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ORIGINAL ARTICLE

APPLICATION OF SRAP MARKERS FOR THE IDENTIFICATION OF GENETIC DIVERSITY WITHIN *SALIX* CLONES

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ABSTRACT: The evolutionary history of *Salix* genus and the relationships among the species and accessions are controversial and not well understood. In this study, SRAP markers were used to establish the level of genetic relationships and polymorphism across eleven clones of two *Salix* species collected from 9 different regions of West Azarbaijan province. The SRAP analysis with 22 anchored primers generated 116 scorable loci, of which 84 were polymorphic (77%). Jaccard similarity coefficient ranged from 0.22 to 0.74. The Complete linkage cluster was performed and dendrogram drawn by help of NTSYS pc 2.02 software which revealed three main clusters. According to our results, there is a relatively high genetic distance across *Salix* clones in West Azarbaijan province of Iran. Furthermore, it could be inferred that SRAP markers are suitable tools for the evaluation of genetic diversity and relationships within the *Salix* genus.

Key words : Genetic diversity, PCR, polymorphism, *Salix,* SRAP

INTRODUCTION

The family Salicaceae is a natural taxon consisting of 3 genera and 620 species distributed mainly in Northern hemisphere (Qureshi et al., 2007). The genus *Salix* is a large, taxonomically complex, with 350 to 500 species worldwide in form of deciduous and dioecious trees or shrubs (Argus, 1997). *Salix* occurs largely in the Northern Hemisphere with center of abundance in China and former Soviet Union, where there are about 270 and 120 species, respectively. There are 103 species in North America and 65 species in Europe (Argus, 1999*).* Recently, 31 species of the *Salix* genus were reported in Iran (Maassoumi, 2009). Willow trees are one of the important biomass products that named as biological filter in order to metal accumulation and destruction of organic pollutions in soil (Kuzovkin and Volk, 2009). Interest in growing of willows for energy, production of high-value chemicals and other bio-based products have been increased in the United States during the past two decades (Abrahamson et al., 1990). The leaves and especially barks are a rich source of salicylates. Therefore, the use of natural salicylates derived of willow bark is necessary for the economic fabrication of herbal drugs in the pharmaceutical industry (Förster et al., 2009). Morphological identification of willow clones and species is frequently problem due to intra-specific variation, outward similarities and developmental variability. Hybridization is an important source of variability within *Salix* species that occurs naturally and also may be induced through cross – pollination (Zalesny and Bauer, 2007).

However, identifying the genetic basis of agronomic traits presents a formidable challenge in willow. There are 19 chromosomes in the haploid set and the genus is very heterogeneous. Many species are polyploids and numerous inter-specific hybrids occur at ploidy levels.

Different DNA-based molecular marker systems have been used for marker assisted selection in plant breeding (Mukhlesur et al., 2010). An interesting modified marker technology termed as sequence-related amplified polymorphism (SRAP) (Li and Quiros, 2001) is similar to RAPD marker, but with preferential random amplification of coding regions in genome. The SRAP technology is a newly developed method, which is based on two-primer amplification and preferentially amplifies open reading frames (ORFs) (Li and Quiros, 2001). With the unique primer design, SRAP markers are more reproducible, stable compared with other molecular marker techniques. SRAP markers have been demonstrated to be more powerful for revealing genetic diversity among closely related cultivars than SSR, ISSR and RAPD markers (Budak et al., 2004).

However, up to now, the SRAP molecular marker had not been used in the genealogical classification of *Salix*. Therefore, we employed SRAP markers to examine their potential for genetic diversity study in *Salix* and

to determine relationships among *Salix* clones landraces in west Azarbijan province.

MATERIALS AND METHODS

PLANT MATERIALS

Leaves of *Salix* clones were collected from 9 different regions of West Azarbaijan province of Iran and transferred to -80 °C freezer in Biotechnology Research Center of Urmia University (Table 1).

