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Identification and Utilization of Polymorphic SSR Markers for Genetic Diversity Studies in Oil Palm

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A B S T R A C T

Oil palm (*Elaeis guineensis* Jacq.) having chromosome number 2n=32 and belongs to the family Arecaceae and it is a rich source of perennial vegetable-oil in India. To sustain the edible oil requirement in the country, oil palm is one of the best options due to its high oil-yield (4-6 t/ha) potential compared to other annual oil-yielding crops. Polymorphic simple sequence repeat (SSR) markers play an important role in genetic diversity and mapping studies in a crop like oil palm. In the present study, total eight genotypes were screened using 110 SSR markers. With of these, 42 were found to be polymorphic and 68 were monomorphic and the number of alleles ranged from two to six. The highest Polymorphism Information Content (PIC) value was observed with the primer, mEgCIR0779 (0.76), while the lowest with mEgCIR3288 (0.11), at an average value of 0.38. Genetic diversity ranged from 0.12 (mEgCIR3288) to 0.79 (mEgCIR0779), with an average value of 0.45. Based on PIC and other genetic parameters, four highly-polymorphic markers, *viz.*, mEgCIR0779, mEgCIR0782, mEgCIR2347 and mEgCIR2595 were identified. The identified polymorphic SSR loci can be effectively used in mapping and genetic diversity studies of oil palm crop improvement programme. Totally 42 polymorphic SSRs identified and grouped the eight genotypes into two major clusters and the clustering pattern observed was that based on geographical origin.

Introduction

Oil palm (*Elaeis guineensis* Jacq.) known for its rich source of perennial vegetable-oil in India and it belongs to the Arecaceae family with chromosome number 2n=32. To meet the edible oil requirement of the country, it is one of the best option due to its high oil yield (4-6 t/ha/year) potential as compared to other annual oil yielding crops (MaryRani, 2015). The oil palm produces five times more oil per hectare per year than the annual oil yielding crops. It has 16 pairs of chromosomes with a genome size of 1.8 Gb (Singh *et al.*, 2013).

Oil palm is highly heterozygous in nature, genetic studies aimed at improving the efficiency of oil palm cultivation. Evaluating genetic diversity and characterizing oil palm germplasm plays a crucial role in the genetic improvement of oil palm.

Genetic diversity among germplasm can be measured using morphological, biochemical and molecular techniques (Mohamadi and Prasanna, 2003). Morphological variables have been routinely employed to evaluate genetic diversity among NIFOR oil palm breeding programme (West, 1976, Okwuagwu *et al.*, 2008). Morphological markers are not sufficiently reliable due to low polymorphism, vulnerability to environmental factors and confounding effect of plant developmental stage (Smith and Smith, 1992). Molecular markers are markers of choice duo to their repeatability, high polymorphism, not influenced by environmental factors and developmental stages of plants (Zane *et al.*, 2002). Molecular marker technique includes Isozyme markers (Protein based) and DNA based markers. DNA based markers classified into Hybridization based (RFLP) and PCR based (RAPD, SSR and AFLP). In isozyme analysis, separation of different forms of an enzyme is based on charge. It is having inherent disadvantages like limited number of enzyme loci and developmental and seasonal dependent enzyme expression. The most reliable markers are those based on DNA; these dependent on distinctive structure of the genetic material, and have largely replaced protein markers in genetic studies (Corley and Tinker, 2003).

