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Development of SSR Markers and Assessment of Genetic Diversity in Medicinal *Chrysanthemum morifolium* Cultivars

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Chrysanthemum morifolium, is a well-known flowering plant worldwide, and has a high commercial, floricultural, and medicinal value. In this study, simple-sequence repeat (SSR) markers were generated from EST datasets and were applied to assess the genetic diversity among 32 cultivars. A total of 218 *in silico* SSR loci were identified from 7300 *C. morifolium* ESTs retrieved from GenBank. Of all SSR loci, 61.47% of them (134) were hexa-nucleotide repeats, followed by tri-nucleotide repeats (17.89%), di-nucleotide repeats (12.39%), tetra-nucleotide repeats (4.13%), and penta-nucleotide repeats (4.13%). In this study, 17 novel EST-SSR markers were verified. Along with 38 SSR markers reported previously, 55 *C. morifolium* SSR markers were selected for further genetic diversity analysis. PCR amplification of these EST-SSRs produced 1319 fragments, 1306 of which showed polymorphism. The average polymorphism information content of the SSR primer pairs was 0.972 (0.938–0.993), which showed high genetic diversity among *C. morifolium* cultivars. Based on SSR markers, 32 *C. morifolium* cultivars were separated into two main groups by partitioning of the clusters using the unweighted pair group method with arithmetic mean dendrogram, which was further supported by a principal coordinate analysis plot. Phylogenetic relationship among *C. morifolium* cultivars as revealed by SSR markers was highly consistent with the classification of medicinal *C. morifolium* populations according to their origin and ecological distribution. Our results demonstrated that SSR markers were highly reproducible and informative, and could be used to evaluate genetic diversity and relationships among medicinal *C. morifolium* cultivars.

Keywords: ***Chrysanthemum morifolium*, EST-SSR, marker development, genetic diversity, phylogenetic relationship**

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INTRODUCTION

Chrysanthemum morifolium (Ramat.) Kitam is an important member of the family Asteraceae. A large number of *C. morifolium* cultivars are ornamental and medicinally important plants that are planted all over the world (Teixeira da Silva, 2003; Bhattacharya and da Silva, 2006). In addition to their esthetic value, some *C. morifolium* cultivars are used medicinally for their curative

effects, particularly for treating common cold, headache, and dizziness (Chinese Pharmmacopoeia Editorial Committee, 2010). Based on growing regions and processing methods, the main domestic varieties of medicinal *C. morifolium* are divided into "Hang-ju," "Bo-ju," "Qi-ju," "Gong-ju," "Ji-ju," "Chu-ju," "Huai-ju," and "Chuan-ju" (Shao et al., 2010; Zhao et al., 2013). In China, some regions have become important producing areas, such as Tongxiang (Zhejiang Province), Yancheng (Jiangsu Province); Wuzhi (Henan Province), Chuzhou, and Sexian (An'hui Province) (Shao et al., 2010; Wang T. et al., 2013; Chen et al., 2014).

For a long time, the genetic improvement of *C. morifolium* has been impeded because of its genome complexity, high level of heterozygosity, and the occurrence of both inbreeding depression and self-incompatibility (Anderson, 2006). Understanding genetic diversity is very important in plant breeding programs and the conservation of genetic resources. Molecular markers have potentials to reveal the genetic diversity among medicinal *C. morifolium* germplasms. Recently, a few studies have been reported on genetic diversity in cultivated chrysanthemums (Bhattacharya and da Silva, 2006; Xu et al., 2006; Shao et al., 2010; Zhang et al., 2011b), preliminary genetic linkage map construction, and QTL detection (Zhang et al., 2010, 2011a) using RAPD, AFLP, ISSR, and SRAP markers.

Simple-sequence repeats (SSRs), also known as microsatellites, are short tandem repeated motifs that may vary in the number of repeats at a given locus (Tautz, 1989). SSR markers have many advantages over other molecular markers, such as genetic co-dominance. They are multi-allelic, relatively abundant, widely dispersed across the genome, and easily and automatically scored (Powell et al., 1996). Over the past few years, SSR markers have been used in genetic diversity analysis (Dirlewanger et al., 2002; Hasnaoui et al., 2012; Shiferaw et al., 2012; Emanuelli et al., 2013; Ren et al., 2014), parentage assessment (Malysheva et al., 2003), species identification (Shirasawa et al., 2013), and mapping genetic linkage (Temnykh et al., 2000; Olmstead et al., 2008; Lu et al., 2012, 2013). In the genus *Chrysanthemum*, SSR markers have been reported

TABLE 1 | List of medicinal *Chrysanthemum morifolium* samples included in this study.

