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Distribution and Frequency of SSR Motifs in the Chrysanthemum SSR-enriched Library through 454 Pyrosequencing Technology

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국화 SSR-enriched library에서 SSR 반복염기의 분포 및 빈도

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ABSTRACT: Chrysanthemums, often called mums or chrysanths, belong to the genus *Chrysanthemum*, which includes about 30 species of perennial flowering plants in the family Asteraceae. We extracted DNA from *Dendranthema grandiflorum* ('Smileball') to construct a simple sequence repeat (SSR)-enriched library, using a modified biotin-streptavidin capture method. GS FLX (Genome Sequencer FLX System which provides the flexibility to perform the broad range of applications) sequencing (at the 1/8 run specification) resulted in 18.83 mega base pairs (Mbp) with an average read length of 280.06 bp. Sequence analyses of all SSR-containing clones revealed a predominance of di-nucleotide motifs (16,375, 61.5%) followed by tri-nucleotide motifs (6,616, 24.8%), tetra-nucleotide motifs (1,674, 6.3%), penta-nucleotide motifs (1,283, 4.8%), and hexa-nucleotide motifs (693, 2.6%). Among the di-nucleotide motifs, the AC/CA class was the most frequently identified (93.5% of all di-nucleotide types), followed by the GA/AG class (6.1%), the AT/TA class (0.4%), and the CG/GC class (0.03%). When we analyzed the distribution of different repeat motifs and their respective numbers of repeats, regardless of the motif class, of 100 SSR markers, we found a higher number of di-nucleotide motifs with 70 to 80 repeats; we also found two di-nucleotide motifs with 83 and 89 repeats, respectively, but their product lengths were within optimum size (297 and 300 bp). In future work, we will screen for polymorphisms of possible primer pairs. The results will provide a useful tool for assessing molecular diversity and investigating the population structure among and within *Chrysanthemum* species.

***Key words*:** Chrysanthemum, SSR, enriched library, pyrosequencing

The name *Chrysanthemum* is derived from the Greek words *chrysos* (gold) and *anthemom* (a flower) (Linneaus, 1753). Chrysanthemums, often called mums or chrysanths, belong to the genus *Chrysanthemum*, which includes about 30 species of perennial flowering plants in the family Asteraceae. Chrysanthemums were first recorded by Confucius in 500 BC in China. In the late fourth century AD, they were introduced to Japan from China (Elzer, 2000). Japan considered chrysanthemum a national flower, and the

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flowers were often used in centerpieces during national festivals and holidays. Chrysanthemum then spread to Europe, America, and eventually throughout the world. By the time the Massachusetts Horticultural Society was incorporated in 1829, 17 varieties of chrysanthemum were recognized in the United States (http://faculty.ucc.edu/biology-ombrello/pow/chrysanthemum.htm). Widespread cultivation in Europe began in the 19th century when nurseries started to specialize in growing varieties developed by hybridization and importation from the Orient (http://faculty.ucc.edu/biology-ombrello/pow/chrysanthemum.htm).

The Chrysanthemum is valued and cultivated around the world and soon became the second most sold cut flower at Dutch flower auctions after the rose. Other than for aesthetic purposes (garden plants and commercial cut flowers), chrysanthemum flowers were thought to have healing properties and were used as symbols of health and long life (Yeung, 1983). Therefore, chrysanthemum flowers are used extensively in traditional Chinese medicine for treating a wide range of ailments, including upper respiratory infections, allergies, headaches, red eyes, and hypertension (Yeung, 1983). Chrysanthemum flowers effectively reduce irritation and inflammation in the lungs, nasal passageways, and throat. Chrysanthemum flowers have antibacterial, antifungal, and antiviral activities (Yeung, 1983). They also have a calming, antihypertensive effect. White chrysanthemum is considered slightly superior to other forms for nourishing the liver. Yellow chrysanthemum is used most often to treat eye redness and headache (Yeung, 1983). Besides its medicinal value, a chrysanthemum extract can also be used as raw material in dye and tea production, particularly in China. Chrysanthemum leaves are steamed or boiled and used as greens in Chinese cuisine. Although there are endless uses for chrysanthemum, there have been relatively few systematic genetic analyses, compared to other crops.

The genome of chrysanthemum is composed of multiple sets of chromosomes that range from diploid to decaploid. The basic monoploid number is nine, but other sets include diploid (2n = 18), tetraploid (4n = 36), hexaploid (6n = 54), octaploid (8n = 72), and decaploid (10n = 90) (Hartl and Jones, 2009). Moreover, a long period of cultivation, natural selection, and artificial crossing has led to numerous cultivars, abundant morphological variation, powerful suitability, wide distribution, and an extremely complicated genetic background. Consequently, the investigation, classification, and identification of chrysanthemum cultivars has become very difficult, further restricting breeding and practical applications.

