J Wood Sci (2005) 51:77–82
DOI 10.1007/s10086-003-0618-7

NOTE

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Cladistic relationships among the *Pleurotus ostreatus* complex, the *Pleurotus pulmonarius* complex, and *Pleurotus eryngii* based on the mitochondrial small subunit ribosomal DNA sequence analysis

Received: August 1, 2003 / Accepted: December 5, 2003

Abstract The cladistic analysis of the V4 domain sequences, performed by UPGMA, the neighbor-joining, and parsimony methods, revealed that the 19 *Pleurotus* strains tested in this study evolved along three lineages, each corresponding to a separate biological species: the *Pleurotus ostreatus* complex, the *Pleurotus pulmonarius* complex, and *Pleurotus eryngii*. Moreover, the cladistic positions of the 3 biological species show that the *P. ostreatus* complex and *P. eryngii* were derived from a common ancestor at a later stage of evolution, and that the common ancestor had diverged from the lineage of the *P. pulmonarius* complex during an earlier evolutionary event. The sequences of the 5′ portion of the mt SSU rDNA among the strains of the *P. ostreatus* complex had 99.2%–99.6% homology. All test strains in the *P. pulmonarius* complex had completely identical sequences. The homology of the strain sequences between the *P. ostreatus* complex and the *P. pulmonarius* complex ranged from 96.0% to 96.3%. The sequence of the strain of *P. eryngii* showed 97.8%–98.3% and 96.5% homologies with those of the strains in the *P. ostreatus* and the *P. pulmonarius* complexes, respectively.

Key words Biological species · mt SSU rDNA · Phylogenetic relationship · *Pleurotus*

Introduction

Most of the *Pleurotus* mushrooms are edible fungi. In 1986, Singer[1] described 38 morphological species in this genus, and some new species were designated as *Pleurotus* mushrooms based on morphological features in recent reports by Buchanan,[2] Zervakis and Balis,[3] and Segedin et al.[4] However, the taxonomic identification and phylogenetic relationships among the genus *Pleurotus* are still ambiguous.[3,5]

In our previous studies, we used mating tests to identify intersterility groups (biological species)[6] and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of the partial 26S rDNA for phylogenetic analysis.[7] We identified 12 biological species among 25 *Pleurotus* morphological species in this genus.[6] Phylogenetic analyses based on the PCR-RFLP data of the partial 26S rDNA revealed that 9 of the biological species, the *Pleurotus cornucopiae* complex, the *Pleurotus cystidiosus* complex, the *Pleurotus salmoneostramineus* complex, *Pleurotus calyptratus*, *Pleurotus corticatus*, *Pleurotus dryinus*, *Pleurotus nebrodensis*, *Pleurotus smithii*, and *Pleurotus ulmarius*, were congruent with independent phylogenetic lineages.[7] However, the remaining 3 biological species (the *Pleurotus ostreatus* complex, the *Pleurotus pulmonarius* complex, and *Pleurotus eryngii*) had identical RFLP types.

Different rDNA regions are used to analyze phylogenetic relationships among organisms at the different taxonomic levels.[8] Bruns and Szaro[9] reported that the nucleotide substitution rate in the mitochondrial small subunit ribosomal gene (mt SSU rDNA) is 16 times greater than in the homologous regions of nuclear SSU rDNA for ten members of the order Boletales. Mitochondrial SSU rDNA has also been used to investigate the interspecies relationships in the genera *Amylostereum*[10] and *Polyporus*.[11] Furthermore, Gonzalez et al.[12] reported that three variable domains (V4, V6, and V9) exist within the whole mt SSU rDNA region for *Agrocybe aegerita*. These three domains are suggested to be good species-specific markers for the phylogeny of Basidiomycetes, and have already been used for phylogenetic analysis of interspecies relationships in genera *Agrocybe*[13] and *Pleurotus*.[5]

In the present study, we have tried to resolve the phylogenetic relationships among the closely related biological species of genus *Pleurotus* described above by using the partial mt SSU rDNA sequences.

