Restriction Fragment Length Polymorphisms in the Nuclear Ribosomal DNA of Four *Laccaria* spp.: *L. bicolor, L. laccata, L. proxima*, and *L. amethystina*

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ABSTRACT

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Restriction fragment length polymorphisms (RFLPs) were examined in the nuclear ribosomal DNA (rDNA) of 29 isolates of *Laccaria bicolor*, eight of *L. laccata*, three of *L. proxima*, and two of *L. amethystina*. Whole-cell DNAs were digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I and probed with cloned rDNA from *Armillaria ostoyae* by Southern hybridizations. The observed variability suggests that rDNA restriction fragment phenotypes can be used as additional taxonomic characters for comparing *Laccaria* spp., since the four *Laccaria* spp., as well as four biological species within *L. laccata*, were distinguishable. North American isolates of *L. bicolor* appear to constitute a relatively homogenous group that is distinguishable from European isolates. Large variations in RFLP patterns are detected among isolates of *L. laccata*, reflecting the morphological, physiological, and genetic heterogeneity of this species. The nuclear ribosomal repeat unit has apparently diverged in *L. bicolor* and *L. amethystina* on the two continents and among biological species of *L. laccata*. Most of the intraspecific polymorphism in rDNA restriction fragment length was observed after digestion with *Bgl*II and *Eco*RI. Mixed rDNA types that differ at least in repeat unit length were also detected with these two enzymes within seven dikaryotic isolates of *L. bicolor* collected from the field.

The agaric genus *Laccaria* Berk. & Br. (Basidiomycetes, Tricholomataceae) is a complex of more than thirty species, many of which are ectomycorrhizal symbionts of numerous shrub and forest tree species of the temperate and tropical regions. This symbiosis plays a fundamental role in determining the rate of plant growth, nutrient uptake, water absorption, and protection against pathogens (15). Certain *Laccaria* spp. (e.g. *L. laccata* Cooke, *L. proxima* (Boud.) Pat., and *L. bicolor* (Maire) Ort.) were reported to be pioneer species found often in recently disturbed areas and young forest stands (8,9). This interesting characteristic, as well as the relative ease with which *Laccaria* spp. can be manipulated in pure culture, has contributed to the selection of *Laccaria* spp. as models for applied and fundamental research on ectomycorrhizae (4,14).

The identification of *Laccaria* spp. by morphological and physiological characters remains problematic in many cases. The separation of *L. laccata, L. proxima*, and *L. bicolor* can only be achieved with difficulty by using criteria such as spore size and shape (19). Moreover, crossing studies have shown that, within the taxonomic species *L. laccata* and *L. bicolor*, fully and partially intersterile populations exist (10,12,14, G. M. Mueller, *unpublished results*). Thus, additional identification criteria are needed, and the present study was undertaken to compare closely related taxa of *Laccaria* at a molecular level.

Restriction fragment length polymorphisms (RFLPs) in the nuclear and mitochondrial genomes of many different organisms have been used for taxonomic (27), phylogenetic (5), and population studies (7,11,25). Polymorphisms in ribosomal DNA (rDNA) have been shown to be informative at the species level for many fungi (1,2,13,21,23,27). The presence of highly conserved regions in the ribosomal genes allows the use of probes from another fungus in the examination of the *Laccaria* isolates. This report presents an analysis of RFLPs in the nuclear rDNA between and within four *Laccaria* spp.: *L. bicolor, L. laccata, L. proxima*, and *L. amethystina* Cooke, and discusses the possibility of their utilization as species- and isolate-specific markers. More emphasis was placed on one species, *L. bicolor*, in order to assess the level of intraspecific variability.

MATERIALS AND METHODS

The *Laccaria* isolates and their respective geographical and biological origins are listed on Table 1. The isolates were maintained on 2% Difco malt agar. Mycelium for DNA extraction was grown as static cultures in the modified Melin-Norkrans (MMN) liquid medium of Marx (18), except that glucose was used in place of sucrose and a ferric citrate solution in place of ferric chloride.

