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Assessment of genetic diversity among soursop (*Annona muricata*) populations from Java, Indonesia using RAPD markers

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Abstract. *Suratman, Ari Pitoyo, Sri Mulyani, Suranto. 2015. Assessment of genetic diversity among soursop (*Annona muricata*) populations from Java, Indonesia using RAPD markers. Biodiversitas 16: 247-253.* The objective of this study was to determine genetic diversity of the soursop (*Annona muricata* L.) populations from Java (Indonesia) using RAPD markers. A total of 70 individuals were collected from 7 soursop populations, distributed along the geographical range of natural distribution in Java. Genetic diversity was estimated by RAPD technique using 6 arbitrary selected primers. Those primers produced 151 polymorphic bands with the percentage of polymorphism for each primer ranged from 95% to 100%. The genetic diversity value (h) within each population ranged from 0.0418 to 0.0525. The highest h value (0.0525) was found in the KRA population whereas the lowest h value was observed in the PCT population. The highest genetic distance value (0.0410) was observed in SKH-GKD populations pair whereas the lowest genetic distance value (0.02448) was estimated in KRA-PCT populations pair. Based on the dendogram, the seven soursop populations were segregated into three major clusters. The first cluster consisted of SKH, KRA, PCT, NGW, and KPG populations. The GKD population was then grouped into second cluster. In the third cluster, the BGR population was grouped separately and more genetically distant than the others. However, the relationship dendrogram showed that the grouping did not always indicate the geographical origins similarity, but possibly showed the genetic similarity.

Key words: assessment, genetic diversity, Java, RAPD, soursop

INTRODUCTION

Soursop *(Annona muricata* L.) is a species of the genus *Annona* belonging to family Annonaceae, which is known mostly for its edible fruit. Soursop is distinguished by its conspicuous spiny fruit and its obovate leaves with domatia on the underside (Kerrigan et al. 2011). Soursop is native to the Caribbean and Central America but is now widely distributed in the tropics and subtropics of the world, so it can be found in the West Indies, North and South America, lowlands of Africa and Pacific islands (Badrie and Schauss 2009). Today the soursop has spread throughout the world so it is also grown in Southeast Asia included Malaysia and Indonesia (Hasni 2009).

This species was recorded not only useful in the food industries but also for medical purposes. Soursop can be eaten freshly and also used as raw material for puree, juice, jam, jelly, powder fruits bars and flakes. It is also excellent for making refreshing drinks, sherbets and flavoring/ topping for ice-cream and dessert (Bates et al. 2001; Abbo et al. 2006; Hasni 2009). For medical purpose, 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 and Schauss 2009).

Information about the extent of genetic diversity of soursop in Java (Indonesia) is still incompletely understood. Better understanding of genetic diversity and its distribution is essential for rational utilization of germplasm in crop improvement (Piyasundara et al. 2009). Evaluation of genetic diversity among various accessions of crop species is fundamental for plant breeding programs (Tanksley 1983; Ikbal et al. 2010). This information can provide a predictive estimation of genetic variation within crop species thus facilitating breeding material selection (Qi et al. 2008).

A variety of molecular, chemical and morphological markers are used to characterize the genetic diversity among and within crop species (Ozkaya et al. 2006). Morphological markers are routinely used for estimating genetic diversity but are not successful due to strong influence of environment. Hence, the use of molecular markers has complemented the classical strategies (Tanksley 1983). Molecular markers provide a better estimation of genetic diversity than morphological marker that is dependent of effect of various environmental factors. Various molecular markers techniques based on Polymerase Chain Reaction (PCR) amplification have become increasingly important at the study of genetic diversity. Random Amplified Polymorphic DNA (RAPD) is one of various approaches which available for DNA fingerprinting (Lakshmi et al. 2011).

