DEVELOPMENT OF AN EFFECTIVE SSR-BASED FINGERPRINTING SYSTEM FOR COMMERCIAL PLANTING MATERIALS AND BREEDING APPLICATIONS IN OIL PALM

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ABSTRACT

*DNA fingerprints provide the basis for genetic diversity studies and molecular breeding. However, routine and commercial scale DNA fingerprinting in oil palm (*Elaeis guineensis *Jacq.) remains challenged by the need for a robust yet low cost method. In addition, there is also a need for a precise and reliable marker set capable of distinguishing oil palm genotypes within narrow and mixed genetic population bases at the individual and population level. With the rapid evolution of genotyping and sequencing technologies, a wide range of genotyping tools have been developed but are not yet commercially feasible for oil palm. Consequently, we tested the feasibility of using simple sequence repeat (SSR) markers to genotype oil palm breeding and commercial planting materials of restricted and mixed parentage from Advanced Agriecological Research Sdn Bhd (AAR). The results obtained from screening 33 palms using a selected set of 17 highly polymorphic and informative SSR markers obtained from an initial set of 23 SSR, showed that clones, crosses and sibs within crosses could be separated by their DNA fingerprints. However, as expected palms within clones could not be separated. In another test exercise at the population scale, the four variable but related populations: Deli; Dumpy Deli x Yangambi x AVROS; Dumpy Deli x AVROS; Dumpy Deli x AVROS x La Me; were distinctly separated using 11 SSR from the same pool of 23 polymorphic SSR. There is good potential for this set of 11 SSR markers to be developed for general applicability across all* E. guineensis *oil palm genetic materials and for use in the Malaysian Plant Variety Protection Act. The set of SSR markers also serve as the base set for the development of marker-assisted and genome-wide selection at AAR.*

Keywords: SSR fingerprinting system, plant variety protection, genotype authentication.

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INTRODUCTION

Simple sequence repeats (SSR) provide the molecular basis of DNA fingerprinting by making use of pairs of primers to amplify repetitive DNA. These SSR genomic regions are abundant in plant genomes with high variation in repeat numbers between different lines within a species (Wang *et al*., 1994). They are short nucleotide sequences (usually 2-6 bp) repeated many times and found in both the coding, non-coding and usually non-translated regions of the genome (Morgante and Olivieri, 1993; Wang *et al*., 1994; Powell *et al*., 1996). The usefulness of SSR markers in crop and particularly oil palm genetic research is determined by their highly polymorphic nature and co-dominance, equipment availability, low development cost, ease of use and transferability between laboratories.

The early attempts in developing oil palm SSR markers have laid the foundation of using molecular markers for oil palm genetic studies. Billotte *et al*. (2001) published the results of screening the microsatellite-enriched libraries of (GA)n, (GT)n, and (CCG)n types and assessing the efficiency of using the characterised 21 SSR loci to reveal the *Elaeis* genetic diversity. In the following years, continued development of the SSR markers had led to the success of producing the first oil palm microsatellite-based linkage map (LM2T x DA10D genotypes) which consists of 16 independent linkage groups, as well as revealing the locus of shell gene that controls the presence or absence of the shell (Billotte *et al*., 2005). With a total set of 390 SSR markers developed in the previous studies (Billotte *et al*., 2001; 2005), an integrated SSR linkage map was established and a quantitative trait locus (QTL) analysis was carried out for oil palm production traits and bunch components (Billotte *et al*., 2010).

In 2007, Rajinder *et al*. reported an effective method to develop SSR markers where a set of 12 informative SSR markers was identified and found suitable for fingerprinting the oil palm tissue culture clones. Since then, SSR markers have been used by others (Rajinder *et al*., 2008; Cochard *et al*., 2009; Durand-Gasselin, 2009) for germplasm diversity studies and for differentiating oil palm genetic populations. It is also widely acceptable by oil palm scientists to use for molecular breeding improvement (Billotte *et al*., 2010; Seng *et al*., 2011) and agronomic trait studies (Rajinder *et al*., 2009; Tranbarger *et al*., 2012; Montoya *et al*., 2013) but need to be optimised to make the approach commercially viable. The first and perhaps most important application of oil palm genomics is in genotype authentication for breeding (Corley, 2005), tissue culture cloning (Soh *et al*., 2011; Wong *et al*., 2011), and eventually plant variety protection (PVP). The last requires palm genetic differentiation at the individual plant level especially with oil palm materials that are genetically narrow and related (Rosenquist, 1986). This is important, as there can be a real probability of identity confusion when one individual palm is randomly chosen from a large sample size (Tessier *et al*., 1999; Zhang *et al*., 2006). Plant breeder's rights for oil palm were endorsed by the Malaysian Plant Variety Protection Board in 2010 allowing the seed producers in Malaysia to officially register their elite planting materials (website: http://pvpbkkt.doa.gov.my). The guidelines for the conduct of tests for Distinctness, Uniformity and Stability: Oil Palm (DUS-OP Guidelines, Plant Varieties Board Malaysia, 2009) states that DNA fingerprints will only be used in case there is a dispute and not used as the basis to differentiate a palm from other varieties when the phenotype is the same. The Malaysian Palm Oil Board (MPOB) has recommended a preliminary set of SSR markers (*Table 1*) for this purpose (Rajinder *et al*., 2007).

Currently oil palm DNA profiling is constrained by the requirements for molecular data reproducibility and genotyping capacity. Lack of reproducibility of banding patterns between and within samples can be a consequence of laboratory and/or field errors. In the laboratory, poor DNA template quality, inconsistent interpretation of mixed-intensity banding patterns due to high background noise for gel-based scoring methods and generation of primer-derived or non-specific PCR amplification products are the probable causes. In the field, palm misidentification is often the cause as sample mix-up can arise at any point along the palm's production (seed or ramet nursery) and field planting and material sampling chain.

Our main interest focused on testing the efficiency of a set of SSR markers for DNA fingerprinting. This was achieved through studying the ability of the SSR markers to distinguish between oil palm clones, within clones, at the family/population level and differentiating palm variety (hybrids, clones). In addition, the applicability of the SSR markers to determine authenticity/illegitimacy/piracy and quality control in seed and ramet production was also investigated. The ultimate goal of this study is to establish an integrated analysis environment using a high resolution gel-based separation platform for oil palm SSR marker analysis. This will enable the geneticist to investigate the origin, relationship among species or populations and the genetic structure of a breeding line.

