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In silico analysis of diverse endophytic fungi by using ITS1-5,8S-ITS2 sequences with isolates from various plant families in Brazil

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ABSTRACT. Brazil has a great diversity of plants, and considering that all plant species studied to date have endophytic microorganisms (bacteria or fungi), the country is a resource in the search for bioactive compounds. Endophytes live within plants without causing damage and may be in dynamic equilibrium with the health of the plant. Endophytic fungi can be identified by sequencing the region corresponding to internal transcribed spacer 1-5,8S-internal transcribed spacer 2 ribosomal DNA, and carrying out phylogenetic analyses of these sequences helps to identify species. The objective of this research was to perform *in silico* phylogenetic analysis of fungi isolated from various plant families in Brazil. For this study, we chose 12 articles published between 2005 and 2012 that examined endophytes isolated in Brazil. We analyzed sequences deposited in the National Center for Biotechnology Information GenBank database and carried out alignment to determine the genetic distance of strains using the Molecular Evolutionary Genetics Analysis version 5 program. The articles yielded 73 plant species belonging to 13 families found in the Brazilian States of Amazonas, Bahia, Minas Gerais, Paraná, and São Paulo. The use of

GenBank and the Molecular Evolutionary Genetics Analysis program for phylogenetic observation revealed that several endophytes had been incorrectly identified because inconsistencies were apparent in their location in the phylogenetic tree. However, approximately 98% of the sequences deposited in GenBank were consistent with the identification of related genera, indicating that the database is sufficiently robust to support future studies, in which molecular identification of endophytes is made via analysis of ribosomal DNA sequences.

Key words: Endophytic fungi; Phylogenetic analysis; ITS1-5,8S-ITS2; Family plants; Brazil; DNA barcoding

INTRODUCTION

Brazil has the richest flora in the world. It includes more than 56,000 species of plants - nearly 19% of the world's flora (Ministério do Meio Ambiente, 2002). Currently, 100,000 fungus species have been described, and this number is increasing by approximately 1.2% per year. In every plant studied thus far, the presence of at least one endophyte has been confirmed, signaling broad biological diversity and the discovery of new species. Therefore, endophytic fungi are a promising area of study in Brazil and other tropical areas.

The endophyte-plant interaction may have arisen because of co-evolutionary processes during the appearance of higher plants hundreds of millions of years ago (Strobel et al., 1996; Pamphile and Azevedo, 2002). Strobel (2003) reported that evidence of these associations has been discovered in fossilized tissues of stems and leaves. These endophytic microorganisms may have developed genetic systems that allowed the transfer of information to and from higher plants. Jasinski and Payette (2007) reported microorganisms associated with higher plants in fossilized conifer tissues, suggesting that these organisms may have co-evolved with them.

These microorganisms provide many advantages to their hosts related to plant growth and protection against illness, insect attack, phytopathogenic fungi, and other pests. These effects are due to substances produced by endophytes that occupy ecological niches similar to those occupied by plant pathogens. The interactions between plants and microorganisms have been known for a long time; however, the presence of endophytes within the plant opens new perspectives for studies of these interactions, because unlike pathogens, these organisms do not cause disease in plants. Although endophyte-host interactions are not yet well understood, many cases of symbiosis may be characterized by neutral or antagonistic interactions (Azevedo et al., 2000; Souza et al., 2004).

Several endophytic fungi have been isolated and studied in Brazil, in which the tropical climate is a positive selective natural force for endophyte diversity and a richness of flora is present. Although knowledge of the ecology, life history, and phylogeny of endophytic fungi has increased and accumulated rapidly in recent decades, details about their evolutionary origin, speciation, and ecological roles have not yet been fully revealed (Saikkonen et al., 2004). Therefore, knowing the number of species of fungi and their phylogenetic distribution is an important tool for elucidating the pattern and time of fungal diversification as well as the complexity of ecosystems (Arnold et al., 2007; Higgins et al., 2007; Arnold et al., 2009).

Endophytes are being intensively studied because they have properties that can be applied in various areas, and they are potentially useful in agriculture, biological control, and the development of bioactive compounds, studies of which have indicated that their presence in large numbers can reduce attacks by insects (Kogel et al., 2006; Koulman et al., 2007) and pathogenic fungi in host plants (Azevedo et al., 2000). Information has been uncovered through bioprospecting, which includes the critical stage of identifying endophytes with molecular techniques as internal transcribed spacer (ITS)1-5,8S-ITS2 sequencing analysis and the study of diversity using the National Center for Biotechnology Information (NCBI) GenBank database, the Basic Local Alignment and Search Tool (BLAST), and the Molecular Evolutionary Genetics Analysis (MEGA) program. Arnold et al. (2007) concluded that GenBank matches, if based on well-identified taxa, can be sufficient for estimating species richness and upper-level taxonomic placement. However, the prevalence of unnamed samples in GenBank, the presence of misidentified taxa, and the rapid growth of the database - which translate to highly divergent matches at the genus and family levels when BLAST results from year to year are compared - underscore the need for caution when estimating taxonomic composition based on BLAST results alone. According to Palsson (2000), the term *in silico* biology refers to the use of computers to perform biological studies. Several researchers have used *in silico* approaches for genetics analysis (Chen et al., 2005; Bellemain et al., 2010; Gilbert et al., 2011; Victoria et al., 2011). Bellemain et al. (2010) explored the potential amplification biases that various commonly used ITS primers might introduce during the amplification of various parts of the ITS region in samples containing mixed templates ("environmental barcoding"). They have performed *in silico* polymerase chain reaction analyses with commonly used primer combinations using various ITS datasets obtained from public databases as templates. They conclude that ITS primers have to be selected carefully, especially when used for high-throughput sequencing of environmental samples.

The objectives of the present study were to analyze the diversity of endophytic fungus isolated from plants in Brazilian territories by using phylogenetic analysis from sequences deposited in GenBank, to correlate the endophytes with the families of plants in which they were isolated, and to validate the use of ITS1-5,8S-ITS2 sequencing. We sought to evaluate the effectiveness of ribosomal DNA (rDNA) sequencing for fungal identification and molecular diversity studies through *in silico* analysis.

