ycologia, 87(2), 1995, pp. 223–238.

Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences

-Marc Moncalvo[1]
-Hua Wang
-Shyang Hseu
Applied Microbiology Laboratory, Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan, Republic of China

tract: Over 250 species have been described in *noderma*. Species identification and species circumption are often unclear and taxonomic segregation the genus remains controversial. In this study we uenced the 5′ half of the 25S ribosomal RNA gene the internal transcribed spacers to determine apriate regions to i) discriminate between *Ganod*species and ii) infer taxonomic segregation of *noderma s. lato* (Ganodermataceae) on a phylogebasis. We studied 19 *Ganoderma* isolates repreing 14 species classified in 5 subgenera and sec, one isolate of the related genus *Amauroderma*, one isolate of *Fomitopsis* which served as the outin parsimony analysis. Results showed that a ition bias was present in our data, and that rates ucleotide divergence in the different ribosomal ns varied between lineages. Independent and bined analyses of different data sets were perd and results were discussed. Nucleotide seces of the internal transcribed spacers, but not e of the coding regions, distinguished between *Ganoderma* species, and indicated that isolates of *G. tsugae* group were misnamed. Phylogenetic sis of the combined data sets of the divergent ain D2 of the 25S ribosomal RNA gene and of internal transcribed spacers indicated that sub*Elfvingia* was monophyletic, whereas sections *noderma* and *Phaeonema* were not. Combined data these regions is useful for infrageneric segreof *Ganoderma* on a phylogenetic basis. Phylotic analysis from data of the D2 region alone ngly supported *Amauroderma* as a sister taxon of *noderma*. This suggested that the D2 region should suitable for systematics at higher taxonomic ranks the Ganodermataceae. The low sequence variation rved in the 25S ribosomal gene within *Ganoderma* es suggested that the genus is young.

sponding author.
ed for publication December 27, 1994.

Key Words: *Ganoderma*, internal transcribed spacers, nucleotide sequence, phylogeny, 25S ribosomal RNA gene

INTRODUCTION

Ganoderma Karst. is a cosmopolitan genus of wood decaying polypore fungi of economic importance. Several species cause severe diseases in plantations or in forests (Steyaert, 1967, 1972; Mahmood, 1971; Ross, 1976; Bakshi et al., 1976). Some of them have been shown to selectively delignify wood and are recognized as a potentially important source of lignin-degrading enzymes (Otjen and Blanchette, 1987). In the Orient, *Ganoderma* species are used in folk medicine to cure various diseases, and strains are commercially cultivated for the preparation of health tablets or drinks. The many medicinal benefits of *Ganoderma* were reviewed by Jong and Birmingham (1992).

The genus *Ganoderma* was established by Karsten (1881) for the laccate and stipitate white rot fungus *Polyporus lucidus* W. Curt. It was later amended by Patouillard (1889) to include all polypores having double-walled basidiospores. Ryvarden (1991) noted that the genus is presently in taxonomic chaos. Species identification and species circumscription are often unclear, and the infrageneric segregation is controversial (Steyaert, 1972, 1980; Corner, 1983; Zhao, 1989).

Over 250 *Ganoderma* species have been described worldwide, most of them from the tropics. Numerous species have been described from pleomorphic characters. As a consequence, there are many synonyms and several species complexes have been recognized (Steyaert, 1972, 1980; Bazzalo and Wright, 1982; Adaskaveg and Gilbertson, 1986; Yeh, 1990). For instance, Chen (1993) and Hseu (unpubl.) demonstrated in several species that the shape of the basidiocarp was greatly influenced by environmental factors. Also, Steyaert (1975) demonstrated in *G. tornatum* (Pers.) Bres. that basidiospore size varies with latitude and altitude, and observed in *G. lucidum* (W. Curt.: Fr.) Karst. that context color was darker in collections from more southern latitudes on the European continent (Steyaert, 1972).

Patouillard (1889) divided the genus into two sections, sect. *Ganoderma* and sect. *Amauroderma*. The latter was created to separate species characterized by

subspherical or spherical basidiospores with the wall uniformly thickened from those having basidiospores truncated at the apex. Karsten (1889) established the genus *Elfvingia* Karst. for nonlaccate *Ganoderma* species, and Murrill (1905a) raised *Amauroderma* Pat. to generic rank. Murrill (1905b) created *Tomophagus* for *P. colossus* Fr. but the genus was considered a synonym of *Ganoderma* by Furtado (1965), Steyaert (1972) and Ryvarden (1991). *Elfvingia* was emended to include all species lacking pilocystidia and reduced to subgeneric rank by Imazeki (1939), and was considered to be a synonym of *Ganoderma* by Furtado (1965). Imazeki (1952) raised to generic level sect. *Trachyderma* Imaz. (Imazeki, 1939), which was created to accommodate *G. tsunodae* (Yasuda) Trott. Steyaert (1972) proposed three new genera, *Haddowia, Humphreya* and *Magoderma,* for a mixture of *Ganoderma* and *Amauroderma* species. Later, he divided *Ganoderma* into four subgenera and two sections on the basis of the cutis anatomy (Steyaert, 1980), but Furtado (1965) and Corner (1983) noted intermediacy between the described types of cutis and argued that alleged distinctions break down in practice. Zhao et al. (1979) segregated subgen. *Ganoderma* on the basis of context color.

Besides morphological traits of fruitbodies, additional taxonomic characters have been investigated for systematics of *Ganoderma.* Cultural studies were conducted by Nobles (1948), Stalpers (1978), Bazzalo and Wright (1982), Adaskaveg and Gilbertson (1986), Hseu (1990), and Wang and Hua (1991). Isozyme electrophoretic phenotypes were used by Hseu (1990). These methods produced new characters for studies at the species level, but their use was not investigated at higher taxonomic levels. Intercompatibility studies have been reported in the *G. lucidum* complex by Adaskaveg and Gilbertson (1986), Peng (1990) and Hseu (1990), and in the *G. applanatum* group by Yeh (1990).

In this work, we studied ribosomal RNA genes (rDNA) to infer their applicability for systematics of *Ganoderma.* Ribosomal genes were chosen because they form a mosaic pattern of conserved and variable regions which makes them attractive for taxonomic investigation at many levels (Bruns et al., 1991; Hibbett, 1992). However, Taylor et al. (1990) showed that levels of sequence variability in a given region are different in different fungal taxa, and that no unique region can be used to identify all fungal species nor to address phylogenetic relationships among all fungi. The most variable regions are used for systematics at lower taxonomic levels. These are the intergenic regions (IGR), the internal transcribed spacers (ITS), and the divergent domains of the large ribosomal subunit (25S rRNA) or of its gene (25S rDNA), which were named D1 to D7 in the model of Michot et al. (1984). In *Armillaria,* the IGR 1 region was suitable to distinguish between biological species and to infer their phylogenetic relationships, while variation in the ITS region was too low (Anderson and Stasovski, 1992). The IGR 1 region was useful for race identification in *Puccinia* (Kim, 1992). The ITS regions were used for systematics in *Phytophthora* (Lee and Taylor, 1992), in the Sclerotiniaceae (Carbone and Kohn, 1993), in boletes (Bruns et al., 1992), in rusts (Zambino and Szabo, 1993), and in *Talaromyces* and *Penicillium* (LoBuglio et al., 1993). Divergent domains in the 25S rDNA were used for species identification and phylogenetic studies in yeasts (Kurtzman, 1992a, b), in *Fusarium* (Guadet et al., 1989), in *Lyophyllum* (Moncalvo et al., 1993) and in *Lentinus* (Hibbett and Vilgalys, 1993). The latter study included *G. lucidum* and other polypores.

We sequenced the ITS regions and the 5′ half of the 25S rDNA to determine appropriate regions to i) discriminate between *Ganoderma* species and ii) infer segregation of *Ganoderma s. lato* (Ganodermataceae Donk) on a phylogenetic basis. A comparison was also made of rates and modes of evolution of the different rDNA regions studied, and phylograms produced from independent and combined analyses of different data sets were critically examined.

MATERIALS AND METHODS

Organisms studied.—The strains used in this study, their origins, and their classification in Steyaert's (1980) or Zhao's (1989) system are presented in TABLE I. Isolate CBS 428.84 was received from Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands, as *G. valesiacum* Boud., but it was named *G. tsugae* Murr. by its collector. Since the synonymy of both names proposed by Stalpers (1978) has not been widely accepted, we named this isolate in TABLE I as it was identified by its collector. The names of other isolates followed those given in the strain catalogues of CBS or of American Type Culture Collection (ATCC), or those given by the collectors for strains of other origins. Identification of *Ganoderma* isolates collected in Taiwan and in the Philippines was based on Sawada (1931, 1943), Hseu (1990) and Yeh (1990). Most strains used in this study were chosen because they had available morphological, cultural or mating data for comparison with molecular data. The only *Amauroderma* species available to us was included in this study. An isolate of the genus *Fomitopsis* was added to serve as outgroup for the phylogenetic analysis of the sequence data because this genus was regarded as the closest relative of *Ganoderma s. lato* by Corner (1983). The *Fomitopsis* isolate was identified as a member of the *F. rosea* complex, a group requiring taxonomic revision.

