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The molecular diversity analysis of *Auricularia auricula-judae* in China by nuclear ribosomal DNA intergenic spacer

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ABSTRACT

Background: For the crossbreeding of *Auricularia auricula-judae*, selecting the appropriated parents in hybridization is very important. However, the classification and diversity analysis of *A. auricula-judae* has been equivocal, due to the similarity of the fruiting body morphology and its susceptibility to environmental influences. For this purpose, the molecular diversity of 32 *A. auricula-judae* commercial cultivars in China was analyzed by using the nuclear ribosomal DNA intergenic spacer.
Results: The complete nuclear rDNA gene complex of *A. auricula-judae* isolate is 11,210 bp long, and contains the 18S, 5.8S, and 28S rRNA gene as well as the ITS and IGS regions. Based on the sequence data, four more effective primer combinations for the IGS region of *A. auricula-judae* were designed. Nucleotide sequence variation in the IGS among 32 *A. auricula-judae* commercial cultivars in China sorted into three strongly supported clades, which is correlated with geographical regions. Most strains originated from the same area were with a narrow genetic basis and could possibly be domesticated from the local wild-type strains.
Conclusion: The grouping information obtained in the present work provides significant information for further genetic improvement in *A. auricula-judae*, and suggested that the IGS region can be used as an excellent tool for identification of genetic variation.

1. Introduction

Auricularia auricula-judae (Bull.) Quel. which has a global distribution in tropical, sub-tropical and temperate region, has been first cultivated in China more than one thousand years ago. It is an important edible and medical mushroom, and the annual production is fourth in the world, following *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes* [1].

To protect the rights of mushroom breeders, it is very important to discriminate among main cultivars of *A. auricula-judae*. However, the classification and diversity analysis of edible mushroom has been equivocal, due to the similarity of the fruiting body morphology and its susceptibility to environmental influences. Thus, problems frequently arise if the analysis is based entirely on morphological characteristics.

Fortunately, molecular biology techniques provide a useful methodology for systematic analysis of genetic polymorphism. The spacer regions of ribosome DNA genes are useful for examination of close relationships between organisms, because of the divergence in their nucleotide sequences. The internal transcribed spacer (ITS) between the 18S and 25S rRNA genes is moderate and has been widely applied in phylogenetic studies of basidiomycetes [2]. The intergenic spacer (IGS) between the 25S and 18S rRNA genes is evolving fastest in the rDNA complex [3,4], and has been applied in the polymorphism analysis of edible fungi, such as *Agaricus bisporus* [5], *Laccaria bicolor* [6], *Hebeloma cylindrosporum* [7], *Lentinula edodes* [8], *Pleurotus eryngii* [9], *Tricholoma matsutake* [10], *Ferula sinkiangensis* [11], *Tuber borchii* [12] and *Rhodocollybia laulaha* [13]. However, sequence analysis of the IGS regions of *A. auricula-judae* has not been reported.

In this study, the complete rDNA repeat unit of *A. auricula-judae* was firstly sequenced and analyzed, particular emphasis was placed on the IGS region and the more effective primer combinations were designed. Based on the nucleotide sequence variation in the IGS, the genetic polymorphism of 32 *A. auricula-judae* commercial cultivars in China will be discussed.

2. Materials and methods

2.1. Mushroom strains

Thirty-two main cultivars of *A. auricula-judae* in China were used throughout this study. All cultivars were collected from local professional

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Table 1
Designation, source, and floristic regions of *A. auricula-judae* test strains.

