Species delimitation in *Pleurotus eryngii* species-complex inferred from ITS and EF-1α gene sequences

Stefania M. Mang* and Giovanni Figliuolo

*Dipartimento di Biologia, Viale dell’Ateneo Lucano 10, Università degli Studi della Basilicata, Potenza 85100, Italy*

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Taxon delimitation in *Pleurotus eryngii* species-complex was investigated using an independent loci-based approach. A collection of 47 *Pleurotus* strains was analyzed through nucleotide sequences of the internal transcribed spacer region (ITS1, 5.8S and ITS2) and M13 minisatellite fragments. A sub-sample made of 12 clones representative of the ITS phylogenetic clusters was sequenced to elucidate variations in the elongation factor 1 alpha gene (EF1-α). A unique clade made of three evolutionary units, i.e. Chinese *nebrodensis*, Sicilian *nebrodensis* and *eryngii-ferulae* from the Mediterranean region, was revealed. The evolutionary genetic divergences were highly correlated (r = 0.86) for both genes. The average number of base substitutions per site was higher for EF1-α gene (0.013) than for ITS (0.001). M13 minisatellite polymorphism marked a clear association between strains and geographic origin but its resolution in taxa delimitation was low. Data from ITS and EF1-α genes supported the hypothesis that the species-complex is a unique gene-pool. Within the species-complex, the *nebrodensis* type is characterized by a level of nucleotide substitutions lower than as expected for its ranking at a species level.

**Keywords:** elongation factor 1-alpha; ITS; M13 marker; Pleurotus; taxonomy

**Introduction**

*Pleurotus eryngii* species-complex includes widely distributed basidiomycetes occurring in Europe, the Mediterranean region and Asia, specific to natural and semi-natural grasslands (*Festuco–Brometea* and *Thero–Brachypodietea* associations), and also grown as edible mushrooms on artificial substrates mainly in Europe, Israel, China and Japan.

Systematics of *P. eryngii* (DC.:Fr.) Quél based on morphological (Linnaeus 1758), biological (Mayr 1942), and geographical and ecological (van Valen 1976) data lead to different classifications of the *P. eryngii* populations. Moreover, within the species-complex, taxa were grouped in botanical varieties (Inzenga 1863; Quelét 1872; Lanzi 1894; Hilber 1982; Mou et al. 1987; Lewinsohn et al. 2002; Venturella et al. 2002), sub-species (Zervakis and Balis 1996) or species (Candusso and Basso 1995).

The *P. eryngii* species-complex classification has been driven by the “host specificity” approach considering potential host plants, all belonging to the Apiaceae and Asteraceae families, on which the fungus performs as a weak parasite on stems and roots (Zervakis et al. 2001a; Lewinsohn et al. 2002). Based on this approach, *P. eryngii* (DC: Fr.) Quél. var. *eryngii* has been associated to root residues of *Eryngium campestre* L., *P. eryngii* var. *ferulae* Lanzi to *Ferula communis* L. subsp. *communis*, *P. eryngii* var. *tingitanus* Lewinshon et al. to *Ferula tingitana*, *P. nebrodensis* (Inzenga) Sacc. to *Cachrys ferulacea* (L.) Calestani, *P. eryngii* var. *elaeoselini* Venturella et al. to *Elaeoselium asclepium* (L.) Bertol. subsp. *asclepium*, *P. eryngii* var. *thapsiae* Venturella et al. to *Thapsia garganica* L. and *P. eryngii* var. *touliensis* Mou to *Ferula sinkinagensis* Shen (Lewinsohn et al. 2002; Venturella et al. 2000, 2002; Kawai et al. 2008).

In a greenhouse-replicated trial, De Gioia et al. (2005) found lower morphological differences among the a priori classified vars. *ferulae*, *eryngii*, and major differences with respect to the *nebrodensis* type. Taking into account morphological, biological and molecular data, the *nebrodensis* type has also been classified as a distinct species (Zervakis et al. 2001b).

