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Marker-Trait association for fruit characters in mango (*Mangifera indica* L.) cultivars

B. Padmakar[1], M.R.Dinesh[2], K.V. Ravishankar[1*]
[1]Division of Biotechnology, [2]Division of Fruit Crops
ICAR-Indian Institute of Horticultural Research
Hessaraghatta Lake Post, Bengaluru 560089, Karnataka, India
*Corresponding author: E-mail: kvravi@iihr.res.in, Fax: +91-80-28466291
Ph: +91-80-28466420 Extn: 406 (Off.), +91-9480050523 (Mob.)

ABSTRACT

In the present study, putative marker-trait associations were identified within a core collection of mango cultivars by simple-sequence-repeat marker based association study. A panel of 48 mango varieties which represented the core collection of the South-West region of India, were characterized at the molecular level using 31 simple sequence repeat markers. Morphological characterization included important fruit characteristics viz., fruit weight, total soluble solids (TSS), pulp content and acidity. The study on population structure revealed two sub-groups in the core collection. Association analysis, computed by General Linear Model (GLM), using TASSEL resulted in the identification of seven markers being associated with the trait titrable acidity where as one marker each of the traits fruit weight and TSS. These trait-specific markers were highly significant at $p<0.05$ and explained a good amount of phenotypic variation by exhibiting substantial R^2 values ranging from 0.71 to 0.86 for acidity, 0.61 for TSS and 0.59 for fruit weight. This is the first report on marker-trait associations (MTA) in mango.

Key words: Marker-Trait association, fruit characters, mango, *Mangifera indica* L.

INTRODUCTION

The genus *Mangifera* belongs to the family Anacardiaceae, and *Mangifera indica* L. (mango) is the most important species in this genus for commercial fruit production. Mango has been under cultivation in India for more than 4000 years (De Candolle, 1884; Mukherjee, 1951). India is considered as the world's richest diversity centre for mango. The narrow genetic base of modern crop cultivars is the most serious obstacle to sustain and improve crop productivity due to the rapid vulnerability of genetically uniform cultivars by potentially new biotic and abiotic stresses (Van Esbroeck *et al*, 1999). Plant genetic resources comprising of wild plant species, modern cultivars and their crop wild relatives, which are the important reservoirs of natural genetic variations, originated from a number of historical genetic events as a response to environmental stresses and selection through crop domestication (Ross-Ibarra and Gaut, 2007; Meilleur and Hodgkin, 2004). An efficient exploiting of these *ex situ* conserved genetic diversities is vital to overcome future problems associated with the narrowness of the genetic base of modern cultivars.

Association mapping was first introduced into plant genetics in 2001 (Thornsberry *et al*, 2001). This depends on the population structure and linkage disequilibrium (LD) pattern in plants (Flint-Garcia *et al*, 2003). Many important crops have a long and complex domestication and breeding history, together with the limited gene flow in most wild plants and populations exist as complex population structures (Sharbel *et al*, 2000; Flint-Garcia *et al*, 2003). When performing association analysis based on these populations without considering the effects of population structure, spurious association between genotype and phenotype variation may be detected because of the unequal allele frequency distribution between subgroups (Knowler *et al*, 1988). Recently,

with the development of statistics, independent markers that are distributed through whole genome are successfully used to detect population structures (Pritchard *et al*, 2000a, b). The resolution of association mapping depends on the extent and distribution of LD across the genome within a given population (Remington *et al*, 2001).

In mango, information on genetic diversity, population structure, and LD is very meagre. Hence, the objectives of our present research were to assess the genetic diversity in the germplasm collection, to investigate the population structure of the germplasm and, finally, to detect the putative marker-trait associations.

MATERIAL AND METHODS

Plant material

A set of 48 mango cultivars, from a pool of 269 cultivars of the Indian peninsular region were selected as core-collection, which are being maintained in the field genebank collection of the Indian Institute of Horticultural Research, Bengaluru, India, were taken for the study. The total genomic DNA was extracted from leaf material by modified CTAB method (Ravishankar *et al*, 2000). The DNA quantification was carried out using a Genequant UV-Spectrophotometer (GE Health Care Bio-sciences Ltd) and integrity was examined by agarose gel electrophoresis (1.0%).

