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Cytogenetic and Flow Cytometry Analysis of Iranian *Rosa* spp.

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ABSTRACT

Since the ploidy level and nuclear DNA amounts are less exposed to natural selection than most morphological traits, and are more stable in the stages of evolution, they are used to study variation among different species. In order to study the diversity of Iranian *Rosa* spp., chromosome counting and flow cytometry (FCM) analyses were carried out on 10 species collected from different parts of the country. For each plant, cuttings were rooted and young leaves were collected. Root tips were pretreated in 8-hydroxyquinolin, fixed in Lewitsky solution and macerated in cellulase. Chromosome number and ploidy level of the investigated species ranged from diploid ($2n = 2x = 14$) to hexaploid ($2n = 6x = 42$). FCM analysis was conducted with propidium iodide (PI) on *R. moschata*, *R. beggeriana*, *R. persica*, *R. foetida*, *R. hemisphaerica*, *R. pimpinellifolia*, *R. canina*, *R. boissieri*, *R. orientalis* and *R. pulverulenta*. The mean 2C-value of different species showed a varying degree from 0.83 pg (s.d. ± 0.08 pg) in *R. persica* from subgenus *Hulthemia* to a four-fold higher amount of 3.54 pg (s.d. ± 0.08 pg) in *R. pulverulenta* from subgenus *Rosa* (=*Eurosa*). Finally, the PI measurements were compared with measurements obtained from 4', 6-diamidino-2-phenylindole (DAPI) staining, which indicated that DAPI ratios (p_1/p_2) were lower, but closely correlated ($r^2 = 0.98$) with PI ratios (p_1/p_2).

Keywords: chromosome number, nuclear DNA amount, ploidy level, wild rose species
Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; **FCM**, flow cytometry; **PI**; propidium iodide

INTRODUCTION

The comparison of chromosomes is a traditional way of studying the genomes of different species. A prevalent measurement of chromosomes is defining the chromosome number in cell nuclei and determining the ploidy level of a plant. In the genus *Rosa*, the reported basic chromosome set is $x = 7$ and the ploidy levels generally include diploid, triploid, tetraploid, pentaploid, hexaploid and octaploid (Darlington and Wylie 1955), however, rare higher ploidy levels such as 11 x for an interspecific hybrid has also been reported by Zeilinga (1969).

Although the accuracy and confidence of chromosome counts are indisputable, this conventional method is laborious, time consuming and tissues containing actively dividing cells (e.g. root tips) may not be promptly available. Furthermore, since the chromosomes of roses are small (1-3 μm long) (Crane and Byrne 2003) and, as woody plants, they produce high amounts of tannins, polyphenols and other secondary metabolites, preparation of high-quality chromosome spreads are difficult and need more experience. Therefore, considering other methods of determining the ploidy level merits exploring. Flow cytometry (FCM) is a promising alternative which has several advantages over other methods of measuring ploidy, including: (i) quick sample preparation, (ii) non-damaging sampling, (iii) non-reliance to mitotically active dividing cells, (iv) rapid recognition of mixoploidy, (v) low costs of operating once equipment has been procured (Dolezel 1997; Suda *et al.* 2007).

Since Bennett (1972) declared that the total amount of genomic DNA has effects on an organism other than that of the encoded genetic information, i.e. 'nucleotype theory', there is a broad interest towards identifying the genome size of individual plants. The nuclear DNA content is usually constant between cells in an individual and relatively constant between individuals of the same species (Ben-Sade and Samach 2003), but it varies considerably between species. This variation is about 13,000-fold, and the 1C values differ from 0.01 pg in unicellular algae *Cyanidium caldarium* (Muravenko *et al.* 2001) to 127.4 pg in tetraploid angiosperm *Fritillaria assyriaca* (Bennett and Leitch 2005). The knowledge of nuclear DNA amounts can be used for comparative studies of genome organization (intron size), taxonomy and systematics, genome evolution, phylogeny and plant breeding (Ben-Sade and Samach 2003). Its uses are common in breeding strategies such as haploidization, polyploidization and further detection and disposal of mixoploids (Allum *et al.* 2007; Khosravi *et al.* 2008).

In the present investigation in order to illustrate the assumed diversity among different species of Iranian wild roses, we hereby report for the first time, chromosome counts, ploidy levels and nuclear DNA amounts of 10 Iranian *Rosa* spp.

