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POPULATION DIVERSITY OF *AELUROPUS LAGOPOIDES*: A POTENTIAL CASH CROP FOR SALINE LAND

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Abstract

Aeluropus lagopoides is a salt tolerant grass which propagates both through genets and ramets. Six disjunct populations of *A. lagopoides* from Pakistan were selected to test the hypothesis that genetic diversity would be low within but higher among populations. Genetic diversity was investigated using RAPD markers. AMOVA showed higher genetic diversity within population (74%) and lower among population (26%). Furthermore, there were no genetic differences between coastal and inland populations. However, substantial (11%) genetic variation existed among populations of Sindh and Balochistan. Higher genetic diversity within populations are possibly due to physical disturbances that may provide more opportunity for establishment of seeds and increase the possibility of out crossing. Low diversity among populations or between coastal and inland populations indicates fragmentation of a single meta-population due to anthropogenic activity. Geographical barrier between Sindh and Balochistan, appears to mediate gene flow among populations of *A. lagopoides*.

Introduction

Aeluropus lagopoides (Linn.) Trin. ex Thw. (Poaceae) is a salt-secreting, rhizomatous perennial grass which dominates both the seasonally inundated coast as well as inland salt flats of arid Pakistan (Khan & Gulzar, 2003). It has a wide range of distribution from northern part of Africa to the Middle East (Cope, 1982) and appears to propagate mainly through rhizomes in mono-specific stands or through seed while colonizing open niches (Gulzar & Khan, 1998). It is a good candidate for saline agriculture because of its perennial habit (Torbatinejad *et al*., 2000). Ecologically, its clonal characteristics also help in reducing soil erosion (Khan & Gul, 1999). Adaptive features of *A. lagopoides* like slow vegetative propagation, vigorous seed production, strong network of roots, epicuticular wax, salt secreting habit and small leaves help the species to survive both in coastal and inland stressful habitats (Mohsenzadeh *et al*., 2006). Gulzar & Khan (1998) reported considerable variation in environmental conditions between coastal and inland regions of Pakistan with variable effects on water relations of three halophytes. Coastal populations were subjected to harsher environmental conditions in comparison to inland ones. Stresses such as salinity, light and temperature have inhibitory effects on seed germination of *A. lagopoides*. Consequently, it depends on vegetative propagation to maintain its population (Khan & Gulzar, 2003) under stressful environments which may lead to a decrease in the genetic diversity and fitness (Hensen & Oberprieler, 2005).

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Random amplified polymorphic DNA (RAPD) markers are useful in diversity studies of plant populations (Rodriguez-Gacio *et al*., 2009). In comparison to microsatellites, these markers have some limitations, such as marker allele dominance (Lynch & Milligan, 1994) and sometimes low reproducibility, which may have discouraged many investigators from using RAPD. However, high reproducibility can be obtained under carefully controlled conditions (Rodriguez-Gacio *et al*., 2009). RAPD analysis also has certain advantages like high levels of polymorphism, are inexpensive, faster and easier when compared to microsatellite analysis and also does not require prior DNA sequence information of the target species (Rodriguez-Gacio *et al*., 2009). Application of cytochrome P450 gene-analogues (PBA) as a RAPD marker is an efficient way for analyzing the plant functional genetic material (Yamanaka *et al*., 2003). Inui *et al.* (2000) designed some P450 based analogues (PBA) primers based on the conserved domains of P450 mono-oxygenase gene. PBA markers were also successfully used as RAPD marker for 51 plant species from 28 taxonomic families by Yamanaka *et al.* (2005). Previously, PBA markers were used at individual species or accession level for genetic diversity studies (Yamanaka *et al*., 2005). Gilani *et al*., (2009) used PBA markers for evaluating population diversity of *Withania coagulans*, due to its higher reproducibility and greater number of polymorphic bands. Microsatellites, known as simple sequence repeats (SSRs), can be used as RAPD markers resulting in higher number of reproducible alleles (Jatoi *et al*., 2006). SSRs have also been utilized as efficient markers as they are in abundance and well dispersed in genome with high frequency of polymorphism (Jatoi *et al*., 2006). They also reported the successful utilization of rice-SSRs markers, as RAPD markers for exploring genetic diversity in Zingiberaceae.

