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SSR Analysis of Genetic Relationship and Classification in Chrysanthemum Germplasm Collection

*LUO Chang, CHEN Dongliang, CHENG Xi, LIU Hua, LI Yahui, and HUANG Conglin**

Beijing Agro-Biotechnology Research Center, Beijing Academy of Agriculture and Forestry Sciences, Beijing Engineering Research Center of Functional Floriculture, Beijing Key Laboratory of Agricultural Genetic Resources and Biotechnology, Beijing 100097, China

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A B S T R A C T

To further test the accuracy and rationality of the Chinese classification system for chrysanthemum (*Chrysanthemum morifolium*), 10 simple sequence repeat (SSR) markers were used to identify a collection of 88 chrysanthemum and its related genera accessions. In total, 42 effective alleles across 88 accessions were detected; 3 429 bands were obtained by PCR amplification, including 2 630 polymorphic bands. The similarity coefficient ranged from 0.53 to 0.88. Cluster analysis based on UPGMA illustrated that the wild species and large-flower cultivars were first divided into two clusters, then the large-flower cultivars formed five distinct groups according to petal type, indicated that petal type can be a classification criterion. In the wild species cluster, *C. vestitum* and *C. zawadaskii* grouped with *A. trilobata*, suggested that the *Ajania* genera was closely related to the *Chrysanthemum* genera. 'Hangbaiju', 'Gongju' and 'Chuju' were grouped together, and 'Boju', *O. longilobus* and *C. mongolicum* constituted another branch, showed a correlation with geographic region of origin. Population structure analysis was subsequently performed with *K* values ranging from 2 to 10, and the most likely estimate for the population structure is five subpopulations, which is nearly consistent with the clustering results. Principle component analysis was further performed to verify the classification results. The results showed that these SSR markers are very powerful for studying genetic relationships and will be useful tools in the identification and classification of chrysanthemum.

Keywords: chrysanthemum; SSR; polymorphism; genetic relationship; classification

1. Introduction

Chrysanthemum (*Chrysanthemum morifolium*), one of the most popular ornamentals throughout the world, has approximately 1 600 years of cultivation history in China and is noted for ornamental, edible, tea and medicinal uses. Chinese traditional chrysanthemums have ornamental values in their abundant diversity of flower type, color and architecture (Zhang et al., 2010). Such a rich diversity is in accordance with the genomic complexity and allohexaploid background of cultivated chrysanthemum worldwide (Dowrick and El-Bayoumi, 1966). Although the origin of chrysanthemum has remained uncertain, the generally accepted hypothesis is that cultivated chrysanthemums are derived from natural hybridizations among species of *Chrysanthemum sinense*, *C. erubescens*, *C. ornatum*, *C. japonese*, *C. makinoi*, *C. chanetii*, *C. vestitum*, *C. indicum*, *C. lavandulifolium* and *C. zawadskii* (Dai et al., 1998; Yang et al., 2006). Cultivated chrysanthemums are highly heterozygous due to their out-breeding and self-incompatibility, and this has delayed the genetic improvement of chrysanthemum (Drewlow et al., 1973). Chinese traditional chrysanthemums as hybrids owned very complicated genetic background coming from multiple species over hundreds of years of chrysanthemum breeding. During the long period of cultivation and natural evolution, Chinese traditional chrysanthemums have confronted serious phenomenon of homonyms and synonyms because of their high similarity in phenotypic characteristics (Zhang et al., 2014a). This problem has impeded plant breeding and international communication.

* Corresponding author. Tel.: +86 10 51503801
E-mail address: conglinh@126.com

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The study of chrysanthemum classification is popular in China, USA, UA and Japan and they developed several classification systems for chrysanthemum (Garibaldi et al., 2009; Yu et al., 2009; Barakat et al., 2010; Zhang et al., 2013a; Yuan et al., 2017). Both the Japanese and American classification criteria involve flower diameter and the flower head type. The same as the Chinese system, America classification gives priority to the flower diameter as the first criterion. In Japan, the flower diameter is used as the second criterion. The English classification system is relatively simple: flower head type is the only code. However, these classification systems are not competent to classify Chinese traditional chrysanthemums. Although the Chinese classification system for chrysanthemum is more systematic than others, there still needs more scientific evidence to support this system. Compared to morphological trait classification systems, molecular markers can reveal genetic differences at the DNA level between plants without environmental effects and are effective for evaluating the genetic diversity of germplasm in breeding programs (Jia, 1996). Over the past few years, various molecular markers have been developed and applied in chrysanthemum, such as random amplified polymorphic DNA (RAPD) (Wolff, 1996; Huang et al., 2000; Martín et al., 2002; Sehrawat, 2003), amplified fragment length polymorphism (AFLP) (Klie et al., 2013), inter-simple sequence repeats (ISSR) (Miao et al., 2007; Shao et al., 2010), sequence-related amplified polymorphism (SRAP) (Fei et al., 2011), and simple sequence repeats (SSR) (Zhang et al., 2014b; Yuan et al., 2017). SSR markers were testified to be a more advanced tool than all of these markers. The strengths of SSR markers include their high numbers in eukaryotes, the co-dominance of alleles, and their arbitrary dispensation throughout the genome with special consortium within low-copy regions (Morgante et al., 2002; Song et al., 2016). Basic on these advantages, SSR markers are very suitable for the identification and classification of Chinese traditional chrysanthemum.

