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RESEARCH ARTICLE

SSR assisted identification of mango (*Mangifera indica* L.) hybrids and development of DNA barcodes

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Abstract

The importance of varietal identification has grown dramatically on a global scale, particularly in the context of plant variety protection. To address the same, 100 new hyper-variable mango simple sequence repeats (HMSSRs) markers were screened for polymorphism among 24 mango hybrids bred at ICAR- IARI, New Delhi. Out of which, 89 polymorphic HMSSRs were used for the generation of DNA barcodes of mango hybrids. A total of 1,861 alleles with an amplicon size of 130 to 450 bp have been detected. The average number of alleles was 2.60 per locus, and PIC ranged from 0.04 (HMSSR1382) to 0.72 (HMSSR1289) with an average of 0.39. UPGMA analysis grouped these mango hybrids into two major clusters broadly representing the parental influence in hybrids. Cluster II comprised of 14 hybrids and cluster I had 10 hybrids. Model-based structure analysis revealed two gene pools, and AMOVA indicated that higher molecular variation is due to individuals (82%). The generated allelic variations of these polymorphic markers were translated into DNA barcodes by separating the allele size for each polymorphic HMSSR locus. A total 11 unique and 35 rare alleles from amplified alleles have been observed. Validation of these hybrid-specific alleles was attempted on a set of four tree replicates of the same genotypes grown at different places, of which seven hybrid-specific alleles could be validated in the present study. Overall, the present set of HMSSRs convincingly revealed to be highly informative and useful for future molecular research in mango. Furthermore, identifying hybrid-specific alleles would be highly useful in identifying and protecting mango hybrids.

Keywords: DNA barcode, hybrid-specific alleles, gene diversity, mango, variety identification.

Introduction

Mango (*Mangifera indica* L., 2n= 40) belongs to the family *Anacardiaceae*, and is acclaimed as the "King of the fruits" in India. It is a delicious fruit that possesses anti-oxidant, anti-diabetic, gastroprotective, cardiotonic, anti-viral and anti-inflammatory properties (Shah et al. 2010). It also contains ample pro-vitamin A (4800-16800 I.U.), minerals and is fairly rich in other nutrients. Annually, India produces 20.44 million tonnes of mangoes from an area of 2.29 million hectares with a productivity level of 9.31 MT/ha (NHB, 2019-20). Indian mangoes are exported to UAE, UK, Saudia Arabia, Yemen, Kuwait, Qatar, USA, Nepal, Bangladesh, China and Germany. The total export of fresh mango fruit from India is around 27,872.77 MT valuing Rs. 3.27 billion (APEDA, 2021-22). Asia's share in global mango production is nearly 77%, while rest comes from the African and American continents.

There are 69 species in the genus *Mangifera*, including many tropical trees (Kostermans and Bompard 1993). The extensive genetic diversity and extremely heterozygous nature of *M. indica* is result from a lengthy era of domestication, alloploidy, and outcrossing (Singh, 1960). It has a relatively medium genome size of nearly 439 Mbp (Singh et al. 2016). The phenological and morphological characteristics of flowers, foliage, fruits, and seeds have traditionally been used to categorize the genus *Mangifera*. However, many of these characters are under the influence of the environment, leading to frequent deceptive parallel selection and

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making these methods less precise to characterize mango germplasm. Mango is considered as a difficult plant species to improve by the conventional methods of breeding (Iyer and Dinesh, 1997; Iyer and Schnell, 2009), owing to its perennial nature, high level of heterozygosity, single seed per fruit, low fruit set and poor understanding about genetics of important horticultural traits.

At ICAR-IARI, New Delhi, mango hybridization work is in progress and several hundred hybrids have been developed, evaluated, and promising hybrids have been identified and released for commercial cultivation. Molecular profiling of these hybrids using DNA markers is of utmost importance to safeguard the protection of these valuable genetic materials. In mango, different marker systems have been used, *viz*., random amplified polymorphic DNA (RAPDs) (Schnell et al. 1995; Ravishankar et al. 2000; Karihaloo et al. 2003; Bajpai et al. 2008); amplified fragments length polymorphism (AFLP) (Eiadthong et al. 2000; Kashkush et al. 2001); inter-simple sequence repeats (Eiadthong et al. 1999; Pandit et al. 2007) and simple sequence repeats (Duval et al. 2005; Viruel et al. 2005; Schnell et al. 2006; Ravishankar et al. 2011). Among these, SSRs markers are receiving more attention due to their codominant nature, polymorphic behavior, multiallelic nature, high reproducibility and comprehensive genome coverage (Kalia et al. 2011; Kumari et al. 2020; Arogundade et al. 2022; Ukoskit 2007; Honshoet al. 2005; Galvez-Lopez et al. 2009; Schnell et al. 2006; Duval et al. 2006; Ramachandra et al., 2021; Srivastav et al. 2021)).

The present study was aimed to know the molecular identities of mango hybrids developed at the Indian Agricultural Research Institute, New Delhi. One-hundred novel SSRs designed from Amrapali whole genome sequence data were used to understand the genetic relationships among mango hybrids, generation of DNA barcodes and identification of hybrid specific-alleles for protection of these hybrids in the future.

