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Full Length Article

Correlation between Mating Compatibility and the Phylogenetic Relationship of a Rare Edible Mushroom, *Pleurotus nebrodensis*, with Different *Pleurotus* Species

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Abstract

Pleurotus nebrodensis is a newly developed, rare, edible mushroom, with increasing economic value owing to its nutritional content, excellent shape and firm texture. Currently, its industrial development is strongly limited because of low biological efficiency, strict cultivation conditions, as well as unstable fruiting. To provide a base for *P. nebrodensis* breeding *via* hybridization, its mating compatibility with various *Pleurotus* species was determined. Additionally, a phylogenetic tree was constructed using Neighbor-joining method with internal transcribed spacer (ITS) region sequences. Mating tests demonstrated that nine dikaryons in 16 pairings were formed between four monokaryons showing different mating-types of *P. nebrodensis* with those of *P. eryngii*; however, only one successful mating was observed with *P. ferulae*. Strikingly, *P. cornucopiae*, a distant related species of *P. nebrodensis*, produced seven dikaryons with *P. nebrodensis*. *P. pulmonarius*, *P. abalonus*, *P. djamor* and *P. citrinopileatus* were found to be incompatible with *P. nebrodensis*. On this basis, *P. eryngii*, *P. ferulae* and *P. cornucopiae* can be employed as parental strains for hybridization breeding of *P. nebrodensis* because of their compatibility. The phylogenetic tree showed a close relationship of *P. nebrodensis* with *P. eryngii* and *P. ferulae*, followed by *P. cornucopiae*, *P. pulmonarius*, *P. abalonus*, *P. djamor* and *P. citrinopileatus*. Combination of phylogenetic analysis and mating tests indicated that the mating compatibility of *P. nebrodensis* with tested *Pleurotus* species was closely related to their genetic relationship. Undoubtedly, these study findings will accelerate hybridization breeding of *P. nebrodensis* because it provides a strain spectrum for parental selection.

Keywords: *Pleurotus nebrodensis*; Mating compatibility; Phylogenetic relationship; Hybridization breeding

Introduction

Pleurotus nebrodensis, belonging to Basidiomycetes, is a newly developed edible mushroom with excellent flavor and firm texture. Its distribution is restricted to Xinjiang in China and Sicily in Italy, where it is found on *Cachrys ferulacea* and *Ferula sinkiangensis*, respectively (Mao, 2000; Venturella, 2000). The *Pleurotus nebrodensis* fruiting body is rich in nutrients, including sub-oleic acid, non-saturated fatty acids, proteins, amino acids and many micro elements such as calcium, zinc and manganese (Alam *et al*., 2009). In terms of medicinal value, it contains a number of biologically active compounds like polysaccharides with therapeutic activities such as modulation of the immune system, inhibition of tumor growth and inflammation, hypoglycemic and antithrombotic activities, decreasing blood lipid concentration and prevention of high blood pressure and atherosclerosis (Wang and Ng, 2004; Choi *et al*., 2005; Lv *et al*., 2009). Apart from nutritional and medicinal values, *P. nebrodensis* has a fat body, is pure white color and crisp and has a delicious taste, making it a preferred choice of consumers. Owing to these excellent attributes, *P. nebrodensis* is viewed as having great promise for further expansion of the mushroom industry. Currently, *P. nebrodensis* is receiving increasing attention in East Asia and other countries.

The successful cultivation of *P. nebrodensis* on a substrate of spruce wood chips, cottonseed hulls and brans, achieved in China in 1983 (Le *et al*., 2007), created a new opportunity for the mushroom industry. Several studies have demonstrated that long cropping cycles and strict environmental conditions potentially required for the fruiting of *P. nebrodensis*, makes its industrial cultivation difficult and its production expensive (Chang and Miles, 1988; Tan *et al*., 2005; Shen *et al*., 2005). Another disadvantage is the unstable fruiting observed in commercial cultivation, with no identifiable cause (Chen and Liu, 2007; Wang *et al*., 2011), resulting in a high economic risk for growers. Hence, commercial cultivation of *P. nebrodensis* is not attractive to most growers, although it has a relatively

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high market price. Therefore, novel strains with desirable cultivation characteristics such as stable fruiting, high yield and ease of cultivation should be developed.

