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Identification of Cocoa (*Theobroma cacao* L.) Genetic Uniformity Through RAPD Molecular Markers

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Abstract

Theobroma cacao L. has been propagated through either generative or vegetative techniques. One of the vegetative methods is somatic embryogenesis. Somatic embryogenesis has been employed by the Indonesian Coffee and Cocoa Research Institute for producing cocoa seedlings. Plant breeding activities are hampered by the significant level of plant heterogeneity among their progeny. Mislabelling of genetic impurities can be an issue. Molecular markers can be used to detect genetic variation at an early stage. The most common marker is the random amplified polymorphic DNA (RAPD) molecular marker. The study aims to determine the polymorphic RAPD primers in the analysis of genetic uniformity between mother plants and the seedlings derived from somatic embryogenesis (SE). The analyzed samples consisted of twelve individuals: six mother plants and six seedlings derived from SE. The results revealed that the percentage of polymorphic bands was 100% with band sizes ranging from 295-2785 bp for primer GY169 while for primer GY107 percentage of polymorphic bands was 80% with band sizes ranging from 345-1678 bp. Primer GY169 and primer GY107 can be amplified and used for cocoa similarity and heterogeneity.

Keywords: *Theobroma cacao*, RAPD markers, polymorphism

INTRODUCTION

Indonesia is the world's 7th largest cocoa producer with a capacity of 200.000 tonnes (ICCO, 2023) over an area of ±1,44 million hectares (BPS, 2022). Cocoa plant development has been attempted both generatively and vegetatively (Wahyudi, 2015). Somatic embryogenesis (SE) is a commonly used technique for propagating cocoa seedlings (Nappu *et al.,* 2014).

The Indonesian Coffee and Cocoa Research Institute has conducted various experiments particularly tissue culture in producing superior cocoa seedlings through the SE technique (Winarsih *et al.,* 2003: Avivi *et al.,* 2010). Plant breeders' attempts to produce improved cocoa plants are further hampered by high plant heterogeneity among their progeny. The existence of cocoa seed propagation technology, specifically SE, makes it easier to produce cocoa seedlings (Pancaningtyas & Susilo, 2022).

Somatic embryogenesis is a strategy for propagating embryos formed from somatic cells without gamete fusion (Dwiyani, 2015). The advantages of cocoa seedlings derived from somatic embryogenesis include uniform

plant growth, high productivity, and rapid fruiting (Nappu *et al*., 2014). The cocoa seeds grown also have superior characteristics and are identical to their mother trees (Wahyudi, 2015).

Epigenetic variations can occur in tissue culture without changing the DNA sequence, generally associated with non-permanent (temporary) activation or silencing of genes (Debnath & Amrita, 2022). Epigenetic variations in SE cocoa can occur, this is related to cell rejuvenation (callus, primary embryo, secondary embryo, and plantlet regeneration) which causes DNA methylation levels to change (Gyamfi *et al.,* 2016). Previous research regarding epigenetic variations in blueberry plants regenerated through tissue culture showed an increase in antioxidant levels and SSR was analyzed molecularly to determine whether there was genetic variation, the results showed there were no genetic changes in the regenerants (Goyali *et al*., 2015).

Genetic impurity and mislabelling are issues in massive seedling production (Pancaningtyas & Susilo, 2022). High-yielding cocoa clones used as mother trees in plant propagation are required to maintain their genetic purity (genetic homogeneity) and clone characteristics (Cruz-Martinez *et al.,* 2017; Sulistiyorini *et al.,* 2018). Molecular markers can be used to identify genetic stability in plants since they are stable, located in all regions of the plant, and are not influenced by the environment (Sulistiyorini *et al.,* 2018). The RAPD molecular marker is used in random selection to determine polymorphic DNA fragments (Syahri *et al.,* 2019).

Cocoa seedlings propagated by somatic embryogenesis must be genetically identical to the mother tree. Based on this, this research aimed to determine the genetic uniformity between cocoa seedlings (SE) and mother cocoa plants through RAPD molecular markers with several primer selections. Research on primer cocoa selection is required to obtain direct reference information in analyzing cocoa genetic uniformity.

