academicJournals

Vol. 12(42), pp. 6055-6060, 16 October, 2013
DOI: 10.5897/AJB2013.12473
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Random amplified polymorphic DNA (RAPD) markers reveal genetic diversity in bael (*Aegle marmelos* Correa) genotypes of Andaman Islands, India

Dipak Nayak[1]*, D. R. Singh[2], Sabarinathan, P.[3], Shrawan Singh[2] and Tarama Nayak[4]

[1]Indian Agricultural Research Institute- Regional Station, Kalimpong, West Bengal, India- 734 301.
[2]Central Agricultural Research Institute, Port Blair, A and N Islands, India- 744 101.
[3]Bharat College of Science and Management, Thanjavur, Tamil Nadu, India- 613 005.
[4]Department of ASEPAN, Palli Siksha Bhavan, Visva Bharati, Santiniketan, West Bengal, 731 235- India.

Accepted 20 September, 2013

The present study evaluated genetic variability of superior bael genotypes collected from different parts of Andaman Islands, India using fruit characters and random amplified polymorphic DNA (RAPD) markers. Genomic DNA extracted from leaf material using cetyl trimethyl ammonium bromide (CTAB) method was subjected to polymerase chain reaction (PCR) with 12 polymorphic primers. A total of 476 polymorphic loci were identified with mean value of 39.66 bands per primer and 63.99% polymorphism. Application of unweighted pair group method using arithmetic average cluster analysis generated three genotypic groups. 'Bael-5' and 'Bael-8' were most similar genotypes whereas 'Bael-7' and 'Bael-1' were extreme divergent. The clusters based on molecular data were not in agreement with the morphological traits in most of the cases as low level of correlation was observed between the classification methods based on fruit characteristics and RAPD markers. The bael genotypes were found to have considerable genetic variability, demonstrating the importance of RAPD markers to analyse each genotype in a collection in order to efficiently maintain the germplasm collection for genetic improvement of bael.

Key words: Aegle, Bael, random amplified polymorphic DNA (RAPD) and genetic diversity.

INTRODUCTION

Aegle marmelos Correa, the bael, belongs to the Rutiaceae family and indigenous to India. It is one of the most important underutilized fruits of India, Burma and Ceylon (Srivastava et al., 1998). The bael fruit pulp contains important bioactive compounds such as carotenoids, phenolics, alkaloids, pectins, tannins, coumarins, flavonoids and terpenoids. It has several ethnomedicinal applications and source of traditional medicines such as anti-diarrhea and anti-dysentery (Brijesh et al., 2009), hepatoprotective activity (Rajasekaran et al., 2009) and antifertility effect (Chauhan et al., 2008). The leaves are also used in several traditional formulations used in diabetes and ophthalmic disorder.

*Corresponding author. E-mail: dipakiari@gmail.com.

Abbreviations: RAPD, Random amplified polymorphic DNA; **CTAB,** cetyl trimethyl ammonium bromide; **EST,** expressed sequence tags; **SCAR,** sequence-characterized amplification regions; **SSR,** simple sequence repeats; **SNP,** single nucleotide polymorphism.

Table 1. The studied bael genotypes and their place of collection and fruit quality traits.

