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GENETIC DIVERSITY ANALYSIS OF NAOMI AND SENSATION MANGO CULTIVARS USING RAPD AND ISSR POLYMERASE BASED PCR

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ABSTRACT

The present investigation aimed to use RAPD and ISSR techniques that depend on the polymerase chain reaction technique (PCR) to analyze the genetic variation between Naomi and Sensation mango cultivars. RAPD revealed a total number of 105 DNA band, fifteen bands of them were polymorphic and the average number of the polymorphic bands were seven bands / primer. ISSR primers clarify a wide range of variance between the studied cultivars, ISSR was able to recognize 124 DNA bands and thirty-two out of them were polymorphic with polymorphism percentage 25.8% and average polymorphic bands 10.3 / primer. Also, RAPD detected a high genetic similarity between Naomi and Sensation (91.4); while, ISSR detected 82.4%.

RAPD and ISSR identify unique DNA bands distinguish between the two studied cultivars and could be used as a fingerprint for both cultivars. Naomi and Sensation cultivars were distinguished by 15 positive unique markers. The Naomi cultivar was identified by 14 positive markers and 4 negative markers. On the other hand Sensation was able to detect 14 negative unique markers and 4 positive one. The four negative unique markers resulted from three primers (OPO09, OPG06, OPG09), the size of these markers ranged from 200bp -280 bp.

Keywords **:** RAPD, ISSR, Genetic diversity, Mango cultivars

Introduction

Mango belongs to Anacardiaceae family, consisting of at least 69 species and distributed in several countries in tropical region. Germplasm characterization and identification of genetic relatedness among accessions are important for conservation and breeding programs (Mansour *et al*., 2014). Traditional identification of mango mostly relies on morphological features such as locality, leaf-flower-flesh color, growth habit and other characteristics of plants.

Molecular markers for assessment of genetic variation in plants have shown many advantages. They are neutral, not related to age and tissue type, and not influenced by the environmental conditions and are more informative than morphological markers (Marshall, 1997). Thus, molecular markers can be considered to be more effective approach compared to morphological markers to identify plant genotypes in a germplasm or fruit trees collection. Numerous molecular markers have been developed such as isozyme, Restriction Fragment Length Polymorphism (RFLP), Random Amplified of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple sequence Repeat (SSR), Inter-Simple sequence Repeat (ISSR), and Single Nucleotide Polymorphisms (SNP). Among which, RAPD and ISSR are highly preferred because they are PCR based markers and possess common advantages such as simple, rapid, economic, require minimum laboratory skill, require small DNA quantity, high number of fragments in each reaction, and do not require prior knowledge of genetic genome of targeted plants (VIET and NGAN TRAM 2019). However, RAPD has its own limitations such as low reproducibility due to reactive conditions such as DNA concentrations, the concentration of PCR components, and the number of cycles of the reaction (Williams *et al*., 1990, Mbwana *et al*., 2006). ISSR is a preferred alternative as a highly variable, reproducible marker leading to expanded use in genetic diversity research, population genetic studies, genetic markers, crop identification, source analysis, identification, genetic change identification, and cross-breeding in different plants such as potato (Bornet *et al*., 2002); sugar beet (Izzatullayeva *et al*., 2014); bitter gourd (Singh *et al*., 2015); millet (Dvořáková *et al*., 2015) and mango (Mansour *et al*., 2014).

This study was undertaken to assess the genetic diversity and genetic relationship of Naomi and Sensation to provide basic information for future biodiversity conservation and management program of the *Mangifera* genetic resources.

Materials and Methods

DNA fingerprint

DNA Extraction

Total genomic DNA was isolated from Mangifera leaves (2-3 g) using CTAB method as described by Williams *et al*. (1990) with slight modifications.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD- PCR Reactions

A set of fifteen random 10- mer primers (Table 1) was used in the detection of polymorphism among the two mango genotypes. RAPD-PCR was carried out according to the procedure given by Williams *et al*. (1990) with minor modifications. The amplification reaction was carried out in 50 µl reaction volume containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM primer, 1U Taq DNA polymerase and 25 ng templates DNA.

