

MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *Pisolithus* IN SOIL UNDER EUCALYPTUS PLANTATIONS IN BRAZIL⁽¹⁾

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SUMMARY

Eighteen *Pisolithus* basidiomes were collected from *Eucalyptus* plantations in the state of Minas Gerais, Brazil. These basidiomes were characterized morphologically and molecularly. The basidiomes varied in shape, color and size. One of them was found underground, indicating a hypogeous fungus. The main morphological distinctive characteristic was spore ornamentation, which distinguished two groups. One group with short and erect spines was identified as *Pisolithus microcarpus*, and the other with long and curved spines as *Pisolithus marmoratus*, after analyzing the cladogram obtained by phylogenetic relationship based on internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA of these isolates.

Index terms: ectomycorrhizal fungus, phylogenetic analysis; internal transcribed spacer (ITS), nuclear ribosomal DNA, taxonomy.

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RESUMO: *CARACTERIZAÇÃO MORFOLÓGICA E MOLECULAR DE *Pisolithus* OCORRENDO EM PLANTAÇÕES COMERCIAIS DE EUCALIPTOS NO BRASIL*

*Dezoito basidiocarpos de *Pisolithus* foram coletados em plantações comerciais de Eucalyptus no Estado de Minas Gerais, no Brasil. Esses basidiocarpos foram caracterizados morfológica e molecularmente. Eles variaram na forma, cor e tamanho. Além disso, um deles foi encontrado abaixo do solo, sugerindo que seja um hipógeo. A principal característica morfológica distintiva foi a ornamentação dos esporos, que foram separados em dois grupos: um apresentou espículas curtas e eretas, e o outro, espículas longas e curvas. Eles foram identificados, respectivamente, como *Pisolithus microcarpus* e *Pisolithus marmoratus*, depois da análise do cladograma obtido pela relação filogenética baseada no espaçador interno transcrito (ITS) do DNA ribossomal nuclear desses isolados.*

Termos de indexação: fungos ectomicorrízicos, análise filogenética, espaçador interno transcrito (ITS), DNA ribossomal nuclear, taxonomia.

INTRODUCTION

Pisolithus is a group of gasteromycetes with worldwide distribution that form ectomycorrhizas with a wide range of angiosperm and gymnosperm tree species (Marx, 1977).

The dispersal of ectomycorrhizal trees, such as eucalyptus and pine, outside their natural range, has resulted in the introduction of a narrow range of exotic ectomycorrhizal fungi into forest plantations in many countries of Europe, South America, Africa and Asia (Garbaye et al., 1988; Martin et al., 1998; Gomes et al., 2000; Díez et al., 2001; Singla et al., 2004; Reddy et al., 2005). Whether the distribution of these fungi reaches beyond these areas depends on the host range of the fungi and their ability to compete with indigenous species.

Since *Pisolithus* was described, several taxa have been proposed based on distinctive carpophore and basidiospore morphology (Marx, 1977). There is considerable polymorphism in terms of basidiomes, spore and culture morphology in *Pisolithus* strains (Burgess et al., 1995; Watling et al., 1995; Anderson et al., 1998; Kasuya et al., 2008). Large variations in colony growth rates, enzyme activity, polypeptide patterns, and mycorrhizal ability have been reported (Kope & Fortin, 1990; Lamhamedi et al., 1990; Burgess et al., 1994, 1995). Based on basidiospore morphology and mating incompatibility tests, Kope & Fortin (1990) proposed that the genus *Pisolithus* comprises several biological species.

DNA-based methods have provided further support for this hypothesis. Junghans et al. (1998) reported that RAPD analysis (Random Amplification of Polymorphic DNA) clustered *Pisolithus* isolates in two main groups, according to host and geographical origin. Using the same isolates, these data were confirmed by Restriction Fragment Length

Polymorphism (RFLP) in the internal transcribed spacer (ITS) regions amplified by Polymerase Chain Reaction (PCR) and on mitochondrial DNA (Gomes et al., 1999, 2000). Based on RFLP analysis of the ITS sequences of several *Pisolithus* isolates, Farmer & Sylvia (1998) suggested that this taxon represents a complex species. At least 11 different clades have been reported, of which the major were recognized as phylogenetic species (Martin et al., 2002).

