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Agronomic Traits and Genetic Fidelity of Four Cocoa Clones Derived from Somatic Embryogenesis Culture

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Abstract

Morphological and genetic characterization of MCBC1, PBC230, KKM22 and KKM4 cocoa clones derived from staminode and immature zygotic embryo culture were compared with those conventionally grafted. Somatic embryogenesis culture successfully produced true-to-type progenies of elite cocoa clones of MCBC1, PBC230, KKM22 and KKM4 (from staminode culture). Phenotype variations ($p < 0.05$) were observed only in KKM4 clone from immature zygotic embryo culture which exhibited lower quantities in the fresh pod weight, number of flat beans per pod, seed length, seed width and individual seed weight. The genetic stability of the cultured clones was tested using fragment analysis with 12 SSR primers to validate these results. Eleven of these SSR primers detected mutations only in the allelic profiles of KKM4 clone from immature zygotic embryo. These results validated those variations in KKM4 clones of immature zygotic embryo culture were due to interactions between genotypic and explant types. Unfortunately, these variations were negative attributes to cocoa productivity. Thus, it is suggested that successful production of true-to-type KKM4 cocoa clone should consider other means of propagation including modification of the culture conditions.

Keywords: *Theobroma cacao*, Field performance, Somaclonal variation, DNA sequence, Micropropagation

Introduction

Theobroma cacao L. or cocoa is a diploid fruit tree species that belongs to the Malvaceae family. Cocoa is exclusively cultivated in the tropical area for its beans which used as a main raw material for both confectionary and cosmetic making industries. About ~4 million tons of cocoa beans produced by cocoa producing countries from West Africa, South America and Southeast Asia are imported to the European countries for such industries in 2018 [1]. Thus, it is crucial to produce a mass quantity of high quality and uniform cocoa planting materials to meet the industrial demands. This could be potentially achieved via plant tissue culture technique such as somatic embryogenesis. In this culture techniques, a single somatic cell obtained from leaf, flower or stem explants undergoes several differentiation processes before developed into the whole plants after culture [2]. Since the regenerated plants are formed from bipolar structure of root and shoot of the somatic cells and without fusion of gametes [2], the incidence of variation is expected to be lower in cocoa plant. For cocoa, the protocols for somatic embryogenesis culture have been developed by several researchers [3-7].

Despite this, the variations in plants following tissue culture in general has been reported and represents a drawback in the adoption of this technology. Its possible causes and detection methods have been extensively reviewed by [8]. In most cases, variations in the regenerated plants exhibited abnormalities in both morphological and genetic characteristics. However, the variations in the genetic constituents are not always expressed as an altered morphological characteristic. Thus, it is crucial to use both morphological and genetic markers to detect somaclonal variation in tissue culture derived plant. Morphological detection requires extreme monitoring throughout the growth and development of the regenerated plants to identify any mutant. This is carried out by visual comparison of plant morphometrics of agronomic parts such as leaf, stem, branch, root, flower, fruit and seed. In contrast, molecular analysis aimed to assess the genetic stability of micropropagated plants.

At present, various molecular markers such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP) have been used to screen plants regenerated from tissue culture for somaclonal variations [8,9]. Among these molecular markers, microsatellite SSR and ISSR were reported to have high genomic abundance throughout genome, co-dominant, locus specific, greater reproducibility, high level of polymorphism, informative and strong discriminatory power [10,11]. Notably, the SSR marker which is formed from the tandemly repeated units of short sequence motifs, enables easy detection of mutational events from the alteration in the size of the new DNA sequence [8]. In addition, 15 SSR primers were selected as international molecular standards for DNA fingerprinting of *T. cacao*, making them suitable candidates to test for genetic homogeneity and detect somaclonal variations in tissue cultured progenies.

Specifically, for cocoa plants derived from somatic embryogenesis culture, molecular studies were reported for genotypes from Indonesia [11] and Brazil [12,13] using SSR markers, cleaved amplified polymorphic sequence, freeware Artbio and 386-methylation-sensitive amplified polymorphism respectively to determine somaclonal variation in plantlets and embryogenic callus. However, these findings were limited to plants at the early stage of plantlet regeneration and may not be directly applicable to mature trees in the field conditions. A detailed assessment of the regenerated plants is crucial especially for perennial species which exhibit a long pre-bearing growth and development periods. Furthermore, the genetic stability of cocoa genotypes from Malaysia following somatic embryogenesis culture have not been examined to conform its homogeneity. Thus, this paper aimed to quantify agronomic traits and assess genetic stability of somatic embryogenesis cultured cocoa clones following 2 years of field planting. The findings will validate the efficacy of somatic embryogenesis culture technique in producing true-to-type plants for commercial production of cocoa in Malaysia. Equally, field screening may assist plant scientists in selecting superior clones with good agronomic traits for future crop breeding programs.