DNA isolation, PCR amplification and sequencing.—DNAs were isolated from mycelia grown in potato

TABLE I. List of taxa used in this study, their source and their classification in Steyaert's (1980) and Zhao's (1989) system

Species	Locality	Abbr[a]	Source and collection numbers of the strains[b]	Classification in	
				Steyaert's system[c]	Zhao's system[c]
Ganoderma lucidum (W. Curt.: Fr.) Karst.	Taiwan	TW	Hseu, R. S., RSH RZ	sect. *Ganoderma*	sect. *Ganoderma*
	India	IN	Bakshi, B. K., FRI 55, ATCC 32471		
	Philippines	PH	Moncalvo, J. M., JM P93.1		
G. tsugae Murrill	USA	US	Adaskaveg, J. E., CBS 428.84	sect. *Ganoderma*	sect. *Ganoderma*
	Japan	JP[d]	Sato, J. H., RSH J2		
	Taiwan	TW[d]	Tseng, T. C., RSH 1109		
	China	CH	Zhang, X. Q., ZHANG 0981		
G. microsporum Hseu	Taiwan	TW	Hseu, R. S., RSH 0821	sect. *Ganoderma*	sect. *Ganoderma*
G. weberianum (Bres. & Henn.) Stey.	Philippines	PH	Humphrey, C. J., CBS 219.36	sect. *Ganoderma*	sect. *Ganoderma*
G. subamboinense Henn. var. *laevisporum* Bazz. & Wright	Argentina	AG	Bazzalo, N. E., BAFC 247, ATCC 52419	sect. *Ganoderma*	sect. *Ganoderma*
G. resinaceum Boud. apud Pat.	Netherlands	EC	Stalpers, J. A., CBS 194.76	sect. *Ganoderma*	sect. *Ganoderma*
G. boninense Pat.	Taiwan	TW	Hseu, R. S., RSH RS	sect. *Ganoderma*	sect. *Phaeonema*
G. formosanum Chang & Chen	Taiwan	TW	Hseu, R. S., RSH 0109	sect. *Ganoderma*	sect. *Phaeonema*
G. pfeifferi Bres.	Netherlands	EC	Stalpers, J. A., CBS 747.84	sect. *Characoderma*	sect. *Phaeonema*
G. oerstedii (Fr.) Torr.	Argentina	AG	Del Busto, E., BAFC 218, ATCC 52410	sect. *Characoderma*	sect. *Ganoderma*
G. australe (Pers.) Pat.	Taiwan	TW	Hseu, R. S., RSH 0705	subg. *Elfvingia*	subg. *Elfvingia*
G. gibbosum (Blum. & Nees) Pat.	China	CH	?, ACCC 5.151	subg. *Elfvingia*	subg. *Elfvingia*
G. lobatum (Schw.) Atk.	USA	US	Nobles, M. K., DAOM 8008, CBS 222.48	subg. *Anamixoderma*	subg. *Elfvingia*
G. adspersum (Schulz.) Donk	Europe	EC	Demoulin, V., CBS 351.74	subg. *Anamixoderma*	subg. *Elfvingia*
Amauroderma rude (Berk.) Torr. var. *intermedium* (Bres. & Pat.) Furt.	Taiwan		Moncalvo, J. M., JM ASP.1	gen. *Amauroderma*	gen. *Amauroderma*
Fomitopsis cf. *rosea*	Taiwan		Moncalvo, J. M., JM T92.10		

[a] Abbreviations used in the FIGURES and in the TABLES to indicate the locality of the strains.

[b] Strains from CBS (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands), ATCC (American Type Culture Collection, Rockville, USA) and ACCC (Agriculture Culture Collection Center, Beijing, China) were purchased via the Culture Collection and Research Center in Hsinchu, Taiwan.

[c] Section *Ganoderma*, section *Characoderma* Stey. and section *Phaeonema* Zhao et al. of subgenus *Ganoderma*; subgenus *Anamixoderma* Stey. and subgenus *Elfvingia* (Karst.) Imazeki; genus *Amauroderma* (Pat.) Murrill.

[d] Commercially cultivated strains.

dextrose medium for 1–2 wk prior to harvesting, except for strains JM-P93.1 and JM-ASP.1 in which DNAs were extracted from wild collected basidiocarps. The isolation procedure was essentially as described in Raeder and Broda (1985), but for better removal of polysaccharides and pigments centrifugation times were increased up to 45 min at 15 000 × *g* after addition of phenol : chloroform : isoamylalcol or chloroform : isoamylalcol, and cleaning steps using 70% ethanol after DNA precipitation were repeated up to 5 times for some samples. Reagents and conditions for PCR amplification were as described in Vilgalys and Hester (1990). Amplified DNA fragments were purified using a Promega kit (A7170). Direct DNA sequencing of double-stranded PCR products followed the method of Sanger et al. (1977) using a cycle-sequencing kit (Promega Q4100) and following the manufacturer's protocol, except that concentration of template DNAs was 50–100 times higher than indicated. Sequencing primers were end-labelled with [γ-^{32}P] ATP (>5000 Ci/mmol). Primers were chosen from a list compiled by Rytas Vilgalys at Duke University and were prepared for us there. Primer sequences not given below were in Vilgalys and Hester (1990). The rDNA region targeted for PCR amplification and sequencing encompasses the internal transcribed spacers ITS 1 and ITS 2, and the 5′ half of the 25S rDNA. Primers BMB-CR (5′-GTACACACCGCCCGTCG-3′, position 1624–1640 in *Saccharomyces cerevisiae* 17S rRNA) and LR1 were used to generate ITS templates for sequencing. The ITS 1 and ITS 2 regions, respectively, were sequenced with primers 5.8S and 5.8SR. Primer combinations 5.8SR/LR21 (5′-ACTTCAAGCGTTT-CCCTTT-3′, position 424–393 in *S. cerevisiae* 25S rRNA), and LROR (5′-ACCCGCTGAACTTAAGC-3′, position 26–42 in *S. cerevisiae* 25S rRNA)/LR7, were used to generate sequencing templates for the 5′ half of the 25S rDNA. The former products were sequenced with primers LR21 and LR15 (5′-TAAAT-TACAACTCGGAC-3′, position 154–138 in *S. cerevisiae* 25S rRNA), while the latter were sequenced with LR7, LR6, LR5 and LR3. Sequences were read by eye from autoradiographs produced by exposure of Kodak X-OMAT film to the dried gel.

Phylogenetic analysis of the sequence data.—Sequences were manually aligned and gaps were introduced into the sequences to increase their aligned similarity. Only cladistically informative sites as defined in the Phylogenetic Analysis Using Parsimony (PAUP) manual (Swofford, 1990) were used in the phylogenetic analysis. Parsimony analysis was performed from separate ITS 1, ITS 2 and 25S rDNA data sets and from combined data sets. First sets of analyses included only the positions unambiguously aligned for all taxa. In the second sets of analyses, these positions were added but were recoded as described in Bruns et al. (1991) except that nonalignable nucleotides were not scored as missing data, but instead were with a symbol that indicated which nucleotide could occupy the given position. For instance, we used the symbol D for {AG-T} if either A, C or a gap, but neither G nor T could be aligned in a given position for a given taxon. This method allowed us to extract a maximum number of phylogenetic characters from the data set without generating cladistically informative sites by arbitrary decisions during sequence alignment. The relevance of recoding characters in variable regions that do not align unambiguously throughout the sample relies on the fact that these regions often align unambiguously for subsets of taxa and, therefore, provide phylogenetic information relative to these subsets. Phylogenetic analysis of the data was by PAUP version 3.0i (Swofford, 1990) configured for the Macintosh. Gaps were set as "fifth base" and the five character states were considered unordered. All characters were weighted equally. We used the equate definition in PAUP to set symbols scoring uncertain nucleotides and recoded positions. When two or more taxa shared identical nucleotide sequences after removal of the phylogenetically uninformative sites, only one of them was chosen for analysis. We performed heuristic searches using TBR branch swapping on starting trees generated with 20 random addition sequences. Search settings were as follows: MULPARS option was in effect, steepest descent option was not in effect, MAXTREES was set to 100, zero length branches were set to collapse to yield polytomies, and multistate taxa were interpreted as uncertainty. The robustness of the internal branches of the tree was evaluated by 100 bootstrap replications using a heuristic search with simple addition sequences. The decay index (Mishler et al., 1991) was also used to estimate relative robustness of individual branches in the combined analysis by using a heuristic search with simple addition sequences and MAXTREES set to 10 000.

RESULTS

PCR amplification and sequencing.—Sequencing templates consisted of single PCR products as determined on agarose gels. Primer BMB-CR with LR1 produced PCR products of about 850 base pairs (bp). This indicated that the size of the ITS regions in our sample was approximately 400 bp. The sizes of sequencing templates produced by primer combinations 5.8SR/LR21 and LROR/LR7 were of about 750 bp and 1400 bp, respectively. In *Ganoderma, Amauroderma* and *Haploporus* species, there are sequences showing a high level of complementarity with primer 5.8SR that are located

the D2 region (FIG. 1). We believe that these sences were responsible for nonspecific attachment primer 5.8SR, which resulted in two PCR products hen this primer was used with those whose complementary positions are located beyond D2 (data not shown). This made us prepare two different templates sequencing the complete 5′ half of the 25S rDNA.

