J. Microbiology. September 1996 p. 215-219

Vol. 34, No. 3

Phylogenetic Study of *Trichaptum* Species Based on the RFLP Analysis of Mitochondrial DNA

Misun Kim and Hack Sung Jung*

Department of Microbiology, College of Natural Sciences, and Research Center for Molecular Microbiology, Seoul National University, Seoul 151-742, Korea

(Received May 6, 1996/Accepted August 30, 1996)

Eight strains of *Trichaptum* (Polyporaceae), two strains from each species of *T. abietinum*, *T. biforme*, *T. fusco-violaceum*, and *T. laricinum* were examined to see their phylogenetic relationships by digesting mitochondrial DNAs with *Eco*RV, *Hind*III, *Xba*I, and PstI, and then analyzing fragmentation patterns with the methods of Nei and Li. *T. abietinum*, *T. biforme*, and *T. laricinum* developed an independent phylogenetic lineage, respectively, but *T. fusco-violaceum* FP-133997-sp showed a close relationship with two strains of *T. biforme*, and *T. fusco-violaceum* HHB-4016-sp barely grouped with those of *T. laricinum*. Based on the results of the RFLP analysis of mtDNA, it is concluded that *T. fusco-violaceum* is under way to differentiation into two different subgroups.

Key words: *Trichaptum*, RFLPs, mitochondrial DNA, phylogeny

Trichaptum is a genus of white rot fungi in the Polyporaceae (Hymenomycetes, Basidiomycotina) and occurs in the north of temperate zone of the Northern Hemisphere including Korea and Japan, infecting conifers and hardwoods and causing damages in forests. Eight species of *Trichaptum* are known in North America and four species have been reported in Korea. However, because of lack of the distinct macroscopic and microscopic characters, there have been arguments between taxonomists about whether the members of *Trichaptum* should be treated as individual species, subspecies, or varieties of a species based on fruitbody morphology.

Trichaptum was erected as a genus by Murrill in 1904 and later accepted by Gilbertson and Ryvarden (4) but has been placed under *Hirschioporus* until recently. *Trichaptum* is a cosmopolitan group of white wood-rotting polypore fungi. Some of its species are economically important because they are active decomposers of dead conifers like balsam firs, Douglas firs, and pine trees (1, 6, 8). Members of *Trichaptum* are characterized by the purple to violet pore surface in actively growing fruitbodies which pales to buff or brownish color on aging or drying. Microscopic observation shows that these fungi produce cylindrical basidiospores and characteristic cystidia in the hymenium and that their trama is composed of di- to trimitic hyphal systems. Generative hyphae with clamps and dominant skeletal hyphae are regularly observed but binding hyphae are rarely present or seemingly absent. And also there is an imperforate parenthosome in the dolipore septum of *Trichaptum* (15), which has been reported only in polyporoid fungi of the Hymenochaetaceae so far, but its phylogenetic significance is still unknown (4, 11).

Mitochondrial DNA (mtDNA) has a great diversity in size and organization among organisms. Fungi especially have various mtDNAs in size, mostly ranging from 19 kb to 121 kb, and in structure, consisting of introns and noncoding spacer sequences in many parts (5). Mitochondrial genomes usually include genes for large subunit ribosomal RNA (LSU rRNA), small subunit ribosomal RNA (SSU rRNA), subunits I, II, III of cytochrome oxidase complex, cytochrome b, and subunit 6 of ATP synthetase complex (5). And also a complete set of tRNA genes for mitochondrial protein synthesis is usually included in most organisms. In general, there are great similarities in mtDNAs among closely related organisms, which are useful enough to compare their phylogenetic relationships.

For the search of phylogenetic relationships between taxonomically important species of *Trichaptum*, restriction fragment length polymorphisms (RFLPs) of mtDNAs extracted from eight strains of four *Trichaptum* species were studied by digesting mtDNAs with restriction enzymes. Fragmentation patterns of mtDNAs by

* To whom correspondence should be addressed

the RFLP analysis, digestion profiles in other words, were examined and compared each other and one another among eight strains. And these patterns were analyzed by calculating corresponding nucleotide sequence divergence values (9), which were transformed into a matrix to evaluate evolutionary distances and construct a phylogenetic tree using a computer software package. Based on the obtained informations and compared with the present classification, the phylogeny of *Trichaptum* was discussed in this study.