Materials and methods

Establishment of somatic embryogenesis cultured and grafted cocoa clones

The somatic embryogenesis culture was initiated from both staminode and immature zygotic embryo explants collected from 4 elite cocoa clones, i.e., MCBC1, PBC230, KKM22 and KKM4 of Trinitario group. These clones were selected due to their superior traits of high yield potential and resistant to many pests and diseases. The protocols for callus induction, development of somatic embryos and plantlet regeneration were described by a previous study [14]. The plantlets were sub-cultured into a new fresh medium containing the same composition of nutrients every 4 weeks until they were ready for acclimatization. Healthy plantlets with height between 150 to 200 mm were selected for hardening process. At the initial stage of hardening process, the plantlets were transferred into Jiffy-7 peat pellets and kept at 25 - 26 °C in the Plant Tissue Culture Laboratory. To maintain high humidity condition (80 - 90 %), individual plantlet was covered in a plastic bag and manually misted with 100 mL water twice daily. This condition also protects the plantlets against mechanical damage and insect attack. After 14 days, the plantlets were transferred into 100×100 mm^2 perforated polybags filled with potting mix soil. The polybags were placed on the balcony outside the Plant Tissue Culture Laboratory with light intensity approximately 1,420 lux. The plants were maintained for 4 weeks with manual irrigation at 150 mL, twice daily. For each plant, 0.25 g of compound fertilizer (NPK 15:15:15) was applied once a week. Meanwhile, grafted plants were established from scions of the mother plant of the same cocoa clones as described previously [14] and placed in the greenhouse nursery for another 6 months period.

Nursery establishment and field planting

After 4 weeks of acclimatization process, the bare rooted somatic embryogenesis with height averaged 0.3 m were relocate to a greenhouse nursery at Malaysian Cocoa Board Research Station, Sarawak, Malaysia (1° 27'N, 110° 29'E, 27 m a.s.l.) with grafted plants. In this location, the greenhouse was sheltered with double layers of black netting with light penetration of 1,560 lux and relative humidity between 27 and 30 %. All plants were transplanted into 140×140 mm^2 polybags filled with sand and soil mixture at 1:1 ratio by volume. The plants were watered manually twice a day with 250 mL tap water. Fertilizer was applied once a week with 0.42 g of NPK 15:15:15 per plant. All cocoa trees were maintained at the nursery for 4 months before field planting. In the field, the experimental design was conducted based on a randomized complete block design (RCBD). Four selected elite cocoa clones namely 1) MCBC1, 2) KKM22, 3) KKM4 and 4) PBC230 were transplanted in the field condition after

regenerated from 3 propagation types of 1) grafting (as a control), 2) immature zygotic embryo culture and 3) staminode culture. Around 84 cocoa trees (7 replicates from each cocoa clones) were planted at 3×3 m spacing on a Red Yellow Podzolic Soil (USDA) in this study. In the field area, the mean annual rainfall was 4,130 mm with January being the wettest month (690 mm) and July as the driest month (195 mm). The mean daily air temperature ranged from 30 - 34 °C. The management of cocoa trees was previously described by [14].

Assessment of agronomic traits

The cocoa agronomic traits were quantified from the first to 24 months from transplanting. The stem diameter was measured at 10 mm from the ground using a calliper (OEMTOOLS 25363, Malaysia) with an accuracy of 0.1 mm whereas stem height was measured using a measuring tape (McKenic measuring tape, Malaysia). Time to first flowering was measured using calendar days. The weight of fresh pod and individual seed were determined using a digital analytical balance (Mettler TOLEDO XS105DU, USA) and number of flat beans per pod was counted manually. Statistical analyses were performed using VassarStats online Statistical Computation (http://vassarstats.net/) [15]. For each trait, means were reported, and comparison was established by Tukey's HSD (honestly significant difference) test at $p < 0.05$. A 2-way ANOVA was conducted to test the difference and interaction among cocoa clones and propagation methods.

Assessment of genetic variability

Plant materials

For comparative study, the grafted cocoa clones of the same origin were selected for molecular analysis. The materials for this fragment analysis were collected from 3 young healthy leaves (at the second flush behind the shoot apex) of both somatic embryogenesis culture and grafting derived cocoa clones. In the laboratory, these leaves were cleaned with 70 % ethanol before washing with distilled water thrice. The leaves were then cut into small pieces (0.5×0.5 cm^2) and crushed to a fine powder in liquid nitrogen using a mortar and pestle for subsequent DNA extraction.

DNA extraction

The DNA was extracted using a CTAB-SDS method [16,17] with a slight modification in the incubation procedure. The DNA extraction was conducted in triplicate for each sample of plant tissues. Approximately 50 mg of the plant tissue powder was transferred into 1.5 mL Eppendorf tube containing 1 mL of extraction buffer (0.1 M Tris-HCl, 0.05 M EDTA (pH 8.0), 0.5 M NaCl, PVP (0.4 % w/v) and 0.7 µL β-mercaptoethanol) and 50 µL SDS (10 %, w/v). The tubes were then incubated in a water bath at 65 °C for 30 min before centrifuged at 13,000 rpm for 10 min. The supernatant was collected from the tube and transferred into a new 1.5 mL Eppendorf tube which have been added with 800 µL isopropanol.

