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MOLECULAR CHARACTERIZATION OF THE SUGARCANE CULTIVAR GMP3 MUTANTS INDUCED THROUGH COLCHICINE USING PCR-RAPD MARKERS

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SUMMARY

Plant breeding's rewarding polyploidy analysis can happen by observing molecular characteristics. Previously obtained 21 sugarcane mutants had used colchicine induction on the GMP3 cultivar at the PT Gunung Madu Plantations, Lampung, Indonesia. However, little information emerged related to the informative molecular markers and analysis of molecular characteristics in sugarcane mutants. The presented research complements previous studies regarding the characterization of superior sugarcane mutants based on agronomic and anatomical attributes. The said research ran through PCR-RAPD amplification using five primers. The DNA band pattern diversity analysis ensued using MVSP program version 3.2. The cluster analysis through UPGMA also estimated the genetic distance. Polymorphic band assessment employing the polymorphism information content (PIC) also helps determine the informative level of the used primers. The PCR-RAPD markers' screening results showed that four informative primers (OPN-07, OPB-19, OPA-04, and OPA-07) have PIC values ranging from 0.30 to 0.39. The molecular characterization showed that 21 GMP3 mutants had 35–60 DNA bands with 28 polymorphic bands and a similarity index of 0.47–1.00. The promising results would help increase sugar production and improve quality by inducing mutation in the sugarcane cultivar GMP3.

Keywords: Sugarcane (*Saccharum officinarum* L.), sugarcane cultivar GMP3, cluster analysis, genetic distance, markers, molecular analysis, PCR-RAPD, polymorphism information content (PIC)

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Key findings: In analyzing the molecular traits in colchicine-induced sugarcane mutants, the PCR-RAPD markers OPN-07, OPB-19, OPA-04, and OPA-07 appeared relatively informative, with a PIC value of 0.30–0.39. The genetic variation analysis of 21 GMP3 mutants had 35–60 DNA bands with 28 polymorphic bands. The phylogenetic analysis showed that all sugarcane mutants have a similarity index value ranging from 0.47 to 1.00, with groupings based on mutagen concentration and soaking time.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is a C4 plant commonly cultivated in tropical areas, including Indonesia, with a prime use for sugar production (Windiyani *et al*., 2022; Wiangwiset *et al.*, 2023). With the relatively low sugarcane productivity, the sugar industry cannot fulfill the world's sugar needs (CBS, 2021). However, overcoming this hindrance by assembling superior sugarcane seeds could meet food needs and realize sugar self-sufficiency.

The sugar industry works on increasing sugarcane production and conducting a research program for assembling promising sugarcane cultivars at the PT Gunung Madu Plantations (GMP), Lampung region, Indonesia. The released commercial cultivars at the PT GMP, Indonesia, comprised GMP1, GMP2, GMP3, GMP4, GMP5, GMP6, and GMP7. Sugarcane commercial cultivar GMP3 is a widely planted variety by PT GMP with a crop area of 30% of the total cultivated area in Indonesia. Based on data, the cultivar GMP3 is leading in production per hectare compared with other cultivars, probably due to its larger crop area with resistance to pests and diseases. However, based on field observations, the cultivar GMP3 has small stems, minimum leaf width, and low sugar yield with poor quality (Mahfut *et al*., 2023).

In sugarcane cultivars, quality improvement can progress through colchicine-induced mutation, followed by a precision selection of these mutants (Fathurrahman, 2023). Colchicine is an alkaloid compound that has a strong affinity for tubulin that can inhibit cell division and develop genetic variations. This compound is often practical in plant breeding to induce mutations that produce crop plants with different ploidy levels and to improve plant characteristics. Mutation breeding has been a widely used technique for various trait enhancement, such as agronomic (Aristya *et al*., 2019), anatomical (Mahfut *et al*., 2023), and molecular traits (Kasiamdari *et al*., 2019). Characterization is also vital in determining the morphological (Mahfut 2021, 2023; Mahfut *et al*., 2021), agronomic (Windiyani *et al*., 2022), anatomical (Mahfut *et al*., 2023a, b), and genetic characteristics of the sugarcane (Mahfut *et al*., 2016, 2020, 2023c).

