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ORIGINAL PAPER

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# Molecular characterization of *Pisolithus* spp. isolates by rDNA PCR-RFLP

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**Abstract** Variation within ribosomal DNA (rDNA) genes of 19 isolates of *Pisolithus* from different geographic origins and hosts was examined by polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) analysis. The primers utilized amplify rDNA regions in a wide range of fungi. One amplified region includes the internal transcribed spacer (ITS), which has a low degree of conservation. The ITS amplification products (640–750 bp) were digested with a variety of restriction endonucleases. Cluster analysis based on the restriction fragments grouped the isolates into three distinct groups: group I contained isolates collected in the northern hemisphere, except Pt 1, group II contained those collected in Brazil and group III contained isolate Pt 1. Additional analysis of other rDNA regions, IGS, 17 S and 25 S rDNA, resulted in similar groups. The data suggest that the taxonomy and systematics of this ectomycorrhizal fungus should be revised.

**Key words** DNA polymorphism · Ectomycorrhizal fungi · PCR-RFLP · *Pisolithus* spp. · Ribosomal DNA

## Introduction

*Pisolithus tinctorius* (Pers.) Coker and Couch isolates are some of the most commonly used fungi for inoculation in controlled mycorrhization programs (Marx et al. 1982; Burgess et al. 1995). These fungi associate with many tree species, both angiosperms and gymnosperms, have a worldwide distribution, adapt to diverse environmental conditions, and are easily propagated in vitro (Marx et al. 1982; Cline et al. 1987).

Kope and Fortin (1990) suggested the occurrence of biological species within the *Pisolithus* complex after observing homokaryotic incompatibility and diversity in basidiospore spine morphology. Burgess et al. (1995) observed considerable heterogeneity among *Pisolithus* isolates collected from different regions of Australia when they analyzed phenotypic traits such as expression of polypeptides, culture type, basidiospore, and basidiome characteristics. They concluded that geographic origin, not host, was the main cause of phenotypic variation among Australian isolates.

In a previous paper, we reported extensive genetic diversity among 20 isolates of *Pisolithus* based on RAPD-PCR analysis (Junghans et al. 1998). The isolates were grouped according to their host and geographic origins, suggesting the existence of two genetically distinct groups, and showing the need for future investigations of taxonomy and systematics of these fungi. In this present work, we looked at the genetic variation among *Pisolithus* isolates by analysis of ribosomal DNA (rDNA) by RFLP (restriction fragment length polymorphisms).

The polymerase chain reaction (PCR) amplification of the rDNA region, coupled with RFLP can be used in taxonomic studies of ectomycorrhizal fungi, as it has already been shown to be a useful tool for distinguishing among fungus isolates at the species level (Egger and Fortin 1990; Gardes et al. 1990; Lobuglio et al. 1991). It has also been successfully applied in the identification of ectomycorrhizal fungi directly from mycorrhizal roots (Gardes et al. 1991; Henrion et al. 1992, 1994).

The rDNA genes in most eucaryote nuclear genomes are highly repeated on specific chromosomes, and contain regions that evolved at distinct rates which provide areas with varying degrees of sequence divergence for analysis. Genes coding the 17 S, 5.8 S, and 25 S (or their equivalents) are separated by spacer se-

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quences to form a single repeat unit which is arranged head-to-tail in multiple copies (Ibrahim et al. 1994). While the rDNA sub-units are highly conserved, the intergenic spacer (IGS) and internal transcribed spacer sequences (ITS) are polymorphic, and provide useful tools for taxonomic and phylogenetic studies (Henrion et al. 1994).

The main goal of the present study was to characterize 19 isolates of *Pisolithus*, from three different regions of the world, by the rDNA PCR-RFLP technique.

## Materials and methods

### Sample sources and growth conditions

Isolates of *Pisolithus* were obtained from the fungus collection at the Laboratory of Mycorrhizal Associations of the Department of Microbiology at the Federal University of Viçosa, MG, Brazil. These isolates, their respective geographical origins, and hosts are described in Table 1.

