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Full Length Research Paper

Molecular phylogeny of *Trametes* and related genera based on internal transcribed spacer (ITS) and nearly complete mitochondrial small subunit ribosomal DNA (mt SSU rDNA) sequences

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Some species of *Trametes* and their related genera are so similar in microstructure characteristics that it is difficult to identify and separate them by traditional taxonomy. In this study, we elucidated relationships among *Trametes* through comparison of the nuclear internal transcribed spacer (ITS) and the nearly complete mitochondrial small subunit ribosomal DNA (mt SSU rDNA) sequences. Finally, phylogenetic trees were built. Phylogenetic analysis of the ITS and mt SSU rDNA sequences showed consensus results; most of the *Trametes* species clustered within a single clade and formed a main clade together with species of *Pycnoporus*. The genera *Coriolopsis* and *Cerrena* could be separated clearly from the main clade. The determination of secondary structure of the ITS1 and ITS2 domains of the representative species indicated that the conservative structure of some helices might be useful for resolving the phylogenetic relationship among some related species. The secondary structures of the variable domains V4 and V6 in mt SSU rDNA were also determined in this study. We found that both the conserved domains and the variable domains all contained informative sites which could significantly contribute to resolving the phylogenetic relationships within the *Trametes* group. The length and sequences of the V4 and V6 domains were found to be constant within a species, but very different among species with significant inter-species variations. The major advantage of these species-specific markers is that we only need to study one isolate from one species to determine their phylogenetic position. Secondary structure information of the V4 and V6 domains is found to be a useful marker to resolve phylogenetic problems. Based on our study, we considered that the genus *Pycnoporus* had a close relationship with *Trametes*; *Trametes trogii* was grouped with *Coriolopsis gallica*, indicating that it might belong to *Coriolopsis*; *Cerrena*, as a separate genus, was placed far away from *Trametes*.

Key words: *Trametes*, internal transcribed spacer (ITS) sequences, nearly complete mitochondrial small subunit ribosomal DNA (mt SSU rDNA), secondary structure, phylogenetic analysis.

INTRODUCTION

Trametes, as a representative genus of Polyporaceae, was established by Fries in 1835. It is widely distributed in nature and some species of *Trametes* have been used as traditional medicines in China. However, it is difficult to identify many species of *Trametes* and their related genera due to their highly similar morphological characteristics (Gilbertson and Ryvarden, 1987). Using hyphal tapes, Ryvarden classified 16 genera (*Cerrena*, *Coriolopsis*, *Cryptoporus*, *Daedaleopsis*, *Datronia*, *Earliella*, *Elmerina*, *Fomitella*, *Hexagonia*, *Lenzites*, *Megasporoporia*, *Microporus*, *Mollicarpus*, *Pycnoporus*, *Trametes* and *Trichaptum*) as belonging to the *Trametes* group (Ryvarden, 1991). However, based solely on such

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morphological characteristics, the general circumscription of *Trametes* and their related genera have always been confused (Ko and Jung, 1999a). Thus, *Trametes* is regarded as one of the most confused group of genera in Polyporaceae. Actually, the current taxonomy of *Trametes* has been questioned by Zhang et al. (2006).

In recent years, mycologist have attempted to use sequence data to resolve the taxonomic problems in *Trametes* and in the related genera (Ko and Jung, 1999a,b; Tomšovský et al., 2006; Zhang et al., 2006; Miettinen and Larsson, 2010). The internal transcribed spacer (ITS) and the partial sequences of mitochondrial small-subunit ribosomal DNA (mt SSU rDNA) had been analyzed to infer the phylogenetic position of various species of *Trametes*. In their phylogenetic study, Ko and Jung (1999a) considered *Trametes consors* as a species of *Cerrena*. They also considered *Trametes* and *Cerrena* as representing distinct genera in contrast to Corner (1989) who treated *Cerrena* as a synonym of *Trametes*. Based on ITS and large-subunit ribosomal DNA (LSU) sequence data, Tomšovský et al. (2006) found that all *Trametes* species formed a single clade except for *Trametes cervina*, and that the genus *Pycnoporus* grouped inside the *Trametes* clade. The species *Trametes gibbosa* grouped so close to *Lenzites betulina* that Tomšovský et al. (2006) reclassified *Trametes gibbosa* as *Lenzites gibbosa*. After comparing *Trametes versicolor*, *Trametes hirsuta* and *Trametes pubescens* based on ITS sequences, Zhang et al. (2006) noted that the results based on molecular phylogenetic analysis are consistent with morphology in *Trametes*, and that ITS sequences are useful in distinguishing the genera with similar morphological characteristics.

The ITS sequence is a significant value for classification in fungi because of its appropriate evolutionary rate (Su et al., 2007). However, the role of ITS maker has been debated because the domains ITS1 and ITS2 are removed in the course of rRNA maturation. The other main reason was due to the strong conservation of the core structure in the mature rRNA molecules, the ITS domain is much diverse in both sequence and size (Lalev and Nazar, 1998). Nevertheless, research in recent years has shown that the ITS domain plays a crucial role during rRNA maturation. Sande et al. (1992) found that some regions of ITS sequence formed a steady secondary structure which is critical to the RNA maturation. Good et al. (1997) showed that the ITS2 domain was not only critical to the maturation of the large subunit but also had severe effects on the levels of the 18S rRNA. Mai and Coleman (1997) proposed that the secondary structure possessed information that participates and direct endonucleolytic enzymes to the proper cut sites.

Sometimes, ITS domain is so variable that it is often used in the systematics of species within a genus (Moncalvo et al., 1995 a, b; Yan et al.,1995). Thus, new markers are needed to resolve phylogenetic problems at a family level. The mt SSU rDNA has a moderate evolutionary rate, and it is considered to be a good marker to resolve phylogenetic conflict, however, mt SSU rDNA was reported to contain 9 variable domains, and it has been difficult to design primers to amplify these domains (Hong et al., 2002). Because of this, only partial sequences of mt SSU rDNA could be used in phylogenetic analysis. Unfortunately, the sequence length of these domains is short and they contain few informative sites, so they are not commonly used among molecular systematists (Hong et al., 2000). In recent years, Gonzalez and Labarère (2000) found that three variable domains (V4, V6 and V9) possessed long nucleotide extensions, and that kind of mutation seemed to be species-specific. Hong et al. (2002) designed universal primers for polypore fungi to amplify the nearly complete sequences of mt SSU rDNA, and the sequences of *Ganoderma* and related species were amplified successfully by using those primers. Hong et al. (2002) proposed that phylogenetic information was contained both in the variable domains and the conserved domains, and that the secondary structure information of variable domains was also a useful marker in delineation of phylogenetic groups.

