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Genetic Diversity of Indonesian Pineapple (*Ananas comosus* (L.) Merr.) Cultivars Based on ISSR Markers

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ABSTRACT

Pineapple (*Ananas comosus* (L.) Merr.) is the third-most important tropical fruit traded and widely cultivated in Indonesia with various cultivars. This study identifies genetic diversity and determines the phenetic relationship of nine pineapple accessions based on inter-simple sequence repeat (ISSR) markers. Four ISSR primers were utilized for genetic diversity analysis and diversity relationships using POPGENE 1.31 and MVSP 3.2 to form a dendrogram. The results showed that nine pineapple accessions revealed the successful amplification of 50 DNA bands, 46 polymorphic, with a percentage average of 89.38%. The calculation result of the effective alleles (Ne), Nei's gene diversity (h), Shannon information index (I), and polymorphism information content (PIC) showed that ISSR 1 had the highest value and ISSR 16 had the lowest value. The average of the Ne value was 1.44; the average of h was 0.28, which indicated low genetic variation; the average of (I) was 0.43, which indicated that not all groups had the same frequency; the averages of the PIC value of 0.28 which showed that the four ISSR primers used were somewhat informative. The results of the phenetic relationship based on ISSR molecular markers showed two clusters that separated the accession of 'Spanish' from the accession of 'Queen' and 'Cayenne.' This resulting study showed that ISSR analysis was suitable for studying genetic diversity among pineapple cultivars.

Keywords: *Ananas comosus*, cultivar, Indonesia, ISSR, phenetic relationship

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INTRODUCTION

The pineapple (*Ananas comosus* (L.) Merr.) is a plant belonging to the Bromeliaceae family and produces significant fruit for the economy. It was in agreement with the statements made by Hassan et al. (2011) and Wang et al. (2017), who stated that

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after bananas and oranges, pineapple was the third-most important tropical fruit traded. As a tropical nation with a variety of agroclimates, Indonesia can support the growth of pineapple production and is currently the largest pineapple-producing nation in the world (Budianingsih et al., 2017; Statista, 2024). A total of 336,102 and 261,769 tons of pineapples were produced in Central Java and Riau Provinces, respectively (Badan Pusat Statistik Provinsi Jawa Tengah, 2022; Badan Pusat Statistik Provinsi Riau, 2023).

Pineapple cultivars have been divided into groups based on differences in morphological characteristics, especially in their leaves and fruits (Wang et al., 2017). Pineapple cultivar groups that have been cultivated in Indonesia were 'Queen', 'Cayenne', 'Abacaxi', and 'Spanish' (Amda et al., 2020; Hernosa et al., 2022; Rosmaina et al., 2021). Analysis of morphological characteristics has become a classic approach to distinguishing plant cultivars, so this character became the main character used in the classification and initial identification of taxa, both phenotypic and genotypic (Martiwi et al., 2020; Valencia & Alcasid, 2014). However, this character has a weakness, which can be plasticity, because environmental factors influenced it, so other characters are needed for support and comparison (Singh, 2010).

Molecular characters are one of the supporting and comparative characters that can be used in the classification and identification of taxa. This character is stable, can be detected in all plant tissues, is not influenced by environmental factors, and can be detected in all phases of growth (Pabendon et al., 2007; Zulfahmi, 2013). Due to their speed, accuracy, and strong results, molecular marker techniques currently appear to be the method of choice for a variety of applications, displaying excellent reliability to supplement and enhance conventional approaches (Ismail et al., 2020). Furthermore, molecular markers are now frequently employed as a tool to assess the accuracy of plant taxonomy classifications. Through genetic characterization and fingerprinting, genetic analysis, relationship mapping, and molecular breeding, molecular marker technology is also effective in improving crops (Valencia & Alcasid, 2014).