MATERIALS AND METHODS

The research was conducted from November 2022 to June 2023 at the Biotechnology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences , University of Jember; Bioscience Laboratory, Jember State Polytechnic; and Biotechnology Laboratory, Indonesian Coffee and Cocoa Research Institute. The research was carried out through several stages. The first of which is the morphological identification of the sample (flush color), followed by a series of DNA isolation, genomic DNA measurement, target DNA amplification, and target DNA visualization for PCR products

The examination required cocoa leaf samples (ICCRI 07, ICCRI 03 and Sulawesi 03) collected from the Indonesian Coffee and Cocoa Research Institute, Jember, and Quick-DNATM Plant/Seed Miniprep Kit (Zymo Research, D6020). Distilled water, agarose 1,25%, 100 bp DNA marker, and 2 RAPD primers (Table 1.). DNA quantification was performed to determine the purity of the acquired DNA (Pancaningtyas &Susilo, 2022). The absorbance ratio for DNA quantification is at λ 260 nm and λ 280 nm in the range of 1.8-2.0, values in this range indicate the high quality of DNA with the absence of contamination (Turahhmi *et al.,* 2021).

Selection of cocoa clones ICCRI 07, ICCRI 03 and Sulawesi 03 tested had high productivity and resistance to CPB pests and black pod disease. The three clones also continued previous research where DR 02, MCC 02, ICCRI 09, Sulawesi 01 and

Table 1. List of RAPD primer sequences.

Primer	Sequence 5'- 3'	Reference
GY169	CTAAGCTGCTTTTGTTTGAGC	Das *et al.,* 1996
GY107	GTTCAGGGCTGTTTATAG	Das *et al.,* 1996

Sulawesi 02 clones had been tested using the RAPD molecular marker by Pancaningtyas & Susilo (2022). The mother tree used must have been selected both in terms of tree morphology, age of the mother tree, and tree health (disease-free) with an age of about six years and accompanied by a plant breeder. Meanwhile, seedling selection is also considered regarding seedling morphology, seedling age, and seedling health with an age of about four months. Leaf sampling (flush) was carried out at leaf sitting positions 2 or 3 for both mother trees and seedlings and were selected to be good morphologically.

Genomic DNA isolation was carried out using liquid nitrogen with a sample of 0.2 g according to the manual protocol specified by the Quick-DNATM Plant/Seed Miniprep Kit. The optimized concentration of the RAPD primer used was 10 uM, and to get many polymorphic bands, temperature gradient polymerase chain reaction (PCR) was carried out first to get the right annealing temperature.

DNA amplification through a PCR method begins with making a cocktail. The required PCR cocktail is 20 µL. The cocktail preparation is by preparing the PCR *HS Red* master mix, adding 2 µl of RAPD primer (Table 1), adding 2 µL of template DNA, and 6 µL of ddH_2O was added. DNA amplification includes pre-denaturation (95 ^{0}C for 5 minutes), denaturation (94 ^{0}C for 30 seconds), annealing (50 ^{0}C), extension (72 ^{0}C for 60 seconds), and final extension (72 ^{0}C for 5 minutes) with 45 repetitions of the denaturation, annealing, and extension processes.

Analysis of the result of DNA amplification using electrophoresis, which has two stages: agarose gel preparation and running electrophoresis. The electrophoresis time conditions required 30 minutes at 100 volts. Following the completion of the process, DNA visualization is performed with an ultraviolet (UV) transilluminator to confirm the presence or absence of DNA bands.

Scoring data is employed in the data analysis. The data analyzed using the NTSYS-pc program (Hadiati *et al.,* 2018; Budi & Mawardi, 2021). The resulting DNA fragment (band) will be assigned a score of 1, while unformed DNA bands will be assigned a score of 0. The data will be evaluated for the percentage of polymorphic alleles created in each primer to reveal the percentage of polymorphic alleles formed in each primer. The band visible on the gel corresponds to the RAPD allele (Sitepu *et al.,* 2019). For measuring the molecular weight of DNA bands using the Gel Analyzer program, the formula for estimating the rate of polymorphic bands is as follows (Ahmed, 2021).

$$\% \text{ Polymorphic band} = \frac{Number\ of\ polymorphic\ loci}{Total\ number\ of\ loci} \times 100\%$$

RESULTS AND DISCUSSION

Morphological Identification of *T. cacao* L.