Materials and Methods

Strains and culture

Eight strains of *Trichaptum*, two strains for each of four species, *T. abietinum* FP-101819-sp and MJL-1247-sp, *T. biforme* FP-86522-sp and HHB-7316-sp, *T. fusco-violaceum* FP-133997-sp and HHB-4016-sp, and *T. laricinum* RLG-4665-sp and RLG-6936-sp, donated by Dr. K. K. Nakasone (Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, Wisconsin) were used in this study. They were grown on agar plates and in shaking cultures, for colony observation and DNA isolation, respectively, of MED medium (malt extract 1%, yeast extract 1%, dextrose 3%, agar 1.5%) and MEA medium (malt extract 2%, peptone 0.5%, dextrose 0.5%, agar 2%) at 24°C for two weeks under dark condition.

Isolation of mitochondrial DNA

Liquid cultures of mycelium were harvested by filtration on gauze and rinsed with distilled water (13), resuspended in cold lysis medium (0.6 M sorbitol, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4), and macerated with an omni-mixer for 30 seconds. The mitochondrial pellet was collected from the supernatant by differential centrifugation (10) and purified further on sucrose step gradients of 1.0 M, 1.3 M, 1.6 M, and 2.0 M (7). For the isolation of mtDNA, the mitochondrial pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and lysed by adding SDS to a concentration of 0.2 %. Mitochondrial DNA was extracted with phenol, phenol: chloroform, and chloroform consecutively and precipitated with ethanol, and then was redissolved in TE buffer.

Restriction enzyme digestions

Four enzymes, *Eco*RV, *Hind*III, *Xba*I, and *Pst*I (Boehringer Mannheim, Kosco, Amersham), were used in this experiment. Reactions were carried out according to the supplier's instructions. DNA fragments were separated by horizontal agarose gel electrophoresis in TAE (400 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.2) (12) with lambda DNA digested by *Hind*III as a size marker. After electrophoresis, mtDNAs were stained with EtBr for observation and then photographed.

Data analysis

Digestion profiles generated by restriction enzymes were compared in pairs to determine the number of DNA fragments in common between strains. According to the fragment comparison method of Nei and Li (9), the proportion of fragments in common (F value) was calculated by

$$F = 2N_{xy}/(N_x + N_y)$$

where N_x and N_y are the number of fragments for each compared strain and the N_{xy} is the one of fragments in common, and then the F value was converted into the nucleotide sequence divergence estimate (p value) by

$$p = (-\ln F)/r$$

in which r is the number of nucleotide base pairs for the restriction endonuclease recognition site, that is six for the restriction enzymes of this experiment.

With p values, a p value distance matrix was made and, based on the matrix table, an unrooted phylogenetic tree was constructed using the UPGMA option of the Neighbor program from the PHYLIP 3.5c package (Joseph Felsenstein, University of Washington) to interpret phylogenetic relationships and evolutionary distances and to compare the results with the classical taxonomy between strains and species of *Trichaptum*.

Results and Discussion

Cultures of *Trichaptum*

When eight strains of four *Trichaptum* species were grown on agar plates of a MED medium, they developed distinct cultural characteristics (Table 1). Results of drop-tests for the tyrosinase activity of hyphae were erroneous in *T. abietinum*, *T. fusco-violaceum*, and *T. laricinum*, but it is known that the presence or absence of tyrosinase is sometimes difficult to judge because tyrosinase is an intracellular enzyme (14). Each strain of *T. abietinum* and *T. fusco-violaceum* showed apparently different physiology in terms of growth rate, which seems to be ascribed to a phenomenon of physiological variation occurring between strains of a same species.

RFLPs of *Trichaptum*

When the mitochondrial pellet was collected by differential centrifugation and purified further on sucrose step gradients, most of mitochondria were obtained from

Table 1. Cultural characters and host specificities of *Trichaptum* strains.

