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Genetic polymorphism and taxonomic infrastructure of the *Pleurotus eryngii* species-complex as determined by RAPD analysis, isozyme profiles and ecomorphological characters

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The *Pleurotus eryngii* species-complex includes populations of choice edible mushrooms, growing in the greater Mediterranean area in close association with different genera of plants of the family Apiaceae. Their distinct host-specialization served as the principal criterion for the discrimination of several taxa; however, the genetic relationships among the various *P. eryngii* ecotypes remain ambiguous. In the present study, 46 *Pleurotus* strains with a wide range of geographical origins were isolated from *Eryngium* spp., *Ferula communis*, *Cachrys ferulacea*, *Thapsia garganica* and *Elaeoselinum asclepium* subsp. *asclepium*, and were subjected to isozyme and random amplified polymorphic DNA-PCR (RAPD) analysis. The 16 enzyme activities tested were controlled by 28 loci, 11 of which were monomorphic. Host-exclusive zymograms for the Aph (acid phosphatase) and Phe-1 (dopa-phenoloxidase) loci were obtained from *Pleurotus* strains associated with *C. ferulacea*. Allele frequencies, genetic diversity and mean diversity were high for isolates from *Eryngium* spp. and *Ferula communis*. In RAPD analysis, the use of five primers allowed the production of 45 (out of 48) polymorphic bands, while four molecular markers specific for the identification of *Pleurotus* strains growing on *E. asclepium* subsp. *asclepium* and *C. ferulacea* were obtained. The *Pleurotus* strains produced 35 distinct electrophoretic types and 42 RAPD patterns, which independently permitted the separation of the fungal populations into five clusters in accordance with their host-specificity. In addition, the evaluation of the principal ecological and morphological characters provided further evidence for discriminating between *P. nebrodensis* growing on *C. ferulacea* and the rest of the host-associated populations. The latter represent taxa at the varietal level: *P. eryngii* var. *eryngii*, *P. eryngii* var. *ferulae* and *P. eryngii* var. *elaeoselini*. The position of taxa of dubious validity, such as *P. hadamardii* and *P. fossulatus*, is discussed in relation to the new findings. All Mediterranean *Pleurotus* populations growing on umbellifers seem to have recently diverged through a sympatric speciation process, that is based on both intrinsic reproductive barriers and extrinsic ecogeographical factors.

Keywords: *Pleurotus nebrodensis*, Apiaceae, mushroom systematics, fungal speciation and evolution, host-specificity

Abbreviations: ET, electrophoretic type; RAPD, random amplified polymorphic DNA.

INTRODUCTION

Fungi of the genus *Pleurotus* (Basidiomycotina, Poriales) are tetrapolar heterothallic; dikaryons produce edible reproductive structures (i.e. basidiomata) on a large array of lignocellulosic substrates. The only group of this genus growing in association with living plants is *P. eryngii sensu lato*. This species-complex includes populations which are weak parasites on the roots and stems of umbellifers (family Apiaceae, genera *Eryngium*, *Ferula*, *Ferulago*, *Cachrys*, *Laserpitium*, *Diplotaenia* and *Elaeoselinum*), appearing mostly in groups from autumn until late spring. Their distribution is located within a rather well-defined area of the northern hemisphere, extending westwards to the Atlantic coasts of France and Morocco, and along a zone lying within 30–50° N from central Europe to the Mediterranean coast of Africa, and eastwards to Kazakhstan and India.

There is much controversy on the proper assignment of host-specialized populations within the *P. eryngii* complex. For example, some authors consider most or all ecotypes as distinct species (Boisselier-Dubayle, 1983; Joly *et al*., 1990), some view them as varieties of *P. eryngii* (Bresinsky *et al*., 1987; Hilber, 1982), and others express intermediate arguments (Venturella, 2000; Zervakis & Venturella, 1998). In addition, there are several other names that have been proposed to accommodate taxa of dubious validity, like *P. fossulatus* (Cooke) Sacc. or *P. hadamardii* Constantin, growing also on umbellifers (Joly *et al*., 1990; Pegler, 1977). This confused situation presents a major challenge for fungal taxonomy, speciation and co-evolution studies. Its clarification would also be of substantial benefit for applied research, since the use of *Pleurotus* fungi is linked to several agro-industrial activities of great economic importance, e.g. conversion of lignocellulosic residues to food and feed, biocontrol of plant diseases, degradation of noxious pollutants, production of enzymes and medicinal compounds, etc. (Heinfling *et al*., 1998; Philippoussis *et al*., 2001; Ruiz-Duenas & Martinez, 1996; Wasser & Weis, 1999; Zervakis *et al*., 1996).

There are significant problems in classifying *Pleurotus* isolates using only morphological characters (which are often unreliable or inconclusive mainly due to the large influence exerted by environmental factors) or compatibility experiments (which are based on the application of the controversial 'biological species concept'). Therefore, the application of molecular criteria is essential for providing a thorough insight into the taxonomic relationships between *Pleurotus* populations and in pertinent speciation processes (Iraçabal *et al*., 1995; Petersen & Hughes, 1999; Vilgalys *et al*., 1996). Isozyme analysis has been successfully applied to several taxonomic studies in mycology (Micales *et al*., 1986). Interpretation of zymograms has been useful in identifying genetic variability within and between fungal species (Gottlieb *et al*., 1998; Urbanelli *et al*., 1998; Zervakis *et al*., 1994), and for revealing the extent of variation in diverse populations from numerous hosts (Harrington *et al*., 1996; Surve-Iyer *et al*., 1995; Yoon *et al*., 1990) and geographical origins (Bonde *et al*., 1993; Stanosz *et al*., 1999; Zervakis & Labarère, 1992). On the other hand, the random amplified polymorphic DNA-PCR technique (RAPD-PCR) has permitted the study of the population structure of many fungi that are difficult to characterize with other markers. This technique allows rapid generation of reliable and reproducible DNA fingerprints and has been used to investigate the genetic variation within several fungal groups (Bryan *et al*., 1999; Paavanen-Huhtala *et al*., 1999; Raina *et al*., 1997), or to clarify systematics based on traditional criteria (Assigbetse *et al*., 1994; Holmes *et al*., 1994).

The aim of this study was to elucidate the systematics and assess the diversity of European *Pleurotus* taxa associated with umbellifers, and determine speciation processes under way. Forty-six dikaryotic strains belonging to the *P. eryngii* species-complex isolated from five different host-plant genera were examined by the use of RAPD-PCR and isozyme techniques, and evaluated in conjunction with ecological observations and morphology of basidiomata.

