Phylogenetic relationships of *Pleurotus* species according to the sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6 and V9 domains

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A comparative study of the V4, V6 and V9 domains of the mitochondrial small-subunit (SSU) rRNA was conducted to evaluate the use of these sequences to investigate phylogenetic relatedness within the genus *Pleurotus*. The PCR products encompassing these regions from 48 isolates belonging to 16 *Pleurotus* species were sequenced and compared. From this comparison, the length and sequence of the three domains were found to be constant within a species. Significant inter-species variations due to insertion/deletion events were found, in most cases occurring in regions not directly involved in the maintainance of the standard SSU rRNA secondary structure. Phylogenetic analysis based upon these mitochondrial sequences was in agreement with relationships previously established by morphological descriptions and with previous studies based upon the nuclear genome or isozymes; moreover such analysis resolved some ambiguities in earlier analyses. It was confirmed that *P. ostreatus* and *P. florida* represent a single species, as well as *P. pulmonarius* and *P. sajor-caju*. The phylogenetic analysis also made it possible to assess the relative positions of *P. rattenburyi*, *P. lampas*, *P. sapidus*, *P. colombinus* and *P. eryngii*. The results clearly showed that sequences of the V4, V6 and V9 domains of the mitochondrial SSU rRNA could provide good markers for use in the taxonomy and phylogeny of species of Basidiomycota. Because of their nucleotide conservation, the major advantage of these species-specific markers was the possibility to study only one isolate from each species to determine phylogenetic relatedness.

Keywords: Basidiomycota, mitochondrial SSU rRNA, *Pleurotus*, taxonomy, phylogeny

INTRODUCTION

Most taxomonic and phylogenetic studies of Basidiomycota have been based on the analysis of morphological characters. In the last 10 years, relationships among species in several genera of Basidiomycota have often been established by RFLP studies of the nuclear genome, and by amplification of nuclear sequences by PCR. RFLP analysis allowed separation of species belonging to the genera *Laccaria* (Gardes *et al.*, 1990), *Armillaria* (Smith & Anderson, 1989) and *Sclerotinia* (Khon *et al.*, 1988). PCR investigations have mainly focused on nucleotide sequences of the internal transcribed spacer (ITS) located between the nuclear rDNA 18S and 28S subunit genes, and made it possible to determine the relationships between fungal species from the genus *Ganoderma* (Molcalvo *et al.*, 1995).

Little use has hitherto been made of extrachromosomal genetic information constituted by the mitochondrial DNA in Basidiomycota. The mitochondrial genomes usually contain structural genes for subunits involved in the respiratory chain, and genes for components of the mitochondrial protein synthesis machinery, i.e. a complete set of tRNAs, the large and the small subunits of the rRNA (LSU and SSU rRNAs) (for a review see Barroso & Labarère, 1995). Mitochondrial

Abbreviations: ITS, internal transcribed spacer; SSU, small subunit.

The GenBank accession numbers for the sequences reported in this paper are given in Methods.

DNA has been used in several studies to determine phylogenetic relationships of the coleopteran genus *Hegeter* (Juan *et al.*, 1996), the dipteran *Drosophila buzzatiis* (Spicer, 1995), the Ascomycota *Allomyces macrogynus* (Paquin *et al.*, 1995) and the protist *Euglena gracilis* (Teissier *et al.*, 1997). All these phylogenetic analyses based upon mitochondrial genes (*cox1*, *cyt b* and SSU rRNA) have given congruent results when compared to results obtained from nuclear genomes.

Recently the sequence and secondary structure of the mitochondrial SSU rRNA from the Basidiomycota *Agrocybe aegerita* was determined (Gonzalez *et al.*, 1997). Analysis indicated that in this SSU rRNA, three domains, V4, V6 and V9, possessed unusually long nucleotide extensions. Comparisons of these sequences with partial 5′ sequences overlapping the V4 domain of 80 other Basidiomycota species, available in databases (Bruns & Szaro, 1992; Hibbet & Donoghue, 1995), showed that length variations of the V4 domain seemed to be species-specific. We previously found that sequences of the three domains are species-specific in the genus *Agrocybe* and could be used to determine phylogenetic relationships between species (Gonzalez & Labarère, 1998). These results suggested that such mitochondrial sequences could constitute a new approach in the taxonomy and phylogeny of microorganisms. Because of the invariability of these sequences within a species, the major advantage of this approach is the need for only one isolate from each species to determine the phylogenetic relationship. Unfortunately, no other phylogenetic study was available in the literature on the genus *Agrocybe* to infer confidence in the use of these sequences in phylogeny of Basidiomycota.

In this study, we investigated the lengths and sequences of the V4, V6 and V9 domains among the genus *Pleurotus* (order *Poriales*). *Pleurotus* species are widespread mushrooms that usually grow on wood; they include cultivated species of important economic value such as *P. ostreatus*, *P. pulmonarius*, *P. cornucopiae* and *P. cystidiosus*. Moreover, the genus *Pleurotus* has been more thoroughly investigated than the genus *Agrocybe*, and numerous phylogenetic studies based upon the analysis of molecular markers are available; they have been based upon the comparison of isozyme patterns (Zervakis *et al.*, 1994), the analysis of the nuclear genome by RFLP (Iraçabal *et al.*, 1995) and sequence comparisons of the ITS (Vilgalys & Sun, 1994). More recently, intercompatibility studies have been carried out for some species of *Pleurotus* (Zervakis & Balis, 1996). So it appeared particularly interesting to compare our analysis based upon mitochondrial sequences with previous phylogenetic analysis based upon nuclear markers. Moreover, all these previous studies have failed to resolve some phylogenetic ambiguities, such as the taxonomic position of *P. sapidus*, *P. eryngii*, *P. colombinus* and *P. flabellatus*. In addition, most previous studies classify the species *P. pulmonarius* and *P. sajor-caju* in the same species complex (Zervakis *et al.*, 1994; Iraçabal *et al.*, 1995), and *P. florida* has been described as a probable subspecies of *P. ostreatus* (Guzman *et al.*, 1994). In this context, it was interesting to investigate whether the use of mitochondrial sequences could resolve some of these ambiguities.

In this work we used specific primers flanking the V4, V6 and V9 domains of the mitochondrial SSU rRNA (Gonzalez & Labarère, 1998) to amplify mitochondrial DNAs from 48 isolates from 16 *Pleurotus* species (Table 1); isolates were chosen according to their different geographical origin. PCR products were purified and sequenced. Alignment of the resulting sequences allowed us to deduce nucleotide sequence variations and the putative evolution of each mitochondrial domain. We determined the secondary structures of each domain and used these to regroup species within subgroups. We established a consensus tree based upon the concatemerized sequences of the V4, V6 and V9 domains using the neighbour-joining, the parsimony and the UPGMA methods. We then compared the resulting trees to previously reported phylogenetic analysis of *Pleurotus*.

METHODS

***Pleurotus* strains and culture medium.** All the *Pleurotus* strains used were dikaryotic (Table 1). They were grown in Petri dishes (90 mm) containing 30 ml potato dextrose agar (PDA) medium (39 g l^{-1}; Sigma), in the dark at 26 °C (FPN 70 incubator, Friga-Bohn) for 8–14 d. The geographical origins of the strains used are reported in Table 1.

Genomic DNA purification. Total DNA was extracted from vegetative mycelia with the *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) method adapted for a small quantity of mycelium (Barroso *et al.*, 1995). Mycelium (around 0·2 g) was collected with a scalpel from an 8 d culture, frozen in liquid nitrogen and crushed in a mortar. The crushed mycelium was resuspended in 0·7 ml extraction buffer (100 mM Tris/HCl pH 8, 2%, w/v, CTAB, 20 mM EDTA, 1·4 M NaCl, 2%, v/v, β-mercaptoethanol) and incubated for 20 min at 56 °C. Then 0·7 ml chloroform/isoamyl alchohol (24:1, v/v) was added and the two phases were mixed to obtain an emulsion. After centrifugation (9000 ***g***, 15 min, 20 °C), the aqueous phase was removed and again subjected to a second extraction with 0·7 ml chloroform/isoamyl alcohol, as described above. Nucleic acids were precipitated by 0·7 ml precipitation buffer (50 mM Tris/HCl pH 8, 1%, w/v, CTAB, 10 mM EDTA, 1%, v/v, β-mercaptoethanol) for 30 min at room temperature. The precipitate was recovered by centrifugation (9000 ***g***, 15 min, 20 °C), dried, resuspended in 0·5 ml 1 M NaCl and incubated for 20 min at 56 °C. Nucleic acids were then precipitated at room temperature by adding 2 vols absolute ethanol. After centrifugation (11000 ***g***, 15 min, 20 °C), the pellet was washed three times with 1 ml 70% (v/v) ethanol to completely eliminate the excess of CTAB. The pellet was dried and then resuspended in sterile distilled water. Nucleic acids were used directly for PCR amplifications or stored at 4 °C.

