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Assessment of genetic diversity in mango using inter-simple sequence repeat markers

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ABSTRACT

Genetic diversity among 63 mango genotypes (*Mangifera indica* L.) was investigated using Inter-Simple Sequence Repeat (ISSR) markers. A total of 334 scorable amplification products were detected with 28 ISSR primers of which 331 (99.10%) were polymorphic. Most of the primers (89.29%) exhibited 100% polymorphism. Primers UBC-812 and UBC-891 identified with the highest number of genotypes with unique fingerprints (53). The highest number of different fingerprints (58) was obtained with primer UBC-812, while the lowest number of different fingerprints (17) was obtained with primer ISSR-5. Primers UBC-812, UBC-891, UBC-808 and UBC-836 were found to be of high value for fingerprinting in mango as they were able to resolve 58, 57, 55 and 55 of 63 mango genotypes selected for the study, respectively. The Jaccard's similarity values ranged from 0.25 (between 'Cambodiana' and 'Mombasa') to 0.79 (between 'K-1' and 'K-3') with a mean of 0.53. UPGMA tree constructed on ISSR data on the basis of Jaccard's similarity coefficient clustered 55 of 63 mango genotypes into six major groups, however eight mango genotypes remained unclustered. The Cluster 1 comprised of mainly south Indian genotypes, while Cluster 2 comprised of mainly man-made hybrids and genotypes from the Northern and Eastern regions of India. Genotypes indigenous to Western parts of India grouped in Cluster 3. Cluster 4 had the exotic genotypes introduced from Brazil, while Clusters 5 and 6 comprised of Floridan mango genotypes. UPGMA clustering of ISSR data showed good correspondence with pedigree, geographical separation and embryo types, i.e. mono- or poly-embryonic.

Key words: Diversity analysis, mango, ISSR.

INTRODUCTION

Mango (*Mangifera indica* L.), considered "The King of Fruits" in Indian sub-continent is an important member of family Anacardiaceae and is believed to have originated in the Indo-Myanmar region. ember of family Anacardiaceae and is believed to have originated in the Indo-Burma region.It has been cultivated for more than 4,000 years and a wide genetic diversity exists in this crop in India. Allopolyploidy, out-breeding and wide range of agro-climatic conditions prevailing in this country contributes to the enormous diversity of the cultivated mango. In addition, there has been widespread hybridization and recombination of characters in mango germplasm. Over the thousands of years of its cultivation and domestication, the wide genetic diversity of the plant has been fixed in many varieties. The present day cultivars are mainly seedling selections and are maintained through vegetative propagation. Presently, India has over 1,000 mango cultivars and represents the biggest mango gene-pool in the world. Precise information on the genetic relationships within such diverse germplasm is needed for efficient and effective management of plant genetic resources as well as for their utilization to carry out future breeding programmes. Therefore, it is of paramount importance to collect, characterize and conserve land races that are regionally and locally important.

Traditional methods of cultivar identification are based on objective description of tree, flower and fruit characteristics. However, these observations are time consuming and error prone due to environmental variations affecting expression of these characteristics. Often, phenotypically indistinguishable trees may be genotypically similar or *vice-versa*. Characterization based on horticultural traits needs complementation with molecular markers as they can contribute greatly to the utilization of genetic diversity through descriptive information of structure of genotypes, analyses of relatedness, the study of identity and location diversity. In order to assess the genetic diversity and to identify the cultivars in mango, PCR-based DNA markers are

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considered to be best tools. A range of DNA markers, *viz.* AFLP, RAPD, SSR and ISSR have been used for exploring the diversity (Bally *et al*., 4; Eiadthong *et al.*, 5,6; Kumar *et al*., 9; Karihaloo *et al*., 7; Lopez-Valenzuela *et al*., 10; Ravishankar *et al*., 14; Schnell *et al*., 17; Srivastava *et al*., 18). Among these, Inter Simple Sequence Repeat (ISSR) (Srivastava *et al*., 18) is a reproducible semi-arbitrary primed PCR method that uses simple sequence repeats as primers, combining most of the advantages of microsatellites and AFLP, to the universality of Randomly Amplified Polymorphic DNA (RAPD). ISSRs offer greater probability than any other PCR marker system in the repeat regions of the genome, which are the most potent regions for producing cultivar-specific markers. Automated PCR base makes ISSRs the marker of choice for screening genotypes for ascertaining genetic diversity. The present study was conducted to asses the genetic diversity in mango germplasm and to study the genetic relationship among the mango varieties.

