Characterization and Development of EST-SSR Markers from Transcriptome Sequences of *Chrysanthemum × morifolium* Ramat.)

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Abstract. Simple sequence repeat (SSR) markers are valuable for genetic and breeding applications, but SSR resources for the ornamental genus chrysanthemum (*Chrysanthemum × morifolium* Ramat.) are still limited. Expressed sequence tags (ESTs) are sources of SSRs that represent an opportunity to develop SSRs to accelerate molecular breeding in chrysanthemum. In total, 4661 SSR loci were identified from 3823 SSR-containing unigenes in the chrysanthemum transcriptome with an average of one SSR per 6.98 kb. Of these SSR sequences, trinucleotide repeats (30.0%) predominated, followed by dinucleotide repeats (17.9%). In total, 1584 primer pairs were subsequently synthesized. By screening the parents and six individuals of the F₁ progeny, 831 (52.5%) valid EST-SSR markers were identified, of which 361 (43.4%) were polymorphic. The annotation of unigenes containing polymorphic SSRs indicated that 330 (93.5%) demonstrated significant homology to other plant protein sequences. Twenty-five polymorphic EST-SSR markers were further selected for transferability analysis and exhibited 93% amplification in six *Ajania* species and six other *Chrysanthemum* species. Based on genotyping of the 59 samples, neighbor-joining analysis revealed six phylogenetic groupings, which was confirmed by population structure analysis and principal component analysis (PCA). Phylogenetic relationships among the 59 samples revealed by SSRs were highly consistent with the traditional taxonomic classification of *Chrysanthemum* and *Ajania*. The polymorphism information content (PIC) values ranged from 0.29 to 0.86, with a mean of 0.67, indicating high levels of informativeness. This research reveals the SSR distribution characteristics of chrysanthemum and provides a large number of new EST-SSR markers for further genetic diversity studies, genetic mapping, and molecular marker-assisted selection breeding for chrysanthemum.

Chrysanthemums (*Chrysanthemum × morifolium* Ramat.) are common flowers that possess substantial aesthetic value. They are cultivated all over the world and are important economic ornamentals that comprise a considerable proportion of the flower industry in many southeast Asian and European countries (Zhang et al., 2011). Cultivated chrysanthemums are generally believed to be a hybrid complex derived from natural hybridization involving several species, such as *Chrysanthemum vestitum*, *Chrysanthemum indicum*, *Chrysanthemum lavandulifolium*, and *Chrysanthemum*

zawadskii (Dai et al., 1998; Yang et al., 2006). They are classified as segmental allohexaploid with a most frequent somatic chromosome number of 54 ($2n = 6x = 54$) (Klie et al., 2014) and an extremely large genome size of ≈9.4 Gb (<http://data.kew.org/cvalues/>). Cultivated chrysanthemums possess high genetic variation, as is evident in the various cultivars. The accumulated genetic variability provides an important resource of alleles to improve important ornamental traits; however, conventional breeding programs may not be sufficient to improve complex traits in chrysanthemum. Modern molecular breeding tools, such as molecular marker-assisted selection (MAS) breeding, could enhance important chrysanthemum ornamental traits. Hence, development of polymorphic markers is urgently needed.

Molecular markers are valuable tools used in genetic linkage map construction and MAS breeding. Among molecular markers, SSR markers are useful genetic markers because they are hypervariable, co-dominant, and highly informative. Further-

more, compared with genomic SSRs, expressed sequence tag SSRs (EST-SSRs) will likely provide more possibility of finding associations with functional genes because they occur within the coding region and may affect gene function. EST-SSR markers also exhibit high transferability among closely related species and can be exploited as anchor markers for comparative mapping or evolutionary studies. However, EST-SSR markers have been developed for only a limited number of *Chrysanthemum* species (Feng et al., 2016; Han et al., 2018; Jo et al., 2015; Khaing et al., 2013; Li et al., 2013; Liu et al., 2015; Park et al., 2015; Wang et al., 2013; Zhang et al., 2014). As a result, anonymous dominant markers were predominantly used in earlier studies, e.g., earlier genetic maps of chrysanthemum were constructed using random amplified polymorphic DNA, intersimple sequence repeats, amplified fragment length polymorphism (Zhang et al., 2010), and sequence-related amplified polymorphism markers (Zhang et al., 2011). More recently, a single nucleotide polymorphism (SNP)-based map was constructed (Van Geest et al., 2017). However, because of the lack of available sequence information and the need for special instrumentations, SNP markers are difficult to use for most researchers. In addition, SSR markers are more informative than SNP markers, which are generally biallelic. Therefore, SSR marker technology remains important because of its simplicity and convenience combined with its sequencing-free assay, in particular for species that lack genome sequence data.

