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Determination of the genetic diversity in populations of halophytic grass *S. ioclados* using PBA markers

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Abstract

The genetic structure of salt-tolerant halophytic grass *Sporobolus ioclados* collected from inland and coastal areas of Pakistan was determined using P450 based analog, the functional genomic markers. Our data revealed high polymorphism (96%) in the collected germplasm. The high genetic variability could be attributed to outcrossing and sexual reproduction. An analysis of molecular variance depicted higher genetic diversity (80%) within the populations and lower (20%) among the populations. The low genetic diversity among the inland and coastal populations might be due to fragmentation and long physical distances. The dendrogram and principal component analysis clearly distinguished the genotypes of the two populations into two distinct clusters. Our data demonstrated that P450 based analog markers are reliable and reproducible for assessing genetic diversity in halophytic grass and provide valuable insight for future breeding and conservation programs.

Keywords: Coastal populations; genetic diversity; halophytes; inland; *S. ioclados.*

1. Introduction

Sporobolus ioclados is salt tolerant, stoloniferous perennial halophytic grass of the family Poaceae that dominates inland and coastal salt beds of arid Pakistan (Gulzar *et al*., 2005). This grass species is widely distributed on various inhospitable soil types such as saline wet patches, dry sandy areas, and salt marshes, including the coastal dunes and saline deserts of Africa, the Middle East, and India (Cope, 1982). It mainly propagates through stolons and scarcely through seeds, enabling this grass species to adapt to saline, dry and wet habitats (Khan, 1993). Roots of *S. ioclados* can sustain 500 mM salinity, making it a suitable saline agriculture candidate to be used as a forage crop (Gulzar *et al.,* 2005; Gulzar & Khan, 2003). Its solid roots and clonal propagation network can help reduce soil erosion, especially in coastal areas. Plantations of salt-tolerant plant species such as *S. ioclados* are beneficial for the reclamation and subsequent management of saline soils (Khan & Duke, 2001). Halophytes, in general, are non-conventional

pastures that can utilize not only the degraded saline lands for agriculture but some of these species have aromatic and medicinal uses while others can be used as fuels, forages, and oil production (Akinshina *et al.,* 2016; Ksouri *et al.,* 2012).

Assessment of the richness of the gene pool is essential for the sustainable utilization and conservation of any plant species to meet future demands and to know the potential to cope with future unknown environmental stresses. Despite their economic and ecological values, few molecular studies have been undertaken in halophytes. Lambertini *et al.* (2008) used RAPD and AFLP primers to decipher genetic variability in *Phragmites australis* populations. Ahmed *et al.* (2011) used RAPD and PBA primers in *Aeluropus lagopoides* and emphasized the importance of starting and extending molecular-level studies in more halophytic species.

Several PCR-based DNA markers, including RAPD, AFLP, and SSRs, are available for the analysis of plant genetic variability in neutral regions of plant genomes (Aladadi *et al.,* 2018; Al-Salameen *et al.,* (2014); Karp *et al.,* 1997; Powell *et al.,* 1996). The RAPD primers due to mismatch annealing have reproducibility issues. AFLP marker system technique is relatively laborious and time-consuming. On the other hand, SSRs are species-specific primers and require prior knowledge of genome sequence (Garcia *et al.,* 2004). P450-Based Analog (PBA) markers (also called functional genomics markers) are relatively new. They are based on cytochrome P450 and are used for assessing polymorphism in many plant species (Yamanaka *et al.,* 2003). PBA markers were initially developed for poorly studied genomes and have been successfully utilized in many crop species such as *Aerluropus lagopoides* (Ahmed *et al.,* 2011), *Curcuma amada* (also called mango ginger) (Jatoi *et al.,* 2010), *Withania coagulans* (Gilani *et al.,* 2009), and *Musa* (banana) (Wan *et al.,* 2005).

Considerable effort was made to determine the genetic diversity of different halophytes, but the genetic structure of *S. ioclados* was poorly studied. In the present study, PBA primers were applied to investigate the genetic diversity and relationships of two Pakistani populations of *S. ioclados*. The PBA primers amplification in functional genomic regions and high reproducibility are expected to give a reliable picture of genetic diversity.

