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Analysis of Cocoa Clonal Seedlings Purity Through Deoxyribonucleic Acid (DNA) Barcoding and Random Amplification of Polymorphic DNA (RAPD) Fingerprinting

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Abstract

The genetic purity of a plant indicates the similarity properties between seedlings in the field and the description of the plant in the database. Plant identification through fingerprinting or the use of molecular markers and deoxyribonucleic acid (DNA) barcoding by comparing variation among DNA sequenceses was developed to investigate plant purity. The primers used to identify barcodes on cocoa were derived from the chloroplast genome, including *rbc*L and *mat*K. This research aimed to study the consistency of the *rbc*L primer when applied to other cocoa clones, and to analyse the polymorphic diversity of each cocoa clone using RAPD fingerprinting. DNA extraction for barcoding and DNA fingerprinting was carried out at the Bioscience Laboratory, Jember State Polytechnic. DNA barcoding was tested on DR 1, DR 2, Sulawesi 01, Sulawesi 02, ICCRI 03, and ICCRI 09 clones, which were propagated using somatic embryogenesis and cocoa leaf samples of mother plants from the field. The stages include DNA extraction, sequencing, analysis of the sequencing results, and phylogenetic analysis. Meanwhile, RAPD used DR 2, MCC 02, Sulawesi 01, Sulawesi 02, and ICCRI 09 clones. The 260/280 absorbance ratio from this experiment was in the range of 1.77–2.40. The results of seedlings uniformity analysis using DNA barcoding on cocoa plants produced from in vitro propagation showed that the multiplied seedlings did not show any difference in sequence with the parent plant (DR 2, Sulawesi 01, Sulawesi 02, and ICCRI 09). The analysis of the diversity of cocoa clones Sulawesi 01, Sulawesi 02, MCC 02, ICCRI 03, and ICCRI 09 through RAPD fingerprinting showed that the OPA 15 primer produced more apparent polymorphic band than the other three primers (OPP 08, OPW 11, and M 29).

Keywords: DNA sequences, *rbc*L, *mat*K, clonal uniformity, RAPD, molecular marker

INTRODUCTION

The successful development of cocoa mass propagation technology through the somatic embryogenesis (SE) method is a long process of plant breeding activities to produce superior cocoa planting materials. Plant breeders and cocoa farmers face the problem of the high level of plant heterogeneity among progenies. Vegetative propagation of plants using the SE technique is one of the efforts to obtain genetically uniform plants in the field.

Genetic impurity or mislabelling is a problem that often occurs in commercial seedlings production. Plant genetic purity indicates the similarity of properties between

the plants tested and their descriptions. Identifying plant purity through a morphological approach has several drawbacks, including taking a long time, being influenced by the environment, and limited and inconsistent diversity. This condition encourages the development of markers that can directly access the part of the material that controls the character or characteristics of an individual, which are known as DNA molecular markers. There are various types of markers, including morphological, biochemical, and DNA-based molecular markers (Kumar *et al*., 2009).

Another method used for plant identification is DNA barcoding by paying attention to variations in DNA sequences. This sequence can be identified after amplification using a primer pair, for example, a primer to amplify the atpB-*rbc*L intergenic interrupter region of the chloroplast genome. According to Kang *et al*. (2017), this region of the chloroplast genome has been used for interspecific phylogenetic studies in tree species in the tropical cloud forest and several other plants such as in medicinal plants: red jabon and gofasa (Sundari *et al*., 2019), a molecular marker for selection in *Camellia oleifera* (Chen *et al*., 2015) and several plant populations of *Kelloggia chinensis* and *Kelloggia galioides* Torrey ex Benth (Nie *et al*., 2005). Two short regions encoding plastid DNA (*mat*K and *rbc*L) have been identified and selected as alternative regions for use in plant identification (CBOL, 2009). The DNA sequences obtained were then aligned for sequence variation and phylogenetic analysis. Sequence alignment was performed using the ClustalW program (Ganz, 2021). Alignment was performed to determine the level of homology of the DNA base sequences analyzed.

