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Genetic variation and structure of rubber population based on microsatellites

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Abstract

Information towards genetic variation and structure of rubber leads to a proper utility of rubber clones for a varietal development. Rubber population represented by nine Asian, 10 South American and nine West African clones was described by indices for genetic variation such as number of alleles (N_a), number of effective alleles (N_e), observed heterozygosity (H_o), unbiased heterozygosity (uH_e), fixation index (F), Shannon's information index (I) and gene diversity (GD); and population structure such as analysis of molecular variance (AMOVA), structure analysis and principal component analysis (PCA) based on 13 microsatellites. Microsatellites derived 60 alleles in all with means N_a, N_e, H_o, uH_e, F, I and GDof4.615, 2.997, 0.619, 0.686, 0.051, 1.174 and 0.647 per microsatellite, respectively. AMOVA revealed 4%, 16% and 80% genetic variation among groups, among and within clones, respectively. The initial three groups based on geographical origin were reassigned into four based on the structure analysis.PCA supported the grouping through the distribution of clones on the scatterplot's projection. PCA also detectedPC1 clones: GW5, IAN873, IRCA22, PB311, RRIM600 and USM1as the most variable clones based on squared cosines. Microsatellite data showed a rich genetic variation is within clones and confirmed rubber is genetically heterogenous. PCA result suggested PC1 clones can be effective parents while unbiased structure grouping will serve as heterotic groups as basis for the hybridization and development of new rubber varieties.

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Introduction

Hevea brasiliensis (Willd. *ex* A. Juss) Muell. Argor rubber plant produces natural and flexible latex as raw material for more than 40,000 end-products such as tires, slippers, shoes, etc., including 400 devices for medicine (Mooibroek and Cornish, 2000).Rubber's unique properties *such as* resilience, elasticity and resistance towards impact, abrasion, heat and cold temperatures (Cataldo, 2000; Cornish,2001) makes the plant irreplaceable by any alternative sources of rubber. Rubber thus is considered as one of the most valued and beneficial industrial crops for the past 100 years (Priyadarshan and Gonçalves, 2002).

Stable and high-latex yielding rubber clones however, were used intensively in any *Hevea* breeding programs (Kinnarat and Rattanawong, 2002) which led to the development of clones with a narrow genetic constitution (Yu *et al.,* 2011).Molecular marker techniques provided efficient and effective methods for characterizing the genetic variation without environmental interaction (Morgante and Olivieri, 1993).

Microsatellites among themhave been dubbed as an ideal tool for genetic variation and structure analyses because it is multi-allelic, reproducible, codominant, and abundant within the genome of any crop (Gupta and Varshney, 2000).

Latest studies used microsatellites to analyze rubber genetic variations and structure in South America (de Souza *et al.,* 2015) and in the Philippines along with multivariate statistics (Cantila *et al.,* 2016).

This study aimed to describe the genetic variation and structure of rubberusing indices for genetic variationand population structure based on microsatellites.

Materials and methods

DNA extraction

Young leaves of 28 rubber clones (Table 1) were extracted using the modified Diversity Arrays Technology (DArT) protocol (Jaccoud *et al.,* 2001) with extraction buffer [0.35 M sorbitol, 0.1 M tris-hydrochloride (Tris HCl) pH 8.0, 5 m Methylenediaminetetraacetic acid (EDTA) pH 8.0, and water]. Deoxyribonucleic acid (DNA) was preheated and incubated at 65°C for 30 minutes. The liquefied suspension was taken with an amount equal to chloroform: isoamyl (24:1) mixture and was centrifuged within5-8 minutes at 13,000 rpm. Liquid in the upper phase was then transferred to a new tube. Cold ethanol (95%) precipitated the DNA in the aqueous phase. DNA then was centrifuged for two times. DNA pellets were washed with cold ethanol (75%) and were dried. Two µl of ribonuclease (RNAse) was added to DNA for one hour incubation at 37°C. DNA pellets were dissolved in 1xTE with the composition of 10mM TrisHCl pH 8.0, 1 mM EDTA pH 8.0. DNA concentrates was finally quantified by gel electrophoresis (0.8%agarose) and visualized by ethidium bromide staining.

