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Comparison of PCR based marker systems for genetic analysis in different cultivars of mango

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Abstract

Native diversity is well represented in northern and eastern parts of India for mango. We evaluated three important polymerase chain reaction (PCR) based marker techniques *viz*., random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR) and directed amplified mini satellite DNA (DAMD) and examined their suitability for depicting genetic relationships and discrimination among closely related group of 46 mango varieties grown in the different agro-ecological zones in Uttar Pradesh, Bihar and West Bengal. Nine RAPD, eleven ISSR and four DAMD primers generated 110, 160 and 43 discrete fragments, respectively, accounting for polymorphism of 87.3, 79.83 and 83.72%, respectively. Cumulative analysis of these markers resulted in comprehensive UPGMA based dendrogram where in native mangoes representing important breeding lines and varieties from Uttar Pradesh fall more or less in separate cluster, while Bihar and West Bengal cultivars represent genetically different lineage forming distinct separate cluster. The prime focus on the study was towards identification of genetic variability that warrants establishing origin and molecular evolution of mango cultivars of eastern and northern India because they are the rich gene pool for conservation. Highest diversity index (DI) and polymorphic information content (PIC) values were found in DAMD indicating it to be more informative than others. Similarly, high effective multiplex ratio (EMR) and marker index (MI) were recorded by ISSR reflecting ability to simultaneously detect a large number of bands. The study accomplished establishing genetic relationship and also DNA fingerprint development. The data is also useful for mapping studies for gene identification.

Key words

Mango, Genetic relationship, Polymorphic information content, Marker index, Effective multiplex ratio, Diversity index

Introduction

Mango (*Mangifera indica* L.) has been cultivated, praised and even revered in its homeland as "King of fruits" since ancient times. It is reported to be an allotetraploid that evolved after interspecific hybridization and subsequent doubling of chromosome number (Mukherjee, 1953). In India, it is grown in almost all the states and shares about 56% of total production in the world. As on date in India, more than 1,000 varieties exist which have tremendous diversity in taste, aroma, size, fiber content and pulp *etc*. Almost all the present commercial cultivars in India are a result of selection from diversity available in seedling germplasm domesticated for commercial orchard over thousands of years. As a consequence, though the geographical distribution of the mango under cultivation is vast, the genetic variation may not be so well distributed. Mango gene pool has attracted lot of interest for molecular diversity analysis and several markers *viz*., RAPD (random amplified polymorphic DNA), RFLP (Restriction fragment length polymorphism), AFLP (Amplified fragment length polymorphism) and SSR (Simple sequence repeat) markers have been utilized by several workers (Schnell and Knight, 1993; Schnell *et al*., 1995; Lopez-Velanzuela *et al*., 1997; Eiadthong *et al*., 1999, 2000; Ravishanker *et al*., 2000; Hemanth Kumar

et al., 2001; Karihaloo *et al*., 2003; Pandit *et al*., 2007) to characterize germplasm accessions world over.

Among the PCR based DNA marker systems, RAPD, DAMD and ISSR are commonly and extensively used tools for assessment of variability in crops. These marker systems are efficient due to their ease, rapidity and reliability, for analysis of molecular differentiation and for resolving taxonomic problems in plants (Ranade *et al*., 2006). We have tried to compare three marker systems by estimating discriminatory power of matrices *viz*; PIC, EMR, DI and ultimately MI. The EMR defines number of loci (bands) simultaneously analyzed per experiment. A single parameter, MI is a universal matrix that represents amount of information obtained per experiment in a given marker system. Another aspect in which marker systems differ is their ability to determine relationship between accessions based on genetic similarity estimation. This has importance for breeding program, germplasm conservation and genetic resource management where relationship and distance estimates give insight into genetic makeup of related accessions. Additionally, genetic relationships among mango accessions determined by three dominant marker assays were compared for congruency to arrive conclusive placement in the relationship tree.

Materials and Methods

Plant materials: Leaf sample of 46 varieties of mango collected from eastern (29 cultivars) and northern (17 cultivars) regions of India were taken for the study.