Studies based on DNA sequences serve to reevaluate earlier classifications and delimit species more accurately (Taylor et al., 2000). The progress of phylogeny studies and their implications for understanding the biogeography of ectomycorrhizal fungi has been significant (Jarosh & Bresinsky, 1999; Wu et al., 2000; Kanchanaprayudh et al., 2003; Moyersoen et al., 2003; Singla et al., 2004).

Although inoculation is not a rule in tree nurseries in Brazil, it is rather common to find *Pisolithus* basidiomes under commercial eucalyptus plantations. However, with exception of the Southern region, where *P. microcarpus* was found (Giachini et al., 2000), *Pisolithus* basidiomes in other areas have not been identified at the species level. The objective of this study was to identify the *Pisolithus* species found in eucalyptus plantations in the state of Minas Gerais.

MATERIAL AND METHODS

Sampling and sites

Basidiomes of 18 *Pisolithus* isolates, collected from the soil of commercial plantations of *Eucalyptus* spp., between February/2002 and February/2004, in different areas of CENIBRA - Celulose Nipo-Brasileira S.A., in Minas Gerais, Brazil, were used in this study.

Electron microscopic observation of basidiospores

Basidiospores from mature basidiomes were air-dried and sputter-coated with gold+palladium before examination under a JSM5310 scanning electron microscope, low vacuum, at an accelerating voltage of 15 kV. Mean basidiospore diameter ($n = 20$) was determined for each isolate using scanning electron micrographs and basidiospore spine morphology classified by the terminology of Kope & Fortin (1990).

DNA manipulations

Samples of *Pisolithus* spores or internal parts of dried mushrooms were used for DNA extraction. DNA was isolated using DNAeasy Kit (Qiagen, Düsseldorf, Germany). The ITS region of rDNA (ITS1-5.8S-ITS2) was amplified with ITS1f (Gardes & Bruns, 1993) and ITS4 primers (White et al., 1990). Amplifications were performed on a GeneAmp PCR System 2400 (Perkin-Elmer). Samples were initially denatured for 30 s at 94 °C, followed by 40 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C.

Samples of PCR products were purified using QIAquick® PCR purification kit (Qiagen, Düsseldorf, Germany) and sequenced by the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA), according to the manufacturer's instructions. Sequences were generated for both strands using the ITS1f and ITS4 primers for comparison and to ensure reliability.

Sequence analysis

The sequences were deposited in a GenBank database (Table 1), and compared with other sequences (Table 2). The sequences were imported into MEGA, version 3.1 (Kumar et al., 2004), and aligned using the program Clustal W (Higgins et al., 1994). The resulting multiple alignments were optimized visually. The final alignment of this study is available on request. ITS sequences of *Paxillus involutus* (AF167700) and *Suillus luteus* (L54110) were used as outgroup taxa. A neighbor joining (NJ) algorithm method was used for phylogenetic analysis, applying a p-distance model with software MEGA (Kumar et al., 2004). The robustness of each branch was determined using the nonparametric bootstrap test (Felsenstein, 1985) with 1000 replicates.

RESULTS

The basidiomes shape and size varied (Table 1). Furthermore, isolate VIC 30600 was found underground, indicating a hypogeous fungus. Two distinctive spore ornamentations were observed, forming one group with the isolates VIC 30596, VIC 30597 and VIC 30600, with long and curved spines, and spore diameter from 7.24 to 7.91 μm (Figure 1, Table 1). The other isolates had short and erect spines and a spore diameter from 6.2 to 8.62 μm (Figure 1, Table 1). As many as eight basidiospores per basidium were observed in all cases (data not shown).