MATERIALS AND METHODS

Leaf samples of 63 mango genotypes (Table 1) were collected from the scientifically maintained orchards of Mango Germplasm Block of Indian Agricultural Research Institute, New Delhi and Horticulture Research Centre, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. For each genotype, 5 g of tender and healthy leaves were harvested, washed under tap water followed by rinse with sterile distilled water and midribs and thick veins were removed. Samples were wrapped in aluminum foil, fixed by dipping briefly in liquid nitrogen and stored at -70°C till DNA extraction.

Extraction of total genomic DNA was carried out using C-TAB method given by Saghai-Maroof *et al*. (16) with minor modifications as suggested by Porebski *et al.* (13), and Khanuja *et al.* (8). The NaCl (5 M) treatment at the DNA precipitation step (Porebski *et al*., 13) helped to overcome the co-precipitation of polysaccharides. The use of polyvinyl pyrrolidone (PVP) @ 2% enabled freedom from phenolic compounds and gave rise to clear DNA pellets. Part of each DNA sample was diluted with sterilized double-distilled water to yield a working concentration of 25 ng/µl and stored at 4^0C for immediate use while the original stocks were kept for long term storage at -20°C.

Amplification of DNA was performed using 28 ISSR primers (Table 2). All PCR reactions were carried out in a final volume of 20 µl reaction mixture containing 1 µl template DNA (25 ng/µl), 1 µl ISSR primer (5 pM), 2 µl of buffer (10X), 1.6 µl $MgCl_2$ (25 mM), 0.3 µl dNTP (10 mM), 0.2 µl *Taq* polymerase (5 U/µl) and 13.9 µl MilliQ water.

Table 1. List of mango genotypes used in the study.

Sl. No.	Genotype	Native place	Place of collection
G1	Janardhan Pasand	South India	New Delhi
G2	Totapari	South India	Pantnagar
G3	Totapari Red Small	South India	New Delhi
G4	St. Alexandrina	Brazil	New Delhi
G5	Iturba	Brazil	New Delhi
G6	Rosari	Brazil	New Delhi
G7	Bappakai	South India	New Delhi
G8	Chandrakaran	South India	New Delhi
G9	K-1	South India	New Delhi
G10	K-2	South India	New Delhi
G11	K-3	South India	New Delhi
G12	K-4	South India	New Delhi
G13	K-5	South India	New Delhi
G14	Kurukkan	South India	New Delhi
G15	Sabre	South East Asia	New Delhi
G16	Cambodiana	South East Asia	New Delhi
G17	Moovandan	South India	New Delhi
G18	Peach	South Africa	New Delhi
G19	Olour	South India	New Delhi
G20	Suvarnrekha	South India	New Delhi
G21	Lal Sundari	South India	New Delhi
G22	Neelum	South India	New Delhi
G23	Ratna	South India	New Delhi
G24	Bhadauran	North India	New Delhi
G25	Alphonso	Western India	New Delhi
G26	H-1-1	North India	New Delhi
G27	H-11-2	North India	New Delhi
G28	Pusa Arunima	North India	New Delhi
G29	Mallika	North India	New Delhi
G30	Amrapali	North India	New Delhi
G31	H-4-12	North India	New Delhi
G32	Fernandin	Western India	Pantnagar
G33	Langra	North India	Pantnagar
G34	Gorakhpur Langra	North India	Pantnagar
G35	Samar Bahist Alibagh	North India	New Delhi
G36	Pant Sindhuri	North India	New Delhi
G37	Khas-ul-Khas	North India	Pantnagar
G38	Gulab Khas	North India	Pantnagar
G39	Gulab Khas Green	North India	New Delhi
G40	Dashehari	North India	New Delhi
G41	Turpentine	West Indies	New Delhi
G42	Mombasa	North India	New Delhi
G43	Bombay Green	Eastern India	Pantnagar
G44	Bombay Yellow	Eastern India	Pantnagar
G45	Tamancha	North India	Pantnagar
G46	Sona Tol	North India	Pantnagar
G47	Thanking Amadi	North India	Pantnagar
G48	Kesar	Western India	Pantnagar
G49	Tommy Atkins	Florida	New Delhi
G50	Edward	Florida	New Delhi
G51	Eldon	Florida	New Delhi
G52	Zill	Florida	New Delhi
G53	Sensation	Florida	New Delhi
G54	Elard	Florida	New Delhi
G55	Hathijhul	North India	Pantnagar
G56	Husnara	North India	New Delhi
G57	Malviya Bhog	Eastern India	New Delhi
G58	Ram Kela	North India	Pantnagar
G59	Bijauragarh	North India	Pantnagar
G60	Chausa	North India	Pantnagar
G61	Fajli	North India	Pantnagar
G62	Rataul	North India	New Delhi
G63	Bara Malda	Eastern India	Pantnagar

