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Study on Diversity of Sapodilla (*Manilkara zapota*) by Molecular Marker in the Special Region of Yogyakarta

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ABSTRACT

The objective of this research was to determine the diversity and relationship of sapodilla accession derived from districts in DIY based on DNA profiles. From the screening of 20 total primers, five primers (OPA 20, OPB 5, OPB 6, OPB 8, and OPC 19) producing polymorphic bands in RAPD analysis were selected. Different-shaped samples of sapodilla randomly collected from Bantul, Gunungkidul, Kulonprogo, Sleman and Yogyakarta city were used in this research. The analysis of molecular data was performed by using NTSys pc-2.02 and GenAlex 6.1 program. The clustering indicated that the accessions tend to group by their locations. However, there was no corelation between DNA marker and the fruit shape. The first group comprised samples from Yogyakarta city (Keraton Yogyakarta), Bantul and Kulonprogo while the second group consisted of samples from Sleman and Gunungkidul with genetic similarity of 53% and 37% respectively. The genetic diversity was high (PLP = 98.85% of 87 loci and H=0.280±0.14) comprising the genetic diversity within population (53%) which was greater than genetic diversity between populations (47%). Having the highest genetic diversity, Gunungkidul should be potential for conservation and selection of sapodilla in DIY.

INTRODUCTION

Sapodilla (*Manilkara zapota* (L.) van Royen) is known originated from southern Mexico and the Spanish invaders took it from Mexico to the Philippines and then spread to Southeast Asia, including Indonesia (Soerianegara & Lemmens, 2002). Sapodilla is a tropical fruit tree belonging to Sapotaceae widely grown in the lowland rainforest especially in a moist hot climate (Peiris, 2007). This species is also well adapted in Indonesia's marginal areas. Sapodilla fruit was consumed in fresh. The flavor and fruit shape of sapodilla fruit is vary varied. Sapodilla, like most of perennial crops, contains high levels of polysaccharides, sap, polyphenols, some of pigments, and other secondary metabolites, it making sapodilla can be used as medicine for cough, fever, antibiotics and antimicrobial (Chanda & Nagani, 2010). According to Peiris (2007), sapodilla is one of the most under utilized fruits which then resulted in the limited information available about this fruit including in Indonesia.

The type pollination of sapodilla is outcrossing. Hu et al. (2014) revealed that perennial plant with *outcrossing* type usually have greater genetic diversity. Genetic diversity guarantees the survival of species. This variation allows species to change over time, to survive according to changing environmental conditions. The magnitude of diversity within a species depends on the number of individuals, the range of geographical distribution, the degree of population isolation and the breeding system (Lowe, Harris, & Ashton, 2004).

Genetic diversity in a population reflects the magnitude of genetic resources that can be utilized in selection activities and increase the collection of germplasm (Rahayu & Handayani, 2010). Hapsoro, Warganegara, Utomo, Sriyani, & Yusnita (2015) describes that parental selection was carried out not only based on phenotypic performance, but also genetic diversity among parents.

Finkeldey (2005/1998) proposed that genetic diversity exists in two levels, namely diversity in population and diversity between populations.

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Genetic diversity in the population is a measure of genetic variation in a single population, whereas genetic diversity among populations is a diversity caused by diversity between two or more populations.

The special region of Yogyakarta (DIY) is one of the biggest production centers of sapodilla in Indonesia. It consists of four districts, namely Kulon Progo, Bantul, Gunungkidul, Sleman and the city of Yogyakarta. There is diversity of sapodilla in DIY, either in morphologies or in flavors. Based on the ration of the diameter and length of the fruit, sapodilla fruit is classified into three types, i.e. round, oval and elliptical (Rozika, Murti, Purwanti, & Setyastuti, 2013).