DNA molecular techniques were used to investigate plant genetic variability to detect and establish relationships at the cultivar and species levels (Souza et al., 2017). One of the molecular markers frequently used to analyze genetic diversity and identify phenetic relationships was inter-simple sequence repeat (ISSR). ISSR is a polymerase chain reaction (PCR)-based genetic marker that has been developed as an anonymous and one of the markers with repeated sequences in the form of DNA fragments with a size of 100-3,000 bp located between microsatellite regions (Mohamed et al., 2014; Napitu et al., 2016). ISSR is also a molecular marker with a number of benefits, including being simple, quick, and affordable like the random amplified polymorphic DNA (RAPD) molecular marker, not requiring sequence data during primary construction, requiring

only PCR for analysis, and randomly distributing the process across the genome (Godwin et al., 1997; Vijayan, 2005; Semagn et al., 2006). ISSR is a molecular marker frequently employed to examine plant genetic variability, and it is more reproducible than RAPD since it can be used in most situations and has been very effective in genetic fingerprinting and diversity analysis (Arif et al., 2020; Godwin et al., 1997).

Previous studies have effectively used ISSR molecular techniques to analyze genetic variation in pineapple cultivars. In a study by Wang et al. (2017) on the genetics of pineapple accessions from 10 pineapple-producing countries, 13 ISSR primers amplified 96 bands, 91 of which were polymorphic. A total of 56 DNA bands were amplified in the study of Vanijajiva (2012) about the assessment of genetic diversity and relationships in pineapple cultivars from Thailand using ISSR markers, of which 27 were polymorphic. A study by Harahap et al. (2022) about genetic similarities in plantlet pineapples in vitro from North Sumatra using ISSR molecular markers reported that out of the six primers from the 192 amplified bands, 23 bands of polymorphic loci were obtained. This study identifies genetic diversity and determines phenetic relationships of pineapple accessions from Central Java and Riau Provinces based on molecular characters using ISSR markers.

MATERIALS AND METHODS

Sampling Collection

Five districts were chosen for sample collection: Wonosobo, Magelang, and Pemalang Regencies in Central Java Province: Kampar and Siak Regencies in Riau Province (Figure 1). The samples collected were pineapple leaves from farmers' plantations with the criteria for pineapple plants to be sampled 1–3 years old and flowering. The leaves were placed in ziplock plastic, labeled, and stored at -20°C in the freezer. Nine pineapple

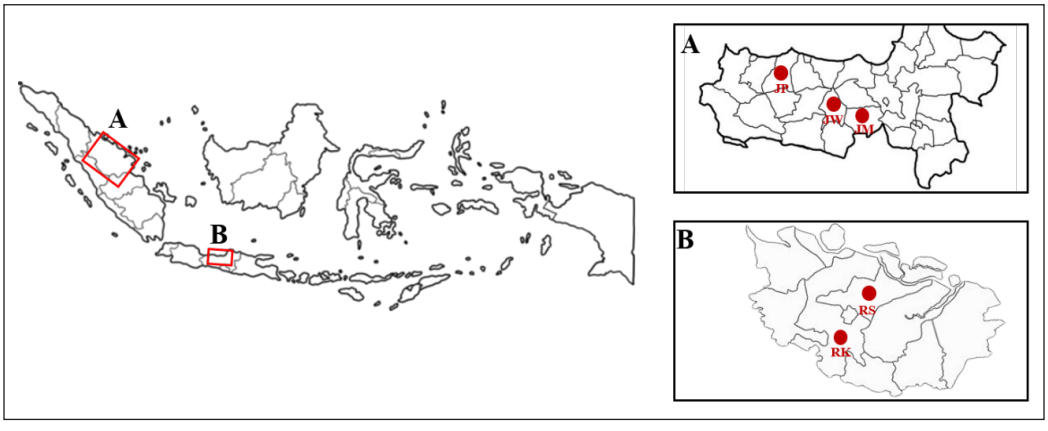

Figure 1. Five pineapple sampling locations in Indonesia: (A) Central Java Province (JW = Wonosobo: 7°22'12.7"S 109°52'32.4"E, JM = Magelang: 7°35'46.8"S 110°10'55.5"E, and JP = Pemalang: 7°09'36.9"S 109°18'39.3"E); (B) Riau Province (RK = Kampar: 0°25'15.7"N 101°14'44.8"E and RS = Siak: 1°05'12.4"N 102°07'52.5"E)
Note. S = South; W = West; E = East; N = North

