Jour. Microbiol. June 1996 p. 117-123

Phylogenetic Relationship of *Ganoderma* Species with the Polyporaceae Based on RFLP Analysis of the Nuclear ITS Region

Hong-Je Park, Kee-Sun Shin, Cheol-Sik Yoon, Dong-Hun Lee, and Kyung Sook Bae*

Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, KIST, 52 Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea

(Received January 30, 1996/Accepted April 22, 1996)

Restriction-polymorphic patterns of nuclear-ITS were examined for the genetic relationships among 12 basidiomycetous mushrooms belonging to Aphyllophorales and Agaricales. The taxonomic affinity of *Ganoderma* species with the family Polyporaceae also was examined. With 13 restriction endonucleases, 159 restriction characters were generated from the 12 species examined. UPGMA and neighbor-joining analyses separated the 12 species into two genetically distinct groups that correspond to orders (Agaricales and Aphyllophorales) where each species is included. This result indicates that there is clear genetic demarcation between Agaricales and Aphyllophorales. Dendrograms constructed by several data analyses showed that even though *Ganoderma* species are somewhat in intermediate taxonomic position between the Polyporaceae and families of the Agaricales, they are genetically more related to the Polyporaceae. These results are consistent with morphological characters observed in those mushrooms. However, it is premature to conclude taxonomic status of *Ganoderma* species in the present study employing small sample size.

Key words: Taxonomy, Aphyllophorales, Agaricales, Ganodermataceae, Polyporaceae, ITS-RFLPs

Mushrooms have attracted great interest because of their economic and industrial potential. However, application of these mushrooms to economic or industrial utility is limited due to lack of efficient systems for identification and taxonomy of mushrooms. Taxonomic studies of mushrooms have been mainly based on morphological features of fruitbodies (1). However, it is difficult to obtain fruitbodies at the time required because production of fruitbodies depends on the season and is limited to certain species under artificial condition. As a result, the state of mushroom taxonomy has remained unsatisfactory (19). Taxonomic confusion can also be found in the genus *Ganoderma*. The genus *Ganoderma* has been known as a group of fungi whose pileus and stem are covered by a crust with the consistency and appearance of lacquer (1). Even though previous studies (13, 16, 20) have described many *Ganoderma* species, there have been conflicting species concepts among taxonomists due to the highly varying features encountered and the different criteria used (1). Karsten (7) had placed the genus *Ganoderma* in the Polyporaceae. However, Donk (3) erected a new family Ganodermataceae for *Ganoderma* species because they have different morphological features from those of typical Polyporaceae members. However, Donk's view of these species has not been supported by all taxonomists because each taxonomist has different concepts and criteria to distinguish the genus *Ganoderma*. In order to resolve such disagreements, molecular investigative approach should be used.

Advances in molecular biology have provided new tools for examining genetic variations in fungi (2, 8, 10, 22). DNA restriction fragment length polymorphisms (RFLPs) represent heritable differences in the lengths of DNA fragments that are generated by digestion with a restriction endonuclease (6, 11, 15, 17, 21, 23). In particular, RFLPs of nuclear internal transcribed spacer (ITS) have been successfully used for the taxonomy of mushrooms (2, 5, 12) in conjunction with polymerase chain reaction (PCR).

The present study attempts to resolve disagreements regarding taxonomic affinity of the *Ganoderma* species with the Polyporaceae by characterizing restriction pat-

* To whom correspondence should be addressed

Table 1. The strains used in this study and size of PCR products

Order	Family	Strains	Size of PCR products	KCTC No.
Aphyllophorales		*Fomes fomentarius*	678	6363
		Lenzites betulina	660	6354
	Polyporaceae	*Fomitella fraxinea*	"	6355
		Gloeophyllum abietinum	"	6355
		Coriolus versicolor	"	6356
	Ganodermataceae	*Ganoderma lucidum*	"	6365
		Ganoderma adspersum	632	6366
Agaricales		*Pleurotus ostreatus*	728	6359
	Pleurotaceae	*Hohenbuehelia* sp.	"	6362
	Agaricaceae	*Agrocybe semiorbicularis*	"	6364
	Bolbitiaceae	*Macrolepiota procera*	771	6361
	Tricholomataceae	*Flammulina velutipes*	833	6367

terns of nuclear ITS1-5.8S-ITS2. Thus, we examined species in the Polyporaceae as well as Ganoderma species. This study also examined the genetic relationships among 12 mushrooms belonging to Aphyllophorales and Agaricales.

