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ANALYSIS OF THE INHERITANCE OF AFLP MARKERS IN AN INTERSPECIFIC CROSS OF OIL PALM USING THE PSEUDO-TESTCROSS STRATEGY

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The newly developed technique of amplified fragment lengthpolymorphism (AFLP) was used to study the polymorphism and segregation of AFLP markers in an interspecific cross of oil palm. The technique revealed a high level ofpolymorphism between the Colombian oleifera *(female parent) and Nigerian guineensis (male parent) used to generate the cross. The segregation of AFLP markers was studied in a population of 77 F, individuals and analysed as a pseudo-testcross. Using only 10 primer pair combinations, we were able to amplify 674 bands out of which 91 show segregation. All markers detected were scored as dominant, and the segregation ratios indicated that majority of these markers (80%) were inherited in a Mendelian manner. The results also showed that the male parent (palm T128, a Nigerian* guineensis) *was* more *heterozygous than the female parent (UP1026, a Colombian* oleifera). *The AFLP technique was also found to be suitable for detecting contaminants and would therefore be useful for assessing the fidelity of controlled crosses.*

INTRODUCTION

Molecular markers are indispensable tools for generating genetic linkage maps. Linkage maps, in turn, serve many useful purposes such as in studying evolutionary relationships in related species (Bennetzen and Freeling, 1993) and for improved selection in breeding programmes (Kazan *et al., 1993).* They also provide starting points for the positional cloning of genes. For plants with large genomes such as oil palm, these maps must be of

sufficient resolution and marker density to be useful in crop improvement programmes. For this reason, construction of higher density maps is necessary. This requires the analysis of a large number of DNA markers by using techniques which can detect minor sequence differences.

The development of techniques such as restriction fragment length polymorphism (RFLP) (Tanksley et al., 1989) and random amplified polymorphic DNA (RAPD) (Williams et al., 1990) has generated useful tools that serve well as molecular markers. Co-dominant and locus specific RFLP markers are unequalled for many applications and have been successfully used in construction of detailed linkage maps in many plant species including oil palm (Mayes et al., 1997). RFLP mapping is often used to place cloned complementary DNAs (cDNAs) on genetic linkage maps. However, RFLP analysis requires the 'up-front' development of probe libraries and involves Southern blot hybridization – procedures that are time consuming and labour intensive. RAPD markers, on the other hand, are generated using short DNA primers and the polymerase chain reaction (PCR). RAPD markers, being easily generated, can be rapidly analysed using only small amounts of DNA. RAPD analyses do not require prior sequence information and can be viewed directly after gel electrophoresis. Since there is no need for specific probe libraries and radioisotope detection, this saves much time in comparison to the standard RFLP approach. Maps of RAPD markers have been reported for several plant species (Reiter et al., 1992; Grattapaglia and Sederoff, 1994). Unfortunately, repeatability of the RAPD technique has been a source of concern in many studies (Pillay and Kenny, 1996). This has, to a certain extent, hindered the usefulness of this marker system. Furthermore, RFLP or RAPD can only detect a few loci in each experiment.

The recent development of the technique of amplified fragment length polymorphism (AFLP) has introduced an efficient method for identification of large numbers of molecular markers (Vos et al., 1995). This technique has the capacity to assess a much larger number of loci than other currently available marker techniques. The AFLP technique is based on selective amplification of a subset of genomic restriction fragments using PCR. In assaying for AFLP, genomic DNA is digested with restriction endonucleases, ligated to adapters and amplified by PCR, using primers that contain the common sequences of the adapters and one to three arbitrary nucleotides as selective sequences. Although AFLP is a PCR based assay, all current evidence suggest that the technique is as reproducible as RFLP and does not suffer some of the shortcomings associated with RAPD. The reproducibility of AFLP protiles is, in fact, assured by the use of restriction site-specific adapters, adapter-specific primers and stringent amplification conditions (Vos et al., 1995).

High reproducibility, rapid generation and high frequency of identifiable AFLP polymorphism make AFLP analysis very useful for saturation mapping. In fact, the AFLP technique has been shown to identify more markers in soyabean than RFLP and RAPD (Lin et *al.*, 1996). This technique has been used successfully to generate sufficient markers rapidly to construct linkage maps in potato (van Eck et al., 1995) and rice (Nandi et al., 1997). The technique has also been shown to have potential in mapping quantitative traits (Powell *et* al., 1997) and in marker assisted breeding (Vos et *al.*, 1995). Before making extensive use of AFLP markers in linkage map construction in the oil palm, the suitability and inheritance of these markers from parents to offspring need to be evaluated. It was thus, the intention of the present study to determine the suitability of the AFLP technique in the development of markers for mapping the oil palm genome. The segregation patterns and inheritance of AFLP bands in an oii palm mapping population derived from an interspecific cross were studied.

