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Studies on genetic variability and relationship of bael (*Aegle marmelos* (L) Correa) using morphological and molecular markers

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ABSTRACT

Bael (*Aegle marmelos* (L) Correa) is an important underutilized fruit crop of India. A total of 25 bael trees were selected from 356 bael trees of Sakharayapattana in Chikkamagalur district, Karnataka, India based on the fruit morphological traits (fruit weight, pulp weight, skull thickness, seed weight per fruit, No. of seeds per fruit, No. of locules per fruit, No. of seeds per locule, pulp wt. : seed wt.). These 25 trees were evaluated for phenotypic and genotypic variations using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. RAPD and ISSR markers showed significant polymorphism among the trees. Jaccard's genetic similarity value of RAPD and ISSR was found in the range of 0.00–0.95 and 0.06–0.56, respectively suggesting a moderate level of genetic diversity. The present study revealed that molecular markers can be successfully utilized for determining genetic diversity and relationship of bael trees for further varietal improvement.

Keywords: Bael, genetic variability, morphology and molecular markers

INTRODUCTION

Bael (*Aegle marmelos*(L) Correa) belongs to the family Rutaceae and is an important underutilized indigenous fruit crop of India and has high medicinal and nutritional values. Since pre-historic times, it was found as wild in Sub-Himalayan tract and dry deciduous forests of Central and Southern Indian region. Therefore, a large number of landraces are available in different diversity regions (Pandey *et al.*, 2013) Each tree is genetically different from others as most of them are of seedling origin. Traditionally, morphological characters have been used to identify and characterize the bael. However, there is a high level of genetic variability which can sometimes be used accurately to distinguish each tree. When the morphological traits are used for determining diversity and relationships among plant species, they are not sufficient because of environmental influences. Thus, the usefulness of molecular markers has been investigated as a means of characterizing and discriminating against different species more precisely (Benharrant *et al.*, 2002). The introduction of molecular biology techniques, such as DNA-based markers, allows for direct comparison of different genetic materials independent of environmental influences. The viability and purity of accessions can be analysed by utilization molecular markers. This process can increase both the quantity and quality of plant (Mujeeb *et al.,* 2017) Molecular characterization would be more rewarding in terms of accurate identification and characterization of most closely related trees at the intra-specific level. The degree of similarity between the banding patterns provides information about genetic similarity and relationships between the samples studied. The application largely depends on the type of markers employed, distribution of markers in the genome, type of loci they amplify, level of polymorphism and reproducibility of the products (Virk *et al.,* 2001 and Fernandez *et al.,* 2002). Among the molecular markers, RAPD and ISSR markers have been extensively used to study genetic diversity and relationship. These markers can detect polymorphism in a single reaction. The main objective of the study was to characterise bael trees using morphological molecular markers, to evaluate the genetic diversity and relationship.

MATERIALS AND METHODS

Among 356 trees, 76 fruiting trees were subjected to study of variation in fruit morphological traits like fruit weight, pulp weight, skull thickness, seed weight per fruit, No. of seeds per fruit, No. of locules per fruit, No. of seeds per locule, pulp wt. : seed wt. Based on the fruit morphological traits the best 25 trees were selected for molecular marker analysis. Plant material (leaves) of 25 bael trees were collected for genomic DNA isolation using standardized cetyl trimethyl ammonium bromide (CTAB) extraction protocol (Benharrant *et al.*,2002) and thenthe DNA was quantified using a spectrophotometer and the quality of the DNA was checked on 0.7% agarose gel.

RAPD-PCR Amplification

Twelve RAPD primers were used for RAPD analysis of 25 bael trees. PCR amplification was carried out using 1X Taq buffer solution and 1 U Taq DNA polymerase (Bangalore Genie Pvt. Ltd.), 1.25 mM $MgCl_2$, 0.8 mM dNTP mix, 5 µM of a single decamer primer and 50 ng genomic DNA and the volume made up to 20 µl using sterilized double-distilled water. The amplification was performed in VWR Peqlab thermocycler with initial pre-denaturation at 94 °C for 4 min followed by 40 cycles of denaturation at 92 °C for 2 min, at annealing temperature (Table 1.) for 1 min, and extension at 72 °C for 2 min. Final extension was performed for 5 min at 72 °C. Amplification products were separated by electrophoresis on 1.5 % Agarose gel stained with ethidium bromide at 80 V. Bands were visualized and photographed in a gel documentation unit.

