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EVOLUTIONARY RELATIONSHIPS OF *LENTINUS* TO THE POLYPORACEAE: EVIDENCE FROM RESTRICTION ANALYSIS OF ENZYMATICALLY AMPLIFIED RIBOSOMAL DNA

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ABSTRACT

Evolutionary relationships of *Lentinus* to the Tricholomataceae and Polyporaceae were studied using restriction analysis of nuclear-encoded ribosomal RNA genes (rDNA). Five species of *Lentinus*, three species in the Polyporaceae and two species in the Tricholomataceae were examined. Ribosomal DNA phenotypes were determined by restriction endonuclease digestion of enzymatically amplified rDNA (PCR fingerprinting). This method generates restriction fragment length polymorphism data without Southern blotting or autoradiography. With five different four-base restriction enzymes, one hundred and one unique restriction fragments were resolved. Among the 16 individuals studied, there were twelve different rDNA types. A similarity matrix based on presence or absence of comigrating restriction fragments was analyzed with UPGMA, complete and single-linkage analysis (using the NTSYS computer package), and Fitch-Margoliash and KITSCH analysis (using the PHYLIP computer package). Results of all analytical approaches were highly consistent and strongly suggest that *Lentinus tigrinus* is more closely related to the Polyporaceae than to the Tricholomataceae. Morphological characters (dimitic hyphae and hyphal pegs) also support this hypothesis. The other species of *Lentinus* appear to be intermediate between the Polyporaceae and Tricholomataceae and could not be clearly assigned to either family. The results also suggest that *Lentinus* is paraphyletic and, therefore, that lamellae have arisen repeatedly by convergent evolution.

Key Words: *Lentinus*, rDNA, polymerase chain reaction, molecular systematics, evolution

The genus *Lentinus* Fr. shows similarities to both the Aphyllophorales and the Agaricales (*sensu* Fries). Traditionally, *Lentinus* has been placed in the agaric family Tricholomataceae because it possesses a lamellate hymenophore and white spore print (Miller, **1973**). However, in most modern treatments it has been placed in or near the Polyporaceae (Singer, **1986**; Pegler, **1983**; Kühner, **1980**; Moser, **1978**). Characters used to support this placement include the presence of dimitic and amphimitic hyphal systems in both the Polyporaceae and *Lentinus*, i.e., both thin-walled generative hyphae and thick-walled skeletal or ligative hyphae in the sporocarp context (Corner, **1966**, **1981**; Pegler, **1975**, **1983**). In addition, hyphal pegs, fascicles of sterile hyphae emerging from the hymenium surface, occur in some genera of the Polyporaceae and *Lentinus* subg. *Lentinus* (Pegler, **1983**; Corner, **1981**).

Pegler (**1983**) suggested that *Lentinus* together with *Pleurotus* (Fr.) Kummer and *Panus* Fr. are derived from a polyporoid ancestry. If this is true, then the presence of lamellae in both *Lentinus* and the Agaricales *s.str.* is the result of convergent evolution. Corner (**1981**) proposed an alternate phylogeny in which the tribe Lentinieae (*Lentinus, Panus,* and *Pleurotus*) are a basal, paraphyletic group from which the Agaricales *s. str.* and the Polyporaceae arose. In this scenario, gills arose only once (although they have been independently lost in several lineages). Because *Lentinus* possesses characters that are intermediate between the Polyporaceae and the Agaricales *s. str.*, an understanding of the phylogenetic relationships of *Lentinus* is critical to a natural classification scheme for the hymenomycetes.

The question addressed in this study was: how are *Lentinus*, the Polyporaceae, and the Tricholomataceae related to each other? Molecular evidence was sought because morphological evidence alone has been insufficient to conclusively answer this question. Molecular characters were generated by an application of the polymerase chain reaction (PCR) that has been used to detect restriction fragment length polymorphisms (RFLPs) for diagnosis of genetically based human diseases (Kogan *et al.*, **1987**; Wallace *et al.*, **1988**). As a taxonomic tool, this method has been called 'PCR fingerprinting' (Vilgalys and Hester, **1990**). The method permits very rapid identifi-

TABLE I
TAXA EXAMINED

Lentinus	
subg. *Lentinus*	
sect. *Tigrini*	
Lentinus tigrinus	DUKE RV88, VT 296
subg. *Panus*	
sect. *Panus*	
Lentinus strigosus	DUKE DH1, VT 343, VT 340
Lentinus torulosus	VT 1502
sect. *Squamosi*	
Lentinus lepideus	DUKE 484, VT 306
Lentinus ponderosus	VT 302, VT 303, VT 304
Polyporaceae	
Polyporus arcularius	VT 959
Polyporus squamosus[a]	SAR 89-468
Grifola frondosa[a]	SAR 89-478
Tricholomataceae	
Mycena galericulata	SAR 198
Collybia species	SAR 39

[a] DNA isolated from field-collected sporocarps. Taxa with "VT" numbers from Dr. Orson K. Miller, Virginia Polytechnic Institute, Blacksburg, Virginia. All other collections from Duke University herbarium and culture collection.

cation of restriction enzyme digestion phenotypes for homologous genes from a large number of individuals. Results from PCR fingerprinting are similar to those from Southern hybridization. However, PCR fingerprinting does not involve transfer of DNA to hybridization filters or autoradiography and requires only several nanograms of DNA from each individual studied.

Ribosomal RNA genes (rDNA) were chosen for analysis because they contain both highly conserved regions that code for mature ribosomal RNA and more variable intergenic sequences (Elwood *et al.*, **1985**; Hillis and Davis, **1985**). Ribosomal DNA has been used in systematic studies of several groups of fungi including *Agrocybe* Fayod (Rehner and Ammirati, **1987**) in which RFLPs were used, and *Fusarium* Link *ex* Fr. (Gaudet *et al.*, **1989**) and *Rhizopogon* Fr. (Bruns *et al.*, **1989**) in which nucleic acid sequence data were used. In this study, presence or absence of comigrating restriction fragments between taxa were scored and used to compute a similarity matrix for distance-based analyses. Similar approaches using random RFLPs have been used in *Neurospora* Shear & Dodge (Taylor and Natvig, **1989**; Natvig *et al.*, **1987**) and *Armillaria* (Fr.) Kummer (Anderson *et al.*, **1987**; Smith and Anderson, **1989**).

MATERIALS AND METHODS

Material studied.—The organisms used in this study (TABLE I) represent the two subgenera of *Lentinus* (five species total), the Polyporaceae *s.str.* (three species), and the Tricholomataceae (two species). Multiple isolates of several species of *Lentinus* were examined so that intraspecific rDNA variability could be assessed (TABLE I).

Cultures were maintained on MEA slants (1.5% malt extract, 1.5% agar) at 4 C then transferred to 50 ml MYG liquid media (1% malt extract, 0.4% yeast extract, 1% glucose) for approximately two wk prior to harvesting.

DNA isolation and PCR amplification.—DNA isolation was performed essentially as described by Raeder and Broda (**1985**). DNA was isolated from cultured mycelium or from field collected sporocarps (TABLE I). Surface tissues of the pileus were removed prior to DNA isolation from sporocarps.

