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Molecular characterization of mesquite (*Prosopis juliflora*) genotypes that varied in histamine content using microsatellite markers

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In many arid and semi-arid regions, *P. juliflora* has an enormous importance in the protection of this fragile environment, and of a very economical importance. In those areas, *P. juliflora* is regarded as a renewable natural resource of multiple uses, where they are used in the production of wood for many purposes and for fuel. This research carried out many studies on the genetic structure of some *P. juliflora*genotypes that exist in Saudi Arabia. This study presented the characterization of *P. juliflora* genotypes using 14 polymorphic microsatellite markers. SSR markers showed that the number of alleles varied from one to 5. The analysis of SSR markers has been shown to have most abundant motif of dinucleotide AG/CT followed by mononucleotide A/T. The genetic cluster tree, using UPGMA program, divided the genotypes to 4 main groups at similarity coefficient between 0.56 and 0.91. In addition, two restriction enzymes, Bam-HI and Eco-RI have been used in conjunction with six SSR primers to obtain more distinguishable differences. Genetic cluster tree for similarity coefficients of SSR primers using only Eco-R1 enzyme showed that the enzyme gave very clear genetic variation among the genotypes under study. However, the enzyme BamH1could be useful in identifying mesquite genotypes had free or low content of histamine.

Keywords: *Prosopis juliflora*, Molecular genetics, SSR markers, Restriction enzymes.

INTRODUCTION

Prosopis juliflora (Mesquite) is a species of flowering plants belonging to the Fabaceae family which is found in arid and semi-arid regions. Prosopis species vary widely in their productivity, and their use and utilization by humans (Pasiecznik et al. 2001). They constitute a very important natural resource for dry zones due to their multi-purpose nature, with the potential to provide a wide range of products, and their ability to grow on the poorest soils where few other useful species can survive. Prosopis trees are harvested for pods, fuel or timber wood, and for many other products, such as medicinal extracts or foliage for animal fodder. Prosopis species are seen to fulfill very important roles both in production and protection in many arid and semi-arid regions of the world. Samples of *Prosopis juliflora* collected from different areas may have incipient races due to geographical locations and morphological differences.

Previous studies of genetic resources of Prosopis have been performed by means of morphological characters (Cony, 1995; Mantovan,

2004), isozymes (Saidman and Vilardi, 1993; Bessega et al. 2000a) or RAPD markers (Saidman et al. 1998; Bessega et al., 2000b). However, actual understanding of genetic resources in Prosopis forests in Saudi Arabia is still rudimentary. Therefore, highly polymorphic molecular markers are indispensable for the study of the genetic resources of *P. juliflora*. Microsatellite markers, also known as SSRs (Simple Sequence Repeats), have proven to be an extremely valuable molecular tool for a wide range of genetic studies in many organisms. In the last years, these markers have become one of the most popular molecular markers used with application in many different fields, such as population genetics, conservation and management of biological resources, genome mapping and also forensic studies (Natalya, 2008). Their high polymorphism, co-dominant mode of expression and the relative ease of scoring represent the major features that make microsatellites of large interest for many genetic studies. SSR markers revealed a broad cross-species affinity when tested in *Prosopis* species (Mottura et al. 2005; Pomponio et al. 2015). Sablok and Shekawat (2008) described the identification of the SSR in the *P. juliflora spp*. The partial sequencing of cDNA clones (expressed sequence tags, ESTs) has become the method of choice for the rapid and cost-effective generation of data on the coding capacity of genomes. Over the past few years a large number of large-scale EST sequencing projects have been completed successfully. There are approximately 30 752 757 EST entries in the National Center for Biotechnology Information. The ESTs were useful in identifying the gene content of the organism (Mahalingam et al. 2003). Moreover, Karaca and İnce (2011) showed that restriction enzymes were suitable in the conversion of monomorphic microsatellite markers to polymorphic markers which segregated in co-dominant Mendelian fashion.

In Saudi Arabia, large saline desert areas, and limited suitable land for crop production, and, inadequate availability of good quality water are the major limitations for agriculture. There is also a great concern regarding water resources and its use in agriculture in Saudi Arabia. This may have potential for prosopis cultivation since it can grow and flourish in a very limited supply of water, and tolerate soil and water salinity. However, for successful and profitable production of *P. juliflora* as a multipurpose tree, and for consideration as a reliable natural resource in Saudi Arabia, it is very important to study genetic variability within a population of *P. juliflora* and measuring differentiation among populations using SSR markers. Also, the objective of this study was characterization of mesquite genotypes vary in histamine content using SSR in conjunction with restriction enzymes.

