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RESEARCH ARTICLE

Genetic Diversity among five Species of Willow (Salix spp.) from Duhok region- Kurdistan\ Iraq Based on AFLP and SRAP Markers

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ABSTRACT

The paper explores the genetic diversity and taxonomic classification of the genus Salix (willows), a taxonomically complex group in the Salicaceae family. It employs molecular markers, including AFLP (Amplified Fragment Length Polymorphism) and SRAP (Sequence-Related Amplified Polymorphism), alongside chloroplast sequences, to analyze genetic relationships among five Salix species (S. alba, S. babylonica, S. purpurea, S. acmophylla, and S. aegyptiaca). The findings reveal significant genetic variability and propose a revised infrageneric classification of Salix. Key results include: • S. alba and S. purpurea are closely related, forming a cluster, while S. babylonica is genetically distinct. S. acmophylla and S. aegyptiaca show close genetic similarity, likely due to geographic and environmental adaptations. The combined molecular markers (AFLP and SRAP) demonstrate high polymorphism rates, confirming their effectiveness for studying genetic diversity and phylogenetic relationships in Salix. The research also underscores the influence of environmental factors on species clustering, contributing to a better understanding of Salix taxonomy and genetic relationships.

INTRODUCTION

The genus Salix belongs to the family Salicaceae, which **Linnaeus, (1753)**, described and divided into two genera: Populus and Salix. **Nakai, (1920)**, later introduced Chosenia as a new genus. Salix comprises deciduous and dioecious trees or shrubs **(Watson & Dallwitz, 1992)**. Willow trees are dioecious, meaning that flowers of only one sex occur on a single individual. Chromosome numbers in Salix species range from 2n = 38, 57, 76, 95, 114, to 152 **(Suda & Argus, 1968)**.

Morphological characteristics have long been used to identify species, genera, and families. They are also employed to evaluate systematic relationships and discriminate cultivars and breeding lines. However, identifying Salix species is challenging due to their high morphological variability. Diagnostic features such as growth form, leaf shape, hairiness, bark characteristics, catkin size, and ovary hairiness often vary. To address this variability, identifications should focus on "normal" growth or population-level analysis rather than individual specimens **(Argus, 2008)**. Willows, whether trees or shrubs, have single buds and simple leaves that alternate in various shapes and sizes. Leaf margins range from glandular-toothed to entire, with short petioles. Willow plants are erect, spreading, or somewhat pendulous. Salix species are dioecious, and flowering occurs before or as the leaves emerge **(Argus, 2004)**. Male flowers (staminate) have two distinct stamens without either calyx or corolla, while female flowers have one or two nectaries and are grouped into catkins **(Newsholme, 2002)**. Stamens may be free or partially to completely connate.

The pistillate flowers feature a unilocular, stipitate ovary, and fruits are obclavate capsules with two valves that disperse small seeds surrounded by a "parachute" of hairs **(Argus, 2004)**.

MATERIAL AND METHODS

1. PCR amplification of AFLP primers

Eleven AFLP selective compensation primers were used in this study. DNA Restriction and Ligation: Digest genomic DNA with restriction enzymes (True 91 and MseI). The ligate adaptors to the digested fragments by using T4 DNA ligase. Then the Pre-selective Amplification conducted by using primers complementary to adaptor sequences. The PCR perform to amplify a subset of fragments, and the last step Selective were amplification of primers with additional selective nucleotides at the 3′ end. To analyze the Separate amplified fragments the polyacrylamide gel had been used, and the Visualize fragments by silver staining.

2. SRAP (Sequence-Related Amplified Polymorphism)

PCR Amplification were prepared the PCR mix with DNA, primers, and reagents. The PCR products Run on an agarose. The Visualize of the DNA bands used by SYBR Green.

The Data Analysis Scored the bands for presence/absence across samples to generate a binary matrix.

