APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1996, p. 1354–1363 Vol. 62, No. 4
0099-2240/96/$04.00+0

Differentiation and Grouping of Isolates of the *Ganoderma lucidum* Complex by Random Amplified Polymorphic DNA-PCR Compared with Grouping on the Basis of Internal Transcribed Spacer Sequences

RUEY-SHYANG HSEU, HSI-HUA WANG, HUEI-FANG WANG, AND JEAN-MARC MONCALVO*

Applied Microbiology Laboratory, Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, Republic of China

Received 29 June 1995/Accepted 19 January 1996

Laccate polypores of the *Ganoderma lucidum* species complex are widespread white rot fungi of economic importance, but isolates cannot be identified by traditional taxonomic methods. Parsimony analysis of nucleotide sequences from the internal transcribed spacers (ITS) of the ribosomal gene (rDNA) distinguished six lineages in this species complex. Each ITS lineage may represent one or more putative species. While some isolates have identical ITS sequences, all of them could be clearly differentiated by genetic fingerprinting using random amplified polymorphic DNA (RAPD). To investigate the suitability of RAPD markers for taxonomic identification and grouping of isolates of the *G. lucidum* complex, RAPD fragments (RAPDs) were used as phenotypic characters in numerical and parsimony analyses. Results show that data from RAPDs do not distinguish the same clades as ITS data do. Groupings based on analysis of RAPD data were very sensitive to the choice of the grouping method used, and no consistent grouping of isolates could be proposed. However, analysis with RAPDs did resolve several robust terminal clades containing putatively conspecific isolates, suggesting that RAPDs might be helpful for systematics at the lower taxonomic levels that are unresolved by ITS sequence data. The limitations of RAPDs for systematics are briefly discussed. The conclusion of this study is that ITS sequences can be used to identify isolates of the *G. lucidum* complex, whereas RAPDs can be used to differentiate between isolates having identical ITS sequences. The practical implications of these results are briefly illustrated.

Ganoderma lucidum (W. Curt.: Fr.) P. Karsten and allied species are widespread polypore fungi causing white rot of hardwoods, conifers, and palms. In the natural forest, these fungi attack preferably old and declining trees and decay dead wood and stumps, but severe diseases have been reported for plantations of khair (8), grapevines (3), oil palm (32, 42, 48), betel palm, rubber, tea, and other economically important tropical crops (27, 32, 54). The type of decay caused by *Ganoderma* species is influenced by several parameters, including the *Ganoderma* species and type of wood, and can range from simultaneous decay of all wood components to selective delignification (1, 6, 7). The potential importance of identifying these species and the practical applications of white rot fungi have been reviewed (6, 18, 23), white rot fungi constituting the only group of microorganisms shown to be effective in the biological degradation of lignin.

In the Orient, *Ganoderma* species are regarded as the herb of longevity. These fungi have been used in folk medicine for hundreds of years, and strains are commercially cultivated for preparation of health tablets. Medicinal benefits of *Ganoderma* spp. were reviewed by Jong and Birmingham (19). Isolates used in pharmaceutical and medicinal studies and, consequently, commercially cultivated isolates are generally named *G. lucidum*. However, as used in the pharmaceutical literature, this name encompasses several laccate *Ganoderma* species that might differ in their bioactive compounds.

The *G. lucidum* species complex includes *Ganoderma tsugae* Murr., *Ganoderma valesiacum* Boud., *Ganoderma oregonense* Murr., *Ganoderma resinaceum* Boud., *Ganoderma pfeifferi* Bres., *Ganoderma oerstedii* (Fr.) Torr., *Ganoderma ahmadii* Stey., and several other taxa that are restricted to tropical areas (2, 9, 14, 37–39, 43). The use of traditional taxonomic methods has been inconclusive for establishing a stable classification of the group, and these methods are useless for characterization of individual strains. However, an accurate identification system and a phylogenetically based classification of *Ganoderma* taxa together with the development of genetic markers for individual strains would have practical implications in epidemiology studies, the wood industry, and pharmacology. For instance, it would help in the monitoring fungal propagation within and between fields and in bioprospecting for new genes and new metabolites and would provide useful information for genetic engineering or breeding of commercial strains.

In a previous study using parsimony analysis of sequences of DNA coding for rRNA (rDNA), six monophyletic groups of the *G. lucidum* complex were identified (34). Delineation of these groups was in agreement with other sources of taxonomic data. Intragroup rDNA sequence variation was too low for further taxonomic segregation, but several lines of evidence suggested that each group is composed of more than one species. Some isolates had identical nucleotide sequences in the internal transcribed spacers (ITS) and other rDNA regions. The results also indicated that several strains were misnamed.

In this work, the PCR (40) was used with arbitrary primers to randomly amplify DNA fragments (random amplified polymorphic DNA [RAPD]-PCR) (50, 52) of 36 isolates of the *G. lucidum* species complex. The purpose of the study was to investigate the use of RAPD profiles in the species complex for (i) differentiation of individual strains, (ii) grouping and iden-

* Corresponding author. Present address: Mycology Laboratory, Department of Botany, Duke University, Durham, NC 27708. Electronic mail address: jeanmarc@acpub.duke.edu.

TABLE 1. Fungal isolates and nucleotide sequence accession numbers in the European Molecular Biology Laboratory (EMBL) data library

Phyletic group[a]	Isolate no.[b]	Given name(s) (reference or source of information)[c]	Geographical origin	EMBL accession no.	
				ITS 1	ITS 2
I.1	FWP 14329[d]	*G. ahmadii* (43)	Pakistan	Z37047	Z37098
I.1	CBS 282.33[d]	*G. valesiacum* (CCRC)	United Kingdom	Z37056	Z37081
I.1	CBS 223.48[d]	*G. tsugae* → *G. valesiacum* (CCRC)	Canada	Z37054	Z37079
I.1	CBS 428.84[d]	*G. tsugae* → *G. valesiacum* (CCRC)	United States	X78735	X78756
I.1	ATCC 46754[d]	*G. lucidum* → *G. tsugae* (CCRC)	United States	Z37055	Z37080
I.1	ATCC 46755[d]	*G. lucidum* (CCRC) → *G. valesiacum* aggregate (34)	Canada	Z37052	Z37076
I.1	CBS 177.30[d]	*G. oregonense* (CCRC)	United States	Z37060	Z37100
I.1	ATCC 46750[d]	*G. oregonense* (CCRC)	Canada	Z37061	Z37101
I.2	RYV 33217[d]	*G. lucidum* (37a)	Norway	Z37096	Z37073
I.2	CBS 270.81[d]	*G. lucidum* (CCRC)	France	Z37049	Z37099
I.2	ATCC 52409[d]	*G. oerstedii* (CCRC)	Argentina	Z37058	Z37083
I.2	ATCC 52410[d]	*G. oerstedii* (CCRC)	Argentina	X78739	X78760
II.1	ATCC 52411[d]	*G. oerstedii* (CCRC) → *G. resinaceum* (52a) → *Ganoderma* sp. 1 (34)	Argentina	Z37059	Z37084
II.1	RSH RZ[d]	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan[e]	X78743	X78764
II.1	RSH G001	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	X87345	X87355
II.1	RSH 0630[f]	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	X87346	X87356
II.1	RSH 0708[f]	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	X87347	X87357
II.1	RSH 0926[f]	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	X87348	X87358
II.1	RSH 0922	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	X87350	X87360
II.1	RSH 0815	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	ND[g]	ND
II.1	RSH 0709	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	X87349	X87359
II.1	RSH 0626[d]	*G. lucidum* (17) → *Ganoderma* sp. 2 (34)	Taiwan	Z37048	Z37072
II.1	ATCC 32471[d]	*G. lucidum* (CCRC) → *Ganoderma* sp. 2 (34)	India	X78744	X78765
II.1	ATCC 32472	*G. lucidum* (CCRC) → *Ganoderma* sp. 2 (34)	India	X87351	X87361
II.2	RSH J2	*G. lucidum* → *G. tsugae* (17) → *Ganoderma* sp. 3 (34)	Japan[e]	X78746	X78767
II.2	RSH BLC[d]	*G. lucidum* → *G. tsugae* (17) → *Ganoderma* sp. 3 (34)	Taiwan[e]	Z37097	Z37078
II.2	RSH 1109[d]	*G. lucidum* → *G. tsugae* (17) → *Ganoderma* sp. 3 (34)	Taiwan[e]	X78747	X78768
III.1	CBS 430.84[d]	*G. lucidum* (CCRC) → *Ganoderma* sp. 4 (34)	United States	Z37051	Z37075
III.1	RSH TEX.1[d]	*G. lucidum* (26a) → *Ganoderma* sp. 4 (34)	United States[e]	Z37053	Z37077
III.2	CBS 194.76[d]	*G. resinaceum* (CCRC)	Netherlands	X78737	X78758
III.2	CBS 152.27[d]	*G. resinaceum* (CCRC) → *G. pfeifferii* (34)	United Kingdom	Z37062	Z37085
III.2	CBS 747.84[d]	*G. pfeifferi* (CCRC)	Netherlands	X78738	X78759
	ZHANG 0932	*G. tsugae* (53a)	China	ND	ND
	RSH J1	*G. lucidum* (40a) → *Ganoderma* sp. (R. S. Hseu)	Japan[e]	X87352	X87362
	ACCC 5.65	*G. lucidum* (ACCC)	China	X87354	X87364
	ACCC 5.75	*G. lucidum* (ACCC)	China	ND	ND

