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Analysis of Genetic Diversity Among Chinese *Auricularia auricula* Cultivars Using Combined ISSR and SRAP Markers

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Abstract DNA polymorphism among 34 Chinese *Auricularia auricula* cultivars was analyzed using inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers. Thirty ISSR primers amplified a total of 129 DNA fragments of which 125 (96.9%) were polymorphic, whereas 11 SRAP primer combinations amplified 154 fragments of which 148 (96.1%) were polymorphic. Both methods were highly effective in discriminating among the test strains. Phylogenetic trees constructed on the basis of ISSR, SRAP, and combined ISSR/SRAP analyses using the Unweighted Pair-group Method with Arithmetic Averages (UPGMA) method distributed the 34 strains into four or five major groups. Clustering analysis based on all the three data sets indicated a high level of genetic diversity among *A. auricula*, although the combined ISSR/SRAP data were more concordant with the main agronomic characters of strains and their geographical centers of cultivation. Our findings will facilitate future *A. auricula* breeding programs and the development of bioactive products from this commercially important medicinal mushroom.

Introduction

Auricularia auricula, the jelly ear fungus, is widely considered to have been the earliest artificially cultivated edible mushroom, and annual production of *Auricularia* species worldwide is now the fourth highest of all the industrially cultivated culinary and medicinal mushrooms. In China alone, which has two main cultivation areas located in the northeast provinces of Heilongjiang, Jilin, and Liaoning, and in the central provinces of Hubei, Sichuan, and Shanxi, the estimated output of this mushroom in 2003 (latest figures available) was almost 1.655 million tonnes [1].

In addition to their nutritional and organoleptic attributes, *A auricula* and the closely related *Auricularia polytricha* have been used as medicines for many centuries in China and other parts of Asia in the treatment of such widely varying conditions as hemorrhoids, hemoptysis, angina, diarrhea, and gastrointestinal upsets [2]. More recently, *Auricularia* mushrooms have been reported to prevent blood clotting [3], strokes, and heart attacks [4], to lower blood cholesterol and triglycerides [5], exhibit antioxidant activities [6], and to be effective in the treatment of diabetes [7] and certain cancers [8].

The major problems currently facing growers of *A. auricula* is the random labeling of strains and the introduction into different regions of identical strains under different designations. Incorrectly designated strains result in huge economic losses, have a negative impact on mushroom breeding program, and cause confusion with regard to the protection of intellectual property rights. Therefore, precise identification and classification of commercial *A. auricula* cultivars is of major importance for Chinese and overseas markets.

Unlike other mushrooms, the use of fruit body morphology to identify individual *A. auricula* strains is not feasible due to the relatively simple structure of the sporophore. However, strain identification can now be achieved effectively using a selection of DNA marker

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technologies available for this purpose [9], and which have also been used to study genetic diversity and population genetics in a variety of agricultural products [10, 11] including mushrooms [12–14]: Two such technologies, inter-simple sequence repeats (ISSR) [15] and sequence-related amplified polymorphism (SRAP) [16], have proven especially reliable and effective, especially when data from both types of analyses have been combined.

In this study, combined ISSR and SRAP procedures were adopted for DNA fingerprinting of 34 strains of *A. auricula* cultivated in China. Our data demonstrate that the combined methodologies were superior in identifying *A. auricula* strains and for analyzing genetic diversity among Chinese cultivars, and facilitate efficient evaluation, management, and utilization of *A. auricula* germplasm resources.

Materials and Methods

Mushroom Strains

Thirty-four *A. auricula* strains collected from different regions of China, and maintained at the Institute for Edible Fungi, Shanghai Academy of Agricultural Sciences, were used in the study.

DNA Extraction

Total DNA was extracted from 1 g of wet mycelia using the cetyl trimethylammonium bromide (CTAB) method described by Sambrook et al. [17], and the quality confirmed by 1.0% (w/v) agarose gel electrophoresis. DNA concentrations were determined with a BioPhotometer 6131 (Eppendorf, Germany), and samples were diluted to 50 ng μl^{-1} for PCR amplification.

