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Phylogenetic analysis of oyster mushrooms (*Pleurotus* spp.) based on restriction fragment length polymorphisms of the 5′ portion of 26S rDNA

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Abstract Polymorphism analysis of the 5′ portion of 26S rDNA from 34 *Pleurotus* strains (12 intersterility groups) collected mainly from Asia was performed. By combining the restriction fragment length polymorphism (RFLP) patterns obtained from digestions with seven restriction enzymes, the 34 *Pleurotus* strains were assigned to 11 RFLP types. Ten RFLP patterns corresponded with biological species, but one pattern was found in intersterility groups I (*P. ostreatus* complex), II (*P. pulmonarius* complex), and VIII (*P. eryngii*). The phylogenetic tree suggests that *Pleurotus* species have evolved in two patterns based on 26S rDNA RFLP data. One major cluster comprising the "*P. ostreatus* clade" is separated by relatively short branches, suggesting that the *P. ostreatus* complex, the *P. pulmonarius* complex, *P. eryngii*, and *P. nebrodensis* (intersterility groups I, II, VIII, and IX, respectively), share a recent common ancestor. RFLP data did not distinguish the species in the intersterility groups I, II, and VIII. The other major cluster apparently divided into five sublevel clusters in early stages of evolution and these clades split into terminal nodes in late stages of evolution: the *P. calyptratus-salmoneostramineus* clade, the *P. cornucopiae-ulmarius* clade, the *P. dryinus* clade, the *P. corticatus* clade, and the *P. cystidiosus-smithii* clade.

Key words *Pleurotus* · RFLP type · Phylogenetic tree · Intersterility group · Biological species

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Introduction

Pleurotus mushrooms include important commercial species that are widely cultivated throughout the world for their good taste, flavor, and ease of cultivation. *Pleurotus* species are also the subject of many taxonomic studies.[1] However, many problems in taxonomic nomenclature and phylogenetic relationships of the *Pleurotus* species remain unresolved. According to Zervakis and Balis,[2] the taxonomic disagreements over *Pleurotus* species have risen for the following reasons: initial misidentification, absence of type specimens, instability of morphological characters due to environmental changes, limited reports on physiological characteristics, and the lack of mating compatibility studies. Thus, to clarify the taxonomic status of species in the genus *Pleurotus*, and to accurately determine the names of mushrooms in scientific literature, many researchers have identified biological species among the *Pleurotus* morphological species by applying various sets of criteria. To date, many studies on the mating compatibility of species have identified intersterility groups among *Pleurotus* species. Vilgalys and Sun[3] reported eight intersterility groups (biological species) among different geographic origins of *Pleurotus* strains. Petersen and Hughes[4] reported six intersterility groups among seven *Pleurotus* species. Recently, Zervakis and Balis[2] reported eight intersterility groups among thirteen *Pleurotus* species. In our previous study, we demonstrated 12 intersterility groups among 25 *Pleurotus* species that were collected mainly from Asia.[5]

In recent decades, biochemical and molecular criteria, including isoeletric focusing analysis and isozyme electrophoresis, have been used to determine the intraspecific and interspecific relationships among *Pleurotus* species.[6,7] Molecular analyses based on restriction fragment length polymorphism (RFLP) of total DNA,[8] mitochondrial DNA,[9] and ribosomal DNA,[10,11] sequence and structure analysis of mitochondrial rRNA,[12] and sequence analysis of ribosomal DNA[3,13] have also been useful for understanding phylogenetic relationships as well as the taxonomical identification of *Pleurotus* species.

In this study, the polymorphism of the 5′ portion of 26S ribosomal DNA (26S rDNA) from 34 *Pleurotus* strains, including 12 intersterility groups (biological species) collected mainly from Asia, were examined by polymerase chain reaction-RFLP (PCR-RFLP). Phylogenetic trees constructed using RFLP data by the unweighted pair group method with arithmetic mean algorithm (UPGMA),[14] and the neighbor-joining (NJ) method[15] were used to examine the relationships among 34 *Pleurotus* strains and were compared to the relationships derived from their classifications in 12 biological species.

