GENETIC VARIABILITY OF 15 ROBUSTA COFFEE GENOTYPES SELECTED BY FARMER BASED ON SSRs MARKERS

KERAGAMAN GENETIK 15 GENOTIPE KOPI ROBUSTA HASIL SELEKSI PETANI BERBASIS PENANDA SSRs (Simple Sequence Repeats)

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ABSTRACT

Robusta coffee (*Coffea canephora*) has been grown widely in Indonesia, especially in Bengkulu Province. For the last few decades, some farmers have been selected and developed several Robusta clones through plagiotropic shoot grafting technique to replace earlier coffee populations which were derived from seed. Hence, it would reduce the genetic diversity of Robusta coffee at farmer's field. To understand the genetic variability among 15 Robusta coffee genotypes selected by farmer, it is important to perform molecular analysis. Leaf samples of 15 Robusta coffee genotypes selected by farmer were collected from smallholder Robusta coffee plantations in Bengkulu Province. Genetic diversity analysis was conducted in the Germplasm, Breeding, and Biotechnology Laboratory of Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), and Molecular Biology Laboratory, Indonesian Cereals Research Institute (ICERI). DNA samples were amplified using 34 SSRs markers. The result showed that 23 out of 34 SSRs markers had high polymorphism levels. Allele number per locus ranged from 2-8 with an average of 4 alleles per locus. Dendrogram analysis based on genetic similarity was obtained with score of about 0,44-0,79, and r score = 0,92 (good fit). Based on cluster analysis as well as PCoA analysis, there are three distinct groups of genotypes. Those three groups can be distinguished by specific character of leaf morphotype. Nevertheless, the majority of genotypes were clustered together into the single group. This indicates narrow genetic diversity among Robusta genotypes that selected by farmer.

Kata kunci: *Coffea canephora*, plagiotropic clones, genetic drift

ABSTRAK

Kopi Robusta telah dikembangkan secara luas di Indonesia, khususnya di Provinsi Bengkulu. Beberapa dekade terakhir sebagian petani telah menyeleksi dan mengembangkan beberapa genotipe dengan teknik sambung tunas plagiotrop untuk merehabilitasi populasi kopi Robusta asal biji. Oleh sebab itu, terdapat peluang terjadinya penurunan keragaman genetik kopi Robusta di lahan petani. Analisis molekuler perlu dilakukan untuk mengevaluasi keragaman genetik antar 15 genotipe kopi Robusta hasil seleksi petani. Kegiatan analisis keragaman genetik dilaksanakan di Laboratorium Plasma Nutfah, Pemuliaan, dan Bioteknologi, Balai Penelitian Tanaman Industri dan Penyegar (Balittri), Sukabumi dan Laboratorium Biologi Molekuler, Balai Penelitian Tanaman Serealia (Balitsereal), Maros. DNA diamplifikasi dengan menggunakan 34 marka SSR. Hasil penelitian menunjukkan bahwa 23 dari 34 marka SSR yang digunakan mampu menghasilkan tingkat polimorfisme yang tinggi. Jumlah alel berada pada kisaran 2-8 alel per lokus dengan rata-rata 4 alel per lokus SSR. Analisis dendrogram berdasarkan kemiripan genetik diperoleh dengan skor sekitar 0,44-0,79 dan skor r = 0,92 (good fit). Berdasarkan hasil analisis gerombol dan analisis komponen utama diketahui bahwa terdapat tiga kelompok genotipe. Masing-masing kelompok dapat dibedakan berdasarkan karakter morfotipe daun. Meskipun demikian, sebagian besar genotipe diklasifikasikan ke dalam satu kelompok. Ini menandakan bahwa keragaman genetik klon-klon kopi Robusta hasil seleksi petani cenderung rendah.

Keywords: Coffea canephora, *klon plagiotropik, kehilangan genetik*

INTRODUCTION

Coffee is one of the most important commercial crop-plant, and the second most valuable international commodity after fossil fuel. Million people in the world depend on coffee plant for their livelihoods. Today, coffee is cultivated in about 80 countries with 70% of its production come from Arabica species, whereas the remaining production are from Robusta species (Anthony *et al.*, 2001; Anthony *et al.*,2002; Stieger *et al.*, 2002; Taye, 2006).

