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Research Article

Genetic Diversity among Eight Species of Willow (*Salix* spp.) from Iran Based on SRAP Markers

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Abstract

This study reports the application of sequence-related amplified polymorphism (SRAP) technique in characterization of 8 species of *Salix* (spp) from Iran by screening 22 primer combinations (PCs). Twenty two SRAP primer combinations could amplify 116 scorable loci, of which 107 bands were polymorphic. The amplified DNA fragments were used for calculation and statistical analysis. The Complete linkage cluster was performed and dendrogram drawn with the help of NTSYS pc 2.02 software which revealed two main clusters and several sub-clusters. This investigation showed that genetic distance was relatively significant among these species. The Jaccard similarity coefficient ranged from 0.18 to 0.55. The results also propose that the SRAP marker is a useful tool for evaluation of genetic diversity and relationships among different *Salix* species.

Keywords: Genetic diversity, PCR, polymorphism, Salix spp, SRAP

Introduction

The genus *Salix* belongs to the Salicaceae family with 350 to 500 species worldwide. *Salix* occurs largely in the Northern Hemisphere with a centre of abundance in China and the former Soviet Union (Argus, 1997). They are compatible to various environmental conditions, in common propagated in cool and modest regions, but they also found in tropical and mid-tropical regions (Kuzovkinaand Volk, 2009). Recently, 31 species of the *salix* genus were reported in Iran (Maassoumi, 2009). The plant is distributed in different regions of Iran such as Alborz, Karaj, Gachsar, Chaloos, Azarbayjan, Hamedan and so on. The Salicaceae family especially the *Salix* genus has economic importance. Leaves and especially barks of *Salix* species like *Salix alba* are a rich source of salicylic acid, which has antipyretic and analgesic properties. Therefore, willow has potential in the pharmaceutical industry and its natural salicin derivative can be used as medicine (Förster et al., 2009). Willows in general are perennial, outcrossing, insect pollinated species with a long life history and

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overlapping generations, all contributing to a relatively high degree of heterozygosity and intra- as well as inter-population genetic variation (*Kopp* et al., 2002). The genus is very heterogeneous, although the haploid chromosome number is *n*=19, but many species are tetraploid and higher ploidy levels are common (Hakansson, 1995). Assessment of genetic diversity on the basis of morphological features is time consuming and may be influenced by environmental conditions (Mullis & Falcona, 1987). Therefore, use of genetic markers could serve as a viable alternative to assess genetic diversity because genetic markers can relatively reduce ambiguousness of morphological markers (Paplauskiene & Dabkevicene, 2008). Analysis of genetic diversity and relations between members of the population is important for categorization. Various DNA-based molecular marker systems were used to study genetic diversity (Rahman et al., 2010). Sequence related amplified polymorphism (SRAP) technology preferably amplifies open reading frames (ORFs) (Li & Quiros, 2001).In fact, SRAP works like a random amplified polymorphic DNA (RAPD) marker, but SRAP targets specific regions of the genome (Dalong et al., 2010). With a unique primer design, SRAP markers are more sustainable and are less complex compared to other molecular marker techniques. Up until now, some molecular marker techniques have been applied to determine relationships among species of willows such as Randomly Amplified Polymorphic DNA (RAPD) (Barker et al., 1999, Przyborowski & Sulima, 2010), Amplified fragment length polymorphism (AFLP) (Barker et al., 1999, Hanley et al., 2002, Douhovnikoff & Dodd, 2003) and Simple Sequence Repeat (SSR) (Stamati et al.; 2003, 2007). In earlier studies, SRAP markers have been used in intra-population genetic diversity assessment of two *Salix* species (Daneshvand et al., 2014).This is the second report of the SRAP molecular marker application in the genealogical classification of *Salix* species in the world.

Materials and Methods

Fresh young leaves of 8 species of willow were collected from different regions of West Azerbaijan in the North West of Iran including Urmia, Mahabad, Nagadeh, Oshnavieh and Piranshahr (Table 1).

A modified version of the cetyltrimethyl ammonium bromide (CTAB) method was used to extract genomic DNA. Approximately 0.3 g of 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 minutes. After incubation, the samples were cooled to room temperature and centrifuged at 14,000 rpm for 10 minutes, followed

Table 1. ***Salix*** **spp species with sex and origin of collection**

No	Species	Sex	Origin
1	*S.acmophylla*	Male	Nagadah
2	*S.aegyptiaca*	Male	Urmia
3	*S.alba*	Male	Piranshahr
4	*S.babylonica*	Female	Urmia
5	*S.excelsa*	Female	Oshnavieh
6	*S.elbursensis*	Female	Urmia
7	*S.matsudana*	Male	Urmia
8	*S.triandra*	Male	Mahabad

by two extractions with 0.3 ml chloroform: isoamyl alcohol (24:1), and precipitated with 2 volume 100 % Ethanol at -20°C. The pellet was washed twice with 1ml 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 0.8 % 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.

