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GENETIC DIVERSITY ANALYSIS OF SAPOTA (*Manilkara zapota*) GERMPLASM BY RAPD MARKER

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Abstract

The experiment was carried out at the Department of Horticulture, Patuakhali Science and Technology University (PSTU), Bangladesh, during the period from October 2018 to April 2019. The genetic diversity of eight sapota germplasm *viz*. Gp-1, Gp-2, Gp-4, Gp-6, Gp-7, Gp-9, Gp-15 and Gp-17available at the Germplasm Center of PSTU using PCR based Random Amplified Polymorphic DNA (RAPD) marker were studied.By the PCR, the RAPD primers generated various bands ranging from 3 to 10 and yielded a total of 45 bands of which 42.12% were polymorphic.Comparatively higher genetic distance (0.28) was found between Gp-1 and Gp-9. The inter-population similarity index value was the highest (99%) between Gp-2 and Gp-9, Gp-4 and Gp-15.Among the germplasm, Gp-1 and Gp-17werethe most polymorphic with other sapotagermplasm. Interestingly, the leaf morphological features (length and width) were also found the highest in Gp-1 and Gp-17.

Keywords: Genetic diversity, sapota germplasm, RAPD marker.

Introduction

Sapota [*Manilkara zapota* (L.) van Royen] is an economically important species of the Sapotaceae family native to tropical America. As a rainforest tree known as the "Chicle tree", it has a long history of human use. It has been used for many purposes including latex, fruit and timber. Being originated in the tropical America, it is an important commercially cultivated fruit in several countries including Bangladesh. More area is being brought under this crop every year in Bangladesh. Popularity of this crop is increasing among the farmers due to its high production per unit area, continuous fruiting and thereby income throughout the year, low cost of production, liking to Bangladeshi palate, and very little incidence of diseases and pests. The type of pollination of sapota is outcrossing. Hu *et al.* (2014) revealed that perennial plants with outcrossing type usually have greater genetic diversity. This variation allows species to change over time, to survive according to changing environmental conditions. Genetic diversity of a plant population could be assessed using morphological, biochemical, and also molecular markers. The advantage of molecular markers over morphological and

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biochemical markers is that it is not influenced by environmental factors (Hapsoro *et al.*, 2015). Laserna *et al.* (2012) proposed that the diversity of phenotypes was a form of phenotypic changes associated with genotypic response to changing environmental conditions. In order to obtain accurate information on diversity molecular markers are usually used as general approach.In sapota, most of the work on variability has been carried out using morphological characteristics such as tree shape, structure, leaf color, shape, fruit size and shape (oval and round). So far, only few studies were carried out to estimate the extent of genetic variability in sapota cultivars using either isozymes or DNA markers.

RAPD (pronounced "rapid") stands for Random Amplified Polymorphic DNA. RAPD markers are attractive because of their simplicity, versatility, modest cost and ability to detect even the relatively small amount of variation (Ragot and Hoisington, 1993). Genetic studies had widely used RAPD for estimating the genetic diversity (Aukar *et al.*, 2002), and genetic relatedness among accessions (Bayazit *et al.*, 2011). RAPD was applied for identifying the genetic diversity of sapota in four populations in Mexico (Heaton *et al.*, 1999); in 20 cultivars (Meghala *et al.*, 2005) and in 19 cultivars (Suhasini *et al.*, 2013) in India. RAPD marker is distributed throughout the genome. This marker is also relatively feasible, fast and cheap to produce high polymorphism (Medhi *et al.*, 2014). From the best of our knowledge and available literature, there was no related works done in the southern part of Bangladesh. Thus, a comprehensive study was felt necessary to get information and the present study was undertaken to analyze the genetic diversity of sapota germplasm that could support the sapota breeding program.

Materials and Methods

Experimental materials

Selected eight germplasm *viz*., Gp-1, Gp-2, Gp-4, Gp-6, Gp-7, Gp-9, Gp-15 and Gp-17 available at the Germplasm Center, PSTU were used as treatments for the experiment. All the selected germplasm were seven years old grafted tree that started first flowering and fruiting at the age of four.

