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GENETIC DIFFERENTIATION IN DIFFERENT ENDEMIC *BOSWELLIA SACRA* (BURSERACEAE) POPULATIONS FROM OMAN

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Abstract

Boswellia sacra is an economically important species of family Burseraceae used for frankincense production, an aromatic bark resin that has been a product of local and global trade since prehistoric times in Oman. To help conservation efforts for *Boswellia sacra* populations from Oman, we evaluated 13 populations from diverse ecological locations based on RAPD and SDS-PAGE genetic markers. Genetic diversity based information and the pattern of genetic differentiation across the species habitats helps strategies to conserve wild genetic resources. This is the first in Oman on the genetic evaluation of *Boswellia sacra* populations based on total leaves protein and RAPD analysis. RAPD primers produced a total of 40 RAPD fragments, of which 34 (85%) were polymorphic. Each primer generated 7 to 9 bands with an average of 8 bands per primer. Total leaves protein revealed total 12 polypeptides bands of which 4 (33%) were polymorphic (ranging from 20 to 72 KDa). The levels of expected heterozygosity for RAPD and SDS-PAGE markers were 0.288 and 0.122 respectively. AMOVA analysis showed high level of molecular variance within populations (RAPD - 68% and SDS PAGE - 81%) and low level molecular variance among populations. Isolation by distance model revealed no significant correlation between genetic distances and geographic distances. UPGMA-cluster analysis grouped majority of the populations in the same cluster but some showed high genetic and geographic distances from central and western regions (Thumriat, Dowkha and Mughsil). The results conclude that at present high level of genetic diversity is still preserved in *B. sacra* populations in Dhofar regions.

Key words: Endemic *Boswellia sacra*, Genetic diversity, SDS PAGE, RAPD markers, Oman

Introduction

Boswellia sacra is one of most economically important species of genus Boswellia Roxb, belongs to the family Burseraceae. It is mostly restricted to the Mahra and Hadramawt regions of Yemen and to the southwest regions of Oman and is considered as the only native species to the Arabian Peninsula (Raffaelli & Tardelli, 2006). *Boswellia sacra* are known since ancient times for the production of resins that are economically and culturally quite vital (Khan *et al.,* 2017). Frankincense a yellowish brown gum exuded by the trunk of the tree, used for sterilization by the early Egyptians. Oman and Yemen has been also involved in the collection of frankincens and its trade prospered for many eras. The remains of the prehistoric port of Sumharam (I-III century B.C.), from where the frankincense product was used to be distributed, persisted near Khor Rori east of Salalah (Groom, 2000; Avanzini, 2000). The frankincense have been renowned for their medicinal effects against various diseases such as, viral hepatitis, pathogenesis, cancer, typhoid , and inflammation (Woldeamanuel, 2011; Al-Harrasi *et al.*, 2013; Lin, 2013; Hussain *et al.*, 2013; Al-Harrasi *et al.*, 2014). The collection and production of the resin from *Boswellia sacra* has been a vital activity for majority of the Dhofar people till the years 'seventies to eighties' of the last century, the reason why *Boswellia sacra* is historically important tree and considered as one of the most dynamic genetic resources of Sultanate of Oman (Raffaelli *et al.*, 2006). *B. sacra* populations have shown clear signs of destruction due various unknown and known factors such as, grazing by goats, camels and for firewood purposes (Haile *et al.*, 2011). In spite of its rapid deteriorating status, low seed germination, lesser annual growth rate and economic position (Abiyu *et al.*, 2016; Teshome *et al.*, 2017), a few conservation efforts are ongoing and most the efforts are not supported by genetic data.

Knowing the genetic diversity of plant populations is vital to recognise for future conservation strategies (Moran, 2002; Verde *et al.,* 2013). Genetic variation of plant populations is directed by genetic and demographic practices such as, population size, genetic drift and gene dispersal of the species and breeding system (Booy *et al.,* 2000. Many modern genetic markers, such as Simple Sequences Repeat (SSR), amplified fragment length polymorphism (AFLP), random-amplified polymorphic DNA (RAPD) and biochemical markers (SDS PAGE) are usually use for the assessment of population's genetic diversity (Booy *et al.,* 2000; Coppi *et al.,* 2010; Jan *et al.,* 2016[a,b]; Sharma *et al.,* 2017; Shinwari *et al.,* 2014). Various molecular markers and the biochemical protein profiling of plants have been successfully and usually implied to define the evolutionary traits of a number of crops (Jan *et al.,* 2017 [a,b]; Shinwari *et al.,* 2011; Shinwari *et al.,* 2013; Hameed *et al.,* 2009).