Table 1. Isolate identification, spore diameter including spines, basidiome size, growth habit, and GenBank accession of Brazilian *Pisolithus* isolates used in this study

Isolates	Spores diameter	Basidiome size width x height	Growth habit	GenBank accession
	μm	(stipe height) mm		
VIC 30590	Immature	36 x 43 (20)	Epigeous	-
VIC 30591	Immature	17 x 26 (10)	Epigeous	-
VIC 30592	7.12 \pm 0.60	48 x 42 (18)	Epigeous	-
VIC 30593	Immature	33 x 30 (00)	Epigeous	HQ693094
VIC 30594	6.40 \pm 0.54	28 x 60 (28)	Epigeous	-
VIC 30595	Immature	28 x 38 (16)	Epigeous	-
VIC 30596	7.91 \pm 0.96	57 x 28 (05)	Epigeous	HQ693095
VIC 30597	Immature	28 x 28 (00)	Epigeous	HQ693096
VIC 30598	7.09 \pm 0.76	25 x 29 (05)	Epigeous	HQ693097
VIC 30599	Immature	18 x 37 (00)	Epigeous	HQ693098
VIC 30600	7.24 \pm 0.7	24 x 15 (00)	Hypogeous	HQ693099
VIC 30601	Immature	52 x 50 (27)	Epigeous	-
VIC 30602	Immature	36 x 30 (10)	Epigeous	HQ693100
VIC 30603	Immature	28 x 34 (08)	Epigeous	-
VIC 30604	8.62 \pm 0.65	46 x 58 (34)	Epigeous	HQ693101
VIC 30605	6.20 \pm 0.50	52 x 55 (08)	Epigeous	-
VIC 30606	7.24 \pm 0.51	62 x 86 (22)	Epigeous	HQ693102
VIC 30607	6.82 \pm 0.52	75 x 96 (45)	Epigeous	-

Table 2. *Pisolithus* isolates with information concerning host, geographical origin and GenBank accession number

Isolate	Host plants	Locality	GenBank accession
PT90A	<i>Eucalyptus</i> sp.	Viçosa, Brazil	AF140547 ⁽¹⁾
RS26	<i>Eucalyptus</i> sp.	Viçosa, Brazil	AF142991 ⁽³⁾
MU98/5A	<i>E. globules</i>	Kudardup, Western Australia	AF374644 ⁽³⁾
MU98/9	<i>E. globules</i>	Scott R, Western Australia	AF374649 ⁽³⁾
MU98/2	<i>E. marginata</i>	Augusta, Western Australia	AF374641 ⁽³⁾
MU98/12	<i>Eucalyptus</i> sp.	Manjimup, Western Australia	AF374652 ⁽³⁾
MARX270	<i>P. elliottii</i>	Georgia, USA	AF37 4632 ⁽³⁾
PT301	<i>Pinus</i> sp.	Georgia, USA	AF143233 ⁽¹⁾
Pasoh01 clone 1	<i>Shorea macroptera</i>	Forest Service, Pasoh, Malaysia	AF415226 ⁽³⁾
MURU5134		Western Australia	AY179746 ⁽⁴⁾
PTJap (MH175)	<i>P. pumila/Betula ermanii</i>	Mt Iou, Shiretoko Peninsula, Hokkaido, Japan	AF37 4629 ⁽³⁾
FORBs05006	<i>B. maximowicziana, B. platyphila, Abies sachalinensis, Larix. Leptolepis</i>	Rankoshi	EF192104 ⁽²⁾
FORBs05010	<i>P. pumila, B. ermanii</i>	AkaAkan	EF192107 ⁽²⁾

⁽¹⁾ Gomes et al. (2000). ⁽²⁾ Kasuya et al. (2008). ⁽³⁾ Martin et al. (2002). ⁽⁴⁾ Thomas et al. (2003).