METHODS

Biological material. Details of the 46 *Pleurotus* dikaryons used in this study are listed in Table 1. Strains were maintained on complete yeast medium (CYM) solidified with agar (Raper *et al*., 1972); cultures were stored at 4 °C in vials with sterile distilled water, and are kept in the fungal culture collection of the Institute of Kalamata (NAGREF-IK). All voucher specimens are deposited in the Herbarium Mediterraneum (PAL) and in the Herbarium of the Institute of Kalamata (NAGREF-IK).

Morphological and ecological data. Field surveys and periodical observations on *Pleurotus* taxa growing on umbellifers were carried out in Sicily and Greece from autumn 1995 to autumn 2000. Each taxon was evaluated as regards morphological, anatomical, distributional and ecological characters (Venturella *et al*., 2000; Zervakis & Balis, 1996).

Dikaryotic and homokaryotic fungal cultures. For the establishment of dikaryons in pure culture, small pieces from the basidioma context were taken and placed on Petri dishes with water agar. After a few days, hyphal tips were transferred to fresh medium. For the assessment of the loci and alleles responsible for the enzyme activities tested, *Pleurotus* homokaryotic (monokaryotic) strains were produced from at least three dikaryons per host-associated population. Single-spore isolates were obtained either from naturally occurring basidiomata or from basidiomata grown *in vitro* (Zervakis & Balis, 1995).

Enzyme extraction, starch-gel electrophoresis and analysis of isozyme data. For the production of mycelial cultures, dikaryotic or homokaryotic strains were grown in 250 ml Erlenmeyer flasks containing 100 ml CYM. Culture conditions, mycelium harvest and enzyme extraction were as described by Zervakis *et al*. (1994).

Enzymes were separated by horizontal starch-gel electrophoresis. The five different tray and gel buffer systems used for optimal resolution of isozyme patterns, and preparation of

Table 1. Details of the 46 *Pleurotus* dikaryons used in this study

The acquisition code number of the dikaryons, the host from which they were isolated, their geographical origin, and the electrophoretic type (ET) to which they were assigned after isozyme analysis are shown.

Code no.*	Host	Geographical origin	ET
NAGREF-IK P63	*Eryngium* sp.	Crete, Greece	1
NAGREF-IK P64	*Eryngium* sp.	Crete, Greece	1
NAGREF-IK P65	*Eryngium* sp.	Evros, Greece	2
NAGREF-IK P66	*Eryngium* sp.	Evros, Greece	3
NAGREF-IK P101	*Eryngium* sp.	Andros, Greece	4
NAGREF-IK 2	*Eryngium* sp.	Evoia, Greece	5
UPA10	*E. campestre*	Apulia, Italy	6
UPA11	*E. campestre*	Apulia, Italy	7
UPA12	*E. campestre*	Apulia, Italy	6
UPA13	*E. campestre*	Madonie Mt., Italy	–
UPA16	*E. campestre*	Madonie Mt., Italy	8
ATCC 36047	*Eryngium* sp.	Former Czechoslovakia	9
CBS 10082	*Eryngium* sp.	Former Czechoslovakia	10
NAGREF-IK 851101	*Eryngium* sp.	France	–
NAGREF-IK 81	*Eryngium* sp.	France	13
NAGREF-IK 831101	*Eryngium* sp.	France	11
NAGREF-IK 831102	*Eryngium* sp.	France	–
NAGREF-IK 80	*Eryngium* sp.	France	12
NAGREF-IK P102	*Ferula* sp.	Andros, Greece	14
NAGREF-IK P109	*F. communis*	Ag. Efstratios, Greece	15
NAGREF-AMRU 244	*F. communis*	Crete, Greece	21
NAGREF-AMRU 245	*F. communis*	Crete, Greece	22
UPA1	*F. communis* subsp. *communis*	Pellegrino Mt., Italy	16
UPA17	*F. communis* subsp. *communis*	Madonie Mt., Italy	17
UPA18	*F. communis* subsp. *communis*	Madonie Mt., Italy	18
UPA19	*F. communis* subsp. *communis*	Madonie Mt., Italy	19
UPA20	*F. communis* subsp. *communis*	Madonie Mt., Italy	20
UPA2	*Cachrys ferulacea*	Madonie Mt., Italy	23
UPA3	*C. ferulacea*	Etna Mt., Italy	24
UPA6	*C. ferulacea*	Madonie Mt., Italy	25
UPA7	*C. ferulacea*	Madonie Mt., Italy	26
UPA8	*C. ferulacea*	Madonie Mt., Italy	26
UPA9	*C. ferulacea*	Madonie Mt., Italy	–
UPA23	*C. ferulacea*	Madonie Mt., Italy	27
UPA28	*C. ferulacea*	Madonie Mt., Italy	28
UPA4	*Elaeoselinum asclepium* subsp. *asclepium*	Madonie Mt., Italy	30
UPA24	*E. asclepium* subsp. *asclepium*	Madonie Mt., Italy	29
UPA29	*E. asclepium* subsp. *asclepium*	Mussomeli, Italy	31
UPA30	*E. asclepium* subsp. *asclepium*	Mussomeli, Italy	32
UPA31	*E. asclepium* subsp. *asclepium*	Mussomeli, Italy	32
UPA5	*Thapsia garganica*	Madonie Mt., Italy	–
UPA26	*T. garganica*	Madonie Mt., Italy	33
UPA27	*T. garganica*	Madonie Mt., Italy	–
UPA32	*T. garganica*	Madonie Mt., Italy	34
UPA35	*T. garganica*	Madonie Mt., Italy	34
UPA36	*T. garganica*	Madonie Mt., Italy	35

*ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; NAGREF-AMRU, National Agricultural Research Foundation, Institute of Agricultural Machinery & Constructions, Greece; NAGREF-IK, National Agricultural Research Foundation, Institute of Kalamata, Greece; UPA, Department of Botany, University of Palermo, Italy.

gels, were as described by Zervakis *et al*. (1994). Conditions for electrophoresis and staining protocols for the 23 enzyme activities assayed were as follows (enzyme abbreviations for data-informative loci are defined in Table 2): ADH, GDH, LDH, SOD, XDH (Allendorf *et al*., 1977), APH, ALP, GADH, ME (Loukas & Krimbas, 1980), PHE-DRE, PHE-TRE (Kerrigan & Ross, 1989), DIA, EST, LAP, PGM (Zervakis & Labarère, 1992), and G6PD, HK, PHI, IDH, MDH, PEP-LT, PEP-PHE-PRO, 6PGD (Zervakis *et al*., 1994). The gels were read just after the end of the incubation period, and then preserved for future reference in a water/methanol/acetic acid (50:40:10) solution. All protein extracts were electrophoretically examined at least three times and produced consistent zymograms, which only occasionally showed some fluctuations in their staining intensity (band staining intensity was not taken into account when the zymograms were interpreted).