Original accession	Code	Voucher number	Longitude (E)	Latitude (N)	Location
Hangju "Dayangju"	Dyj-1	CM1	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "Dayangju"	Dyj-12	CM12	120°43′	29°05′	Pan'an, Zhejiang province
Hangju "Dayangju"	Dyj-21	CM21	115°03′	31°18′	Macheng, Hubei province
Hangju "Zaoxiaoyangju"	Zxyj-2	CM2	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "Zaoxiaoyangju"	Zxyj-13	CM13	120°43′	29°05′	Pan'an, Zhejiang province
Hangju "Xiaoyangju"	Xyj-3	CM3	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "Xiaoyangju"	Xyj-20	CM20	115°03′	31°18′	Macheng, Hubei province
Hangju "Yizhongdabaiju"	Yzdbj	CM4	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "Xiaohuangju"	Xhj-5	CM5	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "Xiaohuangju"	Xhj-10	CM10	120°43′	29°05′	Pan'an, Zhejiang province
Hangju "No. 1 of Jinju"	Jj1	CM6	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "No. 2 of Jinju"	Jj2-7	CM7	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "No. 2 of Jinju"	Jj2-14	CM14	120°43′	29°05′	Pan'an, Zhejiang province
Hangju "No. 3 of Jinju"	Jj3	CM8	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "No. 4 of Jinju"	Jj4	CM9	120°32′	30°38′	Tongxiang, Zhejiang province
Hangju "Chidahuangju"	Cdhj	CM11	120°43′	29°05′	Pan'an, Zhejiang province
Hangju "Dabaiju"	Dbj	CM15	120°25′	33°78′	Sheyang, Jiangsu province
Hangju "Xiaobaiju"	Xbj	CM16	120°25′	33°78′	Sheyang, Jiangsu province
Hangju "Changbanju"	Cbj	CM17	120°25′	33°78′	Sheyang, Jiangsu province
Hangju "Hongxinju"	Hxj	CM18	120°25′	33°78′	Sheyang, Jiangsu province
Hangju "Dahuangju"	Dhj	CM19	120°25′	33°78′	Sheyang, Jiangsu province
Machengju	Mcj	CM22	115°03′	31°18′	Macheng, Hubei province
Boju "Daboju"	Dboj	CM23	115°78′	33°85′	Bozhou, Anhui province
Boju "Xiaoboju"	Xbj	CM24	115°78′	33°85′	Bozhou, Anhui province
Gongju "Zaogongju"	Zgj	CM25	118°43′	29°87′	Shexian, Anhui province
Gongju "Wangongju"	Wgj	CM26	118°43′	29°87′	Shexian, Anhui province
Gongju "Huangyaoju"	Hyj	CM27	118°43′	29°87′	Shexian, Anhui province
Chuju	Cj	CM28	118°32′	32°3′	Chuzhou, Anhui province
Huaiju "Huaixiaohuangju"	Hxhj	CM29	113°38′	35°1′	Wuzhi, Henan province
Huaiju "Huaidabaiju"	Hdbj	CM30	113°38′	35°1′	Wuzhi, Henan province
Huaiju "Huaixiaobaiju"	Hxbj	CM31	113°38′	35°1′	Wuzhi, Henan province
Huaiju "Huaizhenzhuju"	Hzzj	CM32	113°38′	35°1′	Wuzhi, Henan province

for *C. nankingense* (Wang H. B. et al., 2013). In addition, SSR markers have been used to identify and classify Chinese traditional ornamental chrysanthemum cultivars (Zhang et al., 2014). Nevertheless, current genetic knowledge is very limited for Chinese traditional medicinal chrysanthemum varieties, which hinders genetic conservation and improvement of these endangered, but economically important Chinese medicinal herbs.

In this study, SSR markers were developed and were applied to investigate genetic diversity and phylogenetic relationships among medicinal *C. morifolium* cultivars, with the aim to provide new information that could be used to improve the utilization and conservation of *C. morifolium* genetic resources.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

A total of 32 cultivars of *C. morifolium* were collected from the main distribution areas in China. The sampled germplasms and voucher specimens are shown in **Table 1**. These varieties were verified and confirmed using the specimens stored in the herbarium at the Institute of Botany, Chinese Academy of Sciences, Beijing, China (http://www.nhpe.org). Voucher samples were deposited in the Zhejiang Provincial Key Laboratory for Genetic Improvement and Quality Control of Medicinal Plants, Hangzhou Normal University, China.

Fresh, young leaf tissues from 10 individuals of each cultivar were randomly collected for genomic DNA isolation. The genomic DNA was isolated as described previously (Feng et al., 2013). The integrity and quality of the DNA were evaluated by electrophoresis on 0.8% agarose gels, and the concentration of the genomic DNA samples was determined using a UV spectrometer.

SSR markers Development

A total of 7300 *C. morifolium* EST sequences (total size 3.7 Mb) were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/), with a mean length of 531 bp. These ESTs were analyzed to identify the perfect SSR loci using the MIcroSAtellite (MISA) software (http://pgrc.ipk-gatersleben.de/misa/), following the set to detect tandem repeats of hexa-, penta-, tetra-, tri-, and di-nucleotides, with a minimum number of 4, 4, 5, 7, and 10 tandem arrays of the core repeat, respectively. SSR loci embedded the ESTs with appropriate flanking sequences were selected for primer design using software Primer 3.0 (Untergasser et al., 2012). The parameters for designing the primers were set as follows: primer length with 20 ± 2 nucleotides, amplification product size of 100–300 bp, GC content of 40–60%, and optimum annealing temperature of at least 50°C.

SSR Analysis

A total of 136 SSR primer pairs, targeting at 86 *C. nankingense* (Asteraceae) EST-SSRs (Wang H. B. et al., 2013) and 50 *C. morifolium* EST-SRRs identified in the present study, were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd (Shanghai, China). After a trial run of 136 pairs of SSR primers, 55 of them with clearly separated bands, stable amplification, and rich polymorphism were chosen for further analysis.

SSR amplification was performed using 20 μL of PCR mixture solution containing 2 μL 10 × PCR buffer (100 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, pH 8.8), 2 μL $MgCl_2$ (20 mM), 0.4 μL dNTPs (10 mM), 1 μL of each primer (10 μM) (forward and reverse), 1 U Taq DNA polymerase (TaKaRa Bio., Kyoto, Japan), and 50 ng genomic DNA templates. PCR amplification was run using a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) with the following program: 94°C for 5 min, followed by 35 cycles of 94°C for 40 s, then 40 s at the annealing temperature of each primer pair, 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. PCR products were separated on 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. Sanger

TABLE 2 | Characterization of EST-SSRs in *C. morifolium* genome.

Parameter	Value
Total number of ESTs searched	7300
Total size of examined sequences (bp):	3,717,958
Total number of ESTs with SSRs	207
Total number of ESTs with a single SSR	197
Total number of ESTs with more than 1 SSR	10
Repeat types	
Di-nucleotide	27 (12.39%)
Tri-nucleotide	39 (17.89%)
Tetra-nucleotide	9 (4.13%)
Penta-nucleotide	9 (4.13%)
Hexa-nucleotide	134 (61.47%)
Total number of SSRs identified	218

TABLE 3 | Distributions of microsatellite motifs observed in *C. morifolium* ESTs in the GenBank database.

