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Full Length Research Paper

Polyploidy levels of Chinese large-flower chrysanthemum determined by flow cytometry

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Flow cytometry was used to determine the ploidy level of 405 Chinese large-flower chrysanthemum (*Chrysanthemum morifolium* Ramat.) cultivars. Sixty-three cultivars are triploid, 175 cultivars tetraploid, 32 cultivars pentaploid, 46 cultivars hexaploid and 1 cultivar heptaploid. Forty-eight cultivars were then randomly selected for confirmation by chromosome-counting; the results are in agreement with the classification of ploidy level by flow cytometry. Most cultivars are aneuploid. The high percentage of tetraploid and triploid, instead of hexaploid in previous studies, represents the first evidence of low ploidy in large-flower chrysanthemum, which indicated a wider range of ploidy variation in this population. The results also offer further insights to the possible evolution and the regulation of flower size of this large-flower population. Additionally, the combination of flow cytometry and chromosome-counting is proved to be efficient and necessary for large-scale ploidy screening of chrysanthemum.

Key words: Chrysanthemum, ploidy level, flow cytometry.

INTRODUCTION

The chrysanthemum (*Chrysanthemum morifolium* Ramat.) originates in China, and is one of the most important flowers in the world. Cultivated chrysanthemum is one the group of chrysanthemum genus, comprising more than 3,000 China native cultivars. Cultivated chrysanthemum is divided into large- and small-flower system, the former refers to cultivars with inflorescence diameter larger than 60 mm (Li and Shao, 1990). Large-flower chrysanthemum is superior to most ornamental flower in terms of abundant diversity in flower type and color, and is regarded as one of two flower spectacle in the world (Chen, 2001).

However, their genetic backgrounds as well as genetic relationships are lack of knowledge. Large-flower chrysanthemum has been cultivated for more than 1, 600 years (Zhao et al., 2009). It experienced long-term artificial and natural cross, and folk breeding work consisted of a considerable part of artificial breeding. Thus, few records were left for us to investigate into its complex genetic backgrounds.

Lack of fundamental information has largely limited large-flower chrysanthemum's further research. Ploidy level of cultivars is thought to be one aspect. The incompatibility within different ploidy background is proved to heavily restrict breeding work (Dai and Chen, 1996) and germplasm evaluation. The basic chromosome number within the genus is x=9 (Dowrick, 1953). Previous findings showed that the chromosome number of large-flower chrysanthemum varies from 45 to about 71, hexaploid and hexaploid-based aneuploid are the most commonly found cytotype (Li et al., 1983; Li and Shao, 1990; Liu and Yang, 2009; Zhu et al., 2011). Aneuploidy variation widely exists and large difference on chromosome number could be generally observed in different root-tip cells of the same individual. Until now, there is still lack of knowledge of ploidy level for most cultivars, which is due to these inherent difficulties with *C. morifolium* cytogenetic, as well as the lack of powerful high throughput determining means.

Ploidy determination of Chinese large-flower chrysanthemum is traditionally performed by chromosome-

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counting. However, concerning its high level of ploidy, numerous chromosomes and aneuploidy variation, counting is laborious and fraught with inaccuracies. Measurement of the nuclear DNA content by flow cytometry is suggested as an alternative (Dolezel, 1998). The method has been increasingly used for large-scale ploidy screening (Dolezel et al., 2007), and has been proved to have a close relationship with chromosome counting on results (Yagi et al., 2009; Roux et al., 2003; Gamiette et al., 1999).

In this study, we aimed to determine the ploidy level of Chinese large-flower chrysanthemum cultivars systematically by flow cytometry, and to develop an efficient system for large-scale ploidy screening of chrysanthemum. The study on ploidy level and high throughput determining means will lead to a better understanding of chrysanthemum's genetic background and a more efficient work of breeding and germplasm evaluation.