Halophytes and their habitats have received special attention for nature conservation especially in Central Europe (Weising & Freitag, 2007). Fewer studies have exploited molecular markers to address issues of genetic diversity and gene flow of halophytes between coastal and inland habitats (Lambracht *et al*., 2007). Several reports are available on the ecological and physiological aspects of *A. lagopoides* (Waghmode & Hegde, 1984) however, population diversity of *A. lagopoides* has not been explored.

Lower genetic diversity within population of *A. lagopoides* would be due to clonal growth, whereas the relatively higher genetic diversity among populations due to disjunct distribution. Based on the above assumptions, we have the following hypothesis; (a) genetic diversity is higher among populations of *A. lagopoides*, (b) coastal and inland populations have lower similarity of genetic material, and (c) difference in gene pool among population of *A. lagopoides* reflects the presence of geographical barriers.

Materials and Methods

Population sampling: Leaves from six populations of *Aeluropus lagopoides* were collected from the coastal and inland habitats of two provinces (Sindh and Balochistan) of Pakistan. GPS positions of all populations were recorded and are listed in Table 1. Sixteen accessions were selected from each population for investigating within population genetic diversity. Leaf samples were placed in plastic bags containing silica gel, transported to the laboratory and stored in a refrigerator.

DNA extraction: Protocol of Doyle & Doyle (1990) with minor modifications was used for the extraction of total DNA from 96 individuals collected from 6 different populations of *A. lagopoides*. A total of 0.25 g air dried leaves of each individual plant were added

separately to 700 µl micro-preparation buffer (300 µl extraction buffer, 5% sarkosyl, 1% sodium bisulphite, 2% PVP (polyvinylpyrrolidone), 1% mercaptoethanol and 300 µl lysis buffer). The extraction buffer was prepared from 0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA (ethylenediaminetetraacetic acid) and lysis buffer from 0.2 M Tris, 0.05 M EDTA, 2 M NaCl (sodium chloride), 2% CTAB (cetyl trimethyl ammonium bromide). After extraction, 700 µl of chloroform : isoamyl alcohol was added in a ratio of 24:1 to remove impurities. DNA was then precipitated from the aqueous phase by adding 400 µl isopropanol and centrifuged. The DNA pellet was washed with 70% ethanol, air-dried, and re-suspended in 100 µl TE buffer (10 mM Tris/HCl pH 8.0; 1 mM EDTA). The quality and quantity of DNA was checked on 1% agarose gel and spectrophotometer at 260/280 nm. After quantification of extracted DNA, working solution of 25 ng μl^{-1} was prepared and stored at 4°C until use in RAPD-PCR reactions with (PBA and rice-SSR) markers.

Random amplified polymorphic DNA (RAPD) analysis: We used 20 markers (14 PBA and 6 rice-SSR) for RAPD analysis details of the markers used are given in Table 2. RAPD-PCRs were carried out in reaction volumes of 25 µl containing 1 × Ex Taq buffer (TaKaRa Japan; including Mg^{+2}), dNTPs (0.16 mM each), 1 unit of Ex Taq Polymerase (TaKaRa, Japan), 1 mM of each forward and reverse primer and 50 ng of DNA template. The PCR was performed in a thermal cycler (GeneAmp, PCR System 9700, Applied Biosystems) with a heated lid and optimized primer dependent conditions for *A. lagopoides*. During application of PBA and rice-SSR markers: initial denaturation (94°C for 5 min.) and final extension (72°C for 10 min.) was performed followed by decrease in temperature to 4°C. After initial denaturation step with PBA markers, performed 40 cycles of 94°C (1 min.), primer specific annealing temperature (Table 3; 2 min.) and 72°C (3 min.) sequentially. While for rice-SSR following conditions were used: 35 cycles of 94°C (1 min.) followed by primer specific annealing temperature (1 min.) and 72°C (1.5 min.). The PCR products were separated on 1.5% agarose gels (100 V; 120 min.) in 0.5% TBE buffer and ethidium bromide was used for staining before taking picture. Øx174 was used as a molecular size marker ranging between 72 to 1,353 bp. The banding pattern was visualized under UV-light of Geldoc System (Biorad, München, Germany) while for band scoring the Quantity One Software (Version 4.6.6) was used.