SSR motif	Number of repeat units in ESTs								
	3	4	5	6	7	8	9	≥10	Total
Di-nucleotide	–	–	–	–	–	–	–	27	27
(AC)n	–	–	–	–	–	–	–	10	10
(AG)n	–	–	–	–	–	–	–	13	13
(AT)n	–	–	–	–	–	–	–	4	4
Tri-nucleotide	–	–	–	–	19	12	5	3	39
(AAC)n	–	–	–	–	4	4	3	–	11
(AAG)n	–	–	–	–		2	–	–	2
(AAT)n	–	–	–	–	3	2	–	2	7
(ACC)n	–	–	–	–	8	2	1	1	12
(ACT)n	–	–	–	–	1			–	1
(AGC)n	–	–	–	–		1	1	–	2
(ATC)n	–	–	–	–	3	1		–	4
Tetra-nucleotide			6	2		1		–	9
Penta-nucleotide		9						–	9
Hexa-nucleotide	110	17	6	1				–	134
Total	110	26	12	3	19	13	5	30	218

TABLE 4 | Polymorphism of 55 SSR primer pairs in medicinal *Chrysanthemum morifolium* samples.

Primer pair (ID)	Primer Sequences (5′-3′)	Repeat motif	T_m	Size	No. of loci	No. of Polymorphic loci	Polymorphic loci %	PIC	References
CMeSSR001	F:ATTCTGTCACTCAAACACCAC R:GGTTCAAACGAGCTAAATTACA	$(CATC)_8$	55.3	251	26	26	100.00	0.978	This study
CMeSSR002	F:CTCACCATTTTCAGACCATTAT R:ACACATCTTGTACCTCTTGGTT	$(ACA)_7$	55.4	251	26	26	100.00	0.976	This study
CMeSSR003	F:CTTTTTCACACACACTCAACAT R:TTGGAGACGTTGTTGTAAAGTA	$(TAA)_7$	54.5	256	26	26	100.00	0.976	This study
CMeSSR004	F:AAAATGTTAGGTGCAGGATTAC R:AAAAACCGTTCCAGATTACAC	$(AAT)_7$	54.3	248	27	27	100.00	0.977	This study
CMeSSR005	F:AAAACCTTCACTAGATCACACC R:TTTCAGTATCTTGGACCAGTCT	$(CAC)_7$	56.3	252	29	29	100.00	0.978	This study
CMeSSR006	F:ATTCTCTTAATTAGCCAGCAAG R;GTGAATCGTAAATTCAGTTGG	$(CAC)_7$	54.3	247	17	17	100.00	0.958	This study
CMeSSR007	F:GTCCTCCTTCAAAGCAAA R:GACGATTAATTATTGGGTAATA	$(CTA)_7$	51.3	157	28	28	100.00	0.976	This study
CMeSSR009	F:AGTGATGATGAATTGAAAGAGC R:CTCTCAAGTGTTGAAGGAACTC	$(AAT)_8$	56.4	258	18	17	94.44	0.959	This study
CMeSSR010	F:CATTTTCTTCATGGTACTCACA R:GTGAGGATGGAAATCTAGTAGG	(CAC)7	56.4	169	27	27	100.00	0.978	This study
CMeSSR011	F:AGGACAACTCAACTGTTAGGAG R:GTTTCTCAACCTCTTCTTCATC	$(CCA)_7$	57.2	255	20	19	95.00	0.964	This study
CMeSSR012	F:ATTCCCAACCTTCTTTAACC R:AACTAAATCACCATCTCTTGCT	$(CA)_{11}$	54.1	254	29	29	100.00	0.979	This study
CMeSSR013	F:ATGAGAGGGAAATAGAAAGTGA R:TACTTGACGCTAACGGAGTAGT	$(GTAATA)_3(TAA)_4$	56.4	219	26	26	100.00	0.973	This study
CMeSSR014	F:CAAAACTTTCAACAGAGTCATC R:AGAAATAACGACTGGTCAGATT	$(CAACAT)_3(CAG)_4$	54.5	281	35	35	100.00	0.983	This study
CMeSSR015	F:TCTTGGTCAGCTTAATTACTCA R:CATCACCTCCTCCTCCTC	$(TGG)_7$… $(TGG)_4(AGG)_4$	57.1	236	30	29	96.67	0.979	This study
CMeSSR016	F:GAATACTAAATGGGTGGAAGAA R:GCAAATAGATGTCCTTTAGGG	$(GGA)_4(GGT)_4$	55.3	250	18	18	100.00	0.967	This study
CMeSSR017	F:TCATGAAATCCGTGTATATGTC R:ACCCTAATTCTCAAAATGAACC	$(AC)_5a(AC)_6$	54.5	229	30	30	100.00	0.980	This study
CMeSSR018	F:ATCTACTATCCAAGCCATGAAC R:TATCCACCACCACCACCA	$(CAC)_5$… $(GGT)_{10}$… $(TGG)_6$	56.8	264	25	23	92.00	0.973	This study
gi298295865	F:ACTCACTTGCCCCATTTGTC R:AGAGAAGCTCTCCAGGGACC	$(AACCCT)_5$	59.8	146	15	15	100.00	0.957	Wang H. B. et al., 2013
gi298300528	F:AGGGCATCGATAATCCATCA R:AGATACGTGCCCATTTGAGG	$(ATATC)_4$	56.8	135	12	12	100.00	0.938	Wang H. B. et al., 2013
gi298295793	F:ATAGAATTCCCCGACGACAA R:GGCGGTTGAGATTGATAGGA	$(CCCTAT)_4$	56.8	111	15	14	93.33	0.948	Wang H. B. et al., 2013
gi298296818	F:ATGTCCAGCTTGATGGGAAG R:GGCCCCTTGCAAATCCTC	$(GTG)_7$	58.8	210	24	23	95.83	0.977	Wang H. B. et al., 2013
gi298298301	F:CTTGACCGAAACACCGAAAT R:TGGCATCCTAGTTAGCAGCA	$(TTG)_9$	56.8	198	17	16	94.12	0.958	Wang H. B. et al., 2013
gi298299323	F:GCACATTTCCTTCATGGGTT R:TCCACGGTTTCAGATGATGA	$(ACA)_9$	57.8	264	33	33	100.00	0.982	Wang H. B. et al., 2013

