



Phylogenetic diversity of endophytic leaf fungus isolates from the medicinal tree *Trichilia elegans* (Meliaceae)

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ABSTRACT. Various types of organisms, mainly fungi and bacteria, live within vegetal organs and tissues, without causing damage to the plant. These microorganisms, which are called endophytes, can be useful for biological control and plant growth promotion; bioactive compounds from these organisms may have medical and pharmaceutical applications. *Trichilia elegans* (Meliaceae) is a native tree that grows abundantly in several regions of Brazil. Preparations using the leaves, seeds, bark, and roots of many species of the Meliaceae family have been widely used in traditional medicine, and some members of the *Trichilia* genus are used in Brazilian popular medicine. We assessed the diversity of endophytic fungi from two wild specimens of *T. elegans*, collected from a forest remnant, by sequencing ITS1-5.8S-ITS2 of rDNA of the isolates. The fungi were isolated and purified; 97 endophytic fungi were found; they were separated into 17 morpho-groups. Of the 97 endophytic fungi, four genera (*Phomopsis*, *Diaporthe*, *Dothideomycete*, and *Cordyceps*) with 11 morpho-groups were identified. *Phomopsis* was the most frequent genus among the identified endophytes. Phylogenetic analysis showed two major clades:

Sordariomycetes, which includes three genera, *Phomopsis*, *Diaporthe*, and *Cordyceps*, and the clade Dothideomycetes, which was represented by the order Pleosporales.

Key words: Endophytes; *Trichilia*; rDNA sequencing; Meliaceae; Molecular identification

INTRODUCTION

The *Trichilia elegans* A. Juss belongs to the family Meliaceae. About 70 species of this genus occur in the American tropical region. In the remnant forests and in the regions near Maringá, Paraná, Brazil, three species of *Trichilia* can be found: *T. catigua* A. Juss, *T. elegans* A. Juss, and *T. pallida* Sw. These species are widely distributed in South and Central America, where *T. elegans* is the most abundant in southern Brazil (de Souza et al., 2001). In Brazil, some *Trichilia* genus plants are used in popular medicine to treat rheumatism and malaria, for inducing vomit and also for having a purgative characteristic (Garcez et al., 1996).

No information concerning the fungi associated with the genus *Trichilia* sp, especially fungal endophytes is available. According to Agusta et al. (2006), many symbiotic relationships are known in nature. Considering the relationship between plants and microorganisms, and they can be divided into two parts, depending on their localization: the ones that dwell inside the plant, called endophytes, and the ones that dwell on the surface of the plant, called epiphytes.

Endophytes or endophytic microorganisms can be found inside organs such as roots, stalks, leaves and seeds. These microorganisms are identified as being fungi or bacteria, which unlike pathogenic microorganisms, do not cause damage to their host; on the contrary, the endophytes play a very important role in plant health, acting as controllers of pathogenic microorganisms and protecting the plant against insect-pests and even domestic herbivores (Azevedo et al., 2000). Most studies focus on the leaf, and even initial studies are able to show an increase in the resistance against pathogens. This group of microorganisms can produce a great and different number of molecular classes which have biological activity (Strobel, 2002). Ding et al. (2010) working with endophytes, showed that the fermentation broth of endophytic fungi of *Camptotheca acuminata* Decne has broad-spectrum antimicrobial activities against important phytofungi and phytobacteria.

The key elements for the evolution of the endophytes are quite complex, involving various types of interactions between the species, numerous levels of happenstance, and multidirectional flows; they are also influenced by random events, such as biotic and abiotic factors, which guide the process of coevolution between fungi and their hosts (Saikkonen et al., 2004). The interaction endophyte-plant host could be at the genotype level (Pamphile and Azevedo, 2002).

Although knowledge regarding the ecology, life cycle and phylogeny of endophytic fungi has quickly increased and accumulated over the last three decades, questions concerning their evolutionary origin, species and ecological role are not yet completely understood (Saikkonen et al., 2004). There is good reason to believe that partnership coevolution was essential for the survival of both, and in this case, the symbiosis was mutualistic (Read et al., 2000).

