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Variation analysis of three Banyumas local salak cultivars (*Salacca zalacca*) based on leaf anatomy and genetic diversity

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Abstract. *Herawati W, Amurwanto A, Nafi'ah Z, Ningrum AM, Samiyarsih S. 2018. Variation analysis of three Banyumas local salak cultivars (*Salacca zalacca*) based on leaf anatomy and genetic diversity. Biodiversitas 19: 119-125.* A variation analysis of three salak cultivars was conducted in Banyumas District, Central Java, Indonesia. The aim of the study was to investigate variation amongst the cultivars based on anatomy and genetic diversity by using RAPD (*Random Amplified Polymorphic DNA*) technique. The result of the study showed that all salak cultivars have similar variation in leaf anatomy. The only distinguishing characteristic is the number of trichomes on cultivars Kedung Paruk and Kalisube per 1 mm^2 unit of leaf area, while the cultivars of Candinegara lacked trichomes. Ten primers could detect 50 polymorphic bands (80,6%) from a total of 62 bands which could be observed clearly. The percentage of polymorphic loci in 'Candinegara' was 64%, 'Kedung Paruk' 62%, and 'Kalisube' 44%. The heterozygosity value of 'Candinegara' was 0.1590, 'Kedung Paruk' 0,1449, and 'Candinegara' (0.0235). Meanwhile, the furthest genetic distance was shown by 'Kedung Paruk' and 'Candinegara' (0.0704). This result gives us important basic information about cultivars of *Salacca zalacca* in Banyumas District which have high genetic diversity and germplasm. These results can be used for further research such as for conservation and genetic engineering.

Keywords: Anatomical characteristics, molecular, snake fruit

INTRODUCTION

Salak (*Salacca zalacca*) is a species of palm tree (family *Arecaceae*) native to Java and Sumatera in Indonesia. The fruits are known as snake fruit due to the reddish-brown scaly skin. Cultivar diversity of salak in Indonesia is relatively high especially at the center of salak cultivation such as in Java island, where there are many different names of salak based on their area of origin (Nandariyah 2010). Two popular cultivars with sweet flavor are salak pondoh from Yogyakarta province and salak Bali from Bali island. . A high demand for these two cultivar increased ,local salak cultivar plantation is decrased and nearly ectinct. Currently, Banyumas local salak which is sour and relatively big is difficult to find and may contribute to diversity losses. Farmers are reluctant to plant Banyumas local salak although it can still be processed into chips snacks, jam, and syrup. Salak fruits contain quite high nutrients and can also be used as an antioxidant (Aralas et al. 2009)

In Banyumas District, there are three salak cultivars based on their origin of growth, namely 'Kedung Paruk'at Kedung Paruk, Ledug Village, Kembaran Sub-district, 'Kalisube'at Kalisube Village, Banyumas Sub-district, and 'Candinegara'at Candinegara Village, Pekuncen Sub-district. Some studies on morphological characters of Banyumas local salak cultivar have been carried out (Herawati et al 2012) but none on leaf anatomy and molecular characters. Molecular markers such as DNA have advantages compared to morphology markers due to their stability, consistency and lack of environmental interference (Zulfahmi 2013). One of the molecular techniques that are frequently used in genetic diversity analysis is RAPD (*Random Amplified Polymorphic DNA*). RAPD is considered practical for use over the other markers since the DNA required is lower, it is less costly, does not require blotting and data can be collected quickly (Gurijala et al. 2015). This marker resulted from DNA amplification process *in vitro* with PCR (*Polymerase Chain Reaction*) (Williams et al. 1990), and it is used to identify diversity in both intra or interspecies levels (Singh et al. 2010; Langga et al. 2012).

Therefore, a study on genetic characteristics such as DNA is important to complete salak cultivar characteristic data that can be used for conservation plan strategies. The morphological, anatomical and molecular characters of salak are essential for the establishment and conservation of germplasm collections, providing valuable information to breeding programs and agronomic studies regarding desirable attributes (Santos et al. 2012). The snake fruit germplasms could be combined through breeding activities to select new varieties which have desirable traits that meet the consumer's demand. Such an ideal fruit is an alternative food for the peoples' choice of vitamins, minerals, and fibers sources. This diversity of food is supported the Indonesian government policy of food diversification (Budiyanti et al. 2015)

The aim of this study was to know the genetic diversity of Banyumas local salak cultivars based on anatomy leaf and RAPD method. Understanding of genetic variation would be highly important information in preserving and utilizing germplasm.

