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Identification of microsatellite markers associated with the horticultural traits in elite mango cultivars

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Abstract
Mango (*Mangifera indica* L.) is considered an important fruit crop in the tropical and sub-tropical world due to its high nutritious quality, rich flavour and high marketing value. Knowledge about the extent of genetic diversity is the major component in designing future breeding strategies for a sustainable mango production. It is important to couple phenotypic characterization with genetic diversity analysis for germplasm conservation in gene bank. In this study, 20 mango cultivars were characterized for flowering and fruit traits using 17 simple sequence repeat (SSR) loci. Sixteen polymorphic markers with their alleles were subjected to marker trait association with 16 phenotypic traits. Step wise linear regression method was followed to study the marker-trait association and only the markers showing significant association in terms of phenotypic variance (R^2) were taken into consideration. The markers having significant association with the economic traits varied from one (fruit volume, titratable acidity, ascorbic acid, reducing sugars, total sugars and total number of flowers) to eight (no. of hermaphrodite flowers). The highest phenotypic variance was obtained for number of hermaphrodite flowers (0.974) which was explained together by eight alleles, with highest contribution by marker mMICIR027. The information about association of markers with the economic traits of mango, after further validation, will be helpful in selecting markers for screening varieties with particular trait and will assist in mango breeding programme.

Keywords: mango, SSR, marker-trait association, genetic diversity

Introduction
Mango (*Mangifera indica* Linn.) one of the choicest and admired fruit crops of the tropical and subtropical areas of the world. Its significance can easily be recognized by the fact that it is known as 'King of Fruits'. The ripe mango fruit is also a good source of dietary fibre, vitamin B6 and vitamin A. The genus Mangifera contains about 70 species mostly restricted to tropical Asia and can be divided into two subgenera (Limus and Mangifera) (Bompard, 2009; Kostermans and Bompard, 1993) [3, 22] with mango belonging to the subgenus Mangifera. Mango originated in the Indo-Myanmar region (De Candolle 1884; Mukherjee 1951) [6, 26].
Earlier, cultivars were differentiated based on morphological features which was inefficient and inaccurate. This problem was further compounded by the perennial nature of the crop. Molecular markers serve an efficient tool for the cultivar identification. Thus, molecular identification of mango cultivars has been carried out with different molecular systems such as isozymes (Degani *et al*. 1990) [7], minisatellites (Adato *et al*. 1995) [1], Inter Simple Sequence Repeats (ISSRs; Eiadthong *et al*. 1999) [11], amplified fragment length polymorphism (AFLPs; Eiadthong *et al*. 2000; Kashkush *et al*. 2001) [10, 20], random amplified polymorphic DNA (RAPDs; Schnell *et al*. 1995; Lopez-Valenzuela *et al*. 1997; Ravishankar *et al*. 2000; Hemanth Kumar *et al*.2001; Karihaloo *et al*. 2003) [31, 24, 29, 14, 19] and SSRs (Honsho *et al*. 2005; Schnell *et al*. 2006; Duval *et al*. 2005; Viruel *et al*. 2005; Ravishankar *et al*. 2011; Dillon *et al*. 2013) [15, 32, 9, 36, 30, 8]. The genetic diversity of Indian mango cultivars has been earlier reported using RAPD markers Ravishankar *et al*. 2000; Hemanth Kumar *et al*. 2001; Karihaloo *et al*. 2003) [29, 14, 19] ISSR markers (Sagar *et al*. 2007) and simple sequence repeats (SSR) loci (Malathi *et al.* 2013; Singh and Bhat 2008) [25, 33] with a small number of genotypes. Microsatellites or SSRs have become the markers of choice for fingerprinting and genetic diversity analysis in many plant species (Gupta and Varshney 2000) [13] due to their high polymorphism, codominant nature and reproducibility (Varshney *et al*. 2005; Rajwant *et al*. 2011) [35, 27]. Microsatellites consist of highly variable tandem repeats of very short motifs (1-6 bp) (Litt and Lutty, 1989) [23].
Knowledge about the extent of genetic diversity is the major component in designing future breeding strategies for sustainability in mango production. It is important to couple phenotypic