[a] Phyletic group numbers are as described by Moncalvo et al. (34).
[b] Strains were from the collections of X. Q. Zhang (ZHANG), R.-S. Hseu (RSH), L. Ryvarden (RYV), the herbarium of the Jardin Botanique de Belgique (Meise, Belgium) under the label Fungi of West Pakistan (FWP), the American Type Culture Collection (ATCC; Rockville, Md.), the Agriculture Culture Collection Center (ACCC; Beijing, China), and the Culture Collection and Research Center (CCRC; Hsinchu, Taiwan).
[c] Original identification is followed by name change(s). Sources of identification were the catalog of the Agriculture Culture Collection Center (ACCC), Beijing, China, the catalog of the Culture Collection and Research Center (CCRC), Hsinchu, Taiwan, and as otherwise indicated.
[d] Isolates used in a previous rDNA systematic study (34).
[e] Commercially cultivated strains.
[f] Hybrid between II.1 RSH RZ and II.1 RSH G001.
[g] ND, ITS sequence not determined.

tification of isolates, and (iii) systematics of taxonomic aggregates that were unresolved by ITS sequence data. RAPD-PCR is one of the most sensitive and efficient methods currently available for distinguishing between different strains of a species (10). For instance, RAPD markers distinguished between individual strains and meiotic progeny of the cultivated mushroom *Agaricus bisporus* (22) and between clinical isolates of the fungal pathogens *Histoplasma capsulatum* (21) and *Cryptococcus neoformans* (31). RAPD markers identified strains of the bacterium *Xanthomonas campestris* pv. pelargonii (29), races of *Gremmeniella abietina* (15), ZG groups of *Rhizoctonia solani* (12), geographically distinct populations of *Phytophthora megasperma* (26), and different populations and species of arbuscular-mycorrhizal fungi (53). RAPD fingerprintings were also used in systematic studies of species aggregates: they delineated three distinct species of *Borrelia* (spirochete) (51), four intersterility groups of low-temperature basidiomycetes (24), and five putative species in the *Stylosanthes guianensis* complex (higher plants) (20).

The suitability of RAPD analysis for systematic studies still needs to be tested by comparison of RAPD data with other systematic data. This study evaluates RAPD grouping in comparison with ITS-based phyletic groups. The suitability of rDNA sequencing for phylogenetic reconstruction and systematics of fungi has been widely demonstrated and reviewed by Bruns et al. (11) and by Hibbett (16). This work is the first study comparing RAPD fingerprinting with rDNA sequencing in fungal systematics. Because RAPD profiles are simpler and faster to produce than DNA sequences, they may present several advantages for taxonomic identification and grouping of isolates in the *G. lucidum* species complex.

MATERIALS AND METHODS

Fungal isolates. Table 1 lists the 36 fungal isolates used in this study. Isolates labelled with an asterisk in Table 1 were classified into six phyletic groups on the basis of a previous phylogenetic study using rDNA sequences (34). In Table 1, these groups are labelled I.1, I.2, II.1, II.2, III.1, and III.2, of which I.1 and I.2,

II.1 and II.2, and III.1 and III.2 were monophyletic (34). Isolates RSH G001, RSH 0630, RSH 0708, RSH 0926, RSH 0922, RSH 0815, RSH 0709, and ATCC 32472 were classified in II.1 because they are interfertile with group II.1 isolate RSH RZ (II.1 RSH RZ) (17). Isolates ZHANG 0932, RSH J1, ACCC 5.65, and ACCC 5.75 were not classified. With the exception of *G. ahmadii* FWP 14329 (fragment of basidiocarp of the type specimen), all isolates were maintained as mycelial colonies on potato dextrose agar (Difco). Mycelia were grown in potato dextrose broth (Difco) for 1 to 2 weeks at 25 to 30°C and then harvested, lyophilized, and stored at −20°C until DNA extraction. Both tissue of the context of the basidiocarp and in vitro-grown mycelia of III.1 RSH TEX.1 were used in the experiment.

DNA isolation, PCR amplification, sequencing, and phylogenetic analysis of ITS sequences. DNA isolation, PCR amplification, and cycle sequencing of the ITS region were done as described elsewhere (33). Nucleotide sequences were aligned by eye.

Determination of nucleotide sequence variation between isolates was by comparison of pairwise distance in the program package PAUP version 3.0 (46) configured for the Macintosh. Phylogenetic analysis based on parsimony was performed by use of PAUP (46) with cladistically informative characters only. When two or more taxa had an identical sequence, only a single sequence was used in the analysis. We performed a heuristic search by use of random taxon addition sequence with the number of replicates set to 20. Other settings in PAUP were as follows: gaps were coded as fifth base, all characters were weighted equally, MAXTREES was unrestricted, the MULPARS option and steepest descent option were in effect, branches having maximum length zero were allowed to collapse to yield polytomies, and tree bisection-reconnection (TBR) branch-swapping was performed on starting trees. Isolates of group III.2 were designated as an outgroup to root the tree. The robustness of the tree was evaluated by 100 bootstrap replications by use of simple addition sequence.

RAPD using arbitrary primers. RAPD profiles were produced by the method of Williams et al. (52) and Welsh and McClelland (50). The oligonucleotide sequences of the primers used are given in Table 2. Reagents were from Perkin-Elmer Cetus. The reaction mixtures were prepared on ice. To determine experimental conditions yielding several amplification products and the reproducibility of RAPD profiles, preliminary experiments were conducted with a limited number of isolates by varying the concentrations of $MgCl_2$, deoxyribonucleotide triphosphates (dNTPs), AmpliTaq DNA polymerase, primer and template DNA, and annealing temperature in the PCR. However, we did not try to create conditions that yielded the highest number of RAPDs for each primer. Amplification reactions were performed with a total volume of 25 μl and standardized as follows for taxonomic analysis of RAPDs: 0.2 to 5 ng of template DNA (in 10 μl of H_2O), 0.6 μM primer, 50 μM each dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.5 U of AmpliTaq DNA polymerase. In each amplification reaction, a control sample without DNA was included. Reaction mixtures were covered with a drop of sterilized mineral oil. Ice-cold samples were quickly transferred in a Perkin-Elmer Cetus thermal cycler (model 480) preheated at 98°C and incubated at this temperature for 2 min to denature the DNA completely. Samples were then subjected to 45 cycles of amplification as follows: 1 min at 95°C to denature the DNA, 1 min at 36°C to anneal the primers, 2 min at 72°C to extend the annealed primers. A final extension step of 10 min was programmed to ensure complete extension of the amplified products. Each DNA was amplified one to three times by use of the conditions described above. The amplified fragments were analyzed by electrophoresis of 10 μl of the amplification reaction mixture in 2% agarose gels run in Tris-acetate-EDTA buffer (28). A molecular size marker (Boehringer Mannheim marker VI) was loaded in the external lanes of the gel for correction of a possible "smiling effect" during electrophoresis and to facilitate comparison of RAPD profiles from gel to gel. RAPDs were visualized after staining with ethidium bromide (28) and photographed with a UV transilluminator. Scanning gels into a computer and using the program package NCSA Gel Reader (University of Illinois at Urbana, Champaign) for measuring fragment size and resolving bands of low intensity did not offer better precision than the hand-and-eye method, and the latter was finally preferred for recording data.

Taxonomic analysis of DNA amplification fingerprints. Amplified fragments were scored 1 for presence and 0 for absence and recorded in a data matrix. Similarity between isolates was determined by the band-sharing coefficient (F) calculated by the formula of Nei and Li (36), i.e., $F = 2N_{xy}/(N_x + N_y)$, where N_{xy} is the number of common amplified fragments between two isolates and N_x and N_y are the number of fragments in isolates X and Y, respectively. The functional form of the relationships between RAPD similarity coefficient and ITS sequence difference was examined by use of Cricket Graph 1.3 (Cricket Software, Malvern, Pa.). Amplified fragments were treated as phenotypic characters, and numerical analyses were performed with SYSTAT 5.1 for the Macintosh (1990–91; SYSTAT Inc.) by use of normalized percent disagreement (NPD) as the distance metric and the average-linkage clustering method. The NPD distance produces a distance index that is the percentage of comparisons of values resulting in disagreement in two profiles and was preferred to the band-sharing coefficient F for cluster analysis because NPD takes in consideration the whole structure of the data set. The sensitivity of the phenogram to the clustering algorithm was determined by use of the complete-linkage and Ward minimum variance methods. Although we did not consider RAPDs to be phylogenetic characters, the data matrix was also subjected to parsimony analysis in PAUP (46) to identify taxonomic groups by use of a character-based method and to determine whether RAPD-based phenetic groups were robust independently of the analytical method used. PAUP settings were as described for parsimony analysis for the ITS data set.

TABLE 2. Oligonucleotide primers used in this study

Primer designation	Sequence	Reference
R1	TGCCGAGCTG	22
R2	AGTCAGCCAC	22
R3	AATCGGGCTG	22
R4	GAAACGGGTG	22
R5	GCGATCCCCA	21

RESULTS

ITS variation and phylogenetic analysis. ITS sequences encompassing the entire ITS 1 and ITS 2 regions were deposited in the European Molecular Biology Laboratory and given the accession numbers shown in Table 1. Sequence alignment was unambiguous except for three small regions showing several deletion or insertion events. Different possible alignments in these small regions had no effect on the overall topology of the phylogenetic trees (34); therefore, all characters were included in this study. ITS sequence variation between isolates is shown in Table 3. No sequence difference was found between II.1 RSH RZ, II.1 RSH G001, and their progeny (II.1 RSH 0630, II.1 RSH 0708, and II.1 RSH 0926) and between other phylogenetically closely related organisms (Table 3). Parsimony analysis of the ITS data set used 76 phylogenetically informative characters and produced 52 equiparsimonious trees of 104 steps in length. The strict consensus tree is depicted in Fig. 1.

