COMPARATIVE ANALYSIS ON THE DIVERSITY OF *AURICULARIA AURICULA-JUDAE* BY PHYSIOLOGICAL CHARACTERISTICS AND TRAP FINGERPRINTING

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ABSTRACT

Phenotypic traits (physiological characteristics) and genotypic traits (target region amplification polymorphism) were used to study the diversity of 32 main cultivars of *Auricularia auricula-judae* in China. 27 important and stable physiological indexes were evaluated; 16 pairs of TRAP primer combinations produced 535 unambiguous and reproducible DNA fragments, of these 524 (97.9%) were polymorphic. Dendrograms were constructed by Unweighted Pair-group Method with Arithmetic Averages (UPGMA) method, and the principal coordinate analysis (PCO) of the two methods exhibited similar clustered patterns, revealing that all the tested strains could be divided into three distinct groups, each of which was correlated with different 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, some strains were suspected to be synonymous. The grouping information obtained in the present work provides significant information for further genetic improvement in *A. auricula-judae*.

Keywords: *Auricularia auricula-judae*, Physiological Characteristics, TRAP, Genotypic diversity, Genetic Diversity

INTRODUCTION

Auricularia auricula-judae (Bull.) Quel. which is also known as wood ear, was subjected to Basidiomycota, Agaricomycetes, Incertae sedis, Auriculariales, Auriculariaceae, *Auricularia* [1]. *A. auricula-judae* is wildly spread in Asia, Europe and Africa, but particularly cultivated in China. Besides its nutritional attributes, *A. auricula-judae* has been used as medicine to treat angina, diarrhea, gastrointestinal and hemorrhoids upsets [2] for many centuries in China and other parts of Asia. In recent years, *A. auricula-judae* has been reported to have the functions of lowering blood cholesterol and triglycerides [3], preventing strokes and heart attacks [4], and effective in treating diabetes [5] and certain cancers [6]. It also exhibits antioxidant activities [7]. Therefore, *A. auricula-judae* enjoys popular favor in Europe, America, Japan, Korea, Malaysia, Indonesia and Thailand. At present, it is the fourth among the industrially cultivated edible fungi in the world. The officially issued statistical data reported that the estimated production of *A.*

auricula-judae in China reached about 1.905 million tons and the export reached about 7.6 million dollars in 2008.

China has abundant cultivated germplasm resources of *A. auricula-judae*. However, a major problem currently facing culativars is that identical *A. auricula-judae* cultivars are frequent introduced into different designations and randomly labeled for commercial purposes. Incorrectly designating strains may lead to negative impacts on breeding, and even cause conflicts in the protection of intellectual property rights. What is more serious is that cultivars could suffer great economic loss from the improper introduction of the strains. Therefore, it will be contributory to systematically study the main cultivar diversity of *A. auricula-judae*.

In recent years, comprehensive studies have focused on combining phenotypic traits with genotypic traits to obtain more reliable results, which have been used frequently for analyzing the diversity of cultivated germplasm sources in plants, such as in pea, wheat, common bean, almond, tomato and *Zinnia elegans* [8-12], but rarely on the cultivated germplasm of edible fungi, except *Pleurotus eryngii*, *Pleurotus ferulae* and *Agaricus subfloccosus* [13-15].

Physiological characteristics analysis is a traditional method to analyze phenotypic diversity, and has been proved to be helpful to achieve reliable results under well-controlled environmental conditions, by reliable measurement methods, and with sufficient repetition. TRAP is a fairly new marker technique which is improved on the basis of sequence-related amplified polymorphism (SRAP). It uses a fixed primer of about 18 nucleotides, which is designed from the target-expressed sequence tag (EST) or gene sequence, as well as an arbitrary primer of about the same length, with either an AT or GC-rich core to anneal with an intron or exon, respectively [16]. As a simple yet powerful technique for estimating genetic diversity, TRAP has been successfully used for sugarcane, sunflower, *Porphyra*, *Pelargonium*, *lettuce*, and *Spinacia oleracea* germplasm diversity [17-22]. Although TRAP has been introduced into fungi and proved to be reliable for analyzing genetic diversity of *Lentinula edodes* in China [23], it was for the first time adopted in the present study for analyzing the diversity of *A. auricula-judae*.

