International Journal of Current Research and Academic Review
ISSN: 2347-3215 Volume 3 Number 3 (March-2015) pp. 195-201
www.ijcrar.com

Genetic diversity of different cocoa clones by RAPD (Random Amplified Polymorphic DNA) markers

Johannes Panggeso[1*], Alam Anshary[1], Sakka Samudin[2] and Zainuddin Basri[2]

[1]Department of Plant Pests and Diseases, Agric. Faculty, Tadulako University, Palu, Central Sulawesi
[2]Department of Agronomy, Agriculture Faculty, Tadulako University, Palu, Central Sulawesi

***Corresponding author**

KEYWORDS

Genetic diversity,
RAPD,
Cocoa

A B S T R A C T

For developing of cacao breeding program and proposing plant variety protection in Central Sulawesi the availability of morphological and molecular descriptions of germplasm collections is necessary. Therefore, genetic diversity studies through morphological and molecular characterization for cacao collections in Indonesia need to be conducted. The use of various types of molecular markers have been used to characterize, analyze the diversity, and describe the genetic mapping of cacao. The research was aimed to provide initial information about the genetic diversity of cocoa clones in Central Sulawesi, which can be used as a potential parent in crossing process to get the new high yielding and resistant cocoa progeny against fruit rot disease caused by *P. palmivora.* Totally ten cocoa clones from two different locations in Central Sulawesi had been collected and molecularly characterize by using of four RAPD primer to describe their polymorphisms. Amplification product of PCR using four different primers resulted 2 to 10 DNA bands. OPW11 primer generated 10 polymorphisms bands, OPM06 produced 5 plymorphic bands, OPK17 with 3 polymorphic bands and OPO20 generated only 2 bands. Based on theese four RAPD primers, 10 cocoa clones from Ampibabo and Palolo could grouped in 3 clusters.

Introduction

Indonesia belong to the one of the most cocoa producer in the world after Ivory-Coast and Ghana (Susilo, 2007). Cocoa production capacity in several other Asia-

Pacific countries such as Papua New Guinea, Vietnam, Philipina including Indonesia is still far below of their capacity production. However if it compared to other countries, Indonesia has some advantages in the development of cocoa, based on the reasons: the availability of land, relatively low in cost production, have a large domestic market potential and a good managed of transportation.

Based on market needs of cocoa beans, mainly from Western European countries and the United States allows great opportunity for Indonesia to cultivate and improve cacao quality in order to meet the market demands. Indonesian cocoa, especially those produced by the farmer, have lowest valued in the international market because of the unfermented beans, seeds with high levels of dirt and contaminated with insects, fungi or mycotoxin.

To improve the quality and to increase the productivity of cocoa, fermentation of cocoa beans, planting of improved varieties, application of cultivation technology as well as, pest and disease control are need to be implemented. Various diseases caused by pathogens can be found in the cacao plant, one of the main obstacles in the cultivation of the cocoa in Indonesia is black pod disease caused by *Phytopthora palmivora* (Prawirosoemardjo and Purwantara, 1992). Black pod disease attack or infect the entire plant from stems, leaves and fruit (McMahon & Purwantara, 2004). Disease severity depend on temperature and humidity of area where cocoa plants are cultivated. Yield losses in the field ranged from 20-30% per year (Wood and Lass, 1985). Even in a particular regions or countries disease intensity can reached up to 40% (Van Der Vosen, 1997). Major strategies to overcome the losses caused by the fungus is use of pesticides. However, the pesticides application have a negative impact to the environment. The use of resistance cocoa clones to control pod rot disease is a right effort based on several grounds, not pollute the environment and has a high adaptability. To obtain a high yielding and resistant clones to *P. palmivora*, selection of clones of existing cocoa field is necessary.

Morphological characterization and analysis of genetic diversity and pathogenicity test clones in cocoa cultivation centers, is one of the selection process for this goal. Analysis of genetic diversity of plants can be done by direct observation of morphological phenotype or by using molecular markers. The use of molecular can more quickly and accurately to confirm the favorable plant genotype because its expression is not affected by environmental conditions and the age of the plant. In addition to providing confirmation of the genotype molecular marker not only infer the genetic diversity and genetic relationship among and between species or varieties but simultaneously can also help to explain the phylogenetic relationship (Weising et al. 1995).

