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Nuclear DNA C-values in 12 species in Nymphaeales

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Abstract — Nuclear DNA C-values are the basic data of species and used in a strikingly wide range of biological fields. Genome size of the most species belonging to the three families Nulembonaceae, Cabombaceae and Nymphaeaceae in Nymphaeales were not assessed, especially none of the species in Cabombaceae was reported so far. In present research, flow cytometry was used to assess the genome size of 12 species belonging to the three families in Nymphaeales and a standard squash technique to count the chromosome numbers of the tested materials. In the tested different species, 2C nuclear DNA contents ranged from 1.55 to 8.11 pg, which is more than fivefold variation. And chromosome numbers varied from 2n=16 to 2n=72. Differences between the species or among populations within a species were also recorded by statistical analysis. The tested species are all small genomes except two species of *Victoria*. The ploidies did not matched with their DNA contents very well in *Nymphaea*. Significant differences were found between the species of *Nymphaea, between those of Victoria* and among wild populations of *Nelumbo nicifera*; while no obvious differences were found between the species of *Nelumbo* and among the cultivars of *Euryale ferox* as well as among those of *Brasenia schreberi,* respectively.

Key words: chromosome numbers; flow cytometry; Nuclear DNA C-values; Nymphaeales

INTRODUCTION

Nymphaeales includes three families, Nelumbonaceae, Nymphaeaceae and Cabombaceae, which are important economic families, especially as aquatic ornamentals. There exist seven genera in water lily family (Nymphaeaceae), two in Cabombaceae and only one in Nulembonaceae. Hundreds of cultivars are planted all over the world in both *Nelumbo* and *Nymphaea*. *N. nucifera* and *Brasenia schreberi* are the most popular and tasty vegetables, and they are also a kind of essential traditional Chinese medicine. Some species' stems of *Nuphar* and *Nymphaea* are used as tanning agent.

Despite the economic values of these plants, Nymphaeale is an old and evolutionarily primitive order based on similarities in floral and vegetative morphology (CRONQUIST 1988; WILLIAMSON and SCHNEIDER 1993). Furthermore, fossil evidence suggests that Nymphaeaceae has not changed much over the past 160 million years (CEVALLOS-FERRIZ and STOCKY 1989; COLLINSON 1980; MULLER 1981). Its taxonomy and genetics are rather confusing. There has been some disagreement about the classification of Nymphaeaceae (DAHLGREN 1980; SOLTIS *et al*. 1999; NANDI *et al*. 1998). Now most people agree with that Nelumbonaceae, Nymphaeaceae and Cabombaceae were the independent families (ANGIOSPERM PHYLOGENY GROUP 2003). And people paid much attention to the morphologic research instead of basic information such as genome size, thus it hampers the detailed genome analysis which is needed to characterize the existing germplasm, including the cash species and varieties.

The DNA content per genome is usually constant between cells within an individual, and relatively constant between individuals of the same species. So nuclear DNA contents are the basic data of species and used in a strikingly wide range of biological fields, including taxonomy, genome

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evolution, ecology and the environment, genomics, plant breeding, cell and molecular biology, conservation, physiology and development (BENNETT *et al*. 2000). As DNA amount is a key biodiversity character, it should be readily available. In Nymphaeale, only three species' DNA contents have been estimated till now. Many methods can estimate the contents, but flow cytometry is the most powerful and efficient tool in detecting DNA content. In this study flow cytometry was used to estimate the DNA contents of twelve species and some populations in Nymphaeale, and the data of the most species were reported for the first time. The results offered a basis for the application of flow cytometry in the taxonomy and biotechnology of these interesting families.

MATERIALS AND METHODS

Plant material - The plants studied were collected in the fields or botanical gardens. Table 1 shows the provenance of all the species and populations investigated. According to the taxonomy there are only two species in the genera of *Nelumbo* and *Victoria* each, and only one species *in Brasenia* and *Euryale,* respectively. Each plant was represented by three extracts obtained from different leaves. The content of chicken red blood cells (2C=2.5pg) (RASCH *et al*. 1970) was used as internal standard for all the tested materials except *Brasenia schreberi* and *Euryale ferox* in which the internal standard was that of *Oriza sativa* (2C=0.9pg) (BENNETT *et al*. 2000).

