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ORIGINAL ARTICLE

Phylogenetic relationship of two popular edible *Pleurotus* in China, Bailinggu (*P. eryngii* var. *tuoliensis*) and Xingbaogu (*P. eryngii*), determined by ITS, RPB2 and EF1α sequences

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Abstract The aims of this study are to assess the utility of the internal transcribed spacer (ITS) region, and partial translation elongation factor (EF1α) and RNA polymerase II (RPB2) genes, for differentiation of Bailinggu, *P. eryngii*, and *P. nebrodensis*; to reconstruct phylogenetic relationships between the three species; and to confirm the taxonomic status of Bailinggu based on ribosomal and protein-coding genes. Pairwise genetic distances between Bailinggu, *P. eryngii*, and related *Pleurotus* strains were calculated by using the p-distance model, and molecular phylogeny of these isolates was estimated based on ITS, RPB2, and EF1α using maximum parsimony and Bayesian methods. Differences in ITS, RPB2, and EF1α sequences show that Bailinggu, *P. eryngii*, and *P. nebrodensis* are distinct at the species level. Phylogenetic analyses reveal that *P. eryngii* is closer to *P. nebrodensis* than to Bailinggu. Sequence analyses of ribosomal and protein-coding genes confirm that *P. eryngii* var. *tuoliensis* is identical to Bailinggu. *P. eryngii* var. *tuoliensis* should be raised to species level or a new name should be introduced for Bailinggu after a thorough investigation into *Pleurotus* isolates from *Ferula* in Xinjiang Province. This study helps to resolve uncertainty regarding Bailinggu, *P. eryngii* and *P. nebrodensis*, improving the resource management of these strains. ITS, EF1α, and RPB2 sequences can be used to distinguish Bailinggu, *P. eryngii* and *P. nebrodensis* as three different species, and *P. eryngii* var. *tuoliensis* should be the scientific name for Bailinggu at present.

Keywords Oyster mushroom · Awei mo · *Ferula* · Speciation

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Introduction

Pleurotus is one of the most diverse groups of cultivated mushrooms. In this group, "Xingbaogu" and "Bailinggu" are two popular edible mushrooms of high commercial value that are widely cultivated in China. Xingbaogu was introduced from Europe about 20 years ago, and it is commonly accepted that *P. eryngii* (DC.) Quél. is the scientific name for this popular edible mushroom. Bailinggu, a famous edible mushroom, was known as "Awei Mo" in Xinjiang Province, where they were first cultivated and widely exported. Bailinggu was a trade name for the cultivated Awei Mo proposed by Mao in 1997 [1]. Although the name has become widely accepted, the taxonomic status of Bailinggu remains uncertain. *P. eryngii* var. *nebrodensis*, *P. eryngii* var. *tuoliensis*, and *P. nebrodensis* have all been used as scientific names for Bailinggu [1–8]. After its successful cultivation, Bailinggu was morphologically identified as *P. nebrodensis* (Inzenga) Quél. by Mao [1]. *P. eryngii* var. *nebrodensis* has also been used for Bailinggu by some researchers because of taxonomic disagreements regarding *P. nebrodensis* [9, 10]. *P. eryngii* var. *tuoliensis*, also known as Awei Mo, was discovered on *Ferula* sp. in Tuoli and Mulei counties of Xinjiang Province by Mou et al. [11], and was first cultivated in that region. Awei Mo is a general designation of *Pleurotus* spp. on *Ferula*, and two different taxa, *P. eryngii*

and *P. eryngii* var. *tuoliensis* were involved [11, 12]. Mao [2] accepted that Bailinggu was actually the cultivated strain of *P. eryngii* var. *tuoliensis*, and concluded that *P. eryngii* var. *tuoliensis* should be a synonym of *P. nebrodensis*, as did Jia and Qin [7]. *P. nebrodensis* has become dominant in the last few decades and the name has appeared in many publications and commercial catalogs by Chinese researchers, which have proliferated since the 1990s. More recently, *P. eryngii* var. *tuoliensis* C. J. Mou was resurrected as the scientific name for Bailinggu at the variety level by Kawai et al. based on analysis of internal transcribed spacer (ITS) sequences [5].

The relationship between Bailinggu, *P. eryngii* and *P. nebrodensis* remains controversial. Prior to this study, some attempts have been made to clarify the relationship between them. Based on PCR–RFLP analysis of partial 28S rDNA and monokaryon–monokaryon mating tests, Bao et al. [13, 14] concluded that *P. eryngii* and "*P. nebrodensis*" (Bailinggu) from China were independent and incompatible species. Zhang et al. [12] investigated the genetic polymorphism of Ferula Mushroom growing on *Ferula sinkiangensis* using ITS sequences and IGS1-RFLP analyses, and their results showed that *P. eryngii* and Bailinggu (as "*P. nebrodensis*") are two different *Pleurotus* species growing on *Ferula sinkiangensis*. Kawai et al. [5] indicated that Bailinggu and *P. nebrodensis* belong to the same species, *P. eryngii*, because hybridization occurred between them.

