Assessment of Genetic Diversity of Barnyard Millet Accessions using Molecular Markers

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(Received: 4 April 2011; Revised: 1 August 2011; Accepted: 25 October 2011)

Present study was conducted to access genetic diversity among 33 accessions of two cultivated species of barnyard millet *i.e.*, *Echinochloa crus-galli* (15 accessions) and *E. frumentacea* (18 accessions). Though crop possesses great nutritional value, little attention has been paid for the improvement of this crop. We assessed genetic diversity on the basis of morphological and RAPD analysis. Morphological analysis suggested that EC-545 is superior for grain yield, whereas VRS-MB-1202 and VRS-MB-889 were found to be good for fodder yield. On the other hand, RAPD primers were able to segregate the accessions in two groups at a similarity level of 34% with clear demarcation of inter and intra species diversity. The data would be important in detailing the level of variation and relationship within and between species to plan future domestication trials and to manage the wild species collection that is available in the gene banks.

Key Words: Barnyard millet, Genetic diversity, RAPD

Introduction

Barnyard millet (*Echinochloa* sp.) is one of the oldest domesticated millets in the semi-arid tropics of Asia and Africa. The genus *Echinochloa* includes some 20 species that are distributed widely in the warmer parts of the world. Two of the main species, *E. crus-galli* and *E. frumentacea* are grown as cereals. In addition to these two domesticated species, the genus includes about 30 annual and biennial wild species distributed worldwide (Clayton and Renvoize, 1986). These millet species are morphologically very dissimilar. Indian barnyard millet (*E. frumentacea*) can easily be distinguished from Japanese barnyard millet (*E. crus-galli*) by its panicle, thinner texture of the glumes and lower lemma (Yabuno, 1971).

The crop is valued for its drought tolerance, good yield and superior nutritional value. It is the fastest growing crop among all millets and can be harvested in a short period of nine weeks. Barnyard millet is an important dual-purpose crop. Its grains contain 6.2% protein, 9.8% crude fiber, 65.5% carbohydrates and are consumed just like rice (Ruiz-santaella *et al.*, 2006). Also it is a nutritive fodder for animals. These aspects make barnyard millet a valuable crop.

Being one of the traditional crops, barnyard millet (*E. frumentacea*) is cultivated at almost all farming situations in Uttarakhand hills even in rainfed areas. But, the cultivation area of the crop has reduced tremendously in past decades because of one or other reasons, among which main are, the poor productivity and susceptibility to diseases like collar rot and seed rot etc. Moreover, in absence of wide genetic diversity among local cultivars and released varieties, the selection for adaptation to cold temperature is discouraging (Gullord *et al*., 1975). On the other hand Japanese millet (*E. crusgalli*) is high yielding, shows better adaptability to cold temperature and disease resistant. So the accessions posses better morphological characters and wide genetic diversity should be evaluated for this region.

Genetic diversity of common morphological traits is difficult to measure in a natural population since the traits are influenced by environmental factors to a large degree. On the other hand, their expression is largely governed by different interacting genes. Assessment of genetic diversity with molecular markers overcomes this problem because these molecular traits have virtually no environmental component and large numbers of variables are produced. As a result, qualitative and quantitative traits can be more efficiently introduced into plant breeding programmes. Molecular techniques for evaluating genetic diversity have been improved in the last decades, complementing the use of morphological markers. Measurement of genetic diversity with molecular markers is relevant to assessment of ecological conditions because it allows estimation of

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important population parameters *e.g.*, characterization of the geographic structure or connectivity of populations. Molecular markers such as SDS-PAGE, isozymes and DNA markers have been found more useful to study genotypic diversity in many plant species (Tanksley *et al.*, 1989; Paterson *et al.*, 1991).

In the present study we evaluated genetic diversity among 33 different accessions of two cultivated species of barnyard millet (*E. crus-galli* and *E. frumentacea*) on the basis of morphological and molecular (RAPD) markers.

Materials and Methods

Plant Material

Two sets of different accessions of both the species (*E. crus-galli* and *E. frumentacea*) were examined. 15 accessions of *E. crus-galli* were procured from ICRISAT, Hyderabad (Andhra Pradesh, India) and 18 accessions of *E. frumentacea* were collected from VPKAS, Almora (Uttarakhand, India).

