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M.D. Fernández-Romero · A.M. Torres · T. Millán
J.I. Cubero · A. Cabrera

Physical mapping of ribosomal DNA on several species of the subgenus *Rosa*

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Abstract The localization of NORs by fluorescent in situ hybridization on metaphase spreads of five diploid (*Rosa gigantea* Coll., *Rosa moschata* Herrm., *Rosa multiflora* Thunb., *Rosa rugosa* Thunb. and *Rosa sempervirens* L., 2n=2*x*=14), one triploid (*Rosa chinensis* 'semperflorens' Koehne., 2n=3*x*=21) and one tetraploid (*Rosa gallica* 'versicolor' L., 2n=4*x*=28) ancestral species of modern roses was studied. Two terminal hybridization signals were observed in all diploid species corresponding to a single NOR per genome in these species. Triploid *R. chinensis* showed three hybridization sites on the short arm of three morphologically similar chromosomes. Six hybridization sites, located at terminal positions of the short arms of three chromosome pairs, were observed in the tetraploid species. These signals corresponded to three pairs of NORs and all of them were located in chromosome pairs of different size. These observations, together with the analysis of meiotic pairing in PMCs, support the view that our specimen of *R. chinensis* is an autotriploid and that *R. gallica* 'versicolor' has an alloploidy nature.

Keywords *Rosa* · *In situ* hybridization · Physical mapping · NORs

Introduction

The genus *Rosa* includes more than 150 species and thousands of cultivars, most of them of complex hybrid origin. This genus exhibits a typical polyploid series with a basic chromosome number of 7. Euploids range from 2n=2*x*=14 to 2n=8*x*=56 (Darlington and Wylie 1955). This genus has been divided taxonomically into four subgenera, with the subgenus *Rosa* (*Eurosa*) including the greater part of species. Of the ten sections into which the subgenus *Rosa* is divided, sections *Synstylae*, *Gallicanae*, *Chinensis*, *Cinnamomeae*, *Pimpinellifoliae* and *Caninae* contain the most-important ancestral roses. In order to clarify the genomic relationships, cytogeneticists and plant breeders have analysed both somatic and meiotic metaphase chromosomes. Cytological reports in rose including chromosome counts, karyotypes and meiotic configuration frequencies have been published (Liu and Li 1985; Subramanian 1987; Ma and Chen 1991,1992; Ma et al. 1997a). On the basis of both arm ratio and chromosome length the authors concluded that karyotypes were largely symmetric and that genomic uniformity existed across the genus. Attempts to apply the C-banding technique to mitotic chromosomes of rose also resulted in a poor resolution of bands (Price et al. 1981). Fluorescent in situ hybridization (FISH) is a powerful technique that could help to identify individual chromosome pairs, to study the phylogenetic relationships within the genus, and to characterise the chromosome complements of hybrids between related wild species and the cultivated roses. Reports about in situ hybridization on chromosomes of roses are scant, mainly due to difficulty in preparing chromosomes suitable for FISH. As far as we know, physical mapping of repetitive DNA sequences on roses has been previously reported by Ma et al. (1997b). In the present work we show the localization by FISH of nuclear organizer regions (NORs) on metaphase spreads of diploids, as well as triploid and tetraploid ancestral species of the subgenus *Rosa*. With the aim to elucidate the polyploid origin, meiotic pairing of the triploid and tetraploid species was also analyzed. We show for the first time cytological data that support the view that *Rosa chinensis* is an autotriploid and *Rosa gallica* 'versicolor' has an alloploid nature.

