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Short Communication: Genetic similarity analysis of in vitro cultivated pineapple (*Ananas comosus*) from Sipahutar, North Sumatra, Indonesia using ISSR markers

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Abstract. *Harahap F, Nusyirwan N, Afiva A, Prasetya E, Suriani C, Hasibuan RFM, Poerwanto R. 2022. Short Communication: Genetic similarity analysis of in vitro cultivated pineapple (Ananas comosus) from Sipahutar, North Sumatra, Indonesia using ISSR markers. Biodiversitas 23: 5623-5628.* Sipahutar pineapple from North Sumatra, Indonesia, is considered difficult to breed, resulting in a lack of seed availability. However, tissue culture allows the emergence of somaclonal variations, especially in plantlets undergoing several subculture stages. This study aimed to identify somaclonal variations in Sipahutar pineapple seedlings generated from in vitro culture using Inter Simple Sequence Repeat (ISSR) markers. Fifteen accessions were used in this study consisting of Sipahutar pineapple grown in its original environment, a laboratory environment, acclimatized, and in vitro cultured. Total of 10 primers, of which seven primers detected polymorphisms among the population. This study resulted in a low genetic similarity among the Sipahutar pineapple population. The genetic diversity of Sipahutar pineapple plants propagated by in vitro culture had a similarity level of 75%-94%, showing somaclonal variations in vitro culture. The genetic similarity of pineapple accessions was divided into two main clusters, namely cluster A consisted of 11 accessions and cluster B consisted of 4 accessions. Cluster separation does not correlate to the source of plant materials studied.

Keywords: ISSR, pineapple, polymorphisms, Sipahutar, somaclonal

INTRODUCTION

Pineapple (*Ananas comosus* (L.) Merr.) is a tropical and subtropical plant indigenous to southeast Brazil and began to be cultivated in Indonesia in the 16th century (Paull and Duarte 2011; Rosmaina et al. 2021). It is the leading commodity fruit crop with abundant production in Indonesia. North Sumatra is one of the areas in Indonesia that contributes a high production of pineapple, up to 15% (Lubis et al. 2014; BPS 2019). One of the most renowned pineapples in North Sumatra is from the Sipahutar area. This pineapple is famous for its distinctive sweetness, firmness, large size, and yellow skin color with a greenish tip (Harahap et al. 2019a). Pineapple production in Sipahutar has decreased gradually because of the limited availability of quality seedlings. Propagation is generally done using vegetative parts in the form of crowns, suckers, and slips. However, the propagation technique is ineffective because the growth time is 12-24 months (Harahap et al. 2018). Although seedlings from the crown result in even growth, they take a long time to grow, whereas seedlings from the slip and sucker produce a large number of leaves but are difficult to plant, and the maturity of the fruit is uneven (Ardisela 2010).

The problem of seedling production can be solved by utilizing in vitro culture. The method can produce superior seeds while maintaining their uniform form in a short period (Harahap 2011). Many studies on the development of pineapple plants from Sipahutar with in vitro culture have been reported, such as Harahap et al. (2013, 2015, 2018, 2019b, 2020a, 2020b), Harahap and Nusyirwan (2014), Sinulingga and Harahap (2014), Hasanah et al. (2018), Insani et al. (2018), and Fernando et al. (2020). However, in vitro culture in plants can allow the emergence of somaclonal variations, especially in plantlets that have undergone subculture stages. In general, factors that can cause somaclonal variations are the composition of chemicals in the media, the length of the callus growth phase, the period of subcultures, the regulatory substances used, the sources of explants, and other physical treatments (Harahap 2011).

Given the possibility of somaclonal variations, pineapple seedlings cannot be commercially produced. There is an urgency to examine this matter to find an effective way to propagate the seedlings. One method to detect somaclonal variations is observing plantlets using Inter Simple Sequence Repeat (ISSR) as molecular markers. The markers are widely used to evaluate genetic similarities in various in vitro culture crops, including *Plantago major* (Ghorbanpour and Khadivi-Khub 2015), *Manihot esculenta* (Vidal et al. 2015), *A. comosus* (Da

Silva et al. 2016; Kohpaii et al. 2017), *Citrus paradisi* (Yulianti et al. 2017), *Cucumis m* (Raji et al. 2018), *Vitis vinifera* (Indaryani et al. 2019), *Artemisia vulgaris* (Jogam et al. 2020), *Taraxacum pieninicum* (Kamińska et al. 2020), and *Morus alba* (Rohela et al. 20). ISSR marker has a high level of producibility and a high level of polymorphism (Reddy et al. 2002). Therefore, this study aimed to analyze the genetic similarity of pineapple plants from Sipahutar cultured in vitro by using the ISSR markers.