Molecular analysis: The DNA was extracted using modified cetyl trimethyl ammonium bromide (CTAB) method from 2 g of leaf tissue as described by Dellaporta *et al*. (1983) and purified by RNase treatment followed by extraction using phenol and chloroform. The DNA samples were checked both qualitatively as well as quantitatively by agarose gel electrophoresis according to Sambrook *et al*. (1989). The PCR reactions were carried out on the total genomic DNA in a final volume of 25µl reaction mixture with the 9 RAPD primers, 11 different ISSR primers and 4 DAMD primers.

The RAPD and ISSR reactions were carried out as described earlier by Bajpai *et al*. (2008). While in DAMD, DNA amplification was carried out according to Zhou *et al*. (1997). The reaction mixture contained 50 ng DNA templates, 200 mM of each dNTPs, 2 mM $MgCl_2$, 200 mM primer, 1x Taq polymerase buffer, 1 unit of Taq DNA polymerase. The amplification was performed in programmable thermal cycler with following programme of initial denaturation at 94°C for 2 min followed by 40 cycles of, 94°C for 1 min, 55°C for 2min, 72°C for 2min and final extension at 72°C for10 min.

The PCR amplified products were electrophoresed on 1.5% (w/v) agarose gel in 1x TBE buffer for three hours at 5V/cm. After completion of electrophoresis, gel was stained with ethidium bromide and visualized on a transilluminator and gel images were acquired under gel documentation system (Alpha Innotech Corp. USA).

Data analysis: The RAPD, ISSR and DAMD bands were scored as presence (1) or absence (0) and only those that was well defined and consistently repeatable in two independent amplification were included in the analysis. Similarity matrix based on Jaccard's similarity coefficient was used to construct unweighted pair group method with arithmetic average (UPGMA) dendrogram. To evaluate the discriminatory power of molecular markers PIC, MI and EMR were calculated. The PIC value was determined by applying the formula (Roldan-Ruiz *et al*., 2000) $PIC_i = 2f_i (1- f_i)$, where f_i is the percentage of the amplified alleles (bands present) and $(1- f_i)$ is the frequency of the null allele (band absent) for i^{th} allele. The MI was calculated as the product of two function that is DI and EMR as described by Prevost and Wilkinson (1999). The DI of the primer is defined as 1- sigma $(pi)^2$ where pi is the frequency of i^{th} allele, while EMR of a primer is defined as the "product of the fraction of polymorphic bands and the number of polymorphic bands for an individual assay" (Milbourne *et al*., 1997).

Results and Discussion

RAPD marker analysis: Of 30 RAPD primer tested, 9 showed clear reproducible band patterns and were chosen for the study. Out of total of 110 discrete fragments of size ranging from 300 to 3500 bp identified, 96 (87.3%) were polymorphic. Four (OPF 15) to nineteen (OPA 19) bands were amplified per primer at the average rate of 12.22 bands (Table 1). The number of polymorphic fragments for each primer varied from 2 (OPF 15) to 19 (OPA 19) with an average of 10.66. The band data were used to generate the similarity matrix. Jaccard's similarity coefficient ranged from 0.88 (between Papia and Safeda Calcutta) to 0.378 (Lucknow Safeda and Anopan). The UPGMA tree constructed on the basis of Jaccard's coefficient clustered the accessions into 3 groups, one comprising majority of North Indian accessions, other having East Indian accessions and third cluster with accessions from both the regions (Fig. 1). The average PIC, DI, MI and EMR were 0.245, 0.27, 25.84 and 83.52, respectively (Table 2).

ISSR marker analysis: Of 30 ISSR primer screened, 11 showing clear reproducible band patterns generated 160 discrete fragments ranging in size from 300 to 3000 bp and 127 (79.38%) were polymorphic. Eight (ISSR 9) to twenty one (ISSR 5) bands were amplified per primer at the average rate of 14.5 bands per primer (Table 1). The number of polymorphic fragments for each primer varied from 7 (ISSR 9) to 19 (ISSR 10) with an average of 11.54. Genetic similarity based on Jaccard's coefficient varied from 0.83 (between Amin Ibrahimpur and Amin Prince) to 0.467 (between Kishan Bhog and Amin Khurd). UPGMA clustering of ISSR data divided genotypes into 2 major sub-clusters, one cluster has all north Indian accessions and few accessions from West Bengal and Bihar and other having eastern accessions except Nisar Pasand which occupied most distal shoot (Fig. 2).The average PIC, DI, MI and EMR were 0.217, 0.27, 34.13 and 100.33, respectively (Table 2).