MATERIALS AND METHODS

Plant Material

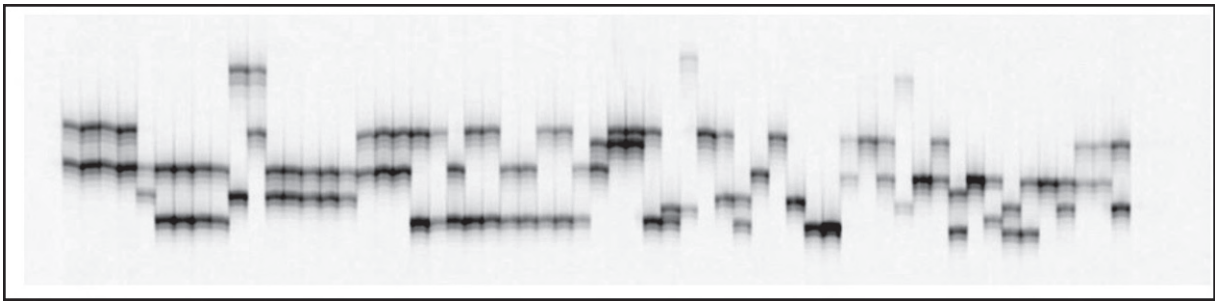
The genus *Elaeis* consists of two oil palm species, *Elaeis guineensis* is of West Africa origin and *Elaeis oleifera* is from the Central and South America.

TABLE 1. DUS-OP PRIMERS FOR SIMPLE SEQUENCE REPEAT (SSR) ANALYSIS

Locus name	M13-tailed primer sequences F: 5′-3′ R: 5′-3′	Expected size of allele attached with M13 tail (bp)	Annealing temperature (°C)
sEG00097 (EAP 03745)	Fwd (left): GGAAACAGCTATGACCATGAAGAAGGGTGTAGATGGTTC Rev(right): CCTGAGCTTCATTGTCTGAT	194	52
sEG00056 (CNI01714)	Fwd (left): GGAAACAGCTATGACCATATACAATATGCTGCCTGAAG Rev (right): TGCAATTTCTTAGCAGAAGC	188	52
sEg00038 (CNH01303)	Fwd (left): GGAAACAGCTATGACCATATCAAGCGGCAGTTATGAGAT Rev (right): ATACATTATTCCCACCACCA	183	52
sEG00098 (EAP03854)	Fwd (left): GGAAACAGCTATGACCATTTACAGTCGGGAACTATTGG Rev (right): CATAAGAAGCAACCTCTGAA	192	52
sEG00167 (CNHP00167)	Fwd (left): GGAAACAGCTATGACCATACAAAAGATGAAAGCTGAAAAG Rev (right): TACCAAACAAGAAAGCAGTTTA	173	52
sEg00032 (CEOP00030)	Fwd (left): GGAAACAGCTATGACCATCTGTTGAGCTGGAGAGACCC Rev (right): CCAACCAGGATCAGTTTGGT	287	55
sEg00080 (EAP01840)	Fwd (left): GGAAACAGCTATGACCATAAGAACTATGACCTCACCAAAA Rev (right): AACTCTATGCTATTGCTACACGA	171	52
sEg00125 (EO02861)	Fwd (left): GGAAACAGCTATGACCATTACCCTTTTCCCTCCCTCCATA Rev (right): CATCATCTCCGTTGCCAGTATT	170	52
sEg00126 (EO02978)	Fwd (left): GGAAACAGCTATGACCATCCGTCTCAAAAGCCCTAAAC Rev (right): TTGTTGTCCCACTCCCTCTT	234	52
sEg00127 (EO03035)	Fwd (left): GGAAACAGCTATGACCATCTAAAATTCCCTCATCGTCTC Rev (right): CTCGAAGCTCATCGTCTCTC	175	52

Note: The content of this table was adopted without amendment from the *Annex 2: Protocols DNA Profiling of Oil Palm: Useful Explanations on Molecular Characteristics Using Simple Sequence Repeat* (Plant Varieties Board Malaysia, 2009).

In South-east Asia, the commercial planting of *E. guineensis tenera* palm is a hybrid between *dura* and *pisifera* palms, where the distinction amongst them can be easily identified through their fruit forms, *i.e*. *dura* fruit has think shell, which is absent in *pisifera* (shell-less) and the *tenera* hybrid has the thin-shell yielding higher mersocarp oil than the parental palms *dura* and *pisifera* (Corley and Tinker, 2003; Rajinder *et al*., 2013a, b). In this study, 59 core genotypes selected from the oil palm germplasm of Advanced Agriecological Research (AAR) were used in the screen for highly polymorphic SSR markers (*Figure 1*). The reliability of these SSR markers (H_e > 0.5 and PIC > 0.5) was tested at two levels using two different groups of oil palm genetic materials. The initial experiment in the first study tested 60 *tenera* palms from five clones: A98 (3/7x OBS4/30), A163 (0250/9 x 406/19), A174 (0250/9 x 406/19), A176 (0105/27 x L238T), A195 (0246/49 x 406/19) and a DxP (*dura* x *pisifera*) control cross DxP/677 using DUS-OPSSR markers (*Table 1*). The following experiment tested 23 SSR markers (*Table 2*) on 33 sibling palms from three DxP crosses: 76/14 x 126/37 (Deli x AVROS), 250/9 x 406/19 (Deli x



Lane No.	Type	Cross source	Parentage
1-4	Clone	ZD'F' x OBS4/30	Dumpy x AVROS x AVROS
5-9	Clone	M766 x OBS4/16	Dumpy x Deli x AVROS
10-12	Progeny/sibs	76/14 x 126/37	Deli x AVROS
13-16	Clone	14/3 x OBS4/16	Deli x AVROS
17-19	Clone	22/7 x 0126/37	Deli x Yangambi x AVROS
20-28	Progeny/sibs	250/9 x 406/19	Deli x Cameroon
29	Progeny	0106/10 x 65/4	Deli x EWS
30-33	Progeny/sibs	0106/10 x 0126/11	Deli x Yangambi x AVROS
34	Progeny	Unidentified	Dumpy AVROS
35	Progeny	Unidentified	Yangambi
36	Progeny	Unidentified	Nigerian
37	Progeny	Unidentified	Dumpy Yangambi AVROS
38	Progeny	Unidentified	Ghana
39	Progeny	Unidentified	Ulu Bernam Deli
40	Progeny	Unidentified	Ulu Remis Deli
41	Progeny	Unidentified	Angola
42	Progeny	Unidentified	Zaire Cameroon
43	Progeny	Unidentified	EWS AVROS
44	Progeny	Unidentified	Dumpy Ulu Remis Deli
45	Progeny	Unidentified	Ulu Remis Ulu Bernam
46	Progeny	Unidentified	Ulu Bernam
47	Progeny	Unidentified	Binga Yangambi AVROS
48	Progeny	Unidentified	Binga Yangambi AVROS
49	Progeny	Unidentified	Ulu Remis La Me Deli
50	Progeny	Unidentified	La Me
51	Progeny	Unidentified	Dumpy AVROS La Me
52	Progeny	Unidentified	Unidentified
53	Progeny	Unidentified	Dumpy AVROS
54	Progeny	Unidentified	Dumpy AVROS
55	Progeny	Unidentified	Cameroon
56	Progeny	Unidentified	Dumpy AVROS Nifor
57	Progeny	Unidentified	Ulu Remis La Me Deli
58	Progeny	Unidentified	La Me Deli
59	Progeny	Unidentified	Yangambi AVROS