Morphological Characters

All the 33 genotypes were sown in the fields in a Randomized Block Design (RBD) at GBPUA&T, Hill Campus, Ranichauri, Tehri Garhwal (Uttarakhand) for two consecutive years 2004 and 2005. Crop management was done according to the recommended agronomic practices. Sowing was done in the plots (3m × 1m), plant to plant distance was maintained 10 cm., while row to row distance was kept 22.5 cm. Eight morphological characters including, germination (%), days to 50% flowering, plant height, number of fertile tillers, length of spikelet, days to maturity, 1000 grain wt and yield/plant were taken to assess genetic variability in accessions.

DNA Isolation and PCR Amplification

Seeds were grown in the green house and leaf material was harvested from 3-4 week old seedlings. Total cellular DNA was isolated from leaf material of individual plants following the procedure of Saghai-Maroof *et al.* (1984) with few modifications. Samples for PCR amplification were diluted to 15 ng/µl with deionized distilled water.

Fifty random decamer primers were used to study polymorphism. Out of these, 21 primers gave polymorphic bands and were used for further study. The PCR amplifications were carried out in a 25 µl reaction mixture containing 15 ng of genomic DNA, 100 ng primer, 1X PCR reaction buffer (Banglore Genie, India), 0.8 mm DNTP mix (Banglore Genie) and 1.5 U Taq polymerase (Banglore Genie). Amplifications were carried out in a thermal cycler (Eppendorf), programmed for one initial denaturation 95°C for 5 min. followed by 49 cycles of 92°C for 10 sec., 36°C for 1 min., 72°C for 2 min. and synthesis 72°C for 2 min. Amplifications were repeated twice in order to check reproducibility.

PCR amplification products were analyzed by electrophoresis on 1.5% agarose gel and run in TBE buffer at 80 Volt for approximately 5hrs. Gels were stained in ethidium bromide solution (0.5 $\mu g/ml^{-1}$) and documentation was done using Gel Doc system (Bio-Rad).

Data Analysis

Field data for morphological characters was evaluated by the analysis of variance (ANOVA) using RBD to calculate the significance by magnitude of F value (P= 0.01) and D^2 statistics as suggested by Rao (1952) using computer software. The calculation of D^2 values involved the steps followed by Murthy and Arunachalam (1996).

RAPD-PCR data was evaluated for pair-wise similarity and cluster analysis was done on the basis of presence and absence of bands. Computer software (Gel-Compar-II, ver. 3.5, Applied Maths, USA) was used to perform the similarity matrix analysis using 'UPGMA' with 'Dice' coefficient of similarity.

Results and Discussion

Estimation of Genetic Diversity Based on Morphological Characters

On the basis of D^2 analysis, all the accessions of two species were grouped into three clusters (Table 1). Cluster-I comprised of accessions of *E. frumentacea,* cluster-II consists of *E. crus-galli*, while cluster-III had two accessions of *E. crus-galli* and rest ten were of *E. frumentacea* (Table 1).

The average intra- and inter-cluster genetic distance (d values) shown in Table 1. Inter-cluster centeroids distance ranged from 0.00 to 3.14 and Intra-cluster distance ranged from 1.76 to 1.9. Comparison of these morphological clusters revealed that maximum intra-cluster distance (D=2.25) was observed in cluster-II whereas, maximum inter cluster centeroids distance (D=3.41) was observed in between cluster-III and cluster-II (Table- 1). It has previously been suggested that genetic drift and selection in different environments can produce greater diversity (Raje and Rao, 2001). We observed that accessions of *E. crus-galli* showed genetic superiority to *E. frumentacea* in different agronomic characters. The findings get support by Bandyopadhyay (1998, 1999). He

Table 1. D^2 statistics* based clustering pattern and average inter and intra cluster d values of the different accessions of two different species of *Echinochloa*

Clusters	Accessions grouped in different clusters	d values of Clusters		
		I	II	III
I	RS-MB-1554, VRS-MB-1839, VRS-MB-886, VRS-MB-846, VRS-MB-1361, VRS-MB-1535, VRS-MB-1377, VRS-MB-871 **(n=8)**	1.76		
II	IEC-530, IEC-531, IEC-540, IEC-545, IEC-546, IEC-547, IEC-548, IEC-549, IEC-555, IEC-556, IEC-542, IEC-533, PRB-9404 **(n=13)**	3.40	2.25	
III	IEC-535, IEC-538, VRS-MB-1202, VRS-MB-893, VRS-MB-889, VRS-MB-1506, VRS-MB-882, VRS-MB-1546, VRS-MB-1508, VRS-MB-1543, VRS-MB-858, VRS-MB-1372 **(n=12)**	2.50	3.41	1.91

* The D^2 analysis was done according to Murthy and Arunachalam, 1996.
IEC and PRB: Accession number for the accessions of *E. crus-galli*, VRS-MB: Accession number for the accessions of *E. frumentacea*.

