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ORIGINAL ARTICLE

Genetic variability and molecular phylogeny of *Pleurotus eryngii* species-complex isolates from Iran, and notes on the systematics of Asiatic populations

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Abstract The *Pleurotus eryngii* species-complex includes taxa of the northern hemisphere growing in association with plants of the family *Apiaceae* (umbellifers). In this study, 45 *Pleurotus* strains were isolated from five different host-plants: *Ferula ovina*, *F. assa-foetida*, *Smyrniopsis aucheri*, *Kellusia odoratissima*, and *Cachrys ferulacea*; all plant species, with the exception of *C. ferulacea*, are reported for the first time as hosts for this fungal group. Random amplified polymorphic DNA-PCR (RAPD) analysis and nucleotide sequence data from the internal transcribed spacer of the nuclear rRNA genes (ITS1, 5.8S and ITS2) were used for assessing genetic diversity and for determining phylogenetic relationships among the populations studied. Results permitted the grouping of the strains studied into three major clusters corresponding mainly to the nature of the host-plant: the first included isolates collected from *Ferula* spp. only, the second included isolates originating from *C. ferulacea* only but from various sampling locations, and the third included all *K. odoratissima* and *S. aucheri* associated strains plus a few isolates collected from *F. ovina* and *C. ferulacea*. The grouping of the Iranian material, in conjunction with the position in the resulting phylograms of other previously obtained *P. eryngii* complex sequences, revealed that the first cluster is related to the asiatic '*P. nebrodensis*' (or to the asiatic *Ferula* spp. associated Pleuroti), the second forms a rather distinct lineage which is linked with reference strains originally classified as *P. fossulatus*, whereas the third cluster falls within the main part (or the "core") of this complex, i.e., *P. eryngii*. *Pleurotus* populations growing on umbellifers in Iran seem either to have recently diverged through a sympatric speciation process based mainly on ecological factors (e.g., *P. fossulatus*), or they form part of a rather wide agglomerate associated with various host-plants where exchange of genetic material is still in progress (i.e., *P. eryngii*).

Keywords *Pleurotus nebrodensis* · *Pleurotus fossulatus* · *Apiaceae* · Host specificity · Edible mushroom · Speciation

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Introduction

Iran is a country with vast mountainous regions, varied climatic and edaphic features, and a particularly rich plant

biodiversity. Especially as regards the family *Apiaceae* (umbellifers), Iran constitutes a major center of diversification where a considerable level of taxa diversity and a high number of endemic species occur; notably, a total of 363 species and 114 genera of *Apiaceae* are recorded in this country, of which 114 species and 12 genera are endemic (Ajani et al. 2008; Pimenov and Leonov 2004). Among them, species of the genera *Cachrys*, *Ferula*, *Smyrniopsis* and *Kelussia* attract significant interest from both local scientists and village people towards their exploitation for various purposes, i.e., human food (e.g., *K. odoratissima*, commonly known as "mountain celery"), folk medicine applications, animal feed, production of essential oils for pharmaceutical and nutritive applications, etc. (Amiri 2007; Faridi et al. 2008; Ghorbani 2005; Khajeh et al. 2005). Of relevant importance is the consumption of *Pleurotus* mushrooms, which grow on umbellifers in Iran, either fresh as food readily available in local markets or in the form of dried powder used in traditional medicine.

The *Pleurotus eryngii* species-complex constitutes the only group of the genus *Pleurotus* whose members are associated with plants of the family *Apiaceae* by developing a facultatively biotrophic mode of growth (Hilber 1982; Joly et al. 1990; Zervakis and Balis 1996). Basidiomata of host-specific populations appear on the roots and stems of umbellifers (e.g., genera *Eryngium*, *Ferula*, *Ferulago*, *Cachrys*, *Laserpitium*, *Diplotaenia*, *Elaeoselinum, Thapsia*, etc.) singly or in groups from autumn until early summer. The distribution range of this species-complex extends through a rather well-defined zone in the Old World ranging from Morocco to the Netherlands in the west and extends to China eastwards, comprising regions of the Middle East and western Asia (e.g., Turkey, Israel, Armenia, Uzbekistan, Afghanistan) (Hilber 1982; Zervakis et al. 2001).

As regards Iran in particular, the existence of *P. eryngii* was first reported by Petrak (1939) [as *P. fuscus* (Batt.) Bres.] and Esfandiari (1948). Later, Heim (1960) recorded the presence of what he called '*Pleurotus nebrodensis*' growing on root and stem residues of *Diplotaenia cachrydifolia* in the Fars mountains (Shiraz area, south Iran). Further information about the occurrence of *P. eryngii* species-complex in the Esfahan, Fars, Boyer-ahmad and Kohkiloyeh, and Tehran provinces was provided by Saber (1990, 1997), who reported that such fungal populations are abundant and widespread in central, southern and western regions. Recently, Abdollahzadeh et al. (2007) studied *P. eryngii* species-complex isolates from the Kurdistan region of northern Iran (at altitudes higher than 2,000 m asl in the areas of Sanandaj, Hane Gelan and Saral) and in association with the host plants *Prangos* sp., *Pimpinella* sp. and *Ferula haussknechtii*.

However, identification of *Pleurotus* isolates and establishment of taxonomic/phylogenetic relationships (together with assessment of intraspecific genetic variability/polymorphism) within this species-complex and among its various host-specific populations ('ecotypes') remain problematic since most morphological characteristics of basidiomata are influenced by environmental conditions, while initial misidentifications of the host-plant and/or of the associated fungal specimens have led to erroneous taxonomic conclusions (Hilber 1982; Zervakis and Balis 1996). Such problems were further aggravated by the introduction of new taxa (without the necessary support from robust taxonomic data originating from the use of additional criteria, e.g., ecomorphological characters, compatibility experiments, molecular analysis, etc.), and the complex processes governing speciation in this group (Zervakis et al. 2001).

The application of integrated approaches, usually employing one or more molecular techniques, have contributed to the elucidation of such ambiguous issues in *Basidiomycota*. Hence, for example, the use of random amplified polymorphic DNA (RAPD) permitted the assessment of population dynamics of *Tricholoma* spp. (Gryta et al. 2006), of the genetic variation in *Hemileia vastatrix* (Gouveia et al. 2005), and of the relationships among *Lepista* spp. (Stott et al. 2005). On the other hand, ribosomal DNA internal transcribed spacers (rDNA ITS-5.8S) sequencing provided valuable data for supporting species delimitations in the genera *Amanita*, *Lactarius*, *Cystoderma* and *Cystodermella* (Moreno et al. 2008; Nuytinck and Verbeken 2007; Saar et al. 2009), for assessing phylogeny within *Cortinarius* and *Polyporus* (Frøslev et al. 2007; Krüger et al. 2006), and for establishing long distance dispersal phenomena in *Ganoderma* and biogeography of *Flammulina* spp. (Ge et al. 2008; Moncalvo and Buchanan 2008).

This work aimed at determining the genetic polymorphism, the taxonomic identity and the phylogenetic relationships of *Pleurotus* isolates growing on several *Apiaceae* host-plants in poorly investigated areas of Iran by evaluating for the first time their host-plant associations and biogeographic distribution in conjunction with the data provided through the use of RAPD and ITS sequencing.

Materials and methods

Biological material

Basidiomata of 45 *Pleurotus* strains were collected directly from the roots and stems of their associated *Apiaceae* host-plants in 12 different locations of western Iran from April to May 2006 (Table 1). Sampling locations, their geographic coordinates and altitudes above sea level appear in Fig. 1.