MATERIALS AND METHODS

Plant materials

Four hundred and five Chinese traditional large-flower chrysanthemum cultivars (*Chrysanthemum morifolium* Ramat) collected from northern China are preserved in the chrysanthemum nursery garden of Beijing academy of agriculture and forestry sciences, Beijing, China. Their source and genetic background is unknown. Young and healthy leaves of stem tips were collected.

Flow cytometric analysis

Diploidy *Chrysanthemum lavandulifolium* was used as reference standard. It was proved by chromosome counting and showed 2n=2x=18 as expected (Figure 1a) (Wang et al., 2010), assuring the reliability of consequent experiments.

Samples were prepared according to Dolezel, (1997). About 1 g fresh leaf was chopped with a sharp razor blade in a Petri dish containing 2 ml pre-chilled lysis buffer (Dolezel et al., 2007). The sample was filtered through a 50 µm nylon mesh and centrifuged for 5 min at 800 rpm at 4°C. The supernatant was removed, and nuclei were re-suspended in 200 µL propidium iodide (PI) (50 µg/mL). The sample was then briefly vortexed and stored in the dark at 4°C for 30 min.

Flow cytometry analysis on 15,000 cells of each sample was carried out using a FACS-Vantage (Becton-Dickinson, USA) flow cytometry. The standard peak was programmed to appear at about channel 50 at 488 nm. The ploidy level was estimated by comparing the mean fluorescence intensity of nuclei of sample material with that of the reference standard. The measurements are triplicate and results with coefficient of variation (CV, %) lower than 5% are finally accepted.

Chromosome counting

In order to verify the results of flow cytometry, 48 cultivars were randomly selected for further determination by chromosome-counting. Young root tips were excised from cuttings, held in ice water for 48 h and then fixed in Carnoy's solution (3:1 ethanol: glacial acetic acid) at 4°C for 48 h. The fixed root tips were squashed under a glass slide in a drop of 45% glacial acetic acid. Chromosome-counts were performed under a phase contrast microscopy (Olympus BH2). At least 30 cells were observed. The most frequent number was finally accepted.

RESULTS

Flow cytometric analysis

Variation of the ratio of sample and reference largely existed in identical ploidy level (partially showed in Table 1). Based on the obtained results, we conducted the deviation of each ploidy level, and defined triploid as the value (sample/reference ratio) between 1.35 to 1.65; tetraploid as 1.80 to 2.20; pentaploid as 2.30 to 2.70; hexaploid as 2.80 to 3.20; heptaploid as 3.30 to 3.70, and others out of range are classified as uncertainty.

According to the above standard, 317 cultivars were preliminarily certained; other eighty-eight were out of range and were classified as uncertainty. With flow cytometric method, 317 cultivars were classified in five clusters corresponding to 3x, 4x, 5x, 6x and 7x ploidy levels (Figure 1). In spite of general variation, the value (sample/reference ratio) for triploid, tetraploid, pentaploid, hexaploid and heptaploid cultivars was basically approximated 1.5:2:2.5:3:3.5, which gave no doubt on the deviation we defined. Sixty-three triploids, one hundred and seventy-five tetraploids, thirty-two pentaploids, forty-six hexaploids and one heptaploid were preliminarily identified. Tetraploid clearly predominated and accounted for 55.21% of all certain cultivars, while the percentage of hexaploid was only 14.51% (Figure 2).

Chromosome-counting

Forty-eight cultivars were randomly selected for confirmation by chromosome-counting. According to the results of flow cytometric, this random population comprised five triploids, thirty-one tetraploids, six pentaploids, and six hexaploids (Table 1). The chromosome-counting results of forty-eight cultivars are in agreement with the classification of ploidy level by flow cytometry. Most cultivars are aneuploid. The chromosome number of 'Fengongge' is 28, that of 'Fuguifengliu' is 36, that of 'Baisongzhen' is 44 and that of 'Jinefeitian' is 53 (Figure 3). The chromosome number ranges from 26 to 29 on triploid level, 33 to 40 on tetraploid level, 44 to 47 on pentaploid level and 50 to 58 on hexaploid level. Therefore, it was demonstrated that the deviation applied in flow cytometric is appropriate.

