

Genetic diversity of shallots (*Allium ascalonicum* L.) from several locations in North Sumatra, Indonesia based on RAPD markers

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Abstract. Hasanah Y, Mawarni L, Hanum H, Lestami A. 2022. Genetic diversity of shallots (*Allium ascalonicum* L.) from several locations in North Sumatra, Indonesia based on RAPD markers. *Biodiversitas* 23: 2405-2410. Shallot (*Allium ascalonicum* L.) is one of the leading horticultural and spice commodities in Indonesia. Assembling shallot varieties to get a good seed quality requires a high genetic diversity which can be analyzed using molecular markers. This study aims to identify the genetic diversity of shallots from several locations in North Sumatra based on Random Amplified Polymorphic DNA (RAPD) markers. This research was conducted at the Biotechnology Laboratory, Faculty of Agriculture, Universitas Sumatera Utara, Medan in September-October 2021. A total of 11 shallot varieties from several locations in North Sumatra were genetically analyzed based on RAPD markers with OPA-13, OPB-07, OPD-20, OPM-01 primers. The results showed that 11 shallot varieties originating from several locations in North Sumatra had a high genetic diversity with the presence of two main groups at a dissimilarity coefficient of 76%. These beneficial results can be used as a complement to morphological markers in the genetic study of shallots for breeders to decide what genotypes will be crossed to make new genetic combinations in the development of shallots. This study reports the success of the shallot varieties' fingerprints using RAPD markers.

Keywords: Genetic diversity, RAPD, shallot, variety

INTRODUCTION

Shallot (*Allium ascalonicum* L.) is one of the leading horticultural plants and spices used in various processed dishes throughout the world (Sulistio et al. 2017; Yao et al. 2017; Tabor 2018). Shallots contain beneficial nutrients for the body such as energy 72 kcal, 79.80 g water, 16.80 g carbohydrates, 2.5 g protein, 0.1 g total fat, 31.2 mg vitamin C, 3.2 g total fiber, 7.87 g of total sugar in every 100 g of shallots (Aryanta 2019).

Based on BPS data (2020), the production of shallots in North Sumatra in 2018 reached 16.337 tons, in 2019, it reached 18.072 tons and increased in 2020 with the production of 29.222 tons. This product has not been able to meet the needs of shallots in North Sumatra (43.000 tons) in 2020. One of the problems in increasing shallot production is the low quality of the seeds. Currently, farmers are still using shallot bulbs from previous plantings that have been set aside (Basuki 2010; Rahayu et al. 2019). According to Sembiring (2017), shallot production is much more influenced by seeds. Besides, seeds from the same variety show different production when planted in different areas, it is suspected that genetic mutations occur that create genetic diversity.

The analysis of genetic diversity in plants can be done by observing morphological and isozyme properties. However, this analysis is not sufficient in determining accurate markers due to environmental influences (Al-Shagir and Salam 2015). Therefore, molecular markers are beneficial complements for morphological and isozyme

properties because they are not influenced by the environment (Ramesh et al. 2020). Several PCR-based molecular markers have been developed to assist genetic diversity studies (Suryadi et al. 2019).

In research to identify genetic diversity of plants, molecular markers in the form of Random Amplified Polymorphic DNA (RAPD) are often used. The RAPD technique is a DNA amplification technique based on Polymerase Chain Reaction (PCR) using a random single oligonucleotide (primary) to form DNA fragments (Dayarani and Dhanarajan 2014). The advantages of RAPD are that the results obtained are faster and easier to perform, the large number of DNA band polymorphisms and the relatively low cost (Weising et al. 1995; Kumar and Gurusubramanian 2011). RAPD is a technique that can be used for polymorphic analysis of unknown genomes (Babu et al. 2014). RAPD analysis using ten base primers is often used for kinship studies and identification of varieties (CIMMYT 1998).

