Research Article

Induction of Synthetic Polyploids of Porang (*Amorphophallus muelerri* Blume) and Assessment of Its Genetic Variability Using Morphological Data and RAPD Molecular Marker

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ABSTRACT

This study uses morphological characteristics and RAPD markers to evaluate the polyploidization of synthetic porang. Seeds of triploid porang (2n=2x=26) were soaked in the different colchicine concentrations for 24 hours. After colchicine treatment, the porang seeds were planted to an MS medium that contained 2.2 μM of 6-benzylaminopurine (BAP), then, 40 days after planting in the MS media, the morphology and molecular of synthetic polyploid porang were characterized. For DNA extraction, a total of 100 mg of young leaves of porang plantlet was collected. One way Anova followed by the Duncan test (95%) was performed for phenotypic characterization. The number of different alleles, number of effective alleles, Shannon's information index, diversity, and unbiased diversity were assessed for genetic diversity. Synthetic polyploid porang has a higher total shoot, root, and wider leaves than normal porang. Polyploidy induction also successfully increased the genetic diversity of porang, and the genetic diversity will increase porang adaptability and sustainability of porang cultivation.

INTRODUCTION

Porang (*Amorphophallus muelerri*) known as tuberous plant is a member of the *Araceae* family. (Wahyudi et al. 2013). Porang tuber contains the highest glucomannan compared to the other genus in family of *Araceae* (Ekowati et al. 2015). Glucomannan is a hemicellulose that is hydrocolloid and easily soluble in water (Nurlela et al. 2021), so it is widely used for food ingredients (Tester & Al-Ghazzewi 2017) and emulsifier in the industrial product (Li et al. 2018). Furthermore, glucomannan is also beneficial in the food sector as used as an ingredient for shirataki, konyaku, edible film, and artificial rice (Tester & Al-Ghazzewi 2017). In the industrial sector, glucomannan is widely used as material for immobilization, fixation support, and encapsulation (Yang et al. 2017). Because of its many benefits, making porang a major export commodity in Indonesia

(Atase Perdagangan KBRI Tokyo 2021).

Through the Ministry of Agriculture, the Indonesian government has instructed to cultivate porang throughout the archipelago. However, most farmers have cultivated the porang vegetatively using bulbils and tubers recently. Unfortunately, these types of cultivation cause porang in Indonesia to have a low genetic variation (Wahyudi et al. 2013; Nikmah et al. 2016). Alternatively, porang farmers use seeds as a seedling. Still, because porang seeds are apomixis, the resulting plants are either genetically similar to their parents or have a limited range of traits. That will have an impact on the sustainability of porang cultivation in Indonesia. Therefore, efforts to increase porang genetic variation either through polyploidy induction (Touchel et al. 2020) or mutation induction are urgently needed.

Naturally, over the course of their evolutionary history, the majority of Angiosperms double their genomes once or more (Aversano et al. 2012). However, polyploidy can be synthetically triggered with the aid of colchicine, oryzalin, and trifluralin (Touchel et al. 2020). Still, polyploid induction with colchicine was more successful than with oryzalin and trifluralin (Talebi et al. 2017). Colchicine is an alkaloid that causes mutations and doubles plant chromosomes (Alkadi et al. 2018) by inhibiting the anaphase stage. Inhibition of anaphase causes spindle fibers to bind to tubulin, resulting in the chromosomes not being separated, which eventually causes cells to contain multiple chromosomes (polyploidy) (Miri 2020). Colchicine has been frequently used as a chemical agent to induce polyploidy and has succeeded in increasing the productivity of flowering plants (Manzoor et al. 2019), herbal plants (Madani et al. 2019) and horticultural plants (Eng & Ho 2019)

According to a report, after polyploidization, synthetic polyploids displayed quick alterations in the genomic organization and gene activity (Song & Chen 2015). Polyploid plants' genotypes can alter as a result of epigenetic and genetic interactions, heterozygosity, gene silence, and gene dosage effects (Miri 2020). Losing duplicated genes, DNA sequence changes, rearrangements of the structural chromosome, and gene conversion are some examples of genomic modifications after polyploidy induction (Ding & Chen 2018). Some morphological characteristics including plant height, leaf and stomata morphology, flowering time, biomass, and tuber size are also the affected trait after polyploidy induction (Sattler et al. 2016).

