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Using RAPDs to study phylogenetic relationships in *Rosa*

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Abstract Nineteen species of rose (*Rosa* sp.) were analysed using Random Amplified Polymorphic DNA markers (RAPD). Each 10-base-long arbitrary primer produced a specific DNA banding pattern that grouped plants belonging to the same species and botanical sections as predicted from their genetic background. One hundred and seventy-five amplification products were examined by cluster analysis to assess the genetic relationships among species and their genetic distances. All of the accessions belonging to 1 species grouped together before branching to other species. Dendrograms constructed for intra- and inter-specific studies showed a good correlation with previous classifications by different authors based on morphological and cariological studies. Our results show that the RAPD technique is a sensitive and precise tool for genomic analysis in rose, being useful in assigning unclassified accessions to specific taxonomic groups or else allowing accessions classified by traditional criteria to be re-classified.

Key words *Rosa* sp. · RAPDs markers · Polymerase chain reaction · Phylogenetic relationships

Introduction

Taxonomy or genetic relationship studies of the genus *Rosa* are complicated due to a large number of spontaneous as well as man-made crosses. Many of the characters used in taxonomic determinations are difficult to evaluate since they are subject to human interpretation. Moreover, many morphological traits belonging to all developmental stages are required in order to assign an individual to a specific taxon. Thus, several wild species (as *canina, pouzinii, squarrosa, corymbifera* etc.) are usually referred to as the "canina group" or "canina complex" as their morphological features suggest that they are close to each other from an evolutionary point of view (Klastersky 1968; Facsar et al. 1991). This fact is also supported by cariological analysis and artificial crosses (Zielinski 1985). The same situation could be inferred with other sections or subsections of the genus, such as the so-called *tomentosa* and *rubiginosa* (Klastersky, 1968) or *micrantha* (Facsar et al. 1991) groups.

Modern roses derive from complex hybridizations that have been performed by breeders since the end of the 18th century. Comprehensive reviews of the history and chromosome structure of the modern artificially propagated roses are given by Hurst (1941) and Wylie (1954 a,b). Our modern varieties contain, at varying extents, genomic portions of some basic species (especially *gallica, multiflora*). This extreme genetic complexity is not well-recorded in most cases as breeders did not keep or did not publish the origin of the parents they chose for their crossing schemes.

Apart from the problems mentioned above, there are two main fields of disagreement among specialists on roses. (1) The names given to the Chinese roses introduced at the end of the 18th and the beginning of the 19th centuries. French rosalists usually identify them as *R. indica* Thory (including several botanical varieties such as *vulgaris, fragrans, sulphurea*, etc.), while British scientists group most of them as *R. chinensis* Jacq. and some cultivars *R.* × *odorata* (Andr.) Sw. A further basis for confusion derives from the several homonyms and synonyms involving not only these basis specific names but also *semperflorens*. (2) The taxonomic treatment of man-obtained roses, which are recognized by some authors as true new species (*R.* × *noisettiana, R.* × *kordesi* etc), whereas others favour the idea of assigning only commercial names while grouping them into a single species, usually *R.* × *hybrida*.

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It is obvious that rapid and reliable methods for clarifying the taxonomic status of any accession are of outmost importance, from both a theoretical and a practical point of view. Recent technological advances in DNA analysis, such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPDs) have greatly increased our ability to understand the genetic relationships in a wide range of plant species including potato (Debener et al. 1990), tomato (Miller and Tanksley 1990), *Rubus* (Waugh et al. 1990), papaya (Stiles et al. 1993), *Musa* (Bhat and Jarret 1995) and others. The molecular approach is especially informative because the markers are phenotypically neutral and not subject to environmental effects. In addition, a large number of markers are available. In previous reports both isozymes and RAPD markers (Williams et al. 1990; Welsh et al. 1991; Welsh and McClelland 1991) have been used to identify modern cultivars of roses for registration and protection purposes (Torres et al. 1993; Torres et al. 1995; Millán et al. 1995). While isozymes proved to be less efficient in detecting genetic variability, the RAPD technique provided an extensive amount of variation leading to clear cultivar identification.

The objectives of the investigation presented here were to estimate the discriminatory power of RAPD markers in describing the variation in both wild and cultivated forms of *Rosa* and to use this new tool in taxonomic, systematic and evolutionary studies of the genus. We have used the RAPD technique to detect intra-specific variation among 5 wild species of *Rosa* and inter-specific variation and genetic relationships among 17 old cultivated species.