Here, we used the large dataset of EST sequences to identify EST-SSR markers for chrysanthemum. The specific aims of our study for chrysanthemum were as follows: 1) to characterize the EST-SSR loci and screen SSR primer polymorphisms in F₁ progeny, 2) to characterize the possible function of polymorphic SSR-containing unigenes, 3) to test cross-species/genus transferability within the genera *Chrysanthemum* and *Ajania*, and 4) to apply EST-SSR markers to phylogenetic relationships analysis.

Materials and Methods

Microsatellite identification, primer design, and polymorphism screening. A total of 88,499 ESTs, obtained from *C. × morifolium* ‘Jinbudiao’ transcriptome sequences of flower buds, were submitted to the sequence read archive database at GenBank (www.ncbi.nlm.nih.gov), where they were combined and given the accession number SRP109613. These sequences were assembled further after redundancy elimination and produced 17,462 unigenes with a mean length of 1864 base pairs (bp). The MicroSATellite program (MISA, <http://pgrc.ipk-gatersleben.de/misa>) was used to detect SSRs, and the search criteria for di-, tri-, tetra-, penta-, and hexanucleotide motifs were a minimum of six, five, five, four, and four repeats, respectively. Two or more SSRs

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separated by no more than 100 bp were defined as compound SSRs. Mononucleotide and complex SSR types were excluded from the study. Compound ones that were separated by 0 bp were considered. To predict the location of the SSR motifs in genes, the ORF Finder software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was used to identify the initiation and termination codons in EST sequences. Primers were designed using Primer 3.0. A subset of 1584 primer pairs were selected for validation of marker assay performance.

Genomic DNA was isolated from fresh young leaves using a DNA extraction kit (Demeter Biotech, Beijing, China). DNA quality was checked on 1% agarose gels and quantified by Unico ultraviolet-visible Spectrophotometer (Unico, Franksville, WI). Polymerase chain reaction (PCR) was performed in 10 µL of reaction mixture containing 25 ng genomic DNA, 5 µL 1 × Power Taq PCR MasterMix (TsingKe, Beijing, China), 0.5 µL of 10 µM each primer, and 3 µL sterile distilled water. The PCR reaction program was as follows: DNA denaturation at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, the appropriate annealing temperature for 30 s; 1 min at 72 °C, and 72 °C for 10 min as a final extension.

All EST-SSR primer pairs were initially screened using genomic DNA from the *C. ×morifolium* 'Jinbudiao'. PCR products were separated by electrophoresis in 2% agarose gels. Generally, a subset of EST-SSR primers was identified as validated markers. All validated markers were screened for polymorphisms among the parents and six individuals of an F₁ mapping population. The use of closely related individuals in a population may enhance the reliability of our results. Amplified products that showed a band of the expected size were separated on 8% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. The primers that were not successfully amplified or produced multiple bands were further tested by reducing or increasing the annealing temperature. The mapping population is maintained in the experimental fields at Xiaotangshan, which belongs to Beijing Forestry University, Beijing, China (lat. 40.0°N, long. 116.3°E).

Functional annotation. To identify the putative function of SSR-containing unigenes, the unigenes harboring polymorphic microsatellite were subjected to BLASTX search against the National Center for Biotechnology Information nonredundant protein database, the Gene Ontology (GO) database, the Cluster of Orthologous Groups (COG) database, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Sequences with E value <10⁻⁶ and scores >50 were annotated.

Cross-species/genera transferability and evaluation of genetic relationships within 59 samples. To assess cross-species/genera transferability, 25 randomly selected polymorphic EST-SSRs were genotyped in 59 samples, including 36 *Chrysanthemum*, 10 *Ajania*, and 13 intergeneric hybrids (Supplemental Table 1). The PCR amplification reaction system was

conducted as mentioned previously. PCR product amplification was directly separated on 8% denaturing polyacrylamide gel electrophoresis and visualized by silver staining. All reliable bands were scored as 1, 0 for presence and absence, and transformed into a binary matrix. The number of alleles (N_a), observed and expected heterozygosities (H_o and H_e , respectively), Shannon index (I), and PIC were calculated for each marker using an R package (an R package that is suitable for hexaploids).

The discriminatory abilities of EST-SSR markers were estimated using cluster analyses to assess genetic relationships among 59 samples. The binary data were analyzed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.10 software with SIMQUAL module to calculate genetic similarity (GS) coefficients based on coefficient for similarity matching (Rohlf, 1998). A neighbor-joining (NJ) dendrogram was constructed using the GS matrix by the unweighted pair group method with arithmetic average (UPGMA). Bootstrapping analysis (1000 resamplings) was carried out using software FREETREE V0.9.1.50 (Pavlicek et al., 1999). Bootstrap values more than 50 are considered significant and provided on the dendrogram. Biplot was computed based on the PCA of molecular data using software MVSP v3.1.3. The data were also analyzed using PCA to further demonstrate the multiple dimensional distributions of the 59 samples in a scatter-plot.