25S rDNA variability and phylogenetic analysis.—Complete nucleotide sequences from the 5′ end of the 25S DNA to the corresponding homologous position 1427 *S. cerevisiae* rRNA (Gutell and Fox, 1988) were termined for the five strains noted with asterisks in g. 1. Only 12 variable positions were found, of which ne were located in the divergent domain D2, two in 1, and none in D1. The remaining variable position as located at position 1293 in *S. cerevisiae* rRNA. Therefore, we sequenced only the D2 region for the her strains of this study. The alignment of the nucleotide sequences in D2 is reported in FIG. 1. It shows at *Fomitopsis rosea* was the most divergent taxon in ur sample, and that *Amauroderma rude* had several nique substitutions. In divergent domain D2, differ- *Ganoderma* species shared identical nucleotide sequences: *G. weberianum*, *G. microsporum* and *G. subamboinense* showed a unique pattern, as did *G. pfeifferi* and *G. boninense*. Also, no variation was found between *G. australe*, *G. adspersum* and *G. gibbosum*, three species classified in subgen. *Elfvingia*. In contrast, one nucleotide substitution that was unique to our sample occurred among strains of *G. lucidum*. This substitution was certainly not a PCR artifact for it was also found in other isolates of this species (data not shown). In comparison with the results above, an unexpected level of divergence was observed among strains named *G. tsugae*: cultivated strains from Japan and Taiwan had an identical sequence that differed in two positions from that of *G. tsugae* collected in China, and in three positions from that of *G. tsugae* from USA.

The semistrict consensus tree produced by parsimony analysis of the nine cladistically informative sites of the D2 region is depicted in FIG. 4. The only external branch supported by bootstrap replications placed *A. rude* as a sister taxon of *Ganoderma* species (70% confidence level). The branch that separated *G. australe*, *G. gibbosum* and *G. adspersum* from other *Ganoderma* species collapsed in the strict consensus tree and in the bootstrap analysis. However, this branch showed that three of the four species in our sample that were classified in subgen. *Elfvingia* formed a separate clade, which was basal to other *Ganoderma* species.

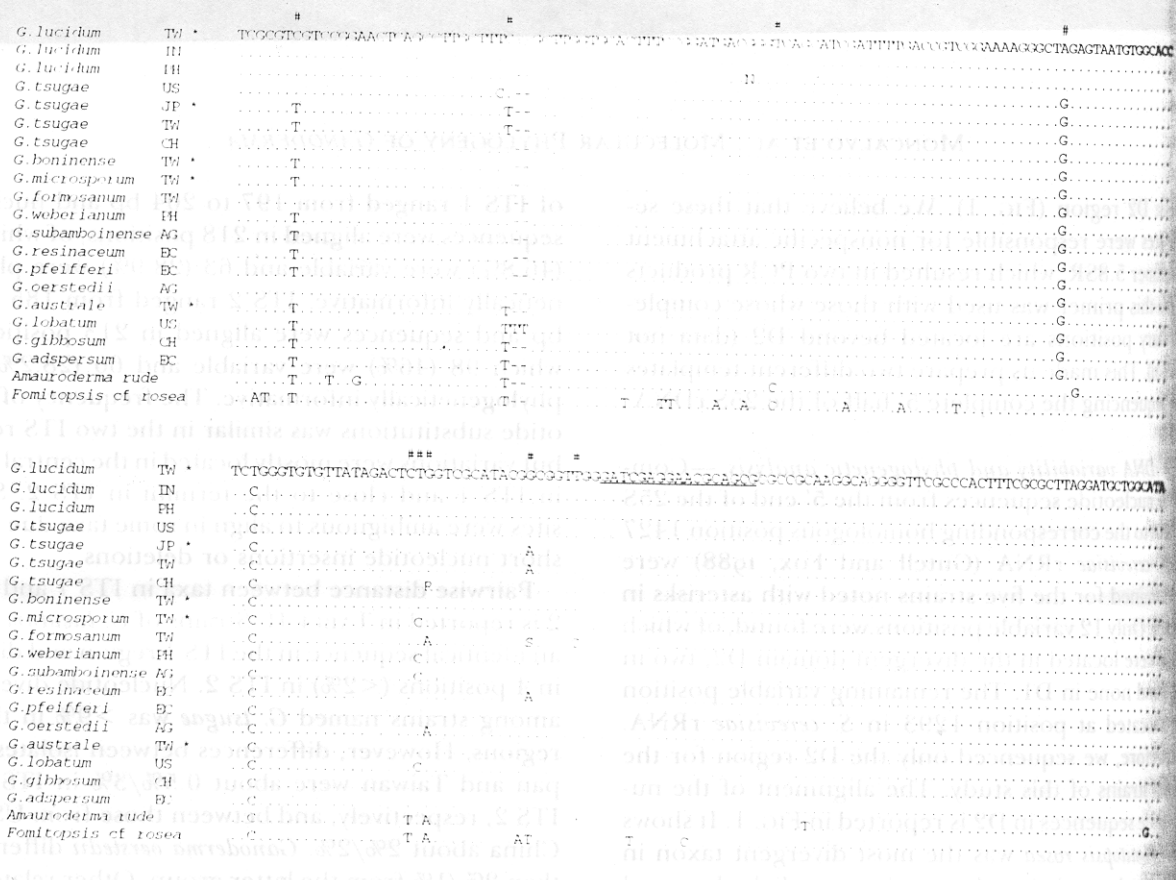
ITS variability.—Alignment of nucleotide sequences in the ITS regions is shown in FIGS. 2 and 3. The size of ITS 1 ranged from 197 to 204 bp and nucleotide sequences were aligned in 218 positions, of which 102 (46.8%) were variable and 63 (28.9%) were phylogenetically informative. ITS 2 ranged from 185 to 196 bp and sequences were aligned in 213 positions, of which 98 (46%) were variable and 60 (28.2%) were phylogenetically informative. The frequency of nucleotide substitutions was similar in the two ITS regions, but variations were mostly located in the central region in ITS 1 and close to the termini in ITS 2. Several sites were ambiguous to align in some taxa due to very short nucleotide insertions or deletions.

Pairwise distance between taxa in ITS 1 and in ITS 2 is reported in TABLE II. Strains of *G. lucidum* shared an identical sequence in the ITS 1 region and diverged in 4 positions (<2%) in ITS 2. Nucleotide divergence among strains named *G. tsugae* was >9% in the two regions. However, differences between isolates of Japan and Taiwan were about 0.5%/3% in ITS 1 and ITS 2, respectively, and between those from USA and China about 2%/2%. *Ganoderma oerstedii* differed less than 2%/1% from the latter group. Other related species were *G. weberianum* and *G. microsporum* (0%/4.5%), *G. pfeifferi* and *G. resinaceum* (1.5%/2%), and *G. australe* and *G. gibbosum* (2.5%/3%). Higher nucleotide divergence between recently diverged taxa was usually observed in ITS 2. However, in some cases interspecies variation in the ITS 1 region was significantly higher than in ITS 2, for instance, between *G. pfeifferi* and *G. boninense* (7%/1.5%), and between *G. lobatum* and *G. adspersum* (15%/4%).

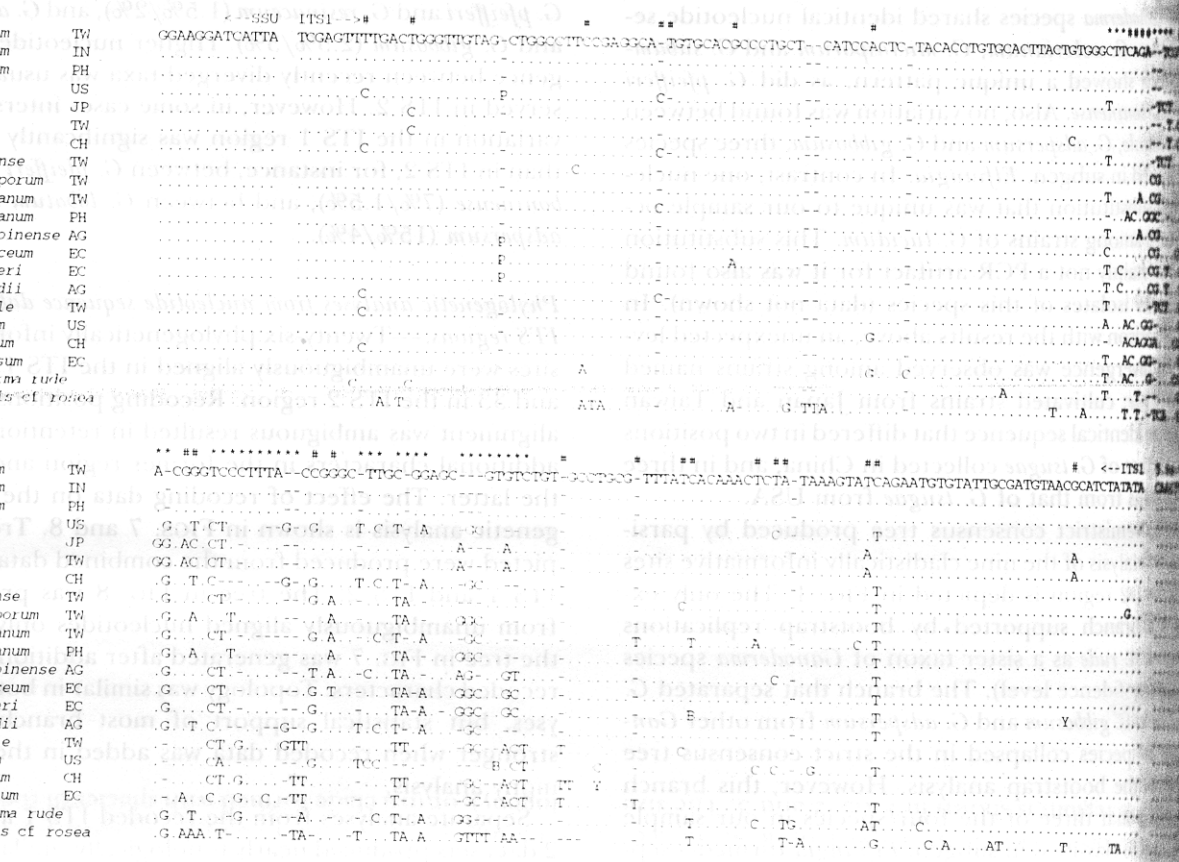
Phylogenetic analyses from nucleotide sequence data of the ITS regions.—Twenty-six phylogenetically informative sites were unambiguously aligned in the ITS 1 region and 33 in the ITS 2 region. Recoding positions where alignment was ambiguous resulted in retention of 37 additional characters in the former region and 27 in the latter. The effect of recoding data on the phylogenetic analysis is shown in FIGS. 7 and 8. Trees depicted were produced from the combined data sets of ITS 1 and ITS 2. The tree in FIG. 8 was produced from unambiguously aligned nucleotides only, while the tree in FIG. 7 was generated after addition of the recoded characters. Topology was similar in both analyses, but statistical support of most branches was stronger when recoded data was added in the parsimony analysis.