RAPD molecular markers have been extensively used to examine genetic diversity of plant populations (Rehman *et al.,* 2015; Pervaiz *et al.,* 2010; Maki and Horie, 1999; Wang *et al.,* 2004; Sreekumar and Renuka, 2006), and to recommend phylogenetic hypotheses in plants (Jolner *et al.,* 2004). The results obtained from RAPD technique are reliable and widely accepted (Iqbal *et al.,* 2014; Collins *et al.,* 2003). In addition, SDS-PAGE

analysis is broadly used owing to its easiness and validity for defining genetic structure of plant germplasm as total storage proteins are mostly free of environmental flux (Hameed *et al.,* 2009). Hence, in the present study it was aimed to evaluate the genetic diversity and genetic population differentiation of 13 populations of *B. sacra* growing wildly and in cultivated areas in Oman using RAPD and PAGE analysis.

Material and Methods

Population sampling, total genomic DNA and Protein extraction: *B. sacra* tree populations growing in 13 different areas of Dhofar region; central, eastern and western regions were selected for sample collection. These are the most representative sites of *B. sacra* population in Oman occupying the central, eastern and western regions of Dhofar. Fresh leaf samples were collected from a total of 35 trees per each 13 populations, each represented by 10 individuals (Table 1).

A modified DNA extraction protocol with Cetyl Trimethyl Ammonium Bromide (CTAB) was used according to the method of Doyle & Doyle (1987). For each sample, 100 mg of fresh leaf was crushed with liquid nitrogen in a mortar and pestle, then 200 µl of DNA extraction buffer [5M NaCl, 1M Tris-HCl (pH 8.0)], 0.5M EDTA, 2% CTAB, 0.2% 2-Mercaptoethanol, were added, mixed thoroughly and incubated at 65°C for 30 min with occasional vortexing after every ten minutes. Then, 600 µl of phenol: chloroform: iso-amyl alcohol (25:24:1) was added, and the tubes were thoroughly mixed for 20 min. The tubes were centrifuged at 13500 rpm for 20 min at 4°C. The supernatant (upper layer) of 400 µl was collected and transferred to a new sterile centrifuge tube, and an equal volume of ice-cold iso-propyl alcohol was added and inverted for 20 min. The tubes were centrifuged at 13500 rpm for 20 min at 4°C and the iso-propyl alcohol (supernatant) was discarded. Then, 250 µl NaAc (3M) and 10 µl RNAase (100 µg/mL) were added and incubated at 37°C for 30 min. This was followed by the addition of ice cool 70% ethanol and incubation for 20 min at -20°C. The tubes were centrifuged at 13500 rpm for 20 min and the supernatant was discarded. Then 100% ice cooled ethanol was added to each tube followed by centrifugation at 13500 rpm for 20 min. Precipitated DNA was collected and the pellet was dried before re-dissolving in 100 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA). Estimated DNA yields were calculated by a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Q33217), and DNA samples were stored at -20°C freezer until further molecular analysis.

Protein extraction from leaves: The already crushed leaves in liquid nitrogen were subjected to further crushing in mortar at 4°C with protein extraction buffer (6 ml per 1 g) which included 0.2M EDTA, Tris-HCL (1M, pH 8.8), SDS (10%), 2-merceptoethanol (8%), glycerol (50%), and 2.5% polyethylene glycol (PEG 20000), 0.2M MOPS (3-N-morpholino propane sulfonic acid, pH 7.0), 5% Polyvinylpyrrolidone (PVP), 200 mM DTT and 1% Triton x-100. Then the extracted protein samples were incubated in boiling water for three minutes and then after cooling, centrifuged (13500 rpm for 20 minutes at 4°C). The supernatant was collected from form each sample in a separate sterile eppendorf tube (300 µl) BPB (3′, 3″, 5′, 5″-tetrabromophenolsulfonphthalein) was added. 15 µl samples as well as Cleaver Scientific Molecular Weights Protein Ladder (kDa 240-8) were used for electrophoresis as endorsed by Le Grange & De Villiers (1996).