Five forward and 8 reverse primers were purchased from a commercial source (Cinnagen Tehran, Iran). After initial tests, 22 out of 30 primer combinations were chosen for amplification of *Salix* genome (Table 2).

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

Primer	Type	Sequence
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'

The total volume of PCR reaction mixture was 25 µl, consisting 30 ngof genomic DNA, 0.75 µl dNTP (10 mM), 1 µl $MgCl_2$ (50 mM), 0.5 µl of each forward and reverse 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 minutes at 94 °C, 5 cycles of three steps: 1 minute of denaturation at 94 °C, 1 minute of annealing at 32 °C and 1.5 minute elongation at 72 °C. In the following 35 cycles, the annealing temperature increased to 55°C, with a final elongation step of 10 minutes at 72 °C. PCR products were separated on 3 % agarose-gel stained with ethidium bromide for 50 minutes. A 100 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 hours at room temperature.

DNA fragments were scored as '1' and '0' where '1' stands for the presence and '0' for the absence of each SRAP fragment. Genetic similarities (GS) were measured by Jaccard's coefficients based on the definition of Jaccard (1908). The similarity matrix and dendrograms were constructed using the numerical taxonomy multivariate analysis system (NTSYS pc 2.02) software package. A tree was constructed based on the similarity matrix using the complete linkage. In order to see how well a cluster analysis represents the similarity matrix, a COPH module was used to transform the tree matrix to a matrix of ultrametric distances (a matrix of distances implied by the cluster analysis). Finally, the MXCOMP module was used to compare these ultra-metric similarities and similarity matrix produced by complete analysis. As such, the marker index was the sum of the polymorphism information content (PIC) values for all the selected markers produced by a particular primer combination. 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

Thirty SRAP primer pairs were tested for their efficacy and 22 primer combinations generated polymorphic bands. A total of 116 amplicons were produced, of which 107 were polymorphic (93.4 %). 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 also ranged from 1 (ME3/EM19) to 10 (ME1/EM4), with an average of 4.8 amplicons/primer set. The percentage of polymorphic markers produced by each primer pair ranged from 57.1 % (ME1/EM20) to 100 % (Table 3). The results obtained with the primer pair ME1-EM3 is shown in Figure 1. Genetic similarity between different species ranged from 0.18 (between *Salix acmophylla* and *Salix triandra*) and (between *Salix triandra* and *Salix excels)* to 0.55 (between *Salix acmophylla* and *Salix excelsa*) (Table 4). These results indicate a high level of genetic diversity among species of willow. Based on

Table 3. Polymorphism based on 22 primer combinations

Primer combination	Total fragments	Number of Polymorphic bands	Polymorphism %	PIC*
ME1-EM3	7	7	100	0.366
ME1-EM4	10	10	100	0.343
ME1-EM6	5	5	100	0.331
ME1-EM17	4	3	75	0.164
ME1-EM18	3	3	100	0.303
ME1-EM19	5	5	100	0.301
ME1-EM20	7	4	57.1	0.125
ME2-EM3	8	7	87.5	0.360
ME2-EM6	7	7	100	0.380
ME3-EM1	9	9	100	0.337
ME3-EM3	4	4	100	0.218
ME3-EM4	9	8	88.9	0.285
ME3-EM6	3	3	100	0.386
ME3-EM17	6	5	83.3	0.250
ME3-EM19	1	1	100	0.375
ME3-EM20	1	1	100	0.219
ME4-EM1	9	8	88.9	0.379
ME4-EM3	7	7	100	0.264
ME4-EM6	4	3	75	0.312
ME4-EM17	1	1	100	0.469
ME4-EM20	1	1	100	0.501
ME8-EM1	5	5	100	0.432
Total	116	107	93.4	-
Average	5.3	4.8	-	0.323