Table 1. List of RAPD primers and their annealing temperatures

Primer	Marker sequence (5' to 3')	Annealing temperature (°C)
OPA-02	TGCCGAGCTG	37
OPN-03	GGTACTCCCC	37
OPN-12	CACAGACACC	37
OPM-05	GGGAACGTGT	37
OPM-06	CTGGGCAACT	38
OPX-17	GACACGGACC	36
OPM-12	GGGACGTTGG	38
OPM-15	GACCTACCAC	36
OPM-20	AGGTCTTGGG	38
OPB-1	GTTTCGCTCC	36
OPA-08	GTGACGTAGG	36
OPA-1	CAGGCCCTTC	38

ISSR-PCR Amplification

Sixteen primers, which gave the best amplification results with the sample DNA, were selected for ISSR-PCR analysis. PCR-amplification was carried out using 1X Taq buffer solution and 1 U Taq DNA polymerase (Bangalore Genie Pvt. Ltd.), 1.40 mM $MgCl_2$, 0.8 mM dNTP mix, 8 µM of a single decamer primer and 50 ng genomic DNA and the volume made upto 25 µl using sterilized double-distilled water. The amplification was performed in VWR Peqlab thermocycler 2 min at 94°C, followed by 40 cycles each of 1 min at 94°C (denaturation), 1 min at 55°C (annealing for ISSR primers), 2 min at 72°C (extension) followed by one final extension of 7 min at 72°. Amplification products were separated by electrophoresis on 1.5 % Agarose gel stained with ethidium bromide at 80 V. Bands were visualized and photographed in a gel documentation unit and analyzed.

Data Analysis

Amplified bands generated from RAPD and ISSR-PCR amplification were scored based on the presence (1) or absence (0) of bands for each primer and used to calculate a genetic similarity matrix using software NTSYS-pc version 2.1. Cluster analysis was performed for molecular data using the "unweighted pair group method using arithmetic means" (UPGMA) algorithm, from which dendrograms depicting similarity among trees were drawn and plotted using NTSYS-pc software.

RESULTS AND DISCUSSION

The variations in fruit morphological traits among the trees are depicted in Table 2. Significant maximum fruit weight was observed in tree SB-353 (320.00 g) and minimum fruit weight was observed in tree SB-115 (54.30 g). Pulp weight was found significantly maximum in tree SB-353 (202.40 g) whereas, minimum pulp weight was observed in SB-71 (22.53 g) and it was on par with the tree SB-148. The difference in fruit weight might be attributed to an increase in pulp weight, seed weight, skull weight of trees. The findings are in agreement with the results of earlier researches (Pandey *et al.,* 2008, Pandey *et*

Table 2. Fruit morphological traits of 25 bael trees

Tree No.	Fruit weight (g)	Pulp weight (g)	Skull thickness (mm)	Seed weight (g)	No. of seeds / fruit	No. of locules / fruit	No. of seeds / locule	Pulp wt. : Seed wt.
SB-353	320.00	202.40	4.95	19.80	40.00	10.67	3.75	10.22
SB-351	136.00	58.37	6.82	8.80	16.00	11.00	1.46	6.64
SB-147	99.10	36.50	4.40	7.70	10.00	8.00	1.25	4.74
SB-90	110.30	45.10	6.92	0.12	1.00	9.00	0.11	392.17
SB-111	146.20	48.10	5.33	8.70	44.00	10.00	4.40	5.53
SB-33	153.50	57.10	3.96	18.00	56.00	8.00	7.06	3.18
SB-80	85.90	27.70	5.54	13.22	26.00	9.00	2.90	2.10
SB-350	91.20	39.50	3.89	5.40	40.00	9.00	4.49	7.33
SB-288	95.50	30.80	5.51	3.50	33.00	10.00	3.32	8.81
SB-161	123.30	47.30	5.47	15.30	34.00	8.00	4.28	3.09
SB-2	162.00	46.00	4.09	12.00	46.00	10.00	4.64	3.85
SB-148	65.70	22.60	4.06	2.40	15.00	7.00	2.14	9.40
SB-16	157.20	48.00	4.48	20.50	62.00	9.00	6.89	2.34
SB-91	125.10	46.07	3.87	0.15	1.00	9.00	0.11	322.12
SB-66	127.30	35.80	7.10	9.96	33.00	9.00	3.67	3.59
SB-1	190.90	49.00	5.60	25.80	84.00	10.00	8.45	1.90
SB-73	159.60	65.50	4.89	15.50	26.00	7.67	3.40	4.85
SB-9	75.70	27.70	4.82	4.71	13.00	9.00	1.46	5.90
SB-115	54.30	10.47	3.99	5.38	22.00	10.00	2.24	1.92
SB-273	96.20	34.00	6.67	7.00	17.00	11.00	1.55	4.92
SB-272	91.90	33.50	4.23	7.10	22.00	9.00	2.46	4.72
SB-146	105.50	42.30	5.40	3.60	9.00	11.00	0.83	11.86
SB-71	56.00	22.53	4.02	5.60	3.00	7.00	0.43	4.04
SB-354	150.00	73.00	4.18	9.60	34.00	12.00	2.83	7.62
SB-175	136.00	53.42	5.93	5.20	25.00	10.00	2.50	10.31
F value	**	**	-	-	*	-	-	**
S. Em±	5.31	1.14	0.06	0.29	0.71	0.43	0.20	15
CD @ 5%	15.08	3.24	0.18	0.82	2.02	1.21	0.56	42.61