DISCUSSION

The reliability and feasibility of ploidy determination of Chinese large-flower chrysanthemum cultivars was carried out with flow cytometry.

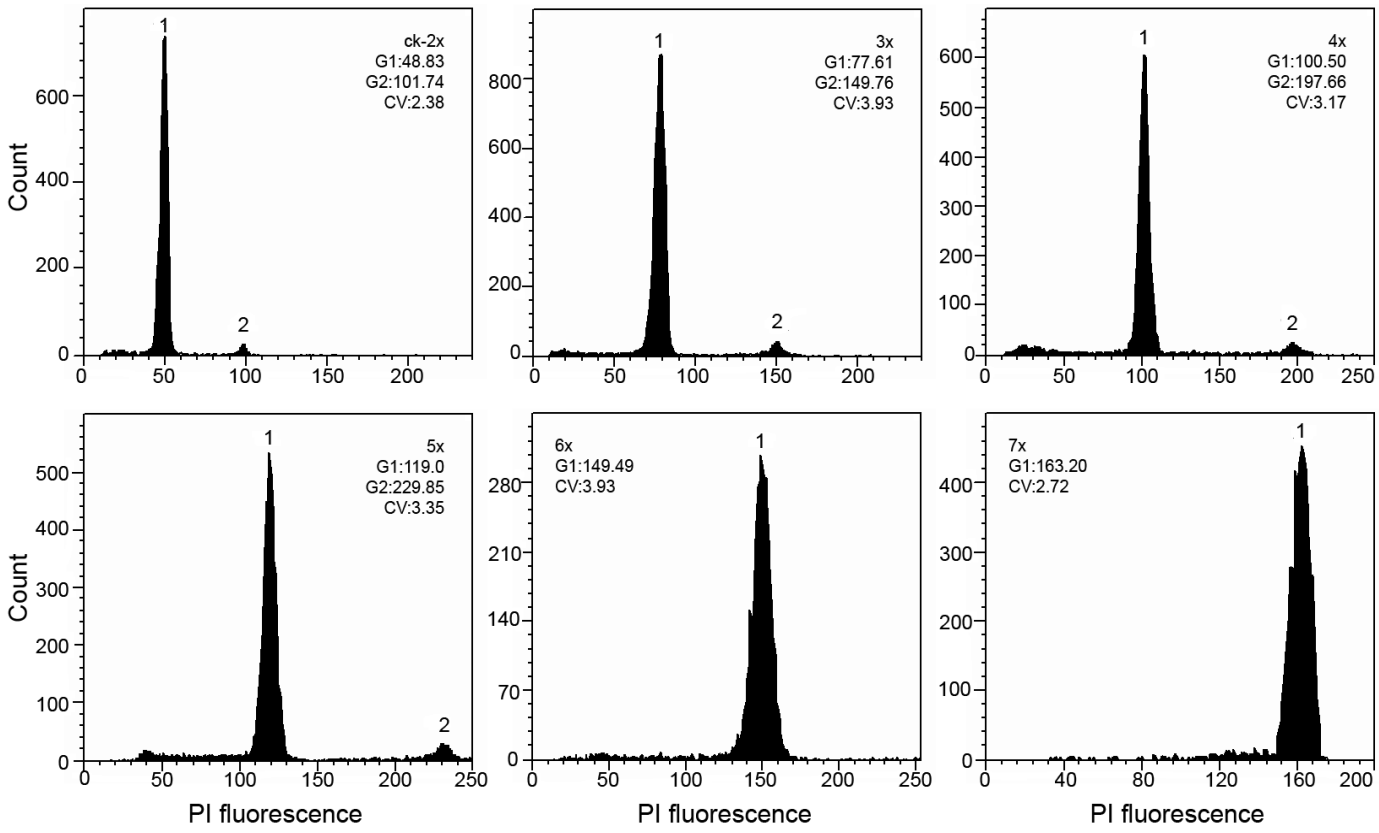

Figure 1. Flow cytometric histogram patterns of nuclei isolated from C. lavandulifolium and typical cultivars of different ploidy. Peak 1 corresponds to nuclei at the G_0/G_1 phase, while peak 2 corresponds to nuclei at the G_2 phase. Coefficient of variation value (CV, %) of the G_0/G_1 peaks are also given.