Genotype	Place of collection	Fruit weight (kg)	Fruit length (cm)	Fruit width (cm)	Shell thickness (mm)	Wt. of shell (g)	No. of seeds	Wt. of seeds (g)
Bael-1	North Andaman	1.80±0.12	26.00±0.58	8.67±0.33	2.87±0.09	429.67±5.78	131.33±5.93	27.33±1.20
Bael-2	North Andaman	1.06±0.08	13.67±1.15	7.38±0.39	3.20±0.06	244.38±16.27	126.46±5.95	24.71±1.10
Bael-3	Middle Andaman	1.10±0.06	16.00±0.58	9.00±0.58	3.47±0.15	265.33±5.7	130.67±2.96	24.67±0.33
Bael-4	Middle Andaman	1.16±0.09	15.00±0.58	10.33±0.33	2.90±0.12	231.33±5.93	126.67±9.28	26.00±1.53
Bael-5	Middle Andaman	1.00±0.012	12.00±1.15	7.67±0.33	3.07±0.09	230.00±7.64	115.33±7.31	22.33±1.20
Bael-6	Middle Andaman	0.93±0.09	11.33±1.2	5.33±0.33	3.37±0.03	228.00±4.36	112.00±1.53	22.33±0.33
Bael-7	Middle Andaman	1.20±0.06	13.00±0.58	6.00±0.58	3.60±0.01	240.33±5.78	120.00±2.89	21.33±0.88
Bael-8	Middle Andaman	0.56±0.03	8.00±0.5	5.33±0.88	3.20±0.05	162.33±6.23	86.00±0.58	17.67±0.33
Mean		0.73±0.06	8.00±0.58	6.67±0.33	3.10±0.05	168.00±10.12	189.67±5.78	36.00±1.15
LSD @ 5%		0.25	2.31	14.99	0.24	19.94	16.00	2.93

Due to the long historic cultivation of bael in India, synonymies and homonymies can be observed among genotypes cultivated in different regions. Thus, diversity of bael germplasm based on morphological characteristics of the fruit has been performed by Nath et al. (2003) and Rai and Misra (2005). However, due to the effects of environmental factors on these attributes, their use can be ambiguous. Therefore, markers independent from the environment are necessary for reliable identification and discrimination of genotypes and cultivars (Sarkhosh et al., 2006). DNA based molecular markers are independent from environmental interaction, developmental stage, unlimited in number and show high level of polymorphism (Sarkosh et al., 2006).

Further, pre-conservation analysis of potential germplasm using DNA markers reduces duplicity in germplasm (Singh et al., 2012). These markers are also used for various purposes in horticultural crops such as, construction of genetic linkage map (Venkateswarlu et al., 2006), phylogenetic (Kafkas and Perl-Treves, 2001) and genetic diversity (Zamani et al., 2007).

Out of the different marker systems, random amplified polymorphic DNA (RAPD) marker system is an easy and informative molecular marker requiring no previous information of DNA sequences and its efficacy had been justified to be the same as other molecular markers in studies on genetic diversity (Zamani et al., 2010). The information about the nature and magnitude of genetic variability as well as relatedness among bael genotypes would be helpful in formulating an effective breeding programme.

The Andaman Islands are situated in Bay of Bengal and 1200 km away from mainland India. This region is rich in diversity of different horticultural crops (Abraham et al., 2008) including bael. However, none of the studies has been carried out on bael diversity from these islands. Therefore, the aim of the present study was to analyse the genetic variability and relatedness among the superior genotypes of bael collected from Andaman Islands, India.

MATERIALS AND METHODS

Plant materials

During survey of Andaman Islands in 2011-2012, great diversity in bael was observed in different villages. From this diversity, a total of eight representative bael genotypes were collected from different habitats in Andaman Islands. These genotypes represented maximum diversity of bael in islands (Table 1). From these, young healthy leaves were collected in plastic bags packed in ice for transport to the laboratory. The leaf samples were washed three times in sterile distilled water, frozen in liquid nitrogen and stored in the freezer at −20°C for further analysis. Five fruits from each genotype were collected for recording morphological characters (Table 1) previously reported to be important in bael evaluation (Nath et al., 2003) and means of these traits were used for statistical analysis.

DNA extraction

Leaves from each accession were macerated separately and genomic DNA was extracted according to the cetyl trimethyl ammonium bromide (CTAB) method suggested by Saghai-Maroof et al. (1984)] with minor modification. The purity and quantity of genomic DNA was determined spectrophotometrically and confirmed by comparative analysis with the intensity of known concentrations of unrestricted lambda DNA after electrophoresis in 0.8% agarose gel and staining with ethidium bromide. Following quantification, a portion of the DNA sample was diluted in TE buffer to final concentration of 10, 20, 30 and 40 ng per µl for subsequent RAPD analysis. The remaining DNA stock samples were stored in the freezer at −20°C.