The result of phylogenetic analysis was similar to that of our previous work, which used a rather similar sample (34), and will therefore not be presented in detail here. In Fig. 1, group III is paraphyletic as a consequence of group III.2 being chosen as an outgroup to root the tree, but group III is monophyletic if the tree is rooted either with I.1, I.2, II.1, or II.2 (34). Bootstrapping the data matrix showed that monophyly of lineages I, II, and III and of groups I.1 to III.2 is strongly supported (91 to 100% confidence level). Strains not used in our previous study (Table 1) were grouped as follows. Isolates that were classified in II.1 from interfertility studies all grouped with other isolates of II.1, and isolates J1 and 5.65 clustered in group II.2. ITS sequences of strains ZHANG 0932 and ACCC 5.75 were not produced, and taxonomic identification of these isolates will be discussed later.

Figure 1 shows that basal relationships between lineages I, II, and III and intragroup relationships of groups I.1 to III.2 remain unresolved. Our sample was biased both toward recently diverged isolates (0 to 10 nucleotide differences in ITS) and distantly related taxa (>25 nucleotide differences) (see Fig. 4), suggesting that there is room for additional taxa in the *G. lucidum* complex, which might help in resolving basal phylogenetic relationships between lineages I, II, and III. Intragroup ITS variation was 0 to 2.5% in I.1 (two to four putative species), 0 to 0.25% in I.2 (two putative species), 0 to 3% in II.1 (two species), 0 to 1.25% in II.2 (including strains RSH J1 and ACCC 5.65), 0.5% between the two isolates of III.1, and 0.5 to 1.75% in III.2 (two species) (Table 1 and 3).

DNA amplification polymorphisms (RAPD-PCR). Preliminary experiments using selected isolates with primers R1 to R5 showed that the concentrations of $MgCl_2$, AmpliTaq DNA polymerase, dNTPs, template DNA, and annealing temperature in the PCR were all critical experimental parameters for

TABLE 3. Pairwise comparison between nucleotide sequence differences in ITS[a] and RAPD similarity coefficients (*F* value) between isolates.

Isolate	Taxa	No. of sequence differences and similarity coefficient (10^2) for isolate[b]:																																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	I.1 FWP 14329		3	4	8	3	6	6	6	9	10	9	9	ND[c]	30	30	35	33	34	37	37	37	37	37	37	ND	37	39	38	37	38	45	42	43	44	44	ND
2	I.1 CBS 282.33	21		1	2	0	3	3	3	6	7	6	6	ND	31	31	34	33	34	34	34	34	34	34	35	ND	35	36	37	35	36	43	40	40	38	38	ND
3	I.1 CBS 223.48	19	78		4	0	2	2	2	5	6	5	5	ND	30	30	33	32	33	33	33	33	33	33	34	ND	34	35	36	34	35	42	39	39	37	37	ND
4	I.1 CBS 428.84	25	42	37		2	2	2	2	5	6	5	5	ND	29	29	31	30	31	31	33	33	33	33	34	ND	34	33	34	34	34	40	37	37	37	37	ND
5	I.1 ATCC 46754	22	89	75	36		0	0	0	3	4	3	3	ND	28	28	31	30	31	31	31	31	31	31	32	ND	32	33	34	32	33	40	37	37	35	35	ND
6	I.1 ATCC 46755	6	35	35	29	38		0	0	3	4	3	3	ND	26	26	33	32	33	33	31	31	31	31	32	ND	32	35	36	32	30	42	39	39	35	35	ND
7	I.1 ATCC 46750	19	20	19	19	18	22		0	3	4	3	3	ND	28	25	31	30	31	31	31	31	31	31	32	ND	32	33	34	32	35	40	37	37	35	35	ND
8	I.1 CBS 177.30	13	22	21	17	20	20	79		3	4	3	3	ND	28	28	34	33	34	30	31	31	31	31	32	ND	32	32	33	32	35	41	38	38	35	35	ND
9	I.2 RYV 33217	25	54	45	18	50	48	33	26		0	0	0	ND	27	27	35	33	34	30	30	30	30	30	31	ND	31	32	33	31	29	41	38	38	36	36	ND
10	I.2 CBS 270.81	25	42	39	22	44	41	33	26	67		1	1	ND	27	27	34	33	34	30	29	29	29	29	30	ND	30	32	33	30	29	41	38	38	37	37	ND
11	I.2 ATCC 52409	21	41	31	21	39	20	27	35	51	56		0	ND	28	28	34	33	34	30	30	30	30	30	31	ND	31	32	33	31	33	41	38	38	36	36	ND
12	I.2 ATCC 52410	20	35	35	24	33	36	23	21	44	53	60		ND	30	30	35	34	35	33	30	30	30	30	31	ND	31	35	34	31	33	41	38	38	36	36	ND
13	ZHANG 0932	24	39	27	24	37	32	42	29	44	41	28	40		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	III.1 CBS 430.84	21	31	41	8	33	43	31	22	25	30	41	49	32		2	17	14	15	32	32	32	32	32	33	ND	33	32	33	33	35	38	35	36	36	34	ND
15	III.1 RSH TEX.1	26	29	28	11	30	29	31	35	54	54	58	45	29	43		17	14	15	32	32	32	32	32	33	ND	33	32	33	33	34	38	35	45	36	34	ND
16	III.2 CBS 194.76	43	19	25	22	24	5	13	27	17	23	23	26	22	30	28		5	7	43	43	43	43	43	43	ND	43	45	46	43	44	48	45	45	44	44	ND
17	III.2 CBS 152.27	15	29	28	21	23	10	20	22	19	26	29	33	33	25	37	27		2	41	41	41	41	41	41	ND	41	42	43	41	45	44	41	43	42	42	ND
18	III.2 CBS 747.84	18	24	18	18	17	5	18	24	7	7	8	23	28	15	11	41	59		43	43	43	43	43	43	ND	43	49	45	43	46	45	42	44	43	43	ND
19	II.1 RSH RZ	35	30	26	16	28	30	39	36	43	49	48	37	33	54	56	32	31	15		0	0	0	0	0	ND	0	1	2	0	9	41	29	29	30	30	ND
20	II.1 RSH G001	26	42	39	22	37	29	25	26	29	26	26	36	36	32	42	35	30	33	46		0	0	0	1	ND	1	1	2	2	9	31	29	29	30	30	ND
21	II.1 RSH 0630	15	33	28	15	35	24	20	22	23	29	25	33	33	44	37	32	30	34	52	68		0	0	0	ND	0	1	2	1	9	31	29	29	30	30	ND
22	II.1 RSH 0708	24	34	27	24	32	31	20	25	33	36	34	41	34	31	43	33	32	31	50	67	71		0	0	ND	0	1	2	1	9	31	29	29	30	30	ND
23	II.1 RSH 0926	26	26	24	21	31	34	12	19	26	33	26	34	30	30	37	37	31	35	46	58	67	76		1	ND	1	1	2	2	9	31	29	29	30	30	ND
24	II.1 RSH 0922	22	19	18	17	20	20	39	36	26	32	23	36	33	29	36	31	42	33	46	43	45	48	58		ND	0	2	3	0	9	32	30	30	30	30	ND
25	II.1 RSH 0815	10	28	26	10	29	22	30	35	34	28	28	35	43	31	18	26	37	32	46	57	45	47	46	46		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
26	II.1 RSH 0709	13	38	31	13	30	24	21	23	31	31	32	32	29	29	29	19	41	29	48	43	44	44	38	52	49		2	3	0	9	32	30	30	30	30	ND
27	II.1 RSH 0626	38	35	38	21	33	32	31	29	43	51	51	43	29	57	56	38	22	12	85	49	48	50	49	39	39	45		3	3	10	32	30	30	31	31	ND
28	II.1 ATCC 32471	35	29	29	15	25	22	32	27	43	51	41	36	33	59	58	25	31	15	79	42	44	43	38	42	42	45	81		4	12	33	31	31	33	33	ND
29	II.1 ATCC 32472	22	29	32	13	27	12	32	30	32	32	39	40	22	32	33	27	30	25	46	40	42	48	35	57	50	52	43	45		10	31	29	29	30	30	ND
30	II.1 ATCC 52411	6	38	33	13	36	24	19	13	22	15	13	15	35	21	15	6	26	30	12	27	21	17	22	23	24	22	13	12	22		33	33	33	31	31	ND
31	II.2 RSH J2	19	46	40	22	45	32	34	41	39	29	31	25	29	31	40	27	26	32	36	35	30	39	36	21	31	29	29	30	26	33		3	5	4	4	ND
32	II.2 RSH BLC	0	33	36	24	35	33	23	33	21	25	29	33	24	30	33	16	35	29	27	26	25	28	26	21	19	21	25	23	17	31	55		2	1	1	ND
33	II.2 RSH 1109	9	36	32	22	38	33	36	43	29	29	23	19	22	26	33	20	19	21	31	34	23	29	27	17	29	23	20	22	17	18	69	57		1	1	ND
34	RSH J1	27	26	21	13	17	21	32	30	35	35	30	44	44	39	42	28	42	34	47	41	42	42	35	37	29	43	47	52	31	9	36	26	31		0	ND
35	ACCC 5.65	20	30	30	8	25	34	26	28	55	60	45	44	31	59	48	22	28	12	58	31	32	36	32	38	30	34	38	60	38	16	28	24	22	44		ND
36	ACCC 5.75	23	23	22	15	21	21	25	29	29	39	32	26	16	32	46	34	23	18	44	27	30	37	27	27	16	35	47	44	30	0	27	19	33	48	45	

[a] ITS length of these isolates being about 400 bp in size (34), four sequence differences between two isolates correspond approximately to 1% of nucleotide variation in ITS.
[b] Numbers of ITS sequence differences are shown in the upper triangle of data. RAPD similarity coefficients (10^2) are shown in the lower triangle of data.
[c] ND, not determined.