No	Cultivar	Source[a]	Floristic region	GeneBank accession no	
				IGS1	IGS2 3′-end
1	HEI-29	MIHL	NE China	JF440716	JF440751
2	8808	MIHL	NE China	JF440706	JF440755
3	CBS-7	JAU	NE China	JF440710	JF440749
4	YM-1	JAU	NE China	JF440728	JF440754
5	HEI-916	JAU	NE China	JF440704	JF440762
6	9809	DCH	NE China	JF440734	JF440735
7	DA-1	DCH	NE China	JF440712	JF440757
8	DA-2	DCH	NE China	JF440713	JF440743
9	DA-3	DCH	NE China	JF440714	JF440758
10	139	HAU	Central China	JF440708	JF440746
11	YE-K3	HAU	Central China	JF440729	JF440761
12	SN-A8	HAU	Central China	JF440724	JF440752
13	XP-10	HAU	Central China	JF440727	JF440753
14	8129	HAU	Central China	JF440705	JF440742
15	SHAN-1	HAU	Central China	JF440723	JF440741
16	AU110	BIH	Central China	JF440707	JF440736
17	DZ-1	BIH	Central China	JF440711	JF440750
18	HME-1	EMIK	SW China	JF440730	JF440745
19	HE-3	SAAS	East China	JF440718	JF440740
20	DP-5	HAU	East China	JF440721	HQ414241
21	XK-1	HSCS	East China	JF440720	HQ414239
22	XE-987	GEMC	North China	JF440726	HQ414242
23	XE-887	GEMC	North China	JF440725	JF440739
24	HE-9	EMIS	North China	JF440715	JF440737
25	JY-1	MIHB	North China	JF440719	JF440759
26	ZJ-310	EMIC	North China	JF440731	JF440760
27	ME-6	EMIC	North China	JF440722	JF440738
28	ZHI-5	HIB	NWChina	JF440732	HQ414243
29	97-1	HIB	NWChina	JF440733	JF440756
30	C21	MIS	NWChina	JF440709	JF440748
31	173	XFH	NWChina	JF440717	JF440744
32	186	XFH	NWChina	JF440703	JF440747

[a] MIHL, Heilongjiang Microbiological Institute; JAU, Jilin Agricultural University; DCH, Dongning County, Heilongjiang Province; HAU, Huazhong Agricultural University; BIH, Biological Institute of HenanScientific Academy; GEMC, Guangda Edible Mushroom Center, Jining; EMIS, Edible Mushroom Institute of Shouguang; MIHB, Microbiological Institute of Heibei Province; EMIC, Edible Mushroom Institute of the Chinese Agricultural University; HIB, Hanzhong Institute of Botany, Shanxi Province; MIS, Microbiological Institute of Shanxi Province; XFH, Xixiang Edible Fungi Institute, Shanxi Province; SAAS, Shanghai Academy of Agricultural Sciences; HSCS, Haibing Spawn Center of Suizhou; EMIK, Edible Mushroom Institute of Kunming.

research institutes in different geographical regions of China. According to different floristic regions, tested strains were divided into six populations (Table 1).

2.2. DNA extraction

Mycelia of all cultivars were separated from spawn and incubated on CYM (complete yeast media) agar plates at 25°C for 10 d. The agar plates with the grown mycelia were cut into small pieces, transferred into 100 ml CYM liquid medium, and cultured at 25°C for 14 d. Genomic DNA was extracted from freeze-dried mycelia of 32 *A. auricula-judae* cultivars by using the method of Tang et al. [14]. The DNA quality was confirmed by 1.0% (w/v) agarose gel electrophoresis, and concentrations were determined with a BioPhotometer 6131 (Eppendorf, Germany), then diluted to 50 ng/μl for PCR amplification.

2.3. PCR amplification

Polymerase chain reaction (PCR) amplifications for rDNA complete region of *A. auricula-judae* strain AU110 were done with combinations of universal primers (Table 2). On the basis of above sequence information, eight more effective primers for amplifying the IGS1 and IGS2 region of *A. auricula-judae* were designed by using the web-derived software "Primer 3" (http://frodo.wi.mit.edu/primer3/ (Table 2)), and their relative position in the ribosomal repeat unit is indicated with arrows in Fig. 1. The main design parameters were as follows: primer optimum size, maximum size, and minimum size were all set to 22, 26 and 18 nucleotides respectively; primer optimum Tm, maximum Tm, and minimum Tm were set to 54°C, 60°C, and 50°C respectively. Their relative binding site in the *A. auricula-judae* ribosomal repeat unit is indicated within Table 2.

Using the new primers, we PCR-amplified the IGS1 and IGS2 domains from total DNA of 32 strains of *Auricularia* in a MyCycler thermal cycler (Bio-Rad, USA), which programmed as follows: initial denaturation 4 min at 94°C; 37 cycles of: denaturation 1 min at 94°C, annealing 1 min (at a temperature corresponding to the T_m of the primers used), extension 3 min at 72°C; and final extension 10 min at 72°C. Each reaction was performed in microfuge tubes 0.2 ml in a volume of 20 μl, including 0.25 mM dNTPs, 0.4 μM of each primer, 1.2 U r*Taq* DNA polymerase (TaKaRa, Japan), 1× PCR buffer, 2.0 mM $MgCl_2$, and 150 ng DNA template. The reaction products were analyzed on a 1% agarose gel in 1× TAE buffer. A 2 kb DNA ladder (Gibco-BRL) was included as DNA size marker. PCR product purification and DNA sequencing were performed by Songon Biotechnology Company and Genecore Biotechnology Company in Shanghai. Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/).

