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Phylogenetic Analysis of *Trichaptum* Based on the RFLP of PCR-Amplified DNAs

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To infer phylogenetic relationships between species of *Trichaptum* (Polyporaceae), RFLP analyses of PCR-amplified DNAs were accomplished. Regions coding for ITSs of nuclear SSU rRNA genes and for mitochondrial SSU rRNA genes from thirteen strains of four *Trichaptum* species (*T. abietinum*, *T. biforme*, *T. fusco-violaceum*, and *T. laricinum*) were amplified and digested with eight restriction enzymes. All the fragmentation patterns were characterized and coded as 0/1 for the absence/presence of fragments. A phylogenetic tree based on the combined data sets was constructed using the Dollo parsimony method. While every two strains of *T. abietinum*, *T. biforme*, *T. fusco-violaceum*, and *T. laricinum* formed an independent group, the other strains of *T. abietinum* and *T. fusco-violaceum* made mixed groupings among compared strains. It is inferred that *T. abietinum* and *T. fusco-violaceum* have more variations, possibly geographic or physiological ones, than other species in the genus.

Key words: *Trichaptum*, ITS, SSU rRNAs, RFLP, phylogeny

The genus *Trichaptum* is a cosmopolitan taxon of white wood-rotting polyporoid fungi that decompose conifers and hardwoods killed by insects or diseases (1, 8). The most common species of *Trichaptum* are *T. abietinum*, *T. fusco-violaceum*, and *T. laricinum*, which are much alike in general appearance and have microscopic similarities. These fungi have few characters to separate them by species and differ only in the configuration of their hymenial surfaces: one being poroid, one irpicoid, and one lamellate (12, 13). Many authors treated these poroid, irpicoid, and lamellate forms in different ways. Some believed them to be three distinct species and others considered them to be subspecies or varieties within the same species.

Morphologically, this genus is characterized by actively growing fruitbodies with purplish to violet pores that become pale buff or pale brown on drying or with age. Microscopically, it has cylindrical spores, diagnostic cystidia, and di- to trimitic hyphal systems. There are clamped generative hyphae and dominant skeletal hyphae in this genus, which rarely has binding hyphae. Ryvarden (17) treated *Trichaptum* as a member of the *Trametes* Group, which includes *Daedaleopsis*, *Fomitella*, *Lenzites*, *Microporus*, and eleven related genera, of the Polyporaceae. *Trichaptum* is quite unique because it has an imperforate parenthosome in the dolipore apparatus (9, 14, 21). This feature has been reported only in the Hymenochaetaceae so far, but its phylogenetic significance is still unclear (5, 17, 18).

Through intersterility test, Macrae (12) once concluded that the three poroid, irpicoid, and lamellate forms of *Trichaptum* are best considered as three distinct species as they can't be crossed with one another. This conclusion was confirmed by Magasi (13) later. Thus, each of three forms is believed to be a separate species. However, according to Macrae and Magasi, the poroid form, *T. abietinum* could be subdivided into two or three different subgroups: two from North America that were incompatible with each other, but were partially compatible with a third one from Europe. The incompatibility between the two subgroups from North America was not correlated with host or geographic range. It may be well to separate *T. abietinum* into two or more subgroups physiologically or phylogenetically someday.

Eukaryotic ribosomal RNA genes are generally arranged as a tandemly repeated array having both variable and highly conserved regions. A variable noncoding

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Table 1. List of *Trichaptum* strains used in this study