Polymerase chain reaction (PCR) conditions

Thirteen microsatellites (Table 2)based from several authors(Seguin *et al.,* 2002; Le Guen*et al.*, 2011;Cantila*et al.,* 2015)were ordered and synthesized at Beijing SBS Genetech Co., Ltd, People's Republic of China. The total PCR mix was 10 µl [0.3 unit *Thermus aquaticus* (Taq) DNA polymerase, 1.0 unit 10 m Mdeoxynucleotide triphosphates (dNTP), 1.0 unit 10x PCR buffer, 0.8 unit microsatellite, 4.1 unit ddH_2O, 2 µl of 10-ng/ul DNA from each 28 rubber DNA]. PCR was done initially with a denaturation for 2 minutes at 94°C(step 1), followed by 30 cycles of second sat 94 °C (step 2),annealing for 1 minute at 56°C (step 3), extension for 1 minute at 72°C (step 4), 29 times repeating step 2 to step 4 (step 5), 5 minutes at 72°C(step 6) and storage at 4°C (step 7). Amplified banding products were viewed using gel electrophoresis (4.5% polyacrylamide) along with silver stain.

Data analyses

Banding results generated by 13 microsatellites were compiled into a data matrix where present=1and absent=0 of the bands. Gen AlEx 6.5 computed

genetic variation with indices as follows: number of observed alleles (N_a), number of effective alleles(N_e), observed heterozygotes (H_o), unbiased heterozygosity (uH_e), fixation index(F), Shannon's information index (I)and heterozygosity or gene diversity (GD) (Peakell and Smouse, 2012). Gen AlEx 6.5 also carried out analysis of molecular variance (AMOVA) where p value was tested with 9999 permutations and F-statistics (F_{ST}, F_{IS} and F_{IT}).Structure 2.3.4 generated probabilistic grouping model on Bayesian approach(Pritchard *et al.,* 2000). Initial values for K were defined from 2 to 7and 10 runs of each K were carried out based on: the admixture model and correlated allele frequencies with 50,000 burn-in (iterations) period and 100,000 Markov Chain Monte Carlo (MCMC) replications.

Structure harvester derived true value of K based on Ad hoc statistics (Earl *et al.,* 2012). All molecular activities were done on the Molecular Biology and Genetics Laboratory of the University of Southern Mindanao Agricultural Research Center (USMARC), Kabacan, Cotabato from January to March 2014.

Results and discussion

Genetic variation

Thirteen microsatellites or loci derived 60 alleles with 4.615 alleles per locus (Table 2) across the population. M124 had the highest alleles of 7 with corresponding highest N_e of 3.682 (Table 3). Higher N_e means a higher number of the heterozygote is expected in accordance to Hartl and Clark's explanation (1989).

Table 1. Twenty-eight rubber clones assigned in groups based on geographical origin.

Geographical origin (N)	Name of rubber
Asian clones (9)	PB235, PB217, PB260, PB330, PB311, RRIM600, RRIM712, RRIM901, and USM1
West African clones (9)	IRCA18, IRCA19, IRCA22, IRCA41, IRCA109, IRCA111, IRCA145, IRCA209 and IRCA230
South American clones (10)	FX1042, GA237, GA308, GA337, GW5, GV21, IAN113, IAN711, IAN713 and IAN873

N_e was between 2.265 (MnSod) and 3.682 while H_o was between 0.358 (M412) and 0.859 (A2736) (Table 3). Slight congruence was observed in N_e and H_o but it can be noted that locus with ≥3.376 N_e had >0.6 H_o. Loci were as follows: M124 with 3.682 and 0.747 H_o, AY486582 with 3.537 N_e and 0.681 H_o, AF221706 with 3.52 N_e and 0.781 H_o and hmct5 with 3.376 Ne and 0.627 H_o. For unbiased heterozygosity (uH_e), the range was between 0.57 (M412) and 0.761 (M124) (Table 3). H_o highest values 0.859 (A2736), 0.781 (AF221706) and0.748 (hmct1) had a lower uH_e counterpart. Drop-out or null alleles (Fukunuga *et al.,* 2014) could be the reason and it is the failure of one allele to amplify during PCR reactions (Wang *et al.,* 2009).