Table 2. Cluster mean for different morphological characters

S. No.	Characters	Clusters		
		I	II	III
1.	Germination (%)	89.93	91.49	92.69
2.	Days to 50% flowering.	71.92	83.65	67.47
3.	Plant height (cm)	173.25	135.40	186.56
4.	Number of fertile tillers	2.74	3.43	3.26
5.	Panicle length (cm)	10.76	15.66	14.54
6.	1000 seed weight (g)	3.51	4.26	2.88
7.	Days to maturity	123.71	128.62	116.94
8.	Yield / plant* (g)	116.00	206.00	125.00

* Data represents average of total grain weight of five plants from each accession.

reported that the accessions of *E. crus-galli* were better adapted for Uttarakhand hills and were promising for high grain yield. Average inter-cluster and intra-cluster distance (D) values presented in Table- 2 indicated that the maximum variability was present in cluster-II and cluster-III for agronomic characters. So the clusters showing greater genetic diversity can be used in further crop improvement programme. Several workers suggested selection of parents for hybridization from two clusters having wide inter-cluster and intra-cluster distance to get maximum heterosis (Pradhan and Rao, 1990; Mehta *et al*., 2005).

Molecular Diversity Based on RAPD Markers

In this study only 21 primers were used after a two-step selection of polymorphic primers. First, 50 random primers were prescreened with the four barnyard millet accessions VRS-MB 1508, VRS-MB 1546, IEC-530 and IEC-540. 21 primers revealed polymorphism and were used in further study. Polymorphic primers detected 7 to 15 bands with an average of 12 bands from each accession.

Genetic diversity among 33 accessions was calculated on the basis of DNA fingerprints generated with a set of 21 oligo-decamer primers using UPGMA method based on 'Dice' similarity coefficient. On the basis of Dice coefficient of similarity (Fig.1) among 33 genotypes, similarity ranged from 20.5 to 78.4. At 34% similarity two major groups are formed, which clearly separate 15 accessions of *E. crus-galli* and 18 accessions of *E. frumentacea*. At 48% similarity 8 clusters were formed and two genotypes remained ungrouped (Fig.1). Six clusters were formed within accessions of *E. crus-galli* and four were in *E. frumentacea*.

The marker system successfully resulted in classifying of the two species of barnyard millet as both the species grouped separately in two different groups (Fig.1). Within species variability revealed by RAPD marker was evident but diversity among accessions of same species was low. Similarity observed among accessions of *E. crus-galli* was up to 75 %, whereas accessions of *E. frumentacea* showed higher genetic similarity (upto 80%). This might be due to the collection of the accessions from a small area. Variability within species may be less due to high inbreeding nature of these crops (Yabuno, 1966). Similarly, Salimath *et al*., (1995) reported only 10% polymorphism in the 17 accessions of *Eleucine coracana* and explained the low polymorphism in finger millet because of high inbreeding nature of the crop. The low degree of variability in RAPD markers was observed within *E. frumentacea*, while high degree of morphological variability was observed in this

