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Assessment of genetic diversity among wild *Auricularia polytricha* populations in China using ISSR markers

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Abstract – The genetic diversity of *Auricularia polytricha* among 27 wild populations in China was analyzed by using inter-simple sequence repeat (ISSR) markers. At the species level, a total of 509 loci were amplified using eleven ISSR primers with the percentage of polymorphic loci (P) = 100%, Nei's gene diversity (H) = 0.1935, Shannon information index (I) = 0.3274, and total genetic diversity (H_t) = 0.1918. At the population level, P = 35.3%, H = 0.1277 and I = 0.1907 whereas diversity within populations (H_s) is 0.1135. A high degree of genetic differentiation (G_{st} = 0.4081) among populations was detected. This genetic structure is likely due to the combined effects of the limited gene flow (N_m = 0.7251), geographical isolation, and random genetic drift. Clustering analysis revealed that a remarkable geographical relatedness exists among strains within population, whereas no distinct geographical pattern occurs in the genetic variation among populations. Furthermore, clustering analysis did not suggest genetic variation to be correlated with the host association.

Basidiomycetes / Gene flow / Genetic distance / Genetic variation / Population genetics

INTRODUCTION

Auricularia polytricha (Mont.) Sacc. belongs to Auriculariaceae, Basidiomycota, and it is known as wood ear, or red ear (Yu *et al.*, 2008). It is distributed world-wide from the temperate to the tropical zone and being found on broad-leaved trees, ranging from decayed branches of living and dead trees to decayed stumps or logs (Imazeki & Hongo, 1965; Reichard *et al.*, 2005). The natural distribution of *A. polytricha* in China ranges from temperate to tropical climate zones, but it is more common in tropical China. *A. polytricha* and its closely related species *A. auricula-judae* (L.) Underw. are widely used in soups and dishes in oriental cuisine and the consumption of *A. polytricha* has increased year by year (Yu *et al.*, 2008, Tang *et al.*, 2010). Being rich in fibers, proteins,

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microelements, but low in fat content, species of *Auricularia* are medically used for the dietetic prevention of hyperlipidemia (Cheung, 1996), and it has been reported that *A. polytricha* has several medicinal effects, such as promoting blood circulation, treating hemorrhoids, analgesic, antitumor etc. (Liu, 1984; Ying *et al.*, 1987; Sheu *et al.*, 2004; Dai *et al.*, 2009). Although widely cultivated in China, the strains for cultivation of *A. polytricha* in China were isolated from limited samples (Du *et al.*, 2011a, 2011b)

Zietkiewicz *et al.* (1994) developed inter-simple sequence repeats (ISSR) markers by using of 3' or 5'-anchored primers to increase the stringency and reproducibility. So far, ISSR has been used in studies on fungal genetic diversity, population genetics, and strain identification (Kazuhisa *et al.*, 2002; Gryta *et al.*, 2006; Zhang *et al.*, 2007; Su *et al.*, 2008; Yu *et al.*, 2008; Fu *et al.*, 2010; Tang *et al.*, 2010). Efforts have been made to understand the genetic diversity of cultivated strains of *A. polytricha* by using random amplified polymorphic DNA (RAPD), sequence-related amplified polymorphism (SRAP), or ISSR markers (Zhang *et al.*, 2006, 2007; Yu *et al.*, 2008), but population level diversity, genetic structure, and relationships among wild *A. polytricha* in China were never fully investigated. The purpose of this study was to assess the levels of genetic variation and relationships among 27 wild populations of *A. polytricha* in China using ISSR markers, and to provide information for further study on cultivation and breeding programs.

MATERIALS AND METHODS

Strains and culture conditions: Wild strains of *A. polytricha* were isolated from different natural forests in China, that were at least 10 km away from farm land in order to avoid contamination with cultivated strains. In total, 145 wild strains of *A. polytricha* representing 27 populations in China (Fig. 1) were isolated from different hosts and geographical regions by tissue isolation (see online supplementary material, Tab. 1). All strains were grown on malt-extract dextrose agar at 25°C in the dark for 7 days and preserved at 4°C, and were deposited at the herbarium of the Institute of Microbiology, Beijing Forestry University (BJFC).

