Taxonomic relationships within the fungal genus *Pleurotus* as determined by isoelectric focusing analysis of enzyme patterns

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Isoenzymes from 23 isolates belonging to the fungal genus *Pleurotus*, subdivision Basidiomycotina, were examined by polyacrylamide gel isoelectric focusing and protein blotting. A numerical analysis was then done based on the zymograms for acid phosphatase, diaphorase, esterases, leucine aminopeptidase, phosphoglucosmutase and phenoloxidas. The resulting dendrogram of taxonomic distances demonstrated the suitability of enzyme isoelectric focusing for use in systematics, as it clearly distinguished the four well-defined species *P. cornucopiae*, *P. cystidiosus*, *P. eryngii* and *P. flabellatus*. The species *P. ostreatus*, whose taxonomy is controversial, was separated from *P. pulmonarius*, and *P. columbinus* was classified as a distinct taxon. The delimitation of *P. sajor-caju* and *P. sapidus* strains appeared to be more difficult as they seemed to be closely related, the former with *P. pulmonarius* and the latter with *P. ostreatus*. Isoenzymes also proved to be valuable for *Pleurotus* strain characterization. Their potential application for providing biochemical markers useful in taxonomy is discussed.

Introduction

The fungal genus *Pleurotus* (Fr.) Quél. is widely distributed throughout the world and is of particular economic importance as it comprises certain edible species that make up a steadily increasing part of the international mushroom market (Chang, 1984). The present taxonomy of the genus, however, is confusing and needs to be resolved.

A recent comprehensive review (Singer, 1986) on the systematics of basidiomycetes estimated the number of *Pleurotus* species to be between 35 and 43. However, this is indicative only, as many controversies were reported; these arise from the fact that the taxonomy of *Pleurotus* has been based mainly on morphology and compatibility relationships. These criteria alone have been used to distinguish species such as *P. cornucopiae*, *P. cystidiosus*, *P. dryinus*, *P. eryngii* and *P. flabellatus*, but in other cases they are insufficient. For instance, *P. ostreatus* was distinguished from *P. sapidus* because the two are intersterile and have a different spore-print colour and host range (Anderson *et al.*, 1973). However, Eger *et al.* (1979) contradicted the previous classification, after a

comparative examination of basidiocarp features and interspecific crosses, stating that *P. ostreatus* and *P. sapidus*, together with *P. pulmonarius* and *P. columbinus*, belong to the same species. Hilber (1982) made a thorough study of the mating reactions as well as the microscopic and macroscopic characteristics of many *Pleurotus* isolates and concluded that *P. pulmonarius* and *P. ostreatus* were different species, *P. sapidus* is more likely to correspond to *P. cornucopiae*, and *P. columbinus* is simply a variety of *P. ostreatus*. There are other disagreements concerning the exact taxonomic position of the cultivar *Pleurotus* sp. *florida* (considered either as a *P. pulmonarius* or a *P. ostreatus* strain) and whether *P. cystidiosus* and *P. abalonus* belong to separate (Han *et al.*, 1974; Bresinsky *et al.*, 1987) or the same species (Jong & Peng, 1975; Moore, 1985).

Meanwhile, biochemical and molecular biological approaches including studies of isoenzymes (Micales *et al.*, 1986) and DNA restriction fragment length polymorphisms (Kozłowski & Stepien, 1982) have been adopted and applied in fungal systematics. Studies utilizing enzyme electrophoresis have attempted to discriminate among species in genera such as *Polyporus* (Shannon *et al.*, 1973), *Fomes* (Harris *et al.*, 1974), *Monilinia* (Willems *et al.*, 1977), *Botrytis* (Backhouse *et al.*, 1984), *Agaricus* (Kerrigan & Ross, 1988) and *Phytophthora* (Oudemans & Coffey, 1991) or to characterize

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strains of *Agaricus brunnescens* (Royse & May, 1982), *Lentinus edodes* (Royse *et al.*, 1983; Ohmasa & Furukawa, 1986), *Volvariella volvacea* (Royse *et al.*, 1987) and *Agaricus bitorquis* (Roux & Labarère, 1990).