Incomplete reproductive barriers are still present between taxa belonging to the *P. eryngii* species-complex (Zervakis and Balis 1996; Taylor et al. 2000; Kawai et al. 2008; Rodriguez-Estrada et al 2010). Mating experiments performed under *in vitro* conditions showed that host-specific varieties were not isolated reproductively from each other and it was argued that mating between different strains could contribute to the homogenisation of genetic diversity within the species-complex (Hilber 1982; Vilgalys et al. 1993; Zervakis and Balis 1996). In addition, no definitive data is yet available about the ecological...
Markers-assisted genomic analysis significantly increased the resolution of genetic relatedness studies within and among broader groups of extended kin (Vilgalys and Sun 1994; Zervakis et al. 1994, 2001b; Reverberi et al. 2000; Zhang et al. 2000, 2006; Venturella 2002; Urbanelli et al. 2002, 2003, 2007; Della Rosa et al. 2004; De Gioia et al. 2005; Ro et al. 2007; Kawai et al. 2008), as well as phylogenetic classification at a greater evolutionary depth (Zervakis et al. 1994, 2004; Gonzales and Labarère 2000; Taylor et al. 2000; Albertò et al. 2002; Rodríguez-Estrada et al. 2010). The degree of genetic separation between the supposed host specific \textit{ferulae} and \textit{eryngii}, however, is still controversial, since different authors (Zervakis et al. 2001b; Urbanelli et al. 2002, 2007; De Gioia et al. 2005) reported phylogenetic relationships at the level of extended kinship within local groups or sub-populations, rather than phylogenetic structures at intermediate or great evolutionary depths.

Several phylogenetic analyses using sequence data from the SSU rRNA mitochondrial gene or nuclear ITS DNA have shed light on inter- and intra-specific classification in \textit{Pleurotus} (Vilgalys and Sun 1994; Gonzales and Labarère 2000; Albertò et al. 2002). These investigations showed that \textit{P. eryngii} is more related to \textit{P. ostreatus} (Jacq.: Fr.) Kummer and \textit{P. populinus} Hilber and Miller than to other \textit{Pleurotus} species. Within the \textit{P. eryngii} gene-pool, the \textit{nebrodensis} type is the most diverging population and the extent of genetic variation does not clearly indicate whether its differentiation is at a “species” or at a “variety” level.

Different nuclear ribosomal RNA genes were extensively exploited in molecular fungal systematics: the small subunit RNA (17-18S) (Bruns et al. 1992; Swann and Taylor 1995; Guarro et al. 1999; Gonzales and Labarère 2000); large subunit RNA (25–28S) (Hibbett 1992; Hopple and Vilgalys 1999; Moncalvo et al. 2000; Maruyama et al. 2005; Padamsee et al. 2008); internal transcribed spacer (ITS) (White et al. 1990; O’Donnell 1992; Gardes and Bruns 1993; Chen et al. 2001; Park et al. 2004; Tuchwell et al. 2005; Zhang et al. 2006; Rodríguez-Estrada et al. 2008) and the intergenic spacer (IGS), including SS RNA (Hibbett 1992; Appel and Gordon 1996; James et al. 2001; Alejandro and Todd 2005; Zhang et al. 2006; Geiser et al. 2007). ITS sequence genetic information performed as the best to discriminate within and between species in several living organisms including fungi.

\textit{Pleurotus} molecular systematics based on both ITS and IGS sequence variation revealed two groups (\textit{P. eryngii} var. \textit{ferulae} and \textit{nebrodensis} collected on \textit{Ferula sinkiangensis}) within the Chinese germplasm (Zhang et al. 2006). In addition, Kawai et al. (2008) used ITS sequences to propose the taxonomic position of the Chinese \textit{Pleurotus eryngii} “Bai-Ling Gu” at variety level (\textit{P. eryngii} var. \textit{touliensis}). Furthermore, phylogenetic relationships have been inferred among varieties of \textit{P. eryngii} using both ITS region and β–tubulin gene. Either allelic polymorphisms within the β-tubulin gene or lack of variation in the ITS region has been detected between the varieties \textit{eryngii} and \textit{ferulace} (Rodríguez-Estrada 2008; Rodríguez-Estrada et al 2008). Thus, these genes proved to be unable to provide a proof for species delimitation within the \textit{P. eryngii} species-complex. Nonetheless, different genes can provide specific genetic information to resolve different biological problems. Among these genes, the translation elongation factor (EF1-α), involved in ribosomal protein synthesis in eukaryotes, was previously used for phylogeny studies on several basidiomycetes (Matheny et al. 2007), ascomycetes, such as \textit{Fusarium} species (Kristensen et al. 2005; Bentley et al. 2006; Maphosa et al. 2006; Steward et al. 2006; Dupont et al. 2007; Mansfield and Kulda 2007), on arbuscular mycorrhizal fungi (Helgason et al. 2003), and \textit{Puccinia} and \textit{Uromyces} spp. (Van der Merwe et al. 2007). Even so, this gene is still less exploited for \textit{Pleurotus} genus (Marongiu et al. 2005; Mang 2008; Mang and Figliuolo 2008; Rodríguez-Estrada et al. 2010).

