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Cytological and Molecular Characterisation of a Collection of Wild and Cultivated Roses

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ABSTRACT

A collection of 112 rose accessions, including wild species as well as ancient and modern cultivars were studied. Chromosome number was determined in 104 accessions. Overall, 33 diploid (2*n*=2*x*=14), 15 triploid (2*n*=3*x*=21), 47 tetraploid (2*n*=4*x*=28), 1 pentaploid (2*n*=5*x*=35) and 8 hexaploid (2*n*=6*x*=42) accessions were found. A selection of 27 accessions with ploidy level ranging from 2*x* to 6*x* were used to localize the NORs by FISH. A single NOR per genome was detected in 16 diploid species, 15 from subgen. *Rosa* (Sects. *Rosa*, *Synstylae*, *Pimpinellifoliae*, *Banksianae*, *Bracteatae* and *Indicae*) and one from subgen. *Platyrhodon*. However, in diploid *Rosa majalis*, two pairs of NORs per genome were found. Differences in hybridization signals in 3*x*, 4*x*, 5*x* and 6*x* polyploid accessions were detected. Based on these differences, the autoploid or alloploid nature of these accessions are discussed. In addition, 17 STMS were used to analyse 29 rose accessions. All markers presented high levels of polymorphism, generating a total of 219 different alleles ranging between 111 and 365 bp. The number of allelic patterns ranged from 9 to 22 and all of them presented unique patterns, with a minimum and maximum frequency of 40% and 95.2%, respectively. Based on PIC values obtained (from 0.778 to 0.935) all the STMS were classified as informative markers (PIC > 0.5). This set of microsatellite markers, with high discriminatory power, was used for cultivar identification and allelic patterns of twelve cultivars of roses. All the molecular and cytological data demonstrate the high genetic variability present in the subgenus *Rosa*. This study will help to clarify the origin and genomic relationships among species from this subgenus.

Keywords: ploidy level, Nucleolar Organizer Regions, Sequence Tagged Microsatellite Sites, varietal identification
Abbreviations: **AFLP**, Amplified Fragment Length Polymorphism; C_j, confusion probably; **DAPI**, 4'6-diamidino-2-phenylindole; D_j, discriminating power; **FISH**, fluorescent *in situ* hybridization; H_E, expected heterozygosity; H_O, observed heterozygosity; **IFAPA**, Instituto de Formación Agraria y Pesquera de Andalucía (Spain); **NOR**, Nucleolar Organizer Region; **ITS**, Internal Transcribed Spacer; **lg**, linkage group; **PCR**, Polymerase Chain Reaction; **PIC**, Polymorphic information content; **QTL**, Quantitative Trait Locus; **RAPD**, Random Amplified Polymorphic DNA; **RFLP**, Restriction Fragment Length Polymorphism; **Sect.**, Section; **STMS**, Sequence Tagged Microsatellite Sites; **Subgen.**, subgenus; **UPGMA**, Unweighted Pair Group Method with Arithmetic means; **UPOV**, International Union for the Protection of New Varieties in Plants

INTRODUCTION

The genus *Rosa* is one of the most economically important of all ornamental plants. Its species are used for the production of cut and garden roses, perfume, and medicine. In spite of the economic importance of roses, available knowledge about their genetics is scarce compared to agronomic crops.

This genus is distributed mainly throughout the Northern Hemisphere (Krüssmann 1981) and only four species are naturally present in the southern holarctic region (Wissemann and Ritz 2007). South East China has been postulated as the center of origin for the whole genus (Atienza *et al*. 2005). *Rosa* includes more than 150 species and thousands of cultivars, most of which are of complex hybrid origin (Gudin 2000; Yan *et al*. 2005).

There is great taxonomic confusion in this genus due to the complex evolutionary history of the wild species combined with a long history of cultivation and interbreeding of selected genotypes (Koopman *et al*. 2008). The most recently updated taxonomy (Wissemann and Ritz 2005; 2007) divides the genus into four subgenera, three of which (*Hulthemia*, *Platyrhodon* and *Hesperhodos*) are monotypic or contain two species. The fourth subgenus (*Rosa*), which contains most species, is divided into nine sections: *Rosa* (=*Cinnamomeae*), *Caninae*, *Synstylae*, *Pimpinellifoliae*, *Banksianae*, *Bracteatae*, *Indicae*, *Laevigatae* and *Gallicanae*) These sections (except *Banksianae*, *Bracteatae* and *Laevigatae*) contain the most important ancestral species from which modern garden roses originate (Gudin 2000).

Wild species and cultivars represented in both private and public rose collections give access to most of the variability present in the genus. Conservation of this germplasm is a basic tool for maintaining genetic variability and for selection and breeding purposes. Morphological descriptions have traditionally been used to distinguish rose species and for cultivar identification (Wissemann 2003). In order to supplement and refine the morphology-based descriptions, isoenzyme patterns were introduced to distinguish rose cultivars (Kuhns and Fretz 1978; Cubero *et al*. 1996; Grossi *et al*. 1997). Later, differences in DNA sequences detected by Restriction Fragment Length Polymorphism (RFLP) analysis helped to identify cultivars (Hubbard *et al*. 1992; Rajapakse *et al*. 1992; Ballard *et al*. 1995). Simple techniques based on PCR (Polymerase Chain Reaction) such as RAPD (Random Amplified Polymorphic DNA) (Torres *et al*. 1993; Millán *et al*. 1996; Matsumoto and Fukui 1996; Atienza *et al*. 2005) or long (20-mer) PCR primers (Martin *et al*. 2001) were also introduced to identify rose cultivars and assist phylogenetic relationship studies.

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Markers with a higher discriminatory power and showing more repeatable patterns, such as ITS (Internal Transcribed Spacer) (Wissemann and Ritz 2005), STMS (Sequence Tagged Microsatellite Sites) (Esselink *et al*. 2003; Kimura *et al*. 2006) and AFLPs (Amplified Fragment Length Polymorphisms) (Zhang *et al*. 2001; Leus *et al*. 2004; Koopman *et al*. 2008) have also been developed in roses.

STMS markers are particularly recommended for genotype identification due to their high polymorphism and reproducibility. They have been widely applied in many plant species, including wheat (Prasad *et al*. 2000), olive (Rayo *et al*. 2003; Noormohammadi *et al*. 2007), grape (This *et al*. 2004), lychee (Viruel and Hormaza 2004), strawberry (Gil-Ariza *et al*. 2006) and roses (Esselink *et al*. 2003; Kimura *et al*. 2006).

Cytological studies are another approach to characterising this complex genus. The knowledge of the chromosome number of a particular rose species or cultivar also has a great practical importance to rose breeders. The information given by cytological studies provides an idea of the evolution of this genus during its long period of cultivation. The genus *Rosa* exhibits a typical polyploid series with a basic chromosome number of 7. Euploids range from diploid to octoploid (Darlington and Wylie 1955, cited by Gudin 2000), even a case of a hendecaploid has been described by duplication of chromosomes in the embryo-sac of *R. canina* L. (Zeilinga 1969). Cytological reports in roses have included chromosome counts, karyotypes and meiotic configuration data (Wylie 1954a; Liu and Li 1985; Subramanian 1987; Ma and Chen 1991, 1992; Ma *et al*. 1997). On the basis of arm ratios and chromosome length these studies concluded that rose karyotypes were largely symmetric and that genomic uniformity existed across the genus.

Fluorescent *in situ* hybridization (FISH) may help to clarify the homology and genome assignment of individual rose chromosomes. Reports about *in situ* hybridization in rose chromosomes have been focused on the location of Nucleolar Organizer Regions (NORs) on the metaphase spreads of diploids (Ma *et al*. 1997; Mishima *et al*. 2002) and meiotic metaphase I in *R. canina* (Lim *et al*. 2005). Physical mapping of 18S-25S rDNA sites within the genus *Rosa* could also provide valuable information about relationships among species and for the identification of the diploid ancestors of polyploid species. Physical mapping with ribosomal genes has also helped to elucidate the polyploid origin of some species of the subgenus *Rosa* (Fernández-Romero *et al*. 2001).