In the present work, enzymes from 23 isolates belonging to nine *Pleurotus* species were examined with the aid of polyacrylamide gel electrophoresis isoelectric focusing (PAGE-IEF) and protein blotting. Eight different activities were tested and a numerical analysis of zymographic patterns was carried out. The aim was to test the suitability of this technique as a potential tool for use in fungal systematics and to contribute to *Pleurotus* taxonomy by providing some objective criteria useful for the species delimitation and characterization.

Methods

Biological material. All fungal isolates used in this study are dikaryotic strains belonging to nine species level taxa of the genus *Pleurotus* (Fr.) Quél., order Agaricales: *P. columbinus* (Quél. apud Bres.) Quél.; *P. cornucopiae* (Paul. ex Pers.) Roll.; *P. cystidiosus* O. K. Miller; *P. eryngii* (DC. ex Fr.) Quél.; *P. flabellatus* (Berk. & Br.) Sacc.; *P. ostreatus* (Jacq. ex Fr.) Kummer; *P. pulmonarius* (Fr.) Quél.; *P. sajor-caju* (Fr.) Sing. and *P. sapidus* (Schulzer & Kalchbr.) Sacc. Details of the *Pleurotus* strains examined are presented in Table 1.

Culture media and conditions. The mycelial cultures were maintained and propagated on complete yeast medium (CYM) (Raper *et al.*, 1972), comprising (g l⁻¹): glucose (20); bacto-peptone (2); yeast extract (2); MgSO₄·7H₂O (0.5); KH₂PO₄ (0.46); K₂HPO₄ (1) and agar (20). For electrophoresis, one Petri dish culture of each isolate was scraped with a scalpel to remove the surface mycelium, which was then transferred into test-tubes containing 10 ml CYM, and disrupted with a Polytron homogenizer (Kinematica). The homogenate served as inoculum for liquid cultures in Roux flasks, containing 100 ml CYM. The incubation lasted 15–20 d and took place under static conditions at 22 °C in the dark. At least two independent cultures were performed for each isolate.

Preparation of protein samples. The mycelial cultures were harvested by filtration on sterile gauze, rinsed several times with cold distilled water and pressed dry between filter papers. The quantity of the mycelium obtained varied from 2 to 4 g per flask, depending on the strain.

Protein was extracted according to Roux & Labarère (1990). After centrifugation (30 min, 4 °C, 9000 r.p.m.), the resulting supernatant was concentrated into a Centriscart I (Sartorius GmbH) tube until a protein concentration of 10 mg ml⁻¹ was achieved. The extracts were further stored at –20 °C as a 10% (v/v) glycerol suspension.

Protein concentrations were determined according to Bradford (1976), using bovine serum albumin (fraction V-BRL) as standard.

Isoelectric focusing. A 7.5% (w/v) polyacrylamide gel, 0.5 mm thick, containing 3.5 mM-glutamic acid and 2% (w/v) ampholytes (Servalyt, pH 3–10) (Righetti, 1986) was fixed on one sheet of Gel Bond (FMC). The electrode buffers used were: for the cathode, 20 mM-arginine,

Table 1. *Species, original host, geographical origin and source of the Pleurotus isolates examined*

