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RESEARCH ARTICLE

Molecular variation and population structure in endangered *Limonium bicolor*: genetic diversity of microsatellite markers and amplified fragment length polymorphism analysis

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Abstract. Knowledge and analysis of the genetic structure of an endangered species is important for its conservation and evolutionary process. Simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs) were used in evaluation of the genetic diversity and population differentiation in *Limonium bicolor* (Plumbaginaceae), an endangered herb with high medicinal and horticulture value. A total of 117 alleles were detected with an average 5.85 alleles per locus using SSR and 222 bands from AFLP were amplified in six populations. It was found that *L. bicolor* was characterized by high levels of genetic polymorphism (100 and 83.78%), low levels of total genetic diversity ($H_t = 0.2824$ and 0.2424), and moderate degrees of genetic differentiation among populations ($\Phi_{ST} = 0.284$ and 0.251). Analysis of molecular variance (AMOVA) revealed that the main variation component existed within populations (71.56%; 74.93%) rather than among populations (28.44%; 25.07%). Four main clusters were displayed in the UPGMA using TFPGA, which was consistent with the result of principal coordinate analysis (PCA) using NTSYS. Mutations or infrequent gene flow among populations can increase the plant slowly, thus *in situ* conservation policies should be implemented first for effective and sustainable development. At the same time, *ex situ* measures, such as those individuals with rare alleles, to maintain the relationships between individuals and populations are also proposed.

Keywords. genetic diversity; simple sequence repeats; amplified fragment length polymorphism; plant conservation; *Limonium bicolor*.

Introduction

Limonium is the most species-rich and widespread genus of Plumbaginaceae, with inhabit inland dry gypsum soils, coastal cliffs and salt marshes (Palacios *et al.* 2000; Palop-Esteban *et al.* 2007). In China, there are only four genera of Plumbaginaceae (Dong 2005). Many of these species inhabit salt marshes, and their range has been reduced because of habitat loss due to human pressure, and eventually isolation formed (Villalba and Lumbreras 1993; Palop *et al.* 2000; Palop-Esteban *et al.* 2007).

Limonium bicolor is an endangered and perennial herb from salt marshes along the coast of China. It has excellent medicinal merits, such as enriching the blood, clearing away 'heat-evil', getting rid of hepatitis, diarrhea, bronchitis and other disorders (Li 1978). *L. bicolor* is also an excellent cut flower material that favoured by domestic and foreign tourists. As it has a variety of uses, the market demand has increased year by year. In recent years, the resource has been extinct by the influence of human activities, especially the overexploitation of its germplasm resources in the flowering season (Zhou *et al.* 1998).

For endangered species, genetic diversity and genetic structure analysis are very important since it reflects the survival potential of populations and can provide the basis

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for species conservation (Lande 1988; Cai *et al.* 2011). Microsatellite, also known as simple sequence repeats, is a simple repetitive sequence that is evenly distributed in the eukaryotic genome and consists of tandem repeats of two to six nucleotides. The number of repetitions of the units is highly variable and abundant in the individual thus it has been shown to be an effective genetic marker and useful in genetic analyses, and genetic diversity of different germplasms (Palop-Esteban *et al.* 2007, 2011; Cai *et al.* 2012; Hou *et al.* 2012; Jiménez *et al.* 2017).

AFLP is based on PCR technology to amplify genomic DNA restriction fragments, which combines the characteristics of RFLP and PCR technologies. It has the advantages of good reliability, strong repeatability and widely used in genetic breeding, genetic diversity, *in situ* and *ex situ* conservation of an endangered plant species (Palacios *et al.* 1999; Sezai *et al.* 2011; Chen *et al.* 2013; Mengesha *et al.* 2013; Lopez and Barreiro 2013; Ye *et al.* 2015).

In the present study, 102 accessions representing six populations of *L. bicolor* were collected from different regions of China to evaluate the genetic diversity and population structure using SSR and AFLP. The main objectives of this study were to assess the level of genetic variation and degree of genetic differentiation among wild populations from different regions, to determine the genetic relationships among the wild populations for assessing the effectiveness of *ex situ* collection of *L. bicolor* diversity, to contribute to a better understanding of the genetic profile of this endangered species and could then be used to develop useful strategies for its conservation and sustainable industrial development.

