

Nuclear DNA Amounts in Roses

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Received: 23 November 1999 Accepted: 16 December 1999

Nuclei isolated from young leaves were stained with propidium iodide (PI) and their fluorescence intensities were measured by flow cytometry. The ratio of fluorescence intensities of four calibration standards and 34 roses to an internal standard, parsley (*Petroselinum crispum*), provided a basis for estimating the DNA amounts of *P. crispum* and rose. The 2C DNA amount of *P. crispum* ($2n = 22$) was estimated as 4.46 pg (s.d. ± 0.08 pg). The 2C DNA amounts of diploid roses ($2n = 14$) varied between subgenera, sections and cultivars, and ranged from 0.78 pg (s.d. ± 0.08 pg) in *Rosa xanthina* and *R. sericea* (section Pimpinellifoliae) to 1.29 pg (s.d. ± 0.08 pg) in 'Félicité et Perpétue' (Hybrid Sempervirens). Within each section, the DNA amounts of diploid species were similar. In the sections Carolinae and Cinnamomeae, DNA amounts were proportional to ploidy numbers. In the Pimpinellifoliae, DNA amounts of tetraploids were disproportionately larger than those of diploids which suggests that they originated as hybrids with species of sections with larger DNA amounts. Ratios of the fluorescence intensities of nuclei of roses to *P. crispum* (internal standard) were also measured using 4',6-diamidino-2-phenylindole (DAPI) which binds preferentially to AT base pairs. These DAPI ratios were lower than, but closely correlated ($r^2 = 0.997$) with PI ratios. Fluorescence intensities of either PI or DAPI-stained nuclei of roses can be used as rapid indicators of ploidy if variation in the DNA amounts between different taxonomic groups is taken into account.

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Key words: Flow cytometry, nuclear DNA amounts, *Petroselinum crispum*, phenolics, *Rosa*, roses.

INTRODUCTION

Chromosome numbers in the genus *Rosa* are based on multiples of seven and range from $2n = 2x = 14$ to $2n = 8x = 56$ (Darlington and Wylie, 1955). Aneuploids are rare (Rowley, 1960). The subgenera, *Hulthemia*, *Platyrrhodon* and *Hesperodos*, have only one species each, in which $2n = 2x$. The fourth subgenus, *Eurosa*, contains over 120 species grouped into ten sections (Rehder, 1960). In the sections Banksianae, Bracteatae, Indicae, Laevigatae and Synstylae $2n = 2x$, in the Gallicanae $2n = 4x$, in the Carolinae and Pimpinellifoliae $2n = 2x$ and $4x$, in the Caninae $2n = 4x$, $5x$ and $6x$, and in the Cinnamomeae $2n = 2x$, $4x$, $6x$ and $8x$ (Darlington and Wylie, 1955).

Cultivars of *R. chinensis* Jacq. and *R. chinensis* \times *gigantea* Coll. (section Indicae) with the recessive gene for 'recurrent-flowering' were introduced into Europe approx. 1800 AD. They were crossed with cultivars of short flowering season, including *R. damascena* Mill. (section Gallicanae), and *R. moschata* Herrm. and *R. multiflora* Thunb. (section Synstylae). Modern roses that possess the recurrent-flowering character originated from these early hybrids. Genes from just a few other species, including *R. wichuraiana* Crép. (section Synstylae) and *R. foetida* Herrm. (section Pimpinellifoliae), were later introduced by introgressive hybridization (Hurst, 1941; Wylie, 1954, 1955a,b). Modern roses are grouped into horticultural classes that include Polyanthas ($2n = 2x$), Hybrid Teas ($2n = 3x$, $4x$),

Floribundas ($2n = 3x$, $4x$) and Miniatures ($2n = 2x$, $3x$, $4x$), the genomic origins of which have been partially obscured by intercrossing. Cultivated roses are popular as plants for house and garden, and cut flowers are sold in substantial numbers; more than 18 000 registered cultivars are listed by Cairns (1993).