The development of RAPD markers, generated by the PCR using arbitrary primers, has provided a rapid and easy tool for the detection of DNA polymorphism. RAPD assay is one of the suitable methods for identifying the genotypes

within a short period. The DNA amplification with RAPD does not require any previous knowledge of natural target DNA sequence, any digestion by restriction enzymes or radioactive probes. This technique also has been widely used to ascertain the genetic diversity in many crops by high levels of polymorphism with only limited amount of genomic DNA (Williams et al. 1990). It has been proved that RAPD can be used as an efficient tool for genetic characterization of many plant species (Hu et al. 1999). The use of RAPD markers in the applied breeding programs can facilitate plant breeders to identify appropriate parents involved for crosses (Abd El-Hady et al. 2010).

The objective of this research was to determine genetic diversity of the soursop populations in Java (Indonesia) using RAPD markers. This is the first report on the assessment of genetic diversity in seven soursop populations originating from Java (Indonesia) using RAPD markers. Thus, information about genetic diversity through RAPD markers obtained in this study could be valuable for breeding strategies of soursop in Java.

MATERIALS AND METHODS

Plant materials

A total of 70 individuals were collected from 7 soursop populations, distributed along the geographical range of natural distribution of *A. muricata* in Java (Table 1 and Figure 1). The material collected consisted of young and healthy leaves of each individual, which were placed in plastic bags and kept in ice box, while transported to the laboratory. In laboratory, samples were subsequently stored at-20°C until DNA extraction.

DNA extraction

Genomic DNA was extracted by the CTAB extraction procedure (Dellaporta et al. 1983) with some modifications. The extracted DNA was quantified in a spectrophotometer measuring absorbance at 260 and 280 nm. The integrity of the DNA was determined by electrophoresis on a 1% (w/v) agarose gel and photographed under ultraviolet (UV) light. The extracted DNA was then kept at-20°C for RAPD analysis.

Table 1. The location of soursop (*Annona muricata* L.) populations studied in Java with climatic data for each locality.

Population	Code	Latitude and longitude	Altitude (m a.s.l.)	Temp. (^oC)	Humidity (%)	Light intensity (lux)
Sukoharjo, Central Java	SKH	S $07^0$41'22,1", E $110^0$54'33,5"	148	34	58	358
Karanganyar, Central Java	KRA	S $07^0$41'28,16", E $110^0$54'38,5"	617	31	59	5.153
Kulonprogo, Yogyakarta	KPG	S $07^0$43'24,8", E 110046'28,6"	77,3	32	62	2.592
Gunungkidul, Yogyakarta	GKD	S $07^0$89'23,2", E 110071'29,7"	235	37,5	49	16.900
Ngawi, East Java	NGW	S $07^0$32'67,1", E $111^0$12'05,7"	735	32	42	4.156
Pacitan, East Java	PCT	S $08^0$08'94,4", E 111001'38,7"	581	34	55	17.240
Bogor, West Java	BGR	S $06^0$20'58", E $106^0$04'68"	155	33	60	237