Materials and methods

Plant sample collection and genomic DNA extraction

Plant samples were collected from six extant populations in different provinces of China (table 1; figure 1). DNA was extracted from fresh leaves using Qiagen DNA extraction kit according to the manufacturer's instructions. The DNA were loaded onto 1% (w/v) agarose gels and subjected to electrophoresis to evaluate the quality of extracted DNA.

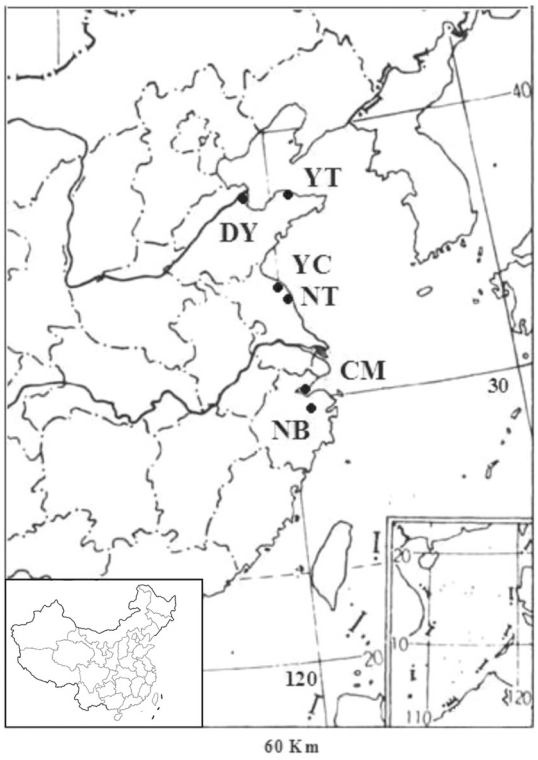

Figure 1. Locations of the sampled *L. bicolor* populations in China.

SSR-PCR amplification

Sau3A I (TaKaRa) was used to digest DNA and fragments ranging from 200 to 800 bp were extracted, and then purified fragments were ligated. Two biotinylated probes $(CTT)_{10}$ and $(TG)_{15}$ were hybridized to DNA fragments and those biotinylated hybrids were captured using streptavidin-coated magnetic beads (Promega, Madison, USA). The products containing microsatellites were ligated to pMD18-T vector (TaKaRa) and transformed into chemically competent cells (*Escherichia coli* DH5α). Subsequently, the microsatellite-enriched genomic library was constructed.

A 10 μL PCR reaction volume was carried out consisting of 10 ng genomic DNA, 2 mM $MgCl_2$, 0.2 mM dNTP, 1 μL PCR buffer, 0.4 μM of each primer, and 0.5 U *Taq* DNA polymerase (TaKaRa). PCR amplification

Table 1. Locations and accession numbers of six *L. bicolor* populations.

Population code	Location	Accession number	Longitude (E)	Latitude (N)
CM	Chongming, Shanghai	16	121°04′	31°62′
YC	Yancheng, Jiangsu province	18	120°13′	33°38′
NT	Nantong, Jiangsu province	18	121°05	32°08′
YT	Yantai, Shandong Province	18	121°39′	37°52′
DY	Dongying, Shandong province	18	118°49′	37°46
NB	Ningbo, Zhejiang province	14	121°56′	29°86′

programme was as follows: initial denaturation at 95°C for 5 min; subsequent 30 cycles of denaturing at 95°C for 30 s, annealing at an optimal temperature (49−61°C) for 30 s, extension at 72°C for 2 min; and a final extension for 10 min at 72°C. Six per cent denatured polyacrylamide gel was used to separate the products and visualized by silver-staining.

AFLP-PCR amplification

AFLP primers that produced polymorphic bands and PCR amplification was performed according to Ding *et al.* (2013a, b). Primer combinations were selected based on reproducibility, clarity of bands, and their highly polymorphic nature.

