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Pleurotus eryngii species complex: Sequence analysis and phylogeny based on partial *EF1α* and *RPB2* genes

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ARTICLE INFO

Article history:
Received 9 June 2009
Received in revised form
1 March 2010
Accepted 4 March 2010
Available online 7 March 2010
Corresponding Editor: Joey Spatafora

Keywords:
Phylogeny
Pleurotus eryngii
RNA polymerase II gene
Species complex
Translation elongation factor gene
Varieties

ABSTRACT

The *Pleurotus eryngii* species complex comprises at least six varieties (var. *eryngii* (DC.: Fr) Quel., *ferulae* Lanzi, *elaeoselini* Venturella *et al.*, *nebrodensis* (Inzenga) Sacc., *tingitanus* Lewinsohn *et al.* and *tuoliensis* C.J. Mou). This species is unique among the genus *Pleurotus* because in nature it is found in association with certain species of the Apiaceae (Umbelliferae) and Asteraceae (Compositae) families. Sequences of partial regions of the translation elongation factor (*EF1α*) and RNA polymerase II (*RPB2*) genes were analyzed in order to detect nucleotide polymorphisms that might unequivocally distinguish varieties *eryngii*, *ferulae*, *elaeoselini* and *nebrodensis*. A phylogenetic analysis was also performed with an aim to establish phylogenetic relationships among those. Sequence analysis of the partial *EF1α* and *RPB2* genes contained nucleotide polymorphisms able to unequivocally distinguish variety *nebrodensis* from the rest. However, distinction among *eryngii*, *elaeoselini* and *ferulae* was achieved only through the *RPB2* gene. The phylogenetic analyses from the combined data sets (*EF1α* and *RPB2*) indicated that *P. eryngii* is a monophyletic group and that varieties *eryngii*, *elaeoselini* and *ferulae* are closely related. *P. eryngii* var. *nebrodensis* was placed in a distinct clade clearly differentiated from the other varieties but still monophyletic with the *P. eryngii* complex. The limited nucleotide variation in partial *EF1α* and *RPB2* among varieties *eryngii*, *ferulae* and *elaeoselini* supports the placement of these groups as varieties and not species within the complex.

Introduction

The genus *Pleurotus* includes several edible species that are known for their exceptional flavor and their relatively low-cost methods of cultivation. Species identification in this genus is often difficult because it is largely based on morphology of the basidiomata. Incorrect naming of commercial strains has also contributed to ambiguity in the taxonomy of the genus (Buchanan 1993). The "species complex" concept is widely applied in fungi to define closely related species that are completely or partially intercompatible (Vilgalys & Sun 1994; Zervakis & Balis 1996; Zervakis *et al.* 2001b; Bao *et al.* 2004). The *Pleurotus eryngii* species complex consists of several varieties and species: var. *eryngii* (DC.: Fr) Quel, var. *ferulae* Lanzi (syn = *Pleurotus fuscus* var. *ferulae*), var. *elaeoselini* Venturella *et al.*, var. *tingitanus* Lewinsohn *et al.*, var. *nebrodensis* (Inzenga) Sacc, var. *tuoliensis* C.J. Mou, *Pleurotus hadamardii* Costantin and *Pleurotus fossulatus* (Cooke) Sacc. (Candusso & Basso 1995; Venturella 2000; Lewinsohn *et al.* 2002; Kawai *et al.* 2008). In nature, members of this group function as

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1878-6146/$ – see front matter © 2010 Published by Elsevier Ltd on behalf of The British Mycological Society.
doi:10.1016/j.funbio.2010.03.003

facultative biotrophs associated with some genera of the Apiaceae (Umbelliferae) and Asteraceae (Compositae) families (Zervakis et al. 2001a). Morphological characteristics used to differentiate individuals within these groups may be ambiguous because of environmental influences or overlap of traits of interest. For example, it was reported recently that white basidiomata, a feature considered distinctive of var. *nebrodensis*, may also be found in some isolates of variety *ferulae* growing in association with *Ferula sinkiangensis* in Xinjiang, China (Zhang et al. 2006). As is true for other fungi, incomplete reproductive barriers exist within the *P. eryngii* group (Bresinsky et al. 1987, Zervakis & Balis 1996, Kawai et al. 2008). Therefore, biological species delimitations often are difficult to establish.

The authors previously evaluated the utility of the internal transcribed spacer region (ITS) of the rDNA gene cluster and partial β-tubulin gene to infer phylogenetic relationships among varieties of *P. eryngii* (Rodriguez Estrada 2008; Rodriguez Estrada et al. 2008). Allelic polymorphisms (intra-isolate polymorphisms) within the *β-tubulin* gene and lack of variation in the ITS region (also reported by Ro et al. 2007) were observed. Therefore, those regions were neither useful for comprehensive phylogenetic studies within this species complex nor to unequivocally distinguish varieties. Other protein-encoding regions that may be useful to determine phylogenetic relationships among closely related taxa are the genes encoding for the translation elongation factor (*EF1α*) and the second largest subunit of the RNA polymerase II (*RPB2*) (Liu et al. 1999; Roger et al. 1999; Matheny et al. 2002; Tanabe et al. 2004; Froslev et al. 2005; Matheny 2005; Matheny et al. 2006).