Materials and methods

Plant material

Seventeen accessions of 4 wild rose species collected in Córdoba (Spain) were used to study intra-specific relationships (Fig. 1): *Rosa canina* (plants 1–4), *R. corymbifera* (plants 5-10), *R. micrantha* (plants 11–15) and *R. pouzinii* (plants 16, 17).

For inter-specific analysis, 17 old cultivars (20 accessions) of roses belonging to seven botanical sections within the subgenus Eurosa were kindly provided by the Royal Botanical Garden of Madrid (Fig. 2): section Pimpinellifolia: *Rosa foetida* (6), *Rosa* × *xanthina* (17); section Banksianae: *Rosa banksiae* (1) and *R. cymosa* (18); section Gallicanae: *R. gallica* (7) and *Rosa* × *centifolia* (13); Gallicanae × Caninae: *Rosa* × *alba* (10, 11); section Caninae: *R. canina* (2,3) and *R. micrantha* (19); section Cassiorhodon: *Rosa* × *paulii* (16); section Synstilae: *R. sempervirens* (8), *R. multiflora* (20) and *R. Wichuriana* (9); section Chinensis: *R. chinensis* (4,5), *Rosa* × *noisettiana* (14), *Rosa* × *odorata* (15) and *R. borboniana* (12).

In the present work we have adopted the nomenclature of the Botanical Garden of Madrid, which is based on the denominations given by Bean (1970) and Klastersky (1968).

Analytical procedures

Young leaf tissue of mature plants was excised, immediately frozen in liquid nitrogen and the leaves stored at −80 °C. DNA was extracted using the method described by Torres et al. (1993). Coprecipitated RNA was eliminated by adding 0.7 units of RNAase per sample. The DNA was dissolved in TE, and the final concentration was determined by agarose gel electrophoresis using known concentrations of λ uncut DNA as standard.

Ten primers for intra-specific and 16 for inter-specific analysis, each 10 bases in length (Operon Technologies), were chosen (Table 1). The selection was made at random from a pool of primers that gave reasonable numbers of strong amplification products using the 4 rose species as a template. Polymerase chain reaction (PCR) was carried out in 25-µl reactions containing 20–40 ng of plant genomic DNA, buffer (50 m*M* KCl, 10 m*M* Tris-HCl, pH 9.0, 0.1% Triton X-100), 1.5 m*M* $MgCl_2$, 100 m*M* of each dNTP, 2–4 m*M* of primer and 0.6 units of *Taq* DNA polymerase (Promega). Amplification was done in a Perkin elmer Cetus DNA Thermal Cycler programmed for 40 cycles with the following temperature profile: 1 min at 94 °C, 2 min at 35 °C, 2 min at 72 °C. Cycling was concluded with a final extension at 72 °C for 8 min. Amplification products were electrophoresed in 1% agarose, 1% Nu-Sieve agarose, 1 × TBE gels, visualized by ethidium bromide staining and photographed under UV light. Controls lacking template DNA were included on each primer reaction mix.

Data analysis

All the plants were scored for presence of absence of RAPD fragments, and the data was entered into a binary data matrix as discrete variables ("1" for presence and "0" for absence of a homologous band). Jaccard's coefficient of similarity was calculated, and the species were grouped by cluster analysis using the unweighted pair-

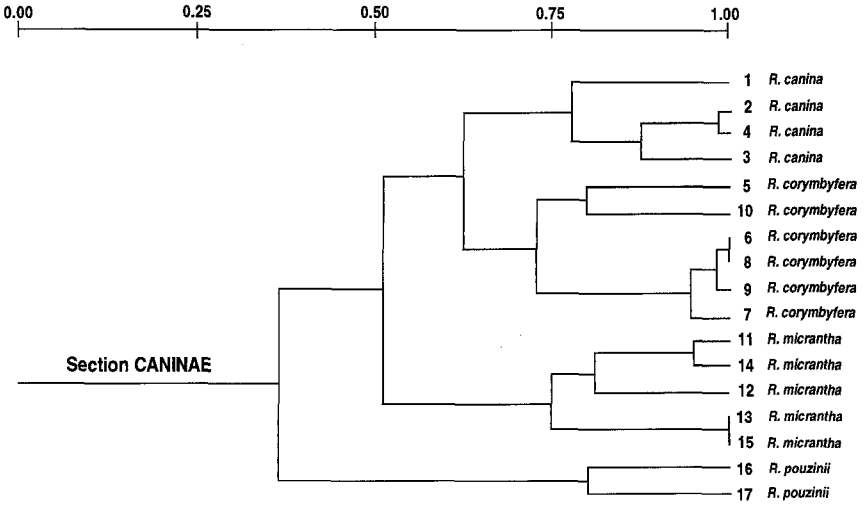

Fig. 1 Phenogram derived from analysis of 17 accessions of 4 wild *Rosa* species using 10 random primers