Figure 1. Fingerprints of 59 Advanced Agriecological Research's core genotypes generated by the simple sequence repeat (SSR) marker sMg00042. Palm No. 1-33: clonal and progeny palms; 34-59: oil palm germplasm.

TABLE 2. LIST OF SIMPLE SEQUENCE REPEAT (SSR) MARKERS USED FOR GENOTYPING AAR'S PLANTING MATERIALS

No.	Marker ID	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)
1.	sEg00035	TTATTGATTGATGCAAGATACAC	TTGATAAAATACAAGAGATAGCA	161
2.	sMg00025	GAGGAGGAGGGGAGAAGAGT	AAATACCATTCAGAGAAAGCAC	198
3.	sMg00042	CCGAATAGAAGAGGAAAGAATA	AGGTTTGGTGGAGAAGTGTT	247
4.	sMg00108	ACGAAACAGAGGCATAGAGACT	ACAATTAAACAGCAACGCTAGA	186
5.	mEgCIR00369	GGGTAGCAAACCTTGTATTA	ACTTCCATTGTCTCATTATTCT	206
6.	mEgCIR03428	GACAGCTCGTGATGTAGA	GTTCTTGGCCGCTATAT	175
7.	mEgCIR03649	TTTAGAGGACAAGGAGATAAG	CGACCGTGTCAAGAGTG	284
8.	mEgCIR03544	AGCAGGGCAAGAGCAATACT	TTCAGCAGCAGGAAACATC	188
9.	mEgCIR02595	TCAAAGAGCCGCACAACAAG	ACTTTGCTGCTTGGTGACTTA	184
10.	mEgCIR03358	CCAAGGAACAACATAGA	GTTCCCATCCTATTAGAC	208
11.	mEgCIR00783	GAATGTGGCTGTAAATGCTGAGTG	AAGCCGCATGGACAACTCTAGTAA	296
12.	mEgCIR03389	GTCCATGTGCATAAGAGAG	CTCTTGGCATTTCAGATAC	93
13.	mEgCIR02600	GGGGATGAGTTTGTTTGTTC	CCTGCTTGGCGAGATGA	277
14.	mEgCIR03808	CCGCTAACTTGGTATAC	ATTTCCAGCAGCTAATC	190
15.	mEgCIR03376	CCCTCCCTGCTACCTTCT	TTATGTGAGTGCCTTTGATG	213
16.	mEgCIR02332	GAAGAAGAGCAAAAGAGAAG	GCTAGGTGAAAAATAAAGTT	204
17.	mEgCIR02492	CATCAAGCATGACTGCAAGTAA	TTCCGAATTTGGATGAATCC	248
18.	mEgCIR03311	AATCCAAGTGGCCTACAG	CATGGCTTTGCTCAGTCA	176
19.	mEgCIR02427	GAAGGGGCATTGGATTT	TACCTATTACAGCGAGAGTG	116
20.	mEgCIR03546	GCCTATCCCCTGAACTATCT	TGCACATACCAGCAACAGAG	286
21.	mEgCIR00521	GTGACTTTGGGCTGAAT	ACAGCATCTCCAACTCTATC	137
22.	mEgCIR00177	TGAATGTGTGTGCAATGTGTAT	ATAGTCAATAATCGTAGGAAAATG	114
23.	mEgCIR03298	GACTACCGTATTGCGTTCAG	GGTTTTGGTTCGTGGAG	137

Note: AAR and MPOB refer to the Advanced Agriecological Research and the Malaysian Palm Oil Board, respectively. SSR No. 1-4 were provided by MPOB; No. 5-23 were selected from Billotte *et al.* (2005).

Cameroon), 106/10 x 126/11 (Deli x Yangambi x AVROS) and clonal palms or ramets of six clones: Clone 5/14C (Dumpy x AVROS x AVROS), Clone 7 (Dumpy x Deli x AVROS), Clone 3 (Deli x AVROS), Clone 12 (Deli x Yangambi x AVROS), Clone 258 (Deli x EWS) and Clone 259 (Deli x Yangambi x AVROS). Both experiments were used to evaluate the ability of selected SSR to distinguish palms within clone, between clones and from different crosses.

Our second study involved testing 40 palms from four different oil palm segregating populations: i) Deli, ii) Dumpy x Yangambi x AVROS, iii) Dumpy x Yangambi x AVROS selfed, and iv) Dumpy x AVROS x La Me selfed. The palms were used to appraise the ability of the selected SSR to discriminate out-crosses and to cluster palms at the population level. Ten palms were sampled from each population for the test. The lineages of the parental palms used are described by Soh *et al*. (2003) and Rosenquist (1986).

DNA Extraction and PCR Amplification

The genomic DNA of oil palm leaf samples was isolated using the iNtron Plant DNA extraction kit (iNtron Biotech., Korea). The modified MPOB DNA extraction protocol (Rahimah *et al.,* 2006) was used whenever a greater quantity of DNA was needed. The quality and quantity of the extracted DNA was checked using a Lambda 25 UV/Vis Spectrophotometer (Perkin Elmer, United Kingdom) prior to dilution and further utilisation for PCR amplification. The forward and reverse SSR primer sequences used for the PCR amplification are listed in *Table 1*. A fusion of the forward SSR primer with a M13 (-21) tail, 5′-TGT AAA ACG ACG GCC AGT-3′ (18bp), at the 5′end was synthesised for PCR amplification. During the PCR amplification, the universal IRDye®-labelled M13 (-21) primer was incorporated into the PCR reaction for labelling the PCR products with the fusion primer and standard reverse primer (Schuelke, 2000). The PCR reaction mixture (10 μl) contained 5.0 μl DyNAzyme II PCR Master Mix (cat# F-508, Finnzymes, Finland), 0.5 μl of each forward (10 μM) and reverse primers (10 μM), 0.5 μl of 1.0 μM IRDye®-labelled M13 (-21) primer, 10-20 ng template DNA and nuclease-free water. The PCR programme used was one cycle of 94ºC for 4 min followed by 35 cycles of 94ºC for 30 s; 52ºC for 90 s; 72ºC for 60 s. Finally, the PCR reaction was completed with an additional extension of 30 min at 60ºC before cooling to 10ºC. The PCR amplification was performed in Veriti 96-well Thermal Cycler (Applied Biosystems, USA). The final IRDye®-labelled product was analysed using an infrared dye detection system, *i.e.* NEN 4300 DNA Analyser (Li-COR Biosciences, USA).

