Cluster C1 included material deriving from a geographically-confined area (Slovenia, Italy, and Hungary), possessing both dark and light coloured pilei, particular basidiospore morphology and were associated only with *Ferulago campestris* host plants. No GenBank deposited material was available for comparison purposes for this particular group.

Separation of groups within clusters D1 and E1 was relatively less robustly supported, e.g. 55 % for specimens originating from Asia and 74 % for material from Europe and Asia respectively. Hence, the former cluster consisted of material isolated in west and central Asia only (Armenia, Iran, Afghanistan, and India) and mainly included GenBank depositions classified as *P. fossulatus* or *Pleurotus* cf. *eryngii*. The latter (E1) consisted of *Pleurotus nebrodensis* strains isolated from Sicily and Greece, which presented a mixed positioning within the core group, and a distantly positioned specimen (H137) from Iran.

When genetic similarities were calculated among representatives of the clusters formed from ITS1-5.8S-ITS2 sequences analysis by employing Kimura's criterion, then the lowest values were obtained when C1 was compared to all other Clusters, i.e. 95.1–97.0 % *vs.* A1, 94.3–96.4 % *vs.* B1, 94.8–96.8 % *vs.* D1, and 95.2–97.3 % *vs.* E1. In contrast, when A1, B1, D1 or E1 were compared against each other higher values were calculated (e.g. A1 *vs.* B1: 97.0–98.4 %, D1 *vs.* E1: 97.5–99.1 %).

Bayesian inference of phylogeny with ITS1-5.8S-ITS2 data, provided a tree very similar to that generated by the Kimura's distance model (Supplementary material, Fig S1). Again, five major clusters with high statistical support (90–100 %) derived corresponding to *P. eryngii* from various hosts in Europe and Asia, *P. eryngii* from *Ferula* spp. in Asia, *P. nebrodensis* from Europe, *Pleurotus* from *Prangos ferulacea* in Asia and European *Pleurotus* growing in association with *F. campestris*.

Phylogenetic analysis based on IGS1 sequences was performed with 46 unambiguously aligned sequences deriving from material examined in the frame of this work plus seven sequences obtained from the GenBank. By using the Kimura's distance method, four major clusters (A2, B2, C2, and E2) were produced with bootstrap values exceeding 57 % (Fig 4). Cluster A2 included strains originating from Europe (with the exception of Chinese isolates associated with *Eryngium* sp.) and assigned to *P. eryngii* varieties. As before, their intracluster grouping did not seem to be particularly related with either the associated host-plant or the geographic origin. Strain PN5 (from Romania on *Laserpitium latifolium* producing light

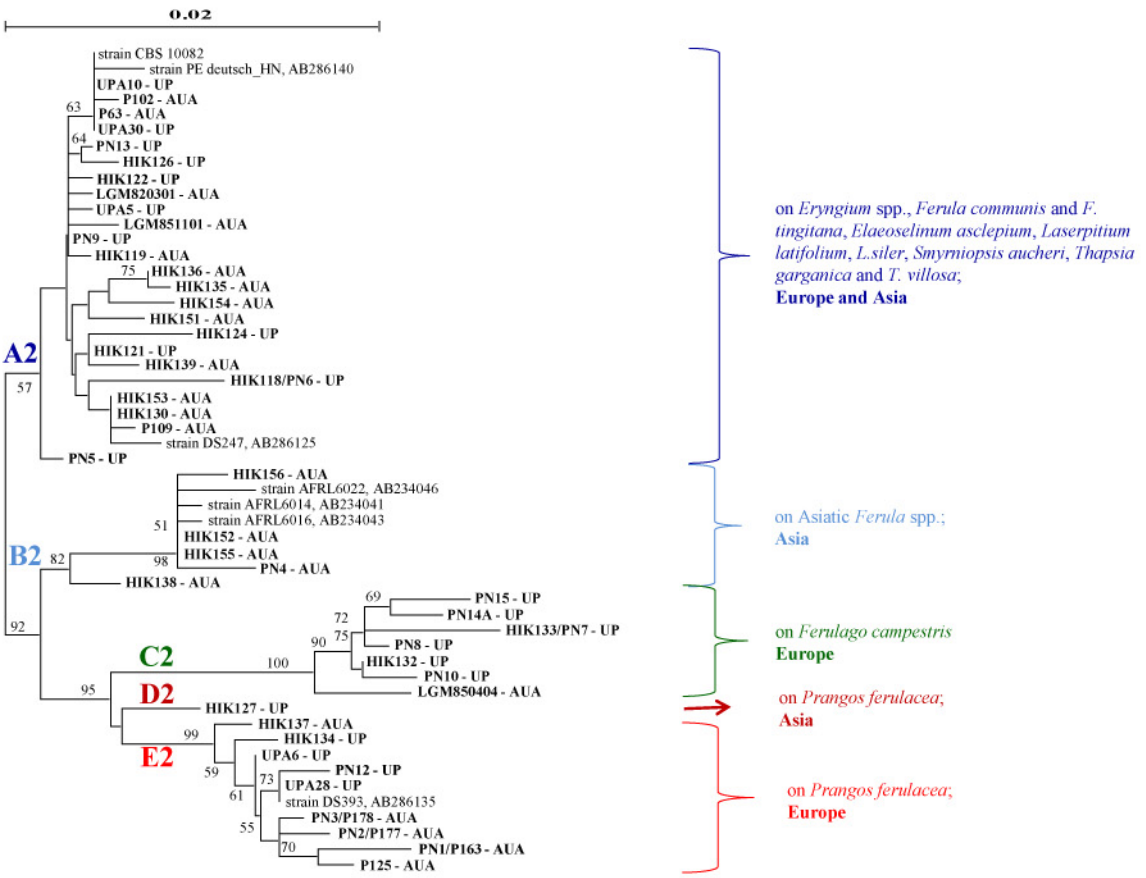

Fig 4 – Phylogenetic tree illustrating the relationships among 46 specimens of the *P. eryngii* complex (marked in bold) based on IGS1 sequences, while additional sequences from the GenBank were included in the analysis (strain code and accession no. are noted). Trees were generated by using the neighbour-joining algorithm and the distance was calculated on the basis of Kimura's two-parameter model. Bootstrap values derived from a total of 1000 replicates, and are noted when they exceeded the 50 % threshold. Each distinct cluster is represented by a different capital letter.

coloured pilei) was rather distantly related with the rest of the isolates in this cluster. In accordance with the ITS1-5.8S-ITS2 results, Cluster B2 included again only Chinese and Iranian material associated with *Ferula* spp. with the exception of strain HIK138 which was not positioned within the main group composed of Chinese isolates. Cluster C2 was robustly supported (100 %) and consisted of European isolates growing on *F. campestris*. D2 and E2 included material associated only with *Pr. ferulacea* and were composed of strains from Asia (HIK127 from Armenia, showing only 44 % support and grouped together with *P. fossulatus* strains in the ITS-5.8S rRNA gene-based tree) and south Europe (with the exception of isolate HIK137 from Iran) respectively.

When genetic similarities were calculated among representatives of the clusters formed from IGS1 sequences analysis employing Kimura's criterion, then the lowest values were obtained when C2 was compared to all other Clusters, i.e., 96.2–97.8 % *vs.* A2, 96.6–97.9 % *vs.* B2, and 96.6–98.0 % *vs.* D2. In contrast, when A2, B2 or D2 were compared against each other higher values resulted (e.g. A2 *vs.* B2: 97.5–99.0 % and D2 *vs.* HIK127: 98.1–98.9 %).

Results from Bayesian analysis of IGS1 sequence data were very similar to those obtained by the Kimura's method, resulting again in a phylogenetic tree composed of four major clusters with very high statistical support (99–100 %) and one individual strain (HIK127) which was positioned separately (Supplementary material, Fig S2). Clusters corresponded to *P. eryngii* from various hosts in Europe and Asia, *P. eryngii* from *Ferula* spp. in Asia, *Pleurotus* from *Pr. ferulacea* in Europe, and European *Pleurotus* growing in association with *F. campestris*.

The concatenated data from combined ITS1-5.8S-ITS2 and IGS1 sequences were analysed with Kimura's distance method and a tree with four distinct clusters (100 % support value each) was produced (Fig 5). As it was the case with IGS1, *P. eryngii* strains from Europe (associated with various hosts), Middle East (on *Ferula tingitana*) and Asia (on various hosts excl. *Ferula* spp.) composed Cluster A3, whereas all *P. eryngii* strains from Asiatic *Ferula* spp. formed a sister group to the previous one (B3, with 84 % support). A third well separated Cluster (C3) included all *F. campestris* related strains from central Europe. Last, D3 and E3 consisted of all *Pr. ferulacea* associated strains; material of south European origin (Sicily and Greece) were closely linked, whereas two isolates from Iran and Armenia were grouped separately presenting different levels of affinity with the core group.

The Bayesian concatenated tree was in full accordance with the results presented above (Supplementary material, Fig S3; parameters estimated in MrBayes runs are included in Supplementary material, Table S1). These results were further confirmed by maximum parsimony analysis conducted

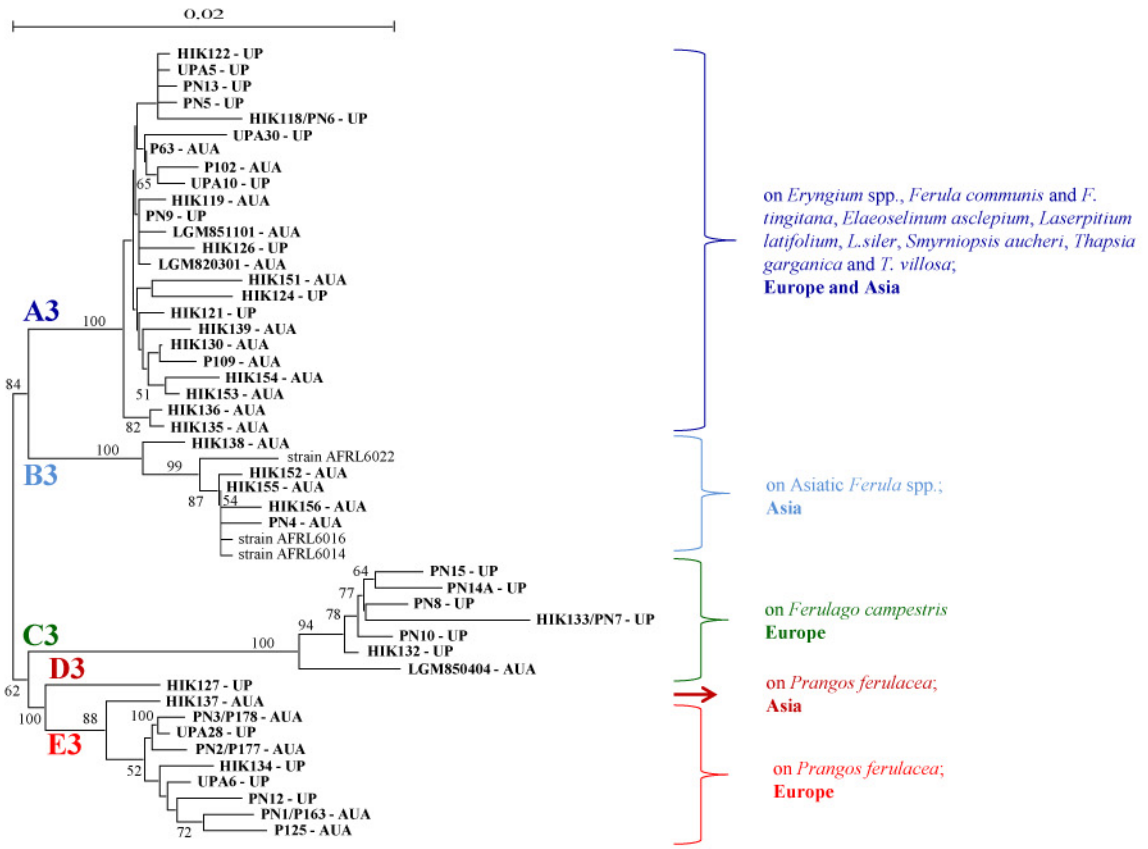

Fig 5 – Concatenated tree illustrating the phylogenetic relationships among 46 specimens of the *P. eryngii* complex (marked in bold) based on combined ITS1-5.8S-ITS2 and IGS1 sequences, while additional sequences from the GenBank were included in the analysis (strain code is noted). Trees were generated by using the neighbour-joining algorithm and the distance was calculated on the basis of Kimura's two-parameter model. Bootstrap values derived from a total of 1000 replicates, and are noted when they exceeded the 50 % threshold. Each distinct cluster is represented by a different capital letter.

for the entire sequence dataset (Supplementary material, Fig S4). Again, noteworthy was the clear separation of *F. campestris* associated *Pleurotus* specimens in respect to strains originating from other associated plant.

In general, concatenated trees revealed relative geographic rather than plant-dependent affinities within the main *P. eryngii* group (Clusters A1, A2, and A3) as it was evidenced by grouping of Asiatic strains (HIK135 and HIK136 from Iran *vs.* HIK153 and HIK154 from China) and to a lesser extend among strains from Italy, Greece, and Israel. They also presented differences between Iranian and Chinese material growing on Asiatic *Ferula* spp. (HIK138 *vs.* the rest of the group) as well as between Hungarian (LGM850404) *versus* Slovenian and Italian specimens from *F. campestris*. On the other hand, no particular differentiation was detected in sequences from Sicilian and Greek *P. nebrodensis*, in contrast to what was observed with material originating from Iran (HIK137) which was distantly related.