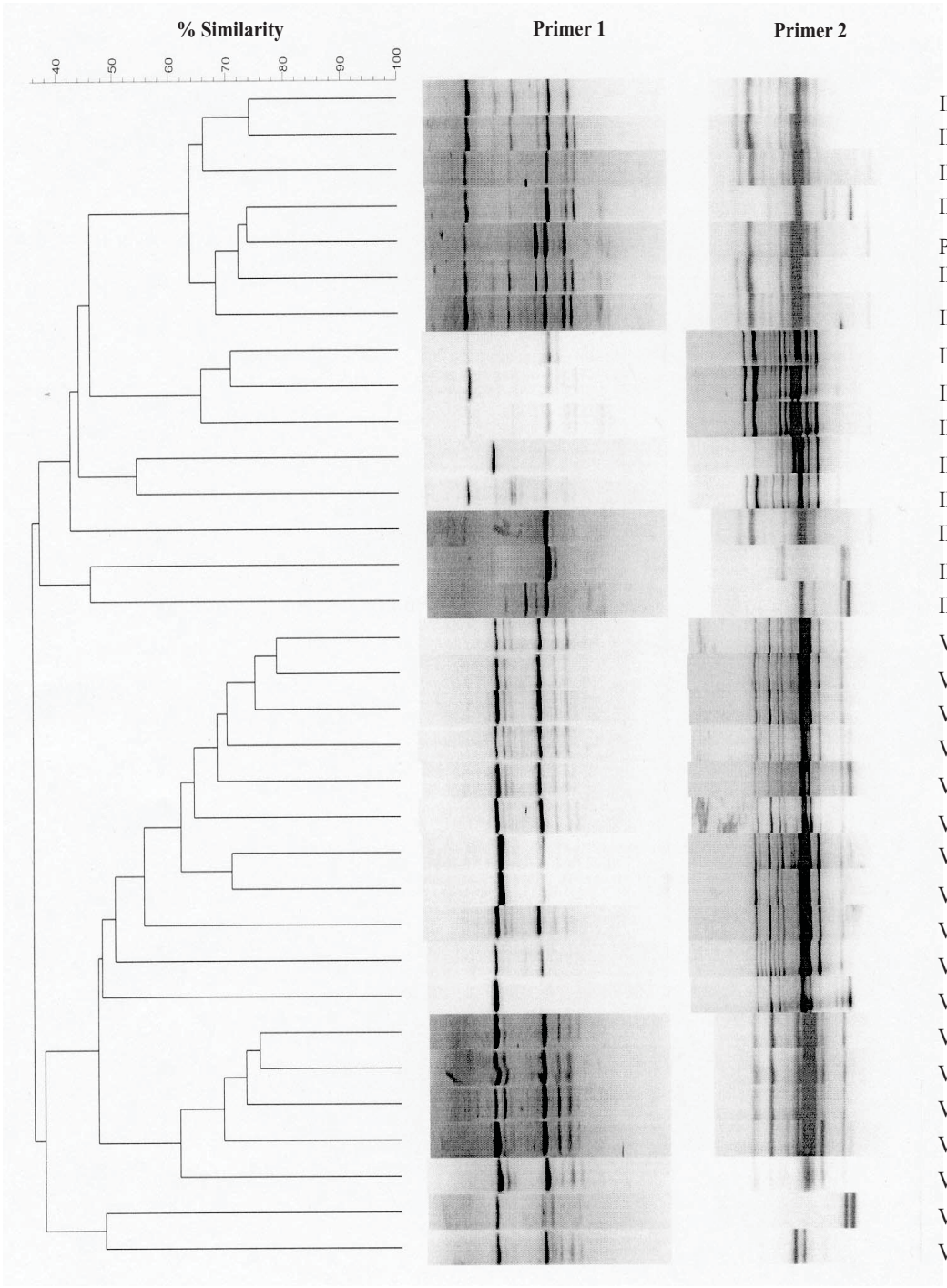

Fig. 1: Random amplified polymorphic DNA cluster analysis of fingerprint patterns generated with a set of 21 oligodecamer primers from genomic DNA of 33 accessions belonging to two cultivable species of Barnyard millet (*Echinochloa crus-galli* and *Echinochloa frumentacea)*

species. Crop domestication represents relatively recent (about 10,000 years) evolutionary process from a few wild populations, so low genetic variability within species of barnyard millet is not surprising (Hilu and Johnson, 1991).

Variability in RAPD marker was evident in both the domesticated species. The similarity index showed about 64% diversity between two species. The study was supported by Hilu (1994). Polymorphism in RAPD markers has been reported in several domesticated species of wheat (Devos and Gale, 1992; He *et al*., 1992) finger millet (Hilu,

1994), *Gossypium* (Rana and Bhat, 2005) and barnyard grass (Roy *et al*., 2000), etc.

The clear-cut segregation of the two species, *E. frumentacea and E. crus-galli* is in marked contrast within considerable overlap in morphological markers, where specimen identification can sometimes become difficult (De Wet *et al*., 1983). The two species differ in inflorescence morphology, spikelet size and texture, and presence of awns.