DNA EXTRACTION

Genomic DNA was extracted from fresh leaf tissue using the cetyltrimethyl ammonium bromide (CTAB) method. For each DNA sample, approximately 0.3 g fresh leaf tissue was placed into liquid nitrogen, crushed rapidly in a mortar, and transferred to a 2.0 ml tube. 0.7 ml 2 × CTAB buffer (0.5 M EDTA, 1 M Tris HCl, pH 8, 5 M NaCl, 2% CTAB, and 2% B-mercaptoethanol) was added to the tubes, mixed, and incubated at 65 C for 60 min. After incubation, the samples were cooled to room temperature and centrifuged at 14,000 rpm for 10 min, followed by two extractions with 0.3 ml chloroform–isoamyl alcohol (24:1), and precipitated with 2 volumes of 100% Ethanol at -20°C. The pellet was washed twice with 1 ml 75% ethanol. The DNA pellet was re- suspended in 50 µl ddH2O and stored at -20°C. The resulting DNA quality was detected with a 1% agarose-gel stained with ethidium bromide and quantified using a spectrophotometer. The DNA was diluted to 30 ng /µl and stored at -20°C to be used as PCR templates.

SRAP AMPLIFICATION CONDITIONS AND DETECTION

In this assay, 30 different primer combinations including 5 forward and 8 reverse (Cinnagen Tehran, Iran) were examined. After initial tests, 22 out of 30 primer combinations were chosen for amplification of *Salix* genome (Table 2). Each 25 µl PCR reaction mixture consisted of 30 ng genomic DNA, 0.75 µl dNTP (10 mM), 1 µl MgCl2 (50 mM), 0.5 µl of each FWD and REV primers (100 µM), 2.5 µl 10 x Taq buffer, and 0.5 µl Taq DNA polymerase (5u / µl). PCR was performed in a Veriti 96 well Thermal cycler (Applied biosystems, USA). The reaction procedure was as follows: One cycle of 5 min at 94 ˚C, 5 cycles of three steps: 1 min of denaturation at 94 ˚C, 1min of annealing at 32˚C and 1.5 min elongation at 72 ˚C. In the following 35 cycles, the annealing temperature increased to 55 ˚C, with a final elongation step of 10 min at 72 ˚C. PCR products were separated on 3% agarose-gel stained with ethidium bromide for 50 min. A 50 bp plus DNA Ladder Marker (Fermentas) was used as the size marker. Electrophoresis conditions were held at a constant voltage of 50 V for 5 h at room temperature.

DATA ANALYSIS

The data obtained by scoring the presence (1) or absence (0) of amplified fragments from the SRAP profiles with different individual as well as collective primers. Data were entered in a MICROSOFT EXCEL spreadsheet to create a binary matrix. The binary matrix was analyzed using the NTSYSpc (Numerical Taxonomy System, version 2.02 Rohlf, 1998). The SIMQUAL program was used to calculate the Jaccard's coefficient. A tree was constructed based on the similarity matrix using the complete linkage. The PIC value was calculated using the formula $PIC=1-\Sigma pi^2$, where Pi is the frequency of the i^{th} allele (Smith *et al.,* 1997).

RESULTS AND DISCUSSION

A total of 116 reproducible fragments were observed (Table 3) which 84 fragments were polymorphic (77%). The number of amplicons produced by each primer set ranged from 1 (ME3/ EM19) to 10 (ME1/EM4), with an average of 5.3 amplicons / primer set. The number of polymorphic amplicons ranged from1 (ME3/EM19) to 8 (ME4/EM1), with an average of 4 amplicons /primer set. The percentage of polymorphic markers produced by each primer pair ranged from 28.5% (ME1/EM20) to 100% (7 pairs in Table 3). The results obtained with the primer pair ME1/EM4 is shown in Figure 1. Genetic similarity between different clones ranged from 0.22 (between clones *Salix acmophylla.1* and *Salix excels*.4) to 0.74 (between clones *Salix excels*.5 and *Salix excels.6*) (Table 4). These results indicate a high level of genetic distance among species of willow. The lowest and highest PIC (polymorphism information content) values were 0.154 and 0.460 for primer combinations of Me1/ Em20 and Me4/Em20, respectively. Polymorphic information is one of the important parameters in terms of discriminatory power. The high content of this criterion implies a high polymorphism and existence of rare alleles in a marker locus, indicating a high discriminatory power of the marker. The value of 0.248 was detected as mean PIC value (Table 3). Based on cluster analysis, three main clusters were developed. The first cluster included S.*acmophylla*.1, *S.acmophylla*.3, S.*acmophylla*.4, *S. excels.*5; *S.excels*.6 The cluster II contained S.*acmophylla.*2, S.*acmophylla*.5 and S.*excels*.4. The S.*excels*.1, S.*excels*.2 and S.*excels*.3 were grouped in cluster III (Figure 2).