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M.D. Fernández-Romero · A.M. Torres · T. Millán
CIFA, Departamento de Mejora y Agronomía, Apdo. 4240, 14080 Córdoba, Spain

J.I. Cubero · A. Cabrera (✉)
Departamento de Genética, ETSIAM, Univ. of Córdoba, Apdo. 3048, 14080 Córdoba, Spain
e-mail: ge1cabca@uco.es
Tel.: +34-957-218-510, Fax: +34-957-218-503

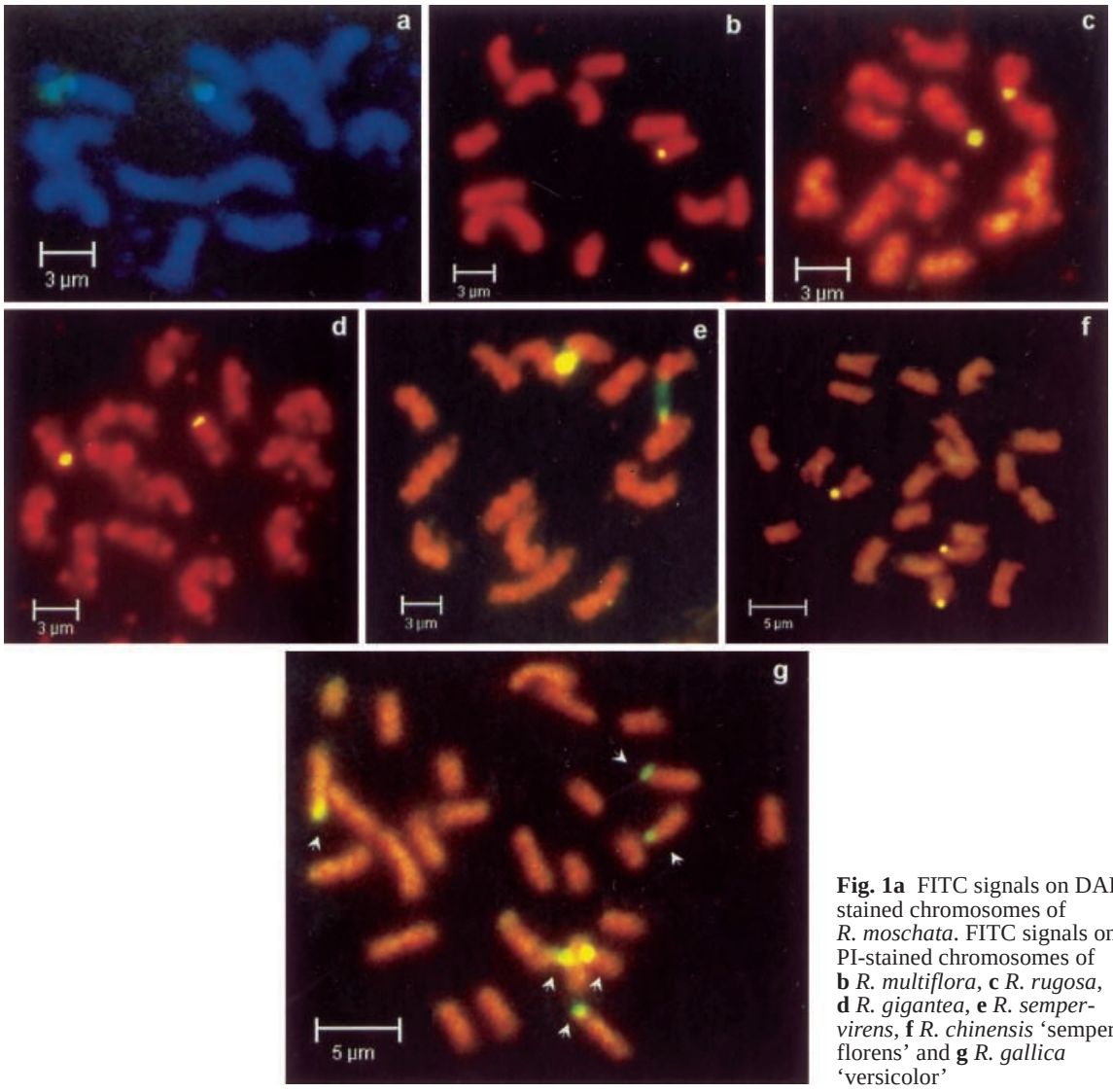

Fig. 1a FITC signals on DAPI-stained chromosomes of *R. moschata*. FITC signals on PI-stained chromosomes of **b** *R. multiflora*, **c** *R. rugosa*, **d** *R. gigantea*, **e** *R. sempervirens*, **f** *R. chinensis* 'semperflorens' and **g** *R. gallica* 'versicolor'