Materials and Methods

Organisms

Cultures of 12 basidiomycetous mushrooms were obtained from Korean Collection for Type Cultures (KCTC) (Table 1). Agar plugs carrying mycelium were transferred to flasks containing 200 ml of 2.5% PDB, and cultured for approximately one week at 28°C. Mycelia were harvested by vacuum filtration after through rinsing in distilled water. Mycelia were freeze-dried overnight for DNA isolation.

Isolation of genomic DNA

Isolation of genomic DNA was performed by modification of previously described methods (4, 24). After lyophilized mycelium was broken into small fragments with a spatular in 1.5 ml tube, 100 μl of lysis buffer (50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol) was added (9), and mixed thoroughly. The samples mixed with lysis buffer were placed in a microwave oven (Little Litton, 400 W) with the lids open and heated on full power for a total of 30~40 sec, step wise, 15~20 sec, 10~15 sec, and 5~10 sec, respectively (4). In order to prevent boiling over, samples were covered with 1 mm beads. Immediately after heating, 400~500 μl of lysis buffer was added. These samples were then incubated at 80°C for 10 min, followed by adding 500 μl of mixture of chloroform and phenol (1 : 1). They were vortexed thoroughly and then centrifuged at 12,000×g for 15 min. The upper phase was transferred into a new tube, followed by centrifugation at 12,000×g for 10 min with one volume of Isopropanol and 10 μl of 3 M sodium acetate. The supernatant was discarded, and the pellet was rinsed with 70% ethanol. DNA pellet was resuspended in 100 μl of RNase solution (100 μg of RNase A/ml) and incubated at 37°C for 2 h to digest RNA. DNA concentration was estimated prior to PCR by comparing the intensity of λDNA bands in 0.8% agarose gel with a series of DNA dilutions, and by viewing them over a UV transilluminator (310 nm) after staining with ethidium bromide.

Table 2. Twenty restriction endonucleases used for digestions

	Restriction endonucleases	Digestion
Four cut enzyme	*Hha*I, *Mbo*I, *Rsa*I, *Taq*Im *Hsp*92II,	digested
	*Acc*II, *Alu*I, *Msp*I, *Hpa*II, *Hae*II	undigested
Five cut enzyme	*Ava*II	digested
	*Hin*fI	undigested
Six cut enzyme	*Acc*I, *Cla*I, *Hind*III, *Sal*I	digested
	*Apa*I, *Eco*RI, *Hinc*II, *Pst*I	undigested

PCR amplification and enzyme digestion

Amplification reaction mixtures were 100 μl in volume, and contained 10 μl of 10×buffer (Promega), 100 μM each of deoxyribonucleotide triphosphates (Perkin-Elmer-Cetus), 50 pmoles of each of ITS1 and ITS4 primers (24), 20~25 ng of genomic DNA, and 0.7 unit of *Taq* DNA polymerase (Promega). The reaction mixtures were overlaid with mineral oil (Sigma) and placed in an MJ Research DNA thermal cycler (Model PTC-100-60), in which the heating/cooling block was preheated to 90°C. Subsequent amplification reactions were performed after 2 min of pre-incubation at 94°C to enhance denaturation

Table 3. Distribution of restriction fragments generated from 13 restriction endonucleases

Line: 1, *L. betulina*; 2, *F. fraxineaea*; 3, *G. abietinum*; 4, *G. adspersum*; 5, *P. ostreatus*; 6, *M. procera*; 7, *Hohenbuehelia* sp.; 8, *F. fomentarius*; 9, *A. semiorbicularis*; 10, *C. versicolor*; 11, *G. lucidum*; 12, *F. velutipes*