According to Avise and Ball (1990), the phylogenetic species concept can contribute to a significant advance in species recognition if the magnitudes of phylogenetic patterns and the historical and reproductive reasons for such patterns are integrated as interpretative tools. Within this context, the goal of this study was to provide additional evidence about taxon delimitation within the \textit{P. eryngii} gene-pool by focusing on ITS and EF1-α gene sequence analysis along with the M13 minisatellite polymorphisms.

**Materials and methods**

**Strains studied**

The fungal strains used in this study were characterised by a brown or white pileus colour, and had different geographic origins – Apulia Region (South Italy), Sicily (Mediterranean Area), Sardinia (Mediterranean Area) and China, and their preliminary classification (vars. \textit{eryngii}, \textit{ferulae} and \textit{nebrodensis}) was based on the host plant (Table 1).

All cultures were maintained on malt extract agar medium (5% malt extract, 1.5% agar), stored at 4°C in vials and the vouchers kept in the fungal culture collection (De Gioia et al. 2005). Samples were analyzed with both ITS gene and M13 marker. A group composed of 12 strains, each one nested within the ITS clusters, was used to clone the EF1-α gene. Furthermore, the selected set of clones
Table 1. *Pleurotus eryngii* sample core (47 strains) characterized via molecular analyses. The a priori classification, based on overall morphology ideotypes, was: type *nebrodensis*, *ferulae* and *eryngii*. The phylogenetic sub-clusters based on ITS and EF-1α gene sequences were: A = *P. nebrodensis* (Sicily), B = *P. nebrodensis* (China) and C = *P. eryngii* (including the a priori type *ferulae* and *eryngii* from Apulia, Sardinia and Sicily). All sequences were generated in this study (for details see GenBank accession numbers).