With attention to genetic diversity importance in survival of valuable genetic reserves of one region and its role in genetic development, study of genetic diversity is necessary. In other words, scientific studies in ecology and evolution are dependent on genetic diversity survey. Better conservation of forests needs the knowledge of genetic diversity between populations. Selection of populations with higher genetic diversity is suitable for genetic conservation. Due to hemisphere changes in the world, trees are threatened because of natural accidents like flood, drought, globe warming and several types of stresses. DNA based markers such as RFLP, RAPD, SSR and AFLP are used for ecological, evolution, categorization, phylogenetic and genetics studies in plant science. Each of the marker systems has advantages and disadvantages. RFLP marker has been hired in genetic mapping and study of diversity in repairing programs and select of homozygotes and heterozygotes. But RFLP technique has limitation because of high primers preservation cost, time consuming reactions, complexity and dangerous (Botstein et al., 1980). The complexity is duo to the fact that each of the digestion, ligation and multiplication are indispensable (Feng et al., 2009). Beside, genomic DNA methylation may cause false polymorphism when a restricted enzyme sensitive to methylation is used. The other concern in selection of a marker system, especially in the case of novel markers, is the separation ability of specific DNA bands (Haanstra et al., 1999). For example, RAPD is a simple method to fingerprint genomic DNA, but poor consistency and low multiplexing output limit its use. SSR has the advantage of producing mostly co-dominant markers; however, the development of these is considerably expensive and time-consuming (Li and Quiros, 2001). Amplified Fragment Length Polymorphism (AFLP) marker system has been extensively used for genomic fingerprinting, because it is rapid and has high reproduction potency and polymorphism. But it has two disadvantages containing length method and high cost (Vos et al., 1995).

SRAP marker is a simple and efficient marker system that contoured in order to reproduction of open reading frames (ORF). In comparison with other molecular markers, SRAP has been demonstrated to be a useful tool for population genetic studies (Li and Quiros, 2001, Ferriol et al., 2003, Esposito et al., 2007). Ferriol et al. (2003) reported that the information given by SRAP markers was more concordant with the morphological variability and the evolutionary history of the morphotypes than that of AFLP markers. This may be because the SRAP preferentially amplifies ORFs, which is significant for gene and genetic diversity. As a relatively new marker system, SRAP has been used in genetic studies on plant species such as *Cucurbita pepo* (Ferriol et al., 2003), *Brassica oleracea* (Li et al., 2003), *Brassica rapa* (Rahman et al., 2007) and *Celosia argentea* (Feng et al., 2009). Feng et al. (2009) studied genetic variation of *Celosia argentea* population by SRAP marker. Ten primers produced 54.2% polymorphism on average with genetic similarity ranging from 0.38 to 0.90 among populations. Talebi et al. (2012) observed 83% polymorphism investigating genetic variation of *Pistacia* and Han et al. (2012) detected 71.85% polymorphism level in genetic variation study of *Paeonia suffruticosa* based on SRAP marker.

Furthermore, in comparison to other randomly amplified markers, the SRAP was found to be distributed more evenly throughout the whole genomes. This is suitable for gene localization, genetic map construction and transcription mapping (Li et al., 2003). Cluster analysis divided the 11 varieties into three groups. The first group contained S.ac.1, S.ac.3, S.ac.4, S.e.5 and S.e.6 clones. From geographical point Urmia, Miandoab and Naghade clones had higher analogy. The S.ac.2, S.ac.5 and S.e.4 were placed in second group. Third cluster contained *S.excelsa* species including S.e.1, S.e.2 and S.e.3 grown in Salmas, Piranshahr, and Khoy. The *Salix.acmophylla.1*-from Urmia and *Salix.excels.4*-from tekab showed the lowest similarity coefficient of 0.22 and was grouped in separate clusters. Due to the geographical distance between these locations, dissociation of these two clones is expected. The highest similarity belonged to *Salix excels.5*– from Oshnavieh- and *Salix excels.6*–from Miandoab with the similarity coefficient of 0.74 compared to the other samples. Therefore, in order to produce higher heterosis level and aggressor segregation, varieties and cultivars belong to different clusters can be chosen for higher yield.

The results obtained from clones and cultivars of *Salix* samples showed suitable genetic diverse for future breeding program. Thus, clones with genetic differences can be used in the hybridization program. *Salix acmophylla.*1 and *Salix excels.*4 clones showed a higher genetic distance in this study, therefore their selection is suggested as parents to produce hybrid cultivar.

