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Influence of Genetic and Morphological Markers in Evaluation of Jatropha Diversity

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ABSTRACT

Assessment of genetic diversity is a key prerequisite for genetic improvement of crops through plant breeding. In this study, genetic diversity analysis of 11 elite lines, developed out of the germplasm accessions of Jatropha curcas L. collected from Central American and Asiatic regions was performed using 279 SSR markers, of these 53 were newly developed from expressed sequence tags (EST) available in NCBI database. The alleles were scored and detected a total of 352 alleles across eleven 11 lines with mean value of 2.5 per locus. Besides, 110 SSRs were found to be polymorphic and the polymorphic information content (PIC) of these loci were in the range of 0.14 to 0.84 with an average of 0.44. The UPGMA cluster analysis of data showed the formation of two groups namely A and B and they exhibited a mean similarity coefficient of 33.8% and 36% respectively. The similarity coefficient range was from 0.14 to 0.58 with a mean of 0.36 at all SSR loci. The lowest similarity co-efficient was observed in EJC-CA1 line by forming a separate clade. The phenotypic correlation study between morphological traits namely number of fruits per bush (NF), number of seeds per bush (NS), seed weight per bush (SW), test weight (TW) and oil content percentage (OP) indicated positive among these traits. Based on average cluster analysis method, these 11 lines were categorised into three groups and the first cluster consisting of six lines found to possess high crop productivity when compared to other two clusters. The 3 clades thus formed were used to avoid crosses within the group while developing and testing hybrid combinations, thus a crossing effort of about 60% was saved due to the guidance provided by these markers.

Key words: *Jatropha curcas, Genetic diversity, Morphological traits, Polymorphism, SSR.*

INTRODUCTION

Jatropha is a valuable perennial plant, bearing high quality of nonedible oil in the seeds. This shrub is widely known as a drought and salinity tolerant crop and has been identified as a potential biodiesel crop. The genus *Jatropha* comprises around 160 to 175 species[1] and almost all the species are diploid in nature with 22 chromosomes[2]. The chromosome number in some species such as *J. cuneata* Wiggins & Rollins, *J. dioica* Sesse and other species including *J. heterophylla* Heyne are tetraploids and *J. tirucalli* L. has 2n=20 chromosomes[3,4,2].

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Jatropha curcas is a potential biofuel resource emerging as an alternative to petroleum diesel owing to its high quantity production of non-edible oil-seeds[5,6]. Efforts have been made for the past two decades to develop *Jatropha* as an industrial crop to meet the growing demand of raw materials for the production of biodiesel. The lack of better high yielding varieties and nonexistence of agronomic information prevents the extensive exploitation of this crop.

The achievement of genetic improvement could be obtained by the selection of available distinct desired traits for crop improvement program[1]. Genetic diversity analysis by using molecular markers has been well established tool in many crops[7]. Among the molecular markers microsatellites or simple sequence repeats (SSRs) are the best and suitable markers because of their high reproducibility, polymorphism and co-dominant inheritance[8]. It is reported that *Jatropha* specific 241 novel EST-SSR and G-SSR markers were developed from NCBI database sequences to utilize in genetic diversity studies[9]. Further, a total of 45 accessions of *J.curcas* were subjected to genetic diversity study by using 36 of EST-SSRs and 20 of genomic SSRs. Later studied the assessment of genetic diversity, genetic structure and genetic differentiation among 32 *Jatropha* germplasm collections using ten microsatellite markers[10]. In 2013, Vischi[11] studied the genetic relationship among the toxic and nontoxic *Jatropha* accessions by using a total of 40 SSR markers, of these 7 showed polymorphism among all the accessions used in the study. Kumari[11] reported the usage of 32 EST SSRs from the database that exhibited polymorphism among the genotypes and compared with the data of phenotypic variations [13,14,15,16]. However, the aim of the current study was to develop novel SSR markers from *Jatropha* specific EST databases, which would differentiate genetically distinct lines through evaluation of genetic diversity within the *Jatropha* lines for conservation and genetic improvement.