The present study aimed to survey the phenotypic and genotypic diversity of 32 main cultivars of *A. auricula-judae* in China. The study was performed in phenotypic traits (physiological characteristics) and genotypic traits (TRAP molecular marker), the usefulness and reliability of the two methods were also compared.

MATERIALS AND METHODS

Mushroom strains. Thirty-two main cultivars were collected from local professional research institutes in different geographical regions of China; and maintained at the Institute for Edible Fungi, Shanghai Academy of Agricultural Sciences. All strains were divided into five populations according to different floristic regions (Table 1).

Physiological characteristics. All strains were incubated on CYM (complete yeast media) for 7 d at 25 ^{o}C and then cultivated by wood-log method in Suixian, Hubei Province to generate fruiting bodies. Table 2 shows the detailed evaluation list of 27 stable and important physiological characteristics indexes, each index was repeated 10 times.

Table 1: Designation, source, and floristic regions of *A. auricula* tested strains

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

[1]MIHL, Heilongjiang Microbiological Institute; JAU, Jilin Agricultural University; DCH, Dongning County, Heilongjiang Province; HAU, Huazhong Agricultural University; 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; 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

TRAP analysis. DNA extraction was conducted by following the method of Tang et al. [24].Sixteen fixed primers were derived from nucleotide sequences of the *Auricularia* genus in Genbank and designed by the web-derived software "Primer 3" (http://frodo.wi.mit.edu/primer3/) (Table 3). The main design parameters were as follows: primer optimum size, maximum size, and minimum size were all set to 18 nucleotides; primer optimum Tm, maximum Tm, and minimum Tm were set to 53 ^{o}C, 55 ^{o}C, and 50 ^{o}C respectively. Sequences of the arbitrary primers with an AT or GC-core sequence were selected from SRAP primers of Tang et al. [24].

PCR amplification of each pair primer was repeated 3 times and then analyzed in 6% denaturing polyacrylamide gel as described by Xiao et al [23]. The gels were directly analyzed to well record the genotypes of the tested strains instead of being analyzed on the photographs. To ensure the reproducibility and reliability, the bands with low intensity were excluded, and the scored polymorphic bands were rescored manually for several times. DNA bands were scored with "1" for presence, and "0" for absence in each genotype.

Table 2: 27 evaluation index of physiological characters

No	Physiological Characteristics	Evaluation Method
1	hyphal density degree under 25 °C	sparse=1/ medium=2/ dense=3
2	mycelium growth rate under 25 °C	≤0.3 slow=1 / 0.3-0.5 medium =2/≥0.5 fast =3
3	mycelium endurance to 40 °C	absent=0/present =1
4	optimum temperature for mycelium growth	24 °C =1/26 °C =2/ 28 °C =3
5	optimum pH for mycelium growth	pH6=1/pH7=2
6	optimum moisture for mycelium growth	55%=1/ 60 %=2/ 65%=3/ 70%=4
7	mycelium assimilation of nutriment	≤ 22 slow=1/22-24 medium=2/≥ 24 fast=3
8	time from inoculation to harvest in log cultivation	≤ 75d short=1/75-90d medium =2/ ≥ 90d long =3
9	occurrence status of fruiting body	≥ 70% tufted =1/ tufted or solitary =2/ ≥ 70% solitary =3
10	shape model of fruiting body	cupped =1/ flaky=2/earlobe=3/ chrysanthemum=4
11	edge model of fruiting body	flat=1/curved=2/nicks=3
12	ventral color of fresh fruiting body	cinnamon=1/brown=2/ brownish black =3
13	ventral color of dried fruiting body	brown=1/ brownish black =2
14	reverse color of fresh fruiting body	cinnamon=1/dust color=2/brown=3
15	reverse color of dried fruiting body	gray=1/ dust color=2/ brown=3
16	wrinkle number of fresh fruiting body	0 absent=1/1-3 light=2/ ≥ 4 heavy =3
17	wrinkle depth of fresh fruiting body	≤ 100 shallow=1/100-300 medium =2/≥ 300 deep =3
18	length of fresh fruiting body	≤ 45.0 short=1/45.0-50.0 medium=2/≥ 50.0 long=3
19	width of fresh fruiting body	≤ 65.0 narrow=1/65.0-80.0 medium=2/≥ 80.0 wide=3
20	longitude to width ratio of fresh fruiting body	≤ 0.6 small=1/0.6-0.8 medium=2/≥ 0.8 large=3
21	root size of fresh fruiting body	≤ 3.0 small=1/3.0-5.0 medium=2/≥ 5.0 large=3
22	back-scrolling degree	absent=1/medium =2/obvious=3
23	thickness of fresh fruiting body	≤ 1.0 thin=1/1.0-2.0 medium=2/≥ 2.0 thick=3
24	The quality of fresh fruiting body	soft=1/medium=2/hard=3
25	single weight of fresh fruiting body	≤ 5.0 low=1/5.0-8.0 medium=2/≥ 8.0 high=3
26	ratio of dry weight to fresh weight	≥ 1/10 low=1; 1/10-1/15 medium=2; ≤ 1/15 high=3
27	dried weight of harvested fruiting body per 100kg logs	≤ 1.5 low=1/1.5-2.5 medium=2/≥ 2.5 high =3