Materials and methods

Five diploid (2n=2*x*=14, *Rosa sempervirens* L., *Rosa moschata* Herrm. and *Rosa multiflora* Thunb. from the section *Synstylae*, *Rosa gigantea* Coll. from the section *Chinensis*, and *Rosa rugosa* Thunb. from the section *Cinnamomeae*), one triploid (2n=3*x*=21, *Rosa chinensis* 'semperflorens' Koehne. from the section *Chinensis*) and one tetraploid (2n=4*x*=28, *Rosa gallica* 'versicolor' L. from the section *Gallicanae*) ancestral species of roses were studied. All species were grown in a space-planted test field in the CIFA facilities in Córdoba (Andalucia, Southern Spain).

Mitotic chromosome preparations from shoot tips were obtained according to the protocol of Ma et al. (1996) with some modifications. Shoot tips of approximately 1 cm were pretreated for $2^1/_2$ h with 0.1% colchicine at room temperature in darkness, and fixed overnight in freshly made 2 acetone:1 glacial acetic acid (v/v) +2% polyvinylpirrolidone (PVP, MWt 40000). After fixation, the shoot tips were soaked in distilled water several times and treated in 1 N HCl for 15 min. After being rinsed for 5 min in distilled water, the materials were equilibrated with 0.01 M sodium citrate at pH 4.6 for 10 min at room temperature. The shoot tips were then enzymatically digested in 8% cellulase (8.4 units per mg solid; SIGMA) +2% pectolyase (3.3 units per mg solid; SIGMA) in 0.01 M sodium citrate, pH 4.6, for 3 h at 37°C, using 50 µl of enzyme mix per tip in a 0.5-ml Eppendorf tube. After digestion the Ma et al. (1997b) procedure for obtaining cellular suspensions and spreading was carried out. The preparations were examined under phase contrast and the best slides were frozen over liquid nitrogen, their coverslips removed and then stored at room temperature until used for FISH.

The probe used was the complete 18/25S rDNA repeat sequence isolated from soybean and inserted in the plasmid pGMr1 (kindly provided by Prof. Weeden of Cornell University, New York) The probe was labelled by nick translation with digoxigenin-11-dUTP (Boehringer) and detected with antidigoxigenin-FITC (Boehringer). The in situ hybridization protocol was according to Cabrera et al. (1999). The hybridization mixture consisted of 50% formamide and 10% dextran sulfate in 2× SSC plus 12 ng/µl of labelled probe, 0.1 µg of sonicated salmon sperm DNA, 0.14 µg of yeast tRNA and 0.005 µg of glycogen. This mixture was denatured for 8 min at 75°C in PCR and cooled on ice for 5 min. A 15-µl aliquot of the mixture was applied to each slide. The chromosomes, together with the hybridization mixture, were incubated in PCR for denaturing the chromosomes and taken to the annealing temperature (37°C) following the temperature series:75°C for 7 min, 55°C for 2 min, 50°C for 30 s, 45°C for 1 min, 42°C for 2 min, 40°C for 5 min, 38°C for 5 min and 37°C for 5 min. The hybridization was carried out overnight at 37°C in a humid chamber. After hybridization, slides were washed twice in