Molecular markers offer great scope for assessing genetic diversity and relationship among natural population because they are impervious to environmental conditions and are detectable in all stages of plant growth and developments (Mondini *et al.*, 2002). Among the likely alternatives, isozymes are not satisfactorily variable due to low polymorphism ((Purba *et al.*, 2000 and Ghesquiere, 1985). Random amplified polymorphism DNA (RAPD) has also been examined (shah *et al.*, 1994), but poor reproducibility of amplification products limits their generalization in genetic diversity studies (Rafalski, 1997). Other more robust molecular markers such as Restriction fragment length polymorphic DNA (RFLP) (Maizura *et al.*, 2006) are complex: requiring relatively large amount of purified and high molecular weight DNA, time consuming and laborious. Finally, Amplified fragment length polymorphism (AFLP) is a dominant marker which rarely detect heterozygosity and is scored as a presence/absence polymorphism. Molecular markers have been used for different applications like genetic diversity, genotype identification, QTL mapping and marker assisted selection. Assessment of the genetic variation and diversity in oil palm has been carried out based on RAPD (Shah *et al.*, 1994; Rajanaidu *et al.*, 2000; Satish and Mohan Kumar, 2007), AFLP (Kularatne, 2002), Isozymes (Hayati *et al.*, 2004) and RFLP (Maizura *et al.*, 2006). Among these, PCR based SSRs are widely used in any crop improvement programmes and also in oil palm germplasm. Very few efforts have been made in India for genetic diversity among the Indigenous germplasm using molecular markers (Satish and Mohan Kumar, 2007). The data also provide sufficient evidence for identifying each variety, dura, pisifera and tenera separately as well as the parental dura and pisifera together. For the first time to check the level of variability in oil palm varieties DNA based polymorphism assay was performed (Satish and Mohan Kumar, 2007). However, till now no reports on the extent of genetic diversity among the Indigenous oil palm germplasm. Identification of more polymorphic SSR markers is indispensable in utilizing them in genetic diversity, mapping and marker assisted selection programmes.

Hence in the present study we used 110 SSR markers for identification of polymorphic SSRs for their use in genetic diversity studies in selected genotypes. The present study conducted with the aim of identification of polymorphic SSR markers among a set of oil palm germplasm and genetic diversity analysis of the selected germplasm using polymorphic SSR markers.

Materials and Methods

Plant materials and DNA extraction

Total eight oil palm accessions were used and the details of the accessions were given in table 1. The leaflet of an unopened spear of oil palm in field gene bank was used for genomic DNA extraction (Gawel and Jarret, 1991).

SSR amplification using PCR

A set of 110 SSR markers were used for amplification in the eight selected genotypes of oil palm. The forward and reverse sequences of the primers were obtained from Billote *et al.,* (2005). Thermal reaction were carried out in a reaction mixture (20 µl) consisting of 10 X buffer (Himedia), 2 µl having 15 mm $MgCl_2$, 0.2 mM of each forward and reverse primer, 2 µl of 2 mMdNTPs, 0.2 µl of 1 U of Taq DNA polymerase (Invitrogen, USA) and about 25-50 ng of template DNA. The PCR amplifications were performed in a Thermocycler (Biorad, USA) programmed for an initial denaturation of 3 min at 95^{o}Cfollowed by 35 cycles of 30s at 95^{o}C, 30s of 50^{o}C annealing temperature, extension of 1 min at 72^{o}C, with a final extension of 10 min at 72^{o}C, and hold at 4^{o}C. The PCR products were fractioned on 3 % super fine resolution (SFR) agarose gel. The electrophoresis was carried at 100 volts for 3h at room temperature. Agarose gel stained with ethidium bromide and visualized using Bio-imaging system (Bio Rad) and scoring was carried out manually based on the size of the 100bp ladder. The statistical analysis of polymorphism and UPGMA analysis for generating dendrogram was done by using power marker v 3.0 (Liu and Muse, 2005). The PIC, heterozygosity, gene diversity, allele frequency and inbreeding co-efficient were calculated using power marker V3.0 software (Liu and Muse, 2005).

Results and Discussion

The genomic DNA of the oil palm genotypes (Table 1) were amplified using 110 SSR markers and yielded scorable bands. All the 110 SSRs were spread across all the chromosome of oil palm evenly. Out of the 110 primers, 42 (38.1 %) loci were found to be polymorphic, and detected 113 alleles with an average of 2.7 alleles per locus while 68 SSR loci (61.8 %) were monomorphic. The number of alleles generated with polymorphic primers ranged from 2 to 6 among the oil palm genotypes. The SSR loci mEgCIR0779 and mEgCIR0782 were found to have maximum number of alleles (6 and 5 respectively) followed by the mEgCIR2347 and mEgCIR0246, mEgCIR0243 and mEgCIR0192. The banding pattern representing the polymorphism of SSR loci were shown in Figure 2a and for SSR loci mEgCIR0779 and mEgCIR0792 in Figure 2b. The PIC value for all the polymorphic primers across eight oil palm genotypes varied from 0.11 to 0.76 with an average value of 0.38 demonstrating their ability to discriminate between individual accessions. The higher the PIC of the marker, the more informativeness of the marker. Out of 42 primers mEgCIR0779 shown highest PIC value of 0.76, followed by mEgCIR0782 (0.73), mEgCIR2347 (0.63) and mEgCIR2595 (0.64) and the lowest PIC value was observed in primers mEgCIR3286 (0.11) and SEG00166 (0.14) and followed by SPSC00033 (0.19) and mEgCIR0774 (0.19) (Table 3).