Genomic DNA templates were gel-purified in low-melting agarose and ribosomal DNA was amplified as described in Vilgalys and Hester (**1990**). The PCR was used to amplify a fragment (*ca* 1.7 kilobases) homologous to a region in *Saccharomyces cerevisiae* rDNA from base position 34 in the 5.8S RNA to base position 1448 in the 25S RNA (see Vilgalys and Hester, **1990**, for primer sequences). This region was chosen because it contains both coding and non-coding (intergenic) sequences which could provide amounts of variation appropriate for taxonomic studies at many levels.

Two sets of control PCR reactions were run: one in which the genomic DNA template was

omitted to control for contamination by exogenous DNA, and another in which one or the other primer was omitted to confirm the identity of the PCR products. PCR is capable of a 10^8 amplification of target sequences (Saiki *et al.*, **1988**). Therefore, template contamination is a serious issue, especially when very small amounts of template are used (Kwok and Higuchi, **1989**).

RFLP analysis of PCR products by PCR fingerprinting.—Two microliters of each PCR reaction were electrophoresed in 0.8% agarose and stained with ethidium bromide (FIG. 1). Bacteriophage lambda DNA digested with *EcoR* I and *Hind* III was used as a molecular weight standard.

The remaining PCR-amplified DNA products were phenol extracted, precipitated, dried, and resuspended as described in Vilgalys and Hester (**1990**). Each PCR product was divided into five aliquots for digestion with the restriction endonucleases *Taq* I, *Hha* I, *Ava* II, *Hinf* I, and *Hae* III according to the manufacturer's protocols (Promega Biotech). Final volumes were 20 μl each.

Restriction digests were electrophoresed in 4% agarose gels (2.25% NuSieve agarose, 1.75% Seakem GTG agarose; FMC Bioproducts) and stained with ethidium bromide. To facilitate the use of 40-well combs, composite gels with a less brittle 0.8% agarose insert for the combs were employed. *Hae* III-digested Phi-X 174 DNA or *Hinf* I-digested pBR 322 were used as molecular weight standards. Fragment sizes were estimated with the MICROGENIE computer package (Beckman Instruments). Restriction patterns were recorded by photographing gels over a UV transilluminator after destaining for one hour in distilled water.

Phylogenetic analysis.—Presence or absence of comigrating restriction fragments was scored for all isolates digested with each enzyme, resulting in a 16 individual by 100 character binary matrix. When redundant individuals with identical RFLP phenotypes for all five enzymes were removed, the matrix condensed to 12 rDNA types by 100 characters (TABLE II).

A distance metric based on a modification of the Dice coefficient (Sneath and Sokal, **1973**) was calculated from the RFLP phenotypes as

$$D = 1 - 2(n_{xy})/(n_x + n_y)$$

in which n_{xy} equals the number of restriction fragments shared between individuals, and n_x and n_y equal the total number of restriction fragments in all digests in individuals x and y, respectively (TABLE III). This coefficient was chosen because it produces a standardized distance measure that is not biased by differences in the total number of fragments in each member of a pair.

Distance methods were used to construct unrooted networks by the algorithm of Fitch and Margoliash and also by KITSCH analysis using the computer package PHYLIP (Felsenstein, **1988**). Phenetic analyses (UPGMA, single linkage and complete linkage) were performed using the computer package NTSYS (Rohlf *et al.*, **1979**).

RESULTS

PCR amplification.—Gel electrophoresis of undigested PCR products from all taxa except *Collybia* revealed a uniform band of approximately 1.67 kilobases, indicating absence of large length mutations among those taxa (FIG. 1). The product from *Collybia* was estimated to be 1.73 kilobases. The actual sizes are probably within one hundred bases of these estimates. A secondary fragment of approximately 0.8 kilobases was obtained in both of the *Lentinus tigrinus* PCR products. The origin of this fragment is unknown. No PCR products were obtained in any of the controls, indicating that template contamination did not occur (data not shown).

Mean values for sums of fragment sizes from all digests are shown in TABLE IV. In general, there was good agreement between size estimates for the undigested PCR products and the sums of fragment sizes, but there were some anomalies: means of sums of fragment sizes in *Lentinus lepideus* isolate 306 and *Grifola frondosa* are both consistently lower than the size estimate for the undigested PCR product, while in *Lentinus ponderosus* it is higher.

Interspecific variation.—RFLP phenotypes for the PCR amplified rDNAs from five digests are shown in FIGS. 2, 3, and 4 and TABLE II. A total of one hundred and one unique bands were resolved among the 16 individuals in 10 species. For *Ava* II and *Taq* I there were 8 different restriction phenotypes. For *Hha* I, *Hae* III, and *Hinf* I there were 10 different restriction phenotypes. When results from all 5 enzymes were pooled, each strain could be assigned to one of 12 unique rDNA types. With one possible exception, no two species shared rDNA tpyes. All three isolates of *Lentinus strigosus* (Schwein.) Fr.

TABLE II

DISTRIBUTION OF RESTRICTION FRAGMENTS

Enzyme	No.	Size[a]	rDNA type[b] 1	2	3	4	5	6	7	8	9	10	11	12
*Hinf*I	1	415	1	1	0	0	0	0	1	1	1	1	0	1
	2	359	1	1	1	1	1	1	1	1	1	1	1	0
	3	316	1	1	0	0	0	0	0	0	0	1	1	0
	4	299	0	0	1	0	0	0	0	0	0	0	0	0
	5	371	0	0	0	1	1	1	0	0	0	0	0	0
	6	307	0	0	0	0	0	0	1	1	1	0	0	0
	7	517	0	0	0	0	0	0	0	0	0	0	0	1
	8	432	0	0	0	0	1	1	0	0	0	0	0	1
	9	217	0	0	0	0	0	0	0	0	0	0	1	1
	10	212	1	1	1	1	1	1	1	1	1	0	1	1
	11	199	1	1	1	1	1	1	1	1	1	0	1	0
	12	174	0	0	1	0	0	0	0	0	0	0	1	0
	13	204	0	0	0	0	0	0	0	0	0	1	0	0
	14	141	1	1	1	1	1	1	1	1	1	1	1	1
	15	88	0	0	0	0	0	0	1	0	0	0	0	0
	16	54	0	0	0	1	1	1	0	0	0	0	0	0
	17	47	1	1	1	1	1	1	1	1	1	0	1	1
	18	35	1	1	1	1	1	1	1	1	1	0	1	1
	19	257	0	0	0	1	1	1	0	0	0	0	0	0
*Hae*III	1	950	1	1	0	0	0	1	0	0	0	0	0	0
	2	1018	0	0	0	0	0	0	0	0	0	0	1	0
	3	1178	0	0	0	0	0	0	0	0	0	0	0	1
	4	699	0	0	1	0	0	0	0	0	0	0	0	0
	5	569	1	0	0	0	0	0	1	1	1	1	0	0
	6	520	0	1	1	1	1	1	0	0	0	0	0	0
	7	442	0	0	0	1	1	1	0	0	0	0	0	0
	8	353	0	0	0	1	1	1	1	1	0	1	0	0
	9	427	0	0	0	0	0	0	1	0	0	0	0	0
	10	478	0	0	0	0	0	0	1	0	0	0	0	0
	11	434	0	0	0	0	0	0	0	0	0	0	1	1
	12	460	0	0	0	0	0	0	0	0	1	0	0	0
	13	363	0	0	0	0	0	0	0	0	1	0	0	0
	14	295	0	0	0	0	0	0	0	1	0	0	0	0
	15	288	0	0	0	0	0	0	0	0	0	1	0	0
	16	221	1	1	0	0	0	0	0	0	0	0	0	0
	17	195	0	0	0	1	1	1	0	0	0	0	0	0
	18	185	0	0	0	0	0	0	1	1	1	1	1	0
	19	226	0	0	0	0	0	0	0	0	0	0	0	1
	20	179	0	0	0	0	0	0	0	1	0	0	0	0
	21	97	1	1	1	1	1	1	1	1	1	1	1	1
	22	70	1	1	1	1	1	1	1	1	1	1	1	1
*Hha*I	1	639	1	1	1	1	1	1	1	1	1	1	1	1
	2	458	1	1	0	0	0	0	0	0	0	0	0	0
	3	401	0	1	0	0	0	0	0	0	0	0	0	0
	4	418	0	0	1	1	1	1	1	0	0	0	0	0
	5	386	0	0	1	1	1	1	1	0	0	0	0	0
	6	415	0	0	0	0	0	1	0	0	0	0	0	0
	7	336	0	0	0	0	0	0	0	1	0	0	0	0
	8	534	0	0	0	0	0	0	0	0	1	0	1	0
	9	447	0	0	0	0	0	0	0	0	1	0	1	0
	10	469	0	0	0	0	0	0	0	0	0	1	0	0
	11	410	0	0	0	0	0	0	0	0	0	1	0	0
	12	238	1	0	0	0	0	0	0	1	0	0	0	0
	13	233	0	0	0	1	0	0	0	0	0	0	0	0
	14	208	0	0	0	1	0	0	0	0	0	0	0	0
	15	203	1	0	0	0	0	0	0	0	0	0	0	0
	16	205	0	0	0	0	0	0	0	1	0	0	0	0