Experimental site and design

The experiment was conducted at the Plant Biotechnology Laboratory, Department of Horticulture, PSTU, during October 2018 to April 2019. The single factor experiment was conducted in a Completely Randomized Design (CRD) with four replications.

Sample preparation

The recently matured leaves that were free from damage caused by pests and diseases and available throughout the year were used for isolation of DNA. The fully open leaves were harvested before 7.00 am to avoid increasing of phenolic compound.

DNA extraction

Genomic DNA was isolated from the leaves by DNAzol reagent formulated for the isolation of genomic DNA from plants. Plant leaves were rinsed with distilled water and 50 mg of younger leaves were taken. Then the leaves were ground into powder with mortar and pestle and 1 ml of DNAzol was added and then homogenized properly. The mixture was centrifuged at 10,000 rpm for 10 minutes and transferred the supernatant to a 2 ml micro tube.

DNA purification and precipitation

Twenty micro liter RNase were added in the tube to degrade RNA. The tube was incubated at 65˚C for 60 minutes with frequent swirling and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into new micro centrifuge tube and an equal volume of (Chloroform: isoamyl alcohol) (24:1) was added to the tube. Then the tube was shaken vigorously to form a complete emulsion. The tube was centrifuged at 12,000 rpm for 15 minutes to separate the phases. The aqueous phase (supernatant) was removed with micropipette, and transferred to a new tube. Purification step was repeated about 3 times. Potassium acetate (Sigma - Germany) 3M (1/10 volume) was added to the supernatant, mixed gently and was precipitated with 2/3rd volume of cold isopropanol. Then it was incubated at 4˚C for 12-24 hours. The precipitated nucleic acids were collected by centrifuging at 12,000 rpm for 15 minutes and washed twice with 70% ethanol. The pellets were air dried and resuspended in TE buffer.

DNA quantification

DNA concentration and purity were measured by NanoDrop™ Spectrophotometer (Thermo Scientific). One micro liter of each DNA extracts was diluted with 99 µl of deionized water and the absorbance at 260 nm/280 nm was measured. The DNA concentration was expressed in ng/ul.

Primer selection

To detect the polymorphism of genotypes of sapota, five 10-mer (OPA 20, OPB 05, OPB 06, OPB 08, OPC 19) and three 12-mer (OPA 00, OPA 02, OPA 03) primers were used for DNA amplification. Primers that have GC content more

than 60% are suitable for RAPD analysis (Anne and Les, 1995). Final subsets of eight primers exhibited good quality banding patterns and sufficient variability was detected for further analysis. List of primers are as follows:

Primer code	Sequence (5' - 3')	GC content (%)	Melting temperature (ºC)
OPA 00	ATCAGCGCACCA	58.3	45.7
OPA 02	GCCAGCTGTACG	66.7	44.7
OPA 03	TGCCTCGCACCA	66.7	49.6
OPA 20	GTTGCGATCC	60.0	33.5
OPB 05	TGCGCCCTTC	70.0	41.1
OPB 06	TGCTCTGCCC	70.0	39.8
OPB 08	GTCCACACGG	70.0	37.3
OPC 19	GTTGCCAGCC	70.0	39.0

DNA amplification

The process suggested by Handayani *et al.*, 2016 was used for PCR amplification of DNA. The total volume of PCR mixture was 14 µl for each sample, where 2 µl genomic DNA, 1 µl RAPD primer, 7 µlPCR reaction mix (Go Taq ^{R} G2 Green master mix, Promega), 4 µl nuclease free water were mixed. PCR consisted of one cycle of initial denaturation at 94˚C for 4 minutes, which was followed by 45 cycles of denaturation at 94˚C for 1 minute; annealing at 37˚C for 1 minute; and extension at 72˚C for 1 minute 30 seconds. PCR was completed with one cycle of 72˚C for 7 minutes and 4˚C for 1 minute. The amplification products were analyzed by electrophoresis using 1.5% agarose gel (Promega Corp.) in 1x TAE buffer for 1.5 hours in 75 Volt. The result was checked by UV transluminator light and documented by digital camera (Alpha Imager mini).