	1	2	3	4	5	6	7	8	9	10	11	12
*Acc*I												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	0	1	0
3	1	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	1	0	0
5	0	0	1	0	0	1	1	0	1	0	0	0
6	0	0	0	0	0	1	0	0	0	0	0	0
7	0	1	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	1	0	0	0	0
9	0	0	0	1	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	1	0	0	0
11	0	0	0	0	0	0	0	1	0	0	0	0
12	0	0	0	0	1	0	0	0	0	0	0	0
13	0	1	1	1	0	1	1	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	1	0	0
*Taq*I												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	1	0	1	1	0	1	1	0	1	1	1	1
3	0	0	0	0	0	0	0	0	1	0	0	0
4	1	0	0	1	0	0	0	0	0	0	0	0
5	0	0	0	0	1	0	0	1	0	0	0	0
6	0	0	0	0	0	1	1	0	0	0	0	0
7	0	1	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	1	0
9	0	0	0	0	1	0	0	0	0	0	1	0
10	0	1	1	0	0	0	0	1	0	0	0	0
11	0	1	0	0	0	0	0	1	0	1	0	0
12	1	0	1	1	1	1	1	0	1	0	0	1
13	0	0	0	0	0	0	0	0	0	1	0	0
*Hha*I												
1	0	0	0	0	0	0	0	1	0	0	0	0
2	0	0	0	0	0	0	0	0	0	1	0	0
3	0	0	0	0	0	0	0	0	1	0	0	0
4	1	1	1	1	1	1	0	0	0	0	0	0
5	0	0	1	1	0	0	1	0	0	0	1	0
6	1	1	0	0	1	1	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	1	0
8	0	0	0	0	0	0	0	0	0	1	0	0
9	0	0	0	0	0	0	1	0	0	0	0	1
10	0	0	0	0	0	0	0	0	0	0	1	0
11	0	0	0	0	0	0	1	0	1	0	0	1
12	0	0	0	0	0	0	0	1	0	0	0	0
13	0	0	0	0	0	0	0	0	0	1	0	1
*Cla*I												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	1	1	1	1	1	1	0
3	1	1	1	1	1	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	1	0
5	0	0	0	0	0	0	0	0	0	1	0	1
6	0	0	0	0	0	0	0	1	0	0	0	0
7	0	0	0	0	0	0	0	1	0	0	0	0

	1	2	3	4	5	6	7	8	9	10	11
4	0	1	0	0	0	0	1	1	1	0	0
5	0	0	0	0	0	0	0	0	0	1	0
6	0	0	1	1	0	0	1	1	1	0	0
7	0	0	0	0	0	1	1	0	0	0	1
8	0	0	0	0	0	1	0	0	0	0	0
9	1	0	1	0	1	0	0	0	0	0	0
10	0	1	0	0	0	0	0	0	0	0	0
11	1	1	1	1	1	1	1	1	1	0	1
12	0	0	0	0	0	0	0	1	0	0	0
13	0	1	0	0	0	0	0	0	0	0	0
*Hsp*92II											
1	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	0	1
3	0	0	0	0	0	0	0	0	0	0	1
4	0	0	0	1	0	0	0	0	0	0	0
5	0	0	0	0	0	0	1	1	1	0	0
6	1	1	0	0	0	1	0	0	0	0	0
7	0	0	1	1	0	0	0	0	0	0	0
8	0	0	1	0	0	0	0	0	0	0	0
9	1	1	0	0	1	1	1	0	0	0	0
10	0	0	0	0	0	0	1	0	0	0	1
11	0	0	0	0	0	0	1	1	1	0	0
12	0	0	0	0	0	0	0	0	1	1	0
13	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	1	0	0	0
15	1	0	0	0	0	0	0	0	0	0	0
*Alu*I											
1	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	1	1	1	0
3	0	0	0	1	0	0	0	0	0	0	0
4	0	0	0	0	0	1	1	0	0	0	0
5	0	1	0	0	0	0	0	0	0	0	0
6	0	0	1	0	1	0	0	0	0	0	0
7	1	0	0	0	0	0	0	0	0	0	1
8	0	0	0	0	0	0	0	0	0	0	1
9	1	0	1	0	1	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	1
*Acc*II											
1	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	1	0
3	0	0	0	0	0	0	0	0	1	0	0
4	0	0	1	0	0	1	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	1
6	0	0	0	0	0	0	0	1	0	0	0
7	0	0	0	1	0	0	0	0	0	0	0
8	1	0	0	0	1	0	0	0	0	0	0
9	0	1	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	1	0	0	0	0