Amplification reactions

In order to select the most polymorphic primers for RAPD analysis, DNA samples from the genotypes Bael-1 and Bael-2 were amplified using 44 primers. Elevated levels of polymorphism were detected with 12 primers and these 12 primers were employed in the subsequent reactions. PCR were carried out in a thermocycler (G-

Table 2. List of selected informative RAPD primers, their sequence and some information about generated bands in this study.

DNA marker	Marker sequence (5' to 3')	Range of amplicon size (bp)	Total No of bands	Average No of bands across genotypes	PIC value
OPA-02	TGCCGAGCTG	200-1200	57	6.33	0.26
OPN-03	GGTACTCCCC	200-1250	45	4.09	0.18
OPN-12	CACAGACACC	100-1000	71	5.92	0.25
OPM-05	GGGAACGTGT	300-1300	70	4.67	0.2
OPM-06	CTGGGCAACT	300-1500	80	5.33	0.22
OPX-17	GACACGGACC	200-1200	75	5.77	0.24
OPM-12	GGGACGTTGG	300-750	40	4.00	0.17
OPM-15	GACCTACCAC	200-600	32	4.57	0.2
OPM-20	AGGTCTTGGG	300-1500	71	5.07	0.21
OPB-1	GTTTCGCTCC	300-1400	70	5.38	0.22
OPA-08	GTGACGTAGG	300-1600	60	5.45	0.23
OPA-1	CAGGCCCTTC	200-1500	62	5.64	0.23

Storm, USA). The reaction mixtures (20 µl) were comprised in 1X buffer solutions, 1.0 mM $MgCl_2$, 1x PCR buffer, 1.75 mM $MgCl_2$, 200 mM dNTPs, 0.5 mM of a single decamer primer and 1 U Taq DNA polymerase (Genaxy Scientific Ltd., India). The reactions were performed in a thermocycler (G-Storm, USA) involved an initial denaturation step of 4 min at 94°C, followed by 35 cycles comprising of denaturation for 1 min at 92°C for 1 min, annealing for 1 min at 37°C, extension at 72°C for 2 min and a final extension at 72°C for 5 min. Amplicons were separated by electrophoresis on 1.5% agarose (Genaxy Scientific Ltd., India) gel in TE buffer at 80 v for 3 h. Gels were treated with 0.5 µl/ ml ethidium bromide for 30 min and bands were visualized and photographed under UV light by a gel documentation system (UVP, Bio Doc. Co., USA).

Data processing

The number of polymorphic bands generated by each primer was determined initial visual examination of the gel photographs, taking into account the resolution and degree of amplification. Only bands of medium and strong intensity were included in the subsequent analysis. For RAPD analysis, presence of band was scored 1 whereas the absence of the band was scored 0. The scored RAPD markers were converted onto a binomial (0/1) matrix. Based on the matrices, the genetic similarities between bael genotypes were determined using Jaccard's coefficients and dendogram was constructed by applying unweighted pair group method with arithmetic average (UPGMA) clustering based on Jacard's similarity index bands. These computations were performed using the programme NTSYS-pc software ver. 2.11 (Rohlf, 2000).

RESULTS

Mean values of the studied fruit characteristics showed considerable variations between genotypes for all characters. Significant differences were recorded between genotypes for all characteristics based on analysis of variance (Table 1). In preliminary experiments of RAPD reproducibility, it was revealed that the concentraton of template DNA was crucial to obtain the maximum number of reproducible bands. Varying the concentration of template DNA (10, 20, 30 and 40 ng) revealed that 20 ng resulted to the maximum number of reproducible bands, and therefore 20 ng was used in all subsequent PCR reactions. Out of 44 RAPD primers, 12 primers which produced good and reproducible polymorphic bands among the 8 bael genotypes were used for further analysis. Polymerase chain reaction of bael genomic DNA using 12 selected polymorphic primers generated a total of 733 amplified bands. Among them, 476 bands were polymorphic in nature. The highest number of polymorphism was observed with primer OPA-01. The size of amplified fragments ranged between 250 and 1600 bp for all primers (Table 2). Comparatively low polymorphic information content (0.17 to 0.26) was shown by selected polymorphic primers. Average number of bands across genotypes were found maximum in primer OPA-02 (6.33) while minimum in primer OPM-12 (4.00). Results of similarity matrix showed the moderate degree of genetic similarity coefficients ranged from 0.49 to 0.72. The lowest similarity (0.49) was between genotypes Bael-7 and Bael-1 which were the most divergent while the highest similarity (0.72) was found between the genotypes 'Bael-5' and 'Bael-8' (Table 3).

