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ISSR based analysis of genetic variability of plantlets culture of pineapple (*Ananas comosus* L.) from Sipahutar, North Sumatera, Indonesia

Fauziyah Harahap[1*], Annisa Afiva[1], Miftahul Jannah[2], Eko Prasetya[1]
[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan
Jl. Willem Iskandar Pasar V Medan Estate, Medan, Indonesia. 20221
*Email: fauziyahharahap@unimed.ac.id
[2]Department of Biology, Faculty of Sciences and Technology, Islamic University of As-syafiiyah
Jl. Jatiwaringin Raya No 12, East Jakarta, Indonesia. 17411

ABSTRACT. Sipahutar pineapple is very popular in North Sumatra, because of the distinctive sweet taste and normal water content. Furthermore, it is large in size and has a yellow fruit skin color with greenish tips. However, the problem with Sipahutar pineapple production is the limited amount of good quality seeds. The utilization of *in vitro* culture techniques on pineapples allows the emergence of somaclonal variations, especially in plantlets that have undergone the subculture stage. This somaclonal variation is one of the problems in commercial seedling production, therefore, it is necessary to conduct an initial examination using the inter simple sequence repeat (ISSR) molecular marker. This study aimed to determine the genetic and primary stability of ISSR, which can be used in pineapple plants native to Sipahutar. The methods used include sample preparation, DNA isolation, primer optimization, ISSR primer amplification by PCR method, and electrophoresis. Furthermore, a total of 15 samples were amplified with six ISSR primers, and the data were analyzed by cluster method using the NTSYS-PC software. The final result was visualized in a dendrogram and analysis of diversity was conducted using GenAlex. The results showed that the level of genetic variability of the Sipahutar pineapple, which has undergone *in vitro* culture using six ISSR molecular markers was 76-97%. Meanwhile, the genetic variability level of the native to Sipahutar pineapple can be influenced by the long culture period and the use of N^6-benzyladenine. The primers can be used to observe the genetic variability, except for ISSR 25 with a PIC value of 0.000.

Keywords: *Ananas comosus* L.; *in vitro* culture; N^6-benzyladenine; PIC; Sipahutar

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INTRODUCTION

Pineapple is one type of fruit with a high level of production in Indonesia (Pusat Data dan Sistem Informasi, 2016). One of the areas that play an essential role in pineapple production is Sipahutar District, North Sumatra (Badan Pusat Statistik Kabupaten Tapanuli Utara, 2019). They have a distinctive sweet taste, not too much water content, large fruit, and yellow fruit skin with greenish tips (Harahap *et al*., 2019). However, the production of Sipahutar pineapple has decreased since 2015 (Badan Pusat Statistik Kabupaten Tapanuli Utara, 2019) due to the limited amount of quality seed (Harahap *et al.*, 2015).

In the Sipahutar area, the propagation technique conducted uses vegetative parts in crowns, slip and sucker with relatively long growth times ranging from 12-24 months as our previous studies (Harahap *et al.*, 2019). In addition, seedlings from crowns require a long time to grow, seeds from slip and sucker have many leaves, uneven fruit maturity, and challenging to plant (Hossain, 2016; Reinhardt *et al.*, 2018). Utilizing *in vitro* culture techniques can solve the seed production, evidenced by our previous studies on the development of Sipahutar pineapple seedlings and has produced plantlets with similar results (Insani *et al.*, 2018; Fernando *et al.*, 2020; Harahap *et al.*, 2020).

The use of *in vitro* culture techniques on pineapple plants can allow the emergence of somaclonal variations, therefore, the seed produced should be uniform (da Silva *et al*., 2016; Kohpaii *et al*., 2017). One of the steps taken is to observe the plantlets using molecular markers. The molecular markers used in the genetic stability analysis are RAPD, AFLP, and ISSR (Scherer *et al*., 2015; da Silva *et al*., 2016;

Kohpaii *et al*., 2017). This study used ISSR molecular markers.