DNA extraction: For total genomic DNA extraction, mycelia were harvested for each strain grown in liquid medium, containing 25 ml malt extract dextrose broth and incubated at 26°C for 10 days. ZR Fungal/Bacterial DNA Kit™ was used to extract DNA. The concentration of DNA was estimated with Smart Spec™ Plus (Bio-rad, USA) and the quality of DNA was checked with 0.8% agarose gel. DNA samples were diluted to working solutions of 25 ng μL^{-1}, confirmed by measuring ultraviolet absorption using a Smart Spec™ Plus (Bio-rad, USA) visible spectrophotometer, and stored at –20°C until use.

ISSR reactions: ISSR reactions, thermal cycling parameters and agarose gel analysis were as previously described (Du *et al.*, 2010). Genotyping was performed with 11 ISSR primers (P1-P3, P10, P12, 808, 823, 826, 834, 841, 842) that were selected based on the results of initial screening of 40 ISSR primers (Du *et al.*, 2010), which were designed following the primer sequences of University of British Columbia (UBC, Zou *et al.*, 2001).

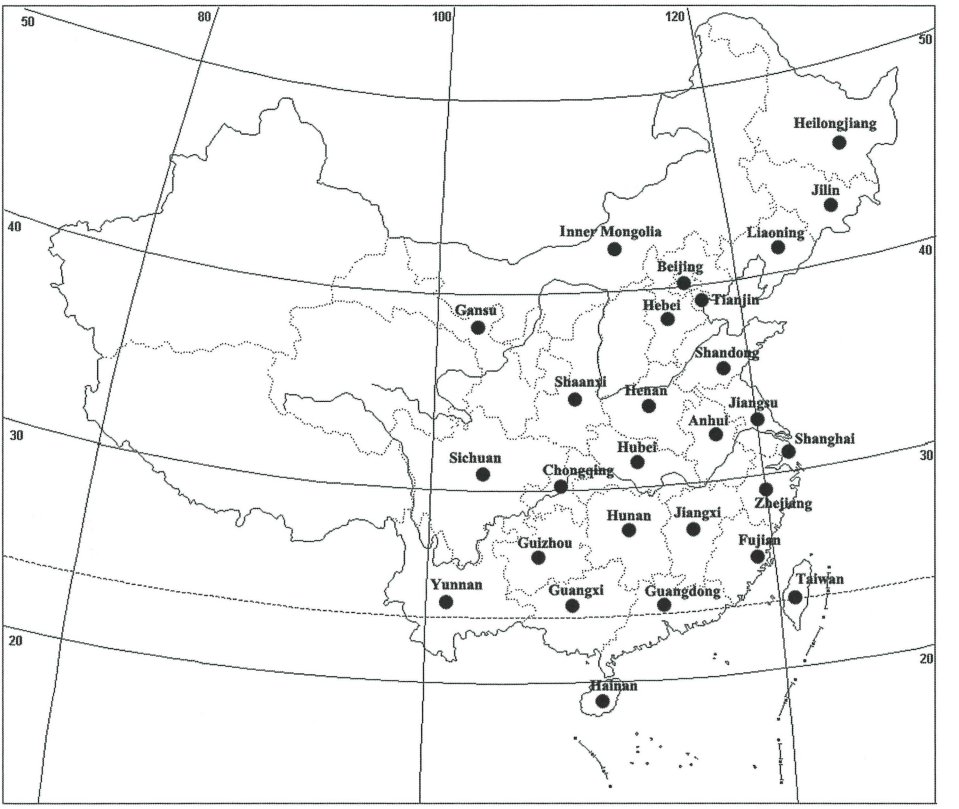

Fig. 1. Locations of the wild populations of *Auricularia polytricha* sampled in China.