PCR Analysis (RAPD): Polymerase chain reactions were performed in a total volume of 25 µl containing 1 µl (15 ng) of DNA, 2 µl of 10 mM primer, 10 µl Dream Taq PCR Master Mix (2X Thermo Scientific) containing Dream Taq DNA Polymerase, 2X Dream Taq buffer, dATP , dCTP dGTP and dTTP, 0.4 mM each and 4 mM $MgCl_2$. Thermocycling was carried out as, initial denaturation at 94°C for 3 min (one cycle), 35 cycles each of denaturation at 94°C for 30 s annealing at 44°C for 30 s and elongation for 45 s at 72°C and final extension at 72°C for 7 min. The amplified products were separated by gel electrophoresis in 2% agarose in TAE buffer (16.6 M acetic acid Tris-base (40.0 mM) and 0.5 M EDTA-pH 8.0), and visualized in a UV gel documentation system (Cleaver Scientific, omniDOC system, UK).

Table 1. List of evaluated populations of *B. sacra* with geographic location.

No.	Central region		
	Populations	**Geographic coordinates**	**Elevations (m)**
P1	Dowkha (wild)	17 21.407’N 54’ 03.637'E	645
P2	Dowkha (cultivated)	17 20.273’N 54’ 04.580'E	663
P3	Mughsil	17 20.260’N 54’ 20.260'E	600
P4	Adonab (wild)	16 52.573’N 53’ 44.516'E	181
P5	Adonab (cultivated)	16 57.893’N 53’ 52.705'E	097
P6	Thumriat	16 57.225’N 53’ 54.839’E	057
P7	Sadah -a	17 06.039’N 55’ 03.390'E	098
P8	Sadah -b	17 04.763’N 55’ 04.052'E	068
P9	Sadah -c	17 00.589’N 54’ 08.170'E	244
P10	Hasik (lower)	17 31.237’N 55’ 12.923'E	215
P11	Hasik (upper)	17 32.102’N 55’ 13.320'E	254
P12	Kabout (lower)	17 44.988’N 55’ 21.143'E	474
P13	Kabout (upper)	17 47.821’N 55’ 24.559'E	031

SDS PAGE electrophoresis: SDS-PAGE analysis was carried out as described by Laemmli (1970), to differentiate between all 13 populations of the studied *B. sacra* leaves by their protein profiles. Electrophoresis was carried out with a Bio-Rad, Hercules, USA-220/2.0 vertical electrophoresis system. The separation gel (12%) contained: 30% acrylamide mixture (Polyacrylamide and N, N′-Methylenebis acrylamide), SDS (0.1%), ammonium per sulphate (10%), Tris-HCL (1.5 M: pH 8.8), and TEMED (Tetramethyl-ethylediamine) (0.06 ml per 100 ml solution). The stacking gel (5%) comprised of: acrylamide mixture (Polyacrylamide and N, N′-Methylenebis acrylamide), bis-acrylamide (30%), SDS (10 %), ammonium per sulphate (0.035%), Tris-HCI (1M pH 6.8), and TEMED (0.071 ml per 100 ml solution). After pouring the stacking gel, it was kept at room temperature to polymerise for one hour. The wells were carefully washed with tank buffer consisting of Tris-HCI (0.021M pH 8.8), glycine (0.2M), and SDS (0.12 %). Electrophoresis was carried out at 180V for 4 hours. Gels were stained at room temperature in staining solution of 0.02% CBB (Commassie Brilliant Blue R250), 5% ethanol, and 6% acetic acid. After the gels had been stained for overnight, the staining solution was replaced by destaining solution and destained the gels for at least 5 hours.

Statistical analysis

Data generated from RAPD and SDS-PAGE analysis were scored as 1 for the presence and 0 for the absence of bands. To analyse the effective number of alleles (N_e), the percentage of polymorphic bands (PPB), observed number of alleles (N_a), Nei's (1973) Shannon's information index (I) and genetic diversity (H_e), we used GenAlEx statistical software, version 6.2 (Smouse and Peakall, 2015). To assess the variation among and within the population, we chose analysis of molecular variance (AMOVA). Constructed a dendrogram using the UPGMA (unweighted pair group method with arithmetic mean) cluster analysis (Lynch & Milligan, 1994) using the algorithm provided by NTSYSpc Software after converting the data matrix of Nei's genetic distances into the NTSYS format. Then POPGENE (v 1.32) (Yeh & Boyle, 1997) was used to determine the percent polymorphic loci, and Nei's unbiased measures of genetic identity/distance (Nei, 1978).