MATERIAL AND METHODS

Sequences

To perform the phylogenetic analysis, we used data from articles recorded in the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES Periodics database. We selected 12 studies from Brazil published between 2005 and 2012. All studies had used sequencing of the ITS1-5,8S-ITS2 region of rDNA to identify endophytes. These 12 studies covered 73 species of plants distributed in 13 families: Orchidaceae, Rubiaceae, Euphorbiaceae, Malvaceae, Meliaceae, Solanaceae, Asteraceae, Fabaceae, Rutaceae, Anacardiaceae, Myrtaceae, Viscaceae, and Poaceae. The plants were from the following Brazilian States: Amazonas, Bahia, Minas Gerais, São Paulo, and Paraná (Figure 1). The ITS1-5,8S-ITS2 sequences of endophytic fungi were acquired through GenBank from NCBI.

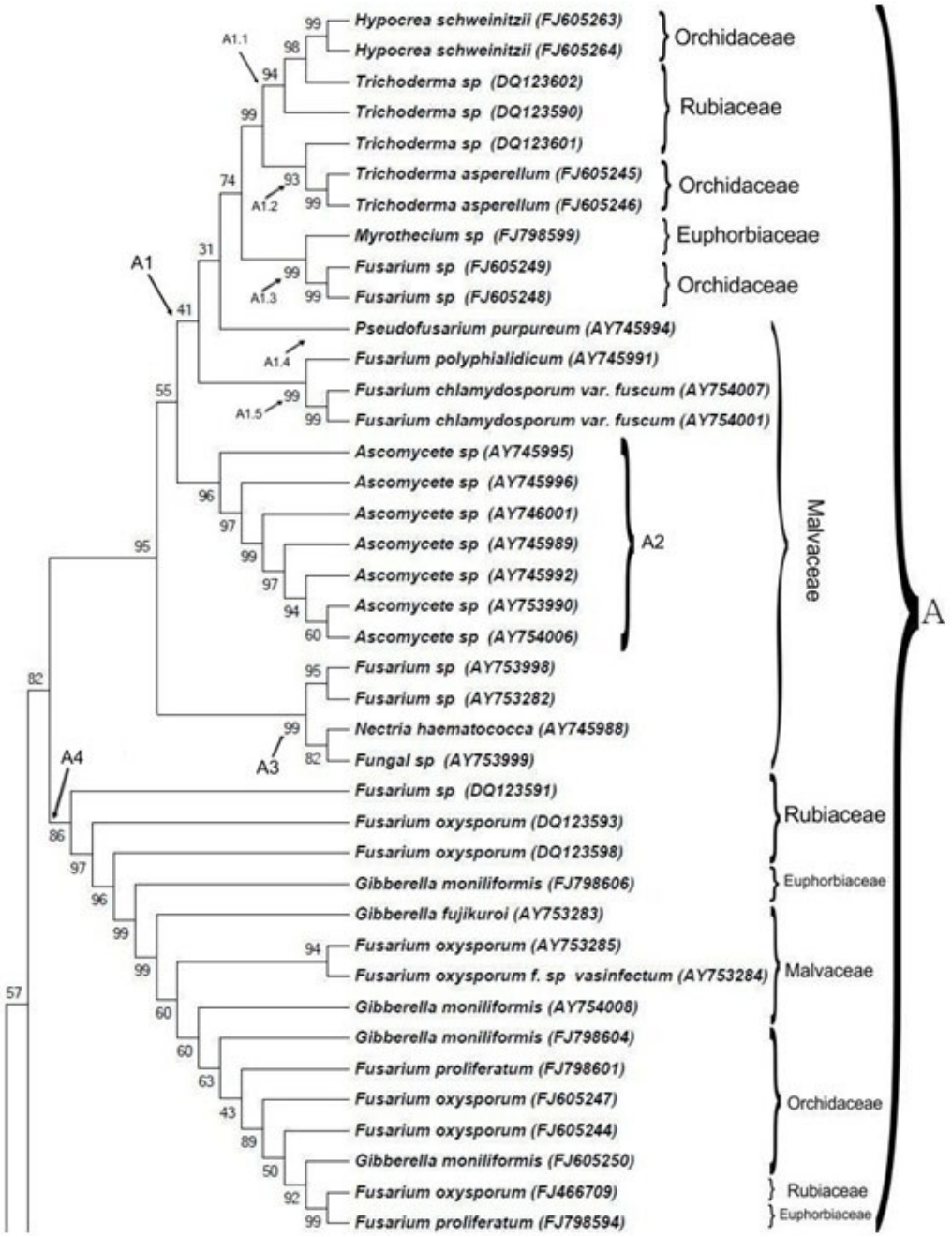

Figure 1. Phylogenetic analysis. The sequences determined were aligned by using the MEGA program (version 5.0; Tamura et al., 2011), with grouping by the NJ method (Saitou and Nei, 1987) using a p-distance matrix for nucleotides with the pairwise gap deletion option adopted and with 1000 bootstrap repetitions.