| Phylogenetic sub-cluster | Strain label | Origin   | Presumed host plant | Pileus colour | GenBank accession No. ITS/EF1-α |
|--------------------------|--------------|----------|---------------------|---------------|-------------------------------|
| B                        | *Pleurotus nebrodensis* | 1 CH China | *Ferula sinkiangensis* | White         | FJ904724/FJ904775            |
|                          |              | 2 CH China | *Ferula sinkiangensis* | White         | FJ904725                      |
|                          |              | 3 CH China | *Ferula sinkiangensis* | White         | FJ904726                      |
| A                        |              | 37 SC Sicily | *Cachrys ferulacea* | White         | FJ904760/FJ904771            |
|                          |              | 38 SC Sicily | *Cachrys ferulacea* | White         | FJ904761                      |
|                          |              | 39 SC Sicily | *Cachrys ferulacea* | White         | FJ904762/FJ904772            |
|                          |              | 40 SC Sicily | *Cachrys ferulacea* | White         | FJ904763                      |
|                          |              | 41 SC Sicily | *Cachrys ferulacea* | White         | FJ904764/FJ904773            |
|                          |              | 42 SC Sicily | *Cachrys ferulacea* | White         | FJ904765/FJ904774            |
| C                        | *Pleurotus eryngii* | 4 PL Apulia | *Ferula communis* | Brown         | FJ904727                      |
|                          |              | 5 PL Apulia | *Eryngium campestre* | Brown         | FJ904728                      |
|                          |              | 6 PL Apulia | *Ferula communis* | Brown         | FJ904729                      |
|                          |              | 7 PL Apulia | *Ferula communis* | Brown         | FJ904730                      |
|                          |              | 8 PL Apulia | *Ferula communis* | Brown         | FJ904731                      |
|                          |              | 9 PL Apulia | *Eryngium campestre* | Brown         | FJ904732                      |
|                          |              | 10 PL Apulia | *Ferula communis* | Brown         | FJ904733                      |
|                          |              | 11 PL Apulia | *Eryngium campestre* | Brown         | FJ904734                      |
|                          |              | 12 PL Apulia | *Ferula communis* | Brown         | FJ904735/FJ904776            |
|                          |              | 13 PL Apulia | *Ferula communis* | Brown         | FJ904736                      |
|                          |              | 14 PL Apulia | *Eryngium campestre* | Brown         | FJ904737                      |
|                          |              | 15 PL Apulia | *Eryngium campestre* | Brown         | FJ904738                      |
|                          |              | 16 PL Apulia | *Ferula communis* | Brown         | FJ904739/FJ904777            |
|                          |              | 17 PL Apulia | *Ferula communis* | Brown         | FJ904740                      |
|                          |              | 18 PL Apulia | *Ferula communis* | Brown         | FJ904741                      |
|                          |              | 19 PL Apulia | *Eryngium campestre* | Brown         | FJ904742                      |
|                          |              | 20 PL Apulia | *Eryngium campestre* | Brown         | FJ904743                      |
|                          |              | 21 PL Apulia | *Eryngium campestre* | Brown         | FJ904744/FJ904778            |
|                          |              | 22 PL Apulia | *Ferula communis* | Brown         | FJ904745                      |
|                          |              | 23 PL Apulia | *Eryngium campestre* | Brown         | FJ904746                      |
|                          |              | 24 PL Apulia | *Eryngium campestre* | Brown         | FJ904747                      |
|                          |              | 25 PL Apulia | *Eryngium campestre* | Brown         | FJ904748                      |
|                          |              | 26 PL Apulia | *Eryngium campestre* | Brown         | FJ904749                      |
|                          |              | 27 PL Apulia | *Eryngium campestre* | Brown         | FJ904750                      |
|                          |              | 28 PL Apulia | *Eryngium campestre* | Brown         | FJ904751                      |
|                          |              | 29 PL Apulia | *Ferula communis* | Brown         | FJ904752                      |
|                          |              | 30 PL Apulia | *Ferula communis* | Brown         | FJ904753                      |
|                          |              | 31 PL Apulia | *Eryngium campestre* | White         | FJ904754/FJ904779            |
|                          |              | 32 PL Apulia | *Eryngium campestre* | White         | FJ904755/FJ904780            |
|                          |              | 33 SC Sicily | *Ferula communis* | Brown         | FJ904756                      |
|                          |              | 34 SC Sicily | *Ferula communis* | Brown         | FJ904757                      |
|                          |              | 35 SC Sicily | *Ferula communis* | Brown         | FJ904758                      |
|                          |              | 36 SC Sicily | *Eryngium campestre* | Brown         | FJ904759/FJ904781            |
|                          |              | 43 SA Sardinia | *Eryngium campestre* | Brown         | FJ904766                      |
|                          |              | 44 SA Sardinia | *Eryngium campestre* | Brown         | FJ904767                      |
|                          |              | 45 SA Sardinia | *Eryngium campestre* | Brown         | FJ904768                      |
|                          |              | 46 SA Sardinia | *Eryngium campestre* | Brown         | FJ904769/FJ904782            |
|                          |              | 47 SA Sardinia | *Eryngium campestre* | Brown         | FJ904770                      |

was composed of at least one specimen from each eco-geographic origin and representative of the a priori defined taxon of the *P. eryngii* species-complex. The *P. ostreatus* nucleotide sequences of the ITS region and EF1-α gene (accession numbers EU520255 and AY883432, respectively) retrieved from the GenBank database (NCBI) were used as reference for a species within the genus for phylogenetic analyses.
**DNA isolation**

DNA was isolated from freshly harvested mycelium scraped off the surface of a growing colony as described in De Gioia et al. (2005).

