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RESEARCH ARTICLE

RAPD MARKER ASSAY BASED GENETIC DIVERSITY IN AONLA (*Emblica officinalis* L.)

Rane, A.A, Bharat Kag, Vinay Hegde* and B.N. Sathyanarayana

Plant Tissue Culture Laboratory and Molecular Biology units, Division of Horticulture, University of Agricultural Science, GKVK, Bangalore- 560 065, Karnataka, India

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ABSTRACT

Aonla (*Emblica officinalis L.*) belongs to the family Euphorbiaceae. The chromosome number of Aonla is 2n=28. Aonla is indigenous to tropical south eastern Asia particularly in central and southern India. The fruit is highly nutritive and it is a rich source of vitamin C. It is valued as antiscorbutic, diuretic, laxative and antibiotic. The investigation was comprised of estimation of genetic diversity of Aonla by using Random Amplified Polymorphic DNA marker. The light green colored leaves harvested from 20 different germplasm were used for DNA extraction by CTAB method. Isolated genomic DNA subjected to PCR amplification using 40 RAPD decamer primers. The data from molecular profiling using RAPD primers were analyzed mainly by scoring for the presence or absence of polymorphic bands through visual interpretation and this binary data was used for statistical analysis. The data was used to generate genetic similarity coefficient similarity matrix on the basis of Jaccard's coefficient. The Dendrogram (cluster diagram) generated by unweighted pair group method with arithmetic average (UPGMA) algorithm using Sequential Agglomerative Hierarchial and Nested (SAHN). Dendrogram revealed that the genotypes could be grouped into four clusters. The dendrogram showed two (A and B) major clusters at 57% similarity while C and D depicted 59% and 60% of homology respectively within the cluster. The cluster information thus enables us in selecting the parent for breeding programme.

INTRODUCTION

Aonla (*Emblica officinalis* L.) is an important minor fruit and a crop of commercial significance. It belongs to the family *Euphorbiaceae* and known as amla, amlaki, amali, ambala and nelli in different parts of India. Aonla is native to India (Singh, 1985) and trees are seen almost throughout the country. Perry (1943) reported chromosome number of Aonla is 2n=28 and variation from 2n=98 to 104 has been observed. Aonla is said to be indigenous to tropical south eastern Asia particularly in central and southern India (Ferminger, 1947). The fruit is highly nutritive and it is a rich source of vitamin C (600mg/100g) among fruits next only to Barbods Cherry (Asenjo, 1953). It is valued as antiscorbutic, diuretic, laxative and antibiotic. It could be used in treating chronic dysentery, bronchitis, diabetes, fever, diarrhea (Dalzell and Gibson, 1861), jaundice, dyspepsia and cough. It is also used in tanning and dyeing industries.

For crop improvement in Aonla, more serious attempts have not been made so far. Surveys have to be made to identify the elite trees having good agronomic and economic traits with commercial and industrial uses. Most of the morphological characters are influenced by environmental factors and many quantitative traits are of polygenic inheritance, which express only after several years of growth (Hamrick *et al*., 1992). In recent years, isozyme and molecular marker techniques have been used to assess the genetic diversity more accurately (Anand, 1998). Genetic diversity is important in plant breeding and is commonly measured by genetic distance or genetic similarity (Weir, 1990). Molecular marker-based genetic diversity analysis has potential for assessing changes in genetic diversity over time and space (Duwick, 1984). Molecular markers represent a powerful and potentially rapid method for characterizing and managing plant germplasm, both *in situ* and *ex situ* (Virk *et al*., 1995). Recent development in DNA technologies have made it possible to uncover a large number of genetic polymorphisms at the DNA level and use them as markers for evaluation of the genetic basis for the observed phenotypic variability. DNA markers are more suitable and ubiquitous in nature. RAPD is one of the powerful molecular techniques which have been applied to a wide range of crops to assess genetic diversity, to evaluate genetic relationships between accessions or cultivars (Williams *et al*., 1993) and estimation of relatedness (Lynch, 1999). This study is an attempt to explore molecular aspects of Aonla to achieve a better understanding of genetic variation and to investigate their inter-relationship and further crop improvement.