Results

Genetic diversity based on RAPD Markers RAPD markers: The five screened primers with good results utilized for genetic diversity assessment of *B. sacra* produced 40 clear visible bands (Table 2). Thirty four (85%) out of forty DNA fragments were polymorphic. All five RAPD primers produced more than 80% polymorphic bands with the highest percent polymorphic bands produced by CB-23 primer (88%) followed by CB-9 primer with 75% polymorphic bands. RAPD markers expected heterozygosity (H_E) was calculated for all the *B. sacra* populations; P7 (Sadah-a) population showed the highest H_E (0.18) followed by P12 (0.07), P4 (0.08) and P3 (0.09) with the lowest expected heterozygosity. Shannon's information index followed a different pattern where two different and geographically apart populations, P2 (Dokha-cultivated) and P11 (Hasik-upper) revealed the highest values of Shannon's information index (0.19, 0.19) respectively, while the population from Kabout (lower) showed a lower value as compared to rest of the populations (0.1). The highest number of effective alleles (Ne) were restricted to only two geographically closest populations, P6 (Sadah) and P7 (Sadah –a) (1.3 for each populations) while rest of populations had 1.1 to 1.2 effective alleles (Ne) (Table 4).

AMOVA analysis provided information regarding the genetic structure of populations (Table 3). A higher level of genetic diversity was observed within the populations (68%) compared to a lower level among populations (32%). All the 13 populations grouped into four main clusters (I-IV), cluster III is a large group with eight populations grouped together, cluster I grouped only P1 population and cluster II and IV grouped two populations each. Cluster analysis showed that P1 (Dowkah-wild) is genetically different from rest of the *B. sacra* populations (Fig. 2). Principal coordinate analysis was also carried out for all populations. Here the first three principal coordinates accounted for 63.73, 30.96 and 3.56%, respectively (Fig. 1). Geographic and genetic distances (p>0.0268) were not significantly correlated between the populations (Table 5).

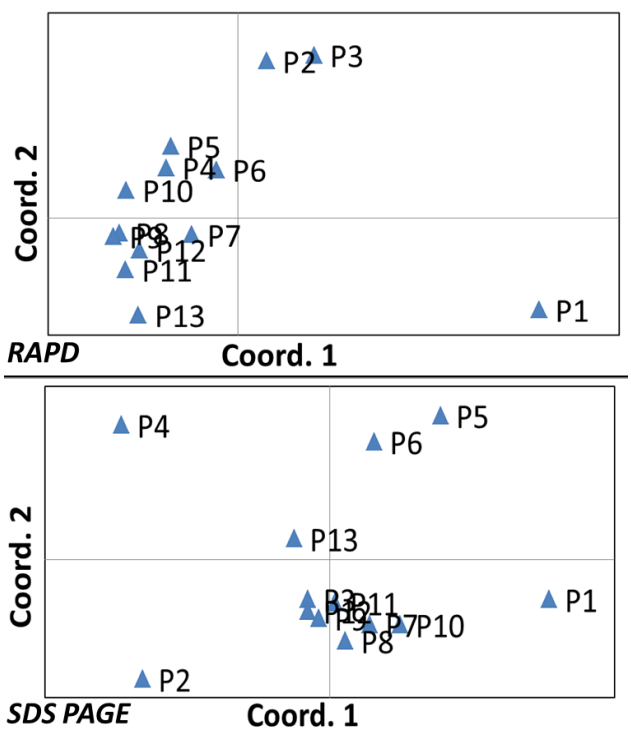

Fig. 1. Principal Coordinate Analysis (PCoA) of RAPD marker and SDS PAGE.

Table 2. RAPD primers set amplifying polymorphic bands and protein bands from 13 populations of *B. sacra*.

