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Nucleotide Sequence Analysis of the 5S Ribosomal RNA Gene of the Mushroom *Tricholoma matsutake*

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From a cluster of structural rRNA genes which has previously been cloned (Hwang and Kim, in submission; *J. Microbiol. Biotechnol.*), a 1.0-kb *Eco*RI fragment of DNA which shows significant homology to the 25S and 5S rRNAs of *Tricholoma matsutake* was used for sequence analysis. Nucleotide sequence was bidirectionally determined using deletion series of the DNA fragment. Comparing the resultant 1016-base sequence with sequences in the database, both the 3′ end of 25S rRNA gene and 5S rRNA gene were searched. The 5S rRNA gene is 118-bp in length and is located 158-bp downstream of 3′ end of the 25S rRNA gene. IGS1 and IGS2 (partial) sequences are also contained in the fragment. Multiple alignment of the 5S rRNA sequences was carried out with 5S rRNA sequences from some members of the subdivision Basidiomycotina obtained from the database. Phylogenetic analysis with distance matrix established by Kimura's 2-parameter method and phylogenetic tree by UPGMA method proposed that *T. matsutake* is closely related to *Efibulobasidium albescens*. Secondary structure of 5S rRNA was also hypothesized to show similar topology with its generally accepted eukaryotic counterpart.

Key words: 5S rRNA, *Tricholoma matsutake*, phylogeny, secondary structure

Tricholoma matsutake (S. Ito et Imai) Singer is an ectomycorrizal fungus which belongs to Agaricales of the Basidiomycotina and grows symbiotically with the pine tree, *Pinus densiflora* (17, 18). Some of the 5S rRNA sequences have been determined from fungi (10, 15, 28), and most of them have been compiled (4, 22, 23). Distribution pattern of 5S rRNA genes in fungi can be sorted into two groups, the first group has most or all of the ribosomal RNA genes as components of rDNA cluster composed of 18S, 5.8S, 25S, and 5S, and the second group does not have 5S rRNA in the same rDNA cluster compared with other rRNA genes (29). In eukaryotes, transcription of 5S rRNA genes are dependent on RNA polymerase III, and involves more complex mechanisms not found in prokaryotes (6).

Phylogenetic analysis based on the 5S ribosomal RNA (rRNA) has long been used to elucidate the origin and evolution of a number of organisms (9, 13, 24). Some limitations exist because of the small amount of information in the 5S rRNA molecule (7); however, the easiness of isolation and sequence determination still enable it to be a good source of phylogenetic analysis. In our previous paper (Hwang and Kim, in submission), we reported the cloning of the ribosomal rDNA containing 18S, 5.8S, 25S, 5S, internal transcribed spacers (ITS1 and ITS2), and intergenic spacers (IGS1 and partial IGS 2). From the results of Northern hybridization, it has been identified that a 1.0-kb *Eco*RI digested fragment contained the 5S rRNA gene.

We here report the nucleotide sequence of 5S rRNA together with 3′ end of 25S rRNA, IGS1 and partial IGS2, phylogenetic analysis of *T. matsutake* based on 5S rRNA sequence, and putative secondary structure of 5S rRNA.

Materials and Methods

Strains and plasmids

The bacterial strains used for subcloning and M13 phage proliferation are *Escherichia coli* JM109 (*recA1, supE44, endA1, hsdR17, gyrA96, relA1, thi* △ (*lac-proAB*)/ F′[*traD36, proAB*$^+$, *lacI*^{q} *lacZ* △*M15*]) (30) and XL-1-Blue (*supE, hsdR, lac* /F′[*proAB*$^+$, *lacI*^{q} *lacZ* △*M15*] (3). The recombinant plasmid pTMr108 derived from the cloned *T. matsutake* rDNA fragment, was used for DNA sequencing. Plasmid vectors used for deletion reaction

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were pUC18 (Ap^r, lacZ, lacI), and two recombinant plasmids, pTMr108 and 108R (16).

Chemicals and enzymes

Most restriction enzymes, RNase, and T4 DNA ligase were obtained from Promega or Boehringer-Mannheim. Enzymes were used according to the recommendations of the manufacturers. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) were obtained from Sigma. The [α-^{35}S] dATP and autoradiography film were obtained from Amersham. For recovery of DNA fragment from agarose gel, GENECLEAN kit (BIO 101 Inc.) was used. Double-strand nested deletion kit was obtained from Pharmacia. DNA sequencing kits were from United States Biochemical (Sequenase version 2.0 kit) and from Boehringer-Mannheim (Taq DNA sequencing kit). All other chemicals and enzymes were reagent grade obtained from commercial sources.