Many studies have been carried out on the cultivation and genetic diversity of shallots using conventional methods, such as the effect of paclobutrazol and sulfur on shallots (Hasanah et al. 2021), the effect of applying Mount Sinabung volcanic ash and rice husk charcoal on shallot production (Purba et al. 2015), physiological characteristics of shallots in the highlands and lowlands (Hasanah et al. 2022), morphology and taxonomic relationships of shallot cultivars from Indonesia (Fitriana and Susadarini 2019), the effect of eco-enzyme on shallot production (Hasanah et al. 2021). However, the study on the genetic diversity of

shallots in North Sumatra, Indonesia based on RAPD has not been widely reported. Therefore, the objective of the study was to analyze genetic diversity using RAPD markers to determine the genetic relationship of shallot varieties origin from several locations in North Sumatra.

MATERIALS AND METHODS

The research was conducted at Biotechnology Laboratory, Faculty of Agriculture, Universitas Sumatera Utara, Medan Indonesia from September to October 2021.

Plant materials and sample collection

The materials used were the leaves of 11 varieties of shallots collected from different locations in North Sumatra, namely Bima Brebes Simalungun, Sumenep Simalungun, Lokananta Langkat, Tajuk Simalungun, Maja Simalungun, Batu Ijo Serdang Bedagai, Lokananta Simalungun, Sanren Berastagi, Lokananta Berastagi, Sanren Medan, and Lokananta Medan. The leaves are put into a plastic sample and stored in an icebox to maintain the freshness of the leaves until analyzed in the laboratory. Other materials used include CTAB buffer, tissue, liquid nitrogen, TE buffer, TAE buffer, Chloroform Isoamylalcohol with ratio of 24:1, NaOH, Na-EDTA, NaCl, 70% and 100% alcohol, HCl, β -mercaptoethanol agarose (Promega V3121), cold isopropanol.

DNA extraction

DNA extraction was carried out according to the CTAB method of Orozco-Castillo et al (1994) which was modified by the addition of Polyvinylpyrrolidone (PVPP) and β -mercaptoethanol (Toruan-Mathius et al. 1997). A total of 0.1-2.0 g of leaves were ground using a mortar and pestle until completely pulverized/smoothed while added liquid nitrogen and PVPP at the end. After it was smooth, the sample was put into a 2 mL tube that already contained 1 mL of CTAB 2% (w/v) and 10 μ L β -mercaptoethanol 2% (w/v) extraction buffer and then vortexed until homogeneous. Then, the samples were incubated in a water bath for 30 minutes at a temperature of 65°C and homogenized by turning the tube every 10 minutes. After that, it was incubated at room temperature for 4-5 minutes, then added 1 mL of chloroform: isoamyl alcohol (24:1).

Samples were centrifuged at 13.000 rpm for 10 minutes at room temperature. After that, the supernatant obtained was transferred to another centrifuge tube, then 1 ml of chloroform: isoamyl alcohol (24:1) was added, shaken with a vortex and centrifuged again at 13.000 rpm at 4°C for 7 minutes. The supernatant obtained was then transferred and added 1 mL of cold isopropanol. The tube was shaken slowly until fine white threads appeared. Moreover, it was incubated for one night in the refrigerator to precipitate DNA pellet.

In addition, it was centrifuged at 13.000 rpm for 5 minutes at 4°C. The supernatant was homogenized by inverting the tube, then stored in a refrigerator (4°C) for 30 minutes, then centrifuged again at 13.000 rpm for 5

minutes at 4°C. The supernatant obtained was then discarded and dried. The dry pellet was dissolved with 100 μ L of TE buffer, then spun manually until homogeneous. Next, 1 mL of absolute ethanol was added, and then inverted until homogeneous. It was then incubated in a freezer (-20°C) for 30 minutes, then centrifuged again at 13.000 rpm at 4°C for 10 minutes. This step was repeated until the pellets were clean. Then, the supernatant was discarded while pelleting using absolute ethanol and dried. The dried DNA pellet was then dissolved with 100 μ L of TE buffer and stored in DNA stock in the freezer (-20°C).

PCR amplification-random amplified polymorphic DNA (RAPD) marker

Amplification was conducted using 4 RAPD primers, namely OPA-13, OPB-07, OPD-20 and OPM-01 (Table 1). Before running the PCR, DNA was diluted by taking 6 μ L of DNA stock and adding 10 μ L of ddH₂O to obtain 16 μ L of DNA aliquots.