The *T. cacao* sample was characterized by comparing leaf flush phenotypes to literature sources from the Indonesian Coffee and Cocoa Research Institute (Wahyudi, 2015; Misnawi *et al.,* 2019). The results of the morphological observations showed that the reddish color of *T. cacao* leaves in the mother tree, ICCRI 07 clone, had a pink color (Figure 1.a). In contrast, the color

difference in the seedlings derived from SE was slightly reddish brown (Figure 1.d). ICCRI 03 clone had a leaf color that was reddish brown (Figure 1.b), while the seedling had a different color, which was light brownish green (Figure 1.e). The Sulawesi 03 clone had dark red leaf color (Figure 1.c), while the seedling had pale green, slightly reddish leaf color (Figure 1.f). The leaves of *T. cacao*, both mother tree and seedlings derived from SE, have the same leaf shape, which is round, elongated, with pinnate type/bone arrangement. The leaves of *T. cacao* have a tapered tip and base. The surface and edges of the leaves of *T. cacao* are rough and wavy (Figure 1). Based on the results of the morphological characterization of *T. cacao* leaves on Bangka Island, Bangka Belitung Islands, are that they have tapered leaf tips and bases with wavy leaf edges (Zasari & Rostiar, 2022). According to Rindu *et al.* (2021), the leaf shape of *T. cacao* in Lima Puluh Kota district of West Sumatra, namely oblong or elongated round.

The results of the research found differences in leaf flush color between the mother tree and seedling (SE) in the three clones (ICCRI 07, ICCRI 03 and Sulawesi 03) and this case was also found. According to Gesty (2022), morphological differences were found in fruit, flowers and leaf flush *T. cacao* resulting from somatic embryogenesis (4 years old) with the mother tree clone BL 50 (4 years old) in Padang Pariaman District, namely the *T. cacao* clone BL 50 plant, has a red color of flowers and leaf flush, while the plant