Recently, intraspecific hybridization by hyphal fusion of monokaryotic mycelia, derived from basidiospores of *P. nebrodensis* was carried out (Zhang *et al*., 2010). However, significant improvements in the cropping cycle and biological efficiency were not observed in the hybrid. This may be mainly due to the limited genetic diversity of *P. nebrodensis*, since this fungus is distributed only in very few areas of the world, resulting in a lack of the synergistic effect of hybridization. To overcome this limitation, introducing genetic material responsible for high yield, fast growth and stable fruiting from other mushroom species into *P. nebrodensis* is becoming essential. To achieve this, a variety of approaches like transgenic breeding, protoplast fusion and interspecific hybridization could be explored. Of these, interspecific hybridization has long been used and still serves as an efficient tool for the breeding of edible mushrooms; considerable success has been achieved in *Pleurotus* breeding (Chakraborty and Sikdar, 2008; Gupta *et al*., 2011).

Pleurotus has a biofactor tetrapolar mating system. This kind of mating is regulated by two mating-type loci, namely *A* and *B*. Both loci A and B are multi-allelic. The present study focuses on the mating compatibility of *P. nebrodensis* with different *Pleurotus* species to provide a theoretical base for reasonable and objective selection of parental strains in the breeding of *P. nebrodensis*. In addition, a phylogenetic tree was constructed using software Mega 5.05 with the internal transcribed spacer (ITS) sequences to gain insights into the correlation of mating compatibility with the phylogenetic relationship between *P. nebrodensis* and different *Pleurotus* species. Undoubtedly, the present study will be beneficial for the field of *P. nebrodensis* breeding because it provides a strain spectrum for selection of parental strain.

Materials and Methods

Mushroom Strains

The eight *Pleurotus* species used in this study are listed in Table 1. These strains were maintained on potato dextrose agar (PDA) medium at the Mycological Research Centre, Fujian Agriculture and Forestry University.

Medium

PDA medium consists of potato extract (200 g boiled in 500 mL distilled water), 20 g glucose and 18 g agar per liter of distilled water. For liquid cultures, agar was not added.

Isolation of Monokaryotic Strains

In a financial program for establishing the germplasm bank of edible mushrooms, a large number of strains comprising different species were collected from most parts in China. To validate whether these strains were possibly misidentified, fruiting and molecular characterizations of the collected strains were carried out. During this process, spore prints of a variety of strains were obtained by placing sulfate papers under mature basidiocarps for 24 h in a sterilized Petri dish that was completely sealed. The spore prints thus obtained were stored and served as a reference for strain identification and as genetic material for further investigation on genetics and breeding.

Single spore isolation was performed as follows: spores on the sulfate papers were scraped into sterile distilled water with an inoculating loop, yielding a final concentration of approximately 1×10^5 spores per mL by appropriate dilution. Aliquots (0.2 mL) of the resulting suspensions were spread on the surface of PDA medium in 9-cm Petri dishes. After incubation at 28°C for 2–5 days, depending on the strains used, the spores germinated and were microscopically examined. The germinated spores were picked up with an inoculating needle while viewing them under a microscope (40× magnification), transferred into PDA slants and incubated at 25°C in the dark.

On complete growth of the isolates, an agar plug cut from the actively growing margin of colony was placed on glass slides, covered with coverslips and carefully pressed to a thin layer. Mycelia growing on agar were examined for the absence of clamp connections under a microscope at 40× magnification. Mycelia of isolates showing absence of clamp connections were considered as monokaryotic strains. In this study, 20 or more monokaryons were isolated for each *Pleurotus* species for use in subsequent experiments.

Mating Type Determination

Mating types were determined by randomly selecting 12 of the original monokaryotic isolates derived from the same strain. These single spore cultures were paired in every possible combination (66 combinations), avoiding reciprocal crosses. Briefly, a single agar plug containing monokaryotic mycelia was placed together with another monokaryon (1 cm apart) on PDA in tubes and incubated at 28°C. After 7 days of growth, a small area of agar at the junction of the two cultures was then examined for the presence of clamp connections on mycelia, using a microscope at 40× magnification. Each cross was considered for mating compatibility (clamp formation) or incompatibility (absence of clamp formation), and mating types were then assigned arbitrarily to each monokaryon.