DAMD marker analysis: Four primers out of six amplified 43 DNA fragments ranging in size from 300 to 3500 bp. Of these, 36 (83.72%) were polymorphic. The number of bands ranged from 8 (HVB) to 17 (33.6 b) with an average rate of 10.75 bands per primer (Table 1). The number of polymorphic fragments for each primer varied from

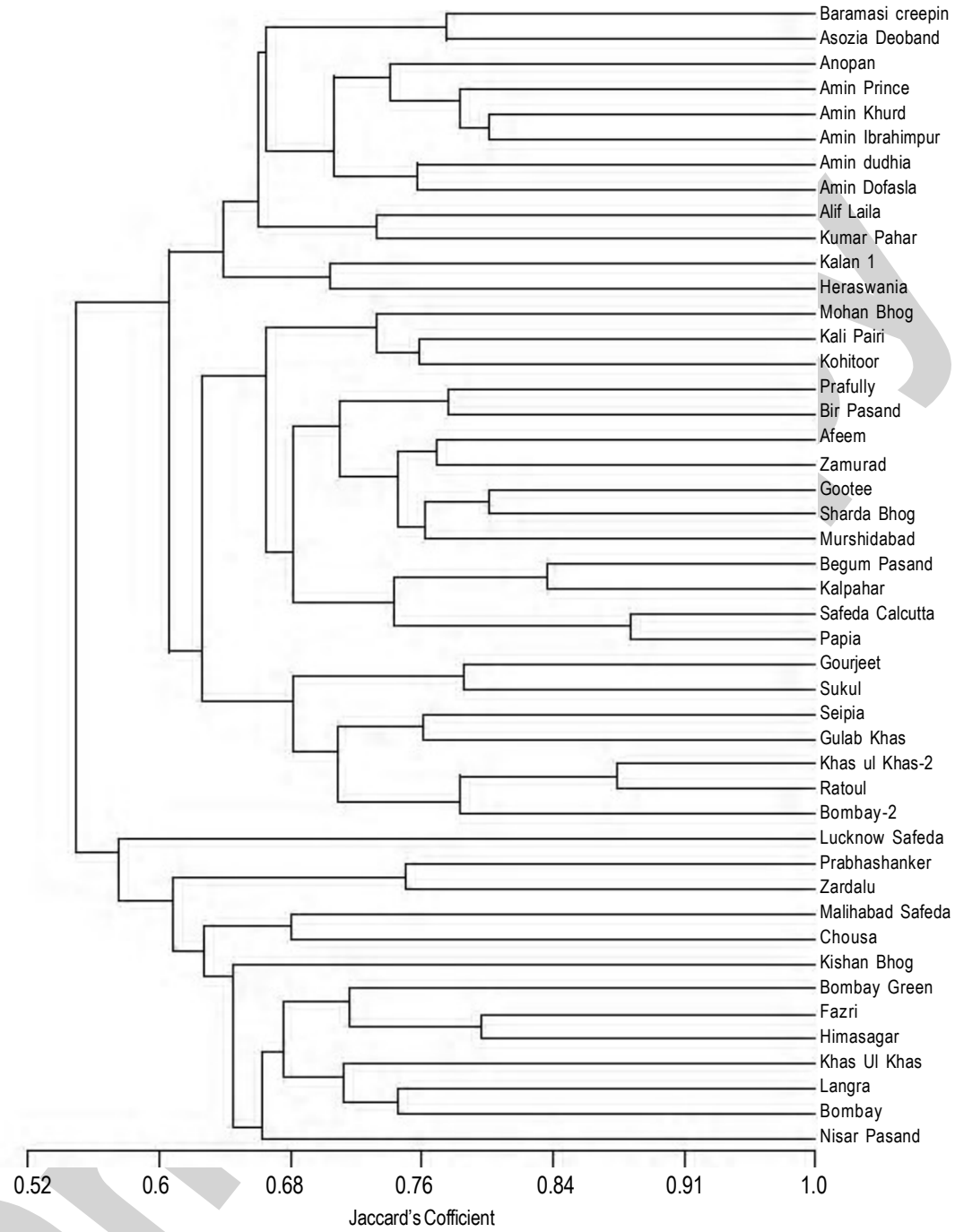

Fig. 1: Dendrogram constructed using UPGMA based on Jaccard's similarity matrix data generated by RAPD analysis, revealing genetic relationship among mango accessions

