Pak. J. Bot., 48(3): 1183-1187, 2016.

CHARACTERIZATION OF FOUR *SALSOLA* SPECIES AND THEIR GENETIC RELATIONSHIP BY AFLP

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Abstract

Amplified length polymorphism (AFLP) technique was used to characterize and detect molecular genetic markers for four *Salsola* species collected from Al Jouf region in the northern of Saudi Arabia and to shed light on their genetic relationships. Three primer combinations were used for AFLP analysis of the four *Salsola* species, they generated a total of 181 fragments of which 133 were species specific markers scored across *Salsola* species. The dendogram produced by Jaccard's coefficient and the UPGMA clustering method showed one main cluster, subdivided into two subclusters. The first sub cluster included *Salsola schweinfurthii* and *Salsola tetrandra*. The second sub cluster included *Salsola villosa* and *Salsola cyclophylla*. It is worth mentioning that this is the first study to use AFLP markers to characterize and detect molecular genetic markers for the four *Salsola* species and their genetic relationships.

Key words: *Salsola*, DNA fingerprinting, Genetic diversity, AFLP.

Introduction

Kingdom of Saudi Arabia has a varied heritage of plant diversity as the climate is generally arid in most parts of the country; nearly 30% of the flora is rare. Significant number of species and plant associations were found in the central and northern regions.

Flora of the northern regions, especially in Al Jouf region is dominated by annual species. However, perennial components such as *Salsola* spp., *Traganum nudatum*, *Haloxylon* spp, *Artemisia* spp., *Zilla spinosa*, etc. are forming large communities in all rage lands. The climate is generally arid with an average annual rainfall of 120 mm and an average maximum temperature of 48ºC during July to September and an average minimum temperature of 8ºC during December-February.

The genus *Salsola* belongs to the tribe Salsoleaes s. str. of the subfamily Salsoloideae S.1. (Kapralov *et al*., 2006; Akhani *et al*., 2007). A common name of various members of this genus and related genera is saltwort, for their salt tolerance. The genus name *Salsola* is from the Latin salsus, meaning "salty"(Mosyakin, 2003).

Salsoleae mainly distributed throughout arid and semiarid regions in which these environments serve as favorable habitats for it due to the morphological, anatomical, and physiological characters (Kadereit *et al*., 2003).With the advance of molecular techniques, several types of DNA markers have been used like RFLP, RAPD, microsatellites (SSRs) and Amplified Fragment Length Polymorphism (AFLP) to characterize species.

AFLP technique is based on the complete endonuclease restriction digestion of total genomic DNA, followed by selective PCR amplification resulting in unique, reproducible fingerprint for each individual and a large number of informative markers (Pompanin *et al*., 2005) and Arrigo *et al*., 2012). It is a very useful technique that does not require prior information of DNA sequence. It is able to detect high levels of polymorphism and resolve genetic relationships among groups that were previously difficult to resolve with morphological characters or other molecular markers (Obae *et al*., 2013).

Flora of Saudi Arabia lacks the genetic and molecular studies; so, this study aimed to the definition of plant species, preservation of germplasm and to detect genetic relationships between different species of genus *Salsola*.

Materials and Methods

Four species of the genus *Salsola* that considered as important grazing plants in Saudi Arabia were chosen; *Salsola villosa* Schult. , *Salsola schweinfurthi* Solms-Laub.*i, Salsola cyclophylla* Baker and *Salsola tetrandra* Forssk. They were collected from Al Jouf region in the northern of Saudi Arabia. The plant specimens have been identified following Migahid, 1996 and Al-Hassan, 2006.

It is the first study to use AFLP technique to characterize and detect molecular genetic markers between the four species of *Salsola* and to shed light on their genetic relationship.

AFLP protocol: The original protocol of Vos *et al*., 1995 was used, while modifications suggested by Kamisugi *et al*., 2008; Huang & He, 2010; Song *et al*., 2011 and Mikulaskova *et al*., 2012 were followed. Adaptors and primers sets were all synthesized by Eurofins (Germany) (Table 1).

Table 1. Primers/adaptors names and sequence of primers at 5´→3´ used to establish the AFLP technique.

