Short Communication

RAPD based genetic variability among cultivated varieties of Aonla (Indian Gooseberry, *Phyllanthus emblica* L.)

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ABSTRACT

Aonla, the Indian Gooseberry (*Phyllanthus emblica*) is widely grown in India due to its neutraceutical properties. Investigations on the use of RAPD markers enabled us to estimate genetic variability among commercially cultivated varieties. This study also enabled us to distinguish these varieties using a set of four decamer primers, which was otherwise difficult by using morphological markers. Cluster analysis revealed three different groups of varieties directly associated to their place of origin. RAPD markers were also able to differentiate varieties of same origin or even selection from same parents. This information can be used for identification of varieties and further crop improvement programme. **[Physiol. Mol. Biol. Plants 2009; 15(2) : 169-173]** *E-mail : dr.subramaniam@jains.com*

Key words : *Aonla, Phyllanthus emblica, RAPD*

INTRODUCTION

Aonla *(Phyllanthus emblica)* is a medium-sized deciduous tree belonging to the family Euphorbiaceae. It is commonly known as Indian Gooseberry and grows in the plains and sub-mountain tracts all over the Indian subcontinent from 200 to 1300 m. altitude (Pathak, 2003). Aonla is well known for its neutraceutical and pharmacological properties (Chatterjee and Sil, 2007; Calixto *et al.,* 1998). Not only it is a wonderful antioxidant (Kumar *et al.,* 2006), but it has also proven anti-fungal, anti-bacterial (Sidhu *et al.,* 2007), anti-viral (Balasubramaniam *et al.,* 2007), anti-mutagenic (Kumar, *et al*, 2006), anti-hepatoxic (Chatterjee and Sil, 2007), anti-inflammatory (Asmawi *et al.,* 1992, Ihantola-Vormisto *et al.,* 1997), anti-histaminic, immuno-modulatory (Neetu *et al.,* 2002), hypolipidemic (Mathur *et al.,* 1996), and hypotensive relieving properties. Amla fruit is rich in Vitamin C (Pathak, 2003; Pillay and Iyer, 1958; Shah and Hamid, 1968) and pectin. However, Ghosal *et al.*, (1996) through comprehensive chromatographic, spectroscopic and crucial chemical analysis of fresh juice and solvent extractives have shown that what is estimated as vitamin C is infact low molecular weight hydrolysable tannins that are implicated in therapeutics. Tannins present in Aonla (Srivastava and Ranjan, 1967) are known to retard the oxidation of Vitamin C. Apart from Aonla fruits, the constituents in leaves (Basa and Srinivasulu, 1987) and bark (Laumas and Seshadri, 1958) are also known to possess neutraceutical properties.

Processed products of Aonla have become very popular in the recent years due to their use in the formulation of Chawanaprash which is marketed in India under different trade names. Varietal/genotype identification of Aonla is therefore important in order to selectively process the variety as demanded by the customer. The conventional method used to identify varieties involves studies on leaf/fruit morphology, fruit colour etc. (Pathak, 2003). However, these are based on physical traits that undergo variations due to environmental and edaphic conditions. In order to develop an efficient identification method, molecular techniques have been used since these are reliable, unaffected by environmental conditions and can aid varietal identification. Genetic polymorphisms naturally prevailing in plants has been studied widely and is used to differentiation varieties that differ even marginally from each other. Polymerase Chain Reaction (PCR)–based methods such as Random Amplified Polymorphic DNA (RAPD) (Bradeen and Havey, 1995; Sambrook and Russell, 2001) can be a powerful tool for the authentication of plant materials under study (Warude *et al.,* 2006).

A large number of reports have appeared in the literature using RAPD patterns for differentiating varieties, species, etc of crop plants. These include

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studies on mango (*Mangifera indica*) (Eidathong *et al.,* 1999, Lowe *et al.,* 2000), pomegranates (*Punica granatum*) (Sarkosh *et al.,* 2006), onion (Storsberg *et al.,* 2004), pea (Koveza and Gostimskii, 2005), Tunisian fig (Salhi-Hannachi *et al.,* 2006), sugarcane (Besse *et al.,* 1998) etc., wherein subtle differences in the banding patterns have been used as an index to differentiate varieties and assess genetic variability. Research on similar lines has been initiated to use molecular marker specifically RAPD to study genetic variability among *P. emblica* cultivars.

