The Pharma Innovation Journal 2022; 11(12): 4895-4899

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(12): 4895-4899

www.thepharmajournal.com
Received: 02-10-2022
Accepted: 06-11-2022

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Molecular screening of alphonso mother plant (*Mangifera indica* L.) and its seedlings through molecular markers

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Abstract
The most valued table cultivar of mango (*Mangifera indica* L.), known as "Alphonso," has been grown in India for more than a century. In order to explore intra cultivar heterogeneity based on microsatellite markers, 15 seedlings of Alphonso mango were grown by the stones taken from identified mother plant from Agriculture Research Station, Shirgaon, District-Ratnagiri (Maharashtra). It was investigated that whether there was genetic heterogeneity or relatedness between the chosen seedlings and their mother plant. The all fourteen verified mango-specific simple sequence repeats (SSRs) were found polymorphic. In the result we found total 224 alleles were produced by polymorphic microsatellites, they showed the 100 % polymorphism. The Jaccard's similarity coefficient ranged from 0.037 to 0.867. The polymorphic information content (PIC) values were observed between 0.218 (SSR-19) to 0.964 (SSR-26). The Microsatellites with a high degree of polymorphism expressed by primer SSR-26, SSR-24, SSR-16 and MngSSR-26, were more effective in differentiating the chosen alphonso samples.

Keywords: Alphonso, PIC, polymorphism, SSR

1. Introduction
Mango (*Mangifera indica* L., 2n=40), a member of the Anacardiaceae family, is one of the world's oldest and most significant tropical fruits. Because of its nutritious value, distinct flavour and attractive aroma, it is appropriately referred to as the "King of Fruits." It is also known as India's "National Fruit." It is believed to have originated in the Indo-Burma region of Southeast Asia and has been grown in India for over 4000 years (Mukherjee, 1953; Kostermans and Bompard, 1993) [22, 23]. India contains different types of mango varieties and the most mango germplasm in Southeast Asia. Mangoes are said to come in over 1,000 different varieties in India (Mukherjee, 1951; Singh, 1996) [12, 13, 19]. Based on cytogenetics, Mango is supposed to have a partly allopolyploid genome (Mukherjee, 1950). Several publications have demonstrated that genetic markers for mango are inherited in a disomic manner (Duval *et al*., 2005; Schnell *et al*., 2005, 2006; Viruel *et al*., 2005) [9, 16, 17, 21], implying that mango should be classified as diploid.

Alphonso mangoes are one of India's most popular varieties. In terms of area and productivity, it is the most widely grown cultivar in the Konkan region and it is known as 'Hapus' locally. Afonso de Albuquerque, a Portuguese General and military specialist who assisted in the establishment of Portuguese colonies in India, was the inspiration for the variety's name. The Portuguese pioneered grafting on mango trees, resulting in exceptional types such as Alphonso. The fruit was later brought to the Konkan area of Maharashtra, Goa, Gujarat and portions of Tamil Nadu, Karnataka and Kerala in the Southern states. Alphonso is well-known both nationally and globally for its appealing fruit form, colour, flavour, taste and aroma, as well as its exceptional post-ripening keeping qualities. In Konkan, Devgad Alphonso (Hapus) is known for its aroma, low of fibre and sweet flavour. These features are attributed to Devgad's geographical environment and are not found elsewhere.

For mango improvement programmes and genetic resource management, molecular analysis is essential. PCR-based DNA markers are potent tools for analysing Alphonso's molecular makeup. Molecular markers, unlike agronomic and morphologic features, are not affected by the environment. Mango characterisation has recently employed DNA markers obtained by PCR techniques (Singh *et al*., 2009; Bhargava and Khorwa, 2011; Begum *et al*., 2012) [20, 5, 2].

Many studies have employed SSR markers to characterize mango. Due to their multiallelic and highly polymorphic character, SSR markers can provide a better genetic diversity spectrum even when used in less number. (Singh *et al*., 2016) [18]

The present research work was undertaken with the following objectives: Fidelity testing of seedlings developed by stones of alphonso mango, Comparative molecular analysis of seedlings and mother plant through molecular markers (SSR markers).

2. Material and Methods

2.1 Plant material and DNA extraction

Mango stones were taken for seedling preparation from a identified mother plant (Plant no.2) grown at Agriculture Research Station, Shirgaon, District Ratnagiri. From the mango stones, seedlings were developed at the Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli. The two to three young leaves per assigned number were collected from each seedling and kept in paper bags. The leaves were wrapped in moist tissue paper and were refrigerated in -80 ^{0}C. The DNA was extracted from seedling leaves which were 13-14 days old, while from the mother plant tender leaves were utilized for DNA isolation.