DNA manipulation

Plasmids were isolated by method of SDS/alkaline lysis procedure used by Birnboim and Doly (2). Endonuclease digestion, ligation, transformation into *E. coli* cells by $CaCl_2$ method, and preparation of single-stranded DNA from M13 phage were done as described by Sambrook *et al.* (20)

Construction of deletion series

A 1.0-kb DNA insert carried on pTMr80, which hybridized to *T. matsutake S rRNA, was digested with Eco*RI and ligated into pUC18 vector, and two recombinant plasmids, pTMr108 and 108R, harboring the inserts in the opposite direction were obtained. Both plasmids were digested with *Pst*I and *Sal*I, and subjected to Exonuclease III and S1 nuclease digestion according to the manufacturer's instructions.

DNA sequence analysis

DNA sequencing was performed using deletion series by the dideoxynucleotide chain termination reaction method (21). In our previous paper, it was shown by Northern hybridization that the plasmid pTMr108 harbor the 5S rRNA gene. BLAST program (1) was used to search the homologous region to 5S rRNA sequences in the database. CLUSTALV, a multiple alignment program, was used extensively to try and locate significant homology in group of 5S rRNA sequences. Using the DNADIST and NEIGHBOR program (version 3.55c) of Felsenstein's PHYLIP package (5), the distance matrix by Kimura's two-parameter method (12, 14) and the phylogenetic trees for the 5S rRNA sequences were made. Secondary structure of 5S rRNA was also formulated according to the energy minimizing algorithm (11, 25).

Results and Discussion

5S rRNA and 3′ end of 25S rRNA

Restriction enzyme mapping and sequencing strategy of pTMr108 are shown in Fig. 1. Performing bidirectional sequencing using deletion series from about 1.0-kb DNA insert gave a 1016-bp DNA sequence (Fig. 2). BLAST program was used to search the sequence that show homology to this sequence. Then the 3′ region of 25S rRNA and 5S rRNA sequences of various organisms was collected (data not shown). The putative terminal sequences of 25S rRNA and 5S rRNA of *T. matsutake* are identified by their structural similarities to highly conserved terminal regions found in other eukaryotes, which are collected in the database. We have predicted a putative 3′ end of 25S rRNA (486-bp in length) and 5S rRNA sequence (118-bp). IGS1 (158-bp) and partial IGS2 are also predicted (Fig. 2). 5S rRNA shows 61.02% of G+C content, consisting of 20.34% A, 30.51% G, 30.51 % C, and 18.64% T. There is no TATA-like sequence upstream of 5S rRNA gene, but a putative transcription termination region (31), which is rich in tracts of T, is found downstream of 5S rRNA gene.

Phylogenetic trees

The putative 5S rRNA sequence of *T. matsutake* has

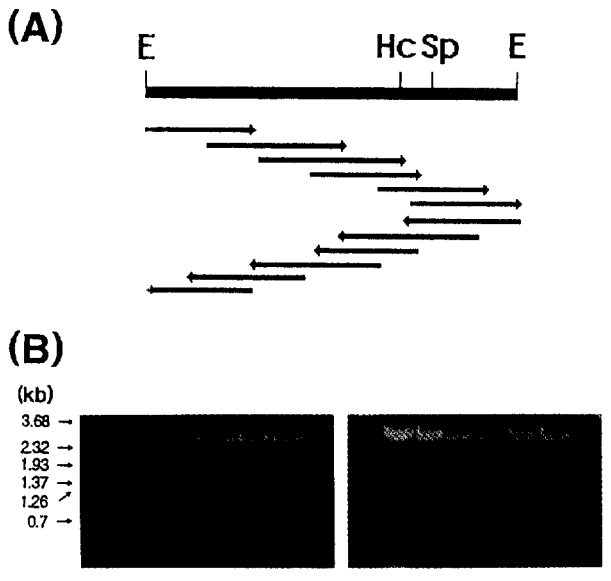

Fig. 1. Sequencing strategy (A) and constructed bidirectional deletion series (B) of pTMr108 containing 1.0-kb DNA fragment. The arrows show the extent and direction of sequence determination from the deleted *T. matsutake* rDNA fragments. E, *Eco*RI; Hc, *Hin*cII; Sp, *Sph*I.

