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GENETIC DIVERSITY AND RELATIONSHIPS OF 48 CULTIVARS OF *GERBERA JAMESONII* ADLAM IN CHINA REVEALED BY MICROSATELLITE MARKERS

ZHENG CHEN, YANHUI SHEN[1], NA LU, MIN WU, CHAOQUN WU AND SHAOYUN HE*

College of Forestry and Landscape Architecture, South China Agricultural University, Guangzhou 510642, China

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Abstract

Gerbera (*Gerbera jamesonii*) is one of the most important floricultural crops for the cut flower trade and ranks the fifth among commercial cut flowers in the world. However, the genetic background of many *G. jamesonii* cultivars is unclear, which limits further breeding programs. In this study, we developed 8 microsatellite markers for *G. jamesonii*by the magnetic beads enrichment method and thenused them to analyze genetic diversity and relationships of 48 cultivars in China. On average, 5.3 alleles (2 - 11) per marker were amplified for these 48 cultivars. The heterozygosity (Ho) ranged from 0.021 to 0.835 for the 8 markers. UPGMA based clustering analysis showed that these cultivars could cluster into four major groups. Principal coordinates analysis (PCoA) showed highly similar results to the UPGMA-based clustering. According to the UPGMA clustering, cultivars in Group I was mainly composed of yellow and pink tubular petals; the trend in Group II was weak, but cultivars with the same colour often cluster together; Group III was composed of only one cultivar with green tubular flower; only three cultivars with yellow tubular petals in Group IV cluster together. Present study revealed relatively high genetic diversity in cultivars of *G. jamesonii* in China and genetic relationships of these cultivars provide useful information for further breeding.

Introduction

Gerbera jamesonii (Compositae), also called African daisy, is native to South Africa and now cultivated all over the world (Danaee *et al.* 2011). It ranks fifth as a cut flower in the world (Bhatia *et al.* 2009, Stephen *et al.* 2011). *Gerbera jamesonii* was first introduced to China in the 1980s and some cultivars have been bred in China subsequently (Yang 2012). Information of genetic diversity and genetic relatedness among these cultivars is a prerequisite for future breeding and improvement programs. Most cultivars of *G. jamesonii* in China were introduced from foreign countries (Xin *et al*. 2010), and the genetic background of most of the cultivars remains unclear.

So far, there are only two reports focusing on the genetic diversity of cultivars of *G. jamesonii* in China. Li *et al.* (2004) reported genetic diversity of 22 cultivars using ISSR markers. Later, Gong *et al.* (2010) identified 89 EST (expressed sequence tag)-SSR markers and further used some of these markers to examine the genetic diversity and relatedness of 40 cultivars (Gong *et al.* 2012). Although these reports provided some useful information, insufficient sampling or unsuitable markers could not give a comprehensive and precise picture of genetic diversity and relatedness of cultivars of *G. jamesonii* in China. Genomic SSR markers are usually more polymorphic than EST-SSR markers because the latter are tightly linked to coding genes, which are often subject to purifying selection (Shokeen *et al.* 2011, Bhatia *et al.* 2009, Spandana *et al.* 2012, Zhang RY *et al.* 2012, Zhao *et al.* 2012). For closely related cultivars, the advantage of genomic SSR markers is more obvious.

*Author for correspondence: <syhe2001@163.com>. [1]College of Horticulture, South China Agricultural University, Guangzhou -510642, China. Zheng Chen and Yanhui Shen have contributed equally to this work.

Forty eight cut flower cultivars of *G. jamesonii* were collected from two biggest cultivation areas in China, Yunnan and Guangdong province. We developed 8 genomic SSR markers with the microsatellite enrichment method (Kandpal *et al.* 1994, Varshney *et al.* 2005) and used these markers to examine genetic diversity and relatedness of 48 cultivars of *G. jamesonii* in China, with a view to providing basic information for breeding new cultivars.

Materials and Methods

Forty eight cultivars of *G. jamesonii* were sampled in this study (Table 1). 28, 12 and 8 cultivars were collected from Yunnan Institute of Flowers (Kunming, Yunnan), Suilong Gerbera Company (Guangzhou, Guangdong) and South China Agriculture University (Guangzhou, Guangdong). Some of the main ornamental characteristics of the 48 gerbera cultivars are shown in Table 1. Genomic DNA was extracted from silica-dried leaves with the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Dolye 1987). A 1.2% agarose gel electrophoresis was used to examine the quality and concentration of total DNA.