MATERIALS AND METHODS

Plant Materials

Fifty genotypes of mesquite plant, *Prosopis juliflora*, were used in this study. The genotypes were collected from Al-Qassem and Eastern regions, Saudi Arabia to study genetic characterization of mesquite genotypes. Mesquite genotypes were labeled (B1 to B25) for Al-Qassim and (E1 to E25) for Eastern regions (Table 3). Voucher specimens were collected by Buraidah and Dammam Municipalities, Saudi Arabia, and were registered (ENV#1064-09) in King Abdulaziz City for Science and Technology (KSA).

DNA Extraction

Total genomic DNA of 50 genotypes of *P. juliflora*will be extracted using the method described by Saghai et al. (1994). The quality and quantity of the DNA were determined using UV-Spectrophotometer at wavelengths of 260 and 280nm.

SSR analysis

Fourteen SSR (microsatelites) markers (Mottuora et al. 2004) were used for PCR screening of Mesquite plant (*Prosopis juliflora*) genomic DNA. In addition, six microsatellites that have been developed for *Prosopis chilensis*, and cross-species amplification (Mottuora et al. 2005) were used in conjunction with restriction enzymes. We characterized the genotypes of our sample of 50 individuals using all SSR analysis was applied in a thermal cycler (Thermolyne Amplitron). The reaction mixture (25 µL) was contained 1x PCR buffer with Mg Cl2 (50 mM K Cl, 10 mM Tris- HCl (pH 9.0), 2 mM Mg Cl2 and Triton X-100), 200 µM each of dATP, dCTP, dGTP, and dTTP, 30 ng template DNA, 25 pM of each primer and 2.5 unit of Taq polymerase. The mixtures will be subjected to the following conditions: hold at 95˚C for 1 min, followed by 35 cycles of 94˚C for 30s, 52˚C for 60s and 72˚C for 2 min, and a final hold at 72˚C for 8 min. PCR products were visualized along with a DNA marker on 2% agarose gel with 1X TAE buffer and were detected by staining with the ethidium bromide.

Restriction enzymes: Ten µl of the PCR-products of the six SSR markers (Mottuora et al., 2005) from all genotypes were digested with each of the four restriction enzymes *Eco-RI, Bam-HI, Hind-III*, and *Msp-I* (5 units g^{-1} DNA) according to Ince et al. (2010). The restriction enzyme digests were carried out for 1 h at the temperature and in the buffer recommended by the manufacturer (Promega, Southampton). Digests were subjected toelectrophoresis at 100 V for 2 h on a 2% DNA agarose (BioWhittaker Molecular Applications, Wokingham), using Tris-acetate buffer (pH=8). A 100 bp DNA ladder (Gibco BRL, Paisley) was used as a size marker. After electrophoresis, the gels were stained in ethidium bromide (5 µg ml-1) for 20 min and photographed under UV light.

Data analysis

SSR data were scored for presence (1), absence (0) or as a missing observation (9), and each band was regarded as a locus. Two matrices, one for each marker, were generated. The genetic similarities (GS) were calculated according to Nei and Li (1979): GS = 2Nij/(Ni+Nj), where Nij is the number of bands present in both genotypes i and j, Ni is the number of bands present in genotypes i, and Nj is the number of bands present in genotype j. Based on the similarity matrix, a dendrogram showing the genetic relationships between genotypes, was constructed using the unweighted pair group method with arithmetic average (UPGMA) though the software NTSYS-pc version 2.11 (Rohlf, 2000).

RESULTS

Genetic Polymorphism in genotypes using SSR markers

Fourteen SSR primers have been used for all genotypes of both Al-Qassim and Eastern regions to study the genetic variation at DNA level (Table 1). 43 bands have been given showing polymorphism among the genotypes. Primers used were mono-nucleotides; thiamine/adenine (T/A), and adenine/thiamine (A/T), and also di-nucleotides adenine guanine/cytosine thiamine (AG/CT). Each primer gave 1 to 5 bands. The results indicated that mono-nucleotide primers T/A gave 9 bands and A/T gave 16 bands, while double base primers AG/CT gave 18 bands (Fig. 1). The genetic distances between the genotypes of *Prosopis juliflora* were studied by the mentioned 14 primers. The genetic cluster tree, using UPGMA program, divided the genotypes into 4 main groups at similarity coefficient between 0.56 and 0.91 (Fig. 2). First main group included the genotypes; B1, B2, B3, B5, B8, B9 and B12 from Al-Qassim Region at similarity coefficients between 0.72 and 0.86. The second main group included the genotypes; B2, B7, B14, B15, B16, B21, and B22 from Al-Qassim Region and only the genotype E2 from Eastern Region at similarity coefficients between 0.69 and 0.86. The third group was divided to three sub-groups. The first sub-group included the genotypes; B4, B10, B18, B19, B20, B23 and B25 from Al-Qassim Region and both E14 and E16 genotypes from Eastern Region at similarity coefficients 0.75 and 0.91. The second sub-group included the genotypes; E6, E7, E8, E9, E10, E12, E17, E18, E23 and E25 from Eastern Region and the genotype B11 from Al-Qassim Region. The third sub-group included the genotypes; B6 and B13 from Al-Qassim Region and the genotypes; E1, E3, E5, E11, E13, E15, E20, E21, and E24 from Eastern Region at similarity coefficients between 0.77 and 0.88. The fourth main group included both the genotypes B17 and B24 from Al-Qassim Region.