The translation elongation factor (*EF1α*) is a binding protein required for ribosomal protein synthesis in eukaryotes. A study performed by Marongiu et al. (2005) revealed that the *EF1α* gene contains nucleotide substitutions that are useful to distinguish two varieties of *P. eryngii*: *ferulae* and *eryngii*. The *RPB2* is a single copy gene that encodes the second largest subunit of the RNA polymerase II, the enzyme that transcribes pre-mRNA (Liu et al. 1999; Matheny et al. 2007). The *RPB2* gene possesses 12 highly conserved domains across kingdoms that have been used to design PCR primers (Liu et al. 1999). The *RPB2* gene was used in combination with other genomic regions to infer phylogenetic relationships at the species level for the genera *Cortinarius* and *Inocybe* (Froslev et al. 2005; Matheny 2005). In the present study, the authors explored use of the *EF1α* and *RPB2* genes to infer phylogenetic relationships among members of the *P. eryngii* species complex.

Materials and methods

Fungal cultures

A total of 39 isolates of *Pleurotus* spp. were used, representing six species (*Pleurotus dryinus* (Pers.: Fr.) Kumm., *Pleurotus ostreatus* (Jacq.: Fr.) Kumm., "*P. sapidus*" (Schulz.) Sacc. [name entered in the Pennsylvania State University Mushroom Culture Collection, PSUMCC], *Pleurotus tuberregium* (Fr.) Sing., *Pleurotus cornucopiae* (Paul.: Pers.) Roll. and *Pleurotus cystidiosus* Miller) and four varieties (*eryngii*, *ferulae*, *elaeoselini* and *nebrodensis*) of the *P. eryngii* species complex (Table 1). Isolates with codes "WC" were obtained from the PSUMCC (Table 1). Cultures in that collection are permanently stored in liquid nitrogen (−196 °C and 10 % glycerol).

Culture conditions and extraction of DNA

Isolates of *Pleurotus* spp. were grown for 15–20 d in 30 ml sterile potato dextrose broth (PDB) contained in 125 ml Erlenmeyer flasks. Broth and mycelium were filtered through miracloth® (Calbiochem). The mycelium was rinsed with sterile-distilled water and squeezed twice, placed in 2 ml eppendorf tubes, lyophilized, and stored in a glass desiccator at room temperature. Lyophilized mycelium was manually ground with a micropestle in a 1.5 ml Eppendorf tube. DNA extractions were performed using a DNeasy® plant mini kit (Qiagen) according to manufacturer's instructions. DNA concentrations were visualized and estimated by gel electrophoresis and a Beckman DU®640B spectrophotometer, respectively. DNA stock solutions were adjusted to 20 ng/μl with sterile, distilled water and stored at −20 °C until used.

PCR amplification of partial EF1α and RPB2 genes

The set of primers EF116OR (5′ CCGATCTTGTAGACGTCCTG 3′) and EF595F (5′ CGTGACTTCATCAAGAACATG 3′) was used to amplify a portion of the *EF1α* gene targeting exons 4–6 and expanding introns 4 and 5 (Wendland & Kothe 1997; Marongiu et al. 2005). Two sets of primers were used to amplify regions of the *RPB2* gene. Primers fRPB2 5F (5′ GAYGAYMG WGATCAYTTYGG 3′) and bRPB2 7.1R (5′ CCCATRGCY TGYTTMCCCAT DGC 3′) amplify a fragment of ~1100 bp targeting exons 3–5 (domains 5–7). Primers b6.9F (5′ TGGAC NCAYTGY GARATYCAYCC 3′) and b11R1 (5′ TGGATYTTG TCRTC CACCAT 3′) amplify a fragment of ~1100 spanning exon 4–5 (domain 7–11) (Liu et al. 1999; Matheny 2006). The schematic structure of the *EF1α* and *RPB2* genes and the target sections for primer recognition are available as Supplementary material (Fig S1). The PCR master mix from Promega (*Taq* DNA polymerase) was used to carry out PCR reactions (25 μl) with primers (10 μM each) and DNA template (60 ng). PCR cycling was done in a PTC-100™ Programmable Thermal Controller (MJ Research). PCR cycle conditions for *EF1α* amplifications were 94 °C/4 min; 36 cycles of 94 °C/1 min, 55 °C/1 min, 72 °C/1 min; and a final extension step of 72 °C/10 min. PCR conditions for amplification of the *RPB2* gene were 94 °C/5 min; 35 cycles of 94 °C/1 min, 57 °C/1 min, 72 °C/90 s; and a final extension step of 72 °C/10 min. PCR products were visualized by electrophoresis in 1 % agarose gels stained with ethidium bromide (0.16 μl/ml). DNA loading buffer (5×; 2 μl) + 5 μl PCR product were loaded on the gel and electrophoresed for approximately 30 min at 2.3–3.3 V/min.