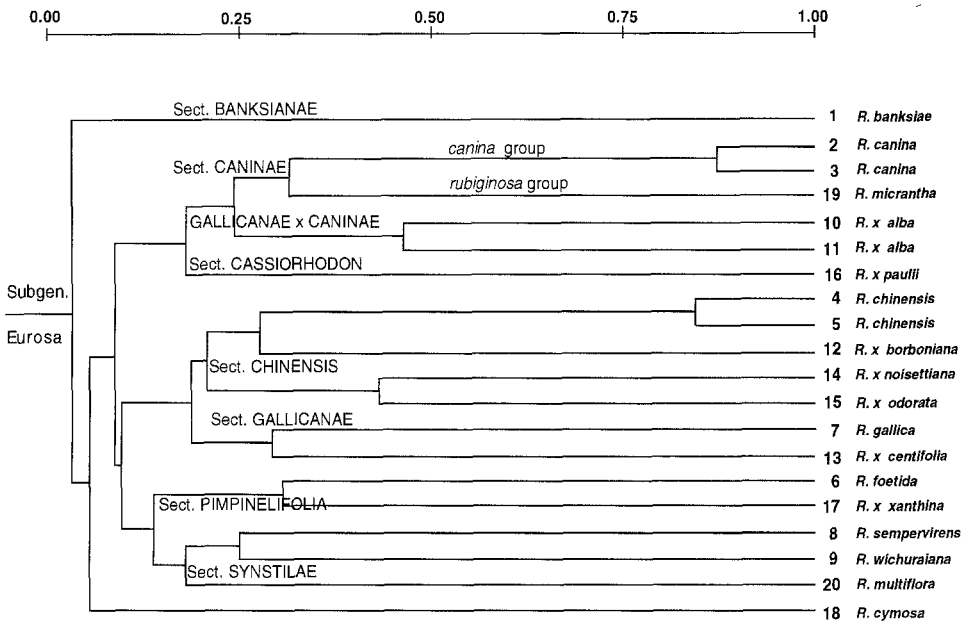

Fig. 2 Phenogram derived from analysis of 20 accessions of 17 *Rosa* species using 10 random primers

Table 1 Sequence of the 16 oligonucleotide primers used for intra- and inter-specific analysis (primer ID following OPERON's recommendations)

Primer ID	Sequence	Number of fragments analysed[a]	
	(5′-3′)	(A)	(B)
OPA-01[b]	CAGGCCCTTC	8	8
OPA-02[b]	TGCCGAGCTG	9	8
OPA-03[b]	AGTCAGCCAC	5	4
OPA-05[b]	AGGGGTCTTG	8	3
OPA-07[b]	GAAACGGGTG	13	6
OPA-08[b]	GTGACGTAGG	5	4
OPA-09[b]	GGGTAACGCC	7	8
OPA-10[b]	GTGATCGCAG	4	7
OPA-11	CAATCGCCGT	–	4
OPA-12[b]	TCGGCGATAG	7	9
OPA-13[b]	CAGCACCCAC	9	5
OPA-15	TTCCGAACCC	–	5
OPB-03	CATCCCCCTG	–	7
OPC-14	TGCGTGCTTG	–	5
OPD-20	ACCCGGTCAC	–	9
OPI-16	TCTCCGCCCT	–	8
Total		75	100

[a] (A), Intra-specific analysis; (B), inter-specific analysis.
[b] Used only for intra-specific analysis

group (UPGMA) method. Phenograms were produced as described by Sneath and Sokal (1973) using the NTSYS-pc package for numerical taxonomy and multivariate analysis systems (Rohlf 1989).

Results

Intra-specific variation

Most of the accessions belonging to the same species produced similar banding patterns. Nevertheless, with only 10 primers and 75 PCR amplification products the technique allowed us to distinguish a particular species or an accession of the same species from the rest. Although the number of scorable polymorphisms yielded by the 10 primers was large (93), only the most consistently reproducible bands from repeated PCR amplifications were included in the statistical analyses. Among the 75 fragments recorded, 12 (17% were common to all accessions, 31 (41%) were unique (particular to a species) and 32 (42%) were phenetically informative since an individual fragment was shared by at least 2 accessions but not by all of them. Thus, accessions known to belong to the same species grouped to a single branch of the tree, as expected. Coefficients of similarity ranged from about 70% to 90%, grouping precisely those species belonging to section caninae (*canina*, *corymbifera*, *micrantha* and *pouzinii*) (Fig. 1).

