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Phylogenetic Study of *Trichaptum* Inferred from Nuclear Ribosomal DNA Sequences

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For the phylogenetic study of the genus *Trichaptum*, nuclear ribosomal DNA sequences from eight strains of four *Trichaptum* species were examined. Phylogenetic trees were constructed using molecular data on 18S rDNA and 5.8S rDNA and their ITSs. Parsimony analyses of the *Trichaptum* species showed that *T. biforme* and *T. laricinum* made a monophyletic group respectively, suggesting that each species is phylogenetically independent. However, *T. abietinum* represented a polyphyletic group and *T. fusco-violaceum* formed a polytomous group, suggesting that these species could be in the process of evolutionary differentiation. Examination of base substitutions of the 18S rRNA gene reveals that the C-T transition is most predominant and that there is a stronger transition bias between closely related organisms rather than between distantly related ones.

Key words: Base substitution, ITS, phylogeny, rDNA, *Trichaptum*

The genus *Trichaptum* belongs to the family Polyporaceae, one of the largest groups in the Aphyllophorales. Since Fries first used this family in 1838, it has been adopted as a catchall group of polyporoid species from different families of different affinities (15, 38). *Trichaptum* is a world-wide taxon of white wood-rotting fungi and used to be well-known by the name of *Hirschioporus* for a long time. The fruitbodies of *Trichaptum* have tints of purple to violet which pale off with age or on drying. Microscopically, they have typical cylindrical spores, fusiform to clavate cystidia, and dimitic hyphal systems of clamped generative hyphae and dominant skeletal hyphae. *T. biforme*, however, rarely has binding hyphae.

Trichaptum species appear every much alike and have few characters to separate them. They largely differ in the configuration of hymenophores, which are poroid, irpicoid, or lamellate (29, 30). The poroid form was originally described as *Boletus abietinus* by Dickson in 1793 and the irpicoid form was given the name *Sistotrema violaceum* by Persoon in 1801, but Donk later proposed the genus *Hirschioporus* to include both fungi in 1933 (29). Now the poroid form is called *T. abietinum* and the irpicoid form is called *T. fusco-violaceum*. The lamellate form was first described by Karsten in 1905 as *Lenzites laricina* and was later transferred to the genus *Hirschioporus* by Teramoto in 1951 (29).

Some authors considered these forms to be too similar to separate and treated them as variations of a same species but others described them as different species. Macrae (29) once reported that the three forms of *Trichaptum* were intersterile with one another and could be treated as three different species, which was confirmed again by Magasi (30). They also concluded that there could be two or three more subgroups in the poroid form *T. abietinum*, because two incompatible subgroups from North America were partially compatible with another one from Europe. The fourth common species of the genus is *T. biforme*, which has similar basidiocarps as those of *T. abietinum* and used to be called *Coriolus pergamenus*. However, *T. biforme* has a narrow base and a glabrescent tomentose surface and has no gelatinous layer beneath the context present in the pileus of *T. abietinum* (16), morphologically differentiating itself from *T. abietinum*.

The ribosomal RNA (rRNA) genes are now commonly used for analyses of phylogenetic relationships in pursuit of evolutionary pathways of organisms because they have been highly conserved and change very slowly. Sequence analyses of 18S ribosomal DNAs (rDNAs) have been widely performed and are useful for the comparison of fungal groups (2, 3, 5, 7, 14, 18, 22, 34, 35, 40, 44). But there have been difficulties in interpretation of taxa diversity within narrow ranges and in phy-

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logenetic classifications of interspecific or intraspecific organisms. This is because of the low base substitution rate of rDNAs, so 18S rDNAs are believed to be more useful for the comparison of distantly related fungal taxa.

However, two variable non-coding regions called internal transcribed spacers (ITS1 and ITS2) separating highly conserved portions of 18S, 5.8S, and 28S RNA genes change more rapidly and have been useful for the study of closely related organisms both within and between species (1, 4, 9, 10, 11, 12, 28, 32, 33, 36, 43, 44, 47, 48). Thus, the ITS1-5.8S-ITS2 region or ITSs are now frequently used for narrow phylogenetic groups of fungi. In relation to taxonomic aspects based on morphological characters, both 18S rDNA and ITS sequences were used here to discuss phylogenetic relationships of *Trichaptum* based on molecular data.