Chromosome counts - The chromosome counts were obtained from root-tips, occasionally from young leaves with a standard squash technique (MUKAI and GILL 1991). The materials were put in fixation in the solution of ethanol and glacial acetic acid at the ratio of 3:1. The hydrolyzing in 1 M HCl at 60°C lasted for 5 to 10 min depending on the toughness of the materials. The materials were stained by a drop of 2% aceto-orcein for a few minutes, and then squashed. At least five fine metaphases were chosen to count the numbers of chromosome.

Sample preparation and cytometric measurements - For flow cytometry analysis, the nucleus suspensions of plant materials were prepared from young leaves. The samples (typically 20 mg) were chopped separately with a sharp razor blade in nucleus extraction buffer (50mM KCl, 10mM $MgSO_4$, 5mM Hepes, 3mM dithiothreitol, 0.25% Triton X-100) as described by Arumuganathan and Earle (ARUMUGANATHAN and EARLE 1991). The polyphenol oxidation was avoided by adding 5% polyvinyl pyrrolidone in the buffer, which was special and very important to Nymphaeales. The solution was filtered through nylon cloth (30 um mesh size),and the suspension was stained with PI (Propidium iodide). In all experiments, a final saturating concentration of 50 $\mu g \cdot mL^{-1}$ *PI*

Table 1 — Provenance of the species studied

Family	Genus	Species and populations	Origin	Source of material [a]
Nelumbonaceae	*Nelumbo*	*N. nucifera*	Asia, Oceania	LRC
		N. lutea	America	LRC
Nymphaeaceae	*Nymphaea*	*N. odorata*	East and north America	WBG
		N. tetragona	Asia	WBG
		N. nouchali var. *caerulea*	North Africa and tropic	WBG
		N. mexicana	America and Mexico	WBG
		N. capensis cv. 'Blue Charies Thomas'	East Africa, Madagascar and south Africa	WBG
Nymphaeaceae	Nuphar	*N. pumila*	Asia, Europe	WBG
Nymphaeaceae	Victoria	*V. cruziana*	Paraguay, North Argentina	NBG
		V. amazonica	Brazil, Bolivia	WBG
Nymphaeaceae	Euryale	*E. ferox* (*E. ferox* 'youchi'; *E. ferox* 'wuchi')	China, south-east Asia, Siberia of Russia, Europe	LRC
Cabombaceae	*Brasenia schrebe*	*B. schreberi* (*B. schreberi*. 'Shuzhou'; *B. schreberi* 'lichuan')	Asia, Africa, Australia, North America	WVR

[a] Plant material obtained from Wuhan Botanical Garden (WBG), Nanjing Botanical Garden (NBG), Lotus Research Center (LRC) and Wuhan Institute of Vegetables (RIV) all in China.

was used for both those dilutions and extract solution.

Chicken red blood cell nuclei were isolated according to VAN PROOIJEN-KNEGT *et al.* (1980). After the isolation they were fixed in ice-cold 70% ethanol and store at 4°C overnight, washed with pre-cooled 1×PBS 2-3 times, and centrifuged for 5 min at 800-1,000 rpm. The supernatant was discarded and the cell pellets were resuspended in 0.5 mL of 0.5% pepsin in 0.1 M HCl. After incubating at room temperature for 20-30 min with gentle shaking, the mixture was centrifuged for 5 min at 800-1,000 rpm, then the supernatant was discarded and the cells were resuspended in 1 mL of 1×PBS containing PI solution (50 $\mu g \cdot mL^{-1}$).

The detection of PI fluorescence intensity was performed with Phoenix Flow Systems (Beckman and Coulter). At least 10,000 cells were measured per sample.

The Nuclear DNA contents of each sample were measured by a formula in which the ratio of the mean of cells from each sample to the mean of cells from internal standard is multiplied by the known DNA content of internal standard. Here the mean refers to the mean PI fluorescence intensity of each nucleus. The fluorescence intensity was proportional to the nuclear DNA content, and was produced automatically by the flow cytometry.

Statistical analysis - The *t*-test was applied to compare the species within the two genera *Nelumbo* and *Victoria*, and the two populations of *Brasenia schraber* and *Euryale ferox*, respectively. The F-test was carried on *Nymphaea* and *Nelumbo nucifera* to analysis the differences among the species or wild populations.