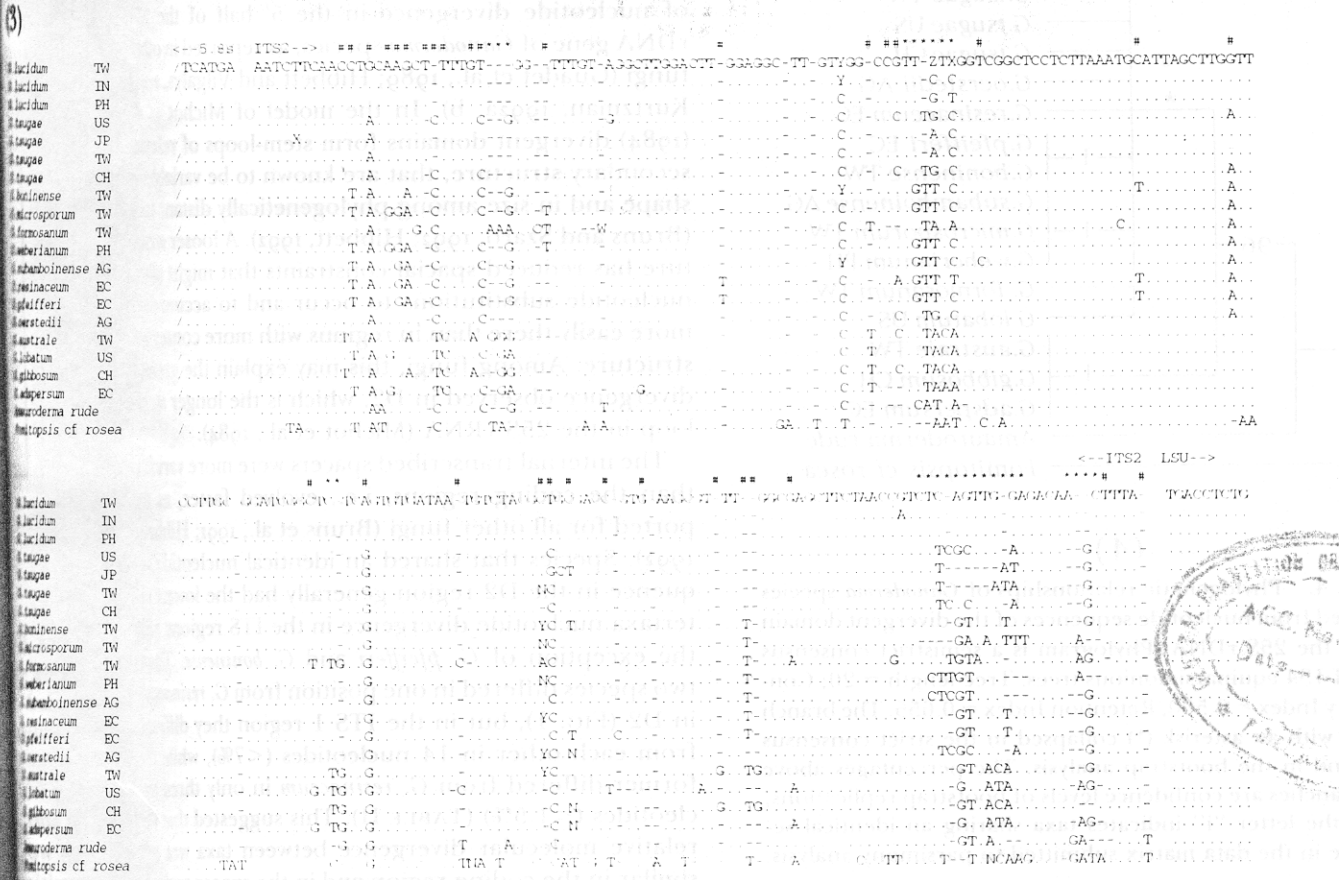
Separate analyses from the recoded ITS 1 and ITS 2 data sets produced nearly topologically similar trees, which are depicted in FIGS. 5 and 6. The main difference between the two analyses was the placement of *G. formosanum*, which either belonged to a clade including species of the *G. tsugae* complex (ITS 1 phylogeny), or to a clade formed with species of the *Elfvin-*

(1)


G.lucidum TW
G.lucidum IN
G.lucidum PH
G.tsugae US
G.tsugae JP
G.tsugae TW
G.tsugae CH
G.boninense TW
G.microsporum TW
G.formosanum TW
G.weberianum PH
G.subamboinense AG
G.resinaceum EC
G.pfeifferi EC
G.oerstedii AG
G.australe TW
G.lobatum US
G.gibbosum CH
G.adspersum EC
Amauroderma rude
Fomitopsis cf rosea

(2)


<-SSU ITS1-->
<--ITS1
G.lucidum TW
G.lucidum IN
G.lucidum PH
G.tsugae US
G.tsugae JP
G.tsugae TW
G.tsugae CH
G.boninense TW
G.microsporum TW
G.formosanum TW
G.weberianum PH
G.subamboinense AG
G.resinaceum EC
G.pfeifferi EC
G.oerstedii AG
G.australe TW
G.lobatum US
G.gibbosum CH
G.adspersum EC
Amauroderma rude
Fomitopsis cf rosea

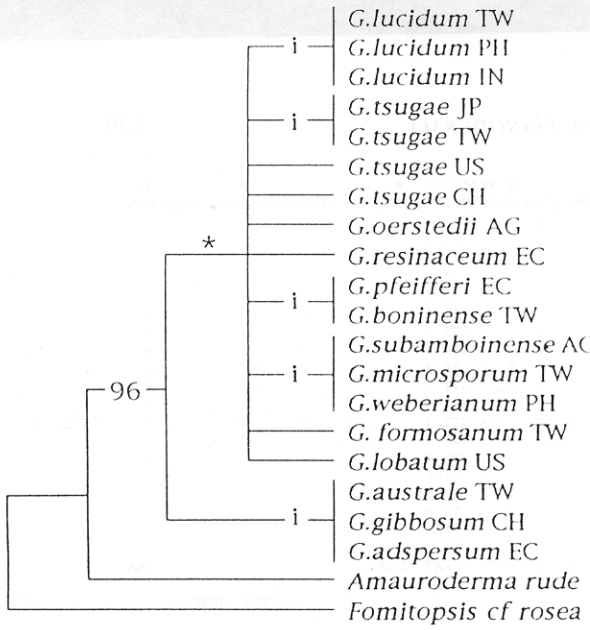


FIGS. 1–3. Aligned sequence data of the divergent domain D2 of the 25S rDNA (FIG. 1) and of the internal transcribed spacers regions ITS 1 (FIG. 2) and ITS 2 (FIG. 3) of taxa listed in TABLE I. Dots (·) in the sequences indicate conserved bases, and dashes (-) indicate gaps. Ambiguities within a strain are as follows: N = {A, T, G, C}, R = {A, G}, S = {A, T}, X = {T, C}, P = {G,-}. Phylogenetically informative sites as defined in Materials and Methods are indicated with pound signs (#) when unambiguously aligned in all taxa, with asterisks (*) otherwise. Asterisks following the taxon name in FIG. 1 indicate that complete nucleotide sequences from position 1 to the corresponding position 1427 in *S. cerevisiae* 25S rRNA have been determined for this taxon (data not shown). Sequence underlining in FIG. 1 shows a high level of similarity with primer 5.8SR (see text).

group (ITS 2 phylogeny). However, in both cases the statistical support of the branch was low (<75% confidence level). In contrast with phylogenetic analysis from the D2 data set (FIG. 4), the placement of *A. rude* as a sister taxon of *Ganoderma* species was strongly supported in trees produced from the ITS data. All trees produced from the ITS data sets indicated that the four species classified in *Elfvingia* were monophyletic. In addition, the tree produced from the ITS 2 data set indicated that *G. resinaceum, G. pfeifferi, G. lucidum, G. subamboinense, G. microsporum* and *G. weberianum* were monophyletic (87% confidence level), while the tree produced from the ITS 1 data set showed a poor resolution of internal branches.

Phylogenetic analysis of the combined data sets.—The result of the parsimony analysis of the combined ITS/5.8S data sets is depicted in FIG. 9. The tree topology produced from the combined data sets was very similar to the tree topolgy produced from the recoded ITS data alone (FIG. 7), but bootstrap values of most internal branches were higher. Decay analysis and bootstrapping gave similar results in evaluating robustness of internal branches: branches supported by 90–100% of bootstrap confidence are still present in trees five to nine steps longer with the exception of the branch grouping *G. resinaceum* and *G. pfeifferi,* which decayed in trees three steps longer, and branches supported by <90% of bootstrap replication collapsed when a tree only one step longer was examined.