Table.1 Details of the eight oil palm indigenous accessions used in the study

Sr. No.	Genotypes	IC number	Place of collection
1	Nellore 1	IC0610025	Suryapalli, Nellore, Andhra Pradesh
2	TTD-1	IC0610027	Theni, Tamilnadu
3	AND-16	IC0610018	Krishna nallah, Andaman and Nicobar Islands
4	MANG-1	ICO610030	Sulia, Mangalore, Karnataka
5	MANG-6	IC0610032	Sulia, Mangalore, Karnataka
6	AND-24	ICO610024	Krishna nallah, Andaman and Nicobar Islands
7	NELLORE-2	IC00610026	Suryapalli, Nellore, Andhra Pradesh
8	MANG-2	IC00610031	Sulia, Mangalore, Karnataka

Table.2 SSR markers used to assess genetic diversity in eight oil palm indigenous genotypes

Sr. No.	SSR MARKER	Forward primer (5' -3')	Reverse primer (3'-5')	Annealing temperature (^{o} C)	Repeat Motif	Linkage group
1	SEG00113	GTCACCGAACCCTAATAAAAT	ATGCAGTTGAGGACAAAAAG			
2	mEgCIR0268	GCAACACCATTAGAGAGA	TCCATGCATCCAAACAG	52	(GA)12	1
3	mEgCIR0163	ATGCATGTGATTTTATTAGGTGAGA	CGACCCTCAGTCAATCAGTAAG	52	(GA)23	8
4	mEgCIR0246	GGTAAGAGATGAGATGGGTTGTC	AGGAATTAAGGGTTGTAGGTGAA	52	(GA)19	8
5	mEgCIR0243	TGGAACTCCTATTTTACTGA	GCCTCGTAATCCTTGTCA	52	(GA)17	10
6	mEgCIR0192	AAGCTAGCGACCTATGATTTTAGA	AAACAAGTAATGTGCATAACCTTTC	52	(GA)18	11
7	mEgCIR0037	CCAGTCTGCTAACCATCCTATAC	TCTCACTTCCTCCCCACATC	52	(GA)17	15
8	mEgCIR0177	TGAATGTGTGTGCAATGTGTAT	ATAGTCAATAATCGTAGGAAAATG	52	(GA)20	15
9	SMG00210	CTTTTCCCTCATCTCTGCTTC	CGTCTACCTTGTTTAGCTGTTGT	nil	nil	nil
10	SMG00217	GGTGGAATTAGTTGCTCAGAAG	CGCAGATGTTTCATAATCGAG	nil	nil	nil
11	SPSC00163	GGTGGAATTAGTTGCTCAGAAG	CGCAGATGTTTCATAATCGAG	nil	nil	nil
12	SPSC00033	ATGGTCCCGTCCTAGGATTT	AACAGCTTGCCTCCTTGGTA	nil	nil	nil
13	mEgCIR0894	TGCTTCTTGTCCTTGATACA	CCACGTCTACGAAATGATAA	52	(GA)18	7
14	mEgCIR0555	TACCATCACTGACCAATAAC	GTCTTTCTTGCTAACTACAC	52	(GA)18	8
15	mEgCIR0774	TGGCCGAGGCAGAAGAAAAT	GCTTGGTGGGTAAGCTGGATTATT	52	(GA)20	8
16	SMG00156	GGTGTCATAACTTCGTTGTTGCT	ATGCTCAAAAGTGGGTTTCTCTC	nil	nil	nil
