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Molecular analysis of sorghum genotypes using functional marker cytochrome P450

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Abstract
In the present study the genetic diversity of thirty sorghum bicolor L. genotypes to drought stress was assessed with the help of Cytochrome P450 markers. Molecular characterization of thirty sorghum genotypes was done by using ten Cytochrome P450 primers. In Cytochrome P450 analysis the total number of 16 bands was observed among the thirty sorghum genotypes. The PIC values, represents a allele diversity and frequency among the varieties, was highest for Cyt 02 and Cyt 06 primer and ranged from 0.25 to 1.00 with an average of 0.73. Primer Cyt 02 having highest power to identify among the identified genotypes as they reflected the resolving power of 1.87. The resolving power varies from 1.00 to 1.87 with a mean value of 1.43. The similarity matrix ranged from 0.2 to 1 in Cytochrome P450 primers. The highest value of similarity matrix was reported in genotypes (Jalana local, MJ-513, HC-260 & Pc-1001), (Rajasthan local & HC-308), (PC-23, SPV-462, PC-1002 & Golden local) and (Pant chari-5, Pc-615, Gwalior local, HC-171, PC-8, HC-136, SPV-1616 & PC-3). While the lowest value of similarity matrix was observed in genotypes Rampur local & SSV-84, Pant chari-7 & Pant chari-4, Rajasthan local & M.P chari and CSV-15 & Pant chari-4. UPGMA method was used for cluster analysis and accordingly the thirty sorghum genotypes were grouped into two different clusters. Major clusters consist of 24 genotypes and rest of the six genotypes are grouped in the other cluster indicating presence of genetic diversity among the genotypes at molecular level.

Keywords: Drought stress, Sorghum, Cytochrome P450, drought tolerance index

Introduction
Sorghum is the king of millets and ranks fifth globally among cereals for economic importance. It is used for food, feed and fodder for human, poultry and cattle. More than half of the world's sorghum is grown in semi-arid tropics of India and Africa, where it is a staple food for millions of poor people (Iqbal *et al*. 2010) [6]. It is an important summer season crop grown both for fodder and grain purposes. The wild distribution and high genetic diversity of sorghum in Africa indicate that this crop must have originated in this continent. Kimber (2000) [7] suggested that sorghum could have been introduced to Asia, in particular India and China, by human migrations around 1045 B.C. To date, Ethiopia and surrounding countries are considered the geographical area of origin (Kimber, 2000; Vavilov, 1951) [7, 19] and these countries are well recognised in world sorghum improvement programs.
Molecular markers are characterized by their abundance and are unaffected by environmental influence (Ram *et al*., 2007) [14]. They provide unambiguous estimates of genetic variability of populations. This information regarding genetic variability at molecular level can help to identify and develop genetically unique germplasm that complement the existing cultivars (Chakravarthi and Nara-vaneni, 2006) [3]. To investigate and develop new genetic tools for assessing genome-wide diversity in higher plant species, polymorphism of gene analogues of cytochrome P450 mono-oxygenases were found very useful. Data mining on *Arabidopsis thaliana* indicated that a small number of primer-sets derived from P450 genes could provide universal tools for the assessment of genome-wide genetic diversity in diverse plant species that do not have relevant genetic markers, or for which there is no prior inheritance knowledge of inheritance traits (Yamanaka *et al.* 2003) [20]. The sequence diversity of P450 gene-analogues in different plants species reflects the diversity of functional regions in the plant genome and is therefore an effective tool in functional genomic studies of plants.

Materials and Methods

Plant material

The plant material used in this study (Table 1) was procured from Department of Genetics and Plant Breeding, College of Agriculture, S.V.P.U.A.T, Modipuram, Meerut (U.P.). A list of all thirty genotypes included in present study is given in Table 1. For classifying finger millet genotypes for their drought tolerance, different seedling traits were used under artificial water stress condition in laboratory condition. Data were recorded on different seedling characters. Drought Tolerance Index (DTI) was calculated by using the formula of Maiti et al. (1996) [8] to determine the drought tolerance responses of the genotypes.