Materials and methods

Strains and culture condition

All strains in this study were dikaryotic strains representing 34 *Pleurotus* strains (Table 1), and were preserved at the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan. Mycelia of each dikaryotic strain were cultivated in 200-ml Erlenmeyer flasks containing 50 ml of GA liquid medium (20 g glucose, 1.5 g $(NH_4)_2HPO_4$, 1.0 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, and 0.5 mg thiamine·HCl per 1.0 l of distilled water). The cultures were incubated at 25°C for 2 weeks. The mycelia were harvested by filtration, washed several times with distilled water, and lyophilized. The dried mycelia were stored at −20°C.

DNA isolation and PCR amplification

Total DNA was extracted from each strain using the Cell and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech). The 5′ portion of 26S ribosomal DNA was amplified using the following pair of primers: LR0R (5′-ACCCGCTGAACTTAAGC) and LR7 (5′-TACTACCACCAAGATCT).[11] PCR amplification was performed in 25-μl reactions containing 1.5 units of *Taq* DNA polymerase (Amersham Pharmacia Biotech), 0.2 mM dNTP mixture, 1X PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0), 12.5 pM primers, and 10–50 ng of total DNA template. Amplification was carried out as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s with a final extension of

Table 1. *Pleurotus* strains used in this study

Stock no.	Species	Intersterility group	Strain	Geographic origin	Acquisition source
1	*Pleurotus ostreatus* (Jacq.: Fr.) Kumm.	I	TD-33	Japan	MBTU
2	*Pleurotus ostreatus* (Jacq.: Fr.) Kumm.	I	MH006008	Japan	HOKUTO
3	*Pleurotus ostreatus* (Jacq.: Fr.) Kumm.	I	Chusei	Japan	NICHINOH
4	*Pleurotus ostreatus* var. *columbinus* (Quél) Pilat	I	ATCC36498	France	ATCC
5	*Pleurotus flabellatus* (Berk. et Br.) Sacc.	I	ATCC62883	Unknown	ATCC
6	*Pleurotus flabellatus* (Berk. et Br.) Sacc.	I	FMC251	Japan	NTFS
7	*Pleurotus djamor* (Fr.) Boedijn	I	IFO32398	Japan	IFO
8	*Pleurotus pulmonarius* (Fr.) Quél	II	MH006043	Japan	HOKUTO
9	*Pleurotus pulmonarius* (Fr.) Quél	II	MH006045	Japan	HOKUTO
10	*Pleurotus eugrammus* (Mont.) Dennis	II	585	China	EFI
11	*Pleurotus eugrammus* var. *brevisporus* (Mont.) Dennis	II	574	China	EFI
12	*Pleurotus opuntiae* (Durieu: Leveille) Sacc.	II	ATCC90202	India	ATCC
13	*Pleurotus sajor-caju* (Fr.) Sing.	II	TD-991	Nepal	MBTU
14	*Pleurotus sajor-caju* (Fr.) Sing.	II	MH006061	Nepal	HOKUTO
15	*Pleurotus sapidus* (Schulz.) Sacc.	II	0601	China	EFI
16	*Pleurotus* sp. *florida* (Fr.) Kumm.	II	TD-002	Thailand	MBTU
17	*Pleurotus calyptratus* (Lindbl.) Sacc.	III	IFO32795	Japan	IFO
18	*Pleurotus cornucopiae* (Paul.:Pers.) Roll.	IV	MH00301	Japan	HOKUTO
19	*Pleurotus cornucopiae* var. *citrinopileatus* (Sing.) Ohira	IV	0579	China	EFI
20	*Pleurotus corticatus* (Fr.) Quél.	V	580	China	EFI
21	*Pleurotus cystidiosus* Miller	VI	4110	Japan	MBTU
22	*Pleurotus cystidiosus* Miller	VI	4072	China	MBTU
23	*Pleurotus abalonus* Han	VI	TD-200	Japan	MBTU
24	*Pleurotus abalonus* Han	VI	4103	Japan	OMI
25	*Pleurotus dryinus* (Pers.: Fr.) Kumm.	VII	ATCC48595	Norway	ATCC
26	*Pleurotus dryinus* (Pers.: Fr.) Kumm.	VII	IFO32797	Japan	IFO
27	*Pleurotus eryngii* (DC.: Fr.) Quél	VIII	MH006062	China	HOKUTO
28	*Pleurotus nebrodensis* (Inz.) Sacc.	IX	TD-021	China	MBTU
29	*Pleurotus salmoneostramineus* Vass.	X	MH00504	Japan	HOKUTO
30	*Pleurotus rhodophyllus* Bres.	X	0597	China	EFI
31	*Pleurotus rhodophyllus* Bres.	X	0595	China	EFI
32	*Pleurotus ostreatoroseus* Sing.	X	ATCC96235	Brazil	ATCC
33	*Pleurotus smithii* Guzman	XI	ATCC46391	Mexico	ATCC
34	*Pleurotus ulmarius* (Bull.: Fr.) Quél	XII	TD-003	Japan	MBTU

