EVALUATION OF INTER-SIMPLE SEQUENCE REPEAT (ISSR) MARKERS FOR GENETIC MAPPING OF AN OIL PALM INTERSPECIFIC HYBRID MAPPING POPULATION

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ABSTRACT

*Inter-simple sequence repeat (ISSR) markers were evaluated for mapping onto the genetic linkage map of an oil palm interspecific hybrid mapping population (*Elaeis oleifera × E. guineensis*). The genetic linkage of the markers was determined using the two-way pseudo-testcross strategy and the parental map was constructed using JoinMap 4.0. A total of 36 informative ISSR markers consisting of 20 single-primers and 16 double-primers were genotyped and generated 41 scorable polymorphic bands. Of these 41 polymorphic markers, 39 segregated in the* E. guineensis *male parent (T128) and only two segregated in the* E. oleifera *female parent (UP1026). As there were insufficient number of markers generated for UP1026, only the linkage map for T128 could be constructed. Twenty-eight of the ISSR markers were successfully mapped on the T128 parental map together with the existing RFLP, AFLP and SSR markers. Overall, 25 linkage groups covering a total map length of 2402.6 cM with an average interval of 7.1 cM were constructed in this study. Eight ISSR markers were incorporated into six of the 18 linkage groups, while 20 ISSR formed seven small groups separately. The ISSR markers were mostly mapped at the distal ends of linkage groups, regions which were generally not accessible by the other marker systems. This study demonstrated that ISSR markers are useful in combination with other markers for genetic mapping in oil palm. The ISSR primers with CT repeats developed from the dinucleotide 3'- anchored sites also showed the highest level of polymorphism. Furthermore, the development of ISSR markers is comparatively simple, fast, cost-effective and shows reproducibility, thus revealing its potential for genetic analyses of oil palm.*

Keywords: inter-simple sequence repeat (ISSR), oil palm interspecific hybrid, genetic linkage map.

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INTRODUCTION

Oil palm is a perennial crop that consists of two species; the African *Elaeis guineensis* Jacq. and the *Elaeis oleifera* Cortez of South American origin. *Elaeis guineensis* is an economically important crop compared to *E. oleifera,* which is less important, due to its low oil content. The oil to bunch ratio in *E. oleifera* is only about 5% compared to more than 25% normally observed in *tenera* (*E. guineensis)* (Rajanaidu *et al*., 2000). The poor yield of *E. oleifera* is due to its large proportion (65%) of parthenocarpic fruits (Sharma, 2005). However, the *E. oleifera* palm has interesting traits such as slow height increment and resistance to certain diseases (Hardon and

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Tan, 1969). Another important attribute is the oil content which has unique fatty acid composition, high iodine value (Singh *et al*., 2009) and high carotene content (Mohd Din *et al.,* 2004). In order to introgress the important characteristics of *E. oleifera* into the *E. guineensis* planting materials, interspecific hybridisation has been utilised by breeders, producing viable offsprings with the aim of improving the oil yield, oil quality, iodine value and also to develop short and compact palms (Chin *et al*., 2003; Escobar and Alvarado, 2003).

The efforts in developing and evaluating oil palm interspecific crosses face many challenges, among which include the long life cycle of 10 to 12 years (Oboh and Fakorede, 1989) and huge resources (*e.g*. land and labour) required for the conventional breeding programmes. The limitations may be eased by implementation of marker-assisted selection (MAS) which will facilitate selection at an early stage of development thus reducing the time to develop hybrids with desirable traits. One of the pre-requisities for MAS is to develop a molecular marker based-genetic linkage map and mapping of markers associated with traits of interest.

The development of specific and arbitrarily primed markers for PCR assays has facilitated the generation of DNA markers which aid in the genetic map construction by targeting specific genomic regions (Michelmore *et al*., 1991). Simple sequence repeats (SSR) or microsatellites (Staub and Serquen, 1996; Gupta and Varshney, 2000) are used to detect polymorphism at or near the repetitive regions. Other types of commonly used PCR-based markers are random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). These markers come with limitations such as low reproducibility (RAPD), expensive cost (AFLP) and require prior knowledge of the target flanking sequences to develop primers (SSR) (Reddy *et al*., 2002).

