



Assessment of genetic diversity in Indian Barnyard millet (*Echinochloa* spp. complex) using morphological and molecular markers

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Abstract: In the present study, morphological and molecular markers (RAPD primers) were used to analyze the genetic diversity and genetic relationships among 21 accessions of *Echinochloa* spp. complex comprising the wild and cultivated species collected from Melghat and adjoining regions of Vidarbha, Maharashtra. The availability of diverse genetic resources is a prerequisite for genetic improvement of any crop including barnyard millet. A high degree of molecular diversity among the landraces was detected. Among the 21 genotypes, two major groups (A and B) were formed, at 67.28 % similarity, which clearly encompasses 15 accessions of *E. frumentacea* and 6 accessions of *E. colona*. Higher similarity was observed in accessions of *E. frumentacea*. The accessions IC 597322 and IC 597323 also IC 597302 and IC 597304 showed more than 94% similarity among themselves. The classification of genetic diversity has enabled clear-cut grouping of barnyard millet accessions into two morphological races (*E. frumentacea* and *E. colona*).

Keywords: Barnyard millet, Genetic diversity, Genetic relationship, RAPD marker

INTRODUCTION

Barnyard millet, *Echinochloa* sp. ($2n=36, 54$) is a self pollinating crop which belongs to family *Poaceae* and is grown as cereal or fodder crop. It is one of the oldest domesticated millets in the semi-arid tropics of Asia and Africa (Holm *et al.* 1977). The genus comprises approximately 50–60 species (Michael 1994) and includes important native species (Watson and Dallwitz 1992), species cultivated as fodder and grain crops, and significant weed species such as *E. crus-galli* (barnyard grass) and *E. colona* (awnless barnyard grass) (Holm *et al.* 1977). *Echinochloa utilis* is a temperate annual hexaploid ($2n=6x=54$) and is cultivated in Japan, Korea etc. *E. frumentacea* ($2n=36, 54$) is cultivated in India, Pakistan and Nepal (Yabuno, 1962). The wild ancestor of *Echinochloa frumentacea* is the tropical grass *Echinochloa colona* (L.) Link. Barnyard millet is the fastest growing crop among all millets and can be harvested in a short period of nine weeks. Its grains contain 11.2(g) protein, 10.1(g) crude fiber, 4.4(g) minerals, 15.2(mg) Iron, 11(mg) calcium per 100gm grain (MINI, India). Also it is a nutritive fodder for animals. Studies on nutrient composition, glycaemic index and health benefits of barnyard millets indicated that it is beneficial for type-II diabetes (Ugare *et al.*, 2011). The crop is valued for its drought tolerance, good yield and superior nutritional value. Study of biodiversity and available natural resources are essential for identifying genotypes having higher

yield potential without compromising on the nutritional front. Measurement of genetic diversity also allows the estimation of important population parameters e.g., characterization of the geographic structure or connectivity of populations (Gupta *et al.*, 2009). 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. One technique which can provide useful data for the comparison of plant types is random amplified polymorphic DNA (RAPD) analysis. This is a modified PCR technique involving the amplification of whole-plant DNA extracted from leaves or other plant organs. In the RAPD technique, multiple 10 base pair (bp) oligonucleotide primers are added each to an individual sample of DNA which is then subjected to PCR. This technique is sensitive, fast, requires the use of no radioactive probes, and is easily performed. In the present paper, an attempt has been made to assess the genetic diversity in Indian Barnyard millet (*Echinochloa frumentacea* (Roxb.) Link) and its progenitor wild species (*E. colona* (L.) Link) from Melghat Biosphere Reserve using morphological and molecular markers.

MATERIALS AND METHODS

Plant material: During a survey and exploration programme during 29th October - 5th November 2012, a total of 21 accessions of barnyard millet and their wild relatives were collected (Table 1). The passport data

(place of collection i.e. village, block, latitude, longitude, altitude) and vernacular name of the species collected are mentioned (Table 2). Two sets of different accessions of both the species (*E. frumentacea* and *E. colona*) were examined. 15 accessions of *E. frumentacea* and 6 accessions of *E. colona* were used for the study.