In conclusion, sequence analysis of the ITS1-5.8S-ITS2 region demonstrated high levels of similarity among closely related strains making thus very difficult to distinguish between *P. eryngii* varieties, whereas it was very useful for discriminating among species or even subspecies of the complex. Similarly, IGS1, although rarely used in the past to elucidate *Pleurotus* phylogeny, is of significant value for distinguishing closely related mushroom taxa even at infraspecific level (Liang *et al.* 2009; Wingfield *et al.* 2009).

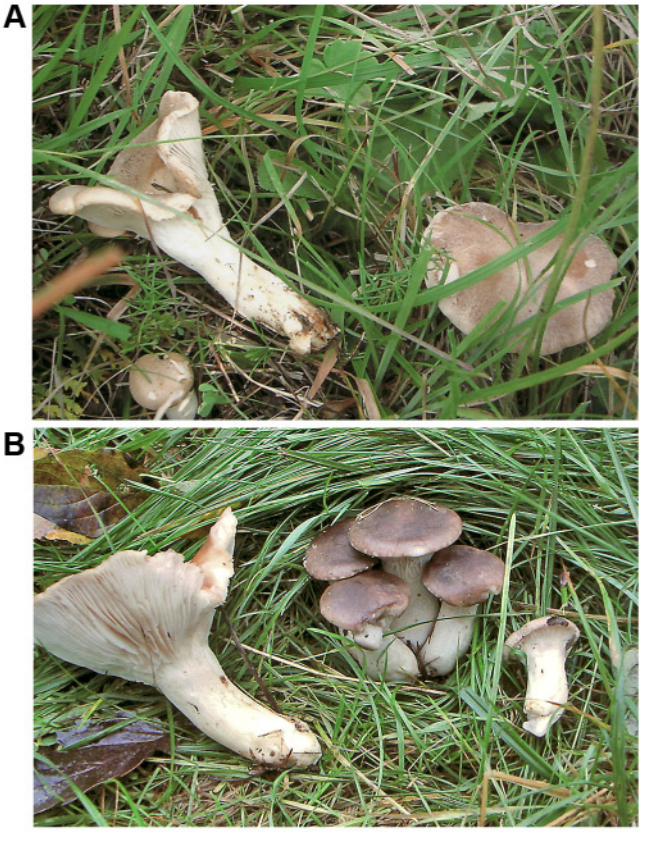

Fig 6 – Basidiomes of *Pleurotus ferulaginis*: (A) with light coloured pilei; (B) with dark coloured pilei. Photos provided by Mrs. Cattarossi. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

New species and combinations

Pleurotus ferulaginis Zervakis, Venturella & Cattarossi, **sp. nov.**, Fig 6A,B.

MycoBank No.: MB807238

This species differs morphologically from the other taxa of the *Pleurotus eryngii* complex by its cylindrical to bacilliform basidiospores, and regarding its habitat, it is associated with *Ferulago campestris* (and possibly with *Pimpinella saxifraga*) plants only.

Type (designated here): Slovenia, Koper, Pomjan, on rotting root residues of *F. campestris* (syn. *Ferulago galbanifera*), date: May 2004, coll. Bersan (holotype in ACAM 2010-0125; isotype in PAL 007-SIC).

DNA sequences ex holotype: HM998831 (ITS1-5.8S-ITS2); HM998794 (IGS1).

Etym.: ferulaginis (Lat.): referring to the genus name of the common host plant, *Ferulago* W.D.J. Koch.

Basidiomes macromorphology: Pileus 3–15 cm in diam, fleshy, initially convex but soon almost flat and later infundibuliform, surface at first smooth, then often unevenly fibrillose or with small brown (15/7C) squamules towards the outer half, shape circular to kidney-like with wavy edges and often inrolled margin, white to cream to ivory to light brown to beige-brown to beige-buff to warm brown to brown (to 10/3C to 10/2B to 15/9C to 13/6E to 12/5E to 15/11E to 15/7C). Lamellae crowded, thin, entire, broad, and dense, decurrent to the top of the stipe, without anastomoses, white to cream to ivory (to 10/3C to 10/2B). Stipe 2.5–10.5 × 1.0–3.0 cm, central and rarely subcentral, robust, cylindric to spindle-shaped and often rounded towards the base, white to cream to ivory (10/3C to 10/2B). Flesh firm and sometimes elastic, solid, white to cream. Odour and taste mild and pleasant. Spore print white to cream.

Basidiomes micromorphology: Spores (10.5) 11.0–16.0(17.0) × 4.0–5.5(6.0) μm, 12.85 × 4.99 μm on average, Q = (2.17)2.20–3.00(3.20), Q_m = 2.54, cylindrical to bacilliform, at most with shallow suprahilar depression, smooth, thin-walled, hyaline, with one or more drops, acyanophylous, inamyloid (Fig 7A). Basidia clavate, four-spored, 35–55 × 7–9 μm, thin-walled and hyaline in KOH; sterigmata up to 6 μm long; basidioles 25–45 × 5–7 μm μm (Fig 7B). Cheilocystidia and pleurocystidia absent but lamellae edge with a pubescence composed of protruding tramal hyphae, ending to some cystidioid elements resembling reduced basidioles, clavate, rostrate, mucronate, flexuosus, lecythiform or fusoid, 25–35 × 4–5 μm (Fig 7C); similar elements occasionally present on lamellae sides as well. Hymenophoral trama irregular, monomitic, made up of cylindrical to flexuosus hyphae, 3–10 μm broad, hyaline and thin-walled. Pileipellis a thin cutis, made up of somewhat intricate, thin to slightly thick-walled, cylindrical hyphae, 3.0–12.0 μm wide (Fig 7D). Pigment parietal, indistinct to pale grayish yellow. Pileus trama monomitic. Clamp connections present at all septa and often prominently swollen.

Distribution and ecology: It appears in May and June. Habitat: meadows by the roots and stems of *F. campestris* (Besser)

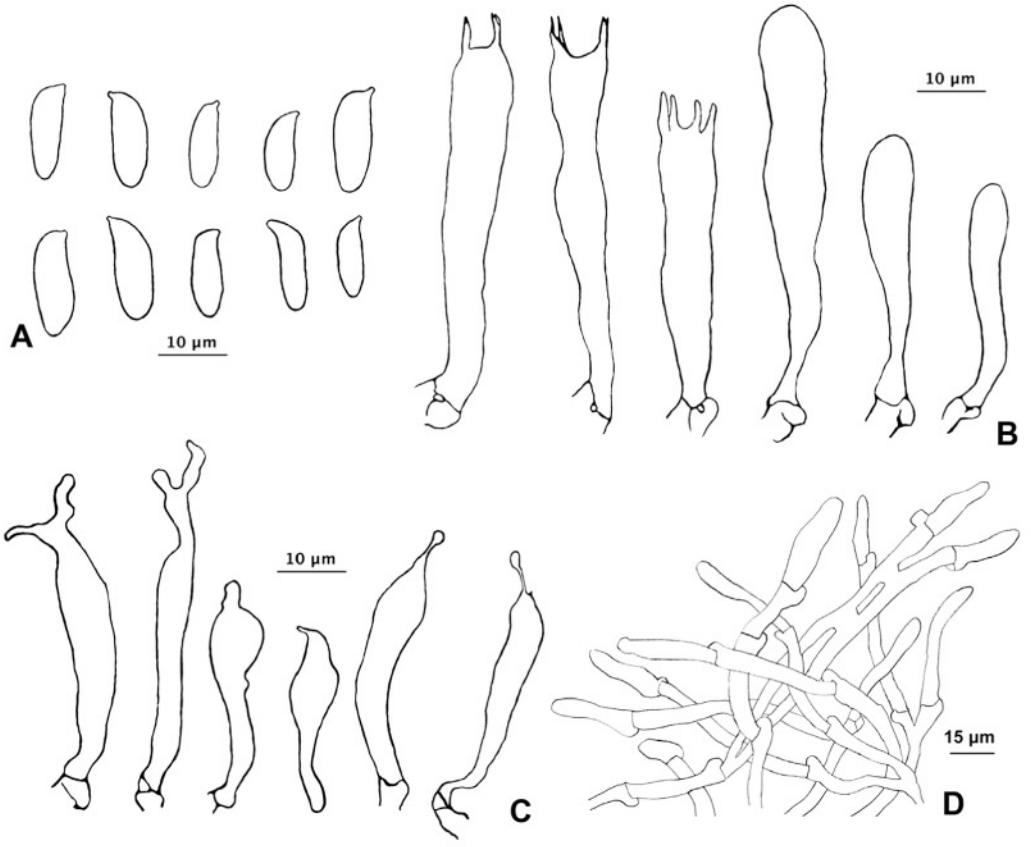

Fig 7 – Microscopic characters of *Pleurotus ferulaginis* pilei (drawn from the holotype): (A) basidiospores; (B) basidia; (C) cystidioid elements resembling reduced basidioles at the edge of lamellae; (D) hyphae from pileipellis.

Grecescu and possibly of *Pimpinella saxifraga* L.; it was found (and also reported) to occur in Slovenia (Postojna, Sežana, Divača, Ilirska Bistrica, etc.) and in NE Italy (Udine and Veneto regions). It is collected and highly appreciated as food by local people.

Other specimens examined: For details see Table 1.

Taxonomic remarks: The epithet 'ferulaginis' was first introduced by Stropnik *et al.* (1988) to describe only by macromorphology a variety of *P. eryngii* growing in association with *F. campestris* (syn. *F. galbanifera*) and *Pimpinella saxifraga* in Slovenia. However, '*P. eryngii* var. *ferulaginis*' is an invalid name (Art. 36.1 of ICBN) since no English or Latin description was provided, while a holotype is missing. In this work, apart of Slovenian specimens, pertinent material from other neighbouring countries (Italy and Hungary) was studied by anatomical, mating compatibility and molecular approaches before arriving at establishing this taxon at the species level within the *P. eryngii* complex. *Pleurotus ferulaginis* is considered as a distinct species on the basis of the very low *in vitro* mating intercompatibility percentages (32 % *vs.* European *P. eryngii* varieties, 8–17 % other taxa of the *P. eryngii* complex; in general, the lowest detected in this study) and its distinct phylogenetic position which clearly separates it from other *Pleurotus* populations growing in association with *Apiaceae* plants (genetic similarities *vs.* other taxa of the complex ranged from 96.0 to 97.7 for the combined genes dataset). ITS1-5.8S-ITS2 and IGS1 sequences of *P. ferulaginis* possess several discriminating regions incl. four large deletions segments. Of diagnostic value are the characteristic cylindrical to bacilliform basidiospores, whose dimensions differ substantially from all other *P. eryngii* complex taxa, and the associated host species *F. campestris* not linked to any other *Pleurotus* fungi.

Pleurotus nebrodensis subsp. **fossulatus** (Cooke) Zervakis & Venturella, **comb. nov.**

MycoBank No.: MB807240.

Basionym: *Pleurotus fossulatus* Cooke, Saccardo's Syll. fung. IX: 49; XII: 585.

Description: Pegler (1977) provided a detailed description of the fungus based on specimens collected from Afghanistan and Pakistan. Later, Puri *et al.* (1981) and Saber (1997) described *P. fossulatus* from India and Iran respectively without diverting from Pegler's description. In addition, examination of material included in this work concurred with previous pertinent reports.

Distribution and ecology: Information on the occurrence of this fungus is rather scarce. It was previously reported from Iran, Afghanistan, Pakistan, and India (Pegler 1977; Puri *et al.* 1981; Ravash *et al.* 2010; Saber 1997). Only Saber (1997) and Ravash *et al.* (2010) provided information about the associated plant by reporting that this taxon appears on *Prangos ferulacea* root and stem residues during spring (April to May) in mountainous regions of Iran. One specimen from Armenia isolated from *Pr. ferulacea* was included in this study and it was found to present high similarity with sequences obtained from NCBI

as 'P. *fossulatus*' (e.g. EU233945 and EU233946), extending thus the known distribution of this taxon further to the northwest of the continent.

Specimen examined: HIK127, from Armenia on Pr. *ferulacea* (Table 1).