Table 1
Collection of pineapple samples from Central Java and Riau Provinces

Cultivar	Local name	Accession code	Accession location	Province
'Cayenne'	Kopyor Pineapple	JW01	Duren Sawit, Wonosobo	Central Java
'Cayenne'	Kopyor Pineapple	JW02	Duren Sawit, Wonosobo	
'Cayenne'	Benggolo Pineapple	JM01	Kembang Limus, Magelang	
'Cayenne'	Benggolo Pineapple	JM02	Kembang Limus, Magelang	
'Queen'	Madu Pineapple	JP01	Beluk, Pemalang	
'Queen'	Kualu Pineapple	RK01	Kualu, Kampar	Riau
'Queen'	Moris Pineapple	RK02	Pagaruyung, Kampar	
'Spanish'	Madu Pineapple	RK03	Pagaruyung, Kampar	
'Queen'	Moris Pineapple	RS01	Sungai Apit, Siak	

accessions from Central Java and Riau Provinces were analyzed molecularly at the Genetic Engineering Laboratory, Center for Biotechnology Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia (Table 1).

Data Collection and Analysis

A total of 0.1 g of leaf samples were used for DNA isolation using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987) with modifications. The liquid nitrogen was used to compress the leaf samples until smooth and then transferred to a 2 ml microtube with 1,400 µl of CTAB buffer (HiMedia, USA). The samples were incubated in a 60°C water bath for 60 min, switching positions every 10 min to maintain homogeneity. After cooling, add 500 µl of chloroform: isoamyl alcohol 24:1 (CIAA, Merck, Germany), and then vortex for 2 min. The sample was then centrifuged for 15 min at 8,050 x *g*. The supernatant was poured into a microtube, 500 µl of CIAA was added, and the microtube was recentrifuged at 8,050 x *g* for 15 min. Removing the second centrifuge supernatant and transferring it to a new 1.5 µl microtube. Then, the supernatant was added with sodium acetate as much as 1/10 of the volume of the supernatant obtained and 2/3 of the total volume. The microtubes were gently turned over so that all solutions were homogeneous and stored in a -20°C freezer for 24 hr. The sample was recently centrifuged for 10 min at 8,050 x *g*. Supernatants were removed, and pellets in DNA deposits were washed using 70% ethanol solution (Merck, Germany). Ethanol was removed from the microtubes, and DNA pellets were dried in the air. In the last stage, 50 µl of Tris-EDTA (TE) buffer (HiMedia, USA) was added to the microtube and stored in the freezer at -20°C. Nanodrop (MaestroGen, Taiwan) was used to test DNA concentration and purity at 260/280 nm absorbance.

Four ISSR sequence primers were used for DNA amplification, as reported by Wang et al. (2017) (Table 2) with the ISSR-PCR method. A total of 25 µl of PCR mix, including 2 µl of DNA template (25 ng), 2 µl of ISSR primer (10 pmol), 12.5 µl

Table 2
Sequences of inter-simple sequence repeat (ISSR) primer used in DNA amplification (Wang et al., 2017)

Primer	Primer sequences (5' – 3')
ISSR 1	5' CACACACACACACACAGT3'
ISSR 3	5' GAGAGAGAGAGAGAGA(CT)C(AG)3'
ISSR 16	5'GTGTGTGTGTGTGTGTC3'
ISSR 24	5'GACAGACAGACAGACA3'

of PCR Kit ready mix MyTaqTM HS Red Mix (Bioline, United Kingdom), and 8.5 µl of nuclease-free water, were used for the PCR reaction.

The following PCR conditions are used to carry out ISSR amplification: initial denaturation at 94°C for 5 min, followed by denaturation with 40 cycles for 30 s at 94°C, annealing at 50–51°C for 45 s, continued with the final extension at 72°C for 90 s. The electrophoresis of DNA bands on 1.8% agarose gel in 50 ml of Tris-borate-EDTA (TBE) 1× buffer (HiMedia, USA) and using florosafe DNA dye (Boca Scientific Inc., USA) as much as 4 µl revealed to determine the results of the DNA amplification. Electrophoresis was performed at 110 V for 30 min and then visualized using an ultraviolet (UV) transilluminator.

