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Genetic Diversity and Structure in a Germplasm Collection of Pansies using SRAP Markers

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Abstract

Pansies are one of the most important bedding flowers worldwide. Investigating the genetic diversity and identifying the genetic structure and relationships among germplasms are useful for geneticists and breeders. In this study, the genetic diversity and genetic structure of forty pansies germplasms from six breeding organizations were analysed by sequence-related amplified polymorphism (SRAP). Twenty-four primer pairs generated 430 polymorphic bands and showed high resolving power by 9.92 on average. The Nei's gene diversity across 40 accessions was 0.271. Compared with the accessions from other organizations, the accessions from Jiuquan Jinqiu Horticulture Seed had high percentage of polymorphic bands and genetic diversity. As for flower size, the polymorphic bands percentage and genetic diversity of medium flower group were large. The genetic distances between mini flower group and others were relatively large. Most of accessions were grouped into three clusters that reflecting the genetic relationships of accessions correlated to their original place using principle coordinate analysis (PCoA) and unweighted pair group method arithmetic means (UPGMA). Bayesian analysis inferred that most pansies accessions had admixed genetic background, and they mainly derived from 'Jonny Jump Up; 'Schweizer Riesen' and cultivars from PanAmerica Seed. These results can be helpful to germplasm introduction and crossing parent selection in pansies breeding.

Keywords: *Viola* × *wittrockiana*; *Viola cornuta*; Genetic background; Genetic relationship; Sequence-related amplified polymorphism

Introduction

Pansies (*Viola*×*wittrockiana*) are popular bedding flowers usually grown in garden beds, pots, borders or landscapes. They are hybrid species in *Melanium* section of *Viola* genus, in which spontaneous hybridization and polyploidization are common and often lead to fertile derivates despite ploidy differences (Clausen, 1927; Erben, 1996; Ballard *et al.*, 1999). Present-day large-flowered garden pansies are thought to have originated from selection and initial crossing between *V. tricolor* (2n=26) and *V. lutea* (2n=48), later hybridized with a large and varied flower colored perennial *V. altaica* Ker-Gwal. (2n=22) and an alpine perennial *V. cornuta* (2n=22) (Horn, 1956). Hybridization among these different species with different chromosome numbers brought various hybrid generations, garden pansies, with a large of phenotypic variations, such as rich flower colors and extended flower size (Huziwara, 1966).

In the last 50 years, the variety of pansies has expanded rapidly with application of many useful breeding methods, like autopolyploidy from induced mutagenesis or allopolyploidy from spontaneous or artificial interspecific hybridization to create new desirable phenotypic traits such as unusual flower colors, plant vigor, compactness, and tolerance to pathogens or environmental stress (Levin, 1983; Bailey *et al.*, 1995; Lagibo *et al.*, 2005; Zhang *et al.*, 2010; Du *et al.*, 2018), which makes the genetic relationship among cultivars of pansies more complex.

Understanding the genetic diversity and population structure of collected germplasms are useful for geneticists and breeders. Morphological traits, biochemical and molecular markers can be used for these purposes. Investigation adopted morphological traits have been published (Du *et al.*, 2010, 2011), but morphological traits are often limited in numbers and are easily influenced by environment. With powerful ability to provide large amounts of information and detecting variation among individuals and populations reliably, molecular markers, such as random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and restriction site amplified polymorphism (RSAP), were employed to analyse genetic relationship of pansies and *Viola* species

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(Ko *et al.*, 1998; Yockteng *et al.*, 2003; Culley *et al.*, 2007; Wang and Bao, 2007; Li *et al.*, 2015; Vemmos, 2015; Ahmad *et al.*, 2019).