Analytical methods: Bands were scored as present (1) or absent (0) and entered into a binomial matrix. ISSR fragment is frequently used without the need to make assumptions regarding Hardy-Weinberg equilibrium (Fontaine *et al.*, 2004). The percentage of polymorphic loci, Nei's gene diversity (H) (Nei, 1973), Shannon index (I) (Lewontin, 1972), observed number of alleles (N_a), effective number of alleles (N_e), total gene diversity (H_t), the genetic diversity of individuals relative to their subpopulation (H_s) and genetic differentiation relative to the total population (G_{st}) (Nei, 1987) were analyzed using POPGENE 32 (Yeh *et al.*, 2000). Gene flow estimates (N_m) were calculated as $N_m = 0.5(1-G_{st})/G_{st}$. An unbiased genetic distance (D) matrix (Nei, 1978) among populations was generated (POPGENE 32) and used to create neighbor-joining (NJ) trees (MEGA 3.1). Molecular variance (AMOVA) described by Excoffier *et al.* (1992) was analyzed by using ARLEQUIN 3.1 (Excoffier *et al.*, 2005). Finally, a principal coordinate analysis (PCoA) was performed based on the genetic similarity matrix of the tested strains using NTSYS pc version 2.10e EIGEN programs (Rohlf, 2000).

RESULTS

Population genetic diversity: 11 selected primers generated a total of 509 loci at an average of 46 loci per primer (Tab. 2), with sizes ranging from 100 to 2000 bp, and few bands were more than 2000 bp. At the species level, the percentage of polymorphic loci (P) was 100%, estimates observed number of alleles (N_a) = 2.0000, the effective number of alleles (Ne) = 1.2705, Nei's gene diversity (H) = 0.1935, Shannon information index (I) = 0.3274, and total genetic diversity (H_t) = 0.1918. At the population level, P = 35.3%, N_a = 1.3533, Ne = 1.2177, H = 0.1277, I = 0.1907, and genetic diversity within populations (H_s) = 0.1135. The samples from Hainan exhibited the highest level of genetic diversity (P = 63.85, H = 0.1735, I = 0.2763), followed by Guangdong (P = 61.69%, H = 0.1687, I = 0.2686), Jiangxi

Table 2. Genetic diversity statistics for all loci within populations of *Auricularia polytricha* in China

Population	*Sample size*	*Polymorphic loci No.*	*Percentage of polymorphic loci (P)*	*Nei's diversity (H)*	*Shannon index (I)*
Anhui	3	128	25.15	0.1118 ± 0.1930	0.1601 ± 0.2764
Beijing	5	178	34.97	0.1336 ± 0.1881	0.1984 ± 0.2753
Chongqing	4	142	27.9	0.1139 ± 0.1857	0.1666 ± 0.2699
Fujian	3	140	27.5	0.1222 ± 0.1987	0.1751 ± 0.2845
Gansu	2	88	17.29	0.0864 ± 0.1893	0.1198 ± 0.2624
Guangdong	13	314	61.69	0.1687 ± 0.1634	0.2686 ± 0.2408
Guangxi	3	133	26.13	0.1161 ± 0.1955	0.1663 ± 0.2799
Guizhou	1	–	–	–	–
Hainan	15	325	63.85	0.1735 ± 0.1631	0.2763 ± 0.2397
Hebei	3	102	20.04	0.0891 ± 0.1781	0.1276 ± 0.2550
Henan	6	205	40.28	0.1411 ± 0.1817	0.2138 ± 0.2685
Heilongjiang	1	–	–	–	–
Hubei	6	178	34.97	0.1305 ± 0.1869	0.1945 ± 0.2726
Hunan	2	98	19.25	0.0963 ± 0.1973	0.1335 ± 0.2736
Inner Mongolia	1	–	–	–	–
Jilin	2	76	14.93	0.0747 ± 0.1784	0.1035 ± 0.2473
Jiangsu	7	218	42.83	0.1427 ± 0.1773	0.2186 ± 0.2632
Jiangxi	11	273	53.63	0.1628 ± 0.1744	0.2535 ± 0.2571
Liaoning	5	181	35.56	0.1358 ± 0.1888	0.2017 ± 0.2764
Shandong	9	238	46.76	0.1457 ± 0.1745	0.2258 ± 0.2580
Shaanxi	3	141	27.7	0.1231 ± 0.1991	0.1763 ± 0.2851
Shanghai	4	163	32.02	0.1302 ± 0.1923	0.1906 ± 0.2798
Sichuan	8	236	46.37	0.1550 ± 0.1830	0.2366 ± 0.2687
Taiwan	8	234	45.97	0.1533 ± 0.1816	0.2343 ± 0.2675
Tianjin	3	128	25.15	0.1118 ± 0.1930	0.1601 ± 0.2764
Yunnan	15	302	59.33	0.1541 ± 0.1598	0.2477 ± 0.2364
Zhejiang	2	95	18.66	0.0933 ± 0.1950	0.1294 ± 0.2703
Mean	5.4	179.8	35.3	0.1277 ± 0.1840	0.1907 ± 0.2660
Total	145	509	100	0.1935 ± 0.1202	0.3274 ± 0.1654