Inter-specific variation

Mutivariate analysis of 16 primers amplifying a total of 100 reproducible fragments grouped plants belonging to the same species and botanical sections as predicted from their genetic background. PCR amplification of total genomic DNA from 17 old cultivars (20 accessions) of roses yielded more than 137 scorable polymorphisms, although only the most reproducible bands (100) were included in the statistical analysis. Figure 2 clearly shows that the 2 accessions of both *R. canina* and *R. chinensis* group together with a similarity coefficient of 85–90%, while in *R. alba* accessions the similarity coefficient detected was significantly smaller (50%). The rest of the species belonging to the same section were precisely grouped. Inter-specific analysis revealed a high degree of genetic diversity; 31 (31%) of the recorded bands were

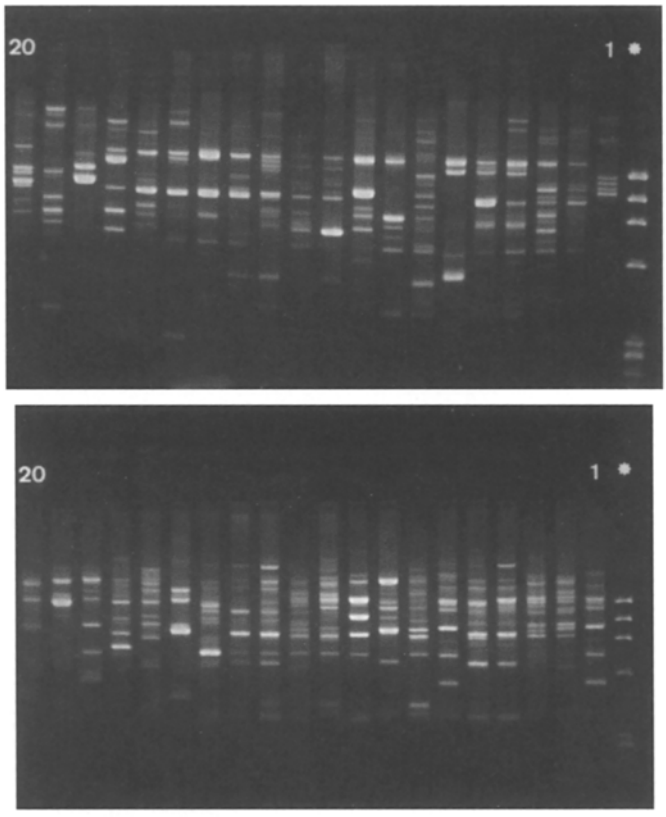

Fig. 3 RAPD polymorphisms in 20 *Rosa* accessions using primers OPA-01 (*upper*) and OPD-20 (*lower*). *Lane(*)* Size marker derived from $\phi \times 174$/*Hae*III digest; *lanes 1–20* accessions described in Materials and methods

specific to each section, and 10 of them (10%) were specific to a species (Fig. 3). Since the species included in this study have very different geographical and taxonomic origins, the low values of the similarity coefficient (less than 50%) were expected.

Discussion

Figures 1 and 2 show an almost perfect grouping of species based on their botanical sections. Even in the taxonomically complex section Caninae DC (Zielinsky 1985) all of the species appear to be related. Unexpectedly, *R. micrantha*, which belongs to group *rubiginosa* within section *Caninae* (Fig. 2), seems to be closer to *canina* and *corymbifera* than *pouzinii*, the latter three belonging to group *canina* (Klastersky 1968). Supporting our results, however, is the fact that crosses between *R. rubiginosa* and *R. canina* were very fertile [8–19 (with an average of 15.1) seeds per hip, versus 19 and 16 for the parents, respectively] and the meiosis of the F_1 showed good pairing (Gustafsson 1944). As mentioned earlier, the separation of these groups is based on morphological characters only, and this has always presented huge problems to taxonomists (Gustafsson 1944; Facsar et al. 1991). Thus, the use of molecular markers can help in establishing the limits among these traditional groups on a more objective basis.

R. × *alba*, a presumably spontaneous cross between *canina* and *gallica* species (Hurst 1941; Darlington, 1963) was also associated with section *Caninae*. The cultivated *R.* × *alba* is nowadays a hexaploid, and two hypotheses have been suggested with respect to its origin: (1) a pentaploid *canina* as mother plant that contributes four genomes (Gustafsson 1944; Hurst 1941) and a tetraploid *gallica* that yields two genomes (Wylie 1954 b); (2) alternatively, *alba* may have arisen from a hexaploid *canina* mother (Gustafsson 1944) providing five genomes and a unknown diploid parental. In both cases there would be a greater proportion of mother genomes than father genomes in *alba*, and its position in Fig. 2 corroborates this fact. It is worth mentioning that the mother plant is a *canina sensu latissimo*, i.e., it may belong to any species of this section. This fact could justify the relatively low coefficient of similarity (about 50%) shared by the 2 *R.* × *alba* accessions. On the contrary, the ancestry of *R. arvensis* in *alba*, a member of the section *Synstilae* DC and also a hypothetical parent of *alba* (Klastersky 1968), does not agree with our results. However, further analyses are necessary to confirm this hypothesis.