MATERIALS AND METHODS

Plant material

Cuttings and shoot tips of one plant from each of the 10 Iranian wild rose species *R. moschata* Herrmann, *R. beggeriana* Schrenk, *R. persica* Michx, *R. foetida* Herrmann, *R. hemisphaerica* Herrmann, *R. pimpinellifolia* L., *R. canina* L., *R. boissieri* Crépin, *R. orientalis* Dupont and *R. pulverulenta* M.B. were collected from different geographical regions of Iran (**Fig. 1**). They were identified according to their morphological traits with the assistance of Mrs. Khatamsaz (botanist) and an identification key which was reported by her (Khatamsaz 1992).

Chromosome counting

The cuttings (15 cm) were rooted in a medium of peat (Klasmann-Deilmann, Geeste, Germany) and perlite (1:1). According to Agayev's procedure (2002): 1 cm end of newly formed roots were ex-

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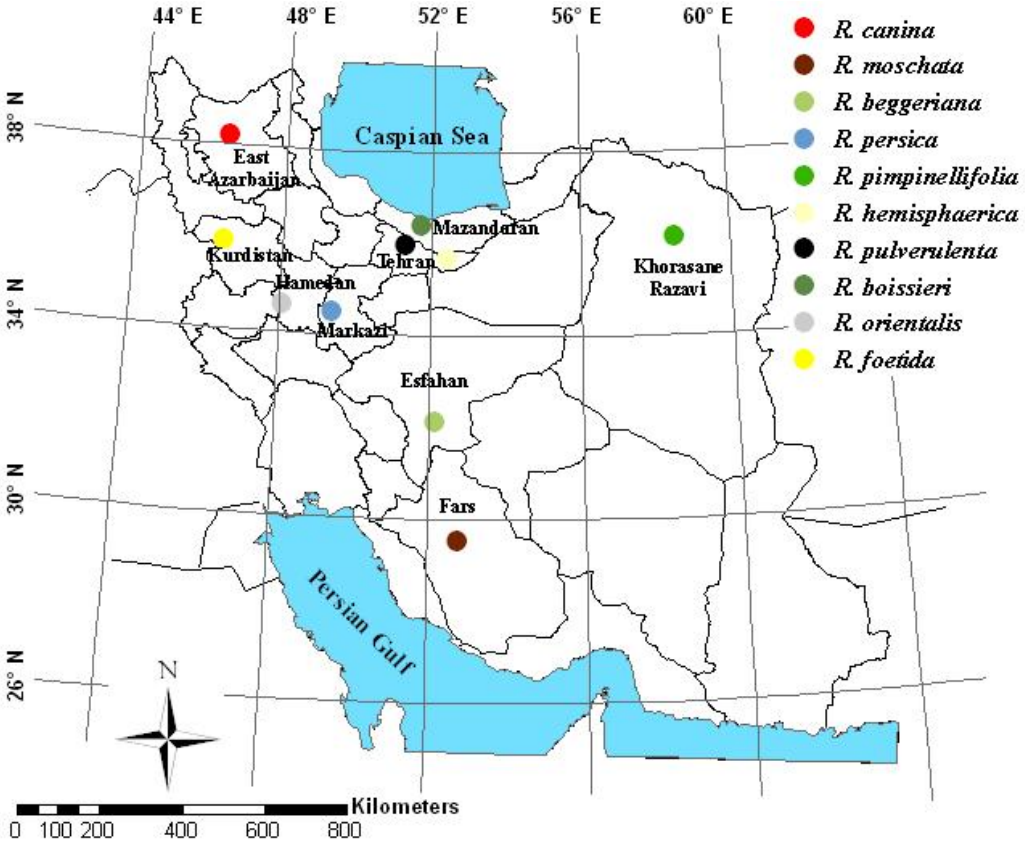

Fig. 1 Site map of collected *Rosa* spp. samples in Iran.

cised and pretreated with 2 mM 8-hydroxyquinoline for 4 hours at 4°C and then fixed with a Lewitsky solution (Agayev 1998) for 36 hrs at 4°C. Root tips were thoroughly washed under running water for 3 hrs and subsequently transferred to 70% ethanol at -20°C for storage. Roots were then hydrolyzed for 10 mins in a previously warmed (60°C) 1N NaOH and then stained with iron hematoxylin for 18 hrs at 32°C. About 1.5 mm of root tips were cut under a binocular with a sharp razor blade followed by maceration with 2% (w/v) cellulose (Onozuka R-10, Japan) for 1.5 hr. Finally roots were moved to a drop of 45% acetic acid on a slide and they were gently squashed with a pressure of thumb on cover slip. At least five samples (from one plant) were examined with a Nikon E-800 microscope and after counting the chromosomes, pictures of the best metaphase spreads were taken.