The DNA was isolated using the Doyle and Doyle's (1990) [8] method with minor changes to the buffer composition and concentration. To avoid contamination, the young newly flushing 13-14 day old leaves were collected and sterilized with 70 percent ethanol. The following process was followed to isolate genomic DNA.

2.2 Screening of mango samples using SSR molecular markers

The mango samples were screened for fidelity testing and comparative molecular analysis using 15 SSR primers over different chromosomes. The SSR primers were acquired from the paper published by Begum *et al*. (2012) [2] and Begum *et al*. (2014) [4]. The details of the primers used are listed in table1.

2.3 Molecular screening of genotypes

The gels were systematically examined, and amplicons that had only been produced once for a certain variant were identified as containing the band for that variant. In addition, two genotypes' fragments were marked, which when combined with additional bands obtained with different primers, constituted the screening.

2.4 Scoring and Data Analysis

As a dominant expression, each amplification result was considered an SSR marker and was scored across all samples. The presence (+/1) or absence (-/0) of bands was determined. The size of each allele was assessed by utilizing software to run a DNA ladder at the same time (Uvi-Tec, Fire-reader software version 15.12). Using the tool MVSP-A (Multivariate Statistical Package - 5785 Version 3.1), the data was used for similarity-based analysis. To produce a dendrogram, similarity coefficients were utilized to construct UPGMA (unweighted pair group method with average).

The distance matrix and dendrogram were created using the UPGMA (Unweighted Pair Group Method of Arithmetic Means), a computer tool for distance estimation, based on the diversity coefficient produced from pooled data.

The following formula was used to compute the % polymorphism of the obtained bands:

$$\text{Percent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \text{ X } 100$$

3.2.5 Polymorphism Information Content

The value of the Polymorphism Information Content (PIC) was evaluated using a formula developed by Powell *et al* (1996) [14]

$$PIC = 1-\sum P_{ij}^2$$

Where,

- Pij is the frequency of the i_{th} and j_{th} loci, summed over all lines in the locus.
- For each profile created among 16 mango samples, PIC values ranging from 0 (monomorphic) to 1 (extremely highly discriminative, with multiple alleles each in equal and low frequency) were estimated.

Table 1: List of Mango Microsatellite markers

Sr.no	Primer Name	Sequences	Amplification Range (bp)	References
1.	SSR- 16	F: GCTTTATCCACATCAATATCC R: TCCTACAATAACTTGCC	150-180	Begum *et al.* 2012 [2]
2.	SSR-19	F: AATTATCCTATCCCTCGTATC R: AGAAACATGATGTGAACC	140-180	Begum *et al.* 2012 [2]
3.	SSR-20	F: CGCTCTGTGAGAATCAAATGGT R:GGACTCTTATTAGCCAATGGGATG	295-310	Begum *et al.* 2012 [2] & Begum *et al.* 2014 [4]
4.	SSR-24	F: GATGAAACCAAAGAAGTCA R: CCAATAAGAACTCCAACC	300-320	Begum *et al.* 2012 [2]
5.	SSR-26	F: GCCCTTGCATAAGTTG R: TAAGTGATGCTGCTGGT	180-230	Begum *et al.* 2012 [2]
6.	SSR-41	F: ATCCCCAGTAGCTTTGT R: TGAGAGTTGGCAGTGTT	210-244	Begum *et al.* 2012 [2] & Begum *et al.* 2014 [4]
7.	SSR-51	F: AAATAAGATGAAGCAACTAAAG R: TTAGTGATTTTGTATGTTCTTG	287	Begum *et al.* 2012 [2] & Begum *et al.* 2014 [4]
8.	SSR-52	F: AAAAACCTTACATAAGTGAATC R: CAGTTAACCTGTTACCTTTTT	207	Begum *et al.* 2012 [2] & Begum *et al.* 2014 [4]
9.	SSR-80	F: TGGTATTCAAGCATGGTCCCTC R: TCCCATCACACACACACAC	244	Begum *et al.* 2012 [2] & Begum *et al.* 2014 [4]
10.	SSR-84	F: TCTATAAGTGCCCCCTCACG	200-260	Begum *et al.* 2012 [2]