**ITS amplification and sequencing**

The entire region of nuclear rDNA composed of ITS1 and 2 spacers and including also the 5.8S RNA gene was amplified using the universal primers: ITS1 (5′-TCCGTAAGGTAGAACCCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATGGATATGC-3′), as reported in White et al. (1990). The same oligos were used as sequencing primers. Each PCR reaction, containing in a 50-μl reaction volume 1X PCR buffer, 2 mM MgCl2, 0.25 mM dNTPs, 1 U Taq Phusion™ High-Fidelity DNA polymerase (Finnzymes, Finland), 0.2 μM of each primer and 20 ng of purified total DNA template, was carried out at least twice. Polymerase chain reactions were performed with I-Cycler thermacycler (BioRad, Hercules, CA, USA) and consisted of the following steps: 30 s at 98°C, 10 s at 98°C for 30 cycles, 30 s at 50°C and 30 s at 72°C, 5 min at 72°C. Amplified DNA fragments were separated in a 1% agarose gel for further analyses. The ITS amplicons yield was sufficient to allow direct sequencing. PCR products were purified and concentrated using standard protocols (Sambrook et al. 2001). DNA was dried by lyophilization, resuspended in 5 μl of sterile water, and then used as template in a sequencing grade reaction performed with BigDye® Terminator v.1.1 Cycle Sequencing Kit (Figliuolo and Di Stefano 2007). Sequencing products were cleaned with Centri-Sep columns (Princeton Separations, Inc., Princeton, NY, USA), lyophilized with Hetosicc apparatus and diluted in formamide. Sequencing was achieved with AB3130 Genetic Analyzer (Applied Biosystems, Inc., 2005). Sequence alignments and SNPs discovery were performed with SeqScape v. 3.1 Program (Applied Biosystems, Inc., 2005). Sequences have been deposited in GenBank/NCBI (accession numbers FJ904774–FJ904782; Table 1). Alignments were carried out using the CLUSTALW (progressive alignment method) into the MEGA 4.0 (Tamura et al. 2007) Evolutionary Genetic Analysis software.

**EF-1α gene amplification, cloning and clones analyses**

The EF-1 α gene region was amplified using Pleurotus genomic DNA as a template and the overlapping degenerated EF1-α primer sequences (Rehner 2001). Primer combination 526F (forward: 5′-GTCGTYGTYATGHCAYGT-3′) × 1567R (reverse: 5′-ACHGTRCRATACCCACRATCTT-3′) was used to amplify 1000 bp of the EF1-α gene. PCR reaction conditions were: 1 min at 98 °C; 10 s at 98 °C for 30 cycles, 30 s at 65 °C, 15 s at 72 °C, 5 min at 72 °C. The amplicons from single locus were directly cloned into PCR Zero Blunt Vector using the Zero Blunt PCR Cloning Kit (Invitrogen USA). Twelve positive colonies were cultured into Luria broth media plus antibiotic (kanamycin 50 μg/ml). Subsequently, plasmid DNA was extracted from each positive colony using a PureLink HQ Mini Plasmid Purification Kit (Invitrogen). The EF1-α gene sequencing was carried out using M13 sequencing primers. Sequencing grade reaction preparation, chromatogram analysis and SNPs discovery were performed as reported above for the ITS region. The EF1-α gene nucleotide sequences deposited in GenBank/NCBI have accession numbers FJ904771–FJ904782 (Table 1).

**Phylogenetic analyses**

Nucleotide sequences were analyzed with MEGA 4.0 software, treating gaps as missing data, excluding ambiguous positions (complete deletion option) and using the CLUSTAL alignment method. Following sequence alignment, the evolutionary distances were calculated as the model of base substitutions per site used for calculating evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Codon positions included were 1st+2nd+3rd+Noncoding. ITS and EF1-α data were analyzed both separately and in combination. For each gene, the evolutionary history was inferred using either the Neighbor-Joining (NJ) method (Saitou and Nei 1987) or the Maximum Parsimony (MP) method (Eck and Dayhoff 1966). A bootstrap consensus un-rooted tree (cut-off value 70%) was inferred from 1000 replicates to represent the evolutionary history of the taxa (Felsenstein 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Since the clustering outputs from the two clustering methods were concordant, only the NJ trees have been presented in this paper. Before combining the ITS and EF1-α loci across 12 different specimens for phylogenetic analyses, the relationship strength between the evolutionary distances of the above genes was tested. Using regression analysis (Proc reg in SAS Software) between the two matrices of genetic distances, the regression coefficient between ITS and EF1-α evolutionary distances and the r² value were computed with SAS v. 9.1 2002-2003 (SAS Institute Inc., Cary, NC, USA).

**Minisatellite M13 amplification and analysis**

Minisatellite variation analysis was carried out using the universal primer M13 (GAGGGTGCCGTTCT) and amplifying the repetitive DNA genomic sequence (Latouche et al. 1997). PCR reaction was performed as indicated by Williams et al. (1990) with minor modifications (25 μl final volume of PCR reaction, containing 1×
PCR buffer, 2 mM MgCl₂, 0.25 mM dNTPs, 1 U Taq DNA polymerase (Invitrogen), 0.2 μM M13 universal primer and 20 ng of genomic DNA template. Amplifications were performed with I-Cycler thermalcycler (BioRad) and consisted of the following steps: 2 min at 95°C, 1 min at 45°C for 35 cycles, 1 min at 45°C, 1 min at 72°C, 5 min at 72°C. Finally, amplified DNA fragments were separated in a 2% agarose gel and visualized with ethidium bromide.

