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Genetic polymorphism of ferula mushroom growing on *Ferula sinkiangensis*

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Abstract Mating tests, internal transcribed spacer (ITS) sequence analysis, intergenic spacer 1–restriction fragment length polymorphism (IGS1-RFLP), IGS1 sequence analysis, and IGS2-RFLP analysis were carried out on isolates of 17 morphologically different *Pleurotus* mushrooms collected on *Ferula sinkiangensis*. The isolates were divided, based on mating tests and ITS sequence analysis, into two groups identical to *P. eryngii* var. *ferulae* and *P. nebrodensis*, respectively. Single spores from these two groups were incompatible, but those from *P. eryngii* var. *ferulae* and *P. eryngii* were compatible and combined to produce 56.25% dikaryon mycelia with clamp connections. The ITS of *P. eryngii* var. *ferulae* and *P. nebrodensis* (GenBank accession no. AY311408) were both 638 bp in size but differed by 3% in sequence. *P. eryngii* var. *ferulae* and *P. eryngii* (GenBank accession no. AY368658) were identical in ITS size and sequence. *P. nebrodensis* was the dominant population of *Pleurotus* mushroom growing on *F. sinkiangensis*. It exhibited genetic diversity. The two species could also be distinguished by IGSI-RFLP, similar to identification by mating tests and ITS sequence analysis. Difference in IGS1-RFLP existed between *P. eryngii* var. *ferulae* and *P. nebrodensis*. The sequence difference reached 2.28%. Both IGS1 size and IGS1-RFLP were similar among the different samples of *P. nebrodensis*. The 17 isolates were separated into five types based on IGS2 size and IGS2-RFLP, with both interspecies and extraspecies differences. *P. nebrodensis* exhibited polymorphism and was divided into four types. These results agreed with macroscopic differences. IGS2 might be the effective domain of genetically polymorphic ribosomal DNA in *P. nebrodensis* mushrooms found in Xinjiang, China.

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Introduction

The various *Pleurotus* species are tetrapolar heterothallic, nonparasitic ligninolytic fungi. Only *P. eryngii* complex and *P. nebrodensis* are weakly parasitic and can live on the root or base stem of living plants in the Umbelliferae family. *P. eryngii* complex is widespread in the Mediterranean, Central Asia, central Europe, and North Africa (Lewinsohn et al. 2002). However, distribution of *P. nebrodensis* is restricted to Xinjiang in China (Mao 2001) on *Cachrys ferulacea* and Sicily in Italy (Venturella et al. 2000) on *Ferula sinkiangensis*. In Xinjiang, *Pleurotus* mushrooms growing on *F. sinkiangensis* are all called ferula mushrooms. There is considerable diversity in the fruiting body morphology of wild ferula mushroom and ferula mushroom cultured from isolates.

Research has indicated abundant genetic diversity in *P. eryngii* complex (Venturella 2000). Various genera belonging to family Umbelliferae are hosts of *P. eryngii* complex. They are named, respectively, as various species or variants according to their host. *Pleurotus* mushroom growing on *Cachrys* was named as *P. nebrodensis* by Venturella (2000) and as *P. eryngii* var. *nebrodensis* by Bresinsky et al. (1987). *Pleurotus* mushroom occurring on *F. sinkiangensis* was first reported as *P. ferula* and later described as *P. eryngii* var. *tuoliensis* (Mu et al. 1987), *P. eryngii* var. *ferulae*, *P. eryngii* var. *nebrodensis* (Huang 1996), and *P. nebrodensis* (Mao 2001), respectively. The classification has been equivocal due to the parallel existence of morphological similarities and differences in culture characteristics. The fruiting body morphology is susceptible to environmental influences. Thus, problems frequently arise if the classification is based entirely on morphological characteristics. Molecular biology tech-

