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Isolation and characterization of microsatellite markers for *Dendranthema morifolium* (Asteraceae) using next-generation sequencing

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ABSTRACT. *Dendranthema morifolium* (Asteraceae) is a perennial herbaceous plant native to China. A long history of artificial crossings may have resulted in complex genetic background and decreased genetic diversity. To protect the genetic diversity of *D. morifolium* and enabling breeding of new *D. morifolium* cultivars, we developed a set of molecular markers. We used pyrosequencing of an enriched microsatellite library by Roche 454 FLX+ platform, to isolate *D. morifolium* simple sequence repeats (SSRs). A total of 32,863 raw reads containing 2251 SSRs were obtained. To test the effectiveness of these SSR markers, we designed primers by randomly selecting 100 novel SSRs, and amplified them across 60 cultivars representing five different petal shape groups. Sixteen SSRs were polymorphic with the number of alleles ranging from 6 to 19, and their expected and observed heterozygosities ranging from 0.477 to 0.848, and 0.250

to 0.804, respectively. The polymorphism information content ranged from 0.459 to 0.854 and the inbreeding coefficient ranged from -0.119 to 0.759. An unweighted pair-group method arithmetic average analysis was performed to survey the phylogenetic relationships of these 60 cultivars and five clusters were identified. These markers can be used for investigating genetic relationships and identifying elite alleles through linkage and association analyses.

Key words: SSR; Roche 454; Phylogenetic relationship; *Dendranthema morifolium*

INTRODUCTION

Dendranthema morifolium (Asteraceae) is a perennial herbaceous plant native to China. Its flowers have been used in traditional Chinese medicine for centuries. *D. morifolium* has been cultivated in China and Japan for at least 3000 years. Breeding through multiple artificial crossings has led to the formation of a large number of *D. morifolium* cultivars, including cut-flower, potted, garden, and ground-cover types (Zhang et al., 2011). A long history of artificial crossing and selection might result in a complex genetic background and decreased genetic diversity (Khaing et al., 2013). To protect the genetic diversity of *D. morifolium* and breed new *D. morifolium* cultivars, a set of molecular markers should be developed for investigating this species' genetic diversity, examining the genetic relationships of its cultivars, and identifying elite alleles through linkage and association analyses (Yamasaki et al., 2005; Khaing et al., 2013). Simple sequence repeats (SSRs) are considered excellent molecular markers for their abundance, high mutation rates, co-dominance, and relative ease of scoring (Kalia et al., 2011; Yuan et al., 2015; Zhao et al., 2016). SSRs are frequently used to survey phylogenetic relationships and select loci associated with agronomic traits in *D. morifolium* cultivars (Chen et al., 2016). Previous studies have reported some *D. morifolium* SSR markers (Moe et al., 2011; Khaing et al., 2013; Liu et al., 2015; Shim et al., 2015). However, the limited number of SSR markers available for *D. morifolium* fails to meet the requirements for genetic diversity evaluation, as well as linkage and association analyses. In this study, we used the Roche 454 FLX+ platform to obtain SSR markers from an enriched library for *D. morifolium*. These novel SSRs were then used to analyze the genetic relationships of 60 major *D. morifolium* cultivars from Kaifeng.

MATERIAL AND METHODS

Plant material and DNA extraction

All *D. morifolium* samples were collected from Henan University campuses. The 60 cultivars represented five different petal shape groups (Table 1 and Figure 1). Vouchers are deposited in the herbarium of Institute of Chinese Materia Medica in Henan University (Herbarium accession No. HCMM-201-260). Genomic DNA was extracted from fresh leaves using genomic DNA extraction kit v. 3.0 (TaKaRa, Dalian, China) following the manufacturer protocol.

Table 1. Information on the 60 *Dendranthema morifolium* cultivars used in the study.