Species	Isolate no.	Host or substrate	Origin*	Source†
<i>P. columbinus</i>	16	Unknown	France	ATCC 36498 (Vandendries)
	17	Unknown	Italy	CBS 37351 (Baldacci)
<i>P. cornucopiae</i>	18	Unknown	Germany	ATCC 38547 (Eger)
	19	<i>Ulmus</i> sp.	Netherlands	CBS 38380 (van der Poel)
<i>P. cystidiosus</i>	11	<i>Ficus carica</i>	C. Greece	LGAM P50
	12	<i>Populus deltoides</i>	USA	ATCC 28597 (Lombard)
<i>P. eryngii</i>	13	<i>Eryngium</i> sp.	S. Greece	LGAM P63
	14	Field	N. Greece	LGAM P66
	15	Field	USSR	ATCC 36047 (Ginterova)
<i>P. flabellatus</i>	20	Rotting log	Unknown	ATCC 38138 (Graham)
	21	Oil palm waste	Unknown	CBS 66585
<i>P. ostreatus</i>	4	<i>Fagus</i> sp.	C. Greece	LGAM P38
	5	<i>Populus</i> sp.	C. Greece	LGAM P60
	6	<i>Populus</i> sp.	N. Greece	LGAM P61
	7			Commercial strain
<i>P. pulmonarius</i>	8	Unknown	Germany	ATCC 32783 (Haider)
	9	Unknown	Unknown	ATCC 34675 (Esser)
	10	Unknown	Unknown	ATCC 38538 (Eger)
	1	<i>Fagus</i> sp.	C. Greece	LGAM P26
<i>P. sajor-caju</i>	2	Unknown	USSR	ATCC 36050 (Ginterova)
	3	Unknown	USA	CBS 13285 (Graauwman)
	22	<i>Euphorbia royleana</i>	India	ATCC 32078 (Rangaswami)
<i>P. sapidus</i>	23	Hardwood	USA	ATCC 24986 (Petersen)

* The *Pleurotus* strains originating from Greece were isolated from three geographical areas: S, southern; C, central; N, northern.

† ATCC, American Type Culture Collection, Rockville, Maryland, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, Holland; LGAM, Laboratory of General and Agricultural Microbiology, Agricultural University of Athens, Greece. The commercial strain of *P. ostreatus* is widely cultivated in mushroom farms throughout Europe. It is available to research workers from LGAM.

20 mM-lysine and 10% (v/v) ethylene diamine; for the anode, 20 mM-aspartic acid and 20 mM-glutamic acid (Radola, 1980). Isoelectric focusing was carried out at 4 °C on a flat bed apparatus (2117 Multiphor II, LKB) connected to a constant power supply. Prefocusing lasted 1 h at 10 W. Then, 50 µl of protein extract per isolate were deposited on the surface of the gel with 5 × 10 mm filter-paper wicks (3MM, Whatman), along a line 1 cm away from the anode. Focusing was carried out at 10 W for 1 h and 15 W for 2 h.

The pH gradient was determined by cutting out 25 consecutive pieces (5 × 10 mm) from the gel, along the direction of the gradient, incubating them separately in 10 ml of distilled-degassed water overnight and measuring the pH, at 20 °C, with a Knick glass-type electrode. The pH values were plotted against the distance measured from the edge of the gel and the pI values of the enzyme bands were determined by interpolation from this curve.

Protein transfer and isoenzyme staining. The method for protein blotting onto nitrocellulose membranes (BA 85, Schleicher & Schuell) was derived from the 'native blot' method of Reinhart & Malamud (1982), as modified by Roux & Labarère (1990). Phosphoglucosomutase was the only enzyme among those examined that was detected directly on the gel.

After protein transfer (2 h at 4 °C), the nitrocellulose membrane was incubated at room temperature in the appropriate enzyme staining solution, prepared as described below.

Acid phosphatase (APH; EC 3.1.3.2): 100 ml 0.1 M-citrate buffer, pH 5.0; 50 mg α -naphthyl phosphate and 50 mg Fast Garnet GBC salt (Lilly & Charvat, 1987).

Diaphorase (DIA; EC 1.6.4.3): 100 ml 0.2 M-Tris/HCl buffer, pH 8.0; 4 mg 2,6-dichlorophenolindophenol; 10 mg NADH and 20 mg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Allendorf *et al.*, 1977; modified).

Esterases (EST; EC 3.1.1.1). Two types of esterase were detected. (a) Acetate esterase (EST-AC): 100 ml 0.05 M-Tris/HCl buffer, pH 7.1; 50 mg α -naphthyl acetate; 50 mg β -naphthyl acetate and 100 mg Fast Blue RR salt. (b) Butyrate esterase (EST-BUT): as for acetate esterase, but with α -naphthyl butyrate and β -naphthyl butyrate as substrates (Goullet & Picard, 1984; modified).