MATERIAL AND METHODS

A total of 11 *Jatropha* elite entities, native of Asiatic and Central America regions were experimented in Randomized Block Design (RBD) in three replicates during the year 2016-17 at Samalkota, Andhra Pradesh. The healthy young leaf samples were collected from the plants for genomic DNA isolation. These entities were developed from out of the germplasm accessions collected from these regions and subsequent development cycles.

Mining of ESTs database and primer design

The Expressed Sequence Tag (EST) sequences of Jatropha were retrieved from National Centre for Biotechnology Information (NCBI) database and screened for sequences with repeats using WebSat[17] software (www. http://wsmartins.net/websat). The microsatellite markers were designed and developed by screening of each of these repeat containing sequences and filtered out based on a criteria of minimum number of repeat motifs such as di, tri, tetra, penta, and hexanucleotide (Figure. 1).

Table 1: Lines used for studying the properties of newly developed SSRs and morphological markers

Region	Accessions	Number
Asiatic	EJC-AS-1, EJC-AS-2, EJC-AS-3, EJC-AS-4, EJC-AS-5, EJC-AS-6, EJC-AS-7 and EJC-AS-8	8
Central America	EJC-CA-1, EJC-CA-2 and , EJC-CA-3	3

Genomic DNA extraction

Total genomic DNA was isolated by following the protocol of Vijay Y[18] and quality and quantity were measured using Nanodrop® ND-1000 spectrophotometer (Saveen Werner, Sweden) and subjected for PCR analysis.

PCR and Electrophoresis

DNA amplification was performed in a reaction volume of 10 µl containing 50 ng template DNA, 1x PCR reaction buffer [15 mM Tris-HCl, 2 mM dNTPs, 1U Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific corporation, USA.), 10µM of forward and reverse primers of each and sterile distilled water (4.8 µL). PCR was carried out in a 96- Well Thermal Cycler (Biorad, CA, USA). The DNA was initially denatured at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing (Tm°C) for 45s, extension 72°C for 1 min, and then a final extension at 72°C for 10 min. The PCR products were fractionated on QIAxcel Advanced System using QIAxcel DNA high resolution cartridge. The allele sizes were visualized and calculated using QIAxcel Screen gel software. QIAxcel is one of the robust, fast and semi-automated system which increases the resolution power up to 3-5bp and helps to detect minor alleles that are difficult to identify in traditional agarose gel electrophoresis. The time taken to analyse 96 samples is less than 1 hour and results are highly reproducible (Fig. 3).

Screening for polymorphism:

The synthesized markers were used or employed to study the genetic diversity analysis of 11 *Jatropha* lines. The SSR band profiles were scored as '1' for the 'presence' and '0' for the 'absence' of band for each of the locus across the 11 genotypes. The molecular size of each fragment was determined by comparing with known molecular size ladders. UPGMA cluster analyses was performed using NTSYSpc vers 2.1 program[19]

Phenotypic characterization

Phenotypic evaluation of the selected five traits as listed in the Table 2 were conducted for the 11 *Jatropha* lines in the second year of the trial. The coefficient of simple correlation, statistical multivariate analyses were carried out for the identification and grouping of genotypes into different clusters with the help of Minitab version 16.