Genetic diversity of a plant population could be assessed using morphological, biochemical, and also molecular markers. The advantage of molecular markers than morphological and biochemical markers is not influenced by environment factors (Hapsoro, Warganegara, Utomo, Sriyani, & Yusnita, 2015). Sera et al. (2003) concluded from the research that inter-artificial varieties of arabica coffee have morphological differences, but not much different at the level of DNA, is possible because some of these varieties originated from the same early generations (ancestors). The estimation of genetic diversity of sawo is based on geographical location, referring to Holsinger & Mason-Gamer (1996) statements that genetic differences may exist between individuals in one population, between populations within the same geographic area, between populations of different geographical regions, geographic. Laserna, Maddonni, & López (2012) proposed that the diversity of phenotypes was a form of phenotypic changes associated with genotypic response to changing environmental conditions. In order to obtain information on diversity which is more accurate, molecular markers are usually used as general approach.

The utilization of molecular markers for new sawo plants began in the late 1990s. The markers are used for identification, phylogenetic analysis and diversity.Genetic studies have widely used RAPD for estimating the genetic diversity (Aukar, Lemos, & Oliveira, 2002), and genetic relatedness among accessions (Bayazit, Imrak, Küden, & Kemal Güngör, 2011).

Heaton, Whitkus, & Gomez-Pompa (1999) have used RAPD molecular markers on sapodilla breeding program. According to Inoue et al. (2006), RAPD marker was linked to fruit skin color in Japanese pear. RAPD was also applied for identifying the genetic diversity of sapodilla using RAPD markers in four populations in Mexico (Heaton, Whitkus, & Gomez-Pompa, 1999) and in 20 cultivars in India (Meghala, Ravishankar, Anand, & Rekha, 2005). RAPD marker is distributed throughout the genome. This marker is also relatively feasible, fastand cheap to produce high polymorphism (Medhi, Sarmah, Deka, & Bhau, 2014).

Due to limited information about sapodilla in DIY, this research aimed to obtain information about the genetic diversity of different-shaped sapodilla accessions collected from several districts in DIY and to perform clustering analysis for the accessions based on RAPD marker. Therefore, the results of this research were expected to support the sapodilla breeding program.

MATERIALS AND METHODS

The experiment was conducted in the Laboratory of Genetics and Plant Breeding, Faculty of Agriculture from December 2013 to April 2014. Fully expanded mature leaves of 26 different-shaped accessions were randomly collected from four districts and Yogyakarta city in DIY (Table 1). Extraction of 0.1 g leaf sample was done using the CTAB (Cetyl trimethyl ammonium bromide) method (Doyle & Doyle, 1990) modified by Sari & Murti (2015). The quantity test of DNA was performed using spectrophotometer (Gene Quant 1300), while the quality test of DNA was performed using electrophoresis.

Five µl DNA genomes and 2 µl loading buffers were put in agarose well (1%) after added with DNA staining 1 µl, and it was performed in electrophoresis for approximately 1 hour in 80 V using TBE 1X reagent. The results of electrophoresis were transferred to UV trans-illuminator and photographed by a digital camera.

Twenty primers of RAPD (Heaton, Whitkus, & Gomez-Pompa, 1999) produced by Operon Technologies Inc. were screened and the primers producing polymorphism were used for further analysis (Table 2). DNA amplification was carried out by adding the samples into PCR tube (1x PCR reaction mixture consists of 5 mL, NFW 3.25 ml, 0.25 ml RAPD primer and 3 ml DNA). Then, the samples were inserted into the PCR thermocycler with the following setting: one cycle for 4 minutes at 94°C followed by 45 cycles for 1 minute at 94°C (denaturation), 1 minute at 37°C (annealing), 1 minute 30 seconds at 72°C (extension), and it was completed with 7 minutes at 72°C and 1 minute at 4°C. The amplification productwas visualizedby electrophoresis.

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Table 1. Accessions of sapodilla as sampels in each district.