	A1	A2	B1	B2	F1	F2	L1	L2
Laccase	+	+	+	+	+	+	+	+
Tyrosinase	−	±	−	−	−	±		±
Peroxidase	+	+	+	+	+	+	+	+
KOH	−	−	−	−	−	−		
Growth rate	5.2 cm/week	1.2 cm/week	4.0 cm/week	3.7 cm/week	1.3 cm/week	5.4 cm/week	4.6 cm/week	3.8 cm/week
Marginal hyphae	up	down	up	up	down	down	up	down
Colony outline	smooth	smooth	smooth	smooth	smooth	smooth	rough	rough
Colony color	white	white	white	white	white	white	white	brown
Host	*Pinus*	conifer	hardwood	*Prunus*	conifer	*Abies*	*Larix*	unknown

The growth rate was measured after incubation for seven days on MEA medium. Al and A2, *T. abietinum* FP-101819-sp and MJL-1247-sp; B1 and B2; *T. biforme* FP-86522-sp and HHB-7316-sp; F1 and F2, *T. fusco-violaceum* FP-133997-sp and HHB-4016-sp; L1 and L2, *T. laricinum* RLG-4665-sp and RLG-6936-sp.

Table 2. *Eco*RV RFLP data.

	A1	A2	B1	B2	F1	F2	L1	L2
A1	-	6/14	6/14	6/19	6/16	6/14	6/18	8/19
A2	0.1412	-	10/16	8/20	8/18	4/15	8/19	4/20
B1	0.1412	0.0783	-	6/18	8/16	6/13	8/17	6/17
B2	0.1921	0.1527	0.1831	-	6/20	8/19	10/21	4/22
F1	0.1635	0.1352	0.1155	0.2007	-	4/15	16/19	16/20
F2	0.1412	0.2203	0.1289	0.1442	0.2203	-	6/16	4/17
L1	0.1831	0.1442	0.1256	0.1237	0.0286	0.1635	-	14/20
L2	0.1442	0.2682	0.1736	0.2841	0.0372	0.2412	0.0594	-

Table 3. *Hind*III RFLP data.

	A1	A2	B1	B2	F1	F2	L1	L2
A1	-	4/11	6/13	4/13	6/13	6/16	4/13	4/11
A2	0.1686	-	2/14	8/15	2/12	4/15	2/10	4/11
B1	0.1289	0.3243	-	8/15	6/15	6/15	4/12	4/13
B2	0.1964	0.1048	0.1048	-	6/14	6/16	4/12	4/13
F1	0.1289	0.2986	0.1527	0.1412	-	6/18	2/13	2/14
F2	0.1635	0.2203	0.1527	0.1635	0.1831	-	4/15	8/16
L1	0.1964	0.2682	0.1831	0.1831	0.3120	0.2203	-	14/11
L2	0.1686	0.1686	0.1964	0.1964	0.3243	0.1155	0.1686	-

Table 4. *Xba*I RFLP data.

	A1	A2	B1	B2	F1	F2	L1	L2
A1	-	2/11	4/12	2/16	2/13	4/12	2/10	2/13
A2	0.2841	-	6/12	2/14	4/11	6/12	2/9	8/11
B1	0.1831	0.1155	-	6/17	6/13	8/14	2/10	6/14
B2	0.3466	0.3243	0.1736	-	14/17	2/16	4/14	4/18
F1	0.3120	0.1686	0.1289	0.0324	-	4/14	4/11	4/14
F2	0.1831	0.1155	0.0933	0.3466	0.2088	-	4/11	6/13
L1	0.2682	0.2507	0.2682	0.2088	0.1686	0.1686	-	6/11
L2	0.3120	0.0531	0.1412	0.2507	0.2088	0.1289	0.1010	-