Data analyses. For phenotypic traits data, the original matrices based on physiological characteristic indices were standardized by the STAND option, and then constructed into the Euclidean distance matrices by using the SIMINT option [25], while for genotypic traits data, the SIMQUAL option was applied to calculate the simple matching (SM) coefficient and the pairwise genetic similarity (GS) matrix [26]. The construction of Unweighted Pair-group Method with Arithmetic Averaging (UPGMA) dendrograms and Principal Coordinate Analyses (PCO) were carried out by following Tang et al. [24].

Data analyses were performed using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc), version 2.10 (Exeter Software, Setauket, New York) software package [26].

Table 3: 16 pairs primers combinations used in TRAP

Primer name	GeneBank accession no.	Primer sequence		Number of loci	Percentage of polymorphism
		Fix-primer	Arbitrary[a]		
Ras1-L	GQ244321	TCGGAAATACCAGAGGCAGC	EM8	33	100%
Ras2-L	GQ244319	ATCCTGACATTGCAGCCACA	EM8	24	100%
Ras3-L	GQ244318	GGTGGTAAAGTGGAAGCCC	EM5	16	100%
Ste1-L	FJ756942	GGCATGAGAGCACCCACATA	EM5	41	100%
Ste2-L	FJ756941	CCTGGAACTCGGGTAAGTGG	EM5	37	100%
Ste3-L	DQ303127	TCTGAGCCTCGTCTGTCTCA	EM5	38	100%
Fks-L	AY254574	TACGCCCGGAATCAAGAGTG	EM5	25	100%
Lac1-L	AY450405	TCCGAAGTGGATCCAGACCT	EM1	28	100%
Lac2-L	AY450405	CACTTGAGGGTCACGCAAAC	EM8	70	94.59%
Mul-L	AY485828	GAGCAACTTGTCCAGCCAAC	EM8	29	100%
Lac-L	AY616035	GGAACGTACTGGGTCCACTC	EM1	41	95.34%
Cla-L	AY225999	GAGCACAATCAGCTCGAGGA	EM8	27	100%
Ste1-R	FJ756942	GCAGAGGACACAGAGGATGG	ME2	25	92.59%
Ste3-R	DQ303127	AAGGATGGATGAGCTCGCAG	ME2	37	94.87%
Fks-R	AY254574	ATGTTCGCCCATACCAGACC	ME2	26	100%
Lac1-R	AY450405	AGTACGGTCGTCTCGAGGAT	ME3	28	100%

[a] detail information cited from the paper of Tang et al. (2010)

RESULTS AND DISCUSSION

Diversity analysis based on physiological characteristics. The physiological characteristics were found correlated with geographical region where the strains are originated from. The strains from the same regions always had more similar physiological traits, while more divergence was found between those from different regions. The mycelium morphology of majority strains (such as DA-2) from the Northeast grew densely and had the fastest nutriment assimilation; the fruiting body was thick, solitary, cupped or earlobe-shaped in brownish black or brown, with a flat edge and obviously wrinkled with a hard and crispy texture (Fig.1 a-c). In contrast, the mycelium morphology of majority strains (such as DP-5) cultivated in the Southeast region grew sparsely and had the slowest mycelium nutriment assimilation; the fruiting body was thin, solitary or tufted, earlobe-shaped in cinnamon, curved or nicked on its edge, and slightly wrinkled with a soft and smooth texture (Fig.1 d-f). In addition, the mycelium morphology of majority strains (such as XE-887) cultivated in the North region grew densely and had the middle mycelium nutriment assimilation; the fruiting body was moderately thick, solitary or tufted, earlobe-shaped or flaky in brown, curved on its edge, and moderately wrinkled with a moderate hard and smooth texture (Fig.1 g-i).