Mating Compatibility Test

Following the assignment of mating types to all monokaryons isolated from the strains tested, four monokaryotic isolates representing mating types A_1B_1, A_1B_2, A_2B_1, and A_2B_2 from each *Pleurotus* species were

randomly selected. A total of 32 single spore cultures were selected from the eight strains. The mating compatibility was determined by paring the four single spore cultures of *P. nebrodensis* with those of the remaining *Pleurotus* strains in every possible combination (16 parings between *P. nebrodensis* with each one of the remaining strains). In total, there were 112 parings (7 ×16). In 16 combinations between the two strains, if one or more pairings were found to be compatible, the two species were considered as having mating compatibility.

DNA Isolation, ITS Amplification and Sequencing

All tested strains were inoculated into 50 mL PDA liquid medium in a 250-mL shake flask and cultured at 28°C for 10 days at 150 rpm. About 1 g mycelia (wet weight) was collected and ground in liquid nitrogen by using a mortar and pestle, following which DNA was extracted using the CTAB method (Noël and Labarére, 1987).

The ITS region of the rDNA sequence of tested strains was amplified by a polymerase chain reaction (PCR) using universal ITS primers ITS1-5′ TCC GTA GGT GAA CCT GCG G-3′ (forward) and ITS4-5′ -TCC TCC GCT TAT TGA TAT GC-3′) (reverse) (Avin *et al*., 2012). PCR reaction was performed using a thermal cycler (Veriti thermal cycler, Applied Biosystems, USA) with the following cycling conditions: an initial denaturation of 5 min at 95°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, extension for 1 min at 72°C and a final extension for 10 min at 72°C. Amplification products were electrophoresed on a 1.5% agarose gel with DNA Marker DL 2000 (TaKaRa, Japan). The amplified ITS DNA segments in a range of 500–750 bp inferred from the marker bands were purified using the SanPrep Column DNA Gel Extraction Kit (Sangon, China), cloned into pMD19 T-vector (TaKaRa, Japan), and sequenced in both directions (Sangon, China).

Sequence Analysis and Phylogeny

Sequences determined in this study were deposited in the GenBank database. For establishing a reference, the following strains and accession numbers of ITS sequences from GenBank were used for the construction of a neighbor-joining phylogenetic tree: *P. nebrodensis* (GQ456057), *P. eryngii* (FJ904756), *P. ferulae* (DQ077888), *P. cornucopiae* (KF932721), *P. citrinopileatus* (JN234853), *P. pulmonarius* (KF932728), *P. djamor* (KC414259), and *P. abalonus* (AF315810).

Multiple alignment was performed with Clustal X (Version 1.83), and sequences were edited manually in this software to exclude ambiguous alignments. The phylogenetic tree was constructed with the neighbor-joining method with the Kimura 2-parameter model, using the Mega software package (Version 5.05), with *Lentinula edodes* as an outgroup. The reliability of the different phylogenetic groupings was evaluated by a bootstrap test (1000 replications).

Results

Mating Compatibility between *P. nebrodensis* and Different *Pleurotus* Species

The mating results of *P. nebrodensis* with the remaining tested strains representing different *Pleurotus* species are presented in Table 2. Nine crosses of 16 produced clamp connections between *P. nebrodensis* and *P. eryngii*, with a mating frequency of 56.25%, which is unusually high for tetrapolar mushrooms, as the theoretical mating frequency is 25% among monospore cultures derived from a tetrapolar mushroom. However, with *P. ferulae*, a species that is closely related to *P. nebrodensis*, out of 16 combinations, only one cross produced clamp connections. The success rate was at a relative value of 6.25%; nevertheless, this compatibility still offers a chance for successful mating of *P. ferulae* with *P. nebrodensis*.