Next, the Eppendorf tube was inverted and incubated at 4 °C for about 2 h before centrifuged at 13,000 rpm for 10 min. The supernatant was discarded from the tube and the remaining pellet inside the tube was resuspended with 400 µL 0.1 M×TE (Tris-EDTA) buffer and 400 µL CTAB. A volume of 10 µL RNase was added to remove the remaining RNA. Then, the tube was incubated at 65 °C for 15 min before added with 800 µL chloroform: isoamyl alcohol (24:1, v/v). The Eppendorf tube was further centrifuged at 13,000 rpm for 5 min to separate the mixtures. The upper phase of the mixture was transferred into a new 1.5 mL Eppendorf tube and mixed with 1.5 mL ethanol (95 % v/v) to precipitate the DNA. Later, the Eppendorf tube was incubated at room temperature (25 °C) for 15 min and centrifuged at 13,000 rpm for 10 min. The supernatant was again discarded whereas the remaining DNA pellet was washed twice with 700 µL ethanol (70 % v/v). The pellet was air dried at room temperature (25 °C) for 1 h and dissolved in 50 µL of 0.1 M×TE (Tris-EDTA) buffer before storage at 4 °C.

DNA quantification

To determine the DNA quality, about 5 µL of the extracted DNA was subjected to electrophoresis on 1 % agarose gel stained with ethidium bromide and visualized under ultraviolet transilluminator. The DNA concentration was quantified using a NanoDrop® ND - 1,000 UV Vis Spectrophotometer (Thermo Fisher Scientific) at absorbance of 260 nm wavelength (**Table 1**). The absorption at 260 nm gives an estimation of nucleic acids concentration whereas absorption at 280 nm corresponded to the residual protein. The A260/280 ratio was used to determine the DNA purity in which the values between 1.7-2.0 indicates a high-quality DNA. On the other hand, the A260/230 ratio measures the level of salt carryover in the purified DNA. The DNA yield was then calculated by multiplying the DNA concentration by total sample volume.

Table 1 DNA quantification from leaf samples of MCBC1, PBC230, KKM22 and KKM4 cocoa clones propagated from staminode, immature zygotic embryo culture and grafting.

Sample	Absorbance reading		DNA concentration (ng/µL)	Total sample volume (µL)	DNA yield (µg)
	A260/280	A260/230			
STMCBC1	1.96	0.69	2.5	50	0.12
STPBC230	2.91	0.50	3.1	50	0.15
STKKM22	2.00	0.80	3.0	50	0.15
STKKM4	1.60	0.25	6.7	50	0.34
IZMCBC1	1.18	0.90	4.5	50	0.23
IZPBC230	2.46	0.39	3.2	50	0.16
IZKKM22	2.45	0.87	6.0	50	0.30
IZKKM4	2.03	0.40	3.3	50	0.16
GMCBC1	2.04	0.61	7.8	50	0.39
GPBC230	2.05	0.55	6.3	50	0.31
GKKM22	1.79	0.53	5.9	50	0.30
GKKM4	1.90	0.51	3.1	50	0.15

ST, staminode culture trees; IZ, Immature zygotic embryo culture trees; G; Grafted donor trees

SSR markers

Ten internationals standard SSR primers (**Table 2**) commonly used for DNA sequencing of cocoa genotype [18] were chosen for this study. These SSR primers were purchased from the Integrated DNA Technologies (IDT, Coralville, Iowa). The forward primers were labelled at the 5-prime end with fluorescent CEQ blue and green dyes (D4, Beckman Coulter) to allow the detection of PCR products. The primers labelled with 6-FAM (blue dye) were: mTcCIR6, mTcCIR15, mTcCIR26 and mTcCIR11 primers whereas mTcCIR1, mTcCIR37, mTcCIR12, mTcCIR24, mTcCIR60 and mTcCIR8 primers were labelled with HEX (green dye).

Table 2 The characteristics of the SSR primers [24] used for DNA amplification of *Theobroma cacao*. T = Annealing temperature for Polymerase Chain Reaction (PCR) amplification, F = Forward and R = Reverse.

Primer	Primer sequences	Allele size (bp)	T (°C)
mTcCIR1-Y16883	F: GCAGGGCAGGCTCAGTGAAGCA R: TGGGCAACCAGAAAACGAT	128 - 146	51
mTcCIR37-AJ271942	F: CTGGGTGCTGATAGATAA R: AATACCCTCCACACAAAT	136 - 187	46
mTcCIR12-Y16986	F: TCTGACCCCAAACCTGTA R: ATTCCAGTTAAAGCACAT	165 - 256	46
mTcCIR24-Y16996	F: TTTGGGGTGATTTCTTCTGA R: TCTGTCTCGTCTTTTGGTGA	186 - 207	46
mTcCIR60-AJ271958	F: CGCTACTAACAAACATCAAA R: AGAGCAACCATCACTAATCA	190 - 218	51
mTcCIR6-Y16980	F: TTCCCTCTAAACTACCCTAAAT R: TAAAGCAAAGCAATCTAACATA	224 - 253	46
mTcCIR15-Y16988 mTcCIR26-Y16998 mTcCIR11-Y16985	F: CAGCCGCCTCTTGTTAG R: TATTTGGGATTCTTGATG F: GCATTCATCAATACATTC R: GCACTCAAAGTTCATACTAC F: TTTGGTGATTATTAGCAG R: GATTCGATTTGATGTGAG	234 - 263 285 - 310 286 - 321	46 46 46
mTcCIR8-Y16982	F: CTAGTTTCCCATTTACCA R: TCCTCAGCATTTTCTTTC	290 - 307	46