DNA sequencing

PCR products were purified using ExoSAP-IT® (USB) according to manufacturer's instructions. Purified DNA template was concentrated (SPD1010 SpeedVac®System) or diluted to 40 ng/μl for sequencing. The forward primer for *EF1α* (EF595F) and reverse primers for *RPB2* (b11R1 and bRPB2 7.1R) were used for sequencing (1 μM each). DNA template and primer were transferred to 96-well μltraAmp™ PCR plates

Table 1 – Isolates of *Pleurotus* spp. used in this study. GenBank accession numbers corresponding to partial *EF1α* and *RPB2* genes are indicated.

Species	Variety	Isolate code	Original Source[a]	Geographic origin[b]	Host/substrate[b]	*EF1α* Accession Number	*RPB2* Accession Number
P. eryngii	*Eryngii*	Pe-AL1	INRA[c]	Entre Deux Mers, Launay, France	*Eryngium campestre*/ calcareous soil	GQ225115	GQ225114
		Pe-Al11	INRA	Causse Mejean, France	*E. campestre*/limestone plateau	GU139132	GU186821
		Pe-Al20	INRA	Oleron island, France	*E. maritimun*/Litoral of dunes		
		WC888	SEFI[d]	Unknown	Commercial	GU139133	GU186794
		Pe-Al32	Commercial farm	Unknown	Commercial	GU139127	GU186822
		WC968	IBAF[e]	Unknown	Commercial	GU139128	GU186795
		WC967	IBAF	Unknown	Commercial	GU139134	GU186796
		WC957	U. Bari[f]	Sicily, Italy	Unknown	GU139131	GU186831
		WC984	IBAF	Unknown	Commercial	GU139130	GU186797
		WC989	Commercial farm	Unknown	Commercial	GU139129	–[n]
P. eryngii	*Ferulae*	WC966	IBAF	Sardegna, Italy	Unknown	GU139135	GU186823
		WC955	U. Bari	Sicily, Italy	Unknown	GU139141	GU186810
		WC929	U. Haifa[g]	Gilboa Mt., Israel	*Ferula* sp.	GU139140	GU186824
		WC933	U. Haifa	Gevaot Merar, Israel	*Ferula* sp.	GU139139	GU186825
		WC926	U. Haifa	Tabor Mt., Israel	*Ferula* sp.	–	–
		WC927	U. Haifa	Menahemya, Israel	*Ferula* sp.	GU139138	GU186826
		WC981	IBAF	Sicily, Italy	Unknown	GU139144	GU186827
		WC970	IBAF	Puglia, Italy	Unknown	GU139143	GU186828
		WC982	IBAF	Sardegna, Italy	Unknown	GU139145	GU186816
		WC956	U. Bari	Bari, Italy	Unknown	GU139137	GU186798
		WC850	PSU	China	Unknown	GU139142	GU186829
		WC954	U. Bari	Taranto, Italy	Unknown		GU186809
		WC969	IBAF	Sardegna, Italy	Unknown	GU139136	GU186830
P. fuscus	*Ferulae*	WC994	CBS[h]	Unknown	Unknown	GU139146	GU186812
P. eryngii	*Elaeoselini*	WC999	U. Palermo[i]	Unknown	Unknown	GU186799	GU186811
P. eryngii	*Nebrodensis*	WC777	IBAF	Sicily, Italy	Unknown	GU186800	GU186814
		WC976	IBAF	Sicily, Italy	Unknown	GU186803	
		WC979	IBAF	Sicily, Italy	Unknown	GU186801	GU186813
		WC980	IBAF	Sicily, Italy	Unknown	GU186802	GU186815
		WC958[j]	U. Bari	Unknown	Unknown	–	–
P. dryinus		MW-84	Unknown	Unknown	Unknown	GU186807	–
P. ostreatus		WC632	ASI[k]	Unknown	Unknown	–	–
		WC739	Italspawn	Unknown	Unknown	GU186804	GU186817
		WC971	IBAF	Italy	Basilicata	GU186805	–
"P. sapidus"[m]		WC153	Unknown	Unknown	Unknown	–	–
		WC529	PSU[l]	State College, PA, USA	Unknown	–	–
P. tuberregium		WC823	Nigeria	Nigeria	Unknown	GU186793	
P. cystidiosus		WC609	ASI	Unknown	Unknown	GU186808	GU186819
P. cornucopiae		WC608	ASI	Unknown	Unknown	GU186806	GU186820
		WC397	Unknown	Toronto	Unknown	–	–