The gene nomenclature adopted in this paper follows that of May *et al*. (1979). Bands (and hence their genetic basis) were interpreted using the outcome of the electrophoretic runs of the homokaryotic isolates. Encoding of every distinct area of enzymic activity (locus), assignment of numbers to alleles of each locus, and encoding of electrophoretic data for input into the computer software followed Zervakis *et al*. (1994). *Pleurotus* isolates which shared identical multilocus phenotypes were grouped into the same electrophoretic type (ET) (Table 1).

Isozyme data were partitioned by locus and population for further analysis. For all loci, the allele frequencies, the genetic diversity per locus and the mean diversity were calculated using the formulae of Selander *et al*. (1986) and Nei (1978) with a correction for bias in small samples. In addition, for each population (either host-associated or geographical) examined, the percentage of polymorphic loci (0·95 criterion), the mean number of alleles per locus and the mean diversity were determined to provide an estimate of the intrapopulation variability.

DNA extraction and RAPD-PCR analysis. Mycelia, grown on solidified CYM, were frozen in liquid nitrogen in Eppendorf tubes and ground into powder with a micropestle (Kontes Pellet Pestle, Fisher cat. no. K749520-0-000). DNA extraction followed Rogers & Bendich (1988). DNA concentration and dilution were estimated by gel electrophoresis and spectrophotometry. Amplification reactions were performed in a final volume of 25 µl containing 10 ng genomic DNA. The reaction solution consisted of 200 µM each of dATP, dCTP, dGTP and dTTP, 50 µmol oligonucleotide primer (Operon Technologies, Kit OPB) and 2 units *Taq* polymerase (Boehringer Mannheim) in 10 mM Tris pH 8·3, 2 mM $MgCl_2$, 0·001 % gelatin, 0·05 % Tween 20, 50 mM KCl. Amplification was performed in a Techne Progene thermalcycler: one cycle at 94 °C for 3 min, 37 °C for 1 min and 72 °C for 1 min, and 44 cycles at 94 °C for 30 s, 37 °C for 1 min and 72 °C for 1 min. Amplified fragments were resolved on a 1·1 % agarose gel, run under standardized conditions, and stained by ethidium bromide. A 100 bp ladder DNA marker (Pharmacia) was used as a size standard.

Each amplification run included a negative control reaction without the addition of DNA and each reaction was performed at least twice. Initially, a subset of the isolates was used to perform a preliminary screening of 20 decamer oligonucleotide primers to identify those that gave reproducible marker profiles and to exclude those producing very low proportion of polymorphic bands. Five primers were selected: OPB01 (5′-GTTTCGCTCC-3′), OPB02 (5′-TGATCCCTGG-3′), OPB10 (5′-CTGCTGGGAC-3′), OPB14 (5′-TCCGCTCTGG-3′) and OPB18 (5′-CCACAGCAGT-3′). Some variations in RAPD patterns were detected in the duplicate experiments (i.e. approximately 10 % of the total amplified bands were not consistently amplified). However, only distinct, clearly resolved and reproducibly amplified fragments were selected for RAPD analysis and scored as present (1) or absent (0). Comparisons of RAPD profiles were only made between samples that were included in the same run, and which had been separated on the same agarose gel. There was no differential weighting for band intensity. The assumption was made that amplification products of the same size which were present in the profiles generated by different isolates represented products from equivalent loci.

Statistical analysis. Cladistic analysis was performed with the tester version 4.0b4a of PAUP* (Phylogenetic Analysis Using Parsimony) written for PowerPC (Swofford, 2000). All characters (26 from the isozyme data, and 48 from the RAPD data) were unordered and of equal weight. Parsimony settings were as follows: ACCTRAN (accelerated transformation) character-state optimization, stepmatrix option allowed assignment of states not observed in terminal taxa to internal nodes (all states in stepmatrix), and multiple states of taxa were interpreted as polymorphism (in the case of isozymes only). Genetic distances were calculated by the PAUP* software on the basis of mean genetic differences.

Branch robustness of the derived cladograms was evaluated in PAUP* using two different methods: (a) bootstrapping, and (b) jacknife-resampling. Both methods used maximum-parsimony as the optimality criterion and performed an heuristic search with 500 replicates and the following settings: simple addition sequence (reference taxon: *P. pulmonarius*), starting trees obtained via stepwise addition, 10 trees held at each step during stepwise addition, tree-bisection-reconnection (TBR) as branch-swapping algorithm, MULPARS option not in effect, steepest descent option not in effect, MAXTREES setting 500, branches having maximum length zero allowed to collapse to yield polytomies, topological constraints not enforced. In addition, the jacknife-resampling method was set to 50 % of characters deleted in each replicate. The respective cladograms shown in Results derived from the enforcement of the 50 % majority-rule consensus.

RESULTS

Isozyme analysis

For the purposes of this study 23 enzyme activities were examined; for seven of them (ALP, DIA, GADH, GDH, ME, PHE-TRE, XDH) no activity was detected or they were poorly resolved and therefore abandoned. For the rest of the enzymes tested, interpretation of electrophoretic patterns was based on the zymograms produced by the homokaryotic progenies (Table 2). Hence, the production of the other 16 activities was governed by 28 loci, 11 of which were monomorphic (HK, PHI, PEP-LT and PEP-PHE-PRO were monomorphic at all loci). Among the rest of the activities examined, the production of EST and SOD was controlled by three loci in each case; however, only one locus per activity (Est-1 and Sod-1) was clearly resolved for all strains tested. In total, 13 clearly interpretable and polymorphic loci were obtained which permitted the classification of the 40 strains into 35 distinct electrophoretic types (data not

Table 2. Summarized results of isozyme electrophoresis

Results are shown only for data-informative loci: enzyme activities (and their respective EC no.), corresponding loci, alleles with mobility designations, allele frequencies and genetic diversity (*h*) for each locus examined in a sample of 40 *P. eryngii* species-complex strains.

