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Analysis of *Aegle marmelos* (L.) Corr. diversity using citrus based microsatellite markers

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Abstract

Analysis of diversity by citrus based microsatellite set in *Aegle marmelos* (L.) Corr. was undertaken because molecular markers are DNA based markers and reveal the genetic diversity which is more universal. Genetic diversity of *A. marmelos* was measured by using 10 microsatellite markers. A total of 47 alleles were detected in *A. marmelos* across the 10 loci investigated, all these alleles were polymorphic, thus revealing a level of 100% polymorphism. The number of observed alleles assorted flanked by 4 to 7 with mean 4.7± 1.059 alleles at each locus. The experimental no. of alleles intended for every 10 loci gone beyond the effectual no. of alleles that assorted between 1.384 and 3.164 with an average value 1.995±0.11. In present study the mean observed heterozygosity was 0.127+0.06, which was not more than heterozygosity (expected). The expected heterozygosity varied from 0.164 (CCSM147) to 0.685 (CT19) with mean value 0.407±0.177. Since, observed heterozygosity is less than the expected heterozygosity seems to be due to inbreeding.

Key words: Simple sequence repeat, *A. marmelos* (L.) Corr., heterozygosity.

Introduction

Microsatellite DNA includes nucleotide string which is formed by one to six tandem repeatable bases, extensively dispersed in genome. It has advantages for instance codominant, high polymorphism, steady amplification outcomes, excellent repeatability as well as easy recognition (Mengpei *et al.,* 2011). Microsatellite markers are very valuable tools for distinguishing the agricultural crop assortment (Padilla *et al.,* 2014). Conventional expansion of microsatellite markers is very expensive as well as lengthy since it engages library creation as well as sequencing of clone. Genome of plant study has been paying attention on main crops as well as model species (Vogel *et al*., 2010). According to Zhao *et al.* (2011), various open databases related with microsatellite markers, which are accessible in favor of main crops as well as plant species which are used as model. Penjor *et al*. (2013) investigated as well as sequenced for matK genes in the chloroplast with 135 accessions by using 22 genera of Aurantioideae and furthermore evaluated them phylogenetically. Their work incorporated numerous accessions which have not been observed in other investigations. Results signified that the species of citrus can be categorized into 3 groups: a pummelo group, a citron group as well as a mandarin group. Rai *et al.* (2013) investigated the elevated intensity of microsatellites in guava plant as cross genera transferability which is appropriate in favor of the investigation of intra as well as inter specific heritable assortment of intented species, for which no information regarding EST-derived in addition to genomic microsatellite is accessible. Nayak *et al.* (2013) estimated inherent inconsistency of bael of Andaman Islands by using RAPD primers. Present work was proposed to assess genetic variability in bael by using citrus specific microsatellite markers. According to the best of our information this is the first time when citrus specific primers have been utilized in this plant while earlier other molecular markers were utilized in place of SSR.

Materials and methods

Leaves of *A. marmelos* were collected from 40 different places from India (Table 1). Tender leaves of individual plants were used for the extraction of DNA, for diversity analysis. Sampling was done from north and western region of India whihc included five agroclimatic zones. Eight locations were selected from each zone so total samples were 40 (Table 1 and Fig. 1).

Extraction of DNA: Extraction of DNA was done from tender foliages by CTAB technique as given by Doyle and Doyle (1990).

Purification of DNA: Purification of DNA was carried out by phenol extraction and ethanol precipitation method.

Estimation of quantity and quality of DNA: 0.6% Agarose gel was run to determine the quantity as well as quality (in terms of smear depicting degradation) of the DNA in the sample from comparison of intensity of fluorescence yield by Ethidium bromide (Etbr).

Polymerase chain reaction: DNA was amplified by thermal cycler (PCR machine) (Bangalore Genei Pvt. Ltd., India/ Biorad, India), PCR mix was used for one reaction as Genomic DNA (50 ng) 2.0 µL, 1X Buffer (Taq) 2.5 µL, $Mgcl_2$ (25 mM) 0.5 µL, dNTP mix (10 mM) 2.0 µL, Primer (forward-10 pmol) 2.0 µL, Primer (reverse-10 pmol) 2.0 µL, Taq polymerase (1 unit) 0.3 µL, Distilled water (nuclease free) 13.7 µL, total quantity was 25.00 µL. After adding all the ingredients mixed gently and after centrifugation immediately placed the tubes in the thermal cycler which was pre programmed using the following temperature profile: An initial denaturation of 5 min at 94 ^{0}C followed by 35 cycles of 1 min at 94 ^{0}C, 1 min at 57 ^{0}C and 2 min at 72 ^{0}C, then final extension of 10 min at 72 ^{0}C then stored at 4 ^{0}C. After completion of the PCR programme, the PCR products were checked on 2% Agarose for the amplification in electrophoresis unit (Bangalore Genei Pvt. Ltd., India). Before loading into the