Nuclear DNA amounts have been estimated in *R. canina* L. (2C = 2.86 pg) by Greilhuber (1988) using Feulgen microdensitometry, *R. multiflora* (2C = 1.65 pg) by Dickson *et al.* (1992) using flow cytometry, and *R. hybrida* L. 'Mme G. Delbard' (2C = 2.94 pg) and *R. rugosa* Thunb. (2C = 1.33 pg) by Moyne *et al.* (1993) using flow cytometry. DNA amounts of other roses, assessed by Feulgen microdensitometry (El-Lakany, 1972) and by flow cytometry (Jacob *et al.*, 1996), were quoted in relative but not absolute amounts. Greilhuber (1988) showed that the interaction between chromatin and tannins diminishes the staining of DNA by the Feulgen reaction and it is possible that other phenolic substances interact with chromatin in the same way. Tannins bind preferentially to formaldehyde and fixation of shoot meristems in 4 g l^{-1} neutral formaldehyde in Sørensen's phosphate buffer (pH 7.0) substantially increased the estimated DNA amount of *R. canina* (Greilhuber, 1988). Estimates of DNA amounts by Feulgen microdensitometry for *R. acicularis* Lindl., *R. blanda* Ait. and *R. wichuraiana* listed by Bennett and Smith (1991) did not take account of self-tanning and may underestimate the amount of DNA. It is possible that tannins and other phenolics might interfere with the binding to DNA of fluorochromes used in flow cytometry. Polyvinyl

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pyrrolidone (PVP) is known to bind to phenolics (Walker, 1971). O'Brien *et al.* (1996) included PVP-10 in their nuclei isolation buffer to prevent phenolics from interacting with DNA when they investigated DNA amounts in *Pinus* by flow cytometry. Dickson *et al.* (1992) included PVP-40 (10 g l^{-1}) to increase the number of intact nuclei in the initial isolation solution they used for flow cytometry of some Rosoideae. Arumuganathan and Earle (1991) estimated the 2C DNA amount of parsley, *Petroselinum crispum* (Mill.) Nyman ex A.W. Hill, to be 3.98 pg. It was used as an internal standard in the present investigation because its DNA amount was similar to that of roses but was unlikely to give overlapping peaks with roses of any ploidy level.

Flow cytometry enables DNA amounts to be determined rapidly from leaf tissue. Leaves of roses are more readily available than roots because commercially propagated plants are commonly budded onto rootstocks of a different species. The choice of a suitable fluorochrome is important. The fluorochrome 4',6-diamidino-2-phenylindole (DAPI) produces sharper peaks than other widely used DNA fluorochromes, including propidium iodide (PI), but it has a preference for AT bases (Doležel, Sgorbati and Lucretti, 1992). PI shows no base preference and is more suitable than DAPI for the determination of absolute DNA amounts.

The objectives of the present investigation were to develop suitable protocols for the assessment of DNA amounts in roses by flow cytometry, assess variations in DNA amount in a selection of species and cultivars, and consider the validity of using DNA amounts as rapid predictors of ploidy.

MATERIALS AND METHODS

Rose species and cultivars

Representatives of all the subgenera and sections of *Rosa*, and ploidy levels of 2x, 3x, 4x, 5x and 6x were investigated. Specimens for flow cytometry and chromosome studies were obtained from the following UK sources: David Austin Roses Ltd., Wolverhampton; the Royal National Rose Society, St. Albans; Harkness Roses, Hitchin; Chelsea Physic Garden, London; and the University of East London. Herbarium specimens of the roses investigated are deposited at the Herbarium of the Royal Botanic Gardens, Kew.

Chromosome counts

The tips of actively growing roots or shoots were pretreated in a saturated solution of α -bromonaphthalene for 24 h at 4°C, fixed overnight in ethanol-glacial acetic acid (3:1, v:v), hydrolysed in 5 M HCl at 25°C for 30 min, stained in Feulgen reagent for 3 h, then stored in 450 g l^{-1} acetic acid for 1–24 h at 4°C. The terminal 1 mm of the tips were excised, macerated and squashed between slide and coverslip in 45% aceto-orcein. Coverslips were removed from frozen preparations, and the slides dipped in absolute ethanol and flamed. Unstained slides were observed by

phase contrast microscopy. Supplementary staining was obtained with 0.05 g l^{-1} toluidine blue in citrate buffer (pH 4.0). Slides were then air-dried and mounted under a coverslip in histomount (National Diagnostics, New Jersey, USA).

DNA calibration standards

Vigna radiata (L.) Wilczek 'Berken' (2C = 1.06 pg) (Bennett and Leitch, 1995), *Lycopersicon esculentum* Mill. 'Stupicke Polni Rane' (2C = 1.96 pg) (Doležel *et al.*, 1992), *Glycine max* (L.) Merr. 'Polanka' (2C = 2.50 pg) (Doležel, Doleželová and Novák, 1994) and *Hordeum vulgare* L. 'Sultan' (2C = 11.12 pg) (Bennett and Leitch, 1995) were used as calibration standards. *P. crispum* 'Champion Moss Curled' was used as an internal standard to relate the DNA amounts of the calibration standards to those of roses.

