BAR CODING AND GENETIC DIVERSITY ANALYSIS OF MILLET CROP SPECIES OF ELUSINE GENUS USING RAPD MARKERS

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ABSTRACT:

Aim: Millets are a group of highly variable small seeded grasses, grown around the world as cereal crops or grains for fodder and human food. In this study, screening of crop primers with 8 genotypes of millet crop species belonging to genus *Panicum, Eleusine, Echinochloa* and *Brachiaria* was done. Main objective was to understand the genetic diversity among 7 millet crops varieties and a wild grass using RAPD markers by targeting nuclear gene. To find out the relationship between species of the same genus based on RAPD markers and to construct phylogenetic tree using PyElph 1.4 and Past326b softwares.

Methodology: PCR analysis was done using the selected RAPD to identify the polymorphic loci between the 8 genotypes. Primers OPC06, OPD13, OPW04, OPC18 were selected for analysis based on the band patterns, for further RAPD analysis. Bar Coding and Sequencing was done for validation of species with the help of softwares BLAST and Clustal OMEGA. Phylogenetic tree was constructed using all 4 primers with the help of software tools PyElph 1.4 and Past326b. The point parameter, which guided the joining linkage rule, is unweighted pair group average (UPGMA) and the genetic distance was computed from raw data.

Outcome: Among the 4 primers used, OPW04 and OPD13 showed maximum scoring of bands based on the phylogeny were established between the crop species of *Eleusine* species and the

Indian Goose grass. The dendrogram showed that the varieties could be grouped into clusters. The sample C (*Elusine indica*), D (*Elusine coracana*) is diverged from the same root and C, D and G (Indian goose grass) are from the same root. Sample G is found to be very closely related to the C and D.

Keywords: genetic diversity, phylogeny, RAPD analysis, grass species

INTRODUCTION:

Millets are a group of cereal crops which acts as beneficial human food. Millets are important crops in the semiarid tropics of Asia and Africa, with 97% of millet production in developing countries [1] [2]. The crop is favored due to its productivity and short growing season under dry and high temperature conditions. The members of family Poaceae belong to different subfamilies. The most commonly cultivated millets are *Eleusine coracana, Eleusine indica* and *Panicum* species. *Eleusine* is a predominantly African genus with seven of its nine species confined to tropical Africa. The genus can be divided into two separate groups of related species on the basis of annual and perennial habit. Apart from the highly successful pan tropical weed *E. indica* L. and the crop plant *E. coracana* L. the species have very limited distributions most being localized in parts of East. Most of Eleusine species are annual grasses used as ornament and enclosed within a thin membranous pericarp [3]. *Eleusine indica,* the Indian goose grass is a species of grass in the family poaceae. It is a small annual grass distributed throughout the warmer areas of the world to about 50 degrees latitude. It is an invasive species in some areas and is closely related to *Eleuisne coracana*. *Eleuisne coracana* or finger millet is an annual herbaceous plant widely grown as a cereal crop in the arid and semiarid areas in Africa and Asia. It is a tetraploid and self-pollinating species probably

evolved from its relative *Eleusine africana* [4] [5].

Millets have been utilized for human food from prehistoric times. Some studies believe that millets were the first cultivated crops and they are said to have been used in China, India and Egypt before there were written records. Proso or bread millet was grown by the Swiss stone age dwellers and has been extensively cultivated throughout Crimea, Turkestan, Mongolia, Manchuria, Tibet and Siberia as a bread grain up to recent times. This crop was also utilized for food in Europe during medieval times but has been gradually superseded by wheat, rye, rice, maize and potatoes. The switch to other cereals is attributed to the recent demand for yeast-fermented bread and higher yields obtainable from the more improved small grains. Ragi or finger millet was cultivated from the earliest times for food and beverage in India and Central Africa. The expanded use of this cereal may have been relatively recent and associated with the intensified cultivation of marginal lands particularly in the hotter, semi-arid areas.Other work related to this subject has been published more recently by the Central Food Technological Research Institute at Mysore and Shri Avinashi lingam Home Science College at Coimbatore in India. These results and other experiences indicate that millets can be used for preparing a large number of highly palatable and nutritious foods.