Cultures of *Pisolithus* were maintained in Petri dishes containing solid MMN medium (Marx 1969) for 25 days at 28 °C. Mycelia for DNA extraction were obtained by inoculating MMN liquid medium with agar plugs containing mycelia collected from the border of the colonies, followed by incubation for 25 days at 28 °C with no agitation.

### DNA extraction

DNA was extracted from 0.5–1.0 g of fresh mycelium according to procedures of Schäfer and Wöstemeyer (1992) as modified by Junghans et al. (1998). DNA concentration was estimated by comparison with known standards in 1% agarose gels stained with ethidium bromide.

### PCR amplification and RFLP analysis

The primer pairs used to amplify the ITS, IGS, 17 S rDNA, and 25 S rDNA have been described by Henrion et al. (1992) and were synthesized by GIBCO-BRL. All 19 isolates (Table 1) were used for amplification of the ITS and IGS rDNA regions. Five *Pisolithus* isolates from Brazil (IS 83, Pt 90A, Pt 90B, RS 20, and RS 26) and five from the northern hemisphere (Pt 185 R, Pt 301, Pt 1, Pt 5, and PF) were used for amplification of the 17 S region. Four isolates from Brazil (Pt 90A, Pt 90B, RS 20, and RS 26) and four from the northern hemisphere (Pt 301, Pt 1, Pt 5, and PF) were used for amplification of the 25 S region.

Amplification reactions (25 μl) of the ITS, IGS, 17 S, and 25 S were conducted according to Henrion et al. (1992). Amplification of the 25 S rDNA (25 μl) was performed with the following modifications: 120 pmol of each primer, 0.4 mM of each dNTP and 2 units of *Taq* DNA polymerase. The DNA samples were amplified in a thermocycler PTC-100 (MJ Research, Inc.), programmed for 40 cycles (94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min and 30 s). After the 40th cycle, a final extension step at 72 °C for 7 min was performed. For amplification of the 17 S region, the conditions were as follows: 94 °C for 5 min, 30 cycles (94 °C, 1 min; 50 °C, 1 min; 68 °C, 3 min) followed by 68 °C for 10 min. Amplification of the 25 S region was according to the amplification of the 17 S region except that the annealing temperature was 56 °C. DNA products were analyzed in a 1.5% agarose gel immersed in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) or were ethanol precipitated for restriction analysis. The DNA fragments were size-fractionated in 2% agarose gel, which was stained with ethidium bromide (0.5 μg/ml) and photographed under UV light with a Polaroid camera.

### Data analysis

Amplification products after restriction with endonucleases were scored as 1 (presence) or 0 (absence) and used for determination of genetic distances among the isolates (Nei and Li 1979). Genetic distances were used to cluster the isolates by the UPGMA method with the aid of the program Statistica 4.2 for Windows. Pearson correlation analyses were done with the aid of the same statistical program.

## Results

The ITS region, which includes the 5.8 S rDNA, was amplified for all 19 isolates. The sizes of the amplified ITS products were between 640 and 750 bp (Fig. 1A). Restriction enzymes *Hae* III, *Hin*f I, *Sau*3A I, and *Taq* I cleaved the ITS products into smaller polymorphic fragments. Figure 1B shows the pattern obtained with *Hin*f I. No sites for *Rsa* I were present in the ITS. *Hae*

**Table 1** Geographical origin and host tree species of *Pisolithus* isolates

| No | Isolate | Origin | Host |
|---|---|---|---|
| 1 | IS 83 | Viçosa - MG - Brazil | *Eucalyptus* |
| 2 | RV 82 | Viçosa - MG - Brazil | *Eucalyptus* |
| 3 | Pt 90A | Viçosa - MG - Brazil | *Eucalyptus* |
| 4 | Pt 90B | Viçosa - MG - Brazil | *Eucalyptus* |
| 5 | RS 20 | São Roque - Guaíba - RS - Brazil | *Eucalyptus* |
| 6 | RS 23 | Tabatinga -Guaíba - RS - Brazil | *Eucalyptus* |
| 7 | RS 24 | Figueirinha - Guaíba - RS - Brazil | *Eucalyptus* |
| 8 | RS 26 | Barba Negra - Guaíba - RS - Brazil | *Eucalyptus* |
| 9 | RS 27 | Barba Negra - Guaíba - RS - Brazil | *Eucalyptus* |
| 10 | Pt 26 | Santa Catarina - SC - Brazil | *Eucalyptus* |
| 11 | Pt 185 R | Georgia - USA | *Pinus taeda* |
| 12 | Pt 185 6.0R | Georgia - USA | *P. caribaea* |
| 13 | Pt 185 6.5R | Georgia - USA | *P. caribaea* |
| 14 | Pt 301 | Georgia - USA | *Pinus* |
| 15 | Pt 303 | Georgia - USA | *Pinus* |
| 16 | Pt 5 | Oregon - USA | unknown |
| 17 | PF | France | unknown |
| 18 | Pt France | France | unknown |
| 19 | Pt 1 | Oregon - USA | unknown |