Statistical analyses: For statistical analyses, MS Excel binary data sheet was prepared by scoring all DNA fragments as either 1 (present) or 0 (absent). Each band was assumed to represent the phenotype at a single biallelic locus and the RAPD markers represented dominant alleles (Williams *et al*., 1990). Observed number of alleles (Ao), effective number of alleles (Ae), genetic diversity (h; Nei, 1973), Shannon information index (I) and percentage of polymorphic bands were calculated using the statistical software POPGENE 1.31 (Yeh *et al*., 1999) based on Hardy-Weinberg equilibrium. Dendrogram was constructed through POPGENE Ver. 1.31 (Lynch & Milligan, 1994) using the un-weighted pair-group method of arithmetic average (UPGMA; modified from NEIGHBOR procedure of PHYLIP Version 3.5) based on genetic distances (Nei, 1972). Analysis of molecular variance (AMOVA), population banding pattern, Mantel test and principal coordinate analysis (PCA) was performed using GenAlEx 6.1 (Peakall & Smouse, 2006). AMOVA analyses were performed at three hierarchical levels to categorize the distribution of genetic variation when 1) all populations were considered as a region, 2) coastal vs. inland regions and 3) Sindh vs. Balochistan regions (computing 999 permutations). Mantel test was performed between genetic and geographical distances of selected populations. PCA was conducted on the basis of Nei unbiased genetic distance.

Table 1. Locations of six *A. lagopoides* populations selected from Pakistan.

Site of collection	Province (Region)	Code	Sample size	Latitude (North)	Longitude (East)
Ship Breaker*	Balochistan	SB	16	25° 01′ 57.09″	66° 42′ 56.46″
Gadani*	Balochistan	GB	16	25° 56′ 11.15″	66° 43′ 55.16″
Hub	Balochistan	HB	16	25° 01′ 55.24″	66° 43′ 19.13″
Sands Pit*	Sindh	SS	16	24° 50′ 56.47″	66° 56′ 13.43″
Clifton*	Sindh	CS	16	24° 47′ 57.27″	67° 02′ 18.01″
Karachi University	Sindh	KS	16	24° 56′ 06.11″	67° 07′ 18.41″

* = Populations from coastal region

Table 2. List of rice-SSR and PBA primers.

Primer	Sequence (5' to 3')	Reference
Rice-SSR		
RM 1	F: GCGAAAACACAATGCAAAAA R: GCGTTGGTTGGACCTGAC	Panaud *et al*., (1996)
RM 125	F: ATCAGCAGCCATGGCAGCGACC R: AGGGGATCATGTGCCGAAGGCC	Temnykh *et al*., (2000)
RM 135	F: CTCTGTCTCCTCCCCCGCGTCG R: TCAGCTTCTGGCCGGCCTCCTC	Akagi *et al*., (1996)
RM 153	F: GCCTCGAGCATCATCATCAG R: ATCAACCTGCACTTGCCTGG	Akagi *et al*., (1996)
RM 171	F: AACGCGAGGACACGTACTTAC R: ACGAGATACGTACGCCTTTG	Akagi *et al*., (1996)
RM 278	F: TTCCCTGTTAAGAGAGAAATC R: GTGTATTTGGTGAAAGCAAC	Temnykh *et al*., (2000)
PBA		
CYP1A1F	GCC AAG CTT TCT AAC AAT GC	Inui *et al*., (2000)
CYP2B6F	GAC TCT TGC TAC TCC TGG TT	Inui *et al*., (2000)
CYP2C19F	TCC TTG TGC TCT GTC TCT CA	Inui *et al*., (2000)
CYP1A1R	AAG GAC ATG CTC TGA CCA TT	Inui *et al*., (2000)
CYP2B6R	CGA ATA CAG AGC TGA TGA GT	Inui *et al*., (2000)
CYP2C19R	CCA TCG ATT CTT GGT GTT CT	Inui *et al*., (2000)
heme2B6	ACC AAG ACA AAT CCG CTT CCC	Kiyokawa *et al*., (1997)
heme2C19	TCC CAC ACA AAT CCG TTT TCC	Kiyokawa *et al*., (1997)

Results

In the current study, we used 27 sets of primers to test our hypotheses and among these 20 primer combinations (6 rice-SSR and 14-PBA) showed two or more sharp bands (Table 3).