Calculation of genetic distance and genetic similarity

Estimates of genetic distance and genetic similarity were calculated all possible pair-wise by the following formula (Nei, 1979):

Genetic distance, $d_{xy} = 1 - \{2n_{xy} / (n_x + n_y)\}$

Genetic similarity, $S_{xy} = \{2n_{xy} / (n_x + n_y)\} \times 100\%$

Where, n_x and n_y are the numbers of bands amplified in individuals x and y, respectively, and $2n_{xy}$ is the number of bands shared by those individuals.

Observation of leaf morphological features

Leaf morphological features of eight sapota germplasm like length and width of leaves were measured by a slide calipers and the mean value was determined in millimeter (mm). The final readings were expressed in centimeter (cm).

Statical analysis

Data were analyzed statically and means were separated by least significant difference (LSD) at 5% level of probability.

Results and Discussion

RAPD analysis

In the experiment, the primers generated various bands ranged from 3 to 10 and yielded a total of 45 bands of which 42.12% were polymorphic (Table 1). The highest percent of polymorphic bands (67%) was observed for primer OPA 20 and the lowest (17%) was for primer OPA 02.The amplification patterns of representative samples of sapota with eight different primers have been shown in the Fig. 1 to 8.The highest 10 bands were observed by OPC19 primer, where only two bands were polymorphic that were in Gp-1 and Gp-17. Moreover, the highest four polymorphic bands were found by OPA20 within total 6 bands, where two polymorphic bands in Gp-1 and another 2 in Gp-17. On the other hand, the lowest 1 polymorphic band was observed by OPA02, which was in Gp-1. So in most of the cases, the maximum polymorphism was observed in Gp-1 followed by Gp-17 in respect of other studied six germplasm.

Table 1. RAPD primers with corresponding bands scored and their size range together with polymorphic bands observed in 8 sapota germplasm

Sl. No.	Primer code	No. of total band	No. of common band	No. of polymorphic band	Proportion of polymorphic band (%)	Molecular weight range (kb)
1	OPA00	5	2	3	60	0.6 to 1.5
2	OPA02	6	5	1	17	0.5 to 1.0
3	OPA03	4	2	2	50	0.3 to 1.2
4	OPA20	6	2	4	67	0.4 to 1.5
5	OPB05	3	2	1	33	0.6 to 1.3
6	OPB06	6	3	3	50	0.35 to 1.0
7	OPB08	5	3	2	40	0.3 to 1.1
8	OPC19	10	8	2	20	0.3 to 1.3
Total	8 primers	45	25	18	337	--
Average	--	5.63	3.23	2.25	42.12	--

Sarker (2012) found that the primers generated banding patterns in sapota ranging from 3 to 5 and yielded a total of 57 band patterns having 48.89% polymorphic bands. She observed that, the highest percent of polymorphic bands (66.67%) was from primer OPA 03 and the lowest (20%) from primer OPS 07. Increased number of bands was obtained in fungi with increased GC content of the primer (Kubelik and Szabo, 1995).

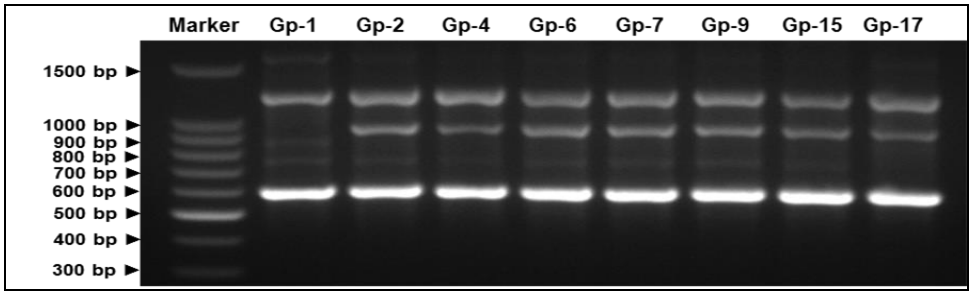

Fig. 1: RAPD pattern of sapota germplasm generated by the primer OPA 00.