The ribosomal DNA (rDNA) is present in all organisms and its evolution is rapid, so it is used to discriminate related species or even varieties of the same species. The ITS regions

are flanked by preserved segments (18S, 5.8S and 28S genes). These preserved regions provide the information about the phylogeny and the taxonomic level, since their evolution is slow and they are highly similar within different taxa. On the other hand, the variable regions, the ITS, show many mutations during the process of evolution, making it possible to use them in intra-specific classification. The enlargement of the transcribed spacer region (ITS1-5.8S-ITS2) of rDNA through the polymerase chain reaction (PCR) along with the sequencing and the analysis of similarity between the sequences obtained and those deposited in databases is often used for the identification of fungal species.

Considering the importance of the *T. elegans* as a medicinal plant, the aim of the present work was to determine the diversity endophytic fungi by sequencing the ITS1-5.8S-ITS2 region of rDNA, and to carry out a phylogenetic analysis and comparison with other sequences deposited in NCBI database.

MATERIAL AND METHODS

Biological materials and collection place

For the present work, we used leaves from two *T. elegans* trees which were about 300 m apart. The trees are in “Horto Florestal Dr. Luiz Teixeira Mendes”, within the city of Maringá, located at 51° 57' W, 23°26' S, and at an altitude of 556 m. The collected leaves did not show blotches or herbivore damage. In this forest remnant, there are *T. elegans* bushes and small trees. The temperature during the collection month (May, 2007) varied between 18° and 31°C. The relative humidity was at about 75%. The material was immediately processed after the collection.

Isolation of endophytic fungi

The superficial disinfection of the collected leaves was carried out using sodium hypochlorite (NaOCl), according to the protocols described by Bernardi-Wenzel et al. (2010), which were slightly modified. The NaOCl concentration used was 5%, and the time was 5 min.

After the superficial disinfection, the epiphytic community was removed by first aseptically cutting the leaves into small fragments of about 5 mm². Afterwards, four leaf fragments were placed onto each Petri plate containing potato dextrose agar (PDA) half-strength medium, with 50 µg/mL tetracycline to prevent bacterial growth. The plates were incubated at 28°C for seven days. The fungi grown were transferred to new PDA plates and purified.

Grouping, characterization and identification of endophytic fungi based on morphology

The isolated fungi were sown by the prick technique in solid medium (PDA) and incubated at 28°C. After seven days, they were grouped according to the colony appearance, color and the mycelium type. The micro-culture plate technique was used for the identification of the microscopic structures using a light microscope, according to Kern and Blevins (1999). The data from the macroscopic and microscopic (cytological) analyses were compared with identification keys and molecular analysis (sequencing of rDNA).

DNA extraction

The endophytes were grown on a dialysis membrane, which remained on PDA medium in a Petri plate at 28°C for seven days. The method for DNA extraction was described by Pamphile and Azevedo (2002). After one week growth, the mycelium was removed from the membrane with a sterilized spatula, and about 300 mg mycelium was homogenized in a mortar with liquid nitrogen, resulting in a fine powder. This powder was transferred to a 1.5 mL Eppendorf tube, and then 700 µL pre-heated extraction buffer (1 g sodium dodecyl sulfate (SDS)/100 mL 25 mM EDTA, 200 mM Tris-HCl, pH 8.0, 200 mM NaCl) at 65°C were added. The homogenized reaction mixture was incubated in a water bath at 65°C for 45 min. From this stage on, the samples were kept on ice at 4°C.