In large-flower chrysanthemum, we found that variation of DNA content within identical ploidy level brought some difficulties to distinguish the two contiguous ploidy levels by flow cytometric. Abundant aneuploidy variation was thought to be one of the reasons; another was the highly complex genetic background. The flow cytometric detection of ploidy is based on a simplified assumption that all the chromosomes have the same DNA content (Roux et al., 2001), which may not be the case in chrysanthemum (Dowrick et al., 1969).

Therefore, defining an appropriate deviation on DNA content of each ploidy level which was expressed as ploidy index in this study is important in ploidy determination. In *Dioscorea alata* L., Obidiegwu et al. (2010) accepted 10% deviation on fluorescence and successfully detected tetraploid, hexaploid and octaploid. But when applied to large-flower chrysanthemum that comprised a series of continuous ploidy, 10% deviation led to an overlap of one high ploidy level by another. So we conducted the deviations of each ploidy level and demonstrated its reliability. Although the ploidy level of 88 cultivars remained a further estimation, we have rapidly locked their distribution and have known they are aneuploids.

It is also suggested that the combination of flow cytometric and chromosome-counting is highly efficient in large-scale ploidy screening for large-flower chrysanthemum. The use of flow cytometric as preliminary screening, and, if need be, chromosome-counting is useful as further estimation.

The possible origination of low ploidy large-flower chrysanthemum

Through the survey on cytometry of Chinese large-flower chrysanthemum, lower ploidy such as triploid has not been reported so far. Zhu et al. (2011) analyzed the karyotype parameter of 38 Chinese large-flower chrysanthemum cultivars and showed the fewest chromosome number found in 'Mojianrong' is 46. Even in small-flower chrysanthemum, the fewest chromosome number was reported in Japanese small-flower 'YS' with 2n=2x=36 (Endo et al., 2004).

The occurrence of triploids and tetraploids indicates a lower ploidy level which exist in large-flower population. What are the evolutionary status of these low ploidy cultivars? Whether they occur before hexaploid; these

Table 1. Ploidy level of 48 random selected cultivars of Chinese large-flower chrysanthemum using flow cytometric analysis.

