



Diversity of endophytic fungal community associated with *Piper hispidum* (Piperaceae) leaves

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ABSTRACT. Tropical and subtropical plants are rich in endophytic community diversity. Endophytes, mainly fungi and bacteria, inhabit the healthy plant tissues without causing any damage to the hosts. These fungi can be useful for biological control of pathogens and plant growth promotion. Some plants of the genus *Piper* are hosts of endophytic microorganisms; however, there is little information about endophytes on *Piper hispidum*, a medicinal shrub used as an insecticide, astringent, diuretic, stimulant, liver treatment, and for stopping hemorrhages. We isolated the fungal endophyte community associated with *P. hispidum* leaves from plants in a Brazilian forest remnant. The endophytic diversity was examined based on sequencing of the ITS1-5.8S-ITS2 region of rDNA. A high colonization frequency was obtained, as expected for tropical angiosperms. Isolated endophytes were divided into 66 morphogroups, demonstrating considerable diversity. We identified 21 isolates, belonging to 11 genera (*Alternaria*, *Bipolaris*, *Colletotrichum*, *Glomerella*, *Guignardia*, *Lasiodiplodia*, *Marasmius*, *Phlebia*, *Phoma*, *Phomopsis*, and *Schizophyllum*); one isolate was identified only to the order level (Diaporthales). *Bipolaris* was the most

frequent genus among the identified endophytes. Phylogenetic analysis confirmed the molecular identification of some isolates to genus level while for others it was confirmed at the species level.

Key words: Endophytes; Piperaceae; Molecular identification; rDNA sequencing; Phylogeny

INTRODUCTION

The medicinal plant *Piper hispidum* Swartz (Piperaceae family) is a shrub distributed throughout the Antilles, and Central and South America, including all Brazilian geographic regions (Guimarães and Giordano, 2004). It is commonly known as “platanillo-de-cuba” and “bayuyo” (Cuba), “cordoncillo” (Mexico), “jaborandi” and “falso-jaborandi” (Brazil) and its properties include use as an astringent, diuretic, stimulant, for unblocking the liver, and stopping hemorrhages (Roíg y Mesa, 1945).

It is reported that other *Piper* species, such as *P. barbatum* and *P. nigrum*, are hosts of endophytic microorganisms (Yandry et al., 2006; Aravind et al., 2009) that inhabit healthy plant tissues, which after infection remain at least transiently symptomless (Ding et al., 2010). Endophytes are useful in the biological control of pathogens or plant growth promotion; nevertheless, some of them are commensal species that cause no direct effect on the host plants (Procópio et al., 2009).

The largest diversity of endophytic species can be found in tropical and subtropical rainforests because these ecosystems are the richest in plant diversity (Banerjee, 2011). Endophyte-plant interactions become more complex in regions with a greater variety of organisms (Pamphile et al., 2004) and include a co-evolution process involving endophytic isolates and the host plants, as shown by Pamphile and Azevedo (2002). Endophytic fungi can be associated with inter- and intracellular colonization of plant host tissues (Bernardi-Wenzel et al., 2010).

Most studies have focused on foliar endophytes, since they are especially diverse and abundant (Arnold et al., 2000). Ecologically relevant roles of tropical foliar endophytic fungi have been demonstrated, such as the physiological costs in terms of water relations and photosynthesis (Pinto et al., 2000; Herre et al., 2005; Arnold and Engelbrecht, 2007) and anti-pathogen protection of hosts mediated by endophytes (Hanada et al., 2010; Rocha et al., 2011), indicating a defensive mutualism that can be detected by the control of natural herbivores and pathogens in laboratory and greenhouse experiments (Saikkonen and Helander, 2010).

Culture-dependent studies of endophytic fungi, those beginning with their isolation from surface-disinfected plant samples and cultivation on appropriate culture medium, have been widely employed (Araújo et al., 2001; Sánchez Márquez et al., 2007; Ding et al., 2010; González and Tello, 2011).

