Journal of Environmental Biology
© Triveni Enterprises, Lucknow (India)
Free paper downloaded from: www. jeb. co. in

November 2010, 31(6) 883-890 (2010)
For personal use only
commercial distribution of this copy is illegal

Isozyme, ISSR and RAPD profiling of genotypes in marvel grass (*Dichanthium annulatum*)

Raghvendra Saxena[1] and Amaresh Chandra*[2]

[1]Institute of Allied Science and Computer Application (IASCA), Institute of Technology and Management (ITM), University, Gwalior - 474 001, India
[2]Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi - 284 003, India

(Received: April 04, 2009; Revised received: March 08, 2010; Accepted: March 25, 2010)

Abstract: *Genetic analysis of 30 accessions of marvel grass (Dichanthium annulatum Forsk.), a tropical range grass collected from grasslands and open fields of drier regions, was carried out with the objectives of identifying unique materials that could be used in developing the core germplasm for such regions as well as to explore gene (s) for drought tolerance. Five inter-simple sequence repeat (ISSR) primers [$(CA)_4$, $(AGAC)_4$, $(GACA)_4$]; 27 random amplified polymorphic DNA (RAPD) and four enzyme systems were employed in the present study. In total, ISSR yielded 61 (52 polymorphic), RAPD 269 (253 polymorphic) and enzyme 55 isozymes (44 polymorphic) bands. The average polymorphic information content (PIC) and marker index (MI) across all polymorphic bands of 3 markers systems ranged from 0.419 to 0.480 and 4.34 to 5.25 respectively. Dendrogram analysis revealed three main clusters with all three markers. Four enzymes namely esterase (EST), polyphenol oxidase (PPO), peroxidase (PRX) and superoxide dismutase (SOD) revealed 55 alleles from a total of 16 enzyme-coding loci. Of these, 14 loci and 44 alleles were polymorphic. The mean number of alleles per locus was 3.43. Mean heterozygosity observed among the polymorphic loci ranged from 0.406 (SOD) to 0.836 (EST) and accession wise from 0.679 (IG3108) to 0.743 (IGKMD-10). Though there was intermixing of few accessions of one agro-climatic region to another, largely groupings of accessions were with their regions of collections. Bootstrap analysis at 1000 iterations also showed large numbers of nodes (11 to 17) having strong clustering (>50 bootstrap values) in all three marker systems. The accessions of the arid and drier regions forming one cluster are assigned as distinct core collection of Dichanthium and can be targeted for isolation of gene (s) for drought tolerance. Variations in isozyme allele numbers and high PIC (0.48) and MI (4.98) as observed with ISSR markers indicated their usefulness for germplasm characterization.*

Key words: *Cluster analysis, Dichanthium annulatum, Genetic resources, Genetic similarity, Marvel grass, Polymorphism*
PDF of full length paper is available online

Introduction

Dichanthium annulatum (Forsk.) commonly known as Marvel grass is an important perennial grass species of tropical and subtropical regions. It is an important component of two major grass covers of India *i.e.*, *Dichanthium-Cenchrus-Lasiurus* and *Sehima-Dichanthium* (Dabadghao and Shankarnarayan, 1973). To date around 20 species of the genus have been reported, eight of these found in India in various agro-ecological zones (Arora *et al.,* 1975). Two species *viz.*, *D. annulatum* and *D. caricosum* are widely used for forage production. The basic chromosome number in *Dichanthium* is 10, however *D. annulatum* complex shows different ploidy levels with chromosome number 2n = 2X= 20, 4X = 40 and 6X = 60 with distinct morphological characters (Mehra, 1961). It is largely apomictic in nature but line showing facultative apomixes is also observed. Despite of this, a high level of polymorphism has been reported (Chandra *et al.,* 2004, 2006) along with considerable agro-morphological variations among accessions collected from similar eco-geographical situations (Agarwal *et al.,* 1999). In India a collection of more than 250 accessions (collected from different parts of the country) is maintained at Indian Grassland and Fodder Research Institute (IGFRI), Jhansi. Despite such germplasm holding, only one variety (Marvel-8) has been released in India.

* **Corresponding author:** amaresh62@yahoo.com

The characterization of germplasm is required to maintain identity and purity for proper conservation and management. Further, this helps to identify unique lines of crop species, especially those growing unabatedly under harsh environmental conditions. Morphological, phenological and agronomical characteristics are often used for estimating genetic variations. These traits however, are often polygenic and/or influenced by environmental conditions. Despite high variability at morphological level variations in isozyme alleles in *Dichanthium* germplasm of the drier regions has not been studied. The allozyme technique (Hunter and Markert, 1957) provides an opportunity to establish genetic relationships among crop species and cultivars, and used to estimate genetic diversity in many crops (Freitas *et al.,* 2000; Bhandari *et al.,* 2006; Jain *et al.,* 2006).

