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GENETIC DIVERSITY ANALYSIS OF MILLET CROP SPECIES OF PANICUM GENUS USING RAPD MARKERS

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ABSTRACT

Millets are a group of diversified crops and highly nutritional. Random amplified polymorphic DNA (RAPD) was evaluated as a source of genetic markers for studying variation among species of *Panicum.* Screening of crop primers with 7 genotypes of millet crops species belonging to various genus was done. Four primers OPC06, OPC18, OPD13 and OPW04 were selected for PCR based analysis of these RAPD markers. Gel electrophoresis documentation was used for scoring of binary matrices by software PyElph 1.4. Further, the binary scores were analyzed by PAST 325 software to construct the dendrogram based on Euclidean distance. The primers OPC06 and OPD13 showed the highest polymorphism (83%) and maximum scoring of bands based on which phylogeny was established between the crop variants of *Panicum* species and the wild natal buffalo grass. Wild grass roots from the different clade and distant relative of millet crops of Panicum genus, showing its diverse genetic makeup. Natal buffalo grass is considered as millet but not included in crops and cereals, because of the evolutionary changes and environmental conditions.

KEYWORDS: genetic diversity, polymorphism index, RAPD markers.

INTRODUCTION

Millet is an important group of seeded cereal crops. Millets are among the member of grass family and native of mostly Africa and Asia. Millets are well known for their adaptability to agro-ecological environments in the world.[1] *Panicum miliaceum* is a grain crop with many common names including proso millet, broom corn millet, common millet, hog millet,

kashfi millet, red millet and white millet. Proso millet is efficiently drought-resistant, which is seen in regions with low water availability and longer periods with no rain.[2][3][4] Little millet appears close to proso but is generally shorter, has smaller panicles and seeds, and is grown on a low scale voluntarily or with minimum care on less fertile lands.[5][6] Less genetic diversity occurs in the world collections of this species than appears among the other species. Perhaps very little of this species is grown in places other than that of India.[7] *Eleusine indica*, the Indian goose grass, yard-grass, goose grass, wiregrass, or crowfoot grass, is a species of grass belonging to family Poaceae. Though usually considered an annual, it may survive for more than a year in climates which is not subjected to frost.[8]

Finger millet or ragi has a relatively wide range of adaptation within moderate temperatures and moisture ranges. It is grown mainly in the South in Karnataka and Tamil Nadu where approximately two-thirds of the two-million-ton annual crop is produced. Brown top millet, a native of India, has relatively limited cultivation. In the United States as much as 100,000 acres are grown annually chiefly in Georgia, Florida and Alabama for hay and pasture; while the seed provides feed for quail, doves and other game birds. Even with broadcasting method, the crop yields about 7 to 8 quintal grains per acre and four tractor loads of good quality fodder which cattle savor.[9][10][11]

Echinochloa crus-galli is a type of wild grass originating from tropical Asia that was formerly classified as a type of panicum grass. Individual plants can produce up to 40,000 seeds per year. Water, birds, insects, machinery and animal feet disperse it, but contaminated seed is probably the most common dispersal method.[12] [13] One of the consequences of low genetic variability could be the inability to cope with abiotic and biotic stresses. From the growing knowledge on the genome sequences of organisms it becomes evident that all forms of diversity have their origin at genetic level. Genetic diversity analysis provides vital and powerful data that helps for a better understanding of genetic variation and improved conservation strategies.[14]

Thus, this study involves analysis of genetic diversity among 7 genotypes of millet crops of family Gramineae, belonging to various genuses in order to provide better knowledge for breeding programs in future studies. By determining and calculating Polymorphic Index Content (PIC) using software PIC Calc, and to understand the genetic diversity among 7 millet crops varieties and a wild grass using RAPD markers by targeting nuclear gene has

been done through this study. Construction of phylogenetic trees to see the relation among species of the same genus based on RAPD markers has been recorded.

MATERIALS AND METHODS

Sample collection

The Millet crop samples were procured from 3 different local field regions including Bidadi (Ramnagara) of Bengaluru and linked borders Tumkur (Arkere). The sample collection was done in the first week of February 2019 and temperature recorded was around 28^0C-33^0C with humidity of 54%. Leaves collected were stored at -20^0C and used as required. DNA isolation was done from leaves of the collected plants after washing and drying.