Cultivar name	Ploidy level	Mean value of ploidy index ± SE	CV range
C. morifolium 'Yudiechi'	3x	1.48±0.05	4.21-4.27
C. morifolium 'Baoxingtangjin'	3x	1.52±0.01	4.36-4.96
C. morifolium 'Lechengchunxue'	3x	1.61±0.03	4.19-4.27
C. morifolium 'Tianxiayipin'	3x	1.51±0.02	3.77-3.90
C. morifolium 'Fengongge'	3x	1.50±0.02	3.49-3.57
C. morifolium 'Pengzeziyan'	4x	2.14±0.02	3.99-4.04
C. morifolium 'Jinhongjiaohui'	4x	1.95±0.02	3.15-3.45
C. morifolium 'Changanyanhuo'	4x	1.94±0.03	3.35-3.40
C. morifolium 'Tangyulongyun'	4x	1.97±0.03	3.67-3.81
C. morifolium 'Laomoju'	4x	1.97±0.04	3.29-4.01
C. morifolium 'Fensongzhen'	4x	2.13±0.06	3.49-3.88
C. morifolium 'Qiufengshuiyue'	4x	2.00±0.04	3.62-3.72
C. morifolium 'Huangjinzhuangshi'	4x	2.01±0.04	4.08-4.34
C. morifolium 'Longshe'	4x	1.84±0.04	3.86-4.10
C. morifolium 'Daidaizhaoxia'	4x	2.12±0.01	3.26-3.50
C. morifolium 'Huanglongge'	4x	1.84±0.02	3.59-3.77
C. morifolium 'Bolinqing'	4x	2.01±0.05	3.70-4.18
C. morifolium 'Xiahui'	4x	1.96±0.03	3.75-4.00
C. morifolium 'Xueyan'	4x	1.87±0.04	4.03-4.17
C. morifolium 'Xishiqiaozhuang'	4x	1.99±0.02	3.67-3.92
C. morifolium 'Mokui'	4x	2.00±0.03	3.19-3.25
C. morifolium 'Feiliuzhixia'	4x	2.13±0.03	2.91-3.05
C. morifolium 'Yunxiahuancai'	4x	1.98±0.04	3.33-3.41
C. morifolium 'Xuridongsheng'	4x	1.86±0.04	4.24-4.37
C. morifolium 'Tangyulongyun'	4x	1.88±0.01	4.01-4.25
C. morifolium 'Tangyushouweng'	4x	2.14±0.02	3.22-3.31
C. morifolium 'Dahongmaoju'	4x	1.83±0.02	3.36-3.84
C. morifolium 'Bihaiyinlong'	4x	2.09±0.03	3.91-4.15
C. morifolium 'Changheluori'	4x	2.11±0.03	4.38-4.72
C. morifolium 'Xintaoranzui'	4x	1.85±0.05	3.12-3.24
C. morifolium 'Chuchishifei'	4x	1.82±0.01	3.89-4.01
C. morifolium 'Taohuahong'	4x	1.87±0.04	3.65-3.83
C. morifolium 'Huangjingyishou'	4x	2.02±0.03	3.76-4.01
C. morifolium 'Yulanhe'	4x	1.96±0.02	4.30-4.76
C. morifolium 'Fuguifengliu'	4x	1.91±0.03	3.75-3.93
C. morifolium 'Feiniaomeiren'	4x	1.86±0.02	3.84-4.01
C. morifolium 'Baisongzhen'	5x	2.51±0.02	3.42-3.57
C. morifolium 'Qingshanbaozhu'	5x	2.45±0.02	3.46-3.61
C. morifolium 'Jinguanling'	5x	2.38±0.06	3.47-3.64
C. morifolium 'Huanshuimingzhu'	5x	2.45±0.03	3.29-3.35
C. morifolium 'Tangyuaoshi'	5x	2.44±0.02	4.14-4.36
C. morifolium 'Qingshangusha'	5x	2.43±0.03	3.31-3.82
C. morifolium 'Hualouyufeng'	6x	2.98±0.01	3.78-4.01
C. morifolium 'Ziyun'	6x	3.03±0.04	3.60-3.79
C. morifolium 'Ziruyi'	6x	2.99±0.02	4.35-4.63
C. morifolium 'Caoanqingdan'	6x	2.98±0.03	4.74-4.87
C. morifolium 'Tangyuhewu'	6x	2.82±0.04	4.54-4.90
C. morifolium 'Jinefeitian'	6x	2.88±0.02	3.15-3.33

