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Genetic diversity of the Chinese traditional herb *Blumea balsamifera* (Asteraceae) based on AFLP markers

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ABSTRACT. *Blumea balsamifera* is a commercially important medicinal herb in China and other parts of Asia. It is used to produce borneol. This plant grows in the wild, but resources have diminished greatly in recent years. We examined the genetic diversity of this species to help develop conservation strategies; 35 plants from five provinces were analyzed using AFLPs. Eight AFLP primer combinations generated 1367 fragments, giving a mean of 172 fragments per primer combination. Polymorphism in the germplasm analysis was found for 1360 (99.48%) of the fragments, of which 264 (19.27%) fragments were unique (accession specific) and 423 (25.33%) of the fragments were rare (present in less than 10% of the accessions). The polymorphic

fragments were used to group the accessions in a UPGMA phenogram. Most grouping was geographical. In general, accessions coming from Guizhou and Guangxi showed higher diversities as these accessions were scattered in different groups. The genetic distance estimated by Jaccard similarity coefficient index showed low variability among genotypes (coefficient value ranged from 0.60 to 0.95). More attention should be given to the study and conservation of the biodiversity of this economically important genus.

Key words: *Blumea balsamifera*; Genetic diversity; AFLP; Conservation genetics

INTRODUCTION

Blumea balsamifera is not only used as an herbal in Chinese traditional medicine but also as economic materials for L-borneol extraction, which is widely used in the cosmetic and medical industry (Donkin, 1999; State Pharmacopeia Committee of China, 2010). In contrast to synthetic borneol, borneol from *B. balsamifera* is non-poisonous and safer, also having a more natural aroma. In contrast to borneol from *Dryobalanops aromatica* Gaertn. f., it is easier to get in large amounts. Also, some other bioactive chemicals have been discovered in *B. balsamifera*. For example, a sesquiterpenoid isolated from its leaves has shown mild cytotoxic activity (Hasegawa et al., 2006, Norikura et al., 2008a,b), icthyothereol acetate and cryptomeridiol have exhibited moderate antifungal activities (Ragasa et al., 2005), and its methanol extracts or water extracts have shown plasmin inhibitory activity, xanthine oxidase inhibitory activity, hepatoprotective activity, etc. (Nguyen et al., 2004; Nessa et al., 2004; Osaki et al., 2005).

In recent years, due to the characteristics of rapid, accurate, polymorphism which are not affected by recessive characters, molecular markers are widely used in medicinal plant genetic diversity analysis. Li et al. (2004) used random amplified polymorphic DNA (RAPD) to study the genetic diversity of *Sargentodoxa cuneata,* and the results indicated that the same herbal in different areas exhibited obvious differences, where cluster analysis showed that there was a direct correlation between genetic distance and geographic distance. Zhang et al. (2010), Wu et al. (2007) and Guan et al. (2013) obtained the same results in their research of *Rhodiola rosea* L., *Cibotium barometz* (L.) J. Sm. and *Gastrodia elata* Blume.

B. balsamifera is found in the tropical and sub-tropical regions of Asia, especially in China and Southeast Asia. Owing to complicated and unique climate characteristics of China, after millions of years of evolution and artificial selection, *B. balsamifera* has gained abundant germplasm resources. These resources provided a base for developing new varieties. However, hitherto, research on *B. balsamifera* germplasm resources is still limited to the stage of morphological variation, where genetic diversity or variations revealed by molecular markers is still rare (He et al., 2005). In this study, 35 germplasm resources of *B. balsamifer*a from five provinces of China were investigated by amplified fragment length polymorphism (AFLP) markers. The genotyping data were then analyzed using the unweighted pair group method with arithmetic average (UPGMA) and principal component analysis (PCA) to understand the pattern of variation.

MATERIAL AND METHODS

Plant material collection and total DNA extraction

B. balsamifera mostly grows in semi-tropical areas of China, such as Yunnan, Hainan, Guangxi, Guizhou, and Guangdong. A total of 35 *B. balsamifera* individuals were collected from five provinces, according to the actual quantity in different localities. Details of collected samples are given in Table 1.