PCR amplification. Amplifications were performed using the previously described primer pairs V4U/V4R, V6U/V6R and V9U/V9R (Gonzalez & Labarère, 1998) to amplify the regions containing, respectively, the variable domains V4, V6 and V9 of the mitochondrial SSU rDNA. These primer pairs were complementary to regions flanking the V4, V6 and V9

Table 1. Length of the variable domains V4, V6 and V9 of the mitochondrial SSU rDNA from 49 strains belonging to 16 species of *Pleurotus*

Pleurotus groups, species and strains*	Origin†	V4 domain				V6 domain			V9 domain		
		Length (nt)‡	Secondary structures§			Length (nt)‡	Secondary structures§		Length (nt)‡	Secondary structures§	
			P23-2	P23-1	P23-3		P37-1	P37-2		P49-2	P49-1
GROUP A											
A1											
P. citrinopileatus											
SC 97 12 13	MUCL 28684	77	+	–	–	266	+ +	–	236	–	+
P. euosmus											
SC 97 12 22	CBS 307.29	71	+	–	–	264	+ +	–	223	–	+
A2											
P. cornucopiae											
SC 96 06 01	Japan[b]	77	+	–	–	82	+	–	225	–	+
SC 96 06 02	Japan[b]	77	+	–	–	82	+	–	225	–	+
A3											
P. cystidiosus											
SC 91 04 13	CBS 615.80	69	+	–	–	75	+	–	208	–	‖
SC 96 09 02	Thailand[b]	69	+	–	–	75	+	–	208	–	‖
SC 91 04 15	CBS 298.35	69	+	–	–	75	+	–	208	–	‖
A4											
P. dryinus											
SC 97 12 32	CBS 278.90	69	+	–	–	195	+	+	256	–	+
SC 97 12 28	CBS 804.85	69	+	–	–	195	+	+	256	–	+
A5											
P. smithii											
SC 97 12 30	CBS 703.94	183	+	–	+	91	+	–	230	–	+
GROUP B											
P. colombinus											
SC 97 12 14	MUCL 31682	107	+	+	–	83	+	–	261	+	+
P. eryngii											
SM 81	France (south-west)[b]	106	+	+	–	83	+	–	241	+	+
SM 83 11 02	France (south-east)[b]	Id	+	+	–	Id	+	–	Id	+	+
SM 85 11 01	France (north-west)[b]	Id	+	+	–	Id	+	–	Id	+	+
SC 87 10 24	Spain (Encantad)[b]	106	+	+	–	83	+	–	241	+	+
SC 92 05 10	Spain[b]	Id	+	+	–	Id	+	–	Id	+	+
SM 90 10 04	Greece (Oriestiada)[b]	106	+	+	–	83	+	–	241	+	+
SM 90 10 17	Greece (Lasithi)[b]	Id	+	+	–	Id	+	–	Id	+	+
SM 90 10 28	Greece (LGAM P 66)[a]	Id	+	+	–	Id	+	–	Id	+	+
SM 90 10 29	Greece (LGAM P 63)[a]	Id	+	+	–	Id	+	–	Id	+	+
SC 91 04 04	CBS 100.82	106	+	+	–	83	+	–	241	+	+
SC 91 04 26	ATCC 36047	Id	+	+	–	Id	+	–	Id	+	+
P. flabellatus											
SC 97 12 09	MUCL 31687	90	+	+	–	82	+	–	267	+	+
SC 97 12 12	MUCL 28912	90	+	+	–	82	+	–	267	+	+
P. ostreatus											
SM 84 11 01	France (south-west)[b]	104	+	+	–	83	+	–	267	+	+
SM 91 04 07	CBS 291.47	Id	+	+	–	Id	+	–	Id	+	+
SM 96 09 13	Italy[b]	104	+	+	–	83	+	–	267	+	+
Sm 85 10 01	Czechoslovakia[b]	104	+	+	–	83	+	–	267	+	+
SM 90 10 07	Greece (Vardoussia)[b]	Id	+	+	–	Id	+	–	Id	+	+
SM 90 10 31	Greece (LGAM P 38)[a]	104	+	+	–	83	+	–	267	+	+
SM 40	India (Kashmir)[b]	Id	+	+	–	Id	+	–	Id	+	+
SM 96 09 07	Thailand[b]	104	+	+	–	83	+	–	267	+	+

Table 1 *(cont.)*

Pleurotus groups, species and strains*	Origin†	V4 domain				V6 domain			V9 domain		
		Length (nt)‡	Secondary structures§			Length (nt)‡	Secondary structures§		Length (nt)‡	Secondary structures§	
			P23-2	P23-1	P23-3		P37-1	P37-2		P49-2	P49-1
P. florida											
SC 97 12 03	MUCL 31688	104	+	+	−	83	+	−	267	+	+
SC 97 12 08	MUCL 31686	104	+	+	−	83	+	−	267	+	+
P. pulmonarius											
SM 92 05 12	Spain[b]	90	+	+	−	78	+	−	233	+	+
SM 92 05 23	Hungary[b]	90	+	+	−	78	+	−	233	+	+
SM 90 10 01	Greece (Vardoussia)[b]	90	+	+	−	78	+	−	233	+	+
SM 90 10 12	Greece (Vardoussia)[b]	Id	+	+	−	Id	+	−	Id	+	+
SM 90 10 22	Greece (Vardoussia)[b]	Id	+	+	−	Id	+	−	Id	+	+
SM 96 06 06	Japan[b]	Id	+	+	−	Id	+	−	Id	+	+
SM 96 06 07	Japan[b]	Id	+	+	−	Id	+	−	Id	+	+
SM 92 05 42	CBS 132.85	90	+	+	−	78	+	−	233	+	+
P. sajor-caju											
SM 37	Mauritius[a]	90	+	+	−	78	+	−	233	+	+
SC 96 09 05	Thailand[b]	90	+	+	−	78	+	−	233	+	+
SC 96 09 06	Thailand[b]	90	+	+	−	78	+	−	233	+	+
SC 97 12 05	MUCL 28685	Id	+	+	−	Id	+	−	Id	+	+
P. sapidus											
SC 97 12 23	CBS 195.92	102	+	+	−	84	+	−	262	+	+
GROUP C											
C1											
P. lampas											
SC 97 12 26	CBS 323.49	71	+	−	−	100	+	−	284	+	+
C2											
P. rattenburyi											
SC 97 12 24	CBS 175.94	67	¶	−	−	82	+	−	415	−	+

* The strain numbers correspond to the nomenclature of the Laboratory of Molecular Genetics and Breeding of Cultivated Mushrooms.

† CBS, Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands; MUCL, Mycothèque de l'Université Catholique du Louvain-la-Neuve, Belgium; ATCC, American Type Culture Collection, Manassas, VA, USA. Isolates previously used by Iraçabal *et al.* (1995) are marked '*a*'; isolates used only in this study are marked '*b*'.

‡ Id, length determined by agarose gel electrophoresis.

§ +, Presence; −, absence; + +, long P37-1 helix; ‖, truncated P49-1 helix; ¶, truncated P23-2 helix.

domains; they were determined by comparison with partial sequences available in databases and from the *A. aegerita* mitochondrial SSU rRNA sequence (Gonzalez *et al.*, 1997). PCR reactions contained 50 mM KCl, 10 mM Tris/HCl pH 9, 0·1% Triton X-100, 0·2 mM of each dNTP, 2·5 mM $MgCl_2$ and 3–5 µl purified DNA in a final volume of 25 µl. Forty cycles were performed in a PTC 100 thermal cycler (MJ Research) as follows. DNA was denatured for 30 s at 95 °C, annealing of primers was obtained at a temperature corresponding to $T_m - 2$ °C (i. e. 42, 54 and 50 °C for amplification of the regions overlapping the V4, V6 and V9 domains, respectively) for 30 s, and an elongation step was performed for 30 s. PCR products were then analysed in a 1·5% agarose gel, or in a 5% polyacrylamide gel, and observed after ethidium bromide staining.

