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Research Article

Polymorphism in the internal transcribed spacer (ITS) of the ribosomal DNA of 26 isolates of ectomycorrhizal fungi

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Abstract

Inter- and intraspecific variation among 26 isolates of ectomycorrhizal fungi belonging to 8 genera and 19 species were evaluated by analysis of the internal transcribed sequence (ITS) of the rDNA region using restriction fragment length polymorphism (RFLP). The ITS region was first amplified by polymerase chain reaction (PCR) with specific primers and then cleaved with different restriction enzymes. Amplification products, which ranged between 560 and 750 base pairs (bp), were obtained for all the isolates analyzed. The degree of polymorphism observed did not allow proper identification of most of the isolates. Cleavage of amplified fragments with the restriction enzymes *Alu* I, *Hae* III, *Hin*f I, and *Hpa* II revealed extensive polymorphism. All eight genera and most species presented specific restriction patterns. Species not identifiable by a specific pattern belonged to two genera: *Rhizopogon* (*R. nigrescens*, *R. reaii*, *R. roseolus*, *R. rubescens* and *Rhizopogon* sp.), and *Laccaria* (*L. bicolor* and *L. amethystea*). Our data confirm the potential of ITS region PCR-RFLP for the molecular characterization of ectomycorrhizal fungi and their identification and monitoring in artificial inoculation programs.

Key words: DNA fingerprinting, ectomycorrhizal fungi, internal transcribed spacer (ITS), PCR-RFLP, ribosomal DNA (rDNA).

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Introduction

Ectomycorrhizal fungi have an important role in forest ecosystems because they mutualistically associate with different plant species, thereby increasing nutrient absorption while protecting the host plant against pathogens and abiotic stresses (Smith and Read, 1997; Allen, 1991). Taxonomically diverse, these fungi include between 5,000 and 6,000 species, mainly basidiomycetes (Molina *et al.*, 1992).

Phenotypic differences between two isolates of the same species of an ectomycorrhizal fungus may be as pronounced as the differences between two distinct species, and for this reason identification of these fungi is not clear-cut (De la Bastide *et al.*, 1995). Identification of ectomycorrhizal fungi is mainly based on the analysis of sporocarps whose presence is triggered by specific environmental conditions (Egger, 1995; Gardes and Bruns, 1996). Usually the identification of the mycosymbiont by morphological analyses of the mycorrhiza is more feasible than sporocarp analyses, although special skills are required to directly analyze a root-attached symbiont (Karén *et al.*, 1997).

DNA-based molecular techniques combining the polymerase chain reaction (PCR) with analysis of restriction fragment length polymorphisms (RFLP) represent new tools to aid in properly identifying ectomycorrhizal fungi. The PCR-RFLP technique, coupling two known procedures to detect polymorphisms in DNA regions which have been amplified by specific oligonucleotide primers and restricted with different endonucleases, has been successfully used to analyze regions of ribosomal DNA of ectomycorrhizal fungi (Gardes *et al.*, 1991; Erland *et al.*, 1994; Henrion *et al.*, 1994; Farmer and Sylvia, 1998; Gomes *et al.*, 1999; Glen *et al*., 2001a). The internal transcribed spacer (ITS) region separating genes 17S and 25S, can be amplified by specific primers anchored in these two units. Since the ITS region is highly conserved intraspecifically but variable between different species it is often used in taxonomy (Bruns *et al.*, 1991; Hillis and Dixon, 1991), but ITS region polymorphism for identifying ectomycorrhizal fungi species has been determined for only a restricted number of species, leaving its full potential as a taxonomic tool as yet unexplored (Karén *et al.*, 1997).

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In the work published in this paper, 26 isolates of ectomycorrhizal fungi belonging to 8 genera and 19 different species were analyzed using the PCR-RFLP technique as applied to the ITS regions of these fungi. These analyses aimed to confirm the classification of these fungi and also to find markers able to facilitate the identification of specific isolates for breeding purposes.

Materials and Methods

Fungal isolates and culture conditions

Ectomycorrhizal fungi isolates (Table I) were obtained from the fungal collection maintained at the Laboratory of Mycorrhizal Associations, Department of Microbiology, Federal University of Viçosa, Minas Gerais, Brazil. Cultures were maintained in Petri dishes containing modified Melin-Norkrans agar medium (MMN) (Marx, 1969) for 25 days at 28 °C. Mycelia for DNA extraction were obtained by inoculating MMN broth with agar plugs containing mycelia collected from actively growing colony margins, followed by 25 days static incubation at 28 °C. Ectomycorrhizae were obtained as described by Junghans *et al.* (1998).

