

**Genetic similarity and taxonomic relationships
within the genus *Pleurotus* (higher Basidiomycetes)
determined by RAPD analysis**

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Abstract—Random amplified polymorphic DNA polymerase chain reactions (RAPD-PCR) were used to assess the genetic diversity among 37 strains of 10 *Pleurotus* species from different geographical and ecological areas worldwide, as well as to show the extent of accordance between these molecular data and previously obtained morphological and physiological data. The RAPD dendrogram obtained by using a UPGMA program, grouped the investigated strains into 6 clusters. One strain of *P. cystidiosus* and 3 strains of *P. smithii*, all originating from Israel, were grouped together, while another strain of *P. cystidiosus*, which originated from the USA, was clearly separated, which can be explained by the fact that ecological factors and geographical separation have a strong effect on the outcome of similar studies. Strains of *P. pulmonarius* and *P. djamor* showed also that geographical separation causes genetic differences. Cultivated strains with unknown morphologies were included in the study and caused serious difficulties in the interpretation of results.

Key words—genomic DNA polymorphism

Introduction

The genus *Pleurotus* is a heterogeneous group of increasing economic value. Several species are of nutritional and/or medicinal importance (Gunde-Cimerman 1999, Guzman 2000, Cohen et al. 2002). Some *Pleurotus* species have the ability to absorb microelements from the different cultivation media, and thus they may present an excellent dietary source (Stajić et al. 2002).

Due to *Pleurotus*' ability to efficiently degrade natural lignin by excreting four ligninolytic enzymes [laccase (Lac), manganese-dependent peroxidase (MnP), versatile peroxidase (VP), and aryl-alcohol oxidase (AAO)] (Muñoz et al. 1997), some species of the genus *Pleurotus* are commercially cultivated on different raw lignocellulosic materials (sawdust, paper, agricultural wastes, and other potential environmental pollutants) produced in enormous amounts worldwide.

However, the systematic position of some *Pleurotus* taxa was not well determined for a long time. Some taxonomic groups classified previously by morphological criteria were later shown, by the aid of molecular methods, to include several taxonomic entities (Brasier 1997).

Recently, several new taxonomic achievements in the genus *Pleurotus* were made by adding both mating and molecular studies. According to morphological characters, the subgenus *Coremiopleurotus* includes six morphological taxa (*P. cystidiosus*, *P. abalonus*, *P. smithii*, *P. fuscusquamulosus*, *P. gemmellari*, and *P. australis*). After testing their mating compatibility, three intersterility groups were revealed [*P. australis*, *P. cystidiosus* sensu lato, and *P. smithii* (Zervakis 1998)]. However, according to analysis of internal transcribed spacer 2 regions (ITS) sequences of ribosomal DNA (rDNA) (Zervakis et al. 2004), five phylogenetic species were presented within the subgenus *Coremiopleurotus* (*P. australis*, *P. abalonus*, *P. fuscusquamulosus*, *P. smithii*, and *P. cystidiosus*). Previously defined mating groups were found to be either paraphyletic (Zervakis & Balis 1996, Brasier 1997) or to contain several distinct molecular lineages (Vilgalys & Sun 1994, Isikhuemhen et al. 2000). However, for a long time, it was unclear, whether *P. salignus* and *P. ostreatus* were different species or if they should have been regarded as synonymous (Bresinsky et al. 1977, Hilber 1982, Moser 1983, Watling & Gregory 1989).

The aim of this study was to investigate molecular-genomic polymorphism among selected *Pleurotus* species and strains in order to confirm the accuracy of their systematic position, previously obtained on the basis of their morphology and ability of ligninolytic enzyme production.

Materials and methods

Organisms and growth conditions

The investigated species and strains of the genus *Pleurotus* and their origin are shown in Table 1. These cultures are preserved on worth agar medium (WA) in the culture collection of the Institute of Evolution, University of Haifa (HAI), and they are documented in the Catalogue of Cultures (Wasser et al. 2002).

