African Journal of Biotechnology Vol. 9(37), pp. 6091-6095, 13 September, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

Inter simple sequence repeat (ISSR) markers as reproducible and specific tools for genetic diversity analysis of rose species

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Accepted 30 April, 2010

Rose is one of the most important cultivated ornamental plants in the world. A molecular approach using inter-simple sequence repeat (ISSR) markers was applied to seven species of *Rosa*. To obtain clear and reproducible bands on 2% agarose gels, 9 ISSR primers and 5 parameters (annealing temperature, DNA concentrations, primer concentrations, *Taq* DNA polymerase and $MgCl_2$ concentrations) were screened. The resolution of six ISSR markers was performed, with optimal annealing temperature (Ta) varying from 45 to 50°C. A total of 66 DNA fragments were amplified, of which 50 were polymorphic. The optimal conditions for ISSR system were determined as follows: $MgCl_2$ concentration was 2 mM, the quantity of *Taq* DNA polymerase 1 U, template DNA 30 ng and the concentration of primer was 1 µM and the reaction program was: initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 30 s at 94°C, annealing for 45 s at specific annealing temperature for each primer, extension for 2 min at 72°C and a final 10 min extension at 72°C.

Key words: Inter-simple sequence repeat marker, rose species, genetic diversity, optimization.

INTRODUCTION

The genus *Rosa* contains more than 150 species, but only ten species have contributed to the modern commercial roses (Gudin, 2000). The genetic background of modern roses is therefore narrow compared to the available *Rosa* species (Gudin, 2000; Koopman et al., 2008; Takeuchi et al., 2000). Genetic relationships within the genus *Rosa* are confusing due to the variability of species and the weak barriers to intraspecific hybridization (Koopman et al., 2008).

An understanding of species relationships is a prerequisite for the effective utilization of the genetic variation available to a breeder. The value of grouping into sections all the known species and hybrids derived is apparent, as the procedure simplifies the study of rose development. There is a need to improve our understanding of the biological relationship between *Rosa* species (Matsumoto et al., 1998).

The genus *Rosa* has been classified into four subgenera: *Hulthemia*, *Platyrhodon*, *Hesperhodos*, which are monotypic or include two species and *Eurosa*, which contains all the remaining species grouped into 10 sections (Wissemann and Ritz, 2005). The classification of the genus *Rosa* has presented many problems to taxonomists, as gradation between many subdivisions are slight and in many instances, segregation is a matter of opinion (Takeuchi et al., 2000).

Recently, as an adjunct to the morphological and

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Abbreviations: ISSR, Inter-simple sequence repeat; **Ta,** annealing temperature; **RFLPs,** restriction fragment length polymorphism; **PCR,** polymerase chain reaction; **RAPD,** random amplified polymorphic DNA; **SSRs,** simple sequence repeats; **AFLP,** amplified fragment length polymorphism; **Tm,** melting temperature.

physiological methods, identification tests based on isozyme patterns have been introduced to fingerprint *Rosa* species (Grossi et al., 1997; Kim and Byrne, 1996; Kuhns and fretz, 1978), but the information derived from isozyme markers has been limited by the number of loci for which assays are available. Conventional detection of restriction fragment length polymorphism (RFLPs) is expensive, time consuming and demand probes, radiochemical and qualified personnel (Rajapakse et al., 1995).

The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently, simple sequence repeats (SSRs) or microsatellites (Reddy et al., 2002). The major limitations of these methods are low reproducibility of RAPD, high cost of amplified fragment length polymorphism (AFLP) and the need to know the flanking sequences to develop species specific primers for SSR polymorphism (Belaj et al., 2003). ISSR-PCR is a technique that overcomes most of these limitations (Wu et al., 1994). It is rapidly being used by the research community in various fields of plant improvement (Reddy et al., 2002). The technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of plant species. 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 (Bornet and Branchard, 2001, 2004). The technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD (Bornet and Branchard, 2001, 2004). As a result of these advantages and their universality and easiness of development (no need to sequence data), ISSR markers are more and more requested.