Isolation of DNA

Leaf tissues (100 mg) were homogenized in 500 µl of CTAB Extraction Buffer (2% Cetyl trimethylammonium bromide, 1% Polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, or CTAB Extraction Buffer). The homogenate was incubated at 60°C for 30 minutes, followed by centrifugation (14,000 x g) for 5 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) was added, vortexed and then centrifuged (14,000 x g) for 10 minutes, to separate the phases. The aqueous upper phase was used to precipitate the DNA by adding 0.7 ml cold isopropanol and incubate at -20°C for overnight. The pellets were pooled out and washed with ice cold 70% ethanol. Ethanol was decanted and residual ethanol was removed by drying in a SpeedVac. Dry the pellet long enough to remove alcohol. DNA was dissolved in TE buffer and then treated with RNase solution A in order to remove any possible contamination of RNA and incubate at 37°C for 30 minutes. The quantity and purity of DNA was confirmed by electrophoresis, using agarose (0.8%) gel. DNA concentration can be calculated based on standard ladder which was 1Kb. Column purification was done using spin column-based tube (cat. No. 69702) with a solid phase of silica layer (0.45 µm pore size) to get rid of salt contamination.

Random Amplified Polymorphic DNA (RAPD) Analysis

A total of four primers (OPW04, OPC06, OPC18 and OPD13) (**Table 2**) were used for RAPD analysis. The primers were procured from Genei Laboratories Pvt. Ltd, Bangalore, India. The PCR amplifications were performed in a Eppendorf Mastercycler Gradient (Germany) and carried out in a final volume of 25µl in a reaction mixture containing 2 µl (20-40 ng) of template DNA, 30 pm of random primer, 2.5 Mm dNTPs each, 1 PCR assay buffer (containing 100 mM KCl, 20 mM Tris-HCl (pH 8), 1 mM DTT, 0.5% Igepal, and 0.5%

Tween 20, 50% glycerol, 100 mM MgCl2), 0.5 unit of DNA Taq polymerase (Genei Laboratories Pvt. Ltd, Bangalore, India). The PCR program consisted of 1 cycle of 5min at 94 ºC, 29 cycles of 1 min at 94 ºC, 1 min at 40 ºC, 2 min at 72 ºC, and a final extension of 6 min at 72 ºC, the temperature was ultimately kept at 16^0C. The PCR products were separated on 2% agarose gel (Sigma, St. Louis, MO) and stained with ethidium bromide (0.001%), and the number of bands was recorded using a gel documentation system. Standard ladder used was 100bp.

Final gel electrophoresis is done which is used for construction of dendrogram and taxon sampling. The tree was computed based on the information extracted from the gel image. The genetic distances are also displayed on the branches. The distance matrix corresponding to the gel image was computed and saved using PyElph. Further, the binary score value was used by PAST 325 software and PIC calc to construct the final dendrogram showing diversity among all species with all primers and polymorphism, respectively.

Table 1: Annealing temperature and sequences of all primers taken for study.

PRIMERS	ANNEALING TEMPERATURE	PRIMER SEQUENCES
OPW 04	27°C	5’CAGAAGCGGA 3’
OPC 06	25°C	5’GAACGGACTC 3’
OPC 18	25°C	5’TGAGTGGGTG 3’
OPD 13	27°C	5’GGGGTGAGA 3’

Polymorphic information content

Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Marker systems fluctuate in their information content, which vastly depends on polymorphism. The concept of polymorphism is used to define genetic variation in a population, which has been significantly studied in recent years by several established scientific restraints, for example, genetics, ecology, zoology, and microbiology.[15][16][17] There are two processes of the quality of a polymorphism as a genetic marker: heterozygosity (H) and polymorphic information content (PIC). Since its first application by Botstein et al., PIC has become the most widely applied formula for genetic studies to measure the information content of molecular markers.[18] To illustrate its wide application, we surveyed DNA fingerprinting publications of the last few years. This search revealed that more published papers utilized the PIC

formula. The program, PIC calc, can calculate these values from manually uploaded allelic frequencies or from a given file containing binary data.

Statistical analysis

Analysis was conducted by PAST 325 software. The program used was cluster analysis joining (tree clustering) with raw input data of each population separately. The main parameter, which guided the joining process linkage rule, is unweighted pair group average (UPGMA) and the genetic distance was estimated from raw data. Genetic distance denotes the genetic divergence between species or between populations within a species. It is measured by a variety of factors. Smaller genetic distances indicate relativeness whereas large genetic distances indicate a more detached genetic relationship. In its simplest form, the genetic distance between two populations is the difference in frequencies of the traits. Genetic distance is a quantitative measure of genetic difference between individuals, population or species at the allelic level.[19]

RESULTS AND DISCUSSION

Polymorphism index content evaluation

The binary matrix obtained by band matching was also calculated using PICcalc software (SJK from University of Liverpool). Polymorphism Information content (PIC) showed that all primers generated value more than the 0.5 which is the range to predict high diversity among any population.

Table 2: Polymorphism Index Content (PIC Percentage).

