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Genotyping of Sumatera local variety of citrus using random amplified polymorphism DNA (RAPD) technique

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Abstract

Indonesia has local varieties of citrus that are no less than imported citrus, especially in terms of fruit freshness. However, people are more interested in the color of citrus peel so people prefer imported citrus to local citrus, especially in Sumatera. Therefore, it is necessary to carry out efforts to conserve and improve the characteristics of these citrus to improve their quality through plant breeding. This study aims to optimize DNA isolation methods for citrus fruit samples with Chelex-TE and to determine the genetic profile of local Sumatera citrus and imported citrus using the genotyping RAPD. The samples used were several local citrus in Sumatera (Citrus Siam Mountain Omeh, Citrus Madu, Citrus Keprok Maga, Citrus Keprok Brastepu and Citrus Pasaman) and imported Citrus (Citrus Sunkist, Citrus Clemengold, Citrus Murkot and Citrus Wokam). DNA was isolated using the 10% Chelex-TE method which was optimized for several parameters such as grain size, fruit skin and leaves. RAPD was performed using 10 RAPD primers. The results showed that the optimum 10% Chelex Chelex-TE isolation method was a sample size of 1 grain. The amplification of local Sumatran citrus and imported citrus using 10 single primers produced polymorphic bands. The value of jaccard's similarity indicates that the five samples of Sumatera local variety of citrus and imported citrus have high genetic variation. Indonesia has local varieties of citrus that are no less than imported citrus, especially in terms of fruit freshness. However, people are more interested in the color of citrus peel so people prefer imported citrus to local citrus, especially in Sumatera. Therefore, it is necessary to carry out efforts to conserve and improve the characteristics of these citrus to improve their quality through plant breeding. This study aims to optimize DNA isolation methods for citrus fruit samples with Chelex-TE and to determine the genetic profile of local Sumatera citrus and imported citrus using the *genotyping* RAPD. The samples used were several local citrus in Sumatera (Citrus Siam Mountain Omeh, Citrus Madu, Citrus Keprok Maga, Citrus Keprok Brastepu and Citrus Pasaman) and imported Citrus (Citrus Sunkist, Citrus Clemengold, Citrus Murkot and Citrus Wokam). DNA was isolated using the 10% Chelex-TE method which was optimized for several parameters such as grain size, fruit skin and leaves. RAPD was performed using 10 RAPD primers. The results showed that the optimum 10% Chelex Chelex-TE isolation method was a sample size of 1 grain. The amplification of local Sumatran citrus and imported citrus using 10 single primers produced polymorphic bands. The value of jaccard's similarity indicates that the five samples of Sumatera local variety of citrus and imported citrus have high genetic variation.

Introduction

Citrus fruits are generally favored by the world community, including Indonesia, especially during the COVID-19 pandemic, people's demand for citrus is increasing. This is because citrus fruit has a taste, aroma, freshness and a source of vitamins for the body. The demand for citrus fruits has increased followed by an increase in fruit imports. From fresh fruit import data in 2020 it reached 638,556.3, then in 2021 it increased to 775,422.4 (Statistics Central, 2022). The increase in the availability of imported citrus fruits has an effect on local consumption of citrus fruits.

Some types of citrus that are developing in Indonesia include citrus siam, citrus keprok, citrus citrus (citrus bali), citrus manis (citrus iris), limes, lemons, and kaffir limes. Citrus siam is the most popular local citrus in Indonesia, followed by tangerines which have a fresh sweet and sour taste (Balitjestro, 2022). According to data from the Central Statistics Agency, the productivity of citrus siam/keprok in Indonesia has increased from 2019. Data for 2019 shows that the total production of citrus siam/keprok produced from all over Indonesia is 2,444,518 tons and in 2020 it has increased to 2,593. 384 tons, but then in 2021 it will drop to 2,401,064 tons. Regions with productivity levels above 100,000 tonnes include East Java, Bali, North Sumatra, South Kalimantan, Riau, West Sumatra and West Kalimantan (Central Bureau of Statistics, 2022).