Population structure was investigated using a model-based approach, which is implemented in the software package STRUCTURE 2.3.4. Different population genetic clusters ($K = 1-10$) were evaluated with five runs per K value. For each run, the initial burn-in period was set to 100,000 with 2000,000 MCMC iterations, under the admixture model and independent allele frequencies, with no prior information on the origin of individuals. To determine the most probable value of K , the deltaK method described by Evanno et al. (2005) was used as implemented in Microsoft Excel 2016. STRUCTURE results were displayed in the program STRUCTURE PLOT (Ramasamy et al., 2014).

Results

Characteristics of EST-SSR markers in chrysanthemum transcriptome. By sequenc-

ing the transcriptome of chrysanthemum, we identified 4661 EST-SSR loci located in 3823 (21.9%) unigene sequences from 3.26 Mb total sequence, with an average of 1 SSR per 6.98 kb (Table 1). The SSRs were not evenly distributed, with 688 (3.9%) being unigenes containing more than one SSR locus (Table 1). Analysis of all 4661 EST-SSR loci showed that trinucleotide (30.04%, 1400) and dinucleotide repeat motifs (17.89%, 834) predominated, followed by tetranucleotide (1.57%, 73), pentanucleotide (0.13%, 6), and hexanucleotide repeat motifs (0.09%, 4) (Table 1).

The frequency distribution of 35 major types with di- and trinucleotide repeat motifs and repeat number of EST-SSRs are shown in Fig. 1. Of the identified SSRs, the dinucleotide repeat AC/GT (31.29%, 261) was the most plentiful motif, followed by CA/TG (27.82%, 232), AT/TA (17.27%, 144), TCA/TGA (10.2%, 143), ATC/GAT (9.3%, 130), and AG/CT (12.35%, 103) (Fig. 1A and B). SSR length was mostly 12 to 20 bp, accounting for 93.7% of the SSRs. The largest microsatellite identified was 78 bp, with a hexanucleotide motif repeated 13 times (Fig. 1C).

SSR primers screening and polymorphism testing. Using *C. ×morifolium* 'Jinbudiao', 831 (52.5%) primer pairs were successfully amplified (Supplemental Table 2). Moreover, 180 primer pairs amplified bands larger than expected, or produced multiple bands. The remaining 573 primer pairs did not generate PCR products after subjecting them to various PCR programs.

These 831 primer pairs were further subjected to screening for polymorphisms in the F₁ progeny, including the parents and six individuals. A total of 361 primer pairs (43.4%) amplified reproducible polymorphic bands, including 95 di-, 257 tri-, 8 tetra-, and 1 compound motif loci. A total of 1396 alleles were detected, and the number of alleles per locus ranged from 2 to 8, with an average of 3.87 (Supplemental Table 3).

We also investigated the distribution of microsatellites in the transcript (coding sequence [CDS], 5' untranslated region [UTR], and 3'UTR). Of the 831 validated SSR markers, 400 (48%), 340 (41%), and 91 (11%) were located in the CDS, 5'UTR, and 3'UTR, respectively. In addition, 96% of di-repeats and 100% of tetra-repeats were located within the UTR, whereas 67% of the tri-repeats occurred within the CDS.

Table 1. Summary of expressed sequence tag-simple sequence repeat (EST-SSR) markers identified in the chrysanthemum transcriptome.

Searching items	Numbers
Total number of sequences examined	17,462
Total size of examined sequences (Mb)	3.26
Total number of identified SSRs	4,661
Number of SSR-containing sequences	3,823
Frequency of SSRs	1/6.98 kb
Number of sequences containing more than 1 SSR	688
Number of SSRs present in compound formation	305
Dinucleotide	834
Trinucleotide	1,400
Tetranucleotide	73
Pentanucleotide	6
Hexanucleotide	4

Fig. 1. Characterization of sample sequence repeats (SSRs) in chrysanthemum transcriptome. (A) Frequency distribution of dinucleotide SSRs based on motif type. (B) Frequency distribution of trinucleotide SSRs based on main motif type. (C) Number of different repeat motifs.

Functional annotation. Of the 361 polymorphic SSR-containing unigenes, 330 (93.5%) possessed sequence similarities and could be assigned putative functions (Supplemental Table 3). Several unigenes encoded proteins similar to important transcription factors, such as CEM190-containing unigenes encode proteins similar to transcription factor *APETALA2* (*AP2*), CEM505-containing unigenes encode proteins similar to transcription factor *bHLH*, and CEM512-containing unigenes encode proteins similar to zinc finger protein *CONSTANS* (*CO*). In addition, 246, 83, and 71 SSR-containing unigenes were assigned to GO annotations, COG classifications, and the KEGG database, respectively (Supplemental Fig. 1A–C). Annotation with GO confirmed that the highest proportion of unigenes were related to cell, cell part, and metabolic process. COG classi-

cation of the unigenes revealed that the most highly represented functional group was “general function,” followed by “replication, recombination, and repair.” KEGG pathway enrichment analysis showed that the pathways with the most abundant unigenes are spliceosome and messenger RNA surveillance pathway. However, the exact function for these SSR-containing unigenes needs further research.