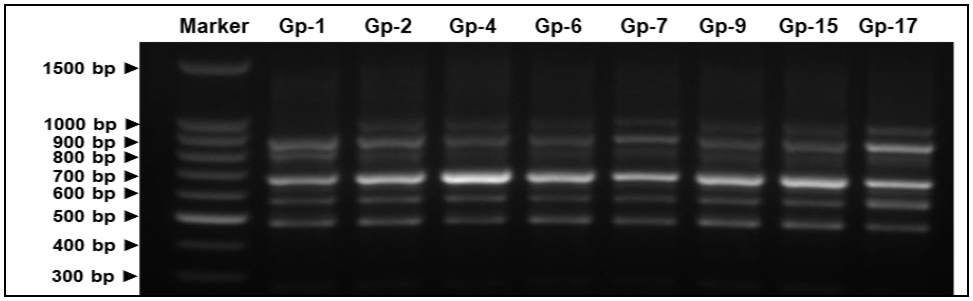

Fig. 2: RAPD pattern of sapota germplasm generated by the primer OPA 02.

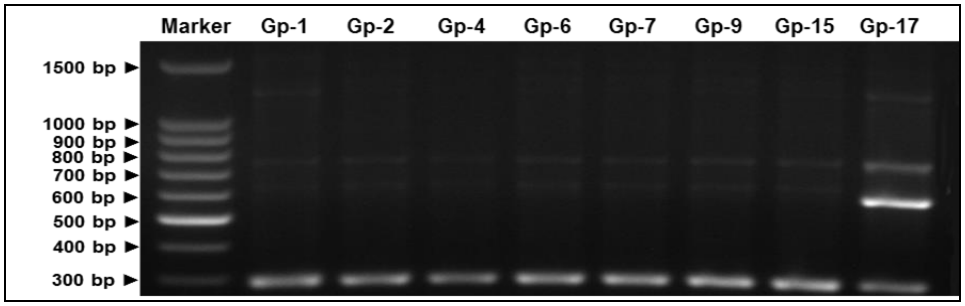

Fig. 3: RAPD pattern of sapota germplasm generated by the primer OPA 03.

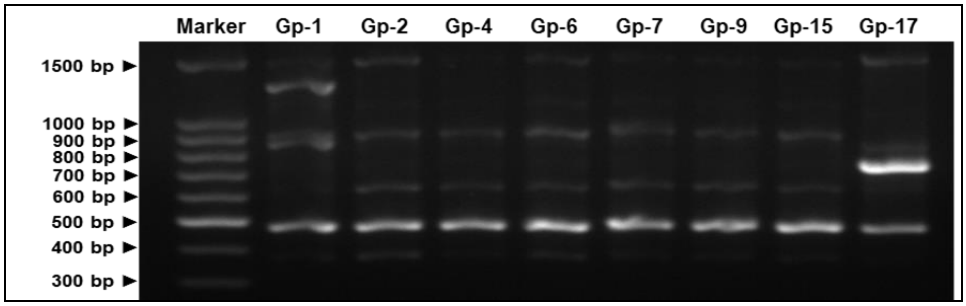

Fig. 4: RAPD pattern of sapota germplasm generated by the primer OPA 20.

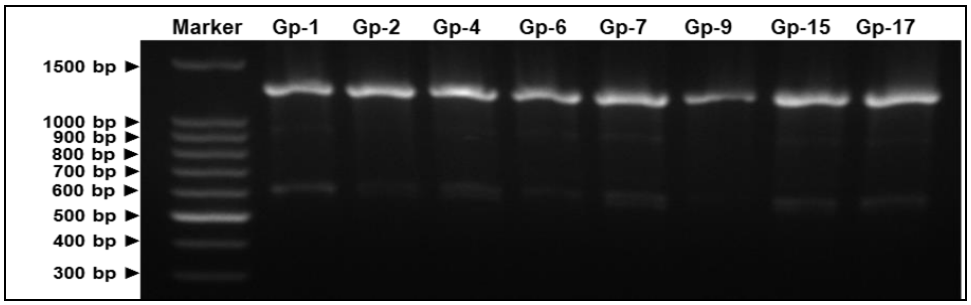

Fig. 5: RAPD pattern of sapota germplasm generated by the primer OPB 05.