Genetic polymorphism by using SSR markers with restriction enzymes

Six SSR markers with four restriction enzymes have been used to obtain more distinguishable differences. Only two restriction enzymes, *Bam-HI* and *Eco-RI* gave polymorphism among prosopis genotypes (Table 2). The restriction enzymes gave 21 bands, 20 of them showed polymorphism (97.6%) among the genotypes. *Eco-RI* gave 10 bands, all of these bands showed polymorphism (100%). While, *Bam-HI* gave 11 bands, 10 of them gave polymorphism among the genotypes (90.9%). Genetic cluster tree for similarity coefficients of SSR primers using only *Eco-RI* enzyme showed that the enzyme gave very clear genetic variation among the genotypes under study (Fig. 3 & Fig. 4). The enzyme could distinguish between the genotypes of the two regions. The genetic cluster tree was divided into two main groups within the genetic coefficients ranged from 0.51 to 1.0. The first main group included the genotypes; B1, B3, B4, B5, B7, B8, B9, B14, and B23 from Al-Qassim Region only at genetic similarity coefficients ranged from 0.64 to 1.0. The second main group was divided into three sub-groups.

Table 1: SSR (microsatelites) markers used for PCR screening of Mesquite plant (*Prosopis juliflora*) genomic DNA

NO	Primer	Repeat Motifs/ folds	Sequence Forward & Reveres Primer		Total	Poly morphic	Mono morphic	% Poly morphic
AT repeat motif								
1	Mtgsp_004G08	AT-11	F	GTGGAAGTTTGATCATTTCG	3	3	0	100
			R	CAAATCTCACCCACTACA				
2	Mtgsp_003F07	AT-11	F	CAGGAGGACAAACACCAACTC	4	4	0	100
			R	TCCACGCTTTGAGATAAAATGA				
3	Mtgsp_003C03	AT-16	F	CCGGACACCCCACTTATTTA	1	1	0	100
			R	CCTCACAGTGCATTGGAAAA				
4	Mtgsp_001G08	AT-12	F	CGGACAAAACAGATTGTCCTT	0	0	0	0
			R	GAAGGTGCGTTTAGCAACA				
5	Mtgsp_001C04	AT-19	F	TGGCAAAGTGATGAGAGGGT	1	1	0	100
			R	ATATACCACCACAGCCGGAG				
TA repeat motif								
6	Mtgsp_001B05	TA-32	F	TTGTCTTAAATCGGATGGCT	2	2	0	100
			R	AGCGTTACAGGGTGTTCCTG				
7	Mtgsp_005B08	TA-10	F	GCGTTAGCATGGGTTAATGG	5	5	0	100
			R	GCAAACAATGGTGTGTCGAG				
8	Mtgsp_001E07	TA-8	F	GCCCTAAGGACTGCATTTTG	5	5	0	100
			R	CCCCTCCTAAACCCTCAATC				
9	Mtgsp_004B09	TA-16	F	TATCAGTCATAAGGCAAAAG	0	0	0	0
			R	CTATTTAAGAGTATAATTGGGA				
10	Mtgsp_005h11	TA-24	F	CTATTTAAGAGTATAATTGGGA	4	4	0	100
			R	AAGGTGACCMGGAATGTGG				
AG/CTrepeat moti								
11	Mtgsp_004d03	AG-14	F	AAGGTGACCMGGAATGTGG	5	5	0	100
			R	GCTTGTTGGGTTGGTCTGTT				
12	Mtgsp_001b05	AG-17	F	TGGGTTTACGGAAGGATGAA	5	5	0	100
			R	GCAAGCACTTCCCATAAAGC				
13	Mtgsp_001c10	AG-18	F	CAAATTTGCCCCACACAAAT	5	5	0	100
			R	TTGCCTTTCTGCCACTCTTT				
14	Mtgsp_005g08	AG-21	F	CAAATTTGCCCCACACAAAT	3	3	0	100
			R	CAAATTTGCCCCACACAAAT				
	Total				43	43	0	100