MATERIALS AND METHODS

Plant materials

Pineapple leaf samples from Sipahutar, North Sumatra, Indonesia were used as parental plants, which were replanted at the Education Foundation of YAHDI Tissue Culture Laboratory. For comparison, plants were cultured in different environments, along with the acclimatized plants and plantlets from in vitro culture in MS medium with 2 ppm of vitamins (nicotinic acid, pyridoxine, thiamine, and glycine) and 2 ppm of BA. Fifteen accessions used in this study consisting of: (a) the Sipahutar pineapple grown in its original environment (2 accessions: A1 and A2); (b) the Sipahutar pineapple grown in a laboratory environment (2 accessions: B1 and B2); (c) acclimatized Sipahutar pineapples (2 accessions: C1 and C2); and (d) in vitro cultured Sipahutar pineapples (9 accessions: D1-D9). The research was conducted at the Education Foundation of YAHDI Tissue Culture Laboratory and the Bio Cell Molecular Laboratory, Universitas Negeri Medan, Indonesia, from December 2019 to August 2020.

Procedures

DNA extraction and amplification

DNA samples from pineapple plants were extracted from frozen young leaves at -20°C using the Geneaid Genomic DNA Mini Kit (Plant). Optimization and the primary selection stage were used in this study directly from the DNA isolation stage (Table 1).

Table 1. Sequences and annealing temperatures of ISSR primers

Primer	Primer sequence (5'-3')	Annealing temp. (°C)
ISSR 11	GAG AGA GAG AGA GAG AC	46.8
ISSR 25	CGT GTG TGT GTG TGT GT	53.0
ISSR 27	CYG TGT GTG TGT GTG TGT	54.0
UBC 813	CTC TCT CTC TCT CTC TT	45.7
UBC 814	CTC TCT CTC TCT CTC TA	44.7
UBC 815	CTC TCT CTC TCT CTC TG	46.8
UBC 819	GTG TGT GTG TGT GTG TA	49.4
UBC 821	GTG TGT GTG TGT GTG TT	50.3
ISSRED-17	GAC GAC GAC GAC GAC	52.6
ISSRED-14	-	-

DNA amplification reactions were performed with 25 µL solution consisting of 2.5 µL ISSR primer, 2.5 µL sample DNA, 7.5 µL aquabides, and 12.5 µL PCR Kit. DNA amplification was performed on a PCR gradient tool, Labcycler, programmed to start at 97°C for 4 minutes. Furthermore, each of the 37 consecutive cycles was denatured at 95°C for 45 seconds, annealing at each primary temperature for 50 seconds, and extension at 72°C for 1 minute, followed by post-extension at 72°C for 10 minutes and cooling at 4°C for 10 minutes.

DNA electrophoresis

DNA amplification was followed by separating the DNA band by electrophoresis on 1% agarose gel. In each agarose gel was inserted 1.5 µL of loading dye with 8.5 µL of amplified DNA. The gel was soaked in 0.2x TBE solution for 75 minutes at 70 volts. Electrophoresis results were observed with the gel documentation (Syngene InGenius3). The standard DNA size used was 100 bp of the DNA ladder to determine the size of the DNA band.

Data analysis

The appearance of DNA bands was read and scored using the Gen Pro Analyzer 3.1 software. The scores were 1 (present) and 0 (absent or doubtful existence). Genetic similarity and diversity were analyzed with Similarity Qualitative (SIMQUAL); then, cluster analysis was performed with the Sequential Agglomerative Hierarchical Nested (SAHN) program using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with NTSYS-PC 2.1 software (Rohlf 2000). The result was represented as dendrogram data.

RESULTS AND DISCUSSION

DNA polymorphisms

The electrophoresis results and band size of each primer are displayed in Figure 1 and Table 2.