MATERIALS AND METHODS

The investigation on 'RAPD marker assay based genetic diversity in Aonla (*Emblica officinalis* L.)' was carried out at the department of Horticulture, University of Agricultural

Corresponding author: vinayhegde.bhandimane@gmail.com

Sciences, Bangalore. The present investigation was comprised of two main objectives- Standardization of procedures for RAPD in Aonla and estimation of genetic diversity.

Plant material and sample collection: Tender, healthy, mature and disease/pest free leaves from 20 different varieties of Aonla were collected from different places (University of Agricultural Sciences- Bangalore, University of Agricultural Sciences- Dharwad and IIHR- Bangalore), brought to the lab immediately and preceded with the DNA extraction protocol. (Table 1).

Isolation of genomic DNA: Aonla leaves are rich in polysaccharides and phenols, which interferes with the isolation and purification of DNA samples. The polysaccharides contamination can also cause problems in downstream processing or application by inhibiting certain enzymes in PCR. Extraction of DNA was preferred with CTAB method (Dellapotra *et al*., 1983 modified by Porebski *et al*., 1997 and Shashidhara *et al*., 2003). The DNA pellets were air dried at 37^0C for 15 min and dissolved in 500µl of TE (Prepared 10mM Tris HCl and 1mM EDTA mixed, pH adjusted to 8.0 and autoclaved) buffer and store at -20^0C.

Gel electrophoresis: 5 µl of crude DNA sample was pipetted on to a parafilm and mixed well with 2-3 µl of 10X loading dye by pipetting and is loaded on the gel. The gel was run at 75 V for 1 hour and bands were visualized and documented using a gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA).

DNA quantification using spectrophotometer: Concentration and purity of the DNA samples (5µl of each DNA sample was taken in a quartz cuvette) were estimated by recording absorbance at 260 nm and 280 nm in a UV spectrophotometer (Genequant pro, Bioscience). A good DNA preparation exhibits the spectral properties A260/ A280 = 1.8. DNA concentration was calculated for all samples (using the relationship for double stranded DNA, 1 O.D. at 260 nm = 50µg/ ml). (Table 2)

PCR amplification using RAPD primers: For RAPD screening, 40 primers from Operon technologies, USA, belonging to OPA, C, D, H, K, M, N, Q and Z series were used (TABLE- 3). All the stock solutions were prepared under the laminar flow hood for maintaining sterile conditions. Autoclaved micro-tubes, pipette tips, double distilled and sterile water were used. Amplification was performed with thermal cycler (Eppendorf Germany) using the cycling parameters of Das *et al*., 1999. Genomic DNA isolated from 20 Aonla varieties was subjected to PCR amplification using 40 RAPD decamer primers. RAPD – PCR was set up in 25µl reaction in PCR tubes using the constituent reagents [Reaction buffer (10x) =2.50µl, MgCl2 (25mM) =1µl, dNTPs (2.5mM) =1µl, *Taq* DNA polymerase (3U/µl) =0.3µl, Template DNA (20ng/µl) =02.00µl, Primer (3pm/µl) =03.50µl, Sterile distilled water =14.70µl]. To minimize pipetting errors a master mix excluding the DNA template was prepared including one negative control. DNA template was dispensed (3µl/tube), followed by addition of master mix carefully. The content was mixed gently spun down at 1500rpm for 15 sec. The tubes were placed in a TECHNE, TC 5000 and following PCR programme [Mullis *et al.*, 1986 and Saiki *et al.,* 1988]. At end of PCR, 2.5µl of loading dye was added to tubes and spin down for 2-5 seconds at top speed in microfuge and then store at 4^0C till electrophoresis.

Electrophoresis and visualization of PCR products: The amplified PCR products of each RAPD primers were separated on 1.5% agarose gels with ethidium bromide (0.5µl/ml) and viewed under UV light of the gel documentation system (UVPRO, UK.) and photographed.