As for distant species of *P. nebrodensis*, *P. cornucopiae* produced seven positive matings with *P. nebrodensis*. The compatibility rate reached as high as 43.75%, suggesting that high compatibility exists between the two strains despite the fact that they were not closely related. The reason for this high compatibility needs further investigation, as an elucidation of this phenomenon is significant for breeding studies. In contrast to the above-mentioned results, *P. nebrodensis* was found to be completely incompatible with *P. pulmonarius*, *P. abalonus*, *P. djamor* and *P. citrinopileatus*, possibly due to the higher genetic differences inhibiting the hyphal fusion of *P. nebrodensis* with these strains. Hence, it can be inferred that. *P. eryngii*, *P. ferulae* and *P. cornucopiae* can be used as parental strains for hybridization breeding of *P. nebrodensis*.

Difference in the Length of ITS Sequences among Tested Mushroom Strains

The electrophoretic profiles of the ITS products of eight tested species is shown in Fig. 1. Obviously, there was no visual difference in the length of ITS in the gel. Sequencing revealed that the lengths of the PCR products of the ITS region in eight mushroom strains representing different *Pleurotus* species were in the range of 571 to 667 bp (Table 3). This value was similar to that of most of the *Pleurotus* species. The maximum length was recorded in *P. djamor* as 667 bp, whereas the minimum length was recorded in *P. ferulae* as 571 bp. *P. nebrodensis* was identical to *P. eryngii* and *P. cornucopiae* in terms of ITS size (639 bp); however, the nucleotide sequences were different. The length of ITS of *P. nebrodensis* (639 bp) considerably differed from that of *P. ferulae* (571 bp), although the two species were compatible in terms of mating. Hence, it was concluded that a significant difference in ITS length among the different edible mushrooms did not necessarily lead to incompatibility between them.

Table 1: *Pleurotus* species used in this study

Strains	Species	Sources
Pl. e0041	*P. eryngii*	Shouguang Edible Mushroom Institute
Pl. d0002	*P. djamor*	Sanming Mycological Institute
Pl. a0014	*P. abalonus*	Longhai Jiuhu Edible Fungus Research Institute
Pl. g032	*P. pulmonarius*	
Pl. c0003	*P. citrinopileatus*	Northeastern Institute of Edible and Medicinal Fungi
Pl. co0031	*P. cornucopiae*	Fujian Agriculture and Forestry University
Pl. n0005	*P. nebrodensis*	
Pl. f0003	*P. ferulae*	Qingyuan country, Xinjiang Province (Isolated by Nianlai Huang)

Table 2: Results of mating tests between *P. nebrodensis* with different *Pleurotus* species *via* mating among monokaryotic mycelia

Strains		*P. nebrodensis* Pl. n0005				Positive Mating
		A_1B_1	A_1B_2	A_2B_1	A_2B_2	
P. eryngii Pl. e0041	A_1B_1	–	+	+	+	3
	A_1B_2	–	–	–	+	1
	A_2B_1	–	+	+	+	3
	A_2B_2	–	–	+	+	2
Total						*9*
P. ferulae Pl. f0003	A_1B_1	+	–	–	–	1
	A_1B_2	–	–	–	–	0
	A_2B_1	–	–	–	–	0
	A_2B_2	–	–	–	–	0
Total						*1*
P. cornucopiae Pl. co0031	A_1B_1	+	+	–	+	3
	A_1B_2	–	+	–	–	1
	A_2B_1	–	–	–	–	0
	A_2B_2	+	–	+	+	3
Total						*7*

A_1B_1, A_1B_2, A_2B_1, and A_2B_2 represent the four different mating types of monokaryons. All matings were incompatible between *P. nebrodensis* and *P. pulmonarius*, *P. abalonus*, *P. djamor*, and *P. citrinopileatus*

+, clamp formation (compatibility); –, absence of clamp formation (incompatibility)

Table 3: Lengths of ITS sequences of tested species and their corresponding GenBank accession numbers

