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AFLP Based Analysis of Genetic Diversity in Buffle Grass

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Abstract: Amplified Fragment Length Polymorphism (AFLP) technique was used in the present study to access the genetic diversity within and among populations of buffle grass (*Cenchrus ciliaris* L.). Samples of nine populations comprising of thirty accessions of *C. ciliaris* were collected from 9 sites of Cholistan provenance, Pakistan. Molecular analysis of *C. ciliaris* revealed 27.8% polymorphism among populations. Within population genetic polymorphism ranged from 16.7% to 44.4% for populations from Din Garh Fort, Kalay Pahad and Baghdad Campus respectively. In all nine populations, 24 rare and 6 unique alleles have been observed. Cluster analysis based on Nei's gene diversity was performed on binary data obtained to group accessions in neighbour joining (NJ) pattern to construct a tree. It was found that individuals of the same origin grouped into the same clusters. Based on these results, it is concluded that individuals of *C. ciliaris* represent relict populations, which hold enough genetic diversity. The erosion of genetic diversity may be the result of anthropogenic and uncontrolled grazing and to lesser extent prolonged droughts and salinity. It is suggested that both *ex-situ* and *in-situ* conservation strategies should be adopted to reclaim the dwindling genetic diversity. It will serve as a guideline in setting priorities for reclaiming genetic diversity and consequently habitat loss.

Key words: AFLP · Buffle grass · Genetic diversity · Polymorphism · Conservation · Populations

INTRODUCTION

Buffle grass (*Cenchrus ciliaris* L.) is a tall, much branched and tussock forming grass. Though it grows on different soils in Cholistan desert, yet optimum production is obtained on fertile sandy loam [1]. *C. ciliaris* acts as an efficient sand-binder to stabilize the sand dunes [2]. *Cenchrus ciliaris* among other grasses were found to be most drought resistant grass species in deserts [3]. This grass is resistant to abiotic and biotic stresses, thus reported to be grown with promising forage production [4]. It is an excellent source of fodder due to nutritive proteins and crude fiber contents. However, the heavy grazing pressure may diminish strands of *Cenchrus ciliaris* from the arid rangelands of Cholistan desert. It may inflict shortage of fodder, which would reduce the local herbivorous fauna in this area [4].

The grass has the highest potential for starch and protein production which is comparable to any of the cultivated plant [3]. Numerous studies on genetic variability in perennial grasses showed a great deal of morphological variation in Cholistan desert. The declining genetic diversity in natural rangelands of Cholistan desert might be due to combined anthropogenic, climatic, salinity, drought and over grazing factors [5-8].

Escalating awareness about diminishing biodiversity of *Cenchrus* in Cholistan [4, 7] has enhanced the need to study the grass *Cenchrus*. Though it is an economically important grass but little or no knowledge on molecular level is available from this area. Therefore, it is essential to have basic molecular knowledge on the organization of genetic variation in this grass species to make use of this species for their conservation, future utilizations for breeding programs and the development of genetic maps for quantitative trait locus discovery [9].

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Understanding the population genetic diversity, together with the relationship of ecological factors, is a prerequisite for effective conservation and management of plant species [10]. Recently, AFLP technique [11] has been continuously used to analyze genetic diversity and population structure in diverse plant species [12-16]. AFLP technique has many priorities over other DNA fingerprinting techniques like RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA) and STS (sequence-tagged sites) [17]. AFLP is a high resolution PCR-based molecular marker assay with high reproducibility that requires no prior genome sequence information. It can generate a large number of reproducible loci in wide genome range [18].

The present study based on AFLP technique was designed to assess genetic diversity, genetic structure and genetic relatedness within and among populations of *C. ciliaris* in Cholistan desert. Additionally, genetically diversified populations of this grass could be identified for an effective management regarding this vulnerable species both by *in-situ* and *ex-situ* conservation and utilization of the germplasm in future studies such as stress studies and breeding practices etc.

MATERIALS AND METHODS

Study Site: A number of collecting expeditions were made during March-April and August-September (after moon soon) 2007 at various locations of the Cholistan desert, comprising an area of about 26,000 km^2. Geographic co-ordinates of each population site were collected with a GPS (Global Positioning System) and field notes were taken at the spot (Table 1).

Sampling of the Plant Material: Nine populations of *Cenchrus ciliaris* Retz. were sampled from different habitats of Cholistan desert (Table 2). Due to over grazing and severe environmental conditions, seeds of only few plants were collected. Otherwise, the leaf material of available plants was collected *in-situ* all across the populations according to Reisch *et al.* [19]. Each leaf material of plant was sampled at a distance of 20 to 30 meters to avoid the ramets of the same genet and the sampled leaves immediately dried in silica gel [20].