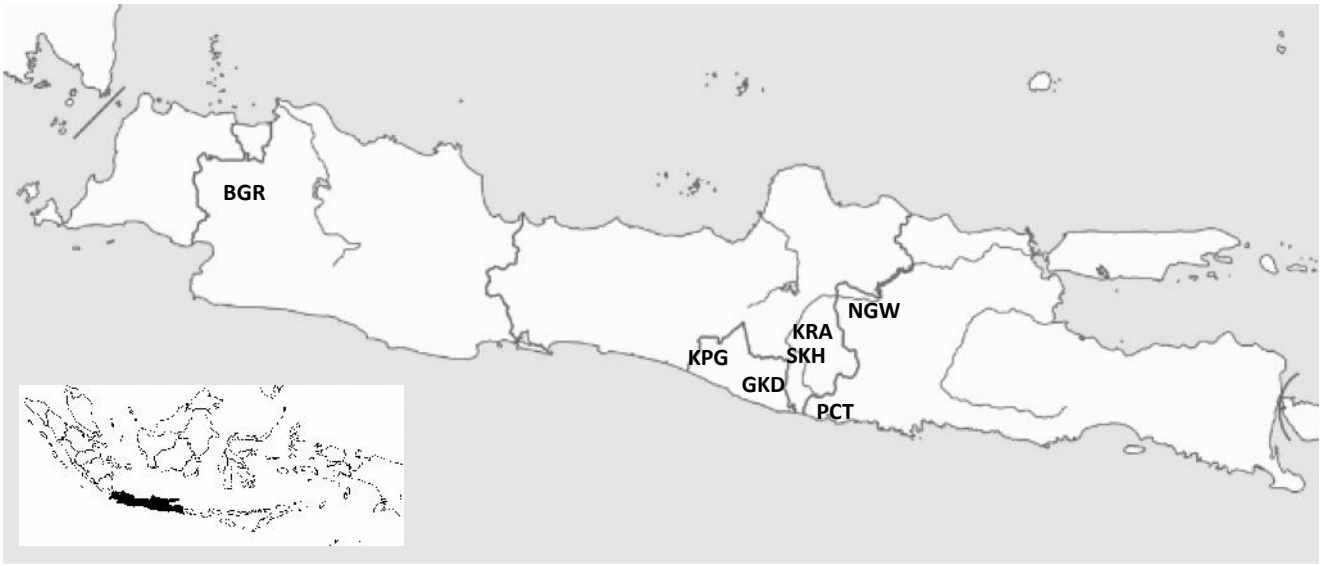

Figure 1. Map of the collection areas for 7 natural populations of soursop (*Annona muricata* L.) in Java. Note: SKH: Sukoharjo, KRA: Karanganyar, NGW: Ngawi, PCT: Pacitan, GKD: Gunung Kidul, KPG: Kulonprogo and BGR: Bogor

RAPD analysis

RAPD reaction and procedures through PCR were carried out as described by Williams et al. (1990). 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 screened initially to find specific diagnostic markers in the tested soursop populations (Table 2).

Electrophoresis

The amplified PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel in 1×TAE buffer at 80 V 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 bands. Gels were then stained with 0.5 µg/mL ethidium bromide and amplification products were visualized and photographed under UV light. The obtained profile image was then saved on a magnetic disc.

Data analysis

All distinct bands (RAPD markers) were analyzed according to their position on gel and visually scored on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using selected primers initially in the RAPD analysis were then pooled to create a single data matrix. In order to estimate polymorphic loci, genetic diversity, and genetic distance (Nei 1978), a computer program, POPGENE (Version 1.31), was used in this study (Yeh et al. 1999, Ghosh et al. 2009). A dendrogram among populations was constructed based on the genetic distance matrix by applying an Unweighted Pair Group Method with Arithmetic Averages (UPGMA) cluster analysis using a computer program, Numerical Taxonomy and Multivariate Analysis System (NTSYS) Version 2.00 (Rohlf 1998).

RESULTS AND DISCUSSION

A total of 15 primers were screened initially for their ability to generate amplified band patterns and to assess polymorphism in the tested populations (Table 2). Arbitrary primers used in the present study were 10 bp in size, had a GC content of 60% to 70% and did not contain any palindromic sequence.

Six random primers (A18, A20, P04, P06, P10, P11) gave optimal results, which yielded comparatively maximum number of amplification products with high intensity and minimal smearing, good resolution and also clear bands. The selected primers were then used to produce RAPD profiles for the references soursop populations. DNA bands resulted by PCR amplification using one of the selected RAPD primers in this study were shown in Figure 1.

Six random primers that we used generated a number of amplified DNA bands. A total of 152 bands were generated, ranging 23 to 31 bands per primer, corresponding to an average of 25.3 bands per primer. The size of bands ranged between approximately 200 and 2950 bp. Six random primers also produced 151 polymorphic bands, with an average of 25.2 polymorphic bands per primer. The degree of polymorphism within each population varied depending on the primer tested. The percentage of polymorphism for each primer ranged from 95% to 100% with an average of 99.3% polymorphism per primer. In all 5 primers (A20, P04, P06, P10, P11) produced 100% polymorphism while primer A18 showed least polymorphism (95.8%) (Table 3). The obtained data indicates that all selected random primers produced high level of polymorphism.