DNA barcodes often used in plants are chloroplast DNA (cpDNA). Izzah (2017) used chloroplast genome structure in cocoa, to determine phylogenetic relationship and develop DNA barcode markers. The term DNA barcode implies a particular sequence characterizes every living species to show genetic variation within a species and between species (Basith, 2015). Over the past few decades, four plant DNA barcode markers, *rbc*L, *mat*K, *trn*H-*psb*A, and ITS2, have been developed, tested, and used to answer basic questions in systematics, ecology, evolutionary biology, and conservation, including community assembly, species interaction networks, taxonomic discovery and environmental conservation. DNA barcodes on plants are used to monitor the purity of commercial products, such as food and herbal supplements (Kress, 2017). DNA barcoding uses specific DNA regions to identify species. Identification through the chloroplast genome provides a higher level of accuracy of analysis results. The quality of the DNA sequence and the selected marker used, can affect the results obtained. Each individual should have DNA markers for each species to check for errors and allow for intraspecific variation (Li *et al*., 2014; de Vere *et al*., 2015). Dong *et al*. (2012) developed 24 regions of the chloroplast genome such as *psb*A-*trn*H, *rbc*L, *atp*H-*atpl*, *pet*A-*psb*J, *ndh*A-*ndh*A, *trn*K-*trn*K, *pet*B-*pet*D, *ndh*C-*trn*V, *trn*S1-*trm*G1, *trn*W-*psa*P, *clp*Pp-*psb*D, *acc*D, *acc*D-*psal*, *ndh*F, *pet*N-*psb*M, *psb*M-*trn*D, *psb*E-*pet*L, Rpl32-*trn*L, *rpo*B-*trn*C, *rps*16-*trn*Q, *trn*H-*psb*A, and *trn*S2-*trn*G2, as candidate markers in plant DNA barcoding. Based on the information, analysis using DNA barcoding method was used to evaluate the performance of different barcode locations and efficiency for the discrimination of different species and cultivars, also trying to find out the highly informative primers designed from the chloroplast genome based on the efficiency of polymerase chain reaction (PCR) amplification in *Oryza sativa* L. (Singh *et al*., 2017). Recent DNA barcoding technologies were developed by Kane *et al*. (2012) through ultra-barcoding to identify all individuals of *Theobroma cacao* (L.) between and within species.

RAPD is the most frequently used genome propagation method because it is simple and requires a small amount of genomic DNA (William, 1990). Plant samples that are true to type will show the same DNA banding pattern as the varietal sample from the parent plant. On the other hand, samples of off-type plants will produce different DNA banding patterns. Genetic diversity obtained from DNA analysis is also valuable for determining kinship relationships between individuals or populations under study. This information can be used to improve plant genetic quality through well-programmed plant breeding. Genetic diversity can occur due to variations in the nucleotides that make up DNA. This variation can affect the individual phenotype of organisms that can be observed directly (Suryanto, 2003). Syahri *et al.* (2019) conducted RAPD research to analyze genetic diversity in cocoa plants using several primers including OPA 15, OPD 3, M 29, and OPP 08 that produce the clear polymorphic band. Suwastika *et al*. (2019) develop analysis of DNA fingerprint (SSR and RAPD) based on nuclear and chloroplast genome independently, also in combination of both approaches on Sulawesi cocoa.

This research aims to determine the consistency of the *rbc*L primer as a DNA barcoding marker to differentiate cocoa clones and the analysis the polymorphic diversity of each cocoa clone using DNA-RAPD fingerprinting.

MATERIALS AND METHODS

DNA Barcode Amplification using PCR

DNA extraction was carried out at the Bioscience Laboratory, Jember State Polytechnic, from June 2020 to August 2020. DNA was isolated using genomic DNA extraction with Quick-DNATM Plant/Seed Miniprep Kit (Zymo Research, D6020). The DNA quality was tested with a spectrophotometer to see its concentration and purity, then ran in agarose gel.