Indonesia has local varieties of citrus that are not inferior to imported citrus, especially in terms of fruit freshness, because local citrus harvested by farmers can be directly marketed and distributed to various regions. Consumers can benefit directly from the freshness of local citrus in a relatively short time compared to imported citrus. Imported citrus requires a long logistics process so that consumers do not get the benefits of fresh fruit. In addition, local citrus also have a lower price compared to imported citrus (Teme, 2006). The taste of local citrus is sweeter and according to consumer tastes and has more water content than imported citrus (Nafisah, 2013). However, people are more interested in citrus peel color so people prefer imported citrus to local citrus. When viewed in terms of color, most of the local citrus come from the Siamese type of citrus citrus. The fruit skin color is green or green mixed with yellow and there are still spots, while imported citrus have almost no spots on the fruit skin (Kiloes, 2012). Local citrus sometimes have a skin surface that is not so smooth, the color of the fruit is not uniform even though it is in the same type, so that when it is displayed it does not attract the attention of consumers (Sadeli & Utami, 2013).

Several local citrus varieties have not yet been widely developed and are even threatened with extinction, such as the citrus keprok maga from Mandailing Natal, North Sumatra. Citrus keprok maga is one of the mainstay fruit commodities in North Sumatra, because it has comparative and competitive advantages with other citrus cultivars or varieties. Based on Decree No. Release of varieties. 216/kpts/TP.240/4/2001, this type of citrus has the advantage of sweet fruit taste, flat round fruit shape, soft and easy to peel, and strong aroma. The marketing areas for this citrus apart from North Sumatra are also marketed to West Sumatra, Riau, Jakarta and even exported to Singapore. Even so, citrus keprok maga is one of the citrus varieties that is not widely known and has not been widely developed. Maga tangerines are still a garden plant in their area of origin in Puncak Sorik Marapi District, Mandailing Natal Regency (Ihsan *et al*., 2019).

The productivity of maga tangerines has decreased very significantly due to CVPD (*Citrus Vein Phloem Degeneration*) disease which caused a large number of deaths in 2009. As a result, local traditional markets are unable to meet consumer demand for these citrus (Ihsan *et al.*, 2019) . One of the factors that causes a large number of deaths from a disease in plants is the low genetic variation in the plant population. We certainly don't want this to happen to other local citrus, especially on the island of Sumatra.

Therefore, it is necessary to carry out efforts to conserve and improve the characteristics of these citrus to improve their quality through plant breeding. To carry out plant conservation and breeding, information on plant genetic variation in a population is needed. Pangaribuan (2018) has used genetic variation analysis of citrus keprok maga using *Single Sequence Repeat* (SSR) molecular markers, but the electroferogram data obtained did not show a polymorphic DNA profile because it was only visualized with agarose gel electrophoresis. Whereas SSR markers in the form of di-, tri, and

tetranucleotide repeats require high-resolution visualization such as polyacrylamide gel electrophoresis to obtain polymorphic DNA profiles.

Another technique that is more efficient and can be analyzed using agarose gel electrophoresis is *Random Amplified Polymorphic* DNA (RAPD) (Amina *et al*., 2022). RAPD molecular markers have been frequently used in analyzing the genetic variation of citrus plants in various publications. One example is the citrus siam gunung omeh, which originates from West Sumatra, which has already been analyzed for its genetic variation using the RAPD technique by Devy & Hardiyanto (2017) but only used 2 RAPD primer types. RAPD analysis with more primers is expected to provide more accurate results because it targets multiple locus.

Citrus classification based solely on morphological characters to support breeding programs is not the right choice. This is due to the high cross compatibility, mutation frequency, heterozygosity, and polyembryony. Differences in morphological characters that appear in different species are caused by genetic diversity. This genetic difference does not only appear between species, even within one species there is also a diversity of genes. variety even to accession (Aviarganugraha, 2012).

In addition, the process of characterizing morphological similarities takes a long time (Ruiz *et al*., 2000). Development of DNA markers based on polymerase chain reaction (PCR) provides an alternative approach in the process of identifying genotypes and studying interspecies polymorphism in one plant population (Baig & Bhagwat 2009; Hussein *et al.*, 2004, in Yulianti *et al.*, 2016).

Based on the description above, it is necessary to *genotyping* several types of local citrus varieties of Sumatra using RAPD molecular markers and comparing them with several types of imported citrus.

Material and Methods

Material

The tools used in this study include autoclaves. 0.5-10 µL micropipette, 10-100 µL micropipette and 100-100 µL micropipette, scalpel, tweezers, ruler, marker, micropestle rack microtube, spatula, scales, centrifuge, vortex, spindown, thermal shaker, water bath, biosafety cabinet, nanophotometer, spindown, PCR workstation, thermal cycler, ice box, electrophoresis chamber and power supply, agar mold, well comb, gel documentation and computer.