ISSR Analysis

Thirteen random primers (Sangon Biotech, Shanghai) were selected for ISSR analysis (Table 1). ISSR amplification was carried out using an Amp9600 Thermal Cycler (PE Company) in 20-µl reaction volumes containing 0.5 U *Taq* DNA polymerase, 2.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTP (TaKaRa Biotech, Dalian), 0.75 µmol/l primer, and 150 ng template DNA. Amplification conditions were: 94°C for 5 min; 35 cycles of 94°C for 30 s, 53.1–56°C (see Table 1 for primer annealing temperature) for 45 s, and 72°C for 90 s; followed by a final extension for 7 min at 72°C. Amplified products were fractionated by electrophoresis in 2% (w/v) agarose/TAE gels, visualized under UV after staining with ethidium bromide, and documented using a gel documentation and image analysis system (Gene Systems, Hong Kong).

SRAP Analysis

Eleven random SRAP primer combinations (Sangon Biotech, Shanghai) were selected for SRAP analysis (Table 2). SRAP amplifications were performed in 25-µl reaction volumes containing 0.2 mmol/l dNTPs, 2.5 mmol/l $MgCl_2$, 1.0 U *Taq* DNA polymerase (TaKaRa Biotech, Japan), 50 ng genomic DNA and 66 ng of each primer. Amplification conditions were: 5 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; followed by a final extension 10 min at 72°C. Amplification products were fractionated by electrophoresis in 6% (w/v) polyacrylamide gels at 60 W for 2 h and visualized by silver staining. Pbr322DNA/Msp I (Tiangen Biotech, China) was used as size markers.

Data Analyses

Data analyses were performed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc), version 2.10 (Exeter Software, Setauket, New York) software package [18], the SIMQUAL program was used to calculate the Dice similarity coefficient [19], a common estimator of genetic identity, as follows:

$$\text{Dice} = 2\text{NAB}/(2\text{NAB} + \text{NA} + \text{NB})$$

where NAB is the number of bands shared by samples A and B, NA represents the number of amplified fragments in

Table 1 ISSR primers and corresponding annealing temperatures

Primer	Sequence (5′ → 3′)	Annealing temperature (°C)	Primer	Sequence (5′ → 3′)	Annealing temperature (°C)
P3	$(GA)_8T$	53.1	P21	$(AG)_8YA$	54.0
P4	$(GA)_8C$	56.0	P22	$(AG)_8YC$	56.0
P5	$(AG)_8G$	56.0	P25	$(GA)_8YC$	56.0
P6	$(AG)_8C$	56.0	P26	$(GA)_8YT$	54.0
P9	$GS(GT)_8$	52.0	P27	$(GA)_8YG$	56.0
P10	$(AG)_8T$	54.0	P32	$(GA)_8A$	54.0
P16	$(TC)_8C$	56.0			

Table 2 Sequences (5′ → 3′) of SRAP forward (me) and reverse (em) primers

Primer	Sequence	Primer	Sequence
me1	5′TGAGTCCAAACCGGATA	em1	5′GACTGCGTACGAATTAAT
me2	5′TGAGTCCAAACCGGAGC	em2	5′GACTGCGTACGAATTTGC
me3	5′TGAGTCCAAACCGGAAT	em3	5′GACTGCGTACGAATTGAC
me4	5′TGAGTCCAAACCGGACC	em4	5′GACTGCGTACGAATTTGA
me5	5′TGAGTCCAAACCGGAAG	em5	5′GACTGCGTACGAATTAAC
me6	5′TGAGTCCAAACCGGACA	em6	5′GACTGCGTACGAATTGCA
me7	5′TGAGTCCAAACCGGACG	em7	5′GACTGCGTACGAATTCAA
		em8	5′GACTGCGTACGAATTCAC

sample A and NB the number of amplified fragments in sample B. The SHAN program was applied to the three distance matrices (ISSR, SRAP, and ISSR + SRAP) to construct the Unweighted Pair-group Method with Arithmetic Averages (UPGMA) dendrograms [20]. The goodness of fit of the clustering to the data matrix was calculated using the COPH and MXCOMP programs. In order to estimate the correlations between the three dendrograms, the correlation between the three genetic matrices was determined using the Mantle test [21]. Finally, a Principal Coordinate Analysis (PCO) was performed using the DCENTER and EIGEN programs to obtain a graphical representation of the relationship between the 34 test genotypes.

Results

The designation, source, and main cultivation area of the 34 *A. auricula* strains used in this study are shown in Table 3.

ISSR Analysis

A total of 129 bands, of which 125 (96.9%) were polymorphic, were scored from the 34 genotypes using the 13 ISSR primers listed in Table 2. For representational purposes, the extent of polymorphism revealed by primer P3 is shown in Fig. 1.