PCR amplification

The PCR amplification was conducted with a mixture of 0.5 µL genomic DNA (20 ng μL^{-1}), 0.1 µL dNTP solution (20 mM), 0.8 µL MgCl2 (25 mM), 0.1 µL of each primer (forward and reverse primers) (10 µM), 2 µL 5×colourless GoTaq® Reaction buffer (Promega) (5×) and 0.125 µL GoTaq® DNA polymerase (Promega) (5U µL-1). The thermal cycling protocol for the amplifications were performed according to [19]. All PCR amplifications were conducted in a GeneAmp® PCR System 9,700 (Applied Biosystems, Inc.).

Fragment analysis and scoring

In each sample, 3 µL of PCR product was mixed with 6.95 µL deionized formamide (SLS, sample loading solution) and 0.05 µL GeneScan 400HD ROX (Applied Biosystems) for the fragment analysis. The mixture was first centrifuged at 13,000 rpm for 5 min before incubated at 95 °C for 5 min. Then, the mixture was placed on ice for another 5 min and subsequently visualized with a capillary electrophoresis on ABI PRISM 310 Genetic Analyser (PE Biosystems). All fragment sizes were scored to 2 decimal places with GeneMapper Software Version 5.0 (Applied Biosystems, Inc.) following the electrophoresis. For DNA samples with difference in fragment size which could possibly be due to mutation, PCR amplification and fragment analysis were repeated thrice for validation. The alleles in somatic embryogenesis cultured clones were considered mutated when > 2 bp variation was detected compared with the size of the original allele from the grafted donor trees.

Results and discussion

Stem height and diameter were continuously increased from the first to 24 months after transplanting. During this period, all clones developed height above 1.0 m and stem diameter exceeded 50 mm (**Table 3**). Similarly, [20] reported comparable field performance for the somatic embryogenesis cultured clones after 2 years of transplanting. However, both stem height and diameter were found to be significantly different among clones in which MCBC1 had the maximum measurements for the traits (stem height = 1.20 m and stem diameter = 60.1 mm). These results were similar with the previous findings [7,20] where variation among cocoa clones was correlated with their genotype background. Furthermore, [21] reported that both stem height and diameter were under a genetic control for the polyclonal variety such as Trinitario. This variety which is a natural hybrid of Criollo and Lower Amazon Forastero family [22] usually inherit the traits of both family groups. Thus, any cocoa clones from Trinitario variety are expected to be heterogeneous in growth characteristics under field conditions and this was observed in MCBC1 clone.

For seed traits, only KKM4 clone from immature zygotic embryo culture produced the highest number of flat beans per pod (4 beans) and the lightest individual seed weight (4.13 g). Consequently, these factors have resulted in the lowest pod weight (339.6 g) borne by this clone. Unfortunately, such traits are unfavorable by the cocoa industries because they indicate low productivity. On a different note, all cocoa trees flowered after 538 days (within 2 years from transplanting) with no difference among clones and propagation source. These findings corroborated the work of [23].

Table 3 The agronomic traits of MCBC1, PBC230, KKM22 and KKM4 cocoa clones derived from somatic embryogenesis culture (staminode and immature zygotic embryo) and grafting after 24 months of field planting at Malaysian Cocoa Board Research.

Cocoa clone	Propagation method	Agronomic traits					
		Stem height (m)	Stem diameter (mm)	Fresh pod weight (g)	No. of flat bean per pod	Fresh individual seed weight (g)	Time to first flowering (days)
MCBC1	ST	1.22	61.4	399.4	2	4.30	527
	IZ	1.20	62.2	403.5	2	4.33	531
	G	1.17	56.8	408.0	2	4.26	555
	Mean	1.20	60.1	403.6	2	4.30	538
PBC230	ST	1.14	57.2	408.9	2	4.28	543
	IZ	1.09	58.1	406.0	2	4.30	536
	G	1.08	55.4	405.0	2	4.26	545
	Mean	1.10	56.9	406.7	2	4.28	541
KKM22	ST	1.14	56.2	396.6	2	4.33	588
	IZ	1.10	58.9	393.1	2	4.34	592
	G	1.10	57.7	380.8	2	4.31	595