Table 1. Investigated *Pleurotus* species and strains

Scientific name of species	HAI number of strains	Origin of strains
<i>P. cornucopiae</i> (Paulet) Rolland	32	Cultivated strain, Belgium.
	586	KW, A. S. Buchalo (88), 1999. V. D. Lozovoy (444), Sochi, Russia, 1979. on <i>Ulmus</i> sp.
<i>P. citrinopileatus</i> Singer	602	Cultivated strain, England
	435	Cultivated strain, England
<i>P. cystidiosus</i> O.K. Miller	95	Israel, Haifa, park, on <i>Schinus terebinthifolius</i> Raddi
	607	KW, A. S. Buchalo (190), 1999. CCBAS, M. Semerdzieva (466), 1981. Mississippi-delta, Louisiana, USA

<i>P. djamor</i> (Rumph. ex Fr.) Boedijn	485	Cultivated strain, Hawaii, Nextlab
	46	Mexico
<i>P. eryngii</i> (DC.) Quél. var. <i>eryngii</i>	193	Ukraine., Kherson region, Chaplinka district, Askania-Nova, on <i>Stipa</i> sp.
	201	Israel, Menahemya, on <i>Ferula</i> sp.
	356	Israel, Gevaot Merar, near Gedera, on <i>Ferula</i> sp.
	507	Cultivated strain, Hawaii, Nextlab
	616	Israel, Tabor, on <i>Ferula</i> sp.
	711	Israel, Tel Hazor, on <i>Ferula</i> sp.
	716	Israel, Gilboa, on <i>Ferula</i> sp.
	728	Israel, Lahav, on <i>Ferula</i> sp.
<i>P. eryngii</i> var. <i>tingitanus</i> Lewinsohn	555	Israel, Sataf, on <i>Ferula tingitana</i> L.
<i>P. ostreatus</i> (Jacq.) P. Kumm.	207	Ukraine, Transcarpathian region, Beregovo district, village Ivanovka, on <i>Quercus robur</i> L.
	221	Israel, Atlit, park, on <i>Salix</i> sp.
	234	Cultivated strain, Hungary.
	290	Cultivated strain, England.
	387	Cultivated strain (HK-35), Yugoslavia.
	493	Cultivated strain, Hawaii, Nextlab
	494	Cultivated strain, Hawaii, Nextlab
	495	Cultivated strain, Hawaii, Nextlab
592	KW, A. S. Buchalo (1300).	
<i>P. florida</i> Eger, nom. nud.	393	Cultivated strain, England.
<i>P. pulmonarius</i> (Fr.) Quél.	509	Cultivated strain, Hawaii, Nextlab
	572	KW, A. S. Buchalo (194), -CCBAS, M. Semerdzieva (478).
	573	KW, A. S. Buchalo (111), -VKM (F-2006). Coll. Russia, Sochi.
<i>P. salignus</i> (Pers.) P. Kumm.	326	Israel, Dan Natural Reserve, Tel Dan, on <i>Salix</i> sp.
	328	Israel, Dan Natural Reserve, Tel Dan, on <i>Salix</i> sp.
<i>P. salmoneo-stramineus</i> Lj. N. Vassiljeva	77	Cultivated strain (M2700), Belgium
	327	Germany
<i>P. smithii</i> Guzmán	138	Israel, Haifa, Carmel park on <i>Morus alba</i> L.
	140	Israel, Haifa, Carmel, Moria St. park, on <i>Morus alba</i>
	141	Israel, Jerusalem, on <i>Schinus terebinthifolius</i>

Isolation of genomic DNA

Strains were incubated on Petri dishes containing WA for 21 days at 25°C. Mycelia were harvested, frozen in liquid nitrogen, and ground to powder in a mortar. The DNA was isolated as described by Weining & Henry (1995). The purity and quality of the DNA was determined spectrophotometrically and by agarose gel electrophoresis. Standard 10 ng μ l⁻¹ DNA working solutions were prepared for each sample in TE buffer and were kept at 4°C for further tests.