Table 3 Designation, source and main cultivation area of *Auricularia auricula* test strains

No.	Cultivar	Source	Main area of cultivation	No.	Cultivar	Source	Main area of cultivation
1	Hei-29	MIHL	NE China	18	Au110	BIH	Central China
2	8808	MIHL	NE China	19	DZ-1	BIH	Central China
3	CBS-7	JAU	NE China	20	XE-987	GEMC	North China
4	Ym-1	JAU	NE China	21	XE-887	GEMC	North China
5	Hei-916	JAU	NE China	22	HE-9	EMIS	North China
6	9809	DCH	NE China	23	JY-1	MIHB	North China
7	DA-1	DCH	NE China	24	ZJ-310	EMIC	North China
8	DA-2	DCH	NE China	25	ME-6	EMIC	North China
9	DA-3	DCH	NE China	26	HE-3	SAAS	SE China
10	139	HAU	Central China	27	DP-5	HAU	SE China
11	YE-K3	HAU	Central China	28	XK-1	HSCS	SE China
12	SN-A8	HAU	Central China	29	Zhi-5	HIB	NE China
13	XP-10	HAU	Central China	30	97-1	HIB	NE China
14	8129	HAU	Central China	31	C21	MIS	NE China
15	HR-901	HAU	Central China	32	C22	MIS	NE China
16	Shan-1	HAU	Central China	33	173	XFH	NE China
17	HME-1	EMIK	South China	34	186	XFH	NE China

MIHL Heilongjiang Microbiological Institute; *JAU* Jilin Agricultural University; *DCH* Dongning County, Heilongjiang Province; *HAU* Huazhong Agricultural University; *EMIK* Edible Mushroom Institute of Kunming; *BIH* Biological Institute of Henan Scientific 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; *SAAS* Shanghai Academy of Agricultural Sciences; *HSCS* Haibing Spawn Center of Suizhou; *HIB* Hanzhong Institute of Botany, Shanxi Province; *MIS* Microbiological Institute of Shanxi Province; *XFH* Xixiang Edible Fungi Institute, Shanxi Province

