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D.T. Junghans · E.A. Gomes · W.V. Guimarães
E.G. Barros · E.F. Araújo

Genetic diversity of the ectomycorrhizal fungus *Pisolithus tinctorius* based on RAPD-PCR analysis

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Abstract Twenty *Pisolithus tinctorius* isolates from different geographic locations and different hosts were characterized by the random amplified polymorphic DNA technique. Thirteen arbitrary primers generated 87 DNA fragments, all of them polymorphic. These data were used to calculate genetic distances among the isolates. The pairwise genetic distances ranged from 1 to 100%, with an average of 58.7%. Cluster analysis based on the amplified fragments grouped the isolates according to their host and geographical origins. Group I contained isolates collected in Brazil and group II those collected in the Northern Hemisphere. In addition to the diversity seen at the molecular level, the isolates also showed host specificity. Greenhouse experiments demonstrated that isolates from the Northern Hemisphere colonized mainly *Pinus* whereas isolates from Brazil colonized only *Eucalyptus*. The molecular data suggest that the *Pisolithus tinctorius* isolates analyzed belong to two distinct groups. The data also suggest new guidelines for future investigations on the taxonomy and systematic of this important fungus species. Furthermore, these results support future experiments aimed at the selection and development of improved isolates of *P. tinctorius*.

Key words DNA polymorphism · Ectomycorrhizal fungi · Genetic diversity · *Pisolithus tinctorius* · RAPD

D. T. Junghans · E. A. Gomes · W. V. Guimarães
E. F. Araújo (✉)
Universidade Federal de Viçosa, Departamento de Microbiologia/BIOAGRO, Viçosa, MG, 36571-000 - Brazil
Fax: +55-31-899-2573; e-mail: ezfa@mail.ufv.br

E. G. Barros
Universidade Federal de Viçosa, Departamento de Biologia Geral/BIOAGRO, Viçosa, MG, 36571-000 - Brazil

Introduction

The importance of the association between roots of trees and ectomycorrhizal fungi is very well established (Harley and Smith 1983). This association leads to increased supply of phosphorus to the host plant and consequently a positive effect on growth (Bougher et al. 1990; Thomson et al. 1994). *Pisolithus tinctorius* (Pers.) Coker and Couch is one of the most widespread ectomycorrhizal fungi. It has been found in 33 different countries and in 38 of North American states (Marx 1977). It is one of the most promising fungi for production of inoculum for forest trees, and has mainly been used in controlled inoculation programs of *Eucalyptus* (Garbaye et al. 1988) and *Pinus* (Marx and Bryan 1971). It can be cultivated easily in the laboratory, and the golden-brown mycorrhiza can be scored easily in the field. Additionally, its adaptation to high temperatures is an advantage in relation to other mycorrhizal fungi (Le Tacon et al. 1987). *Pisolithus tinctorius* has been classified as a basidiomycete in the order Sclerodermatales and family Sclerodermataceae (Hutchison 1991). The taxonomy of the genus is unclear, and it may comprise a number of species (Kope and Fortin 1990; Burgess et al. 1995). Because of its ample geographic distribution and morphological diversity, at least 47 different denominations have been used to refer to this fungus (Grand 1976).

Pisolithus tinctorius has demonstrated substantial intraspecific variability for a number of traits, including colony morphology, growth rate, enzyme activity, phytohormone production (Ho 1987a), optimal temperature for axenic growth (Cline et al. 1987), cultural characteristics, and mycorrhiza formation ability (Marx 1981; Burgess et al. 1994). Such diversity is extremely important for the adaptability of the species, and provides a means for sustained forest productivity under different environmental conditions (Martin et al. 1994). For this reason, knowledge of the genetic diversity of this fungus is of importance.

