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INVESTIGATION OF NUCLEAR DNA CONTENTS OF LYCORIS SPECIES (AMARYLLIDACEAE) WITH DIFFERENT CHROMOSOME NUMBER BY FLOW CYTOMETRY

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Abstract

The chromosome number and karyotype of *Lycoris* genus display great variability. Flow cytometry was used to estimate 1Cx-values with the aim to analyze the genome size of *Lycoris* species with different basic chromosome numbers. Three *Lycoris* species with x=11, three *Lycoris* species with x=8 and two hybridization origination species, *L. straminea* (2n=19) and *L. haywardii* (2n=22) were quantified by flow cytometry in this study. The results demonstrated that: (1) the 1Cx-values of *Lycoris* lines with x=11 ranged from 20.22 pg to 25.46 pg, among which, *L. radiata* var. *pumila* and *L. radiata* (triploid) are markedly smaller than the other species with x=11. (2) The 1Cx-values of *L. aurea*, *L. chinensis* and *L. longituba* with x=8 were close, which were 30.40 pg, 32. 42 pg and 31.40 pg respectively, much larger than those with x=11, suggesting different origin between *Lycoris* species with x=11 and x=8. (3) The 1C DNA contents of *L. straminea* and *L. haywardii* were 26.97 pg and 23.96 pg respectively, which were close to the averages of their hypothetic parental lines, well proofing their hybridization origination. To our knowledge, the data may be helpful for the evolution studies of *Lycoris* genus.

Key words: *Lycoris*, Genome size, Flow cytometry.

Introduction

Knowledge of genome size is helpful for plant scientists working in the area of genome analysis, biotechnology, plant breeding, and physiology. In addition, genome size (Cx-value) could be applied to investigate the relationships within genera, and valuable information could be obtained from such work for species under studies on biodiversity (Bennett & Leitch, 2005). It has been successfully used to investigate the origin of plants with different basic number (Lavia & Fernández, 2008) and different karyotype (Poggio *et al*., 2007).

Flow cytometry (FCM) is accepted as the method of choice for the measurements of genome size, which is accurate, dynamical, low cost, and also with the advantages of high throughput and general applicability (Kurita & Hsu 1998; Lavia & Fernández, 2008). Nowadays, a large number of angiosperm taxa belonging to several genera had been estimated by FCM, such as *Miscanthus* (Li *et al*., 2013), *Pongamia pinnata* (Ramesh *et al*., 2014), *Turnera ulmifolia* (López *et al*., 2011), *Hepatica* (Mabuchi *et al*., 2005), *Narcissus* (Zonneveld, 2008), *Galanthus* (Zonneveld *et al*., 2003). In consideration of an easy reference, scientists have worked together to produce pooled lists of plant DNA C-value in electronic form (Bennett & Leitch, 2005; Bennett & Leitch, 1995; Bennett & Leitch, 1997; Zonneveld *et al*., 2005). Now researchers can easily search the plant DNA C-value at http://www.kew.org/genomesize/homepage.

The genus *Lycoris*, a member of the family Amaryllidaceae, consists of 30 species around the world, of which 15 species are native to China. Polyploidization and hybridization have been considered as important modes of speciation within *Lycoris* (Bose & Flory, 1963; Kurita 1988a, b; Kurita & Hsu 1996). To date, triploid and tetraploid of *L. radiata* and triploid *L. sprengeri* have been reported (Kurita 1987a, Zhou *et al*., 2007; Zhang *et al*., 1999). Some species are originated from inter-specific hybridization, such as, *L. haywardii* and *L. straminea*, which were deduced as the hybrid of *L. radiata* var. *pumila* and *L. sprengeri* and the hybrid of *L. radiata* var. *pumila* and *L. chinensis* respectively (Bose & Flory 1963; Kurita 1987b, c). The most important feature of *Lycoris* genus is that the basic chromosome number displays great variability, including x=6, x=7, x=8, and x=11. And among those, x=11 and x=8 are common, and x=11 was considered as the most primitive basic chromosome number within *Lycoris* (Hsu *et al*., 1994). Speciation and phylogenetic relationships in *Lycoris* genus have been extensively studied by approaches of morphology, cytology, and molecular biology (Hsu *et al*., 1994; Bose & Flory, 1963; Kurita 1988a, b; Kurita & Hsu, 1996; Kurita 1987a, b, c; Shi *et al*., 2006; Chung, 1999; Hayashi *et al*., 2005). And genome size is a new criterion to investigate the relationships within genera. Investigation of genome size in *Lycoris* genus may help to uncover the relationships of *Lycoris* species with different basic chromosome number. Till now, only the genome size of *L. aurea* was estimated by FCM, which is 23961 Mb/1C (Zonneveld *et al*., 2005).