Here, to further test the accuracy and rationality of the Chinese classification system for chrysanthemum, we identified 88 chrysanthemum and its related genera accessions based on SSR markers using clustering, population structure and principal component analyses (PCA). This research can contribute to an improved understanding of the genetic basis of classification of chrysanthemum, providing more information on the evolution of Chinese traditional chrysanthemum.

2. Materials and methods

2.1. Plant materials

The collection of 88 accessions included 52 Chinese traditional chrysanthemum cultivars, 27 Japanese chrysanthemum cultivars, which has a flower diameter greater than 16 cm, four cultivars of medicinal *C. morifolium* and five wild species belonging to *Chrysanthemum* and related genera, which has a flower diameter less than 16 cm. All accessions are maintained by Chrysanthemum Nursery of Beijing Agro-Biotechnology Research Center, Beijing, China. Uniform chrysanthemum seedlings were raised in a greenhouse under natural light at ~22 °C, and a relative humidity of 70%–75%. Eight plants from each cultivar were used for phenotypic investigation. After flower bud appearance, flower traits were measured according to the handbook of distinctiveness, uniformity and stability test of new plant varieties of the People's Republic of China (Chrysanthemum) (Table 1 and Fig. 1).

2.2. DNA isolation

Fresh leaf samples were collected from Chrysanthemum Nursery of Beijing Agro-Biotechnology Research Center, Beijing, China, frozen in liquid nitrogen, and stored at −80 °C. Genomic DNA was extracted based on a CTAB method of Moisan-Thiery et al. (2005). DNA quality was examined by electrophoresis in 1% agarose, and DNA concentration was quantified with a BioPhotometer (Eppendorf, USA). For SSR analysis, DNA was diluted to 60 ng μL^{-1} and stored at −20 °C.

2.3. SSR analysis

We obtained 23 SSR primer pairs with high polymorphism by primary screening in previous study (Li et al., 2013). Ten SSR markers (Table 2) with higher polymorphism were applied to identify and classify 88 chrysanthemum and its related genera accessions. The PCR reaction system was based on Huang et al. (1999) and optimized through an orthogonal diagram L_{16} (4^5) experiment designed to evaluate five factors (template DNA, Mg^{2+}, dNTP, primer and *Taq* DNA polymerase) at four different levels and selected the optimal level for each factor by fully random single factor experiment. The optimal SSR-PCR system is: 60 ng of DNA template, 2.0 mmol·L^{-1} Mg^{2+}, 0.1 mmol·L^{-1} dNTP, 0.3 μmol·L^{-1} primer, 1 U *Taq* DNA polymerase in a 25 μL reaction system. The optimal annealing temperature for the SSR-PCR reaction system was determined to be 53.1 °C by gradient PCR. The DNA amplification was performed as follows: an initial melting at 95 °C for 5 min followed by 35 cycles of 94 °C for 50 s, 53.1 °C for 50 s, 72 °C for 50 s, and the reaction mixture was held at 4 °C after a final extension step of 72 °C for 8 min. The DNA amplification products were preliminarily analyzed by electrophoresis in 3% agarose gels in 1 × TAE buffer under 6 V·cm^{-1} and visualized by means of ethidium bromide and UVP Gel Documentation. PCR products were loaded on 6% polyacrylamide gels in 1 × TEB buffer and the electrophoresis was carried out at 80 W for 1.5 h. The bands were visualized by silver staining the gel using the method of Bassam et al. (1991).

2.4. Data analysis

The number of alleles (N_a), effective numbers of alleles (N_e), Nei's gene diversity (*H*) and Shannon index (*I*) were calculated using POPGENE version 1.32 (Yeh et al., 1999), and polymorphism information content (*PIC*) was analyzed using PowerMarker version 3.25 (Liu and Muse, 2005). The SSR amplification bands were scored 1 for presence and 0 for absence at the same mobility forming the original matrix. The similarity of qualitative data was calculated using the SM similarity index. Cluster analysis of genetic similarity data was performed using the unweighted pair group method of arithmetic averages (UPGMA) and a dendrogram was obtained using NTSYS version 2.1 (Applied Biostatistics, Port Jefferson, NY, USA) software.

Population structure analysis was performed using STRUCTURE software version 2.3.4 (Stanford University, CA, USA) with