Molecular systematics of the genus *Pleurotus*: analysis of restriction polymorphisms in ribosomal DNA

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Part of the nuclear ribosomal DNA unit of *Pleurotus cornucopiae*, including most of the intergenic sequences, was used as a probe to hybridize with DNA from eleven *Pleurotus* taxa (29 isolates), digested with nine restriction endonucleases. For all digests, a high level of rDNA heterogeneity between and within species was detected, which is in agreement with the phenetic variability already reported in previous studies on *Pleurotus* taxa. Numerical analysis of the results, performed by use of two different tree-making methods, clearly distinguished among well-defined species, i.e. *P. dryinus*, *P. eryngii* and *P. flabellatus*, which presented large phenetic distances with the rest of the taxa tested. *P. abalonus*, despite morphological similarity and interfertility with *P. cystidiosus*, appears to be a discrete taxon. Although they showed relative affinity, *P. cornucopiae*, *P. columbinus* and *P. ostreatus* presented large distances among their representative isolates, supporting the idea that they correspond to distinct taxa. All strains of *P. pulmonarius*, *P. sajor-caju* and *P. sapidus* having small phenetic distances were closely positioned on dendrograms; in relation to results from recent interfertility and isozyme studies, these results support the common genetic background of *P. pulmonarius* and *P. sajor-caju*; the position of *P. sapidus* remains controversial. Furthermore, the rDNA analysis identified ten fragments suitable for species identification: eight allowed the characterization of five taxa (*P. cornucopiae*, *P. ostreatus*, *P. sajor-caju*, *P. abalonus* and *P. columbinus*) and two distinguished among clusters including related taxa. By a combination of just two restriction enzymes, the rDNA probe used seems to be the appropriate tool for both varietal characterization and protection of commercially valuable strains.

Keywords: rDNA polymorphism, RFLP, *Pleurotus*, systematic markers

INTRODUCTION

The widely distributed fungal genus *Pleurotus* (Fr.) Qué., order Agaricales (Singer, 1986), is of increasing economic interest (Chang & Miles, 1991) owing to its ability to readily form fruitbodies of good organoleptic quality on inexpensive agricultural and industrial by-products. In the past most *Pleurotus* species were dealt with in systematic studies based on morphological and physiological criteria and the outcome of mating tests (Anderson *et al.*, 1973; Eger *et al.*, 1979; Hilber, 1982; Bresinsky *et*

al., 1987); however the authors failed to arrive at a common conclusion. More recently, mating compatibility studies in strains of the *Pleurotus ostreatus* complex from North American collections permitted a clear determination of the North American species of oyster mushrooms (Vilgalys *et al.*, 1993). Furthermore, protein electrophoresis and isozyme analysis have been employed to offer more accurate information on the phenetic relationships between and within the *Pleurotus* species (May & Royse, 1988; Zervakis & Labarère, 1992; Zervakis *et al.*, 1994).

Restriction fragment length polymorphisms (RFLPs) of mitochondrial or nuclear rDNA have been adopted for species delineation and phylogenetic analysis in fungal genera such as *Aspergillus* (Kozłowski & Stéprien, 1982),

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Abbreviation: RFLP, restriction fragment length polymorphism.

Sclerotinia (Kohn *et al.*, 1988), *Phytophthora* (Förster *et al.*, 1988), *Verticillium* (Carder & Barbara, 1991) and *Fusarium* (Kistler *et al.*, 1987). Moreover the use of RFLPs in the systematics of basidiomycetes has demonstrated the suitability of this approach for the elucidation of ambiguities in the genera *Agaricus* (Hintz *et al.*, 1988), *Armillaria* (Anderson *et al.*, 1987) and *Laccaria* (Gardes *et al.*, 1991). When using RFLPs in systematics, it is necessary to select a probe which is able to detect phenetic variations in both distant and related species. In fungi, rDNA units are repeated and thus are easily detectable sequences, showing variability at the species level (Walsh *et al.*, 1990; Kohn *et al.*, 1988) and at the strain level (Specht *et al.*, 1984; Cassidy *et al.*, 1984). Indeed, the rDNA unit appears to be especially well suited as a molecular marker because it is composed of conserved gene sequences and variable intergenic sequences which evolve more rapidly than many other parts of the genome (Rogers *et al.*, 1989).

The main aim of this study was to determine whether an RFLP method based on ribosomal polymorphism was suitable for application to the systematics of the genus *Pleurotus*. Thus, rDNA variability in 29 isolates belonging to eleven *Pleurotus* taxa from distant geographic areas was investigated by using nine endonucleases which had either none, one or several sites in the ribosomal probe. Most of the isolates have been already characterized by morphological or physiological criteria (Zervakis & Balis, 1991) and all by isozyme analyses (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994). The resulting RFLP patterns, detected by the *P. cornucopiae* ribosomal probe (composed mainly of coding regions of 18S and 5S genes, part of the 25S gene and two flanking intergenic sequences), were numerically transformed to determine the phenetic distances between strains and the relationships among species. Other goals of this work were to identify molecular markers characteristic of species and to determine whether RFLP could be used in routine examination to identify species, by comparison with results obtained previously using morphological and biochemical criteria.