Thermocycling Profile and Detection of the PCR Products:

PCR amplification was performed in a Perkin-Elmer/ DNA Thermal Cycler 2400 (Norwalk, CT) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94oC. Each cycle consisted of a denaturation step at 94°C for 1 min, an annealing step at 37°C for 2 min, and an elongation step at 72°C for 2 min. The primer extension segment was extended to 7 min at 72°C in the final cycle. The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5µg/ml) in 1X TBE buffer at 95 volts for two hrs. PCR products were visualized on UV light.

ISSR-PCR reaction and thermos-cycling profile:

Twelve ISSR primers were used (Table 1). DNA was amplified according to the following protocol. Each PCR reaction mix of 25 µL contained the 30 ng template DNA, 2.5 µL of 10X PCR buffer, 1.5 µL of 25mM MgCl2, 2.5 µL of the dNTPs mix, 30 pmol of ISSR primer, 1.0 U Taq DNA polymerase (Promega, WI, USA). The amplification was performed in a thermal cycler (Applied Bio Systems, USA) programmed for initial denaturation of 5 min at 94°C; 40 cycles of 2 min denaturation at 94°C, 45 Sec. annealing at 50°C and 2 min extension at 72°C; and final elongation step at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel containing ethidium bromide 0.5µg/mL in TBE buffer for 2 h at 100 V. After electrophoresis, the gels were observed under an UV-transilluminator, documented in Gel-Doc XR (Bio-Rad) and photographed. The size of the amplicons was determined using 100 bp DNA ladder plus.

Table 1 : Sequence of the RAPD and ISSR primers

Fifteen decamer arbitrary RAPD primers		Sequence of the primers used in ISSR detection	
Primer	**Sequence (5'-3')**	**Primer**	
OPC10	TGTCTGGGTG	ISSR1	AGAGAGAGAGAGAGAGYC
OPC11	AAAGCTGCGG	ISSR2	AGAGAGAGAGAGAGAGYG
OPC16	CACACTCCAG	ISSR3	ACACACACACACACACYT
OPC17	TTCCCCCCAG	ISSR4	ACACACACACACACACYG
OPG01	CTACGGAGGA	ISSR5	GTGTGTGTGTGTGTGTYG
OPG04	AGCGTGTCTG	ISSR6	CGCGATAGATAGATAGATA
OPG05	CTGAGACGGA	ISSR7	AGACAGACAGACAGACGC
OPG06	GTGCCTAACC	ISSR8	GATAGATAGATAGATAGC
OPG09	CTGACGTCAC	ISSR9	GACAGACAGACAGACAAT
OPG11	TGCCCGTCGT	ISSR10	ACACACACACACACACYA
OPO01	GGCACGTAAG	ISSR11	ACACACACACACACACYC
OPO03	CTGTTGCTAC	ISSR12	AGAGAGAGAGAGAGAGYT
OPO04	AAGTCCGCTC		
OPO07	CAGCACTGAC		
OPO09	TCCCACGCAA		

Data Analysis

The banding patterns generated by RAPD-PCR and ISSR-based markers analyses were compared to determine the genetic relatedness of the tested mango genotypes. The amplified fragments were scored either as present (1) or absent (0). Bands of the same mobility were scored as identical. The genetic similarity coefficient (GS) between two genotypes was estimated according to Dice coefficient (Sneath and Sokal, 1973).

