Pelita Perkebunan 34 (1) 2018, 23–32 ISSN: 0215-0212 / e-ISSN: 2406-9574

Genetic Diversity Analysis of Edel Cocoa Clones Parental Cross Using Maturase K (*matK*) Gene

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Received: 18 September 2017 / Accepted: 26 November 2017

Abstract

Economically, cacao bean products are divided into fine flavor and bulk cocoa. Fine flavor cocoa has white color beans while bulk cocoa has purple color beans. Bean color of fine-flavor cocoa beans is determined by the presence of a double recessive gene which is inherited from Criollo cocoa type. Analysis of the genetic background in this study used maturase K (matK) gene to ascertain the identity of the genotypes which will be used as a parent in crossing of fine flavor cocoa plants. The study aimed to investigate the genetic background of the promising clones that will be used as a parent in breeding program on fine flavor cocoa based on maturase K (*matK*) gene in order to ensure the identity of the genotype that will be used in parent crossing and it had Criollo ancestor. DNA analysis was conducted at Agency for the Assessment and Application Technology (BPPT), Serpong, West Java. DNA analysis was conducted on eight genotypes consisting of four genotypes of fine flavor cocoa (ICCRI 02, DRC 16, PNT 16 and DR 2) and four genotypes of bulk cocoa (MCC 01, MCC 02, Sulawesi 1, and KW 617). The results showed that Maturase K (*matK*) was one of chloroplast gene which could be used to study phylogenetic and evolution on cocoa. Two primers Mac 02 and Mac 09 were used for amplification of matK gene on cocoa with a rate of homology 99-100% with position 872 bp for Mac 02 and 1153 bp for Mac 09. The results of the phylogenetic analysis showed that the cocoa genotypes would be used as parent crossing included DR 2, ICCRI 02, DRC 16, PNT 16, MCC 01, MCC 02, Sulawesi 1, KW 617 and HJ 2 tended to have ancestral Criollo as female parent.

Keywords: Maturase K, fine-flavor cocoa, genetic variation, crossing parent

INTRODUCTION

Cocoa plants are classified into three types, namely Criollo, Forastero, and Trinitario. Forastero consists of three groups including Amelonado, Upper Amazona and Lower Amazona (Motamayor *et al.*, 2008). Forastero is the most-used cocoa reaching up to 80-90% of total cocoa production. Criollo is a type of cocoa which has fine taste, yet it is vulnerable to pest and disease (Mawardi, 1982). Trinitario is a crossed cocoa plant between Criollo and Forastero which results to excellent vigor variation, characteristics and production (Motamayor *et al.*, 2008). Based on economic point of view, cocoa plants are classified as edel cocoa and bulk cocoa. Bulk cocoa is the one that has fresh purple beans, while edel cocoa has fresh white beans (Anita-Sari *et al.*, 2016).

Cocoa plants in Indonesia are mostly the breeds of Trinitario cocoa which is the crosses plants between Criollo Venezuella (introduced in 1888) and Forastero Venezuela (introduced in 1880). Cocoa breeding program was done at the first time in 1912 by Dr. C.J.J. van Hall using the selection method to the parental plants in Djati Roenggo cocoa farm, Central Java, Indonesia. The selection method resulted in 12 numbers of cocoa including DR 1 and DR 2. Whilst, DRC 16 is one of the selected plants from the DR 1 illegitimate beans (Marwadi, 1982). Repeated selection was done on edel cocoa population in Penataran cocoa farm, East Java, which resulted in quality cocoa beans genotype of ICCRI 01 and ICCRI 02 which are resistant to pod rot (Suhendi *et al*., 2004). DRC 15 is an edel cocoa, a quality genotype that is resistant to VSD (vascular streak dieback), obtained from the selection method carried out in Kaliwining Experimental Station which was later renamed with ICCRI 05 (Susilo *et al*., 2009). Individual selection to Trinintario population planted in 1938 in Penataran cocoa farm was conducted based on several criteria including yield potency, susceptibility to VSD and quality beans (color of the beans). The selection resulted six varieties of cocoa breeds code PNT 8, PNT 12, PNT 16, PNT 17, PNT 18 and PNT 33B (Anita-Sari *et al.*, 2015).