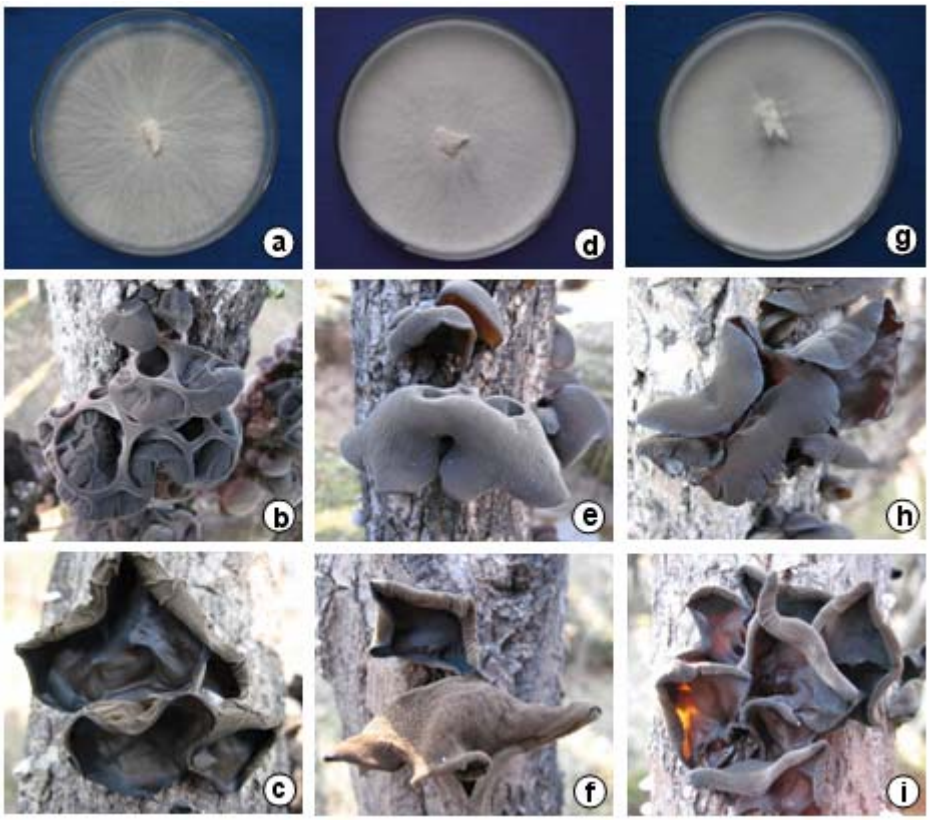

Figure 1: The mycelium and morphology characters of the DA-2 (a-c), DP-5 (d-f) and XE-887 (g-i) strains. Strains DA-2, DP-5 and XE-887 originated from Northeast, Southeast and North region respectively.

Euclidean distance similarity coefficients among the 32 tested strains was ranged from 4.27 (XK-1 and DP-5) up to 11.33 (C21 and DP-5); the co-phenetic correlation between clustering and the data matrix was estimated at 0.77, corresponding to a good fit. UPGMA dendrogram grouped all strains into six main clusters at a Euclidean distance index value of 6.78 (Fig. 2). Cluster II, III and IV comprised of 7, 12 and 10 strains respectively, while Cluster I, V and VI respectively contained a single strain. Partial strains demonstrated higher similarity coefficients and clustered into sub-clusters, such as DA-1 and DA-3, 97-1 and SHAN-1, 9809 and 139, DP-5 and XK-1, XE-987 and XE-887, XP-10 and YE-K3, and ME-6 and ZHI-5.

PCO was performed to more directly visualize the association among accessions (Fig.3). It showed that the three most principal coordinates explained 45.18% of the total variation. 32 tested strains were divided into 3 groups while Group I contained 7 strains, Group II contained 14 strains and the rest 11 strains were in Group III. The results of PCO analysis closely corresponded to those obtained through UPGMA cluster.