As a comparison, Cota et al. (2011) used 15 selected primers to evaluate genetic diversity of *Annona crassiflora*. These primers generated 140 bands of which 123 (87.8%) were polymorphic. In contrast, Ronning et al. (1995) reported 15 selected primers to analyze genetic variation between *A. cherimola, A. squamosa* and their hybrids. A total of 92 bands were produced while 86 bands (93.5%) were polymorphic. Although our study used a lower number of selected primers compared than Ronning et al. (1995) and Cota et al. (2011) but the total bands produced (152) and the average of percentage of polymorphism (99.3%) were comparable. In all cases, both the total bands produced and the average of percentage of polymorphism of Ronning et al. (1995) and Cota et al. (2011) were a bit lower than our results. Thus, the number of random primers and polymorphic bands generated were not similar range for species. They can vary significantly in different plant species.

According to Upadhyay et al. (2004), this is understandable that product amplification depends upon the sequence of random primers and their compatibility within genomic DNA. The number of markers detected by each primer depends on primer sequence and the extent of genetic variation, which is genotype specific. Poerba and Martanti (2008) described that each primer has its own attaching site, so the DNA band resulted by each primer was different, both in size of multiple basa pairs and in amount of DNA bands. Due to this fact, the selection of primers in RAPD analysis then affected the resultant band polymorphism.

Table 2. Parameters of the random primers used in the present study for initial screening.

Primers code	Nucleotide sequences (5'-3')	GC content (%)	References
A3	AGTCAGCCAC	60	Ronning et al. 1995
A18*	AGGTGACCGT	60	Ronning et al. 1995
A20*	GTTGCGATCC	60	Ronning et al. 1995
B18	CCACAGCAGT	60	Ronning et al. 1995
C11	AAAGCTGCGG	60	Ronning et al. 1995
D8	GTGTGCCCCA	70	Ronning et al. 1995
D11	AGCGCCATTG	60	Ronning et al. 1995
P02	AGATGCAGCC	60	Cota et al. 2011
P03	ATGGCTCAGC	60	Cota et al. 2011
P04*	CAGGCCCTTC	70	Cota et al. 2011
P05	CTCTTGGGCT	60	Cota et al. 2011
P06*	ACCACCCGCT	70	Cota et al. 2011
P07	ACCCCCACAC	70	Cota et al. 2011
P10*	GGCTCATGTG	60	Cota et al. 2011
P11*	GTCAGGGCAA	60	Cota et al. 2011

Note: * Selected for RAPD analysis for all samples of the seven soursop populations in Java