In order to investigate whether *Lycoris* species with different basic chromosome number have different genome size, three *Lycoris* species with x=11, three *Lycoris* species with x=8 and two species of hybrid origin with 2n=19 and 2n=22 were analyzed by FCM in this study. The results will facilitate the investigation of the origin of relationships among *Lycoris* species.

Materials and Methods

Plant materials: Plant materials were obtained from the nursery of Nanjing Botanical Garden Mem.Sun Yat-Sen. They were *L. sprengeri* Comes ex Baker, (2n=22), *L. radiata* (L' Héritier) Herbert, (two diploid lines (2n=22),

one triploid lines (2n=33)), *L. haywardii* Traub, (2n=22), *L. radiata* var. *pumila* Gery, (2n=22), *L. aurea* (L' Héritier) Herbert, (2n=16), *L. chinensis* Traub, (2n=16), *L. longituba* Y. Xu & G. J. Fan, (2n=16) and *L. straminea* Lindley, (2n=19). Seeds of *Triticum aestivum* L. cv. Chinese Spring were kindly supplied by Jizhong Wu (Jiangsu Academy of Agriculture Sciences, Nanjing, China), which was used as standard (2C = 34.90 pg) (Zonneveld *et al*., 2005).

Each *Lycoris* species, composed of stems from three individuals, were collected in the summer 2015, when they were flowering. And leaves of *Triticum aestivum* L. cv. Chinese Spring were collected when the seedlings were cultivated in incubator for two weeks. All samples were packed in plastic bags, stored at 4°C until use.

Chromosome preparation: The root tips were used for cytogenetic analysis. Firstly, root tips about 1-2 cm long were gathered and soaked in 0.002 M 8-hydroxyquinoline for 6 h at 4 °C. These tips were fixed in the solution of absolute ethanol and glacial acetic acid at the ratio of 3:1 for 24 h at 4°C. Subsequently, the root tips were washed with tap water and then treated with1 M hydrochloric acid at 60 °C for 6 min. After that, the root tips were stained with phenol-fuchsin for 12 h. Finally, the stained root tips were tapped in 45% acetic acid and pressed by a microscope slide (Zhou *et al*., 2007). The chromosome numbers were counted by a photomicroscope (Nikon Eclipse 50i, Japan). The chromosome counts were determined using at least 10 well-spread metaphase cells for each species.

FCM analysis: All samples were investigated by flow cytometry (BD. Accuri™ C6) at a laser wave length of 488 nm. The G's buffer (Galbraith *et al*., 1983) was used for nuclear isolation. Samples were chopped on ice with 1 ml G's buffer. Immediately, the nuclear suspensions were filtered through a 33 µm Nylon filter, then RNase A (50 µg/ml) was added into the suspensions. The mixed-nuclear suspensions were incubated at 37°C for 15 min, and stained with PI stock at 50 µg/ml working concentration. They were kept at 4°C until analysis.

Each sample was assayed 3 times and each time 5,000 nuclei were analyzed. Meanwhile, the coefficient of variation (CV) values was under 5%. And the following formula: 1C nuclear DNA content of test sample (pg) = 2C peak mean of test sample x 1C nuclear DNA content of *Triticum aestivum* L. cv. Chinese Spring (17.5 pg) / 2C peak mean of *Triticum aestivum* L. cv. Chinese Spring was used to calculate the genome size of the *Lycoris* species. The results were subjected to SPSS 17.0. One-way ANOVA was performed for all the data. A significance level of 1% was selected for 1 Cx value of species with x=11.

Results and Discussion

Lycoris genus has large chromosome and large genome (Kurita 1986; Kurita 1987b; Go *et al*., 2012), while only the 1C DNA content of *L. aurea* was reported (Zonneveld *et al*., 2005). In this study, the genome sizes of six diploid species (*L. aurea*, *L. chinensis*, *L. longituba*, *L. sprengeri*, *L. radiata* and *L. radiata* var. *pumila*), one triploid species and two species of hybridization origin (*L. haywardii*, and *L. straminea*) were quantified by FCM. The species, ploidy, chromosome number, DNA contents and 1Cx-values are listed in Table 1. The results demonstrated that each *Lycoris* species surveyed has a large genome, larger than that of *Triticum aestivum* L. cv. Chinese Spring. Interestingly, the 1C DNA contents of all diploid species varied from 20.22 pg to 32.42 pg, demonstrating great differences in genome size.

