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 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 involved in evaluation of 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 of 5.85 alleles per locus with SSR and two hundred and twenty two bands from AFLP were amplified from the 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 (Ht= 0.2824 and 0.2424), and moderate degrees of genetic differentiation among populations (Φ_{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, so in situ conservation policies should be implemented firstly 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.

Key words *Limonium bicolor*, genetic diversity, SSR, AFLP, plant conservation

Introduction

The *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). There are only four genera of Plumbaginaceae in China (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 (Palop et al., 2000; Villalba and Lumbreras, 1993; 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 favored 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 over-exploitation of its germplasm resources in the flowering season (Zhou et al., 1998).

For endangered species, genetic diversity and genetic structure analysis is very important, because it reflects the survival potential of populations and can provide the basis 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 2 to 6 nucleotides. The number of repetitions of the units is highly variable and abundant in the individual, so it has been shown to be an effective genetic marker and useful in genetic analyses and genetic diversity of different germplasms (Cai et al., 2012; Hou et al., 2012; Jiménez et al., 2017; Palop-Esteban et al., 2007; Palop-Esteban et al., 2011).

AFLP is based on PCR technology to amplify genomic DNA restriction fragments, which combines the characteristics of RFLP and PCR technology. 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 (Ye et al., 2015; Sezai et al., 2011; Mengesha et al., 2013; Chen et al., 2013; Lopez et al., 2013; Palacios et al., 1999).

In the current study, 102 accessions representing 6 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 research 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

As shown in Table 1 and Fig. 1, the plant samples were collected from six extant populations in different provinces of China. Fresh leaves were used as material for DNA extraction 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.

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). The products containing microsatellites were ligated to pMD18-T vector (TaKaRa) and transformed into chemically competent cells (*Escherichia coli* DH5α). By now, 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 program 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 percent 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. 2013. Primer combinations were selected for the present study 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 (version 1.32; Yeh et al., 1999) was used to estimate the percentage of polymorphic loci (PPL), total gene diversity (Ht), gene diversity within population (Hs), expected heterozygosity (H, Nei's gene diversity), Shannon's information index (*I*), observed number of alleles (Na), effective number of alleles (Ne) and the coefficient of genetic differentiation (G_{st}) (Nei, 1973; Lewontin, 1972; Kimura and Crow, 1964; McDermott and McDonald, 1993). The dataset was analyzed and assumed that populations were in Hardy-Weinberg equilibrium (HWE).

Genetic relationship of the populations was investigated with the UPGMA (unweighted pair group method with arithmetic mean) method (Sneath and Sokal, 1973) based on the genetic similarity matrix using TFPGA software version 1.3 (1000 permutations, Miller, 1997) and bootstrapping values were calculated. Mantel test was performed with NTSYSpc version 2.1 (1000 permutations, Rohlf, 2000) 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 $Nm = (1-\Phi_{ST})/ 4\Phi_{ST}$ (Wright 1951). Principal coordinate analysis (PCA) as implemented in NTSYS-pc was 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 6 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 level and species level were 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 (Ne) 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 analyzed by using POPGENE 3.2 program and there was a moderate genetic differentiation (Gst = 0.2983/0.2597) among populations. As shown in Table 4, analysis of molecular variance (AMOVA) was performed to study genetic differentiation among six populations from different geographical regions and to estimate the percentage of intra and inter-population genetic variation. It showed that the main molecular variance occurred within populations (71.56% by SSR, 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 (Nm = 1.176/1.425) between all populations.

Genetic distances and geographical distances between all pairs of the populations were listed in Table 5. The result of the Mantel test revealed that no significant positive correlation was found between the genetic distances and the 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 in four major clusters (Fig. 2). Cluster I contained populations YC, NT and Cluster II included population YT, DY, the other 2 populations (NB, CM) clustered together with the previous populations in turn. Principal component analysis (PCA) of 102 individuals was carried out using NTSYS program in order to have a deeper understanding of the diversity of *L. bicolor* (Fig 3). It revealed that six populations were separated into four clusters, including YT, DY and YC, NT clustered into two clusters, and individuals of NB and CM separately clustered into the other clusters.