Morphological characterization

The genotypes were characterized for traits such as fruit weight (g), TSS (ºB), acidity (%) and pulp content (%). Mature, naturally ripened fruits were used for recording observations on fruit weight, TSS, acidity and pulp content. The mean of five randomly chosen fruits in each variety was used for analysis. TSS was determined using a refractometer. Pulp content was calculated using the following formula:

{Total fruit wt (g) - [Stone wt (g) + Peel wt (g)]} x 100/Total fruit wt (g)

Titrable acidity was determined by titration in terms of citric acid with NaOH 0.1N where 10ml of the sample was taken and dissolved in water to make up the volume to 100ml and filtered through muslin cloth. 10ml of the aliquot was titrated against 0.1N NaoH using a few drops of 1% phenolphthalein indicator till the pink colour persisted as an end-point (Ranganna, 1977). Acidity was calculated as per cent anhydrous citric acid as per the formula given below:

$$\% \text{ Acidity} = \frac{\text{Titre} \times \text{Normality of alkali} \times \text{Volume made up} \times \text{Eq. wt. of acid} \times 100}{\text{Volume of sample taken for estimation} \times \text{Wt./Volume of sample} \times 1000}$$

Molecular characterization

A set of 31 SSR loci containing 20 SSRs developed in our laboratory (Ravishankar *et al*, 2011) was used for genotyping the core mango germplasm. The remaining 11 SSR markers were selected from other studies (Schnell *et al*, 2005; Duval *et al*, 2005; Viruel *et al*, 2005). PCR amplification was performed using labelled forward primers with fluorophores FAM and HEX at their 5' end. PCR reaction conditions were employed as per Ravishankar *et al* (2011). Amplification products were initially screened on 3% Agarose gel electrophoresis and, then, HEX and FAM amplified products were pooled. Pooled PCR products were separated to determine product-size by capillary electrophoresis using an automated DNA Sequencer ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA) at the facility at ICRISAT, Hyderabad.

Existing genetic diversity within the core collection was assessed by analyzing the following parameters: number of alleles per locus (A), observed heterozygosity (Ho, direct count), expected heterozygosity ($He=1-\sum pi^2$ where, pi is the frequency of the *i*th allele) (Nei 1973) and Polymorphic Information Content (PIC) was arrived at as per Botstein *et al* (1980). This was done using CERVUS 3.0.3 software (Kalinowski *et al*, 2007). Additionally, genetic relationship among genotypes was calculated by computing the dissimilarities through simple matching co-efficient and a dendrogram was constructed using Ward's Minimum Variance method and DARWin 5.0 software (Perrier *et al*, 2003).

Population structure analysis

STRUCTURE 2.3.1 **(**Pritchard *et al*, 2000a) was used for evaluating the structure, i.e., to identify the admixture among cultivars, and, to predict the number of populations present among the 269 cultivars under study. The number of subgroups (K) was set from 2

to 10. For each K, six runs were performed separately by setting the burn-in length and MCMC iterations to 100,000. The model choice criterion implemented in the structure to detect true *K* is an estimate of the posterior probability of data for a given *K*, Pr(*X* | *K)* (Pritchard *et al*, 2000a). This value, called 'Ln P(D)' in structure output, is obtained by first computing the log likelihood of data at each step of the MCMC. From this ΔK, which is the second order rate of change of likelihood, the function with respect to K was computed and plotted against K, for detecting the number of true populations (Evanno *et al*, 2005). Structure harvester (Earl, 2009), which harvests data from STRUCTURE results and generates ΔK (Evanno *et al*, 2005), was used to predict the true number of populations. Cultivars with membership probabilities e" 0.8 were assigned to the corresponding sub-group and lines while membership probabilities < 0.8 were assigned to a 'mixed' sub-group.

Association analysis

With available genotyping, phenotyping and structure-based Q-matrix data, association analysis was computed using the General Linear Model (GLM) module by selecting F-test with 1000 permutations, with software Trait Analysis by Association, Evolution and Linkage (TASSEL) 2.10 (Bradbury *et al*, 2007).

RESULTS AND DISCUSSION

Morphological characterization

Fruit weight ranged from 20g to 898g, with a mean of 288.9g per fruit. TSS values (ºBrix) ranged from 8.85 to 23.9, with a mean value of 17.6. Per cent titrable acidity values ranged from 0.12 to 6.02, with a mean value of 0.714. Pulp content in the fruit ranged from 26.6% to 79.8%, with a mean value of 65.07% (Table 1), which showed considerable variation for various traits among the varieties studied. A wide variability, existing for different traits in mango varieties from different regions, has been reported earlier (Lakshminarayana, 1980).