The ISSR marker has a high degree of reproducibility over RAPD markers (Mei *et al*., 2015), less costly use and the absence to use radioactivity in AFLP markers (Costa *et al*., 2016). Furthermore, it is used to observe genetic diversity, phylogenic studies, gene tagging, genome mapping, and observe the evolution of various species (Farajpour *et al*., 2012; Lu *et al*., 2012). ISSR marker analyzes the genetic stability of various *in vitro* crops (Pathak & Dhawan, 2012; Poerba *et al*., 2012; Ghorbanpour & Khadivi-Khub, 2015; Saha *et al*., 2016; Jogam *et al*., 2020; Kamińska *et al*., 2020; Rohela *et al*., 2020).

This study aims to determine the genetic stability of Sipahutar pineapple from *in vitro* culture with plants from the plantations to avoid somaclonal variations. It is possible to commercially produce *in vitro* culture seed pineapple to improve the economy of the community in Sipahutar District.

MATERIALS AND METHODS

This study was conducted at the YAHDI Medan-Marelan Plant Tissue Culture Laboratory for the propagation of pineapple plants by *in vitro* culture and The Biocell and Molecular Laboratory at Universitas Negeri Medan. Furthermore, a total of 15 mother and culture samples were used. The mother plant leaf samples came from the Lumban Matio hamlet, Onan Runggu I Village, Sipahutar District, North Tapanuli Regency, North Sumatra Province (L1 and L2). Meanwhile, the pineapple leaf samples cultured *in vitro* were planted for six months with one subculture. The first was conducted for three months on Murashige and Skoog (MS) media enriched with vitamin 2 ppm and N^6-benzyladenine (BA) 2 ppm (M1-M6), as well as MS medium enriched with BA 2 ppm (N1-N7). Subculturing was conducted but on MS media supplemented with vitamin 2 ppm and BA 2 ppm with a subculture frequency of three months.

The DNA from the native to Sipahutar pineapple used was extracted from leaves frozen at -20°C using a plant DNA isolation kit (Geneaid™, GEC150). Furthermore, the DNA isolation process was conducted according to the protocol of the kit, followed by primary optimization. This stage was carried out to determine the type of primer and annealing temperature that may amplify the DNA of the Sipahutar pineapple.

Furthermore, the DNA amplification stage was conducted using selected primers and the reaction was carried out on a 25 µl volume consisting of 2.5 µl primer, 2.5 µl DNA samples, 7.5 µl nuclease-free water, and 12.5 µl KIT PCR (MyFi™ Mix Bioline ® Master Mix). In addition, it was conducted using a gradient PCR (SensoQuest Gradient Labcycler) tool which was programmed to pre-denature at 97°C for 4 min in 1 cycle. The denaturation was performed in 37 consecutive cycles at 95°C for 45 s while the annealing stage was carried out at the melting temperature (Tm) of each primer (Table 1) for 50 s, extension at 72°C for 1 min. This was then followed by post extensions at 72ºC for 10 min and ended with cooling at 4°C.

Table 1. List of six primers used in the study with varying melting temperatures.

Primer	Sequence (5'-3')	Annealing temp. (°C)
ISSR 11	GAGAGAGAGAGAGAC	46.8
ISSR 25	CGTGTGTGTGTGTGT	53.0
ISSR 27	CYGTGTGTGTGTGTGT	54.0
UBC Primer 813	CTCTCTCTCTCTCTT	45.7
UBC Primer 814	CTCTCTCTCTCTCTA	44.7
UBC Primer 815	CTCTCTCTCTCTCTG	46.8

The results of DNA amplification were followed by separation of DNA bands by electrophoresis method using 1% agarose gel (1 g agarose, 100 ml TBE buffer, 5 µL red gel strain). In each well of agarose gel, 1.5 µl of loading dye was inserted with 8.5 µl of amplified DNA and was ran in a TBE solution for 75 min with a strong current of 70 volts. Furthermore, the electrophoresis results were observed using a UV transilluminator (Biostep GmbH) and documented using a camera. The standard DNA size used was a 100 bp DNA

ladder (Geneaid™, GEC150) to determine the size of the amplified band.