However, the aforementioned studies were mainly based on a single ribosomal gene, and the results did not resolve the uncertainty regarding Bailinggu, *P. eryngii*, and *P. nebrodensis*. There is not as yet a unified scientific name for Bailinggu, and many strains labeled "*P. eryngii*" or "*P. nebrodensis*" in culture collection centers are actually representatives of Bailinggu. These have caused much confusion for phylogenetic, genetic, and breeding programs, which hindered strategies for scientific research, breeding, and commercial activities of Bailinggu. In contrast, the utility of ITS sequences for species identification in *Pleurotus* has been questioned, and we hesitate to agree with the treatment of Bailinggu and *P. nebrodensis* as different varieties of the same species *P. eryngii*. It is important to clarify the taxonomic status of Bailinggu and the relationship between Bailinggu, *P. eryngii*, and *P. nebrodensis* based on multiple molecular markers.

ITS sequences have been widely used to reconstruct phylogenetic relationships and for species discrimination in some Agaricales groups at or below the species level. According to previous studies [15, 16], ITS sequences cannot be used to unequivocally distinguish varieties in the genus *Pleurotus*, whereas a more recent study revealed that ITS might be a desirable DNA barcode for cultivated oyster mushroom [17]. The two protein-coding genes RPB2 and EF1α have been used in combination with other genomic regions to infer phylogenetic relationships at the species level for some mushrooms [18], and their utility for discrimination and phylogenetic reconstruction among members of *Pleurotus* at the species or variety level has also been explored [19]. Furthermore, RPB2 yielded better resolution than ITS for species discrimination in fungal groups [20]. Nevertheless, RPB2 and EF1α have not been used to distinguish Bailinggu from *P. eryngii* and *P. nebrodensis* in previous studies.

In the present study, ITS, RPB2, and EF1α sequences of Bailinggu, *P. eryngii*, *P. nebrodensis*, and related species were analyzed: (1) to assess the utility of the three genes for differentiation of Bailinggu, *P. eryngii*, and *P. nebrodensis*; (2) to clarify relationships between the three species; and (3) to confirm the scientific name of Bailinggu based on ribosomal and protein-coding genes. This study would help to improve the resource management of Bailinggu, *P. eryngii*, and *P. nebrodensis*, and provide a theoretical basis for phylogenetic, genetic, and breeding research.

Materials and methods

Sampling, GenBank data

Three wild Awei Mo strains ("Awei", "Pnh529" and "HM777041"), and cultivated Bailinggu and P. eryngii strains were tested in this study (Table 1). The wild strains were isolated from Awei Mo on *Ferula* in Xinjiang Province, and the rest cultivated Bailinggu and *P. eryngii* strains were obtained from Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences (SAAS Table 1). Isolates of Bailinggu and *P. eryngii* were grown on potato dextrose agar medium in a Petri dish at 25 °C for 7–10 days. These strains were stored at 4 °C.

Accession numbers of the tested sequences with codes "GU" and "GQ" presented in Table 1 were from Rodriguez Estrada et al. [19].

DNA extraction, PCR and sequencing

Genomic DNA was extracted from mycelia using a Biospin Fungus Genomic DNA Extraction Kit according to the manufacturer's instructions. ITS sequence was amplified using the primers ITS4 and ITS5 [21]. The primers used for RPB2 were fRPB2 5F (5′-GAYGAYMG WGATCAYTTYGG-3′) and bRPB2 7.1R (5′-CCCATRGCYTGYTTMCCCATDGC-3′), and b6.9F (5′-TGGAC NCA YTGY GARATYCAYCC-3′) and b11R1 (5′-TGGATYTTG TCRTC CACCAT-3′) [22, 23]. Primers b6.9F and b11R1 were used to amplify a region between domains

Table 1 List of strains, source and GenBank accession numbers for phylogenetic analysis in this study