MATERIALS AND METHODS

Study area

Fresh *S. zalacca* specimens used in this study were obtained from the fields at Salak center in Banyumas District, Central Java, Indonesia. Samples of three Banyumas local salak cultivars were Kalisube', 'Kedung Paruk' and *S. zalacca* 'Candinegara'.

Anatomical characters

Leaf anatomy was observed from longitudinal and transverse sections. The anatomy preparations used the paraffin method (Sass,1958). The variables studied were cuticle thickness, mesophyll thickness, epidermis thickness, stomata size, the number of stomata, and trichomes. All of the parameters were measured with light microscope (CH20) with the ocular micrometer inside of 400x magnified.

Molecular character

RAPD analysis steps included isolation, purification, quantification, dilution, amplification of DNA, electrophoresis, and visualization.The analysis was done in Laboratory of Molecular Genetics, Balai Besar Penelitian Bioteknologi dan Pemuliaan Tanaman Hutan (BBPBPTH) Yogyakarta, Indonesia.

DNA extraction used the modified CTAB method (Doyle and Doyle, 1990). The extract was purified using Gene Clean III Kit (Q-biogene). DNA concentration was quantified using the "NanoVue" (GE Healthcare) and expressed in the ratio of the absorbance at 260 and 280 nm ($A_{260/280}$). Then, DNA was diluted to 2.5 ng/µL for the PCR reaction. DNA was amplified using a controlled programmed machine GeneAmp thermocycler PCR System 9700 (Perkin Elmer) Applied Biosystems. Ten primers were used in this study namely OPA-9, OPA-10, OPA-11, OPA-16, OPA-18, OPC-8, WTO-18, OPM-10, the OPW-13 and OPX-17. Modified amplification method followed Nandariyah (2010). PCR began with pre-denaturation at a temperature of 95 °C for 5 minutes, followed by 45 cycles of reaction consisting of denaturation at 94 °C for 30 seconds, annealing at 37 °C for 30 seconds, the elongation primer (extension) at a temperature of 72 °C for 1 minute 30 seconds, the final extension at 72°C for 7 minutes, and was finalized with holding at 4 °C. PCR amplification products were run in electrophoresis tank on a 1.2% agarose gel at a voltage of 120 Volts in 1x TBE buffer solution for ± 3 hours. Electrophoresis results were photographed using Fotodyne Image Analyzer (BIO-RAD Gel DocTM EQ) equipped with UV light.

Data analysis

Data were analyzed descriptively based on the presence or absence of DNA fragments generated by primers at each locus. Quantitative analysis results of the binary data was performed using the program of POPGEN 1:32 (Yeh et al. 1999) for genetic diversity and genetic distance.

