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MOLECULAR MARKERS ASSISTED GENETIC CHARACTERIZATION OF SOME SELECTED WILD *POACEAE* SPECIES

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Abstract

Genetic diversity of ten selected wild species belonging to family *Poaceae* was assessed by using RAPD markers. The species consisted of *Tragus roxburgii*, *Eragrostis poaeoides*, *Brachiaria distachya*, *Dactyloctenium aegyptium*, *Setaria glauca*, *Setaria verticillata*, *Chrysopogon aucheri*, *Heteropogon contortus*, *Saccharum spontaneum* and *Themeda anathera*. In total 10 primers of OPC series were used, of which 9 primers gave reproducible amplifications. Out of 139 bands produced, 92 bands were shown to be polymorphic leading to 66% polymorphism across the species. NTSYS cluster analysis showed 59% similarity among the selected species indicating their common phylogenetic origin and more genetic stability during evolution. The genetic similarity index value ranged from 0.51 to 0.89. The results indicated high level of genetic diversity among grass species on the basis of RAPD technique. There is need to study other members of family *Poaceae* from different populations by using other molecular markers.

Introduction

The family *Poaceae* includes about 10,000 species divided into 12 subfamilies and over 700 genera (Grass Phylogeny working Group, 2001; Chen *et al.*, 2006). In Pakistan this family is represented by 158 genera and 492 species distributed in 26 tribes (Cope, 1982). Grasses are the most successful monocots (seed-bearing plants with single seed leaves), providing highly nutritional grains and livestock forage, and preventing soil erosion. Corn, barley, potatoes, rice, wheat and potatoes provide almost two-third of the food we eat, of these except potatoes the remaining four are grains belonging to family *Poaceae* (Devender & Dimmitt, 2009). Many grass species are among the most destructive weeds. Their slender and apparently fragile forms contradict their ability to compete with most other plants (Dekker, 1991).

In Pakistan no artificial/natural grasslands are present. Only in patches these grasses are reported in Sindh, Punjab, Khyber Pakhtunkhwa and Azad Kashmir. In Khyber Pakhtunkhwa, Punjab and Azad Kashmir moisture is available for their growth while most have wide amplitude. The cattle were with chance to graze on pockets/patches of seasonal grasses, which after rain fall appeared on the banks of canals, nallahs and barren areas etc. (Husain *et al.*, 2009). The increase in human population is biological living process and world population is tremendously increasing with the passage of time, study of pasture grasses can improve live-stock and poultry etc. In western and other developed countries like Japan, sufficient work has been done and finally they have developed improved varieties of these grasses for drought, frost, and shade and temperature resistance (Tompsett, 1976; Kidokoro, 2009). Looking towards the need of Pakistan research was conducted related to genetic diversity of wild grasses.

The purpose of present study was to examine the level of genetic diversity among selected wild species i.e. *Tragus roxburgii*, *Eragrostis poaeoides*, *Brachiaria distachya*, *Dactyloctenium aegyptium*, *Setaria glauca*, *Setaria verticillata*, *Chrysopogon aucheri*, *Heteropogon contortus*, *Saccharum spontaneum* and *Themeda anathera*, using random amplified polymorphic DNA (RAPD). RAPD is simple, easy to perform and do not require prior sequence information, it is widely used in phylogenetic studies (Rath *et al.*, 1998; Mahmood *et al.*, 2010; Mahmood *et al.*, 2011a; Mahmood *et al.*, 2011b; Nazar & Mahmood, 2011; Shinwari *et al.*, 2011) and population genetics (Kwon & Morden, 2002; Pervaiz *et al.*, 2010).

Material and Methods

Plant Material: Ten wild grass species belonging to family *Poaceae* were randomly collected from the same ecological zone of Islamabad.

