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Biology, morphology, and phylogeny of some strains of the *Pleurotus eryngii* species complex

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Abstract: Effective methods of preserving the gene pool of valuable edible and medicinal mushrooms are to maintain them in *in vitro* culture collections and to correctly identify and verify the preserved strains. It is necessary to consider not only the results of molecular genetic studies but also cultural, morphological and physiological characteristics as additional criteria. This article presents data on the colony and mycelial morphology, growth characteristics and temperature tolerance, and phylogenetical placement of four strains of edible and medicinal mushroom from the *P. eryngii* species complex received into the IBK Mushroom Collection as *P. nebrodensis* strains. All the studied strains are mesophiles with the fastest growth rate of 11.0 mm/day at 26°C and a lethal temperature of 40°C. In addition to common anastomoses, mycelial strands and clamp connections, the vegetative mycelium of the studied strains formed single colorless round excretory cells on the lateral hyphal ramifications. All cultures were able to form primordia and fruit bodies on agar media. Phylogenetic analysis suggests that all four strains do not belong to *P. nebrodensis*, but two of them, IBK 1947, 2035 are *P. eryngii* var. *ferulae*, and two strains, IBK 1855 and 1927, belong to *P. tuoliensis* (*P. eryngii* var. *tuoliensis*).

Keywords: *Pleurotus eryngii* var. *ferulae*; *P. tuoliensis*; mycelium; pure culture; phylogenetic identification

Abbreviations: IBK – Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine

INTRODUCTION

An urgent requirement is the preservation of the diversity of living organisms, which is noted in the relevant international conventions and documents (Convention on Biological Diversity), legislative acts of Ukraine, and in particular in the Law of Ukraine "On the Red Book of Ukraine". Preservation of the natural diversity of macromycetes *ex situ* is currently evaluated by the world mycological community as a promising and effective approach. The main idea is to preserve the gene pool of macromycetes in pure culture in specialized collections [1-3]. Based on culture collections, work is underway to preserve the mycobiota gene pool, study the biological properties of fungal species, find reliable means of maintaining them under artificial conditions and develop fundamental research in systematics, phylogenetics and other areas of mycology. Recently, considerable resources have been devoted to the conservation of edible and medicinal macromycetes *in vitro*. A significant number of macromycete species are widely used in modern biotechnology (production of fruiting bodies, mycelial biomass, various pharmaceutical substances, nutraceuticals, enzymes, etc.) as model objects in fundamental mycological research [4]. The IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany includes a total number of 1340 strains belonging to

265 species of Basidiomycota and Ascomycota and it is the largest official specialized culture collection of macromycetes in Ukraine. Activities of the IBK are focused on the preservation of the gene pool of macrofungi *in vitro* and fundamental research into the systematics, phylogeny, biology and biotechnology of edible and medicinal mushrooms [1-8].

Many mushroom species are reported as producers of different groups of bioactive compounds and have been recommended for a variety of therapeutic applications. *Pleurotus* species produce the high diversity of bioactive compounds with different pharmacological activities. *P. eryngii* (DC.) Quél. is highly prized as a culinary medicinal choice mushroom, also known as the king oyster. It is a valuable cultivated edible mushroom with high nutritional value and medicinal properties, providing a source of some dietary fibers with various health benefits to humans [9-12]. Recent studies have shown that the *P. eryngii* content of carbohydrates, the main components of the fungal fruiting body, is the highest among *Pleurotus* species (75.4%) as well as highest in fat content (3.5%), but these do not exceed the limit of 6% indicated by the FDA to comply as a low-fat food [10,13]. The polysaccharides isolated from *P. eryngii* have a variety of biological activities, including antioxidant, antihyperlipidemic, antitumor, immunoregulatory and bacteriostatic [9,13-15]. Cateni et al. [12] reported the antioxidative effect of polysaccharides that inhibited cell viability in colorectal adenocarcinoma cells. The cold-water extract of *P. eryngii* var. *ferulae* has an anticancer effect on human colon cancer cells [16]. The ethanolic extract of *P. eryngii* var. *ferulae* inhibited the growth and proliferation of stomach (BGC 823) and melanoma (B16F10) cancer cells [10], and triterpenes of *P. eryngii* have inhibitory activity against breast cancer MCF-7 cell lines *in vitro* [16]. The antiinflammatory effect of pleuran from *P. eryngii* which suppressed inflammation in the delayed type (type IV hypersensitive) allergy response in mice was described [14]. Antiviral, antimicrobial effects showed water-soluble sulfonated polysaccharides of *P. eryngii*, which inhibited the growth of pathogenic *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* [10]. It has been also reported that polysaccharides and vitamins of *P. eryngii* have antidepression properties and anti-aging activity [17].