RESULTS

Genetic diversity by SSR markers

A total of 46,947 *Jatropha* EST sequences were downloaded from the NCBI's database and WebSat[17] software was used to screen each sequence for the presence of microsatellites. Upon analysis, 310 EST sequences were found to have repeats containing sufficient flanking DNA sequence regions. Fifty three randomly selected designed primers were synthesized (Supplementary Table 1) along with 226 SSR markers mined from open source. The genetic characterization of 11 *Jatropha* lines were performed with a total of 279 SSR markers The automated high-resolution capillary electrophoresis of QIAxcel advanced system was used to run the DNA samples. Aanalysis of the run revealed 110 SSRs to be polymorphic out of 279 SSRs screened and this resulted in detection of 355 alleles. (Figure.2). We found that the QIAxcel gel run system was found to be better than agarose gel electrophoresis with regard to speed, accuracy and resolution (Figure.3). The data were scored, computed and a phylogenetic tree was generated with the use of UPGMA cluster analysis. The similarity coefficient was found to be ranged from 0.14 to 0.58 with a mean of 0.36 across all SSR loci. The analysis further revealed the formation of two groups namely, 'A' and "B' with similarity coefficient of 33.8%, and 36% respectively. This diversity analysis revealed that group A consisting of two Central American and four Asiatic lines (EJC-CA2, EJC-CA3, EJC-AS1, EJC-AS-2, EJC-AS-3 and EJC-AS-4) with close genetic similarity among themselves whereas group B includes other Asiatic lines such as EJC-AS5, EJC- AS6, EJC-AS7 & EJC-AS8, which were found genetically nearer among themselves but far away from Central American lines(Figure. 4). Additionally, one of the the central American line (ELCA1) showed lowest similarity co-efficient by forming a separate clade, which indicates that this line is genetically distant from both Asiatic lines and other Central American lines.

Genetic diversity by morphological markers

Morphological studies showed a considerable level of variation among all the 11 *Jatropha* lines, for the five quantitative traits as enumerated in Table 2. The variation observed among these eleven lines for the five selected phenotypic characters were found to be different in each of the lines. The results presented in Table 3 reveals a wide range of variation for different parameters from 6.3% to 37.5%, highest being in seed weight (gram) followed by number of seeds and lowest in oil yield. Data showed the range of variation in Number of fruits, Number of seeds, seed weight(gram), test weight(gram) and oil percent are from 655 to 3026, 1216 to 7573, 568 to 4189, 458.6 to 646.6 and 29.4% to 36.2 with a mean value of 2169, 5396, 2859, 522 and 32%.

Accessions EJC-CA-1, EJC-AS 1, EJC-AS-5 and EJC-AS-7 had high percent of oil content than the mean value of 32%; EJC-AS-7 was found possess higher oil content among all the accessions. EJC-CA-1, EJC-CA-2, EJC-AS 1, EJC-AS-5, EJC-AS-6, EJC-AS-7 had more seed yield (g) than the mean value of 2859 g. The accession EJC-AS-7 having higher seed yield and more oil percent than their mean value is found to be a best elite among all the accessions. These phenotypic traits showed extensive variation within the lines and such type of variation could be used for the identification of suitable lines in breeding programs aiming to improve the yield.

Table 2: Descriptors for 5 morphological traits recorded in 11 *Jatropha* lines

S.no	Traits	Description
1	No of fruits/Bush	The total number of fruits /plant
2	No of seeds/Bush	The total number of seeds/plant
3	Test weight	weight of 100 seeds in grams
4	Seed yield/Bush (grams)	Total seed weight harvested per plant
5	Oil content (%)	Hexane extractable lipids after extraction by n-Hexane for 4 Hours.

Table 3: Mean Standard deviation (SD) and coefficient of variation (%) of five morphological characteristics for the 11 *Jatropha* lines

Variables	Mean	SD	CV (%)	Minimum	Maximum
No of Fruits	2169	674.2	31.1	655	3026
No of Seeds	5396	1782.4	33.0	1216	7573
Seed Weight (gram)	2859	1073.2	37.5	568	4189
Test weight (gram)	522	61.1	11.7	458.6	646.6
Oil%	32	2.0	6.3	29.4	36.2

Analysis of correlation with phenotypic data

Correlation coefficient for five metric traits is presented in Table 4. All of the correlations were significant at 5% level of significance. Seed yield had a positive correlation with all the characters, highest being with number of fruits (r=0.956) followed by number of seeds (r=0.953). Oil percent had a positive correlation with all characters, significant positive correlation with seed yield, number of fruits, number of seeds and test weight (r=0.758, 0.687, 0.665, 0.610) respectively.