The objective of this study was to examine the phylogenetic relationships among some species of the *Trametes* group based on nuclear ITS and the nearly complete mt SSU rDNA sequences. The secondary structure of some domains in the two sequences was constructed to help us find a useful marker to resolve the phylogenetic problems with the *Trametes* group.

MATERIALS AND METHODS

rTaq DNA polymerase, PCR purication kit, pMD-18T vector and *Escerichia coli* JM109 strain were purchased from TaKaRa (Dalian, China). All other chemicals were of analytical grade or highest quality.

All isolates used in this study are listed in Table 1. The sequences of species analyzed in this study are marked with asterisks, the other sequences previously published were retrieved from the database of GenBank. All the fungi were cultured on PDA (20% potato juice, 20 g/L glucose, 3.0 g/L KH_2PO_4, 1.5 g/L $MgSO_4 \cdot 7H_2O$) at 28°C for 7 to 10 days. Then, mycelia were transferred to the PDA liquid medium and cultured at 28°C with agitation of 150 r/min for another 7 days. After centrifugation, mycelia were harvested and then washed with phosphate buffered saline (PBS) twice and dried in sterile air.

DNA isolation and PCR amplification

For extraction of fungal DNA, 50 mg of mycelia were ground into a powder in liquid nitrogen. Afterwards, each sample was transferred to a 1.5 ml centrifuge tube and then resuspended in 0.8 ml of lysis buffer (100 mM Tris-HCl [pH = 8.0], 2 mM EDTA [pH = 8.0], 1.4 M NaCl, 2% CTAB) and incubated at 65°C for 30 min. Then, 0.8 ml phenol-chloroform (1:1) was added to each tube and the suspension was shaken vigorously. After centrifugation at 10000 g for 10 min, the supernatant was transferred to a new tube and extracted again using phenol-chloroform. The samples were suspended in two volumes of ethanol and kept at -40°C for 1 h; then centrifuged at 10000 g for 10 min. The resulting final precipitate was dissolved in Tris-EDTA (TE) buffer.

Table 1. Fungal isolates for ITS and mt SSU rDNA sequences analysis.

Species	Origin	GenBank accession no.
Trametes suaveolens	CFCC	EU661885* /FJ591060*
Trametes velutina	CFCC	EU661886* /FJ591062*
Trametes versicolor	Our laboratory	EU661891* /FJ591061*
Trametes versicolor	Our laboratory	FJ591065*/ FJ591059*
Trametes versicolor	Our laboratory	FJ591066*/ FJ591057*
Trametes elegans	Our laboratory	EU661879* /FJ591058*
Pycnoporus sanguineus	Our laboratory	EU661890* /FJ591063*
Cerrena unicolor	Our laboratory	EU661887* /FJ591064*
Coriolopsis trogii	CGMCC	EU661876* /FJ591056*
Trametes versicolor	—	AY309016 / AF042324
Pycnoporus cinnabarinus	—	AF363757 / AF214465
Pycnoporus sanguineus	—	AF363758 / —
Pycnoporus sanguineus	—	AF363759 / —
Pycnoporus coccineus	—	AF363760 / —
Pycnoporus coccineus	—	EU520116 / —
Trametes suaveolens	—	AY684180 / U27079
Trametes suaveolens	—	EF491003 / —
Trametes hirsuta	—	AF516556 / AF042154
Trametes hirsuta	—	AY968077 / —
Trametes villosa	—	— / AF042325
Trametes elegans	—	AY684178 / —
Trametes ochracea	—	AY684177 / —
Trametes ochracea	—	EU162063 / —
Trametes orientalis	—	EU771082 / —
Trametes maxima	—	AB158315 / —
Cerrena consors	—	— / AF042821
Cerrena unicolor	—	DQ056858 / AF089822
Cerrena unicolor	—	EF577058 / —
Coriolopsis gallica	—	AY684172 / AF042152
Coriolopsis trogii	—	AJ438139 /AF358744
Coriolopsis trogii	—	DQ912696/ —
Coriolopsis caperata	—	EU030180 / —
Coriolopsis caperata	—	EU030179 / —
Auricularia auricular-judae	—	EU427047 / —
Ganoderma lucidum	—	— /AF214468

The GenBank accession number marked with asterisks were determined and submitted in this study, the others were download from GenBank; the first number refers to the ITS sequence and the second one the mt SSU rDNA sequence.

The nuclear rDNA ITS region was amplified using the primers ITS1/ITS4 (White et al., 1990), and the PCR amplification was performed as follows: 94°C for 3 min followed by 30 amplification cycles consisting of a denaturation step of 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. At the same time, primers BMS05/BMS173 were used to amplify the most complete sequences of the mitochondrial small-subunit rDNA (Hong et al., 2002), the PCR amplification was performed as follows: denaturation at 94°C for 3 min followed by 30 amplification cycles consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR products were purified and then cloned into pMD18-T vectors. Cloned DNAs were sequenced by the dideoxy chain termination method (Sanger et al., 1977).

DNA sequence analysis

DNA sequences were aligned with the program of Clustal X 1.81. Phylogenetic analyses were performed using programs contained in Phylip 3.67. The software RNA structure 4.6 was used to predict the secondary structure of ITS1 and ITS2 domain in ITS sequences, and V4, V6 domain in mt SSU rDNA sequences.