The need for thorough research of the potential pharmaceutical and medicinal properties of *Pleurotus* species is facilitated by the study of the biology of their strains in pure culture. One of the critical species of this genus is *P. nebrodensis*, whose geographical range and strain composition have often been misinterpreted. Most published works deal with the geography, ecology and conservation status of this species in nature. Fungal culture collections, as well as GenBank data, contain dozens of strains and records of *P. nebrodensis*, which need to be sorted out and given their taxonomical affinity. Hence, correct identification of the isolates in pure culture, which belong to this fungal group, is of critical importance. Our work aimed to study and identify strains of the IBK culture collection in their vegetative stage of development using molecular, genetic and morphological-cultural methods.

MATERIALS AND METHODS

Mushroom strains

Four dikaryotic pure cultures of the IBK: IBK 1927, IBK 1947 and IBK 2035, obtained as *P. nebrodensis* by exchange with another collection (International Center for Cryptogamic Plants and Fungi, Institute of Evolution, University of Haifa (HAI), Israel, 2006), and also IBK 1855, isolated by the tissue plug method from a carpophore and identified as *P. nebrodensis* (Ukraine, Crimea, 2010), were the focus of this research [1]. The taxonomical affiliation of the cultures was determined by cultural-morphological, physiological and molecular-genetic methods.

Morphological, cultural, and physiological studies

Morphological and cultural studies of IBK cultures were performed in Petri dishes using four standard and modified nutrient agar media: beer wort agar (WA – liquid beer wort, diluted to a density of 8° Balling scale with distilled water and 20.0 g/L agar-agar), glucose-peptone-yeast agar (GPYA, in g/L: 25 glucose, 5 peptone, 3 yeast extract, 20 agar-agar, pH 6.0), malt extract agar (MEA; 2% sugar, Difco, USA, pH 4.7), potato dextrose Agar (PDA; Oxoid, Great Britain), The radial growth speed was calculated according to a previously described method [18]. To determine the optimal temperature for mycelial growth, surface cultivation was performed on PDA, found initially as

the best media for these strains at 22°C, 26°C, 28°C, 30°C and 34°C. To check viability, the culture was incubated on PDA at 4°C and 35-40°C. After the 3rd day of incubation, the presence or absence of mycelial growth was considered. The preservation or loss of viability of the cultural mycelium was checked during subsequent incubation at 26°C. The micro- and macromorphological features of the vegetative mycelium were observed and recorded according to standard methods [19]. Micromorphological characteristics included a description of the characteristic features of the hyphal system and the presence of clamps and anamorphs on the hyphae. The microstructures of the vegetative mycelium were examined using a JSM-35C scanning electron microscope (Japan) according to the modified method [20].

DNA isolation

Molecular genetic studies were conducted at the M.G. Kholodny Institute of Botany (Kyiv, Ukraine). The source of DNA in mycological research was a pure axenic culture [21]. In our study, a vegetative mycelium of IBK strains 1855, 1927, 1947, 2035 on day 20 of cultivation was used. DNA extraction of the studied cultures was conducted according to an express method described below. The mycelial biomass was aseptically transferred to a 200-μL plastic Eppendorf PCR tube previously injected with 50 μL of sterile lysis buffer, which contained 0.01 M Tris and 0.005 M EDTA in 100 mL of sterile H_2O, pH 8.0 [22].

The PCR tubes containing the mycelium were tightly closed and then transferred to a microwave oven for 1 min under conditions of maximum heating and heated twice with an interval of seveal minutes to prevent thermal deformation of the tubes. The DNA was eluted into a lysis buffer. Using a dispensing pipette, 25 μL of the supernatant was transferred in duplicate to sterile 50-μL PCR tubes. The finished product was stored at -20°C until further use. PCR was performed in a T100TM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, USA) using a standard protocol for internal transcribed spacer (ITS) sites in fungi, using primers ITS1f: CTTGGTCATTTAGAGGAAGTAA, and ITS4: TCCTCCGCTTATTGATATGC [23,24]. To confirm the success of the amplification, gel electrophoresis of amplified DNA fragments in a 1% agarose gel-based on 1×SB buffer, 0.2 M NaOH and 0.75 M H_3BO_3 in 1 L of deionized water was performed at a voltage of 120 V in a PowerPac™ Basic (Bio-Rad Laboratories, Inc., Hercules, USA). Visualization of the PCR products was performed in a trans-porthole Universal Hood 75S (Bio-Rad Laboratories, Inc., Hercules, USA). The amplified PCR product was purified from the residues of the polymerase mixture using 0.1 units of shrimp alkaline phosphatase (GE Healthcare, Princeton, USA) and 0.23 units of exonuclease I (New England Biolabs, Ipswich, USA) per sample [25]. To determine the nucleotide sequences of the studied fragment of the genome, the purified PCR products were sequenced by the Sanger method using primers ITS1f and ITS4 at the PrimBio Research Institute (Exton, USA). The sequences were edited, trimmed from primer sequences, and aligned using the software package Geneious v. 8.1.8 (Biomatters LTD, Auckland, New Zealand).