In this study, ISSR markers are evaluated to overcome the technical limitations observed in other PCR-based marker systems (Gupta *et al*., 1994; Wu *et al*., 1994; Zietkiewicz *et al*., 1994). The technique involves amplification of the DNA fragments between neighbouring or; opposite-oriented SSR loci (Zietkiewicz *et al*., 1994) using a single or double primers. The primers range from 16 - 25 bp containing SSR motifs of di-, tri-, tetra- or penta nucleotide repeats with 5′ or 3′ anchored base(s). ISSR is technically inexpensive, simple, fast, requires a small quantity of DNA and no prior primer sequence information is required (Casasoli *et al*., 2001; Reddy *et al*., 2002; Lakshmanan *et al*., 2007). In fact, ISSR markers have also been proven useful in tagging specific traits such as the restorer gene in rice (Akagi *et al*., 1996) and the *Fusarium* wilt resistance gene in chickpea (Ratnaparkhe *et al*., 1998a). Although ISSR are dominant markers, they reveal a reasonable rate of polymorphism, multi-banding profiles and highly reproducible. These characteristics make ISSR a good choice for genetic mapping studies. They helped in extending and saturating linkage maps in larch (Arcade *et al*., 2000) and *Citrus* (Sankar and Moore, 2001).

In oil palm, ISSR markers can be equally useful in accessing the polymorphism or genetic variation since the repetitive DNA are abundantly distributed throughout the genome (Cheah *et al*., 1995). A number of marker systems have been applied in the genetic mapping of oil palm. Moretzsohn *et al*. (2000) constructed a linkage map from a single controlled cross using RAPD. Later, Billotte *et al*. (2005) reported high density linkage map based on AFLP and SSR markers. The map represents the first high density linkage map with 16 independent linkage groups in line with the 16 haploid chromosomes in oil palm. The genetic maps have also been proven useful in mapping markers linked to the shell thickness gene and QTL for yield components (Billotte *et al*., 2010). Singh *et al*. (2009) also reported on a linkage map for an interspecific hybrid population (O×G) using AFLP, RFLP and SSR markers. The map consisted of 21 linkage groups which have revealed markers associated with QTL for iodine value (IV) and fatty acids composition (palmitic, myristic, stearic, oleic, linoleic and palmitoleic acids). The efforts to find markers more closely linked to the genes of interest are still on-going and the linkage analysis will be greatly assisted by additional molecular markers that can help to saturate the map. In this study, the usefulness of ISSR as an additional marker system to further saturate the O×G genetic linkage map was evaluated.

MATERIALS AND METHODS

Plant Materials and Genomic DNA Preparation

A total of 117 hybrid palms from an interspecific mapping population were used in this study. The cross was created between a Colombian *E. oleifera* palm (UP1026) as female parent and a Nigerian *E. guineensis tenera* (T128) as the male parent. The mapping population was planted at United Plantation, Teluk Intan, Perak, Malaysia (Singh *et al*., 2009).

ISSR-PCR Analysis

A total of 112 ISSR primers were used, of which 100 primers were obtained from the University of British Colombia Biotechnology Laboratory, Vancouver, Canada (UBC set #9) and 12 additional degenerate primers were custom-designed by Invitrogen™ based on Phoon (2009). The initial screening of a subset of the mapping population

(12 palms inclusive of both parents) was carried out by using the single primers. The informative primers were used to genotype the 117 palms. In addition, ISSR double primers were also used by combining two informative single primers in a PCR reaction. The PCR amplification was carried out in a 15 μl reaction mixture containing 10X PCR standard buffer, 0.2 mM dNTP, 1.0 U *Taq* DNA polymerase (all New England Biolabs® Inc, UK) with 50 ng μl^{-1} genomic DNA, 0.6 μM primer(s) and deionised distilled water. The amplification was performed in a Thermal Cycler GeneAmp® PCR System 9700 (Applied Biosystems, USA) at an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C (for 45 s), 30 s at T_a (for double primers, T_a used was 55°C), extension at 72°C for 2 min and a final 7 min of extension at 72°C. Amplification products were analysed by electrophoretic separation on 2% agarose gel at 100 V for 3 hr. The bands were visualised by staining with ethidium bromide using FlouChem software (Alpha Innotech Imaging System, USA). The size of each band was determined using the 1 kb DNA ladder (GeneRuler, Fermentas™, USA). For each primer, PCR analysis was carried out in duplicate to ensure the consistency and reproducibility of the results.