Table 2. List of ISSR primers used for characterization of 63 mango genotypes.

Sl. No.	Primer	Primer sequence ((5'-3')	Ta (°C)
1.	ISSR-1	ACT ACT ACT ACT ACT	48.2
2.	ISSR-2	TCT CTC TCT CTC TCT CAG G	51.0
3.	ISSR-3	ACA CAC ACA CAC ACA CCT A	53.8
4.	ISSR-4	GGA GAG GAG AGG AGA	47.5
5.	ISSR-5	CGT AGT AGT CAC ACA CAC ACA CA	49.0
6.	ISSR-6	ACG ACT ACG GTG TGT GTT TGT GT	49.0
7.	ISSR-7	GTG AGT GAG TGA GTG A	47.5
8.	ISSR-8	GAC AGA CAG ACA GAC A	47.5
9.	UBC-807	AGA GAG AGA GAG AGA GT	49.0
10.	UBC-808	AGA GAG AGA GAG AGA GC	48.8
11.	UBC-809	AGA GAG AGA GAG AGA GG	45.0
12.	UBC-810	GAG AGA GAG AGA GAG AT	46.7
13.	UBC-811	GAG AGA GAG AGA GAG AC	48.2
14.	UBC-812	GAG AGA GAG AGA GAG AA	48.2
15.	UBC-823	TCT CTC TCT CTC TCT CC	49.0
16.	UBC-830	TGT GTG TGT GTG TGT GG	49.0
17.	UBC-834	AGA GAG AGA GAG AGA GYT	49.0
18.	UBC-835	AGA GAG AGA GAG AGA AGA GYC	47.5
19.	UBC-836	AGA GAG AGA GAG AGA GYA	49.0
20.	UBC-841	GAG AGA GAG AGA GAG AYC	48.8
21.	UBC-842	GAG AGA GAG AGA GAG AYG	48.8
22.	UBC-856	ACA CAC ACA CAC ACA CYA	48.8
23.	UBC-876	GAT AGA TAG ACA GAC A	47.5
24.	UBC-878	GGA TGG ATG GAT GGA T	47.5
25.	UBC-881	GGG TGG GGT GGG GTG	47.5
26.	UBC-886	VDV CTC TCT CTC TCT CT	49.0
27.	UBC-890	VHV GTG TGT GTG TGT GT	49.0
28.	UBC-891	HVH TGT GTG TGT GTG TG	49.0

