Phylogenetic Analysis of *Pleurotus* Species Based on the Nuclear SSU rRNA Sequences

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The internal regions of nuclear small subunit rRNA from 6 *Pleurotus* species and 5 *Pleurotus ostreatus* strains were amplified by PCR and sequenced. The DNA sequences of 8 *Pleurotus* strains (*P. ostreatus* NFFA2, NFFA4501, NFFA4001, KFCC11635, *P. florida*, *P. sajor-caju*, *P. pulmonarius*, and *P. spodoleucus*) were identical, but *P. cornucopiae* differed from them in two bases out of 605 bases. However, phylogenetic analysis of the sequences by DNA-distance matrix and UPGMA methods showed that *P. ostreatus* NFFA2m1 and NFFA2m2, known as mutants of *P. ostreatus* NFFA2, belonged to another group of Basidiomycotina, which is close to the genus *Auricularia*. The difference of the SSU rDNA sequences of *P. cornucopiae* from other *Pleurotus* species tested corresponds to the difference of mitochondrial plasmid type present in *Pleurotus* species as observed by Kim *et al.* (1993, *Korean J. Microbiol.* 31, 141-147).

Key words: *Pleurotus*, SSU rRNA, phylogeny

The oyster mushroom *Pleurotus ostreatus* and its related species are among the more conspicuous fungi causing wood decay in terrestrial ecosystem worldwide and are widely collected and cultivated as edible fungi. Many aspects concerning lignin degradation by this organism are being extensively studied (6, 8) and the molecular cloning of genes encoding lignin-degrading enzymes has yielded information on the structure and expression of the enzymes (1, 2, 3). The development of vector for replicative transformation made this fungus more useful in many aspects (5).

In our previous study (4), we observed that plasmid DNAs were present in the mitochondrial fraction of most *Pleurotus* species. However, *P. ostreatus* NFFA2m1 and NFFA2m2, putative mutants of *P. ostreatus* NFFA2, did not contain any plasmid. *P. cornucopiae*, on the other hand, contained a plasmid which did not hybridize with others in *P. osteratus*, *P. florida*, *P. sajor-caju*, *P. pulmonarius*, and *P. spodoleucus*. For further studies we had to analyze and confirm the phylogenetic position and the relatedness of *Pleurotus* species we used.

For the classification of many fungi, the morphology of sexual structures has been mainly used as one of the key criteria. However, morphology alone is not reliable for the classification of organisms since a sexual stage has never been observed for some fungi and fruitbody morphology can converge or may vary with environmental conditions. The chromosomal DNA sequences coding for the ribosomal RNAs (rDNA) are ideal for phylogenetic studies and the presence of highly conserved regions enables the amplification of selected portions of the sequence by PCR (7). Analysis of rDNA sequences has been useful in clarifying some of the problems that fungal classification presents. The 18S rDNA sequences are recently used to study the phylogenetic relationship of the fungi.

In order to get information on the phylogenetic relationship among *Pleurotus* species, we amplified the internal region of small subunit (SSU, 16S-like) rDNA from 11 *Pleurotus* species. Table 1 lists the *Pleurotus* species examined in this study. All the *Pleurotus* species were maintained and grown in malt medium as described previously (4). DNA was isolated and the SSU rDNA was amplified by PCR with fungus-specific oligonucleotide primers, NS3 (5'-GCAAGTCTGGTGCCAGCAGCC-3') and NS4 (5'-CTTCCGTCAATTCCTTTAAG-3') as described by White *et al.* (7). The major product of about 600 bp was mainly amplified with some minor products which showed species specific pattern coincident with

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Table 1. A list of *Pleurotus* species examined in this study

Species	Strains	Sources
Pleurotus ostreatus	NFFA2	NFFA[1]
	NFFA4001	NFFA
	NFFA4501	NFFA
	KFCC11635	KFCC[2]
	NFFA2m1	S. O. Kang
	NFFA2m2	S. O. Kang
Pleurotus sajor-caju	ASI2139	ASI[3]
Pleurotus florida	ASI2013	ASI
Pleurotus pulmonarius	ASI2091	ASI
Pleurotus cornucopiae	ASI2011	ASI
Pleurotus spodoleucus	ASI2104	ASI