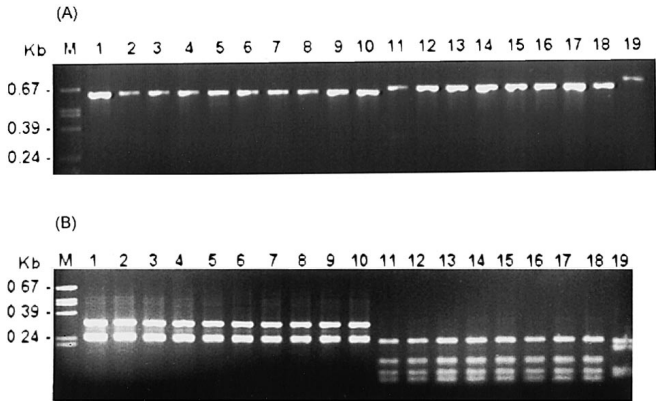


**Fig. 1** Gel electrophoresis of the amplified ITS rDNA region (**A**) and this region digested with the restriction enzyme *Hin*f I (**B**) of 19 isolates of *Pisolithus*. Lane numbers correspond to isolate identification given in Table 1. Fragment size markers in base pairs are indicated by M (pUC 19 DNA digested with *Hin*f I-*Rsa* I)

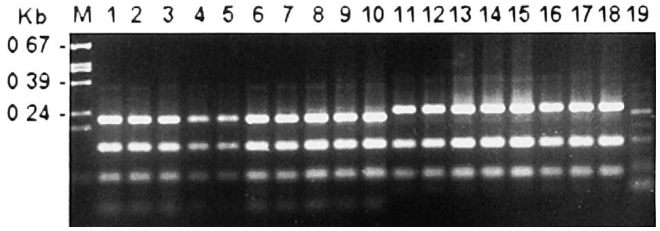


**Fig. 2** Gel electrophoresis of the amplified IGS rDNA region of 19 isolates of *Pisolithus* digested with the restriction enzyme *Rsa* I. Lane numbers correspond to isolate identification given in Table 1. Fragment size markers in base pairs are indicated by M (pUC 19 DNA digested with *Hin*f I-*Rsa* I)

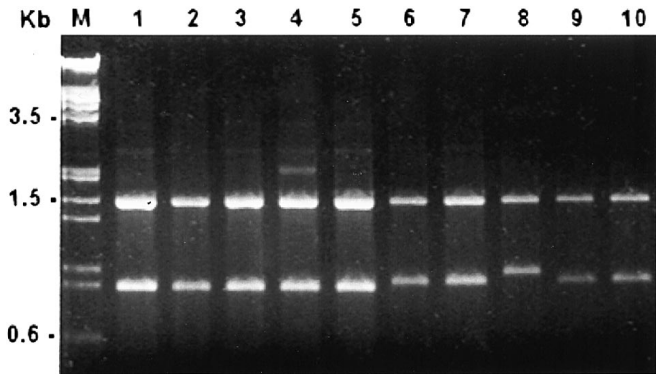


**Fig. 3** Gel electrophoresis of the amplified 17 S plus ITS rDNA regions for 10 isolates of *Pisolithus*. Amplified regions were digested with the restriction enzyme *Eco*R I. (1) IS 83, (2) Pt 90A, (3) Pt 90B, (4) RS 20, (5) RS 26, (6) Pt 185 R, (7) Pt 301, (8) Pt 1, (9) Pt 5, (10) PF. Fragment size markers in base pairs are indicated by M (Lambda DNA digested with *Eco*R I-*Hin*d III)

III cleaved all isolates from the northern hemisphere except Pt 1, but cleaved none of the isolates collected in Brazil. *Hin*f I, *Sau*3A I, and *Taq* I cleaved the DNA of all 19 isolates (Table 2).