*Polymorphism Information Content

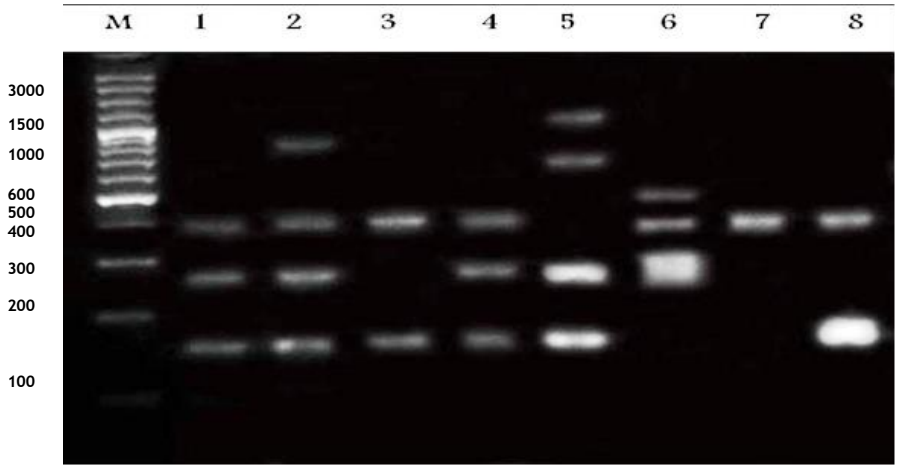

Figure 1. PCR products of *Salix* species using ME1-EM3 primer combination. Numbers on the gel are indicative of willows listed in table 1.M: DNA ladder 100 bp plus

cluster analysis, two main clusters were developed. The first cluster included *S.acmophylla*, *S.excelsa* and *S.aegyptiaca*. The second cluster contained *S.alba*, *S.babylonica*, *S.triandra*, *S.elbbursensis* and *S.matsudana* (Figure 2). The mean of polymorphism information content (PIC) of SRAP PCs was 0.323 with a range of variation from 0.125 (Me1-Em20) to 0.501 (Me4-Em20) (Table 3).

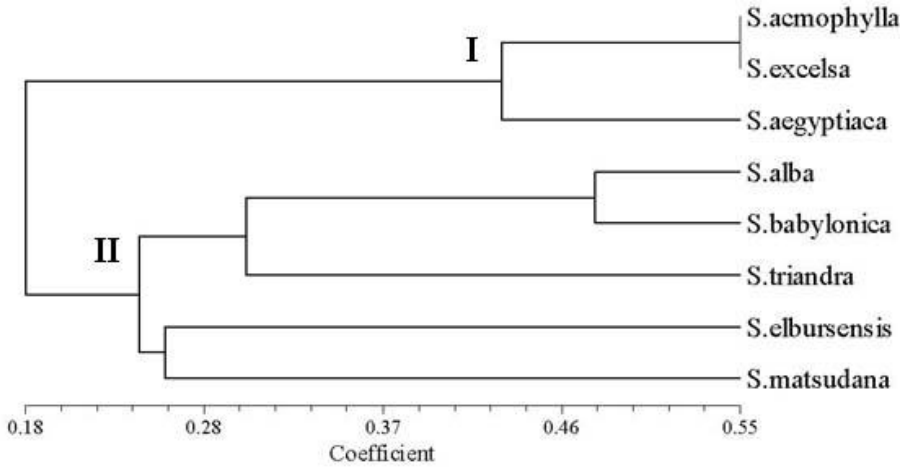

Figure 2. Complete linkage dendrogram of genetic similarity among the analyzed species of willow

Willow genus is compatible with heterogeneous life conditions during its long life period. The use of molecular markers is useful in determining genetic variation (Englbrecht et al., 2000; Whitehead et al., 2003; Liu et al., 2006). The DNA based markers such as RFLP, RAPD, SSR and AFLP are used for ecological, evolution, categorization, phylogenetic and genetics studies in plant science. The SRAP markers proved that they are potent and vigorous markers in identifying genetic diversity between species and homogeneous varieties compared to SSR, ISSR and RAPD markers. Therefore, the SRAP marker is suitable for genetic diversity studies because of simplicity, high confidence, high patient valence and simple sequence to selected bands (Li & Quiros, 2001). Bodek et al.,(2004) compared four markers based on genetic diversity potency in *Valeriana Officinalis*, based on their study that four markers potencies in genetic diversity definition was respectively: SRAP> SSR> ISSR> RAPD. Very few studies have been led to analyze the genetic variation of the genus *Salix* and its species. Barker et al., (1999) used RAPD and AFLP analyses to characterize the genetic diversity in potential biomass of willow.

