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COLCHICINE-INDUCED GENETIC VARIABILITY IN PORANG (*Amorphophallus muelleri*): A STUDY USING SRAP MARKERS

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ABSTRACT

Mutation induction of porang both physically and chemically is widely used to increase the genetic variation of porang. However, mutation induction chemically by using colchicine is more effective than the other mutagens. Therefore, this study aims to assess the genetic diversity of porang after mutation induction using colchicine. Polyploidy induction is performed 30 days after planting of the porang plantlet. 0%, 0.01%, 0.02%, 0.03% and 0.04% colchicine was added to 12.5 ml of MS medium using a 0.22 µm Millipore filter. After 53 days, porang plantlets were ready for DNA isolation. A total of 10 SRAP primer combinations were used for DNA amplification. Genetic diversity in and between the porang population was performed using POPGENE software. Clustering analysis using PAST Software was also performed to identify genetic variation. Mutation induction using colchicine significantly increased the genetic diversity of porang. Among the SRAP primer combinations, ME2-EM2 is the most effective in detecting the induced genetic variation in porang. Mutation induction using 0.03% colchicine is highly recommended as it produces significant genetic changes compared to normal porang. Porang mutant resulted from 0.03% colchicine treatment holds great potential for porang breeding programs, as it can generate novel phenotypic variations and increase the chances of obtaining superior genotypes with desirable agronomic traits.

Keywords: *colchicine, genetic variation, mutation induction, porang*

INTRODUCTION

Porang (*Amorphophallus muelleri* Blume) is a tuberous plant belonging to the Araceae family [1]. Porang tubers serve as an alternative food source due to their high nutritional content, comprising 76.5% starch, 9.2% protein, 25% fibre, and 0.2% fat [2]. One of the major starch components in the porang tuber is glucomannan (50.84%-70.7%) [3]. The glucomannan content in porang tubers is significantly higher compared to other *Amorphophallus* species, such as *A. variabilis* (47.56%) [4], *A. campalatus* (3.2%) and *A. paeoniifolius* (2.52%) [5].

Recently, farmers have been cultivating porang both generatively using seeds and vegetatively using tubers [6] and bulbils [7]. Vegetative propagation produces seedlings that are genetically identical to the parent plant [8], whereas generative propagation through seeds results in apomictic seeds [9], which also exhibit genetic uniformity with the parent plant. Both generative and vegetative propagation lead to low genetic diversity in porang [10]. Therefore, efforts to enhance the genetic diversity of porang are necessary where one of which is through mutation induction [11].

The mutation induction in porang has been successfully conducted using both physical and chemical mutagens. Physical mutation using gamma-rays by [12] has been proven to affect morphological character (such as root number, leaf number, plant height, and leaf colour) as well as anatomical characteristics (including stomatal features and oxalate crystal density). Meanwhile, chemical mutation induction using colchicine resulted in polyploid plants, which were characterised by a larger plant size compared to normal porang [13]. These findings highlight the importance of performing genetic variation studies to support the development of improved porang varieties.

Genetic variation can be identified using both direct and indirect approaches [14]. Direct identification involves morphological observations [14, 16, 15], whereas Indirect identification can be performed using cytological [16] and molecular approaches [17]. The morphological approach does not produce consistent results [18], while the cytological approach is less effective when dealing with large sample sizes and is challenging due to the short duration of mitosis [19]. Therefore, molecular identification serves as an alternative method [17].

Molecular identification using DNA profiling is commonly used in genetic diversity analysis [20], with techniques such as RAPD [21], ISSR [22], SRAP [23], and SSR [24]. Each method has distinct principles and applications. RAPD is simple and fast using short random primers [25], but less consistent [21, 26]. ISSR targets non-

coding regions between microsatellite loci [27, 28] and uses primers with additional sequences [29]. SSR, a microsatellite marker, offers high polymorphism and consistent results [30], though it requires high initial costs for primer development [31].