(Continued)

TABLE 4 | Continued

Primer pair (ID)	Primer Sequences (5′-3′)	Repeat motif	T_m	Size	No. of loci	No. of Polymorphic loci	Polymorphic loci %	PIC	References
gi298297301	F:TCAAACACCACCACCAACAC R:ATGTCACCAAGTCCTGGTCC	$(CATC)_8$	58.8	167	25	25	100.00	0.973	Wang H. B. et al., 2013
51	F:CCCCCTCTTCTTCTTCAACC R:CAATAGAAAGCGCGTGACAA	$(CCAA)_4$	57.8	202	22	22	100.00	0.974	Wang H. B. et al., 2013
53	F:TCGAAGACAATCAGCACCTG R:TAAGTGTTCTTCCAGCGCCT	$(ATG)_7$	57.8	233	18	17	94.44	0.961	Wang H. B. et al., 2013
64	F:GGCGATGGATGATGATGATT R:GAAAGAGGTGGATCGGATGA	$(TTC)_9$	56.8	267	22	22	100.00	0.991	Wang H. B. et al., 2013
86	F:AAACCACCAAACCCATCAAA R:AACTTTGCCAGCATCGACTT	$(TGG)_8$	54.7	223	25	25	100.00	0.990	Wang H. B. et al., 2013
135	F:CATTCCTACCCATCCCTCCT R:CGCATGAGTGAGCCTAATGA	$(GTGGAG)_4$	58.8	100	31	31	100.00	0.993	Wang H. B. et al., 2013
204	F:TGAGCTTCATCCGCTTCTTT R:TGGTCGTATTCCGTCCATTT	$(TGA)_8$	55.8	262	10	10	100.00	0.985	Wang H. B. et al., 2013
219	F:AAAAGGTTGTGAGTGGGTCG R:CCTCGGTCGATAAATCTCCA	$(GGGAAG)_4$… $(TGAGGG)_4$	57.8	228	26	25	96.15	0.976	Wang H. B. et al., 2013
221	F:AACCATGAATCCAGACACCC R:ACCAAGCCAGTCGAGTTTTG	$(TCA)_7$	57.8	181	20	20	100.00	0.964	Wang H. B. et al., 2013
235	F:GCCCCAATTTATTCACTCCA R:GCTCTTCCTCGTAAGCATCG	$(AAC)_6$	57.8	257	30	30	100.00	0.979	Wang H. B. et al., 2013
262	F:TCTGCCAGCTTTGGGTAACT R:GTGCGCCTGTATTGACTTGA	$(CTTTTT)_4$	57.8	260	13	13	100.00	0.946	Wang H. B. et al., 2013
270	F:AGGTGGAAAATACTGTGCGG R:TGTTTCTGCACCTCAACAGC	$(ATAGTA)_4$	57.8	139	19	19	100.00	0.966	Wang H. B. et al., 2013
285	F:CCGGTGTTCGGTATAAATGG R:ACAATTCGCTTCGGCTCTAA	$(GTG)_7$	56.8	116	11	11	100.00	0.965	Wang H. B. et al., 2013
312	F:GGCCCAAGTTTGAGACAAAA R:TCGGTATAAGTGCACCACGA	$(AAG)_7$	56.8	219	36	36	100.00	0.985	Wang H. B. et al., 2013
313	F:GGCGTTCTCTTCCATTTCAA R:GTTTTGGACCTTGCTTCTGC	$(GAA)_7$	56.8	253	37	37	100.00	0.984	Wang H. B. et al., 2013
320	F:GGTCCTTCGTTTCATTTGGA R:CGGGGGTAGGAATAGAAAGC	$(TGG)_7$	57.8	235	21	21	100.00	0.973	Wang H. B. et al., 2013
327	F:GAATGCAGCCTCAACAACAA R:GAGCCGCCATTGTCATATTT	$(TCAAAG)_4$	55.8	219	27	27	100.00	0.979	Wang H. B. et al., 2013
357	F:ACCCAACCTGAACAAGATGC R:ATACTGCTGCCACTGACCCT	$(GGGTCA)_4$	58.8	252	24	24	100.00	0,990	Wang H. B. et al., 2013
581	F:CCAATCCCAAACACTCCCTA R:GCCGTTACCACTGCTCTTTC	$(CA)_{16}$	58.8	223	24	24	100.00	0.976	Wang H. B. et al., 2013
984	F:TCAAAACCCATCATCACCCT R:CGGCGTTTGTATCTTGGTTT	$(ACA)_7$	55.8	186	28	27	96.43	0.979	Wang H. B. et al., 2013
995	F:TTGTTCCACGTGACGAGATT R:CTCCCAAATGACCCATCATC	$(TGTTGG)_4$	56.9	244	16	16	100.00	0.961	Wang H. B. et al., 2013
1036	F:CTTTGGTAAGCGAAGGCTGT R:GCCATTTGTAAGCGGTTTGT	$(AATG)_7$	55.7	153	16	16	100.00	0.955	Wang H. B. et al., 2013
1187	F:GAAAGCGATCATTGGGAAAA R:TTACCCGTACATTCGGGATT	$(GAGAAG)_4$	54.7	112	19	19	100.00	0.963	Wang H. B. et al., 2013
1424	F:TAAAATCCATCCGTCCATCC R:CTTCCATATCTGCCAGTGGG	$(CAA)_7$	57.8	278	26	26	100.00	0.975	Wang H. B. et al., 2013