TABLE II

DISTRIBUTION OF RESTRICTION FRAGMENTS

Enzyme	No.	Size[a]	rDNA type[b] 1	2	3	4	5	6	7	8	9	10	11	12
	17	130	1	1	0	0	0	0	0	0	0	0	1	1
	18	81	0	0	0	0	0	0	1	1	0	0	0	0
	19	64	1	1	1	1	1	1	1	1	1	1	0	0
	20	57	1	1	1	1	1	1	1	1	1	1	1	1
	21	26	0	0	1	1	1	1	1	1	1	0	0	0
*Ava*II	1	731	0	0	0	0	0	0	0	0	0	0	0	1
	2	546	1	1	1	0	0	0	1	1	1	1	1	1
	3	450	0	0	0	1	1	1	0	0	0	0	0	0
	4	431	1	1	1	1	1	1	0	0	1	0	0	0
	5	344	1	1	1	1	1	1	1	1	1	1	0	0
	6	295	1	1	1	1	1	1	1	1	1	1	1	0
	7	271	0	0	0	0	0	0	0	0	0	0	1	0
	8	257	0	0	0	0	0	0	0	0	0	0	1	0
	9	153	0	0	0	0	0	0	0	0	0	0	1	1
	10	88	1	1	1	1	1	1	1	1	1	1	0	0
	11	73	1	1	0	0	0	0	0	0	1	1	0	0
	12	33	0	0	1	1	1	1	0	0	0	0	0	0
	13	51	0	0	0	0	0	0	0	0	0	0	1	0
	14	29	1	1	1	1	1	1	1	1	1	0	0	0
*Taq*I	1	700	0	0	0	0	0	0	0	0	0	0	0	1
	2	578	1	1	0	0	0	0	0	0	0	0	0	0
	3	447	1	1	0	0	0	0	0	0	0	0	0	0
	4	476	1	1	1	1	1	1	1	1	1	0	1	1
	5	324	0	0	1	0	1	1	1	1	1	0	0	0
	6	296	0	0	0	0	0	0	1	1	1	1	0	0
	7	262	0	0	0	0	0	0	0	0	1	0	0	0
	8	434	0	0	0	0	0	0	0	0	0	1	0	0
	9	381	0	0	0	0	0	0	0	0	0	0	0	1
	10	279	0	0	0	0	0	0	0	0	0	1	0	0
	11	268	0	0	0	0	0	0	0	0	0	0	0	1
	12	239	0	0	1	1	1	1	1	1	0	0	0	0
	13	226	0	0	0	0	0	0	0	0	0	1	0	0
	14	213	0	0	0	0	0	0	0	0	0	0	1	0
	15	183	0	0	0	0	0	0	1	1	0	1	0	0
	16	140	0	0	1	1	1	1	0	0	0	0	0	0
	17	110	0	0	1	1	1	1	1	1	1	1	1	0
	18	153	0	0	0	0	0	0	0	0	0	0	1	0
	19	70	0	0	0	0	0	0	0	0	0	0	1	0
	20	45	0	0	0	0	0	0	0	0	0	1	0	0
	21	48	0	0	1	1	1	1	1	1	0	1	0	0
	22	27	1	1	1	1	1	1	1	1	1	1	1	1
	23	12	1	1	1	1	1	1	1	1	1	1	0	0
	24	274	0	0	0	0	0	0	0	1	0	0	0	0

[a] Fragment sizes (base pairs) estimated by comparison to molecular weight standards.

[b] rDNA types: 1 = *Lentinus strigosus*, 2 = *Lentinus torulosus*, 3 = *Lentinus lepideus* isolate 306, 4 = *Lentinus lepideus* isolate 484, 5 = *Lentinus ponderosus* isolates 302 and 304, 6 = *Lentinus ponderosus* isolate 303, 7 = *Lentinus tigrinus*, 8 = *Polyporus arcularius*, 9 = *Polyporus squamosus*, 10 = *Grifola frondosa*, 11 = *Mycena galericulata*, 12 = *Collybia* sp.

were identical. However, two of these isolates correspond to *Panus rudis* Fr., while the other one corresponds to *Panus fragilis* Miller; both were placed as synonyms of *L. strigosus* by Pegler, **1983**.