2. Materials and methods

2.1 Sample collection/plant material

A total of 48 leaf samples of *S. ioclados* were collected from two different populations designated as "an inland and a coastal population." The inland population was from the University of Karachi (24° 55' 50" N, 67° 6' 55" E), consisting of a calcareous soil type. The coastal population was obtained from the Hawks Bay area of Karachi (24° 50' 24" N 66° 54' 24" E), which has a soil type composed of fine and coarse particles of sand. The distance between the two sampling locations was 33 km, and a small hilly range exists between the locations. The area experiences an arid subtropical climate with high temperatures throughout the year. The average annual rainfall is 130 mm, and the mean yearly temperature is 32°C.

The leaf samples were air-dried and packed in bags filled with silica gel. Then, the dried leaf samples were brought to the Gene Research Center, University of Tsukuba, Japan, for studying the genetic diversity of this halophytic grass species.

2.2 Isolation of DNA and PCR reaction

The genomic DNA was extracted from leaf samples according to Doyle & Doyle (1990) with slight modifications (using higher concentration (1%) of β-mercaptoethanol). Fifteen PBA markers were used to assess the genetic variation within and among plant populations of *S. ioclados*. The final volume of the PCR reaction mixture was 25µl, containing approx. 25ng of extracted DNA, 1 X Taq buffer, dNTPs (0.16mM), 1µM of each forward and reverse primers, and 1 unit of Taq Polymerase (TaKaRa, Japan). The PCR initial denaturation was 94°C for 5 minutes, followed by 32 cycles of denaturation (94°C for 1 minute), annealing (depending upon the primer set), and elongation (72°C for 3 minutes) in a thermal cycler machine (Applied Biosystems). The Yamanaka *et al.* (2003) method was followed for PCR assay. Eight primers (Table 1) were used in this study. The reaction products of the PCR were separated by gel electrophoresis in an agarose gel (1.5 %) run at 100 volts for 2 hours, followed by ethidium bromide staining for visualization.

Table 1. List of PBA primers used for the study.

Primers	Sequence (5' to 3')	Reference
CYP1A1F	GCCAAGCTTTCTAACAATGC	Inui et al. (2000)
CYP2B6F	GACTCTTGCTACTCCTGGTT	Inui et al. (2000)
CYP2C19F	TCCTTGTGCTCTGTCTCTCA	Inui et al. (2000)
CYP1A1R	AAGGACATGCTCTGACCATT	Inui et al. (2000)
CYP2B6R	CGAATACAGAGCTGATGAGT	Inui et al. (2000)
CYP2C19R	CCATCGATTCTTGGTGTTCT	Inui et al. (2000)
heme2B6	GCCAAGCTTTCTAACAATGC	Kiyokawa et al. (1997)
heme2C19	TCCCACACAAATCCGTTTTCC	Kiyokawa et al. (1997)

2.3 Statistical data analysis

The polymorphic bands obtained with PBA primers were scored as present (1) or absent (0) and used for further statistical analysis. A measure of genetic similarities was determined by the Jaccard (1908) coefficient using Numerical Taxonomy System, version 2.0 (NTSYS-pc) software (Rohlf, 2000). A dendrogram was then constructed based on the unweighted pair group

method of the arithmetic average (UPGMA). Principal component analysis (PCA) was used to reveal the grouping pattern of the genotypes further. To partition the total genetic variance, the study of molecular conflict (AMOVA) was performed using GENALEX 6 software (Peakall & Smouse, 2006).

3. Results

3.1 PCR amplification and polymorphism

15 primer combinations were tested using 3 forward P450 primers and 5 reverse primers to amplify extracted genomic DNA samples. Each primer combination successfully amplified the DNA fragments of *S. ioclados*. The total amplified fragments using the 15 PBA primer sets ranged from 1 to 8 among the individuals of both populations. In the genotypes of the inland population, PBA primer sets yielded 101 fragments, having 95 polymorphic fragments. Out of these, 11 primer sets showed 100% polymorphism of DNA fragments in the inland population. However, in the coastal population, 101 pieces were produced. Among 15 primer sets, 13 showed 100% polymorphism. On average, 11.2 fragments and 11.5 fragments were amplified in the genotypes of the inland and coastal populations, respectively (Table 2). In addition, the reproducibility of the primers was also checked with selected DNA samples.

