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Assessment of Population Genetic Diversity of cocoa (*Theobroma cacao* L.) using RAPD Markers: The Case of cacao Agroforestry's System in East Kolaka, Indonesia

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ABSTRACT: Knowing the vast variety of species and their potential uses in conventional agroforestry systems is critical to increasing tropical area efficiency and ensuring the sustainability of the natural resource base. One of important species for agroforestry system in Southeast Sulawesi is cocoa. The biggest cocoa production center in Southeast Sulawesi can be found in East Kolaka. The sustainability of Cocoa plantation in East Kolaka are constrainted by pest attack, low soil fertility and seed quailty. Therefore improvement effort are needed, one of which through identification of genetic resources using molecular tools. Molecular marker using RAPD have been successfully reported for this purpose. This research aim was to determined the population genetic of cocoa from three population from Agroforestry system in Kolaka. The research consist of three activities those were samples preparation, DNA isolation and amplification and data analysis. Four polymorphic primers OPA 15, OPD 03, OPP 08and M29 were used for amplification. The annealing temperature for OPA15, OPD 03, OPP 08 and M 29 were 34.2°C, 40.8°C, 37.6°C, and 34°C, respectively. The result of this research showed that three different sub cluster population of cocoa were found in East Kolaka. The first sub-cluster generally consisted of individuals from population three. The second sub-cluster consisted of one population, while the three sub-clusters generally only from population two. These results provide information will contribute to the sustainable use and conservation of these cacao genetic resources.

Keywords: Agroforestry system, Genetic diversity, Molecular marker, RAPD, *Theobroma cacao*.

I. INTRODUCTION

East Kolaka is known as the center of cocoa production in Southeast Sulawesi. The available data shows that Southeast Sulawesi produces the highest cocoa productivity in 2003 (1,287.81 kg ha^{-1} $year^{-1}$) and the lowest in 1996 (891.23 kg ha-1 $year^{-1}$). The average production of cocoa in Southeast Sulawesi was 966.01 kg ha^{-1}, higher than the national cocoa productivity, which was 950.90 kg ha^{-1}. The East Kolaka is designated as a cocoa production center in Southeast Sulawesi. The consideration for choosing this location due to the average planting area for cocoa was increasing 6.31% (7.176 ha) every year. The cocoa production also tend increase from 59,899 tons in 2003 to 74,614 tons in 2007, but in 2012 the cocoa production was decrease to 48,823 tons [1].

The cocoa production decline in East Kolaka is caused by the plant pest organisms attack, low seed quality, decreased soil fertility that is possible due to inappropriate land use. For small-scale farmers who cultivate cocoa culturally, the decline in cocoa production has affected the family's main income source. Changes in technical cultivation and management of monoculture-based cocoa to agroforestry systems are needed to improve cocoa production sustainability and create alternative sources of income. This change is also able to improve the livelihoods of the macro and local economies. Farmers in Konawe and Kolaka, Southeast Sulawesi, state the cocoa as one of the main livelihood sources that reaches 35% of the overall livelihood source choices [2]. In terms of land-use systems, [3] found that land use in Southeast Sulawesi was quite diverse with various types of plants such as food crops, mixed gardens, mixed woods, and monocultures. However, for the land use system for cocoa, farmers in Southeast Sulawesi cultivate cocoa with a system of monoculture and agroforestry. Agroforestry as a strategic synergy between agriculture, forestry, and the environment, is very important for renewing natural resource management that is more in line with the principles of sustainability and also the accelerating of the prosperity achievement.

The RAPD technique requires the amplification of certain genome regions of an organism, and a dexterous marker system used in revealing the genetic diversity in population and conservation genetics [4]. This amplification requires specific primers (specific oligonucleotide sequences). This technique success is based on the primer suitability and optimization of the PCR process. Non-specific primers can cause amplification of other areas in the genome that are not targeted or otherwise there is no amplified genome area. PCR optimization is needed to produce the target character. This optimization concerns the denaturation temperature and DNA annealing in PCR machines. The DNA banding pattern of RAPD results is influenced by several factors, namely the component of PCR reaction (DNA template concentration, polymerase enzyme concentration, primer concentration, and number of thermal cycles), cycle temperature of PCR (*denaturation* and *annealing*). Random primer concentrations for DNA amplification in some plants varies depending on the primer and plant types, so it needs specific research to obtain the optimum amplification products [5].