RESULTS

Phylogenetic analysis based on the ITS sequences

An approximately 600 bp ITS rDNA fragment was

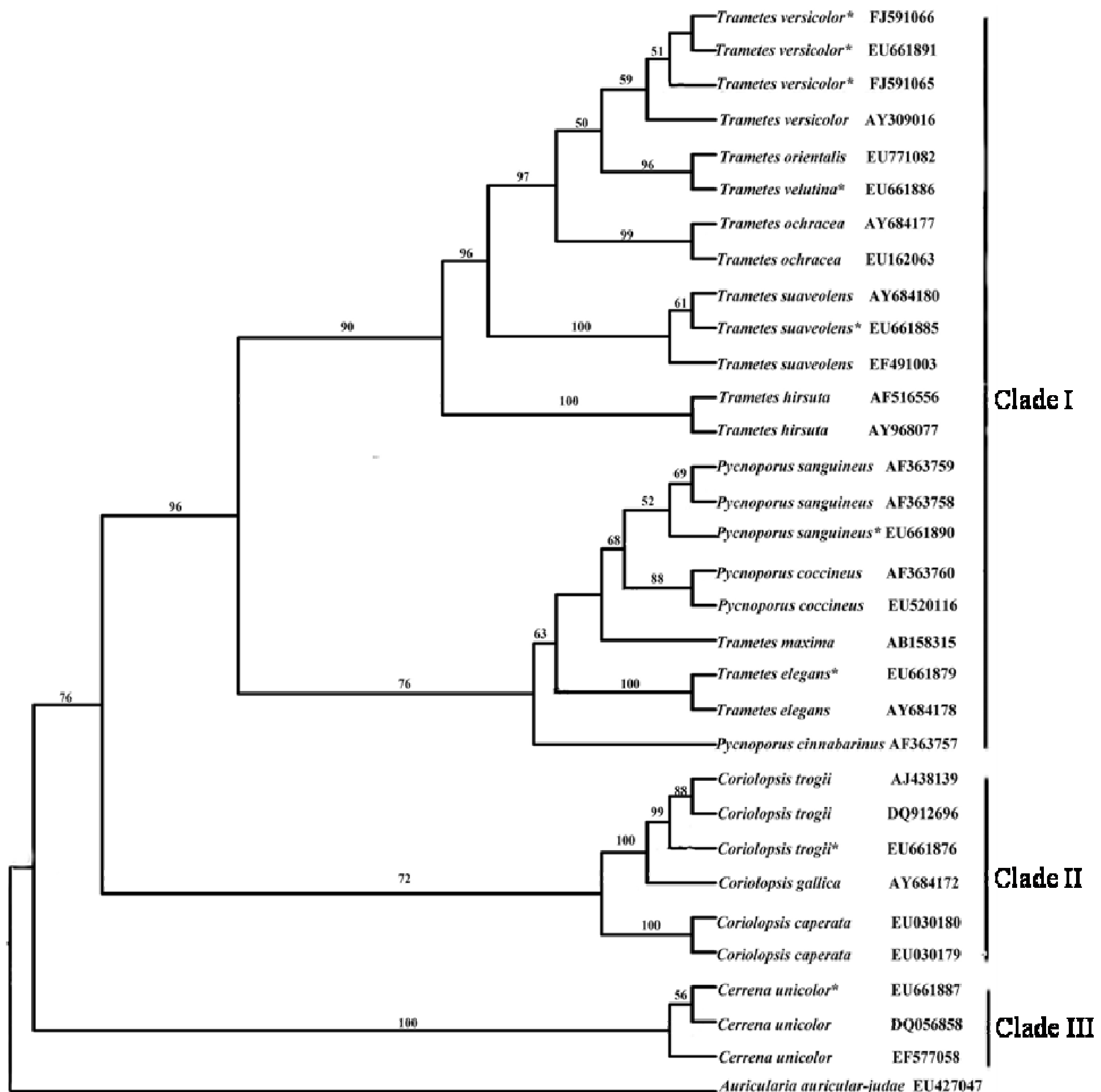

Figure 1. Consensus tree obtained by the maximum parsimony method based on the ITS sequences. *Auricularia auricular-judae* was used as an outgroup to root the tree. Bootstrap values, more than 50% from 1000 replications, are shown in the branches. The sequences from 9 strains determined and submitted in this study are marked with asterisks at the end of the species names.

sequenced from all isolates used in this study. Besides these, another 22 sequences obtained from GenBank were also used to construct the tree, and the sequence of *Auricularia auricular-judae* was used as an out-group. The consensus tree was built using the maximum parsimony method. In the tree, 31 isolates formed three main clades. In the first clade, all 18 strains from *Trametes* and 6 strains from *Pycnoporus* grouped together and could be separated from the groups of *Cerrena* and *Coriolopsis*. *Trametes versicolor*, *Trametes orientalis*, *Trametes velutina*, *Trametes ochracea*, *Trametes suaveolens* and *Trametes hirsuta* grouped together and formed the first sub-group in the main clade. In the second sub-group, all the strains from *Pycnoporus* were grouped with *Trametes maxima* and *Trametes elegans*, and the bootstrap value for this branch was 76%. The genus *Pycnoporus* was very close to *Trametes*. In the second clade, three species from *Coriolopsis* grouped together and the bootstrap value for this branch was 72%. *Coriolopsis* could be separated from *Trametes* very clearly. *Cerrena unicolor* grouped in the third clade, and was phylogenetically distinct from *Trametes* (Figure 1).