Species name	collection number	KCTC accession	host	locality	collector	determiner
T. abietinum	MJL-1247-Sp	6237	conifer	Idaho	Larsen, M.J.	-
T. abietinum	FP-101819-Sp	6238	*Pinus*	Mississippi	Nakasone, K.K	Larsen, M.J.
T. abietinum	DAOM F7426	-	*Picea rubra*	Adirondack Mts., New York	Welch & White	-
T. abietinum	DAOM 72245A	-	*Tsuga canadensis*	Gatineau Park, Quebec	Macrae & McLain	Macrae, R.
T. abietinum	DAOM 73811	-	-	Gustrow, Germany	Schult, F.	-
T. biforme	HHB-7316-Sp	6239	*Prunus serotinus*	Madison, Wisconsin	Burdsall, H.H. Jr	Burdsall, H.H. Jr.
T. biforme	FP-86522-Sp	6240	hardwood	Beltsville, Maryland	Lombard, F.F.	Stevenson, J.A.
T. fusco-violaceum	HHB-4016-Sp	6241	*Abies fraseri*	Swain, Tennessee	Burdsall, H.H. Jr	Larsen, M.J.
T. fusco-violaceum	FP-133997-Sp	6242	conifer	Hungry Horse, Montana	Larsen, M. J.	Larsen, M.J.
T. fusco-violaceum	DAOM 53117	-	*Abies balsamea*	Canwood, Saskatchewan	Macrae & Vaartaja	Macrae, R.
T. fusco-violaceum	DAOM 53128	-	*Pinus sylvestris*	Hellandsfeltel, Norway	Bauger, E.	Robak, H.
T. laricinum	RLG-4665-Sp	6243	*Larix*	Bigfork, Montana	Gilbertson, R.L.	Larsen, M.J.
T. laricinum	RLG-6936-Sp	6244	-	Lake Placid, New York	Gilbertson, R.L.	

region, the internal transcribed spacers (ITS1 and ITS2), has high resolving power for intraspecific analysis. And the mitochondrial genome is known to evolve about 10 times faster than the nuclear genome. Both of them have been used as useful DNA regions for the study of closely related fungal organisms. The polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) analysis is now widely applied in typification, taxonomy, and phylogeny of fungi (2, 7, 15).

To search for phylogenetic relationships between the species of *Trichaptum*, internal transcribed spacer regions (ITS1-5.8S-ITS2) of nuclear small subunit rRNA genes (nuclear SSU rRNA) and parts of mitochondrial small subunit rRNA genes (mitochondrial SSU rRNA) were PCR-amplified and digested with *Hae*III, *Hpa*II, *Cla*I, *Nsi*I, *Cfo*I, *Hin*fI, *Dra*I, and *Ssp*I. The RFLP profiles of digested DNAs were examined and analyzed by characterizing the fragmentation patterns as 0/1 codes for the absence/presence of fragments. Based on the combined data set, a phylogenetic tree was constructed using the Dollo parsimony method of PAUP and the possible phylogeny of *Trichaptum* was discussed in this study.

Materials and Methods

Strains, culture, and isolation of total DNAs

Thirteen strains of four *Trichaptum* species (5 strains for *T. abietinum*, 2 strains for *T. biforme*, 4 strains for *T. fusco-violaceum*, and 2 strains for *T. laricinum*) received from the Center for Forest Mycology Research (USDA Forest Products Laboratory, Madison) and bought from the Canadian Collection of Fungus Cultures (Center for Land and Biological Resources Research, Ottawa) were used for the study (Table 1). Cultures were maintained on MED agar (malt extract 1%, yeast extract 1%, dextrose 3%, agar 1.5%) or on ME agar (malt extract 2%, peptone 0.5%, agar 1.5%) at 24°C for two weeks under dark conditions and then stored at 4°C. For DNA extraction, mycelium was grown in shaking cultures of liquid ME media for 5 days and then harvested by filtration on gauze. DNAs were isolated according to the protocol of Raeder and Broda (16) with some modifications.

PCR amplification of DNAs

Using ITS5 and ITS4 primers designed for the ITS region of fungal nuclei and MS1 and MS2 primers designed for the SSU rRNA gene of fungal mitochondria (22), DNA amplifications were performed in a 10 mM reaction mixture containing Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 2.5 μl of each primer, with about 200 ng of template DNA and 2 units of DynaZyme (POSCOCHEM). Total volume was adjusted at 100 μl which was overlaid with one droplet of mineral oil. The reaction was cycled 30 times to the profile of 94°C for 1 min 30 sec, 50°C for 1 min 30 sec, and 72°C for 1 min 30 sec, with 1 sec extended for each cycle, and then followed by a final extension at 72°C for 10 min. A sample of each amplification reaction was electrophoresed on 0.7% agarose gel (TAE buffer) and bands were visualized with ethidium bromide under UV light. The amplified DNA solution was extracted by chloroform and precipitated by 1/10 volume of 3 M sodium acetate and 3 volume of EtOH. And to confirm the identify of amplified ITS regions, secondary PCR amplifications were performed using first PCR products as templates and the JTS2 primers as internal primers, and then the sizes of

