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Assessment of Genetic Relatedness in Roses by ISSR Markers

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Abstract: The genus *Rosa* contains more than 200 species and 18000 cultivars. Genetic relationships within the genus *Rosa* are confusing due to the variability of species and the weak barriers to intraspecific hybridization. Inter-simple sequence repeat (ISSR) markers were used to study phylogenetic relationships among 47 rose genotypes. Among 15 ISSR primers, 11 primers showed acceptable polymorphism and amplified 193 bands which 173 of them were polymorphic. Banding patterns were transformed into binary data of presence-absence and matrices were processed using NTSYS Ver. 2.02 software program. The dendrogram was constructed using Jaccard coefficient and UPGMA algorithm. The 47 rose genotypes were classified into 4 major groups with, within-group similarity values of >0.52. Group 1 included *R. banksiae* Ait., group 2, *R. canina* L., *R. damascena* Mill. and *R. moschata* Herm. with >53% within-group similarity values, group 3 included *R. foetida* Herm. and *R. hybrida* L. and group 4 included *R. Chinensis* Jacq. None of the species used in this study wereclustered within group 1, indicating that there was no direct relationship between these species. In this phylogenetic tree *R.chinensis*clusters in the lower part of the tree with 46% similarity with group 2 and 3.

Key words: Rose species · ISSR marker · Phylogeneticrelationship

INTRODUCTION

There are almost 200 species and more than 18000 cultivars in the genus *Rosa* [1]. They are mostly shrubs, distributed in the temperate zones of the northern hemisphere [2]. An understanding of species relationships is a prerequisite for the effective utilization of the genetic variations available to breeders. The value of grouping into sections all the known species and theirhybrids is apparent, as the procedure simplifies the study of rose development [3]. There is a need to improve our understanding of the biological relationships between the *Rosa* spp.

Since the mid1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. The widely-used PCR-based markers are RAPDs [4], SSRs or microsatellites [5] AFLPs [6, 7] and ISSRs [8].

In the 1990s, molecular markers were developed for rose cultivar identification [9, 10] and several of these were tested for identifying species relationships in *Rosa*. Debener *et al.* [9] and Millan *et al.* [10] used RAPDs to examine the relationshipsamong cultivars and a limited number of wild species. Thework of Debener *et al.* [9] showed a distinction between agroup of cultivars and a group of wild species. In a study onwild accessions, Millan *et al.* [10] showed a clustering largelyaccording to the sectional affinities. The latter was also found by Jan *et al.* [11]. Wu *et al.* [12] used RAPDs to study the relationships with in sect. *Synstylae*, but obtained a tree withlittle resolution. More recently, Wen *et al.* [13] employed RAPDs to study the relationships of *R. roxburghii* and relatives and Bruneau *et al.* [14] (2005) used them to study *R. blanda* andsegregates. However, problems with the reproducibility of RAPD markers[6] may render them less suitable as phylogeneticmarkers, although there has been some improvement in recentyears [15]. Mitochondrial and chloroplast RFLPswere used by

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Matsumoto *et al.* [16] and Takeuchi *et al.* [3] to study the relationships among wild *Rosa* species. Althoughthey were able to draw conclusions on certain groups, the plastid RFLP data showed a general lack of resolution. To overcome the drawbacks of RAPDs and RFLPs, alternative markers were developed, most notably microsatellites or simple sequence repeats (SSRs) [5] and AFLPs [17]. Both marker types combine high reproducibility with high variability, potentially increasing both reliability and resolution of phylogenies. On top of that, both AFLPs and microsatellites enable a genome-wide sampling, increasing the chance that the data sets and phylogenies represent the evolutionary affinities of the species rather than that of theindividual characters,but these techniques havetheir own disadvantages. AFLP has medium reproducibility but is labour intensive and has high operational and development costs [6]. Microsatellites require knowledge of the genomic sequence to design specific primers and thus are limited primarily to economically important species.

Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available. ISSR markers are DNA sequences delimited by two inverted SSR composed of the same units which are amplified by a single PCR primer, composed of few SSR units with or without anchored end. ISSR-PCR gives multilocus patterns which are very reproducible, abundant and polymorphic in plant genomes [5, 8, 18]. Comparison of ISSR and other PCR-based markers have shown their efficiency in plant breeding [19]. As a result of these advantages and their universality and easiness of development (no needs to sequence data), ISSR markers are more and more requested. The aim of this study was to evaluate the use of ISSR markers to characterize and to estimate genetic diversity between anumber of rose species.