Molecular markers, particularly those based on DNA, are a reliable tool for determining genetic diversity among individuals. Studies with random amplified polymorphic DNA–PCR (RAPD-PCR) (Lanfranco et al. 1993; Tommerup et al. 1995), PCR amplification of ribosomal DNA (Henrion et al. 1992, 1994), restriction fragment length polymorphism (RFLP) (Armstrong et al. 1989), amplifield fragment length polymorphism (AFLP) (Majer et al. 1996), microsatellites (Sastry et al. 1995) and other types of DNA-based markers have become increasingly popular for such purposes. RAPD-PCR is a very simple procedure by which specific genomic DNA fragments can be amplified with the aid of oligonucleotide primers of random sequences. Such molecular markers have been also used in studies of taxonomy (Lanfranco et al. 1993) and genetic mapping of an ectomycorrhizal fungus (Doudrick et al. 1995). In the present work, this technique was used to determine the genetic diversity among 20 isolates of *P. tinctorius* collected in *Eucalyptus* and *Pinus* forests in three different countries.

Materials and methods

Genetic material

All isolates were obtained from the culture collection of the Department of Microbiology, Federal University of Viçosa, MG, Brazil. Details of the origin and the original hosts of the isolates are given in Table 1.

Maintenance of isolates

The isolates were initially grown in MMN-agar medium (Marx 1969) for 30 days. Then agar plugs were cut off from the edge of actively growing colonies and transferred to sterile distilled water at 4 °C. Every 3 months, the cultures were renewed by repeating this procedure. For mycelium production, 500-ml Erlenmeyer flasks containing 150 ml of MMN medium were inoculated with four 5-mm agar plugs from 30-day-old cultures, and incubated for 2–3 weeks at 28 °C.

DNA extraction and amplification

DNA was extracted from 0.5–1.0 g mycelium, according to Schäfer and Wöstemeyer (1992), with the following modifications: β-mercaptoethanol was added to the extraction buffer to a final concentration of 1% and the deproteinization step was performed twice with chloroform-isoamyl alcohol. The final pellet was resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing RNAse (50 mg/ml). DNA concentration was estimated by comparison with known standards in 1% agarose gels stained with ethidium bromide. PCR amplification of DNA sequences was performed with each of 20 random primers (Kit AG, 01–20, Operon Technologies Inc., Alameda, Calif, USA). Each 25-µl PCR reaction contained 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 µM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.4 µM of a random primer, 25 ng DNA and 1 unit of *Taq* DNA polymerase. Reactions were performed in a 9600 model thermocycler (Perkin-Elmer Cetus, Norwalk, Conn, USA) programmed for 40 cycles, each consisting of one denaturing step of 15 s at 94 °C, one annealing step of 30 s at 35 °C, and one extension step of 1 min at 72 °C. After the 40th cycle, a final extension step was performed for 7 min. For each primer used, there was a negative control in which target DNA was replaced by TE. Amplification products were loaded onto 1.2% agarose gels immersed in TBE buffer (90 mM Tris-borate, 1 mM EDTA, pH 8.0) and run for 3–4 h at 100 V. The gels were then stained with ethidium bromide (0.5 µg/ml) and photographed under UV light with a Polaroid camera.

Data analysis

Presence (1) or absence (0) of specific DNA amplification products was scored and used to calculate the pairwise genetic distances among the isolates (Nei and Li 1979). Only intense reproducible bands were considered. Cluster analysis based on the genetic distances were calculated using the unweighted pair-group method using arithmetic means (UPGMA) available in the program Statistica 4.2 for Windows.

Table 1 Geographical origin and original host tree species of *Pisolithus tinctorius* 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 27	Santa Catarina, SC, Brazil	*Eucalyptus*
12	Pt 185R	Georgia, USA	*Pinus taeda*
13	Pt 185 6,0R	Georgia, USA	*Pinus caribaea*
14	Pt 185 6,5R	Georgia, USA	*Pinus caribaea*
15	Pt 301	Georgia, USA	*Pinus*
16	Pt 303	Georgia, USA	*Pinus*
17	Pt 306	Georgia, USA	*Pinus*
18	Pt 5	Oregon, USA	unknown
19	Pt France	France	unknown
20	PF	France	unknown