RAPD analysis

Random amplified polymorphic DNA polymerase chain reactions (RAPD-PCR) (Williams et al. 1990) were carried out in 20 μ l reaction volume containing 30 ng genomic DNA, 10 x PCR buffer Mg free, 50 mM MgCl₂, 2 mM dNTP, Taq polymerase (1 U μ l⁻¹) (Biotools, Madrid), 12 ng primer [University of British Columbia (UBC), Canada], and double distilled water. Six primers, which gave well-resolved bands in gel electrophoresis were used: **1.** UBC 155 (CTG GCG GCT G); **2.** UBC 215 (TCA CAC GTG C); **3.** UBC 250 (CGA CAG TCC C); **4.** UBC 310 (GAG CCA GAA G); **5.** UBC 335 (TGG ACC ACC C); and **6.** UBC 458 (CTC ACA TGC C). Amplification was performed in a GeneAmp PCR System 9700 thermal cycler: one cycle at 92°C for 3 min, 45 cycles at 92°C for 30 sec, 42°C for 1 min, and 72°C for 1 min, and one cycle at 72°C for 10 min. Amplified fragments were resolved on a 1.2% agarose gel. We performed several control experiments including (i) separate RAPD-PCR runs on the DNA of the same isolates, (ii) variation of DNA concentration, and (iii) the analyses of RAPD-PCR products in separate agarose gels to ensure the reproducibility of the RAPD patterns. A size marker (Ladder Mix, MBI Fermentas) was used as a reference in all gels. The RAPD patterns were analyzed with the software Gene ImagIR version 3.52 (Scanalytics) and exported into the Treecon software (Van de Peer & De Wachter 1994) to generate an Unweighted Pair Group Method with Average Means (UPGMA) dendrogram from a distance matrix (Nei & Li 1979).

Results

All strains included in this study showed unique banding patterns, as observed from amplification band comparisons a total of 6 RAPD-PCR primers. Altogether, an average of 46 \pm 6 bands per isolate (minimum 25 bands and maximum 59 bands) was obtained in a total of 260 different band positions. Along the topology of an UPGMA dendrogram, we defined six clusters A-F, in order to yield clusters of previously morphologically defined species (Fig. 1). These clusters split from each other at a genetic dissimilarity distance of approximately 0.54-0.64 (Fig. 1). Basically, a good correlation was observed between morphological and molecular grouping of strains. However, several inconsistencies were noticed. Both strains of *P. salignus* (HAI 326 and HAI 328) clustered within the group of *P. ostreatus* (cluster A, Fig. 1). Two strains of *P. ostreatus* (HAI 290 and HAI 592) were in the *P. eryngii* cluster (cluster B). *P. smithii* strains were grouped in the same cluster (cluster C) with one of the *P. cystidiosus* strains (HAI 95 from Israel), which was clearly separated by another lineage made up by one *P. cystidiosus* strain from the USA (HAI 607). All *P. pulmonarius* strains were grouped together (cluster D), with HAI 509 being clearly separated from strains HAI 572 and

573. Both *P. citrinopileatus* strains clustered together with the *P. salmoneostramineus* strain (HAI 77), which is thus clearly separated from the other *P. salmoneostramineus* strain (HAI 327). The two *P. djamor* strains, HAI 46 and 485, were grouped closely together with the *P. cornucopiae* strain, HAI 32 (cluster F), which was then clearly separated from the other *P. cornucopiae* strain, HAI 586.