Different species were observed to have common restriction phenotypes for some enzymes. *Lentinus torulosus* (Pers.: Fr.) Lloyd was identical to *Lentinus strigosus* for *Hinf* I, *Ava* II, and *Taq* I. All three isolates of *Lentinus ponderosus* Miller were identical to one isolate of *Lentinus lepideus* (Fr.: Fr.) Fr. (isolate 484) for *Ava* II, but

TABLE III

DISTANCE MATRIX BASED ON RESTRICTION PHENOTYPES FOR RDNA (VALUES OF THE DICE COEFFICIENT HAVE BEEN ROUNDED FROM SIX DECIMAL PLACES)

rDNA type/species	rDNA type											
	1	2	3	4	5	6	7	8	9	10	11	12
1. *L. strigosus*	0.000											
2. *L. torulosus*	0.079	0.000										
3. *L. lepideus* (306)	0.385	0.344	0.000									
4. *L. lepideus* (484)	0.450	0.412	0.200	0.000								
5. *L. ponderosus* (302,4)	0.450	0.412	0.171	0.054	0.000							
6. *L. ponderosus* (303)	0.429	0.391	0.183	0.067	0.013	0.000						
7. *L. tigrinus*	0.391	0.412	0.314	0.378	0.351	0.360	0.000					
8. *P. arcularius*	0.371	0.420	0.324	0.387	0.360	0.368	0.120	0.000				
9. *P. squamosus*	0.303	0.323	0.313	0.408	0.380	0.389	0.240	0.250	0.000			
10. *G. frondosa*	0.460	0.484	0.531	0.559	0.559	0.565	0.382	0.391	0.415	0.000		
11. *M. galericulata*	0.492	0.484	0.500	0.588	0.588	0.594	0.529	0.536	0.446	0.613	0.000	
12. *C.* species	0.536	0.527	0.614	0.672	0.639	0.645	0.607	0.613	0.586	0.709	0.455	0.000

were identical to the other isolate of *Lentinus lepideus* (isolate 306) for *Hha* I, and *Taq* I. *Polyporus arcularius* Batsch : Fr. was identical to *Polyporus squamosus* Huds. : Fr. for *Hinf* I. *Lentinus tigrinus* was identical to *Polyporus arcularius* for *Ava* II, and *Taq* I, despite the extra amplification product in *Lentinus tigrinus*.

Intraspecific variation.—Intraspecific heterogeneity was detected in *Lentinus lepideus* for *Taq* I, *Ava* II, *Hae* III, and *Hinf* I. In total, there were 12 bands that were present in only one of the two isolates sampled from *Lentinus lepideus*: 5 unique to isolate 306 and 7 unique to isolate 484 (FIGS. 2–4).

Minor intraspecific heterogeneity was also observed among the three isolates of *Lentinus ponderosus*. The 950 base pair fragment present in the *Hae* III digest of isolate 303 of *Lentinus ponderosus* is not present in the other two isolates from that species although the three isolates are otherwise identical (FIG. 3). Since the PCR amplification products from all three *Lentinus ponderosus* isolates are the same size, this result is most likely due to intragenomic variation within the rDNA itself. The unique *Hae* III fragment in isolate 303 comigrates with partial digestion products in the other two isolates. These results, together with the unequal staining of certain bands, suggest that one or more *Hae* III sites have been lost, or were never gained, in some copies of the tandemly repeated rDNA of isolate 303 but are present in the other copies and in all copies in the other two isolates.

The remaining species for which multiple isolates were scored were invariant: *Lentinus strigosus,* three isolates; *Lentinus tigrinus,* two isolates.

Cluster analyses and Fitch-Margoliash network.—Dendrogram topologies (FIGS. 5, 6) were highly consistent regardless of the method used to construct them (UPGMA, complete linkage, single linkage, KITSCH, and Fitch-Margoliash). In every case, the following results were obtained: 1, *Mycena* and *Collybia* were consistently placed as sister taxa, separate from *Lentinus* and the polypores; 2, within the *Lentinus*-plus-polypores cluster *Lentinus strigosus* and *Lentinus torulosus* formed a distinct group; 3, *Lentinus lepideus* and *Lentinus ponderosus* formed a distinct group; and 4, *Lentinus tigrinus* consistently clustered with *Polyporus arcularius* and *Polyporus squamosus*.

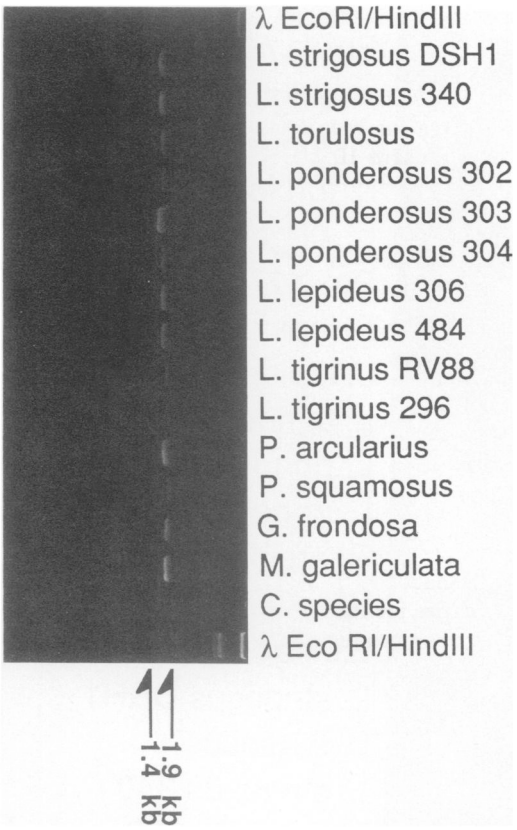


FIG. 1. PCR products of rDNA electrophoresed on 4% agarose and stained with ethidium bromide. *Lentinus strigosus* 343 not shown. Estimated size of fragments is 1.67 kilobases (kb) except *Collybia* which is 1.73 kb. λ = molecular weight standard. Selected marker fragment sizes in kilobases.

There was some slight variability in the results of the different clustering methods. *Grifola frondosa* (Dicks.: Fr.) S. F. Gray was placed in the *Lentinus tigrinus–Polyporus* cluster by the Fitch-Margoliash algorithm, but in KITSCH analysis and each of the phenetic analyses it was placed as sister group to the rest of the *Lentinus*-plus-polypores cluster (FIGS. 5, 6).

The results of the three phenetic methods of analysis were all topologically identical, except that single linkage placed the *Lentinus tigrinus-Polyporus* cluster closest to the *Lentinus strigosus–L. torulosus* cluster while complete linkage and UPGMA placed it closest to the *Lentinus lepideus–L. ponderosus* cluster. The dendrogram produced by KITSCH analysis was topologically identical to the UPGMA and complete linkage results.

TABLE IV

SIZE ESTIMATES OF PCR PRODUCTS FROM SUMS OF FRAGMENT SIZES IN ALL DIGESTS

rDNA type	Sum of fragment sizes[a]	Standard deviation
1	1753	121.6
2	1735	107.7
3	1517	146.3
4	1621	316.3
5	1684	237.9
6	1874	445.3
7	1723	283.8
8	1680	221.5
9	1708	104.7
10	1528	120.6
11	1587	282.1
12	1852	218.8

[a] Mean values of sums of fragment sizes from all digests.

The cophenetic correlation coefficients for UPGMA, single linkage, and complete linkage were 0.962, 0.956, and 0.957, respectively. This indicates that while each phenogram is a good representation of distances in the original distance matrix, the UPGMA phenogram provides a slightly better fit to the data. The sum of squares for the Fitch-Margoliash and KITSCH analyses were 0.372 and 1.583 with average percent standard deviations of 5.349 and 11.034, respectively. This indicates that the Fitch-Margoliash analysis introduces less distortion of distances in the original distance matrix than does KITSCH.