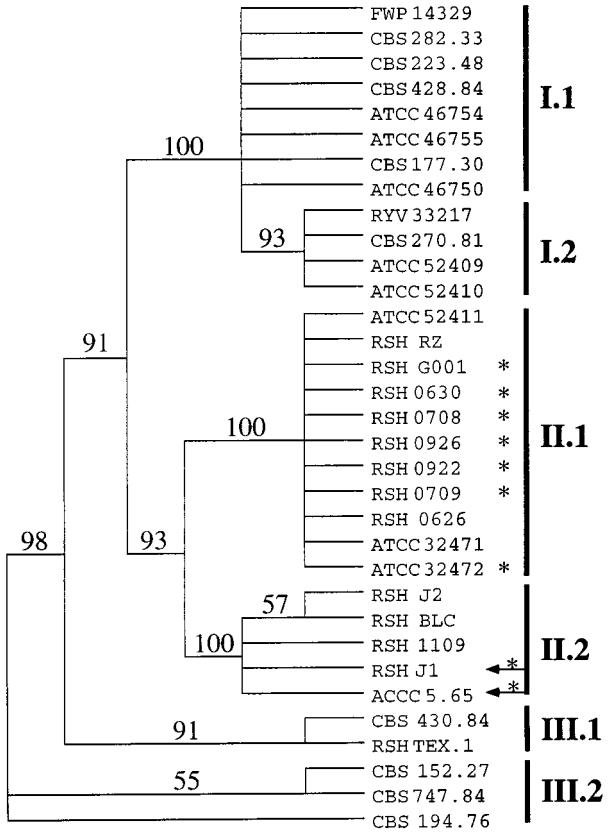

FIG. 1. Phylogenetic relationships between isolates listed in Table 1 inferred by ITS nucleotide sequence data. The tree depicted represents the strict consensus tree of 52 equiparsimonious trees by use of a heuristic search in PAUP (46) from 76 cladistically informative characters. Tree length = 104; consistency index = 0.808; retention index = 0.923. Values above branches are confidence levels estimated by 100 bootstrap replicates. Asterisks indicate the taxa not used in our previous molecular phylogeny for the *G. lucidum* species complex (34), and arrows indicate isolates not classified in Table 1 for which ITS sequences have been produced in this work.

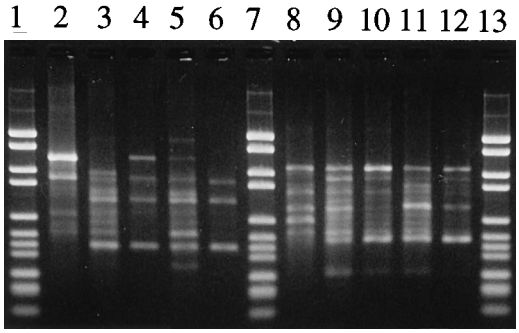

FIG. 2. RAPD fingerprinting of dikaryotic strains II.1 RSH RZ and II.1 RSH G001 and their progeny by use of primers R3 (lanes 2 to 6) and R5 (lanes 8 to 12). Lanes: 1, 7, and 13, molecular size marker VI (Boehringer GmbH, Mannheim, Germany); 2 and 8, strain II.1 RSH RZ; 3 and 9, strain II.1 RSH G001; 4 and 10, strain II.1 RSH 0630; 5 and 11, strain II.1 RSH 0708; 6 and 12, strain II.1 RSH 0926.

producing RAPDs (data not shown). Amplification products were detected only in the range of 1 to 4 mM $MgCl_2$. RAPD profiles were not routinely reproducible at enzyme concentrations higher than 1 U/25 μl. Concentrations of template DNA in the range 0.5 to 5 ng (a twofold higher dilution than that used to amplify ITS) yielded higher numbers of bands, but in some samples, a two- to fivefold-higher dilution enabled better visualization of bands of lower intensity in agarose gels stained with ethidium bromide. Higher DNA concentrations resulted in the loss of bands of low intensity, as did DNA concentrations lower than 0.1 ng. We therefore routinely used 0.5 to 5 ng of template DNA in our standardized procedure and ran a twofold-diluted sample when the interpretation of a profile was unclear. We observed that some DNAs yielded more bands when the $MgCl_2$ and dNTP concentrations were increased to 3 mM and 200 μM, respectively (data not shown). In the comparative analysis, we scored only bands visualized by the standardized amplification procedure. This point will be discussed later.

With the standardized amplification procedure, primers R1 to R5 (Table 2) yielded 2 to 11, 2 to 10, 2 to 10, 1 to 11, and 1 to 10 amplification products, respectively. RAPD profiles were reproducible for all isolates. In all experiments, the RAPD profile of strain RSH TEX.1 generated from DNA prepared from dry basidiocarp tissue was identical to that produced from DNA isolated from cultivated mycelium. Each primer-DNA combination produced a distinct PCR fingerprint, with the exception of R5-II.1 RSH G001 and R5-II.1 RSH 0630 (Fig. 2). Figure 2 shows RAPD fingerprints produced by primers R3 and R5 with II.1 RSH RZ and II.1 RSH G001 and their progeny. All strains employed in this study were clearly distinguished when RAPD profiles produced by the five primers were pooled.

No bands or profiles were diagnostic for groups I.1 to III.2. Taken together, the five primers yielded 125 scorable bands (polymorphic DNA fragments) that ranged from 0.1 to 2.83 kb in size. The presence or absence of bands was scored for each isolate to produce the data matrix shown in Fig. 3. All visible bands were recorded. It was sometimes difficult to score bands of lower intensity correctly, but the overall topology of RAPD phenograms was not affected by minor differences in the data matrix that could result from misinterpretation of bands of lower intensity (data not shown).

Comparison of RAPD similarity and ITS sequence divergence. The RAPD similarity coefficients (F value) between isolates ranged from 0 to 0.89 and are given in Table 3 together with ITS sequence differences. To facilitate pairwise comparison, RAPD similarity and ITS divergence are plotted in Fig. 4. The figure shows that isolates having higher levels of RAPD similarity (0.60 to 0.89) also have lower ITS sequence divergence (0 to 5 nucleotide substitutions, ca. 98.75 to 100% ITS sequence similarity). Figure 4 also indicates that there is little overall relationship between ITS and RAPD similarities throughout our sample: RAPD similarity coefficients of strains having identical ITS sequences or differing by 1 to 5, 6 to 25, and 26 to 49 nucleotides largely overlap (F = 0.18 to 0.89, 0.05 to 0.85, 0.06 to 0.55, and 0 to 0.60, respectively). As a consequence, intraphylum RAPD similarity coefficients (F_{intra}) overlap with interphyla similarity coefficients (F_{inter}): F_{intra} and F_{inter} values were 0.09 to 0.78 and 0 to 0.54, respectively, in I.1 (8 isolates), 0.44 to 0.67 and 0.07 to 0.58, respectively, in I.2 (4 isolates), 0.12 to 0.85 and 0.10 to 0.56, respectively, in II.1 (12 isolates), 0.22 to 0.69 and 0 to 0.60, respectively, in II.2 (6 isolates), 0.43 and 0.08 to 0.59, respectively, in III.1 (2 isolates), and 0.27 to 0.59 and 0.05 to 0.43, respectively, in III.2 (3 isolates) (Table 3). The interfertility group (biological species) labelled *Ganoderma* sp. 2 in Table 1 included 11 isolates from India and Taiwan that differed by only 0 to 3 nucleotides, and RAPD similarity coefficients were 0.35 to 0.85 (Table 3).