Sequence-related amplified polymorphism (SRAP) is a simple and reliable marker technique. It used two-primer with special core sequences to amplify open reading frames (ORFs). The core sequences in the forward primer including the sequence CCGG to target exons and AATT as the core sequences in reverse primer to aim introns. The primers are 17 or 18 nucleotides long to assure amplification reproducibility. In order to gain moderate throughput ratio, SRAP employ two-step PCR program, the first five cycles are run setting the annealing temperature at 35°C, but following 35 cycles are run with the annealing temperature at 50°C. Finally, it combines simplicity, reliability, moderate throughput ratio for genetic analysis (Li and Quiros, 2001). With these advantages, it has been popular since it was developed, and widely used to analyze genetic relationships in many species, like *Cucurbita pepo*, buffalograss, petunia, yam, purslane, cotton, *Saccharum spontaneum, etc.* (Ferriol *et al.*, 2003; Budak *et al.*, 2004; Xu *et al.*, 2008; Wu *et al.*, 2014; Jia *et al.*, 2017; Sheidai *et al.*, 2018; Yu *et al.*, 2019). Wang *et al.* (2012) used SRAP to study the genetic relationships among 43 cultivars of *V. tricolor* and *V. cornuta.* But the genetic diversity and genetic backgrounds of pansies cultivars haven't been mentioned and unclear till now. For this reason, we employed SRAP to investigate the genetic diversity and genetic structure of a germplasm collection in order to provide reference information for the germplasm management and crossing parent selection in pansies breeding.

Materials and Methods

Plant Material

A total of 40 pansy accessions, including 22 cultivars widely grown nowadays and 18 breeding lines bred of Key Lab of Flower Breeding, Henan Institute of Science and Technology, were selected for genetic diversity analysis. These accessions represented two popular garden pansies: *V.×wittrockiana* and *V. cornuta*. Two aspects were considered when selecting accessions available in the pansies collection of Henan Institute of Science and Technology. Firstly, cultivars with different flower sizes which generally be used for pansies classification were selected to investigate the genetic difference among these groups. Secondly, cultivars from different seed companies or public sectors were selected to investigate the genetic diversity of pansies within these organizations and genetic relationship of pansies among them. Description of pansies accessions and their origins are presented in Table 1. All plants were provided by Key Lab of Flower Breeding, Henan Institute of Science and Technology. Fresh young leaves were collected from the five or six leaves seedlings of each accession for DNA isolation.

DNA Extraction

DNA was extracted from 0.2 g fresh leaves using SDS method (Wang and Fang, 2009). The DNA quality was checked on a 0.7% (w/v) agarose gel and the concentration was measured by UV visible (Thermo Scientific NanoDrop2000, U.S.A.). All DNA samples were diluted to 20 ngL^{-1} and stored at -20ºC prior to PCR amplification.

SRAP Genotyping

Eleven sequence-related amplified polymorphism (SRAP) primers including five forward primers and six reverse primers as described by Li and Quiros (2001) (Table 2) produced thirty primer combinations to test DNA amplification. Twenty-four primer combinations showed high ability to detect polymorphism (Table 3). The amplification mixture of SRAP with a total volume of 25 μL consisted of 2.5 μL of 10×buffer (with Mg^{2+}), 2 μL of dNTP (2.5 m*M*), 1.5 μL of left primer (10 m*M*), 1.5 μL of right primer (10 m*M*), and 2 μL of DNA template (20 ng μL^{-1}), 0.3 μL of Taq polymerase (5 U μL^{-1}). The amplification was programmed for an initial pre-denaturation step of 5 min at 94ºC, then 5 cycles of 1 min denaturation at 94ºC, 1 min annealing at 36ºC and 1 min extension at 72ºC, followed by 35 cycles of 1 min at 94ºC, 1 min annealing at 50ºC, and 1 min extension at 72ºC, ending with a final extension of 10 min at 72ºC.

All PCR products were separated on 6% (w/v) denaturing polyacrylamide gels in 1×TBE buffer solution at 70 w power for 2.5 h, and then stained using the fast silver staining protocols (Du *et al.*, 2010). The separated DNA bands were visualized and estimated by comparing with 2,000 bp ladder molecular size standard (Solarbio). To reduce deviation, only clear DNA bands were utilized in SRAP analysis.