Leucine aminopeptidase (LAP; EC 3.4.11.1): 100 ml 0.1 M-citrate buffer, pH 5.2; 30 mg L-leucine β -naphthylamide.HCl; 10 mg MgCl₂; 10 mg MnCl₂ and 30 mg Black K salt (Shaw & Prasad, 1970; modified).

Phenoloxidases (PHE; EC 1.10.3.2). Two types of phenoloxidase were detected. (a) Dopa-reacting phenoloxidase (PHE-DRE): 100 ml 0.1 M-phosphate buffer, pH 6.5 and 1.6 g DL-dihydroxyphenylalanine (dopa) (Choi *et al.*, 1987). (b) Tolidine-reacting phenoloxidase (PHE-TRE): as for dopa-reacting phenoloxidase, but with 800 mg *o*-tolidine as substrate (Kerrigan & Ross, 1988).

Phosphoglucosomutase (PGM; EC 2.7.5.1): 100 ml 0.1 M-Tris/HCl buffer, pH 8.0; 20 mg glucose 1-phosphate; 10 mg NADP; 40 units glucose-6-phosphate dehydrogenase; 20 mg MgCl₂; 10 mg nitroblue tetrazolium (NBT) and 4 mg phenazine methosulphate (PMS) (Allendorf *et al.*, 1977; modified).

The incubation period was 20 min for EST-AC and LAP, 2 h for APH, EST-BUT and PGM and 8 h for DIA, PHE-DRE and PHE-TRE. Stained nitrocellulose sheets were photographed, and after drawing zymograms the sheets were preserved by drying. Each staining procedure was repeated at least three times, on separate gels, for each isolate.

Interpretation of data and statistical analysis. Isoenzyme banding patterns were recorded according to their migration along the polyacrylamide gel with a pH gradient, and each band was assigned a pI value.

A binomial matrix was employed to analyse variability and to transform band patterns for input into the STAT-ITCF (version 4.0,

October 1988), software for statistical analysis written by the Institut Technique des Céréales et des Fourrages, (manual and software available upon request from, ITCF, 8 avenue du Président Wilson, 75116 Paris, France). The presence of a band at a particular position (pI) was designated '1', its absence at the same position was designated '0' (Smith & Anderson, 1989). Variations in staining intensity, which certain bands occasionally presented, were not taken into account for the construction of this matrix. Taxonomic distances were computed according to Jaccard coefficients (Sneath & Sokal, 1973). The distance values were further subjected to agglomerative hierarchical weighted group-average clustering to produce a dendrogram.

Results

Reproducibility and appearance of banding patterns

Each protein extract of a *Pleurotus* isolate was independently focused and stained at least three times, on separate gels, the extracts originating from at least two different mycelial cultures. All replicates of each strain produced invariant zymograms, apart from small differences concerning the intensity of certain bands. This electrophoretic technique proved to be particularly sensitive for enzyme detection and at the same time it offered good resolution for the majority of the activities tested. Only toluidine-reacting phenoloxidase bands had a tendency to diffuse, but they remained interpretable. Protein blotting onto nitrocellulose membranes prior to staining resolved many of the difficulties. Representative patterns of four enzyme activities are presented (Fig. 1).

Zymograms and pI ranges of the 23 Pleurotus isolates

Acid phosphatase. A total of fifteen bands was detected within a pH range of 5.16–6.69 (Fig. 2a). The 23 isolates were found to possess from one to seven bands each. Only strains 13 and 15 shared the same pattern. The *P. eryngii* phenotype was characterized by the appearance of three distinct bands at pH values of 6.15, 6.45 and 6.69. *P. cystidiosus* isolates alone (11 and 12) had a band with pI = 6.30. Strains 20 and 21, belonging to *P. flabellatus*, had a unique combination of bands with pH values of 5.70 and 6.06 that permitted this species to be distinguished from the others studied.

