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BRIEF COMMUNICATION

R-ISSR marker as a useful tool for detection of new genomic loci in *Arthrocnemum macrostachyum*

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Abstract

Arthrocnemum macrostachyum, is a perennial halophytic shrub typical of Mediterranean salt marshes. The present study aims to investigate some combinations of inter simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) primers applied in real PCR. Thereby, the potential of R-ISSR markers to detect new genomic loci in 3 genotypes of *A. macrostachyum* grown in the Western coast of Syria was examined. Different combinations of RAPD and ISSR primers produced bands that were absent when single ISSR or RAPD primers were used. The results have demonstrated that ISSR primer $(AG)_8TC$ gave more informative pattern when combined with different RAPD primers comparing to other tested primers. In contrast, the tested ISSR primer $(GACA)_4$ gave less informative pattern when used alone. These combinations were successfully applied in real PCR to detect new genomic variability in *A. macrostachyum* genotypes.

Additional key words: PCR, polymorphism, RAPD.

The *Salicornioideae* (14 - 16 genera, *ca*. 90 species) is a remarkable subfamily of *Chenopodiaceae* that is notable for their halophytic nature associated with particular anatomical, morphological, and physiological adaptations (Kadereit *et al*. 2006). *Arthrocnemum macrostachyum* (Moric.) Moris. is a typical species in the salt marshes of the Mediterranean region (Vicente *et al*. 2007). In the Western coast of Syria, it often forms monodominant communities or co-occurs with other species such as *Halimione portulacoides* (L) Aellen, *Inula chritmoides* L. and *Juncus acutus* L. Wilson (1980) proposed that *Arthrocnemum* is a possible intermediate between *Sarcornia* and the Australian endemic genera based on morphological characters shared with both groups. However, the phylogenetic position of *A. macrostachyum* is not fully resolved. Random amplified polymorphic DNA (RAPD) markers (Williams *et al*. 1990, Singh *et al*. 2006, Sikdar *et al*. 2010) and inter simple sequence repeat (ISSR) markers (Joshi *et al*. 2000, Okun *et al*. 2008) are two molecular approaches that have been used to detect variation among plants. Many studies have investigated the combination of different types of markers to assess the genetic variability. For example, Raina *et al*. (2001) have used both RAPD- and ISSR-fingerprinting data, in combination, to examine phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. Many regions in DNA double-strand structure could not be amplified using a single RAPD or ISSR primers, but the combination between these primers (RAPD or ISSR) makes the distance between their binding sites and their orientation more available to obtain an amplification *via* PCR reaction. Chadha *et al*. (2007) have applied REMAP-ISSR combination for genetic polymorphism studies in *Magnaporthe grisea*. In 2008, Behera *et al*. have demonstrated the successful analysis of genetic diversity in Indian bitter gourd (*Momordica charantia* L.) using RAPD and ISSR markers for developing crop improvement strategies. Muthusamy *et al*. (2008) have also reported that both marker systems RAPD and ISSR, either individually or combined, can be effectively used

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Abbreviations: CTAB - cetyltrimethylammonium bromide; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; ISSR - inter simple sequence repeat.

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in determination of genetic relationships among rice and bean landraces. The objective of this study is to investigate some combinations of RAPD and ISSR primers in multiplex PCR reaction and thereby, to match more genetic information and new loci in *A. macrostachyum* species.

Many genotypes of this species can be found in Syria and are located in the Western littoral salines coast at 12 km to the North of Lattakia, providing source of samples for DNA extraction. Samples were collected in spring from plants spread on rocky and sandy soil with salinity estimated at an EC of 70 dS m^{-1}, and annual rainfall ranging from 650 to 700 mm. Plant genomic DNA was extracted by a cetyltrimethylammonium bromide (CTAB). Leaf tissue (150 mg) was ground in liquid nitrogen, the powder was transferred to a 2 cm^3 Eppendorf tube, mixed with 0.9 cm^3 of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.0018 cm^3 β-mercaptoethanol, 2 % CTAB), and incubated at 65 °C for 20 min. DNA was extracted with one volume of a chloroform:isoamyl alcohol mix (24:1, v/v) and centrifuged at 12 000 *g* for 10 min at 4 °C. The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with an equal volume of cold isopropanol and kept at -20°C for 10 min. Then centrifuged at 12 000 *g* for 10 min at 4°C, and the supernatant was discard, DNA was then spooled out and washed with 1 M ammonium acetate and 100 % ethanol. The cleaned DNA pellet was air dried and dissolved in 0.1 cm^3 of 0.1× TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). After an addition of 0.005 cm^3 of RNase (10 mg cm^{-3}), and an incubation for 30 min at 37 °C, the DNA concentration was quantified by DNA fluorimeter and kept at -80 °C until use.