Data Analysis

In terms of the presence (1) or absence (0) with clear and polymorphic bands, a binary data matrix was generated. Resolving power (Rp) (Prevost and Wilkinson, 1999) of primers was to discriminate pansies accessions was calculating according to the formula of Gilbert *et al.* (1999)

$$Rp = \sum Ib \quad (1)$$

$$\mathrm{Ib} = 1 - 2 \times \sqrt{(0.5 - P)^2} \quad (2)$$

where p was the proportion of 40 accessions possessing 1 band. Nei's gene diversity (H), shannon information index (I), and the percentage of polymorphic bands (PPB), were calculated using Popgene32 (Quardokus, 2000). Total genetic diversity of forty accessions and within various cultivar groups were estimated by Popgene32 based on the percentage of polymorphic loci.

To investigate the genetic structure of the selected

Table 1: List of pansy accessions used in this study and their flower size, flower color and their origins

NO	Name	Flower size*	Flower color	Country/Company of origin	Species
1	XXL Yellow Blotch	extra large	yellow with brown blotch	USA/PanAmerican Seed	*V.×wittrockiana*
2	Matrix^{TM} Yellow Clear	large	yellow	USA/PanAmerican Seed	*V.×wittrockiana*
3	Matrix^{TM} Blue Frost	large	blue white with blotch	USA/PanAmerican Seed	*V.×wittrockiana*
4	Matrix^{TM} White	large	white	USA/PanAmerican Seed	*V.×wittrockiana*
5	Panola^{TM} –Blue true	medium	deep blue with purple whiskers	USA/PanAmerican Seed	*V.×wittrockiana*
6	Panola^{TM} Pink Shade	medium	pink shade with purple whiskers	USA/PanAmerican Seed	*V.×wittrockiana*
7	Schweizer Riesen Firnengold	large	yellow with black blotch	Germany/Dehner Seed	*V.×wittrockiana*
8	Schweizer Riesen Alpensee	large	blue with black blotch	Germany/Dehner Seed	*V.×wittrockiana*
9	Johnny Jump Up	mini	purple with yellow blotch	Germany/Dehner Seed	*V. cornuta*
10	Schweizer Riesen coronation gold	large	yellow	NL/Buzzy Seeds	*V.×wittrockiana*
11	Mont Blanc	medium	white	NL/Buzzy Seeds	*V.×wittrockiana*
12	229.05	medium	scarlet with black blotch	China/JiuQuan Jinqiu Horticulture Seed	*V.×wittrockiana*
13	229.07	medium	white	China/JiuQuan Jinqiu Horticulture Seed	*V.×wittrockiana*
14	229.01	medium	yellow	China/JiuQuan Jinqiu Horticulture Seed	*V.×wittrockiana*
15	229.04	small	white	China/JiuQuan Jinqiu Horticulture Seed	*V.×wittrockiana*
16	229.10	small	black	China/JiuQuan Jinqiu Horticulture Seed	*V×wittrockiana*
17	229.14	small	white with blue blotch	China/JiuQuan Jinqiu Horticulture Seed	*V.×wittrockiana*
18	EYO	extra large	yellow	China/Shanghai Academy of Landscape Architecture Science and Planning	*V.×wittrockiana*
19	EWO	large	white	China/Shanghai Academy of Landscape Architecture Science and Planning	*V.×wittrockiana*
20	ERO	medium	scarlet	China/Shanghai Academy of Landscape Architecture Science and Planning	*V.×wittrockiana*
21	EP	medium	purple with black blotch	China/Shanghai Academy of Landscape Architecture Science and Planning	*V.×wittrockiana*
22	EO	mini	scarlet with yellow center	China/Shanghai Academy of Landscape Architecture Science and Planning	*V. cornuta*
23	XXL-YB-1	extra Large	yellow with brown blotch	Breeding line	*V.×wittrockiana*
24	M-YC-1	large	orange	Breeding line	*V.×wittrockiana*
25	M-YB-1	large	yellow with brown blotch	Breeding line	*V.×wittrockiana*
26	PXP-BT-L	medium	light blue with purple whiskers	Breeding line	*V.×wittrockiana*
27	HsY4-1	large	yellow	Breeding line	*V.×wittrockiana*
28	HAR2	large	red	Breeding line	*V.×wittrockiana*
29	HAR2-1	large	red with brown blotch	Breeding line	*V.×wittrockiana*
30	08-NL-5	small	purple	Breeding line	*V.×wittrockiana*
31	G1-X-1	medium	yellow	Breeding line	*V.×wittrockiana*
32	G11-5-1	small	red	Breeding line	*V.×wittrockiana*
33	G11-6-1	small	red	Breeding line	*V.×wittrockiana*
34	G10-B	small	black	Breeding line	*V.×wittrockiana*
35	YP-1	medium	purple and white	Breeding line	*V.×wittrockiana*
36	CW-1	medium	white	Breeding line	*V.×wittrockiana*
37	WH	medium	white	Breeding line	*V.×wittrockiana*
38	WO	medium	white	Breeding line	*V.×wittrockiana*
39	YL	medium	white with purple whiskers	Breeding line	*V.×wittrockiana*
40	YP	medium	purple and white	Breeding line	*V.× wittrockiana*