Sequence analysis

Verification of strains IBK 1855, 1927, 1947 and 2035 was performed using the nucleotide sequence of rDNA genes (ITS1-5,8S-ITS2). A heuristic algorithm on a BLAST server was used to search for closely related sequences (http://www.ncbi.n/m.nih.gov/blast), allowing us to determine the most homologous sequences in the available international nucleic acid database of the National Center for Biotechnological Information of the National Institutes of Health GenBank (http://www.ncbi.n/m.nih.gov/GenBank).

Phylogenetic analysis

We amplified and sequenced the ITS1-5.8S-ITS2 region of rDNA for four IBK strains. For comparison, additional sequences of this DNA region for 103 strains of the genus *Pleurotus* were downloaded from the NCBI Nucleotide database. All sequences were initially aligned and compared in Multalin [26] and ClustalX [27]. The alignment was manually adjusted, and ambiguous regions were excluded from the alignments using Mesquite 3.61 [28]. The analysis included a total of 316 nucleotide characters. The phylogenetic relationships of the remaining 66 sequences were determined by maximum likelihood (ML) using Garli 1.0 [29], and phylogenetic support was assessed by

Table 1. Verification of species of the *Pleurotus eryngii* complex IBK strains by DNA typing >99.49%; query cover >95%, * – not *P. nebrodensis* sequences, ** – isolated from a carpophore, Ukraine, Crimea, 2010, *** – obtained from International Center for Cryptogamic Plants and Fungi. Institute of Evolution, University of Haifa (HAI), Israel, 2006.

Strain, species IBK#, NCBI [54] accession #, length (bp)	Query cover (%)	Identity (%)	Sequence ID	Species, isolates
****1855** *Pleurotus tuoliensis* MZ234121 568	100	99.65	MN822666	**P. nebrodensis* breed XB54
	100	99.47	KF724518	*P. eryngii* var. *tuoliensis* strain CCMSSC01415
	100	99.47	EU570097	*P. eryngii* strain Baiyangdu lxy-2
	100	99.30	MN244440.2	*P. tuoliensis* voucher JFRL62
	100	99.30	MK757153	*P. tuoliensis* isolate CCPF5095
	100	99.30	MK757152	*P. tuoliensis* isolate CCPF5007
	100	99.30	MG694568	**P. nebrodensis* strain 1601
	100	99.30	MG282490	**P. nebrodensis* isolate P151
	100	99.30	KY686287	**P. nebrodensis* isolate Bailinggu1
*****1927** *Pleurotus tuoliensis* MN646249 590	100	99.66	AY540331	**P. nebrodensis* strain S498
	99	99.83	MN646251	*P. eryngii* var. *ferulae* IBK 2035
	97	99.65	AB286163	*P. eryngii* unspecified
	97	99.65	AY581434	**P. nebrodensis* isolate WW
	97	99.65	AY311408	**P. nebrodensis*
	97	99.65	FJ873699	**P. nebrodensis* isolate 924
*****1947** *Pleurotus eryngii* var. *ferulae* MN646250 616	99	99.02	OL687127	*P. eryngii* strain ATCC 90797
	97	99.67	HM561985	*P. eryngii* isolate PET1
	97	99.33	DQ333235	*P. eryngii* clone 2
	96	99.50	MK785235	*P. eryngii* isolate 24
	97	99.33	JF908624	*P. eryngii* voucher 14176
	95	99.49	MT535679	*P. eryngii* voucher Ghobad-Nejhad 137
*****2035** *Pleurotus eryngii* var. *ferulae* MN646251 613	97	100	AY540331	**P. nebrodensis* strain S498
	95	99.83	MN646249	**P. eryngii* IBK 1927
	99	98.20	MT644908	*P. ostreatus* isolate DM1049
	100	98.05	MN646250	*P. eryngii* var. *ferulae* IBK 1947
	99	98.04	MH569608	*P. ostreatus* strain ICMP11678
	93	100	AB286163	*P. eryngii* genes

1000 bootstrap replicates using PAUP* 4.0a109 [30]. The ITS sequences of *P. calyptratus, P. cornucopia* and *P. djamor* served as an outgroup.