DNA markers, such as random amplified polymorphic DNA (RAPD), have been used in genetic and breeding studies in many plant species (Williams *et al.,* 1993). Of the various molecular diagnostic techniques available, RAPD and ISSR are easy to perform and cost-efficient, does not require radioactive compounds and analysis can reveal a high degree of polymorphism. Compared to restriction fragment length polymorphism (RFLP) marker, RAPD and ISSR can generate markers more rapidly but with some loss of information, because

these markers are usually dominant markers. RAPD and RFLP (Smith *et al.,* 1993) have been employed for developing markers for identification and fingerprinting of Napier grass (*Pennisetum purpureum*), however limited information is available on RAPD polymorphism in *Dichanthium*. Our earlier studies in *Dichanthium* indicated a high level of variations with RAPD markers (Chandra *et al.,* 2004, 2006). DNA markers, especially those based on microsatellites are useful in assessing large numbers of accessions, quickly and reliably (Sarala *et al.,* 2002). Inter-simple sequence repeat-PCR (ISSR-PCR) is a simple, cost efficient, robust, multilocus marker method, which is extensively useful in determining genetic variability (Sarala *et al.,* 2002), however choice of primers used in ISSR amplification is critical for obtaining high levels of polymorphism. The objectives of our study are to assess, estimate and compare genetic variability obtained with isozyme, ISSR and RAPD in thirty accessions of *Dichanthium* largely collected from drier regions of the country.

Materials and Methods

Plant materials: Thirty *Dichanthium* accessions used in the present investigation represent collections from the central north (Bundelkhand) and the southern plateau and hills (Dharwad and Bangalore) of India. These accessions were collected from their natural habitats, *i.e.*, grasslands and open fields, and maintained in experimental fields of the Indian Grassland and Fodder Research Institute, Jhansi (25°27'N, 78°35'E). All the accessions used in present study were tetraploid (Saxena and Chandra, 2006). The fresh and young leaves from three plants of each accession were used for enzyme extraction and total genomic DNA isolation.

Enzyme extraction and isozyme analysis: Fresh and young leaves from each accession were homogenized in three (v/w) fold volume of cold extraction buffer (50 mM Tris-HCl, pH 7.5, 10% sucrose, 1.0 mM EDTA and 1 mM 2-mercaptoethanol, added freshly). The extract was centrifuged (10,000 rpm) for 10 min at 4°C and resultant supernatant was used as enzyme source. Isozyme analysis of peroxidase (PRX, E.C. 1.11.1.17), esterase (EST, E.C. 3.1.1.2), polyphenol oxidase (PPO, E.C. 1.14.18.1) and superoxide dismutase (SOD, E.C. 1.15.1.1) was performed using native polyacrylamide gel (10%) electrophoresis (PAGE) at 4°C. Gels were stained as described by Veech (1969) for peroxidase and as described by Wendel and Weedel (1989) for esterase, superoxide dismutase and polyphenol oxidase.

Plant DNA extraction: For DNA extraction fresh and young leaves from three plants were used for isolation of genomic DNA following CTAB method (Iqbal *et al.,* 1997) with suitable modifications (Chandra *et al.,* 2004). DNA was quantified using UV spectrophotometer and also checked on 0.7% agarose gels. The stock DNA was finally diluted to 5 ng μl^{-1} for use in polymerase chain reaction (PCR).

RAPD analysis: RAPD-PCR reactions was performed in 20 µl of reaction mixture containing 67 mM Tris-HCl pH 8.0, 16.6 mM $(NH_4)_2SO_4$, 0.45% v/v BSA, 3.5 mM $MgCl_2$, 150 µM of each dNTP, 7.5 pmoles (15 ng) primer, 0.5 unit Taq polymerase and 25 ng genomic template DNA. The reaction was performed in a thermal cycler (PTC 200, MJ Research, USA) with a cycling program of 94°C for 1 min, 37 °C for 1 min, 72°C for 2 min for 40 cycles, followed by 41st cycle at 94°C for 1 min, 37°C for 1 min and finally at 72°C for 10 min primer extension step. PCR products were separated by electrophoresis on 1.6% agarose gel, and visualized using ethidium bromide.

ISSR analysis: In the present study five ISSR primers with the basic repeats of $(CA)_4$, $(AGAC)_4$, $(GACA)_4$ were used to identify markers with high levels of polymorphism. The reaction mixtures contained 2.5 µl of 10x buffer, 2.0 µl of 25 mM $MgCl_2$, 1.5 µl of 2.5 mM dNTP, 2.0 µl primer (0.5 µM), 0.1% gelatin, 1 unit *Taq* polymerase and 30 ng genomic DNA in a final volume of 25 µl. The PCR reactions were performed on thermal cycler (PTC 200, MJ, Research USA) with cycling program consisting 94°C for 4 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing for 1 min at 50°C, extension at 72°C for 90 s, and final extension at 72°C for 7 min. The amplification product was separated by electrophoresis on 1.6% agarose gel and detected by ethidium bromide staining. The molecular weight marker (100 bp ladder) was used for band sizing.