Up to now, two largest coffee producers worldwide are Brazil and Colombia which then followed by Vietnam, Indonesia, Ethiopia, India and Mexico (International Coffee Organization [ICO], 2014). In Indonesia, coffee is the second most important estate crops after palm oil. According to Indonesian Coffee Exporters and Producers Association, export of Robusta and Arabica coffee in 2010 reached 360.603 and 78.036 tonnes, respectively, with the value of US $ 571.977.000 and US $ 249.162.000, respectively.

Robusta coffee is a species of coffee with the origin in central and western sub-Saharan Africa. It is a flowering plant species that belong to Rubiaceae family (Bha *et al*., 2005). In Indonesia, Robusta coffee has been widely grown for the last decades, especially in Bengkulu Province. Robusta coffee was introduced to farmers in order to replace the Arabica and Liberica species because it has better resistance to leaf rust disease and higher yield.

Formerly, farmers used seedling which derived from the seed of open pollinated plants as planting material. However, Robusta coffee is considered as *self-sterile* species; therefore the new emerging populations showed diverse phenotypic variation. Several creative farmers have selected the best individuals from different Robusta coffee populations and subsequently multiply them clonally by means of pagiotropic grafting techniques. According to the farmers, those selected genotypes showed higher and more stable yield compared to coffee plants that derived from seed. Afterwards, Robusta genotypes selected by farmer being spread immediately to wider areas and replace the population which derived from seed. Hence, this evidence could reduce the genetic diversity of Robusta coffee on farmer fields and it may remove the possible valuable genes for future coffee breeding programs.

In the view of the wide geographical distribution of *C. robusta*, characterization and evaluation of its genepool is necessary for crop improvement programs as well as for conservation and management of genetic resources (Prakash, Combes, Dussert, Naveen, & Lashermes, 2005). The use of morphological techniques for genetic diversity study in plants is limited due to the influence of environmental factors and growth stage of the plant (Weising, Nybom, Wolff, & Kahl, 2005). Therefore, molecular markers become the best choice to analyze the genetic diversity of plants. Recently, many plant scientists used RPAD markers to evaluate genetic variation in plants (Ardiana, 2009; Syafaruddin and Santoso, 2011; Syafaruddin, Randriani, & Santoso, 2011; Syafaruddin & Tresniawati, 2011; Randriani, Listyati, & Syafaruddin, 2011). However, SSRs have many advantages, such as high reproducibility, codominant inheritance, the possibility of automation, and widely used to determine the genetic variation within and between populations (Chaparro, Cristancho, Cortina, & Gaitan, 2004; Vigouroux *et al*., 2005; Masumbuko & Brynggelson, 2006). In addition, another potential of SSRs markers is to clearly differentiate coffee genotypes from different geographical origin. This suggests the possibility of SSRs markers to be use in quality control (DNA-based traceability) of Ethiopian premium specialty coffees by their areas of production in Ethiopia (Teressa, Dominique, Vincent, & Brouhan, 2010).

The objective of this study was to evaluate the genetic variability of 15 Robusta coffee genotypes selected by farmer using 34 SSR markers.

MATERIALS AND METHODS

Leaf samples of 15 Robusta coffee genotypes selected by farmer were collected in May 2013 from smallholder Robusta coffee plantations in Bengkulu Province. Laboratory activities were conducted at Germplasm, Breeding and Biotechnology Laboratory of Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), and Molecular Biology Laboratory, Indonesian Cereals Research Institute (ICERI). A total of 34 SSRs markers were used for DNA amplification (Tabel 1).