The appearance of the DNA band was read using the Gen Pro Analyzer 3.1 application and was translated into binary data with a score of 1 (present), and 0 (absent and for questionable bands). Meanwhile, genetic stability and diversity were analyzed using Similarity for Qualitative Data (SIMQUAL), followed by cluster analysis using the SAHN program and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and the NTSYS-PC 2.1 software (Rohlf, 2000). The results obtained were dendrogram data (phylogenetic tree), and the calculation of the average and an effective number of alleles, heterozygosity, Shannon information index, and number and percentage of polymorphic loci were analyzed using the GenAlex 6.5 program (Peakall & Smouse, 2012).

RESULTS AND DISCUSSION

The results of the visualization (Fig. 1.) showed that the primers used were well amplified, and out of the six used, 192 bands of DNA were identified. In total, 23 bands of polymorphic loci were obtained from the 192 amplified bands. Also, the amplicon size for each primer was different but ranged from 1000-300 bp.

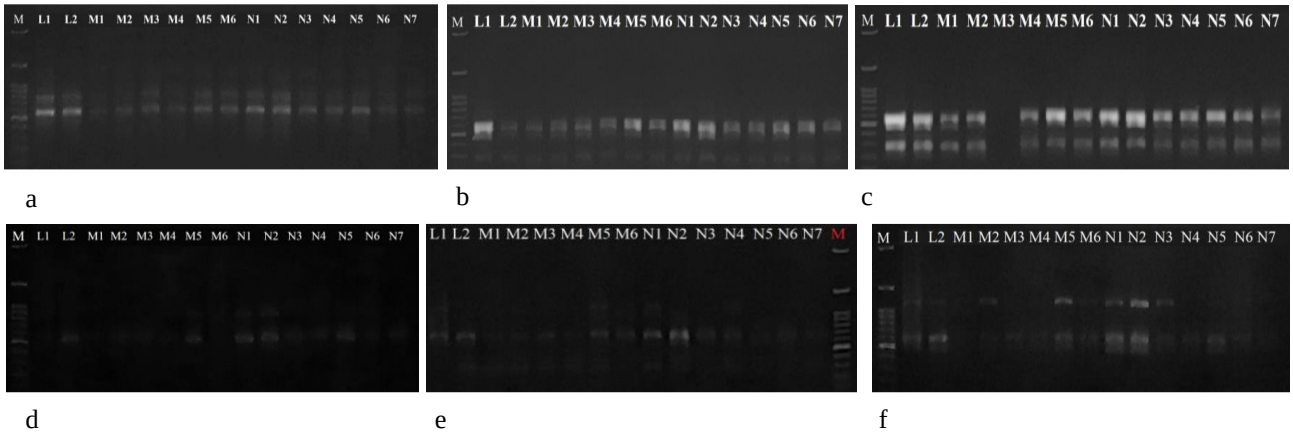

Fig. 1. ISSR primary amplification visualization results in research samples: a. ISSR 11; b. ISSR 25; c. ISSR 27; d. UBC Primer 813; e. UBC Primer 814; f. UBC Primer 815; L1-L2. Samples of pineapple mother plants from plantations in the Sipahutar area; M1-M6 and N1-N7. Samples of pineapple plantlets from Sipahutar; M. Marker 100 bp.

The number of DNA bands and amplicon size in each ISSR primer ranged from 16 (UBC Primer 813) to 60 (ISSR 25) are shown in Table 2.

Table 2. The number of bands and amplicon size of each ISSR primary amplification.

Primer	Result of DNA band amplification	Number of bands	Amplicon size (bp)
ISSR 11	There is an amplified DNA band	29	800, 600, 400
ISSR 25	There is an amplified DNA band	60	600, 550, 500, 300
ISSR 27	There is an amplified DNA band	34	600, 300
UBC Primer 813	There is an amplified DNA band	16	1000, 500
UBC Primer 814	There is an amplified DNA band	27	1000, 700, 300
UBC Primer 815	There is an amplified DNA band	26	1000, 600, 500