Results and Discussions

Polymorphism detected by RAPD marker

Fifteen primers generated reproducible and easily scorable RAPD profiles (Fig. 1). These produced multiple band profiles with a number of amplified DNA fragments ranging from 2 to 16 (Table2). The total number of amplicons produced by the tested primers was 105 with an average number of 7 amplicons/ primer. While the number of polymorphic fragments ranged from zero to three. In this respect, Souza *et al*. (2011) stated that, PCR of mango DNA using the six selected primers generated a total of 55 polymorphic loci with 100% polymorphism and the number of bands per primer varied between 6 (primers A01 and G10) and 14 (primer N05), with an average of 9.16 ± 3.31 bands per primer. Moreover, Samal *et al*. (2012) used fifteen 10-mer arbitrary primers to detect RAPD markers among commercial cultivars, hybrids and local mango, and reported that RAPD primers yielded an average of 10.4 bands per primer. A maximum number of 16 amplicons were amplified

with primer OPC11, while the minimum number of amplicons was amplified with primers OPC17, OPG04, OPG11 and OPO07. The highest number of polymorphic bands (3) was obtained with primer OPG06 and OPG09, representing the highest percentage (42.9%) of polymorphism. The average number of polymorhpic fragments / primer within tested mango genotypes was one amplicon. In this respect, Samal *et al*. (2012) the number of bands produced per RAPD primer within different mango genotypes ranged from eight (RPI-2, OPA-4 and OPA-5), to 15 (RPI-6, OPA-1).

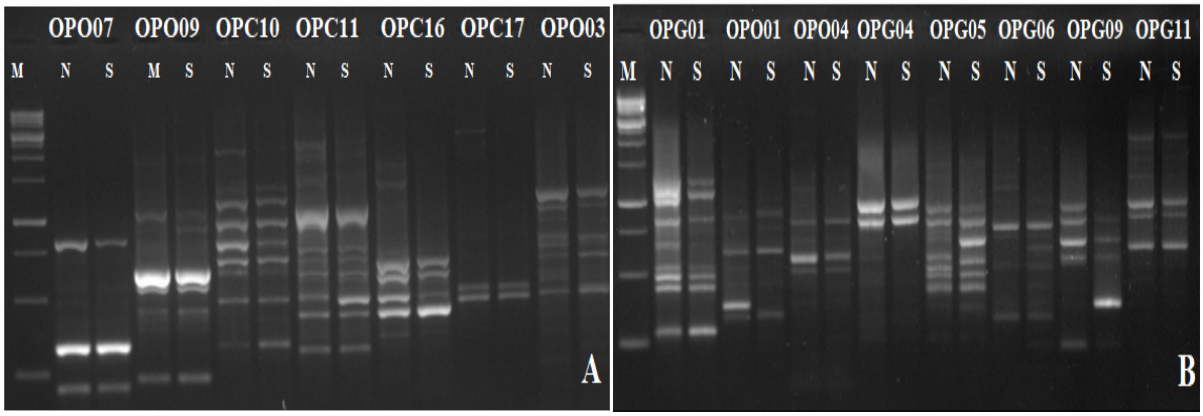

Fig. 1 : RAPD profiles of Naomi (N) and sensation (S) cultivars amplified with RAPD primers (A and B). M: molecular weight marker (1Kb ladder plus.)

Table 2 : Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by RAPD markers within two mango cultivars.

Primer	Total # of amplicons	Monomorphic amplicons	Polymorphic amplicons	% of polymorphism
OPC10	9	8	1	11.1
OPC11	16	16	0	0.00
OPC16	8	6	2	28.0
OPC17	3	2	1	33.3
OPG01	9	8	1	11.1
OPG04	2	2	0	0.00
OPG05	8	7	1	12.5
OPG06	7	4	3	42.9
OPG09	7	4	3	42.9
OPG11	4	4	0	0.00
OPO01	7	6	1	14.3
OPO03	11	4	0	0.00
OPO04	4	10	1	9.09
OPO07	7	7	0	0.00
OPO09	10	9	1	10.0
Total	105	93	15	
Average	7	6.2	1	