MATERIALS AND METHODS

Plant Material

A mapping population of 77 F_1 palms derived from the cross UP1026 x T128 were analysed. The female parent, UP1026, is a Colombian *oleifera* and the male parent, T128, is a Nigerian *guineensis* with high iodine value (I.V.).

Preparation of Genomic DNA

DNA was prepared from young spear leaves by the method of Doyle and Doyle (1990).

AFLP Procedure

The AFLP assay was carried out using the GIBCO BRL AFLP Analysis System 1 essentially as described in the manufacturer's manual, with some minor modifications.

Three hundred and fifty ng of genomic DNA was digested with 3.2µl of EcoRI and *Mse*I (1.25 units μl^{-1} each) at 37°C for 4hr. After heat inactivation of the enzymes at 70°C, the fragments were ligated to the EcoRI and *Mse*I adapters in the presence of T4 DNA ligase at 20°C for 3hr. A preselective amplification was then carried out by amplifying a lo-fold dilution of the ligation mixture.

For selective amplification, a selected EcoRI primer (with three selective nucleotides) was labelled with γ^{33}P-ATP using T4 polynucleotide kinase. The labelled EcoRI primer was mixed with a selected *Mse*I primer (three selective nucleotides containing dNTPs) at a ratio of 1:9 to form a primer master mix. The PCR contained 5µl of a 30 fold diluted pre-amplified DNA, 5µl of primer master mix, 0.5 unit of *Taq* DNA polymerase, 2µl of a 10x PCR buffer in a final volume of 20µl. PCR conditions as recommended by the manufacturer were adopted for use with the Perkin Elmer 9600 thermocycler.

Aliquots of the post PCR mixture were heated with an equal volume of formamide dye [98% (v/v)] formamide, 10mM EDTA, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol at 90°C for 3min. A 5µl sample was electrophoresed in a 6% (w/v) polyacrylamide sequencing gel with 7.5M urea. The gel was dried and exposed to an x-ray film (Kodak XK-1) at -80°C for 2-3 days.

Data Analysis

The AFLP loci were scored as dominant markers, and, as such, segregation was scored on the basis of presence or absence of the amplified band. The parental origin of the marker was also recorded. Two separate data sets were obtained, one for each parent.

The pseudo-testcross strategy (Grattapaglia and Sederoff, 1994) was used for analysis of segregation data. In the pseudo-testcross configuration, each parent is, in turn, considered the heterozygous individual. Segregation is expected to be in the 1:1 ratio for bands present in the heterozygous parent (*Aa*) but absent in the homozygous parent (aa). A χ^2 test ($p = 0.05$) was performed to test the null hypothesis of 1:1 segregation on all scored segregating bands. ***Table 1*** illustrates the types of Mendelian segregation of AFLP patterns obtainable in the offsprings of the interspecific cross scored as a pseudo-testcross.

RESULTS AND DISCUSSION

Primer Screening

This study was carried out to determine the feasibility of the AFLP technique for generating molecular markers in the oil palm. In the initial

TABLE 1. SEGREGATION OF AFLP AMPLIFICATION PRODUCTS IN A PSEUDO-TESTCROSS

Parental genotypes	Expected Mendelian ratio		AFLP phenotype on autoradiogram								Remarks
			Parents		Offspring						
			♀	♂	1	2	3	4	5	6	
Aa x *aa*	1:1	♀	+	−	+	−	+	−	+	+	Segregation data included in the maternal data set
aa x *Aa*	1:1	♂	−	+	−	+	−	−	+	−	Segregation data included in the paternal data set

experiment, UP1026 and T128, the diploid parents of the interspecific mapping population, were tested using 10 randomly chosen primer combinations. This was done in order to identify primer combinations which amplify bands that are: i) informative between the parental palms, and ii) easy to score. Each band is considered to represent a single locus of a dominant marker. On this basis, presence of a fragment in a parent indicates that the parent is either homozygous dominant or heterozygous for that locus.

