Rose Germplasm Analysis with RAPD Markers

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Abstract. The genus *Rosa* consists of more than 100 species classified into four subgenera, *Eurosa*, *Platyrhodon*, *Hesperhodos*, and *Hulthemia*, and distributed widely throughout the northern hemisphere. The subgenus *Eurosa* includes 11 sections. The other subgenera are monotypic. One hundred and nineteen accessions and 213 markers of 36 rose species that include eight sections of the subgenus *Eurosa* and one species each from the subgenera *Hesperhodos* and *Platyrhodon* were used to calculate a similarity matrix, which was clustered with the unweighted pair group method using arithmetic means (UPGMA). The RAPD markers distinguished between all the rose accessions, and species grouped into their respective sections. Therefore, classification of *Rosa* using RAPD data generally supports traditional classification. The Asian rose sections (*Laevigatae*, *Banksianae*, *Bracteatae*, *Pimpinellifoliae*, *Chinenses*, and *Synstylae*) were consistently separated from the primarily North American sections (*Cassiorhodon* and *Carolinae*). The *Cassiorhodon* and *Carolinae* sections were grouped together with the subgenera *Hesperhodos* and *Platyrhodon*. Both subgenera separated out at the same level as sections within the subgenus *Eurosa*, suggesting that they are more appropriately classified as sections within the subgenus *Eurosa*. Sections *Cassiorhodon* and *Carolinae* overlapped, and are probably best grouped as one section as previously suggested.

Roses are one of the most important flower and nursery crops throughout the world. About 60 million rose plants for garden use are propagated in the United States (Streeper, 1990) and \$300 million is generated by the cut flower market annually (U.S. International Trade Commission, 1995). The demand for greater disease resistance has led to the use of more wild rose species in breeding and the development of unique interspecific derived germplasm, such as amphidiploids (Byrne et al., 1996). Unfortunately, this work is limited by the dearth of information available about the genetic relationships among rose species.

The genus *Rosa* consists of more than 100 species, the exact number varying with the classification system (Allen, 1973; Rehder, 1940). Their wide geographic distribution, polyploidy, and frequent interspecific hybridization, together with human involvement, makes this genus diverse and difficult to classify. Traditional identification and classification of plants are based on morphological characteristics (Allen, 1969, 1973; Bean, 1970;

Erlanson, 1934; Flory, 1950; Lewis, 1957a, 1957b, 1958, 1970; Rehder, 1940). Because each species of the genus *Rosa* has a wide and overlapping range of morphological variations that are influenced by environmental conditions, classification based on morphology alone is not adequate (Lewis, 1957b). Chemotaxonomic studies of roses (Mikanagi et al., 1993, 1994; Okuda et al., 1992; Raymond et al., 1995) based on the wide range of variant polyphenolic compounds have been reported. Isozyme markers have also been used for rose identification (Kim and Byrne, 1996; Kuhns and Fretz, 1978a, 1978b; Lee and Kim, 1982; Walker and Werner, 1997; Yoneda et al., 1993) and classification (Kim, 1994; Kim and Byrne, 1994). However, the small number of consistently resolvable loci (Kim, 1994; Kim and Byrne, 1994) limits the utility of isozyme markers. Direct DNA-based diagnostic assays are considered powerful and reliable tools for genetic analysis because the number of scorable loci is greater, and expression is similar in all tissues (Hubbard et al., 1992; Torres et al., 1993). DNA markers, such as restriction fragment length polymorphisms (RFLPs) (Hubbard et al., 1992), random amplified polymorphic DNA (RAPD) (Gallego and Martinez, 1996; Torres et al., 1993; Walker and Werner, 1997), and mini- and microsatellite probes (Vainstein and Ben-Meir, 1994) have been used for rose identification, but only recently has preliminary taxonomic research been done with RAPD analysis (Debener et al., 1996; Millan et al., 1996).

Random amplified polymorphic DNA

(RAPD) markers (Williams et al., 1990) have been widely used for determining variety and parentage identification, construction of high-density genetic maps, targeted mapping for specific traits with bulk segregant analysis or near-isogenic lines, and studying genetic diversity and phylogenetic relationships (He et al., 1992; Hu and Quiros, 1991; Kresovich et al., 1992; Tingey et al., 1993; Welsh et al., 1991). This method uses genomic DNA as a template, is rapid, does not employ radioisotopes, and only requires nanograms of DNA per reaction (Williams et al., 1990).