After incubation, 300 µL saturated phenol were added; the tube was mixed gently and then centrifuged (12,000 g) for 15 min. The aqueous phase was collected and 300 µL chloroform were added, followed by another centrifugation. Afterwards, the aqueous phase was collected and then another 300 µL chloroform added, followed by centrifugation at 12,000 g for 15 min. Next, the aqueous phase was transferred to a clean tube to which 600 µL chloroform were added. The tube was gently mixed and centrifuged again at 12,000 g for 15 min. The aqueous phase was collected and 8 µL RNase (10 mg/mL) were added; the tubes were incubated in a water bath at 37°C for 1 h. Once again, 300 µL chloroform were added and centrifugation was repeated as before. The aqueous phase was collected and added to two volumes of ethanol refrigerated at -20°C. The tube was gently mixed, and then centrifuged for 2 min at 8000 g. The supernatant was discarded. The precipitate was washed using 300 µL 70% ethanol, and the tubes were centrifuged at 8000 g for 2 min. The 70% alcohol was discarded and the tubes were inverted on an absorbent sheet for 30 min at 37°C, until the tubes were completely dry. The DNA was dissolved in 200 µL Tris/EDTA (TE) buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA, pH 8.0).

The DNA concentration was estimated by electrophoresis in a 1.0% agarose gel. The DNA molecular weight standard used was the High DNA Mass Ladder (Invitrogen). After electrophoresis, the gel was photographed and documented.

DNA amplification by polymerase chain reaction (PCR)

The ribosomal DNA amplification, ITS1-5.8S-ITS2 region, was carried out and primers ITS1 and ITS4 were used as described by White et al. (1990).

Purification of the amplification products

The ITS1-5.8S-ITS2 amplified regions of rDNA of the isolates were purified using the GFX PCR DNA and Gel Band Purification (Amersham Biosciences) kit according to the manufacturer's instructions.

After the purification, the DNA of the endophytic isolates was quantified using 1% agarose gel analysis, photographed and documented.

Sequencing of the ITS regions of rDNA

The DNA samples corresponding to the ITS1-5.8S-ITS2 rDNA region were se-

quenced. The sequencing reaction was performed by the PCR technique. For a final volume of 10 μL , 4 μL sequencing solution (DYEnamic™ ET dye Terminator Cycle kit (MegaBACE™), GE Healthcare), 1 μL ITS1 primer, 2 μL (concentration 10 ng/ μL) of the previously purified DNA and 3 μL Milli-Q water were used. Next, the reaction mixture was placed in a thermal cycler (MJ Research, Inc., TTC-100), programmed to carry out 35 cycles after an initial denaturation at 95°C for 2 min. Each cycle consisted of three stages: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 60°C for 1 min. A final cycle was run at 60°C for 5 min. The sequencing was performed in a MegaBACE™ 1000 sequencer (Amersham Biosciences). The injection and electrophoresis conditions were 1 Kv/90 s and 7 Kv/240 min, respectively.

After sequencing, the samples were analyzed and edited. For the identification of the isolates, the nucleotide sequences found were compared to the ones that were deposited in NCBI (National Center for Biotechnology Information website) database. For the species research, the BLAST program was used. Species identification was determined based on the better result obtained for identity.

The nucleotide sequences obtained in this study have been submitted to the GenBank and were assigned accession numbers GQ 461580 to GQ 461592.

Estimation of genetic distance of the isolates

The sequences determined were aligned by using the MEGA program (version 5.0) (Tamura et al., 2011) with grouping by the neighbor-joining (NJ) method (Saitou and Nei, 1987), using a p-distance matrix for nucleotides with the pair-wise gap deletion option adopted and with 10,000 bootstrap repetitions.

RESULTS

Isolation of endophytic fungi from *T. elegans*

All of the 400 leaf fragments from the two *T. elegans* trees showed colonization. Ninety-seven endophytic fungi were randomly selected for isolation on PDA, for further analysis. Those 97 endophytic isolates were grouped into 11 different morph groups according to their cytological and morphological characteristics.

Molecular identification of isolates based on rDNA sequencing

From the 11 morph groups of endophytic fungi, it was possible to identify the genera and the species of 13 isolates, belonging to 11 different morph groups, through the sequencing analysis of the ITS1-5.8S-ITS2 region and through BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast/>), from NCBI database. After sequencing of the fungi, four endophytic fungus genera were identified: *Cordyceps*, *Diaporthe*, *Phomopsis* and *Dothideomycete* (Table 1).