A dendogram was constructed from values of similarity coefficients generated from RAPD data. According to the dendrogram (Figure 1), the genotypes were divided into two major genotypic groups at a 0.54 similarity coefficient, containing 7 and 1 genotypes, respectively, based on unweighted pair group method using arithmetic average cluster analysis. The genotype Bael-7 placed in a distinct cluster while other cluster sub divided in to 2 sub-clusters. Sub-cluster 'a' consisted of two genotypes, 'Bael-1' and 'Bael-2', where these genotypes separated from each other at 0.63 similarity coefficients. The 'sub-cluster b' comprised of five genotypes, 'Bael-4', 'Bael-6', 'Bael-5', 'Bael-8' and 'Bael-3'. Within sub-cluster 'b', genotypes 'Bael-5' and 'Bael-8' placed very closely with 0.72 similarity coefficients. In this study, the genetic

Table 3. Genetic similarity matrix based on RAPD data among 8 bael genotypes.

	Bael-1	Bael-2	Bael-3	Bael-4	Bael-5	Bael-6	Bael-7	Bael-8
Bael-1	1.00							
Bael-2	0.63	1.00						
Bael-3	0.52	0.61	1.00					
Bael-4	0.63	0.61	0.65	1.00				
Bael-5	0.59	0.61	0.66	0.62	1.00			
Bael-6	0.63	0.53	0.51	0.67	0.59	1.00		
Bael-7	0.49	0.53	0.56	0.55	0.53	0.56	1.00	
Bael-8	0.61	0.64	0.66	0.63	0.72	0.60	0.64	1.00