Cocoa breeding program is currently focused on conducting some researches to find for the best edel cocoa that are resistant to pests and diseases especially VSD which has caused huge damage to cocoa farms in Indonesia. The breeding process can be obtained through cross pollination between edel cocoa and bulk cocoa which has strong resistance to pests and diseases. The cross-pollinating to obtain edel cocoa with white beans needs parent seeds from Criollo gene for the white gene produced by two pairs of recessive genes. The white gene is inherited from Criollo cocoa (Bartley, 2005). Thus, by knowing the genetic background of parent cross plants from the Trinitario breed which is originally from the Criollo breed, it is expected that the probability to produce cocoa with higher number of white beans since the Criollo brings recessive white gene (aa) is cross-pollinated with the Trinitario (Aa), that gives higher chance to obtaine the aa genotype. The result would be different if Forastero (AA) is used in the experiment, that is likely to produce purple cocoa beans (Aa).

Analysis of cocoa genetic background can be done using genom chloroplast. According to Chase *et al.* (1993), chloroplast genom is highly suitable to be used for kinship identification in species level. Chloroplast genom (cpDNA) is a paired-DNA that forms circular shape of size 120-160 kb (Didriksen, 2010) which consists of the *rbcL*, *trnH-psbA* and *matK* genes (Kress *et al*., 2007; Kalangi *et al*., 2014). The consortium for the barcode of life (CBOL) recommends *rbcL* and *matK* genes to be used as standard barcodes. The order of nucleotide of the *matK* gene produces approximately 1500 pb (Enan & Ahmed, 2012). Fuse & Tamura (2000) stated that *matK* gene is one of the chloroplast gene which codes the maturase. The eternal plastid that is most effective to be used in the phylogeny and plant evolution studies. The objective of this research was to investigate the genetic background of the parents clones of the cross edel cocoa based on the maturase K (*matK*) gene in order to determine that the genotype of the breeds used in the cross pollination bring the characteristics of Criollo beans.

MATERIALS AND METHODS

In this study, the genetic materials were collected from the collection of cocoa (*Theobroma cocoa* L.) germplasm in Kaliwining Experimental Station of Indonesian Coffee and

Cocoa Research Institute (ICCRI), Jember, East Java, Indonesia. Multiplication of the genetic materials was done using grafting method on the rootstock of plants after reaching the age of 3 months. Good quality scions were used in this study as indicated by brown color in their top parts, and green color in their bases. The plant grafting was done using 2-3 prospective buds. The graft cover was opened after 2 weeks as indicated by the presence of new buds in the upper scion. Graft failure is shown by withered scions or rotten scions. The genetic materials obtained from the process were then brought from ICCRI to Luwikopo Garden, Bogor Agriculture University to make it easier in selecting samples of leaves and in analyzing the DNA. The genetic materials included two types of cocoa plants; bulk cocoa and edel cocoa (Table 1).

Table 1. Genetic meterials used in DNA analysis of *matK* gene of cocoa plant

Genotype	Pedigree
Sulawesi 01	Bulk cocoa, exploration result of South Sulawesi
MCC 01	Bulk cocoa, exploration result of South Sulawesi
MCC 02	Bulk cocoa, exploration result of South Sulawesi
KW 617	Bulk cocoa, selection result of TSH 858 x Sulawesi 01 cross
DR 2	Edel cocoa, selection result of Djati Roenggo Estate
ICCRI 02	Edel cocoa, selection result of Penataran Estate
DRC 16	Edel cocoa, selection result of DR 53 progeny in Djati Roenggo Estate
PNT 16	Edel cocoa, exploration result of Penataran Estate

DNA analysis was conducted in Badan Pengkajian dan Penerapan Teknologi (BPPT), Serpong, West Java. The DNA of eight cocoa genotypes including four genotypes of edel cocoa (ICCRI 02, DRC 16, PNT 16, DR 2) and four genotypes of bulk cocoa were analyzed in this study.