For the establishment of dikaryons in pure culture, small pieces from the context of the isolated basidiomata were removed aseptically and transferred onto Petri dishes

Table 1 Details of the 45 *Pleurotus* dikaryons studied: strain codes, geographic origins (Iran), *Apiaceae* host-plants, and GenBank accession numbers

Strain code	Geographic origin	Host plant	GenBank accession no.
C1	Pirmehran	*Ferula ovina*	FJ514549
C2	Pirmehran	*Ferula ovina*	FJ514550
C3	Pirmehran	*Ferula ovina*	FJ514551
C4	Pirmehran	*Ferula ovina*	FJ514552
C5	Pirmehran	*Ferula ovina*	FJ514553
C6	Felard	*Smyrniopsis aucheri*	FJ514554
C7	Felard	*Smyrniopsis aucheri*	FJ514555
C8	Felard	*Smyrniopsis aucheri*	FJ514556
C9	Felard	unknown	FJ514557
C10	Gharun	*Cachrys ferulacea*	FJ514558
C11	Gharun	*Cachrys ferulacea*	FJ514559
C12	Gharun	*Cachrys ferulacea*	FJ514560
C13	Gharun	*Cachrys ferulacea*	FJ514561
C14	Gharun	*Cachrys ferulacea*	–
C15	Dashtezarrin	*Cachrys ferulacea*	FJ514562
C16	Dashtezarrin	*Cachrys ferulacea*	FJ514562
C17	Dashtezarrin	*Cachrys ferulacea*	FJ514564
C18	Ahmadegharib	*Ferula ovina*	FJ514565
C19	Ahmadegharib	*Ferula ovina*	FJ514566
C20	Ahmadegharib	*Ferula ovina*	FJ514567
C21	Ahmadegharib	*Ferula ovina*	–
C22	Ahmadegharib	*Ferula ovina*	FJ514568
C23	Sepidan	*Cachrys ferulacea*	FJ514569
C24	Sepidan	*Cachrys ferulacea*	FJ514570
C25	Sepidan	*Cachrys ferulacea*	FJ514571
C26	Fereidan	*Cachrys ferulacea*	FJ514572
C27	Fereidan	*Cachrys ferulacea*	FJ514573
C28	Fereidan	*Cachrys ferulacea*	FJ514574
C29	Fereidan	*Cachrys ferulacea*	FJ514575
C30	Fereidan	*Cachrys ferulacea*	FJ514576
C31	Bazoft	*Kellusia odoratissima*	FJ514577
C32	Bazoft	*Kellusia odoratissima*	FJ514578
C33	Bazoft	*Kellusia odoratissima*	FJ514579
C34	Bazoft	*Kellusia odoratissima*	FJ514580
C35	Bazoft	*Kellusia odoratissima*	FJ514581
C36	Kohrig	*Cachrys ferulacea*	FJ514582
C37	Kohrig	*Cachrys ferulacea*	FJ514583
C38	Sardasht	*Cachrys ferulacea*	FJ514584
C39	Sardasht	*Cachrys ferulacea*	FJ514585
C40	Mangase	*Cachrys ferulacea*	FJ514586
C41	Aligudarz	*Smyrniopsis aucheri*	FJ514587
C42	Aligudarz	*Smyrniopsis aucheri*	FJ514588
C43	Aligudarz	*Smyrniopsis aucheri*	–
C44	Aligudarz	*Ferula assa-foetida*	FJ514589
C45	Aligudarz	*Ferula assa-foetida*	FJ514590

containing malt extract agar medium (MEA). After a few days of incubation at 27°C in the dark, hyphal tips were transferred to fresh medium. *Pleurotus* strains were maintained on MEA, and pure cultures were stored at −80°C in the fungal culture collection of the Department of Agronomy and Plant Breeding, Shahrekord University. All voucher specimens are deposited in the Herbarium of the Agricultural Research Centre, Shahrekord.

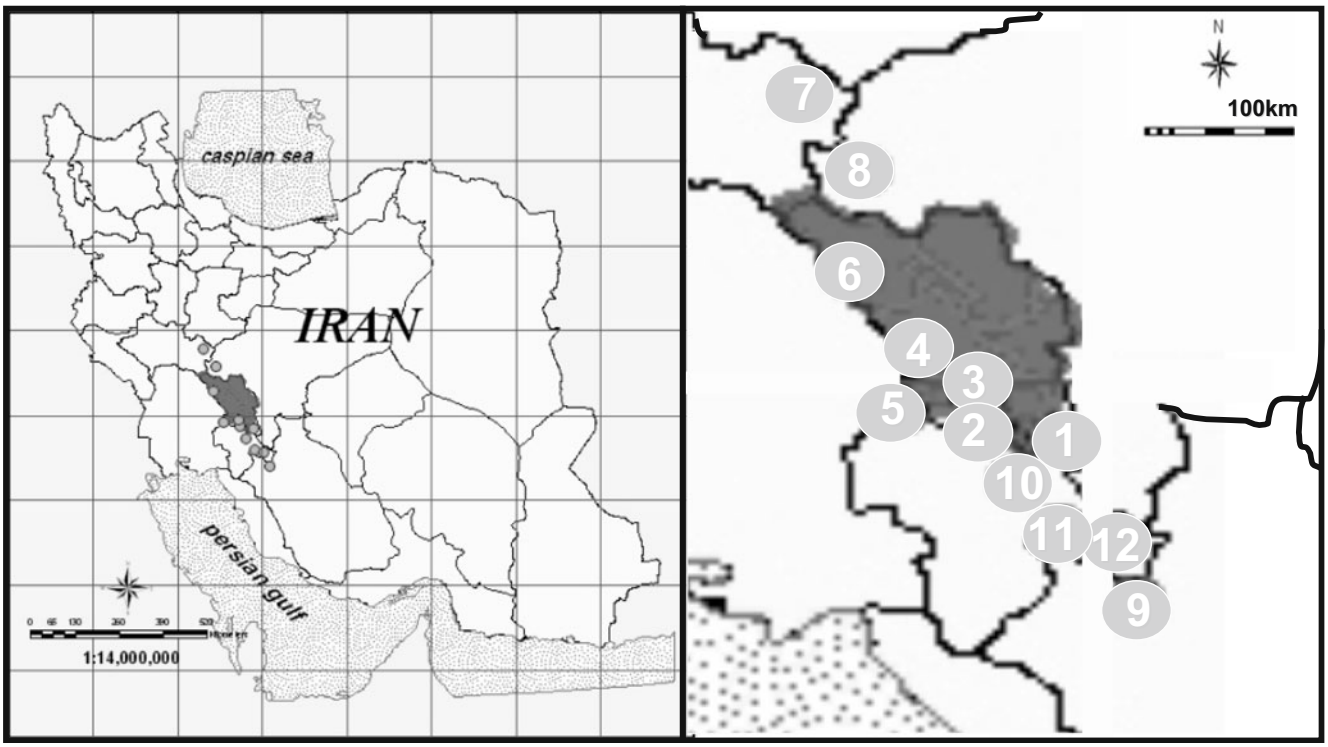

Fig. 1 Map of Iran with the main sampling region and individual sampling localities of the 45 *Pleurotus eryngii* complex strains marked in numbered circles: (1) Felard (host-plant: *S. aucheri*, longitude-latitude (°N–°E): 51.18–31.17, altitude (m): 2267); (2) Kohrig (*C. ferulacea*, 50.53–31.21, 2632); (3) Sardasht (*C. ferulacea*, 50.51–31.22, 2075); (4) Mangase (*C. ferulacea*, 50.49–31.31, 2297); (5) Gharun (*C. ferulacea*, 50.22–31.27, 3200); (6) Bazoft (*K. odoratissima*, 50.07–31.12, 2420); (7) Aligudarz (*S. aucheri* and *F. assa-foetida*, 49.41–32.24, 2034); (8) Fereidan (*C. ferulacea*, 50.06–32.56, 3585); (9) Sepidan (*C. ferulacea*, 51.47–30.17, 1620); (10) Dashtezarrin (*C. ferulacea*, 51.03–30.61, 2873); (11) Ahmadegharib (*F. ovina*, 51.19–30.43, 2860); (12) Pirmehran (*F. ovina*, 51.35–30.40, 1837)

DNA extraction and RAPD-PCR analysis

Each fungal isolate was inoculated in Petri dishes with MEA medium and the resulting culture was incubated for two weeks at 27°C. Then, small pieces of mycelium were transferred into 250 ml flasks containing 100 ml of sterile malt extract and incubated at 23°C for 20 days (the cultures were shaken twice per day). When the fungus colonized the medium, mycelium was harvested through the use of a filter paper placed on the top of a funnel and then washed with sterile distilled water (Zervakis et al. 1994).