Species	Isolate number	Source	Location	GenBank accessions	
				EF1a	RPB2
Pleurotus cornucopiae	WC608	ASI	Unknown	GU186806	GU186820
P. cystidiosus	WC609	ASI	Unknown	GU186808	GU186819
P. elaeoselini	WC999	U. Palermoi	Unknown	GU186799	GU186811
P. eryngii	Pe-AL1	INRAc	Entre Deux Mers, Launay, France	GQ225115	GQ225114
P. eryngii	Pe-Al11	INRA	Causse Mejean, France	GU139132	GU186821
P. eryngii	WC888	SEFId	Unknown	GU139133	GU186794
P. eryngii	Pe-Al32	Commercial farm	Unknown	GU139127	GU186822
P. eryngii	WC968	IBAFe	Unknown	GU139128	GU186795
P. eryngii	WC967	IBAF	Unknown	GU139134	GU186796
P. eryngii	WC957	U. Barif	Sicily, Italy	GU139131	GU186831
P. eryngii	WC984	IBAF	Unknown	GU139130	GU186797
P. eryngii	Pe21	SAAS	China	KU727126	KU727133
P. eryngii	Pe11	SAAS	China	KU727124	KU727132
P. eryngii	Pe16	SAAS	China	KU727125	KU727131
P. eryngii var. *ferulae*	WC966	IBAF	Sardegna, Italy	GU139135	GU186823
P. eryngii var. *ferulae*	WC955	U. Bari	Sicily, Italy	GU139141	GU186810
P. eryngii var. *ferulae*	WC929	U. Haifa	Gilboa Mt., Israel	GU139140	GU186824
P. eryngii var. *ferulae*	WC933 1	U. Haifa	Gevaot Merar, Israe	GU139139	GU186825
P. eryngii var. *ferulae*	WC927	U. Haifa	Menahemya, Israel	GU139138	GU186826
P. eryngii var. *ferulae*	WC981	IBAF	Sicily, Italy	GU139144	GU186827
P. eryngii var. *ferulae*	WC970	IBAF	Puglia, Italy	GU139143	GU186828
P. eryngii var. *ferulae*	WC982	IBAF	Sardegna, Italy	GU139145	GU186816
P. eryngii var. *ferulae*	WC956	U. Bari	Bari, Italy	GU139137	GU186798
P. eryngii var. *ferulae*	WC850	PSU	China	GU139142	GU186829
P. eryngii var. *ferulae*	WC969	IBAF	Sardegna, Italy	GU139136	GU186830
P. eryngii var. *ferulae*	WC994	CBSh	Unknown	GU139146	GU186812
P. eryngii var. *nebrodensis*	WC777	IBAF	Sicily, Italy	GU186800	GU186814
P. eryngii var. *nebrodensis*	WC979	IBAF	Sicily, Italy	GU186801	GU186813
P. eryngii var. *nebrodensis*	WC980	IBAF	Sicily, Italy	GU186802	GU186815
P. eryngii var. *tuoliensis*	Bai2	SAAS	Sichuan, China	KU727119	KU727138
P. eryngii var. *tuoliensis*	Pnh622	SAAS	Sichuan, China	KU727128	KU727134
P. eryngii var. *tuoliensis*	K888	SAAS	Japan	KU727121	KU727136
P. eryngii var. *tuoliensis*	Awei	SAAS	Xinjiang, China	KU727120	KU727137
P. eryngii var. *tuoliensis*	Pnh529	SAAS	Xinjiang, China	KU727127	KU727135
P. ostreatus	WC739	Italspawn	Unknown	GU186804	GU186817
P. ostreatus	WC971	IBAF	Italy Basilicata	GU186805	–

Accession numbers with codes "GU" and "GQ" were from Rodriguez-Estrada et al. [19], and those with "KU" were generated in the present study

3–11; and fRPB2 5F and bRPB2 7.1R were used for amplification of domains 5–11. Primers EF116OR (5′-CCGAT CTTGTA GACGT CCTG-3′) and EF595F (5′-CGTGACTTCAT CAAGAAC ATG-3′) were used to amplify a portion of the EF1α gene [24, 25]. Amplification was performed in 30-μl volumes containing 1 μl template DNA, 12 μl distilled water, 1 μl of each primer and 15 μl PCR mix (DreamTaq™ Green PCR Master Mix [2×], Fermentas). Amplifications for the three genes were carried out under the same conditions: 94 °C/5 min; 35 cycles of 94 °C/1 min, 55 °C/1 min, 72 °C/90 s; and a final extension step of 72 °C/10 min. Primers for sequencing are the same as amplification. Sequences generated in the present study are deposited in GenBank.

Phylogenetic and statistical analyses

The sequences used in phylogenetic analysis were aligned in Muscle 3.6 [26] and ClustalX [27], and manually modified in BioEdit 7.0.9.0 where necessary [28]. Sites judged to be too ambiguous in alignment, as well as spliceosomal introns in RPB2, were excluded from phylogenetic analysis.