M13 fragment analysis
Each M13 marker band was visually detected and relative mobility was recorded using a binary code. To calculate genetic distance indices, each band, at a given level of molecular weight, was interpreted as a single allele and each genotype as a single haplotype. Faint bands were not scored. The Dice’s genetic diversity index (GD), measuring the number of unshared bands from multiple loci profile for each genotypic pairwise comparison (Nei and Li 1979), cluster analysis (Proc cluster, Average method) and tree drawing (Proc tree in SAS Software), were computed (SAS v. 9.1, 2002–2003).

Results
ITS and EF-1α nucleotide sequence variation
The PCR reaction performed with ITS1/4 universal primers yielded a single amplicon of about 700 bp allowing direct sequencing of the ITS region. A set of 47 ITS region nucleotide sequences were obtained and analysed (Table 1) along with the 762-bp ITS sequence of P. ostreatus (EU520255) retrieved from GenBank (NCBI). The average content in four bases of the ITS region for the 47 strains belonging to P. eryngii species-complex was: T = 29.7%, C = 22.4%, A = 26.1% and G = 21.8%, with a transitions/transversions ratio equal to 1.4. The final alignment contained 615 nucleotide sites, of which 598 sites were constant, 17 were variable. Of 17 variable sites, 15 were parsimony-informative and two singletons (parsimony-uninformative). Namely, zero variable sites were recorded in nebrodensis type from China (three strains), one site was recorded in nebrodensis from Sicily (six strains) and six sites were registered in eryngii-ferulae types from Apulia and Sardinia (38 strains). For the outgroup strain P. ostreatus (AY883432), the number of variable sites increased to 57. The distribution of the variable sites over the EF1-α clone set was partitioned between the gene-pools as follows: six sites in nebrodensis from Sicily and 15 sites were observed in eryngii-ferulae types. The average evolutionary divergence for P. eryngii species-complex, without considering P. ostreatus, was higher for EF-1α (d = 0.013) than for ITS (d = 0.001) (Table 2), as shown by the linear regression analysis of pairwise evolutionary genetic distances, computed using the common set of twelve strains for both genes (Figure 4). Also, divergence within nebrodensis was higher (d = 0.002 for ITS and d = 0.009 for EF-1α) than within eryngii-ferulae types (d = 0.000 for ITS and d = 0.007 for EF-1α) (Table 2; Figures 1 and 2). The divergence within the nebrodensis is consistent with the inter-continental geographic distance between Chinese and Sicilian strains. On the other hand, the eryngii-ferulae strains from the Mediterranean area showed a reduced within gene-pool divergence (Table 2; Figures 1 and 2). The average evolutionary divergence between nebrodensis and eryngii-ferulae types was d = 0.002 for ITS and d = 0.017 for EF-1α (Table 2). Including P. ostreatus, the overall mean genetic distance for the ITS was 0.028 and for the EF-1α was 0.016 (Table 2).

The pairwise genetic distances at interspecific level (P. ostreatus vs. nebrodensis or P. ostreatus vs. eryngii-ferulae) for both ITS and EF-1α genes were ~0.6 and ~0.05, respectively. These values are larger than the distance between nebrodensis and eryngii-ferulae by a factor of ~300 for EF-1α and ~2.5 for ITS (Table 2).

Evolutionary trees based on ITS and EF-1α nucleotide variation
A unique clade of P. eryngii species-complex composed of three sub-groups consistently separated from P. ostreatus, which performed as a distinct species, was obtained using the evolutionary molecular ITS distances. Namely, the

|                      | Average evolutionary divergence |
|----------------------|-------------------------------|
|                      | ITS   | EF1-α |
| P. eryngii species-complex | 0.001 | 0.013 |
| Overall mean distance    | 0.002 | 0.017 |
| Nebrodensis vs. eryngii-ferulae | 0.002 | 0.009 |
| Within nebrodensis       | 0.000 | 0.007 |
| Within eryngii-ferulae   |       |       |
| P. ostreatus plus P. eryngii | 0.028 | 0.016 |
| Overall mean distance    | 0.602 | 0.049 |
| P. ostreatus vs. nebrodensis | 0.574 | 0.053 |
| P. ostreatus vs. eryngii-ferulae |       |     |
sub-clades were nebrodensis type from Sicily (A), highly supported with 82% of bootstrap values, nebrodensis type from China (B), also highly supported with the 97% of bootstrap values, and eryngii-ferulae group from Mediterranean region (group C), with a bootstrap value lower than 70% (Figures 1, 2 and 4). The eryngii-ferulae sub-population from Apulia, Sardinia and Sicily showed two variants of the pileus colour (brown and white) (Table 1) and genetic homogeneity for their ITS sequences, as shown in Figure 1.