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

72°C for 10 min. The PCR products were visualized by electrophoresis on a 1.2% agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V for 2 h followed by staining with 0.5 μg/ml ethidium bromide solution.

Restriction enzyme digestion, agarose gel electrophoresis and phylogenetic analysis of RFLP patterns

The PCR products were digested by seven restriction enzymes following manufacturer's instructions (Takara Shuzo): *Msp* I, *Hea* III, *Ava* I, *Hinf* I, *Hha* I, *Alu* I, and *Acc* II. The restriction fragments were separated by 2.5% agarose gel electrophoresis in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 100 V for 4 h. The presence (1) or absence (0) of individual restriction fragments was scored for each strain (Table 2). The RFLP fragments distance matrix was calculated from RFLP patterns according to the method of Nei and Li[16] using the Restdist program in the PHYLIP package.[17] Dendrograms were constructed by the UPGMA method[14] and the NJ method[15] using the Neighbor program in the PHYLIP package.[17]

Results

Polymorphism of the 5′ portion of 26S rDNA among *Pleurotus* biological species

The 5′ portion of 26S rDNA from 34 *Pleurotus* strains was successfully amplified with primers LR0R and LR7. All of the amplified fragments were approximately 1460 base pairs (bp) in length.

Table 2 shows the distribution of the restriction fragments resulting from the digestion of the amplified rDNA fragments. Among the 34 *Pleurotus* strains, enzymes *Msp* I, *Hea* III, *Ava* I, *Hinf* I, *Hha* I, *Acc* II, and *Alu* I produced 7, 6, 4, 4, 5, 9, and 4 RFLP patterns, respectively. When RFLP patterns from each enzyme were combined, 11 RFLP types (a–k, in Table 2) emerged. The most common RFLP type, a-type, was found in 12 *Pleurotus* morphological species (*P. djamor*, *P. flabellatus*, *P. ostreatus*, *P. ostreatus* var. *columbinus*, *P. eugrammus*, *P. eugrammus* var. *brevisporus*, *P. opuntiae*, *P. pulmonarius*, *P. sajor-caju*, *P. sapidus*, *P.* sp. *florida*, and *P. eryngii*). Four RFLP types were found in two or three morphological species: c-type (*P. cornucopiae* and *P. cornucopiae* var. *citrinopileatus*), e-type (*P. abalonus* TD-200 and *P. cystidiosus* 4110), f-type (*P. abalonus* 4103 and *P. cystidiosus* 4072), and i-type (*P. ostreatoroseus*, *P. rhodophyllus*, and *P. salmoneostramineus*). The following 6 RFLP types were each identified in only one species: b-type (*P. calyptratus*), d-type (*P. corticatus*), g-type (*P. dryinus*), h-type (*P. nebrodensis*), j-type (*P. smithii*), and k-type (*P. ulmarius*).