Taxonomic remarks: The evaluation of past morphological descriptions and information on habit and habitat of Asiatic 'P. *fossulatus*' material (Pegler 1977; Ravash *et al.* 2010; Saber 1997) vis-à-vis pertinent data deriving from the study of P. *nebrodensis* specimens (Gargano *et al.* 2011; Venturella 2000; Zervakis *et al.* 2001; this work) revealed significant similarities. The only clearly discriminative character that could be identified was the dimensions of basidiospores: mean width 5.9 μm and quotient 2.42 for Italian specimens of P. *nebrodensis* (6.1 μm and 2.16 respectively for the Greek specimens) *vs.* mean width 5.3 μm and quotient 2.24 for Asiatic material. Furthermore, mating data showed an intercompatibility percentage of 48 % when the only culture, which was available from Asiatic Pr. *ferulacea*, was mated with P. *nebrodensis* strains from Italy and Greece. In contrast, percentages of successful matings for both European and Asiatic populations were notably lower *versus* other host-associated isolates (8–37 %). Additional evidence was provided by comparing ITS1-5.8S-ITS2 gene and IGS1 sequences from *Pleurotus* associated with Pr. *ferulacea* from Europe and Asia since high similarities were detected (97.5–99.1 % from ITS1-5.8S-ITS2, and >98 % from combined sequences datasets). Particularly as concerns Asiatic material, GenBank sequences were available only for ITS1-5.8S-ITS2 (Ravash *et al.* 2010; Vilgalys & Sun 1994) from specimens originating from Iran (FJ514558, FJ514564), India (AY368664) and Afghanistan (EU233946) which were added in the present analysis for covering the entire known distribution of this taxon. All cladograms presented a clear separation of Pr. *ferulacea* associated specimens from the rest of the P. *eryngii* complex, and a significant relatedness between European and Asiatic *Pleurotus* populations growing on this particular plant species; the relatively low bootstrap support (e.g. 55 % and 74 %; Fig 3) for the respective phylogenetic clusters is in line with the taxonomic approach adopted here: one species (P. *nebrodensis*) including populations from Mediterranean Europe (subsp. *nebrodensis*) and west to central Asia (subsp. *fossulatus*) together with individual strains of intermediate/dubious phylogenetic position (H137 and H127). Noteworthy was that all material isolated from Pr. *ferulacea* residues presented close genetic affinity and rather minute anatomical differences. Based on the above evidence, we believe that P. *fossulatus* can not be maintained as a species and, therefore, its relegation to the subspecies level is proposed, i.e. P. *nebrodensis* subsp. *fossulatus*.

Pleurotus eryngii subsp. **tuoliensis** (C.J. Mou) Zervakis & Venturella, **comb. nov.**

MycoBank No.: MB807241.

Basionym: *Pleurotus eryngii* var. *tuoliensis* C.J. Mou, in Mou, Cao & Ma, Acta Mycol. Sin. **6(3)**: 153 (1987).

Description: see P. *eryngii* var. *tuoliensis* Mou by Mou *et al.* (1987).

Distribution and ecology: The original description by Mou *et al.* (1987) was based on material collected in the northwest part of China (Xinjiang, Urumqi) from the root of *Ferula feruloides* during spring. Apart of *Ferula sinkiangensis* and *Ferula haussknechtii* which are also confirmed host plants for P. *eryngii* subsp. *tuoliensis* in China (Kawai *et al.* 2008; Zhang *et al.* 2006), Ravash *et al.* (2010) reported the existence of a distinct phylogenetic group (within the P. *eryngii* complex in Iran) consisting of specimens collected in association with F. *assa-foetida* and *Ferula ovina*. In the frame of the present work, examination of sequences from that last study (GenBank: FJ514549, FJ514565, FJ514590) together with additional pertinent material revealed that P. *eryngii* subsp. *tuoliensis* occurrence is not limited to China as previously thought but extends westwards to Iran. It is also interesting to remember that the Xinjiang region from where this taxon was originally described lies on the border of China with several countries (e.g. India, Pakistan, Afghanistan, and Kyrgyzstan) where other similar looking fungi were reported in the past by different names (Dhancholia 2013; Kaul 1999) despite their close morphological resemblance and related host plants.

Specimens examined: See Table 1.

Taxonomic remarks: In several (mainly Chinese) papers and popular publications this taxon is erroneously referred to as 'P. *nebrodensis*' or '*Pleurotus ferulae*', which is apparently due to the fact that it resembles the morphology of the true P. *nebrodensis* or because it was found growing in association with *Ferula* spp. However, it has nothing to do with *Pleurotus* associated with Pr. *ferulacea* or with P. *eryngii* var. *ferulae* occurring in Europe (i.e., on *Ferula* spp. with European and/or circum Mediterranean distribution only). Specimens of this taxon in China are locally named as either 'A-Wei-Mo' or 'Bai-Ling-Gu', the former presenting grey-brown to dull white and the latter white pilei. Both morphotypes are associated with Asiatic *Ferula* spp. only, and were determined as P. *eryngii* var. *tuoliensis* (Kawai *et al.* 2008; this work). There is no compelling evidence that P. *eryngii* var. *ferulae* exists in China despite contradictory reports based on the study of material for which the identity of the host plant was not always confirmed (Kawai *et al.* 2008; Zhang *et al.* 2006).

The outcome of the present work revealed a limited differentiation of *Pleurotus* specimens associated with Asiatic *Ferula* spp. from P. *eryngii* varieties, with prominent features those of white to cream coloured pilei, basidiospores of relatively low width (5.4 μm), high elevations habitats and spring appearance of basidiomes. Mating experiments showed a rather increased compatibility *vs.* European P. *eryngii* (65 %), which however was much lower than values measured when strains from different European (and Israeli) P. *eryngii* varieties were paired with each other (78–100 %). On the other hand, sequencing of ITS1-5.8S-ITS2 and IGS1 regions conferred at a clear separation of Asiatic *Ferula* spp. associated *Pleurotus* from all other clusters although genetic similarities with the main P. *eryngii* group were high (up to 98.4 % and 99.1 % for the two sequence regions examined). Still, nucleotide differences between sequences of *Pleurotus* growing on Asiatic *Ferula* spp. *vs.* P. *eryngii* varieties were more pronounced than those detected when various P. *eryngii* varieties were compared with each other. Based on these data, we hereby propose to elevate variety *tuoliensis* at the subspecies level, i.e. P. *eryngii* subsp. *tuoliensis*.

Hence, the P. *eryngii* complex in Asia is composed of populations that are either associated with *Ferula* spp. only (they correspond to P. *eryngii* subsp. *tuoliensis*), or with Pr. *ferulacea* only

(*P. nebrodensis* subsp. *fossulatus*), or with several other *Apiaceae* genera (i.e. *Eryngium*, *Smyrniopis*, and *Kellusia*; the 'core' *P. eryngii* group or *P. eryngii sensu stricto*).

Determination key for Pleurotus *taxa associated with plants of the family Apiaceae*

General characteristics of the *Pleurotus eryngii* species-complex: Taxa with a palearctic distribution, growing on dead residues of roots and stems of *Apiaceae* plants (or as facultative biotrophs if conditions permit), producing basidiomes from autumn to early summer; pileus fleshy, off-white to beige to brown to dark brown, usually scattered with numerous darker squamules or fibril-like patches; lamellae off-white to cream, thin, broad, entire, not particularly dense, decurrent and often forming a fine network of anastomoses at the top of the stipe; stipe firm and well-developed, mostly central to subcentral; absence of veil, no formation of coremia or chlamydospores; hyphal system monomitic, basidiospores, basidia and trama hyaline in KOH and Melzer's reagent; mycelial cultures not producing pigments, dikaryotic colonies more or less zonate and radial with even margin and white to cream colour, thin-walled hyphae with abundant clamp-connections; occasional production of nematode-trapping microdroplets singly on short secretory sterigmata in aerial hyphae.

1 Associated only with *Prangos ferulacea*.....2

Associated with other *Apiaceae* hosts.....3

2(1) Occurring in Sicily (Italy) and south continental Greece, basidiomes appearing usually singly during spring or early summer, in altitudes from 1200 to 2000 m, pileus 3.0–14.5 cm, light ivory to cream, basidiospores 12.2–17.4 × 5.5–8.2 µm (Q_m=2.27).....**nebrodensis**

Occurring in west and central Asia, basidiomes appearing in spring, in high altitudes, pileus 3.0–14.0 (–25) cm, whitish to cream to yellow ochraceous, surface initially glabrous later strobiliform, squamulose and often deeply cracked, basidiospores 9.0–14.0 × 4.5–6.0 µm (Q_m=2.24).....**nebrodensis *subsp.* fossulatus**

3(1) Associated with *Ferulago campestris* (and possibly *Pimpinella saxifraga*), occurring in NE Italy, Slovenia and Hungary, basidiomes appearing singly or in small groups, in May and June, from sea level to 700 m, pileus 4.0–15.0 cm, whitish to ochraceous to beige to brown, basidiospores 11.3–13.4 × 4.6–5.1 µm (Q_m=2.54).....**ferulaginis**

Associated with other *Apiaceae* hosts (not previously mentioned)..... 4

4(3) Associated with *Ferula* spp. that present mostly (or only) an Asiatic distribution, occurring only in Asia, basidiomes appearing usually singly during spring to early summer, in mountains and high altitude plains, pileus 4.0–16.5 cm, whitish to cream, basidiospores 8.7–14.3 × 4.5–6.3 µm (Q_m=2.15).....**eryngii *subsp.* tuoliensis**

Associated with other *Apiaceae* hosts (not previously mentioned).....5

5(4) Forming light coloured pilei, i.e. whitish to cream to buff to beige, occurring in Europe and associated with *Elaeoselinum asclepium* subsp. *asclepium*, *Margotia gummifera*, *Laserpitium latifolium*, *L. siler*, *Magydaris panacifolia* and *Thapsia villosa,* basidiomes appearing in autumn and spring, singly or in groups, growing from sea level to alpine environments, basidiospores 10–14 × 4.8–7.1 µm (Q_m=2.09).....**eryngii *var.* elaeoselini**

Dark coloured pilei, i.e. tan brown to brown to dark brown to grey brown..... 6

6(5) Associated only with *Ferula* spp. occurring in the larger Mediterranean area, basidiomes appearing from autumn to spring, singly or in groups, found in garigues, wastelands and pastures, on limestone and silicaceous soils, from sea level to 1200 m, pileus 5–25 (–30) cm, fleshy, dark brown to chestnut brown to grey brown.....**eryngii *var.* tingitanus** (on *F. tingitana*), and

.....**eryngii *var.* ferulae** (on *F. communis*)

Associated with hosts other than *Ferula* spp. (not previously mentioned).....7

7(6) Associated with *Thapsia garganica*, reported so far only from Sicily (Italy), basidiomes appearing in spring and autumn, usually at high elevations (> 1000 m), mostly singly, pileus 2–10 cm, brown to dark or grey brown, basidiospores 10–14 × 5–7 µm (Q_m=2.00).....**eryngii *var.* thapsiae**

Associated with *Eryngium* spp., *Opopanax chironium, Peucedanum* spp., *Smyrniopsis aucheri*, *Kellusia odoratissima* etc., basidiomes appearing from autumn to late winter, occurring mostly in groups from sea level to 1500 m, pileus 4–15 cm, light brown to tan brown to grey brown, basidiospores 9.1–13.5 × 4.6–6.7 µm (Q_m=2.04).....**eryngii *var.* eryngii**

Taxonomic notes and remarks for other related taxa of dubious identity

As previously demonstrated there are significant limitations of applying morphological criteria alone for delimiting among taxa in the *Pleurotus eryngii* complex since most of the anatomical features of basidiomes are overlapping and/or influenced by environmental conditions. This is anticipated in fungi for which speciation processes are primarily driven by physiological adaptation (host specialization), where little or no selection pressure is exerted for qualitative and/or quantitative changes in morphology. Therefore, morphological variability is limited and appears gradually through genetic drift in recently diverged taxa; in this particular case, differentiation of morphological character states follows genetic isolation (Brasier 1987; Kauserud *et al.* 2006; Nilsson *et al.* 2003). A widely used distinguishing character for this particular group of fungi has been the colour of pilei; although this character could be employed, we have shown that intraspecies variability permits both the existence of light and dark coloured pilei in the same species (i.e., *P. eryngii* and *Pleurotus ferulaginis*), while the same character could be representative of different taxa as well (e.g. white to cream pilei in both *Pleurotus nebrodensis* and *P. eryngii* subsp. *tuoliensis*).

On the other hand, quantitative characters such as spore size are very often used for delimiting species and even for defining phylogenetic relations in fungi (Luckow 1995; Parmasto & Parmasto 1992). Dimensions of basidiospores seem to be a more reliable discriminating feature in this complex, at least for clearly separating *P. ferulaginis* from the rest of the taxa. Furthermore, geographic populations of *P. nebrodensis* (Italy *vs.* Greece *vs.* Asia) demonstrate a notable variation in this character; the former two as regards their length, and subsp. *fossulatus* for both length and width. Among *P. eryngii* varieties, basidiospore size is quite variable and could be used as additional criterion in identifying host-associated subspecific taxa in conjunction with habitat and pileus morphology. In general, phylogenetic clades of the complex could be mainly defined on the basis of *Pleurotus* associated hosts and mating compatibility, and secondarily by morphological and/or habitat features (Fig 3).

Mating compatibility tests have been congruent with ecological and molecular differentiation in discriminating taxa within the *P. eryngii* complex. In fact, the biological species concept is commonly employed for species circumscription in basidiomycetes although mating data may not always accurately reflect evolutionary relationships (Vilgalys & Sun 1994). Despite that intersterility barriers between most species are prezygotic and often absolute, there are quite a few cases of partial hybridization reported for wood-decomposing basidiomycetes (Duncan 1972; Harrington *et al.* 1989; Mishler & Donoghue 1982; Petersen 1995), including those in *Pleurotus* spp. (Kawai *et al.* 2008; Zervakis & Balis 1996; Zervakis *et al.* 2004; this work). Although the biological species concept can not be directly applied in these cases, intermediate intercompatibility values are useful for assessing the degree of reproductive isolation.