Data Analysis and Map Construction

Only DNA bands that could be scored clearly were used for data analysis. As ISSR markers are dominant, polymorphic loci were determined by the presence or absence of a band in one parent and not in the other. Segregation ratios observed among progeny were tested using the chi-square test for goodness-of-fit to the expected ratio ($P < 0.05$). The ISSR markers were analysed together with the AFLP, RFLP and SSR markers reported previously (Singh *et al*., 2009) to produce an updated linkage map. The construction of linkage map was carried out using JOINMAP 4.0 programme (Van Ooijen, 2006) with the criteria as described by Singh *et al*. (2009). The markers were grouped by using independence LOD evaluation ranging from 2.8 – 6.6 and were ordered into their respective groups using the regression mapping algorithm. The Haldane mapping function was used for map distance calculation. The final linkage map was drawn using MAPCHART 2.1 (Voorrips, 2002).

RESULTS

Polymorphism of Single and Double Primed ISSR in Oil Palm

Preliminary screening of 112 ISSR primers had identified 20 informative primers. The 20 informative primers generated 24 polymorphic bands, of which 23 were derived from T128 and one from UP1026. All the informative primers and sequences are listed in *Table 1*. The detected polymorphism rates were ranked based on the repeat motifs: 29.2% in CT repeats, 16.7% in $(AC)_n$, 12.5% in $(GT)_n$, 8.3% in each of $(GA)_n$, $(CA)_n$, $(TC)_n$ and $(AG)_n$ and 4.2% in each of the $(TG)_n$ and $(GACA)_n$ motif. The results showed that most of the polymorphic primers (95.8%) contained dinucleotide repeats. This study also found that the 3'-anchored primers that generated the most number of polymorphic bands contained dinucleotide repeats. These dinucleotide primers proved to be the most robust in the current ISSR analysis of oil palm. In contrast, no polymorphic band was observed in tri-, penta- repeats primers and the 5'-anchored ISSR primers. ISSR double primer was generated by combining two informative single primers in a PCR reaction. In the preliminary screening, a total of 190 primer-combinations were tested and 16 proved to be informative. Genotyping these informative primers generated 17 polymorphic bands, which mainly (16 bands) segregated in T128 and one in UP1026 (*Table 1*).

Integration of ISSR Markers into the T128 Parental Linkage Map

A total of 39 ISSR markers were found segregating in the male *E. guineensis* (T128) gametes and only two markers in the female *E. oleifera* (UP1026) gametes. This agreed with the previous study (Singh *et al*., 2009) that the male parent was more heterozygous than the female parent. The markers (in the present and previous study) were insufficient for construction of the UP1026 map. The ISSR markers were combined with the data set comprising of 503 AFLP, RFLP and SSR markers that were previously generated by the Malaysian Palm Oil Board (MPOB) and used to construct the T128 map. Prior to linkage analysis, markers with significant distortion ($P < 0.0001$) or with more than 12 missing data points were removed.

The T128 genetic map was constructed with 337 markers distributed into 25 linkage groups (LG) as illustrated in *Figures 1a, 1b* and *1c*. The marker order in every LG was set at its best estimated position. The total map length was 2402.6 cM with an average distance between two adjacent markers of 7.13 cM. The map consisted of 13 large LG (120.0 – 189.8 cM), nine midsize LG (36.1 – 93.1 cM) and three small LG (10.4 – 11.7 cM). Of the 39 analysed ISSR, 28 (72%) combined well with other types of markers (42 RFLP, 214 AFLP and 53 SSR). Eight ISSR markers (2.37%) were mapped in LG 6, LG 12, LG 13, LG 14, LG 17 and LG 18 that already had AFLP, RFLP and SSR markers. Twenty other ISSR markers (5.93%) mapped separately into seven small LG with sizes ranging from 10.4 – 58.6 cM and labelled as ISSR 1 – 7.

TABLE 1. INFORMATION ON THE INTER-SIMPLE SEQUENCE REPEAT (ISSR) MARKERS SELECTED FOR LINKAGE MAPPING IN THE *E. oleifera* × *E. guineensis* INTERSPECIFIC CROSS