the interlayer of 1.3/1.6 M gradients. Upon the digestion of the mtDNAs, each species of *Trichaptum* produced unique digestion profiles for various restriction enzymes. When a restriction enzyme produces too many fragments, it becomes difficult to count correct numbers due to crowded fragment bands. And when insufficient fragments are generated, it also becomes impractical to calculate F values because there can be no common fragments to be compared. When the numbers and sizes of common and total DNA fragments from the digestion profiles were considered, four enzymes, *Eco*RV, *Hind*III, *Xba*I, and *Pst*I produced most appropriate restriction fragment patterns after digestion of mtDNAs among preliminarily tested restriction endonucleases. *Pst*I generated DNA fragments up to sixteen but other enzymes just up to ten or twelve (data not shown). Numbers of common and total fragments between strains and species were counted and their fraction matrix was made for each digestion enzyme (Tables 2 to 5). The nucleotide sequence divergence values were estimated between mtDNAs of eight strains and p values for pairwise comparisons were tabulated as a p value distance matrix (Table 6) for the construction of a dendrogram.

Table 5. *Pst*I RFLP data.

	A1	A2	B1	B2	F1	F2	L1	L2
A1	-	12/19	10/24	16/25	16/28	8/26	2/15	4/17
A2	0.0766	-	10/23	10/24	12/27	8/25	ND	4/14
B1	0.1459	0.1388	-	14/18	10/26	10/24	ND	4/15
B2	0.0744	0.1459	0.0419	-	6/27	8/23	2/14	4/16
F1	0.0933	0.1351	0.1593	0.2507	-	8/28	ND	2/19
F2	0.1964	0.1899	0.1459	0.1760	0.2088	-	ND	4/17
L1	0.3358	ND	ND	0.3243	ND	ND	-	ND
L2	0.2412	0.2088	0.2203	0.2310	0.3752	0.2412	ND	-

Fractions of common fragment per total fragment numbers (above diagonals of Tables) and their corresponding p values (below diagonals of Tables) were estimated from RFLP data of mtDNAs generated by *Eco*RV (Table 2), *Hind*III (Table 3), *Xba*I (Table 4), and *Pst*I (Table 5) digestions. Symbols for species names and strain numbers are same as in Table 1. ND, not determined.

Table 6. Distance matrix of average *p* values of *Trichaptum* RFLP data.

	A1	A2	B1	B2	F1	F2	L1	L2
A1	-							
A2	0.1676	-						
B1	0.1498	0.1642	-					
B2	0.2024	0.1819	0.1259	-				
F1	0.1744	0.1844	0.1391	0.1563	-			
F2	0.1711	0.1865	0.1302	0.2076	0.2053	-		
L1	0.2459	0.2210	0.1923	0.2100	0.1697	0.1841	-	
L2	0.2165	0.1747	0.1829	0.2406	0.2364	0.1817	0.1097	-

Symbols for species names and strain numbers are same as in Table 1.

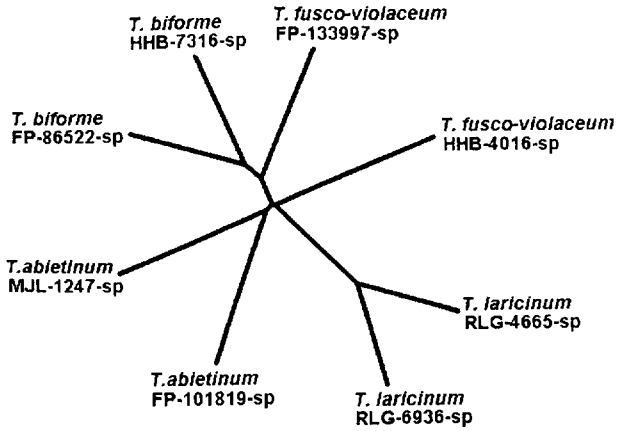

Fig. 1. Unrooted phylogenetic tree of *Trichaptum* based on the *p* value distance matrix of mtDNA RFLPs.

Phylogenetic considerations

Pairwisely compared *p* values ranged from 0.1097 between *T. laricinum* RLG-4665-sp and RLG-6936-sp to 0.2459 between *T. abietinum* FP-101819-sp and *T. laricinum* RLG-4665-sp. Lower *p* values are associated with fewer nucleotide differences, which is an indication of higher similarity of mtDNAs among compared strains and are assumed to correspond to a shorter differentiation time since two organisms shared a common ancestor (2, 3).