Null alleles could be determinedby the fixation index (F) (Hartl and Clark, 1997).Positive F means the occurrence of null alleles.

TA2163, M412 and AY486585 with 0.336, 0.334 and 0.232 F (Table 3)had<0.6H_o. Negative F on the other hand, implies excess heterozygotes (Hartl and Clark, 1997). A2736, AF221700, AF221706, hmct1and M124 with-0.309, -0.201, -0.098, -0.163 and -0.04, respectively had>0.6 H_o. Shannon information index (I) another tool gives values >1.0 and readily translates into heterozygosity (Sherwin *et al.,* 2006). I was between 0.918 (MnSod) and 1.374 (M124).

M124, AY486582, hmct5 and AF221706 with >1.3 I also had >0.6 H_o and uH_e. $GD$is also called polymorphism information content (PIC) in genetics and Simpson or Gini-Simpsonindexin ecology (Nei and Roychoudhury, 1974; Botstein *et al.,* 1980; Rao, 1982;Sherwin *et al.,* 2006;Chesnokov and Artemyeva, 2015).GDdetermines microsatellites' capacity to generate polymorphism information over a pool of genotypes (Anderson *et al.,* 1993; Perseguini *et al.,* 2012).

GD was between 0.538(M412) and 0.718 (M124) (Table 3). Botstein *et al.* (1980) categorized microsatelliteas highly, moderately and lowly polymorphic if its value are>0.5, within 0.25-0.5 and <0.25, respectively. Highest *GD* were 0.718, 0.745 (AF221706), 0.74 (hmct5), 0.733 (AY486582) and 0.701 (hmct1). *GD*>0.7 is regarded as the most ideal selected microsatellites (Botstein *et al.,* 1980).

Table 2. Microsatellite sequences optimized at specific annealing temperature and their allelic sizes and number.

Locus	Microsatellite sequence	Annealing Temp. (°C)	Allele	N_a
A2736	F: 5' gcaacctgatgaataaaga 3' R: 3' aaatgagaaacaagaagacc 5'	52	A=394 B=418 C=432 D=470 E=491	5
AF221700	F: 5' tttggcattgatgttga 3' R: 3' ccaaatatgctgtttcagga 5'	53.2	A=190 B=193 C=196 D=204	4
AF221706	F: 5' tgtgtcctctacttgtcttcatttg 3' R: 3' gcctctacttttctttctcctttat 5'	58.1	A=222 B=229 C=235 D=245 E=247	5
AF221711	F: 5' acaagagatgcgagaagaaataccc 3' R: 3' cataacagctgaatgaaaataaaac 5'	61.3	A=380 B=384 C=412 D=442 E=465	5
AY486582	F: 5'cctgtatgaaatcaagagaaga 3' R: 3' tagaggtagaagccaatgagtt 5'	56.5	A=159 B=168 C=171 D=176 E=180	5
AY486585	F: 5' ggcagtagcacaatcatttttagta 3' R: 3' tttcctcactgttttgtcattcc 5'	58.1	A=142 B=158 C=163 D=172	4
hmct1	F: 5' aaccagaagggtgtcatgct 3' R: 3' ggaatcccatgacaatccac 5'	58.4	A=187 B=197 C=203 D=242 E=296	5
hmct5	F: 5' atgtatgtgtgcgcaggaag 3' R: 3' ctgtagtcatggcagcagga 5'	60.5	A=198 B=210 C=223 D=229 E=243	5
Ma31	F: 5' tcctgccatccttatcct 3' R: 3' tttttgtattgccccagccgtgagt 5'	63	A=248 B=253 C=264 D=268	4
M412	F: 5' cattagttggctgctctttcatttc 3' R: 3' acttatcttatgttccatctaccac 5'	59.7	A=168 B=173 C=187 D=197	4
M124	F: 5' tcatttcaagttcaccgtgcttatt 3' R: 3' agcgcatgtatttgccttatgtctc 5 '	61.3	A=137 B=142 C=147 D=150 E=152 F=158 G=169	7
MnSod	F: 5' tgtgctgcctttgtcttaacatgcc 3' R: 3' gcaaatagcaatgagtttctgactc 5'	63	A=196 B=203 C=221	3
TA2163	F: 5' atgcaacagagtaggaggaga 3' R: 3' tcaaggcaaatgaagtg 5'	52	A=187 B=195 C=200 D=204	4
Total	-	-	-	60
Mean	-	-	-	4.615

F-forward, R-reverse and N_a= number of alleles.