Measurements of fluorescence intensity

Four leaf discs (total 50 mm^2) of young leaves of the calibration standards or the roses under investigation were cut with a cork borer. These, together with a sprig of leaf material of *P. crispum* of similar weight, were chopped with a sharp razor blade in nuclei isolation buffer. The effect on PI and DAPI fluorescence intensities of adding PVP-40 at various concentrations (0, 0.3, 1, 3, 10, 30 g l^{-1}) to nuclei isolation buffer was investigated in the rose 'Félicité et Perpétue' using *P. crispum* as an internal standard. For PI staining, the leaf material was chopped in 0.8 ml of ice-cold nuclei isolation buffer (LB01 lysis buffer; Doležel *et al.*, 1992). This buffer consisted of 15 mM Tris, 2 mM NaEDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM β -mercaptoethanol and 1 ml l^{-1} Triton X-100, pH 7.5. The lysate was made up to 2 ml with buffer and filtered through nylon gauze (pore size $40 \mu\text{m}$). Ribonuclease A ($2.9 \mu\text{l}$ of a 34 mg ml^{-1} solution) and PI ($10 \mu\text{l}$ of a 10 mg ml^{-1} solution) were added and incubated in the dark for 1 h on ice. The samples were filtered through nylon gauze (pore size $20 \mu\text{m}$) and fluorescence intensity was measured with a CA-III flow cytometer (Partec GmbH, Münster, Germany) with a 40×0.80 quartz objective. An Argon laser light source (488 nm wavelength) was used with a TK420 dichroic mirror and a OR610 barrier filter. For DAPI staining, leaf material was chopped at room temperature in 0.4 ml nuclei isolation buffer ('high resolution DNA kit, solution A: nuclei isolation'; Partec GmbH, Münster, Germany). The suspension was filtered through a $40 \mu\text{m}$ nylon gauze and mixed with 1.6 ml of staining buffer (the 'high resolution DNA kit, solution B: DAPI staining' of Partec GmbH, Münster, Germany) for a minimum of 2 min. The suspension of nuclei was analysed by the CA-III flow cytometer with a 100 W high-pressure mercury lamp, KG1, BG38, UG1 filters, TK420 dichroic mirror and a GG435 barrier filter. Data were analysed on DPAC software (Partec GmbH, Münster, Germany). The recorded modal channel numbers were adjusted to compensate for non-linear amplification by the method of Bagwell *et al.* (1989). Estimates of the ratio of fluorescence intensities of each calibration standard and rose to *P. crispum* (p_1/p_2) were

based on the mean of five samples, each with a minimum of 10 000 nuclei giving peaks with a coefficient of variation of less than 5%.

RESULTS

The effect of PVP-40 on fluorescence intensity

The inclusion of PVP-40 in the nuclei isolation buffer did not affect the fluorescence intensity of DAPI-stained nuclei of *P. crispum*. However, when leaves of rose and parsley were chopped together, the fluorescence intensity of the nuclei of both species increased with each increment of PVP-40 up to 10 g l⁻¹ (Fig. 1). The nuclei of rose and parsley were similarly affected (Fig. 1) and the ratio of the fluorescence intensities of rose to parsley (p_1/p_2) did not vary with concentration of PVP-40. These observations indicate that phenolics from the leaves of the rose entered the nuclei of both rose and parsley and interfered with the staining of DNA by DAPI. However, this interference could be offset by the inclusion of PVP-40 in the nuclei isolation buffer and by the use of an internal standard. The fluorescence intensity of PI was not influenced by PVP-40 concentration in either rose or parsley. However, at concentrations of PVP-40 of less than 10 g l⁻¹, nuclear debris was conspicuous in the flow cytometry histograms and the peaks were often undetectable. This indicated that PVP-40 assisted in maintaining the integrity of the nuclear envelope. In all further measurements of fluorescence intensity, PVP-40 (10 g l⁻¹) was included in the nuclei isolation buffer prior to staining with either DAPI or PI and parsley was used as an internal standard.

2C nuclear DNA amounts

A straight-line relationship was observed between the DNA amounts of the four standards (Fig. 2). The coefficient of determination (r^2) was 1.000 and the ratio of the PI fluorescence intensities of the standards to *P. crispum* (p_1/p_2) was defined by the formula:

$$\text{DNA amount (pg)} = 4.474 p_1/p_2 - 0.012 \\ (\text{s.d.} \pm 0.080 \text{ pg})$$

This formula was used to estimate the DNA amounts of *P. crispum* and the roses.