Purification and sequencing of PCR amplification products. Amplimers were purified using the Quiaquick PCR purification kit (QIAGEN), following the manufacturer's instructions to remove all the excess primer prior to sequencing reactions.

Sequencing of PCR products was performed on both strands using the ThermoSequenase sequencing kit (United States Biochemical) according to Sanger *et al.* (1977) with [α-^{33}P]ddNTPs (55·5 TBq $mmol^{-1}$, Amersham). The primer pairs V4U/V4R, V6U/V6R and V9U/V9R were used to sequence PCR products of the variable domains V4, V6 and V9, respectively. The sequencing products were analysed in 6% polyacrylamide gels and revealed after exposure to Kodak X-omat LS films.

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                                                     V4a
P. cit AGCTTTGAAAGTTTATTTTT----------------------------------------------------------------ATACAAA-----
P. euo AGCTTTGAAAGTTTATTTTT----------------------------------------------------------------ATACAAA-----
P. cor AGCTTTGAAAATTTAATTTT----------------------------------------------------------------ATACAAA-----
P. ery AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AAAAAAAA-TTT
P. ost AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AAAAATT--TTT
P. flo AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AAAAATT--TTT
P. col AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AAAAAAAATTTT
P. sap AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AAAAA----TTT
P. fla AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AATAA-----TA
P. pul AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AATAA-----TT
P. saj AGCTTTGAAAATTTAATTTT----------------------------------------------------------------AATAA-----TT
P. cys AGCTTT-AAAGTTTATTTT-----------------------------------------------------------------AAAAA-------
P. dry AGCTTTGAAAGTTTATTTTT----------------------------------------------------------------TAAT--------
P. lam AGCTTT-AAAGTTTATTTTT----------------------------------------------------------------AATAAT------
P. rat AGCTTT-AAAGTTTATTTTT----------------------------------------------------------------AATAAT------
P. smi AGCTTT-AAAGTTTATTTTCTCATCTCCCTCCTCCCACCTCCCATCTCCCACCTTCCCATCTATAGATGATTGGTGCTTTCATCAATGATGGGATG
       ****** *** ***** ***
                   V4b
P. cit ----------------------------------GGCTCT--ATTT-------AAATTAAAAAAA-AAATAGTGAAAATAAAATC-ATTTAAAGCT
P. euo ----------------------------------GGCTCT--ATTT-------AAATAA-------AAATAGTGAAAATAAAATAAATT-AAAGCT
P. cor ----------------------------------GGCTCT--ATTT-------AAATTAAAAAAA-AAATAGTGAAAATAAAATAAATT-AAAGCT
P. ery TTTT----TCTTTTCTAGAAAAAAAGAG-----TAGCTCT--ATTTGTTCAATAAATT--------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. ost TTTT----TCTTTTCTAGAAAAAAAGAG-----TAGCTCT--ATTTGTACA-TAAATT--------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. flo TTTT----TCTTTTCTAGAAAAAAAGAG-----TAGCTCT--ATTTGTACA-TAAATT--------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. col TTTT----TCTTTTCTAGAAAAAAAGAG-----TAGCTCT--ATTTGTTCAATAAATT--------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. sap TTTT----TCTTTTCTAGAAAAAAAGAG-----TAGCTCT--ATTTGTACA-TAAATT--------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. fla --TT----TGTTTT-----AAAATGAT------TAGCTCT--ATTT-----ATTAAATT-------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. pul --TT----TGTTCT-----AAAATGAT------TAGCTCT--ATTT-----ATTAAATT-------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. saj --TT----TGTTCT-----AAAATGAT------TAGCTCT--ATTT-----ATTAAATT-------AAATAGTAAAAATAAAATCAATT-AAAGCT
P. cys ---------------------------------TAGCTCT-CATTT-------TTAATT-------AAATAGTAAAAATAAAATTAATT-AAAGCT
P. dry ---------------------------------TAGCTCT--ATTT-------TTAAAT-------AAATAGTAAAAATAAAATTAATT-AAAGCT
P. lam ---------------------------------TAGCTCT--------------AAAT-GTAATTTAAACAGTAAAAATAAAATTAATT-AAAGCT
P. rat ---------------------------------TAGCTCT-------------------GTAATTTAAACAGTAAAAATAAAATTAATT-AAAGCT
P. smi ATTGGTGCTTGGTGCTTGGTGAATGATGGAAGATAGCTCTTTTTTTA----ATTAAATAGTAA---AAATAGTAAAAATAAAATTAATTTAAAGCT
                                   *****                          *** *** **********  *** ******
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Fig. 1. Alignment of the V4 domain sequences of 16 species of the genus *Pleurotus*. Sequences strictly conserved among the 16 species are indicated by asterisks. The location and the putative size of insertion/deletion events are indicated by dashes. V4a and V4b boxes represent putative insertions/deletions events. *P. cit*, *P. citrinopileatus* SC 97 12 13; *P. euo*, *P. euosmus* SC 97 12 22; *P. cor*, *P. cornucopiae* SC 96 06 01; *P. ery*, *P. eryngii* SM 81; *P. ost*, *P. ostreatus* SM 84 11 01; *P. flo*, *P. florida* SC 97 12 03; *P. col*, *P. colombinus* SC 97 12 14; *P. sap*, *P. sapidus* SC 97 12 23; *P. fla*, *P. flabellatus* SC 97 12 09; *P. pul*, *P. pulmonarius* SM 92 05 12; *P. saj*, *P. sajor-caju* SM37; *P. cys*, *P. cystidiosus* SC 91 04 13; *P. dry*, *P. dryinus* SC 97 12 32; *P. lam*, *P. lampas* SC 97 12 26; *P. rat*, *P. rattenburyi* SC 97 12 24; *P. smi*, *P. smithii* SC 97 12 30.

GenBank accession numbers. For the V4 domain: *P. citrinopileatus*, AF091891; *P. colombinus*, AF091892; *P. cornucopiae*, AF091893; *P. cystidiosus*, AF091894; *P. dryinus*, AF091895; *P. eryngii*, AF091896; *P. euosmus*, AF091897; *P. flabellatus*, AF091898; *P. florida*, AF 091899; *P. lampas*, AF091900; *P. ostreatus*, AF 091901; *P. pulmonarius*, AF-091902; *P. rattenburyi*, AF091903; *P. sajor-caju*, AF091904; *P. sapidus*, AF091905; *P. smithii*, AF091906. For the V6 domain: *P. citrinopileatus*, AF091907; *P. colombinus*, AF091908; *P. cornucopiae*, AF091909; *P. cystidiosus*, AF091910; *P. dryinus*, AF091911; *P. eryngii*, AF091912; *P. euosmus*, AF091913; *P. flabellatus*, AF091914; *P. florida*, AF 091915; *P. lampas*, AF091916; *P. ostreatus*, AF 091917; *P. pulmonarius*, AF-091918; *P. rattenburyi*, AF091919; *P. sajor-caju*, AF091920; *P. sapidus*, AF091921; *P. smithii*, AF091922. For the V9 domain: *P. citrinopileatus*, AF091923; *P. colombinus*, AF091924; *P. cornucopiae*, AF091925; *P. cystidiosus*, AF091926; *P. dryinus*, AF091927; *P. eryngii*, AF091928; *P. euosmus*, AF091929; *P. flabellatus*, AF091930; *P. florida*, AF 091931; *P. lampas*, AF091932; *P. ostreatus*, AF 091933; *P. pulmonarius*, AF-091934; *P. rattenburyi*, AF091935; *P. sajor-caju*, AF091936; *P. sapidus*, AF091937; *P. smithii*, AF091938.

Phylogenetic analysis. Sequences were aligned with CLUSTAL W (version 1.7) software (Higgins & Sharp, 1989). Consensus trees were constructed with the UPGMA, the neighbour-joining and the parsimony methods of the phylogeny inference package PHYLIP (version 3.5). To infer confidence of the branchpoints in the constructed tree, we performed a bootstrap analysis (1000 bootstrap replicates).