Intraspecific variation in both ITS1-5.8S-ITS2 and IGS1 sequences of the *P. eryngii* complex was rather high (1.06–1.99 % as compared to 1.23–5.68 % of interspecific variation for ITS1-5.8S-ITS2, and 1.23–1.54 % as compared to 1.22–3.82 % of interspecific variation for IGS1). Such values are in accordance with previous findings demonstrating high genetic variability within *Pleurotus* populations associated with *Eryngium* and *Ferula* plants (De Gioia *et al.* 2005; Rodriguez Estrada *et al.* 2010) and in various other species or species complexes in Basidiomycota (Hughes *et al.* 2001; Moukha *et al.* 2013; Taylor *et al.* 2006; Tian *et al.* 2013). Growth in temporally heterogeneous or stressful environments and/or the predominance of horizontal transmission through sexual spores are possible explanations for the heterogeneity noted. In addition, overlapping intraspecific and interspecific ITS-5.8S rRNA gene variation was also observed in other groups of closely related species possessing few morphological differences, and was subsequently associated with recent speciation events (Nuytinck & Verbeken 2007).

Phylogenetic data based on the analysis of ITS1-5.8S-ITS2 and IGS1 led to the formation of lineages which were congruent with biogeography patterns and mating relationships. For example, a large cluster (A1 and A2 in Figs 4 and 5) was formed consisting of specimens of considerable diversity as regards host plants and geographic origins. Furthermore, it was clearly separated from other groups (by both intercompatibility and molecular criteria) and constitutes the 'core' of the *P. eryngii* complex ('*P. eryngii sensu stricto*') which includes all known European/Mediterranean host-associated varieties plus material from Asia. The latter originates from various plants (i.e. *Eryngium* sp., *Kellusia odoratissima* and *Smyrniopsis aucheri*) existing in China and Iran. What is most interesting though, is that according to the outcome of a previous work on Iranian strains (Ravash *et al.* 2010), *Ferula ovina* and *Prangos ferulacea* were also shown to be hosts for specimens belonging to *P. eryngii sensu stricto*. In fact, this constitutes the most pronounced discrepancy in respect to what other studies demonstrate (Kawai *et al.* 2008; Zervakis *et al.* 2001; this work), i.e., that these two hosts are exclusively linked with *P. eryngii* subsp. *tuoliensis* and *P. nebrodensis*, respectively. It seems that coexistence of two or more host-plants within a geographically confined area in Iranian mountains permits ongoing gene flow among *Pleurotus* populations growing on different plants; this hypothesis is supported by the observed intercompatibility in matings among *Pleurotus* strains from different plants (*Prangos* sp., *Pimpinella* sp. and *Ferula haussknechtii*; Abdollahzadeh *et al.* 2007) in northern Iran. The particularly high level of diversity and endemism for *Apiaceae* plants in Iran (Ajani *et al.* 2008; Pimenov & Leonov 2004) facilitates exchange of genetic material among *Pleurotus* populations associated with sympatric hosts making thus this particular area a 'species melting pot' or the place where *Pleurotus* fungi growing on *Apiaceae* plants could still 'meet and mate'.

In contrast, strict host-specificity is evidenced for other populations of the *P. eryngii* complex; low intercompatibility values for those growing on *Ferulago campestris*, on Asiatic *Ferula* spp. and on *Pr. ferulacea* (with the previous exception of localized populations in Iran) are concordant with their distinct phylogenetic placement (clusters B1 and B2, C1 and C2, and D1 and D2; Figs 3 and 4). In all these cases reproductive isolation precedes morphological differentiation as a possible consequence of incipient or recent speciation. In addition, short divergence time results in discrepancies between phylogenetic and biological species (Taylor *et al.* 2000), whereas when higher divergence time has elapsed, distinct phylogenetic groups become intersterile regardless of geographic separation (Moncalvo & Buchanan 2008).

As regards pertinent findings and taxonomic names of dubious status, Heim (1960) described a large-sized white-coloured *Pleurotus* from Iran on *Diplotaenia cachrydifolia*, which he identified as *P. nebrodensis* (its distribution area includes the Elburs Mts in northwestern Iran and adjacent regions, i.e. provinces Tehran, Mazanderan, Zanjan; Pimenov *et al.* 2011). Unfortunately, this specimen was not available to confirm its identity, but the basidiomes morphology in conjunction with the size of the spores (10–12.7(–16) × 5.8–6.9(–7.5) μm) are in accordance with Heim's opinion. Although no *Pleurotus* material from *D. cachrydifolia* was ever available for molecular analysis, the affinity (and cooccurrence) of this plant species to *Pr. ferulacea* (Ajani *et al.* 2008) renders quite possible that *Pleurotus* fungi associated with these two plants are conspecific.

On the other hand, the view supporting that mushrooms growing on *Laserpitium* spp. (*L. latifolium*, *L. siler*, and *L. gallicum*) in subalpine zones of central Europe belong to *P. nebrodensis* (Heim 1960; Hilber 1982) is not correct. Initially, Costantin (Offner & Heim 1924) introduced the name *Pleurotus hadamardii* to describe a *Pleurotus* occurring in the French Alps (Vanoise). By mistake, thoroughly explained later by Heim (1960), this fungus was reported to be associated with *Eryngium alpinum* while in fact it was growing on residues of coexisting *L. latifolium* plants. *Pleurotus* mushrooms growing in association with *Laserpitium* spp. produce light coloured pilei and have most of their other morphological characters in common with those growing on *Elaeoselinum asclepium*. As it is evidenced from previous studies employing morphological and/or other approaches (Chinan & Venturella 2012; Venturella *et al.* 2000; Zervakis *et al.* 2001) and by the outcome of this work, such material is grouped within *P. eryngii sensu stricto* (*P. eryngii* var. *elaeoselini*) and therefore '*P. hadamardii*' is a *nomen nudum* since the specimens Costantin originally described (later named *P. nebrodensis* by Heim or *P. eryngii* var. *nebrodensis* by Hilber) belong in fact to *P. eryngii*.

The name *Pleurotus himalayaensis* was recently introduced to describe an allegedly new species of the complex producing basidiomes during May and June, and growing on residues of *Ferula jaeschkeana* Vatke, at high altitudes (2900–4000 m) in Himachal Pradesh, India (Dhancholia 2013). The identity and distribution of the associated plant (occurring in the Himalayas, from Pakistan to Himachal Pradesh and in central Asia) and the habit/habitat of the mushroom in conjunction with its main morphological features (e.g. off white to cream pilei and basidiospore dimensions) fit the description of *P. eryngii* subsp. *tuoliensis*.

Biogeography, speciation, and coevolution

The role of ecological specialization in speciation was emphasized both in theoretical models (Diehl & Bush 1989; Maynard Smith 1966; Kondrashov & Kondrashov 1999) as well as in

case studies, usually concerning host switches in parasitic and mutualistic relationships (Garbelotto et al. 1998; Tedersoo et al. 2013). However, many saprotrophic (lignicolous) fungi are associated to their substrate in much the same way parasites are as regards their host; hence sympatric divergence could occur between such organisms which do not mate with each other because they can not grow together on the same host plant or specialized substrate (Giraud et al. 2006). In fact, niche differentiation and partial (or complete) intersterility among geographically confined populations are principal factors affecting the degree of genetic isolation and the formation of new species in Basidiomycota, and therefore might be significant from a microevolutionary point of view as indication of incipient or recent sympatric speciation events (Brasier 1987; Garbelotto et al. 1996; Kauserud et al. 2006; Petersen & Ridley 1996).

Populations of the *Pleurotus eryngii* complex display marked host/substrate specificity by growing as saprotrophs (or facultative biotrophs) on various *Apiaceae* plants within a rather confined geographical range. In addition, other obstacles in the gene flow could also be present in such microhabitats. For example, allochrony in producing their basidiomes subsequently leads to the establishment of preferential mating between *Pleurotus* homokaryons originating from the same associated plant, given the fact that primary mycelia are generally short-lived. In this way, assortative mating is promoted leading to disruptive selection (Kawecki & Ebert 2004).

Therefore, it seems that taxa within the *P. eryngii* complex gradually developed distinct gene pools through an efficient mechanism of premating barriers, and have recently diverged through a sympatric speciation process based on both mating incompatibility and extrinsic factors such as host/substrate specialization (i.e., *Pleurotus ferulaginis* and *Pleurotus nebrodensis*). In fact, *P. ferulaginis* occurs in a region where *P. eryngii* varieties coexist (although not in close proximity), yet ecological adaptation plays a dominant role in keeping apart populations growing in different niches. For other taxa of the complex (i.e., *P. eryngii* subsp. *tuoliensis* and *P. nebrodensis* subsp. *fossulatus*), incipient speciation seems quite possible since elements like geographic distance and physical barriers, in conjunction with associated plants, constitute essential prerequisites for the genetic isolation of Asiatic populations producing basidiomes during spring or early summer in largely compartmentalized mountainous terrains (albeit geographically adjacent to the limits of *P. nebrodensis* distribution as exemplified by strains HIK127, HIK137, and HIK138 in our study).

Geographic distribution patterns for *Pleurotus* fungi showed that earlier evolved species present a world-wide distribution, whereas recently evolved groups (among which is the *P. eryngii* complex) occur in the Northern Hemisphere only (Vilgalys & Sun 1994). A major factor in recent species radiations within the Palearctic zone is the Pleistocene glaciation (Willis 1996; Stewart et al. 2010). *Pleurotus* taxa associated with *Apiaceae* are evolving since that period by building microevolutionary units in refugia confined in specific plants and particular geographic localities, e.g. *Prangos ferulacea* in high altitudes of Sicily and southern Greece, *Ferulago campestris* in northeast Italy and Slovenia, or various *Apiaceae* species in topographically distinct habitats of China, Pakistan, India, and Iran.

As recently shown by Taylor (2008), taxonomic relatedness among host plants regulates the 'host effect', while host phylogeny determines the extent of variation in both species richness and community composition of ectomycorrhizal fungi and fungal plant pathogens (Gilbert & Webb 2007; Ishida et al. 2007). It is these similarities in both mutualistic and antagonistic interactions which indicate that such associations are governed by common evolutionary pathways (Fontaine et al. 2011). Therefore, speciation in one of the interacting organisms, particularly the host, can lead to speciation in the other provided that some type of reproductive isolation exists (Giraud et al. 2008), much as it is observed for populations of the *P. eryngii* complex. Current disjunct distribution ranges for each individual taxon of this group follow a pattern which is clearly linked to that of the associated plant(s), reflecting thus a possible case of plant-fungus coevolution. Comparative analysis of homologous information (fungal ITS sequences deriving from this study *vs.* plant ITS sequences deriving from GenBank) demonstrated evolutionary relationships between various *P. eryngii* taxa and *Apiaceae* lineages. This was evidenced when comparison of the respective trees resulted in similar topologies, while the phylogenetic positions of *Pleurotus* populations were in accordance with the phylogeny of their associated plants (Fig 8).

When the delimitation of *P. eryngii* var. *ferulae* and var. *tingitanus* (occurring only in the greater Mediterranean region on *Ferula communis* and *Ferula tingitana* respectively) from *P. eryngii* subsp. *tuoliensis* (occurring only in Asia on *Ferula feruloides*, *F. sinkiangensis*, *Ferula assa-foetida*, and *Ferula ovina*) is evaluated vis-à-vis the biogeography of *Ferula* spp., then the congruence observed between the respective phylogenetic trees (Fig 8) is of particular interest. Hence, Asiatic *Ferula* spp. such as *F. ovina* (broadly distributed in the Irano-Turanian floristic region, Jordan is the westernmost limit of its distribution), *F. feruloides* and *Ferula sinkiangensis* (occurring only in Central Asia and in northwest China respectively), as well as *F. assa-foetida* (distributed in west Asia and in Libya) were never reported in Europe or in the Middle East; in contrast, *F. communis* and *F. tingitana* are found only in the circum Mediterranean region (Kurzina-Mlynic et al. 2008; Pimenov & Kljuykov 2002; The Euro+Med PlantBase 2006).

On the other hand, plants of the genus *Eryngium* originated in the western Mediterranean; the 'Old World' clade evolved independently to form subgenus *Eryngium* and includes species like *E. alpinum*, *Eryngium maritimum*, *Eryngium campestre*, and *E. planum* (Calviño et al. 2008). With the exception of the former (confined in south and central Europe), the other *Eryngium* spp. associated with *Pleurotus* present a rather wide distribution extending to Asia as well (especially *E. planum* which is hence considered as the principal host species for Asiatic *P. eryngii* although no detailed reports are available in pertinent literature). As already demonstrated, *Pleurotus* material growing on *E. maritimum* and *E. campestre* was positioned in the same cluster as several other host-associated varieties of *P. eryngii*, but the respective plant species are clearly separated (as expected) from each other and form distinct phylogenetic groups (Fig 8). Of interest was that *P. eryngii* var. *thapsiae* and *P. eryngii* var. *elaeoselini* are linked with the same plant cluster which includes their associated hosts/substrates, i.e., *Elaeoselinum asclepium*, *Laserpitium latifolium*, *Laserpitium siler*, *Thapsia garganica*, and *Thapsia villosa*.