RAPD stands for Random Amplification of Polymorphic DNA. Its resolving power is much lower than targeted, species-specific DNA comparison methods like short tandem repeats. In recent years, RAPD has been used to characterize and trace the phylogeny of diverse plant and animal species. All RAPD markers are dominant i.e. it is not possible to distinguish whether a DNA segment is amplified from the locus that is heterozygous or homozygous [6]. It is a PCR

based technique for identifying genetic variation. Earlier studies have shown that Neighbor joining tree methods through ITS barcoding could successfully identify samples [7]. The secondary structures of the ITS2 region provide dimensionality for species identification. Two-dimensional images will be always better and easier for identification. Previous studies on DNA barcoding concentrated more on the same family, genus or species. However, an ideal barcode should be variable enough to identify closely related species. Meanwhile, the barcodes should also be conservative in identifying distantly related species [8].

The objective of our study is to find the genetic diversity among the millet crop varieties and one wild grass sample through constructing Dendrogram. The identified 4 primers were screened against 8 genotypes accessions with a recommended PCR thermal profile.

MATERIALS AND METHODS:

Sample collection:

The experimental method comprised of a set of 7 genotype varieties. All the samples for genome extraction was collected from the field regions including Ramnagara of Bengaluru and linked borders of Tumkur. **Table 1** depicts the samples taken for study and their respective codings.

Table 1: Shows the samples taken and codes given to the samples

Sl.No.	Common Name	Sample code

1.	Proso millet	PM01 (A)
2.	Little millet	LM02 (B)
3.	Finger millet	FM03 (C)
4.	Finger millet	FM04 (D)
5.	Brown top millet	BM05 (E)
6.	Barnyard millet	BM06 (F)
7.	Indian Goose grass	EIB (G)

Genomic DNA extraction:

Figure 1: Collected samples- washed in salt solution and dried for extraction process

Plant samples can be prepared by cryogenically grinding tissue in a mortar and pestle after chilling in liquid nitrogen. Freeze dried plants can be ground at room temperature. In either case, a fine powder is best for extracting DNA. For each 100 mg homogenized tissue 500 µl of CTAB extraction buffer was added followed by thorough vortexing it was incubated at 60°C for 30 minutes. Following the incubation period, homogenate was centrifuged and equal volume of chloroform/isoamyl alcohol (24:1) was added. Then the mixture was centrifuged for

10 mins at 14,000 x g to separate the phases. The aqueous upper phase was carefully transferred to a new tube. Precipitated the DNA by adding 0.7 ml cold isopropanol and incubated at -20°C for overnight. This solution was passed through DNA binding columns, washed and eluted purified form of genomic DNA. 1 µl of RNase solution A was added and incubated at 37°C for 30 minutes to get rid of RNA contamination.

Quantity check of DNA:

2µl of the DNA solution was mixed with 2µl of Bromo phenol dye loaded on gel. 0.8 % agarose solution in 1 X TAE buffer was prepared and Ethidium bromide solution (0.1 g/ml) was added to visualize DNA bands. The gel slab was observed under UV light to see DNA concentrations, which was calculated based on standard ladder (1kb). A zigzag pattern of a single band near the well indicated intact genomic DNA. This DNA was further taken for amplification process [9].

PCR analysis:

PCR analysis was done using RAPD technique to identify the polymorphic loci between the 8 genotypes. Based on the concentration of DNA during gel electrophoresis reaction mixture for 25 µl prepared as shown in **Table 2**.PCR program was set: Initial denaturation 94°C for 5 minutes, Final denaturation 94°C for 1 minute, Annealing 30°C for 1 minute, Elongation 72 °C for 2 minutes, repeated the steps 2, 3 and 4 for 30 cycles and Final elongation was kept at 72 °C for 8 minutes. Once the program is done the reaction mixture was mixed with dye and subjected to gel electrophoresis. This gel image is futher used for construction of phylogenetic tree and taxon sampling using different softwares.

Table 2: PCR reaction mixture data for single primer

Sample	Specimen	DNA (µl)	Primer(µl)	PCR Master mix(µl)	Water(µl)
1	Proso millet	1	1	12.5	10.5
2	Little millet	5	1	12.5	6.5
3	Finger millet 1	5	1	12.5	6.5
4	Finger millet 2	5	1	12.5	6.5
5	Brown top millet	5	1	12.5	6.5
6	Barnyard millet	5	1	12.5	6.5
B	Goose grass	2	1	12.5	9.5

Genetic diversity analysis:

All genotypes were scored for the presence and absence of the RAPD bands. The data entered into a binary matrix as discrete variable, 1 representing present band and 0 representing absence of the band. It was generated by software PyElph 1.4. Thereafter, this data matrix was subjected to further analysis. The 0/1 matrix was used to construct dendrogram using UPGMA of Past326b computer software to infer genetic relationships and phylogeny.