Ta: Annealing Temperature, A: Adenine, T: Thymine, G: Guanine, C: Cytosine, Y: (C, T), V: (A, C, G), D: (A, G, T), H: (A, C, T)

Polymerase Chain Reaction was carried out in Perkin Elmer Thermocycler with programme as (i) denaturation at 94 °C for 5 min., (ii) denaturation (35 cycles) at Ta (annealing temperature of the particular primer) for 1 min., (iii) primer annealing at 50 °C for 1 min., (iv) Primer extension at 72 °C for 2 min., and (v) Final extension step at 72 °C for 7 min. The PCR amplification products were stored at 4 °C until loading. Amplified DNA fragments were separated out on 2% agarose gel containing ethidium bromide (3 µl/100 ml of agarose gel) in 1X TBE buffer for 4-5 h at 60 V. The resolved amplification products were visualized under UV light on a UV-transilluminator and photographed using gel documentation system (Gel Doc Mega, Biosystematica, UK).

The band profiles of each gel were scored visually and recorded as presence (1) or absence (0) of bands and binary quantitative data matrix was constructed. A pair-wise difference matrix between genotypes was determined using Jaccard's Similarity coefficient. Data analysis was performed using the NTSYS-pc (Numerical Taxonomic System) version 2.11 computer programme package and Winboot software. A dendrogram was constructed by UPGMA method to measure the resulting phenetic groups and original matrix was bootstrapped 1,000 times by employing Winboot to group the genotypes into discrete clusters. To study the informativeness of RAPD in Mango, the number of genotypes with unique fingerprint (NGUF) per primer and the number of different fingerprints (NF) per primer were recorded.

The discriminatory power of ISSR markers was evaluated by two parameters. The Polymorphism Information Content (PIC) for each ISSR marker was calculated as $PIC_i = 2f_i (1-f_i)$, where PIC_i is the Polymorphic Information Content of marker i, f_i is the

frequency of the marker bands present, and ($1-f_i$) is the frequency of absent marker bands. The ability of a primer to distinguish between large numbers of genotypes, i.e. Resolving Power of primer (Rp) of selected 28 ISSR primers were determined..

RESULTS AND DISCUSSION

Initially 75 ISSR primers were used for generating banding profile, out of which 28 primers gave consistent and discrete bands. These 28 primers used in the present study generated a total of 334 scorable bands on amplification (Table 3). Most of the primers (89.29%) exhibited 100% polymorphism, while least polymorphism (85.71%) was shown by primer UBC-876. A typical ISSR profile developed by primer ISSR-8 for 63 mango genotypes is shown in Fig. 1. The number of scorable bands generated by 28 ISSR primers, ranged from 7 (ISSR-5, UBC-830, UBC-876 and UBC-878) to 18 (ISSR-2), with an average of 11.93 bands per primer (Table 3). The range of polymorphic bands per primer was 6 (UBC-876) to 18 (ISSR-2), with an average of 11.82 polymorphic bands per primer. The bands size generated by ISSR primers ranged from 100 to 4,000 bp. Primer UBC-886 produced bands of minimum size and ranged 100 to 1,000 bp, while primer UBC-856 produced bands of maximum size and ranged 400 to 4,000 bp. Two primers, i.e. UBC-812 and UBC-891 were identified with the highest number of genotypes with unique fingerprints

Table 3. Band statistics and related parameters generated by 28 ISSR primers in 63 mango genotypes.