a Original source refers to the immediate isolate supplier.
b Geographic origin and host/substrate refers to the place and conditions where the isolates were found in nature. Commercial usage of the isolates is specified under host/substrate.
c INRA – National Institute of Agronomic Research, MYCSA (Mycology and Food Security), Villenave D'Ornon, France.
d SEFI – Shanghai Edible Fungi Institute, Shanghai, China.
e IBAF – Institute of Agro-environmental and Forest Biology, Rome, Italy.
f U. Bari – University of Bari, Department of Biology and Plant Pathology, Bari, Italy.
g U. Haifa – University of Haifa, Institute of Evolution, HAI Culture Collection, Haifa, Israel.
h CBS – The Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
i U. Palermo – University of Palermo, Department of Botany, Sicily, Italy.
j Misidentified as *P. eryngii* var. *nebrodensis*.
k ASI – Agriculture Science Institute, Suwon, Korea.
l PSU – The Pennsylvania State University, Mushroom Culture Collection, University Park, USA.
m Presumably *P. pulmonarius*.
n Dashes indicate sequences were not submitted to GenBank; no dash indicates sequence not available.

and sealed with ThermalSeal™. Sequencing was done at the Nucleic Acid Facility, The Pennsylvania State University using an ABI Hitachi 3730XL DNA analyzer.

Sequence data analysis

Sequence chromatograms were visualized and edited with Sequencher version 4.7 (Gene Codes Corporation 2006) software. Sequence alignments were done using the clustal W algorithm in MEGA (3.1) and manually edited. Alignments were performed for *EF1α*, two portions of the *RPB2* gene and the two data sets combined. Data retrieved from GenBank [accession numbers AY883432 (*EF1α*) and AY786062 (*RPB2*)] for *P. ostreatus* were included in the alignments. Nucleotide differences among varieties and species within the genus were calculated. A distance matrix based on the combined data set was constructed. Phylogenetic reconstructions for the *EF1α*, *RPB2*, and combined data sets were performed based on the Neighbor-Joining method (Nei & Kumar 2000) and the p-distance (nucleotide substitution) model in MEGA (3.1). The phenetic analysis confidence was assessed by bootstrap tests with 1000 replications.

Results

Sequence data analysis for the partial EF1α *gene in* Pleurotus spp.

Amplification of the *EF1α* gene yielded a single band of approximately 583 bp in agarose gel. A segment of ~548 bp remained after sequences were edited and trimmed. Sequence variation among var. *ferulae*, *eryngii* and *elaeoselini* was less than 1% (Table S1). Inclusion of isolates of the var. *nebrodensis* in the analysis resulted in 1.5 % variation among the four varieties. In contrast, alignment of 38 the isolates belonging to the seven species of *Pleurotus* included in this study showed 34.9 % variation (Table S1). As reported by Marongiu *et al.* (2005), three nucleotide substitutions unequivocally distinguish *P. eryngii* var. *eryngii* from *P. eryngii* var. *ferulae*. However, base substitutions indicative of var. *ferulae* were also found in the var. *elaeoselini* and one isolate of uncertain identity WC958 (received by our collection as var. *nebrodensis*). Base substitutions at positions 95, 116, 188, and 271 discriminated var. *nebrodensis* from the rest (Fig 1 and Fig. S2). No unique variable sites that allowed discrimination of var. *elaeoselini* from the other groups were found. Percentage of nucleotide variation among *Pleurotus* species included in this study was as high as 34.9 % (Table S2). Sequences of the partial *EF1α* gene were deposited in GenBank (see Table 1 for accession numbers).

Sequence analysis of the partial RPB2 *gene in* Pleurotus spp.

Sequences were edited and trimmed for the two fragments of the *RPB2* gene. For primer set f5F/b7.1R, a region of 521 bases remained. This region is referred to as *RPB2-1* in this paper (Fig. S3a). For the primer set b6.9F/b11R1, a region of 731 bases remained. This region is named here as *RPB2-2* (Fig S3b). These segments correspond to positions 370–890 (*RPB2-1*) and 1260–1990 (*RPB2-2*) of the *P. ostreatus* sequence retrieved from GenBank accession number AY786062. *RPB1-1* and *RPB2-2* sequences corresponding to the same isolate were deposited in GenBank as a single entry joined by a string of 369 ''N''s (see Table 1 for accession numbers). Multiple alignments were performed in separate data sets for each portion of the gene. A total of 13 nucleotides were different between isolates of the varieties *ferulae* and *eryngii* for the two segments (Table S2). However, only five sites (410, 965, 974, 1111 and 1501) were able to distinguish the two varieties from each other (Fig 1). Fourteen base substitutions allowed discrimination between var. *nebrodensis* and other members of the species complex (Fig 1 and Fig. S3). Variety *elaeoselini* shared a 100 % identity with the misidentified isolate WC958. A total of three sites differentiated these two isolates from the other members of the *eryngii* complex (11, 910 and