When *F. rosea* was chosen as outgroup in the analysis, *A. rude* was supported with a 90% bootstrap value as a sister taxon of *Ganoderma* species (FIG. 9). A slightly better statistical support of most internal branches among *Ganoderma* taxa was obtained when *A. rude* was chosen as outgroup. Then, species classified in *Elfvingia* formed a monophyletic lineage with a confidence level of 100%, and laccate species that are easily rec-



(4)

FIG. 4. Phylogenetic relationships of *Ganoderma* species inferred from nucleotide sequences of the divergent domain D2 of the 25S rDNA. Phylogram is a semistrict consensus tree of 134 equiparsimonious trees. Tree length = 20; Consistancy Index = 0.500; Retention Index = 0.655. The branch noted with an asterisk (*) collapsed in the strict consensus tree and in the bootstrap analysis. The percentages above the branches are confidence levels of bootstrap replications, while the letter "i" indicates taxa sharing an identical sequence in the data matrix submitted to parsimony analysis.

ognizable from species of the *G. lucidum/G. tsugae* group formed a statistically well supported clade (95% bootstrap value).

Strains of *G. lucidum* from Taiwan, Philippines and India had a monophyletic origin (100% confidence level). Isolates named *G. tsugae* in Japan and in Taiwan grouped with *G. lucidum* isolates. Although statistical support of that branch was low (73%), this indicates that collections named *G. tsugae* in eastern Orient might not be monophyletic with *G. tsugae* from China and North America. The latter group formed an unresolved monophyletic clade including *G. oerstedii* (100% bootstrap value), a species of the *G. lucidum/G. tsugae* complex that was reported from subtropical and tropical America only (Steyaert, 1980).

In FIG. 9, *G. formosanum* was placed in an isolated position among *Ganoderma* species. This taxon clustered in different positions in the former analyses (FIGS. 4–8), and when *A. rude* was the outgroup in the combined analysis, it occupied a basal position to other *Ganoderma* species, but statistical support was very weak (19%) (data not shown).

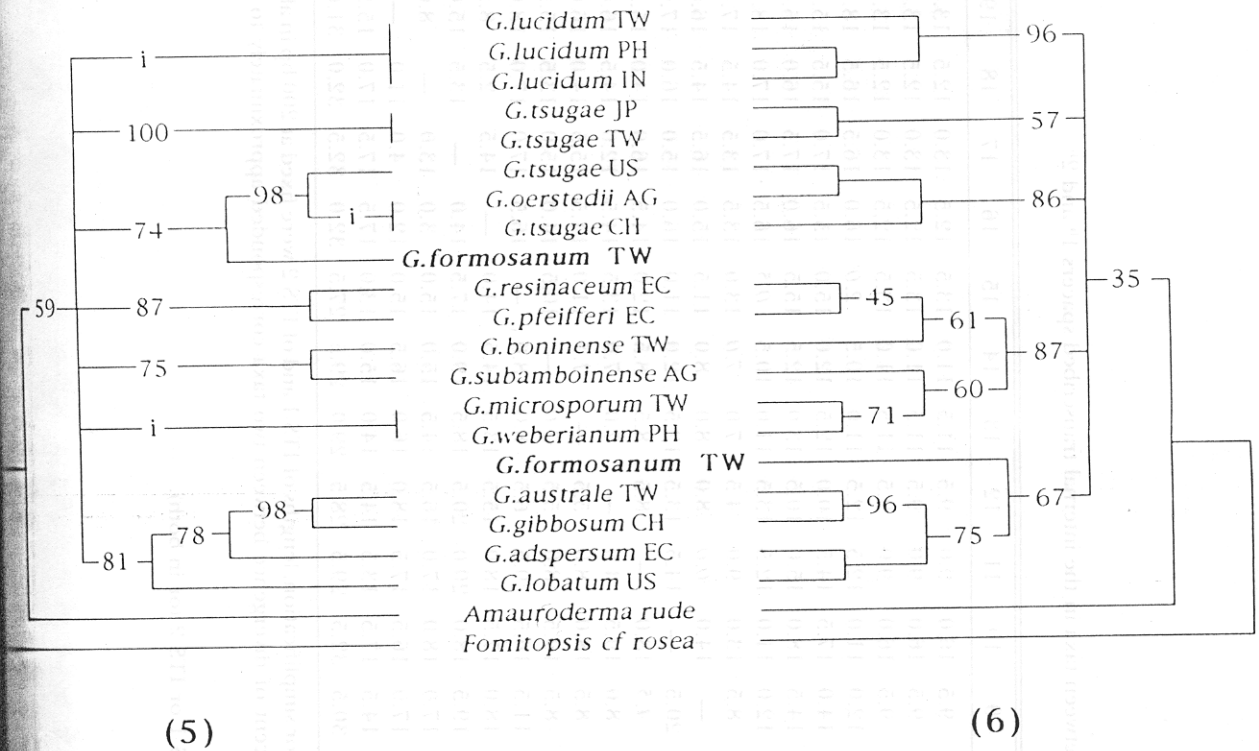
DISCUSSION

Rates and modes of evolution of the rDNA regions studied.—Divergent domain D2 showed the highest level of nucleotide divergence in the 5′ half of the 25S rDNA gene of *Ganoderma* species, as reported in other fungi (Guadet et al., 1989; Hibbett and Vilgalys, 1993; Kurtzman, 1992a, b). In the model of Michot et al. (1984) divergent domains form stem-loops of relaxed secondary structure, that are known to be variable in shape and in size among phylogenetically distant taxa (Bruns and Szaro, 1992; Hibbett, 1992). A looser structure has reduced spacial constraints that might allow nucleotide substitutions to occur and to accumulate more easily there than in regions with more conserved structure. Among fungi, this may explain the greater divergence observed in D2, which is the longer stem-loop in the 25S rRNA (Michot et al., 1984).

The internal transcribed spacers were more variable than the coding regions, i.e., evolved faster, as reported for all other fungi (Bruns et al., 1991; Hibbett, 1992). Species that shared an identical nucleotide sequence in the D2 region generally had the lowest intertaxa nucleotide divergence in the ITS regions with the exception of *G. pfeifferi* and *G. boninense*. These two species differed in one position from *G. resinaceum* in D2 (FIG. 1), but in the ITS 1 region they differed from each other in 14 nucleotides (<7%), while the former differed from *G. resinaceum* in only three nucleotides (<1.5%) (TABLE II). This suggested that the relative molecular divergence between taxa was not similar in the coding region and in the spacer regions.

TABLE II indicated that the more recently diverged taxa usually showed a higher level of nucleotide substitution in ITS 2, suggesting that this region evolved earlier than ITS 1 during speciation processes. But nucleotide divergence in the ITS 1 region was significantly higher than in ITS 2 between *G. pfeifferi* and *G. boninense* (7% vs. 1.5%), or between *G. lobatum* and *G. adspersum* (15% vs. 4%), for instance. TABLE II also indicated that *G. formosanum* and *G. lobatum* usually had higher nucleotide divergence relative to other taxa in the ITS 2 region, while *G. tsugae* US and CH had higher divergence in the ITS 1 region. Taken together, these results indicated that relative rates of nucleotide substitution in the two ITS regions varied differently between different taxa. This meant that the rate of evolution at a particular site was dependent on lineage and was not a property of the site. This was also observed in the small subunit rDNA among distant fungal species (Bruns et al., 1992).

The ratios of transition to transversion base substitutions in ITS 1, ITS 2 and D2 among *Ganoderma* species were 2.22 (20/9), 4.16 (25/6) and 9/0, respectively, and decreased to 1.65 (33/20), 2.00 (8/4),



(5) (6)

FIGS. 5, 6. Phylogenetic relationships of *Ganoderma* species inferred from nucleotide sequences of the internal transcribed spacers regions ITS 1 (FIG. 5) and ITS 2 (FIG. 6). Phylograms were generated from 100 bootstrap replications from data coded as described in Materials and Methods. The percentages above the branches are confidence levels of bootstrap replications, while the letter "i" indicates taxa sharing an identical sequence in the data matrix submitted to parsimony analysis.

and 14 (28/2), respectively, when *F. rosea* and *A.* were included in the calculation (TABLE III). A transition bias among recently diverged species which lineages with longer divergence times was reported by et al. (1992), Bruns and Szaro (1992), and Kauffman et al. (1993). Thus, the much higher bias observed in the D2 region than in the ITS regions could indicate that the former region diverged much later (or much slower), as it already appeared from direct comparison of nucleotide alignment (FIGS. 1–4). The calculated bias in the ITS 2 region among *Ganoderma* species was almost twice that in ITS 1, suggesting that the latter diverged earlier. This was not evident in the overall comparison of base substitutions between taxa (TABLE II), and was in conflict with the observation that recently diverged taxa usually showed higher variation in ITS 2. This suggested that divergence rate may not corroborate divergence time. Finally, Bruns and Szaro (1992) reported a strong bias towards C-T transitions in the nuclear rDNA genes of fungi. In *Ganoderma*, C-T transitions also dominated the D2 region but were approximately equal to A-G transitions in ITS (TABLE III).