Table 3. Analysis of molecular variance (AMOVA) for 145 *Auricularia polytricha* samples from 27 populations

Source of variation	*Degrees of freedom*	*Sum of squares*	*Variance components*	*Percent of total variance*	*P-value**
Among populations	26	1962.595	6.014770 Va	12.05	0
Within populations	118	5179.598	43.89490 Vb	87.95	0
Total	144	7142.193	49.90967		

**P* value was calculated by a permutation procedure based on 1023 replicates.

(P = 53.63%, H = 0.1628, I = 0.2535) and Yunnan (P = 59.33%, H = 0.1541, I = 0.2477). While the least genetic diversity was found in the samples of Jilin, Gansu, Hebei, Zhejiang and Hunan, in which Nei's gene diversity (H) was less than 0.1. Only a single strain was collected from the Guizhou, Heilongjiang and Inner Mongolia sites respectively and thus diversity indices could not be evaluated.

Population genetic differentiation: A high degree of genetic differentiation (G_{st}) = 0.4081 (POPGENE 32) was detected among populations, suggesting that 59.19% of variation was intra-population, but among populations also resided a remarkable genetic differences. The result was further confirmed by AMOVA analysis (Tab. 3), which showed that 87.95% of the variation occurred within populations. Similar phenomenon was also found in previous studies (Wang *et al.*, 2005; Huang *et al.*, 2009). The indirect estimate of gene flow based on genetic distance was lower (N_m = 0.7251) indicating that low levels of gene exchange occur among populations.

Analyses of genetic distances among the 27 populations sampled revealed that Hainan and Guangdong populations had the least genetic variation (D = 0.025; Tab. 4), whereas in Inner Mongolia and Heilongjiang populations (D = 0.195) the highest variability was incurred. The neighbor-joining dendrogram (Fig. 2) based on Nei's genetic distances (1978) showed that the populations can be classified