Taxonomic analysis of RAPD profiles. The data matrix in Fig. 3 was used for numerical and parsimony analyses to iden-

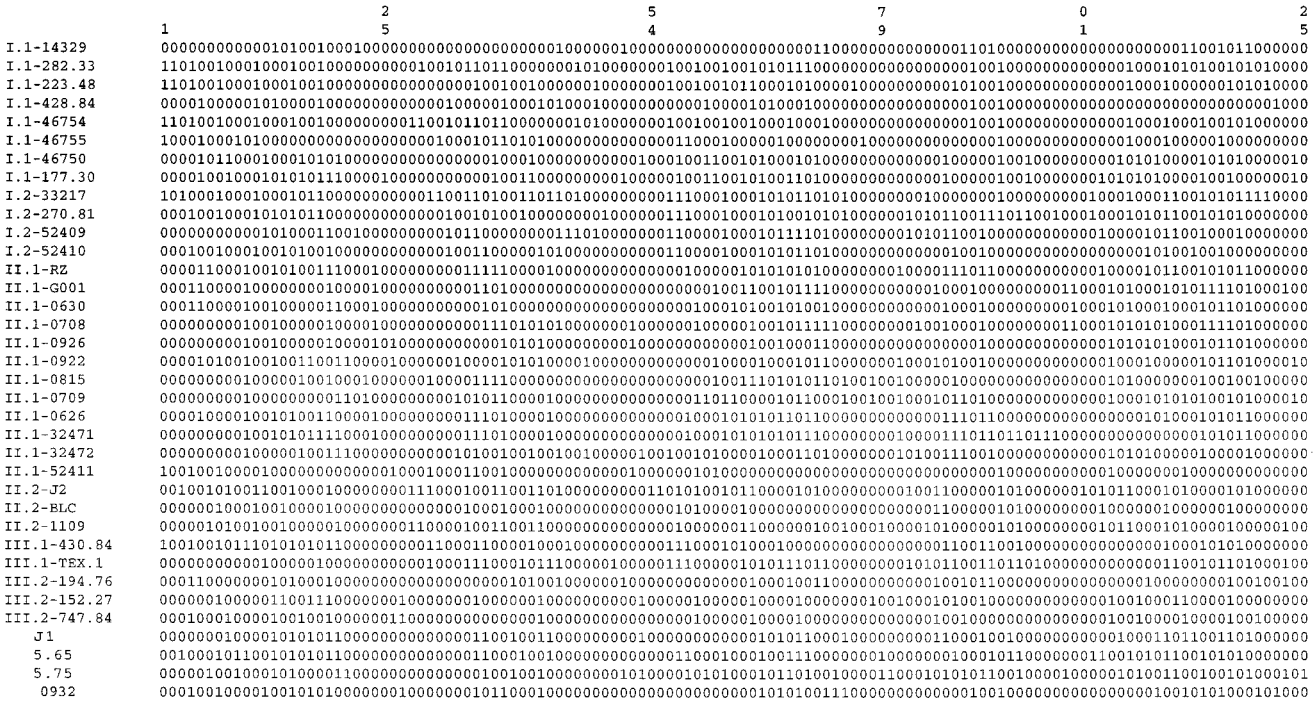

FIG. 3. Matrix of presence (1) or absence (0) of RAPDs for isolates of Table 1 developed by use of primers R1 (lanes 1 to 24), R2 (lanes 25 to 53), R3 (lanes 54 to 78), R4 (lanes 79 to 100), and R5 (lanes 101 to 125).

tify robust taxonomic groups. The phenogram depicted in Fig. 5 shows that average-linkage, complete-linkage, and Ward minimum variance methods produced identical terminal clusters except in their placement of isolate 5.65. Deeper branches of the phenogram, however, were sensitive to the choice of clustering algorithm. The strict consensus tree of parsimony analysis resolved terminal groups that were rather similar to those found in numerical analysis (Fig. 5) and did not resolve deeper branches (data not shown). Parsimony analysis partially summarized the different cluster analyses since it collapsed most branches that were in conflict when different clustering methods were used. By parsimony analysis, 15 groups were detected by bootstrapping the data matrix, four of which were found in 80% or more of bootstrap replications (Fig. 5). The only significant difference between cluster and parsimony analyses was in the placement of isolate I.1 ATCC 46755, which either clustered in I.2 (average linkage and complete linkage clusterings), stood alone (Ward minimum variance clustering), or grouped with I.1 CBS 428.84 in 9% of the bootstrap replications in parsimony analysis. Terminal groups that were present in all analyses or were meaningful for further comparison with ITS-based phyla are labelled groups 1 to 9 in Fig. 5.

Phyletic groups I.1 to III.2 were not differentiated by taxonomic analysis of RAPD profiles (Fig. 5): isolates of I.1 were in groups 2, 7, and 9; isolates of I.2 were all in group 8 but mixed with the two isolates of III.1; isolates of II.1 were in groups 3, 5, and 6; isolates of II.2 were in groups 1 and 4; isolates of III.2 were in groups 6 and 7. However, terminal clades that were present both in cluster and in parsimony analyses of RAPD profiles and that were still present in bootstrapping the data matrix in parsimony analysis grouped isolates of the same ITS-based phyletic group with the exception of I.1 FWP 14329 and III.2 CBS 194.76 in group 7. If we consider RAPD groups found in cluster analyses and in the strict consensus tree of parsimony analysis but not supported by bootstrapping, then strain III.1 RSH TEX.1 can be classified with I.2 ATCC 52409 and I.2 ATCC 52410. If we consider only the results of cluster analysis, then II.1 ATCC 52411 can be classified with isolates of III.2 (group 6), and strains of I.2 and III.1 and I.1 ATCC 46755 composed a well supported group (group 8).

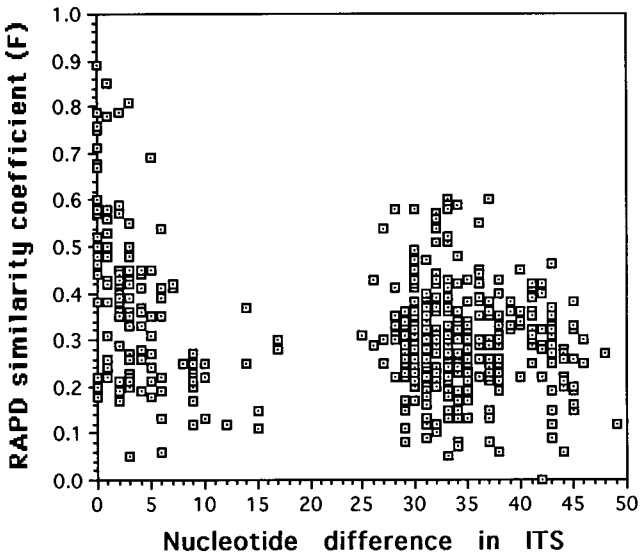

FIG. 4. Relationships between RAPD similarity coefficient and ITS sequence difference. The functional form of the relationships between the two types of measurements is not known but was roughly approximated as logarithmic (log), first-order polynomial (P1), and second-order polynomial (P2). When all data were examined, correlation coefficients (r^2) were 0.242 for log compared with 0.192 for P2 and 0.169 for P1 and 0.440 for P2 compared with 0.434 for P1 and 0.291 for log when the data set was restricted to ITS sequence differences not exceeding 12 nucleotides (<3%).

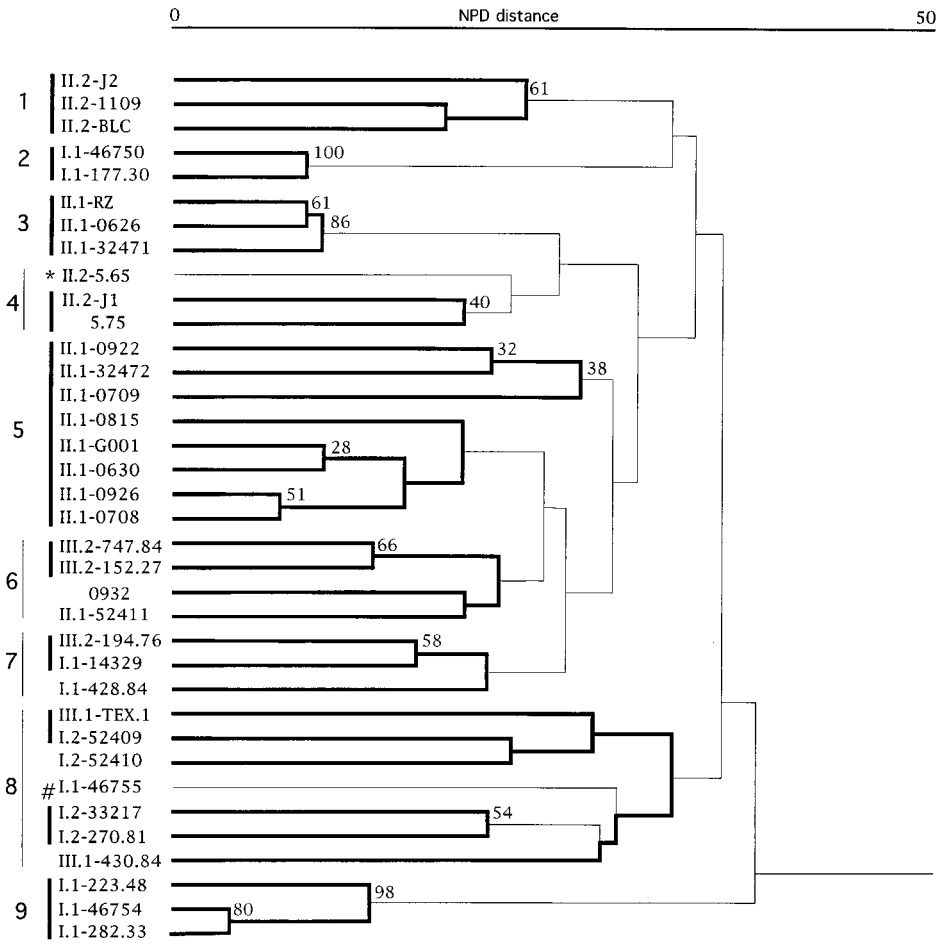

FIG. 5. Taxonomic analysis of RAPD profiles. The phenetic tree depicts average-linkage clustering of NPD distances calculated from the data matix in Fig. 3. Bold lines in the phenogram show branches that were also present in Ward minimum variance and complete-linkage clustering. Vertical bold lines on the left indicate clusters that were found in parsimony analysis (strict consensus of 10 equiparsimonious trees, 539 steps in length; consistency index = 0.212; retention index = 0.490). Branches present in bootstrapping the data matrix in parsimony analysis are indicated by bootstrap values above branches (percentage of 100 replications). Clusters that were present in all analyses or are meaningful for further discussion are labelled groups 1 to 9. Strain II.2 ACCC 5.65 (labelled with an asterisk in group 4) classified with III.1 CBS 430.84 (group 8) in Ward minimum variance clustering and parsimony analysis (28% of bootstrap replications). Bootstrapping the data matrix in parsimony analysis slightly modified group 8 by clustering strain I.1 ATCC 46755 (labelled with #) with I.1 CBS 428.84 (9% of bootstrap replications), while strains III.1 RSH TEX.1 clustered with I.2 ATCC 52409 and I.2 ATCC 52410 stood alone.