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Table 1. *Pleurotus* strains used in this study

Stock no.	Species	Intersterility group	Strain	Geographic origin	Acquisition source
1	*P. djamor*	I	IFO32398	Japan	IFO
2	*P. flabellatus*	I	ATCC62883	Unknown	ATCC
3	*P. flabellatus*	I	FMC251	Japan	NTFS
4	*P. ostreatus*	I	TD-33	Japan	MBTU
5	*P. ostreatus*	I	MH006008	Japan	HOKUTO
6	*P. ostreatus*	I	Chusei	Japan	NICHINOH
7	*P. ostreatus* var. *columbinus*	I	ATCC36498	France	ATCC
8	*P. eugrammus*	II	585	China	EFI
9	*P. eugrammus* var. *brevisporus*	II	574	China	EFI
10	*P. opuntiae*	II	ATCC90202	India	ATCC
11	*P. pulmonarius*	II	MH006043	Japan	HOKUTO
12	*P. pulmonarius*	II	MH006045	Japan	HOKUTO
13	*P. sajor-caju*	II	TD-991	Japan	MBTU
14	*P. sajor-caju*	II	MH006061	Japan	HOKUTO
15	*P. sapidus*	II	0601	China	EFI
16	*P.* sp. *florida*	II	TD-002	Thailand	MBTU
17	*P. eryngii*	VIII	MH006062	Japan	HOKUTO

ATCC, American Type Culture Collection; EFI, Edible Fungi Institute, Shanghai Academy of Agricultural Science; HOKUTO, Hokuto Co. Ltd.; IFO, Institute for Fermentation, Osaka; MBTU, Laboratory of Microbial Biotechnology, Tottori University; NICHINOH, Nippon Nourin Shukin Co.; NIFS, National Institute of Forestry Science, Tsukuba; OMI, Ohita Mushroom Institute, Ohita

Materials and methods

Strains and culture condition

The 17 strains used in this study were dikaryotic strains representing twelve *Pleurotus* morphological species (Table 1). The method for cultivation of mycelia of each dikaryotic strain is described elsewhere.[7] Mycelia were lyophilized after washing with distilled water, and stored at −20°C.

DNA isolation and PCR amplification

Total DNA of each strain was extracted using the Cell and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The 5′ portion of the mt SSU rDNA was amplified using primers MS1 (5′-CAGCAGTCAAGA ATATTAGTCAATG) and MS2 (5′-GCGGATTATCG AATTAAATAAC) designed by White et al.[14] in a 100-μl reaction system composed of 1.5 units of Taq DNA polymerase (Amersham Pharmacia Biotech), 0.2 mM dNTP mixture (Amersham Pharmacia Biotech), 1 X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 12.5 pM primers and 10–50 ng of template total DNA. Amplification was carried out as follows: 1 cycle of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 90 s at 72°C, and finally, 1 cycle of 10 min at 72°C. Amplified PCR products (5μl) and 100-base-pair ladder molecular weight markers (Amersham Pharmacia Biotech) were electrophoresed on a 1.5% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V for 2 h, and visualized by staining with 0.5 mg/ml ethidium bromide solution. The molecular weights of the PCR products were estimated.

Purifying PCR products and cycle sequencing

PCR products were purified using Microcon Centrifugal Filters (Millipore, Bedford, MA, USA). Purified PCR products (2μl) were electrophoresed on a 1.5% agarose gel with molecular weight standards λ/*Hind*III·*Eco*RI (Nippon Gene, Tokyo, Japan) to estimate the concentrations of the PCR products. Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers MS1 and MS2 were used in sequencing reactions for both DNA strands. The extension products were purified with Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ, USA), and analyzed with the ABI Prism 3100 Genetic Analyzer in the Gene Research Center of Tottori University.

Phylogenetic analyses

The sequence data were edited with Genetyx-SV/RC Ver. 6.1 (Software Development, Tokyo, Japan). The multiple alignments of all the sequences were performed using CLUSTAL W (http://www.ddbj.nig.ac.jp/e-mail/clustalw-e.html), followed by manual adjustments. The location of the V4 variable domain in the 5′ portion of the mt SSU rDNA from each strain was determined by comparing the aligned sequences with the corresponding sequences from the GenBank database (http://www.ncbi.nlm.nih.gov./Genbank/index.html). The phylogenetic relationships were analyzed with the UPGMA,[15] neighbor-joining (NJ),[16] and parsimony[17] methods by using the DNADIST, NEIGHBOR, and DNAPARS programs in the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html).[18] The bootstrap test for estimating the reliability of phylogenetic tree topology was performed using 100 replications with the SEQBOOT program.[18] The consensus tree was

obtained by running the CONSENSE program.[18] The TreeView program[19] was used to view the phylogenetic tree.