The main ingredients in this study were several Sumatera local variety of citrus (citrus keprok maga, citrus keprok brastepu, citrus madu, citrus pasaman and citrus siam gunung omeh) and imported citrus (citrus clemengold, citrus murcot, citrus wokam and citrus sunkist). Obtained from fruit stalls and supermarkets in Padang City, while citrus keprok maga and citrus keprok brastepu samples were obtained from tree owners in Mandailing Natal, North Sumatra. Other materials used include Chelex-100, TE buffer pH 8, Proteinase K, wide bore pipette tips, micropipette tips of various sizes, microtubes, PCR tubes, 70% alcohol, Bayclin, Trisbase, HCl, ethylene diamine tetra-acetic acid (EDTA), ultra pure distillated water (ddH_2acetic acid glacial, KOD-Fx Neo Toyobo, dNTPs, PCR buffer, 10 RAPD primers (Table 1), nuclease-free water, powder , TAE buffer, GelRed , Ladder DNA 1 KB, loading dye tissue and heat-resistant plastic.

Table 1. RAPD Primary Sequences Used (Fitriana, 2021)

No	Primary	Order of base
1	OPA-02	TGC CGA GCT G
2	OPA-04	AAT CGG GCT G
3	OPB-12	CCT TGA CGC A
4	OPC-15	GAC GGA TCA G
5	OPE-12	TTA TCG CCC C
6	OPE-14	TGC GGC TGA G
7	OPE-15	ACG CAC AAC C
8	OPJ-20	AAG CGC CCT C
9	OPM-09	GTC TTG CGG A
10	OPN-15	CAG CGA CTG T

Method

1) Isolation of Citrus DNA

DNA isolation was carried out using the 10% chelex-TE method. Prior to working in the laminar, the laminar is prepared and sterilized with 70% alcohol with wipes dryMicrotubes were prepared and the tube was labeled based on the sample isolated, the fruit sample was cut with scissors and the fruit pulp was taken with tweezers as much as 1 grain used for isolation. Chelex powder, tube's plate, microtube,*wide bore pipette tips*, 100-1000 uL micropipette, Tris-EDTA (TE) buffer, proteinase K and *thermal shaker*. The UV laminar was turned on for 10 minutes.

At the time of isolation, the *thermal shaker* was turned on and set the temperature to 56^{o} C. The isolation procedure begins by transferring the 10% chelex to a new microtube which has been labeled with a 10% sample micropipette with wide *bore pipette tips*, then 5 µL of proteinase K is added, then spindown. Make sure the 10% chelex and proteinase K are mixed. The sample of citrus fruit pulp is put into a microtube which already contains a mixture of 10% chelex and proteinase K. and make sure the sample is submerged in the solution. The samples were incubated for 24 hours in a *thermal shaker* 56^{o} C. After that, the samples were incubated again at 99^{o} C for 10 minutes.supernatant was transferred to a microtube which was labeled with the name of the sample and the date of isolation.

2) *Genotyping* RAPD

The components of the RAPD PCR reaction were put into a PCR tube consisting of the DNA polymerase enzyme KOD FX-Neo Toyobo with a total volume of 10 µl consisting of 5 µl PCR buffer, 2 µl dNTPs, 0.2 µl KOD FX, 0.4 µl RAPD primer concentration of 10 µM, 0.5 µl (50-100 ng) DNA template and diluted to 10 µl with the addition of nuclease-free water.

The PCR program used in this PCR reaction, according to Fitriyani (2022), starts with an initial denaturation at 94^{o} C for 3 minutes, then annealing for 45 seconds at 36^{o} C and extension for 45 seconds at 68^{o} C . This cycle is repeated a number of times. 15 times. After the annealing temperature reached 68° C, then proceed with the next 30 cycles consisting of denaturation at 94° C for 1 minute, annealing at 36° C for 45 seconds and elongation at 68° C for 2 minutes. The PCR process ended with the final elongation stage at 68° C for 5 minutes.

3) Electrophoresis

The PCR product mixture was electrophoresed using 1% agarose gel. PCR products were mixed with *loading dye* and *GelRed* before being added to the agarose gel wells. A 1 Kb DNA ladder was used as a DNA size marker. The DNA size marker used was a 1 Kb DNA ladder, with a composition of 3 µl of *ladder* plus 15 µl *red gel* and 1 µl *loading dye*, then put into the well. 5 µl of PCR results of DNA mixed with 5 µl of red gel and 1 µl of loading dye, then put each into the agarose well. Running

electrophoresis was carried out at 50 volts for 120 minutes (Fitriyani, 2022). DNA band detection was carried out using Gel Doc Uvitec.