The NJ analysis of the EF1-α gene yielded a topology close to the ITS cluster, in which all taxa were grouped together into a unique clade, made of three sub-groups, the same as for ITS (A, B and C). Once more, the EF-1α sequence of P. ostreatus, based on its nucleotide sequence variation, was placed outside of the P. eryngii
Figure 2. Evolutionary relationships among 13 taxa of P. eryngii species-complex based on EF1-α gene (∼600 bp) sequence variation. The evolutionary history was inferred using the Neighbor-Joining method and the analyses were conducted in MEGA4. The bootstrap consensus tree inferred from 1000 replicates and bootstrap values greater than 70% are shown. One EF1-α gene sequence (AY883432) belonging to P. ostreatus taken from the NCBI GenBank was included as species reference within the Pleurotus genus.

Nevertheless, the cluster included strains a priori classified as type nebrodensis from Sicily (which formed a sub-group) and strains classified as nebrodensis type from China. Strains from Sardinia (clustered into the same sub-group) and some strains from Apulia region formed the last sub-group of this cluster.

The second cluster was made of strains from Apulia region and contained three sub-groups showing various sub-clusters. The only exception was represented by a few Sicilian strains (33 SC, 34 SC and 36 SC) that were included in the same cluster as those from Apulia (P. eryngii and P. ferulae types) and did not belong to the nebrodensis type (Figure 5).

Discussion

In the 19th Century, the P. eryngii species-complex was classified into botanical varieties (nebrodensis, eryngii and ferulae) (Inzenga 1863; Lanzi 1894 and Quelet 1872). Since then, many methods have been employed to understand taxonomy within this group, usually leading to different results. Most authors have reported the within gene-pool distinction at variety level (Hilber 1982; Mou et al. 1987; Venturella et al. 2000, 2002; Lewinsohn et al. 2002; De Gioia et al. 2005; Zhang et al. 2006; Rho et al. 2007; Kawai et al. 2008). In contrast, Urbanelli et al. (2002, 2003) divided the vars. eryngii and ferulae into two distinct biological species. In addition, the white coloured nebrodensis type was considered a different species in many studies (Candusso and Basso 1995; Zhang et al. 2000, 2006; Zervakis et al. 2001b; Urbanelli et al. 2007). So far, molecular data seems to have supported the splitting of the gene-pool rather than its lumping. Nonetheless, our preliminary findings, based on intra-specific genetic distances within P. eryngii species-complex
Figure 3. Linear regression of pairwise evolutionary genetic distances for ITS and EF1-α genes showing high significant correlation between the two types of evolutionary genetic distances (slope = 0.86; \( p < 0.001 \)) and \( r^2 = 74.8\% \).

Figure 4. Evolutionary relationships among 12 Pleurotus taxa belonging to the \( P. \) eryngii species-complex based on both ITS and EF-1 (1574 bp) sequence variation. The evolutionary history was inferred using the Neighbor-Joining method and the analyses were conducted in MEGA4. The bootstrap consensus tree inferred from 1000 replicates and bootstrap values greater than 70% are shown.

(De Gioia et al., 2005) and phylogeny (Mang and Figliuolo 2008; Mang 2008), support the presence of a single species not yet differentiated in sub-species. The same findings were recently presented by Rodriguez-Estrada et al. (2010).