Materials and methods

Twenty-four mango hybrids belonging to different crosses were used (Table 1) in the present investigation. These hybrids are maintained at the main fruit orchard of the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi.

DNA extraction and SSR genotyping

Genomic DNA was isolated from fresh leaf tissues using CTAB method proposed by Doyle and Doyle (1987) with minor modifications. Agarose gel (0.8%) electrophoresis was used to assess the concentration and integrity of extracted DNA and quantitative assessment was done using Nanodrop™ spectrophotometer (Thermo Fisher, USA). DNA with A260/A280 ratio of 1.5 to 2.0 values was considered a sign of good quality. Further DNA dilution with Milli-Q® water was done to achieve a working concentration of 25-30 ng/µl and was preserved at -20°C for long-term storage. The present investigation utilized one hundred HMSSRs with ≥50 bp SSR length, designed from whole-genome sequences of Amrapali mango.

PCR standardization and amplification

Polymerase chain reaction (PCR) was carried out in a Thermal Cycler (Bio-Rad, USA) using a 10 µL volume of the reaction mixture, containing 2.0 µL of 25 ng DNA template and 5 µL Ready PCR Mix (OnePCR™, GeneDireX), 0.5 µL of 10 µmol/L each of forward and reverse primers and 2.0 µL of molecular grade water. Thermal reactions were performed with initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation (94°C for 60 seconds), annealing (46–56°C for 60 seconds), and extension (72°C for 60 seconds) with a final extension step at 72°C for 10 minutes. The amplified SSR fragments were size separated on a 3% agarose gel containing ethidium bromide (2.0 g/100 mL) in 1X TAE buffer under a constant voltage of 5 V/cm for about 3 hours and photographed on a gel documentation system (Gel. Luminax, Zenith).

SSR data analysis

Amplified distinct and unambiguous bands were scored and converted to a binary number system of 1 for presence, 0 for absence and 9 for missing datum. Data analysis, tree generation and bootstrapping were performed using

Table 1. A list of mango hybrids used in the present study

S. No.	Hybrid		Parentage
1	Mallika	:	Neelum x Dashehari
2	Amrapali	:	Dashehari x Neelum
3	Pusa Arunima	:	Amrapali x Sensation
4	Pusa Pratibha	:	Amrapali x Sensation
5	Pusa Shreshth	:	Amrapali x Sensation
6	Pusa Lalima	:	Dashehari x Sensation
7	Pusa Peetamber	:	Amrapali x Lal Sundari
8	Pusa Deepshikha	:	Amrapali x Sensation
9	Pusa Manohari	:	Amrapali x Lal Sundari
10	H-3-2	:	Amrapali x Sensation
11	H-2-14	:	Amrapali x Alphanso
12	H-1-5	:	Amrapali x Sensation
13	H-1-11	:	Amrapali x Sensation
14	H-4-8	:	Amrapali x Sensation
15	H-7-1	:	Amrapali x Sensation
16	H-12-5	:	Amrapali x Sensation
17	NH-16-2	:	Amrapali x Sensation
18	NH-17-1	:	Amrapali x Sensation
19	NH-17-3	:	Amrapali x Sensation
20	NH-17-4	:	Amrapali x Sensation
21	NH-18-4	:	Amrapali x Sensation
22	NH-19-2	:	Amrapali x Sensation
23	NH-19-3	:	Amrapali x Sensation
24	NH-20-2	:	Amrapali x Sensation

H = Hybrid, NH = New hybrids

PowerMarker 3.25 (Liu and Muse 2004). Unweighted pair group arithmetic average (UPGMA) was used for cluster analysis and a dendrogram was generated to show the relationships among mango hybrids (Liu and Muse 2004). The genetic diversity indices, *viz*., observed heterozygosity, major allelic frequency and polymorphism information content of each SSR locus were calculated using Power Marker 3.25. The genomic organization of mango hybrids was examined using the software STRUCTURE 2.3.3, which uses model-based clustering, where clusters (populations) are identified using a Bayesian technique based on a fit to the Hardy-Weinberg equilibrium and linkage equilibrium. Calculating K, the number of clusters, was the first phase of the investigation. STRUCTURE was randomly run 5 times for each cluster (K) value between 1 and 20, utilizing correlated allele frequencies and the admixture model with 1 million Markov chain Monte Carlo (MCMC) and 100000 burn-in repeats repetitions throughout the analysis. The final population subgroups were deduced using the ΔK statistic and the consistency of grouping patterns across runs (Evanno et al. 2005). The optimal groups were assessed based on each parent's membership percentage. Molecular variance (AMOVA) and Principal Coordinate Analysis were analyzed using GenAlEx 6.1. Polymorphic SSR data was further used for the generation of DNA barcodes of 24 mango hybrids (Harisha et al. 2021).