Intersterility groups (biological species) determined by mating compatibility tests[5] were compared with the RFLP types identified in this experiment. We found that each *Pleurotus* strain in the eight intersterility groups (III, IV, V, VII, IX, X, XI, and XII) was classified as a specific RFLP type: b, c, d, g, h, i, j, and k, respectively (see Fig. 1). In other words, these eight RFLP types may be biological species-specific. Furthermore, the strains of intersterility group VI produced either one of two RFLP types – e-type or f-type. However, the species of intersterility groups I, II, and VIII were all identified as a-type, indicating that the species of intersterility groups I, II, and VIII cannot be distinguished based on PCR-RFLP analysis of the 5′ portion of 26S rDNA.

Phylogenetic analysis of RFLP patterns

The phylogenetic trees of 34 *Pleurotus* strains generated by the UPGMA (Fig. 1a) and the NJ methods (Fig. 1b) each have 11 terminal nodes corresponding to the 11 RFLP types. Both trees are nearly identical except for the position of species in intersterility groups III and X.

The topological shape of the dendrogram produced by the UPGMA method implies that the cluster comprising RFLP a- and h-types is more distant from all other phylogenetic branches. This implies that this cluster arose through a distinct phylogenetic pathway. The h-type RFLP pattern, found in one species of intersterility group IX (*P. nebrodensis*), is on a separate branch from the a-type node. The a-type node contains members from the following multi-intersterility groups: four species from group I (*P. djamor*, *P. flabellatus*, *P. ostreatus*, and *P. ostreatus* var. *columbinus*), seven species from group II (*P. eugrammus*, *P. eugrammus* var. *brevisporus*, *P. opuntiae*, *P. pulmonarius*, *P. sajor-caju*, *P. sapidus*, and *P.* sp. *florida*), and one species from group VIII (*P. eryngii*).

The other major cluster in Fig. 1a containing 12 *Pleurotus* taxa is divided into two subclusters. The first subcluster includes two terminal branches of the RFLP b- and i-types, which correspond to the intersterility groups III (*P. calyptratus*) and X (*P. ostreatoroseus*, *P. rhodophyllus*, and *P. salmoneostramineus*), respectively. The second subcluster is further divided into two clusters: one comprises two terminal nodes of RFLP c- and k-types, corresponding to intersterility groups IV (*P. cornucopiae* and *P. cornucopiae* var. *citrinopileatus*) and XII (*P. ulmarius*), respectively, and the other subcluster contains the terminal node of RFLP g-type, corresponding to group VII (*P. dryinus*), and a subcluster at the fourth level. The subcluster at the fourth level consists of a node of RFLP d-type corresponding to the intersterility group V (*P. corticatus*), and a subcluster that branches into the terminal node of f-type and a subcluster containing the terminal nodes of e- and j-types. RFLP e- and f-types correspond to intersterility group VI (*P. abalonus* and *P. cystidiosus*), while j-type corresponds to intersterility group XI (*P. smithii*). In this major cluster, every terminal node corresponds to a single intersterility group, although the strains of intersterility group VI (*P. abalonus* and *P. cystidiosus*) split into the RFLP e- and f-types.