Scanning electron microscopy (SEM)

The microstructures of the vegetative mycelium were examined according to the modified method [20]. Mushroom cultures were grown on wort agar in Petri dishes. In the inoculation stage, 4 sterilized square (4×4 mm) cover glasses were aseptically placed 1-5 cm away from the inoculum. Strains were incubated at 26°C. Then, after the mycelium grew over the cover glass surfaces, the cover glasses were removed from the surface of the agar media and transferred to microscope slides. The microscope slides were placed into a sealed glass vessel to fix the mycelium with osmium tetroxide vapor (1% solution) for 96 h. The slides were transferred to an empty Petri dish to dry for 72 h for fixation. After drying, the samples were covered with gold in a vacuum spray gun JII-4X with rotation and examined using a JSM-35C scanning electron microscope (Japan) at magnification from 100× to 16.000×.

Statistical analysis

All data were statistically processed for analysis. Values of standard deviations (SD), coefficients of variation and confidence intervals were calculated using the standard statistical packages Microsoft Office Excel and STATISTICA 8.0. Experimental data are expressed based on quintuple measurements as the

mean±SD. Student's *t*-test was applied to determine significance, with P<0.05 considered significant.

RESULTS

Sequence analysis

Confirmation of the species affiliation of *P. nebrodensis* strains was performed with the BLAST search algorithm using the obtained ITS region. For all investigated strains, we sequenced the complete internal transcription spacer: ITS1, 5.8S and ITS2 regions of rRNA, as well as adjacent regions of the 18S and 28S subunits surrounding the ITS. Using the BLAST algorithm, we compared ITS sequences of four IBK strains with those of GenBank. Each search gave a mixture of *P. eryngii* and *P. nebrodensis* sequences. However, with the information about the closest *P. nebrodensis* sequences from GenBank, we observed that none of them belong to the original *P. nebrodensis* from Sicily and adjacent geographical regions. After removing false *P. nebrodensis* records, we identified strains IBK strains 1855 and 1927 as *P. tuoliensis* (or *P. eryngii* var. *tuoliensis*) and strains 1947 and 2035 as *P. eryngii* var. *ferulae*. We deposited the sequences of the *P. eryngii* complex samples, which demonstrated 98 to 99% identity with the existing records, thus confirming the taxonomical classification of the studied strains (Table 1). However, the BLAST algorithm cannot serve as a proof of taxonomical identity since the deposits in NCBI Nucleotide database are not verified, as in the case with wrong *P. nebrodensis* sequences. Therefore, the phylogenetic placement of the four studied strains was performed.

Phylogenetic analysis

For phylogenetic identification of IBK strains we downloaded 96 sequences of *P. eryngii*, *P. tuoliensis* (*P. eryngii* var. *tuoliensis*), *P. eryngii* var. *ferulae*, *P. ferulaginis*, and *P. nebrodensis* from the datasets used previously [31-33]. The alignment's length was 316 positions. Of 316 nucleotide positions of the ITS region, which is at the lower range of the PCR product described for *P. tuoliensis* [34], 63 positions were informative (Fig. 1). None of the IBK strains was placed in the *P. nebrodensis* clade. The maximum likelihood phylogenetic reconstruction confirmed the results of molecular identification.