The materials used were cocoa leaf of DR 1, DR 2, Sulawesi 01, Sulawesi 02, ICCRI 03, and ICCRI 09 clones, 24 leaf samples were used for primary selection (12 from mother boom and 12 samples from cocoa clonal seedlings with three replications). The primary selection was carried out on *rbc*L primers from the chloroplast genome of cocoa (Figure 1).

PCR amplification using MyTaq HS Red Mix (Bioline, BIO-25047), alcohol, aquadest, liquid nitrogen, PVP (polyvinyl pyrrolidone), extraction buffer, b-mercaptoethanol, chloroform solution: isoamyl alcohol, phenol solution, LiCl, DEPC.ddH2O sodium acetate 3 M pH 5.8, absolute ethanol, and 70% ethanol. The extracted DNA was amplified by PCR using specific primer pairs of *rbc*L genes in cocoa plants (Tc-*rbcl*F '5- ATG TCA CCC ACC AAC AGA GAC TAA AGC-3', Tc-*rbcl*R '5- GTA AAA TCA AGT CCA CCR CG -3'). The primary effectiveness of each gene were compared and tested. The materials used for agarose gel electrophoresis include agarose (Sigma), 0.5X TBE buffer, 1% ethidium bromide (w/v), loading buffer (2.5% phenol blue bromine, 40% sucrose), and 1 kb marker. The tools used in this analysis include mortar, micropipette, centrifuge, UV-VIS spectrophotometer, electrophoresis, UV transilluminator, and a thermocycler.

Sequencing: Sequencing aims to read the DNA sequence in the amplified fragment. The extracted DNA (100 mL) was then sequenced in its base sequence at the Genetics Science Lab, using bi-directional sequencing.

Figure 1. Chloroplast DNA of *Theobroma cacao*. The red circle showed the *rbc*L marker in the chloroplast genome (Kane *et al.*, 2012)

Analysis: Sequencing results were analyzed, including assembling, editing, and preliminary alignment with MEGA 5.05 software.

Purity test through DNA barcoding compared cocoa mother boom sequence and cocoa seedling propagated by somatic embryogenesis sequence. In making the phylogenetic tree, *Theobroma cacao* Scavina 6 chloroplast partial genome, *Theobroma cacao* Criollo chloroplast partial genome, and *Theobroma cacao* ICS 39 chloroplast partial genome from the GenBank (www.ncbi.nih.gov) were also used as a comparison.

DNA fingerprinting-RAPD: DNA extraction was carried out at the Bioscience Laboratory, Jember State Polytechnic, from November 2020 to February 2021. RAPD determined the diversity of each cocoa cultivar, whereas fingerprinting by comparing the polymorphic bands of electrophoresis results. The materials used were cocoa leaf of DR 2, MCC 02, Sulawesi 01, Sulawesi 02, and ICCRI 09 clones, each clone used two leaf samples from cocoa clonal seedlings with three replications. The PCR optimization stage was carried out to obtain the most appropriate DNA extraction method. Genomic DNA quantification using nanodrop, with a volume of 30 mLfor each cultivar. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. The 260/280 ratio from this experiment is in the range 1.77–2.40, this indicates that DNA used in this experiment is "pure".

The PCR master mix used is 2x Taq HS Red Mix. In this study, four types of primers were used, including OPA 15 (5'-TTC CGA ACC C -3'), OPP 08 (5'- ACA TCG CCC A -3'), M 29 (5' TGC GTG CTT G -3') and OPW 11 (5'- CTG ATG CGT G -3'). The annealing temperature was optimized for each primer.

RESULTS AND DISCUSSION

DNA Barcoding Analysis on Cocoa Plants

We demonstrated the experiment of two cocoa clones from in vitro propagation compared with the cocoa mother boom (DR 1 and DR 2). Based on morphological observations, there are differences in the flush color of the two clones. DR 1 mother boom has red flush color meanwhile DR 2 mother boom has green color.

Uniformity analysis through DNA barcoding was carried out to ensure that the seedlings produced from in vitro propagation matched the mother boom in the field. Cocoa clonal seedlings from two clones have the same color with DR 2 mother boom, this result was confirmed with phylogenetic tree (Figure 2). Therefore, we can conclude that cocoa clonal seedling 1 and 2 is DR 2 clones. This method can be used as a tool to detect the purity of seedlings using the *rbc*L marker.