To examine whether taxonomic groupings of RAPD profiles at lower taxonomic levels were consistent with those of earlier data (34) (Table 1), lineages I and II were analyzed separately (Fig. 6 and 7) because NPD distance takes into account the whole structure of the data matrix. Groups that were found in all analyses of the entire data set (Fig. 5) were generally also found in analyses of the reduced data sets (Figs. 6 and 7).

Figure 6 shows that RAPD data did not support the taxonomic distinction between I.1 and I.2 within group I, although the four strains of I.2 did group together in all analyses except in complete-linkage clustering. In group I.2, RAPD profiles supported the distinction between *G. oerstedii* and *G. lucidum*, which were not distinguished by ITS sequences (0 to 1 nucleotide difference; Table 3). *G. ahmadii*, a distinctive species (34, 43) and the most divergent taxon of I.1 in ITS sequence differences (Table 3), nested with I.1 CBS 428.84 (*G. valesiacum* complex) in cluster analyses and in the strict consensus tree of parsimony analysis of RAPD data (Fig. 6); the grouping of these two strains, however, had no bootstrap support (Fig. 6). In contrast, RAPD profiles clearly identified isolates labelled *G. oregonense* (Fig. 6) that had an identical ITS sequence with I.1 ATCC 46754 (*G. valesiacum* complex) (Table 3). Distinction or conspecificity between *G. oregonense*, *G. tsugae*, and *G. valesiacum* was largely discussed elsewhere (4, 5, 14, 34, 39, 41), and the observed segregation of these taxa in Fig. 6 will need further scrutiny by use of a larger sampling and reference to host relationships, geographical origin, and mating data.

Figure 7 shows that RAPD profiles did not distinguish between II.1 and II.2 within lineage II. RAPD analysis split II.2 into two groups. The first group (II.2 RSH J2, II.2 RSH 1109, and II.2 RSH BLC) represents a biological species (17) (species 3 in Table 1) and was not distinguished from the second group (II.2 RSH J1 and II.2 ACCC 5.65) by ITS analysis (Fig. 1 and Table 3). To evaluate this delineation, cultural and mating behaviors of these taxa were studied; differences were found in growth rates between isolates of the two groups, and monokaryons of II.2 RSH J2 were incompatible with the dikaryotic culture of II.2 RSH J1. Taken together, these results strongly suggested that RAPD markers might have identified two species in II.2. II.1 ATCC 52411 represents a distinct species (Table 1) and stood alone in the phenogram (Fig. 7). The other isolates of II.1 were interfertile (17) and roughly

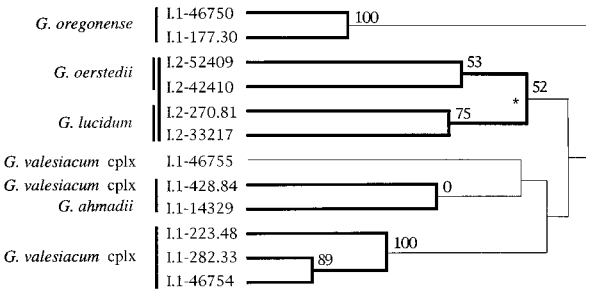

FIG. 6. Taxonomic analysis of RAPD profiles of isolates of groups I.1 and I.2. See the legend to Fig. 5 for an explanation of the bold lines and the values above the branches. The branch marked with an asterisk was not present in the complete-linkage clustering. Parsimony analysis found two equiparsimonious trees, 179 steps in length (consistency index = 0.497; retention index = 0.543). *G. valesiacum* cplx designates strains belonging to the *G. valesiacum* species aggregate.

segregated in three subgroups on the basis of RAPD profiles (Fig. 7). These subgroups were unexplained: they did not separate Indian isolates (II.1 ATCC 32471 and II.1 ATCC 32472) from Taiwan isolates, and they separated strains II.1 RSH G001, II.1 RSH RZ, and their progeny (II.1 RSH 0630, II.1 RSH 0926, and II.1 RSH 0708) that had identical ITS sequences (Table 3). II.1 RSH RZ clustered with II.1 RSH 0626 and II.1 ATCC 32471, with which it differed by 1 and 2 nucleotides, respectively (Table 3).

Lineage III was not analyzed separately, but Fig. 5 showed that III.2 CBS 152.27 strongly grouped with III.2 CBS 747.84 (group 6), while III.2 CBS 194.76 classified apart (group 7). The result was in agreement with differences in ITS sequence (Table 3) and ITS phylogeny (Fig. 1) and supported the change of the name of the former isolate to *G. pfeifferi*, as proposed elsewhere (Table 1).

Identification of isolates ACCC 5.75 and ZHANG 0932. To evaluate whether taxonomic identification of isolates of the *G. lucidum* complex was possible in the absence of ITS data, nucleotide sequences were deliberately omitted for isolates ACCC 5.75 and ZHANG 0932. RAPD fingerprinting strongly suggested that isolate ACCC 5.75 should be classified with II.2 RSH J1 (Fig. 5) together with II.2 ACCC 5.65 (Table 3 and Fig. 7); this seems to be correct, because the three strains have similar cultural characteristics (data not shown) and geographical origins (Table 1). The basidiocarp morphology, culture characteristics, and geographical origin of strain ZHANG 0932 suggested its placement in I.1 or I.2 (data not shown). However, ZHANG 0932 could not be unequivocally placed by RAPD fingerprinting. Higher RAPD similarity coefficients (F = 0.40 to 0.45) of strain ZHANG 0932 were with I.1 CBS 177.30, I.1 ATCC 46750, I.2 CBS 270.81, I.2 ATCC 52410, and II.1 RSH 815 (Table 3), but ZHANG 0932 classified with II.1 ATCC 52411 (group 6) in cluster analysis of NPD distance (Fig. 5), with which it had a similarity coefficient of 0.35 (Table 3), and stood alone in parsimony analysis (data not shown). Therefore, the ITS sequence will be necessary for taxonomic identification of isolate ZHANG 0932.

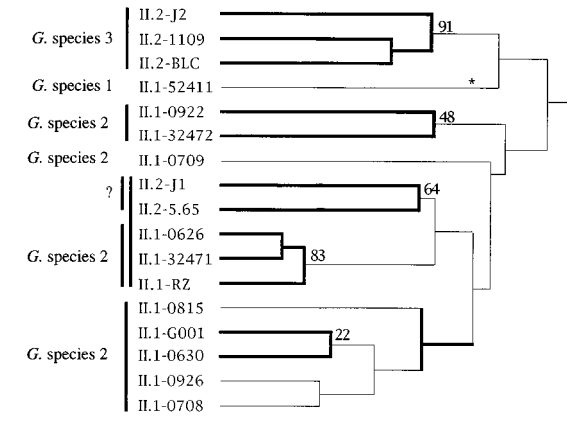

FIG. 7. Taxonomic analysis of RAPD profiles of *Ganoderma* (*G.*) isolates of groups II.1 and II.2. See the legend to Fig. 5 for an explanation of the bold lines and the values above the branches. Parsimony analysis found two equiparsimonious trees, 247 steps in length (consistency index = 0.405; retention index = 0.516). The branch marked with an asterisk collapsed in 90% of the bootstrap replications in parsimony analysis.

DISCUSSION

This study shows that strains of the *G. lucidum* complex can be differentiated by RAPD fingerprinting with the primers listed in Table 2; the potential of RAPDs as genetic markers throughout prokaryotes and eukaryotes was suggested in the original description of the method (50, 52). In contrast, RAPDs produced by primers R1 to R5 did not identify the ITS-based groups of the *G. lucidum* complex which were supported by high confidence levels (Fig. 5 to 7). RAPD groupings were sensitive to clustering methods and generally poorly supported by bootstrapping the data matrix in parsimony analysis, indicating that our RAPD data set lacks internal robustness. Therefore, no alternative taxonomy to that based on ITS data can be proposed on the basis of our RAPD data. It remains possible that lineage sorting, among-site rate variation (44), or other phenomena result in a gene tree that is internally robust but incongruent with the organismal phylogeny. However, there are good reasons to assume that the ITS phylogeny shown in Fig. 1 reflects the correct phylogeny of the taxa investigated in this study: our ITS tree is independently supported by characters developed by mycelia in culture, host relationships, geographical origin of the isolates, and/or mating data (34).

In cluster analysis, the RAPD profiles produced in this work failed to distinguish between phyletic groups I.1 to III.2 because intragroup similarities were not higher than intergroup similarities (Table 3). The lack of resolution of the RAPD phenograms at high taxonomic levels (Fig. 5 to 7) can be attributed to the fact that as genetic divergence between taxa increases, the probability that comigrating RAPDs are homologous (and thus taxonomically informative) rapidly decreases.

