Genetic Diversity Study of *Elaeis guineensis* germplasm using EST-SSRs

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ABSTRACT

MPOB collection of germplasm has covered 11 countries for Elaeis guineensis and these germplasm have been used as genetic resources for development of oil palm breeding programmes.The objectives of this study are to determine the potential use of EST-SSRs markers to study the genetic variability of the germplasm collections. A total of 330 palms originating from 11 countries in Africa were screened using 10 EST-SSRs primers. Data was analyzed using Biosys-1 software to calculate the genetic variability parameters. It was found that the germplasm collections exhibited high level of genetic diversity. Most of the loci tested were 100% polymorphic at 0.95 criterion. A total of 46 alleles were detected across all the germplasm collection. Of these, 3 were considered as rare alleles. Nigeria germplasm showed highest number of alleles per locus and highest number of rare alleles, high percentage of polymorphic loci and high heterozygosity suggesting that Nigeria could be the centre of diversity of wild oil palm. This study also revealed that Madagascar germplasm are unique and different compared to the oil palm populations from the African mainland. Based on the dendrogram constructed, the germplasm collections could be divided into 3 clusters; cluster 1 consisting of Angola, Tanzania, Cameroon, Nigeria, Zaire, Sierra Leone, Guinea and Ghana germplasm, cluster 2 consist of Gambia and Senegal germplasm and the Madagascar germplasm was positioned in cluster 3.

INTRODUCTION

Malaysia currently has the largest collection of oil palm germplasm in the world. Since 1973, Malaysia has collected *Elaeis guineensis* germplasm from 11 countries and *Elaeis oleifera* germplasm from 7 countries (Rajanaidu, 1994a; Rajanaidu and Jalani, 1994).

The objectives of these collections are to broaden the genetic base of the current oil palm breeding materials and to ensure conservation of wide range of oil palm genetic resources for posterity. At present, the *Elaeis guineensis* and *Elaeis oleifera* genetic materials and other economic palms from Africa and Central-South America are maintained in the form of field genebank to safeguard the long term interest of the Malaysian oil palm industry. For conservation purposes, the major issue with respect to oil palm field genebank is the large space needed and high maintenance cost.

Study of genetic diversity using molecular markers among the MPOB oil palm germplasm is important for establishment of a core collection (germplasm with minimal repetitive and maximal genetic diversity).The estimated genetic distance determined using molecular marker data may prove useful in developing appropriate sampling strategies for conservation and also strategies for future collection.

SSRs (also known as microsatellites) are regions of DNA consisting of short, tandemly repeated units (1-6 bp in length) found within the coding or noncoding regions of all eukaryotic organism. The polymorphic level of these markers depends on the variation in the number of repeat units. SSRs are co-dominantly inherited, multiallelic, reproducible, have good genome coverage and are relatively abundant. SSR markers are widely used for purposes of DNA fingerprinting, paternity testing, linkage map construction and population genetic studies. The first report of microsatellites in plants was made by Condit and Hubbel (1991) and Gupta *et al.,* (1999) suggesting their abundance in plant systems.

Genomic SSR markers, developed from genomic DNA libraries, can correspond to either the transcribed region or the nontranscribed region of the genome and thus not very useful in identifying candidate genes. In addition, genomic SSR are frequently not transferable to closely related species (Roder *et al.* 1995; Sourdille *et al*. 2001). In contrast, EST-SSR markers which are sequenced portions of complementary DNA copies of mRNA- represent part of the transcribed region of the genome. The conserved nature of transcribed region may limit their polymorphism, thus EST-SSR have been reported to be less polymorphic compared with genomic SSRs in crop plants (Rajeev et al. 2005). However, they have some advantages over genomic SSRs especially in genetic diversity studies for germplasm collections because of their higher level of transferability to related species. They can often be used as anchor markers for comparative mapping and evolutionary studies (Rajeev *et al*. 2005). These markers enable the variation in express sequences to be assayed and often have putative functions. These gene targeted markers have the potential of representing functional markers, which can enhance the role of genetic markers in germplasm evaluation. In several plant species including oil palm, EST-SSR have been identified, developed and used in a variety of studies. They have proven useful in plant systems such as grape (Scott et al. 2000), sugarcane (Cordeiro *et*

al. 2001), rye (Hackauf and Wehling, 2002), barley (Thiel *et al.* 2003), wheat (Nicot *et al*. 2004), and coffee (Valerie *et al*. 2006).