Diaphorase. Eleven bands with pI values between 4.50 and 6.30 were visualized (Fig. 2b). Each *Pleurotus* isolate gave from one to seven bands of enzyme activity. Common patterns were observed between the 11–12, 13–15 and 22–23 pairs of strains leading to the formation of 20 phenotypic classes. *P. cornucopiae* isolates (18 and 19) were identified by a band with pI = 4.50, while strains belonging to *P. eryngii* (13, 14 and 15) shared a band with a pI value of 4.65. The strains of *P. pulmonarius* (1, 2 and 3) were distinguished on the basis of

Fig. 1. Representative zymograms, detected on nitrocellulose membranes, for different *Pleurotus* isolates. (a) Acid phosphatase; (b) butyrate esterase; (c) acetate esterase; (d) leucine aminopeptidase. Isolates are numbered as in Table 1.

the simultaneous presence of two bands with pI values of 5.91 and 6.30. In addition, a single band at pI = 6.24 appeared to be quite characteristic for *P. cystidiosus*.

Acetate esterase. Forty-one distinct bands were observed for this highly polymorphic enzyme in a pH range of 4.56–7.05 (Fig. 3a). The isolates possessed from 5 to 21 bands each, and all electrophoretic phenotypes were found to be unique. *P. flabellatus* strains were characterized by the presence of a band with pI = 7.05. Isolates 16 and 17 belonging to *P. columbinus* shared a band combination, with pI values of 6.21, 6.45 and 6.60, which did not exist among the rest of *Pleurotus* strains examined.

Butyrate esterase. Fourteen bands were detected with pI values of 4.53–6.90 (Fig. 3b). *Pleurotus* isolates gave from one to eleven bands each. Only *P. cystidiosus* strains shared an identical pattern appearing in the narrow pH region 6.30–6.60. *P. ostreatus* strains (4, 5, 6, 7, 8, 9 and 10) were separated on the basis of a two-band pattern with pI values of 5.70 and 6.78.

Leucine aminopeptidase. Nine bands were recorded ranging in pH from 4.59–4.89 (Fig. 4a). The strains

tested possessed from one to five bands each. The same patterns were found among the following isolates: 4, 10, 18; 5, 6; 7, 16; 9, 23; 13, 14, 15; and 20, 21. Consequently, 15 phenotypic classes were distinguished. A band with a pI = 4.68 characterized the *P. flabellatus* isolates, while *P. eryngii* strains were distinguished by the presence of a single band at pH = 4.59.

Phosphoglucomutase. Six bands were detected within a pH range of 4.65–5.43 and the isolates gave from one to three bands each (Fig. 4b). Identical phenotypes were observed for the following groups of strains: 1, 16; 2, 3, 7, 9; 4, 5, 6, 8; 18, 19, 23; 10, 22; 13, 14, 15; and 20, 21. Consequently, nine distinct electrophoretic groups were identified for this activity. Species such as *P. cystidiosus*, *P. eryngii* and *P. flabellatus* could be identified by bands of pI = 5.43, pI = 5.25 and pI = 4.65 respectively.

Dopa-reacting phenoloxidase. A total of 16 bands were visualized with pI values of 4.65–6.51 and isolates were found to possess from one to ten bands each (Fig. 5a). The sole common band, of pI = 5.16, was shared by strains 18 and 19. This enzyme established specific band combinations for *P. columbinus* (pI = 4.65 and pI = 4.74),

Fig. 2. Diagrammatic representation of zymograms for 23 *Pleurotus* isolates of (a) acid phosphatase and (b) diaphorase. Each protein extract of these strains was independently focused and stained at least three times, on separate gels, originating from at least two independent mycelial cultures. The isolates appear numbered as in Table 1. Dotted lines separate the taxa examined.

P. cystidiosus ($pI = 5.10$ and $pI = 5.22$) and *P. sapidus* ($pI = 4.65$ and $pI = 6.18$). It was also possible to distinguish *P. cornucopiae* strains from their single-band pattern ($pI = 5.16$).

Toluidine-reacting phenoloxidase. Nineteen bands were observed ranging in pI from 4.44–6.60; the isolates gave from 3 to 12 bands each and have unique electrophoretic phenotypes (Fig. 5b). This activity produced one common band for all the strains examined ($pI = 4.50$). *P. eryngii* isolates were characterized by two unique bands with pI values of 5.64 and 5.70.