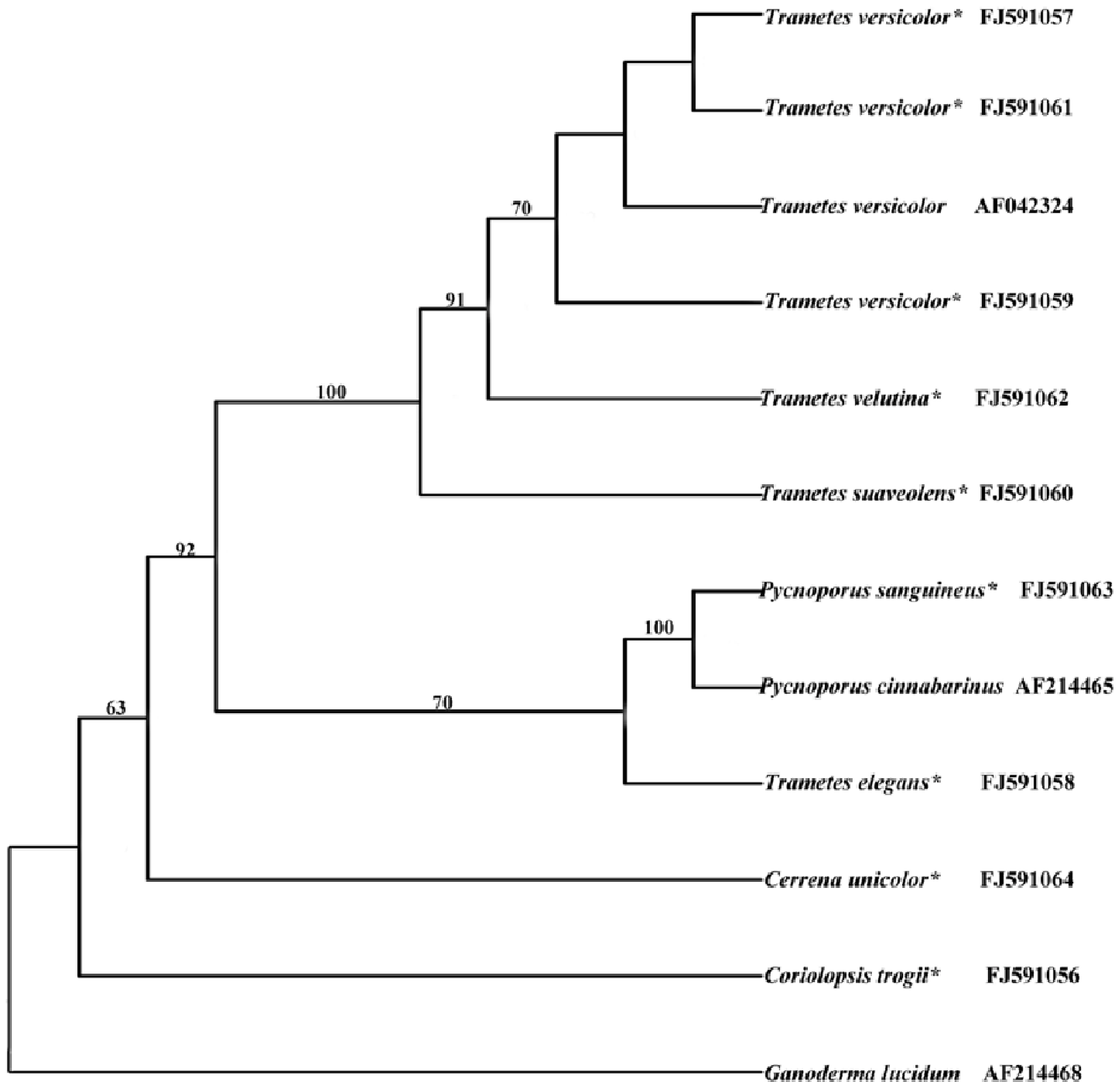

Figure 2. Consensus tree obtained by the maximum likelihood method based on almost full-length of mt SSU rDNA. *Ganoderma lucidum* was used as an outgroup to root the tree. Bootstrap values, more than 50% from 1000 replications, are shown in the branches. The sequences from 9 strains determined and submitted in this study are marked with asterisks at the end of the species names.

Phylogenetic analysis based on both the most complete and partial mt SSU rDNA sequences

The PCR fragments of the mt SSU rDNA regions from the 9 specimens sequenced ranged from 1470 to 1632 bp in length, and the consensus tree was built using the 11 most complete sequences of mt SSU rDNA that were determined by us and obtained from GenBank. However, because the complete mt SSU rDNA sequences data of *Trametes* are currently unavailable, we used partial sequence data with more data in GenBank to construct the tree. Both trees generated used *Ganoderma lucidum* as an outgroup. Phylogenetic analysis of the SSU sequences showed that the relationships among these fungi were similar to those found in the ITS analysis. In the tree based on the most complete sequences of mt SSU rDNA, three *Trametes* species (*T. versicolor*, *T. suaveolens* and *T. velutina*) formed the first sub-group in the first clade. *Pycnoporus cinnabarinus* and *Pycnoporus sanguineus* were grouped with *T. elegans*, as previously noted by Zhang et al. (2002), the genus *Pycnoporus* groups phylogenetically close to *Trametes*. The two species *Coriolopsis trogii* and *Cerrena unicolor*, occurs on a single branch each at the base of the tree separate from *Trametes* and *Pycnoporus* (Figure 2). In the tree based on the partial sequences of mt SSU rDNA, *P.*