The use of various types of molecular markers have been used to characterize, analysis of diversity, and genetic mapping of cacao (Lanaud et al., 1999), but in Central Sulawesi has not been done. The development markers in the selection for cocoa plant breeding programs have been started by Schnell et al. (2007), while the genetic diversity of cacao by using RAPD markers was performed by Williams et al. (1990).

Materials and Methods

Place and Time

DNA isolation of leaf cocoa from Ampibabo and Palolo and molecular analysis was

conducted at the Integrated Laboratory, Faculty of Agriculture, Hasanuddin University started on March 2014 until November 2014.

Research Methods

Material leaves of individual cacao plants were placed into plastic bags and stored on cool box contain ice in the field for transportation and at -80 °C in the laboratory until extraction process. Extraction of total genomic DNA and amplification process with random amplified polymorphic DNA (RAPD) primers were conducted according to the procedure of DNeasy Plant Mini Kit (250) (Qiagen, Cat# 69106). After extraction, the concentration of DNA was estimated by spectophotometer at 260 nm (Sambrook et al., 1989). Bands of total genomic DNA separated by electrophoresis in 0.8% agarose gel were used to check the integrity and purity of the extracted DNA. After quantification, the DNA samples were diluted to 10 ng / μL.

There were ten clones with the code: M01, M06, M45, Ap, S1, S2, Irian, UNTAD 1, Plo, and Local used in this experiment (Table 1.).

Amplification of DNA using PCR machine takes place with the 3-Step cycling stages. Denaturation for 4 minutes at a temperature of 94.0°C, annealing for 2 minutes with a temperature of 35.0° C, extension for 2 minutes at a temperature of 72.0 (1 cycle). 30 seconds denaturation at a temperature of 94.0° C, 1 min annealing at a temperature of 35.0° C, Extension 2 minutes with a temperature of 72.0° C, Final Extension 5 minutes for 72.0 C (35 cycles). After PCR, amplicon were separated on 2% agarose gels run in TRIS-acetate buffer (Sambrook et al. 1989). Strong, clearly defined DNA bands profiles were recorded after staining with ethidium bromide. Bands were identified by their molecular sizes relative to a 100-bp marker ladder on 2% agarose gel. Totally four RAPD primers were used in the experiment (Table 2).

DNA banding pattern of amplification product was translated into binary data, then used to calculate the coefficient of similarity and to create dendrogram. Only loci that showed a clear band, used for scoring. RAPD phenotype binary matrix is then prepared for use in the analysis of individual cluster by using UPGMA (Unweighted Pair Group with Arithmatic Average).

Result and Discussion

The results show that all of four primers were able to amplify all DNA of tested cacao samples with a total of 20 DNA bands (Table 1). The number of bands in each primer varied between 2-10 bands. OPW 11 primer resulted 10 bands, whereas least bands generated by OPO 20 and OPK 17 primer with each of 2 bands and 3 bands. Band size of the amplified DNA ranged from 100-900 bp. The number of bands produced by each primer depends on the distribution of the site that is homologous to the primer sequences in the genome. Differences in the number and size of bands determine the level of genetic diversity of these cocoa clones. The DNA profiles of four consecutive primers presented in Figures 1.

Figure 1. DNA patterns of 10 cocoa clones after PCR amplification using OPO20 (Top Left),OPM06 (Top rightt), OPK17 (Bottom Left) and OPW11 primer (Bottom right). Separation on the 2% of agarose gel. Marker (M), Lokal clones (1) AP (2), UDT1 (3), PLO (4), Irian (5), 45 (6), M06 (7), M01 (8), S1 (9), S2 (10).

Genetic distance between each tested clone is calculated according to Nei (1978) formula and presented in Table 3. Highest genetic distance value was revealed between M01 and S1 clone (0,9163) and the lowest was between M06 and Irian clone (0,1625).