PCR master mix was made in a microtube with a composition for one reaction with a total volume of 15 μ L, including Go Taq PCR 8.5 μ L, nuclease free water 3.5 μ L, primer 1 μ L, and DNA sample 2 μ L. The amplification process was carried out using a PCR machine. Gene Amp PCR Applied Biosystem amplification reaction was designed for time, temperature, and number of the thermal cycles 45 times, with a series of the initial denaturation steps at 94°C for 1 minute, denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 4 minutes.

Electrophoresis of amplification results with RAPD marker was carried out by dissolving 2% agarose gel. The gel was made by dissolving 1.6 g of agarose in 80 ml of 1x TAE buffer, then made to dissolve using a hot plate and stirred using a magnetic stirrer. Next, 1 μ L of gel red was added and poured into the electrophoresis gel concrete which had been equipped with a comb (well) until the gel solidified. The solid gel was transferred to the electrophoresis bath and given 1x \pm 670 mL TAE solution (until submerged). A total 1 Kb DNA Marker (3 μ L) which had been mixed with loading dye (1 μ L) was injected into agarose gel well using a micropipette. Furthermore, DNA samples that had been prepared were inserted into the next agarose gel well as much as 4 μ L each well. After all samples had been injected, the electrophoresis device was connected to a power supply with 75 volts of electricity for 60 minutes. The result of electrophoresis was observed with UV lamp in a transilluminator and documented using gel documentation.

Table 1. Sequence of primer

Name of primer	Sequence (5'-3')
OPA-13	CAGCACCCAC
OPB-07	GGTGACGCAG
OPD-20	ACCCGGTCAT
OPM-01	GTTGGTGGCT

Data analysis

Data analysis was conducted based on the amplified scoring data. The analyzed molecular data were converted into binary data based on the presence or absence of amplified bands. If there is an amplified band, it is coded 1, while if there is no it is coded 0. Calculation of the percentage of polymorphic bands uses the following formula:

$$\% \text{ polymorphic band} = \frac{\Sigma \text{polymorphic band}}{\Sigma \text{total band}} \times 100\%$$

Distance matrix or genetic dissimilarity was analyzed by Unweighted Pair Group Methode Arithmetic (UPGMA), to visualize the grouping of varieties, using Multivariate Statistical Package (MVSP ver 3.2) software (Kovach 1989).

RESULTS AND DISCUSSION

Amplification of shallot DNA with RAPD markers

Shallot DNA was analyzed based on 4 primers of RAPD, namely OPA-13, OPB-07, OPD-20 and OPM-01. Primer selection was carried out to randomly predict genetic diversity in shallots. RAPD analysis was carried out to predict the existence of genetic diversity which could be seen through banding patterns formed.

The highest number of banding patterns was established in OPD-20 primers with 6 band patterns, while the lowest number was found in OPA-13 and OPB-07 primers with 4 banding patterns. The highest band size was established in OPD-20 primer of 900 bp, whereas the band size lowest was established in OPA-13 primer of 100 bp. Recapitulation of DNA band amplification of 11 shallot varieties on 4 RAPD primers can be seen in Table 2. Visualization of the electrophoresis results of 2% agarose gel with 4 RAPD marker primers can be seen in Figures 1-4.

OPA-13 primer showed different band patterns between shallot varieties with a total of 4 bands produced with band sizes from 100-531 bp. The percentage of polymorphic bands is 100%. OPA-13 primer visualization was described in Figure 1.

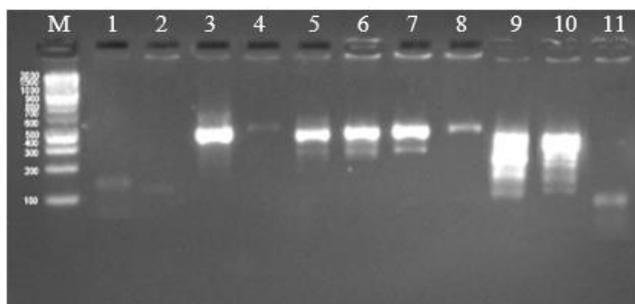


Figure 1. Electrophoregram amplification of 11 shallot DNA with OPA-13 primer; M: Ladder 1 kb Marker; 1: Bima Brebes Simalungun, 2: Sumenep Simalungun, 3: Lokananta Langkat, 4: Tajuk Simalungun, 5: Maja Simalungun, 6: Batu Ijo Serdang Bedagai, 7: Lokananta Simalungun, 8: Sanren Berastagi, 9: Lokananta Berastagi, 10: Sanren Medan, 11: Lokananta Medan

OPB-07 primer showed different band patterns between shallot varieties with a total of 4 bands produced with band sizes from 115-732 bp. Percentage of polymorphic bands is 100%. OPB-07 primer visualization was described in Figure 2.