The molecular data in the form of DNA bands that appear are given a score with the provisions of 0 (invisible DNA band) and 1 (visible DNA band) so that the data obtained is binary. The number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), average number of Ne, average h, and average I were all calculated for each primer using the POPGENE 1.31 program (Yeh et al., 1999). The following formula was also used to compute the PIC (De Riek et al., 2001):

$$PIC = 1 - [f^2 + (1 - f)^2]$$

where, 'f' indicates the marker's frequency within the data set. DNA band scoring results were used to calculate the similarity matrix of pineapple accessions using the simple matching coefficient (SMC) formula. To create a dendrogram, a cluster analysis utilizing unweighted pair group methods with arithmetic averages (UPGMA) and multi variate statistical package (MVSP) software version 3.2 was used.

RESULTS

A general morphological view can distinguish three pineapple cultivars from the nine accessions observed, one of which can be seen in the morphological characters of the fruit, such as the color of the fruit when ripe, fruit shape, and crown shape (Figure 2).

Nine pineapple accessions from Central Java and Riau Provinces, Indonesia, were tested by DNA amplification using four ISSR primers (ISSR 1, ISSR 3, ISSR 16, and ISSR 24), and the result revealed the successful amplification of 50 DNA bands, 46 of which were polymorphic. Each primer produced 8 to 20 DNA bands that were amplified (Table 3 and Figure 3). ISSR 24 produced the highest number of DNA bands (20 bands), whereas ISSR 3 and 16 produced

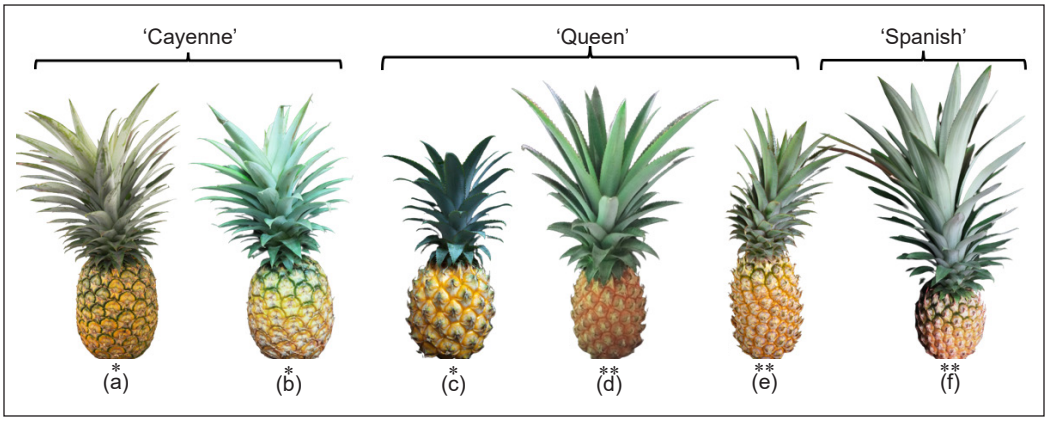

Figure 2. Morphological characters of fruit in three Indonesian pineapple cultivars: (a-b) 'Cayenne', (c-e), 'Queen', and (f) 'Spanish'
Note. * = Central Java Province; ** = Riau Province

the lowest number of DNA bands (8 bands) (Table 3 and Figure 3). However, the primer that showed the highest percentage of polymorphism in this study was in ISSR 1 (100%) and the lowest in ISSR 16 (75%) (Table 3 and Figure 3). The size of the amplified DNA bands in this study ranged from 175–2,000 bp (Table 3 and Figure 3). The analysis indicated that the average percentage of polymorphic DNA (PPB) in four ISSR primers was 89.38%.

The average of Ne, average h, average I, and average PIC values were calculated (Table 3), reported that between ISSR 1, ISSR 3, ISSR 16, and ISSR 24 showed that ISSR 1 had the highest value and ISSR 16 had the lowest value. The average value of Ne was 1.44. At the same time, the low genetic variety was suggested by the average value of h being 0.28, and not all groups had the same frequency, as shown by the average value of I being 0.43. The four ISSR primers used in this study were found to have a PIC value ranging from 0.19 to 0.33, with an average of 0.28, indicating that they were considered somewhat informative.