By substituting p_1/p_2 by 1.0, the DNA amount of *P. crispum* 'Champion Moss Curled' was estimated as 4.46 pg (s.d. \pm 0.080 pg), which is 0.48 pg higher than the estimate by Arumuganathan and Earle (1991) on an unspecified variety of *P. crispum*. Root tip squashes of *P. crispum* 'Champion Moss Curled' indicated a chromosome number of $2n = 22$.

The 2C DNA amount of each rose was estimated as the mean of five replicates and the standard deviation was calculated using the formula:

$$\text{s.d.} = \sqrt{\text{s.d.}_S^2 + \text{s.d.}_R^2}$$

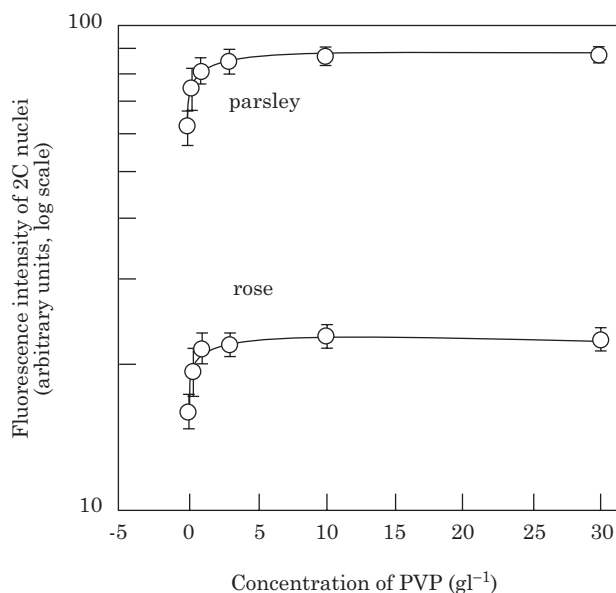


FIG. 1. Mean fluorescence intensities of DAPI-stained 2C nuclei (\pm s.d.) of parsley and rose (leaves of both types were chopped together) in relation to concentration of PVP in the nuclei isolation buffer.

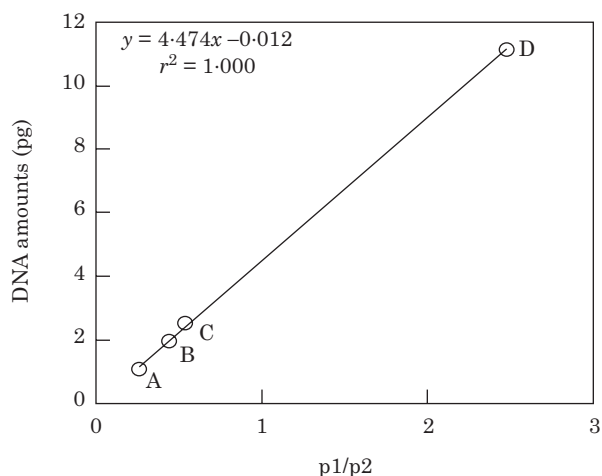


FIG. 2. DNA amounts of four calibration standards in relation to the ratio of fluorescence intensities of PI-stained nuclei of the standards to parsley (p_1/p_2) (A, *V. radiata*; B, *L. esculentum*; C, *G. max*; D, *H. vulgare*).

where s.d._S^2 is the variance of the calibration line (Fig. 2) and s.d._R^2 is the variance of the five replicates. The estimates of 2C DNA amounts in our samples of roses (Table 1) ranged from 0.78 pg (s.d. \pm 0.08 pg) in the diploid species *R. xanthina* Lindl. and *R. sericea* Lindl. to 3.04 pg (s.d. \pm 0.08 pg) in hexaploid *R. acicularis*. Throughout this range, the ratio of the fluorescence intensities of roses to parsley in DAPI-stained nuclei were closely correlated with those of PI-stained nuclei ($r^2 = 0.997$) but smaller (Fig. 3).