In this paper we report the characterisation both cytologically and with molecular markers of a collection of roses maintained in IFAPA of Córdoba (Spain). This collection includes wild species as well as ancient and modern cultivars either collected in Spain or provided by public and private institutions. These roses were characterised for horticultural traits and resistance to several fungal diseases (data not published) and crosses between accessions differing in these traits were used for genetic mapping and location of Quantitative Trait Loci (QTL) (Dugo *et al*. 2005). Our aim was to determine the ploidy level of the collection and the physical location of ribosomal genes in accessions with different ploidy levels. In addition, we analysed the effectiveness of STMS for cultivar identification.

MATERIALS AND METHODS

Plant materials

One hundred and twelve accessions of wild species (one genotype per species), as well as ancient and modern roses cultivars belonging to three of the subgenera of the genus *Rosa* from the rose collection of IFAPA "Alameda del Obispo" Córdoba (Spain) were analysed in this study (**Table 1**). Most entries belong to subgen. *Rosa* and are reported according to Wissemann and Ritz (2007). For the allocation of genotypes to section we followed Beales (1997). Twelve entries from sect. *Rosa*, 12 sect. *Caninae*, 11 sect. *Synstylae*, 10 sect. *Pimpinellifoliae*, 3 sect. *Banksianae*, 1 sect. *Bracteatae*, 25 sect. *Indicae*, 1 sect. *Laevigatae* and 19 sect. *Gallicanae* were studied. Sixteen modern cultivars could not be assigned to a single section.

Chromosome preparations

Chromosome number was determined for 102 accessions from subgenus *Rosa*, one from subgenus *Plathyrhodon* and one from subgenus *Hesperhodos* (**Table 1**). Plant tissue suitable for chromosome counting was not available in the Pretty® family of cut rose cultivars, 'Dallas' and 'Meinelvis' (Sun King®). Mitotic chromosome preparations from shoot tips were obtained according to the protocol of Ma *et al*. (1996) with modifications reported in Fernández-Romero *et al*. (2001). The best slides were frozen over liquid nitrogen, their coverslips removed and then stored at room temperature until used for FISH.

Detection of rDNA sites

The localization of NORs (Nucleolar Organizer Regions) was analysed by FISH (Fluorescence *In Situ* Hybridization) on metaphase spreads in twenty seven accessions. Representative wild roses species and ancient cultivars including multiple ploidy levels were selected. The probe was the complete 18S-25S rDNA repeat sequence isolated from soybean and inserted into the plasmid pGMr1. It was labelled by nick translation with biotin-16-dUTP (Roche, Mannhein, Germany) and detected with Streptavidin-Cy3 conjugate (Sigma, Saint Louis, USA). The *in situ* protocol was performed according to the method of Cabrera *et al*. (2002). The hybridization mixture consisted of 50% formamide and 10% dextran sulphate in 2X 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. Chromosomes were counterstained with DAPI (4'6-diamidino-2-phenylindole) (Sigma) and mounted in Vectashield (Vector, Burlingame, USA). Signals were visualized using a Leica epifluorescence microscope. Images were captured with a SPOT CCD camera using the appropriate SPOT 2.1 software (Diagnostics Instruments, Inc., Sterling Heights, Michigan, USA) and processed with Photoshop 4.0 software (Adobe Systems Inc.) using only processing functions that affect all pixels equally.

STMS analysis

DNA extraction

Twenty nine accessions, including representative wild species, ancient and modern roses cultivars with different ploidy levels (**Table 1**) were analysed using STMS markers. The total genomic DNA was extracted from young leaves using the methodology by Torres *et al*. (1993), after replacing the extraction buffer by one adapted to woody species (Cheng *et al*. 1997).

STMS assay

Twenty four markers obtained by Esselink *et al*. (2003) were amplified in PCR reactions in a total volume 20 µl, containing 20-40 ng of genomic DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 or 2 mM $MgCl_2$ and 0.4U *Taq* DNA polymerase (Biotools, Spain) in standard buffer (75 mM Tris-HCl pH 9, 50 mM KCl and 20 mM $(NH_4)_2SO_4$). The thermal profile for PCR was optimized by Esselink *et al.* (2003) in number of cycles and annealing temperature and consisted of initial denaturation at 94°C for 3 min followed by 30, 35 or 50 cycles of 94°C for 30 s, 50°C or 55°C for 30 s and 72°C for 45 s, concluding with an incubation at 72°C for 3 min. Forward primers were labelled with fluorophores 6FAM or HEX (Sigma-Genosys Ltd.) at the 5′ ends. All the reactions were conducted three times, using DNA of different extractions. PCR products were separated using an automatic capillary sequencer (ABI 3130 Genetic Analyzer Applied Biosystems/HITACHI) at the Unit of Genomics of the Central Service for Research Support of the University of Córdoba (Spain). The size of the amplified bands was calculated based on an internal standard DNA (400HD-

Table 1 Accessions studied including their subgenera, section, accession code in the collection, source of material, chromosome number and total alleles detected with 17 STMS.

Subgenera	Section	Species/cultivar	Code[a]	Source[b]	Chromosome number	Total alleles
II. *Rosa* L.	1. *Rosa* (=*Cinnamomeae*) (DC. Ser.) (incl.. *Carolinae*)					
		'Amadis'	2A-1	RJBM	14	33
		Rosa blanda Ait.	C-28	Loubert	28	-
		R. carolina L.	2A-16	RJBM	28	27
		R. foliolosa Nutt. ex Torr. & Gray	E-20	TAMU	21	-
		R. laxa Retz.	E-18	TAMU	28	-
		R. macrophylla Lindl.	D-4	Loubert	14	21
		R. majalis Herrm.	1A-14	RJBM	14	18
		R. paulii Rehd.	B-25	RJBM	14	-
		R. pendulina L.	2A-8	Loubert	28	-
		R. pendulina pyrenaica (Gouan) R. Keller	C-27	Loubert	28	-
		R. rugosa Thunb.	E-22	TAMU	14	22
		R. virginiana Mill.	B-10	RJBM	28	-
	2. *Caninae* (DC) Ser.					
		'Gil Blas'	1A-6	RJBM	42	-
		R. agrestis Savi	2A-10	RJBM	28	-
		R. canina L.	3A-2	Córdoba (Spain)	42	53
		R. corymbifera Borkh.	3A-6	Córdoba (Spain)	42	-
		R. micrantha Borrer ex Sm.	3A-14	Córdoba (Spain)	35	43
		R. mollis Sm.	2A-27	RJBM	28	-
		R. nitidula Besser	1A-27	RJBM	28	-
		R. pouzinii Tratt.	3A-18	Córdoba (Spain)	42	44
		R. x alba L.	1A-10	RJBM	42	-
		R. x alba 'Maxima'	1A-3	RJBM	42	-
		'Alba Semi-plena'	2A-11	RJBM	42	60
		R. x alba 'Suaveolens'	1A-5	RJBM	42	-
	3. *Synstylae* DC.					
		'Adélaïde d'Orléans'	E-5	RJBM	14	33
		'American Pillar'	1A-24	RJBM	21	32
		'Dundee Rambler'	1A-16	RJBM	21	-
		'Mme Norbert Levavasseur'	1A-2	RJBM	14	-
		R. brunonii Lindl.	E-19	TAMU	28	-
		R. moschata Herrm.	1A-23	Loubert	14	-
		R. multiflora Thunb.	B-18	RJBM	14	-
		R. sempervirens L.	3A-20	Córdoba (Spain)	14	18
		R. setigera Michx.	1A-20	RJBM	14	-
		R. wichurana Crép.	B-11	RJBM	14	-
		R. wichurana 'Basye'	E-15	TAMU	14	28
	4. *Pimpinellifoliae* (DC.) Ser.					
		R. foetida Herrm.	2A-20	RJBM	28	-
		R. foetida bicolor (Jacq.) E. Willm.	1A-25	RJBM	28	-
		R. omeiensis chrysocarpa Rehd.	1A-13	Loubert	14	-
		R. pimpinellifolia altaica (Willd.) Thory	D-8	RJBM	28	-
		R. pimpinellifolia hispida Godet	C-23	Loubert	28	-
		R. pimpinellifolia maxima	D-7	Loubert	28	-
		R. primula Boul.	B-24	RJBM	14	-
		R. sericea Lindl.	E-17	TAMU	14	-
		R. xanthina Lindl.	B-13	RJBM	28	-
		R. xanthina spontanea Rehd.	C-19	Loubert	21	-
	5. *Banksianae* Lindl.					
		R. banksiae Ait.	B-14	RJBM	14	-
		R. banksiae normalis Regel	B-12	RJBM	14	-
		R. cymosa Tratt.	B-9	RJBM	14	-
	6. *Bracteatae* Thory					
		R. bracteata Wendl.	E-13	TAMU	14	-
	7. *Indicae* Thory					
		'Antoine Ducher'	2A-4	RJBM	28	46
		'Blush Noisette'	D-10	RJBM	14	33
		'Mlle Cécile Brünner'	1A-22	RJBM	14	-
		'Champneys' Pink Cluster'	1A-26	RJBM	21	-
		'Hermosa'	2A-22	RJBM	21	-
		'La France'	1A-12	RJBM	21	-
		'Reine Victoria'	2A-13	RJBM	28	-
		'Le Vésuve'	E-6	RJBM	14	-
		'Louise Odier'	2A-9	RJBM	28	-
		'Manettii'	D-11	RJBM	28	-
		'Mrs. Aaron Ward'	1A-1	RJBM	28	-
		'Parks`Yellow Tea-Scented China'	1A-11	RJBM	28	-
		'Pink Peace'	1A-17	RJBM	28	-