EF1α *RPB2*

Sites / Variety	95	116	188	241	271	393	611	152	269	406	410	488	489	494	901	965	974	1099	1108	1111	1144	1234	1297	1417	1501	1544	1497
Ery (PeAL1)	T	C	G	T	G	A	C	G	T	C	A	C	T	C	T	C	A	T	C	G	G	G	C	T	C	A	T	C
Fer (WC982)	.	.	.	C	T	C	.	.	.	.	.	T	.	.	.	.	G	C	.	.	A	.	.	.	.	G	.	.
Ela (WC999)	.	.	.	C	T	C	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.
WC958	.	.	.	C	T	C	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.
Neb (WC980)	C	T	A	C	C	C	T	.	C	T	C	.	A	G	C	.	.	C	T	A	.	A	.	C	G	G	T	G
	&	&	&	*	*	*	&	#	&	&	&	*	&	&	&	#	*	*	&	&	*	&	#	&	&	*	&	&
					&																							

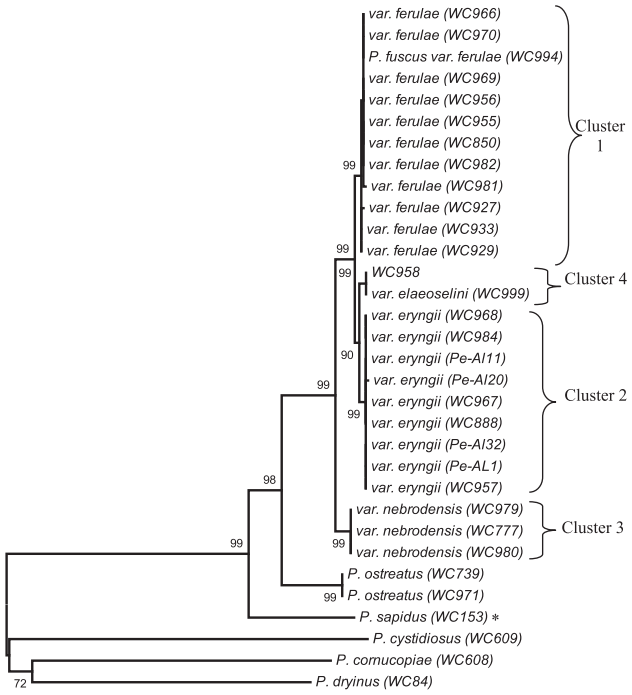
Fig 1 – Variable nucleotide sites found in partial sequences of the *EF1α* and *RPB2* genes among four varieties of *Pleurotus eryngii*: *eryngii* (Ery), *ferulae* (Fer), *elaeoselini* (Ela), *nebrodensis* (Neb), and isolate WC958. Position numbers for *EF1α* and *RPB2* are given according to the sequence of Pe-AL1 (GenBank accession number GQ225115 and GQ225114, respectively). *Corresponds to positions distinguishing varieties *eryngii* and *ferulae* (in *EF1α* this was reported previously by Marongiu *et al.*, 2005). &Unequivocally distinguishes *P. eryngii* var. *nebrodensis* from the other varieties. #Distinguishes var. *elaeoselini* from var. *ferulae*, *eryngii* and *nebrodensis*.

1234, Fig 1 and Fig S3). Percentage of nucleotide variation among *Pleurotus* spp. included in this study was as high as 40 % for RPB2-1 (209 sites) and 30 % for RPB2-2 (222 sites) (Table S2). Sequences of both portions of the *RPB2* gene have been deposited in GenBank (Table 1). Both segments are joined by a string of 369 ''n''s estimated from alignments compared to *P. ostreatus RPB2* sequence (AY786062).

Phylogenetic analysis based on partial sequences of EF1α *and* RPB2 *genes*

Phylogenetic reconstruction, based on the *EF1α* gene, groups members of the *P. eryngii* species complex into three clusters (Figure S4). Cluster 1 contains varieties *ferulae*, *elaeoselini*, and the misidentified isolate WC958. This cluster is supported by a bootstrap value of 91 %. Cluster 2 contains only isolates of the variety *eryngii* and is supported by a bootstrap value of 94 %. A third cluster, with 96 % bootstrap value support, includes isolates of the variety nebro*densis*. As explained above, var. *elaeoselini* and isolate WC958 share nucleotide substitutions with the *ferulae* group. Therefore, var. *elaeoselini* and isolate WC958 clustered along with var. *ferulae* in the tree based on partial sequence of the *EF1α* gene. On the other hand, the tree generated from *RPB2* sequences (Fig S5) clustered the *elaeoselini* and WC958 isolates along with the var. *eryngii* in a group supported by a bootstrap value of 98 %. In this tree the *ferulae* cluster is supported by a bootstrap value of 99 % while the *nebrodensis* cluster by 100 %. The phylogenetic tree based on combined *EF1α* and *RPB2* data sets (Fig 2) shows that the varieties *eryngii*, *ferulae*, *elaeoselini* and *nebrodensis* are grouped in different clusters, each well supported by bootstrap values of 99 %. The lowest genetic distance was observed between varieties *eryngii* and *elaeoselini* (0.005) (Table 2).