Numerical analysis of zymograms

A distance matrix, based on the electrophoretic phenotypes of the *Pleurotus* isolates studied, was generated using the Jaccard coefficient and the levels of taxonomic distance between the strains are shown in Table 2. The resulting dendrogram, produced after group-average

clustering, illustrates the relationships found among *Pleurotus* species (Fig. 6). According to the dendrogram, the strains formed five clusters at $D = 0.68$ (68%) containing four taxonomically well-separated species, i.e. *P. cornucopiae*, *P. eryngii*, *P. flabellatus* and *P. cystidiosus*, together with one larger group including the rest of the taxa examined (the $D = 0.68$ value was selected, as at this particular distance the four previously mentioned species were clearly distinguished). Isolates belonging to *P. cornucopiae*, *P. eryngii* and *P. flabellatus* established tight intraspecific bonds at distance values of 40% and lower. However, *P. cystidiosus* strains proved to be less related ($D = 0.67$). The large, fifth group previously mentioned could be separated into two subgroups. The first contained *P. sajor-caju* and isolate 10 (originally classified as *P. ostreatus*), which separated at a distance of 60%. The second subgroup could be divided into two smaller clusters, one including all *P. pulmonarius* strains, the other *P. ostreatus*, *P. columbinus*

Fig. 3. Diagrammatic representation of zymograms for 23 *Pleurotus* isolates of (a) acetate esterase and (b) butyrate esterase. Details as for Fig. 2.

Fig. 4. Diagrammatic representation of zymograms for 23 *Pleurotus* isolates of (a) leucine aminopeptidase and (b) phosphoglucosutase. Details as for Fig. 2.

Fig. 5. Diagrammatic representation of zymograms for 23 *Pleurotus* isolates of (a) dopa-reacting phenoloxidase and (b) toluidine-reacting phenoloxidase. Details as for Fig. 2.

Table 2. Jaccard's coefficients of taxonomic distance among 23 *Pleurotus* isolates based on electrophoretic banding patterns for eight enzymes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
2	597																					
3	550	439																				
4	727	635	638																			
5	685	639	642	687																		
6	654	573	551	653	348																	
7	620	611	690	594	563	630																
8	662	594	657	574	586	579	446															
9	627	654	620	623	554	494	507	548														
10	757	652	656	705	681	630	632	615	658													
11	776	823	881	914	929	880	868	806	877	862												
12	818	841	859	871	861	833	800	809	813	859	674											
13	743	750	797	857	757	738	712	770	731	863	902	767										
14	686	730	757	833	769	765	693	750	696	808	908	800	340									
15	764	770	817	875	776	756	750	740	765	800	867	790	365	411								
16	736	652	656	683	623	575	594	554	622	677	906	851	721	770	795							
17	694	685	652	656	711	628	684	634	636	729	841	806	750	779	803	475						
18	846	797	833	686	803	827	773	742	771	793	941	827	817	864	909	767	738					
19	867	814	833	776	857	826	825	776	855	811	907	851	857	921	879	759	750	406				
20	848	853	855	867	873	844	794	855	808	836	900	873	875	866	926	810	781	796	818			
21	879	899	887	842	853	840	824	868	819	850	896	824	871	862	891	823	774	787	810	389		
22	698	600	672	787	716	699	687	629	632	600	827	807	836	829	855	754	708	750	792	680	745	
23	646	548	617	545	667	616	597	556	586	683	883	862	817	778	851	574	657	685	745	780	833	607

Fig. 6. Taxonomic relationships among *Pleurotus* isolates as visualized after weighted group-average clustering. This dendrogram was computed from the Jaccard's coefficients of taxonomic distance (Table 2). The dashed line indicates the level of taxonomic distance ($D = 0.68$) that permits the classification of the total *Pleurotus* isolates into five clusters A, *P. flabellatus*; B, *P. cornucopiae*; C, *P. eryngii*; D, *P. cystidiosus*; E, the larger group containing the rest of the taxa studied.

and *P. sapidus*. Within the latter, there was a relatively high degree of similarity ($D < 0.55$) among *P. ostreatus* isolates 5, 6, 7, 8 and 9. *P. columbinus* strains appeared closely related ($D = 0.48$) and further linked with the *P. ostreatus* complex. A relative affinity to the last group was demonstrated also by the *P. sapidus* isolate, primarily classified with strain 4 and subsequently attached to the rest of this species ($D = 0.60$).