The detection of polyploidy level is categorized as direct (Miri 2020) and indirect method (Wibisono et al. 2021). The direct method, which counts the total of chromosomes during metaphase of cell division using cytogenetic techniques, is frequently time-consuming and requires quite specific procedures for every species (Sattler et al. 2016). In addition, high chromosome number and small chromosome size are also a shortcoming of direct method by using cytogenetic (Guo et al. 2016). Indirect methods like morphological, physiological, and molecular markers are additional methods of addressing the direct method's shortcomings in detecting polyploidization in the plant (Sattler et al. 2016). Normally, the morphological assessment considers the quantity, total, and size of leaves and shoots (Salma et al. 2017). The polyploid synthetic plant commonly has a bigger flower, larger fruit, and tuber and ticker leaf than the normal plant (Zang et al. 2018).

Advances in molecular technologies have opened a new perspective for determining polyploidization. For example, Guo et al. (2016) have succeeded in developing 10 single copies of fully informative SSRs for detecting ploidy levels in polyploid willow. With the help of this analytical tools, polyploids plant may be promptly screened, and precise ploidy

levels can be efficiently confirmed by flow cytometry (FCM). Also, Aliyev et al. (2007) used random amplified polymorphic DNA (RAPD) to detect diploid and tetraploid wheat. Furthermore, Wahyudi et al. (2020) successfully used the RAPD marker to detect mutant soybean after being induced by ethyl methyl sulfonate (EMS). Gene redundancy after polyploid induction may be the reason why dominant molecular markers like RAPD is widely used for polyploidy detection (Aversano et al. 2012). Therefore, the current study aims to assess the polyploidization of synthetic porang by using morphological character and RAPD marker.

MATERIALS AND METHODS
Plant Material, polyploidy induction, and in vitro propagation
Six treatments with four replications, and a completely randomized design were used in this study. Seeds of triploid porang (2n=2x=26) were used for chromosome duplication. Total of 72 of porang seeds were soaked in the variation of colchicine concentration, including 0, 0.05, 0.1, 0.15, 0.2, and 0.25 ppm for twenty hours in dark condition. After colchicine treatment, porang seed was transferred to an MS medium supplemented with 2.2 µM of 6-benzylaminopurine (BAP). All cultures were preserved at 25±2°C under 24-hour white illumination (1,500 lux) for 40 days. After 40 days in MS medium plantlets were ready for analysis.

Phenotypic characterization
Forty days after planting in the MS media, the morphology of synthetic polyploid porang was characterized. The total root and shoot, the height of shoot, and the length and width of leaves were calculated to compare normal and synthetic porang. The height of shoot was measured with the ruler from the shoot base to the highest of shoot. The length of leaves was measured with the ruler from the base to the tip of leaves whereas the width of leaves was measured from the widest side of the leaves. Young leaves were then stored for DNA isolation.

DNA Extraction and PCR RAPD
100 mg of young leaves of porang plantlet was collected for DNA isolation. The DNA was extracted using The Wizard® Genomic DNA Purification Kit Promega following the manufacturer's guidelines for plants. The quality of total DNA was then assessed by electrophoresis on 1% agarose gel for DNA isolation and 1.5 % for PCR RAPD and ladder 1-Kb (Thermo Scientific) was used as a marker.

   PCR-RAPD was carried out with PCR Thermocycler (BIORAD) using 17 OPA (Operon Technology Ltd) primers (OPA 1 -17). PCR reaction contains 10 µl mixtures consisting of 3 µl double distilled water (ddH2O), 1 µl primers of OPA 1-18 (10 pmol), 5 µl MyTaq HSRed Mix and 1 µl DNA template (20 ng/µl). The temperature profile of PCR consists of one cycle at 94 °C for 4 minutes, followed by 45 cycles of amplification. Each amplification cycle had denaturing step at 94 °C for 30 seconds, an annealing (the temperatures of OPA 1-17 were followed the research of Probojati et al. (2019)) for 30 seconds and terminated with extension step at 72 °C for 5 minutes. The final extension was performed at 72 °C for 7 minutes. The PCR products were then examined by gel electrophoresis (1.5%) followed by Sybr green staining, with a DNA ladder 100 bp (NexView) for marker. DNA bands were photographed under a UV transilluminator (BioRAD).