Isolation of total genomic DNA followed the protocol of Rogers and Bendich (1988) as modified by Zervakis et al. (2001). Mycelium (1.5–2.5 g) was frozen with liquid nitrogen and quickly homogenized into fine powder. The powder was mixed with 500–700 µl warm (60°C) DNA extraction buffer (50 mM Tris pH 8.0, 50 mM EDTA, 3% w/v SDS and 0.1 mg/ml proteinase K) into 2 ml microfuge tubes and extracted with phenol/chloroform/isoamyl alcohol (25:24:1). The DNA was precipitated with 0.1 vol of 3 M sodium acetate and 0.6 vol of isopropanol, washed with 70% ethanol and resuspended in water. The purity and quality of genomic DNA was determined spectrophotometrically and confirmed by use of 1.2% agarose gel electrophoresis. DNA stock solution was stored at −20°C until needed.

Initially, a subset of the isolates was used to perform a preliminary screening of 55 decamer oligonucleotide primers to identify those that provided reproducible marker profiles and to exclude those producing a very low proportion of polymorphic bands. Ten primers (OPG kit, Operon Technologies Inc., USA) were selected for further analysis of the 45 DNA samples: OPG 05, OPG 07, OPG 08, OPG 10, OPG 19, OPG 44, OPG 47, OPG 52, OPG 58, and OPG 68 (Table 2).

RAPD analysis was carried out by using the method of Williams *et al*. (1990). Amplification reactions were performed in a final volume of 25 µl containing 75 ng genomic DNA; the reaction solution consisted of 200 µM each of dATP, dCTP, dGTP and dTTP, 15 ng oligonucleotide primer (Genset, France) and 1.2 units *Taq* DNA polymerase (Fermentas), 1.5 mM $MgCl_2$ and 1x PCR buffer. Amplification was performed in an Eppendorff Mastercycler gradient thermalcycler as follows: predenaturation stage at 94°C for 3 min followed by 45 cycles at 94 for 1 min, 37°C for 1 min, 72°C for 2 min, followed by an extension stage of 10 min at 72°C. Amplified fragments were resolved on a 1.2% agarose gel and stained

Table 2 Primer identity and sequence, size-range of the bands scored for each primer, and number of polymorphic/total bands observed from the RAPD-PCR analysis of 45 *P. eryngii* complex strains

Primer	Sequence	Size-range of bands scored (bp)	Number of polymorphic bands / no of bands scored
OPG05	5′-AggggTCTTg-3′	300–2700	15/15
OPG07	5′-gAAACgggTg-3′	250–2100	16/16
OPG08	5′-gTgACgTAgg-3′	600–1800	9/9
OPG10	5′-gTgATCgCAg-3′	420–2800	17/17
OPG19	5′-CAAACgTCgg-3′	380–2500	17/17
OPG44	5′-ggACTggAgT-3′	250–3100	21/21
OPG47	5′-ggTgACgCAg-3′	310–2900	12/12
OPG52	5′-CCTTgACgCA-3′	290–1350	15/15
OPG58	5′-CCACAgCAgT-3′	320–2100	13/14
OPG68	5′-gAgggCgTgA-3′	380–1950	13/14
Total			148/150

by ethidium bromide. A 100 bp ladder DNA marker (Fermentas) was used as a size standard.

PCR, cloning and sequencing of the rDNA-ITS region

The entire region of nuclear ribosomal DNA which comprises both internal transcribed spacers ITS1 and ITS2 and the 5.8S subunit was PCR amplified in each of the isolates with primers ITS1 and ITS4 (White et al. 1990). Primer ITS1 (TCC GTA GGT GAA CCT GCG C) binds to the 3′ end of the small subunit (ssu) of the 18 S rDNA gene and primer ITS4 (TCC TCC GCT TAT TGA TAT GC) binds to the 5′ end of the large subunit (lsu) of the 28 S rDNA gene. A reaction mixture (50 μl) containing 10x reaction buffer, 2 mM $MgCl_2$, 250 μM of each dNTP, 0.4 μM of each primer, 1.5 unit of *Taq* DNA polymerase (Fermentas International Inc., Canada) and 50 ng of purified total DNA was prepared. The amplification was performed using an Eppendorff Mastercycler gradient thermalcycler which was set to run at 94°C for 3 min for initial denaturation followed by 35 cycles of 94°C for 30 S, 50°C for 1 min, and 72°C for 1 min. A final extension of 7 min at 72°C was included. After confirming the amplification of single size fragment (~680 bp) as revealed by the presence of a single band on 1% agarose gel, the agarose block containing DNA was excised from the gel with a razor blade. Purified DNA was recovered from the agarose using the DNA extraction kit (#K0513, Fermentas).

To obtain a clean sequencing, the ITS region was cloned. The PCR products of each of one of the 45 isolates were ligated into the pTZ57R/T vector and cloned using the InsT/A cloning kit (#K1214, Fermentas) following the manufacturer's protocol. Recombinant plasmids were identified by colour selection after growth on Luria-Bertani plates containing X-Gal. The positive transformation of the bacterial clones was reconfirmed by using a small portion of a colony as the PCR template. Plasmid minipreps were performed using Genejet clean (Fermentas) following the manufacturer's protocol. The plasmid DNA was used for PCR amplification of the ITS insert with both forward and reverse sequencing primers M13/pUC. Sequencing was performed by the Geneservice Ltd (Cambridge, UK) through the use of an ABI automated sequencer. Resulting chromatograms were assembled and edited with the DNAstar software (DNASTAR Inc., Madison, USA). The sequences have been deposited in GenBank/NCBI (accession numbers: FJ514549 to FJ514590, Table 1). Alignments were performed by using the CLUSTAL W (http://www.ebi.ac.uk/clustalw/). Gap regions with ambiguous alignment were excluded from the analyses.

Data scoring, statistical and phylogenetic analyses

For RAPD analysis, each amplification run included a negative control reaction without the addition of DNA, and each reaction was performed at least twice. Some variations in RAPD patterns were detected in the duplicate experiments. However, only distinct, clearly resolved and reproducibly amplified fragments were selected for RAPD analysis, most of which ranged in size from 0.25 to 3.5 kb (PCR products sized below 250 bp or above 3.5 kb yielded faint and irreproducible bands). Comparisons of RAPD profiles were made only 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. In total, 150 distinct and scorable bands were produced by the 45 *Pleurotus* isolates examined.

The presence or absence of DNA fragments generated by RAPD-PCR was assessed, and scored as present (1) or absent (0). This information was used to compile a binary

data matrix. Genetic similarities were calculated among all possible pairs of strains using Jacard's similarity index, and principal coordinate analysis (PCoA) was performed with NTSYS-PC 2.02 software (Rohlf 1998); PCoA starts with a matrix of similarities between the strains examined and depicts the data in three dimensions in such a way that the distances between points (strains) are as close as possible to the original state (Singh et al. 2006). Partitioning of molecular variance within and among populations was calculated by AMOVA (Excoffier et al. 1992) through the use of the ARLEQUIN software (Schneider et al. 2001).

The RAPD data matrix was also used for the calculation of distances between strains on the basis of mean genetic differences, and for the assessment of their phylogenetic relationships through the use of PAUP* (version 4.0b10) software developed for Macintosh PowerPC (Swofford 2002). All 150 characters were unordered and of equal weight. Cluster analysis was performed by the unweighted pair group method using arithmetic averages (UPGMA) with mean character difference as the distance measure; branch robustness of the derived cladograms was evaluated by PAUP* using two different methods: (a) bootstrapping (Felsenstein 1978), and (b) jackknife-resampling (Farris et al. 1996) with 1000 replicates. In the jackknife analysis, 50% of the characters were deleted in each replicate.