Table 3
Diversity information parameters on the four inter-simple sequence repeat (ISSR) markers

Primers	TNB	NPB	PPB (%)	Ne	h	I	PIC
ISSR 1	14	14	100	1.52	0.33	0.50	0.33
ISSR 3	8	7	87.5	1.46	0.28	0.44	0.28
ISSR 16	8	6	75	1.28	0.19	0.30	0.19
ISSR 24	20	19	95	1.49	0.30	0.46	0.30
Total	50	46	357.50	5.75	1.10	1.70	1.10
Average	12.50	11.50	89.38	1.44	0.28	0.43	0.28

Note. TNB = Total number of bands; NPB = Number of polymorphic bands; PPB = Percentage polymorphic bands; Ne = Average of effective alleles; h = Average Nei's gene diversity; I = Average Shannon information index; PIC = Polymorphism information content

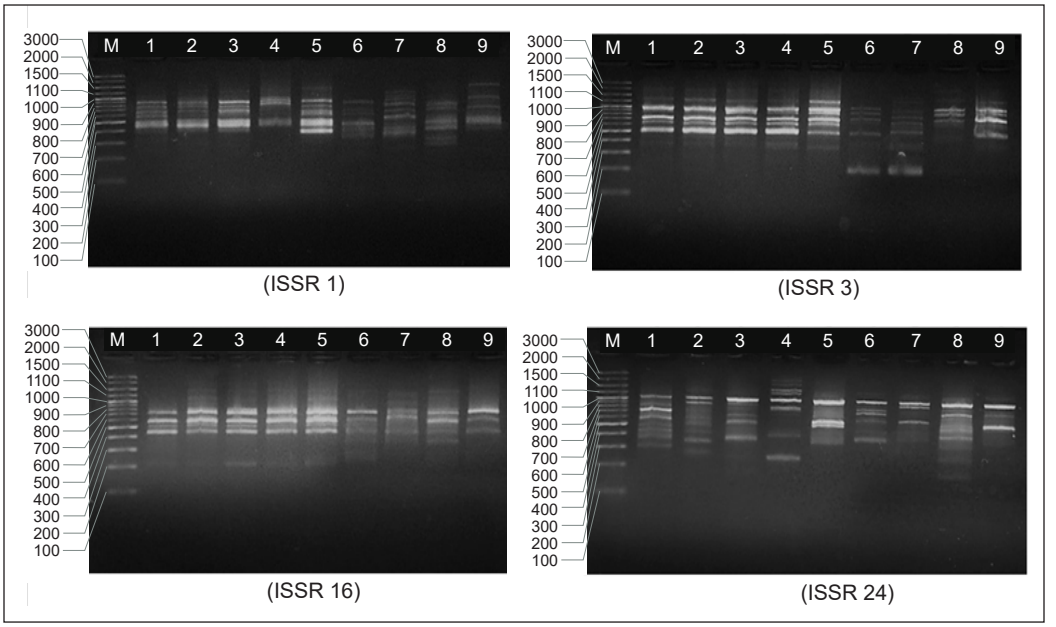

Figure 3. The inter-simple sequence repeat (ISSR) profiles of ISSR1, ISSR 3, ISSR 16, and ISSR 24 on *Ananas comosus*

Note. M = Marker 100 bp; Lane 1 = 'Cayenne' (JW01); Lane 2 = 'Cayenne' (JW02); Lane 3 = 'Cayenne' (JM01); Lane 4 = 'Cayenne' (JM02); Lane 5 = 'Queen' (JP01); Lane 6 = 'Queen' (RK01); Lane 7 = 'Queen' (RK02); Lane 8 = 'Spanish' (RK03); Lane 9 = 'Queen' (RS01)

The similarity value in nine pineapple accessions based on the dendrogram (Figure 4) ranged from 59–86%. Based on cluster analysis using ISSR markers on nine pineapple accessions, there were two primary clusters, namely clusters A and B, at the phenon line of 60% similarity index (Figure 4). Cluster A was an accession from 'Spanish,' while Cluster B was from 'Queen' and 'Cayenne'. Cluster A consisted of one accession from 'Spanish' (RK03), while cluster B consisted of four accessions from 'Queen' (RK01, RK02, RS01, JP01) and four accessions from 'Cayenne' (JW01, JW02, JM01, JM02).