Table 1 (Cont.)

Subgenera	Section	Species/cultivar	Code[a]	Source[b]	Chromosome number	Total alleles
		'Pompon de París'	1A-9	RJBM	14	-
		'Rose du Roi'	2A-19	RJBM	28	-
		'Safrano'	2A-3	RJBM	14	-
		'Soleil d'Or'	1A-4	RJBM	28	-
		'Solfaterre'	B-23	Loubert	14	-
		'Souvenir de la Malmaison'	F-24	RJBM	21	43
		R. chinensis Jacq. 'Mutabilis'	E-7	RJBM	14	-
		R. chinensis 'Semperflorens' (='Slater' Crimson China')	B-17	RJBM	21	42
		R. gigantea Collet	2A-26	RJBM	14	35
		R. borboniana Desportes ('Bourbom Rose')	B-27	RJBM	28	-
		R. x noisettiana	2A-17	RJBM	14	-
		R. x odorata	B-16	RJBM	14	-
	8. Laevigatae Thory					
		R. laevigata Michx.	C-13	Loubert	14	-
	9. Gallicanae DC.					
		'Cardinal de Richelieu'	2A-12	RJBM	21	-
		'Celsiana'	E-25	Loubert	28	-
		'Centifolia Minor'	D-12	RJBM	21	-
		'Félicité Hardy' ('Mme. Hardy')	E-23	RJBM	28	-
		'Kazanlik' (=*R. x damascena trigintipetala*)	D-9	RJBM	28	-
		'Old Cabbage'	1A-29	RJBM	21	-
		'Petite de Hollande'	E-24	RJBM	28	-
		'Portland Rose'	D-14	RJBM	21	-
		'Quatre Saisons Continue' (=*R. x damascena bifera* Regel)	D-6	RJBM	28	-
		'Raubritter'	2-A2	RJBM	28	-
		'Rose de Meaux'	1A-8	RJBM	28	-
		'Rose du Roi'	2A-19	RJBM	28	-
		'White Provence'	1A-28	RJBM	28	-
		R. x damascena versicolor ('York and Lancaster')	1A-19	RJBM	28	-
		R. gallica L.	D-15	CBGC	28	-
		R. gallica incarnata (Mill.) R. Keller	1A-01	Loubert	21	-
		R. gallica versicolor L.	2A-7	RJBM	28	45
		R. gallica x R. Arvensis	D-16	CBGC	21	-
		R. x centifolia ('Cabbage Rose')	1A-18	RJBM	28	-
	Unknown					
		'Cardinal'	D-27	U plantas	28	-
		'Carta Blanca'	-	U plantas	28	48
		'Meidresia' ('Carte d' Or')	-	U plantas	28	39
		'Dallas'	F-17	U Plantas	-	41
		'Dot Ora'	D-29	INIA	28	-
		'Keitaibu' (=Laser™)	B-1	U plantas	28	-
		'Keihatakaho' (=Pretty® Bride)	-	U plantas	-	38
		'Meiblanca' (=Pretty® Girl)	-	U plantas	-	38
		'Meifebink' (=Pretty® Princess)	-	U plantas	-	38
		'Febesa' (=Pretty® Woman)	-	U plantas	-	38
		'Meizepline' ('Red Monarch')	-	U plantas	-	42
		'Meifecham' (=So Pretty®)	-	U plantas	-	38
		'Soleil de Minuit'	D-30	INIA	28	-
		'Meihelvet' ('Sonia Meilland')	B-3	U plantas	28	-
		'Meinelvis' (=Sun King®)	-	U plantas	-	39
		'Vaalon'	D-31	INIA	28	-
III. *Platyrhodon* (Hurst) Rehder		*R. roxburghii* Tratt.	B-26	RJBM	14	-
IV. *Hesperhodos* Cockerell		*R. stellata mirifica* Greene	C-20	Loubert	14	-

[a] Number of the accession in the IFAPA, Centro "Alameda del Obispo" Córdoba (Spain) collection.
[b] **RJBM**: Real Jardín Botánico de Madrid, CSIC (Spain), leg. J. Armada; **Loubert**: Nursery LOUBERT, Les Brettes (France); **TAMU**: Collection at Texas A.M. University, Department of Horticultural Sciences (USA), leg. D. Byrne; **CBGC**: Conservatoire Botanique de Gap Charance (France); **U Plantas**: Universal Plantas S.A. (Spain); **INIA**: INIA reference collection, Alcalá de Henares (Spain).

ROX, Applied Biosystems) with GeneScan 3.x software and the results were interpreted using the program Genotyper 3.7 (both from Applied Biosystems).

Statistical analysis

The alleles were scored as 1 (present) or 0 (absent) in a binary matrix for each STMS. Number of alleles, unique alleles, allelic patterns and unique allelic patterns for each STMS primer were calculated. Confusion probability (C_j) and discriminating power (D_j) of each STMS were estimated according to Tessier *et al*. (1999). Polymorphic information content (PIC) was also computed (Botstein *et al*. 1980). For nine diploid accessions ('Amadis', *R. macrophylla* Lindl., *R. majalis* Herrm., *R. rugosa* Thunb., 'Adélaïde d'Orléans', *R. sempervirens* L., *R. wichurana* 'Basye', 'Blush Noisette' and *R. gigantea* Collet) allelic frequencies, ob-

served heterozygosity (H_O) and expected heterozygosity (H_E) were calculated by using the Excel Microsatellite Toolkit (Dept. of Genetics, Trinity College, Dublin, Ireland).

Genetic distances between all pairwise combinations of the accessions were calculated using Jaccard's coefficients of similarity. Grouping of the genotypes was determined by using UPGMA (Unweighted Pair Group Method with Arithmetic means). The correlation coefficient between the similarity matrix and the cophenetic values matrix was computed to test the goodness of fit of the cluster analysis using the Mantel (1967) test. NTSYS-pc 2.02j software (Applied Biostatistics, Setauket, USA) was used for previous statistical analyses. Branch support values were determined in 100 bootstrap replicates using both PHYLIP 3.67 and PhylTools 1.32 software.

RESULTS

Determination of ploidy level

Mitotic chromosome number of the 104 roses species/cultivars evaluated is given in **Table 1**. Overall, 33 diploid ($2n$=$2x$=14), 15 triploid ($2n$=$3x$=21), 47 tetraploid ($2n$=$4x$=28), 1 pentaploid ($2n$=$5x$=35) and 8 hexaploid ($2n$=$6x$=42) accessions were found. No entries with heptaploid ($2n$=$7x$=49) or octoploid ($2n$=$8x$=56) ploidy levels were in the collection. Specifically, whilst species in sects. *Banksianae*, *Bracteatae* and *Laevigatae* were all diploid, in sects. *Rosa*, *Synstylae*, *Pimpinellifoliae* and *Indicae* accessions with 14, 21 and 28 chromosomes were found. In contrast, in both sects. *Gallicanae* and *Caninae* only polyploid species were observed; accessions of sect. *Gallicanae* had either 21 or 28 chromosomes and those of sect. *Caninae* had 28, 35 or 42 chromosomes.