analysis with genetic diversity for germplasm conservation in gene bank collections. The use of molecular markers supports the study of genetic marker-trait associations of biological and agronomic interest on diverse genetic material. Recently, some studies reported a correlation between morphological and molecular data for the determination of genetic relationships between olive cultivars (Taamalli *et al*., 2006; D'Imperio *et al*., 2011) [34, 5], but in several other studies conducted on olive, genetic diversity assessment of morphological and molecular data was not significantly correlated (Corrado *et al*., 2009; Rao *et al*., 2009; Belaj *et al*., 2011) [4, 28, 2]. Significant associations between eight AFLP markers and fruit traits were identified. While five AFLP markers demonstrated significant negative correlation with fruit and stone weight, width and length and total polyphenols ($P < 0.05$), three AFLP markers displayed significant positive correlation with α-tocopherol and γ-tocopherol ($P < 0.01$) (Ipek *et al*., 2015) [16]. However, there is no report on the association of molecular markers with any morphological and agronomic fruit traits in mango. To our knowledge, this is the first report on association of molecular markers with the horticultural traits in mango cultivars. For breeding and other genetic studies, molecular markers linked to traits of interest are highly desired.

Materials and Methods

A total of 20 mango cultivars maintained on the AICRP (Fruits) garden, Bihar Agricultural University, Sabour, Bhagalpur were used for analyses (Table 1). Since all mango cultivars have been maintained in the same experimental plot with four replicates, the variation due to the environmental factors such as temperature, light and soil that influence morphological and agronomic traits can be considered negligible among the cultivars.

Table 1: List of the cultivars selected for the study

S. No	Cultivars
1.	Langra
3.	Zardalu
4.	Fernandin
5.	Bombay green
6.	Mulgoa
7.	Alphonso
8.	Vanraj
9.	Himsagar
10.	Fazli
11.	Bangalora
12.	Mallika
13	Mankurad
14.	Swarnarekha
15.	Dashehari
16.	Krishnabhog
17.	SB Chausa
18.	Beneshan
19.	Kesar
20.	Bombai

Evaluation of morphological and biochemical fruit characteristics

Phenological traits were recorded by observing the flowering pattern regularly during the flowering period. Different genotypes used in this study were studied for their flowering behaviour by recording the observations for the various traits. Morphological fruit traits were evaluated from three replicates per cultivar. Fruit weights (g), fruit width (mm), fruit length (mm) were measured for the different twenty mango cultivars (Table 3).

SSR Analysis

Total genomic DNA was extracted from young leaves by following CTAB method with some modifications. The DNA sample was diluted with sterilised distilled water in the ratio of 1:10. The diluted samples were then stored at 4°C for immediate use and the original concentrate were stored at -20ºC for long term storage.

PCR procedure

After optimising the concentration of components, PCR amplification was carried out with 25.00 ng of genomic DNA, 5 µl premix Taq ver, 0.50 µl each of forward and reverse primer for 10 µl volume, the details of PCR protocol followed is presented here under. The PCR reaction profile was DNA denaturation at 94°C for 4 min followed by 45 cycle of 94°C for 30 sec; primer annealing at (46- 55°C) for 1 min, 72°C for 2 min, and finally 72°C for a final extension of 5 min. Amplification products were separated by electrophoresis in 2% agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination

Data Analyses

Each band was treated as one SSR marker. The scoring of the band was done by observing the photograph carefully and the homology of the bands was based on their migration distance in the gel. The presence of band was scored as '1' and absence of band as '0'. Single marker analysis was performed to tag potential SSR markers linked to the phenotypic data of fruit morphological and biochemical traits among the diverse mango genotypes using genotypic data of 16 polymorphic markers based on step-wise linear regression method (Haley and Knott, 1992) using SPSS software Version 16.0.

Results and Discussions

All the 16 polymorphic markers with their alleles were subjected to marker trait association with all the 16 phenotypic traits, results of which have been represented in the Table 2. Step wise linear regression method was followed to study the marker-trait association and only the markers showing significant association in terms of phenotypic variance (R2) were taken into consideration. The markers having significant association with the economic traits varied from one (fruit volume, titratable acidity, ascorbic acid, reducing sugars, total sugars and total number of flowers) to eight (no. of hermaphrodite flowers) (Table 4). Cumulative regression value (R2) for fruit weight was 0.651, which was explained by three alleles. The highest phenotypic variance was obtained for number of hermaphrodite flowers (0.974) which was explained together by eight alleles, with highest contribution by marker mMiICIR027. The same marker (mMiICIR027) was found to be associated with flowering intensity and length of panicles, explaining a phenotypic variance of 0.102 and 0.162 respectively. However, no marker associated with total no. of panicles, no. of deformed panicles and days to maturity could be obtained with the set of markers used in this study. With respect to the fruit traits, marker mMiCIR016 was found to be associated with fruit weight and fruit width with R2 value of 0.315 and 0.404 respectively while marker mMiCIR008 was associated with fruit volume with R2value of 0.305. Furthermore, the same allele of maker MiIIHR06 was found to be associated with both total and reducing sugars as well as TSS and interestingly was also