DISCUSSION

PCR fingerprinting of rDNA.—PCR fingerprinting is an extremely rapid method for generating RFLP phenotypes for phylogentic or diagnostic purposes. The entire procedure, from PCR to restriction analysis, can be performed in two days. In its simplest form, as used here, PCR fingerprinting does not permit construction of a detailed, ordered restriction map. Therefore, presence or absence of individual restriction sites cannot be scored for use in a cladistic or maximum likelihood analysis. Instead, distance-based methods of analysis must be used. A modification of PCR fingerprinting using a series of overlapping PCR fragments ("PCR mapping"; Vilgalys and Hester, **1990**) could provide discrete restriction site characters and eliminate this analytical weakness.

Estimates of relatedness based on PCR finger-

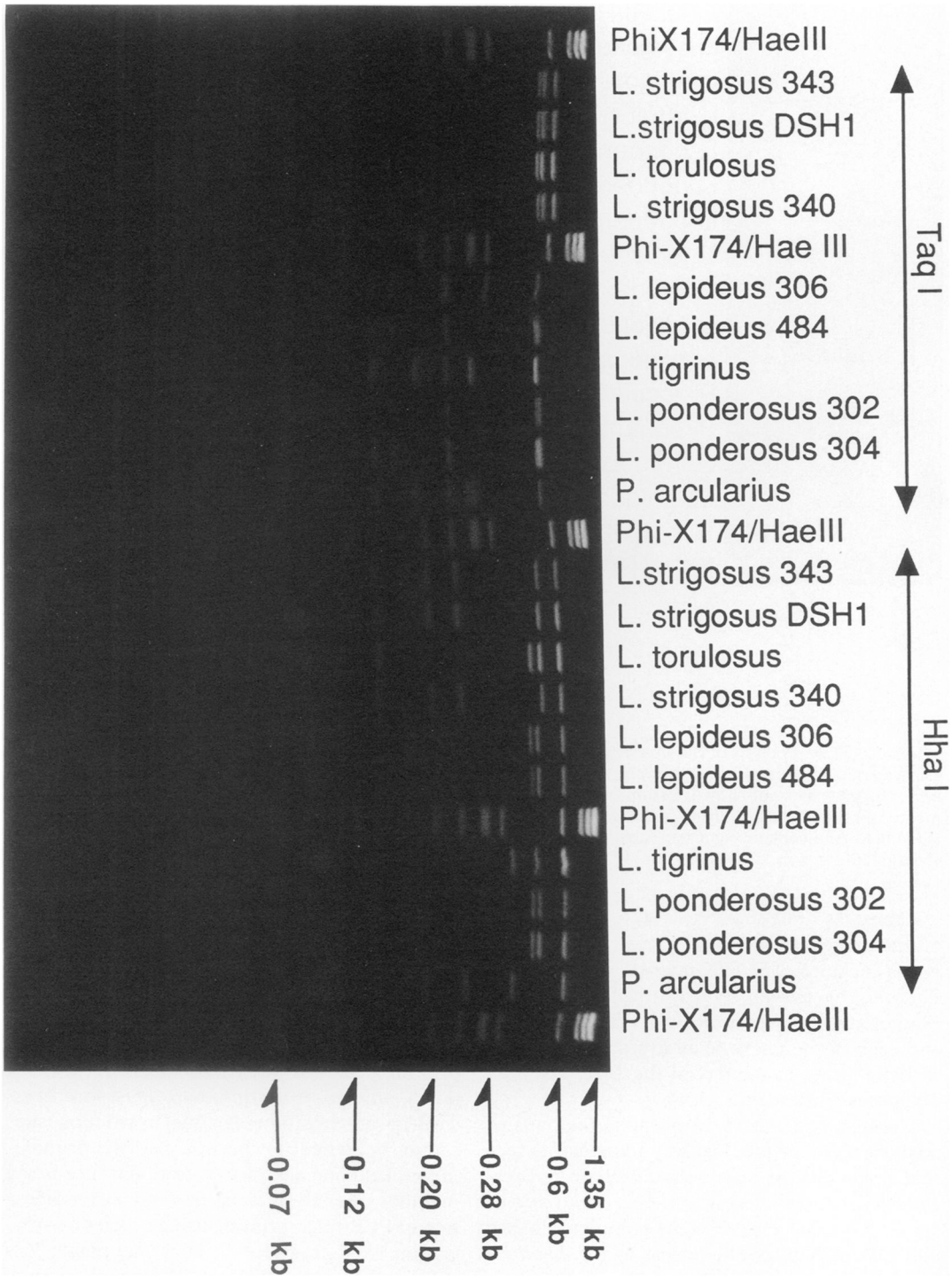


FIG. 2. *Taq* I and *Hha* I restriction digestion products electrophoresed on 4% agarose and stained with ethidium bromide. PhiX174/*Hae* III = molecular weight standard. Selected marker fragment sizes in kilobases (kb).