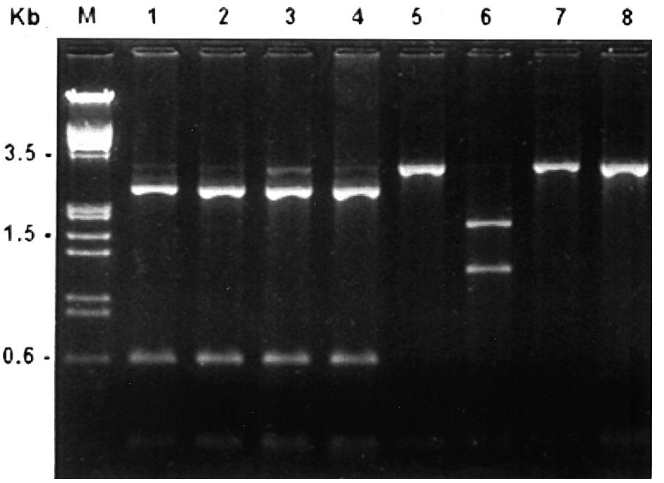


**Fig. 4** Gel electrophoresis of the amplified 25 S rDNA region for 8 isolates of *Pisolithus*. Amplified 25 S was digested with the restriction enzyme *Eco*R I. (1) Pt 90 A, (2) Pt 90B, (3) RS 20, (4) RS 26, (5) Pt 301, (6) Pt 1, (7) Pt 5, (8) PF. Fragment size markers in base pairs are indicated by M (Lambda DNA digested with *Eco*R I-*Hin*d III)

The proximal IGS region was amplified as a monomorphic band of 450 bp in all 19 isolates. Restriction analyses of this band with *Sau*3A I revealed no polymorphism. However, polymorphic fragments were obtained with enzymes *Hin*f I, *Rsa* I (Fig. 2) and *Taq* I (Table 2). Amplification of the 17 S and 25 S rDNA produced fragments of 2300 and 3200 bp, respectively. Cleavage of 17 S with *Eco*R I (Fig. 3), *Hin*d III, *Rsa* I and *Xho* I, and of 25 S with *Bgl* II, *Eco*R I (Fig. 4) and *Hin*f I generated polymorphic fragments (Table 3).

Based on restriction products from all four rDNA regions analyzed, genetic distance matrices were constructed (not shown). These matrices show the genetic distances to be 0–90%, with the greatest distances between the Brazilian isolates and those from the northern hemisphere. The genetic distances among the Brazilian isolates are close to zero. The distances among the isolates collected in France and USA are 0–89%. Cluster analyses based on genetic distances for all four rDNA regions analyzed were in accord (Fig. 5), and separated three groups: group I contains the isolates collected in the northern hemisphere, except Pt 1, group II contains the isolates from Brazil, and group III contains only Pt 1 (Fig. 5).

## Discussion

Ribosomal DNA PCR-RFLP analyses have been used to help resolve taxonomic problems concerning several fungus species (Bruns et al. 1991). rDNA is a convenient region to help characterize ectomycorrhizal fungi in different phases of their life cycles (Henrion et al. 1992; Erland et al. 1994; Henrion et al. 1994; Paolocci et al. 1995). It is a relatively conserved region which can be easily amplified with specific primers anchored in-