Table 2
Primers for the amplification of the rDNA repeat of *A. auricula-judae*.

Primer	Sequence(5′–3′)	Target region	Binding site (bp)	Source
NS1	GTAGTCATATGCTTGTCTC	18S complete sequence	1–19	Sivakumaran et al. [26]
NS8	TCCGCAGGTTCACCTACGGA	18S complete sequence	1776–1757	Sivakumaran et al. [26]
ITS1	TCCGTAGGTGAACCTGCGG	ITS1-5.8S-ITS2	1757–1775	Matsumoto et al. [27]
ITS4	TCCTCCGCTTATTGATATGC	ITS1-5.8S-ITS2	2353–2334	Matsumoto et al. [27]
LROR	ACCCGCTGAACTTAAGC	28S 5′ end	2319–2335	Bunyard et al. [5]
LR7	TACTACCACCAAGATCT	28S 5′ end	3766–3750	Bunyard et al. [5]
ALR7R	AGATCTTGGTGGTAGTA	28S 3′ end	3750–3766	Bunyard et al. [5]
LR12	TTCTGACTTAGAGGCGTTCAG	28S 3′ end	5433–5413	Bunyard et al. [5]
LR12R	CTGAACGCCTCTAAGTCAGAA	IGS1 complete sequence	5413–5433	Bunyard et al. [5]
M-1	AACCACAGCACCCAGGATTCCC	IGS1 complete sequence	7884–7863	Bunyard et al. [5]
5SF	TAGTTACGCCTTATAGACGC	5S complete sequence	7728–7747	Huysmans et al. [28]
5SR	CAGGATTCCCGCGTGGTCCC	5S complete sequence	7870–7851	Huysmans et al. [28]
5SRNAR	ACCGCATCCCGTCTGAT	IGS2 complete sequence	7792–7809	Vilgalys et al. [29]
INVSR1R	ACTGGCAGAATCAACCAGGTA	IGS2 complete sequence	11,210–11;192' 2–1	Vilgalys et al. [29]
IGS1-5F	TATGTCCCGCATGTGTTAGT	IGS1 5′ end	5462–5481	This work
IGS1-5R	CGCCCATTACATTTCGTCA	IGS1 5′ end	6745–6727	This work
IGS1-3F	CGCCCTTTCTAATGAC	IGS1 3′ end	6715–6730	This work
IGS1-3R	GCGTCTATAAGGCGTAACTA	IGS1 3′ end	7747–7728	This work
IGS2-5F	AAGTCCTGGTGGCGTATCTC	IGS2 5′ end	7574–7593	This work
IGS2-5R	CCATCGTGCCCTCGTATTC	IGS2 5′ end	10,274–10,256	This work
IGS2-3F	GAATACGAGGGCACGATGG	IGS2 3′ end	10,256–10,274	This work
IGS2-3R	GAGACAAGCATATGACTACTG	IGS2 3′ end	11,209–11,210; 1–19	This work

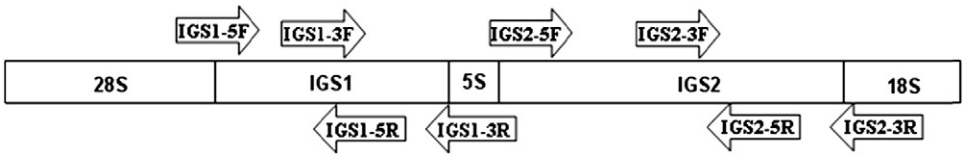

Fig. 1. Primer position used for the amplification of the IGS sequence of *A. auricula-judae* test.

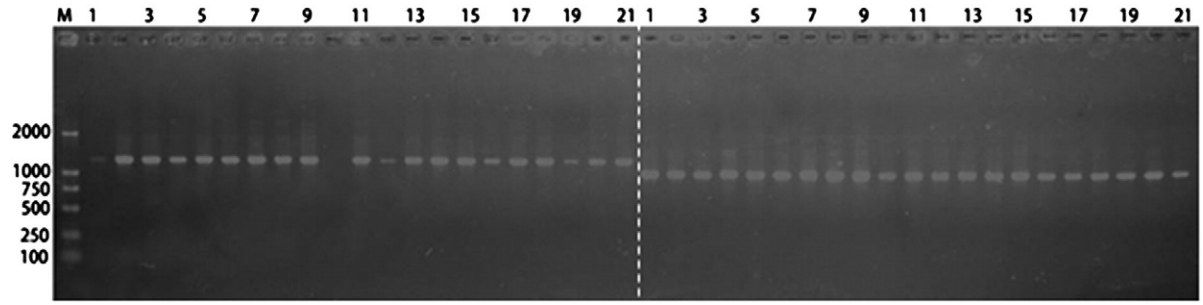

Fig. 2. IGS1 5′ end and 3′ end amplification of partial *A. auricula-judae* test cultivars. Note: 1–21 are the treatments showed in Table 1; the left and right panels represent the amplification result of IGS1 5′ end and 3′ end respectively.