Molecular characterization

A total of 31 SSR loci, randomly distributed across the genome, were used for evaluating genetic diversity in the core mango germplasm consisting of 48 cultivars. All the 31 SSR loci studied were polymorphic across the 48 cultivars, and a total of 380 alleles were detected (Table 2). Average number of alleles per locus was 12.25, ranging from 3 to 22. Mean PIC value was 0.768, with a range of 0.516–0.936. Choice of germplasm is one of the key factors determining resolution in association mapping. For detecting more number of alleles, the selected germplasm should theoretically include all the genetic variation available in a particular species, because, a diverse germplasm includes a more extensive recombination in its history, and allows for high level of resolution. In the present study, 31 SSR loci, likely distributed randomly across the genome, were used for detecting genetic diversity in a germplasm containing 48 mango cultivars. A total of 380 alleles, with an average of 12.25 alleles per locus, were detected in the entire population; the average PIC was 0.768. The genetic diversity detected was much higher than that reported by Singh and Bhat (2009), H_e being 0.651 and PIC 0.483. The main reasons for this difference are the germplasm under study and the SSRs used. The high genetic diversity detected in our study was mainly due to the broad range of germplasm used and more number of dinucleotide-type SSRs used, which are considered to have higher mutation rates than the other types (Vigouroux *et al*, 2002). The dendrogram developed (Fig. 1) clustered the mango varieties into two main groups, which were further sub-grouped into several sub-clusters based on their genetic relationship. These groupings are incongruent with results of the structure.

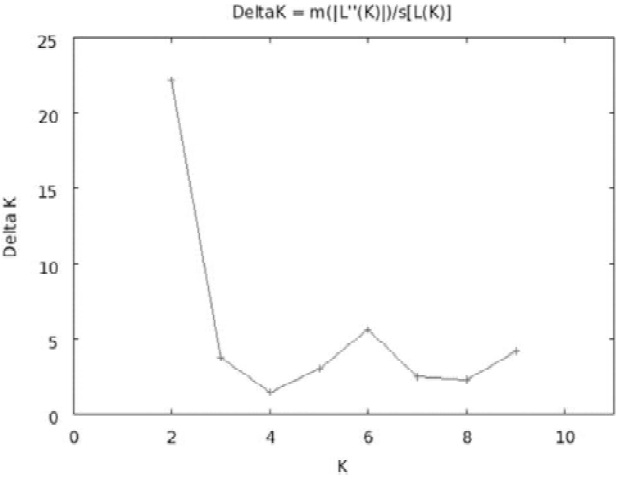

Fig. 1. Dendrogram of 48 mango cultivars generated through Ward's Minimum Variance method using dissimilarity matrix, computed by simple-matching coefficient through DARWin software

Table 1. List of traits and their morphological characteristics within the core collection

Cultivar	Fruit weight (g)	TSS (^{O}Brix)	Acidity	Pulp content (%)
Akhadya	203.70	19.60	0.32	60.10
Allanpurbaneshan	360.00	17.90	0.25	73.19
Alphonso	246.20	19.00	0.32	66.90
Appemidi	111.00	15.50	2.88	63.06
Aryasamaj	281.00	14.90	0.19	69.61
Asiquot	465.00	18.00	0.38	76.93
Bandarbandal	481.20	19.50	0.25	76.66
Banganapalli	440.00	18.50	0.12	61.70
Bombaygreen	350.00	21.50	0.32	65.00
Chandrakaran	56.20	23.70	0.80	26.60
Chettalli	436.50	11.10	0.19	79.80
Cipia	387.00	18.70	0.19	68.09
Dwarfrumani	201.00	14.50	0.12	76.83
Goakodur	183.00	13.20	0.83	67.38
Gulabkhas	231.00	20.00	0.19	75.46
Guruvam	225.50	15.20	0.38	64.10
Halsage	99.00	8.85	3.78	52.30
Hamlet	898.00	14.30	2.43	69.00
Himayatpasand	492.50	20.70	0.12	63.30
Holekoppodaappe	81.90	9.40	6.02	45.06
Hydersaheb	242.50	18.80	0.25	58.20
Jeerige	177.00	16.00	1.47	63.61
Kadikai	292.70	15.90	0.70	75.65
Kalapadi	150.00	19.50	0.44	31.90
Kanaappe-1	22.60	22.10	1.21	46.46
Karol	202.00	22.00	0.64	66.60
Khuddus	178.00	20.40	0.25	69.37
Lazzatbaksh	20.00	13.80	0.13	64.20
Mahamoozda	245.00	20.70	0.13	59.10
Malaimisri	248.00	14.70	0.19	70.60
Malgesh	271.50	14.70	0.25	69.31
Moreh	237.00	17.50	0.51	61.44
Muffarai	351.50	23.90	0.76	70.35
Mulgoa	362.50	20.80	0.27	64.40
Mutwarpasand	837.50	12.60	0.64	77.00
Navneetham	440.00	20.70	0.38	75.07
Neelum	256.00	20.00	0.40	57.00
Nekkare	170.00	15.50	0.38	43.20
Papayarajugoa	467.50	20.80	0.32	75.90
Peddarasam	463.70	14.80	0.12	71.90
Rangoongoa	320.00	20.00	0.61	71.60
Ratnagirialphonso	220.00	20.00	0.32	74.34
Rumani	200.00	19.20	0.25	75.40
Sardar	236.20	19.20	0.14	59.70
Shendriya	225.50	19.00	0.38	64.92
Shidadakkeappe	171.70	10.80	2.63	66.98
Totapuri	471.00	17.50	0.25	70.50
Vellaikulamban	161.00	18.70	0.19	67.60