Liquid fungal cultures were harvested by filtration, rinsed with distilled water, frozen in liquid nitrogen, and lyophilized. DNA was extracted by the procedure of Murray and Thompson (20) and purified in an ethidium bromide-CsCl density gradient (17) or according to the procedure of Lee, et al (16). Whole-cell DNA extracts (0.5–1 μg) were digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I (Pharmacia Ltd., Dorval, Qué., Canada; BRL Laboratories, Gaithersburg, MD) according to the digestion conditions recommended by the manufacturer. Digested DNAs were electrophoresed on 0.8% agarose gels in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 20–50 V for 10–15 hr (for maxigels, 30-cm length). Capillar transfer of DNA fragments from gels to nylon membranes (Genescreen Plus, NEN Research Products, Boston, MA) was done according to the manufacturer's recommendations.

The rDNA probes used (gift of J. A. Anderson) were plasmids pAM1 and pAM2 from *Armillaria ostoyae* Romag. (1). pAM1 contains a 3.4 kb rDNA fragment including the 3′ end of the 26S coding sequence and the 5′ end of the intergenic spacer,

whereas pAM2 contains a 6.8 kb rDNA fragment including the 3′ end of the intergenic spacer, the 16S and 5.8S coding sequences, the internal transcribed spacer, and most of the 26S coding sequence. pAM1 and pAM2 together represent the entire nuclear ribosomal repeat unit. A map of the rDNA from *A. ostoyae* is presented in Fig. 1. Plasmid probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol.; ICN Biomedicals), using a nick translation kit from Bethesda Research Laboratories (Gaithersburg, MD), to a specific activity of about 10^8 cpm/μg DNA. Ten ng of bacteriophage lambda DNA were usually added to 500 ng of plasmid DNA during nick translation in order to visualize the lambda marker as molecular weight standards. Prehybridization was done at 65 C for 2 hr in 6 × SSC, 5 × Denhardt's solution, and 0.5% SDS and subsequent hybridization at 65 C for 16–24 hr in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS, 10% Dextran sulfate, 150 μg of sheared salmon sperm DNA/ml, and 10–20 ng of probe DNA/ml. Just before use for hybridization, the probe was brought to 1 ml with distilled water and denaturated by boiling for 10 min. Blots were then washed twice at room temperature for 5 min in 2 × SSC, twice at 65 C for 30 min in 2 × SSC, 1% SDS, and, finally, twice at room temperature for 10 min in 0.1 × SSC. Autoradiography was performed with Kodak X-Omat AR film (with and without Cronex Lightning-Plus intensifier screen) at −70 C for 3–5 hr.

Fragment mobility was measured as the migration distance from the loading well in the agarose gel to the center of the hybridization

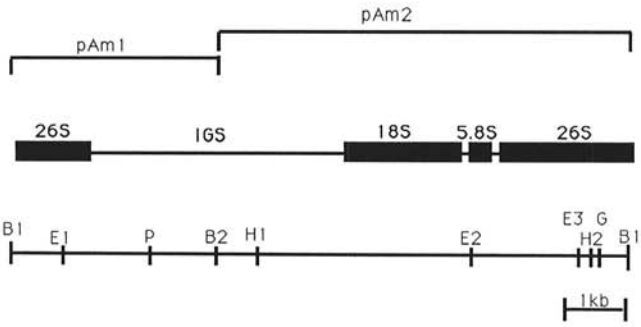

Fig. 1. Restriction site map of the rDNA repeat in *Armillaria ostoyae* strain 300-2 and illustration of the rDNA clones (pAM1 and pAM2) used in this study. pAM1 and pAM2 are subcloned *Bam*HI fragments of rDNA. The map is from Anderson, et al (1). The three *Eco*RI sites are in positions similar to those previously reported for *Agrocybe pediades* (23) and *Coprinus cinereus* (6). Abbreviations: B = *Bam*HI, E = *Eco*RI, G = *Bgl*II, H = *Hin*dIII, P = *Pst*I.