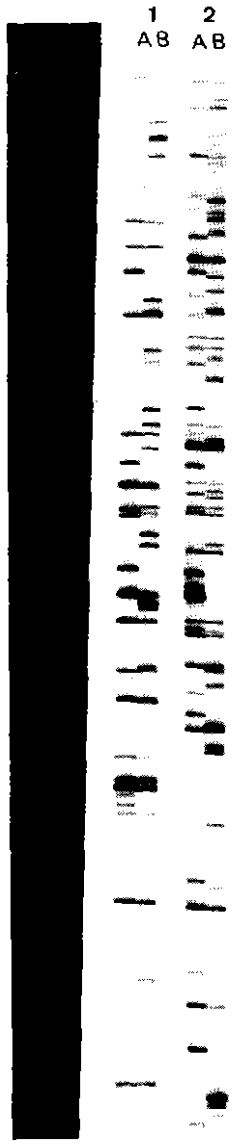

Figure 1. Image of parental AFLP profiles generated with two primer combinations E-ACTIM-CAC (1) and E-ACC/M-CAA (2). A and B refers to UP1026 and T128 respectively.

A survey of 10 different primer pair combinations showed that all gave easy to read AFLP profiles. The AFLP patterns obtained for two of these primer pair combinations are shown in ***Figure 1***. On average, the primer pairs used amplified about 67 bands ranging from 50 to 800bp in size.

The primers examined also generated highly polymorphic AFLP profiles. All 10 pairs of primers identified polymorphic bands between the parental palms. In all cases, each primer pair yielded multiple polymorphic bands. Between the parents, the number of polymorphic amplification products visible on the gel ranged between 27 to 58 (*Table 2*), with a mean of 41.3 polymorphic bands per primer pair used. Taking all 10 primer pair combinations into consideration, 413 of the 674 bands (61%) were polymorphic. A significant correlation ($r = 0.94$) between the total number of bands and number of polymorphic bands was observed. Majority of the polymorphic bands were contributed by the male parent (T128).

For every primer pair combination, the level of polymorphism revealed varied between 54.5% and 68.6%. This high level of polymorphism observed is an indication of the diversity of the two species in the interspecific cross. The fact that the AFLP assay detects single base pair changes throughout a large part of the genome also contributes to high efficiency in scanning for polymorphism.

One of the advantages of the AFLP analysis is that, by using a different primer pair, the AFLP patterns varied and a different set of polymorphic bands could be observed. This indicated that each primer pair used amplified different segments of the genome. Since all primer combinations used in this study were found to produce easy to read polymorphic AFLP profiles, they were all chosen for analyses of individuals of the mapping population.

Patterns of Inheritance of AFLP Bands

The inheritance of AFLP markers in the population progeny derived from the interspecific cross (referred to as OxG) was examined to determine the suitability of the population for genetic mapping in oil palm. The pseudo-

TABLE 2. POLYMORPHISM AND ORIGIN OF AFLP BANDS IN THE PARENTS, UP1026 AND T126

Primer combination	No. of amplified bands	Total No. of polymorphic bands	Origin of polymorphic band		Polymorphism
			UP1026	T128	(%)
E-ACT/M-CTA	57	34	8	26	59.6
E-ACT/M-CAC	46	27	8	19	58.6
E-ACT/M-CAA	99	54	22	32	54.5
E-ACT/M-CAG	61	34	12	22	55.1
E-ACA/M-CTC	52	32	9	23	61.5
E-ACA/M-CAA	86	58	26	32	67.4
E-ACC/M-CTC	49	31	15	16	63.3
E-ACCIM-CAA	67	46	18	28	68.6
E-AAG/M-CTG	87	51	20	31	58.6
E-AAG/M-CAC	70	46	18	28	65.7
Total	674	413	156	257	-
Moan	67.4	41.3	15.6	25.7	61.35

testcross mapping strategy was employed (Grattapaglia and Sederoff, 1994). In this strategy, it is assumed that in a cross between two individuals, markers which are heterozygous in one parent and null in the other, will segregate 1:1 in their F, progeny following a testcross configuration (*Aa* x *aa* or *aa* x *Aa*). The term 'pseudo-testcross' is used because the testcross mating configuration of the markers is only inferred after analysing for segregation of the markers in the offsprings. Either the male or female parent can be fixed as the heterozygous individual contributing the segregating bands, The term 'one-way' or 'two-way' pseudo-testcross is used when one or both parents involved in the analysis are heterozygous, respectively.