Flow cytometry

Propidium iodide (PI) (Partec, Münster, Germany) was used as a stain to determine the absolute amounts of DNA. Leaf discs (total 50 mm^2) of young leaves (2^{nd} or 3^{rd} leaves from the apical meristem of the actively growing plants) of roses were cut together with a similar amount of young leaves of parsley (*Petroselinum crispum* 'Champion Moss Curled') as an internal standard, according to Yokoya *et al.* (2000). Polyvinyl pyrrolidone (PVP) was added to the nuclei isolation buffer at a concentration of 10 g l^{-1}. For PI staining, young leaf discs were cut in 0.4 ml ice-cold nuclei isolation buffer (Partec) on a plastic Petri dish. The volume of the lysate was brought up to 2 ml with a staining buffer (Partec), into which 6 µl/2 ml of ribonuclease A (Partec) and 12 µl/2 ml of PI were added. The solutions were filtered through a Partec 50 µm CellTrics (Partec) and samples were incubated in the dark for 1 hr on ice and subsequently filtered through a Partec 30 µm CellTrics. Fluorescence intensity was measured with a PA-II flow cytometer (Partec) by means of laser beam excitation. Estimates of the ratio of fluorescence intensities of each rose to parsley (p_1/p_2) were based on the mean of five samples (from one plant), each with a minimum of 10,000 nuclei, giving peaks with a coefficient of variation of less than 5%. Further nuclear DNA amounts were calculated by the formula presented by Yokoya *et al.* (2000):

DNA amount (pg) = 4.474 p_1/p_2 – 0.012 (s.d. ± 0.08 pg)

DAPI staining of the nuclei were also conducted for the fresh leaves of six species (*R. beggeriana*, *R. pimpinellifolia*, *R. canina*, *R. boissieri*, *R. orientalis* and *R. pulverulenta*) and their ploidy level were estimated by FCM. The procedure for DAPI staining was similar to PI, with the exception that nuclei isolation and staining buffer (Cystain UV ploidy buffer, Partec) were different from PI solutions. The ratios of fluorescence intensities of rose to parsley (p_1/p_2) in DAPI- and PI-stained 2C nuclei were compared.

RESULTS AND DISCUSSION

Since Heslop-Harrison in 1921 (cited in Crane and Byrne 2003) reported the first chromosome numbers of the genus *Rosa*, the chromosome counts of 147 species of the total 184 were identified. The greatest gaps remained in the Asian species (Crane and Byrne 2003). In the present investigation of Iranian rose species, chromosome counts were as follow: *R. persica*, *R. beggeriana*, *R. moschata* were diploid ($2n = 2x = 14$); *R. foetida*, *R. pimpinellifolia*, *R. hemisphaerica* were tetraploid ($2n = 4x = 28$); *R. orientalis*, *R. canina*, *R. boissieri* were pentaploid ($2n = 5x = 35$); and *R. pulverulenta* was hexaploid ($2n = 6x = 42$) (**Fig. 2**). Chromosome numbers of some of the species collected in other countries have been listed by Bolkhovskikh *et al.* (1969), Ritz *et al.* (2005) and Yokoya *et al*. (2000). Our data is in agreement with their results and helps to confirm the identification of the species investigated. However, to our knowledge, present investigation reports for the first time the chromosome numbers for *R. boissieri* and *R. pulverulenta*.

The 2C DNA amounts estimated for the species studied ranged from 0.83 pg (s.d. ± 0.08 pg) in *R. persica* (subgenus *Hulthemia*) to a fourfold amount of 3.54 pg (s.d. ± 0.08 pg) in *R. pulverulenta* (subgenus *Rosa*) (**Table 1**).