The PAUP* software was also used for ITS1, ITS2 and 5.8S rDNA sequence analysis, and maximum-parsimony was selected as the optimality criterion. The characters were unordered and weighted equally. Heuristic searches used 100 replicates of random addition sequence with tree-bisection-reconnection (TBR) branch-swapping. Other options in PAUP* were set as follows: starting trees obtained via stepwise addition, one tree held at each step during stepwise addition, MULPARS option in effect, steepest descent option not in effect, MAXTREES setting unlimited and branches having maximum length zero allowed to collapse to yield polytomies. Branch robustness was evaluated again by bootstrap and jackknife methods which used 100 replicates of heuristic searches with the same settings as above, except that in each replicate MAXTREES was set to 541 (i.e., number of all most-parsimonious trees derived from the respective heuristic searches).

In addition to the Iranian material, eight sequences obtained from NCBI were included in the analysis as "reference material" (taxon assignment was maintained as appearing in the NCBI information form): *P. eryngii* (strain code: A6690, origin: Austria, NCBI accession no.: AY450347), *Pleurotus* sp. (PHZAU18, DQ077883), *P. eryngii* (ATCC36047, ex-Czechoslovakia, AY368657), *P. fossulatus* (ATCC62885, India, AY368664), *P. fossulatus* (CCRC36238, AY265833), *P. nebrodensis* (S498, China, AY540331), *P. nebrodensis* (strain no. 4, AY720935), and *P. nebrodensis* (ACCC50869, host-plant: *Ferula sinkiangensis*, China, AY311408).

Results

Analyses of RAPD data

Ten primers were selected to assess the genetic diversity within a collection of 45 *P. eryngii* species-complex isolates (Table 2). A minimum of 9 (OPG8) and a maximum of 21 (OPG44) unambiguously amplified bands were generated furnishing a total of 150 bands ranging in size from 0.25 to 3.50 kb; 148 of these bands were polymorphic (98.67%). Only one band (size: 1350 bp) produced by primer OPG58, and one band (size: 510 bp) produced by OPG68 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 *F. ovina* produced two common DNA bands, with approximate sizes 930 and 970 bp (primers OPG10 and OPG19 respectively); the latter was unique for this particular host, while the former was shared with strains growing on *S. aucheri* as well. Furthermore, all strains associated with *C. ferulacea* shared three DNA fragments with approximate sizes 1250, 980 and 850 bp (primers OPB7, OPG7 and OPG8 respectively); however, all of them appeared also in strains isolated from other host-plant species: the first in *S. aucheri*, the other two in *K. odoratissima*. All *Pleurotus* strains associated with *K. odoratissima* produced 16 common DNA bands in total, whereas isolates from *S. aucheri* shared 14 DNA fragments. In such cases, combinations of three or more DNA fragments could potentially discriminate among *Pleurotus* isolates originating from different host-plants.

Statistical (PAUP*) treatment of RAPD data produced a matrix of pairwise distances (not presented) between *Pleurotus* strains based on their mean character differences. It is interesting to note that the lowest distance values were obtained between strains isolated from the same host in the same location, i.e., the group of strains C10, C13 and C14 (distances: 0.013–0.040, *C. ferulacea*, Gharun), C44 and C45 (0.053, *F. assa-foetida*, Aligudarz), and C21 and C22 (0.100, *F. ovina*, Ahmadegharib). In contrast, the highest distances were observed mainly between strains isolated from different hosts and locations, e.g., C2 and C4 vs. C12 (0.500-0.520, *F. ovina* and *C. ferulacea*, Pirmehran and Gharun respectively), and C32 vs. C37 (0.507, *K. odoratissima* and *C. ferulacea*, Bazoft and Kohring respectively), or rarely from the same host but from distant geographic origins (e.g., C12 vs. C29, 0.513, *C. ferulacea*, Gharun and Fereidan).

RAPD data permitted the separation of *Pleurotus* strains into three large clusters: Clusters I, II and III (Fig. 3), which were supported by high bootstrap and jackknife resampling values (>96%/94%) in distance/UPGMA trees. The first (Cluster I) included eight strains (C1, C2, C18, C20, CP21, C22, C44 and C45) which were isolated from *Ferula* host plants only, i.e., six out of a total of ten from *F. ovina*, plus both strains originating from the same host-plant and locality (*F. assa-foetida* in Aligudarz). Positioning of the latter two (C44 and C45) was rather distinct and supported by high statistical value (100%), while strains C20, C21 and C22 associated with *F. ovina* (Ahmadegharib) were also closely related. Pairwise distances within this cluster ranged from 0.053 (C44 vs. C45) to 0.360 (C20 vs. C45). Cluster II included 14 strains isolated exclusively from *C. ferulacea* plants (C10–C15, C17, C24, C36–C40), with the only exception of strain C9 (unknown origin). In particular, strains C10 to C15 (all from *C. ferulacea* in Gharun), and the pairs of strains C36–C38 and C37–C39 (all from *C. ferulacea* and from adjoining geographic localities) demonstrated close affinity combined with relatively high bootstrap support. Pairwise distances within this cluster ranged from 0.013 (C13 vs. C14) to 0.307 (C9 vs. C36, and C12 vs. C24). The rest (23) of the strains investigated were positioned in Cluster III, i.e., all strains isolated from *K. odoratissima* and *S. aucheri*, plus a few strains from *F. ovina* and *C. ferulacea*. Within this cluster several strains were grouped closely together, i.e., C41, C42 and C43 (all from *S. aucheri* in Aligudarz), all but one isolates from *K. odoratissima* (in Bezoft), as well as strains C6 and C7 (from *S. aucheri* in Felard), and C28 and C30 (*C. ferulacea* in Fereidan). Pairwise distances within this cluster ranged from 0.127 (C41 vs. C42) to 0.393 (C27 vs. C5 and C25). "Inter-Cluster" comparisons of pairwise distances revealed considerably higher values, e.g., members of Clusters I vs. II (e.g., 0.520, C2 vs. C12), or II vs III (e.g., 0.513, C12 vs. C29, and 0.507, C32 vs. C37).

Results from application of PCoA separated strains under study into three major groups (Fig. 4) in accordance with the results of the clustering analysis presented above. The first group consisted of eight isolates (C1, C2, C18, C20, CP21, C22, C44 and C45) associated with *Ferula ovina and F. assa-foetida* plant-hosts, the second group was composed of 14 strains isolated from *Cachrys ferulacea* (C10–C15, C17, C24, C36–C40), with the only exception of strain C9 (unknown origin), and the remaining 23 isolates appeared in the third group. Eigenvalues and cumulative values of PCoA were obtained (data not presented); 24.28% of the total variation corresponded to the first principal component alone, while 80.84% of the total variation were obtained by the first 16 principal components.

Based on the average of 148 polymorphic loci, an analysis of molecular variance was performed taking into consideration either the ten geographic origins of the studied populations [Pirmehran, Felard, Gharun, Dashte-zarrin, Ahmadegharib, Sepidan, Fereidan, Bazoft, Lordegan (incl. Mangese, Sardasht and Kohrig) and Aligudarz] or the five associated host-plants. AMOVA revealed that variance within localities accounted for 71.33% of total variance, whereas variance between localities accounted to only 28.67% of the total; the respective values among host-plants was 20.19% and within host-plants 79.81% (Table 3). Genetic diversity within *P. eryngii* complex strains, expressed as AMOVA mean square deviations, was positively correlated ($r=0.95$; $P<0.001$) with percentages of polymorphic markers detected per strain. The Bartlett's test for population heteroscedasticity produced highly significant values indicating different levels of variability within different *P. eryngii* complex strains (Table 3).

ITS sequencing data analysis

Sizes of amplified products ranged from approximately 730 to 750 base pairs. Phylogenetic analyses were executed with 42 unambiguously aligned sequences of 709 base pairs (no sequences were obtained for strains C14, C21 and C43) plus the eight sequences obtained from the NCBI; 641 positions were constant, 48 variable characters were parsimony-uninformative and 20 variable characters were parsimony-informative.