Table 2. ISSR primer amplification of polymorphic marker selection in pineapple plants

Primer identification	Number of amplification bands	Amplicon size (bp)
ISSR 11	26	900, 800, 600
ISSR 25	56	600, 550, 500, 300
ISSR 27	55	600, 550, 500, 100
UBC Primer 813	36	800, 600, 500
UBC Primer 814	21	1000, 600, 300
UBC Primer 815	42	1000, 600, 500, 400
UBC Primer 819	0	0
UBC Primer 821	0	0
ISSRED-14	0	0
ISSRED-17	54	1000, 800, 600, 500, 400, 300

Note: The total number of clearly identified DNA bands was 290. The number of polymorphic loci of all primers used was 30 bands. The amplicon size in each primer ranged from 1000 to 100 bp. The number of DNA bands on each ISSR primer ranged from 26 (ISSR 11) to 56 (ISSR 25)

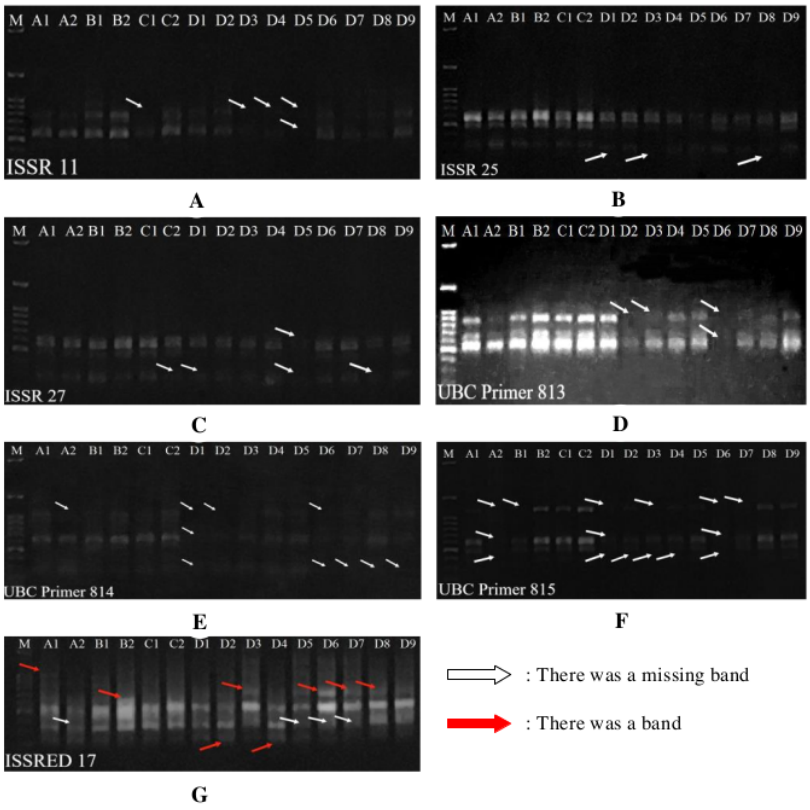

Figure 1. The ISSR profile of the in vitro cultured pineapple from Sipahutar, North Sumatra, Indonesia using primer: A. ISSR 11; B. ISSR 25; C. ISSR 27; D. UBC Primer 813; E. UBC Primer 814; F. UBC Primer 815; and G. ISSRED 17. M: Marker ladder 100 bp; A1-A2: parental plants; B1-B2: pineapple with different environmental growth conditions; C1-C2: pineapple that acclimatized; and D1-D9: plantlets from in vitro culture

Invisible bands were seen at 800 bp of 4 and 600 bp of 1 in Figure 1A. Figure 1B: At 300 bp, 3 invisible bands were found. Invisible band was found in Figure 1C at 600 bp of 1 and 100 bp of 4. Figure 1D shows the detection of invisible bands at 800 bp of 3 and 500 bp of 1. Invisible bands were found in Figure 1E at 1000 bp of 4, 600 bp of 1, and 300 bp of 5. Invisible bands were seen at 1 31 bp of 6, 600 bp of 3, and 400 bp of 5 in Figure 1F. The total number of clearly identified DNA bands was 290. The number of polymorphic loci of all primers used was 30 bands. The 5mplicon size in each primer ranged from 1000 to 100 bp. The number of DNA bands on each ISSR primer ranged from 26 (ISSR 11) to 56 (ISSR 25) (Table 2).

The electrophoresis results of the primers showed several differences in amplified bands in the parental plant sample with the comparison sample (Figure 1). This showed that the genetics in each plant was unstable. The visualization of DNA amplification on ISSRED-17 was unclear. This can be caused by the low number of copies of DNA fragments that can affect the loci to be analyzed (Williams et al. 1993). Primers of UBC 819, UBC 821, and ISSRED-14 could not be amplified because the primers' base sequence was not complementary to the base sequence in the DNA of pineapples from Sipahutar (Grubaugh et al. 2019).