METHODS

Strains, media and culture conditions. All fungal isolates used in this study were dikaryotic strains belonging to eleven *Pleurotus* species (Table 1): *P. abalonus* (Han *et al.*), *P. columbinus* (Qué. apud Bres.) Qué., *P. cornucopiae* (Paul. ex Pers.) Roll., *P. cystidiosus* O. K. Miller, *P. dryinus* (Pers. ex Fr.) Kummer, *P. eryngii* (DC. ex Fr.) Qué., *P. flabellatus* (Berck. & Br.) Sacc., *P. ostreatus* (Jacq. ex Fr.) Kummer, *P. pulmonarius* (Fr.) Qué., *P. sajor-caju* (Fr.) Sing. and *P. sapidus* (Schulzer & Kalchbr.) Sacc. Isolates used in this work included wild strains collected in various geographical areas and three commercial strains (nos 08, 85 and 90); for isolates no. 04 (*P. columbinus*) and no. 82 (*P. pulmonarius*) the wild or commercial origin is unknown (Table 1). Dikaryotic mycelia were cultivated at 25 °C in the dark in Roux flasks containing liquid Complete Yeast Medium (Raper & Hoffman, 1974).

Total DNA isolation. Fungal cultures (15–20 d old) were harvested by filtration on sterile gauze, rinsed with distilled water and quickly frozen in liquid nitrogen. Total DNA was

extracted by using the *N*-cetyl-*N*-*N*-trimethylammonium bromide procedure adapted to basidiomycetes (Noël & Labarère, 1989).

Restriction enzyme digestion, agarose gel electrophoresis, and Southern blotting. Total DNA was digested singly with nine endonucleases (*Ava*I, *Bam*HI, *Cla*I, *Eco*RI, *Kpn*I, *Sca*I, *Sma*I, *Sst*I and *Hind*III) for 3 h using 3–4 U (μ g DNA)⁻¹ according to the supplier's instructions (Gibco-BRL). Digested DNA fragments were separated by electrophoresis on 0.8% (w/v) agarose gels in TEA buffer (40 mM Tris/HCl, 2 mM EDTA, 20 mM sodium acetate, pH 8.0) at 60 V for 12 h (30 cm length) (Maniatis *et al.*, 1982) and transferred onto a nylon membrane (Hybond N+, Amersham) by capillary blotting as described by Southern (1975).

Probe isolation, labelling and hybridizations. The rDNA probe (a pBR322-based recombinant plasmid) is a 6.5 kb *Eco*RI fragment of the rDNA unit of *P. cornucopiae* (Fig. 1) cloned in *Escherichia coli* strain JM 83. Plasmid DNA was extracted from liquid bacterial cultures by alkaline lysis according to the method of Vo-Quang *et al.* (1985). After linearization by *Hind*III, the probe plasmid was radiolabelled with 25 μ Ci [α -³²P]dCTP [3000 Ci mmol⁻¹ (111 kBq mmol⁻¹), Amersham] by using the random primer DNA labelling kit (Gibco-BRL) with a specific radioactivity higher than 10⁸ c.p.m. (μ g DNA)⁻¹. Nylon membranes were treated for prehybridization as specified by the manufacturer (Amersham) and hybridized with the rDNA probe at 60 °C for 12–15 h. Nylon membranes were successively washed in 6 × SSC, 2 × SSC and 0.1 × SSC mixed with 0.1% (w/v) SDS for 10–30 min for each washing step. Autoradiography was performed with Kodak X-Omat AR film at –70 °C for 16–24 h in the presence of an intensifying screen.

Analysis of RFLP data. RFLP analysis was carried out by performing digestion, electrophoresis and Southern hybridizations on total DNA samples from each isolate tested. The molecular size of the detected restriction fragments was evaluated by graphical interpolation using a regression curve, established between the log migration against the log molecular size of lambda DNA digested with *Hind*III.

To transform the RFLP data for numerical analysis, the molecular phenotypes of each *Pleurotus* isolate were coded with the aid of a binary system, where the presence (1) and absence (0) of the hybridizing fragments at a particular position on the autoradiograms were scored for each strain. All bands were considered as single fragments unless their relative thickness suggested otherwise. Variations in staining intensity were not taken into account for the construction of the matrix. The measure of distance between isolates was based on the proportion of mismatches among the DNA fragments as follows: $F = 1 - [2n_{xy}/(n_x + n_y)]$, where n_{xy} is the number of fragments in common between two isolates and n_x, n_y are the total number of fragments displayed by each isolate (Nei & Li, 1979). Then the mean value was calculated for all the tested endonucleases and a distance matrix was established. It should be noted that presence of insertions or deletions, producing length mutation, could disproportionately affect the data analysis.

Dendrograms were constructed, from the distance matrix, with two tree-making methods. The first was the UPGMA (unweighted pair group method using arithmetic means) clustering, which forms groups by successively pairing similar molecular phenotypes according to the magnitude of their observed distances (Sneath & Sokal, 1973). The software package used was the program NT-SYS (numerical taxonomy system using multivariate statistical programs) on a Nixdorf-Siemens model PCD-4H computer (Rohlf *et al.*, 1982). The