DNA extraction

DNA was extracted from 0.5 to 1.0 g of fresh mycelium or ectomycorrhizae, according to the method of Schäfer and Wöstemeyer (1992) as modified by Junghans *et al.* (1998). DNA concentration was estimated by comparison with known standards in 1% (w/v) agarose gels stained with ethidium bromide.

PCR amplification and RFLP analysis

The primer pairs used to amplify the rDNA ITS region (ITS1 and ITS4) have been described by White *et al.* (1990). The cycling parameters were: 40 cycles, each cycle

Table I - The ectomycorrhizal fungi studied and their origin and hosts.

N.º	Species	Isolate	Origin	Host
1	*Rhizopogon nigrescens*	CK 4	Viçosa - MG[a] - Brazil	*Pinus* sp.
2	*R. reaii*	CK 7	Viçosa - MG - Brazil	*P. caribaea*
3	*R. roseolus*	CK 29	Viçosa - MG - Brazil	*P. patula*
4	*R. roseolus*	CK 30	Viçosa - MG - Brazil	*P. patula*
5	*R. roseolus*	CK 31	Viçosa - MG - Brazil	*P. patula*
6	*R. roseolus*	CK 32	Viçosa - MG - Brazil	*P. patula*
7	*R. roseolus*	CK 33	Viçosa - MG - Brazil	*P. patula*
8	*R. vinicolor*	A 153	USA	*Tsuga heterophylla*
9	*R. rubescens*	DR 181	Unknown	*P. resinosa*
10	*Rhizopogon* sp.	RN ITA	Itabira - MG - Brazil	*P. elliottii*
11	*R. subcaerulescens*	RS1USA	Oregon - USA	Unknown
12	*Suillus granulatus*	CK 13	Viçosa - MG - Brazil	*P. caribaea*
13	*S. luteus*	9012	Unknown	*P. nigricans*
14	*S. granulatus*	SGEB	Viçosa - MG - Brazil	*Pinus* sp.
15	*S. granulatus*	SG 91	Viçosa - MG - Brazil	*Pinus* sp.
16	*S. brevipes*	7598	Unknown	*P. contorta*
17	*Laccaria laccata*	S 444	Oregon – USA	*Pseudotsuga* sp.
18	*L. amethystea*	DR 227	USA	Unknown
19	*L. bicolor*	DR 72	USA	Unknown
20	*Scleroderma* sp.	SCL ITA	Itabira - MG - Brazil	*P. elliottii*
21	*S. areolatum*	DR 165	USA	*Quercus* sp.
22	*Cenococcum geophilum*	CGL	France	*Eucalyptus* sp.
23	*C. geophilum*	CGP	USA	*Pinus* sp.
24	*Hebeloma cylindrosporum*	HC	France	Unknown
25	*Paxillus involutus*	PI	France	*E. dalrympleana*
26	*Pisolithus tinctorius*	PT 145*	Florianópolis – SC[b] - Brazil	*Eucalyptus* sp.
27	*P. tinctorius*	PT 90A*	Viçosa – MG - Brazil	*Eucalyptus* sp.

[a]The state of Minas Gerais.
[b]The state of Santa Catarina.
*Used for inoculation of *Eucalyptus grandis*.

consisting of a denaturation step at 94 °C for 1 min, an annealing step at 50 °C for 1 min and an extension step at 72 °C for 1 min 30 s. After the 40th cycle, a final extension step was performed at 72 °C for 7 min. Components for 25 µL PCR reactions were: 10 ng of DNA template, 40 pmoles of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM of each dNTP (dGTP, dCTP, dATP and dTTP) and 1 unit of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Negative controls (no DNA template) were included in every experiment. The amplification reaction was performed in a thermocycler PTC - 100 (MJ Research, Inc., Waltham, MA, USA). After amplification, DNA products were separated by electrophoresis in a 1.5% (w/v) agarose gel immersed in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) or precipitated for RFLP analysis. Precipitation was done by adding 100 mM NaCl and 2.5 volumes of absolute ethanol. Samples were kept at -20 °C for 2 h and centrifuged at 12,000 x g for 30 min. The pellet was washed with 70% (v/v) ethanol, re-suspended in 10 µL of water and digested with different restriction enzymes. The DNA fragments were size-fractionated in 2% (w/v) agarose gel, stained with ethidium bromide (0.5 µg/mL) and photographed under UV light, either with a Polaroid camera or the images were captured and stored using the Eagle Eye II photodocumentation system (Stratagene, La Jolla, CA, USA).