Table 2. List of SSR markers used and their characteristics

Locus	No. of alleles	Observed heterozygosity (Ho)	Expected heterozygosity (He)	Polymorphic Information Content (PIC)
MilIHR03	4	0.543	0.666	0.59
MilIHR04	11	0.532	0.792	0.754
MilIHR05	9	0.63	0.699	0.653
MilIHR12	6	0.646	0.686	0.631
MilIHR13	8	0.646	0.825	0.79
MilIHR15	19	0.596	0.888	0.868
MilIHR17	22	0.708	0.937	0.923
MilIHR18	15	0.25	0.789	0.767
MilIHR19	20	0.688	0.902	0.883
MilIHR23	20	0.354	0.912	0.895
MilIHR24	17	0.479	0.835	0.808
MilIHR26	22	0.479	0.949	0.936
MilIHR28	13	0.667	0.845	0.816
MilIHR30	15	0.729	0.901	0.882
MilIHR31	16	0.563	0.835	0.812
MilIHR32	17	0.688	0.886	0.868
MilIHR34	14	0.75	0.859	0.835
MilIHR35	12	0.583	0.874	0.85
MilIHR36	21	0.75	0.9	0.883
MilIHR37	18	0.604	0.87	0.847
MiSHRS1	13	0.5	0.873	0.835
MiSHRS4	12	0.393	0.877	0.846
MiSHRS18	8	0.595	0.699	0.644
MiSHRS37	9	0.333	0.702	0.668
MiSHRS48	3	0.375	0.642	0.516
MiCR008	4	0.25	0.758	0.658
MiCR009	7	0.649	0.778	0.729
MiCR018	5	0	0.758	0.677
MiCR030	10	0.435	0.859	0.823
LMMA7	4	0.625	0.708	0.616
LMMA11	6	0.13	0.577	0.533

Population structure

In order to understand the genetic structure of the population under study, a model-based approach in the STRUCTURE software was used to subdivide each cultivar to the corresponding subgroup. STRUCTURE software was run for the number of fixed subgroups K from 2 to 10, and six runs were performed for each K. As the STRUCTURE software overestimates the number of subgroups (Pritchard and Wen, 2004), and it is difficult to choose the ''correct'' K from the Ln probability of data, Ln P(D). Thus, the true number of populations in the present study has been predicted by computing the ΔK (Evanno *et al*, 2005), which is the second order rate of change of the likelihood function with respect to K, using Structure Harvester (Earl, 2009). The resulting plot (Fig. 2) between ΔK and K clearly showed the number of populations of K=2 is true. The structure derived subgroups were corresponded as P1 and P2. P1 sub-group was the largest with 25 cultivars, followed by P2 sub-group having 19 cultivars. Additionally, 4 cultivars that had < 0.8 membership in each of the two

Fig. 2. Graph of K (assumed population number) versus DK (second-order rate of change of likelihood function with respect to K) predicting the probability that K=2 is true

(Obverlap of words in the diagram are due to immutable software and may be ignored)

sub-groups, and had a mixture of the two sub-groups, were assigned to a mixed sub-group.