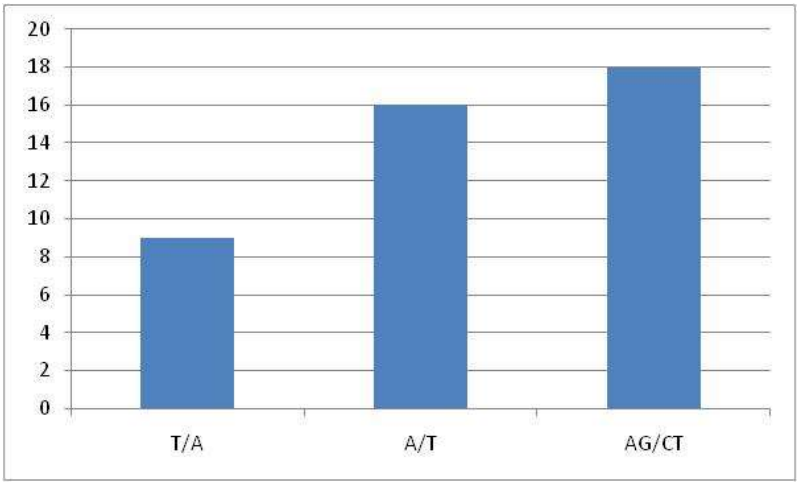


Figure1: Frequency distribution of SSR markers in *P. jouliflora* genotypes.

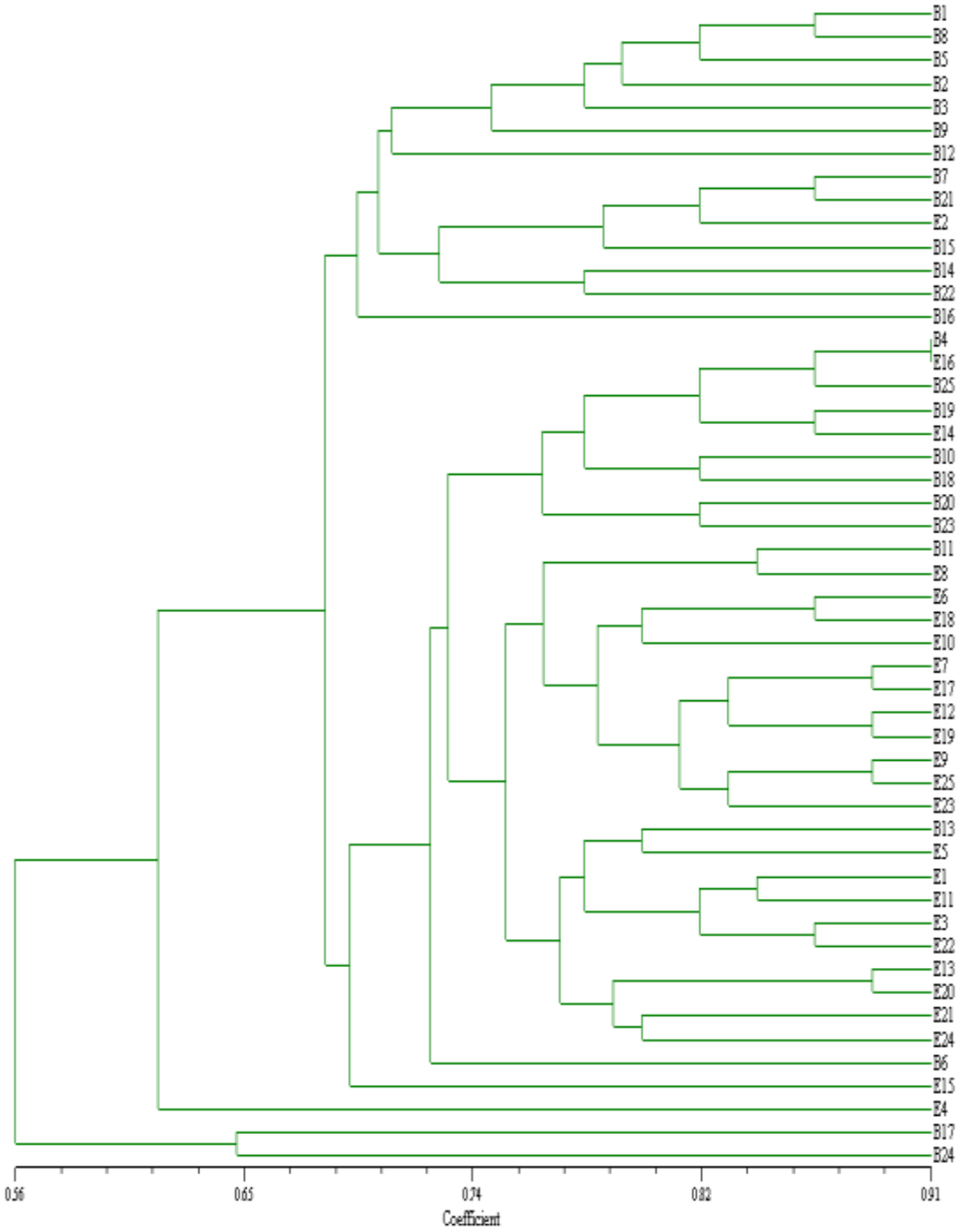
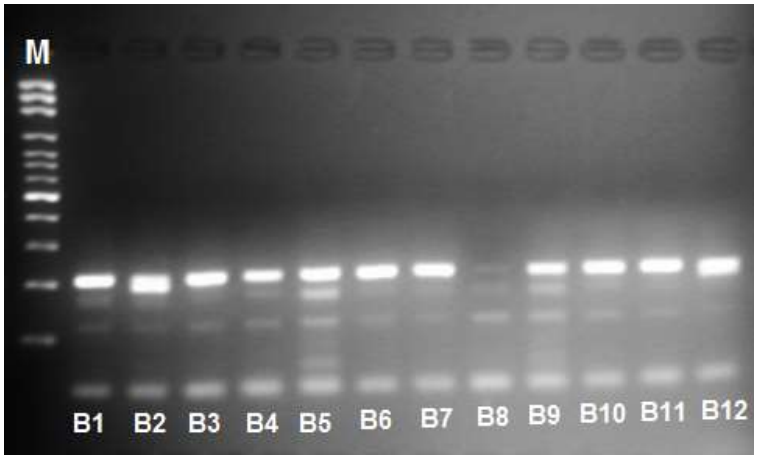