Table 2. The general profile of DNA amplification by using PBA markers across inland and coastal genotypes of *S. ioclados*

Primers	Total bands	Polymorphic bands	Polymorphism (%)	Average fragments	Total bands	Polymorphic bands	Polymorphism (%)	Average fragments
CYP2B6F/CYP1A1R	7	7	100	0.6	7	7	100	0.7
CYP1A1F/CYP2B6R	7	7	100	0.7	7	7	100	0.5
CYP2B6F/CYP2B6R	7	7	100	0.5	7	6	86	0.5
CYP2B6F/heme2B6	8	8	100	0.6	8	8	100	0.8
CYP2B6F/heme2C19	8	8	100	0.5	8	8	100	0.6
CYP2C19F/CYP2B6R	8	6	75	0.6	8	8	100	0.5
CYP1A1F/CYP1A1R	1	1	100	0.1	1	1	100	0.1
CYP1A1F/heme2B6	8	6	75	1.1	8	8	100	0.5
CYP1A1F/heme2C19	5	4	80	0.5	5	5	100	0.2
CYP2C19F/CYP1A1R	7	7	100	0.4	7	7	100	0.6
CYP2C19F/heme2B6	6	6	100	1.2	6	6	100	1.5
CYP2C19F/heme2C19	8	8	100	1	8	8	100	1.1
CYP1A1F/CYP2C19R	8	7	87.5	1.3	8	8	100	1.3
CYP2B6F/CYP2C19R	6	6	100	1.1	6	4	67	1.6
CYP2C19F/CYP2C19R	7	7	100	1	7	7	100	1
Total	**101**	**95**		**11.2**	**101**	**98**		**11.5**

Table 3. Gene diversity analysis of *S. ioclados* between inland and coastal Populations by using different primers combination

Primers	Inland Population	Costal Population	Average
CYP2B6F/CYP1A1R	0.38	0.26	0.32
CYP1A1F/CYP2B6R	0.39	0.28	0.34
CYP2B6F/CYP2B6R	0.30	0.33	0.31
CYP2B6F/heme2B6	0.37	0.34	0.36
CYP2B6F/heme2C19	0.36	0.40	0.38
CYP2C19F/CYP2B6R	0.43	0.31	0.37
CYP1A1F/CYP1A1R	0.06	0.06	0.06
CYP1A1F/heme2B6	0.41	0.41	0.41
CYP1A1F/heme2C19	0.25	0.17	0.21
CYP2C19F/CYP1A1R	0.34	0.23	0.28
CYP2C19F/heme2B6	0.30	0.20	0.25
CYP2C19F/heme2C19	0.37	0.43	0.40
CYP1A1F/CYP2C19R	0.31	0.45	0.38
CYP2B6F/CYP2C19R	0.43	0.36	0.40
CYP2C19F/CYP2C19R	0.38	0.37	0.38
Mean	0.34	0.31	0.32

The estimates of genetic diversity for all the genotypes of inland and coastal populations were determined based on PBA primers amplification (Table 3).

3.2 Genetic variability between two populations

Individuals of the inland population showed a total variation of 0.34 in the presence of all primer sets. Primer set CYP1A1F/CYP1A1R showed the lowest variation of 0.06, and primer sets CYP2C19F/CYP2B6R, and CYP2B6F/CYP2C19R showed the highest 0.43 genetic variation. Among the individuals of the coastal population, the average genetic diversity was 0.31. Primer set CYP1A1F/CYP1A1R again showed the lowest variation (0.06), while primer set CYP1A1F/CYP2C19R showed the highest gene diversity (0.45). Genetic variations ranged from 0.06 to 0.40 within the two different populations, and the total average of gene variation was 0.32. The AMOVA indicated inter and intra- population diversity of *S. ioclados* and quantified the significance of genetic diversity of the two populations (Table 4). Our data showed that 20% of the total variation was due to the difference between the two populations ($P < 0.01$). The variation among the individuals of the same population was 80% of the total variability.

Table 4. AMOVA analysis of PBA- PCR variation of the inland and coastal populations of *S. ioclados*

Source	Among Populations	Within Populations	Total
df	1	46	47
Sum of Square	118.77	783.21	901.98
Mean of Square	118.77	17.03	135.80
Variance components.	4.24	17.03	21.27
Percentage of variation	20%	80%	
***P*- value**	<0.01	< 0.01	

3.3 Genetic relationships of the accessions of the two populations

The dendrogram was derived by subjecting the PBA-PCR data of the polymorphic fragments to multivariate analysis. Dendrogram analysis separated the genotypes of *S. ioclados* into two distinct clusters (Figure 1).