	1	2	3	4	5	6	7	8	9	10	11	12
2	0	1	0	0	0	0	1	1	1	0	0	0
3	0	0	0	1	0	1	0	0	0	0	0	0
4	0	0	1	0	0	0	0	0	0	0	0	0
5	1	0	0	0	1	0	0	0	0	1	0	0
6	0	0	0	0	0	0	0	0	0	0	0	1
7	0	0	0	0	0	0	0	0	0	0	0	1
8	1	0	0	0	0	0	0	0	0	0	0	0
9	0	0	1	0	1	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	1
*Sal*I												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	0	1	0
3	0	0	0	0	0	0	0	0	0	1	0	0
4	0	0	0	0	0	0	0	0	0	1	0	0
5	0	0	0	1	0	1	1	0	0	0	0	0
6	1	0	1	0	1	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	1	0	0	0	0
8	0	1	0	0	0	0	0	0	0	0	0	0
9	0	1	0	0	0	0	0	1	0	0	0	0
*Msp*I												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	1	1	1	0	0
3	0	0	0	0	0	0	0	0	0	0	1	0
4	0	0	0	1	0	0	0	0	0	0	0	0
5	0	0	1	0	0	0	0	0	0	0	0	0
6	1	0	0	0	0	1	0	0	0	0	0	0
7	0	0	0	0	1	0	0	0	0	0	0	0
8	0	1	0	0	0	0	0	0	0	0	0	0
9	0	1	0	0	0	1	0	0	0	0	0	0
10	1	0	0	0	1	0	0	0	0	0	0	0
11	0	0	0	0	0	1	1	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	1	0
13	0	0	0	0	0	0	0	0	1	0	0	0
*Hind*II												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	0	1	0
3	0	0	0	0	0	0	0	0	1	1	0	0
4	0	0	0	0	0	0	1	0	0	0	0	0
5	1	1	1	1	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	1	0	0	0	0
7	0	0	0	0	1	1	0	0	0	0	0	0
8	0	0	0	0	0	1	0	0	0	0	0	0
9	0	0	0	0	1	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	1	0	0	0	0
*Ava*II												
1	0	0	0	0	0	0	0	0	0	0	1	0
2	0	0	0	0	0	0	0	0	1	1	0	0
3	0	1	0	1	0	1	0	0	0	0	0	0
4	0	0	0	0	1	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	1

Table 3. Continued

	1	2	3	4	5	6	7	8	9	10	11	12
8	0	0	0	0	0	1	1	0	0	0	0	0
9	1	1	0	1	0	0	0	0	0	0	0	0
10	0	0	1	0	1	0	0	0	0	0	0	0
*Rsa*I												
1	0	0	0	0	0	0	0	0	0	0	0	1
2	0	0	0	0	0	0	0	0	0	1	0	0
3	0	0	0	0	0	0	0	0	1	0	0	0

	1	2	3	4	5	6	7	8	9	10	11	12
11	1	0	0	1	0	0	0	0	0	0	0	0
12	0	0	0	0	1	0	0	0	0	0	0	0
13	0	1	0	0	0	0	1	1	0	0	0	0
14	0	1	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	1
*Mbo*I												
1	0	0	0	0	0	0	0	0	0	0	1	0

	1	2	3	4	5	6	7	8	9	10	11	12
6	0	0	1	0	0	0	0	0	0	0	0	0
7	1	0	0	0	0	0	1	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	1
9	0	0	0	0	0	0	0	0	0	0	0	1
10	1	0	0	0	0	0	1	0	0	0	0	0
11	0	0	1	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	1	0	0	0

of genomic DNA. The amplification protocol consisted of 35 cycles of 1 min of denaturation at 94℃, 1 min of annealing at 57℃, and 2 min of extension at 72℃. After 35 cycles, 5 min of an additional extension step was run at 72℃..

Twenty restriction enzymes were used for digestions according to manufacturer's instruction (GIBCO BRL) (Table 2). Digested fragments were resolved on 1.5% ultra pure agarose-gel electrophoresis, and detected by staining with ethidium bromide and viewing with UV transilluminator. For each experiment, a 123 bp DNA ladder was also run on the gel to serve as a size marker. Gels were photographed over a UV transilluminator using a Polaroid camera (Model DS-34), and black and white film (Type 667, Polaroid Corp.).

Analysis of RFLPs

Restriction fragments were scored for all samples on two possible character states: 0 (fragment absent) and 1 (fragment present) (Table 3). Estimation of genetic relationships between all pairs of samples was determined using distance values (D) generated from the following formula (7, 20):

$$D = 1 - 2C_{xy}/(N_x + N_y + 2C_{xy})N$$

in which C_{xy} equals the number of fragments in common to samples x and y, and N_x and N_y represent the number of unique bands. D values range from 0 to 1. When the D values for a comparison of two sample are close to 0, a high degree of genetic similarity is indicated. D values close to 1 indicate a low degree of genetic similarity. The goodness of fit of the phenogram was examined on the basis of cophenetic correlation using the program MXCOMP. The average genetic distance value rm($\bar{D}$) was used to assess the degree of genetic variation among samples in each group. These values were calculated using the following formula:

$$\bar{D} = \frac{\sum D_{xy}}{N}$$

in which D_{xy} represents the distance value between samples x and y in a pair of groups to be compared or between samples x and y in a group, and N equals the total number of comparisons between or within groups.

UPGMA and neighbor-joining analyses were used to construct dendrograms by the software package NTSYS (version 1.8) developed by Rohlf *et al.* (18).