To match more loci at the genomic level, eight RAPD and four ISSR primers were tested. The designed RAPD and ISSR selected primers (Table 1) from list of *Operon Technologies* were synthesized in our department using DNA-synthesizer (*Polygen*, Germany).

For RAPD and ISSR markers, the amplification reaction was carried out in 0.025 cm^3 reaction volume containing 1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 25 ng primer, 1.5 U Taq DNA polymerase and 30 ng template DNA. PCR amplification was performed in a *T-gradient* thermal cycler (*Bio-Rad*, Hercules, USA). Programmed to fulfill 42 cycles (for RAPD analysis) or 35 cycles (for ISSR analysis or RAPD-ISSR combinations) after an initial denaturation cycle for 4 min at 94 ºC. Each cycle consisted of a denaturation step at 94 ºC for 1 min, an annealing step for 2 min at 35 ºC (for RAPD analysis) or at 52 ºC (for ISSR analysis) or at 38 ºC (for RAPD-ISSR combination analysis), and an extension step at 72 ºC for to 2 min, followed by extension cycle for 7 min at 72 ºC in the final cycle. The PCR products were separated on a 1.5 % ethidium bromide-stained agarose (*Bio-Rad*) in 0.5× Tris-borate-EDTA (TBE) buffer. Electrophoresis was performed for 2.5 h at 85 V and visualized with a UV transilluminator.

Table 1. RAPD and ISSR primers tested in this study in terms of primer sequence and G+C content.

Primer		Primer sequence 5' - 3'	G+C content [%]
RAPD	OPA18	AGGTGACCGT	60
	OPB01	GTTTCGCTCC	60
	OPC08	TGGACCGGTG	70
	OPC15	GACGGATCAG	60
	OPE04	GTGACATGCC	60
	OPE18	GGACTGCAGA	60
	OPQ18	AGGCTGGGTG	70
	OPR12	ACAGGTGCGT	60
ISSR	ISSR 1	(GACA)4	50
	ISSR 2	(AG)8TC	50
	ISSR 3	(AG)8GTG	53
	ISSR 4	(AC)8T	47

Our results demonstrated that new bands were revealed by proper mixing of ISSR and RAPD primers in multiplex PCR reactions. As shown in Fig. 1, new loci yielded by using the combinations of ISSR $(AG)_8TC$ with RAPD primers (OPE04, OPE18, OPQ18, OPC08, OPC15 and OPR12). ISSR primers used in R-ISSR analysis with