The ITS and the combined EF1α and RPB2 dataset (Table 1) were analyzed respectively using maximum parsimony (MP). MP analyses were conducted in PAUP* version 4.0b10 [29]. All characters were treated as unordered and of equal weight. Gaps were treated as missing data. Bootstrap values were calculated from 1000 replicates.

Bayesian analyses were also performed on ITS and the combined dataset using MrBayes 3.1.2 [30]. Six Markov chains were run for two runs from random starting trees for one million generations and sampled every 10 generations. Every time the diagnostics were calculated, 25 % of the samples from the beginning of the chain were discarded. A majority rule consensus tree of all remaining trees was calculated.

Nucleotide differences between *Pleurotus* species were calculated. Genetic distances were inferred by computing pairwise distances based on the p-distance (nucleotide substitution) model in Mega 4 [31].

Results

ITS sequence analysis of Bailinggu, *P. eryngii*, *P. nebrodensis, and Pleurotus* spp.

ITS sequences obtained in this study consist of 592 bp, comprising 232 bp for ITS1, 145 bp for 5.8S, and 215 bp for ITS2. The genetic distances among Bailinggu, *P. eryngii*, P. *nebrodensis*, and the other *Pleurotus* spp. (Fig. 1) were inferred based on the p-distance (nucleotide substitution) model. The lowest divergence (0.005) among these species was between *P. populinus* (AY 450346) and *P. ostreatus* (AY 450345, epitype of *P. ostreatus*), and the highest (0.178) was observed between *P. eryngii* and *P. djamor* (GU 722277). Among the 18 taxa tested, the divergence value between Bailinggu and *P. eryngii* (0.016) is higher than those between Bailinggu and either *P. populinus* or *P. ostreatus* (0.013 and 0.009 respectively), but identical to that between *P. populinus* and *P. pulmonarius*. The divergence between Bailinggu and *P. nebrodensis* is 0.009, which is identical to the distance between Bailinggu and *P. ostreatus*. The distance between *P. eryngii* and *P. nebrodensis* (0.011) is identical to the divergence between *P. ostreatus* and *P. pulmonarius* but higher than that between *P. ostreatus* and Bailinggu.

Among these tested *Pleurotus* species, 11 nucleotides could be used to distinguish the least divergent species *P. populinus* (AY 450346) and *P. ostreatus* (AY 450345); 12 for *P. nebrodensis* and *P. ostreatus*; 13 for Bailinggu and *P. ostreatus*; and 14 for *P. eryngii* and *P. ostreatus*. Regarding *P. nebrodensis*, Bailinggu, and *P. eryngii*, the ITS similarities of Bailinggu and *P. eryngii* are 97–98 %, and 14–16 nucleotides were found to be different between these isolates (Table 2). A total of 12 different bases were observed in the ITS sequences of *P. eryngii* and *P. nebrodensis*, and 14 base substitutions could distinguish Bailinggu from the European *P. nebrodensis*. Additionally, six base substitutions were also found in partial ITS1 sequences of the epitype of *P. nebrodensis* and Bailinggu.

ITS sequences recovered from wild Awei Mo strains were almost identical to those of the cultivated Bailinggu strains, differing in no more than four bases. However, up to 16 and 14 different nucleotides were found between *P. eryngii* and wild Awei Mo, and between *P. nebrodensis* and wild Awei Mo, respectively. As mentioned previously, two taxa (*P. eryngii* and *P. eryngii* var. *tuoliensis*) are involved in Awei Mo. Therefore, the wild Awei Mo tested in the present study should be *P. eryngii* var. *tuoliensis*, and Bailinggu could be the cultivated strains of *P. eryngii* var. *tuoliensis*.

Sequence analysis of EF1α and RPB2 genes

Two portions of RPB2 sequences were amplified using the two pairs of RPB2 primers, and they were edited and trimmed following Rodriguez Estrada et al. [19]. Of the RPB2 sequences, 1253 sites remained, and the two portions were joined by a string of 369 Ns estimated from alignments with the RPB2 sequence of *P. ostreatus* (AY786062). In total, 33 sites were able to distinguish Bailinggu and *P. eryngii* from each other; 26 base substitutions allowed discrimination between *P. nebrodensis* and Bailinggu; and a total of 20 sites differentiated *P. nebrodensis* from *P. nebrodensis* (Table 3).

Amplification of the EF1α gene yielded a segment of 538 bp. EF1α showed less variation than RPB2. Eleven base substitutions (95, 155, 172, 181, 188, 241, 271, 334, 346, 480, 483) discriminated Bailinggu from *P. eryngii*. Eight base substitutions at positions 116, 155, 172, 181, 334, 346, 394, 483 discriminated *P. nebrodensis* from Bailinggu, and seven sites (95, 116, 188, 241, 271, 394, 480) could distinguish *P. nebrodensis* from *P. eryngii*.