Table 1. *Pleurotus* dikaryons used in this study

Isolate code no.	Species	Origin	Type*	Source†
01	<i>P. abalonus</i>	Japan	Wild	LGMACC 39
02	<i>P. abalonus</i>	Japan	Wild	LGMACC 62
03	<i>P. columbinus</i>	France	Wild	ATCC 36498 (Vandendries)
04	<i>P. columbinus</i>	Italy	—	CBS 37351 (Baldacci)
05	<i>P. cornucopiae</i>	Germany	Wild	ATCC 38547 (Eger)
07	<i>P. cornucopiae</i>	Netherlands	Wild	CBS 38380 (van der Poel)
08	<i>P. cornucopiae</i>	France	Commercial	LGMACC 15
09	<i>P. cornucopiae</i>	France (Dordogne)	Wild	LGMACC 50
10	<i>P. cystidiosus</i>	Greece (Salamina)	Wild	LGAM P50
11	<i>P. cystidiosus</i>	USA (Georgia)	Wild	ATCC 28597 (Lombard)
15	<i>P. dryinus</i>	Germany	Wild	ATCC 36502 (Lyr)
19	<i>P. eryngii</i>	Greece (Orestida)	Wild	LGAM P66
22	<i>P. eryngii</i>	Greece (Lasithi)	Wild	LGAM P63
23	<i>P. eryngii</i>	ex-Czechoslovakia	Wild	ATCC 36047 (Ginterova)
29	<i>P. eryngii</i>	France (Valery sur Somme)	Wild	LGMACC 841043
34	<i>P. flabellatus</i>	—	Wild	ATCC 38138 (Graham)
41	<i>P. ostreatus</i>	Greece (Fthiotida)	Wild	LGAM P60
45	<i>P. ostreatus</i>	Germany	Wild	ATCC 32783 (Haider)
59	<i>P. ostreatus</i>	Spain (Albaladejito)	Wild	LGMACC 871020
61	<i>P. ostreatus</i>	France (Gironde)	Wild	LGMACC 861008
74	<i>P. pulmonarius</i>	Greece (Vardoussia)	Wild	LGAM P26
81	<i>P. pulmonarius</i>	ex-Czechoslovakia	Wild	ATCC 36050 (Ginterova)
82	<i>P. pulmonarius</i>	USA	—	CBS 13285 (Graauwmans)
85	<i>P. pulmonarius</i>	Hungary	Commercial	LGMACC 850403
86	<i>P. sajor-caju</i>	India	Wild	ATCC 32078 (Rangaswami)
88	<i>P. sajor-caju</i>	Mauritius	Wild	LGMACC 37
89	<i>P. sajor-caju</i>	India	Wild	LGMACC 35
90	<i>P. sajor-caju</i>	ex-Czechoslovakia	Commercial	LGMACC 851003
91	<i>P. sapidus</i>	USA (Michigan)	Wild	ATCC 24986 (Petersen)

*—, Unknown.

† The *Pleurotus* isolates used in the present study were obtained from the following fungal culture collections: ATCC, American Type Culture Collection (Rockville, USA); CBS, Centraalbureau Voor Schimmelcultures (Baarn, The Netherlands); LGAM, Laboratory of General and Agricultural Microbiology (Agricultural University of Athens, Greece); LGMACC, Laboratoire de Génétique Moléculaire et d'Amélioration des Champignons Cultivés (Université de Bordeaux, II, France).

Fig. 1. Restriction map of the cloned *P. cornucopiae* rDNA unit. The sites of the endonucleases used in this work are indicated: A, *AvaI*; C, *CmaI*; E, *EcoRI*; M, *SmaI*; Sc, *ScaI* and Ss, *SstI*. The position of the 6.5 kb *EcoRI* insert used as probe is shown.

By reason of the small sample size, the distance values should be considered as rough estimates; nonetheless, as the sample size was similar for all species, the same degree of error should apply. Moreover, the conclusions deriving from RFLP data were compared with previous morphological and biochemical studies performed on the same *Pleurotus* isolates (Zervakis & Balis, 1991; Zervakis & Labarère, 1992; Zervakis *et al.*, 1994).

RESULTS

The rDNA probe was a 6.5 kb *EcoRI* insert of the *P. cornucopiae* rDNA unit containing the 5S and 18S genes, and part of the 25S gene flanking two intergenic sequences (IGS 1 and IGS 2) (Fig. 1). Total DNA of the 29 *Pleurotus* isolates was separately digested by nine endonucleases (each one having a six nucleotide pair recognition sequence): *Bam*HI, *Hind*III, *Kpn*I, *Sca*I, *Sma*I, *Sst*I, *Cl*aI, *Ava*I and *Eco*RI. After blotting on nylon, restriction digests were hybridized with the radiolabelled rDNA probe.

second was the neighbour-joining method, which finds pairs of molecular phenotypes that minimize the total branch length at each stage of clustering of the phenotypes, starting from a starlike tree (Saitou & Nei, 1987). Calculations were executed by a computer program developed by Professor J. Sourdis (Department of Genetics, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece) with the same hardware.

Table 2. Binomial matrix of restriction fragments from hybridizations between the rDNA probe of *P. cornucopiae* and total DNA of 29 *Pleurotus* isolates, digested with nine endonucleases

1, Fragment present; 0, fragment absent; –, not tested.