[1] NFFA; National Federation of Forestry Association, Korea
[2] KFCC; Korean Federation of Culture Collection
[3] ASI; Agricultural Sciences Institute, R.D.A. Korea

our results (data not shown). The main PCR products were purified using Gene Clean II kit (Bio 101) and cloned in pUC18 or pGEM-3Zf(+). Cloned PCR products were sequenced in both directions using universal primers. Among 11 species and strains examined, *P. ostreatus* NFFA2, NFFA4001, NFFA4501, KFCC11635, *P. sajor-caju, P. florida, P. pulmonarius,* and *P. spodoleucus* exhibited identical nucleotide sequences, indicating that all these species are very closely related. *P. cornucopiae* exhibited difference in two bases out of 605 bases. Two putative mutant strains (*P. ostreatus* NFFA2m1 and NFFA2m2), shown to have same nucleotide sequences each other, differed 24 bases from other species (Fig. 1).

For phylogenetic analysis, these sequences have been aligned, using the ClustalV program (D. Higgins, European Molecular Biology Laboratory), with 9 SSU rDNA sequences of Basidiomycotina species from GeneBank and EMBL databases. Phylogenetic analysis of DNA sequences was performed with programs of the PHYLIP package (Joseph Felsenstein, University of Washington). Using the DNADIST program, we turned the base differences of SSU rDNA sequences into distance matrix using Kimura's 2-parameter method. The resulting distance matrix was then paired using the NEIGHBOR program which employs UPGMA method. A phenogram inferred by the UPGMA method was produced using the DRAWGRAM program. The resulting phylogenetic relationship is presented in Fig. 2.

Pleurotus species were found to be closely related to the subgroup belonging to the order Agaricales of the subclass Holobasidiomycetidae among the class Hymenomycetes. But this phenogram indicated that the two strains (*P. ostreatus* NFFA2m1 and NFFA2m2), previously known as mutants of *P. ostreatus* NFFA2, are more closely related with *Auricularia* than *P. ostreatus* and thus belong to other heterogeneous group. Although this group also belongs to the class Hymenomycetes, *Thana-*

