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A PCR/RFLP technique to characterize fungal species in *Eucalyptus grandis* Hill ex. Maiden ectomycorrhizas

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Abstract With the increasing awareness of the significance of mycorrhizas, research is focusing on studies to elucidate the contribution of the symbiosis to ecosystem dynamics. In this sense, molecular biology has acquired great significance. PCR/RFLP techniques were adapted to characterize ectomycorrhizal fungi associated with *Eucalyptus grandis.* The ITS region of the fungal rDNA from pure cultures and from of mycorrhizas synthesized in vitro was amplified. Primers NSA3/NLC2 were used followed by a nested reaction with primers ITS1F/NLB3. Amplicons were then digested with the enzymes *Mbo*I, *Hinf*I and *Taq*I. Amplification resulted in a 1,000-bp fragment for basidiomycetes and a 1,500 bp fragment for *Cenococcum geophillum* (an ascomycete). There was no amplification of the plant DNA. The enzymes *Mbo*I and *Hinf*I were more effective than *Taq*I, resulting in patterns of two to five fragments allowing the identification of the isolates both in culture and in mycorrhizas. *Hinf*I allowed greater differentiation among the isolates and a higher number of polymorphisms. Restriction with *Taq*I resulted in too many fragments. Amplification efficiency for the fungal DNA was 64% in culture and 87% in mycorrhizas. The modified methodology represents a valuable tool to complement traditional approaches in ecosystem studies.

Keywords Ectomycorrhizas · *Eucalyptus grandis* · Polymerase chain reaction · Restriction fragment length polymorphism

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Introduction

Ectomycorrhizal community analysis aims to identify and monitor taxa and their interactions with other members of the entire community in time and space. It is also essential to monitor the survival and persistence of ectomycorrhizal fungi introduced during controlled mycorrhizal inoculation practices. Traditional methods of study include the collection and identification of sporocarps (Dalhberg and Stentröm 1991; Giachini et al. 2000), morphological characterization of mycorrhizas and isolation of fungi (Zak 1973; Ingleby et al. 1990; Agerer 1994; Goodman et al. 1996–1998).

Molecular ecology is a relatively new field in which the polymerase chain reaction (PCR; Mullis and Fallona 1987) has had an important role in the integration of genetic data with morphological data of different species. The key factor in PCR is the determination of specific primers to identify the limits of the target sequence. Using the appropriate primers, different questions at different taxonomic levels can be addressed (Nylund et al. 1995). It is also possible to identify a selected taxon in mixed materials, such as mycorrhizas where fungal DNA is mixed with plant DNA (Gardes and Bruns 1993; Egger 1995).

A variety of primers have been designed to amplify the ITS region (White et al. 1990; Gardes and Bruns 1993; Egger 1995; Martin and Rygiewicz 1999; Glen et al. 2001a, b). Primer specificity determines the kind of materials that can be used for the amplification: DNA from pure cultures or DNA from mixed samples.

To date, the analysis of mycorrhizal communities in eucalypt plantations has been performed through traditional methods of evaluation of sporocarp distribution and quantification (Giachini et al. 2000) and morphotyping of mycorrhizas in different plantations and conditions. Molecular biology techniques have been mostly applied to basic aspects of the symbiosis, and especially to the fungal symbiont (Tagu et al. 1993; Diaz et al. 1997; Junghans et al. 1998; Martin et al. 1998; Glen et al. 2001a, b). There are no references to the application of

these techniques in studies of eucalypt mycorrhizas in nursery and field conditions.

In order to obtain a valuable tool for the study of the composition and dynamics of the mycorrhizal community associated with eucalypt plantations, PCR in combination with restriction fragment length polymorphism (RFLP) techniques were adapted to characterize the fungal partner in pure culture and in symbiosis.

Materials and methods

Approximately 100 mg fresh mycelium of cultures grown for 2 weeks in MMN medium (modified Melin Norkrans; Marx 1969) were used for the DNA extraction. Table 1 presents the isolates used for DNA extraction and for in vitro mycorrhiza synthesis. All isolates were obtained from the ectomycorrhizal fungi collection of the "Laboratório de Ectomicorrizas" of the Universidade Federal de Santa Catarina (UFSC), in Florianópolis, SC, Brazil.