Eleven clades that were supported by 50% or higher bootstrap values (Fig. 5) were produced, whereas eight of these clades were also retained by jackknife-resampling with a statistical support higher than 50%. Four clades had bootstrap support values higher than 83% (and >78% with jackknife). Bootstrapping generally gave slightly higher statistical support than jackknifing, but both statistics were strongly correlated ($R=0.96$; data not shown). *Pleurotus* strains were separated into three major clades (Clusters I, II and III, Fig. 5), or into three well-supported taxa that strongly (but not fully) correspond to the associated host-plants of the strains examined. The first (I) included six strains (C1, C2, C18, C22, C44 and C45) which were isolated from *Ferula* host plants only, i.e., four out of a total of nine from *F. ovina*, plus both strains originating from *F. assa-foetida*. Cluster II included 13 strains isolated exclusively from *C. ferulacea* plants, with the only exception of strain P9 of unknown origin. The rest (23) of the strains investigated were positioned in Cluster III, i.e., all strains isolated from *K. odoratissima* and *S. aucheri*, plus a few strains from *F. ovina* and *C. ferulacea* (as was the case in the RAPD grouping). All three clusters were supported by high bootstrap and jackknife resampling values (Fig. 5). For Cluster I in particular, C44 and C45 (from *F. assa-foetida* in

Table 3 Analysis of molecular variance based on the RAPD-PCR data resulting from the study of 45 *P. eryngii* complex strains from 10 localities and five host-plants

Source of variance	Sum of squares	Variance component (d.f)	Percentage (%) of total variance[a,b]	Bartlett's heteroscedasticity indices (Chi square)
Variance among localities	486.667	54.074 (9)	28.67	1.738 (d.l.=9)
Variance within locality*	675.333	19.295 (35)	70.35/71.33[c]	
Variance among host-plants*	286.461	71.615 (4)	12.41/20.19[c]	0.572 (d.l.=4)
Variance within host-plants	875.539	21.888 (40)	79.81	
Variance among location within host-plants*	200.205	40.041 (5)	17.23	
Total[d]	1162.000	130.951 (44)		

[a] PHI-statistics: PHIst=0.296, PHIsc=0.197, PHIct=0.124

[b] Significance of variance component expressed as the probability of obtaining a more extreme random value computed from nonparametric procedures: $p<0.001$

[c] The first value corresponds to the nested analysis, and the second value corresponds to the analysis within localities or among host-plants

[d] Corresponds to the sum of the values of the components of the nested analysis, indicated with an asterisk (*)

Aligudarz) presented the closest affinity. Moreover, three previously studied strains (ACCC50869, Strain4 and S498), two of them originating from China and identified as *P. nebrodensis*, were grouped within this cluster (supported by bootstrap and jackknife values of 74% and 60% respectively). For Cluster II, identical sequences were obtained for strains C12, C13, C38 and C39 (all originating from *C. ferulacea*, and collected from the neighboring localities of Gharun and Shardasht). In addition, strains C15, C17 and C37, which were collected from the neighboring localities of Dashtezarrin and Kohrig, were grouped closely together. All Iranian strains of this cluster were linked together and were then affiliated with strains ATCC62885 (from India) and CCRC36238 (of unknown origin); the latter two previously identified as *P. fossulatus* appeared closely related. Last, in Cluster III, identical sequences were shared by strains C16, C19, C20 and C33, which originated from different hosts (*C. ferulacea*, *K. odoratissima* and *F. ovina*) but from nearby localities (Dashtezarrin, Bazoft and Ahmadegharib); and by strains C8, C26, C31 and C42, which were again isolated from various hosts (*S. aucheri*, *C. ferulacea* and *K. odoratissima*) and different origins. Eleven of the 23 Iranian strains of this cluster formed a subclade, while the following strains with sequences obtained from previous studies were also grouped within Cluster III: ATCC36047 (*P. eryngii* from former Czechoslovakia), PHZAU18 (deposited as *Pleurotus* sp.) and 6690 (*P. eryngii* from Austria). The whole clade was supported by high bootstrap and jackknife values (97% and 93% respectively), and this was also the case for the closely associated (99%) sister groups corresponding to Clusters II and III.

Partial 18S rRNA gene, the entire ITS1-5.8S rDNA-ITS2 and partial 28S rRNA gene sequences varied in length from 637 bp in strains C29 and C45 to 641 bp in strain C22. The highest divergence measured within one major cluster (I, II or III) or terminal clade was 1.59% (10 bp) for Cluster I (strain C2 vs. ACCC50869); the respective values among strains within Clusters II and III were 0.94% (6 bp, C11 vs. C37) and 1.41% (9 bp, PHZAU18 vs. C4 and C32). When comparisons were performed for pairs of sequences of the entire *Pleurotus* population examined, the highest values were obtained between members of Cluster I vs. those of Cluster III, e.g., 3.34% (21 bp, ACCC50 vs. PHZAU18), 3.03% (19 bp, ACCC50 vs. C27) or 2.35% (15 bp, C2 vs. C28) for Iranian material only. On the other hand, the lowest percentages of base differences in 'inter-Cluster' comparisons were noted between strains of Cluster II vs. those of Cluster III (e.g., 1.57%, 10 bp, C23 vs. C37; 1.41%, 9 bp, ATCC62885 vs. C32 and vs. C4), whereas relatively high values were calculated for comparisons between members of Cluster I vs. those of Cluster II (e.g., 2.39%, 15 bp, ACCC50 vs. ATCC62885 and vs. C40; 1.88%, 12 bp, C40 vs. C2 and vs. C44).

Discussion

Iran is a country which, despite its particularly rich plant biodiversity, remains largely under-investigated as regards the wealth of its plant-associated microbiota. Especially *Pleurotus* taxa, which grow as facultative biotrophs of *Apiaceae* species, present particular interest not only because of their particular growth habits and ecological preferences but also because of their economic importance to local communities. In the frame of the present study, 45 dikaryotic *Pleurotus* strains growing on umbellifers were isolated directly from five different host-plants in 12 locations of western Iran, and they were examined through the use of molecular techniques for determining their taxonomic identity and phylogenetic relationships, and for assessing the existing genetic poly-

morphism. Such novel results combined with ecological preferences and distribution data of the Iranian material could actually form a type of "connecting link" between pertinent information already available from European and East Asian populations of this species-complex (Kawai et al. 2008; Urbanelli et al. 2007; Zervakis and Balis 1996; Zervakis et al. 2001; Zhang et al. 2006).

The geographic distribution for the host-plants of the Iranian *Pleurotus* strains studied coincides with what for European countries is the distribution zone of the *P. eryngii* complex associated with *C. ferulacea* (this host-plant is the only common one among those examined in this work and in other previous relevant studies on this fungal group). In addition, *K. odoratissima* is endemic in Iran (found only in the western part), while *S. aucheri*, *F. ovina* and *F. assa-foetida* occur in central and western Asia only (Pimenov and Leonov 2004); these last four plant species are reported here for the first time as hosts for fungi of the *P. eryngii* complex. It should be noted that all 12 localities investigated for the occurrence of *Pleurotus* specimens growing in association in umbellifers formed part of mountainous regions in western Iran exceeding altitudes of 1620 m (Fig. 1). At these particular sites average annual values of temperatures ranged from 7 to 16°C, rainfalls from 384 to 900 mm, and relative humidities from 41 to 47%.

RAPD-PCR provides the means of assessing polymorphisms at a wide range of loci (Williams et al. 1990). Every strain examined in this study (through the use of 10 selected primers) showed a unique genotype, even within the individual clusters formed from the cladistic analysis of pertinent data (Figs. 2 and 3). Molecular variation among *Pleurotus* isolates yielded up to 98.67% polymorphic bands, which represents a particularly high degree of polymorphism. Similar polymorphism levels (99.4%) were observed in a study on Israeli *P. eryngii* complex strains assessed through the use of 12 primers, which amplified 164 scorable RAPD loci (Lewinsohn et al. 2001). Furthermore, high intraspecific genetic diversity values were noted in the tree endophyte *Gnomonia setacea* (Lappalainen and Yli-Mattila 1999), in the ant-symbiotic basidiomycete *Leucoagaricus gongylophorus* (Doherty et al. 2003), in the grass pathogen *Claviceps purpurea* (Jungehülsing and Tudzynski 1997), etc. Such cases were mainly attributed to the predominance of sexual reproduction, to adaptation to stressful and temporally heterogeneous environments and/or to the horizontal transmission system of propagules (airborne spores). On the other hand, adaptation of biotrophic and symbiotic fungi to different hosts causes accumulation of genetic differences within the same species due to isolation phenomena.