Molecular characterization is also a commonly used approach in the polymerase chain reaction – random amplified polymorphic DNA (PCR-RAPD) technique (Yuniastuti *et al*., 2023). This technique has been successful in the different aspects of the genetic diversity in the sugarcane because it is simple, relatively low cost, and tolerant of DNA purity levels, not requiring previous DNA sequence information in its application (Abekova *et al*., 2022).

Even in the present era, there is little information related to PCR-RAPD marker screening and characterization of sugarcane resulting from mutation breeding with colchicine induction based on molecular trait analysis. Past studies also reported genetic diversity by evaluating the chewing sugarcane (*Saccharum* spp.) cultivars using RAPD markers (Ullah *et al.,* 2013). Tabasum *et al*. (2010) analyzed sugarcane genotypes, including *S. officinarum* and *S. barberi,* which showed a high level of polymorphism using PCR-RAPD markers, with more than one allele identified for each marker. Furthermore, Tolangara *et al*. (2020) also reported that eight of 50 markers used attained detection for a polymorphic level of 16%.

Several studies with molecular characterization and colchicine induction reported that sugarcane mutants manifested with 27 (72.9%) DNA bands from the total 37 DNA bands (Avivi *et al*., 2019). However, these

findings contradicted the research on mutation induction in oranges (Tolangara *et al*., 2020) and taro (Matsuyama *et al*., 2020), which showed varying patterns of genetic diversity. Hapsoro *et al*. (2015) reported genetic similarity, ranging from 17% to 97%, with an average of 57% among the 38 sugarcane accessions collected from Australia, America, Asia, and Africa.

The present research complements previous studies regarding the characterization of superior sugarcane mutants in the colchicine-induced GMP3 cultivar based on agronomic and anatomical analysis (Mahfut *et al*., 2023). The agronomic character analysis results indicate that 21 mutants of the GMP3 cultivar showed a variety of features, including medium-sized leaf width, dark green leaf color, branching leaves, lack of dorsal hairs, cylindrical segment shape, medium segment length (> 13 cm), and medium-sized stem diameter (2.5–3 cm). According to another study, morphological characteristics showed that the sugarcane cultivar GMP3 mutants have stomata of the Graminae type (Mahfut *et al*., 2023). The variation in stomata size among the cultivar GMP3 mutants and controls resulted in a similarity index (0.20), indicating a considerably high level of stomata variation.

Based on this, research related to the molecular characterization of the sugarcane cultivar GMP3 resulting from the colchicine-induced mutation is critical to carry out. It also complements the agronomic and anatomical characterization data that has previously transpired; therefore, the presented study can become the basis for efforts to improve sugar production and quality through colchicine-induced mutation in the sugarcane cultivar GMP3.

MATERIALS AND METHODS

Samples collection

The plant material depended on the sample collection of young leaves from less than six months old genotypes, following the methodology of Mahfut *et al*. (2023) below.

DNA extraction and quantification

The Cetyl Trimethyl Ammonium Bromide (CTAB)-based DNA extraction procedure employed the method of Doyle and Doyle (1987). A quantitative DNA test continued using a UV Vis spectrophotometer (Thermo Scientific Multiskan Sky). The DNA purity level measurement calculated the 260 nm and 280 nm absorbance values; however, the DNA purity values ranged from 1.8 to 2.0 (Sambrook and Russel, 1989).

DNA amplification

DNA amplification progressed using five selected PCR-RAPD markers (OPN-07, OPA-04, OPB-19, OPC-16, and OPA-07). The PCR composition comprised a volume of 20 µl, consisting of 2 µl primer, 10 µl PCR Mix, 6 µl ddH2O, and 2 µl DNA sample. DNA amplification advancement used a T100 Thermal cycler (BioRAD). The initial denaturation transpired at a temperature of 94 °C for 3 min, then followed by 30 cycles consisting of denaturation at a temperature of 94 °C for 1 min, annealing according to the temperature melting, extension at a temperature of 72 °C for 2 min, and final extension at 72 °C for 5 min. Visualizing PCR-RAPD products occurred by using a 1% agarose gel electrophoresis for 90 min at 100 volts (Rajput *et al*., 2020).