Table 2. Coding characters from restriction fragments

	ITS1-5.8S-ITS region				MtSr region				
	*Hae*III	*Hpa*II	*Cla*I	*Nsi*I	*Cfo*I	*Hin*f	*Nsi*I	*Dra*I	*Ssp*I
T. abietinum FP-101819-Sp	0011	100000	0110000	0100	100	000	0000	11010	1010
T. abietinum MJL-1247-Sp	0100	0001000	0001010	0010	000	000	1100	11010	0101
T. abietinum DAOM 73811	1000	0001000	1010000	1000	100	000	1000	01010	0101
T. abietinum DAOM F7426	0100	0001000	0010000	0000	101	000	0100	10101	0000
T. abietinum DAOM 72245A	0100	0001110	1010111	1010	100	100	????	?????	????
T. biforme FP-86522-Sp	0100	0001000	???????	0000	101	001	????	?????	????
T. biforme HHB-7316-Sp	1000	0001000	???????	1000	110	???	0010	10101	1010
T. fusco-violaceum FP-133997-Sp	1000	0011110	0010101	0100	111	100	1000	01010	1010
T. fusco-violaceum HHB-4016-Sp	0100	0000111	0000000	0000	000	000	1000	01010	1010
T. fusco-violaceum DAOM 53117	0011	1001000	0110010	0000	100	001	1000	01010	1010
T. fusco-violaceum DAOM 53128	0011	0001000	0001010	0010	110	000	0100	10110	1010
T. laricinum RLG-4665-Sp	0100	0110000	0010100	0001	110	011	0011	11001	0100
T. laricinum RLG-6936-Sp	0100	0110000	0010100	0001	110	011	0011	11001	0100

Fragments are coded as present (1), absent (0), or not-known (?).

the secondary PCR products were compared with known sizes of ITS regions.

Restriction enzyme digestions

Six enzymes (*Hae*III, *Hpa*II, *Cla*I, *Nsi*I, *Cfo*I, and *Hinf*I) were used for ITS regions of nuclear SSU rRNA genes and three enzymes (*Nsi*I, *Dra*I, and *Ssp*I), adequate for recognition of AT-rich mitochondrial DNAs, for parts of mitochondrial SSU rRNA genes. Amplified DNAs were digested according to the manufacturer's instructions. The products of the restriction fragments were resolved on 0.75% agarose-gel electrophoresis with 1 kb ladder as a size marker. Agarose gels were then stained with ethidium bromide.

Parsimony analysis

The characters used in this study were the presence or absence of digested bands. The presence of band was coded as "1" and the absence as "0". Bands developed in only one strain were discarded because uninformative autapomorphies are not used in the parsimony analysis. In phylogenetic reconstructions for data sets of RFLP, the heuristic search option of parsimony algorithms of PAUP 3.1.1. (19) was accomplished using the stepwise addition option of the heuristic search, with the tree-bisection-reconnection (TBR) branch swapping performed on starting trees, and zero length branches set to collapse to yield polytomies. However, it should be remembered that a convergent gain of an apparently homologous site will be much more rare than the convergent loss of an ancestral site (3). Consequently, many authors (4, 20, 11) recommended that Dollo parsimony, which allows a character to be gained only once while minimizing losses, would be more appropriate for the analysis of restriction site data than the commonly used Wagner method, which counts gains and losses as equal events.