The 2C DNA amounts of diploid roses varied between different subgenera, sections and cultivated roses, and

TABLE 1. Chromosome numbers ($2n$) and 2C DNA amounts of rose species and cultivars estimated by flow cytometry (all estimates based on five replicates)

Group	Species or cultivar	$2n =$	2C DNA amount (pg \pm s.d.)
Subgenus <i>Eurosa</i>			
Sections:			
Banksianae	<i>R. banksiae</i> Ait. var. <i>lutea</i> Lindl.	$2x = 14$	1.04 ± 0.08
Bracteatae	<i>R. bracteata</i> Wendl.	$2x = 14$	1.15 ± 0.08
Caninae	<i>R. canina</i> L.	$5x = 35^*$	2.91 ± 0.08
Carolinae	<i>R. foliolosa</i> Nutt.	$2x = 14$	0.94 ± 0.08
	<i>R. nitida</i> Willd.	$2x = 14$	0.95 ± 0.08
	<i>R. virginiana</i> Mill.	$4x = 28$	1.95 ± 0.08
Cinnamomeae	<i>R. iliensis</i> Chrshan.	$2x = 14^*$	0.95 ± 0.08
	<i>R. rugosa</i> Thunb. var. <i>alba</i> W. Robins	$2x = 14$	0.98 ± 0.08
	<i>R. willmottiae</i> Hemsl.	$2x = 14$	0.92 ± 0.08
	<i>R. bella</i> Rehd. & Wils.	$4x = 28^*$	1.94 ± 0.08
	<i>R. fedtschenkoana</i> Reg.	$4x = 28$	1.94 ± 0.08
	<i>R. latibracteata</i> Boul.	$4x = 28$	1.91 ± 0.08
	<i>R. moyesii</i> Hemsl. & Wils.	$6x = 42$	2.88 ± 0.08
Gallicanae	<i>R. acicularis</i> Lindl. var. <i>engelmannii</i> S. Wats.	$6x = 42$	3.04 ± 0.10
	<i>R. gallica</i> L. var. <i>officinalis</i> Thory	$4x = 28$	2.20 ± 0.08
	<i>R. damascena</i> Mill. var. <i>versicolor</i> West.	$4x = 28$	2.16 ± 0.08
	<i>R. centifolia</i> L.	$4x = 28$	2.23 ± 0.08
Indicae	<i>R. chinensis</i> Jacq.	$2x = 14^*$	1.16 ± 0.08
Laevigatae	<i>R. laevigata</i> Michx.	$2x = 14$	1.14 ± 0.08
Pimpinellifoliae	<i>R. xanthina</i> Lindl. 'Canary Bird'	$2x = 14^*$	0.78 ± 0.08
	<i>R. sericea</i> Lindl. f. <i>pteracantha</i> (Franch.) Rehd. & Wils.	$2x = 14^*$	0.78 ± 0.08
	<i>R. foetida</i> Herm.	$4x = 28$	1.95 ± 0.08
	<i>R. spinosissima</i> L. var. <i>hispida</i> (Sims) Koene	$4x = 28$	1.85 ± 0.08
	<i>R. arvensis</i> Huds.	$2x = 14^*$	1.12 ± 0.08
Synstylae	<i>R. sempervirens</i> L.	$2x = 14$	1.14 ± 0.08
	<i>R. wichuraiana</i> Crép.	$2x = 14^*$	1.13 ± 0.08
	<i>R. stellata</i> Woot. var. <i>mirifica</i> (Greene) Cockerell	$2x = 14$	0.86 ± 0.08
Subgenus <i>Hesperodos</i>	<i>R. persica</i> Michx.	$2x = 14^*$	0.84 ± 0.08
Subgenus <i>Hulthemia</i>	<i>R. roxburghii</i> Tratt. var. <i>hirtula</i> (Reg.) Rehd. & Wils.	$2x = 14$	0.95 ± 0.08
Subgenus <i>Platyrhodon</i>			
Horticultural classes:			
Polyantha	'Ballerina'	$2x = 14^*$	1.18 ± 0.08
Hybrid Sempervirens	'Félicité et Perpétue'	$2x = 14^*$	1.29 ± 0.08
Large-flowered Climber	'New Dawn'	$3x = 21^*$	1.76 ± 0.08
Floribunda	'Frensham'	$3x = 21^*$	1.79 ± 0.08
	'Mountbatten'	$4x = 28^*$	2.30 ± 0.08

* Chromosome counts made in this investigation (all others based on those of Darlington and Wylie, 1961).