niques provide a useful theoretical basis and methodology for systematic classification and analysis of genetic polymorphism in mushrooms. These techniques have enabled the disclosure of the structure and function of ribosomal DNA. The internal transcribed spacer (ITS) and intergenic spacer (IGS) domains have become important DNA fragments for the analysis of biodiversity. ITS and IGS are rDNA domains that evolve at a faster rate. The ITS domain is a molecular marker for comparing different species of the same genus of fungi (Mitchell and Bresinsky 1999). IGS domain is a highly variable domain (Paule and Lofquist 1996), usually containing the effective variable sequence for determining interspecies or individual genetic relationship in fungi (Guidot et al. 1999). ITS and IGS are important molecular markers for extraspecies comparison in the same fungal genus and for analysis of interspecies mutation and genetic polymorphism (Bunyard et al. 1996).

In the present investigation, 17 isolates were prepared from 17 samples collected from *F. sinkiangensis* and reconfirmed by cultivation. They were divided into five groups according to morphological features and then subjected to antagonism test and rapid analysis of polymorphic DNA (RAPD). Isolates with no antagonism response, but with similar morphological characteristics (Huang 1996) and RAPD profiles (spectra), were eliminated. Those with antagonism response and dissimilar morphological characteristics and RAPD profiles were selected and used for a mating test. Intergenic spacer 1–restriction fragment length polymorphism (IGS1-RFLP), IGS2-RFLP, and sequence analysis of ITS and IGSI domains were carried out with *P. eryngii* (ACCC50894) with gray pileus produced in Italy as reference. The aim was to investigate its genetic diversity and, at the same time, provide a molecular biology basis for the classification of *Pleurotus* mushroom on *F. sinkiangensis*. The reasons for using *P. eryngii* (ACCC50894) are as follows: (1) it is far from Xinjiang geographically; (2) *P. eryngii* is a complex close to *P. nebrodensis* and *P. eryngii* var. *ferulae*; (3) to confirm the taxonomic position of *P. eryngii* var. *ferulae* that it belongs to *Pleurotus*; and (4) to confirm the taxonomic position of *P. nebrodensis* that it does not belong to *P. eryngii* complex.

Ferula mushroom has economic and also medicinal value. It has been successfully cultivated in China, France, and India. Correct species identification and protection of the rights of breeders of commercial strains are of paramount importance. The intent of the present study is to demonstrate that IGS analysis provides a convenient and quick technology for identification of strains, and that it has promising prospects for application.

Materials and methods

All of the cultures (see Table 1) are kept in the Agricultural Culture Collection of China (ACCC). The specimens of the fruiting bodies are stored in the Microbiology Research Institute of Academia Sinica and in Sanming Mycological Institute, Fujian Province, China.

Mating tests

The basidiospores were collected at 20°C from fruiting bodies and cultivated in separate houses to prevent contamination. A spore suspension was used to obtain a monokaryon for the mating tests. The mycelium was incubated

Table 1 Isolates tested

Group	Isolates	Scientific name	Place of collection	Macro characteristics
1	50656	*P. eryngii* var. *ferulae*	Tuoli, Xinjiang, China	Colony restricted, fruiting after 35-day running, texture compact, pileus milky white/near white with scattered light-yellow stripes
2	50869, 50914, 50929, 51005, 51067, 51032, 51342, 51343, 51454, 51465, 51466, 51477, 51485	*P. nebrodensis*	Mulei or Tianshan, Xinjiang, China	Colony flat, fruiting after 90-day running or longer, texture compact, pileus white, stipe short, fruiting body shaped like the palm of a hand
3	51004, 51060, 51341, 51963	*P. eryngii* var. *nebrodensis*	A ertai, Xinjiang, China	Colony flat, fruiting after 70-day running, texture soft, pileus white, stipe long, fruiting body shaped like a horse's hoof
4	51326, 1452	*P. nebrodensis*	Tuoli, Xinjiang, China	Pileus white with radiating light-yellow stripes; other characteristics similar to group 2
5	51453	*P. nebrodensis*	Tulin, Beijing Jinxin Mushroom Co. Ltd.	Flat colony, fruiting after 90-day or longer, baby fruiting body light gray, pileus with tiny inconspicuous pustules, texture average, stipe average in length, mature fruiting bodies white, shaped like the palm of a hand
6	50894	*P. eryngii*	Italy	Gray pileus, host is *Eryngii*