MATERIALS AND METHODS

Plant Material: For this study, 47 genotypes of genus *Rosa* including: 3 genotypes of *Rosa banksiae* Ait., 7 genotypes of *Rosa canina* L., 3 genotypes of *Rosa chinensis* Jacq. Var. *minima*, 4 genotypes of *Rosa damascena* Mill., 3 genotypes of *Rosa foetida* Herm, 22 genotypes of *Rosa* × *hybrida* L. (14 genotypes of hybrid perpetual, 4 genotypes of Rambler and 4 genotypes of 'Sanaz') and 5 genotypes of *Rosa moschata* Herm. were collected from several provinces of Orumieh.

DNA Extraction: Samples (fresh mature leaves 4 g) were rinsed in tap water followed by sterilized distilled water. They were then air-dried and stored at -80°C if not used immediately. The leaves were ground in liquid nitrogen in a sterile prechilled mortar and pestle. Extraction buffer (120 mMTris-HCl, pH:8.0; 80 mM EDTA pH:8.0; 4% β-mercaptoethanol; 2% CTAB) (at a ratio of 5 ml per gram of fresh sample) was added to each tube, incubated the tubes at 60°C water bath for 40 min. and vortexed the tubes vigorously in 5 min. intervals. The tubes were centrifuged at 8000g for 5 min. and supernatant was discarded. 20 ml clean up buffer (120 mMTris-HCl pH:8.0; 10 mM EDTA pH:8.0; 2% β-mercaptoethanol; 2% PVP; 1.5 M NaCl; 0.2% CTAB) was added to each pellet and after resuspension, then were incubated at 60°C water bath for 30 min. with occasional inversion. When the tubes were cooled to room temperature, 20 ml of chloroform-isoamyl alcohol (24:1 v/v) and 2 ml of 5M potassium acetate were added and the tubes were shaken vigorously to form an emulsion. Tubes were centrifuged at 8000 g at room temperature for 20 min. After centrifugation the aqueous phase transferred to a new tube and added 20 ml of chloroform-isoamyl alcohol (24:1 v/v), centrifuged and the aqueous phase transferred to a new tube and added 20 ml cold isopropanol with 2 ml of 3M sodium acetate, mixed and incubated at 20°C for 30 min. The DNA was precipitated by centrifugation at 8000 g at room temperature for 10 min.

Resulting pellets were washed 3 times with an equal volume of 70% ethanol.Then the pellets were dried and resuspended in 2 ml of TE buffer with 10 µl of RNAase and incubated at 37°C for 4 h.

Primers: Primers were purchased in lyophilized form from Cinnagen Co. Iran. In this research 15 ISSR primers were tested (Table 1).

DNA Amplification: DNA amplification was carried out in 25 µl reactions consisting of 30 ng of template DNA, 1 U of *Taq*DNA polymerase enzyme (Fermentase Co.), 0.2 mM of each dNTP (Cinnagen Co.), 1X*Taq* amplification buffer (Fermentase Co.), 2 mM $MgCl_2$ and 1µM primer (Cinnagen Co.). Amplifications were carried out by using a DNA thermal cycler (Eppendorf, mastercycler gradient) programmed as: 94°C for 5 min., 35 cycles at 94°C for 30 s, 45-54°C for 45 s and 72°C for 2 min. and a final extension at 72°C for 10 min. PCR products were separated on 1.5% agarose gel and stained in ethidium bromide, observed under UV light and photographed using gel documentation unit.

Table 1: The ISSR primers used to asses genetic variation among forty seven rose genotypes