Data analysis

After restriction with endonucleases the amplification products were scored as 1 (presence) or 0 (absence) of a restriction site and used for determining genetic distances between the isolates (Nei and Li, 1979). These distances were used to cluster the isolates by the unweighted pair group method with averages (UPGMA) method using the Statistica program (version 4.5 for Windows, StatSoft, Inc. 1993, Tulsa, OK, USA).

Results

The internal transcribed spacer (ITS) of the rDNA region of 26 isolates belonging to 8 genera of ectomycorrhizal fungi showed extensive length polymorphism (Figure 1), PCR amplification with specific primers for the ITS region generated bands ranging from 560 to 750 bp. Figure 1 shows that all but two species (*Suillus brevipes* and *Hebeloma cylindrosporum*) presented only one amplification product. No difference was detected between the 11 *Rhizopogon* isolates analyzed, while the amplification patterns for all three *Suillus* species were quite distinct. No polymorphism was observed within the three *Laccaria* species nor between the two *Cenococcum geophilum* isolates, although polymorphism was observed between the two *Scleroderma* isolates.

To detect a wider range of polymorphisms, the PCR products were cleaved with the restriction enzymes *Alu* I, *Hae* III, *Hin*f I, and *Hpa* II (Table II). Figure 2 shows the restriction patterns obtained with *Alu* I. However, restriction patterns with all these enzymes could not distinguish between the nine *Rhizopogon* isolates and for this reason the PCR products from these isolates were further cleaved with three other enzymes (*Ava* II, *Mbo* I, and *Taq* I) but still no polymorphism was detected. Only two of these isolates, belonging to the species *R. subcaerulescens* (RS1USA) and *R. vinicolor* (A 153), showed different patterns compared to the others. *R. subcaerulescens* showed a distinct pattern with all three enzymes and *R. vinicolor*, with enzymes *Alu* I and *Ava* II. All other species, except *L. bicolor* and *L. amethystea*, could be identified by at least one restriction enzyme pattern.

Restriction fragments obtained with all the endonucleases tested were used to determine genetic distances between the genotypes and cluster them into specific groups (Figure 3). In general, isolates grouped according to their genus, *e.g. Rhizopogon*, *Laccaria*, and *Cenococcum* isolates were clustered into three distinct groups. However,

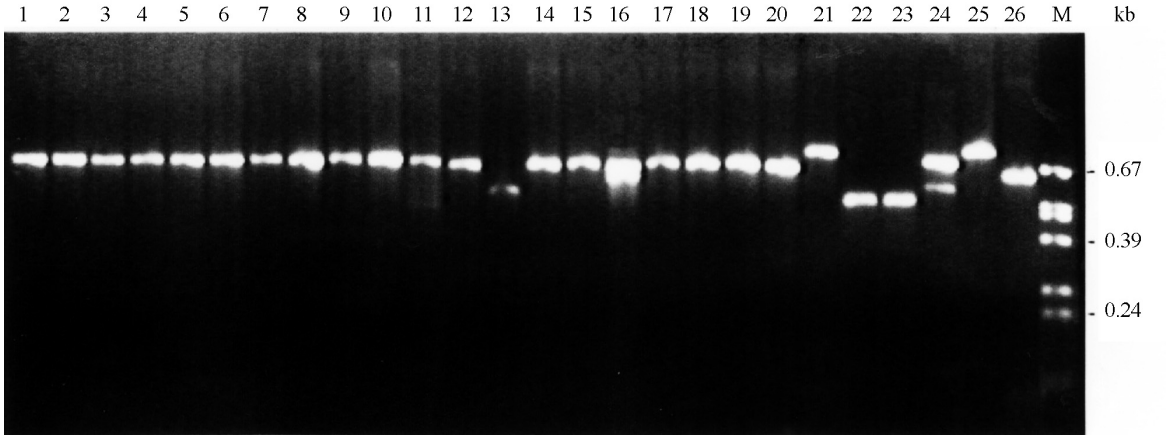

Figure 1 - Gel electrophoresis of the amplified rDNA internal transcribed sequence (ITS) region of 26 ectomycorrhizal fungal isolates. Lanes are numbered according to the isolate identification scheme given in Table I. Size markers (pUC 19 digested with *Hin*f I and *Rsa* I) are indicated by M.