The objective of this study was to use RAPD markers to classify 36 rose species using a phenetic analysis for interpretation.

Materials and Methods

Plant materials. One hundred and nineteen rose genotypes representing 36 species were surveyed. One to 11 species (mainly diploid) were selected to represent eight of 11 sections within the subgenus *Eurosa* and one species each from the subgenera *Platyrhodon* and *Hesperhodos* (Table 1). Young leaves were collected from the greenhouse, screenhouse, and field, put into labeled envelopes, and stored in an ice chest for transport to the laboratory. In the laboratory the leaves were stored at –20 °C in a freezer until their DNA was extracted.

DNA isolation. A miniprep protocol utilizing a cationic hexadecyl trimethyl ammonium bromide (CTAB) method modified from Doyle and Doyle (1987) and Pateron et al. (1993) was used. Modifications were designed to counter the high level of secondary compounds found in rose leaves. These compounds degrade DNA, and inhibit subsequent enzyme digests and PCR reactions. The modifications included the use of 2-mercaptoethanol as an antioxidant, and further purification through phenol extraction and gel filtration.

Young leaf tissue (50–70 mg) was ground with liquid nitrogen in a 1.5-mL microfuge tube. The powder was then mixed with 1 mL 4X CTAB solution and 2.5 µL 2-mercaptoethanol. The homogenate was incubated in a 65 °C water bath for 1–2 h with periodic gentle vortexing, and the DNA was extracted twice with 24 chloroform : 1 isoamyl alcohol (CIA) and twice with 25 phenol : 24 chloroform : 1 isoamyl alcohol. The final pellet was dissolved in 150 µL TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), and further purified by gel filtration with 5% Sephadex G-50 gel column constructed from a 1.5-mL microcentrifuge tube. DNA concentration was determined by 0.8% agarose gel electrophoresis in TE buffer (Sambrook et al., 1989) and comparison of band intensities with lambda DNA standards. All DNA samples were diluted to 0.25 ng·µL^{–1} before use.

RAPD assay. The ten 10-base-long arbitrary primers (Operon Technologies, Alameda, Calif.) that gave the most reproducible and polymorphic patterns were selected from 80 primers (Kit E, F, G, H). Those used were E14, E19, F06, F14, G11, G19, H06, H12, H15, and H19. Amplification reactions were performed

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Table 1. Rose materials used in RAPD study.

Subgenus	Section ^z	Rosa species	Number of accessions
<i>Eurosa</i> L.	<i>Chinenses</i> Sér.	<i>R. chinensis</i> Jacq.	8
		<i>R. ×odorata</i> (Andr.) Sweet.	1
	<i>Banksianae</i> Lindl.	<i>R. banksiae</i> Ait.	9
		<i>R. ×fortuniana</i> Lindl.	1
	<i>Laevigatae</i> Thory.	<i>R. laevigata</i> Michx.	3
	<i>Bracteatae</i> Thory.	<i>R. bracteata</i> Wendl.	5
		<i>R. clinophylla</i> Thory	3
	<i>Synstylae</i> DC	<i>R. brunonii</i> Lindl.	5
		<i>R. filipes</i> Rehd. & Wils.	1
		<i>R. gentiana</i> Rehd. & Wils.	1
		<i>R. luciae</i> Franch. & Rochebr.	2
		<i>R. moschata</i> Herrm.	5
		<i>R. mulliganii</i> Bouleng.	2
		<i>R. multiflora</i> Thunb.	15
		<i>R. setigera</i> Michx.	6
		<i>R. wichuraiana</i> Crép.	5
		<i>R. hugonis</i> Hemsl. & Wils.	2
		<i>R. omeiensis</i> Rolfe	3
		<i>R. primula</i> Bouleng	5
		<i>R. xanthina</i> Lindl.	1
		<i>R. sericea</i> Lindl.	3
	<i>Cassiorhodon</i> Dumort (<i>Cinnamomeae</i> DC)	<i>R. arkansana</i> Porter. ^y	1
		<i>R. blanda</i> Ait.	2
		<i>R. californica</i> Cham. & Schech ^y	2
		<i>R. majalis</i> Herrm.	2
		<i>R. ×paulii</i> Rehd.	1
		<i>R. rugosa</i> Thunb.	19
		<i>R. woodsii</i> Lindl.	4
		<i>R. forrestiana</i> Bouleng.	1
		<i>R. davurica</i> Pallas	1
		<i>R. macrophylla</i> Lindl.	1
	<i>Carolinae</i> Crép. ^y	<i>R. carolina</i> L.	1
		<i>R. foliolosa</i> Torr. & Gray	4
		<i>R. nitida</i> Willd.	1
		<i>R. roxburghii</i> Tratt.	9
<i>Platyrhodon</i> Rehd.			
<i>Hesperhodon</i>			
Cockerell ex Rehder		<i>R. minutifolia</i> Engelm.	3