Extraction of Genomic DNA: The genomic DNA was extracted from 0.2-0.3 g dry leaf material using a method described by Kang and Yang [21] and finally the genomic DNA was resuspended in 50µl TE buffer. Purified DNA was quantified using spectrophotometer (Bio-Rad Instruments, Italy) at 260nm and the purity measured by the ratio of the absorbance at 260 and 280nm. The quality and quantity of the genomic DNA was also inspected by running a small aliquot of genomic DNA on 1% agarose gel.

AFLP Analysis: AFLP fingerprints were generated by performing a method, described by Vos *et al.* [11]. Restriction and ligation were done concurrently by adding 5µl extracted DNA (400-500*ng* DNA) to 45µl buffer (10mM Tris-HCl pH 7.5; 10mM MgAc, 50 *mM* KAc) containing 5 U *Eco*RI, 5 U *Mse*I, 2 U T_4 DNA ligase 5*pmol* *Eco*RI adapter, 50 *pmol* *Mse*I adapter and 0.2 *mM* ATP. The mixture was then incubated at 37°C for 4hrs and diluted 10 times in 0.1X TE (1 *mM* Tris-HCl, 0.1mM EDTA pH 8). Two consecutive PCRs were run to selectively amplify the *Eco*RI-*Mse*I DNA fragments.

Table 1: Sampled populations of *C. ciliaris* from various locations in Cholistan with geographical provenance, abbreviation (Abb.), number of sampled individuals (n) and Pop. size (population size in 100 m^2 area) and the habitat description where the populations were located

					Provenance							
Sr. No.	Site Name	Abb.	n	Pop. Size	Long.°N	Lat.°E	Vegetation	Soil Type	Topography	Temperature Fluctuation	Type of Land use	Nutrient Stress
1	Baghdad Campus	BC	4	50	29.380	71.771	Scrub	Sandy loam	Dunes and humps	Low	None and	Medium
2	Lal Suhanra	LS	2	5	29.395	71.998	Large shrubs	Sandy loam	Dunes and humps	Low	Moderate grazing	Low
3	Derawar Fort	DR	3	5	28.768	71.333	Sparse trees	Clayey	Flat plains	High	Grazing Moderate	High
4	Din Garh Fort	DG	2	10	28.942	71.848	Scrub with Sparse trees	Sandy	Flat plains	Medium	Grazing	High
5	Mauj Garh Fort	MJ	5	5	29.016	72.082	Scrub with Sparse trees	Clayey	Flat plains	High	Grazing	High
6	Khokhran Wala Toba	KWT	4	20	28.084	71.678	Sparse shrubs		Dunes	High	Grazing	Medium
7	Chanan Pir	CHP	4	30	28.967	71.716	Sparse shrubs	Sandy	Flat plains and dunes	High	Grazing	High
8	Kalay Pahar	KP	2	25	27.846	70.814	Sparse shrubs	Sandy	Huge Dunes and humps	High	Grazing	Medium
9	Rahim Yar Khan	RYK	4	2	28.423	70.299	Sparse trees and shrubs		Sandy Flat Area	Medium	Grazing	Medium
	Total		30									

Table 2: Accessions of *C. ciliaris* L. From Cholistan desert

Sr. No.	Site Name	Accession
1	Baghdad Campus	BC-1
2	Baghdad Campus	BC-2
3	Baghdad Campus	BC-3
4	Baghdad Campus	BC-4
5	Lal Suhanra	LS-1
6	Lal Suhanra	LS-2
7	Derawar Fort	DR-1
8	Derawar Fort	DR-2
9	Derawar Fort	DR-3
10	Din Garh Fort	DG-1
11	Din Garh Fort	DG-2
12	Mauj Garh Fort	MJ-1
13	Mauj Garh Fort	MJ-2
14	Mauj Garh Fort	MJ-3
15	Mauj Garh Fort	MJ-4
16	Mauj Garh Fort	MJ-5
17	Khokhran Wala Toba	KWT-1
18	Khokhran Wala Toba	KWT-2
19	Khokhran Wala Toba	KWT-3
20	Khokhran Wala Toba	KWT-4
21	Chanan Pir	CHP-1
22	Chanan Pir	CHP-2
23	Chanan Pir	CHP-3
24	Chanan Pir	CHP-4
25	Kalay Pahad	KP-1
26	Kalay Pahad	KP-2
27	Rahim Yar Khan	RYK-1
28	Rahim Yar Khan	RYK-2
29	Rahim Yar Khan	RYK-3
30	Rahim Yar Khan	RYK-4

The pre-amplification was performed using 5 µl of the above mentioned diluted mixture added to a 15µl mixture giving a final concentration of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM of each dNTPs, 40*ng* of *Eco*RI and *Mse*I adapter-directed primers, each with a single selective base (*Eco*RI+1 and *Mse*I+1 primers) and 1 U of *Taq* DNA polymerase.