TABLE 1. List of *Laccaria* isolates

Species	CRBF isolate designation[a]	Geographical origin	Biological origin[b]	Collector (Alternative accession number)
L. bicolor	0101	Ontario	Basidiomata	C. Godbout (0211)
	0440	Ontario	Basidiospore	B. Kropp (0440)
	0494	Ontario	Basidiospore	B. Kropp (0494)
	0572	Ontario	Basidiospore	B. Kropp (0572)
	0599	Sweden	Cross	B. Kropp (0599)
	0730	Tennessee	Basidiomata	G. Mueller (993)[T#42521]
	0731	Tennessee	Basidiomata	G. Mueller (994)[T#42754]
	0732	North Carolina	Basidiomata	G. Mueller (1014)[T#42752]
	0733	Idaho	Basidiomata	G. Mueller (1225)[T#42607]
	0734	Washington	Basidiomata	G. Mueller (1293)[T#42606]
	0735	Oregon	Basidiomata	G. Mueller (1367)[T#42755]
	0736	Oregon	Basidiomata	G. Mueller (1478)[T#42603]
	0738	British Columbia	Basidiomata	G. Mueller (1264)[T#42604]
	0809	Sweden	Basidiospore	G. Mueller (360-2)
	0810	North Carolina	Basidiospore	G. Mueller (1744-2)[T#44153]
	0811	North Carolina	Basidiospore	G. Mueller (1771-2)[T#44153]
	0812	North Carolina	Basidiospore	G. Mueller (1773-4)[T#44167]
	0813	North Carolina	Basidiospore	G. Mueller (1804-3)
	0814	Michigan	Basidiospore	G. Mueller (1869-2)
	0815	Michigan	Basidiospore	G. Mueller (1873a.6)
	0816	Washington	Basidiospore	G. Mueller (1981-1)
	0817	Washington	Basidiospore	G. Mueller (2008-2)
	0818	Washington	Basidiospore	G. Mueller (2013-4)
	0819	Washington	Basidiospore	G. Mueller (2021-3)
	0820	Washington	Basidiospore	G. Mueller (2061a.6)
	1039	Québec	Basidiomata	B. Kropp (SPR1)
	1040	Québec	Basidiomata	B. Kropp (SPR2)
	1041	Ontario	Basidiomata	R. Summerbell (Dm32)
	1043	Québec	Basidiospore	B. Kropp (62)
L. laccata				
Biological species 1	0737	North Carolina	Basidiomata	G. Mueller (1768)[T#44164]
	0774	North Carolina	Basidiospore	G. Mueller (1751-4)
	0775	Michigan	Basidiospore	G. Mueller (1829-8)
Biological species 2	0777	Ontario	Basidiospore	G. Mueller (1925-1)
(var. *moelleri*)	0778	Ontario	Basidiospore	G. Mueller (1929-1)
Biological species 3	0771	Sweden	Basidiospore	G. Mueller (1560-1)
	0773	Sweden	Basidiospore	G. Mueller (1599-4)
Biological species 4	0769	Sweden	Basidiospore	G. Mueller (369-4)
L. proxima	0511	Ontario	Basidiomata	J. Gagnon (511)
	0780	California	Basidiospore	G. Mueller (2100-5)
	0821	California	Basidiospore	G. Mueller (2131-1)
L. amethystina	0772	Sweden	Basidiospore	G. Mueller (1570-1)
	0776	Michigan	Basidiospore	G. Mueller (1919-1)

[a]CRBF = Centre de Recherche en Biologie Forestière

[b]All isolates initiated from germinated spores are monokaryotic cultures whereas those from basidiomata are dikaryotic cultures. Isolate 0599 is a dikaryotic culture obtained by crossing two monokaryotic cultures 1591-2 and 1591-3, both initiated from germinated basidiospores of a single basidiocarp collected by G. Mueller.

signal. Fragments separated by less than 1 mm on the autoradiogram were not considered different. The molecular size of each fragment was estimated with a standard curve of migration versus log molecular size of *Hin*dIII-digested bacteriophage lambda.

RESULTS

Whole-cell DNAs from 29 isolates of *L. bicolor*, eight of *L. laccata*, three of *L. proxima*, and two of *L. amethystina* were digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I and then hybridized with pAM2. The sizes of detected rDNA fragments on southern hybridizations are given in Table 2.