Table 1: Sorghum genotypes

S.N0.	Name of genotype	S.N0.	Name of genotype
1.	Rampur local	16.	HC-308
2.	Jalana local	17.	PC-1001
3.	Pant chari-7	18.	GPS-5
4.	Rajasthan local	19.	Pant chari-4
5.	CSV-15	20.	CSV-21F
6.	CSV-17	21.	M.P chari
7.	PC-23	22.	Pant chari-5
8.	U.P chari-2	23.	PC-615
9.	SPV-462	24.	Gwalior local
10.	PC-1002	25.	HC-171
11.	Golden local	26.	PC-8
12.	MJ-513	27.	PC-121
13.	Pant chari-6	28.	HC-136
14.	HC-260	29.	SPV-1616
15.	SSV-84	30.	PC-3

Isolation of DNA

The plant leaf samples for each genotype was taken and grind it to fine powder with the help of liquid nitrogen with a pinch of PVP in mortar and pestle. Grounded sample was taken in the autoclaved eppendrof tube (2.0ml) and 1 ml CTAB extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH8), 0.2% v/v 2-β mercaptoethanol) was added and incubate it for 1 hour in water bath at 65° C with intermitted shaking. Then add 600µl of Chloroform: isoamyl: alcohol (24:1) to the eppendrof tube and content is mixed gentely by inverting tubes 2-3 times and then tubes were centrifuged at 12000 rpm for ten minutes at 4° C. after centrifugation the supernatant approx. 400-500µl was transferred to the new autoclaved eppendrof tube and add pre chilled 600µl isopropanol (0.8V) and keep it overnight. Mixed gently the contents of the eppendrof and centrifuge at 10000 rpm for ten minutes at 4° C and now discard the supernatant with the help of micropipette and rinse the pellet 70% ethanol. Again centrifuge the content of the tube at 10000 rpm for ten minutes at 4° C and again discard the supernatant and air dry the pellet at room temperature for fifteen minutes. Now dissolve the pellet in 100 µl of TE buffer and keep it at -20° C for further experiments. The quality and quantity of DNA was checked by BIO-RAD Smart Spec™ Plus Spetrophotometer. The final DNA concentration of each sample was adjusted to 25 ng/µl.

DNA amplification conditions and gel electrophoresis

Ten Cytochrome P450 primers screenining was done on basis of their ability to detect distinct and clearly resolved amplified products. PCR amplification and electrophoresis were undertaken to identify the polymorphism among different genotype. The PCR was performed in a volume of 20 µl containing 2.0 µl of 10x Taq buffer with MgCl2, 2.0 µl of each of the 1 mM dNTPs mix, 1.0 µl of a Cytochrome P450 primer (forward 5µm and reverse 5µm each), 1.0 U of *Taq* DNA polymerase (1U/µl), 2.0 µl of 25 ng/ µl of genomic DNA template and 11.0 µl Mili pore water. Amplification reaction was performed in MY GENE™ Thermal cycler for one initial denaturation cycle at 94°C for 5 min followed by 40 cycles at 94°C for 1 minute for denatuation, at temperature according to primer for 1 min for annealing, at 72° for 2 minute for extension on completion of the 40 cycles final extension was carried out at 72°C for 7 min, then held at 4°C for forever. Now the samples were stored at 40 °C before electrophoresis. The PCR amplified products were separated out through 2 % agarose containing ethidium bromide. Finally visualized and photographed under UV light on gel documentation system. Fig. 1 showing Cytochrome p450 profiling pattern of 30 sorghum genotype with Cytochrome p450-02 primer. Molecular work was done at Molecular Biology Lab of SVP University of Agriculture and Technology, Meerut, Uttar Pradesh.

Data analysis

The data obtained by scoring the Cytochrome P450 profiles using different primer pairs individually were subjected to the construction of similarity matrix using Jaccard's coefficients. Cluster analysis was done on the basis of similarity values. NTSYS-pc software (version 2.02) was used for statistical analysis. UPGMA was used to construct dendograms in order to group genotypes into different clusters. Two parameters, viz. polymorphic information content (PIC) and resolving power (Rp) value were used to evaluate the discriminatory power of Cytochrome P450 markers. The polymorphic information content (PIC) for each pair of Cytochrome P450 marker was calculated as

$$PIC_i = 1 - \sum_{j=1}^{n} P^2_{ij}$$

P_{ij} is the frequency of the jth allele for marker, i and the summation extends over n alleles and the resolving power of the primers was calculated as Rp = ΣIb where, Ib is band informativeness.

Results and Discussion

Molecular characterization of the thirty sorghum genotypes was carried using Cytochrome P450 markers. Ten pair of functional primers Cytochrome P450 were used for initial screening out of which four primers (Cyt 01, Cyt 02, Cyt 03 and Cyt 09) which were highly reproducible and developed more bands were used for analyzing the genetic relationship among the thirty sorghum genotypes. Detailed information about the primers used, number of polymorphic bands (NPB), number of monomorphic bands (NMB), percentage of polymorphism (PP), polymorphism information content (PIC) and resolving power (Rp) are presented in Table 2.

