Genetic Similarity of Local Mandarin Accessions
(Citrus reticulata) Resulted from Endosperm Culture
According to ISSR and Microsatelite Markers

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Abstract. Citrus is the third most important fruit commodity in Indonesia, especially seedless citrus. One technique to get seedless citrus is by endosperm culture possessing triploidy sets of the chromosome. Plants that have triploidy sets of the chromosome will usually become sterile or seedless plants. This research aimed to 1) compare ratio of the length and width of the leaves between three types of local mandarin (Citrus reticulata) as mother plants and mandarin accessions grown from endosperm cultures, 2) identify mother plant and mandarin accessions from endosperm cultures with Inter Simple Sequence Repeats (ISSR) markers and 3) identify mother plant and mandarin accessions from endosperm cultures with Simple Sequence Repeats (SSR) markers. The research was conducted at Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI) Batu started from October 2018 until January 2019. The plants used in the study were Garut mandarin and seven accessions grown from endosperm cultures. Identification of plants with morphological markers has several disadvantages due to environmental factors. Identification with molecular markers is needed to complete the morphological information obtained. SSR markers are more suitable to be used in this study compared to ISSR markers because the results are in accordance with the objectives of this study, which is not expected to have high genetic variation. Identification with SSR molecular markers is more suitable because it showed low genetic diversity or no genetic diversity between mother plants and plants endosperm cultures when compared to ISSR markers. The desired change in the plants produced by endosperm cultures is only in ploidy properties.

Keywords: Mandarin; endosperm culture; genetic uniformity; and molecular marker.

1. Introduction

Citrus was the third most important fruit commodity in Indonesia after Mango when viewed from crop area and production amount per year. Indonesia’s citrus production in 2015 is estimated at 2.40 million tons and continues to increase by 2019 with a
production estimate of 2.77 million tons. The average increase of citrus production over the next five years is estimated at 3.64% per year [1]. It was a challenge as well as opportunities for citrus farmers, citrus entrepreneurs, and governments in an effort to increase citrus production [2]. Genetic diversity played an important role in plant breeding because information and understanding of genetic diversity can help the success of crop breeding programmes. The potential nature of a variety should be known in advance so that the diversity of germplasm can be utilized in the variety Improvement Program [3].

Triploid citrus plants are plants whose chromosome number is a multiple of three of their basic chromosomes resulted in a sterile condition. Breeding techniques used to produce triploid citrus plants are crossing techniques between diploid citrus plants with tetraploid citrus plants and endosperm culture techniques. Citrus plants grown from endosperm culture produce triploid citrus plants even though there are plants with other ploidy levels due to several factors. This technique able to produce seedless fruit that can increase economic value without any other character changes such as leaf shape. Local mandarin used in this research is jerukkeprokGarut (C. reticulata) which is famous for its noble fruit qualities. Not all morphological characteristics can be used as a solid character to distinguish one group from another because there are traits of plants that are strongly influenced by environmental changes that include nutrition, temperature, humidity, and climate [4]. Molecular approach is needed to determine the diversity of tangerines and differentiate groups of oranges in genetic terms and can be seen through the arrangement of DNA bands that appear.

This research aimed to compare ratio of the length and width of the leaves between three types of mandarin (C. reticulata) as mother plants and local mandarin accessions (jerukkeprokGarut) grown from endosperm cultures, identify mother plant and the local mandarin accessions from endosperm cultures with Inter Simple Sequence Repeats (ISSR) markers, and identify mother plant and the local mandarin accessions from endosperm cultures with Simple Sequence Repeats (SSR) markers.

2. Materials and Methods

The research was conducted at Plant Breeding Laboratory Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI) Batu started from October 2018 until January 2019.

Plant materials were the mother plant of the local mandarin (jerukkeprokGarut), GT 12, GT 110, GT 2, GT 113, GT 224, GT 109, and GT 6. The materials used were ISSR
primers: ISSR 4 (HVH(TCC))_5, and ISSR 5 ((TCC)_5RY), SSR primers: TAA15 (Forward sequence: GAAAGGTTACTTGACCAAGGC and Reverse sequence: CTTCCAGCTGCACAAGGC), and TAA41 (Forward sequence: AGGTCTACATTGCATTGTC and Reverse sequence: ACATGCAGTGCTATAATGAATG), distilled water, young leaves of the samples, concentrated HCl, concentrated NaOH, Dream taq (Thermo Fisher Scientific), loading dye, TBE 0.5X, agarose, Ethidium Bromide (EtBr), 70% alcohol, alcohol 96%, ddH₂O, TE 1X, chloroform isooamyl alcohol (Chisam), cold isopropanol, Sodium acetate, β-Merchaptoethanol, extraction buffer, NaCl, DNA marker (100 bp), CetylTrimethyl ammonium bromide (CTAB) solution, Tris-HCl buffer, Ethylene diamine solution tetraacetic acid (EDTA), and Polyvinyl Pyrulidone (PVP).