MATERIALS AND METHODS

Plant Material

Fresh and young leaves of different varieties of *P. emblica* were collected from the Research and Demonstration Farm of Jain Irrigation Systems Limited, Agripark, Jain Hills, Jalgaon, India and authenticated by a senior horticulturist attached to our R and D farm. The leaf material was washed thoroughly in Ultra High Pure Water (TKA water Purification System) and rinsed with 70% ethanol, air dried and used for DNA extraction.

Isolation of Genomic DNA

High molecular weight genomic DNA was extracted from *P. emblica* by adopting the Cetyl Trimethyl Ammonum Bromide (CTAB) method with slight modification considering the nature of highly acidic tissues (Warude *et al.,* 2003). Briefly, 2.5 grams of fresh leaf tissues were ground to a fine powder using liquid nitrogen in the presence of 20 mg polyvinyl pyrrolidone (PVP) and 10 mg activated charcoal. The powder was then transferred to a 50 ml polypropylene tube and 15 ml of warm CTAB buffer (pH 8.0) was added. This was followed by the addition of 0.3 % [v/v] 2-mercaptoethanol in a fume hood, to inhibit action of nucleases. The suspension was mixed well and incubated at 65 ^{o}C for a period of 1 hour. Following this, 5 ml of chloroform : isoamyl alcohol (24:1) was added to the tubes at room temperature (RT), and mixed well by gentle inversion. The tubes were centrifuged at 8000g for 10 minutes at RT. The same was repeated again and the aqueous layer was extracted with phenol : chloroform : isoamyl alcohol (25:24:1) and the resulting aqueous layer was precipitated with 0.7 volume of ice-cold isopropanol and incubated at -20 ^{o}C for 1 hour. The mixture was centrifuged at 8000g for 5 minutes to pelletise the DNA. The DNA pellet was then washed twice with 70 % ethanol and dried under vacuum for 15 minutes. The pellet was dissolved in TE buffer (pH 8.0) and (quantified by subjecting the DNA to a 0.5 % agarose gel electrophoresis stained with ethidium bromide, against a known standard DNA using the alpha imager software).

Primers

The primers used for amplification were purchased from Operon Biotechnologies, GmbH, Nattermannallee, Cologne, Germany. Fifty five random primers of different groups as given in table 1, were screened using Polymerase Chain Reaction (PCR). Nine primers showing good polymorphic bands were used for UPGMA analysis.

RAPD-PCR

Hot-start PCR for the amplification of DNA was carried out in a 25 µl volume of reaction in a 0.2 ml microfuge PCR tube. A reaction tube typically consisted of 100 ng DNA, 0.5 unit *Taq* DNA polymerase (Recombinant, from Fermentas life sciences) 10 mM dNTP, 1X *Taq* DNA Polymerase buffer with KCl, 2.5 mM $MgCl_2$ and 20 pico mole decamer primer. Amplifications were done in a DNA thermal cycler (Applied Biosystems, USA) employing the following conditions: Hot-start at 94 ^{o}C for 2 min, 40 cycles at 94 ^{o}C for 30 secs, 42 ^{o}C for 1 min and 72 ^{o}C for 2 min, with a final extension at 72 ^{o}C for 8 minutes. The PCR products were subjected to 1.8 % agarose (Sigma-Aldrich, USA) gel electrophoresis in 1X TAE buffer along with a DNA ladder (Bangalore Genei). The DNA was stained by adding ethidium bromide directly into the gel mixture, for better visualization. The DNA bands were photographed under ultra violet light using the gel documentation system (Alpha Innotech, USA).

Data Processing

RAPD patterns were analyzed by scoring for presence or absence of band such as (1) or (0) respectively, in each lane. Genetic similarity and genetic distance was estimated as per Nei (1972). The similarity matrix was analysed by the unweighted pair-group method with arithmetic average (UPGMA), using the procedure described by Nei (1972). A Microsoft window-based software 'Popgene (Yeh *et al*., 1997) version 1.31 for population genetics' was used to perform the above statistical analysis.