Principal component analysis (PCA) based on 50 bands ISSR of nine accessions pineapple showed two principal components (PC1 and PC2) (data not shown). The UPGMA clustering (Figure 4) and the distribution pattern from PCA (Figure 5), respectively, were associated. The first principal component (PC1) explained 23.54% of the variation. In comparison, the second principal component (PC2) explained 21.83% of the variation, and the total value of the cumulative variation from PC1 and PC2 was 45.37% (data not shown).

DISCUSSION

This study was the first report on ISSR markers being applied to identify genetic differences in pineapple accessions from Central Java and Riau Provinces. Various pineapple accessions from Riau Province

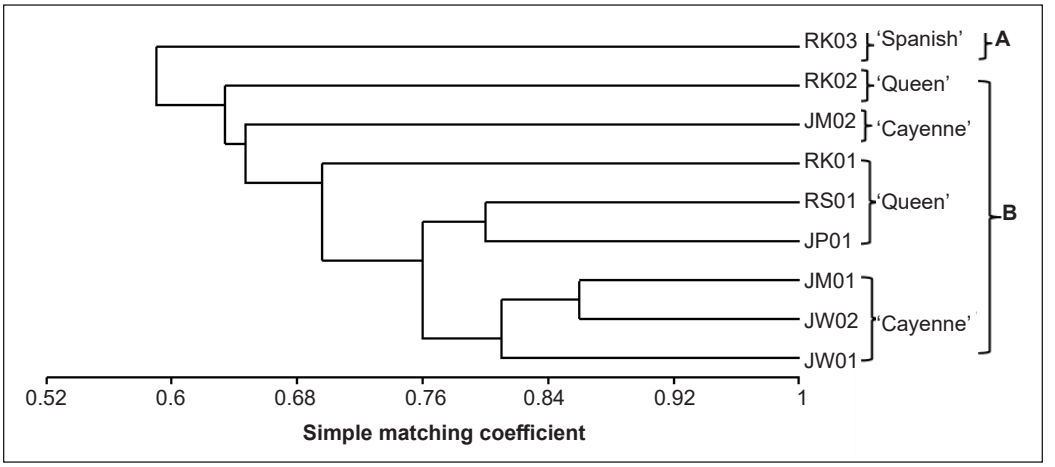

Figure 4. Dendrogram of nine pineapple accessions based on inter-simple sequence repeat (ISSR) markers