Cluster analysis of the 20 DNA banding patterns produced three different cluster groups. First group joined Irian and M06 clone, second group joined AP and the PLO clone, whereas third group joined M01 and S1 clone. Lokal clone and S2 clone separately outside of the three groups. Genetic dendogram obtained from this analysis, indicating that the M01 and S1 clone has a close relationship among 10 cocoa clones tested. Analyze of genetic relationship between 10 cocoa clones according to Nei (1978) with the UPGMA resulted three different cluster groups (Figure 2).

The analysis of genetic diversity on 10 cocoa clones from two districts showed, that DNA amplification total genomic using four RAPD primers OPO20, OPM16, OPK17, and OPW11 generates readable DNA bands, so that the results can be analyzed (Table 3). All of 4 RAPD primer represent a wide variation. The obtained 20 DNA fragments have total genomic ranging size from 100bp to 900bp, with a high level of polymorphic. The maximum number of DNA bands present in the primer OPW11, followed by OPM06, OPO20 and OPK17 respectively. DNA amplification of ten cocoa clones using four random primers above show DNA band with different intensity. The intensity of DNA bands in each primer is strongly influenced by the the purity and concentration of DNA template. The number of bands produced by each primer depends on the distribution of the site that is homologous to the primer sequences in the genome.

The difference in the number of DNA polymorphic bands and its intensity that generated by each primer, depend on the complementary of primer sequences on the DNA template used. The genetic diversity analysis can work well if the choosed primer can display difference bands that reflect the precise and specific polymorphism of DNA among individuals tested. In addition, the quality and purity of DNA template concentration should be enough and not contaminated with phenolic and polysaccharides compounds which may affect the interpretation of the data (Fatchiyah, 2008; Weeden et al., 1992). Selection of RAPD primer for polymorphisms analysis influence the resulting bands, because each primer has a separate annealing sites, that consequently resulting different polymorphic DNA band, both in size and number.

Genetic diversity in cacao has been examined with several markers such as isozymes (Ronning and Schnell 1994; Warren 1994), mitochondrial and chloroplast DNA (Laurent et al. 1993b), nuclear restriction fragment length polymorphisms (Laurent et al. 1993a, 1994; Figueira et al. 1994; Lerceteau et al. 1997) and RAPDs (Russell et al. 1993; Figueira et al. 1994; Lerceteau et al. 1997). These research revealed, that criollo and forastero varieties has different pattern from many of these analyses (Russell et al. 1993; Ronning and Schnell 1994). The previous studies has also concluded that the greatest diversity in cacao is found in South American plants from the upper Amazonian basin (Laurent et al. 1993a, 1994; Russell et al. 1993)

Of these methods, RAPD offers the highest potential for generating large numbers of markers with the greatest ease (Russell et al., 1993; Welsh and McClelland, 1990). RAPD markers have some limitations,

however, including questionable reproducibility of some bands, a requirement for stringent standardization of reaction conditions, comigration of different amplification products, and dominant inheritance (Bachmann, 1994). Factors other than heredity, such as repetitive DNA and genome size, may account for pattern variation (Bachmann, 1994); novel bands arising in offspring of known pedigree have been reported in primates (Riedy et al., 1992). Therefore, the Mendelian inheritance of these markers must be demonstrated.

Table.1 Code of used Cocoa Clones and its origin

No.	Clone Name	Origin
1.	Lokal	Palolo
2.	AP	Ampibabo
3.	UDT 1	Palolo
4.	PLO	Palolo
5.	Irian	Palolo
6.	45	Ampibabo
7.	M 06	Ampibabo
8.	M01	Ampibabo
9.	S1	Palolo
10	S2	Ampibabo

Table.2 Nucleides Base of RAPD Primer

No.	Primer	Nucleotide Base
1.	OPO 20	ACACACGGCTG
2.	OPM 16	CTGGGCAACT
3.	OPK 17	CCCAGCTGTG
4.	OPW 11	CTGATGCGTG