Morphological characters: All the 21 genotypes were sown in the fields in a Randomized Block Design (RBD) at ICAR-National Bureau of Plant Genetic Resources Regional Station, Dr.PDKV campus, Akola (Maharashtra) for two consecutive years 2012 and 2013. Crop management was done according to the recommended agronomic practices (N:P:K 40:20:0). Sowing was done in the plots (3m × 1m), plant to plant distance was maintained at 10 cm, while row to row distance was kept at 30.0 cm. Various morphological characters including, plant height, leaf length, leaf width, flag leaf length, flag leaf width, inflorescence length, inflorescence width, number of racemes / inflorescence, raceme length, color of inflorescence, days to maturity, inflorescence shape, inflorescence compactness, shape of lower raceme, length of peduncle, arrangement of spikelet, degree of branching, degree of lodging and 100 seed weight 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 Doyle and Doyle (1987, 1990) using CTAB with few modifications.

Measurement of DNA purity and quality: The quantity of DNA was measured using a UV Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Sambrook et al., 1989). The DNA concentration was determined by the formula: DNA concentration = OD₂₆₀ × 50 µg/ml × dilution factor (Linacero et al., 1998).

Screening of RAPD primers: Decamer oligonucleotides were used in this study. 20 RAPD primers (Table 3) were screened for polymorphism. Primers that gave clear and polymorphic profiles were chosen for further study.

DNA amplification of selected primers: DNA samples for PCR amplification were diluted to 30 ng/µl with T.E buffer. Twenty random decamer primers were used to study polymorphism. Out of these, 11 primers gave polymorphic bands and were used for further study.

The PCR amplifications were carried out in a 25 µl reaction mixture containing 30 ng of genomic DNA, 0.5µM primer, 1X PCR reaction buffer (Invitrogen), 1.5mM MgCl₂ (Fermentas), 0.2 mM dNTP mix (Fermentas) and 1 U Taq polymerase (Invitrogen). Amplifications were carried out in a thermal cycler (Veriti, Applied Biosystems), programmed for one initial denaturation 95°C for 5 min. followed

by 40 cycles of 92°C for 1 min., 26.8 to 42.4°C depending on primer for 1 min., 72°C for 2 min. and synthesis 72°C for 7 min.

PCR amplification products were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide solution (0.5 µg/ml) and run in TBE buffer at 70-80 Volts. A 1kb ladder plus maker (Fermentas) was used as a molecular weight standard. The documentation was done using Gel Doc system (Alpha innotech).

Data analysis : Field data for morphological characters was evaluated and represented graphically by plotting a graph of the various characters used for the study.

Molecular data was analyzed from the banding pattern of RAPD markers. The presence of band was scored from the photograph. Only clear and reproducible bands were given consideration. These bands were considered as polymorphic, when they were absent in some sample in frequency greater than 1% and change in band intensity were not considered as polymorphism. Clear bands were scored as present (1) or absent (0) at particular position or distance migrated on the gel. The data matrix of 1's and 0's been prepared from the scorable bands and was entered into the data analysis package.

The similarity index was used to calculate the genetic distance values and to construct the dendrogram. The dendrogram provides a visual representation of the differences in the population of various accessions. The NTSYS-pc software ver. 2.02 was used to estimate genetic similarities with the Jaccard's coefficient. The matrix of generated similarities was analyzed by the unweighted pair group method with arithmetic average (UPGMA), employing Sequential, Agglomerative, Hierarchical and Nested Clustering (SAHN) clustering module.

Size of fragments in base pairs was detected with the help of 1 Kb plus ladder (Table 5). Percent polymorphic loci (a locus is defined polymorphic when the frequency of the marker (allele) is <1.) was calculated using the formula:

$$\text{percent polymorphic loci} = \frac{\text{number of polymorphic bands}}{\text{total number of bands compared}} \times 100$$

RESULTS AND DISCUSSION

Estimation of genetic diversity based on morphological characters: During the present study, a wide range of diversity was observed between *E. frumentacea* and *E.colona* accessions. The data was analyzed by plotting a column chart of the two species against different morphological characters viz. plant height, leaf length, leaf width, flag leaf length, flag leaf width, inflorescence length, inflorescence width, number of racemes/ inflorescence, raceme length, color of inflorescence, days to maturity, inflorescence shape, inflorescence compactness, shape of lower raceme, length of peduncle, arrange-

Table 1. Diversity collected in barnyard millet and their wild relatives.