Inferring character homology is a major problem for the use of RAPDs in systematic studies. Lack of homology of comigrating fragments would preclude their use as taxonomic characters in both phylogenetic and phenetic analyses. As currently used in systematic studies, comigrating fragments are often assumed empirically to be homologous; however, many primer site sequences can produce identical bands, and bands of different molecular weights may be homologous and differ in weight because of deletion or insertion. Therefore, assessment of homology of comigrating RAPDs would always remain tentative in the absence of sequence information on the amplified fragments. This could be obtained either by cloning RAPD markers and using these as probes in Southern hybridizations or by sequencing. However, the main advantages of the method (i.e., speed and simplicity) in producing taxonomic data would be lost.

Other difficulties in applying RAPD data in systematics are direct consequences of the extreme sensitivity and mechanism

of RAPD-PCR. Artifactual variations in RAPD profiles and reliability of the method have been discussed over a wide range of parameters (13, 25, 35, 47, 50, 52), and it was shown that experimentation must be set up carefully and rigorously standardized for producing reproducible and consistent profiles. In this work, RAPD profiles were easy to reproduce by use of the standardized protocol, and consistency was shown in producing identical RAPDs from both basidiocarp tissue and culture-grown mycelium of III.1 RSH TEX.1. However, a problem largely overlooked in earlier systematic studies was encountered when RAPDs were used for systematics of the *G. lucidum* complex: it was observed that some isolates produced more RAPDs when higher $MgCl_2$ and dNTP concentrations were used in the amplification reaction. These fragments were qualitatively scorable in our data matrix but could not be included in the comparative analysis because different experimental conditions cannot be set for quantitative comparison. It was found that differences in PCR conditions in producing a higher number of amplification products were taxonomically related (data not shown). It is speculated that for *Ganoderma* spp., these differences arise when DNAs isolated from different taxa carry over different impurities which interfere with RAPD-PCRs. DNA isolation from *Ganoderma* spp. generally yields polysaccharides and phenolic compounds that are difficult to remove. High-quality DNA is not required for conventional PCR amplification targeting specific DNA fragments, but it is not known how important DNA quality is as a parameter in producing RAPDs. If matches between an arbitrary primer and the template DNA in RAPD-PCR (that occur at low stringency) are sensitive to taxon-specific impurities, artifactual RAPDs that are reproducible and taxon specific may be produced. That would not provide a correct estimation of overall sequence relatedness between primer and DNAs and would be misleading for systematic studies.

On the basis of RAPD data, only groups detected consistently by all analyses were considered meaningful. These groups are composed of recently diverged isolates that are putatively conspecific, as indicated by low ITS divergence between these isolates (ca. 0 to 1%). Meaningful groups were better evidenced by parsimony analysis since maximum parsimony collapsed most branches having little taxonomic support. Unlike clustering analysis, parsimony analysis revealed the lack of taxonomic structure in the RAPD data set at higher taxonomic levels while still recognizing robust groups at lower taxonomic levels. This observation suggests that cladistic algorithms can serve as an explicit character-based method to identify groups of isolates within a discrete data set, even if the data set is inappropriate for inferring hierarchical classification of these groups (i.e., for reconstructing phylogenies).

The robust RAPD-based groups detected in all analyses were not detectable or else poorly resolved in the ITS analysis (Fig. 1 and Table 3). Most of these groupings are in agreement with other sources of data. For instance, RAPDs distinguished two morphologically and geographically distinct taxa in group I.2 (*G. lucidum* and *G. oerstedii*) (Fig. 6), identified two putative biological species in group II.2 (II.2 RSH J2, RSH BLC, and RSH 1109 versus II.2 RSH J1, ACCC 5.65, and ACCC 5.75) (Fig. 7), and supported identification of III.2 CBS 152.27 as *G. pfeifferi* instead of *G. resinaceum* (Fig. 5 and Table 1). In contrast, RAPD groupings in interfertility group II.1 were not consistent with the geographical origins of the isolates (India and Taiwan) and did not indicate a close relationship between strains II.1 RSH RZ and II.1 RSH G001 and their progeny (II.1 isolates RSH 0630, RSH 0926, and RSH 0708) (Fig. 7), which have identical ITS sequences (Table 3). A lack of correspondence between RAPD similarities and other sources of taxonomic data in intraspecific groupings was also found, for instance, for *Botrytis cinerea* (49) and *Septoria tritici* (30).

This study shows that while RAPDs cannot serve as a substitute for ITS sequencing for taxonomic identification and groupings in the *G. lucidum* complex, RAPDs might still be useful in identifying taxonomically meaningful groups for the systematics of the more recently diverged taxa having identical or nearly identical ITS sequence. The results indicate that our RAPD data matrix contains taxonomic information for the more related taxa only, also suggesting that character homology inferences for RAPDs might be correct only at the population or variety level in species of the *G. lucidum* complex. More generally, the use of RAPDs in systematics has several limitations, and the relevance and taxonomic meaning of RAPD groupings always need careful comparison with that of other sources of data. Although RAPD markers can serve in distinguishing taxa by producing taxon-specific amplified fragments or grouping closely related taxa in numerical or cladistic analyses (references 12, 15, 20, 24, 26, 29, 49, and 51, and present work), the utility of RAPDs as systematic characters is limited because of difficulties in assessing character homologies. However, RAPD-PCR has the potential to survey entire genomes, and RAPDs may provide insights into organismal evolution that are overlooked by single-gene comparisons. Combining RAPD-PCR and sequencing methods to produce phylogenetic characters thus still may hold some promise in evolutionary genetics and systematics: by determining the nucleotide sequences of randomly amplified products, homologies between RAPDs could be inferred with greater confidence and nucleotide sequences that are variable in homologous RAPD fragments could be used as phylogenetic characters. Such an approach was explored recently in cichlids by Sultmann et al. (45), who showed that the RAPD-PCR technique followed by sequencing of selected fragments produced phylogenetic characters.

The conclusion of this work is that ITS sequencing can be used to identify collections of the *G. lucidum* species complex and RAPD fingerprinting can be used to differentiate between isolates having identical ITS sequences. The nucleotide accession numbers given in Table 1 form an expandable ITS data base for *Ganoderma* spp. with which the ITS sequence of any isolate can be compared. In the *G. lucidum* species complex, strains showing less than 1.5% sequence differences are putatively conspecific (34), but ultimately, conspecificity has to be determined by interfertility studies. Correct naming of most isolates still depends on further taxonomic work. The practical implications of this study are numerous. For instance, ITS phylogeny showed that commercial *Ganoderma* strains in the Orient (and probably also pharmaceutical strains) belong to different species and were largely misnamed in the scientific literature. RAPD data showed that commercial isolates of this study that were conspecific were not from a clonal line. In epidemiological studies of crops infected by *Ganoderma* spp., it has been suggested that the infection might spread by hyphal propagation from tree to tree by root-to-root contact or by the intermediary of vegetation debris (32). Clonal propagation in a field could be monitored with RAPD markers, while ITS sequencing would help in the identification of the pathogen and in determination of host specificity and distribution of virulent *Ganoderma* species.

ACKNOWLEDGMENTS

This work was supported by research grant no. 8209004 from the Microbiological Research Foundation of the Republic of China (Taiwan).

We thank two anonymous reviewers for helpful comments in the revision of the manuscript.