Several DNA analysis techniques have been applied to identify chrysanthemum cultivars. Unrelated cultivars can be distinguished from each other using random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), polymerase chain reaction (PCR), hybridization-based DNA fingerprinting, as well as restriction fragment length polymorphism (RFLP; Zhang *et al*., 2010), and sequence-related amplified polymorphism (SRAP; Zhang *et al*., 2011). Cultivars with different flower colors belonging to one family, i.e., that are vegetatively derived from one cultivar, appear to have the same DNA fragment patterns, regardless of the technique applied. The absence of polymorphisms between different accessions of the same cultivar indicates high stability of the observed patterns (Wolff *et al*., 1995). Therefore, it is important to develop a high-resolution technique for assessing and distinguishing among the more complicated chrysanthemum accessions.

SSRs, or microsatellite markers, have been used to study genetic diversity, phylogenetic relationships, classification, evolutionary processes, and quantitative trait loci in many crops (Moe *et al.*, 2010; *Cho et al.*, 2011; *Zhao et al.*, 2011). SSRs offer several advantages, such as technical simplicity, relatively low cost, high genetic resolution power, and high polymorphism. Moreover, they are reliable and easy to score (Gupta and Varshney, 2000). SSRs are clusters of short tandem repeat nucleotide bases distributed throughout the genome and are co-dominant, multi-allelic, and require a small amount of DNA for scoring. Therefore, they have been recognized as useful molecular tools for marker-assisted selection in various species (Agrama *et al*., 2007). The present study determined the distribution of SSR motifs in the sequence data of an SSR-enriched library of *Chrysanthemum*. Our results can be used to develop SSR markers that may be useful for crop-improvement programs.

MATERIALS AND METHODS

Plant materials and DNA extraction

One chrysanthemum accession, Yesan G7 [*Dendranthema grandiflorum* (Ramat.) Kitam., 6n=54], Smileball was used to develop the genomic libraries. The plant samples were taken from Yesan Chrysanthemum Experiment Station. DNA was extracted from fresh green leaves using the Qiagen DNA extraction kit (Qiagen, Hilden, Germany). The relative purity and concentration of extracted DNA was estimated with Thermo Scientific NanoDrop ND1000 (Shimadzu scientific instruments, Suja Sukumaran, Japan).

SSR-enriched library construction

The library was constructed following the modified biotin-streptavidin capture method of Dixit *et al*. (2005) and Ma *et al*. (2009). Briefly, total DNA was digested in separate reactions with seven restriction enzymes: *EcoRV, DraI, SmaI, PvuI, AluI, HaeIII, and RsaI*. After pooling, the

digested DNA was size-fractionated on 1.4% agarose gels. Fragments ranging from 300–1500 bp were eluted from the gel followed by purification using a gel extraction kit (Qiagen). DNA fragments were ligated to an adaptor (AP11–5'-CTCTTGCTTAGATCTGGACTA-3' and AP12–5'-TAGTC-CAGATCTAAGCAAGAGCACA-3') with T4 DNA ligase at 14°C overnight. The adaptor-ligated DNA was hybridized with a mixture of biotin-labeled SSR probes [(GA)20, (CA)20, (AT)20, (GC)20, (AGC)15, (GGC)15, (AAG)15, (AAC)15, (AGG)15], and the hybridized DNA fragments were captured with streptavidin-coated magnetic beads (Promega, Madison, WI, USA). After stringent washing, the captured DNA fragments were eluted in 50 ml distilled water. Final eluates were amplified by PCR with the AP11 primer (94°C for 7 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min; 72°C for 10 min). The products were used to initiate pyrosequencing (hereafter, 'pyrosequencing') using a Roche 454 GS FLX Titanium sequencer (Genome Sequencer FLX System which provides the flexibility to perform the broad range of applications; Roche/454 Life Sciences, Branford, CT, USA) (Ronaghi, 2001; Elahi and Ronaghi, 2004).