Cocoa clone	Propagation method	Agronomic traits					
		Stem height (m)	Stem diameter (mm)	Fresh pod weight (g)	No. of flat bean per pod	Fresh individual seed weight (g)	Time to first flowering (days)
	Mean	1.11	57.6	390.2	2	4.32	592
KKM4	ST	1.11	57.8	404.0	2	4.32	560
	IZ	1.15	58.4	339.6	4	4.13	610
	G	1.08	58.8	403.0	2	4.31	554
	Mean	1.11	58.3	382.2	3	4.26	575
***p*-value**	Propagation	0.222	0.064	0.720	< 0.001	0.473	0.992
	Tukey's HSD (5 %)	0.07	2.24	20.57	0.31	0.06	94.82
***p*-value**	Clones	< 0.05	< 0.05	0.053	< 0.001	0.139	0.556
	Tukey's HSD (5 %)	0.09	2.85	26.12	0.40	0.08	21.01
***p*-value**	Propagation* Clones	0.952	0.193	< 0.05	< 0.05	< 0.05	0.993
	Tukey's HSD (5 %)	0.20	6.40	58.18	0.88	0.17	23.86

ST = staminode culture trees; IZ = Immature zygotic embryo culture trees; G = Grafted trees

The genetic fidelity analysis with ten SSR primers produced about 206 reproducible fragments or alleles (**Table 4**). These alleles were found within their international standard range as documented in [24] with the range between 127.03 (mTcCIR1-Y16883) to 347.22 bp (mTcCIR18-Y16991). Of these SSR primers, 12 primers (mTcCIR1, mTcCIR37, mTcCIR60, mTcCIR6, mTcCIR40, mTcCIR33, mTcCIR26, mTcCIR11 and mTcCIR8) (**Table 4**) successfully detected polymorphism in the somatic embryogenesis cultured clones when compared with mother clones from grafting. The accuracy of SSR primers to test genetic stability among different cocoa genotypes have been confirmed previously by several researchers [11-13]. Additionally, these SSR primers have been used to screen true-to-type [25,26] as well as off-type plants derived from tissue culture for coconut (*Cocos nucifera* L.) [27], sugarcane (*Saccharum officinarum* L.) [28] and banana (*Musa* sp.) [29].

Table 4 The fragment length (allele sizes) of all fifteen SSR primers for all cocoa clones. (The pink highlight = variation in allele sizes, yellow highlight = allele addition and green highlight = allele deletion. A1 = Allele 1; A2 = Allele 2)

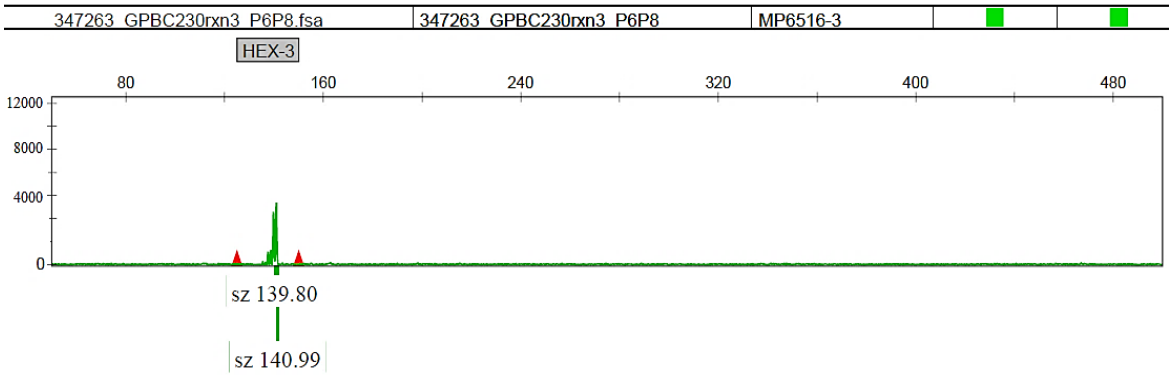
	Locus name	Cocoa clones	Allele	Allele size (bp)			
				ST	IZ	G	[24]
1	mTcCIR37-AJ271942	MCBC1	A1	153.98	154.12	154.12	136 - 187
			A2	177.28	177.34	177.34	
		PBC230	A1	147.46	147.40	147.49	
			A2	158.39	158.48	158.53	
		KKM22	A1	164.71	164.78	164.65	
			A2	185.54	185.57	185.65	
		KKM4	A1	147.21	**158.39**	147.38	
			A2	164.65	**177.23**	164.72	
2	mTcCIR60-AJ271958	MCBC1	A1	191.75	192.02	191.83	190 - 218
			A2	208.76	209.04	208.84	
		PBC230	A1	191.75	191.73	191.93	
			A2	208.77	208.77	208.92	
		KKM22	A1	194.06	193.74	193.96	
		KKM4	A1	193.72	**191.79**	193.72	
			A2	208.74	**212.55**	208.81	
3	mTcCIR6-Y16980	MCBC1	A1	224.24	224.46	224.52	224 - 253
			A2	226.14	226.16	226.11	
		PBC230	A1	226.32	226.31	226.40	
			A2	248.20	248.21	248.19	
		KKM22	A1	226.58	226.19	226.36	
			A2	248.05	248.17	248.20	