Table 1. Primary core collection library of *Blumea balsamifera*.

No.	Code	Location	Province	Geographic Information			Appendix
				Latitude (N)	Longitude (E)	Altitude (m)	
1	HN1	Baisha	Hainan	19°02.372'	109°34.146'	506.5	Wild
2	HN2	Wanning	Hainan	18°43.843'	110°13.591'	10.9	Wild
3	HN3	Qiongzhong	Hainan	18°99.785'	109°82.379'	198.0	Wild
4	HN4	Baoting	Hainan	18°68.983'	109°54.021'	58.0	Wild
5	HN5	Tuichang	Hainan	19°33.009'	110°08.832'	74.0	Wild
6	HN6	Qionghai	Hainan	19°02.456'	109°30.006'	210.5	Wild
7	HN7	Wanning	Hainan	18°44.843'	110°23.391'	20.9	Wild
8	HN8	Wuzhishan	Hainan	18°54.357'	109°40.258'	711.5	Wild
9	HN9	Baisha	Hainan	19°19.759'	109°44.335'	67.0	Wild
10	HN10	Baisha	Hainan	19°19.759'	109°44.335'	67.0	Cultivar
11	HN11	Danzhou	Hainan	19°42.438'	109°16.505'	37.5	Cultivar
12	HN12	Diaoluo	Hainan	18°78.964'	109°89.212'	148.0	Wild
13	YN1	Jinghong	Yunnan	21°57.704'	100°35.446'	1374.0	Wild
14	YN2	Puer	Yunnan	22°41.978'	100°56.818'	1312.0	Wild
15	YN3	Puer	Yunnan	22°43.047'	100°56.273'	1145.0	Wild
16	YN4	Puer	Yunnan	22°37.176'	100°59.661'	959.7	Wild
17	YN5	Funing	Yunnan	23°36.690'	105°37.360'	818.0	Wild
18	GX1	Qingzhou	Guangxi	22°41.610'	109°34.143'	256.4	Wild
19	GX2	Yulin	Guangxi	22°61.401'	110°28.484'	256.6	Cultivar
20	GX3	Nanning	Guangxi	22°75.212'	108°47.625'	147.0	Cultivar
21	GX4	Baise	Guangxi	23°53.890'	106°32.909'	132.0	Wild
22	GX5	Baise	Guangxi	23°54.934'	106°28.102'	126.0	Wild
23	GZ1	Luodian	Guizhou	25°47.360'	105°40.535'	800.0	Wild
24	GZ2	Xingyi	Guizhou	24°52.663'	105°00.628'	845.0	Wild
25	GZ3	Xingyi	Guizhou	25°07.758'	105°55.990'	395.1	Wild
26	GZ4	Xingyi	Guizhou	25°11.031'	106°06.801'	690.0	Wild
27	GZ5	Xingyi	Guizhou	25°01.513'	105°44.089'	934.1	Wild
28	GZ6	Xingyi	Guizhou	25°07.758'	105°55.990'	395.1	Wild
29	GZ7	Xingyi	Guizhou	24°52.663'	105°00.628'	845.0	Wild
30	GZ8	Xingyi	Guizhou	24°51.910'	105°01.428'	805.9	Wild
31	GZ9	Guanling	Guizhou	25°55.619'	105°35.864'	731.5	Wild
32	GZ10	Guanling	Guizhou	25°53.425'	105°39.092'	656.3	Wild
33	GZ11	Xingyi	Guizhou	25°03.267'	105°56.235'	397.7	Wild
34	GD1	Maoming	Guangdong	23°07.973'	113°29.101'	68.0	Cultivar
35	GD2	Guangzhou	Guangdong	21°93.795'	111°35.742'	537.0	Cultivar

Total genomic DNA of these materials was isolated from 0.02 g dry leaves using the QIAGEN DNeasy Plant Minikit according to the manufacturer instructions.