No	Accesion code	Fruit shape	Disctrict/Regency
1.	A1	Elliptical	Bantul (Bantul)
2.	A2	Elliptical	Bantul (Bantul)
3.	A8	Elliptical	Bantul (Bantul)
4.	G1	Elliptical	Yogyakarta city
5.	G3	Elliptical	Yogyakarta city
6.	H1	Elliptical	Pathuk (Gunungkidul)
7.	H4	Elliptical	Pathuk (Gunungkidul)
8.	H10	Elliptical	Pathuk (Gunungkidul)
9.	A10	Oval	Bantul (Bantul)
10.	B2	Oval	Pajangan (Bantul)
11.	C5	Oval	Imogiri (Bantul)
12.	E2	Oval	Sentolo (Kulonprogo)
13.	G2	Oval	Yogyakarta city
14.	H3	Oval	Pathuk (Gunungkidul)
15.	I1	Oval	Karangmojo (Gunungkidul)
16.	J2	Oval	Kalasan (Sleman)
17.	L2	Oval	Cangkring (Sleman)
18.	B9	Round	Pajangan (Bantul)
19.	C1	Round	Imogiri (Bantul)
20.	E6	Round	Sentolo (Kulonprogo)
21.	G4	Round	Yogyakarta city
22.	H2	Round	Pathuk (Gunungkidul)
23.	H8	Round	Pathuk (Gunungkidul)
24.	I8	Round	Karangmojo (Gunungkidul)
25.	J1	Round	Kalasan (Sleman)
26.	L3	Round	Cangkringan (Sleman)

Table 2. Accessions of sapodilla as sampels in each district.

No	Primers	Sequence	No	Primers	Sequence
1.	OPA 2	TGCCGAGCTG	11.	**OPB 8**	GTCCACACGG
2.	OPA 5	AGGGGTCTTG	12.	OPB 9	TGGGGGACTC
3.	OPA 7	GAAACGGGTG	13.	OPB 10	CTGCTGGGAC
4.	OPA 8	GTGACGTAGG	14.	OPC 2	GTGAGGCGTC
5.	OPA 14	TCTGTGCTGG	15.	OPC 5	GATGACCGCC
6.	OPA 15	TTCCGAACCC	16.	OPC 9	CTCACCGTCC
7.	OPA 17	GACCGCTTGT	17.	**OPC 19**	GTTGCCAGCC
8.	**OPA 20**	GTTGCGATCC	18.	OPD 7	TTGGCACGGG
9.	**OPB 5**	TGCGCCCTTC	19.	OPD 8	GTGTGCCCCA
10.	**OPB 6**	TGCTCTGCCC	20.	OPD 13	GGGGTGACGA

Remarks: The bold were selected primers.

Calculation of the percentage of polymorphic loci (PLP), heterozygosity (h), and genetic distance was performed using GenAlEx6.1 program while the NTSYSPC-2.02 program was applied for clustering analysis.

RESULTS AND DISCUSSION

The samples extraction in this research was performed by using Doyle and Doyle method with modification (Sari & Murti, 2015) producing good quality of DNA. Five of twenty primers able to produce clearly visible bands were selected. The clearance of bands depends on the quality of DNA the primary sequence which has more than 60% of GC suitable for RAPD analysis (Khatun, Hossain, & Mahbubur Rahman, 2012). Bands readability is also important in genetic diversity analysis. Selected primers were then applied in RAPD analysis of sapodilla accessions.

The screening of primers aimed to acquire primers that are able to produce polymorphic bands. Polymorphic bands mean several distinguishable DNA bands on some samples that are not found in (or different from) other samples. Five of 20 RAPD

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primers were used in the primers screening (OPA 20, OPB 5, OPB 6, OPB 8, and OPC 19) generated polymorphic band (Table 2). It was found that the number of OPB primers generating polymorphic loci was more than the number of OPA and OPC primers which produced polymorphic loci. Thus, the five primers which are able to generate polymorphic loci were selected to amplify the DNA of leaf samples of all accessions.

RAPD analysis of all accessions was performed by using the selected primers (Table 1). Table 3 shows that the lowest polymorphic loci was in identified Kulonprogo samples; reaching 14 polymorphic loci, while the highest ones was identified in Gunung Kidul samples; achieving 55 polymorphic loci. The results of the genetic diversity analysis of each population consisting of the polymorphism and heterozygosity (h) values are also illustrated in Table 3. The result showed that all selected primers produced 98.85% polymorphism levels of 87 loci and 0.280 ± 0.140 for heterozygosity in DIY. Meanwhile, the highest value of polymorphism (63%, 55 loci) and the greatest heterozygosity (0.204 ± 0.019) were found in sapodilla population in Gunungkidul.

