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Genetic Diversity Analysis of Soursop (*Annona muricata* L.) in West Java Region of Indonesia Using RAPD Markers

A. E. Z. Hasan^{1*}, N. Bermawie², H. Julistiono³, E. I. Riyanti⁴, Hasim¹, I. M. Artika¹
and P. Khana¹

¹Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Jl. Lingkar Akademik, Kampus IPB Darmaga, Bogor, Indonesia.

²Indonesian Spice and Medicinal Crops Research Institute, Indonesian Agency for Agricultural Research and Development, Bogor, Indonesia.

³Biology Research Center, Indonesian Institute of Science, Cibinong Bogor, Indonesia.

⁴Molecular Biology Division, Indonesian Center for Agricultural Biotechnology and Genetic Resources, Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture. Indonesia.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was determine the genetic diversity of *Annona muricata* L. using molecular random amplified polymorphic DNA (RAPD) markers. Amplification of genomic DNA of 9 samples using RAPD analysis yielded 57 fragment, of which 30 were polymorphic, with an average of 6 polymorphic fragment per primer.

Study Design: The data was analysed with SIMQUAL program of NTSYS-pc (Version 2.02), and similarities between accessions were estimated using the Jaccard's coefficient calculated as $J = A / (N - D)$, where A is the number of positive matches (that is, presence of band in both samples), D is

*Corresponding author: E-mail: pakzainalhasan@gmail.com;

the number of negative matches (that is, absence of band in both samples) and N is the total sample size including both the number of matches and unmatched. Dendrogram was created from the resultant similarity matrices using the UPGMA method following SAHN function of NTSYS-pc (Version 2.02).

Place and Duration of Study: The study was conducted from September 2014 to March 2015. The analysis of genetic diversity was done in the Biochemistry Laboratory and the Molecular Biology Laboratory of Plant II, Agronomy and Horticulture Faculty of Agriculture Bogor Agricultural University.

Methodology: Plant material from West Java, DNA extraction after that primer screening and DNA amplification. Data analysis and Electrophoresis of amplified product.

Results: The genetic distance was calculated based on Jaccard's similarity coefficient using SIMQUAL program in NTSYS-pc software to clarify the genetic relationships. An UPGMA dendrogram was constructed using NYSYSpc 2.10 software. A clear divergence between the two related species was found from the cluster analysis. Jaccard similarity coefficient of soursop ranged from 0.451 to 0.902 which revealed a wide range of genetic identity. Based on this value it was noticed that the relationship between soursop sample was closely. High similarity coefficient of 0.902 was measured between; the sample GRT3 with CJR2 and CJR3. According to analysis of the Jaccard similarity coefficient, the sample SKB2 with SKB1 had low similarity coefficient (0.451). This indicated that both the accessions were genetically distant.

Conclusion: Primary elected to amplify DNA *Annona muricata* L. produces two main groups. Group A consists of 4 samples (GRT1, CJR1, SKB1, GRT 2) and group B consisted of 5 samples (GRT3, CJR2, CJR3, SKB2, SKB3).

Keywords: *Annona muricata* L.; genetic diversity; RAPD; West Java Indonesia.

ABBREVIATIONS

RAPD	: Random amplification of polymorphic DNA
PCR	: Polymerase Chain Reaction
DNA	: Deoxyribonucleic acid
GRT1, GRT2 GRT3	: Garut, West Java
CJR1, CJR2, CJR3	: Cianjur, West Java
SKB1, SKB2, SKB3	: Sukabumi, West Java
CTAB	: Cationic Hexa decyl Trimethyl Ammonium Bromide
EDTA	: Ethylene diamine tetra acetic acid