DNA Extraction and Determination

The DNA extraction method refered to a modified model of DNA Mini Kit (Plant) Protocol. In the preparation process, genom was extracted by selecting some samples of leaves which were then cut into smaller size around 1 mm x 1 mm or 0.15 g to make easier in grinding. Liquid nitrogen was added in the grinding process of the leaves in mortal porcelain. The mashed leaves were then put into 1.5 microtube for vortex processing. The next step was adding 400 µL GP1 and 0.5 µL RNAse then incubated in a temperature of 65^{O}C for 10 minutes. After that, it was added with 100 µl GP2 to undergo vortex process until it evenly spread before incubated in ice for 3 minutes. The solvent was then poured into the filter column, centrifuged at 5000 rpm for 2 minutes, and its supernatant was put into 1.5 microtube to be added with GP3 buffer as much as 1.5 times of the supernatant volume. Then, the solvent was moved to the GD column to be centrifuged for 2 minutes at 5,000 rpm twice. After that, the supernatant was left 400 µl of W1 Buffer was added and centrifuged for 30 seconds. The GD collumn was then brought to 1.5 microtube, added with elution buffer which had been incubated within a temperature of 65^{O}C and let sit for 5 minutes, centrifuged for 1 minute at 5000 rpm, and then the GD Collumn was thrown away. The result of the extraction was then tested using nano drop in order to determine its DNA quality and quantity per 1 µl.

The DNA was amplified using 2 pairs of primer which were designed using BLAST program, which results were coded as Mac 02 and Mac 09. Amplification process employed a PCR Takara machine with the total solvent volume of 25 µL per reaction which consisted of 12.5 µL PCR buffer, 0.5 µL primer Mac 09 Forward, 0.5 µl primer Mac 09 Reverse

(or 0.5 µL of Mac 02 Forward; 0.5 µl of primer Mac 02 Reverse); 0.5 µL Terra Polimerase; 1 µL of DNA and 9 µL of ddH_2O. PCR amplification was set as follows; 1) pre-denaturation at 98^{O}C for 2 minutes for 1 cycle; 2) Denaturation at 98^{O}C for 10 for 35 cycles; 3) Anneling TM 57^{O}C for 15 minutes for 30 cycles; 4) Elongation at 68^{O}C for 1 minute for 35 cycles; and 5) Final elongation for 68^{O}C for 1 minute for 1 cycle.

The product of amplification called amplicon was then electrophorized at 0.8% agarose using 1X TAE buffer, dyed using loading dye and then soaked into 1% ethidium bromide for 15 minutes and it was visualized using UV transluminator and digital camera.

Sequencing

The sequencing process required a mixture of 23 µl DNA from the PCR process and 50 µL of amplicon. Purification was done to 300 mg gel from the electrophoresis added with DF buffer, and then incubated in 55-60^{O}C temperature for 10-15 minutes. After that, 800 ml of the sample was put into the DF column to be centrifuged at 14.000 rpm for 30 seconds. Then, the supernatant was thrown away, and 600 µL of wash buffer was poured and centrifuged at 14.000 rpm for 30 seconds and 2 times repetition. Elution buffer or TE was then added and centrifuged at 14.000 rpm for 30 seconds, and the purification result was analyzed using a nanodrop. The result of the purification or the amplicon from the PCR process was finally sent to the Fist Base for the sequencing procedure.

The result of the sequencing process was analyzed using BLAST program and Clustal Omega. The analysis was done in the homology level and sequenced alignment among the eight genotypes. Kinship analysis was administered using the phylogenetic tree test in Clustal Omega program.

RESULTS AND DISCUSSION

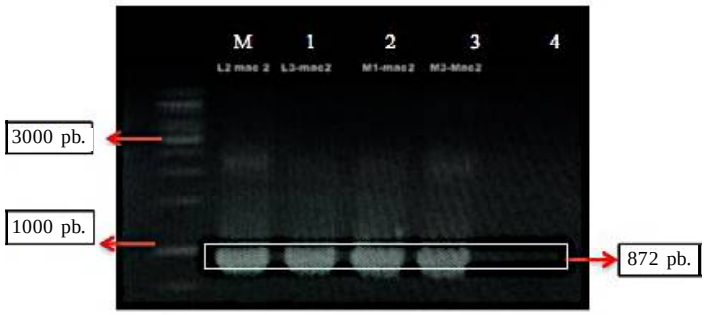
DNA isolation of the cocoa chloroplasts was done to amplified the target gene that is the maturase K (*matK*) found in the chloroplasts. The isolation of the pure chloroplast DNA of the cocoa plants was a challenging task since cacao leaves had smaller cells, and the amount of core DNA in every cell was higher that the cpDNA, besides it also contained high polyphenols which caused the imperfection and absence of restriction endonuclease (REN) enzyme. According to Fatchiyah *et al*. (2011)), DNA extraction from the nucleous, mitochondria, and other organels is a crucial step through lysis processs and homogenization using extraction buffer or lysis buffer to protect the DNA from any damage.