RESULTS

Chromosome counts - Counts for the species in *Nymphaea* and *Victoria* were unobtainable as roots could not be taken without risk of damaging delicate living specimens, so we used the young leaves to test. The chromosome number varies from 2n=16 in *Nelumbo* to 2n=72 in *Brasenia schreberi*, and the *Nelumbo* has the smallest haploid chromosome number, n=8. In *Nymphaea*, the chromosome number varies from 2n= 28 to 2n= 56, which seems varying from diploid to tetraploid according to the chromosome basic number x=14 (WEI *et al.* 1994).

Nuclear DNA amount - The nuclear DNA amount and other cytogenetic data assessed in 12 species belonging to three families were presented in table 2. According to the data of the angiosperm DNA C-values database (http:// www.rbgkew.org.uk/cval/databasel.html), the nuclear DNA

Table 2 — Nuclear DNA amount and chromosome number of the species studied

Genus	Sepecies or subspecies	2n	2C±SD (pg)	2C/2n	1C DNA ($*10^8$bp) [a]	Standard species	*t* or F-test
Nelumbo	*N. nucifera*	16	1.90±0.06	0.12	9.29	CRBC[b]	t_{60}=1.1091, P=0.1359
	N. lutea	16	1.93±0.06	0.12	9.44	CRBC	
Nymphaea	*N. odorata*	56	4.59±0.29	0.08	22.45	CRBC	$F_{6,23}$=320.39, P=5.66×10^{-21}
	N. odorata	56	1.55±0.11	0.03	7.58	CRBC	
	N. odorata	42	4.22±0.20	0.08	20.64	CRBC	
	N. tetragona	28	3.65±0.17	0.13	17.85	CRBC	
	N. nouchali var. *caerulea*	42	1.59±0.03	0.04	7.78	CRBC	
	N. mexicana	56	1.49±0.03	0.03	7.29	CRBC	
	N. capensis cv. 'Blue Charies Thomas'	28	1.56±0.16	0.06	7.63	CRBC	
Nuphar	*N. pumila*	34	5.26±0.21	0.15	25.72	CRBC	
Victoria	*V. cruziana*	24	8.11±0.23	0.34	39.66	CRBC	t_{22}=5.0581, P=2.29×10^{-6}
	V. yamasu	24	7.40±0.40	0.31	36.19	CRBC	
Euryale	*E. ferox*	58	1.89±0.07	0.08	9.24	O.sativa	
Brasenia	*B. schreberi*	72	2.49±0.14	0.03	12.18	O.sativa	

[a] 1 pg=978×10^6 bp (Cavalier-Smith 1985)
[b] Chicken red blood cell (CRBC)

contents of all the tested species have never been reported except those of *Nelumbo nucifera* in Nelumbonaceae and the DNA C-value of *Brasenia schreberi* assessed here filled the blank of DNA contents in Cabombaceae. The 2C DNA contents ranged from 1.55pg in *Nymphaea tetragona* to 8.11pg in *Victoria cruziana*. All of the tested taxa had small genomes (defined as 1C≤3.5 pg) except the two species of *Victoria*. There is an approximal 5-fold variation between the largest and smallest 2C DNA values. The difference is small and only equal to 0.5% of the approximal 1000-fold variation known for angiosperms as a whole. The parameter 2C/2n reflects the mean value of each chromosome: *Nymphaea odorata*, *Nymphaea mexicana and* Brasenia schreberi were the smallest (0.03pg) and *Victoria cruziana* was the largest (0.34pg).

We used *t*-test to compare the DNA contents of the tested species in each of the two genera, *Nelumbo and Victoria*, respectively. Significant differences were observed between the two species of *Victoria* (t=-5.06, P=2.29×10^{-5}). However, we did not find any remarked differences between the two tested species of *Nelumbo* (t=1.11, P=0.14). And the significant difference ($F_{6,23}$=320.39, P=5.66×10^{-21}) was found among the five tested species of *Nymphaea* by F-test.