The sensitivity and specificity of molecular biology techniques for the genetic differentiation of species have promoted great advances in the identification of fungal species. Frequently, amplification of the internal transcribed spacer region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA) by the polymerase chain reaction (PCR) combined with sequencing procedure and similarity analysis between the sequences obtained and those available in GenBank has been employed (Magnani et al., 2005) to answer systematic questions and determine phylogenetic heredity (Ragozine, 2008).

Considering the shortage of information about endophytes from *P. hispidum*, this study aimed to isolate the fungal endophyte community associated with *P. hispidum* leaves from a tropical forest remnant and to describe the endophytic diversity based on sequencing of the ITS1-5.8S-ITS2 region of rDNA and phylogenetic analysis.

MATERIAL AND METHODS

Plant collection

Mature, symptomless, undamaged leaves of *P. hispidum* were randomly collected from "Horto Florestal Dr. Luiz Teixeira Mendes", a 37 hectare forest remnant in the municipality of Maringá, Paraná State, southern Brazil (23°26'5.10"S, 51°57'59.46"W). The temperature of the collection month (January 2009) ranged between 19.8° and 29.4°C with an average temperature of 24.8°C and relative humidity of about 76%. The collected material was immediately processed.

Isolation of endophytic fungi

Leaves were first washed in running tap water, aqueous solution 0.01% Tween 80 (Synth) and autoclaved distilled water (twice) to remove epiphyllous debris. Leaves were rinsed with 70% ethanol for 1 min, surface-disinfected with sodium hypochlorite solution (3% available Cl⁻) for 3 min, and rinsed once in 70% ethanol (30 s) and twice in sterile distilled water. The disinfection process was checked by spreading 100 µL of the last water used on Petri dishes containing PDA (potato dextrose agar) culture medium supplemented with tetracycline (50 µg/mL in 50% ethanol) to prevent bacterial growth.

Leaves were cut into 3-5-mm² fragments and equally placed on PDA dishes with tetracycline added. All dishes were incubated at 28°C in biochemical oxygen demand (BOD) for seven days and periodically checked. Afterward, the colonization frequency (%) was determined: (number of fragments colonized by fungi / total number of fragments) x 100.

For the purification process the isolated fungi were transferred to PDA dishes and grown for seven days, then a 5-mm² fragment of each endophytic culture was macerated in 1 mL 0.01% Tween 80, spread (100 µL) on PDA dishes and incubated for 24 h. Immediately after, monosporic colonies were transferred to new PDA dishes and also grown for seven days. When necessary, the purification process was repeated until generating pure cultures. Endophytic fungi were maintained on PDA.

Molecular identification of endophytic fungus isolates

Genomic DNA was extracted as described by Pamphile and Azevedo (2002), except that endophytes were previously grown for seven days in dishes with potato dextrose broth. The DNA concentration and purity were checked by spectrophotometer Genesys 10UV (OD 260/280 nm) and the DNA integrity was checked by electrophoresis on 1% agarose gel using a High DNA Mass Ladder (Invitrogen) as the DNA molecular weight standard. The final concentration of DNA was adjusted to 10 ng/mL.

PCR amplification of the ITS1-5.8S-ITS2 region of rDNA was performed as described

by Magnani et al. (2005), using primers ITS1 and ITS4 (White et al., 1990). Negative controls, containing all reagents except for genomic DNA, were prepared in each PCR. PCR products were purified with GFX PCR DNA and Gel Band Purification kits (Amersham Biosciences) in accordance with manufacturer instructions. The samples were prepared for sequencing according to Magnani et al. (2005), except that only primer ITS1 was employed, and sequenced in a MegaBACE™ 1000 automated sequencer (Amersham Biosciences) with 1 kV/90 s and 7 kV/240 min as injection and electrophoresis conditions, respectively.

For the identification of fungal isolates, percentages of sequence identity and coverage were compared with available sequences in GenBank (<http://www.ncbi.nlm.nih.gov>) using BLASTN, to search for the closest matched sequences. The sequence data from this study were submitted to GenBank under accession Nos. JF766988 to JF767008.