RESULTS AND DISCUSSION

Seven varieties of *P. emblica*, namely Kanchan, Krishna, NA7, NA-10, BSR-1, Anand-1 and Chakkaya that produced consistent amplifications were analysed. Of

Table 1. List of primers used for screening in RAPD

Primer Code		Primer Code		Primer Code	
Group	**Number**	**Group**	**Number**	**Group**	**Number**
OPD	03, 05, 07, 10 and 17	OPAK	13 and 20	OPAO	13
OPAJ	02,05, 09, 12, 14 and 17	OPAF	12 and 16	OPAP	12
OPAB	11, 14, 16, 19 and 20	OPAD	06, 19 and 20	OPAQ	20
OPAC	09, 13, and 19	OPAH	05, 08 and 15	OPAU	05
OPC	09, 15 and 17	OPAA	12	OPAW	07
OPM	09, 11, 12, 13 and 14	OPE	14	OPB	01
OPJ	07, 12, and 14	OPAE	04	OPA	20
OPAI	15, 17 and 20	OPAS	10	OPAG	19

the total 55 primers screened, four Operon primers namely OPJ-12, OPAU-05, OPJ-14, and OPAQ-20 collectively produced 33 polymorphic bands in these varieties (Figure 1-A to D). It is evident from Figure 1A that OPJ-12 exhibits subtle polymorphism between these varieties and the important polymorphic bands are indicated with an arrow. It is worth noting that the two polymorphic bands in Kanchan are of ~800 bp and ~1.2 kb which are not exhibited in any other variety at this position. Similarly, NA-10 and BSR-1 exhibited polymorphisms at ~400 bp and ~900 bp respectively. Only NA-7 and NA-10 varieties were found to possess the ~300 bp fragment which was not exhibited in any other variety. NA-7 and Anand-1 can be easily distinguished by the total absence of 1.5 kb band which is otherwise present in rest of the varieties. This is depicted in Figure 1B. The RAPD patterns exhibited by NA-7 variety of *P. emblica* with OPJ-14 resulted in the appearance of a unique ~300 bp band which was not found in any other variety used in this study (Figure 1C). The same band was also found to be amplified in NA-7 and NA-10 with OPJ-12, which is of interest. Hence use of primer OPJ-14 can uniquely differentiate the NA type of varieties, (NA-7 from NA-10) which was not otherwise possible with OPJ-12.

As can be observed from Figure 1D, use of primer OPAQ-20 led to the differentiation of NA10 among all varieties by the appearance of a ~900 bp fragment which was unique. In addition, this primer was also able to selectively differentiate Anand-1 variety from others. A high degree of genetic diversity was also noticed among the varieties used in the study when this primer was used for RAPD analysis.

Analysis of Genetic distance (Figure 2) and similarity co-efficient data (Table 2) shows close similarity between Chakaiya and Kanchan with similarity coefficient of 0.81, and hence they are grouped together. As a matter of fact, Kanchan is a chance seedling from Chakaiya. Krishna and NA-10 were also found to be closely related to each other which may be due their parental

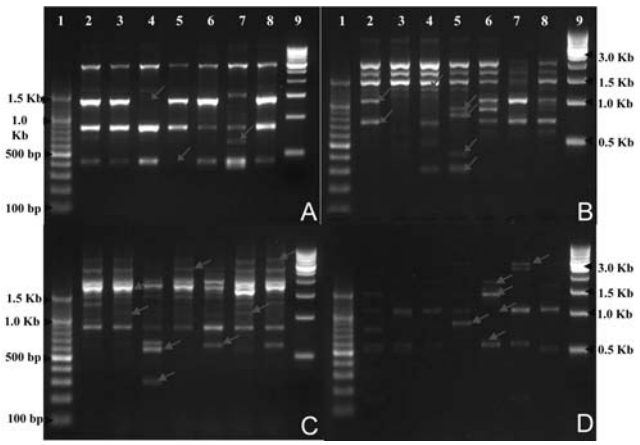

Fig. 1. RAPD pattern of different varieties of Phyllanthus emblica amplified with OPAU-05[A], OPJ-12[B], OPJ-14[C], and OPAQ-20[D], when subjected to 1.8% Agarose Gel Electrophoresis stained with Ethidum bromide. Lane 1 & 9 : 100 bp and 0.50 kb DNA ladders respectively, Lanes 2-8 : Kanchan, Krishna, NA-7, NA-10, BSR, Ananad-1 and Chakaiya, varieties.