^zTaxonomic treatment according to *Trees and Shrubs Hardy in British Isles* (Bean, 1970). The plants used are mainly diploids.

^yTetraploids.

in volumes of 12.5 µL containing 10 mM Tris-HCl (pH 8.3) and 50 mM KCl, 3 mM MgCl₂, 0.1 mM each of dATP, dCTP, dGTP, and dTTP (Promega), 0.1% Triton X-100, 0.01% gelatin, 0.05 ng·µL⁻¹ DNA, 0.004 Units AmpliTaq DNA polymerase (Perkin Elmer, Foster City, Calif.), and 1.25 ng·µL⁻¹ (≈0.36 µM) primer. Amplifications were performed in a Thermal Cycler (PTC-100 Programmable Thermal Controller; MJ Research, Watertown, Mass.) programmed for 41 cycles of 1 min. at 92 °C, 1 min. at 35 °C, 2 min. at 72 °C. Samples were then stored at 4 °C. Amplification products were subjected to electrophoresis in 2% agarose gels and visualized by ethidium bromide staining; 1 kb DNA Ladder (GIBCO BRL, Bethesda, Md.) was used as the size marker. All reactions were repeated at least three times and only reproducible bands were scored.

Data analysis. Each polymorphic band was labeled based upon the primer code and approximate size. For example, F06-0800 represented the 800 bp band amplified by primer F06. Only two character states, present (1) and absent (0), were scored. Ambiguities were scored as missing data. Data were analyzed by the computer program NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System,

version 1.80 for PC; Rohlf, 1994) for phenetic analysis using the Dice Coefficient (Clifford and Stephenson, 1975; Nei and Li, 1979; Rohlf, 1994) for calculating the similarity among all 119 taxa with 213 characters. The formula used was:

$$DICE = 2a/2a + b + c$$

where 'a' was the number of bands present in common, and 'b + c' was the number of bands present in one taxon but absent in the other. The phenogram was constructed with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering procedure (Rohlf, 1994).

Results and Discussion

Two hundred thirteen polymorphic markers generated from 10 primers were used to characterize the 119 rose accessions representing a total of 36 rose species. The number of markers detected for each primer varied from 15 (E14) to 30 (H12), with an average of 21.3. The degree of polymorphism with RAPDs detected in this study was greater than in most other plants (Kesseli et al., 1993; Link et al., 1995; Skroch et al., 1993; Tingey et al., 1993).

The rose has a small genome (up to 0.825 pg per haploid genome) (Dickson et al., 1992) and a high level of polymorphism. The high number of bands per primer may also be related to the direct selection of the primers with more polymorphic bands. The size of markers ranged from 260 (E19) to 2300 bp (G11) (Fig. 1).

Relationships between the sections and subgenera. The phenogram (Fig. 2) places the roses into two divisions. The second division included only the section *Bracteatae*. The first division is divided into two groups. Group 1 includes two subgenera, *Hesperhodon* (group A) and *Platyrhodon* (group B), as well as two sections of the subgenus *Eurosa*, *Cassiorhodon* (group C1 to C4) and *Carolinae* (group C5 and D). Group 2 includes five sections of the subgenus *Eurosa*, the sections *Banksianae* (group E), *Synstylae* (group F), *Chinenses* (group G), *Pimpinellifoliae* (group H), and *Laevigatae* (group I).