For phylogenetic analysis, a dendrogram was made with the sequences obtained in this study and those available in GenBank. Sequences were aligned using ClustalX with the default settings and the dendrogram was made with version 4 of the MEGA program (Tamura et al., 2007) with grouping by neighbor-joining method (Saitou and Nei, 1987), using p-distance matrix for nucleotides with the pairwise gap deletion option and with 10,000 bootstrapping (BP) repetitions.

RESULTS

Isolation and molecular identification of endophytic fungi from *P. hispidum*

From the 500 leaf fragments sampled from *P. hispidum*, a colonization frequency of 96.59% was obtained. The absence of microbial growth in the negative control ensured the efficiency of the surface-disinfection process. From the 483 endophytes that colonized fragments, 138 were randomly isolated and grouped into 66 morphogroups, according to their morphological characteristics, such as colony coloration, pigment formation, development, and growth of mycelial colony on PDA. Ninety-eight endophytes, with at least two fungal isolates from each morphogroup when they existed (some morphogroups were constituted by a single endophyte), were randomly selected and purified for DNA extraction.

At the end of sequencing of the ITS1-5.8S-ITS2 region, it was possible to identify 21 from the 98 fungal endophytic isolates, belonging to 18 different morphogroups and 11 genera (*Alternaria*, *Bipolaris*, *Colletotrichum*, *Glomerella*, *Guignardia*, *Lasiodiplodia*, *Marasmius*, *Phlebia*, *Phoma*, *Phomopsis*, and *Schizophyllum*); one isolate was identified until the ordinal level (Diaporthales) (Table 1). The most frequent genus was *Bipolaris*, with 23.81% of identified endophytes belonging to it, followed by *Colletotrichum* (19.05%), *Alternaria* and *Phomopsis* (9.53% each). Other genera were represented by a single identified isolate (4.76%). The percentages of identity among the obtained sequences and those available in GenBank ranged from 90 to 100%.

Phylogenetic analysis based on data of rDNA sequencing

Phylogenetic analysis separated the fungal endophytes in five clades (Figure 1). The first clade comprises fungi from the phylum Ascomycota, class Sordariomycetes and Hypocreomycetidae *incertae sedis*, with representatives from the genus *Colletotrichum* and its tele-

omorph *Glomerella*. The isolate G56-91 (90% identity with *Glomerella cingulata* FJ904831.1 at BLAST) was grouped to *Glomerella* sp (GQ334409.1) with 93% in BP analysis and grouped to the other *Colletotrichum/Glomerella fungi* with 99% BP. The isolate G10-57 (97% identity with *C. gloeosporioides* GU066653.1 at BLAST) was subgrouped (99% BP) in the subclade that grouped about 73% of *C. gloeosporioides* from NCBI. The isolates G13-109 (95% identity with *C. theobromicola* GU994355.1 at BLAST) and G54-136 (99% identity with *C. boninense* GU994382.1 at BLAST) were grouped with 95% BP and both of them were grouped to the isolate G08-64 (97% identity with *Colletotrichum* sp EU734583.1 at BLAST) with 76% BP. These three isolates were grouped to the other fungi from this clade with 99% BP.

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

Endophytes	Closely related fungal sequence	Identity (%)
G02-02	<i>Guignardia mangiferae</i> HM222959.1	98
G03-90	<i>Bipolaris sorokiniana</i> HM195260.1	96
G08-64	<i>Colletotrichum</i> sp EU734583.1	97
G10-57	<i>Colletotrichum gloeosporioides</i> GU066653.1	97
G13-109	<i>Colletotrichum theobromicola</i> GU994355.1	95
G14-29	<i>Alternaria mali</i> FJ418189.1	98
G14-86	<i>Alternaria</i> sp HQ328034.1	98
G20-20	<i>Lasiodiplodia theobromae</i> GU228527.1	93
G25-51	<i>Schizophyllum commune</i> AF249388.1	98
G25-59	<i>Phlebia</i> sp AB210077.1	90
G25-95	<i>Marasmius cladophyllus</i> HQ248211.1	98
G27-60	<i>Phomopsis</i> sp EU878429.1	91
G29-79	<i>Phomopsis</i> sp FJ827629.1	96
G30-30	<i>Bipolaris</i> sp DQ123600.1	93
G31-31	<i>Bipolaris</i> sp DQ123600.1	96
G32-118	<i>Bipolaris</i> sp GU017499.1	100
G34-52	<i>Phoma herbarum</i> FJ904851.1	96
G54-136	<i>Colletotrichum boninense</i> GU994382.1	99
G55-117	<i>Bipolaris sorokiniana</i> HM195251.1	94
G56-91	<i>Glomerella cingulata</i> FJ904831.1	90
G65-65	<i>Diaporthales</i> sp HQ117860.1	98