six (HBV) to fourteen (33.6b) with an average of 9. Jaccard's similarity coefficient varied from 0.96429 (between mango accessions Bombay and Prafully) to 0.35294 (between Sukul and Sharda Bhog). The UPGMA clustering of DAMD data depicted that accessions are broadly divided into two clusters one cluster comprises eastern varieties of mango whereas other comprises both the accession from eastern and northern regions (Fig. 3).The average PIC, DI, MI and EMR were 0.298, 0.36, 13 and 30.24, respectively (Table 2). Genetic relationship among mango accessions obtained with RAPD, ISSR and DAMD markers.

The genetic relationship among the mango accessions were based on the combined data of these three markers amplifying 259 polymorphic fragments. The clustering was based on similarity matrix generated by computing Jaccard's coefficient ranging from 49 to 83% indicating fairly adequate diversity. Accessions Herasawania and Khas Ul Khas were most distant (similarity being 0.492) while

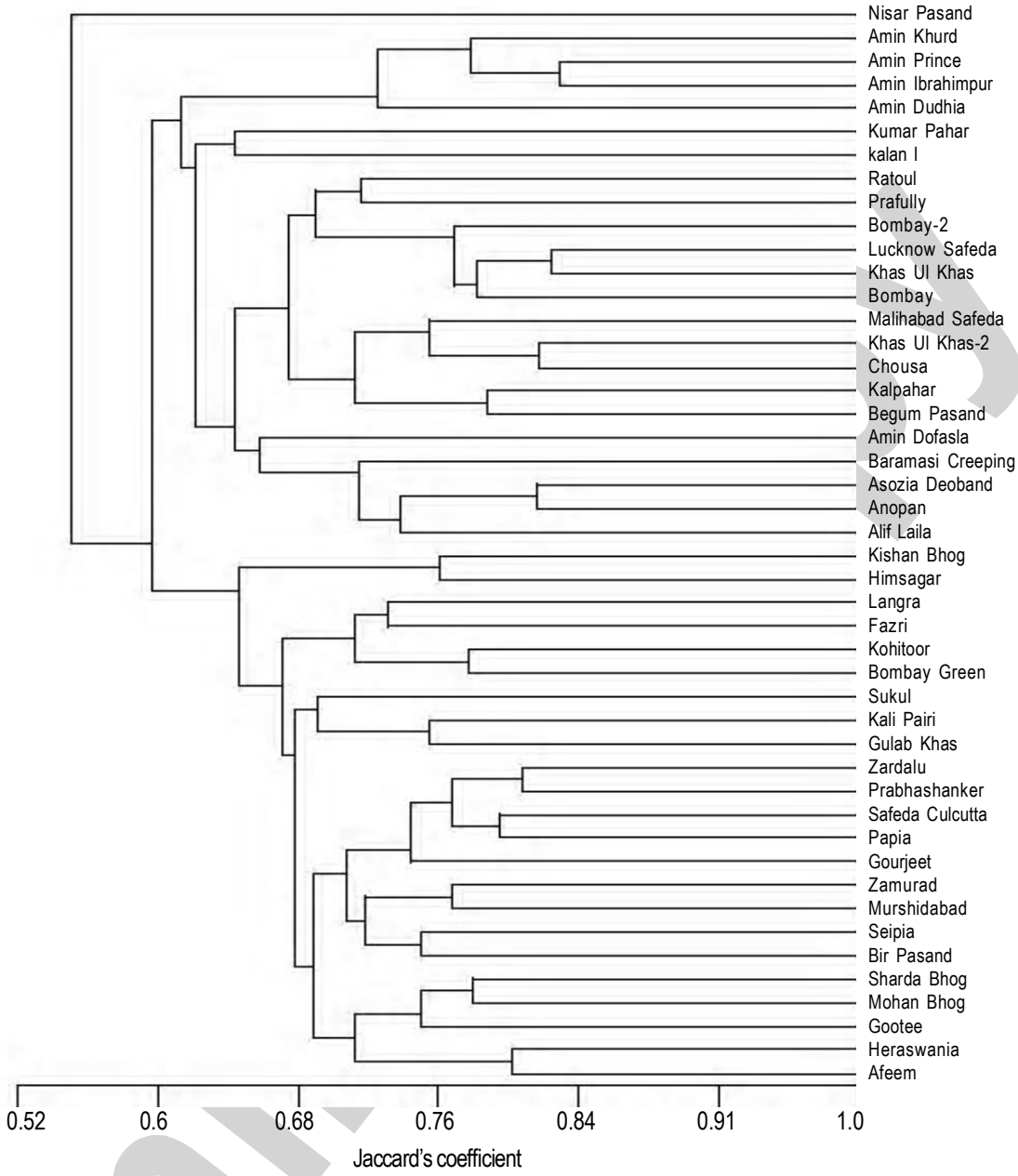

Fig. 2: Dendrogram constructed using UPGMA based on Jaccard's similarity matrix data generated by ISSR analysis, revealing genetic relationship among mango accessions

Papia and Safeda Calcutta were nearest (similarity being 0.831). UPGMA clustering of combined data of these three arrange accessions into two major clusters . One cluster had all East Indian varieties of mango and the other cluster had major accessions of mango from northern regions of India.

Comparison of marker systems: The usefulness of each system was examined in terms of number of loci revealed EMR and the amount of polymorphism detected DI. The ability of different assay to infer genetic relationship was also observed. Highest level of polymorphism (DI) 0.36 was detected for DAMD and 0.27 for both RAPD and ISSR. The EMR was 83.52, 100.33 and 30.24 for RAPD, ISSR and DAMD, respectively. The MI, which is the product of DI and EMR, was used to evaluate the overall utility of each marker system. The ISSR resulted in the highest marker indices of 34.13 followed by RAPD and DAMD (25.84 and 13), respectively. Average PIC was also calculated and DAMD resulted in the highest PIC of 0.298, while the PIC for RAPD and ISSR were 0.245 and 0.217, respectively (Table 2). The ISSR had the highest EMR and DAMD the highest DI and PIC values. Average genetic similarity estimates from the three methods were 0.618 (RAPD), 0.644 (ISSR) and 0.603 (DAMD). Histogram of pairwise distances between 46