The zones of activity were defined for each of four enzyme systems. These activity zones were designated as locus (1 to 5) having different numbers of bands corresponding to alleles. Taking this into consideration the parameters estimated for genetic variations were percentage of polymorphic loci (Pp), mean number of alleles per polymorphic locus (Ap) (Hamrick and Godt, 1997), mean expected heterozygosity based on unbiased estimate of Nei (1978) and allele frequency. Allele frequencies were also used to estimate expected heterozygosity, however the genetic interpretation in terms of individual as heterozygous/ homozygous for a particular locus was not attempted due to apomictic nature of the crop. Genetic diversity (H_e = Hardy-Weinberg expected heterozygosity) (Weir, 1989) was calculated for each locus (including monomorphic and polymorphic loci) by:

$$H_e = 1 - \Sigma x_i^2$$

where x_i is the mean frequency of the *i*th allele pooled across the accessions.

The PIC value was calculated employing the formula of Roldan-Ruiz *et al.* (2000): $PIC_i = 2f_i(1 - f_i)$, where f_i is the frequency of the amplified allele (band present) and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker *i*. MI was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell *et al.,* 1996). The binary data generated on the basis of presence (1) and absence (0) of the bands in both ISSR and RAPD was analyzed for genetic similarity among the accessions based on Dice's similarity coefficient, which was also converted to distance measures (d) using the formula d = 1-s. Amplification failure of a sample or missing data was coded as 9. Dendrogram was constructed by Sequential Agglomerative Hierarchical and Nested

(SAHN) clustering using the Un-weighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. Boot strap analysis was carried out at 1000 scale limit to understand the level of clustering of the different nodes (Yap and Nelson, 1996).

Results and Discussion

Isozyme patterns and cluster analysis: The four enzyme systems generated a total of 16 isozyme loci with 55 alleles. 80% of the alleles were polymorphic (Table 1). Number of loci varied from 3 to 5 whereas alleles at a particular locus from 2 to 7. Locus four of esterase (EST4) was polymorphic in 96.6% of the accessions followed by PPO3 in 90%. PRX1 locus was least polymorphic (16.6%). Although both the SOD2 and PRX2 loci possessed two alleles and both of these were either present or absent in accessions and thus the polymorphism was in the form of a null allele. At the SOD2 locus both alleles were present in 5 and absent in 25 accessions. This was also true with the PRX2 locus but the accessions were different. The polymorphic loci within accessions ranged from 37.50 to 75%. Accessions IG97-121, IG97-244, IG97-245 and IG97-144 depicted the least polymorphism (37.5%) whereas accession IG97-234 showed the maximum polymorphism (75%). The number of monomorphic loci also varied from maximum 10 in 6 accessions and minimum 4 in only one genotype, IG97-234. Minimum allele frequency (0.037) was observed at the EST4 locus for allele 4 whereas the maximum (0.75) was observed at SOD3 for allele 1. Esterase was the most polymorphic enzyme, generating 22 alleles of which 20 were polymorphic. Locus 4 of esterase yielded 7 alleles which were polymorphic in 29 of the accessions examined.

The dendrogram of 30 accessions based on 55 bands of four isozyme systems (EST, PPO, PRX and SOD) showed 3 main clusters (Fig. 1A). The genetic distance ranged from 0.00 to 0.253 between three clusters. Cluster-1 was further subdivided into two sub-clusters comprising 11 accessions in total. Two accessions, *viz*., IG97-241 and IG95-25 originally collected from the close vicinity showed 100% similarity. Sub-cluster 1-1 embodied 5 accessions of which four were collected from one place and one from different region, but they were not highly agro-ecologically diverse. Cluster 1-2 comprised of 6 accessions showed 83-100% similarity among them. Sub-cluster 1-1 was 74.7% similar to Sub-cluster 1-2. Cluster-2 comprised of 13 accessions of which 9 were from central north India and 4 from south India. This cluster was further subdivided into three Sub-clusters. Sub-cluster 2-1 comprised of two accessions belongs to one region with 98.0% similarity among them. Sub-Cluster 2-1 showed 76.0% similarity with Sub-cluster 2-2 which comprised 8 accessions from central north India and one (IGKMFD-1) from south India. Sub-cluster 2-3 embodied 3 accessions of south India. Cluster-3 comprised of 6 accessions of which 4 accessions, *viz*., IG97-121, IG97-244, IG97-245 and IG97-144 of central north India showed 100% similarity among them. The other two accessions of this cluster belong to south India and showed 79.0 and 83.0% similarity with the rest of the members of this cluster. Cluster-3 showed 72.0% similarity with Cluster-2 and these two clusters together exhibited 67.0% similarity with Cluster-1.