Taheri, Abdullah, Abdullah, & Ahmad (2013) suggested that the diversity of a population is determined by the number and percentage of polymorphic band. The polymorphism in this research achieved 98.85%, which was higher than the result of Meghala, Ravishankar, Anand, & Rekha (2005) research in India, which just produced 76% band polymorphism of 20 primers used. The high polymorphism obtained in this research indicated high genetic diversity of sapodilla in DIY.

The value of heterozygosity (h) was measured from the dominant marker ranges 0-0.5 and reaches maximum value when present and absent bands at the same frequency (p = q = 0.5) (Gaudeul, Till-Bottraud, Barjon, & Manel, 2004). The results showed that the population of the sapodilla in Gunungkidul had the highest value of polymorphism (63%, 55 loci) with the greatest heterozygosity (0.204 ± 0.019). This great heterozygosity value reflected the high genetic diversity of sapodilla in Gunungkidul. It implied that the population of Gunungkidul was promising to be selection object since it had greater variety of genotype.

Analysis of genetic diversity by AMOVA based on geographic location (Table 4) showed that genetic diversity within population was higher than genetic diversity between populations amounted to 53% and 47% of the total diversity respectively. The genetic diversity within population (intra-population) was higher than the genetic diversity between populations (inter-population) of sapodilla accessions in DIY; indicating that the population with similar genetic composition came from the same population/district. A similar distribution pattern was also proposed by Hartati, Rimbawanto, Taryono, Sulistyaningsih, & Widyatmoko (2007) who found that out-crossing populations, such as Pulai (*Alstonia scholaris* (L.) R. Br.), had higher genetic diversity of intra-population compared to that of inter-population achieving 85% and 15%, respectively.

Table 3. Polymorphic and variation value.

Population/ District	Number of accesions	Polymorphic Loci	Polymorphic loci percentage (%)	Heterozigosity (h)	
				Means	Standard error (SE)
Bantul	8	29	33.33	**0.126**	0.020
Kulonprogo	4	14	16.09	0.066	0.016
Yogyakarta City	4	29	33.33	0.138	0.021
Gunungkidul	8	55	63.22	0.204	0.019
Sleman	4	23	26.44	0.108	0.020
DIY	26	86	98.85	0.280	0.140

Table 4. AMOVA based on geographic location (district).

Source of variance	df	Sum of Square	Mean Square	Variance component	Total variance (%)
Inter population	4	164.39	41.09	**6.61**	47
Intra population	21	158.37	7.54	7.54	53
Total	25	322.76		14.15	