Primer	No. of bands	Total no. of polymorphic	Percentage of polymorphic bands (%)
OPW 04	19	12	82%
OPC 06	20	10	83%
OPC 18	18	09	83.2%
OPD 13	19	10	82%
Mean	**76**	**4 1**	

Recorded value with primer OPW 04 and OPD 13 were 82% whereas with primers OPC 06 and OPC 18 were 83% (**Table 2**). This shows the high rate of polymorphism leading to higher chances of mutation, which clearly signifies the reason of greater diversity among these millet species. Total of 4 primers were used based on their reproducibility and clarity of bands. All the species of millets showed PIC value of 0.821, which is 82%, is considerably high and determines the diversity among all the species of Poaeceae family. Further

calculated, the PIC value for 3 Panicum species was 0.81 i.e. 81%. Genetic diversity among millets, especially *Panicum* species is high but yet all are considered as panic grasses.

Dendrogram constructed by UPGMA at distant labeling signifies that species *Panicum natalense* is more closely related to the species *Panicum miliaceum* (Proso millet) than *Panicum sumatrense* (Little millet). Though, wild grass roots from the different clade and distant relative of millet crops of *Panicum* genus, showing its diverse genetic makeup. Natal buffalo grass is considered as millet but not included in crops and cereals, because of the evolutionary changes and environmental conditions.

Genetic diversity assessment (RAPD analysis)

In order to study the genetic diversity of a wild grass among *Panicum* genus, UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was done using 4 RAPD markers. The markers OPW 04, OPC 06, OPC 18 and OPD 13 generated DNA bands within the range of 100-200bp under Gel electrophoresis. Band linkage with two of the RAPD markers, OPC 06 of sequence 5'GAACGGACTC 3' and OPD 13 of sequence 5' GGGGTGAGA 3' markers (**Fig 1**) have melting point of 25°C and 27°C respectively and are specific for the millet crops, as observed on the basis of their reproducibility and clarity of band. Band matching was 20% under threshold of 20 showing the similarity percentage, which is considerably low.

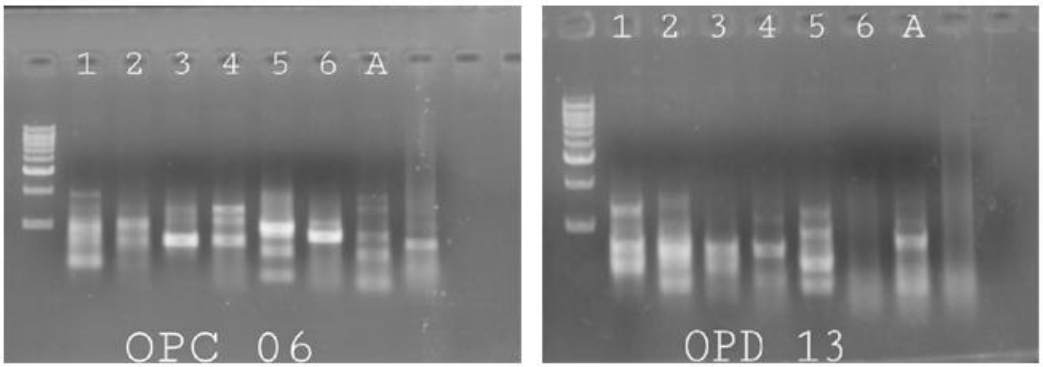

Fig 1: (A) RAPD analysis of millets with marker OPC 06; (B) RAPD analysis of millets with marker OPD 13; Ladder -100bp, Lane 1-6 & A –samples.

The total amplified products of 4 RAPD primers resulted in a total number of **82** bands with an average of 18.2 bands per primer, of which **51%** were polymorphic (**Table 2**). All four primers have shown high polymorphism of average 83% among clusters obtained. Gene cluster obtained shows similarity coefficient of 3.4. Regarding the level of polymorphism at the genera level, the finding confirmed that millets are more or less related but evidently diverged.