Phylogenetic analysis

Polymorphism analysis proceeded based on the bands visible from the gel doc amplification, with the scoring carried out visually. A score of one equals a DNA band appearing, and a score of zero, if a band does not appear (Liu *et al.*, 2006). The scoring analysis of DNA band patterns used the Multivariate Statistical Package (MVSP) version 3.2 program. The genetic distance for cluster analysis utilized the unweighted pair group with the arithmetic average (UPGMA) method. The polymorphic bands resulting from PCR-RAPD amplification calculation ensued for the polymorphism information content (PIC) values to determine

the informative level of the markers used. The PIC values sub-division had three categories, i.e., PIC > 0.60 (Highly informative), PIC > 0.3–0.59 (Moderately informative), and PIC < 0.3 (Less informative) (El-Esawi *et al*., 2022).

RESULTS AND DISCUSSION

DNA extraction and quantification

The quantity test of the genomic DNA isolation from the sugarcane control leaves and cultivar GMP3 mutants appears in Table 1. The results showed that the DNA purity ratio resulting from the A260/A280 absorbance comparison ranged between 1.873 and 1.932. It shows that the DNA obtained has a relatively good quantity. These results were highly analogous to previous findings, which reported that a good DNA purity ratio ranged at 1.8–2.0 (Sambrook and Russel, 1989).

The basic principle of quantitative DNA testing using spectrophotometry was that the sample must be clear and completely dissolved, with no colloidal particles. DNA containing purine and pyrimidine bases can absorb UV light. The DNA double bands can absorb UV light at 260 nm, while protein and phenol contaminants can absorb light at 280 nm. With this difference in UV light absorption, DNA purity measurement can be done by calculating the absorbance value of 260 nm divided by the absorbance value of 280 (A260/A280). However, if the DNA purity value is less than 1.8, it indicates contaminants from protein and UV; if the DNA purity is more than 2.0, it indicates chloroform and phenol contaminants (Sambrook and Russel, 1989).

The test results showed that the ensuing DNA concentration ranged from 1095.8 to 9650.9 μg/ml. However, the lowest concentration obtained was in the mutant M10 (1095.8 μg/ml), and the highest was in the mutant M21 (9650.9 μg/ml). Two factors influence the DNA concentration—the extraction speed at the extraction time and the composition of the addition of lysis buffer (Sophian *et al*., 2021). The DNA extraction speed is the most influential factor because, at the cell lysis and precipitation stages, the supernatant requires extraction for each

Table 1. DNA quantity test results on control and 21 mutants of GMP3 cultivar.

No.	Sample	DNA Purity (A260/A280)	DNA Concentration (x50 ng/ μl)
1.	M / C 0,1/ H/01	1.873	1973.6
2.	M / C 0,1/3H/15	1.932	2118.9
3.	M / C 0,1/3H/16	1.832	2146.8
4.	M / C 0,1/3H/02	1.862	1268.1
5.	M / C 0,1/3H/10	1.876	1384.9
7.	M / C 0,1/3H/04	1.850	1926.2
8.	M / C 0,1/3H/07	1.871	1601.3
9.	M / C 0,1/3H/06	1.864	3259.8
10.	M / C 0,1/3H/03	1.825	1095.8
11.	M / C 0,1/3H/14	1.887	1762.3
12.	M / C 0,1/3H/02	1.964	1940.0
13.	M / C 0,1/3H/08	1.902	1082.4
15.	M / C 0,1/3H/05	1.876	1384.9
16.	M / C 0,1/3H/01	1.860	3007.7
17.	M / C 0,1/1H/01	1.847	1.7156
18.	M / C 0,1/2H/01	1.917	2883.4
19.	M / C 0,2/1H/04	1.911	1496.2
21.	M / C 0,1/3H/09	1.874	9650.9
22.	M / C 0,2/3H/10	1.925	2170.4
23.	M / C 0,1/ 3H/12	1.880	1316.2
24.	M / C 0,1/3H/11	1.848	1320.7

Table 2. DNA bands resulting from PCR-RAPD amplification on control and 21 mutants of GMP3 cultivar.