RESULTS

PCR amplification and sequencing of the V4, V6 and V9 domain within 16 *Pleurotus* species

We extracted total DNA from 48 isolates belonging to 16 *Pleurotus* species (Table 1), and amplified by PCR regions overlapping the V4, V6 and V9 domains of the mitochondrial SSU rRNA gene as previously described (Gonzalez & Labarère, 1998). Agarose gel electrophoresis of the PCR products showed that a single amplimer was obtained for each domain; there were no significant intra-species length variations, whereas large inter-species size variations were observed.

For each species, we purified and sequenced PCR products from one to four isolates of different geographical origin, and precisely determined the lengths of the three domains (Table 1). The results confirmed preliminary analysis of PCR amplimer patterns obtained by agarose gel electrophoresis, i.e. all strains from the same species possessed exactly the same domain length, while there was great inter-species variation in size. The V4 domain ranged from 67 nt (*P. rattenburyi*) to 183 nt (*P. smithii*), the V6 domain from 75 nt (*P. cystidiosus*) to 266 nt (*P. citrinopileatus*), and the V9 domain from 208 nt (*P. cystidiosus*) to 415 nt (*P. rattenburyi*). Some species possessed the same length for one domain: 90 nt

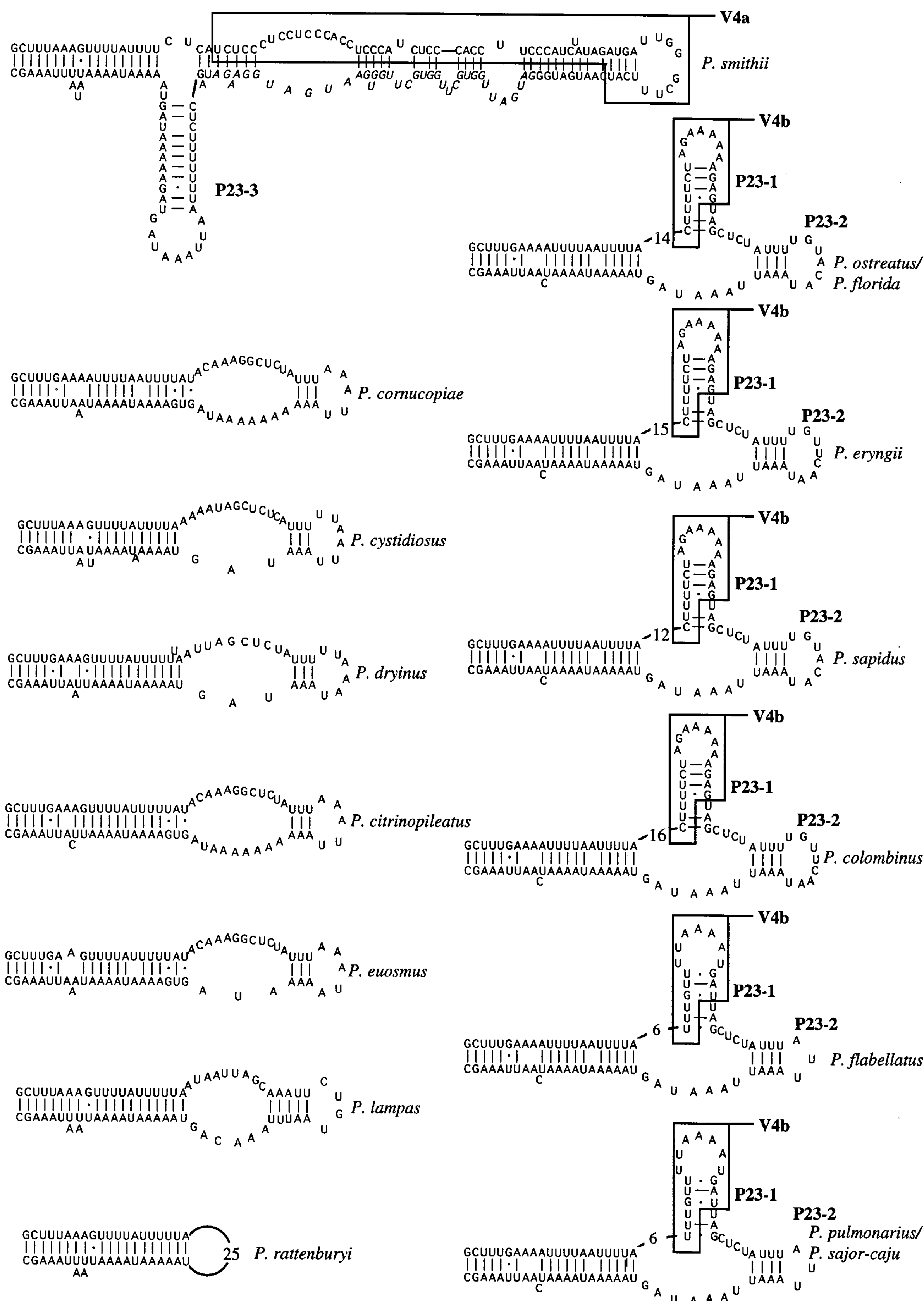

Fig. 2. Proposed secondary structure of the variable domain V4 of 16 species of the genus *Pleurotus*. The number of the additional helix is indicated. Nucleotides constituting the V4a and V4b motifs are framed.

(*P. pulmonarius*, *P. sajor-caju* and *P. flabellatus*) or 77 nt (*P. citrinopileatus* and *P. cornucopiae*) or 71 nt (*P. lampas* and *P. euosmus*) for the V4 domain; 82 nt (*P. cornucopiae*, *P. flabellatus* and *P. rattenburyi*) or 83 nt (*P. colombinus*, *P. florida* and *P. ostreatus*) for the V6 domain; and 267 nt (*P. flabellatus*, *P. florida* and *P. ostreatus*) for the V9 domain. Only two groups of species – *P. pulmonarius* (8 isolates) and *P. sajor-caju* (4 isolates) on the one hand, and *P. florida* (2 isolates) and *P. ostreatus* (8 isolates) on the other hand – shared the same V4, V6 and V9 lengths.

Sequence comparisons of the V4, V6 and V9 domains within the genus *Pleurotus*

Alignments of the nucleotide sequences showed that the sequences flanking the three variable domains were very similar among the 16 *Pleurotus* species studied (78–100% identity of either the V4, V6 or V9 flanking regions). In contrast, lower percentages of identity (<70%) were observed when the V4, V6 and V9 sequences were compared.

For most of the species included in the study, several isolates were studied (2–11), and sequence comparisons showed that all isolates from the same species possessed identical sequences for the V4, V6 or V9 domains, respectively. Moreover, in the case of different species with identical nucleotide length for one of the three domains, these sequences appeared to be species-specific. Indeed, the occurrence of nucleotide changes allowed us to discriminate these species; for example, *P. flabellatus* (2 isolates) and *P. pulmonarius* (8 isolates), which both have a V4 domain of 90 nt, differed by two nucleotide base differences, while *P. flabellatus* (2 isolates) and *P. ostreatus* (8 isolates) (V9 = 267 nt) differed by 35 nucleotide changes. However, two groups of species – *P. pulmonarius* (8 isolates) and *P. sajor-caju* (4 isolates) on the one hand and *P. ostreatus* (8 isolates) and *P. florida* (2 isolates) on the other hand – shared exactly the same nucleotide sequence for each domain studied (Fig. 1).

Sequence alignment showed conserved nucleotide motifs within the genus. Three were located in the V4 domain (Fig. 1): AAARTTTTAWTTT in the 5′ part, GCTCT in the central part, and AAAYAGTRAAAATAAAAT in the 3′ part. Another conserved motif was located in the 5′ part of the V6 domain (CATATYTA) and two in the V9 domain: AWTAWAACTT in the 5′ part, and CATTTRASTMA in the 3′ part.

For each domain the nucleotide motifs constituting the smallest sequence were present in the largest one, suggesting an evolution by sets of insertion/deletion events. For example, *P. smithii*, possessing the largest V4 domain (183 nt), differed from the other species mainly by the presence of the additional nucleotide motifs V4a and V4b. The V4a motif is absent in all other species studied, while the V4b motif is partially found in six species (*P. eryngi*, *P. ostreatus*, *P. colombinus*, *P. sapidus*, *P. flabellatus*, *P. pulmonarius*) (Fig. 1).