Eucalyptus grandis Hill ex Maiden seeds were germinated in tap water medium (TWM, $CaSO_4 2H_2O$, 500 µM; H_3BO_3, 3 µM; glucose, 2 g l^{-1}; tap water, 1 l; agar, 0.8%; pH 5.7; Thomson et al. 1994). Once germinated, seedlings were transferred to test tubes with mineral MNM medium (without sucrose; modified from Fortin et al. 1980). Seedlings and inoculum were held in place between filter paper and the test tube wall. The inoculated seedlings (15 seedlings per fungus) and the controls (15 uninoculated seedlings) were placed in a growth chamber (25°C, 12 h light) and checked weekly for mycorrhiza formation.

For DNA extraction from fungal cultures and mycorrhizas, a combination of protocols was used (Lee and Taylor 1991; Martin 1995). The material was macerated in 100 µl TNE (10 mM Tris, 100 µM NaCl, 1 mM EDTA, pH 8), 0.2% 2-β-mercaptoethanol (2-ME). Homogenization was followed by incubation in 300 µl CTAB lysis buffer [2% CTAB (cetyl trimethyl ammonium bromide), 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, pH 8, 1% 2-ME] for 1 h at 65°C and centrifugation with 1 volume (400 µl) phenol-chloroform-isoamil alcohol (1:1:4%) at 13,000 *g* for 10 min. DNA was precipitated from the supernatant by addition of 20 µl 3 M NaOAc and 0.5 volume of isopropanol (–20°C) and centrifugation for 2 min. The resulting pellet was washed with 80% ethanol and left to dry overnight. DNA was suspended in 50 µl TE (10 mM Tris, 1 mM EDTA, pH 8). The presence of DNA was verified by electrophoresis in 1% agarose gel.

Amplification of the ITS region was achieved with fungi-specific primers that amplify the ITS region from asco- and basidiomycetes. Primers used were NSA3 (5'-AAA CTC TGT CGT GCT GGG GAT A-3') and NLC2 (5'-GAG CTG CAT TCC CAA ACA ACT C-3'), designed by Martin and Rygiewicz (1999), followed by a nested reaction with ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3'; Gardes and Bruns 1993) in combination with NLB3 (5'-GGA TTC TCA CCC TCT ATG A-3', Martin and Rygiewicz 1999).

The conditions for the pair NSA3/NLC2 were 1× reaction buffer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.1 µM each primer, 5 mg/ml BSA, 0.5 U *Taq* polymerase (Promega), in a final volume of 25 µl. In order to increase specificity of the reaction a Hot Start PCR procedure was used (Martin 1995). Amplification was run in a Stuart Scientific thermocycler (Model Gene-Tech, MINI-GENE) with the following conditions: 95°C for 20 s, annealing temperature of 69°C (45 s) for 5 cycles and 68°C for 35 cycles, and an extension at 72°C for 30 s, with a final extension of 4 min.

For the nested reaction with ITS1F/NLB3 conditions were very similar to those used for NSA3/NLC2, except that the annealing temperature was 63°C for the first 5 cycles and 60°C for the following 35 cycles. PCR products were run in 2% agarose gel and DNA products were kept at 4°C until being employed.

For the amplification of the fungal DNA in mycorrhiza, two controls were always included in the reaction: the DNA from the plant as a negative control and DNA of a pure culture as a positive control.

Previous to digestion with restriction enzymes, 200 µl PCR product were precipitated with 1 volume PEG/NaCl, for 15 min at 37°C and then centrifuged for 5 min. The pellet was then washed with 80% ethanol and centrifuged for 3 min and allowed to dry for 2 h. DNA was then resuspended in 50 µl water.

For restriction, 2 U each of the following enzymes were used: *Mbo*I, *Hinf*I and *Taq*I (GIBCO-BRL). For *Mbo*I and *Hinf*I, 10 µl PCR product in a total volume of 20 µl were incubated for 3 h at 37°C following the manufacturer's instructions. For *Taq*I, incubation was at 65°C for 3 h. Restriction products were visualized in a 3% agarose gel (0.5 µg ethidium bromide).