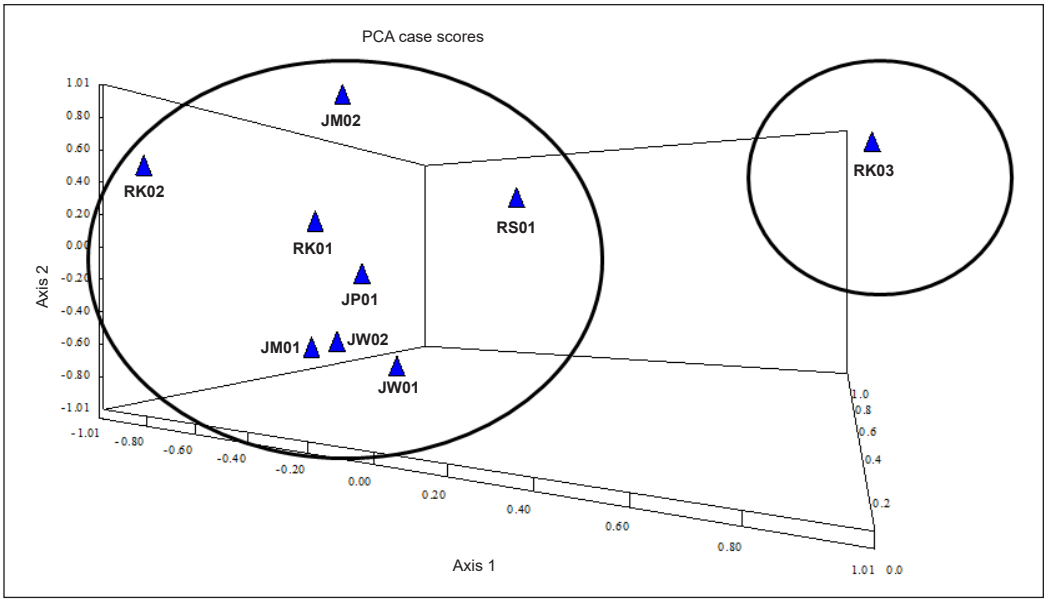

Figure 5. Three-dimensional plot of principal component analysis of nine accessions pineapple based on inter-simple sequence repeat markers

Note. PCA = Principal component analysis; JW01 = 'Cayenne' (Wonosobo); JW02 = Cayenne' (Wonosobo); JM01 = 'Cayenne' (Magelang); JM02 = 'Cayenne' (Magelang); JP01 = 'Queen' (Pemalang); RK01 = 'Queen' (Kampar); RK02 = 'Queen' (Kampar); RK03 = 'Spanish' (Kampar); RS01 = 'Queen' (Siak)

showed a substantial amount of genetic variety. In previous studies, the genetic diversity of pineapple accessions from Riau Province was conducted by RAPD profiles, and the result showed a high polymorphic percentage value of 53–91% (Rosmaina et al., 2022).

The results of this study used four ISSR primers that were previously used by Wang et al. (2017) for pineapple diversity

in ten pineapple-producing countries. The number of DNA bands in the four ISSR primers amplified in the result of this study had a greater value (50 bands) than the result of the study by Wang et al. (2017) (44 bands), with a range from 8–20 and 8–14, respectively. Moreover, the highest number of DNA bands from four ISSR primers was ISSR 24, totaling 20 bands. The result was in agreement with Wang et al. (2017) study results, which showed that the ISSR 24 produced the most DNA bands. It showed that the same primer (ISSR 24) in different studies can produce a high number of bands in pineapple identification. However, the percentage of polymorphic bands showed different results, that were in this study showed a value of 100% only found in ISSR 1, while the study by Wang et al. (2017) used the same four primers and showed that all primers produced percentage polymorphic band value of 100%. It showed that the total percentage value of polymorphic DNA from Wang et al. (2017) was higher than our result.

Each primer produced a varied number of DNA bands and polymorphic bands. It explained that the ISSR molecular marker was a powerful tool for genetic studies of pineapples because it could identify very low levels of genetic variation. Rakoczy-Trojanowska and Bolibok (2004) and Reddy et al. (2002) stated that ISSR primers were molecular markers that can produce high polymorphisms. According to Vanijajiva (2012), a high polymorphism level suggested that the probability of mutation in pineapple DNA is considerable. The extensive geographic distribution and significant ecological variables may be responsible for the high mutation probability. It was also supported by Aradhya et al. (1994), who suggested that ecological isolation may cause morphological differences among the species of *Ananas*. It might also suggest that finding polymorphisms at the species level is a high priority for random primers. Moreover, Gholami et al. (2021) stated that ISSR markers proved useful for distinguishing and identifying the relationships among species and populations collected from geographically different locations.

Although the ISSR molecular marker has several advantages over other molecular markers, it was rarely used to identify the genetic diversity of pineapples in Indonesia. It was also supported by Wang et al. (2017), who stated that ISSR molecular markers were still rarely used in analyzing the genetic diversity of pineapple. The study related to pineapple genetic diversity in Indonesia that used ISSR molecular markers was Harahap et al. (2021, 2022) about the genetic variability of pineapple plantlets and the genetic similarity of pineapples cultivated in vitro from Sipahutar, North Sumatra, reported that a total of six and ten ISSR primers used in both Harahap studies, respectively, the results revealed genetic similarity of plantlets and in vitro culture pineapple had similarity level of 76–97% and 75–94%. This study resulted in a higher percentage of polymorphism, 75–100% from four ISSR primers.