2.4. Sequence analysis

The complete rDNA repeat analysis of strain AU110 was performed by Repeatmasker software (http://www.repeatmasker.org/). Alignments of IGS1 and IGS2 nucleotide sequences were made by using the MegAlign module of the Lasergene software system (DNASTAR, Inc.) implementing the Clustal W algorithm [15], with some manual adjustments.

Based on the IGS1, IGS2 and the combined sequence, phylogenetical tree contains 32 test cultivars were obtained with the Minimun Evolution and Maximum Parsimony methods. Kimura-2-Parameter and close-neighbor-interchange algorithm were used in ME and MP method respectively. Gaps were encoded as complete deletion and thus excluded from analyses; transition and transversion were set as the same weight. Tree topology was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 3.1 [16]. Moreover, the IGS nucleotide sequences of *L. edodes* (GenBank accession numbers AB030581 and AB030582) were set as outgroup in the construction of the combined phylogenetical tree [8].

3. Results

3.1. Cloning and sequencing of the rDNA repeat unit

The complete rDNA region of *A. auricula-judae* cultivars AU110 was amplified, and then submitted to GenBank (GenBank accession number JN712676). The complete region was 11,210 bp long, and contained the 18S, 5.8S, and 28S rRNA genes as well as the ITS and IGS regions. Following DNA sequence alignment with the filamentous fungi [17], the exact size of each gene was estimated as follows: 1805 bp for the 18S rDNA, 513 bp for ITS1,5.8S region and ITS2 sequence, 3135 bp for 28S, 2335 bp for IGS1, 118 bp for 5S and 3304 bp for the IGS2 region.

The IGS1 region has no repeats. In the IGS2 region, the sequence from 8551 bp to 8638 bp in the 5′ end region was G-rich with $(GGGGA)_n$ repeat, and $(TTAGG)_n$ repeat existed in the sequence from 8768 bp to 8799 bp and from 8845 bp to 8880 bp, which increased the sequencing difficulty of complete IGS2 domain.

3.2. Species-specific primer development and PCR-amplification of IGS region

On the basis of the complete rDNA sequence of AU110, four species-specific primer combinations for amplified IGS region of *A. auricula-judae* were designed (Table 2, Fig. 1). The 5′ end and 3′ end of IGS1 region were amplified by using primer pairs IGS1-5F/IGS1-5R and IGS1-3F/IGS1-3R, and the product size were about 1200 bp and 1000 bp respectively (Fig. 2). The 5′ end of IGS2 region was amplified by using primer pairs IGS2-5F/IGS2-5R, and the product size was about 2500 bp, however, the region was G-rich and difficult for sequencing, thus excluded from further analyses. The product size of 3′ end of IGS2 region was about 800 bp (Fig. 3), which was amplified by using primer pairs IGS2-3F/IGS2-3R. The accession numbers of IGS1 and IGS2 3′ end sequence of all test cultivars are listed in Table 1.

The IGS1 fragment of 32 test isolates ranged from 2284 bp (JY-1, HME-1 and 186) to 2312 bp (ME-6), and the average size was 2299 bp; the 3′ end of IGS2 were nearly 800 bp. The detailed information of IGS region can be found in Table 3.