4) Data Analysis

a) Scoring Analysis

Scoring was carried out based on the size of the DNA molecule which was read by the Uvitec software and standardized with a Ladder of 1 kb. If there is a DNA band, it is given a score of 1, whereas if there is no DNA band, it is given a score of 0. Thus, monomorphic bands (no band or locus variations) and polymorphic bands (there are observed band or loci variations) are obtained. The polymorphism value indicates the level of locus variation of each band that is amplified by each primer.

b) Analysis of Genetic Diversity with Jaccard Similarity

The Jaccard Similarity method is one of the methods used to calculate the similarity between two objects. Similarity measure method used to compare the similarity and diversity of 2 sample sets. The results of scoring DNA bands that have been made in excel form, then analyzed using Jaccard similarity analysis. The data were analyzed descriptively to determine the genetic variation with the level of polymorphism and genetic similarity seen in the amplified DNA bands.

Results and Discussion

Citrus DNA samples consisting of several Sumatera local variety of citrus (citrus keprok maga, citrus keprok brastepu, citrus madu, citrus pasaman and citrus siam gunung omeh) and imported citrus (citrus clemengold, citrus murcot, citrus wokam and citrus sunkist) were amplified using 10 RAPD primers. The sequence of primers can be seen in table 1. The amplification results of several Sumatera local variety of citrus and imported citrus using 10 single primers can be seen in Figure 1. The PCR products were electrophoresed using 1% agarose gel which was run for 40 minutes with a current of 100 volts, producing bands polymorphic. The results of Sumatera local variety of citrus and imported citrus that were amplified with ten primers were then analyzed using the Gel Analyzer application to see the DNA bands of each sample.

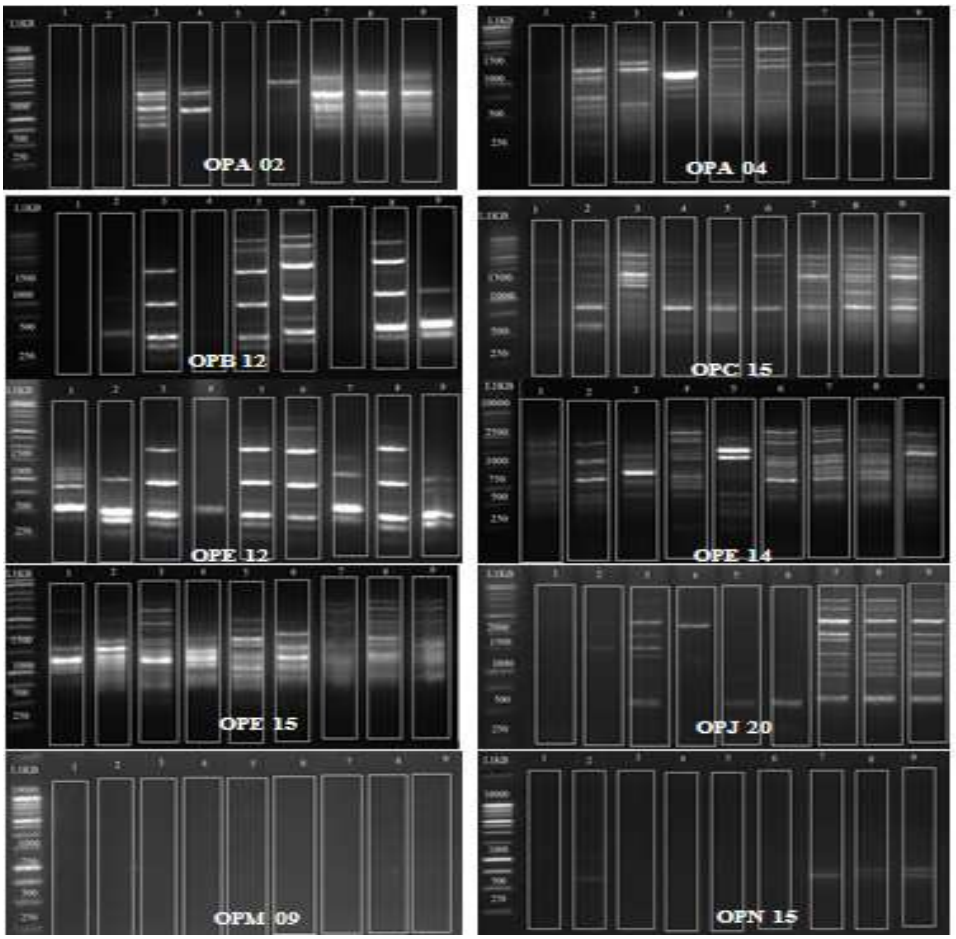

Figure 1. Results of electrophoresis of samples of Sumatera local variety of citrus and imported citrus with 10 primers