Negative controls were included to detect cross contamination in DNA extracts and PCR plates in the amplification reaction of SSR and AFLP. Only clear and reproducible distinguished bands were recorded and used in the following analysis.

Statistical analysis

SSR and AFLP bands in the gel profiles were recorded as present (1) and absent (0) and only the clearest and strongest reproducible bands were scored and used for the analysis. PopGene software (ver. 1.32; Yeh 1999) was used to estimate the percentage of polymorphic loci (PPL), total gene diversity (H_t), gene diversity within population (H_s), expected heterozygosity (H_e, Nei's gene diversity), Shannon's information index (I), observed number of alleles (N_a), effective number of alleles (N_e) and the coefficient of genetic differentiation (G_{st}) (Kimura and Crow 1964; Lewontin 1972; Nei 1973; McDermott and McDonald 1993). The dataset was analysed and assumed that populations were in Hardy–Weinberg equilibrium (HWE).

Genetic relationship of the populations was investigated using unweighted pair group method with arithmetic mean (UPGMA) method (Sneath and Sokal 1973) based on the genetic similarity matrix using TFPGA software ver. 1.3 (1000 permutations, Miller 1997) and bootstrapping values were calculated. Mantel test was performed with NTSYSpc ver. 2.1 (1000 permutations, Rohlf 2002) to estimate the correlation between the genetic distances (Nei 1972) and geographical distances (in km). Genetic differentiation was estimated by the analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) using 1000 random permutations within and among populations. A pairwise Euclidean distance matrix and all input files required by AMOVA were generated through AMOVA-PREP 1.01 (Miller 1998). Pairwise Φ_{ST} values between populations were computed and gene flow (Nm) was calculated from Φ_{ST} values as $Nm = (1\text{-}\Phi_{ST})/4\Phi_{ST}$ (Wright 1951). Principal coordinate analysis (PCA) as implemented in NTSYS-pc was carried out to reveal associations among individuals and genetic distances among the groups.

Results

Twenty microsatellite loci generated a total of 117 alleles in the 102 samples of six different *L. bicolor* populations (table 2). The number of alleles per locus was from 3 to 11, with the average number of 5.85. A total of 222 reproducible AFLP bands were obtained from 102 individual plants and 83.78% was polymorphic. Genetic diversity parameters at both population and species levels are shown in table 3. At the species level, percentage of polymorphism loci (PPL) estimated were 100% and 83.78% respectively. The effective number of alleles (N_e) were 1.4475 and 1.3968, the Shannon information index (I) were 0.4329 and 0.3726, Nei's gene diversity was H = 0.2770/0.2410.

Genetic differentiation was analysed by using PopGene 3.2 and there was a moderate genetic differentiation (G_{st} = 0.2983/0.2597) among populations. As shown in table 4, AMOVA was performed to study genetic differentiation among six populations from different geographical regions and to estimate the percentage of intrapopulation and interpopulation genetic variation. It showed that the main molecular variance occurred within populations (71.56% by SSR and 74.93% by AFLP) and the rest among populations (28.44%/25.07%). This analysis also revealed differentiation in allele frequencies (Φ_{ST} = 0.284/0.251) and the estimated number of migrations per generation (N_m = 1.176/1.425) between all populations.

Genetic and geographical distances between all pairs of the populations are listed in table 5. The result of the Mantel test revealed that no significant positive correlation was found between the genetic distances and geographic distances (r = 0.1515; $P > 0.05$). Nei's genetic distance among populations ranged from 0.0343 to 0.1695 by SSR and AFLP combinations. Cluster analysis was based on a similarity matrix of polymorphic bands and a dendrogram was constructed using UPGMA by TFPGA program, all six populations can be classified as four major clusters (figure 2). Cluster I contained populations YC, NT and cluster II included population YT, DY, the other two populations (NB and CM) clustered together with the previous populations in turn. PCA of 102 individuals was carried out using NTSYS to get a deeper understanding of the diversity of *L. bicolor* (figure 3). It revealed that the six populations were separated into four clusters, including YT, DY. YC and NT clustered into two clusters, and individuals of NB and CM separately clustered into the other clusters.