Table II - Restriction fragments (pair bases) of the internal transcribed spacer (ITS) rDNA amplified regions of ectomycorrhizal fungi isolates produced by different restriction enzymes.

	Alu I	*Hae* III	*Hpa* II	*Hin*f I
Isolate	Fragment sizes (bp)	Fragment sizes (bp)	Fragment sizes (bp)	Fragment sizes (bp)
CK 4	400 290 60	730	460 260	240 210 140 120
CK 7	400 290 60	730	460 260	240 210 140 120
CK 29	400 290 60	730	460 260	240 210 140 120
CK 30	400 290 60	730	460 260	240 210 140 120
CK 31	400 290 60	730	460 260	240 210 140 120
CK 32	400 290 60	730	460 260	240 210 140 120
CK 33	400 290 60	730	460 260	240 210 140 120
A 153	650 60	730	460 260	240 210 140 120
DR 181	400 290 60	730	460 260	240 210 140 120
RN ITA	400 290 60	730	460 260	240 210 140 120
RS1USA	430 300	660	340 170 140	230 200 120 110
CK 13	610 80	460 170 90	330 170 140 90	190 130 70 30
9012	400 140	260 110 70	260 210 190	300 180 100
SGEB	610 80	460 170 90	330 170 140 90	190 130 70 30
SG 91	610 80	460 170 90	330 170 140 90	190 130 70 30
7598	610 380 170 140	540 430 140	420 360 250 180	350 270 210 140
S 444	410 250 60	710	720	360 340
DR 227	410 130 120 60	710	720	360 340
DR 72	410 130 120 60	710	720	360 340
SCL ITA	380 260	330 200 90	660	250 200 120 40
DR 165	450 190 60	330 150 100 80	510 160 60	350 260 130 50
CGL	380 140	430 90	400 100 70	170 150 110 70 30
CGP	360 140	440 90	400 100 70	190 170 120 90 50
HC	510 320 260 200 170	630 450 310	530 360 340 190	360 300 280 180 140 110
PI	510 230	530 130	300 240	380 350
PT 145	360 90 80	620	350 240 60	350 250 40

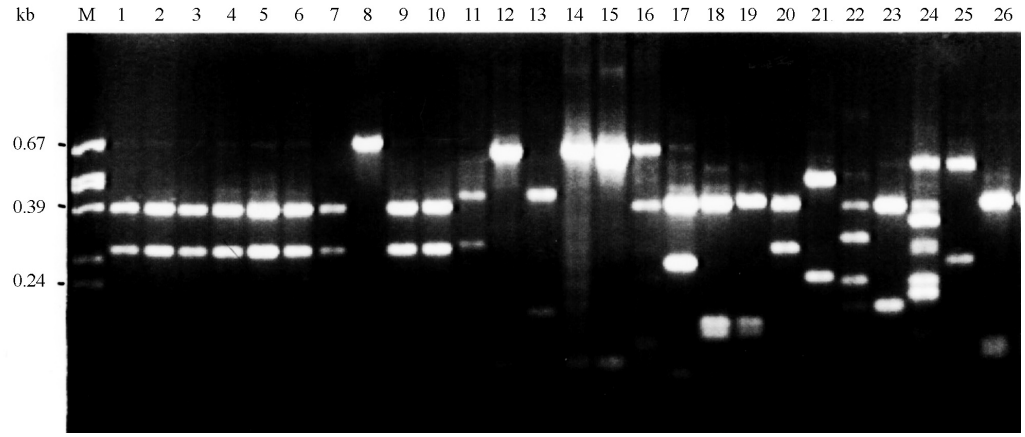

Figure 2 - Gel electrophoresis of the amplified rDNA internal transcribed sequence (ITS) region of 26 ectomycorrhizal fungal isolates digested with the *Alu* I restriction enzyme. Lanes are numbered according to the isolate identification scheme given in Table I. Size markers (pUC 19 digested with *Hin*f I and *Rsa* I) are indicated by M.