Yokoya *et al.* (2000) gave an account of the nuclear DNA amounts of *R. persica, R. foetida* and *R. canina* as 0.84, 1.95 and 2.91 pg (s.d. ± 0.08 pg), respectively. The genome sizes of these three species estimated in the present study were 0.83, 1.91 and 2.95 pg (s.d. ± 0.08 pg), respectively. The differences were negligible and such minor variation could be attributed to the fact that different clones of

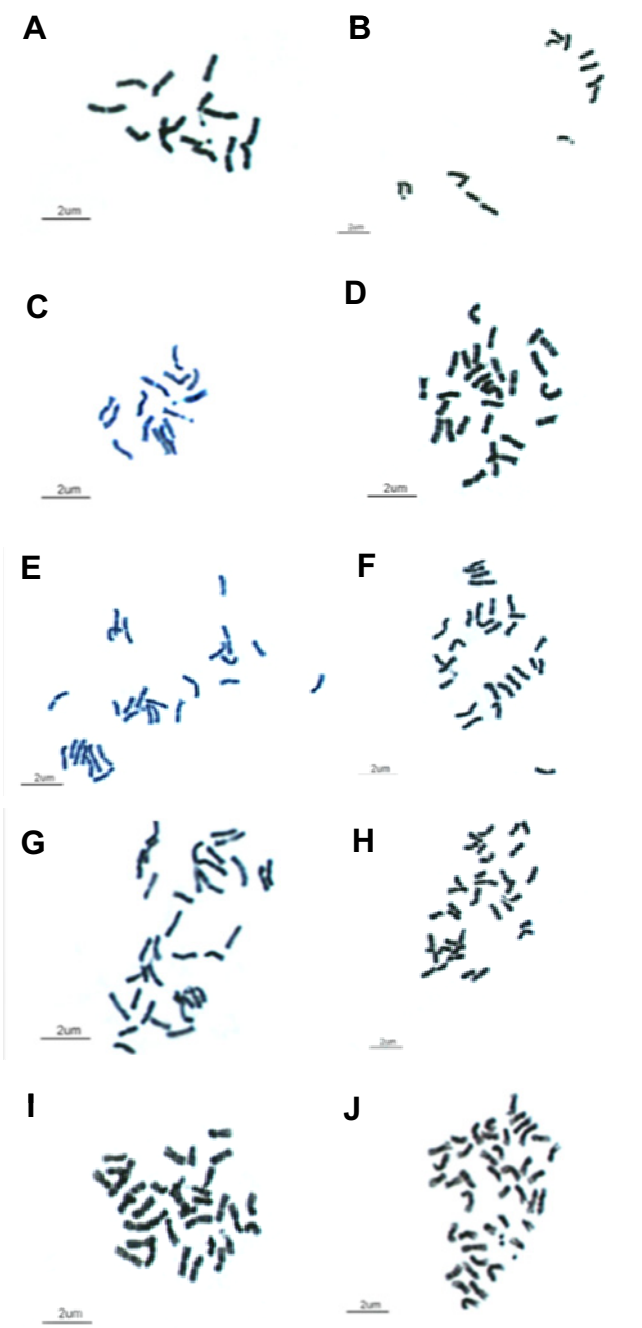

Fig. 2 Chromosome counts of the wild roses. (**A**) *Rosa beggeriana* (2*n* = 2*x* = 14); (**B**) *R. moschata* (2*n* = 2*x* = 14); (**C**) *R. persica* (2*n* = 2*x* = 14); (**D**) *R. pimpinellifolia* (2*n* = 4*x* = 28); (**E**) *R. foetida* (2*n* = 4*x* = 28); (**F**) *R. hemisphaerica* (2*n* = 4*x* = 28); (**G**) *R. boissieri* (2*n* = 5*x* = 35); (**H**) *R. orientalis* (2*n* = 5*x* = 35); (**I**) *R. canina* (2*n* = 5*x* = 35); (**J**) *R. pulverulenta* (2*n* = 6*x* = 42).

species were used in the present investigation. Greilhuber (1988) also estimated the 2C DNA amount of *R. canina* (2.86 pg, s.d. ± 0.34 pg) by feulgen microdensitometry, which is very close to the value estimated by FCM in the present investigation. Moreover, according to the last update of the Plant DNA C-values Database, "Release 4.0" launched in October 2005 (Bennett and Lietch 2005 cited in Gregory *et al.* 2006), our investigation reports the nuclear DNA amounts of the species for the first time for *R. beggeriana*, *R. moschata*, *R. pimpinellifolia*, *R. hemisphaerica*, *R. orientalis*, *R. boissieri* and *R. pulverulenta* (**Table 1**).