Data analysis
One-way Anova followed by the Duncan test (95%) was performed for

phenotypic characterization. The presence or absence of a certain DNA fragment was indicated by a score of 1 or 0 on the RAPD bands. To create the data matrix, only amplification bands that were repeatable and clear were rated. The matrix data (score 0 and 1) was then used to detect the genetic diversity of normal and synthetic polyploid porang. Genetic diversity was analyzed using GenAlEx 6.5 software (Peakall & Smouse 2012). The genetic diversity was assessed based on Na = No. of Different Alleles, Ne = No. of Effective Allele, I = Information Index, h = Diversity, and uh = Unbiased Diversity. Furthermore, the data matrix was also used to cluster normal and synthetic polyploid porang. Unweighted pair group method with an arithmetic mean (UPGMA) algorithm followed by Jaccard's coefficient similarity (Hammer et al. 2001) was used for clustering analysis. Clustering analysis was performed in PAST (Paleontological Statistics) software. The percentage of polymorphism was determined by dividing the polymorphic bands of each primer by the sum of the scored bands x 100. The polyploidy was also assessed by comparing the band thickness of each primer and the presence of a band between normal and synthetic polyploidy of porang.

RESULTS AND DISCUSSION
Effect of colchicine on the morphology of synthetic polyploids of porang
The application of colchicine with different concentrations affects the growth of porang, especially the number of roots and shoots, shoots height and length, and width of leaves (Table 1). The most roots, as well as the longest and widest leaves in synthetic polyploids of porang showed at a concentration of 0.1 ppm, whereas the highest number and height of shoot was observed at a concentration of 0.15 ppm (Table 1). Applying colchicine above 0.1 ppm reduces the number of roots and length and width of leaves, whereas the concentration of colchicine above 0.15 ppm reduces the total and height of shoots (Table 1).

The increasing number of roots and shoots of the synthetic polyploid plant was also observed in *Dracocephalum kotschyi* Boiss (Zahedi et al. 2014) and *Sphagneticola calendulacea* (L.) (Kundu et al. 2018), where Tetraploid plants had much more roots than diploid plants. However, applying colchicine at high concentrations will negatively impact the plants because it will inhibit root growth (Manzoor et al. 2019) and reduce the percentage of root formation (Miri 2020). This phenomenon was also observed in this study, where the concentration of colchicine above 0.1 ppm decreased the formation of roots (Table 1).

Moreover, the length and width of leaves are also impacted by colchicine treatment (Table 1) (Figure 1). Talei et al. (2020) also stated a similar result on polypoid induction of *Stevia rebaudiana*. Different colchicine concentrations significantly affected to the total and length of leaves of *Stevia rebaudiana*. The increasing number of leaves will increase the stomata size and density, providing more efficient water use or transpiration rates (Wei et al. 2019).

Table 1. Colchicine's effect on the growth of synthetic polyploid porang.

Concentration	Roots number	Shoots number	Shoot height	leaf length	leaf width
0 ppm	1.875a	4.6625a	1.93a	1.2875a	0.8125a
0.05 ppm	3ab	4.745a	3.6075ab	3.425ab	2.1626ab
0.1 ppm	**5.1650b**	7,9975ab	6.315bc	**5.655b**	**4.03b**
0.15 ppm	3.9575ab	**10.9950b**	**9.6425c**	4.9325b	3.3b
0.2 ppm	2.665a	6.83ab	5.175ab	3.9ab	2.5ab
0.25 ppm	2a	4.58a	3.2925ab	3.2125ab	2.425ab

Note: Different letters indicate significantly different based on the 5% Duncan's test

Some polyploids are well-known for producing larger organs such as flowers, fruits, and especially the leaves (Eng & Ho 2019). This phenomenon, known as the "Giga" effect, causes cell size to increase as a result of the chromosome duplication (Sattler et al. 2016). As a result, the farmers will invest less in resources (such as fertilizer and plant growth regulators) to generate a larger crop, which can help reduce dangerous chemical residues (Manzoor et al. 2019). As a result, polyploidization could be advantageous for the agronomically-intensive industries of pomiculture, floriculture, and horticulture (Eng & Ho 2019). Likewise, in this study, increasing the number of roots, the size of the leaves, and the height of the shoots are expected to increase the porang tubers' sizes, so the results of this study will be very beneficial for farmers.