The objectives of this project was to study the ability of oil palm derived EST-SSRs in discriminating the various oil palm germplasm collections and to estimate the genetic variability parameters obtained using this marker system.

MATERIALS AND METHODS

Samples: EST-SSR analysis was performed on germplasm collections from 11 countries, as follows;

a) Cameroon
b) Ghana
c) Zaire
d) Tanzania
e) Madagascar
f) Gambia
g) Guinea
h) Nigeria
i) Senegal
j) Sierra Leone
k) Angola

Thirty palms were selected from different populations for each of the country above. Spear leaves were harvested from each sampled palm for DNA extraction

DNA extraction

DNA extraction was carried out using the modified CTAB method (Dellaporta *et al*.1983). Two grams of fresh leaf tissue was ground to fine powder in the presence of liquid nitrogen using mortar and pestle. The powder was then transferred into 30 ml Falcon tube mixed with 20 ml CTAB Buffer and incubated at 60^0C for 30 minutes. An equal volume of chloroform/isomyl alcohol (24:1 v/v) was added and centrifuged at 10,000 rpm for 15 minutes. The supernatant was pipetted into sterile tubes and two volumes of isopropanol was added to precipitate DNA. The DNA was pelleted at 12,000 rpm for 15 minutes. The DNA pellet was washed twice with 70% ethanol containing 10mM ammonium acetate and dissolved in TE buffer (10mM Tris, 1mM EDTA pH 8.0). The DNA was then treated with RNAse (50µg/ml) at 37^0C for 20 minutes. One-tenth volume of 7.5 M ammonium acetate and two volumes of absolute ethanol was added to precipitate the DNA. DNA was spun down at 12,000 rpm for 15 minutes. The resultant DNA pellet was washed twice with 70% ethanol and dissolved in 1 ml of TE buffer.

DNA quantification and qualification

DNA concentration was determined using a spectrophotometer by measuring the absorbance at λ = 260.0 nm, 280.0 nm and 350.0 nm. The optical density (OD) ratio at 260/280 was calculated to determine the DNA purity and OD at λ 260.0 and 350.0 were

used to calculate the DNA concentration. Digestibility test was carried out using *Eco*R1 and *Hae*III restriction enzymes.

SSR analysis

The EST-SSR markers used in this study were developed in MPOB. They are CNH 00938, CNH 0887, CNH 1617, CNI 01937, EAP 03160, EO 02978, MF233-033, CNIP 00421, MF233-056 and CNI 01733. In primer labelling, γ-^{33}p was used to detect amplified fragments. The forward primer was 5' end labeled at 37 ^{0}C for 1 hour and 30 min using T4 polynucleotide kinase. The labeling reactions contained 15 pmoles of primer, 0.1 µl of γ-^{33}p, 1 U of T4 polynucleotide kinase in a total volume of 1.0 µl. PCR reaction consist of 1 U of *Taq* Polymerase, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM dNTPs and 10 X PCR buffer. An aliquot of DNA was diluted to the concentration of 50 ng/µl and used in the SSR analysis.

PCR condition

The following PCR programme was used; predenaturation at 95°C for 1 minutes, denaturation at 95°C for 30 seconds, annealing at 52°C (except for CNH1617 primer, at 54°C) for 30 seconds, and extension at 72°C for 30 seconds. This programme was repeated for 35 cycles, followed by a final extension at 72°C for 5 minutes.

Analysis on Acrylamide Gel

PCR product was mixed with an equal volume (10 µl) of formamide dye (0.3% bromophenol blue, 0.3% xylene cyanol; 10 mM Diaminoethanetetra-acteic acid (EDTA) pH 8.0; 97.5% deionized formamide). Prior to loading and electrophoresis, the samples were heated for 3 minutes at 95^{0}C and then rapidly cooled on ice. The gel was prepared by mixing 100ml of 6% polyacrylamide (20:1 acrylamide:bisacrylamide) containing 7.5 M urea and 1M Tris-borate-EDTA (TBE). 100µl of 10% ammonium persulphate and 20µl of TEMED were also added. 5µl of the heat-treated samples were electrophoresed at constant power of 1600V for 3 hours. The gels were then vacuum dried for one hour and exposed against X-ray film for 3-4 days at –80^{0}C.