Figure 2: Dendrogram constructed from similarity coefficients and showing the clustering of *Prosopis jouliflora* genotypes using SSR markers

Table 2: Characterization of microsatellite loci in *Prosopis jouliflora* genotypes.

NO	Primer Name	Repeat Motif of the cloned allele		SSR Forward & Reveres Primer	Restriction Enzymes	NO. of PCR Bands	Poly Morphic bands	Mono morphic bands	% Poly morphic
1	Mo05	(CT)3T (CT)2	F	AATTCTGCAGTCTCTTCGCC	EcoR1	2	2	0	100
			R	GATCCCTCGTGACTCCTCAG	BamHI	2	2	0	100
2	Mo07	(GC)8	F	GAAGCTCCCTCACATTTTGC	EcoR1	3	3	0	100
			R	CTATTTGCGCAACACACAGC	BamH1	3	3	0	100
3	Mo08	(AC)9	F	TATCCTAAACGCCGGGCTAC	EcoR1	1	1	0	100
			R	TCCCATTCATGCATACTTAAACC	BamH1	2	1	1	50
4	Mo09	(TG)17	F	ATTCCTCCCTCACATTTTGC	EcoR1	1	1	0	100
			R	ATTCCTCCCTCACATTTTGC	BamH1	1	1	0	100
5	Mo13	(GT)10CT(GT)2	F	TTGATTAGAGTTGCATGTGATG	EcoR1	1	1	0	100
			R	TGCAGTCCCAAGTGTCAGAG	BamH1	1	1	0	100
6	Mo16	(CA)12	F	TGCAGTCCCAAGTGTCAGAG	EcoR1	2	2	0	100
			R	GGGTCCATCCAGAGTAGTGG	BamH1	2	2	0	100
		Total			EcoR1	10	10	0	100
					BamH1	11	10	1	90.9



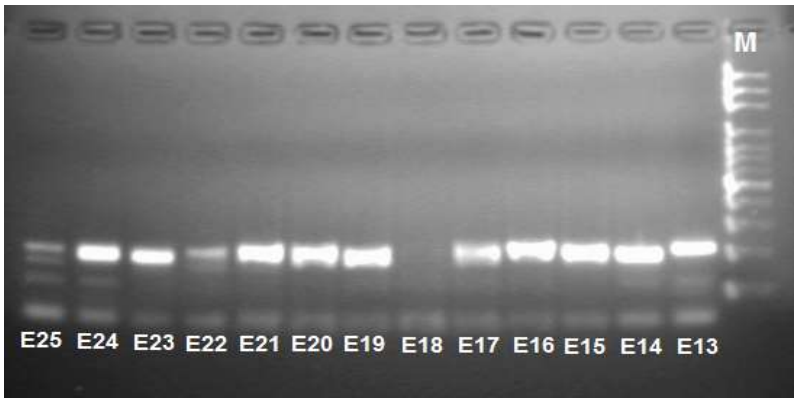


Figure 3: Agarose gels showing restriction patterns of PCR-amplified SSR primer Mo07 digested with *EcoRI*. M. DNA size marker of 100pb ladder