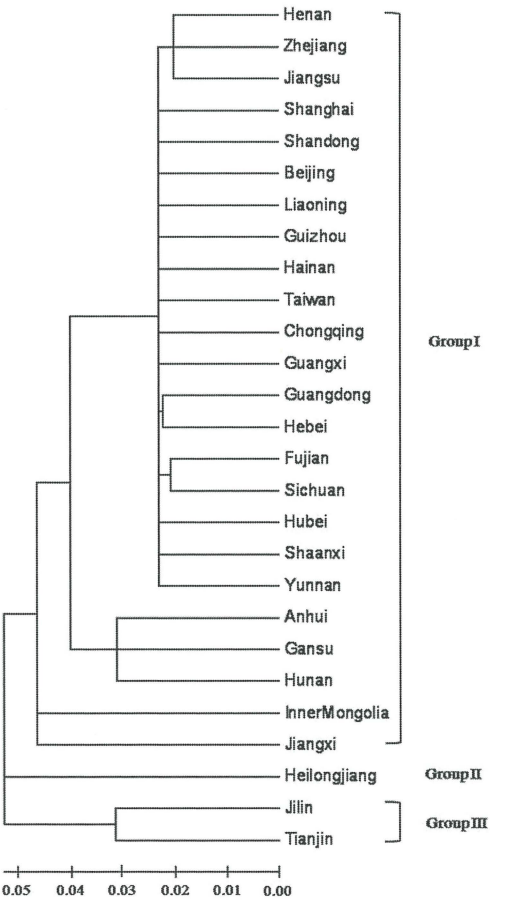

Fig. 2. Neighbor-joining dendrogram based on the genetic distances among 27 populations of *Auricularia polytricha.*

Table 4. Nei's (1978) original measures of genetic distance among 27 populations of *Auricularia polytricha*

Population	*An hui*	*Bei jing*	*Chong qing*	*Fu jian*	*Gan su*	*Guang dong*	*Guang xi*	*Gui zhou*	*Hai nan*	*He bei*	*He nan*	*Heilong jiang*	*Hu bei*	*Hu nan*	*InnerM onglia*	*Ji lin*	*Jiang su*	*Jiang xi*	*Liao ning*	*Shan dong*	*Shaan xi*	*Shang hai*	*Si chuan*	*Tai wan*	*Tian jin*	*Yun nan*
Anhui																										
Beijing	0.090																									
Chongqing	0.082	0.075																								
Fujian	0.078	0.087	0.081																							
Gansu	0.057	0.100	0.103	0.082																						
Guangdong	0.052	0.052	0.061	0.058	0.065																					
Guangxi	0.070	0.053	0.051	0.064	0.084	0.050																				
Guizhou	0.104	0.102	0.104	0.110	0.107	0.072	0.092																			
Hainan	0.047	0.048	0.053	0.051	0.062	0.025	0.047	0.069																		
Hebei	0.082	0.076	0.094	0.076	0.092	0.044	0.073	0.108	0.055																	
Henan	0.057	0.052	0.066	0.068	0.073	0.033	0.058	0.079	0.032	0.059																
Heilongjiang	0.136	0.155	0.154	0.148	0.145	0.123	0.135	0.171	0.118	0.157	0.136															
Hubei	0.063	0.070	0.085	0.060	0.076	0.043	0.066	0.087	0.045	0.067	0.056	0.153														
Hunan	0.068	0.099	0.109	0.102	0.073	0.080	0.094	0.124	0.063	0.089	0.076	0.157	0.088													
Inner Mongolia	0.139	0.141	0.142	0.137	0.148	0.120	0.140	0.180	0.119	0.144	0.123	0.195	0.148	0.157												
Jilin	0.093	0.095	0.109	0.087	0.101	0.075	0.093	0.128	0.068	0.105	0.082	0.133	0.093	0.106	0.147											
Jiangsu	0.073	0.053	0.072	0.064	0.079	0.039	0.055	0.085	0.029	0.053	0.028	0.142	0.059	0.080	0.133	0.085										
Jiangxi	0.058	0.052	0.062	0.055	0.064	0.034	0.043	0.082	0.027	0.036	0.037	0.120	0.052	0.066	0.102	0.072	0.040									
Liaoning	0.087	0.052	0.083	0.073	0.083	0.048	0.061	0.096	0.046	0.072	0.048	0.144	0.068	0.100	0.141	0.085	0.052	0.052								
Shandong	0.066	0.051	0.065	0.066	0.085	0.039	0.054	0.089	0.037	0.063	0.042	0.128	0.055	0.093	0.126	0.084	0.049	0.041	0.046							
Shaanxi	0.074	0.074	0.085	0.070	0.087	0.054	0.065	0.115	0.055	0.079	0.067	0.154	0.061	0.105	0.145	0.107	0.072	0.057	0.075	0.067						
Shanghai	0.067	0.064	0.075	0.072	0.065	0.046	0.066	0.089	0.035	0.061	0.041	0.121	0.062	0.063	0.133	0.84	0.047	0.042	0.064	0.043	0.077					
Sichuan	0.063	0.054	0.072	0.041	0.073	0.034	0.054	0.092	0.034	0.056	0.043	0.140	0.044	0.089	0.132	0.082	0.043	0.044	0.051	0.043	0.054	0.054				
Taiwan	0.064	0.052	0.055	0.055	0.065	0.032	0.045	0.083	0.027	0.060	0.049	0.127	0.055	0.081	0.129	0.084	0.046	0.040	0.054	0.048	0.063	0.050	0.043			
Tianjin	0.087	0.088	0.091	0.078	0.091	0.070	0.079	0.119	0.059	0.094	0.060	0.120	0.089	0.108	0.135	0.063	0.068	0.069	0.079	0.067	0.086	0.073	0.068	0.068		
Yunnan	0.052	0.056	0.057	0.048	0.061	0.031	0.048	0.077	0.028	0.050	0.038	0.118	0.044	0.069	0.110	0.064	0.038	0.030	0.043	0.033	0.057	0.046	0.035	0.038	0.057	
Zhejiang	0.081	0.084	0.097	0.090	0.090	0.067	0.087	0.120	0.056	0.091	0.041	0.156	0.076	0.092	0.144	0.097	0.052	0.065	0.079	0.065	0.097	0.052	0.071	0.078	0.090	0.061