Table 2: SRAP Primers used in this study (Li and Quiros, 2001)

Forward primers (5'→3')		Reverse primers(5'→3')	
me1	TGAGTCCAAACCGG ATA	em1	GACTGCGTACGAATTAAT
me2	TGAGTCCAAACCGGAGC	em2	GACTGCGTACGAATTTGC
me3	TGAGTCCAAACCGGAAT	em3	GACTGCGTACGAATTGAC
me4	TGAGTCCAAACCGGACC	em4	GACTGCGTACGAATTTGA
me5	TGAGTCCAAACCGGAAG	em5	GACTGCGTACGAATTAAC
		em6	GACTGCGTACGAATTGCA

pansies genotypes, unweighted pair group method arithmetic averages (UPGMA) and a principal coordinate analysis (PCoA) based on the simple matching similarity coefficients were used to cluster the samples using NTSYSpc 2.1 (Jensen, 1989). Additionally, the genetic structure among these pansy accessions was investigated using a Bayesian model-based program STRUCTURE 2.3 (Pritchard *et al.*, 2000). The best K value was estimated through the likelihood of the probability of data L (K) (=Ln P(D)), as well as an ad hoc quantity △K (Rosenberg *et al.*, 2001; Evanno *et al.*, 2005). Using the admixture model, the first run was done by replicates for 20 times at each K from 1 to 10 with a burnin length for 1000 and 10,000 for MCMC (Markov Chain Monte Carlo) and the probable number of

Table 3: Amplified parameters of SRAP primer combinations on 40 pansy accessions

Primer combinations	No. bands	No. polymorphic bands	Percentage of polymorphic bands/%	Number of different accessions identified	Resolving power of Primers (Rp)
me1-em1	19	17	89.5	37	7.20
me1-em2	27	24	88.9	39	10.45
me1-em3	27	27	100.0	36	8.45
me1-em5	12	12	100.0	22	4.30
me1-em6	15	15	100.0	37	8.20
me2-em1	4	4	100.0	33	2.95
me2-em2	3	3	100.0	33	2.05
me2-em3	23	23	100.0	40	11.60
me2-em5	6	6	100.0	17	1.65
me2-em6	12	10	83.3	40	5.80
me3-em1	10	10	100.0	29	5.30
me3-em2	40	36	90.0	40	19.75
me3-em3	25	22	88.0	39	14.35
me3-em4	13	12	92.3	40	5.95
me3-em5	16	14	87.5	27	3.70
me4-em1	36	33	91.7	40	14.10
me4-em2	15	13	86.7	37	7.50
me4-em3	26	25	96.2	36	14.45
me4-em4	11	8	72.73	33	2.55
me4-em5	21	21	100.0	40	12.65
me4-em6	13	12	92.3	35	6.15
me5-em1	31	31	100.0	40	15.60
me5-em2	25	24	96.0	40	11.65
me5-em6	30	28	93.3	40	14.85
Total	460	430			109.15
Average	19.2	17.9	93.5		9.92

groups (K = 4 or 5) was estimated by Ln P(D). The second run was performed with 10,000 for burnin length and 100,000 for MCMC by 20 times replicates, at each K from 2 and 8. Distruct was used to summarize the results in a bar plot (Rosenberg, 2004).