Furthermore, the seedling uniformity testing method using the *rbc*L marker was used to differentiate other cocoa clones. Based on the results of the alignment of molecular markers through DNA barcoding on cocoa clones of Sulawesi 01, Sulawesi 02, ICCRI 03, and ICCRI 09, it showed that the sequences from the mother boom (top row) and seedlings derived from somatic embryogenesis (bottom row) did not show any difference (Figure 4). This confirmed that the in vitro propagated seeds matched the label on the mother boom (Figure 3).

Based on the DNA barcoding results identification on several cocoa clones, the *rbc*L marker can be used to amplify cocoa DNA. According to Kane *et al.* (2012), *rbc*L can distinguish varieties from cocoa plants eventhough it only shows one variation difference. As shown in the phylogenetic tree of several cocoa clones (Figure 5), this also confirmed that the cocoa seedlings derived from somatic embryogenesis were identical with the cocoa mother boom.

Figure 2. Leaf morphology of cocoa mother boom and seedlings DR 1 and DR 2 clones

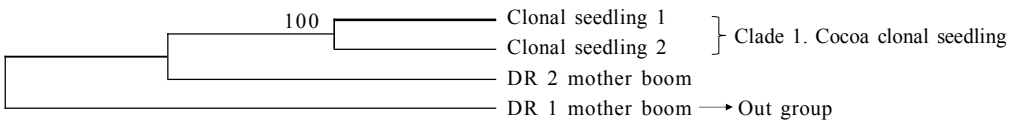

Figure 3. Phylogenetic tree of cocoa clonal seedlings compared with cocoa mother boom

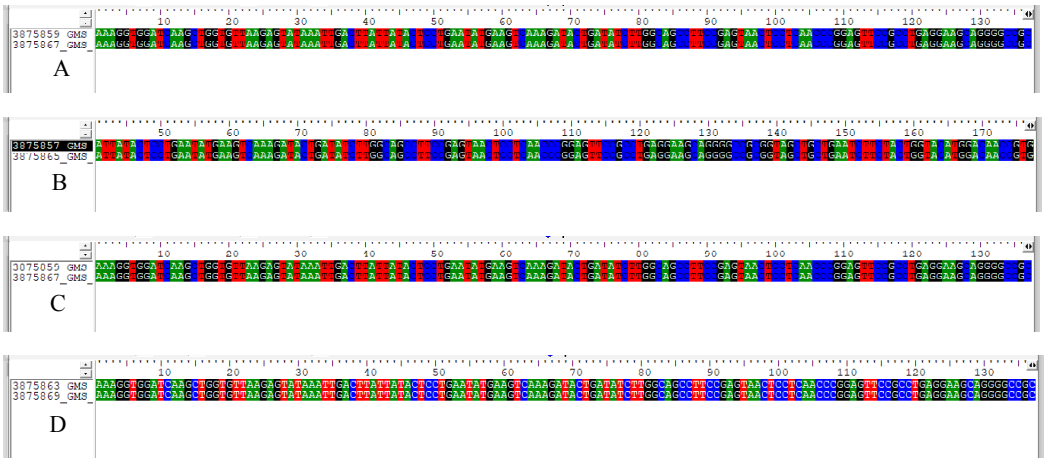

Figure 4. Sequences alignment of Sulawesi 01 (A), Sulawesi 02 (B), ICCRI 03 (C), and ICCRI 09 (D) clones