AFLP analysis

AFLP analysis was performed as previously described by Vos et al. (1995) with the following modifications: 300 ng genomic DNA digested in 20 μL with two combinations of

enzymes (2 µL 10X PstI buffer, 5.0 U PstI, 2.5 U MseI and 0.2 µL 100x BSA) for 16 h, then incubated at 70°C for 15 min to deactivate the restriction enzymes.

PstI (5 µmol) and MseI (50 µmol) adaptors were ligated to the restricted DNA fragments in ligation buffer (10X T_4 DNA ligase buffer, 0.2 U T_4 DNA ligase) and incubated at 16°C for 10 h. All above described chemicals were obtained from TaKaRa (Dalian, China).

The diluted (10-fold) amplified products were used as the template for selective amplification. Selective amplification was carried out with nine selective primer combinations of PstI and MseI with three selective nucleotides (Table 2) in a total volume of 20 µL. The PCR program consisted of two segments. The first segment comprised 12 cycles, each set at 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s. The annealing temperature was lowered by 0.7°C after each cycle during the first 12 cycles. The second segment consisted of 24 cycles set at 94°C for 30 s, 56°C for 60 s and 72°C at 60 s per cycle.

Table 2. Sequences of 8 AFLP selected primer combinations.

No.	Primer combinations	PstI primer (5'-3')	MseI primer (5'-3')
1	PstI-1/MseI-3	GACTGCGTACATGCAGAA	GATGAGTCCTGAGTAACAG
2	PstI-2/MseI-1	GACTGCGTACATGCAGAC	GATGAGTCCTGAGTAACAA
3	PstI-2/MseI-2	GACTGCGTACATGCAGAC	GATGAGTCCTGAGTAACAC
4	PstI-2/MseI-3	GACTGCGTACATGCAGAC	GATGAGTCCTGAGTAACAG
5	PstI-3/MseI-1	GACTGCGTACATGCAGAG	GATGAGTCCTGAGTAACAA
6	PstI-3/MseI-2	GACTGCGTACATGCAGAG	GATGAGTCCTGAGTA ACAC
7	PstI-3/MseI-3	GACTGC GTACATGCAGAG	GATGAGTCCTGAGTAACAG
8	PstI-4/MseI-3	GACTGCGTACATGCAGAT	GATGAGTCCTGAGTAACAG

Data analysis

Genotyping data obtained for the AFLP primer combinations was used for assessing the discriminatory power of AFLP primer combinations by evaluating three parameters: (i) polymorphism information content (PIC), (ii) marker index (MI) and (iii) resolving power (RP) (Roldán-Ruiz et al., 2000).

The PIC value for each AFLP primer combination was calculated as proposed by Roldan-Ruiz et al. (2000):

$$PIC_i = 2\ f_i(\ 1 - f_i\ );$$

where PIC_i is the polymorphism information content of marker i, f_i the frequency of the marker fragments that were present and $1\text{-}f_i$ the frequency of marker fragments that were absent. PIC was averaged over the fragments for each primer combination.

Construction of phenogram

Prominent AFLP fragments for each primer combination were scored visually as present (1) or absent (0). Genetic similarity (GS) was estimated between pairs of accessions according to the Jaccard similarity coefficient (Jaccard, 1908) using the NTSYS-pc 2.02 software package (Rohlf, 1993). The genetic similarity matrix obtained was based on Jaccard similarity. The coefficient was used to prepare the phenogram following the unweighted pair group method with arithmetic average (UPGMA).

RESULTS

For understanding the molecular diversity available for *B. balsamifera* accessions in China, a set of 35 genotypes were selected in such a way that all five provinces from where *B. balsamifera* accessions had been collected were represented. If four accessions had been collected from one town from a particular state of China, only one accession out of four accessions was selected. AFLP markers were employed for molecular profiling since AFLP provides genome-wide fingerprints.