The section *Bracteatae* (2nd division) is considered to be allied with *Banksianae* and *Laevigatae* due to free and caducous stipules (Rehder, 1940), although it is easily distinguished by its deeply incised stipules, large inflorescence bracts, and woolly receptacle (Bean, 1970). Both the isozyme (Kim, 1994) and RAPD analyses indicate that these three sections are distantly related, although the analyses differ from each other in the grouping of these sections with other sections and subgenera. The isozyme data indicated that *Bracteatae* is allied with the sections *Cassiorhodon* and *Carolinae*, and the subgenus *Platyrhodon*, while the RAPD data clearly separated these species groups. According to Lewis and Basye (1961), the F₁ hybrids *R. bracteata* × *rugosa*, *R. bracteata* × *foliolosa* and *R. bracteata* × *roxburghii* are all highly sterile, indicating reproductive barriers between section *Bracteatae* and sections *Cassiorhodon* and *Carolinae* and subgenus *Platyrhodon*.

In both isozyme (Kim, 1994) and RAPD analyses, *Banksianae* is allied to the sections *Chinenses*, *Synstylae*, and *Pimpinellifoliae*, whereas *Laevigatae* is allied with these sections only in the RAPD analyses. The distant relationship among the three sections with free and caducous stipules is further supported by the crossing compatibility studies between *Bracteatae* and *Laevigatae* (hybrid seedlings were inviable) and *Laevigatae* and *Banksianae* (hybrids were sterile) (Lewis and Basye, 1961).

In the first division, group 1 included the subgenera *Hesperhodon* and *Platyrhodon*, and the sections *Cassiorhodon* and *Carolinae*. Both subgenera *Hesperhodon* and *Platyrhodon*, along with the subgenus *Hulthemia*, are traditionally classified as being distinct from other roses by their armed fruits (receptacles) (Bean, 1970). Both the isozyme (Kim, 1994) and RAPD analyses grouped the subgenus *Platyrhodon* with the sections *Carolinae* and *Cassiorhodon*.

In contrast with the isozyme data, the RAPD data indicate that the subgenus *Platyrhodon* is closer to the sections *Cassiorhodon* and *Carolinae* than to the section *Bracteatae*. *Rosa*

Fig. 1. Gel showing polymorphic banding pattern with G19 primer. Eleven polymorphic bands were scored from this gel. Lane 1 and lane 42 represent 1 kb DNA ladder (GIBCO BRL). Lanes 2–41 represent the 40 genotypes. Section/subgenus level: Lanes 2–3 = *Banksianae*; Lanes 4–9 = *Synstylae*; Lanes 10–18 = *Chinenses*; Lanes 19–23 = *Pimpinellifoliae*; Lane 24 = *Laevigatae*; Lanes 25–26 = *Bracteatae*; Lanes 27–35 = *Platyrrhodon*; and Lanes 36–41 = *Cassiorhodon*.

roxburghii (subgenus *Platyrrhodon*) is a distinct rose native to China and Japan, which has peeling bark, ovules inserted on a torus at the base of the receptacle (normally inserted at the bottom and on the walls of the receptacle in *Eurosa*), and a large and spiny fruit with no hint of red in it even when fully ripe (Bean, 1970). Lewis and Basye (1961) reported 60% successful pollination between *R. roxburghii* and *R. bracteata*, but the resulting F_1 hybrids were not fertile (99.8% defective pollen). This further supports the distant relationship between *R. roxburghii* and section *Bracteatae* (second division of Fig. 2) indicated by the RAPD data.

Rosa roxburghii is currently classified in the monotypic subgenus *Platyrrhodon* because of its unique features, such as peeling bark, ovules inserted on a torus at the base of the receptacle, and large, armed, green receptacles (Bean, 1970; Rehder, 1940). This classification has been questioned because of its cross-compatibility with species in subgenus *Eurosa* (Lewis and Basye, 1961). In addition, existence of the spontaneous intersectional hybrid, *R. x micrugosa* Henkel (*R. roxburghii* of the subgenus *Platyrrhodon* with *R. rugosa* of the section *Cassiorhodon*) that appears to be partially fertile (Bean, 1970), suggests an affinity between these two subgenera (Bean, 1970; Verrier, 1991). The isozyme (Kim, 1994) and RAPD data also show that *R. roxburghii* is linked to the sections *Cassiorhodon* and *Carolinae*. Thus, the crossing behavior (Lewis and Basye, 1961), the isozyme data (Kim, 1994), and the RAPD data all support Crépín's (1889) classification system that *R. roxburghii* should be ranked as a section within subgenus *Eurosa*.