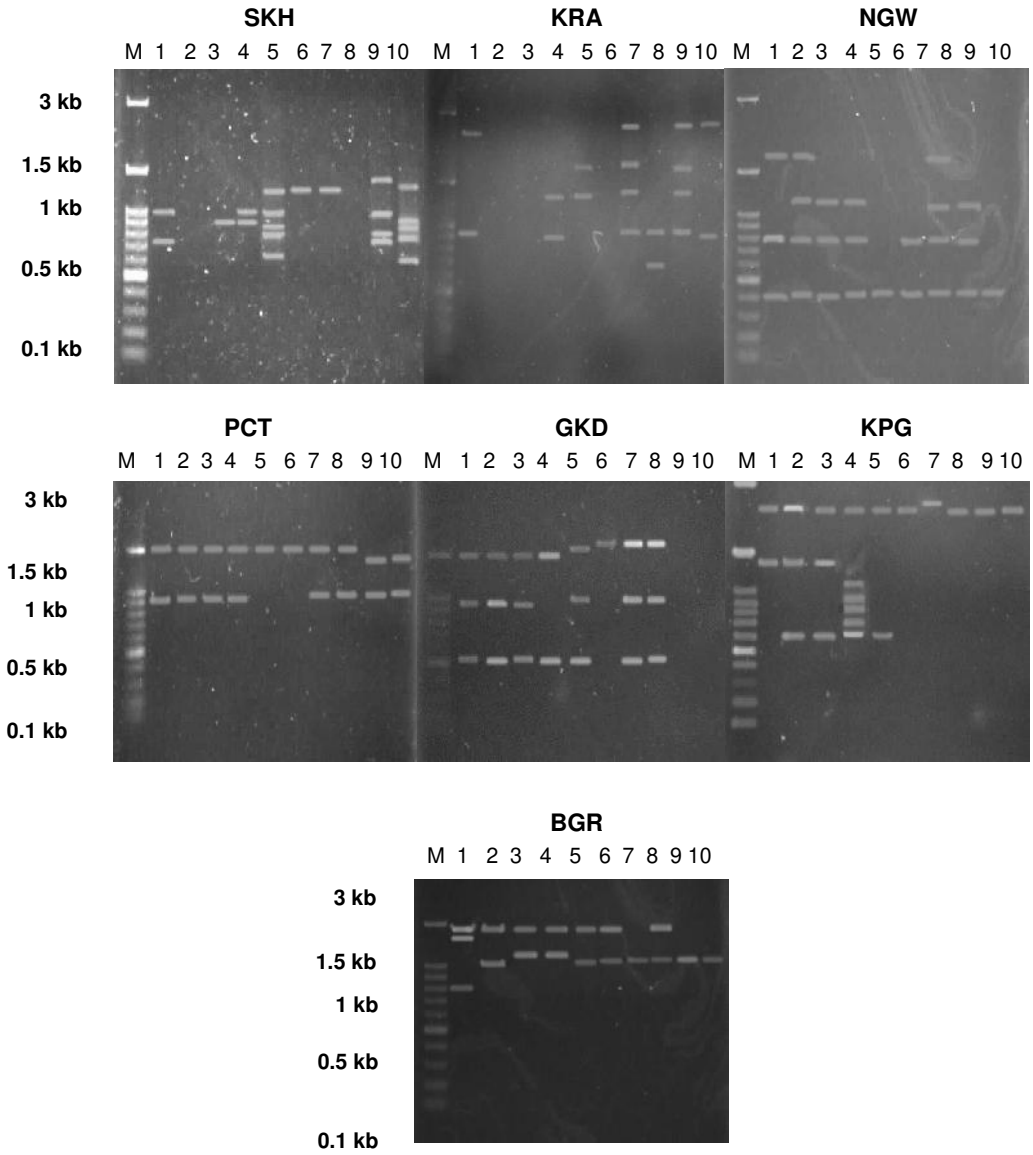

Figure 1. Profile of the RAPD bands amplified by primer P04 for soursop (*Annona muricata* L.) individuals belonging to seven populations in Java. M indicated as Marker (100 bp Ladder) and the number well (1 to 10) in each population indicated number of soursop samples. Note: SKH: Sukoharjo, KRA: Karanganyar, NGW: Ngawi, PCT: Pacitan, GKD: Gunung Kidul, KPG: Kulonprogo and BGR: Bogor

Table 3. Numbers of bands and polymorphic bands, their size range and percentage of polymorphism detected by 6 selected RAPD primers

Primers	Number of bands	Size range (bp)	Number of polymorphic bands	Poly-morphism (%)
A18	24	350-2820	23	95.8
A20	23	500-2950	23	100
P04	27	400-2950	27	100
P06	31	400-2840	31	100
P10	23	200-2040	23	100
P11	24	400-2820	24	100
Total	152		151	595.8
Average	25.3		25.2	99.3

Results showed that each soursop population collected from different localities in Java seemed to have variability in RAPD profiles by using different primers. All RAPD primers selected in this study also showed more than 90% of polymorphism. Percent polymorphism reflects the number of total bands from each primer that distinguish at least one individual. Polymorphism detected by RAPD was determined by the different DNA sequence of the sites, which the primer bound (Lay et al. 2001). It is generally reported that polymorphism between populations also can arise through nucleotide changes that prevent amplification by introducing a mismatch at one priming site; deletion of a priming site; insertions that render priming site too distant to support amplification and insertions or deletions that change the size of the amplified product (Powell et al. 1996).