The relationship between the positions of the samples in the principal coordinate analysis reflected their genetic similarity. The mutation of the first 3 principal coordinates were respectively 30.47%, 11.75% and 9.98%, 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 3 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 3 main eigenvectors was as high as 52.20%, which indicated that the effect of reduction was better.

Discussion

Genetic diversity

It is an important prerequisite for conservation of an endangered species to analyze 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 programs. Comparing with these *Limonium* species of similar life history characteristics (*Limonium narbonense*, PPL=96.36%, H=0.502, H_0=0.446, H_E=0.544, Palop-Esteban et al., 2011; *Limonium dendroides*, PPL=41.8%, H=0.195, Sua'rez-Garcı´a et al., 2009; *Limonium dufourii*, PPL=26.6%, Palacios and Gonza´lez-Candelas, 1997; *Limonium dufourii*, PPL=20.2%, Palacios et al., 1999; *Limonium macrophyllum*, H=0.363, H_0=0.186-0.405, H_E=0.210-0.378, Jiménez et al., 2017; *Limonium sinense*, PPL=49.19%, H=0.1945, Ding et al., 2013), *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 for genetic diversity and population genetic structure analysis (Nybom and Bartish, 2000; Nybom, 2004; Cai et al., 2011). *Limonium* 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 (Yu et al., 2011; Hamrick and Godt, 1996). *L.bicolor* reduced largely in recent years and now is endangered because of 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 Gst 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 to ours, such as SSR (Palop-Esteban et al., 2011) and allozyme (Sua´rez-Garcı´a 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 was 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 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 (Wang et al., 2010; Zhou et al., 1998). 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 fifty years because of conversion of wetlands to farmland and urbanization construction.

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), so 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 firstly 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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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′

Table 2 Twenty polymorphic microsatellite loci isolated in *Limonium bicolor*

Locus	Primer sequence (5′−3′)	Repeat motif	Ta(°C)	Expected Size (bp)	Na	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

Ta annealing temperature, Na 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[a]	H[b]	*I*[c]	Na[d]	Ne[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

a Percentage of polymorphism loci.

b Nei's (1973) gene diversity.

c Shannon's information index.

d Observed number of alleles.

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.

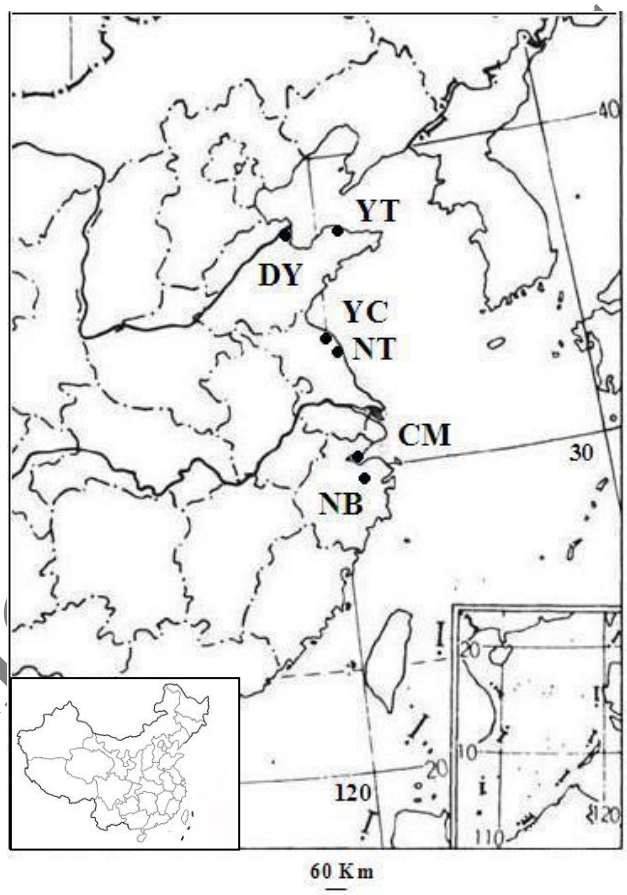

Fig. 1. Locations of the sampled *L.bicolor* populations in China.