Only two different sites were observed between RPB2 sequences of wild Awei Mo strains and the cultivated Bailinggu strains; and four different bases for EF1α

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 Bailinggu																	
2 eryngii	0.016																
3 nebrodensis	0.009	0.011															
4 AY450345 ostreatus	0.009	0.018	0.014														
5 AY450346 populinus	0.014	0.023	0.018	0.005													
6 AY450349 pulmonarius	0.021	0.030	0.025	0.011	0.016												
7 AY450340 opuntiae	0.161	0.173	0.167	0.153	0.153	0.148											
8 GU722277 djamor	0.169	0.175	0.175	0.161	0.164	0.155	0.101										
9 AY450338 calyptratus	0.163	0.168	0.168	0.154	0.155	0.146	0.077	0.067									
10 AF345661 albidus	0.032	0.042	0.037	0.023	0.018	0.028	0.153	0.167	0.158								
11 AF345656 abieticola	0.023	0.032	0.027	0.020	0.021	0.021	0.144	0.161	0.149	0.034							
12 AY450341 cornucopiae	0.142	0.154	0.148	0.137	0.142	0.129	0.113	0.108	0.104	0.151	0.140						
13 AY540319 citrinopileatus	0.134	0.146	0.140	0.129	0.135	0.121	0.113	0.113	0.107	0.143	0.132	0.020					
14 AY450342 australis	0.134	0.141	0.135	0.129	0.132	0.124	0.134	0.121	0.131	0.135	0.126	0.112	0.115				
15 AY315808 abalonus	0.137	0.146	0.142	0.134	0.137	0.135	0.131	0.121	0.127	0.142	0.134	0.118	0.126	0.056			
16 EF514244 cystidiosus	0.143	0.154	0.148	0.140	0.143	0.135	0.120	0.121	0.127	0.137	0.129	0.115	0.118	0.049	0.037		
17 GU722278 dryinus	0.154	0.166	0.159	0.146	0.151	0.146	0.136	0.152	0.139	0.157	0.148	0.117	0.120	0.107	0.094	0.091	
18 AF345662 levis	0.143	0.154	0.148	0.135	0.138	0.132	0.126	0.116	0.119	0.143	0.129	0.084	0.087	0.084	0.076	0.073	0.096

Fig. 1 Distances between Bailinggu, *P. eryngii* and related *Pleurotus* species based on p-distance model

Table 2 Variable nucleotide sites found in complete sequences of the internal transcribed space (ITS) among Bailinggu, Xingbaogu (*Pleurotus eryngii*) and *P. nebrodensis*

Species	Sites																				
	9	84	99	108	132	137	155	160	161	165	166	210	211	212	256	317	465	466	638	648	677
Bailinggu	G	C	G	C	C	G	T	C	A	C	G	–	A	T	T	A	T	T	A	T	T
eryngii	A	T	A	T	T	G	G	C	G	C	G	A	T	A	A	A	–	–	G	C	C
nebrodensis	A	C	A	T	C	C	T	T	G	T	A	–	A	T	T	G	–	–	G	C	C

sequences. The results based on protein-coding genes are in accord with those of ITS sequence analysis.

Phylogenetic analysis inferred from *ITS*, and the combined partial sequences of *EF1α and RPB2* genes

The parsimony analysis of ITS sequences was conducted using 48 taxa, with *Hohenbuehelia grisea* (Peck) Singer and *H. mastrucata* (Fr.) Singer designated as outgroups. The aligned dataset contained 694 nucleotide sites, among which 355 characters were constant, 250 were parsimony-informative, and 89 variable characters were parsimony-uninformative. Parsimony analysis resulted in 15 most parsimonious trees, and one of them is shown in Fig. 2.

The combined dataset of RPB2 and EF1α consisted of 2152 nucleotide sites, among which 1751 characters were constant, 199 were parsimony-informative, and 202 variable characters were parsimony-uninformative. Parsimony analysis resulted in eight most parsimonious trees, and one of the most parsimonious trees is shown in Fig. 3. Bayesian analyses produced topologies almost identical to those from parsimony analyses.