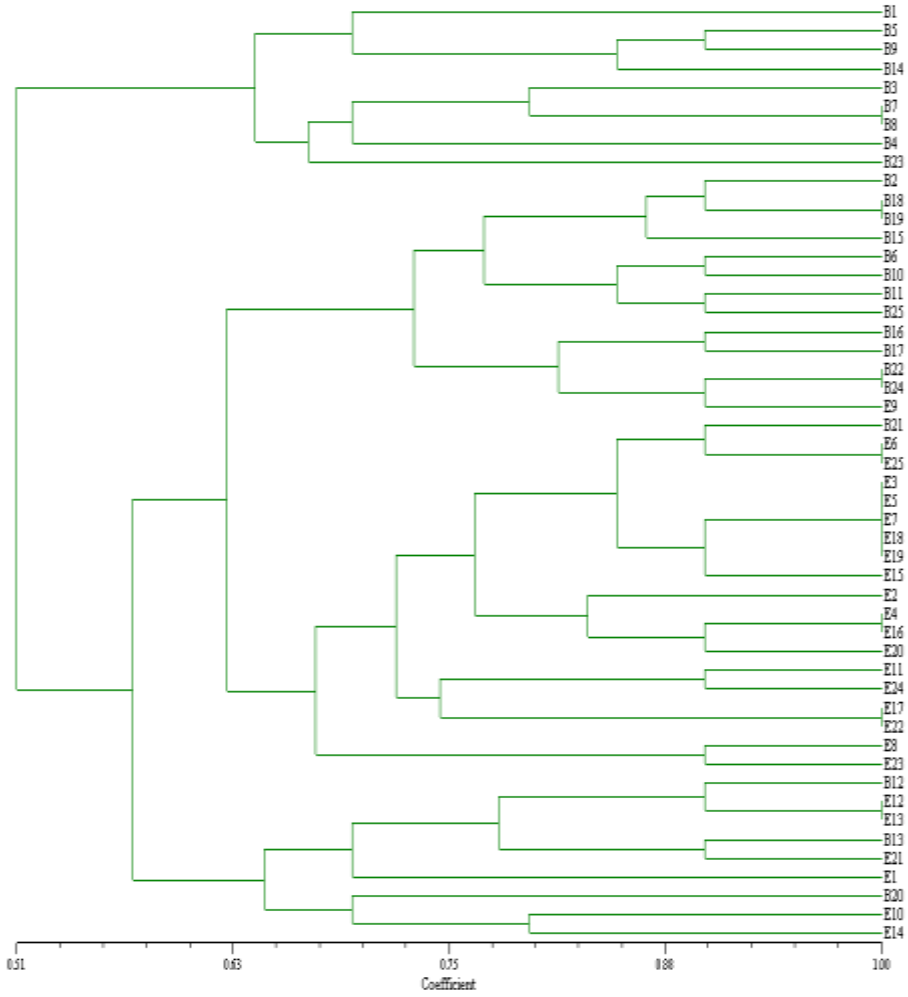


Figure 4: Dendrogram constructed from similarity coefficients and showing the clustering of the genotypes of *Prosopis jouliflora* using SSR with restriction enzyme Eco-RI.

The first sub-group included the genotypes

B2, B6, B10, B11, B15; B16, B17, B18, B19, B22, B24, and B25 from Al-Qassim Region and only the genotype E9 from Eastern Region at genetic similarity coefficients ranged from 0.74 to 1.0. The second sub-group included the genotypes; E2, E3, E4, E5, E6, E7, E8, E11, E15, E16, E17, E18, E19, E20, E24, and E25 from Eastern Region and the genotype B21 from Al-Qassim Region at genetic similarity coefficients ranged from 0.67 to 1.0. Regarding the third sub-group, it included the genotypes; B12, B13, and B20 from Al-Qassim Region and the genotypes; E1, E10, E12, E13, E14, and E21 from Eastern Region at genetic similarity coefficients ranged from 0.64 to 1.0.

Results of the genetic distances for SSR primers using *Bam-HI* enzyme showed that the genotypes which have low histamine contents or no histamine had 100% genetic similarity, such as the genotype B23 from Al-Qassim and the genotype E21 from Eastern Region, both genotypes B19 and B18 from Al-Qassim Region, and both genotypes E14 and E3 from Eastern region (Table 3 & Fig. 5). The genetic cluster tree divided the genotypes into three main groups. The first main group included greater number of genotypes and was divided into two sub-groups. The second main group included the genotypes; B3, B7, B8, B15, and B25 which were all from Al-Qassim Region at genetic similarity coefficients ranged from 0.64 to 1.0. The third main group included only two genotypes; E2 and E16 from Eastern Region only at genetic similarity coefficients ranged from 0.64 to 1.0.