	Locus name	Cocoa clones	Allele	Allele size (bp)			
				ST	IZ	G	[24]
		KKM4	A1	226.22	**230.06**	226.30	
			A2	230.07	**248.07**	248.20	
4	mTcCIR11-Y16985	MCBC1	A1	288.24	288.30	288.32	286 - 321
			A2	308.70	308.84	308.96	
		PBC230	A1	296.25	296.26	296.59	
			A2	315.10	315.06	314.37	
		KKM22	A1	300.18	300.18	300.18	
		KKM4	A1	306.62	**296.27**	306.58	
			A2	315.05	**300.18**	315.01	
5	mTcCIR8-Y16982	MCBC1	A1	288.22	288.21	288.33	290 - 307
		PBC230	A1	289.24	289.23	289.31	
		KKM22	A1	287.57	287.16	287.20	
			A2	304.03	303.50	303.51	
		KKM4	A1	289.22	**287.11**	289.19	
			A2	303.54	**288.06**	303.51	
6	mTcCIR1-Y16883	MCBC1	A1	141.04	140.52	140.80	128 - 146
			A2	-	**139.51**	-	
		PBC230	A1	140.83	140.84	140.99	
			A2	-	139.51	139.80	
		KKM22	A1	128.25	127.03	127.93	
		KKM4	A1	-	**139.51**	-	
			A2	141.54	140.52	141.14	
7	mTcCIR26-Y16998	MCBC1	A1	294.49	294.57	294.51	285 - 310
			A2	300.50	300.70	300.70	
		PBC230	A1	296.60	296.46	296.60	
		KKM22	A1	296.51	296.52	296.53	
			A2	302.71	302.78	302.69	
		KKM4	A1	296.48	296.43	296.42	
			A2	-	**302.61**	-	
8	mTcCIR12-Y16986 (Monomorphic)	MCBC1	A1	187.76	187.98	187.92	165-256
			A2	204.33	204.54	204.48	
		PBC230	A1	212.64	212.62	212.79	
		KKM22	A1	200.26	199.84	200.26	
			A2	212.62	212.21	212.62	
		KKM4	A1	199.83	200.17	200.26	
			A2	212.18	212.64	212.29	
9	mTcCIR24-Y16996 (Monomorphic)	MCBC1	A1	185.70	185.78	185.89	186 - 207
			A2	191.65	191.70	191.77	
		PBC230	A1	185.75	185.78	185.97	
		KKM22	A1	185.10	185.75	185.19	
			A2	197.56	197.46	197.56	
		KKM4	A1	185.52	185.95	185.67	
10	mTcCIR15-Y16988 (Monomorphic)	MCBC1	A1	234.26	234.31	234.30	234 - 263
			A2	238.24	238.33	238.29	
		PBC230	A1	234.19	234.22	234.41	
			A2	242.37	242.36	242.57	
		KKM22	A1	232.79	232.24	232.28	
			A2	252.83	252.26	252.32	
		KKM4	A1	234.14	234.14	234.60	
			A2	242.39	242.32	242.83	

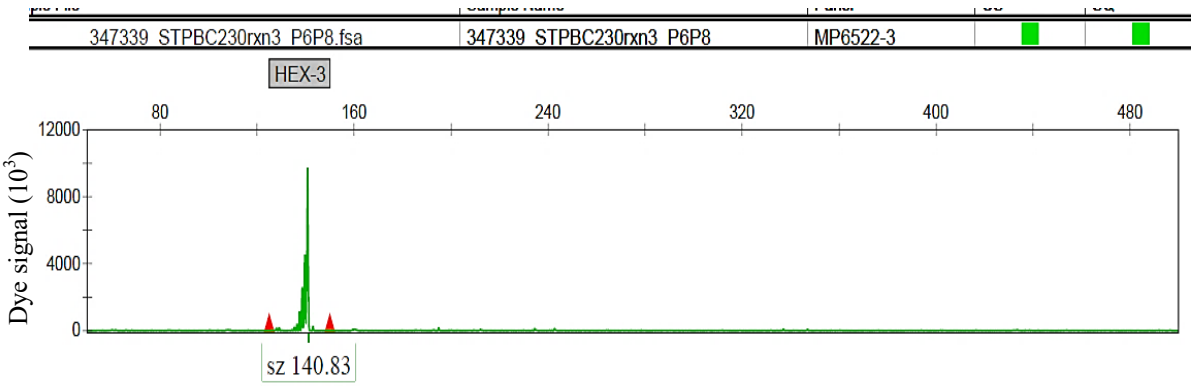
ST: staminode culture, IZ: Immature zygotic embryo culture, G: Grafted donor

In comparison among cocoa clones, no variation was observed in the allelic profiles of KKM22 derived from both staminode and immature zygotic embryo cultures. In contrast, 1 SSR primer identified an allele deletion in PBC230 propagated from staminode culture and an allele addition in MCBC1 propagated from immature zygotic embryo culture. In KKM4 derived from immature zygotic embryo culture, 12 SSR primers showed genetic instabilities. The instabilities of these 3 cocoa clones are caused

