**RESEARCH ARTICLE**

# MICROSATELLITE MARKERS (SSR'S) FOR REVEALING POLYMORPHISM AND IDENTIFICATION AMONG WILLOWS CLONES

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**Abstract:** Microsatellite markers (SSR's) were found effective in revealing polymorphisms among twenty two different species/clones of Willows. Out of 10 SSR primers only seven primers produced SSR profiles with intense banding pattern and generated a unique set of amplification products. Out of the total 24 scorable bands, 16 showed polymorphism and eight bands exhibited monomorphism with an average of 3.43 bands per primer. A unique band of approximately 100 bp was generated by SB-80 for *S. udensis*.Jaccard's similarity correlation coefficient values was highest value (0.98) between two male genotypes of *Salix tetrasperma* [*S. tetrasperma* (TFB) and *S. tetrasperma* (LNM)] and lowest (0.64) between *S. udensis* and *S. nigra*, *S. udensis* and *S. tetrasperma* (TWE) and between *S. matsudana* (PN-722) and *S. pierotii* and 799.The dendrogram exhibited six clear clusters with *S. udensis* coming out as an outline. SSR primer namely SB-80 produced unique band present only in *S. udensis*. This information could be used for characterizing particular genotypes.

**Keywords:** Clones, Microsatellite markers, Willows

## INTRODUCTION

Genetic markers are now days routinely used to monitor the efficiency of various tree improvement activities and are necessary for the construction of genetic maps aiding breeding (White *et al*., 2007, Choudhary *et al*., 2014). Molecular markers specially RAPDs and SSRs are being applied to a greater extent in forest trees to study genetic diversity and contribute about 25 per cent and 19 per cent respectively of the total molecular markers used in forest biotech activities (FAO, 2004). Application of molecular approaches to willow tree improvement may accelerate the rate of genetic gain. Recently developed molecular techniques may enable breeders to pool of candidate breeding trees, so that time is not wasted on making crosses that have low probability of producing desirable progeny. In *Salix* various molecular markers viz.,, RAPD, SSR, AFLP have been used to assess genetic diversity for germplasm characterization (Barker *et al*., 1999). PCR based RAPDs markers can provide a significant advance in the construction and saturation of genetic maps. Many microsatellites in forest tree species have been reported for *Populus* (Cervera *et al*., 2005), *Eucalyptus* (Brondani *et al*., 2002), and *Salix* (Barker *et al*., 2003). Molecular markers can help to characterize populations, estimate genetic variability within and among wild or generated populations, and identify individuals at a young age that will express a trait at maturity. Microsatellites (SSR's) and amplified fragment length polymorphism (AFLP) have proven extremely useful in discriminating between natural populations of *S. alba*, *S. fragilis* and their hybrid *S.* x *rubens* (Beismann, 1997). Recently, a consensus linkage map for *Eucalyptus* was developed exclusively on inter-specific transferable microsatellites (Brondani *et al*., 2006).

With recently developed molecular tools, it becomes possible to identify molecular markers that are closely associated with traits of interest (QTLs). In order to understand the genetics behind variation in important traits of genetic markers (AFLPs, RAPDs, SSR, and RFLP) to *Salix* breeding a linkage map to identify QTLs affecting biomass and adaptability traits was constructed in Sweden. This was first ever report on *Salix* (Tsarouhas *et al.,* 1999; Choudhary, 2011). Later on RFLP, AFLP linkage map for *S. viminalis* and the hybrid *S. viminalis* x *S. schwerini* covering about 70 per cent of the *Salix* genome was developed (Tsarouhas, 2002). With ease of vegetative propagation, *Salix* holds a valuable advantage for QTL mapping since clonal replications could essentially increase the heratibilities of traits related to growth and biomass (Bradshaw and Foster, 1992). Similar studies can be undertaken with our indigenous tree willow and other species/clones of interest for mapping QTLs on desirable traits once data on progeny (phenotyping) on these traits become available in subsequent years. However, the polyploid nature of many willow species and difficulties achieving cross-amplification may limit the application of micro-satellites in diversity studies encompassing the wide range of species. The combination of traditional breeding fortified with advances in molecular research could advance genetic improvement of the genus and as such used in this study.

## MATERIAL AND METHODS

DNA isolation amplification was carried out for SSR analysis using standard Doyle J J and Doyle J L. 1990, protocol. A total of ten SSR primers

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synthesized by M/S Banglore Genei, India Limited were used in the present study.