The isolated strains were divided into five groups based on macro characteristics, colony characters, fruiting body morphology, and running period before fruiting. The scientific names are based on fruiting body morphology (Huang 1996; Mao 2001)

on potato dextrose agar medium in a Petri dish at 25°C. When small colonies were visible, a monokaryon with nonclamp connection was selected for microscopic examination. Four mating types were determined for various isolates (*P. eryngii* and *P. eryngii* var. *ferulae* have four mating types of basidiospores because *Pleurotus* is heterothallic with bifactorial control, i.e., exhibits tetrapolarity). The mating tests were then carried out. The existence of clamp connection was used as evidence for compatibility. Compatibility was indicated as "+" and incompatibility was marked as "–." In general, compatible organisms belong to the same species, whereas incompatible organisms do not.

Extraction of DNA

The mycelium grew on PDA Petri dish with celluloid film at 25°C for 8 days. Then, it was collected, and DNA was extracted as described by Lee and Taylor (1990). The restriction endonucleases used were *Bsu*RI, *Hin*6I, *Hpa*II, *Rsa*I, and *Bsh*1236I (Sangon Co., Shanghai, China). IGS1-PCR reaction conditions were 94°C for 1 min (denaturation), 54°C for 1 min (renaturation), and 72°C for 30 min (extension), for a total of 35 cycles, followed by 72°C for 7 min (extension). ITS-PCR and IGS2-PCR reaction conditions were 60°C (renaturation) and 3 min (extension), with other conditions being identical to those for IGS1-PCR. The digestion system comprised 1-μl restriction endonuclease (10 U/μl,), 6 μl PCR product, and 1 μl 10× buffer, and the volume was made up to 10 μl with water. The reaction was carried out at 37°C for 4 h. Agarose gel (2%) was used for electrophoresis. Results of the enzyme digestion were observed under an ultraviolet gel imaging system.

ITS, IGS1-RFLP, and IGS2-RFLP

The amplification system of ITS-, IGS1-, and IGS2-PCR consisted of 4 μl dNTP (2.5 mM each), primers 1 and 2 (2.5 μl each), *Ex Taq* DNA polymerase (1.25 U), PCR buffer, template (25 ng), and double-distilled water to make up the volume to 50 μl. The sequences of the PCR primers are shown in Table 2.

Table 2 ITS, IGS1, and IGS2 primers

	Sequence
ITS primers	
ITS1	(5′-TCC GTA GGT GAA CCI GCG G-3′)
ITS4	(5′-TCC TCC GCT TAT TGA TAT GC-3′)
IGS1 primers	
LR12R	(5′-GAA CGC CTC TAA GTC AGA ATC C-3′)
5SRNA	(5′-ATC AGA CGG GAT GCG GT-3′)
IGS2 primers	
Inv SRIR	(5′-ACT GGC AGA ATC AAC CAG GTA-2′)
5SRNAR	(5′-ACC GCA TCC CGT CTG AT-3′)

Table 3 Results of mating tests using different strains

		P. eryngii var. *ferulae* (50656)			
		A_xB_x	A_xB_y	A_yB_x	A_yB_y
P. eryngii (50894)	A_xB_x	−	+	−	+
	A_xB_y	+	+	+	−
	A_yB_x	+	+	+	−
	A_yB_y	+	−	−	−

A_xB_x, A_xB_y, etc. represent the four mating types. Incompatibility was observed between *P. nebrodensis* (50869) and *P. eryngii* (50894) and between 50869 and *P. eryngii* var. ferulae (50656)
+ Compatible, − incompatible

ITS and IGSI sequence determination

Both ITS and IGSI were amplified as mentioned above. The amplification products were subjected to direct sequencing (Dunham et al. 2003).