Natural and synthetic polyploid plants are thought to respond to environmental change more effectively than diploid ones. Nonetheless, specific reactions are more typical than predicted tendencies (Mtileni et al. 2021). Notably, polyploid plants are frequently more resistant to stressors such as drought (Li et al. 2021) and salinity (Chao et al. 2013). As a result, chromosome doubling induction is a common technique for increasing both phenotype and genotype traits (Breseghello et al. 2013). Polyploidy induction has been utilized with a variety of plants *Agastache foeniculum* (Talebi et al. 2017), *Musa* spp. (Khamrit & Jongrungklang 2022), *Physalis peruviana* (Comlekcioglu & Ozden 2019), and *Capsicum annuum* (Tammu et al. 2021) and proven to improve their agronomic value.
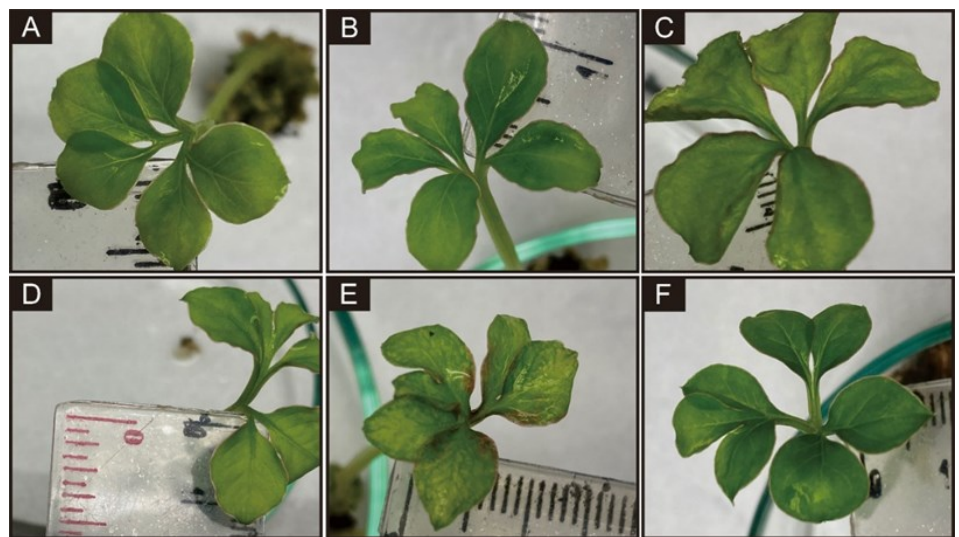

Figure 1. Effect of various concentrations of colchicine on length and width of leaves. a. 0 ppm b. 0.05 ppm c. 0.1 ppm d. 0.15 ppm e. 0.2 ppm f. 0.25 ppm.

Colchicine application also affects the number and height of shoots (Table 1) (Figure 2). However, colchicine application of more than 0.15 ppm decreased shoot formation and height of the shoot. Differences in shoot growth in synthetic polyploids of porang may be caused by the reorganization and restructuring of the polyploid plant genomes, resulting in alteration in genes activation (Soltis et al. 2015). The increased cell dimensions after genome duplication can be described as a sequence of downstream effects (Ruiz et al. 2020). However, it is yet unknown whether these cellular modification affect the overall phenotype and function of the organism (Madani et al. 2021).

Polyploid is a natural phenomenon and has long been acknowledged as an important factor in the diversification of Angiospermae (Soltis et al. 2015). The more genetic variation present in a single polyploid individual than in their diploid ancestors is frequently cited as the

reason for polyploids' "success" (Soltis et al. 2014). Moreover, this genetic diversity might result in novel biochemical, physiological, morphological, and ecological traits, giving polyploids an advantage over their diploid parents in the short term (Mtileni et al. 2021).

Polyploidy is an essential tool in plant breeding and has been commonly used by breeders to increase agronomic interest (Julião et al. 2020). The increase of agronomic interest was signed by the change in plant's morphological characteristics that can be accomplished by modification both chromosome and gene numbers in a cell. Polyploidy is a common phenomenon that is found in over 80% of plants and contributes for 2–4% in flowering plants speciation (Madani et al. 2021).

Figure 2. Effect of various concentrations of colchicine on the number and length of the shoot. a. 0 ppm b. 0.05 ppm c. 0.1 ppm d. 0.15 ppm e. 0.2 ppm f. 0.25 ppm.

There are three principal advantages of polyploidy induction, including heterosis (Bansal et al. 2012), gene redundancy (Roulin et al. 2013), and asexual reproduction (Manzoor et al. 2019). In contrast to their diploid parents, heterosis on synthetic polyploids produces robust polyploid plants (Bansal et al. 2012). Gene redundancy, on the other hand, preserves polyploid synthetic plants from the harmful effects of mutation. However, the induction of polyploid plants has a disadvantage, including the interrupting effects of nuclear and cell enlargement, the epigenetic instability that leads to gene regulation changes, and meiotic errors (Madani et al. 2021).

