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Genetic Diversity in *Salix viminalis* in the Kashmir Valley, India

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ABSTRACT

In Kashmir, *Salix* is cultivated on large scale. There are diverse cultivars of *Salix* found in Kashmir Valley but they are not well demarked due to similarities in certain morphological characteristics. *Salix* species is an important source of aspirin and salicylic acid. It has tremendous economic importance. Its wood is used for manufacturing different types of furniture, bats etc. Therefore, in the present study a fingerprinting technique has been used to differentiate cultivars of *Salix* found in Kashmir Valley. Among *Salix* cultivars, genetic diversity has been ascertained in the *S. viminalis* using Amplified Fragment Length Polymorphism (AFLP) technique. AFLP analysis of different *Salix viminalis* cultivars which were difficult to identify morphologically revealed that they are different cultivars of the same species. AFLP markers were able to reveal that the four *Salix* cultivars taken for study were highly diverse at genetic level. The 4 primers used generated a total of 240 bands of which 197 (82%) were polymorphic. The broad genetic base can be attributed to their out-crossing nature. Our study may be useful in identifying diverse genetic stock of *S. viminalis*, which may be conserved on priority basis.

Key words: Aspirin, salicylic acid, genetic diversity, AFLP, primer, polymorphic, genetic base, out-crossing

INTRODUCTION

The family *Salicaceae* includes three genera, *Salix, Populus* and *Chosenia* with more than 200 species in the world (Rehder, 1940). *Salix* is most abundant in the temperate regions of Northern Hemisphere. Most of the Kashmir *Salix* species have been introduced and planted from cuttings nearly all of which are represented by male plants, which make identification more difficult. The various *Salix* species were introduced primarily for wicker works and sports goods in the valley. Cricket bats are obtained from *S. alba* L. sp. *coerulea* (Sm.) Rech. and *S. fragilis* L. The important wicker willows are *S. triandra* L., *S. purpurea* L. and *S. viminalis* L.; from the twigs of these plants the baskets are made (Javeid, 1972). The bark and leaves of *Salix* also contain substantial amounts of salicylic acid, which has antipyretic and analgesic properties.

The documented history of *Salix* culture in North America started during the period from 1840-1850. The importance of selecting clones with desirable form and wood qualities that were adapted to local soils and pests was recognized in the earliest days of willow culture (Hubbard, 1904).

Growing willows (*Salix* species) in intensive culture systems for biomass can be used for energy or conversion to high value products. It is gaining increasing worldwide interest. Willows in general

are perennial, out-crossing, insect pollinated species with a long life history and overlapping generations, all contributing to a relatively high degree of heterozygosity and intra- as well as inter-population genetic variation (Kopp *et al.,* 2002). Interest in growing and using willows for energy and to produce high-value chemicals and other bio-based products increased in the United States during the past two decades (Abrahamson *et al.,* 1990).

Modern techniques, such as Amplified Fragment Length Polymorphism (AFLP) is a genetic fingerprinting technique that may be useful in predicting the likelihood that parental combinations will yield highly variable progeny. AFLP has been effectively used to fingerprint willows for genetic distance estimates and clone identity verification (Barker *et al.,* 1999) and to identify willow hybrids in natural stands (Beismann *et al.,* 1997).

The AFLP approach, which enables simultaneous analysis of a large number of marker loci throughout the genome, appears to be remarkably powerful. The objective of this work is to study the genetic relationships among different *S. viminalis* cultivars through AFLP markers.

MATERIALS AND METHODS

Plant material: For the study entitled Genetic Diversity in *Salix viminalis* in Kashmir Valley, India, a total of 4 accessions of *Salix viminalis* were collected in the year 2005 from the valley for evaluation of genetic diversity (Fig. 1). The samples studied are cultivated in different regions of the valley. These samples were also being cultivated in the Kashmir University Botanical Garden.