OPD-20 primer showed different band patterns between shallot varieties with a total of 6 bands produced with band sizes from 120-900 bp. The percentage of polymorphic bands is 100%. OPD-20 primer visualization was described in Figure 3.

OPM-01 primer showed different band patterns between shallot varieties with a total of 5 bands produced with band sizes from 129-700 bp. The percentage of polymorphic bands is 100%. OPM-01 primer visualization was described in Figure 4.

Generally, the results showed the banding pattern produced by four primers used showed different banding patterns and some similarities in some varieties. Based on four primers used, the number of band patterns is 4-6 DNA bands per primer. The size of the result DNA bands varied between 100-900 bp. The amplification results showed that several DNA bands did not only appear in one variety but also appeared in another variety, where the banding pattern varied in each variety. This variation is known as polymorphism. According to Karki et al (2015), the polymorphism is diversity in the DNA sequence that arise in a population of 1% or more frequency. Polymorphism occurs because there are nucleotide changes due to DNA mutations.

Table 2. Recapitulation of DNA band amplification of 11 shallot varieties

Name of primer	Band size (bp)	Number of band	Number of polymorphic band
OPA-13	100-531	25	25
OPB-07	115-732	30	30
OPD-20	120-900	36	36
OPM-01	129-700	36	36
Total	127	127 (100%)	

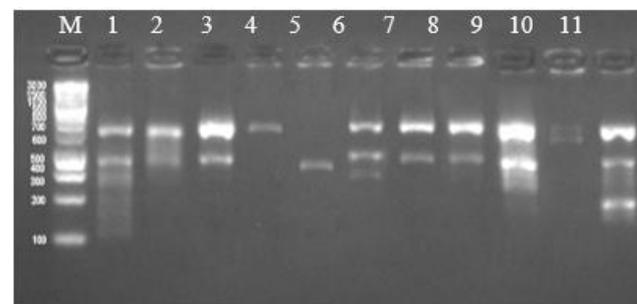


Figure 2. Electrophoregram amplification of 11 shallot DNA with OPB-07 primer; M: Ladder 1 kb Marker; : Bima Brebes Simalungun, 2: Sumenep Simalungun, 3: Lokananta Langkat, 4: Tajuk Simalungun, 5: Maja Simalungun, 6: Batu Ijo Serdang Bedagai, 7: Lokananta Simalungun, 8: Sanren Berastagi, 9: Lokananta Berastagi, 10: Sanren Medan, 11: Lokananta Medan

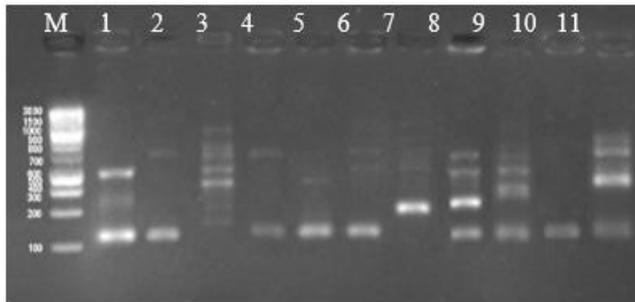


Figure 3. Electrophoregram amplification of 11 shallot DNA with OPD-20 primer; M: Ladder 1 kb Marker; 1: Bima Brebes Simalungun, 2: Sumenep Simalungun, 3: Lokananta Langkat, 4: Tajuk Simalungun, 5: Maja Simalungun, 6: Batu Ijo Serdang Bedagai, 7: Lokananta Simalungun, 8: Sanren Berastagi, 9: Lokananta Berastagi, 10: Sanren Medan, 11: Lokananta Medan