Table 1. Profile data of 34 SSRs markers used to analyze 15 Robusta coffee genotypes selected by farmer in Bengkulu Province
Tabel 1. Profil 34 penanda SSR yang digunakan untuk menganalisis 15 genotipe kopi Robusta hasil seleksi petani di Provinsi Bengkulu

No	Primer name	Primer sequences	No	Primer name	Primer sequences
1.	SSRCa 003	F: ATG ATT CGT AGG TGG AGT GG R: CTA AGC CGC AAA TGA CAG A	18.	Car M049	F: TAC TGG GGA AGA ATT TAT ACT C R: TTA GGC CAT CCA AGA GTA TTC
2.	SSRCa 016	F: AGC AGA TTC CAT CCT TAT CCT R: CCA CTA ATC CAT TCC ATT CC	19.	Car M051	F: GAT GTG GAG GAG GCT GCT GCT GAA R: TAG GGC GCC ATC TGG TAG GGT TGT
3.	SSRCa 019	F: GGG TTA GAT AGA GCA AGA ATG A R: CTG TGA AGG TGT GGA GTT TT	20.	Car M052	F: AGC AGC TGC AGC CACAAC A R: GAG TAA AG CCC CAG AGC GTA ACC T
4.	SSRCa 023	F: GAC CCT TGC CTT TTG TTG R: GCC ATT CAT CCA TTC ATT C	21.	Car M092	F: AGG CCA GAC TTG TTT GAT TTT G R: GGC CCT TCT CGC TTT AGT TG
5.	SSRCa 026	F: GAA TCT GGT GGG CTT TGA R: AAG GAG AGG GGA AGA AAA TG	22.	Car M048	F: CCA GCA ATC CTC CCT CCC ACC AC R: TAC CGT ATG CAG AGA CAA CAA TG
6.	SSRCa 052	F: GAT GGA AAC CCA GAA AGT TG R: TAG AAG GGC TTT GAC TGG AC	23.	Car M105	F: TGC TCC TAC TAA ATA CCC AAA CA R: ATA TGC CCA AGA AAA TTA GAT GAA A
7.	SSRCa 062	F: AAG TTA TTA GGG CAA GAG TGG A R: AAGCTCCAAGACCAAAGATG	24.	M20	F: CTT GTT TGA GTC TGT CGC TG R: TTT CCC TCC CAA TGT CTG TA
8.	SSRCa 068	F: ATGTTGTTGG AGG CATTTTC R: AGG AGC AGT TGT TGT TTT CC	25.	M24	F: GGC TCG AGA TAT CTG TTT AG R: TTT AAT GGG CAT AGG GTC C
9.	SSRCa 081	F: ACC GTT GTT GGA TAT CTT TG R: GGT TGA ACC TAG ACC TTA TTT	26.	CFGA189-NED	F: CAT CCA TCC GAA AAC TTG TAA CG R: CAG CAC TGG CAA ATA GCA ACT CTT
10.	SSRCa 083	F: TCC AAC AAC ATT AAG CGT ATT C R: GAC AAA CCT GAG GGA AAA GA	27.	CFGA502-FAM	F: AAG CCA CCC AGA AAA CAG CAC ATC R: ATT TGC TTC TCA TGT TCC CTT TCA
11.	SSRCa 087	F: TCA CTC TCG CAG ACA CAC TAC R: GCA GAG ATG ATC ACA AGT CC	28.	CFGA547a-VIC	F: AAG GCA TGC GGC GGG AGT AT R: TCG TCA AGG ACA ATC CTA AAG C
12.	SSRCa 088	F: TAC CTC TCC TCC TCC TTC CT R: ATT TCT ATG GAC CGG CAA C	29.	SSRCa 080	F: GTT CTT TCC GCC GTC AAT R: GAG AAG AGA GAG GAA GGG AAA
13.	SSRCa 092	F: ATA GCC TGA GCC GTA ACC A R: GGG TAA TTA TGA CGA GGG ACA	30.	SSRCa 082	F: GCT TGT TTC CAT CGC TAA A R: TTA CAC GTC AAC CCA CAA AC
14.	SSRCa 094	F: GTG TCC TAG GGA AGG GTA AG R: GAG TGC TAG GAG AGG GAG AG	31.	CM2 –FAM	F: TGT GATG CCA TTA GCC TAGC R: TCC AAC ATG TGC TGG TGA TT
15.	SSRCa 095	F: GAG AGA GCC GAG TGA AGA GA R: GAG AGA GAA GCC ATG ATT TGA	32.	CM8-FAM	F: GCC AAT TGT GCA AAG TGC T R: ATT CATG GGG CCT TTG TCT T
16.	Car M096	F: TAC TGG GGA AGA ATT TAT CAT C R: TTA GGC CAT CCA AGA GTA TTC	33.	CM16-HEX	F: TGG GGA AAA GAA GGA TAT AGA CAA GAG R: GAG GGG GGC TAA GGG AAT AAC ATA
17.	Car M101	F: TAT GTC TCT AAC TTT CTA TTT T R: AGA GAC TAC ATT TAC ACA CAG AAG A	34.	SSRCa091	F: CGT CTC GTA TCA CGC TCT C R: TGT TCC TCG TTC CTC TCT CT