Cross-species transferability of 25 polymorphic EST-SSR markers. To assess the utility of the EST-SSR markers beyond *C. ×morifolium*, we selected 25 markers to evaluate whether they were amplifiable in other related species/genera, including *Chrysanthemum* and *Ajania* species/genera (Supplemental Table 1, codes 1–12). All the EST-SSR primers demonstrated successful cross-species amplification (Sup-

plemental Table 4). Of these, 16 (64%) were amplified in all species. Transferability of the primers among *Chrysanthemum* species was identified in *C. indicum* (ind1) (100%), *Chrysanthemum malingense* (100%), *Chrysanthemum chanetii* (96%), *C. indicum* (ind2) (96%), *C. zawadskii* (96%), and *C. indicum* var. *aromaticum* (92%). In *Ajania*, the rate of amplification was slightly lower, and transferability was present with *Ajania potaninii* (100%), *Ajania kharvensis* (92%), *Ajania fruticulosa* (92%), *Ajania achiloides* (88%), *Ajania purpurea* (88%), and *Ajania scharnhorstii* (76%). These data suggest that the EST-SSR markers developed in this study could be efficiently applied to the genus *Chrysanthemum* and *Ajania*.

Evaluation of genetic relationships within 59 samples. The 25 representative EST-SSR markers also were selected to estimate their polymorphism in 59 samples. The allelic variation in these samples was assessed to determine whether these EST-SSR markers could be effectively used to identify genetic variability. In total, 151 alleles were detected with an average of 6 alleles per marker, ranging from 2 at CEM081 to 13 at CEM814 (Table 2). The PIC values ranged from 0.29 for CEM081 to 0.86 for CEM814, with a mean value of 0.67. The other parameters (N_a , H_o , H_e , Shannon index) are shown in Table 2.

In additional analysis, we tested the discriminatory capacity by determining whether the 25 markers were able to recapitulate the known phylogenetic relationships among the 59 samples. The NJ tree (Fig. 2A), based on the shared allele coefficients, clustered them into six main groups. Cluster I comprised three species (ind1, cha, ind2) and cluster II contained nine species, of which three species (man, ind3, zaw) belong to *Chrysanthemum* and six species (fru, pot, ach, sch, kha, pur) belong to *Ajania*. The relationships between cluster I and cluster II are very close. Cluster III included four samples (pac1, 2, 3, 4) belonging to *Ajania pacifica*. There are eight samples (chr1, 2, 3, 4, 5, 6, 7, 8) in cluster IV, which are all cultivars of large chrysanthemum. Cluster V consisted of 13 intergeneric hybrids of the *C. ×morifolium* ‘Pudidanfen’ (male parent, hexaploid) and *A. pacifica* (female parent, decaploid), and cluster VI was composed of 22 samples, including groundcover chrysanthemum and escarpment chrysanthemum.

Population structure estimation for the 59 samples was done using the Bayesian clustering approach implemented in STRUCTURE 2.3.4. Given that the log likelihood values increased progressively as K increased, the method of Evanno et al. (2005) was applied as a criterion to infer the most likely K value. The maximum delta K was detected at K = 6, inferring that all 59 individuals can be grouped into six groups with the highest probability (Fig. 2B). Cluster A was composed of man, ind2, ind3, zaw, fru, pot, ach, sch, kha, pur, and ind1; cluster B was composed of cha, chr1, chr2, and sm; cluster C was the same as cluster V in the NJ tree; cluster D was the same as cluster III and was composed of pac1, 2, 3, and 4; cluster E consisted of chr3, 4, 5, 6, 7,

Table 2. Polymorphism information of 25 expressed sequence tag-simple sequence repeat (EST-SSR) markers in 59 chrysanthemum samples, including the following parameters: the number of alleles (N_a), observed and expected heterozygosities (H_o and H_e), PIC, and Shannon index.