Enzyme (EC no.)	Abbrev.	Loci	Alleles* (frequencies)†	Genetic diversity
Acid phosphatase (3.1.3.2)	APH	Aph	2 = 108 (0·3750), 3 = 86 (0·1750)	0·615
Alcohol dehydrogenase (1.1.1.1)	ADH	Adh	2 = 80 (0·0750), 3 = 65 (0·0500), 0 = silent (0·3250)	0·571
Esterase (3.1.1.1)	EST	Est-1	2 = 120 (0·1125), 3 = 60 (0·4375)	0·583
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6PD	G6pd-1	2 = 85 (0·0625), 0 = silent (0·1500)	0·336
Isocitric acid dehydrogenase (1.1.1.42)	IDH	Idh	2 = 98 (0·2750)	0·383
Lactate dehydrogenase (1.1.1.27)	LDH	Ldh	2 = 80 (0·2000), 3 = 70 (0·0500), 0 = silent (0·4250)	0·663
Leucine aminopeptidase (3.4.11.1)	LAP	Lap-1	2 = 92 (0·4000), 3 = 108 (0·1000)	0·569
Malate dehydrogenase (1.1.1.37)	MDH	Mdh-1	2 = 95 (0·1500), 3 = 58 (0·3625)	0·598
Dopa-phenoloxidase (1.10.3.x)	PHE-DRE	Phe-1	0 = silent (0·2500)	0·359
		Phe-2	2 = 108 (0·0625), 0 = silent (0·3125)	0·495
Phosphoglucomutase (2.7.5.1)	PGM	Pgm-1	2 = 96 (0·2125), 0 = silent (0·3250)	0·626
6-Phosphogluconate dehydrogenase (1.1.1.41)	6PGD	6Pgd	2 = 117 (0·1250)	0·200
Superoxide dismutase (1.15.1.1)	SOD	Sod-1	2 = 108 (0·0750)	0·117

* The most common allele for each locus was assigned the value of 100 (1 = 100), and the rest of the alleles were designated with their relative mobilities with respect to the allele '1'.

† The frequency of allele '1' is equal to the difference of the sum of the frequencies of the other alleles from 1.

Table 3. Number of strains per population, mean number of alleles per locus, percentage of polymorphic loci and mean diversity for all loci examined in the five populations of the *P. eryngii* species-complex based on host preference

Population	No. of strains	Mean no. of alleles per locus	Polymorphic loci (%)	Mean diversity
Eryngium	15	2·92 ± 0·51	100·0	0·261 ± 0·039
Ferula	9	2·15 ± 0·28	84·6	0·288 ± 0·041
Cachrys	7	1·46 ± 0·15	38·5	0·122 ± 0·014
Elaeoselinum	5	1·77 ± 0·21	38·5	0·123 ± 0·011
Thapsia	4	1·46 ± 0·18	38·5	0·099 ± 0·007

shown). Only five pairs of isolates produced identical zymograms for all loci: IKP63 and IKP64 from eastern Crete on *Eryngium* sp. (ET1), UPA10 and UPA12 from Apulia on *E. campestre* (ET6), UPA7 and UPA8 from Madonie Mt. on *C. ferulacea* (ET26), UPA30 and UPA31 from Mussomeli on *E. asclepium* (ET32), and UPA32 and UPA35 from Madonie Mt. on *T. garganica* (ET34). Host-exclusive zymograms were produced only from *Pleurotus* strains growing in association with *C. ferulacea*, for the Aph and Phe-1 loci.

Allele frequencies, genetic diversity and mean diversity for all 13 isozyme loci provided an indication of the overall variation that existed among the ETs produced by this study (Table 2). The genetic diversity varied significantly among the different loci, ranging from 0·117 (Sod-1) to 0·663 (Ldh). The mean diversity for all strains was 0·470. Partition of isozyme bands into loci and grouping of strains by host allowed estimation of the mean number of alleles per locus, the percentage of polymorphic loci, and the mean diversity within each host-associated population (Table 3). Increased percentages of polymorphic loci and high mean diversity values were detected for *Eryngium*- and *Ferula*-associated strains (mean diversity 0·261 and 0·288 respectively), whereas isolates growing on *Cachrys*, *Elaeoselinum* and *Thapsia* plants yielded significantly lower values (0·122, 0·123 and 0·099 respectively). Furthermore, a similar type of evaluation performed within groups of strains of identical geographical origin (Italy and Greece, only for *Eryngium*- and *Ferula*-associated specimens) indicated higher diversity for Greek isolates irrespective of the host plant they were collected from: e.g. mean no. of alleles per locus 2·231 vs 1·769 when

Table 4. Genetic distances resulting from isozyme (above the diagonal) and RAPD-PCR (below the diagonal) analysis among five different populations of *Pleurotus* isolated from different plant hosts

Host	*Eryngium*	*Ferula*	*Cachrys*	*Elaeoselinum*	*Thapsia*
Eryngium	0·291/0·262	0·491	0·589	0·531	0·462
Ferula	0·354	0·365/0·217	0·578	0·451	0·512
Cachrys	0·446	0·440	0·128/0·221	0·514	0·398
Elaeoselinum	0·416	0·393	0·441	0·173/0·226	0·321
Thapsia	0·357	0·352	0·387	0·309	0·109/0·178

data from both host plants are compared, or 2·000 vs 1·462 for *Ferula* only. However, these values were consistently lower than those previously obtained when strain partitioning was performed on the basis of the host plant.

A matrix of genetic distances among all *Pleurotus* strains examined was generated by the PAUP* software on the basis of mean character differences. Evaluation of isozyme data produced infrahost distance values ranging from 0·109 (strains growing on *T. garganica*) to 0·365 (strains growing on *Ferula* spp.) (Table 4). As expected, pairwise comparisons between strains associated with different hosts produced higher genetic distance values, with the exception of isolates from *Elaeoselinum* and *Thapsia*. In almost all cases, particularly high values were obtained among strains growing on *C. ferulacea* and the rest of the isolates growing on other hosts ($D >$ 0·514; only *Cachrys* vs *Thapsia* resulted in lower values, $D = 0{\cdot}398$). Specimens collected from *Eryngium* hosts were relatively closer to *Thapsia* ($D < 0{\cdot}491$), and the same was observed between *Ferula* and *Elaeoselinum* strains ($D = 0{\cdot}451$).