The selection markers development for the cocoa breeding program has begun [6], while the cocoa genetic diversity using RAPD markers has been done [7].

The cocoa genetic diversity in the research location will be carried out using RAPD markers. By using molecular markers, the genetic diversity of cocoa germplasm as a prospective parent to be used in plant breeding programs will be determined. A cross between two parents that have a high genetic distance needs to be done to get a new superior cultivar. Molecular markers can identify the parents with a high genetic distance so this method can improve the effectiveness and efficiency of breeding programs to be carried out.

II. MATERIAL AND METHODS

A. Samples exploration

The genetic material exploration of this research was carried out in a cocoa-pepper-coconut mixed farm of Poli-Polia District, East Kolaka Regency.

B. DNA Isolation and amplification

Cocoa DNA isolation was carried out based on the Genomic DNA Mini Plant (Geneaid) kit protocol with little modifications [8]. The collected cocoa leaf samples from field exploration were cut about 10 cm long and then stored in a -20°Cin freezer and ready for DNA isolation process.

DNA amplification with PCR machine sensoquest thermal cycler and Mix Hotstar Qiagen PCR. The volume for one DNA amplification reaction was 12.5 µL, which consisted of 0.625 µL Mix Hotstar Qiagen PCR, 3 µL ddH_2O, 2 µL DNA, and 1.25 µL RAPD primer. The PCR step started by 35 cycles of initial denaturation at 95ºC for 3 minutes, denaturation at 95ºC for 15 seconds, annealing at 51-55 ºC for 15 seconds (different temperatures for each primer), and elongation at 72ºC for a second, and one cycle offinal elongation at 72ºC for 10 minutes. PCR amplification was stored -20°C until electrophoresis process. The PCR products were separated on 2% agarose gel with SB 1x Buffer using horizontal electrophoresis.

The bands obtained appeared on agarose. The band represents alleles in the certain locus. Each primer was used to present a particular locus. The present band was given a scoring number according to the band size.

C. Data Analysis

At the first observation in primer selection process 16 RAPD primers were used [9]. The selection was done by amplifying 12 DNA samples chosen randomly. The primer selection is conducted to select polymorphic primers and determine the appropriate annealing temperature. Determining the right temperature of annealing greatly influences the results of the application of all samples. This is due to the application process of all samples using the same temperature. From 16 observed primer in selection process, only four primer produce polymorphic and clear band. Then this primer were using in this research. The selected primer and it annealing temperatures shown in Table 1.

Table 1: Polymorphic primers and annealing temperatures.

S. No.	Primer	Primer Sequence (5'-3')	Tm (ºC)
1.	OPA 15	TTC CGA ACC C	36
2.	OPD 03	GTC GCC GTC A	43
3.	OPP 08	ACA TCG CCC A	39.2
4.	M 29	CCGGCCTTAC	37.7

D. Genetic Diversity Identification

To identify the genetic diversity, the PCR product of RAPD was scored based on the presence of DNA bands. The bands obtained were translated into allelic data. If there was a band, it was given 1 and 0 if the band not found. All existing bands were tabulated then identified using Darwin software 6 [10]. Identification of genetic diversity was carried out by UPGMA (*Unweighted Pair Group Method Arithmetic*) method. Identification of bootstrap values with UPGMA at 1000 replications to determine the grouping of the evaluated samples. DNA band measurements were determined based on ladder standards using semilogarithmic graph paper. The Polymorphic Information Content (PIC) calculation was done to determine the level of primer informative, PIC was calculated by the formula PIC = 1 - 2, where P_{ij} is the frequency of the j pattern generated by the i primer which is then summed for the overall patterns produced by the primer.