Results

SSR descriptive statistics

One hundred new HMSSRs designed from whole-genome sequences of Amrapali have been used to identify polymorphic markers among 24 mango hybrids. Out of 100 HMSSRs, 89 were polymorphic, five were monomorphic and six HMSSRs did not amplify. A total of 1,861 alleles were detected using polymorphic markers with an amplicon size ranging from 130 (HMSSR965) to 450 bp (HMSSR888 and HMSSR1526). The number of alleles ranged from 2 to 5, averaging 2.60 alleles per primer pair. The PIC value ranged from 0.04 (HMSSR1382) to 0.72 (HMSSR1289). Twenty SSR loci *viz*., HMSSR634, HMSSR888, HMSSR803, HMSSR1839, HMSSR1427, HMSSR1778, HMSSR1758, HMSSR1653, HMSSR1196, HMSSR767, HMSSR478, HMSSR1771, HMSSR405, HMSSR2082, HMSSR1338, HMSSR1980, HMSSR786, HMSSR1326, HMSSR1349 and HMSSR1289 exhibited PIC value ≥0.50, and 10 HMSSRs had PIC value between 0.40 to 0.49 indicating their usefulness in discriminating mango genotypes (Table 2). The observed heterozygosity (H_o) ranged from 0.00 to 1.00, averaging 0.54. The gene diversity (H_e) also referred as expected heterozygosity ranged from 0.04 (HMSSR1382) to 0.77 (HMSSR1289) with a mean value of 0.47. Major allelic frequency ranged from 0.30 (HMSSR1289) to 0.98 (HMSSR2048).

UPGMA analysis of mango hybrids

Unweighted pair group arithmetic average (UPGMA) analysis broadly grouped 24 mango hybrids into two major nodes (Fig. 1). Nod I had 10 mango hybrids. However, node II comprised of 14 mango hybrids. Mango hybrids, namely, Pusa Peetamber, H-2-14, Pusa Manohari, Amrapali, Mallika, NH-17-3, NH-18-4, NH-19-2, NH-19-3 and NH-20-2 grouped in node I at different coefficient of similarity. In the second node, the remaining 14 hybrids have Amrapali and Sensation as parents except for Pusa Lalima, a cross of

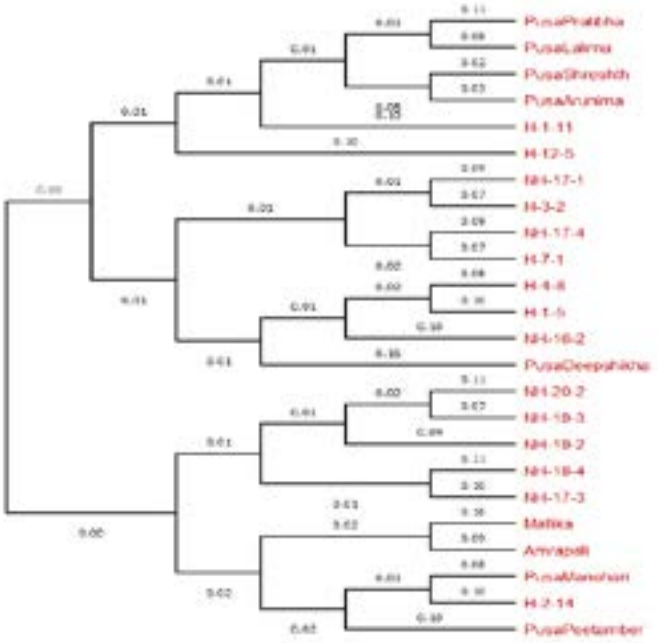

Fig. 1. Dendrogram of mango hybrids based on Neighbor-joining tree method

Table 2. Details of polymorphic HMSSR loci used for genotyping of mango hybrids along with their expected heterozygosity (H_e), observed heterozygosity (H_o), major allelic frequency (M_{af}) and the polymorphism information content (PIC)