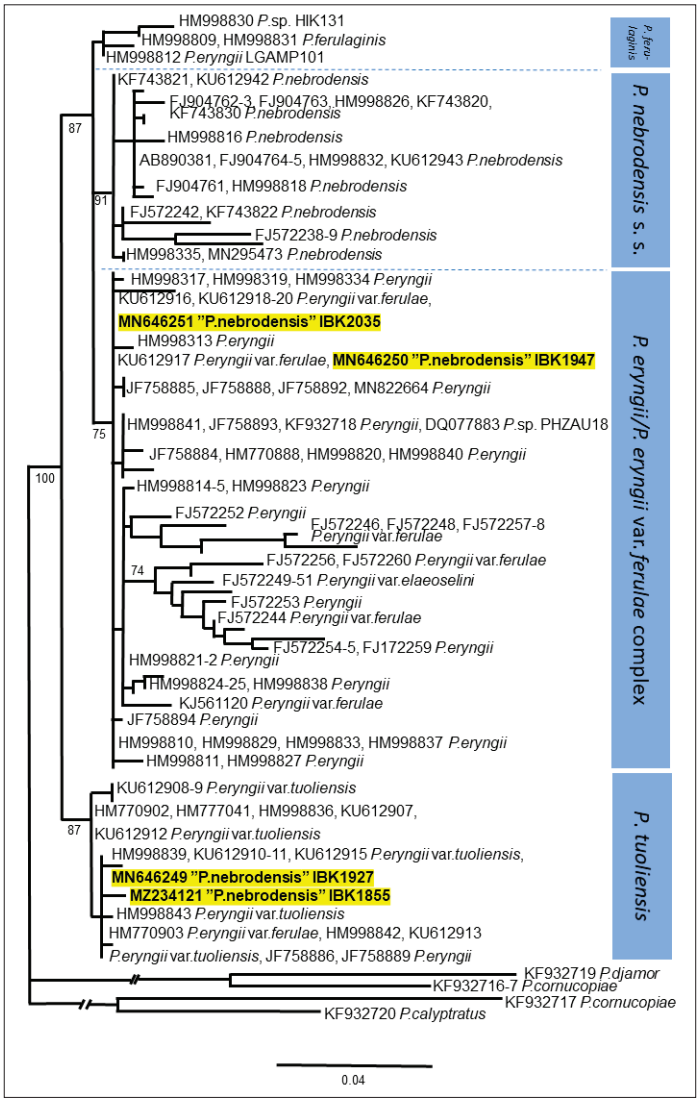

Fig. 1. ML tree used for phylogenetic identification of *P. eryngii* strains in the IBK culture collection. Bold on yellow background are four strains of "*P. nebrodensis*", identified as *P. eryngii* var. *tuoliensis* (IBK 1855, 1927) and *P. eryngii* var. *ferulae* (IBK 1947, 2035). In rectangular boxes to the right are the names of species or varieties of *P. eryngii* species complex. Delimitation of *P. nebrodensis sensu stricto* clade with dashed lines. Outgroup branching is shortened -//- due to long branches. Bootstrap support is indicated below the branches.

According to the data in electronic databases such as Species Fungorum (http://www.speciesfungorum.org/), Mycobank (https://www.mycobank.org/), we found strains IBK 1855 and 1927 to belong to *Pleurotus eryngii* var. *tuoliensis* C.J. Mou with the current name *P. tuoliensis* (C.J. Mou) M.R. Zhao & J.X. Zhang. At the same time, information about the other two studied strains differed: *Pleurotus eryngii*

var. *ferulae* (Lanzi) Sacc. is the synonym of *P. eryngii* (DC.) Quél., as indicated in the Species Fungorum database, but in Mycobank this species continues to be separated as *P. eryngii* var. *ferulae.* In our study, we further focused on Mycobank data specification and accepted that the strains IBK 1947 and 2035 belong to the species *P. eryngii var. ferulae.*

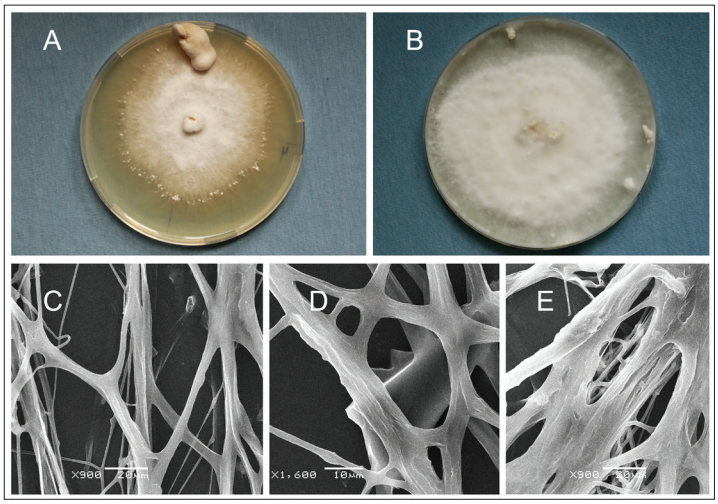

Fig. 2. Teleomorph of *Pleurotus tuoliensis* IBK 1927 *in vitro* on PDA (25 days of cultivation) (**A**); primordia of *Pleurotus eryngii* var. *ferulae* IBK 2035 *in vitro* on PDA (20 days of cultivation) (**B**). Scanning electron microscopy; micromorphological structures of *Pleurotus tuoliensis* IBK 1855: (**C**-**E**) hyphae with anastomoses; (**D**) mycelial strands. Size bars correspond to (C, E) 20 μm and (D) 10 μm.

Macromorphological characteristics

Verification of the studied strains based on classical morphological and cultural characteristics was performed. Namely, the micro- and macromorphology of vegetative mycelium was studied according to the set of features on standard nutrient medium according to the traditional Stalpers classification [19], consisting of growth indexes (radial growth rate), temperature and the presence of a generative stage on certain nutrient media.

Cultural and morphological studies of *P. eryngii* complex strains were performed on standard nutrient agar media of different compositions: GPYA, MEA, PDA, WA. According to the influence of the media on fungal growth, PDA and GPYA media provided the maximum effect. All colonies displayed a similar morphology on each medium. On WA medium, the colonies were dense, cottony and white, with an aerial mycelium at the center and along the edge of the colony. The colony edges were smooth, with a non-pigmented reverse side. On GPYA, the colonies were maximally dense, cottony, white, with concentric lines and a margin with strands. They were unevenly pressed onto the medium surface, with granular formations at the edge. On PDA the colonies were less dense, cottony, white, unexpressed, with a non-pigmented reverse side. On all the above media, concentric circles due to different densities were observed. Not dense, white colonies, prostrate and aerial were observed on MEA. Complete overgrowth of the plates with the substrate required 10 days, after which they were maintained under daylight conditions at 20-22°C. The primordia began to form after 20 (*P. eryngii* var. *ferulae* IBK 2035) to 25 (*P. tuoliensis* IBK 1927) days, after which they developed into fruiting bodies (Fig. 2A, B).