Results

DNA was successfully obtained from isolates UFSC-Am161, C36, C2, UFSC-Ch98, UFSC-Hg93, Pax4, UFSC-Pt145, UFSC-Pt24, H4937, H1234, PT16, ET1, UFSC-Sc122, and UFSC-Sc124 (Table 1).

Table 1 Species and geographical origin of the ectomycorrhizal isolates used for DNA extraction and *Eucalyptus grandis* inoculation in vitro

No.	Isolate	Fungus	Origin
1	UFSC-Am161	*Amanita muscaria* (L. ex. Fr.) Pers. ex. Hooker	Correia Pinto, Brazil
2	C36	*Cenococcum geophilum* Fr.	Amance, France
3	C2	*Cen. geophilum*	Georgia, USA
4	UFSC-Ch98	*Chondrogaster angustisporus* Giachini, Castellano, Trappe & Oliveira	Três Barras, Brazil
5	UFSC-Ch163	*Cho. angustisporus*	Correia Pinto, Brazil
6	UFSC-Hg93	*Hysterangium gardneri* Fischer	São João do Triunfo, Brazil
7	Pax4	*Paxillus involutus* (Batsch) Fr.	Nancy, France
8	UFSC-Pt145	*Pisolithus* sp. Alb & Schwein	Florianópolis, Brazil
9	UFSC-Pt24	*Pisolithus* sp.	Florianópolis, Brazil
10	H-4937	*Pisolithus* sp.	Queensland, Australia
11	H-4943	*Pisolithus* sp.	Queensland, Australia
12	H-1234	*Pisolithus* sp.	WA, Australia
13	PT16	*Pisolithus* sp.	Treinta y Tres, Uruguay
14	ET1	*Pisolithus* sp.	Treinta y Tres, Uruguay
15	UFSC-Sc65	*Scleroderma* sp. (Persoon) Fries	Três Barras, Brazil
16	UFSC-Sc122	*S. citrinum* Pers.	Florianópolis, Brazil
17	UFSC-Sc123	*S. citrinum*	Rio Vermelho, Brazil
18	UFSC-Sc124	*S. citrinum*	Três Barras, Brazil

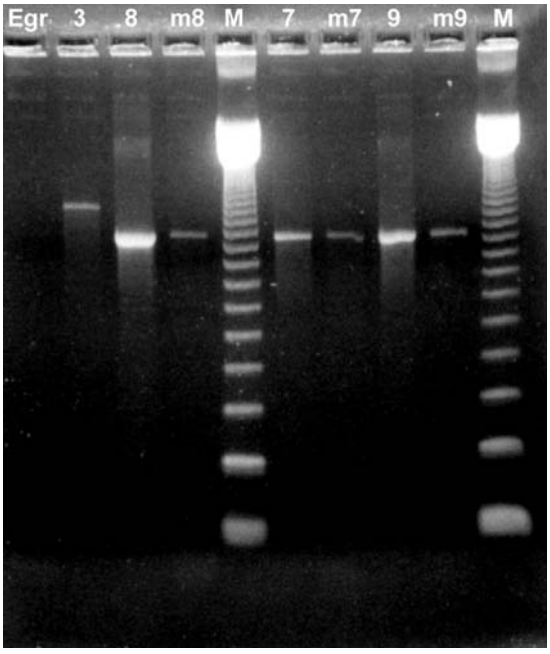

Fig. 1 PCR products of fungal DNA from pure culture and from their mycorrhiza after amplification with primers NSA3/NLC2. *Lane numbers* correspond to the isolate as presented in Table 1. *Egr Eucalyptus grandis* (no amplification), *M* molecular marker 123 bp ADN Ladder (Gibco Brl)

The in vitro mycorrhization system allowed the colonization process to be followed without disturbing the roots. After 60 days, *Pisolithus* isolates were very effective in colonizing roots. *Cenococcum geophilum* isolates (C2 and C36), however, formed few mycorrhizas as did the *Paxillus involutus* isolate (Pax 4). No mycorrhizas were observed on seedlings inoculated with the other isolates.