Endonuclease	Fragment size (kbp)	Isolate:																												
		01	02	03	04	05	07	08	09	10	11	15	19	22	23	29	34	41	45	59	61	74	81	82	85	86	88	89	90	91
<i>Bam</i> HI	25·5	0	0	0	1	1	1	1	1	1	1	–	1	1	1	1	–	0	1	0	0	1	1	1	1	1	1	1	1	1
	23·1	0	0	1	1	1	1	1	1	0	0	–	0	1	0	1	–	0	0	0	0	0	0	0	0	0	0	0	0	0
	19·2	1	1	1	1	0	0	0	0	0	0	–	0	1	0	1	–	1	1	1	1	1	1	1	1	1	1	1	1	1
	17·0	1	1	0	0	0	0	0	0	0	0	–	0	1	0	0	–	0	0	0	0	0	0	0	1	1	0	0	0	0
	12·2	0	0	0	0	0	0	0	0	0	0	–	1	1	1	1	–	0	0	0	0	1	0	1	0	0	1	1	1	0
	10·7	0	0	1	1	0	0	0	0	0	0	–	1	0	1	1	–	1	1	1	1	0	1	0	1	1	0	0	0	1
	9·4	1	1	0	0	0	0	0	0	0	1	–	0	1	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Hind</i> III	23·1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0
	20·8	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	–	0	0	0	0	0	0	1	0	1	1	0	1	0
	12·2	0	0	0	0	0	0	0	0	0	0	1	0	1	1	–	0	0	0	0	1	0	1	1	1	1	1	1	1	0
	9·4	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	–	0	0	0	0	1	0	0	0	0	0	0	0	1
	8·4	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0	–	0	0	0	0	1	1	1	1	1	1	1	1	1
	7·4	0	0	1	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
	6·4	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	0
	6·1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0
	3·3	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
	2·8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	1	0	1	0	0	1	1	1	0
	2·6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	–	1	1	0	0	0	1	1	1	1	1	1	1	0
	<i>Kpn</i> I	28·8	0	0	–	–	0	0	0	0	0	0	0	1	1	1	1	–	0	1	1	0	1	1	1	1	1	1	1	1
23·1		0	0	–	–	1	1	1	1	0	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0
20·8		0	0	–	–	1	1	1	1	0	0	0	0	1	0	1	–	0	1	1	0	1	1	1	1	0	0	1	0	1
18·4		0	0	–	–	1	1	1	1	0	0	0	0	0	0	0	–	0	0	1	0	0	1	1	1	1	1	1	1	1
15·1		0	0	–	–	1	0	0	1	0	0	0	0	0	0	0	–	0	0	1	0	0	1	1	0	1	1	1	1	1
13·3		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
10·7		0	0	–	–	0	0	0	0	0	0	0	0	0	1	1	–	0	0	0	0	1	1	1	1	1	1	1	1	0
9·8		0	0	–	–	0	0	0	0	0	0	0	0	1	1	0	–	1	1	1	0	1	1	0	0	0	0	1	0	1
9·0		1	1	–	–	0	0	0	0	1	1	0	0	0	0	0	–	0	0	1	0	1	1	0	0	0	0	1	1	0
8·6		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
8·2		0	0	–	–	1	0	0	0	0	0	0	0	0	1	0	–	0	0	1	0	1	1	1	1	1	1	1	1	1
7·8		0	0	–	–	0	0	0	0	0	0	1	1	1	0	1	–	1	1	1	1	0	1	0	0	0	1	0	0	1
4·7		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
4·3		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
3·4		0	0	–	–	1	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0	0	0	1	1	1	0	0	1	0
3·1		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
2·7		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
2·4		0	0	–	–	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0	0	1	1	0	1	1	0	1	0
2·0		0	0	–	–	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	1	0	1	0	1	0	0	0	0	0
1·7		0	0	–	–	0	0	0	0	0	0	1	0	0	0	0	–	0	0	0	1	0	1	0	1	0	0	0	0	0
1·5	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	
<i>Sca</i> I	25·5	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	–	0	0	0	0	0	0	0	0	0	0	0	0	1
	23·1	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	1
	19·2	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
	18·4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0	0	0	0	1	1	0	0	1	1
	17·0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0
	11·2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	–	0	0	0	0	0	0	0	0	0	0	0	0
	10·2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	–	0	0	0	0	1	1	1	0	1	1	1	0	0
	9·4	0	0	1	1	1	1	1	1	0	0	0	0	1	1	1	–	1	1	1	1	0	1	0	1	0	0	0	1	1
	9·0	1	1	0	0	0	0	0	0	1	0	1	0	0	0	0	–	0	0	0	0	0	1	0	0	0	0	0	0	0
	8·4	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	–	0	0	0	0	0	0	0	0	0	0	0	0	0
	7·8	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
	6·6	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

Table 2 (cont.)

Endonuclease	Fragment size (kbp)	Isolate:																														
		01	02	03	04	05	07	08	09	10	11	15	19	22	23	29	34	41	45	59	61	74	81	82	85	86	88	89	90	91		
SssI	6.2	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	0	0		
	5.9	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	0	0		
	28.8	1	1	1	1	0	1	0	0	1	1	1	0	1	0	1	1	0	1	0	0	0	0	1	1	1	1	—	1	—		
	25.5	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	0	1	1	1	0	—	0	—		
	19.9	0	0	1	0	1	1	1	1	0	1	0	0	1	1	1	1	0	1	1	0	0	0	1	1	1	1	—	1	—		
	17.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	—	0	—		
	16.3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	0	—		
	15.1	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	—		
	11.7	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	1	1	0	0	0	—	0	—		
	10.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	—	1	—	
	10.2	0	0	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	—	0	—	
	9.4	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0	1	—	0	—	
	8.6	0	0	1	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	—	0	—	
	7.4	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	—	0	—	
	6.8	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	0	0	1	—	0	—	
SmaI	27.0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	—		
	17.7	0	0	0	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	—		
	15.7	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	—		
	11.7	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	—		
	10.7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	—		
	9.8	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	1	—	
	9.4	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	1	0	1	1	1	0	1	0	1	0	0	0	0	—		
	8.8	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—		
	8.6	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	—		
	8.2	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	0	1	—
	7.8	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	0	0	—	
	7.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	—	
	6.6	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	
	5.6	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	
	4.8	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	
4.5	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—		
3.8	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—		
2.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	—		
2.3	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	0	—		
AvaI	28.8	0	0	—	—	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
	23.1	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	0	1	1	
	19.9	0	0	—	—	1	0	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	1	1	0	0	
	16.3	0	0	—	—	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	
	14.5	0	0	—	—	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	
	10.2	0	0	—	—	1	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	
	9.0	0	0	—	—	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1	0		
	8.4	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1	0	1	1	0		
	7.8	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	1	
	7.2	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	0	0	1	0	0	1	0	1	
	6.3	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	1	1	1	1	1	1	
	6.0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	1	1	0	1	0	
	5.8	0	0	—	—	0	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	
	5.5	0	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	1	
	4.3	0	0	—	—	0	0	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	
3.8	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0		
3.5	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0		
3.2	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0		
3.0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0		
2.7	1	1	—	—	0	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0		

Table 2 (cont.)