Estimation of genetic diversity (Statistical/data analysis): The data from molecular profiling using RAPD primers was analyzed mainly by scoring for the presence (1) or absence (0) of polymorphic bands through visual interpretation. Only clear bands were taken into account for scoring. The band sizes were determined by comparing with the 1kbp ladder, which was run along with the amplified products. The experiments were repeated once to several times to check for the consistency. Binary characters scored as 1 for presence and 0 for absence are converted to binary matrix for analysis using Numerical Taxonomy and Multivariate Analysis System (NTSYS pc, ver. 2.02), (Rohlf, 1998). The data was used to generate genetic similarity coefficient similarity matrix on the basis of jaccard's coefficient with SIMQUAL option. The Dendrogram (cluster diagram) generated by unweighted pair group method with arithmetic Average (UPGMA) algorithm using sequential Agglomerative Hierarchial and Nested (SAHN).

RESULTS

Standardization of procedures for RAPD in Aonla: 2-3g of sample (fresh tender leaves) with 10ml of extraction buffer (6% CTAB, 100mM tris pH8.0, 20mM EDTA pH 8.0, 1.4mM NaCl) with 2% PVP and 2% β-Mercaptoethanol (30µl) for 6ml of extraction buffer resulted in good quality and fair quantity of DNA. To assess the quality, electrophoresis was done with 1µl of crude DNA sample on agarose gel (0.8%) stained with ethidium bromide and bands appeared in the gel were documented using Alpha Imager 1200 (*Alpha Innotech Inc., USA*) (Plate 1).

The absorption ratio of DNA with moderate quality for RAPD analysis was observed. The quantity of DNA present in each sample was determined by Spectrophotometer based on absorbance at 260 nm. The quantity of DNA in different samples varied from150 ng/µl to 850ng/µl (TABLE-2). Good quality of DNA was reflected according to A260 / A280 ratio 1.8 from LOCAL-10, NA-10. After quantification, all the samples were diluted to 25ng/µl as per requirement. For PCR amplification of twenty germplasm lines of Aonla 40 RAPD primers screened (TABLE-3). The PCR parameters were optimized *viz;* 25ng/µl concentration of genomic DNA, 1U Taq DNA polymerase (Bangalore Genei Pvt. Ltd. India) 5pM primer concentration, $MgCl_2$ at 25mM, dNTPs at 10mM. A cycling regime of 45 cycles with initial denaturation of 94^0C for 4 min and after each cycle consisting of denaturation of 94^0C for 1 min, annealing at 38^0C for 1 min, extension at 72^0C for 1 min with a final extension of 72^0C for 5 min was found optimal for the amplification of Aonla DNA. The amplified products were separated on 1.5% agarose gel having 0.5 µg/ml of ethidium bromide. The gels were documented under UV light source in a gel documentation unit Alpha Imager 1200

Table 1: List of Aonla germplasms with their geographical origin

Sr.No.	Germplasm Code	Cultivar Name	Place of collection
1	V1	NA-7	UAS GKVK
2	V2	LOCAL-1	UAS GKVK
3	V3	NA-5	IIHR
4	V4	LOCAL-5	UAS GKVK
5	V5	NA-9	IIHR
6	V6	LOCAL-7	UAS GKVK
7	V7	LOCAL-4	UAS GKVK
8	V8	BANARASI LOCAL	IIHR
9	V9	LOCAL-2	UAS GKVK
10	V10	ANAND-1	IIHR
11	V11	NA-10	UAS DHARWAD
12	V12	BNR-1	UAS GKVK
13	V13	LOCAL-9	UAS GKVK
14	V14	LOCAL-10	UAS GKVK
15	V15	LOCAL-6	UAS DHARWAD
16	V16	LOCAL-8	UAS GKVK
17	V17	LOCAL-3	IIHR
18	V18	NA-4	IIHR
19	V19	NA-6	UAS DHARWAD
20	v20	local-11	iihr

Table 2. Spectrophotometric quantification of DNA of the genetic material used in the study

Sl. No.	Accession	OD260/OD280	Concentration (ng/µl)
1	NA-7	1	180
2	LOCAL-1	1.1	150
3	LOCAL-2	1.1	370
4	LOCAL-3	0.8	190
5	LOCAL-4	1.3	300
6	LOCAL-5	0.8	310
7	LOCAL-6	1.2	220
8	LOCAL-7	1	360
9	LOCAL-8	1	300
10	LOCAL-9	1.2	270
11	LOCAL-10	1.8	850
12	LOCAL-11	1.4	210
13	ANAND-1	1.4	160
14	BNR-1	1.3	300
15	NA-4	1.7	340
16	NA-6	1.5	270
17	NA-5	1.5	420
18	NA-9	1.4	390
19	NA-10	1.6	400
20	BANARASI LOCAL	1.5	670