into three groups. Group I is composed of 24 populations with close relationships, group II comprised exclusively the Heilongjiang population, and group III included the Jilin and Tianjin populations. Clustering analysis revealed that no distinct geographical relationship occurred in the genetic variation among populations.

DISCUSSION

In this study, the genetic diversity of wild populations of *Auricularia polytricha* in China was examined based on ISSR fingerprinting, and the tested strains covered a wide distribution ranging from the temperate to the tropical zone. The percentage of polymorphic loci was 100%, an average of 46 polymorphic loci were amplified per primer, as a whole the genetic polymorphism was slightly higher than that in previous ISSR analysis in Hainan, southern China (99.8, 41.6, Du *et al.*, 2011b). However, the genetic diversity level of *A. polytricha* in China was obviously lower than that in Hainan Province (H_t = 0.2346, H_s = 0.1532, Du *et al.*, 2011b).

The diversity at natural population level evidently was higher than that of cultivated strains examined earlier (Zhang *et al.*, 2006; Yu *et al.*, 2008) as also has been observed for *Agaricus bisporus* (J. E. Lange) Imbach and *Lentinula edodes* (Berk.) Singer (Loftus *et al.*, 1988; Kerrigan, 1990; Chiu *et al.*, 1996; Zhang *et al.*, 2007).

Generally, species with small geographic ranges tend to maintain less genetic diversity than geographically widespread species (Hamrick & Godt, 1989), and population genetic theory predicts that large populations tend to maintain higher allelic diversity (Hedrick, 1985; Ellstrand & Elam, 1993). In the present study, wide distribution of *A. polytricha* was correlated with a higher genetic diversity (e.g. Guangdong, Hainan, Jiangxi, Yunnan etc.) than that for a narrow distribution (e.g. Hebei, Jilin, Gansu etc.). Population genetic diversity may be correlated with the sample sizes, but our sample sizes were relatively small for several sites, and it is possible that higher genetic diversity might be found with more extensive sampling. For example, the genetic diversity of *A. polytricha* in Yunnan (southwestern China) was lower than that in Guangdong and Jiangxi, although Yunnan has a wide range of climates and habitats and is considered as one of the biodiversity hotspots in the world. The lower genetic diversity of *A. polytricha* in Yunnan might be due to exceptional drought in the year that we collected the samples.