Primer pair	Sequences (5'-3')	TB	PB	PPB%
RAPD primers				
OPA04	AAT CGG GCT G	7	6	85
CB9	GGT GAC GCA G	8	6	75
CB12	AGT CGA CGC C	8	7	87
CB13	ACG CAT CGG A	8	7	87
CB23	CTG GGC ACG A	9	8	88
Total		40	34	85.00%
SDS PAGE (Polypeptides				
Total		**12**	**4**	**33%**

TB; Total bands, PB; polymorphic bands, PPB; percentage of polymorphic bands

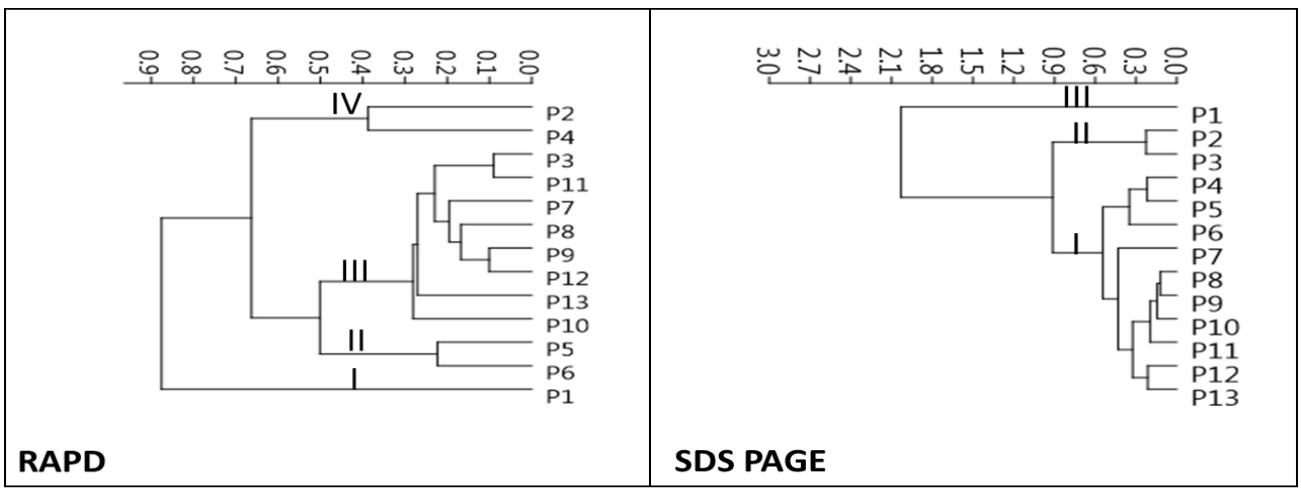

Fig. 2. UPGMA dendrogram generated by similarity coefficients based on RAPD and SDS PAGE analysis.

Table 3. Summary of the AMOVA results for within and among genetic variations of *B. sacra* using SDS PAGE and RAPD markers.

Source	Df	SS	MS	Est. V	%	P
	RAPD Analysis					
Among Pops	12	128.4	10.7	2.2	32	0.001
Within Pops	22	109.3	4.8	4.8	68	
Total	**35**	**237.7**		**6.9**	**100**	
	SDS PAGE Analysis					
Among Pops	12	17.1	1.4	0.2	19	0.018
Within Pops	23	20.0	0.9	0.9	81	
Total	**35**	**37.1**		**1.1**	**100**	

SDS PAGE analysis: Genetic diversity based on Protein polypeptides (SDS-PAGE) analysis showed lower variations in all population of *B. sacra* (Table 2). A total of 12 polypeptides fragments were observed in all 13 populations and only 4 out of 12 were polymorphic in nature (33%). All populations showed less than 33% polymorphic bands (PB) and three populations (P1, P4 and P8) showed 33% PB while two populations (P8 and P9) exhibited only 8% PB. The expected heterozygosity (He) was highest for P3 (0.9) followed by P2, P4 and P6. Two populations showed lowest He (0.1) and these two population were from Dowkah-wild (P1) and Sadah-a (P8). Shaman's information index varied from0.1 to 0.8 for all the populations. The number of effective alleles (Ne) was highest in P4 population (1.6) and it was in the range of 1.1-1.2 in the other 12 populations (Table 4). AMOVA analysis for SDS PAGE showed highest genetic diversity within the populations (81%) as compared to among the populations (19%) (Table 3). All three molecular markers used here for genetic diversity assessment of *B. sacra* showed similar AMOVA results i.e., revealed highest genetic variations within the population and lowest genetic diversity among the *B. sacra* populations.