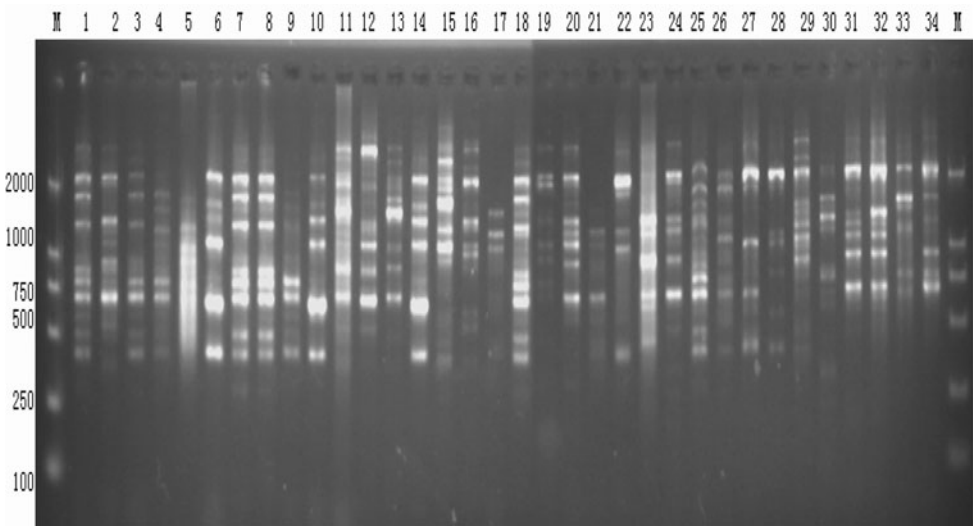

Fig. 1 Representative ISSR amplification profile using primer P3

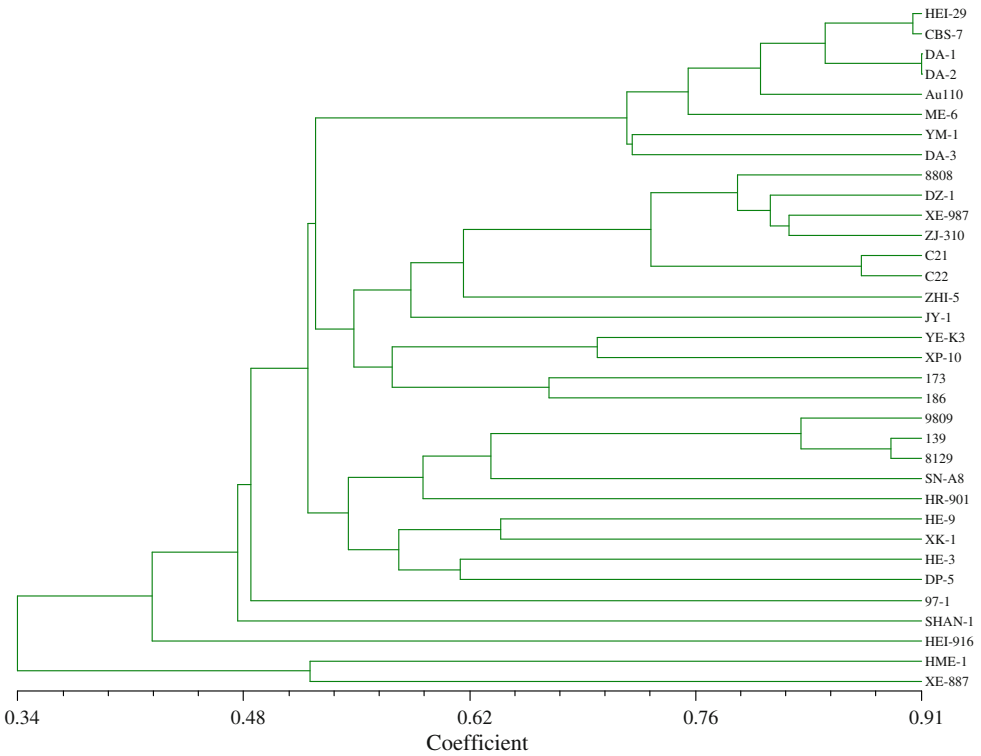

Fig. 2 UPGMA dendrogram of 34 *A. auricula* strains constructed using genetic similarity analysis based on molecular profiles revealed by ISSR markers

Genetic similarities among the 34 genotypes ranged from 0.34 up to 0.91. The co-phenetic correlation for the ISSR dendrogram was estimated at 0.85, corresponding to a good fit. UPGMA grouped the 34 genotypes into five main clusters at a similarity index value of 0.50 (Fig. 2). Cluster I comprised 29 genotypes that were delineated into five sub-clusters at a similarity index value of 0.56. Within Cluster I, strains DA-1 and DA-2 (with a 0.91 similarity coefficient) appeared to be closely related genetically, as were the strain doublets HEI-29 and CBS-7, C21 and C22, and 139 and 8129. Clusters II, III, and IV each comprised a single genotype, while Cluster V consisted of two strains (HME-1 and XE-887) that appeared to be distinct from all the the other genotypes. Lane numbers correspond to the genotypes listed in Table 3. Lane M: DL2000 molecular size markers.

Principal coordinate analysis (PCO) data based on the genetic similarity matrix are shown in Fig. 3. These revealed similar groupings to UPGMA, and confirmed the genetic distinctiveness of genotypes HME-1 and XE-887. The three most informative PCO components accounted for 65.4% of the variations observed.

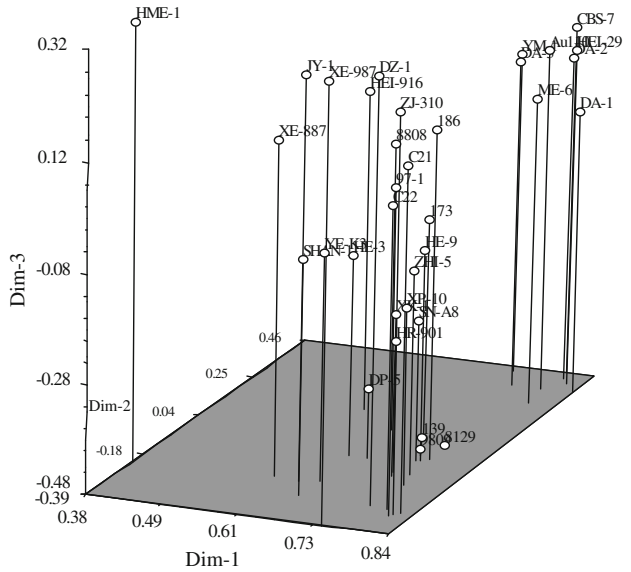

Fig. 3 Relationships among the 34 genotypes of *A. auricula* visualized by Principal Coordinate Analysis (PCO) of ISSR data