1. INTRODUCTION

Soursop (*Annona muricata* L.) is one type of fruit plants originating from tropical South America, and then spread to the mainland of South Asia and Southeast Asia, including Indonesia. One of the medicinal plants that are currently being developed is Soursop (*Annona muricata* L.). Compounds in soursop leaves among other steroid / terpenoids, flavonoids, coumarin, alkaloids, and tannins. Flavonoids act as antioxidants for cancer, antimicrobial, antiviral, regulating photosynthesis and plant growth regulators [1,2]. Indonesian people use soursop leaves as an herbal medicine for treating cancer,

in particular by drinking boiled water fresh soursop leaves. Soursop leaf is widely used as an herbal medicine for treating various diseases, such as: asthma in the Peruvian Andes, diabetes and seizures in Amozania Peru [3]. Soursop contains acetogenin were able to fight 12 types of cancer cells. The many benefits of soursop make people start switching to consume soursop as an alternative to conventional prevention and treatment [4].

Knowledge of genetic variation is essential for breeding programs because it provides information about the molecular structure of plant genetics, so it can be used as a basis for the selection of crops to be cultivated. Genetic variation can be used as material selection desired sample. Development of the field of molecular DNA analysis these days is often used to characterize genetic variation and relationship in a genus, species, cultivars, or accessions. Information on genetic diversity is the basis for the breeding and population genetics experts in the development and improvement of plants, especially as a first step in the selection of plants. This step is especially important to distinguish between individuals within a species as well as the precise identification of samples and the identification of genes that are potential carriers of a single character.

RAPD analysis is one method of molecular analysis used to evaluate the genetic relationships populations of organisms. Basic RAPD analysis is the use of PCR machine that is capable of amplifying the DNA sequences in vitro. PCR techniques involving temperature, DNA denaturation, annealing to the DNA, and primer extension by a DNA polymerase enzyme into a fragment of DNA in various sizes [5]. The use of RAPD markers are relatively cheaper, easier, faster results, it does not require knowledge of the background of the genome being analyzed and easily obtain the random primer necessary to analyze the genomes of all kinds of organisms [6].

Based on data obtained from the data Department of Food Crops of West Java province in 2014, is known production centers in West Java's leading commodity soursop plants located in Sukabumi, Cianjur and Garut. Regional differences cause DNA polymorphism or genetic diversity in these plants [7]. The existence of DNA polymorphism in a soursop plant in West Java, especially in the area of production of major commodities soursop has not been identified. Therefore, the research needs to be conducted to determine the genetic pattern of soursop plant in West Java, Indonesia.

2. MATERIALS AND METHODS

2.1 Plant Material and DNA Extraction

Fresh leaf of soursop were collected from the Garut, Cianjur, and Sukabumi district of West Java. This included 9 soursop samples comprising 3 Garut district (GRT1, GRT2, GRT3), 3 Cianjur district (CJR1, CJR2, CJR3), and 3 Sukabumi district (SKB1, SKB2, SKB3). Genomic DNA was isolated from freshly collected young leaves using the CTAB (cetyltrimethyl ammonium bromide) method of [8] with a few modifications. 0.5 g of young leaf tissue was ground in 2% Polyvinyl pyrrolidone to fine powder using sterile pestle and mortar and suspended in 700 μ l of preheated 2% CTAB buffer (2% CTAB, 0.1 M Tris pH 8.0, 20 mM EDTA; 1.4 M NaCl, and 1% β -mercapto ethanol). The suspension was incubated at 65°C for one hour with occasional inversion. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) mixture was added to the suspension and centrifuged at 11,000 rpm for 5 minutes. The aqueous phase was transferred to a new microfuge tube and added with equal volume of chloroform: isoamyl alcohol (24:1) mixture at 10,000 rpm for 15

minutes. The Chloroform: IAA extraction step was repeated twice. The aqueous phase was transferred to a fresh tube containing 0.6 volumes of ice-cold isopropanol. After centrifugation. The pellet was washed in 70% ethanol and air dried and dissolved in TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0). DNA was quantified using UV-spectrophotometer and diluted to 50 ng/ μ l and used in PCR.

2.2 Primer Screening

Fifteen Operon primers (Operon Technologies Inc., USA) were initially screened using two samples of soursop to determine the suitability of each primer for the study. Five primers were selected for further RAPD-PCR analysis based on their ability to generate clear and distinct polymorphic bands. The primers which generate smeared bands were discarded.