In this study, estimated gene flow (N_m) was 0.7251 individuals per generation. The level of gene flow via dispersal of sexual basidiospores and fruitbodies is constrained by the low migratory capabilities of carriers. The gene flow distance between local populations in *Cycas* was estimated to be only 2-7 km in a study by Yang and Meerow (1996). In this study the samples taken in natural forest were at least 10 km away from farm land and the distance between populations of *Auricularia polytricha* were mostly > 30 km, so the geographical isolation might restrict the migratory capabilities of carriers, and the gene flow was lower. Genetic drift changes the distribution of genetic variation in two ways, by reducing variation within populations and by increasing differentiation between populations (Ellstrand & Elam, 1993). In this study, the high level of genetic variation among populations could be caused mainly by the limited gene flow, geographical isolation, and the genetic drift.

The genetic distance coefficients among 145 strains were also estimated using MEGA 3.1(data not shown), and ranged from 0.039 with the least dissimilarity between strains 116 and 117 (strains from the Beijing site showing a close relationship) to 0.259 with the greatest dissimilarity between strains 83 (Taiwan) and 132 (Sichuan), whereas the genetic distance varied from 0.148 to 0.309 (Du *et al.*, 2011b) in the genetic diversity analysis of 40 *A. polytricha* strains from Hainan Province. The genetic variation of wild *A. polytricha* was much lower than for the closely related *A. auricula-judae* (0.0676-0.7984 in Li *et al.*, 2007; 0.09-0.66 in Fu *et al.*, 2010).

A dendrogram was constructed by the neighbor-joining method based on genetic distance (D) of individuals; all strains were divided into five distinct groups (Fig. 3) when the D was 0.08. Group II comprised the three populations from Shandong, Liaoning and Beijing, in which individuals of each population formed separate clades. Group III was composed of all strains from the Hubei, Shaanxi, Sichuan and Fujian provinces. Group IV mainly comprised the strains from Guangdong except 75 and 76, the two strains clustering within the Taiwan population. Group V consisted of the populations from Guizhou, Taiwan, Jiangsu, and some Hainan strains. The remaining strains were classified in group I. That the strains from Hainan were distributed over four groups except group II, was in