Sl.No.	Primer	BS (bp)	NSB	NMB	NPB	PPB (%)	NGUF	NF	PIC	Rp
1	ISSR-1	200-1000	11	0	11	100.	14	23	0.23	3.33
2	ISSR-2	200-3000	18	0	18	100	13	19	0.15	3.05
3	ISSR-3	300-3000	14	0	14	100	40	49	0.30	6.57
4	ISSR-4	250-3000	17	0	17	100	38	48	0.32	8.38
5	ISSR-5	250-3000	07	0	07	100	07	17	0.30	2.95
6	ISSR-6	300-3000	10	0	10	100	10	19	0.24	3.40
7	ISSR-7	300-3000	11	0	11	100	39	47	0.31	5.21
8	ISSR-8	200-3000	12	0	12	100	28	37	0.27	4.41
9	UBC-807	200-4000	12	0	12	100	42	51	0.38	6.76
10	UBC-808	300-3000	11	0	11	100	49	55	0.40	6.83
11	UBC-809	200-2000	11	0	11	100	29	38	0.35	5.30
12	UBC-810	100-2000	13	0	13	100	23	36	0.29	5.37
13	UBC-811	300-3000	12	0	12	100	25	31	0.23	3.68
14	UBC-812	200-3000	14	0	14	100	53	58	0.32	6.60
15	UBC-823	100-4000	12	0	12	100	47	54	0.39	7.59
16	UBC-830	300-4000	07	0	07	100	17	33	0.41	4.57
17	UBC-834	200-4000	13	0	13	100	43	52	0.35	6.54
18	UBC-835	200-3000	12	0	12	100	43	52	0.34	6.38
19	UBC-836	100-3000	14	1	13	92.86	48	55	0.32	7.21
20	UBC-841	100-4000	13	0	13	100	40	49	0.35	6.98
21	UBC-842	100-2000	08	0	08	100	13	21	0.28	3.08
22	UBC-856	400-4000	15	0	15	100	37	48	0.36	7.62
23	UBC-876	500-3000	07	1	06	85.71	08	22	0.31	3.46
24	UBC-878	300-3000	07	0	07	100	12	24	0.34	3.40
25	UBC-881	300-4000	15	1	14	93.33	41	51	0.30	6.44
26	UBC-886	100-1000	14	0	14	100	41	51	0.38	6.06
27	UBC-890	200-2000	13	0	13	100	35	46	0.33	6.63
28	UBC-891	200-2000	11	0	11	100	53	57	0.41	7.21
Average	-	-	11.93	-	11.82	99.10	31.71	40.82	0.32	5.54
Total	-	-	334	-	331	-	888	1143	-	-

BS: Band Size, bp : base pair, NSB: No. of Scored Bands, NMB : No. of Monomorphic Bands, NPB: No. Polymorphic Bands, PPB: Percentage of Polymorphic Bands, NGUF: No. of Genotypes with Unique Fingerprint, NF: No. of different Fingerprints, PIC: Polymorphism Information Content, Rp: Resolving Power.