SRAP Analysis

A total of 154 bands, of which 148 (96.1%) were polymorphic, were scored from the 34 genotypes using the eleven SRAP primer pairs listed in Table 3. The number of bands amplified with the different SRAP primer combinations varied from 7 to 24. A representative set of amplification profiles obtained with primer combination me1 + em8 is shown in Fig. 4. Lane numbers correspond to the genotypes listed in Table 3. Lane M: Pbr322DNA/Msp I molecular size markers.

Genetic similarities among the 34 genotypes ranged from 0.23 up to 0.90, and the co-phenetic correlation for the SRAP dendrogram was estimated at 0.90, which corresponded to a very good fit. UPGMA grouped the 34 genotypes into five main clusters at a similarity index value of 0.48 (Fig. 5). Cluster I comprised 29 genotypes that were further delineated into five sub-clusters at a similarity index value of 0.53. Within Cluster I, strains DA-1 and DA-2 (with a 0.90 similarity coefficient) appeared to be closely related, as were the strain doublets CBS-7 and YM-1, DZ-1 and XE-987, and C21 and C22, and 139 and 8129. Clusters II, IV, and V each comprised a single genotype (SN-A8, DP-5, and HEI-916, respectively), while Cluster III consisted of two strains (HME-1 and HE-9). Groupings identified by UPGMA analysis were confirmed by PCO data which also revealed that strains HEI-916 and DP-5 were genetically very distinct from the other genotypes. The three most informative PCO components accounted for 63.0% of the variation observed (Fig. 6).

Combined ISSR and SRAP Analysis

In an analysis of combined ISSR and SRAP data, genetic similarities among all the 34 genotypes ranged from 0.34 up to 0.91. The co-phenetic correlation for the combined ISSR/SRAP dendrogram was estimated 0.89, corresponding to a very good fit. UPGMA grouped the 34 genotypes into four main clusters (Fig. 7). Cluster I comprised 29 genotypes that were further delineated into four sub-clusters at a similarity index value of 0.53. Within cluster I, DA-1 and DA-2 were again revealed to be closely related (similarity coefficient 0.91), as were the strain doublets C21 and C22, and 139 and 8129. Genotypes HE-3, DP-5,

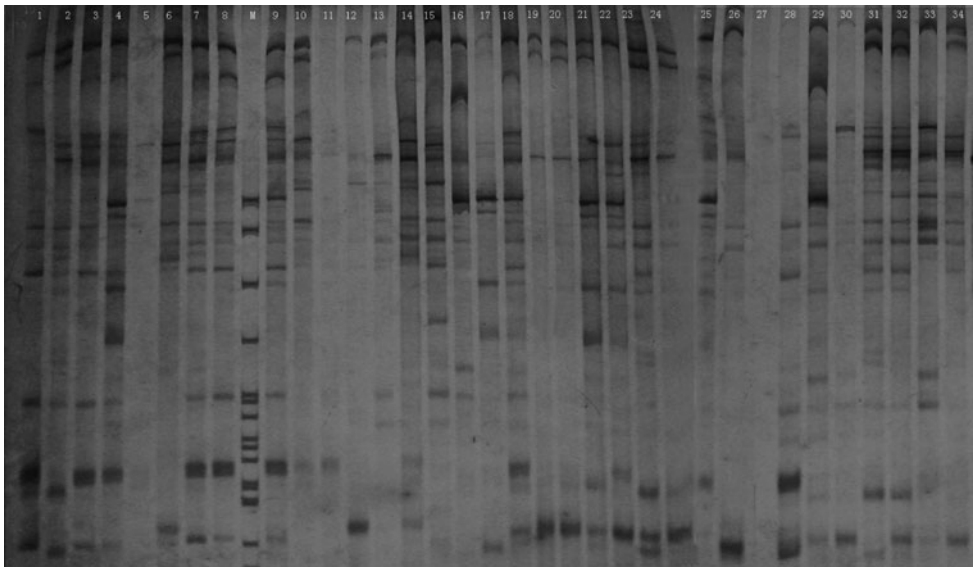

Fig. 4 Representative SRAP amplification profile generated using the primer combination me1 + em8