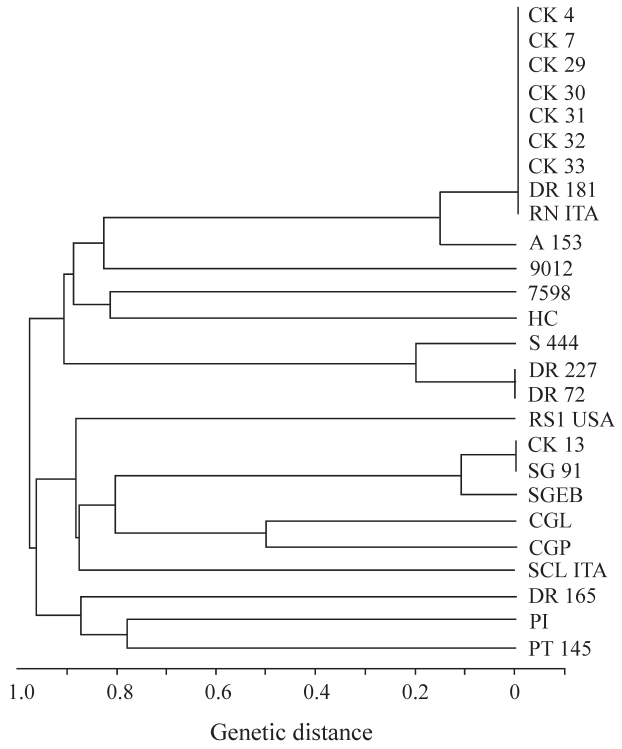

Figure 3 - Unweighted pair group method with averages (UPGMA) cluster diagram of the relationships between 26 ectomycorrhizal fungi isolates. The dendrogram was based on genetic similarity coefficients determined by the presence and absence of rDNA internal transcribed sequence (ITS) region restriction enzyme patterns.

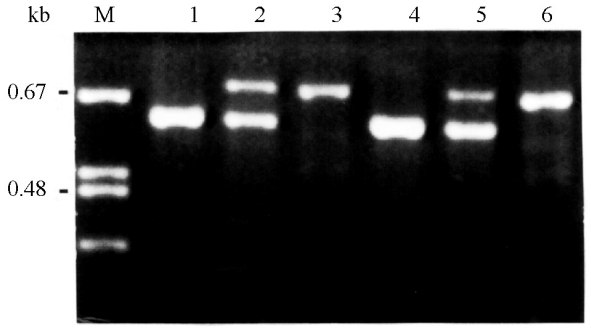

Figure 4 - Gel electrophoresis of the amplified rDNA internal transcribed sequence (ITS) region of the *Eucalyptus grandis* - *Pisolithus tinctorius* ectomycorrhizae. (1) *P. tinctorius* (PT 90A); (2) *E. grandis* - *P. tinctorius* (PT 90A) ectomycorrhizae; (3) *E. grandis*; (4) *P. tinctorius* (PT 145); (5) *E. grandis – P. tinctorius* (PT 145) ectomycorrhizae; (6) *E. grandis*. Size markers (pUC 19 digested with *Hin*f I and *Rsa* I) are indicated by M.

as shown in Figure 3, one *Rhizopogon* (RS1USA) and two *Suillus* (7598 and 9012) isolates were clustered in groups distinct from those specific for their genera and each of the two *Scleroderma* isolates formed a separate group.

The rDNA ITS region of ectomycorrhizae amplified successfully with the ITS1/ITS4 primer pair, the electrophoretic pattern of the amplification products showing two bands (Figure 4).