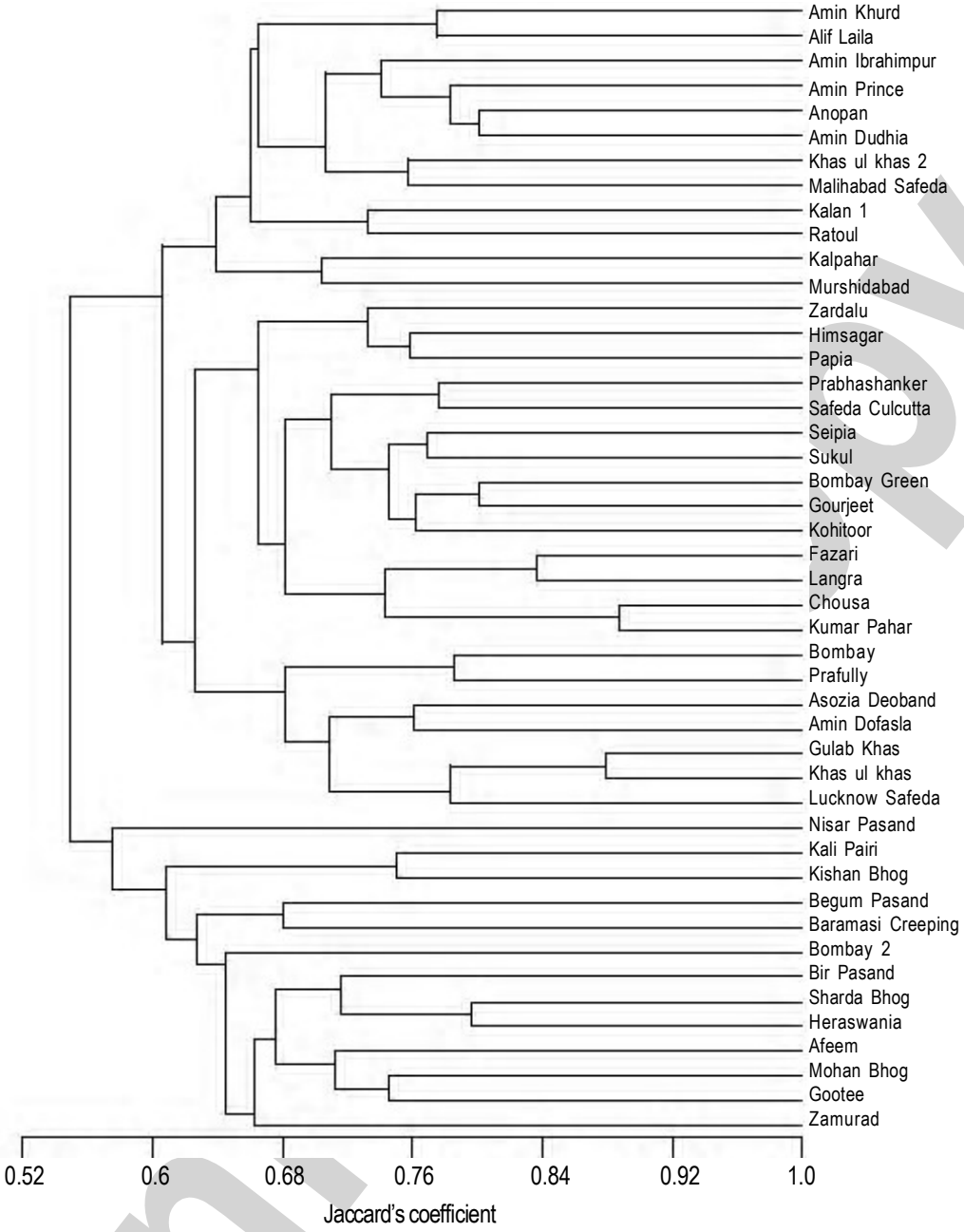

Fig. 3: Dendrogram constructed using UPGMA based on Jaccard's similarity matrix data generated by DAMD analysis, revealing genetic relationship among mango accessions

genotypes shows that genetic similarities obtained by DAMD are fairly spread out and display a wide range in comparison to RAPD and ISSR, which have clustered frequency histograms (Fig. 4).

India has immense wealth of mango germplasm and mango is the most important fruit crop of the country. There exists tremendous diversity in mango in term of taste, aroma, size, fiber content and pulp characters etc. There is a need to document the rich biological diversity in mango for proper identification of mango genotypes. PCR based methods using single primers have been used extensively to determine and analyze genetic diversity, genetic relationship, elimination of duplicates in germplasm, gene tagging and genome mapping in plants. These PCR derived markers obtained with non-species specific primers have gained popularity since prior sequence information is not needed and measure of genetic diversity at DNA level in all tissues at all age of