Table 5. Summary of Nei's (1978) genetic distance values (below diagonal) between soursop population in Java

Population	SKH	KRA	NGW	PCT	GKD	KPG	BGR
SKH	****						
KRA	0.0330	****					
NGW	0.0367	0.0298	****				
PCT	0.0288	**0.02448**	0.0260	****			
GKD	**0.0410**	0.0366	0.0391	0.0316	****		
KPG	0.0347	0.0293	0.0335	0.0269	0.0351	****	
BGR	0.0406	0.0389	0.0382	0.0343	0.0400	0.0323	****

Note: SKH: Sukoharjo, KRA: Karanganyar, NGW: Ngawi, PCT: Pacitan, GKD: Gunung Kidul, KPG: Kulonprogo and BGR: Bogor

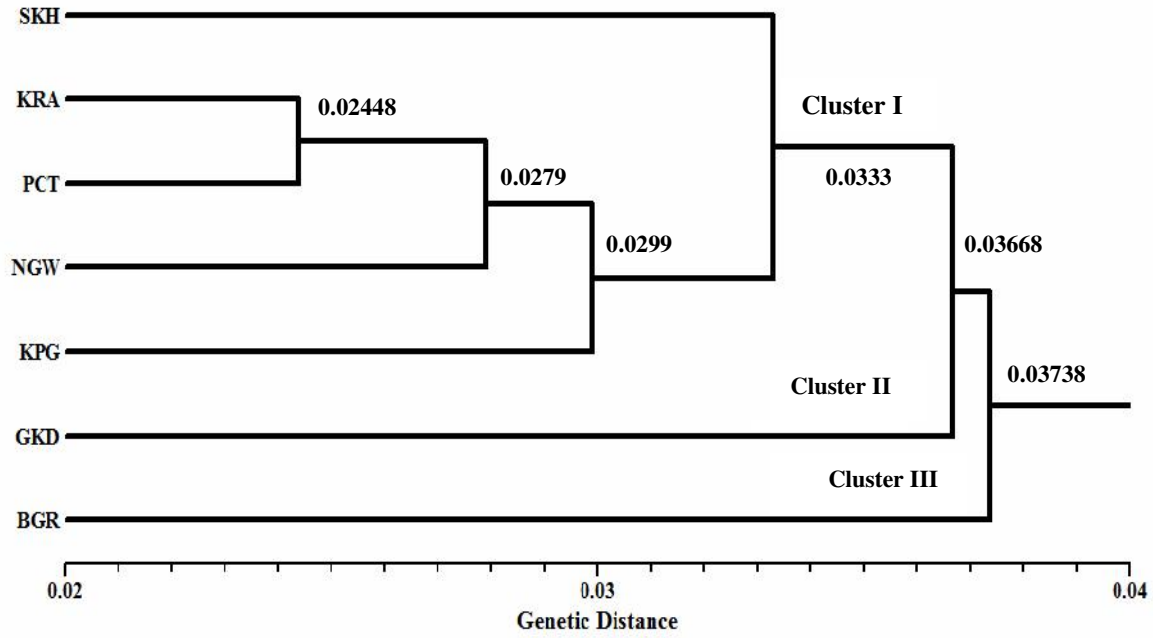

Figure 3. Dendrogram produced using UPGMA cluster analysis based on Nei's (1978) genetic distance indicating genetics relatedness among 7 soursop populations in Java using 6 selected RAPD primers. The average of genetic distance value of the nodes are indicated

Table 4. Genetic diversity on soursop population in Java using 6 selected RAPD primers

Code	Population	h ± SD
SKH	Sukoharjo	0.0454 ± 0.1105
KRA	Karanganyar	**0.0525 ± 0.1095**
NGW	Ngawi	0.0440 ± 0.1158
PCT	Pacitan	**0.0418 ± 0.1079**
GKD	Gunung Kidul	0.0439 ± 0.1184
KPG	Kulonprogo	0.0474 ± 0.1162
BGR	Bogor	0.0459 ± 0.1128
	Average	**0.0458 ± 0.1130**