The tools used in identifying the genetic diversity of citrus are micro rulers, analytical scales, mortars, pastles, Eppendorf 2 µl tubes, Eppendorf 1.5 µl tubes, types (various sizes), micropipets, erlenmeyers, measuring cups, stirrers, hot plates, vortices, centrifuge, water bath, PCR plate, electrophoresis tank, UV Transilluminator, pH meter, autoclave, freezer, fridge, fume hood, gloves, masks, and sprayer.

The research process consisted of characterization of morphological marker, DNA isolation, quantitative and qualitative analysis of extracted DNA, PCR amplification, and data analysis. Total of seven plants grown from Garut mandarin endosperm culture and Garut mandarin mother plants were measured by the length and width of the leaves. The measurement results are calculated ratio. The ratio obtained can be analyzed using Statistical Package for the Social Sciences (SPSS) software. The results of the analysis of leaf length and width ratio are presented in the form of dendogram. DNA extraction and isolation use the CTAB method to obtain pure DNA. Quality test results using electrophoresis with 0.8% agarose and 0.5X TBE produce DNA with insignificant protein and RNA contamination, so that it can still be used in the PCR process. Quantity test results are performed by comparing DNA obtained with the ladder. The bands resulted from electrophoresis was scored and then analyzed using the Sequential Agglomerative Hierarchial and Nested-Unweighted Pair-Group Method with Arithmetic (SAHN-UPGMA) program in NTSYS software version 2.1. The analysis results were presented in the dendrogram.

3. Result and Discussion

3.1. Characterization Based on Morphological Markers
Morphological observations were made by measuring the length and width of the leaves of each citrus accession.

The ratio of leaf length showed that the Garut mother plant is in one group with the accession of GT 224 and GT 6 because they have the same leaf length and width ratio of 2. This showed that the accession of GT 224 and GT 6 has a kinship with the Garut mother plant. Other accessions of Garut mandarins do not indicate any kinship with the mother plant of Garut mandarin. Bani et al. stated that morphological markers can be influenced by environmental factors such as the temperature and nutrition [5]. Therefore, identification of molecular characters was needed in testing plant diversity to complement morphological information.

3.2. Genetic Diversity Based on Microsatellite Markers

Molecular markers were effective in genetic analysis and have been widely applied in plant breeding programs. The ISSR markers are PCR-based molecular markers, which amplify the area between two short nucleotide (microsatellite) replications [6]. The amount of DNA needed in the ISSR markers is 5-50 ng. The SSR or better known as microsatellite markers are molecular markers consisting of repetition units of 1-6 pairs of DNA bases with high variations. The use of SSR markers is relatively easy, very informative, locus specific, and able to read codominant traits, so that it can be applied for genetic diversity analysis [7].
Figure 2. Amplification of the mother plant and the accessions grown from the endosperm cultures by using ISSR 4. M = ladder, G= Mother plant of Garut, 1 = the mother plant (jerukkeprok Garut), 1-7 = the accessions grown from the endosperm cultures, 1= GT 12, 2= GT 110, 3= GT 2, 4= GT 113, 5= GT 224, 6= GT 109, 7= GT 6.

Visualization of PCR results from Garut ISSR 4 produced 7 bands, 3 polymorphic bands and 4 monomorphic bands. The percentage of polymorphism that formed was 42.85%. Visualization of the results of the PCR Garut tangerine ISSR 5 produces 7 polymorphism bands so that the percentage of polymorphism from the Garut tangerine ISSR 5 formed is 100%. Based on these results it can be concluded that the ISSR markers produced high levels of polymorphism. This is consistent with the opinion of Yasin et al. which stated that ISSR markers produced higher levels of polymorphism and showed a higher level of genetic diversity [8].

Figure 3. Amplification of the mother plant and the accessions grown from the endosperm cultures by using ISSR 5. M = ladder, G= Mother plant of Garut, 1 = the mother plant (jerukkeprok Garut), 1-7 = the accessions grown from the endosperm cultures, 1= GT 12, 2= GT 110, 3= GT 2, 4= GT 113, 5= GT 224, 6= GT 109, 7= GT 6.