Cluster analysis of populations of *B. sacra* by means of UPGMA analysis procedures grouped all populations into three main clusters, I, II and III. Cluster 1 consisted of most of the populations (10) and was further divided into two sub clusters, while cluster II consisted only of two populations (P2 and P3) while cluster III grouped only one populations P1 (Fig. 2). The first three principal coordinates contributed for 87.3% of the total variation. The first three principal coordinates, contributed for 44.77% 29.56% 13.16% respectively (Fig. 1). SDS PAGE markers showed the lowest significant correlations among geographic and genetic distances (p>0.0002) as compared to other genetic markers (Table 5).

Table 4. Genetic variations within *B. sarca* populations using RAPD and SDS PAGE markers.

Populations	Na	Ne	I	He	uHe	PPB
	RAPD Analysis					
P1	1.7 (0.15	1.2 (0.05)	0.2 (0.04)	0.14 (0.030	0.17 (0.03)	40
P2	1.1 (0.06)	1.2 (0.04)	0.19 (0.03)	0.13 (0.03)	0.15 (0.01)	35
P3	0.91 (0.05)	1.1 (0.05)	0.13 (0.05)	0.09 (0.00)	0.12 0.03)	22
P4	1.1 (0.08)	1.1 (0.05)	0.12 (0.00)	0.08 (0.10)	0.11 (0.03)	20
P5	1.2 (0.06)	1.2 (0.03)	0.18 (0.04)	0.12 (0.03)	0.16 (0.04)	30
P6	1.3 (0.09)	1.3 (0.06)	0.24 (0.04)	0.16 (0.03)	0.20 (0.00)	40
P7	1.4 (0.05)	1.3 (0.05)	0.27 90.06)	0.18 (0.00)	0.22 (0.00)	50
P8	1.2 (0.09)	1.2 (0.00)	0.21 (0.01)	0.15 (0.00)	0.18 (0.00)	35
P9	1.3 (0.050	1.2 (0.05)	0.22 (0.00)	0.14 (0.01)	0.17 (0.06)	40
P10	1.1 (0.09)	1.2 (0.05)	0.18 (0.06)	0.12 (0.00)	0.14 90.0)	32
P11	1.2 (0.09)	1.2 (0.07)	0.19 (0.01)	0.13 (0.00)	0.15 (0.00)	32
P12	1.5 (0.08)	1.1 (0.06)	0.10 (0.00)	0.07 (0.00)	0.09 (0.00)	17
P13	1.2 (0.07)	1.1 (0.04)	0.14 (0.03)	0.09 (0.06)	0.11 (0.00)	27
	SDS PAE Analysis					
P1	1.3 (0.14)	1.1 (0.10)	0.1 (0.14)	0.1 (0.05)	0.1 (0.02)	33
P2	1.9 (0.10)	1.1 (0.10)	0.1 (0.04	0.8 (0.01)	0.9 (0.02)	16
P3	1.1 (0.02)	1.1 (0.05)	0.1 (0.01)	0.9 (0.04)	0.8 (0.03)	16
P5	1.5 (0.15)	1.1 (0.05)	0.8 (0.06)	0.7 (0.01)	0.5 (001)	16
P6	1.2 (0.18)	1.2 (0.11)	0.8 (0.01)	0.4 (0.01)	0.6 (0.05)	33
P7	1.2 (0.14)	1.1 (0.12)	0.6 (0.02)	0.4 (0.03)	0.3 (0.01)	25
P8	1.3 (0.01)	1.2 (0.01)	0.5 (0.10)	0.19 (0.02)	0.2 (0.01)	33
P9	1.0 (0.03)	1.1 (0.02)	0.3 (0.01)	0.6 (0.02)	0.4 (0.03)	8
P10	1.1 (0.04)	1.1 (0.02)	0.3 (0.01)	0.6 (0.02)	0.4 (0.03)	8
P11	1.2 (0.03)	1.1 (0.11)	0.8 (0.02)	0.4 (0.00)	0.1 (0.00)	25
P12	1.2 (0.03)	1.1 (0.11)	0.8 (0.02)	0.4 (0.00)	0.1 (0.00)	16
P13	1.2 (0.03)	1.1 (0.11)	0.8 (0.02)	0.4 (0.00)	0.1 (0.00)	25