Table 2. Twenty polymorphic microsatellite loci isolated in *L. bicolor*.

Locus	Primer sequence (5′–3′)	Repeat motif	T_a (°C)	Expected size (bp)	N_a	H_O	H_E	GenBank accession no.
ES-01	F: CGAAGAGCCTGACGAATC R: GAATCTCGGAAGAAGTCG	$(AAG)_{32}$	55	256	10	0.81	0.65	MF671922
ES -02	F: GGGACTACTCGGACATAA R: ATTTCAACGACGACGATA	$(CTT)_{16}$	51	176	5	0.56	0.78	MF671923
ES -03	F: GAGACCGTTTGATATGTAT R: ATCAAACCTTAGGCACCC	$(CTT)_{13}$	49	205	11	0.86	0.71	MF671924
ES -04	F: TGGAAATTACTCGGACAT R: ATAGAGGCGATTCTTTGG	$(CTT)_{18}$	54	177	4	0.63	0.49	MF671925
ES -05	F: CACCAGCAGCAGCAGCTT R: ATAAAACCAACCGACCAG	$(TTC)_{16}$	58	256	3	0.62	0.74	MF671926
ES -06	F: GCCGCCAACTTCTTCTTC R: GAGAACAGGCGAGAAGAA	$(CTT)_{10}$	55	155	3	0.31	0.45	MF671927
ES -07	F: CCAAGCGGAAGGTCCAGA R: TCCTTCCTGCCGAACTCA	$(GAA)_9$	61	140	6	0.78	0.84	MF671928
ES -08	F: TCGTGTCTATGGCGTCCT R: GGGAGAAATGGCTGAGAT	$(CTT)_{15}$	56	167	6	0.52	0.64	MF671929
ES -09	F: ATAAACCGACGATCCTCTC R: TCAAGGATTCGCCGGTCT	$(CTT)_8$	55	156	5	0.36	0.61	MF671930
ES -10	F: ATCAGTTG CCACGCCACC R: GAACCAGGAAAGAATGAGA	$(CTT)_{10}$	54	130	5	0.68	0.72	MF671931
ES -11	F: TTCCTCCAACCCACGCA R: GTGCGAATCGTTGCTTTGGT	$(GAA)_8$	59	217	6	0.59	0.66	MF671932
ES -12	F: AGGCTGTCATCCGATTTCC R:CGAGAGTAGAGTGGTGATTCC	$(CTT)_9$	57	173	7	0.70	0.75	MF671933
ES -13	F: AAAGCCGGTCAGATGATG R: CGACGCTTCTGAGGATTA	$(GAA)_{15}$	55	221	4	0.14	0.58	MF671934
ES -14	F: TGGGAGAGTATGTAGAAG R: TTGGCTTTGGAAGCAGAT	$(TG)_9$	51	181	4	0.41	0.44	MF671935
ES -15	F: TCTCTTTCCGCCCGATCCT R: TCCGTCCCACTACAACCT	$(TTC)_9$	57	264	7	0.65	0.67	MF671936
ES -16	F: TTTGTTGAAGGTCGCCGG R: ATCCTGACCCGACCCAAT	$(GA)_9$	58	283	3	0.23	0.28	MF671937
ES -17	F: GTGGA GGTGACGATGATG R: AGAGCCTTGTGGTTGGAC	$(AGC)_5$	54	174	8	0.39	0.42	MF671938
ES -18	F: CTCAAAACATAACCAAAGT R: CTGATGGGTCACAACCTC	$(CAT)_{13}$	49	173	9	0.50	0.75	MF671939
ES -19	F: GTCGTGGCAGTGCTCTAT R: GTACAGGAGGTCGTCATT	$(GCA)_6$	50	126	6	0.41	0.47	MF671940
ES -20	F: ACCTTACATTCTTCTGCCAT R: TGACAATCAACACCCAAT	$(AT)_5(GT)_{11}$	52	179	5	0.69	0.74	MF671941

T_a, annealing temperature; N_a, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity.