2.3 DNA Amplification

PCR reaction was performed as described by [6] with 5 selected primers. Each PCR reaction volume of 20 μ l contained 2.5 mM MgCl₂, 250 μ MdNTPs, 1 μ M of primer, 1 unit of Taq polymerase and 50 ng of genomic DNA. The PCR was performed in thermal cycler of Esco. Amplification conditions were: Initial denaturation at 94°C for 5 min followed by 44 cycles each of denaturation for 1 min at 94°C, annealing for 1 min at 35°C and extension for 1 min at 72°C and finally an 8 min extension at 72°C.

2.4 Data Analysis

For RAPD analysis, the bands were converse to matrix data. In the data matrix, the presence of a band was coded as 1 and absence was marked as 0, and estimation of similarity among all samples was calculated according to the study of [9]. The data was analysed with SIMQUAL program of NTSYS-pc (Version 2.02), and similarities between accessions were estimated using the Jaccard's coefficient calculated as $J = A / (N - D)$, where A is the number of positive matches (that is, presence of band in both samples), D is the number of negative matches (that is, absence of band in both samples) and N is the total sample size including both the number of matches and unmatches [10]. Dendrogram was created from the resultant similarity matrices using the UPGMA method [11] following SAHN function of NTSYS-pc (Version 2.02) [9].

2.5 Electrophoresis of Amplified Product

RAPD-PCR product was detected in 1.5% agarose gel in 1× TAE buffer (pH 8.0, 242 g Tris Base, 57.1 ml glacial acetic acid, 0.5 M EDTA per litre of the buffer) and run in same buffer for 95 minute with voltage at 85V. Gels were stained with 0.5 µg/ml of ethidium bromide for 20 seconds and washed in distilled water for 15 minutes. DNA profiles were visualized on a UV transilluminator and photographed by using gel documentation system (Alpha imager, USA).

3. RESULTS AND DISCUSSION

Nine soursop samples obtained from West Java region were amplified using 5 random primer for RAPD analysis (Table 1). Amplification products of 9 samples yielded a total 57 bands, out of which 30 were found to be polymorphic. The size of the amplification product ranged from 150 to 1000bp. The highest number of bands (15) were obtained with primer OPE-4, while the lowest number of bands (9) were obtained with primer OPH-13. The percentage of polymorphism ranged from 40 to 81.82%. Primer OPE-19, revealed the highest polymorphism (81.82%) while primer OPE-4 exhibited the lowest polymorphism (40%).

The primer OPE-4 showed 9 monomorphic bands were in the range of 200bp to 1500bp (Fig. 1). The primer OPE-7 has amplified a total number of 11 bands in the ranges from 250bp to 1100bp. The primer OPH-5 generated 1 polymorphic bands of size 2400bp while 1 band was absent in GRT1, GRT2, CJR1, SKB1, and present in the other samples. A band size of ~1100bp was absent in GRT2 and SKB1, while present in the other 7 samples. The primer OPH-19 showed 2 monomorphic bands and 9 polymorphic bands were in the range of 300bp to 1300bp. In the case of primer OPH-13 it amplified a total number of 9 bands. All the 9

samples reveal monomorphic and polymorphic bands ranged from ~200bp to 1400bp.

A dendrogram (Fig. 2) based on UPGMA analysis grouped the 9 samples into 2 main groups, with similarity coefficient ranging from 0.60 to 0.94. The dendrogram obtained 2 main groups (A and B) with 4 and 5 samples respectively. The main groups A has 2 clusters (I and II). Cluster I has GRT1, CJR1 and SKB1. Clusters II contained GRT2. The main groups B with clusters I has GRT3, CJR2, CJR3 and SKB3. Clusters II from main group B contained SKB2.