Table 3: Percentage of polymorphism in RAPD banding pattern in *Aonla germplasm*

Sl. No.	Primer Name	Primer Sequence	Total number of Band	Polymorphic Band	Monomorphic Band	Percentage of Polymorphism
1	OPA-9	GGGTAACGCC	9	8	1	89
2	OPK-17	CCCAGCTGTG	8	8	0	100
3	OPQ-20	TCGCCCAGTC	10	9	1	90
4	OPZ-1	TCTGTGCCAC	4	4	0	100
5	OPZ-2	CCTACGGGGA	7	7	0	100
6	OPZ-3	CAGCACCGCA	11	8	3	73
7	OPZ-4	AGGCTGTGCT	11	10	1	91
8	OPZ-5	TCCCATGCTG	10	9	1	90
9	OPZ-6	GTGCCGTTCA	8	8	0	100
10	OPZ-7	CCAGGAGGAC	9	7	2	78
11	OPZ-8	GGGTGGGTAA	9	9	0	100
12	OPZ-9	CACCCCAGTC	8	8	0	100
13	OPZ-10	CCGACAAACC	7	4	3	57
14	OPZ-11	CTCAGTCGCA	4	3	1	75
15	OPZ-12	TCAACGGGAC	4	2	2	50
16	OPZ-13	GACTAAGCCC	8	3	5	37
17	OPZ-14	TCGGAGGTTC	11	11	0	100
18	OPZ-15	CAGGGCTTTC	7	6	1	86
19	OPH-3	AGACGTCCAC	8	6	2	75
20	OPH-6	ACGCATCGCA	11	11	0	100
21	OPH-9	GTAGCTGGG	6	5	1	83
22	OPH-13	GACGCCACAC	5	4	1	80
23	OPH-14	ACCAGGTTGG	7	5	2	71
24	OPH-16	TCTCAGCTGG	7	6	1	85
25	OPH-18	GAATCGGCCA	7	7	0	100
26	OPH-20	GGGAGACATC	9	9	0	100
27	OPC-1	TTCGAGCCAG	10	10	0	100
28	OPC-3	GGGGGTCTTT	7	7	0	100
29	OPC-8	TGGACCGGTG	5	5	0	100
30	OPC-9	CTCACCGTCC	9	9	0	100
31	OPC-10	TGTCTGGGTG	4	3	1	74
32	OPC-13	AAGCCTCGTC	8	8	0	100
33	OPC-15	GACGGATCAG	3	1	2	33
34	OPC-17	TTCCCCCCAG	6	4	2	66
35	OPN-1	CTCACGTTGG	7	6	1	86
36	OPN-5	ACTGAACGCC	3	1	2	33
37	OPN-10	ACAACTGGGG	2	1	1	50
38	OPN-16	AAGCGACCTG	5	4	1	80
39	OPN-19	GTCCGTACTG	7	5	2	71
40	OPM-8	TCTGTTCCCC	4	3	1	75
		Total	285	243	42	81.95