**Table 1.** Nucleotide sequences of 10 SSR primers

| m., | Primer name | Sequences |
|---|---|---|
| 1. | SB-24 | FP-ACT TCA ATC TCT CTG TAT TCT |
| | | RP-CTA TTT ATG GGT TGG TCG ATC |
| 2. | SB-38 | FP-CCA CTT GAG GAG TGT AAG GAT |
| | | RP-CTT AAA TGT AAA ACT GAA TCT |
| 3. | SB-199 | FP-CTA TTT GGT CTC AAT CAC CTT |
| | | RP-CTT TAC CTC AGA AAA TCC AGA |
| 4. | SB-85 | FP-CTC AGC AAC TTA ATC CAA CTA |
| | | RP-GTT TGT TAG GGG AGG TAA GAA |
| 5. | SB-80 | FP-TAA TGG AGT TCA CAG TCC TCC |
| | | RP-ATA CAG AGC CCA TTT CAT CAC |
| 6. | | FP-TAT TGC TTT GAT GGC GAC TGC |
| | | RP-CAG CAA CGG AAA TAG CAA CAG |
| 7. | SB-196 | FP-CTG TTT CCT GCC ACT ATT ACC |
| | | RP-TAT AAT CTG TCT CCT TTT GGC |
| 8. | SB-93 | FP-GAC GCA CAT ACA CCA TTA CAC |
| | | RP-TGT TAG AAA ATT AGG CAC GGA |
| 9. | SB-201 | FP-CCT CTT TTT CTA TTG TGG TCT |
| | | RP-GGC ATG TAT TTT TAC TCC AAC |
| 10. | PHTR-3 | FP-ATT TGC ATC CAG TCT TCA GTA ATT |
| | | RP-CTC AAA GAA AGT GCA TAG AGA TTT CAT |

**Standardization of PCRamplification conditions**

Tests were performed for standardizing polymerase chain reaction amplification conditions mainly the annealing temperature. PCR amplification conducted at different annealing temperatures, which was calculated based on $T_m$ (melting temperature) of both the primers.

| Sr. No | Reactions | | Temperature and Time specifications | Number of cycles |
|---|---|---|---|---|
| 1 | Initial Denaturation | | $94^0$C-for 3min | 1 |
| 2 | A | Denaturation | $94^0$C-for 45 sec | 35 |
| | B | Annealing of Primer | 55-$60^0$C-for 1min | |
| | C | Primer amplification | $72^0$C-for 2min | |
| 3 | Final amplification | | $72^0$C-for 20min | 1 |
| After completion | | | $4^0$C- till electrophoresis | |

**Amplification of DNA by PCR Using SSR primers**

The amplification reactions were performed in a total volume of 25 µl containing

| Sr. No | Constituents | Qty |
|---|---|---|
| 1 | Sterile distilled water | 15.1 µl |
| 2 | Taq buffer (10X) | 2.5 µl |
| 3 | dNTPs (2.5 mM) | 1.25 µl |
| 4 | Primer (10 ng) | 2.0 µl |
| 5 | Taq DNA Polymerase (3U/ µl) | 0.15 µl |
| 6 | DNA (5ng/ µl) | 4.0 µl |
| | **Total for 1 reaction** | **25.0 µl** |

The reagents were mixed thoroughly in a 2 ml Eppendorf tube and vortexed for few seconds. 21 µl of mixture was distributed to each PCR tube and 4.0 µl of template DNA (5 ng/µl) was added to each tube for each amplification reaction in thermal cycler (Corbett Thermal Cycler) programmed as above

**Electrophoresis using agarose gel**

Amplification products were separated on 2.5 per cent agarose gel using 1X TBE buffer (Tris HCI pH 8.0, Boric Acid, Ethylene diamine-tetra acetic Acid) on horizontal gel electrophoresis apparatus (Banglore Genei, India Limited). Ethidium bromide at a concentration of (5 mg/ml) was used as intercalating agent. SSR amplification products were mixed with 3 µl (10X orange dye) of loading dye and loaded onto

the gel. Gel was run according to 5 V/cm of the length of gel till the bands separate. 100 bp DNA Mass Ladder Marker (100-1500 bp) was used as a standard in the first well of each gel.

**Analysis of SSR fingerprints and Scoring of bands**

The amplified bands after separation were visualized using Gel Documentation system. Further the bands were scored for percentage polymorphism for each set of primer amplified product using NTSYS 2.2. The scored bands were analyzed in the form of binary system to prepare the similarity index. The bands with same molecular weight and mobility were treated as identical fragments. Data matrices were prepared in which the presence of a band was coded as 1 whereas the absence as 0. The data matrices were analyzed by the SIMQUAL Program of NTSYS-PC (Version 2.2) and similarities between genotypes were estimated using Jaccard similarity coefficient, calculated as J = A /(N-D), where A is the number of positive matches (i.e. presence of band in both samples), D is the number of negative matches (i.e. absence of band in both samples) and N is the total sample size including both the number of matches and unmatches. Dendrogram was produced from the resultant similarity matrices using the UPGMA method.