** Significant @ 5% and 1%, * Significant @ 5%, - Non significant

al., 2013 and Mitra *et al.,* 2010).The maximum number of seeds per fruit was found in tree SB-1 (84.00) and minimum in SB-90 and SB-91. The difference in seed weight may be attributed to differences in the number and size of seeds among the trees. The results are in conformity with the earlier findings (Pandey *et al.,* 2008, Pandey *et al.,* 2013; Singh and Misra, 2010). Pulp weight : Seed weight was found maximum in SB- 90 (392.17) and it was minimum in SB-1 (1.90). The decrease in seed number per locule has a positive correlation with higher pulp content. Findings are in agreement with the results of earlier researches (Pandey *et al.,* 2013 and Singh and Misra, 2010). The traits like skull thickness, seed weight per fruit, no. of locules per fruit and no. seeds per locule were observed non-significant among the trees.

RAPD analysis

The simplicity of laboratory assay for RAPD markers makes them an attractive method for obtaining intraspecific distinctions. This technique is already used for cultivar identification and genetic variability analysis of several underutilized fruit crops like tamarind (Diallo *et al.,* 2007) and bael (Nayak *et al.,* 2013). In this study, a set of RAPD primers were used for distinguishing the superior trees of bael. The comparatively higher percentage of polymorphic bands detected in the present study indicated that RAPD fragments are moderately polymorphic and particularly informative in the estimation of the genetic relationship of bael trees studied. The polymerase chain reaction of bael genomic DNA using 12 selected RAPD primers generated a total of 1,399 amplified bands (Table 3.). The highest number of bands was observed with primer OPX-17. The size of amplified fragments ranged between 300 and 1800 bp and the lowest number of bands was observed with primer OPN-03. The size of amplified fragments ranged between 500-900 bp. Comparatively, moderate level of polymorphic information content (0.39 to 0.77) value was seen in selected polymorphic primers. The highest PIC value (0.77) was observed for primer OPM-12 whereas, the lowest PIC value (0.39) was observed for OPM-06. It was observed that DNA primers showed an average PIC value of >0.5, which confirms that the primers are highly informative. The maximum average number of bands across trees was found for primer OPX-17 (7.88) while minimum was in primer OPN-03 (1.68).The highest genetic similarity coefficient of 0.95 was found between the SB-147 and SB-90 may be due to their same place of origin. The trees SB-175 and SB-66, SB-9 and SB-1 showed the lowest similarity coefficient (0.00). But the molecular diversity was not in agreement with most of the morphological diversity as reported in *Colocasia esculenta* (Singh *et al.,* 2012). Comparatively high amplitude of the genetic similarity coefficient established in the present study confirms the

Table 3. List of RAPD primers, their sequence and generated bands

Primer	Marker sequence (5' to 3')	Range of amplicon size (bp)	Total No. of bands	Average no. of bands across trees	PIC value
OPA-02	TGCCGAGCTG	200-1400	102	4.08	0.74
OPN-03	GGTACTCCCC	500-900	42	1.68	0.56
OPN-12	CACAGACACC	100-1000	196	7.84	0.58
OPM-05	GGGAACGTGT	300-1000	150	6.00	0.45
OPM-06	CTGGGCAACT	300-900	154	6.16	0.39
OPX-17	GACACGGACC	300-1800	197	7.88	0.47
OPM-12	GGGACGTTGG	300-750	78	3.12	0.77
OPM-15	GACCTACCAC	300-1200	61	2.44	0.61
OPM-20	AGGTCTTGGG	600-1000	136	5.44	0.50
OPB-1	GTTTCGCTCC	500-1200	111	4.44	0.58
OPA-08	GTGACGTAGG	600-1000	55	2.20	0.65
OPA-1	CAGGCCCTTC	300-1200	117	4.68	0.50