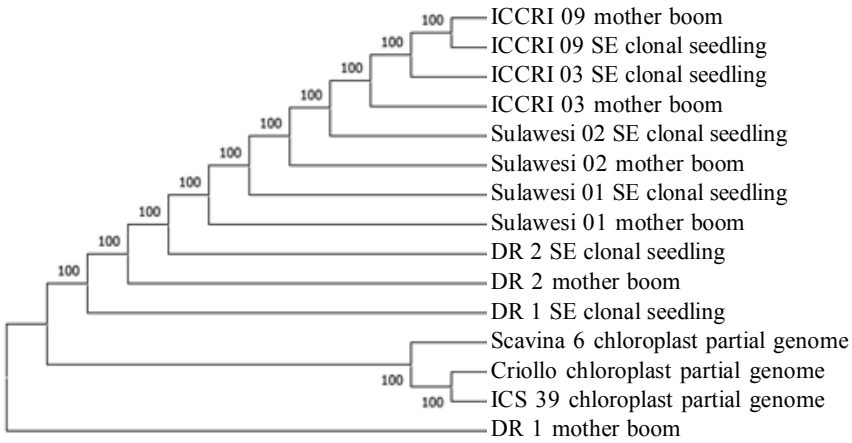

Figure 5. Phylogenetic tree of several cocoa clones compared with cocoa mother boom

Analysis of DNA Fingerprinting Using RAPD

Analysis using DNA barcoding was confirmed based on the comparison of the sequences between the mother boom and the clonal seedlings but could not differentiate between cultivars/clones. Detection of DNA polymorphisms using molecular markers is based on plant genetic traits only, not influenced by environmental factors. Random amplified polymorphic DNA (RAPD) is the most frequently used for genome propagation method because it is straightforward and requires a small amount of genomic DNA (William, 1990). Plant samples that are true to type will show the same DNA banding pattern as the varietal sample from the parent plant; on the other hand, samples of off-type plants will produce different DNA banding patterns.

RAPD is a PCR-based molecular marker that is widely used to identify plant diversity. Based on Rifka *et al*. (2014) experiments identified cocoa (Sulawesi 01, Sulawesi 02, Panter, ICS 60, ICRRI 01, ICCRI 02, ICCRI 03, ICCRI 04, M 01, M 05, Asahan, Local Palolo, Local Sidondo and Fine Cocoa) diversity