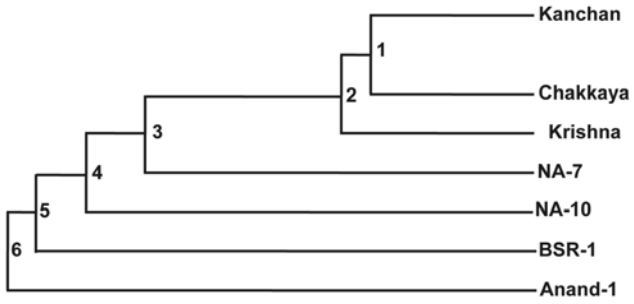

Fig. 2. UPGMA dendrogram of 7 varieties of *P. emblica* based on 9 random RAPD primers.

relationship as both are chance seedlings from cultivar Banarasi. NA-7 which is a seedling selection from Francis variety appeared in a different group from Krishna, Kanchan, Chakaiya and NA-10, Though Krishna, Kanchan, Chakaiya, NA-7 and NA-10, all of North Indian origin, can be differentiated from each other but they were comparatively closer to each other than the BSR-1 and Anand-1, which are essentially of South Indian and Western origin cultivars respectively.

Warude *et al.* (2006) have reported on the development and application of RAPD-SCAR marker for the identification of *Phyllanthus emblica* (at the species level), however studies on the genetic variability of Aonla varieties employing molecular markers have not been attempted. It is very important to characterize the genetic variation using fool-proof methods such as DNA fingerprinting (Sharon *et al.,* 1995) that can generate vital information on the varietal authentication especially in processing as this fruit has a prominent place in neutraceutical industry (Pathak, 2003). This study also finds immense application at the field level by way of molecular characterization of germplasm. This method can in turn help in eliminating duplication of varieties which exhibit similar phenotypic traits. One of the other applications of our study is in breeding and varietal improvement wherein parents possessing two distinctly different traits can be identified and used for breeding purpose. The data presented here provide possibilities of identifying and differentiating most commonly cultivated varieties of *P. emblica* using 4 commercially available primers. It is possible to develop SCAR primers based on the data given here for authentication of specific varieties. This aspect is being pursued in our laboratory.

The RAPD patterns obtained from our study can serve as a vital input to the conventional method of varietal identification that relies solely on morphological characters. Techniques such as DNA fingerprinting (Khanuja *et al.,* 1998) can thus provide valuable information on the genetic basis of variations that exists between different varieties of *P. emblica* undertaken in this study. To the best of our knowledge this is the first report on the characterization of Phyllanthus genotypes based on commercially available primers. The powerful capability of molecular technique to distinguish closely related varieties based on their RAPD patterns has been brought out by this study.

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Table 2. Similarity co-efficient and Genetic Distance among 7 varieties of *P. emblica* based on 4 primers.

Cultivar	Kanchan	Krishna	NA-7	NA-10	BSR-1	Anand-1	Chakkaya
Kanchan	0	0.78	0.54	0.60	0.63	0.60	0.81
Krishna	0.23	0	0.63	0.63	0.54	0.63	0.78
NA-7	0.60	0.45	0	0.45	0.54	0.45	0.66
NA-10	0.50	0.45	0.78	0	0.36	0.33	0.60
BSR-1	0.45	0.60	0.60	1.01	0	0.48	0.57
Anand-1	0.50	0.45	0.78	1.09	0.72	0	0.60
Chakkaya	0.20	0.23	0.40	0.50	0.55	0.50	0

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