S. No.	Code	H_e	H_o	M_{af}	PIC
1	HMSSR 1326	0.6658	0.2857	0.4643	0.6083
2	HMSSR 1619	0.5356	0.9583	0.5208	0.4301
3	HMSSR 298	0.2491	0.2917	0.8542	0.2181
4	HMSSR 556	0.4363	0.4737	0.7105	0.3765
5	HMSSR 634	0.5694	0.6667	0.5833	0.5045
6	HMSSR 865	0.4800	0.8000	0.6000	0.3648
7	HMSSR 390	0.5000	0.7143	0.5000	0.3750
8	HMSSR 436	0.2188	0.2500	0.8750	0.1948
9	HMSSR 724	0.4986	0.6316	0.5263	0.3743
10	HMSSR 1350	0.4907	0.3182	0.5682	0.3703
11	HMSSR 1585	0.5000	0.0000	0.5000	0.3750
12	HMSSR 1978	0.5605	0.6087	0.5435	0.4733
13	HMSSR 266	0.5000	1.0000	0.5000	0.3750
14	HMSSR 312	0.1528	0.1667	0.9167	0.1411
15	HMSSR 1205	0.4985	0.7222	0.5278	0.3742
16	HMSSR 1289	0.7684	0.9130	0.3043	0.7298
17	HMSSR 1551	0.5694	1.0000	0.5000	0.4768
18	HMSSR 1839	0.6050	0.1000	0.5000	0.5270
19	HMSSR 1918	0.4983	0.2353	0.5294	0.3741
20	HMSSR 2082	0.6589	0.5833	0.3958	0.5847
21	HMSSR 408	0.2676	0.3182	0.8409	0.2318
22	HMSSR 622	0.4012	0.5556	0.7222	0.3207
23	HMSSR 807	0.4444	0.4167	0.6667	0.3457
24	HMSSR 912	0.3400	0.2000	0.8000	0.3142
25	HMSSR 1218	0.3924	0.4167	0.7500	0.3414
26	HMSSR 1455	0.4861	0.8333	0.5833	0.3680
27	HMSSR 1491	0.5000	0.3333	0.5000	0.3750
28	HMSSR 1706	0.4962	0.8261	0.5435	0.3731
29	HMSSR 1761	0.4688	0.7500	0.6250	0.3589
30	HMSSR 180	0.5000	1.0000	0.5000	0.3750
31	HMSSR 203	0.3047	0.3750	0.8125	0.2583
32	HMSSR 643	0.4395	0.6522	0.6739	0.3429
33	HMSSR 917	0.4082	0.4286	0.7143	0.3249
34	HMSSR 1062	0.2188	0.2500	0.8750	0.1948
35	HMSSR 1586	0.2778	0.3333	0.8333	0.2392
36	HMSSR 1683	0.2449	0.2857	0.8571	0.2149
37	HMSSR 1980	0.6632	0.9167	0.3750	0.5891
38	HMSSR 317	0.4990	0.9545	0.5227	0.3745
39	HMSSR 405	0.6588	0.7826	0.3696	0.5843
40	HMSSR 419	0.4474	0.6316	0.6842	0.3681
41	HMSSR 457	0.5000	1.0000	0.5000	0.3750
42	HMSSR 478	0.6361	0.4762	0.5000	0.5754
43	HMSSR 1116	0.5000	1.0000	0.5000	0.3750
44	HMSSR 1226	0.5000	0.2308	0.6154	0.4078
45	HMSSR 1338	0.6605	0.6111	0.3889	0.5864
46	HMSSR 1349	0.7355	0.6842	0.3421	0.6871
47	HMSSR 1427	0.5952	0.7143	0.5476	0.5274
48	HMSSR 1531	0.4783	0.7917	0.6042	0.3639
49	HMSSR 1758	0.6078	0.4783	0.5217	0.5394
50	HMSSR 535	0.5747	0.7917	0.4792	0.4831
51	HMSSR 965	0.5148	0.8750	0.5417	0.4020
52	HMSSR 1306	0.4898	0.8571	0.5714	0.3698
53	HMSSR 1344	0.4911	0.7333	0.5667	0.3705
54	HMSSR 1429	0.4861	0.5833	0.5833	0.3680
55	HMSSR 1430	0.4523	0.2083	0.6875	0.3810
56	HMSSR 1498	0.5000	1.0000	0.5000	0.3750
57	HMSSR 1629	0.4783	0.0417	0.6042	0.3639
58	HMSSR 1653	0.6125	0.8696	0.5217	0.5433
59	HMSSR 1778	0.6094	0.3750	0.4375	0.5301
60	HMSSR 1786	0.4834	0.6316	0.6316	0.3893
61	HMSSR 422	0.4888	0.8500	0.5750	0.3693
62	HMSSR 454	0.4575	0.7083	0.6458	0.3528
63	HMSSR 690	0.5000	1.0000	0.5000	0.3750
64	HMSSR 803	0.5981	0.5417	0.4792	0.5141
65	HMSSR 1421	0.4234	0.1739	0.6957	0.3338
66	HMSSR 1426	0.3741	0.3333	0.7708	0.3363
67	HMSSR 2125	0.4523	0.5833	0.6875	0.3810
68	HMSSR 470	0.4783	0.7917	0.6042	0.3639
69	HMSSR 767	0.6424	0.9167	0.4583	0.5697
70	HMSSR 937	0.4939	0.6250	0.6875	0.4616
71	HMSSR 1196	0.6215	1.0000	0.5000	0.5534
72	HMSSR 1325	0.5530	0.4583	0.6042	0.4918
73	HMSSR 1382	0.0408	0.0417	0.9792	0.0400
74	HMSSR 1389	0.4234	0.1739	0.6957	0.3338
75	HMSSR 1526	0.3084	0.0000	0.8095	0.2608
76	HMSSR 1735	0.5391	0.7083	0.6042	0.4672
77	HMSSR 2048	0.0408	0.0417	0.9792	0.0400
78	HMSSR 191	0.5000	1.0000	0.5000	0.3750
79	HMSSR 1313	0.5000	0.0000	0.5000	0.3750
80	HMSSR 2040	0.1189	0.1250	0.9375	0.1151
81	HMSSR 309	0.4297	0.6250	0.6875	0.3374
82	HMSSR 563	0.4444	0.0000	0.6667	0.3457
83	HMSSR 786	0.6528	0.2500	0.5000	0.5994
84	HMSSR 821	0.5304	0.0417	0.5833	0.4414
85	HMSSR 888	0.5408	0.7083	0.6458	0.5048
86	HMSSR 901	0.2491	0.2917	0.8542	0.2181
87	HMSSR 1141	0.4609	0.2917	0.6875	0.3977
88	HMSSR 1771	0.6554	0.6667	0.4167	0.5817
89	HMSSR 1829	0.4991	0.9583	0.5208	0.3746
	Mean	0.4757	0.5487	0.6078	0.3940