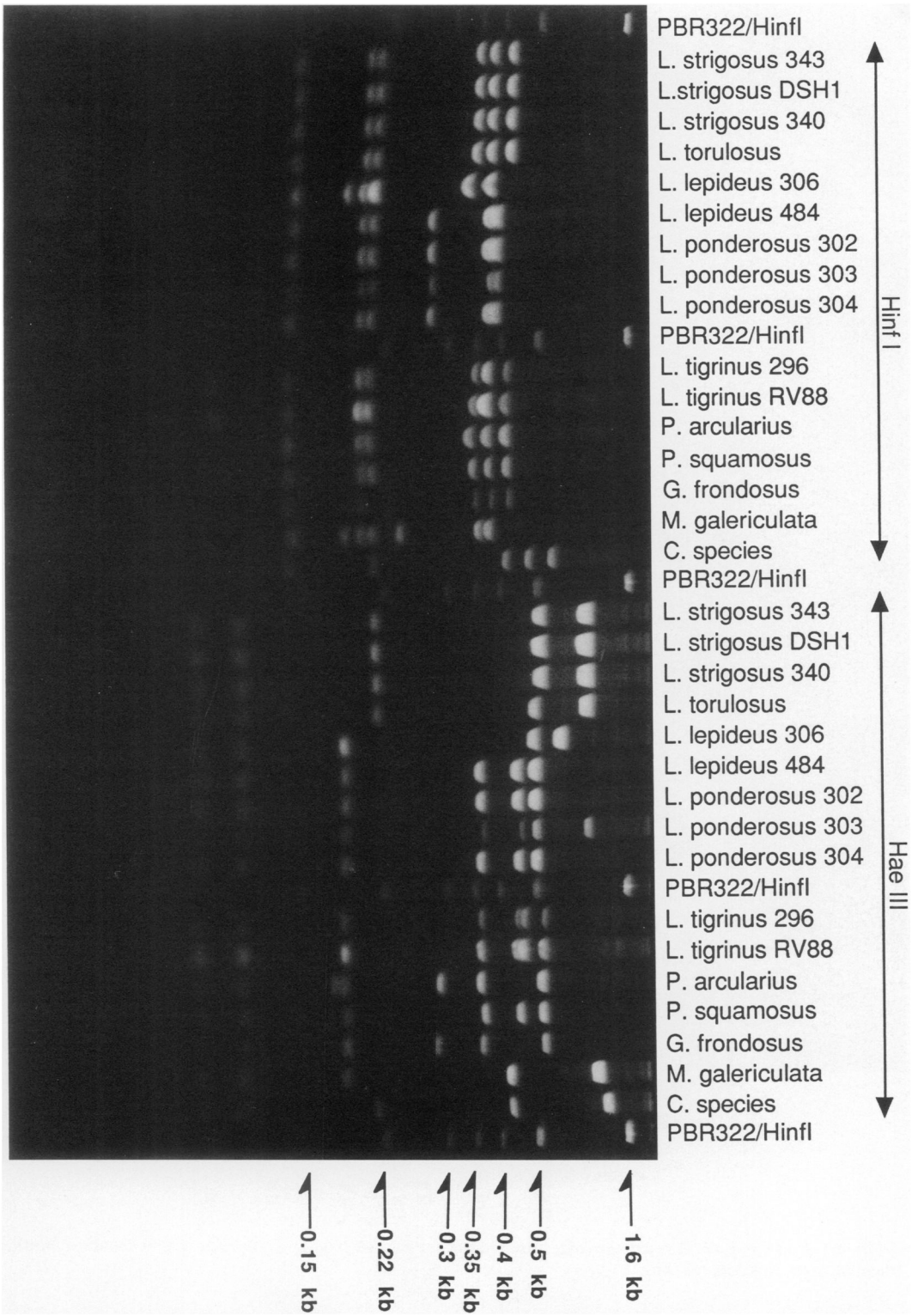


FIG. 3. *Hinf* I and *Hae* III restriction digestion products. PBR322/*Hinf* I = molecular weight standard. Marker fragment sizes in kilobases (kb).

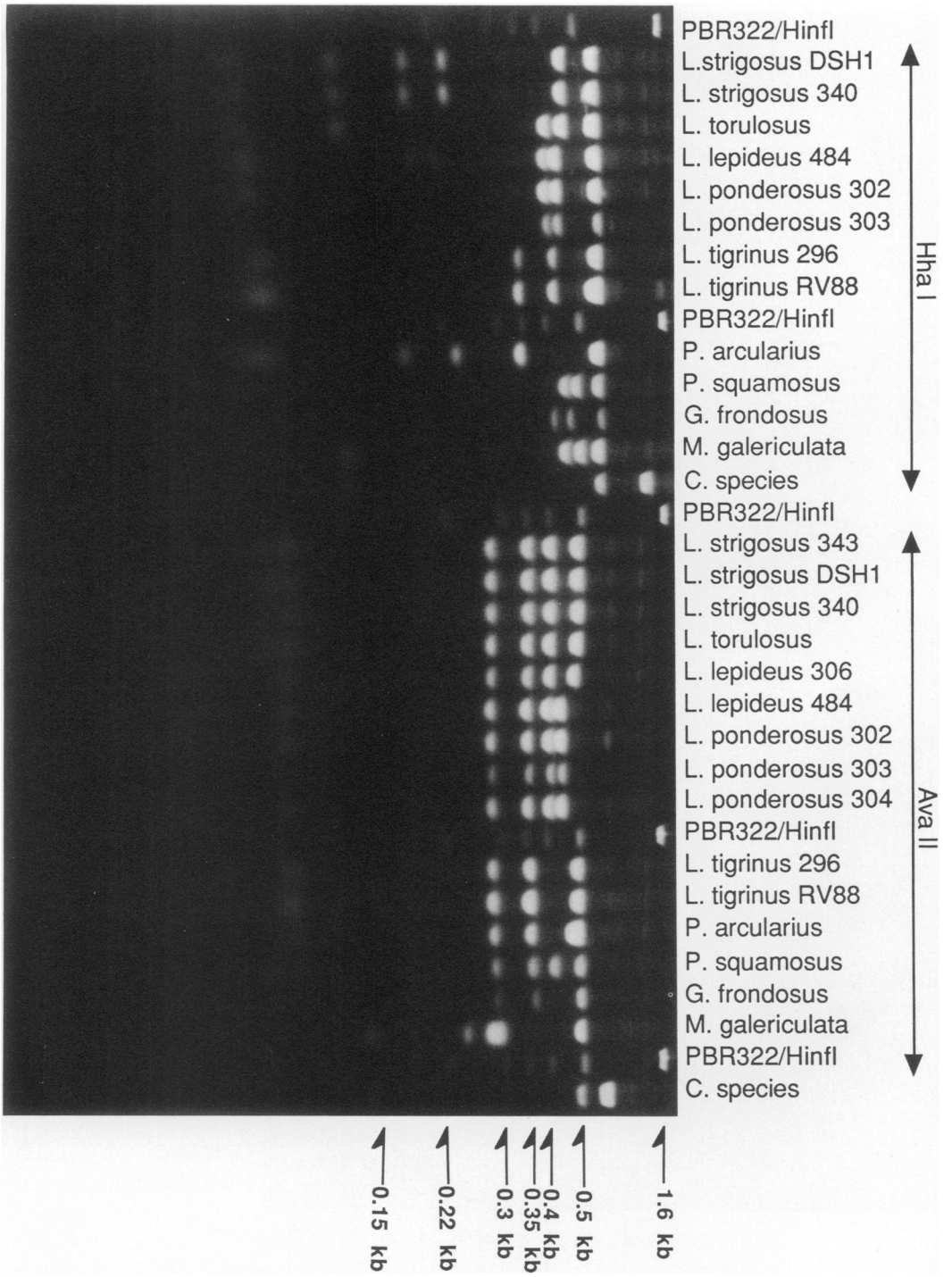


FIG. 4. *Hha* I and *Ava* II restriction digestion products. PBR322/*Hinf*I = molecular weight standard. Marker fragment sizes in kilobases (kb).