Unfortunately, no GenBank ITS sequences were available for *Pr. ferulacea* and *F. campestris* material originating from

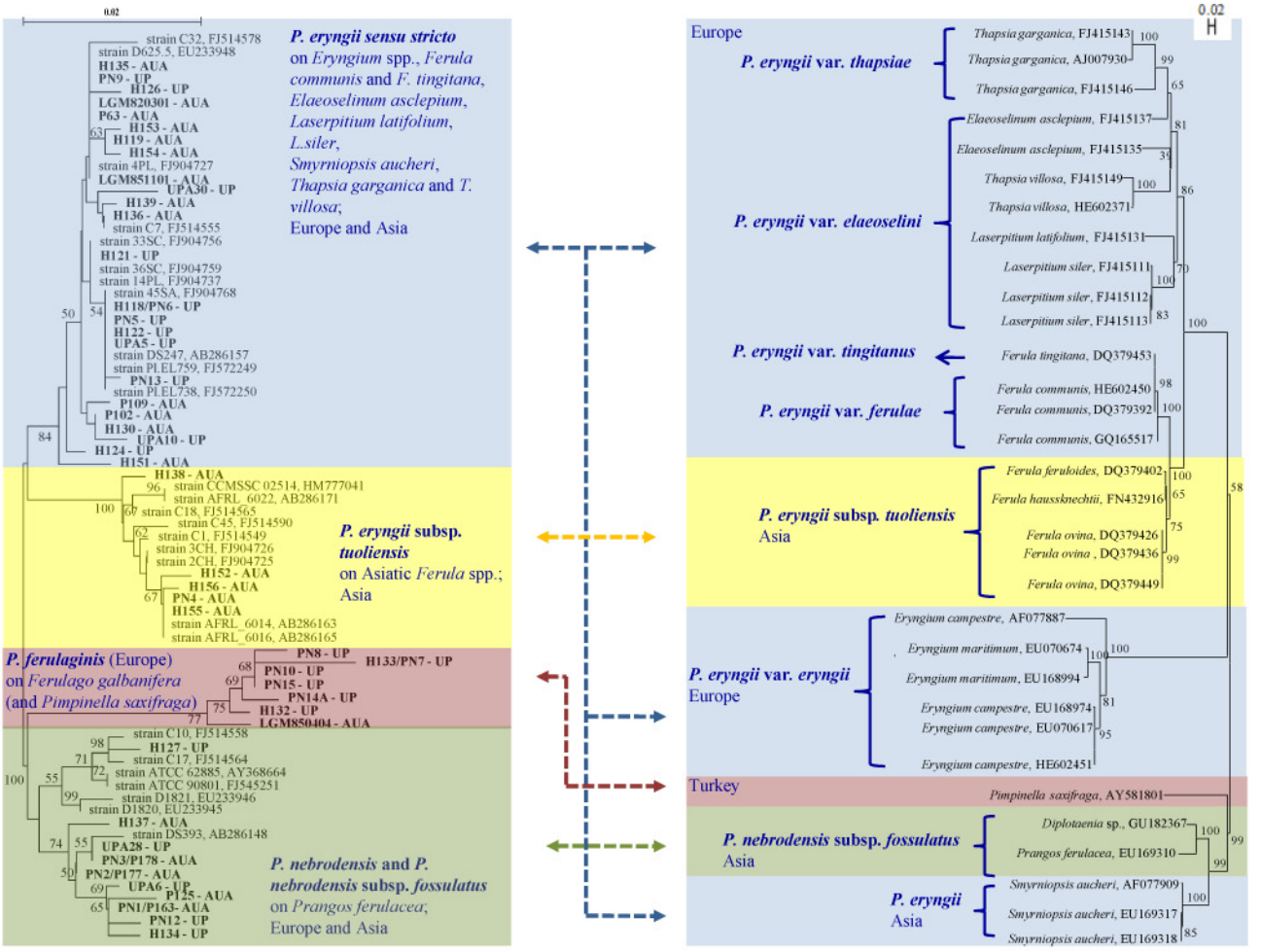

Fig 8 – Relationships between specimens of the *P. eryngii* complex examined in the frame of this study and their associated *Apiaceae* plants as illustrated through the respective phylogenetic trees, which were based on ITS1-5.8S-ITS2 sequences (sequences of plant species derived exclusively from the GenBank). Trees were generated by using the neighbor-joining algorithm, and the distance was calculated on the basis of Kimura's two-parameter model. Bootstrap values derived from a total of 1000 replicates. Identical colour zones are indicative of possible evolutionary linkages between fungal and plant organisms, whereas double-pointed arrows connect the respective clusters of the two trees. Names of associated plants and geographic origins are noted on the *Pleurotus* tree, whereas corresponding *Pleurotus* taxa are indicated on the appropriate positions of the *Apiaceae* tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Europe in order to compare their phylogenetic positions with those of *P. nebrodensis* and *P. ferulaginis* specimens respectively. Instead, material from both *Pr. ferulacea* and *Diplotaenia* sp. (a plant genus associated with white-pilei forming *Pleurotus*; Heim 1960) originating from Asia was used, and it demonstrated a distinct positioning from other *Apiaceae* plants as did *Pleurotus* specimens isolated from *Pr. ferulacea* in the same region (*P. nebrodensis* subsp. *fossulatus*) (Fig 8). Furthermore, an ITS sequence from *Pimpinella saxifraga* (occurring throughout most of Europe, and extending as far as east Siberia and central Asia) was included in the same analysis for indicative purposes only; this species was reported (but not confirmed) as a host/substrate for *P. ferulaginis* (Stropnik *et al.* 1988), and it was clearly separated from the rest of plant material as its allegedly associated fungus was from the other *P. eryngii* taxa (Fig 8).

Based on the above, a notable tendency of *Pleurotus* species to coevolve with their hosts was detected. Possible exceptions involve localized populations that are not host/substrate-specialized yet or may have hybrid origins (e.g. *P. eryngii* occurring in Iran and associated with several plants: *S. aucheri*, *K. odoratissima*, *Pr. ferulacea*, and *F. ovina*; Ravash *et al.* 2010). Such concordant plant and fungus phylogenies involving most taxa of the *P. eryngii* complex suggest high coadaptation with their associated organisms, which in turn is indicative of widely occurring clonal (vegetative) propagation through existing dikaryotic mycelia. Although vertical transmission promotes coevolving of plant and fungus, this type of reproduction alone is not adequate in long-term evolution and should be in fact combined with basidiospores dispersal and crosshybridization with other populations growing on nearby plant communities. In fact, a synthesis of such events (incl. limited long distance dispersal, niche conservation and low vagility) seems to be the more plausible scenario for this group of basidiomycetes explaining the high degree of provincialism observed.

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A reappraisal of the *Pleurotus eryngii* complex – New species and taxonomic combinations based on the application of a polyphasic approach, and an identification key to *Pleurotus* taxa associated with *Apiaceae* plants

Georgios I. ZERVAKIS[a,*], *Spyridon NTOUGIAS*[b], *Maria Letizia GARGANO*[c], *Maria I. BESI*[d], *Elias POLEMIS*[a], *Milton A. TYPAS*[e], *Giuseppe VENTURELLA*[c]

[a]*Agricultural University of Athens, Laboratory of General and Agricultural Microbiology, Iera Odos 75, 11855 Athens, Greece*

[b]*Democritus University of Thrace, Department of Environmental Engineering, Laboratory of Wastewater Management and Treatment Technologies, Vas. Sofias 12, 67100 Xanthi, Greece*

[c]*Università di Palermo, Department of Agricultural and Forest Sciences, Viale delle Scienze, 11, 90128 Palermo, Italy*

[d]*John Innes Centre, Department of Disease and Stress Biology, Colney Lane, Norwich NR4 7UH, UK*

[e]*National and Kapodistrian University of Athens, Department of Genetics and Biotechnology, Faculty of Biology, Panepistemiopolis, Athens 15701, Greece*

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ABSTRACT

The *Pleurotus eryngii* species-complex comprises choice edible mushrooms growing on roots and lower stem residues of *Apiaceae* (umbellifers) plants. Material deriving from extensive sampling was studied by mating compatibility, morphological and ecological criteria, and through analysis of ITS1-5.8S-ITS2 and IGS1 rRNA sequences. Results revealed that *P. eryngii sensu stricto* forms a diverse and widely distributed aggregate composed of varieties *elaeoselini*, *eryngii*, *ferulae*, *thapsiae*, and *tingitanus*. *Pleurotus eryngii* subsp. *tuoliensis* comb. nov. is a phylogenetically sister group to the former growing only on various *Ferula* species in Asia. The existence of *Pleurotus nebrodensis* outside of Sicily (i.e., in Greece) is reported for the first time on the basis of molecular data, while *P. nebrodensis* subsp. *fossulatus* comb. nov. is a related Asiatic taxon associated with the same plant (*Prangos ferulacea*). Last, *Pleurotus ferulaginis* sp. nov. grows on *Ferulago campestris* in northeast Italy, Slovenia and Hungary; it occupies a distinct phylogenetic position accompanied with significant differences in spore size and mating incompatibility *versus* other *Pleurotus* populations. Coevolution with umbellifers and host/substrate specificity seem to play key roles in speciation processes within this fungal group. An identification key to the nine *Pleurotus* taxa growing in association with *Apiaceae* plants is provided.

* *Corresponding author*. Tel.: +30 2105294341; fax: +30 2105294344.
E-mail address: zervakis@aua.gr (G. I. Zervakis).

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Conclusions

The outcome of this study shows that *Pleurotus* fungi growing in association with *Apiaceae* plants could be classified into three phylogenetic species: *Pleurotus eryngii* (or *P. eryngii sensu stricto*), *Pleurotus nebrodensis* and *Pleurotus ferulaginis*. The former includes populations from almost the entire known geographic distribution of the complex, growing on a wide diversity of plants. This species includes several established varieties (i.e., vars. *eryngii*, *ferulae*, *elaeoselini*, *thapsiae*, and *tingitanus*), all primarily distinguished by the identity of the associated host/substrate and by a few ecomorphological characters. Strains isolated from *Laserpitium* plants are classified within *P. eryngii*, and they are not related with *P. nebrodensis* as it was reported in the past. White-pilei producing *Pleurotus* fungi, distributed from Iran to China and growing on *Ferula* spp. occurring in Asia, form a distinct phylogenetic cluster and show low intercompatibility *versus* most other groups of the *P. eryngii* complex. However, their relatively high genetic relatedness to *P. eryngii* justifies their positioning at a subspecific level, *P. eryngii* subsp. *tuoliensis* comb. nov. (instead of *P. eryngii* var. *tuoliensis*, which was previously used to describe Chinese material from a narrower host/substrate range).

Despite the fact that *P. nebrodensis* still demonstrates a (low) *in vitro* intercompatibility with *P. eryngii*, ecomorphological characters and sequencing results advocate for its placement as a distinct species. It is associated with *Prangos ferulacea* only, and its distribution is not restricted in Sicily (as previously believed) but extends to southern continental Greece and most possibly to west Asia as well on the basis of molecular data. The name '*Pleurotus fossulatus*' previously used to accommodate white-pilei *Pleurotus* growing on *Pr. ferulacea* from western and central Asia should be replaced by *P. nebrodensis* subsp. *fossulatus* comb. nov. since specimens described under the former name are linked to European *P. nebrodensis* material after the comparative evaluation of morphological, mating compatibility and molecular results.

Last but not least, *P. ferulaginis* sp. nov. is well discriminated from all other *Pleurotus* taxa growing on umbellifers as evidenced by morphology, mating compatibility and sequence data. It grows in association with *Ferulago campestris* (and possibly with *Pimpinella saxifraga*), and its distribution is limited to Slovenia, NE Italy, and Hungary.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2014.07.001.

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Introduction

The *Pleurotus eryngii* species-complex (*Basidiomycota*, *Agaricales*) 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 & Balis 1996). Basidiomes of host/substrate-specific populations grow on plant roots or lower parts of stems singly or in small groups from September until June. The distribution range of this complex covers a rather well-defined zone in the Old World ranging from Morocco to the Netherlands in the west and extends to China eastwards, comprising most parts of south-central Europe and many regions of the Middle East and west Asia (Hilber 1982; Zervakis *et al.* 2001).

The significance of these fungi as choice edibles is growing fast (Chang 2005) and today they are among the highest priced cultivated mushrooms. In addition, several studies assessed their potential in a wide range of applications, e.g. exploitation of various lignocellulosic residues as cultivation substrates (Philippoussis *et al.* 2001; Rodriguez Estrada *et al.* 2009; Zervakis *et al.* 2013) and the subsequent use of spent material as animal feed (Kwak *et al.* 2008; Okano *et al.* 2007), production of biomass with valuable dietetic and medicinal properties (Chen *et al.* 2012; La Guardia *et al.* 2005; Synytsya *et al.* 2009), biological pretreatment of lignocellulosics for ethanol production (López-Abelairas *et al.* 2013), biodegradation of industrial effluents and toxic pollutants (Gómez-Toribio *et al.* 2009; Ntougias *et al.* 2012; Koutrotsios & Zervakis 2014), etc.