## RESULTS AND DISCUSSION

Microsattelites or simple sequence repeats (SSRs) have gained considerable importance in plant genetic and breeding owing to many desirable genetic attributes including multi allelic nature and codominant inheritance. These can be used in genetic fingerprinting, clonal fidelity applications, genetic mapping marker aided selection and assessments of genetic diversity and phylogeny (Tuskan *et al.,* 2004). Beingcodominant in nature SSR markers are more reliable and reproducible.

A summary of amplified, polymorphic and monomorphic fragment obtained from microsatellite study revealed that seven out of ten primers were found to be polymorphic and amplified the genomic DNA of different species/clones of willows successfully. The number of SSR markers generated per primer varied from 2 to 7 because of primer sequence and due to individual genotype (Table 2&3). The present studies are parallel to the findings of Barker *et al.,* (2003), Lin and Lawrence (2003), Lin and Smart (2005) Christop *et al.,* (2007), Lin *et al.,* (2009) and Singh *et al*., (2011) on willows, Tuskan *et al*., (2004) on Poplar and Steane *et al*., (2006) and Delaporte *et al*., (2001) and Danielle *et al*., (2010) and on *Eucalyptus*.

All the seven SSR primers were found to produce distinct banding pattern for twenty two species/clones of *Salix.* Twenty four amplified products were detected out of which 66.66 per cent were found to be polymorphic. A unique band of approximately 100 bp was found present only in *S. udensis* when amplified with primer SB-80.

Jaccard's similarity correlation coefficient value ranged from 0.64 to 0.98 indicating a fair range of similarity between genotypes. The highest (0.90) was exhibited for two male genotypes (*S. tetrasperma* TFB) and *S. tetrasperma* (LNM) of the same species. The minimum similarity coefficient value was observed between *S. udensis* and *S. nigra*. This result supports the finding of Zsuffa *et al.,* (1984) and Skvortsov (1999) in classification and distribution of *Salix* in new world. The minimum similarity between *S. udensis* and *S. nigra* may be ascribedto the facts that *S. udensis* belong to completely different subgenus i.e. *Vetrix* where as *S. nigra* and *S. tetrasperma* belongs to sub genus *Salix.*

The UPGMA dendrogram based on SSR exhibited five clear clusters (Fig. 1). *S. udensis* came out as outliner and was found to be most divergent. Cluster I comprised of four genotypes viz., *S. babylonica* from India. *S. excelsa* from Turkey *S. pierotti* from USA and NZ-1002 from New Zealand. All the four genotypes belonged to vary closely placed section *Salix* and *Subalbae* of same subgenus *Salix* while, Clone NZ-1002 is a hybrid between *S. alba* of section *Salix* and *S. matsudana* of section *Subalbae*. Cluster II comprised of four genotypes viz., Clone 799, female *S. alba* (SI-64-017) and two male genotypes of *S. alba* (Kashmiri and SI-63-007). All the four genotypes are rightly placed in the same cluster as they belong to same section *Salix.* Clone 799 is a female hybrid of *S. matsudana* and *S. alba* belonging to closely related section i.e. *Subalbae* and *Salix* respectively. Placement of two species (*S. jessonensis* and *S. matsudana*) insame cluster i.e. Cluster III is in agreement with the phylogenetic placement of two species in same section i.e. *Subalbae*.

Cluster IV comprised of only one species *S. udensis* belonging to completely different subgenus i.e. *Vetrix* Cluster V comprised of seven genotypes which are further placed under two sub cluster. Sub cluster one comprises of two male genotypes of *S. tetrasperma* [(*S. tetrasperma* (TFB) and *S. tetrasperma* (LNM)] from India which also registered maximum similarity (0.98) among themselves. Sub cluster two consisted of two female genotypes [*S. tetrasperma* (LG) and *S. tetrasperma* (LNF)] and clone NZ-1179 (*S. alba* and *S. matsudana*) belonging to same subgenus *Salix.* ClusterVI consisted of three genotypes viz., *S. nigra* from USA, *S. tetrasperma* (TNE) and *S. tetrasperma* (LN) from India belonging to same subgenus *Salix.*

The placement of above species/clones by SSR (Microsatellites) is in agreement with the findings on phylogenetic and taxonomic placement of same different species of *Salicaceae* by Zsuffa *et al*., (1984), Skvortsov (1999), Argus (1999) Singh *et al*., 2013 and Singh et al., 2013.