Mycelial growth rates

Given that the study of the physiological characteristics of macromycete cultures is needed to preserve them as valuable biotechnological objects, we examined the influence of the incubation temperature on the growth rate on various media. We investigated the growth of the four strains on different agar media: GPYA, MEA, PDA, WA, and observed the mycelial growth on all the tested media. The studied strains in terms of the radial growth rate belonged to the group of fungi growing at a moderate rate in the range of 2.7-11.0 mm/day (Table 2). All studied strains showed the highest radial growth rate on PDA. Concurrently, the growth rate of IBK 1855, IBK 1927 and IBK 1947 strains on PDA was

Table 2. Rate of radial growth of *Pleurotus eryngii* var. *ferulae* and *Pleurotus tuoliensis* strains (V_R, mm/day) on various agar nutrient media at 26°C.

Strain IBK#	Nutrient medium			
	WA	MEA	GPYA	PDA
1855	3.6±0.2	1.2±0.1	4.2±0.2	6.8±0.3
1927	2.8±0.1	1.7±0.2	4.0±0.1	6.8±0.1
1947	2.7±0.1	1.9±0.1	4.5±0.1	6.8±0.2
2035	4.9±0.3	5.0±0.3	7.4±0.2	11.0±0.2

WA – wort agar, GPYA – glucose-peptone-yeast-agar, PDA – potato-dextrose agar, MEA -malt extract agar. Data are the mean ± standard error for five replications. P<0.05 was considered as statistically significant.

Table 3. Rate of radial growth of *Pleurotus eryngii* var. *ferulae* and *Pleurotus tuoliensis* strains (V_R, mm/day) on PDA at different temperatures.

Strain, IBK#	Temperature, °C						
	4	22	26	28	30	34	38
1855	GI	4.8±0.1	6.8± 0.3	6.4±0.1	5.2±0.2	2.4±0.1	NG
1927	GI	3.7±0.2	6.8±0.1	6.5±0.1	5.1±0.3	2.2±0.1	NG
1947	GI	4.5±0.1	6.8±0.2	5.9±0.1	4.8±0.1	2.5±0.2	NG
2035	GI	7.7±0.3	11.0±0.2	10.3±0.1	6.6±0.4	3.1±0.2	NG

GI – growth on inoculum, NG – no growth. Data are the mean±standard error for five replications. P<0.05 was considered as statistically significant.

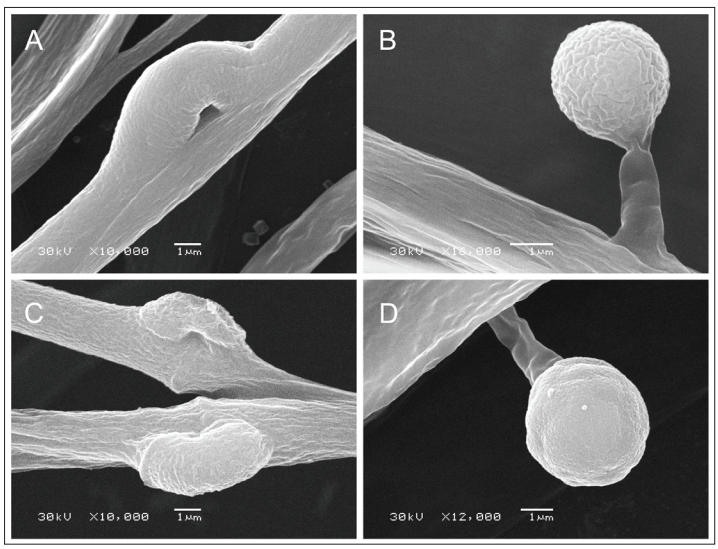

Fig. 3. Scanning electron micrographs of *Pleurotus eryngii var ferulae* IBK 2035: (**A**, **C**) generative hyphae with a medallion clamp connection; *Pleurotus eryngii var ferulae* IBK 1947: (**B**, **D**) excretory cells. Size bars correspond to 1 μm.

about 1.6-5.7-fold faster than that on GPYA, WA and MEA. However, the strains differed in terms of growth rate on the other studied nutrient media. Thus, there was no significant difference between the growth rate of strain IBK 2035 on MEA and WA.