The second clade comprises fungi from the phylum Ascomycota, class Sordariomycetes and order Diaporthales, with representatives from the genus *Phomopsis* and its teleomorph *Diaporthe*. The isolate G27-60 (91% identity with *Phomopsis* sp EU878429.1 at BLAST) was grouped to *Phomopsis* sp (EU878429.1 and EU878433.1) with 59% BP. The isolate G29-79 (96% identity with *Phomopsis* sp FJ827629.1 at BLAST) was grouped to *Phomopsis* sp (EU256482.1) with 50% BP and to the other fungi from this clade with 79% BP. The isolate G65-65 (98% identity with *Diaporthales* sp HQ117860.1 at BLAST) was grouped to *Diaporthales* sp (HQ117860.1) with 91% BP.

The third clade comprises fungi from the phylum Ascomycota, class Dothideomycetes and order Botryosphaerales, with representatives from the genera *Guignardia* and *Phyllosticta* (teleomorph/anamorph), *Lasiodiplodia* and *Botryosphaeria* (anamorph/teleomorph). The isolate G02-02 (98% identity with *Guignardia mangiferae* HM222959.1 at BLAST) was grouped to *G. mangiferae* (HM222958.1) with 93% BP and both of them were grouped with *G. mangiferae* isolates from this clade with 72% BP. The isolate G20-20 (93% identity with *L. theobromae* GU228527.1 at BLAST) was subgrouped to the *L. theobromae* isolates with 99% BP.