Table 1. 1C DNA content for *L. radiata* in diploid, in triploid, *L. radiata* var. *pumila*, *L. sprengeri*, *L. aurea*, *L. chinensis*, *L. longituba*, *L. haywardii* and *L. straminea*, obtained from flow cytometry, ploidy, chromosome number and 1Cx-value.

Basic chromosome number	Species	Ploidy	Chromosome number	Mean genome size(Mbp/1C)	1C DNA (pg) content mean ± SE	1Cx-Value
x=11	*L. radiata* (diploid 1)	2x	2n=22	22960	23.50 ± 1.62	23.50[ab]
	L. radiata (diploid 2)	2x	2n=22	24884	25.46 ± 0.59	25.46 [a]
	L. radiata (triploid)	3x	2n=33	32594	33.35 ± 0.41	22.24 [b]
	L. radiata var. *pumila*	2x	2n=22	19755	20.22 ± 0.17	20.22 [c]
	L. sprengeri	2x	2n=22	23800	24.36 ± 0.56	24.36 [a]
x=8	*L. aurea*	2x	2n=16	29718	30.41 ± 0.60	30.40
	L. chinensis	2x	2n=16	31682	32.42 ± 0.33	32.42
	L. longituba	2x	2n=16	30675	31.39 ± 0.55	31.39
hybrid	*L. haywardii*		2n=22	23416	23.96 ± 0.11	
	L. straminea		2n=19	26354	26.97 ± 0.23	

*a–c: Means following the same letter in a column are not significantly different. Tukey test (P = 0.01)

As shown in Table 1, the 1C DNA contents of species with x=8, close to that of triploid *L. radiata* (2n=33), are much larger than those of species with x=11. *L. aurea*, *L. chinensis* and *L. longituba*, with x=8. Former researches demonstrated that *L. aurea*, *L. chinensis* and *L. longituba* are very similar in morphology, cytology, and pollen characters (Zhou *et al*., 2007). And the 1C DNA contents of these species are 30.40 pg, 32.42 pg and 31.39 pg respectively, also indicating a close relationship among them. In this study, *L. radiata* (diploid and triploid), *L. sprengeri* and *L. radiata* var. *pumila* were selected as representative species with x=11 for genome size analysis. The results demonstrated that 1C DNA content of diploid *L. radiata* and *L. sprengeri* are close. And *L. radiata* var. *pumila* is the smallest among those with 2n=22 (Table 1), consistent with its morphological characters, which is smaller in bulbs, narrower and shorter in leaves than other species (Fig. 1). Leaves of *L. sprengeri* appear in spring, while leaves of *L. radiata* var. *pumila* and *L. radiata* appear in autumn, the plant morphology and leaves of species with x=11, except *L. sprengeri*, were shown in Fig. 1. 1C DNA content of triploid *L. radiata* is 33.35 pg, which increased with the chromosome number, but not in the expected proportion with that of *L. radiata* in diploid or that of *L. radiata* var. *pumila*. The result is acceptable for that triploid species of *L. radiata* is not a simple autotriploid (Kurita 1987a; Hayashi *et al*., 2005).

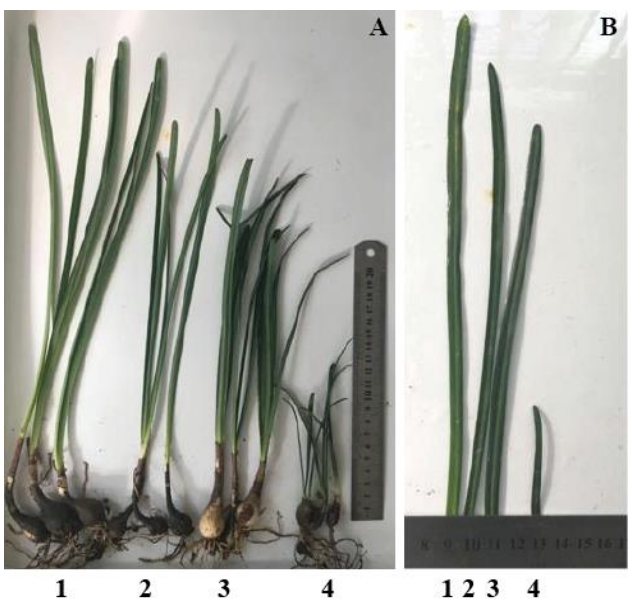

Fig. 1. The whole plants of *L. radiata* and *L. radiata* var. *pumila*, including leaves, bulbs and roots, leaves (A); Leaves cut from *L. radiata* and *L. radiata* var. *pumila* (B) (1for triploid *L. radiata*, 2 for *L. radiata* P1, 3 for *L. radiata* P2 and 4 for *L.radiata* var. *pumila*).