17	SEG00166	CATGCGTCGTCAATAAATGG	TGCTACCAACAATCCAGAGAAG	nil	nil	nil
18	SMG00155	AACCCAACCCAATCAACATTAG	GACACAGATAAAAAGGTCCAG	nil	nil	nil
19	mEgCIR0886	GATCTGCCGGTGCTCCTA	CTCAGTTTAGTCGATCCTTCCATTG	52	(GA)9	8
20	mEgCIR0878	CAAAGCAACAAAGCTAGTTAGTA	CAAGCAACCTCCATTTAGAT	52	(GA)22	11
21	mEgCIR0465	TCCCCCACGACCCATTC	GGCAGGAGAGGCAGCATTC	58	(CCG)4	12
22	mEgCIR0790	TTGGTGGTCCTTTTGAATATC	ACAAACCCAGCACTTAAAATAAC	52	(GA)19	12
23	mEgCIR0779	AATGCAGACCAAGCTAATCATATAC	GTTCAGGTGATGGTGACTCAGATAG	52	(CA)11 (GA)22	14
24	mEgCIR0773	GCAAAATTCAAAGAAAACTTA	CTGACAGTGCAGAAAATGTTATAGT	52	(GT)7 (GA)8	15
25	mEgCIR0782	CGTTCATCCCACCACCTTTC	GCTGCGAGGCCACTGATAC	56	(GA)20	16
26	mEgCIR1713	GCTGAAGATGAAATTGATGTA	TTCAGGTCCACTTTCATTTA	52	(GTAT)3 (GT)12	1
27	mEgCIR2575	GGGACTTCGCAAACTGTAGCA	CGGTGGCGTATGGTGGATT	52	(GA)5	2
28	mEgCIR2347	ATTTTGCATGTGTTGAGAGC	CAACCAATTGCACCCTAAAG	52	(GA)15	3
29	mEgCIR2518	GATCCCAATGGTAAAGACT	AAGCCTCAAAAGAAGACC	52	(GT)6 (GA)32	3
30	mEgCIR2595	TCAAAGAGCCGCACAACAAG	ACTTTGCTGCTTGGTGACTTA	52	(GA)16	4
31	mEgCIR2813	GCTTTGTTGCAGTTTGACTA	GTTTAGGATGTTGCGTGAT	52	(GT)7 (GA)11	5
32	mEgCIR1773	ATGACCTAAAAATAAAATCTCAT	ACAGATCATGCTTGCTCACA	52	(CT)14 (GT)21	12
33	mEgCIR3286	GTTTATCATTTTGGGGTCAG	CGGTGTCCCTCAGGATGTA	52	(GA)19	4
34	mEgCIR3232	GTGAGCGATTGAGGGGTGTG	GGGGCTTGATTGAGTATTTCCA	56	(GA)9	4
35	mEgCIR3281	TTTCTTATGGCAATCACACG	GGAGGGCAGGAACAAAAAGT	52	(GA)17	6
36	mEgCIR3358	CCAAGGAACAACATAGA	GTTCCCATCCTATTAGAC	52	(GA)15	6
37	mEgCIR3383	AGCAAGACACCATGTAGTC	GACACGTGGGATCTAGAC	52	(GA)21	6
38	mEgCIR3293	ACAACCACAAGAGTCCTAAC	CTGCGAAATCATAAAAAGTA	nil	nil	nil
39	mEgCIR3111	TTTCTCATGGTGGGTAGGTG	TCAGATTGCGGTGGATGTAT	52	(GA)15	12
40	mEgCIR3328	GAGGGGGTTGGGACATTAC	TAGCTCACAACCCAGAATCTAT	52	(GA)22	8
41	mEgCIR3376	CCCTCCCTGCTACCTTCT	TTATGTGAGTGCCTTTGATG	52	(GA)19	8
42	mEgCIR3305	ACTTGCACCACTACTTCTAT	CTTTTAGGCATTCTCTTGTAG	52	(GA)15	9

Table.3 Parameters for genetic analysis of 42 SSR loci across the eight oil palm genotypes