The phylogenetic trees placed *P. ostreatus* and ''*P. sapidus*'' as the closest relatives of the *P. eryngii* species complex while *P. cystidiosus*, *P. dryinus*, *P. tuberregium* and *P. cornucopiae* are located at the base of the tree. Sequence analyses of other isolates deposited in our collection as *P. ostreatus*, *Pleurotus pulmonarius* and ''*P. sapidus*'' commonly retrieved incongruent phylogenies. For example, isolate WC632, identified as *P. ostreatus* clustered along with *P. sapidus* (Fig S4). This was also observed in a phylogenetic study performed with the partial *β-tubulin* gene where this isolate clustered together with *P. pulmonarius* (GenBank accession number ASI2020 registered as *Pleurotus sajor-caju*) (Rodriguez Estrada 2008). Therefore, we suspect that the isolates included in this study as ''*P. sapidus*'' (*nomen dubiae*, Vilgalys *et al.* 1993) might belong to the *P. pulmonarius* species. Sequences of the *RPB2* gene for isolates WC632 and WC823 (*P. tuberregium*) were problematic and those isolates were not included in the *RPB2* and combined analyses.



* *Nomen dudiae* but presumably *P. pulmonarius*

Fig 2 – Phylogenetic tree constructed for four varieties of *P. eryngii* and other species within the genus *Pleurotus* based on combined partial sequences of the *EF1α* and *RPB2* genes. The Neighbor-Joining method and the p-nucleotide model were used to construct the tree. Bootstrap values were based on 1000 replications.

Discussion

The *EF1α* and *RPB2* genes have been used separately or in combination to elucidate evolutionary relationships at high and low levels of relatedness in phylogenetic studies of fungi (Roger *et al.* 1999; Tanabe *et al.* 2004; Froslev *et al.* 2005; Matheny 2005; Matheny *et al.* 2007). In the present study, the authors sought to examine phylogenies of the *P. eryngii* species complex and allied taxa by using portions of these genes individually and in combination. We also were interested in detecting specific nucleotide polymorphisms that will allow unequivocal identification of these varieties. Three varieties (*eryngii*, *ferulae* and *nebrodensis*) were clearly separated in the *RPB2* and *EF1α* individual and combined analyses. However, placement of the var. *elaeoselini* (WC999) and isolate WC958 (erroneously identified as var. *nebrodensis*) was ambiguous (Figures S4 and S5). Marongiu *et al.*, 2005 showed that nucleotide substitutions in *EF1α* were discriminatory for varieties *ferulae* and *eryngii*. However, in this study the authors demonstrated that var. *elaeoselini* and isolate WC958 shared the same bases that distinguish the *ferulae* group from *eryngii*. As a consequence, the phylogenetic tree groups in one clade the *ferulae* and *elaeoselini* isolates. Regarding this result, the authors stress that extreme caution should be exercised when designing molecular markers to differentiate varieties within this species complex. As found in this research, sites within the *EF1α* gene that should be able to differentiate the group *eryngii* and *ferulae* are unable to differentiate the var. *elaeoselini* resulting in a misleading placement of this group within the *ferulae* group. Still, sequencing of the *EF1α* gene and identification of the three nucleotides reported by Marongiu *et al.*, 2005 were extremely useful here to perform a first screening and confirm identification of the isolates included in this and previous studies.

Table 2 – Distance matrix constructed in MEGA (p-distance model) for the combined data sets of *EF1α* and *RPB2* genes. Matrix includes four varieties of *Pleurotus eryngii*, *P. ostreatus*, *P. cornucopiae*, *P. dryinus*, "*P. sapidus*" and *P. cystidiosus*.