1C values of two diploid *L. radiata* are 23.50 pg and 25.46 pg respectively, demonstrating a little differences. For there are some differences in karyotypes of *L. radiata* (Bose & Flory 1963; Kurita 1988a, b; Zhou *et al*., 2007; Kurita 1987a; Mookerjea 1955; Shao *et al*., 1994; Qin *et al*., 2004a, b; Zhou *et al*., 2004; Liu *et al*., 2016), we deduced that differences in 1C DNA content between two diploid *L. radiata* may result from material differences. And another phenomenon in this study may also result from the differences in materials, which is that the 1C value of *L. aurea* here is different from that listed by Zonneveld (Zonneveld *et al*., 2005). And also differences of karyotypes in *L. aurea* have been reported (Bose & Flory 1963; Kurita 1987c).

In this study, 2 species of hybrid origin, *L. haywardii* and *L. straminea*, were analyzed. *L. haywardii* with 2n=22, was deduced as the hybrid of *L. radiata* var. *pumila* and *L. sprengeri*, and *L. straminea* with 2n=19, was deduced as the hybrid of *L. radiata* var. *pumila* and *L. chinensis* (Bose & Flory 1963; Kurita 1987b, c). The 1C DNA contents of *L. straminea* and *L. haywardii* are 26.97 pg and 23.96 pg respectively, close to the average of the deduced parent lines, confirming the hybrid origin hypothesis, which is consistent with the results of Shi's ITS analysis (Shi *et al*., 2006).

1Cx-value refers to the amount of DNA in the unreplicated monoploid (x) chromosome set. The 1Cx-values of species with x=11 and x=8 were analyzed (Fig. 2). As shown in Fig. 2, the 1Cx-values of species with x=8 clustered together, markedly larger than those of species with x=11, indicating that different originations between *Lycoris* species with x=11 and species with x=8. 1Cx-values of species with x=11 have a little difference, the 1Cx-value of *L. radiata* var. *pumila* is smaller than diploid *L. radiata* and *L. sprengeri* with x=11, indicating that some evolution any events may take place in the origin of *L. radiata* var. *pumila*. Further investigation using molecular and *in situ* hybridization methods may uncover the evolution events in origin of *L. radiata* var. *pumila*, as well as the origin of triploid *L. radiata*.

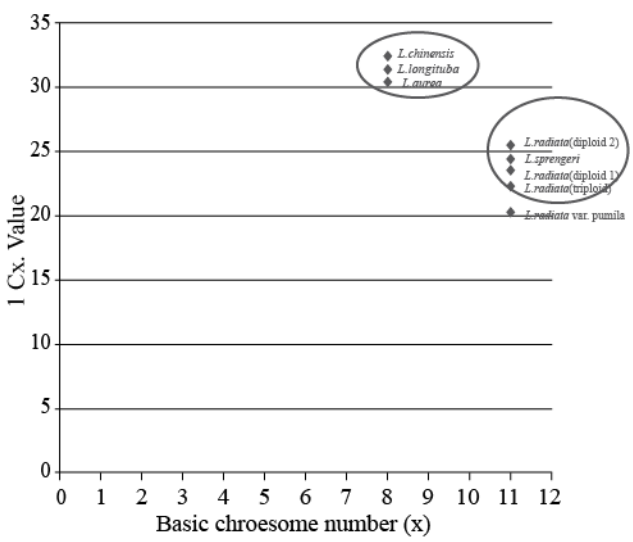

Fig. 2. Scatter plot between 1Cx-value and basic chromosome number. Circles denote groups with close 1Cx values.

In conclusion, DNA content is an important aspect for estimating the phylogenetic relationships of plants, especially for analyzing the relationships among plants belonging to the same genus (Mabuchi *et al*., 2005; Lysak *et al*., 2009). Our results revealed that 1) genome size varies greatly in *Lycoris* genus, and genome sizes of *Lycoris* species with x=8 are much larger than those with x=11. 2) 1C DNA contents of *Lycoris* species with x=8 are very close, while 1C DNA contents of *Lycoris* species with x=11 are also close except that of *L. radiata* var. *pumlia*, which is much smaller than

other species with x=11. To our knowledge, this is the first time to quantify the DNA contents of *Lycoris* species. The data may be helpful for the evolution any studies of *Lycoris* genus, the relationship within the *Lycoris* genus, and also for scientists working in the areas of biodiversity, genome analysis and plant breeding.

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