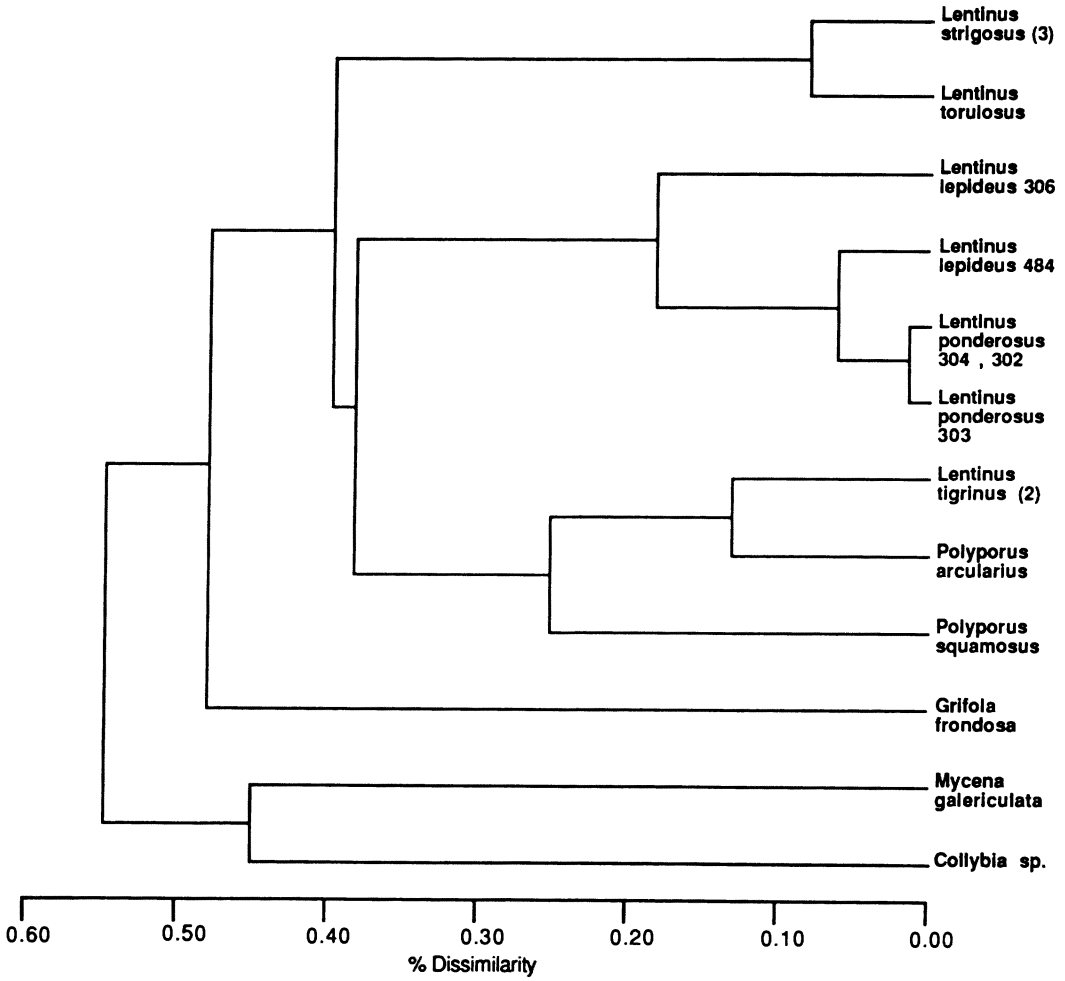
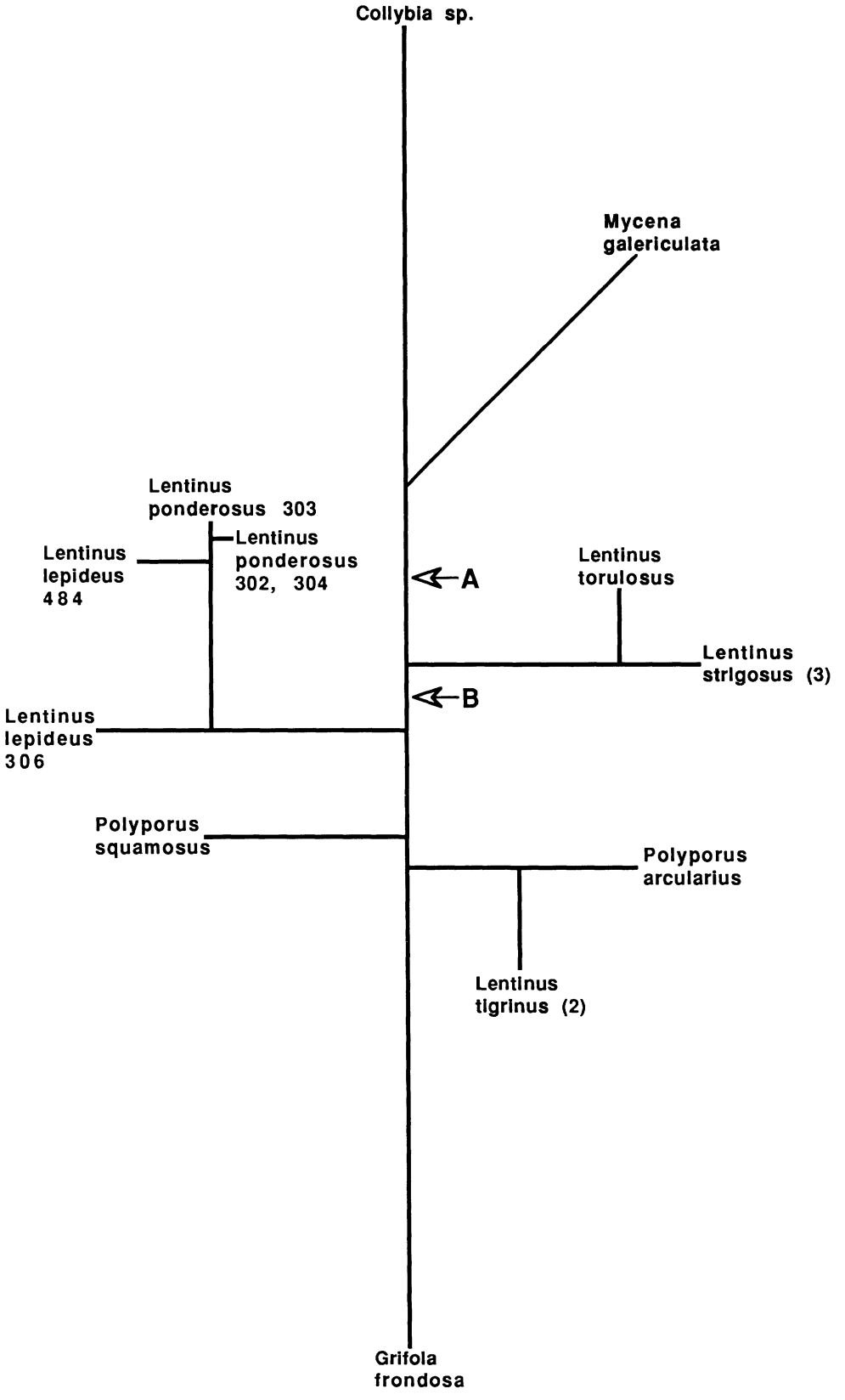


FIG. 5. Phenogram based on rDNA restriction phenotypes analyzed by UPGMA. This unrooted network is topologically identical to the Fitch-Margoliash network (Fig. 6) except for the placement of *Grifola frondosa*.

printing are sensitive to length mutations. Single insertions or deletions may be scored numerous times, thereby artificially inflating the estimate of the Dice coefficient. Error can also be introduced when nonhomologous fragments of similar size in a single digest comigrate and are scored as a single fragment. When this happens, the sum of fragment sizes will be an underestimate of the actual size of the undigested PCR product. Very small fragments may be unresolvable and will also contribute to underestimates of the size of the undigested PCR product. Heterogeneity among the rDNA copies within a genome is also a potential source of error. Variation among rDNA copies in size or presence of restriction sites can result in extra bands in a single digest. This will affect Dice coefficient values and will also cause the sum of fragment sizes to be an overestimate of the actual size of the undigested PCR product. These sources of error may be responsible for the discrepancies between the size estimates of the undigested PCR products and the sums of fragment sizes reported in TABLE IV.

PCR fingerprinting's greatest strength is its ability to survey many individuals rapidly, thus making it particularly useful for preliminary surveys of variation in different regions of DNA prior to sequence analysis or restriction mapping.