RESULT AND DISCUSSION

SRAP Marker:

In *Salix* spp, primer EM13-ME5, EM13-ME3 and EM16-ME1 gave higher number of bands that were 23, and primer EM16-ME5 gave a lower number of bands that were 13 bands, a total number of bands were 223, the polymorphism was 223 with the rate 100% in **Table (1)**

Table 1. The number of bands and degree of polymorphism revealed by each SRAP primer combination of five species of *Salix* genus

Primer combination	Total number of bands	Number of polymorphic bands	Polymorphism rate (%)
EM4-ME5	19	19	100
EM5-ME1	19	19	100
EM5-ME2	19	19	100
EM16-ME6	18	18	100
EM16-ME8	21	21	100
EM13-ME5	23	23	100
EM13-ME3	23	23	100
EM5-ME4	15	15	100
EM16-ME5	13	13	100
EM17-ME12	20	20	100
EM16-ME1	23	23	100
EM9-ME2	20	20	100
Total	233	233	100

Sequence-Related Amplified Polymorphism (SRAP) products of the twelve primer combinations used to determine the genetic diversty of five species of genus *Salix (Salix alba*, *Salix babylonica, Salix purpurea*, *Salix acmophylla*, *Salix aegyptiaca*) were analyzed on 1.5% agarose gel electrophoresis.

The genetic relationship between species was determined using-calculated genetic distances data **(Table 2).** Genetic distance ranged from the lowest value 0.30 (between *S. aegyptiaca* and *S.acmophylla*) to the highest value 0.49 (between *S. aegyptiaca* and *S. purpurea*). The genetic

relationships among *Salix* genotypes were estimated using NTSYS- pc program which is based on Nei's (1972) standard genetic distance.

Table 2. Genetic distance between studied *Salix* spp by SRAP markers

***Salix* spp**	***S. alba***	***S. babylonica***	***S. purpurea***	***S.acmophylla***
S. babylonica	0.43			
S. purpurea	0.39	0.43		
S. acmophylla	0.42	0.45	0.46	
S. aegyptiaca	0.46	0.48	0.49	0.30

AFLP RESULTS:

In *Salix* spp the number of bands generated for each selected primer, MCGT/EAAC gave highest number of bands (163), and primer MCCT/EATA gave lower number of bands (62) with the total number of bands being 1161 with the rate 95.69 **(Table 3)**.

Table 3. The number of bands and degree of polymorphism revealed by each AFLP primer combination of five species of *Salix* genus

Primer combination	**Total number of bands**	**Number of Polymorphic Bands**	**Polymorphism rate (%)**
MCCT/EAAT	97	92	94.85
MCCT/EATA	62	57	91.93
MCCT/EAAC	92	92	100
MCCT/EAAG	83	78	93.98
MCGA/EAGA	73	73	100
MCGA/EAGT	85	85	100
MCGA/EACA	162	147	90.74
MCGA/EACT	78	78	100
MCTA/EACA	160	155	96.88
MCGT/EAAG	106	106	100
MCGT/EAAC	163	148	90.97
Total	1161	1111	95.69

Estimates of genetic similarity matrices based on the AFLP molecular marker data for all pairwise combinations of the five species of genus *Salix* accessions are presented in table 4 (*S. alba*, *S. babylonica, S. purpurea, S. acmophylla* and *S. aegyptiaca***)**. The genetic similarity varied from 0.41 to 0.79. Genetic distance ranged from the lowest value 0.41 (*S. alba* and S*. purpurea*) to the highest value 0.79 (between *S. babylonica* and *S. purpurea*.

Table 4. Genetic distance between studied *Salix* spp by AFLP markers

***Salix* spp**	***S. alba***	***S. babylonica***	***S. purpurea***	***S.acmophylla***
S. babylonica	0.74			
S. purpurea	0.41	0.79		
S. acmophylla	0.54	0.77	0.48	
S. aegyptiaca	0.64	0.69	0.64	0.71

According to this calculation model a phylogenetic tree was constructed to determine the relationship between these varieties as shown in **Table (4)**. The analysis based on AFLP markers, the dendrogram generated a dendrogram based on Dice genetic similarity coefficients using UPGMA cluster analysis. The dendrogram scale value among individual samples ranged from 0.79 to 1.00 on the Dice index, the minimum similarity among five species of genus *Salix* was 79% whereas the maximum similarity between them was 100%. AFLP marker data revealed two main clusters. First, main cluster split into two subclusters, upper included *S. alba* and *S. purpurea* and lower included only *S. babylonica*, the second main cluster included *S. acmophylla* and *S. aegyptiaca*.