We observed similar differences in the V6 and the V9 domains. Indeed, in the V6 domain, a TATAGC motif found in *P. lampas* was absent in all other species and two sequences of 129 nt and 39 nt, found in *P. citrinopileatus*, *P. dryinus* and *P. euosmus* were absent in the other species. In the V9 domain a 10 nt motif present in *P. rattenburyi* and *P. lampas* was absent in the remaining species and a 136 nt motif found in *P. rattenburyi* was absent in all other species except *P. flabellatus* and *P. citrinopileatus*, in which a few residues from this sequence were present (15 nt and 6 nt, respectively).

Comparison of the putative secondary structures of the V4, V6 and V9 domains within the genus *Pleurotus*

With the aim of precisely locating where the insertion/deletion events had occurred, the putative secondary structures of all domains for each species were determined. It should be noted that *P. pulmonarius* and *P. sajor-caju* on the one hand, and *P. ostreatus* and *P. florida* on the other hand, sharing the same sequences for the three domains, possessed the same secondary structures.

For the V4 domain, four groups of putative secondary structures were observed; they were characterized by the presence or absence of additional helices V4-P23-1 and V4-P23-3 and by the length of the major helix V4-P23-2 (Fig. 2). In one group (*P. citrinopileatus*, *P. euosmus*, *P. cornucopiae*, *P. cystidiosus*, *P. dryinus*, *P. smithii*, *P. lampas*), the V4 domain possessed a canonical topology (Neefs *et al*., 1993) without additional helices. Another group (*P. colombinus*, *P. eryngii*, *P. flabellatus*, *P. ostreatus*/*P. florida*, *P. pulmonarius*/*P. sajor-caju*, *P. sapidus*) was characterized by the presence of the additional helix V4-P23-1. Interestingly, the sequence of this helix was strictly conserved among the species *P. colombinus*, *P. eryngii*, *P. ostreatus*/*P. florida* and *P. sapidus* on the one hand, and in *P. flabellatus* and *P. pulmonarius*/*P. sajor-caju* on the other hand. Another group (*P. smithii*) possessed the additional helix V4-P23-3. The last group (*P. rattenburyi*) possessed a truncated V4-P23-2 helix. From the comparison of these secondary structures, it appeared that sequence variations in the V4 domain were not randomly scattered; they were essentially due to (i) differences in the number of nucleotides in the loops, (ii) the presence of additional helices and (iii) the reduction of the V4-P23-2 helix.

Three types of secondary structures were found for the V6 domain (Fig. 3). In most species (*P. cornucopiae*, *P. cystidiosus*, *P. smithii*, *P. colombinus*, *P. eryngii*, *P. flabellatus*, *P. ostreatus*/*P. florida*, *P. pulmonarius*/*P. sajor-caju*, *P. sapidus*, *P. lampas*, *P. rattenburyi*), this domain possessed the single and common V6-P37-1 helix. One variant group, including *P. euosmus* and *P. citrinopileatus*, possessed an unusually large V6-P37-1 with more than 120 base-paired nucleotides. The third

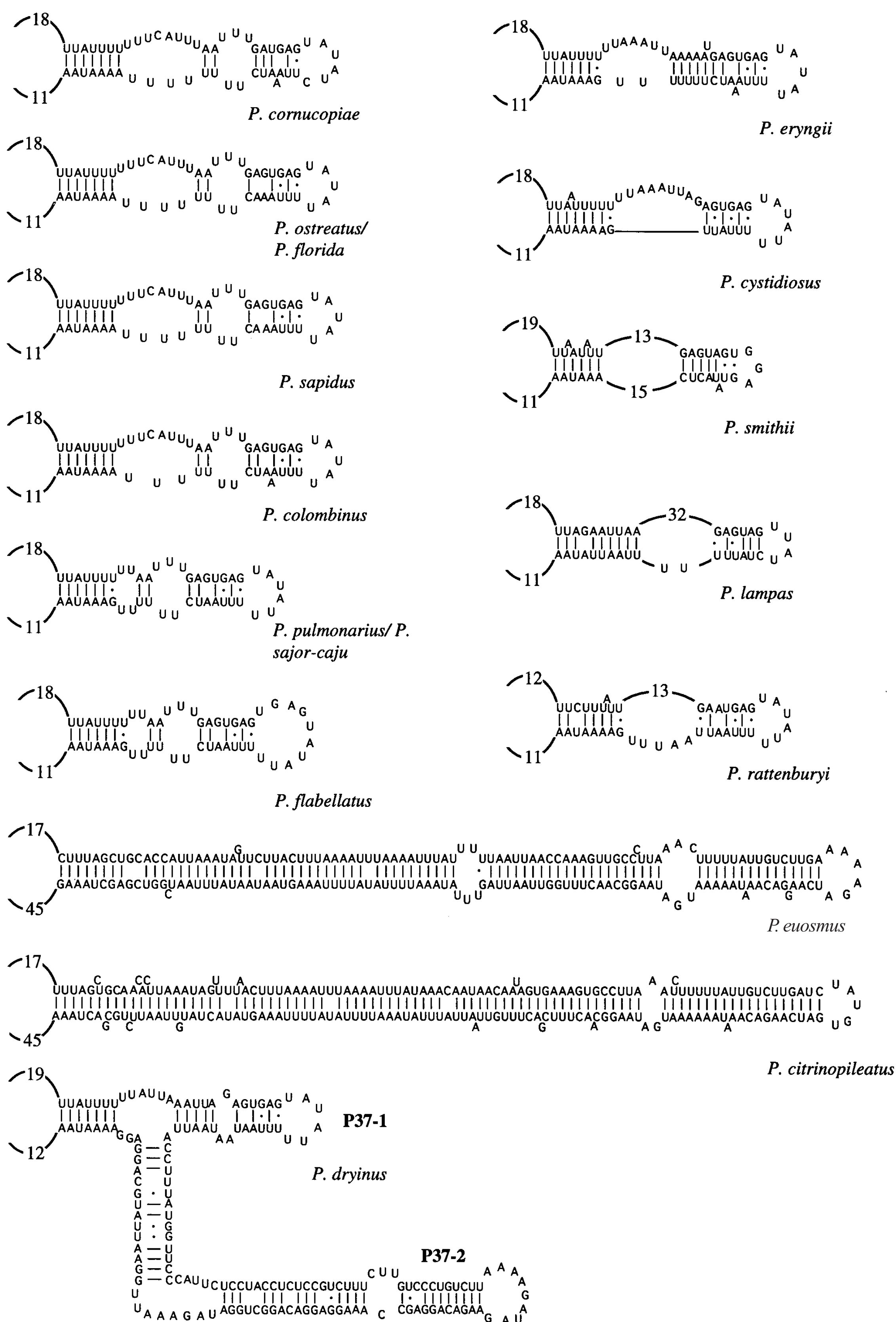

Fig. 3. Proposed secondary structure of the variable domain V6 of 16 species from the genus *Pleurotus*. The number of the additional helix is indicated.

group included only one species (*P. dryinus*), which possessed the additional helix V6-P37-2. As in the V4 domain, length variations in the V6 domain were due to variations in the length of the V6-P37-1 helix and of the loops, and to the occurrence of the V6-P37-2 helix.

We observed three groups of secondary structures for the V9 domain (Fig. 4): one possessed a classical V9-P49-1 helix (*P. citrinopileatus*, *P. euosmus*, *P. cornucopiae*, *P. dryinus*, *P. smithii*, *P. rattenburyi*), one was characterized by a truncated V9-P49-1 helix (*P. cystidiosus*), and one by an additional V9-P49-2 helix (*P. colombinus*, *P. eryngii*, *P. flabellatus*, *P. ostreatus*/*P. florida*, *P. pulmonarius*/*P. sajor-caju*, *P. sapidus*, *P. lampas*).