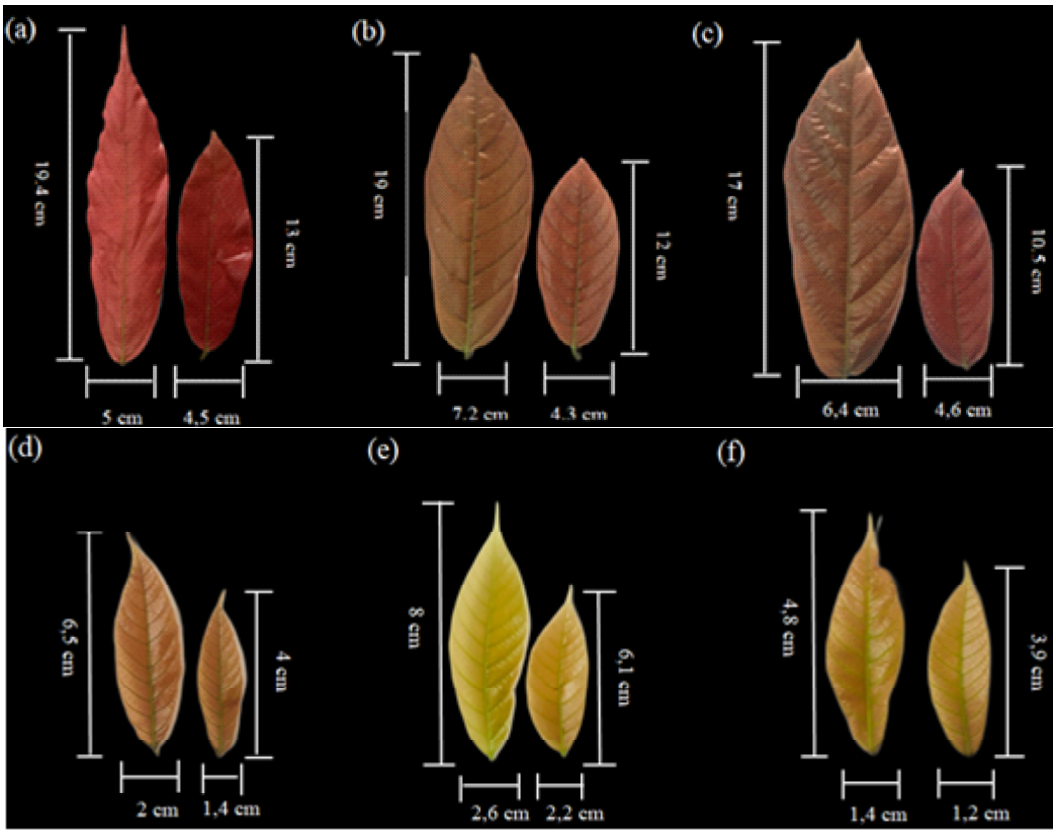

Figure 1. Morphology of young leaves on *T. cacao*; Mother trees aged 6 years: ICCRI 07 (a), ICCRI 03 (b), Sulawesi 03 (c); Cocoa seedlings (SE) aged 4 months: ICCRI 07 (d); ICCRI 03 (e) and, Sulawesi 03 (f)

resulting from somatic embryogenesis of the BL 50 clone is green. Although there are differences in the flush morphology of *T. cacao* leaves from clones ICCRI 07, ICCRI 03 and Sulawesi 03 between mother tree and seedling, in this study it was also reviewed molecularly to determine whether or not there is genetic uniformity in mother tree and seedling using the RAPD molecular marker.

Genome DNA Quantification

The acquired DNA was examined using a UV-Vis spectrophotometer with a wavelength of 260 nm and 280 nm. The results of DNA concentration and purity can be seen in Table 2. The concentration of *T. cacao* DNA ranged from 34.1 to 68.0 ng μL^{-1}, indicating that the amount of DNA collected can be impacted by several factors, including sample conditions and extraction stages.

Fatchiyah *et al.* (2011), stated the factors that can affect DNA extraction are, (1) The process of removing DNA from the nucleus by adding lysis buffer (homogenization); (2) The process of separating DNA from contaminants (cell debris); and (3) DNA precipitation using absolute ethanol/isopropanol. The composition of the addition of lysis buffer when grinding the samples also determines the activity of extracting DNA from leaf samples (Syafaruddin *et al*., 2011). Young leaf samples have a high DNA content because they have active cell development and growth activities (Gusmiati *et al.,* 2021). The purity level was estimated by dividing by the ratio of the absorbance values of 260/280 nm. The purity level because it contains purine and pyrimidine bases, pure DNA can absorb UV light at a wavelength of 260 nm, whereas protein/phenol contaminants can absorb UV light at a wavelength of 280 nm (Fatchiyah *et al.*, 2011).

DNA Amplification Using GY169 and GY107 Primers

The PCR methods were used to amplify the DNA of *T. cacao* by applying two RAPD primers (long primers), GY169 and GY107. The amplification results from the electrophoresis process using 1.25% agarose gel are shown in Figure 2. The amplification products of the two primers, (1) GY169 ranged from 295 to 2785 bp in the parent sample, while in the daughter samples it ranged from 370 to 1692 bp; (2) GY107 ranged from 345 to 1678 bp in the parent samples, while in the juvenile samples, it ranged from 345 to 1330 bp (Table 3). Amplification using primers GY169 and GY107 can produce polymorphic bands in all *T. cacao* samples; this indicates that the two primers are non-specific primers (random amplification).