Combined marker (SRAP and AFLP) analysis

The combined data were used to evaluate the extent of genetic diversity among these different species. It had been used for revealing relationship among willow. Genetic variations of five morpho geographic taxa of willow were examined using Nie's analysis of pairwise similarities between genotypes, as shown in **Table (4)**. Similarity values ranged between all species from 0.34 to 0.50. A higher genetic distance was 0.50 found between *S. babylonica* and *S. aegyptiaca*, and between *S. purpurea* and *S. aegyptiaca* and the lowest genetic distance was 0.34 between *S. aegyptiaca* and *S. acmophylla***.**

Taxonomic relationships in these genuses, according to dendrogram based on Nei's, to express the results of cluster analysis based on data obtained from the AFLP, and SRAP combined. The dendrogram scale varied between 0.78 and 1.00, thus, the minimum similarity between five *Salix* spp was 78%, whereas the highest similarity between them was 100% **Table (5)**.

Table 5. Genetic distance between studied *Salix* spp by combined markers

Salix spp	***S. alba***	***S. babylonica***	***S. purpurea***	***S.acmophylla***
S. babylonica	0.46			
S. purpurea	0.39	0.46		
S. acmophylla	0.43	0.47	0.46	
S. aegyptiaca	0.48	0.50	0.50	0.34

Theresult discovered that the combined markers were able to cluster these species all together and the genetic relationships among them, led to the separation of these species into two main clusters. In the first main cluste, genotypes were divided in two branches, upper included *S. alba* and *S. purpurea* and lower was included. The current study advances understanding of genetic diversity in the genus Salix, complementing recent research that employs molecular markers to evaluate genetic relationships among species. This work aligns with other findings that highlight the utility of AFLP and SRAP markers for genetic analyses, which are critical for phylogenetic studies and breeding programs.

The SRAP analysis showed a high polymorphism rate (98.98%), indicating substantial genetic variability within the genus. Similarly, the AFLP analysis yielded a polymorphism rate of 95.69%, confirming the utility of molecular markers in distinguishing closely related species of Salix **(Barker *et al*., 1999).** These high rates of polymorphism suggest that the Salix genus has undergone significant evolutionary divergence, potentially influenced by environmental factors and geographic distribution **(Chen *et al*., 2010)**. The phylogenetic analyses using both SRAP and AFLP markers produced consistent clustering patterns. For instance, S. alba and S. purpurea were grouped in the same cluster, indicating close genetic similarity. This finding aligns with previous studies that identified S. purpurea and S. alba as closely related species within the same subgenus **(Trybush *et al*., 2012; & Przyborowski & Trybush, 2013)**. On the other hand, S. babylonica was genetically distinct, forming its own cluster, suggesting significant divergence from other species. Such differentiation may reflect environmental adaptations, as S. babylonica is native to different ecological regions compared to S. alba and S. purpurea **(Argus, 2008)**.

Recent studies emphasize the importance of combining multiple molecular markers to improve the resolution of genetic diversity analyses. For instance, a study on Salix purpurea using AFLP, RAPD, and ISSR markers revealed high genetic diversity within populations but low diversity between regions, typical of woody species. This highlights the potential for molecular markers in breeding programs and species conservation efforts **(Sulima *et al*., 2023)**. Further, whole-genome resequencing of Salix species has shown that ancient hybridization and ecological factors significantly influence genetic diversity. These insights suggest that evolutionary processes and geographic adaptations play a pivotal role in shaping genetic relationships within the genus **(Sanderson *et al*., 2023)**.

This study's clustering of species such as S. alba and S. purpurea into close genetic groups is consistent with broader phylogenetic patterns observed across Salix. For instance, recent phylogenomic studies confirm that geographic proximity and ecological niches significantly influence species clustering. Such clustering aids in understanding evolutionary adaptations and informs conservation strategies **(Sanderson *et al*., 2023; & Przyborowski *et al*., 2022)**. The taxonomic implications of these findings are significant, as they further refine the classification of subgenera within Salix. These results, supported by studies on chloroplast genomes and SNP-based methods, suggest the need for more integrative approaches to resolve taxonomic challenges in this complex genus **(Sulima *et al*., 2023; & Sanderson *et al*., 2023)**.

CONCLUSION

In conclusion, the integration of molecular markers and genome sequencing enhances our understanding of Salix taxonomy and genetic diversity. These tools not only facilitate breeding and conservation programs but also help address broader ecological and evolutionary questions. Future research should focus on leveraging advanced genomic tools to explore genetic diversity in more detail, particularly in understudied or threatened species of the genus.

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