Total DNA genom from the isolation process can be seen from its quantity and quality using nanodrop. The DNA which had good quality and quantity were then amplified using primer Mac 02 and Mac 09. The electrophoresis from the PCR using 100 volt potential was done for 35 minutes with agarose concentration of aorund 0.8–1%. The agarose gel resulted from the electrophoresis was soaked in etidium bromide 1% for 15 minutes. Etidium bromide contained fluorescent compound which was chained to the DNA, making the DNA fluorescent inside the agarose gel if it hit the UV light. The PCR *matK* gene amplification produced DNA fragments as seen in electrophoregram (Figure 2, 3, and 4).

The visualization of Terra™ PCR *Polymerase* product using primer Mac 02 and Mac 09 showed the appearance of DNA *matK* gene ladders which were the target gene. Primer Mac 02 was found in 872 pb and the primer Mac 09 was at 1153 pb. The result of the visualization indicated an effect of agarose concentration used in the experiment on the thickness of the ladders. 1% concentration

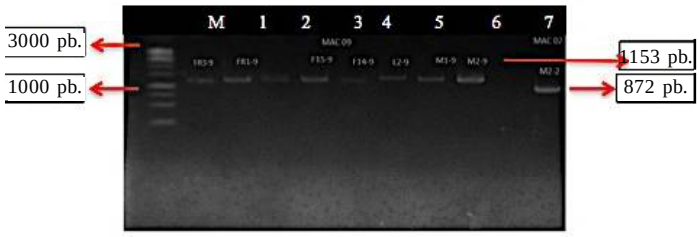
Agarose gel 1%, 100 V, 30

Notes:
M : Marker DNA 1 kb
Column 1 : MCC 02 - Mac 09
Column 2 : PNT 16 - Mac 09



Agarose gel 1%, 100 V, 30

Notes:
M : Marker DNA 1 kb
Column 1 : KW 617 - Mac 02
Column 2 : MCC 02 - Mac 02
Column 3 : ICCRI 02 - Mac 02
Column 4 : PNT 16 - Mac 02



Agarose gel 1%, 100 V, 30

Notes:
M : Marker DNA 1 kb
Column 1 : ICCRI 02 x Sulawesi 01 - Mac 09
Column 2 : ICCRI 02 x Sulawesi 01 - Mac 09
Column 3 :
Column 4 : Sulawesi 01 x ICCRI 02 - Mac 09
Column 5 : Sulawesi 01 x ICCRI 01 - Mac 09
Column 6 : KW 617 - Mac 09
Column 7 : ICCRI 02 - Mac 09
Column 8 :
Column 9 : DRC 16 - Mac 09

Figure 1. Product visualization of Terra™ PCR Polymerase with Mac 02 dan Mac 09 primer on *matK* area of cocoa plant

Table 2. Blast result of *matK* area of cocoa plant

Description of *Theobroma cacao*	Max score	Total score	Query cover	E value	Ident	Accession
Genotype ICS-39 chloplast, partial genome	2918	2918	100 %	0.0	100 %	JQ228387.1
Genotype Pentagonum chloroplast, partial genome	2918	2918	100 %	0.0	100 %	JQ228386.1
Genotype Stahel chloroplast, partial genome	2918	2918	100 %	0.0	100 %	JQ228385.1
Genotype Criollo-22 chloroplast, partial genome	2918	2918	100 %	0.0	100 %	JQ228379.1
*C*hloroplast, partial genome	2913	2913	100 %	0.0	100 %	JQ228389.1
Genotype ICS-01 chloroplast, partial genome	2913	2913	100 %	0.0	100 %	JQ228181.1
Genotype Amelonado chloroplast, partial genome	2913	2913	100 %	0.0	99 %	JQ228380.1
*C*hloroplast, complete genome	2907	2907	100 %	0.0	99 %	HQ336404.2
Genotype ICS-06 chloroplast, partial genome	2907	2907	100 %	0.0	99 %	JQ228383.1
Genotype Scavina-6 chloroplast, partial genome	2907	2907	100 %	0.0	99 %	JQ2283832.1

of agarose showed thicker ladders than the ones from agarose 0.8%.