Discussion

General congruence of molecular and morphological classification

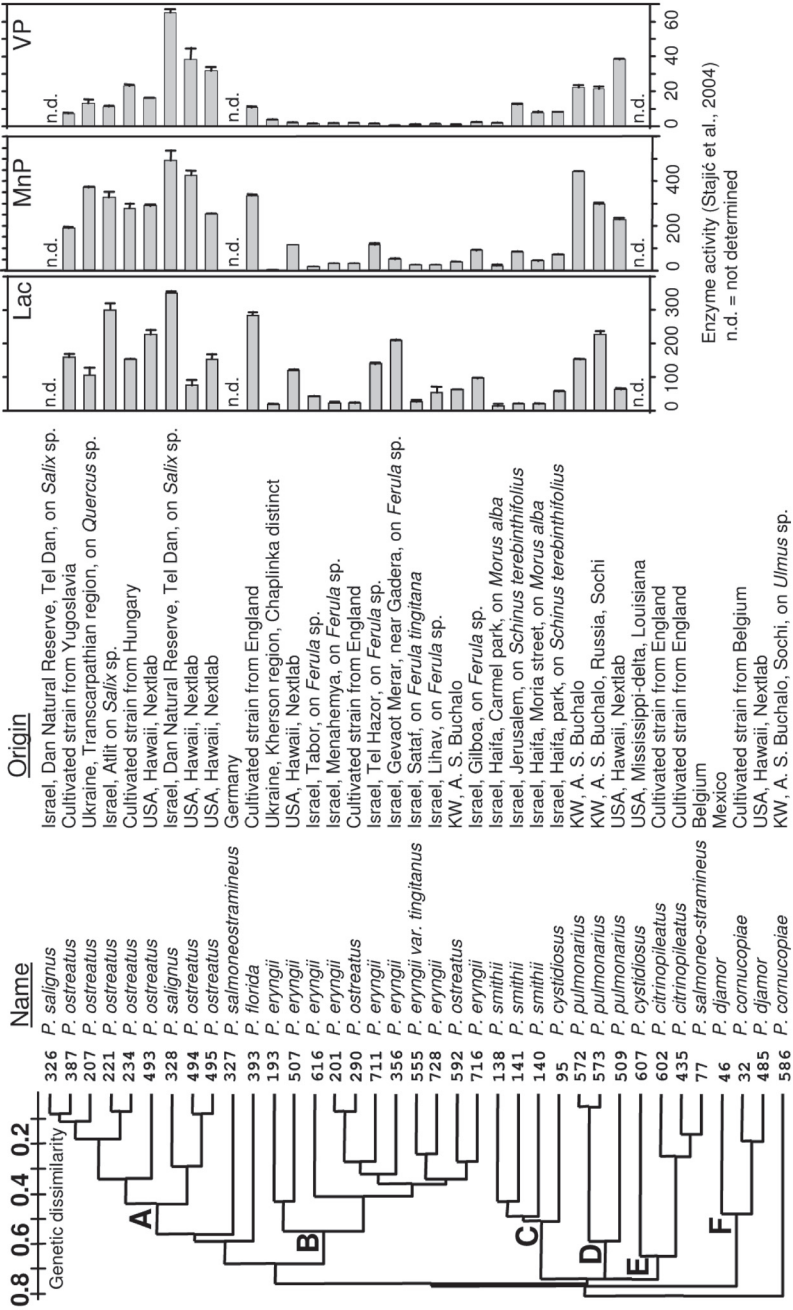
Basically, a strong correlation was found between morphological and molecular criteria, supporting previous studies (Iracabal et al. 1995, Zervakis et al. 2004). We found both *P. salignus* strains (HAI 326 and 328) to cluster within the group of *P. ostreatus* (cluster A, Fig. 1). This supports the conclusions of Hilber (1982) and Watling & Gregory (1989) that *P. salignus* is in fact a synonym of *P. ostreatus*.

In a few cases, the molecular-genomic relationships among strains did not match the morphological groupings. Our data showed that two strains of *P. salmoneostramineus* (HAI 77 and 327) as well as two strains of *P. cornucopiae* (HAI 32 and 586) were split into different genomic clusters representing basically different morphospecies. Additionally, two *P. ostreatus* strains, HAI 290 and 592, clustered not only apart from all other *P. ostreatus* strains (cluster A, Fig. 1), but grouped nicely within the *P. eryngii* cluster B (Fig. 1). The latter case could be explained by previous misidentification. These strains have been received as mycelia culture and their classification history is unknown. As shown in *P. eryngii* species-complex, identification mistakes are easily made due to variations in morphology (Lewinsohn et al. 2002), isoenzymes (Zervakis et al. 1994), and genetic characteristics (Lewinsohn et al. 2001) in response to geographical and/or ecological differences in their environments, i.e., geographic variation.