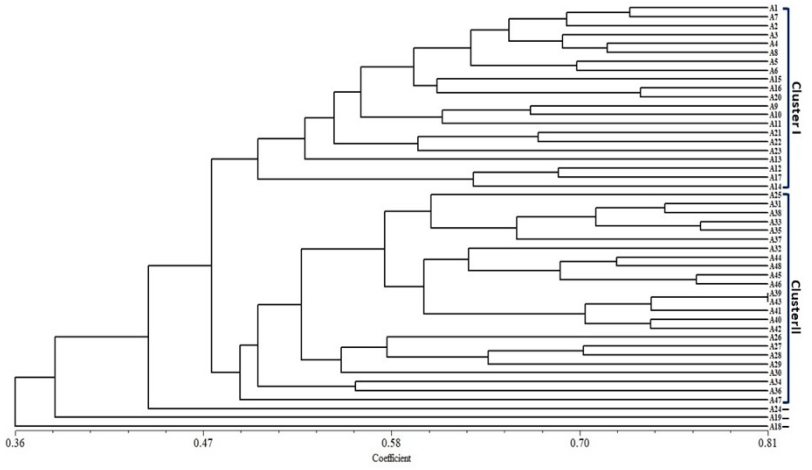

Fig. 1: Dendrogram based on PBA markers indicating the genetic relationship. A1-A24 numbers represent inland accessions, whereas A25-A48 numbers represent coastal accessions.

Cluster I consisted mainly of the individuals of the inland population (A1-A24 represent inland accessions), and cluster II consisted of the individuals of the coastal population (A25-A48 represent coastal accessions). Furthermore, within the inland population, all genotypes were different from each other, but within the coastal population genotypes, A-39 and A-43 were much closer to each other than to other genotypes. The individuals A-24, A-19, and A-18, did not fit into any cluster (Figure 1).

The PCA also classified the population into two distinct groups (Figure 2). The contribution of PC1 was 31.40%, PC2 was 10.30%, and the cumulative contribution was 55 %. In the scatter plot of PCA, most of the genotypes of each population also contributed to the separate groups.