Materials and Methods

Strains and cultures

Eight strains of *Trichaptum abietinum, T. biforme, T. fusco-violaceum,* and *T. laricinum* received from the Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, Wisconsin, were used for the study (Table 1). They were inoculated on a cellophane disc placed on media of malt extract-dextrose agar (MEDA; malt extract 1%, yeast extract 1%, dextrose 3%, agar 1.5%) or malt extract agar (MEA; malt extract 2%, peptone 0.5%, agar 1.5%) and then maintained at 24°C in the dark. Liquid ME medium was used for harvesting mycelium.

DNA isolation and PCR amplification

For DNA isolation, the mycelium grown on the cellophane disc was recovered into an Eppendorf tube using a spatula, frozen in liquid nitrogen, and thawed in an incubator repeatedly until thorough breakage of cells according to the method of Lecellier and Silar (26). Using primers NS1 and ITS4 (Table 2), DNA amplifications were performed in a reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, and 0.2 mM dTTP, 1 μl of each primer, 200 ng of template DNA, and 2 units of *Taq* DNA polymerase (POSCOCHEM). Thirty cycles of reactions were performed with the following program: 1 min 30 sec at 94°C for denaturation, 1 min 30 sec at 50°C for annealing, and 1 min 30 sec at 72°C for extension, with 1 sec extended each cycle. Amplified samples were electrophoresed on 0.7% agarose gel and bands were visualized with EtBr under UV light. DNA was then extracted by chloroform and pre-

Table 1. Fungal species, taxonomic affiliations, and accession numbers of 18S rDNA sequences of *Trichaptum* and compared taxa

fungal species	taxonomic affiliations	accession number
Trichaptum abietinum FP-101819-Sp	Polyporaceae, Aphyllophorales	U63474
MJL-1247-Sp	Polyporaceae, Aphyllophorales	U63475
Trichaptum biforme FP-86522-Sp	Polyporaceae, Aphyllophorales	U63473
HHB-7316-Sp	Polyporaceae, Aphyllophorales	U63476
Trichaptum fusco-violaceum FP-133997-Sp	Polyporaceae, Aphyllophorales	U63472
HHB-4016-Sp	Polyporaceae, Aphyllophorales	U63478
Trichaptum laricinum RLG-4665-Sp	Polyporaceae, Aphyllophorales	U63471
RLG-6936-Sp	Polyporaceae, Aphyllophorales	U63477
Athelia bombacina	Corticiaceae, Aphyllophorales	M55638
Boletus satanas	Boletaceae, Agaricales	M94337
Bulleromyces albus	Filobasidiaceae, Sporobolomycetales	X60179
Coprinus cinereus	Coprinaceae, Agaricales	M92997
Cronartium ribicola	Melampsoraceae, Uredinales	M94338
Filobasidiella neoformans	Filobasidiaceae, Sporobolomycetales	X60183
Leucosporidium scottii	Sporobolomycetaceae, Sporobolomycetales	X53499
Schizophyllum commune	Schizophyllaceae, Aphyllophorales	X54865
Spongipellis unicolor	Polyporaceae, Aphyllophorales	M59760
Sporobolomyces roseus	Sporobolomycetaceae, Sporobolomycetales	X60181
Thanatephorus praticola	Corticiaceae, Aphyllophorales	M92990
Trichosporon cutaneum	Filobasidiaceae, Sporobolomycetales	X60182
Ustilago maydis	Ustilaginaceae, Ustilaginales	X60396
Xerocomus chrysenteron	Boletaceae, Agaricales	M94340
Saccharomyces cerevisiae	All taxa outgroup	M27607

Table 2. Primers and their sequences used for the PCR amplification and sequencing of the 18S-ITS1-5.8S-ITS2 region of *Trichaptum* species

primer	primer sequence (5'→3')				
NS1[a]	GTAGT	CATAT	GCTTG	TCTC	
NS2	GGCTG	CTGGC	ACCAG	ACTTG	C
NS3	GCAAG	TCTGG	TGCCA	GCAGC	C
NS4	CTTCC	GTCAA	TTCCT	TTAAG	
NS5	AACTT	AAAGG	AATTG	ACGGA	AG
NS6	GCATC	ACAGA	CCTGT	TATTG	CCTC
NS7	GAGGC	AATAA	CAGGT	CTGTG	ATGC
NS8	TCCGC	AGGTT	CACCT	ACGGA	
NS19SNU[b]	CCGGA	GAGGG	AGCCT	GAGAA	AC
ITS2[c]	GCTGC	GTTCT	TCATC	GATGC	
ITS3	GCATC	GATGA	AGAAC	GCAGC	
ITS4[a]	TCCTC	CGCTT	ATTGA	TATGC	
ITS5	GGAAG	TAAAA	GTCGT	AACAA	GG