Comparisons of genome size between species and populations within a species - The *t*-test was carried out within two species, *Brasenia schreberi and* Euryale *ferox,* and each of them contained two tested cultivars for detecting effects of population factors (table 3). No significant differences were observed between the two *Brasenia schreberi* populations (*t*=0.40 P=0.35), and between the two *Euryale ferox* populations (*t*=0.02, P=0.49). 2C DNA values of five wild *Nelumbo nucifera* populations were from1.85 pg to 1.96pg. The mean value was 1.89pg. When we used the F-test for the independent samples, we observed significant differences among the five populations of *Nelumbo nucifera* ($F_{4,53}$=10.61, P=2.17×10^{-6}). It could be deduced that the wild populations originated and diverged more early and under different ecological environments stronger genetic variations occurred while different cultivars were produced mainly by artificial selection and they only received less selection press and their differentiation history was shorter, thus their genetic differences were not significant.

Table 3 — Genome size comparisons between populations in three species

species	population	DNA content	*t* or F-test
B. schreberi	Lichuan	2.51±0.09	t_9=0.4018, P=0.3486
	shuzhou	2.48±0.17	
E. ferox	youchi	1.90±0.02	t_9=0.0185, P=0.4928
	wuchi	1.90±0.11	
N. nucifera	Liangzi lake	1.96±0.04	$F_{4,53}$=10.61, P=2.17×10^{-6}
	Baoan lake	1.86±0.05	
	Niushan lake	1.90±0.03	
	Longgan lake	1.90±0.07	
	Chang lake	1.85±0.07	

DISCUSSION

The chromosome numbers of the most species were consistent with the results, which have been published before (Wei *et al*. 1994; Gupta 1978; Gupta 1980; Harada 1952). But the chromosome numbers are not stable within a species of *Nymphaea*. We found that in *Nymphaea odorata*, the chromosome numbers were either 28 or 56, and in *Nymphaea tetragona,* the chromosome number we observed was 28 in this study, while in previous publication it was 84, 112 and 120 (Wood *et al*.1959), respectively. It is interesting to note that some water lily species showed their variable chromosome numbers. Such intraspecific cytological variation is especially frequent in species that reproduce obligatorily, vegetatively. For example, in *Stylidium crossocephalum* (Coates *et al*. 1995) and *Scilla autumnalis* (Ainsworth *et al*. 1983) there exist huge cytotypic differentiation. The reason is that although some populations of certain cytotypes are geographically separated, the plants have great variability in their cytological aspects to adapt the environment for the conservation of existing populations.

It is the first time that the DNA contents of the tested eleven species in Nymphaeales were estimated. The DNA content of the tested *Nelumbo nucifera* has already reported before. Comparing our result with the previous publications, it shows certain differences in DNA values for this species. Hanson (Hanson *et al*. 2001) reported surprisingly low value for *Nelumbo nucifera* (0.49pg/2C). He used *Vigna radiata* 'Berken' (4C=2.12 pg) as a calibration standard for *Nelumbo nucifera* by Feulgen microdensitometry. As pointed out by Dolezel et al.. (Dolezel *et al*. 1998) use of different DNA reference standards may result in different estimates for the same material. However we used *Oriza sativa* as an internal standard to test our results and got proximate results of *Nelumbo*

nucifera, showing that the technique and the results are consistent. Therefore they should be reliable. It is evident that the differences between the results of Hanson and us may be caused by the different methods. Compared with conventional methods, flow cytometry analysis should be a more reliable and advanced method.

Our results showed that in *Nymphaea*, the DNA contents from 1.5pg/2C to 4.5pg/2C were not consistent with the variations that chromosome number was 28 (diploid) to 56 (tetraploid), respectively. BENNETT *et al.* (1997) had reported the DNA amount did not increase in direct proportion at ploidy level. And the mean DNA content of each chromosome varied significantly according to the parameter 2C/2n in this genus. The mechanisms involved are not well understood. Probably unequal crossing over, gene amplification, gene conversion and replicative transposition were the important effected factors.

BENNETT *et al.* (2000) emphasized the contribution of cytology and cytogenetics, and particularly genome size, on plant systematics and phylogeny. Using DNA amount as an adaptive character it can throw light on evolution ships. DNA gain and loss have occurred during speciation, and the comparison of C-values provides a natural way to explain phylogenetic relationships and systematics of narrow taxonomic groups. The results of the present paper showed that most of them were small genomes and these were the ancestral characters according to the evolution of genomes in plants. It further verified the results of the morphological analysis by which Nymphaeales has been thought to be more original evolutionally. There were many viewpoints and suggestions on the relationship and the systematic positions of the families and their genera. Our results only offered some cytological proofs. Certainly, it needs to be deeply studied at the molecular levels.

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