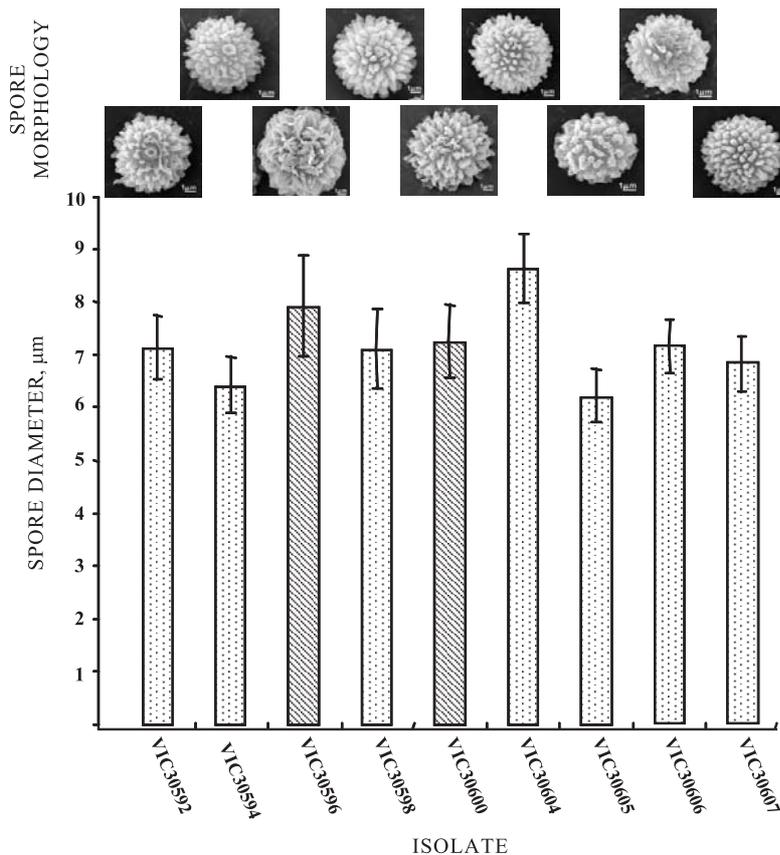


Figure 1. Morphology and spore diameter (n=20) of Brazilian *Pisolithus* isolates.

PCR amplification with the specific primers for the ITS region generated only band with 700 bp. In the phylogenetic analysis, all sequences in the

cladogram were divided into three major clades. The structures were similar to the phylogenetic cladogram in Martin et al. (2002). The sequences of all isolates

used in this study were distributed in clades 1 and 2, with strong bootstrap support (99 %) (Figure 2).

Clade 1 consisted of four species groups. The first species included isolates VIC 30596, VIC 30597 and VIC 30600 that grouped with MU98/5A and MU98/9, identified as *Pisolithus marmoratus*, isolated from *Eucalyptus globulus* in a forest of Australia (Martin et al., 2002). The second species group was the hypogeous *Pisolithus* called *P. hypogaeus*. The isolates MARX270 and PT301 formed a species group identified as *Pisolithus tinctorius*, both from the United States and isolates from *Pinus* forests. The last species group contained other species, all from Japan (Figure 2, Table 2).

The isolates were separated into two monophyletic groups with strong bootstrap support (100 %) in clade 2. One group included the isolates VIC 30593,

VIC 30598, VIC 30599, VIC 30602, VIC 30604, and VIC 30606, together with PT90A and RS26, also from Brazil (Figure 2, Tables 1 and 2), and was identified as *Pisolithus microcarpus* (Martin et al., 2002). The other group that included MU98/2 and MU98/12, was identified as *Pisolithus albus*, native to Australia (Martin et al., 2002) (Table 2, Figure 2).