DNA Extraction: The genomic DNA was extracted from grass blades by using CTAB method (Richard, 1997). One blade of the grass was taken, washed with distilled water and kept in aluminium foil at -20 °C overnight, for good crushing. The blade was crushed in 1 ml of preheated (65 °C) 2X CTAB buffer (Cetyl trimethy amonium bromide, containing 100 mM Tris HCl (pH 8.0), 20 mM Ethylene diamine tetra acetic acid (EDTA) pH 8.0, 1.4 M Sodium Chloride (NaCl) and 1% Merceptoethanol. The mixture was kept for incubation in water bath at 65 °C for 45 minutes. The supernatant was taken and shifted to another 1.5 ml eppendorf an equal amount of chloroform isoamylalchohol was added to the supernatant and tubes were inverted gently 10-15 times and then centrifuged at 10,000 rpm for 20 minutes, the chloroform isoamylalchohol treatment was repeated when needed. After centrifugation the supernatant was collected and an equal volume of chilled isopropanol was added to it and kept at -20 °C for overnight. Then centrifugation was done at 12,000 rpm for 10 minutes. The DNA pellet was washed with 70% chilled ethanol. The pellets were air dried and re-suspended in the mixture of 40-50 µl 0.1X TE (Tris EDTA) buffer. The DNA samples were stored at -20 °C till further use. DNA quality was checked in 1% agarose gel electrophoresis prepared in 0.5X TAE buffer.

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RAPD analysis: Ten primers of OPC (Gene Link) series (Table 1) were used and 9 gave reproducible amplifications. Amplifications were performed in 25 µl reaction mixture containing 1 µl DNA, 1 µM primer (50 pmol), 12.50 µl master mix (Fermentas) and 10.50 µl of PCR water (Fermentas). The thermal cycler conditions in a Labnet MultiGene thermocycler were: 5 min at 94°C, followed by 40 cycles of amplification, and each cycle included denaturation at 94 °C for 1 minute, annealing at 37°C for 30 seconds and extension at 72 °C for 2 minutes followed by an extended elongation step at 72°C for 7 minutes then a hold at 4 °C temperature. The amplified products were resolved in 1.5% agarose gel, in 0.5X TAE buffer at 80 V for 1.5 hours and then stained with ethidium bromide (0.5 μgmL^{-1}) for 15 minutes. The results were observed and photograph was taken with the help of gel documentation system (Wealtec Dolphin Doc^{Plus}).

Table 1. Nucleotide sequences of ten RAPD primers.

Sr. No.	Primer Code	Primer Sequence	Nucleotide Length (bp)
1.	OPC1	TTCGAGCCAG	10
2.	OPC2	GTGAGGCGTC	10
3.	OPC3	GGGGGTCTTT	10
4.	OPC4	CCGCATCTAC	10
5.	OPC5	GATGACCGCC	10
6.	OPC6	GAACGGACTC	10
7.	OPC7	GTCCCGACGA	10
8.	OPC8	TGGACCGGTG	10
9.	OPC9	CTCACCCTCC	10
10.	OPC10	TGTCTGGGTG	10

Cluster analysis: The amplicons obtained with different RAPD primers were scored as present (1) or absent (0) in each species. Cluster analysis was performed based on the similarity coefficient between samples, based on molecular data using Numerical Taxonomy and Multivariate Analysis System (NTSYS) pc version 2.01. Similarity index were generated by the similarity for Quantitative Data (SIMQUAL) subroutine while cluster analysis along with their corresponding dendrogram was generated with the Sequential Agglomerative, Hierarchical and Nested clustering (SHAN) (Rohlf, 2005).

Results and Discussion

In total, out of 10 RAPD primers nine have shown amplifications and out of nine, eight primers revealed some degree of polymorphism among selected species. A total of 139 scorable RAPD markers were produced using 9 primers (Table 2). The molecular size of amplified fragments ranged from 110bp to 200bp. The number of markers generated by each primer ranged from 10 to 27 fragments. On average, 14 bands were produced by each primer. Maximum number of bands (6) for single primer was revealed by *Themeda anathera* for OPC 2. The highest number of amplified loci by all primers was 20 observed in *Tragus roxburgii* while the lowest number (10) was found in *Heteropogon contortus*. In total, 47 monomorphic bands were produced, which lead to 4% monomorphism. Out of 139 bands, 92 bands were shown to be polymorphic leading to 66% polymorphism, which is in agreement with the findings of Aseefa *et al*. (2009) where 94% polymorphism was found among *Cynodon* accessions, while Mene *et al*., (2005) revealed 24% variations among populations of *Chloris roxburghiana*. On the whole, 12 unique bands were found for five species which can act as markers for species authentication. Recently, Khan *et al*., (2010) has reported 14 unique bands for the two species of *Cuscuta* using OPC series of RAPD markers. The similarity index values ranged from 0.48 to 0.89 with the mean value of 0.68. Highest similarity (0.89) was found between *Saccharum spontaneum* and *Dactyloctenium aegyptium* while, *Themeda anathera* and *Brachiaria distachya* showed the lowest (0.48) value of genetic similarity.