**Table 2** Restriction fragments (bp) of the ITS and IGS rDNA amplified regions of *Pisolithus* isolates produced by different restriction enzymes. Identification of the isolates corresponds to Table 1

| Amplified region/ enzyme | Isolates | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| ITS/*Hae* III | 620 | 620 | 620 | 620 | 620 | 620 | 620 | 620 | 620 | 620 | 530 | 530 | 530 | 530 | 530 | 530 | 530 | 530 | 690 |
| | | | | | | | | | | | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | |
| ITS/*Hin*f I | 350 | 350 | 350 | 350 | 350 | 350 | 350 | 350 | 350 | 350 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 240 |
| | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 160 | 160 | 160 | 160 | 160 | 160 | 160 | 160 | 210 |
| | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 130 | 130 | 130 | 130 | 130 | 130 | 130 | 130 | 130 |
| | | | | | | | | | | | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 120 |
| | | | | | | | | | | | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 |
| ITS/*Sau*3A I | 240 | 240 | 240 | 240 | 240 | 240 | 240 | 240 | 240 | 240 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 320 | 250 |
| | 220 | 220 | 220 | 220 | 220 | 220 | 220 | 220 | 220 | 220 | 170 | 170 | 170 | 170 | 170 | 170 | 170 | 170 | 160 |
| | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 140 |
| | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | 80 | | | | | | | | | 100 |
| ITS/*Taq* I | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 180 |
| | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 150 | 110 |
| | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 90 |
| | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 60 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 60 |
| | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | 40 | | | | | | | | | |
| IGS/*Hin*f I | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 170 | 170 | 170 | 170 | 170 | 170 | 170 | 170 | 250 |
| | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 160 | 160 | 160 | 160 | 160 | 160 | 160 | 160 | 170 |
| | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 | 120 |
| IGS/*Rsa* I | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 250 | 240 |
| | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 140 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 210 | 150 |
| | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 60 |
| IGS/*Sau*3A I | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 |
| | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 | 230 |
| IGS/*Taq* I | 280 | 280 | 280 | 280 | 280 | 280 | 280 | 280 | 280 | 280 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 | 260 |
| | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 130 | 130 | 130 | 130 | 130 | 130 | 130 | 130 | 160 |
| | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 30 | 50 |

**Table 3** Restriction fragments (bp) of the 17S and 25S rDNA amplified regions of *Pisolithus* isolates produced by different restriction enzymes

| Amplified region/ enzyme | Isolates | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | IS 83 | Pt 90A | Pt 90B | RS 20 | RS 26 | Pt 185 R | Pt 301 | Pt 5 | PF | Pt 1 |
| 17S/*Eco* RI | 1530 | 1530 | 1530 | 1530 | 1530 | 1490 | 1490 | 1490 | 1490 | 1490 |
| | 820 | 820 | 820 | 820 | 820 | 870 | 870 | 870 | 870 | 890 |
| 17S/*Hin*d III | 2360 | 2360 | 2360 | 2360 | 2360 | 2160 | 2160 | 2160 | 2160 | 2580 |
| | | | | | | 310 | 310 | 310 | 310 | |
| 17S/*Rsa* I | 1490 | 1490 | 1490 | 1490 | 1490 | 1540 | 1540 | 1540 | 1540 | 1540 |
| | 810 | 810 | 810 | 810 | 810 | 850 | 850 | 850 | 850 | 890 |
| 17S/*Xho* I | 1710 | 1710 | 1710 | 1710 | 1710 | 1710 | 1710 | 1710 | 1710 | 2610 |
| | 710 | 710 | 710 | 710 | 710 | 730 | 730 | 730 | 730 | |
| 25S/*Bgl* II | | 1660 | 1660 | 1660 | 1660 | | 1660 | 1660 | 1660 | 1660 |
| | | 1420 | 1420 | 1420 | 1420 | | 1420 | 1420 | 1420 | 1420 |
| 25S/*Eco* RI | | 2440 | 2440 | 2440 | 2440 | | 2970 | 2970 | 2970 | 1720 |
| | | 570 | 570 | 570 | 570 | | 250 | 250 | 250 | 1170 |
| | | 250 | 250 | 250 | 250 | | | | | 250 |
| 25S/*Hin*f I | | 520 | 520 | 520 | 520 | | 480 | 480 | 480 | 480 |
| | | 480 | 480 | 480 | 480 | | 450 | 450 | 450 | 410 |
| | | 470 | 470 | 470 | 470 | | 440 | 440 | 440 | 370 |
| | | 430 | 430 | 430 | 430 | | 410 | 410 | 410 | 360 |
| | | 380 | 380 | 380 | 380 | | 370 | 370 | 370 | 330 |
| | | 330 | 330 | 330 | 330 | | 320 | 320 | 320 | 310 |
| | | 310 | 310 | 310 | 310 | | 310 | 310 | 310 | 260 |
| | | 270 | 270 | 270 | 270 | | | | | |