Since the amount of DNA extracted in the case of mycorrhizas was not sufficient to be visualized in the agarose gel, the presence of DNA was confirmed only after successful PCR amplification.

Primers selectively amplified fungal rDNA from in vitro synthesized mycorrhizas. Figure 1 shows the result of the electrophoresis run for some of the isolates and their mycorrhizas. It can be observed that for the amplified product of the isolate C2 of *C. geophilum*, an ascomycete, the band had an approximate size of 1,500 bp, while for the other isolates, basidiomycetes and their mycorrhizas, the size of the band was approximately 1,000 bp.

Enzymes *Mbo*I and *Hinf*I were effective in the restriction of the amplified fragments, resulting in patterns of two to five fragments that allowed the identification of the different isolates in culture and in the mycorrhiza. The restriction with *Taq*I resulted in too many fragments, making the results very difficult to interpret. Figure 2 shows the result of restriction with *Mbo*I and *Hinf*I for some of the isolates and their mycorrhizas.

Table 2 Results of the different stages of the methodology for each isolate: DNA extraction, amplification with NSA3/NLC2 and ITS1F/NLB3 and restriction with *Mbo*I and *Hinf*I

Isolates	DNA extraction	PCR	RFLP
UFSC-Am161	+	+	+
C36	+	–	–
C2	+	+	+
UFSC-Ch98	+	+	+
UFSC-Ch163	–	–	–
UFSC-Hg93	+	–	–
Pax4	+	+	+
UFSC-Pt145	+	+	+
UFSC-Pt24	+	+	+
H-4937	+	+	+
H-4943	–	–	–
H-1234	+	+	+
PT16	+	+	+
ET1	+	+	+
UFSC-Sc65	–	–	–
UFSC-Sc122	+	–	–
UFSC-Sc123	–	–	–
UFSC-Sc124	+	–	–

Table 3 Results of the different stages of the methodology applied to *Eucalyptus grandis* in vitro synthesized ectomycorrhizas

Isolate	DNA extraction	PCR	RFLP
C2	–	–	–
Pax4	+	+	–
UFSC-Pt145	+	+	+
UFSC-Pt24	+	+	–
H-4937	+	+	+
H-1234	+	+	+
PT16	+	+	+
ET1	+	+	+

Table 2 summarizes results obtained with the different isolates used for the procedures of DNA extraction, amplification and restriction. Table 3 presents the results for those isolates forming mycorrhizas in vitro with *Eucalyptus grandis*.

Amplification efficiency for the fungi in culture was 71% (considering the 14 isolates whose DNA was extracted). In the case of mycorrhizas the efficiency was 87%.

Discussion

Amplification efficiency in this study was not markedly different from those obtained by different authors applying similar methodologies to conifer mycorrhizas. For *Pinus sylvestris* mycorrhizas, Jonsson et al. (1999) obtained a 67% amplification success rate (number of samples amplified/total attempted) with the universal primers ITS1/ITS4. Kårén et al. (1996), using the same primers, obtained an amplification success rate of 90% for *Picea abies* mycorrhizas.

It must be noted that the data described in the literature refer to conifer mycorrhizas presenting generally abundant mycelium. Eucalypt mycorrhizas are very small in