Dashehari x Sensation. Node I was further divided into 2 sub-groups. The first sub-group I_a had Pusa Manohari and Pusa Peetamber, having common parentage of Amrapali and Lal Sundari. Similarly, Amrapali and Mallika hybrids resulting from reciprocal crosses of common parents Dashehari and Neelum were grouped together. Sub-group I_b comprised of NH-17-3, NH-18-4, NH-19-2, NH-19-3 and NH-20-2 obtained from cross of Amrapali with Sensation. Node II also grouped into 2 sub-groups. Pusa Pratibha, Pusa Lalima, Pusa Shreshth, Pusa Arunima, H-1-11 and H-12-5 grouped in subgroup II_a, while, NH-17-1, H-3-2, NH-17-4, H-7-1, H-4-8, H-1-5, NH-16-2 and Pusa Deepshikha grouped together in II_b.

Population structure analysis

The number of populations and the level of membership of each individual in each cluster might be deduced by a model-based study of the genetic structure of the mango. The Evano method (ΔK value) suggested two distinct genetic groups suggesting two populations among the selected 24 mango hybrids (Fig. 2). These distinct two populations I and II had 12 mango hybrids in both. The mango hybrids were differentiated into two populations, which were further categorized as the pure and hybrid types based on the membership fractions (Fig. 3). Mango hybrids with a probability score ≥0.80 was considered as pure, while a score ≤0.80 as admixture type. In the first population, H-2-14, Pusa Manohari, Pusa Peetamber, Pusa Deepshikha, H-1-5, Mallika, H-3-2, NH-17-4, and H-7-1 scored ≥ 0.80, suggesting they were pure. However, hybrids, namely, NH-17-1, H-4-8, and H-1-11, had a score of less than 0.80, suggesting admixture. Similarly, in the second population, Pusa Shreshth, Pusa Arunima, Pusa Pratibha, NH-19-3, NH-17-3 and NH-18-4 had probability score of ≥0.80 while hybrids, namely, Pusa Lalima, NH-20-2, H-12-5, Amrapali, NH-19-2, NH-16-2 had score less than 0.80.

The mean values for Fst among population I (Fst_1) and population II (Fst_2) were 0.0091 and 0.1165, respectively with a mean alpha value of 0.2805. The allelic frequency divergence among populations I and II of the studied mango hybrids was 0.0124.

AMOVA and PCoA

The 24 tested mango hybrids were divided into two (Pop1–Pop2) populations based on parent contribution in a hybrid generation (Fig. 4). The AMOVA test showed that there was a low (3%) variation among the populations. Whereas 15% among the individuals and 82% within the individuals. Analysis of molecular variance revealed that a major (82%) proportion of the variation was seen within the individuals ($p < 0.01$). This finding suggests that the population genetic difference is modest. The examined mango hybrids showed moderate to high genetic diversity, as the first three axes of the principal coordinate analysis (PCoA) explained 26.37% cumulative variation. PCoA showed spatial distribution among hybrids into three distinct groups (Fig. 5). The maximum dissimilarity was observed in hybrids of groups A and B, whereas the minimum dissimilarity was observed in group C.

Generation of DNA barcodes

The SSR profile data of 24 mango hybrids using 89 polymorphic markers were translated into DNA barcodes by separating the alleles by size for each HMSSR locus. DNA barcodes have been generated in two ways, *i.e.*, based on all amplified alleles across all HMSSRs in all 24 mango hybrids and considering total allele count having same size observed during SSRs profiling of mango hybrids. Then, these allele size bars were converted to a linear scale for each of the examined hybrids. The molecular profiles of all polymorphic markers of all mango hybrids can be examined using DNA barcode (Fig. 6). Different colors have been used for the representation of unique, rare and common alleles. However, another approach for the generation of DNA barcodes has given weightage to the total allele count of same size (Fig.