Data analysis

Amplified DNA fragments were scored and each band within each locus was identified according to alphabetical order. The most anodal migrating band designated as allele A, and the next band was allele B. Data were scored and analysed using Biosys1 software to calculate genetic variability parameters such as mean sample size per locus, mean number of allele per locus, percentage of polymorphic loci, observed and expected heterozygosities (Nei, 1978). The genetic distance and construction of dendrogram was carried out as described by Roger, 1972.

RESULTS AND DISCUSSIONS

Forty six bands were generated from 10 primers used in this study (Table 1). The highest number of alleles were observed using primers MF233056, CNH00938 and CNIP00421 (seven alleles). This was followed by MF233033 (six alleles), CNH01617 (five alleles), CNH00887 (four alleles), CNI01733 and CNI01937 (three alleles), EAP03160 and EO02978 (two alleles).

TABLE 1: NO. OF ALLELES PRODUCED BY SSR PRIMERS USED IN THE STUDY

No.	Primer	No. of alleles produced	Name of Alleles
1	EAP03160	2	A, B
2	EO02978	2	A, B
3	CNH00887	4	A, B, C, D
4	CNH01617	5	A, B, C, D, E
5	MF233033	6	A, B, C, D, E, F
6	MF233056	7	A, B, C, D, E, F, G
7	CNI01733	3	A, B, C
8	CNI01937	3	A, B, C
9	CNIP00421	7	A, B, C, D, E, F, G
10	CNH00938	7	A, B, C, D, E, F, G
	Total	46	

According to Brown (1978) alleles can be classified as either common (allele frequency more than 0.100 for at least one sample) or rare (never exceed frequency more than 0.100). Common alleles are divided into three classes, namely widespread (occurrence in more than two regions) or sporadic (occurrence in two regions) or localized (occurrence in only one region). Rare alleles can be classified as either widespread (occurrence in more than one region) or localized (occurrence in only one region). Result of this screening showed that of the 46 alleles, 43 alleles were common, of which 40 (87.0%) were common-widespread, 1 (2.2%) was common-sporadic and 2 (4.3%) were common-localized. Of the 3 rare alleles detected, 2 were rare-widespread and 1 was rare-localized. Table 2 summarizes the occurrence of rare alleles in the oil palm germplasm collections.

TABLE 2: RARE ALLELES DETECTED IN THE OIL PALM GERMPLASM COLLECTIONS INCLUDED IN THE STUDY

Germplasm	Rare allele/Locus		
	A/CNH00887	B/CNH00938	G/CNH00938
Nigeria	√	√	√
Ghana	√	√	
Guinea	√	√	
Sierra Leone	√	√	
Tanzania	√	√	
Zaire	√	√	
Angola	√		
Cameroon	√		
Gambia		√	
Senegal		√	

Three rare alleles were detected that is in locus CNH00887 (allele A) and CNH00938 (allele B and G). The rare alleles occured in all germplasm collections except for the collection from Madagascar. Allele A/CNH00887 was detected in eight germplasm collections and the exceptions being Gambia, Senegal and Madagascar germplasm. Allele B/CNH00938 also occurred in eight germplasm collections and the exceptions were Angola, Cameroon and Madagascar collections. G/CNH00938 was found only in the Nigeria germplasm. The Nigeria germplasm possess all the rare alleles detected in the study.

Table 3 summarizes the estimates of genetic variability parameters for the germplasm analysed in this study. All the loci were 100% polymorphic (0.95 criterion) for the germplasm analyzed except for the materials from Madagascar and Gambia, which the alleles were 90% and 70% polymorphic respectively. The mean percentage of polymorphism was high (96.4%). The mean number of alleles per locus (A) is 3.7 ranging from 2.7 (Gambia) to 4.2 (Nigeria).The mean expected heterozygosity (H_e) was 0.503 ranging from 0.363 (Gambia) to 0.582 (Tanzania). The mean observed heterozygosity (H_0) was 0.398 with values ranging from 0.162 (Madagascar) to 0.513 (Angola). The highest heterozygosity (H_e) was observed in Tanzania (H_e = 0.582) followed by Zaire (H_e = 0.574) and Nigeria (H_e = 0.569). The lowest genetic diversity was observed in collections from Gambia (H_e = 0.363) followed by Madagascar (H_e = 0.372) and Ghana (H_e = 0.480).