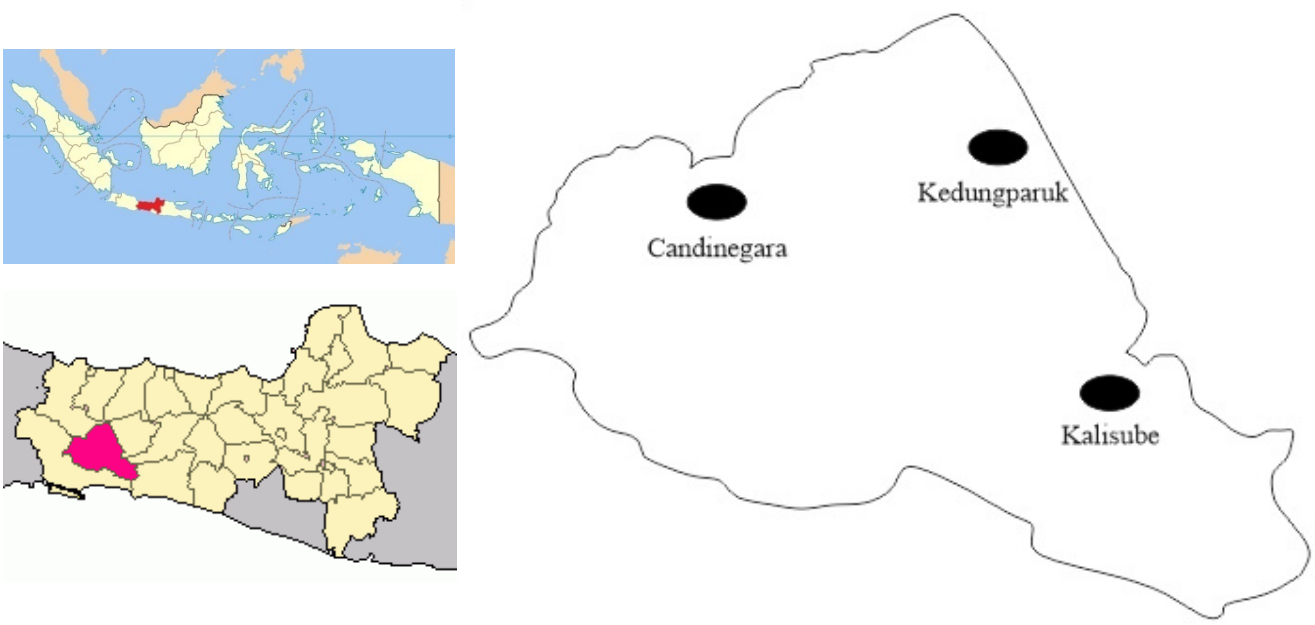

Figure 1. The location of Banyumas local *S. zalacca* cultivar in Banyumas District, Central Java, Indonesia

RESULTS AND DISCUSSION

Leaf anatomy characteristics

The results showed that three cultivars have the same leaf anatomy structure but differ in size (Figure 2). The Kalisube cultivar has a cuticle 5.48 µm thick, while Candinegara is 0.5 µm. Cultivars of Candinegara had an epidermal layer 13.13 µm thick, while Kalisube was thinnest at 11.63 µm. A cultivar of Kedung Paruk had a f mesophyll 133.50 µm thick while Candinegara had the thinnest mesophyll at 115.50 µm. The leaf tissue layer thickness (epidermis thickness, mesophyll thickness, and cuticle thickness) exhibited variation among all examined cultivars. Stomata sizSe, varies between 14 to 16.50 µm long and 3.20 to 3.35 µm wide. Kalisube had the highest number of stomata per 1 mm^2 unit of leaf area, 28.80 Kedung Paruk had the fewest number of stomata,e 20.80. The density of trichoma on Kedung Paruk and Kalisube were the only one trichomes per mm^2 unit of leaf area while the cultivars of Candinegara did not have trichomes (Table 1). There was a significant variation in leaf stomata frequency amongstcultivars.The higher stomatal density or stomatal index can be used as an indicator for higher transpiration rate, higher metabolism, and absorption of mineral and water (Muradoglu and Gundogdu 2011). Micromorphological characters of leaf surfaces can assist in taxonomic identification and classification because of their high structural diversity (Solangi et al. 2010; Gari 2011).

RAPD diversity

Based on the results of measurements using NanoVue, the value of total purity of DNA samples ranged from 1.679 to 1.995 at concentrations ranging from 73.5 to 239.5 ng/mL, while after the purified DNA purity values expressed in $A_{260/280}$ ranged from 1.810 to 2.122 with DNA concentrations ranging from 17.5 to 83.0 ng/mL.