SSR marker development in this study followed the method of Liu (2010). In brief, approximately 250 ng MseI-digested genomic DNA was ligated to an MseI adapter and the ligation product was diluted and amplified with an MseI-N primer. The PCR products were denatured and hybridized with each of the two 5′-biotinylated probes, $(AG)_{15}$ and $(AC)_{15}$ to construct two independent microsatellite libraries. Streptavidin magnesphere paramagnetic particles (Promega Corporation, Madison, Wisconsin, USA) were used to separate the DNA fragments hybridized to the probes. The fragments were washed twice and the recovered DNA fragments were amplified. The PCR products were purified and then ligated into pMD18-T vector (TaKaRa Biotechnology Co., Dalian, China), and then transformed into competent cells JM109 by thermal treatment. Transformants were selected on Luria-Bertani (LB) agar medium containing 100 μg/ml ampicillinum natricum. Two hundred eighty eight positive clones were selected and tested by PCR using $(AG)_{10}$, $(AC)_{10}$, and M13 universal primers, of which 84 clones contained potential microsatellite motifs. These positive clones were sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA).

Clones containing five or more repeats were selected for primer design using primer 3 (http://frodo.wi.mit.deu/primer3/). To test these SSR primers, PCR amplifications were conducted using three cultivars in a final volume of 20 μL, containing 25 ng of genomic DNA, 10× PCR buffer (with Mg^{2+}), 2.5 mM of each dNTP, 10 μM of each primer set, and 1U TaqEx DNA polymerase (TaKaRa Biotechnology Co., Dalian, China). The PCR reactions were carried out for all primers with the following cycling conditions: 5 min of denaturation at 95ºC, followed by 30 cycles of 45s at 94 min, 60s at 55 min, and 60s at 72 min, with a final extension of 8 min at 72ºC. Amplification products were first electrophoresed through 1% agarose gels to assess whether amplification was successful and the expected sizes were obtained.

At last, we selected 8 SSR primers for genotyping of the 48 cultivars (Table 2). Forward SSR primers were labeled with fluorescent dye FAM or HEX and fragment length polymorphisms were visualized on an ABI 3730 DNA analyzer and scored using GeneMapper 3.7 (Applied Biosystems, Foster City, CA, USA).

Genetic diversity analysis of the 48 cultivars was conducted in POPGENE (Yeh *et al.* 1999). We calculated the number of alleles (Na), the number of average effective alleles (ne*)(Kimura M and Crow JF 1964), the observed heterozygosity (Ho) and Shannon information diversity index (I*) (Lewontin 1972). Genetic similarity was calculated on the basis of Jaccard's similarity coefficient (Sneath and Sokal 1973). The resulting matrix of genetic similarity was used to construct the dendrogram through the unweighted pair group method with arithmetic mean

(UPGMA) implemented in package NTSYS-pc (Version 2.10) (Rohlf 1998). Principal coordinate analysis (PCoA) was also carried out using NTSYS-pc (Version 2.10).

Table 1. Forty eight cultivars of *G. jamesonii* used in this study.

Cultivar	Color of ligulate petal	Color of tubular petal	Flower type
13	Orange (yellow edge)	Green	Double
88	Aurantium	"	"
2P6	Purple pink	"	"
2P8	Pink	Dark brown	"
A	Pink	Green	"
C3	Yellow	"	"
C4	Yellow	Dark brown	"
CH	Yolk yellow	"	Semi-double
DC	Red	Green	"
DL	Purple pink	"	"
DN	Orange (yellow edge)	Dark brown	Single-valve
F3	Peach pink	Green	Double
FZ	Purple	"	Semi-double
HJ	Dark yellow	Brown	Double
HW	Orange yellow	Dark brown	Semi-double
HY	Red	Green	"
R6	Prunosus	Black purple	Single-valve
S-1	Orange	Dark brown	Double
S-10	Peach pink	Green	"
S-11	Red	"	"
S-12	Orange	"	Single-valve
S-13	Orange red	"	Double
S-14	Pink	"	"
S-15	Light yellow	Dark brown	"
S-16	Orange	"	"
S-17	Orange	Green	"
S-18	Red heart, white edge	Dark brown	"
S-2	Pink	"	"
S-20	Orange yellow	Green	Single-valve
S-21	Pink	Dark brown	Double
S-22	Yellow	"	"
S-23	The lion pink	Dark brown	"
S-24	Pink	Green	"
S-25	Water pink	"	"
S-3	Yolk yellow	"	"
S-4	Yellow	"	"
S-6	Pink	Dark brown	"
S-7	Pink	Green	"
S-8	Orange	Dark brown	"
S-9	Red	Green	Semi-double
SN	Blush over yellow	Dark brown	"
SZ	Pink	"	Double
W4	White	"	"
YD	White	Green	Semi-double
YR2	Gold yellow	Dark brown	Double
S-19	Red heart, yellow edge	"	"
2P5	Pink	Green	Semi-double
S5	Peach pink	"	Double