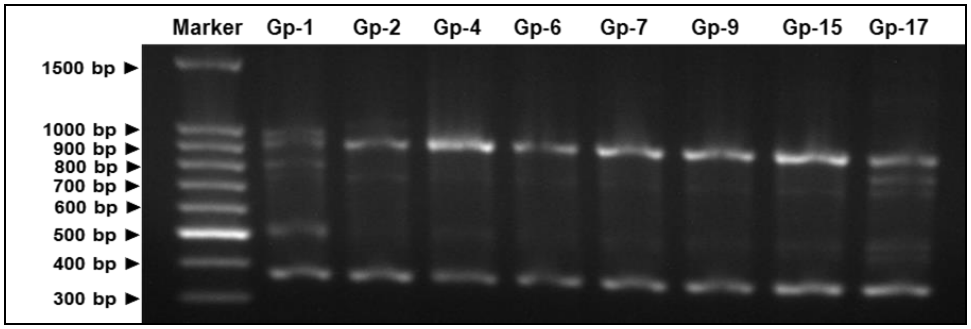

Fig. 6: RAPD pattern of sapota germplasm generated by the primer OPB 06.

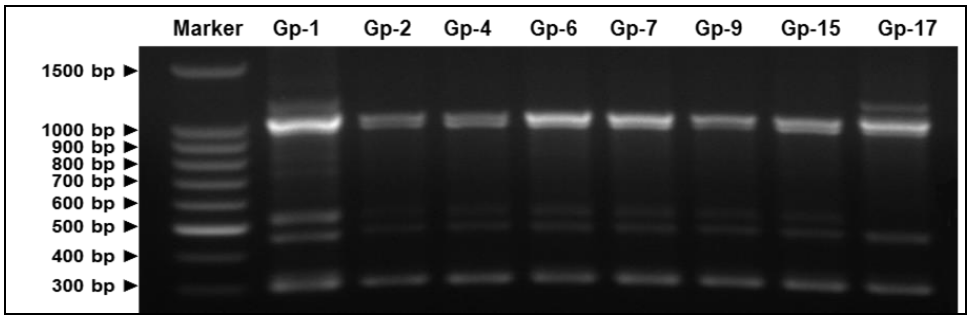

Fig. 7: RAPD pattern of sapota germplasm generated by the primer OPB 08.

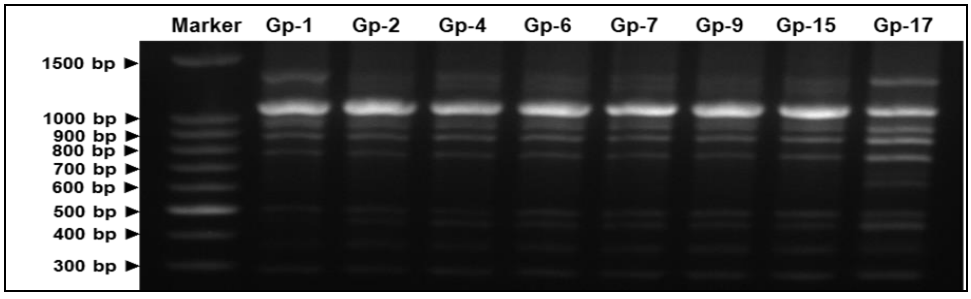

Fig.8: RAPD pattern of sapota germplasm generated by the primer OPC 19.

Genetic distance

The values of pair-wise comparisons of Nei's (1979) genetic distance among eight sapota genotypes were computed from 0.01 to 0.28 (Table 2). Comparatively higher genetic distance (0.28) was found between Gp-1 and Gp-9. The lowest genetic distance (0.01) was found between Gp-2 and Gp-9, Gp-4 and Gp-15.Sarker (2012) reported that genetic distance among 15 germplasm ranged from 0.11 to 0.4. There was genetic variation among the studied sapota germplasm as indicated by the proportion of polymorphic loci.Among the germplasm, the genetic diversity index was the maximum for seven germplasm with Gp-1, which ranged from 0.19 to 0.28. Except Gp-1, genetic diversity index was the maximum for six germplasm with Gp-17, which ranged from 0.18 to 0.26. These results were complementary to the band patterns of the germplasm.