Marker polymorphism

A total of eight primer combinations from four PstI and three MseI selective primers were used to generate AFLP profiles on 35 *B. balsamifera* accessions (Table 1). While analyzing the genome-wide AFLP fingerprint profiles, only prominent fragments were considered. All eight primer combinations generated a total of 1367 fragments, of which 1360 (99.48%) were polymorphic and eight (0.52%) of the polymorphic fragments were unique and accession specific (Table 3). The total number of fragments (monomorphic and polymorphic) for each assay ranged from 124 to 198 with an average of 170.9 per primer combination. The percentages of polymorphic fragments varied from 98.86 to 100% with an average of 99.48% per primer combination.

Table 3. Degree of polymorphism and information content for 8 AFLP primer combinations used.

No.	Primer combinations	NTF	NMF	NPF	% Polymorphism	NUF	NRF	NSF	NSIF
1	PstI-1/MseI-3	188	0	188	100%	36	64	65	23
2	PstI-2/MseI-1	176	2	174	98.86%	29	65	66	14
3	PstI-2/MseI-2	124	1	123	99.19%	35	40	43	5
4	PstI-2/MseI-3	133	0	133	100%	40	37	47	9
5	PstI-3/MseI-1	198	0	198	100%	27	57	102	12
6	PstI-3/MseI-2	181	3	179	98.90%	31	61	64	23
7	PstI-3/MseI-3	191	1	190	99.48%	40	49	87	14
8	PstI-4/MseI-3	176	1	175	99.43%	26	50	82	17
9	Total	1367	8	1360	99.48%	264	423	556	117
10	Minimum	124	0	123	98.86%	26	37	43	5
11	Maximum	198	3	198	100%	40	65	102	23
12	Average	170.9	1	170	99.48%	33	52.9	69.5	14.6

NTF = total number of fragments generated; NMF = number of monomorphic fragments; NPF = number of polymorphic fragments; NUF = total number of unique fragments; NRF = total number of rare fragments; NSF = total number of shared fragments; NSIF = total number of similar fragments.

Marker informativeness for the eight AFLP primer combinations was analyzed using several parameters (Table 3). The fragments generated by the eight primer combinations varied from 124 (PstI-2/MseI-2) to 198 (PstI-3/MseI-1) with an average of 170.9 per primer combination, and percentage of polymorphic fragments varied from 98.48% (PstI-3/MseI-3) to 100% with an average of 99.48% per primer combination. The unique fragments (NUF), present in only one accession for the given primer combination, varied from 26 (PstI-4/MseI-3) to 40 (PstI-3/MseI-3) with a total of 264 eight primer combinations.

AFLP fragments observed in less than 10% of the accessions with a given primer combination were considered rare fragments (NRF). A total of 423 rare fragments were observed with an average of 52.9 rare fragments per primer combination (Table 3). Highest number of

NRF (65) was generated by the primer combination of PstI-2/MseI-1, followed by 61 NRF generated by primer PstI-3/MseI-2. The fewest NRF (37) were generated by the primer PstI-2/MseI-3. Shared fragments (NSF) were those that were scored in 70% of accessions. A total of 556 shared fragments were observed ranging from 43 (PstI-2/MseI-2) to 102 (PstI-3/MseI-1). Similar fragments (NSIF) were the fragments present in more than 70% of accessions, where eight AFLP primer combinations in total generated 117 similar fragments with an average of 14.6 per primer combination.

AFLP features

PIC, MI (a feature of a marker and therefore MI calculated for all primer combinations) and RP (a feature of the primer combination that indicates the discriminatory potential of the primer combination) are three important indices of DNA molecular markers. These indices were analyzed with POPGENE version 1.31 (Yeh et al., 1999). The PIC value of 1360 polymorphic fragments ranged from 0.05 to 0.50, with an average of 0.1914 per primer combination. MI ranged from 19.57 to 50.81 with an average of 33.08 per primer combination. The highest value (50.81) was scored with PstI-3/MseI-1 primer pair and the minimum value (19.57) for PstI-2/MseI-2 (Table 4). A positive correlation was observed between MI and PIC values ($r^2 = 0.78$, $P < 0.005$). RP ranged from 37.20 to 75.87 with an average of 58.31 per primer combination. The highest value (75.37) was found in the primer combination for PstI-3/MseI-1 and the lowest value (37.20) for PstI-2/MseI-2 (Table 4). RP values were found in positive correlation with MI ($r^2 = 0.93$, $P < 0.005$).