Strains	Species	ITS size (bp)	GenBank accession no.
Pl. e0041	*P. eryngii*	639	KJ561114
Pl. d0002	*P. djamor*	667	KJ561115
Pl. a0014	*P. abalonus*	650	KJ561116
Pl. c0003	*P. citrinopileatus*	656	KJ561117
Pl. g032	*P. pulmonarius*	631	KJ561118
Pl. co0031	*P. cornucopiae*	639	KJ561119
Pl. f0003	*P. ferulae*	571	KJ561120
Pl. n0005	*P. nebrodensis*	639	KJ561121

Phylogenetic Analysis

Fig. 2 shows the topography of the phylogenetic tree built with the ITS region sequences (their GenBank accession numbers are shown in Table 3) of the eight tested *Pleurotus* species and their corresponding species from the NCBI GenBank database. The constructed tree consisted of two clades, I and Π, which were further divided into I A, I B, and Π A, Π B. As a variant of *P. eryngii*, *P. ferulae* Pl. f0003 initially clustered with *P. eryngii* Pl. e0041, further clustered with *P. eryngii* 33SC (FJ904756) and *P. nebrodensis* Pl. n0005 and subsequently clustered with *P. nebrodensis* CCMSSC1582 (GQ456057), *P. ferulae* (DQ07788), *P. cornucopiae* Pl. co0031 and *P. pulmonarius* Pl. g0032. These strains formed a tight clade (Clade I A). This clade further clustered with *P. abalonus* in Clade I B. In Clade Π, strains *P. djamor* Pl. d0002 and its corresponding species in the database formed a branch, Clade Π A, whereas *P. citrinopileatus* Pl. c0003 was clustered with the same

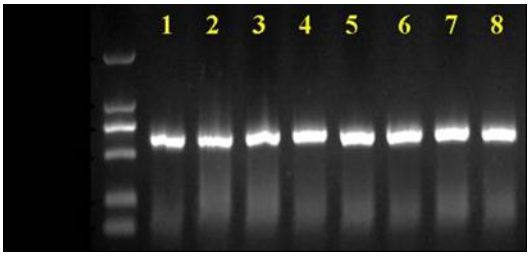

Fig. 1: ITS-PCR electrophoretic profiles of the eight different *Pleurotus* species tested (lines 1, 2, 3, 4, 5, 6, 7, and 8 for strains Pl. e0041, Pl. n0005, Pl. f0003, Pl. d0002, Pl. g0032, Pl. co0031, Pl. c0003, and Pl. a0002, respectively)

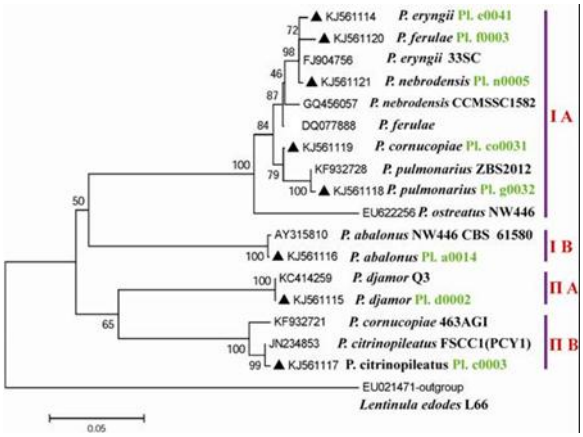

Fig. 2: Phylogenetic tree constructed using the neighbor-joining method, with 1000 bootstrap replicates, based on ITS region sequences of the eight tested *Pleurotus* species and their corresponding species from the NCBI GenBank database

Fig. 3: Mature fruiting bodies of the eight tested *Pleurotus* species showing different shape and color

species in the database and then clustered with *P. cornucopiae* (KF932721), forming another branch, Clade Π B. Based on this phylogenetic tree, it was concluded that *P. nebrodensis* had closer genetic relationships with *P. eryngii*, *P. ferulae* and *P. cornucopiae* than with the other tested *Pleurotus* species.