DISCUSSION

The characteristic that distinguished the two species found in the studied area was spore ornamentation (Figure 1), as confirmed by molecular analysis (Figure 2). One group with short and erect spines was identified as *Pisolithus microcarpus*, and

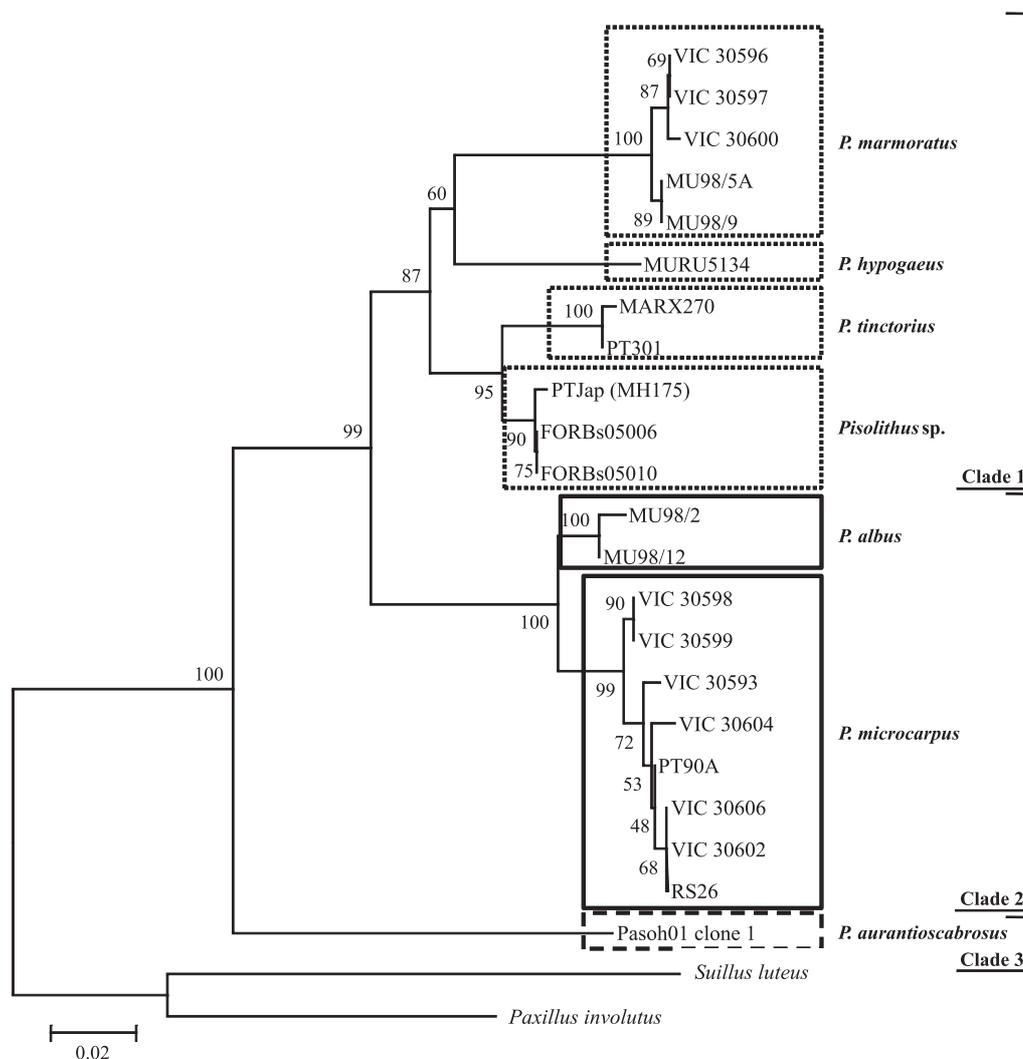


Figure 2. Cladogram obtained by phylogenetic relationships in *Pisolithus* based on the rDNA ITS sequence. The tree was rooted using *Paxillus involutus* and *Suillus luteus* as outgroups. Numbers in the branches indicate the bootstrap support based on 1000 replicates (if > 50%). The species were named as proposed by Martin et al. (2002).

the other with longer and curved spines as *Pisolithus marmoratus*.