RAPD and ISSR fingerprints and cluster analysis: 27 selected random decamer primers (Chandra *et al*., 2003) generated a total of 269 RAPD bands (putative loci). Of the total 269 bands, 16 monomorphic and 253 polymorphic bands were observed. Among the 27 primers, nine have produced one or two monomorphic bands and rest of them generated polymorphic bands. Average PIC values ranged from 0.21 to 0.50 and MI from 1.24 to 7.46 (Table 2). The pair-wise genetic distance based on Dice similarity coefficients of 269 RAPD bands revealed three main clusters in UPGMA module (Fig. 1B). Additionally, a genotype representing a separate cluster joined with rest of the genotypes at the similarity of 47%. The genotype in question IGBANG-D-2 is of south Indian region and observed close to the main Cluster-3 formed by four of six accessions of south India. Cluster-1 comprised of four accessions of central north India showing intra-cluster similarity ranging from 81 to 91%. Cluster-2 comprised of 25 accessions of which only one accession (IGKMFD-1) was from south India and rest from central north India. The intra-cluster similarity in this cluster ranged from 72

Table - 1: Number of alleles at different locus and percentage (%) of accessions polymorphic to the locus

Locus	Number of alleles detected	Number of polymorphic locus	Mean number of alleles / polymorphic locus	% of accessions polymorphic to the locus
EST1	7	4	1.90	80.00
EST2	3	4	2.00	46.66
EST3	5	4	3.06	60.00
EST4	7	4	3.51	96.66
PRX1	2	3	1.00	16.66
PRX2	2	3	0.00	0.00
PRX3	4	3	2.60	83.33
PPO1	3	5	2.00	43.33
PPO2	3	5	1.43	23.33
PPO3	4	5	1.85	90.00
PPO4	3	5	1.00	46.66
PPO5	2	5	1.00	50.00
SOD2	2	2	0.00	0.00
SOD3	3	2	1.00	80.00

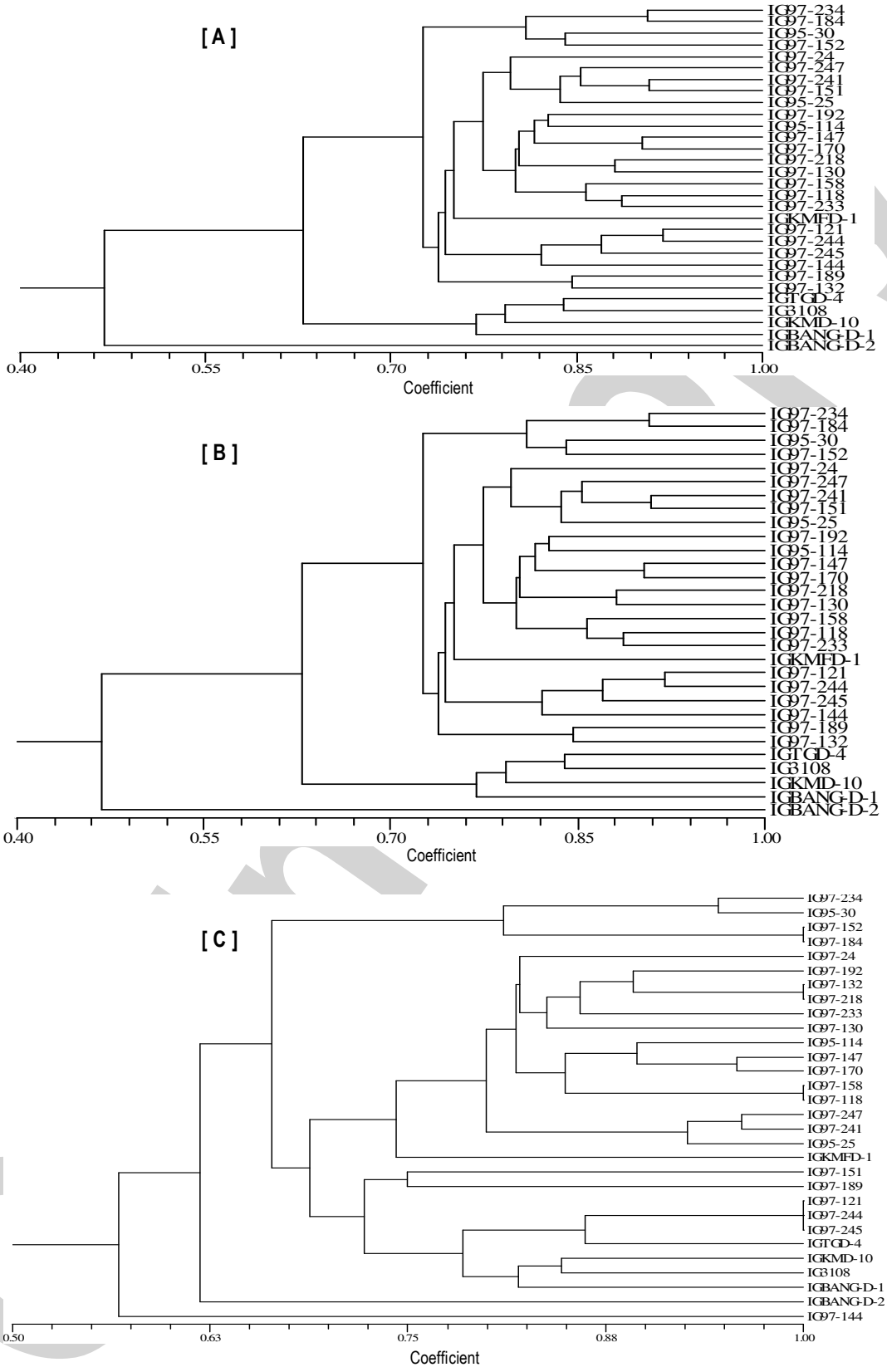

Fig. 1: Dendrogram of 30 *Dichanthium* accessions derived from Dice similarity values based on isozyme (A), RAPD (B) and ISSR (C) markers data. In total, 15 nodes in isozyme, 17 in RAPD and 11 in ISSR were observed having strong clustering (>50 bootstrap values)