Most of previous procedure established for ISSR-PCR analysis in plants involved the use of radioisotope and polyacrylamide sequencing gels (Blair et al., 1999; Shahsavar et al., 2007). The merit of ISSR analysis applied to this research project was its simplicity; however the technique was applied with minimum inexpensive agarose gels and non-radioactive detection, a deviation from the normal practice of most studies that employ microsatellite-based markers. In the present study, experimental conditions for the use of ISSR in genetic diversity analysis of rose species were explored.

MATERIALS AND METHODS

Plant materials

For this study, seven species of *Rosa* including: *Rosa banksiae* Ait., *Rosa canina* L., *Rosa chinensis* Jacq. Var. *minima*, *Rosa damascena* Mill., *Rosa foetida* Herm, *Rosa × hybrida* L. and *Rosa moschata* Herm. were used.

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 mM Tris-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, the tubes were incubated at 60°C water bath for 40 min and vortexed vigorously at 5 min interval. The tubes were centrifuged at 8000 g for 5 min and the supernatant discarded. 20 ml clean up buffer (120 mM Tris-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, were incubated at 60°C water bath for 30 min with occasional inversion. After the tubes were cooled to room temperature, 20 ml of chloroform-isoamyl alcohol (24: 1 v/v) and 2 ml of 5 M potassium acetate was 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 was transferred to a new tube and 20 ml of chloroform-isoamyl alcohol (24: 1 v/v) were added and centrifuged. The aqueous phase obtained after centrifugation was transferred to a new tube and 20 ml cold isopropanol with 2 ml of 3 M sodium acetate were added, mixed and incubated at 20°C for 30 min. The DNA was precipitated by centrifuging at 8000 g at room temperature for 10 min.

Resulting pellets were washed 3 times with an equal volume of 70% ethanol and the pellets were dried and resuspended in 2 ml of TE (Tris + ethylenediaminetetraacetic acid (EDTA)) 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). 9 ISSR primers were tested in this research (Table 1).

PCR conditions and gel electrophoresis

To optimize the reaction conditions, several PCR parameters, including DNA concentration (10, 20, 30, 40, 50 ng/reaction), primer concentration (0.5, 1.0, 1.5, 2.0 µM), $MgCl_2$ concentration (0.5, 1.0, 1.5, 2.0, 2.5 mM) and *Taq* DNA polymerase (0.5, 1.0, 1.5, 2.0 unit/reaction) were tested. The optimum annealing temperature was determined for each primer. Amplification 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, specific annealing temperature for 45 s and 72°C for 2 min and a final extension at 72°C for 10 min. PCR products were separated using 2% agarose gel and stained by 0.5 µg/ml ethidium bromide and observed under UV light and photographed using gel documentation unit.

RESULTS AND DISCUSSION

Influences of annealing temperatures

Among the 9 tested primers, 3 [ISSR-3 (5′-CCA$(CT)_8$-3′), ISSR-5 (5′-CCA$(AG)_8$T-3′) and ISSR-6 (5′-$(CT)_8$AC-3′)] gave no or few amplified bands between the tested species and the rest of the primers gave rich and highly polymorphic fingerprints of which only the latter ones were used. Consequently, a total of 66 clear and reproducible bands were amplified from seven species using the 6

Table 1. The ISSR primers used to assess genetic variation among seven rose species.

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′-CCACTCTCTCTCTCTCTCT-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	HB11	5′-$(GT)_6$CC-3′	5′-GTGTGTGTGTGTCC-3′
9	HB15	5′-$(GTG)_3$GC-3′	5′-GTGGTGGTGGC-3′

Y = Pyrimidine.

Table 2. List of primers, their sequence motifs, melting temperatures, annealing temperatures, number of the amplified fragments generated by ISSR primers in seven rose species.