The genetic distance was calculated based on Jaccard's similarity coefficient using SIMQUAL program in NTSYS-pc software to clarify the genetic relationships (Table 2, similarity with Nei's index [9]). An UPGMA dendrogram was constructed using NYSYSpc 2.10 software. A clear divergence between the two related species was found from the cluster analysis. Jaccard similarity coefficient of soursop ranged from 0.451 to 0.902 which revealed a wide range of genetic identity. Based on this value it was noticed that the relationship between soursop sample was closely. High similarity coefficient of 0.902 was measured between; the sample CJR2 with GRT3 and CJR3 with GRT3. According to analysis of the Jaccard similarity coefficient, the sample SKB2 with SKB1 had lowest value of similarity coefficient (0.451). This indicated that both the accessions were genetically distant.

Based on the genetic diversity, known that GRT2 has a genotype that is most different from the others. Results of RAPD marker analysis illustrate that GRT1 and GRT3 equally in the region have a different genotype. Although the samples taken are in one district, but the genetic diversity can produce a large diversity. It is affected because the specific environment in each region.

Table 1. Details of amplification obtained with different RAPD primers

No	Name of primer	Sekuen (5'-3')	No. of total bands	No. of polymorphic bands	Polymorphism percent (%)	Total no. of bands amplified
1	OPE4	GTGACATGCC	15	6	9	40.00
2	OPE7	AGATGCAGCC	11	6	5	54.55
3	OPH5	AGTCGTCCCC	11	5	6	45.45
4	OPH13	TGTAGCTGGG	9	4	5	44.44
5	OPH19	GTGACCAGCC	11	9	3	81.82
Total			57	30	28	52.63