Na = No. of Different Alleles, Ne = No. of Effective Alleles, I = Shannon's Information Index, He = Expected Heterozygosity, uHe = Unbiased Expected Heterozygosity and Percentage of Polymorphic Loci

Table 5. Pairwise population matrix of Nei genetic distance and geographic distance-km (above the diagonal), (B) RAPD and (A) SDS PAGE.

	Tum	Dok-c	Dok-w	Muh	Ado-w	Ado-c	Sad-a	Sad-b	Sad-c	Has-l	Has-u	Kab-l	Kab-u
	A												
Thumrait	0.0	35	53	57	39	61	139	126	49	163	147	165	181
Dowkha-c	0.33	0.0	19	63	29	45	104	92	28	132	116	140	156
Dowkha-w	0.39	0.33	0.0	78	43	52	86	73	36	113	97	124	139
Mughsil	0.43	0.35	0.16	0.0	34	36	145	140	44	187	171	201	217
Adonab-c	0.31	0.33	0.35	0.38	0.0	22	117	109	13	154	138	167	182
Adonab-w	0.37	0.32	0.35	0.34	0.27	0.0	110	105	18	154	139	173	187
Sadah-a	0.36	0.29	0.33	0.33	0.27	0.00	0.0	20	104	61	53	103	109
Sadah-b	0.35	0.32	0.33	0.32	0.29	0.33	0.30	0.0	96	52	39	87	96
Sadah-c	0.40	0.35	0.38	0.36	0.31	0.00	0.28	0.30	0.0	143	127	159	174
Hasik-L)	0.39	0.36	0.33	0.38	0.30	0.00	0.31	0.33	0.31	0.0	16	48	49
Hasik-U)	0.30	0.33	0.38	0.43	0.30	0.34	0.37	0.34	0.36	0.27	0.0	50	57
Kabaut-L)	0.40	0.39	0.46	0.48	0.40	0.04	0.43	0.42	0.40	0.31	0.27	0.0	18
Kabaut-U)	0.39	0.38	0.37	0.36	0.32	0.00	0.27	0.30	0.34	0.30	0.28	0.36	0.0
	Tum	**Dok-c**	**Dok-w**	**Muh**	**Ado-w**	**Ado-c**	**Sad-a**	**Sad-b**	**Sad-c**	**Has-l)**	**Has-u)**	**Kab-l)**	**Kab-u)**
	B												
Thumrait	0.0	35	53	57	39	61	139	126	49	163	147	165	181
Dowkha-c	0.45	0.0	19	63	29	45	104	92	28	132	116	140	156
Dowkha-w	0.39	0.09	0.0	78	43	52	86	73	36	113	97	124	139
Mughsil	0.62	0.32	0.31	0.0	34	36	145	140	44	187	171	201	217
Adonab-c	0.58	0.23	0.28	0.07	0.0	22	117	109	13	154	138	167	182
Adonab-w	0.44	0.21	0.21	0.06	0.08	0.0	110	105	18	154	139	173	187
Sadah-a	0.49	0.23	0.32	0.28	0.18	0.16	0.0	20	104	61	53	103	109
Sadah-b	0.70	0.33	0.43	0.21	0.19	0.19	0.12	0.0	96	52	39	87	96
Sadah-c	0.72	0.34	0.44	0.26	0.20	0.26	0.09	0.04	0.0	143	127	159	174
Hasik-L)	0.70	0.28	0.39	0.26	0.17	0.25	0.11	0.10	0.07	0.0	16	48	49
Hasik-U)	0.68	0.37	0.44	0.24	0.19	0.21	0.14	0.15	0.14	0.16	0.0	50	57
Kabaut-L)	0.68	0.38	0.38	0.23	0.20	0.23	0.21	0.21	0.21	0.24	0.14	0.0	18
Kabaut-U)	0.66	0.42	0.46	0.15	0.20	0.21	0.22	0.21	0.23	0.21	0.20	0.18	0.0