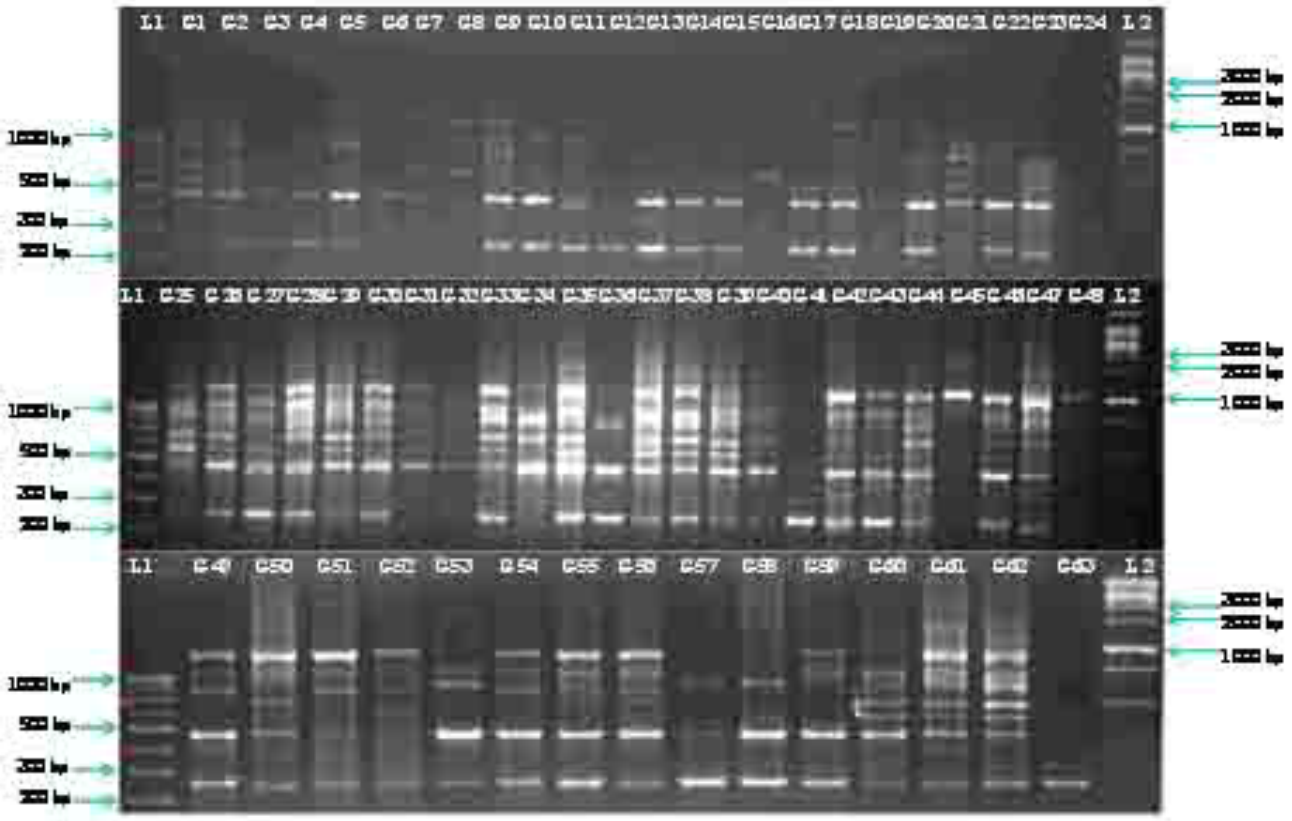

Fig. 1. Amplification profile of 63 mango genotypes (G1-G63) employing primer ISSR-4, Ladder (L1: 100 bp; L2: kb).

(53). The highest number of different fingerprints (58) was obtained with primer UBC-812, while the lowest number of different fingerprints (17) was obtained with primer ISSR-5. Primers UBC-812, UBC-891, UBC-808 and UBC-836 were found to be of high value for fingerprinting in mango because they were able to resolve 58, 57, 55 and 55 of 63 mango genotypes selected for the study, respectively (Table 3).

The Polymorphism Information Content (PIC) expresses the discriminating power of the locus taking into account not only the number of alleles that are expressed, but also their relative frequencies and frequency of alleles per locus. The highest PIC (0.41) was recorded for primers UBC-830 and UBC-891, whereas the lowest PIC (0.15) for primer ISSR-2 (Table 3). Dominant markers as ISSR have a maximum PIC value of 0.5, when half of the accessions have the band and the other half does not have the band. The primers employed in the present study showed high Rp values, which were able to distinguish most of the mango genotypes (Table 3). The Rp values of 28 primers ranged from 2.95 (ISSR-5) to 8.38 (ISSR-4) with an average value of 5.54.

For analysis of genetic relatedness amongst 63 mango accessions, the binary matrix of ISSR bands was used to calculate pair-wise Jaccard's similarity coefficients. The values ranged from 0.25 (between 'Cambodiana' and 'Mombasa') to 0.79 (between 'K-1' and 'K-3') with a mean of 0.53. The matrix based on Jaccard's similarity values was subjected to UPGMA analysis to construct a dendrogram, in which at a similarity value of about 0.56, six major clusters were formed (Fig. 2). Cluster 1 and Cluster 2 were further grouped into 2 sub-cluster each (fig.2). Eight genotypes namely 'Zill', 'Sabre', 'Mombasa', 'Malviya Bhog', 'Turpentine', 'Bhadauran', 'Tamancha' and 'Cambodiana' did not share any similarity with other mango genotypes and were ungrouped. The most similar accessions were 'K-1' and 'K-3' (80% similarity).