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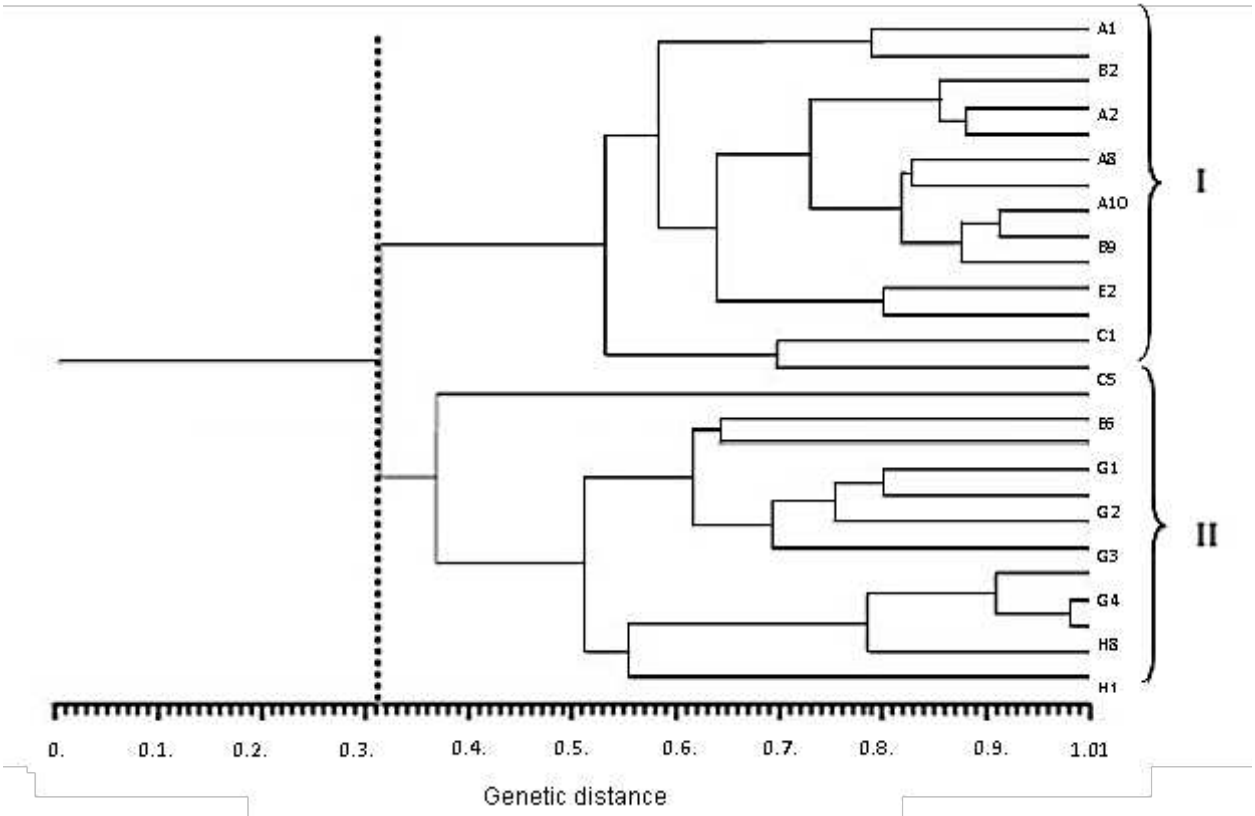

Fig. 1. Clustering of sapodilla accesions based on RAPD markers.

Table 5. Genetic similarity and Nei genetic distance.

Population	Bantul	KP	Yogyakarta	G. Kidul	Sleman
Bantul		0.91	0.85	**0.78**	0.69
Kulonprogo	**0.08**		0.88	0.72	0.64
Yogyakarta City	**0.16**	**0.12**		0.75	0.67
Gunungkidul	**0.24**	0.32	**0.28**		0.87
Sleman	**0.36**	**0.44**	0.38	**0.13**	

Remarks: Genetic similarity (unbold characters); Genetic distance (bold characters)

DNA band-based dendogram clustered the 26 sapodilla accessions into two large groups at the similarity value of 31.5% (Fig. 1). The first group consisted of sapodilla accessions from Bantul, Kulon Progo and Yogyakarta city with similarity value of 53%, while the second group consisted of sapodilla accessions of Gunungkidul and Sleman with 37%. Table 5 shows the value of genetic distance and genetic similarity between populations. It was found that the neighboring locations had lower genetic distance or greater similarity. For example, the genetic distance of sapodilla populations from Kulonprogro-Bantul (relatively close) was lower than that from Kulonprogro-Sleman (farther) reaching 0.08 and 0.36, respectively.

DNA bands-based clustering resulted in grouping of 26 accessions of sapodilla into 2 clusters based on their location of origin instead of morphological characters such as fruit shape. It might be due to human or natural intervention. There were no samples having a similarity value of one (100% similar). This result implied that the accessions of sapodilla were propagated by seeds which then spread in or out to neighboring district by humans. Differences in conditions between districts do not restrict the spreading of sapodilla as the plant is widely adaptable and grows well in tropical climate conditions (Meghala, Ravishankar, Anand, & Rekha, 2005).