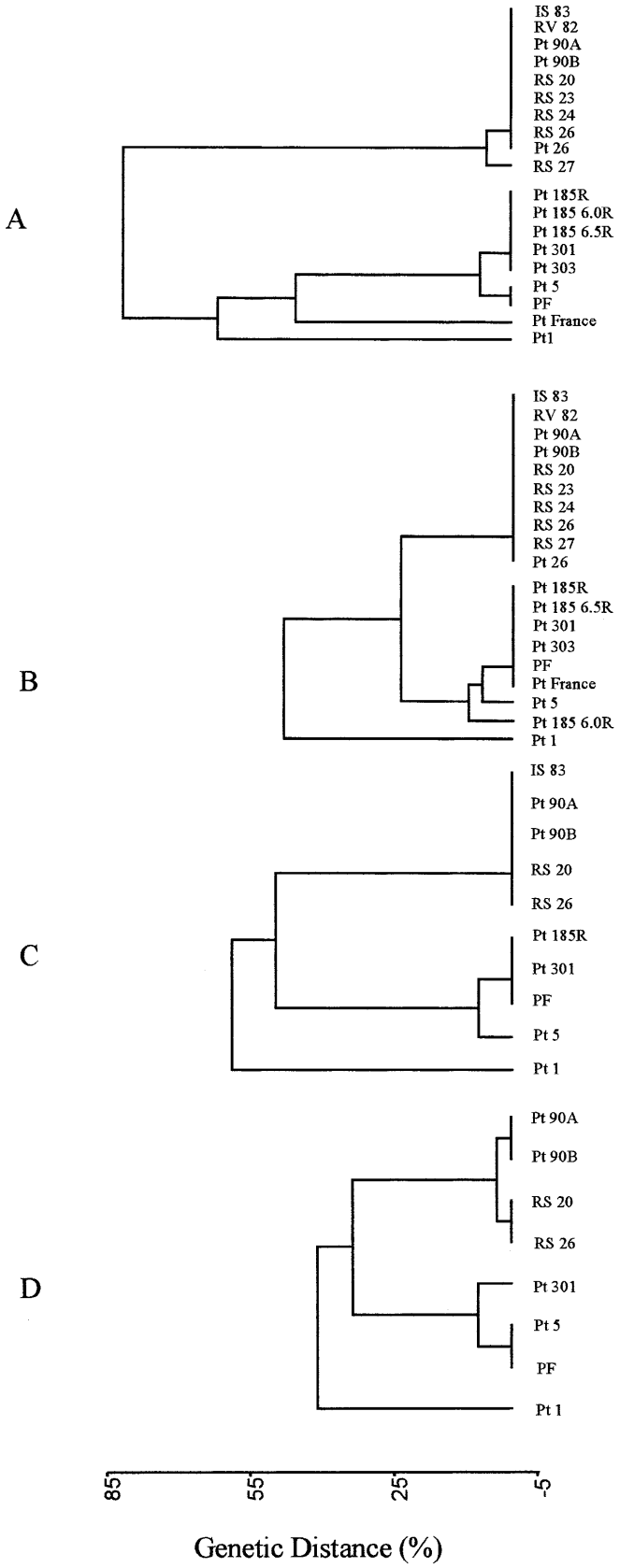


**Fig. 5** UPGMA cluster diagram of relationships among *Pisolithus* isolates. The dendrograms are based on genetic similarity coefficients determined from restriction enzyme patterns of rDNA. (A) ITS, (B) IGS, (C) 17 S, (D) 25 S

side the highly conserved regions of the structural genes. The multicopy nature of the rDNA also makes the ITS region easy to amplify even from small amounts of DNA (Henrion et al. 1994).