Except for isolate 0814, which had a 10.2 kb *Bam*HI fragment, no *Bam*HI restriction site was found in the ribosomal repeat unit of the isolates of *L. bicolor*. Two out of the five enzymes tested (*Hin*dIII and *Pst*I) always yielded a single fragment. All North American isolates of *L. bicolor* had corresponding fragments for these two enzymes. The two Swedish isolates (0599 and 0809) of *L. bicolor*, collected 400 km apart, could be distinguished from the North American isolates of *L. bicolor* by the absence of *Pst*I restriction site (Table 2) as well as by the occurrence of a larger *Hin*dIII fragment for isolate 0599 (9.5 kb in size compared to 6 kb for all the North American isolates of *L. bicolor*) (Table 2, Fig. 2).

Polymorphisms in the *Bam*HI, *Hin*dIII, and *Pst*I fragments were observed for isolates of *L. laccata*. More similarities were detected within biological species than between, regardless of the geographical origin of the isolates. For example, biological species 3, which includes two Swedish isolates (0771 and 0773) from different geographical areas, had corresponding *Bam*HI, *Hin*dIII, and *Pst*I patterns. However, both isolates differed from the other Swedish isolate 0769 (biological species 4) by the *Pst*I fragments and by the absence of *Hin*dIII restriction sites. A similar trend was also observed for the North American isolates of *L. laccata*, although polymorphism was also noticed within biological species 1 with *Bam*HI and *Pst*I. Of interest was the 6 kb *Hin*dIII fragment in biological species 1, which appeared similar in size to a fragment found in all North American isolates of *L. bicolor*.

A *Bam*HI fragment of approximately 10.2 kb was found in all three isolates of *L. proxima* from North America (Table 2). Two isolates (0511 and 0780, from Ontario and California, respectively) had a corresponding *Hin*dIII fragment but differed

TABLE 2. Size in kilobases of restriction DNA fragments hybridizing to pAM2

Species	Isolates	Restriction enzyme				
		*Bam*HI	*Bgl*II	*Eco*RI[a]	*Hin*dIII	*Pst*I
L. bicolor	0101	>25[b]	(7.0,6.7)[c],3.4,0.7	(7.8,7.5),2.2,1.7	6	14
	0440	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0494	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0572	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0599	>25	(6.2),3.4,0.7	(7.0),1.8,1.7	9.5	>25
	0730	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0731	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0732	>25	(7.0,6.7),3.4,0.7	(7.8,7.5),2.2,1.7	6	14
	0733	>25	(7.0,6.5),3.4,0.7	(7.8,7.2),2.2,1.7	6	14
	0734	>25	(7.0,6.7),3.4,0.7	(7.8,7.5),2.2,1.7	6	14
	0735	>25	(7.0,6.7),3.4,0.7	(7.8,7.5),2.2,1.7	6	14
	0736	>25	(7.0,6.7),3.4,0.7	(7.8,7.5),2.2,1.7	6	14
	0738	>25	(6.7),3.4,0.7	(7.5),2.2,1.7	NT[d]	14
	0809	>25	(6.2),3.4,0.7	(7.0),1.8,1.7	NT	>25
	0810	>25	(7.0),3.4,0.7	NT	6	14
	0811	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	NT
	0812	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0813	>25	(7.0),3.4,0.7	NT	6	14
	0814	10.2	NT	(7.8),2.2,1.7	6	14
	0815	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0816	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0817	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0818	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	0819	>25	(6.7),3.4,0.7	(7.5),2.2,1.7	6	14
	0820	>25	(7.0),3.4,0.7	NT	6	14
	1039	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	1040	>25	(7.0),3.4,0.7	(7.8),2.2,1.7	6	14
	1041	>24	(7.0,6.7),3.4,0.7	(7.8,7.5),2.2,1.7	6	14
	1043	>25	(6.5),3.4,0.7	(7.2),2.2,1.7	6	14
L. laccata						
Biological species 1	0737	>25	(7.6),3.4	(7.8),2.2,1.7	6	9.4
	0774	>25	(7.6),3.4	(7.8),2.2,1.7	6	NT
	0775	10.4	(7.6),3.4	(7.8),2.2,1.7	6	14
Biological species 2	0777	>25	(6.7),3.4	(7.0),1.8,1.7	9.5	>25
	0778	>25	(6.7),3.4	(7.0),1.8,1.7	9.5	>25
Biological species 3	0771	>25	(6.7),3.4	NT	>25	13.8
	0773	>25	(6.7),3.4	(7.0),2.2,1.7	>25	13.8
Biological species 4	0769	>25	(6.7),3.4,0.7	(7.5), 2.2,1.7	6.6	14
L. proxima	0511	10.2	(6.5),3.4,0.7	3.2,1.8,2.7	9.5	>25
	0780	10.2	(6.5),3.4,0.7	3.2,2.2,1.7	9.5	13.8
	0821	10.2	NT	3.2,2.2,1.7	NT	13.8
L. amethystina	0772	>25	(7.0),3.4,0.7	(7.5),1.8,1.7	4.4,3.2,2.3	>25
	0776	>25	(6.5),3.4,0.7	(6.7),1.8,1.7	9.3	>25

