

Strain specific differences in ribosomal DNA from the ectomycorrhizal fungi *Laccaria bicolor* (Maire) Orton and *Laccaria laccata* (Scop ex Fr) Br

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Summary — The restriction fragment length polymorphism patterns of the ribosomal RNA genes of 14 isolates belonging to various ectomycorrhizal fungus species including the related basidiomycetous ectomycorrhizal fungi *Laccaria laccata* (Scop ex Fr) Br and *Laccaria bicolor* (Maire) Orton have been determined. The isolates were obtained from various geographical sources in France, the United Kingdom and North America. Total DNA of vegetative mycelium was cleaved with a series of restriction enzymes, electrophoretically separated and probed with radiolabelled rDNA gene from *Coprinus cinereus* (Schaeff: Fr) SF Gray. Results indicate that isolates belonging to different species had different restriction enzyme sites in the rDNA. Although distinct patterns were observed within species, a core of common bands could be discerned within each species. Since various patterns were observed within *L. bicolor* and *L. laccata*, rRNA gene restriction patterns may have epidemiological as well as taxonomic interest.

***Laccaria bicolor* / *Laccaria laccata* / restriction fragment length polymorphism / RFLP / ribosomal DNA / taxonomy / epidemiology**

Résumé — Étude du polymorphisme de l'ADN ribosomal chez différentes souches de champignons ectomycorhiziens *Laccaria bicolor* et *Laccaria laccata*. Afin de caractériser la diversité génétique au sein des champignons ectomycorhiziens appartenant aux espèces *Laccaria bicolor* et *L. laccata*, une étude du polymorphisme de l'ADN ribosomal (ADNr) de 14 souches appartenant à plusieurs espèces et de provenances géographiques variées a été entreprise. Dans un premier temps, nous avons développé une méthode d'extraction de l'ADN total du mycélium végétatif simple et rapide. Les régions intergéniques de l'ADNr des champignons présentant des variations importantes à la fois au niveau du nombre de sites de restriction des endonucléases et au niveau de la taille des séquences, une analyse du polymorphisme de longueur des fragments de restriction (RFLP) a été conduite sur l'ADN total de ces champignons mycorhiziens. Il apparaît que le polymorphisme de longueur des fragments de restriction est très important entre des genres différents (fig 1A), modérés entre espèces d'un même genre (figs 2A et B) et restreint avec les isolats d'une même espèce (figs 2A et B). En général, on observe une bonne conservation du nombre de sites de restriction au niveau du gène de l'ADNr des Laccaires. Les fragments de restriction EcoRI de 1.45, 8.0, et 9.4 kpb se rencontrent chez la plupart des souches de *Laccaria* que nous avons analysées (tableau II). La comparaison des profils de restriction EcoRI des souches de *L. bicolor* et *L. laccata* permet l'attribution aisée d'une souche à l'une ou l'autre de ces deux espèces. De plus, le polymorphisme des fragments de restriction est suffisant pour distinguer les souches de provenances géographiques différentes (figs 2A et B).

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Il est particulièrement intéressant de noter que le profil de restriction de *L. laccata* S238 que nous avons obtenu est similaire à celui des isolats américains de *L. bicolor* CRBF581 et CRBF569. Ces résultats confirment ceux publiés par Armstrong *et al* (1989) et conduisent à reclasser la souche américaine *L. laccata* S238 dans l'espèce *bicolor*.

En conclusion, l'étude du polymorphisme des fragments de restriction de l'ADNr des champignons ectomycorrhiziens nous a permis de : 1) montrer que le gène codant pour les ARNr de *Laccaria* présente une homologie élevée avec le gène de *Coprinus cinereus* confirmant une conservation importante de l'ADNr au sein des Agaricales; 2) démontrer qu'il existe un polymorphisme des fragments de restriction de l'ADNr au sein des isolats des différentes espèces analysées; et 3) discriminer un certain nombre de souches appartenant aux espèces *Laccaria bicolor* et *L. laccata*. La RFLP de l'ADNr peut donc s'appliquer avec succès à l'étude des divergences génétiques et à l'identification de champignons ectomycorrhiziens. L'amplification préalable de l'ADNr à l'aide de la PCR (Polymerase Chain Reaction), en évitant l'emploi de radioisotopes, devrait conduire à une simplification considérable de l'analyse du polymorphisme des fragments de restriction.