No.	Markers	Sequence	Band size (bp)	Bands sum	Polymorphic bands sum	PIC value (%)
1	OPN-07	CAGCCCAGAG	650-900	55	6	0.39
2	OPB-19	ACCCCCGAAG	650-900	35	5	0.33
3	OPA-04	ATTCGGGCTG	600-850	60	5	0.30
4	OPC-16	CACACTCCAG	700-950	45	5	0.28
5	OPA-07	GAAACGGGTG	650-950	48	7	0.35

Notes: bp = base pairs; PIC = Polymorphic Information Content.

sample for the DNA precipitates in some samples. According to Wulan *et al*. (2021), the DNA concentration will considerably affect the quality of the amplified fragments. A DNA concentration that is too low will produce thinner splinters on the gel and may not even be visible. However, if the DNA concentration is too high, it will cause the fragments to appear thick, making it difficult to differentiate one fragment from another.

Screening of PCR-RAPD markers

The DNA amplification using five PCR-RAPD markers in the control and 21 mutants of the sugarcane cultivar GMP3 obtained 28 DNA polymorphic bands. The DNA bands resulting from PCR-RAPD amplification from the 22 samples are available in Table 2. The amplification results showed that all PCR-RAPD markers could recognize and amplify the DNA sequences in the sugarcane mutant’s genome. The amplification produces 35–60 DNA bands with 28 polymorphic bands having 600–950 bp band size.

The resulting number of DNA bands mostly depends on the marker’s ability to recognize the complementary DNA sequence on the template. Therefore, in the PCR-RAPD analysis, the marker selection influences the level of polymorphism of the DNA bands produced (Ahmed and Obeid, 2010). It may refer to the fact that each marker has its attachment site, resulting in a different DNA band. Slameto (2023) explained that polymorphism is a genetic variation in the DNA chain. The ratio of polymorphic DNA bands determines the diversity of a population because it describes the state of the plant genome.

The number of amplified DNA bands also varies among the markers used. It could be due to differences in the nucleotide sequence of each marker, which reflects variations in the marker attachment to segments along the plant genome. Assessing the polymorphism DNA bands pattern can progress by comparing the appearing and non-appearing DNA bands (Amiteye, 2021). Abdel-Rhman and Rizk (2021) also reported differences and similarities in DNA band patterns amplified by markers, which can formulate the percentage of DNA band patterns with polymorphisms and monomorphisms. The more regions of the genome amplified by markers, the higher the level of polymorphism.

The analysis of the polymorphism level for each marker is visible in the PIC values. The highest PIC values were evident for four markers, i.e., OPN-07, OPA-07, OPB-19, and OPA-04, with values of 0.39, 0.35, 0.33, and 0.30, respectively. However, the lowest PIC appeared for marker OPC-16 (0.28). Based on the presented results, four PCR-RAPD markers had a PIC value of ≥ 0.3, which showed that all the markers used had the molecular marker category, moderately informative in the analysis of molecular characteristics in sugarcane mutants.

The pertinent research had several markers as relative representatives, which also served in previous studies. Ahmed and Khaled (2009) reported that the use of representative markers (OPA-04 and OPA-07), as well as five other marker visualizations (OPA-01, OPB-07, OPB-10, OPO-10, and OPO-14), produced 44 fragments in each sugarcane genotype. Kawar

et al. (2009) also stated the use of the representative marker OPA-04 and six other markers (OPA-17, OPAB-17, OPC-08, OPA-16, OPG-05, and OPG-17), which were successful in measuring the genetic diversity of 17 sugarcane cultivars.

DNA amplification

The visualization results of PCR-RAPD amplification of the sugarcane cultivar GMP3 mutants occur in Figure 1. PCR-RAPD amplification with the primer OPA-04 produced 60 DNA bands and 5 polymorphic bands of 600–850 bp (Figure 1A). Primer OPB-19 produced 35 DNA bands and 6 polymorphic bands of 650–900 bp (Figure 1B). Primer OPC-16 revealed 45 DNA bands with five polymorphic bands ranging from 700 to 950 bp (Figure 1C). Primer OPN-07 exhibited 55 DNA bands and 6 polymorphic bands with a range of 650–900 bp (Figure 1D), and the OPA-07 primer showed 48 DNA bands, with seven polymorphic bands ranging from 650 to 950 bp (Figure 1E). The amplification results of the five primers showed bands of varying sizes and polymorphic patterns. The DNA band profiles produced from the five primers have incurred testing, which can be suitable for analyzing the genetic variations and relationships in sugarcane genotypes.