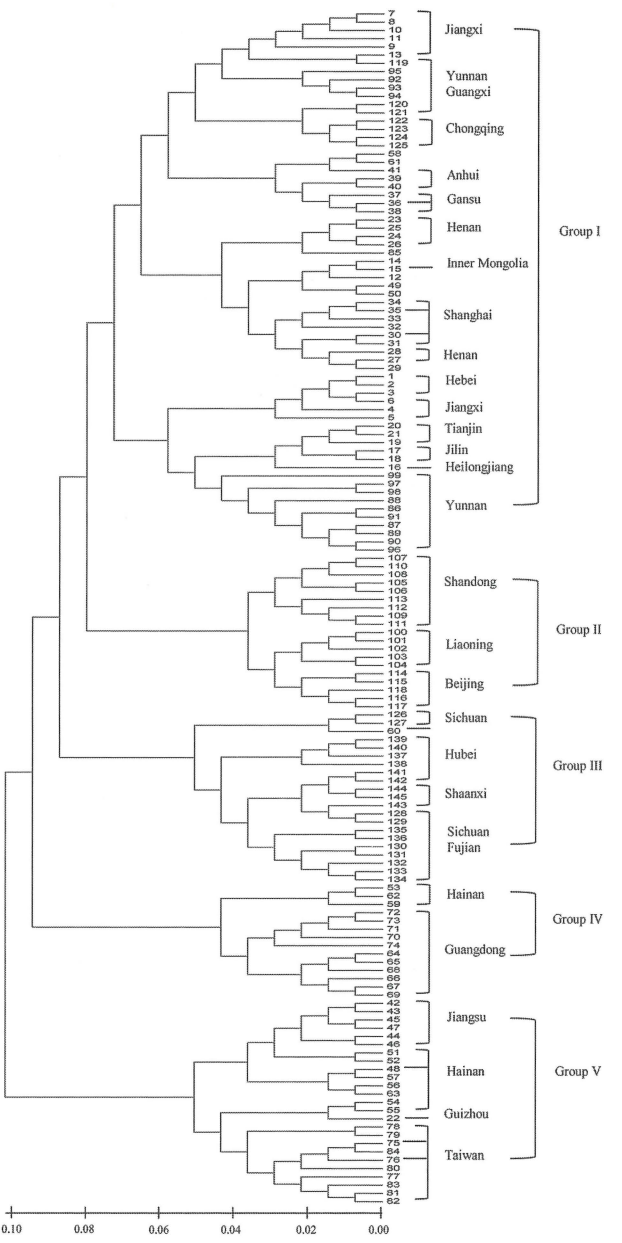

Fig. 3. Neighbor-joining dendrogram based on the genetic distances of 145 *Auricularia polytricha* strains.

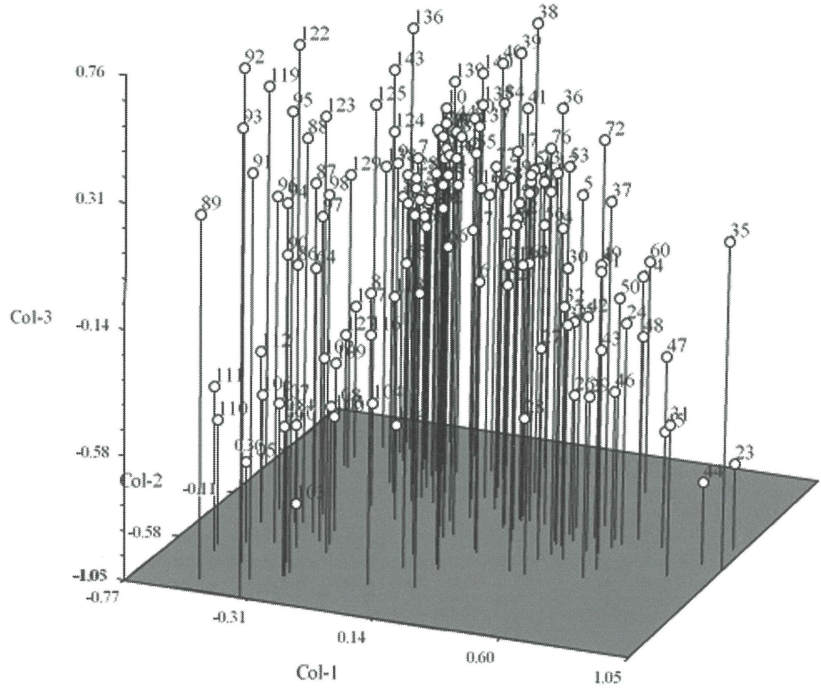

Fig. 4. Relationships among the 145 *Auricularia polytricha* strains visualized by principal coordinate analysis (PCoA) of ISSR-based genetic similarities. Coordinates 1, 2, and 3 are indicated.

accordance with the result of genetic diversity of *A. polytricha* in Hainan Province (Du *et al.*, 2011b), suggesting that individuals of Hainan have a richer genotypic variability. Clustering analysis indicated that most of the individuals of a specific population were arranged in population-specific clusters, which was further confirmed by a principal coordinate analysis (PCoA) (Fig. 4) that revealed a remarkable geographical relatedness among strains within populations, whereas no distinct geographical relationship was observed in the genetic variation among populations. Furthermore, clustering analysis did not suggest genetic variation to be correlated with the host association. For example, four strains (106, 110-112) sampled from *Populus* did not cluster but intermixed in the dendrogram with strains on other hosts from the Shandong population.

The information on the genetic relatedness of strains will be useful for future breeding programs in the same way as a better knowledge on the influence of environmental and physiological factors will contribute to the cultivation of edible fungi in China (Guo *et al.*, 2011, Hou *et al.*, 2011). Genetic depression is a common phenomenon in cultivated strains of *A. polytricha*, and based on the present study, genetic diversity of commercial strain could be improved by introducing new isolates into breeding programs.

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