[a] A faint hybridization signal (compared to the other fragments in the hybridization pattern) was occasionally detected around 3.5 kb in *L. bicolor* strains 0101, 0440, 0494, 0572, 0599, 0733, 0738, 0811 and 1041 and *L. laccata* isolates 0737, 0775, 0777 and 0778. This signal was not included in the analysis.

[b] >25 = no restriction site.

[c] The sizes of the fragments within parentheses are rough approximates.

[d] NT = not tested.

by their *Pst*I patterns. No *Bam*HI nor *Pst*I restriction sites were detected for the two isolates of *L. amethystina*, one from Sweden (0772) and the other from North America (0776). *L. amethystina* 0772 was unique in having three *Hin*dIII fragments.

*Eco*RI and *Bgl*II gave complex fragment patterns (Table 2, Fig. 3) with pAM2, which reflect the presence of two or more restriction sites in the ribosomal repeat unit. Polymorphism existed within *Laccaria* spp. with respect to the large *Bgl*II and *Eco*RI fragments (>6.2 kb) (Table 2). Similar to the results obtained with *Bam*HI, *Hin*dIII, and *Pst*I patterns: differences were observed between Swedish and North American isolates of *L. bicolor* and polymorphisms within *L. laccata* and *L. amethystina*.

Two fragments, between 6.5 and 7.0 kb in size, occurred in the *Bgl*II pattern of seven isolates of *L. bicolor* (Table 2). All the isolates of *L. bicolor* with these double *Bgl*II fragments also showed double *Eco*RI fragments greater than 7.3 kb (Table 2). It should be noted that all these isolates were dikaryotic. When three monokaryons (0440, 0494, and 0572, all from a progeny of the dikaryon 0101) were analyzed, a single 7.0 kb *Bgl*II fragment and a single 7.8kb *Eco*RI fragment were found instead of the doublet in the corresponding digests of their parental strain.

All three isolates of *L. proxima* had an *Eco*RI fragment of 3.2 kb instead of one greater than 7 kb. The strong hybridization signal observed suggests that this fragment might be a doublet. With respect to the other *Laccaria* spp., this result could be indicating the presence of at least one additional *Eco*RI site in the ribosomal repeat unit in *L. proxima*.

A strong hybridization signal at 3.4 kb was detected with *Bgl*II in all the *Laccaria* isolates tested. A *Bgl*II fragment of 0.7 kb, which gave a faint hybridization signal in comparison to the other fragments in the hybridization pattern, was present in all the isolates of *L. bicolor, L. proxima*, and *L. amethystina* but in only one out of the four biological species of *L. laccata*. Since no hybridization signal was observed at 3.4 and 0.7 kb when pAM1 was used as probe, it appears that certain *Bgl*II sites were located in the ribosomal region corresponding to pAM2.