In the ITS tree (Fig. 2), all the *Pleurotus* species we tested formed a monophyletic group with a bootstrap value of 100 %, divided into two different clades. Clade I was composed of *P. cystidiosus* var. *abalonus*, *P. cystidiosus*, *P. australis*, *P. dryinus*, *P. levis*, *P. djamor*, *P. calyptratus*, *P. opuntiae*, *P. cornucopiae*, and *P. citrinopileatus*. Bailinggu, *P. eryngii*, and *P. nebrodensis* clustered in Clade II with *P. abieticola*, *P. pulmonarius*, *P. ostreatus*, *P. sapidus*, *P. ostreatus* var. *florida*, *P. populinus*, and *P. albidus*. Isolates of Bailinggu, *P. eryngii*, and *P. nebrodensis* clustered together in the same clade with low support (bootstrap value 65 %), and the latter two groups (*P. eryngii*, and *P. nebrodensis*) were nested in an internal clade with a bootstrap value of 81 %. Isolates of cultivated Bailinggu, the wild Awei Mo strains collected from Xinjiang,

Table 3 Variable nucleotide sites found in partial sequences of RPB2 among Bailinggu, Xingbaogu (*Pleurotus eryngii*) and *P. nebrodensis*. Position numbers are given according to GQ225114

Species	Site																				
	6	20	41	95	152	269	296	456	463	478	488	494	518	928	958	961	974	1093	1096	1099	
Bailinggu	T	G	T	C	T	C	C	T	C	A	C	C	T	C	G	C	C	A	A	T	
eryngii	C	C	C	T	T	C	A	A	T	T	C	T	C	T	A	T	T	T	T	C	
nebrodensis	T	C	C	T	C	T	C	A	T	A	G	C	C	T	G	T	C	T	T	T	
Species	Site																				
	1108	1111	1144	1162	1219	1222	1279	1297	1312	1417	1468	1501	1504	1507	1546	1558	1594	1615	1617		
Bailinggu	A	A	A	G	G	C	T	C	C	G	G	G	C	C	A	A	C	A	G		
eryngii	G	A	G	A	C	T	C	T	T	C	C	A	C	C	G	G	T	G	–		
nebrodensis	A	G	A	A	C	T	T	C	T	G	C	G	T	G	G	G	G	G	–		

"GQ456052, *P. nebrodensis*" and three Iranian "*P. nebrodensis*" (FJ514550, FJ514589, FJ514568) were clustered in the same clade with strong support (92 %). *P. eryngii* var. *eryngii*, *P. eryngii* var. *ferula*, and var. *elaeoselini* were grouped as a monophyletic group (bootstrap value 94 %), while the different varieties could not be separated by their ITS sequences. Additionally, *P. nebrodensis* from Italy was nested in another clade with a support of 87 %, and it was placed as the sister species to the *P. eryngii* complex.

Topologies of the *P. eryngii* complex, *P. nebrodensis*, and Bailinggu in the trees inferred from the combined EF1α and RPB2 are similar to those from ITS sequences. The three groups formed a monophyletic clade with a support of 95 %, while isolates of Bailinggu, *P. eryngii*, and *P. nebrodensis* were nested in different subclades with strong support. The phylogenetic trees placed *P. nebrodensis* as the closest relative of the *P. eryngii* species complex. Furthermore, the analysis showed that the representatives of *P. eryngii* var. *eryngii* and *P. eryngii* var. *ferula* could be separated based on the combined partial sequences of EF1α and RPB2, as previously reported by Rodriguez-Estrada et al. [19].

The results showed that Bailinggu, *P. eryngii*, and *P. nebrodensis* are three distinct species rather than varieties within the same species *P. eryngii*, and that *P. nebrodensis* is the species most closely related to the *P. eryngii* complex.

Discussion

Because the application of an incorrect scientific name for Bailinggu may hinder strategies for breeding and create complications for publications, patents and products, the application of a correct scientific name for commercial and research-oriented Bailinggu strains is important. Although doubts concerning the relationship between Bailinggu, *P. eryngii*, and *P. nebrodensis*, and the identity of Chinese Bailinggu, have been expressed by some researchers [5, 13, 14], none of them have proved that Bailinggu is a distinct species separate from *P. eryngii*; or that *P. nebrodensis* is closer to *P. eryngii*. There is no doubt that more breeding and scientific research materials of Bailinggu could be uncovered based on the analyses of ITS, RPB2, and EF1α sequences. This study would improve the uncertainty around Bailinggu, *P. eryngii*, and *P. nebrodensis*, promoting scientific research and breeding programs for these mushrooms.