Good quality of DNA ladders from the electrophoresis using PCR can be seen from the cleanliness of the ladder. The selected DNA as then sequenced to see the formation of nucleotide base from each ladder according to the primer used in the process. Based on the result, the nucleotides were merged in order from each sequencing of each primer Mac 02 and Mac 09 to form chunked *matK* gene from each genotype. The merging was done using BLAST program which the pairing results were later manually merged.

Base Nucleotide Order Homology

The determination of base nucleotide order was carried out using Nucleotide Basic Local Allignment Search Tool (Nucleotide BLAST). Referring to the result of a research done by Imanissa (2016), it was stated that the sequencing result was confirmed using the Blast program (nucleotide blast) in NCBI. This program was used to obtain organism information with similar base nucleotide order to the base nucleotide order of the samples. The homology of base nucleotide order obtained using *blastn* showed that the *matK* area from the sequencing result had high homology level at 99-100% in the *matK* area from the *Theobroma cocoa* genotype ICS-39, Pentagonum, Shahel, Criollo-22, ICS -01, ICS-06 and Scavina-6 (Table 2). Base nucleotide order obtained from the sequencing was the *matK* area of *Theobroma cocoa* L. (NCBI:2).

Multiple Allignment and Philogenetic Analysis

Multiple alignment was administered to the sequenced results from two primaries Mac 02 and Mac 09 which had been previously merged. The pairing of the sequencing results of the two primaries using BLAST program were then manually merged based on the base nucleotides pairing order. The multiple alignment was done to eight genotypes and some sequenced area of the *matK* from several sources such as the ones mentioned by Dermawan (2016) including HJ 2 and sequenced *matK* area from a number of cocoa genotypes obtained from GeneBank NCBI. Some of the secondary data used in this study were also obtained from the GeneBank NCBI including Amelonado, ICS-39, Scavina-6, ICS-06, Criollo-22, EET-64, and ICS-01.

The result of the analysis showed that the genotype Sulawesi 1 had some differences from the DRC 16, and it showed an obvious difference between the two nucleotide bases compared to other genotypes. It was found that the other genotypes have the base order AG (*adenine-guanine*) while Sulawesi 1 has a

GA (*guanine-adenine*). The genotype DRC 16 also showed a different base nucleotide from other genotypes for DRC 16 has a TT (*thymine-thymine*). Whereas, other genotypes have an AT (*adenine-thymine*). The quality of those bases have been analyzed using a software namely Sequnced Scanner 2.0 which result showed that they were high quality bases. This result goes in line with the ones pointed out by Immanisa (2016) in which it was stated that the Sulawesi 1 had the highest difference in number of the bases compared to other samples.

Phylogenetic analysis was performed based on the sequencing or the order of nucleotide bases from the result of the merge between two primaries. The result of the analysis showed that the genotyes formed three groups. The group I consisted of ICS 06, Sca 6 and ICS 01 with a genetic gap of 0.0. The group II consisted of ICS-39, HJ 2, DR 2, MCC 01, Sulawesi 1, PNT 16, KW 617, DRC 16, MCC 02, ICCRI 02, EET-64 and Criollo-22. Whilst, the group III only consisted of Amelonado.

The result of the phylogenetic analysis showed that cocoa genotypes in Indonesia are DR 2, ICCRI 02, DRC 16, PNT 16, MCC 01, MCC 02, Sulawesi 1, KW 617 and HJ 2 which have closer relationship and genetic background to Criollo rather than Forastero which in this experiment was represented by Amelonado, Upper Amazona and Lower Amazona. This result supports the report on cocoa planting released by Mawardi (1982) who mentioned that the first