Accurately identified biological material with well-understood phylogenetic relationships within and among closely related taxa are essential prerequisites for mushroom strain selection, breeding, and exploitation. In the past, taxonomic studies for members of the *P. eryngii* complex were mainly based on host-specificity and anatomy often accompanied by mating compatibility data (Hilber 1982; Lewinsohn *et al.* 2002; Mou *et al.* 1987; Venturella *et al.* 2000, 2002; Zervakis & Balis 1996), and led to the confirmation of the status of *P. eryngii* var. *eryngii* (DC.: Fr.) Quél. and var. *ferulae* Lanzi, or the establishment of several new varieties under *P. eryngii*: var. *tuoliensis* C.J. Mou, var. *elaeoselini* Venturella *et al.*, var. *thapsiae* Venturella *et al.*, and var. *tingitanus* Lewinsohn *et al.* In addition to well-known taxa like *Pleurotus nebrodensis* (Inzenga) Quél. and *Pleurotus fossulatus* (Cooke) Sacc., other names were also introduced to accommodate related entities at specific or varietal level, e.g. *Pleurotus hadamardii* Costantin and *P. eryngii* var. *ferulaginis* Stropnik *et al.* (Gargano *et al.* 2011; Offner & Heim 1924; Pegler 1977; Venturella 2000; Stropnik *et al.* 1988).

However, in many cases delimitation of species proved to be problematic, while pertinent reports were often contradictory; hence, conclusions on the correct identity of several taxa and their affinities were ambiguous and difficult to rely on. Indicative examples are the confusion in naming the white-coloured forms of *Pleurotus* mushrooms growing on *Apiaceae* plants irrespectively of their geographic origin or host (Heim 1960; Hilber 1982; Saber 1997), the dubious taxonomic position of *Pleurotus* populations associated with *Laserpitium* spp. (Chinan & Venturella 2012; Joly *et al.* 1990), the simultaneous use of three different names (i.e., *P. eryngii* var. *tuoliensis*, *P. nebrodensis*, and *Pleurotus ferulae*) to describe the same taxon in China (Choi *et al.* 2009; Mao 2000; Mou *et al.* 1987; Zhang *et al.* 2005), the misapplication of names (e.g. *P. hadamardii*; Offner & Heim 1924), the conflicting arguments on the taxonomic status of the epithet 'nebrodensis' at either species or varietal level (Kawai *et al.* 2008; Rodriguez Estrada *et al.* 2010; Zervakis *et al.* 2012), the establishment of new taxa without adequate supporting evidence such as *Pleurotus himalayaensis* (Dhancholia 2013), etc.

Recently the use of molecular approaches shed some light into this perplexed situation by confirming the taxonomic status of varieties *eryngii*, *ferulae*, and *elaeoselini* (Rodriguez Estrada *et al.* 2010; Zervakis *et al.* 2001), by identifying 'Bai-Ling-Gu' (widely used in China to describe '*P. nebrodensis*' specimens) as *P. eryngii* var. *toluensis* (Kawai *et al.* 2008), and by studying the largely unexplored relevant material originating from Iran (Ravash *et al.* 2010). Nevertheless, significant discrepancies still exist regarding the phylogeny of this species complex since the examined material in previous studies represented only part of its known distribution, while data on the taxonomic status and phylogenetic relationships for and among 'key-taxa' such as *P. nebrodensis*, *P. fossulatus*, and *P. eryngii* var. *tuoliensis* (and in respect to *P. eryngii*) are still missing together with much needed information on other understudied populations associated with *Ferulago* and *Laserpitium* associated plants.

In this study, sequences from the nuclear internal transcribed spacer (ITS1-5.8S-ITS2) and intergenic spacer (IGS1) regions of the ribosomal RNA repeat from 46 specimens of the *P. eryngii* complex, covering to a significant extent (its occurrence both in terms of geographic distribution and associated-plant diversity), were examined in conjunction with morphology, ecology, and compatibility data. Phylogenetic analysis was used to infer relationships within this *Pleurotus* group, to define evolutionary paths in accordance with biogeography and to provide insight about speciation processes for plant-associated populations.

Materials and methods

Biological material

Forty-six (46) specimens representing most of the known world-wide distribution range of *Pleurotus eryngii* species-complex populations were included in this study; in addition, this material was associated with different *Apiaceae* plants, i.e., *Prangos ferulacea*, *Elaeoselinum asclepium* subsp. *asclepium*, *Margotia gummifera*, *Eryngium amethystinum*, *Eryngium campestre*, *Eryngium maritimum*, *Peucedanum cervaria*, *Ferula assa-foetida*, *Ferula communis*, *Ferula feruloides*, *Ferula ovina*, *Ferula sinkiangensis*, *Ferula tingitana*, *Laserpitium latifolium*, *Laserpitium siler*, *Thapsia garganica*, and *Thapsia villosa*, in order to more accurately reflect the pertinent diversity. Details on the identity of the fungal material used appear in Table 1. No type specimens were included in the study; however, care was taken to ascertain a wide/diverse representation of the *Pleurotus* taxa examined. All material is deposited in the herbaria of the Laboratory of General and Agricultural Microbiology of the Agricultural University of Athens (ACAM) and of the

Table 1 – Biological material used in this study: identity of each *Pleurotus* specimen examined (as finally established), associated plant, geographic origin, specimen code, GenBank accession numbers, and cluster designation (based on the outcome of the phylogenetic analysis; Figs 3 and 4).

a/a	Taxon	Associated plant	Geographic origin	Specimen code	ITS accession no.	IGS accession no.	Cluster designation
1.	*P. eryngii* var. *eryngii*	*Eryngium maritimum*	Greece, Ag. Nikolaos (Crete)	P63 – AUA[a]	HM998811	HM998776	A1 and A2
2.	*P. eryngii* var. *eryngii*	*Eryngium campestre*	Italy, Apulia	UPA10 – UP[a]	HM998817	HM998782	A1 and A2
3.	*P. eryngii* var. *eryngii*	*Eryngium* sp.	Ukraine, Crimea	HIK119 – AUA[a]	HM998820	HM998786	A1 and A2
4.	*P. eryngii* var. *eryngii*	*Eryngium* sp.	France	LGM851101 – AUA[a]	HM998810	HM998775	A1 and A2
5.	*P. eryngii* var. *eryngii*	*Eryngium* sp.	China	HIK153 – AUA	HM998840	HM998804	A1 and A2
6.	*P. eryngii* var. *eryngii*	*Peucedanum cervaria*	Italy, Remanzacco (Udine)	PN9 – UP	KF743828	KF743840	A1 and A2
7.	*P. eryngii* var. *elaeoselini*	*Laserpitium siler*	Italy (north)	HIK124 – UP[a]	HM998825	HM998789	A1 and A2
8.	*P. eryngii* var. *elaeoselini*	*Laserpitium latifolium*	Italy, Lago di Cei – Rovereto (Trento)	HIK126 – UP[a]	HM998827	HM998791	A1 and A2
9.	*P. eryngii* var. *elaeoselini*	*Laserpitium latifolium*	Romania, Potoci (Neamt)	PN5 – UP	KF743824	KF743838	A1 and A2
10.	*P. eryngii* var. *elaeoselini*	*Laserpitium latifolium*	Italy, Bellamonte (Trento)	HIK118/PN6 – UP[a]	KF743825	HM998785	A1 and A2
11.	*P. eryngii* var. *elaeoselini*	*Elaeoselinum asclepium*	Italy, Mussomeli (Sicily)	UPA30 – UP[a]	HM998819	HM998784	A1 and A2
12.	*P. eryngii* var. *elaeoselini*	*Elaeoselinum asclepium*	Italy, Rocca Ramusa (Sicily)	HIK122 – UP[a]	HM998823	HM998788	A1 and A2
13.	*P. eryngii* var. *elaeoselini*	*Elaeoselinum asclepium*	Italy, Sicily	PN13 – UP[a]	KF743831	KF743843	A1 and A2
14.	*P. eryngii* var. *elaeoselini*	*Margotia gummifera*	Spain, Alhaurín el Grande (Malaga)	HIK121 – UP	HM998822	HM998787	A1 and A2
15.	*P. eryngii* var. *elaeoselini*	*Thapsia villosa*	Spain	HIK151 – UP	HM998838	HM998802	A1 and A2
16.	*P. eryngii* var. *ferulae*	*Ferula communis*	Greece, Andros island	P102 – AUA[a]	HM998813	HM998778	A1 and A2
17.	*P. eryngii* var. *ferulae*	*Ferula communis*	Greece, Ag. Efstratios island	P109 – AUA[a]	HM998814	HM998779	A1 and A2
18.	*P. eryngii* var. *ferulae*	*Ferula communis*	France	LGM820301 – AUA[a]	HM998808	HM998773	A1 and A2
19.	*P. eryngii* var. *tingitanus*	*Ferula tingitana*	Israel, Sata Springs – Judean Mts.	HIK130 – AUA	HM998829	HM998793	A1 and A2
20.	*P. eryngii* var. *thapsiae*	*Thapsia garganica*	Italy, Madonie Mt. (Sicily)	UPA5 – UP[a]	HM998815	HM998780	A1 and A2
21.	*P. eryngii* subsp. *tuoliensis*	*Ferula* sp.	Iran, Esfahan	HIK138 – AUA	HM998836	HM998800	B1 and B2
22.	*P. eryngii* subsp. *tuoliensis*	*Ferula sinkiangensis*	China	HIK152 – AUA[a]	HM998839	HM998803	B1 and B2
23.	*P. eryngii* subsp. *tuoliensis*	*Ferula sinkiangensis*	China	HIK155 – AUA[a]	HM998842	HM998806	B1 and B2
24.	*P. eryngii* subsp. *tuoliensis*	*Ferula feruloides*	China, Sichuan	HIK156 – AUA[a]	HM998843	HM998807	B1 and B2
25.	*P. eryngii* subsp. *tuoliensis*	commercial	China	PN4 – AUA	KF743823	KF743837	B1 and B2
26.	*P. eryngii*	Apiaceae	Iran, Khansar	HIK135 – AUA	HM998833	HM998797	A1 and A2
27.	*P. eryngii*	Apiaceae	Iran, Savers Mt.	HIK136 – AUA	HM998834	HM998798	A1 and A2
28.	*P. eryngi*	*Prangos* sp.	Iran, Kordestan	HIK139 – AUA	HM998837	HM998801	A1 and A2
29.	*P. eryngii*	commercial (Huairou)	China	HIK154 – AUA	HM998841	HM998805	A1 and A2
30.	*P. ferulaginis*	Apiaceae	Hungary	LGM850404 – AUA	HM998809	HM998774	C1 and C2
31.	*P. ferulaginis*	*Ferulago campestris*	Slovenia, Pomjan (Koper)	HIK132 – UP[a]	HM998831	HM998794	C1 and C2
32.	*P. ferulaginis*	*Ferulago campestris*	Italy, Campoformido (Udine)	HIK133/PN7 – UP[a]	KF743826	HM998795	C1 and C2
33.	*P. ferulaginis*	*Ferulago campestris*	Italy, Remanzacco (Udine)	PN8 – UP[a]	KF743827	KF743839	C1 and C2
34.	*P. ferulaginis*	*Ferulago campestris*	Italy, Remanzacco (Udine)	PN10 – UP	KF743829	KF743841	C1 and C2
35.	*P. ferulaginis*	*Ferulago campestris*	Italy, Paisan di Prato (Udine)	PN14A – UP	KF743832	KF743844	C1 and C2
36.	*P. ferulaginis*	*Ferulago campestris*	Italy, Fagagna (Udine)	PN15 – UP	KF743833	KF743845	C1 and C2
37.	*P. nebrodensis* subsp. *fossulatus*	*Prangos ferulacea*	Armenia, Gegharkunik (Sevan)	HIK127 – UP[a]	HM998828	HM998792	D1 and D2
38.	*P. nebrodensis*	*Prangos ferulacea*	Iran, Kordestan	HIK137 – AUA	HM998835	HM998799	E1 and E2
39.	*P. nebrodensis*	*Prangos ferulacea*	Greece, Kyllini Mt. (Peloponnese)	P125 – AUA[a]	HM998826	HM998790	E1 and E2
40.	*P. nebrodensis*	*Prangos ferulacea*	Greece, Parnassos Mt. (Sterea Ellas)	PN1/P163 – AUA[a]	KF743820	KF743834	E1 and E2

41.	*P. nebrodensis*	*Prangos ferulacea*	Greece, Chelmos Mt. (Peloponnese)	PN2/P177 – AUA[a]	KF743821	KF743835	E1 and E2
42.	*P. nebrodensis*	*Prangos ferulacea*	Greece, Chelmos Mt. (Peloponnese)	PN3/P178 – AUA	KF743822	KF743836	E1 and E2
43.	*P. nebrodensis*	*Prangos ferulacea*	Italy, Monte Maletto – Etna (Sicily)	HIK134 – UP	HM998832	HM998796	E1 and E2
44.	*P. nebrodensis*	*Prangos ferulacea*	Italy, Madonie Mt. (Sicily)	UPA6 – UP[a]	HM998816	HM998781	E1 and E2
45.	*P. nebrodensis*	*Prangos ferulacea*	Italy, Madonie Mt. (Sicily)	UPA28 – UP[a]	HM998818	HM998783	E1 and E2
46.	*P. nebrodensis*	*Prangos ferulacea*	Italy, Piano della Madonna (Sicily)	PN12 – UP	KF743830	KF743842	E1 and E2

a Strains used for mating compatibility experiments (maintained in AUA – LGAM Culture Collection).