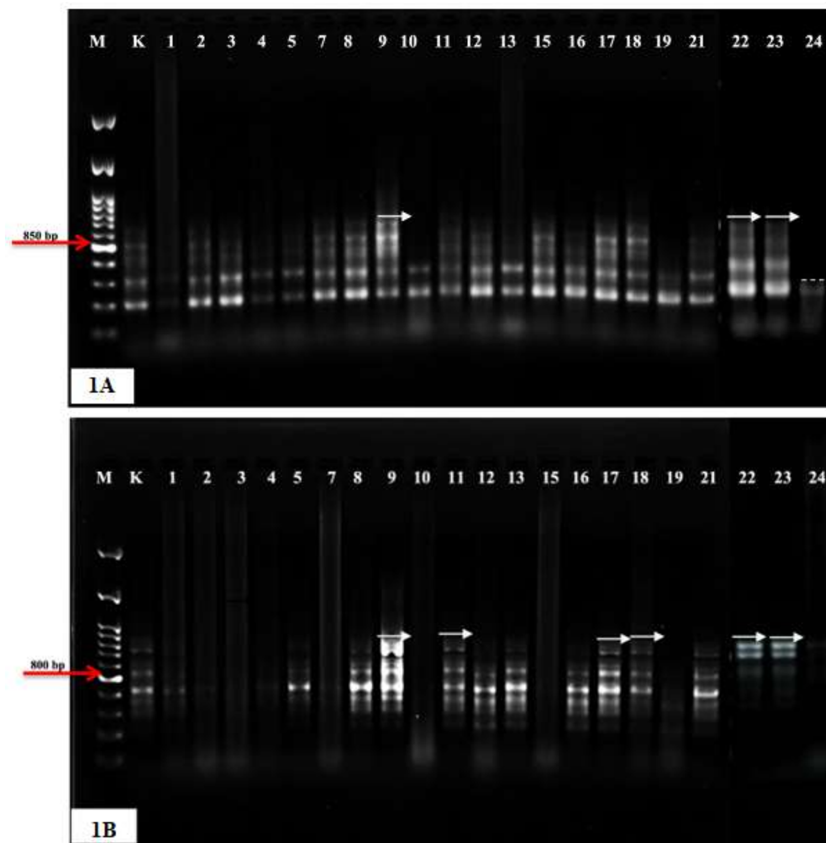

Figure 1 (A,B). DNA band patterns resulting from amplification of RAPD markers of GMP3 cultivars and 21 GMP3 cultivar mutants based on molecular characteristics. Description: 1A: OPA-04, 1B: OPB-19. locus that has an additional DNA band, locus that has lost a DNA band, K: Control, M: 1000 bp marker.