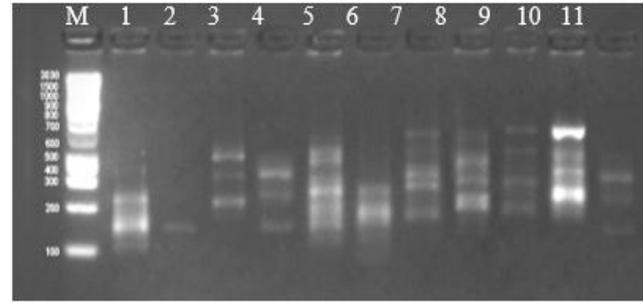


Figure 4. Electrophoregram amplification of 11 shallot DNA with OPM-01 primer; M: Ladder 1 kb Marker; 1: Bima Brebes Simalungun, 2: Sumenep Simalungun, 3: Lokananta Langkat, 4: Tajuk Simalungun, 5: Maja Simalungun, 6: Batu Ijo Serdang Bedagai, 7: Lokananta Simalungun, 8: Sanren Berastagi, 9: Lokananta Berastagi, 10: Sanren Medan, 11: Lokananta Medan

The primer used is known to be the most effective primer in showing genetic diversity in plants, whereas the results also showed high polymorphism in shallot plants. According to Maesaroh et al (2014), selection of RAPD primers affects polymorphism of the sample because the primer has its attachment site. So, the resulting polymorphic DNA bands differ in the number of DNA bands and the size of base pairs.

Success of a primer in amplifying a DNA template is determined by the presence or the absence of homology of primer nucleotide sequence with DNA template nucleotide sequence. It is also influenced by DNA quantity and quality, the concentration of $MgCl_2$, *Taq polymerase* DNA enzymes, and attachment temperature primer (Martida and Pharmawati 2016; Mubarak et al. 2020). Unspecific primers can lead to amplification of other regions at the genome that is not targeted or have no other amplified genomic regions. The optimization of PCR is also required to generate the desired trait that focused on denaturation and annealing temperature in the PCR machine. Low denaturation temperature can cause the double-stranded DNA not open so that new DNA polymerization can't occur (Wang et al. 2014). Optimum temperature is required in the process of attaching the primer to exposed DNA strand because too high a temperature can cause amplification not to occur, too low a temperature causes the primer to attach to the other side of the genome that is not homologous side, as a result, many nonspecific regions of the genome can be amplified. The temperature of annealing is determined by the primer used which is influenced by the length and composition of the primer (Porta and Enners 2012).

Primer concentration affects the intensity of PCR-RAPD products. In line with Susilowati et al (2019), the concentration of genomic DNA is the most important factor in the amplification reaction, where DNA concentrations that are too high can increase contaminants that interfere with amplification reaction (Setiati et al. 2019). The research result of Rozana (2021) primer concentrations that are too low or too high cause amplification not to occur accurately. Akzad et al (2021) stated that the low ratio

between primer and template DNA can cause inconsistent RAPD products. Besides, magnesium is an important component in PCR reaction and affects the quality of the resulting RAPD profile (Serec et al. 2016). According to Hu et al (2019), a high $MgCl_2$ concentration also affects the band's number produced and decreases the intensity of certain bands.

The way primer recognizes complementary DNA sequence on DNA template used determined the number and intensity of DNA band produced after DNA amplification by PCR (Kalle et al. 2014). The results of DNA amplification do not always get the same band and intensity, wherein the amplified DNA band intensity at each primer is highly influenced by the concentration and purity of the DNA template (Panggeso et al. 2015). DNA template consists of phenolic compounds and polysaccharides, so the low concentration of DNA often results in dimmer amplified DNA bands (Sahu et al. 2012). Distribution of primer attachment sites at the DNA template and competition for primer attachment sites at the DNA template caused one fragment to be amplified in large numbers and the other fragments a little. The amplification process may be initiated in several regions, but only certain sets obtain detected as DNA bands after amplification (Probojati et al. 2019).

DNA band pattern resulting from the amplification indicates the presence of polymorphism. The number of amplified polymorphic bands is various. The more polymorphic bands are produced, the easier it is to observe variations. DNA band polymorphism can be influenced by individual variations number at population (Shukumsirichart 2018). In this study, high band polymorphism indicates high genetic diversity of shallot plants.