Results

Mating test

It was revealed by the mating tests that compatibility existed between *P. eryngii* var. *nebrodensis* and all of the isolates designated *P. nebrodensis*. The isolates of the latter were also compatible. This suggests that all of them belong to the same species, and the differences are just at the strain level, despite the fact that morphological differences in fruiting bodies between the former and the latter were greater than those among all of the latter designated *P. nebrodensis*. The mating tests showed that *P. nebrodensis* (ACCC50869) was incompatible with both *P. eryngii* var. *ferulae* (ACCC50656) and *P. eryngii* (ACCC50894). However, *P. eryngii* var. *ferulae* (ACCC50656) was compatible with the reference strain *P. eryngii* (ACCC50894), with which 56.25% dikaryon (9 dikaryons in 16 pairings) was formed (Table 3).

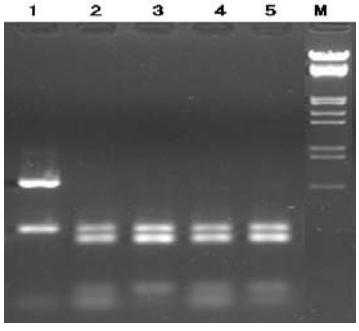

Fig. 1 IGS1-RFLP electrophoretic profiles. Results of *Hpa*II cleavage of *P. nebrodensis*. *1* Isolate number 1, ACCC50656; *2* isolate number 2, ACCC50869; *3* isolate number 3, ACCC51060; *4* isolate number 4, ACCC51452; *5* isolate number 5, ACCC51453. *M* molecular marker: (λDNA/*Hin*dIII and *Eco*RI). Numbers *1–5* and *M* also apply to Figs. 2 and 3

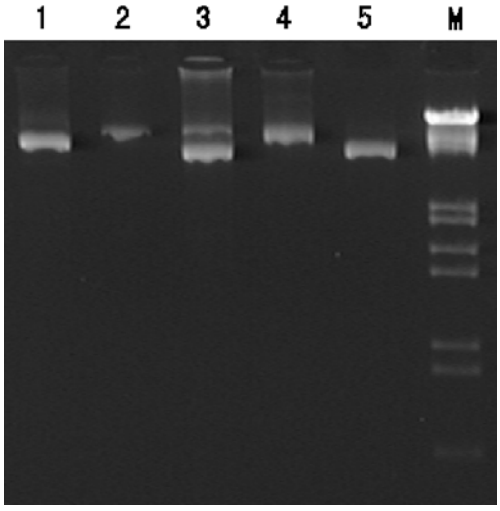

Fig. 2 IGS2 amplification results

ITS

The results revealed that the size of the ITS of all isolates was identical, being 638 bp, but the nucleotide sequences were different. This difference existed only between incompatible isolates, i.e., only between *P. eryngii* var. *ferulae* and *P. nebrodensis*, and there were no differences in ITS sequence among the compatible isolates of *P. nebrodensis*. The isolate named *P. eryngii* var. *ferulae* was identical to *P. eryngii* in ITS sequence (GenBank accession no. AY368658). The others were identical to *P. nebrodensis* in ITS sequence (GenBank accession no. AY311408). The results indicated that the difference of the ITS domain lies mainly in changes in nucleotide sequence and not in variations in length of the region. The ITS sequences showed differences in 19 nucleotides, i.e., a 3% difference between two species.