Table 1. Latitude and longitude of places of sample collection

Sample Id	Name of place	Latitude	Longitude	Sample Id	Name of place	Latitude	Longitude
1	Aligarh	26.90	80.94	21	Kota	25.21	75.86
2	Bareilly	28.36	79.43	22	Morena	26.49	77.99
3	Bulandshahr	28.42	78.01	23	Sheopur	25.67	76.69
4	Farrukhabad	27.38	79.59	24	Shivpuri	25.43	77.66
5	Hardoi	27.39	80.13	25	Ahmadnagar	19.09	74.74
6	Kannauj	27.05	79.91	26	Aurangabad	19.87	75.34
7	Kanpur	26.41	80.23	27	Jalna	19.82	75.88
8	Unnao	26.53	80.48	28	Nashik	19.99	73.78
9	Amritsar	31.63	74.87	29	Pune	18.52	73.85
10	Ganganagar	29.49	73.50	30	Sangli	16.85	74.58
11	Hisar	29.14	75.72	31	Satara	17.68	74.01
12	Jalandhar	31.32	75.57	32	Solapur	17.65	75.90
13	Jind	29.32	76.30	33	Ahmedabad	23.02	72.57
14	Ludhiana	30.90	75.85	34	Anand	22.54	72.95
15	New Delhi	28.65	77.16	35	Jamnagar	22.47	70.05
16	Rohtak	28.89	76.60	36	Junagadh	21.52	70.45
17	Banasthali	26.39	75.86	37	Mahesana	23.58	72.36
18	Bundi	25.27	75.41	38	Morvi	22.81	70.82
19	Guna	24.63	77.29	39	Patan	23.84	72.12
20	Gwalior	26.21	78.18	40	Rajkot	22.30	70.80

Fig. 1. Sample collection sites

wells, gel-loading dye (Xylene-cyanol and Bromophenol blue in glycerol) was added to the product. After loading the products into the wells, the products were run under constant voltage conditions (100 V) till the two dyes got separated. Amplified products appeared as sharp pink color bands under UV transilluminator due to the intercalation of Ethidium bromide.

Agarose (ultra resolution) gel electrophoresis: The amplified samples were identified on ultra resolution agarose and results were recorded for further analysis on 3% Ultra resolution agarose.

Microsatellite genotyping: The allelic data was fed in excel sheet to use in input files of population analysis software POPGEN32.

Results and discussion

Ten citrus specific microsatellite primers were selected for genetic characterization of *A. marmelos*, details of these are given in Table 2**.** Each of the ten citrus specific microsatellite markers augmented fine with *A. marmelos*, which have been documented to be polymorphic in Rutaceae family, after amplification, generated specific banding prototype from which assessing was done for individual genotypes. A total of 47 alleles were detected in *A. marmelos*, across the 10 loci investigated, all these alleles

Table 2. List of primers

Locus Name	Repeat	Forward Primer	Reverse Primer	GC (%)	Allele
AG14	AG	AAAGGGAAAGCCCTAATCTCA	CTTCCTCTTGCGGAGTGTTC	49	4
ATC09	ATC	TTCCTTATGTAATTGCTCTTTG	TGTGAGTGTTTGTGCGTGTG	40	4
CT19	(CT)n	CGCCAAGCTTACCACTCACTAC	GCCACGATTTGTAGGGGATAG	53	5
CT21	CT	CGAACTCATTAAAAGCCGAAAC	CAACAACCACCACTCTCACG	48	7
CTT01	CTT	TCAGACATTGAGTTGCTCG	TAACCACTTAGGCTTCGGCA	49	4
TC26	(TC)n	CTTCCTCTTGCGGAGTGTTC	GAGGGAAAGCCCTAATCTCA	53	4
CCSM2	(AG)n	TTGCCCACTTGGTCACTCAC	GGAACGTCAGCATCGAAGAA	53	4
CCSM13	(AG)n	CTAGAGCCGAATTCACC	AACAGCTACCAAGACACC	51	5
CCSM147	(AG)18	AGACTCACGTAACCTACTTC	GCTATGTTATGATACGTCTG	43	4
CCSM17	(AG)n	ACATGGACAGGACAACTAAG	GTTATGATACGTCTGTGTCC	45	6