Silver Staining and Infrared Dye Detection

The initial screening for polymorphic SSR markers using the core genotypes was carried out utilising a 6% polyacrylamide gel electrophoresis

and banding profiles were observed using the silver staining protocol described by Gustavo and Peter (1994). Subsequently, the DUS-OP SSR markers (*Table 1*) and selected 23 polymorphic SSR markers (*Table 2*), were converted into IRDye®-detection markers for genotyping studies. The positions of the SSR markers listed in *Table 2* were predetermined based on the published high density oil palm linkage map constructed using 255 SSR and 688 amplified fragments length polymorphisms (AFLP) (Billotte *et al.*, 2005). Presumably, the selected 23 SSR markers should distribute across the 16 linkage groups published. Higher resolution of amplified SSR was achieved using the NEN 4300 DNA Analyser (Li-COR Biosciences, USA). Binary matrix scoring was carried out manually to avoid misinterpretation of mixed intensity SSR fragment patterns.

Despite the use of robust SSR protocols and the preparation of high quality DNA samples, fingerprinting errors have been reported causing duplicate samples to give apparently different profiles. Therefore, repeated fingerprinting (Taberlet and Luikart, 1999; Bonin *et al.*, 2004) and use of the multitubes approach (Valiere *et al.*, 2002) were introduced in other crops to minimise technical errors. In this study, each sample was duplicated and the experiment was repeated at least twice to ensure that the results were reproducible.

Selection of Informative SSR Markers and Cluster Analysis

The CERVUS software, version 3.0.3 (Marshall *et al.*, 1998; Kalinowski *et al.*, 2007) was used to analyse the informativeness of SSR markers *i.e.* number of alleles (K) generated from genotyped accessions, observed heterozygosity (H_o), expected heterozygosity (H_e) and polymorphism information content (PIC) were calculated using formulae developed by Nei (1987), Botstein *et al.* (1980) and Tessier *et al.* (1999). Cluster analysis was carried out using the multivariate statistical package MVSP (Kovach Computing Services, Anglesey, Wales).

RESULTS AND DISCUSSION

Selection of SSR Markers for Oil Palm Fingerprinting

The informativeness of the SSR markers was assessed by calculating the PIC (Botstein *et al.*, 1980) and discrimination power, D_j (Tessier *et al.*, 1999). PIC is a measure of informativeness related to expected heterozygosity (H_e), and likewise is calculated from allele frequencies and commonly used to select markers for linkage mapping (Botstein *et al.*, 1980; Hearne *et al.*, 1992). According to Taamalli *et al.* (2008), SSR markers with PIC values greater than 0.5 were classified as informative markers suitable for individual identification, whereas loci with PIC values greater than 0.7 would be useful for linkage mapping. The polymorphism rate of SSR used in the study were also evaluated according to Diaz *et al.* (2007), who reported that SSR markers with D_j (>0.85) and PIC (>0.77) values were most suitable for application in olive cultivar selection and identification.

In our study, PIC and D_j values were calculated for each SSR marker (*Table 3*) from the 59 core genotypes of AAR's oil palm germplasm (*Figure 1)*. Among the 23 SSR markers analysed, 22 markers (the exception was sMg00025 with PIC of 0.444), qualified as highly polymorphic markers. The mean number of alleles per locus was 3.04. The H_e was calculated using an unbiased formula from allele frequencies assuming a Hardy-Weinberg equilibrium (Nei, 1987). The H_e is a marker informativeness measure of a locus where loci with H_e of 0.5 or less are less useful for large-scale genetic relationship analysis. The overall H_e (mean expected heterozygosity) across the 59 core genotypes' analysed was 0.651, generally considered high, with a minimum of 0.528 and a maximum of 0.806 (*Table 3*).

In *Table 3*, some markers exhibited a big difference between H_o and H_e. For example, H_e of mEgCIR03649 and mEgCIR02492 was high but their observed heterozygosity (H_o) value was zero. This observation inferred that the 59 genotypes must be homozygous for different alleles (three alleles were detected for mEgCIR03649 and mEgCIR02492, respectively), despite the targeted locus was deviated from Hardy-Weinberg equilibrium due to the segregating of null allele or genotyping errors (Marshall *et al.* 1998). At the course of determining the SSRs' polymorphism, this type of SSR marker was not excluded from downstream analysis; unless the null allele frequency is more than 0.05 detected by Cervus software during the fingerprinting exercise.

However, marker polymorphism can vary with sample size and origin of genotypes screened (Taamalli *et al.*, 2008). This was demonstrated in another SSR fingerprinting study, where similar statistical approaches were used to analyse SSR bands produced by 26 samples originating from wider genetic origins, *i.e.* Dumpy x AVROS, Yangambi, Nigerian, Dumpy x Yangambi x AVROS, Ghana, Ulu Bernam Deli, Ulu Remis Deli, Angola, Zaire x Cameroon, EWS x AVROS, Dumpy x Ulu Remis Deli, Ulu Remis Deli x Ulu Bernam Deli, Binga x Yangambi x AVROS, Ulu Remis Deli x Dabou Deli, La Me, Dumpy x AVROS x La Me, Cameroon, Dumpy x AVROS x Nifor, Dabou Deli and Yangambi x AVROS. The average non-exclusion probability of two unrelated individuals (NE-I) estimated from this set of 26 genotypes was ranging from 0.081 to 0.401, whereas, for previous study using the 59 core genotypes, the NE-I for individual marker was

TABLE 3. THE INFORMATION OF MARKER IDENTITY (ID), ALLELE NUMBER (K), NUMBER OF GENOTYPE TESTED (N), OBSERVED HETEROZYGOSITY (H_O), EXPECTED HETEROZYGOSITY (H_e), POLYMORPHISM INFORMATION CONTENT (PIC) AND DISCRIMINATORY POWER (Dj)