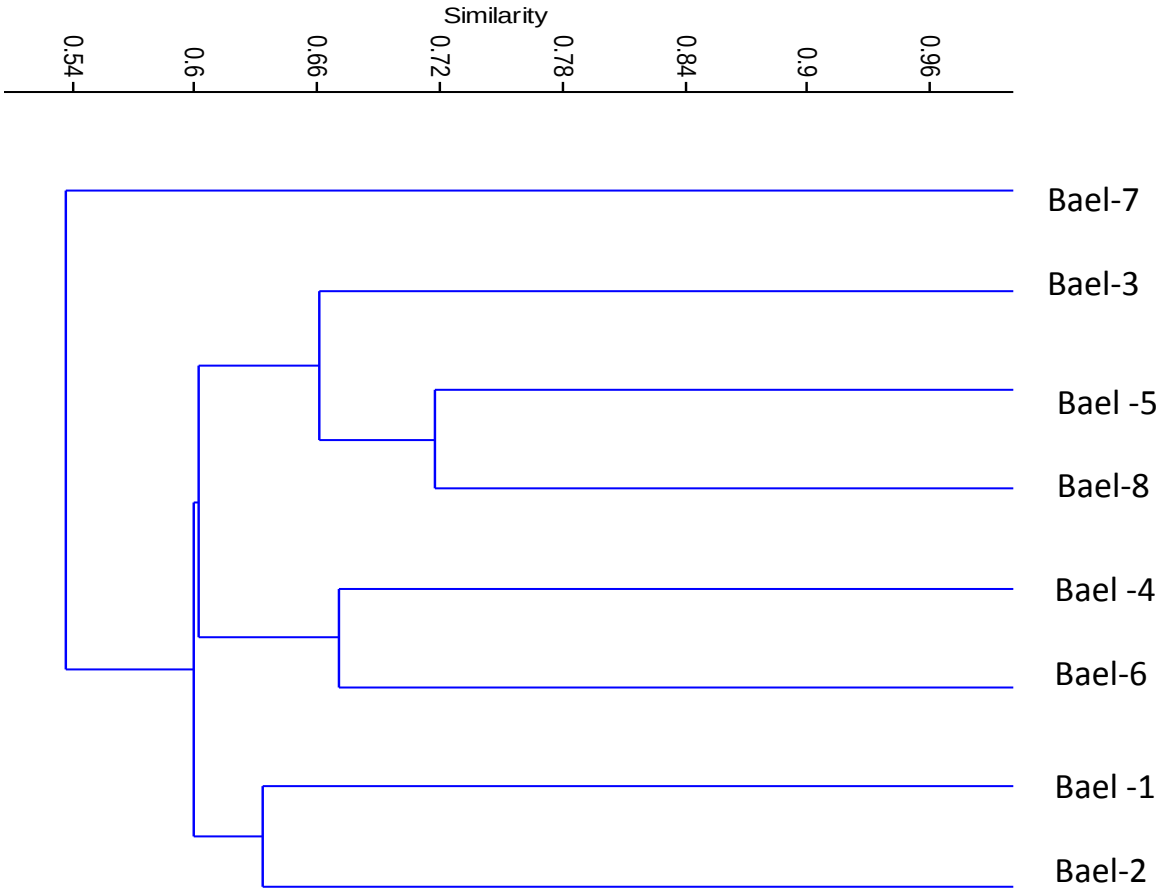

Figure 1. The UPGMA dendogram depicted by NTsys software based on RAPD data derived from similarity coefficients, showing the relationship of bale genotypes.

diversity results from RAPD markers differ from the morphological diversity. The cluster analysis of morphological characteristics, 'Bael-2' was the most dissimilar genotypes while in molecular analysis showed 'Bael-7' as the most divergent genotype. The high level similarity detected between 'Bael-5' and 'Bael-6' in the morphological analysis was not corroborated by the RAPD analysis that showed a large genetic distance between them.

DISCUSSION

Characteristics showing greater quantity range had higher coefficient of variation (CV), meaning a higher selection possibility for those characteristics in improvement programme of bael. Fingerprinting and assessment of genetic variability among accessions is of interest in terms of genetic resource conservation but also for practical application of breeding. Phenotypic des-

criptor can identify accessions but are environmentally affected. They do not allow the quantification of the genotypic similarity between accessions as do genetic distances from DNA polymorphism. The simplicity of laboratory assay for RAPD markers makes them an attractive method for obtaining intraspecifc distinctions. This technique is already used for cultivar identification and genetic variability analysis of several underutilized fruit crops like loquat (Badenes et al., 2004), tamarind (Diallo et al., 2007) and for protecting plant patents (Baird et al., 1996), although some question regarding reliability of RAPDs have been raised. In this study, a set of RAPDs was used for distinguishing the superior genotypes of bael. The comparatively higher percentage of polymorphic bands detected in the present study clearly indicated that RAPD fragments are moderately polymorphic and particularly informative in the estimation of genetic relationship of bael genotypes. According to Faleiro et al. (2009), the number and percentage of polymorphism in RAPD fragments depends on the number and variability of the genotypes and/or accessions analysed. Comparatively high amplitude of the genetic similarity coefficient established in the present study confirms the occurrence of considerable genetic variability among bael genotypes. However, variation was higher than that reported by Rajwana et al. (2008) for 25 cultivars of mango (range 0.69-0.89) but lower than that reported by Karihaloo et al. (2003) for mango cultivars (0.32 -0.75). The highest genetic similarity coefficient of 0.72 between the 'Bael-5' and 'Bael-8' might be due to their same place of origin (North Andaman) or occurrence of an intense gene flow between these genotypes.