PIC value was calculated using a formula written in an article by [8], Polymorphic Information Content Calculator, an Online Program.

$$PIC = 1 - \sum_{i=1}^{I} P_i^2 - \sum_{i=1}^{I-1} \sum_{j=i+1}^{I} 2 P_i^2 P_j^2$$

III. RESULTS AND DISCUSSION

DNA Isolation and Amplification: DNA isolation is the basic step in molecular analysis. High purity and quantities of DNA will determine the result of Polymerase Chain Reaction (PCR) process [11, 12]. DNA isolation of tree species is rather difficult than herbaceous species due to the occurrence of polysaccharides, phenols and tannins that can inhibit the activity of DNA polymerase during PCR [13]. Our result show, Genomic DNA Mini Plant (Geneaid) kit protocol able to produce good yield and quality of DNA. The result of PCR-RAPD given in Fig. 1-4.

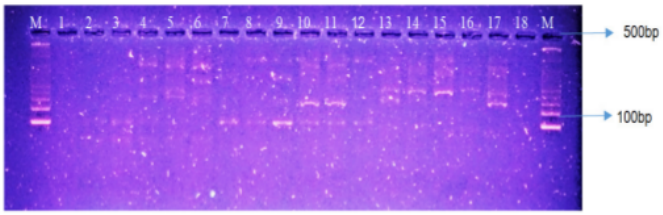

Fig. 1. Electropherogram of DNA amplification products of Cocoa using OPA-15 RAPD primers. Notes: M = 100 bp marker, 1-18 = DNA amplification products.

The amplification of Cacao DNA using four RAPD primers generated polimorphic bands. Fig. 1 presents DNA bands pattern amplified by OPA15. This primer did not produce any band on individual #1, #2, and #18. Fig. 2 shows sample #1 and #2 had amplified bands using primer OPD 03. Fig. 3 depicts band with the smallest size, 200bp, whereas Fig. 4 presents bands that were generated successfully by primer M-29 and had the smallest size of 500bp. Based on Fig. 1-4, all primer produce better polymorphic DNA band and satisfactory result. The good result of amplification can be affected by many factor. As known that RAPD amplification is performed in conditions resembling those of polymerase chain reaction using genomic DNA from the species of interest and a single short oligonucleotide primer (usually a 10-mer). The highest diversity from four observed primer found in primer OPP-08 (6 band polymorphic) and the lowest found in primer OPD-03 (3 band polymorphic) [9]. The difference in DNA band pattern was because of the amplification of DNA sequence at certain positions [14]. Differences in base pairs (bp) in the DNA sequence cause amplification not to be performed due to incompatibility between the primer and complementary DNA sequences.

Genetic diversity of cacao (Theobroma cacao L.): A dendrogram of cocoa originated from cocoa-pepper-coconut mixed farm in Poli-Polia District, East Kolaka Regency given in Fig. 5. The three population were divided into three cluster whereas the individual of each population randomly joined with each other.

The results showed that cocoa individuals from 3 populations tended to group randomly so that the individual's genetic relationship in one population was low. The lower genetic relationship, the higher the value of genetic diversity. Future tree breeding efforts require genetic variation within and between populations. The effort was made with the construction of seed orchard with plant material originating from different populations and seeds from superior parents. This aims to increase genetic variation in the population so that it will produce seeds that are of high quality and genetically superior. High genetic variation will support a population to adapt to changes that occur in the surrounding environment and biodiversity will be maintained [15, 16].

This research was consistent with the analysis of the genetic similarity cluster [17]. Suweg (*Amorphophallus muelleri*) shows the separation of accessions into two main clusters which are partially grouped based on population and others cluster randomly. Based on the identification results, the first sub-cluster generally consisted of individuals from population three, while the second sub-cluster consisted of individuals from one population, and the three sub-clusters generally consisted of individuals from population two. Genetic diversity research in Durum wheat lines showed the results of principle coordinate analysis (PCoA) based on ISSR data showed a good congruency with cluster analysis and supported the clustering pattern of UPGMA dendrogram too [18].