No.	Primers	5´-3´ motif	Melting temperature	Annealing temperature	Number of polymorphic bands	Total number of bands amplified
1	ISSR-1	$AG)_8$YT	42.3	45	6	8
2	ISSR-2	$(GA)_8$C	43.3	45	8	11
3	ISSR-4	$(AC)_8$YT	48.3	50	8	10
4	ISSR-7	$GA)_8$ACC	51.5	50	11	12
5	HB11	$(GT)_6$CC	42.6	45	10	15
6	HB15	$(GTG)_3$GC	43.5	45	7	10
Total					66	50

Y = Pyrimidine.

selected ISSR primers, of which 50 were polymorphic (Table 2).

Clear, intensive and thin bands were observed with annealing temperatures (Ta) ranging from 45 to 50°C (Table 2). In this research, except for primer 7 whose Ta was lower than melting temperature (Tm), in other primers, using high stringency (Ta higher than Tm), clear and reproducible bands were observed (Figures 1 and 2). Modifications of annealing temperature are known to have a great impact on the richness and legibility of fingerprints. According to Bornet and Branchard (2001), Ta is primer specific and always superior to Tm because of the need of high stringency to facilitate annealing of primers. These authors reported that Ta values for optimal hybridization was 2 to 14°C higher than Tm. Similar results were also reported by Charters and Wilkinson (2000). Although in many papers, unique and low annealing temperatures have been used for ISSR amplifications with different primers (Gilbert et al., 1999; Sanchez de la Hoz et al., 1996), however, a low annealing temperature may increase nonspecific amplification, leading to artifact bands. The discrepancy in trends toward optimal values of Ta in comparison with Tm indicated the need of optimization. In our study, no particular trends in optimal hybridization temperature were found.

Influences of PCR conditions

We investigated several parameters that could affect pattern quality and reproducibility of ISSR fingerprints. The genomic DNA at 30 ng was found to be optimum for PCR amplification. More spurious products were amplified at lower concentration with low intensity and were difficult to score. Some products that were amplified at 30 ng were not amplified at higher concentration (50 ng). Many studies showed that 5 – 500 ng template DNA could offer an amplification result (Huang and Sun, 2000). The optimal content of DNA used for ISSR-PCR mainly depends on the kind of material and purity of template DNA (Hua et al., 2006). Among the four concentration of *Taq* DNA polymerase, 1 U yielded sharp and consistent bands, whereas, other concentrations produced either faint bands or smears. Sharpness of bands was improved by increasing $MgCl_2$ concentration to 2 mM. Mg^{2+} was an important factor in stimulating *Taq* DNA polymerase, opening double chains of prime and template DNA and annealing temperature, so the appropriate Mg^{2+} concentration was essential to the ISSR (Bhatia et al., 2009; Dje