Fig. 2. ΔK plotfrom the structure analysis of mango hybrids

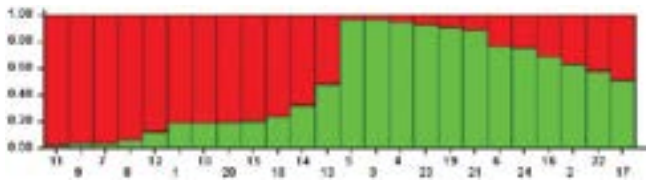

Fig. 3. Population structure analysis of mango hybrids

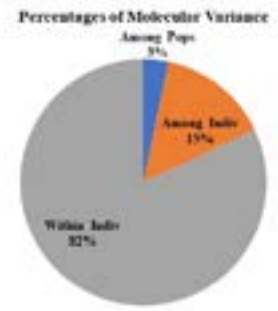

Fig. 4. Percent molecular variance among mango hybrids based on SSR data

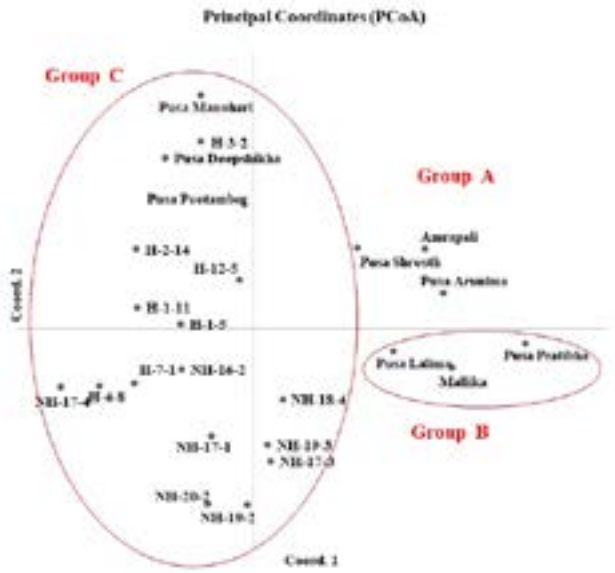

Fig. 5. Principal Coordinates Analysis of mango hybrids using SSR markers

7). These DNA barcodes have been found useful in the identification of hybrid-specific unique alleles. It is also worth mentioning here that there is a dearth of information on DNA barcodes library for fruit crops like mango. Therefore, the attempt made here to develop DNA barcodes based on unique HMSSRs have significant value.

Identification and validation of hybrid-specific alleles

Analysis of data revealed 11 polymorphic HMSSRs amplifying unique/specific alleles for eight different mango hybrids, which can distinguish specific hybrid from the rest. Hence, these unique HMSSRs serve as important resources for ascertaining the molecular identity of eight hybrids. Based on allelic frequency statistics, a total of 11 unique and 35 rare alleles have been identified. The unique allele showed an allelic count of 1 and rare alleles had 2-5 counts. These 11 alleles, *viz.*, HMSSR965 (~150 bp), HMSSR2048 (~300 bp), HMSSR1382 (~300 bp), HMSSR1218 (~260 bp), HMSSR888 (~450 bp), HMSSR1430 (~380 bp), HMSSR419 (~300 bp), HMSSR1226 (~290 bp), HMSSR535 (~280 bp), HMSSR1786 (~200 bp) and HMSSR2040 (~330 bp) amplified unique hybrid-specific alleles for Mallika, Mallika, NH-17-1, Pusa Deepshikha, Pusa Peetamber, Pusa Peetamber, Pusa Manohari, Amrapali, Pusa Lalima, H-2-14 and Pusa Peetamber, respectively (Fig. 8). Validation of hybrid-specific alleles was attempted using a set of four tree replicates of the same genotype grown at different locations. Out of 11 hybrid-specific unique alleles, only seven, *i.e.*, HMSSR965 (150 bp; Mallika), HMSSR1382 (300 bp; NH-17-1), HMSSR2048 (300 bp; Mallika), HMSSR2040 (330 bp; Pusa Peetamber), HMSSR888 (450 bp; Pusa Peetamber), HMSSR1218 (260 bp; Pusa Deepshikha) and HMSSR1430 (380 bp; Pusa Peetamber) could be validated (Table 3) and amplified same allele across all tree replicates (Fig. 9). This fact confirmed the usefulness of these HMSSRs in hybrid identification, germplasm protection and determining duplicates in the germplasm.

Discussion

The usefulness of a DNA marker to detect even a small amount of polymorphism indeed has a significant value in distinguishing diversity between closely related genotypes. SSRs have been recommended as the choicest marker due to their codominant nature, polymorphic behavior, and high reproducibility (Rajwana et al. 2011; Kumari et al. 2020). A total of 89 polymorphic markers were used for profiling of 24 mango hybrids. Most of the HMSSRs were bi-allelic. However, a few HMSSRs amplified more than two alleles in a few genotypes. This variation may be attributed to the allopolyploid nature of the mango (Mukherjee, 1953). Twenty HMSSRs exhibited PIC value ≥ 0.50, and 10 HMSSRs had PIC value of 0.40 to 0.49, indicating their usefulness in discriminating closely related mango genotypes and their future usefulness in molecular studies, construction of genetic linkage maps and QTL mapping in mango (Srivastava et al. 2021). The genetic diversity observed among the hybrids may be attributed to the heterozygous nature of the parents. Heterozygosity is simply calculated as the percentage of heterozygous individuals in the population. If the observed heterozygosity is lower than expected, we attribute this to forces such as inbreeding. If heterozygosity is higher than expected, we might suspect and isolate-breaking effect and mixing of two previously isolated populations. Our study suggests that observed heterozygosity is slightly higher (0.548) than the expected (0.475) and not in agreement with Srivastava et al. (2021), where they reported less value, *i.e.*, 0.40 for H_o compared to H_e(0.78). This might be attributed to different set of HMSSRs, size and type of population studied. The present study obtained hybrids from crosses of highly diverse parental genotypes. However, our results are in conformity with the observations of Riaz et al. (2018) who reported more expected heterozygosity than observed heterozygosity. NJ tree analysis grouped 24 mango hybrids into 2 major clusters. Cluster I had 10 mango hybrids. However, cluster II comprised of 14 mango hybrids. The grouping of the mango hybrids was much related to their evolutionary history and parentage. Our study showed that Pusa Manohari and Pusa Peetamber are grouped together having common parentage. Similarly, Amrapali and Mallika has common parentage of Dashehari and Neelum were also grouped together. It was also observed that the clustering of mango hybrids influenced by the genetic contribution of both female and male parents. It was evident that hybrids in cluster I had affinity to female parent Amrapali. However, cluster II had hybrids with common male donor parent Sensation.