In this work, RFLP analyses of approximately 6.6 kb of the rDNA region revealed extensive polymorphism among isolates of *Pisolithus* collected in different regions of the world. The amplified ITS region was between 640 and 750 bp (Fig. 1A), which is in accordance with the sizes obtained for other ectomycorrhizal fungi. Gardes et al. (1991) studied the ITS region of 10 species of ectomycorrhizal fungi and found sizes varying from 600 to 800 bp.

Among analyzed regions, only the ITS showed polymorphism before treatment with restriction enzymes (Fig. 1A). This type of polymorphism has been observed in several ectomycorrhizal fungi (Gardes et al. 1991; Henrion et al. 1992, 1994). However, according to Rogers and Bendich (1987), the IGS in most fungi is the most polymorphic region because of unequal crossing-over involving sub-repetitive sequences within the IGS. Nevertheless, even after cleavage with restriction enzymes, the IGS in our work showed a lower degree of polymorphism than the ITS (Table 2).

The IGS in *Pisolithus* could be amplified with a primer anchored in the 5 S rDNA, indicating that this gene is within the IGS region. This has been observed in other fungi of the subdivision Basidiomycotina (Vilgalys and Gonzales 1990). This arrangement also occurs in some ascomycetes (Bell et al. 1977), although in *Neurospora crassa* and other ascomycetes the 5 S gene is organized in a complex way spread along the genome (Kim et al. 1992).

We also observed polymorphism in the 17 S region cleaved with restriction enzymes (Fig. 3; Table 3). It is important to note that the primers used to amplify 17 S also amplify the ITS region (Henrion et al. 1992). However, there are no sites for the enzymes *Eco*R I, *Rsa* I and *Xho* I in the ITS. Consequently all of the polymorphisms revealed by these enzymes reflect variation present specifically within the 17 S region.

Cluster analysis based on these data divided the isolates into three distinct groups (Fig. 5), which roughly coincide with their geographic origins and the type of host from which the isolates were collected. However, because geographic origin and host affinity co-vary for these isolates, we can draw no conclusion regarding their relative effect. Nevertheless, Burgess et al. (1995) compared 85 Australian isolates from a range of hosts, basidiocarp types, and geographic locations, and found four groups using numerical taxonomic analysis of 30 soluble polypeptides identified by SDS-PAGE. They observed a relationship between the groups defined by polypeptide analysis and mycorrhiza development and function, and suggested that such a grouping technique could be used to select *Pisolithus* isolates likely to stimulate eucalypt growth.

Our study confirms the findings of Junghans et al. (1998) in which the genetic diversity of most of the iso-

lates analyzed in this work was studied by RAPD-PCR analysis. Pearson correlation analyses between the data we obtained and those obtained by Junghans et al. (1998) showed coefficients of 0.81 (RAPD/ITS) and 0.78 (RAPD/IGS) ($P<0.05$). Similar groups were defined by both works, although the RAPD analysis yielded greater genetic distances among the isolates than found in this work. In both studies, the degree of genetic variation between *Pisolithus* isolates collected in Brazil and those collected in the northern hemisphere is high, indicating that these fungi are extremely heterogeneous. However, among Brazilian isolates, the genetic distance is close to 0% (Fig. 5). It is possible that the reason for the low genetic diversity among isolates collected in Brazil is that only a small number of *Pisolithus* isolates were introduced from Australia together with *Eucalyptus* plants at the beginning of this century. In accord with this, Burgess et al. (1995) observed that *Pisolithus* isolates collected from *Eucalyptus* plantations in Brazil and South Africa are geneticall y very close to Australian isolates.

rDNA PCR-RFLP analysis is an appropriate and sensitive method for ecological and taxonomic studies of *Pisolithus*. Further analysis of rDNA clusters, using intensive restriction mapping or sequencing of amplified rDNA regions and alignment of these sequences, will allow the design of an appropriate DNA probe for a specific isolate. This could then be used to investigate the success of inoculation in the field. Moreover, several authors have suggested a need for taxonomic revision of the genus *Pisolithus*. Our work supports this idea and demonstrates a useful molecular tool for its implementation.

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