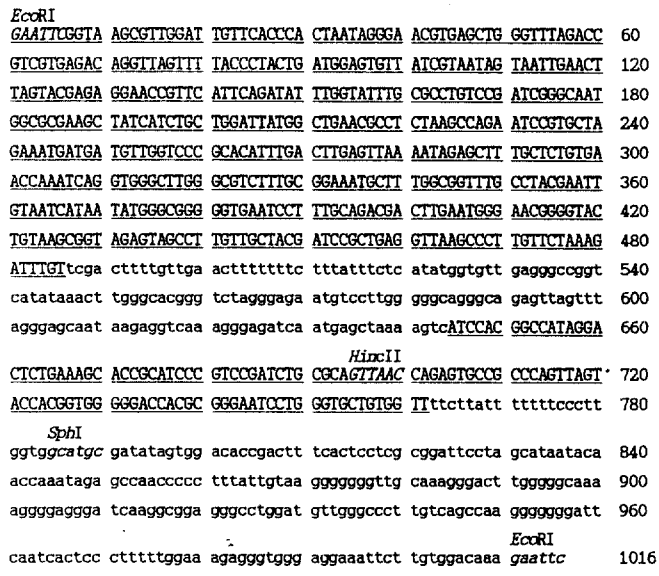

Fig. 2. Nucleotide sequence of pTMr108 shows putative 3′ end of 25S rRNA and 5S rRNA. The coding sequences are represented in underlined capital letters, and spacer sequences (intergenic spacers, IGS 1 and 2) are in lower case letters. Restriction enzyme sites are also depicted in italic letters. Accession number is Z49275 (EMBL).