occurrence of considerable genetic variability among bael trees. However, variation was higher than that reported for 25 cultivars of mango (range 0.69-0.89) (Rajwana *et al.,* 2008). A dendrogram (Fig 1.) was constructed from values of similarity coefficients generated from RAPD data. The trees were divided into six major genotypic groups at a 0.446 similarity coefficient, containing 6 clusters respectively, based on the unweighted pair group method using arithmetic average cluster analysis. The trees SB-2, SB-351, SB-161, SB-353 placed in a distinct cluster while other clusters subdivided into sub-clusters. Cluster 'a' consists of 19 trees, where these trees separated from each other at 0.57 similarity coefficients forming a distinct cluster for SB-175. This cluster was further divided at 0.614 forming a distinct cluster for SB-80. Cluster 'b' comprised of two trees SB-123 and SB-273. It was observed that SB-147 and SB-90 were placed very closely at a similarity co-efficient of 0.95.

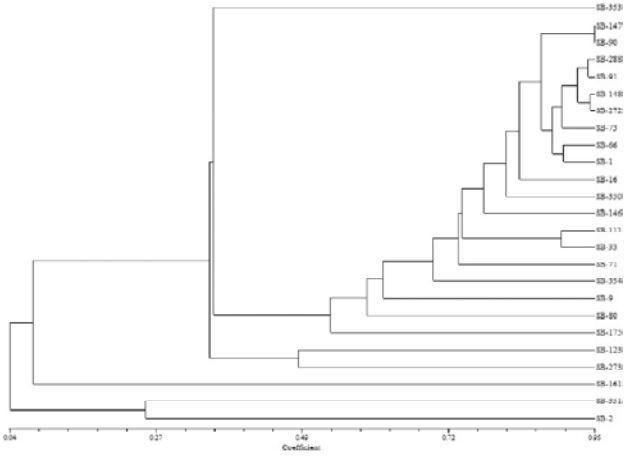

Fig. 1. Dendrogram deviding the 25 trees of bael based on Jaccard genetic similarity coefficient from analysis.

ISSR analysis

Polymerase chain reaction of bael genomic DNA using 16 selected ISSR primers generated a total of 1,496 amplified bands (Table 4.). The highest number of bands was observed with primer UBC-807 and the lowest number of bands was observed with primer UBC-890. Comparatively higher polymorphic information content (0.83 to 0.99) was shown by selected polymorphic primers. The highest PIC value (0.99) was observed in primer UBC-888 whereas, lowest PIC value (0.83) was observed in UBC-815. Average number of bands across trees were found maximum in primer UBC-807 (7.28) while minimum in primer UBC-890 (1.60). The highest genetic

Table 4. List of ISSR primers, their sequence and generated bands

Primer	Marker sequence (5' to 3')	Total No. of bands	Average No. of bands across trees	PIC value
UBC 807	AGA GAG AGA GAG AGA GT	182	7.28	0.90
UBC 810	GAG AGA GAG AGA GAG AT	125	5.00	0.88
UBC 811	GAG AGA GAG AGA GAG AC	59	2.36	0.97
UBC 815	CTC TCT CTC TCT CTC TG	63	2.52	0.83
UBC 824	TCT CTC TCT CTC TCT CG	97	3.88	0.94
UBC 825	ACA CAC ACA CAC ACA CT	137	5.48	0.94
UBC 834	AGA GAG AGA GAG AGA GYT	103	4.12	0.95
UBC 836	AGA GAG AGA GAG AGA GYA	76	3.04	0.96
UBC 840	GAG AGA GAG AGA GAG AYT	65	2.60	0.98
UBC 841	GAG AGA GAG AGA GAG AYC	108	4.32	0.92
UBC 842	GAG AGA GAG AGA GAG AYG	117	4.68	0.94
UBC 859	TGT GTG TGT GTG TGT GRC	88	3.52	0.98
UBC 888	BDB CAC ACA CAC ACA CA	56	2.24	0.99
UBC 889	DBD ACA CAC ACA CAC AC	96	3.84	0.84
UBC 890	VHV GTG TGT GTG TGT GT	40	1.60	0.97
UBC 891	HVH TGT GTG TGT GTG TG	57	2.28	0.96

similarity coefficient of 0.56 between the SB-1 and SB-73may be due to their same place of origin and occurrence of an intense gene flow between these trees. But the molecular diversity was not in agreement with most of the morphological diversity as reported in *Colocasia esculenta* (Singh *et al.,* 2012). Comparatively high amplitude of the genetic similarity coefficient established in the present study confirms the occurrence of considerable genetic variability among bael trees. A dendrogram was constructed from values of similarity coefficients generated from ISSR data. According to the dendrogram (Fig. 2.), the trees were divided into nine major genotypic groups at a 0.30 similarity coefficient, containing nine clusters respectively, based on unweighted pair group method using arithmetic average cluster analysis. The trees SB-354, SB-351, SB-175, SB-353 placed in a distinct cluster while other clusters sub divided in to sub-clusters. Cluster 'a' consists of five trees, where these trees separated from each other at 0.57 similarity coefficients forming a distinct cluster for SB-175. This cluster was further divided at 0.33 forming a distinct