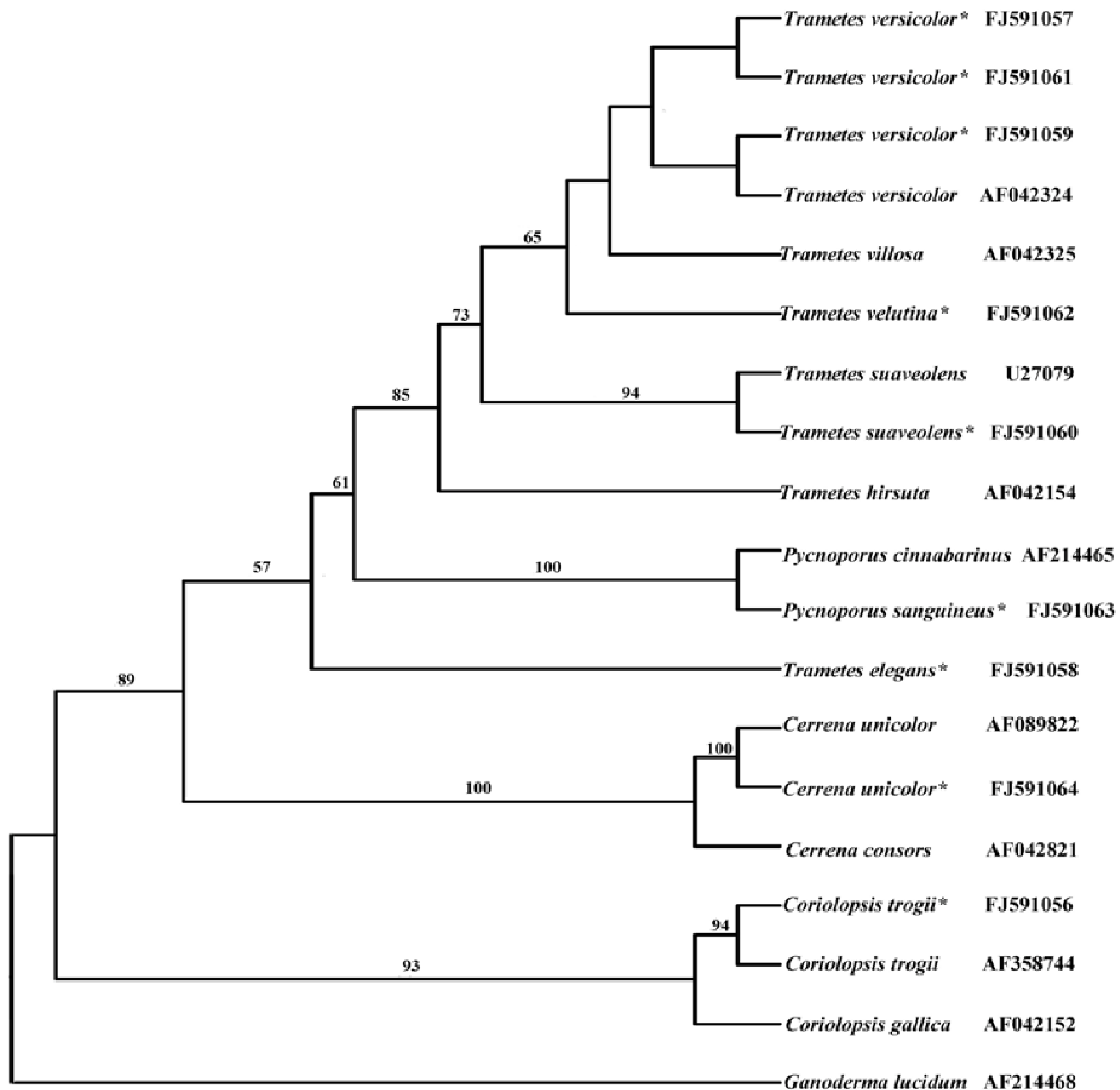

Figure 3. Consensus tree obtained by the maximum likelihood method based on partly sequences of mt SSU rDNA. *Ganoderma lucidum* was used as an outgroup to root the tree. Bootstrap values, more than 50% from 1000 replications, are shown in the branches. The sequences from 9 strains determined and submitted in this study are marked with asterisks at the end of the species names.

cinnabarinus and *P. sanguineus* grouped with *Trametes* taxa. *Cerrena unicolor* and *T. consors* fell outside of the large *Trametes/Pycnoporus* clade, *T. consors* was considered to pertain to *Trametes*, however, in this study, it grouped with *C. unicolor* with a high bootstrap value (100%). *C. trogii* and *Coriolopsis gallica* constituted the final clade, with a bootstrap value of 96% (Figure 3).

Comparison of the secondary structures of the ITS1 and ITS2 domains

Three groups of putative secondary structures were observed in the ITS domain and were characterized by the presence or absence of the helices C2 and C4 and by the structure and length of the helix C3. In the first group (using *T. versicolor* and *P. sanguineus* as representative species), *T. versicolor* and *P. sanguineus* had a similar structure consisting of three helices, however, *P. sanguineus* had a slightly longer C3 helix. The helices C2 and C4 were absent in *C. trogii*, while the C3 helix in it was longer and more complex than in other genera, the 14 nucleotides from positions 148 to 161 provided *C. trogii* an additional small helix in the C3 domain. *C. unicolor* had a C2 helix, and this structure was not found in other genera (Figure 4).