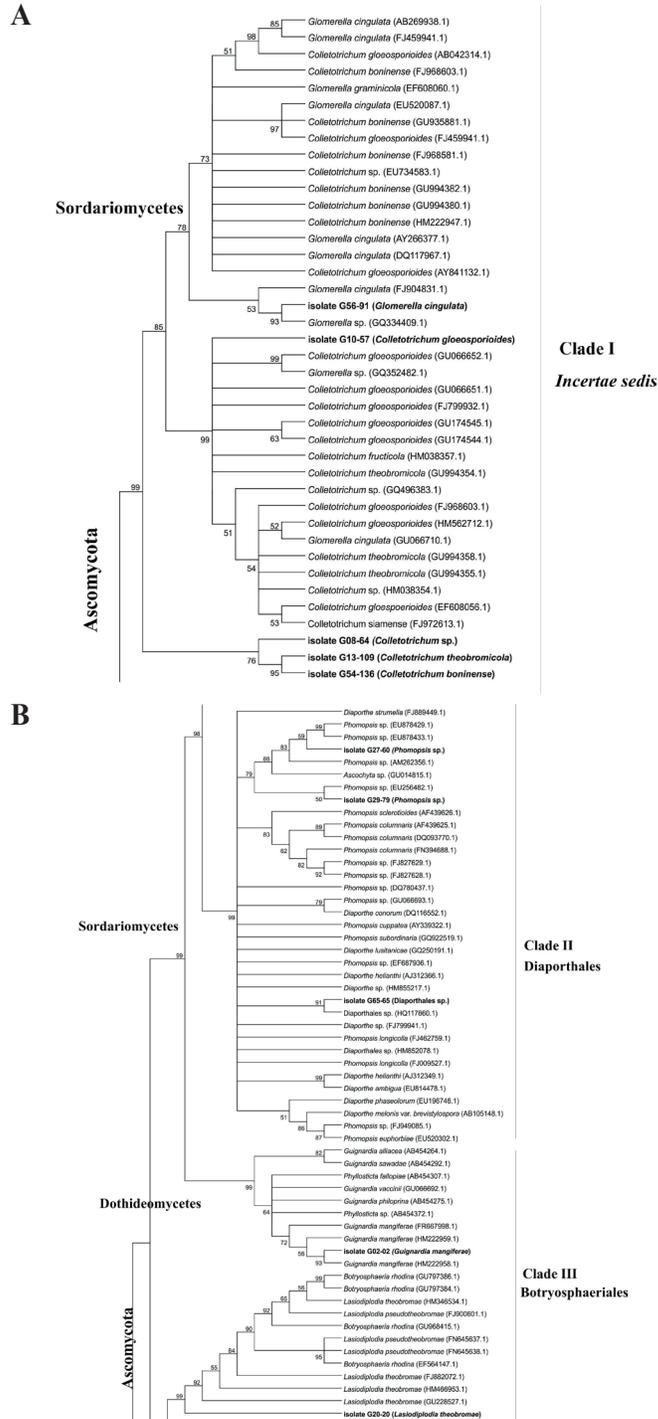


Figure 1. Continued on next page.