Results and Discussion

PCR amplification

Approximately 50~100 μg of DNA was obtained from 1 mg of fresh mycelia. Size of ITS1-5.8S-ITS2 fragments amplified from each of 12 strains ranged from 632 to 833bp (Table 1), suggesting existence of length mutations. None of PCR products was observed in any of controls without the template DNA. As shown in Table 1, PCR products from strains of Polyporaceae and Ganodermataceae were approximately 660bp in size except for those from *F. formentarius* (678 bp) and *G. adspersum* (632 bp). Five strains belonging to the Agaricales, however, resulted in varying size of PCR products ranging from 728 to 833 bp. This result indicates that members in the Agaricales are genetically more variable than those in the Aphyllophorales. More genetic variation in the Agaricales may be ascribed to use of taxonomically more diverse families in the Agaricales than in the Aphyllophorales. Strains within a family, however, resulted in similar size of PCR products except for *Ganoderma* species. Two *Ganoderma* species showing different length of PCR products might have resulted from genetically heterogeneous origins.

Polymorphic Restriction Patterns

Among 20 restriction endonucleases used, 13 endonucleases generated polymorphic restriction patterns (Table 2). The sum of fragments digested by each enzyme was close to the size estimate of PCR products generated from each of 12 species. A 159 phenotypes by 12 strain matrix was generated from 13 restriction endonucleases and 12 strains (Table 3). As shown in Fig. 1a and b, *Taq*I-digest revealed more polymorphic patterns than *Cla*I-digest did. *Ava*II, *Hind*III, and *Sal*I generated sufficient polymorphisms but they did not digest

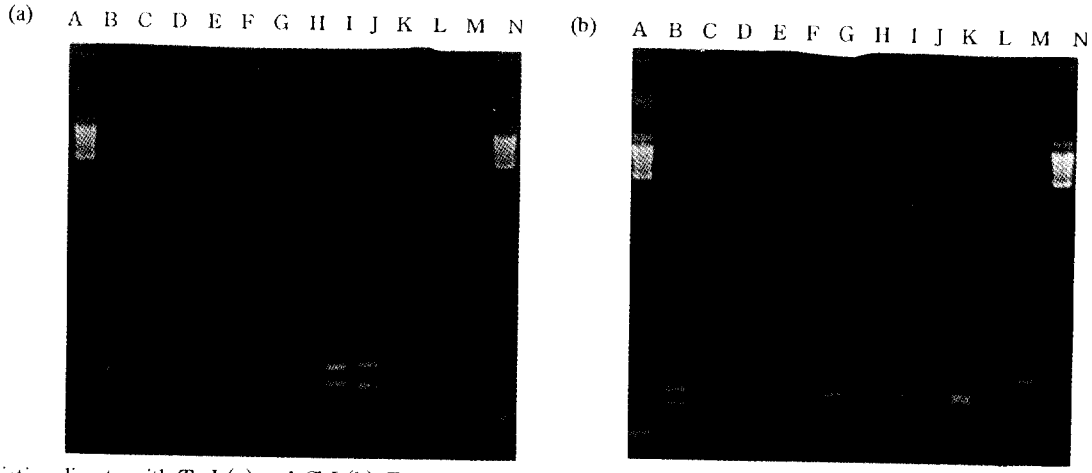

Fig. 1. Restriction digests with *Taq*I (a) and *Cla*I (b). Fragments were electrophoresed on 1.5% ultra pure agarose in 1×TAE buffer at 7.8 volts/cm for 2.5 h and stained with ethidium bromide. A, 123 bp DNA ladder; B, *F. fomentarius;* C, *L. betulina;* D, *F. fraxinea;* E, *G. abietinum;* F, *C. versicolor;* G, *G. lucidum;* H, *G. adspersum;* I, *P. ostreatus;* J, *Hohenbuehelia* sp.; K, *A. semiorbicularis;* L, *M. procera;* M, *F. velutipes;* N, 123 bp DNA ladder

Table 4. Distance matrix based on restriction phenotypes of ITS

Line : 1, *L. betulina;* 2, *F. fraxinea;* 3, *G. abietinum;* 4, *F. fometarius;* 5, *C. versicolor;* 6, *G. adspersum;* 7, *G. lucidum;* 8, *Hohenbuehelia* sp.; 9, *P. ostreatus;* 10, *A. semiorbicularis;* 11, *M. procera;* 12, *F. velutipes*