ISSR primer	Sequence (5′ - 3′)	T_a (°C)	Polymorphic bands (bp)	
			E. oleifera **(UP1026)**	*E. guineensis* **(T128)**
Single primer				
UBC811	GAGAGAGAGAGAGAGAC	53		240
UBC814	CTCTCTCTCTCTCTCTA	53		550
UBC815	CTCTCTCTCTCTCTCTG	53		200
UBC818	CACACACACACACACAG	53	400	330
UBC822	TCTCTCTCTCTCTCTCA	52		1500
UBC825	ACACACACACACACACT	50		750
UBC827	ACACACACACACACACG	54		450
UBC828	TGTGTGTGTGTGTGTGA	50		750
UBC836	AGAGAGAGAGAGAGAGYA	53		200 & 450
UBC840	GAGAGAGAGAGAGAGAYT	55		200
UBC843	CTCTCTCTCTCTCTCTRA	55		450
UBC845	CTCTCTCTCTCTCTCTRG	51		1600
UBC849	GTGTGTGTGTGTGTGTYA	54		625 & 870
UBC850	GTGTGTGTGTGTGTGTYC	54		450
UBC853	TCTCTCTCTCTCTCTCRT	53		1100
UBC856	ACACACACACACACACYA	55		650
UBC857	ACACACACACACACACYG	54		1100
UBC873	GACAGACAGACAGACA	52		260
ISSR12	CTCTCTCTCTCTCTCTYG	55		1300 & 1700
ISSR13	CTCTCTCTCTCTCTCTYA	55		375
Double primers				
UBC811×UBC849	$(GA)_8C \times (GT)_8YA$	55		230
UBC811×UBC850	$(GA)_8C \times (GT)_8YC$	55		200
UBC811×UBC873	$(GA)_8C \times (GACA)_4$	55		450
UBC814×ISSR13	$(CT)_8A \times (CT)_8YA$	55		375
UBC815×UBC822	$(CT)_8G \times (TC)_8A$	55		300
UBC815×UBC843	$(CT)_8G \times (CT)_8RA$	55		450
UBC818×UBC825	$(CA)_8G \times (AC)_8T$	55		780
UBC818×UBC840	$(CA)_8G \times (GA)_8YT$	55		450
UBC822×ISSR12	$(TC)_8A \times (CT)_8YG$	55		1300
UBC825×UBC836	$(AC)_8T \times (AG)_8YA$	55	240	
UBC825×UBC856	$(AC)_8T \times (AC)_8YA$	55		650
UBC825×UBC873	$(AC)_8T \times (GACA)_4$	55		630
UBC827×UBC856	$(AC)_8G \times (AC)_8YA$	55		900
UBC836×UBC873	$(AG)_8YA \times (GACA)_4$	55		490
UBC843×UBC857	$(CT)_8RA \times (AC)_8YG$	55		430 & 1060
UBC856×ISSR13	$(AC)_8YA \times (CT)_8YA$	55		690
	Total polymorphic bands		**2**	**39**

Note: B=(C,G,T)(not A); D=(A,G,T)(not C); H=(A,C,T)(not G); N=(A,G,C,T); R=(A,G)(not T,C); V=(A,C,G)(not T); Y=(C,T)(not G,A).