Endonuclease	Fragment size (kbp)	Isolate:																												
		01	02	03	04	05	07	08	09	10	11	15	19	22	23	29	34	41	45	59	61	74	81	82	85	86	88	89	90	91
ClaI	2.4	0	0	—	—	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	2.0	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	0	0
	1.8	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	0	0	0	1
	1.6	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	1	0	0	0	0	1
	1.1	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1
	1.0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
	24.2	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	—	0	1	1	0	0	1	1	1	1	1	1	1	1
	20.8	0	0	0	0	1	1	1	1	0	1	0	0	0	1	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0
	17.7	0	0	0	0	0	1	1	1	0	0	1	1	1	1	1	—	0	1	1	1	0	1	1	1	1	1	0	1	0
	16.3	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
	13.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	0	1	1	1	0	0	0	0	0	0	0	0	0
	12.2	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	—	0	0	0	0	0	1	1	0	0	0	0	0	0
	9.8	0	0	1	1	1	1	1	1	0	0	1	0	0	1	1	—	1	1	1	1	1	1	1	1	1	1	0	1	0
	9.2	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	—	0	0	0	0	0	1	1	0	0	0	0	0	0
	8.8	0	0	0	1	1	1	1	1	0	0	1	0	0	0	1	—	0	1	1	1	0	1	1	1	1	1	0	1	1
	8.4	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0
	7.8	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	—	0	0	0	0	0	0	0	0	0	0	0	0
	6.8	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	—	0	1	1	1	1	1	0	1	1	1	1	1	0
	6.4	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	—	0	1	0	1	0	1	0	1	0	0	0	0	1
	6.0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	—	0	0	1	1	0	0	0	0	0	0	0	0	0
	5.2	0	0	0	0	1	1	1	1	0	1	0	0	0	1	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0
	4.4	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	—	0	1	1	1	0	0	0	0	0	0	0	0	0
	3.8	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	—	1	1	1	1	0	0	0	0	0	0	0	0
	3.5	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0
	3.2	0	0	0	1	0	0	0	0	0	1	0	0	1	1	0	—	0	0	0	1	0	0	0	0	0	0	0	0	0
	2.7	0	0	0	1	1	1	1	1	0	1	0	1	1	1	0	—	1	1	1	1	0	0	0	0	0	0	0	0	0
	2.2	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	—	1	1	1	1	0	0	0	0	0	0	0	0	0
	1.9	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	—	1	0	0	0	0	0	0	0	0	0	0	0	0
1.1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	
0.7	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0	0	0	0	0	0	0	
0.6	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	1	—	1	1	0	0	0	0	0	0	0	0	0	0	
EcoRI	25.5	1	0	1	1	1	1	0	0	1	1	0	0	1	0	1	0	0	1	0	0	1	1	1	1	0	0	1	1	0
	19.9	1	0	1	0	0	0	0	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0	1	1	0
	17.7	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	1	1	1	1	0	1	0	0
	17.0	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	
	15.7	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	
	10.2	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	
	9.4	0	0	1	0	1	1	1	1	0	1	1	0	1	1	0	1	0	1	0	0	1	1	0	1	0	0	0	1	
	8.2	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	8.0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	1	1	1	1	0	1	1	
	7.6	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	0	
	7.2	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	1	
	6.8	0	0	1	1	1	1	1	1	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	
	6.4	1	1	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	0	
	6.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	
	5.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	
	5.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0	
	4.6	0	1	1	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	
	4.5	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	0	
	4.2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	
	3.8	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	1	
3.6	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1		
3.3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0		
3.0	1	1	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		
2.7	1	1	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

Fig. 2. Representative RFLP patterns produced by hybridization of *Pleurotus* DNA with the rDNA probe. Total DNA was digested by *Hind*III (a), *Sma*I (b), and *Eco*RI (c). Strains are numbered as in Table 1. Fragment sizes are in kb.

To study the rDNA polymorphism, the RFLP fragments were examined on the basis of (i) their strain specificity by comparison of patterns obtained for the nine endonucleases tested, (ii) their similarity between isolates within a single species and (iii) their relatedness among different species. The resulting RFLP autoradiograms were used to construct a matrix of phenetic distances, generated by the method of Nei & Li (1979), which was then computed to produce the dendrograms. Furthermore, RFLP patterns were compared in order to find whether some RFLP fragments could characterize a species or a cluster of species and might therefore be suitable as systematic markers.

The rDNA polymorphism within the genus *Pleurotus*

Hybridizations of the *P. cornucopiae* rDNA probe with total DNA of the 29 *Pleurotus* isolates revealed 160 fragments showing 186 RFLP patterns after performing a

total of nine digestions (Table 2); representative hybridization patterns are presented in Fig. 2. When all the digestions were considered, each of the 29 isolates studied possessed a characteristic pattern. The highest number of bands (21, 24, 25 and 26) was obtained when total DNA was digested by *Ava*I, *Eco*RI, *Kpn*I or *Cla*I (Table 3). The highest degree of discrimination was obtained with digestions by *Ava*I, *Cla*I, *Eco*RI, *Kpn*I or *Sst*I. The *Bam*HI, *Hind*III, *Sma*I and *Sca*I digestions appeared to be less polymorphic, thus forming numerous groups of strains sharing the same pattern. *Sst*I was the only endonuclease which presented entirely unique phenotypes among the *Pleurotus* strains examined (with the sole exception of *P. abalonus* isolates), although it produced a relatively low total number of DNA fragments. In the same way, when total DNA was digested by *Eco*RI, 25 among the 29 isolates studied possessed different patterns. The *Eco*RI-unidentified isolates 41 and 61 on the one hand and 10 and 29 on the other hand could