Primer name	N_a	H_o	H_e	PIC	Shannon index
CEM076	6	0.92	0.69	0.64	1.31
CEM080	6	0.88	0.79	0.75	1.63
CEM081	2	0.29	0.35	0.29	0.54
CEM082	5	0.59	0.75	0.70	1.43
CEM088	8	0.69	0.80	0.77	1.75
CEM091	4	0.90	0.70	0.65	1.27
CEM093	3	0.48	0.62	0.55	1.03
CEM371	8	0.85	0.75	0.71	1.57
CEM372	6	0.88	0.63	0.55	1.18
CEM380	7	0.73	0.83	0.81	1.86
CEM496	10	0.81	0.80	0.77	1.83
CEM501	5	0.95	0.75	0.71	1.49
CEM504	4	0.71	0.70	0.64	1.28
CEM505	5	0.93	0.79	0.76	1.59
CEM508	5	0.54	0.70	0.64	1.30
CEM512	5	0.73	0.62	0.55	1.14
CEM513	8	0.91	0.83	0.80	1.87
CEM514	4	0.46	0.65	0.57	1.11
CEM515	7	0.88	0.74	0.70	1.51
CEM519	5	0.78	0.74	0.70	1.46
CEM685	7	0.76	0.85	0.83	1.92
CEM723	4	0.66	0.66	0.59	1.17
CEM780	8	0.46	0.78	0.76	1.75
CEM782	6	0.14	0.39	0.37	0.89
CEM814	13	0.92	0.88	0.86	2.26
Mean	6.04	0.71	0.71	0.67	1.45

and 8; and cluster F was composed of 21 cultivars. The results were quite similar to the NJ tree, with the exception of chr1, 2, and sm.

The genotyping data were also subjected to PCA analysis to obtain an alternative perspective of the phylogenetic relationships among the 59 samples. PCA showed similar results with the NJ tree, except that the major groups I and II formed one cluster in the two-dimensional plot (Fig. 2C).

Discussion

Next-generation sequencing technology provides a simple and effective way to develop molecular markers such as SSRs and SNPs, of which few are available for chrysanthemum. Before this research, only 7300 *Chrysanthemum* ESTs (http://www.ncbi.nlm.nih.gov/genbank/dbest/dbest_summary/) had been generated, and Feng et al. (2016) developed 17 novel EST-SSR markers from them. The EST-SSRs developed in the current study will prove directly useful for chrysanthemum, and readily transferable to closely related species, as illustrated here between *Chrysanthemum* and *Ajania*.

In the chrysanthemum transcriptome, the mean length of the unigenes was 1864 bp, which was much longer than reported in previous transcriptome studies of the tree peony (mean length 698 bp) (Wu et al., 2014) and *Neolitsea sericea* (mean length 733 bp) (Chen et al., 2015). It revealed that the overall sequence data from *C. ×morifolium* 'Jinbudiao' were effectively assembled to create longer DNA sequences or contigs. Longer sequences provide longer lengths for selection of SSR flanking sequence, which have been found to assist in designing SSR

primers (Zalapa et al., 2012). A survey on dicotyledonous species reported that the proportion of ESTs containing SSRs ranged from 2.65% to 16.82% (Varshney et al., 2005). In this study, ≈21.9% of 17,462 ESTs contained at least one SSR, which was higher than the upper limit of the range, as well as other reported values, such as *Stylosanthes guianensis* (9.96%) (Ding et al., 2015). Furthermore, the distribution density of EST-SSRs was one SSR in every 6.98 kb. This value was higher than the frequency reported in the tree peony (1/9.24 kb) (Wu et al., 2014) and *Pinus dabeshanensis* (1/23.08 kb) (Xiang et al., 2015), but lower than that in *N. sericea* (1/3.8 kb) (Chen et al., 2015) and taro (1/5.90 kb) (You et al., 2015). Chrysanthemum may have a higher frequency of EST-SSRs, but alternative explanations, such as the size of the data set, SSR search criteria, and database-mining tools also should be considered (Varshney et al., 2005).

Among the various sequence repeats, trinucleotides were predominant (Table 1), consistent with studies in plants such as *C. indicum* (Han et al., 2018), rice (Lawson and Zhang, 2006), *Brassica rapa* (Zheng et al., 2016), and *Helianthus annuus* (Pramod et al., 2014). This observation is common for EST-derived SSRs, as changes in trinucleotide repeat number will not cause frameshift mutations. Our analysis of EST-SSRs found that SSRs with AC/GT motifs (31.29%) were the most frequent (Fig. 1A), which agrees with results reported in *Chrysanthemum nankinense* (Wang et al., 2013) and *C. indicum* (Han et al., 2018). The most prevalent trinucleotide motif type often differs among species. In chrysanthemum, TCA (10.2%) predominated, unlike those in *Dysosma versipellis*, *S. guianensis*, and *N. sericea* (AAG

(Chen et al., 2015; Ding et al., 2015; Guo et al., 2014); taro (CCG) (You et al., 2015); *P. dabeshanensis* (AGC) (Xiang et al., 2015); and tree peony (CCA) (Wu et al., 2014). In contrast, CCG/CGG (0.6%) motifs were very rare (see Fig. 1), found mostly in monocots such as maize, barley, and taro (Morgante et al., 2002; Toth et al., 2000; You et al., 2015). The observed rarity of CCG/CGG repeat units has been reported in some dicots, such as the common bean, *D. versipellis*, *S. guianensis*, *N. sericea*, *P. dabeshanensis*, and the tree peony (Chen et al., 2014, 2015; Ding et al., 2015; Guo et al., 2014; Wu et al., 2014; Xiang et al., 2015). The extremely low number of SSR motifs containing C and G could be attributed to the composition of dicot genes being less rich in G+C.