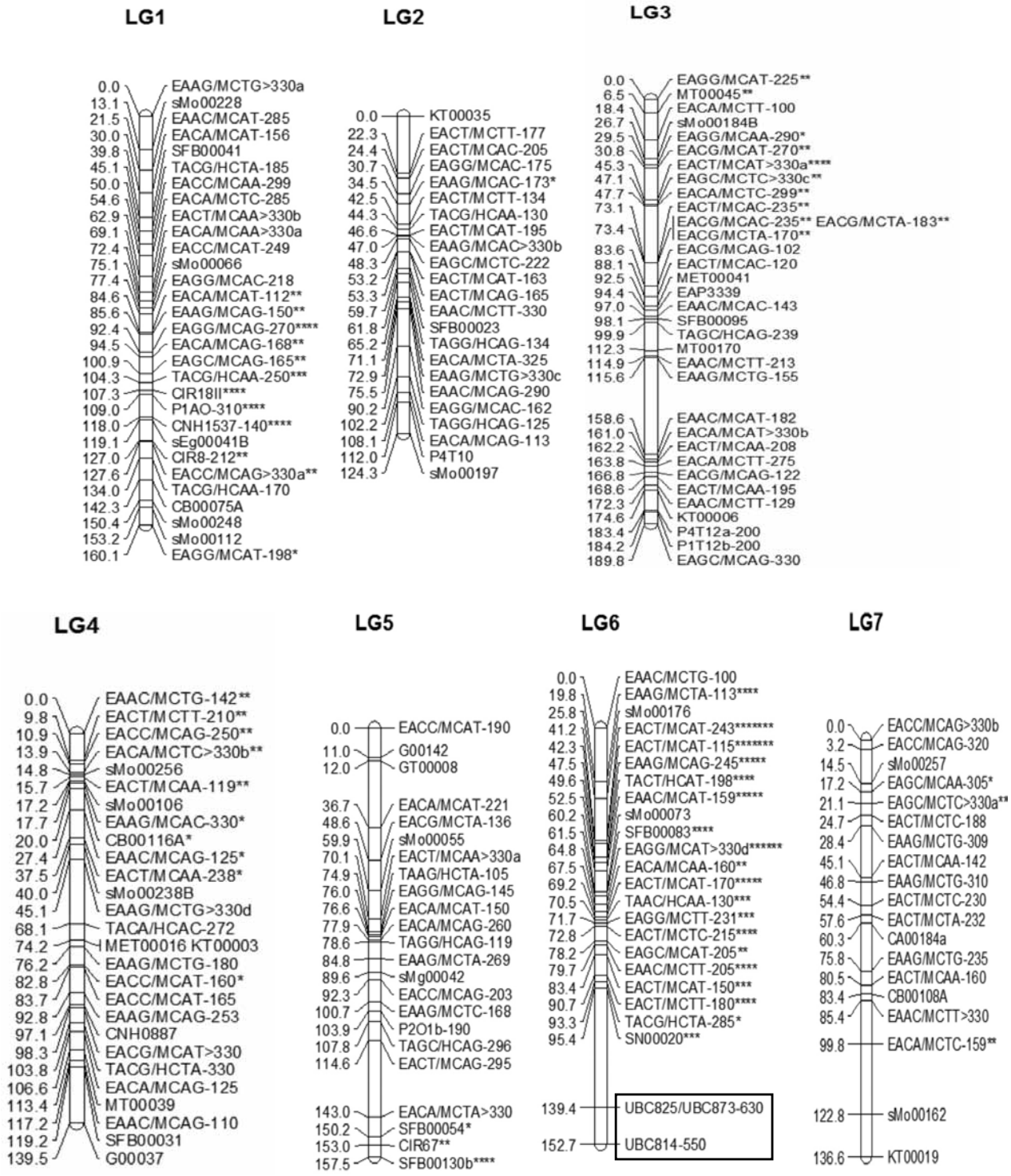

*Figure 1a. The T128 linkage map consisting of amplified fragment length polymorphism (AFLP), RFLP, simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers (indicated in box). Linkage groups (LG) are labelled as 1 – 7. Skewness of markers indicated by asterisk: * (P<0.1); ** (P<0.05), *** (P<0.01), **** (P<0.005), ***** (P<0.001), ****** (P<0.0005)*