Rice-SSR as a RAPD marker: During application of rice-SSR markers as RAPD, number of observed bands was 44, while the average number of bands per accession were 5.1 (Table 3). Number of polymorphic bands were 42 out of 44 which exhibited 95% polymorphism. Moreover, four rice-SSR markers displayed 100% polymorphism (Table 3). RM135 and RM278 markers yielded maximum (7.8) and minimum (3.2) numbers of average bands per accession, respectively.

PBA as a RAPD marker: The observed number of bands was 114 when we used P450 primer combinations. However, number of bands per accession were 5.2 (Table 3). Among 114 bands, 105 bands were polymorphic and rate of polymorphism was 92%. Among all P450 markers, Cyp2B6F / Cyp1A1R showed maximum average numbers (9.7) of bands per accession.

Table 3. General profile of DNA amplification by 6 rice-SSR and 14 PBA primers set in *A. lagopoides*.

Primer Set	Annealing Temperature	Total band	PL[a]	PPL[b]	Ave. bands[c]
RM 1	55	5	4	80	3.6
RM 125	55	6	6	100	5.2
RM 135	63	10	9	90	7.8
RM 153	55	9	9	100	5.5
RM 171	55	9	9	100	5.3
RM 278	55	5	5	100	3.2
Total		**44**	**42**	**95**	**5.1**
CYP1A1F/ CYP2C19R	46.5	11	11	100	6.1
CYP1A1F/ heme2C19	46.5	6	6	100	3.9
CYP2B6F/ CYP2C19R	46.5	10	10	100	6.2
CYP2C19F/ CYP2C19R	46.5	10	10	100	6.3
CYP2C19F/ CYP2B6R	52	6	4	67	3.0
CYP2B6F/ heme 2C19	52	4	3	75	2.6
CYP2B6F/ heme 2B6	52	8	8	100	4.8
CYP1A1F/ CYP2B6R	52	8	7	88	6.3
CYP1A1F/ heme2B6	52	9	9	100	6.2
CYP2B6F/ CYP1A1R	52	12	10	83	9.7
CYP2B6F/ CYP2B6R	52	12	11	92	9.1
CYP1A1F/ CYP1A1R	56	2	1	50	1.6
CYP2C19F/ heme 2B6	56	11	11	100	4.1
CYP2C19F/ heme 2C19	56	5	4	80	2.5
Total		**114**	**105**	**92**	**5.2**

[a]PL = Polymorphic loci
[b]PPL = Percentage of polymorphic loci
[c]Ave. Bands = Average number of bands

Population diversity based on both PBA and rice-SSR: On the basis of all markers (rice-SSR and PBA), the total number of polymorphic bands was 147 with 93.04% polymorphic loci (Table 4). Total polymorphic loci and percent polymorphic loci were highest (TPL: 121; PPL: 76.58%) in Clifton population (CS) and lowest (TPL: 79; PPL: 50%) in Sands pit (SS) population (Table 4). The Shannon information index (S) and Nei's genetic diversity varied from 0.294 to 0.426 and 0.202 to 0.291, respectively at population level (Table 4). Among selected populations, Sands pit population showed the least values of all genetic variability parameters (h = 0.202; S = 0.294; Table 4). The size of amplified band ranged between 200-1800 bp.

Dendrogram constructed on the basis of UPGMA analysis, divided all six populations into two clusters (Fig. 1). All the three populations from Balochistan were grouped in Cluster I. Cluster II consisted of populations from Sindh region (Fig. 1) whereas Sands pit population (SS) was separated from all other populations.

Principal coordinate analysis was conferring the result of UPGMA by two separate groups of Sindh and Balochistan populations (Fig. 2). However coastal and inland populations were mixed with each other (Fig. 2). The sharing of PC1, PC2 and PC3 were 41.64%, 33.36% and 11.23% respectively, whereas the cumulative contribution was 86.23%. Results of Mantel test revealed weak correlation between genetic and geographical distances (R^2 = 0.181; p<0.010; Fig. 3).

Table 4. Genetic diversity indices of *A. lagopoides* populations.