Polymorphism Detected by ISSR Unanchored Primers

Twelve unanchored primers were used in the present study; all of these tested primers produced good, reproducible and scorable patterns. The amplification patterns produced by primers ISSR1to ISSR6 and ISSR7 to ISSR12 are shown in Fig.(2A and B respectively). The total number of amplified amplicons by the tested primers was 124 fragments, and the number of amplified DNA fragments by each primer ranged from 4-15 fragments. ISSR2 and ISSR7 amplified the highest number of fragments (15 bands) while, ISSR8 produced the lowest number of amplicons (4bands). The average number of fragments/ primer was 10.3, and the size of these fragments ranged from 180-700 bp. All the used primers produced polymorphic bands (Table 3). The number of polymorphic bands ranged from 1 to 8 resulting in an average polymorphism/ primer of 2.7. ISSR2 revealed the highest number of polymorphic bands (8) while the lowest number of polymorphic amplicons was detected by ISSR3, ISSR4, ISSR8 and ISSR11 (1 band). Therefore, the percent of polymorphism revealed by the different primers ranged from 11.1% to 53.3%. In this context, Viet and Ngan (2019) used ten ISSR primers to evaluate genetic composition of 10 mango genotypes, the tested primers showed clear and reproducible bands. A total 145 bands were generated; out of which 143 bands were polymorphic, accounting for 98.1%. The sizes of amplified bands range from 200 to 2000 bps. The number of bands varied from 10 (primer UBC853) to 18 (primer UBC811). The average of amplified bands and polymorphic bands per primer were 14.5

and 14.3, respectively. Polymorphic percentage ranged between 92.3% (primer UBC825) to 100% (primer UBC880; UBC855; UBC813; UBC853; UBC809; UBC814; and UBC810), with an average of 98.1%.

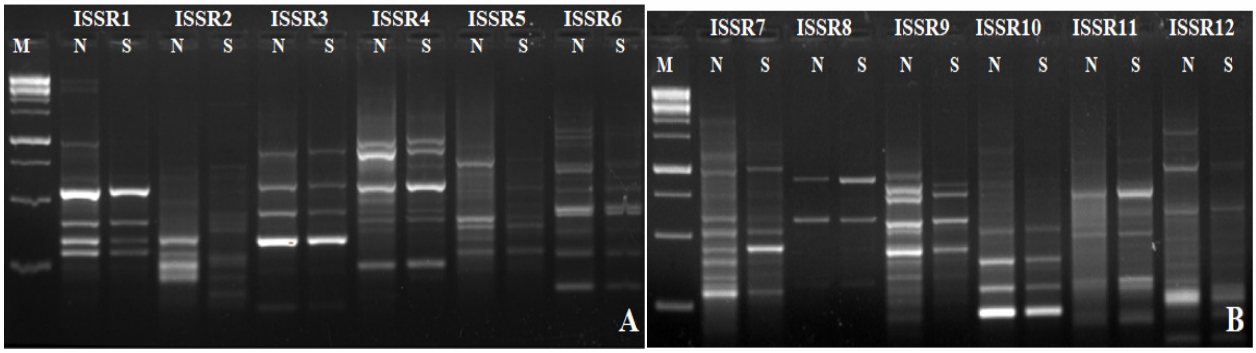

Fig. 2 : Electrophoretic separation pattern of the ISSR- PCR products (as revealed on 1.8% agarose gel) using the primers (A and B). M: 1Kb ladder plus DNA marker. Lanes 1 and 2 represent the two mango genotypes: Naomi (N) and sensation (s).

Table 3 : Total number of amplicons, monomorphic amplicons, polymorphic amplicons and percentage of polymorphism as revealed by ISSR markers within two mango cultivars

Primer	Total # of amplicons	Monomorphic amplicons	Polymorphic amplicons	Percent of polymorphism
ISSR1	8	5	3	37.5
ISSR2	15	7	8	53.3
ISSR3	7	6	1	14.3
ISSR4	9	8	1	11.1
ISSR5	10	6	4	40.0
ISSR6	13	11	2	15.4
ISSR7	15	12	3	20.0
ISSR8	4	3	1	25.0
ISSR9	12	10	2	16.7
ISSR10	9	6	3	33.3
ISSR11	9	8	1	11.1
ISSR12	13	10	3	23.1
Total	**124**	**92**	**32**	
Average	**10.3**	**7.7**	**2.7**	

Genetic Relationships as Revealed by RAPD and ISSR Markers:

In the present study, to determine the genetic relationships between Naomi and Sensation genotypes, the scoring data (1 for presence and 0 for absence) resulting from the **15** tested primers was used to compute the similarity matrices according to Dice (Sneath and Sokal, 1973).The genetic similarity between Naomi and sensation was 91.4%(Table 4) with RAPD marker. While, it was 82.4% (Table 4) with ISSR marker. Souza *et al*. (2011) found that, genetic similarity of RAPD markers were in the range 0.07 (Sensation and Rosa 18) and 0.94 (Rosa 41, Rosa 48 and Rosa 49), with an average value of 0.36 ± 0.18. Meanwhile, Zulhairil *et al*. (2015) stated that, genetic similarity of ISSR markers ranged from 0.0938 to 0.8046 among the Mangifera species. Moreover, Samal *et al*., (2012) calculated average Jaccard's similarity among 65 mango genotypes and it was found to be 62.44%. Two reciprocal mango hybrids 'ArkaAruna'('Banganpalli' 9 'Alphonso') and 'Arka Puneet' ('Alphonso' 9 'Banganpalli') showed the maximum similarity coefficient (0.88) among all 65 genotypes. Pruthvish and Chikkaswamy (2016) mentioned that genetic analysis of RAPD markers based on Ward s method of Euclidean distance showed genetic similarity among the collection of Mango varieties. The collection of Alphonso & Bangalora was closest with the maximum similarity (95%) whereas Mulgoa and Neelum forms separate cluster than other varieties.

Table 4 : Genetic similarity (GS) matrices computed according to Dice coefficient from RAPD and ISSR

Genetic similarity detected by RAPD marker			**Genetic similarity detected by ISSR marker**		
Naomi	Naomi	Sensation	Naomi	Naomi	Sensation
	100 %			**100 %**	
Sensation	**91.4 %**	**100 %**	Sensation	**82.4 %**	**100 %**

Genotype Identification by Unique RAPD Markers:

As shown in Tables (5), the RAPD assay permitted the identification of the two mango cultivars by unique positive and/or negative markers. Two mango genotypes were characterized by 15 positive unique RAPD markers.

Among the tested primers, 10 exhibited negative markers and 10 revealed positive markers. These identified 30 unique markers across the two mango cultivars. The RAPD primers generating the different markers and the markers approximate size are listed in Table (5). Naomi was characterized by the highest number of positive unique

markers (14) and lowest negative markers (4). Meanwhile, Sensation revealed 4 positive and 14 negative unique markers; the four negative markers resulted from three primers (OPO09, OPG06 and OPG09). The size of these unique markers ranged from 200 to 850 bp. Certain primers, were more informative than the others e.g., OPC16, OPG06 and OPG09 since they identified the highest number of unique positive and negative markers (6),these primers together had the potential to identify the two mango genotypes. In this respect, Ibrahim *et al*. (2016) stated that RAPD assay permitted the identification of 9 mango genotype and elven hybrids by unique positive and/or negative markers. Eight out of the nine genotypes were characterized by 11 positive unique RAPD markers. Among these eight genotypes, two were also characterized by negative RAPD markers. Among the tested primers, three exhibited negative markers and six revealed positive markers. These identified 18 unique markers across the twenty tested genotypes. Hybrid H2 was characterized by the highest number of unique markers; three are negative and one was positive marker. This was followed by H1 which revealed three positive unique markers, one of these markers resulted from primer OPZ18 and two from OPO16. On the other hand, the lowest number of unique markers was detected in H3, H4, H6, H11 and Sensation genotype which were identified by only one unique positive marker. The size of these unique markers ranged from150 to 1306 bp. Certain primers, were more informative than the others e.g., OPC17, and OPO16 since they identified the highest number of genotypes (3).The presence of unique.

Table 5 : The two mango cultivars characterized by unique positive and / or negative RAPD markers, marker size and total number of markers identifying each cultivar.