Although Bailinggu is the cultivated strain of *P. eryngii* var. *tuoliensis*, and that molecular analyses suggest that it is a distinct species from *P. nebrodensis* and *P. eryngii*, we hesitate to raise *P. eryngii* var. *tuoliensis* to the species leve

Fig. 2 Phylogenetic relationship of Bailinggu, *P. eryngii*, *P. nebrodensis* and related species inferred from ITS sequences. Maximum parsimony bootstrap values (BS > 50%) are indicated above or below the branches

or to introduce a new name for the well-known Bailinggu, because the holotype of *P. eryngii* var. *tuoliensis* is lost, and neither additional materials from the type locality nor from adjacent areas are available for morphological or molecular phylogenetic comparison. For the time being, we propose that the name *P. eryngii* var. *tuoliensis* be used for Bailinggu.

P. eryngii var. *tuoliensis* has been recorded on *Ferula sinkiangensis* and *F. ferulaeoides* (Steud.) Korov. in Xinjiang Province, and some researchers traced the cultivation history of Bailinggu and concluded that it is the cultivated strain of *P. eryngii* var. *tuoliensis*. Zhang et al. [12] found that there are only two different *Pleurotus* species, *P. eryngii* and Bailinggu (as "*P. nebrodensis*"), on *Ferula sinkiangensis*. In the present phylogenetic analysis based on ITS sequences, a voucher specimen HM77041 labeled as *P. eryngii* var. *tuoliensis* was collected on *Ferula* in Xinjiang Province, and its morphological characters correspond to the original descriptions of *P. eryngii* var. *tuoliensis* except for variation in stipe length. Additionally, ITS, RPB2, and EF1α sequences of this voucher specimen and other two wild Awei Mo strains are almost identical with the cultivated Bailinggu. It seems obvious that Bailinggu should be the cultivated *P. eryngii* var. *tuoliensis*. Three Iranian "*P. nebrodensis*" were also nested in the Bailinggu clade, which were collected on *Ferula* [32]. In conjunction with previous studies [5, 12, 33], it can be deduced that the distribution of wild Bailinggu is not