Table 4. Maker attributes for AFLP combinations used.

No.	Primer combinations	PIC	EMR	MI	RP
1	PstI-1/MseI-3	0.1813	188	34.08	56.86
2	PstI-2/MseI-1	0.1860	174	32.36	58.57
3	PstI-2/MseI-2	0.1591	123	19.57	37.20
4	PstI-2/MseI-3	0.1626	133	21.63	37.31
5	PstI-3/MseI-1	0.2566	198	50.81	75.37
6	PstI-3/MseI-2	0.1798	179	32.18	67.60
7	PstI-3/MseI-3	0.1993	190	37.87	69.49
8	PstI-4/MseI-3	0.2066	175	36.16	64.11
9	Minimum	0.1591	123	19.57	37.20
10	Maximum	0.2566	198	50.81	75.37
11	Average	0.1914	170	33.08	58.31

PIC = polymorphism information content; EMR = effective multiplex ratio; MI = marker index; RP = resolving power.

Genetic diversity and phylogenetic analysis

Genotyping data obtained for all 1360 polymorphic fragments from eight primer combinations were used to estimate pairwise similarity comparisons between the accessions. The genetic similarity matrix was determined using the Jaccard similarity coefficient using the NTSYS-pc program. Subsequently, the genetic similarity matrix was used for constructing the UPGMA phenogram with the help of the NTSYS-pc program. The similarity coefficient values of the phenogram ranged from 0.66 to 0.91, suggesting a broad genetic base (Figure 1).

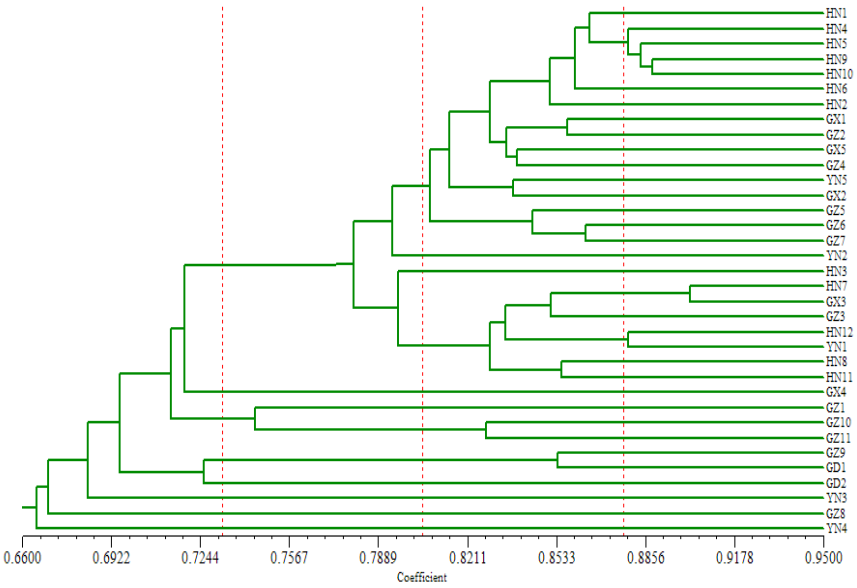

Figure 1. Dendrogram of 35 *Blumea balsamifera* accessions based on AFLP analysis using UPGMA.

On the basis of the UPGMA cluster diagram (Figure 1), all accessions were classified into four major clusters (Cluster I, II, III and IV). Cluster I contained 18 accessions, with a coefficient of 0.7953, and could be divided into four subclusters. Subcluster "i" contained seven accessions from Hainan (HN1, HN2, HN4, HN5, HN6, HN9 and HN10), with a coefficient of 0.8533. Subcluster "ii" contained four accessions, with two accessions from Guangxi (GX1 and GX5) and the others from Guizhou (GZ2 and GZ4), with a coefficient of 0.8339. Subcluster "iii" contained two accessions, from Yunnan (YN5) and Guanxi (GX2), respectively. Subcluster "iv" contained three similar accessions from Guizhou (GZ5, GZ6 and GZ7) with a coefficient of 0.8469. Excluding those subclusters, one accession from Yunnan (YN3) also belonged to Cluster I.