Determination of the critical and optimal temperature for vegetative mycelial growth of the four IBK strains was performed at 4°C, 22°C, 26°C, 28°C, 30°C, 34°C, 38°C and 40°C (Table 3). The critical temperature for growth of the vegetative mycelium was found. All strains showed growth on inoculum at 4°C and terminated growth at 38°C, but they did not lose their viability and resumed growth when the temperature dropped to 26°C within a week. The three-day exposure to 40°C was lethal to all studied strains.

The optimal temperature for the mycelial growth of all strains on PDA was 26°C. The highest rates of radial growth in the temperature range from 26 to 30°C were characteristic of strain IBK 2035. At 28°C, the mycelial growth rate decreased slightly, and at 22°C it decreased dramatically. According to our data, the radial growth rate did not decrease gradually under the temperature change from 22 to 30°C, but it reached its maximum value from 26 to 28°C, which is an important condition for the development of cultivation technologies.

Micromorphological characteristics

Based on the obtained results, criteria for the identification *in vitro* were determined. The vegetative mycelium consisted mainly of moderately branched, regularly septate, colorless generative hyphae with a diameter from 2.5 to 5.0 μm. They formed anastomoses and mycelial strands as shown for *P. tuoliensis* IBK 1855 (Fig. 2 C-E). The vegetative mycelium of *P. eryngii* var. *ferulae* consisted of hyphae with regular one-sided medallion clamp connections (Fig. 3A, C). The formation of the excretory cells was typical for this species. Single spherical hyaline cells with a diameter of 3.0×3.0 μm formed on the simple unbranched lateral hyphal ramifications (Fig. 3B, D). The size of excretory cells ranged from 2.4×2.4 μm to 4.8×4.8 μm and of elliptical ones it was 1.6×2.0 μm.

DISCUSSION

According to Nobles [35], isolates of the same species are very similar in color, the texture of colonies and micromorphological features of the hyphal system, whereas the growth rate and relation to temperature might vary. In addition, the growth rate of the vegetative mycelium, along with the ability to biosynthesize certain metabolites, is an important strain characteristic. The growth rate can be significantly affected by the composition of the nutrient media and the incubation temperature. Information on the growth characteristics of *P. eryngii* on agar nutrient media is quite limited. The ability of *P. eryngii* to develop a generative stage *in vitro* on certain media can be used

for the verification of strains of this species in culture collections.

It should be noted that the formation of the generative stage in pure culture could be one of the most reliable criteria for confirming species identity because it is species specific [20,36]. Thus, while preserving pure cultures in the collection, the formation of fruiting bodies is given special attention. Species of the genus *Pleurotus* form the teleomorph stage very well *in vitro* [37]. The most studied species are well-known edible medicinal and cultivated mushrooms: *Pleurotus ostreatus* (Jacq.) P. Kumm., *P. djamor* (Rumph ex Fr.) Boedijn, *P. cystidiosus* O.K. Mill. and *P. eryngii* [38]. However, it is impossible to obtain fully developed fruit bodies on the agarized media. We observed abortive primordia, lacking gills and basidiospores, mostly in the form of deformed mycelial aggregations of various sizes.

In our investigation, we observed the maximal level of linear growth rates in strain *P. eryngii* var. *ferulae* IBK 2035 – 11.0 mm/day on the PDA medium, while under the same conditions the other strains grew a maximum of 6.8 mm/day. A similar tendency was also demonstrable from the results with three *P. eryngii* strains in our previous experiments [18] where we indicated that PDA was most suitable for their mycelium growth. However, the growth rate on different media may differ depending on the species and strains, for example, for *P. eryngii* strains are indicated at the optimal media: at the same time with PDA authors reported glucose peptone, mushroom complete (MCM), MEA [38], yeast malt extract agar media [34], Czapek Dox agar (CZA) [39]. A similar tendency is also demonstrable from the results by Ryu et al. [40] – 27 strains of *P. eryngii* cultivated on solid media (PDA and MCM) as well as different broth media (PDB broth, MCM and YMG broth).

Examination of the optimal and critical temperatures of vegetative growth were carried out on PDA medium. The best incubation temperature in the study was found at 26°C for all the strains. Studies reported the temperature optimum for *P. eryngii* vegetative growth rate at 20°C [39-41], 25°C [38, 42-45] and 30°C [46]. On the other hand, some had the same high growth level at different temperatures, at 20°C, 25°C, 30°C [40], 26°C, 28°C and 30°C [18]. The temperature at 40°C was found to be lethal for all studied strains. At the same time, there are data showing that no growth was observed at 35°C [42] or 37°C [18] but mycelium continued to grow after their transfer to optimal conditions. On another side, some authors reported that they observed the lowest growth rate of wild *P. eryngii* strains at 40°C [41].