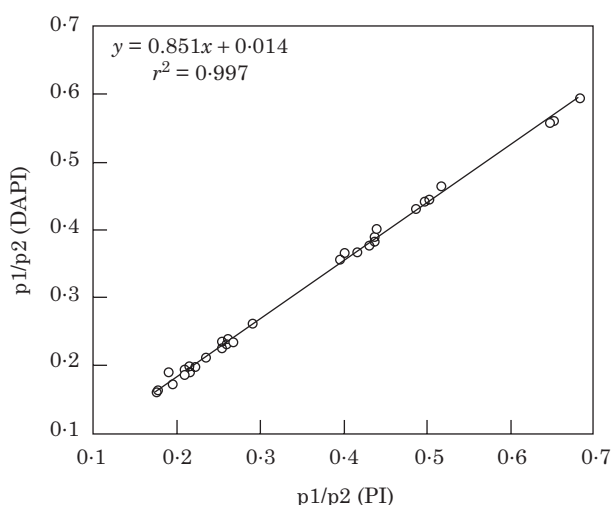


FIG. 3. Relationship between ratios of fluorescence intensities of parsley to rose (p_1/p_2) in DAPI and PI-stained 2C nuclei (roses listed in Table 1).

ranged from 0.78 pg (s.d. \pm 0.08 pg) in the diploid Pimpinellifoliae to 1.29 pg (s.d. \pm 0.08 pg) in the diploid cultivar 'Félicité et Perpétue' (Hybrid Sempervirens). Within the sections, the 2C DNA amounts of diploid species were similar, and in the Carolinae and Cinnamomeae the 2C DNA amounts were approximately proportional to ploidy number. However, in the Pimpinellifoliae, the 2C DNA amounts of tetraploids were disproportionately larger than those of the diploids which suggests that they arose as hybrids between the Pimpinellifoliae and species of sections with larger DNA amounts. The 2C DNA amounts of the cultivars are larger, particularly in the case of 'Félicité et Perpétue', than might be expected from combinations of the genomes of the sections Gallicanae, Indicae and Synstylae from which they were derived.

DISCUSSION

Two of the roses listed in Table 1 had previously been investigated by other authors but in neither case were the

plants of the same clone. The 2C DNA amount of pentaploid *R. canina* was estimated by Greilhuber (1988) to be 2.86 pg (s.d. \pm 0.34 pg) which is within the 95% confidence interval of our estimate (2.91 pg, s.d. \pm 0.08 pg). The 2C DNA amount of *R. rugosa* was estimated by Moyne et al. (1993) to be 1.33 pg (s.d. \pm 0.22 pg), whereas our estimate for *R. rugosa* var. *alba* W. Robbins was 0.98 pg (s.d. \pm 0.08 pg). The estimate by Moyne et al. (1993) was based on fluorescence of nuclei stained with Hoechst 33258 that has a preference for AT-rich regions and may have been influenced by the AT:GC ratio of the rose relative to their calibration standard (chicken red blood cells).

Information about the ploidy levels of breeding stocks is widely used by rose breeders and there is interest in deducing this information from rapid estimates of DNA amount obtained by flow cytometry (Jacob et al., 1996). On the basis of our small sample of cultivars (Table 1), it seems likely that diploid, triploid and tetraploid cultivars can be distinguished by flow cytometry. However, 'Frensham' ($2n = 3x$) has a similar amount of DNA to *R. spinosissima* L. ($2n = 28$, section Pimpinellifoliae) and *R. canina* ($2n = 5x$, section Caninae) has more DNA than *R. moyesii* Hemsl. & Wils. ($2n = 6x$, section Cinnamomeae). Thus errors may arise in predicting ploidy if comparisons are not based on roses of similar taxonomic origin. With this reservation, it is anticipated that flow cytometry of roses, using DAPI or PI fluorescence, will prove to be a valuable method of studying variations in ploidy. Applications include the determination of the ploidy of parents and hybrids in breeding programmes, the selection of plants with doubled chromosome numbers after treatments with spindle inhibitors, the selection of haploids from anther cultures and investigations of ploidy in wild populations.

The present investigation indicates greater variation in DNA amount between, rather than within, sections of the genus *Rosa*. The possible association of these differences and similarities with karyotypic differences would be an interesting subject for further investigation.

ACKNOWLEDGEMENTS

The authors thank David Austin Roses Ltd., Harkness Roses, Chelsea Physic Gardens and the Royal National Rose Society for supplying samples of rose material, Prof. J. Doležel for tomato and soybean calibration standards, and acknowledge financial support from the Ministry of Agriculture Fisheries and Food. R. Lewis acknowledges the collaborative support of the Science and Engineering Research Council and Horticulture Research International (East Malling).

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