	B[b]	C[c]	D[d]	E[e]	F[f]	G[g]	H[h]	I[i]
A. *P. eryngii* var. *eryngii* (Pe-AL1)[a]	0.007	0.005	0.014	0.041	0.065	0.193	0.188	0.204
B. *P. eryngii* var. *ferulae* (WC982)		0.007	0.014	0.042	0.064	0.193	0.187	0.205
C. *P. eryngii* var. *elaeoselini* (WC999)			0.013	0.042	0.065	0.194	0.189	0.203
D. *P. eryngii* var. *nebrodensis* (WC980)				0.038	0.059	0.189	0.187	0.199
E. *P. ostreatus* (WC739)					0.055	0.191	0.188	0.199
F. *P. sapidus* (WC153)						0.196	0.188	0.196
G. *P. cornucopiae* (WC608)							0.161	0.187
H. *P. dryinus* (WC84)								0.187
I. *P. cystidiosus* (WC609)								

a *P. eryngii* var. *eryngii*.
b *P. eryngii* var. *ferulae*.
c *P. eryngii* var. *elaeoselini*.
d *P. eryngii* var. *nebrodensis*.
e *P. ostreatus*.
f "*P. sapidus*" (presumably *P. pulmonarius*).
g *P. cornucopiae*.
h *P. dryinus*.
i *P. cystidiosus*.

The *RPB2* phylogeny placed isolates WC958 and WC999 within the *P. eryngii* var. *eryngii* cluster. However, when the *EF1α* and *RPB2* data sets were combined, a cluster supported by a bootstrap value of 99 % emerged. Zervakis *et al.*, 2001b states that var. *elaeoselini* possesses an intermediate position since successful mating between this group and var. *eryngii* and *ferulae* were higher (45–70 %) than positive mating between var. *ferulae* and *eryngii* (40 %). In this study, the authors support the idea of an intermediate position for var. *elaeoselini* since this taxon clustered with either *eryngii* or *ferulae* varieties. Previous analysis of a fragment of the *β-tubulin* gene showed that the sequence of isolate WC958 was identical to one of the alleles found in var. *ferulae* (Rodriguez Estrada 2008). The intermediate position of var. *elaeoselini*, the higher ability to mate successfully with var. *eryngii* and *ferulae* and the fact that WC958 share identical sequence of the *β-tubulin* gene, might be an indication that *elaeoselini* may be the result of a hybridization event between the two varieties *eryngii* and *ferulae*. In initial observations, the authors questioned the identity of isolate WC958. This isolate was originally identified as *P. eryngii* var. *nebrodensis* and, in fact, this isolate produces white basidiomata when cultured on a cottonseed hulls-based substrate (Rodriguez Estrada 2008). Venturella, 2002 states that entirely white mushrooms growing on *Elaeoselinum gummiferum* and *Thapsia villosa* in Salamanca, Spain, were misidentified as var. *P. nebrodensis* by several amateur mycologists. However, microscopic features indicated that such isolates are in fact *P. eryngii* var. *elaeoselini*. Therefore, it is not surprising that isolate WC958 is placed in the same cluster with isolate WC999 suggesting that it belongs to the var. *elaeoselini*. Isozyme and RAPD analyses performed by Zervakis *et al.*, 2001b showed a relative similarity of var. *elaeoselini* especially to the var. *ferulae*. In the present study, however, the tree constructed from the combined data sets revealed that *P. eryngii* var. *elaeoselini* may be more closely related to var. *eryngii* than to *ferulae*.

Varieties *eryngii* and *ferulae* appear not to be regularly mating in nature since both *EF1α* and *RPB2* clearly differentiate both groups and tree topologies are congruent. De Gioia *et al.*, 2005 performed a genetic study of the *P. eryngii* complex based on qualitative morphological features (i.e. color of pileus and cuticle, pileus shape, stipe position and surface, etc.) and molecular markers (RAPD and minisatellite profiles). De Gioia *et al.*, 2005 suggest that the *eryngii* and *ferulae* groups should remain as varieties since low genetic distances observed between these groups supported a categorization below the species level. Another phylogenetic study based on the ITS and IGS1 regions also grouped these two varieties within a same clade but well distinguished from var. *nebrodensis* and var. *tuoliensis* (Kawai *et al.* 2008). In this study, a distance matrix based on the combined *EF1α* and *RPB2* data sets (Table 2) shows low values among var. *ferulae*, *eryngii* and *elaeoselini* (0.005–0.007). However, distances among nebrodensis and the rest of the varieties were higher (0.013–0.014).

It has been demonstrated through *in vitro* experiments, that individuals from different hosts retain the ability to interbreed, but hybridization and development of dikaryons produced by individuals from different ecotypes may be reduced (Cailleux *et al.* 1981; Zervakis & Balis 1996). Hybrids between closely related species of fungi are rarely found in nature since adaptations to substrates may generate low rates of hybrid survival (Kauserud *et al.* 2007). In the case of *P. eryngii*, it was observed *in vitro* and *in vivo* that var. *eryngii* is a better substrate colonizator and competitor than var. *ferulae* (Urbanelli *et al.* 2002). The spatial distribution of *P. eryngii* is roughly restricted and overlaps in ecological niches might be found. In Sicily, for example, *P. eryngii* var. *eryngii* is found between 0 and 1500 m above see level while *P. eryngii* var. *ferulae* is distributed below 1300 m. These two varieties can be found as sympatric populations. In contrast, *P. nebrodensis* grows at 1200–2000 m and *P. eryngii* var. *elaeoselini* is found between 0 and 1200 m (Venturella 2000; Zervakis *et al.* 2001b). Some phylogenetic studies on the genus *Pleurotus* have shown that evolution of the species is driven by geographical separation (allopatric speciation) (Vilgalys & Sun 1994; Zervakis *et al.* 2004). However, *P. eryngii* is not distributed world wide, but