IGSI-RFLP

All of the tested isolates did not differ in IGS1 size, being about 920 bp and existing in multiple copies. When *Bsu*RI, *Hin*6I, *Hpa*II, *Rsa*I, and *Bsh*1236I were used as endonucleases, only the IGS1-RFLP after *Hpa*II digestion indicated a difference between *P. eryngii* var. *ferulae* and others (Fig. 1). The IGS1-RFLP profile was identified to reference *P. eryngii* (data not shown). The IGS1-RFLP profiles of all isolates generated by the other four endonucleases were identical, indicating IGS1 difference only in extraspecies in the present study. It is suggested that no mutations of nucleotide sequence have arisen in various interspecies populations of *P. nebrodensis*. Sequence analysis disclosed that the estimate of 920 bp for IGS1 length is accurate. *P. eryngii* var. *ferulae* had a sequence in IGS1 distinct from *P. nebrodensis*, and its sequence has been registered in GenBank (accession no. AY463033). The isolates of *P. nebrodensis* and *P. eryngii* var. *nebrodensis* exhibited the same sequence in IGS1. The sequence, as represented by ACCC50869, has also been registered in GenBank (accession no. AY463034). There was a difference in 21 nucleotides (i.e., 2.28% difference) between *P. eryngii* var. *ferulae* and *P. nebrodensis*.

IGS2-PCR and IGS2-RFLP

Intergenic spacer 2 amplification results indicated that the IGS2 domain was longer and variable in length. The length of IGS2 of different isolates manifested polymorphism (Fig. 2). *P. eryngii* var. *nebrodensis* generated two bands, 7.8 and 3.5 kb, respectively, and the others gave rise to only one band but of different sizes, which was 3.5 kb for isolate ACCC51453, 7.8 kb for isolates ACCC50869 and ACCC51452 (*P. nebrodensis*), and 4.4 kb for ACCC50656 (*P. eryngii* var. *ferulae*), respectively.

With the exception of *Hpa*II, the 17 isolates were divided into five groups based on IGS2-RFLP profiles by digestion of the four endonucleases *Bsu*RI, *Hin*6I, *Rsa*I, and *Bsh*1236I. They acted on different restriction sites in the domain, and this resulted in a polymorphic IGS2-RFLP profile after electrophoresis (Fig. 3, Table 4). This indicates that IGS2 represents the domain with the most abundant polymorphism in ribosomal DNA for ferula mushroom population that belongs to two species in Xinjiang, China.

Discussion

Based on the mating test results obtained in the present study and the Linnaeus concept of species separation, there

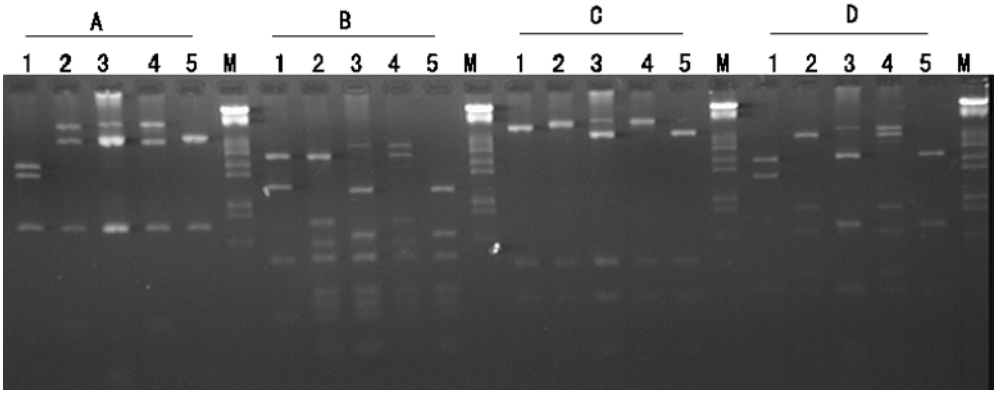

Fig. 3 IGS2-RFLP electrophoretic profiles. **a***Bsh*1236I; **b***Bsu*RI, **c***Hin*6I, **d***Rsa*I