Statistical analysis:

Analysis was conducted on Past326b Software tool. The programme used was cluster analysis joining (tree clustering) with raw input data of each population separately. The main parameter which guided the joining process is linkage rule, unweighted pair group average (UPGMA) and the genetic distance was computed from raw data. In unweighted pair group the average distance between two clusters is calculated as the average distance between all pairs of objects in the two different clusters. The grass family includes all the major cereals and most of the minor grains [10]. The phylogeny of the family helped in increasingly the knowledge about their evolutionary relationship. In the present study, 7 genotypes from Karnataka were subjected to diversity analysis based on variation in RAPD molecular profiles. DNA bands

were scored for DNA fingerprinting analysis with the RAPD profile generated using 4 RAPD primers. The results obtained were observed for 4 primers which is used for analysis.

Sanger sequencing:

Identification of species is very much important while carrying out any studies. So all 7 genotypes was bar coded using Sanger sequencing technique with ITS02 nuclear gene. The internal transcribed spacer (ITS) region belonging to the nuclear genome is a non-functional RNA sequence located between18S and 25S rRNA coding regions. The ITS1 is present between 18S and 5.8S rRNA and *ITS*2 is present between 5.8S and 25S rRNA. ITS is the transcriptional unit situated between the structural ribosomal RNAs during rRNA maturation, the ITS spacers are excised and non-functional maturation products are rapidly degraded. The total length is around 700 bp including the 5.8S rRNA region, which has constant length of 163 or 164 bp. Similar to the RAPD PCR mixture, using ITS02 primers Sanger sequencing reaction mixture was made as in **table 3**.

Table 3: PCR reaction mixture for Sanger sequencing with ITS02 primer.

Sl.No	COMPONENTS	CONCENTRATIONS
1	Forward primer	10pM
2	DNA template	200-300ng
3	Big dye terminator(BDT)	0.5 µL of each dNTP´S and ddNTP´S
4	10X buffer	2 µl
5	Nuclease free water	Make up to 10 µl

The PCR program used was Initial denaturation 95°C for 2 minutes, Final denaturation 95°C for 30 seconds, Annealing 55°C for 30 seconds, Elongation 72 °C for 1 minute, Repeat steps 2,3 and 4 for 30 cycles and Final Elongation 72 °C for 10 minutes. Further the amplified DNA fragment was purified using gel purification method. Obtained purified DNA was sequenced

by Sanger sequencing using genetic analyzer. The .ab1 files were analyzed with chromatogram peaks in Finch TV and using Seq Scanner .ab1 files were converted to fasta and PDF files for further analysis. BLAST was used for comparing primary biological sequence information, such as the nucleotides of DNA sequences. It helped to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. The obtained 10 hits with less E value and maximum identity and coverage were selected for phylogenetic analysis.

RESULTS AND DISCUSSION:

The gel pattern obtained indicated that the *Eleusine* species were clearly separated and scattered throughout the dendrogram, which might indicate an implemented sorting of the *Eleusine* species. RAPD analysis gave transparent results with primers OPC06 and OPC18 (**figure 2**). Both the Gel plates showed clear bands with high polymorphism, which show that the primer tagged sequences are located on different loci in each species. However, fewer similarities were also seen in band matching with software PyElph 1.4. the diversity analysis was done using the binary matrix generated by the same software. This matrix was used to construct a Dendrogram with the help of software Past326b to show the diversity among species collected.

Eleusine genus belongs to a relatively small genus but the classification of the genus has been difficult, not only at the intra-generic level, where considerable disagreements on species delimitation and their relationships have persisted [11]. Hence, these findings could be of value towards a better characterization of the genus. These findings are in agreement with a comparative study performed by Salimath et al. [12]. *Elusine* genus showed close relation to the wild grass (Indian Goose grass). Sample EIB was identified as *Elusine indica* although it

was a wild species and not preferred as edible by the locals. This showed the diversity among the species. Since the two crop species and the wild grass species emerged from the clade they pose the similar genetic composition and traits but not considered as a food source.

The first report involving molecular diversity analysis of a large set of little millet genotypes also showed grouping of the genotypes according to their collection centers reflects the presence of a higher level of genetic diversity among genotypes collected from geographically distinct areas. The overall genetic diversity of a taxon has great implications for its long-term survival and evolution. Therefore, the knowledge of the degree and pattern of genetic diversity is important for designing conservation strategies [13]. Knowledge of genetic diversity and potential heterotic relationships among parental lines is of significant importance in hybrid breeding programs [14]. Thus, in future breeding can be done between these two plants to gain the crop with longevity and efficient growing trait of wild grass species.