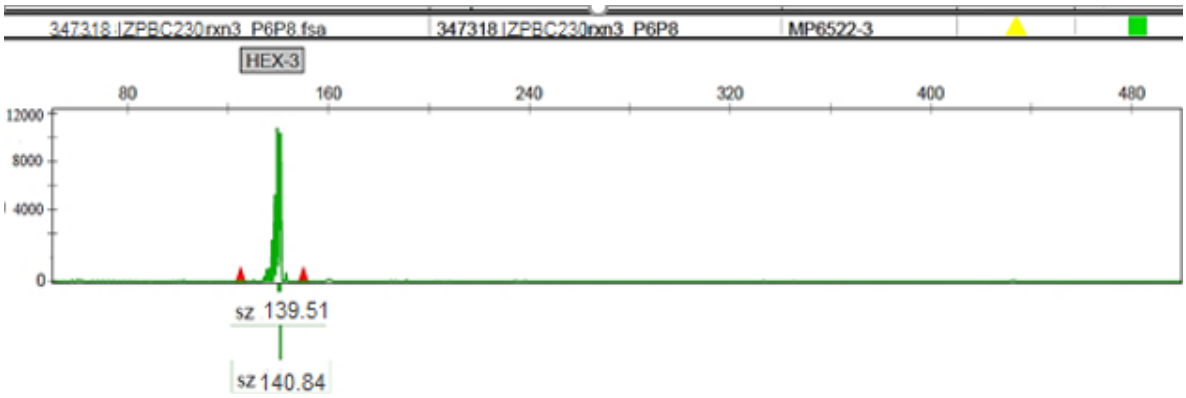
by the slippage mutations which can be indicated by allele deletion, allele addition (**Figure 1**) and allele size change (**Figure 2**).