18S-25S rDNA

The number of NOR signals detected by FISH with the 18S-25S rDNA probe is presented in **Table 2**. A total of 34 accessions were selected including all levels of ploidy found in our collection. Seven of these accessions were previously analysed by our group (Fernández-Romero *et al*. 2001). Two hybridization signals located at terminal positions of one chromosome pair were detected in 16 diploid species: 15 from subgen. *Rosa* (sects. *Rosa*, *Synstylae*, *Pimpinellifoliae*, *Banksianae*, *Bracteatae* and *Indicae*) and one from subgen. *Platyrhodon* (**Table 2**). These results indicated a single NOR per genome in these species. **Fig. 1A** shows diploid *R. chinensis* Jacq. 'Mutabilis' displaying two hybridization signals. However, *R. majalis*, a diploid, showed four hybridization signals (**Fig. 1B**), two of them of strong intensity and the other two with weak signals. This result indicates that this diploid species carries two pairs of NORs per genome. As far as we know, this is the first report showing a diploid rose with two pairs of NORs per genome.

In the triploid 'Champneys' Pink Cluster', three hybridization signals were detected at terminal positions of three subtelocentric chromosomes (**Fig. 1D**). However, the triploid *R. foliolosa* Nutt. Ex Torr. & Gray displayed six hybridization signals on six different chromosomes, indicating that this accession carried two pairs of NORs per genome (**Fig. 1E**). Triploid 'Centifolia Minor' showed four hybridization signals at terminal positions of four chromo-

Table 2 Number of NORs signals detected by FISH in rose species or cultivars with different ploidy level.

Ploidy level	Subgen.	Section	Species/cultivar	No. of NORs signals
Diploid				
	Rosa	*Rosa* (=*Cinnamomeae*)	*R. majalis*	4
			R. paulii	2
			R. rugosa	2[a]
		Synstylae	*R. moschata*	2[a]
			R. multiflora	2[a]
			R. sempervirens	2[a]
			R. wichurana	2
		Pimpinellifoliae	*R. primula*	2
			R. sericea	2
		Banksianae	*R. banksiae*	2
			R. cymosa	2
		Bracteata	*R. bracteata*	2
		Indicae	*R. gigantea*	2[a]
			R. chinensis 'Mutabilis'	2
			R. x noisettiana	2
			R. x odorata	2
	Platyrhodon		*R. roxburghii*	2
Triploid				
	Rosa	*Rosa*	*R. foliolosa*	6
		Indicae	'Champneys' Pink Cluster'	3
			R. chinensis 'Semperflorens'	3[a]
		Gallicanae	'Centifolia Minor'	4
Tetraploid				
		Rosa	*R. blanda*	8
			R. carolina	8
		Synstylae	*R. brunonii*	4
			R. xanthina	4
		Indicae	'Rose du Roi'	6
		Gallicanae	'Kazanlik'	6
			'Quatre Saisons Continue'	6
			R. x damascena versicolor	6
			R. gallica versicolor	6[a]
Pentaploid				
		Caninae	*R. micrantha*	5
Hexaploid				
		Caninae	*R. canina*	6
			R. corymbifera	6
			R. pouzinii	6

[a] According to Fernández-Romero *et al*. (2001)

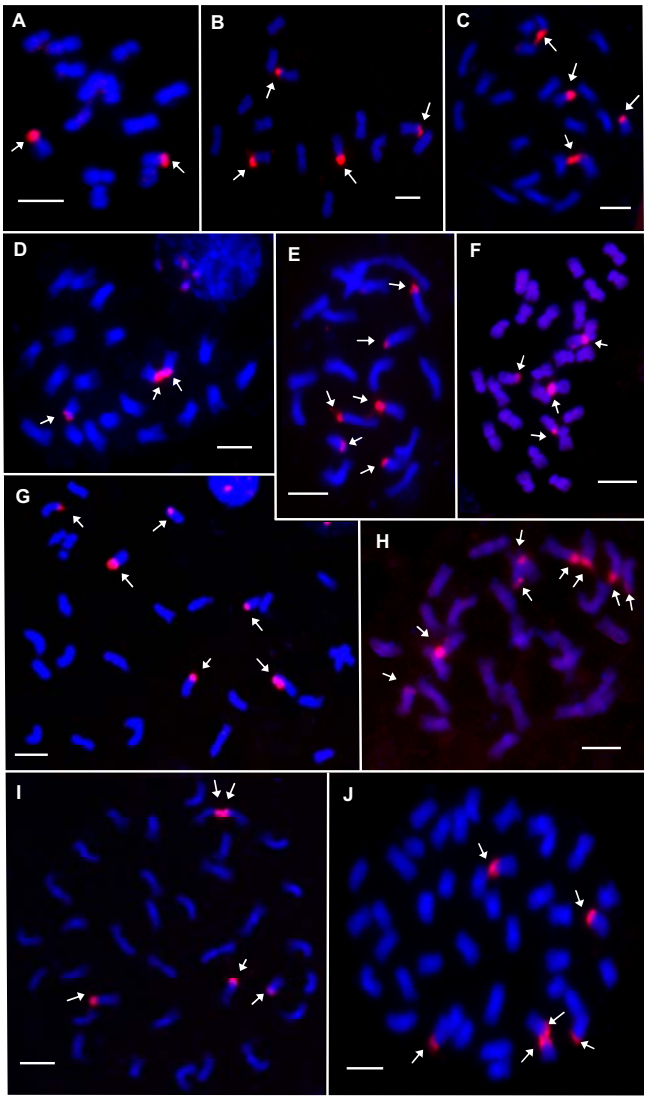

Fig. 1 Fluorescence *in situ* hybridization (FISH) with 18S-5.8S-26S rDNA sequence on metaphase chromosomes of (**A**) *R. chinensis* 'Mutabilis' (2*n*=2*x*=14), (**B**) *R. majalis* (2*n*=2*x*=14), (**C**) 'Centifolia Minor' (2*n*=3*x*=21), (**D**) 'Champneys' Pink Cluster' (2*n*=3*x*=21), (**E**) *R. foliolosa* (2*n*=3*x*=21), (**F**) *R. brunonii* (2*n*=4*x*=28), (**G**) 'Quatre Saisons Continue' (2*n*=4*x*=28), (**H**) *R. blanda* (2*n*=4*x*=28), (**I**) *R. micrantha* (2*n*=5*x*=35) and (**J**) *R. canina* (2*n*=6*x*=42). Chromosomes were counterstained with DAPI. Bar represents 5 µm. Hybridization signals were indicated by arrows.

somes (**Fig. 1C**). Consequently, it contains two genomes with one 18S-25S rDNA locus and one genome with two 18S-25S rDNA loci.

In tetraploid *R. brunonii* Lindl. and *R. xanthina* Lindl., four hybridization signals were found (**Fig. 1F**) indicating a single rDNA locus per genome in these tetraploid species. However, tetraploids *R. blanda* Ait. and *R. carolina* L. showed eight hybridization sites located at terminal positions of four chromosome pairs. Four signals were of strong intensity and the other four displayed weak signals (**Fig. 1H**). We can conclude that these tetraploid species contained two pairs of NORs per genome. Tetraploids 'Rose du Roi', 'Kazanlik', 'Quatre Saisons Continue' and *R.* × *damascena versicolor* showed six hybridization sites at terminal positions on six chromosome arms (**Fig. 1G**). These results indicate that these ancient roses contain a pair of genomes with two 18S-25S rDNA loci per genome in addition to a pair of genomes with one 18S-25S locus per genome.