Results

Polymorphism and Primer Evaluation

The twenty-four SRAP primer combinations generated a total of 460 bands, of which 430 (93.5%) bands were polymorphic (Table 3). The number of polymorphic bands per primer combination was 17.9 averagely. The primer combination, Me3–Em2, produced the most polymorphic bands with 36, but Me2–Em2 generated the least polymorphic bands with 3.

The resolving power of the SRAP primers combinations ranged from 1.65 by Me2-Em5 to 19.75 by Me3-Em2 with a mean of 9.92. Six SRAP primers combinations (Me3-Em2, Me5-Em1, Me5-Em6, Me4-Em3, Me3-Em3 and Me4-Em1) possess the highest Rp values (19.75, 15.60, 14.85, 14.45, 14.35, and 14.10, respectively) and each can distinguish all 40 pansy accessions. As we expect, most of primer combinations produced more polymorphic bands and had higher resolving power.

Genetic Diversity Analysis

The percentage of polymorphic bands (PPB), the Nei's gene diversity (H) and the Shannon information index (I) across 40 pansy accessions and within groups classified by origin and flower size, are showed in Table S1. The Nei's gene diversity and the Shannon information index across 40 accessions were 0.271 and 0.424 respectively. Except breeding lines, the pansy accessions from Jiuquan Jinqiu Horticulture Seed had the highest PPB, genetic diversity and information than those from other companies. With respect to flower size, the highest PPB, genetic diversity and information were observed in medium flower groups, and the lowest in mini flower group.

The largest genetic distance (0.217) was exhibited between the cultivars from Dehner Seed and those from Buzzy Seeds. But the genetic distances among cultivars from PanAmerican Seed, Jiuquan Jinqiu Horticulture Seed, Shanghai Academy of Landscape Science and Planning and breeding lines were very low. The distances among groups of large flower, medium flower and small flower were relatively low. The largest genetic distances were among mini flower group to others. Extra-large flower group showed intermediate distances to others (Table S2).

The differentiation of all individuals was investigated using PCoA (principal coordinate analysis) (Fig. 1). The first and second coordinates accounted for 30.1% and 24.8% of the total variance and most of accessions were grouped into three groups as shown in the Fig. 1.

Program STRUCTURE presented the genetic structures and the ancestry of individuals. The L (K) value reached its maximum at K = 4 and the maximum $\triangle$K also presented at K = 4, suggesting that the genetic background of these accessions could be classified into four. In the