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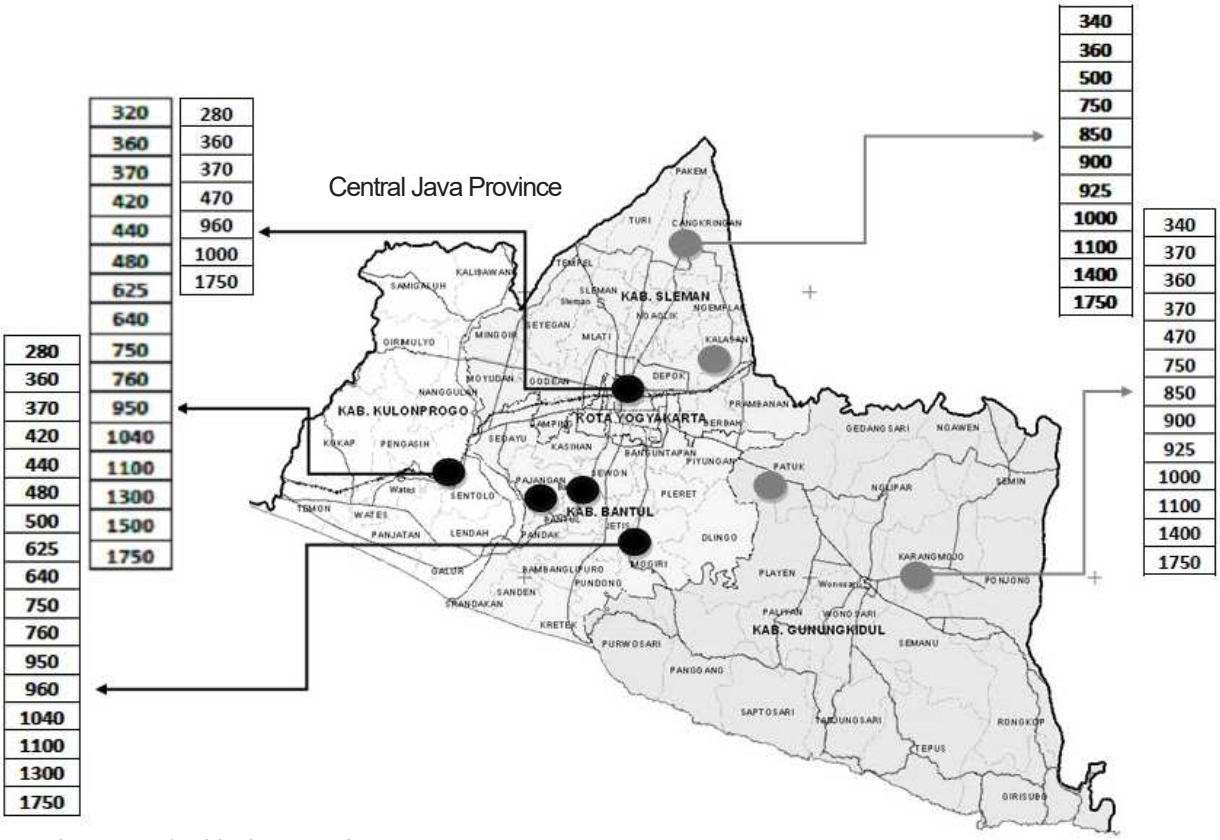

Remarks: group 1 = black; group 2 = gray

Fig. 2. DNA band in each location.

According to Fig. 2, populations showing large genetic similarity tend to have a banding pattern or the same locus. The scheme in the figure excluded the specific band in each location which means only the bands owned by two or more populations were included. The banding pattern showed that the bands possessed by the population from Yogyakarta city were owned by other populations. This result is in accordance with the expectation that sapodilla accessions spreading in DIY is originated from Yogyakarta city.

The result of this research indicated that the genetic distance is highly correlated with the geographical distance. Similar result also happened to the research of Widyatmoko, Rimbawanto, & Chasani (2013) on the Jati (*Tectona grandis*, Linn. F.) population in Sulawesi. Accessions originated from the same area or in neighboring geographical locations tend to have greater genetic similarity. Pollens and seeds deployment among neighboring locations allow the gene flow from one population to other populations. Similarity among accessions within population is probably caused by gene derived from the same ancestor (co-Ancestry) in certain populations. Since sapodilla is a cross or open pollinated plant, there might be pollen mixing between members of the population. Sapodilla pollination requires insects such as bees as pollinators (Salinas-Peba & Parra-Tabla, 2007). Hence, those pollinators also contribute the pollen deployment in or out to neighboring locations. While Heaton, Whitkus, & Gomez-Pompa (1999) revealed that bats and other animals help the spreading of sapodilla seed.