No.	Marker ID	K	N	H_o	H_e	PIC	Dj
1.	sEg00035	4	56	0.393	0.615	0.560	0.6205
2.	sMg00025	3	57	0.368	0.533	0.444	0.5376
3.	sMg00042	4	58	0.672	0.735	0.678	0.7412
4.	sMg00108	3	52	0.154	0.634	0.549	0.6403
5.	mEgCIR00369	3	36	0.528	0.528	0.644	0.6537
6.	mEgCIR03428	4	59	0.983	0.674	0.607	0.6802
7.	mEgCIR03649	3	59	0.000	0.625	0.546	0.6306
8.	mEgCIR03544	4	59	0.763	0.606	0.531	0.6112
9.	mEgCIR02595	6	59	0.763	0.745	0.692	0.7513
10.	mEgCIR03358	6	59	0.678	0.806	0.770	0.8136
11.	mEgCIR00783	3	59	0.559	0.660	0.581	0.6655
12.	mEgCIR03389	8	59	0.966	0.780	0.738	0.7866
13.	mEgCIR02600	3	57	0.018	0.638	0.561	0.6437
14.	mEgCIR03808	5	59	0.915	0.723	0.665	0.7293
15.	mEgCIR03376	4	59	0.763	0.673	0.597	0.6787
16.	mEgCIR02332	3	59	0.881	0.618	0.530	0.6231
17.	mEgCIR02492	3	59	0.000	0.648	0.570	0.6541
18.	mEgCIR03311	3	59	0.542	0.623	0.536	0.6281
19.	mEgCIR02427	5	59	0.576	0.564	0.514	0.5691
20.	mEgCIR03546	3	56	0.304	0.601	0.509	0.6067
21.	mEgCIR00521	4	58	0.207	0.593	0.539	0.5984
22.	mEgCIR00177	5	59	0.186	0.661	0.591	0.6661
23.	mEgCIR03298	4	59	0.695	0.683	0.625	0.6889

TABLE 4. THE EFFECT OF SAMPLE SIZE (N) AND ORIGIN OF GENOTYPES ON MARKER POLYMORPHISM

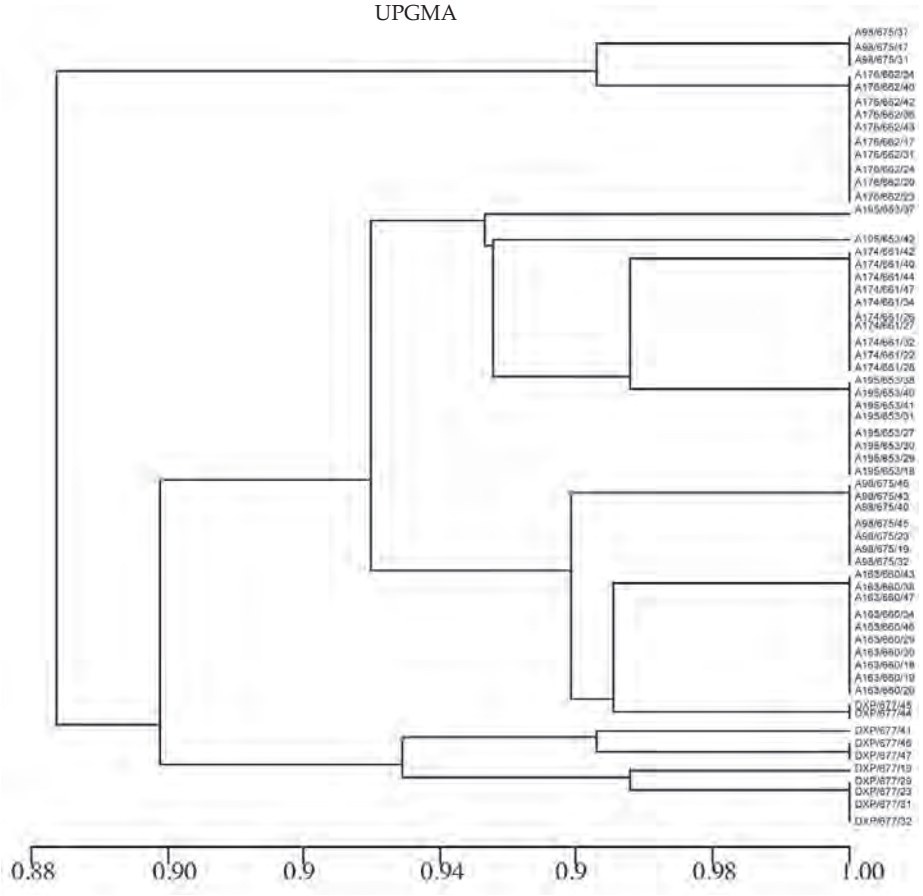
No.	Marker ID	*N=59*			*N= 26*		
		H_e	PIC	NE-I	H_e	PIC	NE-I
1.	sEg00035	0.615	0.560	0.202	0.600	0.522	0.235
2.	sMg00025	0.533	0.444	0.306	0.582	0.496	0.259
3.	sMg00042	0.735	0.678	0.124	0.751	0.688	0.118
4.	sMg00108	0.634	0.549	0.217	0.636	0.548	0.217
5.	mEgCIR00369	0.644	0.561	0.207	0.685	0.587	0.189
6.	mEgCIR03428	0.674	0.607	0.172	0.678	0.602	0.176
7.	mEgCIR03649	0.625	0.546	0.219	0.667	0.580	0.193
8.	mEgCIR03544	0.606	0.531	0.229	0.511	0.438	0.312
9.	mEgCIR02595	0.745	0.692	0.115	0.713	0.647	0.142
10.	mEgCIR03358	0.806	0.770	0.070	0.789	0.739	0.086
11.	mEgCIR00783	0.660	0.581	0.193	0.615	0.535	0.226
12.	mEgCIR03389	0.780	0.738	0.086	0.799	0.749	0.081
13.	mEgCIR02600	0.638	0.561	0.207	0.598	0.504	0.253
14.	mEgCIR03808	0.723	0.665	0.132	0.759	0.699	0.111
15.	mEgCIR03376	0.673	0.597	0.181	0.666	0.586	0.187
16.	mEgCIR02332	0.618	0.530	0.232	0.657	0.568	0.203
17.	mEgCIR02492	0.648	0.570	0.200	0.649	0.559	0.210
18.	mEgCIR03311	0.623	0.536	0.227	0.601	0.523	0.235
19.	mEgCIR02427	0.564	0.514	0.239	0.537	0.487	0.264
20.	mEgCIR03546	0.601	0.509	0.250	0.423	0.356	0.401
21.	mEgCIR00521	0.593	0.539	0.219	0.657	0.568	0.203
22.	mEgCIR00177	0.661	0.591	0.183	0.711	0.649	0.140
23.	mEgCIR03298	0.683	0.625	0.156	0.541	0.493	0.258

Note: H_e: Expected heterozygosity; PIC: polymorphism information content; NE-I: average non-exclusion probability for identity of two unrelated individual palms (Marshall *et al*., 1998).

ranging from 0.070 to 0.306 (*Table 4*, calculation was described by Marshall *et al*., 1998).