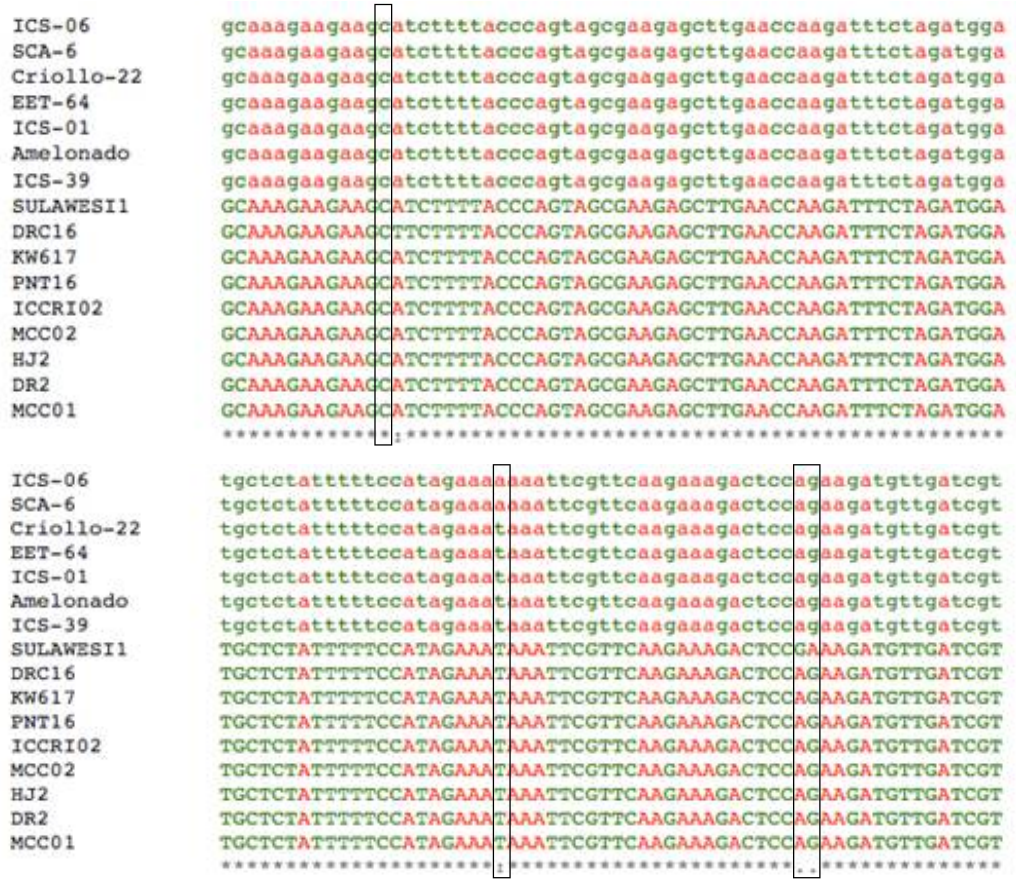


Figure 2. Differences in the nucleotide base arrangement based on the multi alignment of the *matK* on the cocoa plant

Table 3. Difference in the number of bases between genotypes based on cacao results *Multiple Alignment*

No. genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	***															
3	**	*														
4	**	*														
5	**	*														
6	**	*														
7	**	*														
8	**	*														
9	**	*														
10	***	**	*	*	*	*	*	*	*							
11	***	**	*	*	*	*	*	*	*	*						
12	**	*								*	*					
13	**	*								*	*					
14	**	*								*	*					
15	**	*								*	*					
16	**	*								*	*					

Notes: No. genotypes: (1) Sulawesi 1, (2) DRC 16, (3) KW 617, (4) PNT 16, (5) ICCRI 02, (6) MCC 02, (7) HJ 2, (8) DR 2, (9) MCC 01, (10) ICS-6, (11) Sca-6, (12) Criollo-22, (13) EET-64, (14) ICS-1, (15) Amelonado, (16) ICS-39.