found to be associated with fruit weight although with a different allele. In a similar study, association analysis of SSR and ISSR markers with fruit characteristics in sweet cherry cultivars revealed 14 SSR alleles negatively or positively correlated with different morphological traits by using MRA (Ganopoulos *et al*., 2011) [12]. Also, three ISSR markers were identified to be correlated with fruit harvest time and soluble solids and four ISSR markers were found to be correlated with fruit skin colour. In addition, study on cherries, 38 SSR alleles and 135 RAPD markers were associated with 14 fruit characters using MRA analysis (Khadivi-Khub, 2013) [21]. Similarly, four ISSR markers were associated with protein content and four with sugar content by using stepwise MRA in mulberry (Morus spp.) (Kar *et al*., 2008) [18]. The association of three ISSR markers with high antioxidant activity in *Valeriana jatamansi* was also reported by using simple-regression analysis, and it was suggested that these markers can be utilized for the selection of genotypes with high antioxidant activity in breeding programs (Jugran *et al*., 2013) [17]. In a study done by (Ipek *et al*., 2015) [16] associations of AFLP markers with fruits were determined using a multiple-regression analysis with stepwise addition of AFLP markers. Significant associations between eight AFLP markers and fruit traits were identified. While five AFLP markers demonstrated significant negative correlation with fruit and stone weight, width and length and total polyphenols ($P < 0.05$), three AFLP markers displayed significant positive correlation with α-tocopherol and γ-tocopherol ($P < 0.01$). However, since the number of genotypes used in our present study as well as the number of polymorphic markers used for screening was less, further validation of these markers and their association with traits mentioned herein is needed in larger germplasm or a mapping population. Moreover, this information about the association of these markers and the horticultural traits in mango cultivars may prove useful for further researches by students or scientists who are interested in carrying out research in this aspect.

Table 2: Primer name and primer sequences for the 16 SSR loci found in twenty mango cultivars

Locus	Primer Sequence(5'-3')
MIAC-2F	GCTTTATCCACATCAATATCC
MIAC2-R	TCCTACAATAACTTGCC
MIAC3-F	TAAGCTAAAAAGGTTATAG
MIAC-3R	CCATAGGTGAATGTAGAGAG
MIAC-5F	AATTATCCTATCCCTCGTATC
MIAC-5R	AGAAACATGATGTGAACC
MIAC-6F	CGCTCTGTGAGAATCAAATGGT
MIAC-6R	GGACTCTTATTAGCCAATGGGATG
MIAC-11F	GTGCGAGGAGATATCTGT
MIAC-11R	CTGGTTCTTCATTGTTGAGATG
MICA231-1F	TGGAAGGACCATGCTTGAAT
MICA231-1R	GGTCACACACACACACACA
mMiCIR005F	GCCCTTGCATAAGTTG
mMiCIR005R	TAAGTGATGCTGCTGGT
mMiCIR008F	GACCCAACAAATCCAA
mMiCIR008R	ACTGTGCAAACCAAAAG
mMiCIR016F	TAGCTGTTTTGGCCTT
mMiCIR016R	ATGTGGTTTGTTGCTTC
mMiCIR027F	ACGGTTTGAAGGTTTTAC
mMiCIR027R	ATCCAAGTTTCCTACTCCT
MMiCIR030F	GCTCTTTCCTTGACCTT
mMiCIR030R	TCAAAATCGTGTCATTTC
MiIIHR06F	CGCCGAGCCTATAACCTCTA
MiIIHR06R	ATCATGCCCTAAACGACGAC
MiIIHR20aF	CCTAACGCGCAAGAAACATA
MiIIHR20aR	ACCCACCTTCCCAATCTTTT
MiIIHR12F	GCCCCATCAATACGATTGTC
MiIIHR12R	ATTTCCCACCATTATTGTCGTTG
MiSHRS-36F	GTTTTCATTCTCAAAATGTGTG
MiSHRS-36R	CTTTCATGTTCATAGATGCAA
MiSHRS-37F	CTCGCATTTCTCGCAGTC
MiSHRS-37R	TCCCTCCATTTAACCCTCC