DNA concentration illustrates the amount of DNA contained in the RNA solution. DNA obtained from the extraction called total DNA is DNA that is not clean because it is mixed with contaminants such as RNA and others. Weeden et al. (1992) stated that the DNA purification that is not perfect may still contain polysaccharides, phenolic compounds or other contaminants so that with increasing concentration, the contaminant DNA also increases. Purification process needs to be done to remove contaminants since they can prevent the formation of DNA suspension that interferes the amplification process. DNA concentration after purification in this study ranged from 17.5 to 83.0 ng/µL. The amount of DNA produced was more than sufficient for use in PCR analysis. sDNA purity can be determined using the ratio of the optical density values at various wavelengths. $A_{260/280}$ as value ratio of the absorption at a wavelength of 260 nm and 280 nm is commonly used as an indicator of the purity of DNA. DNA is pure if the ratio of absorption at 260 nm and 280 nm is 1.8. Khosravinia et al. (2007) stated that if $A_{260/280}$ is smaller than 1.8 indicated the presence of phenol or protein contamination on the extraction, meanwhile if $A_{260/280}$ is 2 or greater showed the presence of RNA. In this study, DNA quantification of purified DNA resulted in the score that ranged from 1.810 to 2.122. This shows that the DNA samples of all three cultivars bark have a sufficient purity value for the analysis of PCR-RAPD.

DNA dilution was made to obtain a uniform concentration for using in PCR analysis. DNA concentration required for PCR-RAPD amplification in this study is 2.5 ng/mL. If DNA concentration is too high or too low it will affect the results of amplification. This is confirmed by Langga et al. (2012) that if the DNA dilution is less pure and less precise, primers will not attach to the target DNA. Young et al. (2000) also stated that RAPD genetic markers is very sensitive to the reaction conditions and the quality of the DNA template. Therefore, concentration and purity of DNA, primers and genomic DNA preparation procedures need to be consistent.

Analysis of three Banyumas cultivars, namely *S. Zalacca* 'Kalisube', *S. Zalacca* 'Kedung Paruk' and *S. Zalacca* 'Candinegara' using ten primers capable of detecting 50 clear polymorphic bands (80.6%) from a total of 62. The length of polymorphic band ranging from 300-2300 bp. The resulting number of polymorphic bands ranged from 2-11 with an average of 6 bands per primer. A primer that produces the highest amount of polymorphism is OPA-16, while one that produces the lowest amount of polymorphism is OPD-18 (Table 2).

Polymorphism of DNA fragments generated in this study (80.6%) is in line with several previous studies using RAPD techniques. RAPD analysis from Nandariyah (2010) using 12 salak cultivars in Java with six random primers resulted in 68.4% polymorphism. Roslim et al. (2003) analyzed the genetic relationship of coconut type in Banyuwangi, Lubuk Pakam, and Paslaten using ten primers produces 80% polymorphism. Karsinah et al. (2002) detected the genetic diversity in citrus germplasm using ten primer generated 91.8% polymorphism. The results showed that the ten primers showed G + C content between 60-70% indicated that primers are good enough to use for PCR-RAPD. This is consistent with the statement of Poerba andYuzammi (2008) that the successful amplification of genomic DNA using PCR-RAPD technique is also determined by the base sequence of the primers used and the quality or content of the primer in each reaction. RAPD markers obtained with random amplification of DNA segments from a single arbitrary primer. Primers used are typical of 10 bp. G + C content of bases in primer sequences used for PCR-RAPD should contain more than 40% G + C bases (usually containing 50-80% G + C) and does not contain sequences that are palindromic.