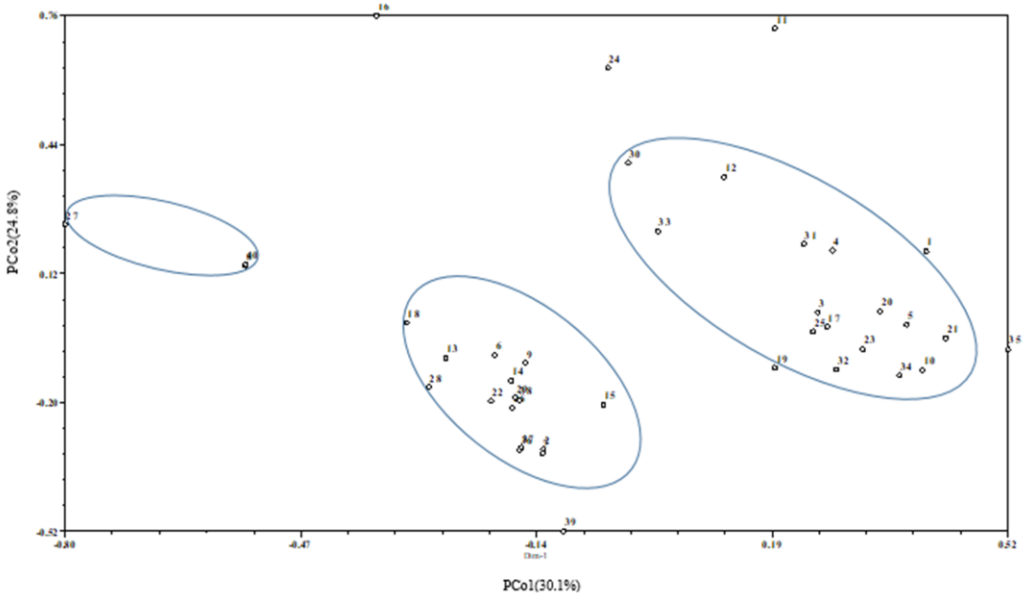

Fig. 1: Principal coordinates of PCoA based on genetic similarity of genetic distances obtained from SRAP markers

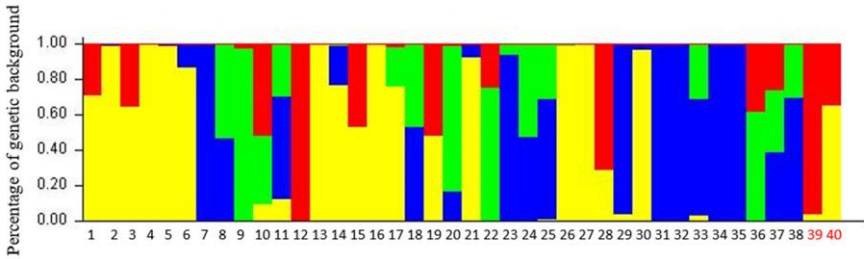

Fig. 2: Inferred genetic structure of pansies by STRUCTURE based on SRAP posterior probability. Numbers below the figure represent the code of accessions which were as same as those in table 1. Different colors different genetic background

barplot (Fig. 2), most accessions were observed having two or more ancestry genetic backgrounds. All cultivars from PanAmerica Seed shared similar genetic backgrounds with most cultivars from JiuQuan JinQiu Horticulture Seed, four breeding lines (No.26, 27, 30, 40) and a cultivar from Shanghai Academy of Landscape Architecture Science and Planning (No.21) (yellow). 'Jonny Jump Up' (No. 9), 'Schweizer Riesen Alpensee' (No.8), 'Schweizer Riesen coronation gold'(No.10), two cultivars from Shanghai Academy of Landscape Architecture Science and Planning (No.20, 22) and two breeding line (No.36, 24) shared a common ancestry (green). 'Schweizer Riesen Firnengold' (No.7) and 'Schweizer Riesen Alpensee' (No.8) shared a common ancestry with 'Mont Blanc' (No.11), cultivars from Shanghai Academy of Landscape Architecture Science and Planning (No.18) and seven breeding lines (No.23-25, 29, 31–35, 38) (blue). A cultivar (No.12) from JiuQuan JinQiu Horticulture Seed and two breeding lines (No.28, 39) presented from direction selection from 'Schweizer Riesen coronation gold'(No.10) (red).

Discussion

SRAP has been widely used for genetic analysis in many species, such as *Brassica oleracea*, *Cucurbita pepo*, buffalograss, petunia, chrysanthemum, *V. tricolor*, yam, purslane, cotton, and *Saccharum spontaneum, etc.* (Li and Quiros, 2001; Ferriol *et al.*, 2003; Budak *et al.*, 2004; Xu *et al.*, 2008; Zhang *et al.*, 2010; Wang *et al.*, 2012; Wu *et al.*, 2014; Jia *et al.*, 2017; Sheidai *et al.*, 2018; Yu *et al.*, 2019). In this research, twenty-four primer combinations of SRAP generated a total of 430 polymorphic bands, with 17.9 polymorphic bands per primer combination averagely. More polymorphic bands generating by similar primers in pansies than in *B. oleracea and* pepper reflected that pansies have more diversity. By the same pansies germplasm collection, RSAP presented to generate more polymorphic bands (21.81% polymorphic bands per primer pairs) (Li *et al.*, 2015) than SRAP did. However, SRAP primers could generate more polymorphic bands after primers were assayed according to Wang *et al.* (2012) in which twenty-one primer pairs