In the OxG progeny examined, the inheritance pattern was studied in a total of 77 F, individuals using 10 primer pair combinations. As shown in ***Table*** 3, all 10 primer pair combinations revealed scorable multiple segregating markers. Some primer pairs (e.g.,

TABLE 3. SEGREGATION OF AFLP MARKERS IN THE PSEUDO-TESTCROSS OBTAINED BY USING 10 PRIMER COMBINATIONS

Primer combination	Total No. of amplified bands	Segregation according to model		Total No. of segregating bands	No. of markers meeting goodness of fit to 1:1 ratio
		Aa x *aa* (♀ UP1026 heterozygous)	*aa* x *Aa* (♂ T128 heterozygous)		
E-ACT/M-CTA	57		11	11	8
E-ACT/M-CAC	46		6	6	6
E-ACT/M-CAA	99		10	10	6
E-ACT/M-CAG	61	2	5	7	6
E-ACA/M-CTC	52	1	7	8	7
E-ACA/M-CAA	86	4	8	12	10
E-ACC/M-CTC	49	1	4	5	4
E-ACCIM-CAA	67	1	9	10	5
E-AAG/M-CTG	87	-	14	14	13
E-AAG/M-CAC	70		8	8	8
Total	674	9	82	91	73
Mean	61.4	0.9	8.2	9.1	7.3

E-AAG/M-CTG) revealed more segregating markers than others. The number of segregating markers observed for each primer combination ranged from 5 to 14. On average, the number of informative bands per primer pair was 9.1. This shows that the method provides a much higher multiplex ratio in oil palm than RFLP (Cheah et al., 1990) or RAPDs which provided only 1-2 segregating loci per reaction per gel lane.

The number of segregating bands obtained was also found to be significantly correlated with the total number of bands obtained (r=0.72). This clearly indicates that an increase in the pattern complexity of the total amplified DNA bands corresponds to a significant increase in informative markers for mapping. The AFLP profiles obtained for a number of F_1 individuals using two different primer pair combinations are shown in *Figure* 2.

In this study, we detected 91 segregating markers using only 10 primer combinations. Chi-square analysis was performed for each of the segregating bands scored to determine if segregation deviated from the expected 1:1 ratio. At a significance level of p=0.05, 73 of these markers (about 80%) segregated in the expected ratio. For some of the primer pair combinations examined (e.g. E-ACT/M-CAC and E-AAG/M-CAC), all the segregating markers