Figure 1. Young and healthy leaf of Robusta coffee used for DNA isolation
Gambar 1.Daun pucuk kopi Robusta yang diambil untuk bahan isolasi DNA

DNA Isolation

Genomic DNA of Robusta coffee genotypes was isolated according to CTAB method used by Doyle & Doyle (1987). Approximately 0.5 g of young and healthy coffee leaves (Figure 1) were used for DNA isolation. DNA quality and quantity were estimated using standard DNA lambda through electhrophoresis. DNA concentration was quotation by using standard DNA lambda. Template DNA of each sample was dissolved into 25 ng for PCR analysis.

PCR Amplification and Amplicon Visualization

DNA amplification was conducted by using method of Williams, Kubelik, Livak, Rafalski, & Tingey (1990). PCR reactios were performed in a total volume of 25 µl that contained 12.5 µl Go taq mix, 10.5 µl water (ion free), 1 µl random primer, and 1 µl of DNA.

The PCR amplification was carried out at 94 °C for 4 min, then 40 cycles each were performed at 94 °C for 30 s, 36 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min. Amplified products were first checked on 1.2% agarose gel in 1x TBE buffer to confirm the amplification on each sample. Afterwards, the amplified PCR products were electrophoretically separated on 6% polyacrylamide gels in 1x TBE buffer. Staining of the gels was done using ethidium bromide for 20 min. Subsequently, DNA banding patterns were visualized under UV light using the gel documentation system.

Data Analysis

The resulted polymorphic bands on each SSRs markers in different Robusta coffee genotypes were scored and coded in a binary format: 1 for presence and 0 for absence, respectively. The data were subsequently analyzed using NTSYS-PC version 2.1 (Rohlf, 2000) to obtain cluster dendrogram, principal coordinate analysis (PCoA) and genetic distance value among coffee genotypes.

RESULTS AND DISCUSSION

The level of genetic variation among coffee genotypes was figured out by the degree of DNA polymorphism. According to McGregor, Lambert, Greyling, Louw, & Warnich (2000) and Poncet *et al*. (2004), polymorphism is defined by the description of difference amplification of DNA fragments, which subsequently scored and analyzed based on the presence or absence of bands. In this study, these 34 SSR markers were able to generate a high degree of polymorphism (0.57), which effectively differentiate each of 15 Robusta coffee genotypes selected by farmer. This degree of DNA polymorphism was considerably higher compared to the result done by Cubry *et al*. (2008), due to they used only 7 SSRs markers to analyze 519 coffee genotypes.

Table 2. Polymorphic level and allele number of 34 SSRs markers on 15 Robusta coffee genotypes selected by farmer in Bengkulu province

Tabel 2. Tingkat polimorfik dan jumlah allel dari 34 penanda SSR yang digunakan untuk menganalisis 15 genotipe kopi Robusta hasil seleksi petani di Provinsi Bengkulu