(Continued)

TABLE 4 | Continued

Primer pair (ID)	Primer Sequences (5′-3′)	Repeat motif	T_m	Size	No. of loci	No. of Polymorphic loci	Polymorphic loci %	PIC	References
1428	F:AACGCCCAAAACACCAACT R:TAGAACCTTGTGCCCCCATA	$(AC)_9$	56.6	224	26	26	100.00	0.978	Wang H. B. et al., 2013
1520	F:AAATCACGGATCCCCTTCTT R:TTATCATCTTGGGGAGTGGC	$(ATAGA)_4$	57.8	158	32	32	100.00	0.982	Wang H. B. et al., 2013
1584	F:CCTCCTCAAAACGACCATGT R:CGTCCCCATTACAATATCCG	$(ACA)_7$	57.8	263	24	23	95.83	0.973	Wang H. B. et al., 2013
1742	F:AAGTGATAAGATGGGTGGCG R:GGTGGAGGCTCATTCAAATC	$(TGG)_7$	57.8	166	28	28	100.00	0.974	Wang H. B. et al., 2013
1762	F:GCGTCAAATTACTGGTGGCT R:GTCTCATTTTCCGGCGATAA	$(AAC)_6$	56.8	259	22	21	95.45	0.972	Wang H. B. et al., 2013
1773	F:CAAATGGGGTCGTTACGAAT R:AATCCCCGAATTCCCAATAG	$(CAT)_6$	55.8	215	17	17	100.00	0.965	Wang H. B. et al., 2013
1774	F:TCACCACCACCACTGTCACT R:TGTGGGCTCTAGAGGTTTGG	$(CACCGG)_4$	59.8	276	38	38	100.00	0.984	Wang H. B. et al., 2013
1779	F:AAAGTCCCCTTGCTTGTTCC R:CGACTCCATTTGATCCACCT	$(AACC)_5$	57.8	148	25	25	100.00	0.975	Wang H. B. et al., 2013
Total					1319	1306			
Average					23.98	23.75	98.90%	0.972	

sequencing was used to confirm SSRs in amplified genomic DNA fragments as described previously (Lu et al., 2013).

Data Analysis

Only reproducible and consistent SSR fragments were scored as present (1) or absent (0) for each of the SSR markers. The polymorphism information content (PIC) of each pair of SSR primers was calculated using the formula:

$$PIC = 1 - \left(\sum_{i=1}^{n} p_i^2\right) - \left(\sum_{i=1}^{n-1}\sum_{j=i+1}^{n} 2q_i^2 q_j^2\right)$$

Where n is the number of alleles (marker), q_i is the ith allele frequency, and q_j is the jth allele frequency (Botstein et al., 1980), The cluster analysis was conducted by NTSYS-pc version 2.10e software (Rohlf, 2000). A dendrogram was constructed using the unweighted pair group method with an arithmetic mean (UPGMA) based on similarity matrices calculated using the simple matching (SM) coefficient (Nei and Li, 1979). The data was also analyzed using principal coordinate analysis (PCoA) (Gower, 1966) to further demonstrate the multiple dimensional distributions of the chrysanthemum cultivars in a scatter-plot.

RESULTS

SSR markers Development and primer design

In total, 218 microsatellites were detected in 207 ESTs (**Tables 2, 3**). Among them, 10 (4.83%) ESTs contained more than one SSR loci (**Table 2**). Information about 218 SSR loci was showed in Supplementary Material. Of all detected SSR loci, hexa-nucleotide repeats were the most abundant with 134 loci, (61.47% of the total), followed by tri-nucleotide repeats with 39 loci (17.89% of the total), di-nucleotide repeats with 27 loci (12.39% of the total), tetra-nucleotide repeats with 9 loci (4.13% of the total), and penta-nucleotide repeats with 9 loci (4.13% of total) (**Tables 2, 3**). After removal of those ESTs with too short or inappropriate flanking sequences for primer design, 50 EST-SSRs were selected for primer design (**Table 4**).

A total of 136 SSR primer pairs, including 50 *C. morifolium* EST-SSRs identified above and 86 *C. nankingense* EST-SSRs (Wang H. B. et al., 2013), were screened using three genomic DNA samples. Fifty-five of the primer pairs (40.44%) generated reproducible polymorphic DNA amplification products. The amplified bands with clear and expected size were sequenced. The corresponding repeat motifs were validated for 50 EST loci by Sanger sequencing. Finally, 17 novel *C. morifolium* EST-SSRs were developed successfully, and 38 *C. nankingense* EST-SSRs were confirmed with transferability for application in a related species. These 55 pairs of SSR primers were used for further genetic diversity analysis in *C. morifolium* cultivars (**Table 4**).