The primers GY169 and GY107 have been studied on grapes and pears which showed a lot of banding profiles so that they have a fairly high level of polymorphism (Das *et al*., 1996). The use of primers GY169 and GY107 was also carried out in the analysis of consanguinity on several grape cultivars which showed that the similarity of the band

Table 2. Quantification results of *T. cacao* genomic DNA

	Sample	DNA Purity λ 260/280	DNA Concentration (ng μL^{-1})
Mother tree	ICCRI 07	2.01	48.3
	ICCRI 03	1.94	36.6
	Sulawesi 03	1.96	68.0
Seedlings (SE)	ICCRI 07	1.98	36.2
	ICCRI 03	1.92	60.7
	Sulawesi 03	1.98	34.1

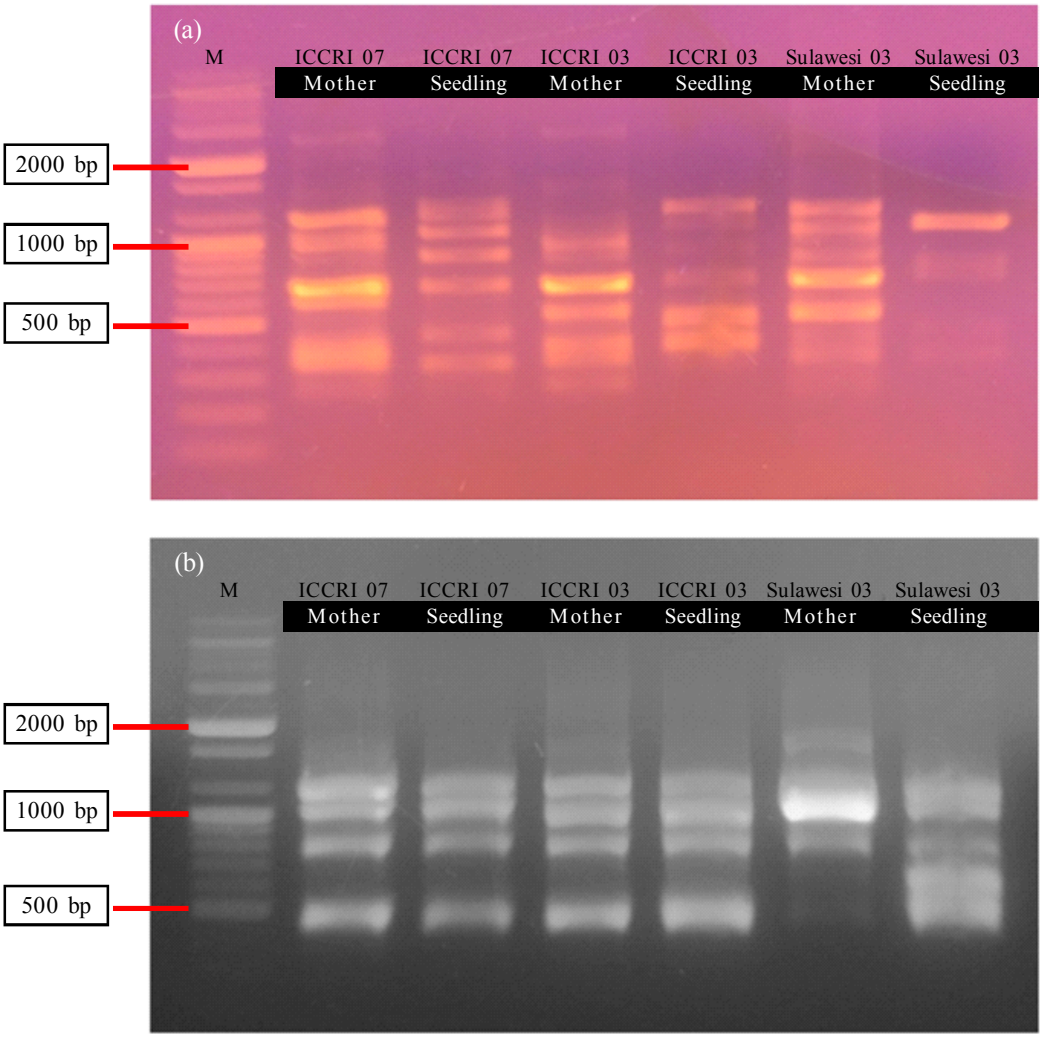

Figure 2. Band profile of cocoa DNA amplified using two primers: GY169 (a) and GY107 (b); M: ladder marker 100 bp

patterns produced was 97% but the error rate of 2-3% in scoring RAPD data was at bright or medium intensity bands (Ye *et al*., 1998). The use of GY107 primer was also carried out on two types of palm (Ajwa & Barhi) in Malaysia which showed a fairly uniform banding pattern with a high polymorphism level of 100% (Ismail & Farida, 2022).