Palermo University (PAL), and/or it is available in pure cultures preserved in the pertinent collection of the former Institution.

Morphology

Data presented here are based on specimens either collected through the authors' fieldwork performed in various European countries (with emphasis placed in the Mediterranean area), or they were kindly provided by several individuals for the purposes of this study.

Morphological characteristics of fresh and dried basidiomes were examined by following the methods described elsewhere (Hilber 1982; Zervakis & Balis 1996), using light and phase-contrast microscopy at magnifications of up to × 1000. All sections were mounted and observed in 3 % KOH and selectively in cotton blue and Melzer's reagent. Particular emphasis was placed on microscopic features like basidiospores size, basidia size (length, width), and presence of cystidia or cystidia-like elements, measured with the aid of a micrometre in a Leitz microscope. In all cases, a minimum of 30 measurements were conducted for each character. Particularly as regards the size range of spores, 5 % of the measurements were excluded from each end of the range and are presented in parentheses, while the quotient (Q) of their dimensions was calculated as the ratio of spore length over spore width. Line drawings were prepared by using tracing paper with digital photomicrographs. Colour description and encoding terminology are from Maerz & Paul (1930).

Mating compatibility experiments

Selected dikaryotic strains (Table 1) representing various hosts and geographic areas were further processed for obtaining mating compatibility data between populations of different origin. *In vitro* production of basidiomes, single-spore isolation and pairing tests between monokaryons were performed as previously described (Zervakis 1998). Agar plugs (3 mm in diam) from 12 monokaryotic isolates per *Pleurotus* dikaryon were placed in pairs, about 15 mm apart, in all possible combinations in 90 mm Petri dishes with malt extract agar. Interpretation of mating results was performed 1–2 weeks after inoculation for ensuring an established nuclear migration and a well-formed contact zone between growing colonies. Additional pairings were carried out when these self-crosses failed to reveal all four mating types for a given dikaryon. One tester-strain was selected out of each one of the four incompatibility groups for conducting pairings among different collections, in the same way as explained above. Matings were interpreted as compatible when true clamp connections could be microscopically observed on the hyphae of the contact zone of the two colonies and away from it, under the microscope. The values given represent the percentage of successful matings (over the total of the matings performed) between the monokaryotic tester-strains of the dikaryons confronted. For inter-population or inter-taxa mating experiments, the mean of all individual results among strains from the two populations/taxa was calculated (Zervakis *et al.* 2004).

DNA isolation, PCR amplification, and sequencing

DNA isolation from pure cultures followed a modification of the protocols described by Zervakis et al. (2004) and Ravash et al. (2010). In brief, mycelia were grown on malt extract agar in Petri dishes, harvested using a sterile scalpel, frozen with liquid nitrogen and quickly homogenized into fine powder. The powder was mixed with warm (60 °C) DNA lysis buffer (3 % sodium dodecyl sulfate (SDS), 1 % 2-mercaptoethanol, and 50 mM Tris–EDTA, pH 7.2) and then 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. Genomic DNA purity and quality was determined spectrophotometrically. For DNA isolation from herbarium specimens (dried basidiomes), the same process was followed but instead of the SDS, cetyl trimethyl ammonium bromide (CTAB) was used in the lysis buffer (1 % CTAB, 10 mM Na_2EDTA, 0.7 M NaCl, and 50 mM Tris–HCl pH 8) since its use improved the quality of the DNA obtained.

Primers ITS1 and ITS4 (White et al. 1990) were used for the amplification of the ITS1-5.8S-ITS2 region (Vilgalys & Hester 1990). The IGS1 region (incl. part of the 3′-end of 28S rDNA, IGS1 and part of the 5′-end of the 5S rDNA) was amplified using primers LR12R and 5SRNA (Vilgalys lab, Duke University). The polymerase chain reaction was performed by using a PTC-200 thermocycler (MJ Research Inc., USA) at the following conditions: an initial denaturation step with 35 cycles of 30 s at 94 °C, 30 s primer annealing at 55 °C and 30 s DNA chain extension at 72 °C for the amplification of the gene regions examined. All amplifications were completed by 10 min DNA chain extension at 72 °C. PCR products were visualized in agarose gels stained with ethidium bromide; DNA was eluted from the gel by using Ultrafree-MC 0.45-μm filter units (Millipore, Bedford, MA, USA).

Clones for each isolate sequenced were obtained by cloning the respective amplicons (ITS1-5.8S-ITS2 or IGS1 regions) into the pGEM-T Easy Vector (Promega, USA) before insertion into DH5a competent cells. Plasmid DNA was purified using the NucleoSpin Plasmid QuickPure kit (Macherey-Nagel, Germany). Sequencing reactions were performed at the Institute of Molecular Biology and Biotechnology (Heraklion, Greece) and at CEMIA Inc. (Larissa, Greece).

Resulting chromatograms were assembled and edited with the CAP3 software (http://pbil.univ-lyon1.fr/cap3.php) (Huang & Madan 1999). Sequences were deposited in the GenBank and their accession numbers are presented in Table 1.

Phylogenetic analyses

Sequences were aligned through the aid of the ClustalW software (http://www.ebi.ac.uk/clustalw/). TREECON for Windows (Version 1.3b) was used for constructing phylogenetic trees from distance-matrices (Van de Peer & De Wachter 1993). Evolutionary distances (and the respective genetic similarities expressed as percentages) were calculated by using Kimura's two-parameter model (1980), and trees were generated by the neighbour-joining algorithm (Saitou & Nei 1987). Bootstrap values were derived from a total of 1000 replicates.

Bayesian Markov chain Monte Carlo (MCMC) analysis was implemented by MrBayes ver. 3.2.1 (Ronquist et al. 2012) by using the GTR + I + C model. Two runs were performed for 10 000 000 generations for ensuring that the average standard deviation of split frequencies was less than 0.01. Sampling of Markov chains was performed every 1000 generations, and the first 25 % of the sampled trees were discarded as burn-in. Moreover, evolutionary history was inferred by using the Maximum Parsimony method with the MEGA 6 software (Tamura et al. 2013). Phylogenetic analyses were performed for each DNA region sequenced and for the combined dataset. Both ITS1-5.8S-ITS2 and IGS1 regions were concatenated through Mesquite software ver. 2.75 (Maddison & Maddison 2011).

In addition to the biological material examined in this study, 27 additional *Pleurotus* sequences for ITS1-5.8S-ITS2 and seven for IGS1 obtained from the GenBank were included in the analysis for comparison purposes (their selection was made on the basis of the additional information they could confer as regards higher representation of associated plant and geographic origins).

For assessing a possible coevolution between taxa of the *Pleurotus eryngii* complex and their associated *Apiaceae* species, the phylogram prepared from sequences of the ITS1-5.8S-ITS2 region of fungal specimens was compared with a phylogenetic tree constructed on the basis of ITS1-5.8S-ITS2 sequences of the respective plants deriving from the GenBank. Sequences of *Apiaceae* species were selected in accordance to their relevance to *Pleurotus* taxa studied (i.e., their reported fungus–plant association) and their availability in the NCBI database. Evolutionary distances for the *Apiaceae* tree were calculated by using Kimura's two-parameter model, and the pertinent tree was generated by the neighbour-joining algorithm.

Results and discussion

The taxonomy and phylogeny of *Pleurotus eryngii* species complex is evaluated through the examination of specimens deriving from most of their known associated plants and geographic origins. Mating compatibility experiments performed among representative monokaryotic isolates from 27 selected dikaryons in conjunction with a thorough study of distinctive ecological and morphological features, and the outcome of the phylogenetic analysis performed for 46 specimens through the analysis of two nuclear DNA regions allowed an up-to-date account of the systematics in this complex.

Morphology

All specimens included in this study were examined as regards their macroscopical and microscopical characteristics, which are summarized in the key provided below together with habitats details. In most cases, observed features coincided with pertinent morphological descriptions available for *Pleurotus eryngii* and most of its well-established varieties (i.e. var. *eryngii*, var. *elaeoselini*, var. *ferulae*, var. *tingitanus*, and var. *thapsiae*) as well as for *Pleurotus nebrodensis*

(Hilber 1982; Gargano *et al.* 2011; Lewinsohn *et al.* 2002; Venturella *et al.* 2000; 2002; Zervakis & Balis 1996). On the other hand, relatively scarce information was available on Asiatic populations identified as *P. eryngii* var. *tuoliensis* and *Pleurotus fossulatus*. For these two groups, as well as for *Pleurotus* strains growing in association with *Ferulago campestris* in Europe, more detailed accounts follow.

Light-coloured pilei (white/off-white to cream to light beige) were present throughout the known distribution of the complex and characterize populations growing on Asiatic *Ferula* spp., *Prangos ferulacea* (both in Asia and Europe), as well as material associated with *Eleaoselinum* and *Laserpitium* spp. In contrast, pilei with dark colours were typical of taxa growing on *Eryngium*, *Thapsia*, and European *Ferula* spp. Furthermore, the rare and practically non-studied *Pleurotus* growing on *F. campestris* residues demonstrated a relatively wide variation in pilei colours ranging from off-white to brown. However, this morphological feature could be of use (albeit limited) in grouping among plant-related populations in contrast to other macroscopical characters which are rather constant. On the other hand, basidiospores dimensions proved to be of diagnostic value since they vary markedly among the *Pleurotus* taxa studied. For facilitating grouping of strains on the basis of their associated plants, basidiospores mean width was plotted against their quotient (length over width dimensions) on the same graph (Fig 1). In this way, one particular group was clearly separated from the rest, i.e. European specimens growing on *F. campestris* presented respective values of 5.0 μm and 2.54 (spore dimensions: 11.3–13.4 × 4.6–5.1 μm). Of interest was the variability

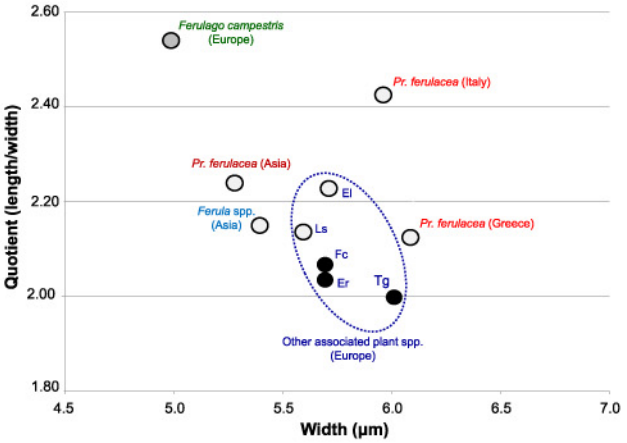

Fig 1 – Basidiospores mean width presented in respect to their mean quotient values for *Pleurotus* material examined in this study and organized on the basis of their associated plant species and geographic origin. Empty (white) circles represent taxa/populations with light-coloured pilei and filled (black) circles represent taxa/populations with dark-coloured pilei. Within the larger dotted area (which includes data from *P. eryngii* varieties occurring in Europe) individual circles account for material associated with *Elaeoselinum asclepium* subsp. *asclepium* [El], *Eryngium* spp. [Er], *Ferula communis* [Fc], *Laserpitium* spp. [Ls], and *Thapsia garganica* [Tg].

demonstrated by *Pr. ferulacea* associated material, whose basidiospore dimensions differed on the basis of their geographic origin; Sicilian specimens presented width and quotient values of 6.0 μm and 2.42 and Greek material showed values of 6.2 μm and 2.15 (spore dimensions of European specimens growing on *Pr. ferulacea*: 12.2–17.4 × 5.5–8.2 μm), while specimens from Asia presented values of 5.3 μm and 2.24 (spore dimensions: 9.0–14.0 × 4.5–6.0 μm). On the other hand, *P. eryngii* varieties represented on the basis of their associated plant showed relatively wide variation ranging from mean width values of 5.4 (var. *tuoliensis*, spore dimensions: 8.7–14.3 × 4.5–6.3 μm) to 6.1 μm (vars. *tingitanus* and *elaeoselini*) and from quotients of 2.00 (var. *thapsiae*) to 2.21 (var. *tingitanus*).

Ecology

The importance of ecological factors should be also emphasized since both habitat diversification and allochrony in producing basidiomes play significant roles in the ability of the fungus to cross-hybridize in nature with other populations. For example, our long-term observations showed that *Pleurotus* isolates growing in association with *Prangos ferulacea* form basidiomes from spring to early summer as do populations associated with *Thapsia garganica*, *Elaeoselinum asclepium* and to a lesser extend *Ferula* spp. However, *Pr. ferulacea* is the only associated plant among the aforementioned which appears in altitudes exceeding 1300 m, whereas *E. asclepium* distribution is limited to a rather narrow altitudinal zone (i.e. 400–700 m). In addition, *T. garganica* occurs at a wider zone (sea-level to 1400 m) and *Ferula* spp. at rather low altitudes (sea-level to ca. 700 m). On the other hand, *Pleurotus* associated with *Eryngium* spp. do not form basidiomes during spring or summer; their appearance being prominent from autumn to early winter at altitudes ranging from sea-level to 1100 m.