Table 3. Genetic diversity of *L. bicolor* populations revealed by SSR and AFLP.

Population code	PPL	*H*	*I*	N_a	N_e
SSR					
CM	62.16	0.2188	0.3290	1.6216	1.3656
YC	51.35	0.1828	0.2742	1.5135	1.3083
NT	56.76	0.1859	0.2832	1.5676	1.3107
YT	61.26	0.1959	0.2993	1.6216	1.3279
DY	72.97	0.1962	0.3102	1.7297	1.3073
NB	57.66	0.2096	0.3066	1.5766	1.3904
Population average	60.36	0.1982	0.3004	1.6051	1.3350
Species-level	100	0.2770	0.4329	2.0000	1.4475
AFLP					
CM	48.65	0.1850	0.2730	1.4865	1.3219
YC	46.85	0.1745	0.2613	1.4685	1.2949
NT	54.05	0.1725	0.2647	1.5405	1.2843
YT	55.03	0.1823	0.2746	1.5043	1.3114
DY	56.55	0.1695	0.2629	1.5642	1.2721
NB	51.35	0.1928	0.2826	1.5123	1.3513
Population average	52.08	0.1794	0.2699	1.5127	1.3060
Species-level	83.78	0.2410	0.3726	1.8378	1.3968

PPL, percentage of polymorphism loci; *H*, Nei's (1973) gene diversity; *I*, Shannon's information index; N_a, observed number of alleles; N_e, effective number of alleles.

Table 4. AMOVA results for *L. bicolor* populations.

Source of variation	d.f.	Sum of squares	Mean squares	Variation components	Total variation (%)	*P* value
SSR						
Among populations	5	150.2388	30.048	1.5418	28.44	$P<0.001$
Within populations	96	372.3690	3.879	3.8788	71.56	$P<0.001$
AFLP						
Among populations	5	117.3443	23.469	1.1757	25.07	$P<0.001$
Within populations	96	337.4008	3.515	3.5146	74.93	$P<0.001$

Table 5. Nei's genetic distances and geographical distances among six populations of *L. bicolor.*

Pop. ID	CM	YC	NT	YT	DY	NB
CM	–	0.0357	0.0570	0.1385	0.1553	0.0835
YC	229.3467	–	0.0343	0.1124	0.1429	0.1007
NT	60.9709	168.4167	–	0.0626	0.1096	0.0801
YT	656.7856	474.8005	606.3736	–	0.0514	0.1287
DY	702.6282	477.9047	642.9452	256.2267	–	0.1695
NB	196.5194	414.6138	251.8768	852.8522	892.4094	–

Nei's genetic distances are given above the diagonal and geographic distances (km) are given below the diagonal.

The relationship between the positions of the samples in the PCA reflected their genetic similarity. The mutation of the first three principal coordinates were 30.47%, 11.75% and 9.98%, respectively, and the corresponding cumulative contribution rates were 30.47%, 42.22% and 52.20%, respectively. It is generally believed that if the variance of the first three main eigenvectors accounts for more than 40% of the total variance, the ranking effect is satisfactory. The cumulative contribution rate of variance of the first three main eigenvectors was as high as 52.20%, which indicated that the effect of reduction was better.

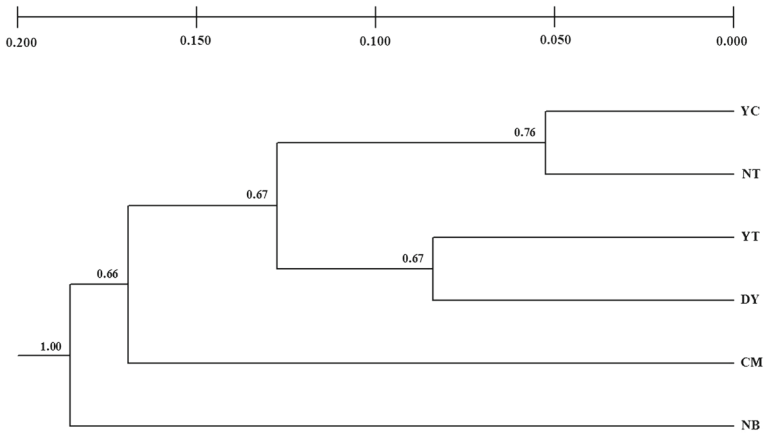

Figure 2. An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of genetic relationships among six populations of *L. bicolor* calculated on the basis of genetic similarity by means of SSR and AFLP primer combinations.

