

ANALYSIS OF GENETIC VARIABILITY IN SOURSOP (*Annona muricata* L.) POPULATIONS FROM CENTRAL JAVA AND EAST JAVA BASED ON RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKER

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ABSTRACT

The objective of this research was to determine genetic variability of the soursop (*Annona muricata* L.) populations from Central Java and East Java based on RAPD markers. Leaves of 40 individuals were collected from 4 soursop populations in Central Java and East Java, include : Sukoharjo, Karanganyar (Central Java), and Ngawi, Pacitan (East Java). Genomic DNA was extracted from the leaves by the CTAB extraction procedure with some modifications. A total of 15 RAPD primers were purchased from commercial source and tested to find specific diagnostic markers for each individuals by RAPD-PCR. The measurement of soursop population genetic distance was based on similarity coefficient using method of Group Average Clustering and Unweight Pair Group Method Arithmetic (UPGMA) of NTSYS program version 2.02i. Results showed that each soursop population collected from different localities seemed have variability in RAPD profiles by using different primers. Four RAPD polymorphic primer was selected from 15 RAPD primers, namely A18, A20, P10 and P11. A total of 58 bands produced, varying from 9 to 20 bands per primer. The selected four RAPD primers produced 57 polymorphic bands, whereas polymorphism for each primer ranged from 95 % to 100 %. Dendrogram indicated that four soursop populations tend to segregate form two separated clade. The sample collected from Sukoharjo formed a separate cluster while the sample collected from Ngawi, Pacitan and Karanganyar grouped together in other cluster and diverged from population Sukoharjo.

Keywords : Central Java, East Java, Genetic Variability, RAPD, Soursop (*Annona muricata* L.)

INTRODUCTION

The genus *Annona* contains approximately 120 species and distributed mainly in the tropical and subtropical regions of the World (Mabberley, 1997). Numerous species produce edible fruits with a taste that is highly appreciated, such as soursop (*Annona muricata* L.) and sugar apple (*Annona squamosa* L.). Soursop is distinguished by its conspicuous spiny fruit and its obovate leaves with domatia on the undersurface (Kerrigan *et al.*, 2011). It produces large heart-shaped edible fruit of various sizes (average fruit weight is about 0.5 kg) yellow green in color with white flesh. Soursop, like other tropical fruits, serves as a potential source of raw materials for fruit products such as juice, beverages, wine, jellies, jam puree, power fruit bars, and flakes (Bates *et al.*, 2001; Abbo *et al.*, 2006). Thus, soursop has many therapeutic properties; the juice is diuretic while the other parts have antibacterial, anticancerous, astringent, and other properties (Asprey and Thornton, 1995). Soursop is used as an antispasmodic, emetic, and sudorific in herbal medicine. A decoction of the leaves is used to kill head lice and bed bugs while a tea from the leaves is well known to have sedative properties. The juice of the fruit is taken orally for hematuria, liver complaints, and urethritis (Badrie *et al.*, 2009).

Although *A. muricata* belongs to an important group of plants and traditionally been used for many diseases, information about the extent and the nature of genetic variability this species is still poorly understood. Various molecular markers techniques based on PCR amplification have become increasingly important at the study of genetic variability and relationships among plants. Molecular markers provide the best estimates of genetic variability that are independent of effect of various environmental factors. Various approaches are available for DNA fingerprinting such as Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Simple Sequence Repeats (SSRs) and Random Amplified Polymorphic DNA (RAPD) (Lakshmi *et al.*, 2011).

RAPD markers have been used for numerous applications in plant molecular genetics research despite having disadvantages of poor reproducibility and not generally being associ-

ated with gene regions (Williams *et al.*, 1990; Welsh and McClelland, 1991). RAPD is an established technique commonly used to provide information regarding DNA-level variability for many applications in genetic analyses (Ferreira and Grattapaglia, 1995).

The wide use of these markers in conservation programs can be credited to their usability for any kind of organism and to their rapid results (Lopes *et al.*, 2002). RAPD not only can reveal high levels of polymorphism, but also has advantages of speed, low cost and requirement for only minute amounts of plant materials. This method has been successfully used in identification of many edible fruit species.