In situ hybridization with the ribosomal DNA probe revealed signals on five chromosomes of pentaploid *R. micrantha* Borrer & Sm. (**Fig. 1I**). Similarly, six hybridization sites were found in three chromosome pairs of hexaploids *R. canina*, *R. corymbifera* Borkh. and *R. pouzinii* Tratt. (**Fig. 1J**). These results indicate that these four *Caninae* species have a single rDNA site per genome.

STMS variability and cultivar characterisation

Seventeen out of the 24 STMS analysed, with clear amplification patterns, were selected to evaluate 29 accessions of the IFAPA collection differing for ploidy level (**Table 1**). Three replications per sample, derived from different DNA extractions, were analysed using seven different STMS assays revealing identical amplifications patterns. Due to the observed repeatability, only two repetitions per sample were performed in the ten remaining STMS assays. The amplification obtained with these markers showed patterns similar to the ones obtained by Esselink *et al*. (2003), low or no stutter bands and similar locus scorability.

The parameters of variability analysed for STMS markers are presented in **Table 3**. All STMS presented high levels of polymorphism, generating a total of 219 different alleles with fragment size ranging between 111 and 365 bp. The average value of alleles/STMS was 12.9, fluctuating from 5 (RhM405) to 24 (RhAB26). All the STMS markers, except RhM405 and RhE2b, showed unique alleles with an average value of 5/locus. The markers RhD206 and RhAB22 revealed the highest number of unique alleles with 12 and 10 alleles, respectively. The number of allelic patterns ranged from 9 (RhAB1) to 22 (RhEO506) and all STMS presented unique allelic patterns, with a minimum and maximum number of 4 (RhE2a) and 20 (RhJ404), respectively (**Table 3**). High values of discriminating power (*Dj*) were obtained for the STMS evaluated, with values ranging from 0.812 (RhAB1) to 0.968 (RhEO506) and an average value of 0.92. As expected, the values of confusion probability (*Cj*) were low and ranged from 0.032 (RhEO506) to 0.188 (RhAB1). Based on PIC values obtained (from 0.778 to 0.935) all the STMS were classified as informative markers (PIC > 0.5), indicating the potential use of this set of microsatellite markers for cultivar identification (**Table 3**).

Markers analysed and results obtained with nine diploid accessions are shown in **Table 4**. As expected, a maximum of two alleles/STMS were obtained in diploid accessions with all the markers, except for RhJ404. The latter presented more than two alleles, suggesting the amplification of at least two different loci, so its statistical parameters were not calculated. STMS fragments size ranged from 111 bp to 365 bp. The total number of alleles was 116, varying from 3 (RhAB1) to 12 (RhAB22 and RhAB26). Minimum and maximum allelic frequencies were 5.56 and 70.00, respectively. The heterozygosity observed varied from 0.22 (RhAB1 and RhB303) to 1.00 (RhAB22) with an average value of 0.52. Except for markers RhAB22, RhAB26 and RhP524, the observed heterozygosity was lower than expected (with a value mean of 0.82). The heterozygosity deficiency found in these markers (**Table 4**), might be attributable to the presence of null alleles.

Amplification with two informative markers (i.e. RhAB26 and RhEO506) permitted the identification of 24 genotypes. Only the group of the Pretty® family of cut flower rose cultivars ('Febesa', =Pretty® Woman; 'Keihatakaho', =Pretty® Bride; 'Meiblanca', =Pretty® Girl); 'Meifebink', =Pretty® Princess; and 'Meifecham', =So Pretty®) remained undistinguishable showing identical banding patterns with the 17 STMS. Such cultivars differ in flower colour and have the same genetic background. **Table 5** displays allelic pattern profiles of twelve genotypes with different ploidy levels using six markers chosen because of their discriminatory power (*Dj* ≥ 0.950).

STMS data were also used to assess their reliability to establish genetic relationships among 29 accessions shown in **Table 1**. A dendrogram was obtained from the UPGMA analysis based on the Jaccard similarity index (**Fig. 2**). A high correlation between similarity and cophenetic matrices was obtained (r=0.96) indicating good fit of the original data to clustering. Except for the group of the Pretty®

Table 3 Size range, number of alleles, number of allelic patterns, discriminating power (*Dj*), confusion probability (*Cj*) and polymorphic information content (PIC) observed in 29 accessions of a roses collection studied with 17 STMS primers.

STMS	Size range (bp)	No. of alleles	No. of unique alleles	No. of unique allelic patterns (%)	No. of allelic patterns	*Dj*	*Cj*	PIC
RhAB1	141-197	8	5	6 (66.7)	9	0.812	0.188	0.778
RhAB13	130-179	14	4	14 (77.8)	18	0.922	0.078	0.887
RhAB22	137-194	20	10	17 (85.0)	20	0.950	0.050	0.916
RhAB26	161-299	24	7	19 (90.5)	21	0.954	0.046	0.919
RhAB40	185-252	18	9	19 (95.0)	20	0.950	0.050	0.912
RhB19	129-149	8	2	10 (71.4)	14	0.863	0.137	0.833
RhB303	111-140	10	2	13 (72.2)	18	0.952	0.048	0.918
RhBK4	156-239	14	6	16 (84.2)	19	0.946	0.054	0.913
RhD206	178-365	22	12	17 (85.0)	20	0.926	0.074	0.894
RhE2a	158-189	6	3	4 (40.0)	10	0.874	0.126	0.844
RhE2b	160-195	10	0	16 (88.9)	18	0.862	0.138	0.832
RhEO506	187-259	18	5	18 (81.8)	22	0.968	0.032	0.935
RhI402	192-211	7	2	8 (66.7)	12	0.850	0.150	0.820
RhJ404	126-165	12	1	20 (95.2)	21	0.967	0.033	0.928
RhM405	152-176	5	0	7 (53.8)	13	0.882	0.118	0.851
RhO517	239-262	9	2	13 (72.2)	18	0.943	0.057	0.911
RhP524	112-228	14	5	14 (77.8)	18	0.942	0.058	0.908
Total		***219***	***75***	***231 (79.4%)***	***291***			
Mean		***12.9***	***4.4***	***13.6***	***17.1***	***0.915***	***0.084***	***0.882***

Table 4 Size range, number of alleles, allelic frequencies, observed heterozygosities (H_O) and expected heterozygosities (H_E) in nine diploid accessions of a roses collection studied with 17 STMS primers.

STMS	Size range (pb)	No. of alleles	Allelic frequencies (min-max)	H_O	H_E
RhAB1	141-165	3	10.00 - 70.00	0.22	0.51
RhAB13	130-165	8	5.56 - 16.67	0.44	0.91
RhAB22	140-186	12	6.25 - 18.75	1.00	0.96
RhAB26	161-299	12	6.25 - 18.75	0.87	0.95
RhAB40	185-233	7	8.33 - 25.00	0.33	0.91
RhB19	131-149	6	7.14 - 21.43	0.29	0.88
RhB303	111-140	7	5.56 - 22.22	0.22	0.88
RhBK4	156-225	8	5.56 - 50.00	0.44	0.75
RhD206	193-365	7	5.56 - 16.67	0.56	0.89
RhE2a	158-189	5	5.56 - 38.89	0.44	0.74
RhE2b	160-188	6	5.56 - 44.44	0.55	0.77
RhEO506	187-244	10	5.56 - 16.67	0.67	0.93
RhI402	192-211	5	5.56 - 44.44	0.44	0.71
RhJ404	-[nd]	-	-	-	-
RhM405	157-176	4	16.67 - 38.89	0.55	0.76
RhO517	250-262	7	5.56 - 27.78	0.55	0.84
RhP524	112-134	9	5.56 - 22.22	0.78	0.88
Mean		***7.25***	***6.89-30.80***	***0.52***	***0.83***

[nd] not determined

Table 5 Allelic patterns (in bp) of twelve roses varieties obtained with six STMS primers chosen on account of their *Dj* values (≥ 0.950). Unique alleles and unique allelic patterns are shown in bold.