Sequence-related amplified polymorphism (SRAP) is a PCR-based marker that generates multiple codominant markers for each amplification of open reading frames [23]. SRAP utilises both forward and reverse primers [32]. The SRAP forward primer, 17 bp in length, consists of a 14-nucleotide core region rich in C and G bases, along with three selective nucleotides at the 3' end, which enhance binding specificity to exon regions [33]. Conversely, the reverse primer, 18 bp in length, contains a 15-nucleotide core rich in A and T bases, with three selective nucleotides at the 3' end, increasing binding specificity to intron, spacer, and promoter regions [33, 34]. According to Jahangirzadeh et al [35], polymorphic variation primarily arises from differences in intron length, promoter regions, and the spacing between them. The combination of forward and reverse primers has the potential to create optimal pairings to maximise primer efficiency. In addition, SRAP markers have been used to detect genetic variation in *Vigna unguiculata* [23], *Mallotus oblongifolius* [36], and *Coffea canephora* [37]. Therefore, this study aims to assess the genetic diversity of porang after mutation induction using colchicine. Understanding and enhancing genetic diversity is essential for porang cultivation, as it provides a foundation for developing superior varieties with improved adaptability, resilience, and productivity.

METHODS

Sample preparation and in vitro propagation. Porang shoot explants (2n=26) were cultured on Murashige and Skoog (MS) medium supplemented with 2 mg.L^{-1} benzyl adenine (BA). The explants were kept at 21°C under continuous fluorescent light at an intensity of 1500 lux. After 30 days, the explants were prepared for polyploidy induction.

Polyploidy induction. Polyploidy induction was performed by supplementing colchicine into the culture medium at concentrations of 0%, 0.01%, 0.02%, 0.03% and 0.04%. Colchicine was added to 12.5 ml of MS medium using a 0.22 µm Millipore filter. After 7 days of colchicine treatment, the explants were moved to a colchicine-free MS medium. Following 53 days in the colchicine-free medium, the explants were prepared for observation.

DNA isolation. Fresh young porang leaves were used for DNA isolation. Total genomic DNA was extracted using the Tiangen Genomic DNA, which offers a detailed protocol for plant DNA isolation. The extracted DNA was then assessed for both quality and quantity. DNA quality was analysed through electrophoresis on a 1% agarose gel, while the AE-Nanodrop 200 Nucleic Acid analyser was utilised to measure DNA concentration.

DNA amplification. DNA Amplification was carried out with a PCR Thermocycler (BIORAD) using a 10 SRAP Primer combination (Table 1). PCR reaction contains 10 µl mixtures consisting of 3 µl double distilled water (ddH2O), 1 µl primers forward and reverse (10 pmol), 5 µl Go Taq Master Mix and 1 µl DNA template (20 ng/µl).

The SRAP-PCR amplification was performed with an initial denaturation at 94°C for 5 minutes, followed by five cycles of denaturation at 94°C for 1 minute, annealing at 35°C for 1 minute, and extension at 72°C for 1 minute. Subsequently, 35 cycles were performed with an annealing temperature increased to 50°C. A final extension step at 72°C for 7 minutes was included before holding the reaction at 4°C.

Data analysis. Genetic diversity analysis was conducted using POPGENE software version 1.32. Intra-population genetic diversity was assessed using parameters such as the number of observed alleles (Na), the number of effective alleles per locus (Ne), allele frequency, polymorphic loci, Nei's genetic diversity, expected heterozygosity (He), and the Shannon index. The Ne value was calculated using the formula:

$$Ne = \frac{1}{(1-h)} = \frac{1}{\sum \mathrm{pi}^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where pi represents the frequency of the i-th allele at a locus, and h = 1 - ∑pi² represents heterozygosity within the locus. Inter-population genetic diversity analysis included parameters such as total genetic diversity (HT), genetic differentiation among populations (GST), gene flow (Nm), and genetic distance.

Clustering analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and Jaccard similarity index in PAST software version 4.03. Primer efficiency analysis was conducted using the parameters Polymorphism Information Content (PIC), Effective Multiplex Ratio (EMR), and Marker Index (MI).