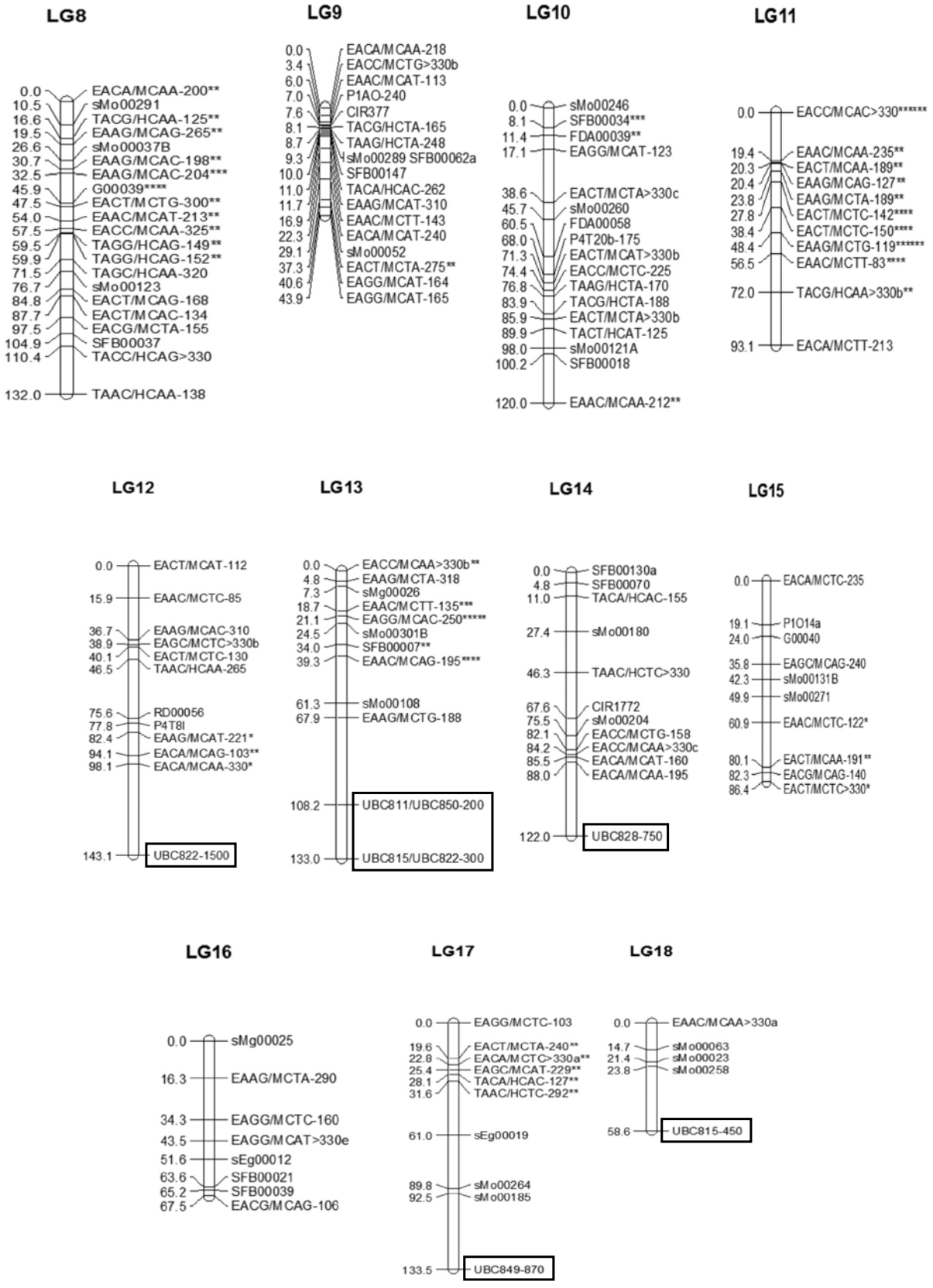

Figure 1b. Linkage groups (LG) 8 – 18 of the T128 map consisting of amplified fragment length polymorphism (AFLP), RFLP, simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers (indicated in boxes).

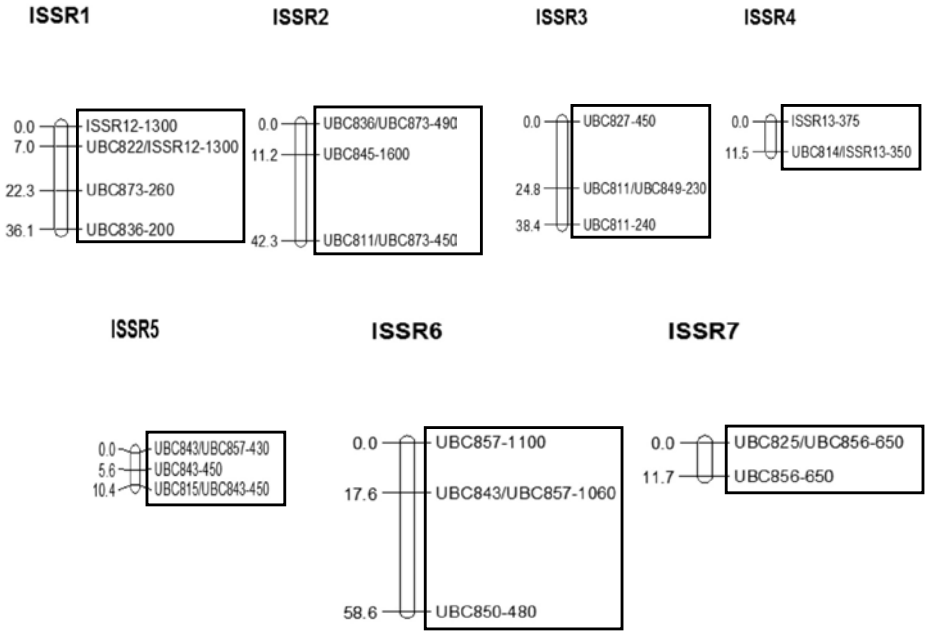

Figure 1c. Seven small linkage groups (ISSR1 – 7) of T128 consisting of inter-simple sequence repeat (ISSR) markers only (indicated in boxes).