to 94%. This big cluster appeared to be a bridge between two other main clusters. Accessions from both north and south India were observed in this cluster and showed 73% similarity with Cluster-1 and 63% with Cluster-3 (Fig. 1B). Cluster-3 comprised of four south India accessions with intra-cluster similarity of 77 to 84%. This cluster was joined with an accession of south India. Even accession IGKMFD-1 of south India clustered with 24 members of central north India in Cluster-2, showed only 25% dissimilarity with the majority of the accessions of this cluster.

Five ISSR primers having di and tetranucleotide repeats generated 61 bands. Of these, 52 were polymorphic in nature. As expected primers like $GC(CA)_4$ and $GT(CA)_4$ of 10 nucleotides base pairs with dinucleotide repeats were comparatively more polymorphic (93.33 to 100%) than those of primers having long nucleotides sequence (71.42 to 83.33%). Polymorphic information content (PIC) ranged from 0.405 to 0.500. The Marker index (MI) values of individual primers ranged from 4.00 to 6.98 (Table 3). Like isozyme and RAPD, ISSR markers also generated 3 major clusters which together constitute 28 accessions and rest of the two accessions namely IGBANG-D-2 and IG97-144 formed a separate node and showed 62.21 and 56.77% genetic similarity with rest of the accessions respectively. Cluster-1 separated from Cluster-2 and 3 at 66.6% genetic similarity where as Cluster-2 and Cluster-3 separated from each other at 68.43% genetic similarity. Cluster-1 embodied four accessions of the same region *i.e*., north India central plateau and variability among them ranged from 81.08 to 100%. Winboot analysis also showed strong clustering at 11 different nodes where bootstrap values were more than 50. Cluster 2 was the largest cluster possessed 17 accessions. As Cluster-1, most of the accession grouped in this cluster belongs to north India central plateau with one accession (IGKMF-D-1) of south India. Intra-cluster similarity in this cluster ranged from 72.91 to 100%. Of the 17 accessions, two sets of accessions forming two sub-clusters showed 100% similarity to each other (Fig. 1C). Rest of the 13 accessions showed the genetic similarity between 72.91 to 96.10%. Cluster-3 embodied seven accessions, four belongs to south India and rest three namely IG 97-121, IG 97-244, IG 97-245 from north India and showed 100% genetic similarity to each other. Accession IGTGD-4 of south India was observed closest (86.21%) to the three north Indian accessions of this cluster.

Mean heterozygosity among the polymorphic loci in *Dichanthium* germplasm studied presently was comparatively high (0.406-0.836) to that reported in crops like sunflower and guar (Kongkiatngam *et al.,* 1995). However, Napier grass where three enzyme systems reported to identify more than 90% accessions indicated a high level of genetic diversity (Bhandari *et al.,* 2006). Isozyme analysis in *Cenchrus,* a tropical grass also depicted a high level of polymorphism. Of 16 isozyme loci observed in present work, EST1 with maximum heterozygosity (0.836) indicated the importance of a particular locus in determining the genetic diversity in *Dichanthium*. Similarly, at accession level, maximum heterozygosity (0.743) was observed in IGKMD-10. Other three accessions *viz*., IGKMFD-1, IGTGD-4, IGBANGD-2 of the same region (South India), depicted heterozygosity more than 0.70.

An average PIC value of 0.46 across all scored RAPD bands, as well as an average MI of 4.34 across all primers obtained with *Dichanthium* accessions was different than that of AFLP-based genetic diversity studies in various crops (Powell *et al.,* 1996; Muminovic *et al.,* 2004). Though both AFLP and RAPD are dominant markers, the easiness associated with RAPD analysis as well as high PIC and MI obtained with *Dichanthium* justifies its use for fingerprinting and identification of cultivars for different agroclimatic zones. ISSR yielded high MI (5.50) with each primer which was more than RAPD (4.3) indicating more usefulness of such marker (Table 4).

All three marker systems (Isozyme, RAPD and ISSR) generated three main clusters. Cluster-2 obtained with two DNA-based markers was observed largest cluster among three clusters. Isozyme based clusters also indicated two groups each comprising 4 and 2 accessions depicting 100% similarity. Such situation was not observed with RAPD and ISSR presumably because of high number of markers generated from both coding and non-coding regions of the genome. Though the level of polymorphism was high with DNA-based markers, the clustering patterns of accessions were comparable between isozyme and DNA-based markers. Even bootstrap values also indicated strong clustering at comparable number of nodes (11 to 17) with three sets of markers. However, the difference lies with the placement of accessions of south India particularly in Cluster-2 and 3. The dendrogram based on DNA-based markers data-set indicated a closer relationship between accessions of south India. At inter-specific level, the reliability of DNA-based data is comparable to that of isozyme data indicated such markers are more valuable in assessing the genetic variations in populations (Liu and Furnier, 1993).