Independent vs combined analyses.—Comparison between individual gene phylogenies showed that the poorly supported branch in the D2 phylogram was indicative of phylogenetic relationships, and that phylogenetic resolution from the ITS 1 data set is intermediate between those observed from the D2 data set and the ITS 2 data set (FIGS. 4–6). Topologies of individual gene phylogenies were in agreement except in the placement of *G. formosanum* (FIGS. 4–6).

Bull et al. (1993) and de Queiroz (1993) stated that it is inappropriate to combine data sets in a single analysis if the trees that result from separate analyses of these data sets are positively incongruent with each other, an opinion that was not shared by Chippindale and Wiens (1994), however. Conflicts between individual phylogenies might suggest that individual data sets have different histories, therefore, that branches in positive conflict may be correct for each data set. A consequence of the latter proposition would be that gene phylogenies, for instance, might not reflect organismal phylogenies.

The ITS 1 phylogeny (FIG. 5) and the ITS 2 phylogeny (FIG. 6) are in conflict in the placement of *G. formosanum*. Conflicting branches had moderate bootstrap support (67% and 74%). These branches collapsed when trees one step longer were examined (data not shown). In the combined ITS analyses (FIGS. 7, 8), *G. formosanum* clustered with species of *Elfvingia*

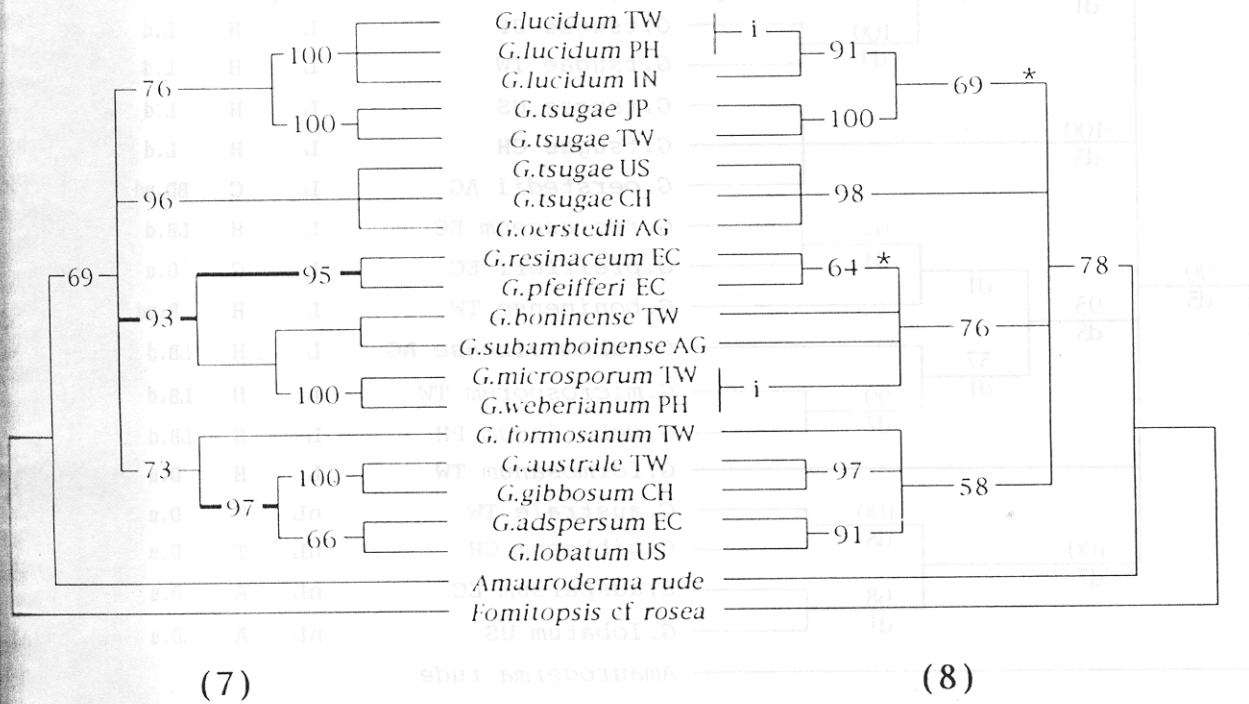
TABLE II. Percentage[a] of nucleotide divergence between taxa in the internal transcribed spacers 1[b] and 2[b]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. *G. lucidum* TW	—	*0.0*	*0.0*	13.5	8.5	9.0	14.0	8.5	9.5	16.0	9.0	9.5	11.5	11.0	13.5	12.5	13.0	12.5	13.5	14.0	27.0
2. *G. lucidum* IN	*1.0*[c]	—	*0.0*	13.5	8.5	9.0	14.0	8.5	9.5	16.0	9.0	9.5	11.5	11.0	13.5	12.5	13.0	12.5	13.5	14.0	27.0
3. *G. lucidum* PH	*0.0*	*2.0*	—	13.5	8.5	9.0	14.0	8.5	9.5	16.0	9.0	9.5	11.5	11.0	13.5	12.5	13.0	12.5	13.5	14.0	27.0
4. *G. tsugae* US	9.0	10.5	9.5	—	15.0	15.5	*2.0*	13.0	12.0	11.0	12.5	12.5	11.0	10.5	*2.0*	16.0	16.5	16.5	18.5	16.5	28.5
5. *G. tsugae* JP	8.0	9.0	8.5	10.0	—	*0.5*	15.0	11.0	14.0	17.5	14.5	10.0	12.5	12.0	15.0	15.5	17.0	15.5	15.5	14.5	28.5
6. *G. tsugae* TW	7.0	8.0	7.5	8.0	*3.0*	—	15.5	11.5	14.5	18.0	15.0	10.5	13.0	12.5	15.5	16.0	17.5	16.0	16.0	15.0	29.0
7. *G. tsugae* CH	8.0	8.5	8.5	*2.0*	9.5	7.5	—	13.0	12.0	11.0	12.5	13.5	11.0	10.5	*0.5*	16.5	17.0	17.0	18.5	15.5	28.5
8. *G. boninense* TW	10.5	11.5	11.0	7.0	11.0	10.0	8.0	—	8.5	13.0	9.0	4.5	7.0	*7.0*	13.0	13.5	13.5	14.5	17.0	16.0	28.5
9. *G. microsporum* TW	14.0	15.0	14.5	11.5	15.5	14.0	12.5	8.0	—	14.0	*0.0*	8.0	8.0	8.0	11.5	15.0	16.5	14.5	16.0	12.5	26.5
10. *G. formosanum* TW	18.5	19.0	18.5	17.5	20.5	18.5	17.5	17.5	20.5	—	14.5	13.5	12.5	12.0	11.0	16.0	15.0	16.0	17.0	18.0	32.5
11. *G. weberianum* PH	13.0	14.0	13.5	9.5	15.5	14.0	10.5	7.5	*4.5*	18.0	—	8.5	8.5	8.5	12.0	14.5	16.0	14.0	15.5	13.0	26.0
12. *G. subamboinense* AG	12.5	13.5	13.0	6.5	13.0	11.5	7.5	5.0	8.0	19.5	4.0	—	7.0	7.0	13.5	14.5	12.0	14.5	16.0	15.5	28.5
13. *G. resinaceum* EC	11.5	13.0	11.5	8.0	13.5	11.5	9.0	2.5	8.5	18.0	8.0	5.5	—	*1.5*	11.0	14.5	15.5	14.0	16.0	15.5	27.0
14. *G. pfeifferi* EC	12.0	13.0	12.5	8.5	12.5	11.5	9.5	*1.5*	8.5	19.5	8.0	5.5	*2.0*	—	10.5	14.0	15.0	13.5	15.5	15.0	27.0
15. *G. oerstedii* AG	8.0	9.5	8.5	*1.0*	8.5	7.0	*1.0*	6.5	11.5	16.5	9.5	6.5	8.0	8.0	—	16.5	17.0	17.0	18.0	15.0	28.5
16. *G. australe* TW	15.0	16.0	15.0	14.0	14.5	13.5	15.0	13.0	18.0	17.5	18.0	15.5	13.5	14.5	14.0	—	14.5	2.5	8.5	18.5	30.0
17. *G. lobatum* US	18.5	18.5	18.5	18.0	18.0	17.5	17.5	17.5	19.5	18.0	20.0	20.5	18.5	19.0	17.5	14.0	—	13.5	15.0	20.5	33.0
18. *G. gibbosum* CH	14.0	14.0	14.0	15.0	15.0	14.5	15.0	13.5	17.0	18.0	17.0	16.5	14.5	15.0	15.0	3.0	13.0	—	8.0	18.0	29.5
19. *G. adspersum* EC	16.0	16.0	16.0	15.5	15.5	15.0	15.0	15.0	17.0	16.5	17.5	18.0	16.0	16.5	15.0	12.0	4.0	11.0	—	18.5	30.5
20. *Amauroderma* sp.	14.5	15.5	14.5	13.0	17.0	15.5	14.0	13.5	14.5	17.5	13.5	14.5	14.0	15.0	13.0	17.5	17.5	17.0	15.5	—	28.5
21. *Fomitopsis rosea*	29.5	31.0	29.5	29.0	27.5	27.5	28.5	28.0	30.5	32.5	29.5	28.5	29.0	29.5	27.5	32.0	32.5	32.0	31.0	29.0	—

[a] Calculated from the pairwise distances between taxa as determined in PAUP. For simplification, lengths of ITS 1 and of ITS 2 were fixed at 200 bp in all combinations. Calculated values were therefore slightly above true values (see text). One percent of divergence between two taxa corresponded approximately to two nucleotide substitutions in the ITS region.