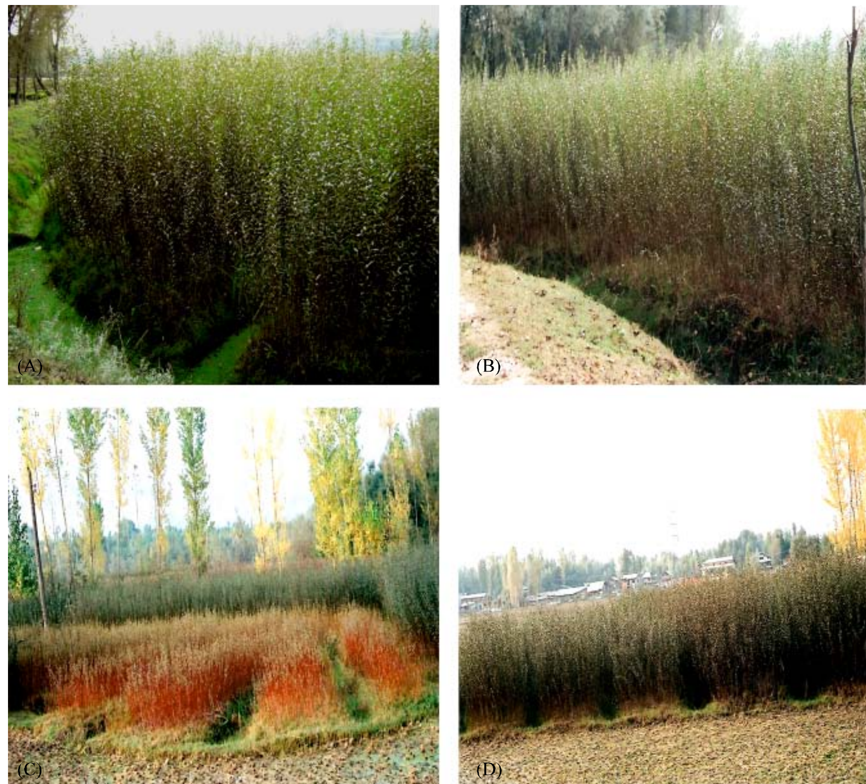

Fig. 1: General morphology of *Salix viminalis* species/cultivars in Kashmir Valley; (A) *Salix viminalis,* (B) *Salix viminalis* (white) , (C) *Salix viminalis* (red) and (D) *Salix viminalis* (black)

The analysis was carried out in order to estimate the genetic variation within the different cultivars of *Salix viminalis*.

DNA isolation: Young *Salix* leaves were collected and lyophilized (in liquid nitrogen at -196°C) over a period of 10-15 days for DNA extraction. The genomic DNA was extracted from 70-75 mg of the freeze dried leaf sample using QIAGEN Dneasy Plant Mini Kit (QIAGEN GmbH. Max-Volmerstrasse 4, 40724 Hilden, Germany). Lyophilized tissue was ground to a fine powder in pestle and mortar using liquid nitrogen. Ground tissue was transferred to an appropriately sized centrifuge tube to which 400 µL of lysis buffer (1% cetyltrimethyl ammonium bromide (CTAB), 5% polyvinyl pyrrolidone (PVP), 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl (pH-8.0) and 350 mM 2-mercaptoethanol) and 4 µL of RNase stock solution was added. Samples were mixed thoroughly by vortexing and were incubated for 10 min. at 65°C. 150 µL of precipitation buffer was added to each tube and again incubated for 10 min at -20°C. The lysate was then centrifuged at 15000 rpm for 5 min. After centrifugation the supernatant was transferred to the QIA shredder spin column and centrifugation was carried out at 15000 rpm for 2 min. Flow through fraction from the above step was transferred to a new tube without disturbing the cell debris pellet and 400 µL of precipitation buffer was added to the lysate and mixed gently. The mixture was then transferred to mini spin columns (binding columns) and centrifuged for 1 min at 8000 rpm. The flow through fraction was discarded and to the retained fraction 500 µL of wash buffer (70% ethanol) was added and centrifuged again at 8000 rpm for 1 min. Ten microliter of elution buffer was then added and incubated for 5 min at room temperature. Centrifugation at 8000 rpm for 1 min was done to elute. DNA concentration was estimated by comparison to serial dilution of a lambda DNA standard in a 1.0% agarose gel (Fig. 2).

AFLP analysis: AFLP analysis was performed according to procedure described by Vos *et al.* (1995), with a commercially available kit (AFLP®Analysis System I, Invitrogen Life Technologies, Carlsbad, CA). Approximately 200 ng DNA of each sample was digested with Eco R1/Mse1

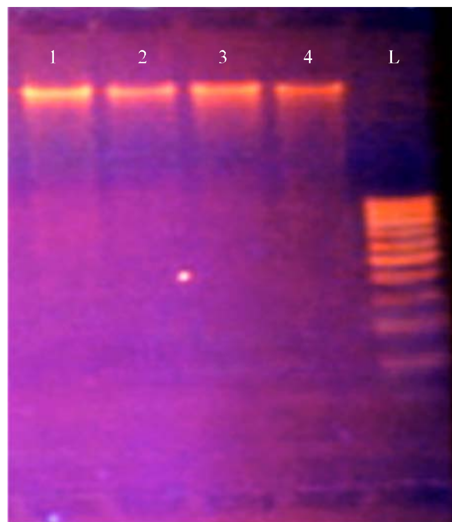

Fig. 2: Isolated DNA from 4 *Salix viminalis* species/cultivars resolved on 1.0% agarose. L represents Lambda phage DNA