		R: ACTGCCACCGTGGAAAGTAG		
11.	SSR-85	F: GCTTGCTTCCAACTGAGACC R: GCAAAATGCTCGGAGAAGAC	250-310	Begum *et al.* 2012 [2]
12.	SSR-89	F: CGCCGAGCCTATAACCTCTA R: ATCATGCCCTAAACGACGAC	110-140	Begum *et al.* 2012 [2]
13.	MngSSR-14	F: TCATTAAGCTGTGGCAACCA R: CATTGCATAGATGTGGTCATT	160-192	Begum *et al.* 2012 [2] & Begum *et al.* 2014 [4]
14.	MngSSR-26	F: ACCTTGGTCAGGACAAAATCC R: GACTTCATAAGAAGAGGCGTC	135-150	Begum *et al.* 2014 [4]

3. Results and Discussion

3.1 Marker Analysis of the mango samples with mother plant

3.1.1 SSR analysis in between mango samples and mother plant

The SSR profile of 15 samples of alphon so mango seedlings developed from stones and one sample of mother plant were analyzed individually for each primer and were used for further analysis. The following individual samples were taken to assess the genetic variation present in between selected 15 leaves samples of mango seedlings developed from mango stones and one leaves sample of mother plant. The SSR patterns of genomic DNA of these samples were analyzed with respect to the fragments, in formativeness of the markers and polymorphism for the assessment of genetic diversity present in between selected samples are presented in Plate 1.

3.1.2 Percent polymorphism and Genetical distance between selected samples

In the present study we found total of 224 scoreable DNA fragments and all DNA fragments were polymorphic. The percent polymorphism detected across the 14 primers in 16 samples was found 100%. The product size ranged from 0.110 kb (SSR-89) to 0.320kb (SSR-24). The somewhat similar kind of findings observed by Begum *et al*., (2013) [3] in their study on intravarietal heterogeneity in thirty-one 'Beneshan' cultivar, they found a total of 58 polymorphic alleles using 23 SSR markers obtained 51.72% polymorphism rate. In thirty-one accession the PCR product size (kb) ranged from 0.100 (SSR-52) to 0.310 (SSR- 20).

In our study genetic distance between 16 samples of Alphonso was 0.037 to 0.867. The highest genetic distance was found between S11 and S13 (0.867), while the lowest genetic distance was found between S1 and S6, S1and S10, S1 and S14, S2 and S14 (0.037). Our results are on the line of the finding of de Souza and Lima, 2004 [6] worked on 25 accessions of 'Rosa a polyembryonic cultivar of mango using RAPD markers. They reported 55% of genetic dissimilarity.

Cluster analysis of selected samples

After analysing the banding patterns produced by 16 samples of the Alphonso mango using 14 SSR primers, a dendrogram based on Jaccard's similarity coefficient was constructed using UPGMA it was presented in Fig. 1. The dendrogram divided the 16-mango sample into I and II, two major clusters (Table 2).

The major cluster-I comprised 12 mango samples, and was further found to be divided into two sub clusters (IA and IB).

- Sub Cluster IA was further subdivided into two sub-sub clusters [IA (a) and IA (b)].
  - Sub-sub cluster IA (a) included only one sample *i.e*., S_{15}.
  - Sub-sub cluster IA (b) further subdivided into two sub-clusters [IA (ba) and IA (bb)].
  - Sub-sub cluster IA (ba) included six samples *i.e*., S_{14}, S_{13}, S_{11}, S_{10}, S_{12}, S_8.
  - Sub-sub cluster IA (bb) included three samples *i.e*., S_9, S_7, S_5.
- Sub Cluster IB included two samples *i.e.*, S_6, S_4.

The major cluster-II comprised 4 mango samples, and was further found to be divided into two sub clusters (IIA and IIB).

- Cluster IIA included of one sample *i.e*., S_3.
- Sub Cluster IIB included of one sample *i.e*., S_1, S_2, M.

From a foregone discussion, it can be observed that the major cluster II (sub cluster IIA and IIB) showed close affinity towards their mother plant M particularly S_1seedling (64.7%). Existence of fifteen seedlings individual accession (S_1 to S_{15}) and leaf sample collected from mother plant accession (M). From 16 leaf samples sub groups indicate that there was heterogeneity among the seedling, which could not give any insight about the homogeneity within samples. This information can be extremely useful to the mango nurseries for helping in the correct choice of mango multiplication material.

Similar results were obtained by Begum *et al*., (2013) [3] in his study genetic relationship among the "Beneshan" accessions were assessed by the cluster analysis of similarity matrix. A similarity matrix based on the proportion of shared SSR alleles was used to established the level of relatedness between accessions samples. All of the three accessions collected from the mother block nursery are genetically dissimilar, it is evident that there is heterogeneity within the mother block at the DNA level.