Table 1. SRAP primer combination used in this study

Primer	Sequence (5'-3')	Tm (°C)	Ta (°C)	GC (%)
SRAP ME1-EM1	TGAGTCCAAACCGGATA (F)	50	50	47.1
	GACTGCGTACGAATTAAT(R)	50		38.9
SRAP ME1-EM2	TGAGTCCAAACCGGATA (F)	50	50	47.1
	GACTGCGTACGAATTTGC (R)	54		50
SRAP ME2-EM3	TGAGTCCAAACCGGAGC (F)	54	50	58.8
	GACTGCGTACGAATTGAC(R)	50		38.9
SRAP ME2-EM2	TGAGTCCAAACCGGAGC (F)	54	50	58.8
	GACTGCGTACGAATTTGC (R)	54		50
SRAP ME2-EM1	TGAGTCCAAACCGGAGC (F)	54	50	47.1
	GACTGCGTACGAATTAAT(R)	54		50
SRAP ME3-EM2	TGAGTCCAAACCGGAAT (F)	50	50	47.1
	GACTGCGTACGAATTTGC (R)	54		50
SRAP ME3-EM3	TGAGTCCAAACCGGAAT (F)	50	50	47.1
	GACTGCGTACGAATTGAC(R)	52		44.4
SRAP ME3-EM4	TGAGTCCAAACCGGAAT (F)	50	50	58.8
	GACTGCGTACGAATTTGA (R)	54		50
SRAP ME4-EM2	TGAGTCCAAACCGGACC (F)	54	50	58.8
	GACTGCGTACGAATTTGC (R)	54		50
SRAP ME4-EM3	TGAGTCCAAACCGGACC (F)	54	50	58.8
	GACTGCGTACGAATTGAC(R)	52		44.4

Note: Tm = Melting temperature, Ta = Annealing temperature, GC % = Guanine & Cytosine composition, F = Forward primer, R = Reverse primer.

Each primer was analysed to determine its PIC value, which was calculated using the formula:

$$PIC = 2(f)(1-f) \dots (2)$$

where PIC represents polymorphism information, f is the frequency of the observed band fragment, and (1-f) is the frequency of the absent band marker.

The Effective Multiplex Ratio (EMR) was used to determine the effective ratio of the total observed bands to the number of polymorphic bands. It is calculated using the formula:

$$EMR = \beta \dots (3)$$

where β represents the total number of bands per primer, and the value of β corresponds to the number of polymorphic bands.

The Marker Index (MI) is calculated using the formula:

$$MI = PIC \times EMR \dots (4)$$

RESULTS AND DISCUSSION

Increased genetic diversity of porang after mutation induction with colchicine. The induction of mutations using colchicine has successfully increased the genetic diversity of Porang. This improvement is evidenced by the number of polymorphic alleles, with 22 out of 33 alleles (66.6%) showing polymorphism. Furthermore, the increase in genetic diversity is supported by the percentage of polymorphic bands, which rose from 3.03% in the control group to 54.55% in the colchicine-treated group (Table 2). A higher proportion of polymorphic bands reflects greater genetic variation.

Table 2. The effect of colchicine on the genetic variation of porang

	Normal porang	Colchicine-induced porang
P (%)	3.03	54.55
Total sample	3	10
Na	1.0303 ± 0.1741	1.5455 ± 0.5056
Ne	1.0130 ± 0.0745	1.3025 ± 0.3911
h	0.0091 ± 0.0522	0.1735 ± 0.2036
I	0.0144 ± 0.0830	0.2617 ± 0.2864

Note: P (%) = percentage of polymorphic bands; N = number of samples; Na = number of alleles; Ne = number of effective alleles; h = genetic diversity index; I = Shannon's index.

The increasing genetic diversity of porang after mutation induction using colchicine was also detected via RAPD [13] and ISSR [38] molecular markers. This aligns with studies showing colchicine disrupts spindle formation, causing polyploidy and chromosomal rearrangements [39] that introduce new alleles and DNA sequence variations and subsequently increase the genetic diversity of porang. A similar phenomenon was also detected in soybean [40] and hard wheat [41], where colchicine-induced mutation led to severe chromosomal damage, causing increasing genetic variation.

The improvement in Porang's genetic diversity is further demonstrated by the increase in both the number of alleles (Na) and the number of effective alleles (Ne) in the treatment group relative to the control. The Na value rose from 1.0303 to 1.5455, representing a 50% increase, while the Ne value grew from 1.0130 to 1.3025, corresponding to a 28.52% rise. The upward trend in both parameters indicates that the treatment successfully enhanced allele abundance, leading to greater genetic variation [42].

The heterozygosity index (h) increased from 0.0091 in the control group to 0.1735 following colchicine-induced mutation. Based on Nei's [43] classification, the control group exhibited very low heterozygosity ($h<0.30$), indicating a predominantly homozygous population. In contrast, although the treated group's heterozygosity value remained within the low category, the increase to 0.1735 signifies a measurable improvement in genetic variation. This elevated heterozygosity suggests that colchicine treatment contributed to greater genetic variability compared to the untreated population [44].