No.	Primer	Sequences (5´-3´)	Sequences (5´-3´)
1	ISSR-1	5'-$(AG)_8$YT-3'	5'-AGAGAGAGAGAGAGAGYT-3'
2	ISSR-2	5'-$(GA)_8$C-3'	5'-GAGAGAGAGAGAGAGAC-3'
3	ISSR-3	5'-CCA$(CT)_8$-3'	5'-CCACTCTCTCTCTCTCT-3'
4	ISSR-4	5'-$(AC)_8$YT-3'	5'-ACACACACACACACACYT-3'
5	ISSR-5	5'-CCA$(AG)_8$T-3'	5'-CCAAGAGAGAGAGAGAGAGT-3'
6	ISSR-6	5'-$(CT)_8$AC-3'	5'-CTCTCTCTCTCTCTCTAC-3'
7	ISSR-7	5'-$(GA)_8$ACC-3'	5'-GAGAGAGAGAGAGAGAACC-3'
8	ISSR-8	5'-$(TC)_8$CC-3'	5'-TCTCTCTCTCTCTCTCCC-3'
9	ISSR-9	5'-$(CT)_8$RG-3'	5'-CTCTCTCTCTCTCTCTRG-3'
10	ISSR-10	5'-$(CT)_8$G-3'	5'-CTCTCTCTCTCTCTCTG-3'
11	ISSR-11	5'-$(GT)_8$T-3'	5'-GTGTGTGTGTGTGTGTT-3'
12	ISSR-12	5'-$(AAG)_6$C-3'	5'-AAGAAGAAGAAGAAGAAGC-3'
13	ISSR-13	5'-$(CT)_8$YA-3'	5'-CTCTCTCTCTCTCTCTYA-3'
14	ISSR-14	5'-$(GT)_6$CC-3'	5'-GTGTGTGTGTGTCC-3'
15	ISSR-15	5'-$(GTG)_3$GC-3'	5'-GTGGTGGTGGC-3'

Y = Pyrimidine

R = Purine

Data Analysis: The observed bands in the gel were evaluated based on the presence (coded 1) or absence (coded 0) of polymorphic fragments for each primer. Cluster analysis was performed with NTSYS-pc Version 2.02, a numerical taxonomy and multivariate analysis software package using an unweighed pair-group method, arithmetic average (UPGMA).

RESULTS

ISSR Amplification: Fifteen primers were initially tested using rose DNA (Table 1). Four primers amplified no products at all. The other 11 primers gave clear, species-specific fingerprint patterns with all samples (Figs. 1 and 2). The amplified fragment sizes ranged from 100 to 3000bp with the scoreable region being from 150 to 2500bp. Fingerprint patterns consisted of 193 fragments which 173 of them were polymorphic among the samples (Table 2). Althoughwe did not do an extensive comparison between 3´- and 5´-anchored primers using the same core repeat, but in our study 5´-anchored primers did not amplify any product. These results may be useful for selecting ISSR primers for other species of genus *Rosa*.

We studied many factors of PCR amplifications to achieve clear fingerprint patterns. High-quality DNA template was essential to obtain a large number of well-resolved fragments. RNA in DNA template preparations interfered with the PCR reaction and reduced the number of fragments that could be scored.

Variation Between Species: ISSR clustering from Jaccard similarity matrix (Fig. 3) have a cophenetic coefficient equal to 0.93. Cluster analysis resulted in grouping of the 47 genotypes into 4 distinct groups (Fig. 3). Group 1 consisted of *Rosa banksiae* genotypes, which was placed, independently, very distantly from the rest of the species.

Group 2 included genotypes of *R. canina*, *R. damascena* and *R. moschata* species with >55% within-group similarity values (Fig. 3). In this research, genotypes of *R. damascena* and *R. moschata*have 53% within-group similarity.

In present phylogenetic tree, genotypes of *R. hybrida* and *R. foetida* were placed in group 3 with >52% within-group similarity values.

In group 4 of this phylogenetic tree, genotypes of *R. chinensis*were placed. The similarity value between these genotypes was >70%, but they have 46% similarity with genotypes of group 2 and 3.

In this research, there was 46% similarity value between *R. hybrida* and other roses (unless *R. banksiae*).

DISCUSSION

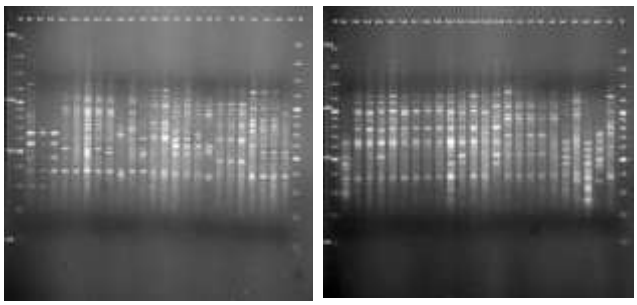
None of the species used in this study clustered within group 1, indicating that there is any direct relationship between these species. This species was the most dissimilar in the dendrogram of Matsumoto *et al.* [20] and Atineza *et al.* [4]. By RAPD-analysis Millan *et al*. [10] assigned *R. banksiae*as a member of subgenus *Rosa*. This species is belonging to the Sect. *Banksianae*. Morphologically the section is characterized by free and deciduous stipules, nonpubescent receptacles and branchlets and reflexed and deciduous sepals [21].