Crop / grass name	Botanical name	Biological status	Chromosome Number	CK*	DN**	BP***	Total
Barnyard millet	<i>Echinochloa frumentacea</i> Link.	Crop	2n=36,54	3	11	1	15
Jungle rice	<i>Echinochloa colona</i> (L.) Link.	Wild	2n=18,36,54	3	3	-	6

*CK- Chikhaldara, **DN- Dharni, ***BP- Balapur (adjoining Melghat)

Table 2. Passport data of *Echinochloa* species collected from Melghat Biosphere, Maharashtra.

S. N	Accession No.	Botanical Name	Vernacular Name	Village	Block	Latitude	Longitude	Altitude(ft)
1	IC 597287	<i>E.frumentacea</i>	Bhagar	Doma	CK	21° 33' 187''	77° 33' 369''	2010
2	IC 597298	<i>E.frumentacea</i>	Sawa	Rehetiakheda	CK	21° 39' 16''	77° 15' 776''	1045
3	IC 597302	<i>E.frumentacea</i>	Sawa	Kekdabad	DN	21° 37' 01''	76° 56' 44''	1001
4	IC 597304	<i>E.frumentacea</i>	Sawa	Kutanga	DN	21° 42' 162''	77° 05' 520''	1005
5	IC 597307	<i>E.frumentacea</i>	Safed Sawriya	Chatwavod	DN	21° 37' 644''	76° 58' 297''	1107
6	IC 597308	<i>E.frumentacea</i>	Lal Sawriya	Kekdabod	DN	21° 37' 490''	76° 56' 954''	1117
7	IC 597310	<i>E.frumentacea</i>	Sawa	Tembli	DN	21° 30' 738''	76° 54' 557''	1100
8	IC 597315	<i>E. colona</i>	Saweli ghas	Jutpani	DN	21° 30' 762''	76° 56' 071''	1000
9	IC 597320	<i>E. colona</i>	Bada Sawriya	Kara	DN	21° 30' 372''	77° 06' 224''	1102
10	IC 597322	<i>E.frumentacea</i>	Sawriya	Lawada	DN	21° 31' 961''	77° 01' 496''	1295
11	IC 597323	<i>E.frumentacea</i>	Safed Sawriya	Bod	DN	21° 31' 961''	77° 01' 496''	1295
12	IC 597324	<i>E.frumentacea</i>	Chota sawriya	Bod	DN	21° 31' 792''	76° 59' 445''	1209
13	IC 597329	<i>E.frumentacea</i>	Sawan	Tingriya	DN	21° 33' 280''	76° 52' 025'	1093
14	IC 597336	<i>E.frumentacea</i>	Sawriya	Jilpi	DN	21° 27' 974''	76° 48' 578''	1183
15	IC 597339	<i>E.frumentacea</i>	Bhagar	Patharpur	DN	21° 26' 595''	76° 50' 304''	1262
16	IC 597347	<i>E.frumentacea</i>	Sawa	Kamapur	CK	21° 24' 675''	77° 23' 414''	2935
17	D 2010-5	<i>E.frumentacea</i>	Bhagar	Bahadura	BP	20° 51' 279	76° 48' 281	879
18	IC 597289	<i>E. colona</i>	Saweli ghas	Baramacha	CK	21° 30' 095''	77° 31' 373''	2021
19	IC 597290	<i>E. colona</i>	Saweli ghas	Amjhari	CK	21° 25' 411''	77° 20' 642''	2005
20	IC 597292	<i>E. colona</i>	Saweli ghas	Bhawai	CK	21° 16' 330''	77° 23' 155''	2100
21	IC 597314	<i>E. colona</i>	Saweli ghas	Tembli	DN	21° 31' 497''	76° 54' 160''	1100

*Refers to Block (Chikhaldara-CK and Dharni- DN, BP- Balapur (adjoining Melghat)

ment of spikelet, degree of branching, degree of lodging, 100 seed weight etc. The data of different morphological characters is placed in Table 4 and Fig.2. The two cultivable species showed wide genetic diversity and narrow

relationship with each other based on morphological characters used in the study.