***Laccaria bicolor* / *Laccaria laccata* / polymorphisme des fragments de restriction / RFLP / ADN ribosomal / taxonomie / épidémiologie**

INTRODUCTION

Laccaria laccata (Scop ex Fr) Br and *L. bicolor* (Maire) Orton species are ectomycorrhizal fungi belonging to the Tricholomataceae. Despite many common properties, there is a high degree of variation in morphological, physiological, and biochemical characteristics among species as revealed by growth behaviour, mycorrhizal competence (Kropp *et al*, 1986; Kropp and Fortin, 1988; Wong *et al*, 1989) and electrophoretic polypeptide patterns (Hilbert and Martin, unpublished data). Thus, it appears that distinct subgroups of *L. laccata* and *L. bicolor* are present, but the biological status of these subgroups and their interrelationships are poorly known. However, it is important to accurately differentiate these subgroups because, within isolates of *L. laccata* and *L. bicolor*, some are more efficient than others at increasing tree growth under nursery and field conditions (Le Tacon *et al*, 1988).

The increased incidence of sylvicultural use of ectomycorrhizal species has stimulated interest in the use of epidemiological markers to fingerprint and compare isolates. Morphological methods rely upon

the anatomy of fruitbodies and spores for accurate identifications. While *Laccaria* B and Br (Agaricales) is well described, several taxonomic and nomenclatural problems have persisted within the genus (Mueller and Vellinga, 1986). An alternative identification method which would be more rapid and specific is therefore desirable. Biochemical approaches, such as isoenzyme patterns, 2-dimensional gel electrophoresis and immunochemical techniques are currently under investigation. Recent studies have demonstrated the use of relatively large DNA fragments complementary to sequences of the 17S and 25S ribosomal RNA molecule as group-specific probes in hybridization tests using fungi (Wu *et al*, 1983; Specht *et al*, 1984; Klassen *et al*, 1987; Hintz *et al*, 1989).

The use of RFLP (restriction fragment length polymorphism) analysis of DNA as an aid in ectomycorrhizal fungus taxonomy has been recently reported (Armstrong *et al*, 1989; Rogers *et al*, 1989; Gardes *et al*, 1990, 1991). These studies demonstrated the potential usefulness of the RNA gene restriction pattern as a taxonomic tool and that restriction enzyme patterns of the rDNA from many ectomycorrhizal fungi in-

cluding *Laccaria* species were different. We report here on rDNA polymorphisms among *L. bicolor* and *L. laccata* isolates from various geographical sources in France, the United Kingdom and North America. In addition, a rapid micropreparation method to extract high molecular weight DNA from small amounts of ectomycorrhizal mycelia is described.

MATERIALS AND METHODS

Strains and culture conditions

Isolates were obtained from various geographical sites in France, the United Kingdom and North America (table I). The identification of sporocarps collected in France was confirmed by Prof Lamoure at the University Claude Bernard (Lyon, France) and those collected in North America by G Mueller (Department of Botany, Field Museum of Natural History, Chicago, USA). Media and methods for the routine culturing of all isolates were as described by Martin *et al* (1990).

Isolation of DNA

Whole-cell DNA from vegetative mycelium was prepared as follows: fungal mycelium from a 250-ml culture was collected in a sieve and dried in several portions onto filter papers (Whatman No1, in a Büchner funnel connected to a water pump). The resulting "cakes" were peeled off, frozen in liquid nitrogen and lyophilized overnight. About 50 mg of the lyophilized material was ground with a mortar and pestle until it had the consistency of fine sand. Ground tissue was suspended in 500 μ l 20 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 250 mM NaCl, 0.5% SDS and 0.1 mg proteinase K for 4 h at 55 °C. The fungal suspension was centrifuged at 32 000 *g* for 30 min at 4 °C to pellet the cellular debris. Proteins in the supernatant were denatured and removed by sequential extractions with 500 μ l Tris-saturated phenol-chloroform-

isoamyl alcohol (24/24/2, v/v/v) and chloroform-isoamyl alcohol (24/1, v/v) (Maniatis *et al*, 1982). The phases were separated by centrifugation for 15 min at 7 500 *g*. The aqueous phase was taken off carefully and was incubated with 10 units RNase A (5 mg/ml, Sigma Type IIIA, preincubated for 15 min at 65 °C in 50 mM Na acetate pH 6.5 to denature DNAase activity) for 2 h at 37 °C. The solution was then mixed with 50 μ l 3 M Na acetate and 1.5 ml cold absolute ethanol, followed by gentle mixing. DNA was then pelleted by centrifugation at 7 500 *g* for 10 min, washed with 70% (v/v) ethanol, pelleted again, and dried in a vacuum dessicator for 5 min. Finally, the DNA pellet was rehydrated in 20 to 200 μ l of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and stored at -20 °C until use.