This research is the first attempt to study the genetic variability of *A. muricata* from Central Java and East Java using random amplified polymorphic DNA markers. Information on genetic diversity and relationship among and between individuals, populations, plant varieties, and species of plant are important for plant breeders in guiding the improvement of crop plants (Dharmar and De Britto, 2011). The objective of this research was to determine genetic variability of the soursop populations from Central Java and East Java based on RAPD markers.

MATERIAL AND METHODS

Plant Material

Leaves of 40 individuals were collected from 4 soursop populations, with 10 individuals representing a population along the geographical range of natural distribution of *A. muricata* in Central Java and East Java (Table 1 and Figure 1). The material collected consist of young and healthy leaves of each individual, which were placed in plastic tubes and kept in ice while transported to the Laboratory of Molecular Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Sebelas Maret University (UNS), where they were stored in a freezer at -20°C until the time of DNA extraction.

DNA Extraction

Genomic DNA was extracted by the CTAB extraction procedure (Dellaporta *et al.*, 1983) with some modifications. Young fresh leaves of soursop were ground in mortar and then

Table 1. Location of the soursop (*Annona muricata* L.) populations studied with climatic data for each locality

Population	Code	Altitude (m)	Temperature (°C)	Humidity (%)	Light Intensity (lux)
Sukoharjo, Central Java	SKH	148	34	58	358
Karanganyar, Central Java	KRA	617	31	59	5153
Ngawi, East Java	NGW	735	32	42	4156
Pacitan, East Java	PCT	581	34	55	17240

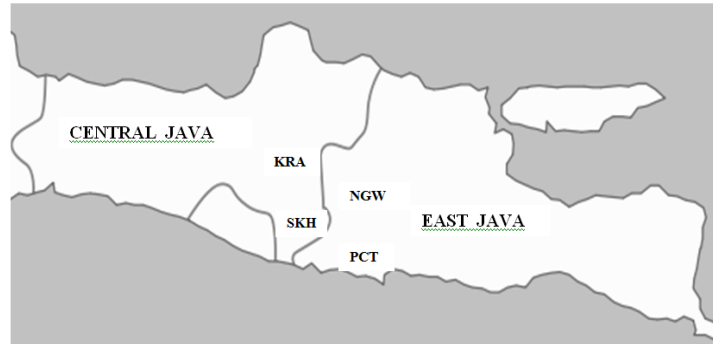


Figure 1. Map of the collection areas for 4 natural populations of soursop (*Annona muricata* L.) in Central Java and East Java. SKH (Sukoharjo), KRA (Karanganyar), NGW (Ngawi), PCT (Pacitan).

transferred to a 1.5 ml tube, to which 0.5 ml pre-heated (65°C) 2×CTAB (2%(w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl (2%-mer-captoethanol)) extraction buffer was added. Samples were incubated for 60 min at 65°C and then added 0.5 ml chloroform: isoamyl alcohol (24:1) and mixed for about 5 min. Samples were centrifuged at 9500 g for 10 min, and supernatant was transferred to fresh tube. An equal volume of isopropanol was added, and high mw DNA spooled out upon mixing. Samples were centrifuge at 9500 g for 10 min. After the remaining supernatant were poured off, the spooled DNA was rinsed with 70% ethanol, air dried, and 0.1 ml TE, containing 20 ug/ ml RnaseA was added. The extracted DNA was kept at 4°C for RAPD analysis. The concentration and quality of each DNA sample were estimated by comparing the intensity of bands of the analyzed DNA with a 100 bp DNA ladder, after electrophoresis in 1 % agarose gel.