Fungal inoculation in the greenhouse

Seeds from *Eucalyptus grandis* and *Pinus elliottii* were sterilized in a 30% (v/v) hydrogen peroxide solution for 10 min, washed thoroughly with sterile distilled water and germinated in a washed and autoclaved quartz sand bed. The seedlings were watered daily until transplantation. Twenty days after germination, the seedlings were transferred to plastic pots containing the same substrate and were inoculated with 10 agar plugs containing *P. tinctorius* mycelium (isolates Pt 90A, RS 20, Pt 303, Pt 5, and Pt France) from cultures grown for 20 days at 28 °C in MMN solid medium. Uninoculated control seedlings were produced in pots without mycorrhizal cultures. The pots were arranged in five randomized complete blocks. A second inoculation was performed 30 days later using slurry inoculum. The plants were watered daily with distilled water and once a week with a nutritive solution (Clark 1975). After 3 months, the plants were harvested. The roots were washed, cut into 1- to 2-cm-long pieces and stored in 50% ethanol. These segments were assessed for mycorrhizal colonization under a binocular microscope (×40) using the grid-line intercept method of Giovanetti and Mosse (1980).

Results

Out of 20 primers tested, 13 (OPAG 03, 04, 06, 07, 08, 09, 11, 13, 14, 15, 17, 18 and 20) produced strong reproducible bands and were used in this study. These primers amplified a total of 87 DNA bands. No bands were present in negative controls. All fragments considered for analysis were polymorphic. Figure 1 shows the amplification pattern obtained with primer OPAG03. The number of fragments per primer varied between 4 and 8 with an average of 6.7. The sizes of the bands were between 320 and 2,900 base pairs.

Pairwise genetic distances among the isolates (Table 2) ranged from 1% (isolates 12 and 13; 13 and 15, from Georgia, USA) to 100% (isolates 6 and 20, one from Brazil and the other from France).

Cluster analysis based on these data grouped the isolates into two large groups: group I formed by all isolates collected in Brazil, except Pt 27 (No. 11) and group II formed by all isolates from the Northern Hemisphere, except Pt France (No. 19) (Fig. 2). The shortest genetic distance within group I was 8% and the largest 58%, with an average distance of 34.7%. The shortest genetic distance within group II was 1% and the largest 45%, with an average of 21.2% (Table 2). Considering all isolates used in this study, the mean genetic distance was 58.7%, demonstrating the great diversity among the 20 isolates of *P. tinctorius* analyzed.

Within group I there was a strong tendency for isolates from the same Brazilian regions, e.g. Minas Gerais (east) and Rio Grande do Sul and Santa Catarina (south), to form clusters (Fig. 2). In group II, all isolates from Georgia-USA could be separated from those collected in France or Oregon, USA. Isolate Pt 27, col-

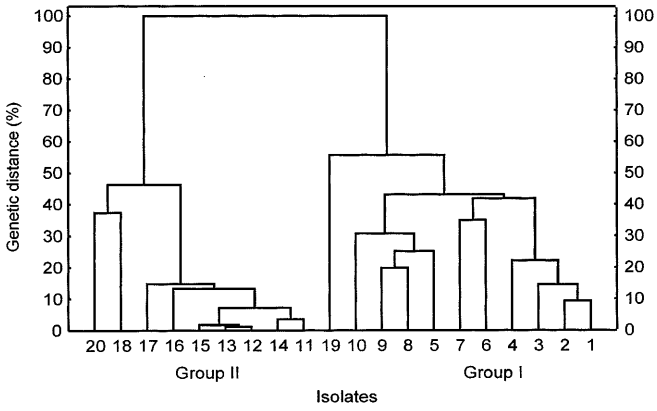

Fig. 2 Cluster analysis of 20 isolates of *Pisolithus tinctorius* based on RAPD-PCR data. The dendrogram was generated from genetic similarity coefficients obtained from the presence or absence of 87 DNA bands based on the unweighted pair-group method using the arithmetic average. Isolates are identificated in Table 1