Restriction endonuclease digestion and agarose gel electrophoresis

One to 2 μ g DNA were digested overnight with 5–10 units of various restriction enzymes (*Bam*HI, *Eco*RI, *Pvu*II, *Hind*III) (Pharmacia Fine Chemicals, St Quentin/Yvelines, France) or Gibco-BRL (Cergy Pontoise, France) according to the manufacturers' instructions. The restriction fragments were size-fractionated on a 5 x 10 cm 1.0% agarose gel in TBE (89 mM Tris-HCl; 89 mM boric acid; 2 mM EDTA, pH 8.0) as described by Maniatis *et al* (1982). The DNA was electrophoresed at 75 mA for 1 h. Bacteriophage λ , digested with *Hind*III, was used as a size standard.

Southern blotting and hybridization

After electrophoresis, agarose gels were sequentially soaked in 0.25 M HCl for 5 min, distilled water for 15 min, twice in 1.5 M NaCl, 0.5 M NaOH for 30 min and twice in 1.0 M Tris-HCl (pH 8.0), 1.5 M NaCl for 30 min. Southern blotting (Southern, 1975) was carried out on Hybond-N nylon membrane (Amersham France, Les Ulis) according to Maniatis *et al* (1982). The blotted DNA was fixed by UV irradiation at 312 nm for 3 min. Plasmid pCct1 (courtesy of P Pukila, University of North Carolina) encoding one complete repeat of the rDNA from *Coprinus ci-*

Table 1. Isolates of ectomycorrhizal fungi used in the study of restriction fragment length polymorphism of rDNA.

Isolate No	Isolate identification	Collection site	Host plant	Source ^a
003	<i>Laccaria laccata</i>	Cumbria, UK	<i>Picea sitchensis</i>	1
83-222	<i>L. laccata</i>	Grand Grammont, Creuse, France	<i>Picea excelsa</i>	2
S106	<i>L. laccata</i>	USA	Unknown	.
S1023	<i>L. laccata</i>	Oregon, USA	Unknown	3
Cham3	<i>L. laccata</i>	Chamet, Creuse, France	<i>Pseudotsuga menziesii</i>	2
Peyrat	<i>L. laccata</i>	Peyrat-le-Château, Creuse, France	<i>Picea excelsa</i>	4
CRBF569	<i>Laccaria bicolor</i>	Quebec, Canada	Unknown	5
CRBF581	<i>L. bicolor</i>	Quebec, Canada	Unknown	5
S238b	<i>L. bicolor</i>	Oregon, USA	<i>Tsuga mertensiana</i>	6
81-306	<i>L. bicolor</i>	Barbaroux, Creuse, France	<i>Pseudotsuga menziesii</i>	2
83-216	<i>L. bicolor</i>	Grand Grammont, Creuse, France	<i>Picea excelsa</i>	2
Sivrite	<i>Hebeloma crustuliniforme</i>	Meurthe and Moselle, France	<i>Picea excelsa</i>	1
270	<i>Pisolithus tinctorius</i>	Florida, USA	<i>Pinus taeda</i>	7
Sivrite	<i>Cenococcum geophilum</i>	Meurthe and Moselle, France	<i>Picea excelsa</i>	4

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nereus (restriction map in Cassidy *et al*, 1984), was labelled with [α - 32 P]dCTP (3000 Ci/mol) using a nick-translation kit (Amersham France, Les Ulis) according to the manufacturers' instructions. The prehybridization, hybridization and washing steps were performed under high stringency conditions as described previously (Armstrong *et al*, 1989). The blots were dried for 30 min at 60 °C in the Biorad Model 543 gel dryer and exposed to Hyperfilm-MP (Amersham France, Les Ulis) at -70 °C for 24 h to several days.