Cladograms resulting from the isozyme data and evaluated by the bootstrap and jacknife-resampling methods are presented as one in Fig. 1(a). In general, *Pleurotus* strains were clustered according to their associated hosts and formed five major groups. Robustness for clades corresponding to isolates growing on *Eryngium* and *Ferula* plants was low, and only strains IKP65 and IKP66 from NE Greece, and ATCC 36047 and CBS 10082 from the former Czechoslovakia showed relatively good bootstrap support ($> 40\%$). In contrast, higher robustness was observed within groups associated with the rest of the hosts: isolates UPA24, UPA4 and UPA29 from *Elaeoselinum*, and UPA32, UPA35 and UPA36 from *Thapsia*, were supported by values exceeding 54%. Furthermore, three clusters included more than four strains of the same geographical origin: UPA1 and UPA17–20 (*Ferula*, Sicily), UPA26, UPA32, UPA35 and UPA36 (*Thapsia*, Madonie Mt.), as well as the entire *Cachrys* group from Sicily. High values were also obtained for strains AMRU244 and AMRU245 isolated from *Ferula* sp. in Western Crete, which clustered outside the respective host group. Of interest was the poor statistical support among host-based clusters; thus the relative position of the groups of strains associated with *Eryngium*, *Ferula*, *Elaeoselinum* and *Thapsia* was not particularly solid. In contrast, higher robustness was apparent in the comparisons between the *Cachrys* cluster with the rest of the groups.

RAPD analysis

Five primers were selected to survey the genetic diversity within a collection of 42 *P. eryngii* species-complex isolates. A minimum of 6 (OPB10) and a maximum of 14 (OPB01) unambiguously amplified bands were generated, furnishing a total of 48 bands ranging in size from 150 to 1900 bp. Forty-five of these 48 bands (93·75%) were polymorphic. Only two bands with sizes of 750 and 1050 bp produced by primer OPB1, and one band with a size of 950 bp produced by OPB10 were monomorphic. All banding patterns were unique for each strain studied; in addition, a number of bands could be used as molecular markers for the identification of host-specific *Pleurotus* strains. Thus, all *Pleurotus* strains growing in association with *E. asclepium* were distinguished by the presence of one DNA fragment with an approximate size of 1600 bp produced by OPB1. Accordingly, all specimens isolated from *C. ferulacea* produced three distinct bands of 150 bp (OPB2), 500 bp (OPB10) and 1200 bp (OPB14).

Evaluation of RAPD-PCR data produced infrahost genetic distance values ranging from 0·178 (strains growing on *T. garganica*) to 0·262 (strains growing on *Eryngium* spp.) (Table 4). In contrast, significantly higher values were obtained by interhost pairwise comparisons. As in the case of the isozyme analysis, *Pleurotus* strains growing in association with *C. ferulacea* were the most distant within the *P. eryngii* species-complex. Specimens isolated from *Eryngium* hosts were relatively closer to *Ferula* and *Thapsia*; in general, *T. garganica* revealed consistently lower distance values with all other hosts ($0{\cdot}309 < D < 0{\cdot}387$). Noteworthy also was the relative affinity between strains from *Ferula* and *Elaeoselinum* hosts.

The cladograms produced by the 42 RAPD phenotypes were evaluated by the bootstrap and jacknife-resampling methods, and are presented as one (Fig. 1b). All *Pleurotus* strains were arranged in five major clusters in accordance with their associated host-plant. As previously, isolates collected from *Eryngium* spp. were