No.	Accessions	Petal shape	No.	Accessions	Petal shape
1	Shuiqi	Flat	31	Wanguanjinzhu	Tubular
2	Fenshiba	Flat	32	Chixianjinzhu	Tubular
3	Mohe	Flat	33	Xingshitu	Tubular
4	Jinbeidahong	Flat	34	Wuguangshise	Tubular
5	Qiujiewanhong	Flat	35	Jinzhongzhenyu	Tubular
6	Nijinbao	Flat	36	Huowu	Tubular
7	Lvmudan	Flat	37	Qiansiwanlv	Tubular
8	Yipinhong	Flat	38	Zhuyi	Tubular
9	Zixiuqiu	Flat	39	Zisongzhen	Tubular
10	Xiaohonggui	Flat	40	Caihumeiyu	Tubular
11	Yuezhiguang	Flat	41	Taohuaxian	Tubular
12	Chunrijianshan	Flat	42	Yulingguan	Tubular
13	Guohuaxueshan	Flat	43	Huangxiangli	Tubular
14	Fenghuolun	Spoon	44	Guandongdaxia	Tubular
15	Biyugoupan	Spoon	45	Shizhangzhulian	Tubular
16	Qiushuichangliu	Spoon	46	Gudufochen	Tubular
17	Baixiangli	Spoon	47	Dahongtuogui	Anemone
18	Huangpingpang	Spoon	48	Zituogui	Anemone
19	Fenhuangxiaori	Spoon	49	Huanshuifengui	Anemone
20	Tangyuaoshi	Spoon	50	Huanshuimingzhu	Anemone
21	Xiangyunwuhe	Spoon	51	Ruizhugong	Anemone
22	Taizhentu	Spoon	52	Oufentuogui	Anemone
23	Yizhinongyan	Spoon	53	Yuemingxingxi	Anemone
24	Shibafenghuan	Spoon	54	Qianshouguanyin	Abnormal
25	Denghongjiulv	Spoon	55	Fenluhua	Abnormal
26	Xiaguangsishe	Spoon	56	Yucuilongzhao	Abnormal
27	Zongchanfochen	Spoon	57	Zilongzhao	Abnormal
28	Ziyunfeiyue	Spoon	58	Jinlongzhao	Abnormal
29	Shengguanghongshanhu	Tubular	59	Baimaoci	Abnormal
30	Baiou	Tubular	60	Jinfenghuanchao	Abnormal

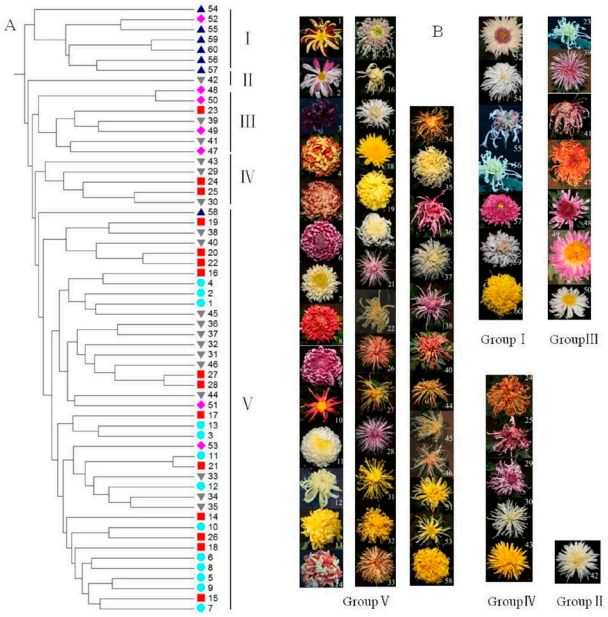

Figure 1. Phylogenetic analysis of 60 *Dendranthema morifolium* cultivars using 16 SSR markers. **A.** Dendrogram of 60 *D. morifolium* cultivars resulting from UPGMA cluster analysis. **B.** Images of the flowers of *D. morifolium* cultivars in each assigned group. Blue arrowhead, gray arrowhead, red square, pink diamond, and light blue circle stand for Abnormal, Tubular, Spoon, Anemone, and Flat petal types, respectively.

DNA sequencing and microsatellite locus search

Approximately 2 μg genomic DNA was digested and separated on a 2% agarose gel. Fragments of 400-700 bp were extracted. Forward (*Eco*RI: 5'-CTCGTAGACTGCGTACC-3', *Mse*I: 5'-GACGATGAGTCCTGAG-3') and reverse (*Eco*RI: 5'-AATTGGTACGCAGTCTAC GAG-3', *Mse*I: 5'-TACTCAGGACTCATCGTC-3') adapters were mixed to prepare double-stranded adapters. Hybridization of biotinylated oligonucleotides [$(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, $(ATCT)_6$, and $(AGG)_8$] and adapter-ligated genomic DNA was performed. Enrichment libraries were purified using the amplicon library preparation protocol. The Lib-L kit and FLX titanium sequencing kit XLR70 (Roche) were used for the library sequencing on the Roche GS-FLX+ system.

MISA v. 1.0 (Thiel et al., 2003) was used to identify SSR loci. The di-, tri-, tetra-, penta-, and hexanucleotide motifs with a minimum of five repeats were considered as SSRs. Primer design parameters were set as follows: length range, 18-24 nucleotides; product size range, 100-500 bp; melting temperature, 55-65°C; GC content, 40-60%; and GC clamps, no more than 3 Gs or Cs in the last five bases at the 3' end using Primer3 (Rozen and Skaletsky, 2000).