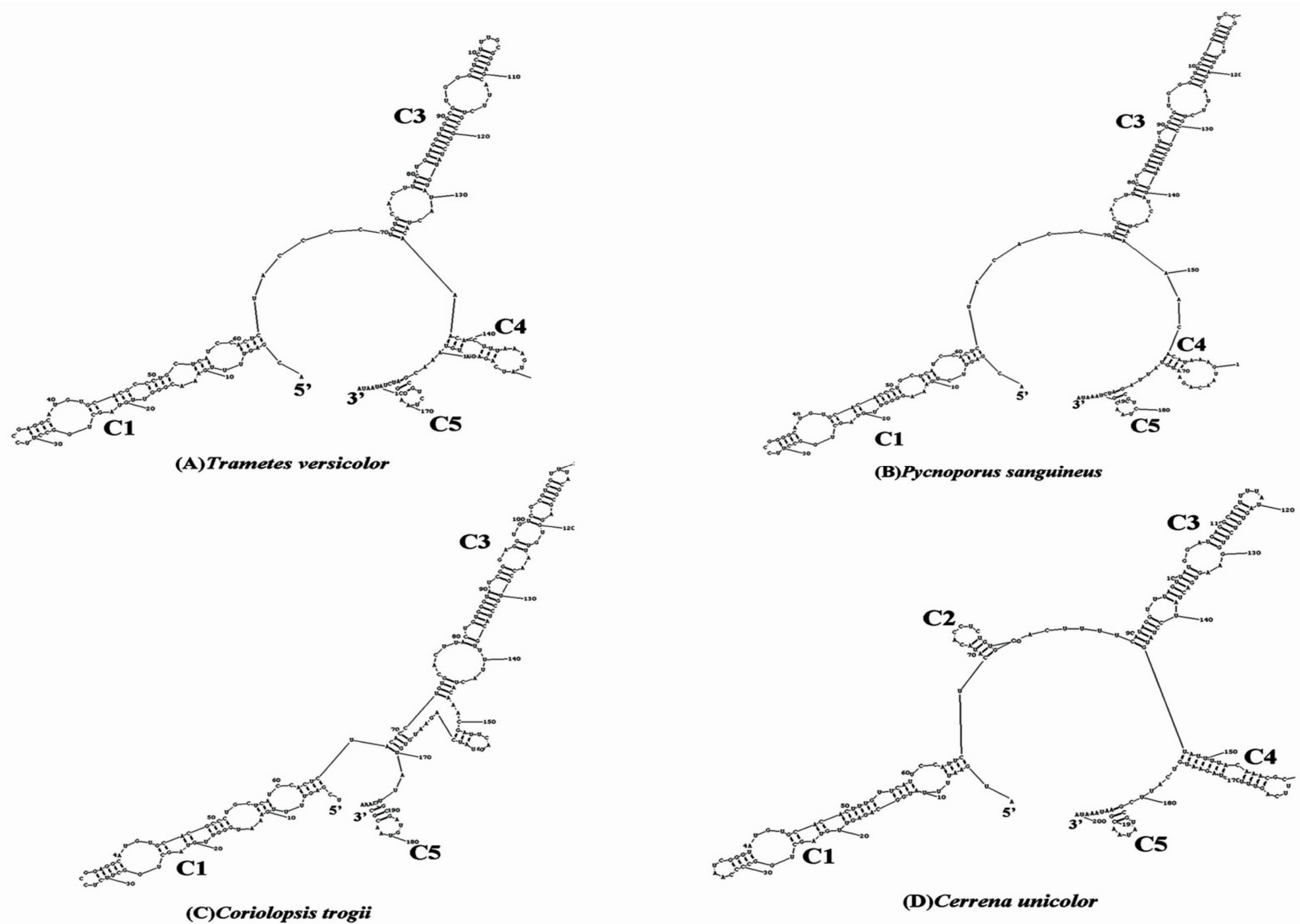

Figure 4. Secondary structures of the ITS1 domain of the 4 strains designated as the representative species of the 4 genera.

The secondary structure of the ITS2 domain also segregated all the genera into three groups. The first group (consisting of *T. versicolor* and *P. sanguineus* as the representative species) had three helices. Helices D1 and D4 were simple and conserved, while helix D3 was so complex and had many internal loops. Remarkably, the D3 domain had three small helices at the top of the domain that were constituted by nucleotides from positions 70 to 140. In the second group (using *C. trogii* as the representative species), the D3 domain was different from the first group in that the nucleotides from positions 73 to 97 were unpaired and formed a large loop, and only two helices were present at the top of the D3 domain. The third group (using *C. unicolor* as the representative species) had an additional D2 domain with a simple structure of D3 domain (Figure 5).

Comparison of the secondary structures of the V4 and V6 domains

The length of the V4 domain ranged from 45 to 53 bp, and the sequences of this domain were conserved within the same species but variable among species because of multiple insertion/deletion events. Unfortunately, the length of this area is so short that the secondary structures of all strains possessed a canonical topology without additional helices. However, some differences could also be observed (Figure 6). In the four *Trametes* species (*T. versicolor, T. suaveolens, T. velutina* and *T. elegans*), the helix was constructed by two loops and stems. The secondary structure of *Pycnoporus* (*P. sanguineus* and *P. cinnabarinus*) was very similar to *Trametes*, small differences were observed that *Pycnoporus* had a smaller inter-loop than *Trametes*, and one nucleotide was unpaired in the second stem. For *C. trogii*, the nucleotides were composed of a hairpin loop that is very distinct from the *Trametes* species; for *C. unicolor*, the length of the V4 domain was longer than other genera, with a longer stem and larger internal loop. At the top of the structure, three consecutive pairs of nucleotides were composed than other genera, so it had longer stem and bigger internal loop, at the top of the structure, three consecutive pairs of nucleotides

Figure 5. Secondary structures of the ITS2 domain of the 4 strains designated as the representative species of the 4 genera.

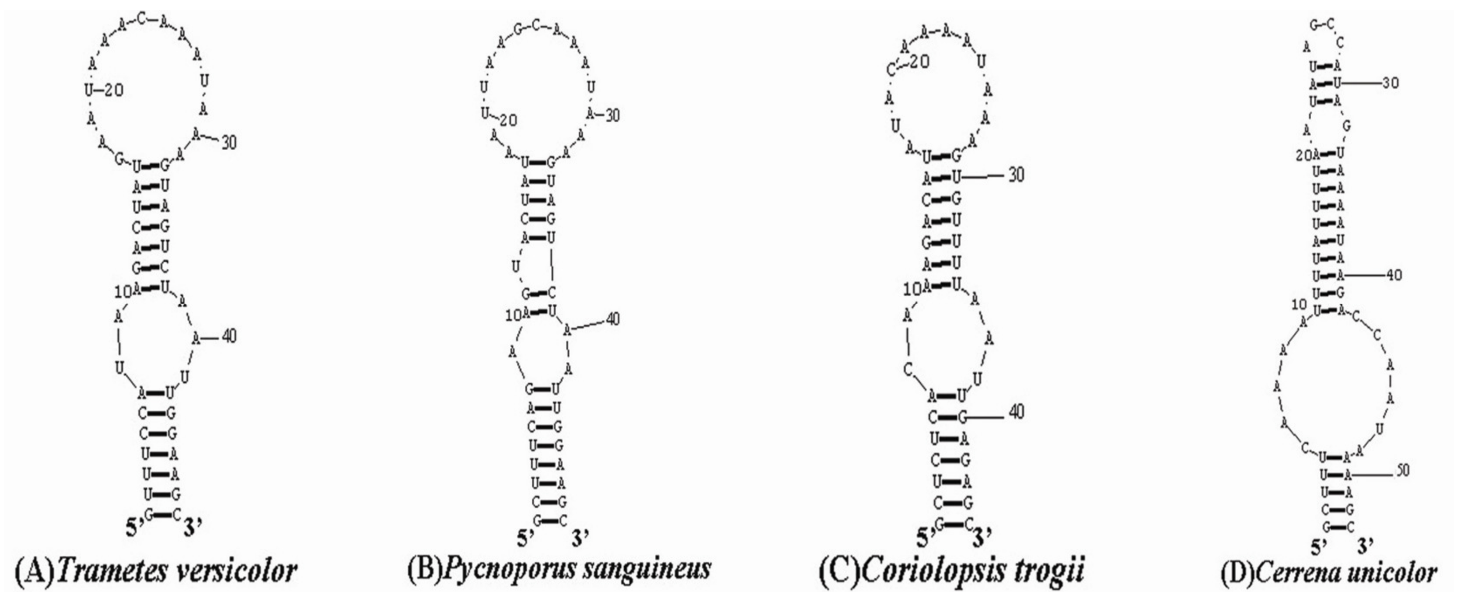

Figure 6. Secondary structures of the V4 domain of the 4 strains designated as the representative species of the 4 genera.