REFERENCES

1. **Adaskaveg, J. E., and R. L. Gilbertson.** 1985. *In vitro* decay studies of selective delignification and simultaneous decay by the white rot fungi *Ganoderma lucidum* and *G. tsugae*. Can. J. Bot. **64:**1611–1619.
2. **Adaskaveg, J. E., and R. L. Gilbertson.** 1986. Cultural studies and genetics of sexuality of *Ganoderma lucidum* and *G. tsugae* in relation to the taxonomy of the *G. lucidum* complex. Mycologia **78:**694–705.
3. **Adaskaveg, J. E., and R. L. Gilbertson.** 1987. Infection and colonization of grapevines by *Ganoderma lucidum*. Plant Dis. **71:**251–253.
4. **Adaskaveg, J. E., and R. L. Gilbertson.** 1988. Basidiospores, pilocystidia, and other basidiocarp characters in several species of the *Ganoderma lucidum* complex. Mycologia **80:**493–507.
5. **Adaskaveg, J. E., and R. L. Gilbertson.** 1989. Cultural studies of four North American species in the *Ganoderma lucidum* complex with comparisons to *G. lucidum* and *G. tsugae*. Mycol. Res. **92:**182–191.
6. **Adaskaveg, J. E., and R. L. Gilbertson.** 1995. Wood decay caused by *Ganoderma* species in the *G. lucidum* complex, p. 79–93. *In* P. K. Buchanan, R. S. Hseu, and J. M. Moncalvo (ed.), *Ganoderma*—systematics, phytopathology and pharmacology. Proceedings of Contributed Symposium 59A,B, 5th International Mycological Congress, August 14–21, 1994, Vancouver, Canada.
7. **Adaskaveg, J. E., R. L. Gilbertson, and R. A. Blanchette.** 1990. Comparative studies of delignification caused by *Ganoderma* species. Appl. Environ. Microbiol. **56:**1932–1943.
8. **Bakshi, B. K., M. A. R. Reddy, and S. Singh.** 1976. *Ganoderma* root rot mortality in khair (*Acacia catechu* Willd.) in reforested stands. Eur. J. For. Pathol. **6:**30–38.
9. **Bazzalo, M. E., and J. E. Wright.** 1982. Survey of the Argentine species of the *Ganoderma lucidum* complex. Mycotaxon **16:**293–325.
10. **Berg, D. E., N. S. Akopyants, and D. Kersulyte.** 1994. Fingerprinting microbial genomes using the RAPD or AP-PCR method. Methods Mol. Cell. Biol. **5:**13–24.
11. **Bruns, T. D., T. J. White, and J. W. Taylor.** 1991. Fungal molecular systematics. Annu. Rev. Ecol. Syst. **22:**525–564.
12. **Duncan, S., J. E. Barton, and P. O'Brien.** 1993. Analysis of variation in isolates of *Rhizoctonia solani* by random amplified polymorphic DNA assay. Mycol. Res. **97:**1075–1082.
13. **Ellsworth, D. L., K. D. Rittenhouse, and R. L. Honeycutt.** 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. BioTechniques **14:**214–216.
14. **Gilbertson, R. L., and L. Ryvarden.** 1986. North American polypores. Fungiflora, Oslo, Norway.
15. **Hamelin, R. C., G. B. Ouellette, and L. Bernier.** 1993. Identification of *Gremmeniella abietina* races with random amplified polymorphic DNA markers. Appl. Environ. Microbiol. **59:**1752–1755.
16. **Hibbett, D. S.** 1992. Ribosomal RNA and fungal systematics. Trans. Mycol. Soc. Jpn. **33:**533–556.
17. **Hseu, R. S.** 1990. An identification system for cultures of *Ganoderma* species. Ph.D. dissertation. National Taiwan University, Taipei, Taiwan, Republic of China.
18. **Jong, S. C., and J. M. Birmingham.** 1992. Edible mushrooms in biotechnology, p. 18–35. *In* Proceedings of the Asian Mycology Symposium, Oct. 1–4, 1992, Seoul, Korea. Mycological Society of the Republic of Korea, Seoul, Korea.
19. **Jong, S. C., and J. M. Birmingham.** 1992. Medicinal benefits of the mushroom *Ganoderma*. Adv. Appl. Microbiol. **37:**101–134.
20. **Kazan, K., J. M. Manners, and D. F. Cameron.** 1993. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. Genome **36:**43–49.
21. **Kersulyte, D., J. P. Woods, E. J. Keath, W. E. Goldman, and D. E. Berg.** 1992. Diversity among clinical isolates of *Histoplasma capsulatum* detected by polymerase chain reaction with arbitrary primers. J. Bacteriol. **174:**7075–7079.
22. **Khush, R. S., E. Becker, and M. Wach.** 1992. DNA amplification polymorphisms of the cultivated mushroom *Agaricus bisporus*. Appl. Environ. Microbiol. **58:**2971–2977.
23. **Kirk, T. K., T. W. Jeffries, and G. F. Leatham.** 1983. Biotechnology: applications and implications for the pulp and paper industry. TAPPI J. **66:**45–51.
24. **Laroche, A., D. A. Gaudet, G. B. Schaalje, R. S. Erickson, and J. Ginns.** 1995. Grouping and identification of low temperature basidiomycetes using mating, RAPD and RFLP analyses. Mycol. Res. **99:**297–310.
25. **Leal, S. C. M., D. J. Bertioli, T. M. Butt, and J. F. Pederby.** 1994. Characterization of isolates of the entomopathogenic fungus *Metarhizium anisopliae* by RAPD-PCR. Mycol. Res. **98:**1077–1081.
26. **Liew, E. C. Y., and J. A. G. Irwin.** 1994. Comparative studies on *Phytophthora megasperma* isolates from chickpea collected in Australia and in Spain. Mycol. Res. **98:**1284–1290.

26a. **Lewis, P.** Personal communication.

27. **Mahmood, T.** 1971. *Ganoderma lucidum*: a virulent incitant of basal stem rot, a malady of hardwoods in West Pakistan. Plant Dis. Rep. **55:**1130–1131.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Manulis, S., L. Valinsky, A. Lichter, and D. W. Gabriel.** 1994. Sensitive and specific detection of *Xanthomonas campestris* pv. pelargonii with DNA primers and probes identified by random amplified polymorphic DNA analysis. Appl. Environ. Microbiol. **60:**4094–4099.
30. **McDonald, B. A., and J. P. Martinez.** 1991. DNA fingerprinting of the plant pathogenic fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*). Exp. Mycol. **15:**146–158.
31. **Meyer, W., T. G. Mitchell, E. Z. Freedman, and R. Vilgalys.** 1993. Hybridization probes for conventional DNA fingerprinting used as single primers in the polymerase chain reaction to distinguish strains of *Cryptococcus neoformans*. J. Clin. Microbiol. **31:**2274–2280.
32. **Miller, R. N. G., M. Holderness, P. D. Bridge, R. R. M. Paterson, M. Sariah, M. Z. Hussin, and E. J. Hilsley.** 1995. A multi-disciplinary approach to the characterization of *Ganoderma* in oil-palm cropping systems, p. 57–66. *In* P. K. Buchanan, R. S. Hseu, and J. M. Moncalvo (ed.), *Ganoderma*—systematics, phytopathology and pharmacology. Proceedings of Contributed Symposium 59A,B, 5th International Mycological Congress, August 14–21, 1994, Vancouver, Canada.
33. **Moncalvo, J. M., H. H. Wang, and R. S. Hseu.** 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. Mycologia **87:**223–238.
34. **Moncalvo, J. M., H. F. Wang, and R. S. Hseu.** 1995. Gene phylogeny of the *Ganoderma lucidum* complex based on ribosomal DNA sequences. Comparison with traditional taxonomic characters. Mycol. Res. **99:**1489–1499.
35. **Muralidharan, K., and E. K. Wakeland.** 1993. Concentration of primer and template qualitatively affects products in random-amplified polymorphic DNA PCR. BioTechniques **14:**362–363.
36. **Nei, M., and W. H. Li.** 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76:**5269–5273.
37. **Ryvarden, L.** 1983. Type studies in the Polyporaceae 14. Species described by N. Patouillard, either alone or with other mycologists. Occasional papers of the Farlow Herbarium of Cryptogamic Botany. Harvard University, Cambridge, Mass.

37a. **Ryvarden, L.** Personal communication.

38. **Ryvarden, L.** 1985. Type studies in the Polyporaceae 17. Species described by W. A. Murrill. Mycotaxon **23:**169–198.
39. **Ryvarden, L., and R. L. Gilbertson.** 1993. European polypores. Fungiflora, Oslo, Norway.
40. **Saiki, R., D. Gelfand, S. Stoffel, S. Scharf, R. Higuchi, G. Horn, K. Mullis, and H. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–494.

40a. **Sato, H.** Personal communication.

41. **Stalpers, J. A.** 1978. Identification of wood-inhabiting fungi in pure culture. Stud. Mycol. **16:**1–248.
42. **Steyaert, R. L.** 1967. Les *Ganoderma* palmicoles. Bull. Jard. Bot. Natl. Belg. **37:**465–492.
43. **Steyaert, R. L.** 1972. Species of *Ganoderma* and related genera mainly of the Bogor and Leiden herbaria. Persoonia **7:**55–118.
44. **Sullivan, J., K. E. Holsinger, and C. Simon.** 1995. Among-site rate variation and phylogenetic analysis of 12S rRNA in Sigmodontine rodents. Mol. Biol. Evol. **12:**988–1001.
45. **Sultmann, H., W. E. Mayer, F. Figueroa, H. Tichy, and J. Klein.** 1995. Phylogenetic analysis of cichlid fishes using nuclear DNA markers. Mol. Biol. Evol. **12:**1033–1047.
46. **Swofford, D. L.** 1990. PAUP 3.0: phylogenetic analysis using parsimony. Illinois Natural History Survey, Champaign, Ill.
47. **Tommerup, I. C., J. E. Barton, and P. A. O'Brien.** 1995. Reliability of RAPD fingerprinting of three basidiomycete fungi, *Laccaria*, *Hydnangium* and *Rhizoctonia*. Mycol. Res. **99:**179–186.
48. **Turner, P. D.** 1965. Infection of oil palms by *Ganoderma*. Phytopathology **55:**937.
49. **Van der Vlugt-Bergmans, C. J. B., B. F. Brandwagt, J. W. Van't Klooster, C. A. M. Wagemakers, and J. A. L. Van Kan.** 1993. Genetic variation and segregation of DNA polymorphisms in *Botrytis cinerea*. Mycol. Res. **97:**1193–1200.
50. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. **18:**7213–7218.
51. **Welsh, J., C. Pretzman, D. Postic, I. Saint Girons, G. Baranton, and M. McClelland.** 1992. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. Int. J. Syst. Bacteriol. **42:**370–377.
52. **Williams, J. G., A. R. Kubelik, J. L. Kenneth, J. A. Rafalski, and V. T. Scott.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. **18:**6531–6535.

52a. **Wright, J. E.** Personal communication.

53. **Wyss, P., and P. Bonfante.** 1993. Amplification of genomic DNA of arbuscular-mycorrhizal (AM) fungi by PCR using short arbitrary primers. Mycol. Res. **97:**1351–1357.

53a. **Zhang, X. Q.** Personal communication.

54. **Zhao, J. D.** 1989. The Ganodermataceae in China. Bibl. Mycol. **132:**1–176.