Table 4 Genetic polymorphism of IGS1-RFLP and IGS2-RFLP for ferula mushroom

	Band (bp)	50656	50869	51060	51452	51453
IGS1-RFLP *Hpa*	580	+	−	−	−	−
	340	+	+	+	+	+
	300	−	+	+	+	+
	140	−	+	+	+	+
IGS2-RFLP	3,391	−	+	+	+	−
*Bsh*12461	2,207	−	+	+	+	+
	1,580	+	−	−	−	−
	1,380	+	−	−	−	−
	733	+	+	+	+	+
IGS2-RFLP *Bsu*RI	2,203	−	−	+	+	−
	1,986	+	+	−	+	−
	941	+	−	+	−	+
	730	−	+	−	+	−
	663	−	−	+	−	+
	607	−	+	−	+	−
	504	+	+	+	+	+
	336	−	+	+	+	+
	286	−	+	+	+	+
	248	+	+	+	+	+
	165	+	+	+	+	+
IGS2-RFLP *Hin*6I	2,815	−	+	+	+	−
	2,614	+	−	−	−	−
	2,304	−	−	+	−	+
	460	+	+	+	+	+
	322	+	+	+	+	+
IGS2-RFLP *Rsa*I	2,571	−	−	+	+	−
	2,314	−	+	−	+	−
	1,670	+	−	+	−	+
	1,207	+	−	−	−	−
	818	−	+	−	+	
	688	−	−	+	−	+
	630	−	+	−	+	−
	403	−	+	−	+	−
	347	+	+	+	+	+

Sequences have been registered with GenBank under accession nos. AY368658, AY311408, AY463033, and AY463034
50656 P. eryngii var. ferulae, group 1; *50869 P. nebrodensis*, group 2; *51060 P. eryngii* var. *nebrodensis*, group 3; *51452 P. nebrodensis*, group 4; *51455 P. nebrodensis*, group 5; + present, − absent

should be two *Pleurotus* species growing on *F. sinkiangensis*, taking into consideration ecological and morphological classification (Venturella 2000; Venturella et al. 2000; Mao 2001), namely, *P. eryngii* var. *ferulae* and *P. nebrodensis*. The difference between them represents extraspecies or species level and not variety level. The sample shaped like a goof (ACCC51060) is the same species as that one shaped like a palm. Both of them should belong to *P. nebrodensis* and not to the *P. eryngii* complex due to their incompatibility with *P. eryngii* and *P. eryngii* var. *ferulae*. *P. eryngii* var. *ferulae* growing on *F. sinkiangensis* should be included in the *P. eryngii* complex due to their compatibility with each other.

Results of ITS sequence analysis concur with the mating test results. ITS sequences are identical between compatible strains. ITS sequence difference is as high as 3% between incompatible strains, much higher than the previously reported interspecies differences of less than 1% (Sugita et al. 1999). Isolates with different ITS sequences are incompatible with each other. Closely related species growing on the same host *F. sinkiangensis* can be distinguished using one or both of the mating tests and ITS sequence analysis.

The IGSI domains of all strains were relatively conserved, but the IGS2 showed abundant polymorphism. Although all of the *P. nebrodensis* strains were identical in ITS and IGS1 sequences, there were obvious differences in IGS2 that exhibit size or RFLP profile. IGS2 is a useful domain for investigating interspecies polymorphism in the population of *P. nebrodensis*. IGS2-RFLP could be used as a DNA molecular marker for identification and distinction between *P. nebrodensis* strains.

The ITS domain is a ribosomal rDNA region that evolves at a relatively fast pace. It has been used as one of the most efficacious markers of phylogeny for comparing species of the same genus. Álvarez and Wendel (2003) found that in the past 5 years, ITS sequence analysis was conducted in plants in 66% of the papers, and that 34% of the papers were completely based on the analysis of ITS sequence for phylogeny investigation. Similarly, ITS sequence analysis plays an important role in phylogeny studies at or below the genus level and in the identification of macrofungi (Dunham et al. 2003).