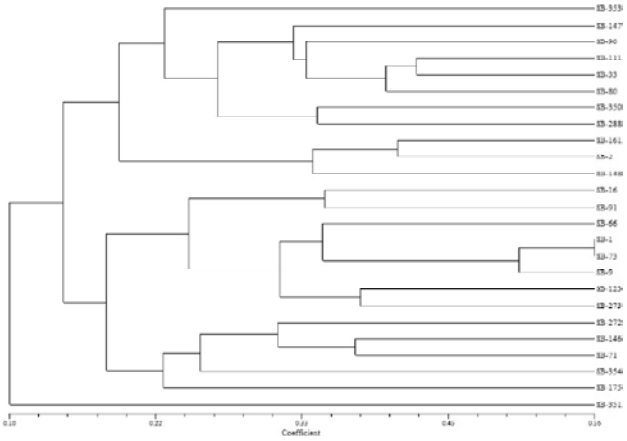

Fig. 2. Dendrogram of 25 trees of bael based on Jaccard genetic similarity coefficient ISSR markers analysis.

cluster for SB-147. Cluster b comprised of two trees SB-350 and SB-288. Cluster c comprised of three trees SB-161, SB-2 SB-148. Cluster d, e, f comprised of two, six and three trees respectively. At a similarity co-efficient of 0.56, it was observed that SB-1 and SB-73 were placed very closely.

RAPD and ISSR combined analysis

A dendrogram was constructed using values of similarity coefficients generated from RAPD and ISSR data. According to the dendrogram (Fig. 3.), the trees were divided into nine major genotypic groups at a 0.51 similarity coefficient, containing nine clusters respectively, based on unweighted pair group method using arithmetic average cluster analysis. The treesSB-353, SB-80, SB-175, SB-123, SB-273, SB-161, SB-2, SB-351 placed in a distinct cluster while other clusters sub divided in to sub-clusters. Cluster 'a' consists of four trees, where these trees separated from each other at 0.59

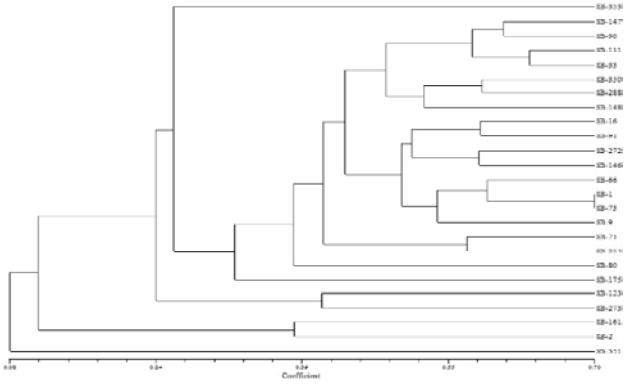

Fig. 3. Dendrogram of 25 trees of bael generated based on combined RAPD and ISSR data

similarity coefficients. Cluster 'b' comprised of three trees SB-350, SB-288 and SB-148. Cluster 'c' comprised of four trees SB-16, SB-91, SB-272 and SB-146. Cluster 'd' and 'e' comprised of four and two trees respectively. At similarity co-efficient of 0.70 it was observed that SB-1 and SB-73 were placed very closely.

CONCLUSION

Both the molecular markers analysis showed a high degree of variation among the selected bael trees. The present study revealed that both the molecular markers can be successfully utilized for inferring genetic diversity and genetic relationship of bael trees. The similarity between SB-1 and SB-73 confirmed the importance of these markers for distinguishing the bael trees based on environmental and genetic factors. Findings of this study indicate that identification of trees from various locations mainly based on morphological characteristics may have encountered the mismatches and mistakes. This indicates the importance of characterisation of trees both at morphological and molecular level for efficient maintenance and exploitation of precious germplasm and to determine groups of high genetic similarity and dissimilarity, which is the key for

establishing breeding strategies in genetic improvement programme of bael.

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