Table 3. Restriction sites detected within the rDNA probe and numbers of RFLP fragments obtained from 29 *Pleurotus* isolates with the use of nine endonucleases

Enzyme	Restriction sites in the probe	Fragments obtained		Specific fragments		
		Total	Mean per strain	Strain	Species	Cluster
<i>Bam</i> HI	0	7	3	0	0	0
<i>Hind</i> III	0	11	2	0	3	0
<i>Kpn</i> I	0	21	5	6	1	0
<i>Sca</i> I	1	14	2	4	0	0
<i>Sst</i> I	1	13	3–4	2	0	0
<i>Sma</i> I	1	19	3–4	4	1	1
<i>Cla</i> I	1	25	7–8	1	0	0
<i>Ava</i> I	2	26	6	2	0	0
<i>Eco</i> RI	2	24	5–6	2	3	1

be distinguished by *Hind*III, *Kpn*I, *Sca*I, *Sst*I, *Sma*I, *Ava*I or *Cla*I digestions.

Numerical analysis of RFLP patterns

The distance matrix obtained according to the method of Nei & Li (1979) from RFLP patterns was then analysed either by UPGMA with the program NT-SYS (Rohlf *et al.*, 1982) or by the neighbour-joining method according to Saitou & Nei (1987).

On the dendrogram obtained by UPGMA (Fig. 3a) nine clusters were distinguished at a distance level of more than 50% ($D > 50\%$). Seven of them corresponded to separate *Pleurotus* species, i.e. *P. flabellatus* (A), *P. dryinus* (B), *P. sapidus* (D), *P. cornucopiae* (F), *P. eryngii* (G), *P. columbinus* (H) and *P. ostreatus* (I). The other two clusters included two species each: *P. cystidiosus*–*P. abalonus* (cluster C) and *P. pulmonarius*–*P. sajor-caju* (cluster E).

Among the eleven species, *P. flabellatus* and *P. dryinus*, which presented distance values well over 75% in all comparisons, appeared clearly distant from other species. Isolates belonging to *P. columbinus* and *P. cornucopiae* formed tight intraspecific groups ($D < 30\%$ in each case, the French strains of *P. cornucopiae* being in close proximity: $D = 15\%$), whereas *P. ostreatus* isolates seemed to be less related ($D = 18$ – 42%). Strains of *P. eryngii*, despite their placement into a unique cluster, had relatively large distances between them [$D = 41$ – 63% ; isolates from northeastern Greece (no 19) and Crete (no 22) being the most distant].

P. ostreatus and *P. columbinus* had closer interspecific relationships, and this was also the case with *P. sapidus* (cluster D) and the compound cluster of *P. pulmonarius*–*P. sajor-caju* (cluster E). In cluster E, strains appeared to be particularly related, although originally assigned to different species, as their distance values did not exceed 55%. Similarly, strains of cluster C (*P. cystidiosus* and *P. abalonus*) showed an increased affinity ($D = 27$ – 45%), and seemed to be well discriminated from the other clusters ($D > 60\%$); the two isolates of *P. abalonus* having the

tightest bonds ($D = 3\%$) among all strains examined; while *P. cystidiosus* isolates exhibited higher intraspecific distance values ($D < 35\%$).

When the results were analysed by the neighbour-joining method, which is considered the best for obtaining accurate topology among the other distance matrix and parsimony methods (Sourdis & Nei, 1988), the dendrogram (Fig. 3b) presented only one major difference in comparison with the one obtained by UPGMA: *P. ostreatus*, *P. columbinus* and *P. cornucopiae* isolates, previously separated into three clusters, were joined into a common larger cluster, *P. ostreatus* and *P. columbinus* being the most related. Relative positions of all other strains remained identical, notably *P. pulmonarius*–*P. sajor-caju* forming one cluster as well as *P. abalonus* and *P. cystidiosus*.

RFLP fragments usable as molecular markers for *Pleurotus* species identification

In order to obtain molecular markers for characterization of *Pleurotus* species, a detailed analysis of patterns was carried out to find RFLP fragments which allowed single species or clusters of species to be distinguished.

In the composite clusters, the 8.8 kb *Sma*I fragment was exclusively present in strains of cluster C (*P. abalonus* and *P. cystidiosus*); the 5 kb *Eco*RI fragment was specifically detected in strains of cluster E (*P. pulmonarius* and *P. sajor-caju*). Eight species-specific fragments allowed differentiation among five species. All the *P. ostreatus* isolates examined shared the 6.4 kb *Hind*III and 2.3 kb *Sma*I bands, while strains of *P. cornucopiae* could be characterized by the 23.1 kb *Hind*III and 23.1 kb *Kpn*I fragments, and *P. sajor-caju* by the 4.5 kb *Eco*RI band. Likewise, *P. columbinus* could be characterized by the 6.1 kb *Hind*III fragment and *P. abalonus* by the 2.7 kb and 3.0 kb *Eco*RI fragments.

In the search for patterns specific to a cluster, it is worth noting that *P. abalonus* isolates showed interspecies affinities with *P. cystidiosus* by featuring common patterns (*Hind*III, *Kpn*I and, partly, *Sst*I and *Cla*I). Moreover, the

Fig. 3. Relationships among 29 *Pleurotus* isolates as visualized by two tree-making methods: (a) the UPGMA clustering method – the dashed line indicates the level of distance ($D = 0.50$) that permitted the classification of the *Pleurotus* isolates into nine clusters (A to I); (b) the neighbour-joining clustering method. Both dendrograms were computed with the use of a distance estimator (Nei & Li, 1979).

two isolates of *P. abalonus* presented five species-specific patterns (*Bam*HI, *Sca*I, *Ava*I and *Sma*I) while *P. cystidiosus* strains presented identical phenotypes for *Sma*I.