Section *Cassiorhodon* Dumort (syn. *Cinnamomeae* Crépin) was represented by only 1 species, *R.* × *paulii*; (*R. rugosa* was not included in the analysis). These are diploid species (2n = 14), but *rugosa* at least can cross with both *rubiginosa* and *canina* (Gustafsson 1944). Although the fertility is very low (2–8 seeds per hip versus 15–40 for the parents) and the meiosis of F_1 was observed to deviate from that of *canina*, a certain similarity between these species is apparent. Gustafsson (1944) proposed the existence of a genome common to both *canina* (aa acd) and *rugosa* (*cc*), enough at least to explain the position found by us of *R.* × *paulii* being closer to sect. *Caninae* than to any other section. This is also in agreement with the results obtained at the morphological level by Zielinsky (1985).

Both *R. chinensis* accessions lie very close to each other (Fig. 2). Bourbon Rose is known to arise from spontaneous crosses between Chinese roses (the "Parson's Pink China" according to Wylie 1954a) and autumn damask roses, i.e. *R. damascena* var 'semperflorens' (Loisel.) Rowley, both of which were used as fences in the Réunion (then Bourbon) Island (Bean 1970; Hurst 1941). Noisette roses have a similar structure, with *R. moschata* substituting for the damask rose, but they come from a conscious crossing performed at the beginning of the 19th century. *R.* × *odorata* (Andr.) is considered by Hurst (1941) to be derived from crosses between *R. chinensis* and *R. gigantea*. Whether the Noisettes have also *odorata* ascendency cannot yet be concluded, but the grouping together of all the species belonging to the sect. *Chinensis* reflects a strong similarity among them. Unfortunately, neither the damask roses nor *moschata* could be analysed.

The closest neighbour to the sect. *Chinenses* is sect. *Rosa* (= *Gallicanae*) (Fig. 2). This could explain why specimens from both sections were crossed as early as the end of the 18th century, that is, following the introduction of the chinese roses into Europe (Hurst 1941; Wylie 1954 a,b; Bean 1970). Although it was not an easy task to obtain fertile descendants, their progenies have been the origin of all modern roses. *R.* × *centifolia* is supposed to have been selected from *gallica* itself (Darlington 1973), from natural crosses between autumn damasks and *alba* (both of them descending from *gallica*) (Krüssman 1986) or from even more complex crosses involving *gallica*, *phoenicia*, *moschata* and *canina* (Hurst 1941). In any case, its position as the closest one to *R. gallica* is consistent with the previously mentioned hypotheses. The RAPD technique may be able to solve the problem of its origin.

Two species of sect. *Pimpinellifolia* group together, as do 3 of sect. *Synstilae* DC. *R. foetida*, *R. wichuraiana* and *R. multiflora*, have been used since the end of the past century in breeding new roses, but their progenies were not analysed in the present study.

Finally, *R. banksiae* and *R. cymosa* were placed, independently, very distantly from the rest of the species (Fig. 2). The position of *R. cymosa*, which is considered to be a relative of *banksiae* (Bean 1970), did not fit the botanical classification of the genus. Whether this position is an artifact or, whether both species are more alike from a morphological point of view than from a molecular one cannot be clarified from our results.

It is obviously necessary to enlarge both the number of species and the number modern cultivate groups to obtain a deeper insight within the genus *Rosa*. Nevertheless, the present study was not meant to be an exhaustive taxonomic analysis but only a preliminary exploration of the use of RAPDs in the genus *Rosa*. Roses were chosen because of their extreme variability, their genetic complexity, especially in modern roses, and their relatively well-known recent history. On the whole, the species' relationships based on our molecular data concur with the classical taxonomic groupings and, in some cases, even help to sharpen the focus on the problem of ancestries. Our results suggest that RAPD technology is a reliable, rapid and sensitive technique by which to estimate relationships between closely and more distantly related species, sections and groups. This method can also help to find natural hybrids in complex groups such as the *canina* one (a fact that can ease the taxonomic treatments), to adscribe genomic formulae to different species and to suggest new wild species as breeding materials.

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