The primers GY169 and GY107 can be used to detect an individual's genetic diversity of variation. The amplification products can be seen in full in Figure 3 and Table 3. The results of the GY107 primer amplification showed a fixed band pattern on the mother cocoa for all clones (mother tree detection), compared to the GY169 primer (Figure 3.b). The results of the amplification of the GY169 primer in all cocoa clones, both mother and seedling (SE), showed high polymorphism (Table 3), indicating 100% genetic diversity.

The use of GY169 primer resulted in a large number of bands and different band

Table 3. Percentage of polymorphic bands in primers GY169 and GY107

Sample name/primer		Band Size (bp)	Σ Band	Σ Band Polymorphic	Σ Band Monomorphic	% Band Polymorphic
Mother tree / GY169	ICCRI 07	295-2785	9	9	0	100%
	ICCRI 03	295-2785	9	9	0	100%
	Sulawesi 03	295-1211	8	8	0	100%
Seedling/GY169	ICCRI 07	370-1692	8	8	0	100%
	ICCRI 03	407-1252	6	6	0	100%
	Sulawesi 03	370-1188	5	5	0	100%
Mother tree / GY107	ICCRI 07	345-1491	5	4	1	80%
	ICCRI 03	365-1572	5	4	1	80%
	Sulawesi 3	345-1678	5	4	1	80%
Seedling / GY107	ICCRI 07	345-1235	4	3	1	75%
	ICCRI 03	365-1330	4	3	1	75%
	Sulawesi 03	345-1330	5	4	1	80%
		Total	73	67	6	1070%
		Average	6.08	5.58	0.5	89.16%

profiles between the mother tree and seedling (SE) in the three clones, namely ICCRI 07, ICCRI 03, and Sulawesi 03. Therefore, the use of GY169 primer was more appropriate in detecting genetic diversity in the three cocoa clones. However, to see genetic uniformity, it is hoped that this study shows that from the three clones, namely ICCRI 07, ICCRI 03, and Sulawesi 03, the mother trees and seedlings (SE) can use the primer GY107 by looking at the band profile produced which is quite uniform.

The amplification of the GY107 primer showed that the polymorphism in the mother trees of ICCRI 07, ICCRI 03, and Sulawesi 03 genotypes was 80%. The major amplification of GY107 is 75% polymorphism in ICCRI 07 and ICCRI 03 clones, and 80% for Sulawesi 03 clone. The use of the GY169 primer is more appropriate for analyzing the genetic diversity of cocoa, which shows more polymorphic bands (Figure 3.a) than GY107. According to Sitepu *et al.* (2019) research, specifically banding pattern analysis on date palm genotypes were successfully amplified with polymorphism levels above 65% had a high level of genetic diversity.

Previous studies have been carried out on *T. cacao* plants. Clones of DR 02, MCC 02, Sulawesi 01, Sulawesi 02, and ICCRI 09 were tested using RAPD primers (10 bp short primers), namely OPA 15, OPW 11, OPP 08, and M 29 which showed that of the four RAPD primers, not all produced polymorphic bands which is real, only found in primary OPA 15 (Pacaningtyas & Susilo, 2022). DNA polymorphism is needed to analyze genetic variation/diversity in an individual by showing the resulting band pattern (Gusmiaty *et al.*, 2021).

The use of primer GY169 for mother and derived from SE of ICCRI 07 clones found four monomorphic bands at sizes 370 bp, 700 bp, 1040 bp, and 1200 bp. ICCRI 03 clone found one monomorphic band at 1017 bp. Sulawesi 03 clone found one monomorphic band at 370 bp. Analysis of genetic uniformity of the mother and derived from SE using primer GY107 was found. Four monomorphic bands of ICCRI 07 were found at sizes 345 bp, 794 bp, 1087 bp, and 1235 bp. Three monomorphic bands of ICCRI 03 were found at sizes 365 bp, 794 bp, and 915 bp. Sulawesi 03 found four monomorphic bands at sizes 345 bp,