Results and Discussion

Authors sequenced 84 clones from the SSR libraries and found that 53 clones contained expected simple sequence repeats (SSR). Among which, 30 sequences were suitable for primer design. For 21 of the 30 primer pairs, successful amplification was observed in three selected cultivars of *G. jamesonii*, and 8 of the 21 SSR loci were polymorphic in the three cultivars. These 8 polymorphic SSR loci (Table 2) were used to examine the genetic diversity of 48 cultivars in China.

Table 2. Eight microsatellite loci of *Gerbera jamesonii* developed in this study.

Locus	Primers sequence (5'–3')	Repeat motif	Size (bp)	Ta (%)	GenBank ID
AG-29	F:5'-AACTGCATTCA CCCTCCCTA-3'	$(CT)_{20}$	245-277	58	KF018570
	R:5'-TGCCATTGATCT CTCTTTATGG-3'				
AC-49	F:5'-TGCTAGCACACC AATGGAGA-3'	$(CA)_3$-$(TG)_8$	170-176	64	KF018564
	R:5'-CATGAAGATGTG AAAGCTGG-3'				
AC-109	F:5'-GTACCCCCATGG AGACACAC-3'	$(GT)_8$	151-153	61	KF018569
	R:5'-TTTGAAGATCT CTTGACAGCA-3'				
AC-120	F:5'-TAACCCTCATCG TGATATATTGG-3'	(GA)7-$(AG)_3$-$(AG)_6$	200-220	52	KF018565
	R:5'-ATTTTGATCCCG CCTCAGTT-3'				
AG-4	F:5'-TTTGGCATGCTT ACAGACGA -3'	$(GT)_8$	207-215	58	KF018568
	R:5'-GAACCACCATCC GCATACTC-3'				
AG-134	F:5'-CCGAGATGTTTC CGATGATG-3'	$(GT)_7(AC)_3$	213-217	60	KF018563
	R:5'-TGAAAAGGGCAA GCTCATCT-3'				
AG-62	F:5'-GGTCCATATCCC GATCAATG -3'	$(CA)_6$-$(CA)_3$-$(CA)_3$	247-259	60	KF018567
	R:5'-CATGGCGTCAAT GAAACAAG-3'				
AC-21	F:5'-ATCATGGCTTGG AGAACCAC-3'	$(TG)_6$	230-240	59	KF018566
	R:5'-CGGAACTTCCCT TCTTGACA-3'				

Ta = Annealing temperature.

Using 8 pairs of SSR primers, 42 alleles from the 48 cultivars of *G. jamesonii* were amplified, with an average of 5.3 alleles per locus. The number of alleles (Na) and the number of average effective alleles (ne*) ranged from 2 to 11, and from 1.02 to 5.76, respectively (Table 3). The observed heterozygosity (Ho) ranged from 0.021 to 0.917, with an average of 0.529. The Shannon

information diversity index (I*) was 0.058 to 1.956 (Table 2). Based on these diversity statistics, there was relatively high genetic diversity in the 48 cultivars in China.

Table 3. Genetic diversity in 48 cultivars of *Gerbera jamesonii* in China.

Primers	Na	Ho	ne*	I*
G-AG-29	11	0.917	5.760	1.956
G-AC-49	2	0.146	2.000	0.693
G-AC-109	2	0.021	1.021	0.058
G-AC-120	8	0.708	2.309	1.234
G-AG-4	4	0.646	2.324	0.983
G-AG-134	3	0.188	1.947	0.807
G-AG-62	6	0.813	3.411	1.432
G-AG-21	6	0.792	3.254	1.373

Na = Number of alleles; Ho = Observed heterozygosity; ne* = Effective number of alleles; I* = Shannon's Information index.