Statistical analysis of the RAPD data revealed that variation within host-specific populations was high as compared to variation between populations of the *P. eryngii* complex, which confirms previous results of a study with

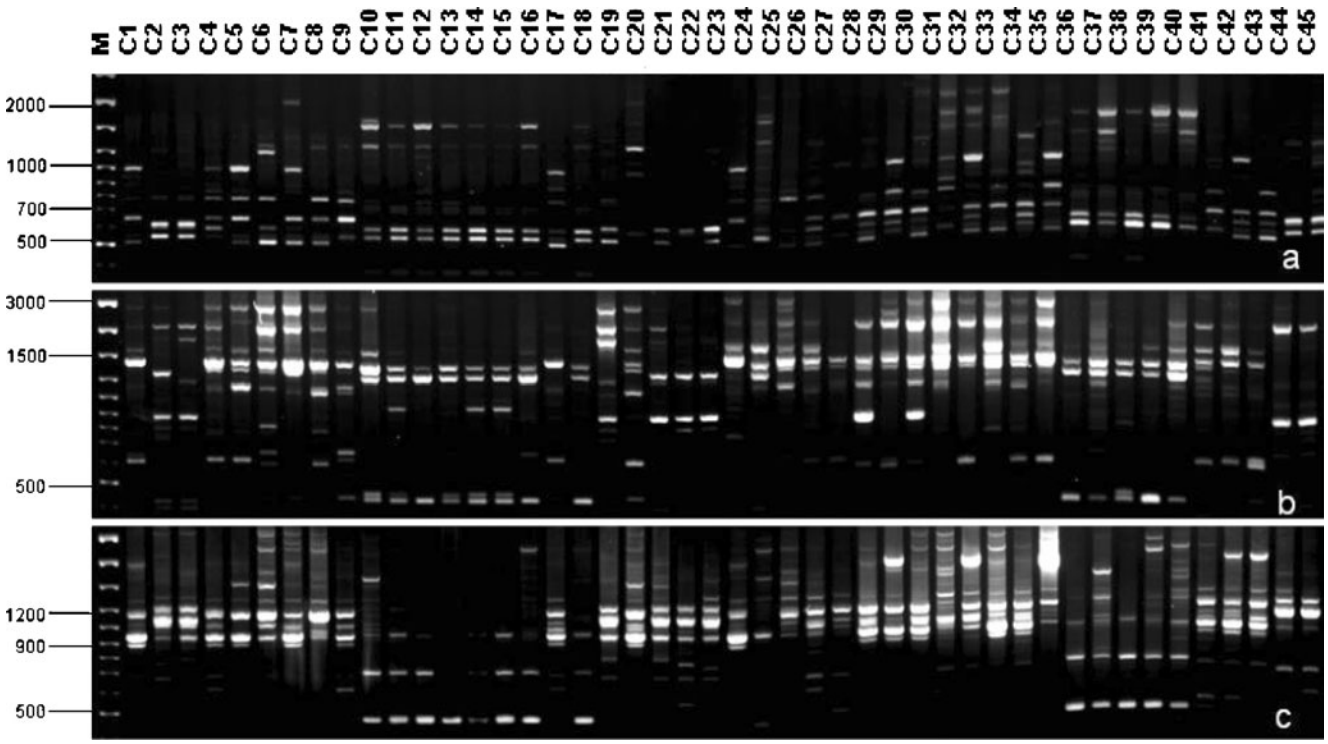

Fig. 2 Indicative RAPD-PCR patterns generated by primers (**a**) OPG68, (**b**) OPG44 and (**c**) OPG10 for the *Pleurotus* strains examined in this study. M, 100 bp DNA ladder used as a molecular weight marker (Fermentas)

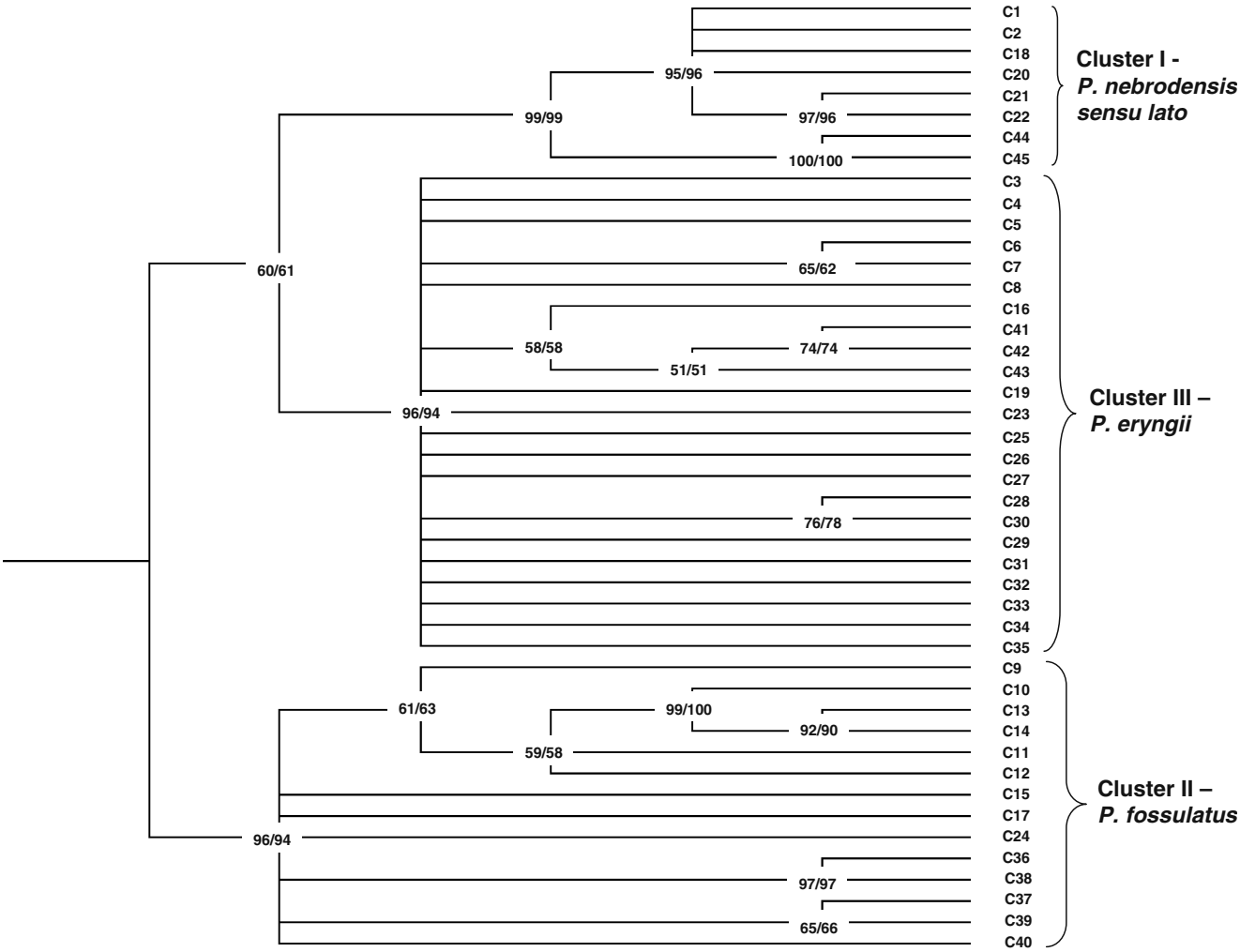

Fig. 3 Tree based on the RAPD-PCR data from the analysis of 45 *Pleurotus eryngii* complex strains produced with PAUP* by UPGMA and mean character difference as distance measure. The numbers at branch points represent bootstrap/jackknife support (values appear only when exceeding 50%)

Italian strains of this complex (De Gioia et al. 2005). The relatively high genetic diversity detected within groups is probably due to an efficient gene flow among intercompatible strains within each group. Such levels of genetic variability are usually observed in populations which are of wild origin, reproduce sexually, have broad ecological niches and/or a wide geographical distribution (Babbel and Selander 1974; Hamrick and Godt 1990; James et al. 1999); populations of the *P. eryngii* complex share several of the above characteristics. In addition, what is more important, the congruence of RAPD data was tested against other sources of information (i.e., host-plant preferences and ITS sequence data), and they were demonstrated to adequately reflect relationships within the *P. eryngii* complex.