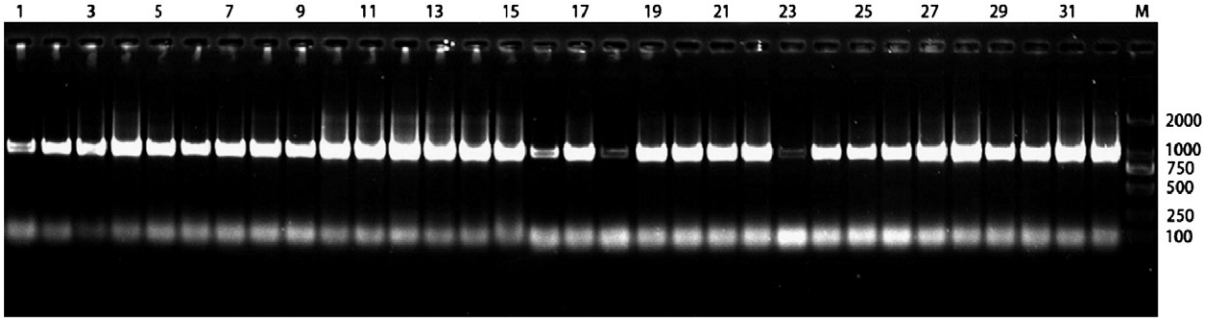

Fig. 3. The amplification result of IGS2 3′ end of *A. auricula-judae* test strains. Note: 1–32 are the treatments showed in Table 1.

Table 3
IGS sequence information of test *A. auricula-judae* cultivars.

Sequence information		IGS1 entire region	IGS2 3′ end region	Complete IGS1 and IGS2 3′ end region
Alignment matrix length (bp)		2312	801	3114
Average region length (bp)		2299	800	3100
Nucleotide composition	A	20.4	22.2	20.9
	T	27.6	25.9	27.2
	C	23.1	21.7	22.8
	G	28.8	30.2	29.2
transitional/transversional (si/sv)		2.7	4.7	3.1
Conserves sites, C		2205	757	2963
Variable sites, V		109	44	152
Parsimony-informative site, Pi		32	23	55
Singleton sites, S		77	21	97

3.3. The phylogenetic analyses based on IGS1 complete sequence

The trees constructed by ME and MP methods based on the IGS1 alignment showed the same clustering patterns (Fig. 4). Five major clades were labeled; cluster I consisted of 8 strains cultivated in the East and Central regions; cluster II contained 6 strains, which were mainly cultivated in the Northeast, Northwest and Central regions; cluster III included 4 strains cultivated in North and Southwest regions; cluster IV merely consisted of the strains C21 and 8808, while cluster V consisted of 12 strains cultivated in the Northeast, North and Central regions.

3.4. The phylogenetic analyses based on IGS2 3′ end sequence

The 32 test strains were divided into 6 similar groups based on ME and MP methods (Fig. 5). Cluster I consisted of 10 strains mainly cultivated in the East and Central regions; clusters II and III contained 8 and 3 strains respectively, which were mainly cultivated in the