The results of this study demonstrate that the amplified region of rDNA contains sufficient variation for this taxonomic problem. It is likely


Collybia sp.
Mycena galericulata
Lentinus ponderosus 303
Lentinus ponderosus 302, 304
Lentinus lepideus 484
A
Lentinus torulosus
Lentinus strigosus (3)
B
Lentinus lepideus 306
Polyporus squamosus
Polyporus arcularius
Lentinus tigrinus (2)
Grifola frondosa

that some of the variation observed exists within the intergenic sequences (Hillis and Davis, **1985**), but without an ordered restriction map or sequence data it is not possible to identify the most variable regions. Sequence analysis of this region of rDNA in lentinoid fungi is currently underway in our laboratory.

Taxonomic conclusions.—Since all the dendrograms were constructed from the same distance matrix, it is not surprising that they are highly congruent. Except for the placement of *Grifola frondosa,* the results of UPGMA and Fitch-Margoliash analysis are topologically identical (FIGS. 5, 6). The Fitch-Margoliash results are preferred for two reasons: first, the Fitch-Margoliash topology placed *Grifola frondosa* with the *Polyporus* species which is a more parsimonious interpretation of hymenophore evolution than the UPGMA topology implies, i.e., the Fitch-Margoliash network implies two character state changes from gills to pores, or vice versa, while the UPGMA phenogram implies three such changes; and, second, the sum of squares and average percent standard deviation are both lower in Fitch-Margoliash analysis than in the KITSCH analysis. KITSCH analysis and UPGMA assume constancy of evolutionary rates and produced identical topologies.

In this analysis phenetic similarity was used to infer phylogenetic relationship. Because the dendrograms presented in FIGS. 5 and 6 are unrooted, it is not possible at this time to make statements about polarities of character evolution or identify monophyletic groups. Nevertheless, certain phylogenetic conclusions are warranted. *Lentinus tigrinus* is very similar to the *Polyporus* species and they are probably closely related. However, the other *Lentinus* species are intermediate between the Tricholomataceae and the Polyporaceae (FIG. 5). If the remaining *Lentinus* species are to be placed into one of these families, then the choice depends on where the dendrograms are rooted. If the root is placed just below the node connecting *Mycena* and *Collybia* (point A, FIG. 6) then *Lentinus* plus the polypores form a monophyletic group, with the Tricholomataceae as an outgroup. The Tricholomataceae is not necessarily the best outgroup, however; there may be more closely related candidates. This interpretation of FIG. 6 is consistent with the hypothesis that dimitic hyphae and hyphal pegs are synapomorphies that unite *Lentinus* with the Polyporaceae. Hyphal pegs occur in some genera of polypores (*Polyporus* Fr., *Pycnoporus* Karst., etc.) and in *Lentinus* subg. *Lentinus.* In this study, subg. *Lentinus* was represented by *Lentinus tigrinus* which consistently clustered with *Polyporus arcularius* and *P. squamosus.* Hyphal pegs may prove to be an important character for identifying the species in *Lentinus* that are most closely related to the polypores although a broader range of taxa will have to be examined before their phylogenetic significance can be determined.

If mid-point rooting is employed (point B, FIG. 6) then *Lentinus* subg. *Panus sensu* Pegler becomes a basal, paraphyletic group from which the Tricholomataceae and Polyporaceae were derived. Under both rooting options *Lentinus* is polyphyletic. A corollary of this is that the character-state transition between gills and pores must have occurred more than once in the evolution of *Lentinus.*

Lentinus strigosus isolates 343 and DH1 (= *Panus rudis*) had the same restriction phenotypes as *Lentinus strigosus* isolate 340 (= *Panus fragilis*) for every enzyme. *Panus fragilis* was differentiated from *P. rudis* by its smaller size, lighter color and delicate habit (Miller, **1965**). Both species have been placed in synonymy with *Lentinus strigosus* by Pegler (**1983**). Although the results of this study are congruent with Pegler's treatment, it is still possible that the part of *Lentinus strigosus* that corresponds to *Panus fragilis* may in fact be a monophyletic species which has diverged morphologically but which has not yet diverged for the rDNA characters surveyed. Indeed, morphological evolution appears to have occurred very quickly in some groups of basidiomycetes. For example, using molecular sequence data from mitochondrial DNA, Bruns *et al.* (**1989**) showed that the false truffle, *Rhizopogon,* previously classified in the Gasteromycetes, is very closely related to the bolete genus

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FIG. 6. Fitch-Margoliash network based on rDNA restriction phenotypes. A and B are rooting options. Rooting at A implies that *Lentinus* and the polypores form a monophyletic group. Mid-point rooting places root at B and implied that *Lentinus* is a basal paraphyletic group from which both the Tricholomataceae and Polyporaceae were derived.

Suillus. Among the other agaricoid Gasteromycetes (Miller and Miller, **1988**) there is a gasteroid form of *Lentinus tigrinus* that has anastomosed gills with a membranous covering (*Lentodium squamulosum* Morgan). This form is still interfertile with typical *Lentinus tigrinus* (Rosinski and Robinson, **1968**).

Lentinus lepideus and *L. ponderosus* formed a very distinct cluster but were poorly resolved from each other. The close placement of the two agrees with both Pegler (**1983**) and Miller (**1973**) who thought the species to be closely related. *Lentinus lepideus* is known to be morphologically variable, and because of this was divided into five forms by Pilát (**1946**). In the current study *Lentinus lepideus* displayed the highest degree of intraspecific variation of all taxa in the study. The distance between isolates 306 and 484, from Montana and North Carolina, respectively, of *L. lepideus* was 18% (FIG. 5). The distance between *L. lepideus* isolate 484 and the *Lentinus ponderosus* cluster was 6%; for comparison, the distance between *Lentinus tigrinus* and *Polyporus arcularius* was 14% (FIG. 5). This amount of divergence, along with the morphological variability, suggests that *Lentinus lepideus* and *Lentinus ponderosus* may actually represent a number of independent lineages in a species complex similar to that in *Armillaria mellea* (Anderson *et al.*, **1989**) or *Collybia dryophila* (Vilgalys and Miller, **1987**).

Lentinus lepideus and *Lentinus ponderosus* have been transferred to the genus *Neolentinus* by Redhead and Ginns (**1985**) along with seven other species in *Lentinus* subg. *Panus sensu* Pegler. The species in *Neolentinus* all cause a brown rot whereas those in *Lentinus s.str.* all cause a white rot. The results of this study are consistent with Redhead and Ginns' treatment and indicate that *Lentinus lepideus* and *Lentinus ponderosus* are distinct from the other *Lentinus* species examined. However, it will be necessary to examine more species in *Neolentinus* and *Lentinus s.str.* to determine if the former is monophyletic.

The results from this preliminary study support the view that *Lentinus*, at least in part, is more closely related to the polypores than to certain agarics. Additional data will be required to resolve specific patterns of evolution in these groups. For instance, the primitive state of the hymenophore is still unclear. To better address phylogenetic problems in these groups it will be necessary to examine more taxa in the Lentinieae and Polyporaceae *s.str.* Sequence or restriction mapping data will also be needed to allow discrete character-based cladistic analyses of molecular characters. The results of this study indicate that the Tricholomataceae may be an appropriate outgroup for such an analysis.

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