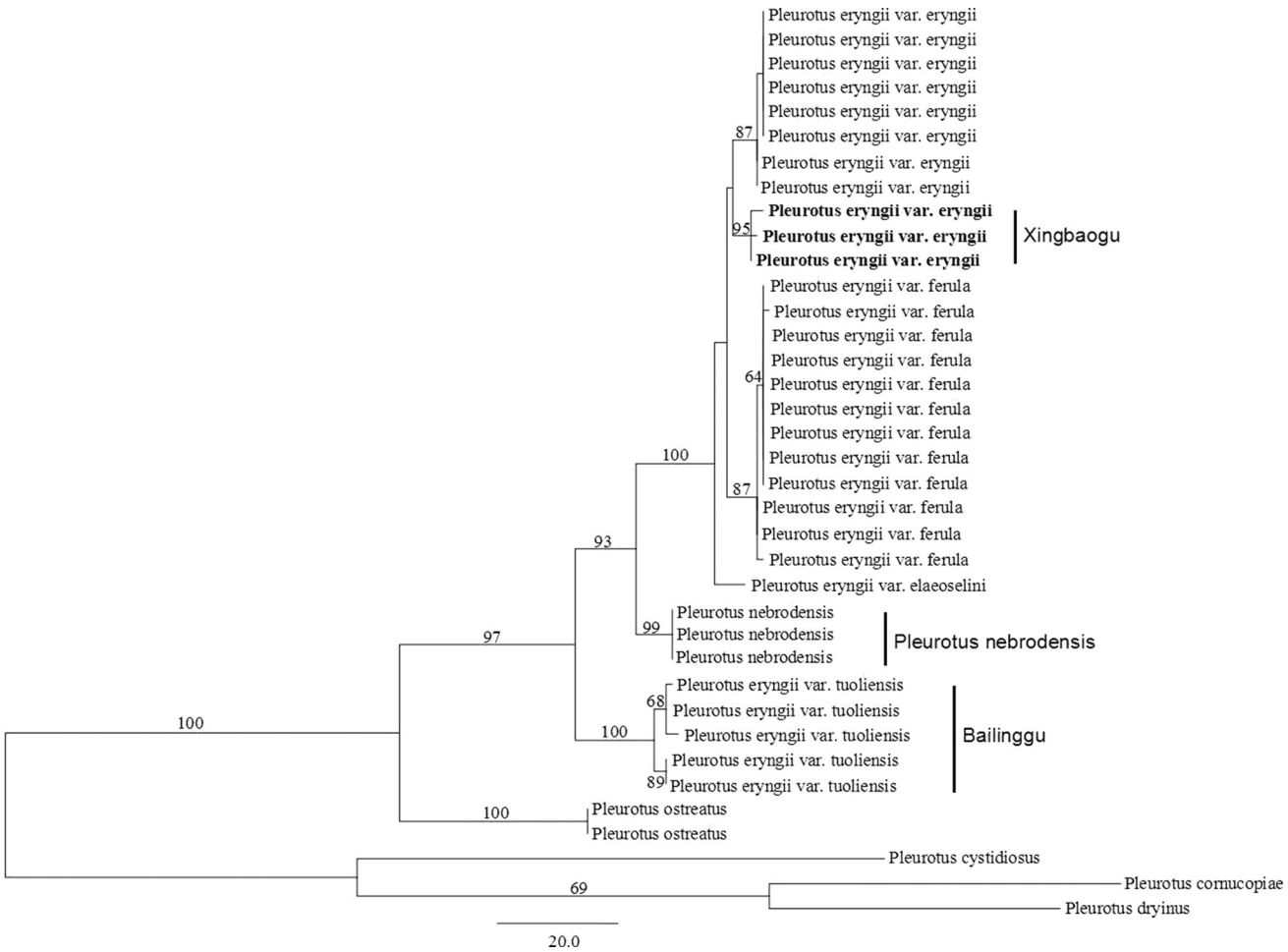

Fig. 3 Phylogenetic reconstruction of Bailinggu, *P. eryngii* and *P. nebrodensis* based on the combined partial EF1α and RPB2 sequences. Maximum parsimony bootstrap values (BS > 50%) are indicated above or below the branches

restricted to China, but that it is also found in other Asiatic areas on *Ferula* host-plants. Unlike Bailinggu, which is restricted to *Ferula*, the European *P. nebrodensis* is associated only with *Cachrys ferulacea*, and they were placed in a distinct "*nebrodensis*" clade in the analysis (Fig. 3). *P. eryngii* has lower host specificity than *P. nebrodensis* or *P. eryngii* var. *tuoliensis*, and various host-plants of *P. eryngii* have been reported in previous studies [32, 34]. In contrast, the morphological traits of Bailinggu are also different from *P. eryngii*. Bailinggu always has a white cap, while *P. eryngii* has a pale brown cap; and the basidiospores of Bailinggu are larger than those of *P. eryngii*. Differences in habitat, morphological characters, and sequences of ribosomal and protein-coding genes distinguish Bailinggu from *P. nebrodensis* and *P. eryngii*.

The relationship among *P. eryngii* var. *tuoliensis*, *P. nebrodensis*, and *P. eryngii* has been controversial. The present results uncover that they are three different species, and *P. nebrodensis* rather than *P. eryngii* var. *tuoliensis* shows the closest relationship to the *P. eryngii* species complex. Despite previous studies based on morphological evidence and RAPD analysis supporting the separation of *P. nebrodensis* from the *P. eryngii* complex and its classification within a distinct taxonomic entity at the species level [32, 33, 35], Kawai et al. [5] indicated that *P. eryngii* var. *tuoliensis*, *P. eryngii* var. *eryngii*, and *P. eryngii* var. *nebrodensis* should be regarded as different varieties of the same species *P. eryngii* because propagation occurred among them. In their study, Kawai et al. [5] over-emphasized the biological species concept, but reproductive barriers may not be absolute, and two different species may be compatible when the divergence time between them is short [36]. Additionally, mating rates observed in the laboratory could be greater than those that occur in nature, because gene flow can be reduced because of host specificity and allochrony. The molecular evidence furnished by this work and that of Rodriguez Estrada et al. [19] have further confirmed that *P. nebrodensis* should be considered a different species rather than a variety. In our phylogenetic analysis, *P. eryngii, P. nebrodensis*, and *P. eryngii* var. *tuoliensis* form a monophyletic group, showing a close relationship between them. This result suggests that the

morphological or habit similarity may be a consequence of recent speciation events, and relatively minor genetic differences were found between these species.

In conclusion, ITS, RPB2 and EF1α sequences can be used to discriminate between Bailinggu, Xingbaogu (*P. eryngii*), and *P. nebrodensis*. *P. eryngii* is more closely related to *P. nebrodensis* than to Bailinggu. Although the name *P. nebrodensis* has been widely applied to the Chinese species of Bailinggu in recent decades, it is a misapplied name. *P. eryngii* var. *tuoliensis* should be recognized as the scientific name for Bailinggu for the time being. *P. eryngii* var. *tuoliensis* represents a distinct species, but thorough investigation into *Pleurotus* isolates from *Ferula* in Xinjiang Province are needed before raising the taxonomic status of *P. eryngii* var. *tuoliensis* or introducing a new name for Bailinggu. Additional specimens of *P. eryngii* var. *tuoliensis* collected in the type locality or its adjacent areas need to be examined.

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