On the other hand, the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat unit has proved to be a valuable tool for species delimitation and subgeneric phylogenetic inference in pertinent fungal studies (Kretzer et al. 1996; Taylor et al. 2000). The region is known to show certain variability even within species, and it might be also used to distinguish among strains of the same species (Chiu 2007; Healy et al. 2004). However, RAPD fingerprinting analysis seems superior in distinguishing closely related *Pleurotus* strains as was evidenced from this and previous studies (Ro et al. 2007).

ITS sequence divergence within host-associated isolates was lower than between host-associated populations. The highest intrataxon divergence measured was 1.59% (Cluster I) followed closely by 1.41% (Cluster III), which are similar to pertinent values noted for *Lactarius deterrimus* (1.35%; Nuytinck and Verbeken 2007) or *Collybia s.str.* (up to 1.22%; Hughes et al. 2001), higher than in *Amanita porphyria* (0.50%; Zhang et al. 2004) or in *Pleurotus pulmonarius* (0.58%; Vilgalys and Sun 1994), and significantly lower than in *Pleurotus cystidiosus* (up to 6.9%; Zervakis et al. 2004) or *Suillus granulatus* (6.04%; Kretzer et al. 1996). On the other hand, intertaxon sequence divergence

values ranged from 1.41% to 3.34%, which are comparable to those noted between the very closely related *Lactarius sanguifluus* vs. *L. vinosus* (1.42 %) and *L. deterrimus* vs. *L. fennoscandicus* (1.37 %), and to the average interspecific distance (3.46%) within *Lactarius* sect. *Deliciosi* (Nuytinck and Verbeken 2007).

The use of RAPD-PCR and ITS sequence analyses permitted grouping of Iranian *P. eryngii* complex isolates into three principal clusters-lineages mainly in accordance with the separation of individual populations on host specialization (Figs. 3, 4 and 5). Two of the three large groups (Clusters I and II) were composed of strains isolated from *Ferula* spp. and *C. ferulacea* respectively, from various sampling locations; in addition, *Pleurotus* strains associated with the former host-plant produced distinctly higher mycelium growth rates (data not presented), possibly as a result of adaptation on this particular substrate type. In the derived phylograms, three strains originally classified as *P. nebrodensis* (two of them originating from China, and one of them collected from *F. sinkiangensis*) were included into a distinct subgroup of Cluster I, whereas two sequences of strains previously identified as *P. fossulatus* (one originating from India) also formed a distinct terminal clade within Cluster II. Therefore, the separation of Cluster I and II members from the rest of the Iranian specimens examined, and their classification as two distinct taxonomic entities at the species level, i.e., *P. nebrodensis sensu lato* (Cluster I) and *P. fossulatus* (Cluster II), seems well justified.

Specimens from several geographic origins were grouped in Cluster III, including all *K. odoratissima* and *S. aucheri* associated strains plus the few remaining isolates collected from *F. ovina* and *C. ferulacea*. Three sequences obtained from previous studies were also grouped within Cluster III, two of them from *P. eryngii* var. *eryngii* from Europe (the third, PHZAU18, was deposited as *Pleurotus* sp.). Hence, Cluster III corresponds to *P. eryngii*, which is therefore expanded by engulfing *Pleurotus* isolates associated with additional previously unreported host-plants, such as *K. odoratissima*, *S. aucheri* and *F. ovina*. Especially as regards *C. ferulacea*, it has been until now exclusively linked with the appearance of *P. nebrodensis* in Europe and in western Asia (Hilber 1982; Joly et al. 1990; Zervakis et al. 2001). However, it seems that some of the *Pleurotus* mushrooms growing on the roots and stems of this plant in Iran share (at least partly) common gene pools with strains from other *Apiaceae* hosts as well. In the particular case of Cluster III, gene exchange is apparently under way among fungi associated with more than one host-plant (as our results demonstrated) irrespective of its nature and geographic origin. This hypothesis is confirmed by the findings of Abdollahzadeh et al. (2007), who demonstrated intercompatibility in the matings performed among *P. eryngii* strains isolated from three different host-plants (*Prangos* sp., *Pimpinella* sp. and *Ferula haussknechtii*) in northern Iran.

On the other hand, a more 'typical situation' for the *P. eryngii* complex, depicting strict host-specificity for distinct *Pleurotus* populations or taxa ('ecotypes'), is the case for strains of Clusters I and II (*P. nebrodensis s.l.* and *P. fossulatus*), which seem to be reproductively isolated from members of the other groups examined due to their association with *Ferula* spp. and *C. ferulacea* plants respectively. Furthermore, *P. nebrodensis* and *P. fossulatus* are phenotypically similar taxa (morphological differentiation seems to follow genetic isolation, as usually happens with *Pleurotus* populations growing on umbellifers; Zervakis et al. 2001), albeit representing different phylogenetic lineages. This morphological and habit similarity may be a consequence of recent speciation events. In fungi, biological species do not necessarily correspond with phylogenetic species, at least when divergence time is short (Taylor et al. 2000), while with higher divergence times distinct phylogenetic groups are intersterile regardless of geography (Moncalvo and Buchanan 2008). Relevant cases of populations with low percentages of base differences in the ITS regions, which apparently have not had enough time to diverge genetically and develop distinct phenotypic characteristics, were observed in other closely related mushroom species as well, e.g., *Collybia tuberosa*, *C. cookie* and *C. cirrhata* (Hughes et al. 2001), and *Lactarius* spp. of the sect. *Deliciosi* (Nuytinck and Verbeken 2007).

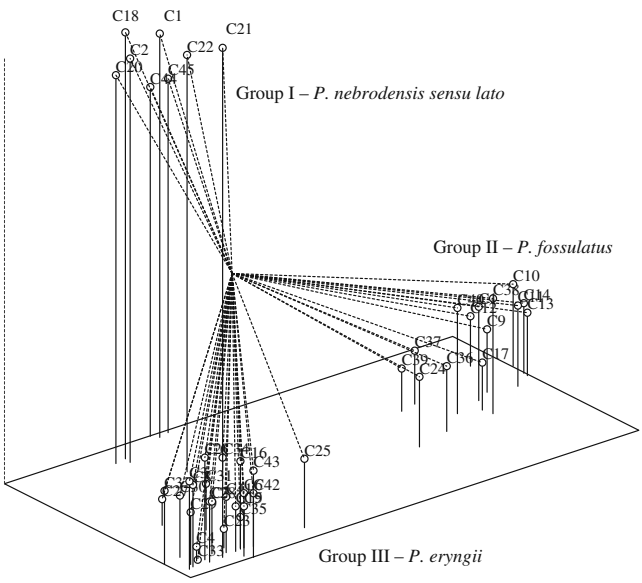

Fig. 4 Principal coordinates map for the first, second and third principal coordinates estimated for 150 RAPD loci using Jacard's similarity index for 45 *P. eryngii* complex strains from Iran