[a] Primers used for both PCR amplification and DNA sequencing.
[b] NS19SNU was a modified primer by replacing A with G at the eighth position of NS19UCB.
[c] ITS1 was omitted in the experiment because it is located so close to ITS5 that the product overlaps with that of ITS5.

cipitated by 1/10 volume of 3 M sodium acetate and 3 volume of EtOH.

DNA cloning and sequencing

PCR-amplified products were cloned into T-vectors to secure high purity DNA. T-vectors were constructed by digesting a Bluescript plasmid KS(+) with *Eco*RV and incubating with *Taq* polymerase (POSCOCHEM) in the presence of dTTP (17, 31). The plasmid was self-ligated by T4 DNA ligase and the resulting ligated mixture was electrophoresed on a low-melting temperature agarose gel. This mixture was used for transformation by the method of Sambrook *et al.* (41) and the transformed DNA was extracted by alkaline lysis method.

The dideoxy chain termination procedure (42) was applied to sequence extracted DNAs, using primers designed by White *et al.* (46) and the primer NS 19SNU (Table 2), a modification of NS19UCB (13). And the Sequenase version 2.0 (United States Biochemical) was used and reaction conditions were according to supplier's instructions.

DNA sequence analyses

Eight 18S rDNA sequences from *Trichaptum* strains were compared with sequences of 15 species retrieved from the EMBL/GenBank databank using *Saccharomyces cerevisiae* as an outgroup (Table 1). Sequences were first aligned using CLUSTAL V (19) and, for ITS1-5.8S-ITS2 sequences, the alignment was visually optimized and gaps were treated as missing data. In phylogenetic reconstructions for 18S rDNA and ITSs, the heuristic search option of parsimony algorithms of PAUP 3.1.1 (45) was applied using the stepwise addition option of the heuristic search. The calculations were done with the tree-bisection-reconnection (TBR) branch swapping performed on starting trees, the mulpars option put in effect, the steepest descent option not put in effect, the initial max-trees set to 100, and zero length branches set to collapse to yield polytomies.

Bootstrap replications were done 500 times to evaluate confidence levels on individual branches of trees. The skewness of tree-length distributions of random trees based on g_1 statistics (20, 21) was examined to measure the quality of sequence data and to estimate the meaningfulness of phylogenetic information. Assessments of transition and transversion base substitutions in sequence data from 23 fungi and from *Trichaptum* species only were done using the list of changes option in DESCRIBE TREES of parsimony algorithms.

Results and Discussions

DNA extraction

The method of Lecellier and Silar (26) was compared with the old protocol of Raeder and Broda (37), in which mycelium harvested from liquid medium is lyophilized and ground in liquid nitrogen to break cells with a pestle. The former one proved to be much more effective in rapid and complete maceration of rigid fungal cell walls under a microscope and assured a good yield of extracted DNA for the experiment.

18S rDNA variability

PCR products covered all the regions from 18S rDNA to ITS2 and ranged between 2333 and 2555 kb in lengths. Aligned sequences of the 18S rDNA region from eight *Trichaptum* strains contained 1741 nucleotides and had a total of 549 variable sites (31.5%) and 341 informative sites (19.6%). They were aligned with 14 basidiomycetes using *Saccharomyces cerevisiae* as an outgroup and then 13 equally parsimonious trees were obtained, each requiring 892 nucleotide substitutions. The bootstrap consensus tree of the parsimony method was identical to the strict consensus tree of 13 equally parsimonious trees, except for the branch for *Thanatephorus praticola*, which collapsed in the bootstrap analysis (Fig. 1).