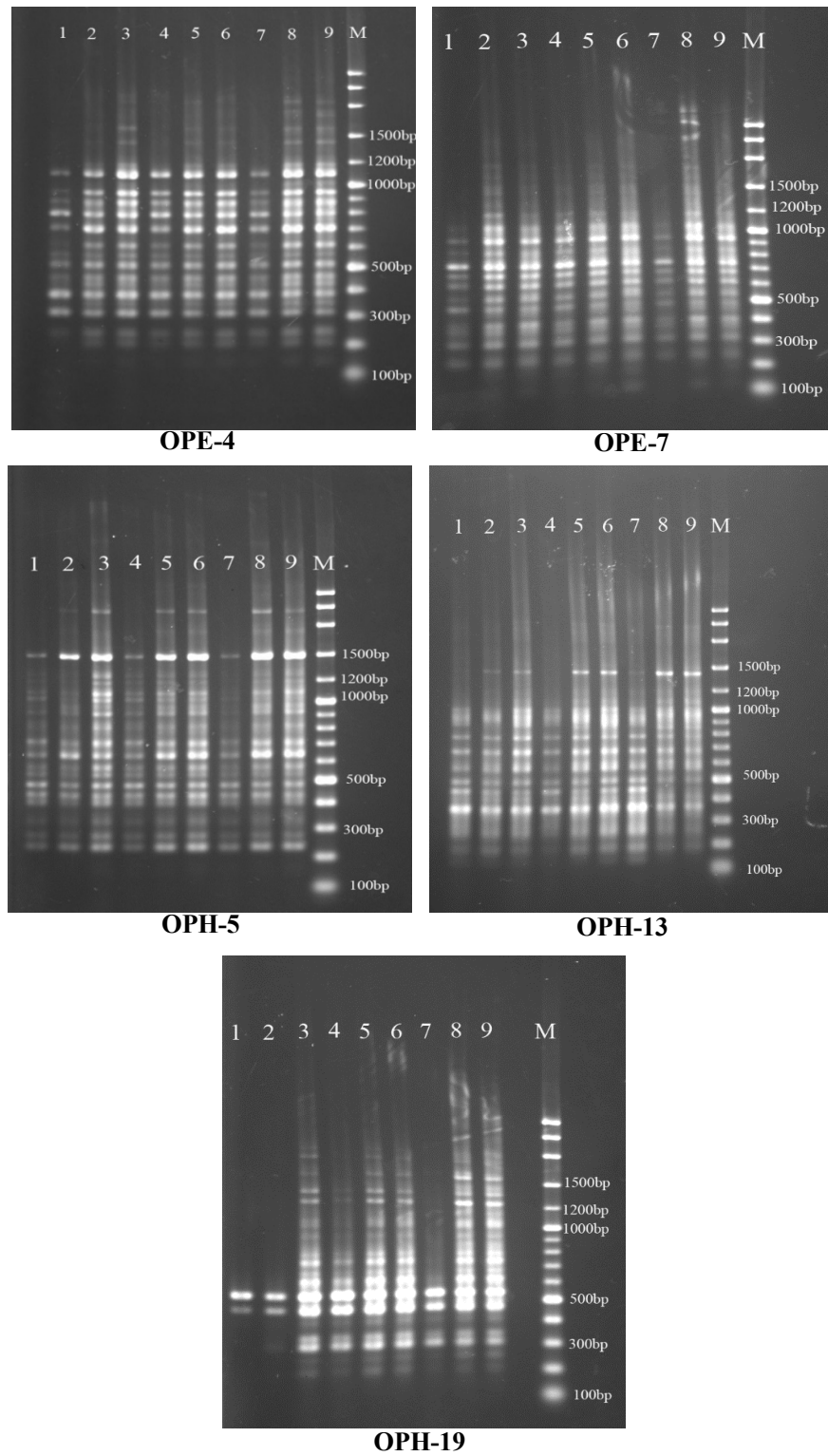


Fig. 1. RAPD profile of 9 soursop sample from 5 primer. From left to right, Garut 1-3 (GRT1, GRT2, GRT3), Cianjur 5-6 (CJR1, CJR2, CJR3), Garut 7-9 (GRT1, GRT2, GRT3), M is DNA marker

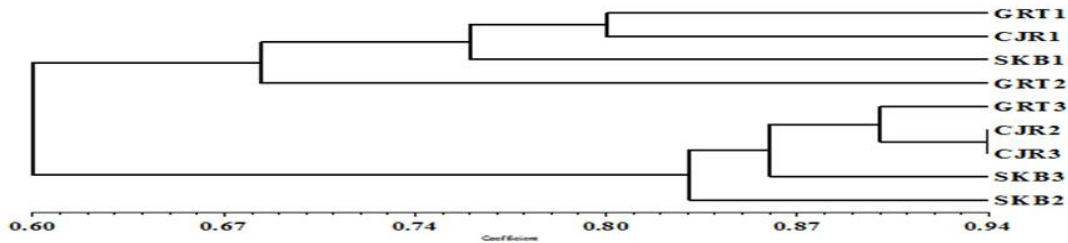


Fig. 2. Dendrogram of 9 sampels based on RAPD analysis

Table 2. The similarity coefficient by Jaccard in NTSYSpc 2.10e software for 9 sample of soursop

	GRT1	GRT2	GRT3	CJR1	CJR2	CJR3	SKB1	SKB2	SKB3
GRT1	1.000								
GRT2	0.686	1.000							
GRT3	0.569	0.725	1.000						
CJR1	0.804	0.686	0.706	1.000					
CJR2	0.529	0.686	0.902	0.686	1.000				
CJR3	0.529	0.686	0.902	0.686	0.941	1.000			
SKB1	0.765	0.686	0.549	0.745	0.569	0.608	1.000		
SKB2	0.471	0.647	0.804	0.627	0.843	0.824	0.451	1.000	
SKB3	0.510	0.608	0.843	0.667	0.882	0.863	0.490	0.863	1.000

RAPD has been widely used for various purposes such as studying genetic diversity in turmeric and ginger [12], *Ganoderma* spp. [13], turmeric [12], *Xylocarpus* spp [14], date palm [15], fig Tunisia barbary [16], cherry [17], almonds [18].

4. CONCLUSION

Primary elected to amplify DNA *Annona muricata* L. produces two main groups. Group A consists of 4 samples (GRT1, CJR1, SKB1, GRT2) and group B consisted of 5 samples (GRT3, CJR2, CJR3, SKB2, SKB3).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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