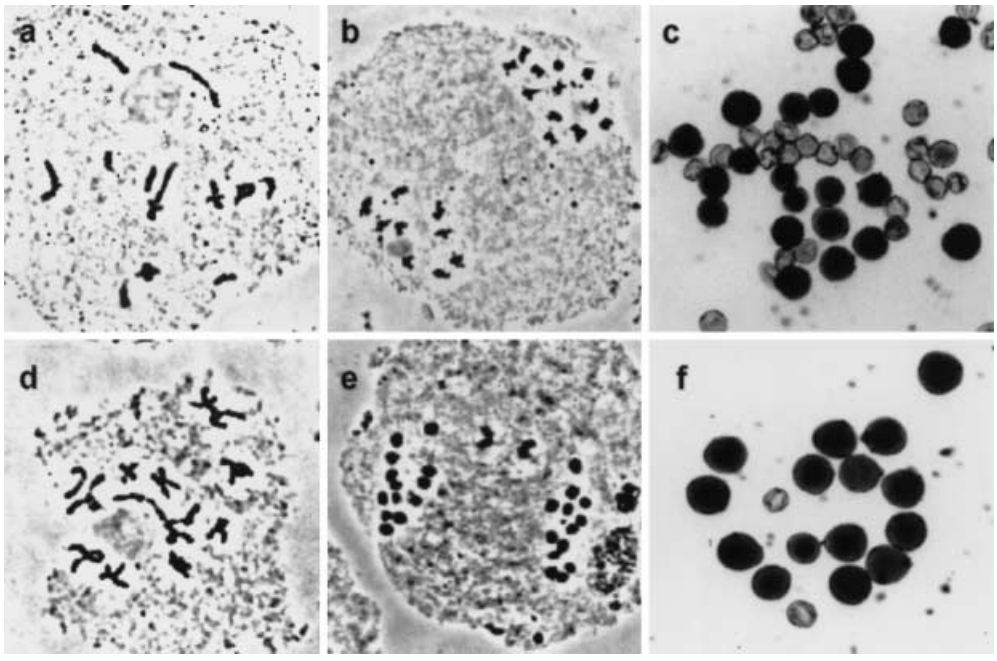

Fig. 2 Meiosis in PMCs of triploid *R. chinensis* 'semperflorens:' **a** diakinesis showing 3 univalents, 3 bivalents and 4 trivalents, **b** telophase-I with an irregular segregation of chromosomes, and **c** pollen grains stained with acetocarmine. Meiosis in PCM of tetraploid *R. gallica* 'versicolor:' **d** diakinesis showing 12 bivalents and 1 tetravalent, **e** telophase-I showing two daughter cells with 13 chromosomes plus 1 retarded element and **f** pollen grains stained with acetocarmine

Table 1 Meiotic pairing in PMCs of *R. chinensis* 'semperflorens' (3×) and *R. gallica* 'versicolor' (4×) (range in brackets)

Species	No. of PMCs examined	I	II	III	IV
R. chinensis 'semperflorens'	33	3.78 (1–6)	3.78 (1–6)	3.21 (1–6)	0 –
R. gallica 'versicolor'	23	0.87 (0–3)	11.22 (8–14)	0.87 (0–3)	0.52 (0–2)

2× SSC at 37°C for 5 min, twice in 1× SSC at room temperature for 5 min and subsequently rinsed in TNT (100 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) at room temperature for 5 min. Then, slides were blocked with 0.5% blocking reagent in 4× SSC at 37°C for 20 min. Slides were counterstained with 2.5 ng/µl of DAPI and 1.5 ng/µl of propidium iodide (PI). Signals were visualized using a LEICA microscope. Images were captured with a SPOT CCD camera using the appropriate SPOT software (Diagnostics Instruments, Inc.) and processed with the PhotoShop 4.0 software (Adobe) using only processing functions that affect all pixels equally. Images were printed on a Mitsubishi sublimation full-color printer.

Meiotic chromosome preparations were obtained from young anthers fixed in 3 ethanol:1 glacial acetic acid (v/v), then squashed in acetocarmine and photographed by phase-contrast microscopy. Pollen grains were treated with acetocarmine for several seconds in order to visualized the viable and inviable grains.