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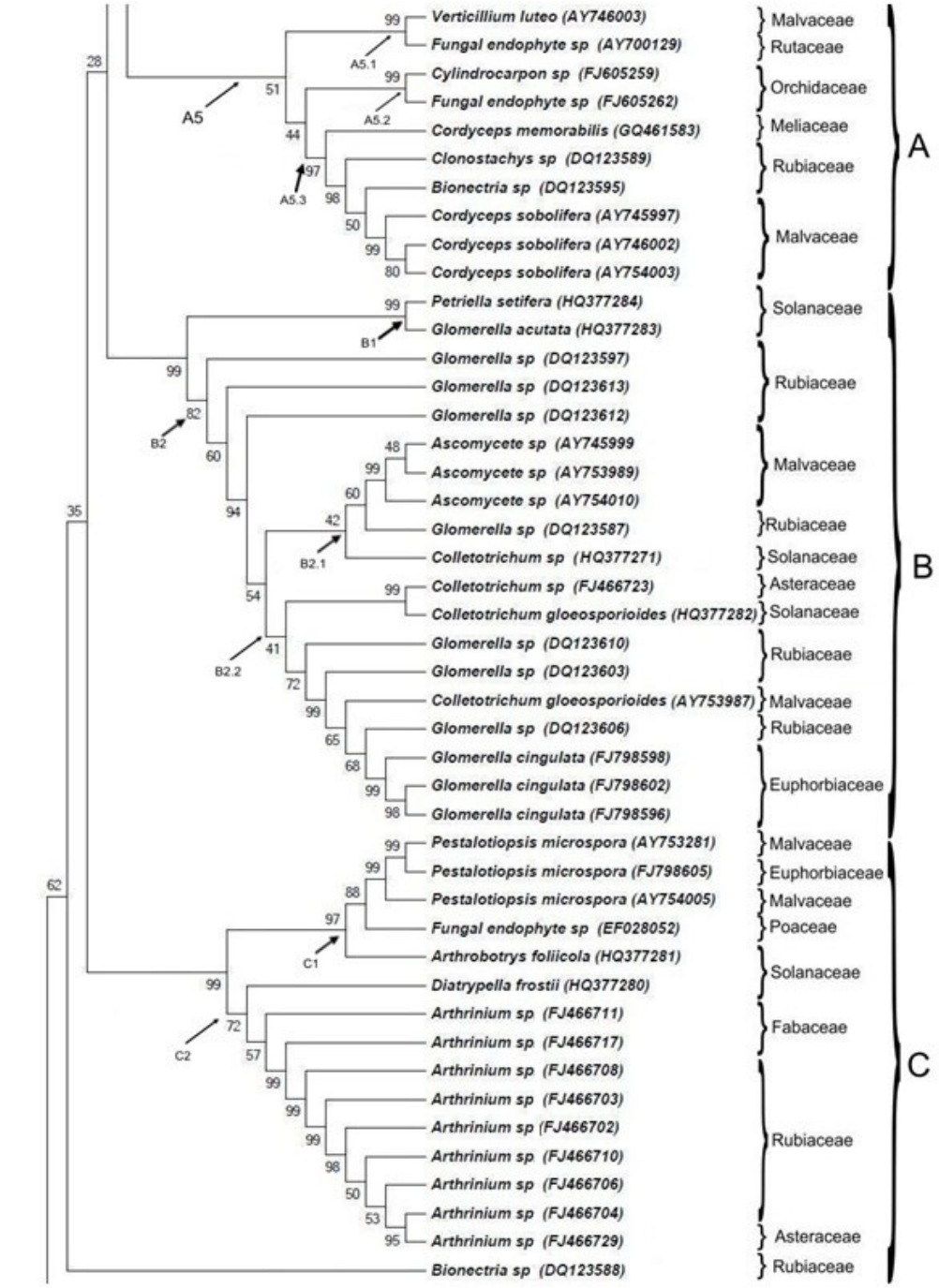

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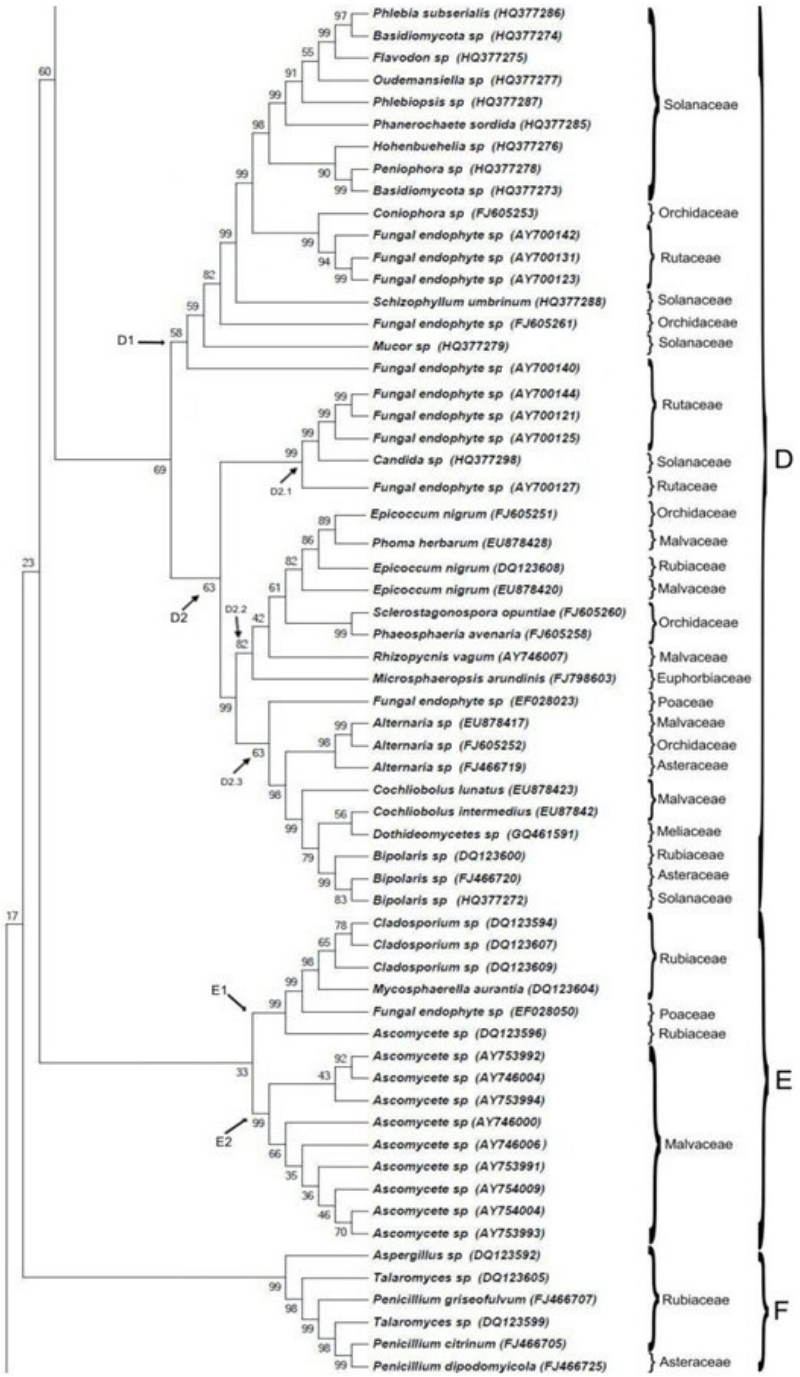