Note: h: gene diversity (Nei 1978); SD: Standard Deviation

Genetic diversity measures were calculated according to Nei's index using POPGENE software and results were depicted in the Table 4. Within each population, the genetic diversity was limited, as indicated by the genetic diversity value (h) that ranged from 0.0418 to 0.0525 with an average of 0.0458 per population. The lowest genetic diversity value (0.0418) was observed in population Pacitan (PCT) whereas the highest genetic diversity value (0.0525) was found in population Karanganyar (KRA). According to Stansfield (1991), the intra-population genetic diversity value (h) was considered low if the value of h was below point two (<0.2). Thus, the soursop populations originated from Java was exhibited low levels of genetic diversity within populations. This occurrence may possibly due to the very limited chances of gene flows among populations.

The existence of low genetic diversity within soursop population possibly has been mostly attributed to self pollination (Archak et al. 2002). Although soursop flowers are apparently adapted to cross pollination despite being anatomically hermaphrodite, the bunched arrangement of stamens does not result in available fertile pollen. There is a period from 36 to 48 hours in which both sexual organs

are ripe simultaneously. However, soursop flowers function as physiologically protogyneous where its pistil and pollen are not ripen in the same time. No insect genera has any influence on pollination because insects unlike the scent of soursop flowers. It is assumed that generally fruits are formed by autogamy after stigmas get in contact with stamens retained by lower petals. Due to those facts, Escobar et al. (1986) argued that pollination would rather sporadic occurred and sometimes stigmas shed after pollination.

Inter-population genetic distance value also were calculated and ranged from 0.02448 to 0.0410 as shown in Table 5. The highest Nei's (1978) genetic distance values (0.0410) was observed in Sukoharjo (SKH)-Gunungkidul (GKD) population pair whereas the lowest genetic distance (0.02448) was estimated in Karanganyar (KRA)-Pacitan (PCT) population pair.

In order to study the relationship between populations, UPGMA algorithm based on Nei's (1978) genetic distance was used to predict a dendrogram for the soursop populations in Java using NTYSYS software. Based on the dendogram, it showed distinct separation of the collected sample from seven soursop populations into three major clusters (Figure 3). The first cluster consisted of Sukoharjo (SKH), Karanganyar (KRA), Pacitan (PCT), Ngawi (NGW), and Kulonprogo (KPG) populations. It was interested that within first cluster, the KRA population from Central Java and the PCT population from East Java, which were separated by geographical position, showed very closed relationship, with 0.02448 of their genetic distance. The NGW population from East Java, the KPG population from Yogyakarta and the SKH population from Central Java which were originated from different provinces also grouped in this cluster. The Gunungkidul (GKD) population was then grouped into second cluster. Geographically, the GKD population from Yogyakarta was far away from West Java and East Java. In the third cluster, the Bogor (BGR) population was grouped separately and more genetically distant than the others. The grouping in the third cluster was very good because the geographical position of the BGR population was separated with another population in Java. However, the relationship dendrogram showed that the grouping was inappropriate with geographical origins in the first cluster. At least, the second and third clusters were grouped in accordance with the geographical consideration. In our study, the grouping did not always indicate the geographical origins similarity, but possibly showed the genetic similarity.

One of the main applications of these clusters is the estimation of the genetic distance among populations and the identification of parents to perform appropriate crosses, and reaching maximum heterosis in hybridization programs. In this study, RAPD markers show that this method is informative and can be used to determine the relationships among populations. The data obtained here confirmed the efficiency of the RAPD technique as a valuable DNA marker for determination and estimation of genetic similarity among different plant genotypes in some populations. The information about genetic similarity will be helpful to avoid any possibility of elite germplasm becoming genetically uniform (Fadoul et al. 2013). Information on genetic diversity and relationship among and between individuals, populations, varieties, and species of plant are also important for plant breeders in guiding the improvement of crop plants (Dharmar and De Britto 2011).Thus, information about genetic diversity through RAPD markers obtained in this study could be valuable for breeding strategies of soursop in Java. This information also indicates that RAPD markers are a suitable marker to assess genetic diversity of crop species.

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