Pyrosequencing of the SSR-enriched library

Single copies of template species from the DNA library were hybridized to DNA capture beads. The immobilized library was re-suspended in amplification solution, and the mixture was emulsified, followed by PCR amplification. After amplification, the DNA-conjugated beads were recovered from the emulsion and enriched. The second strands of the amplification products were removed, leaving the amplified single-stranded DNA library bound to the beads. The sequencing primer was annealed to the immobilized amplified DNA templates. After amplification, a single DNA-carrying bead was placed in each well of a PicoTiterPlate (PTP) device. The PTP was inserted into the FLX genome titanium sequencer for pyrosequencing (Ronaghi, 2001; Elahi and Ronaghi, 2004), and sequencing reagents sequentially flowed over the plate. Information from the PTP wells was captured simultaneously by a camera, and the images were processed in real-time by an onboard computer.

Investigation of SSR motifs

All sequences generated by pyrosequencing were investigated for SSR motifs using the ARGOS program 1.46 (SSRManager) at the default setting (Kim *et al*., 2007). The SSRs detected were categorized as perfect di-, tri-, tetra-, penta-, or hexa-nucleotide motifs. Of the identified motifs, only those with sufficiently large flanking sequences could be used to design primer pairs.

RESULTS

GS FLX titanium sequencing

A summary of the genomic DNA sequencing results is presented in Table 1. About 96.7% (122,228) of the total raw wells (126,347) passed the key step. However, only 53.2% (67,246 wells) of the total raw wells could be successfully sequenced as they passed the filter. GS FLX sequencing (at the 1/8 run specification) resulted in 18.83 mega base pairs (Mbp) with an average read length of 280.06 bp. The read length ranged from 40 to 741 bp with a medium read length of 290 bp. The standard deviation of the read length among all sequence reads was 144.37.

SSR repeat motif identification

All sequences were entered into the SSRManager ARGOS program 1.46 (Kim et al. 2007) to identify the SSR motifs. Analysis of all SSR-containing reads revealed a predominance of di-nucleotide repeat motifs (16,375, 61.5%) followed by tri-nucleotide motifs (6,616, 24.8%), tetra-nucleotide motifs (1674, 6.3%), penta-nucleotide motifs (1283, 4.8%), and hexa-nucleotide motifs (693, 2.6%). Among the di-nucleotide motifs, the AC/CA class was the most frequently identified (93.5% of all di-nucleotide types), followed by the GA/AG class (6.1%), the AT/TA class (0.4%), and the CG/GC class (0.03%).

The AAC class (50.5%) was the highest of the tri-nucleotide motif types followed by ACT (23.7%), ACG (15.6%), ACC (3.8%), AAG (3.6%), and other classes (2.7%). The ACGT class (43.6%) dominated the other classes in the tetra-nucleotide motif type; ACCT (12.5%), AACT (10.0%), AACG (8.9), and other classes (25.0%), whereas in the penta-nucleotide motif type, the AACGT class (27.2%) was found at a higher number than other classes; ACACT (20.6%), ACCGT (10.8%), ACACG (10.1%),

Table 1. GS FLX titanium sequencing results for *Chrysanthemum* (1/8 of PicoTiterPlate).

Item	Number	Percent (%)
Raw Wells	126,347	
Key Pass Wells	122,228	96.7
Passed Filter Wells	67,246	53.2
Total Bases	18,830,109	
Average Length	280.06	
Std Deviation	144.37	
Longest Read Length	741	
Shortest Read Length	40	
Median Read Length	290	