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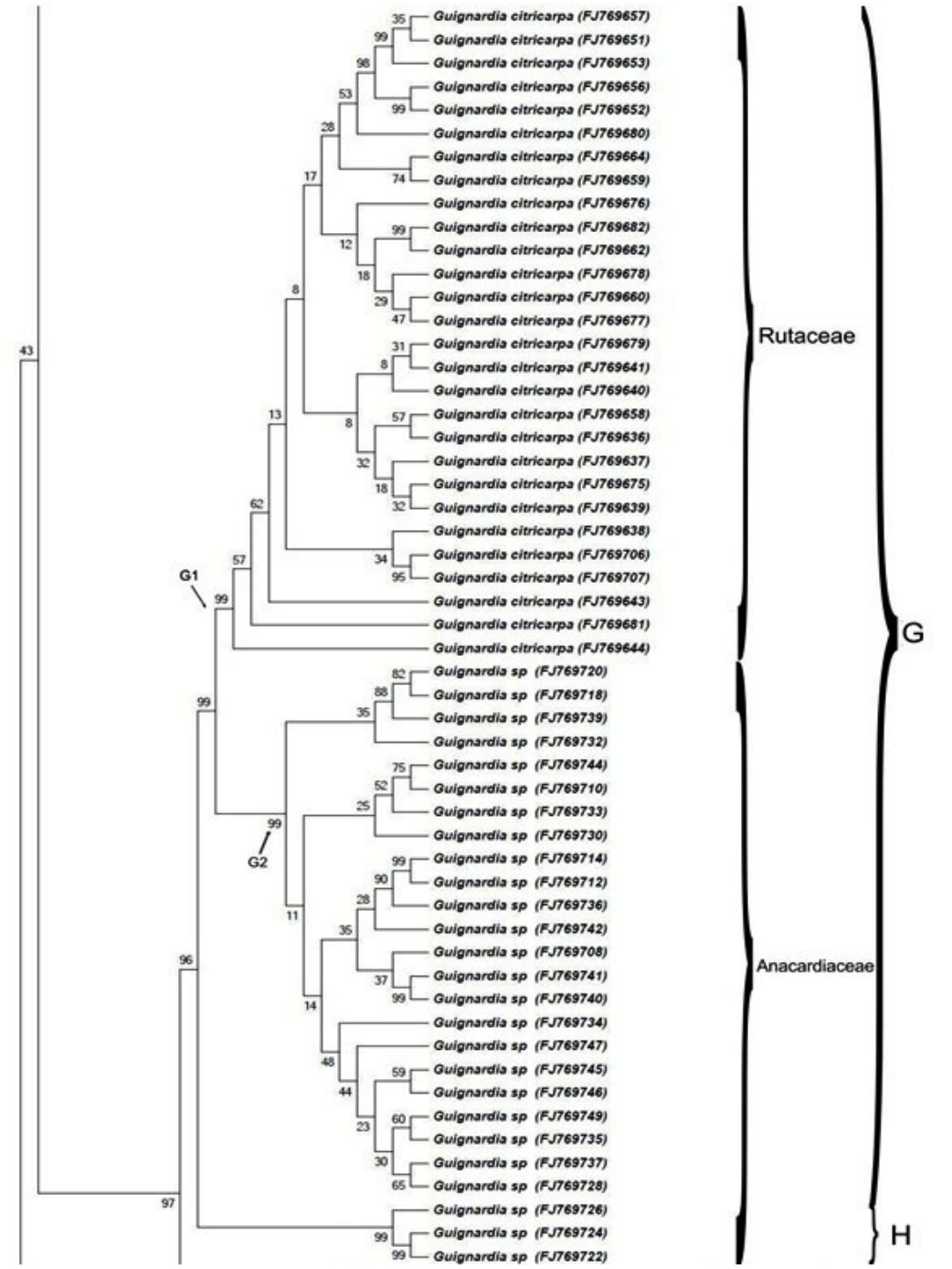

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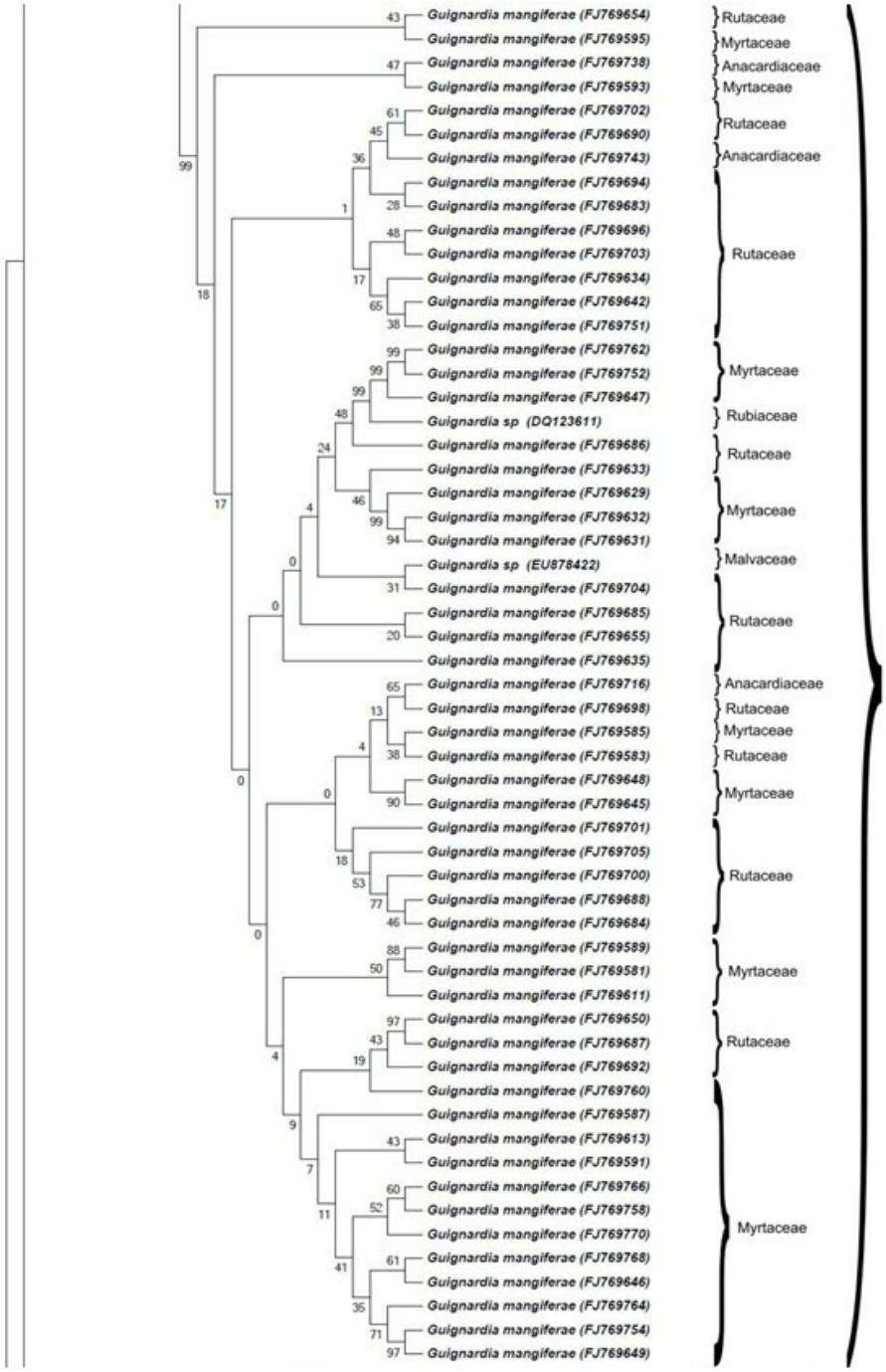