Isozyme and RAPD analyses differ as to the relationship of section *Bracteatae* with sections *Cassiorhodon* and *Carolinae* and subgenus *Platyrrhodon*. Morphological studies (Bean, 1970; Rehder, 1940), the sterility of hybrids with *R. bracteata* and three species: *R. roxburghii* (subgenus *Platyrrhodon*), *R. rugosa* (subgenus *Cassiorhodon*), and *R. folialosa* (subgenus *Carolinae*) (Lewis and Basye, 1961) and the RAPD analyses indicate that *Bracteatae*

is distantly related to both sections (*Cassiorhodon* and *Carolinae*) and the subgenus *Platyrrhodon*, whereas the isozyme study (Kim, 1994) indicates a closer relationship than traditionally reported (Bean, 1970). Although the preponderance of the evidence supports the traditional viewpoint, further research is needed.

Rosa minutifolia of the subgenus *Hesperhodos* is a desert species endemic to western Baja California and is distinguished by its five to seven, small, incised leaflets, bractless pedicels, pinnate and erect sepals, narrow stipules with dilated and divergent auricles, tomentose hypanthia with many long prickles, and a few, basally inserted achenes (Lewis, 1965). The species was first classified as the section *Minutifoliae* by Crépín (1889) and subsequently classified by Parmentier (1898) as a minor branch of section *Cinnamomeae* (*Cassiorhodon*), as part of section *Spinosissimae* Thory (= *Pimpinellifoliae*) (Baker, 1902, 1905), as a separate genus (Hurst, 1928), and, most recently, as a subgenus (Lewis, 1965; Rehder, 1940).

In the RAPD analysis, the three accessions of *R. minutifolia* (subgenus *Hesperhodos*) have 0.29 similarity level with other roses included in group 1. Section *Bracteatae* is the most distant group in the analysis, <0.10 similarity with all other roses (2nd division). All roses in group 1 have <0.20 similarity with those in group 2 (1st division). Within group 2, sections *Laevigatae* and *Banksianae* are the most distant (at the 0.20 level). The sections *Pimpinellifoliae*, *Chinenses*, and *Synstylae* follow these. Thus, the RAPD data indicate that *Hesperhodos* should be ranked as a section within the subgenus *Eurosa* rather than as a separate subgenus. Since the three other species, (*R. mirifica* Green, *R. stellata* Wootton, and *R. vernonii* Greene) of the subgenus *Hesperhodos* were not assayed, further studies are needed to confirm its rank and relationships with other roses.

The sections *Cassiorhodon* and *Carolinae* appear closely related to each other (Fig. 2) and are considered within the same section by some authors (Lewis, 1957a). The RAPD data

of this study supports combining these two sections. Rehder (1940) classified them together based on the characters of adnate and persistent stipules, styles little or not exerted from receptacles, corymbose inflorescence, usually straight prickles and bristles at base of stems, and entire sepals. *Cassiorhodon* is the only taxonomic section that is widely represented by species in both the Old and the New Worlds. The close relationship between these two sections is also supported by the high fertility among some F_1 intersectional hybrids (Lewis and Basye, 1961) and isozyme data (Kim, 1994).