Our results indicated that EST-SSRs in the chrysanthemum transcriptome do not evenly distribute across CDS and UTR regions, with a greater frequency in the latter. The result was consistent with that of other transcriptome surveys (Liu et al., 2016; Pramod et al., 2014). The density of microsatellites in the chrysanthemum unigene database may be dependent on two factors: 1) evolutionary constraints due to the harmful effect of microsatellite mutation on gene function, and 2) the directional selection on microsatellites with adaptive roles (Liu et al., 2016). As microsatellites are highly prone to indel mutations by means of slip-strand mispairing (Levinson and Gutman, 1987), it seems that microsatellites in the CDS regions are more inclined to obstruct gene function, leading to higher evolutionary constraints on microsatellites in the CDS region. Besides, microsatellites in the UTRs may confer evolutionarily adaptive roles on genes with regard to tuning ability. Thus, the directional selection may favor microsatellite expansion in UTRs.

In the current study, 52.5% of the EST-SSR primers amplified the target sequence, which is lower than the success rate of 60% to 90% amplification reported in previous studies, such as *Pinus dabeshanensis* (81.71%), *Stylosanthes* (83.34%), sweetpotato (84.6%), and pigeon pea (71.24%) (Ding et al., 2015; Dutta et al., 2011; Wang et al., 2011; Xiang et al., 2015), but higher than in tree peony (47.3%), *Dysosma versipellis* (42.5%), and *Neolitsea sericea* (16%) (Chen et al., 2015; Guo et al., 2014; Wu et al., 2014). The success ratio may result from primer design, selecting the best performing loci from the total number of sequences available is a key component in the successful development of SSR markers (Zalapa et al., 2012).

Of the 831 valid EST-SSR markers, 361 (43.4%) polymorphic markers were obtained, thereby providing powerful tools for constructing an SSR-based linkage map in chrysanthemum. Research showed that when SSRs ≥20 bp tend to be hypervariable markers, 12 bp ≤ SSRs < 20 bp tend to be potentially variable markers (Temnykh et al., 2001). The lengths of SSRs in this study were mostly 12 to 20 bp (93.7%), which could guarantee a medium degree of polymorphism.

Fig. 2. (A) Genetic relationships among 59 samples based on expressed sequence tag-simple sequence repeat (EST-SSR) markers. The dendrogram shows the genetic relationships among 59 samples. The scale at the bottom of the dendrogram indicates the level of similarity between the genotypes, bootstrap values (>50) were labeled on the branches from 1000 resamplings. The information of the abbreviations represented in the tree can be seen in Supplemental Table 1. (B) STRUCTURE analysis at K = 6 for 59 samples. The colors represent different clusters and the colors in each accession represent the average proportion of alleles that placed each accession under two or more clusters. The text below the figure refers to accession names. (C) Two-dimensional plot of principal component analysis (PCA) of 59 samples. The circles indicate the group of samples that are similar to each other in the PCA analysis (A, B, C, D, and E are the five major clusters).

Moreover, the number of samples and the geographic origin of samples used may have led to a different ratio of polymorphisms. If more samples were screened, the number of polymorphic SSRs in this study would most likely increase.

SSRs are usually considered as “junk DNA” or evolutionarily neutral DNA markers in the genome. Recently, microsatellites have increasingly been identified and characterized within protein-coding genes and their UTRs, providing numerous lines of evidence for the potential functions of microsatellites. When microsatellites locate in a functional gene, their repeat motif variation can influence gene regulation, transcription, translation, and protein function. In the current study, a number of polymorphic SSR-containing unigenes have transcription factor activity or other functions. A similar investigation of the function of microsatellite in *Arabidopsis* (Lawson and Zhang, 2006) and *Sargassum thunbergii* (Liu et al., 2016) showed that some transcripts harboring microsatellites were also related to the transcription factors. In *Elaeis guineensis* (Tranbarger et al., 2012), microsatellite polymorphisms were also found in sequences encoding *AP2-like*, *bZIP*, zinc finger, *MADS-box*, and *NAC-like* transcription factors, as well as other transcriptional regulatory proteins and several RNA interacting proteins. SSRs in transcripts encoding proteins related to transcription and other functions may provide pertinent markers for MAS.

