiopsis brachystegiae* and *Perisporiopsis fusispora* are known only from legumes in Africa and Tropical America, respectively. *Perisporiopsis megalospora* is known from various genera in the *Malpighiales*, and *Perisporiopsis melioloides* from *Myrtaceae*. There is only one other species that has been found on Euphorbiaceae, *Perisporiopsis kwangensis*. In our phylogenetic analyses of ITS (Fig 1), Clade A contained sequences of endophytic fungi from different plants, e.g. *Cecropia*, *Dactylis*, *Dendrobium* and *Holcus*. Thus, it is not clear if Clade A represents one species, based on the definition of "phylogenetic species" or Operational Taxonomic Unit (OTU) using a 98 % sequence identity in ITS (Nilsson *et al.* 2008). A number of studies have demonstrated that ITS is not sufficient for species identification, especially in rapidly evolving or highly diverse genera or species complexes (Chaverri *et al.* 2003; Chaverri & Samuels 2003; Cai *et al.* 2009; Pavlic *et al.* 2009). Therefore, it is possible that there is more than one species of *Perisporiopsis* in Clade A; taxonomic studies are needed.

Due to the high diversity and abundance of endophytes, most studies characterize endophytic fungi using DNA sequences. Until now, the preferred molecular marker has been ITS because of its extensive database in GenBank (*ca.* 600 000 sequences). In addition, the ITS region is easy to amplify and, in many cases, provides enough variation to identify species (Nilsson *et al.* 2008; Seifert 2009). Unfortunately, the rate of submission of correctly identified, named and vouchered sequences in GenBank has not kept pace with the number of easily obtained, unidentified sequences from environmental samples (Nilsson *et al.* 2006). Consequently, many, even most, endophyte sequences in GenBank remain unidentified. For example, when querying the GenBank nucleotide database using the keyword "endophyte", almost 50 % were unidentified, labelled as "fungal endophyte", "uncultured endophytic fungus", or similar. Many others were identified to family or to genus, but few were identified to species.

One of the goals of the Barcode of Life Initiative (BOLI) is to provide diagnostic reference DNA sequences for accurately named species (www.dnabarcodes.org). This Initiative will enable biologists to rapidly and correctly identify unknown specimens/isolates/sequences. Especially, it will contribute to studies involving highly diverse environmental samples, such as soil fungi, endophytes and airborne fungi. Thus, achieving the goals of BOLI will provide an essential identification tool for those conducting environmental sampling. The taxonomic approach taken in the present study to identify and name BLAST-unidentifiable endophytes by sampling and accurately identifying the *ex planta* fungi suggests a method to be used for future fungal diversity studies. This approach includes discovering the substrata or niches a fungus can exploit, and the stages in the life cycle of fungi, including being an endophyte for part of their lives and a saprotroph when its plant host dies. Connecting a DNA sequence with a name that has biological meaning enables microbial (i.e. fungal) biologists to more accurately infer the functional ecology of a particular species in a community or ecosystem.

Acknowledgements

We appreciate the assistance of Ikenna Okafor, an undergraduate student at UMD, in laboratory duties, and Samuel Cuno and Yanet Barrios in field work in Peru. We also thank Dr. Gary J. Samuels and Dr. Amy Y. Rossman for the comments on this paper. This project was funded by a NSF grant to P. Chaverri "Biodiversity of fungal endophytes in rubber trees: Towards understanding their role as plant protection agents" (DEB-0925672); and an Amazon Conservation Association graduate research grant to R. Gazis.

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