Table 2: List of primers, their sequence motifs, melting temperatures, annealing temperatures, number of the amplified fragments generated by ISSR primers in 47 rose genotypes

No.	Primers	5´-3´ motif	Melting temperature	Annealing temperature	Number of polymorphic bands	Total number of bands amplified
1	ISSR-1	$(AG)_8YT$	42.3	45	15	16
2	ISSR-2	$(GA)_8C$	43.3	45	19	20
3	ISSR-4	$(AC)_8YT$	48.3	50	13	16
4	ISSR-6	$(CT)_8AC$	43.3	54	8	10
5	ISSR-7	$(GA)_8ACC$	51.5	50	13	14
6	ISSR-8	$(TC)_8CC$	52.9	54	11	14
7	ISSR-9	$(CT)_8RG$	42.3	54	20	23
8	ISSR-10	$(CT)_8G$	44.9	54	20	21
9	ISSR-12	$(AAG)_6C$	51.8	54	15	16
10	ISSR-14	$(GT)_6CC$	42.6	45	12	14
11	ISSR-15	$(GTG)_3GC$	43.5	45	27	29
Total					173	193

Y=Pyrimidine

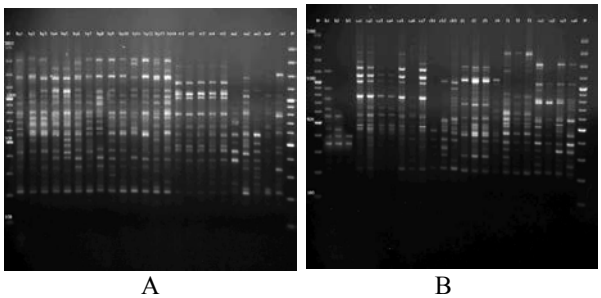
R= Purine



A B

b1, b2, b3=*Rosa banksiae,*ca1, ca2, ca3, ca4, ca5, ca6, ca7= *Rosa canina,*ch1, ch2, ch3= *Rosa chinensis*, d1, d2, d3, d4= *Rosadamascena*, f1, f2, f3= *Rosa foetida,*ra1, ra2, ra3, ra4= *Rosa* × *hybrida*(Rambler), hy1, hy2, hy3, hy4, hy5, hy6, hy7, hy8, hy9, hy10, hy11, hy12, hy13, hy14=*Rosa* × *hybrida*, m1, m2, m3, m4, m5= *Rosa moschata*, sa1, sa2, sa3, sa4= *Rosa* × *hybrida* (Sanaz)andM: Weight marker.

Fig. 1: ISSR-PCR band profiles generated by the primer ISSR- 2 with the sequence 5'-$(GA)_8$C-3' used in seven rose species studied in this investigation



A B

b1, b2, b3=*Rosa banksiae,* ca1, ca2, ca3, ca4, ca5, ca6, ca7= *Rosa canina,* ch1, ch2, ch3= *Rosa chinensis*, d1, d2, d3, d4= *Rosadamascena*, f1, f2, f3= *Rosa foetida,* ra1, ra2, ra3, ra4= *Rosa* × *hybrida*(Rambler), hy1, hy2, hy3, hy4, hy5, hy6, hy7, hy8, hy9, hy10, hy11, hy12, hy13, hy14=*Rosa* × *hybrida*, m1, m2, m3, m4, m5= *Rosa moschata*, sa1, sa2, sa3, sa4= *Rosa* × *hybrida* (Sanaz) and M: Weight marker.

Fig. 2: ISSR-PCR band profiles generated by the primer ISSR-9 with the sequence 5'- $(CT)_8$RG-3' used in seven rose species included in this study