A 1.7 kb *Eco*RI fragment (Fig. 3) was found in all the *Laccaria* isolates when pAM2 was used as a probe but not when pAM1 was used as probe, indicating that this fragment was also located in the portion of the repeat corresponding to pAM2. A 2.2kb *Eco*RI fragment (Fig. 3) was detected in the North American isolates of *L. bicolor*, in the North American isolates of *L. laccata* (biological species 1), in the Swedish isolates of *L. laccata* (biological species 3 and 4), and in two of the three North American isolates of *L. proxima* (0780 and 0821). When this 2.2 kb fragment was absent, a 1.8 kb fragment was present (Fig. 3). These two fragments also hybridized strongly with pAM1.

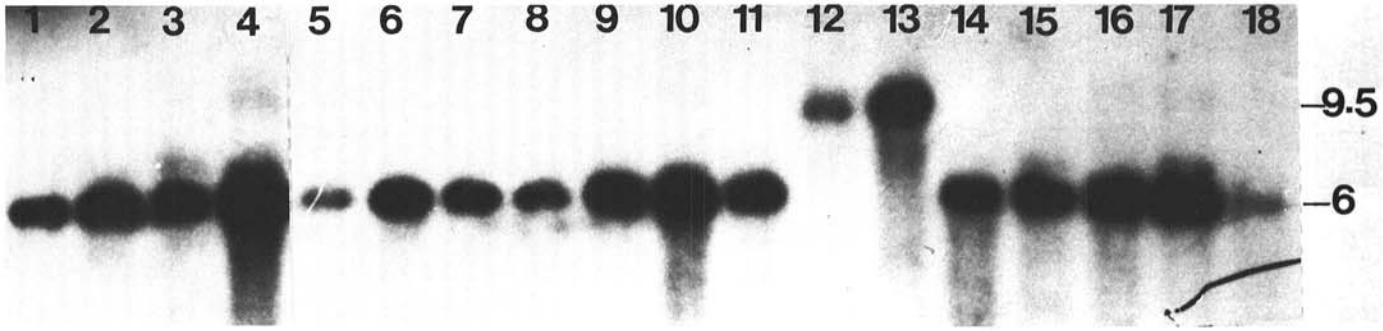

Fig. 2. Southern hybridizations of whole-cell DNAs of *Laccaria* digested with *Hin*dIII and probed with ^{32}P-labelled cloned rDNA from *Armillaria ostoyae*, pAM2. Lanes: 1–12, *L. bicolor* 0730, 0731, 0732, 0733, 0734, 0735, 0736, 0101, 0440, 0494, 0572, and 0599; 13, *L. proxima* 0511; 14 and 15, *L. bicolor* 1039 and 1040; 16, *L. laccata* (1) 0737; 17 and 18, *L. bicolor* 1041 and 1043.