SSR Marker	Major Allele Frequency	Allele Number	Gene Diversity	Heterozygosity	PIC
SEG00113	0.69	2.00	0.43	0.13	0.34
mEgCIR0268	0.86	2.00	0.24	0.00	0.21
mEgCIR0163	0.86	2.00	0.24	0.00	0.21
mEgCIR0246	0.69	2.00	0.43	0.13	0.34
mEgCIR0243	0.38	3.00	0.66	0.25	0.58
mEgCIR0192	0.44	3.00	0.63	0.38	0.56
mEgCIR0037	0.75	2.00	0.38	0.00	0.30
mEgCIR0177	0.44	3.00	0.65	0.38	0.57
SMG00210	0.50	2.00	0.50	0.33	0.38
SMG00217	0.63	3.00	0.53	0.00	0.47
SPSC00163	0.64	2.00	0.46	0.71	0.35
SPSC00033	0.88	2.00	0.22	0.00	0.19
mEgCIR0894	0.63	2.00	0.47	0.00	0.36
mEgCIR0555	0.50	3.00	0.55	0.88	0.46
mEgCIR0774	0.88	2.00	0.22	0.00	0.19
SMG00156	0.79	3.00	0.36	0.14	0.33
SEG00166	0.92	2.00	0.15	0.17	0.14
SMG00155	0.50	2.00	0.50	0.00	0.38
mEgCIR0886	0.58	2.00	0.49	0.50	0.37
mEgCIR0878	0.56	2.00	0.49	0.13	0.37
mEgCIR0465	0.56	2.00	0.49	0.13	0.37
mEgCIR0790	0.57	2.00	0.49	0.00	0.37
mEgCIR0779	0.31	6.00	0.79	0.63	0.76
mEgCIR0773	0.75	3.00	0.40	0.13	0.35
mEgCIR0782	0.29	5.00	0.77	0.43	0.73
mEgCIR1713	0.64	4.00	0.54	0.14	0.50
mEgCIR2575	0.75	3.00	0.41	0.25	0.37
mEgCIR2347	0.43	4.00	0.68	0.14	0.63
mEgCIR2518	0.75	4.00	0.41	0.38	0.39
mEgCIR2595	0.43	4.00	0.69	0.00	0.64
mEgCIR2813	0.83	2.00	0.28	0.00	0.24
mEgCIR1773	0.81	3.00	0.32	0.13	0.29
mEgCIR3286	0.94	2.00	0.12	0.13	0.11
mEgCIR3232	0.81	3.00	0.32	0.13	0.29
mEgCIR3281	0.86	2.00	0.24	0.00	0.21
mEgCIR3358	0.56	3.00	0.59	0.38	0.52
mEgCIR3383	0.50	3.00	0.62	0.13	0.54
mEgCIR3293	0.75	2.00	0.38	0.00	0.30
mEgCIR3111	0.81	3.00	0.32	0.13	0.29
mEgCIR3328	0.63	2.00	0.47	0.00	0.36
mEgCIR3376	0.69	2.00	0.43	0.13	0.34
mEgCIR3305	0.50	3.00	0.59	0.00	0.51