Primer/Adaptor	Sequence 5´→3´
EcoRI– A1	CTCGTAGACTGCGTACC
EcoRI– A2	AATTGGTACGCAGTC
Mse I– A1	GACGATGAGTCCTGAG
Mse I– A2	TACTCAGGACTCAT
Eco + A	GACTGCGTACCAATTCA
Mse + C	GATGAGTCCTGAGTAAC
Eco + ACA	FAM-GACTGCGTACCAATTCACA
Eco + AGG	HEX-GACTGCGTACCAATTCAGG
Eco + ATA	CY3-GACTGCGTACCAATTCATA
Mse + CTC	GATGAGTCCTGAGTAACTC

Preparation of adaptors and primers

a. Adaptors preparation: *Eco*R1 and *Mse*1 adaptors were re-suspended to 100µM with distilled water. After mixing the adaptors, they were heated at 95°C for 5 min to denature, and then left to cool slowly at room temperature to re-nature completely for at least 1 hour.

b. Primers preparation: The primers were re-suspended to 100 µM with distilled water.

Restriction R and ligation L of adaptors

a. Preparation of RL mix: 10 µl of DNA were added to a mix containing the restriction enzymes *Msel* (Biolabs, R0525S), *EcoR1* (Fermentas, ER0271), both *Msel* and *EcoR1* adaptors, ATP (Fermentas, R0441), T4 ligase (Fermentas, EL0015) and Tango buffer (Fermentas, BY5) that allow the activity of all the added enzymes. Finally distilled water was added up to 20 µl.

b. The RL reactions were incubated at 37°C for 4 hours and then placed on ice.

c. The RL reactions were tested by 1% (1g/100ml) agarose gelelectrophoresis and visualized with 1X RedSafe®. Five µl of RL reactions containing the 5 xGreenGoTaq®Flexi buffer were loaded onto the gel, additionally one lane was loaded with 2.5 µlof GeneRuler™ 100bp DNA ladder plus (Fermentas, SM1153).

Pre-selective PCR: PCR reactions were performed using the adaptor specific primer with one base extension. A master mix was prepared and distributed depending on the number of PCR reactions. GreenGoTaq®Flexi DNA polymerase (Promega, M8295) was applied into 25µl mix/reaction. In sterile, nuclease–free micro-centrifuge tubes, the following components were added on ice:

Component	Stock concentration	Reaction concentration
GoTaq® Flexi buffer	5X	1X
$MgCl_2$	25 mM	4 mM
dNTPs	10mM	0.2mM
Eco + A primer	10µM	1µM
Mse + C primer	10µM	1µM
GoTaq™	5 U/ µl	1.25 U
RL mix	-	2.0 µl
Distilled water	-	added up to 25 µl total volume

RL mix (0.2 µl) for every 25µl pre–selective PCR reaction was added. Micro–centrifuge tubes were placed in a Flex Cycler (Analytic Jena) 96 Thermo-cycler. Negative and positive controls were included. PCR reactions were tested by 1% (1 g/100 ml) agarose gel electrophoresis and visualized with 1X Red Safe® (5 µl/100ml), 5 µl of PCR reactions containing the 5 x GreenGoTaq® Flexi buffer were loaded onto the gel directly after amplification, additionally one lane was loaded with 2.5 µl of GeneRuler™ 100bp DNA ladder plus (Fermentas, SM1153).

Three different labeled PCR products of the same sample were mixed together (triplex analysis) as follow:

Selective PCR product	To be added
FAM labeled	2 µl
HEX labeled	2 µl
CY3 labeled	2 µl
Dist. water	4 µl
Total	10 µl

All samples were multiplexed and stored by order in a sterile 96–well PCR-plate and sent to the fragment analysis service.

AFLP data analysis: Band scoring and assortment by Peakscanner Applied biosystems & Microsoft Excel 2013 FAMD software (Schluter & Harris, 2006) was used to perform the phylogenetic analysis and Treegraph2 programs to visualize the produced tree (Stover & Muller, 2010).

The analysis of the AFLP data was performed in two known approachs. The first is based on the band binary criterion (i.e., codifying the detected bands to 1 when presence and 0 when absence) and the other is based on the allele frequancy (i.e., number of a band presence relatively to the number of all individuals). Each has its advantages and dis- advantages, therefore, both forms were combined to obtain the maximum number of valuable indices according to Bonin *et al*., 2007 methodology.

Results

Morphological characters: The four species are all perennial and small shrubs; however, there are some differences in the morphological characters between them (Table 2).

Table 2. The differences of morphological characters among the four *Salsola* species.