Polymerase chain reaction (PCR) amplification and genotyping

Of the 2251 identified SSR markers, we randomly selected 100 microsatellites and blasted them in NCBI GenBank. No similar sequences were found in published data. Subsequently, the primer pairs of the selected SSRs were used to test primer amplification efficiency. Twenty microliters PCR amplification solution consisting of 50 ng genomic DNA, 0.5 μM each primer, and 10 μL 2X Taq PCR MasterMix (0.1 U/μL Taq polymerase, 0.5 mM dNTPs, 20 mM Tris-HCl, pH 8.3). The PCRs were performed using an S1000 thermal cycler (Bio-Rad, USA). The PCR amplification conditions were as follows: pre-denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 60 s and 45 s at the primer-specific annealing temperature; extension at 72°C for 90 s; and a final elongation step at 72°C for 8 min. The PCR products were separated on 8% native polyacrylamide gels and visualized by silver nitrate staining.

Data analysis

The polymorphism information content (*PIC*) was estimated using Cervus v. 3.0.7 (Kalinowski et al, 2007). PopGene v. 1.32 (Yeh et al., 2000) was used to analyze the number of alleles per locus (N_A), expected heterozygosity (H_E), observed heterozygosity (H_O), Shannon's information index (I_S). The significance of the inbreeding coefficient for each locus was tested using FSTAT v. 2.9.3.2 (Goudet, 1995). The Hardy-Weinberg equilibrium for all loci was calculated using GENEPOP 4.2 (Rousset, 2008). The similarity matrix of Dice's coefficient was used to construct a dendrogram based on the unweighted pair group method arithmetic average (UPGMA) using PowerMaker v. 3.25. The significance of the partitioning of genetic variance among different petal shapes was further estimated by analysis of molecular variance using GENALEX 6.41 (Peakall and Smouse, 2006).

RESULTS AND DISCUSSION

A total of 32,863 raw sequence reads were obtained, ranging from 347 to 978 bp with

an average length of 566 bp. Of these raw reads, 2251 were found to contain SSRs and were deposited in GenBank (GenBank accession Nos: KP331842 to KP334092). One hundred SSR primers were randomly selected to design primer pairs and perform PCR amplifications across the 60 *D. morifolium* cultivars, yielding 16 polymorphic amplification products. A total of 160 bands were amplified, with an average of 10 amplified fragments per primer. Fingerprints of all accessions could be amplified from 6 (JH35) to 19 (JH41) bands per primer pair, and PCR product sizes ranged from 156 to 365 bp (Table 2).

Table 2. Primer sequences and characterization of 16 microsatellite loci isolated from *Dendranthema morifolium*.

Primer	Primer sequence (5'-3')	Repeat motif	Ta (ºC)	Allele size (bp)	GenBank accession No.
JH19	F:GGAGGGTTTGAAAACAAGCA R:ATCGCTATTACCATCCGCAC	$(AGAT)_{14}$	63	296-348	KP331860
JH25	F:AGCCTTTTGGAGACGAACAA R:CGACGAAGAAGATGAAGACG	$(ATCTTC)_{10}$	65	233-305	KP331866
JH35	F:TTTTTCATCCTCTGGTTGCC R:CCCGATGCCATATTATCCAC	$(CCTTTT)_{5}$	60	294-336	KP331876
JH39	F:GAACAAAGGGTTTGATCAAGATG R:GGGGCTCAAGAGTGGAATA	$(GA)_{14}$	63	190-212	KP331880
JH40	F:GTGACACGTGGCTTCTCTGA R:CCAACCCATTCATCTCTCTTC	$(GA)_{21}$	58	289-307	KP331881
JH41	F:AAACAGCACAAACCCAAAGG R:CTTACACCGCAAAAGAAGGC	$(GA)_{8}(AG)_{13}$	60	156-199	KP331882
JH42	F:TCTTGACAAAGACGAAGACGAA R:GCACCTCTACCCCATCAACA	$(GACGAT)_{15}$	60	242-342	KP331883
JH47	F:TATCCACGGCCCATAGAAAG R:GAAGGAGGGACCCTGTAGGA	$(GT)_{9}$	62	312-334	KP331888
JH56	F:AGGAGTGATAGTGCACGGCT R:CCAAGGTCTCATTCGATGCT	$(GTT)_{10}$	56	244-268	KP331897
JH57	F:CCCATTTCCTTTTGTTGGTG R:CCCCATCTATGGTTTTGGAC	$(GTT)_{12}$	60	240-264	KP331898
JH70	F:TGAGAGCTTGGTGTGACCTG R:AGTTAGCGTGAATGTGCCCT	$(TG)_{10}(GA)_{18}$	64	265-295	KP331911
JH72	F:CGAGCTTATTTGGTTTTGCC R:ACGAAAGATCGGAGTAAGCG	$(TG)_{13}(ATT)_{5}$	57	300-342	KP331913
JH81	F:GCCGATAGACTATTTGGGCA R:TTGGTATTTCGGCCTCTCAC	$(TGG)_{6}(TGT)_{12}(TTG)_{7}$	58	285-311	KP331922
JH86	F:CAAATACCTCATGTACCTTAAACCTT R:ACTACATTTGCTGGGGTTCG	$(TGT)_{5}(TAT)_{12}$	58	337-365	KP331927
JH87	F:ATGATGAACCACCCGTTTGT R:TCGTCCAAACCAACAAATCA	$(TGT)_{6}(GTT)_{12}$	60	237-261	KP331928
JH99	F:AGGAGTGATAGTGCACGGCT R:CAAGGTCTCGTTCATGCTCA	$(TTG)_{9}(TTG)_{12}$	63	273-297	KP331940