DISCUSSION

In this study, the inheritance of ISSR markers in an oil palm interspecific hybrid mapping population was evaluated. The CT repeats were found to be abundantly present in the oil palm genome. This observation is in agreement with Singh *et al*. (2007) that both oil palm species, *E. guineensis* and *E. oleifera* were rich in GA/CT repeats. However, there was no amplification product generated for AT/TA motifs in the present study. The primers with AT/TA and GC/CG repeat motifs did not produce any amplification or variation probably because the motif sequences had high tendency to form dimer or 'hair-pin' structures. This is the likely explanation why AT repeats, which are abundant in plants (Fang and Roose, 1997; Cekic *et al*., 2001; Reddy *et al*., 2002) did not show any amplification.

Generally, ISSR primers with dinucleotide repeats such as AG, GA, CT, TC, AC and CA showed high polymorphism rate compared to the tri-, tetra- or penta- repeats. In the study of rice germplasm, ISSR primers from AG and GA repeats were reported to be informative in determining the genetic relationships among diverse accessions (Olufowote *et al*., 1997; Garland *et al*., 1999; Davierwala *et al*., 2000; Joshi *et al*., 2000; Sarla *et al*., 2003; 2005) and had been useful in the breeding programmes (Reddy *et al*., 2002). In the present study, AG and GA repeats were also found to be reasonably informative, while a total of 16.7% of the polymorphic bands were derived from $(AC)_n$ motif. Similarly, Nagaoka and Ogihara (1997) reported that AC repeats generated many polymorphic bands in wheat. In the study of *Phaseolus* genus, Hamann *et al*. (1995) reported that the $(GATA)_n$ and $(GACA)_n$ were better distributed in *P. vulgaris* and *P. lunatus* compared to $(CA)_n$ motif. However, in this study, the polymorphism revealed by $(CA)_n$ was higher than the $(GACA)_n$ motif.

The level of polymorphism revealed by ISSR also depends on the types of primers either non-anchored, 3'-anchored or 5'-anchored. Anchoring with single-, two-, three- or four-bases at the 3'- or 5'- termini is to allow annealing at the microsatellite end in the DNA template thus preventing internal annealing and smeary amplicons. The anchor allows only a subset of the repeat sequences to serve as priming sites. The 3'-anchored primers generated the most polymorphic bands because it could detect short repeat motif sequences better than the 5'-anchored primers. Similar results were also reported in other studies (Fang and Roose, 1997; Sankar and Moore, 2001; Yeboah *et al*., 2007). However, further analysis is needed on additional 5'-anchored ISSR primers before a definitive conclusion can be made for oil palm. This is because, Zietkiewicz *et al*. (1994) reported that a broader specificity is obtained with 5'-anchored primers compared to 3'-anchored primers. The differences between 5'- and 3'-anchors could also be based on the number of anchor bases present in the primer sequences. Most of the 3'-anchors tested in the

current study contained single and two-bases, while the 5'-anchors were three-bases which may have contributed to differences in amplification.

There are studies which report that ISSR markers reveal higher polymorphism compared to RFLP and RAPD (Zietkiewicz *et al*., 1994; Kojima *et al*., 1998; Ratnaparkhe *et al*., 1998a, b). However, in this study, ISSR markers generated lower polymorphism compared to other types of markers. This is probably due to the choice of primers or repeat motifs targeted in this study. Another possible explanation could be due to the fact that the *E. oleifera* parent in the hybrid cross appeared to be very homozygous. In other studies, the lack of polymorphic markers could also be due to the fact that only a small region of the genome had been explored (Mulcahy *et al*., 1993; Wolff and Peter-Van Rijn, 1993).

The generation of additional polymorphism using the ISSR double primer can increase the ability to differentiate between closely-related genotypes and simultaneously minimise the necessity to screen more primers. The main purpose of using double primers in this study was to identify additional ISSR polymorphic markers. However, the utilisation of double primers from two single primers in one PCR reaction provides additional room for the generation of PCR artifacts. This is due to the competition between primers, different effects of elongation on the chosen T_a or the formation of heteroduplexes between primers (Cekic *et al*., 2001). In this study, artifacts were avoided as much as possible by carrying out two independent PCR analysis of the same set of samples, and only consistent banding profiles were scored. However, generally the level of polymorphism observed using double primer is lower than that generated by single ISSR primer design.