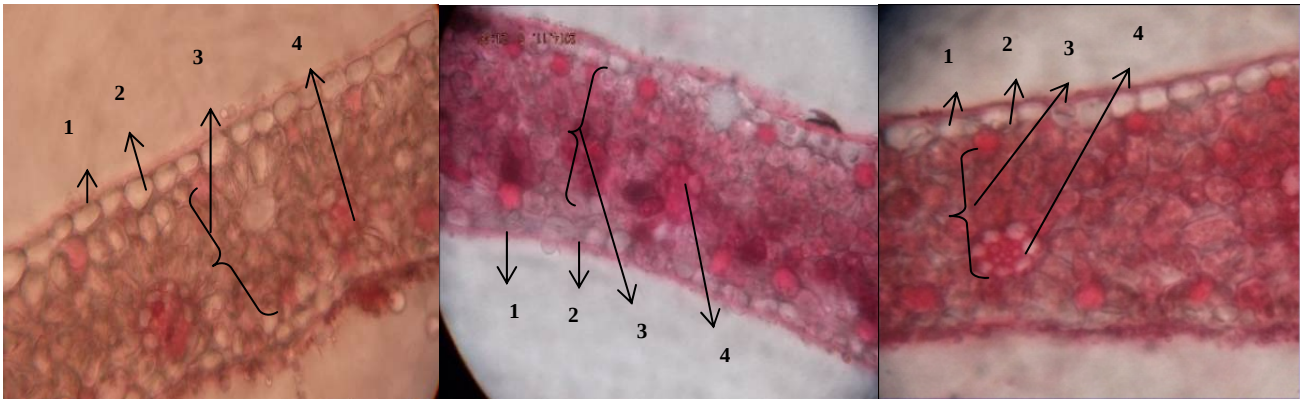

Figure 2. The anatomical structure of leaves (400x). Note: A. Kedung Paruk, B. Kalisube, and C. Candinegara. Cuticle (1), epidermis (2), mesophyll (3), vascular bundle (4)

Table 1. Average anatomical character of leaf (n = 3)

Cultivar	Thickness (µm)			Size stomata (µm)		Density (mm^2)	
	Cuticle	Epidermal	Mesophyll	Length	Width	Stomata	Trichome
Kedung Paruk	5.15	12.38	133.50	15.50	3.30	20.80	1.00
Kalisube	5.48	11.63	115.50	16.50	3.20	28.80	1.00
Candinegara	5.00	13.13	126.50	14.00	3.35	21.60	-

Table 2. List of primers, sequences, loci, number of bands and G+C content in RAPD analysis

Primer	Sequence (5' - 3')	Locus (bp)	No. polymorphic bands	No. monomorphic bands	Total
OPA-9	GGGTAACGCC	300-1000	6	2	8
OPA-10	GTGATCGCAG	500-1300	4	3	7
OPA-11	CAATCGCCGT	700-1300	3	0	3
OPA-16	AGCCAGCGAA	500-2200	11	2	13
OPA-18	AGGTGACGGT	750-1500	3	1	4
OPC-8	TGGACCGGTG	750-1200	5	1	6
OPD-18	GAGAGCCAAC	900-1500	2	1	3
OPM-10	TCTGGCGCAC	850-2300	3	1	4
OPW-13	CACAGCGACA	590-1300	7	1	8
OPX-17	GACACGGACC	550-1200	6	0	6
Total			50 (80,6%)	12 (19,3%)	62 (100%)

Visual observations in the bands of RAPD amplification product of Salak Banyumas local cultivars with the same primer showed the individual variation of the number, length, and intensity of the band. Certain individual bands showed large numbers of amplifications while others showed small bands amplification. Similarly, the length of the bands produced varied greatly among individuals. One was the result of amplification using the primer of OPA-16 (Figure 4) in which the length of the bands produced varied greatly among individuals. Variation in the amount and length of the bands was due to differences in the annealing of primer to the genomic DNA strands. According to Harkingto (2007) the genomic DNA sequence differences between one individual or another causing DNA variation or polymorphisms among individuals, while the primer regions with the same sequence lead to monomorphism DNA among individuals. The intensity of band variations, such as thick and clear, thin and vague were due to the differences in the copy number of amplification resulted from the presence of repetition of certain sequences in the genome, such as the order sequence of tandem repeats

The analysis of PCR-RAPD generated polymorphic bands showed variations in the number, length, and intensity of the resulting bands. Gusmiaty et al. (2012) stated that the DNA fragment size differences or polymorphisms of DNA fragments were due to primer annealing location or site at nucleotide bases that scattered throughout the genome. DNA amplification occurred when primer attached to adjacent two complementary sites with reverse orientation. The distance of amplification sites caused DNA fragments with various sizes.