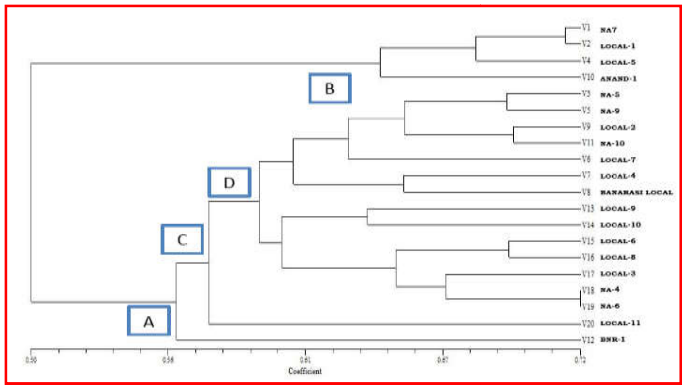

Figure 1: UPGMA dendrogram of Aonla germplasm based on RAPD primers

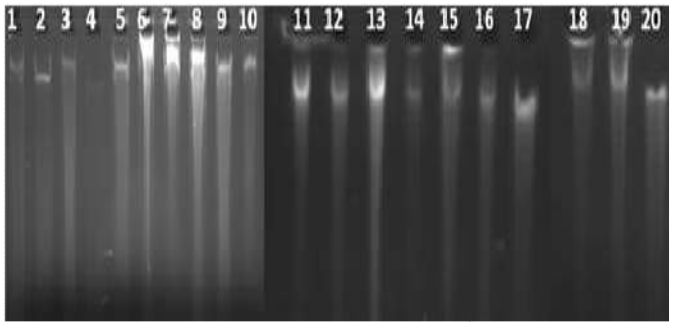

Plate 1: Genomic DNA of Aonla accession in the same order as listed in Table 1

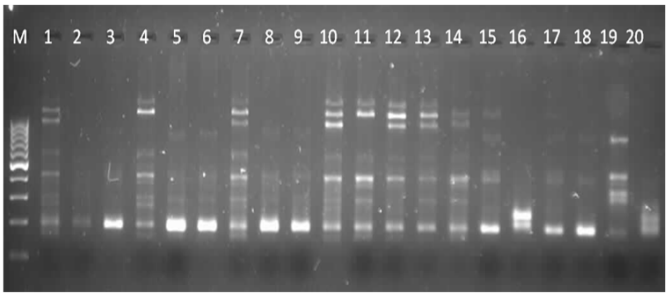

Plate 2: Profiles of OPA-9 primer with Aonla accessions same order as listed in Table1

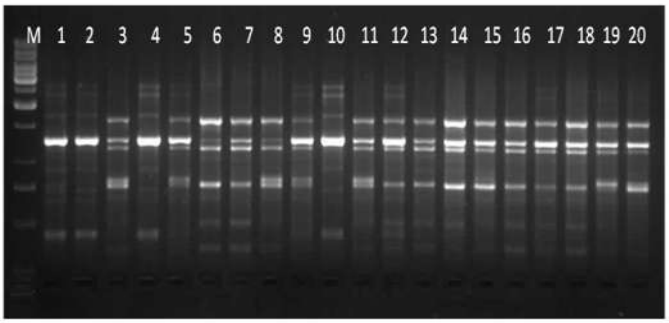

Plate 3: Profiles of OPZ-3 primer with Aonla accessions same order as listed in Table 1

(*Alpha Innotech Inc., USA*) which shows good resolution to score the bands. Gel documentation and gel banding pattern analysis worked out by computer application software to generate Jaccard's co-efficient of similarity matrices. The molecular marker analysis of 20 Aonla germplasms was carried out using 40 RAPD primers. RAPD bands were scored

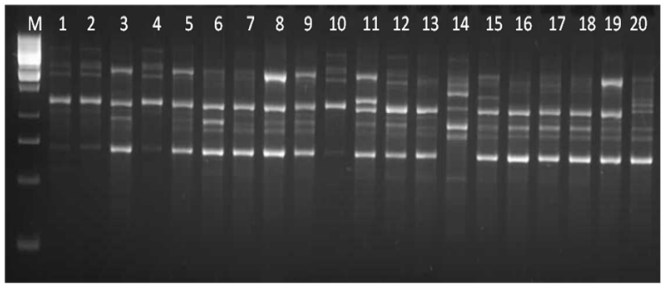

Plate 4: Profiles of OPC-5 primer with Aonla accessions same order as listed in Table1

as present (1) or absent (0) for each sample manually. All the 40 primers exhibited good amplification with clear, sharp, reproducible bands (PLATES 2, 3 and 4). They were polymorphic and generated a total of 285 bands (TABLE-3), out of which 243 were polymorphic across the accessions (81.95%). The number of bands generated by each primer varied from 2 (OPN-10) to 8 (OPZ-3, 4, 14 AND OPH-6). The polymorphism ranged from 33 to 100% among the primers which may be due to the difference in primer sequence. The amplicons size varied from 250bp to 1200bp.