The clustering of sapodilla accessions was not based on morphological trait like fruit shapes (oval, round, and elliptical). This is probably because fruit shape belongs to the qualitative trait which is commonly controlled by one-two or multiple genes or it might because RAPD primers do not amplify genes (or parts of genes) encoding fruit shape. This finding was in accordance with Heaton,

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Whitkus, & Gomez-Pompa (1999) who reported that the high polymorphism generated was not correlated with distinctive phenotypes. According to Hartati, Rimbawanto, Taryono, Sulistyaningsih, & Widyatmoko (2007), RAPD primers amplify DNA in the coding and non-coding regions. Fruit shape character was controlled by one gene and partially dominant producing elliptical, oval and round fruit (Sultana & Rahman, 2013). Alternative to reduce the disadvantages of RAPD is by expanding the number of primers used to amplify most of the genome samples. Hartati, Rimbawanto, Taryono, Sulistyaningsih, & Widyatmoko (2007) stated that many primers were required to obtain an accurate grouping. Inoue et al. (2006) used 200 primers to identify relatedness between RAPD markers and fruit skin color of Japanese pear. Susantidiana, Wijaya, Lakitan, & Surahman (2009) added that a lot of primers used in RAPD method quite affect the number of characters that appear.

Out of line between fruit shape marker and RAPD-DNA profile were amplified resulting the outcome of two markers that do not constitute a part of internassociated, or only partially. The way how RAPD obtained DNA encoding the phenotypic characters was observed (Robi'ah, Sobir, & Surahman, 2005; Susantidiana, Wijaya, Lakitan, & Surahman, 2009). This result was consistent with the result of Heaton, Whitkus, & Gomez-Pompa (1999) mentioning that the phenotype differences of sapodilla were not in accordance with the genetic differences (based on RAPD analyzed).

Based on the dendogram indicating the origin of sapodilla in DIY, G3 and G4 samples were suspected of being elders of the early generations while the other represents the next generation. It is commonly known that sapodilla is a plant widely grown in the complex of Keraton Yogyakarta. Keraton relatives or abdi dalem (Keraton employees) usually have sapodilla plants in front of their house as a symbol of abdi dalem. This might be the cause of sapodilla distribution from the Yogyakarta city to Bantul and Kulon Progo. Source of genes derived from the samples at Keraton were G3 and G4 having low similarity with other samples in the first group. Widyatmoko, Rimbawanto, & Suharyanto (2005) proposed that the gene flow intensity was greater between nearby locations.

The success of a breeding program cannot be apart from the genetic diversity of germplasm used and the relationship between accessions to be used as a parent. Based on the analysis, it was found that the genetic diversity of 26 sapodilla in DIY was high ($h = 0.2854 \pm 0.1490$) which is in accordance with Hu et al. (2014) who reported that perennial plants (out-crossing) had a great diversity. According to this research, sapodilla selection based on locationis more effective due to the tendency of sapodilla accessions in DIY to be clustered according to geographical location. Sapodilla population in Gunungkidul with the highest genetic diversity can be potential object for selection.

CONCLUSION

Sapodilla accessions spreading in DIY tend to cluster into 2 groups based on their location. The first group was the accessions collected from Bantul, Kulon Progo, and Yogyakarta while the second group consisted of accessions from Gunungkidul and Sleman which were clustered with genetic similarity each of 53% and 37%. Genetic diversity of sapodilla in DIY was high with the value of PLP reaching 98.85% of the 87 loci and h of 0.28±0.14. Genetic diversity within population was found to be greater than that between populations amounted to 53% and 47%, respectively. Due to its highest diversity, Gunungkidul was recommended to be potential site for sapodilla selection to support breeding program.

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