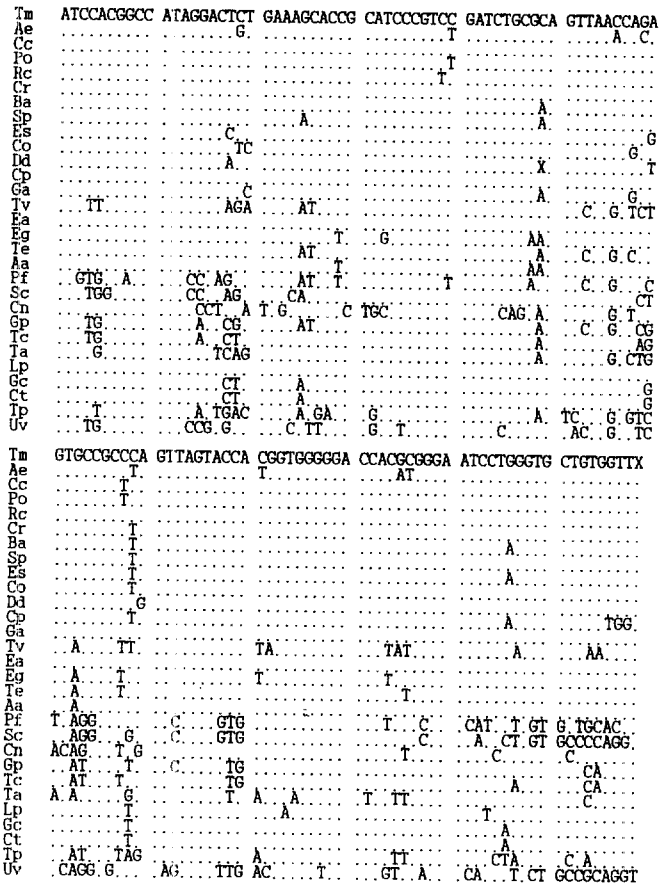

Fig. 3. Comparison of the sequence of the 5S rRNA gene of *T. matsutake* shown in Fig. 2 with the homologous sequences from other 28 Basidiomycotina species. Base differences are indicated in capital letters, the same bases are in dots, and missing base in 'X'. Abbreviations and accession numbers of GenBank (GB) or EMBL: Tm, *Tricholoma matsutake*; Ae, *Agaricus edulis*, K03161 (GB); Cc, *Coprinus cinereus*, K02344 (GB); Po, *Pleurotus ostreatus*, K03168 (GB); Rc, *Russula cyanoxantha*, X01570 (EMBL); Cr, *Coprinus radiatus*, X00074 (EMBL); Ba, *Bjerkandera adusta*, X06854 (EMBL); Sp, *Schizopora paradoxa*, X73890 (EMBL); Es, *Endophyllum sempervivi*, X00574 (EMBL) ; Co, *Ceratobasidium cornigerum*, M 35577 (GB); Dd, *Dacrymyces deliquescens*, X00073 (EMBL); Cp, *Christiansenia pallida*, M58383 (GB); Ga, *Ganoderma applanatum*, X 73589 (EMBL); Tv, *Tulasnella violea*, M35576 (GB); Tp, *Trimorphomyces papilionaceus*, X67494 (EMBL); Ea, *Efibulobasidium albescens*, M58382 (GB); Eg, *Exidia glandulosa*, M58368 (GB); Te, *Tremella mesenterica*, X06852 (EMBL); Aa, *Auricularia auricula-judae*, K03160 (GB); Pf, *Phleogena faginea*, M35574 (GB); Sc, *Septobasidium carestianum*, M35572 (GB); Gc, *Gymnosporangium clavariaeforme*, K03166 (GB); Ct, *Coleosporium tussilaginis*, X00573 (GB); Cn, *Cryptococcus neoformans*, L14753 (GB); Gp, *Graphiola phoenicis*, M35575 (GB); Tc, *Tilletiaria controversa*, X00071 (EMBL); Ta, *Tilletiaria anomala*, X00072 (EMBL); Lp; *Lycoperdon pyriforme*, M58384 (GB); Uv, *Ustilago violacea*, X06847 (GB).

been aligned, on the basis of similarity, using CLUSTALV program, with twenty-eight 5S rRNA sequences of Basidiomycotina species from GenBank or EMBL data base (Fig. 3). With two exceptions, 5S rRNAs of *Dacrymyces deliquescens* of 117 nucleotides and *Ustilago violacea* of 119 nucleotides, all other species harbor 118 nucleotides. Since the 5S rRNAs of *D. deliquescens* and *U. violacea* have a base deletion at nucleotide position 49 and a base insertion at 119, respectively, these gaps of sequences were filled with '–' (dashes) and realigned to juxtapose evolutionary homologous regions. Especially, the 5S rRNA gene of *T. matsutake* maintains a complete homology with the corresponding gene of *Efibulobasidium albescens* which belongs to the Basidiomycotina, with one base pair difference from *Coprinus cinereus, C. radiatus*, and *Russula cyanoxantha*. However, many base differences are present with the corresponding genes of *U. violacea* (47 different bases), *Phleogena faginea* (38 different bases), *Septobasidium carestianum* (33 different bases), and *Trimorphomyces papilionaceus* (30 different bases). Depending on the 5S rRNA sequence similarity, *T. matsutake* is most closely affiliated to *E. albescens*.

Using the DNADIST program, we turned the base differences (substitutions) of 5S rRNA sequences into distance matrix using the Kimura's 2-parameter method (12, 14). Table 1 shows the structural distance (number substitution per site) for comparisons of 5S rRNA genes from *T. matsutake* with those of 28 species of the Basidiomycotina. The resulting distance matrix was then inputted into the NEIGHBOR program which employs the UPGMA method. The DRAWGRAM program was used

Table 1. Distance matrix (Knuc value) calculated by the Kimura's 2-parameter method using DNADIST program. The abbreviations of species used were as described in Fig. 3.