The microculture technique was employed to help the confirmation of some of the results obtained by the sequencing of the ITS1-5.8S-ITS2 region.

Table 1. Isolated endophytes and identification in relation to the genus or species and the percentage identity found in NCBI (National Center for Biotechnology Information website).

Endophytic isolate	Fungal genus/species with greatest similarity	Accession No. in NCBI of greatest similarity	Identity (%)
G1-12	<i>Diaporthe helianthi</i>	AJ312356	98
G4-2	<i>Cordyceps memorabilis</i>	AY245632	99
G5-24	<i>Phomopsis</i> sp	EU002916	97
G5-32	<i>Dothideomycete</i> sp	EU680530	98
G6-1	<i>Diaporthe helianthi</i>	AJ312356	97
G7-3	<i>Phomopsis</i> sp	AY622993	92
G7-36	<i>Phomopsis</i> sp	AY622993	96
G9-10	<i>Phomopsis longicolla</i>	FJ462759	93
G9-38	<i>Phomopsis</i> sp	EU256482	99
G10-5	<i>Diaporthe helianthi</i>	AJ312356	97
G10-20	<i>Phomopsis</i> sp	EU256482	98
G11-14	<i>Phomopsis</i> sp	EU256482	99
G17-27	<i>Diaporthe helianthi</i>	AJ312356	96

Phylogenetic analysis of *T. elegans* endophytes based on data of rDNA sequencing

Phylogenetic analysis by rDNA sequencing permitted us to state that there is genetic variation among the isolates from the genus *Phomopsis*. The greatest genetic distances were between *Phomopsis* and *Dothideomycete* sp, followed by the genus *Cordyceps*. The major clade, Sordariomycetes, includes the two orders Diaporthales and Hypocreales. In the other clade Dothideomycetes, only the order Pleosporales was found.

DISCUSSION

Isolation of *T. elegans* endophytes

The colonization frequency of the fungi from *T. elegans* leaves was high, where 100% of the fragments sampled showed fungal growth. From these fragments, 97 endophytes were randomly isolated and separated into morph groups. In another Meliceae plant, *Guarea guidonia*, a tropical timber tree, Gamboa and Bayman (2001) observed that over 95% of leaf pieces had endophytes. Other authors working with other plants found isolation frequencies near 95-100% (Durán et al., 2005; Pimentel et al., 2006; Arnold et al., 2007). The leaves used for this work were collected in May, 2007. During that month, the level of rainfall was 80-120 mm and the relative humidity was 75-80%. Due to the pluviometric and relative humidity characteristics, it is possible that *T. elegans* was in the best conditions to afford the maximum colonization. Chareprasert et al. (2006) stated that ripe leaves show a greater colonization by endophytes. The same was also found by other authors showing that endophytic colonization frequencies tend to increase indicating that rain, humidity and temperature can influence the occurrence of endophytes (Arnold and Herre, 2003; Chareprasert et al., 2006).

Molecular identification of endophytic fungi isolated from *T. elegans*

In the present work, small differences were found in the length of the ITS1-5.8S-ITS2 region, which varied between 494 and 645 bp. From the 97 *T. elegans* isolated endophytes. It was possible to identify 13 endophytes, belonging to 11 different morph groups, by rDNA

sequencing and morphologic analysis. The most frequent genus found was *Phomopsis* (53.5% isolations), followed by *Diaporthe* (30.8% isolations) and by *Dothideomycete* and *Cordyceps* (7.7% isolations each). Similarly, Gamboa and Bayman (2001), when working with another Meliaceae, *G. guidonia*, obtained thirty eight morphospecies of endophytes, and *Phomopsis*, *Colletotrichum*, *Xylaria* and *Rhizoctonia*-like fungi were the most abundant taxa. Sakayaroj et al. (2010) working with the tropical seagrass *Enhalus acoroides* in Thailand, was able to identify 10 genera and two species of endophytes, while others could only be identified at the family and order levels. In spite of this, Sakayaroj et al. (2010) consider that a major limitation for molecular identification of endophytes is the limited number of sequences and lack of named sequences from the GenBank database for comparison.