Cultivar names	Ploidy level	Allelic patterns (in bp)					
		RhAB40	RhB303	RhAB22	RhAB26	RhEO506	RhJ404
'Adélaïde d'Orléans'	2x	**226**	**111, 140**	**151, 175**	**184, 236**	**223, 232**	**129, 132, 146, 148, 154**
'Amadis'	2x	**230**	**116**	**175, 177**	**182, 236**	**187, 226**	**129, 132, 146, 154**
'American Pillar'	3x	**200, 202, 219**	**114, 116, 140**	**157, 194**	**170, 176, 203**	**217, 238**	-[a]
'Antoine Ducher'	4x	**217, 226**	**116, 120, 140**	**157, 165, 169, 177**	-[a]	**223, 226, 259**	**126, 129, 137, 146, 154**
'Blush Noisette'	2x	**206, 230**	**116, 118**	**151, 157**	**161, 170,**	220, 223	**129, 132, 148, 154**
'Carta Blanca'	4x	**202, 230, 233**	114, 124	**151, 163**	**164, 189, 271**	**205, 220, 226**	**126, 129, 132, 148, 154, 161**
'Carte d'Or'	4x	**200, 230, 233**	114, 124	**163**	166, 170, 271	205, 223	**126, 129, 132, 148, 154**
'Dallas'	-[nd]	202, 217, 233	114, 124	151, 163	**166, 203, 236**	205, 223, 226	**126, 132, 137, 154, 161**
'Meinelvis'	-[nd]	**200, 230**	114, 124, 140	**157, 163**	**166, 203**	**205, 223, 226**	**129, 132, 137, 148, 154, 161**
'Meizepline'	-[nd]	**202, 217, 226**	114, 124, 140	**151, 163**	166, 203, 236, 271	**205, 223**	**126, 129, 132, 137, 148, 154**
'Souvenir de la Malmaison'	3x	**202, 208, 230**	**120, 124, 140**	**151, 157, 163**	166, 170, 271	**205, 208, 223**	**126, 132, 144, 154**
The Pretty® family of cvs.	-[nd]	202, 217, 233	116, 120, 124, 140	163	166, 203, 236, 271	223, 226	132, 137, 148, 154

[a] Missing date
[nd] not determined

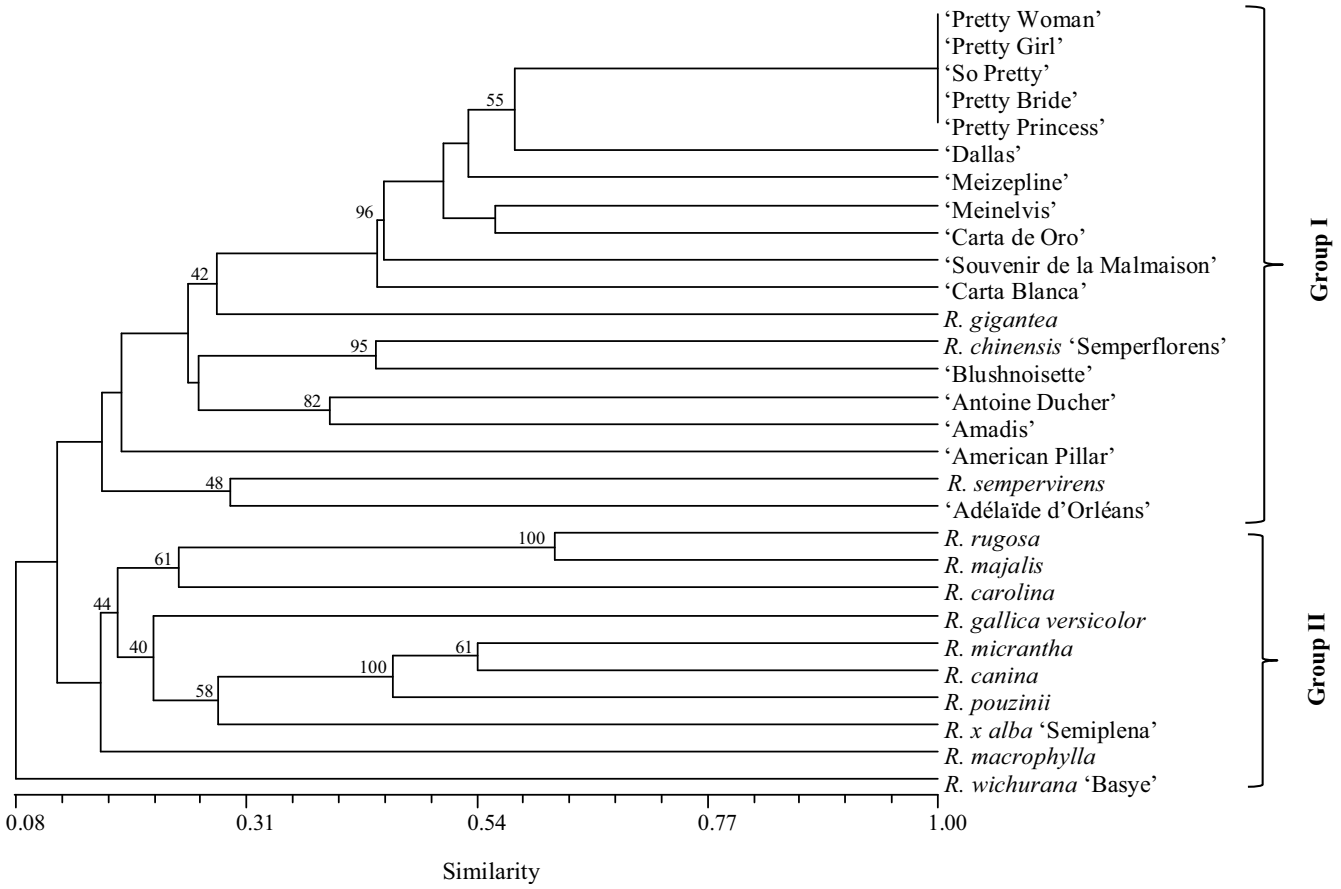

Fig. 2 UPGMA dendrogram obtained from cluster analysis of 29 rose accessions based on Jaccard's coefficient of similarity using 17 STMS**.** Bootstrap values ≥40% are noted above the branches.

family of cultivars, similarity values were low, with minimum and maximum values of 0.02 (*R. wichurana* 'Basye' - *R. rugosa*) and 0.62 (*R. rugosa* - *R. majalis*), respectively.

In the dendrogram obtained (**Fig. 2**), most of the accessions could be classified into two main groups. Group I is comprised mainly by modern (Pretty® family of cultivars, 'Dallas', 'Meizepline', 'Meinelvis', 'Carte d' Or' and 'Carta Blanca') and ancient ('Souvenir de la Malmaison', 'Blush Noisette', 'Antoine Ducher', 'Amadis', 'American Pillar' and 'Adélaïde d'Orléans') cultivars. Group II includes species from sect. *Rosa* (*R. rugosa*, *R. majalis*, *R. carolina* and *R. macrophylla*), *Gallicanae* (*R. gallica versicolor* L.) and *Caninae* (*R. micrantha*, *R. canina*, *R. pouzinii* and 'Alba Semi-plena'). A third, more distant, branch includes only *R. wichurana* 'Basye'. Variability within groups reveals the wide variability present in the plant material under study. Evidence of relationships for most of the accessions according to their sections were found.

DISCUSSION

Ploidy level

The number of chromosomes in all accessions evaluated in the present work (**Table 1**) is in general accordance with that found by other authors in the literature (Wylie 1954a; Darlington and Wylie 1955, cited by Gudin 2000; Krüssmann 1981; Cairns 1993). *Rosa roxburghii* Tratt. from subgen. *Plathyrhodon* and *R. stellata mirifica* Greene from subgen. *Hesperhodos* were diploids ($2n=2x=14$). Also within the large subgen. *Rosa*, only diploid species were found in sections *Banksianae*, *Bracteatae* and *Laevigatae*. On the other hand, in both sections *Caninae* and *Gallicanae* all accessions evaluated were polyploid, and both diploid and polyploids were found in sections *Rosa*, *Synstylae*, *Pimpinellifoliae* and *Indicae*. However, we found some discrepancies among the chromosome numbers of some accessions that are described in the literature. For instance, *R. foliolosa* and *R. xanthina spontanea* Rehd. used in this study were triploid accessions with 21 chromosomes and *R. brunonii* and *R. xanthina* were both tetraploid accessions with 28 chromosomes. These four species have been described previously as diploid species (Krüssmann 1981; Joly *et al*. 2006) and *R. foliolosa* was also described as a tetraploid species (Gudin 2000). All this variability in chromosome number reveals the complexity in roses. The *Rosa carolina* complex, of which *R. foliolosa* belongs, is especially confusing and highlihts the need to confirm the identity of roses in the group within worldwide rose collections using recent taxonomic information (Joly *et al.* 2006). Similarly, the *R. agrestis* Savi accession used in this study was tetraploid, whereas pentaploid or hexaploid accessions of this species have been described (Krüssmann 1981).