[b] ITS 1 and ITS 2 values are given above and below the diagonal, respectively.

[c] Italicized numbers indicate that nucleotide divergence was <2% in either ITS 1 or ITS 2 (or in both).



FIGS. 7, 8. Phylograms produced from the combined data sets of the internal transcribed spacers ITS 1 and ITS 2 with (FIG. 7) or without (FIG. 8) inclusion of recoded nucleotide positions that were ambiguously aligned. Ambiguously aligned positions were recoded as described in Materials and Methods. The tree in FIG. 7 represents the strict consensus tree of six equiparsimonious trees. Tree length = 149 steps; Consistency Index = 0.614; Retention Index = 0.703. The tree in FIG. 8 is a semistrict consensus of 94 equiparsimonious trees. Tree length = 113; Consistency Index = 0.582; Retention Index = 0.690. Branches noted with an asterisk (*) collapsed in the strict consensus tree. The percentages above the branches are confidence levels from 100 bootstrap replications, while the letter "i" indicates taxa sharing identical sequence in the data matrix submitted to parsimony analysis. Bold lines in FIG. 7 indicate branches which were statistically significantly better supported when recoded data were included in the analysis.

TABLE III. Frequency of transition and transversion base substitutions in ITS 1[a], ITS 2[a] and D2[b] determined from unambiguously aligned positions only[c]

	Frequency of base substitutions					
	ITS 1		ITS 2		D2	
Type of substitution	*Ganoderma* species only	All taxa included	*Ganoderma* species only	All taxa included	*Ganoderma* species only	All taxa included
Transition						
C<>T	11	18	13	19	6	16
A<>G	9	15	12	17	3	12
Transversion						
A<>C	4	5	1	3	0	1
G<>T	2	4	0	2	0	1
A<>T	1	7	0	6	0	0
G<>C	2	4	5	7	0	0
TS/TV[d]	2.22	1.65	4.16	2.00	9/0	14

[a] Internal transcribed spacers 1 and 2.
[b] Divergent domain D2 of the 25S rDNA.
[c] Including cladistically uninformative characters.
[d] Transition to transversion ratio.

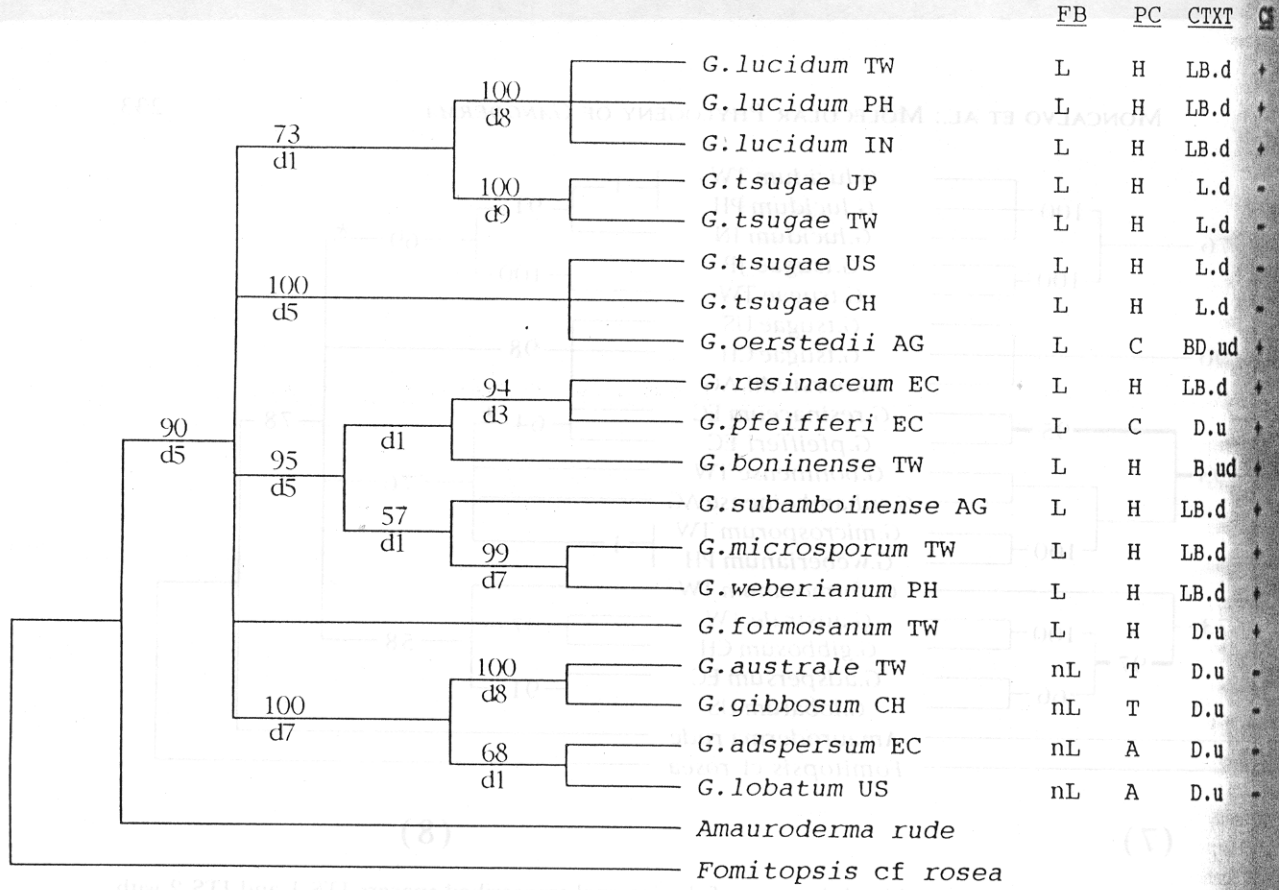


FIG. 9. Phylogenetic relationships of *Ganoderma* species inferred from nucleotide sequences of the combined data set from divergent domain D2 of the 25S rDNA and the internal transcribed spacers ITS 1 and ITS 2. The phylogram represents the strict consensus tree of 54 equiparsimonious trees. Tree length = 303 steps; Consistency Index = 0.588; Retention Index = 0.707. Confidence values from 100 bootstrap replications that are higher than 50% are given above branches. Decay indexes are given below branches. Statistical support for the more external branch was determined from an analysis including all taxa, while statistical support for internal branches was determined after excluding *Fomitopsis* cf. *rosea* from the data matrix and designing *A. rude* as outgroup taxon in the analysis (see text). Morphological characters shown in the figure are abbreviated as follows: basidiocarp (FB) laccate (L) or not (nL); pilear crust (PC) a hymenioderm (H), a characoderm (C), a trichoderm (T) or an anamixoderm (A); context color (CTXT) light (L) to light brown (LB), or brown (B) to dark brown (D), duplex (d) or uniformly colored (u); chlamydospores (CS) present (+) or absent (−) in culture.

as in the ITS 2 phylogeny (FIG. 6), whereas it stood in an isolated position in the combined analysis of the ITS/D2 data sets (FIG. 9). Placement of this strain was sensitive to the choice of the outgroup, and in some analyses *G. formosanum* was placed basal to *Amauroderma,* or between *Amauroderma* and *Ganoderma,* but statistical support was then very low (data not shown).

Examination of individual equiparsimonious phylograms used to produce the conflicting consensus trees depicted in FIGS. 5 and 6 showed that the placement of *G. formosanum,* either with the *G. oerstedii* clade (ITS 1 phylogeny) or with the *Elfvingia* clade (ITS 2 phylogeny), was in all trees from a long, isolated branch (data not shown). Unequal branch lengths may result from sampling unequally distant related taxa (Swofford and Olsen, 1990), and that violates fundamental assumption of parsimony analysis that stipulates similar rates of change along branches of the tree (Felsenstein, 1978). We therefore consider that our data set is inappropriate to resolve the correct placement of *G. formosanum,* and that there is no incongruence in combining the different data sets.

Branches had higher statistical support in analyses from the recoded data matrix, indicating that phylogenetic information was best extracted after recoding characters (FIGS. 7, 8). Resolution and robustness of internal branches was better from analysis of the combined data set (FIG. 9) than from analyses of individual data sets (FIGS. 4–6). However, robustness of the more external branch (*A. rude*) was slightly higher in the

nalysis from the D2 data set alone (96%) than from e combined data sets (90%), and dramatically deased in the ITS 1 phylogeny (59%) and ITS 2 phyeny (35%). This suggested that several sites in the S regions are rapidly evolving and have been satued with multiple substitutions. These sites are therere no longer phylogenetically informative for the re diverged taxa (Mishler et al., 1988). These obrvations suggest that combining rapidly evolving aracters with slowly evolving characters may not be propriate in resolving the more external branches a tree; however, these characters are essential in olving terminal branches. Chippendale and Wiens 94) propose differential character weighting to re- different rates of change or transformational obability between characters within a data set. In is work, we did not attempt to weight characters in simony analysis, because we were unable to proe a rational method to assign weights, and we are aware of the existence of such a method.

Overall, the combined tree depicted in FIG. 9 that aced *G. formosanum* in an isolated position represents r best phylogenetic hypothesis on the basis of riomal DNA sequences.