Molecular diversity based on RAPD markers: In this study 20 RAPD primers were employed, out of

Table 3. Code, sequence and nucleotide length of primers used in the RAPD analysis.

S.No.	Primer code	Primer sequence 5' to 3'	Nucleotide length	Tm °C	Ta °C
1	OPA1	CAGGCCCTTA	10-mers	35.2	30
2	OPA10	GTGATCGCAG	10-mers	29.8	26
3	OPA13	CAGCACCCAC	10-mers	34.8	29
4	OPA15	TTCCGAACCC	10-mers	38.7	34
5	OPA16	AGCCAGCGAA	10-mers	40.5	35
6	OPA17	GACCGCTTGT	10-mers	33.7	28
7	OPA18	AGGTGACCGT	10-mers	31.6	25
8	OPA19	CAAACGTCGG	10-mers	36.6	32
9	OPA2	TGCCGAGCTG	10-mers	42.4	37
10	OPA20	GTTGCGATCC	10-mers	34.2	29
11	OPA3	AGTCAGCCAC	10-mers	26.8	21
12	OPA4	AATCGGGCTG	10-mers	39.3	35
13	OPA5	AGGGGTCTTG	10-mers	31.8	25
14	OPA6	GGTCCCTGAC	10-mers	30.8	25
15	OPA8	GTGACGTAGG	10-mers	22.9	18
16	OPA9	GGGTAACGCC	10-mers	38.7	34
17	OPF1	ACGGATCCTG	10-mers	32.0	27
18	OPP10	TGCCGAGCTG	10-mers	34.0	29
19	OPP2	CCCCGGTAAG	10-mers	34.0	29
20	OPP7	GAAACGGGTG	10-mers	34.5	29

Tm, melting temperature ;Ta, annealing temperature ; OPA series, Sigma; OPF and OPP series, Genaxy

Table 4. Morpho-agronomic traits of *Echinochloa frumentacea* and *E. colona*.

S. No.	Morphological traits	<i>Echinochloa</i> spp.	
		<i>E. frumentacea</i>	<i>E. colona</i>
1	Plant height (cm)	70.38-115.88	22.5-67.5
2	Leaf length (cm)	18.07	15.7
3	Leaf width (cm)	0.65	0.6
4	Flag leaf length (cm)	15.6	6.2
5	Flag leaf width (cm)	1.0	0.25
6	Inflorescence length (cm)	10.39	6.75
7	Inflorescence width (cm)	1.8	0.3
8	Number of racemes/inflorescence	16.0	5.0-6.0
9	Raceme length (cm)	1.7	1.5
10	Colour of inflorescence	Green	Green
11	Days to maturity	78-98	50-60
12	Inflorescence shape	Elliptical	Straight
13	Inflorescence compactness	Intermediate	Open
14	Shape of lower raceme	Straight/slightly curved	Straight
15	Length of peduncle (cm)	10.3	11.0
16	Arrangement of spikelet	Round the axis	One side
17	Degree of branching	Low-medium-high	Low
18	Degree of lodging	Low	Intermediate
19	Seed shattering	Low	High
20	100 seed weight (g)	0.25-0.37	0.11-0.13
21	Seed colour	White	White

which 11 primers revealed polymorphism and were used for further analysis. Polymorphic primers detected 5 to 9 bands with an average of 7 bands from each accession (Table 5). Genetic diversity among 21 accessions was calculated on the basis of DNA fingerprints generated with a set of 11 oligo-decamer primers using UPGMA method based on 'Jaccard's' similarity coefficient. Based on the similarity index, genetic dis-

tance values were calculated and used to construct the dendrogram (Fig.1). Among the 21 genotypes, similarity ranged from 67.28 to 94.0%. Two major groups (A and B) were formed, at 67.28 % similarity, which clearly encompasses 15 accessions of *E. frumentacea* and 6 accessions of *E. colona* (Fig.1). Further, the group of *E. frumentacea* was divided into two major clusters (A₁ and A₂) also *E. colona* was divided into

Table 5. RAPD analysis for 21 Barnyard millet accessions using the following primers.