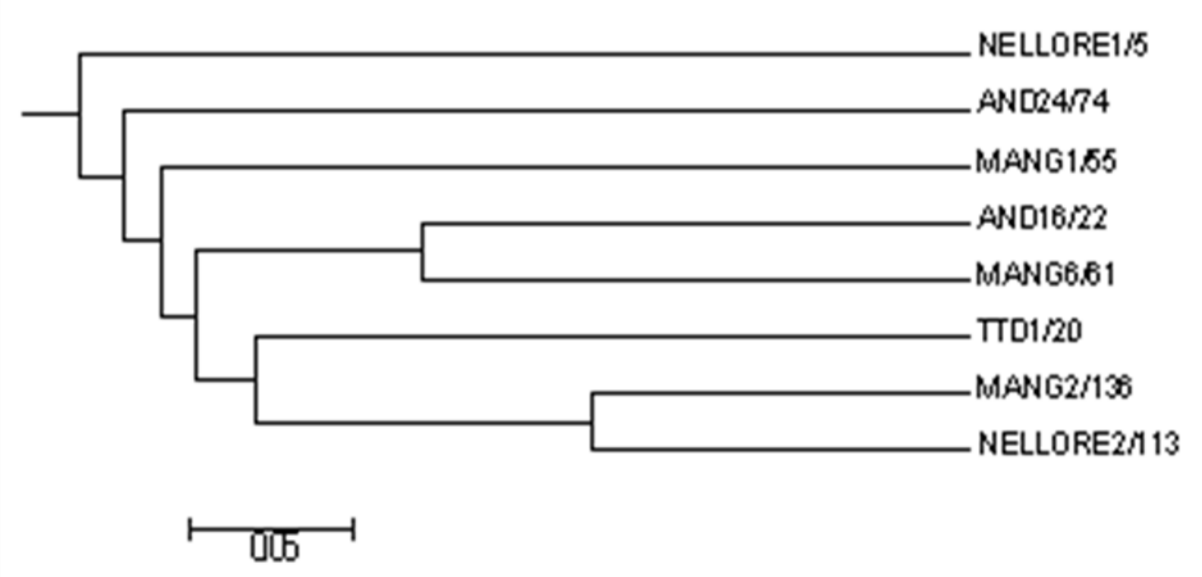


Fig. 1: The dendrogram of eight oil palm genotypes as obtained from POWER Marker software based on UPGMA analysis

Fig.2a The SSR banding profile of mEgCIR0246, mEgCIR0243 and mEgCIR0192 loci among the eight oil palm genotypes. M-Marker (100bp), lane (1-8) oil palm genotypes (for label please refer Table 1)

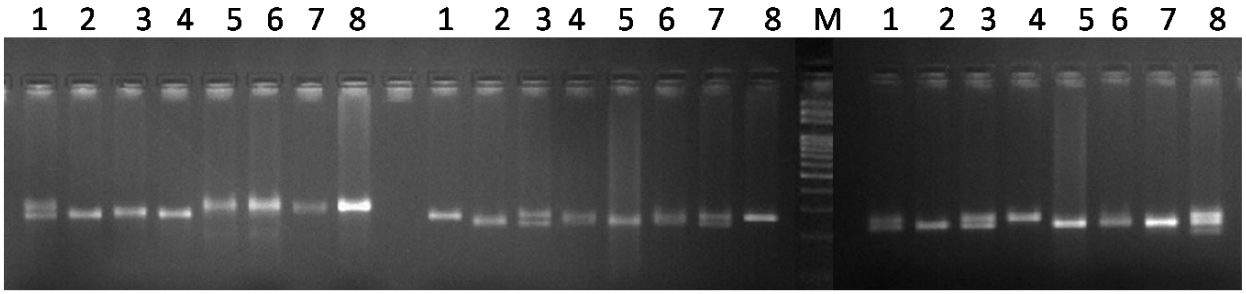
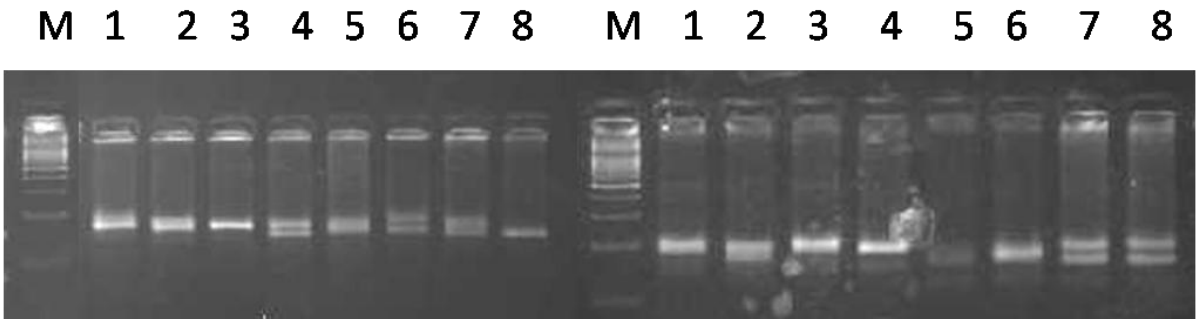


Fig.2b The SSR banding profile of mEgCIR0779 and mEgCIR0782 loci among the 8 oil palm genotypes. M-Marker (100bp), lane (1-8) oil palm genotypes (for label please refer Table 1)