Due to less discriminatory power of isozyme marker, 18 of 30 *Dichanthium* accessions yielded more than 90% similarity and 12 accessions from 67 to 90%. All 18 accessions in question were from central north India. Out of twelve genotypes that have shown similarity from 67 to 90%, six accessions were from south India exhibited a high level of diversity among them. Two closest accessions of this region were IG3108 and IGBANG-D-1 having 88% similarities. Clustering patterns based on isozyme banding patterns clearly indicated that accessions of south India clustered together though having higher level of diversity among them. Four accessions belonging to central north India possessing 100% similarity intermixed with the members of south India. All six accessions belonging to south India were highly divergent and two nearest accessions in this category was IG3108 and IGBANG-D-1. A high level of diversity in isozyme banding patterns have been reported in guinea grass (Jain *et al.,* 2006), where authors emphasized evolution of new types through sexual recombination or other means namely hybridizing of indigenous materials with sexual/apomictic exotic lines. Guinea grass (*Panicum maximum* Jacq.) consists largely of apomictic population, although many sexual plants are

Table - 2: Sequence and amplified products of twenty seven arbitrary primers (Operon) used to generate RAPD markers in *Dichanthium*

Primer	Sequence	No. of bands	No. of polymorphic bands	PIC	MI
OPE01	5'CCCAAGGTCC3'	10	10	0.487	4.87
OPE08	5'TCACCACGGT3'	7	7	0.464	3.25
OPF01	5'ACGGATCCTG3'	12	10	0.500	5.00
OPF04	5'GGTGATCAGG3'	7	6	0.206	1.24
OPF06	5'GGGAATTCGG3'	10	10	0.482	4.82
OPG02	5'GGCACTGAGG3'	6	6	0.499	2.99
OPG12	5'CAGCTCACGA3'	5	5	0.496	2.48
OPH04	5'GGAAGTCGCC3'	8	7	0.296	2.07
OPH05	5'AGTCGTCCCC3'	7	7	0.448	3.14
OPH09	5'TGTAGCTGGG3'	10	10	0.481	4.81
OPH13	5'GACGCCACAC3'	9	9	0.462	4.16
OPI07	5'CAGCGACAAG3'	21	19	0.377	7.18
OPI08	5'TTTGCCCGGT3'	8	7	0.485	3.40
OPI14	5'TGACGGCGGT3'	10	10	0.497	4.97
OPI18	5'TGCCCAGCCT3'	6	5	0.468	2.81
OPAB01	5'GAGCGCCTTG3'	13	13	0.495	6.42
OPAE04	5'CCAGCACTTC3'	8	6	0.492	2.95
OPAE07	5'GTGTCAGTGG3'	15	14	0.497	7.46
OPAH03	5'GGTTACTGCC3'	11	11	0.500	5.50
OPAH09	5'AGAACCGAGG3'	10	10	0.491	4.91
OPB05	5'TGCGCCCTTC3'	11	11	0.443	4.87
OPN04	5'GACCGACCCA3'	9	7	0.487	3.41
OPN06	5'GAGACGCACA3'	8	7	0.460	3.24
OPP09	5'GTGGTCCGCA3'	8	8	0.438	3.57
OPQ06	5'CCGTCGGTAG3'	12	11	0.472	5.19
OPR07	5'ACTGGCCTGA3'	13	12	0.483	5.19
OPR08	5'CCCGTTGCCT3'	15	15	0.488	7.33
Total		269	253	12.494	117.19
Average		9.962	9.370	0.463	4.340

PIC = Polymorphic information content, MI = Marker index

Table - 3: ISSR primers used in 30 *Dichanthium* accessions

Primer	Sequence 5'to 3'	Total bands	Polymorphic bands	% Polymorphic bands	Monomorphic bands	PIC	MI
ISSR80	$GC[CA]_4$	15	14	93.33	01	0.499	6.99
ISSR81	$GT[CA]_4$	08	08	100	-	0.500	4.00
ISSR82	$[AGAC]_4GC$	12	10	83.33	02	0.405	4.05
ISSR83	$AC[GACA]_4$	12	10	83.33	02	0.499	4.90
ISSR84	$[GACA]_4GT$	14	10	71.42	04	0.500	5.00
Total		61	52		09		
Mean		12.2	10.4	85.24	1.8	0.480	4.98

PIC = Polymorphic information content, MI = Marker index

Table - 4: Comparative analysis of banding patterns generated by ISSR, RAPD and isozyme markers system for 30 *Dichanthium* accessions