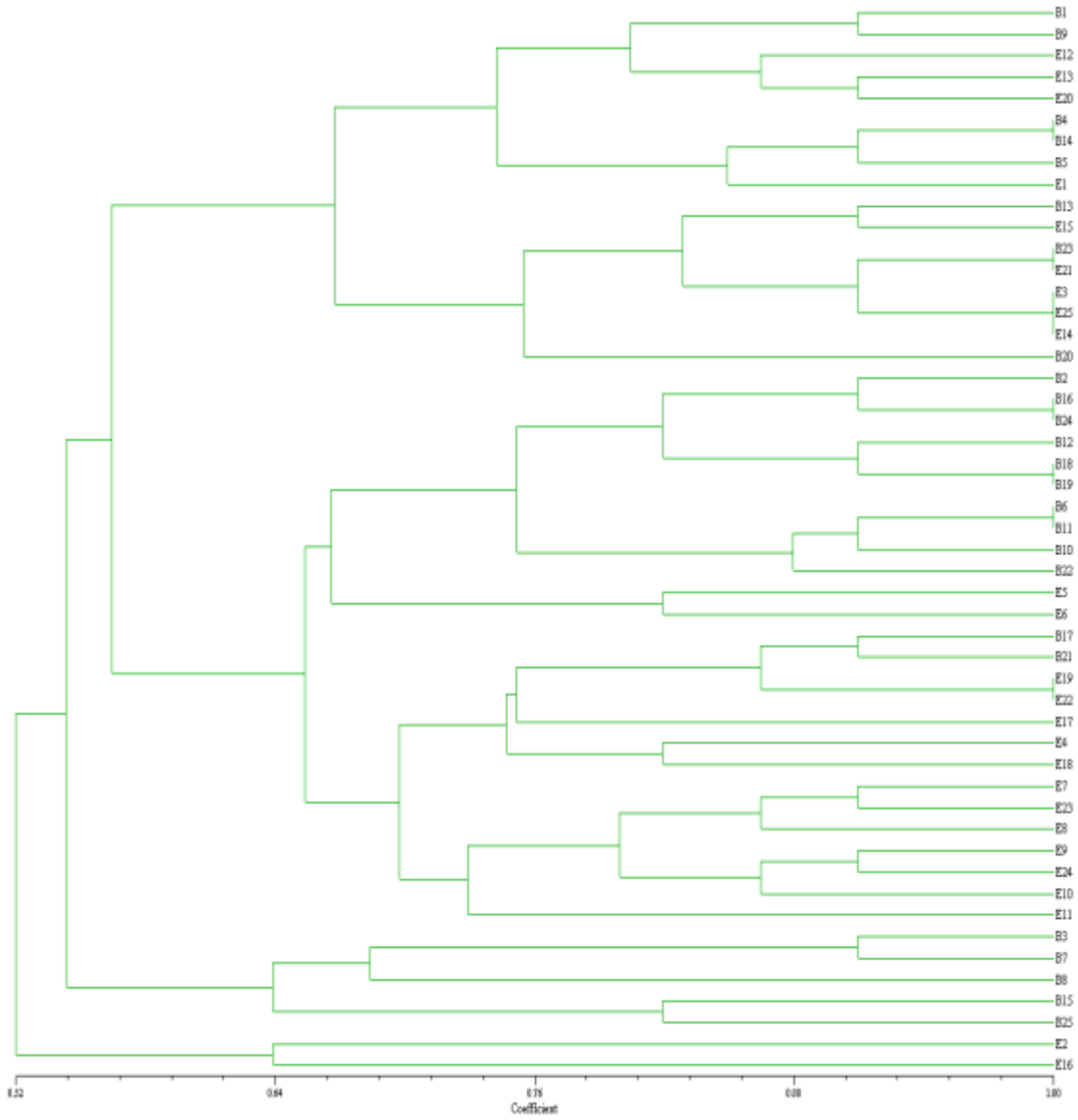


Figure 5:Dendrogram constructed from similarity coefficients and showing the clustering of the genotypes of *Prosopis jouliflora* using SSR with restriction enzyme Bam-HI.