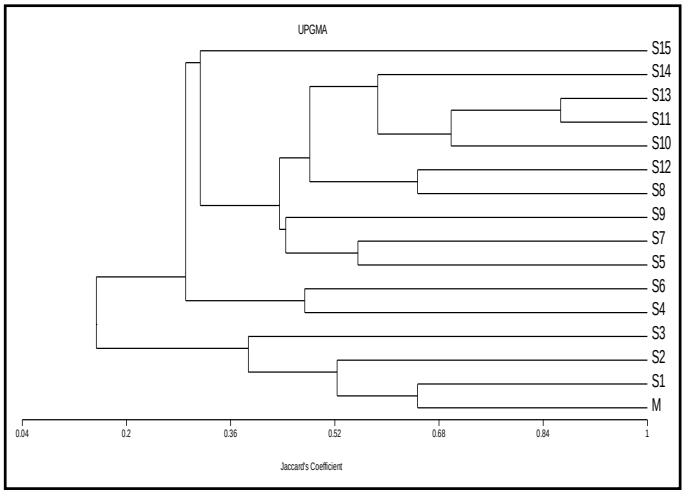

Fig 1: Dendrogram depicting Alphonso samples based on the genetic distance by 14SSR primers.

Table 2: SSR clustering pattern of selected mango samples

Cluster	Sub cluster	Sub-sub cluster		No. of samples	Samples
I	IA	IA(a)		1	S15
		IA(b)	IA(ba)	6	S_{14}, S_{13}, S_{11}, S_{10}, S_{12}, S_8
			IA(bb)	3	S_9, S_7, S_5
	IB			2	S_6, S_4
II	IIA			1	S_3
	IIB			3	S_1, S_2, M

Polymorphic Information Content (PIC)

The PIC values for the 14 SSR primers were evaluated. The primer SSR-26 produced the highest PIC value in the present study (0.964), whereas the primer SSR-19 produced the lowest PIC value (0.218). The average polymorphism information content of all samples was observed 0.533. The primer's in formativeness value was enhanced by its higher PIC value. The best markers for molecular profiling and genetic diversity analysis are those that can identify a large number of distinguishable alleles. For all of the tested SSR loci, the polymorphism information content (PIC) value of each marker, which may be assessed based on its alleles, showed significant variance. The PIC demonstrates the locus's ability to discriminate by taking into account not only the number of expressed alleles but also their relative frequencies and the frequency of alleles per locus (Samant *et al*., 2010) [24].

Begum *et al*., (2013) [3] studied intracultivar heterogeneity-based microsatellite markers. They reported that the polymorphic information content values varied from 0.03 (SSR-59) to 0.72 (SSR-87). Highly polymorphic microsatellites like SSR-80, SSR-87, SSR-28 and SSR-89 were more useful in differentiating the 'Beneshan' accessions.

In nutshell, these data extend the knowledge of SSR application as a molecular tool in intravarietal improvement of mango as reported by Bally *et al*. (1996) [1], de Souza and Lima (2004) [6] and Rocha *et al*. (2012) [15], who have used ISSR and RAPD markers for molecular characterization of intravarietal heterogeneity in different cultivars of mango.

This investigation is an attempt in demonstrating the usefulness of molecular markers in the similarity as well as the band sharing studies in mango seedlings and their mother plant. This study revealed that SSR markers are useful not only for deriving the molecular profiles of the mango seedlings and their mother plant but also in the efficient selection mango seedlings at initial stage.

4. Conclusion

This study indicated that SSR Markers are suitable for the fidelity testing of seedlings developed from stones of identified mother plant and sample of mother plant of Alphonso mango. In this study fidelity testing of seedling of mother plant stone and identified mother plant of Alphonso mango was established. The SSR analysis revealed that the 100 percent polymorphism between seedlings developed from stones of identified mother plant and sample of mother plant. The results of present study indicated the efficiency of SSR Markers in investigating the genetic variation at molecular level. Such study is important for detecting the distinctness present in seedling developed from stones of identified mother plant and sample of mother plant. This study is also important for identification of desire samples and its utilization for further breeding programs. Thus, the variations among the mother plant and their developed seedling revealed the presence of certain distinguishing characteristics in the seedling and mother plant. This study helps in screening the planting material at initial stage of planting which could save the heavy losses as well as resources of the mango growers and also extends the knowledge to the mango breeders for developing authentic planting material.

Acknowledgements

Authors are thankful to, Plant Biotechnology centre, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli- 415712, Dist. Ratnagiri, Maharashtra (India), for providing necessary facilities and valuable suggestion during investigation.

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