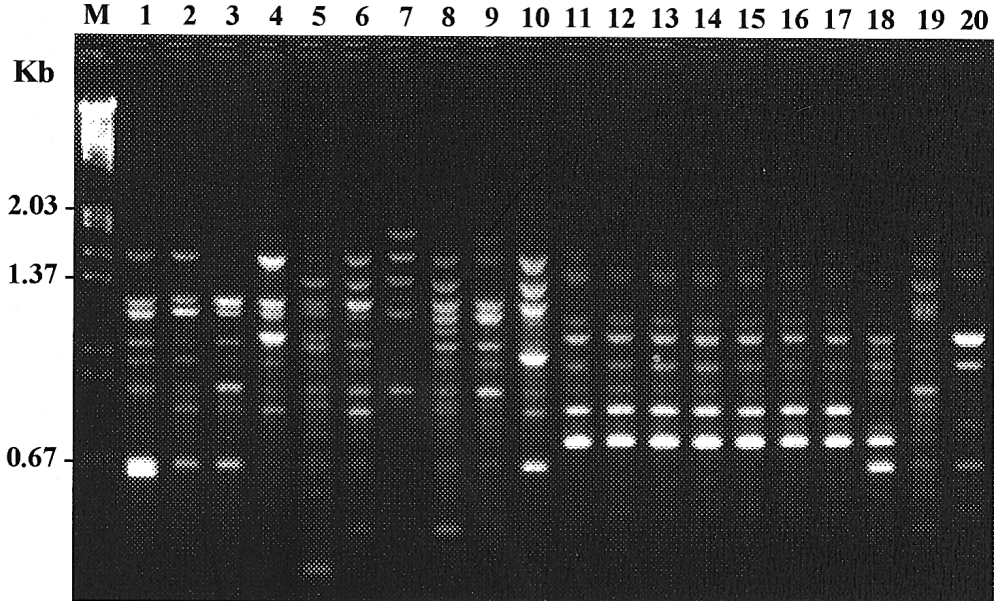

Fig. 1 Gel electrophoresis of random amplified polymorphic DNA (RAPD) fragments obtained with primer OPAG03 for 20 isolates of *Pisolithus tinctorius*. Lane numbers are identical to the isolate identification numbers in Table 1. Molecular weight size markers are indicated by *M*

Table 2 Pairwise genetic distances (%) among 20 isolates of *Pisolithus tinctorius.* Matrix calculated from 87 DNA bands yielded by 13 primers. Isolate details are given in Table 1

2	8																	
3	12	13																
4	22	19	16															
5	32	29	29	37														
6	40	40	36	42	28													
7	35	31	35	28	31	30												
8	42	42	39	47	23	43	35											
9	42	38	42	43	20	43	31	17										
10	30	33	33	38	33	44	36	24	22									
11	83	84	84	85	92	88	85	84	84	77								
12	88	92	89	91	92	94	94	83	86	76	5							
13	86	89	86	88	89	90	91	81	84	74	4	1						
14	84	84	84	86	89	88	86	82	82	78	3	8	7					
15	86	89	86	88	89	91	91	81	84	74	5	2	1	8				
16	90	91	91	93	82	89	93	81	82	82	14	9	10	15	9			
17	85	83	86	89	93	92	88	83	87	77	8	13	13	12	11	19		
18	93	86	90	92	86	88	92	83	84	77	37	38	39	40	40	41	33	
19	48	55	51	58	37	33	53	51	44	47	77	77	77	78	77	79	83	74
20	91	88	92	91	88	100	91	84	81	78	42	44	45	42	42	42	30	32	82
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

lected in the state of Santa Catarina, Brazil under a *Eucalyptus* plantation, did not show the ability to colonize *E. viminalis* and *E. dunnii* (Oliveira et al. 1994) or *E. grandis* and *E. urophylla* (M.C.M. Kasuya personal communication), which could explain its position in group II.

To determine both the ability to form mycorrhiza and the host specificity, two different host species, *Eucalyptus* and *Pinus*, were inoculated with five of the isolates. Interestingly, the three isolates collected in the Northern Hemisphere colonized mainly *Pinus* whereas the two isolates collected in Brazil colonized *Eucalyptus* only (Table 3).