P[a]	*Ao*[b]	*Ae*[c]	*h*[d]	*S*[e]	TPL[f]	PPL[g]
SB	1.746 (0.436)	1.515 (0.382)	0.291 (0.197)	0.426 (0.277)	118	74.68
GB	1.620 (0.486)	1.449 (0.410)	0.249 (0.216)	0.362 (0.305)	98	62.03
HB	1.658 (0.475)	1.437 (0.386)	0.250 (0.205)	0.368 (0.290)	104	65.82
SS	1.500 (0.501)	1.361 (0.405)	0.202 (0.215)	0.294 (0.307)	79	50.00
CS	1.765 (0.424)	1.499 (0.368)	0.286 (0.191)	0.423 (0.268)	121	76.58
KS	1.658 (0.475)	1.409 (0.374)	0.238 (0.200)	0.354 (0.284)	104	65.82
Total	**1.930 (0.255)**	**1.575 (0.330)**	**0.333 (0.150)**	**0.496 (0.203)**	**147**	**93.04**

[a] P = Population codes (see Table 1)
[b] *Ao* = Observed number of alleles
[c] *Ae* = Effective number of alleles
[d] *h* = Genetic diversity according to Nei (1973)
[e] *S* = Shannon's information index
[f] TPL = Total polymorphic loci
[g] PPL = Percentage of polymorphic loci
* Values within parenthesis indicate standard error

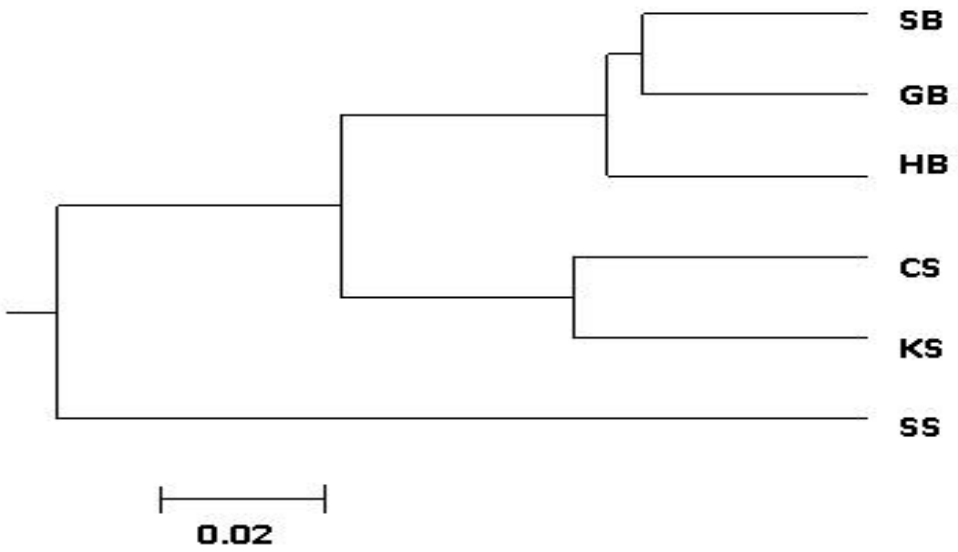

Fig. 1. UPGMA dendrogram based on Nei (1972) genetic distance among the 6 populations of *A. lagopoides* (for population codes see Table 1).

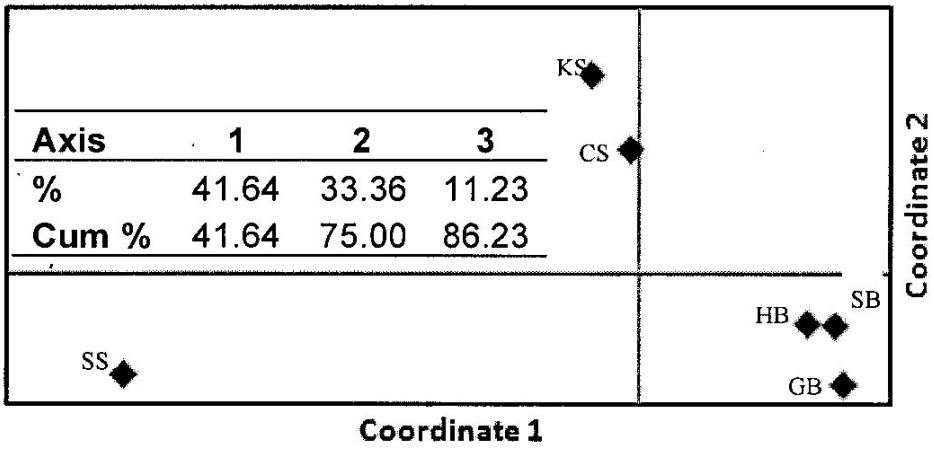

Fig. 2. Principal coordinate analysis (PCA) illustrating genetic differences among 6 populations of *A. lagopoides*. (for population codes see Table 1).