Cluster analysis of shallot

Cluster analysis and calculating the genetic distance between varieties resulted in a dendrogram showing the phylogenetic relationships between shallot varieties from several locations in North Sumatra. The dendrogram is shown in Figure 5.

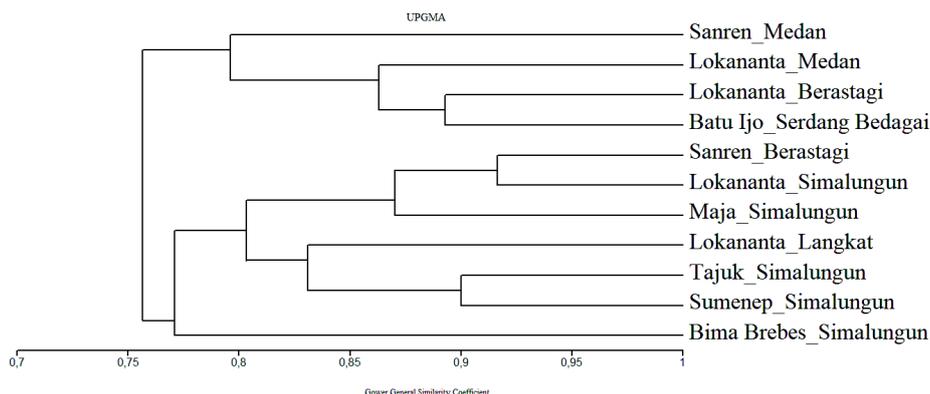


Figure 5. Dendrogram of genetic dissimilarity of shallot varieties origin several locations North Sumatra, Indonesia

The results of molecular analysis of 11 shallot varieties based on dendrogram using UPGMA showed that the coefficient of genetic dissimilarity in 11 varieties tested was 76%. At 76% dissimilarity coefficient, all varieties were divided into 2 groups. Group I consisted of 4 varieties, namely Sanren Medan, Lokananta Medan, Lokananta Berastagi, and Batu Ijo Serdang Bedagai while group II consisted of 7 varieties, namely Sanren Berastagi, Lokananta Simalungun, Maja Simalungun, Lokananta Langkat, Tajuk Simalungun, Sumenep Simalungun, and Bima Brebes Simalungun. The grouping was depend on the pattern of DNA bands formed and bands number that appeared during amplification using 4 RAPD primers (OPA-13, OPB-07, OPD-20, OMP-01).

Cluster analysis shows the grouping of varieties into two main groups. An interesting phenomenon from the analysis cluster result is grouping between the Lokananta variety originating from different locations and grouping with other varieties into one group. This indicates that there is genetic diversity in the Lokananta variety, which may be due to genetic recombination or mutation. Maia and Campos (2021) state that generally genetic diversity in population occurred cause of gene migration, mutation, and recombination. According to Govindaraj et al (2015), plant genetic diversity resources gives chance for plant breeders to develop and improve new cultivars with desired traits, namely yield potential and large seed (farmer-preferred traits) and pest or disease resistance and photosensitivity (breeders preferred traits). Genetic diversity knowledge support management of germplasm efficiently and parents' selection in crossing (Chinnappareddy et al. 2013).

RAPD markers are used as equipment for determining the extent of genetic diversity among shallot varieties. Genetic analysis of shallot by molecular markers supports understanding extent of varietal identification and genetic diversity. Cevik et al (2015) stated that molecular markers have evident to be powerful equipment for the evaluation of genetic diversity and the explanation of genetic relationships between inter and intraspecies. Further, Toker et al (2013), genetic relationships knowledge between shallot varieties can be used to introgression of beneficial traits into the cultigen in plant breeding programs.

In conclusion, analysis of genetic diversity of shallots based on RAPD markers showed that shallot varieties origin in several locations in North Sumatra had high genetic diversity with the presence of two main groups at a dissimilarity coefficient of 76%. This result can be used as a complement to morphological markers that are very useful in the genetic study of shallots for breeders to determine the parents of cross in produce new genetic combinations to effort shallot develop.

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