RAPD Analysis

A genetic analysis was performed, using the RAPD methodology (Williams *et al.*, 1990; Welsh and McClelland, 1991), through PCR. Final volume for PCR reaction was 25 µl, with 3 unit of Taq DNA polymerase, 20 ng of template DNA, 0.1 µM of random primer, 0.4 mM each of dATP, dTTP, dGTP, and dCTP, 1 mM MgCl₂, commercial Taq DNA polymerase buffer. Amplifications were performed in a DNA thermal cycler (Veriti Thermal Cycler, Applied Biosystem). Amplifications were performed as follows: 94°C 5 min, 30°C 1 min, 72°C 1 min, 1 cycle; 94°C 1 min, 30°C 1 min, 72°C 1 min, 45 cycles; 94°C 1 min, 30°C 1 min, 72°C 5 min, 1 cycle. Each amplification reaction was conducted with one unique primer. A total of 15 RAPD primers were purchased from commercial source (1st BASE Custom Oligos, Singapore) and tested to find specific diagnostic markers. The PCR products were electrophoresed on 1.5 % (w/v) agarose gels in 1×TAE buffer at 80V for 2 h. A 100 bp DNA ladder (Geneaid Biotech Ltd, Taiwan) was included in all gels as a reference, to estimate the size of the amplified fragments. Gels were stained with 0.5 µg/ml ethidium bromide, and photographed in UV illumination. Photographs were taken and the profile image was saved on a magnetic disc (Photo-print, Version 98.02, Vilber Lourmat, France).

Data Analysis

Amplified fragments were recorded as absent (0) or present (1) in all individuals for each fragment. These two possible states were considered as the molecular phenotypes, resulting from the expression of two alleles at a single locus, one dominant and one recessive; the dominant being the one that determines the presence of the band (Ardi *et al.*, 2012). The frequency of the two alleles at each locus can be inferred from the frequency of presence and absence of the band (Gonzalez-Rodriguez *et al.*, 2005). Molecular diversity among population was assessed by calculating the percentage of polymorphic fragments (P %). A cluster analysis, using an unweighted pair group method with arithmetic averages (UPGMA) was performed. The analysis was plotted in the form of a dendrogram. All computations were carried out using the NTSYS-Pc, Version 2.02 package (Rohlf, 1993).

RESULT

DNA profiling through RAPD technique has been used for the analysis of variability even within the large germplasm populations. Genetic variability in *A. muricata* in different locations of Java has been carried out using RAPD markers. Results showed that each population collected from different localities seemed have variability in RAPD profiles by using different primers. The fragments observed in this study was clear and scorable for interpretation and then conversion into molecular data for the populations can be conducted. DNA samples fragment resulted by PCR amplification using one of the selected RAPD primers were shown in Figure 2.

While about 15 primers were used initially, four primers have generated reproducible, informative and easily scorable RAPD profiles (Table 2). Four random primers that we used generated multiple band profiles with a number of amplified DNA fragments. A total of 58 bands produced, ranging 9 to 20 bands per primer, corresponding to an average of 14.5 bands per primer. Four random primers also produced 57 polymorphic bands, whereas polymorphism for each primer ranged from 95 % to 100 % (Table 2).

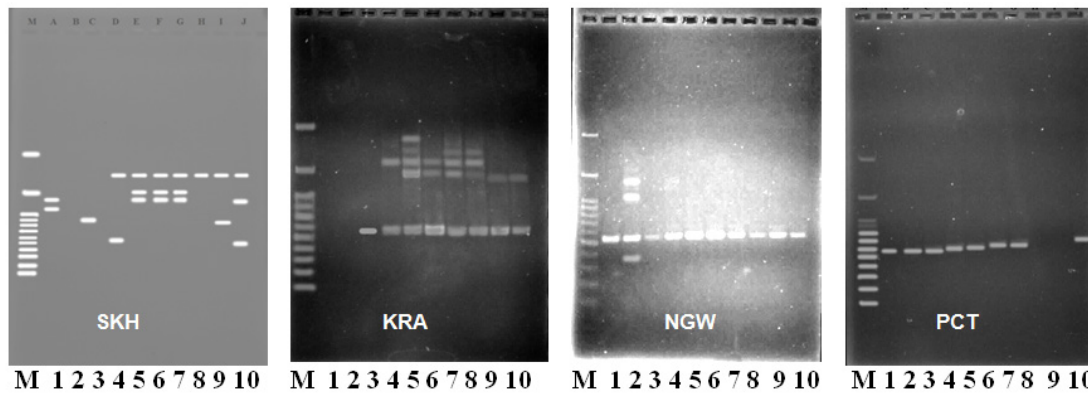


Figure 2. Profile of the RAPD fragments amplified by primer A18 for soursop (*Annona muricata* L.) individuals belonging to four populations in Java. M indicated as Marker (100 bp Ladder) and the number well (1 to 10) in each population indicated number of soursop samples.