plant is seldom influenced by environmental conditions as in the case of morphological markers. Consequently, these methods are important for perennial fruit tree, where molecular biology research has lagged behind compared to food crops. Some reluctance due to RAPD-

Table - 1: RAPD, ISSR and DAMD primers performance for genetic diversity analysis

Primer name	Sequence 5'à3'	Loci scored	No. of polymorphic loci	Percentage polymorphism
RAPD				
OPA 1	CAGGCCCTTC	7	5	71.43
OPA 15	TTCCGAACCC	9	9	100
OPA 18	AGGTGACCGT	12	10	83.33
OPA 19	CAAACGTCGG	19	19	100
OPA 20	GTTGCGATCC	17	16	94.12
OPC 6	GGGGGTCTTT	18	17	94.44
OPF 6	GGGAATTCGG	16	15	93.75
OPF 13	GGCTGCAGAA	8	3	37.50
OPF 15	CCAGTACTCC	4	2	50
ISSR				
ISSR 1	$(AG)_8YC$	13	9	69.23
ISSR 2	$(GA)_8YC$	14	9	64.29
ISSR 3	$(CT)_8RC$	14	11	78.57
ISSR 4	$(CA)_8RG$	12	9	75
ISSR 5	$(GAA)_6$	21	14	66.67
ISSR 6	$(GACA)_4$	16	15	93.75
ISSR 7	$(GGGTG)_3$	14	9	64.29
ISSR 8	$(GGAT)_4$	17	14	82.35
ISSR 9	$(GATA)_4$	8	7	87.50
ISSR 10	$(AAC)_5$	19	19	100
ISSR 12	$(GACA)_4$	12	11	91.67
DAMD				
M13	GAGGGTGGCGGTTCT	9	8	88.89
HVR	CCTCCTCCCTCCT	10	8	80
HVB	GGTGTAGAGAGAGGGGT	7	6	85.71
33.6b	AGGGCTGGAGG	17	14	82.35