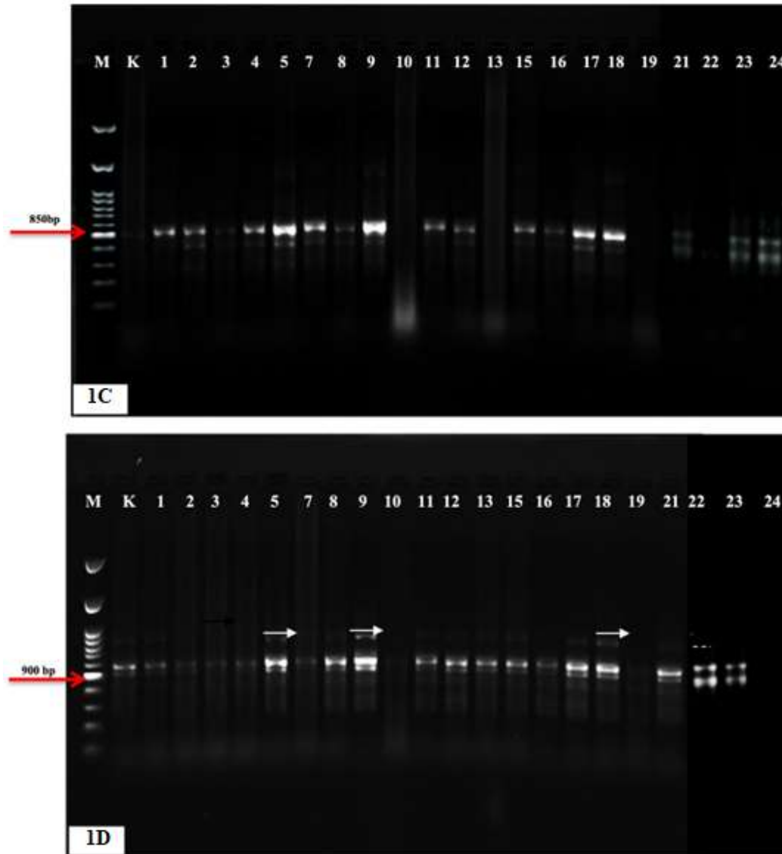

Figure 1 (C,D). DNA band patterns resulting from amplification of RAPD markers of GMP3 cultivars and 21 GMP3 cultivar mutants based on molecular characteristics. Description: 1C: OPC-16, 1D: OPN-07, locus that has an additional DNA band, locus that has lost a DNA band, K: Control, M: 1000 bp marker.

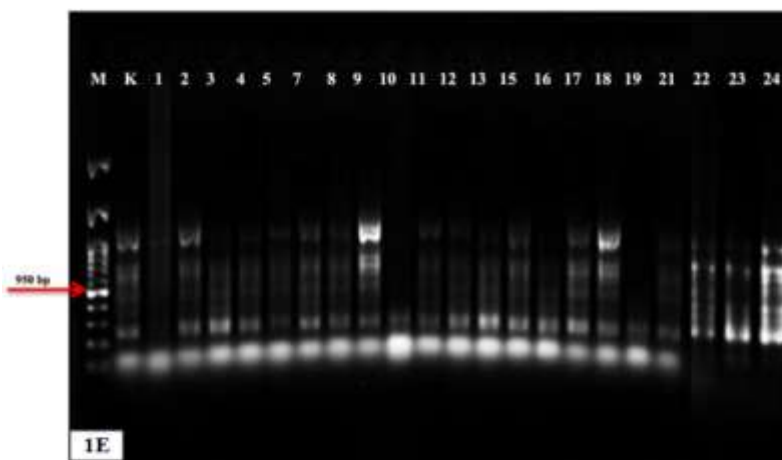

Figure 1 (D, E). DNA band patterns resulting from amplification of RAPD markers of GMP3 cultivars and 21 GMP3 cultivar mutants based on molecular characteristics. Description: 1E: OPA-07, locus that has an additional DNA band, locus that has lost a DNA band, K: Control, M: 1000 bp marker.