Because of the relatively simple structure of the *A. auricula-judae* fruiting body, the use of morphology characteristics to analyze the germplasm diversity was naturally limited. In our research, 27 relatively stable and highly heritable physiological characteristics were chosen for the first time to explore the phenotypic diversity of *A. auricula-judae* (Table 2), and to minimize subjective error and ensure the accuracy of analyses. The physiological characteristics comparison proved that the strains in the same group had more similar physiological traits than in different clusters, and this result could be the useful reference for breeding.

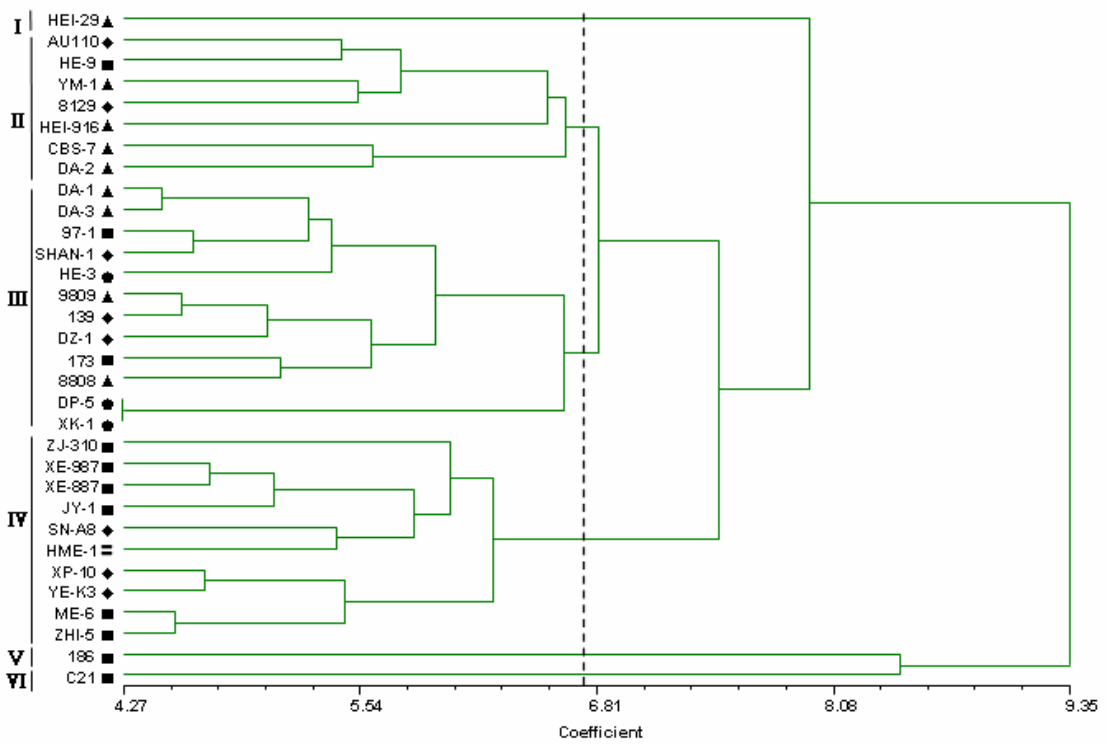

Figure 2: Dendrogram based on the physiological characteristics cluster analysis of *A.auricula-judae*. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