Results and discussion

In situ hybridization with the 18/25S rDNA probe revealed signals on one chromosome pair of all diploid species, corresponding to a single NOR per genome in these species (Fig. 1a-e). The signals were located at terminal positions on the short arm of one submetacentric chromosome pair. Intensity of the signals was higher in both *R. moschata and R. sempervirens* than in *R. multiflora*, *R. gigantea* and *R. rugosa*. A single rDNA locus per genome has been also found previously by Ma et al. (1997b) in the diploid taxa *Rosa chinensis*, *Rosa odorata*, *Rosa×fortuniana*, *Rosa laeviata*, and *Rosa roxburghii*.

Although usually described as a diploid with 2n=14 chromosomes, the *R. chinensis* used in this work was a triploid accession with 2n=3*x*=21 chromosomes. In situ hybridization with the 18/25S rDNA probe detected signals at terminal positions on the short arm of three subtelocentric chromosomes (Fig. 1f). The three chromosomes were similar in size. Cytological analysis on PMCs of triploid *R. chinensis* showed a frequency of 3.21 trivalent formation with a maximum degree of association of six trivalents and three univalents (Table 1). This frequency of trivalent formation provided clear evidence of homology between the three genomes suggesting that this accession is an autotriploid of *R. chinensis*. Segregation at anaphase-I was highly irregular leading to the formation of umbalanced gametes (Fig. 2b). Variation in pollen size and a high percentage of infertile pollen was recorded in the triploid accession after acetocarmine staining (Table 2). As expected, the triploid was very sterile producing very few hips and among them only one seed was found. These results are in agreement with those found by Wulff (1954) and Rowley (1960) who reported that although triploid roses are usually infertile, they occasionally give rise to fertile diploid or tetraploid offspring.

In situ hybridization with the ribosomal DNA probe revealed signals on three morphologically distinct chro-

Table 2 Viability percentage of pollen grains of *R. chinensis* 'semperflorens' (3×) and *R. gallica* 'versicolor' (4×) after treatment with acetocarmine

Species	Total pollen	Fertile	Infertile
R. chinensis 'semperflorens'	557	55.8	44.2
R. gallica 'versicolor'	749	76.5	23.5

mosome pairs of tetraploid *R. gallica*. In all of them, the signals were located at terminal positions on the short arms of different-sized submetacentric pairs. The intensity of the signals was higher in the small chromosome pair than in the other two pairs. (Fig. 1g). These data suggest an allopolyploid origin of *R. gallica* with one NOR in one genome and two NORs in the other one. On the basis of the intra-individual variation of the nrDNA marker ITS1, Wissemann (1999) suggests that tetraploid *R. gallica* possesses two types of chromosomes sets (A, B), thus supporting the allopolyploid origin of *R. gallica*. In a recent paper (Ma et al. 1997b) two pairs of chromosomes with rDNA loci were reported for the commercial tetraploid rose cultivar 'Angel Face', thus resulting in one NOR per genome. However, these authors suggest that the size dimorphism in the tetraploid cultivar was consistent with a 2:2 (AABB) pattern of genome affinity, at least for the NOR chromosomes.

In order to confirm the allotetraploid origin of *R. gallica*, meiotic chromosome pairing was evaluated in this species (Table 1). Univalent chromosomes and multivalent associations were found. Multivalent associations in a tetraploid plant might be due either to heterozygosity for a translocation or to segmental alloploidy. Chromosome associations were mainly bivalents, and cells with up to 14 bivalents were found supporting the allotetraploid origin of *R. gallica*. These frequencies of bivalents formation suggest that either the genomes of the putative parental species were sufficiently distinct for pairing to occur preferentially between genomes of common origin or that after the initial tetraploid was formed, genomic or genotypic changes took place which stabilized meiosis ensuring a regular formation of bivalents. Nevertheless, the maximum number of trivalents and tetravalents were 3 and 2 respectively (Table 1), indicating that some grade of homology already exists among both genomes. Segregation at anaphase-I was quite regular (Fig. 2e) leading to the formation of a high percentage of viable pollen (Table 2) and an average of 6.5 seeds per hip.

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