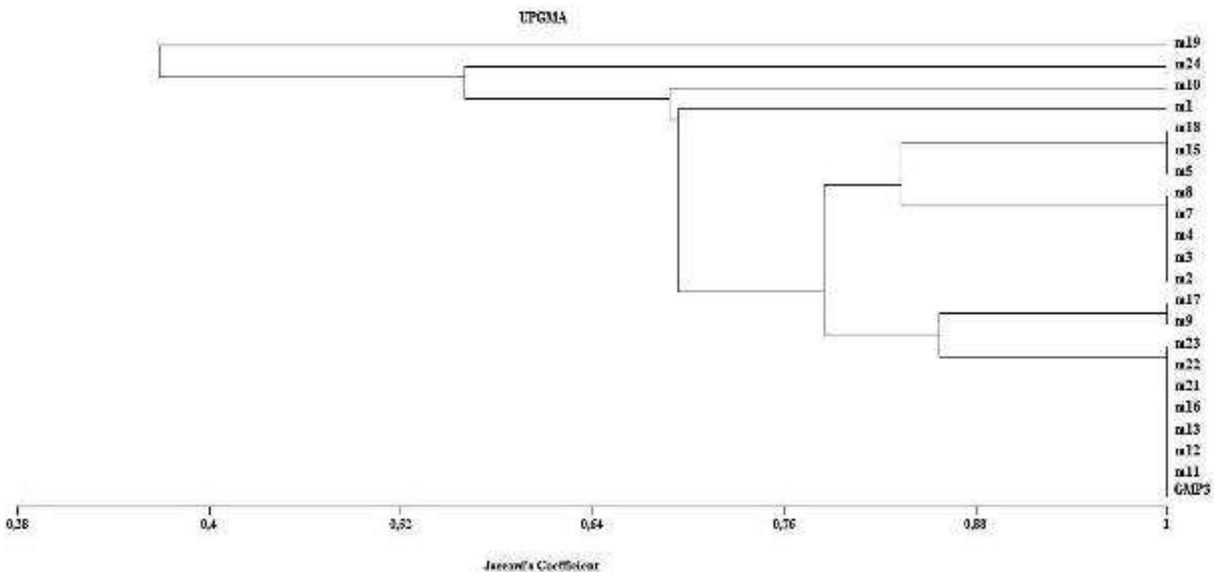

Figure 2. Dendrogram of phylogenetic relationships on control and 21 mutants of GMP3 cultivar using Jaccard's Coefficient.

Based on the molecular analysis, amplification with PCR-RAPD markers also produced several specific DNA bands. The cultivar GMP3 mutants had several DNA band patterns, i.e., the same DNA band pattern, loss of DNA bands, and the addition of new bands. In molecular analysis, the possible cause of band loss is a deletion. After exposing plant tissues to the mutagen colchicine, the primers can undertake annealing, duplication, substitution, insertion, and translocation of nucleotide bases (Viana *et al*., 2019; Mahfut *et al*., 2023).

Phylogenetic analysis

Phylogenetic analysis between the sugarcane cultivar GMP3 control and 21 mutant accessions based on scoring of the DNA bands appeared in Figure 2. The control and 21 mutant accessions of the cultivar GMP3 have a similarity index value ranging from 0.47 to 1.00, reaching a grouping into two clusters. Cluster A has a similarity index of 0.33, while Cluster B has an index value of 0.55.

Cluster A comprised only one accession, namely, M19, and the grouping in cluster A relied upon a concentration of 0.3, with a soaking time of three days. However, other branches in cluster A attained grouping based on differences in soaking time only. Cluster B consists of 21 accessions, namely, M24, M10, M1, M18, M15, M5, M8, M7, M4, M3, M2, M17, M9, M23, M22, M21, M16, M13, M12, M11, and GMP3, with the grouping dependent on the differences with concentration. Based on the cluster analysis, the results further revealed that the sugarcane cultivar GMP3 control treatment and 21 mutants can be in one group. It further revealed that the genetic diversity between the control treatment and the mutants of GMP3 exhibited a close relation. The formation of the clusters and branching showed that the mutants of the cultivar GMP3 had undergone a mutation effect that was not massive.

The cultivar GMP3 mutant grouping depended on concentration and soaking time, considerably influencing the genetic grouping. This grouping was possible because the DNA band pattern resulting from the PCR-RAPD marker amplification will be alike in the same treatment. According to Yasmeen *et al*. (2020), the DNA band patterns grouping showed differences caused by chemical mutagens' concentration and soaking time, which raise

the diversity in sugarcane mutants. This genetic diversity has the mutation causing it that can improve the plant quality. Based on cluster analysis, the control treatment and several mutants of the cultivar GMP3 attained plotting in different groups, indicating that these individuals were genetically diverse. It might be due to the influence of point and silent mutations. The mutation alters the one nucleotide base pair without causing a replacement of the type of amino acid produced (Abdel-Rhman and Rizk, 2021; Sukmawati *et al*., 2021; El-Esawi *et al*., 2022).

The findings from Hapsoro *et al.* (2015) also showed the genetic diversity through 38 sugarcane genotypes procured from Australia, Africa, America, and Asia by PT GMP, Indonesia, with an observed genetic similarity range of 17% to 97%, with an average of 57%. Splitting the said population into three main groups relied on the UPGMA dendrogram. These groupings contained genotypes of 23, 10, and 5, correspondingly. The genotypes in each major group came from various parts of the world. According to the dendrogram, every group has varied sub-groups. The identified eight subgroups included two subgroups in Group 2, two subgroups in Group 3, and four subgroups in Group 1.

CONCLUSIONS

In the sugarcane cultivar GMP3 mutants, the genetic variation based on molecular characteristics has 35–60 DNA bands with 28 polymorphic bands. The polymorphism level of each marker indicated by the PIC values ranged between 0.28–0.39, with various DNA band patterns.

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