S.No.	Primer	Size of fragments(bp)	Total no. of fragments	Number of polymorphic fragments	Percent polymorphic loci
1	OPP2	200-1000	6	4	66.66
2	OPP10	200-700	5	4	80.00
3	OPP7	150-600	5	3	60.00
4	OPA13	200-1000	7	5	71.42
5	OPA4	200-900	9	8	88.88
6	OPA3	100-600	6	4	66.66
7	OPA6	150-700	6	5	83.33
8	OPA5	200-850	5	3	60.00
9	OPA2	200-1000	5	4	80.00
10	OPA18	150-900	7	6	85.71
11	OPF1	100-600	6	4	66.66
Total			67	50	74.62

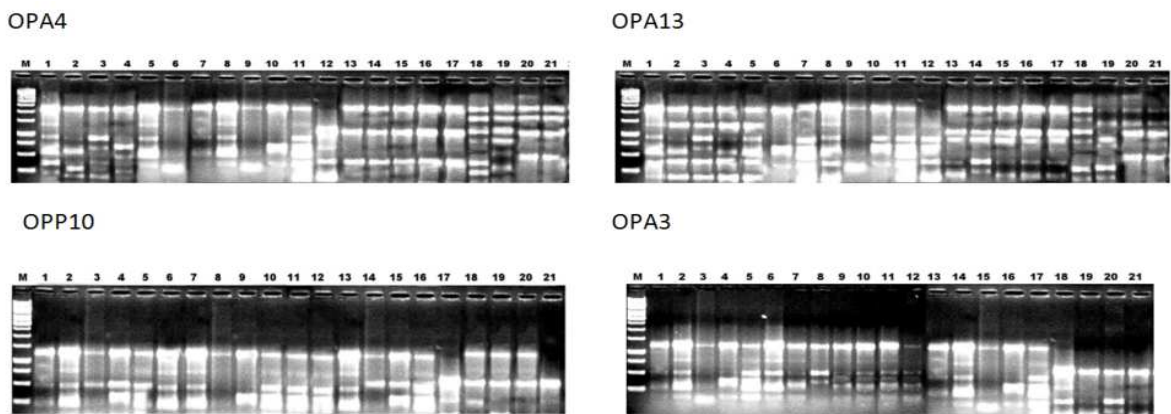


Plate 1. (a) Barnyard millet (*Echinochloa frumentacea* (Roxb.) Link.) (b) Amplification profile of 21 Barnyard millet accessions using RAPD primers, M= DNA ruler plus, numbers on top are accessions as given in Table 2.

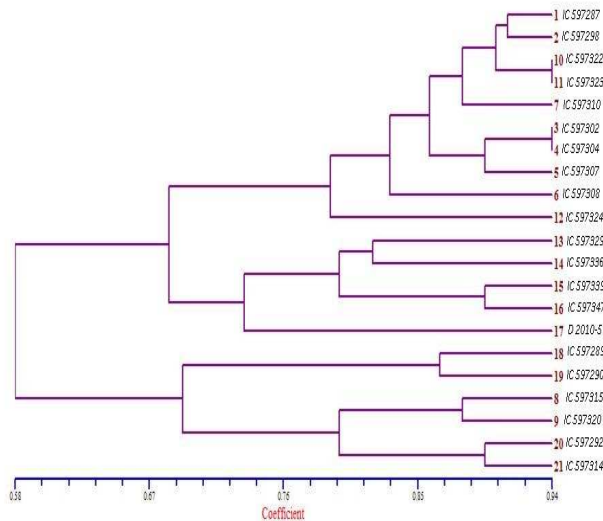


Fig. 1: Random amplified polymorphic DNA cluster analysis of fingerprint patterns generated with a set of 11 oligodecamer primers from genomic DNA of 21 accessions belonging to two cultivable species of Barnyard millet (*Echinochloa colona* and *Echinochloa frumentacea*) on the basis of banding pattern. The unweighted pair-group method, arithmetic mean (UPGMA) algorithm was applied using computer software NTSYS-pc software ver. 2.02.

two major clusters (B₁ and B₂). Higher similarity was observed in accessions of *E. frumentacea*, i.e. up to 94%. The accessions IC 597322 and IC 597323 also IC 597302 and IC 597304 showed more than 94% similarity among themselves. Greater similarity among *E. frumentacea* may be due to repeated cultivation of same genotypes year after year, inbreeding nature of the crop and/or due to the collection of the accessions from a small area.