Gene diversity also known as expected heterozygosity (He) was in the range of 0.12 (mEgCIR3286) to 0.79 (mEgCIR0779) with an average value of 0.45. The heterozygosity, known as observed heterozygosity (Ho) was observed with an average of 0.18 and range of 0.00 to 0.88 (mEgCIR0555). Major alleles with highest frequency were observed for the locus mEgCIR3286 (100 bp) at 94 % followed by the locus SEG00166 (100 bp) at 92%. The ability to provide distance measures between the genotypes that reflect pedigree relatedness ensures a more stringent evaluation of the adequacy of a maker profile data. The fact that minimum genetic distance revealed during the study is a good indication confirming the power of SSR markers to distinguish between geographically similar genotypes and closely related genotypes. The average gene diversity existing among all the genotypes were relatively high (45%), indicating existence of high levels of polymorphisms among the genotypes.

The dendrogram generated through UPGMA analysis grouped all the eight oil palm genotypes into two major groups. Nellore 1 genotype formed a separate cluster and other all *viz.* And-2, And-16, Mang-1, Mang-6 and Mang-2, TTD1 and Nellore 2 formed another cluster (Fig. 1).

Allelic frequencies at each locus varied from population to population and some alleles occurred only in one or some populations. In an out-crossing plant like oil palm where random mating is expected, genetic drift and reproductive isolation are the most common factors that affect allele frequencies (Bakoume *et al.,* 2009).

The expected heterzygosity of 0.79 was similar to 0.78 reported as high by Okoyo *et al.,* (2016a) for *Elaeis guineensis*. The high genetic diversity observed might have resulted from the out crossing behavior of oil palm as earlier reported on *Quercus petraea* (Cottrell *et al.,* 2003). In genetic diversity analysis He and Ho results are in close agreement with the findings reported among oil palm genotypes using SSR markers by Okoyo *et al.,* (2016a). They reported range of He and Ho of 0.167-0.778 and 0.153 to 0.643 respectively, from Nigeria and Malaysia. Okoyo *et al.,* (2016b) reported He of 0.70 and Ho of 0.69 with NIFOR oil palm germplasm. Out of 110 SSRs 42 were Polymorphic and 68 were monomorphic. The gel picture shows the banding pattern of polymorphic SSRs given in figure 2.

The number of SSR loci based on PIC value with more than the average was 16 in number. Among them, SSR loci mEgCIR0779, mEgCIR0792, mEgCIR2347 and mEgCIR2595 were noteworthy due to their relatively highly level of polymorphism. A total of 16 SSR loci came under the PIC range of 0.38-0.76 with an average value of 0.54 while 26 loci came within the PIC range of 0.11-0.37 with an average value of 0.29. The higher the PIC of the marker, the more informativeness of the marker. Out of 42 primers mEgCIR0779 shown highest PIC value of 0.76, followed by mEgCIR0782 (0.73), mEgCIR2347 (0.63) and mEgCIR2595 (0.64), the PIC obtained in this study is within the range of the previous studies in oil palm using SSR markers. Okoyo *et al.,* (2016a) obtained an extremely high mean percentage polymorphism (85.09 %) and Arias *et al.,* (2012) reported maximum PIC value with 0.822 in commercial oil palm material.

The dendrogram developed eight different genotypes into two major group, this may be due to same parentage might be involved in crossing of the oil palm breeding programme and these geographical zones might have derived from one or similar genetic background (s), similar results were found in Bakoume *et al.,* (2009) study in oil palm (*Elaeis guineensis*) natural population using SSR markers. Oil palm is highly heterozygous and it has originated from only four oil palm initially so same genes might have played role in this clustering pattern.

Oil palm is an important crop for vegetable oil and there is need of identification of polymorphic markers for identification of important QTLs in germplasm for further usage

in breeding programme for improving oil yield. In my study identified four highly polymorphic SSR markers (mEgCIR0779, mEgCIR0782, mEgCIR2347 & mEgCIR2595) based on the parameters like PIC value of ≥ 63, gene diversity of ≥ 68 & polymorphic alleles of ≥4. These polymorphic primers can effectively be used in further molecular breeding programs and QTL mapping studies of oil palm since they exhibited very high polymorphism over other loci in the oil palm breeding programme.

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