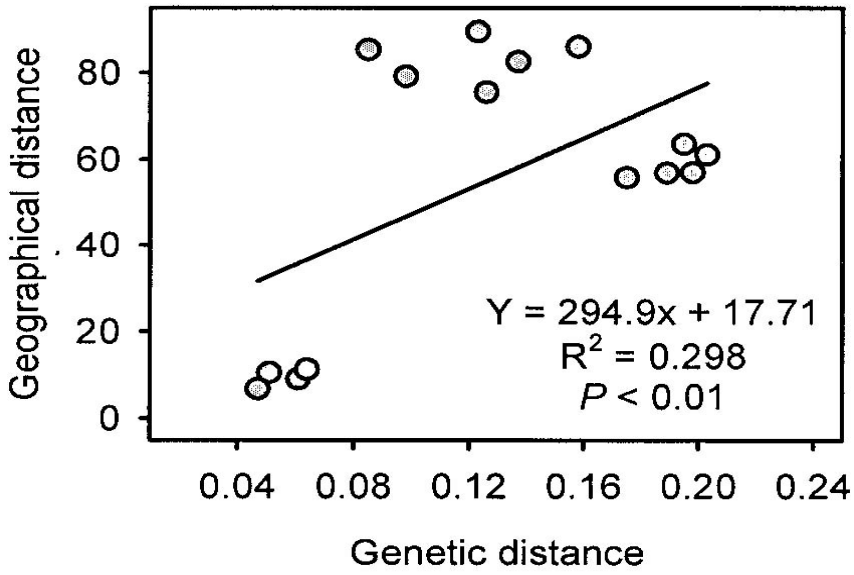

Fig. 3. Graph showing correlation between genetic distance (X-axis) and geographic distance (Y-axis) of *A. lagopoides* populations in Pakistan.

Table 5. AMOVA at the three hierarchical levels based on the populations region *of A. lagopoides*.

Source of variation	df[a]	SS[b]	MS[c]	Variance component	% Variance	*p*-value
Population level						
Among Pops.	5	624.823	124.965	6.647	26%	$p<0.001$
Within Pops.	90	1675.563	18.617	18.617	74%	$p<0.001$
Total	**95**	**2300.385**		**25.264**	**100%**	
Coastal vs. inland						
Among Regions	1	98.432	98.432	0.000	0%	$p<1.000$
Among Pops.	4	526.391	131.598	7.061	27%	$p<0.001$
Within Pops.	90	1675.563	18.617	18.617	73%	$p<0.001$
Total	**95**	**2300.385**		**25.679**	**100%**	
Sindh vs. Balochistan						
Among Regions	1	235.781	235.781	2.886	11%	$p<0.001$
Among Pops.	4	389.042	97.260	4.915	19%	$p<0.001$
Within Pops.	90	1675.563	18.617	18.617	70%	$p<0.001$
Total	**95**	**2300.385**	***G_{ST} = 0.241**	****Nm=1.571**		

[a] Degree of freedom.
[b] Sum of squared deviation.
[c] Mean squared deviation.
[*] Coefficient of population differentiation.
[**] Migration rate

The partitioning of genetic variation during AMOVA analyses (based on all population as a region) indicated that genetic variation among 96 accessions of *A. lagopoides* was lower among populations (26%; $p<0.001$) and higher (74%; $p<0.001$) within populations (Table 5). Similarly AMOVA showed that there was no genetic variation between coastal and inland populations (Table 5). Furthermore, the genetic variation between Sindh and Balochistan regions was 11% ($p<0.001$). The estimated number of migration (*Nm*) among populations was 1.571 per generation (Table 5). Whereas the value of genetic differentiation (*Gst*) among population was 0.241 (Table 5).

Table 6. Unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) among populations of *A. lagopoides* through Nei (1972).