Table 3. Allele Frequencies at ten SSRs in *A. marmelos*

Locus/Allele	A	B	C	D	E	F	G	H	I
AG14	0.05	0.862	0.062	0.025					
ATC09	0.1	0.037	0.812	0.05					
CT19	0.337	0.162	0.412	0.05	0.037				
CT21	0.025	0.025	0.637	0.162	0.062	0.037	0.05		
CTT01	0.05	0.837	0.087	0.025					
TC26	0.1	0.075	0.8	0.025					
CCSM2	0.8	0.125	0.05	.025					
CCSM13	0.037	0.462	0.175	0.287	0.037				
CCSM147	0.037	0.912	0.025	0.025					
CCSM17	0.025	0.712	0.2	0.025	0.025	0.012			

were polymorphic, thus revealed a level of 100% polymorphism (Table 3).

Observed alleles were from 4 (AG14, ATC09, CTT01, TC26, CCSM2, CCSM147) to 7 (CT21) with an overall mean of 4.7 ± 1.059 alleles per locus (Table 4). Padilla *et al.* (2014) found an overall of one hundred ten amplified fragments, with mean 7.9 alleles at every locus. 12 of the SSR loci deemed as extremely useful, with the expected heterozygosity more than 0.5. While, Wang *et al.* (2014) made the study on *Prunus sibirica* L., with 31 nuclear SSRs, they explored the intensity of hereditary assortment as well as inhabitant organization of Siberian apricot which were trialed from twenty two populations transversely in China. Allele's amount at each locus varied between five and thirty three and mean value was 19.323 alleles. Walvekar and Kaimal (2014) used 13 ISSR primers for preliminary screening regarding polymorphic summary produced 83 bands transversely 4 mutagenic variant of *A. marmelos*, out of which 59 were polymorphic bands showed 71% polymorphism. Rai *et al.* (2013) examined that alleles assorted between one to nine, and mean values of alleles were 4.6, 4.5, 4.8 and 2.6 in *S. aromaticum, C. lanceolatus, E. citriodora,* and *E. camaldulensis*, correspondingly. Fan *et al*. (2013) utilized sixty seven microsatellite markers, and identified 277 alleles with mean of 4.13 alleles at each locus. Nayak *et al.* (2013) projected genetic inconsistency of bael of Andaman Islands by using 12 RAPD primers. Total 476 polymorphic loci were recognized with an average of 39.66 bands for every primer as well as 63.99% polymorphism detected. Present results were in agreement with Sharma and Sharma (2012). Hao *et al*. (2015) studied the genetic multiplicity of white birch (*Betula platyphylla*), two hundred fifteen primers demonstrated polymorphism among 5 genotypes as well as one hundred eleven primers to facilitate discernible bands of 41 *Betula platyphylla* plants which were composed from 6 diverse geographical areas. Total 717 alleles were achieved at 111 loci between 2 to 12 alleles at each locus. Zhao *et al.* (2011) identified 162 alleles by using 49 microsatellite markers from *Miscanthus sinensis*. The SSRs markers extended by the proportional genomic approach can be practical for genetic study, germplasm assessment as well as marker-assisted propagation in *Miscanthus species*. Cristofani *et al*. (2011. showed the polymorphism level of the twenty four SSRs loci in twelve citrus varieties. Entire sets of primer augmented microsatellites for anticipated magnitude or range which were earlier used for citrus cultivars, out of which twelve were polymorphic. The number of recognized alleles at every locus varied between one and six with mean value of alleles 2.9 at each locus. The average number of alleles per locus in *A. marmelos* was well and accordingly indicative of adequate polymorphism and their appropriateness for assessing genetic variation. The alleles observed no. gone beyond the effectual alleles no. assorted from 1.197 (CCSM147) to 3.18 (CT19) with an average of 1.875±0.712.