Interestingly non-Indian mango genotypes were separated from Indian mango genotypes. All the Indian mango genotypes were observed to be grouped together, however, four Indian mango genotypes ('Mombasa', 'Malviya Bhog', 'Bhadauran' and 'Tamancha') did not form cluster with others and were ungrouped. Among exotic mango genotypes Brazilian cultivars ('St. Alexandrina', 'Rosari' and 'Iturba') formed a separate cluster (Cluster 4), whereas, Floridan cultivars were grouped into two clusters (Clusters 5 and 6). 'Tommy Atkins', 'Edward' and 'Eldon' formed one cluster (Cluster 5), while

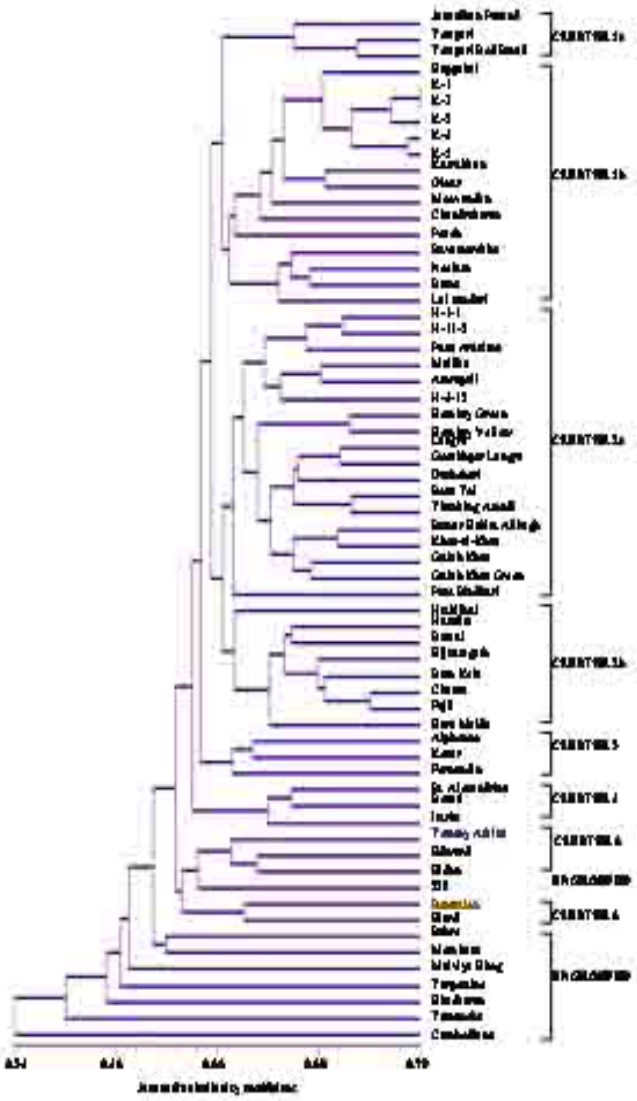

Fig. 2. UPGMA dendrogram based on Jaccord's similarity coefficient revealing clustering in 63 mango genotypes based on ISSR data.