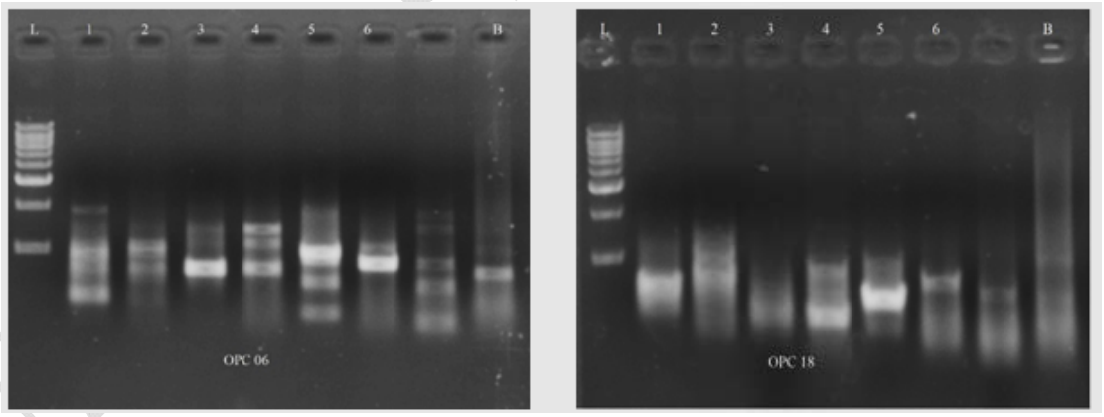

Figure 2: RAPD profiles of varieties obtained with the RAPD Primer OPC 06 and OPC 18 [L=Marker (100bp ladder); Lane no: 1- 6, B = SAMPLES]

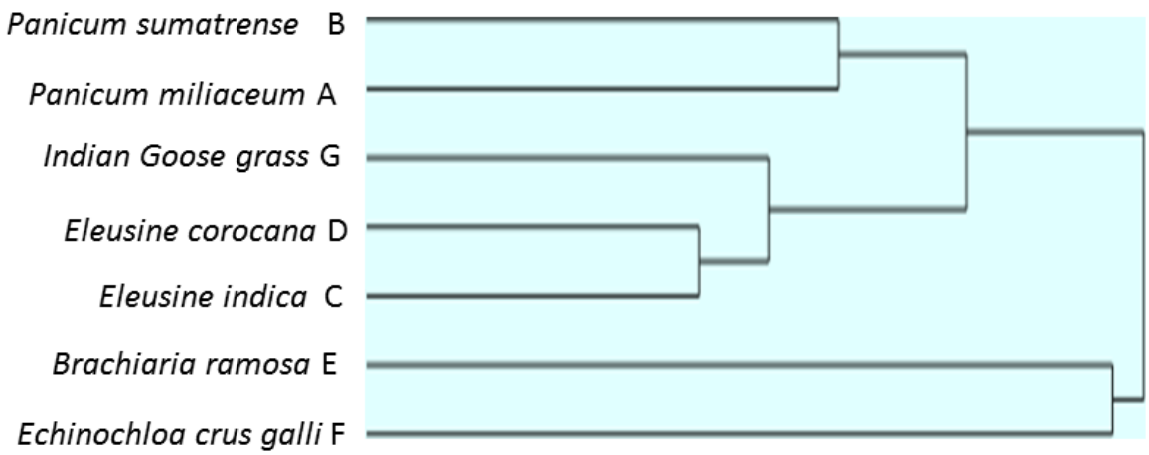

Figure 3: Dendrogram showing clustering of millet varieties constructed using UPGMA based on Euclidean distance obtained from RAPD analysis.

On the other hand, authenticity of the samples taken for study was done using Sanger sequencing method. ITS gene was amplified and the sequence obtained was subjected to nucleotide BLAST, where query samples were searched for similar hits already present in database. The obtained .AB1 files from sequencer were analysed for individual nucleotide bases and peaks using Finch TV software (**figure 4**); and further converted to fasta and pdf formats for data analysis. These chromatograms are the graphical result of Automated Sanger DNA Sequencing. Chromatogram showed variable peaks and highly bound nucleotides. Queries were identified and similarity was noted as listed in **table 4**. The top 10 hits in BLAST were taken further and phylogenetic tree was constructed using Clustal Omega along with our query sequences (**figure 5**). Barcoding tools give authentication to any plant species and can support conservation measures of the plants in several ways. Species delimitation and identification is the first critical step in an accurate assessment of distribution, population

abundance and threats of target species [15] [16]. Barcoding can speed up the identification process of collections, including sterile material and increase knowledge on species distributions and abundance.