The genotypes 'Bael-1' and 'Bael-7' showed the lowest similarity coefficient, which could be due to their differences in fruit characteristics and genetic back ground. But, the molecular diversity was not in agreement with the most of the morphological diversity as also reported by Singh et al. (2012) in *Colocasia esculenta*. These findings support the view that morphopomological characteristics are not the only reliable tools in estimating genetic relationships among large and diverse groups of genotypes/cultivars and should be used mainly for discrimination. However, genetic diversity of some *Rosa* genotypes which were analyzed by RAPD, amplified fragment length polymorphism (AFLP) and morphological characteristics showed a significant correlation among the different marker systems (Wen et al., 2004).

Dif-ferences in results obtained in grouping by using RAPD markers and grouping by phenotaxonomical characters were also reported in strawberry and banana (Garcia et al., 2002; Uma et al., 2004). This can be related to many reasons; one is the effects of grouping by phenol-taxonomical characters were also reported in different climatic conditions on morphological traits, which do not influence RAPD markers (Kumar, 1999; Gupta and Rustgi, 2004). The fragments of genome amplified with RAPD primers may not be part of codons for morphological characteristics (Sarkhosh et al., 2009). It should also be noted that post-transcriptional modifycations and non-nuclear inheritance of some characterristics can cause the lack of fitting of morphological markers with molecular markers (Gupta and Rustgi, 2004). Most of the developed DNA markers are based on the genomic DNA and therefore could belong to either the transcribed regions or the non-transcribed regions of the genome. These markers derived from any region of the genome have also been described as random DNA markers (Sarkosh et al., 2006).

However, during the recent past, research emphasis shifted towards the development of molecular markers from the transcribed region of the genome. The availability of a large number of cDNA clones in a variety of plant systems, and also accumulation of a large number of expressed sequence tags (EST) have made this possible. So far, not a single report has been published on genetic diversity analysis of bael using advanced molecular marker systems viz. sequence-characterized amplification regions (SCAR), simple sequence repeats (SSR), single nucleotide polymorphism (SNP) and EST due to lack of genetic information in bael. Therefore, future research programme should be concentrated to develop advanced marker systems for genetic diversity analysis and identification of candidate marker linked desirable traits and genes.

Conclusions

RAPD analysis has been shown to be useful technique for providing information concerning the degree of polymorphism and diversity of bael as well as for characterizing the germplasm. Findings of this experiment indicate that identification and collection of genotypes from various locations of the country / province, which was mainly based on morphological characteristics, may have encountered the mismatches and mistakes. This emphasizes the importance of characterization both at molecular and morphological 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.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Director, Central Agricultural Research Institute, Port Blair for providing financial assistance and necessary facilities for the study.

REFERENCES

Badenes ML, Canyamas T, Romero C, Martınez-Calvo J, Giordan E, Llacer G (2004). Characterization of under-utilized fruits by molecular markers. A case study of loquat. Genet. Resour. Crop Ev. 51:335–341

Baird WV, Ballard RE, Rajapakse S, Abbott AG (1996). Progress in Prunus mapping and aplication of molecular markers to germplasm improvement. Hort. Sci.1099–1106.

Brijesh S, Daswani P, Tetali P, Antia N, Birdi T (2009). Studies on the antidiarrhoeal activity of *Aegle marmelos* Corr. unripe fruit: Validating its traditional usage, BMC Complement. Alter. Med. 9 (47):9-47

Chauhan A, Agarwal M, Kushwaha S, Mutreja A (2008). Antifertility studies of *Aegle marmelos* Corr., an Indian medicinal plant on male albino rats, Egypt. J. Biol. 10:28-35

Diallo BO, Joly HI, Mckey D, Mckey MH, Chevalier, MH (2007). Genetic diversity of *Tamarindus indica* populations: Any clues on the origin from its current distribution? Afr. J. Biotechnol. 6 (7):853-860

Faleiro FG, Pinto ACQ, Cordeiro MCR, Andrade, SEM (2009). Genetic variability of mango landraces from Mexico based on molecular markers. Acta Horticult. 820:177-182.

Garcia MG, Ontivero M, Diaz Ricci JC, Castagnaro A (2002). Morphological traits and high resolution RAPD markers for the identification of the main strawberry varieties cultivate in Argentina. Plant Breed. 121:76-80.