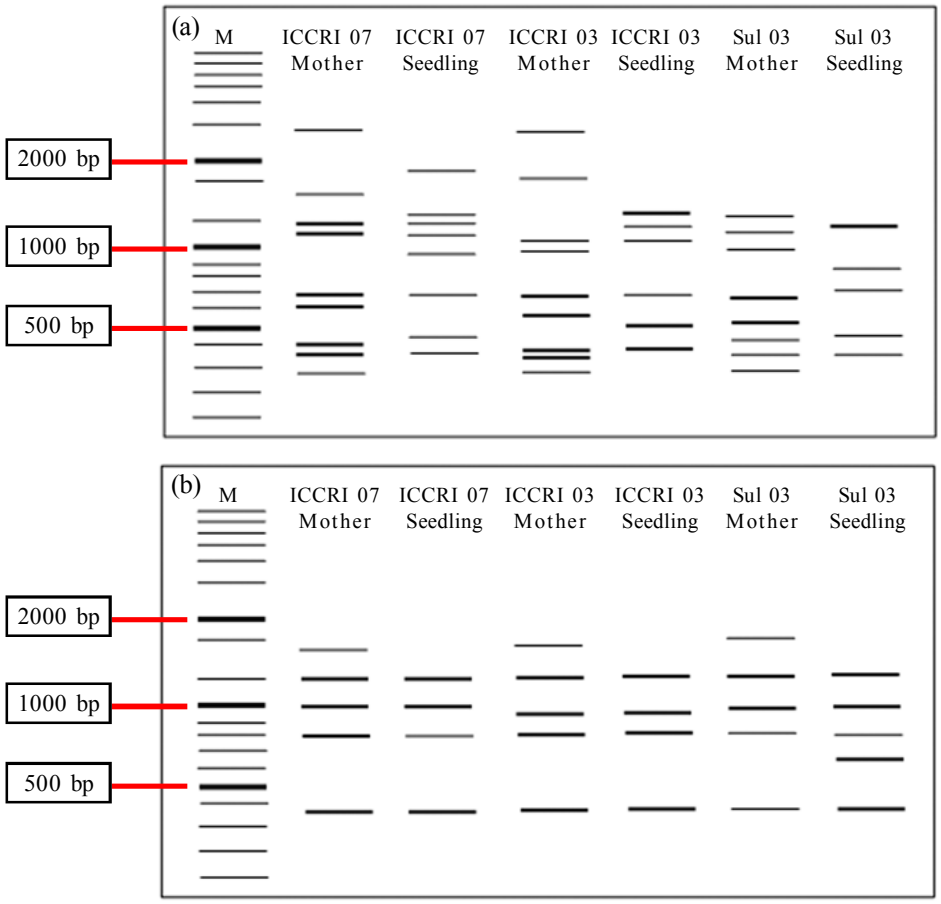

Figure 3. Profile of zymogram and amplified cocoa using two primers: (a) GY169 and (b) GY107; M: ladder marker 100 bp

794 bp, 1119 bp, and 1330 bp. It indicated that the amount of monomorphic bands produced by using the primer GY107 is greater, implying that the primer GY107 is more appropriate in analyzing cocoa uniformity between mother trees and seedlings. This demonstrates that the mother tree and the seedlings derived from somatic embryogenesis at the Research Institute are genetically uniform. This is also shown in the dendrogram of both primer which shows that the GY169 primer results in high genetic diversity (Figure 4.), while the primer dendrogram GY107 shows more genetic uniformity of cocoa between mother trees and seedlings.