Tetraploids, pentaploids and hexaploids were found in sect. *Caninae*. Pentaploids have been reported to be the most common for species within this section (see review in Wissemann 2003). However, in our collection, in a total of 12 accessions evaluated, only one was pentaploid, three were tetraploids, and eight hexaploids. Hexaploid was the highest level of ploidy found in our collection. In the literature, species with the highest level of ploidy have been described in sect. *Rosa*, i.e. *R. acicularis* Lind. ($2n=8x=56$) (Krüssmann 1981; Gudin 2000).

Auto- and alloploidy inferred by the number of NORs

Reports about the physical location of 18S-25S rDNA sites on chromosomes of roses by FISH are scant. Ma *et al* (1997) published the first results in the diploid taxa *R. chinensis*, *R. odorata* (Andr.) Sweet, *R. x fortuniana* Lindl., *R. laevigata* Michx. and *R. roxburgii*. More recently Fernández-Romero *et al*. (2001) analysed both diploid (*R. sempervirens*, *R. moschata* Herrm., *R. gigantea*, *R. multi-*

flora Thunb. and *R. rugosa*) and polyploid taxa (*R. chinensis* 'Semperflorens' and *R. gallica* L.). *Rosa multiflora* (Mishima *et al*. 2002) and a pentaploid *R. canina* (Lim *et al*. 2005) have also been evaluated using both 5S and 18S-25S rDNA probes.

In this study, we show results for the first time of 11 diploid species, belonging to six sections of the subgenus *Rosa* (**Table 2**). In all sections, except for *Rosa*, a single rDNA locus per genome was detected, which is in agreement with results previously reported in other diploid species. Among the three diploid species studied in sect. *Rosa*, two hybridization signals were found in two of them (*R. paulii* Rehd. and *R. rugosa*), whereas *R. majalis* showed four hybridization sites (**Fig. 1B**), indicating that the genome of this latter species carries two NORs. These results suggest that at least two distinct genomes are present in sect. *Rosa*, one type with a single NOR per genome and another one with two NORs per genome.

Rosa is the largest section within the genus. Using different approaches, several authors suggest that sect. *Carolinae* should be included in sect. *Rosa* (=*Cinnamomeae*) (Erlanson 1934; Lewis 1957; Gudin 2000; Wissemann and Ritz 2005; Joly *et al*. 2006; Koopman *et al*. 2008). After inclusion of *Carolinae, Rosa* contains about fifty percent of all rose species and the differentiation within the section is high with considerable variability among the described species (Wissemann and Ritz 2005). Grossi *et al*. (1998) indicated that from a phylogenetic point of view *Cinnamomeae* (=*Rosa*) is the largest section and appears to be central in the evolution of the genus. Also in this section, species have been classified into five different groups according their anthocyanin constituents (Mikanagi *et al*. 2000), and a wide distribution of species has been found in a dendogram based on RAPDs markers (Atienza *et al*. 2005). Therefore, the differences in the number of NORs per genome among *Rosa* species found in the present study seems in accord with the high variability and differentiation found in this section. *Rosa carolina* and *R. foliolosa* were included in sect. *Carolinae* until the updated classification of Wissemann (2003) who included *Carolinae* in sect. *Cinnamomeae*. These two species contain two pairs of NORs per genome as well as *R. majalis* (=*R. cinnamomea plena*) supporting the inclusion of sect. *Carolinae* into sect. *Rosa*.

In polyploids derived from diploids, duplicated rDNA sites corresponding to their duplicated genomes are expected. Evidences of the conservation of 18S-5.8S-26S rDNA sites after polyploidization during evolution in *Sanguisorba* (Rosaceae) has been reported (Mishima *et al*. 2002). In roses, in the triploid accession *R. chinensis* 'Semperflorens' three hybridization sites were found on three morphologically similar chromosomes. The autotriploid nature of this accession was confirmed by meiotic chromosome pairing analysis (Fernández-Romero *et al*. 2001). Triploid *R. foliolosa* and both tetraploid *R. blanda* and *R. carolina*, from sect. *Rosa*, carried two pairs of NORs per genome. The number of NORs, intensity of hybridization signals and morphology of chromosomes carrying NORs indicated that these three species are autopolyploids and probably share the same genome as the sect. *Rosa* diploid *R. majalis*.

Triploid 'Centifolia Minor' showed four hybridization signals (**Fig. 1C**) indicating that this accession could be an allotriploid with two genomes carrying one NOR each and two NORs in the third genome. Similarly, six hybridization sites, located at terminal positions of three chromosome pairs, were observed in ancient tetraploid roses 'Rose du Roi', 'Kazanlik', 'Quatre Saisons Continue' (**Fig. 1G**) and *R. × damascena versicolor*. These observations suggest an allotetraploid origin of these cultivars containing a pair of genomes with two 18S-25S rDNA loci in addition to a pair with one 18-25S locus. We found the same results in tetraploid *R. gallica versicolor* in which analysis of meiotic chromosome pairing supports the view of an allotetraploid nature of this species (Fernández-Romero *et al*. 2001). Consequently, our results support *R. gallica* being one of the ancestors of damask roses 'Kazanlik', 'Quatre Saisons Continue' and *R. × damascena versicolor* and the Portland 'Rose du Roi' as has been previously reported (Hurst 1941; Wylie 1954b; Iwata *et al*. 2000). In order to confirm the autopolyploid or allopolyploid nature of all these accessions, meiotic chromosome pairing should be analysed as previously carried out in both autotriploid *R. chinensis* 'Semperflorens' and allotetraploid *R. gallica versicolor* (Fernández-Romero *et al*. 2001).

One NOR per genome has been found in the triploid noissette rose 'Champneys' Pink Cluster', tetraploids *R. brunonii* and *R. xanthina*, pentaploid *R. micrantha* and hexaploids *R. canina*, *R. corymbifera*, and *R. pouzinii*. Hurst (1941) and Wylie (1954b) proposed that the old rose 'Champneys' Pink Cluster' was derived from a cross between *R. chinensis* and *R. moschata*. FISH analysis with an rDNA probe showed one pair of NORs in these three accessions in agreement with the proposed origin. With regard to the species under study belonging to sect. *Caninae* (**Table 2**), all were also found to have one NOR per genome as has been found previously by Lim *et al*. (2005) in pentaploid *R. canina*.

The genomes in *Rosa*

Hurst in 1925 (cited by Krüssmann 1981) proposed that in the genus *Rosa* there are five distinct genomes (A, B, C, D and E) each one with seven chromosomes, according to some determining morphological characters. Representative species belonging to these genomes are *R. sempervirens* and *R. chinensis* (genome A) from sects. *Synstylae* and *Indicae*, respectively; *R. sericea* Lindl. (genome B) from sect. *Pimpinellifoliae*; and *R. rugosa* (genome C), *R. majalis*, *R. carolina*, *R. foliolosa* (genome D) and *R. macrophylla* (genome E) from sect. *Rosa*. In this study, representative species from all these genomes, except genome E, have been analysed by the number of rDNA sites. We have presented the results for 34 accessions of *Rosa* covering eight of the nine sections and ranging from diploid to hexaploid. So, we can expect that these five genomes should be represented within the taxa analysed. According to our results, all diploid species studied carried a single NOR per genome, except the representative species for genome D with two NORs per genome.

Assuming that genome D is the only one which carries two NORs in the genus *Rosa*, and taking into account the number of rDNA sites found on polyploid species or cultivars in the present study, we can suggest a tentative genomic constitution of some of the polyploid species analysed. Thus, *R. foliolosa* (3*x*) with six hybridization sites (**Fig. 1E**) should be written, DDD. Similarly, *R. blanda* (4*x*) and *R. carolina* (4*x*) both showing eight rDNA hybridization sites (**Fig. 1H**) should be DDDD.