Furthermore, overall genetic diversity among populations also showed an upward trend. The total genetic diversity (Ht) reached 0.2161 ± 0.0428, while the within-population heterozygosity (Hs) was 0.0913 ± 0.0111 (Table 3). The disparity between Ht and Hs values indicates that approximately 42.27% of the total genetic variation resides within populations, whereas 57.73% is distributed among different populations. The greater Ht relative to Hs underscores that genetic differentiation among populations is more pronounced than within-population diversity [45].

Table 3. The effect of colchicine on genetic variation between the porang population

Parameter	Value
Ht	0.2161 ± 0.0428
Hs	0.0913 ± 0.0111
Gst	0.5775
Nm	0.3658

Note: Ht = total genetic diversity; Hs = heterozygosity within the population; Gst = genetic diversity coefficient among populations; Nm = gene flow

The rise in genetic diversity is further reflected by the genetic diversity coefficient among populations (Gst), which was found to be 0.5775. This indicates that 57.75% of the genetic variation exists between populations, while 42.25% is found within individual populations. The elevated Gst value suggests a significant level of genetic variation between populations [46]. In contrast, the gene flow (Nm) between populations was recorded at 0.3658, suggesting that only 36.58% of alleles migrate among populations, whereas 63.42% face movement barriers. This low gene flow contributes to the elevated Gst, implying a degree of genetic isolation among the populations [47].

Mutation induction using colchicine also led to the formation of new alleles that contribute to increased genetic variation. The appearance of new alleles in the treated samples indicates DNA polymorphism, as evidenced by differences in banding patterns between the control samples and those subjected to colchicine induction. The SRAP primer combination ME4-EM2 produced a DNA band at 500 bp that was absent in the control samples (A1, A2, A3) but present in the treated samples (B1-E1) (Figure 1A).

A similar result was also observed in primer combination ME2-EM3, where the control samples (A1, A2, A3) did not show a DNA band at the 200 bp locus, while the treated samples (B1-E1) displayed a different banding pattern (Figure 1B). The disappearance of DNA bands or the emergence of new bands is most likely due to changes in the nucleotide sequence caused by colchicine-induced mutation [48]. These findings further confirm that colchicine treatment effectively induces genetic variation at the molecular level. The appearance of unique DNA fragments in treated samples highlights the potential of colchicine as a mutagenic agent for plant breeding and genetic studies.

In this study, colchicine-induced mutations in porang resulted in the emergence of new alleles and distinct DNA banding patterns, reflecting an increase in genetic variation at the molecular level. These results align with findings from *Calendula officinalis* [49], where colchicine application not only modified morphological characteristics but also enhanced genetic diversity, with a genetic diversity coefficient (Gst) reaching 30%. The increase in variation observed in both cases is likely due to colchicine's ability to disrupt spindle fibre formation during mitosis, leading to chromosome doubling and the generation of new genetic variants. Overall, these findings demonstrate colchicine's potential as an effective mutagenic agent for promoting genetic diversity.

Clustering analysis of porang after colchicine mutation induction. Clustering analysis confirms the results that colchicine induction is able to increase the genetic diversity of porang both within and between populations. Clustering analysis of porang following colchicine mutation induction resulted in five clusters (Figure 2). Cluster I includes the 0.01% colchicine treatment group (B1, B2, and B3), which