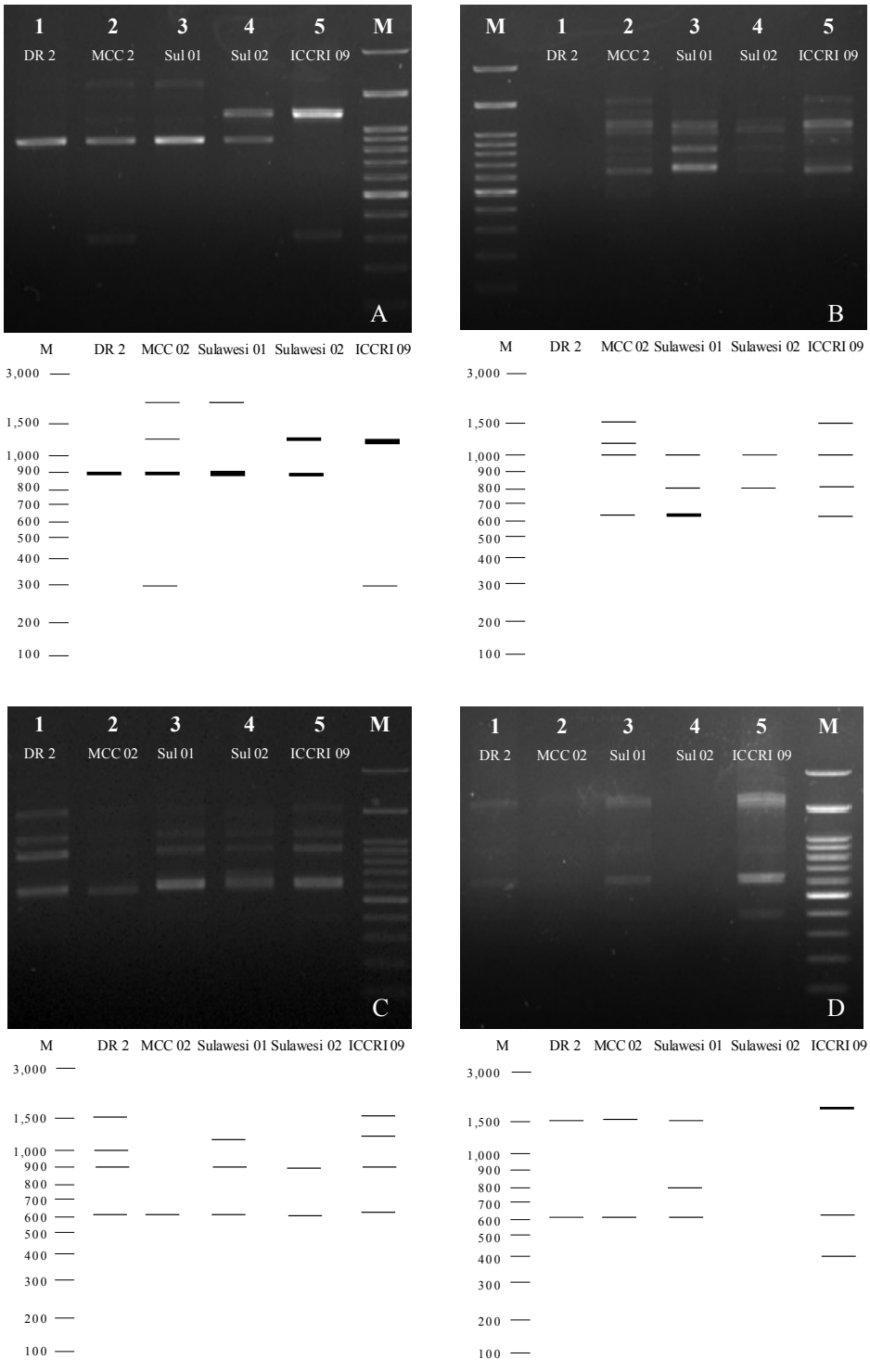

Figure 6. DNA amplification on five cocoa clones (1. DR 2, 2. MCC 02, 3. Sulawesi 01, 4. Sulawesi 02, 5. ICCRI 09) using several primers. Primer OPA 15 with annealing temperature 41.4°C (A), Primer OPP 08 with annealing temperature 37.0°C (B), Primer OPW 11 with annealing temperature 37.0°C (C), Primer M 29 with annealing temperature 36.0°C, M. Marker (D)

in Central Sulawesi using three primers, i.e. TCH 05, AS 9870, and TCM 20 can be used to detect genetic variation of each clone, except for the Sulawesi 01 and Sulawesi 02 clones.

Based on the results of DNA amplification, the four primers used can amplify cocoa DNA and produce polymorphic bands with a size of about 300 to 1500 bp (Figure 6). Primer OPA 15 showed the most apparent RAPD markers for the five cocoa clones. This primer can differentiate among cocoa cultivars. Primers that show polymorphic bands but smear or even no bands cannot be used for diversity analysis. Smear bands occur due to variations in DNA amplification that have a dense difference in size (Jamsari *et al*., 2007). RAPD is still used for genetic diversity analysis because of its ability to amplify various plant species, including pine (Gusmiaty *et al*., 2016), bamboo (Larekeng *et al*., 2018; Makmur *et al*., 2020), and oil palm (Tarigan, 2016). DNA fingerprinting using OPA 15 primer can be used to distinguish several cocoa cultivars, including DR 2, MCC 02, Sulawesi 01, Sulawesi 02 and ICCRI 09, based on the polymorphic band formed. Sulawesi 01 and Sulawesi 02 show almost the same bands, this indicates that these two clones have a close relationship.

CONCLUSIONS

DNA barcoding using the *rbc*L marker can be used to confirm cocoa clonal seedling (Sulawesi 01, Sulawesi 02, ICCRI 03, and ICCRI 09) purity, compared with cocoa mother boom based on sequence alignment. Still, DNA barcoding cannot be used to differentiate among cocoa cultivars. Therefore, RAPD fingerprinting analysis is used, which can be used to distinguish between cocoa cultivars (DR 2, MCC 02, Sulawesi 01, Sulawesi 02, and ICCRI 09) based on the DNA polymorphic bands. Primer OPA 15 showed the most apparent RAPD markers for the five cocoa cultivars.

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