Table 4. Genetic variation in *A. marmelos*

Locus	Na* (Number of alleles observed)	Ne* (Number of alleles effective)
AG14	4	1.331
ATC09	4	1.483
CT19	5	3.180
CT21	7	2.263
CTT01	4	1.404
TC26	4	1.523
CCSM2	4	1.518
CCSM13	5	3.030
CCSM147	4	1.197
CCSM17	6	1.819
Mean	4.7	1.875
SD.	1.1	0.712

According to the UPGMA dendrogram (Fig. 2) based on similarity matrix, sample 25 and 34 were highly similar (similarity index 1.00) with minimum genetic distance 0, which was followed by sample 8 and 19, sample 13 and 31, sample 26 and 30, sample 28 and 26 with genetic distance 0.05, 0.05, 0.07, 0.08, respectively. Sample 5 and 24 were least similar (similarity index 0.17) with maximum genetic distance 1.76, which was followed by sample 3 and 24, sample 16 and 24, sample 17 and 24 with genetic distance 1.55 each, respectively. This variation in similarity may be due to different geographical as well as different ecological conditions of the area from where samples were collected for the estimation of diversity. Based on this study, the large range of similarity values for related samples using microsatellites markers provided greater confidence for the assessment of diversity and relationships.

According to Lia (2013), other parameter heterozygosity (observed) was considered for the estimation of diversity, which is a significant indicator of diversity, since each heterozygote carries different alleles and best represents the variation in autogamous and allogamous populations. Hetrozygosity parameters are indicative of population variability of *A. marmelos* is given in Table 5.

Table 5. Observed and average heterozygosity in *A. marmelos*

Locus	Observed heterozygosity	Average heterozygosity
AG14	0.175	0.249
ATC09	0.15	0.325
CT19	0.05	0.685
CT21	0.2	0.558
CTT01	0.1	0.287
TC26	0.05	0.343
CCSM2	0.05	0.341
CCSM13	0.2	0.67
CCSM147	0.15	0.164
CCSM17	0.15	0.45
Mean	0.127	0.407
SD	0.06	0.177

The polymorphic loci number is: 10
The polymorphic loci percentage is: 100.00%

Table 5 shows the mean observed heterozygosity was 0.127±0.06, which was not more than heterozygosity (expected). The expected heterozygosity (average) varied from 0.164 (CCSM147) to 0.685 (CT19) with the mean of 0.407±0.177. The expected heterozygosity is also called as gene diversity which can be calculated from individual allele frequencies. If observed heterozygosity is less than the expected heterozygosity it may be due to inbreeding *i.e.,* self-fertilization, the most extreme form of inbreeding possibilities, and it is the characteristic of many flowering plants so it may be due to this reason we have got such results (Holsinger, 1990). Results were in consensus with the mean observed heterozygosity 0.359 as well as expected heterozygosity 0.430 in hop (Jakse *et al*., 2011). Hao *et al*. (2015) studied the multiplicity of *Betula platyphylla* and found expected heterozygosity in between of 0.22 to 0.54 with mean 0.46, heterozygosity (observed) was in between of 0.02 and 0.95 with mean 0.26. The observed heterozygosity found for cultivars and wild accessions was 0.587 and 0.568, respectively according to Feng *et al.* (2012). Padilla *et al.* (2014) investigated 12 SSR markers and found the values of heterozygosity (expected) more than 0.5. Inbreeding estimate based on within population f (F_{IS}) was considerably affirmative on the basis of table extensive

Table 6. Fixation index (F_{IS})

Locus	F_{IS}
AG14	0.297
ATC09	0.539
CT19	0.927
CT21	0.641
CTT01	0.652
TC26	0.854
CCSM2	0.853
CCSM13	0.701
CCSM147	0.089
CCSM17	0.666
Mean	0.622