Table 4: Phenotypic correlation of four traits of 11 *Jatropha* lines

	No of fruits	No of seeds	Seed weight (gram)	Test weight (gram)
No of seeds	0.999*			
Seed weight (gram)	0.956*	0.953*		
Test weight	0.418	0.413	0.663*	
Oil%	0.687*	0.665*	0.758*	0.610
***Significant at level=0.05**				

Cluster analysis is a well-known tool for classifying the lines into distinct groups based on variation of single or multiple characters. The identification and grouping of elite lines were performed based on relative genetic distances in this investigation. Among the methods, the Average Linkage Method is commonly preferred due to the less subjectivity involved in the cluster formation[20]. The objective of this study is to classify the 11 elites into optimum number of clusters and there after reduce the crossing load by avoiding crosses between genetically closer parents.

In the cluster analysis, all the 5 traits of 11 lines were used and grouped into 3 different categories with dendrogram (Figure. 5). The comparative data analysis for these five traits are depicted in Table 5. The data illustrated that the group of lines in cluster 1 had high potential in diversity than the other two group of lines found in cluster 2 and 3. A single line named EJC- AS- 8 formed in a separate cluster (cluster 3) found to have poor trait diversity.

Table 5: Comparison of lines by cluster analysis using five morphological traits of 11 *Jatropha* lines

Cluster No.	Line Codes	No of fruits	Mean	No of seeds	Mean	Seed weight	Mean	Test weight (gram)	Mean	Oil%	Mean
1	**EJC-CA-1**	2534	2631	6336	6588	4095.2	3635.5	646.6	553.2	33.4	32.8
	EJC-CA-2	2325		5812.8		3476.6		598.1		31.3	
	EJC-AS-1	2952		7380.8		3787.8		513.2		33.2	
	EJC-AS-5	2538		6342		3207.3		505.6		32.1	
	EJC-AS-6	2411		6082		3057.4		502.5		30.8	
	EJC-AS-7	3026		7573		4188.7		553.2		36.2	
2	**EJC-CA-3**	1634	1855	4084.8	4652	2294.8	2266.6	561.8	489.6	32	30.4
	EJC-AS-2	2108		5291		2524		477		30.4	
	EJC-AS-3	1987		4971		2292.3		461.1		29.6	
	EJC-AS-4	1690		4263		1955.1		458.6		29.4	
3	**EJC-AS-8**	655	655	1216	1216	568.2	568.2	467.3	467.3	30.1	30.1

DISCUSSION

One of the objective of this study is to find the genetically and morphologically distinct *Jatropha* lines for breeding program based on genetic diversity analysis. In early days, RAPD markers were used in *Jatropha* [21,22,23,24] to assess the genetic diversity. Similarly, ISSR markers were used to study the genetic relatedness among 55 *Jatropha curcas* accessions[23, 25]. Gupta[23] characterized *Jatropha* genotypes by performing comparative analysis with help of RAPD and ISSR markers. Use of AFLP markers for assessing the genetic diversity among 38 populations of *Jatropha* was reported using three out of 9 AFLP polymorphic markers and observed low genetic diversity

In the current study, we have used SSR markers to analyse the genetic diversity of 11 *Jatropha* lines, of which 3 are from Central American origin and the remaining eight were from Asiatic regions. All the lines segregated into two clusters with the exception of EJC-CA1, which formed a separate clade. The mean of similarity coefficient 0.38 is comparable with the data obtained by Wen[9], from the evaluation of 45 accessions of Indonesia, Grenada, South America and two Chinese provinces. Further Yadav[27] evaluated 25 Indian accessions with 50 EST based SSR markers and average heterozygosity was found to be 0.30. While Phumichai[7] exploited the genetic diversity among 32 accessions and obtained the average coefficient of 0.43 for the improvement of breeding strategies. In this study, the high resolution (3-5bp), reproducibility, speed and accurate analysis in QIAxcel advanced system (used in the current study) enhanced the number of polymorphic markers, which resulted in detection of more

number of alleles and increased PIC values. The efficiency of each SSR marker will be determined by the PIC value; reflects allele's diversity frequency and marker having such PIC values nearer to 1 are much desirable[28]. The average PIC values of the 110 loci in our study was found to be 0.44 and ranging from 0.14 (RJM 27) to 0.84 (RJM 504).