RESULTS

From 50 mg lyophilized fungal tissue 25–40 μ g of high molecular weight DNA were purified depending on the isolate. The DNA averaged from 25–30 kilobases (kb) in length with little degradation evident (data not shown). Restriction patterns of the purified DNA were obtained from all but one fungus (*Pisolithus tinctorius* Coker and Couch). It is significant to note that the DNA purification method used in the present study was rapid and relatively inexpensive. The time and cost of isopycnic CsCl ultracentrifugation were not necessary.

Ribosomal RNA genes are conserved (Garber *et al*, 1988) and have been extensively used as probes for rDNA of phylogenetically diverse fungi (Reader and Broda, 1984; Specht *et al*, 1984; Klich and Mullaney, 1987; Garber *et al*, 1988; Hintz *et al*, 1989; Laaser *et al*, 1989) including ectomycorrhizal species (Armstrong *et al*, 1989; Rogers *et al*, 1989). Therefore, we used the rDNA probe of the basidiomycete *Coprinus cinereus* to survey the extent of interstrain and interspecies variation in the rDNA of 14 isolates from 5 species of ectomycorrhizal fungi. Labelled-rDNA of *Coprinus cinereus* was hybridized to Southern transfers of restricted DNA of the ectomycorrhizal fungi *Cenococcum geo-*

philum Fr, *Hebeloma crustuliniforme* (Bull ex Pt Am) Q, *Pisolithus tinctorius*, *Laccaria laccata* (Scop ex Fr) Bk-Br and *L bicolor* (Maire) Orton. Hybridization patterns confirmed that *C cinereus* rDNA had strong sequence homology with rDNA of the investigated mycorrhizal fungi (fig 1).

The rDNA of these species was restricted with the endonucleases *Hind*III, *Pvu*II, and *Eco*RI. Of the 4 species assayed for their *Eco*RI rDNA hybridization patterns, *C geophilum*, *L laccata*, *L bicolor* and *P tinctorius* exhibited patterns that appeared characteristic for that genera (fig 1A). *Hind*III yielded 2 homologous bands with *Laccaria bicolor* and *L laccata* isolates (fig 1B) and 1 with the other species (data not shown). *Pvu*II gave rise to 1 band for *C geophilum*, *L bicolor*, *Paxillus involutus*, and *Pisolithus tinctorius*, and 4 bands for *H crustuliniforme* (data not shown). *Hind*III

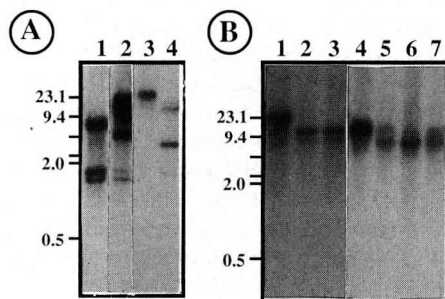


Fig 1. Autoradiogram of *Eco*RI-(A) and *Hind*III-(B) digests of DNA from ectomycorrhizal fungi, separated by electrophoresis and hybridized with 32 P-labelled rDNA (plasmid pCc1). A), lane 1, *Laccaria bicolor* 81-306; lane 2, *Laccaria laccata* S106; lane 3, *Pisolithus tinctorius* 270; lane 4, *Cenococcum geophilum* Sivrite. B) lane 1, *L bicolor* 81-306; lane 2, *L bicolor* CRBF581; lane 3, *L bicolor* CRBF569; lane 4, *L laccata* 83-222; lane 5, *L laccata* Peyrat; lane 6, *L bicolor* S238 (accessioned and distributed as *L laccata* S238); lane 7, *L laccata* Cham3. To the left a molecular mass scale as determined by the position of *Hind*III-cut λ DNA standard.

and *PvuII* were thus not sufficient to discriminate among the fungal genera. However, as pointed out previously (Armstrong *et al*, 1989), it was possible to make genus-specific identifications when the RFLPs produced by all enzymes were compared collectively.

EcoRI rDNA hybridization patterns were employed for investigating the extent of interspecific and intraspecific variations in the rDNA of 12 isolates of *L. laccata* and *L. bicolor* from different geographical locations. Isolates belonging to different *Laccaria* species did not share the same pattern (fig 2). Each species, however, could be characterized by a core of common rDNA gene restriction fragments which constituted a species-specific pattern. Most *L. laccata* isolates had major *EcoRI* fragments at 1.45, 4.0 and 8.0 kb (fig 2A) whereas the *L. bicolor* isolates had major bands at 1.45, 2.0 and 8.0 kb (fig 1A, lane 2 and 2B). The 4.0-kb fragment appeared characteristic for *L. laccata* isolates, whereas the 2.0 kb fragment could be detected in some isolates of both species. However, in spite of these restriction polymorphisms, the sizes of the rDNAs were similar. When fragment sizes of the digested rDNAs were summed, the gene was estimated to be in the same size range as those of other fungi, *ie* 11–14 kb (Garber *et al*, 1988).