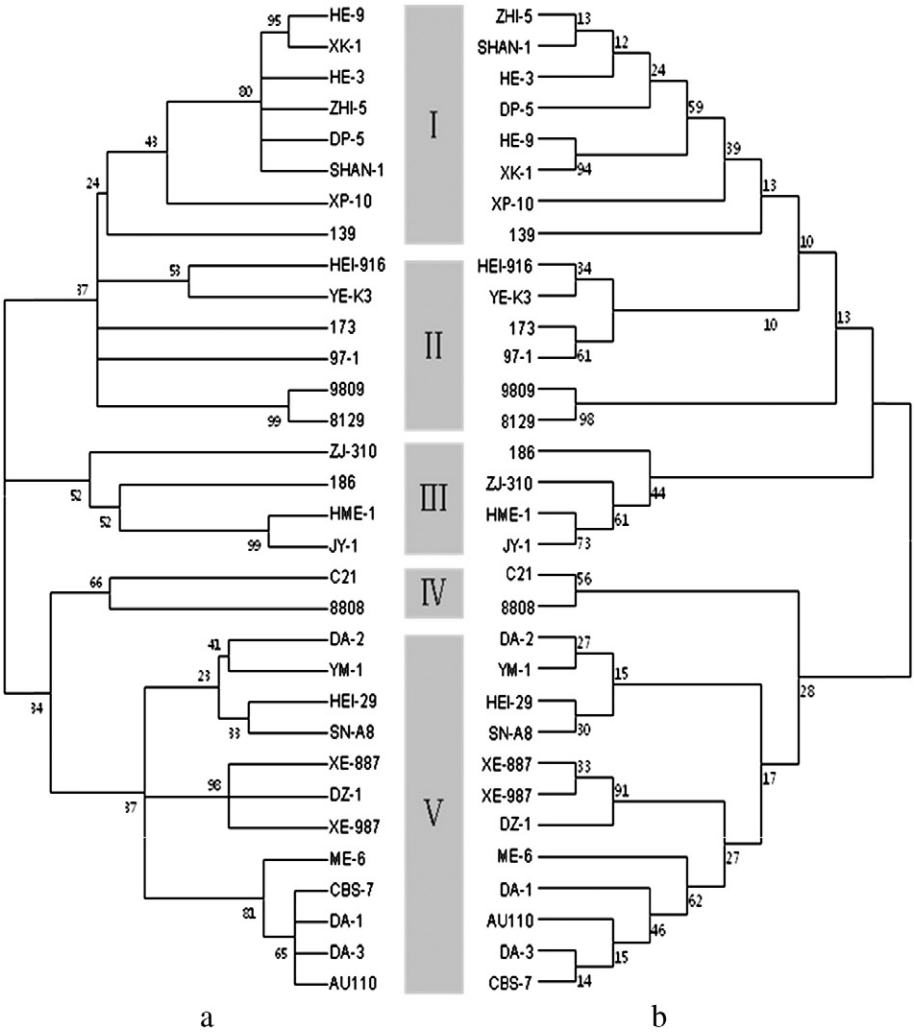

Fig. 4. The phylogenetic tree of *A. auricula-judae* test strains based on IGS1 sequence (a: ME tree; b: MP tree).

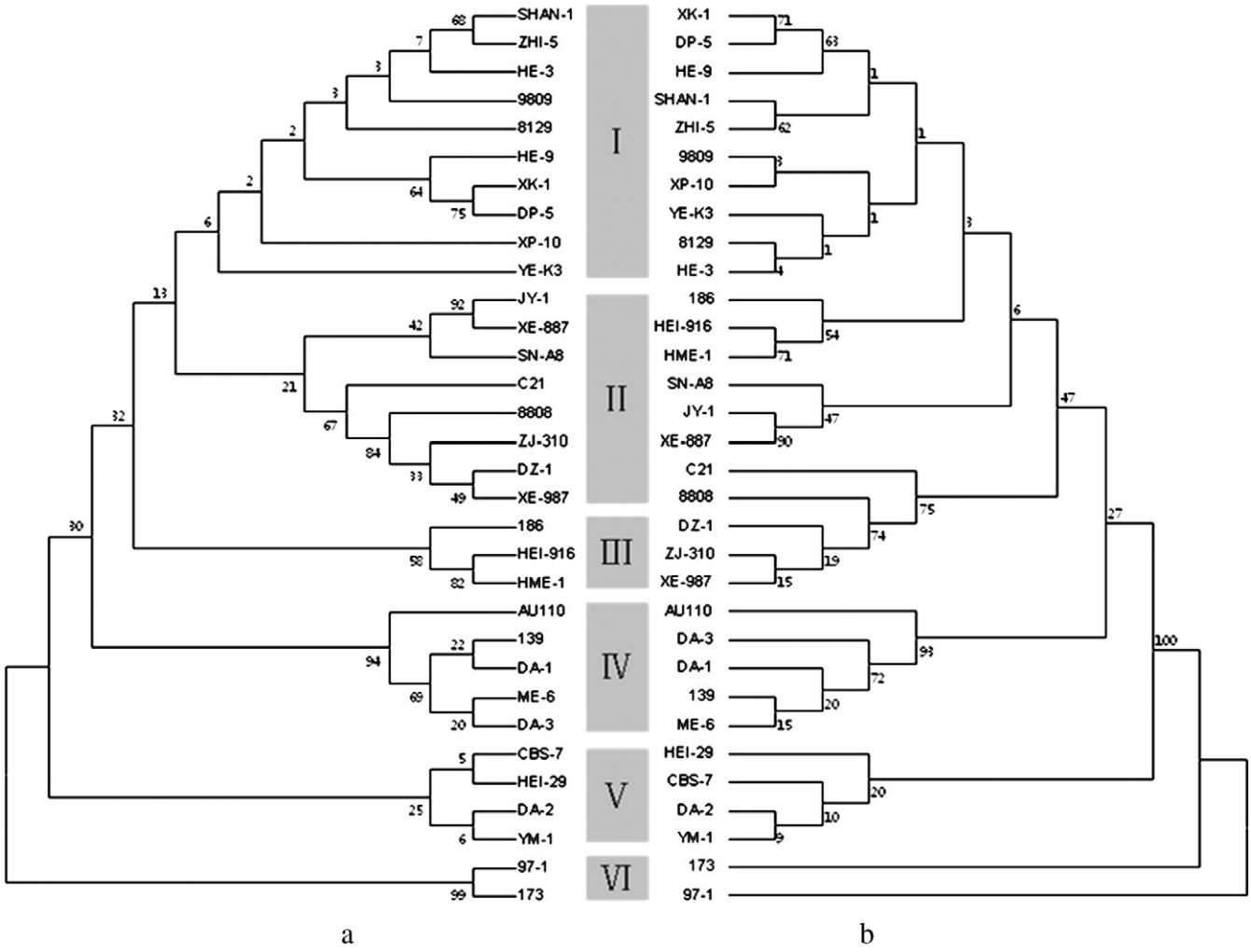

Fig. 5. The phylogenetic tree of *A. auricula-judae* test strains based on IGS2 3′ end rDNA sequence (a: ME tree; b: MP tree).