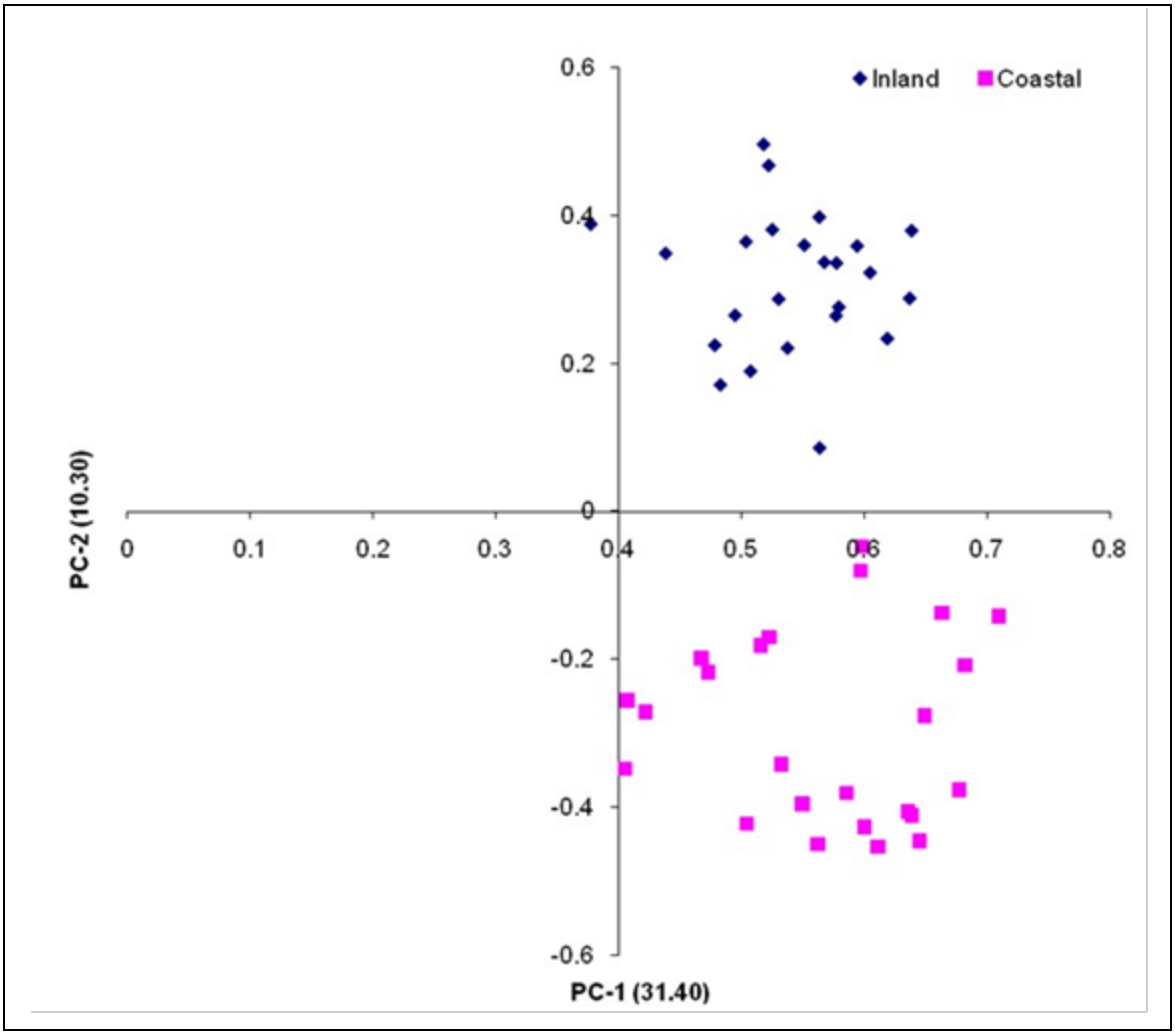

Fig. 2. PCA scatter plot of the genotypes based on PBA markers.

4. Discussion

This was one of the first studies that focused on the genetic variability and relatedness of *S. ioclados* collected from Pakistan's coastal and inland areas. We used PBA markers that amplify functional regions of a genome and are highly reproducible (Jatoi *et al*., 2010). In our study, all 15 PBA primer combinations produced successful PCR amplifications that detected 95 % polymorphism in the studied genomes. However, when the same primers were used in 6 populations of another grass species (*Aerluropus lagopoides*) only 14 combinations produced successful PCR amplification with an average 88% polymorphism rate (Ahmed *et al.,* 2011). In contrast, when RAPD primers were used to study the genetic diversity of 13 different salt-tolerant plant species, including two halophytic grass species, only 91 % polymorphism was revealed (Mahmood *et al.,* 2013). This showed the strong efficiency of PBA markers in this grass species. Though *S. ioclados* is predominantly a perennial clonal species, it showed a high level of polymorphism. Genetic diversity represented by the variability profile showed a broad genetic base of *S. ioclados*.

The genetic diversity observed in the two *S. ioclados* was high but similar in both populations. While studying the genetic diversity of coastal and inland people of *Aeluropus logophiles*, Ahmed *et al.* (2011) also observed a similar pattern using the same PBA primers. The genetic diversity observed in this study may be associated with the wind-pollinated outcrossing and seed production in these populations. The molecular variance analyzed by PBA primers indicated diversity at both *S. ioclados* germplasm acquisition sources. The existence of high genetic diversity within populations was widely reported in other plants, including halophytes (Ahmed *et al.,* 2011; Jatoi *et al.,* 2010; Lambertini *et al.,* 2008).

Interestingly, the dendrogram distributed the germplasm in two large clusters, and the PCA plot scattered the members of each population into two different groups. This indicated the close relatedness of the genotypes of one habitat to each other, thus suggesting that likely, the individuals of a particular habitat share a common ancestry. There is also a possibility that the populations are slowly evolving but independent of each other due to the reproductive barrier in the form of a small hilly range limiting pollen dispersion through the wind (Ahmed *et al.,* 2011). The isolation and fragmentation of habitats and changes in vegetation structure may hinder gene flow between isolated populations, lowering their genetic diversity and influencing the genetic system (Leonardi *et al.,* 2012; Odat *et al.,* 2004; Shuyskaya *et al.,* 2017). Our data suggested that PBA markers are reliable and reproducible for detecting genetic diversity in halophytic grass and provide valuable insights for future improvement and conservation studies.

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