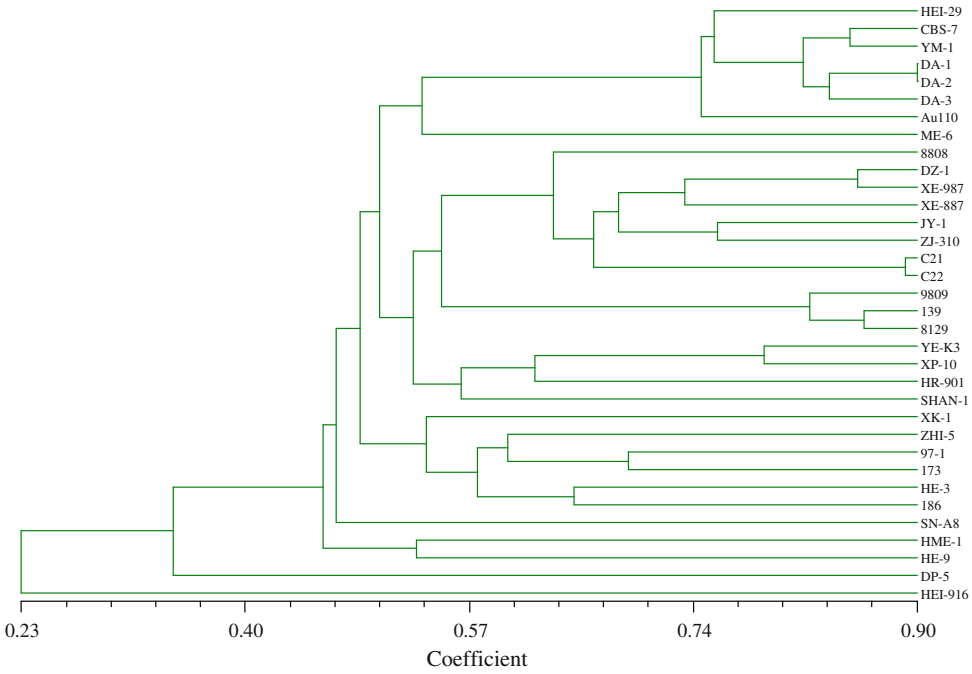

Fig. 5 UPGMA dendrogram of 34 *A. auricula* strains constructed using genetic similarity analysis based on molecular profiles revealed by SRAP markers

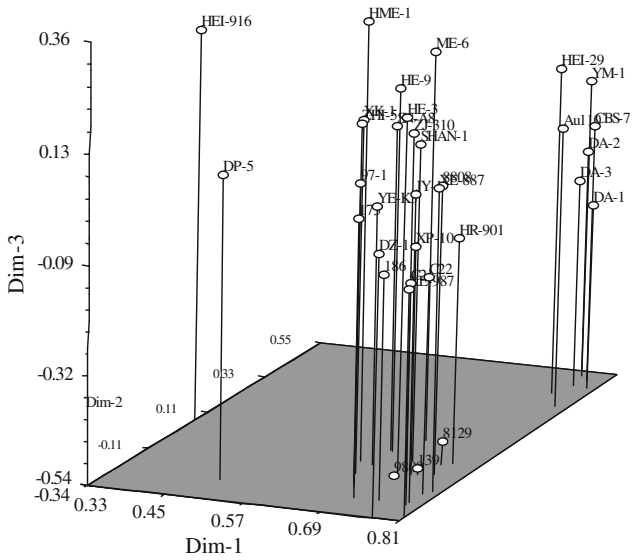

Fig. 6 Relationships among the 34 genotypes of *A. auricula* visualized by Principal Coordinate Analysis (PCO) of SRAP data

and XK-1 were grouped together in cluster II, whereas clusters III and IV consisted of genotypes HME-1 and HEI-916, respectively. Groupings identified by UPGMA analysis were confirmed by PCO data which also revealed that strains HME-1 and HEI-916 were genetically very distinct from the other genotypes (Fig. 8). The three most informative PCO components accounted for 63.0% of the variation observed. The correlation between ISSR and SRAP dendrograms was low ($r = 0.52$), compared with the markedly higher correlations recorded between ISSR and ISSR + SRAP dendrograms ($r = 0.86$), and between SRAP and ISSR + SRAP dendrograms ($r = 0.88$) ($P < 0.05$).