Table 2. Primary analysis of PCR amplification products using selected primers

Primers	Nucleotide Sequences	GC Content (%)	Number of bands	Number of polymorphic bands	Polymorphism (%)
A18	5'-AGGTGACCGT-3'	60	20	19	95
A20	5'-GTTGCGATCC-3'	60	12	12	100
P10	5'-GGCTCATGTG-3'	60	17	17	100
P11	5'-GTCAGGGCAA-3'	60	9	9	100
Total			58	57	98.75

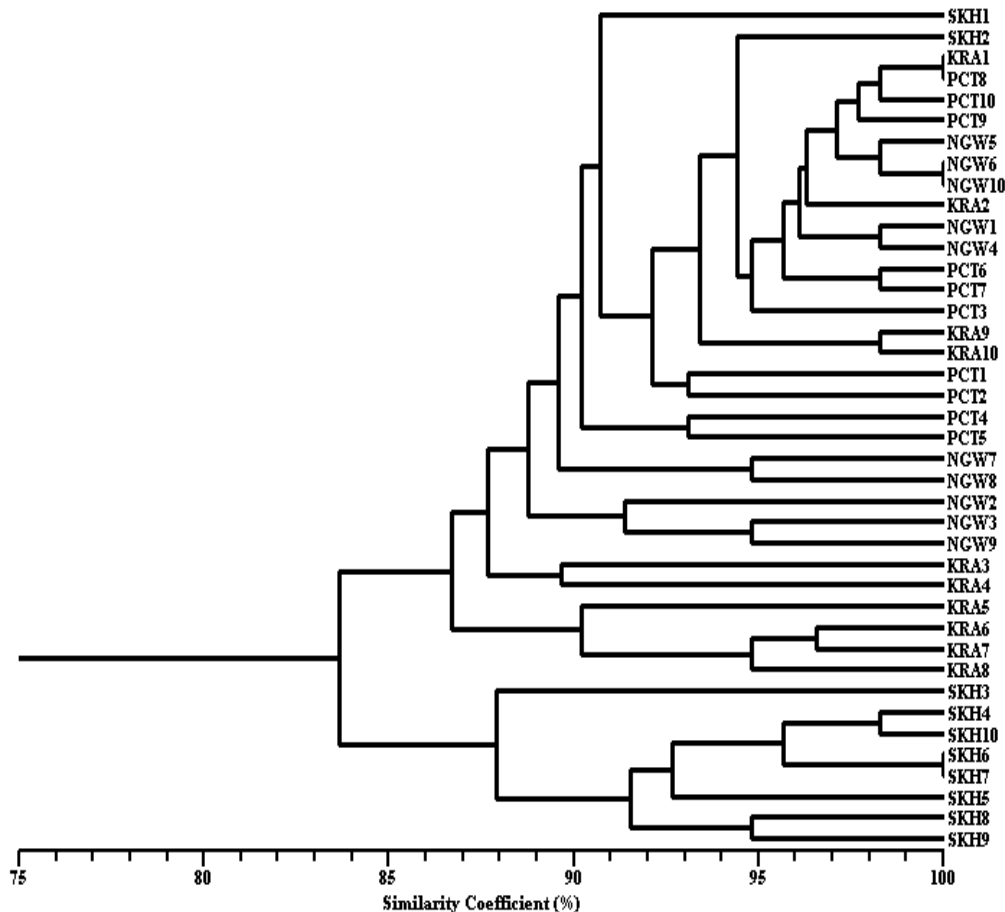


Figure 3. Relationship among 40 samples of *Annona muricata* which included 4 populations in Central Java and East Java using RAPD marker based on UPGMA method.