Similarity observed among accessions of *E. colona* was less than that of *E. frumentacea* i.e up to 88.8 %. This clearly indicates the wild biological nature of the species. The similarity index showed about 32.8% diversity between two species. Polymorphism in RAPD markers has been reported in barnyard grass (*Echinochloa crusgalli*) (Roy *et al.*, 2000).

The two species *E. frumentacea* and *E. colona* differ in inflorescence morphology, plant height, leaf length, leaf width, flag leaf length, flag leaf width, inflorescence length, inflorescence width, number of racemes / inflorescence, raceme length, colour of inflorescence, days to maturity, inflorescence shape, inflorescence compactness, shape of lower raceme, length of peduncle, arrangement of spikelet, degree of branching and degree of lodging etc.

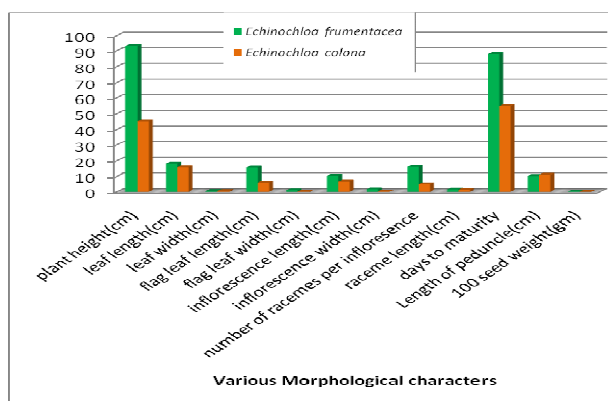


Fig. 2. Comparison of morphological characters between the two species.

The classification of genetic diversity has enabled clear-cut grouping of barnyard millet accessions into two morphological races. Two species of barnyard millet are showing wide genetic diversity. Accessions of *E. colona* showed more genetic diversity within species on the basis of RAPD molecular markers. Also some accessions of *E. frumentacea* showed more than 90% similarity, which allows breeders and farmers for precise selection of better genotype for cultivation. The genetic similarity of the *E. frumentacea* and *E. colona* samples correlated with their close geographic locations. This phenomenon was also observed in the morphological characteristics of barnyard grass (*Echinochloa crus-galli*) in Indonesia (Tasrif, et al., 2003).

Studies on genetic diversity analysis are important in detailing the level of variation and relationship within and between species depending on their geographical location as small millet species are important in view of food and fodder requirement of the native tribal people.

The study can also prove helpful to the farmers in selecting cultivars for greater yields in diverse agronomic conditions. In the context of increasing demand of nutritional crops and ever increasing cases of diabetic and other metabolic disorders, the need of the hour is to make millet farming more lucrative and popular. Melghat biosphere reserve is a rich source of genetic diversity of several millets. Small millet species are important in view of food and fodder requirement of the native Korku and Gond tribal people. Barnyard millet is of high importance in tribal people due to its drought tolerance, good yield and superior nutritional value. Also efforts are to be made in multi-location evaluation of barnyard millet germplasm for high yielding genotypes along with its nutritive characterisation from diverse geographic locations for its proper utilization in crop improvement programmes.

Conclusion

A high degree of molecular diversity among the land-races was detected for barnyard millet. Among the 21 genotypes, two major groups (A and B) were formed,

at 67.28 % similarity, which clearly encompassed 15 accessions of *E. frumentacea* and 6 accessions of *E. colona*. Higher similarity was observed in accessions of *E. frumentacea*. The accessions IC 597322 and IC 597323 also IC 597302 and IC 597304 showed more than 94% similarity among themselves. It enabled clear-cut grouping of barnyard millet accessions into two morphological races (*E. frumentacea* and *E. colona*). The study may be helpful to the farmers in selecting millet cultivars for greater yields in diverse agronomic conditions.

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