Discussion

In common with data obtained for other fungi, including *Beauveria bassiana* [22] and *Ganoderma* species [23], ISSR and SRAP methodologies were highly effective in demonstrating genetic diversity among the 34 cultivated strains of *A. auricula* examined in this study. Although the percentage polymorphism based on SRAP markers was marginally lower compared to ISSR markers (96.9 and 96.1%, respectively), the number of polymorphic bands generated by an individual SRAP primer pair and the total number of polymorphic bands generated by all the 11 SRAP primer pairs employed were both higher. Furthermore, maximum and minimum coefficients of genetic difference among the genotypes were both lower according to the SRAP data indicating that SRAP markers were more effective in revealing genetic diversity within *A. auricula* populations. SRAP methodology was similarly reported to be highly effective in revealing polymorphism and variance among 19 strains of *A. polytricha*, a closely related mushroom species [13], and in samples of buffalo grass germplasm [24].

The correlation between ISSR and SRAP markers was low ($r = 0.52$), which is consistent with the observed differences in the ISSR- and SRAP-based groupings of the 34 *A. auricula* genotypes. ISSR and SRAP techniques target different parts of the genome. ISSR marker amplification

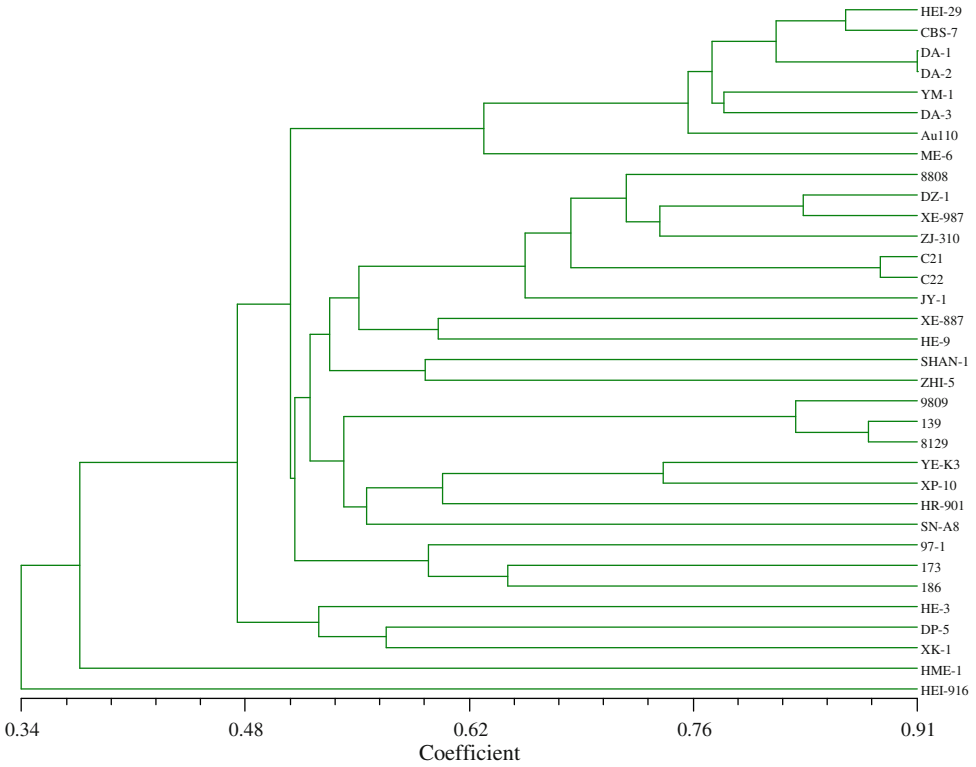

Fig. 7 UPGMA dendrograms of the 34 *A. auricula* strains constructed using genetic similarity analysis based on molecular profiles revealed by combined ISSR/SRAP markers