Table.3 Value of Genetic Distance between 10 cocoa clones (Nei, 1978)

pop ID	1	2	3	4	5	6	7	8	9	10
1	****									
2	0.4308	****								
3	0.5108	0.2877	****							
4	0.4308	0.2231	0.2877	****						
5	0.6931	0.4308	0.9163	0.4308	****					
6	0.5108	0.2877	0.3567	0.2877	0.3567	****				
7	0.5978	0.3567	0.7985	0.3567	0.1625	0.4308	****			
8	0.5108	0.5978	0.9163	0.5978	0.3567	0.5108	0.2877	****		
9	0.5108	0.5978	0.9163	0.5978	0.5108	0.5108	0.2877	0.2231	****	
10	1.2040	0.7985	0.6931	0.4308	0.6931	0.6931	0.4308	0.6931	0.5108	****

Figure.1 DNA patterns of 10 cocoa clones after PCR amplification using OPO20 (Top Left),OPM06 (Top rightt), OPK17 (Bottom Left) and OPW11 primer (Bottom right). Separation on the 2% of agarose gel. Marker (M), Lokal clones (1) AP (2), UDT1 (3), PLO (4), Irian (5), 45 (6), M06 (7), M01 (8), S1 (9), S2 (10)

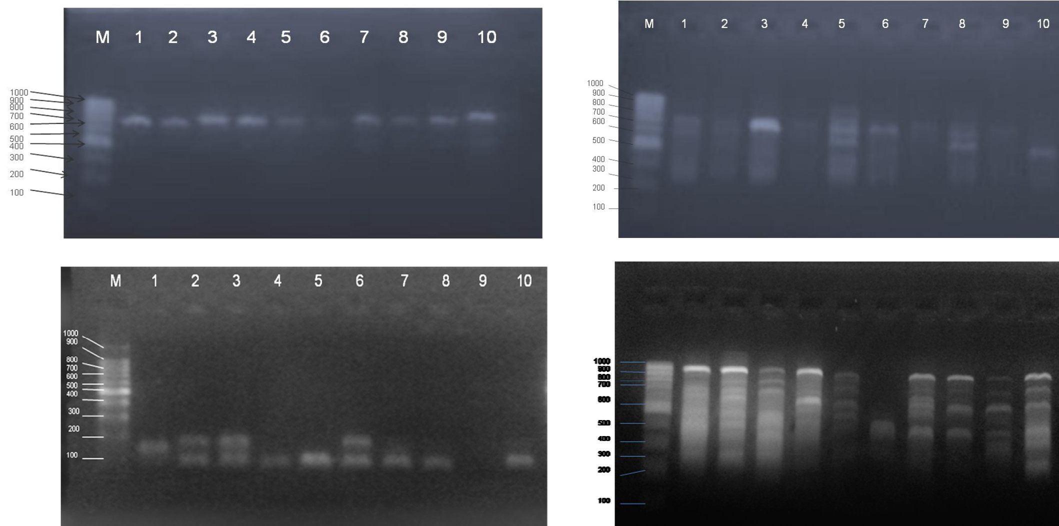
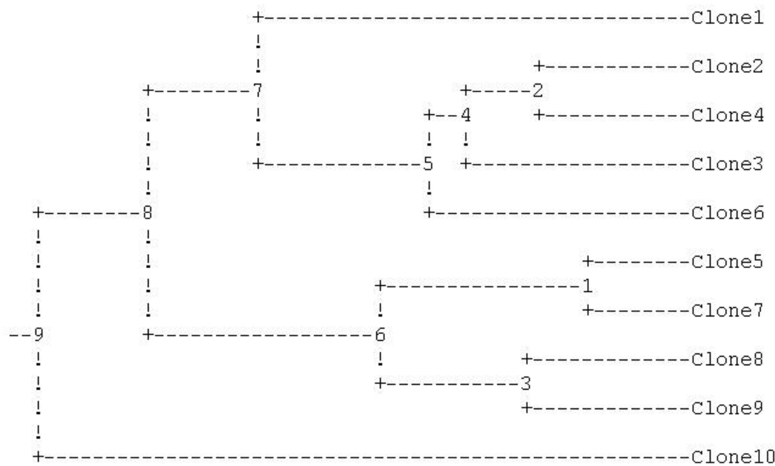


Figure.2 Dendogram of 10 cocoa clones based on genetic distances Nei (1978). Lane (1) Lokal clone, (2) AP, (3) UDT1, (4) PLO, (5) Irian, (6) 45, (7) M06, (8) M01, (9) S1, (10) S2



References

Bachmann, K. 1994. Tansley review no. 63: Molecular markers in plant ecology. New Phytol. 126:403-418.

de la Cruz, M., Whitkus, R., Mota-Bravo, L., 1995b. Tropical tree DNA isolation and ampliÞcation. Mol Ecol 4 : 787-789.