SSR Analysis

The 55 SSR primer pairs generated a total of 1319 fragments with an average of 23.98 fragments per primer pair and a range of 10 (primer pair ID. 204) to 38 (primer pair ID. 1774) (**Table 4**). A total of 1306 were polymorphic. The percentage of polymorphic bands across the primer pairs varied from 92.00 to 100.00% (**Table 4**), with an average 98.90%. Three representative

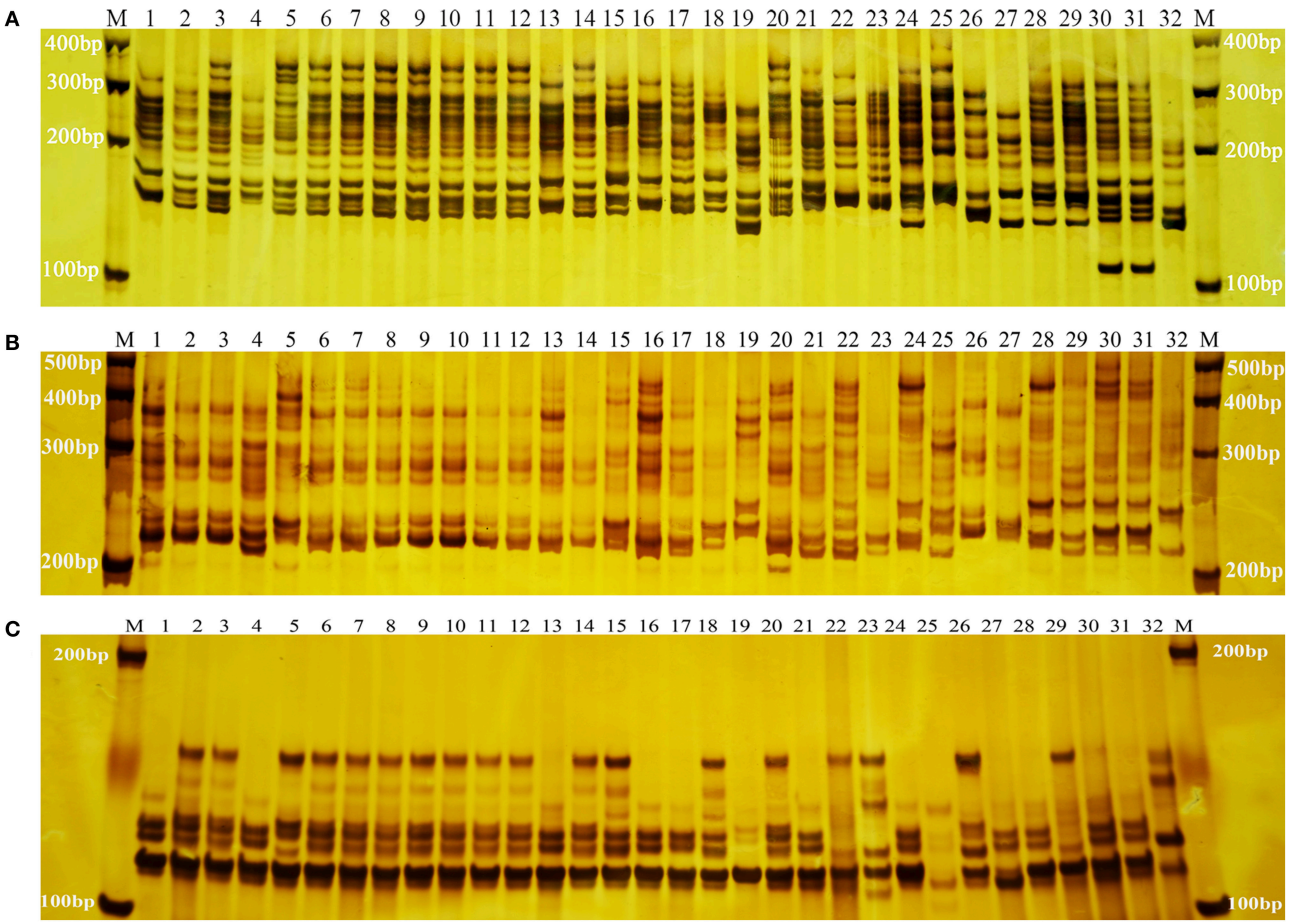

FIGURE 1 | SSR amplification profiles of primer pairs CMeSSR001 (A), 219 (B), and 285 (C). *Lane M*: DNA molecular standards with length (bp) on left and right. *Lanes 1–32*: genotypes of the 32 *Chrysanthemum morifolium* samples (CM1–CM32) in **Table 1.**

profiles (primer pair ID. CMeSSR001, 219 and 285) are shown in (**Figures 1A–C**). The PIC value varied from 0.938 to 0.993 with an average of 0.972 (PIC > 0.5), which indicated that these loci contained a considerable amount of genetic information that could be used in genetic diversity studies on Chrysanthemum germplasms.

Genetic Diversity and Relationships Among Genotypes

A total of 1319 loci were accounted to calculate the genetic diversity among the 32 Chrysanthemum cultivars. Binary data matrices produced by SSRs were used to estimate the genetic similarity of the genotyped Chrysanthemum samples. The pairwise similarity coefficient among the 32 cultivars ranged from a maximum of 0.809 (between Huaiju "Hdbj" and Huaiju "Hxbj") to a minimum of 0.533 (between Huangju "Jj3" and Gongju "Wgj").

A dendrogram using UPGMA analysis was constructed based on the corresponding genetic similarity coefficient among the tested 32 *C. morifolium* populations (**Figure 2**). In this study, all the *C. morifolium* samples could be grouped into two main clusters, with a similarity index of 0.584. Cluster I consisted of 22 cultivars, including all the "Hangju" and "Machengju" samples. This cluster was further subdivided into three subgroups. Subgroup "I-1" consisted of 13 samples, all of which belong to Hangju cultivars. Machengju "Mcj" and eight Hangju cultivars were assigned to subgroup "I-2." Group II comprised of 10 cultivars, which belonged to "Boju," "Huaiju," "Chuju," and "Gongju." Among them, the "Boju," "Chuju," and "Gongju" cultivars were classified into subgroup II-1, while four "Huaiju" cultivars constituted subgroup II-2.

Principal Coordinate Analysis

The SSR data were subjected to PCoA in order to obtain an alternative view of the phylogenetic relationships among the cultivars (**Figure 3**). In the two-dimensional PCoA plot, *C. morifolium* cultivars were divided into two groups (**Figure 3**), which was similar to the pattern as shown by the UPGMA dendrogram. The first two principal axes explained 10.60 and 6.70% of the total molecular variation observed, respectively.