DNA Data Bank of Japan (DDBJ) accession numbers

Sequences of the 5′ portion of the mt SSU rDNA from the following strains were submitted to DDBJ with the following accession numbers: *Pleurotus djamor* IFO 32398, AB113031; *Pleurotus flabellatus* ATCC62883, AB113032; *P. flabellatus* FMC251, AB113033; *Pleurotus ostreatus* TD-33, AB113034; *P. ostreatus* var. *columbinus* ATCC36498, AB113035; *Pleurotus eugrammus* 585, AB113036; *Pleurotus eugrammus* var. *brevisporus* EFI574, AB113037; *Pleurotus opuntiae* ATCC90202, AB113038; *Pleurotus pulmonarius* MH006043, AB113039; *Pleurotus sajor-caju* TD-991, AB113040; *Pleurotus sapidus* EFI0601, AB113041; *Pleurotus* sp. *florida* TD-002, AB113042; *Pleurotus eryngii* MH006062, AB113043.

Results

The length of the 5′ portion of the mt SSU rDNA PCR products among 17 *Pleurotus* strains

Based on agarose gel electrophoresis, the PCR products from all 17 *Pleurotus* strains were single fragments of approximately 650 bp in length. The sequence data showed that the 5′ portion of mt SSU rDNA (not including the sequences of the primers) ranged from 582 to 598 nucleotides (nt) in length.

The sequence homology of the 5′ portion of the mt SSU rDNA among *Pleurotus* strains

The aligned sequences of the 5′ portion of the mt SSU rDNA had a high degree of homology among the *Pleurotus* test strains belonging to the *Pleurotus ostreatus* complex, the *Pleurotus pulmonarius* complex, and *Pleurotus eryngii* (96%–100% homology of the 5′ portion of the mt SSU rDNA) (data not shown).

We used four morphological species (*Pleurotus djamor*, *Pleurotus flabellatus*, *P. ostreatus*, and *P. ostreatus* var. *columbinus*) of the *P. ostreatus* complex in this study. The three strains of *P. ostreatus* (Chusei, MH006008, and TD-33) in the *P. ostreatus* complex had an identical 5′ portion of the mt SSU rDNA sequences, although the sequence datum from the GenBank database (accession number: AF091901) was different from the above three with addition or deletion of four nucleotides (99.3% homology). On the other hand, one strain (ATCC62883) of *P. flabellatus* had 99.3% homology with the sequences of the three strains of *P. ostreatus*, while another strain (FMC251) had a completely identical sequence to those of *P. ostreatus*. The strains of *P. djamor* and *P. ostreatus* var. *columbinus* had identical sequences, with 99.6% homology with the sequence of the three strains of *P. ostreatus*, and 99.2% homology with one of the strain sequences of *P. flabellatus* (ATCC62883). It is probably involved in the misidentified morphological strain(s) in the present study because morphological characteristics regarding taxonomic identification are strongly influenced by the climate, cultivation substrate, and environmental conditions in *Pleurotus* mushrooms.[20]

All the sequences of the strains in the *P. pulmonarius* complex—*P. pulmonarius*, *Pleurotus eugrammus*, *P. eugrammus* var. *brevisporus*, *Pleurotus sajor-caju*, *Pleurotus sapidus*, *Pleurotus* sp. *florida*, and *Pleurotus opuntiae*—were identical. The homology of sequences between the *P. ostreatus* and the *P. pulmonarius* complexes ranged from 96.0% to 96.3%. The strains of *P. eryngii* showed 97.8%–98.3% and 96.5% homology with strains in the *P. ostreatus* and the *P. pulmonarius* complexes, respectively.

The phylogenetic analyses based on the V4 domain sequences

Phylogenetic analyses were carried out using sequence data of the highly variable V4 domain from the *P. ostreatus* complex, the *P. pulmonarius* complex, *P. eryngii*, and the corresponding GenBank data (Table 2). The corresponding V4 variable domain sequences of genus *Pleurotus* from the GenBank database aligned between nucleotide positions 189 and 298 (alignment position) of the 5′ portion of the mt SSU rDNA sequences determined in the present study. The length of the V4 domain ranged from 92 nucleotides for all the members of the *P. pulmonarius* complex to 106 nucleotides for *P. djamor* in the *P. ostreatus* complex. The aligned length of the V4 variable domain was 111 nucleotides.