In order to study the correlation between populations, UPGMA algorithm was used to predict a dendrogram for the four populations of soursop in Central Java and East Java using NTYSYS software (Figure 3). It showed distinct separation of the collected sample from four locations into two major clusters. In general, the sample collected from Sukoharjo (SKH) formed a separate cluster in the dendrogram while the sample

collected from Ngawi (NGW), Pacitan (PCT) and Karanganyar (KRA) were genetically more related, they group together in other cluster and diverged from population Sukoharjo (SKH).

Furthermore, it is suggested that the highest relatedness between the samples collected from Ngawi (NGW), Pacitan (PCT) and Karanganyar (KRA) seemed to depend on the nearby location and perhaps, the same environmental conditions.

Moreover, the highest diversification of the sample collected from Sukoharjo (SKH) however could be reflected by both the global variation and reproductive biology of the species.

DISCUSSION

The success of any molecular genetic studies involving tree species is mainly depend on the efficiency of DNA extraction, which is largely determined by the number of steps involved in the extraction procedure (Tibbits and McManus, 2006). A good DNA extraction procedure should be simple, rapid, efficient and yielding appropriate level of quality DNA suitable for molecular analysis. The genomic DNA samples extracted in this study was enough satisfactory for further molecular analysis.

All RAPD primers selected showed more than 90% of polymorphism. Polymorphism detected by RAPD was determined by the different DNA sequence of the sites, which the primer bound, or by the insertions and deletions occurring between these sites (Lay *et al.*, 2001). According to Ferreira and Grattapaglia (1995), RAPD markers are sensitive and may generate different quantities of amplified fragments depending on the quality and quantity of the DNA used, as well as the amplification conditions.

There is a large number of polymorphic loci in the populations studied and that these populations showed a high level genetic variability, with more than 90% of polymorphism. This high level of genetic variability was found in the populations of soursop, allowing the adaptation of the possible environmental changes and the maintenance of their evolutionary potential. This information is important, because low levels of variability can cause serious effects on the population such as deleterious effects on the adaptation and persistence of these populations.

Genetic variability studies can identify alleles that might affect the ability of the organism to survive in its existing habitat, or might enable it to survive in more diverse habitats. This knowledge is valuable for germplasm conservation, individual, population, variety or breed identification (Duran *et al.*, 2009). Therefore, genetic variability and polymorphism shows the potential use for tree improvement of the populations. The use of RAPD markers in the applied breeding programs can facilitate appropriate choice of the parents involved for crosses (Abd El-Hady *et al.*, 2010).

The variation rates indicate isolation by distance, which emphasizes the importance of maintaining the levels of genetic variation within and among *Annona muricata* L. populations. The utility of RAPD markers in estimating genetic variability also has been demonstrated in *Annona* cultivars (Ronning *et al.*, 1995).

The clustering results of different population suggest that *A. muricata* undergoes major part of genetic variation by environmental factors. Genetic variation in a population is measured by the heterozygosity or the degree of polymorphism. Genetic variability refers to the variation at the level of individual genes (polymorphisms), and provides a mechanism for populations to adapt to their ever-changing environment (Dharmar and De Britto, 2011). The genetic variability in *A. muricata* may be partly explained as a result of abiotic and biotic factors. From the work done in this plant which collected at different geographical locations, it was understood that each location varied with respect to environmental factors and genetic parameters. Geographical, climatic or reproductive variables explain the partitioning of the diversity observed which may aid in

improving the strategies for maximizing the efficiency of germplasm collection and preservation for breeding of *A. muricata*.

Analysis of RAPD could be useful to detect genetic differentiation of *A. muricata* among four populations in Central Java and East Java. From the work done in this species which collected at different geographical locations, it was understood that each location varied with respect to environmental factors and genetic parameters. The information obtained here could be valuable for devising strategies for breeding of *A. muricata*.

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