The class Hymenomycetes formed one cluster and the family Polyporaceae a smaller one within the class and the statistical support for these clust-

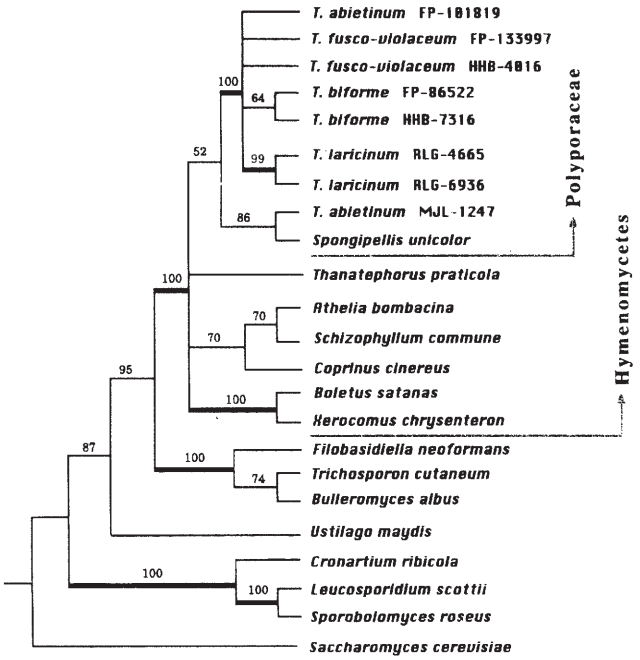

Fig. 1. Phylogenetic tree constructed using parsimony analysis with the heuristic search method of PAUP 3.1.1. It is a bootstrap consensus tree generated from 500 bootstrap replications of 18S rDNA sequences. This tree is identical to the strict consensus tree of 13 equally parsimonious trees requiring 892 nucleotide substitutions, except for the branch for *Thanatephorus praticola*, which collapsed in bootstrap analysis. The tree has a tree length of 1136 steps, a consistency index of 0.659, and a retention index of 0.650. Bootstrap values are given above branches supported by more than 50% and bold lines are used where branches were significantly supported by more than 90%.

ers was 100% and 52 % confidence level for each. All the strains of *Trichaptum* were grouped together and strongly supported by 100% bootstrap values, except *for T. abietinum* MJL-1247, which made up a different group with another species of the Polyporaceae, *Spongipellis unicolor*. However, the branch separating *T. abietinum* MJL-1247 from other *Trichaptum* species was weakly supported in bootstrap analyses.

The skewness of tree-length distributions has been suggested as an indicator of phylogenetic information and a strong left skewness ($g_1<0$) as a good indication of the presence of phylogenetic information (20, 21). When the tree-length distribution of 1,000,000 random trees was analysed, the 18S rDNA data set was highly skewed to the left as estimated by $g_1=-1.33$ (Fig. 2). This suggested that the included data contained good phylogenetic information and that the most parsimonious trees were close to the real tree.

ITS1-5.8S-ITS2 region variability

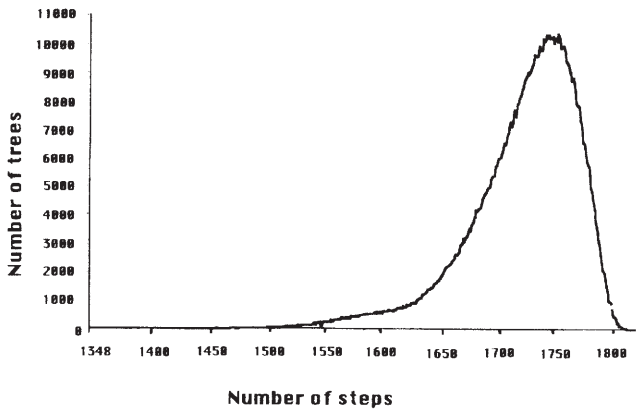

Fig. 2. Tree-length distribution of 1,000,000 random trees generated from possible 5.91×10^{10} rooted trees using the exhaustive and random trees option of PAUP 3.1.1. Tree lengths ranged from a minimum of 1400 to a maximum of 1820. The left skewness measured by the g_1 statistics was -1. 33, which was noticeably more skewed than random data whose critical value was -0.09 at P=0.01.

Sequences of the ITS1-5.8S-ITS2 region from *Trichaptum* species are presented in Fig. 3. They were very variable in lengths and especially the ITS1 region of *T. fusco-violaceum* was 180 bp longer than other species on the average. The ITS1 region eventually increased the length of its PCR products. Sequences of the ITS1 region were 202 to 441 bp in length and aligned in 460 positions, of which 101 positions were optimally aligned with 40 variable sites (39.6%) and 17 informative sites (16.8%). Sequences in the ITS2 region were 215 to 265 bp in length and aligned in 278 positions, of which 227 positions were optimally aligned with 101 variable sites (44.5%) and 60 informative sites (26.4%). Variation in ITS sequences was conspicuously small or almost absent within the same species of *Trichaptum*, suggesting a practical application of ITS sequences for the characterization of species. Sequences of the 5.8S rDNA region were 101 to 105 bp in length with only two variable sites and no single informative site, suggesting that this region had no phylogenetic significance.