Population structure

Population structure is the variant's distribution that can be explained by allelic/genotypic variation. AMOVA-based *F*-statistics (Wright, 1951; Excoffier *et al.,* 1992) such as F_{ST} had 0.036or3.6% which means almost 4% genetic differentiation/variation found among groups (Table 4).

F_{IS} and F_{IT} had0.165 and 0.195 (Table 4), respectively indicate less inbreeding occurrence, which is usual to rubber. The percentage of variation within clones, 80% supported both inbreeding coefficient indices (F_{IS} and F_{IT}) which confirm that rubber Is genetically heterogenous. Asian, South American and West African groups were differentiated by genetic variation indices.

N_e was between 2.625 (West Africa) and 3.513 (South America), H_o between 0.579 (Asian) and 0.644 (South America), uH_e between 0.632(West Africa) and 0.745(South America), I between 1.05 (West Africa) and 1.338 (South America) and GD between 0.640 (West Africa) and 0.706 (South America) across the three groups (Table 5). Despite less significance found among groups (Table 4), South American group still had a highest genetic variation.

Table 3. Thirteen microsatellites and their corresponding indices detected enough genetic variation for the study.

Locus	N_e	H_o	uH_e	F	I	GD
A2736	3.056	0.859	0.694	-0.309	1.19	0.657
AF221700	2.804	0.648	0.674	-0.021	1.123	0.635
AF221706	3.520	0.781	0.752	-0.098	1.327	0.712
AF221711	2.769	0.598	0.669	0.048	1.213	0.628
AY486582	3.537	0.681	0.741	0.029	1.346	0.702
AY486585	3.103	0.519	0.715	0.232	1.159	0.675
hmct1	2.804	0.748	0.680	-0.163	1.169	0.643
hmct5	3.376	0.627	0.745	0.109	1.334	0.703
Ma31	2.779	0.567	0.668	0.104	1.123	0.632
M412	2.637	0.358	0.570	0.334	0.968	0.538
M124	3.682	0.747	0.761	-0.04	1.374	0.718
MnSod	2.265	0.5	0.592	0.103	0.918	0.557
TA2163	2.624	0.41	0.657	0.336	1.016	0.617
Mean	2.997	0.619	0.686	0.051	1.174	0.647
SE	0.118	0.041	0.016	0.052	0.041	0.016

N_e=number of effective alleles, H_o=observed heterozygosity, uH_e=unbiased heterozygosity, F=fixation index, I=Shannon's information index, GD=Nei's gene diversity and SE=standard error of the mean.

Table 4. Analysis of molecular variance (AMOVA) and its derived F-statistics revealed the structure of the rubber population.

Source of variation	df	SS	MS	Estimated Variance	Percentage	F-statistics	P value
Among groups	2	17.393	8.696	0.175	4%	F_{ST}= 0.036	0.002
Among clones	25	135.750	5.430	0.769	16%	F_{IS}= 0.165	0.000
Within clones	28	109.000	3.893	3.893	80%	F_{IT}= 0.195	0.000
Total	55	262.143		4.837	100%		

This study supports Luo *et al.,* (1995) findings that South American clones had higher DNA polymorphism over any clones as they used mitochondria as the basis for genetic variation.