Gupta PK, Rustgi S (2004). Molecular markers from the transcribed/ expressed region of genome in higher plants. Funct. Integr. Genom. 4:139-162.

Kafkas S, Perl-Treves R (2001). Morphological and molecular phylogeny of *Pistacia* species in Turkey. Theor. Appl. Genet. 102:908-915.

Karihaloo JL, Dwivedi YK, Archak S, Gaikwad AB (2003). Analysis of genetic diversity of Indian mango cultivars using RAPD markers. J. Hort. Sci. Biotechnol.78:285-289

Kumar LS (1999). DNA markers in plant improvement. Biotechnol. Adv. 17:143 -183.

Nath V, Pandey D, Das B (2003). Diversity of Bael (*Aegle marmelos* Corr.) in East Central India, Indian J. Plant Genet. Resour. 16 (3):222-225

Rai D, Misra KK (2005). Studies on genetic divergence in Bael (*Aegle marmelos* Correa), Indian J. Hort. 62:152-154.

Rajasekaran C, Kalaivani T, Ramya S, Jayakumararaj R (2009). Studies on Hepatoprotective Activity of Ethanolic Extracts of Fruit pulp of *Aegle marmelos* (L.) Corr. J. Pharmacy Res. 2(8):1419-1423

Rajwana IA, Tabbasam N, Malik AU, Malik AS (2008). Assessment of genetic diversity among mango (*Mangifera indica* L.) genotypes using RAPD markers. Sci. Hort. 117:297-301

Rohlf, FJ, NTSYS - PC Numerical Taxonomy and multivariate analysis system Ver. 1. 60 (2000). Exeter Publ. Ltd., Setauket, New York.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proceeding of National Academy of Science, USA, 81 (24):8014-8018.

Sarkhosh A, Zamani Z, Fatahi R, Ebadi A (2006). RAPD markers reveal polymorphism among some Iranian pomegranate (*Punica granatum* L.) genotypes. Sci. Hort. 111: 24-29.

Sarkhosh A, Zamani Z, Fatahi R, Ranjbar H, (2009). Evaluation of genetic diversity among Iranian soft-seed pomegranate accessions by fruit characteristics and RAPD markers. Sci. Hort. 121, 313–319.

Singh S, Singh DR, Faseela F, Kumar N, Damodaran V, Srivastava RC (2012). Diversity of 21 taro *(Colocasia esculenta* L.) Schott) genotypes of Andaman Islands. Genet. Resour. Crop Ev. 59:821-829

Srivastava AK, Singh HK, Prasad J (1998). Genetic diversity Bael (*Aegle marmelos* Correa). Indian Hort. 42 (2): cover page-ll.

Uma S, Sudha S, Saraswathi, MS, Manickavasagam M, Selvarajam R, Durai P, Sathiamoorthy S, Siva SA (2004). Analysis of genetic diversity and phylogenetic relationships among indigenous and exotic Silk (AAB) group of bananas using RAPD markers. J. Hort. Sci. Biotechnol. 79:523-527.

Venkateswarlu M, Raje US, Surendra NB, Shashidhar HE, Maheswaran M, Veeraiah TM, Sabitha MG (2006). A first genetic linkage map of mulberry (*Morus* spp.) using RAPD, ISSR and SSR markers and pseudotestcross mapping strategy. Tree Genet. Geno. 3:15-24.

Wen XP, Pang XM, Deng XX (2004). Characterization of genetic relationships of *Rosa roxburghi* Tratt. and its relatives using morphological traits, RAPD and AFLP markers. J. Hort. Sci. Biotechnol. 79:189-196.

Zamani Z, Sarkhosh A, Fatahi R, Ebadi A (2007). Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. J. Hort. Sci. Biotechnol. 82: 11-18.

Zamani Z, Zarei A, Fatahi R (2010). Characterization of progenies derived from pollination of pomegranate cv. Malase-Tourshe-Saveh using fruit traits and RAPD molecular marker. Sci. Hort. 124:67-73.