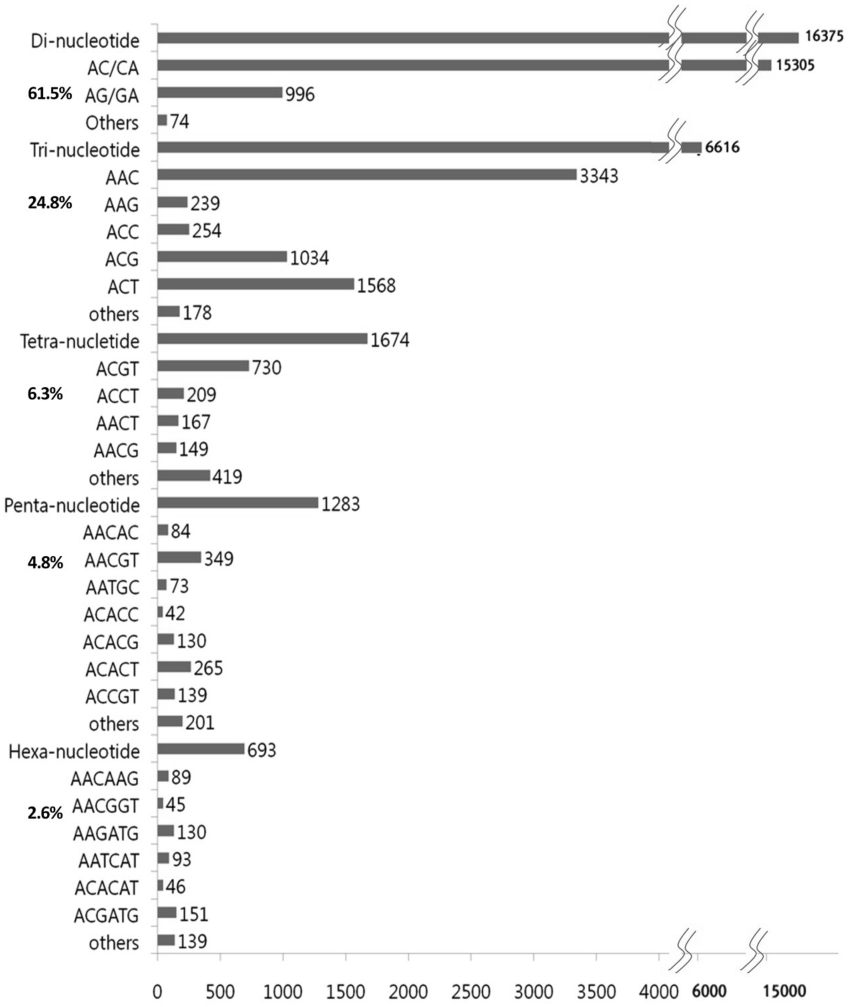

Fig. 1. Characteristics of the simple sequence repeat sequences identified from an enriched library of *Dendranthema grandiflorum* ('Smileball').

AACAC (6.5%), AATGC (5.7%), ACACC (3.3%), and others (15.7%). The ACGATG class (21.8%) was the highest of the hexa-nucleotide motif type followed by AAGATG (18.8%), AATCAT (13.4%), AACAAG (12.8%), ACACAT (6.6%), AACGGT (6.5%), and other classes (20.1%).

When we analyzed the distribution of different repeat motifs and their respective numbers of repeats, regardless of the motif class, of 100 SSR markers, di-nucleotide motifs with less than five repeats were the most abundant, followed by di-nucleotide motifs with less than 10 repeats and those with less than 15 repeats. There was also a higher number of di-nucleotide motifs with 70 to 80 repeats, and two markers had a particularly high number of repeats (83 and 89 repeats, respectively). The abundances of other motifs were less than those of di-nucleotides, in the following order: tri-, tetra-, and other types (Fig. 2). These results make it clear that the higher the nucleotide number, the lower the number of repeats. In other words, it was difficult to obtain a high number of repeats when there were many nucleotides in a motif.

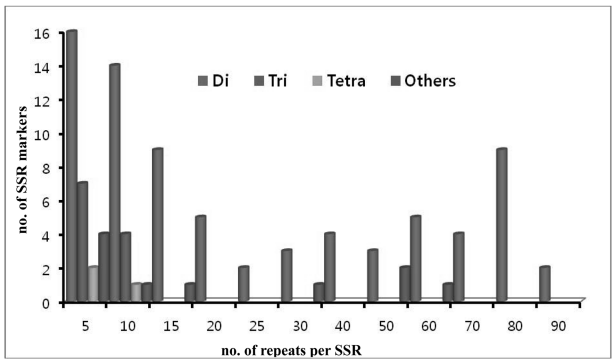

Fig. 2. Distribution of simple sequence repeat (SSR) motifs in 100 designed SSR markers. Colored bars show the number of markers from di-nucleotide, tri-nucleotide, tetra-nucleotide, and other nucleotide categories with different numbers of repeats.