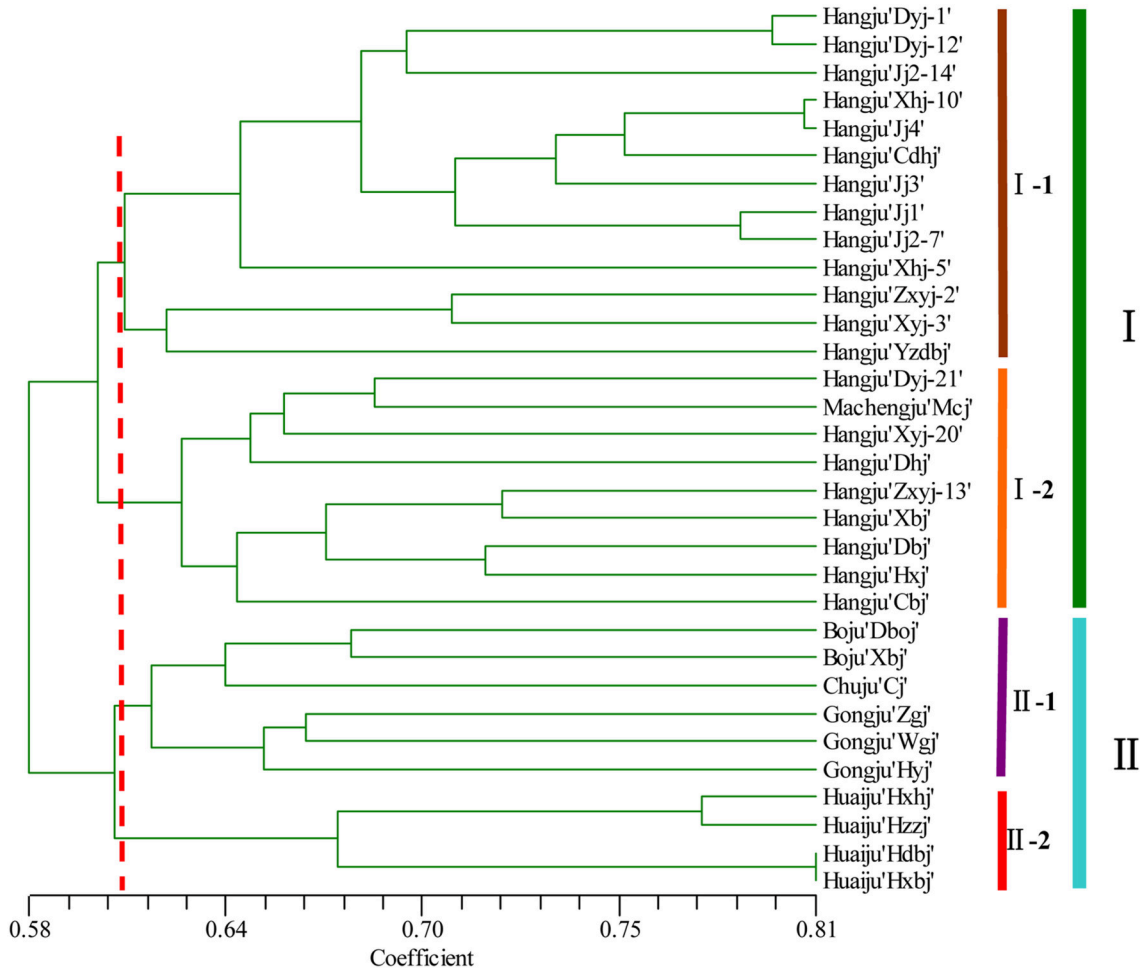

FIGURE 2 | Relationships among *Chrysanthemum morifolium* varieties based on the genetic similarities between DNA fingerprinting patterns from SSR markers used in the UPGMA dendrogram.

DISCUSSION

Compared with anonymous markers, SSR markers, as a type of co-dominance markers, may yield more accurate estimates of genetic diversity. SSRs have been used successfully to determine genetic diversity among many plants (Dirlewanger et al., 2002; Hasnaoui et al., 2012; Shiferaw et al., 2012; Emanuelli et al., 2013; Ren et al., 2014). SSRs were previously identified in *C. nankingense* and *C. nankingense* SSRs were proved to be useful for genetic analysis in the genus *Chrysanthemum* and its related genera (Wang H. B. et al., 2013). In the present study, we found that 44% (38 out 86) of *C. nankingense* SSRs were also proved to be useful for genetic diversity study among medicinal *C. morifolium* cultivars (**Table 4**).

A previous study used 20 SSR markers for identification and classification of Chinese traditional ornamental Chrysanthemum cultivars (Zhang et al., 2014). However, few studies have explored development and application of SSR markers for genetic diversity among medicinal *C. morifolium* cultivars. The diversity and genetic relationship among 29 *C. morifolium* populations were investigated using the types of dominant molecular markers (Shao et al., 2010). The present study report discovery of novel SSRs in *C. morifolium*.