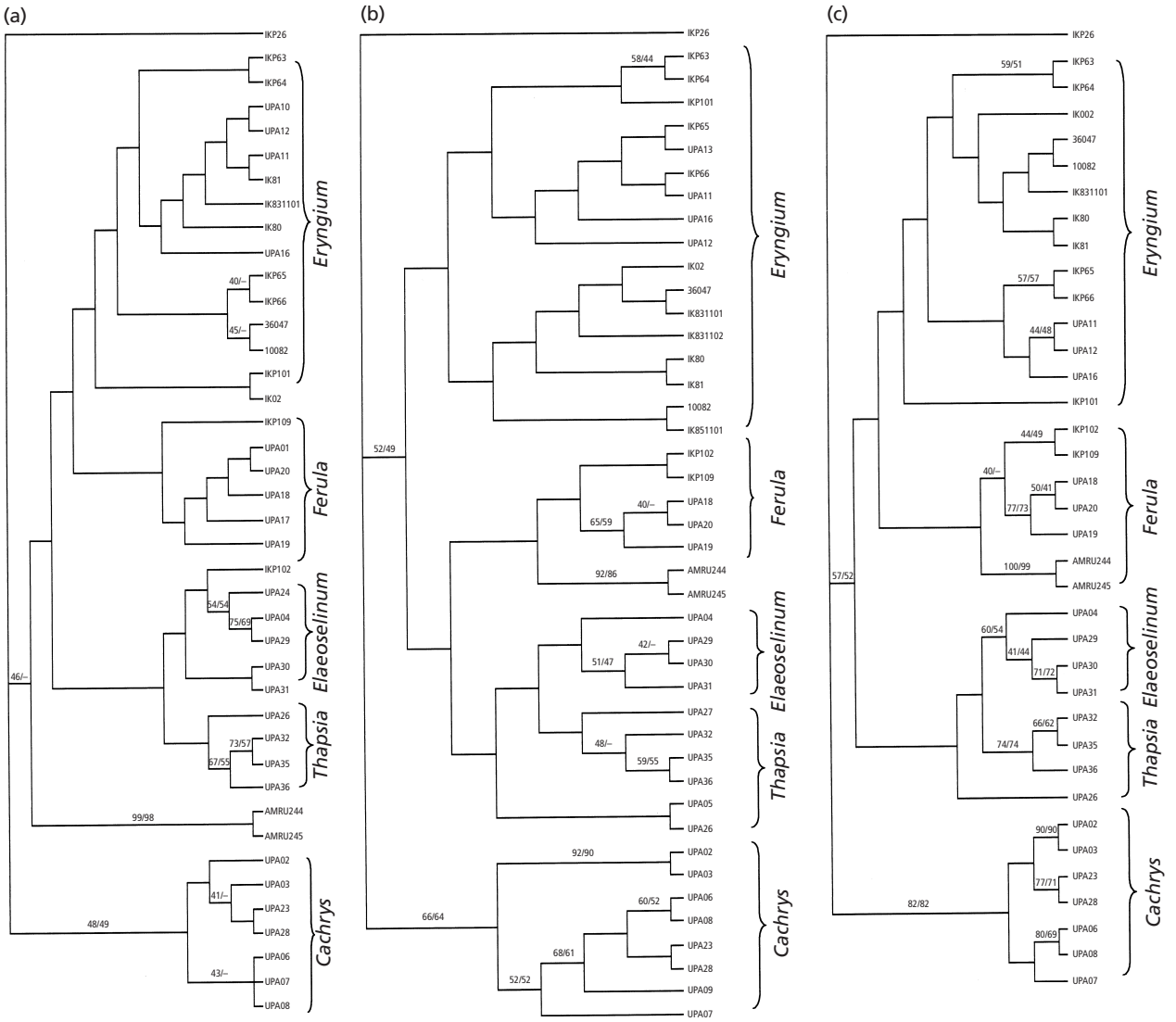

Fig. 1. Cladograms based on the results of (a) the isozyme analysis of 40 dikaryons, (b) the RAPD-PCR analysis of 42 dikaryons and (c) the combination of the isozyme and RAPD-PCR datasets depicting 36 dikaryons, assigned in the *P. eryngii* species-complex originating from five distinct plant-hosts: *Eryngium* spp., *Ferula communis*, *Elaeoselinum asclepium* subsp. *asclepium*, *Thapsia garganica* and *Cachrys ferulacea*. The numbers at branch points represent bootstrap and jacknife values (they are shown only when they exceed 40 %). The numbers at the ends of the branches correspond to *Pleurotus* strain numbers (Table 1). One strain of *P. pulmonarius* (IKP26) was used for rooting of the *P. eryngii* species-complex clade.

linked together with low support, with the exception of IKP63 and IKP64 from eastern Crete (> 44 %). In contrast, clades within the other clusters were of high robustness: *Ferula* (between the isolates of Italian origin), and between all Sicilian specimens collected from *Elaeoselinum*, *Thapsia* and *Cachrys*. Especially in the case of *C. ferulacea*, high statistical support was observed both within the group and externally with the larger cluster formed by all other groups. As was noted in the case of the cladograms produced by the isozyme analysis, individual clusters belonging in *P. eryngii sensu stricto* (i.e. including all host groups except the *C. ferulacea* one) showed poor statistical support with one another (< 10 %).

Combination of isozyme and RAPD-PCR data

When the two isozyme and the RAPD-PCR datasets were combined into one, clusters of the resulting tree showed higher statistical support than those of the individual datasets (Fig. 1c). Grouping of populations was again in accordance with the associated plant-hosts. All populations, with the exception of the isolates from *Eryngium* spp., demonstrated high intragroup bootstrap and jacknife values. Furthermore, the majority of the strains within the *Eryngium* and *Ferula* clusters were positioned with respect to their geographical origin. The statistical support within isolates growing on *C. ferulacea* was high, whereas the coherence within *Ferula*,

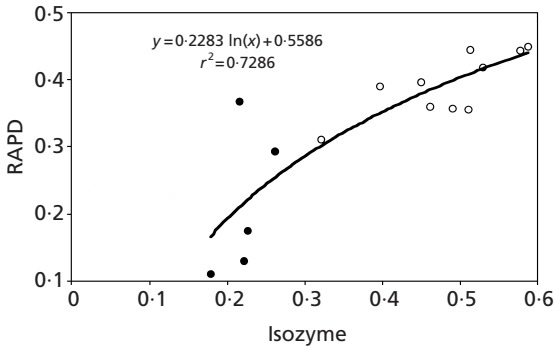

Fig. 2. Correlation between the genetic distances obtained from isozyme and RAPD-PCR datasets. Filled and open circles indicate within-host and between-host distances respectively.

Elaeoselinum and *Thapsia* related strains was satisfactory. Of interest was again the fact that the relative positioning of the host-associated groups within *P. eryngii* showed weak support, which is indicative of their close affinity.

In an attempt to evaluate the correlation between the genetic distances obtained from isozyme and RAPD-PCR datasets, a logarithmic curve was produced demonstrating high correlation ($r^2 = 0{\cdot}73$) between the values which derived from the application of the two approaches (Fig. 2). This graph indicates the lower distances obtained within populations, and illustrates a relatively faster saturation of RAPD data at high genetic distances.

Morphological and ecological features

All specimens used for the purposes of this work were studied as regards their morphological characters and ecological preferences. The main discriminating features of strains originating from different hosts are presented in Table 5.

DISCUSSION

Evaluation of the biochemical and molecular data

This work forms part of a research project on the systematics and phylogeny of Mediterranean *Pleurotus* taxa. Forty-six dikaryotic *Pleurotus* strains growing on umbellifers were selected to reflect the geographical and host range of the populations under examination.

Levels of isozyme variation found within the *P. eryngii* species-complex are high whether measured in terms of percentage of polymorphic loci, alleles per locus, or genetic diversity. They are indicative of high genetic differentiation among populations, with the largest proportion of diversity resulting from differences among individuals from different hosts rather than from different locations. Since most allelic variation at isozyme loci is unlikely to be subject to strong selection (Nei & Graur, 1984), the levels of isozyme variability within populations are primarily determined by effective population size (Crow & Kimura, 1970). In the Mediterranean region, *Pleurotus* populations are unlikely to have passed through severe bottlenecks over the past. Thus, the history of populations, the reproductive

Table 5. Summary of the most distinctive discriminating morphological characters and ecological features of various *Pleurotus* taxa associated with umbellifers