Polyploidy induction using colchicine increase genetic diversity of synthetic polyploids of Porang

A total of 36 loci were successfully amplified using 16 RAPD primers. OPA 15 produced the fewest band (1 band), and OPA 11 produced the most bands (4 bands) (Figure 3). The distinctive band between normal and synthetic polyploid porang was observed in OPA 7 (600 and 700 bp) and OPA 13 (1000 bp) (Figure 3). The appearance of a new band (new

allele) in synthetic polyploids of porang was detected in OPA 11 (600 bp) (Figure 3). The thickening band between normal and synthetic polyploid porang was also detected (600 and 700 bp in OPA 7 and 13) (Figure 3).
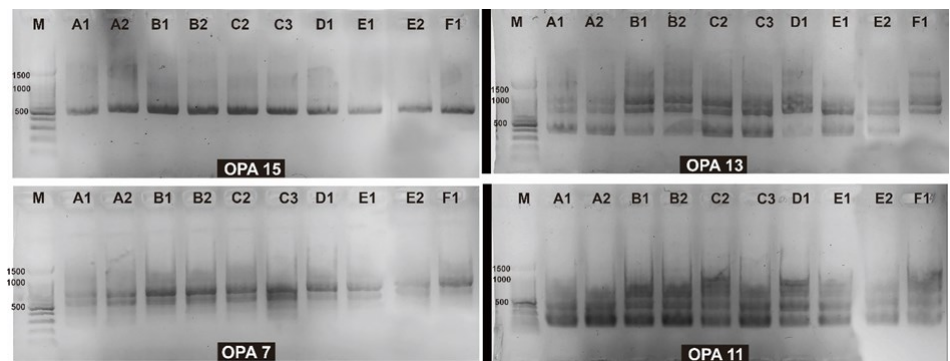

Figure 3. RAPD amplification profile of normal and synthetic polyploid porang. A1 and A2: 0 ppm, B1 and B21: 0.05 ppm, C1 and C2: 0.1 ppm, D1 and D2 0.15 ppm, E1 and E2: 0.2 ppm and F1: 0.25 ppm.

Polyploids are able to be identified based on characteristic of morphology (Miri 2020) and physiology (Fu et al. 2021) with limited precision. Otherwise, we can count the chromosome number under a microscope to directly identify polyploids (Moghbel et al. 2015) or use a flow cytometer to assess DNA concentration (Guo et al. 2016). However, these procedures are laborious and time-consuming, especially when working with many samples. In contrast to these traditional methods, molecular markers offer a highly effective and trustworthy way to select polyploids on a wide scale from natural stands (Aversano et al. 2012).

The emergence of the new allele in synthetic polyploid was also detected in *Lippia alba* (Julião et al. 2020) and *Glycine max* L (Roulin et al. 2013). Genome duplication or redundancy, that enables one of the copies of a chromosome to accumulate mutations (del Pozo & Ramirez-Parra 2015), might explain why synthetic polyploid porang has a new allele and thickening band. In addition, of course, these genetic and epigenetic changes result in the evolution of novel features, which boosts adaptability (Roulin et al. 2013).

A total of 36 loci were then used to detect the genetic diversity of normal and synthetic polyploid (mutant) porang. The Gene diversity index and the percentage of polymorphic loci are general parameters used as indicators of the genetic richness of the taxa. Synthetic polyploid porang has a higher Shannon index value and a percentage of polymorphic loci than normal porang (Table 2). Furthermore, synthetic polyploid porang also has higher diversity and unbiased diversity value than normal porang (Table 2). This result indicated that polyploid induction using colchicine successfully increases the genetic diversity of porang.

Polyploidy induction has been shown to promote genetic diversity by establishing breeding lines in short order and rehabilitating hybrid fertility (Pereira et al. 2014). The increasing genetic diversity after polyploid induction seems to be a general phenomenon that has also been observed in *Lippia alba* (Julião et al. 2020), *Gerbera hybrida* (Bhattarai et al. 2021) and *Pogostemon Cablin* (Afifah et al. 2020). Increasing genetic diversity will bring several benefits and improvements in various contexts, including plant adaptability, disease resistant and increasing productivity and yield.