Group 2 includes sections *Banksianae*, *Synstylae*, *Chinenses*, *Pimpinellifoliae*, and *Laevigatae* of the subgenus *Eurosa*. The species are grouped well within each section. Two unidentified accessions that appear to be hybrids with *Rosa multiflora* clustered with the section *Synstylae*. Selections from the section *Chinenses* have contributed more to the formation of modern garden roses than any other section (Bean, 1970) because of their repeat flowering, climbing or trailing habit, and disease resistance (Shepherd, 1954). No systematic study of the crossing behavior between this section and others have been made, but some crosses have been attempted. *Rosa calocarpa* (André) Willm., a hybrid between *R. chinensis* and *R. rugosa*, is a strong-growing shrub with good fertility (Krüssmann, 1981; Svejda, 1975). Other crosses made between *R. rugosa* and *R. chinensis* to combine the hardiness of *R. rugosa* with the everblooming habit of *R. chinensis*, resulted in F_1 hybrids that were mostly sterile (Svejda, 1975, 1978). Another intersectional hybrid, *R. x manettii* Crivelli, is a hybrid between *R. chinensis* and *R. moschata* (*Synstylae*), also known as *R. x noisettiana* Theory (Krüssmann, 1981). *Rosa x manettii* is a vigorous plant (Cairns, 1993) and is often used as a budding understock, as it can be propagated easily from cuttings (Krüssmann, 1981). The hybrid between *R. multiflora* x *R. chinensis*, one of the origins of the polyantha roses, is a low-growing bush with many small flowers (Krüssmann, 1981). Crosses between the species in the sections *Synstylae* and *Chinenses* showed 8% seed setting and 35% seeds with mature embryos (Ratsek et al., 1940). The isozyme data (Kim, 1994), like the phenogram from the RAPD data, showed that the section *Synstylae* is allied to *Chinenses*. Morphologically, the sections *Synstylae* and *Chinenses* are related to each other based on adnate and persistent stipules and exerted styles (Rehder, 1940).

Although the genus *Rosa* is highly variable, the grouping of the species by RAPD data largely agree with the traditional rose taxonomy, cytology, crossing behavior, and isozyme data. Both the subgenera *Hesperhodos* and *Platyrrhodon* are grouped with the sections *Cassiorhodon* and *Carolinae* in the dendrogram of RAPD data. Therefore, sectional ranks are suggested for these two subgenera. The section *Carolinae* has been classified either as a separate section or as a part of the section *Cassiorhodon*, based on their morphology and crossing behavior. The isozyme and RAPD

Fig. 2. Phenogram derived from 119 rose accessions and 213 RAPD markers using Dice similarities (also Nei and Li) and UPGMA analysis. Species level: arka = *Rosa arkansana*; bank = *R. banksiae*; blan = *R. blanda*; brac = *R. bracteata*; brun = *R. brunonii*; cali = *R. californica*; caro = *R. carolina*; chin = *R. chinensis*; clin = *R. clinophylla*; unkn = unknown taxa (*R. multiflora* hybrid); davu = *R. davurica*; fili = *R. filipes*; foli = *R. foliolosa*; forr = *R. forrestiana*; fort = *R. ×fortuniana*; gent = *R. gentiana*; hugo = *R. hugonis*; laev = *R. laevigata*; luci = *R. luciae*; macr = *R. macrophylla*; maja = *R. majalis*; minu = *R. minutifolia*; mosc = *R. moschata*; mull = *R. mulliganii*; mult = *R. multiflora*; niti = *R. nitida*; odor = *R. ×odorata*; omei = *R. omeiensis*; paul = *R. ×paulii*; prim = *R. primula*; roxb = *R. roxburghii*; rugo = *R. rugosa*; seri = *R. sericea*; seti = *R. setigera*; wich = *R. wichuraiana*; wood = *R. woodsii*; xant = *R. xanthina*. Section level: Bank = *Banksianae*; Brac = *Bracteatae*; Caro = *Carolinae*; Cass = *Cassiorhodon*; Chin = *Chinenses*; Laev = *Laevigatae*; Pimp = *Pimpinellifoliae*; Syns = *Synstylae*. Subgenus level: Euro = *Eurosa*; Hesp = *Hesperhodon*; Plat = *Platyrhodon*.

data support the combination of section *Carolinae* with section *Cassiorhodon*. The sections *Bracteatae*, *Banksianae*, and *Laevigatae* are considered closely related to each other based on morphology. However, neither crossing behavior nor isozyme data support a close relationship. The dendrogram (phenetic analysis) of RAPD data in this study indicated that these three sections are distantly and almost equally related to each other. The section *Chinenses* is grouped with the section *Synstylae* based on morphology, isozyme, and RAPD data.

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