Genetic similarity analysis

Analysis of each primer resulted in a dendrogram of the genetic diversity of pineapple plants (Figure 2), which had a similarity level of 75%-94%. The genetic diversity of pineapple accessions was divided into 2 main clusters at a similarity coefficient of 75%, namely cluster A and cluster B. Cluster A consisted of 11 accessions in the form of one parental plant accession, two comparison plants growing in environmental conditions, two comparison plant accessions from in vitro culture which was acclimatized, and six comparison pineapple plantlets from in vitro culture. Group B consisted of 4 accessions: one accession of the parental plant and 3 accessions o 24 neapple plantlets from in vitro culture. Each grou 2 was further divided into two subclusters: I and II. Cluster A was divided into s 2 clusters I and II with a similarity coefficient of 77.5%. Cluster B

was divided into subclusters I and II with a similarity coefficient of 76%.

Field plant samples (A1), comparison samples for different growth environments (B1 and B2), comparison accessions for acclimatized in vitro culture plants (C1 and C2), and pineapple plantlet accessions from in vitro culture (D4) had a similarity level of 85.5%. Pineapple plantlet accessions from in vitro cultures (D7, D8, and D9) had a similarity level of 89.5%. Field plant accessions (A2) and pineapple plantlet accessions from in vitro cultures (D1 and D2) had a similarity level of 97.2%. Accession D3 had a similarity level of 78% with accessions A1, B1, B2, C1, C2, D4, D7, D8, and D9, and these samples had a similarity level of 77.5% with accession D5. Accession D6 had a similarity level of 76% with accessions A2, D1, and D2.

Da Silva et al. (2016) reported the genetic similarity of pineapple after years of culture in an in vitro environment. In addition, Kohpaii et al. (2017) showed results with in vitro cultured plants that entered the acclimatization stage. The optimization and primary selection results showed that 7 of 10 primers used in this study could amplify the DNA of pineapples from Sipahutar (Figure 1).

The dendrogram revealed that acclimatized plants had higher genetic diversity than plants grown in different environments. However, the genetic similarity between in vitro cultured plants and acclimatized parental plants was low (Ilczuk and Jacygrad 2016; Sherif et al. 2018; Jogam et al. 2020). This was affected by the source of acclimatization plant extracts from plant samples grown in different environments and the same environmental growth factors in the YAHDI Tissue Culture Laboratory. Genetic differences in parental plants originating from the Sipahutar area, in plants grown in different environments, and in acclimatized plants cultured in vitro could occur because of altitude, growth area, and environmental conditions (Rodríguez-Bernal et al. 2013; Sharma 2015; Youssef and Mahgoub 2015).

Genetic similarity testing is one of the requirements in the production of plants by using in vitro culture techniques to ensure that seed production is true to type. The early detection of genetic diversity in plants from in vitro culture is important to avoid developing plants that could have changed genetically. This in vitro culture as the propagation method results in a high similarity of the cultured plant to the parent plant and is effective and efficient (Kohpaii et al. 2017). Molecular markers used to observe the genetic similarity of pineapple plants from in vitro culture were RAPD (Roostika et al. 2015; Pratiwi et al. 2017), AFLP (Scherer et al. 2015), and ISSR (Da Silva et al. 2016; Kohpaii et al. 2017). The main reason why chose ISSR markers in this study was they have a higher reproducibility rate than the RAPD markers, which are cheaper than the AFLP markers (Reddy et al. 2002).

The genetic differences between in vitro cultured plants, field plants, and other comparison plants can be caused by various factors, such as the source of explants, concentration and type of growth regulators, duration of culture, frequency of subculture, and sensitivity of the DNA markers used (Harahap 2011; Leva et al. 2012; Agbidinoukoun et al. 2017). The source of explants from tissues still actively dividing, such as the cambium, generally does not vary. In contrast, differentiated tissues, such as roots, leaves, chimera, and callus tissues, generally have large variations (Leva et al. 2012). In this study, the plantlets had an extracted source from the callus.