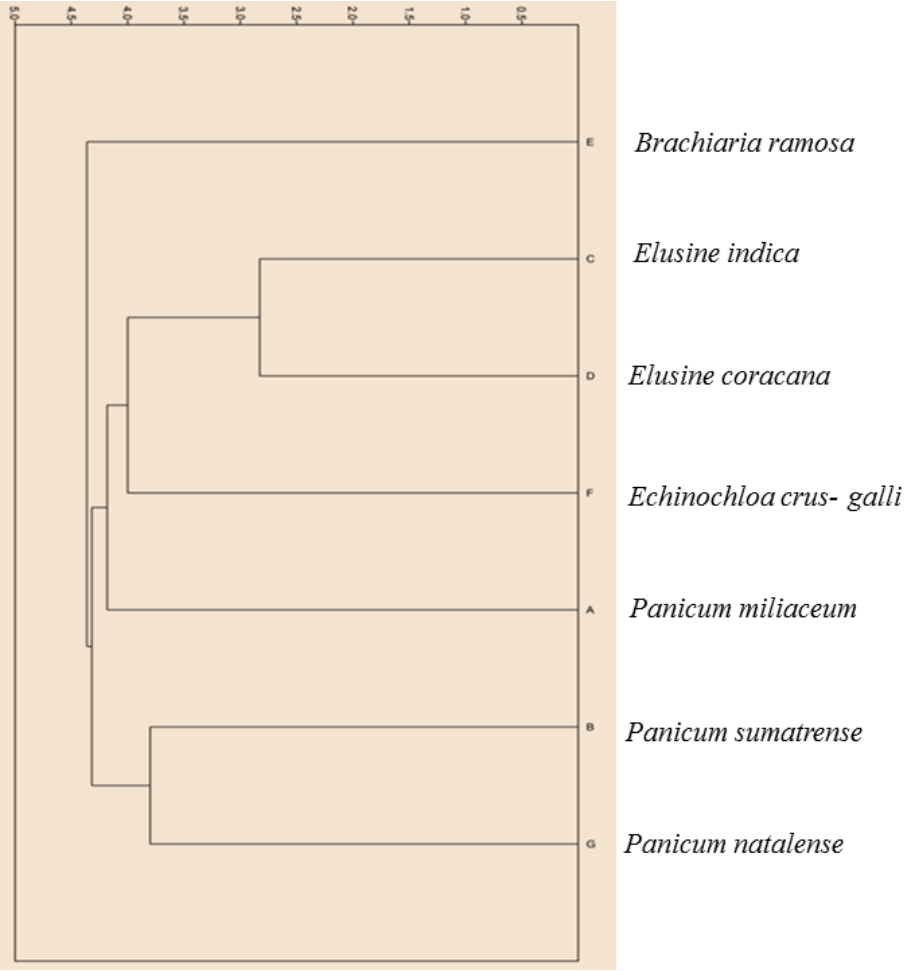

Fig 2: Dendrogram showing clustering of 7 genotypes of millets constructed using UPGMA based on Euclidean distance obtained from binary scores of RAPD analysis.

OPC 06 of sequence 5'GAACGGACTC 3' and OPD 13 of sequence 5' GGGGTGAGA 3' markers have melting point of 25°C and 27°C respectively and are specific for the millet crops, as observed on the basis of their reproducibility and clarity of band. Polymorphism Information content (PIC) showed that all primers generated value more than the 0.5 which is the range to predict high diversity among any population. Recorded value with primer OPW 04 and OPD 13 were 82% whereas with primers OPC 06 and OPC 18 were 83%. This shows

the high rate of polymorphism leading to higher chances of mutation, which clearly signifies the reason of greater diversity among these millet species. The dendrogram constructed shows the close relativeness of wild grass with the species of same genus i.e. little millet (*Panicum sumatrense*). This study can be widely used for breeding programs in future with the genetics make up of wild grass and the adaptively trait to produce healthy millet crops with fast growing properties.

The binary matrix obtained was manually recorded in software PAST 325, which by clustering gave output in Taxonomic representation (**Fig 2**). The phylogenetic tree depicts the relation between samples. Panicum genus has approximately 500 species that have a wide distribution in the world, indicating a rich, but currently under-appreciated gene pool available for switch grass breeding.[20] In this study, divergence from single root shows the link of all common millet, whereas Finger millet varieties are from same clade which also roots Barnyard millet *(Echinochloa crus-galli)*. Proso millet (*Panicum miliaceum)* shows closest relation with Brown Top millet (*Brachiaria ramosa*) clarifying their evolutionary relation. A high level of genetic diversity among *P. sumatrense* genotypes was observed using the RAPD markers. The findings can help in the identification of diverse parents for genetic improvement of *P. sumatrense* using hybridization program.[21] Whereas, in this study the wild grass/natal buffalo grass (*Panicum natalense*) being from same genus as of proso and little millet i.e. *Panicum* genus, is closely associated with the genetics of little millet (*Panicum sumatrense*).

CONCLUSION

Poaeceae and Gramineae families of grass consist of almost 850 genera with more than 1500 species due to their easy growing nature being small in length. Millets crops makes up approximately 70% of the widely cultivated cops in India. This includes Rice, Bajra (pearl millet), rye etc. Thus, with the need of increase in cultivation of these millet crops, wild species of millets can be considered for its fast-growing properties and used in breeding.

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