As expected, the coding regions appear to be highly conserved among the two species, while the spacer regions exhibited larger diversity. The 1.45 kb *EcoRI* fragment including the 5' end of the 25S rDNA gene (fig 3; see also Garber *et al*, 1988) was present in all *Laccaria* isolates examined (fig 2). The 1.70-kb fragment containing over half of the 25S rDNA gene was observed in isolates 81306 and 83216 of *L. bicolor* and in isolates Cham3, 83222 and 003 of *L. laccata*. By contrast, a band at 2.0 kb was observed in isolates devoid of the 1.7-kb fragment. The band presu-

ably results from a 0.3-kb insertion into the 25S rDNA as observed in several fungal species (*eg*, *Hebeloma mesophaeum*, *Galericina autumnalis*) (Rogers *et al*, 1989). In addition, there are 2 bands visible at 3.8 kb and 4.0 kb in *EcoRI*-restricted DNA of

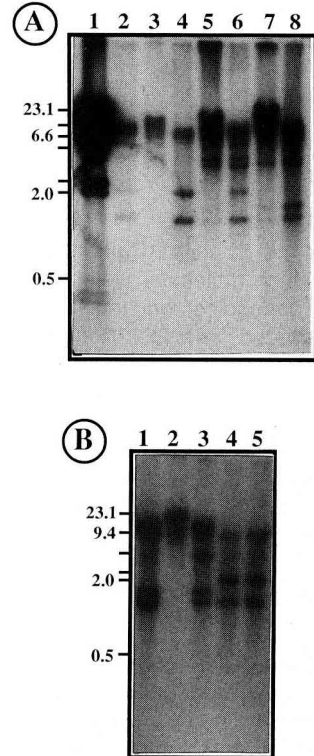


Fig 2. Autoradiogram of *EcoRI*-digests of DNA from *Laccaria laccata* (A) and *L. bicolor* (B) isolates, separated by electrophoresis and probed with ^{32}P -labelled rDNA (plasmid pCc1). A), lane 1, molecular mass standard (*HindIII*-digested λ DNA), lane 2, *Laccaria laccata* S1023; lane 3, *L. laccata* S106; lane 4, *L. bicolor* S238 (accessioned and distributed as *L. laccata* S238); lane 5, *L. laccata* 003; lane 6, *L. laccata* Peyrat; lane 7, *L. laccata* 83-222; lane 8, *L. laccata* Cham3; B), lane 1 *L. bicolor* 81-306; lane 2, *L. bicolor* 83-216 (uncut DNA); lane 3, *L. bicolor* 83-216; lane 4, *L. bicolor* CRBF569; lane 5, *L. bicolor* CRBF581. To the left a molecular mass scale as determined by the position of *HindIII*-cut λ DNA standard.

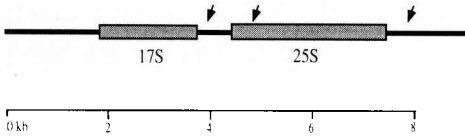


Fig 3. Organization of the rDNA repeat units in *Coprinus cinereus* showing one of the tandem repeats starting from the 3' end of one 25S gene, through the intergenic transcribed spacer (ITS), an 17S gene, another intergenic spacer. *EcoRI* restriction sites are designated by arrowheads (after Cassidy *et al*, 1984).

most *L. laccata* isolates suggesting that there are 2 sets of the rDNA repeat which are similar, but have slight sequence divergence.

DISCUSSION

Morphological, physiological, and biochemical data have suggested that *L. laccata* and *L. bicolor* comprise subspecies. On the basis of the electrophoretic pattern of total proteins, large variations in polypeptide accumulation within *Laccaria* isolates have been distinguished (Hilbert and Martin, unpublished data). Previous studies, which compared RFLPs of rDNA genes from North American isolates of *Laccaria* demonstrated the usefulness of this approach, and rDNA gene restriction patterns have thus been proposed as a taxonomic aid and epidemiological marker for ectomycorrhizal fungi (Armstrong *et al*, 1989; Rogers *et al*, 1989; Gardes *et al*, 1990). Therefore, it was pertinent to evaluate whether such polymorphisms of RFLP patterns could be found for European isolates.