Cluster II contained eight accessions. Five of them were from Hainan (HN3, HN7, HN8, HN11 and HN12), and the others were from Guangxi (GX3), Yunnan (YN1) and Guizhou (GZ3), respectively. In this cluster, except for HN3, all other accessions showed a high degree of homology (85% similarity). Cluster III contained three accessions from Guizhou (GZ1, GZ10 and GZ11), with a coefficient of 0.7349. Cluster IV also contained three accessions, one from Guizhou (GZ9) and the other two from Guangdong (GD1 and GD2).

Excluding those accessions of four major clusters, there were also three accessions, two of them from Yunnan (YN2 and YN4) and the other from Guizhou (GZ8). These accessions showed a low degree of homology (0.6664~0.6988) with the accessions classified into four major clusters. These results indicated that those accessions would be important germplasm resources for hereditary improvement of cultivated *B. balsamifera.*

DISCUSSION

In the case of *B. balsamifera*, there are many chemical constituents and various phar-

macological studies have been carried out, but few studies have been conducted to assess molecular diversity and germplasm collection. Compared to RAPD, inter-simple sequence repeats, restriction fragment length polymorphism and microsatellites, AFLP is an information-rich marker system due to its ability to generate a large number of polymorphic/informative loci simultaneously in a single lane with a single-primer combination, it has been successfully used to investigate the diversity in several tropical plant species, such as Hevea, cassava, etc. In our research, the AFLP method was chosen to assess the genetic similarity of *B. balsamifera* from five provinces of China.

The efficiency of a molecular marker technique depends upon the amount of polymorphism it can detect among the set of accessions under investigation. High quality marker profiling was obtained with eight AFLP primer combinations in 35 accessions collected from provinces of China. All primer combinations used were found to be polymorphic and yielded 1360 of 1367 (99.48%) polymorphic fragments. The level of polymorphism in the present study was higher compared to another AFLP diversity study (88.2-97.2%), each assay ranged from 124 to 198 with an average of 170.9 per primer combination. Also, the AFLP features PIC, MI and RP were determined. PIC ranged between 0.05 and 0.50 with an average of 0.1914 per primer combination, MI ranged from 19.57 to 50.81 with an average of 33.08, and RP ranged from 37.20 to 75.87 with an average of 58.31, A positive correlation was observed between RP, MI and PIC ($r^2 = 0.93$, $r^2 = 0.78$).

Genetic similarity between 35 accessions was calculated using the NTSYS-pc program with the Jaccard similarity coefficient. The results showed that similarity coefficient values of the phenogram ranged from 0.66 to 0.91. Excluding two accessions from Yunnan (YN2 and YN4) and one accession from Guizhou (GZ8), other accessions could be classified into four major clusters (Cluster I, II, III and IV). Excluding those accessions of four major clusters, there were also three accessions, two of them from Yunnan (YN2 and YN4) and one from Guizhou (GZ8), which showed a low degree of homology (0.6664-0.6988) with those accessions of the four major clusters.

In summary, the present study provided a larger number of reliable and reproducible fingerprint profiles for an elite collection of 35 accessions of *B. balsamifera* collected from five provinces of China. On the one hand, similarity analysis of *B. balsamifera* showed lower genetic diversity and higher similarity of *B. balsamifera*, and this included the necessity of protecting the germplasm resources of *B. balsamifera*. On the other hand, higher genetic diversities were documented in the accessions of Hainan and Guizhou. The availability of unique or rare fragments present in different accessions together with genetic dissimilarly data would be very useful for improvement of the species through conventional breeding methods as well as molecular breeding approaches such as marker assisted selection.

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