Potential SSR Markers for Distinguishing Oil Palms between Clones and within Clones

Experiment 1: testing of DUS-OP SSR markers on AAR's clonal materials. MPOB preliminary DUS-OP SSR markers were originally developed from expressed sequence tags (EST) and tested on AAR clonal materials. The same set of SSR markers were evaluated on another set of AAR clonal materials with a breeding cross DxP/677 progeny palms used as a control. Results showed that the fingerprints of clones A176, A174 and A163 were distinct and distinguishable from each other. However, the ramets of clone A195 and A98 were scattered across the clades (*Figure 2*). The fruit form and palm morphology were subsequently examined to further verify the DNA fingerprinting results obtained for clone A195 and A98 (*Figures 3* and *4*). The main purpose is to assess the usefulness and reliability of molecular techniques proposed for DUS-OP in detecting field misidentification errors especially involving palm materials that are closely related and which exhibit similar phenotypes.



Notation	Type	Cross source	Parentage
A98	Reclone	3/7 x OBS4/30	Ulu Remis x AVROS
A163	Clone	0250/9 x 406/19	Ulu Remis x Cameroon
A174	Clone	0250/9 x 406/19	Ulu Remis x Cameroon
A176	Clone	0105/27 x L238T	Ulu Remis x Yangambi
A195	Clone	0246/49 x 406/19	(Dumpy AVROS x Ulu Remis) x Cameroon
DxP/677	Progeny	Unidentified	Unidentified

Note: Clones A163 and A174 were derived from sibling palms.

Figure 2. UPGMA clustering of five clonal and one DxP cross planting material to test the effectiveness of DUS-OP SSR markers to protect breeder's rights.

As shown in *Figure 3*, palms A195/653/37 and A195/653/42 were genetically different from the bulk ramets of Clone A195 and this was also confirmed by the cut-fruit result. However, a different observation was found with Clone A98, where the fruit forms of A98/675/37, A98/675/17 and A98/675/31 were phenotypically the same as the bulk ramets of Clone A98. This led to the need to determine differences in palm morphology and vegetative growth measurements between the anomalies and A98 normal bulk. There were distinct palm morphological differences for A98/675/37, A98/675/17 and A98/675/31 from Clone A98 ramets generally (*Figure 4*). This illustrates the importance of corroborative field evidence in such studies.

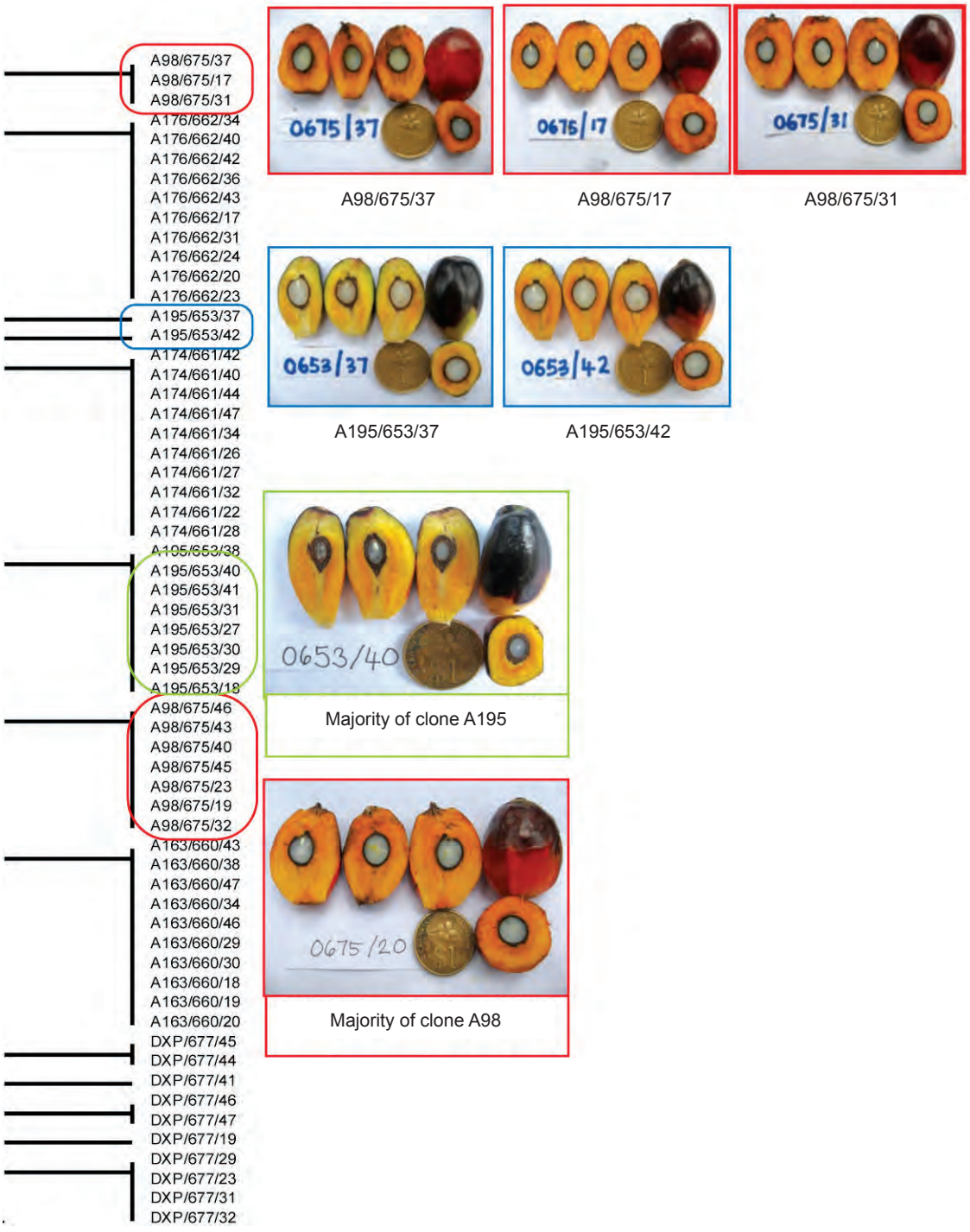


Figure 3. The genetic profile showed that A195/653/37 and A195/653/42 are genetically distinct (blue box) from the majority of Clone A195 (green box) which is also confirmed by the cut-fruit results by mean of shell thickness as well as other phenotypic data collected from the field. Different results were obtained for Clone A98, where the A98/675/37, A98/675/17 and A98/675/31 (red box) had a moderately thin-shell fruit form as similar with the majority of Clone A98, but there were distinct differences in palm morphology between these anomalies and the normal ramets of Clone A98 as shown in Figure 4.