F: forward primer; R: reverse primer; Ta: PCR annealing temperature.

The genetic diversity indices are shown in Table 3. The H_E and H_O ranged from 0.477 to 0.848 and from 0.250 to 0.804, respectively. The average H_E and H_O were 0.788 and 0.535, respectively. The I_S and *PIC* ranged from 1.149 to 2.442 and from 0.459 to 0.854, respectively. The average I_S was 1.829 (Table 3). The inbreeding coefficient ranged from -0.119 (JH47) to 0.759 (JH86). Five of the 16 loci had negative F_{IS} values (all $P > 0.05$), indicating a slight excess of heterozygotes. Total variance was partitioned into components due to differentiation within and among the five petal shape groups. The overall distribution pattern of molecular variation within the cultivars suggested that approximately 91.0% of the total variance could be accounted for by the within group component. The remaining 9.0% of the variation was found among groups. The variance component was shown to be highly significant ($P < 0.05$).

The UPGMA dendrogram was generated to illuminate genetic relationships among the 60 cultivars (Figure 1). Five main clusters (I to V) were observed. Group I comprised five cultivars of the Abnormal petal group (54, 55, 56, 57, 59, and 60) and one cultivar of the Anemone petal group (52). One cultivar of the Tubular petal group (42) constituted Group II.

Table 3. Genetic diversity parameters for 60 *Dendranthema morifolium* cultivars using 16 newly developed microsatellite loci.

Primer	N_A	N_E	H_E	H_O	*PIC*	I_S	F_{IS}
JH19	11	6.2470	0.848	0.571	0.821	2.023	0.497
JH25	9	5.3193	0.816	0.684	0.786	1.865	0.615*
JH35	6	3.9534	0.753	0.373	0.720	1.484	0.182
JH39	9	4.9793	0.806	0.567	0.764	1.827	0.438
JH40	9	3.9929	0.756	0.655	0.725	1.732	0.546*
JH41	19	7.3290	0.871	0.552	0.854	2.442	0.488
JH42	9	4.9057	0.804	0.707	0.776	1.851	0.638*
JH47	10	1.8976	0.477	0.467	0.459	1.149	-0.119
JH56	9	5.5215	0.826	0.300	0.799	1.924	0.153
JH57	8	4.1614	0.766	0.661	0.722	1.601	0.559*
JH70	12	4.5444	0.787	0.525	0.752	1.812	0.398
JH72	14	5.1465	0.813	0.350	0.787	1.993	0.201
JH81	9	5.8489	0.836	0.583	0.806	1.863	0.503
JH86	9	5.0785	0.810	0.804	0.782	1.872	0.759*
JH87	9	6.1909	0.846	0.250	0.819	1.962	0.113
JH99	9	4.6690	0.793	0.517	0.765	1.854	0.393
Mean	10	4.9831	0.788	0.535	0.759	1.829	

N_A: allele number; N_E: effective numbers of alleles; H_E: expected heterozygosity; H_O: observed heterozygosity; *PIC*: polymorphism information content; I_S: Shannon's information index; F_{IS}: inbreeding coefficient; *Significant deviation from Hardy-Weinberg equilibrium.

Group III consisted of four cultivars of the Anemone petal group, two cultivars of the Tubular petal group, and one cultivar of the Spoon petal group. Group IV included three cultivars of the Tubular petal group and two cultivars of the Spoon petal group. Group V contained the remaining 40 cultivars. The clusters were inconsistent with the petal shape grouping (Figure 1), indicating that traditional classification based on petal shapes does not reflect the true phylogenetic relationships of these cultivars.

The present study provides 16 new SSR markers of *D. morifolium*. These SSRs may facilitate conservation strategy development, evolutionary biology research, and molecular breeding of *D. morifolium*. Most of the microsatellite markers obtained in the present study however need further validation. Furthermore, constructing of hybrid groups and recording of phenotypic traits are all future studies on *D. morifolium*, which will enable location of the position of developed SSR markers and construct the linkage map of *D. morifolium*.

Conflicts of interest

The authors declare no conflict of interest.

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