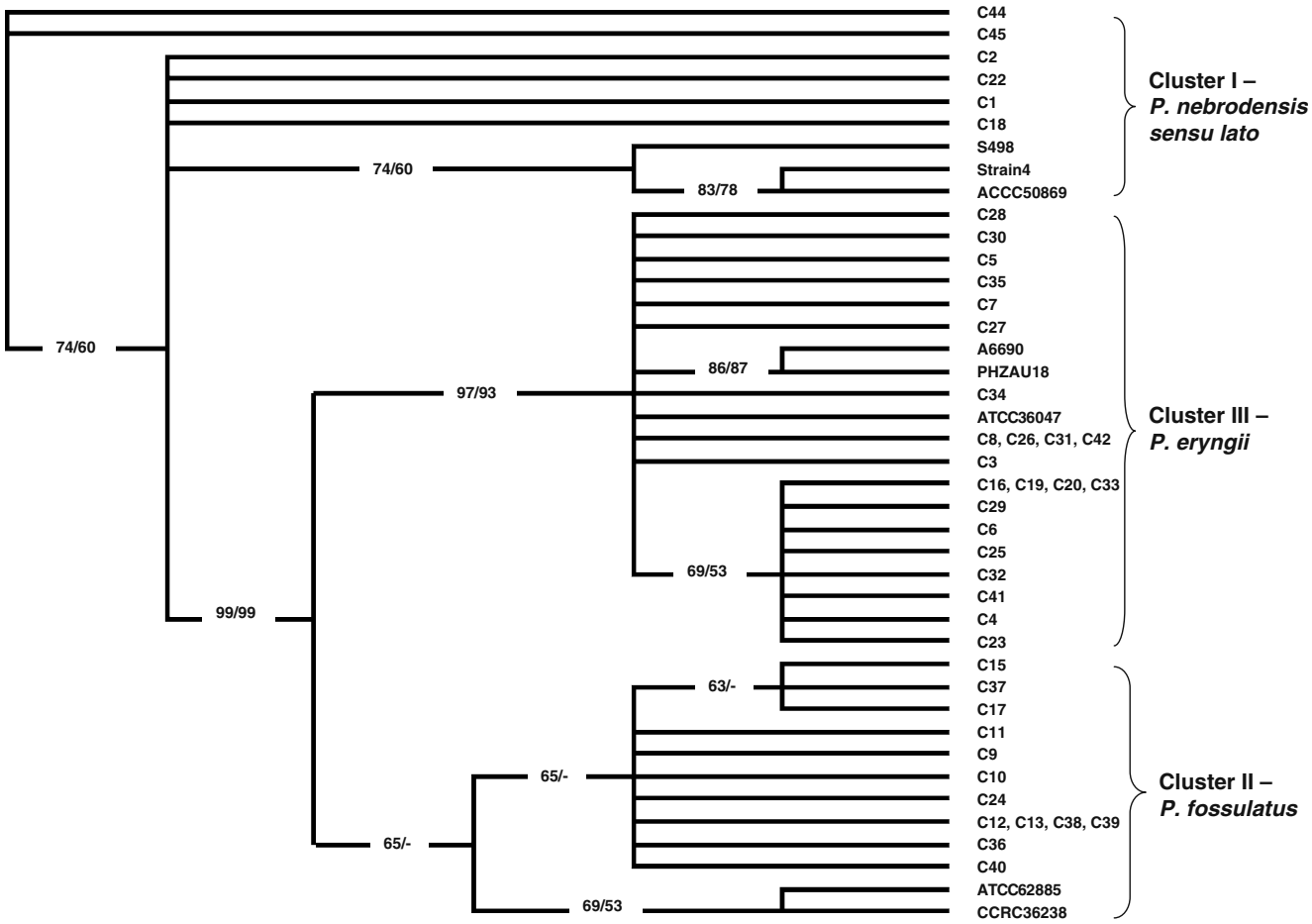

Fig. 5 One of the 541 equally parsimonious trees found by parsimony analysis of the ITS data from the analysis of 42 *Pleurotus eryngii* complex strains produced with PAUP* (length: 77, CI: 0.935, RI: 0.965, HI: 0.065); the depicted tree coincides with the strict consensus tree. The numbers at branch points represent bootstrap/jackknife support (values appear only when exceeding 50%). Eight additional sequences were obtained from NCBI and included in the analysis as "reference material": *P. eryngii* (strain code: A6690), *Pleurotus* sp. (PHZAU18), *P. eryngii* (ATCC36047), *P. fossulatus* (ATCC62885), *P. fossulatus* (CCRC36238), *P. nebrodensis* (S498), *P. nebrodensis* (strain no. 4), and *P. nebrodensis* (ACCC50869) (for more details see Materials and Methods)

Hence, the existence in Iran of three taxa of the *Pleurotus eryngii* complex is established:

(i) *P. nebrodensis sensu lato*, which grows in association with *Ferula assa-foetida* and *F. ovina* only. Previous reports linked its occurrence in Iran with host-plants such as *Diplotaenia cachrydifolia* (Heim 1960), *Ferula communis* and *Prangos* sp. (Saber 1997; reported as *P. eryngii* var. *nebrodensis*). The morphological characteristics provided from the former author for the *D. cachrydifolia* specimen (with whitish-cream colored pilei) fits with the current notion of the species and with the pertinent features characterizing our isolates of Cluster I. On the other hand, for Saber's (1997) specimens, no morphological description is provided, apart from the information that the *F. communis* associated strain was identified by Dr David Pegler. If these data are combined with the outcome of previous studies on *P. nebrodensis* (Kawai et al. 2008; Zervakis et al. 2001; Zhang et al. 2006), it can be deduced that the Asiatic populations of *P. nebrodensis s. l.* are associated with *Ferula* host-plants only (e.g., *F. assa-foetida*, *F. communis*, *F. ovina*, *F. sinkiangensis*), with the possible exception of Heim's (1960) *D. cachrydifolia*. In contrast, European *P. nebrodensis* is related with *C. ferulacea* only (Zervakis et al. 2001).

(ii) *P. fossulatus*, which grows in association with *C. ferulacea* only. Very limited information is presently available for this fungus with whitish to cream pilei, which grows in western and central Asia, i.e., Iran, Afghanistan, Pakistan, and India (Pegler 1977; Saber 1997); only Saber (1997) has reported an associated host-plant (*Prangos* sp., *Prangos ferulacea* is a synonym for *C. ferulacea*). Furthermore, Vilgalys and Sun (1994) included in their phylogenetic analysis two *P. fossulatus* strains originating from India (ATCC

62885 and 52666), which were reportedly intercompatible with *P. eryngii* strains; these two taxa formed distinct – albeit neighbouring – clusters in the resulting phylograms. Strain ATCC 62885 was also used in our study; it is clearly separated from what we here name *P. eryngii* (Cluster III), whereas it presented high affinity with CCRC 36238 (deposited at the NCBI as *P. fossulatus* too) and grouped into the same larger group (Cluster II) with Iranian isolates from *C. ferulacea*. Hence, *P. fossulatus* seems to form a distinct taxon (at the species level according to the phylogenetic species concept) associated in Iran with *C. ferulacea* only; local material forms a sister clade with *P. fossulatus* strains of other origins.

(iii) *P. eryngii*, which grows in association with *S. aucheri*, *K. odoratissima, C. ferulacea*, and *F. ovina*. This taxon is characterized by the typical morphological features described elsewhere (Hilber 1982; Zervakis and Balis 1996); however, lighter coloured pilei (light brown to beige to buff) are present in many of the specimens examined as a result of the phenotypic plasticity due to local environmental conditions and habitat nature. Despite the fact that European *P. eryngii* strains produce basidiomata from autumn to early winter at low altitudes, i.e., from sea level to ca. 1,500 m (depending on the host plants, which are usually species of the genera *Eryngium*, *Ferula*, *Eleoselinum*, *Thapsia*, etc.) (Hilber 1982; Venturella et al. 2000; Venturella et al. 2002; Zervakis and Balis 1996), *P. eryngii* specimens from Iran are generally collected at high altitudes (often exceeding 2,000 m) in spring months only.

In conclusion, strong patterns of host-dependent subdivision in the genetic structure of the *P. eryngii* complex (*P. nebrodensis s.l.* and *P. fossulatus* cases) were evidenced, but occasional deviation from this situation seems to occur as well; it could be mainly attributed to ongoing gene-flow between certain populations in neighbouring areas irrespective of the host they are associated with (e.g., *P. eryngii* case). This is in contrast to what was observed in European populations of the *P. eryngii* complex, where molecular studies revealed strict host-dependent delimitation among different 'ecotypes' of the complex (Urbanelli et al. 2007; Zervakis et al. 2001). In our opinion, it is still early to draw conclusions about the systematics of the Asiatic populations of *P. nebrodensis* (or, of *Pleurotus* fungi growing in association with *Ferula* spp. in Asia) and of *P. fossulatus*, before results of a study including representative host-associated populations from the entire distribution range of the complex are evaluated.

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