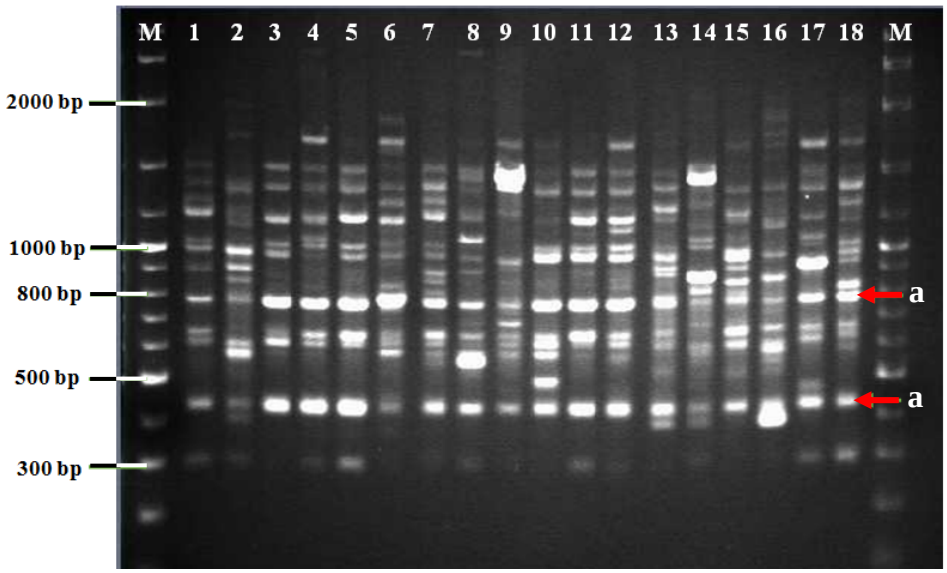

Figure 4. Amplification results of RAPD using OPA-16. Note: No. 1-6 = Kalisube, 7-12 = Kedung Paruk, 13-18 = Candinegara with monomorphic band (a)

Absence of band amplification on some primers could result from (i) reagent components used are not correct, (ii) the nucleotide sequences of the primers are not complementing with the nucleotide bases in the DNA template, so that primers are not able to amplify DNA fragments, (iii) complementary nucleotide sequences of the DNA template has a distance amplification that is too far away, and (iv) the same amplification direction between two annealing positions (Roslim et al. 2003).

Annealing is also one important step that will determine the success of PCR. This is confirmed by Gusmiaty et al. (2012) in which the annealing temperature phase regulation of the PCR process is very influential in the process of primer annealing. Change in temperature of one degree in the primer annealing phase will cause failure attachment. Prana and Hartati (2003) also stated that in addition to determination of the base sequence and quantity of primer, RAPD-PCR amplification success is also determined by the suitability of PCR conditions that include temperature annealing and extension.

Genetic diversity of local Banyumas salak cultivar

Parameters used to determine the genetic diversity in a population are percentage of polymorphic loci (ppl), the number of alleles observed (na), effective number of alleles (ne) and the genetic variation (h) (Finkeldey 2005).

Table 3. The results of variation measurement in local Banyumas salak cultivar

S. zalacca cultivar	No. samples	na	ne	H	PPL
Kalisube	6	1.4400	1.1731	0.1136	44.00%
Kedung Paruk	6	1.6200	1.2133	0.1449	62.00%
Candinegara	6	1.6400	1.2360	0.1590	64.00%

Note: na = number of alleles observed; ne = number of effective alleles, h = genetic variation, PPL = percentage of polymorphic loci

Based on Table 3, it can be seen the values of the effective number of alleles and heterozygosity on each of the different cultivars so that genetic diversity in each of salak cultivars is different. The value of genetic diversity of S. zalacca 'Candinegara' occupied the highest position with a mean value of the effective allele of 1.2360 and the value of heterozygosity of 0.1590. This is possible because of the occurrence of crossbreeding and the possible distribution of seeds in the population of *S. zalacca* 'Candinegara' with the help of humans so that the genetic diversity increased. If it is considered from the magnitude of genetic diversity in *S. zalacca* 'Candinegara' then the population of *S. zalacca* 'Candinegara' is more suitable for breeding material when compared to other sampling locations. Sobir et al. (2005) stated that high genetic diversity is a source of genes that can be utilized to form a recombination so that there are opportunities to improve characters of a plant and to form new varieties that are superior.

Value of genetic diversity and the percentage of polymorphic loci are used to describe genetic diversity. The genetic diversity of each salak cultivar can be viewed from the percentage of polymorphic loci (Table 3). The highest percentage of polymorphic loci was shown by *S. zalacca* 'Candinegara' by 64%, followed by *S. zalacca* 'KedungParuk' by 62% and the lowest was *S. zalacca* 'Kalisube' by 44%.