TABLE 3: ESTIMATES OF GENETIC VARIABILITY PARAMETERS FOR 11 OIL PALM GERMPLASM COLLECTIONS

Germplasm	Mean sample size per locus	Mean no. of allele per locus	Percentage of loci Polymorphic*	Mean Heterozygosity	
				Direct-count (H_o)	Hardy-Weinberg expected(H_e)
1. Nigeria	29.5	4.2	100.0	0.490	0.569
2. Cameroon	30.0	3.8	100.0	0.503	0.556
3. Zaire	30.0	3.8	100.0	0.477	0.574
4. Tanzania	30.0	3.9	100.0	0.460	0.582
5. Madagascar	29.6	3.7	90.0	0.162	0.372
6. Angola	30.0	3.5	100.0	0.513	0.537
7. Senegal	30.0	3.6	100.0	0.387	0.495
8. Gambia	30.0	2.7	70.0	0.327	0.363
9. Sierra Leone	30.0	4.0	100.0	0.343	0.490
10. Guinea	29.8	4.0	100.0	0.341	0.515
11. Ghana	29.5	3.8	100.0	0.376	0.480
Mean	29.85	3.7	96.4	0.398	0.503

* A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95

The polymorphism level (P) revealed by the EST-SSRs was high that is 100% for most of the germplasm except for Madagascar and Gambia germplasm. This result is in agreement with that observed using RFLP analysis (Maizura *et al.* 2006), where the lowest level of polymorphism was reported in populations from Madagascar and Gambia. EST-SSR markers showed a higher level of polymorphism compared to those found using RAPD (P = 88.6%) (Rajanaidu et al. 2000), AFLP (P = 93.4%) (Kularatne, 2000), isozyme, (P =54.5%) (Hayati, 2002), RFLP, (P =56.3%) (Maizura *et al.* 2006). Screening of oil palm germplasm using genomic SSR markers also indicated high level of polymorphism among the germplasm populations, P = 93.1% (Bakoume, 2006). The oil palm germplasm collection showed the high values of A and P indicating that they have high genetic variability which can be used for improvement programmes.

Germplasm from Tanzania, Zaire, Nigeria, Cameroon and Angola exhibited the highest heterozygosity. Heterozygosity refers to the fraction of loci within a palm that are heterozygous. This indicated that these collections are a rich source of genes that can be used for oil palm improvement. These collections may possess unique genes associated with traits which may not be found in the current oil palm planting materials. The current germplasm collection has indeed proved useful in developing new and improved planting materials. For example, the planting materials known as PS1 (dwarf with high yield)(Rajanaidu 1994b), PS2 (high iodine value with high yield)(Rajanaidu 1994c) and PS3 (high kernel content)(Rajanaidu 1996) were developed from selected palms from Nigeria. Selected palms from Tanzania were introduced as thin-shelled *tenera* (PS5) (Kushairi *et al.* 2003a) and high bunch index breeding populations (PS7) (Junaidah *et al.* 2004). The Angolan palms with large fruit *dura* were selected as PS6 (Kushairi *et al.* 2003b). Individuals from Tanzania and Angola collections are being used in the long stalk breeding programme, namely as PS10 (Noh *et al.* 2005). Besides that, materials from Tanzania also showed high carotene content and the Cameroon x Zaire progenies show tolerance to Ganoderma. High vitamin E content is observed in materials from Zaire, Tanzania, Angola, Cameroon and Nigerian.

Chi-square test was employed to determine the differences between values of the observed heterozygosity and Hardy-Weinberg heterozygosity. The observed heterozygosity values showed no significant difference to the Hardy-Weinberg expectation (in H-W equilibrium), except for Guinea and Madagascar collections. With respect to the Madagascar collection, only a limited number of populations were available for sampling in this study. These populations probably only carry part of the total alleles present in samples from Madagascar, explaining the Hardy-Weinberg deviation observed. As for the Guinea samples, the deviation from Hardy-Weinberg was surprising but could be caused by the relatively small sample size analyzed in this study. Generally the high heterozygosity obtained in this study as well as that by Bakoume (2006) indicate the adaptability of oil palm germplasm analyzed to various environments and its large distribution area.