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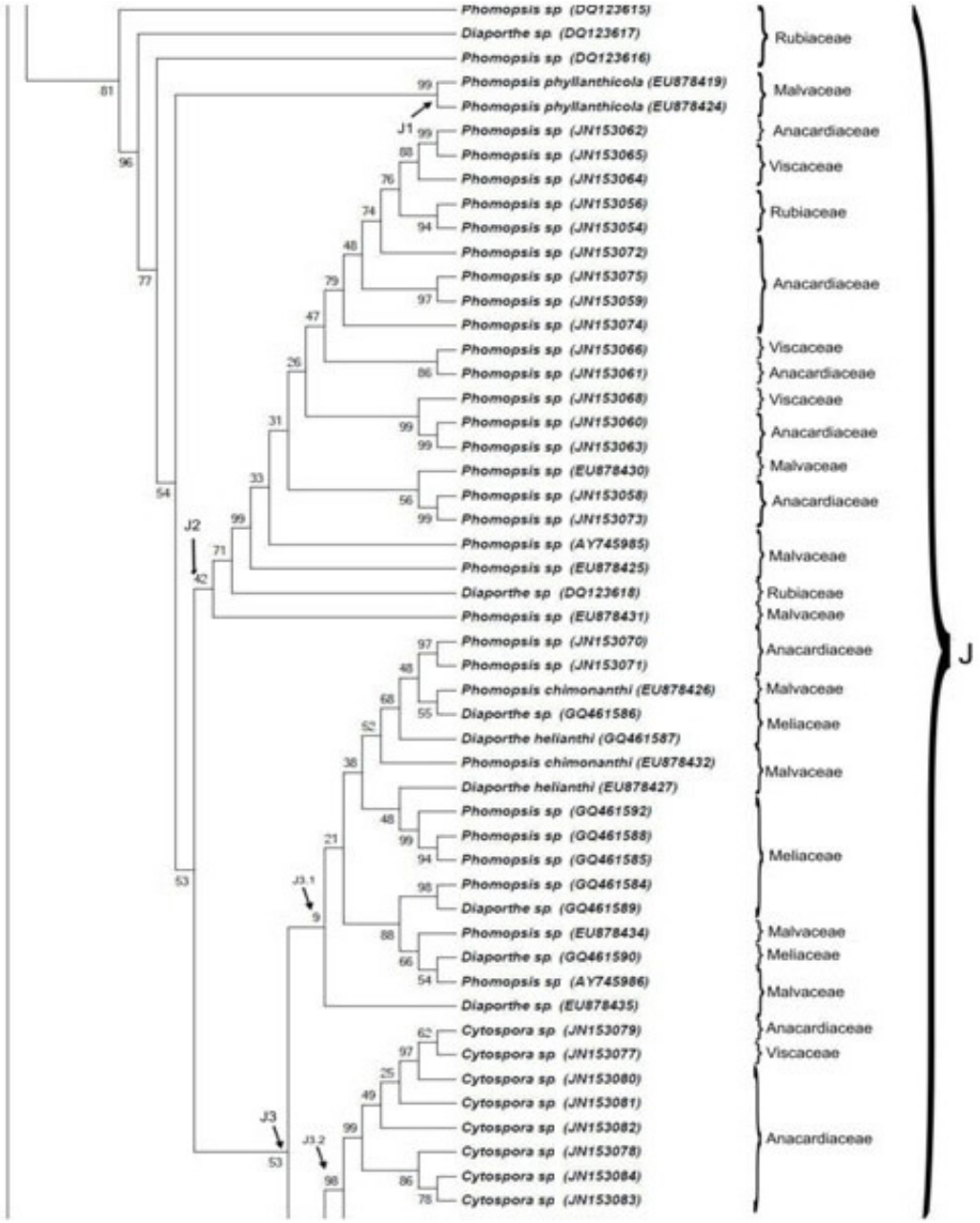