Table 2. Total number of monomorphic and polymorphic bands that were produced from nine RAPD primers from ten grass species.

S. No.	Primers	Total bands	Monomorphic bands	Polymorphic bands	Unique bands	Polymorphism %
1.	OPC-1	15	10	5	0	13.3
2.	OPC-2	27	8	15	4	44
3.	OPC-4	14	10	3	1	28
4.	OPC-5	14	9	5	0	36
5.	OPC-6	10	10	0	0	0
6.	OPC-7	21	0	20	1	100
7.	OPC-8	15	0	15	0	100
8.	OPC-9	10	0	8	2	100
9.	OPC-10	13	0	9	4	100

UPGMA cluster analysis revealed that all the ten species had least 59% and highest 89% similarity level among species collected from same ecological zone (Table 3). Earlier Chandra *et al.*, (2004) reported that accessions of *Dicanthium annulatum* collected from single location scattered in different clusters. According to the dendrogram, ten grass species mainly split into two clusters at 59% similarity level. Cluster 1 (C 1) consisted of *Tragus roxburgii* (1), *Eragrostis poaeoides* (2), *Heteropogon contortus* (8), *Dactyloctenium aegyptium* (4), *Saccharum spontaneum* (9), *Setaria glauca*, (5), *Setaria verticillata* (6), *Chrysopogon aucheri* (7) and *Brachiaria distachya* (3), while Cluster 2 (C 2) was represented only by *Themeda anathera* (10), which formed a cluster by itself. Cluster 1 diverged to form two sub clusters i.e. sub cluser 1 (Sb 1) and sub cluster 2 (Sb 2) (Fig. 1). The members of both clusters shared 66% similarity. Sub cluster 1 consisted of only *Tragus roxburgii* (1), while sub cluster 2 contained eight species namely *Eragrostis poaeoides* (2), *Heteropogon contortus* (8), *Dactyloctenium*aegyptium (4), *Saccharum spontaneum* (9), *Setaria glauca*, (5), *Setaria verticillata* (6), *Chrysopogon aucheri* (7) and *Brachiaria distachya* (3). Sub cluster 2 further divided into two groups i.e. group 1 (G 1) and group 2 (G 2). Both groups exhibited 75 % similarity. Group 1 contained seven species namely *Eragrostis poaeoides* (2) *Heteropogon contortus* (8), *Brachiaria distachya* (4), *Saccharum spontaneum* (9), *Setaria glauca*, (5), *Setaria verticillata* (6) and *Chrysopogon aucheri* (7), whereas group 2 contained *Brachiaria distachya* (3) which formed a separate group. Group 1 further diverged to form sub group 1 (Sbg 1) and subgroup 2 (Sbg 2). Subgroup 1 consisted *Eragrostis poaeoides* (2) and *Heteropogon contortus* (8) both were 81% similar and formed separate lines, while subgroup 2 contained *Dactyloctenium aegyptium* (4), *Saccharum spontaneum* (9) *Setaria glauca*, (5), *Setaria verticillata* (6) and *Chrysopogon aucheri* (7) and all the members of subgroup 2 showed 82% similarity. *Dactyloctenium aegyptium* (4) and *Saccharum spontaneum* (9) showed maximum (89%) similarity. *Setaria glauca* and (5), *Setaria verticillata* (6) also showed close relationship.

Table 3. Genetic similarity coefficients of ten grass species based on Euclidean distance.