Table 3: Histamine concentrations in *Prosopis juliflora* genotypes from Al-Qassim and Eastern regions

Genotypes	Histamine µg/g	Genotypes	Histamine µg/g
B1	18.6	E1	11.3
B2	4.7	E2	7.9
B3	37.0	E3	13.1
B4	6.6	E4	23.7
B5	17.7	E5	9.4
B6	9.4	E6	11.5
B7	1.3	E7	4.2
B8	9.6	E8	8.1
B9	30.2	E9	1.3
B10	9.2	E10	8.7
B11	8.3	E11	1.6
B12	2.9	E12	6.3
B13	9.2	E13	12.8
B14	19.3	E14	12.1
B15	7.7	E16	3.6
B16	10.1	E17	2.0
B17	11.0	E18	ND
B18	12.5	E19	0.8
B19	15.5	E20	ND
B20	4.1	E21	ND
B21	7.9	E22	7.6
B22	17.2	SE*	±1.3
B23	0.9		
B24	10.1		
B25	4.3		
SE*	±1.8		

*SE= Standard Error ; each difference between two means ≥ SE has a significant difference.

DISCUSSION

The results of SSR markers indicated that double base nucleotides AG/CT were more abundant, followed by single base nucleotides T/A. There is an appreciated resemblance between our results as compared with previous studies. AL-Asbahiet al. (2011) reported 13 different genotypes were identified in 46 prosopis trees randomly selected through the use of SSR primers in microsatellite technique. Sablok and Shekawat (2008) found that there are genetic variability and genetic contrast among prosopis trees. They concluded also that there was a contrast in the number of nucleotides and the mono-nucleotide primers exceeded di-nucleotide primers. The genetic cluster tree, using 14 SSR markers, divided the genotypes to 4 main groups at similarity coefficient between 0.56 and 0.91. The study of Elameer and Almalki (2011) concluded that from 109 bands resulted from 29 ISSR primers, 19 bands were from 7 primers with 99% differences among the prosopis genotypes. Also, Motturaet al.(2005) studied two species of genus *Prosopis* (*P. chilesis and P. flexuuosa*). They found that six microsatellite primers gave genetic variability with similarity coefficients between 0.14 and 0.85 and the number of alleles was 2 and 13 in both species. Simple repeated sequences (SSR) technique has a great importance because of its prevailing in the plant genomes, its high level in polymorphism, co-dominance inheritance, and its ability to variation. Therefore, it is the best technique adapted to plant genomes.

In the present study, six SSR markers were

further analyzed using restriction enzymes. Karaca and Ince (2011) showed that restriction enzymes were suitable in the conversion of monomorphic SSR markers to polymorphic markers. Our results indicatedthat *Eco-R1* enzyme is the only enzyme that can distinguish between the genotypes of each region. The ratio reached up to around 90% and 100% in 19 different genotypes. This conclusion is due to that the genome of *P.Juliflora* species contains the AG/CT di-nucleotide as it was explained when the 14 SSR primers were used. The ability of *Eco-R1* enzyme to distinguish between the different genotypes could be based on that this enzyme cuts between the nucleotides A-G. The high genetic similarity ratios could be explained by the cross pollination system in *P. Juliflora* trees. Besides, SSR primers can react properly with prosopis genotypes in a way that results in changes in the number of repeated units and nucleotide sequences through causing drop mutations (Natalya, 2008). The genetic similarity cluster tree of *Bam-HI* enzyme showed a considerable interference between the genotypes of both regions. It is noted that the genotypes which have low histamine contents or no histamine (Table 3) had 100% genetic similarity, such as the genotype B23 from Al-Qassim and the genotype E21 from Eastern Region (Al-Sooqer et al. 2017). Histamine concentrations in these genotypes were 0.9 and 0.0 µg/g, respectively (Table 3). Also, the genotypes B24 and B16 had 100% genetic similarity and their histamine concentrations were (10.1µg/g) (Table 3) (Al-Sooqer et al. 2017). Both genotypes B19 and B18 from Al-Qassim Region and Both genotypes E14 and E3 from Eastern region had similar results. This result indicates the importance of the enzyme *BamH1* in identifying the genotypes free or had low content of histamine. Bruneau et al. (1992) found that the enzyme histidine decarboxylase was responsible for changing histidine to histamine and it was detectable by the restriction enzyme *Bam-HI*.

CONCLUSION

SSR markers showed that *Prosopis juliflora* have most abundant motif of dinucleotide AG/CT followed by mononucleotide A/T. *Eco-R1* enzyme is the only enzyme that can distinguish among *Prosopis juliflora* genotypes. However, the enzyme *BamH1*could be useful in identifying mesquite genotypes had free or low content of histamine.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

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AUTHOR CONTRIBUTIONS

AAA supervised the proposed project. AAA, MIM, and HMM conceived and designed the experiments. MIM supervised the molecular analysis contributed to data interpretation and prepared the manuscript. QDA analyzed molecular analysis and interpreted these data with assistance from MIM. All authors read and approved the final version.

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