The DxP/677 progeny palms were derived from non-inbred parents and genetic segregation among individual palms is expected (*Figure 2*). However, individual palm separation was achieved for only two out of 10 palms. Also, palms 44 and 45 were genetically closer to Clone A163 than Clone A174 even though the latter pairs were siblings. These results implied that the DUS-OP SSR markers while capable of distinguishing most clones of different genetic origins, they, however, may not be discriminatory enough to separate palms of narrow and related lineages, especially sibs. Where clones are being examined, highly discriminatory markers can give added confidence that identical fingerprints truly represent clonal material.

Experiment 2: verification of SSR markers suitable for genotype authentication. Of all the 23 SSR markers (*Table 3*) used to distinguish, 33 oil palms between clones and within clones, the most efficient SSR markers were mEgCIR03428, mEgCIR03649, mEgCIR03544, mEgCIR02595, mEgCIR03358, mEgCIR00783, mEgCIR03389, mEgCIR02600, mEgCIR03808, mEgCIR03376, mEgCIR02332, mEgCIR02492, mEgCIR03311, mEgCIR02427, mEgCIR00521, mEgCIR00177 and mEg CIR03298.

The results from the use of these 17 SSR on six clones and three DxP progenies are shown in *Figure 5*. The progeny palms of the crosses 250/9 x 406/19, 106/10 x 126/11, and 76/14 x 126/37 were clearly separated into their individual cross clades, as expected with separation among palms within crosses or hybrids derived from non-inbred parents. The ramets within clones were well-separated (but not perfectly) into their individual clone clades (Clones A259, A258, 5/14C, 12, 3, 7). One ramet each from Clone 7 (Palm 5) and Clone 3 (Palm 13) showed apparent separation from its co-ramets with significant genetic similarity of 0.691 and 0.981, respectively. This result suggested that there was possibly incorrect lines (*i.e*. Palm 5 and Palm 13) were detected from the Clone 7 and Clone 3 using the indicated set of SSR, without the inclusion of their ortet palms. While, the observed closer genetic distance between the clades of Clone 7 and Clone 3 in comparison with other palms could be an effect of the original ortet palms were being sibs (*Figure 5*).

Separation of Introgressed and Related but Segregating Oil Palm Populations using SSR Markers

Major oil palm breeding programmes have progressed to a stage where introgressed and recombinant populations of advanced breeding parents are commonplace. The ability to separate such populations using markers would be useful to the breeder to facilitate recurrent selection for population improvement and hybrid production. It is also of benefit to seed producers using introgressed parents to be able to distinguish their commercial hybrid seeds from others using molecular markers.

With this aim in mind, we compared the capability of DUS-OP SSR and AAR's selected SSR markers (*Table 2*) to distinguish four different introgressed and related segregating oil palm populations, *i.e.* Dumpy Yangambi AVROS, Dumpy Yangambi AVROS selfed, Deli *Dura* and Dumpy AVROS La Me selfed. Of the 40 palms genotyped, there were 12 alleles from five loci, *i.e.* sEG00056, sEg00038, sEg00080, sEg00125 and sEg00126 produced by the DUS-OP SSR fingerprinting marker set. This small number of loci would not have sufficient discriminating power to differentiate oil palms at the population level (unpublished data). The result obtained using AAR's selective SSR markers (*Table 2*) clearly demonstrated that a minimum of 27 alleles from the 11 loci were able to distinguish the segregating populations (*Figure 6*). The selected loci were sMg00042, mEgCIR3649, mEgCIR3544, mEgCIR2595, mEgCIR3358, mEgCIR2600, mEgCIR3808, mEgCIR2492, mEgCIR2427, mEgCIR3546 and mEgCIR3298.

This study has indicated that using a combination of AAR's 23 selective SSR markers, it is possible to genetically authenticate palms either at the individual, family or population level. In fact, the marker set can also separate clones (even derived from siblings), hybrids and populations with pure or mixed/introgressed and related genetic backgrounds. Since the breeding parents in AAR's introgression and recombinant breeding programmes are similar to those across the major oil palm breeding programmes, the combinations of the 23 selective markers can be readily used to authenticate the various commercial varieties of oil palm. In fact, this combination of markers represents an improved version of the DUS-OP SSR marker set. Also, since the screening method developed is rapid, precise, reliable and cost-effective and amenable to automation, the development of a commercial service is possible.

Nevertheless, the screening for more highly polymorphic and informative SSR markers is a continuing exercise as marker selection and database construction are critical for breeding. The applications are not restricted to genotype authentication and diversity/heterotic group differentiation but can be extended to marker-assisted selection (MAS). For effective use in MAS, markers should be strongly linked to agronomic (*e.g.* ease of harvesting) physiological (*e.g.* resource use efficiency), biotic (*e.g. Ganoderma* disease resistance) and abiotic (*e.g.* drought) (pers. comm. Button, 2011; UPOV/INF/17/1, 2010; UPOV/INF/18/1, 2011). The bulk of SSR markers suitable for genotyping