P*	SB	GB	HB	SS	CS	KS
SB	–	0.943	0.929	0.812	0.906	0.862
GB	0.059	–	0.940	0.815	0.874	0.845
HB	0.073	0.062	–	0.820	0.896	0.873
SS	0.208	0.205	0.199	–	0.830	0.809
CS	0.099	0.135	0.110	0.186	–	0.927
KS	0.149	0.169	0.136	0.212	0.076	–

* = Populations codes (SB, GB, HB, SS, CS, KS; see Table 1)

The unbiased measures of genetic distances among populations ranged between 0.059 (GB-SB) to 0.212 (KS-SS) (Table 6). Genetic distances were lower within a region (Sindh and Balochistan) in comparison to among regions except for those between Sands pit and University of Karachi (SS-KS; 0.212) (Table 6). The unbiased measures of genetic identity among populations varied from 0.809 (KS-SS) to 0.943 (GB-SB) (Table 6). The Sands pit population showed higher (0.199-0.212) genetic distances and lower genetic identity (0.812-0.830) in compariosn with all other population in this study (Table 6).

Discussion and Conclusions

The higher percentage (93%) of total polymorphic bands indicates high genetic diversity in *A. lagopoides*. It is predominantly a clonal perennial species which also produces numerous seeds but maintains a transient seed bank (Khan & Gul, 1999). Vegetative growth has been deemed as a short-term survival strategy whereas, viable seeds may occassionally colonize nearby open spaces in exceptionally good years with higher than average precipitation as a long-term strategy to maintain fitness (Khan & Gulzar, 2003). Frequent disturbances such as grazing or cutting by local inhabitants for cattle feed (Khan & Gulzar, 2003) could also contribute to the availability of such open spaces. *Aeluropus lagopoides* seeds can germinate under highly saline conditions with 30% germination at 500 mM NaCl which could possibly increase the chances of seedling recruitment (Khan & Gul, 1999; Khan & Gulzar, 2003). Genetic diversity within all selected populations of *A. lagopoides* was higher (more than 50%) but varied with populations (CS: 75.68% to SS: 50%). Higher genetic diversity within population was previously reported in *Phragmites australis*, a perennial halophyte from Poaceae, which also propagates vegetatively in marshy conditions (Lambertini *et al.,* 2008). Similar results were previously reported for *Suaeda maritima* and *Withania coagulans* populations (Gilani *et al.,* 2009; Prinz *et al.,* 2009). Among six selected populations, the maximum diversity was found in Gadani and Clifton, which are faced with increasing disturbance due to human interventions such as trampling etc. (Clevering, 1999). However, out-crossing through wind pollination, more profuse seed production in these populations besides gap formation could also be involved. Genetic variation was low (50%) in Sands pit population in comparison with all other populations. Similarly, Sands pit population was clustered separately from other populations possibly due to more frequent tidal inundation, wind erosion and soil nutrient deficiency not conducive for seed germination and/or seedling establishment of *A. lagopoides*. AMOVA revealed that the most (74%) of genetic variation was present within populations that show higher genetic variation among accessions of a population. The 26% genetic variation among population indicates a week population structure. In addition,

AMOVA of coastal and inland populations indicates lack of genetic variation between coastal and inland areas. The higher genetic identity (0.811-0.942) and lower genetic distances (0.059-0.211) among populations support the AMOVA results. Similar reports are also available for *P. australis* in Europe (Lambertini *et al.,* 2008). Prinz *et al.*, (2009) also observed that there was no genetic difference among coastal and inland populations of the obligate halophyte *Suaeda maritima* using AFLP markers. The absence of genetic differentiation among coastal and inland populations of *A. lagopoides* indicates that all the fragmented populations are members of a single meta-population (Gilani *et al.,* 2009). Low genetic variation could be due to: 1. Propagation through rhizome, 2. Perennial habit of the grass, 3. Adequate migration rate (*Nm* = 1.571) and 4. Low genetic differentiation (*Gst*: 0.241). *Gst* value is similar to the average of other Poaceae members (0.28; Lambertini *et al*., 2008).

No molecular studies are reported concerning the extent or mechanism of gene flow of *A. lagopoides*. Previously, Lambertini *et al*., (2008) mentioned the percent of polymorphic loci (62.4 and 62.7) in out-crossing and wind-dispersed members of Poaceae. Our test populations showed similar mean polymorphic loci (65.82%) reported for out-crossing species with wind pollination mechanism. AMOVA of Sindh and Balochistan populations displays lower (11%) but significant (p<0.001) genetic diversity of *A. lagopoides* probably due to the geographical barrier which separates them. It is possible that populations of both these regions may be slowly evolving independent of each other because of a weak reproductive barrier in the form of a low (~100 m high) hilly range which could reduce the pollen dispersal through wind.

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