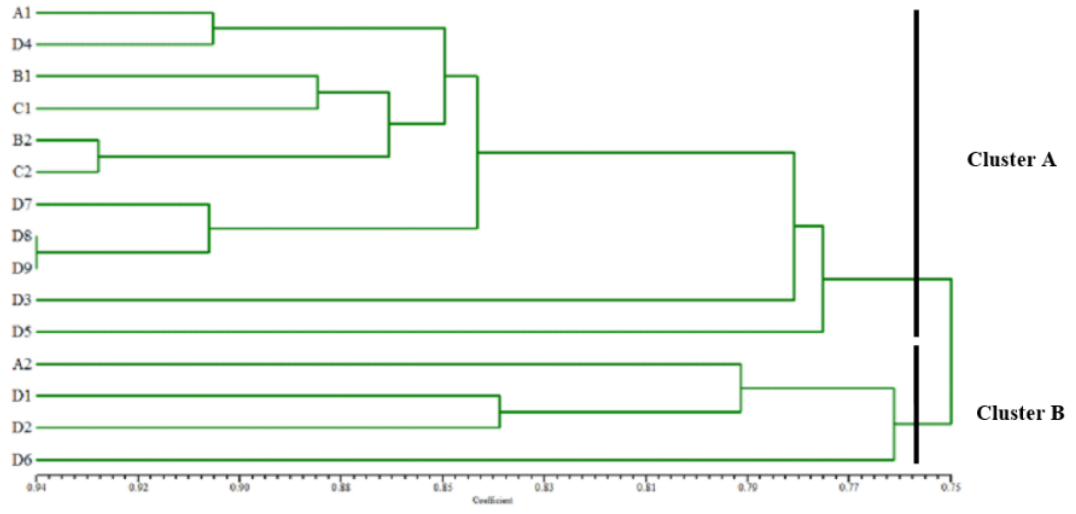

Figure 2. A dendrogram among seven primers. Accessions of the in vitro cultured pineapples from Sipahutar, North Sumatra, Indonesia generated by the UPGMA cluster analysis. A1-A2: Parental plants; B1-B2: Pineapple with different environmental growth conditions; C1-C2: Acclimatized pineapple; and D1-D9: Plantlets from in vitro culture

The addition of growth regulators (both auxins and cytokinins) could cause variations. High BA concentrations (15-30 mgL^{-1}) could cause somaclonal concentrations in bananas and rice by increasing the number of chromosomes and stimulating morphogenesis by destroying the cell cycle (Bairu et al. 2011). BA concentration of 5-8 mgL^{-1} causes small amounts of somaclonal variations in bananas (<6%) (Bhalang et al. 2018), and 3 mgL^{-1} BA in media could cause somaclonal variations of pineapple plants cultured in vitro compared with parental plants (Kohpaii et al. 2017). In this study, genetic diversity in plants cultured in vitro could be compared with parent plants because of the media that included BA, a growth regulator, at a high concentration of 2 ppm.

The culture period's duration and subcultures' frequency also affect the genetic similarity of plants cultured in vitro. This is because the higher the frequency of subculturing, the longer the duration of the cells being exposed to various factors that can cause mutations and genetic diversity when cultured (Peng et al. 2015). For example, genetic diversity was observed in pineapple plants grown from in vitro culture for 8 months without subculture (Kohpaii et al. 2017). Genetic diversity of pineapple plants resulting from in vitro culture for 8, 12, and 24 months with fi[34] subculture treatments with each subculture for 45 days on MS medium without any increase in Plant Growth Regulator was low (Da Silva et al. 2016). Another study reported a genetic diversity level of 85%-89% in grapes subcultured 14 times, whereas grapes subcultured 15 times had a genetic diversity level of 66% (Indaryani et al. 2019). The plantlets were subcultured every 4 months. In this study, the period of plantlet culture was 12 months, with two subcultures on MS with 2 ppm of vitamins and 2 ppm of BA.

In conclusion, the genetic similarity of the Sipahutar pineapple was low. The genetic diversity of pineapple plants from Sipahutar has a similarity level of 75%-94%. The genetic dissimilarity between parental plants, plants are grown in different environments, and the different growth environments caused acclimatized plants. The parent plant was taken directly from its original Sipahutar area. Whereas pineapple plants grown in differe[11] environments and acclimatized plants were both grown in the YAHDI Tissue Culture Laboratory. The genetic dissimilarity of parent plants and propagated pineapple plants was the result of in vitro culture, which could be caused by the plantlet planting medium containing a concentration of 2 ppm of BA, 12 months of the culture period, and the frequency of subculture (2 times) using the same medium. The genetic diversity of pineapple samples was divided into two main clusters, namely cluster A consisted of 11 accessions and cluster B consisted of 4 accessions.. Cluster separation does not correlate to the source of plant materials studied.

[12] ACKNOWLEDGEMENTS

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