Primer	Polymorphic level	Number of alleles per locus
CarM048	0.67	3
CarM049	0.67	5
CarM051	0.48	2
CarM052	0.36	3
CarM092	0.61	3
CarM096	0.61	4
CarM101	0.87	8
CarM105	0.61	3
CFGA189NED	0.24	3
CFGA502FAM	0.42	3
CFGA547Avic	0.30	3
CM16HEX	0.51	3
CM2FAM	0.80	6
CM8FAM	0.55	3
M20	0.72	4
M24	0.75	5
SSRCa003	0.50	4
SSRCa016	0.06	2
SSRCa019	0.78	5
SSRCa023	0.42	3
SSRCa026	0.70	3
SSRCa052	0.73	4
SSRCa062	0.59	4
SSRCa068	0.18	3
SSRCa080	0.49	3
SSRCa081	0.73	5
SSRCa082	0.34	3
SSRCa083	0.83	7
SSRCa087	0.54	3
SSRCa088	0.68	3
SSRCa091	0.79	4
SSRCa092	0.62	3
SSRCa094	0.66	3
SSRCa095	0.63	4
Total	19.44	127
Averages	0.57	4

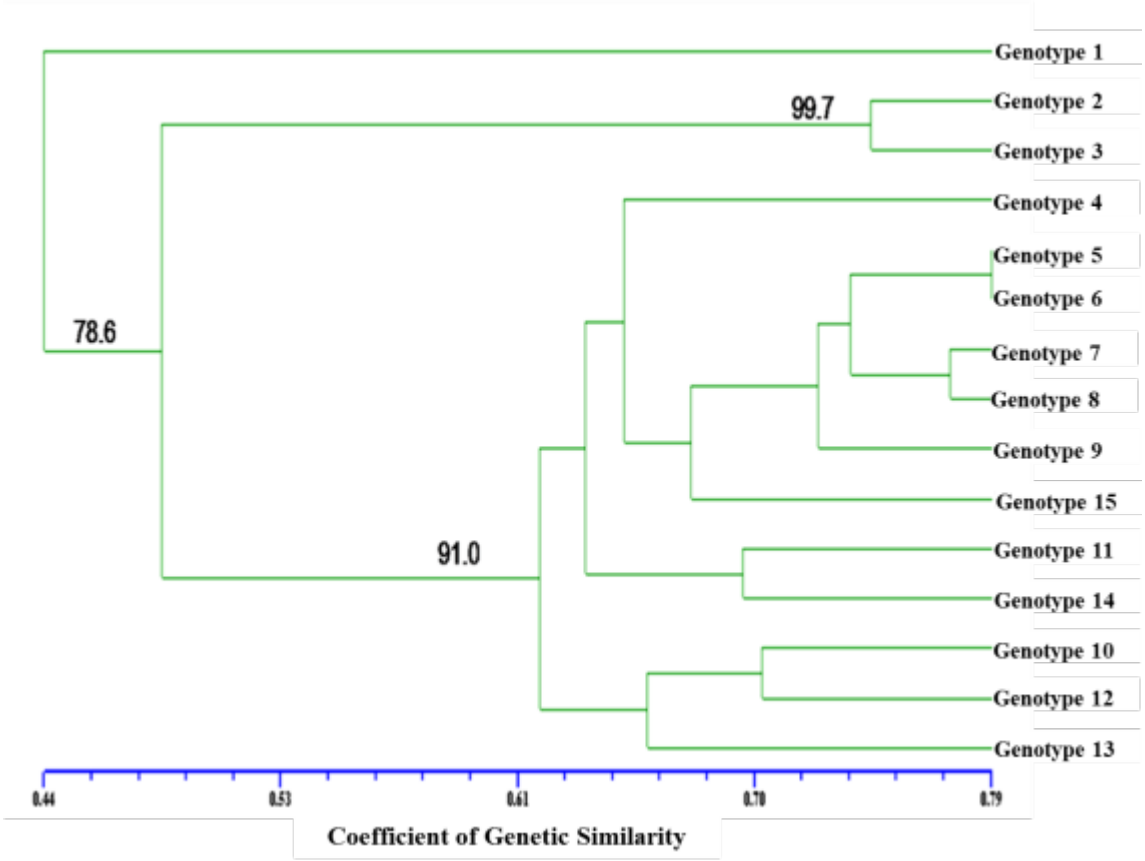

Figure 2. Dendrogram of 15 Robusta coffee genotypes selected by farmer in Bengkulu Province based on genetic similarity value
Gambar 2. Dendrogram 15 genotipe kopi Robusta hasil seleksi petani di Provinsi Bengkulu berdasarkan nilai kesamaan genetik