Components	ISSR	RAPD	Isozyme
Number of assay units	5	27	4
Total number of bands	61	269	55
Mean number of bands / per assay unit	12.2	9.96	13.75
Total number of polymorphic bands	52	253	44
Number of polymorphic bands par assay unit	10.4	9.37	11.0
Mean (%) polymorphism par assay	85.24	94.07	74.51
Number of monomorphic bands par assay	1.8	0.59	2.75
Mean PIC per assay	0.480	0.463	0.419
Mean Marker Index (MI)	4.98	4.34	5.25

ISR = Inter simple sequence repeat, RAPD = Random amplified polymorphic DNA, PIC = Polymorphic information content

also available in nature (Assienan *et al.,* 1993). In case of *Dichanthium* though being largely apomictic in nature the increased variability in sub-humid dry regions of south India might be due to the presence of major grasslands as well as favorable climate for natural crossing. Assienan and Noirot (1995) have reported that apomixis does not lead to the reduction in the diversity of grasses. The *Dichanthium annulatum* complex, having different ploidy levels and growing wild in natural habitats, exhibits large phenotypic variations. Even diverse forms have been reported from material collected at the same locations and the clustering patterns based on agro-morphological attributes in marvel grass indicated independent groupings with their geographical distribution (Agarwal *et al.,* 1999). Genetic variation in *Dichanthium annulatum* genotypes by RAPD and ISSR corroborated that diversity was independent of geographical distribution as genotypes from different areas clustered in the same group and vice versa (Chandra *et al.,* 2004). However, in present study when accessions of only two contrasting regions were analyzed, most of the accessions of South India centered to one part of the phenogram (Fig. 1).

Contrary to isozyme, RAPD revealed eight accessions possessing more than 90% similarity and rest from 47 to 90%. In Cluster-2, single accession of south India got mixed otherwise this cluster as well as Cluster-1 comprised accessions only from central north India. The minimum level of similarity in case of RAPD was 47% whereas in case of isozyme 67%. This difference clearly provided edge of RAPD over isozyme in diversity study as RAPD has the potential to detect polymorphism at more loci and detect both coding and non-coding sequences in the genome. However, the estimates of genetic variation as obtained with RAPD may be higher than those actually existing because of the dominant nature and one-band/one-locus assumption of these markers. Higher estimates of genetic diversity with RAPD markers over isozyme have been reported in other crops also (Liu and Furnier, 1993; Kongkiatngam *et al.,* 1995).

When results of all marker systems were taken together, accessions namely IGKMFD-1, IGTGD-4 and IGBANG-D-2 of south India and IG97-189, IG97-132, IG97-121, IG97-244, IG97-245 and IG97-144 of central north India were identified as distinct as they have depicted high level of variations and unique clustering patterns to the rest of the accessions. The three south Indian accessions (IGKMFD-1, IGTGD-4 and IGBANG-D-2) identified as distinct, either got intermixed with the rest of the north Indian genotypes or formed separate cluster. With RAPD, IGBANG-D-2 accession of south India showed 47% similarity to the rest of the genotypes, whereas with isozyme it has shown 70 and 81% similarity with two other clusters. Both marker systems revealed that genotype IGKMFD-1 of south India was closest to the central north Indian accessions. Study resulted the grouping of accessions based on their genetic distances and both protein and DNA-based markers categorized *Dichanthium* accessions based on their agro-climatic regions. The closely related genotypes as well as diverse and unique accessions can be used for evaluation under specific ecological and environmental conditions.

Acknowledgments

Authors are thankful to the Head, Crop Improvement Division and Director for facilities. RS is thankful to Council of Scientific and Industrial Research (CSIR), New Delhi for providing fellowship (SRF).

References

Agarwal, D.K., S. Gupta, A.K. Roy and S.R. Gupta: Study on agro-morphological variation vis-a-vis geographical distribution in Marvel grass [*Dichanthium annulatum* L. (Stapf)]**.** *Plant Genetic Resour. Newsletter*, **118**, 27-29 (1999).

Arora, R.K., K.L. Mehra and M.W. Hardas: The Indian gene center, prospect for exploration and collection of herbage grasses. *Forage Res.*, **1**, 11 (1975).

Assienan, B., M. Noirot and Y. Gnagne: Inheritance and genetic diversity of some enzymes in the sexual and diploid pool of the agamic complex of maximae (*Panicum maximum* Jacq., *P. infestum* Anders and *P. trichocladeum* K. Schum.) *Euphytica,* **68**, 231-239 (1993).

Assienan, B. and M. Noirot: Isozyme polymorphism and organization of the agamic complex of the maximae (*Panicum maximum* Jacq., *P. infestum* Anders. and *P. trichocladeum* K. Schum.) in Tanzania. *Theor. Appl. Genet*., **91**, 672-680 (1995).

Bhandari, A.P., D.H. Sukanya and C.R. Ramesh: Application of isozyme data in fingerprinting Napier grass (*Pennisetum purpureum* Schum.) for germplasm management. *Genet. Resour. Crop Evol*., **53**, 253-264 (2006).

Chandra, A., A.K. Roy, R. Saxena and A. Dubey: Identification of suitable primers to develop DNA fingerprints of marvel grass. *Ind. J. Agric. Biochem*., **16**, 23-27 (2003).