A critical component of any study concerning genetic

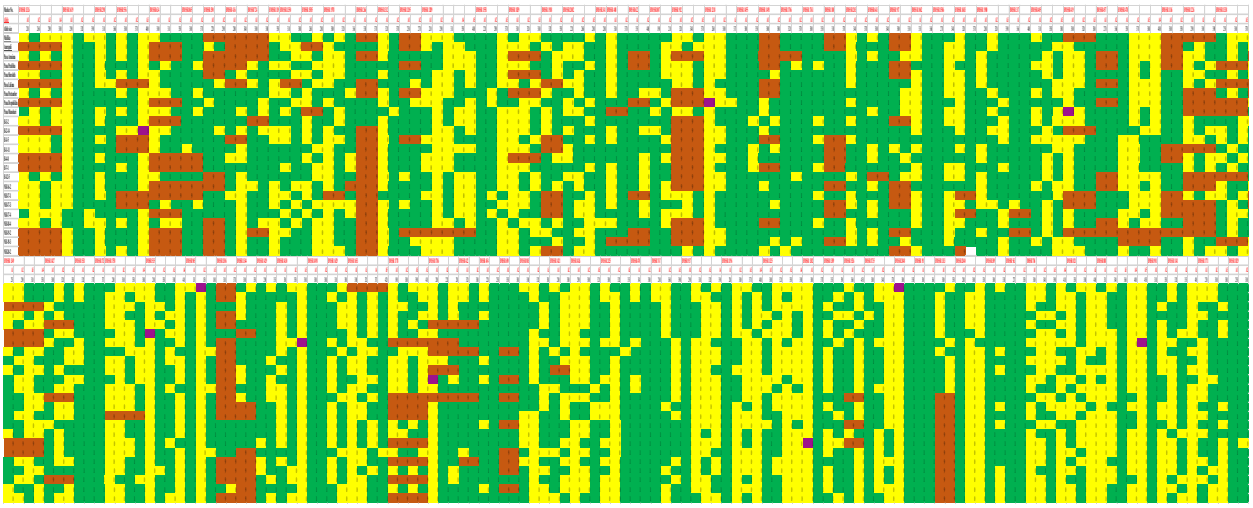

Fig. 6. HMSSR (89) based **Barcode of 24 mango hybrids.**

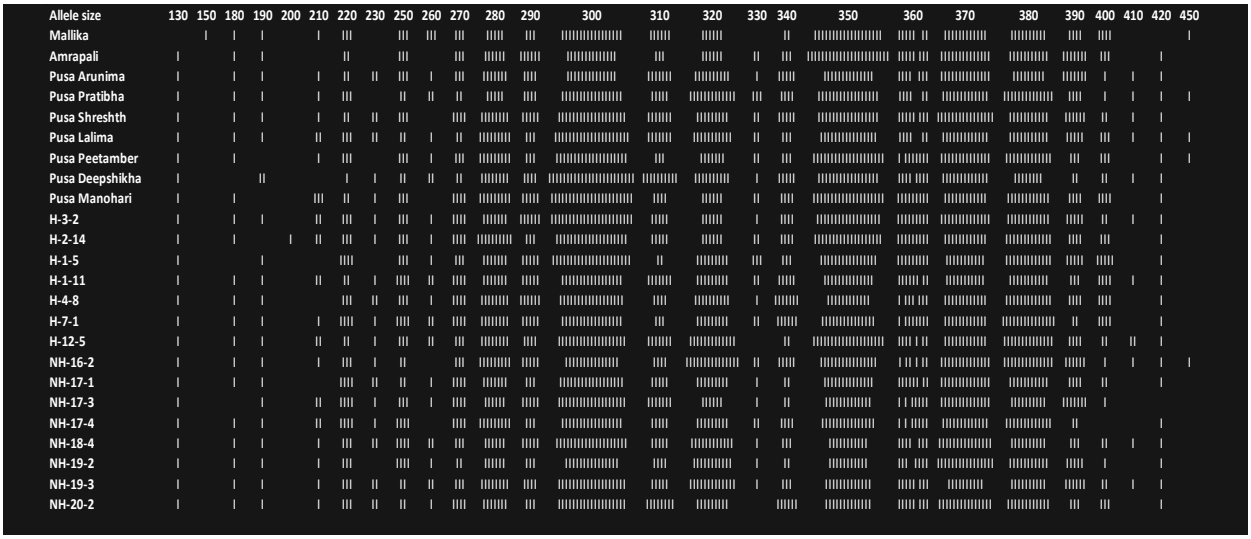

Fig. 7. **DNA Barcode based on the amplification of alleles of same bp in 24 mango hybrids**

Table 3. **Details of unique alleles identified in mango hybrids**

S. No.	Locus	Allelic size (bp)	Hybrid	Validation status
1	HMSSR419	300	Pusa Manohari	-
2	HMSSR1226	290	Amrapali	-
3	HMSSR535	280	Pusa Lalima	-
4	HMSSR965	150	Mallika	+
5	HMSSR1786	200	H-2-14	-
6	HMSSR1382	300	NH-17-1	+
7	HMSSR2048	300	Mallika	+
8	HMSSR2040	330	Pusa Peetamber	+
9	HMSSR888	450	Pusa Peetamber	+
10	HMSSR1218	260	Pusa Deepshikha	+
11	HMSSR1430	380	Pusa Peetamber	+

Note- + indicates unique alleles validation among replicated samples from different trees, - not validated.