Table 2. Genetic diversity index from RAPD data of 8 sapota germplasm

Germplasm	Gp-1	Gp-2	Gp-4	Gp-6	Gp-7	Gp-9	Gp-15	Gp-17
Gp-1	--							
Gp-2	0.19	--						
Gp-4	0.24	0.05	--					
Gp-6	0.27	0.10	0.02	--				
Gp-7	0.20	0.02	0.03	0.05	--			
Gp-9	0.28	0.01	0.04	0.06	0.08	--		
Gp-15	0.23	0.06	0.01	0.16	0.16	0.15	--	
Gp-17	0.26	0.20	0.18	0.21	0.18	0.23	0.25	--

Genetic similarity

Inter-population genetic similarity indices for different germplasm have been presented in the Table 3. In the present study, the inter-population similarity index was the highest (99%) between Gp-2 and Gp-9, Gp-4 and Gp-15. The lowest genetic similarity (72%) was found between Gp-1 and Gp-9. Among the germplasm, genetic similarity was the minimum for seven germplasm with Gp-1, which ranged from 72 to 81%. Except Gp-1, the genetic similarity index was the minimum for six germplasm with Gp-17, which ranged from 74 to 82%. These results were also complementary to the band patterns of the germplasm. Other genotypes were more similar than that of Gp-1 and Gp-17. The higher genetic similarity among these germplasm clearly indicated the genetic relatedness among the genotypes. Sarker (2012) found the highest genetic similarity as 88.8% and the lowest genetic similarity as 60% among the germplasm.

Table 3. Genetic similarity (%) index from RAPD data of 8 sapota germplasm

Germplasm	Gp-1	Gp-2	Gp-4	Gp-6	Gp-7	Gp-9	Gp-15	Gp-17
Gp-1	--							
Gp-2	81	--						
Gp-4	76	95	--					
Gp-6	73	90	98	--				
Gp-7	80	98	97	95	--			
Gp-9	72	99	96	94	92	--		
Gp-15	77	94	99	84	84	85	--	
Gp-17	74	80	82	79	82	77	75	--

Observation of leaf morphological features

Leaf morphological features (length and width) of eight sapota germplasm were determined (Table 4). Both leaf length and width varied significantly among the sapota germplasm. The highest leaf length (12.80 cm) was observed in Gp-17, which was statistically different with other germplasm. However, the second highest leaf length (10.68 cm) was observed in Gp-1. On the other hand, the highest leaf width (4.98 cm) was also observed in Gp-17, which was statistically identical to Gp-1 (4.60 cm). Ramadoss and Arivazhagan (2016) reported that leaf length of sapota ranged from 11.70 cm to 15.60 cm and width ranged from 3.10 cm to 5.80 cm.

Table 4. Leaf morphological features (length and width) of 8 sapota germplasm

Germplasm	Leaf length (cm)	Leaf width (cm)
Gp-1	10.68 b	4.60 a
Gp-2	10.50 b	3.55 b
Gp-4	10.73 b	3.62 b
Gp-6	10.27 b	3.57 b
Gp-7	10.52 b	3.77 b
Gp-9	10.38 b	3.30 b
Gp-15	10.52 b	3.35 b
Gp-17	12.80 a	4.97 a
LSD ($P \geq 0.05$)	0.99	0.55

Common letter(s) within the same column do not differ significantly at 5% level of significance by LSD.

Conclusion

Genetic diversity analysis of eight germplasm (Gp) of Sapota with isolated genomic DNA were done using PCR based Random Amplified Polymorphic DNA (RAPD) marker. Primer OPA 20 produced the maximum visible polymorphic banding patterns. By the PCR, the RAPD primers generated various bands ranging from 3 to 10 and yielded a total of 45 band patterns of which 42.12% were polymorphic. Comparatively higher genetic distance (0.28) was found between Gp-1 and Gp-9. The inter-population similarity index value was the highest (99%) between Gp-2 and Gp-9, Gp-4 and Gp-15.However, among the germplasm, Gp-1 and Gp-17 were most polymorphic with other sapota germplasm. Interestingly, the leaf morphological features (length and width) were also found the highest in Gp-1 and Gp-17 indicating OPA 20 might be specifically linked with leaf features.

Acknowledgements

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