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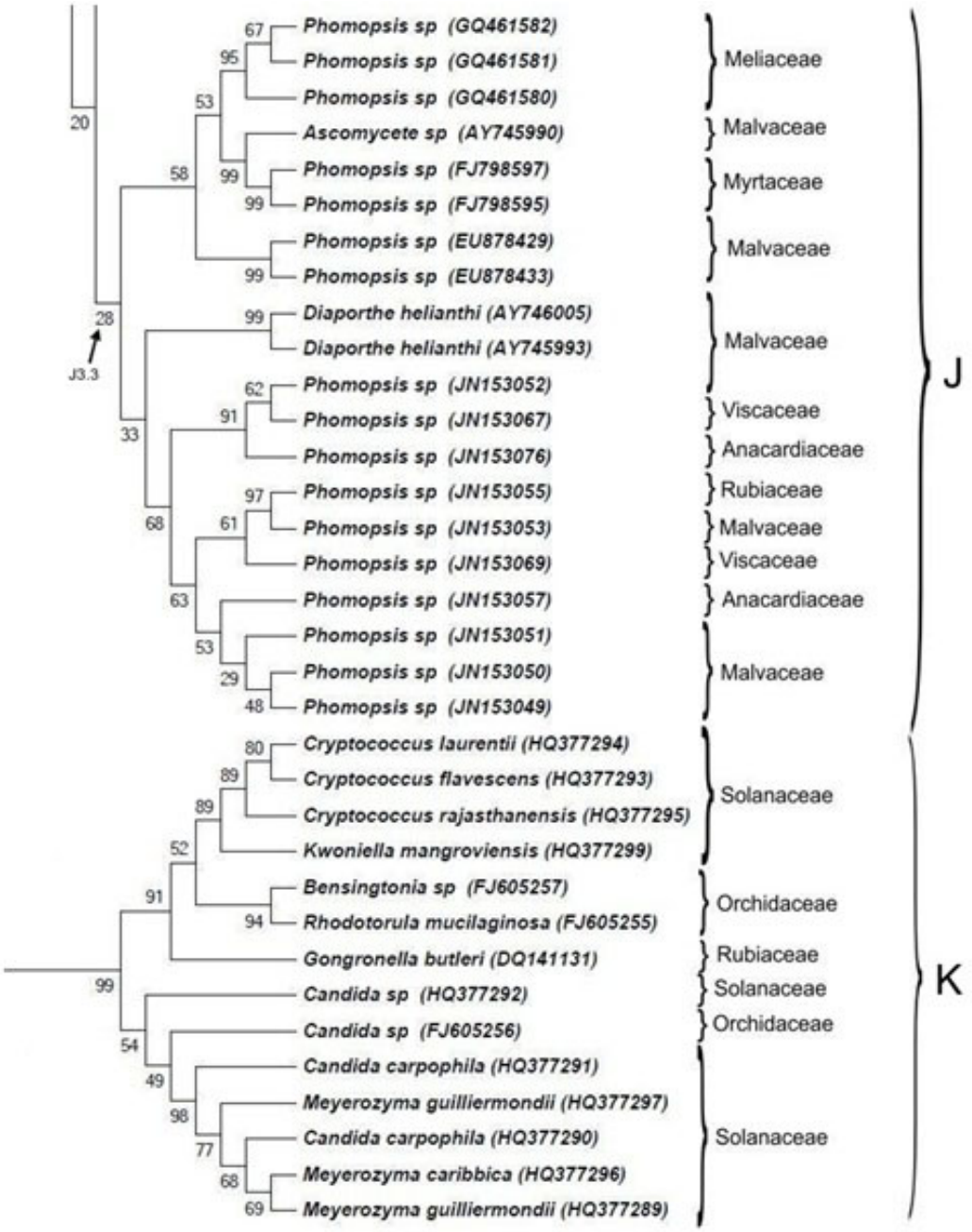

Sequence alignment

The sequences selected from GenBank were submitted to multiple alignment using Clustal-W (Altschul et al., 1990), MEGA 5 (Tamura et al., 2011), and the grouping neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrapping (BP) repetitions for the construction of cladograms (Felsenstein, 1985).

RESULTS AND DISCUSSION

The plant families as well as the references used in endophytic diversity studies are listed in Table 1.

Table 1. Genera of endophytic fungi isolated from each family plant in Brazil.

Family	Isolated fungi	Reference
Orchidaceae	*Hypocrea*, *Trichoderma*, *Fusarium*, *Gibberella*, *Coniophora*, *Epicoccum*, *Sclerostagonospora*, *Bensingtonia*, *Rhodotorula*, and *Candida*	(Vaz et al., 2009)
Rubiaceae	*Trichoderma*, *Fusarium*, *Clonostachys*, *Bionectria*, *Glomerella*, *Arthrinium*, *Epicoccum*, *Phaeosphaeria*, *Cladosporium*, *Mycosphaerella*, *Aspergillus*, *Talaromyces*, *Penicillium*, *Guignardi*, *Phomopsis*, *Diaporthe*, and *Gongronella*	(Sette et al., 2006; Rosa et al., 2010; Abreu et al., 2012)
Euphorbiaceae	*Fusarium*, *Myrothecium*, *Gibberella*, and *Glomerella*	(Rocha et al., 2011)
Malvaceae	*Fusarium*, *Gibberella*, *Nectria*, *Cordyceps*, *Colletotrichum*, *Phoma*, *Epicoccum*, *Rhizopycnis*, *Alternaria*, *Diaporthe*, *Cochliobolus*, *Guignardia*, and *Phomopsis*	(Rubini et al., 2005; Bernardi-Wenzel et al., 2010; Abreu et al., 2012)
Meliaceae	*Cordyceps*, *Phomopsi*, and *Diaporthe*	(Rhoden et al., 2012)
Solanaceae	*Petriella*, *Glomerella*, *Colletotrichum*, *Diatrypella*, *Phlebia*, *Flavodon*, *Oudemansiella*, *Phlebiopsis*, *Phanerochaete*, *Hohenbuehelia*, *Peniophora*, *Schizophyllum*, *Mucor*, *Candida*, *Cryptococcus*, *Kwoniella*, *Bensingtonia*, and *Meyerozyma*	(Vieira et al., 2012)
Asteraceae	*Colletotrichum*, *Arthrinium*, and *Penicillium*	(Rosa et al., 2010)
Fabaceae	*Arthrinium*	(Rosa et al., 2010)
Rutaceae	*Guignardia*	(Gai et al., 2009; Wickert et al., 2009)
Anacardiaceae	*Guignardia*, *Phomopsis*, and *Cytospora.*	(Abreu et al., 2012)
Myrtaceae	*Guignardia* and *Phomopsis*	(Wickert et al., 2009)
Viscaceae	*Phomopsis* and *Cytospora*	(Abreu et al., 2012)
Poaceae	*Fungal endophyte*	(Stuart et al., 2010)

Phylogenetic analysis through ITS sequencing

The few studies on tropical endophytes that have been carried out are primarily descriptive, with some attention to their impact on estimates of global fungal diversity (Arnold et al., 2009). Cultured Ascomycota endophytes are distributed among the Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Leotiomycetes. Arnold (2008) reviewed "designating functional taxonomic units", highlighting that BLAST searches of the NCBI GenBank database of ITS1-5,8S-ITS2 sequences are frequently used to identify endophytes and that ITS data are valued for species-level systematics. However, according to the author, these data may obscure cryptic species; therefore, caution is warranted in assigning identities to endophytes based on BLAST searches. As a result, using phylogenetic analysis with multiple information loci and, where possible, induction of sporulation is important.

The present *in silico* study showed an existing high diversity in fungal endophyte isolates from various host plants in diverse climatic regions in Brazil ranging from the most tropical zone of the Amazon region (Amazonas State) to a more temperate zone (São Paulo State).

Arnold (2008) pointed out that many of the genera shared between temperate zones and the tropics represent fast-growing taxa that are rapidly and easily isolated using standard media.

The phylogenetic analysis in the present study (see Figure 1) was divided into 11 groups represented by keys (A, B, C, D, E, F, G, H, I, J, and K).