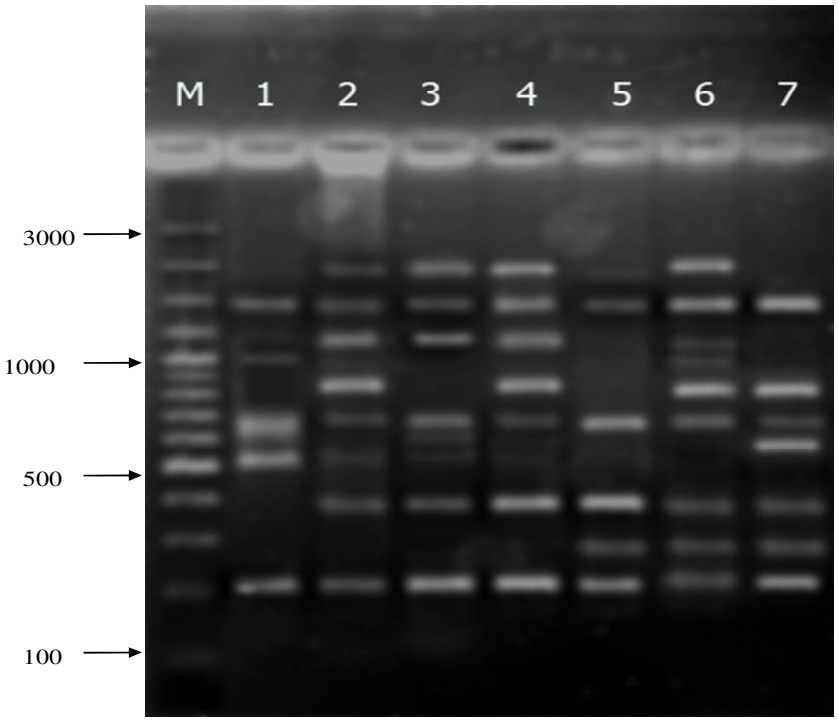

Figure 1. ISSR-PCR band profiles generated by the primer ISSR-2 with the sequence 5′-$(GA)_8$C-3′ used in seven rose species included in this study. Lane 1: *Rosa banksiae,* lane 2: *Rosa canina*, lane 3 *Rosa chinensis*, lane 4: *Rosa damascena*, lane 5: *Rosa foetida*, lane 6: *Rosa × hybrida* and lane 7: *Rosa moschata*; M: weight marker.

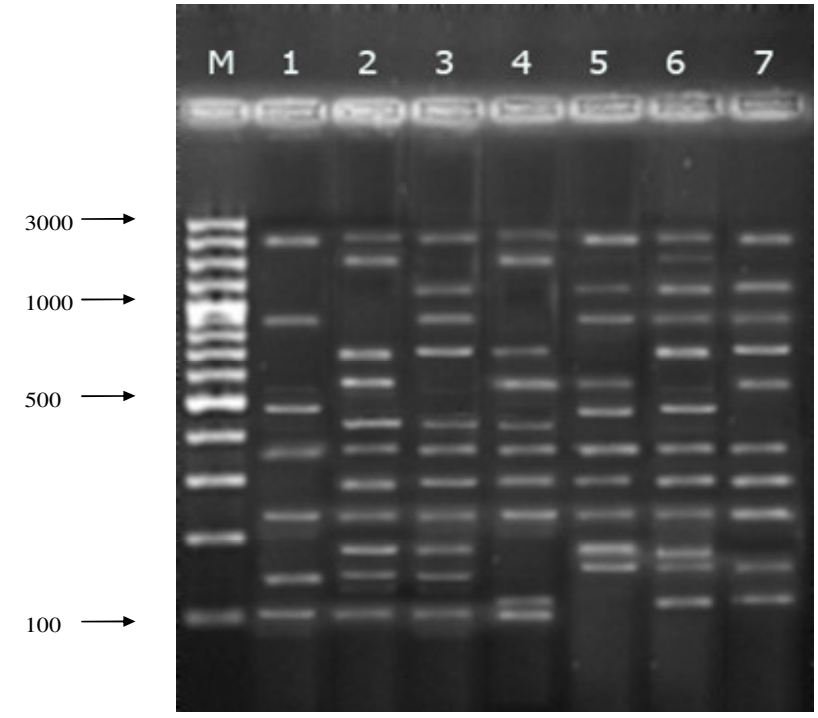

Figure 2. ISSR-PCR band profiles generated by the primer ISSR-15 with the Lane 1: *Rosa banksiae,* lane 2: *Rosa canina*, lane 3: *Rosa chinensis*, lane 4: *Rosa damascena*, lane 5: *Rosa foetida*, lane 6: *Rosa × hybrida* and lane 7: *Rosa moschata*; M: weight marker.

et al., 2006; Hua et al., 2006).

Our results show no modification of patterns when primer concentrations were changed. Concentrations lower than 1 µM per reaction produced complete fingerprints, but the band intensity was low. The optimum concentration of PCR components was standardized on the basis of reproducibility of the bands after repeating the experiments three times.

In conclusion, ISSR markers were chosen because the technique is very simple, fast, cost effective, highly discriminative, reliable and require small quantity of sample DNA. They also do not need any prior primer sequence information and are non-radioactive.

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