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Development of EST-derived SSR markers using next-generation sequencing to reveal the genetic diversity of 50 chrysanthemum cultivars

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ABSTRACT

Chrysanthemum plants are popular worldwide as cut flowers, potted, and in gardens. Several hundred cultivars have been commercialized, indicating that there is substantial genetic variations that can be manipulated under cultivations to produce a wide array of phenotypic variation. To study the genetic diversity of chrysanthemum cultivars in Korea, we first identified simple sequence repeats from chrysanthemum expressed sequence tags generated by FLX 454 sequencing. A total of 1109 ESTs out of 18,226 chrysanthemum ESTs were identified to carry SSRs. A total of 16 out of 46 primer pairs exhibited several polymorphisms among 50 chrysanthemum cultivars. The number of alleles per locus varied from 1 to 15, with an average of 6.25 alleles. The expected heterozygosity ranged from 0 to 0.8958, whereas polymorphism information content ranged from 0 to 0.8872. Based on polymorphisms using 16 SSR markers, a phylogenetic tree was generated revealing four groups within the 50 cultivars showing various levels of genetic diversity. The 16 polymorphic chrysanthemum SSR markers generated in this study would be useful for studies of the genetic conservation, diversity, and population structure of commercial chrysanthemum cultivars as well as closely related species.

1. Introduction

Chrysanthemums are popular plants as cut flowers, potted, and in gardens throughout the world. In many countries including the Netherlands, Japan, Colombia, Spain, and Italy, chrysanthemums are very commercially important for the floral industry (Tomassoli et al., 2004). In addition, some chrysanthemum species, such as *Chrysanthemum morifolium* and *Chrysanthemum cinerariaefolium*, have been widely used as a medicinal tea and in the production of the insecticide pyrethrin, respectively (Hitmi et al., 2000; Miyazawa and Hisama, 2003).

Chrysanthemums have been cultivated in China and Japan for at least 3000 years (Wolff and Peters-Van Rijn, 1993). It has been reported that in 385 A.D., Korea presented Japan with a gift of chrysanthemum seeds; the resultant seedlings had flower colors of blue, red, white, black, and yellow (Ackerson, 1957). In the past decade, the University of Minnesota garden chrysanthemum breeding program has been pursuing studies of the reproductive biology and creation of acceptable, uniform

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seed-propagated cultivars by developing inbred lines homozygous for important phenotypic traits (Anderson et al., 1995; MacDonald and Kwong, 2005). The hybridization of florist or greenhouse chrysanthemums began after 1850 when the practice of culturing chrysanthemums in greenhouses began. Commercial greenhouse chrysanthemum cultivars are 8–14-week short day flowering response groups (Larson and Armitage, 1980). Greenhouse chrysanthemums include both cut flower types and flowering potted plant cultivars, each with specific production protocols (Dole and Wilkins, 1999). As many as 200 species were originally contained within the genus *Chrysanthemum*, but the majority have been subdivided into 38 satellite genera of the chrysanthemum complex (Anderson, 1987). Several hundred cultivars have been commercialized, indicating that there are substantial genetic variations that can be manipulated under cultivations to produce a wide array of phenotypic variation. Because the natural constraints limiting hybridization and the detrimental effects of some phenotypes are no longer determining plant survival. Diverse characters for various chrysanthemum cultivars have been evaluated including flower size, color, shape and blooming period (http://www.mums.org). The Germplasm Resources Information Network (GRIN) recognizes a total of 124 species of chrysanthemums taking synonyms into account, though only 45 species were recorded with accepted names (http://www.ars-grin.gov). In addition, a recent study suggests that hybridization and polyploidy influence phenotypic variations, especially flower size (Liu et al., 2012).