CONCLUSION

In general, study of *Salix* clones using SRAP marker showed that marker can be useful in polymorphism identification and germplasm

management. The levels of polymorphism revealed by these clones can be tagged to genetic mapping of populations for economically important traits and can be useful for selecting parents for breeding. Furthermore, genus *Salix* has a widespread geographic distribution duo to widely dispersed seeds and both sexual and asexual reproduction which are characteristics that tend to be associated with large genetic diversity (Hamrick et al., 1992). We hope our findings could be beneficial in *Salix* germplasm management in Iran.

Table 1: *Salix* clones, sex and origin of collection

N0.	Species	Code	Sex	Origin
1	*S.acmophylla.1*	S.ac.1	Female	Urmia
2	*S.acmophylla.2*	S.ac.2	Male	Mahabad
3	*S.acmophylla.3*	S.ac.3	Male	Miandoab
4	*S.acmophylla.4*	S.ac.4	Male	Nagadah
5	*S.acmophylla.5*	S.ac.5	Female	Oshnavieh
6	*S.excelsa1*	S.e.1	Female	Salmas
7	*S.excelsa2*	S.e.2	Male	Piranshahr
8	*S.excelsa3*	S.e.3	Male	Khoy
9	*S.excelsa4*	S.e.4	Male	Takab
10	*S.excelsa5*	S.e.5	Female	Oshnavieh
11	*S.excelsa6*	S.e.6	Male	Miandoab

Table 2: Forward and reverse SRAP primers used for this study

Primer	Type	Sequence (5'-3')
ME1	Forward	5'-TGAGTCCAAACCGGATA-3'
ME2	Forward	5'-TGAGTCCAAACCGGAGC-3'
ME3	Forward	5'-TGAGTCCAAACCGGAAT-3'
ME4	Forward	5'-TGAGTCCAAACCGGACC-3'
ME8	Forward	5'-TGAGTCCAAACCGGTGC-3'
EM1	Reverse	5'-GACTGCGTACGAATTCAAT-3'
EM3	Reverse	5'-GACTGCGTACGAATTCGAC-3'
EM4	Reverse	5'-GACTGCGTACGAATTCTGA-3'
EM6	Reverse	5'-GACTGCGTACGAATTCGCA-3'
EM17	Reverse	5'-GACTGCGTACGAATTCGAG-3'
EM18	Reverse	5'-GACTGCGTACGAATTCGCC-3'
EM19	Reverse	5'-GACTGCGTACGAATTCTCA-3'
EM20	Reverse	5'-GACTGCGTACGAATTCTCC-3'

Table 3: Polymorphism based on 22 primer combinations

Primer combination	Total fragments	Polymorphic bands	%Polymorphic fragments		PIC
ME1-EM3	7	5		71.42	0.217
ME1-EM4	10	7	70		0.302
ME1-EM6	5	4	80		0.296
ME1-EM17	4	3	75		0.169
ME1-EM18	3	3	100		0.218
ME1-EM19	5	2	40		0.172
ME1-EM20	7	2	28.5		0.154
ME2-EM3	8	6	75		0.292
ME2-EM6	7	4	57.14		0.168
ME3-EM1	9	7	78		0.314
ME3-EM3	4	2	50		0.163
ME3-EM4	9	7	78		0.217
ME3-EM6	3	2	67		0.218
ME3-EM17	6	6	100		0.313
ME3-EM19	1	1	100		0.192
ME3-EM20	1	1	100		0.394
ME4-EM1	9	8	89		0.280
ME4-EM3	7	4	57.14		0.216
ME4-EM6	4	3	75		0.198
ME4-EM17	1	1	100		0.192
ME4-EM20	1	1	100		0.460
ME8-EM1	5	5	100		0.405
Total	116	84	77		-
Average	5.3	4	-		0.248