Discussion

Genetic diversity

It is an important prerequisite for conservation of an endangered species to analyse the genetic diversity and structure as it reflects the status and survival potential of populations (Lande 1988). It is known that genetic diversity is at the heart of biodiversity, which reflects the ability of a given species to adapt to the environment and the potential long-term duration of the survival and development of environment changes (Tripathi *et al.* 2012; Chen *et al.* 2013; Ye *et al.* 2015). In this paper, SSR and AFLP markers were applied to assess the level and pattern of genetic diversity in six populations of *L. bicolor*. The genetic diversity at species level ($H = 0.2770/0.2410$) was slightly higher than the average genetic diversity level between populations ($H = 0.22$ or 0.23) estimated by Nybom (2004), this level of gene diversity may be related to the *Limonium* germplasm and its breeding programmes. Comparing with these *Limonium* species of similar life history characteristics (*L. narbonense*, PPL = 96.36%, $H = 0.502$, $H_0 = 0.446$, $H_E = 0.544$, Palop-Esteban *et al.* 2011; *L. dendroides*, PPL = 41.8%, $H = 0.195$, Suárez-García *et al.* 2009; *L. dufourii*, PPL = 26.6%, Palacios and González-Candelas 1997; *L. dufourii*, PPL = 20.2%, Palacios *et al.* 1999; *L. macrophyllum*, $H = 0.363$, $H_0 = 0.186–0.405$, $H_E = 0.210$-0.378, Jiménez *et al.* 2017; *L. sinense*, PPL = 49.19%, $H = 0.1945$, Ding *et al.* 2013a, b), *L. bicolor* possesses high genetic diversity at species level but lower genetic diversity at population level.

The characteristics of the life history of the species, especially the breeding system, have important implications on genetic diversity and population genetic structure analysis (Nybom and Bartish 2000; Nybom 2004; Cai *et al.* 2011). *L.* species are pollinated by insects and have the sporophytic heteromorphic self-incompatibility system (Baker 1966; Palop-Esteban *et al.* 2011), however self-incompatibility is thought to take important part in the maintenance of the high levels of the genetic variability of species (Eduardo *et al.* 2001; Cai *et al.* 2011). In most cases, widespread species tend to possess high genetic diversity than narrowly distributed ones (Hamrick and Godt 1996; Yu *et al.* 2011). *L. bicolor* reduced drastically in recent years and is now endangered because of the habitat deterioration, human overexploitation and urban development (Zhou *et al.* 1998), although it was once distributed widely in China.

Population structure and gene flow

Gene differentiation and gene flow are important index to evaluate the population genetic structure of a species (Song *et al.* 2010). The value of G_{st} in this study were 0.2983 and 0.2597 based on SSR and AFLP markers, indicating that the major proportion of the total variation of *L. bicolor* existed within populations and the minor variation existed among populations. The other studies have reported similar estimates of genetic structuring as ours, such as SSR (Palop-Esteban *et al.* 2011) and allozyme (Suárez-García *et al.* 2009). *L. bicolor* is an insect-pollinated, outcrossing species due to the sporophytic self-incompatibility system, the current genetic structure may be caused by reproductive system types, habitat fragmentation and human overexploitation in recent years.