Principal coordinates analysis (PCoA) based on the genetic distances indicated that 48 gerbera cultivars were grouped into four clusters, and they were designated as cluster A, B, C and D (Fig. 1). Cluster A included 15 cultivars (88, S-3, S-24, S-18, S-1, YD, S-20, S-15, F3, 2P5, S-25, R6, CH, C4 and FZ); Cluster B included 18 cultivars (S-14, A, S-7, 13, S-12, S-5, DN, SZ, DL, S-11, 2P6, S19, C3, S-10, DC, YR2, HW and S-9); Cluster C included 12 cultivars (S-17, S-16, S-23, SN, S-4, S-22, HY, W4, 2P8, S-21, S-2 and S-6); and Cluster D included 3 cultivars (S-13, S-8 and HJ). Clustering analysis with the UPGMA method generated similar result to the PCoA analysis, and if taking 0.76 as the genetic similarity coefficient threshold, the 48 cultivars were divided into 4 groups (I, II, III and IV). Group I matched with Cluster C in the PCoA analysis, Group II corresponded to Cluster A and B, and Group IV was identical with Cluster D. Group III included only one cultivar (DC). Both Group I and Group II could be further divided into 3 subgroups (Fig. 2).

In this study, we used 8 genomic polymorphic SSR markers to determine genetic diversity of 48 *G. jamesonii* cultivars in China. The average number of SSR alleles, the average observed heterozygosity (Ho) and the average Shannon information diversity index were 5.25, 0.529 and 1.067, respectively, which were higher than those in Gong's (2012) study (3.19, 0.386 and 0.843, respectively). Hence, our genomic SSR markers developed in this study have higher polymorphisms than the previous EST-SSR markers. The primers developed here should be suitable for investigating the genetic diversity of all cultivars of *G. jamesonii*. Relatively high diversity in these cultivars may be caused by multiple introductions. As an important cut flower, *G. jamesonii* was first introduced to China in the 1980s (Yang 2012) and after that different cultivars have been introduced for several times (Xin *et al*. 2010). This would be the reason for a high level of genetic variation in the cultivars in China. In this study, 48 cut flower cultivars of *G. jamesonii* were collected from two biggest cultivation areas in China, Yunnan and Guangdong province. Compared to Gong *et al.* (2012), we focused only on cut flower cultivars and much more cut flower cultivars were used. The cultivars we used are relatively rich in flower color.

The genetic relationships among cultivars of *G. jamesonii* are valuable and important for its cross breeding. The genetic similarity coefficient between 48 cultivars in China ranges from 0.595 to 1.000, indicating remarkable genetic disparity between some cultivars, despite high similarity between others. For example, the genetic similarity coefficients between HJ and DL, between S-13

and CH, and between S-14 and DL are relatively small. In contrast, genetic similarities between cultivars C4 and CH, between A and S-7, and between S2 and S6 are high.

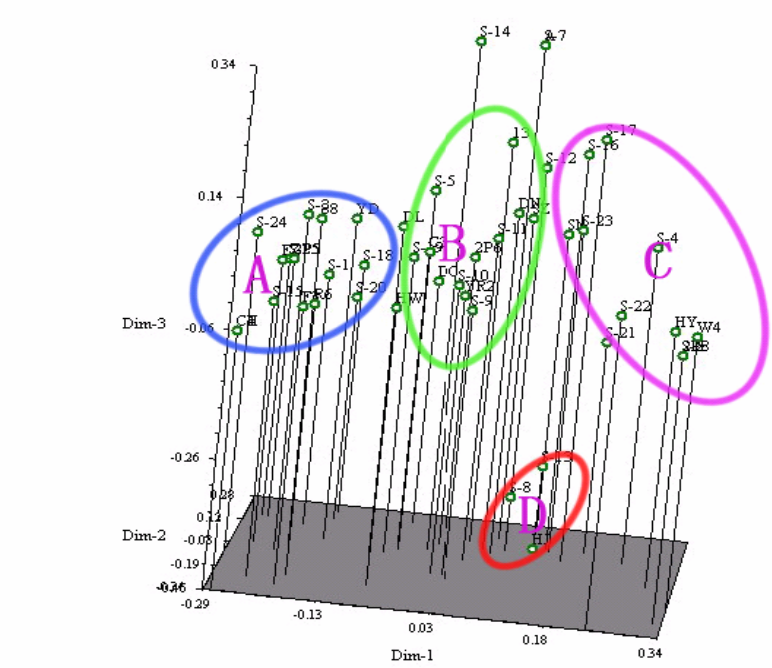

Fig. 1. Three dimensional scatter plot of principal coordinates analysis (PCoA) of 48 gerbera cultivars.