The number of effective alleles is measured to identify the genetic variation in a population through interactions between alleles per locus. Generally, the number observed was greater than the number of effective alleles. The smaller the difference between the value of the number of effective and observed alleles, the more evenly the distribution in a population (Yuan *et al*., 2007). This study showed that the highest effective allele value was 1.731 in primary UBC Primary 815. The lowest detected effective allele was in primary ISSR 25 of 1.000. Heterozygous genotypes is one of the parameters used to determine the level of genetic variation in a population (Sim *et al.*, 2012; Lohmueller, 2014). The highest value

was shown by primary UBC Primary 815 of 0.415. Meanwhile, the lowest was shown by ISSR 25 primers of 0.000 and the average heterozygosity value for all primers was 0.265. Furthermore, the molecular markers have a high level of polymorphism when the PIC value = > 0.5, moderate when the PIC = 0.25 < PIC < 0.5, and low when the PIC value = < 0.25 (Botstein *et al*., 1980). In this study, the PIC values obtained ranged from 0.000-0.497 with an average value of 0.382. Therefore, the ISSR primers used produce low to moderate polymorphic alleles. In this study, the index ranged from 0.000 to 0.603 with the highest shown by primary UBC Primary 815 with a value of 0.603 and the lowest indicated by primary ISSR 25 with a value of 0.000. Table 3 showed that there is no correlation between the number of effective alleles, heterozygosity, PIC, and the Shanon information index. This is consistent with the study of Monfared *et al*. (2018), Ismail *et al*. (2019), and Kimaro *et al*. (2020).

Table 3. Analysis of genetic variability of pineapples from the Sipaphutar region using ISSR markers of the GenAlex 6.5 program.

Primer	Number of effective alleles	Heterozygosity	Polymorphic information content (PIC)	Shannon's information index
ISSR 11	1.481	0.279	0.458	0.405
ISSR 25	1.000	0.000	0.000	0.000
ISSR 27	1.402	0.243	0.369	0.388
UBC Primer 813	1.556	0.356	0.497	0.540
UBC Primer 814	1.544	0.296	0.480	0.424
UBC Primer 815	1.731	0.415	0.487	0.603
Average	1.452	0.265	0.382	0.393

The scoring data obtained from DNA amplification with six ISSR markers were used for cluster analysis using the NTSYS version 2.1 program. Furthermore, the cluster analysis was performed by classifying the resulting genotypes into a dendrogram. The results showed that there are genetic differences found in the samples since the genes in each plant were unstable. Fig. 2 showed the level of similarity of each study sample.

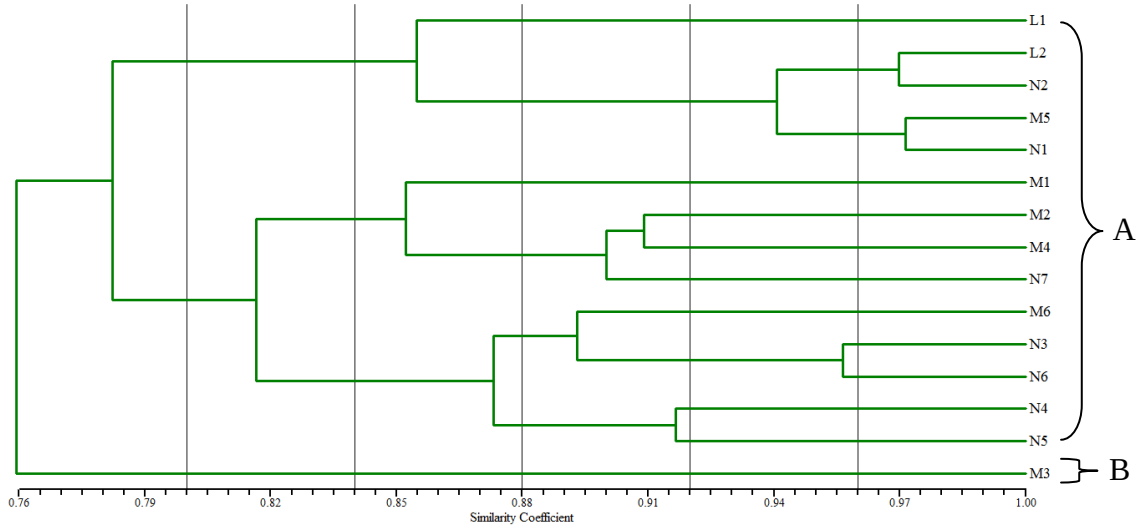

Fig. 2. Dendrogram of the diversity relationship between research samples analyzed with NTSYS 2.1 software: L1-L2. Samples of pineapple mother plants from plantations in the Sipahutar area; M1-M6 and N1-N7. Samples of pineapple plantlets from Sipahutar.