Salsola villosa	*Salsola schweinfurthii*	*Salsola cyclophylla*	*Salsola tetrandra*
Perennial	Perennial	Perennial	Perennial
Dwarf shrub	Small shrub	Dwarf shrub	Dwarf shrub
Stem height 70cm	Stem height 20-50cm	Stem height 20-40cm	Stem height 60cm
Old stem woody, young stems whitish Stem and branches not striate	Old stem woody, young stems purplish	Old stem woody, young stems fresh green	Woody purplish stem
Narrow, linear and alternate leaves sessile, terete or semiterete, bracts more or less leaf-like and successively shorter	Narrow, linear and alternate or opposite, succulent leaves with pointed tip, bracts more or less leaf-like and successively shorter	Leaves with triple sites in nodal branches, bracts more or less leaf-like and successively shorter	Opposite and succulent leaves, bracts more or less leaf-like and successively shorter
Solitary, small and axillary flower	Flowers in clusters and axillary	Paniculate spikes in forming a spicate or paniculate inflorescence on upper part of branches bract axils	Glomerulate in bract axils, forming a spicate or paniculate inflorescence on upper part of branches flower
Winged fruit	Winged fruit	Winged fruit	Small winged fruit
Collected from Hammad area	Collected from Nayal valley	Collected from Hammad area	Collected from Nayal valley

Heemstra *et al*. (1999), Collenette, (1999) and Al-Hassan (2006)

AFLP results: The AFLP analysis of the four *Salsola* species with the three primer combinations generated a total of 181 fragments (Table 3) of which 133 were polymorphic, corresponding to 71.90% level of polymorphism (Table 4). The number of total bands produced by each primer combination ranged from 35 to 84 with an average of 60.33 bands. The percentage of polymorphism varied considerably among the primer combinations. The highest ratio of polymorphism generated by Eco + ATA / Mse + CTC (79.03%), while the Eco + ACA / Mse + CTC produced the lowest polymorphism (62.86%).

Species specific molecular markers for different *Salsola* species: The total number of species specific markers scored across *Salsola* species is 133. Sixty two markers were positive species specific markers and 71 were negative species specific markers. The highest number of species specific markers generated from AFLP analysis for a given combination was 62 (primer combination Eco + AGG / Mse + CTC), while the lowest number of species specific markers 22 was generated from primer combination Eco + ACA / Mse + CTC). (Table 3)

Genetic relationships: The genetic similarities among the four *Salsola* species based on Nei's method (Nei, 1978) and (Saitou & Nei, 1987). The highest pairwise similarity indices resulted from AFLP were between *Salsola schweinfurthii* and *Salsola tetrandra*, while the lowest similarity indices were between *Salsola schweinfurthii* and *Salsola cyclophylla*. (Table 4).

The dendogram produced by Jaccard's coefficient and the UPGMA clustering method showed one main cluster, subdivided into two clusters (Fig. 1). Cluster 1 included *Salsola schweinfurthii* and *Salsola tetrandra*. The second cluster combined *Salsola villosa* and *Salsola cyclophylla*. Distance scale bar is shown FAMD software (Schluter & Harris, 2006) was used to perform the phylogenetic analysis and Treegraph2 programs to visualize the produced tree (Fig. 1). (Stover & Muller, 2010).

Table 3. Number of total bands, polymorphic bands and polymorphism ratio of the four *Salsola* species generated by three AFLP primer combinations.

Enzyme – primer combinations	Polymorphic bands		Total No. of bands	Polymorphism %
	Species specific bands	Non DNA markers		
Eco + ACA / Mse + CTC	22	13	35	62.86
Eco + AGG / Mse + CTC	62	22	84	73.81
Eco + ATA / Mse + CTC	49	13	62	79.03
Total	133	48	181	-
Mean	44.33	24	60.33	71.90

Table 4. Similarity matrices based on molecular data for the four *Salsola* species generated by three AFLP primer combinations.

Species	*Salsola villosa*	*Salsola schweinfurthii*	*Salsola cyclophylla*	*Salsola tetrandra*
Salsola villosa	1.00			
Salsola schweinfurthii	0.41	1.00		
Salsola cyclophylla	0.46	0.39	1.00	
Salsola tetrandra	0.45	0.56	0.53	1.00