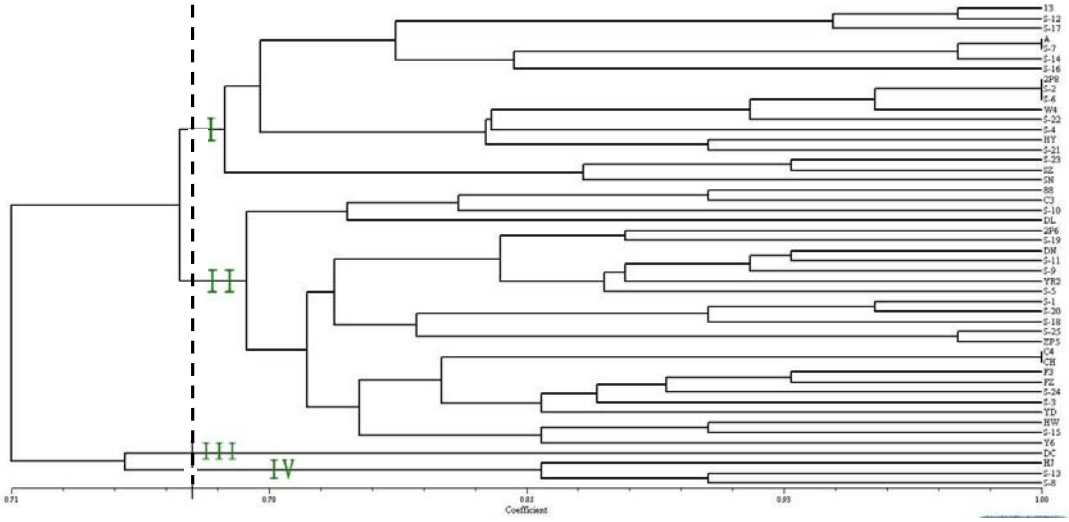

Fig. 2. Clustering analysis of 48 gerbera cultivars using UPGMA method based on 8 SSR markers. Taking similarity coefficient of 0.76 (the dash line in the figure) as the threshold, 48 gerbera cultivars can be divided into 4 groups.

According to the UPGMA analysis, genetic relatedness of these cultivars is sometimes associated with the color of ligulate flower and tubular flower. In Group I, cultivars usually cluster with the color of the ligulate flower. For example, cultivars 13, S-12 and S-17 have orange ligulate flowers and cultivars S-14, S-7 and A have pink ligulate flower. In Group II, the trend of clustering with petal color is weak. Cultivars with the similar color of ligulate petals also have the trend of clustering together. For example, cultivars 88 and C3 with aurantium and yellow petals cluster together. Cultivars 2P5 and S-25 with pink petals cluster together. Group III contains only one cultivar DC with green heart and red semi-double petals, very distint from others. In the Group IV, three cultivars HJ, S-13 and S-8 with yellow to orange petals cluster together, while in other cultivars the association is not obvious.

Group I could be divided into 3 subgroups. In the first subgroup of Group I, cultivars 13, S-12, S-17 with green tubular petals cluster together; cultivars A and S-7 with green tubular petals cluster together; while only the cultivar S-16 with dark brown tubular is kept alone. In the second subgroup of Group I, genetic relatedness with the color of tubular petal is not strong, but cultivars 2P8, S-2, S-6, W4 and S-22 with dark brown tubular petals cluster together. In the third subgroup of Group I, cultivars S-23, SZ and SN with dark brown tubular petals cluster together. As for the three subgroups of Group II, cultivars 88, C3, S-10 and DL have green tubular petals in the first subgroup. In the second subgroup, cultivars 2P6, S-11, S-9, S-5, S-20, S-25 and 2P5 have green tubular petals; cultivars S-19, DN, YR2, S-1 and S-18 have dark brown tubular petals. In the third subgroup, cultivars C4 and CH with dark brown tubular petals cluster together; cultivars F3, FZ, S-24, S-3 and YD with green tubular petals cluster together; cultivars HW, S-15 and R6 with dark brown tubular petals cluster together.

Principal coordinate analysis on these cultivars is largely consistent with the UPGMA-based clustering analysis. The majority of cultivars in Cluster D have yellow ligulate petals, while those in Cluster C have pink ones. In Cluster A and Cluster B, cultivars with red ligulate petals are much more than those with yellow ligulate petals. Our clustering results differ from those of Gong *et al.* (2012), which divided the cultivars of *G. Jamesonii* into 2 clusters. Long-term cross breeding between cultivars with different petal colors may make the genetic relationships more complex. The petal color is not always a very effective indicator of genetic relatedness of these cultivars. Therefore, genetic relationships should be analyzed before cross breeding.

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