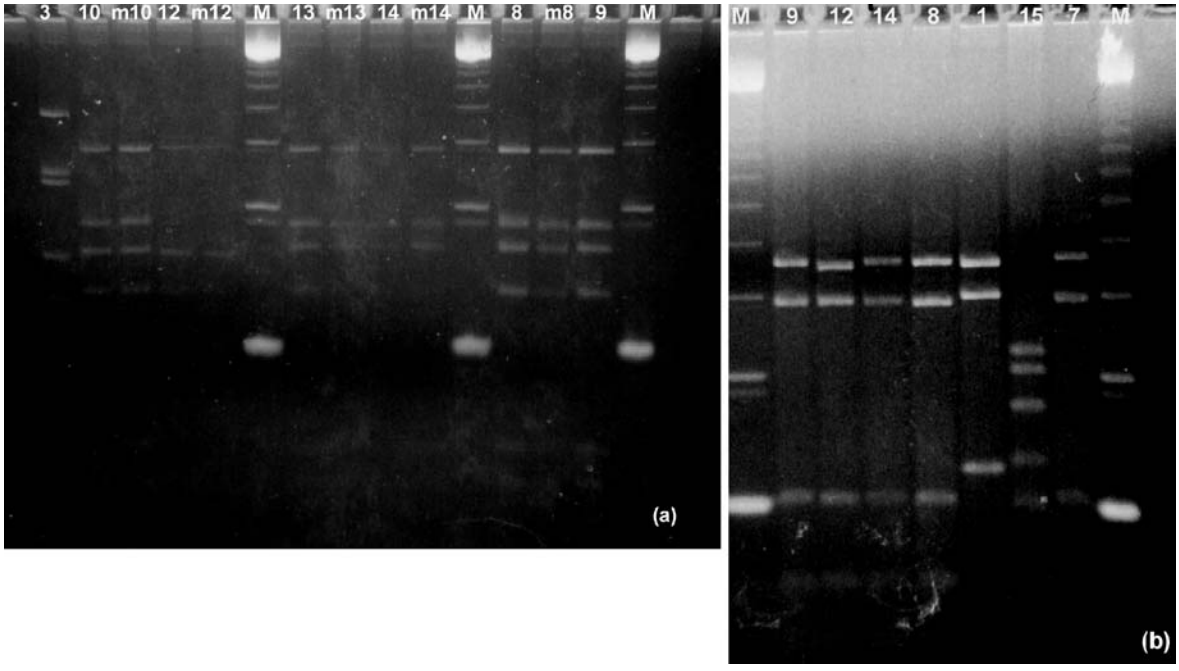

Fig. 2 Restriction products of the amplified ITS region of ectomycorrhizal fungi and their mycorrhiza with enzymes *Mbo*1 (**a**) and *Hinf*1 (**b**). *Lane numbers* correspond to the isolate as presented in Table 1. *M* Molecular marker pGEM (Promega). Band sizes of marker: 2,645+1,605 bp (not separated in gel), 1,198, 676, 517, 460, 396, 350, 222, 179 and final visible band 126 bp

comparison with conifer mycorrhizas, with much less mycelium. They are also superficial mycorrhizas with the Hartig net restricted to the epidermis (Smith and Read 1997). Primers used for the studies on conifer mycorrhizas were universal primers, not specific for fungi, so the reaction conditions had to encompass a wide range of target DNA. In the present case, primers required more stressful conditions (higher annealing temperatures, different reagent conditions) in order to increase specificity, but with diminishing chances of amplification of the target DNA. Taking these facts into consideration, the efficiency obtained was satisfactory.

Amplification of the ITS region with primers NSA3/NLC2 and ITS1F/NLB3 is specific for fungal DNA, and therefore very useful in studies of fungal communities in association with roots like mycorrhizal fungi. It also presents the advantage of amplifying both ascomycetes and basidiomycetes so there is no need to have a previous knowledge of the fungus forming the mycorrhiza, nor to change primers during the reaction according to the fungal partner. There seems to be a difference in the size of PCR products between asco- and basidiomycetes, even though this aspect needs to be verified in a wider variety of fungi (*C. geophilum* might present an intron which could explain the bigger size of its band; F. Martin, personal communication). If the size difference is observed in other ascomycetes, the first step in the technique will indicate to which fungal class the symbiont belongs.

Of the three restriction enzymes, *Hinf*I and *Mbo*I were more effective than *Taq*I in the characterization of the fungi, repeating the polymorphism pattern of the fungus in pure culture and in symbiosis. *Hinf*I was the enzyme that allowed greater differentiation among the isolates, presenting a higher number of polymorphisms.

The efficiency of the method will be further evaluated with other isolates and mycorrhizas from natural communities.

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