DISCUSSION

Polymorphism in rDNA was scored in 29 isolates previously assigned to eleven *Pleurotus* species from various geographical areas throughout the world. All isolates have been previously studied and characterized by the use of morphological and physiological criteria (Zervakis & Balis, 1991), and/or by isozyme analysis (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994).

All the isolates had specific RFLP patterns and presented a high molecular polymorphism (with the sole exception of *P. abalonus*) comparable to the polymorphism evidenced by isozyme analysis (Zervakis & Labarère, 1992; Iraçabal & Labarère, 1993; Zervakis *et al.*, 1994). In comparison

with the DNA polymorphism of species of the order Agaricales previously described in the genera *Armillaria* (Anderson *et al.*, 1987), *Agaricus* (Horgen *et al.*, 1984; Hintz *et al.*, 1989), *Laccaria* (Armstrong *et al.*, 1989; Gardes *et al.*, 1990) and *Lentinus* (Kulkarni, 1991), the rDNA polymorphism evidenced in the *Pleurotus* genus is higher. This could be attributed (i) to the efficiency of the probe used which was a part of the *P. cornucopiae* rDNA unit mainly constituted by intergenic sequences and (ii) to the loss or creation of new restriction sites and/or of length mutations as already evidenced in *P. cornucopiae* rDNA (Iraçabal & Labarère, 1994). The probes used for other studies in Agaricales were either a heterologous rDNA or the entire rDNA unit consisting mostly of conserved sequences, apparently offering a less sensitive detection.

In genus *Pleurotus*, the rDNA probe showed higher polymorphism when DNA was digested by *Ava*I, *Cla*I,

EcoRI or *SstI*; the combination of two digestions (*EcoRI* with one of the endonucleases *HindIII*, *KpnI*, *ScaI*, *SstI*, *SmaI*, *AvaI* or *ClaI*) allowed discrimination of all isolates examined. It is of interest to note that the high variability evidenced in the *EcoRI* digestion as well as the low polymorphism exhibited in *BamHI* and *HindIII* digestions were previously reported in other fungal genera (Kohn *et al.*, 1988; Smith & Anderson, 1989; Gardes *et al.*, 1990; Kulkarni, 1991).

The ribosomal probe used allowed detection of fragments of high molecular size, whose size was larger than that estimated for the rDNA unit (over 10–12 kb). These fragments could correspond to: (i) rDNA fragments produced by partial digestions [this depends on phenomena affecting the endonuclease's specificity and digestibility according to reaction conditions (salt concentration, DNA purity, etc.) and to rDNA methylation (Buckner *et al.*, 1988; Specht *et al.*, 1984; Vilgalys & Gonzalez, 1990; Li & Horgen, 1993]; (ii) rDNA fragments located at the extremities of the rDNA cluster flanked by non-ribosomal sequences whose restriction sites differ according to species; (iii) non-ribosomal fragments possessing homology with some sequences of the probe. When these large fragments were omitted from the numerical analysis of RFLP patterns, interspecific relationships of *Pleurotus* species derived from the dendrograms based on all detected fragments were not affected; only minor changes were observed concerning intraspecific relationships.

Numerical treatment of RFLP data from the 29 *Pleurotus* strains was performed according to two tree-making methods (UPGMA and the neighbour-joining method), which showed single-species clusters for *P. flabellatus*, *P. dryinus*, *P. sapidus* and *P. eryngii* and two two-species clusters: *P. abalonus*–*P. cystidiosus* and *P. pulmonarius*–*P. sajor-caju*. One difference was that *P. ostreatus*, *P. columbinus* and *P. cornucopiae* were separated into three clusters by UPGMA analysis and joined into a common larger cluster by the neighbour-joining method. The species *P. flabellatus*, *P. dryinus* and, to a lesser degree, *P. eryngii* are clearly discriminated by RFLPs. This is in accordance with the anatomical and physiological characteristics (basidiocarp structure, presence of velum and formation of chlamydospores, host specialization, etc.) of these species, which permitted their assignment into discrete categories within the genus *Pleurotus* (Singer, 1986) and with the two previous isozyme analyses (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994); these data indicate the suitability of using RFLPs in studies of fungal systematics.

The two *Coremiopleurotus* species (*P. abalonus* and *P. cystidiosus*), which are grouped in cluster C, presented large phenetic distances with the other *Pleurotus* taxa examined; this is in accordance with their anatomical features, which are rather rare in the genus (production of synnematoïd fructifications from the haploid and the dikaryotic mycelium) and with the previous isozyme data showing the clear separation of *P. cystidiosus* from other *Pleurotus* taxa (Zervakis & Labarère, 1992). Although grouped in the same cluster, these two taxa had high phenetic diversity

and seem to be quite unrelated to each other, suggesting that *P. abalonus* and *P. cystidiosus* evolved as discrete taxa.