Estimation of genetic diversity (Data analysis): The binary data were analyzed using Numerical Taxonomy and Multivariate Analysis System, (NTSYS-pc version 2.02) computer software package to generate pair wise band similarities for 20 Aonla germplasm lines (TABLE-1). The simple matching coefficients between each pair of accessions were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) and sequential agglomerative hierarchical nested clustering (SHAN) by using NTSsys pc 2.02 computer software. Molecular profiles of 20 germplasm lines of Aonla were generated using 40 RAPD primers dendrogram constructed on the binary data (Figure 1).

DISCUSSION

The result of this research is discussed here. The yield and quality of DNA were good with CTAB method (Barnwell *et al*. 1998). The browning that occurred due to binding of phenols to DNA was avoided by the inclusion of PVP (2%) in the extraction buffer. Similar findings were made by (John 1992) in crops containing high phenols. The use of β-mercaptoethanol helped to keep the nucleic acids in non-oxidative environment and to denature endonuclease activities which were similarly reported by Lefort and Douglas (1999). Repeated treatment with chloroform: isoamylalcohol (24:1) resulted in clean DNA, which was consistently amplifiable by PCR. The removal of RNA and proteins was achieved by treating the samples with 5 µl of Rnase and keeping at room temperature over night. Similarly the proteins could be removed by treating samples with phenol: chloroform: isoamylalcohol (25:24:1) and centrifuged at 14,000 rpm for two minutes. The yield of DNA in Aonla types ranged from 150 to 850 ng/µl of sample.

The PCR is an enzymatic method of making multiple copies of a predicted segment of DNA. PCR reaction should be conducted as an experiment complete with control to test purity and viability of reagents (Elrich *et al.,* 1991). 20 ng of

Genomic DNA gave good and consistent amplification. A primer of 5 pm was found optimal. Higher primer concentration may promote impairing and accumulation of non specific products (Innis and Gelfand, 1990). The optimum Mgcl2 concentration was found to be 1.5mM. A successful PCR was performed by a cycling consisting of 45 cycles with initial denaturation of 94^0C for 4 min and after each cycle consisting of denaturation of 94^0C for 1 min, annealing at 38^0C for 1 min, extension at 72^0C for 1 min with a final extension of 72^0C for 5 min. A final selection of primers producing a higher level of polymorphism and more reproducible banding patterns was facilitated by first performing a preliminary screening using pooled DNA. Such preliminary screening has also given successful results in other crops (Demek and Adams 1992, Schnell *et al.,* 2003). The molecular marker analysis of 20 Aonla germplasm was carried out using 40 RAPD primers. RAPD bands were scored as present (1) or absent (0) for each sample manually. All the 40 primers exhibited good amplification with clear, sharp, reproducible bands. They were polymorphic and generated a total of 285 bands (Table-3), out of which 243 were polymorphic across the accessions (81.95%). The number of bands generated by each primer varied from 2 (OPN-10) to 8 (OPZ-3, 4, 14 and OPH-6). The polymorphism ranged from 33 to 100% among the primers which may be due to the difference in primer sequence. The amplicons size varied from 250bp to 1200bp. The binary data were analyzed using Numerical Taxonomy and Multivariate Analysis System, (NTSYS-pc version 2.02) computer software package to generate pair wise band similarities for 20 aonla germplasm lines (Table-1). The simple matching coefficients between each pair of accessions were used to construct a dendrogram using the unweighted pair group method with arithmetic averages (UPGMA) and sequential agglomerative hierarchial nested clustering (SHAN) by using NTSsys pc 2.02 computer software. Dendrogram revealed that the genotypes could be grouped into four clusters (Figure- 1). The dendrogram showed two (A and B) major clusters at 57% similarity. The BNR-1 variety showed high genetic variability with varieties of cluster B which is better for crop improvement. Next level of cluster B four accessions showed 67% similarity. Next level of clustering at 59% similarity was in the C cluster. In the cluster of D, sub cluster showed the 60% similarity in between the clustered accessions. The cluster information thus enables us in selecting the parent for breeding programme.

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