Three main clusters were divided into normal porang and induced polyploid porang (Figure 4). The first cluster comprises normal porang (control), whereas the second and third clusters contain induced poly-

Table 1. Colchicine's effect on the growth of synthetic polyploid porang.

Parameter	Normal porang	Synthetic polyploid porang
P (%)	27.78	88.89
Na (mean ± SE)	1.278 ± 0.076	2.167 ± 0.102
Ne (mean ± SE)	1.278 ± 0.076	1.846 ± 0.083
I (mean ± SE)	0.193 ± 0.052	0.635 ± 0.049
He (mean ± SE)	0.139 ± 0.038	0.413 ± 0.030
uHe (mean ± SE)	0.278 ± 0.076	0.472 ± 0.035

ploid porang (Figure 4). The second cluster consists of induced polyploid porang with a concentration (0.05-0.15 ppm) and is observed to be morphologically different compared to normal porang (control) (Figure 1). This result indicated the RAPD marker's capability for genotyping synthetic polyploid plants. This result also recommends using 0.05-0.15 ppm of colchicine when inducing polyploid in porang.
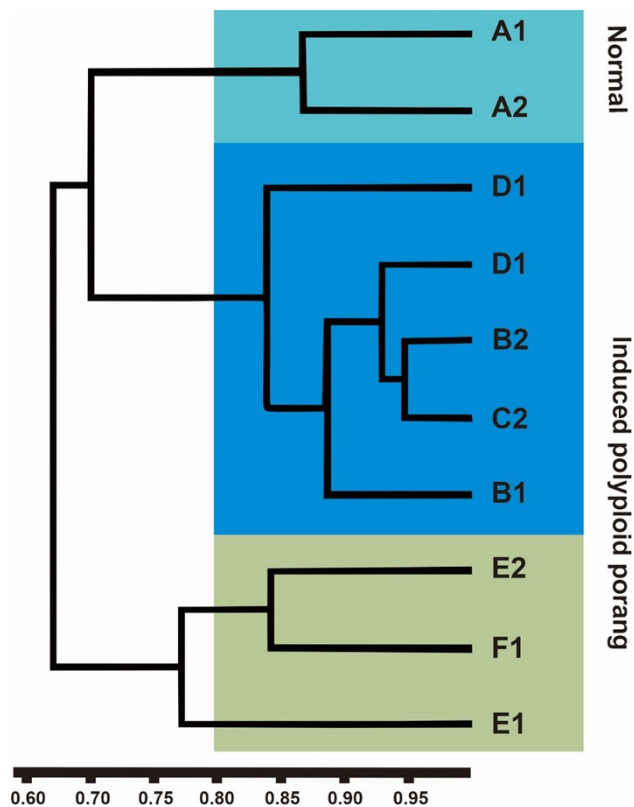

Figure 4. Clustering analysis of normal and synthetic polyploid porang. A1 and A2: 0 ppm, B1 and B21: 0.05 ppm, C1 and C2: 0.1 ppm, D1 and D2 0.15 ppm, E1 and E2: 0.2 ppm and F1: 0.25 ppm. Note: Number below the scale shows the similarity value.

Clustering analysis (Figure 4) strengthens the finding that polyploidy induction is proven to increase genetic diversity (Table 2). This is undoubtedly profitable for porang cultivation since porang cultivation has recently been practised by most vegetative farmers using tuber and bulbils (Lontoh et al. 2019), so porang has low genetic diversity (Wahyudi et al. 2013). In addition, although porang produces seed, the seed is apomixis which causes porang seedlings to have the same character as their parent. Therefore, polyploidization is the only way to enhance the genetic diversity of porang.

CONCLUSIONS
In conclusion, polyploidy induction is successfully achieved in porang, resulting in porang with a higher total shoot, root, and wider leaves.

However, a colchicine concentration above 0.1 decreases the productivity of porang. Polyploidy induction also successfully increased the genetic diversity of porang; of course, the genetic diversity will improve porang adaptability and support the sustainability of porang cultivation. In addition, RAPD is a reliable marker capable of determining the polyploid on porang.

AUTHOR CONTRIBUTION
S., D.W., and R.S.R. conceived the presented idea, developed the theory, and performed the computations. I.T.I. and F. contribute in practical lab and data collection. All authors discussed the results and contributed to the final manuscript.

CONFLICT OF INTEREST
No Conflict of interest

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