Phylogenetic analysis of *Pleurotus* species based on the V4, V6 and V9 domain sequences

The sequences of the three variable domains of each species were all joined together in the order V4-V6-V9 and used to construct consensus trees with the UPGMA, the neighbour-joining and the parsimony methods (PHYLIP package, version 3.5). Similar phylogenetic trees were obtained using the neighbour-joining and the parsimony methods, whereas minor changes were apparent in the consensus tree constructed with the UPGMA method. In the UPGMA tree, three groups (A, B, C) of species were clearly recovered (Fig. 5a). Group A was divided into two subgroups: one included *P. citrinopileatus* and *P. euosmus*, closely related to each other and associated with *P. cornucopiae*; the other included *P. dryinus* and *P. smithii*, strongly associated with *P. cystidiosus*. Group B also contained two subgroups related to *P. ostreatus*/*P. florida*: one subgroup included *P. pulmonarius*/*P. sajor-caju*, closely related to *P. eryngii*; the other subgroup included *P. sapidus* associated with *P. colombinus* and themselves related to *P. flabellatus*. Group C consisted of *P. rattenburyi* and *P. lampas*.

The major differences observed in the neighbour-joining/parsimony tree concerned the relationships among group B species and the relative position of group C. In this tree (Fig. 5b) *P. ostreatus*/*P. florida* were more closely related to *P. sapidus* and *P. colombinus*, instead of being considered as the most divergent species of this group (Fig. 5a). Also, *P. flabellatus* appeared related to *P. pulmonarius*/*P. sajor-caju* and *P. eryngii*. Moreover, group B was not so clearly separated from the other species as in the UPGMA tree. Group C appeared to be inserted between groups A and B in this tree, while it was considered as the most divergent group in the UPGMA tree. However, it should be noted that in the neighbour-joining/parsimony tree, the divergent positions were less supported by the bootstrap analysis than those observed in the UPGMA tree. For example, the phylogenetic position of *P. ostreatus*/*P. florida* possessed a bootstrap support of 67·5% in the UPGMA analysis and only 53–58% in the other analysis.

DISCUSSION

Species-specificity of the V4, V6 and V9 sequences within the *Pleurotus* genus

Previous studies have shown nucleotide conservation in the sequences of the V4, V6 and V9 domains within the genus *Agrocybe* (Gonzalez & Labarère, 1998). Analysis of these sequences in the present study for several isolates of *P. eryngii* (11 isolates), *P. ostreatus* (8 isolates), *P. pulmonarius* (8 isolates) or *P. sajor-caju* (4 isolates) confirmed that sequences of the three domains are species-specific. Regarding the results obtained with about 90 strains in either *Agrocybe* or *Pleurotus* genera, the mitochondrial SSU rRNA V4, V6 and V9 domains do not seem to have evolved since speciation. This nucleotide conservation within a species might be correlated with the ability of these domain sequences to bind specific nucleus-encoded riboproteins involved in obtaining the efficient three-dimensional folding of the 30S subunit (Noller & Lake, 1984; Stern *et al*., 1989; Mueller & Brimacombe, 1997). In the light of our results, it could be hypothesized that these sequences are under strong physiological and/or molecular constraints that avoid significant sequence modifications within species. Thanks to their nucleotide conservation, these sequences constitute useful species-specific markers for taxonomy and phylogeny of Basidiomycota. Consequently, the major advantage of using these mitochondrial sequences is the need for only one isolate of each species to determine phylogenetic relationships. By comparison, in nuclear ITS sequences, which are to date the most investigated sequences in phylogenetic studies, numerous intra-species nucleotide variations were found. For example, 10 nucleotide variations have been described in the ITS1 sequences and four in the ITS2 sequences from two isolates (D383 and D1136) of *P. cornucopiae* (Vilgalys & Sun, 1994). So, when using nuclear ITS sequences to determine phylogenetic relatedness it is necessary to study numerous isolates from the same species.

Inter-species variations and secondary structure of the V4, V6 and V9 domains within the genus *Pleurotus*

As previously described for the genus *Agrocybe*, the three domains show inter-species variations, mainly due to point mutations and to insertion/deletion of nucleotide motifs that strengthen the hypothesis of an evolution of mitochondrial SSU rRNA sequences by insertion/deletion events.

Determination of the secondary structure of each domain showed that insertion/deletion events are not randomly scattered, but essentially located in loops not directly involved in obtaining the mitochondrial SSU rRNA secondary structure. Based upon the V4, V6 and V9 secondary structures, *Pleurotus* species are organized in eight subgroups (Table 1). It is interesting to note that these subgroups are in agreement with previous morphological studies (Singer, 1986). For example, subgroup

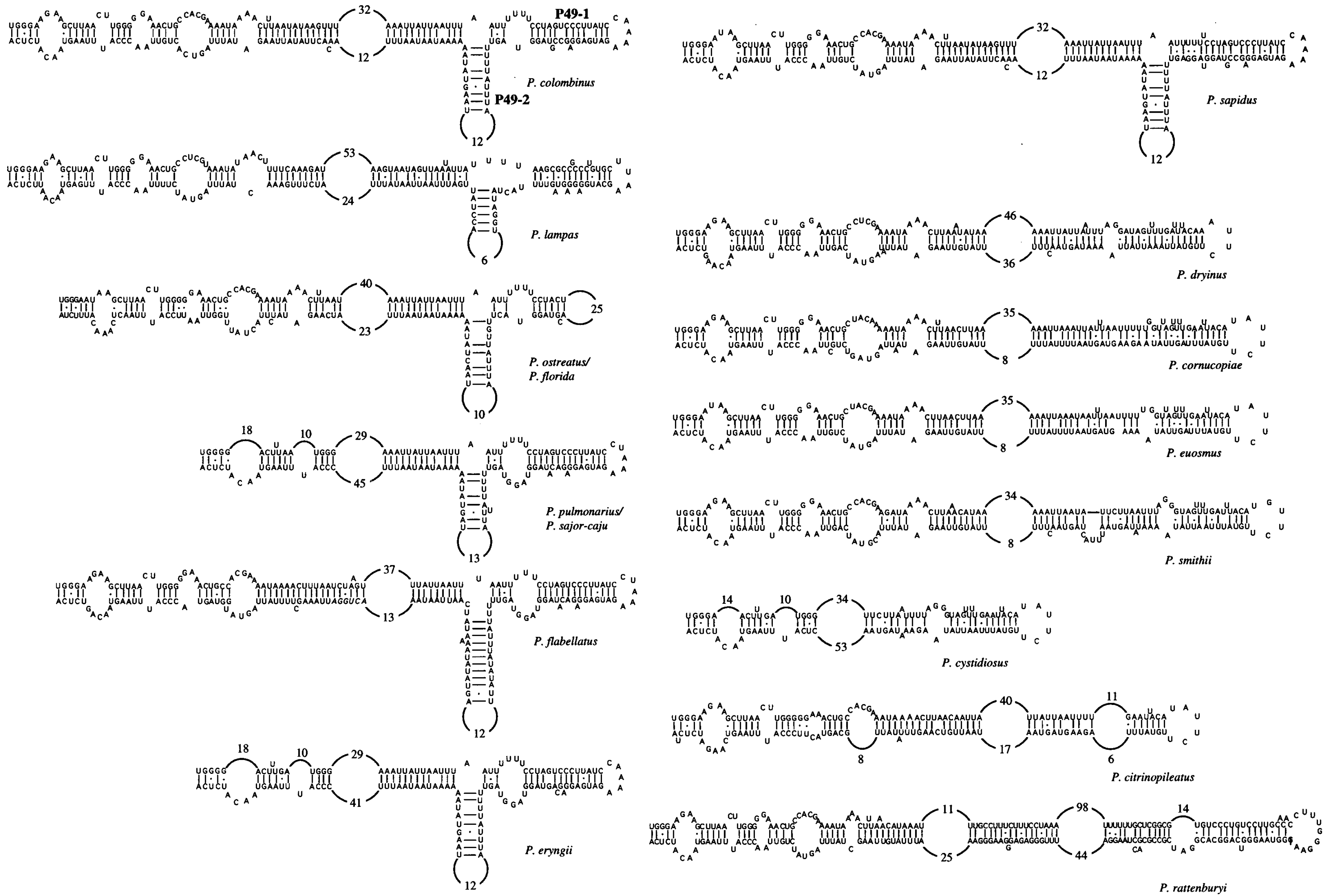

Fig. 4. Proposed secondary structure of the variable domain V9 of 16 species from the genus *Pleurotus*. The number of the additional helix is indicated.