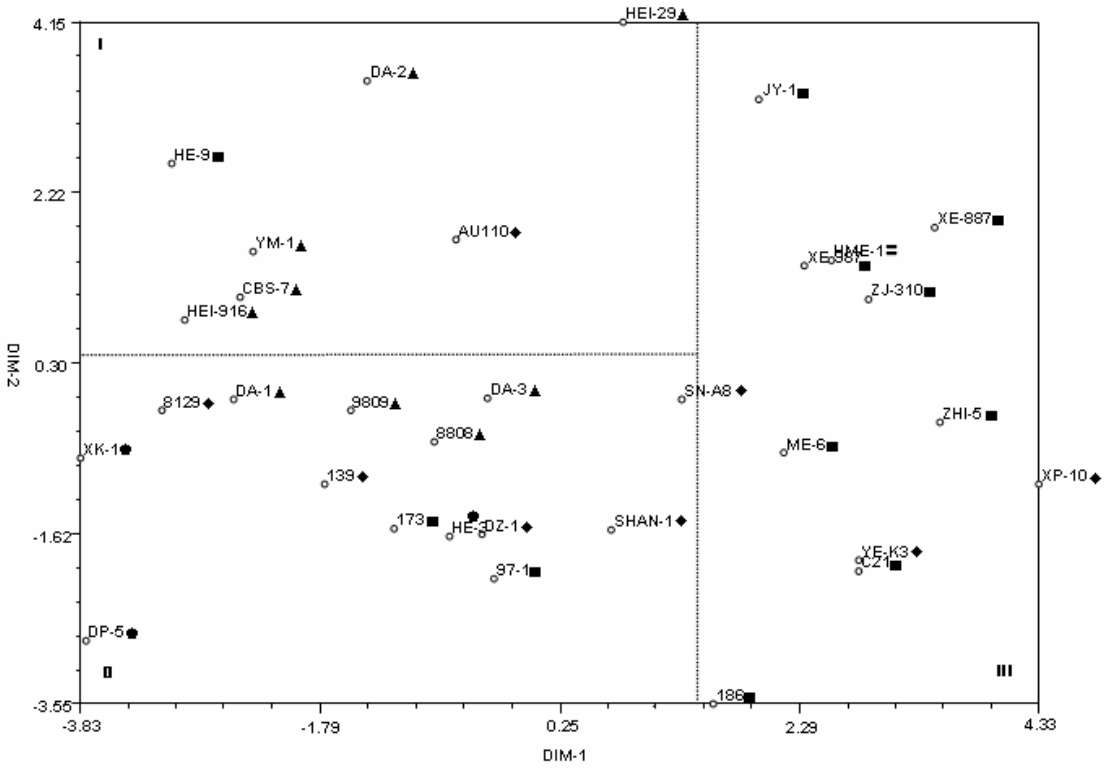

Figure 3: Relationships visualized through the PCO of physiological characteristics data. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

Diversity analysis based on TRAP data. A total of 535 unambiguous and reproducible DNA fragments, of which 524 (97.9%) were polymorphic, were scored from the 32 genotypes, using the 16 TRAP primer pairs listed in Table 3. Most amplified fragments were ranged in different sizes from 100 to 1000 bp, and the number of fragments detected by each primer combination were ranged from 16 to 70, with an average of 34 (Table 3).

Genetic similarities by SM coefficients among the 32 genotypes varied from 0.567 (8808 and SHAN-1) to 0.922 (139 and 8129). The co-phenetic correlation between the clustering and the data matrices was estimated at 0.92, corresponding to a very good fit. UPGMA dendrogram grouped the 32 genotypes into four main clusters at a similarity index value of 0.67 (Fig. 4). Cluster II consisted of 14 genotypes and was further divided into 2 sub-clusters at a similarity index value of 0.70; Cluster I and III each comprised of 7 and 10 genotypes, while Cluster IV composed of a single strain SHAN-1. Several strains demonstrated higher similarity coefficients and were further delineated into sub-clusters, such as AU110 and ME-6, 139, 8129 and 9809, DP-5 and XK-1, and C21 and XE-987.