Table 2. Results of digestions of the 5′ portion of 26S rDNA with seven restriction enzymes

Restriction enzyme	Fragment size (bp)	Stock number																																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Msp I	1300	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
	600	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0
	550	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
	490	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
	440	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	420	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1
	280	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hea III	950	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
	500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	1	0	1	1	0	0	1	1	1	1	1	0
	430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
	330	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	220	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
	190	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1
	170	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	120	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	100	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ava I	1460	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1
	780	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0
	720	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0
	700	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
	530	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
	170	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Hinf I	430	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1
	400	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
	390	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	1	1
	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
	220	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	200	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	160	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1
	140	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	0	0	1	1	1	1	0	0
	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0

Hha I	1200	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1
	700	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
	630	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0
	580	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	0
	440	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
	170	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0
	120	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
	90	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1
Acc II	1000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0
	750	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0
	650	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	450	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0	1	0
	420	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	1	0	0	0	0	0	0	1	0
	380	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	290	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0
	280	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
	270	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	200	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	0	0	0	0	1	1
	150	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
	90	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1
Alu I	680	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1
	650	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0
	520	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
	400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
	380	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	190	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
	90	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	RFLP type	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	b	c	c	d	e	f	e	f	g	g	a	h	i	i	i	i	j	k

bp, Base pairs; RFLP, restriction fragment length polymorphism

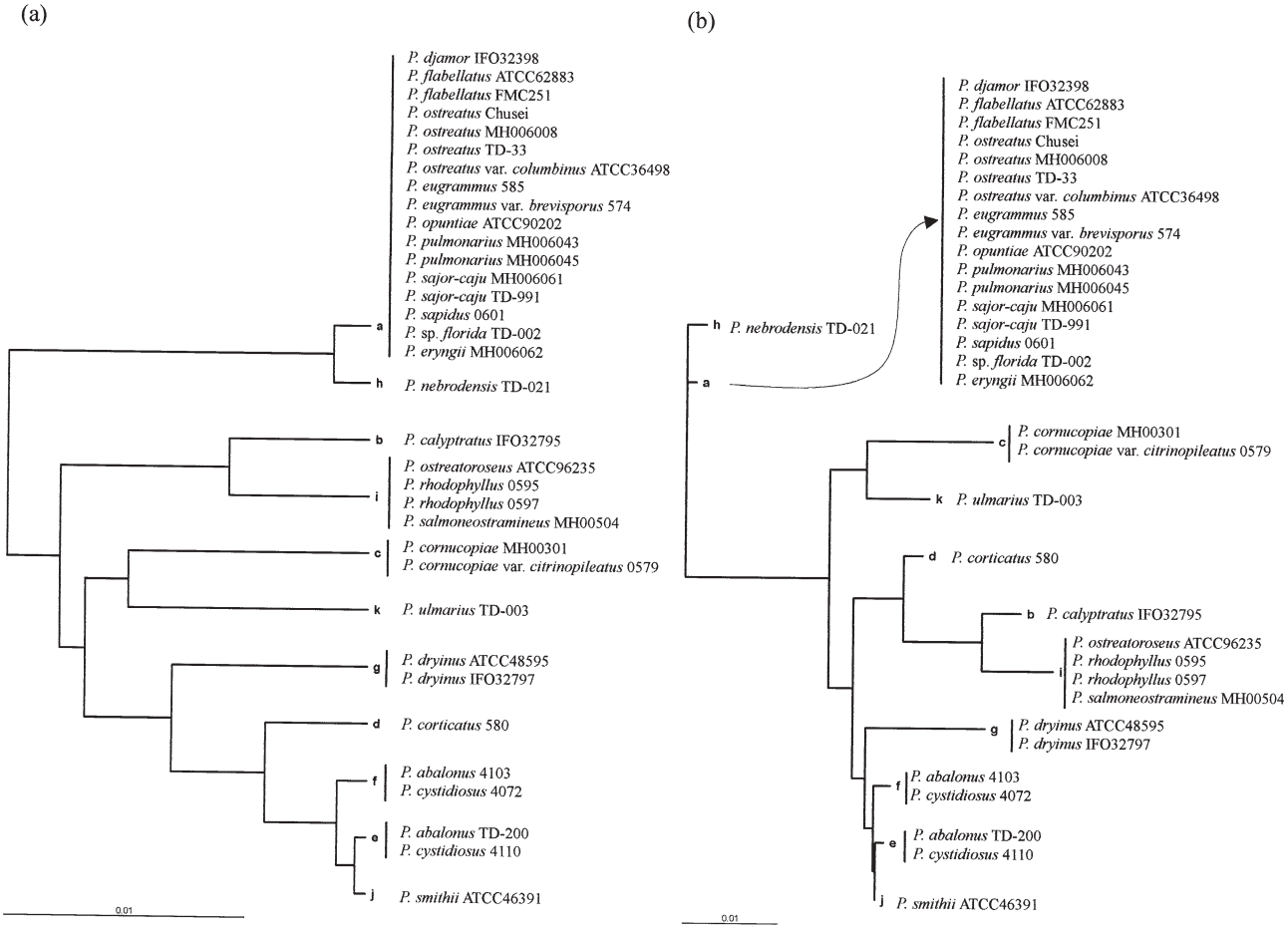

Fig. 1a,b. Dendrograms showing the phylogenetic relationships among the twelve intersterility groups in *Pleurotus* spp. The phylogenetic trees were constructed by the unweighted pair group method with arithmetic mean algorithm method (**a**) and the neighbor-joining method (**b**) based on restriction fragment length polymorphism (RFLP) data. *Lowercase letters* correspond to the eleven RFLP types shown in Table 2

Discussion

In most genomes, the multiple copies of the nuclear ribosomal RNA genes (rDNA) are arranged in tandemly repeated clusters.[18] In eukaryotes, each cluster contains three genes that code for the rRNA small subunit (SSU, 18S), the 5.8S subunit, the large subunit (LSU, 25–28S), and two internal transcribed spacers (ITS).[18] The intergenic spacer (IGS), which contains the 5S rRNA gene, is found between the gene clusters.[18] The different regions of rDNA evolve at variable rates, making them useful for phylogenetic studies among closely or distantly related organisms. Molecular phylogenetic studies in mushrooms have been largely based on RFLP and sequence data from LSU rDNA, especially the 5′ portion of LSU rDNA,[18,19] which encompasses several divergent domains with resolution adequate for analyzing species complexes at the genus level. Recently, Dahlman et al.[20] used phylogenetic analysis of 5′ end LSU rDNA sequence data to distinguish the identity of some species between *Cantharellus* and *Craterellus* genera. A similar region of rDNA was also used to study the phylogenetic relationships in *Agricus*,[21] *Amanita*,[22] *Coprinus*,[23] *Ganoderma*,[24] and *Pleurotus*.[11]

Iracabal et al.[10] generated UPGMA and NJ trees for the *Pleurotus* species based on LSU rDNA RFLP data. They found only one major difference between the two trees: *P. ostreatus*, *P. columbinus*, and *P. cornucopiae* isolates were separated into three clusters on the UPGMA tree, but were joined into a common larger cluster on the NJ tree. On the other hand, Bunyard et al.[11] constructed phylogenetic trees of LSU rDNA RFLP data by both methods for *Pleurotus* strains and concluded that the UPGMA tree was nearly identical to the NJ tree, matching at 17 of 20 nodes including the *P. ostreatus* complex, the *P. pulmonarius* complex, *P. eryngii*, *P. dryinus*, and *P. cystidiosus*. We also used both methods of preparing trees of 34 *Pleurotus* strains and found both trees to be identical, except for the phylogenetic position of the pink oyster mushrooms (*P. ostreatoroseus*, *P. rhodophyllus*, and *P. salmoneostramineus*), to other *Pleurotus* species. However, we do not have any strong evidence supporting one tree over the other as the suitable phylogenetic arrangement of the "*P. calyptratus-salmoneostramineus* clade" among other groups of *Pleurotus* mushrooms.