The Ne, I, h, and PIC calculations (Table 3) showed that ISSR 1 had the highest value in each parameter. This result was different

from Wang et al. (2017), which indicated that ISSR 24 had the highest value on each of those parameters. The average Ne calculation represented the number of Ne derived from each population. It was the homozygosity's reciprocal or inverse value. The higher the value of Ne, the more the individuals are heterozygous (Solin et al., 2014). The h value was utilized to measure genetic diversity and genetic divergence and analyze population relationships. The calculation of gene frequencies at each locus produces heterozygosity. (Terryana et al., 2020). According to Nei (1978), if the heterozygosity (h) value ranges from 0.1–0.4, it indicates low genetic variation; if it ranges from 0.5–0.7, it indicates moderate genetic variation; and if it ranges from 0.8–1.0, it indicates high genetic variation. The heterozygosity (h) value ranges from 0 (zero) to 1 (one). The I value was the most suitable estimator to describe variation at multiallelic loci such as microsatellites (Konopiński, 2020). Then, according to Ramezani (2012), the Shannon diversity index is normalized to have a value between 0 and 1: lower values represent greater diversity, while higher values represent less diversity. A value of 1 for the index indicates that all groups experience the same frequency. PIC is a parameter gauges how well it can spot polymorphism among a population's members. The better this ability, the higher the parameter's value. It provides one of genetic research's markers' quality indicators (Serrote et al., 2020). The four ISSR primers used in this study were shown to have an average PIC value of 0.28, indicating that they are thought to be somewhat informative. In contrast, a study by Wang et al. (2017) revealed an average PIC value of 0.22, indicating that they are seen to be not very informative. According to a study by Serrote et al. (2020), PIC values greater than 0.5 are regarded to be very informative for primers, while values between 0.25 and 0.50 are regarded to be somewhat informative, and values less than 0.25 are regarded to be not very informative. According to Kaki et al. (2020), the high PIC suggested rare alleles and high polymorphism in a single gene locus, which can be utilized to distinguish genotypes.

The result of cluster analysis (Figure 4) and PCA (Figure 5) using ISSR markers on nine pineapple accessions showed that the accession of 'Spanish' (Cluster A) separated from the accession of 'Queen' and 'Cayenne' (Cluster B). This result was supported by Hadiati et al. (2018), Rattanathawornkiti et al. (2016), and Rosmaina et al. (2022), which reported that 'Queen' and 'Cayenne' grouped into one cluster based on molecular characters. Using different ISSR primer types, similar results were obtained where 'Queen' and 'Cayenne' were grouped in one cluster and separated with 'Spanish.' Genetic variations can be influenced by a number of factors, including the quality and intensity of DNA electrophoresis bands, according to Harahap et al. (2022). The purity and concentration of the DNA separated, and the location of the main attachment

sites can impact the strength of DNA bands between samples. Furthermore, it was impacted by the competition for primer attachment sites on the isolated DNA, resulting in one band amplifying in multiplex while others are not amplified (Bilodeau et al., 2012). The similarity value in nine pineapple accessions based on the dendrogram (Figure 2) ranged from 59–86%. According to Jannah et al. (2022), populations with low genetic similarity showed how isolation increases genetic variety, which leads to a certain characteristic's emergence. Moreover, Poerba et al. (2019) added that the isolation drove the evolution of a particular trait with similar genetic properties, as opposed to the distinct environment or geographic condition that gave rise to other adaptation patterns and genetic characteristics.

CONCLUSION

Nine pineapple accessions used to study the genetic diversity showed the average Ne value of 1.44 and h value of 0.28, indicating low genetic variation and a percentage value of polymorphic bands in the four ISSR (ISSR 1, ISSR 3, ISSR 16, ISSR 24) primers of 89.38%. The I value was 0.43, indicating that not all groups had the same frequency, and the PIC average was 0.28, indicating that the four ISSR primers employed were thought to be somewhat informative. The results of the phenetic relationship based on ISSR molecular markers showed two clusters that separated the accession of 'Spanish' from the accession of 'Queen' and 'Cayenne.'

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