**Table 2.** Summary of SSR amplified products obtained from 22 genotypes of willow

| Sr. No | Parameters | |
|---|---|---|
| **1** | **Total number of primers examined** | 10 |
| **2** | **Total number of polymorphic primers** | 7 |
| **3** | **Total number of monomorphic primers** | 3 |
| **4** | **Total number of bands amplified from polymorphic primers** | 24 |
| **5** | **Total number of polymorphic bands identified** | 16 |
| **6** | **Total number of monomorphic bands** | 8 |
| **7** | **Average number of bands per primer** | 3.43 |
| **8** | **Per cent of total polymorphic bands** | 66.66 per cent |
| **9** | **Size range of SSR markers** | 50-800 bp |
| **10** | **Number of amplification products per primer** | 2 (SB-85 & SB-196) to 7 (SB-38) |

**Table 3.** Similarity coefficient values of SSR data using Jaccard's Similarity correlation coefficient

| Genotypes | S.ba | S.ex | S.ud | S.ac | S.pa | S.js | 799 | S.gr | S.mm | S.mf | 1002 | S.af | Kas | 007 | TLg | TFB | TLnm | Tlnf | S.ni | 1179 | Twe | Ln |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **S.ba** | 1.00 | | | | | | | | | | | | | | | | | | | | | |
| **S.ex** | 0.92 | 1.00 | | | | | | | | | | | | | | | | | | | | |
| **S.ud** | 0.80 | 0.80 | 1.00 | | | | | | | | | | | | | | | | | | | |
| **S.ac** | 0.88 | 0.88 | 0.76 | 1.00 | | | | | | | | | | | | | | | | | | |
| **S.pa** | 0.88 | 0.88 | 0.84 | 0.92 | 1.00 | | | | | | | | | | | | | | | | | |
| **S.js** | 0.76 | 0.76 | 0.80 | 0.80 | 0.80 | 1.00 | | | | | | | | | | | | | | | | |
| **799** | 0.84 | 0.84 | 0.88 | 0.80 | 0.88 | 0.84 | 1.00 | | | | | | | | | | | | | | | |
| **S.gr** | 0.84 | 0.84 | 0.72 | 0.96 | 0.88 | 0.76 | 0.76 | 1.00 | | | | | | | | | | | | | | |
| **S.mm** | 0.80 | 0.80 | 0.84 | 0.76 | 0.84 | 0.96 | 0.88 | 0.72 | 1.00 | | | | | | | | | | | | | |
| **S.mf** | 0.80 | 0.80 | 0.76 | 0.84 | 0.84 | 0.96 | 0.88 | 0.80 | 0.92 | 1.00 | | | | | | | | | | | | |
| **1002** | 0.88 | 0.88 | 0.84 | 0.84 | 0.92 | 0.80 | 0.88 | 0.80 | 0.84 | 0.84 | 1.00 | | | | | | | | | | | |
| **S.af** | 0.84 | 0.84 | 0.80 | 0.80 | 0.80 | 0.76 | 0.92 | 0.76 | 0.80 | 0.80 | 0.88 | 1.00 | | | | | | | | | | |
| **Kas** | 0.88 | 0.88 | 0.84 | 0.84 | 0.92 | 0.88 | 0.96 | 0.80 | 0.92 | 0.92 | 0.92 | 0.88 | 1.00 | | | | | | | | | |
| **007** | 0.88 | 0.88 | 0.76 | 0.92 | 0.92 | 0.80 | 0.88 | 0.88 | 0.84 | 0.84 | 0.84 | 0.88 | 0.92 | 1.00 | | | | | | | | |
| **Tlg** | 0.80 | 0.80 | 0.76 | 0.92 | 0.84 | 0.72 | 0.80 | 0.96 | 0.68 | 0.76 | 0.76 | 0.80 | 0.76 | 0.84 | 1.00 | | | | | | | |
| **TFB** | 0.84 | 0.84 | 0.72 | 0.96 | 0.88 | 0.76 | 0.76 | 0.94 | 0.72 | 0.80 | 0.80 | 0.76 | 0.80 | 0.88 | 0.96 | 1.00 | | | | | | |
| **Tlnm** | 0.84 | 0.84 | 0.72 | 0.96 | 0.88 | 0.76 | 0.76 | 0.94 | 0.72 | 0.80 | 0.80 | 0.76 | 0.80 | 0.88 | 0.96 | **0.98** | 1.00 | | | | | |
| **Tlnf** | 0.76 | 0.76 | 0.72 | 0.88 | 0.80 | 0.68 | 0.76 | 0.92 | 0.64 | 0.72 | 0.72 | 0.76 | 0.72 | 0.80 | 0.96 | 0.92 | 0.92 | 1.00 | | | | |
| **S.ni** | 0.84 | 0.76 | **0.64** | 0.80 | 0.80 | 0.68 | 0.76 | 0.84 | 0.72 | 0.72 | 0.80 | 0.74 | 0.80 | 0.88 | 0.80 | 0.84 | 0.84 | 0.76 | 1.00 | | | |
| **1179** | 0.76 | 0.76 | 0.72 | 0.88 | 0.80 | 0.76 | 0.84 | 0.82 | 0.72 | 0.80 | 0.72 | 0.74 | 0.80 | 0.88 | 0.96 | 0.92 | 0.92 | 0.92 | 0.84 | 1.00 | | |
| **Twe** | 0.76 | 0.76 | **0.64** | 0.88 | 0.80 | 0.76 | 0.76 | 0.82 | 0.72 | 0.80 | 0.80 | 0.74 | 0.80 | 0.88 | 0.88 | 0.92 | 0.92 | 0.84 | 0.92 | 0.92 | 1.00 | |
| **Ln** | 0.72 | 0.72 | 0.68 | 0.84 | 0.76 | 0.76 | 0.72 | 0.88 | **0.68** | 0.76 | 0.76 | 0.80 | 0.76 | 0.84 | 0.84 | 0.88 | 0.88 | 0.80 | 0.88 | 0.88 | 0.96 | 1.00 |