Based on rDNA sequence data, Vilgalys and Sun[3] reported two major patterns in the phylogenetic relationships of *Pleurotus* species corresponding to the geographic spe-

ciation of oyster mushrooms, ancient and recent. The tree produced by the UPGMA method in the present study based on RLFP data (Fig. 1a) was similar to the phylogenetic tree of *Pleurotus* species produced by Vilgalys and Sun.[3] The cluster containing RFLP a- and h-types was subdivided by relatively short branches, suggesting that the *P. ostreatus* complex, the *P. pulmonarius* complex and *P. eryngii* (intersterility groups I, II, and VIII), and *P. nebrodensis* (intersterility group IX) share a recent common ancestor, a hypothesis suggested by Vilgalys and Sun.[3] Although the *P. ostreatus* clade including the *P. ostreatus* complex, the *P. pulmonarius* complex, and *P. eryngii* has a similar genetic background based on 26S rDNA, incompatibility factors have evolved among these species. In addition, we have added the independent biological species, *P. nebrodensis*[5] of the intersterility group IX to the *P. ostreatus* clade based on RFLP data.

Vilgalys et al.[1] also reported that the *P. djamor-cornucopiae* clade and the *P. cystidiosus* clade were the two major components, in addition to the *P. ostreatus* clade, of their phylogenetic tree. They suggested that there was a common ancestor of these two clades, and that the two subancestors diverged at a very early stage. However, we propose that the lower major cluster in Fig. 1a divided into five sublevel clusters at an early stage of evolution: the *P. calyptratus-salmoneostramineus* clade, the *P. cornucopiae-ulmarius* clade, the *P. dryinus* clade, the *P. corticatus* clade, and the *P. cystidiosus-smithii* clade. Furthermore, these clades appear to have split into terminal clusters at a later stage in evolution. Based on our results, it appears that the *P. cornucopiae* complex and *P. ulmarius* may have split at a relatively early time. Similar divergences of biological species may have occurred for *P. dryinus*, *P. corticatus*, and *P. abalonus-cystidiosus* (with *P. smithii*) at relatively early stages in the evolution of *Pleurotus* species. In addition, we assume a similar division separated the *P. salmoneostramineus* complex and *P. calyptratus* in a late stage of evolution based on the relatively short branch lengths separating them. Therefore, based on these analyses, the species, and species complexes of this major cluster – the *P. salmoneostramineus* complex, *P. calyptratus*, the *P. cornucopiae* complex, *P. ulmarius*, *P. dryinus*, *P. corticatus*, the *P. cystidiosus* complex, and *P. smithii* – evolved into independent biological species in a manner consistent with the independent evolution of incompatibility factor genes.

A strain of *P. djamor* used in this study was identified as a member of intersterility group I (the *P. ostreatus* complex), but Vilgalys et al.[1] placed this strain among the *P. djamor-cornucopiae* clade in their phylogenetic tree. This discrepancy in *P. djamor* taxonomy might be due to misidentification of the strain(s) included in this taxon as discussed in our previous article.[5]

Although the *P. cystidiosus* complex and *P. smithii* were found in different phylogenetic lineages, they probably are recently diverged species based on the relatively short branches that separate them. Their rare morphological character (the production of the synnemata from mycelia) also infers the close relationships.[25] According to the mating incompatibility tests by Zervakis and Balis[2] and the present authors,[5] the *P. cystidiosus* complex and *P. smithii* were designated as different biological species.

In this study, we determined the phylogenetic relationships among the *Pleurotus* biological species. We found that the majority of intersterility groups (III, IV, V, VI, VII, IX, X, XI, and XII), but not intersterility groups I, II, and VIII were congruent with the independent phylogenetic lineages. These results confirm the conclusion of Vilgalys and Sun[3] that the intersterility groups appear to be independent evolutionary units in *Pleurotus* populations. Although Bunyard et al.[11] and Gonzalez and Labarere[12] did not test the incompatibility of strains used in their studies, the phylogenetic positions among the *Pleurotus* species in both studies were similar to parts of the phylogenetic tree constructed by the UPGMA method in this study.

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