Mating compatibility

Results of intra-stock crosses were in accordance to the bifactorial heterothallic mode of sexual reproduction for all 27 *Pleurotus* dikaryons examined (selected on the basis of best possible representation of each fungal taxon, and its host-plant and/or geographic origin diversity). This tetrapolar system with multiple alleles was also evidenced in previous compatibility studies with *Pleurotus eryngii* material (Hilber 1982; Zervakis & Balis 1996). The outcome of the matings performed in the frame of the present work among all monokaryotic isolates is presented in Fig 2.

Results demonstrated notably higher percentages of successful pairings (i.e., resulting in hybrid-dikaryon formation) among isolates of *P. eryngii* from various European origins; no common incompatibility factors were detected. Within this group of 14 parental strains, matings between monokaryons associated with the same host plant provided almost complete intercompatibility (94–100 %), whereas matings between monokaryons from different hosts varied from 78 % (*Eryngium* spp. *vs.* *Laserpitium* spp.) to 97 % (*Eryngium* spp. *vs.* *Thapsia garganica*). In general, relatively lower values (albeit high as concerns determination of affinity within populations)

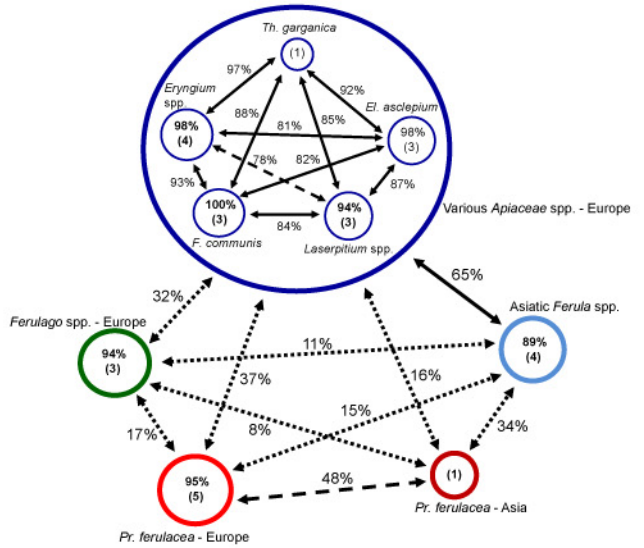

Fig 2 – Schematic representation of the mating compatibility results (expressed in percentages of successful pairings) among *Pleurotus* monokaryotic tester-strains from selected isolates (their number-size appears in parentheses within each circle denoting the particular taxon/population; details on their identity are presented in Table 1) appearing on the basis of their associated plant species and geographic origin. Compatibility percentage values are represented by either solid lines (when exceeding 50 %), dashed lines (when between 20 and 50 %) and dotted lines (when less than 20 %). Values within each circle represent respective results for intrataxon/intrapopulation parings.

were observed in matings between isolates associated with *Laserpitium* spp. *vs.* other European host plants, e.g. *Ferula communis*, *T. garganica*, and *Elaeoselinum asclepium* (78–87 %) and between *E. asclepium* and the rest (81–92 %).

On the other hand, when monokaryons from these 14 *P. eryngii* strains were paired against strains associated with other hosts and/or distant geographic origins, percentages of successful matings decreased considerably, ranging from as low as 16 % (*vs.* an Asiatic strain growing on *Prangos ferulacea*) to 65 % (*vs. P. eryngii* var. *tuoliensis* isolates from Asiatic *Ferula* spp.). Of interest were the rather low values of intercompatibilty obtained among the *P. eryngii* strains and isolates originating from nearby localities on *Ferulago campestris* and *Pr. ferulacea* (32 % for the former originating from Slovenia and Italy, and 37 % for the latter from Italy and Greece). As regards the rest of the 'intrahost' pairings among strains associated with the same host and geographic regions, mating percentages were again high (89–95 %); the lowest percentage was noted in pairings between monokaryons *P. eryngii* var. *tuoliensis* from China. However, noteworthy were again the 'interhost' compatibilities which in most cases were very low, e.g. 8–17 % in pairings of *F. campestris* associated isolates *vs. Pleurotus* from other plant spp. In contrast, relatively higher values were detected between *P. eryngii* var. *tuoliensis* and European *P. eryngii* varieties (65 %), and between isolates associated from *Pr. ferulacea* in Europe and Asia (48 %).

Sequence data analysis of ITS1-5.8S rRNA gene-ITS2 and IGS1 regions

Pleurotus eryngii species-complex specimens shared a similar ITS1-5.8S-ITS2 region with a size ranging from 622 to 641 nucleotides (nt). This size variation was mainly due to the presence of two deletions, one of eight nt beginning at position 235 and the other of 11 nt beginning at position 420 of the ITS1-5.8S-ITS2 rRNA gene, both of them detected only in *Pleurotus* strains associated with *Ferulago campestris* from Slovenia and Italy (the second deletion was also present in the Hungarian strain associated with *F. campestris*). In addition to these deletions, this particular group presented four point mutations (positions 99, 108, 418, and 505) and three insertions of one nt each (after positions 505, 559, and 591). Isolates representing *P. eryngii* varieties could be distinguished from the rest of the material studied by a single point mutation (at position 183), and *Pleurotus* associated with *Prangos ferulacea* from Europe also by a single point mutation (at position 154). As regards strains assigned to *Pleurotus fossulatus* (*Pr. ferulacea* from Asia), they were discriminated by a 2 nt insertion (after position 115) and a single nt deletion (at position 189). On the other hand, *Pleurotus* associated with *Ferula* spp. from Asia presented two point mutations (at positions 93 and 149), a single nt deletion (position 183) and 1–2 nt insertions (after position 426).

The size of IGS1 region ranged from 902 to 924 nt, a variation attributed to the presence of two deletions, one of ten nt beginning at position 506 and the other of 12 nt beginning at position 554, both of them detected only in *Pleurotus* strains associated with *F. campestris*. In addition to these deletions, this particular group contained five point mutations (positions 264, 384, 504, 567, and 876) and three insertions of 1–2 nt each after positions 271, 334, and 687. Isolates from different *P. eryngii* varieties could be distinguished from the rest of the material studied by three point mutations at positions 523, 569, and 657, while Asiatic *Pleurotus* associated with *Ferula* spp. presented four point mutations (positions 257, 652, 679, and 817) and one insertion of one nt after position 553. Furthermore, *Pleurotus* growing on *Pr. ferulacea* (i.e., *Pleurotus nebrodensis*) demonstrated six point mutations (positions 264, 277, 390, 507, 565, and 891) and a one nt insertion after position 687. On the other hand, the only *P. fossulatus* strain examined presented two point mutations (positions 334 and 565) and a one nt insertion after position 687. In all cases, position numbers for ITS-5.8S rRNA gene and IGS1 region are given according to the sequences of *P. eryngii* var. *eryngii* strain P63 (GenBank accession numbers HM998811 and HM998776, respectively), which served as 'consensus sequences' possessing the most frequently found nt for each position of the alignment.

Phylogenetic analysis based on ITS1-5.8S-ITS2 and IGS1 sequencing data

Phylogenetic analysis of ITS-5.8S-ITS2 data was performed with 46 unambiguously aligned sequences deriving from material examined in the frame of this work plus 27 sequences obtained from the GenBank.

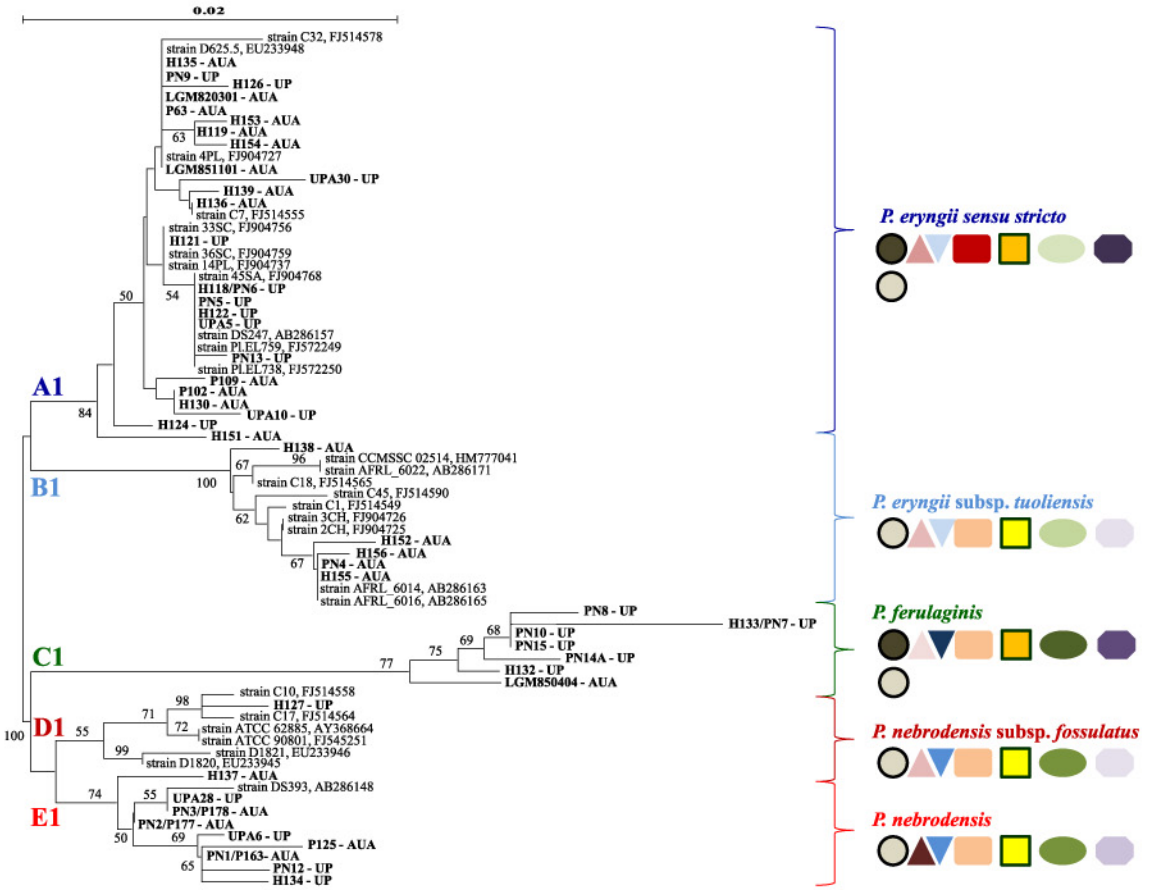

Fig 3 – Phylogenetic tree illustrating the relationships among 46 specimens of the *P. eryngii* species complex (marked in bold) based on ITS1-5.8S-ITS2 sequences, while additional sequences from the GenBank were included in the analysis (strain code and accession no. are noted). Trees were generated by using the neighbour-joining algorithm and the distance was calculated on the basis of Kimura's two-parameter model. Bootstrap values derived from a total of 1000 replicates, and are noted when they exceeded the 50 % threshold. Each distinct cluster is represented by a different capital letter, while the respective associated plants are noted together with the geographic origin of the *Pleurotus* material. In addition, symbols are used for indicating principal/diagnostic morphological and ecological characters as follows: Circle, dark, and light colours: dark and light coloured pilei respectively; Triangle, darker to lighter colours correspond to basidiospores mean width: >6.1 μm, 5.6–6.1 μm, 5.0–5.5 μm, <5.0 μm; Reverse triangle, darker to lighter colours correspond to basidiospores mean quotient: >2.50, 2.22–2.49, <2.22; rectangle, dark, and light colours correspond to basidiomes period of appearance: from autumn to late spring, and from spring to early summer respectively; Square, dark, and light colours correspond to altitude zone of habitats: low to medium altitudes (0–1200 m) and high altitudes (>1200 m) respectively; Oval, darker to lighter colours correspond to associated host plant: *Ferulago campestris*, *Prangos ferulacea*, Asiatic *Ferula* spp., other *Apiaceae* spp.; Octagon, darker to lighter colours correspond to geographic origins: Europe and greater Mediterranean region, Slovenia/N.E. Italy/Hungary, Sicily and Greece, Asia. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

By using the Kimura's distance method, five major clusters (A1, B1, C1, D1, and E1) were produced with bootstrap values exceeding 55 % (Fig 3). A1 included all varieties occurring in Europe and Middle East (i.e., var. *elaeoselini*, var. *eryngii*, var. *ferulae*, var. *thapsiae*, and var. *tingitanus*) plus isolates from continental Asia (Iran and China); it was thus composed of specimens bearing basidiomes with both white and dark coloured pilei from a wide habitats range. However, their individual positioning within cluster A1 was performed without evidencing any particular relation in respect to their host or geographic origin despite the fact that the material studied originated from 14 different plant species and nine countries. Noteworthy was the grouping of strain D625.5 in this particular cluster (deposited as *Pleurotus fossulatus* in GenBank database), and the rather distant positioning of strains HIK124 (from N. Italy on *Laserpitium siler*) and HIK151 (from Spain on *Thapsia villosa*) from the rest of the *Pleurotus eryngii* specimens examined. Cluster B1 consisted of strains with white to cream coloured pilei occurring in Iran and China, and growing in association with *Ferula* plant-hosts only, i.e. *Ferula assa-foetida*, *F. ferulaeoides*, *Ferula ovina*, *Ferula sinkiangensis*, and *Ferula* sp. (all originally assigned either as *P. eryngii* or as *P. eryngii* var. *tuoliensis*).