Table 4: Jaccard similarity coefficient among *Salix* clones

	S.ac.1	S.ac.2	S.ac.3	S.ac.4	S.ac.5	S.e.1	S.e.2	S.e.3	S.e.4	S.e.5	S.e.6
S.ac.1	1										
S.ac.2	0.3953	1									
S.ac.3	0.6071	0.5116	1								
S.ac.4	0.5357	0.5000	0.5484	1							
S.ac.5	0.2537	0.5294	0.3485	0.2985	1						
S.e.1	0.3077	0.4490	0.3659	0.3171	0.3768	1					
S.e.2	0.3265	0.4912	0.4286	0.3077	0.4533	0.5200	1				
S.e.3	0.2679	0.4444	0.3818	0.2759	0.5753	0.4643	0.6000	1			
S.e.4	0.2258	0.5238	0.3065	0.3167	0.5190	0.3182	0.4638	0.5507	1		
S.e.5	0.6154	0.4419	0.5161	0.5517	0.2836	0.3250	0.3137	0.2586	0.3220	1	
S.e.6	0.5517	0.4773	0.5625	0.5484	0.3485	0.4359	0.4000	0.3571	0.3729	0.7407	1

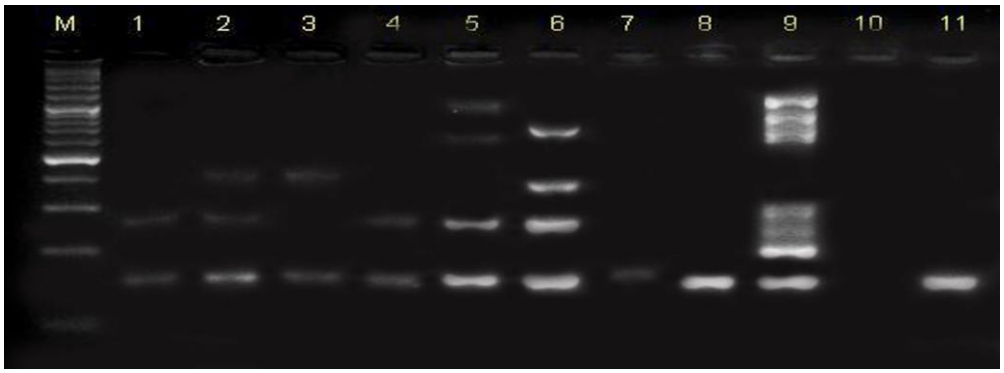

Figure 1 : Agarose gel of PCR-SRAP products among 11 clones of salix using ME1-EM4. M=Ladder 100-3000 bp. Numbers on the gel are indicative of willows listed in table 1.

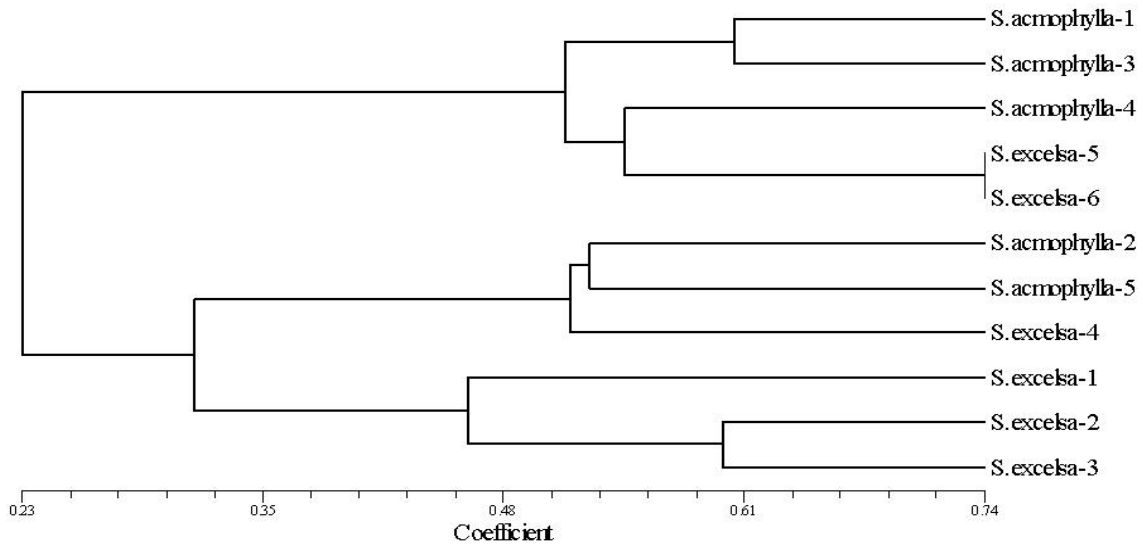

Figure 2 : Complete linkage dendrogram of genetic relationships among the analyzed clones of willow

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