	Tm	Ae	Cc	Po	Rc	Cr	Ba	Sp	Es	Cc	Dd	Cp	Ga	Tv	Ea	Eg	Tm	Aa	Pf	Sc	Cn	Gp	Tc	Ta	Lp	Gc	Ct	Tp	Uv
Ae	0.0716	–																											
Cc	0.0085	0.0809	–																										
Po	0.0171	0.0716	0.0085	–																									
Rc	0.0171	0.0716	0.0258	0.0345	–																								
Cr	0.0085	0.0623	0.0171	0.0258	0.0085	–																							
Ba	0.0259	0.0814	0.0347	0.0436	0.0259	0.0172	–																						
Sp	0.0347	0.0909	0.0436	0.0526	0.0347	0.0259	0.0258	–																					
Es	0.0345	0.0904	0.0434	0.0523	0.0345	0.0258	0.0259	0.0526	–																				
Cc	0.0347	0.1005	0.0436	0.0526	0.0526	0.0436	0.0620	0.0620	0.0708	–																			
Dd	0.0174	0.0726	0.0261	0.0350	0.0174	0.0086	0.0174	0.0261	0.0261	0.0533	–																		
Cp	0.0441	0.1214	0.0532	0.0623	0.0623	0.0532	0.0535	0.0814	0.0441	0.0814	0.0633	–																	
Ga	0.1407	0.1102	0.1307	0.1407	0.1407	0.1307	0.1299	0.1299	0.1509	0.1399	0.1319	0.1855	–																
Tv	0.2476	0.2312	0.2359	0.2476	0.2595	0.2476	0.2734	0.2494	0.2476	0.2343	0.2367	0.2898	0.2459	–															
Ea	0.0000	0.0716	0.0085	0.0171	0.0171	0.0085	0.0259	0.0347	0.0345	0.0347	0.0174	0.0441	0.1407	0.2476	–														
Eg	0.0712	0.1307	0.0620	0.0712	0.0899	0.0805	0.0800	0.0893	0.1089	0.1095	0.0812	0.1207	0.1207	0.2877	0.0712	–													
Tm	0.0809	0.1299	0.0716	0.0809	0.0999	0.0904	0.0899	0.0805	0.1095	0.1200	0.0912	0.1214	0.0993	0.2030	0.0809	0.0999	–												
Aa	0.0347	0.1102	0.0436	0.0526	0.0526	0.0436	0.0434	0.0523	0.0708	0.0712	0.0440	0.0814	0.1399	0.2734	0.0347	0.0347	0.0805	–											
Pf	0.4566	0.5407	0.4720	0.4566	0.4876	0.4720	0.5080	0.4762	0.5034	0.4989	0.4737	0.5175	0.5574	0.5739	0.4566	0.4648	0.4453	0.4302	–										
Sc	0.3926	0.4340	0.4075	0.4225	0.4075	0.3926	0.4110	0.3960	0.4075	0.4040	0.3794	0.3749	0.5225	0.4940	0.3926	0.4940	0.4821	0.4262	0.2734	–									
Cn	0.2559	0.3185	0.2443	0.2559	0.2796	0.2677	0.2658	0.2777	0.2917	0.2696	0.2705	0.3106	0.2777	0.4050	0.2559	0.2677	0.2281	0.2542	0.7426	0.7241	–								
Gp	0.1914	0.1983	0.2021	0.2128	0.1914	0.1809	0.1798	0.1491	0.1914	0.2142	0.1721	0.2507	0.2115	0.2213	0.1914	0.2347	0.1592	0.1902	0.3749	0.3079	0.4069	–							
Tc	0.1473	0.2128	0.1374	0.1473	0.1674	0.1573	0.1563	0.1464	0.1674	0.1582	0.1486	0.2021	0.2237	0.2259	0.1473	0.1674	0.1787	0.1464	0.4453	0.3381	0.3515	0.0904	–						
Ta	0.1977	0.1939	0.2087	0.2199	0.2087	0.1977	0.1964	0.1855	0.1977	0.2101	0.1886	0.2476	0.1939	0.2394	0.1977	0.2312	0.1832	0.1964	0.6103	0.4987	0.3311	0.2034	0.1964	–					
Lp	0.0259	0.0814	0.0347	0.0436	0.0259	0.0172	0.0349	0.0439	0.0436	0.0620	0.0263	0.0720	0.1518	0.2734	0.0259	0.0999	0.1102	0.0620	0.4762	0.4262	0.2938	0.2034	0.1787	0.2213	–				
Gc	0.0523	0.0999	0.0613	0.0704	0.0523	0.0434	0.0436	0.0347	0.0345	0.0616	0.0440	0.0809	0.1715	0.2476	0.0523	0.1283	0.1193	0.0893	0.4876	0.3780	0.3287	0.1602	0.1473	0.1977	0.0616	–			
Ct	0.0523	0.0999	0.0613	0.0704	0.0523	0.0434	0.0436	0.0347	0.0345	0.0616	0.0440	0.0809	0.1715	0.2476	0.0523	0.1283	0.1193	0.0893	0.4876	0.3780	0.3287	0.1602	0.1473	0.1977	0.0616	0.0000	–		
Tp	0.1528	0.2296	0.1632	0.1737	0.1737	0.1632	0.1518	0.1518	0.1737	0.1642	0.1523	0.1990	0.2796	0.3061	0.1528	0.2170	0.2073	0.1726	0.5857	0.4733	0.3152	0.2622	0.2156	0.2715	0.1855	0.1632	0.1632	–	
Uv	0.6360	0.7073	0.6554	0.6751	0.6751	0.6554	0.7033	0.7033	0.6952	0.6873	0.6576	0.6291	0.6873	0.6412	0.6360	0.7328	0.6628	0.6829	0.3829	0.3520	0.8432	0.5613	0.6045	0.7033	0.7033	0.7156	0.7156	0.7638	–