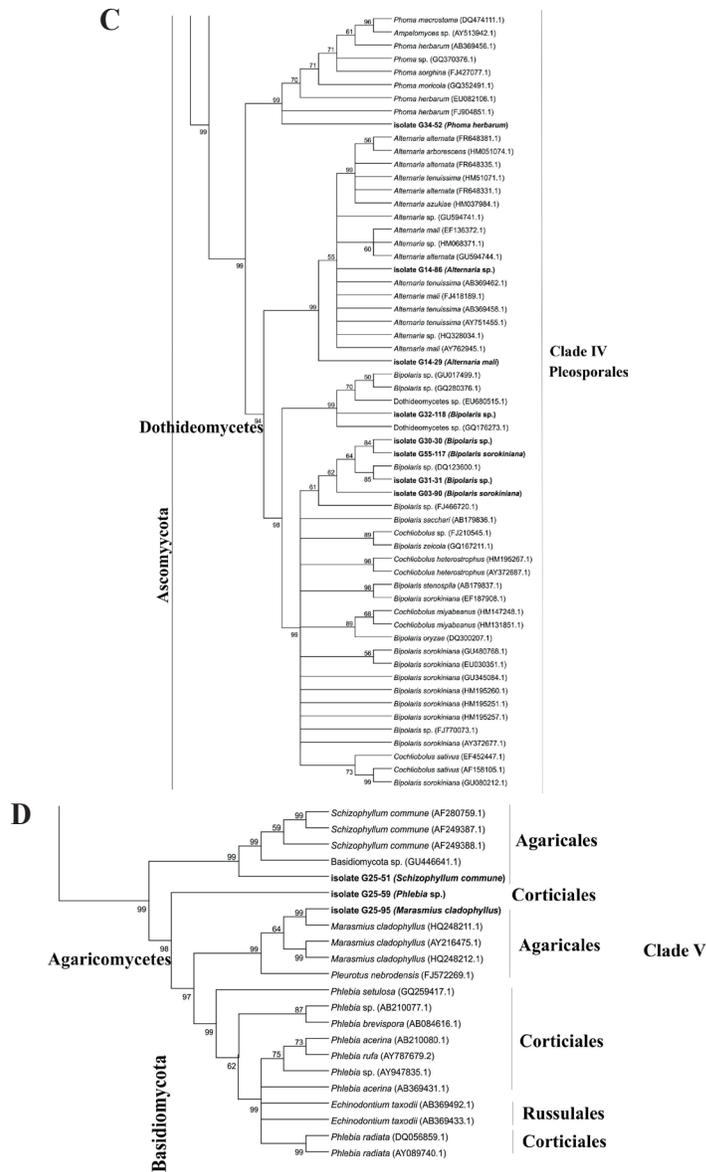


Figure 1. Phylogenetic tree constructed with endophytic sequences from *Piper hispidum* (in bold) and sequences from GenBank (indicated by database code), using the neighbor-joining method using p-distance for nucleotides, with the pairwise gap deletion option. The numbers above and underneath each knot indicate the frequency (in percentage) of each branch in bootstrap analyses of 10,000 replicates. **A.** Clade I comprises the phylum Ascomycota, class Sordariomycetes, with endophytes identified as *Colletotrichum* and *Glomerella*. **B.** Clade II comprises the phylum Ascomycota, class Dothideomycetes and order Diaporthales, with endophytes identified as *Phomopsis* and *Diaporthales*; clade III comprises the phylum Ascomycota, class Dothideomycetes and order Botryosphaeriales, with endophytes identified as *Guignardia mangiferae* and *Lasiodiplodia theobromae*. **C.** Clade IV comprises the phylum Ascomycota, class Dothideomycetes and order Pleosporales, with endophytes identified as *Phoma*, *Alternaria* and *Bipolaris*. **D.** Clade V comprises the phylum Basidiomycota and class Agaricomycetes, with endophytes identified as *Schizophyllum*, *Phlebia* and *Marasmius*.

The fourth clade comprises fungi from the phylum Ascomycota, class Dothideomycetes and order Pleosporales, with representatives from the genera *Phoma*, *Alternaria*, *Bipolaris*, and its teleomorph *Cochliobolus*. The isolate G34-52 (96% identity with *Phoma herbarum* FJ904851.1 at BLAST) was grouped to *P. herbarum* (FJ804851.1) with 99% BP. The isolate G14-86 (98% identity with *Alternaria* sp HQ328034.1 at BLAST) was grouped with 99% BP to the *Alternaria* isolates, including G14-29 (98% identity with *A. mali* FJ418189.1 at BLAST).

The isolate G32-118 (100% identity with *Bipolaris* sp GU017499.1 at BLAST) was grouped to some *Bipolaris* and Dothideomycetes isolates with 99% BP. The isolate G30-30 (93% identity with *Bipolaris* sp DQ123600.1 at BLAST) presented a major proximity with the endophyte G55-117 (94% identity with *B. sorokiniana* HM195251.1 at BLAST) with 84% BP. The isolates G31-31 (96% identity with *Bipolaris* sp DQ123600.1 at BLAST) was grouped to *Bipolaris* sp (DQ123600.1) with 85% BP and the isolate G03-90 (96% identity with *B. sorokiniana* HM195260.1 at BLAST) was grouped to the *Bipolaris* fungi already cited with 62% BP.

The fifth clade comprises fungi from the phylum Basidiomycota and class Agaricomycetes. The isolate G25-51 (98% identity with *Schizophyllum commune* AF249388.1 at BLAST) was subgrouped to the other *S. commune* fungi with 99% BP. The isolate G25-59 (90% identity with *Phlebia* sp AB21000077.1 at BLAST) was subgrouped to the other *Phlebia* fungi with 98% BP, whereas the isolate G25-95 (98% identity with *Marasmius cladophyllus* HQ248211.1 at BLAST) was grouped to *M. cladophyllus* (HQ248211.1) with 99% BP.

DISCUSSION

According to Petrini et al. (1982) and Carroll (1988), cultures of surface-disinfected leaves and stems from healthy plants reveal a high diversity of fungal species growing subcuticularly or within host tissues. Sánchez Márquez et al. (2007) consider that surface-disinfection methods should be used in all endophyte studies because they are efficient at eliminating epiphytes, maintaining the viability of fungi with an endophytic growth habit. The surface-disinfection process with sodium hypochlorite solution (3% available Cl⁻) for 3 min was also used by Araújo et al. (2001) and Procópio et al. (2009), as it is efficient on surface disinfecting plant samples for isolation of both endophytic fungi and bacteria.

Bernardi-Wenzel et al. (2010) state that the foliar fragment size may interfere in the number of endophytic isolates, since if the fragment is large, the microorganisms of the fringes may impair the growth of the most distant ones; therefore, the use of small fragments (about 5-7 mm²) is more efficient for isolating studies. Similarly to these authors, in the current study foliar fragments of up to 5 mm² were employed.