Germplasm from Gambia, Madagascar and Ghana exhibited low heterozygosity. Kathleen *et al.* (2002) stated that isolated populations of widespread species can serve as models of the likely effects of decreased population size due to habitat fragmentation.

Generally the populations near the center of a species range are contiguous and genetically diverse, whereas marginal populations are isolated, smaller, and less genetically variable. In case of Madagascar which is isolated from the mainland, the limited gene flow among populations may contribute to their low heterozygosity. Maizura *et al* (2000), Rajanaidu *et al*. (2000) and Hayati *et al.* (2004) also reported low genetic heterozygosity for Madagascar germplasm. However, Kularatne (2000) reported high heterozygosity in populations from Madagascar. The dissimilarity observed could be due to the different marker techniques employed. There are two possible mechanisms operating in small and isolated populations that could influence the level of heterozygosity in Gambia and Madagascar germplasm. In small populations, genetic drift may occur whereby allele frequency changes more rapidly than that in large populations. In small populations, there is a limited number of individuals that represent the alleles in the gene pool. The absence of some individuals will directly affect the allele frequencies. The limited number of individuals in a small population may also increase tendency of imbreeding (mating among related individuals). Exchange of common alleles results in low heterozygosity. In the study, populations from Ghana also showed low heterozygosity, although the country in situated at the Central and West Africa. Similar result was reported by Kularatne (2000) and Hayati *et al.* (2004). As elaborated by Hartley (1988), the human activities of collecting alcoholic beverage from oil palm have caused tremendous effort of felling oil palm in the wild and establishment of cocoa plantation had caused massive forest clearing and wiping out wild oil palms.

The Nigeria oil palm germplasm collection exhibited high level of polymorphism, high (He), highest mean number alleles per locus (A) and number of rare alleles suggesting that this collection may likely be the centre of diversity for oil palm. The fossil finding similar to oil palm pollen in the Miocene and Niger delta further supports the suggestion. Similar results were reported by Maizura *et al.* (2006) and Hayati (2002). In addition, Nigeria is located in Central Africa and may have experienced massive gene flow from palms in neighboring countries, thus explaining high level of genetic diversity observed.

In the genetic distance study, Biosys1 software was used to calculate distance matrix (Table 4) and construct the dendrogram (Figure 1). The mean genetic distance across the MPOB germplasm collections was 0.230. The lowest genetic distance was observed between Tanzania and Zaire germplasm (0.096). Similar observation was reported by Hayati (2000). This could be due to the fact that Tanzania and Zaire are neighbouring countries. Madagascar and Guinea which are separated by long geographic distance showed the highest genetic distance (0.502).

The dendrogram constructed using Biosys 1 software revealed three genetic groups (Figure 1). The largest cluster, Cluster 1 consists of Angola, Tanzania, Zaire, Cameroon, Nigeria, Sierra Leone, Guinea and Ghana germplasm. In the Cluster 1, there are 2 sub-clusters: sub-cluster 1 consists of Angola, Tanzania and Zaire germplasm. Sub-cluster 2 consists of Nigeria, Cameroon, Sierra Leone, Guinea and Ghana germplasm. Gambia and Senegal germplasm were grouped in Cluster 2 whereas Madagascar germplasm formed a separate cluster (Cluster 3).

TABLE 4: MATRIX OF GENETIC DISTANCE (ROGER, 1972) AMONG MPOB GERMPLASM COLLECTIONS

	Population	1	2	3	4	5	6	7	8	9	10	11
1	ANGOLA	***										
2	CAMEROON	.204	***									
3	GAMBIA	.351	.391	***								
4	GHANA	.269	.249	.354	***							
5	GUINEA	.250	.245	.408	.235	***						
6	MADAGASCAR	.401	.438	.418	.441	.502	***					
7	NIGERIA	.239	.143	.369	.218	.233	.398	***				
8	SENEGAL	.303	.337	.261	.264	.313	.401	.293	***			
9	S.LEONE	.262	.214	.338	.267	.204	.455	.188	.301	***		
10	TANZANIA	.155	.227	.317	.274	.249	.389	.229	.281	.241	***	
11	ZAIRE	.155	.184	.300	.254	.250	.406	.218	.271	.253	.096	***