1	0.0000											
2	0.6863	0.0000										
3	0.6400	0.7551	0.0000									
4	0.6250	0.7021	0.5652	0.0000								
5	0.5385	0.8039	0.5600	0.7917	0.0000							
6	0.6735	0.7083	0.7021	0.6444	0.7551	0.0000						
7	0.7692	0.8039	0.7200	0.7093	0.8846	0.4694	0.0000					
8	0.9608	0.7600	0.8776	0.9149	0.9216	0.9167	0.7647	0.0000				
9	0.8776	0.9167	0.7872	0.8222	0.9184	0.7826	0.6327	0.6250	0.0000			
10	0.9184	0.9583	0.9575	0.9111	0.9184	0.9130	0.8776	0.7500	0.6522	0.0000		
11	0.9184	0.9167	0.8723	0.9111	0.9592	0.8261	0.7551	0.8750	0.9130	0.9130	0.0000	
12	0.8868	1.0000	0.9216	0.9184	0.9623	0.9200	0.8491	1.0000	0.8800	0.8400	0.9600	0.0000

PCR products amplified from certain strains. This result indicates that ITS1-5.8S-ITS2 fragments amplified from certain strains do not have recognition sites for those enzymes. Nevertheless, polymorphic restriction patterns observed on 12 strains appeared to correspond with the taxonomic relatedness among strains. In general, similar restriction patterns were observed between strains of the Polyporaceae and the Ganodermataceae in the Aphyllophorales except for *G. adspersum* (Fig. 1a). Distinct restriction patterns of *G. adspersum* may be a consequence of smaller size of the PCR products than those from other strains. Such similar patterns were also observed among families in the Agaricales. However, *M. procera* of the Bolbitiaceae and *F. velutipes* of the Tricholomataceae exhibited distinct restriction patterns due to length mutations occurring in the region amplified. In general, restriction patterns generated from each of 13 enzymes were similar among strains within the same family.

Genetic relationships among 12 species

In order to determine genetic relationships among 12 strains, dendrograms were constructed by UPGMA and neighbor-joining analyses on the basis of distance values (Table 4). The cophenetic correlation coefficients of the UPGMA and neighbor-joining analyses were 0.8514 and 1.0000, respectively. These results show that the dendrogram constructed by neighbor-joining analysis provides the best fit to the data. In the phenogram from UPGMA analysis, 12 strains fell into two distinct groups, corresponding with orders where each strain belongs to (Fig. 2). Group-A consists of seven strains in the Aphyllophorales including *Ganoderma* species, and group-B is composed of five strains in the Agaricales. *Fomitella*

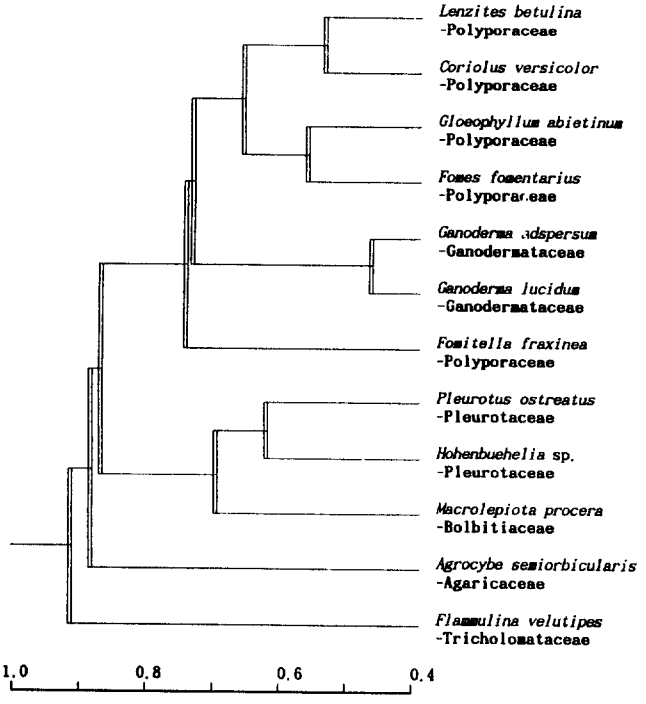

Fig. 2. Dendrogram based on ITS-restriction phenotypes generated from 13 restriction endonucleases and 12 basidiomycetous mushrooms belonging to Aphyllophorales and Agaricales. The dendrogram was constructed from distance values using the UPGMA method in the software package NTSYS-pc. Two distinct groups were delimited, corresponding with orders where each species belongs to.