The colonization of endophytes may be influenced by many factors, such as climatic conditions and plant age (Arnold and Herre, 2003). The colonization frequency of 96.59% obtained in *P. hispidum* leaves is similar to other recent studies about tropical angiosperms (Bernardi-Wenzel et al., 2010; Gazis and Chaverri, 2010). The high rates of infection in mature leaves from tropical trees are considered as a result of the apparent universal receptivity of tropical plants to colonization by endophytic fungi, in addition to the exposure to a high abundance of inoculum in the air column (Van Bael et al., 2005; Arnold, 2002, 2008), since environments with a reduced exposure to aerial fungal inoculum may present a low incidence of endophytic infection (Sánchez Márquez et al., 2011).

Some endophytes isolated in the present study have been also observed in several other studies (Araújo et al., 2001; Sieber, 2007; Ding et al., 2010; González and Tello, 2011; Sun et al., 2011). On the contrary, some genera isolated in this study are rarely reported as endophytes, such as the genus *Phlebia*, which is more associated with soil contamination of specimens (Ragozine, 2008).

In the current study, eight genera were represented each by only a single identified endophyte. Sánchez Márquez et al. (2011) recently emphasized that many species are isolated only once in an endophyte survey, being considered rare in the host plant. The interactions between plants and rare species may represent unstable associations that possibly only occur when a given plant and fungal phenotype are confronted.

There is a shortage of information about the identification of endophytic fungi from *Piper* plants. Fungi from the genus *Alternaria* were also isolated as endophytes from *P. barbatum*, by Yandry et al. (2006), using morphological techniques of identification. Other genera isolated by these authors were *Aspergillus* and *Epicoccum*; endophytes from the classes Coelomycetes and Zygomycetes were also isolated; however, most of the isolates (70%) were *Mycelia sterilia*.

In a study with the tropical seagrass *Enhalus acoroides*, Sakayaroj et al. (2010) identified 10 genera and two species of endophytes while others could only be identified at the family and ordinal levels. Similar to our study, the *Bipolaris* genus was found. These authors pointed to the fact 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. Moreover, some authors stated that sequences can be blasted in GenBank without absolute certainty that these species have been correctly identified (Cai et al., 2009; Pinruan et al., 2010; Botella and Diez, 2011).

However, Albrechtsen et al. (2010) considered that where ITS information is available in the database a correct taxonomical match is more likely. In the analyses of these authors the BLAST results for the 18S sequences were, in most cases, sufficiently strong to suggest affinity even to the genus. In our analyses it was possible to identify, at species levels, 52.38% from the total of endophytes molecularly identified, with BLAST identity ranging from 90 to 100%. Considering a safety margin of isolates that are at least 95% identical to sequences available in GenBank, the percentage of *P. hispidum* isolates identified at the species level was approximately 38.1%.

P. hispidum endophytes may be divided into two main groups: the first one comprises 18 ascomycetous isolates (85.71%) whereas the second group comprises three basidiomycetes (14.29%). A predominance of ascomycetes was reported in other studies (Sánchez Márquez et al., 2007; Albrechtsen et al., 2010; Gazis and Chaverri, 2010; Sakayaroj et al., 2010; González and Tello, 2011; Rocha et al., 2011).

Sieber (2007) pointed out that representatives of both the bitunicate ascomycetes (orders Dothideales, Pleosporales and Mycosphaerellales) and the Xylariales order can be dominant in endophytical communities from angiosperms and gymnosperms. Pleosporales was the most frequent order (38.1% of identified endophytes) in the *P. hispidum* leaves sampled. Most of identified endophytes belong to the class Dothideomycetes (42.86%).

The current study indicated a large endophytical colonization of *P. hispidum* leaves, as expected for angiosperms from tropical areas. The division of endophytes into 66 morpho-groups suggests that there is a diversity of endophytes in the sampled leaves, although it was

possible to identify only 21 of the 98 endophytic isolates. The sequencing and phylogenetic analysis revealed the presence of common and uncommon endophytes in *P. hispidum* leaves. For phylogenetic analysis, the bootstrapping percentages higher than 90% confirmed the molecular identification of some isolates (G56-91, G10-57, G13-109, and G54-136) at the genus level. The molecular identification of other isolates (G34-52 and G25-95) was confirmed at the species level, with rates of 99% BP.

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