* Shows difference in number of nucleotude bares.

cocoa breeding procedure was done by Van Hall in 1912 using the Criollo as the female parent and Forastero as the male parent. The *matK* gene is a chloroplast gene which is maternally inherited, resulting to a progenic closeness and genetic background between the samples and the female parent beans. Character inheritance from the parents to the scions might occur in two mechanisms; chromosomal (nucleous) and extrachromosomal. Extrachromosomal inheritance is controlled by the gene outside the nucleous. Extrachromosomal inheritance might occur in the form of cytoplasmic inheritance if the genetic factors come from outside the nucleous. Genetic factors can be only transferred through cytoplasm and they last for several generations (Sastrosumarjo *et al.*, 2013). The plastids in plants can be inherited from the parent plants to the scions in three ways; maternal, parental and biparental, yet the plastids in plants with flower is mostly inherited maternally (Mogensen, 1996). The DNA plastids inheritance in plants is controlled by methylation process within a specific site of a DNA in a maternal organel that prevents degradation form occurring in the zygote (Hu *et al.*, 2005).

It is important to deterime the genotype identity of the parent clones of cocoa plants to make sure that they bring Criollo genes that produce white cocoa beans. Bartley (2005) explained that white cocoa beans are produced by two pairs of recessive genes (*aa*). It is believed that most of cocoa plants in Indonesia come from Trinitario type which is known to bring Criollo gene (*a*) and Forastero (*A*), which make it feasible to obtain gene *aa* that procudes white beans cocoa through cross-pollination. Furthermore, if the the identity of the parent plants is not identified, it would be difficult to obtain cocoa plants with white beans because when the genotype of Forestero (*AA*) meets the Triniatrio with *Aa* genotype, double recessive genes would not be obtained and white beans cannot be produced.

Beside genetic determination, genetic gap is also an important factor to consider

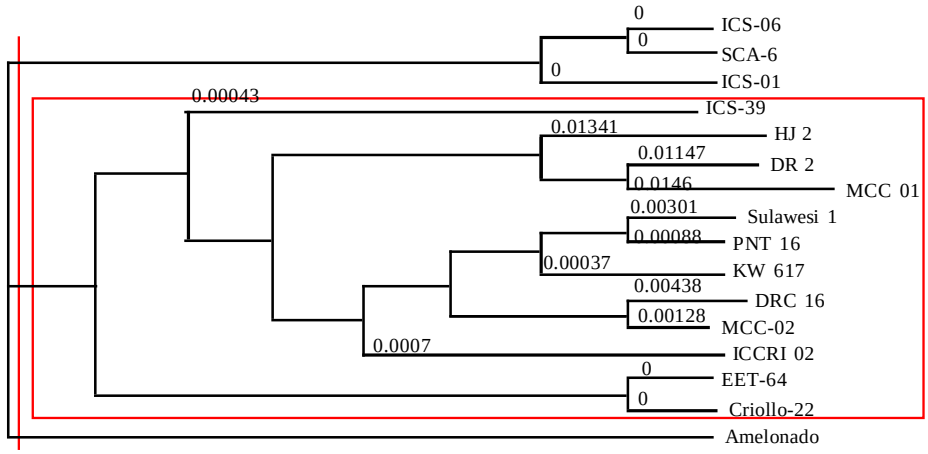


Figure 3. Phylogenetic chart of several cocoa genotypes based on the sequence base nucleotide *matK*

in determining the parent plants. Result of previous research done by Susilo *et al.*, (2011) showed that PNT 23 was a genotype of Java Criollo group which had the closest relationship with Criollo 22. Phylogenetic analysis using *matK* gene was intended to obtain better edel cocoa seeds with better resistance to VSD using parent plants with the closest genetic gap that were DR 2 x MCC 01, PNT 16 x Sulawesi 1/KW 617, DRC 16 x MCC 02. On the other side, to produce better genetic variety, parent plants should have the most distant genetic gap. It is also important in selecting the best parent plants, the characteristics of the plants including the taste of the beans should be highly considered in order to maintain the quality of the edel cocoa which is famous for its fine flavor taste.

CONCLUSIONS

The primer Mac 02 and Mac 09 can be used to amplify the maturase K gene (*matK*) in cocoa plants with a relatively high homology level of around 90-100%. Most of cocoa plants in Indonesia inherit their genetic background from the female Criollo as shown by the result of maturase K gene analysis (*matK*). Parent plants selection to improve the plant resistance to VSD can be carried out using the parent plants with a close genetic gap, namely DR 2 x MCC 01, PNT 16 x Sulawesi 1/ KW 617, DRC 16 x MCC 02. On the other hand, to obtain various genetic diversity, plants with distant genetic gap can be used as the parent plant but the quality taste of the beans should remain the main consideration.

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