DISCUSSION

The polyploidy complex makes chrysanthemum morphologically different. As a result, chrysanthemum is difficult to identify genetically and systematically. Although unrelated cultivars can be distinguished from each other using RAPD analysis, ISSR analysis, hybridization-based DNA fingerprinting, and restriction fragment length polymorphism analysis, cultivars with different flower colors belonging to the same family, i.e., that are vegetatively derived from one cultivar, appear to have the same DNA fragment patterns, regardless of the technique applied (Wolff *et al*., 1995). Wolff *et al*. (1995) suggested that the absence of polymorphism between different accessions of the same cultivar is due to high stability of the patterns. Difficulties in systematically classifying chrysanthemum were also reported by Yang *et al*. (2006). They stated that lineage recombination is due to extensive sharing of chloroplast haplotypes among tetraploid populations with different origins. Multiple differentiation and hybridization/polyploidization cycles have led to evolutionary reticulation in chrysanthemums. Several researchers have investigated the genetic diversity and phylogeny of chrysanthemum using different molecular markers such as isozymes (Ding *et al.*, 2007) and DNA markers (RAPDs) (Wolff 1995, Chatterjee *et al*., 2006, Zhang *et al*., 2010), sequence-characterized amplified regions (Chatterjee *et al.*, 2006), chloroplast SSR markers (Yang *et al*., 2006), amplified fragment length polymorphisms (Zhang *et al*., 2010), ISSRs (Ouyang *et al*., 2010) and sequence-related amplified polymorphisms (Zhang *et al*., 2011). However, Silan *et al*. (2002) pointed out that the results based on morphology, cytology, inter specific hybridization and molecular systematics indicated that there are still lots of work need to be done on chrysanthemum. Therefore, an intensive classification of inter- and intra-species variation must be performed in *Chrysanthemum*. The present study identified the distribution of SSR motifs in a *Chrysanthemum* SSR-enriched library through pyrosequencing technology to develop a more powerful analytical tool for identifying SSR markers in chrysanthemum. GS FLX sequencing yielded (18.83 Mbp), because we inserted the DNA sample in only 1/8 of the PTPs. Although the average read length of 280.06 bp was much lower than the potential average read length (450 bp) of first-class next-generation sequencing services based on the long read (Roche 454 GS FLX Titanium) of the pyrosequencing system, satisfactory sequencing results were achieved.

The pattern of nucleotide repeat motifs found in chrysanthemum was similar to findings for other crops (Cho *et al*., 2010, Dixit *et al*., 2010, Moe *et al*., 2010 and 2011,), revealing a predominance of the di-nucleotide motif compared to the tri-, tetra-, penta-, and hexa-nucleotide motifs. Among the di-nucleotide motifs, the AC/CA class was most frequently identified. The AAC class was the most abundant tri-nucleotide motif, whereas the ACGT class was the most dominant tetra-nucleotide motif.

When we analyzed the distribution of different repeat motifs and their respective numbers of repeats, regardless of the motif class, of 100 SSR markers, we found a higher number of di-nucleotide motifs with 70 to 80 repeats; we also found two di-nucleotide motifs with 83 and 89 repeats, respectively, but their product lengths were within optimum size (297 and 300 bp). Our results confirm the difficulties of obtaining a high number of repeats when there are many nucleotides in the repeat motif. The present results will support the development of SSR markers, which will be useful for applying, classifying, identifying, protecting, and breeding cultivars.

적 요

국화과(Compositae)는 현화식물 중 세계에서 가장 넓게 분포하고, 쌍자엽식물 중 가장 진화된 식물분류군이며, 우리나라에는 약 300여종이 존재하는 것으로 알려져 있다. 구절초, 감국, 쑥, 쑥갓, 개미취, 참취, 곰취 등 국화과 식물들은 예로부터 민간에서 약용 및 식용 소재로써 다양하게 사용되어왔다. 본 연구는 국화 및 국화근연종 유용유전자원 선발을 통하여 육종 소재를 확대하고, 중간모본 및 신품종 육성기반을 구축하고자 DNA 마커시스템의 개발을 위해 수행되었다.

1. 화단국인 Smileball(Dendranthema grandiflorum) 품종을 사용하여 SSR-enriched library를 작성하였고, GS FLX 분석을 통해 18.83Mbp의 염기서열 결과를 얻었으며, read의 평균 길이는 280.06bp로 나타났다.

2. 단순반복염기서열(SSR) 부위를 포함하는 26,780개 clones 중 di-nucleotide motifs가 16,375개(61.5%)로 우세하였고, tri-nucleotide motifs(6,616개, 24.8%), tetra-nucleotide motifs(1,674개, 6.3%), penta-nucleotide motifs(1,283개, 4.8%), hexa-nucleotide motifs(693개, 2.6%) 순으로 나타났다.

3. 얻어진 di-nucleotide motifs들 중에서는, AC/CA class가 93.5%로 대부분이었고, tri-nucleotide motifs에서는 AAC class가 50.5%, tetra-nucleotide motifs는 ACGT class가 43.6%이고, penta-nucleotide motif에서는 AACGT class 27.2%이며, hexa-nucleotide motif에서는 ACGATG class 21.8%였다.

4. 얻어진 염기서열 결과를 토대로 다양한 motif를 갖는 100개의 SSR 마커를 제작하였고, 차후 이를 활용하여 국화 유전자원의 다형성 및 유전자형 분석을 통해 분자유전학적 다양성 및 집단의 구조분석이 가능하고, 국화의 분자육종기반 구축을 위한 유용한 도구가 될 것 이다.

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