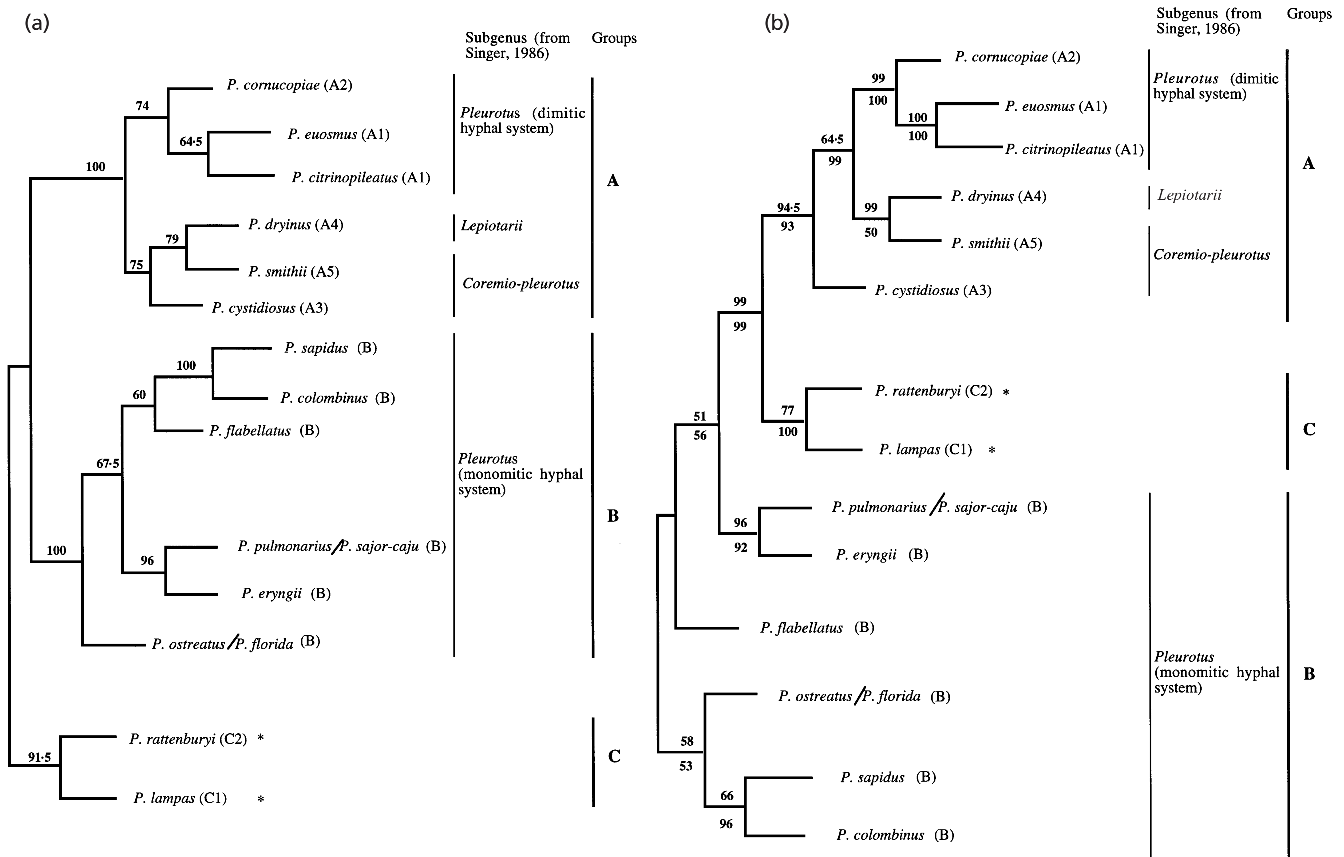

Fig. 5. (a) Consensus tree obtained by the UPGMA method. Bootstrap values greater than 50% are shown. Groups and subgroups defined by comparison of the secondary structures, and the subgenus defined by Singer (1986), are indicated. Asterisks indicate species not described by Singer. (b) Consensus tree obtained by the neighbour-joining and the parsimony methods, based upon the sequences of the variable domains V4, V6 and V9 among 16 *Pleurotus* species. Bootstrap values greater 50% obtained from the neighbour-joining and the parsimony methods are shown above and under branches, respectively. In each tree, subgroups are indicated.

B corresponds to species from the subgenus *Pleurotus* with a monomitic hyphal system, while subgroups A1 and A2 correspond to species from the same subgenus possessing a dimitic hyphal system. Subgroups A3 and A5 include species from the subgenus *Coremio-pleurotus*. Species included in subgroups C1 and C2 were not described by Singer (1986).

Moreover, the three major groups (Table 1) possess structural specificities: (i) group A includes species without additional helices in both V4 and V9, (ii) group B includes species possessing the V4-P23-1 and the V9-P49-2 additional helices, and (iii) group C includes species possessing a truncated V4-P23-2 helix.

Relationship between *Pleurotus* species based on the V4, V6 and V9 sequences

The concatenated V4-V6-V9 sequences were used to construct phylogenetic trees as described by Paquin *et al.* (1997). The use of such concatenated sequences gives congruent results with those obtained with separated data from V4, V6 and V9. However, the bootstrap values, and consequently the validity of these phylogenetic trees, were higher when concatenated sequences were used.

Relationships between species within the three major groups clearly discriminated in the UPGMA phylogenetic tree, and largely recovered in the neighbour-joining/parsimony consensus tree, are in agreement with the subgenera previously described by Singer (1986) and with previous phylogenetic analysis of this genus based upon nuclear markers (Vilgalys *et al.*, 1993; Vilgalys & Sun, 1994; Iraçabal *et al.*, 1995; Zervakis & Balis, 1996) or isozyme markers (Zervakis *et al.*, 1994). However, analysis of the V4, V6 and V9 domains of the mitochondrial SSU rRNA helped us to resolve some ambiguities in species designation. Indeed, sequence comparisons clearly indicated that the *P. pulmonarius* and *P. sajor-caju* isolates studied on the one hand, and the *P. ostreatus* and *P. florida* isolates studied on the other hand, shared identical sequences in their V4, V6 or V9 domains. Previous studies have shown that some *P. pulmonarius* and *P. sajor-caju* strains are interfertile (Hibber, 1982) and would probably have been misidentified (Guzman *et al.*, 1994). Moreover, phylogenetic investigations based upon nuclear RFLP analysis were

not able to differentiate isolates from these two species (Iraçabal *et al*., 1995). So, in accordance with morphological and molecular analysis, our results confirmed that the *P. sajor-caju* strains studied probably belong to the species *P. pulmonarius*. Similarly, the *P. florida* and *P. ostreatus* strains studied have identical nucleotide sequences. Interestingly, it has been previously suggested that strains described as *P. florida* were geographical isolates from the *P. ostreatus* complex (Guzman *et al*., 1991, 1994).

Previous phylogenetic studies have given rise to various ambiguities in the genus *Pleurotus*. According to the molecular markers used, *P. ostreatus*, *P. colombinus* and *P. cornucopiae* have been in turn associated in the same clade (Iraçabal *et al*., 1995) or clearly separated (Zervakis *et al*., 1994), and the position of *P. sapidus* has remained uncertain (Zervakis & Balis, 1996). The phylogenetic analysis based upon the V4, V6 and V9 sequences reported here helps to resolve some of these ambiguities. All resulting trees show that *P. cornucopiae* on the one hand, and *P. colombinus* and *P. ostreatus* on the other hand, are clearly included in two distinct clades. It is to be noted that the close relationship between *P. colombinus* and *P. ostreatus* has been previously reported (Vilgalys *et al*., 1993) and that they have recently been described as sexually compatible species (Zervakis & Balis, 1996). In another way, our results clearly demonstrate close relationships between *P. pulmonarius* and *P. eryngii* (96% bootstrap support) and between *P. sapidus* and *P. colombinus* (100% bootstrap support) (Fig. 5a), while in previous studies *P. eryngii* has been associated either with *P. cystidiosus* (Zervakis *et al*., 1994) or with *P. ostreatus* and *P. colombinus* (Iraçabal *et al*., 1995). Finally, the association of *P. cornucopiae* and *P. citrinopileatus* in the same clade in both consensus trees is supported by recent compatibility assays (Zervakis & Balis, 1996).