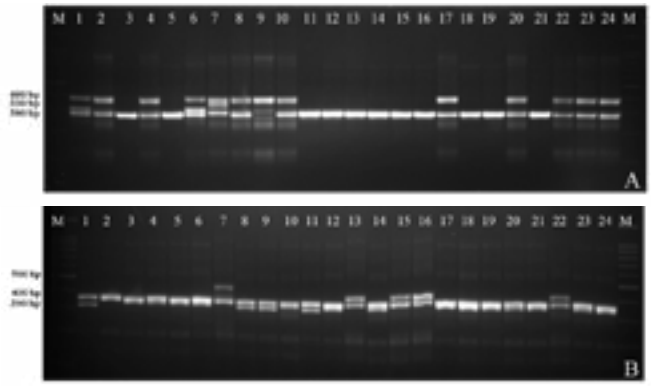

Fig. 8. **HMSSR2040 (A) and HMSSR888 (B) profiles of 24 mango hybrids**

1 = Mallika, 2 = Amrapali, 3 = Pusa Arunima, 4 = Pusa Pratibha, 5 = Pusa Shreshth, 6 = Pusa Lalima, 7 = Pusa Peetamber, 8 = Pusa Deepshikha, 9 = Pusa Manohari, 10 = H-3-2 , 11 = H-2-14,12- H-1-5, 13- H-1-11, 14- H-4-8, 15- H-7-1, 16 = H-12-5, 17 = NH-16-2, 18 = NH-17-1, 19 = NH-17-3, 20 = NH-17-4, 21 = NH-18-4, 22 = NH-19-2, 23 = NH-19-3, 24 = NH-20-2

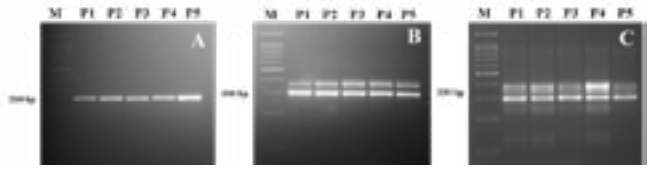

Fig. 9. Validation of unique alleles in replicated samples from different plants HMSSR1218 (A), HMSSR2048 (B) and HMSSR2040 (C)

data requires precise insight into the underlying genetic population sub-structure. A population is a group of individuals sharing a common gene pool and can inter-breed. It can be inferred from the result that there were two distinct genetic groups suggesting two different populations among the mango hybrids. Dillona et al. (2013) reported five mango populations for mango genotypes collected from different locations of Australia which showed a co-mixture type. Hirano et al. (2010) did not find any significant difference while comparing the Myanmar mangoes with mangoes from India and south-east Asia). In the present investigation, the depiction of two populations may also be attributed by the fact that parental combination used in majority of the hybrids was the same. It was further confirmed by AMOVA, which denoted that a major proportion of the variation was within the individuals (82%). Similarly, the first three axes of the principal coordinate analysis explained 26.37% cumulative variance, thus indicating moderate to high genetic diversity among the studied mango hybrids.

DNA barcodes based on allele size variation would serve as a useful resource to resolve discrepancies that may arise during duplication and intellectual property rights issues. The results of the present study were in corroboration with the work carried out by Bajpai et al. (2016). Dinesh et al. (2018) also suggested the usefulness of DNA barcodes in studying the phylogenetic relationship among mango species. Eleven unique and 35 rare alleles were recorded, out of which 7 have been validated. The validated HMSSRs *i.e.*, HMSSR965, HMSSR1382, HMSSR2048, HMSSR2040, HMSSR888, HMSSR1218 and HMSSR1430 are extremely valuable in hybrid identification, germplasm protection and determining duplicates. Our results are in confirmation with the findings of Singh et al. (2014) who generated five unique fingerprints utilizing microsatellite markers in mango. The presence of a novel allele is a sign of diversified genetic background and might be due to specific recombination events. The involvement of five parents, such as Neelum, Dashehari, Alphanso, Lal Sundari and Sensation, represented diverse gene pools and vast genetic backgrounds. The hybrid-specific alleles observed and validated in this study may be further strengthened by sequencing of specific amplicons and designing of primers for amplification of hybrid-specific genomic regions.

To conclude, the present set of new HMSSRs from Amrapali genome have immense value in diversity studies and varietal identification and can be used for marker-assisted breeding in mango. The DNA barcodes developed may serve as useful resource for identification, conservation, and protection purposes. In addition, hybrid-specific alleles and corresponding HMSSR markers have immense value in varietal identification, protection and avoiding duplicates in germplasm.

Authors' contributions

Conceptualization of research (MS, GK, SKS); Designing of the experiments (MS, GK, SKS, V, JP); Execution of experiments and data collection (GK, SHS), Data analysis and interpretation (GK, MS, CK); Preparation of the manuscript (GK, MS).

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