A dendrogram was constructed using data of five morphological traits that divided eleven *Jatropha* lines broadly into three clusters based on the Euclidean distance (Table 5). The first cluster consisting of six lines showed average number of fruits, number of seeds, seed weight, test weight and percent of oil content as 2631, 6588, 3635.5, 553.2 and 32.8 respectively. The second cluster comprising four lines revealed average number of fruits, number of seeds, seed weight, test weight and oil percent as 1855, 4652, 2266.6, 489.6 and 30.4 respectively. The third cluster which contains one line showed average number of fruits, number of seeds, seed weight, test weight and oil% as 655, 1216, 568.2, 467.3, and 30.1 respectively. This phenotypic variability and the difference in productivity and factors contributing thereof presents a good potential and could be exploited to enhance the productivity of future lines and hybrid combinations derived thereof.

This study indicated that there is no direct reconciliation between the data derived from molecular and morphological markers selected in the study of diversity among all the *Jatropha* lines. However partial relationship was observed between lines EJC-AS-5 and EJC-AS-6 where they are very similar in both morphological and molecular diversity analysis. The genetic and phenotypic similarity matrices obtained using both molecular and phenotypic data was found to be negligible. Several studies have earlier reported a similar weak relationships between the data obtained from the molecular and morphological markers[29,30,31,32]. Sunil[33] identified a weak relationship between phenotypic and molecular markers in a group of *Jatropha* lines wherein, the most distinct lines identified by phenotypic traits were not supported by markers data wherein different diverse lines identified. Moreover it could be the fact that the genotypic sequence variation observed in markers study have not exhibited the corresponding phenotypic variation as observed in field.