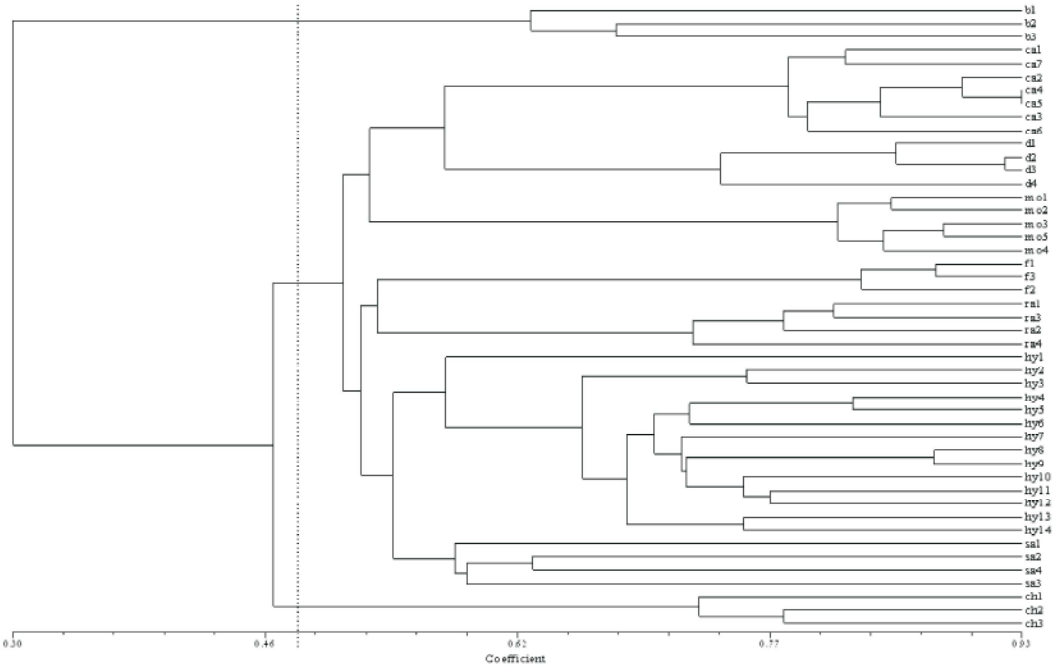


Fig. 3: Dendrogram represents the genetic relatedness among 47 rose genotypes studied in this investigation

In group 2, *R. damascena* and *R. canina*were laid much closed to each other. Koopman *et al.* [7] in their research showed similarity between these two species, but with different similarity values from us.

In this research genotypes of *R. canina* and *R. moschata* species have >53% within-group similarity values. These results were in agreement with the results of Matsumoto *et al*. [16]. They showed that there was 61% similarity between *R. canina* and *R. moschata* but they didn't have notable similarity with *R. banksiae* and *R. foetida*. In this research, genotypes of *R. damascena* and *R. moschata* have 53% within-group similarity. This is in accordance with previous results about these two species. For example, Takeuchi *et al.* [3] in their molecular research showed that *R. moschata* and *R. damascena* were grouped into a cluster. Koopmann *et al.* [7] with AFLP analysis reported that the species of Sect. *Rosa* (which *R. damascena*was placed in this Sect.) and the species of Sect. *Synstylae* (which *R. moschata* was placed in this Sect.) were in the same clade.

In this study, genotypes of group 3 have >52% within-group similarity values. Such similarity between these species was logical because *R. foetida* is one of the parents of modern roses. Such relationship was seen in researches of Bruneau *et al*. [14]. Koopman *et al*. [7], Matsumoto *et al*. [16], Millan *et al*. [10] and Wissemann and Ritz [21].

The results of the similarity between species of group 4 were in disagreement with the results of other researchers e.g. Matsumoto *et al.* [16] in their molecular classification of wild roses using organelle DNA probes showed that there was 61% similarity between *R. canina* and the species of Sect. *Indicae* (*R. chinensis* was placed in this Sect.). Also, Millan *et al.* [10] in their studies with RAPD markers reported that there was 80% similarity between these two species. This incoherence was possible, because molecular marker type or accessions under study were different in various researches.

In this research, there was 46% similarity value between *R. hybrida* and other roses (unless *R. banksiae*). These results showed that these rose species (*R. canina*, *R. chinensis*, *R. damascena*, *R. foetida* and *R. moschata*) had have role in creation of modern hybrid roses. Considering that Hurst and Wylie (cited in Gudin (1)) investigated the origin of modern garden roses and indicated that all known cultivars originated from only 10 species including: *R. foetida* Herm., *R. gallica* L., *R. gigantea* Colett ex Crep., *R. moschata* Herm., *R. multiflora* Thunb., *R. phoenicea* Boiss, *R. rugosa* Thunb. and *R. wichuraina* Crep. Such similarities were expected. Also, the species that have 46% similarities with *R. hybrida* are the ancestors of *R. hybrida* and because of other species except these 5 species having contribution to the gene pool of modern garden roses, 46% similarities between these roses in this study seems to be true.

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