Groups A and B were represented by isolates of class Sordariomycetes and order Hypocreales. Group A (Sordariomycetes) was divided into 4 clades (A1, A2, A3, and A4), and these clades were divided into subclades. Class Sordariomycetes was represented by the largest monophyletic clades in the Ascomycota, with more than 600 genera and 3000 known species (Kirk et al., 2008). Members of the Sordariomycetes are cosmopolitan and function in almost all ecosystems as pathogens and endophytes of plants, arthropods, and mammals and in mycoparasites and saprobes involved in decomposition and nutrient cycling. Most plant pathogens in the Sordariomycetes are distributed in the orders Diaporthales, Hypocreales, Microascales, Ophiostomatales, Phyllachorales, and Xylariales (Alexopoulos et al., 1996).

The clade represented by arrow A1 is divided into 5 subclades (A1.1-A1.5). The subclade represented by arrow A.1.1 includes 2 fungi of genus *Hypocrea* isolated from family Orchidaceae and 2 of genus *Trichoderma* isolated from family Rubiaceae. The subclade A1.2 [93% BP], includes 3 endophyte fungi of genus *Trichoderma* isolated from families Rubiaceae and Orchidaceae.

Subclade A1.3, with 99% BP, includes genus *Myrothecium* isolated from family Euphorbiaceae and *Fusarium* isolated from family Orchidaceae. The branch represented by arrow A1.4 (31% BP) identified the fungus *Pseudofusarium purpureum* isolated from family Malvaceae. Subclade A1.5 (99% BP) groups 3 isolates from genus *Fusarium* isolated from family Malvaceae. Clade A2 (96% of BP) is represented by species denominated *Ascomycete* from *Theobroma cacao* (Malvaceae).

Clade A3 (99% BP) is represented by genera *Fusarium*, *Nectria*, and *Fungal* sp, all isolates from family Malvaceae. Clade A4 (86% BP) has endophytes of genus *Fusarium*. These fungi were isolated from Rubiaceae, Malvaceae, Orchidaceae, and Euphorbiaceae. The isolates of genus *Gibberella* were obtained from Euphorbiaceae, Malvaceae, and Orchidaceae.

Clade A5 includes an isolate of genus *Verticillium* (order Glomerales) from family Malvaceae and an isolate denominated fungal endophyte from family Rutaceae with 99% BP. Subclade A5.2 contains 2 fungi isolated from family Orchidaceae: 1 *Cylindrocarpon* (Hypocreales) and 1 denominated fungal endophyte sp subclade A5.3 (97% BP) is represented by genus *Cordyceps* (Hypocreales order) isolated from families Meliaceae and Malvaceae. This subclade also includes isolates from genera *Clonostachys* (Hypocreales order) and *Bionectria* (Hypocreales order) from family Rubiaceae.

Group B (class Sordariomycetes; 99% BP) is divided into 2 clades. Clade B1 (99% BP) is represented by isolates *Petriella setifera* (order Microascales) and *Glomerella acutata* (order Glomerales), both isolated from family Solanaceae. Clade B2 constitutes 3 fungi from genus *Glomerella* (isolated from family Rubiaceae), 3 *Ascomycete* sp isolated from family Malvaceae, genus *Glomerella* isolated from family Rubiaceae, and *Colletotrichum* (order Glomerales) isolated from family Solanaceae. Subclade B2.2, with 41% BP, includes genus *Colletotrichum* isolated from Asteraceae, Solanaceae, and Malvaceae and genus *Glomerella* isolated from Rubiaceae and Euphorbiaceae.

Group C, with 99% BP, is subdivided into 2 clades (C1 and C2). Clade C1 includes 3 fungi of genus *Pestalotiopsis* (order Xylariales) isolated from families Malvaceae and Eu-

phorbiaceae. In this clade, one fungus denominated fungal endophyte from family Poaceae and another isolate *Arthrobotrys* (order Orbiliaceae) from family Solanaceae were also found.

Clade C2, with 72% BP, has 10 isolates: 1 of genus *Diatrypella* (order Xylariales) isolated from Solanaceae and 9 of genus *Arthrinium* (family Apiosporaceae) isolated from families Fabaceae, Rubiaceae, and Asteraceae. The isolate *Bionectria* sp (order Hypocreales) from family Rubiaceae was not linked to group C.

Group D (69% BP) is divided into 2 main clades. Clade D1 is represented by *Phlebia* (order Corticiales), *Flavodon* (order Polyporales), *Oudemansiella* (order Agaricales), *Phlebiopsis* (order Polyporales), *Phanerochaete* (order Agaricales), and 1 *Basidiomycota* isolated from family Solanaceae. These 5 genera belong to class Agaricomycetes. *Hohenbuehelia* (order Agaricales) and *Peniophora* (order Russulales), belonging to class Agaricomycetes and Basidiomycota isolated from family Solanaceae were also found. Genus *Coniophora* (order Boletales) and 3 fungi denominated fungal endophyte were isolated from family Rutaceae (99% BP). Four additional isolates, 1 of each genera *Schizophyllum* (order Agaricales) and *Mucor* (order Mucorales), both isolated from family Solanaceae, and 2 isolates denominated fungal endophyte isolated from families Orchidaceae and Rutaceae are also included.

Clade D2 (63% BP) is divided into 3 subclades (D2.1, D2.2, and D2.3). D2.1 is composed of 3 fungi denominated fungal endophyte isolated from family Rutaceae and 1 *Candida* (order Saccharomycetales) isolated from family Solanaceae. Subclade D2.2 (83% BP) is formed by 8 isolates: 3 fungi of genus *Epicoccum* (order Pleosporales) isolated from families Orchidaceae, Rubiaceae, and Malvaceae; 1 genus *Phoma* (order Pleosporales) isolated from family Malvaceae; 1 genus *Sclerostagonospora* (order Pleosporales) isolated from family Orchidaceae, 1 genus *Rhizopycnis* (class Dothideomycetes) isolated from family Malvaceae; and 1 genus *Microsphaeropsis* (Pleosporales) isolated from family Euphorbiaceae. Rhoden et al. (2012) isolated endophyte fungi of *Trichilia elegans* belonging to the classes Sordariomycetes and Dothideomycetes, but the main genera isolated were *Phomopsis* and *Cordyceps.*

Subclade D2.3 is composed of 10 isolates: 1 fungal endophyte isolated from family Poaceae and 3 phylogenetically related fungi from genus *Alternaria* (order Pleosporales) isolated from families Malvaceae, Orchidaceae, and Asteraceae. In sequence, the genus *Cochliobolus* (order Pleosporales), with 2 isolates from family Malvaceae, 1 denominated *Dothideomycete* sp isolated from family Meliaceae, and 3 genus *Bipolaris* (order Pleosporales) isolated from families Rubiaceae, Asteraceae and Solanaceae were found.