restriction enzyme solution. After the ligation of the digested DNA, the reaction mixture was diluted 10 fold with TE buffer containing 10 mM Tris-HCL, pH 8.0, 0.1 mM EDTA. The number of cycles for pre-amplification reaction and selective amplification reaction was 20 and 23 cycles, respectively. The pre-amplification product was also diluted 1:50. The amplification products were separated on 6% (w/v) polyacrylamide gels containing 29:1 acrylamide : Bis-acryl-amide (Fisher Scientific, Chicago, IL), 7.5 M urea and 1x TBE buffer (1, 1, 2, 2-tetra bromoethane) used with 0.4 mm spacers and a shark-tooth comb. The gels were electrophoresed for about 20 min. with 1x TBE. Electrophoresis was performed at constant temperature and wattage (45-50°C, 100 w) for about 2.5 h. The gels were stained by silver staining process and the bands were visualized with a transilluminator (Fisher Scientific, Chicago, IL) (Fig. 3A, B and 4A, B).

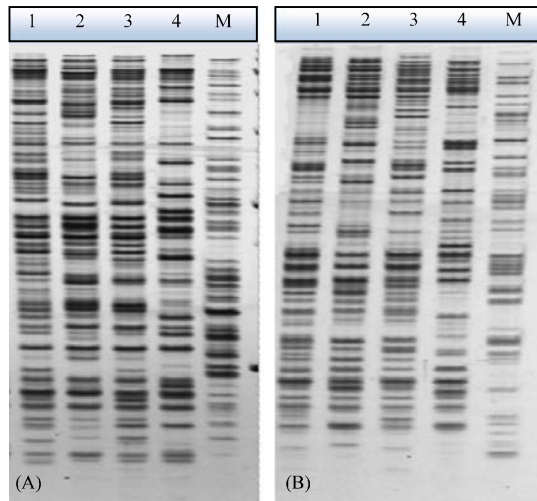

Fig. 3: AFLP fingerprints of four *Salix viminalis* species/cultivars using primers (A) 73 and (B) 74. M represents 1 kb DNA marker

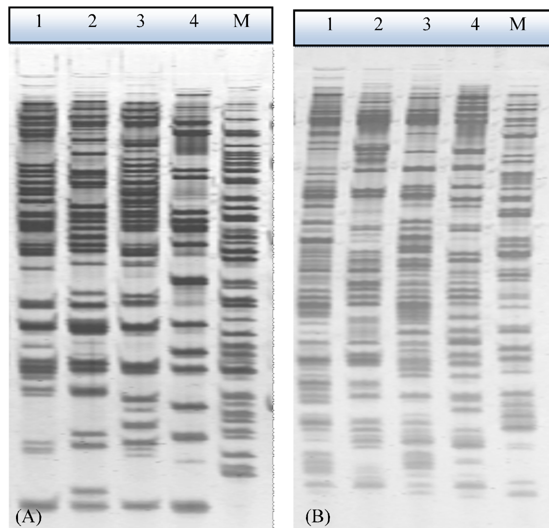

Fig. 4: AFLP fingerprints of four *Salix viminalis* species/cultivars using primers (A) 75 and (B) 76. M represents 1 kb DNA marker

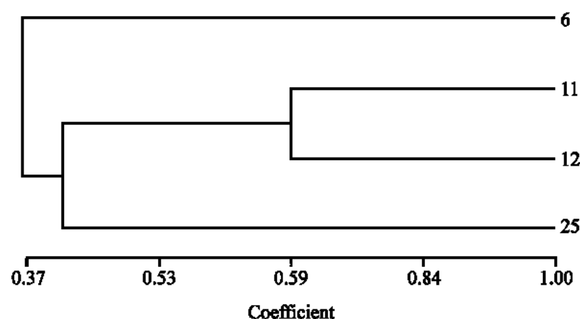

Fig. 5: Dendrogram showing diversity in *Salix viminalis* species/cultivars in Kashmir valley

Data analysis: Polymorphic AFLP markers were manually scored as binary data with presence as 1 and absence as 0. Monomorphic markers were not scored. The data was compiled by $NTSYS_{PC}$ (Numerical Taxonomy and multivariate analysis System) software version 2.0 (Exeter Software, New York). Similarity matrix was computed by Jaccard's coefficient. This analysis was also used for phylogenetic tree estimation, which was visualized as a graphical dendrogram. The resulting dendrogram provides a good estimate of the phylogeny of a particular group of organisms (Fig. 5).