RAPD = Random amplified polymorphic DNA, ISSR = Inter simple sequence repeat, DAMD = Directed amplified minisatellite DNA

Table - 2: Comparison of the average polymorphic information content (PIC), expected heterozygosity for polymorphic markers (DI), of the fraction of polymorphic markers (β), the effective multiplex ratio (EMR) and marker the marker index (MI) for each marker class

Marker system	Average PIC	DI	β	MI	EMR
RAPD (9)*	0.245	0.27	0.87	25.84	83.52
ISSR (11)	0.217	0.27	0.79	34.13	100.33
DAMD (4)	0.298	0.36	0.84	13	30.24

*Number in bracket represents number of primers used in different marker system

reproducibility has been largely overcome by improved lab techniques (Weising *et al*., 1995).

In mango, genetic variation had been studied by various workers using RAPD, ISSR, SSR and AFLP (Schnell and Knight, 1993; Schnell *et al*., 1995; Lopez-Velanzuela *et al*., 1997; Eiadthong *et al*., 1999, 2000; Ravishanker *et al*., 2000; Hemanth Kumar *et al*., 2001; Karihaloo *et al*., 2003; Pandit *et al*., 2007, Bajpai *et al*., 2008). However, all these used only one method at a time, and indicated presence of wide genetic diversity in mango. Srivastava *et al*. (2007) used three different PCR methods among commercial accessions of India. Our study has investigated three different PCR methods RAPD, DAMD and ISSR and the results are interpreted independently as well as collectively among 46 Eastern and Northern Indian mango accessions. Based on EMR, ISSR markers are more efficient tools than RAPD or DAMD markers for mango accessions. This comparison is valid also for MI an estimate of marker utility proposed by Powell *et al*. (1996), as ISSR markers have higher values among the three set of system.

The high MI is the reflection of efficiency of ISSR-PCR markers to simultaneously analyze a large number of bands, rather than level of polymorphism detected. The results have shown that DAMD have higher DI in comparison to others thus highlighting its efficiency as locus with high discriminating power. The large number of PCR assay is due to abundance of di-nucleotide, trinucleotide and tetra-nucleotide repeat motifs in mango genome. The ISSR primers designed to anchor such microsatellite motifs and amplify genome sequences lying between them. Ukoskit (2007) reported 39 unique clones containing di-nucleotide repeats in mango, which was utilized for developing microsatellite markers. Xinhua *et al*. (2005), Sharon *et al*. (1995) and Eiadthong *et al*. (1999) have