Table 2: Molecular Profiling using Cytochrom p450 primers of 30 Sorghum genotypes

S. No.	Primer	No. of polymorphic bands	No. of monomorphic bands	Polymorphism %	Diversity in value of PIC	Resolving power
1.	Cyt 01	4	0	100%	0.31	1.67
2.	Cyt 02	2	0	100%	1.00	1.87
3.	Cyt 03	2	0	100%	0.72	1.07
4.	Cyt 04	1	0	100%	0.96	1.60
5.	Cyt 05	1	0	100%	0.96	1.60
6.	Cyt 06	1	0	100%	1.00	1.53
7.	Cyt 07	1	0	100%	0.72	1.07
8.	Cyt 08	1	0	100%	0.64	1.20
9.	Cyt 09	2	0	100%	0.75	1.00
10.	Cyt 10	1	0	100%	0.25	1.73

In Cytochrome analysis the total number of 16 bands was observed among the thirty sorghum genotypes with 10 primers. All the bands produced were polymorphic and the percentage of polymorphism was 100 % (Table 2). PIC is the value of a marker for detecting polymorphism within a population and primers. In Cytochrome P450 analysis PIC values varies from 0.25 (Cyt 10) to 1.00 (Cyt 02 and Cyt 06) with average of 0.73. The resolving power varies from 1.00 (35 ME 10T23) to 1.87 (Cyt 02) with an average of 1.43. All the bands, generated from 10 Cytochrome P450 primers, were subjected to calculate the genetic similarity index among the 30 genotypes. The similarity matrix varied from 0.2.to 1 among these sorghum genotypes. The highest value of similarity matrix was registered by genotypes (Jalana local, MJ-513, HC-260 & Pc-1001), (Rajasthan local & HC-308), (PC-23, SPV-462, PC-1002 & Golden local) and (Pant chari-5, Pc-615, Gwalior local, HC-171, PC-8, HC-136, SPV-1616 & PC-3). While the lowest value of similarity matrix was recorded by genotypes Rampur local & SSV-84, Pant chari-7 & Pant chari-4, Rajasthan local & M.P chari and CSV-15 & Pant chari-4. The observed similarity matrix in present study was then used to construct a dendrogram with UPGMA method (Fig.2).

Cluster analysis of Cytochrome p450 markers separated sorghum genotypes into two distinct main clusters. Cluster A was included 6 genotypes. Clister A is further divided into sub clusters on the basis of this hierarchical pattern obtained viewing the dendogram genotypes Rajasthan local and HC-308 showed maximum similarity indicating the same line of origin. Cluster B included rest of 24 genotypes and again sub divided into two sub clusters. Cluster B1 comprises eleven genotypes and cluster B2 consist of thirteen genotypes. Cluster B1 is further divided into sub clusters on the basis of this hierarchical pattern obtained viewing the dendogram genotypes where genotype SSV-84 is placed separately thus depicting minimum similarity with rest ten genotypes and four genotypes i.e. (Jalana local, Pant chari-6, HC-260 and PC-1001) showed maximum similarity indicating the same line of origin and four genotypes i.e. (PC-23, SPV-462, PC-1002 and Golden local) showed maximum similarity indicating the same line of origin. Cluster B2 is further divided into sub clusters on basis of this hierarchical pattern obtained viewing the dendogram genotypes. Eight genotypes i.e. (pant chari-5, PC-615, Gwalior local, HC-171,PC-8, HC-136, SPV-1616 and PC-3) showed maximum similarity indicating the same line of origin.

Molecular characterization and detection of genetic variability among sorghum genotypes using Cytochrome P450 technique have not been reported yet but similar work is done in other crops by Yamanka *et al.*, (2003) [20], Panwar *et al.*, (2010) [12] and Saini *et al.*, (2013) [15]. These workers studied different parameters and suggested the use of Cytochrome markers to reveal the polymorphism. In our research the usefulness of Ctochrome P450 markers to detect genetic variation was confirmed and was in agreement with work of earlier workers. These results clearly indicate that Cytochrome p450 is found more suitable to identify highly diverse genotypes of sorghum which can be utilized in future for breeding program in sorghum but Cytochrome p450 is not able to distinguish between closely related diverse genotypes.