Discussion

RAPD-PCR analysis has already been employed effectively for assessing the degree of genetic variation in a range of mycorrhizal fungi (Lanfranco et al. 1993; Perotto et al. 1995; Wyss and Bonfante 1996). In addition to markers located at active regions of the genome, this type of analysis considers a large number of neutral DNA markers which are independent of gene expression. Consequently, it is a sensitive, potent tool for genetic variability studies. The use of randomly selected primers allowed evaluation of the genetic diversity among *P. tinctorius* isolates sampled from different regions and hosts (Table 1). The polymorphisms identified were extensive. No monomorphic band was observed among the 20 isolates with the 13 primers used (87 bands). Considering all 20 isolates in this study, a high level of genetic distance was observed (58.7% on average, Table 2). However, *P. tinctorius* isolates from the same regions tended to be grouped in the same cluster (Fig. 2).

Measures of genetic diversity in fungi, specially ascomycetes and basidiomycetes producing spores with potential for long-distance dispersal, often show a low level of differentiation (Hamelin et al. 1995). Isolates of *Tuber magnatum* Pico, an ascomycete ectomycorrhizal fungus, from the Central and North Italy shared 80–90% of fragments amplified (Lanfranco et al. 1993). A band-sharing index of 65–80% was found among strains of basidiomycetes in the genera *Amanita*, *Boletus* and *Lactarius*, reflecting their low variability (Haudek et al. 1996).

The high degree of genetic diversity in the case of *P. tinctorius* may be explained by a close interaction with hosts and soil conditions leading to localized dispersal of a single genotype. This specificity is confirmed by the low capacity of isolates collected under *Eucalyptus* to colonize *Pinus* seedlings and vice versa (Table 3). Host or geographic origin appeared to be important in the grouping of *P. tinctorius.* All isolates originally associated with *Pinus* (Northern Hemisphere) or *Eucalyptus* (Southern Hemisphere) fell into two distinct groups, showing that the distribution of genetic variability could be related to geographic origin and host specificity. Association of individual isolates with specific host genera has been reported previously to be a major source of phenotypic variation for *Laccaria* (Ho 1987b), *Suillus* (Jacobson and Miller 1992) and *Pisolithus* (Lei et al. 1990).

Table 3 Percentage mycorrhizal colonization of *Eucalpytus grandis* and *Pinus elliottii* root segments upon inoculation with *Pisolithus tinctorius* isolates

Isolate	*Eucalyptus grandis*	*Pinus elliottii*
Pt 90A	60.0	0
RS 20	50.8	0
Pt 303	2.8	97.1
Pt 5	5.2	92.5
Pt France	0	95.1

Another possible reason for the presence of a high level of genetic diversity among *P. tinctorius* isolates is the sharing of distant ancestors. In Brazil, this fungus is a very common inhabitant of *Eucalyptus* plantations, an exotic tree from Australia which is uncommon in the Northern Hemisphere, where *P. tinctorius* is found among pines and oaks (Trappe 1962). Further studies with Brazilian, Australian and North American isolates using rDNA could explain phylogenetic relationships within the genus *Pisolithus*. Martin et al. (1996) used ITS (rDNA internal transcribed spacers) and IGS (rDNA intergenic spacers) to assess the genetic variability of three *Pisolithus* morphotypes in Kenya and found high variability (34–45%), suggesting the absence of interbreeding among those morphotypes of *Pisolithus*.

Recently, great genetic diversity was observed among 85 Australian and 15 non-Australian *Pisolithus* isolates, which were classified according to morphological and cultural characteristics and polypeptide patterns on 1D SDS-PAGE (Burgess et al. 1995). The polypeptide patterns alone, or in combination with basidiospore and culture characteristics, resulted in groups corresponding to host species and geographic location. The authors pointed out that the taxonomy of this genus is currently unclear, and that it may be composed of a number of species.

To our knowledge, this is the first report of a study on the genetic diversity of *P. tinctorius* from different hosts and geographical origins using RAPD-PCR markers. These markers could also be used to select individuals for a large genetic base in controlled mycorrhization programs, and to determine the survival, growth, and dissemination of introduced fungi within a microbial community.

Our results suggest that the *P. tinctorius* isolates analyzed belong to two genetically distinct groups, and provide for future investigations on the taxonomy and systematics of this important fungal species. Only the elucidation of the species concept can lead to an understanding of biodiversity within *P. tinctorius* and thus its ecological importance.

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