The Asian group followed as highest with 2.852 N_e, 0.64H_e and1.133 I, which imply genetically diverse over West Africa. Asian group is composed of clones having high-latex yield and highly recommended in the Philippines (DTI-RODG, 2012). Kinnarat and Ratannong (2002) reported about the extensive parental utility of Asian clones particularly PB217, PB235, PB260 and RRIM600 (Priyadarshan and Goncalves, 2002) for other *Hevea* breeding programs. Nouy and Nicolas (1985) and Baudouin *et al.* (1997) in fact revealed that IRCA clones (West Africa) were bred from 28 Asian clones that could be PB and RRIM-based series. This might be the basis why West African group had a lower genetic variation over Asian group. Their constitution of genes could be in duplication and genetically narrowed.

Table 5. Different genetic variation indices used in differentiating three groups of rubber.

Groups	N_e	H_o	uH_e	I	GD
SA	3.513	0.644	0.745	1.338	0.706
WA	2.625	0.634	0.632	1.050	0.596
As	2.852	0.579	0.680	1.133	0.640
Mean	2.997	0.619	0.686	1.174	0.647
SE	0.266	0.020	0.033	0.085	0.032

SA=South American, WA=West African, As=Asian, N_e=number of effective alleles, H_o=observed heterozygosity, uH_e=unbiased heterozygosity, F=fixation index, I=Shannon's information index, GD=Nei's gene diversity and SE=standard error of the mean.

Table 6. Summary of values used to determine true value of K using Ad hoc statistics (Evanno*et al.,* 2005) from Structure harvester (Earl *et al.,* 2012).

K	Reps	Mean LnP (K)	SD LnP (K)	Ln'(K)	[Ln" (K)]	Delta K
2	10	-1415.84	1.92	-	-	-
3	10	-1306.27	9.72	109.57	9.39	0.97
4	10	-1206.09	8.58	100.18	27.97	3.26
5	10	-1133.88	8.28	72.21	15.4	1.86
6	10	-1077.07	3.84	56.81	-	-

Another, structure analysis based probabilistic model grouping assigns rubber clones using probabilities without prior assumption of its geographical origin (Pritchard *et al.,* 2000).

K2 to K5 patterns (Figure 1) were not straightforward that is why ad hoc statistics with delta K computation were done to identify the true value of K (Evanno *et al.,* 2005).

Table 7. Squared cosines from the PCA used to determine the most variable clones.

Groups	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	2.61	2.06	1.57	1.54	1.20	1.03
Variability (%)	20.09	15.85	12.07	11.87	9.21	7.90
Cumulative %	20.09	35.94	48.01	59.88	69.09	76.98
FX1042	0.04	0.27	0.00	0.03	0.12	0.00
GA237	0.06	0.32	0.21	0.29	0.03	0.01
GA308	0.18	0.04	0.31	0.24	0.03	0.02
GA337	0.11	0.00	0.18	0.04	0.01	0.09
GW5	0.48	0.00	0.13	0.21	0.02	0.00
GV21	0.09	0.01	0.05	0.23	0.17	0.00
IAN113	0.13	0.66	0.01	0.05	0.00	0.09
IAN711	0.00	0.42	0.13	0.14	0.01	0.03
IAN713	0.00	0.05	0.07	0.73	0.00	0.03
IAN873	0.32	0.20	0.03	0.03	0.02	0.00
IRCA18	0.00	0.52	0.02	0.03	0.01	0.14
IRCA19	0.17	0.10	0.50	0.02	0.02	0.04
IRCA22	0.31	0.00	0.00	0.06	0.22	0.00
IRCA41	0.19	0.10	0.02	0.07	0.22	0.03
IRCA109	0.02	0.28	0.03	0.07	0.02	0.00
IRCA111	0.07	0.02	0.39	0.10	0.00	0.24
IRCA145	0.21	0.00	0.11	0.00	0.10	0.05

IRCA209	0.03	0.17	0.13	0.07	0.15	0.29
IRCA230	0.01	0.02	0.07	0.00	0.31	0.14
PB217	0.21	0.02	0.08	0.00	0.37	0.04
PB235	0.21	0.08	0.01	0.30	0.09	0.21
PB260	0.14	0.11	0.08	0.08	0.25	0.20
PB311	0.74	0.05	0.00	0.01	0.11	0.05
PB330	0.00	0.02	0.02	0.35	0.03	0.12
RRIM600	0.38	0.00	0.08	0.08	0.24	0.00
RRIM712	0.20	0.14	0.27	0.06	0.01	0.00
RRIM901	0.03	0.32	0.15	0.00	0.23	0.06
USM1	0.39	0.08	0.21	0.00	0.07	0.05
Most variable	GW5	FX1042	GA308		Residuals	
	IAN873	GA237	IRCA19			
	IRCA22	IAN113	IRCA111			
	PB311	IAN711	RRIM712			
	RRIM600	IRCA18				
	USM1	RRIM901				

Values in bold correspond for each observation to the factor for which the squared cosine is the largest.