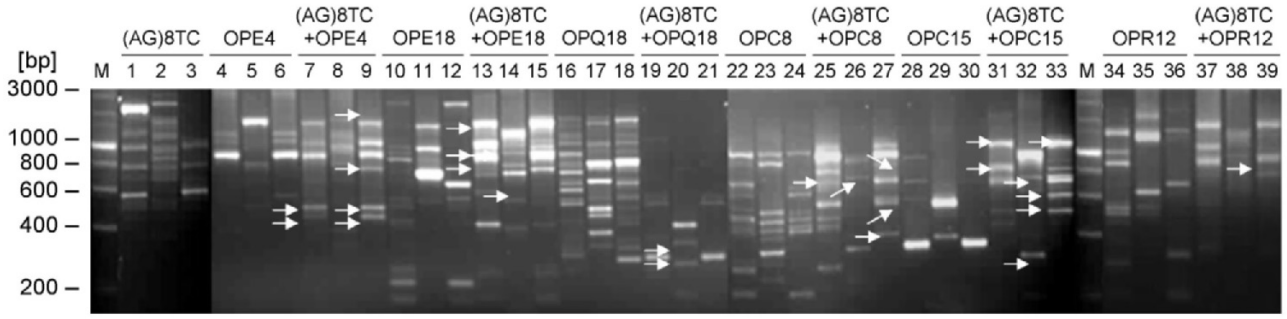

Fig. 1. Combined use of different RAPD primers with ISSR primer $(AG)_8TC$ for the detection of polymorphisms among the three genotypes of *A. macrostachyum* (1, 2 and 3) to show that primer mixing could reveal new genomic loci other than those revealed by ISSR and RAPD alone. M is DNA molecular mass marker. *Arrows* indicate new bands produced as a result of primer mixing.

RAPD primers could be easily developed from di- and tetra-nucleotides with 1, 2 or 3 anchoring bases with approximately 16 - 19 bp. ISSR primer $(AG)_8TC$ gave a highly informative pattern when combined with different RAPD primers comparing to other tested primers (Fig. 1). An especial attractive feature of ISSR analysis is its flexibility in terms of experimental design: the number of generated amplicons may be optimized by changing the number of the core repeat units and anchoring bases (Liu and Wendel 2001). Ye *et al.* (2005) hypothesized that if RAPD primers were included with ISSR primers in the same PCR reactions, new amplicons will be produced from extensions of ISSR and RAPD primers because they represent two different kinds of information about genomic sequences. There may be regions in which a single RAPD or ISSR primer will fail to amplify the DNA, but when they are combined, the distance between their binding sites and their orientation are both correct for effective amplification by PCR. The concept is based on the fact that primers for ISSR and RAPD elicit different genomic information, and the combination between these two kinds of primers in the same PCR reactions might reveal new genomic loci that could not be detected with either technique alone. The combined using of ISSR primer and RAPD primers produced a new band in the background of the former bands (Ye *et al*. 2005). It is interesting to note that the highest informative pattern was amplified by the combination of ISSR primers (for the four tested primers) with RAPD OPE04. However, the less informative profile was observed in the case of ISSR $(AG)_8TC$ and $(GACA)_4$ primers combined with RAPD OPR12. There was no band found to be polymorphic when ISSR primer $(AG)_8GTG$ was combined with RAPD OPR12. Since primer specificity determinants are located within the first eight nucleotides at the 3' end (Caetano-Anollés 1994), anchoring primers at their 3' ends will lower the number of sequences which have homology to the primers, thus producing distinct bands (Parsons *et al*. 1997). The success in specific amplified bands also depends on the anchoring motif. For example, while $(AG)_8T$, $(AG)_8C$ and $(AG)_8G$ primers produced smeared profiles, the AG repeat primers anchored with YT or YA produced clear and distinct PCR products (Pharmawati *et al*. 2005). In our results we found that the AG repeat primers anchored with GTG or TC produced clear and distinct bands. These results were in accordance with Bornet *et al*. (2002) who found that even though $(AG)_8GTG$ and $(AG)_8TC$ primers were composed of the same unit $(AG)_8$, ISSR amplifications gave completely different patterns in potato. ISSR primer $(GACA)_4$ tested in this study gave a less informative patterns when it was used alone [% polymorphic level was 100, 93.75, 93.33 and 90.91 for ISSR primers $(AG)_8GTG$, $(AC)_8T$, $(AG)_8TC$ and $(GACA)_4$, respectively (unpublished)]. Comparing to other arbitrary primers like RAPDs, ISSRs offer enormous potential for resolving intra- and intergenomic relationships (Zietkiewicz *et al*. 1994, Behera *et al*. 2008). As reported in the present study, RAPD primers could be successfully used with ISSR primers for detection of new genomic loci that could not be revealed in the case of using each fingerprint alone. In conclusion, R-ISSR is a powerful tool to assess the genetic diversity of *A. macrostachyum* species in Syria. Future research on the population and evolutionary genetics of the *A. macrostachyum* species is needed since these accessions present considerable interest for genetic studies and plant improvement. Moreover, they have a potential use for reclamation of salt affected lands, a major problem in Mediterranean basin.

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