Several articles mention spherical capitate structures, which are called excretory cells [20,47-50], head-like cell offshoots [51], or nematode-trapping spherical blastic structures [36]. Thorn and Barron [48] described how the hyphae of *Pleurotus* mushrooms grew into the mouthpart of the nematode and digested its contents. It has been established that excretory cells secrete drops of a toxin that causes the immobilization of nematodes upon contact with the mycelium. After immobilization, the hyphae of the fungus are wrapped around the nematode and completely lysed it within 12 h. It was reported [52] that *P. eryngii* fungus has predatory activity against *Panagrellus* sp. larvae due to toxin production and negatively affects *M. javanica* eggs and the development of juveniles due to chitinases and proteases production, demonstrating this fungus' potential to be used in biological control. The study of the properties of these structures showed that they represent the evagination of the lateral cell wall, which is differentiated into a stalk and an expanded apical part and performs a specific function of secretion. In some cases, a nucleus can be observed at the base of the stem. Rhodamine staining showed the presence of several mitochondria at the base of the stem. This nuclear-free structure, functionally dependent on the mother cell, does not take part in reproduction: it is not a conidium [51].

Vegetative hyphae of macromycetes in culture are characterized by the presence of various microstructures, some of which are specific to certain species, while others do not carry additional taxonomic information. A significant variety of modified hyphae has been described, which may have taxonomic significance. Researchers have attempted to classify hyphae based on branching or according to their functions [19,35]. Conidia on the heterokaryotic mycelia of *P. cornucopiae* (Paulet) Rolland, *P. eryngii* (DC.) Quél. and *P. pulmonarius* were described [47], and globose

and elliptical conidia on the hyphae of ten species of oyster mushrooms were recorded [53].

A detailed study of clamp connections, anamorphs and other structures of the vegetative mycelium supports the most accurate identification and morphological characterization of the taxon *in vitro*, which is especially important for its preservation in the collection and for biotechnological application. Using some barcode genes, especially the ITS region of the rDNA, is still the most efficient method for molecular identification of most basidiomycetes, including the genus *Pleurotus*. However, there is a large amount of wrongly identified and labelled ITS sequences of the *P. eryngii* species complex despite several successful species boundary studies [32,34]. These records should undergo a proper taxonomical revision, which is a goal of our further study. Until then, placement of the ITS sequences on the phylogenetic tree remains the most trustful method to identify *P. ferulae*, *P. ferulaginis*, *P. nebrodensis* and *P. tuoliensis* using their molecular data.

CONCLUSION

We have studied the main cultural and morphological features of four strains from the *P. eryngii* species complex, which were identified or received as *P. nebrodensis* strains. The studied features were growth rate on various media at different temperatures, abundant one-sided and medallion paired clamp connections on hyphae, excretory cells and primordia formation on agar media of different compositions. These features can serve as strain-specific additional characteristics of *P. eryngii sensu lato* pure cultures. However, for identification to the species level, neither these features nor molecular identification using barcoding of the ITS region was sufficient, mostly because of the abundant presence of false *P. nebrodensis* sequences in GenBank. Only phylogenetic reconstruction with the elimination of those wrongly identified sequences allowed us to identify strains IBK 1855 and 1927 as *P. tuoliensis* (or *P. eryngii* var. *tuoliensis*) and strains IBK 1947 and 2035 as *P. eryngii* var. *ferulae*. The physiological and morphological characteristics can serve as additional criteria for the monitoring of pure cultures and may have a certain importance for strain preservation and cultivation.

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Author contributions: N. Bisko planned the work, investigated growth and morphological characteristics, contributed to the article's conception, made critical revisions of the manuscript; M. Lomberg performed the literature review and article writing and editing, translated the text of the manuscript into English, designed the figures and tables; O. Mykchaylova isolated the pure culture from a carpophore, investigated the vegetative mycelium by scanning electron microscopy, together with M. Lomberg isolated DNA of investigated strains, wrote the first draft of the manuscript; N. Mytropolska investigated growth characteristics and temperature tolerance, performed the statistical data analysis; V. Kutovenko obtained and provided the new literature data for review, entered the received sequences to GeneBank; A. Gryganskyi coordinated the work, performed the molecular experiments, including PCR, and phylogenetic analysis, analyzed and interpreted the results of RNA sequencing, created the ML tree used for phylogenetic identification, made critical revisions of the manuscript. All authors read and approved the final version of the manuscript.

Data availability: Data underlying the reported findings are deposited in the NCBI database at: GenBank https://www.ncbi.nlm.nih.gov/genbank/ as follows: MN646251.1 – isolate IBK2035 and MN646250.1 – IBK1947; MN646249.1 – isolate IBK1927 and MZ234121.1 – strain IBK 1855. The data are also available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Bisko%20et%20al_7804_Data%20Report.pdf

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