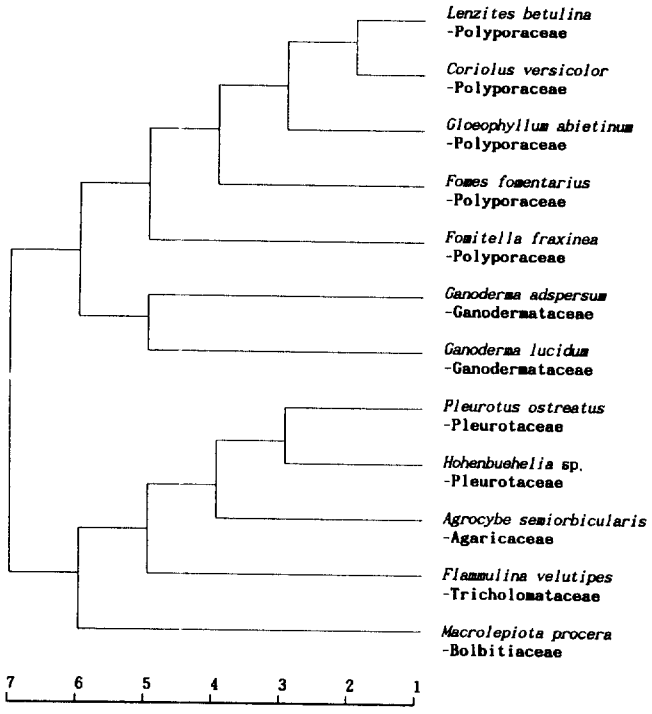

Fig. 3. Dendrogram based on ITS-restriction phenotypes generated from 13 restriction endonucleases and 12 basidiomycetous mushrooms belonging to Aphyllophorales and Agaricales. The dendrogram was constructed by neighbor-joining method in the software package NTSYS-pc. Two distinct groups were delimited, corresponding with orders where each species belongs to.

fraxinea in Polyporaceae was distantly related to other members in Polyporaceae. The average distance value between group-A and group-B was 0.8836, indicating quite distant relationship between the two groups. The average distance values within group-A and group-B were 0.6908 and 0.8408, respectively. Neighbor-joining analysis also distinguished 12 strains into two distinct groups (Fig. 3). Dendrograms constructed from both of UPGMA and neighbor-joining analyses showed that *Ganoderma* strains are closely related to the family Polyporaceae. This results conflict with Donk's (21) view which transferred *Ganoderma* species from the Polyporaceae to the Ganodermataceae.

As shown in the phenogram by the UPGMA analysis (Fig. 2), *F. fraxinea* in the Polyporaceae is more closely related to *Ganoderma* species than other strains of the same family. Even though the dendrogram (Fig. 3) by neighbor-joining analysis separated *Ganoderma* species from the Polyporaceae, they were placed in a close affinity within the same group. These results indicate that there is no clear genetic demarcation between the Polyporaceae and the Ganodermataceae. In order to draw a clear conclusion about taxonomic status of *Ganoderma* species, additional strains from both families should be examined using varying molecular approaches.

Evolutionary relationship

As shown in the phenogram (Fig. 2), strains in the Agaricales were placed at the base of a cluster of strains in the Aphyllophorales. Such a topology indicates that members in the Agaricales are evolutionally more primitive than those in the Aphyllophorales. However, our data from the present study are not sufficient to conclude an evolutionary relationship between the two groups because of the limited number in tested sample size. In order to resolve specific patterns of evolutionary history, additional strains and approaches need to be investigated.

In conclusion, RFLP analyses of nuclear ITS indicate that 12 strains used in this study were clearly distinguished into two genetically distinct groups which correspond with morphology-based classification (Aphyllophorales and Agaricales). These results indicate that RFLPs of ITS could be used as an aid in classifying mushrooms at orders as well as at families. Because there is no clear genetic demarcation between the Polyporaceae and the Ganodermataceae, these families

seem to be in a close taxonomic affinity. Branching patterns seen in the dendrograms indicate that members in the Agaricales are evolutionally more primitive than those in the Aphyllophorales are.

Acknowledgment

This study was supported by Korean Ministry of Science and Technology.