Out-breeding and the wide range of agro-climatic conditions prevalent in this country continue to contribute to diversity in this crop that has a complex genetic background. Therefore, understanding population structure and relationships in mango germplasm is of significant importance for mango improvement and association analysis. Evaluation of population structure is of considerable interest, as, it is a prerequisite for answering several other questions such as estimation of migration, identifying conservation units, and, specifying phylo-geographical patterns (Manel *et al*, 2005). For statistical inference, model-based approaches such as Bayesian clustering are more suitable (Pritchard *et al*, 2000a or 2000b). In the present study, analysis of ΔK (which is the second-order rate of change of likelihood function with respect to K) clearly predicted the probability of a number of populations of K=2 as true.

Association analysis

In plant systems, linkage mapping has traditionally been the most commonly employed method to explain natural phenotypic variation in terms of simple

changes in DNA sequence: experimental crosses are made to generate a family with known relatedness, and, attempts are made to identify co-segregation of genetic markers and phenotypes within this family. In vertebrate systems, association mapping (also known as linkage disequilibrium mapping) is increasingly being used as the mapping method of choice. Association mapping involves looking for genotype-phenotype correlation in unrelated individuals, and, is often more rapid and cost-effective than traditional linkage mapping. Association mapping is not a controlled experiment, but rather, a natural experiment. Genotype and phenotype data are collected from a population in which relatedness is not controlled by the experimenter, and correlations between genetic markers and phenotype are sought within the population. This open-system-design provides higher mapping resolution than the closed system of controlled crosses; but it is difficult to infer as to when and where the recombination occurred. Unlike in vertebrates where controlled crosses may be expensive or impossible, the scientific community in plants can exploit advantages of both controlled crosses and association mapping to increase statistical power and mapping resolution. Besides, this strategy contributes to detection of genetic basis of variation exhibited in various traits of economic importance.

Just a few reports are available on association-mapping-based identification of marker-trait associations in plants such as maize (Harjes *et al*, 2008;Kump *et al*, 2011; Tian *et al*, 2011), *Arabidopsis* (Brachi *et al*, 2010), barley (Ramsay *et al*, 2011), wheat (Zhang *et al*, 2010; Letta *et al*, 2013) and rice (Huang *et al*, 2011). In the case of perennial tree crops (where development of mapping populations is a major constraint), association mapping is considered as a pivotal strategy for dissecting complex traits and for identifying genes responsible for trait-variations.

Therefore, in the present study, we selected a diverse panel of 48 mango cultivars as a core from a set of 269 cultivars. The core was exploited for phenotyping, genotyping and for also identifying population structure. Data thus generated was used for identifying putative marker-trait associations. Of the 31 SSR markers screened, nine showed significant association with three traits (Table 3); seven were associated with the trait, acidity; whereas, there was one marker for fruit weight, and one for TSS. These trait-specific markers were highly significant at $p<0.05$ and explained considerable amount of phenotypic variation by exhibiting substantial R^2 values, ranging from 0.71 to 0.86 for acidity, 0.61 for TSS and 0.59 for fruit weight. We could not conduct linkage disequilibrium and linkage decay studies, as no linkage map is available for mango yet. In a recent study in peach and nectarine cultvars, significant association was observed between markers and pomological traits (Forcada *et al*, 2013). The marker BPPCT015 showed significant association with harvest date, flavonoid and sorbitol content, Also, two genotypes of CPPCT028 showed association with harvest date, total phenolics and total sugars. To the best of our knowledge, this is the first report on identification of marker-trait association using association studies in mango. Thus, identification of trait-specific markers can play a vital role in marker-assisted selection (MAS) for accelerating mango improvement programs.

Table 3. List of Identified Marker-Trait Associations and their characteristics

Trait	Locus	*p*-value	R^2_Marker_value
Acidity	MiIIHR17	9.9×10^{-4}	0.885
Acidity	MiSHRS1	0.004	0.864
Acidity	MiIIHR36	9.9×10^{-4}	0.841
Acidity	MiIIHR30	0.033	0.781
Acidity	MiIIHR23	0.025	0.751
Acidity	MiIIHR24	9.9×10^{-4}	0.712
Acidity	MiIIHR12	9.9×10^{-4}	0.533
Fruit weight	MiIIHR04	0.047	0.593
TSS	MiIIHR37	0.048	0.614

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