Our results clearly indicate that, at molecular and morphological levels, the \( P. \) eryngii species-complex can be divided in two main gene-pools: \( \text{eryngii-ferulae} \) type and \( \text{nebrodensis} \) type. The former group performs as a unique, homogenous gene-pool, and genetic differences between regional geographic origins can be dictated by recently evolved pedigree relationships, as demonstrated by the M13 marker. On the contrary, sequence variation displayed by the ITS and EF-1\( \alpha \) genes, that predates the recent ancestry, operates as a taxonomic value marker. However, the higher rate of substitution observed within the species-complex for EF-1\( \alpha \) was no longer noticed at species level when \( P. \) ostreatus was used as a reference.
Furthermore, phenotypes showed that some morpho-
logical markers, such as the absence or the presence of
melanin, are not yet completely fixed within the gene-pool.
In this study, strains labelled as *P. eryngii* 31_PL and *P.
eryngii* 32_PL (*eryngii-ferulae* group), have white basidi-
omata colour. Our results agree with those of Zhang et al
(2006), which proved that the white basidiomata colour is
not exclusively linked to var. *nebrodensis* but may also be
found in some var. *ferulae* in China.

The *nebrodensis* type is the most differentiated sub-
population at either molecular or morphological levels, but
we consider that the number of fixed mutations is still too
low to differentiate the lineage at species level within the
genus. Thus, the phylogenetic species concept should inte-
grate the magnitude of the threshold of nucleotide substi-
tutions required for species differentiation. We also found
that the *nebrodensis* group (either Sicilian or Chinese)
had two fixed single nucleotide substitutions over the ITS
region that could be used as a future molecular diagnos-
tic tool. It is likely that the *nebrodensis* type from China
is the most diverging gene-pool owing to the isolation by
distance. This consideration is supported in this work by
the average evolutionary divergence between the groups
within the species-complex and the species within the
genus. For instance, for ITS gene, the distance between
*nebrodensis* and *eryngii-ferulae* is 0.002, while between
*P. ostreatus* and *nebrodensis* it is 0.602, and a similar trend is
obtained when comparing the *P. ostreatus* with the *eryngii-
ferulae* types. However, a statistical molecular threshold
for species recognition within the *Pleurotus* genus is not
available. Vilgalys and Sun (1994) showed that the lev-
els of nucleotide substitution for the ITS dataset can reach
0.41 in the case of eight different species of the *Pleurotus*
genus. Sreenivasaprasad et al. (1992) considered that 6%
variation in the ITS nucleotide sequence is acceptable as a
threshold for species delineation of *Colletotrichum*, while

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**Figure 5.** M13 tree based on Dice’s genetic distance and the average method clustering criterion of 47 strains belonging to *P. eryngii* species-complex.
Vinnere (2004) illustrated that variation among isolates that are clustering in the *Colletotrichum acutatum* sensu lato can reach 9.6%, using the same genetic region. Zervakis and Balis (1996) demonstrated that the percentage of compatible matings between ecotypes was variable (∼45% in pairing-analysis including *nebrodensis* and ∼60% between *eryngii* and *ferulae*). It seems that a genetic differentiation is underway, probably promoted by ecological forces (climate and biological substrates) and geographic isolation. In addition, the in situ biological and genetic performance of the *P. eryngii* is still unclear given that it is not possible to exclude saprotrophism or growth preference of the mycelia straw also on different host residues of the poly-specific herbageous coat composing the natural and semi-natural *Festuca-Brometalia* prairies. In several cases, the supposed host plants (e.g. *Ferula communis* and *Eryngium campestris*) are present in the same habitat (Forte et al. 2005).

De Gioia et al. (2005) showed that variation for morphological traits and molecular markers within and among Mediterranean populations of *P. eryngii* was able to identify the population genetic structure of *P. eryngii* species-complex. This can be explained by the out-crossing performance associated with a high genetic compatibility within and between a priori defined *eryngii* and *ferulae* types. Besides, in different reports, the *nebrodensis* type was later classified as different vars. *touliensis* and *elaeoselini* (Inzenga 1863; Venturella et al. 2000; Kawai et al. 2008).

In conclusion, the results of this study, based on the multi-locus analysis, provided sufficient molecular evidence separating macroevolution (ITS and EF-1α genes) from regional patterns of recent variation (M13 marker) and supported a single evolutionary unit represented by *P. eryngii*. The overall molecular analyses clarified species delineation in the *P. eryngii* gene-pool and demonstrated that a separation of *P. nebrodensis* from *P. eryngii* at a species level would require a higher degree of evolutionary divergence. Also it has been confirmed that the *eryngii* and *ferulae* types are belonging to the same taxon (*P. eryngii*). Our conclusions support the hypothesis that, within the species *P. eryngii*, the *nebrodensis* type is better ranked at a variety level.

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