When an unrooted phylogenetic tree was constructed from the *p* value distance matrix (Fig. 1), two strains from each species of *T. abietinum*, *T. biforme*, and *T. laricinum* made an independent lineage of same phylogenetic affinity, respectively, but *T. fusco-violaceum* FP-133997-sp showed a rather close relationship with two strains of *T. biforme*, and *T. fusco-violaceum* HHB-4016-sp, on the other hand, barely grouped with those of *T. laricinum*.

All the strains were keeping rather same evolutionary distances from one another and were branching in a radial direction, suggesting that they have been evolving for almost same periods since the divergence from a common ancestor, except those of *T. laricinum* which evolved a slightly longer time. Judging from distances and directions of phylogenetic branches of the present tree, it is concluded that *T. fusco-violaceum* is under way to differentiation into two different subgroups, which can be new physiological or geographic variations within a species, but the other three species, *T. abietinum*, *T. biforme*, and *T. laricinum* have been evolving as apparently different independent species.

Acknowledgements

The authors are grateful to Dr. K. K. Nakasone, Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, Wisconsin, who generously donated *Trichaptum* strains for this study. The present research was supported by the KOSEF research grant for SRC (Research Center for Molecular Microbiology, Seoul National University).

References

1. **Arora, D.,** 1986. Mushrooms demystified, 2nd ed. Ten Speed Press, Berkeley, California. 959 pp.
2. **Fitch, W.M. and E. Margoliash,** 1967. Construction of phylogenetic trees. *Science* **155**, 279-284.
3. **Förster, H., T.G. Kinscherf, S.A. Leong, and D. P. Maxwell,** 1988. Estimation of relatedness between *Phytophthora* species by analysis of mitochondrial DNA. *Mycologia* **80**, 466-478.
4. **Gilbertson, R.L. and L. Ryvarden,** 1987. North American polypores, Vol. 2. Fungiflora, Oslo, Norway. pp. 437-885.
5. **Grossman, L.I. and M.E.S. Hudspeth,** 1985. Fungal mitochondrial genome, pp. 65-103. *In* Bennett J.W. and L.L. Lasure (eds.) Gene manipulations in fungi. Academic Press, New York.
6. **Jung, H.S.,** 1991. Fungal flora of Kwanak Mountain. *Proc. Coll. Natur. Sci., SNU* **16**, 35-71.
7. **Hecker, L.I. and S.D. Schwartzbach,** 1979. Isolation of *Neurospora* mitochondrial tRNA. *Methods Enzymol.* **59**, 203-

421.

8. **Magasi, L. P.,** 1976. Incompatibility factors in *Polyporus abietinus*, their numbers and distributions. *Mem. N.Y. Bot. Gard.* **28**, 163-173.
9. **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.
10. **Rickwood, D., M.T. Wilson, and V.M. Darley-Usmar,** 1987. Isolation and characteristics of intact mitochondria, pp. 1-16. *In* Darley-Usmar, V.M., D. Rickwood, and M.T. Wilson (eds.) Mitochondria : A practical approach. IRL Press, Oxford.
11. **Ryvarden, L. and R. L. Gilbertson,** 1994. European polypores, Vol. 2. Fungiflora, Oslo, Norway. pp. 394-743.
12. **Sambrook, J., E.F. Fritsch, and T. Maniatis,** 1989. Molecular cloning : A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
13. **Specht, C.A., C.C. DiRusso, C.P. Novotny, and R.C. Ullrich,** 1982 A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. *Anal. Biochem.* **119**, 158-163.
14. **Stalpers, J.A.,** 1978. Identification of wood-inhabiting Aphyllophorales in pure culture. *Studies in Mycology* **16**.
15. **Traquair, J.A. and W.E. McKeen,** 1978. Ultrastructure of the dolipore septum in *Hirschioporus pargamenus* (Polyporaceae). *Can. J. Microbiol.* **24**, 767-771.