The delta K computed was four, the true value of K (Table 6, Figure 2). Rubber clones thus, were grouped into four: K1 by IAN711, IAN 873, GV21, GW5, RRIM600, RRIM712 and RRIM901; K2 by GA237, GA308, GA237, PB235, IRCA18, IRCA19 and IRCA209; K3 by USM1, PB311, IAN113, IAN713 and FX1042; and K4 BY IRCA22, IRCA41, IRCA109, IRCA111, IRCA145, PB217, PB260 and PB330 (Figure 1).

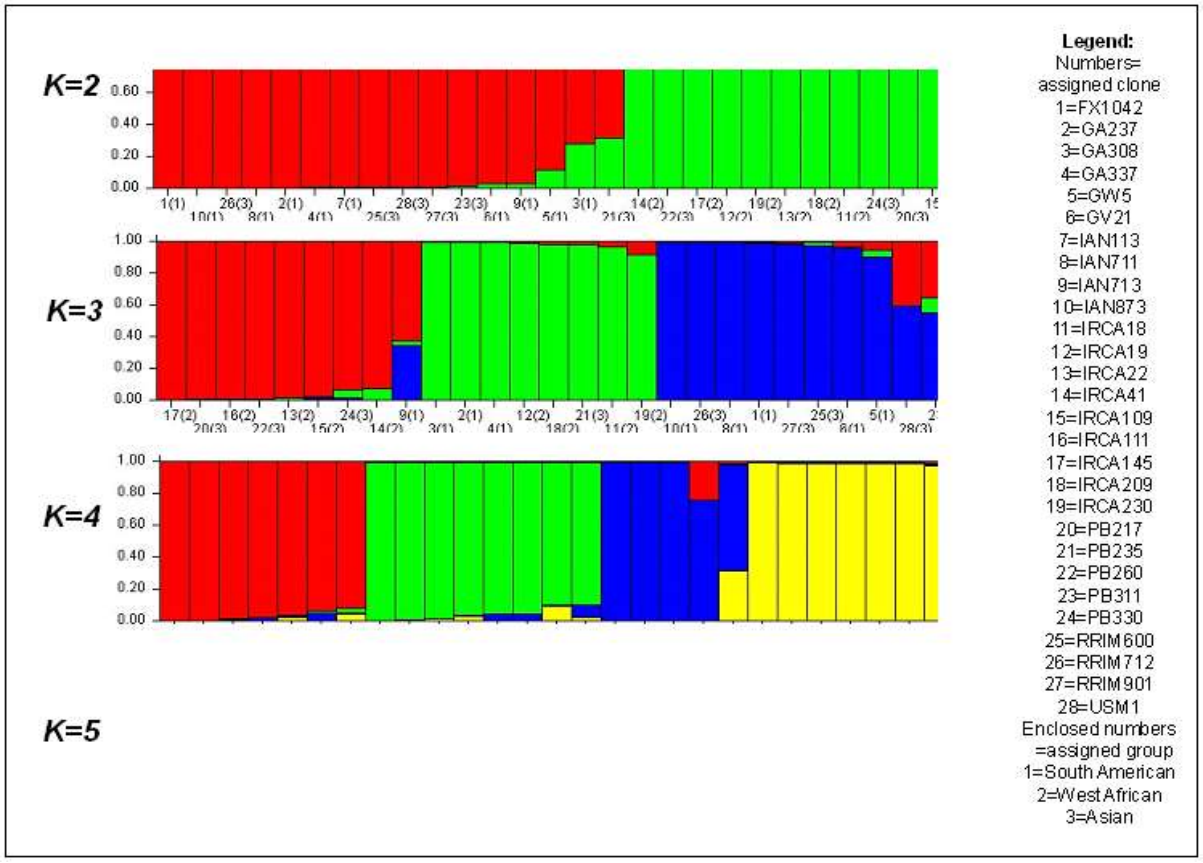

Fig. 1. Population structure patterns (K2 to K5) of 28 rubber clones assignment based on 13 microsatellites using Structure 2.3.4.