Completely identical sequences were merged into one input sequence when running the computer programs to generate the phylogenetic trees constructed by UPGMA, NJ, and parsimony methods shown in Fig. 1. A strain of *Pleurotus dryinus* from the GenBank database (accession number: AF091895) was used as an outgroup to root the trees. The rooted UPGMA tree (Fig. 1a) shows two phylogenetic lineages arising from the root *P. dryinus*: one leads to a terminal node containing all members of the *P. pulmonarius* complex and a strain of *P. pulmonarius* from the GenBank database (accession number: AF091902), and the other subdivides further into a terminal branch leading to *P. eryngii*, and a third-level subcluster. The third-level subcluster divides into a terminal branch containing one *P. flabellatus* sequence (ATCC62883) and one *P. ostreatus* sequence from GenBank database (accession number: AF091901), and a fourth-level subcluster. The fourth-level subcluster comprises two terminal nodes: *P. djamor* and *P. ostreatus* var. *columbinus* on one node and another strain of *P. flabellatus* (FMC251) and three strains of *P. ostreatus* (Chusei, MH006008, and TD-33) on the other node. The NJ and parsimony trees (Fig. 1b) were similar to each other and both show a topological shape identical to the UPGMA tree.

Table 2. Aligned sequences of the 5′ portion of the V4 domain of mt SSU rDNA from 20 strains of 12 *Pleurotus* morphological species

Stock number	V4 domain
(AF091895)	AGCTTTGAAAGTTTTATTTTTTAA——————————TTAGCTCTATTT——TTAAAT-AAATAGTAAAAATAAAATTAATTAAAGCT
1	AGCTTTGAAAATTTTAATTTTAAAAAAAA—TTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTTCAATAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
2	AGCTTTGAAAATTTTAATTTTAAAAA--TTTTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTACA-TAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
3	AGCTTTGAAAATTTTAATTTTAAAAAA--TTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTTCAATAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
4	AGCTTTGAAAATTTTAATTTTAAAAAA--TTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTTCAATAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
5	AGCTTTGAAAATTTTAATTTTAAAAAA--TTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTTCAATAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
6	AGCTTTGAAAATTTTAATTTTAAAAAA--TTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTTCAATAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
(AF091901)	AGCTTTGAAAATTTTAATTTTAAAAA--TTTTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTACA-TAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
7	AGCTTTGAAAATTTTAATTTTAAAAAAAA—TTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTTTGTTCAATAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
8	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
9	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
10	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
11	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
12	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
(AF091902)	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
13	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
14	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
15	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
16	AGCTTTGAAAATTTTAATTTTAATAA——TTTTTGTTCT——AAAATGATTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
17	AGCTTTGAAAATTTTAAATTTAAAAAAAAA-TTTTTTTTTCTTTTCTAGAAAAAAGAGTAGCTCTATTT--ATTAAATTAAATAGTAAAAATAAAATCAATTAAAGCT
	********** ***** ** ***** **** **** ** ********** * **********

Asterisks indicate nucleotides that are completely identical in 19 species belonging to the *Pleurotus ostreatus* complex, the *Pleurotus pulmonarius* complex, or *Pleurotus eryngii*. Dashes indicate the putative insertion–deletion events. The stock numbers in parentheses show the downloaded sequences of AF091895 (*Pleurotus drynus*), AF091901 (*P. ostreatus*), or AF091902 (*P. pulmonarius*) from the GenBank database