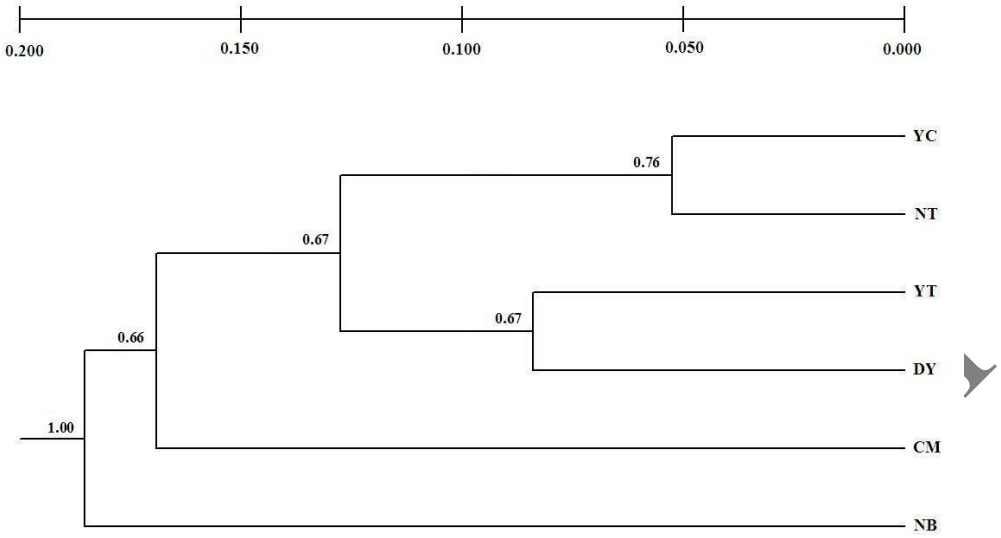

Fig. 2. An unweighted pair-group method with arithmetic averages (UPGMA) dendrogram of genetic relationships among 6 populations of *L.bicolor* calculated on the basis of genetic similarity by means of SSR and AFLP primer combinations

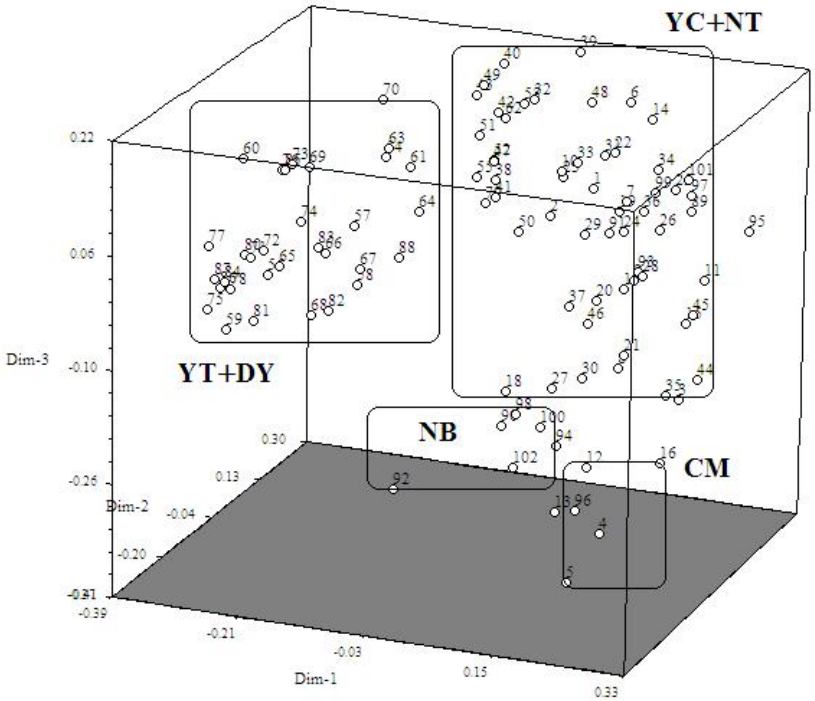

Fig. 3. Relationships among the *L.bicolor* individuals visualized by SSR and AFLP markers based PCA