Asian clones were distributed across four groups while West African clones were only to two groups, K2 and K4. PB and RRIM series of Asian group had dominated on separate K groups. RRIM clones grouped to K1 while PB clones to K4.

The results were supported by Triwitayakorn *et al.* (2011) and Oktavia *et al.* (2011) when they had used 47 EST-SSRs and 12 RAPDs for rubber genetic variation, respectively. South American clones on the other hand dominated groups like K1, K2 and K3.

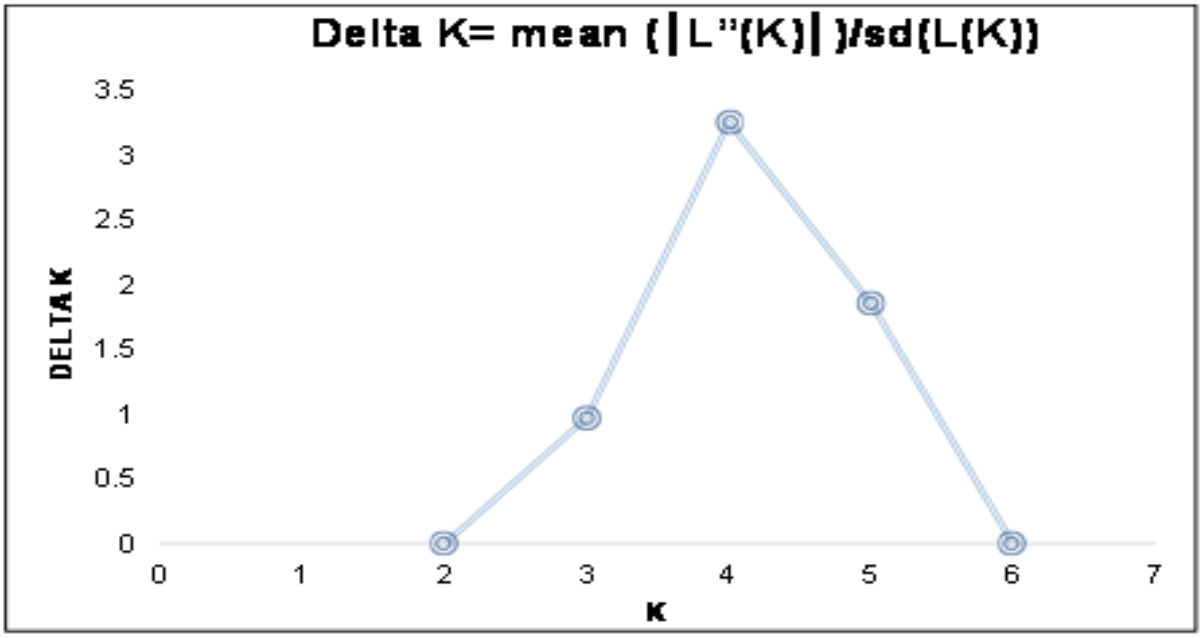

Fig. 2. Determination of the true value of K expressed in a graph using Ad hoc statistics (Evanno *et al.*, 2005) from the Structure harvester (Earl *et al.*, 2012).