RESULTS

The summary of AFLP markers produced by four primer pairs across all genotypes is given in Table 1. The four primers generated a total of 240 bands of which 197 (82%) were polymorphic over all the genotypes. The capability of different primers to generate polymorphic AFLP markers varied significantly, ranging from 40-55 polymorphic bands per primer over all the genotypes. Thereby it confirms the high multiplex ratio expected from the AFLP technique.

Similarity index was estimated using the Jaccard coefficient which revealed accessions SL-11 (*Salix viminalis* white cultivar) and SL-12(*Salix viminalis* red cultivar) sharing a similarity value of 0.69 which is highest among the four accessions studied. Accessions SL-6 (*Salix viminalis*) and SL-25 (*Salix viminalis* black cultivar) are the most diverse genotypes as revealed by the AFLP analysis showing a similarity value of 0.37. Based on genetic similarity values, a phenetic dendrogram was constructed using UPGMA (Unweighed Pair Group Method Arithmetic mean) analysis. On examination of dendrogram, it is clear that there is a large genetic variation. Accessions SL-11 and SL-12 show genetic similarity value of 0.69 while the comparison of accession SL-25 with SL-11 and SL-12 show the genetic similarity value of about 0.40 (Table 2).

AFLP results mostly confirmed that the four *Salix* genotypes studied are distinct from one another as is evident from the large genetic distances among them. However, as revealed from the study that morphologically there is lot of similarity but when these cultivars were analyzed using AFLP technique there was found difference among these cultivars.

DISCUSSION

Molecular fingerprint data have been used to identify particular parent combinations that are likely to yield desirable clones based on maximizing the amount of molecular diversity among crossed genotypes (Kopp *et al.*, 2001).

Results of AFLP analysis in this study suggest that there was in general, large variability among *Salix viminalis* cultivars. One hundred and ninty seven polymorphic loci were detected in the four *S. viminalis* cultivars using only four AFLP primer pairs. Eighty two percent of the fragment types were polymorphic. Every cultivar could be distinguished with either primer pair

Table 1: List of AFLP primers used in DNA fingerprinting of *Salix viminalis* cultivars in Kashmir valley

S. No.	Code	Primer combination	Total bands	Polymorphic bands	Polymorphism
1.	73	E-AAC, M-CAC	57	49	86.0
2.	74	E-AAG, M-CAC	65	51	78.4
3.	75	E-ACA, M-CAC	56	44	78.5
4.	76	E-ACT, M-CAC	62	53	85.4
Total			240	197	82.0

Table 2: Similarity matrix of *Salix viminalis* cultivars in Kashmir valley

	SL-6	SL-11	SL-12	SL-25
SL-6	1.00			
SL-11	0.38	1.00		
SL-12	0.38	0.69	1.00	
SL-25	0.37	0.40	0.40	1.00

alone. These results were similar to those reported for clones of *Salix viminalis* and its hybrids grown for bio-energy in Europe, where AFLP analysis of 29 willow clones with five AFLP primer pairs yielded 919 different fragment types, of which 752 (81.8%) were polymorphic (Barker *et al.*, 1999). The high percentage of polymorphic bands detected in willows in AFLP experiments reflects the low level of domestication in this genus.

Although, the number of polymorphic bands used to estimate similarity among *Salix viminalis* cultivars was relatively small in this study, there appears to be sufficient polymorphism to provide reliable estimate of genetic diversity. In a study by Zhu *et al.* (1998) about 82 to 140 polymorphic AFLP markers were sufficient to accurately estimate similarities among rice accessions. Similarly, Pejic *et al.* (1998) obtained genetic similarity between inbred maize lines which was most accurately estimated with only 150 polymorphic AFLP bands.

The number of polymorphic bands necessary to accurately estimate similarity among willows should be relatively low because of the broad geographic range from which parents were selected and the high degree of genetic variation that exists in this species (Kopp *et al.*, 2002).

Pejic *et al.* (1998) reported that 150 polymorphic bands make it possible for a researcher to reliably estimate genetic similarities among genotypes within the same species. In confirmation to this, it was found that with four primer pairs, generating 197 polymorphic bands, it was possible to fingerprint all of the four species/varieties/cultivars included in this study.

Large molecular genetic variation was observed in the current study because it includes outcrossing species and maintains large genetic variation within populations and high individual-tree heterozygosity. The same results were also reported by Aravanopoulos *et al.* (1998). Further, genus *Salix* has a widespread geographic distribution, 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).

The number of polymorphic bands necessary to accurately discriminate among individuals is determined by the degree of relatedness among individuals being compared, with distantly related individuals requiring relatively few bands, confirming the studies carried out by Tivang *et al.* (1994).

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