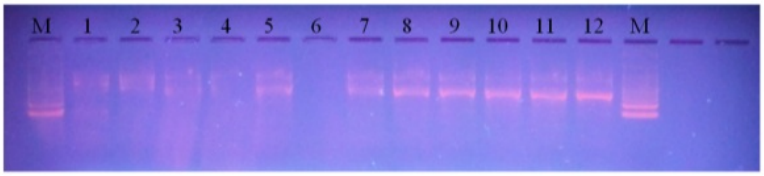

Fig. 2. Electropherogram of DNA amplification products of Cocoa using OPD-03 RAPD primers. Notes: M = 100 bp marker, 1-12 = DNA amplification products.

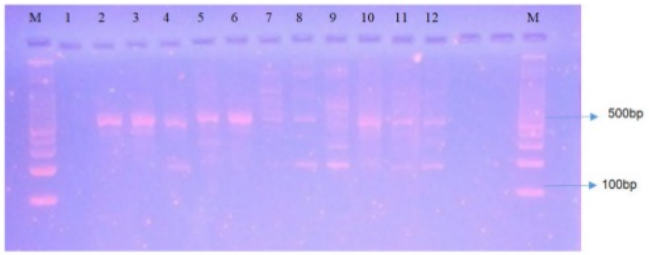

Fig. 3. Electropherogram of DNA amplification products of Cocoa using OPP-08 RAPD primers. Notes: M = 100 bp marker, 1-12 = DNA amplification products.

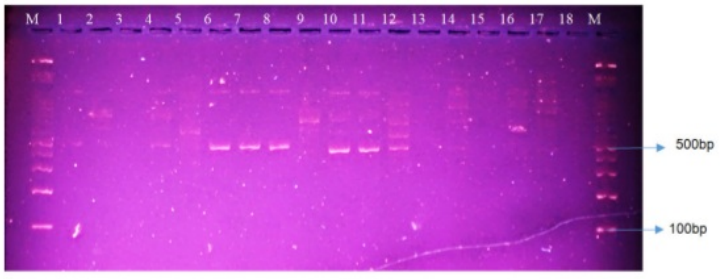

Fig. 4. Electropherogram of DNA amplification products of Cocoa using M-29 RAPD primers. Notes: M = 100 bp marker, 1-18 = DNA amplification products.

Agroforestry is recognized as a possible partial solution for biodiversity conservation and improvement. Some research to know effect agroforestry in cacao study shows a positive result. In North Toraja Regency, plants in research location were Robusta coffee, cocoa, banana, mangos-teen, Buangin (pine-mountain), sugar palm, Betung bamboo, and *Elmerilla ovalis* [19]. In order to sustain and improve cocoa production, activity is needed to exploit genetic variation in performance under differing environmental conditions and breed new genotypes suited to the local conditions [20].

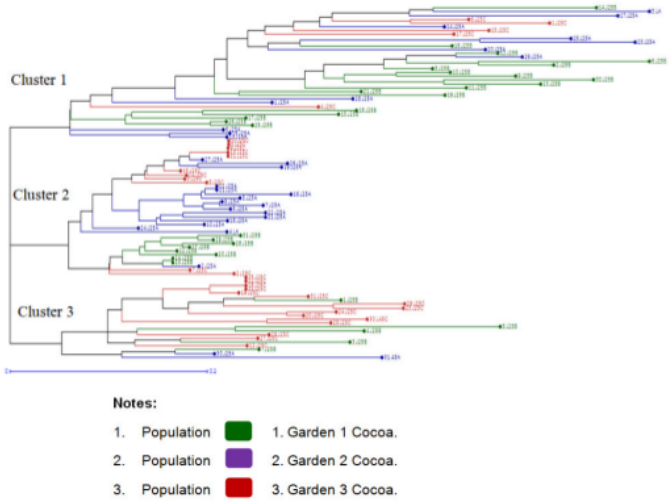

Fig. 5. Dendrogram of Cocoa Genetic Diversity in 3 Population

In On farm management of *Acca sellowiana* (Myrtaceae) at Brazilia showed the management used by farmers, in addition to conserving molecular diversity, tend to increase the genetic variability, an important element for sustainable agricultural designs such as agroforestry systems, and better use of agroecosystem resources [21]. These results provide information will contribute to the sustainable use and conservation of these cacao genetic resources.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5