The most unusual position of *Pleurotus* isolates in the RFLP dendrograms concerned the *P. pulmonarius*, *P. sajor-caju* and *P. sapidus* isolates. The mixed positioning of strains of the first two species in the same cluster accords with the numerous morphological similarities (Zervakis & Balis, 1991), the significant interfertility (Hilber, 1982; G. Zervakis & C. Balis, unpublished results) and the close proximity revealed from biochemical studies (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994); this supports the hypothesis that *P. sajor-caju* is apparently a mistaken name to describe a *Pleurotus* taxon, as was previously used for a similar-looking *Lentinus* species (*L. sajor-caju* Fr.). It seems that the isolates obtained for the purposes of this study, and assigned as *P. sajor-caju*, belong to an Asiatic population of *P. pulmonarius*. The four isolates (three European and one American) assigned as *P. pulmonarius* were clearly separated from the *P. ostreatus* and *P. cornucopiae* isolates; they probably coincide with the European and North American *P. pulmonarius* intersterility groups II, defined by Vilgalys *et al.* (1993). The single *P. sapidus* strain, although clearly separated from both these species, showed strong affinity with them; it was more related to *P. pulmonarius* than to *P. sajor-caju*, for which common morphological and physiological features have been described (Zervakis & Balis, 1991). Positioning of the *P. sapidus* isolate was difficult. An isozyme study performed by starch-gel electrophoresis suggested, as did the RFLP data, more affinity of *P. sapidus* with *P. pulmonarius* than with *P. ostreatus*. Based on the closer relationships of the *P. sapidus* isolate with *P. pulmonarius* isolates, these isolates probably belong to the North American intersterility group II as defined by Vilgalys *et al.* (1993). Moreover, previous works based on morphological and compatibility criteria (Eger *et al.*, 1979) or isozyme data (May & Royse, 1988) suggested that *P. sapidus* was conspecific with *P. ostreatus*. The confusing positioning of the *P. sapidus* isolate could be attributed to the high heterogeneity of the *P. sapidus* group as pointed out by Hilber (1982).

By comparing the dendrograms from the two tree-making methods, only one difference was observed: *P. ostreatus*, *P. columbinus* and *P. cornucopiae* belonged to three clusters by UPGMA analysis and were joined into a common larger cluster by the neighbour-joining method. This can be explained by the fact that the neighbour-joining method minimizes the total branch length at each stage of clustering strains or groups of strains. However, the three species were clearly separated within the cluster, suggesting that they had a common ancestor rather than that they form a unique species. Indeed, *P. cornucopiae* presented distance values greater than 55% in all comparisons with other species, and was distinguished more readily from *P. columbinus* and *P. ostreatus* in both dendrograms. Previous systematic studies based on isozyme data also allowed *P. cornucopiae* to be separated clearly from the other *Pleurotus* species (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994). In accordance with findings of morphological and compatibility studies

(Bresinsky *et al.*, 1977; Hilber, 1982) and of isozyme analyses (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994), all these data are consistent with *P. cornucopiae* being a distinct species. Accordingly, the four European isolates assigned as *P. cornucopiae* certainly belong to the European group IV described by Vilgalys *et al.* (1993). Moreover, the *P. ostreatus* and *P. columbinus* species had large phenetic distances (> 52%) among their individual members. This induced Vilgalys *et al.* (1993) to define a European *P. ostreatus* group I, composed of both *P. ostreatus* and *P. columbinus* isolates. We presumed that our *P. ostreatus* and *P. columbinus* isolates, separated in two neighbouring clusters, could be associated with this European group I. However, the RFLP data confirmed recent biochemical studies (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994) and demonstrated that, although possessing relationships, the *P. ostreatus* isolates studied were clearly separated from *P. columbinus*; these data suggested that *P. ostreatus* and *P. columbinus* are discrete taxa and have independent evolution processes. Furthermore, others (Hilber, 1982; Zervakis & Labarère, 1992; Petersen & Hughes, 1993; Vilgalys *et al.*, 1993; Zervakis *et al.*, 1994) have demonstrated that *P. ostreatus*, *P. columbinus* and *P. cornucopiae* are not related to *P. pulmonarius*, which was clearly separated from these species in spite of the conclusions drawn previously by Eger *et al.* (1979).

The close phenetic relationships as demonstrated by the relative position on the dendrograms of *P. pulmonarius*, *P. sajor-caju* and *P. sapidus*, and the clear separation of the *P. flabellatus*, *P. dryinus* and *P. eryngii* species are evidenced both by the present RFLP study and by previous works based on isozyme analysis (Zervakis & Labarère, 1992; Zervakis *et al.*, 1994). A similar phylogenetic congruence of data obtained from protein electrophoresis and DNA hybridizations has been reported for *Agaricus bisporus* (Royse & May, 1982; Loftus *et al.*, 1988) and *Lentinus edodes* (Royse *et al.*, 1983; Kulkarni, 1991). Comparison of the *Pleurotus* systematics based on RFLP with previous isozyme data (Zervakis & Labarère, 1992) showed that phenetic distances were greater when determined from zymograms; detection of more differences by isozyme analysis can be explained by the influence of environmental factors on protein conformation and activity, which constitute the major drawback in anatomical or biochemical studies.

In conclusion, systematic data based upon rDNA polymorphism analysis are in accordance with those based upon morphological and physiological criteria as well as with compatibility and biochemical methods; they furnish additional information on the relatedness and possible origins of individual species. Moreover, recovery of RFLP fragments (mainly *Hind*III and *Eco*RI fragments) characteristic of a species or of related species allowed five taxa, i.e. *P. cornucopiae* (2 fragments), *P. ostreatus* (2 fragments), *P. abalonus* (2 fragments), *P. columbinus* and *P. sajor-caju*, and two clusters, C (*P. abalonus* and *P. cystidiosus*) and E (*P. sajor-caju* and *P. pulmonarius*) to be distinguished, and to provide efficient molecular markers for a rapid identification of some species for a routine examination in

Pleurotus. These species-specific bands are of an indicative importance only, as a limited number of isolates per species were studied, but they could be used for further systematic studies on larger samples of *Pleurotus*.

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