RAPD marker represents an efficient and inexpensive way to generate molecular data and have been used successfully in various taxonomic and phylogenetic studies (Wilkei *et al*., 1993; Kazan *et al*., 1993). RAPD marker was used for *Hordeum* phylogeny and as a result two main branches were identified at 20% similarity. One was containing sections of *Anisolepis* and *Hordeum* and the second containing sections *Criterion* and *Stenostachys*. Similarly, RAPD markers were more efficient than the ISSR marker with regards to polymorphism detection in *Jatropha curcas*, as 84.26% polymorphism was detected by RAPD marker as compared to 76.54% by ISSR markers (Gupta *et al.*, 2008).

Thus, the RAPD approach is particularly useful to assess genetic diversity in germplasm resources of agricultural crops at intra-specific and inter-specific levels. The molecular character provided by the RAPD approach outlined a pattern of genetic affinities among the species of *Echinochloa* that is well supported by molecular data from cytogenetic, isozymes, chloroplast and nuclear genomes (Yabuno, 1962, 1966; Roy *et al*., 2000). Morphological markers grouped the accessions in three clusters, but some overlapping was observed. RAPD bands, which could mark individual plants, subspecies and species, are very valuable for germplasm resource studies and plant breeding. Two species of barnyard millet are showing wide genetic diversity. Accessions of *E. crus-galli* showed more genetic diversity within species on the basis of morphological and molecular markers. Similar findings were reported by Tabacchi *et al*., (2006). They revealed a good correlation between AFLP and morphological traits in the classification of *Echinochloa* species present in Italian rice fields. Also they showed considerable genetic diversity.

Clear demarcation of inter and intra species diversity among 33 accessions of two species *E. frumentacea* and *E. crusgalli* was observed. When these results were compared with morphological markers we found that the set of 21 RAPD primers were able to differentiate the two species according to their plant hight, no. of fertile tillers, panicle length and 1000 seed weight (Table 3). All the four characters are the major characters in respect to fodder and grain yield. On the basis of yield accessions IEC-530, IEC-533 and PRB-9404 and on the basis of fodder yield accessions VRS-MB-1202, VRS-MB-889 and VRS-MB-1508 were found good. The accession showed both the characters up to optimum level should be selected as the cultivated land is decreasing we need the maximum output from the minimum input. Accession IEC-555 was alone in group 5; this accession shows the average value of all the studied morphological characters. IEC-555 was the best accession for yield as well as for fodder.

With such improvements, primers can be utilized to develop specific marker system to identify the species from the admixture. Selection of better genotypes can be made for species improvement on the basis of percent similarity with other species. Two more similar but

Table 3. Groups formed on the basis of RAPD are differentiated on the basis of morphological characters

Groups*	Germination (%)	50% Flowering (Days)	Plant height (cm)	No. of fertile tiller	Panicle length (cm)	1000-seed weight (g)	Days to maturity	Yield/ plant (kg)
1.	92.70	91.24	135.25	3.21	15.91	4.05	133.48	0.20
2.	90.55	81.11	141.67	2.97	16.79	4.83	126.55	0.18
3.	92.83	63.50	130.53	4.40	15.92	4.22	114.12	0.15
4.	89.00	76.33	134.67	4.60	15.10	3.30	122.67	0.15
5.	92.66	79.33	140.00	3.10	14.10	4.10	123.00	0.18
6.	94.33	64.00	156.00	3.60	14.23	3.47	112.00	0.11
7.	91.79	65.19	187.06	3.02	14.57	3.56	123.53	0.15
8.	90.00	73.33	220.00	2.63	14.60	1.42	126.00	0.12
9.	91.80	67.66	184.87	3.01	13.01	2.67	112.80	0.14
10.	92.17	74.83	171.83	2.99	11.79	3.22	127.67	0.12

Data shown in the table is average of triplicates. Accessions were grown in the field in the plots of 3m x 1m. Plant to plant distance was maintained 10 cm., while row to row distance was kept 22.5 cm. Crop was harvested after maturity.
*Groups were made at similarity of 48% according to Dice coefficient of similarity.
†Data represents average of total grain weight of five plants from each accession.

possessing distinct characters can be chosen for the purpose. Studies of the kind presented here are important in detailing the level of variation and relationship within and between species in order to plan future domestication trials and to manage the wild species collection, which is available in the gene banks.

Acknowledgment

We are thankful to Dr R Prasad, Dr Arun Kumar for providing seed material, Usha for assistance.

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