North, Southwest and Northwest regions; clusters IV, V and VI consisted of 5, 4 and 2 strains respectively, which were cultivated in the Northeast, Northwest and Central regions.

3.5. The phylogenetic analyses based on IGS1 and IGS2 3′ end combined sequence

The ME and MP analyzing result were nearly same based on the IGS1 with IGS2 3′ end combined sequence (Fig. 6), and one strain of *L. edodes* was set as the outgroup. All the test cultivars were divided into 3 main groups; group I consisted of 10 strains, DA-1, DA-2, DA-3, CBS-7, HEI-29, YM-1, ME-6, 139, AU110 and SN-A8, which were cultivated in the Northeast and Central regions; group II contained strains JY-1, XE-887, C21, 8808, ZJ-310, XE-987, DZ-1 and HME-1, which originated from the North and Southwest regions; while group III contained SHAN-1, ZHI-5, HE-3, 9809, 8129, HE-9, XK-1, DP-5, XP-10, YK-K3, 186, HEI-916, 97-1 and 173, cultivated in the Central, Northwest and East regions.

4. Discussion

As the primary genetic material, DNA sequence can reflect the genetic relationship more directly, accurately and objectively than the morphological characteristics. To data, the DNA analyses of fungi focus on the ribosomal DNA sequence. Due to the different selective pressures of rDNA domains, the conservative degree of each rDNA domain was unequal. The 18S, 5.8S, 28S and 5S rDNA gene regions are highly conserved, and always used in phylogeny studies of the order and family [18,19]. ITS region is moderate variable, mainly for the identification of species and varieties [2]. The IGS domain, which evolved the fastest, was always applied for the analysis of the genetic diversity within species. In recent years, the IGS region has been successfully applied to study the genetic diversity of several edible mushrooms, such as *Ferula sinkiangensis* [11], *Armillaria mellea* [20], *Pleurotus eryngii* [21], *Tuber borchii* [12] and *Rhodocollybia laulaha* [13].

According to traditional theory [6], unequal exchange frequently occurred in mitosis, resulting in an amount of repetitive or sub-repetitive units existing in IGS region, whose structure and numbers were variable among different organisms, and represented as the IGS length differences. Saito et al. [8] have studied the IGS region of 16 *L. edodes* commercial cultivars in Japan, and found that there were significant differences within species. The IGS1 sequence number of different mushroom strains is 1–3 bands, and the sizes were between 0.9 kb and 1.5 kb; the IGS2 sequence number varied by 1–4 bands, and the sizes were between 2 kb and 3 kb; IGS1 and IGS2 domains of *L. edodes* contained intricate sub-repeat regions SR1 and SR2 respectively, while SR1 and SR2 have 6 kinds of short sub-repeats units, the structure and number in different strains were distinct, which caused the polymorphism of *L. edodes* IGS sequence. Similar phenomenon of *Lentinula* species has been observed in Nicholson et al. [22]. Huang et al. [9] analyzed the IGS2 region of *P. eryngii* and confirmed that the length and number of the IGS2 sequence amplified by different test cultivars demonstrated high polymorphism, and that the number of IGS2 fragment was among 1–4.

However, the opposite phenomenon also observed in other fungi, the IGS sequences of one certain strain amplified only a single band,