Discussion

The level of polymorphism among the *B. sacra* populations was high with RAPD markers, (85%) as compared to SDS PAGE (33%). This high level of RAPD markers polymorphism in *B. sacra* populations is in agreement with the results of Tsivelikas *et al.,* 2009.The degree of RAPD polymorphism detected in the present study was somewhat higher than that reported in other genetic diversity studies by using RAPD markers in different plants, such as 84%, (Hadia *et al.,* 2008), 79% (Khan *et al*., 2009), 37% (Behera *et al*., 2008), and 72% (Yildiz *et al*., 2011). Comparing to total leaves storage protein, RAPD markers exposed high level polymorphic fragments. This is likely, as proteins markers reproduce only dissimilarity in the coding parts of the genome; this is naturally more conservative and thus shows less polymorphism. Whereas RAPD markers can identify variation in both coding and non-coding parts of the genome, and the size of the primers sequence allows the implication of many DNA fragments with a single primer (Guadagnuolo *et al.,* 2001). The presence of unique RAPD bands by primer CB-12 in some populations from the Thumriat region as well as unique protein fragments the same populations, possibly show the result of eco-geographic alterations in gene diversity of plant populations, where plants from the similar geographic area are closely related (Du *et al.,* 2011). The average effective allele (Ne) is the allele producing polymorphic loci which is 1.19 (RAPD) and 1.167 (SDS PAGE) among all 13 populations. The numbers of effective allele (Ne) are directly linked with expected heterozygosity (He). The higher the number of effective allele (Ne) is the greater the value of expected heterozygosity (He), in other words the higher is the genetic diversity. The expected heterozygosity (HE) for RAPD was 0.127, among thirteen populations, which was comparatively higher than the earlier report of (Coppi *et al.,* 2010).

The AMOVA analysis exhibited higher molecular variance within the populations and lowest molecular variance among the populations for both RAPD and SDS PAGE markers. These results were in line with the earlier studies when molecular markers were used on populations of *B. sacra* from the same geographic regions in Oman (Coppi *et al*., 2010). It has been shown that the higher degree of genetic diversity within populations is a general trend in out-crossed and wind pollinated species as shown in common grasses and *Trifolium virginicum* (Durka *et al*., 2017; Addisalem *et al*., 2016). The Nei's unbiased pair-wise genetic distances revealed large genetic distance between the eastern (Sadah, Kabout and Hasik) and central region (Thumriat) populations, which are geographically most isolated. Hasik and Thumriat populations were genetically the most isolated populations from each other (0.68) followed by Kabout and Thumriat populations (0.66). The range of genetic identity and genetic distance both with RAPD and SDS PAGE showed the occurrence of variability among the populations of Thumriat region. With both RAPD and SDS PAGE markers, populations originating from the similar regions (Dokkha & Mughsil regions) were the closest in genetic distance range, hence had the highest degree of resemblance. However, those from detached areas were the furthermost, maybe because low or no anthropogenic activities. (Yuan *et al.,* 2007). The genetic diversity evaluation among *B. sacra* populations based on RAPD and SDS PAGE markers through dendrogram was low except from two to three populations which grouped separately (P1, P2 and P3 and P4). Similar low genetic variability investigations have been studied by many other researchers in different plant groups (Hadia *et al.,* 2008). This low genetic diversity dendrogram was probably due to high gene flow which was identified by these two kind of markers. The dendrogram showed that populations from Dowkha, Adonab and Mughsil regions clustered separately with both RAPD and SDS PAGE markers. There may be various factors could have led to this separate grouping such as, the coastal ecotype (Jury *et al.,* 2009), might have changed the genetic make-up of these populations. This clustering revealed that populations were grouped according to their agro-ecological areas instead of selfing effect (Tsivelikas *et al.,* 2009; Yuan *et al.,* 2007).

This was possibly due to their close ecogeographical locations of these separately grouped populations may be the key factors.

Conclusion

Our results of genetic diversity evaluation show that in comparison to total leaves protein markers, RAPD markers are good option for genetic diversity assessment in *B. sacra*. Overall all populations showed reasonable level of genetic diversity but some populations showed high level of genetic variations and revealed high genetic and geographic distances (Thumriat, Dowkha and Mughsil), therefore ex-situ and in-situ conservation program are required.

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