However, the phylogenetic position of *P. flabellatus* remains unresolved. This species is related to *P. sapidus* and *P. colombinus* (60% bootstrap support) by the UPGMA method and to *P. pulmonarius*/*P. sajor-caju* (51% bootstrap support) by the neighbour-joining method. In an analysis based upon nuclear markers, *P. flabellatus* has been associated with *P. cornucopiae* (Vilgalys & Sun, 1994), or considered as an intermediate between the *P. ostreatus* and the *P. eryngii* clades (Zervakis *et al*., 1994), or suggested to be the most divergent species of this genus, constituting an outgroup (Iraçabal *et al*., 1995). In the same way, *P. rattenburyi* and *P. lampas* are associated in the same clade in both consensus trees, but the phylogenetic position of this clade varied according to the method used to construct the tree.

Concluding remarks

Our results obtained from species of *Pleurotus* show that the V4, V6 and V9 domains of the mitochondrial SSU rRNA represent efficient molecular markers for the taxonomy of Basidiomycota. These results support previous findings in the genus *Agrocybe* (Gonzalez & Labarère, 1998) and demonstrate the validity of the use of these species-specific mitochondrial sequences in the taxonomy of Basidiomycota. Moreover, nucleotide sequences of these mitochondrial domains appear to be a good alternative for defining phylogenetic relationships between species.

One of the major problems in microbiology is to identify markers strictly specific to a species. Hence, the morphological and molecular study of numerous isolates is necessary to define a species. The sequences of the V4, V6 and V9 domains of the mitochondrial SSU rRNA seem to provide such species-specific markers. The use of these sequences could have numerous applications: for example to discriminate misidentified species in culture collections, to determine the taxonomic position of unidentified species, or to give objective and unvariable criteria to determine a species. However, our studies to date are limited to two genera and future investigations would have to be extended to other genera of Basidiomycota with the aim of establishing a database for these mitochondrial molecular data.

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REFERENCES

Barroso, G. & Labarère, J. (1995). Molecular organization and heredity of the mitochondrial genome in Basidiomycetes. *Mycoscience* **36**, 135–142.

Barroso, G., Perennes, D. & Labarère, J. (1995). A 'miniprep' method for RFLP analysis and dsRNAs detection perfected in the cultivated fungus *A. aegerita*. In *Science and Cultivation of Edible Fungi*, vol. 1, pp. 87–94. Edited by T. J. Elliot. Rotterdam: A. A. Balkema.

Bruns, T. D. & Szaro, T. M. (1992). Rate and mode differences between nuclear and mitochondrial small subunit rRNA genes in mushrooms. *Mol Biol Evol* **9**, 836–855.

Gardes, M., Fortin, A., Mueller, G. M. & Kropp, B. R. (1990). Restriction fragment length polymorphism in the nuclear ribosomal DNA of four *Laccaria* spp.: *L. bicolor*, *L. laccata*, *L. proxima*, and *L. amethystina*. *Phytopathology* **80**, 1312–1317.

Gonzalez, P. & Labarère, J. (1998). Sequence and secondary structure of the mitochondrial small-subunit rRNA V4, V6 and V9 domains reveal highly species-specific variations within the genus *Agrocybe*. *Appl Environ Microbiol* **64**, 4149–4160.

Gonzalez, P., Barroso, G. & Labarère, J. (1997). DNA sequence and secondary structure of the mitochondrial small subunit ribosomal RNA coding region including a group-IC2 intron from the cultivated Basidiomycete *Agrocybe aegerita*. *Gene* **184**, 55–63.

Guzman, G., Bandala, V. M. & Montoya, L. (1991). A comparative study of teleomorphs and anamorphs of *Pleurotus cystidiosus* and *Pleurotus smithii*. *Mycol Res* **95**, 1264–1269.

Guzman, G., Montoya, L., Mata, G. & Salmones, D. (1994). Studies in the genus *Pleurotus*. III. The varieties of *P. ostreatus*-complex based in interbreeding strains and in the study of Basidiomata obtained in culture. *Mycotaxon* **50**, 365–378.

Hibbet, D. S. & Donoghue, M. J. (1995). Progress toward a phylogenetic classification of the *Polyporaceae* through parsimony analysis of mitochondrial ribosomal DNA sequences. *Can J Bot* **73** (Suppl. 1), S853–S861.

Higgins, D. G. & Sharp, P. M. (1989). Fast and sensitive multiple alignment sequence on a microcomputer. *CABIOS* **5**, 151–153.

Hilber, O. (1982). Die Gattung *Pleurotus* (Fr) Kummer unter besonderer Berücksichtigung des *Pleurotus eryngii*-Formenkomplexes. *Bibl Mycol* **87**, 1–448.

Iraçabal, B., Zervakis, G. & Labarère, J. (1995). Molecular systematic of the genus *Pleurotus*: analysis of the restriction polymorphism in ribosomal DNA. *Microbiology* **141**, 1479–1490.

Juan, C., Oromi, P. & Hewitt, G. M. (1996). Phylogeny of the genus *Hegeter* (Tenebrionidae, Coleoptera) and its colonization of the Canary islands deduced from cytochrome oxidase I mitochondrial DNA sequence. *Heredity* **76**, 392–403.

Khon, L. M., Petsche, D. M., Bailey , S. R., Novak, L. A. & Anderson, J. B. (1988). Restriction fragment length polymorphism in nuclear and mitochondrial DNA of *Sclerotinia* species. *Phytopathology* **78**, 1047–1051.

Molcalvo, J. M., Wang, H. H. & Hseu, R. S. (1995). Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacer and 25S ribosomal DNA sequences. *Mycologia* **87**, 223–238.

Mueller, F. & Brimacombe, R. (1997). A new model for the three-dimensional folding of *Escherichia coli* 16S ribosomal RNA. II. The RNA–protein interaction data. *J Mol Biol* **271**, 545–565.

Neefs, J. M., Van de Peer, Y., De Rijk, P., Chapelle, S. & De Wachter, R. (1993). Compilation of small ribosomal subunit RNA structures. *Nucleic Acids Res* **21**, 3025–3049.

Noller, H. F. & Lake, J. A. (1984). Ribosome structure and function: localization of rRNA. In *Membrane Structure and Function*, pp. 217–297. Edited by E. Bittar. New York: Wiley.

Paquin, B., Forget, L., Roewer, I. & Lang, B. F. (1995). Molecular phylogeny of *Allomyces macrogynus*: congruency between nuclear ribosomal RNA- and mitochondrial protein-based trees. *J Mol Evol* **41**, 657–665.

Paquin, B., Laforest, M. J., Forget, L., Roewer, I., Wang, Z., Longcore, J. & Lang, B. F. (1997). The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression. *Curr Genet* **31**, 380–395.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Singer, R. (1986). *The Agaricales in Modern Taxonomy*, 4th edn. Koenigstein, Germany: Koeltz Scientific Books.

Smith, M. L. & Anderson, J. B. (1989). Restriction fragment length polymorphism in mitochondrial DNAs of *Armillaria*: identification of North American biological species. *Mycol Res* **93**, 247–256.

Spicer, G. S. (1995). Phylogenetic utility of the mitochondrial cytochrome oxidase gene: molecular evolution of the *Drosophila buzzatii* species complex. *J Mol Evol* **41**, 749–759.

Stern, S., Powers, T., Changchien, L. M. & Noller, H. F. (1989). RNA–protein interactions in 30S ribosomal subunit: folding and function of 16S rRNA. *Science* **244**, 783–790.

Teissier, L. H., Van der Speck, H., Gualberto, J. M. & Grienenberger, J. M. (1997). The *cox I* gene from *Euglena gracilis*: a protist mitochondrial gene without introns and genetic code modifications. *Curr Genet* **31**, 208–213.

Vilgalys, R. & Sun, B. (1994). Ancient and recent patterns of geographic speciation in the oyster mushroom *Pleurotus* revealed by phylogenetic analysis of the ribosomal DNA sequence. *Proc Natl Acad Sci USA* **91**, 4599–4603.

Vilgalys, R., Smith, A., Sun, B. & Miller, O. K. (1993). Intersterility groups in the *Pleurotus ostreatus* complex from the continental United States and adjacent Canada. *Can J Bot* **71**, 113–128.

Zervakis, G. & Balis, C. (1996). A pluralistic approach in the study of *Pleurotus* species with emphasis on compatibility and physiology of the European morphotaxa. *Mycol Res* **100**, 717–731.

Zervakis, G., Sourdis, J. & Balis, C. (1994). Genetic variability and systematics of eleven *Pleurotus* species based on isozyme analysis. *Mycol Res* **98**, 329–341.

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