Discussion

In the present study, eight isoenzyme activities were analysed, following isoelectric focusing and protein blotting, to provide valid biochemical criteria useful for taxonomic application in the fungal genus *Pleurotus*. Strains were obtained from various geographical areas throughout the world, in order to ensure a genetic diversity representative of the species concerned. Each isolate used had already been identified by application of morphological and physiological criteria (Zervakis & Balis, 1991). It should also be noted that these findings were in agreement with the original identifications made by those supplying the isolates (Table 1).

According to the results obtained, most of the enzyme activities demonstrated considerable levels of intra-

specific variability. The highest diversity between and within species was observed for acetate esterase patterns. All isolates produced distinctive zymograms capable of discriminating among them. Zymograms of similar appearance, though slightly less complex, were recorded for butyrate esterase. The high polymorphism found for esterases seems to be a quite common phenomenon in basidiomycetes (Ohmasa & Furukawa, 1986; Roux & Labarère, 1991). Apparently, such activities might potentially serve as biochemical markers for the registration and protection of commercial *Pleurotus* strains, as already demonstrated for *Lentinus edodes* (Royse *et al.*, 1983) and *Agaricus bitorquis* (Roux & Labarère, 1990). Phenoloxidases also appeared to produce characteristic patterns at the intraspecific level; they could thus be used for systematic studies after numerical analysis. This accords well with the findings of Kerrigan & Ross (1988) concerning laccases of the genus *Agaricus*. The other enzymes tested presented less variability; therefore, their taxonomic value would seem to be in the provision of simple-to-interpret electrophoretic phenotypes. Leucine aminopeptidase and phosphoglucumutase, in particular, were found to be largely invariant within species such as *P. flabellatus*, *P. eryngii* and *P. cornucopiae*.

Statistical treatment of the zymograms revealed large taxonomic distances among the isolates studied, particu-

larly within the species *P. ostreatus* (e.g. $D > 0.65$ for strains 4–5 and 5–10). The large diversity among *P. ostreatus* strains has been pointed out previously (Kulkarni *et al.*, 1986; May & Royse, 1988). In the former study, the levels of similarity were found to be as low as 0.15 among two isolates and the rest of the 30 strains examined (this value could, however, be attributed to the broad species concept adopted by the authors). In the latter study, a high divergence was found in spite of the fact that only loci with minimal intraspecific variation were taken into account. Within species of other genera, distances of similar order have been reported for *Agaricus campestris* (May & Royse, 1982), whilst lower values occur more often, as demonstrated in fungi such as *Lentinus edodes* (Royse *et al.*, 1989), *Botrytis cinerea* (Backhouse *et al.*, 1984) and *Phytophthora cambivora* (Oudemans & Coffey, 1991). The relatively high polymorphism obtained here is due to the resolving power of the electrophoretic method applied (Righetti, 1986), the nature of the isoenzymes examined, and most of all to the wide geographical distribution and the wild origin of the isolates used.