Fatchiyah. 2008. Amplifikasi DNA: Fungsi Dasar dan Aplikasinya. Disampaikan pada Kursus Singkat Analisis Variabilitas Genetik Tanaman MenggunakanPCR (RAPD). Laboratorium Sentral Ilmu Hayati Universitas Brawijaya, Malang. pp.9.

Figueira A., Janick J., Levy M., Goldsbrough P., 1994. Reexamining

the classiÞcation of Theobroma cacao L. using molecular markers. J Am Soc Hortic Sci 119 : 1073Ð1082

Lanaud C *et al*.1999. Isolation and characterization of microsatellites in *Theobroma cacao* L. Mol Ecol. 8: 2141-2143

Laurent, V., A-M. Risterucci, and C. Lanaud. 1993. Variability for nuclear ribosomal genes within Theobroma cacao. Heredity 71:96–103.

Lerceteau E., Robert T., Petiard V., Crouzillat D., 1997. Evaluation of the extent of genetic variability among Theobroma cacao accessions using RAPD and RFLP markers. Theor Appl Genet 95 : 10-19.

MacMahon P dan A. Purwantara . 2004. *Phytophthora* on cacao. *In*: Drenth A & D.I. Guest (eds.). *Diversity and Management of Phytophthora in Southeast Asia*. ACIAR, Canberra, Australia p. 104-115

Nei, M. 1978. Estimation of average heterogycosity and genetic distance from a small numbers of individuals. Genetics 89: 583-590.

Prawirosoemardjo, S. dan A. Purwantara. 1992. Laju infeksi dan intensitas serangan *Phytophthora palmivora* (Butl). Pada buah dan batang pada beberapa varietas kakao. Menara Perkebunan. 60:67-72.

Riedy, M. F., W.J. Hamilton, III, and C.F. Aqadro. 1992. Excess of nonparental bands in offspring from known primate pedigrees assayed using RAPD PCR. Nucleic Acids Res. 20:918.

Ronning C.M., Schnell R.J., 1994. Allozyme diversity in a germplasm collection of *Theobroma cacao* L. J. Hered. 85 : 291-295.

Russell, J. R., F. Hosein, E. Johnson, R. Waugh, and W. Powell. 1993. Genetic differentiation of cocoa (*Theobroma cacao* L.) populations revealed by RAPD analysis. Molec. Ecol. 2:89–97.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Schnell RJ et al. 2007. Development of amarker assisted selection program for cacao. *Phytopat*. 12: 1664-1669.

Susilo, A.W. Akselerasi program pemuliaan kakao (Theobroma Cacao L.) melalui pemanfaatan penanda molekuler dalam proses seleksi. Warta Pusat Penelitian Kopi dan Cacao Indonesia 2007, 23(1), 11-24.

Van Der Vossen Ham, 1997. Strategies of variety improvement in cacao with emphasis on durable disease resistance. Intern. Crop for Genetic Improvement of Cacao, pp. 9-18.

Weeden,N.F., G.M. Timmerman, M.Hemmat, B.E. Kneen, M.A. Lodhi. 1992
reliability of RAPD marker. In Application of RAPD technology to plant breeding , Symposium Proceedings, pp. 12-17.Crop Science Sosiety of America, Medison, WI.

Weising K, Nybom H, Wolff K, and Meyer W. 1995.DNA Fingerprinting in Plant and Fungi. Boca Raton: CRC Press. 472 pp.

Welsh, J. and M. McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:72 13–7218.

Williams, J. G. K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitari primers are useful as genetic markers. Nucleic Acid Res. 18:6531-6535.