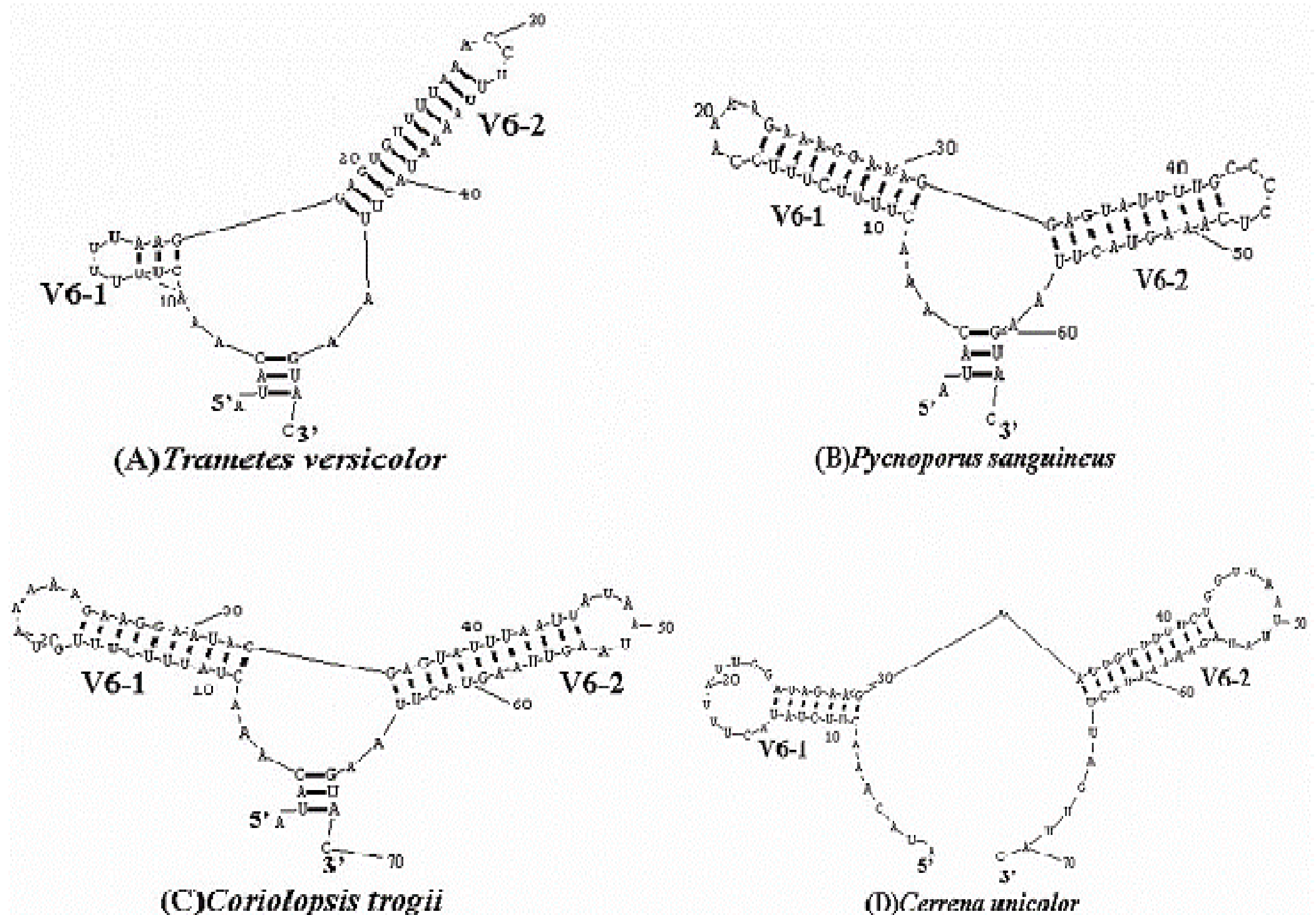

Figure 7. Secondary structures of the V6 domain of the 4 strains designated as the representative species of the 4 genera.

constituted a short stem, and the hairpin loop was smaller than in others (Figure 6).

The length of the V6 domain ranged from 49 to 71bp, and the secondary structure of this domain was characterized by the length and configuration of the two helices V6-1 and V6-2 (Figure 7). The first group contained species of *Trametes* (using *T. versicolor* as the representative species), the secondary structure of *T. versicolor* was simple and conserved because only 49 nucleotides formed the V6 domain. The helix V6-1 was only constituted by a stem with 3 base pairs and a small hairpin loop. The stem of the V6-2 was longer than others. A characteristic of the second group (*P. cinnabarinus* and *P. sanguineus*) was a longer stem in V6-1 that was composed of 10 bp and the structure was more stable. The third group (*C. trogii*) was similar to the first group, but had a longer stem in the helix V6-2, and the nucleotides, constituting the hairpin loop in the V6-2 domain were different from those in the first group. In the forth group (*C. unicolor*), the nucleotides in the 5' and 3' terminal were unpaired, and the hairpin loops in V6-1 and V6-2 were larger than those in other groups (Figure 7).

DISCUSSION

Relationship between *Trametes* and their related species based on the the ITS sequences and mt SSU rDNA sequences

The phylogenetic relationships between *Trametes* and their related genera were examined based on the ITS and the mt SSU rDNA sequences. The results obtained from both datasets were consistent, providing two lines of evidence to support conclusions about some of the species' new phylogenetic placing.

Maximum parsimony analyses based on ITS sequences and the maximum likelihood analyses based on mitochondrial sequences showed that most of the *Trametes* species grouped together with species of *Pycnoporus*. Secondary structure based on both sequence datasets showed that most species in *Trametes*

also had the same secondary structure as *Pycnoporus*. Our study confirmed that the *Pycnoporus* groups were phylogenetically close to *Trametes*. *C. trogii*, once considered to be a *Trametes* sp., grouped within the genus *Coriolopsis* in both the ITS and mt SSU rDNA tree, and bootstrap values of the respective branches were both above 95%, providing a clear separation from the *Trametes* clade. The secondary structure of ITS1 and ITS2 showed that *C. trogii* had a very different structure from *Trametes*. Based on these findings, our results confirmed that *C. trogii* does not group with *Trametes*, and that species in *Trametes* and *Coriolopsis* are pylogenetically distinct.