Figure 4. Amplification of the mother plant and the accessions grown from the endosperm cultures by using SSR TAA15. M = ladder, G= Mother plant of
Garut, 1 = the mother plant (jerukkeprokGarut), 1-7 = the accessions grown from the endosperm cultures, 1= GT 12, 2= GT 110, 3= GT 2, 4= GT 113, 5= GT 224, 6= GT 109, 7= GT 6.

Figure 5. Amplification of the mother plant and the accessions grown from the endosperm cultures by using SSR TAA41. M = ladder, G= Mother plant of Garut, 1 = the mother plant (jerukkeprokGarut), 1-7 = the accessions grown from the endosperm cultures, 1= GT 12, 2= GT 110, 3= GT 2, 4= GT 113, 5= GT 224, 6= GT 109, 7= GT 6.

The results of PCR amplification and visualization of PCR results showed that the primary SSR TAA15 and TAA41 primers mostly produced monomorphic and uniform banding patterns. Suryanto states that samples that are not amplified with the primers used can be caused by several things such as primary mismatches, efficiency, and optimization of the PCR process [9].

The diversity between plant accessions can be seen from the number of bands and the thickness of DNA bands produced [8]. Genetic diversity or polymorphism has the meaning that there are two or more alleles at a locus in the population. Genetic diversity can be observed by observing through genetic characters with observed characteristics in the form of DNA, which is difficult to be influenced by the environment [10].

Molecular characterization results ISSR and SSR that have been presented show that molecular characterization with ISSR produces high genetic diversity. This can be seen based on the polymorphism band formed in the visualization of PCR results. Characterization with molecular SSR does not produce high genetic diversity. This is because the band formed in the visualization of PCR results is monomorphic.

Data analysis of PCR results visualization from ISSR and SSR primers was performed using the NTSYS 2.1 application. Tape scoring is done based on the formed band, the visible ribbon is given a value of 1 while the non-visible ribbon is given a value of 0.
Figure 6. Dendogram of Garut mandarins with primer ISSR. 39 = Mother Plant of Garut, 1 = GT 12, 2 = GT 110, 3 = GT 2, 4 = GT 113, 5 = GT 224, 6 = GT 109, 7 = GT 6.

Figure 7. Dendogram of Garut mandarins with primer SSR. 39 = Mother Plant Garut, 1 = GT 12, 2 = GT 110, 3 = GT 2, 4 = GT 113, 5 = GT 224, 6 = GT 109, 7 = GT 6.
Dendogram analysis on Garut mandarin with ISSR primers at a coefficient of 0.80 resulted in three groups. The first group is the mother plant of Garut, GT 2, GT 113, GT 224, and GT 109. The second group is the accession of GT 12 and GT 6. The third group is the accession of GT 110. Dendogram analysis results on Garut mandarins with SSR primers on the coefficient 0.83 there are two groups. The first group is the mother plant of Garut, GT 2, GT 113, GT 224, and GT 6. The second group is the accession of GT 12, GT 110 and GT 109.

Most of the mandarins produced by endosperm are in the same group as their parent plants. This shows that the mandarin plants resulting from endosperm culture produce low genetic diversity or do not produce genetic diversity. Desired changes in plants resulting from endosperm culture are only in the ploidy nature. Endosperm mandarins are expected to produce fruit without seeds (seedless). Kosmiatin and Ali explain the use of triploid plants to be important because they do not involve genetic modification, so they are more easily accepted by the market. The endosperm culture technique is the most efficient technique for getting seedless citrus. The regeneration system has been mastered and segregation is low so that the chances of getting the same seedless oranges with the parents are higher [11]. Based on the dendogram and the previous explanation, SSR markers are more appropriate to be used in this study because it is seen from the visualization of the results of the PCR and the dendogram results of data analysis show a low level of genetic diversity. ISSR markers are included in dominant inheritance, while SSR markers are included in codominant inheritance. Purnomo and Rejekistate that codominant markers data are generally more precise than dominant one [12].

4. Conclusion

a. Identification of plants with morphological markers has several weaknesses due to environmental factors. Identification with molecular markers is needed to complete the morphological information obtained and more accurate the results.

b. Identification with molecular markers SSR is more suitable because it shows a low genetic diversity or no genetic diversity between the mother plants and plants resulting from endosperm culture when compared with the ISSR markers.

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