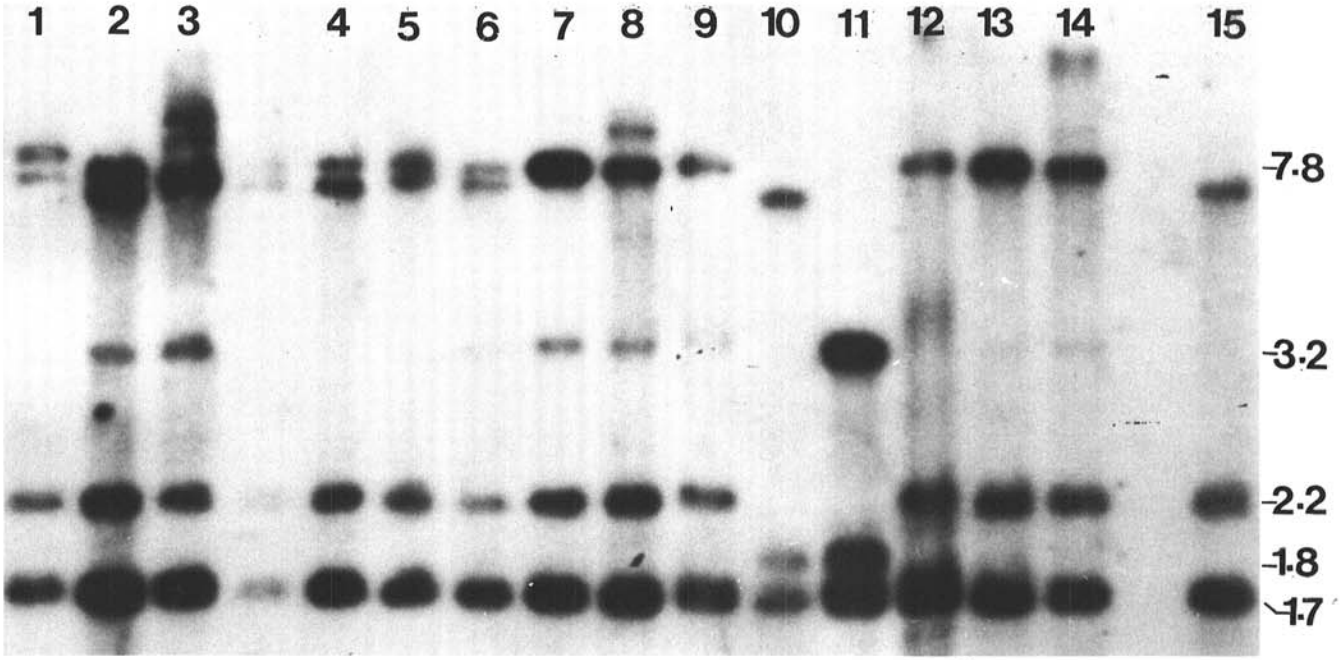

Fig. 3. Southern hybridizations of whole-cell DNAs of *Laccaria* digested with *Eco*RI and probed with ^{32}P-labelled cloned rDNA from *Armillaria ostoyae*, pAM2. Lanes: 1–10, *L. bicolor* 0732, 0733, 0738, 0734, 0735, 0736, 0101, 0440, 0494, 0572, and 0599; 11, *L. proxima* 0511; 12 and 13, *L. bicolor* 1039 and 1040; 14, *L. laccata* (1) 0737; 15, *L. bicolor* 1043. Partial digests were observed in lanes 3 and 8, this autoradiogram giving extra bands >10 kb.

DISCUSSION

Restriction and length polymorphisms in nuclear rDNA were detected between and within four *Laccaria* spp.: *L. bicolor, L. laccata, L. proxima*, and *L. amethystina*. However, North American isolates of *L. bicolor* appear to form a homogenous group that is distinguishable from European isolates of *L. bicolor* using *Bgl*II, *Eco*RI, *Hin*dIII, or *Pst*I as restriction endonucleases. Both groups also are distinguishable from isolates of the other three species using RFLP phenotypes. Although the two European isolates of *L. bicolor* are interfertile with most of the North American isolates (G. M. Mueller, *unpublished results*), the ribosomal repeat unit seems to have diverged on the two continents. A similar trend was observed for *L. amethystina*. The apparent homogeneity of *L. bicolor* in North America is highlighted by the similarities found between isolate 0101 (and its progeny) and 0345, two isolates that belong to different biological species (II and I, respectively) (14). However, the development of biological barriers in *L. bicolor* may have been relatively recent, because partial intersterility is common among North American populations of *L. bicolor* (14, G. M. Mueller, *unpublished results*).

Based on morphological, physiological, and mating characteristics, *L. laccata* has been subdivided into at least four biological species that are completely intersterile (12, G. M. Mueller, *unpublished results*), and which were included in our analysis. RFLP in the nuclear rDNA illustrates the heterogeneity among these biological species and indicates a divergence of their ribosomal repeat unit. Further polymorphism was detected within biological species 1. More isolates for each biological species would be required to determine a representative pattern for species identification purposes.

Characteristic patterns were found among the three North American isolates of *L. proxima*, including a strong hybridization signal at 3.2 kb with *Eco*RI and a 10.2 kb *Bam*HI fragment. The relationship between the 10.2 kb *Bam*HI fragment in *L. proxima* and that in *L. bicolor* isolate 0814 should be examined by restriction-site mapping or sequence analysis.