Discussion

Detection of polymorphism using PCR-RFLP analyses of the rDNA ITS region has been successfully used for identifying several species of fungi (Amicucci *et al.*, 1996). This simple technique requires only minute amounts of DNA and two specific primers flanking the ITS region. We found that the amplification products for the ITS region of 26 isolates of ectomycorrhizal fungi collected in Brazil, the United States and France ranged from 560 to 750 bp, coinciding with the sizes obtained for other ectomycorrhizal fungi (Gardes *et al.*, 1991; Káren *et al.*, 1997). Despite the length polymorphism observed for many of the isolates, ITS analysis alone was not able to separate all the genotypes, the exceptions being *S. brevipes* and *H. cylindrosporum* which presented two amplification products allowing their identification in relation to the other isolates (Figure 1). These two bands may be due to either polymorphism within the rDNA repetitive unit or the presence in the same isolate of more than one nucleus bearing different rDNA sequences (Sanders *et al.*, 1996).

A low degree of ITS region intraspecific polymorphism in ectomycorrhizal fungi has been reported by other authors (Gardes *et al.*, 1991; Erland *et al.*, 1994). According to Karén *et al.* (1997) ITS region intraspecific polymorphism varies among different genera and species, although no general conclusion can be drawn due to the small number of genera and species analyzed.

On the other hand, length polymorphism analysis of the ITS region followed by endonuclease restriction was sufficient to categorize most of the 26 isolates studied by us and place them in their correct species (Figure 2). RFLP analysis of the ITS region has been suggested by several authors as a means for discriminating between ectomycorrhizal fungi at species level (Gardes *et al.*, 1991; Bruns *et al.*, 1991). At genus level, ITS region fragment length polymorphism separated the 26 isolates into their 8 genera with any of the enzymes used.

Cleavage of the ITS region with *Alu* I (Figure 2) allowed differentiation of 12 out of the 19 species studied. However, none of the restriction enzymes produced a distinct pattern for five *Rhizopogon* species (*R. nigrescens*, *R. reaii*, *R. roseolus*, *R. rubescens*, and *Rhizopogon* sp.) and two *Laccaria* species (*L. bicolor* and *L. amethystea*).

Martin *et al.* (1998) analyzed the nuclear rDNA ITS region of *Rhizopogon*, in order to reexamine its present taxonomic classification in terms of the presence of specific DNA polymorphisms, and found that, based on both gel electrophoresis of digested PCR products and previous morphological observations, the five different species represent only one species, *R. villosulus*.

Cleavage of the ITS region of *Pisolithus tinctorius* isolate PT 145 resulted in the same DNA fragment pattern presented by other *Pisolithus* isolates collected in Brazil (Gomes *et al.*, 1999). Indeed, cluster analysis based on RAPD-PCR analysis grouped the Brazilian isolates, while

isolates collected in the USA and France have been shown to be quite distinct (Junghans *et al.*, 1998).

The two bands observed by us on the PCR amplified ITS region of ectomycorrhizae correspond to plant and fungi patterns, this result confirming that ITS1/ITS4 primers are not fungi specific (Paolocci *et al.*, 1995 and Amicucci *et al.*, 1996). However, in some cases, heterologous DNA from the plant material did not interfere with PCR amplification (Gardes *et al.*, 1991; Henrion *et al.*, 1992; Erland, 1995). Glen *et al*. (2001b) tested six primers pairs (targeting three nuclear and three mitochondrial regions) for specificity, sensitivity and species discrimination on identified collections of fungi. Two sets of these primers, one newly designed and targeting the ITS region and the other amplifying a ribosomal DNA fragment of the large mitochondrial subunit met the requirements of high specificity and sensitivity, amplifying DNA from a broad range of the larger basidiomycetes, with no amplification of plant, bacterial or ascomycete DNA. These specific primers discriminated fungi to species level for 91 fungal species from 28 families and are a potential practical PCR-RFLP tool for identifying basidiomycetes in plants from field samples.

The results outlined in this paper show that while interspecific variation of the ectomycorrhizal fungi ITS region is relatively high, intraspecific variability is very limited and that ITS restriction fragment analysis has potential for developing species-level markers for many, but not necessarily all, ectomycorrhizal fungi species. It appears that ITS-RFLP is a potent tool for the taxonomic study of ectomycorrhizal fungi, with the minute amounts of DNA required and the high reproducibility of this procedure making it an ideal method both for studying population heterogeneity in the field and the identification and monitoring of specific strains introduced into the soil in controlled mycorrhization programs.

Acknowledgments

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References

Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, Cambridge.

Amicucci A, Rossi I, Potenza L, Zambonelli A, Agostini D, Palma F and Stocchi V (1996) Identification of ectomycorrhizae from *Tuber* species by RFLP analysis of the ITS region. Biotechnol Lett 18:821-826.