The PIC values were used to estimate the informativeness of the markers, which was categorized as high (PIC > 0.5), moderate (0.5 < PIC < 0.25), and low (PIC < 0.25), respectively (Botstein et al., 1980). In the present research, 23 of 25 primers exhibited high PIC values, the mean PIC value of 0.67 was lower compared with a previous report for medicinal *Chrysanthemum* cultivars (0.97) (Feng et al., 2016), but higher than *Phaseolus vulgaris* (0.47), *Ipomoea nil* (0.41 ± 0.14), and faba bean (0.19) (El-Rodeny et al., 2014; Garcia et al., 2011; Ly et al., 2012). Both UPGMA method and STRUCTURE analyses revealed that the 59 samples were divided into six clusters (Fig. 2A and B). Our results indicated that samples of *Chrysanthemum* had a closer genetic relationship with those of *Ajania*, which is in agreement with previous findings that *Ajania* should be combined into the genus of *Chrysanthemum* (Luo et al., 2018; Ohashi and Yonekura, 2004; Zhao et al., 2009). There was significant genetic differentiation between wild species and cultivars in *Chrysanthemum*. In the case of cultivars, eight large chrysanthemums were clustered together and are closely related to *A. pacifica*, indicating that large chrysanthemums may have a higher ploidy. And the intergeneric hybrids were grouped together (Liu et al., 2015). These results suggest there is substantial differentiation in the genetic relationships between different genera and various cultivars. The incongruence between the dendrogram and the division of cultivars may be attributed to a recording error during the data-collecting process.

In conclusion, 831 valid and 361 polymorphic EST-SSR markers have been developed for chrysanthemum, substantially increasing the molecular marker repository for chrysanthemum. The transferability analysis and phylogenetic relationships analysis have validated the universality of the 25 randomly selected polymorphic EST-SSRs. They were transferable to *Ajania* species and can distinguish the phylogenetic relationships between wild species and cultivars. Some SSR-containing sequences were assigned putative functions, which will require further study. These EST-SSR markers provide a valuable genomic resource for cultivar identification and characterization, genetic diversity assessment, genetic linkage mapping, and MAS in *Chrysanthemum* species and related taxa.

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Supplemental Fig. 1. (A) The classification of 361 unigenes containing polymorphic microsatellite based on the Gene Ontology (GO) annotation. (B) The classification of 361 unigenes containing polymorphic microsatellite based on the clusters of orthologous groups (COG) annotation. (C) The top 10 enriched pathways involved the unigenes containing polymorphic microsatellite. mRNA = messenger RNA.

C

Supplemental Fig. 1. (Continued).

Supplemental Table 1. Abbreviation and origin of 59 samples in this study.

Code	Accession	Abbreviation	Ploidy	Species or cultivar types/origin	Notes
1	<i>Chrysanthemum indicum</i>	ind1	2x	Species/Korea	
2	<i>Chrysanthemum chanetii</i>	cha	4x	Species/Beijing	
3	<i>Chrysanthemum nankingense</i>	man	2x	Species/Jiangsu Province	
4	<i>Chrysanthemum indicum</i>	ind 2	4x	Species/Hubei Province	
5	<i>Chrysanthemum indicum</i> var. <i>aromaticum</i>	ind 3	2x	Species/Hubei Province	
6	<i>Chrysanthemum zawadskii</i>	zaw	6x	Species/Heilongjiang Province	
7	<i>Ajania fruticulosa</i>	fru		Species/Qinghai Province	
8	<i>Ajania potaninii</i>	pot		Species/Sichuan Province	
9	<i>Ajania achilloides</i>	ach		Species/Inner Mongolia	
10	<i>Ajania scharnhorstii</i>	sch		Species/Xinjiang Province	
11	<i>Ajania khatensis</i>	kha		Species/Yunnan Province	
12	<i>Ajania purpurea</i>	pur		Species/Tibet	
13	<i>Ajania pacifica</i>	pac 1	10x	Species/Beijing	
14		pac 2		Species/Beijing	
15		pac 3		Species/Beijing	
16		pac 4		Species/Beijing	
17	<i>Chrysanthemum morifolium</i>	chr1		Cultivars/Beihai Park in Beijing	Large chrysanthemum
18		chr2		Cultivars/Beihai Park in Beijing	Large chrysanthemum
19		chr3		Cultivars/Beihai Park in Beijing	Large chrysanthemum
20		chr4		Cultivars/Beihai Park in Beijing	Large chrysanthemum
21		chr5		Cultivars/Beihai Park in Beijing	Large chrysanthemum
22		chr6		Cultivars/Beihai Park in Beijing	Large chrysanthemum
23		chr7		Cultivars/Beihai Park in Beijing	Large chrysanthemum
24		chr8		Cultivars/Beihai Park in Beijing	Large chrysanthemum
25	<i>Ajania pacifica</i> hybrids	aja1		Intergeneric hybrids/Beijing	
26		aja2		Intergeneric hybrids/Beijing	
27		aja3		Intergeneric hybrids/Beijing	
28		aja4		Intergeneric hybrids/Beijing	
29		aja5		Intergeneric hybrids/Beijing	
30		aja6		Intergeneric hybrids/Beijing	
31		aja7		Intergeneric hybrids/Beijing	
32		aja8		Intergeneric hybrids/Beijing	
33		aja9		Intergeneric hybrids/Beijing	
34		aja10		Intergeneric hybrids/Beijing	
35		aja11		Intergeneric hybrids/Beijing	
36		aja12		Intergeneric hybrids/Beijing	
37		aja13		Intergeneric hybrids/Beijing	
38	Baiou	bo		Cultivars/Beihai Park in Beijing	Large chrysanthemum
39	Lvyishang	lys		Cultivars/Beihai Park in Beijing	Large chrysanthemum
40	Fanxing	fx		Cultivars/Beihai Park in Beijing	Escarpment chrysanthemum
41	Junziyu	jzy		Cultivars/Beihai Park in Beijing	Escarpment chrysanthemum
42	Shiyongju	syj		Cultivars/Beijing Forestry University	Edible chrysanthemum
43	Fenditan	fdt		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
44	Pudifendai	pdfd		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
45	Ziyunqinfang	zyqf		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
46	Fanhuasijin	fhsj		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
47	Zhaoyanghong	zyh		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
48	Jinzhu	jz		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
49	Nongfenzhaoxia	nfzx		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
50	Baisha	bs		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
51	Danhanfen	dhf		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
52	Jinjihongling	jhl		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
53	Maoxiangyu	mxy		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
54	Xiangfei	xf		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
55	Mibaizao	mbz		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
56	Pudidafen	pddf		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
57	Yulong	yl		Cultivars/Beijing Forestry University	Groundcover chrysanthemum
58	HJJ	hjj		Cultivars/Beijing Forestry University	
59	SN*MX	sm		Cultivars/Beijing Forestry University	