P. nebrodensis, *P. eryngii* and *P. ferulae* were generally clustered with their corresponding species in the NCBI GenBank database. The distant species of *P. nebrodensis*, which includes *P. pulmonarius*, *P. abalonus*, *P. djamor* and *P. citrinopileatus*, were separate from each other and clustered with their corresponding species in the database. Contrary to this, *P. cornucopiae* Pl. co0031 was distinctly separated from *P. cornucopiae* 463 AGI (KF932721) which was obtained from the database. This may be possibly due to the misidentification of this strain, since this species is often confused with other *Pleurotus* species in taxonomic studies (Gonzalez and Labarère 2000).

Discussion

Both *P. ferulae* and *P. nebrodensis* are often considered as variants of *P. eryngii* and have been demonstrated as being closely related based on ITS sequence alignment, mating tests and molecular analysis (Zervakis and Balis, 1996; Bao *et al*., 2004; Zhang *et al*. 2006; Kawai *et al*., 2008). In the present study, the phylogenetic tree also showed that these three strains were genetically closely related. In view of the mating compatibility (Zervakis and Balis, 1996; Kawai *et al*., 2008), *P. nebrodensis* mated with both *P. eryngii* and *P. ferulae*. Present results are found similar compatibility. Conversely, according to Bao *et al*. (2004) and Zhang *et al*. (2006), *P. nebrodensis* was incompatible with both *P. eryngii* and *P. ferulae*. These conflicting results might be due to the fact that a very limited number of crosses had been performed in these studies, which would result in an untrustworthy conclusion.

One of the valuable findings of the present study is that high compatibility was found between *P. nebrodensis* and *P. cornucopiae*. To confirm these results further, additional *P. cornucopiae* strains were examined for compatibility with *P. nebrodensis* and similar results were obtained (data not shown). In contrast, a low mating frequency of 6.25% was observed between *P. nebrodensis* and its closely related species *P. ferulae*. This value was significantly lower than that of the mating frequency between *P. nebrodensis* and *P. cornucopiae* (43.75%) (Table 2). Therefore, it was concluded that the mating compatibility among edible mushrooms is not directly related to genetic relationships. For a better understanding of this phenomenon, an in-depth investigation into the mating mechanisms is warranted. Following this cue, for the creation of successful novel strains in breeding practice, extensive intra- and/or interspecies hybridization appears to be essential. Indeed, the findings on mating compatibility between *P. nebrodensis* and *P. cornucopiae* are particularly valuable for the breeding of *P. nebrodensis* because this mushroom can offset the major shortcomings of *P. nebrodensis*.

The characteristics of mushroom fruiting bodies have been traditionally used for taxonomic study, although these are significantly affected by cultivation substrate, environmental conditions and genotypes. Fig. 3 shows mature fruiting bodies of all tested strains. Significant morphological differences were observed in shape, size and color among these strains. The differences in the morphology of fruiting bodies of *P. cornucopiae* were more pronounced than that of fruiting bodies of *P. ferulae* in relation to *P. nebrodensis*; however, a significantly higher mating frequency was observed between *P. nebrodensis* and *P. cornucopiae*. It is believed that the similarity in the morphology was not directly linked with the mating compatibility frequency among edible mushrooms.

Given that the pairing of monokaryons of *P. nebrodensis* with those of *P. eryngii*, *P. ferulae* and *P. cornucopiae* could produce dikaryons, which usually have the ability to form fruit bodies, the three species can be employed as parental strains for the breeding of *P. nebrodensis* by using the duel culture hybridization technique *via* hyphal fusion. However, as the biological efficiency and cropping cycle of *P. cornucopiae* were excellent in comparison to those of *P. eryngii* and *P. ferulae* (Royse, 2004) and because this strain was easier to cultivate, the use of *P. cornucopiae* as a parental strain may be a desirable choice for *P. nebrodensis* breeding. Further hybridization studies, with a special focus on using *P. cornucopiae* as a parental strain, are in progress, with the major objectives of improved yield, shorter cultivation period and generally maintained *P. nebrodensis* fruit body characteristics.

In conclusion, the mating compatibility of *P. nebrodensis* with different *Pleurotus* species was correlated with the phylogenetic relationships among them. Strains *P. eryngii*, *P. ferulae* and *P. cornucopiae* could be used as parental strains for hybridization breeding of *P. nebrodensis* owing to the mating compatibility.

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