Note* L1KB = Ladder 1 KB, 1 = citrus clemengold, 2 = citrus murkot, 3 = citrus wokam, 4 = citrus sunkist, 5 = citrus keprok maga, 6 = citrus keprok brastepu, 7 = citrus madu, 8 = citrus pasaman dan 9 = citrus siam gunung omeh

Scoring analysis begins by counting the DNA bands and giving a score of 1 to the band that appears and a score of 0 to the band that does not appear. Based on Figure 1, the 10 RAPD primers used show the degree of polymorphism between Sumatera local variety of citrus and imported citrus.

It can be seen that there is genotyping of the DNA banding pattern that is formed in each sample which is well amplified by 5 RAPD primers, namely: OPA 04, OPC 15, OPE 12, OPE 14 and OPE 15. However, there are several RAPD primers (OPA 02, OPB 12 , OPJ 20, OPN 15 and OPM 09 which cannot amplify certain samples.

The scoring results on the amplified bands of each DNA sample that had been electrophoresed with ten RAPD primers were then analyzed for genetic distance based on the Jaccard similarity value generated from the PAST 4.08 program. If the Jaccard similarity value gets closer to 1 then the samples are more similar or identical. Conversely, if the Jaccard similarity value is close to 0, it indicates that there is high genetic variation between samples. Genetic variation can be known by looking at the genetic distance which shows how close the genetic kinship of the sample being tested is. The value of Jaccard's similarity or distance indices is shown in Table 2.

Table 2. Score Jaccard's similarity sample Sumatera local variety of citrus and imported citrus

	Similarity or Distance								
Sampel	**1**	**2**	**4**	**4**	**5**	**5**	**7**	**8**	**9**
1	1								
2	0,090	1							
3	0,082	0,154	1						
4	0,123	0,177	0,241	1					
5	0,111	0,087	0,165	0,136	1				
6	0,098	0,088	0,180	0,176	0,261	1			
7	0,065	0,093	0,201	0,180	0,168	0,171	1		
8	0,081	0,107	0,231	0,140	0,174	0,166	0,422	1	
9	0,081	0,097	0,187	0,163	0,198	0,178	0,366	0,37	1

Note* 1 = citrus clemengold, 2 = citrus murkot, 3 = citrus wokam, 4 = citrus sunkist, 5 = citrus keprok maga, 6 = citrus keprok brastepu, 7 = citrus madu, 8 = citrus pasaman dan 9 = citrus siam gunung omeh

Based on the data (Table 2), the sample Sumatera local variety of citrus and imported citrus used in this study are known that each sample has a large genetic distance between one sample and another because the similarity value is close to 0. Genetic closeness between the samples (Figure 1) . Based on this, citrus wokam sample with citrus sunkist still has close genetic diversity than the other samples, as well as the citrus madu sample with citrus pasaman, citrus keprok maga and citrus keprok brastepu.