The SSR markers selected in this study yielded reproducible polymorphic bands in 32 *C. morifolium* cultivars and showed that they provide a powerful and reliable molecular tool for analyzing genetic diversity and relationships among *C. morifolium* cultivars. In this study, 98.90% of the bands generated by the SSR assay were polymorphic, which was higher than the polymorphic proportions of 53.85% detected by SSR among celery cultivars (Fu et al., 2014), 97.40% among grass pea from different regions (Shiferaw et al., 2012), and 97.50% among melon accessions (Kacar et al., 2012). Molecular markers with higher PIC values have a greater ability to identify cultivars. A locus with a PIC greater than 0.5 is considered to be highly diverse, as a previous study reported (Botstein et al., 1980). The PIC values of the SSR markers used in the Chrysanthemum cultivars analysis ranged between 0.938 and 0.993, with an average of 0.972, which indicated that the highly informative SSR markers

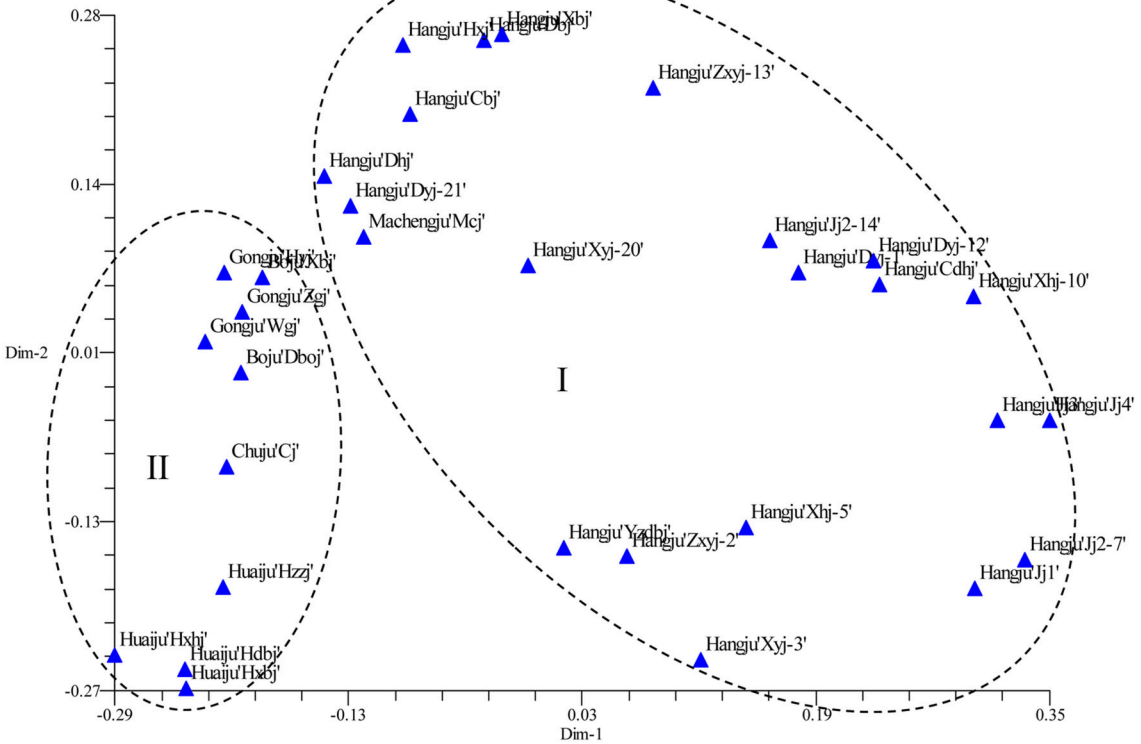

FIGURE 3 | Two-dimensional projection of the PCoA of 32 *Chrysanthemum morifolium* samples based on SSR markers along the first two principal axes.

could be employed in genetic diversity studies of medicinal Chrysanthemum cultivars.

Evaluation of genetic diversity and relationship among plant populations is the foundation of selective breeding programs. Using SSR markers, our study found considerable diversity among Chrysanthemum cultivars, which could be used in breeding programs for Chrysanthemum improvement. According to their origin and ecological distribution, 32 *C. morifolium* cultivars were classified into six sources: Hangju, Machengju, Chuju, Boju, Gongju, and Huaiju (**Table 1**). A dendrogram constructed with SSR data using the UPGMA method indicated that the *C. morifolium* cultivars were grouped into two main groups. All the Hangju cultivars were clustered in the first group along with Machengju "Mcj" (Group I), which means that the genetic relationship between Hangju cultivars and Machengju "Mcj" is very close, consistant with a previous study (Shao et al., 2010). The main growing regions for Boju, Gongju and Chuju are Bozhou (115°78′, 33°85′), Shexian (118°43′, 29°87′), and Chuzhou (118°32′, 32°3′) in Anhui Province, China. In theory, the genetic relationships between the cultivars of these three *C. morifolium* populations may be closer than between other *C. morifolium* populations (Hangju, Machengju and Huaiju). In our study, all the Boju, Chuju and Gongju cultivars were grouped together within subgroup II-1, which confirmed the inference above (**Figures 2**, **3**). The Huaiju cultivars collected from Wuzhi, Henan Province (113°38′, 35°1′), were grouped into subgroup II-2. Geographically, Henan Province is adjacent to Anhui Province, which may explain why the four Huaiju cultivars have a close relationship with other three *C. morifolium* populations (Chuju, Boju, and Gongju) (**Figure 2**). The results of the present study showed that cluster analysis using SSR markers mainly supported the classification of medical *C. morifolium* accessions according to their origin and ecological distribution.

Increased urbanization has meant that *C. morifolium*' cultivation has greatly declined and some populations are rare. Therefore, it is imperative to undertake effective measures to protect *C. morifolium* germplasms. Our study found that there was a high level of genetic diversity between Chrysanthemum populations. As the study by Shao et al. (2010) suggested, a priority for *in situ* conservation should be to rescue and conserve the core populations.

In conclusion, our study demonstrates that SSR technology is a powerful tool for evaluating genetic diversity and relationships among the medical *C. morifolium* cultivars. SSR analysis showed that medicinal *C. morifolium* populations could be classified according to their origin and ecological distribution. In future studies, more medicinal *C. morifolium* cultivars will be included to verify whether these findings are true for more closely related taxa.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: SF, HW. Collected plant samples XS, ZW. Performed the experiments: SF, RH, MJ. Analyzed the data: SF, RH, JL, YJ. Wrote the manuscript: SF, HW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fgene.2016.00113

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