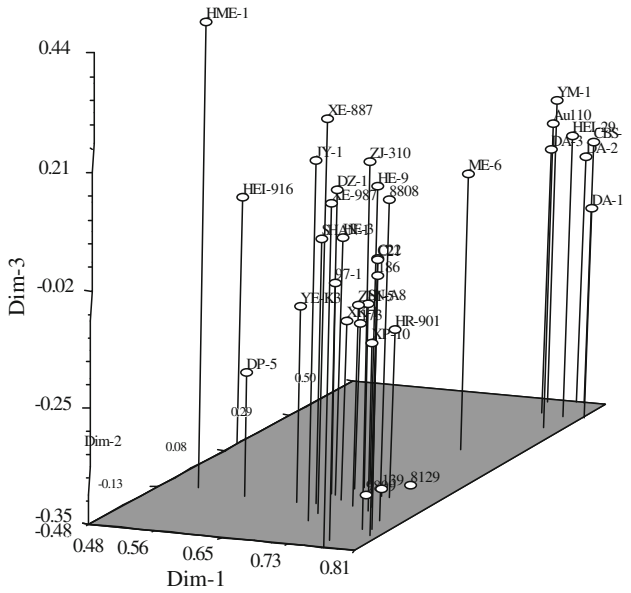

Fig. 8 Relationships among the 34 genotypes of *A. auricula* visualized by Principal Coordinate Analysis (PCO) of combine ISSR/SRAP data

targets are located in the region between simple sequence repeats (SSRs) whereas microsatellite DNA is not a general transcription region. In the case of SRAP, the Open Reading Frame (ORF), including the intron(s) and promoter region, is the amplification target and therefore better reflects the diversity of the genotypes studied.

High correlations were evident between ISSR + SRAP and ISSR dendrograms, and between ISSR + SRAP and SRAP dendrograms, demonstrating that the information revealed by the two sets of markers had not been lost after integration. The correlation coefficient between ISSR + SRAP and SRAP was higher than the value recorded for ISSR + SRAP and ISSR, which is consistent with the clustering data. However, compared with the clustering results based solely on ISSR or SRAP markers, combined ISSR + SRAP data more closely related to cultivation area as well as morphological and agronomic characteristics. Therefore, integration of ISSR and SRAP markers derived from different amplification regions is more effective in detecting genomic variation and in realizing a more complete analysis of resource diversity.

ISSR, SRAP and ISSR + SRAP dendrograms of the 34 test strains generally exhibited highly similar clustering patterns. For example, strains HEI-29, CBS-7, DA-1, DA-2, YM-1 and DA-3 from the Changbai mountain area in Northeast China clustered together in each case, as did strains 8808, DZ-1, XE-987, ZJ-310, C21, C22 and JY-1 from North China. However, small differences were observed, for example in the case of the major cultivated strains (139, 8129, YE-K3 and XP-10) from the Shennongjia mountain area of central China, where the clustering patterns based on SRAP and combined ISSR-SRAP analyses were slightly different from the pattern generated using ISSR markers alone. However, based on the above

analysis, strain 9809 from Changbai clustered together with the relevant strains from Shennongjia. Strain 9809 is widely cultivated in both of these areas, but its actual origin is uncertain.

In summary, our data showed that the 34 *A. auricula* strains cultivated in China are related to some degree according to regional distribution. Most strains originating from same area, for example Northeast China or Central China, clustered together at a high similarity level, possibly indicating that these strains, with a narrow genetic basis, had been domesticated from local wild-type strains. In fact, most cultivated *A. auricula* strains were originally derived from the domestication of wild-type strains growing in China. Following selection over an extended period, the main strains grown in the Changbaishan and Shennongjia mountain areas (the two major cultivation regions) showed considerable variation due to prevailing differences in climate, cultivation methods and cultivation seasons. *A. auricula* cultivated on sawdust logs in the Changbaishan region fruit during Spring and produce thick, brownish black or brown, and slightly wrinkled fruit bodies (lugs) with a hard and crispy texture. Conversely, *A. auricula* cultivated on wood logs in the Shennongjia mountain region fruit in Spring and Autumn and produce thin, tan or canary yellow, chrysanthemum-shaped or slightly wrinkled fruit bodies with a soft and smooth. In North and Southeast China, there is a shorter history of *A. auricula* cultivation following introduction of the cultivation technology and strains from the Changbaishan and Shennongjia regions. According to the clustering analysis, strains from different areas show high similarity coefficients, indicating frequent strain introduction. Also, there is a high probability that strains obtained from the same area and which have high similarity coefficients, including Hei29 and Changbeishan7, Dong A-1 and Dong A-2, YanerK3 and Xueping10, 139 and 8129, and C21 and C22, are actually synonyms even though they have been assigned different designations. According to information relating to strain origins, DA-2 and C22 were, respectively, derived from DA-1 and C21 through tissue isolation and screening.

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