The percentage of polymorphic loci of each cultivar is high enough to show the high diversity in cultivars, while the differences in the percentage of polymorphic loci are small as seen in *S. zalacca* 'Candinegara' and *S. zalacca*' Kedung Paruk' showing the low diversity among cultivars or almost the same genetic structure. Genetic diversity is a key in plant breeding. Plant breeding program is a way to increase the genetic variability in a population. Studies of genetic diversity have important benefits in helping artificial selection, preparing test provenances (source/

origin) as well as controlling a cross. Selection is the first step in getting new superior cultivars and it needs high genetic diversity for its effectiveness (Olivia and Siregar 2012).

Conservation of genetic resources is necessary to maintain genetic diversity. Cultivars that have higher genetic diversity will have the ability to adapt and avoid extinction. High genetic diversity will likely to produce some individuals that are resistant to extreme environmental conditions and to some diseases Olivia and Siregar (2012).

Genetic distances among local salak Banyumas cultivars

Analysis of genetic relationship was done to get an overview of the genetic information on local Banyumas salak cultivars . Results are presented in Table 4. It was found that the genetic distance between the local Banyumas salak cultivars showed a range between 0.0235 to 0.0704. The closest genetic distance was indicated by *S. zalacca* 'Kalisube' and *S. zalacca* 'Candinegara' at 0.0235 while the farthest genetic distance was indicated by *S. zalacca* 'KedungParuk' and *S. zalacca* 'Candinegara' at 0.0704. High genetic distance indicated that relationship between both cultivars was far enough and that the small genetic distance value indicated that the relationship was close. Cluster analysis was performed using UPGMA method (*Unweight Pair Group Method with Arithmetic*) to produce dendrogram. Dendrogram was created based on genetic distances of Nei (1972) in POPGEN 1.32 that illustrated the relationship between three local Banyumas salak cultivars (Figure 5).

Based on Figure 3, it caan be seen that local Banyumas salak cultivars are divided into two groups The first group includes *S.zalacca* 'Kalisube' and *S. zalacca* 'Candinegara'. The second group comprises *S.zalacca*' KedungParuk'. The low genetic distance among cultivars in a large geographical distance between cultivars indicates that the two, namely *S. zalacca* 'Kalisube' and *S. zalacca* 'Candinegara' came from the same source.

The tendency of such grouping is also shown in the results in Olivia and Siregar (2012) in the population of Sengon at community forests in Java. Ağaoğlu et al. (2006) stated that grouping not related to the geographical location was due to RAPD markers used showed good DNA variations in coding and noncoding regions. To obtain an accurate grouping, it is necessary to analyze DNA using more primers and more samples. The more distance between samples, the smaller the success of the cross, but it is possible to obtain superior genotypes when the crossing is successful. Crossing between individuals within a close genetic relationship will increase homozygosity as a consequence of meeting bad alleles. Crossing between individuals with a large genetic distance has the effect of an increase in heterozygosity. Information genetic relationship may be useful for the process of making good quality of seeds (Julisaniah et al. 2008).

Table 4. Genetic distance among three local Banyumas salak cultivars

S. zalacca cultivar	Kalisube	Kedung Paruk	Candinegara
Kalisube	*	0.0444	0.0235
Kedung Paruk	0.0444	*	0.0704
Candinegara	0.0235	0.0704	*

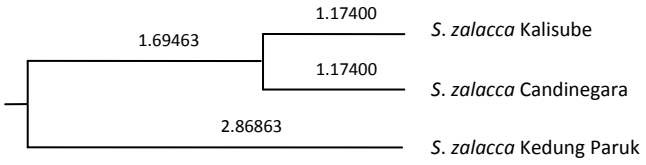

Figure 5. Dendrogram of genetic relationship among local Banyumas salak cultivars

In conclusion, based on the results and discussion, it can be concluded that the local Banyumas salak cultivars have high genetic diversity. Detection of genetic differences and discrimination of genetic relationship between salak cultivars are for sustainable utilization and conservation of plant genetic resources.

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