selected out of 88 to produced 23.8 polymorphic bands per primer in *V. tricolor* and *V. cornuta*.

Pansies were usually classified by flower size such as extra-large, larger, medium and small to facilitate planting. Does this classification in accordance with the genetic differentiation of pansies? In this study, the clusters of pansies accessions by PCoA and STRUCTURE analyses based on SRAP markers were not either in line with the flower size types or flower colors, but reflected the original places of accessions doing important influence. Wang and Bao (2007) analyzed genetic relationship between eighteen pansy inbred lines by RAPD and found UPGMA cluster results were consistent with the flower size types and the original places of the inbred lines. But, Wang *et al.* (2012) found flower color was a principal factor for the cluster result when using SRAP marker for genetic relationship analysis of pansies cultivars.

Genetic diversity of pansy cultivar groups was described previously based on morphological characters such as flower size, flower color, presence/absence of blotch, number of flowers per plant, plant height and width (Baloch *et al.*, 2009; Du *et al.*, 2010), as well as molecular markers like SRAP and RSAP (Wang *et al.*, 2012; Li *et al.*, 2015). The Nei's gene diversity across the same germplasm collection of pansies using SRAP markers in the studied (H = 0.27) is very close to that by RSAP marker in Li *et al.* (2015) (H = 0.25), demonstrating reliability of the diversity detected by SRAP. The diversity of the pansies collection in this study was like that of rose (0.27) (Xu *et al.*, 2011), but higher than that of tobacco (H = 0.16) (Qi *et al.*, 2012). Since sample size is an important factor in influencing genetic diversity. This could explain the relatively lower genetic diversities of cultivars from Dehner Seed and Buzzy Seeds than those from others place in this study.

Bayesian inference is an effective method to study materials human disturbed such as cultivars, for the assemblage of these materials cannot be strictly regarded as natural populations. One important application of the Bayesian model based software STRUCTURE is to identify admixed individuals and present the ancestry of individuals (Tang *et al.*, 2013). In this study, most pansy accessions presented as an assemblage of two or more ancestries (Fig. 2). The original ancestries of these accessions could be traced according to the breeding history of pansies. 'Johnny jump up' has been regard as the earliest cross parent in pansies breeding in England in the early of eighteenth century. In the middle of 20th century, 'Schweizer Riesen' series cultivars were bred in Sweden and then become popular (Zhang and Gu, 2009). In this study, 'Johnny jump up' showed to influence on the genetic background of 'Schweizer Riesen Alpensee; 'Schweizer Riesen coronation gold', and some cultivars of Shanghai Academy of Landscape Architecture Science and Planning. 'Schweizer Riesen Firnengold' owned another genetic origin and did important influence on the genetic background of 'Mont Blanc; a cultivar of Shanghai Academy of Landscape Architecture Science and Planning and most of breeding lines. Cultivars of PanAmerican Seed owned another ancestry and provided genetic basis for most of cultivars of JiuQuan JinQiu Horticulture Seed and some breeding lines.

Conclusion

This study indicated that SRAP was a powerful tool in genetic diversity analysis in pansies with a high resolving power. In the germplasm collection of pansies, the medium flower group displayed the highest genetic diversity that means the genetic background of them was broader than that of others. The clustering of the accessions reflected the original places had largely influenced on the genetic relationships among accessions. The genetic backgrounds of most pansies accessions were admixed. This study will be helpful to crossing parent selection in pansies breeding.

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