Main Figure and Legends

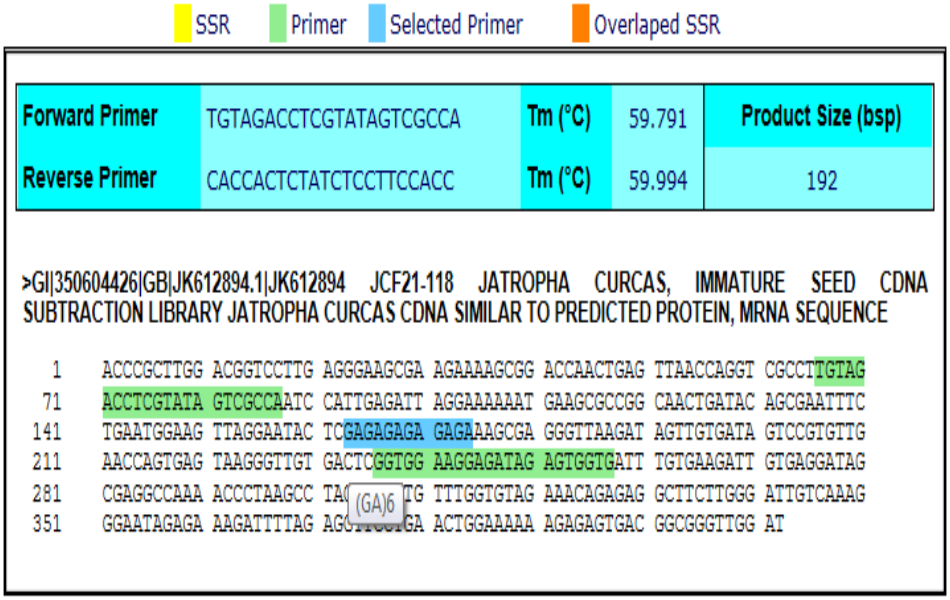

Fig. 1: Primer designing for SSRs by using WebSat software

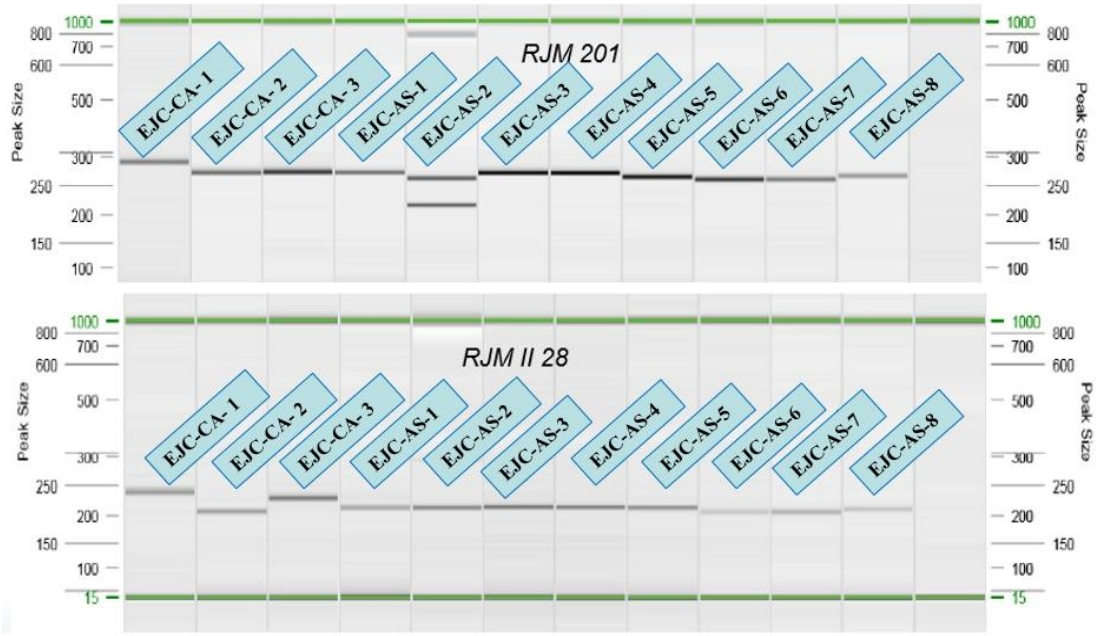

Fig. 2: Amplification and polymorphic potential of selected SSRs in 11 elite *Jatropha* lines

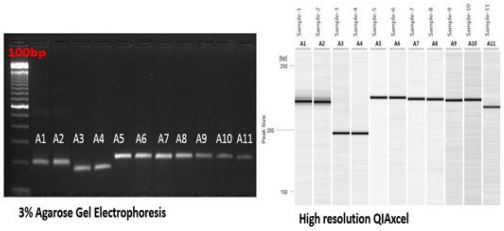

Fig. 3: Comparison between Agarose gel electrophoresis and QIAxcel advaned system (Amplified products of *Jatropha* Lines - A1 to A11)