'Sensation' and 'Elard' were grouped into another cluster (Cluster 6). However, 'Zill' remained ungrouped. Among polyembryonic genotypes 'Peach' of South Africa clustered with polyembryonic varieties of south India. However, South East Asian polyembryonic mango genotypes ('Sabre' and 'Cambodiana') remained ungrouped. Our results are in strong conformity with the findings of Pandit *et al.* (12) who have reported separation of non-Indian cultivars from Indian cultivars. Among Floridan cultivars 'Tommy Atkins', 'Edward' and 'Eldon' formed a single cluster (Cluster 5). The genetic relatedness among these genotypes was expected because they are related with each other by their pedigree. 'Haden' a seedling of Indian Variety 'Mulgoba' gave rise to several Floridan mango varieties like 'Tommy Atkins', 'Edward', 'Eldon' and 'Zill'. A study using RAPD markers supported 'Haden' parentage of 'Eldon', 'Tommy Atkins' and 'Zill' (Schnell *et al.*, 17). Further, Olano *et al.* (11) confirmed the 'Haden' as the maternal parent of 'Edward' using microsatellite markers. However 'Haden' and 'Edward' were placed into separate groups in a study done by Eiadthong *et al.* (5). Separation based on their geographical location was evident among the Indian mango cultivars. Mango genotypes of south Indian origin and north Indian origin were placed separately in the Cluster 1 and Cluster 2 respectively. Separation of genotypes on the basis of geographical location was also reported by Ravishankar *et al.* (14). Similarly Lopez-Valenzuela *et al.* (10) based on RAPD data, grouped mango cultivars into 4 categories according to their geographical origin. The results from this study showed that the cultivars might have evolved from existing mango gene pool in that geographical location. These cultivars were selected by local people and later domesticated by cultivation in large areas. Based on panicle emergence, canopy density and the emergence of a second flush of panicles, Indian mango cultivars were classified into two different ecotypes (Yadav and Singh, 19). The dendrogram analysis also showed two major groups (north Indian cultivars and south Indian cultivars) of Indian mango. Therefore, it appears that the majority of present day commercial cultivars in different parts of the Indian sub-continent have originated from mango germplasm existing in that particular geographical area. However, Pandit *et al.* (12) reported no geographical separation of south and north Indian mango cultivars. Bajpai *et al.* (3) reported that cultivar pool from Northern and Southern regions of India is considerably homogenous. 'Bombay Green', 'Bombay Yellow' and 'Bara Malda' cultivars from eastern regions of India formed a group with north Indian cultivars (Cluster 2). Since the geographic distance between east and north Indian genotypes is comparatively less and there were considerable exchange of material in past, therefore it seems logical to have overlapping and closeness amongst the germplasm. Our results were in strong agreement with the findings of Bajpai *et al.* (3) who carried out RAPD and ISSR analysis of mango accessions. IARI evolved hybrids were placed together in the Cluster 2. Hybrids 'H-1-1', 'H-11-2' and 'Pusa Arunima', sharing similar parentage ('Amrapali' x 'Sensation') were placed together. Hybrids 'Amrapali', 'Mallika' and 'H-4-12' were clustered together in the UPGMA dendrogram. This was expected as 'Amrapali' and 'Mallika' are hybrids derived from 'Neelum' and 'Dashehari' crossed reciprocally. Moreover, 'Amrapali', 'Mallika' and 'H-4-12' had one parent in common ('Dashehari'), and thus showed a high

degree of similarity. Separation of mango genotypes on the basis of embryo type was also revealed by the present investigation. Polyembryonic varieties of south Indian origin ('Bappakai', 'K-1', 'K-2', 'K-3', 'K-4', 'K-5', 'Kurukkan', 'Olour', 'Moovandan' and 'Chandrakaran') were placed together. Out of four exotic polyembryonic mango genotypes, three genotypes, 'Sabre', 'Turpentine' and 'Cambodiana' did not share similarity with any other mango genotype used in the study and were found ungrouped. However, South African polyembryonic cultivar 'Peach' formed group with Indian polyembryonic cultivars. Grouping of mango cultivars based on nature of embryony have been reported by Ravishankar *et al.* (15) and Abirami *et al.* (1). Arnon *et al.* (2) suggested that both monoembryonic and polyembryonic genotypes are inter-crossable and polyembryony is governed by a single dominant gene. The grouping observed in our study indicated that polyembryonic and monoembryonic genotypes have different genetic base with different geographical origin. Thus, the clustering of mango cultivars on the basis of ISSR markers showed good correspondence with pedigree, geographical separation and embryo type.

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