	Eryngium spp.	*Ferula communis* subsp. *communis*	*Cachrys ferulacea*	*Elaeoselinum asclepium* subsp. *asclepium*	*Thapsia garganica*
Pileus size (cm)	5–15	6–25 (30)	4–20 (25)	4–14	2–10
Pileus colour	Brown-red brown, warm brown, light beige to beige brown	Grey-brown to slate grey to beige brown	White-cream to white-ochraceous, alutaceous tones	Whitish to white-cream to light beige, alutaceous tones	Dark brown to warm brown
Pileus surface	Squamules scattered	Innatae fibrillae	Smooth, broken (when mature)	Smooth	Squamules scattered
Cuticle	Velvety, pruinose, with pigments and terminal club-like cells	Thick, velvety, pruinose, heavily pigmented, with terminal club-like cells	Thin, no pigments, velvety, opaque, lacerated in small crackings	Thick, no pigments, velvety, opaque, lacerated in small appressed areolae	Thin, with pigments, velvety, pruinose
Lamellae	Decurrent, cream to light beige, anastomoses at stipe	Decurrent, cream to light beige anastomoses at stipe	Deeply decurrent, whitish to pale yellow, reticulum at stipe	Deeply decurrent, whitish to pale yellow	Deeply decurrent, greyish white anastomoses at stipe
Stipe position	Central to eccentric	Central to eccentric	Central to eccentric, radicating	Central to eccentric, radicating	Central
Stipe size (cm)	2–4 × 1–3	3–10 × 1–4	4–9 × 2–4	4–8 × 1–3	3–5 × 1–3
Spores (μm)	9·1–13·5 × 4·8–6·7	9·6–13·8 × 4·7–6·9	13·2–17·4 × 5·5–8·2	10·1–14·0 × 5·2–7·1	9·8–13·6 × 4·6–7·3
Basidia (μm)	34–51 × 7–9	32–48 × 7–10	33–53 × 7–11	30–50 × 8–12	32–54 × 6–9
Cheilocystidia (μm)	Rare	As reduced hymenial elements	7–11 × 38–62	8–12 × 40–65	Rare
Habit	Early autumn to winter, singly or in clusters	Autumn to spring, singly or in clusters	April–June, singly	March–May, October–November, mostly in clusters	April–May, mostly singly
Habitat	Arid pastures, on limestone and silicaceous soils	Garigues, wastelands and pastures, on limestone and silicaceous soils	Pastures, on limestone and volcanic soils	Pastures, on limestone	Pastures, on limestone, mixed with *Eryngium*
Altitude (m)	0–1500	0–1300	1200–2000	0–1200	1000–1200
Growth rate	Medium	Medium	Slow	Slow	Medium
Virulence*	Strong	Strong	Weak	ND	ND
Chromosome no.†	12 or 14	12	13	ND	ND

*Data from Hilber (1982) (ND, no data).

†Data from Slézec (1984) (ND, no data).

system of *P. eryngii*, allochrony, and co-evolution phenomena with the host plant favour high intraspecific heterogeneity. Similar isozyme diversity has been reported in populations of *Puccinia graminis* (Burdon & Roelfs, 1983), *Morchella esculenta* (Yoon *et al*., 1990), *Crumenulopsis sororia* (Ennos & Swales, 1991), *Agaricus bitorquis* (Roux & Labarère, 1990) and *Pleurotus* spp. (Zervakis *et al*., 1994).

While isozyme analysis provides an indication of variation in the products of certain genes, RAPD-PCR is a means of assessing polymorphisms at a wide range of loci (Williams *et al*., 1990). In this study, the resolution of the RAPD-PCR analysis was better than that of isozyme analysis. Every examined strain of the *P. eryngii* species-complex showed a unique genotype. Previous studies have shown comparable high levels of intraspecific genetic diversities for the tree endophytes *Rhabdocline parkeri* (McCutcheon *et al*., 1993) and *Gnomonia setacea* (Lappalainen & Yli-Mattila, 1999), and for plant pathogens like *Claviceps purpurea* (Jungehülsing & Tudzynski, 1997) and *Stagonospora nodorum* (McDonald *et al*., 1994). Such phenomena were mainly attributed to the predominance of sexual reproduction, whereas in biotrophic fungi adaptation to different hosts causes accumulation of genetic differences within the same species due to isolation phenomena. For example, RAPD-PCR permitted the differentiation of pathogenic races of *Fusarium oxysporum* f. sp. *vasinfectum* (Assigbetse *et al*., 1994), *F. solani* f. sp. *cucurbitae* (Crowhurst *et al*., 1991), *Gremmeniella abietina* (Hamelin *et al*., 1993), and isolates of *A. alternata* f. sp. *citri* (Weir *et al*., 1998) based on host specialization, without any apparent correlation with geographical origin. Such types of cases might be broadened to include some facultative parasites like *Sphaeropsis sapinea* (Stanosz *et al*., 1999), which have a restricted group of long-lived hosts belonging in a single gymnosperm family, or the *Pleurotus* populations growing on umbellifers.

The use of parsimony analysis for RAPD-PCR data has been criticized in the past, especially above the species level (Adams & Demeke, 1993; Backeljau *et al*., 1995). This could be justified because none of the commonly used parsimony analyses provide an appropriate model for RAPD character state change, while a few non-homologous characters can more drastically affect the topology of trees produced than in phenetic analyses based on similarity (Adams & Demeke, 1993). On the other hand, trees obtained with UPGMA and other phenetic clustering methods do not always accurately represent the phylogeny of closely related organisms (Hillis *et al*., 1992), they offer no optimality criteria for choosing between different topologies, and they reduce the rich character-based matrix to abstract distance values offering no possibility of ancestral state reconstruction (Paavanen-Huhtala *et al*., 1999). Hence, phenetic clustering methods should be seen merely as a means of constructing an initial tree for more thorough analysis, not as a method for choosing the final tree (Swofford *et al*., 1996). Along this line of approach, we started our data analysis from NJ and UPGMA trees (data not shown), before proceeding with parsimony analysis. The phenetic trees of RAPD-PCR and isozyme data do not differ substantially from one another, and both are congruent with parsimonious trees (minor differences detected between them could be attributed to the smaller size of the isozyme data matrix). In general, the parsimonious trees deriving from isozyme or RAPD-PCR and from combined isozyme and RAPD-PCR data were very similar to each other, confirming the agreement of the approaches. In contrast, previous phylogenetic studies demonstrated that the use of additional molecular characters such as the mitochondrial small-subunit rRNA and/or of the nuclear rRNA ITS sequences were of particular value only above the species level (Gonzalez & Labarère, 2000; Wu *et al*., 2000; Vilgalys & Sun, 1994).

Systematics and speciation

The use of RAPD-PCR and isozyme analyses permitted grouping of the *P. eryngii* complex isolates into five main clusters in accordance with the separation of individual populations on host specialization; pairwise genetic distances within host isolates were lower than those between host populations. *Pleurotus* isolates growing on *C. ferulacea* formed a distinct group with relatively high statistical support. Therefore their separation from the rest of the populations examined and their classification within a distinct taxonomic entity at the species level, i.e. *P. nebrodensis* (Inzenga) Quél., seems well justified and confirms reports based on morphology (Venturella, 2000). In previous studies, *P. nebrodensis* showed intercompatibility values as low as 6–18 % in crosses with *P. eryngii* var. *eryngii* and var. *ferulae* (Cailleux *et al*., 1981; Hilber, 1982; Zervakis & Balis, 1996). In addition, Cailleux *et al*. (1981) demonstrated that among the successful inter-ecotype matings, a large percentage of hybrid-dikaryons showed disturbed morphogenesis and abnormal reproductive physiology. Moreover, the pleuroti from *C. ferulacea*, which appear in Sicily from late spring to early summer at altitudes exceeding 1200 m, are in the process of morphological differentiation, already showing distinct characters in pileus colour and texture, cuticle, spore size, cheilocystidia and chromosome number (Table 5).