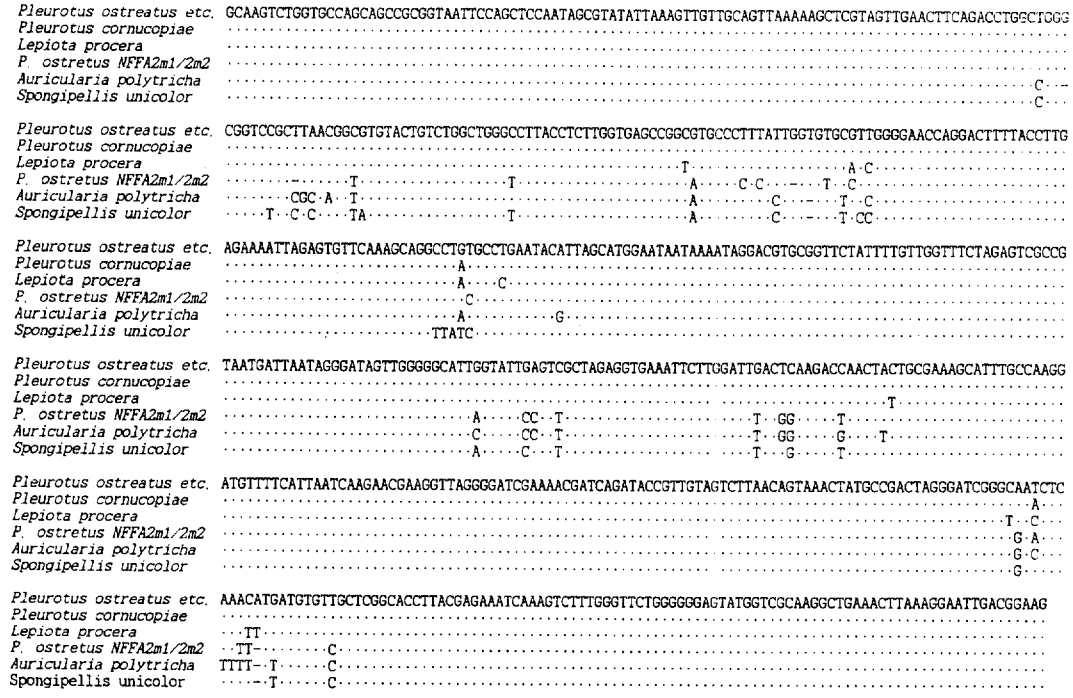

Fig. 1. Comparison of the homologous regions of 605 bases from partially sequenced nuclear SSU rRNA genes. Base differences were indicated in capital letters, identical bases with dots, and missing bases with −. *Pleurotus ostreatus etc.* represents the nucleotide sequences of *P. ostreatus* NFFA2, NFFA4001, NFFA4501, KFCC11635, *P. sajor-caju, P. florida, P. pulmonarius,* and *P. spodoleucus.*

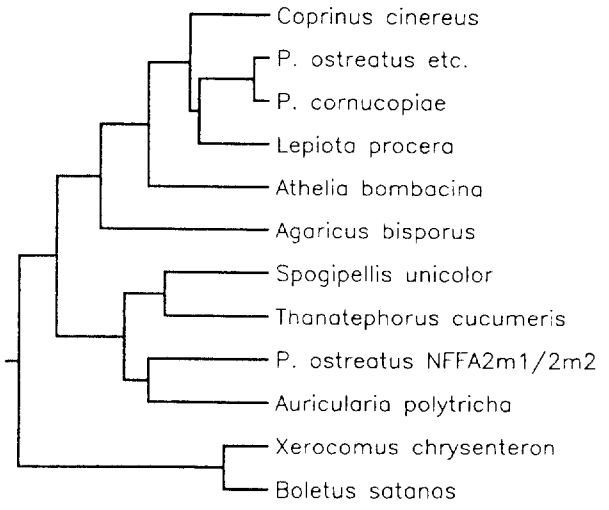

Fig. 2. Phylogenetic relationship of *Pleurotus* species and some basidiomycetes based on the partially sequenced nuclear SSU rDNAs. Abbreviation is the same as in Fig. 1 and the sequences were retrieved from GeneBank and EMBL databases with accession numbers in parentheses; *Agaricus bisporus* (L36658), *Athelia bombacina* (M55638), *Auricularia polytricha* (L22255), *Boletus satanas* (M 94337), *Coprinus cinereus* (M92991), *Lepiota procera* (L36659), *Spongipellis unicolor* (M59760), *Thanatephorus cucumeris* (M92990), *Xerocomus chrysenteron* (M94340).

tephorus cucumuris and *Auricularia polytricha* belong to the subclass Heterobasidiomycetidae. From the results of nucleotide sequences alignment and the phylogenetic tree, it is difficult to believe NFFA2m1 and NFFA2m2 to be strains of *P. ostreatus* but rather members of the Heterobasidiomycetidae.

It was already known that the two strains NFFA2m1 and NFFA2m2 show much different physiological characteristics like the pattern of enzyme production, growth rate, and multiploidy (S. O. Kang, personal communication). Since they do not produce a fruitbody under laboratory conditions, it is not affordable to determine whether they morphologically belong to the genus *Pleurotus* or not. This study clarifies the phylogenetic identity of these two mutants and their relationship among *Pleurotus* species. The present result is also consistent with our previous observation of linear mitochondrial plasmids in *Pleurotus* species (4). The more closely related *Pleurotus* species, containing identical sequences in 605 bp portion of SSU rDNA, all share the homologous linear plasmids, whereas the less closely related *P. cornucopiae*, containing nonhomologous linear plasmid, do not have this "universal" type of plasmid. NFFA2m1 and NFFA2m2 do not have any linear plasmid at all, suggesting a fundamental difference in genetic make-up. Therefore the presence and the type of mitochondrial plasmids correlates well with the phylogenetic relationship obtained through SSU rDNA sequences.

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