Table 3: Physiological parameters of twenty mango cultivars

S. No.	Cultivars	FW(g)	FB(cm)	FL(cm)	FV(cc)
1.	Langra	170.50	6.24	7.66	135.00
2.	Zardalu	211.25	6.48	10.25	145.00
3.	Mankurad	143.00	6.03	7.03	116.50
4.	Mulgoa	306.25	8.92	10.10	285.00
5.	Fernandin	158.00	6.12	7.28	122.50
6.	Swarnarekha	298.00	8.10	11.43	252.50
7.	Baneshan	478.00	8.51	10.97	440.00
8.	Alphonso	217.00	6.59	8.03	172.50
9.	Dashehari	115.00	4.41	6.34	59.00
10.	Bombai	217.00	6.50	8.62	173.75

11.	Bombai Green	195.00	6.43	8.09	172.50
12.	Fazli	552.00	10.75	15.50	412.50
13.	SB Chausa	183.00	5.93	9.40	156.25
14.	Neelum	182.00	6.29	8.31	161.25
15.	Kesar	215.50	6.59	8.61	180.00
16.	Vanraj	406.50	8.29	9.04	395.00
17.	Hemsagar	247.50	6.83	8.98	210.00
18.	Bangalora	419.50	7.78	13.16	392.50
19.	Mallika	362.75	7.44	12.23	305.00
20.	Krishnabhog	277.00	7.01	8.13	275.00
C.D. (at 5%)		46.21	0.46	0.84	43.65
C.V.		12.16	4.61	6.26	13.48

*FW=Fruit weight; FB= Fruit breadth; FL=Fruit length; FV= Fruit volume

Table 4: Comparison of linear regression (R^2 values) analysis for marker-trait association for selected economic traits from 16 polymorphic markers in 20 genotypes

Sl. No.	Traits	Markers	R^2 Value	Sig. F Change
1	Fruit weight	mMiCIR016_a	0.315	0.012
		MiIIHRo6 _c	0.199	0.021
		mMiCIR008 _e	0.137	0.028
2	Fruit width	mMiCIR016_a	0.404	0.003
		mMiCIR008 _e	0.177	0.019
3	Fruit Volume	mMiCIR008 _e	0.305	0.014
4	TSS (°Brix)	MiIIHRo6 _c	0.294	0.016
		MICA231-1 _a	0.252	0.002
		MICA231-1 _c	0.193	0.026
5	Titratable acidity (%)	MICA231-1 _c	0.273	0.018
6	Ascorbic acid (mg/100g)	MIAC11 _e	0.341	0.007
7	Reducing sugars	MiIIHRo6 _d	0.199	0.049
8	Total Sugars	MiIIHRo6 _d	0.323	0.009
9	Total number of flowers	MiIIHR20a _a	0.253	0.028
10	Hermaphrodite flowers	mMiCIRo27_a	0.261	0.025
		MiIIHR12 _b	0.229	0.016
		MIAC11 _c	0.196	0.008
		MIAC3 _b	0.127	0.008
		mMiCIR008 _c	0.069	0.016
		MIAC11 _e	0.042	0.025
		MIAC3F _a	0.036	0.009
		MiIIHRo6 _c	0.014	0.038
11	Flowering Duration	mMiCIR008 _a	0.222	0.042
		MiSHRS37 _a	0.200	0.032
12	Flowering Intensity	mMiCIR030 _a	0.239	0.029
		MiCIR008 _b	0.236	0.013
		MiSHRS37 _a	0.143	0.026
		mMiCIRo27_f	0.102	0.034
		MICA231-1 _b	0.074	0.042
		MICA 231-1 _a	0.092	0.007
13	Length of panicle	MiCIR008 _c	0.266	0.020
		mMiCIRo27_a	0.162	0.042
14	Total no. of panicles	-	-	-
15	No. of deformed panicles	-	-	-
16	Days to maturity	-	-	-

Conclusions

From the present study, it can be concluded that the markers used in this research work showed significant association with the horticultural traits. To the best of our knowledge this is the first report on the associations of microsatellite markers with the traits in mango cultivars and this study would be beneficial in selecting superior mango genotypes in future mango breeding programme. However association of these markers needs to be confirmed in large germplasm for validation and for some effective conclusions.

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