The polymorphic level obtained in this research ranged from 0.06 to 0.87, with an average of 0.57 (Table 2), higher than those obtained by Missio *et al*. (2009) which is 0.46 but slightly less than those of the previous study on Robusta coffee germplasms in which 0.60 (Hendre & Aggrawal, 2014). Of which, the polymorphic level value higher than 0.5 considered as highly informative (Prabakaran, Paramasivam, Rajesh, & Rajarajan, 2010; Lekgari & Dweikat, 2014). Twenty three (67.65%) out of 34 SSR markers used in present study that showed high degree of polymorphism were potentially selected for future assessment of Robusta coffee germplasms. The total number of allele was 127, ranged from 2 to 8 alleles per locus, with an average of 4 alleles per locus (Table 2). Those values were similar to the results obtained by Missio *et al*. (2010) on three coffee species by using of 33 SSR primers, which generate a total of 122 alleles and the average of 5.1 alleles per SSR locus.

Cluster dendrogram was obtained based on genetic similarity value of 0.44-0.79, and supported by a high cophenetic coefficient correlation (r) = 0,92 (good fit). Based on dendrogram analysis, those 15 Robusta coffee genotypes selected by farmer in Bengkulu province can be divided into 3 clusters with highly confidential level of bootstrapping (Figure 2 and 3). The first cluster had only one member, that is genotype 1, while the second cluster consisted of genotype 2 and 3. The third cluster comprised 12 other genotypes. PCoA analysis also revealed that genotype 1 as well as genotype 2 and 3 were stand at distinct points and clearly separated from the rest of genotypes (Figure 3).

Three groups obtained by UPGMA cluster dendrogram could not explain the geographical distributions of each genotype, this is because some of them are distributed in the same areas, but are grouped separately. It might be related to uncontrolled transfer of planting materials among farmers from different regions. In addition, many farmers have been grafting more than one genotype at the same rootstock, so it is quite difficult to distinguish each genotype of Robusta coffee in farmers' fields.

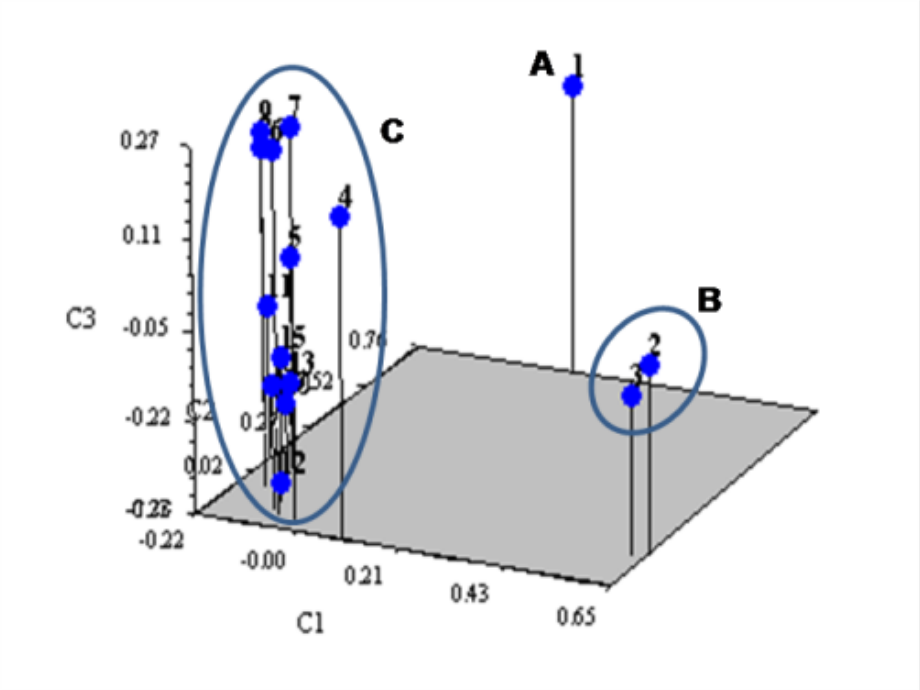

Figure 3. Relative position of 15 Robusta coffee genotypes selected by farmer in Bengkulu province based on PCoA (Principal Coordinate Analysis) analysis with 3 dimensions