Bruns TD, White TJ and Taylor JW (1991) Fungal molecular systematics. Annu Rev Ecol Syst 22:525-564.

De la Bastide, PY, Kropp BD and Piché Y (1995) Mechanisms for the development of genetically variable mycorrhizal in the ectomycorrhizal fungus *Laccaria bicolor*. Appl Environ Microbiol 61:3609-3616.

Egger KN (1995) Molecular analysis of ectomycorrhizal fungal communities. Can J Bot 73:1415-1422.

Erland S (1995) Abundance of *Tylospora fibrilosa* ectomycorhizas in a South Swedish spruce forest measured by RFLP analysis of the PCR-amplified rDNA ITS region. Mycol Res 99:1425-1428.

Erland S, Henrion B, Martin F, Glover LA and Alexander IJ (1994) Identification of the ectomycorrhizal basidiomycete *Tylospora fibrillosa* Donk by RFLP analysis of the PCR-amplified ITS and IGS regions of ribosomal DNA. New Phytol 126:525-532.

Farmer DJ and Sylvia DM (1998) Variation in the ribosomal DNA internal transcribed spacer of a diverse collection of ectomycorrhizal fungi. Mycol Res 102:859-865.

Gardes M and Bruns TD (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can J Bot 74:1572-1583.

Gardes M, White TJ, Fortin JA, Bruns TD and Taylor JW (1991) Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. Can J Bot 69:180-190.

Glen M, Tommerup IC, Bougher NL and O'Brien PA (2001a) Interspecific and intraspecific variation of ectomycorrhizal fungi associated with *Eucalyptus* ecosystems as revealed by ribosomal DNA PCR-RFLP. Mycol Res 105:843-858.

Glen M, Tommerup IC, Bougher NL and O'Brien PA (2001b) Specificity, sensitivity and discrimination of primers for PCR-RFLP of larger basidiomycetes and their applicability to identification of ectomycorrhizal fungi in *Eucalyptus* forests and plantations. Mycol Res 105:138-149.

Gomes EA, Barros EG, Kasuya MCM and Araújo EF (1999) Molecular characterization of *Pisolithus* spp. isolates by rDNA PCR-RFLP. Mycorrhiza 8:197-202.

Henrion B, Chevalier G and Martin F (1994) Typing truffle by PCR amplification of the ribosomal DNA spacers. Mycol Res 98:37-43.

Henrion B, Le Tacon F and Martin F (1992) Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. New Phytol 122:289-298.

Hillis DM and Dixon Mt (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. The Quat Rev of Biol 66:411-453.

Junghans DT, Gomes EA, Guimarães WV, Barros EG and Araújo EF (1998) Genetic diversity of the ectomycorrizal fungus *Pisolithus tinctorius* based on RAPD-PCR analysis. Mycorrhiza 7:243-248.

Karén O, Hogberg N, Dahlberg A, Jonsson L and Nylund J-E (1997) Inter- and intraspecific variation in the ITS region of rDNA of ectomycorrhizal fungi in Fennoscandia as detected by endonuclease analysis. New Phytol 136:313-325.

Martin MP, Högberg N and Nylund J-E (1998) Molecular analysis confirms morphological reclassification of *Rhizopogon*. Mycol Res 102:855-858.

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153-163.

Molina R, Massicotte H and Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen MF (ed) Mycorrhizal Functioning - An Integrated Plant - Fungal Process. Chapman and Hall, New York.

Nei M and Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Nat Acad Sci USA 76:5269-5273.

Paolocci F, Angelini P, Cristofari E, Granetti B and Arcioni S (1995) Identification of *Tuber* spp. and corresponding ectomycorrhizae through molecular markers. J Sci Food Agric 69:511-517.

Sanders IR, Justin PC and Wiemken AW (1996) The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems - a key to understanding the ecology and functioning of the mycorrhizal symbiosis. New Phytol 133:123-134

Schäfer C and Wöstemeyer J (1992) Random primer dependent PCR differentiates aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*). J Phytopathology 136:124-136.

Smith SE and Read DJ (1997) Mycorrhizal symbiosis. 2 ed. Academic Press, London.

White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR protocols. A guide to methods and applications. Academic Press, San Diego, pp. 315-322.