The ploidy level estimation of the investigated species by FCM confirmed the chromosome counts. Compared to chromosome counting of root tip meristems, it proved to be a quick and more readily available method with our germplasm and all of the ploidy levels (di-, tetra-, penta- and hexaploid) were able to be distinguished by means of FCM. As the 2C DNA amounts of polyploids are expected to increase with a direct rise of ploidy level, the tetra-, penta- and hexaploid species in the present investigation also showed an approximate increase of 2-, 3- and 3.5-fold of the mean genome size of diploids respectively (**Table 1**).

The ratio of the fluorescence intensities of six species of roses to parsley in DAPI-stained nuclei were closely correlated with those of PI-stained nuclei (r^2 = 0.98) but smaller (**Fig. 3**) which is in accordance with Yokoya *et al.* (2000). This could be attributed to the preferential binding of fluorochrome DAPI to only the AT-rich regions of DNA (Portugal and Waring 1988) compared to quantitative intercalating of PI into double stranded DNA (Le Pecq and Paoletti 1967). This was shown more precisely in the hexaploid species (*R. pulvelulenta*), where the p_1/p_2 ratio for DAPI was lower than that of PI (**Fig. 3**). Therefore, the selection

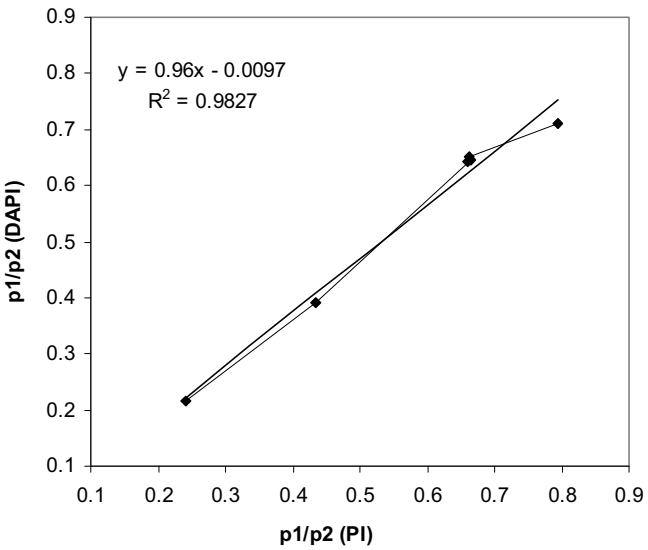

Fig. 3 Relationship between ratios of fluorescence intensities of rose to parsley (p_1/p_2) in DAPI- and PI-stained 2C nuclei.

Table 1 Chromosome numbers (2*n*) and 2C DNA amounts of rose species measured by flow cytometry (all estimates based on five replicates).

Taxa	Species	2*n* chromosome counts	2C DNA amount (pg ± s.d.)
Subgenus *Hulthemia*	*R. persica*	2*x* = 14	0.83 ± 0.08
Subgenus *Rosa* (=*Eurosa*)			
Sections:			
Synstylae	*R. moschata*	2*x* = 14	1.21 ± 0.08
Cinnamomeae	*R. beggeriana*	2*x* = 14	1.07 ± 0.08
Pimpinellifolia	*R. pimpinellifolia*	4*x* = 28	1.93 ± 0.08
	R. hemisphaerica	4*x* = 28	2.05 ± 0.08
	R. foetida	4*x* = 28	1.91 ± 0.08
Caninae	*R. canina*	5*x* = 35	2.95 ± 0.08
	R. orientalis	5*x* = 35	2.94 ± 0.08
	R. boissieri	5*x* = 35	2.96 ± 0.08
	R. pulverulenta	6*x* = 42	3.54 ± 0.08

of a suitable fluorochrome for staining the nuclei in FCM analysis is critical, if not, the ploidy levels will be miscalculated.

In conclusion, the value of cytogenetic studies in association with FCM analysis for determination of the ploidy level was demonstrated. Moreover, the native *Rosa* spp. in Iran showed a great diversity of chromosome numbers and nuclear DNA amounts, which could be beneficial in introducing new gene resources in the breeding of roses. Knowledge of the genome size may help in studying the phylogenetic relationships, correlation of the physiological and agronomical characters to the genome size, determination of the genetic fingerprinting approaches in investigating the population structure, gene flow, and so on.

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