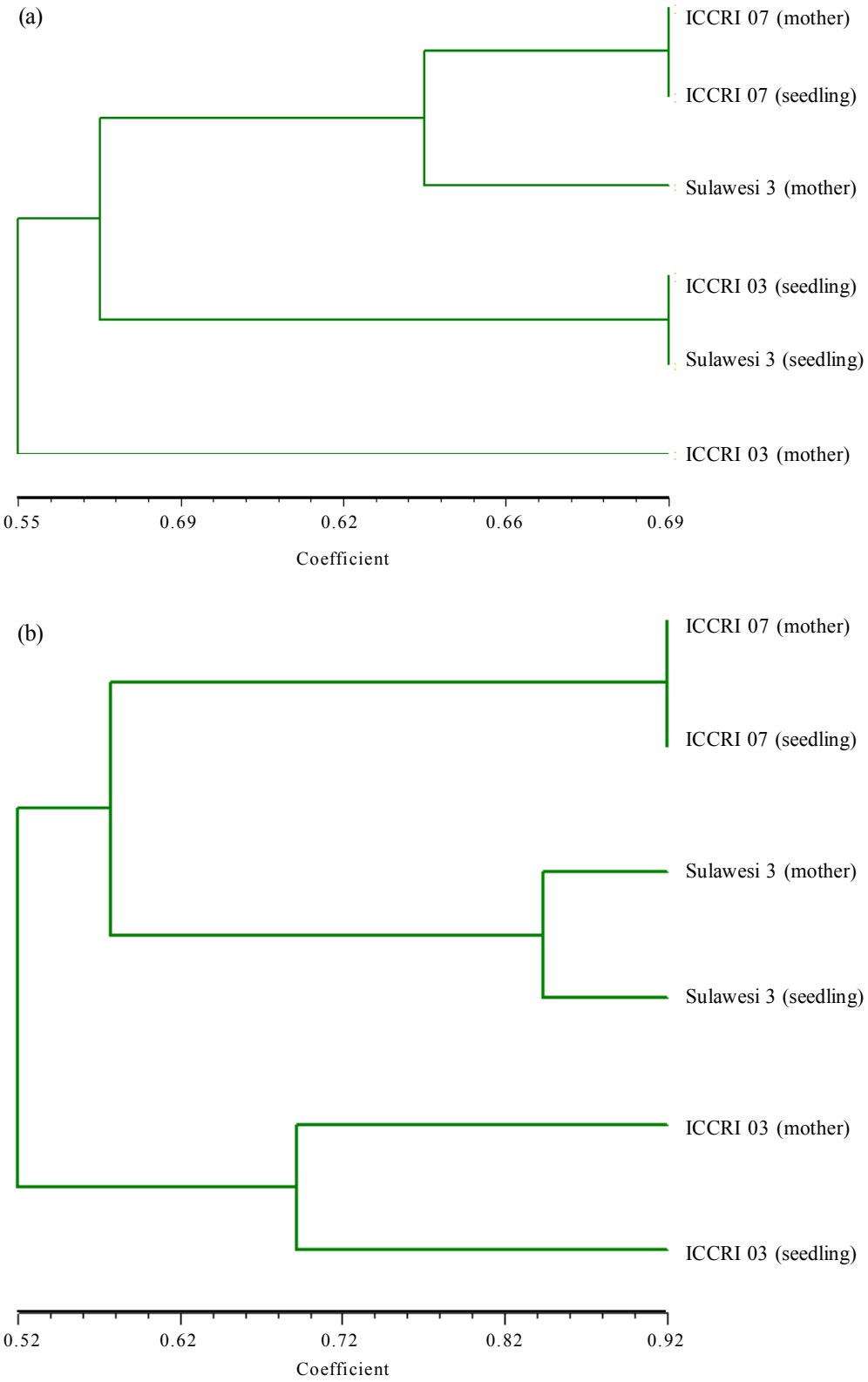

Figure 4. Profile of dendrogram cocoa using two primers: (a) GY169 and (b) GY107.

CONCLUSIONS

T. cacao mother trees and seedlings samples of ICCRI 07, ICCRI 03, and Sulawesi 03 clones were amplified using two RAPD primers (long primers), GY169 (21 bp) and GY107 (18 bp). The GY169 primer produces more polymorphic fragments than the GY107 primer, so it can be utilized to study genetic variation in cocoa, this can be seen from the percentage of polymorphic bands with a score of 100%. In evaluating the genetic homogeneity of cocoa in mother trees and seedlings (SE), the primer GY107 produced more monomorphism bands (same band location) than the primer GY169. It was concluded that the genetic uniformity of *T. cacao* in the mother trees and seedlings derived from SE was found to be uniform.

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