confined to the Mediterranean and surrounding areas. Since non-obvious geographic barriers exist within the area of distribution of *P. eryngii*, it may be feasible to hypothesize that altitude is playing an important role in speciation of var. *nebrodensis*. However, it is important to mention that Zhang *et al*., 2006 recently reported that *P. eryngii* var. *ferulae* and *nebrodensis* both grow in association with *F. sinkiangensis* in sympatric populations in Xinjiang, China. Divergence among var. *eryngii*, *ferulae* and *elaeoselini* is recent since limited genetic variation exists in regions of the genome that are usually useful to elucidate phylogenetic relationships between and among species. Speciation in other basidiomycetes such as *Collybia dryophila*, *P. cystidiosus* complex, *P. tuberregium*, *S. commune*, *Lentinula* spp. and *Grifola* spp. is driven by isolation of populations by allopatry (geographic separation) (Vilgalys 1991; Thon and Royse 1999; Isikhuemhen *et al*. 2000; James *et al*. 2001; Shen *et al*. 2002; Zervakis *et al*. 2004). In the case of *P. tuberregium*, for example, it was estimated that divergence of the African and Australasian–Pacific populations occurred as consequence of the separation of the land 150 million years ago (Isikhuemhen *et al*. 2000). In contrast to this, the geological and climatic history of the Mediterranean area points out to a relatively recent establishment of the current scenario (only 5 million years ago) (Blondel & Aronson 1999). Grove & Rackham, 2001 stated that ecology in the Mediterranean is dominated by environmental alterations rather than evolution since most conditions have not existed long enough in evolutionary terms. For instance, the climate in that region has prevailed only for a few thousand years.

The phylogenetic trees constructed in this study resembles to some extent the outputs presented by Vilgalys & Sun, 1994 where *Pleurotus* speciation follows a pattern of ancient origins in the Southern hemisphere and a recent divergence for species distributed in the Northern hemisphere. In this regard, *P. dryinus* and *P. cystidiosus* are found in both hemispheres while *P. tuberregium* is distributed in the majority of the equatorial areas of Africa, India, Sri Lanka, Southeastern Asia, Northern Australia and Southern Pacific (Corner 1981; Pegler 1983; Singer 1986). Those three species are placed at the bases of the phylogenetic trees.

Members of the genus *Pleurotus* are unique basidiomycetes that present particularly interesting modes of speciation: allopatric and sympatric speciation. To our knowledge, the last is represented only by *P. eryngii* species complex that has developed a certain degree of host specificity resembling evolutionary pathways found usually in plant pathogenic fungal species (Bakkeren *et al*. 2000; Jimenez-Gasco *et al*. 2004).

Previous analyses (Rodriguez Estrada 2008) based on the ITS and β-tubulin regions showed that sequences of varieties *eryngii* and *ferulae* were almost identical between each other (99.7 % similarity for the ITS and 99.9 % similarity for the β-*tubulin* gene). These two varieties were not separated in different cluster in the phylogenetic trees constructed. *P. eryngii* var. *nebrodensis* was 97.6 % similar to the other varieties and unequivocally clustered as a different group (Rodriguez Estrada 2008). Therefore, the authors support the idea that the *P. eryngii* species complex is currently under speciation process as stated by Zervakis *et al*., 2001b and that the varieties *ferulae*, *elaeoselini* and *eryngii* still should be considered varieties and not different species of the complex. *P. eryngii* var. *nebrodensis*, on the other hand, might be considered a different species. This work provides valuable information regarding nucleotide polymorphisms found in the *RPB2* gene that are able to unequivocally distinguish varieties within the *P. eryngii* species complex. In future studies it will be worth while to include other varieties of this group.

Acknowledgements

The authors are thankful to Drs Daniele Sisto and Teresa de Gioia, Department of Biology and Plant Pathology, University of Bari; Dr. Solomon P. Wasser, Institute of Evolution, University of HAIFA; Dr. Ilio Rapana, Institute of Agro-environmental and Forest Biology; Drs Jacques Guinberteau and Philippe Callac, MycSA, INRA Bordeaux; Dr. Giuseppe Venturella, Department of Botany, University of Palermo and Gourmet Mushrooms, Inc. for providing us with the *P. eryngii* strains included in this research.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.funbio.2010.03.003

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