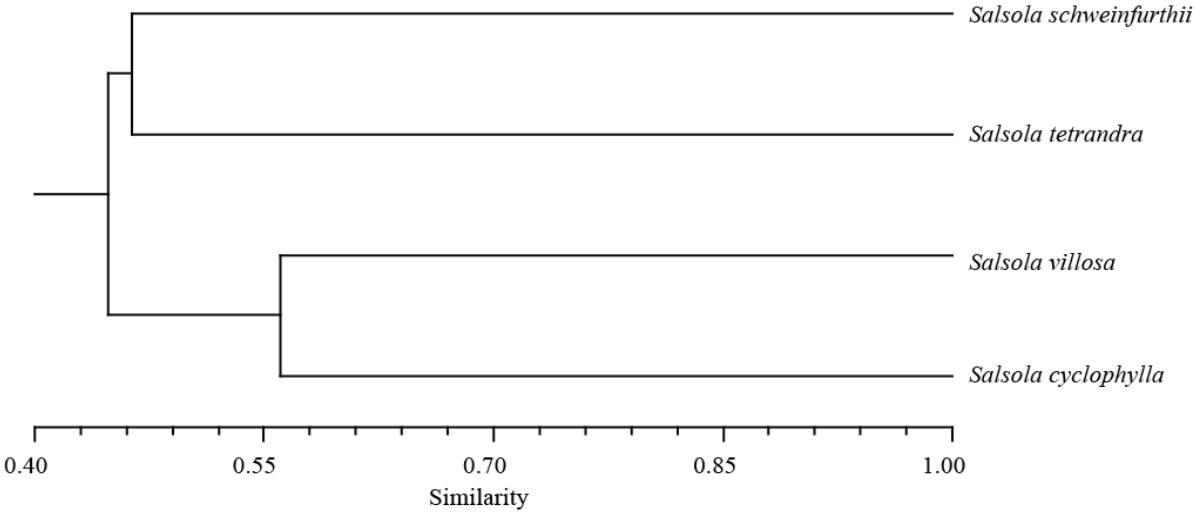

Fig. 1. Neighbor-joining (NJ) tree based on Jaccard similarity coefficient between the four *Salsola* species.

The relationships among species were further examined with principal coordinate analysis (PCoA). The PCoA analysis confirms the distribution of the grouping produced by the UPGMA analysis. Four separate groups were identified (Fig. 2).

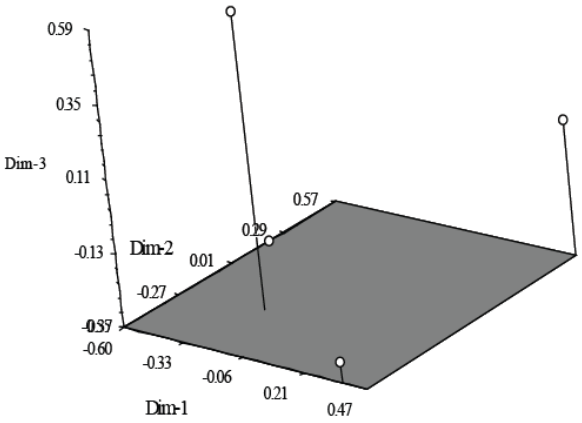

Fig. 2. Analysis of the four *Salsola* species with principal coordinate analysis (PCoA).

The UPGMA tree produced by the AFLP data divided the four species of *Salsola* into two distinct groups at a distance of 0.45. However, the analysis of molecular data revealed more diversity among the four species compared to morphological criteria.

Discussion

The AFLP analysis of the four *Salsola* species with the three primer combinations generated a total of 181 fragments (Table 3) of which 133 were polymorphic. This is an indication that AFLP is a powerful technique and can be successfully used for discrimination of species used in this study. The AFLP bands generated large number of polymorphisms which can be effectively used inter- and intra- species identification.

The *Salsola schweinfurthii* and *Salsola tetrandra* was clearly distinguished as a separate identity at a distance of 0.47 on the UPGMA tree scale they are collected from Nayal area. In the other group comprising *Salsola villosa* and *Salsola cyclophylla* have an overall distance of 0.56 on the distance scale of the UPGMA tree and are delimited into two main clusters (Fig. 1) they are collected from Hammad valley.

So, the higher genetic polymorphism in species is dependant of the amount of sexual reproduction, whereas low levels of genetic variation as often associated with a sexual propagation (Mustafa *et al*., 2005).

The analysis of AFLP polymorphism showed wider diversity among the *Salsola schweinfurthii* compared to the *Salsola cyclophylla*, *Salsola schweinfurthii* growing in Nayal valley and *Salsola cyclophylla* growing in hammad area These indicate that the molecular genetic variability may be due to contribution of local ecological and geographic conditions; these agreed with Badr *et al*., 2012.

Conclusions

The results revealed that AFLP technique generated a large number of polymorphisms so, it is considered to be a useful tool for characterization and distinguishing between the different species of *Salsola* and giving a clear view about the genetic relationships between them.

Acknowledgments

I am grateful to Aljouf University, Sakaka, Saudi Arabia for funding this research under the project number 334/35 and appreciate to Prof. Dr. Eman Fahmy Department of Genetics, Faculty of Agriculture, Ain Shams University for revising the manuscript.

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(Received for publication 27 March 2015)