According to Lacap et al. (2003), the analysis of ribosomal DNA is valid for the taxonomy of the morphologic characteristics of the groups. Genus identification is important to determine which isolates can be potentially biological control agents (Crozier et al., 2006).

Similar to the present research, Murali et al. (2006), working with foliar endophyte assemblages of teak trees growing in dry deciduous and moist deciduous forests of Nilgiri Biosphere Reserve, observed that the species of *Phomopsis* dominated the endophyte assemblages of teak. Also, in accordance with those authors, using the internal transcribed spacer sequence analysis of 11 different isolates, *Phomopsis* fungus is not host restricted and the species concept of *Phomopsis* needs to be redefined, which reinforces the previous conclusions reported by other research involving pathogenic *Phomopsis* species.

In the present study, focusing on *T. elegans* endophytes, the strain G4-2 could be identified as *Cordyceps memorabilis* by ITS sequence analysis. The ITS1-5.8S-ITS2 sequence matched with 99% identity the NCBI sequence of this species used by Kuo et al. (2005) in their study. According to these authors, fungi belonging to the *Cordyceps* species have long been used as food and herbal medicines in Asia and are especially popular as powdered supplements. Despite its acceptance and use, little is known about the phylogenetic relationships of the genus. Because of this, Kuo et al. (2005) used the neighbor-joining method based on the ITS1, 5.8S rRNA, and ITS2 regions to build a phylogenetic tree of 17 *Cordyceps* isolates. Five major groups were evident. *Cordyceps sinensis* was less related to 15 *Cordyceps* species but shared a closer relationship with *Cordyceps agriota*, *C. memorabilis* and *Podostroma cordyceps*, forming a group with a bootstrap value of 71%.

In relation to the percentage of the isolates, the data are consonant with other findings showing that *Phomopsis* is quite frequently found as an endophyte in several plants (Chareprasert et al., 2006; Bernardi-Wenzel et al., 2010).

Phylogenetic analysis of *T. elegans* isolates

Phylogenetic analysis, based on rDNA sequencing, enabled us to show that there is genetic variability among the isolates of the genus *Phomopsis* and that the greatest genetic distances are between the genus *Phomopsis* and *Dothideomycete* sp, followed by *Cordyceps*. Bernardi-Wenzel et al. (2010), with *Luhea divaricata* isolates and phylogenetic analysis by rDNA sequencing, also observed the presence of intraspecific variability of the genus *Phomopsis*. The endophytes of *T. elegans* could be grouped into 2 class clades (Figure 1). The first clade, class Sordariomycetes (100% bootstrapping (BP) analyses), showed the endophytes of genera *Phomopsis* and *Diaporthe* belonging to the order Diaportales and genus *Cordyceps* belonging

to the order Hipocreales. The clade class Dothiideomycetes included the genera *Dothiideomycete*, *Cochliobolus* and *Curvularia*. The endophytes G11-14 (97% identity with *Phomopsis* EU256482 at BLAST), G10-20 (98% identity with *Phomopsis* sp, EU256482 at BLAST) and G9-38 (99% *Phomopsis* sp identity with EU256482 at BLAST) were grouped with another *Phomopsis* sp sequences with 99% BP, confirming the genus identification based on rDNA sequencing. The endophyte strains G10-5 and G6-1 (both with 97% identity with *Diaporthe helianthi*, AJ312356 at BLAST) and G9-10 (93% identity with *Phomopsis longicolla*, FJ462759 at BLAST) fell into a clade comprising another *Phomopsis* sp isolate with 94% BP. The endophyte G1-12 (98% identity with *Diaporthe helianthi*, AJ312356 at BLAST) was grouped with other *Phomopsis*/*Diaporthe* strains with 93% BP. The endophyte G17-27 (96% identity with *Diaporthe helianthi*, AJ312356 at BLAST), was grouped with sequences of another *Diaporthe helianthi* strain with 98% BP. In that case, species classification was confirmed.