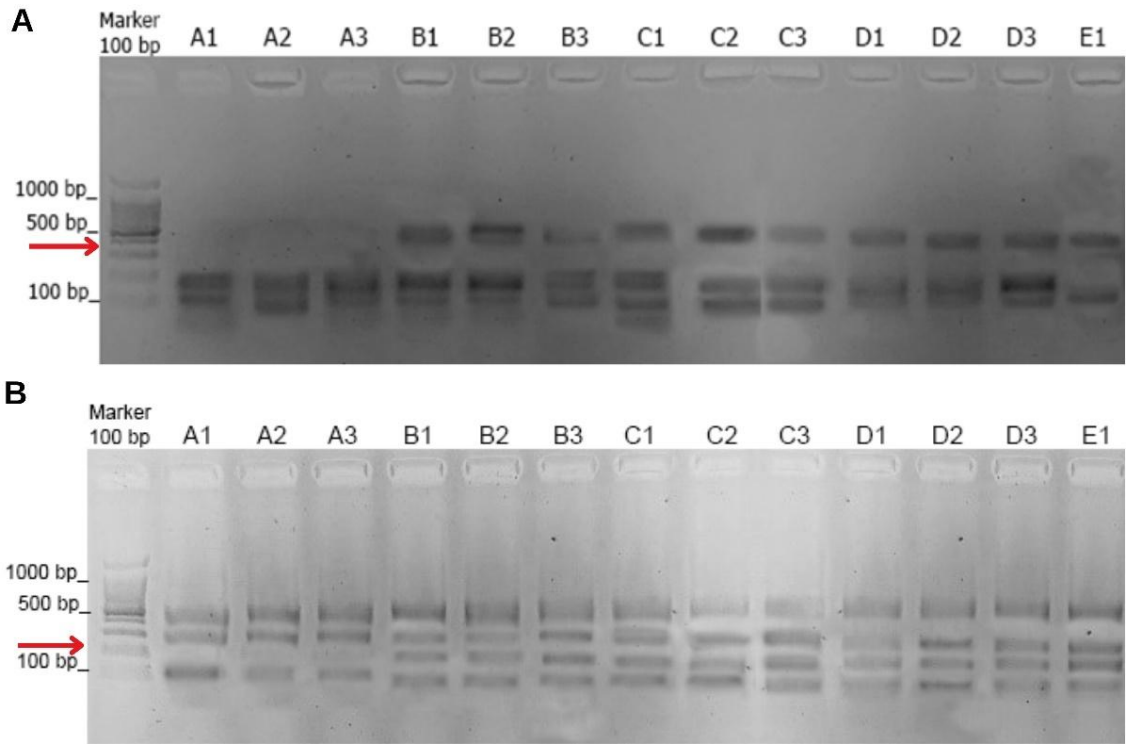

Figure 1. Polymorphic bands after mutation induction using colchicine. (A) ME4 – EM2 (B) ME2 – EM3. Arrows indicate polymorphic bands where bands appear in some loci but do not appear in others. A1: Control (A1-S3) Colchicine 0.01% (B1-B3), Colchicine 0.02% (C1-C3), Colchicine 0.03% (D1-D3): Colchicine 0.04% (E)

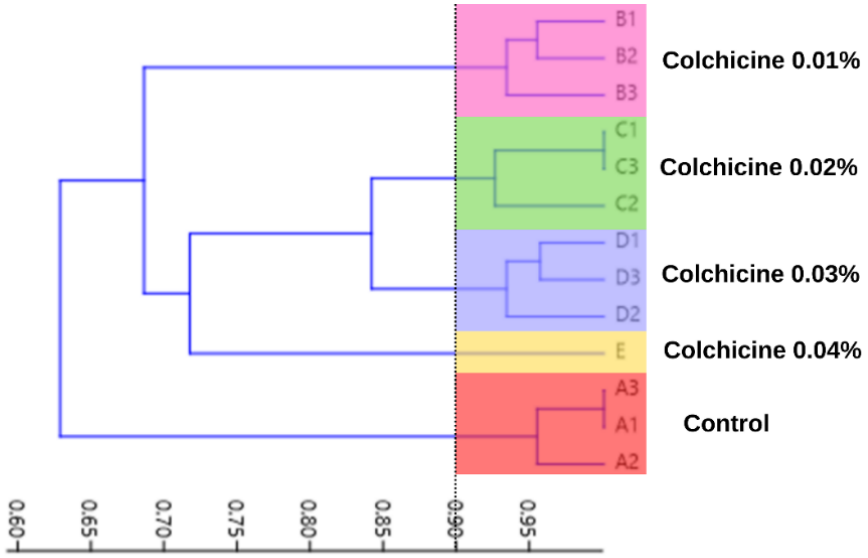

Figure 2. Cluster analysis of porang after mutation induction using colchicine

clustered together with a similarity value of around 0.90.

This clustering further supports the Jaccard similarity analysis (Table 4), which indicates a high genetic similarity (0.91–0.95) among the samples treated with 0.01% colchicine. Cluster II, which consists of the 0.02% colchicine treatment group (C1, C2, and C3), shows consistency with the previous similarity results, as these samples exhibit high similarity values (0.93–1.00).