In order to better clarify the observed discrepancies between morphological and molecular-genomic criteria, we added physiological data available for nearly all strains for comparison (Fig. 1). Stajić et al. (2004) have determined the activities of the ecologically important ligninolytic enzymes: laccase (Lac), manganese-dependent peroxidase (MnP), and versatile peroxidase (VP). Basically, in a comparison of all three enzymes, *P. ostreatus* strains showed high-enzyme activities compared to *P. eryngii* (Fig. 1). Strains HAI 290 and 592, morphologically classified as *P. ostreatus*, however, showed the same low-enzyme activities of the *P. eryngii* strains they clustered with (Fig. 1), compared to the high-enzyme activities of the *P. ostreatus* group. These results support the grouping obtained by RAPD analysis, and thus we propose that the *P. ostreatus* strains, HAI 290 and 592, should be reclassified as *P. eryngii* strains.

The enzyme activities of strain HAI 328, classified as *P. salignus*, shows similar high-enzyme activities as the *P. ostreatus* strains with which it genomically clusters. This additionally confirms that *P. salignus* and *P. ostreatus* should be considered synonyms of the same species (Hilber 1982, Watling & Gregory 1989).

Strain HAI 393, which was morphologically described as *P. florida*, clusters close to, but yet separated from all other *P. ostreatus* strains, indicating an ongoing evolutionary separation process of these two lineages (A; Fig. 1). This was confirmed by mating experiments (Hilber 1982, Bresinsky et al. 1987). Likewise, Gonzalez & Labarère (2000) stated that *P. ostreatus* and *P. florida* represent a single species according to



obtained results by investigations of sequences of the V4, V6, and V9 domains of the mitochondrial small subunit rRNA.

Geographical and ecological influences on genetic characters

In the case of three strains of *P. pulmonarius* as well as of two strains of *P. djamor*, it was shown that both geographical separation and different ecology factors cause genetic differences. Although both strains of *P. pulmonarius* and *P. djamor* strains made up each unique cluster, D and F, respectively, the within-species distance between strains was large (D~40%), especially between *P. pulmonarius* strain HAI 509 and two other strains (HAI 572 and 573), and two strains of *P. djamor* (HAI 46 and 485), respectively. The observed large within-species genomic distance correlates with geographic separation and probably also with ecological divergence, and might finally terminate in speciation.

Despite molecular techniques, the taxonomic relationships between *P. cystidiosus* and *P. smithii* are not yet completely resolved. In the RAPD study of Capelari & Fungaro (2003), the three strains of *P. smithii* (from Mexico) intermingled with 9 strains of *P. cystidiosus* (from Brazil, China, Greece, Japan, Thailand, and the USA), supporting the proposal of Hilber (1997) that *P. smithii* should be considered synonymous with *P. cystidiosus*. In another study, however, Zervakis et al (2004) clearly separated *P. cystidiosus* and *P. smithii* strains on the basis of ITS sequences, yet the two species were closely related to each other in comparison to other species of the *Coremiopleurotus* taxa. Recently, we reported for the first time, on a morphological basis, the presence of *P. cystidiosus* (1 strain) and *P. smithii* (3 strains) in Israel (Stajić et al. 2003). In our RAPD study, these four strains grouped together in a unique cluster (C; Fig. 1), while the *P. cystidiosus* strain from Haifa (HAI 95) was clearly separated from the *P. cystidiosus* (HAI 607), isolated in the USA. Linking these results to the results of Capelari & Fungaro (2003) and Zervakis et al (2004), the following might describe the relationship between *P. cystidiosus* and *P. smithii*. Both taxa are very similar to each other, and the origin (i.e., biogeography and ecology) of the strains might have a strong impact on the outcome of similar studies. Lewinsohn et al (2000) showed the strong impact of ecology on different strains of *P. eryngii*, which, via selection, might influence genetic characteristics. Thus, only a large study of all available isolates of *P. cystidiosus* and *P. smithii*, comprising a variety of geographical and ecological origins, might give more insight into the relationship between *P. cystidiosus* and *P. smithii*.

Conclusions

Results of these investigations showed some incompatibilities between the morphological determination, on the one hand, and the physiological-genomical determination, on the other hand, of *Pleurotus* species and strains. This confirms the current view that for a precise affiliation of strains to a taxonomic group or evolutionary lineage, morphological, behavioural, and molecular criteria should be applied.

Fig. 1. Taxonomic relationships among investigated *Pleurotus* species and strains based on the results of: a) RAPD-PCR analysis; b) study of Lac production; c) study of MnP production, and d) study of VP production by them.

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