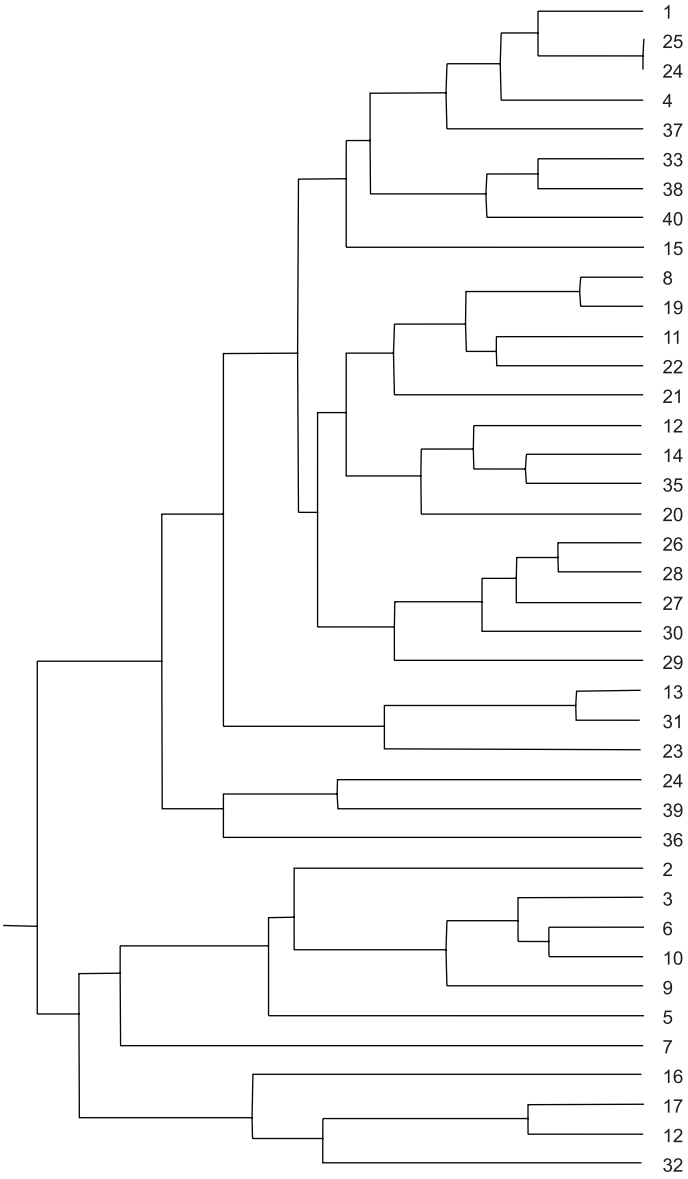

Fig. 2. Dendrogram based on Nei's (1972) genetic distance: Method = Unweighted Pair Group Method with Arithmetic Mean (UPGMA) modified with DendroUPGMA: A dendrogram construction utility (URL address is http://genomes.urv.cat/UPGMA/draw/tree.php) Garcia *et al*. (1999)

randomization (P<0.05). f-estimate varied between 0.089 and 0.927 and mean value is 0.622 (Table 6).

Thus on an average significant deficit of 62.2% of heterozygote exists in the *A. marmelos* population. All the microsatellite markers contributed to the observed heterozygote shortage. The noteworthy heterozygote insufficiency established in *A. marmelos* may possibly be due to various reasons like Wahlund consequences (existence of inhabitants substructure), locus in choice (hereditary hitch-hike), separation of non-amplifying (unacceptable) alleles, scoring preconception (heterozygotes scored inaccurately like homozygotes) or inbreeding. However, unacceptable alleles are mainly implausible to be separated out at each and every locus. There is no physical barrier which can create sub populations in the bael, ruling out the contribution of Wahlund effect. All loci were established as neutral in Ewen Watterson neutrality analysis, hence it can be accomplished that moderate homozygosity is not due to selection.

Allelic richness was estimated by the total number of alleles present. A total of 47 alleles were detected in *A. marmelos* across the 10 loci, all these alleles were polymorphic, which revealed a level of 100% polymorphism. Number of alleles observed diverse between 4 and 7 with mean 4.7±1.059 alleles per locus. Observed number of alleles for every 10 loci exceeded the effective number of alleles which varied between 1.384 and 3.164 with mean 1.995±0.11. Other parameter heterozygosity (observed) was considered for the estimation of diversity, which is a significant indicator of diversity, since each heterozygote carries different alleles and best represents the variation in autogamous and allogamous populations. The expected heterozygosity is also called as gene diversity which can be calculated from individual allele frequencies. In this study the mean observed heterozygosity was 0.127+0.06, which was not more than heterozygosity (expected), this may be due to the inbreeding. The expected heterozygosity varied from 0.164 (CCSM147) to 0.685 (CT19) with mean of 0.407±0.177. Inbreeding is the characteristic of many flowering plants and possible explanation for getting observed heterozygosity less than the expected heterozygosity. The results indicate that diversity conservation is needed to maintain the heterozygosity.

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