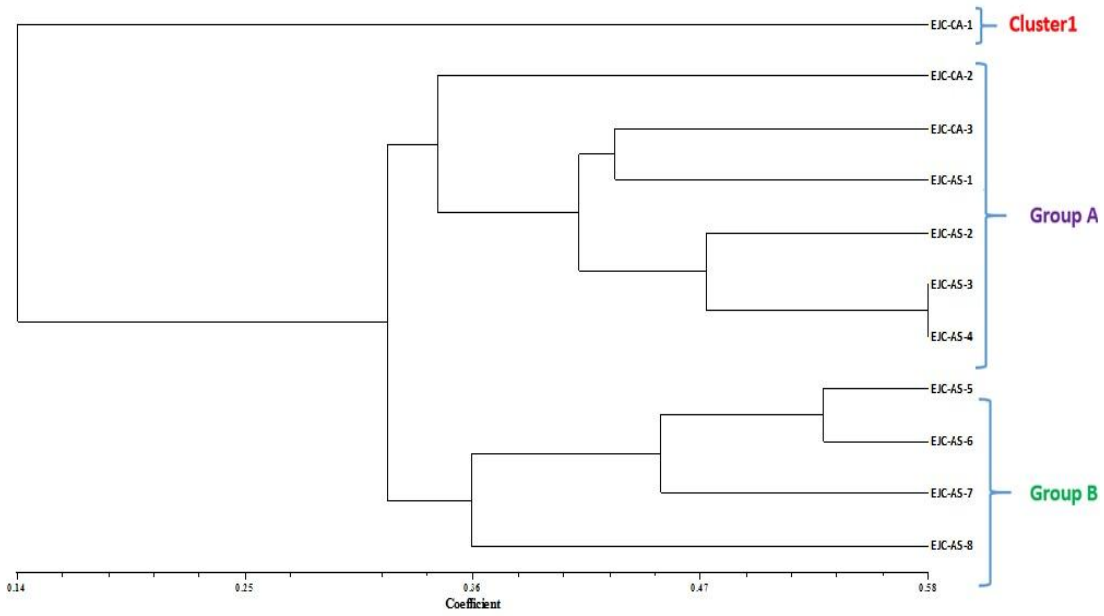

Fig. 4: Dendrogram of 11 *Jatropha* lines derived from UPGMA cluster analysis

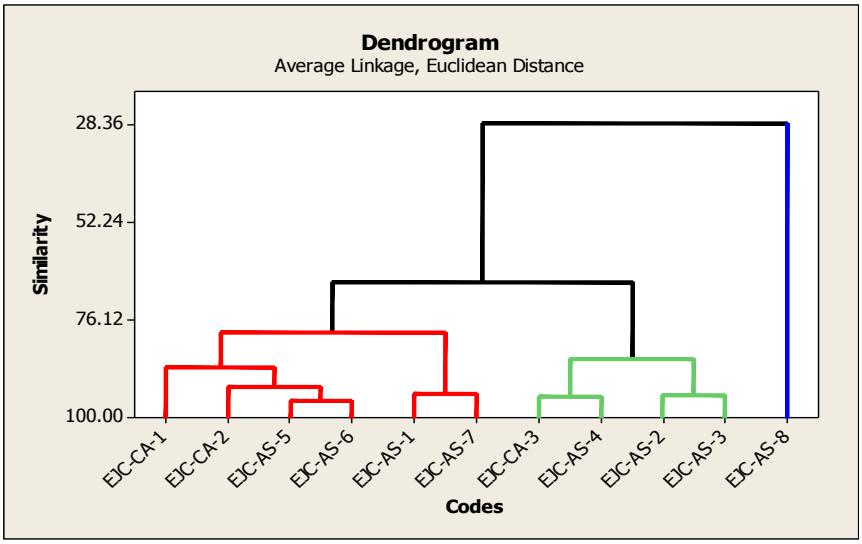

Fig. 5: Hierarchical cluster analysis for morphological data of 11 Jatropha lines.

CONCLUSION

It can be also concluded from the present investigation that more number of unique morphological traits need to be considered for genetic diversity analysis of entities for correlating trait variation with molecular markers. This may result in identification of unique morphological indicator which could accord positive relationship between genotypic and phenotypic variations observed in genetic diversity study of *Jatropha* lines.

The data set derived from this analysis was applied in breeding trials by the avoidance of crossing the entities within the group considering genetic proximity. This resulted in avoidance of crosses and thereby crossing load to the extent of 60%. Thus a cost and effort saving in the top cross trials of classical breeding was obtained due to this marker assisted breeding.

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