Cluster III is formed by the colchicine 0.03% treatment group (D1, D2, and D3), which separates from clusters I and II with a similarity value of around 0.70. This indicates that increasing the colchicine concentration to 0.03% results in more significant genetic changes compared to lower concentrations. The colchicine 0.04% treatment (E) forms a separate cluster IV, which is distinct from the other clusters with a similarity value of around 0.65, suggesting that this highest concentration produces the most distinct genetic variation compared to the other concentration groups.

Table 4. Jaccard similarity coefficient between normal and mutated porang

	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3	E
A1	1.00												
A2	0.95	1.00											
A3	1.00	0.95	1.00										
B1	0.65	0.63	0.65	1.00									
B2	0.68	0.65	0.68	0.95	1.00								
B3	0.65	0.69	0.65	0.91	0.95	1.00							
C1	0.70	0.68	0.70	0.74	0.77	0.74	1.00						
C2	0.66	0.63	0.66	0.69	0.71	0.69	0.93	1.00					
C3	0.70	0.68	0.70	0.74	0.77	0.74	1.00	0.93	1.00				
D1	0.57	0.55	0.57	0.67	0.69	0.67	0.85	0.85	0.85	1.00			
D2	0.56	0.54	0.56	0.65	0.68	0.65	0.84	0.78	0.84	0.91	1.00		
D3	0.59	0.57	0.59	0.69	0.72	0.69	0.88	0.81	0.88	0.96	0.95	1.00	
E	0.57	0.60	0.57	0.55	0.57	0.60	0.76	0.71	0.76	0.69	0.68	0.71	1.00

Note: A1: Control(A1-S3) Colchicine 0.01% (B1-B3), Colchicine 0.02%(C1-C3), Colchicine 0.03% (D1-D3): Colchicine 0.04% (E)

The comparison between colchicine concentration groups shows a structured Jaccard similarity pattern. The observed pattern indicates that as the colchicine concentration increases, the similarity value decreases. The similarity value between the control group (A1-A3) and the 0.01% colchicine group (B1-B3) ranges from 0.63 to 0.68, while the 0.02% colchicine group shows a similarity value between 0.91 and 0.95. The colchicine 0.03% group (D1-D3) decreases to 0.55-0.59. The Jaccard similarity pattern suggests that increasing colchicine concentration is directly proportional to the level of induced genetic change. The colchicine 0.04% treatment group (E) shows relatively low similarity values (0.55-0.76) compared to all other treatment groups, indicating that this highest concentration induces more significant genetic changes.

Normal and colchicine-induced porang are grouped based on the treatments applied (Figure 2). The clustering of 13 porang samples into 5 clusters, with similarity values ranging from 0.54 to 0.90, indicates significant genetic differences due to colchicine induction. The resulting dendrogram structure also shows a systematic variation in genetic changes, where the formed clusters are organised according to the colchicine dose applied to the samples (Figure 2). This pattern provides strong evidence that mutation induction using colchicine can generate measurable and controlled genetic variation in porang, with the variation level being predictable based on the colchicine concentration and dose used. Research conducted by [48] showed that using colchicine mutagen at different dose concentrations can influence the level of genetic variation in sugarcane. Determining the colchicine dose is crucial as a basis for porang breeding strategies, particularly in identifying the optimal colchicine concentration to generate the desired genetic variation and obtain superior porang.

Cluster analysis using SRAP in this study produced the same cluster topology with random amplified polymorphic DNA (RAPD) [13] and inter-simple sequence repeats (ISSR) [38], where the three markers were able to distinguish between normal porang and porang resulting from colchicine-induced mutation. However, SRAP is more effective in grouping porang between different colchicine treatments. SRAP's ability to distinguish grouping porang based on colchicine concentration, likely due to its targeting of open reading frames (ORFs) in exon regions [50]. These functional genomic areas are more prone to structural variations from colchicine's spindle disruption.

Nine out of ten primer combinations successfully amplified polymorphic DNA bands (Table 4). A total of 33 DNA bands with lengths ranging from 50 to 1000 bp were successfully amplified. The primer combination ME2-EM2 produced the highest number of DNA bands (6 bands), while ME1-EM2 produced the fewest (2 bands). A total of 22 polymorphic bands (64.6%) were formed, while 11 bands (35.4%) were monomorphic. The primer combination that produced the highest number of polymorphic bands was ME2-EM2 (Figure 3).