Distance

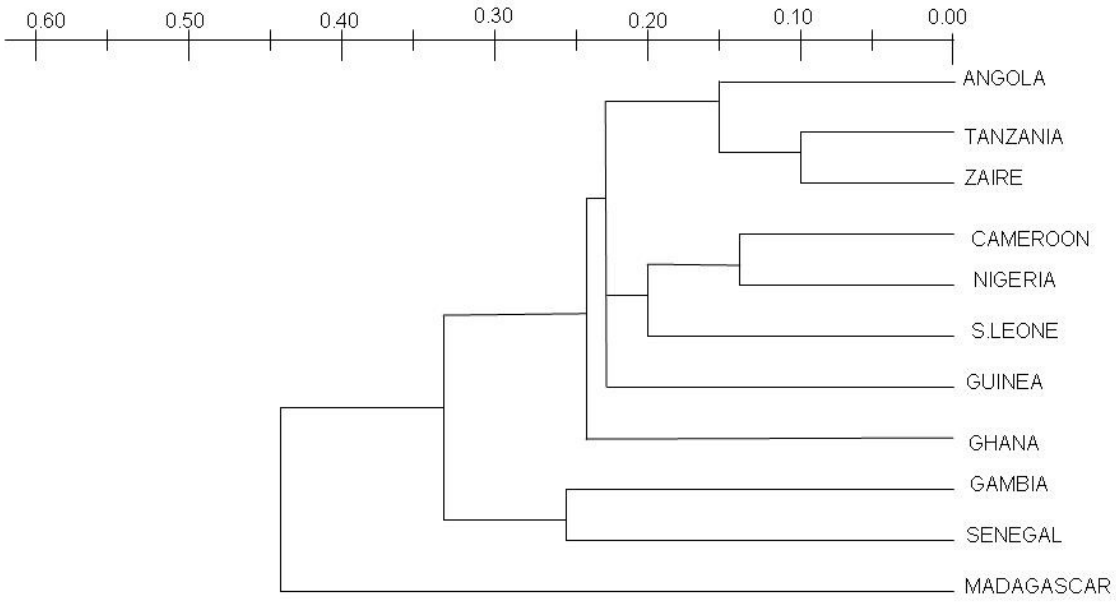

Figure 1: *Dendrogram constructed based on Roger's distance (Roger, 1972) for 11 MPOB oil palm germplasm collections included in the study*