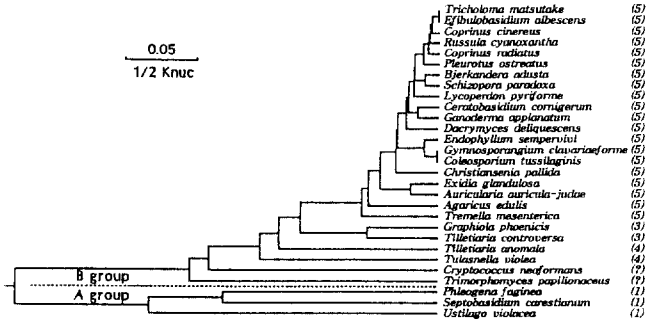

Fig. 4. Phylogenetic trees of some members of the subdivision Basidiomycotina based on distance data in Table 1. NEIGHBOR (UPGMA method) and DRAWGRAM programs were used for this phenogram. The numbers of clusters are indicated in parentheses following the species names, and group A and B are also indicated.

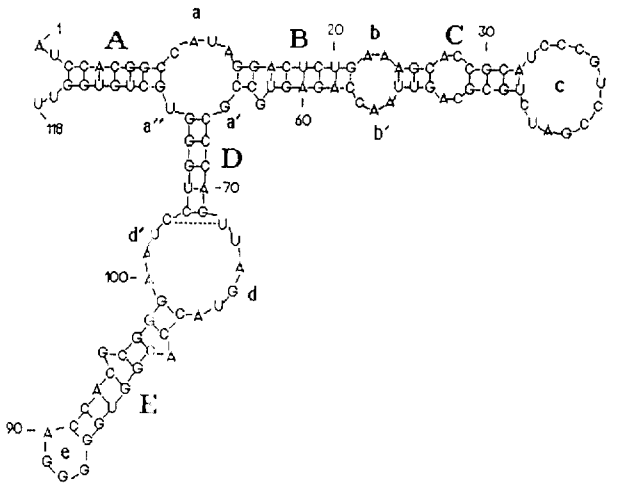

Fig. 5. Possible secondary structure of *T. matsutake* 5S rRNA. The G : C, A : U, and G : U pairs predicted from the program (PC-FOLD) of Turner *et al.* (25) are indicated by bars. The possible helix extensions are also indicated by dotted lines. The odd base-pair (U : U) and deletion of base are not shown.

to produce a phenogram (Fig. 4) inferred by the UPGMA method. *T. matsutake* was found to be closely related to *E. albescens* (Knuc value of 0.0) belonging to the order Tremellales of the Basidiomycotina, and *C. cinereus* (0.0085), *C. radiatus* (0.0085), and *R. cyanoxantha* (0.0085) belonging to the order Agaricales of the Basidiomycotina, which are evolutionarily close on the basis of phenotypic characteristics. The species of Basidiomycotina can be divided into five major phylogenetic clusters as proposed by Walker and Doolittle (28), and *T. matsutake* fall within the cluster 5 (Fig. 4). Together with the species mentioned above, *T. matsutake* can be classified into the type B group as noted (8). *T. matsutake* also forms a closely related group with most members of Agaricales except *Agaricus edulis*. Therefore more extensive investigations are needed to clarify the phylogenetic relatedness of *T. matsutake* to *E. albescens* and *A. edulis*. These phylogenetic analyses show some reproducible informations on the phylogeny of basidiomycetous fungi, but because of their small size, these molecules are unlikely to provide statistically accurate measurements for very close relationships.

Secondary structure of 5S rRNA

A minimal energy model of the secondary structures for RNA (11, 25) was used for prediction. Secondary structure of *T. matsutake* 5S rRNA has been constructed by using PCFOLD program (version 4.0) (25), and compared with that of other eukaryotes (4). The secondary structure of 5S rRNA is composed of five helices, named A through E, which are connected by loops, designated as a through e (22). As shown in Fig. 5, the basic features of these structures are approximately identical to those of the proposed structure (22, 23). There were common features of the Basidiomycotina: the odd base-pair at position 2 : 117 (U : U) in helix A (not shown in this figure), deletion of pyrimidine base between positions 38 and 39 in loop c, and possible helix extensions at 72 : 103 and 21 : 57 indicated by dotted lines. G : U pairs are also detected at position 7 : 112 and 25 : 52. However, compared with secondary structural model for phylogenetic group (23), insertion of base, which is a typical characters of the Basidiomycotina type A, is not detected between position 106 and 107, and odd base-pair (A : C) at position 96, which is common features of type B, is not found either (23, 28).

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