All other strains were positioned within the larger *P. eryngii* group, which was further divided into four main clusters corresponding to *Eryngium*, *Ferula*, *Elaeoselinum* and *Thapsia* hosts. *Pleurotus* strains growing on *T. garganica* show a very narrow distribution range (Madonie Mt., Sicily) and hence their intrapopulation genetic distances and diversities are relatively low. They are characterized by their distinct pileus size and colour, cuticle, habit and habitat (Table 5). Although this population seems to constitute a new variety, additional specimens need to be examined before any definite conclusions are drawn. In contrast, *Pleurotus* isolates from *Eryngium* spp. and *F. communis* plants (originating from various geographical areas) are very

heterogeneous, showing high infrahost genetic distances and diversities. These populations could be discriminated on the basis of ecomorphological characters from the rest of the taxa examined (Table 5). However, the results from previous mating studies which provided intercompatibility percentages exceeding 40% (Hilber, 1982; Zervakis & Balis, 1996), in conjunction with the molecular evidence furnished by this work, support their currently accepted status: *P. eryngii* (DC.: Fr.) Quél. var. *eryngii*, and *P. eryngii* (DC: Fr.) Quél. var. *ferulae* Lanzi.

Pleurotus growing on *E. asclepium* subsp. *asclepium* seems to hold an intermediate position, demonstrating relatively high variability, which could be probably explained by the restricted gene exchange with the other *P. eryngii* taxa. In fact, isolates from these populations give higher percentages of positive results when mated with *P. eryngii* var. *eryngii* and var. *ferulae* (45–70% of successful matings; G. Zervakis & G. Venturella, unpublished results). Isozyme and RAPD data confirm the relative affinity of this group to *P. eryngii sensu stricto*, and especially to var. *ferulae*; co-evaluation of ecomorphological characters such as pileus size, colour, surface and cuticle as well as spores and cheilocystidia size support its taxonomic assignment as a new variety, *P. eryngii* var. *elaeoselini* Venturella *et al.* (Venturella *et al.*, 2000). As it is the case with all other *Pleurotus* growing on umbellifers, morphological differentiation seems to follow genetic isolation for this taxon as well; this is in accordance with previous reports on synnematoid *Pleurotus* taxa (Zervakis, 1998), or other Basidiomycetes (Kemp, 1975).

As regards taxa of ambiguous validity, *P. hadamardii* Constantin should be considered as a *nomen nudum*, since the original description by Constantin referred to a fungus isolated from *Eryngium alpinum*, but it proved to be erroneous (Heim, 1960). Another plant associated with '*P. hadamardii*' is *Laserpitium latifolium* (Joly *et al.*, 1990), which has also been regarded as principal host for *P. nebrodensis* (Heim, 1960; Hilber, 1982). However, *Pleurotus* strains growing on *Laserpitium* spp. in central Europe and Northern Italy show identical microscopical characteristics to *P. eryngii* var. *elaeoselini* (G. Venturella & G. Zervakis, unpublished data), and differ substantially from the original descriptions of *P. nebrodensis* based on biological material collected from Sicily (Inzenga, 1863). Therefore, *P. nebrodensis* should only include strains isolated from *C. ferulacea*, at least until isolates from *Laserpitium* can be thoroughly investigated. In addition, the taxonomic position of *Pleurotus* isolates growing in association with *Diplotaenia cachrydifolia* at high elevations in Iran (Heim, 1960; Saber, 1990) remains dubious, as does that of strains collected from wood in Afghanistan and classified under the name *P. fossulatus* (Pegler, 1977). Both of them apparently belong to the *P. eryngii* complex and morphologically resemble the descriptions of *P. eryngii* var. *elaeoselini* and *P. nebrodensis*.

The level of genetic variation is generally considered adaptive and related to the breadth of geographical ranges and/or to the ecological heterogeneity within the ranges (Lewinsohn *et al.*, 2000; Nevo, 1988). Speciation and the development of species richness appear to be facilitated by restricted gene flow and isolation of small populations (Lande, 1984). Hence, the high diversity in many infraspecific taxa that are trophically highly specialized (e.g. the *P. eryngii* species-complex) suggests that ecologically specialized populations are particularly prone to speciation (Futuyma, 1986a). However, if those populations are brought into contact, much of the divergence they have accomplished will be lost by interbreeding. On the other hand, if they have become different species they can retain their diverse adaptations, and refine them even while sympatric (Futuyma, 1986b). In many cases sympatric populations are in an intermediate stage of speciation (i.e. partially reproductively isolated), and they usually interbreed along a hybrid zone that can persist for long periods (Futuyma, 1986a). This explains the mating behaviour between ecotypes of *P. eryngii*, where ecological or seasonal barriers can readily break down, unless some type of isolation factor(s) reduces gene flow to a very low level.

We believe therefore that the *P. eryngii* species-complex includes many host-associated taxa, which maintain distinct gene pools through an efficient mechanism of premating barriers. These populations have recently diverged through a sympatric speciation process based on both intrinsic reproductive barriers (i.e. partial compatibility in inter-ecotypes matings) and extrinsic factors (host specialization). In addition, ecogeographical parameters such as allochrony in the appearance of basidiomata (and hence discharge of basidiospores), elevation and host plant distribution hinder, to a certain extent, gene exchange among *P. eryngii* ecotypes. This hypothesis is clearly supported in the present study by the distinct clustering of the *P. eryngii* taxa when subjected to isozyme and RAPD-PCR analysis. Similar phenomena of sympatric speciation based on habitat specialization within a common area, resembling the 'microevolutionary units' (Duncan, 1972), were noted in the past for other homobasidiomycetes such as *Hirschioporus abietinus* (Macrae, 1967), *Heterobasidion annosum* (Worrall *et al.*, 1983), *Paxillus involutus* (Fries, 1985) and *Peniophora cinerea* (Chamuris, 1991). In these cases, population divergence was promoted by locally strong ecological discontinuities (e.g. habitat or temporal isolation), and was accompanied by partial or total reproductive isolation.

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