Based on intra-individual variation of the rDNA marker ITS1, the genomic constitution of *R. gallica* has been proposed as AABB by Wissemann (1999). But the presence of a pair of genomes with two 18S-25S rDNA loci in addition to a pair with one 18-25S locus found by Fernández-Romero *et al*. (2001) in *R. gallica versicolor* (2*n*=4*x*=28) suggests that the genomic constitution of this species should be written AADD. On the other hand, according to the number of NORs sites found in allotriploid 'Centifolia Minor' (4 signals, **Fig. 1C**) and the allotetraploids 'Rose du Roi', 'Kazanlik', 'Quatre Saisons Continue' (**Fig. 1G**) and *R. × damascena versicolor* (all of them showing 6 signals), these cultivars should contain both A and D genomes in their constitutions. The presence of the D genome from sect. *Rosa* (= *Cinnamomeae*) in all these accessions belonging to sects. *Gallicanae* and *Indicae* suggest a high crossability among these sections. This hypothesis is supported by the fact that sect. *Rosa* is distributed across the whole geographic range of the subgenus *Rosa* and shows the widest levels of crossability with other sections, as already proposed by Atienza *et al*. (2005). On the other hand, *R. moschata* (genome A) and *R. gallica* have been identified by sequencing the ITS of rDNA as parental species of the

original hybridization that contributed to forming three of the oldest Damask cultivars ('Kazanlik', 'Quatre Saisons Continue' and *R.* × *damascena versicolor*) (Iwata *et al*. 2000).

Molecular evidence presented by analysis of nucleolar ribosomal DNA data (ITS-1) (Ritz *et al*. 2005) suggests that dogroses are a polyploid complex resulting from multiple hybridization events. *Caninae* is characterised by a special type of meiosis in which only seven chromosomes (from 7 bivalents) are transmitted through the pollen, whereas 21, 28 or 35 chromosomes (from 7 bivalents and 14, 21 or 28 univalents) come from the egg cells (Täckholm 1922; Hurst 1931). We found five and six NORs signals in one pentaploid and three hexaploid *Caninae* species, respectively (**Table 2**), corresponding to a single NOR per genome and hence, these species should not contain a D genome. It is considered that tetraploid, pentaploid and hexaploid *Caninae* species contain three, four or five different genomes, respectively (Lim *et al*. 2005). Assuming that there are five genomes and that the D genome is not present in dogroses analysed in this study, a hexaploid *Caninae* genotype with a maximum of four genomes means that either, one genome is triplicated or two genomes are duplicated. "Internal autotriploidy" was postulated by Blackhurst (1948) for some *Caninae* species, and a unique genome configuration in polyploid dogroses has been proposed using microsatellite DNA markers (Nybom *et al*. 2006).

Both polyploidization from diploid to tetraploid as well as formation of allopolyploids species through the formation of interspecific hybrids have occurred in the genus *Rosa*. The production of 2*n* gametes in plants is considered to be the dominant process involved in the origin of polyploid plants (Bretagnolle and Thompson 1995). The production of unreduced pollen cells has been reported in dihaploid *R. hybrida* L. genotypes (El Mokadem *et al*. 2002). Unreduced gametes (both male and female) producing hexaploid seedlings have also been reported in pentaploid *Caninae* species (Nybom *et al*. 2006). This explains that the number of genomes could be maintained in higher ploidy levels; thus we propose that genomic constitution of hexaploid *Caninae* could be explained with a maximum of four genomes. However, further work is needed to clarify the genomic constitution of the complex *Caninae* section and to elucidate the nature of polyploidy in roses. Particularly, *R. x alba* would be a valuable rose to determine the number of NORs in order to confirm its possible intersectional origin with a *Gallicanae* species (Hurst 1941). It could be expected more than six signals if genome D from *Gallicanae* was included in *R. x alba*. Analysis of 5S rDNA sites, together with meiotic pairing studies, could provide valuable information about relationships among species.

Cultivar identification

Construction of databases containing molecular profiles of cultivars have been a major task in recent years. Guidelines for DNA profiling have been proposed by UPOV (International Union for the Protection of New Varieties in Plants) with the aim of generating high quality molecular data for cultivar identification. Proposed criteria are: (a) useful level of polymorphism (b) repeatability within and reproducibility between laboratories (c) known distribution of the markers through the genome and (d) avoidance of markers with null alleles (http://www.upov.int).

The STMS used in this research were developed by Esselink *et al*. (2003). The same authors employed these markers to identify hybrid tea (*R. hybrida*) and rootstock genotypes and could demonstrate their high discrimination power. In the present study, we characterised 29 accessions with very different origins including ancient and modern cultivars as well as wild species. The high level of polymorphism found in our material (PIC >0.5) agrees with the first recommended criterion for the selection of markers proposed by UPOV. Our results also showed highly reproducible patterns in different replications and scorability, similar to that obtained by Esselink *et al*. (2003). Hence, the second general criterion was also met.

Seven of the markers analysed in this study were located in their respective linkage groups by Debener *et al*. (2001). Two of them (RhD206 and RhEO506) were in linkage group (lg) 2, three (RhAB13, RhAB40 and RhJ404) in lg4 and one in lg3 (RhI402) and lg6 (RhAB22). We have also located RhB03 in lg2 (unpublished data). Thus, at present the localizations of 8 markers distributed along four different linkage groups are known, satisfying the third criterion concerning the genome distribution of the markers.

The presence of null alleles is not desirable (the fourth UPOV marker selection criterion). In the present study, the number of alleles could not be estimated since the roses under study included different ploidy levels. Consequently, heterozygosity values were only evaluated for diploid species. Differences between the observed and expected heterozygosity suggest the presence of null alleles (**Table 4**). However, we should take into account that only 9 diploid species have been included in this study; thus further studies using more species and genotypes within a species are necessary to confirm these results.

In **Table 5**, an example of allelic patterns obtained with the most informative markers is presented. It should be noted that because small size differences might be observed in different electrophoretic runs, the final size for a given allele derives from multiple data analyses. We support the suggestions of Esselink *et al*. (2003) that an agreement should be reached to propose reference rose cultivar or species genotypes in order to produce informative allelic ladders. Reference samples have been assigned for species with a higher number of studies in cultivar identification such as olives (Noormahammadi *et al*. 2007) and grapes (This *et al*. 2004). In this way, comparison of fingerprinting obtained in different laboratories would be facilitated.

The discriminatory power of STMS studied was revealed in the UPGMA dendrogram (**Fig. 2**). Only the Pretty® family collection of cut rose cultivars could not be differentiated. Roses analysed included a wide pool of genotypes, and therefore a high degree of variation was obtained. In general, boostrap values were low separating branches within the area of low similarity index. Accessions within sect. *Rosa* show a high level of variability in agreement with the rDNA data obtained in this section. Clear association between *R. rugosa* and *R. majalis* indicate a close relationship between these two species.

The most informative marker was RhEO506 with 81.8% unique allelic patterns and 22 allelic patterns (**Table 3**). This marker combined with any other one could identify all genotypes, except cultivars within the Pretty® family collection. Esselink *et al*. (2003) also considered this marker one of the most informative to fingerprint both hybrid tea and rootstock genotypes.

The proposal prepared by scientists from the Netherlands of the biochemical and molecular techniques group from UPOV in 2002 (proposal 5, TC/38/14-CAJ/45/5) indicates that two sets of seven STMS markers should be used to be able to distinguish a new rose cultivar. In this study, it was not possible to distinguish cultivars within the Pretty® family of cut roses by using 17 STMS markers. We can suggest that those cultivars originated by mutations tracing back to an original ortet, so they could be considered Essentially Derived Varieties. Polymorphic markers that distinguish between sports and an original genotype are very rare to find (Debener *et al*. 2000; Esselink *et al*. 2003). Therefore, current molecular genetics techniques are not reliable tools for this purpose. At present, these cultivars could only be distinguished by phenotypic analysis of flower colour.

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