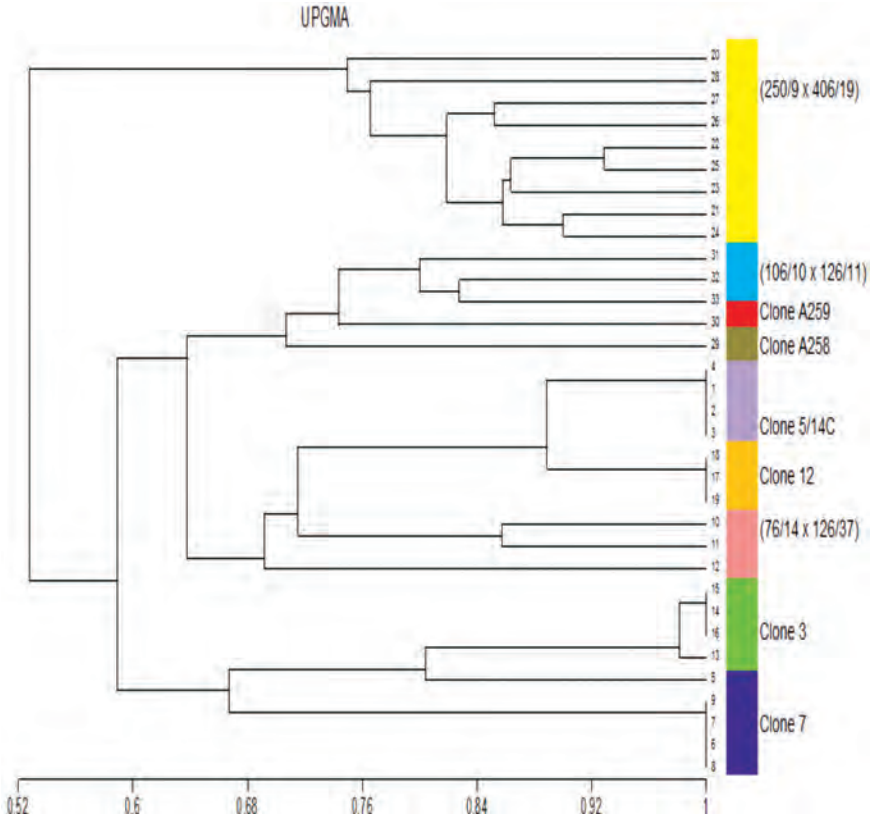
a) A98/675/37 b) A98/675/17 c) A98/675/31

Oil palm morphology for (a), (b) and (c):
1) Taller than the normal ramets of A98; 2) small girth; 3) thin petiole and short frond; 4) petiole colour is green; 5) small bunch size; 6) erect.

d) Normal ramets of Clone A98

Oil palm morphology for normal ramets (d):
1) Palm is slightly short; 2) average girth; 3) average petiole; 4) petiole is orange in colour; 5) bigger bunch; 6) normal, not erect.

Figure 4. Comparison of oil palm morphology between the anomalies (A98/675/37, A98/675/17 and A98/675/31) and the majority of Clone A98.



Notation	Type	Palm sampled	Breeding cross	Parentage
Clone A259	Clone	30	106/10 x 126/11	Deli x Yangambi x AVROS
Clone A258	Clone	29	106/10 x 65/4	Deli x EWS
Clone 5/14C	Clone	1, 2, 3, 4	ZD'F' x OBS4/30	Dumpy x AVROS x AVROS
Clone 12	Clone	17, 18, 19	22/7 x 126/37	Deli x Yangambi x AVROS
Clone 3	Clone	13, 14, 15, 16	14/3 x OBS4/16	Deli x AVROS
Clone 7	Clone	5, 6, 7, 8, 9	M766 x OBS4/16	Dumpy x Deli x AVROS
(250/9 x 406/19)	Progeny	20, 21, 22, 23, 24, 25, 26, 27, 28	250/9 x 406/19	Deli x Cameroon
(106/10 x 126/11)	Progeny	31, 32, 33	106/10 x 126/11	Deli x Yangambi x AVROS
(76/14 x 126/37)	Progeny	10, 11, 12	76/14 x 126/37	Deli x AVROS

Figure 5. UPGMA-based dendogram generated from fingerprinting oil palm clonal materials using 17 candidate simple sequence repeat (SSR) i.e. *mEgCIR03428, mEgCIR03649, mEgCIR03544, EgCIR02595, mEgCIR03358, mEgCIR00783, mEgCIR03389, mEgCIR02600, mEgCIR03808, mEgCIR03376, mEgCIR02332, mEgCIR02492, mEgCIR03311, mEgCIR02427, mEgCIR00521, mEgCIR00177 and mEgCIR03298.*

AAR planting materials have also been used for our pilot exercise on genome-wide selection, adapting the simulation model of Wong and Bernado (2008).

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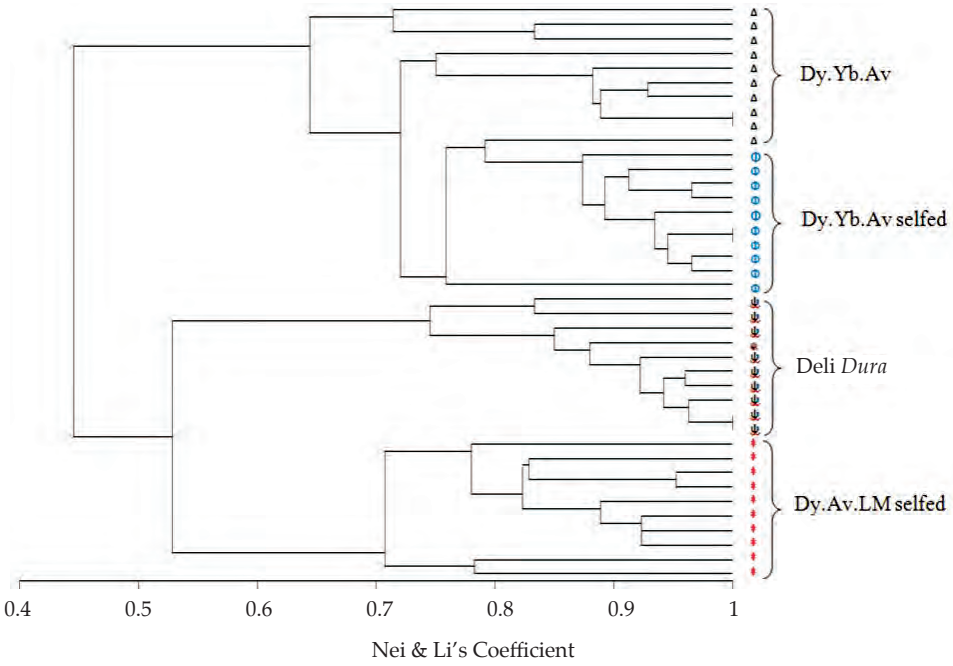

Figure 6. UPGMA clustering of four different segregating populations i.e. *Dumpy Yangambi AVROS, Dumpy Yangambi AVROS selfed, Deli* Dura *and Dumpy AVROS La Me selfed.*

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