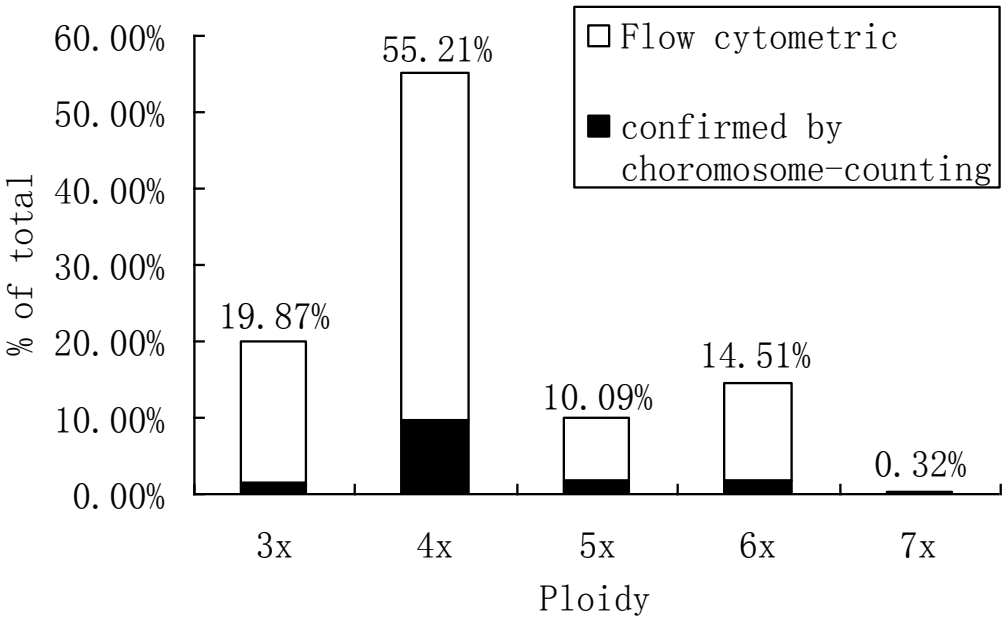

Figure 2. Ploidy distributions of 317 *C. morifolium Ramat* cultivars by flow cytometry and chromosome-counting.

Fig.3 Chromosome-counting of *C. lavandulifolium* and partial cultivars. a *C. lavandulifolium* (2n=2x=18). b 'Fengongge' (2n=28). c 'Fuguifengliu' (2n=36). d 'Baisongzhen' (2n=44). e 'Jinefeitian' (2n=53). Bars=3μm.

are valuable to be studied.

It is generally considered that evolution in the genus Chrysanthemum has several rounds of allopoly-ploidization from low ploidy to high ploidy (Fedorov, 1969). In large-flower chrysanthemum, most previous data of morphological characteristic investigation seem to support the view of evolving from simple to complex. For petal type, flat petal type is the most popular type (Wang et al., 2010) and is generally considered to be the basic type. Spoon and quilled type are advanced, and they further evolve to other types (Miao et al., 2007). For color, yellow color is primitive. White, purple and red are thought to evolve from yellow sequentially. Orange, pink and complex color form at last (Wang et al., 2010).

The origin of cultivated chrysanthemum is still a matter of debate but it is generally accepted that it might be originated by long-term natural and artificial hybridizations within several wild species, which comprised a series of polyploidy namely diploidy, tetraploidy, hexaploidy and others (Kondo et al., 2003). Accordingly, the triploidy and tetraploidy large-flower chrysanthemum may generate from hybridizations within diploid, tetraploid and hexaploid wild species and occur before hexaploidy cultivar. They might act as a transitional population from wild species to hexaploidy cultivar and have a closer relationship with wild species. If so, these new evidence should be of considerable significance to studying the evolution and genetic background of large-flower chrysanthemum.

On the other hand, it is also possible that they occur after hexaploidy cultivars. One way may be crossing between hexaploidy cultivar and diploidy wild species. It is suggested that in the genus *Chrysanthemum* and closely related genus, there are many wild species that are able to cross successfully with modern chrysanthemum (Yang et al., 2010). Another way might be regulated by self-mechanism. In the analysis of karyotype of both traditional and modern large-flower chrysanthemum, Zhu et al. (2011) found that increasing and decreasing of symmetry coexist in modern cultivars. Then they issued the view of multi-direction evolution of karyotype in modern population. It is still unknown whether if the multi-direction mode also exist in ploidy evolution of traditional cultivar. Further evidence needs the support of both cellular and molecular analysis.

We preliminarily developed flow cytometric in Chinese large-flower chrysanthemum and detected a low ploidy population predominant with tetraploid for the first time, which provides new insight to the evolution and genetic background of chrysanthemum. Flow cytometry has been suggested to combine with chromosome counting for large ploidy screening in chrysanthemum.

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