(a) PBC230 clone derived from grafted donor trees

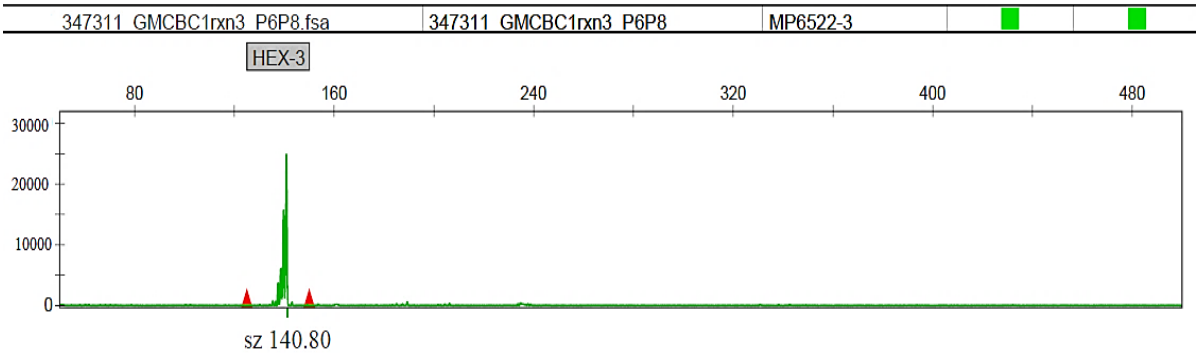


(b) PBC230 clone derived from staminode culture

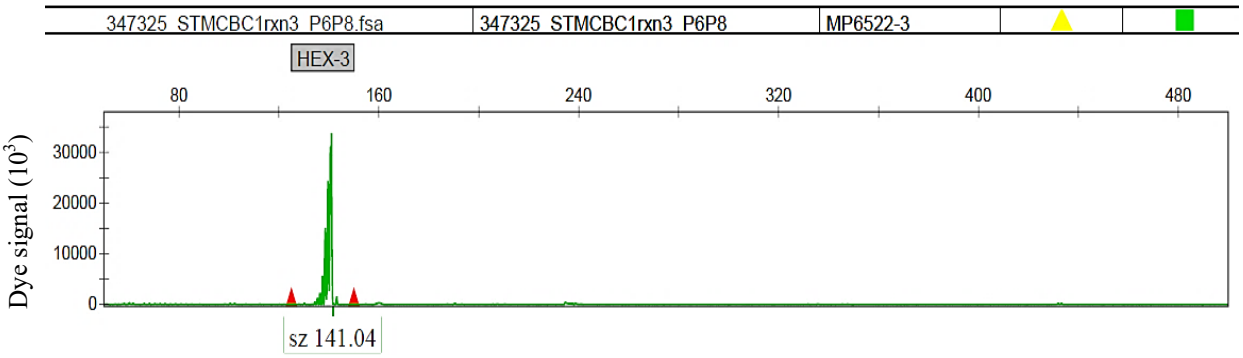


(c) PBC230 clone derived from immature zygotic embryo culture

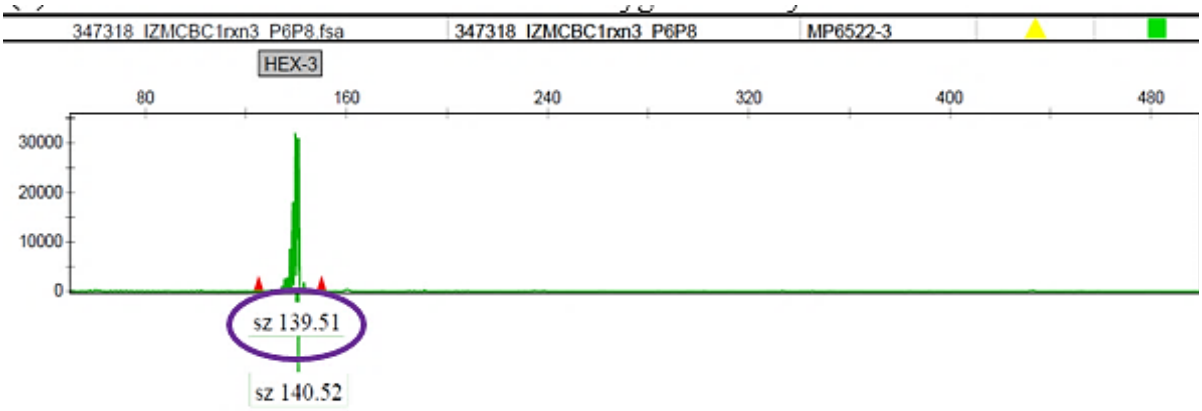
Figure 1 The amplification profiles of mTcCIR1-Y16985 (HEX-3) primer for PBC230 clone. The electropherogram corresponded to PBC230 clone of grafted donor trees and immature zygotic embryo culture are similar which showed heterozygous individual with 2 alleles averaged 139.66 and 140.92 bp. The middle electropherogram corresponded to PBC230 clone of staminode culture which showed homozygous individual with only 1 allele of 140.83 bp. Top scale indicated fragment size in nucleotide. Left scale indicated fluorescence intensity measured in relative fluorescence units.



(a) MCBC1 clone derived from grafted donor trees



(b) MCBC1 clone derived from staminode culture



(c) MCBC1 clone derived from immature zygotic embryo culture

Figure 2 Amplification profiles of MTcCIR1-Y16883 (HEX-3) primer for MCBC1 clone. The electropherogram corresponded to MCBC1 clone of grafted donor trees and staminode culture are similar which showed homozygous individual with 1 allele averaged 140.92 bp. The lower electropherogram corresponded to MCBC1 clone of immature zygotic embryo culture which showed heterozygous individual with 1 allele addition of 139.51 bp. Top scale indicates fragment size in nucleotide. Left scale indicated fluorescence intensity measured in relative fluorescence units. Purple circle = allele addition.

The incidence of allele addition in both KKM4 and MCBC1 clones from immature zygotic embryo culture were similar with those observed in other somatic embryogenesis cultured cocoa clones by [11] and [12]. The researchers reported that allele addition was produced by the extra repeat units at the SSR locus of cocoa clones which was formed from the abnormal mitotic recombination including interchromatid unequal crossing over and intrachromatid exchange. These usually occurred during a long callus proliferation period of indirect somatic embryogenesis culture [30,31]. In the other tissue cultured

crops such as areca nut (*Areca catechu* L.) [32] and asparagus (*Asparagus officinalis* L.) [33], the occurrence of somatic mutation can be avoided by a direct formation of somatic embryo from the explant source without the callus induction stage. Hence, it is recommended to initiate somatic embryo directly from the immature zygotic embryo explant without callus induction for KKM4 clone.

Similarly, a slippage mutation in term of allele size change also constituted the highest form of variation in earlier report on somatic embryogenesis culture derived cocoa clones [13]. According to the researchers, such variation was caused by both sample contamination and locus duplication. Other *in vitro* propagation of cork oak (*Quercus suber* L.) [31], coffee (*Coffee arabica* L.) [34] and pineapple (*Ananas comosus* (L.) Merr.) [35] have indicated that the change in allele size was activated by a cell lineage mechanism. This mechanism reduces the cellular competence for the proofreading and maintenance of DNA sequence integrity during somatic embryogenesis process [36], resulting in alteration of allele size. In another report, the failure of DNA sequence proofreading and alteration in their integrity had caused the allele size change in tissue cultured maize (*Zea mays* L.) [37]. Thus, this might be a similar process which occurred at the DNA cellular level for the variants from KKM4 cocoa clone of immature zygotic embryo culture.

Conclusions

From these findings, given the unstable genetic constituents of KKM4 clone, the use of immature zygotic embryo explant was believed to account for the variation in the agronomic performance of this clone. Twelve SSR markers were successfully used to detect the variations which were observed in the form of slippage mutations such as allele addition and allele size change. Therefore, for cocoa clones which are prone to variation due to their unstable genome, it is suggested to optimize the protocols for somatic embryogenesis which includes reducing the culture period and using a lower concentration of plant growth regulators. It is also suggested that Principal Coordinates Analysis (PCoA) conducted in the future study to find Genetic diversity among somatic embryogenesis cultured cocoa clones.

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