ISSR markers were further evaluated by mapping into the *E. guineensis* (T128) genetic linkage map that had been reported previously by Singh *et al*. (2009) using AFLP, RFLP and SSR markers. In plants, ISSR markers proved useful for map saturation in crops such as barley (Becker and Heun, 1995), *Einkorn* wheat (Kojima *et al*., 1998) and *Citrus* (Sankar and Moore, 2001). In this study, stringent criteria were applied in map construction and appropriate LOD had been applied to incorporate the ISSR markers into the LG. The T128 map provides the best placement for the ISSR markers. The marker order of the previous map was fairly preserved with minimal changes in some instances due to the integration of ISSR markers into the LG. The tendency to lose the original markers order was lower in LG with smaller number of markers, as also observed by Sankar and Moore (2001). Most of the ISSR markers mapped at the end of the LG where recombination was low, probably reflecting the difficulties in positioning the ISSR markers. This also most likely resulted in seven LG consisting of ISSR markers only (*Figure 1c*). Nevertheless, the inclusion of ISSR markers at the end of these LG resulted in large gaps (> 34 cM) as observed in LG 6, 12, 13, 14 and 17.

As oil palm consists of n=16 chromosomes, the number of LG in the T128 map was higher than the expected 16 groups despite a large number of markers used. Among other studies that found similar results were in *Pisum sativum*, *Avena sativa*, European and Japanese larch and *Cicer arietinum* (Weeden *et al*., 1996; Laucou *et al*., 1998; Arcade *et al*., 2000; Loridon *et al*., 2005; Weising *et al*., 2005). The small sample size of the F_1 progeny used in this study probably contributed to this problem. Another possible factor was the insufficient number of polymorphic markers present in particular chromosomal regions of the T128 genome (Singh *et al*., 2009). Adding more polymorphic markers could help to unite the smaller groups and merge them into the expected n=16. According to Weising *et al*. (2005), the exceeded number of LG in *Cicer arietinum* could be due to the presence of large gap between markers. It is expected that some of the LG would merge when a wider range and larger number of markers are used to develop a more detailed linkage map.

Based on the present oil palm map, approximately 29.7% of the markers showed segregation distortion which is slightly higher than reported previously (21%) by Singh *et al*. (2009) but much higher than the 10% reported by Billotte *et al*. (2005) for a *E. guineensis* cross. The distortion rate is almost similar to that reported for coffee (30%) (Ky *et al*., 2000). The LG 3, 6 and 11 contained high number of distorted markers (indicated by asterisks*) (*Figures 1a, 1b* and *1c*). Clustering of these distorted markers were mostly at the centre and towards the end of LG. This could be due to the reduction of recombination rate around centromere and some telomeric effects (Debener and Mattiesch, 1999). The distortion was most pronounced for AFLP which had also been observed in other species such as potato and tomato (Tanksley *et al*., 1992), cocoa (Risterucci *et al*., 2000) and rubber (Lespinasse *et al*., 2000). However, Winter *et al*. (2000) was of the opinion that segregation distortion was not marker type dependent, but a reflection of the region the markers resided on. Thus, the differences in the segregation behaviour of the various marker types could be due to the different genomic regions where they were located. Segregation distortion frequently occurs in interspecific populations, probably due to differences in the genes controlling reproduction processes (Zamir and Tadmor, 1986) or the meiotic drive in gametes or zygotes (Lyttle, 1991; Buckler *et al*., 1999). According to Plomion *et al*. (1995), other reasons that could contribute to the segregation distortion include statistical bias, genotyping and scoring errors, and biological causes (*e.g.* chromosome loss, viability and genetic load) (Bradshaw and Stettler,

1994). Segregation distortion can cause problems in map-based cloning because it reflects suppression of recombination at specific genomic regions. This can lead to underestimation of the physical distance between desirable genes and markers located next to it (Winter *et al*., 2000).

CONCLUSION

In this study, although the ISSR markers showed low polymorphism, it certainly contributed to the existing linkage map. The results could be further improved by additional polymorphic ISSR and other markers such as single nucleotide polymorphism (SNP). The saturated linkage map could help to further facilitate the detection of QTL for fatty acid composition and other agronomic traits in the oil palm for purposes of MAS.

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