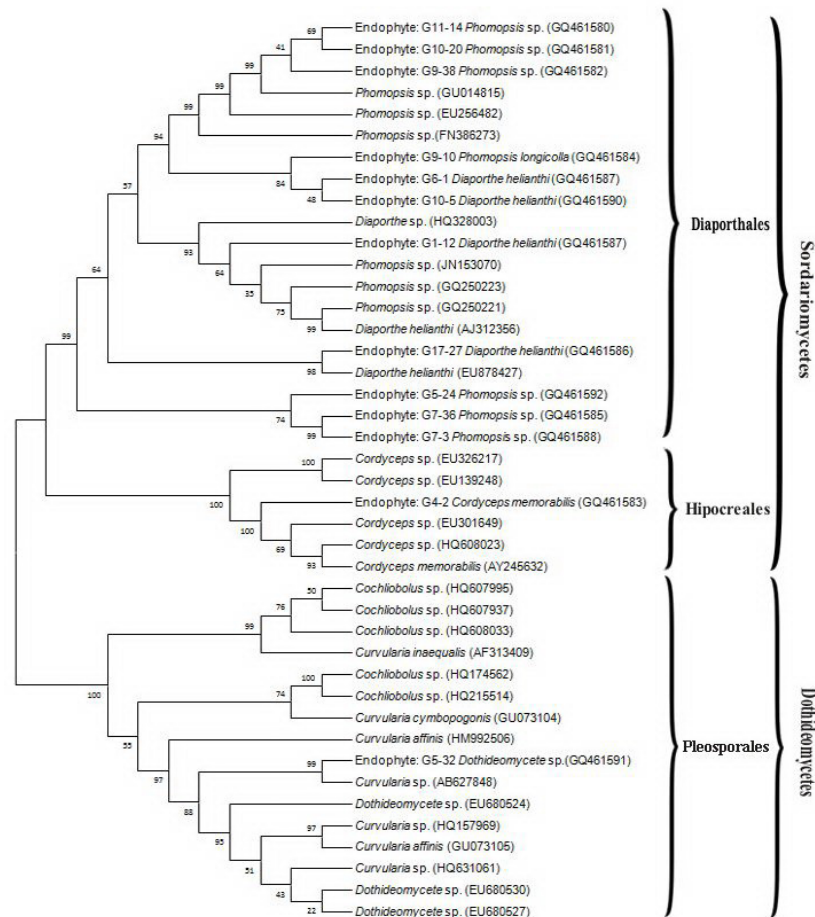


Figure 1. Phylogenetic tree constructed with sequences of the ITS1-5.8S-ITS2 of rDNA region of endophytic fungi from *Trichilia elegans* and sequences from GenBank (indicated by database code), using the neighbor-joining method and using p-distance for nucleotides, with the pairwise gap deletion option. The numbers above and underneath each knot indicate the frequency (in percentage) of each branch in bootstrap analyses of 10,000 replicates.

The endophytes G5-24, G7-36 and G7-3 (97, 96 and 92% identity to *Phomopsis* sp, respectively), belong to a *Phomopsis/Diaporthe* clade with 99% BP, confirming the genus *Phomopsis* identification. The endophyte G4-2 (99% sequence identity with *Cordyceps memorabilis*, AY245632 at BLAST) belongs to *Cordyceps* sp clade with 100% BP, confirming genus classification. The endophyte G5-32 (98% identity with *Dothideomycete* sp, EU680530 at BLAST) fell into a *Curvularia* sp clade with 97% BP, belonging most probably to the genus *Curvularia*.

Future studies will be of benefit to multiple concurrent analytical methods, especially to link traditional morphological species concepts to extensive molecular datasets. It was observed that such clades are also consistent with the diversification of fungi through endophytic symbiosis.

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