Molecular identification of Ganoderma *species.*—Nucleide variation in the ITS regions is useful in distinishing between *Ganoderma* species, but variation in e 25S rDNA gene is too low. TABLE II shows that traspecific variation in the combined ITS regions was 2%, while interspecific nucleotide divergence was ually >6%, with a few exceptions as discussed below. pically, 0 to 2% intraspecific variation in ITS has en reported among fungal species (Anderson and anovski, 1992; Lee and Taylor, 1992; Carbone and ohn, 1993), with one known exception in *Fusarium* O'Donnell, 1992). It is clear, however, that sequence riation alone cannot distinguish between species unvariation in a given region for a given species is tablished.

Nucleotide divergence between *G. resinaceum* and *pfeifferi* was <2% in both ITS (TABLE II). However, veral morphological characters clearly distinguish tween the two species, which were even classified in fferent sections by Steyaert (TABLE I). *Ganoderma crosporum* and *G. weberianum* differed by about 2% one combined the data of the two ITS regions (TAE II). *Ganoderma microsporum* (Hseu et al., 1989) is mainly distinguished from *G. weberianum* (Steyaert, 72) by having smaller basidiospores. Di-mon mating ts were conducted and showed that these two taxa re intersterile (Hseu, unpubl.). *Ganoderma gibbosum* H and *G. australe* TW are two species of the *G. apanatum* complex, a taxonomically difficult group in ich names given to isolates remain tentative. The cleotide divergence of about 2.5% in the ITS regions suggested that the two strains used in this work might belong to different species. ITS sequence data might be helpful in resolving the taxonomy of the complex; however, as for species of the *G. lucidum* complex, comprehensive studies will be necessary prior to any taxonomic conclusion.

The G. lucidum *species complex.*—A high level of taxonomic confusion has always been associated with *G. lucidum* and allied species, such as *G. tsugae, G. valesiacum, G. resinaceum* (Adaskaveg and Gilbertson, 1986) and *G. oerstedii* (Bazzalo and Wright, 1982). Therefore, isolates of this group might have been misnamed.

Our results suggested that concepts of *G. lucidum* in India, in Taiwan and in the Philippines were similar as evidenced by the low level (<1%) of nucleotide divergence observed in the combined ITS regions between isolates from these localities (TABLE II). In addition, Hseu (1990) demontrated that the Indian isolate and the Taiwanese isolate are intercompatible in a di-mon mating experiment. However, it is still not clear whether or not this species is conspecific with *G. lucidum* in North America or in Europe, where the species was described. For instance, Adaskaveg and Gilbertson (1986) reported that North American *G. lucidum* and the European isolate of *G. resinaceum* used in this study belong to the same biological species, but phylogenetic relationships (FIG. 9) and nucleotide variation (TABLE II) clearly separated Asiatic collections labelled *G. lucidum* from *G. resinaceum*.

The commercial isolates from Taiwan and Japan used in this study were identified as *G. tsugae* by one of us (RSH) on the basis that basidiocarps were undistinguishable from those labelled *G. tsugae* from the USA, from where the species was first described. In addition, cultural features of these isolates were similar to those reported for American *G. tsugae* by Adaskaveg and Gilbertson (1986), and they were intersterile with Asiatic collections of *G. lucidum* (Hseu, 1990). However, our results strongly suggested that Japanese and Taiwanese strains are not conspecific with North American *G. tsugae* (>9% nucleotide divergence in the ITS regions), but that the isolate from China might be (<2% difference). Phylogenetic relationships even suggest that collections named *G. tsugae* in our sample might not be monophyletic (FIG. 9).

Surprisingly, neither nucleotide sequence variation in the ITS regions nor phylogenetic analysis distinguished Argentinean *G. oerstedii* from *G. tsugae* US and CH (TABLE II and FIG. 9). However, Bazzalo and Wright (1982) reported two morphological characters that distinguished this isolate from *G. tsugae*: context color brown, with a thin darker band over the tubes, or uniformly dark brown, and a dermis of the "characodermis" type. Bazzalo and Wright (1982) did not

see the small and rare chlamydospores formed in culture by the mycelia of this strain, which were observed by Wang and Hua (1991) and by us. Chlamydospores were not reported from *G. tsugae* isolates (Stalpers, 1978; Adaskaveg and Gilbertson, 1986; Hseu, 1990). Therefore, there are several characters that separate *G. tsugae* and *G. oerstedii*, but there was no molecular evidence in the rDNA region studied to distinguish between the two species.

In conclusion, the taxonomy of the *G. lucidum* complex appears challenging. From the perspective of a phylogenetically based classification of these species, studies within a biogeographic framework should be undertaken. Integration of morphological, physiological, mating and molecular data will be necessary to understand speciation processes in this group. To solve nomenclatural problems, we would suggest the study of the DNA of type specimens. Finally, it might be necessary to determine in *G. lucidum s. lato* a genomic region with greater nucleotide divergence than in the ITS regions to resolve the systematics of the complex using molecular characters.

Molecular phylogeny of Ganoderma.—Phylogenetic analysis of nucleotide sequence data from the D2 region showed that *A. rude* was statistically strongly supported (96% bootstrap value) as a sister taxon of *Ganoderma* species (FIG. 4), while statistical support significantly decreased in the analyses from ITS data (FIGS. 5–7). This suggested that variation in the divergent domain D2 of the large ribosomal subunit might be appropriate to intergeneric segregation in *Ganoderma s. lato* (Ganodermataceae) on a phylogenetic basis. Thus, questions such as whether or not *Amauroderma, Haddowia* and *Humphreya* are natural, and what is the preferred position of *G. tsunodae* (the only species included in *Trachyderma*) and of *G. colossum* [a peculiar species for which Murrill (1905b) created the genus *Tomophagus*] in *Ganoderma s. lato*, might be investigated on a molecular basis from nucleotide sequence data of the D2 region.

Phylogenetic analysis of the combined ITS/D2 data sets indicated that subgenus *Elfvingia* was monophyletic with a confidence level of 100%. As reported in FIG. 9, synapomorphic characters along this branch are the nonlaccate basidiocarp (which is usually sessile), and the cutis not forming a palisade layer, i.e., neither of hymenioderm nor characoderm type. The use of the cutis type as a taxonomic character to segregate *Elfvingia* into three natural subgenera, as proposed by Steyaert (1980), will need further scrutiny.

The laccate *Ganoderma* species having a cutis formed of a palisade layer (hymenioderm or characoderm type) segregated into four branches in the combined ITS/D2 analysis, but relationships between these branches were not resolved (FIG. 9). Two branches included species of the *G. lucidum* complex, and results have been discussed above. *Ganoderma formosanum* stood alone; it was a morphologically distinctive species in our sample for it combined a black and shiny pileus with a uniformly dark brown context. A statistically well supported branch (95%) included the remaining species. Our results do not support Steyaert's (1980) nor Zhao's (1989) infrageneric segregation of *Ganoderma*, for neither subgen. *Characoderma* Stey. nor sect. *Phaeonema* Zhao et al. were monophyletic. This indicated that the transition between hymeniodermis and characodermis and the transition between uniformly dark colored context and a duplex context, both occurred several times during the evolution of *Ganoderma* species.

Overall, our results are in agreement with the opinions of various authors, which were based on morphological observation alone. For instance, the phylogram in FIG. 9 supports the distinction between *Amauroderma* and *Ganoderma*, and monophyly of *Elfvingia* within *Ganoderma*, as predicted by Donk (1964). Also, results agree with Furtado (1965), Corner (1983) and Zhao (1989), since they do not support a division of *Ganoderma* based on pilocystidia shape (sect. *Characoderma*). Finally, it is not very surprising that *Phaeonema*, based on context color solely, is not natural. Therefore, we assume that the gene phylogeny depicted in FIG. 9 reflects natural relationships between the isolates of this study.

We believe that most of the controversy which has been associated with *Ganoderma* systematics in the past might be resolved with the use of molecular techniques to generate novel taxonomic characters and with the use of phylogenetically based classification methods. Eventually, a better understanding of modes and rates of nucleotide substitutions would enable development of a method for appropriate character weighting to improve molecular resolution (Chippendale and Wiens, 1994), and integration of morphological data with molecular data (Hibbett and Vilgalys, 1993; Mishler, 1994; Moncalvo, 1995) would be necessary for complete resolution of the phylogenetic relationships of *Ganoderma* species.

The low nucleotide variation observed in the 25S rDNA (FIG. 1), together with a very high transition bias in this region (TABLE III), suggests that *Ganoderma* species have recently diverged. By comparison, variation in D2 was much higher in the *Lyophyllum decastes* complex (Tricholomataceae) (Moncalvo et al., 1995) than in the whole genus *Ganoderma*. Thus, molecular data supports the opinion of Ryvarden (1991) against that of Corner (1983). The former author suggested that *Ganoderma* may be an advanced group of polypores because of the complex microstructure and the

mogeneity of the genus together with strong variions in some groups, while the latter argued that *noderma* basidiocarps have primitive features from ich other polypores derived. An ongoing phylogeic study of polypores inferred from mitochondrial osomal DNA sequences also support the hypothesis at *Ganoderma* is a young genus (Hibbett, pers. mm.).

AKNOWLEDGMENTS

e thank Rytas Vilgalys at Duke University, North Carolina o prepared the primers for us, Peter K. Buchanan, Landcare Research, Auckland, New Zealand for proof-reading e manuscript, and David S. Hibbett, Harvard University erbaria, Massachusetts and an anonymous reviewer for ghtful comments in the revision of the manuscript. We e grateful to D. S. Hibbett for sharing unpublished data. is work was supported by a postdoctoral grant from the ion National Science Foundation to the first author (JMM), d in part by a grant from the National Science Council Taiwan (NCS82-0409-B-002-425) to the last author (RSH).

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