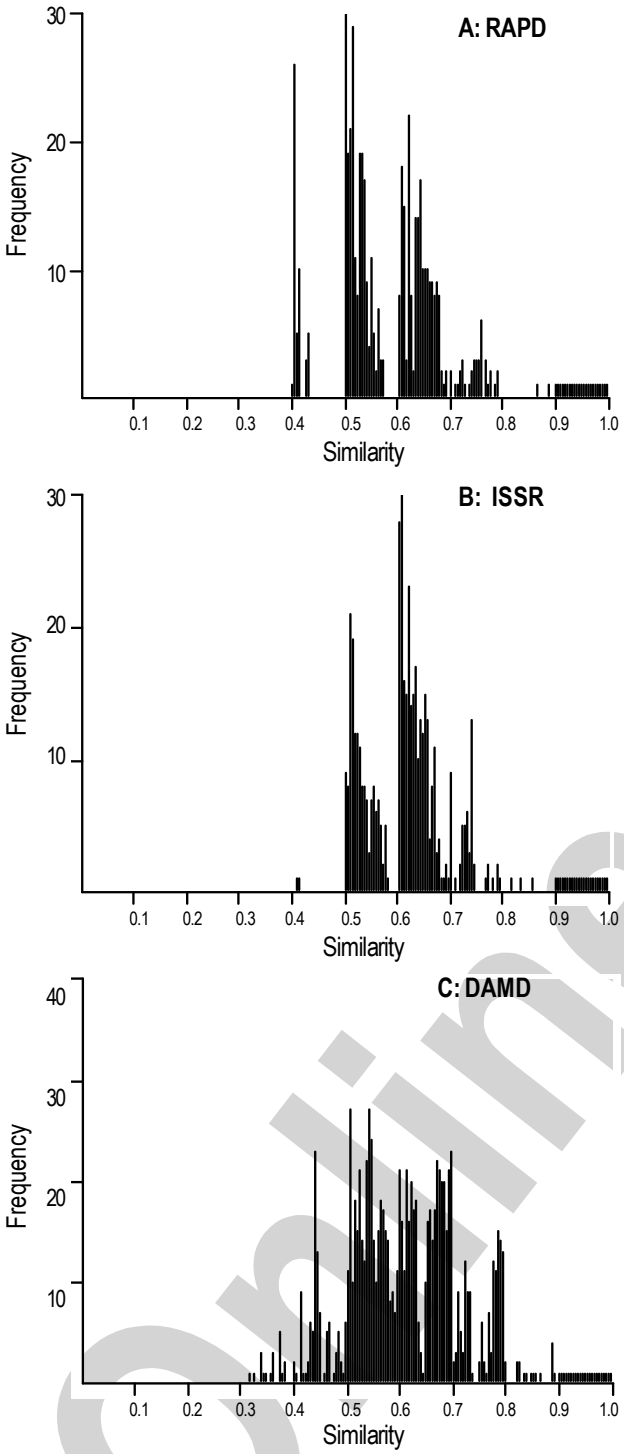

Fig. 4: A histogram similarities between the forty six mango accessions estimated by RAPD, ISSR and DAMD. The height of bars indicated that the number of similarities falling within each 0.02 interval from 0.1 to 1.0

earlier utilized tri and tetra-nucleotide rich markers for polymorphism detection in their study. Higher PIC with tetra-nucleotide repeats GAGA and GACA containing primers indicates presence of these motifs in mango genome, as earlier reported by Xinhua *et al*. (2005).

The cumulative band data revealed genetic differentiation (Jaccard's distance) to be narrow 0.17-0.51 which is understandable, as the distance between two regions is less and overlapping. As earlier workers reported very high level of polymorphism, selected accessions were taken from widely different geographic regions including exotic ones. The UPGMA tress for 46 accessions using RAPD, ISSR and DAMD methods, resulted in different clustering of the genotypes. The non congruence of the UPGMA tree for different method viz., RAPD, ISSR and DAMD reflected the presence of several sites of genomic divergence and heterogeneity. This was reported by earlier worker too (Powell *et al*., 1996, Parsons *et al*., 1997). Recently, some workers (Srivastava *et al*., 2007, Saxena *et al*., 2005) analyzed genetic diversity in different fruit crops using RAPD, ISSR and DAMD methods, individually as well collectively and found cumulative data resulted more comprehensive than that resulted by individual methods. All the three types of marker assay have different properties thus combination of theses three resulted more genome coverage. The UPGMA tree generated by combined data efficiently clustered the mango accessions into two different regions of India. One cluster had all (maximum) East Indian accessions of mango and the other cluster had major accessions from northern regions of India. Based on individual as well as collective methods UPGMA tree depicted congruence among RAPD and cumulative data based tree, while ISSR and DAMD derived tree is largely non-congruent. The North Indian accessions i.e. Amin Prince, Amin Khurd, Amin Ibrahimpur, Amin Dudhia, Amin Dofasla, Asozia Deoband, and Anopan showed a close relationship, reflecting they are genetically very close to each other.

The most important criteria determining choice of assay are informativeness and ease of genotyping. All three assays differed not only in underlying principle but also amount of polymorphism detected. The number of polymorphic bands per primer, the widest range of distance displayed by spread of histogram of frequency (Fig. 4) and the high level of PIC value of DAMD data suggested that a small number of primers would be adequate to distinguish between mango accessions. High genetic diversity indicates fixation of new alleles in specific population along with recombination and segregation of alleles by open-pollination of genetically different in mangoes in varied agro-climates (Rivera-Ocasio *et al*., 2000). Our results clearly demonstrated that PCR based assays of dominant markers targeting 259 polymorphic bands, is a good tool for the genetic analysis of mango accessions. Here critical number of loci and sample size considerations are met with precision (Staub *et al*., 2000; Krauss, 2000). In addition, the result based on cumulative data was found to be reproducible as observed from repeated analysis of studied mango accessions.

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