Using this method, species- or subspecies-specific cores of restriction fragments,

homologous to *Coprinus cinereus* rDNA, have been observed. Among the restriction endonucleases tested, *EcoRI* provided a simple method to distinguish isolates of *L. laccata* and *L. bicolor*. When total DNA from isolates collected from various geographical locations was digested with *EcoRI* and subjected to gel electrophoresis and rDNA hybridization, a different fractionation pattern was associated with each species and most isolates within a species. Thus, species such as *Laccaria* species, usually considered as difficult to distinguish using phenotypic characteristics could be differentiated. Our results confirm that isolate S238 formerly accessioned and distributed as *L. laccata* belongs to *L. bicolor*, and support its recent reclassification (Armstrong *et al*, 1989).

Taken collectively, our work and that of Rogers *et al* (1989) and Armstrong *et al* (1989) demonstrates the evolutionary conservation and utility of ribosomal gene probes for identifying ectomycorrhizal fungi. Rogers *et al* (1989) and Armstrong *et al* (1989) isolated DNA using CTAB-based procedures, used the same *L. laccata* isolate (GM1774), and some of the same endonucleases, but hybridized the RFLPs with different ribosomal gene probes. The former used a non-specific plasmid probe from a non-filamentous fungus (pBD4: containing *Saccharomyces cerevisiae* ribosomal genes; Bell *et al*, 1977) and the later group hybridized RFLPs with the non-specific ribosomal gene plasmid probe pCc1. The hybridized RFLPs for the *EcoRI* digest of isolate Gml1774 was identical for both probes (table II). In the present study, we hybridized RFLPs with pCc1 and used a SDS-DNA extraction method whereas Armstrong *et al* (1989) used a CTAB-based DNA extraction method. Hybridized RFLPs of the *EcoRI* digest of *L. bicolor* S238 for both DNA extraction methods were identical, indicating the compatibility of results among DNA extraction methods.

Table II. Hybridization fragment sizes of *EcoRI*-digested ribosomal DNA from *Laccaria laccata* and *Laccaria bicolor* isolates from various geographical locations in France and North America.

	Fungal isolates			<i>EcoRI</i> r DNA fragments (kb)		
<i>Laccaria laccata</i> S1023	1.45	2.0		4.0	8.0	9.4
<i>L laccata</i> S106	1.45	1.7		3.8	4.0	8.0 9.4
<i>L laccata</i> 003	1.45	1.7		3.8	4.0	8.0 9.4
<i>L laccata</i> Peyrat	1.45	2.0		3.8	4.0	8.0 9.4
<i>L laccata</i> 83-222	1.45	1.7		3.8		8.0 9.4
<i>L laccata</i> Cham3	1.45	1.7		3.8		9.4
<i>L laccata</i> GM1774 ^a	1.45	2.0	3.0		7.6	
<i>Laccaria bicolor</i> S238	1.45	2.0			8.0	9.4
<i>L bicolor</i> S238 ^b	1.45	2.0		4.0	8.0	9.4
<i>L bicolor</i> 81-306	1.45	1.7				9.4
<i>L bicolor</i> 83-216	1.45	1.7		4.0		9.4
<i>L bicolor</i> CRBF 569	1.45	2.0			8.0	9.4
<i>L bicolor</i> CRBF 581	1.45	2.0			8.0	9.4
<i>L bicolor</i> MC: 10/24/86-1 ^a	1.45	2.0	3.0	7.2	7.9	

^a After Rogers *et al* (1989). ^b After Armstrong *et al* (1989).

Our results strongly suggest that *EcoRI* restriction digest patterns of total DNA provide a useful adjunct to other taxonomic criteria to distinguish isolates of the 2 economically important species *L laccata* and *L bicolor*. However, cost, technical skill required, and utilization of radioactive isotopes could prevent the spread of RFLPs in identifying ectomycorrhizal isolates. Polymerase chain reaction (PCR) is being widely used for efficient amplification of specific sequences of genomic DNA (Saiki *et al*, 1988; Gardes *et al*, 1991). Amplification of rDNA and gene restriction patterns of the amplified DNA from ectomycorrhizal fungi and ectomycorrhizas are now under study in our laboratories.

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