Table 4. Jaccard's similarity (upper part)/distance (lower part) coefficient among *Salix* species.

	S.acmophylla	*S.aegyptiaca*	*S.alba*	*S.babylonica*	*S.excelsa*	*S.elbursensis*	*S.matsudana*	*S.triandra*
S.acmophylla	1							
S.aegyptiaca	0.423	1						
S.alba	0.340	0.446	1					
S.babylonica	0.364	0.391	0.477	1				
S.excelsa	0.552	0.475	0.294	0.345	1			
S.elbursensis	0.239	0.392	0.279	0.344	0.333	1		
S.matsudana	0.233	0.264	0.254	0.302	0.300	0.255	1	
S.triandra	0.183	0.291	0.297	0.313	0.185	0.241	0.253	1
S.triandra	0.817	0.709	0.703	0.687	0.815	0.759	0.747	0
S.matsudana	0.767	0.736	0.746	0.698	0.7	0.745	0	
S.elbursensis	0.761	0.608	0.721	0.656	0.667	0		
S.excelsa	0.448	0.525	0.706	0.655	0			
S.babylonica	0.636	0.609	0.523	0				
S.alba	0.660	0.554	0					
S.aegyptiaca	0.577	0						
S.acmophylla	0							

They found both RAPD and particularly AFLP to be useful in the identification of varieties. In addition, Przyborowski et al. (2010) applied RAPD markers to evaluate the genetic diversity of the collection materials of *S.viminalis*, selected for energy generation purposes. Hanley et al. (2002) used AFLP and microsatellite markers to construct a genetic linkage map of *Salix viminalis*. The results presented in this study confirmed the efficiency of SRAP markers in determining the genetic diversity of willows. It was difficult to classify the *Salix* due to abundant hybridization in this genus. There are numerous species of *Salix* and *populous* which have not been identified definitely in Iran, as these are dioecious and their flowers are inaccessible. Such taxonomic discordance in *Salix* at the generic and subgeneric levels is caused by scarceness of informative morphological characters that can be used for systematic studies. On the other hand, because of interspecies hybridization, accurate species identification is required for determination of their range. In this study, the fingerprint patterns of the 8 willow species were analyzed (Table 1) using SRAP primers and high levels of polymorphism were detected. The nearmost genetic similarity (0.18) was obtained between species *Salix acmophylla* and *Salix triandra* followed by *Salix triandra* and *Salix excelsa*. Therefore, the primers used in our study will be useful in genetic analysis of willow accessions in germplasm holdings programmes. In dendrogram, 8 species were clustered into two main groups. Grouping of related species in one cluster presents the efficiency of SRAP marker in identification of closely related genotypes. For example, in cluster II, *S.alba, S.babylonica* and *S.triandra* species showed a close relationship. These species were all categorized in subgenus *Salix* (Maassoumi, 2009). The genetic diversity shown in this study can also be applied for identification of differences at intra-species levels. The lowest genetic similarity obtained between *Salix acmophylla* and *Salix triandra* (0.18) could be considered for breeding and germplasm conservation programmes. The results also indicate sufficient amount of genetic distance (0.45 to 0.82) among different species of the genus in Iran. Moreover, S.acmophylla collected from Naghade had high similarity to *S. exelsa* collected from Oshnavieh. These regions are geographically close.

The average of PIC for 22 primer combination obtained 0.323 ranging from 0.125 to 0.501. This criterion depends not only on the number of the polymorphic alleles in each of primer pairs, but also on the abundance of polymorphic alleles. The high value of PIC indicates the appropriate ability of selected SRAP primers in the polymorphism identification of the evaluated plant species. This value appeared to be relatively higher than other SRAP based studies, e.g. 0.14, evaluating genetic profile of *Morus* genotypes (Zhao et al., 2009) and 0.15, studying genetic variation in Saffron (Keify et al.,

2012).The high level of the PIC indicates high efficiency in differentiation of the used samples in this study that can be proposed for other similar researches. The study of dendrogram generated from cluster analysis showed high diversity among species probably due to overcrossing and separation of male and female flowers. Obviously, further investigation is needed to exploit large population collections having diverse agronomic traits in breeding improvement programmes (Feng et al., 2009).

Conclusion

In conclusion, the SRAP marker system was a simple and efficient marker system that had several advantages over other systems: simple, with a reasonable throughput rate, allows easy isolation of bands for sequencing and, most importantly, it targets ORFs, which could be adapted for a variety of purposes in different *Salix* including SCAR marker, map construction and gene chip.

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