PCR reactions were performed with the following profile: 94°C for 60 sec., 25 cycles of 30 sec. denaturing at 94°C, 30 sec. annealing at 55°C and 60 sec. extensions at 72°C and 10 min at 72°C to complete extension. After checking for the presence of a smear of fragments (100-1000 bp in length) by agarose electrophoresis, the amplification product was diluted 40 times in 0.1X TE. Subsequently selective amplification (second PCR) was carried out using primers with three selective nucleotides. Initially, 16 primer pairs, originated by the combination of 4 *Eco*RI primers and 4 *Mse*I primers, were tested in 30 accessions of *C. ciliaris*. The primer combination (*EcoR*1-AAC/*Mse1*-CAA) was selected in this study while selecting the reproducible bands in electrophoretic patterns and applied to all samples. Selective PCR reactions were performed with the following profile: 94°C for 60sec., 36 cycles of 30 sec. denaturing at 94°C, 30 sec. annealing and 60 sec. extension at 72°C, ending with 10 min at 72°C to complete extension. Annealing was initiated at 65°C which was then reduced by 0.7°C for the next 12 cycles and maintained at 56°C for the subsequent 23 cycles. Reproducibility for each primer pair was checked by running the AFLP protocol at different DNA concentrations; a threshold of 20*ng* DNA/µl before digestion was the lowest concentration which avoided appearance of artifacts or disappearance of some bands.

Electrophoresis of the PCR Product: Amplified products were mixed with 0.2µl of 6x loading-dye (Fermantas), 6% resolving polyacrylamid sequencing gels (10.0ml of 30% acrylamid mix, 26.5ml of H_2O, 0.5ml of ammonium persulfate (APS), 0.04ml of TEMED in 37.04 ml of 10x TBE buffer) were prepared (Table 4). The gel was pre-run for about 30 min before 1.2µl of the mixture was loaded.

Table 3: Genetic diversity within and among populations of *C. ciliaris* Retz. measured as percentage of polymorphic bands per population, rare and unique alleles per populations. 'n' number of accessions in each population

Sr. No.	Population	n	Total No. of Markers	Polymorphic Bands	Polymorphic Bands (%)	Rare Alleles	Unique Alleles
1	BC	4	9	4	44.4	1	0
2	LS	2	6	2	33.3	5	2
3	DR	3	7	2	28.6	0	0
4	DG	2	6	1	16.7	0	0
5	MJ	5	11	3	27.3	4	0
6	KWT	4	9	2	22.2	4	2
7	CHP	4	8	3	37.5	3	0
8	KP	2	6	1	16.7	5	0
9	RYK	4	10	2	20.0	2	2
	Total	30	72	20		24	6
			Among Populations		27.8%		

Table 4: Band sharing among the populations (X population)

X	1	2	3	4	5	6	7	8	9
1	-								
2	4	-							
3	5	4	-						
4	5	4	4	-					
5	5	5	5	3	-				
6	5	5	6	4	7	-			
7	6	5	7	5	6	6	-		
8	5	4	4	6	3	4	5	-	
9	5	4	5	4	7	7	6	4	-

Amplified products along with loading dye were loaded in the gel wells. The gel was run at 80 V for about 4-5 hours. Gel was stained with silver nitrate [22] and finally the gel was analyzed in gel documentation system (Bio Rad Instruments, Italy).

Data Analysis: In the data matrix, the presence of a band was scored as 1, whereas the absence of the band was scored as 0. The basic data structure finally consisted of a binary (0/1) data, representing the scored AFLP markers. The binary data were analyzed in *MS Excel* and genetic diversity within populations calculated as the percentage of polymorphic bands using Nei's gene diversity ($He=1-\Sigma p_i$) for each population using program *PHYLIP* 3.6. The distance matrices based on Nei's genetic distance were used as input file in *PHYLIP* 3.6 [23], using NEIGHBOR, which cluster the populations according to the neighbour-joining (NJ) method. The consensus tree was edited in TreeView 1.6.6 [24] and Microsoft Word for Windows. Banding composition and sharing among accessions and accession groups were recorded. For each group monomorphic and polymorphic bands were counted. Similarly, rare and unique loci were also counted.