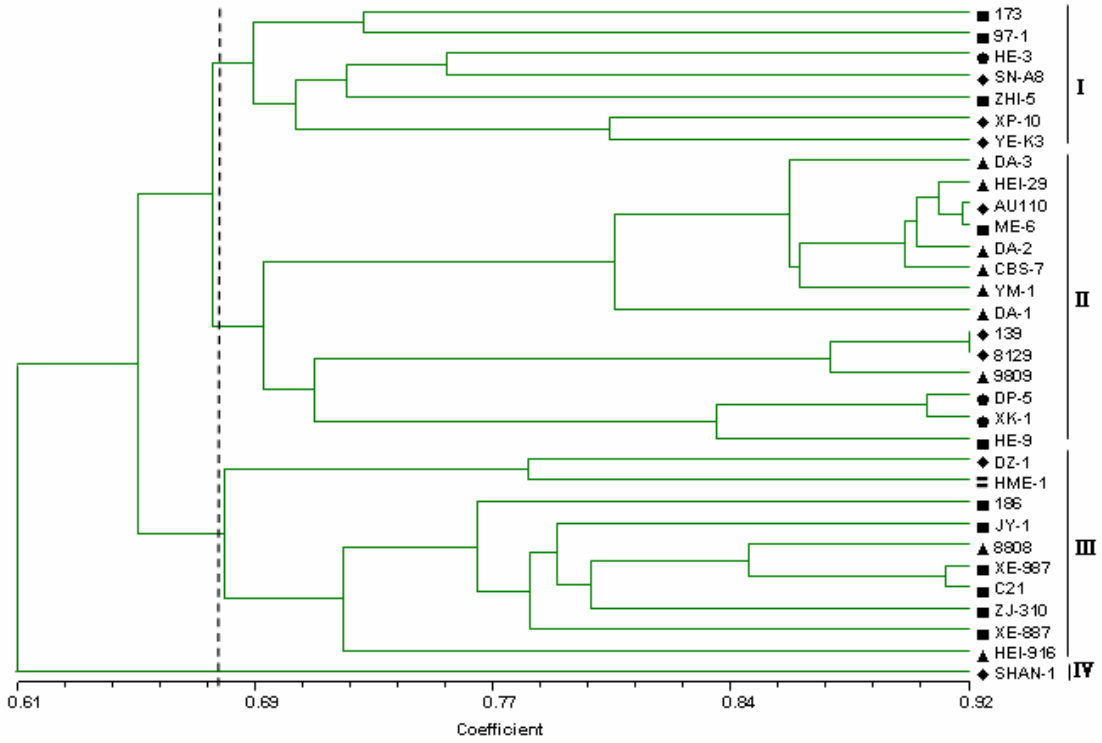

Figure 4: Dendrogram based on TRAP fingerprinting cluster analysis of *A. auricula-judae*. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

Groupings identified by UPGMA analysis were confirmed by PCO data in Figure 5. The three most principal coordinates accounted for 35.40 % of the total variation. Similar to cluster analysis, the PCO result showed that the 32 strains were distinctly divided into 3 groups. Group I contained 8 strains, Group II contained 14 strains and the remaining 10 strains were in Group III.