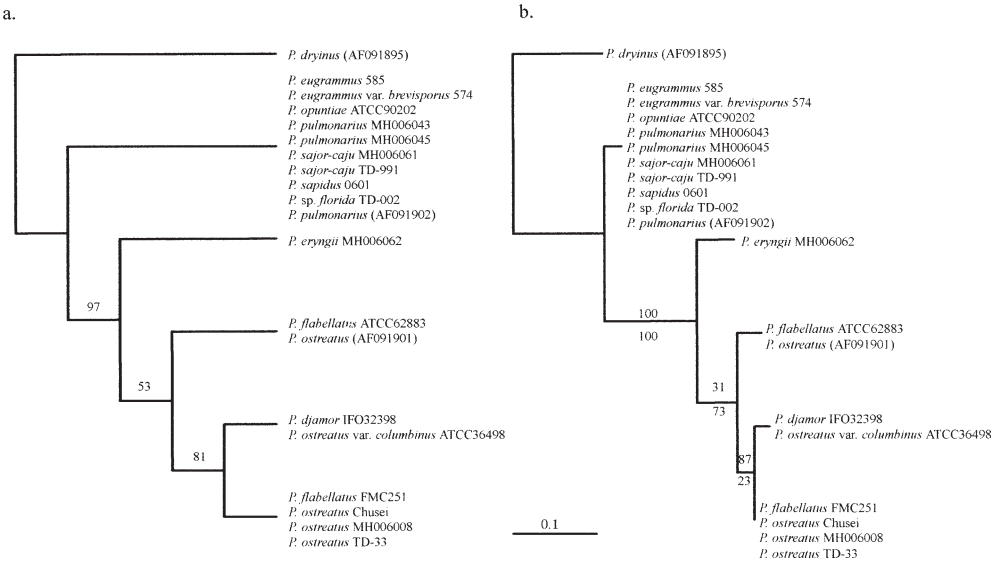

Fig. 1a,b. Phylogenetic trees constructed by UPGMA, neighbor-joining (NJ), and parsimony methods based on the sequence data of the variable V4 domain in the mt SSU rDNA. **a** UPGMA analysis. The bootstrap values from 100 replicates are shown. **b** NJ and parsimony analyses. The bootstrap values from 100 replicates by NJ and parsimony methods are shown *above* and *below* branches, respectively. The sequence data accessed from GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) included *Pleurotus ostreatus* (AF091901), *Pleurotus pulmonarius* (AF091902), and *Pleurotus dryinus* (AF091895)

Discussion

In a previous paper,[6] we reported the identification of 12 biological species among 25 *Pleurotus* morphological species from mating compatibility tests. Furthermore, phylogenetic analyses based on the PCR-RFLP data of the partial 26S rDNA in another paper[7] revealed that 9 of the biological species, the *Pleurotus cornucopiae* complex, the *Pleurotus cystidiosus* complex, the *Pleurotus salmoneostramineus* complex, *Pleurotus calyptratus*, *Pleurotus corticatus*, *Pleurotus dryinus*, *Pleurotus nebrodensis*, *Pleurotus smithii*, and *Pleurotus ulmarius*, were congruent with independent phylogenetic lineages. However, we could not resolve the phylogenetic relationships by using RFLP analysis of the conserved nuclear 26S rDNA among the species of the *Pleurotus ostreatus* complex (*P. ostreatus,* *P. ostreatus* var. *columbinus*, *Pleurotus djamor*, and *Pleurotus flabellatus*), the *Pleurotus pulmonarius* complex (*P. pulmonarius*, *Pleurotus eugrammus*, *P. eugrammus* var. *brevisporus*, *Pleurotus* sp. *florida*, *Pleurotus opuntiae*, *Pleurotus sajor-caju*, and *Pleurotus sapidus*), and *Pleurotus eryngii*. In the present study we demonstrated the phylogenetic relationships among the *P. ostreatus* complex, the *P. pulmonarius* complex, and *P. eryngii* by the sequence analyses of variable V4 domain in the mt SSU rDNA. It is also demonstrated that the application of RFLP analysis of 26S rDNA with a combination of mt SSU rDNA sequence analysis to the *Pleurotus* test strains is very useful for identification of biological species in the genus *Pleurotus*.

The cladograms based on the sequences of the V4 variable domain constructed by UPGMA, NJ, and parsimony methods showed that the *P. ostreatus* complex, the *P. pulmonarius* complex, and *P. eryngii* diverged at an earlier stage of the evolutionary process and then developed independently along these three lineages. The cladistic positions of the three biological species also showed that the *P. ostreatus* complex and *P. eryngii* were derived from a common ancestor at a later stage of the evolution, and that the common ancestor had diverged from the lineage of the *P. pulmonarius* complex during an earlier evolutionary event.

Although some members in the *P. ostreatus* complex underwent subsequent evolutionary divergence, all members of the *P. ostreatus* complex are still gathered in a single phylogenetic cluster. This assumption is supported by a bootstrap value of 73% (64.9/98 trees) in the parsimony tree. On the other hand, the sequences of the 5′ portion of the mt SSU rDNA among all test strains in the *P. ostreatus* complex were very similar (the homologies were greater than 99%). These data support placing the *P. ostreatus* complex in a clade independent of the species of the *P. pulmonarius* complex and *P. eryngii* in the cladogram.

The morphological species belonging to the *P. pulmonarius* complex seem to comprise more closely related taxa, because all members in this biological species do not show any variation in the 5′ portion of the mt SSU rDNA and are placed in a common clade. On the other hand, the high homology of the sequences of the 5′ portion of the mt SSU rDNA (>99%) in the *P. ostreatus* complex shows that the 5′ portion of the mt SSU rDNA is a relatively highly conserved region that has hardly evolved since the biological speciation event.

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (12460079).

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