For populations of the same species, ITS sequence variation is smaller than IGS domain, especially much smaller than IGS2 region because of a lot of repeat sequences and subrepeat sequences in IGS2. The variation of repeat sequence or subrepeat sequence generates the polymorphism in IGS2 length. It was demonstrated that ITS sequence difference was smaller than 1% in populations of the same species (Sugita et al. 1999). There was only a difference of three nucleotides in the ITS sequences of three pathogenic strains of *Cryptococcus neoformans* (Xu et al. 2000). There were greater differences in the nucleotide sequence of the IGS1 and IGS2 domains in the three strains (Diaz et al. 2000), which can be used effectively to distinguish the three strains. ITS difference between strains could not be found, and it just existed in different species in the present study. IGS2 domain is a highly variable region in ribosomal DNA as evidenced by the abundant polymorphism of IGS2-RFLP in populations of *P. nebrodensis*. The difference was much more remarkable than the difference in fruiting body morphology. It is an effective domain for analysis of genetic polymorphism in *Pleurotus* species, in agreement with reports on other species (Bunyard et al. 1996; Saito et al. 2002). *P. nebrodensis* is a delicious cultivated mushroom with great economic value. The IGS2-RFLP will probably become a DNA marker for patented strains and protection of breeder's right. It is much easier

and much more accurate to use for the identification of cultivars than fruiting body morphology.

Interestingly, two closely related species, *P. eryngii* var. *ferulae* and *P. nebrodensis*, grow on the same species of host plant under the same climatic conditions. These two species manifest obvious differences in ribosomal DNA despite similar fruiting body morphology. The former was separated from the ferula mushroom population and was included in *P. eryngii* complex based on incompatibility with *P. nebrodensis* by mating tests or difference in ITS sequence. However, its fruiting bodies are not gray like regular *P. eryngii* var. *ferulae* as previously reported by taxonomists, but are nearly white, more closely resembling *P. nebrodensis* growing on the same host plant. It is probably attributed to gene flow of the dominant species *P. nebrodensis* growing in the same host under the same ecological environment. It is possible that some genes linked with the fruiting body coloration flow from *P. nebrodensis* into *P. eryngii* var. *ferulae*, bringing it close to the dominant population. Nevertheless, genetically, ITS and IGS1 sequence and IGS1-RFLP of *P. eryngii* var. *ferulae* were identical to those of *P. eryngii* (data not shown). However, the length of its IGS2 was also similar to both the reference strains *P. eryngii* from Italy and some of *P. nebrodensis*. Young fruiting bodies of *P. eryngii* var. *ferulae* and some of those of *P. nebrodensis* are both light gray and become milky white, nearly white, or white, respectively, when they grow up. It can thus be surmised that *P. eryngii* var. *ferula* with gray cap might exist in Xinjiang, China. However, to this date, it has not yet been confirmed.

The prevailing consensus has been that there is only one *Pleurotus* species, *P. eryngii*, growing on *F. sinkiangensis*. It is disclosed in this study that there are, in fact, two *Pleurotus* species, *P. eryngii* and *P. nebrodensis*. The pileus of *Pleurotus* mushroom found here is nearly white and not the classical gray color. The fruiting body morphology of macrofungi is affected by environmental conditions. Thus, it is difficult to distinguish between different strains based on fruiting body morphology. Various methods including ITS, IGS1, and ISSR have been used in analyzing the polymorphism of *Pleurotus* mushroom. However, the results are not satisfactory. ITS can only distinguish between two species; IGS1 cannot distinguish between two species and between different strains of the same species. No clear results are obtained by using ISSR. Clear results were obtained by using IGS2 in the present study. The results were consistent with those based on physiological characteristics and morphological features.

Thus, IGS2 can be used as a means of identification to protect the rights of people engaged in cultivating commercial cultivars of ferula mushroom on *F. sinkiangensis* that grows in crowded and acid places. As a consequence, the fruiting bodies of *Pleurotus* species on *F. sinkiangensis* are very few in number and difficult to collect. The *Pleurotus* species exhibit polymorphism and grow only on *F. sinkiangensis* and have good prospects for commercial cultivation. The other *Pleurotus* species growing in Xinjiang are found in rotten wood of woody plants but have not been found on *F. sinkiangensis*.

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