Group E (33% BP) is subdivided into 2 clades (E1 and E2). Clade E1 (99% BP) has 6 isolates: 3 fungi of genus *Cladosporium* (order Capnodiales) isolated from family Rubiaceae, 1 genus *Mycosphaerella* (order Capnodiales) isolated from family Rubiaceae, 1 fungus denominated fungal endophyte isolated from family Poaceae, and 1 *Ascomycete* isolated from family Rubiaceae. Clade E2 (99% BP) comprised 9 fungi denominated *Ascomycete* sp isolated from family Malvaceae.

Group F (99% BP) is represented by 6 isolates: 1 genus *Aspergillus* (order Eurotiales) isolated from family Rubiaceae, 2 genus *Talaromyces* (order Eurotiales) isolated from family Rubiaceae, and 3 *Penicillium* (order Eurotiales) isolated from families Rubiaceae and Asteraceae. Group G (99%BP) is subdivided into 2 clades (G1 with 99% BP, and G2 with 99% BP). G1 contains isolates of *Guignardia citricarpa* (order Botryosphaeriales) isolated from family Rutaceae. Clade G2 contains several endophytes belonging to *Guignardia* sp isolated from Anacardiaceae. Group H (99% BP) contains other *Guignardia* sp isolated from family Anacardiaceae.

Group I is represented by genus *Guignardia* (order Dothideomycete) isolated from families Rutaceae, Anacardiaceae, Myrtaceae, Rubiaceae, and Malvaceae. Group J is divided into 3 main clades (J1, J2, J3). Clade J1 is formed by 2 endophytic fungi of genus *Phomopsis* isolated from family Malvaceae. The genera *Phomopsis* and *Diaporthe* (order Diaporthales) isolated from families Rubiaceae and Malvaceae were not close to J1. Clade J2 is composed of *Phomopsis* isolated from families Anacardiaceae, Viscaceae, Rubiaceae, and Malvaceae.

Abreu et al. (2012) investigated 36 strains of *Phomopsis* spp and *Cytospora*-like fungi and endophytes obtained from various host plants in Brazil using metabolite profiling based on high-performance liquid chromatography-ultraviolet/liquid chromatography-mass spectrometry and cluster analysis. Strains were also subjected to phylogenetic analyses based on ITS rDNA. Tree topologies generated with a Bayesian consensus phylogenetic tree from a nucleotide alignment of the ITS rDNA region (using MEGA 5) and maximum parsimony analyses (using the close-neighbor-interchange algorithm in MEGA 5) were coincident in most parts.

Clade J3 (53% BP) is subdivided into subclades J3.1 (9%BP), J3.2 (99% BP), and J3.3 (28% BP). Subclade J3.1 is organized by genera *Phomopsis* and *Diaporthe* isolated from families Anacardiaceae, Malvaceae, and Meliaceae. Subclade J3.2 is formed by *Cytospora* (order Diaporthales) isolated from families Anacardiaceae and Viscaceae. Subclade J3.3 is represented by genus *Phomopsis* isolated from families Meliaceae, Myrtaceae, Malvaceae, Viscaceae, Anacardiaceae, and Rubiaceae.

Group K is constructed with 14 fungal endophytes: 3 genus *Cryptococcus* (order Tremellales) isolated from family Solanaceae, 1 genus *Kwoniella* (order Tremellales) isolated from family Solanaceae, 1 genus *Bensigtonia* (class Agaricostilbomycetes) isolated from family Orchidaceae, 1 fungal endophyte of genus *Rhodotorula* (order Microbotryomycetes), and 1 genus *Gongronella* (order Mucoromycotina). Also included were 4 endophytes belonging to genus *Candida* (order Saccharomycetales) isolated from families Rubiaceae, Solanaceae, and Orchidaceae and 3 additional fungi of genus *Meyerozyma* (order Saccharomycetales) isolated from family Solanaceae. Notably, the *Candida* sp isolate (HQ377298) from family Solanaceae was not clustered with the other *Candida* spin group K; however, all isolates were obtained from the same host plant.

The majority of studies of endophytic fungi have used leaves, and the proportion of endophyte infection in leaves appears to increase from the arctic to the tropics, although most plant communities have not yet been sampled. In temperate zones, the frequency of endophyte infection is influenced by precipitation, humidity, elevation, irradiance, and air pollution, but the roles of these factors have not been fully assessed in the tropics - in particular, tropical savannas and dry forests. In addition, the canopies of moist or wet forests represent unique environmental conditions characterized by high irradiance, high temperature, and geographic congruence with endophyte-rich forests.

Bernardi-Wenzel et al. (2010) verified that rDNA sequence analysis associated with the traditional method of fungus identification via cytological analysis can identify several endophytic fungi isolated from *Luehea divaricata*. This finding demonstrates that both tools are effective in the study of endophyte taxonomy. With the dendrogram generated with the unweighted pair group method with arithmetic means using the Yule coefficient (using MEGA 5 program), Abreu et al. (2012) classified 36 strains into 6 chemotypes and showed that sequences corresponding to strains classified in various chemotypes formed single phylogenetic lineages or closely related groups in chemical and molecular analyses. Accordingly, metabo-

lite profiling and chemical classification may be used to support phenotypic species recognition in *Phomopsis* and closely related *Diaporthales*.

In this study, using *in silico* studies of phylogenetic analyses of rDNA sequences (from a diverse group of ITS1-5,8S-ITS2 sequences obtained from endophytes isolated in different regions of Brazil and published and submitted to GenBank between 2005 and 2012), we concluded that the GenBank database is sufficiently robust for application in future studies in which the molecular identification of endophyte based on rDNA sequences is necessary. Our data corroborate that studies using ITS sequences obtained via sequencing methodology are sufficient for fungal diversity analyses. Although the current study showed that a few endophytes could not have been correctly identified because of inconsistencies in their location in the phylogenetic tree, more than 98% of the ITS sequences deposited in GenBank were consistent with the identification of related genera, as shown by the tree generated with MEGA 5. This study highlights the importance of future phylogenetic studies in the conservation of endophytic diversity in Brazil.

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