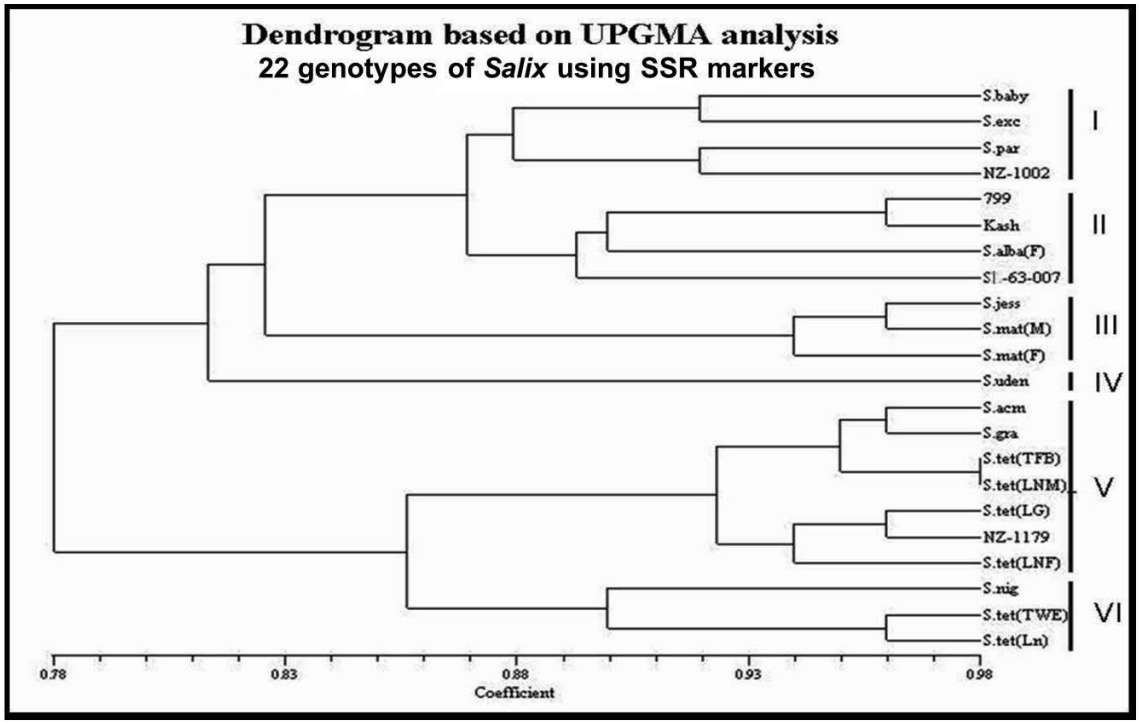


**Figure: 1 Dendrogram based on UPGMA analysis of 22 genotypes of *Salix* using SSR markers**

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