Supplemental Table 4. Transferability of 25 chrysanthemum expressed sequence tag–simple sequence repeat (EST-SSR) markers to other *Chrysanthemum* and *Ajania* species.

Primer name	Species											
	<i>Chrysanthemum indicum</i> (ind1)	<i>Chrysanthemum chanetii</i>	<i>Chrysanthemum mankingense</i>	<i>Chrysanthemum indicum</i> (ind2)	<i>Chrysanthemum indicum</i> var. <i>aromaticum</i>	<i>Chrysanthemum zawadskii</i>	<i>Ajania fruticulosa</i>	<i>Ajania potaninii</i>	<i>Ajania achilloides</i>	<i>Ajania scharnhorstii</i>	<i>Ajania kharlensis</i>	<i>Ajania purpurea</i>
CEM076	+	+	+	+	+	+	+	+	+	+	+	+
CEM080	+	+	+	+	+	+	+	+	+	+	+	+
CEM081	+	+	+	+	+	+	+	+	+	+	+	+
CEM082	+	+	+	+	+	+	+	+	+	+	+	+
CEM088	+	+	+	+	+	-	-	+	-	-	+	+
CEM091	+	+	+	+	+	+	+	+	+	+	+	+
CEM093	+	+	+	+	+	+	+	+	+	+	+	+
CEM371	+	+	+	+	+	+	+	+	+	-	+	-
CEM372	+	+	+	+	+	+	+	+	+	+	+	+
CEM380	+	+	+	+	+	+	+	+	+	+	+	+
CEM496	+	+	+	+	+	+	+	+	+	+	+	+
CEM501	+	+	+	+	+	+	+	+	+	+	+	+
CEM504	+	+	+	+	+	+	+	+	+	+	+	+
CEM505	+	+	+	+	+	+	+	+	+	+	+	+
CEM508	+	+	+	+	-	+	-	+	+	+	+	+
CEM512	+	+	+	+	+	+	+	+	+	+	+	+
CEM513	+	+	+	+	+	+	+	+	+	+	+	+
CEM514	+	+	+	+	-	+	+	+	-	+	+	+
CEM515	+	+	+	+	+	+	+	+	-	+	+	+
CEM519	+	+	+	+	+	+	+	+	+	-	+	-
CEM685	+	+	+	-	+	+	+	+	+	+	+	+
CEM723	+	+	+	+	+	+	+	+	+	+	+	+
CEM780	+	+	+	+	+	+	+	+	+	+	-	-
CEM782	+	+	+	+	+	+	+	+	+	+	+	+
CEM814	+	+	+	+	+	+	+	+	+	-	+	+
Transferability (%)	100	96	100	96	92	96	92	100	88	76	92	88

+ = present of polymerase chain reaction (PCR) amplicons; – = absent of PCR amplicons.