Phylogenetic relationships of *Trichaptum* species

Based on sequences of 18S rDNA, the genus *Trichaptum* made up a single cluster and two strains of *T. biforme* and of *T. laricinum* made up a monophyletic group, suggesting that each of these *Trichaptum* species is phylogenetically separate and independent. However, two strains of *T. abietinum* that separated from each other made up a polyphyletic group and the branch for those of *T.*

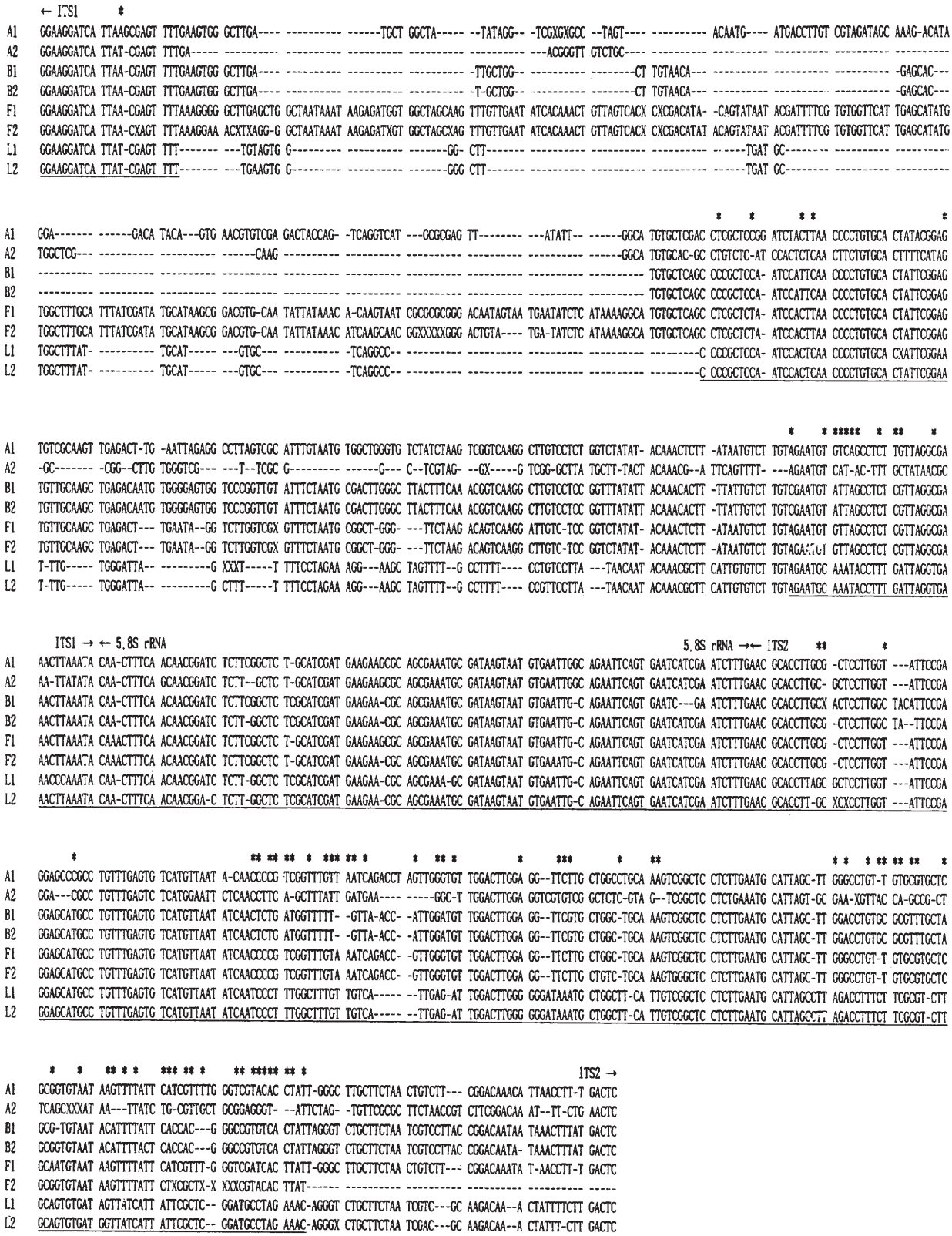

Fig. 3. Aligned sequences of the ITS1-5.8S-ITS2 region. Underlined parts indicate optimally alignable characters included in phylogenetic analyses. Phylogenetically informative sites were designated with asterisks, gaps during the alignment with dashes, and ambiguities within a strain with Xs. A1, *T. abietinum* FP-101819-Sp; A2, *T. abietinum* MJL-1247-Sp; B1, *T. biforme* FP-86522-Sp; B2, *T. biforme* HHB-7316-Sp; F1, *T. fusco-violaceum* FP-133997-Sp; F2, *T. fusco-violaceum* HHB-4016-Sp; L1, *T. laricinum* RLG-4665-Sp; L2, *T. laricinum* RLG-6936-Sp.

fusco-violaceum collapsed, forming a polytomous group. Present results are generally congruent with conclusions from recent studies for the phylogeny of *Trichaptum* based on RFLP analyses of mitochondrial DNA and PCR-amplified DNAs (23, 25). In case of the mitochondrial DNA, *T. fusco-violaceum* was believed to be under way to differentiation into two subgroups (23). Out of the PCR-amplified nuclear and mitochondrial DNAs, *T. abietinum* and *T. fusco-violaceum* were concluded to have more variations than other species of the genus (25).

T. abietinum strains apparently showed phylogenetic uncertainty and *T. abietinum* MJL-1247 consistently grouped with *S. unicolor* of the Polyporaceae in 18S rDNA analyses. It is true that *Trichaptum* and *Spongipellis* are partially related in that both have poroid hymenophores and cause white rot. However, they are fundamentally different in several ways. Members of the genus *Trichaptum* form abundant cystidia, while those of *Spongipellis* do not. *Trichaptum* has a dimitic or exceptionally trimitic hyphal system, but *Spongipellis* has a monomitic hyphal system of generative hyphae only. The spore shape of *T. abietinum* is cylindrical (16, 39), while it is ovoid to ellipsoid in *S. unicolor*. The above-mentioned characters are keys of prime importance in classification and may not vary easily from the viewpoint of evolution.

However, it is assumable that *T. abietinum* is a putative species and can be subdivided into two subgroups, possibly either subspecies or varieties. Macrae (29) and Magasi (30) once concluded that there are two or more incompatible groups within *T. abietinum*. Unfortunately, there are presently no anatomical characters available to discriminate poroid forms of *T. abietinum* from one another. When present and recent results were integrated together for a comprehensive understanding, it is predictable that *T. abietinum* and also *T. fusco-violaceum* could be in the process of evolutionary differentiation or divergence into more subdivisible physiological or geographic taxa. Thus, it can be noted that the current taxonomy of *Trichaptum* needs to be reexamined on the basis of new classification systems in the future.

Table 3. Frequency of unambiguous transition and transversion base substitutions in data sets of 18S rDNA and aligned ITS1-5.8S-ITS2 regions

base substitution type		18S rDNA		ITS1-5.8S-ITS2
		all taxa	*Trichaptum* only	*Trichaptum* only[a]
Transition				
G-A				22 (17.9)
	G>A	81 (12.8)[b]	2 (7.7)	
	A>G	53 (8.3)	3 (11.5)	
C-T				42 (34.1)
	C>T	145 (22.8)	8 (30.8)	
	T>C	98 (15.4)	7 (26.9)	
Transversion				
G-T				16 (13.0)
	G>T	35 (5.5)	–	
	T>G	27 (4.3)	1 (3.8)	
G-C				14 (11.4)
	G>C	32 (5.0)	–	
	C>G	27 (4.3)	–	
A-T				19 (15.4)
	A>T	37 (5.8)	–	
	T>A	49 (7.7)	2 (7.7)	
A-C				10 (8.1)
	A>C	21 (3.3)	1 (3.8)	
	C>A	30 (4.7)	2 (7.7)	
Transition/Transversion		2.92	6.67	2.17

[a] The substitution direction could not be determined due to lack of the appropriate outgroup for the ITS1-5.8S-1TS2 region.