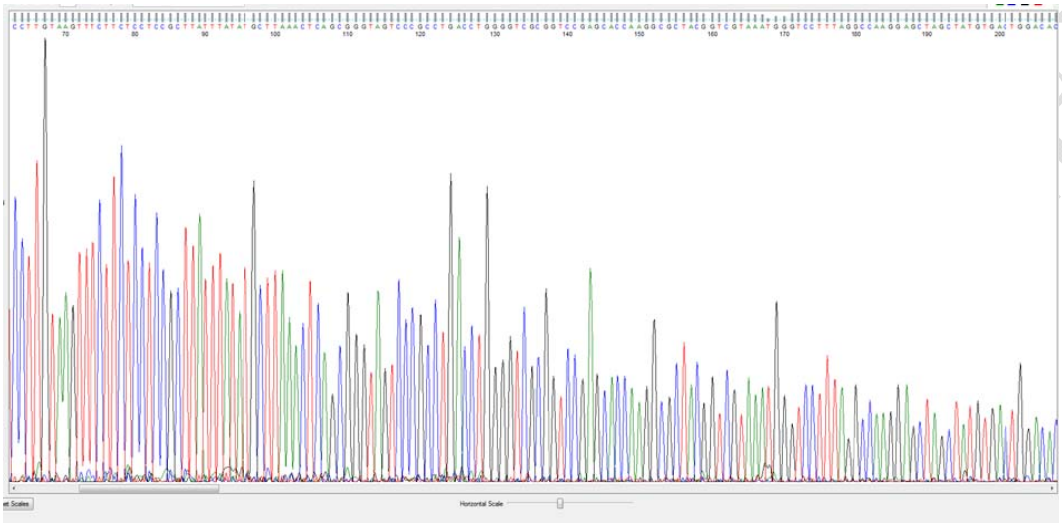

Figure 4: Representative Electropherogram generated by Finch TV showing trace data from Sanger Sequencing.

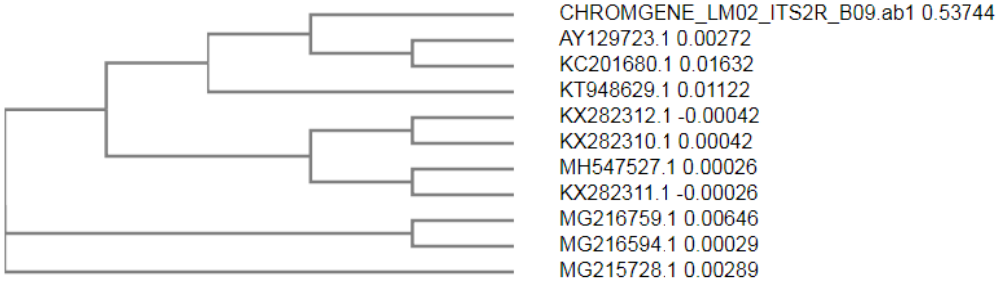

Figure 5: Clustal Omega Phylogenetic tree of query sequence showing the closest related or identical species. LM02 (Little Millet) was found identical to *Panicum sumatrense*.

Table 4: Millets crops species as identified by the BLAST program.

Sl No.	Plant Name	Common Name	Sample

			code
1	*Panicum miliaceum*	Proso Millet	PM01
2	*Panicum sumatrense*	Little Millet	LM02
3	*Elusine indica*	Finger Millet (var 1)	FM03
4	*Elusine corcana*	Finger Millet (var 2)	FM04
5	*Brachiaria ramosa*	Brown Top Millet	BM05
6	*Echinochloa crus- galli*	Barnyard Millet	BM06
7	*Elusine indica*	Indian Goose grass	EIB

CONCLUSION:

The present study indicated that they can be used to assess genetic diversity of millets accessions. These traits of economic importance may be used for genetic potential in the improvement programs of millets landraces. Cluster analysis has proved to be an effective method in grouping millets accessions from different parts of Karnataka that may facilitate the conservation, management, and utilization of plant genetic resources by selecting accessions with good economic traits.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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