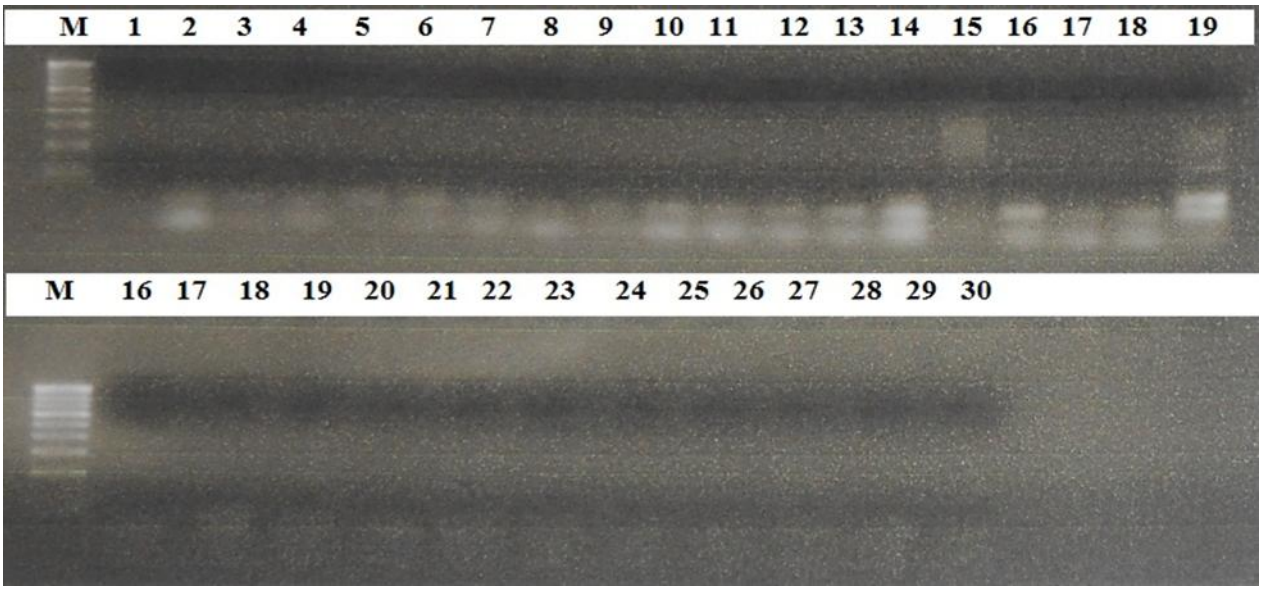

Fig 1: Showing Cytochrome p450 profiling pattern of 30 sorghum genotype with Cytochrome p450-02 primer

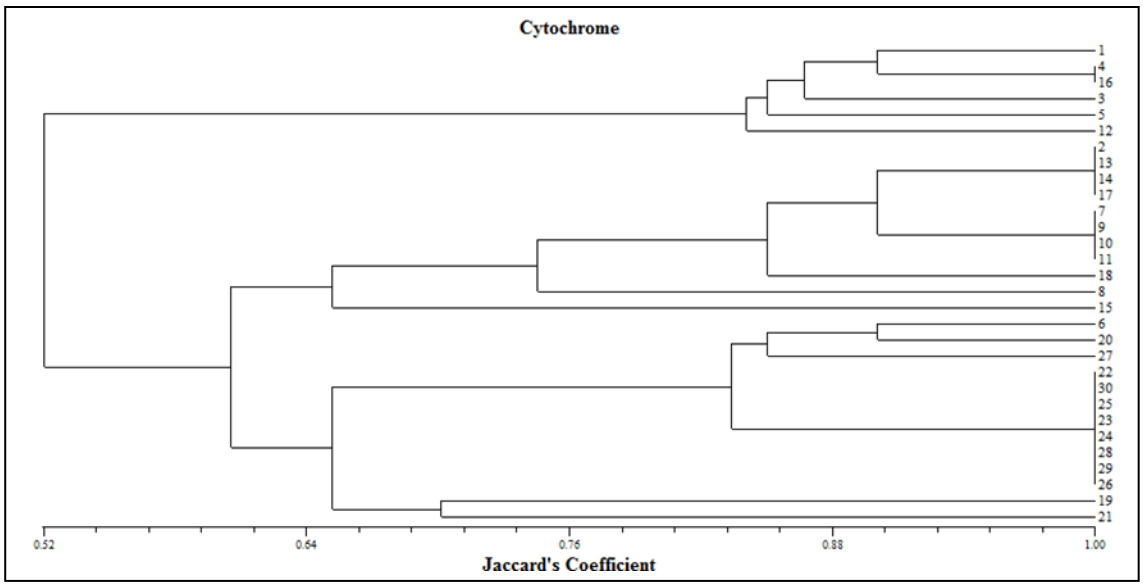

Fig 2: Dendogram showing clustering of 30 sorghum genotypes constructed using UPGMA based jaccard's similarity coefficient obtained from Cytohrome p450 markers based analysis

Conclusion

Present study proves the efficacy of Cytochrome P450 markers in analysing genetic relationship at molecular level, which is important for germplasm conservation and varietal identification. Cytochrome P_{450} genes are widely found in microorganisms, animals and plants (Schalk *et al*., 1999) [16] and play important role in oxidative detoxification and secondary metabolite production (Ohkawa *et al*., 1998) [11]. Ctochrome P_{450} gene analogues have been successfully used as new genetic markers for genetic diversity studies in plants, which reflects both functional and genomic-wide regions (Yamanka *et al*., 2003) [11].

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