Ryvarden (1991) considered *C. unicolor* to be representative of species in the *Trametes* group, and Corner (1989) regarded *Cerrena* as a synonym of *Trametes*. However, Ko and Jung (1999a) found that *C. unicolor* was distinct from the genus *Trametes*. Miettinen and Larsson (2010) found that *C. unicolor* belonged to the Meruliaceae, and it grouped with *Merulius tremellosus*. In this study, *C. unicolor* could be clearly separated from the *Trametes* clade, a result strongly supported by the high bootstrap value present in all the phylogenetic trees. The secondary structure of ITS1 and ITS2 domains showed that *C. unicolor* possessed more helices than other species. The structure of V4 and V6 domains were also distinct from others. All the evidences indicate that *Cerrena* evolved independently from the lineage forming the *Trametes* genus.

The feasibility about the ITS secondary structure

In this study, the secondary structure of ITS sequences was consistent with the phylogenetic tree. Sequence mutations in the ITS domain are problematic during sequence alignment. However, these mutations do not affect the secondary structure. The length variation of the ITS domain might lead to a slight change of the structure of the helical structure which does not affect the stability of the entire structure. Both A and G can be paired with U in the secondary structure model, meaning that the secondary structure is not modified when a base mutation A to G occurs. These characters are obviously noted when comparing the ITS1 structure of *T. versicolor* and *P. sanguineus*. The length of the ITS1 domain in *P. sanguineus* was 195 bp, while it was only 184 bp in *T. versicolor*. The primary reason for the difference in the sequences was two insertion events at nucleotide positions 100 to 105 and 114 to 118 in *P. sanguineus*. However, these two insertion events were paired exactly in the C3 helix in *P. sanguineus*, so that those base pairs that increased the stem length in the C3 domain did not affect the secondary structure stability. Simultanously, the base mutation between A to G did not affect the structure of ITS1 either. Based on the earlier findings, we concluded that the secondary structure of ITS domain is useful in resolving the phylogenetic conflict.

Advantage of using the almost complete sequences of mt SSU rDNA to reserve the phylogenetic problems with *Trametes* group

The mt SSU rDNA is considered as an important molecular marker to resolve phylogenetic relationships at a familial level. However, only partial sequences of this domain have been used in most studies because there are 9 variable domains in the sequence and it is difficult to find universal primers that amplify the entire sequence. Unfortunately, phylogenetic analysis using partial sequences of mt SSU rDNA proved unsatisfactory because of the lack of phylogenetic informativeness. For this reason, mt SSU rDNA sequences are not in common use among molecular systematists. In recent years, however, Hong and Jung (2004) designed pairs of primers for polypore fungi and almost the entire full-length mt SSU rDNA could be amplified.

In the mt SSU rDNA sequence, site variations of the variable domains were more abundant than the conserved domains, so the phylogenetic informativeness was concentrated in the variable domains. Gonzalez and Labarère (1998) considered the V4, V6 and V9 as the most variable domains where the insertion/deletion events could always be observed. However, the 9 strains that we investigated had many sequence mutations in the V4 and V6 domains but few base changes in the V9 domain. Gonzalez and Labarère (2000) found in *Pleurotus* that the V4, V6 and V9 domains in all strains of the same species possessed exactly the same domain length, while there were always large mutations among species. A similar conclusion was also drawn in this study. For example, the V6 domain in 9 strains ranged from 49 to 71 bp, and there was a high rate of mutation in this domain. However, the four strains of *T. versicolor* had identical sequences for the V6 domain and no mutation was observed. *T. suaveolens* and *T. velutina* also had the same nucleotide length as *T. versicolor*, but point mutations were observed in the nucleotides at positions 31 and 33 in the respective species. The variable domain was shown as a useful species-specific marker for taxonomy and phylogeny of Basidiomycota. As is already known, the ITS domain was so variable that point mutations and the insertion/deletion events are easy observed in the same species. Thus in many cases, numerous isolates from the same species are required to resolve the phylogenetic conflict. Because of the nucleotide conservation in the V4 and V6 domain of the same species, only one isolate of each species is required to determine phylogenetic relationships among species.

The analysis of the secondary structure of the V4 and V6 domains showed that structural information was useful in describing the relationship of *Trametes* and related species. Although the point mutations were

always found in the loop areas, they did not directly affect the stability of the secondary structure. The variety structure models could also be found because the insertion/deletion events occurred frequently in the variable domain. In this study, *Cerrena* could be separated from *Trametes* based on helical length and structure.

The primary differences in variable domains among species that had close phylogenetic relationships were point mutations. Thus, the secondary structure of these closely-related species was similar. For example, comparison of the secondary structure of the V4 domains between *T. elegans* and *T. versicolor* showed that there were several point mutations such as the second nucleotide position (U→C) and the sixth nucleotide position (C→U). Interestingly, those two mutations all occured in the stem domain and because both U and C can be paired with G, the mutations did not affect structure stability. Besides those two mutations, three base changes were found in the nucleotides at positions 18, 21 and 25. All three nucleotides were found in the loop area and also did not affect the secondary structure. However, for species with distant phylogenetic relationships, both point mutations and insertion/deletion events could be observed. For example, comparison of the secondary structure of the V6 domains between *T. versicolor* and *Cerrena unicolor* showed that there were three insertions in *Cerrena unicolor*, the first mutation from positions 15 to 29 changed the structure of the V6-1 helix: lengthening the stem and enlarging the loop so that it was bigger than *T. versicolor*. The second mutation at nucleotide positions 42 to 47 resulted to more unpaired bases in the V6-2 domain and decreased the secondary structure stability of the V6 domain. Both mutations affected the structure of the V6 domain, and it can help delineate *C. unicolor* from *Trametes*.

Another advantage of the use of the mt SSU rDNA sequences was that the conserved domains were helpful in resolving phylogenetic relationships. Since the sequences of variable domains had so many mutations and aligning the sequences proved difficult, the conserved domains were concatenated. Informative sites in these domains helped in sequence alignment and resolving phylogenetic relationships.

In conclusion, both ITS and mt SSU rDNA sequences contained valuable phylogenetic information and could be used as molecular markers to resolve phylogenetic problems among *Trametes* and related genera. Furthermore, the mt SSU rDNA could be used in both higher and lower taxonomic ranks as this region is composed of both conserved and variable domains. The V4 and V6 domains, which appear to be the species-specific markers, have the potential for wider use in subsequent studies. Secondary structure could be another useful marker and assisted greatly in the phylogenetic studies. We proposed that it should be considered as a very accessible method in determining phylogenetic relationships.

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