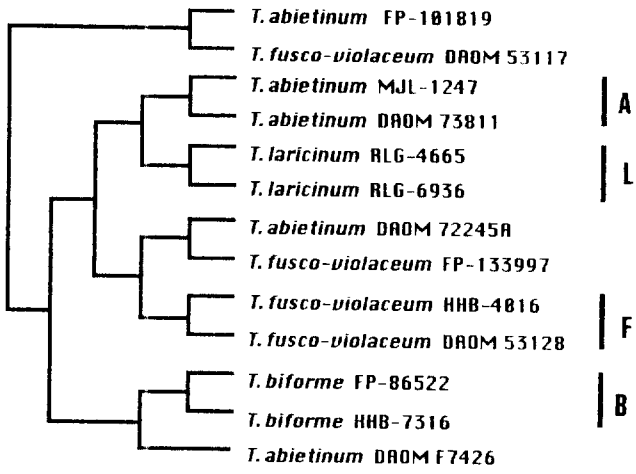

Fig. 1. Most parsimonious tree derived from RFLP data sets. This tree, which is unrooted, is constructed by Dollo parsimony method. It requires 118 changes on the branches (CI=0.331, RI=0.685).

Results and Discussions

PCR amplification

Nuclear DNA fragments amplified using the primers ITS5 and ITS4 ranged from about 600 to 800 bp in size. Lengths of PCRs products were different for each species, which indicates length mutations. However, sizes of mitochondrial DNA fragments amplified using the primers MS1 and MS2 were much the same within about 600 bp.

Parsimony analysis of the data

The total number of restriction fragment characters used in this study were 41 and are listed in Table 2. Characters not determined are coded as "?". As a result of Dollo parsimony analysis based on this combined data set, one most parsimonious tree (MPT) was obtained (Fig. 1). This parsimony tree had 118 steps, a consistency index of 0.331, and a retention index of 0.685.

Phylogenetic considerations on *Trichaptum*

In the previous study for the RFLP analysis of the mitochondrial DNA (10), every two strains of *T. abietinum*, *T. biforme*, *T. fusco-violaceum*, and *T. laricinum* formed a distinct group and the other strains of *T. abietinum* and *T. fusco-violaceum* made mixed groupings among compared strains. But the parsimonious tree of the present extended study shows that the strains of *T. biforme* and *T. laricinum* form independent groups, respectively, while the strains of *T. abietinum* and *T. fusco-violaceum* do not form monophyletic groups.

The strains of *T. biforme* (B group in Fig. 1) and *T. laricinum* (L group in Fig. 1) were not divided into subgroups nor mixed with other groups. And *T. abietinum* MJL-1247 and DAOM 73811 (A group in Fig. 1), and *T. fusco-violaceum* HHB-4016 and DAOM 53128 formed one cluster (indicated as F group in Fig. 1), respectively. On the other hand, *T. abietinum* DAOM 72245A and *T. fusco-violaceum* FP-133997 are closely related with the F group and *T. abietinum* DAOM F7426 with the B group. But *T. abietinum* FP-101819 and *T. fusco-violaceum* DAOM 53117 form a separate group.

According to Macrae (12) and Magasi (13), *T. abietinum* have two or more intersterile groups. But Hallenberg *et al.* (6) reported that sterility barriers are not always distinct boundaries between species, but may be the result of a propagation strategy within the limits of a species in some cases. Though genetic differences are very small between intersterile groups, the sterility barriers can cause speciations in evolutionary pathways. Thus, it seems that *T. abietinum* is composed of at least two or more physiological or geographic taxa. In addition, there is a possibility that *T. fusco-violaceum* is composed of a few subdivisible taxa, too. Therefore, it can be inferred that both of *T. abietinum* and *T. fusco-violaceum* are polyphyletic groups and have two or more geographic or physiological variations.

Especially, *Trichaptum* species have imperforate parenthosomes as in those of the Hymenochaetaceae and are considered as very unique taxa in the Aphyllophorales (5). But *Trichaptum* species do not have enough morphological characters to be subdivided into more varieties and to be connected to the species of the Hymenochaetaceae by the present taxonomy. Considering all available data, some of the *Trichaptum* species need to be subdivided phylogenetically and may have certain relationships even with the taxa of other families. But the PCR-RFLP data alone is not sufficient to infer exact phylogenetic relationships within or between comparable taxa. For more solid and reasonable conclusions about intra- and inter-relationships of *Trichaptum* species, additional molecular data obtained from more strains and sufficient morphological, physiological, and geographic data are required.

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