RESULTS

AFLP Banding and Levels of Polymorphism: An *ECoR*1-AAC/*MSe1*-CAA primers pair combination was used to generate AFLP profile of 30 individuals of *C. ciliaris* representing 9 populations. Among populations polymorphism was 27.8% while within populations the range of polymorphism varied from 16.7% in DG and KP populations to 44.4% in BC population. Six unique and 24 rare alleles were also identified (Table 3). Two unique alleles were found each in accessions of KWT, LS and RYK populations. The range of rare alleles was counted and maximum five rare alleles were present in each of LS and KP populations while one rare allele in BC population was observed (Table 3).

Allele sharing was also counted among populations of *C. ciliaris* (Table 4). The individuals of BC population shared 5 loci with that of MJ, 5 with DG population, 5 with DR population, 5 bands with KWT population, 4 with individuals of LS, 5 with RYK population, 5 KP and 6 with CHP population. The individual of MJ population shared 3 bands with DG, 5 bands with DR population, 7 with KWT population, 5 with LS population, 7 with RYK population, 3 with KP and 6 with CHP population. Individuals of DG population shared 4 bands each with RD, KWT, LS and RYK populations while 6 and 7 alleles with KP and CHP populations respectively. DR population shared 6 alleles with KWT, 4 alleles each with LS and KP populations, 5 with RYK and 7 alleles with CHP populations. KWT population has share 5 alleles LS, 7 alleles with RYK, 4 alleles with KP and 6 alleles with CHP population. LS population of *Cenchrus* shared 4 alleles each with RYK and KP populations while on the other hand 5 populations with CHP population. RYK population shared 4 and 6 alleles with KP and CHP populations. Finally the KP population has shared 5 alleles with CHP population.

CLUSTERING

Cluster analysis (Neighbour Joining) (NJ) based on Nei's genetic distance revealed clusters which group 30 accessions of *C. ciliaris* (Fig. 1). Except four individuals all other individuals of *Cenchrus* were resolved into groups. The major groups were further divided into 7 sub-groups (Figure 1). The exceptional (unresolved) four accessions (1, 2, 3 & 4) of *Cenchrus* were all collected from Baghdad Campus, could not be resolved inspite of being collected from the same habitat. Major group 1 combines individual number 5 & 6 collected from Lal Sohanra, accession number 17 from Khokhran Wala Toba with all the individuals of population from Mauj Garh Fort. Group 2 was constituted of the 3 individuals from Khokhran Wala Toba with these collected from Chanan Pir population.

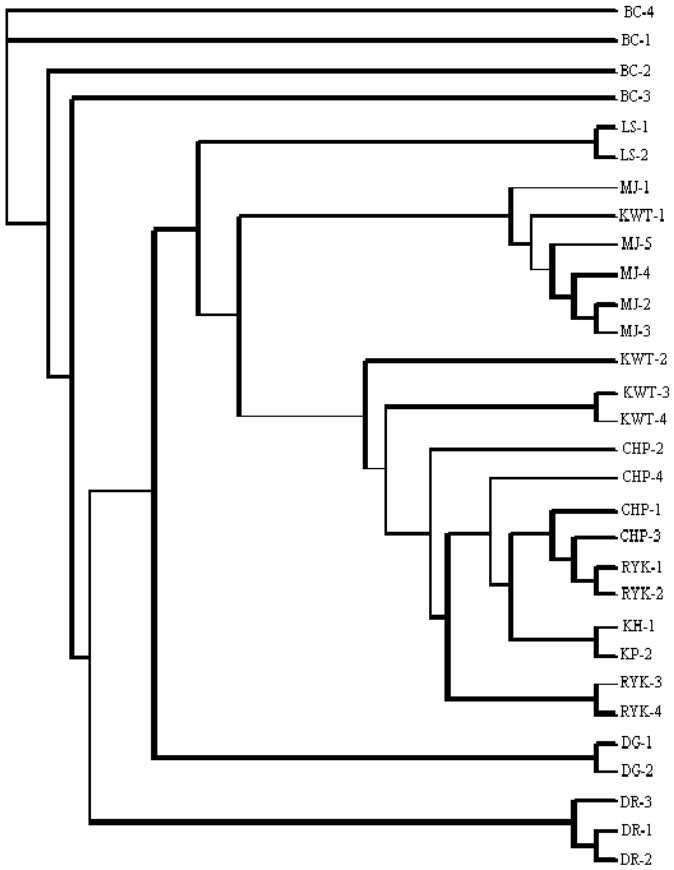

Fig. 1: Neighbour joining (NJ) dendrogram grouping the 30 accessions of *C. ciliaris* L. based on Nei's genetic distance

This group shared the individuals (27, 28, 29 & 30) from Rahim Yar Khan population and individuals of population from Kalay Pahad. In group 3 members of Derawar Fort population and the members of the Din Garh Fort population clustered together.