Genotype	Unique positive markers			Unique negative markers			Grand Total
	Size of the marker band (bp)	*Primer*	Total # of mar- kers/ accession	Size of the marker band (bp)	*Primer*	Total # of markers/ accession	
Naomi	700 550, 620 850 500 360 240 340 540 200, 340	OPC10 OPC16 OPC17 OPO03 OPG01 OPO01 OPG05 OPG06 OPG09	11	450 290, 350 260	OPO09 OPG06 OPG09	4	15
Sensation	450 290, 350 260	OPO09 OPG06 OPG09	4	700 550, 620 850 500 360 240 340 540 200, 340	OPC10 OPC16 OPC17 OPO03 OPG01 OPO01 OPG05 OPG06 OPG09	11	15
Total			**15**			**15**	**30**

Genotype Identification by Unique ISSR Markers:

In the present study, genotype–specific ISSR unique markers could distinguish both Naomi and Sensation cultivars. The ISSR-primers generating the markers and the markers approximate size are shown in Table (6). Both of the tested genotypes were characterized by both positive and negative unique ISSR markers. Naomi genotype was characterized by 24 unique markers with marker size ranged from 180-900 bp. The same unique markers (24) were observed with sensation genotype; however, these markers were negatively unique for sensation genotype. Moreover, eight unique negative markers were detected with Naomi genotypes, these markers were positively unique with sensation genotype and recording molecular weight ranged from 170-500 bp.

Primer ISSR2 represents putative informative primer that could score 5 unique positive and negative markers with Naomi and sensation, respectively, followed by ISSR1, ISSR5 and ISSR10 that scored three unique primers with both of the tested genotypes. While, remaining primers were able to identify one or two unique markers (Table 6). The obtained results were in agreement with the findings of Samant *et al*. (2010), who identified fifty three mango genotype out of sixty – three with two ISSR primers (UBC812 and UBC- 891), meanwhile, ISSR-5 primer identify the lowest number of genotype (17). Moreover, 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 out of 63 selected mango genotypes for the study. Moreover, Fang *et al.* (1998) concluded that accessions with many unique ISSR fragments could be useful for expanding the germplasm base of breeding programs.

Table 6 : The two mango cultivars characterized by unique positive and / or negative ISSR markers, marker size and total number of markers identifying each cultivar.

Genotype	Unique positive markers			Unique negative markers			Grand Total
	Size of the marker band (bp)	*Primer*	Total # of markers/ accession	Size of the marker band (bp)	*Primer*	Total # of markers/ accession	
Naomi	470, 800, 900	ISSR1		170, 210, 270	ISSR2		
	200, 230, 240,	ISSR2		270	ISSR5		
	290, 300	ISSR2		500	ISSR7		
	400	ISSR3		280	ISSR8	8	32
	300	ISSR4	24	410	ISSR11		
	190, 220, 450	ISSR5		230	ISSR11		
	380, 430	ISSR6					
	490, 700	ISSR7					
	180, 480	ISSR9					
	450, 480, 510	ISSR10					
	240, 550	ISSR12					
Sensation	170, 210, 270	ISSR2		470, 800, 900	ISSR1		
	270	ISSR5		200, 230, 240,	ISSR2		
	500	ISSR7		290, 300	ISSR2		
	280	ISSR8	8	400	ISSR3	24	32
	410	ISSR11		300	ISSR4		
	230	ISSR11		190, 220, 450	ISSR5		
				380, 430	ISSR6		
				490, 700	ISSR7		
				180, 480	ISSR9		
				450, 480, 510	ISSR10		
				240, 550	ISSR12		
Total			32			32	64

Conclusion

In summary, this study showed that RAPD and ISSR are useful markers in genetic diversity studies based on the very high polymorphism level detected by the primers in *Mangifera* genotypes. The RAPD and ISSR markers are also suitable for determining the genetic similarity among the *Mangifera* species. It is also recommended that these *Mangifera* accessions need to be conserved on farm and also collected for germplasm collection for conservation programmes. Perhaps these accessions may become useful as a genetic material in the breeding research in the future, especially the wild relatives of *Mangifera* species which are capable of developing resistant factors to disease and pest of mango cultivation.

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