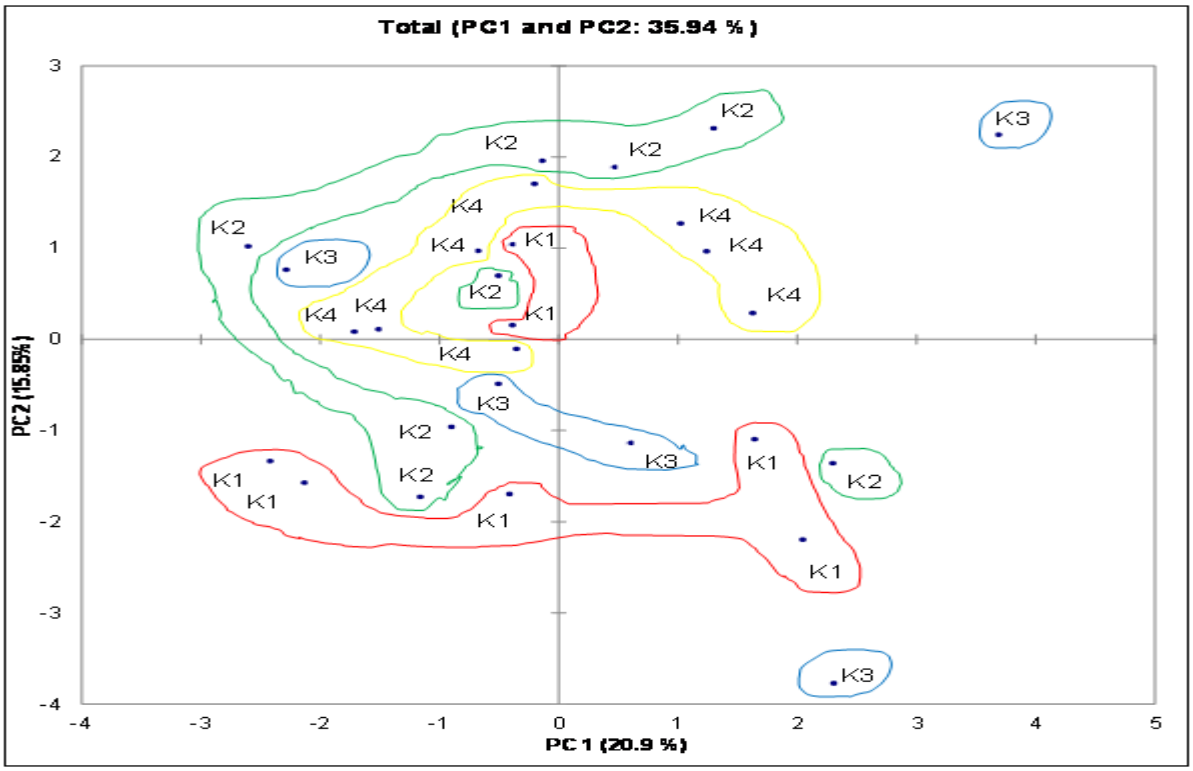

Fig. 3. Distribution of 28 rubber clones in the principal component analysis' scatter plot projections. Groups are indicated with different color lines (K1=red, K2=green, K3=blue and K4=red).

West African clones in the same way dominated K4. Grouping through structure analysis was supported by PCA's scatterplot projection. K1 member clones were distributed on the negative projections of PC2 while both K2 and K4 member clones on the projections of PC2 (Figure 3). K3 member clones however, were far to each other. Few members of K3 group such as FX1042 and IAN 713 were admixtures based on structure analysis (Figure 3).

Admixtures are variables (clones in this study) having <0.80 probability (Garris *et al.,* 2005). PCA however accounted 35.94% total variation for the first two PCs (Figure 3, Table 7). PCA could also detect clones manifesting high genetic variability (Cantila *et al.,* 2016). Highly variable clones were GW5, IAN873, IRCA22, PB311, RRIM600 and USM1 based on squared cosines in PC1 with corresponding 20.09% of the variation (Table 7).

Conclusion

Genetic variation and population structure indices had detected enough information in explaining variation and distribution of genes among groups, among and within clones based on 13 microsatellite data. AMOVA found high genetic variation within clones of 80%, indicating that rubber is highly heterogenous and cross-pollinated in nature.

Twenty-eight rubber clones were assigned into four groups based on the structure analysis, which supported by PCA's scatter plot projection. GW5, IAN873, IRCA22, PB311, RRIM600 and USM1 were among the clones that could possibly give progenies with high heterosis and lead in the effective development of new rubber varieties.

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