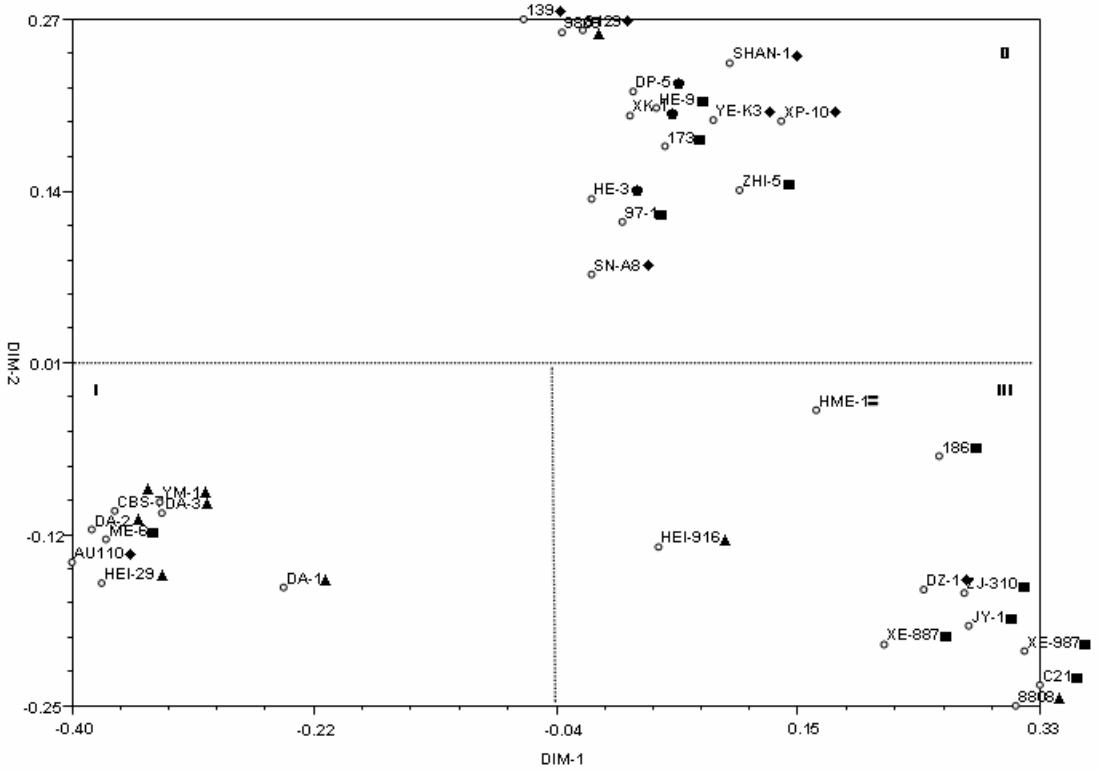

Figure 5: Relationships visualized through the PCO of TRAP date. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

Inter-simple sequence repeats (ISSR) and SRAP had been used to analyze the similar tested strains in our previous study [24], in which ISSR and SRAP generated 129 and 154 polymorphic bands of 34 strains by all 13 ISSR primers and 11 SRAP primer pairs. The percentage of polymorphism of ISSR and SRAP were 96.9% and 96.1%, and the related co-phenetic correlation was 0.85 and 0.90. Compared with ISSR and SRAP, TRAP had a much higher value for evaluating genetic diversity of *A. auricula-judae*. Its advantages presented not only in the total amplified DNA fragments (535 bands), but also in the percentage of polymorphic fragments (97.9%). Although the strains of the Central region have been divided into 2 groups in the clustering, PCO analysis revealed that all the tested strains could be distinctly divided into three groups. The value for estimating genetic diversity was represented by TRAP (0.92) > SRAP (0.90) > ISSR (0.85) in *A. auricula-judae*, which is similar with vegetative biotype buffalograss [27] and *Lentinula edodes* [23].

The comparison and combined diversity analysis based on two methods. The co-phenetic correlation was represented by TRAP (0.92) > physiological characteristics (0.77). The combined PCO analysis of the two methods exhibited similar clustered patterns and revealed that all the tested strains could be divided into three groups: Group I, Group II and Group III, which contained the majority strains originated from the Northeast, the Southeast and the Central, and the North and South regions, respectively.

High correlations between physiological characteristics and molecular marker have been proven in common bean, and Tunisian winter barley [10, 28]. The present study compared the

usefulness and reliability of the two methods, and suggests that to accurately estimate the diversity solely, TRAP demonstrated the higher value in *A. auricula-judae*. Genetic traits analysis could provide more diverse information and help to obtain more reliable results than phenotypic traits. Therefore, in order to achieve a more reliable evaluation and robust characterization of the species diversity, this study suggested that phenotypic traits and genotypic traits should be analyzed and complemented with each other, which is meaningful for the further fungal diversity analyses.

On the other hand, PCO analysis largely corresponded to those obtained through cluster analysis, but could provide more effective and visible information than the UPGMA clustered dendrogram, which could be widely applied in analyzing the fungal diversity in the future.

CONCLUSIONS

In the present study, all the two analytical methods provided important information on phenotypic and genetic diversity of *A. auricula-judae* germplasm. By various methods, all the tested strains could be divided into three groups corresponding to the Northeast, the Southeast and the Central, as well as the North and South regions, respectively, which also proved that the result was reliable.

Most strains originated from the same area clustered together at a high similarity level, for example from Northeast China and North China, which may indicate that these strains were with a narrow genetic basis and could possibly be domesticated from the local wild-type strains. In contrast, the diversity in the Central regions of China was relatively greater due to the frequent introduction from broad scales. The correlations between the diversity and geographical data could further demonstrate the tendency, which was in accordance with our previous results based on ISSR and SRAP [24].

However, partial strains demonstrated higher similarity coefficients in all analysis methods and were suspected to be synonymous, strain doublets DP-5 and XK-1, XE-887 and XE-987 originated from the same region, probably were domesticated from the same fruiting body, but were labeled by different names; strain doublets such as 139 and 9809 originated from different regions could be probably caused by sharing strains in human communication. Herein, the phenotypic and genetic diversity of main cultivars in China were comparatively low, which indicates that the domestication of wild-type strains should attract more attention.

As is well known that, for the improvement of strains, selecting parents in hybridization is very important. Hybridization program involving phenotypic and genetic diverse belonging to different distant clusters will facilitate 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 the other countries.

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