Pisolithus microcarpus and *P. marmoratus* were described by Bougher & Syme (1998). Although native to Australia, isolates of this species were collected in exotic plantations worldwide (e.g. Brazil, China, Portugal, Senegal, and South Africa). *P. marmoratus* is considered the southern hemisphere counterpart of *P. tinctorius*. They are morphologically very similar (Bougher & Syme, 1998), but unlike *P. tinctorius*, *P. marmoratus* is associated with eucalypts.

The clustering of all *Pinus* isolates together is consistent, from the point of view that some degree of host specificity may exist within the *Pisolithus* taxa (Martin et al., 1998). Several previous studies have suggested that *Pisolithus* isolated from basidiomes occurring under *Pinus* spp. are poor colonizers of *Eucalyptus* spp. (Burgess et al., 1994; Junghans et al., 1998; Pereira et al., 2005). Moreover, in a molecular study of *Pisolithus* in Kenya, three taxa were identified as occurring only in either pine plantations, eucalyptus plantations or native *Azelia* vegetation (Martin et al., 1998).

Basidiome morphology has been used in *Pisolithus* taxonomy (Watling et al., 1995; Kanchanaprayudh et al., 2003; Thomas et al., 2003), but size and shape were not useful due to the possible influence of soil and environmental conditions and could not identify *Pisolithus* in Hokkaido (Kasuya et al., 2008). Although isolate VIC 30600 was found to be hypogaeous (Table 1), it was not included in the *P. hypogaeus* species, described occurring in Australia (Thomas et al., 2003). Instead, the isolate VIC 30600 presents similarity with the *P. microcarpus* spores and this date was confirmed by phylogenetic analysis.

Genetic diversity and host specificity of *Pisolithus* was reported in exotic *Eucalyptus* and native hosts in the western Mediterranean region. The ITS sequences suggested the occurrence of several ecological species adapted to exploit different soil types (basic, acid and clayey slate-derived soils), with specificity for particular indigenous hosts. Isolates from eucalypt plantations in Brazil, Kenya and the Mediterranean region grouped together with eucalypt-associated Australian isolates. No transfer to native hosts occurred; the host specificity range of these exotic strains may prevent out-competition and interbreeding with local species. The origin of *Pisolithus* spp. in eucalypt plantations in the Mediterranean basin is Australia; the co-introduction of the ectomycorrhizal fungi might explain the success of these exotic forest plantations (Díez et al., 2001).

In Thailand, the presence of at least three *Pisolithus* species was suggested. *Pisolithus* basidiomes collected in pine forests and under some *Shorea roxburghii* trees in a pine-dipterocarp forest corresponded to species 5, as previously described by

Martin and collaborators in 2002. The basidiomes collected under *S. roxburghii* and *Dipterocarp alatus* trees in the dipterocarp forests did not match any previously reported species. Basidiomes collected from eucalyptus plantations were all identified as *Pisolithus albus* (Kanchanaprayudh et al., 2003).

Australian eucalypts was introduced into Southeast Asia, India, Africa, South America, South Africa, and southern Europe in the last 200 year in form of seeds and saplings, which facilitated the dispersal of Australian *Pisolithus* (Martin et al., 2002).

The ITS sequences of rDNA of *Pisolithus* isolates were also determined to provide information on the genetic variability among isolates and to compare the sequences to other isolates described in the literature. This study should provide a better understanding of the taxonomy and variability of *Pisolithus* in Brazil, underlying the selection of fungal genotypes for forestry inoculation programs. Such programs are of key importance, since forest management is an essential part of Brazilian agriculture.

CONCLUSIONS

1. The trait spore ornamentation is a criterion to distinguish the *Pisolithus* species.
2. Molecular analysis confirmed the presence of two *Pisolithus* species in the State of Minas Gerais.
3. The two *Pisolithus* species in the study area were *Pisolithus microcarpus* and *Pisolithus marmoratus*.

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