Chandra, A., R. Saxena, A.K. Roy and P.S. Pathak: Estimation of genetic variation in *Dichanthium annulatum* genotypes by the RAPD technique. *Trop Grasslands*, **38**, 245-252 (2004).

Chandra, A., R. Saxena and A.K. Roy: Polymorphism and genotype-specific markers for *Dichanthium* identified by random amplified polymorphic DNA. *Genetic Resour. Crop. Evol.*, **53**, 1521-1529 (2006).

Dabadghao, P.M. and K.A. Shankarnarayan: The grass cover of India. ICAR publication, India. pp. 105-106 (1973).

Freitas, N.S.A., T.M.M. De Falco, A. De, H.A. Burity, J.N. Tabosa and M.V.D. Silva: Characterization and genetic diversity of elephant grass cultivars and their hybrids with millet through isozymatic patterns. *Pesquisa Agropecuaria Brasileiria*, **35**, 1125-1133 (2000).

Hamrick, J.L. and M.J.W. Godt: Allozyme diversity in cultivated crops. *Crop Sci*., **37**, 26-30 (1997).

Hunter, R. and C. Markert: Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science*, **125**, 1294-1295 (1957).

Iqbal, J., N. Aziz, N.A. Saeed, Y. Zafar and A. Malik: Genetic diversity evolution of some elite cotton verities by RAPD analysis. *Theor. Appl. Genet*., **94**, 139-144 (1997).

Jain, A., A.K. Roy, P. Kaushal, D.R. Malaviya and S.N. Zadoo: Isozyme banding pattern and estimation of genetic diversity among guinea grass germplasm. *Genet. Resour. Crop Evol.*, **53**, 339-347 (2006).

Kongkiatngam, P., M.J. Waterway, M.G. Fortin and B.E. Coulman: Genetic variation within and between two cultivars of red clover (*Trifolium pratense* L.): Comparisons of morphological, isozyme and RAPD markers. *Euphytica*, **84**, 237-246 (1995).

Liu, Z. and G.R. Furnier: Comparison of isozyme, RFLP and RAPD markers for revealing genetic variation within and between population trembling aspen and big tooth aspen. *Theor. Appl. Genet*., **87**, 97-105 (1993).

Mehra, K.L.: Chromosome number, geographical distribution and taxonomy of the *Dichanthium annulatum* complex. *Cytologia*, **17**, 176 (1961).

Muminovic, J., A.E. Melchinger and T. Lubberstedt: Prospect form celeriac (*Apium graveolens* Var. *rapaceum*) improvement by using genetic resources of *Apium*, as determined by AFLP marker and morphological characterization. *Plant Genetic Resour*., **2**, 189-198 (2004).

Nei, M.: Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics*, **89**, 583-590 (1978).

Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey and A. Rafalski: The comparison of RFLP, RAPD, AFLP and SSR (microsatellites) marker for germplasm analysis. *Mole. Breed*., **2**, 225-238 (1996).

Roldan-Ruize, I., E. Calsyn, T.J. Gilliand, R. Coll, M.J.T. Vaneijk and M. De Loose: Estimating genetic conformity between related ryegrass (*Lolium*) varieties. 2. AFLP characterization. *Mole. Breed*., **6**, 593-602 (2000).

Sarala, N., S. Bobba and E.A. Siddiq: ISSR and SSR markers based on AG and GA repeats delineate geographically diverse *Oryza nivara* accessions and reveal rare alleles. *Curr. Sci.*, **84**, 683-690 (2002).

Saxena, R. and A. Chandra: RAPD and cytological analysis and histological changes caused by moisture stress in *Dichanthium annulatum* accessions. *Cytologia*, **71**, 197-204 (2006).

Smith, R.L., M.E. Schweder, M.K.U. Chowdhury and J.C. Seib: Development and application of RFLP and RAPD DNA markers in genetic improvement of *Pennisetum* for biomass and forage production. *Biomass Bioenergy*, **5**, 51-62 (1993).

Veech, J.A.: Localization of peroxidase in infected tobaccos susceptible and resistant to black shank. *Phytopath*., **59**, 556-571 (1969).

Weir, B.S.: Sampling properties of gene diversity. *In*: Plant population genetics: Breeding and Genetic Resource (*Eds*.: A.H.D. Brown, M.T. Clegg, A.L. Kahler, A.H.D. Weir and B.S. Brown). Sinauer Press, Sunderland, MA. pp. 23-42 (1989).

Wendel, J.F. and N.F. Weeden: Visualization and interpretation of plant isozymes. *In*: Isozymes in plant biology (*Eds*.: D.E. Soltis and P.S. Soltis). Dioscorides Press, Oregon, Portland. pp. 5-45 (1989).

Williams, J.G.K., M.K. Hanafey, A. Rafalski and S.V. Tingey: Genetic analysis using random amplified polymorphic DNA markers. *Methods in Enzymol.*, **218**, 704-740 (1993).

Yap, I.V. and R.J. Nelson: Winboot: A program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms, IRRI, Philippines (1996).