Population genetic structure may be caused by different factors, such as habitat fragmentation, population isolation and gene flow (Ge *et al.* 1998; Palop-Esteban *et al.* 2011). In population genetics, evolutionary biology, conservation biology and ecological sciences, gene flow is important (Qu *et al.* 2004; Ye *et al.* 2015). The *Limonium* seeds are light and may be dispersed by wind for long distance, which may be important in promoting gene flow. In fact, the gene flow estimate was 1.1760/1.4251

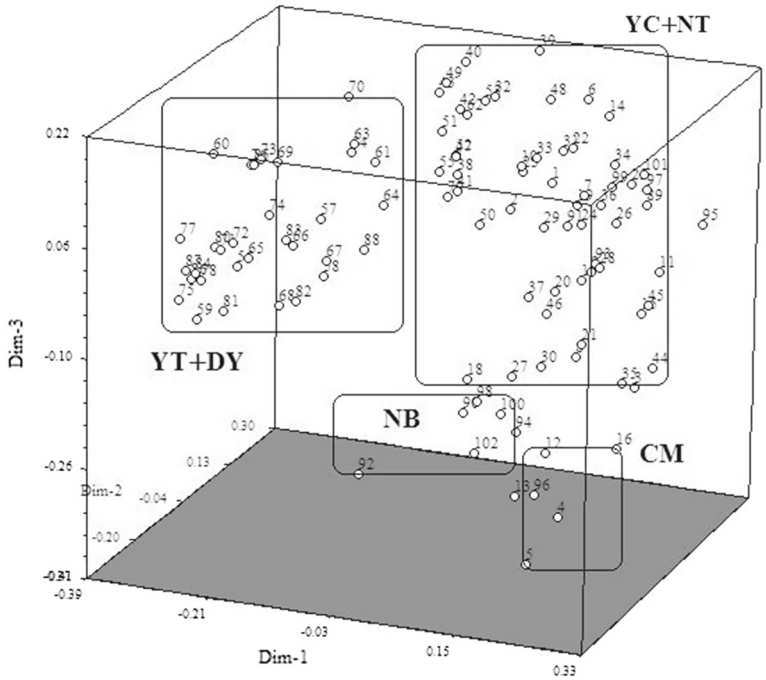

Figure 3. Relationships among the *L. bicolor* individuals visualized by SSR and AFLP markers based PCA.

among populations and this would not suffice to prevent continued divergence among populations and genetic drift in the long run (Wright 1951; Jiménez *et al.* 2017), which may be related to low seed germination rate (Zhou *et al.* 1998; Wang *et al.* 2010). In *Limonium* species, it is most likely associated with a self-incompatibility mechanism as reported in other members of the Plumbaginaceae (Baker 1966; Dulberger 1975; Vekemans *et al.* 1990; Richards 1997; Jiménez *et al.* 2017). The insects that pollinate *Limonium* rarely fly over long distance, which are not expected to cover the distances of the isolated population (Palop-Esteban *et al.* 2011; Jiménez *et al.* 2017). The once continuous landscape of salt marshes along the coasts of China has been subjected to increasing fragmentation for more than 50 years because of conversion of wetlands to farmland and urbanization construction.

In conclusion, High level of genetic diversity is an important condition for a species to survive for a long time (Ye *et al.* 2015). *L. bicolor* presents a strong population differentiation with a lower variation distributed among populations than within populations, and this pattern may be influenced by increasing urban development, industrial pollution and its good medicinal value (Zhou *et al.* 1998).

Genetic diversity is a very important parameter for species management and conservation, how to maintain the genetic diversity is a major goal of conservation for threatened and endangered species. There are several factors which can affect species extinction, such as habitat loss, overexploitation, pollution and climate change (Frankham *et al.* 2014; Ye *et al.* 2015), thus conservation genetics research in rare and endangered species is very important (Al-Qurainy *et al.* 2013).

L. bicolor has been listed as a good restorative drug by the 'Shanxi Chinese herbal medicine' and its current distributions are fragmented and discontinuous, so how to develop management practice in the conservation is very important. *In situ* conservation policies should be implemented first for achieving effective and sustainable development. Habitat preservation is usually the best strategy to keep endangered species for long-term existence (Jiménez *et al.* 2017). For *ex situ* conservation, it is needed to collect the germplasm resources from different populations and construct the seed bank for this species. As many plant samples as possible should be collected from different populations, especially those with high genetic diversity, such as DY and CM populations.

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