Gambar 3. Posisi relatif 15 genotipe kopi Robusta hasil seleksi petani di Provinsi Bengkulu berdasarkan analisis PcoA dengan 3 dimensi

Table 3. Genetic distance matrix of 15 Robusta coffee genotypes selected by farmer in Bengkulu Province

Tabel 3. Matriks jarak genetik 15 genotipe kopi Robusta hasil seleksi petani di Provinsi Bengkulu

Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	0.00														
2	**0.63**	0.00													
3	**0.61**	0.25	0.00												
4	**0.60**	0.51	0.42	0.00											
5	0.55	0.48	0.46	0.26	0.00										
6	0.53	0.44	0.48	0.26	**0.21**	0.00									
7	0.54	0.51	0.50	0.39	0.35	0.22	0.00								
8	0.51	0.53	0.50	0.35	0.27	**0.21**	0.22	0.00							
9	0.52	0.55	0.56	0.41	0.33	**0.21**	0.31	0.25	0.00						
10	0.55	0.57	0.59	0.44	0.34	0.38	0.41	0.39	0.36	0.00					
11	0.53	**0.60**	0.56	0.43	0.41	0.36	0.35	0.29	0.33	0.39	0.00				
12	0.57	0.55	0.49	0.44	0.31	0.33	0.41	0.36	0.41	0.29	0.34	0.00			
13	0.57	0.58	0.53	0.46	0.38	0.38	0.37	0.34	0.37	0.37	0.37	0.31	0.00		
14	**0.62**	0.53	0.50	0.40	0.39	0.31	0.39	0.34	0.37	0.43	0.30	0.34	0.38	0.00	
15	0.56	0.51	0.51	0.41	0.25	0.29	0.37	0.31	0.38	0.33	0.34	0.32	0.43	0.32	0.00

Based on the morphotype, the first group characterized by small-size and elliptical leaves, while the second group shows thick-broad leaves and the third group has a medium-size and ovate leaves feature. According to farmer's experience, Robusta genotypes that belong to the third group also exhibited higher and more stable yield. Therefore, farmers tend to replace those genotypes which belong to the first as well as the second group with the genotypes that clustered in the third group. This indicates a high tendency for losing of some valuable alleles on farmer's field. Hence, the genetic variability among commercial Robusta varieties

tend to be lower in the future as already happened in Kenya (Hue, 2005; Kathurima *et al*., 2012). The similar phenomena was also shown in commercial Arabica varieties due to human interventions (Teressa *et al.*, 2010). However, those superior genotypes might further use as a new source of planting materials.

The genetic distance matrix showed an estimation of genetic distance value between genotypes. Of which, the highest genetic distance value between genotypes are considered as the best parental combination. Several combinations that showed high genetic distance value are: 1 vs 2 (0.63), 1 vs 3 (0.61), 1 vs 4 (0.60), 1 vs 14 (0.62), and 2 vs 11 (0,60) (Table 3). Those combinations may generate novel and wider genetic variations. The Progenies derived from those combinations were subsequently characterized and evaluate individually to obtain new elite clones.

CONCLUSION

Profile data of the majority of SSR markers used in this study showed a high polymorphic level. Twenty three out of 34 SSR markers were potentially used for future Robusta coffee germplasm studies. The number of alleles ranged from 2-8 alleles per SSR locus with an average of 4 alleles per locus. Dendrogram analysis was obtained based on genetic similarity value with the score of about 0,44-0,79, and r score = 0,92 (good fit). Based on cluster analysis as well as PCoA analysis, three distinct groups of Robusta coffee genotypes were obtained. Those three groups also showed specific morphotype character. However, the majority of genotypes were clustered together into the same group. Therefore, the genetic diversity among Robusta genotypes selected by farmer is considerably low. Furthermore, those superior genotypes could be characterize and evaluated as source of planting materials or as parents in hybridization programs.

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