Classification based on cluster analysis confirmed, up to a certain point, the original identification of the *Pleurotus* isolates by assigning them into five larger taxonomic groups. Four of them corresponded to well-defined species such as *P. cornucopiae*, *P. flabellatus*, *P. eryngii* and *P. cystidiosus*. Their constituting members formed tight groups ($D < 0.40$) that were well-separated from each other and from the rest of the species studied. A possible exception could be the two *P. cystidiosus* isolates which appeared to share a relatively high value for taxonomic distance ($D = 0.67$). Nevertheless, the presence of synnematoïd fructifications in both mycelial cultures (Zervakis & Balis, 1991) leaves no doubt as to whether they have been properly assigned and any numeric dissimilarity they possess should be attributed to their distant origins. The last wide cluster contained five taxa, *P. sajor-caju*, *P. ostreatus*, *P. pulmonarius*, *P. sapidus* and *P. columbinus*, that demonstrated closer zymographic relationships and their delimitation was more complicated. Among them, *P. sajor-caju* was the first to be distinguished from the other *Pleurotus* species, despite presenting a certain affinity to *P. pulmonarius* isolates. Its existence in the same dendrogram class as a *P. ostreatus* strain was due rather to the tendency of the clustering process to form groups and to the high polymorphism demonstrated by this latter species. Previous morphological and compatibility studies concerning the rest of the *Pleurotus* species examined had been often controversial and a definitive conclusion had been hard to reach (Anderson *et al.*, 1973; Manning, 1977; Eger *et al.*, 1979; Bresinsky *et al.*, 1987). The present investigation, however, permitted the separation of *P. pulmonarius*

from *P. ostreatus* isolates as their respective clusters were found to be distinct. This outcome is in accordance with the findings of a preceding taxonomic study which applied morphological and physiological criteria to distinguish strains from these two species (Zervakis & Balis, 1991). As concerns *P. columbinus*, the results demonstrated that it could be regarded as a separate taxon. Up till now it was considered as a variety of *P. ostreatus* (Singer, 1986), mainly because the two are sexually compatible with each other (Eger *et al.*, 1979; Hilber, 1982). Nevertheless, morphological differences do exist among them (Hilber, 1982), as well as intersterile matings (Bresinsky *et al.*, 1977). Thus, although it is certain that these two taxa are closely related, whether they belong to the same species or not remains in doubt. The taxonomic position of *P. sapidus* appeared to be bewildering. Isoenzyme patterns suggested a slightly higher similarity with *P. ostreatus* than with *P. pulmonarius*. As this taxon has been reported to exist only in the American continent (Anderson *et al.*, 1973; Manning, 1977), it is probably on its way to speciation. The assumptions made for *P. sajor-caju* and *P. sapidus* were further supported by the outcome of interspecific matings concerning the species in question (C. Balis, personal communication).

The results of the present study indicate another potential application of enzyme isoelectric focusing in fungal systematics. Electrophoretic phenotypes distinguished by particular enzyme bands with species-specific pI values would be demonstrated. Thus, *P. ostreatus* was characterized by a two-band pattern (pI = 5.70 and pI = 6.78) for butyrate esterase activity. As concerns the rest of the *Pleurotus* species examined, the observations made have an indicative value only due to the relatively limited number of isolates used. In this latter case, for instance, *P. eryngii* isolates were identified by bands of activities such as acid phosphatase (pI = 6.15; pI = 6.45 and pI = 6.69), diaphorase (pI = 4.65), leucine aminopeptidase (pI = 4.59), phosphoglucosyltransferase (pI = 5.25) and toluidine-reacting phenoloxidase (pI = 5.64 and pI = 5.70). The presence of similar isoenzyme markers has already been demonstrated in the genus *Agaricus* (May & Royse, 1981; Kerrigan & Ross, 1988). These data indicate that this method might be appropriate for the development of molecular markers useful in systematics. The establishment of appropriate taxonomic criteria, through the right selection and combination of activities producing constant biochemical characters, is feasible and of importance.

The use of isoelectric focusing analysis of zymograms in fungal taxonomy has rarely been reported, but the present work clearly demonstrates its suitability. It presented a clear delimitation of unambiguous species (*P. cornucopiae*, *P. cystidiosus*, *P. eryngii* and

P. flabellatus), and at the same time permitted a thorough insight within the taxa in question. Thus, *P. ostreatus* was distinguished from *P. pulmonarius* and *P. sajor-caju*, the last two appearing related. Throughout this study, *P. sapidus* was linked with *P. ostreatus* to a greater extent than was *P. columbinus*; this latter formed a distinct taxon. The banding patterns obtained proved further that they could provide the means to characterize *Pleurotus* strains with precision and dependability.

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