The results of the scoring analysis for each primer showed that the dendrogram of genetic diversity of pineapple plants from Sipahutar which was the sample of this study (Fig. 2) has a similarity level of 0.76-0.97. The genetic diversity was divided into two main clusters with a similarity coefficient of 0.76, namely group A and B. Group A consisted of 14 samples consisting of two parent plants and 12 pineapple plantlets from *in vitro* culture. Group B consisted of one sample of pineapple plantlets from *in vitro* culture. Furthermore, group A was divided into two sub-clusters, I and II, with a similarity level of 0.785. Sub-cluster I consists of parent plants (L1 and L2) and pineapple plantlets (N1, N2, and M5) with a similarity level of 0.87. Sub-cluster II consists of pineapple plantlets (M1, M2, M4, M6, N3, N4, N5, N6, and N7) with a similarity level of 0.82.

Based on the results of the dendrogram, there are genetic differences in each sample. In line with Roostika *et al*., (2015) study, where the genetic stability of pineapple plants made in 3 different treatments had a similarity level from 0.32-0.93. One of the factors that can influence the genetic differences between the samples is the quality of the intensity of the DNA bands electrophoresis results. The intensity of DNA bands between samples can be affected by the purity and concentration of isolated DNA, and the distribution of the primary attachment sites. Furthermore, it is also affected by the competition for places where the primer is attached to the isolated DNA and causes one fragment to be amplified in multiplexed and others not to be amplified at all (Bilodeau *et al*., 2012). Explant sources originating from meristematic tissue, such as the pericycle, procambium, and cambium parts of the plants, can reduce the likelihood of variation. Meanwhile, highly differentiated tissues such as roots, leaves, stems, and shoots are more likely to develop variations (Bairu *et al*., 2011; Krishna *et al*., 2016). In this study, the plantlets used were sourced from shoots, in the form of differentiated tissue.

The addition of growth regulators such as auxin and cytokinin can cause variations. BA concentrations of 3 mg/L can cause somaclonal variations of pineapple plants cultured *in vitro* with mother plants (Kohpaii *et al*., 2017). Bairu *et al*. (2011) stated that BA concentrations around 15-30 mg/L can cause genetic instability in banana and rice plants by increasing the number of chromosomes and stimulating morphogenesis through the destruction of the cell cycle. There was genetic instability in the plantlets produced by pineapples and the parent plant because the growth medium for the explant used BA growth regulators with a concentration of 2 ppm.

The length of culture time and the frequency of subcultures also affect the genetic stability of the plants cultured *in vitro*. This is because the higher the subculture frequency, the longer the duration of the plant cells being exposed to various factors, which may cause mutations and genetic diversity (Peng *et al*., 2015). Da Silva *et al*. (2016) and Kohpaii *et al*. (2017) stated that there was genetic diversity of pineapple plants grown from *in vitro* culture for more than eight months with multiple subcultures. In this study, the length of time for plantlet culture was 12 months, with two subcultures on the same media, namely MS with the addition of vitamin 2 ppm and BA 2 ppm for samples B1, B2, B3, B4, B5, and B6. Meanwhile, samples B7, B8, B9, B10, B11, B12, and B13 were grown for 6 months on MS media with 2 ppm of BA. The samples were then transferred to MS medium with 2 ppm of vitamin and 2 ppm of BA for six months.

CONCLUSION

The level of genetic stability of Sipahutar pineapple which has undergone *in vitro* culture using six ISSR molecular markers is 76-97%. The level of genetic stability of the pineapple from Sipahutar can be influenced by the long culture period, which is 12 months, and the use of growth media of MS with a concentration of 20 ml/L of BA. Furthermore, the primers can be used in observing the genetic stability of the Sipahutar pineapple plants resulting from *in vitro* culture. The best primer used was UBC 813 with a PIC value of 0.497. Meanwhile, the ISSR 25 primer was not suitable for observing the genetic stability of pineapple plants from

Sipahutar because of the low PIC value of 0.000.

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