	1	2	3	4	5	6	7	8	9	10
1.	1.00									
2.	0.70	1.00								
3.	0.59	0.78	1.00							
4.	0.70	0.83	0.78	1.00						
5.	0.64	0.78	0.67	0.78	1.00					
6.	0.67	0.81	0.75	0.86	0.86	1.00				
7.	0.64	0.78	0.78	0.83	0.78	0.86	1.00			
8.	0.62	0.81	0.75	0.81	0.70	0.72	0.81	1.00		
9.	0.70	0.78	0.72	0.89	0.78	0.86	0.78	0.75	1.00	
10.	0.51	0.54	0.48	0.70	0.54	0.67	0.59	0.62	0.64	1.00

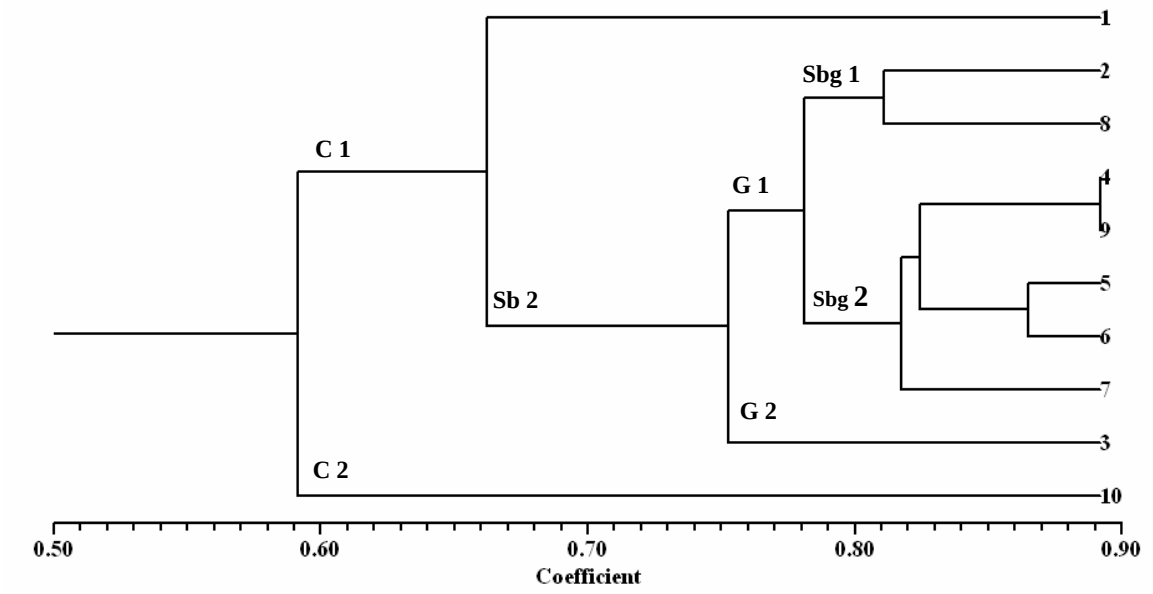

Fig. 1. Dendogram represented the genetic relationships among ten grass species generated from nine RAPD primers, using NTSYS clustral analysis.

C: Cluster, Sb: Sub cluster, G: Group, Sbg: Sub group.

Key for Fig. 1. 1: *Tragus roxburgii*, 2: *Eragrostis poaeoides*, 3: *Brachiaria distachya*, 4: *Dactyloctenium aegyptium*, 5: *Setaria glauca*, 6: *Setaria verticillata*, 7: *Chrysopogon aucheri*, 8: *Heteropogon contortus*, 9: *Saccharum spontaneum*, 10: *Themeda anathera*.

Conclusion

The observed results demonstrate that RAPD markers can be applied to study genetic diversity among grass species, and to identify closely related species. Presently, 66% genetic variation has been observed among the ten wild grass species collected from same geographical and climatic region. Several unique bands have been obtained which can act as species specific markers. The dendrogram showed 59% similarity in all the selected grass species which showed their common origin. It also indicated that these members of *Poaceae* have been diverged from their common ancestor at different time. These results show that molecular markers can assist us in designing our future breeding programs, however, this data can be verified using other techniques too, e.g. DNA bar codes, *rbc*L gene sequences etc. (Shinwari & Shinwari, 2010).

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