DISCUSSION

DNA markers provide a desirable means of determining population structure and genetic diversity. This is the first molecular survey of genetic diversity and population structure for *C. ciliaris* from Cholistan desert Pakistan, using molecular markers. The AFLP analysis is appropriate to study fine scale genetic diversity, utilized here for 30 accessions of *C. ciliaris*, an autogamous grass species. The collection of only few individuals per population was due to the heavy grazing pressures and consecutive droughts over the years. Arshad *et al.* [7] reported that strands of this grass species are on decline.

The lower level of polymorphism was observed within DG and KP populations of *C. ciliaris* (16.7%) which were comparable to the findings of Renganayaki *et al.* [25] who studied the dioecious Texas bluegrass (*Poa arachnifera*). Moreover, it was found that the genetic diversity was not correlated with population size. Among populations genetic diversity was higher in *C. ciliaris* upto 27.80%. The level of genetic diversity is comparable to the cross pollinating grass species e.g. ryegrass (*Lolium* spp.) [26] and smooth broom grass (*Bromus inermis* Leyss.) with high polymorphism (83-90%), pointing their possible outcrossing behaviour [14]. In a study of the widespread *Tanacetum vulgare*, 85% polymorphic bands were observed [27], in the rare *Vicia pisiformis* 7.2% [28] and in apomictic species of *Rosa* section *Caninae* only 3% polymorphism has been reported [29]. These differences are not astonishing, since the level of genetic variability strongly depends on the plants traits [30]. In an analysis of *Yushania niitakayamensis* from grassland and forest undergrowth, 2.91-7.99% variation was found in populations from different habitats [31] and for *Schizachyrium scoparium* 3.18% among populations variation from different soil fertility levels was observed [32].

The habitats colonized by *C. ciliaris* differ to some extent from each other with regard to the availability of nutrients, light, water and type of land used. Different land practices within the Cholistan desert caused the fragmentation of the populations, hence reductions in the gene flow among the populations. For this reason the allele sharing among the populations is done that shows the overall genetic structure of *C. ciliaris* in Cholistan. Interestingly BC population is the most anthropogenically disturbed population among all the nine populations showed 44.4% polymorphism within population hence it is most diverge population adapted to this site in Cholistan.

As known from previous investigations, genetic variation within and among populations is strongly affected by ecological factors [33] and geographically restricted species/populations show little genetic diversity [34]. The ecotypic development of populations depends on climatic and edaphic conditions. The differences among populations referring to these factors can cause microgeographic differentiation, as reported for many other grass species [35, 36, 37]. Furthermore, different types of land use are thought to make a contribution to the differentiation among and within populations [38]. Ecological differences among meadows and pastures in fertilization and defoliation can cause micro environmental adaptations of plant populations [39] and the development of ecotypic variants [40].

As the populations of perennial grass species are on decline in arid rangelands of Cholistan desert, 24 rare and 6 unique alleles have been identified in *C. ciliaris*. This showed that there is a serious threat of genetic erosion of the species due to presence of rare and unique alleles. But at the same time there is a possibility that these rare and unique alleles may be due to founder events of the populations in newly colonized territories of Cholistan desert. The later case seems less true because the populations are relict and no migration is evident to other areas of the Cholistan desert, hence the unique alleles are likely to be called new induction in the pre-existing populations. Whatever the case may be these unique and rare alleles deserve to be conserved to maximize the speciation process as the selection pressure is too high in extreme environments of the Cholistan desert.

Based on our observations it is concluded that *C. ciliaris* found in Cholistan desert are a relict populations holding enough diversity to initiate both *in-situ* and *ex-situ* conservation projects. The study provides a clear evidence of human, grazing, prolonged droughts and salinity impacts on declining the populations causing population fragmentation, ultimately the loss of valuable gene pool. Extensive germplasm collections should be done and an *ex-situ* nursery must be developed where the germplasm is multiplied and properly stored. The best suited accessions should be reintroduced to grow wild to restore the natural glory of the degraded arid rangelands of Cholistan desert. Further investigations concerning other species in such habitat or area are, therefore required.

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