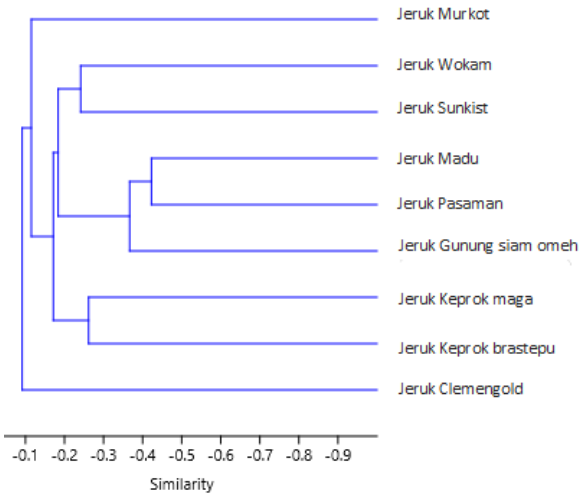

Figure 2. Dendrogram sample Sumatera local variety of citrus and imported citrus

Analysis of the genetic distance of Sumatera local variety of citrus, with the inter-species jaccarrd similarity coefficient between citrus Keprok maga with citrus Keprok brastepu ranging from 0.261, still have close genetic kinship compared to the other samples, as well as the citrus Madu with citrus pasaman. The jaccarrd similarity coefficient between species between citrus Madu with citrus pasaman. ranges from 0.422, between citrus Madu with citrus Siam gunung omeh. ranges from 0.366, between Pasaman oranges and Siam Gunung Omeh oranges ranges from 0.367. The range of genetic distance obtained in this study was higher for Honey oranges and Pasaman oranges with a range of 0.422.

Genotyping Random Amplified Polymorphic DNA (RAPD) is one of the most widely used PCR-based molecular markers for identification at the intraspecies and interspecies levels.Genotyping

RAPD has the ability to quickly detect polymorphisms in nucleotide segments in DNA using a single primer that has a random sequence of nucleotides. The primers used in this study were 10 RAPD primers. Genotyping RAPD effectively presents genetic information in the form of characteristic banding patterns that identify genotype differences based on differences in DNA bands that can be amplified with universal random primers (Azizah, 2009). RAPD has been widely used, including to analyze the genetic diversity of tangerines (Pessina et al., 2011), some citrus rootstocks and scions (Baig & Bhagwat., 2009; Hussein *et al*., 2004), C. limon (Siragusa *et al*., 2008, in Yulianti *et al*., 2016). RAPD was also carried out on Sankrar's *et al*., (2014) to detect the diversity of an citrus variety.

DNA amplification results fromgenotyping RAPD will then be electrophoresed which will then be used for scoring analysis and genetic distance analysis between local Sumatran citrus. Based on Fitriana's research (2021), electrophoresis of the optimum PCR product was carried out on 1% agarose gel with a current of 50 volts for 2 hours, but this was not successful so it was tried with a current of 100 volts for 40 minutes. The use of an electric current of 100 volts aims to separate the DNA bands properly and neatly, so it takes quite a long time. The success of the electrophoresis technique is influenced by the selection of the separating medium and other supporting conditions such as the amount of electric current and the length of time the electrophoresis takes. Agarose gel is a standard method for identifying and purifying DNA and RNA fragments. The band sizes to be separated in this study range from 250-3000 bp (Pratiwi, 2001). Therefore, the band to be separated has a large enough size, so the use of agarose gel electrophoresis is more suitable than polyacrylamide gel.

The RAPD PCR product will produce many DNA bands as shown in Figure 1. The primers are polymorphic because each produces a different number of DNA bands. Each primer produces a number of bands that appear to have varying base sizes and band intensities. The difference in the intensity of the DNA bands is influenced by the distribution of the primary attachment sites in the genome, the purity and concentration of the genome in the reaction. The number of bands produced by each primer depends on the distribution of homologous sites in the genome (Williams *et al*., 1990). The differences in banding patterns based on the number and size of the bands indicate the presence of a very complex plant genome (Grattapaglia *et al*., 2011). Electrophoresis results were scored by giving a value of 1 to the DNA band that appeared and a value of 0 to the band that did not appear. The scoring of the sample amplification results with each primer is shown in Appendix 1.

There were samples that were not amplified by several samples as shown in Figure 2. The primers did not adhere completely to the template DNA, which could be due to the incorrect concentration of the PCR-RAPD components. In addition, the quality of the template DNA also has an effect (Padmalatha & Prasad, 2006).

Genetic distance analysis in this study used analysisjaccard's similarity ordistance indeces. Based on the data shown in Table 2. it can be seen that the local Sumatran citrus and imported citrus have a distant similarity, that is, they have a similarity value close to 0. If the valuejaccard's similarity the closer to 0 the accession is more genetically different and has the potential to be used as parents in plant breeding (Fitriana, 2021). Diversity in plant populations has a very important meaning for the development of genetic resources needed in plant breeding (Karsinah *et al*., 2002). The level of individual diversity in the population describes the status of the species in nature.

Conclusion

Samples of Sumatera local variety of citrus and imported citrus have high genetic variation. None of the samples had genetic similarities. Citrus Wokam with citrus Sunkist still have a closer genetic relationship than the other samples, as well as citrus Madu with citrus Pasaman and citrus Keprok Maga with citrus Keprok Brastepu.

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