Length polymorphisms in the large (>6 kb) *Bgl*II and *Eco*RI fragments were detected within and between the *Laccaria* spp. These length variations are probably due to small insertions or deletions in the intergenic spacer of the ribosomal repeat unit. Such polymorphisms in the *Eco*RI pattern have also been detected in many other species of fungi (13,23). Armstrong, et al (3) have indicated that *Eco*RI RFLPs of rDNA are isolate-specific probes for *L. bicolor*. The survey of many isolates, as was done in our study, suggests that similar patterns are observed among the isolates of *L. bicolor*. However, pattern diversity may have been underestimated in our study because only fragments separated by more than 1 mm on the autoradiogram were considered to be different. Migration over longer distances could help to resolve this point. In any case, we would like to caution against hasty conclusions and classifications of doubtful isolates by rDNA polymorphisms. For example, *L. bicolor* 0599 could have been classified as *L. proxima* if the 9.5 kb *Hin*dIII alone was considered. However, this isolate differs from *L. proxima* with *Bam*HI, *Bgl*II, and *Eco*RI. It is dangerous to base classification or reclassification of isolates on only one marker without any knowledge of the general pattern existing in the species considered.

Seven dikaryotic isolates of *L. bicolor* collected from the field contained mixed rDNA types, indicating that their parental monokaryons differed in their rDNA length. Similarly, Petes and Botstein (22) found a laboratory diploid strain of *Saccharomyces ceverisiae* that contained roughly equal amounts of two rDNA repeat units that differed by a number of small insertions, deletions, and point mutations in the intergenic spacer. The rDNA of this strain was inherited in a Mendelian fashion but with a significant suppression of recombination, a phenomenon that has also been observed in *Coprinus* (6) and *Neurospora* (24). The three monokaryotic isolates from a progeny of *L. bicolor* 0101 are identical in their *Eco*RI and *Bgl*II patterns, but the small sample size does not allow extrapolations concerning the general inheritance and recombination pattern of rDNA in *Laccaria*.

Certain *Laccaria* isolates have a 1.8 kb *Eco*RI fragment, whereas others have a 2.2 kb fragment instead. The 0.4 kb of difference between these two fragments was most likely due to an insertion or deletion at the 3′ end of the 26S. Similar observations also have been reported in *Armillaria ostoyae* (1) and in certain genera in the Cortinariaceae (23). The sequence and function of this insertion as well as its phylogenetic significance remain unknown. It is interesting that all North American isolates of *L. bicolor*, but not the two Swedish isolates, have this insertion. The screening of more European isolates is necessary to determine if the lack of this 0.4 kb insertion is a general phenomenon among European populations of *L. bicolor*. This insertion is apparently also present in one North American isolate of *L. proxima*, in European isolates of *L. laccata*, and in some North American biological species of *L. laccata* but not in two other North American isolates of *L. proxima* nor in the two North American isolates of *L. laccata* in biological species 2.

For any given isolate, the *Hin*dIII fragment observed was consistently shorter than the *Pst*I fragment, the sum of the *Bgl*II fragments, or the sum of the *Eco*RI fragments. It is possible that a *Hin*dIII fragment was not detected because it was located completely within the rDNA region not covered by pAM2. Alternatively, certain *Hin*dIII fragments may be nonhomologous to the *Armillaria* rDNA probe because of evolutionary divergence in the intergenic spacer.

This study suggests that four *Laccaria* spp. and four biological species within *L. laccata* can be distinguished. The nuclear rDNA restriction fragment phenotypes could be used as additional taxonomic characters for the *Laccaria* complex. It also appears that the nuclear rDNA have diverged in *L. bicolor* and in *L. amethystina* on the two continents and among biological species of *L. laccata*. However, additional data on a larger number of isolates, especially from Europe, are required in order to corroborate this trend. Furthermore, detailed restriction site mapping or nucleotide sequencing of a portion of the ribosomal repeat unit should be used to examine the evolutionary history of the *Laccaria* complex.

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