[b] Numbers in parentheses are percentages of given base substitution types within corresponding taxon columns.

Base substitution biases

Distributions of observed base substitutions by examining character-state changes inferred only from common changes in 13 equally parsimonious trees and in the ITS1-5.8S-ITS2 data set are listed in Table 3 and diagrammed in Fig. 4. The observed transition to transversion ratio (T ratio) was greatly different between all taxa (T=2.92) and between *Trichaptum* species only (T=6.67) but slightly variable between 18S rDNA (T=2.92) and ITS1-5.8S-ITS2 regions (T=2.17). The observed values of G-A and C-T transitions in 18S rDNA sequences significantly deviated from the expected values (chi square, $P<0.01$). The C-T transition predominated among base substitution types. A C-T transition bias has been reported from ITS regions of *Gastrosuillus* and *Suillus* (1), *Fusarium* (36), *Talaromyces* (28), and from nuclear and mitochondrial small subunit rRNA genes of ten mushrooms (8). Such a bias was also observed in *Trichaptum* species and was considerably stronger than in other distantly related taxa.

In frequencies of base substitutions calculated from 18S rDNA sequences, the observed ratio of transition to transversion demonstrates that all taxa are slightly biased toward transitions while *Trichaptum* species appear even more biased. Brown *et al.* (6) and LoBuglio *et al.* (28) showed that an increased rate of transitions exists among recently diverged species and that this rate declines with extended divergence times. Such a de-

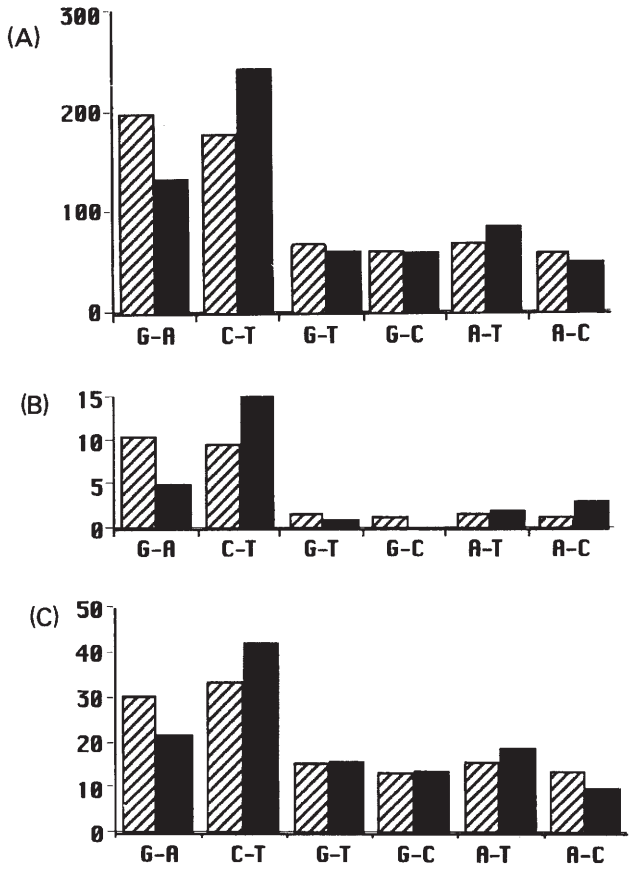

Fig. 4. Distribution of expected and observed base substitution values. (A) all taxa of 18S rDNA sequences. (B) *Trichaptum* species only of 18S rDNA sequences. (C) *Trichaptum* species only of ITS1-5.8S-ITS2 sequences. Observed values were inferred from character-state changes of 13 equally parsimonious trees and of the ITS1-5.8S-ITS2 data set. Expected values were calculated by the weighting method of Knight and Mindell (24), which assumes that the probability of a particular type of substitutions is proportional to the frequency of the bases involved. ▨; Expected base substitution values, ■; Observed base substitution value.

cline may be attributed to the fact that transversions destroy the prior record of transitions as they accumulate (1). This explains why strong transition biases are only observable when sequence divergence is low, i.e. among closely related organisms. When sequence divergence is assumed to predate species divergence (27), the predominance of C>T and T>C (unidirectionally C-T) transition substitutions throughout the examined sequences implies that *Trichaptum* species are closely arelated taxa so far, undergoing recent divergence in consequence of short-term evolution.

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