Many different molecular DNA markers have been developed for plants in general; these can be applied for various purposes such as molecular breeding, genetic conservation, diversity, and evolutionary studies (Ovesna et al., 2002; Staub et al., 1996). Of the available molecular markers, simple sequence repeats (SSRs), or microsatellites composed of tandemly repeated units, are widely used because they are very polymorphic and present in a wide range of plant genomes (Kalia et al., 2011; Powell et al., 1996). In the case of chrysanthemums, several markers have been developed including sequence-related amplified polymorphism (SRAP) (Zhang et al., 2011), simple sequence repeat (Wang et al., 2013), random amplified polymorphic DNA (RAPD) (Barakat et al., 2010), inter simple sequence repeat (ISSR) (Palai and Rout, 2011) and amplified fragment length polymorphism (AFLP) (Morin and Woodruff, 1996; Zhang et al., 2010).

With the rapid development of sequencing technology, numerous ESTs have been obtained for many plant species (Kurata et al., 1994; Qi et al., 2004; Ronning et al., 2003). Furthermore, ESTs can be good resources to generate SSR markers (Ellis and Burke, 2007; Varshney et al., 2005). These SSRs are useful as molecular markers and as tools for biodiversity studies because their development is inexpensive providing high levels of genetic diversity (Morin and Woodruff, 1996; Ovesna et al., 2002; Varshney et al., 2005). Thus far, only a few SSR markers for polyploidy chrysanthemums are currently known due to the limitation of available chrysanthemum gene sequences (Liu et al., 2012; Wolff and Peters-Van Rijn, 1993). Moreover, no study related to the genetic diversity of chrysanthemum cultivars has been reported. In this study, we developed a total of 16 microsatellite markers from chrysanthemum ESTs generated by FLX 454 sequencing. Using the 16 developed microsatellite markers, we successfully demonstrated the genetic diversity of 50 chrysanthemum cultivars.

2. Materials and methods

2.1. Plant materials and genomic DNA extraction

A total of 50 commercial chrysanthemum cultivars used in this study were purchased from a flower market in October 2012. Chrysanthemum cultivars were randomly chosen regardless of their characteristics. Images were taken of the flowers, and five phenotypic characteristics were examined. Leaves from each cultivar were immediately frozen using liquid nitrogen. For the expressed sequence tag analysis, the chrysanthemum cultivar Shinma, which is the most popular cultivar in Korea, derived from the National Institute of Horticultural and Herbal Science, Rural Development Administration, Republic of Korea, was used. Total genomic DNA was extracted from leaves of each cultivar using a Qiagen DNeasy Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The quality and quantity of extracted genomic DNA were measured using a NanoPhotometer (Implen, Munich, Germany) and visualized in 1.8% agarose gel. The concentration of genomic DNA was adjusted to 100 ng/μL.

2.2. Identification of chrysanthemum SSRs from EST data and primer design

After removing redundant EST sequences, a total of 18,226 sequences obtained from 7280 ESTs deposited in the National Center for Biotechnology Information on June 1, 2012, and 11,600 ESTs from a previous study (Jo et al., 2014) were used for the identification of simple sequence repeats (SSRs). SSRs were identified using a MIcroSAtellite identification program (MISA) implemented in PrimerPro v.1.0 (http://webdocs.cs.ualberta.ca/~yifeng/primerpro/) with the default parameters. Primer pairs were designed using MISA (http://www.pgrc.ipkgatersleben.de/misa) implemented in PrimerPro v.1.0 with the default parameters.

2.3. Amplification of SSR alleles by PCR

PCR was performed in 50 μL volumes containing 0.5 μL EX Taq DNA polymerase (5 units/μL) (Takara, Shiga, Japan), 1 pmol of forward primer, 1 pmol of reverse primer, 2 μL dNTP mixture (2.5 mM each nucleotide), 10 μL of 5× EX Taq DNA buffer, 1 μL of DNA template (0.1 μg/μL) and deionized water up to a total volume of 50 μL. PCR conditions included an initial denaturation at 94 °C for 10 min, 35 cycles of 30 s at 94 °C for denaturation, 30 s at 56 °C for annealing and 30 s at 72 °C for extension, and a