The ME2-EM2 primer combination proved to be the most effective, as it demonstrated an optimal balance between the total number of bands, the level of polymorphism, and high values of informative parameters, including PIC, EMR, and MI (Table 5). These findings are consistent with the study by [51], which emphasised that primer effectiveness should be evaluated based on multiple parameters such as PIC, EMR, and MI,

Table 5. Polymorphism analysis of SRAP molecular markers

No	Primer	TNB	NPB	PB (%)	PIC	EMR	MI
1	ME1-EM1	3	2	66.67	0.28	1.33	0.38
2	ME1-EM2	2	0.0	0.00	0.00	0.00	0.00
3	ME2-EM1	4	1	25.00	0.09	0.25	0.02
4	ME2-EM2	6	5	83.33	0.24	4.17	1.02
5	ME3-EM2	3	2	66.67	0.24	1.33	0.32
6	ME3-EM3	3	3	100	0.21	3.00	0.64
7	ME3-EM4	3	2	66.67	0.19	1.33	0.25
8	ME3-EM4	3	1	33.33	0.17	0.33	0.06
9	ME4-EM2	3	3	100	0.28	3.00	0.85
10	ME4-EM3	3	3	100	0.33	3.00	0.99
	Averages	3.3	2.2	64.17	0.20	1.78	0.45
	Total	33	22	641.67	2.04	17.75	4.53

Note: total number of bands (TNB), number of polymorphic bands (NPB), polymorphic band percentage (PB%), polymorphic information content (PIC), effective multiplex ratio (EMR), marker index (MI).

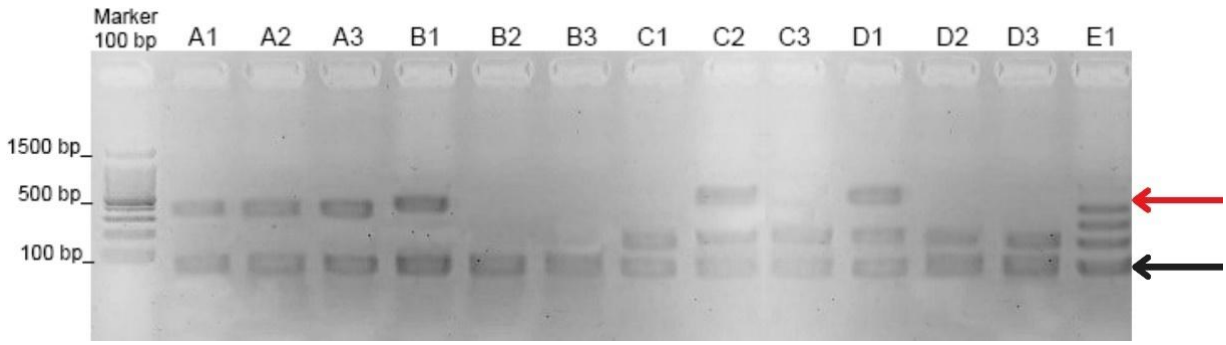

Figure 3. Polymorphic bands generated by the SRAP ME2-EM2 primer combination

rather than solely on the number of bands produced. However, the SRAP primer combination ME2-EM3 and ME4-EM2 (Figure 1) is more effective in evaluating allelic change through observation of DNA bands.

CONCLUSION

Colchicine-induced mutation effectively enhanced the genetic diversity of porang, as reflected by the increase in polymorphic alleles (66.6%), the genetic diversity coefficient among populations (57.75%), and gene flow between populations (36.58%). The increase in genetic diversity was further supported by clustering analysis, which showed that normal porang and colchicine-treated porang clustered based on the colchicine treatments. The SRAP primer combination ME2-EM2 proved to be the most efficient in detecting the induced genetic variation in porang. Mutation induction using 0.03% colchicine is highly recommended as it produces significant genetic changes compared to normal porang. The porang mutant resulting from 0.03% colchicine treatment holds great potential for porang breeding programs, as it can generate novel phenotypic variations and increase the chances of obtaining superior genotypes with desirable agronomic traits.

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