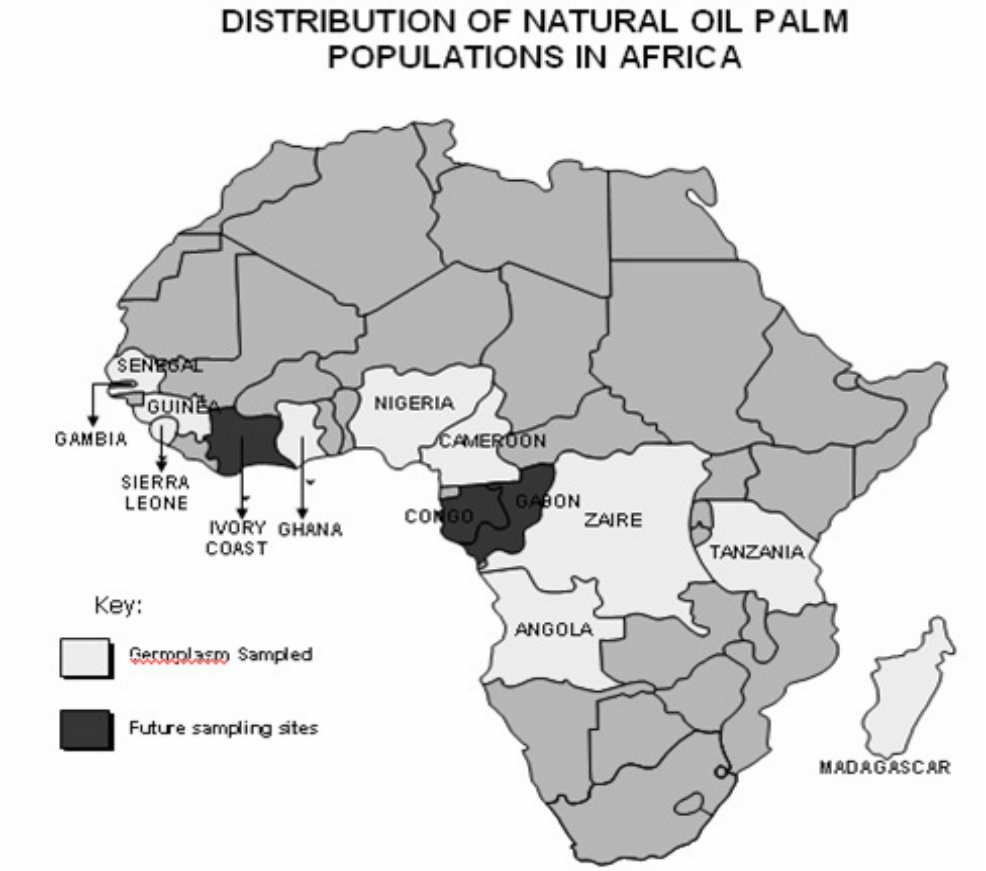

Figure 2: *Map of Africa indicating the distribution of oil palm germplasm*

There is a strong association between genetic distance and geographical location. With the exception for Guinea and Sierra Leone, populations from Central Africa (Nigeria, Cameroon, Angola, Tanzania, Zaire and Ghana) formed one cluster. Similar observation was also reported by Shah *et al*. (1994), Rajanaidu *et al.* (2000) and Maizura *et al.* (2000). It was surprising because Guinea and Sierra Leone (located in West Africa) were grouped together with the materials from Central Africa. However, Zeven (1967) considers the main oil palm belt to begin in Guinea spreading south through Sierra Leone, Ghana and other Central Africa countries and this may explain the genetic similarity between these countries. The grouping of Gambia and Senegal in Cluster 2 reflect the fact that Gambia is actually a subset of Senegal. Gambia is entirely surrounded by Senegal and gene flow may occur between the populations of the two countries contributing to higher genetic similarity. The Madagascar germplasm formed single region cluster implying the unique characteristic of the palms as compared to those in the mainland. Palms from Madagascar are generally short with high iodine value and high linoleic acid (C18:2) oils (Rajanaidu *et al.* 2000).

Study of genetic variation within MPOB germplasm collection or natural population is crucial for effective conservation and exploitation of genetic resources. Introgression

study can be done using genetic materials from germplasm to advanced planting materials for crop improvement programs. Evaluation of germplasm with SSRs derived from ESTs is useful for breeders in the selection of germplasm palm to be conserved in core collection development. Mohammadi *et al*. (2003) has reported on the usefulness of molecular markers for assessment of genetic variation in germplasm collections. EST-SSRs have some advantages compared to genomic SSR and other markers such as RFLP and AFLP because of their potential as markers representing genes linked to traits of agronomic interest. This has important applications in diversity and phylogenetic analysis of germplasm collections. However, the functions of the SSR motif and genes that contain SSRs in the plant genome need to be studied further by localizing the ESTs with traits of interest on genetic linkage maps. EST- SSR markers also have been useful for integrating phenotypic and genotypic variation and have provided breeders and geneticists with an efficient tool to link both characteristic (Gupta *et al*. 2000).

CONCLUSION

The result of this study indicates the usefulness of EST-SSR markers in revealing genetic variability and relationship among MPOB germplasm collection. The study will be extended in the future to estimate genetic variability and relationship for individual populations in each of the germplasm collections. This will help breeders to identify redundancies in the collection and develop strategies for field conservation. Selection and conservation should take into account the oil palm populations with high rare alleles and H_e as these populations may posses interesting and unique genes to be exploited further.

The 10 EST-SSR primers used in this study successfully differentiated and clustered the germplasm collections from the various countries. The estimated genetic variability parameters for germplasm collections were obtained and high genetic diversity was observed among the MPOB germplasm collections.

The high genetic diversity showed in this study revealed that the germplasm collections are good sources for new genes related to novel traits which can be introgressed into the current planting materials. New genes introgressed will increase the genetic variation and may contribute many valuable economic traits.

Based on the estimated genetic parameters (P, A and H_e) it can be concluded that generally, natural African oil palm population assayed maintained high levels of genetic variation. The high percentage of polymorphism loci, highest mean number of alleles per locus, high H_e and number of rare alleles was obtained among the populations from Nigeria. This suggests that these populations may represent the centre of diversity of wild oil palm.

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