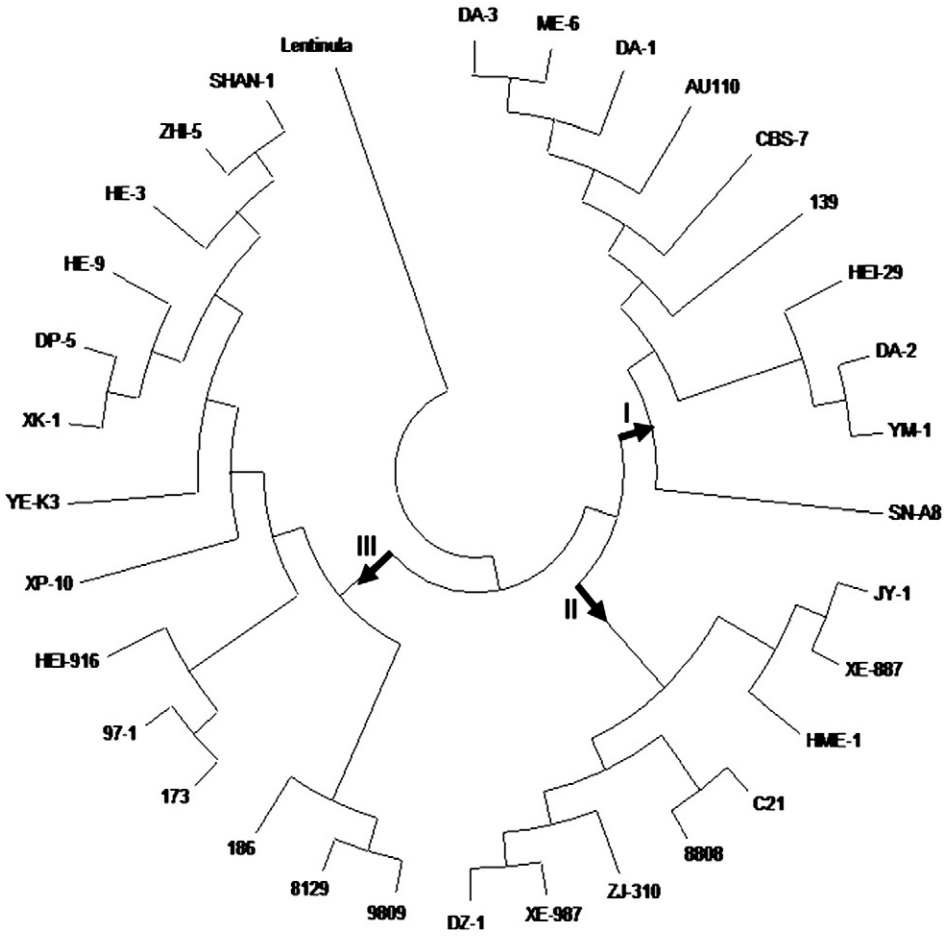

Fig. 6. The phylogenetic tree of *A. auricula-judae* test strains based on IGS1 and IGS2 3′ end rDNA sequence with Minimum Evolution methods.

and there were no polymorphic bands among different materials. For example, Kim et al. [10] analyzed the IGS1 sequence of 29 North American *Armillaria* strains and found that the IGS1 fragment length of all test strains was 606 bp and contained 147 variable sites, and that the phylogenetic results based on ITS and IGS1 were nearly same; a similar phenomenon has also been found in *Tilletia* sp. [23] and *Penicillium marneffei* [24]. The research result demonstrated that there were obvious differences among different fungi, which means the IGS region required further in-depth analysis.

In this study, we firstly studied the complete rDNA sequence of *A. auricula-judae*, especially on the IGS region. Same as the amplified phenomenon of *Armillaria* [10], the amplification of IGS1 and IGS2 regions in *A. auricula-judae* obtained merely one specific band, and few length polymorphisms have been found among test cultivars. The IGS1 have no repeats, oppositely, the 5′ end of IGS2 region contained the rDNA transcription initiation site and terminator, have been found rich in GC and difficult for sequencing, which was same in the plant [25].

The phylogenetic analysis based on IGS1, IGS2 3′ end sequence and the combined sequence, as well as by ME or MP method, get the similar results, which demonstrated that the IGS sequence were highly effectively for analyzing the genetic variation among *A. auricula-judae* strains. What is more is that the combined IGS analysis result, which contains more nucleotide information, can divide all the strains more apparently; 32 test strains have divided into 3 groups, corresponding to the Northeast and Central regions; North and Southwest regions; as well as Central, Northwest and East regions respectively, which reflect the relationship between the genetic characteristics and geographical distribution. Most strains originated from the same area, were with a narrow genetic distance and could possibly be domesticated from the local wild-type strains. According to the grouping information, the genetic diversity of main *A. auricula-judae* cultivars in China was comparatively low, which indicates that the domestication of wild-type strains should attract more attention.

For the improvement of strains, selecting the appropriated parents in hybridization is very important. Hybridization program involving genetic diverse belonging to different distant clusters will facilitate the breeding program. Fortunately, the grouping information obtained in the present work provides significant information for further genetic improvement in *A. auricula-judae*, and is expected to be the reference for the similar studies in other countries.

Conflict of interest

No conflict of interest among the authors.

Acknowledgments

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