References

1. **Bazzalo, M.E. and J.E. Wright**, 1982. Survey of the Argentine species of *Ganoderma lucidum* complex. Mycotaxon **16**, 293-325.
2. **Castle, A.J., P.A. Horgen, and J.B. Anderson**, 1987. Restriction fragment length polymorphisms in the mushrooms, *Agaricus brunnescens* and *Agaricus bitorquis*. *Appl. Environ. Microbiol.* **53**, 816-822.
3. **Donk, M.A.**, 1993. A conspectus of the families of Aphyllophorales. *Perssoonia* **3**, 199-324.
4. **Goodwin, D.C. and S.B. Lee**, 1993. Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. *BioTechniques* **15**, 441-444.
5. **Hibbet, D.S. and R. Vilgalys**, 1991. Evolutionary relationships of *Lentinus* to the polyporaceae: Evidence from restriction analysis of enzymatically amplified ribosomal DNA. *Mycologia* **83**, 425-439.
6. **Hillis, D. and S. Davis**, 1985. Evolution of ribosomal DNA: fifty million years of recorded history in the frog genus *Rana*. *Evolution* **40**, 1275-1288.
7. **Karsten, P.**, 1881. Enumeratio Boletinarum et *Polyporarum Fennicarum* systemate novo dispositorum. *Rev. Mycol.* **3**, 16-18.
8. **Kulkarn, R.K.**, 1991. DNA polymorphisms in *Lentinus edodes*, the Shiitake mushroom. *Appl. Environ. Microbiol.* **57**, 1735-1739.
9. **Lee, S.B., and J.W. Taylor,** 1990. Isolation of DNA from fungal mycelia and single spores, p. 282-287. *In* Innis, M.A., D.H. Gelfand., J.J. Sninsky, and T.J. White (ed.), PCR protocols, a guide to methods and applications, Academic Press, New York.
10. **Loftus, M.G., D. Moore, and T.J. Elliott,** 1988. DNA polymorphisms in commercial and wild strains of the cultivated mushroom, *Agaricus bisporus*. *Theor. Appl. Genet.* **76**, 712-718.
11. **Mark, D.C., G. John, and P.O. Richard,** 1994. The phylogeny of the tomato leaf mould fungus *Cladosporium fulvum* syn. *Fulvia fulva* by analysis of rDNA sequences. *Curr. Genet.* **25**, 318-322.
12. **May, B. and D.H. Royse,** 1981. Application of the electrophoretic methodology to the elucidation of genetic histories of edible mushrooms. *Mushroom Sci.* **11**, 799-817.
13. **Murrill, W.A.,** 1908. Agaricales (Polyporaceae). *North Amer. Flora* **9**, 73-131.
14. **Nei, M. and W.H. Li.,** 1979. Mathematical model for studying genetic variations in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **76**, 5269-5273.
15. **O'Donnell, K.,** 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Curr. Genet.* **22**, 213-220.
16. **Patouillard, N.,** 1889. Le genre *Ganoderma*. *Bull. Soc. Myc. France* **5**, 64-80.
17. **Rancis, I.M., I. Jong, and S.-C. Jong,** 1992. Restriction polymorphisms in the internal transcribed spacers and 5.8S rDNA of *Saccharomyces*. *Curr. Microbiol.* **25**, 251-255.
18. **Rohlf, F., J. Kishpaugh and D. Kirk,** 1979. *NTSYS numerical taxonomy system of multivariate statistical programs.* State University of New York, Stony Brook.
19. **Sagawa, I., M. Tanaka, and Y. Nagata,** 1992. Discrimination of mushrooms in genus *Pleurotus* by DNA restriction fragment length polymorphisms. *J. Gen. Appl. Microbiol.* **38**, 597-603.
20. **Steyaert, R.L.,** 1961. Note on the nomenclature of fungi and incidentally of *Ganoderma lucidum*. *Taxon* **10**, 251-252.
21. **Suh, Y., L.B. Thien, H.E. Reeve, and E.A. Zimmer,** 1993. Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. *Ameri. J. Botany* **80**, 1042-1055.
22. **Toyomatsu, T., H. Takazawa, and A. Zenyoji,** 1992. Restriction fragment length polymorphisms of mitochondrial DNAs from the Basidiomycetes Pleurotus species. *Biosci, Biotech. Biochem.* **56**, 359-361.
23. **Vilgalys, R. and M. Hester,** 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus species*. *J. Bacteriol.* **172**, 4238-4246.
24. **White, T.J , T. Bruns, S. Bruns, and J. Taylor,** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. *In* Innis, M.A., D. H. Gelfand, J.J. Sninsky, and T.J. White (ed.), PCR protocols, a guide to methods and applications. Academic Press, New York.
25. **Yoon, C.S., D.A. Glawe, and P.D. Shaew,** 1991. A method for rapid small-scale preparation of fungal DNA. *Mycologia*, 835-838.
26. **Yoon, C.S. and D.A. Glawe,** 1993. Association of random amplified polymorphic DNA markers with stromatal type in *Hypoxylon truncatum sensu* Miller. *Mycologia* **85,** 369-380.