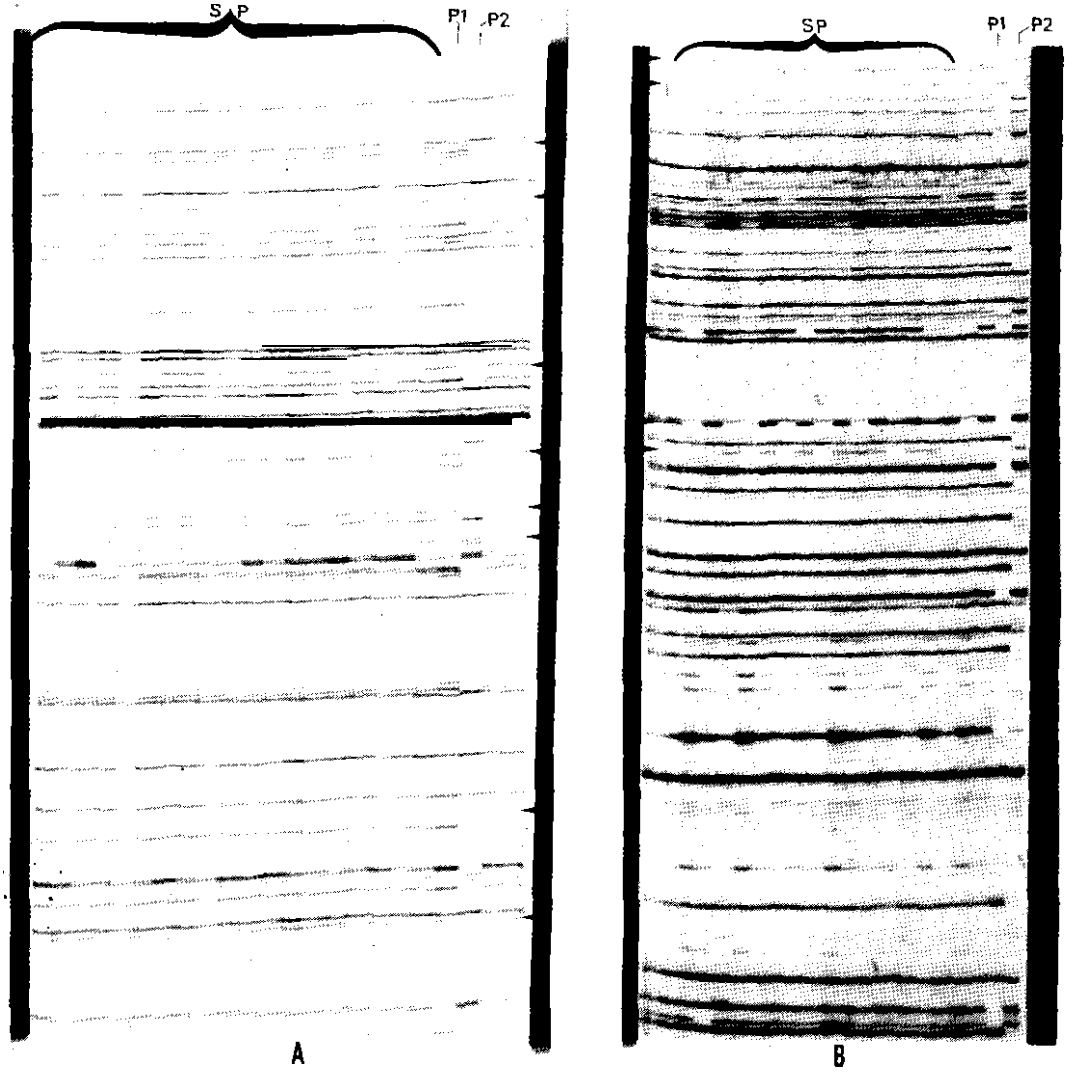

Figure 2. Segregation of AFLP loci in a interspecific cross (UP1026xT128) for the primer combination E-ACA/CTC (A) and E-ACCIM-CTC (B). PI and P2 refer to parents UP1026 and T128 respectively. Black arrows indicate segregating markers. S P is segregating population.

TABLE 4. SEGREGATION ANALYSIS OF AFLP MARKERS BY USING TWO PRIMER COMBINATIONS IN OFFSPRINGS OF THE CROSS UP1026XT128

Primer combination	Size of fragment (bp)	Observed ratio		χ^2 value
		Band present	Band absent	
E-ACA/M-CTC	600	45	30	3.00
	500	30	45	3.00
	370	44	31	2.25
	290	40	35	0.33
	270	36	39	0.12
	250	40	35	0.33
	160	48	27	5.88*
	140	42	33	1.08
E-ACC/M-CTC	840	37	37	0.00
	800	35	39	0.22
	410	47	27	5.40*
	330	37	37	0.00
	310	36	38	0.05

* Significant at 0.05 level (d.f.=1)

detected were in the 1:1 ratio. This shows the suitability of AFLP markers for use in constructing a genetic linkage map for the interspecific cross by using the pseudo-testcross strategy. Results of Chi-square analysis of goodness of fit to 1:1 segregation ratio for 13 of the markers obtained using two primer pair combinations are shown in *Table 4.*

Distorted segregation ratios were observed for a number of AFLP markers (about 20%). While the small progeny size may be a possible explanation for such deviations from the expected ratio, this phenomenon is known to occur in RFLP, RAPD and isoenzyme analyses as well (Carlson et al., 1991). Reduced recombination (Patterson et al., 1990) or chromosomal rearrangement (Tadmor et al., 1987) has been said to be a possible source of segregation distortion in interspecific crosses. Diversity of the parental species used in the interspecific cross could have also contributed to the segregation distortion.

Table 3 shows that the majority of the segregating bands were inherited from the male parent (palm T128, *E. guineensis*). Of the 91 segregating markers obtained, 82 (90.1%) came from T128 and only 9 (9.9%) came from UP1026. This implies that the male parent is more heterozygous than the female parent. A high proportion of polymorphic markers present in the female parent (palm UP1026, *E. oleifera*) did not segregate, most probably because they were homozygous at those loci. As such, sufficient markers could only be generated under the present experimental procedure to enable development of a preliminary linkage map for the male parent. It is therefore concluded that it would be more appropriate to analyse this cross as a 'one-way pseudo-testcross' in which the male, *E.* guineensis, is considered the heterozygous parent and the Colombian *E. oleifera* the homozygous parent.

As for the female parent, either more primers have to be screened or a different set of enzyme combination (e.g. *Pst*I/*Mse*I or *Taq*I/*Hind*III) may have to be used to scan different regions of the genome to generate sufficient markers which are informative for mapping. The low level of heterozygosity detected in the Colombian *oleifera* could be explained by the fact that *E. oleifera* is found in scattered areas in the South American country (Rajanaidu, 1985). This could have encouraged inbreeding, resulting in a relatively high homozygous genome.

Detection of Contaminants

In this experiment, apart from detecting segregating markers, the AFLP technique also

proved useful in detecting individuals with new bands not found in either parents. These bands could have arisen in several ways, among which are the results of i) recombination which produced new restriction sites in the DNA, or ii) pollen contamination when the crosses were generated when pollen from another palm could have contributed to the pollination. Although, in this experiment, a controlled cross was used, pdlen contamination due to perhaps poor pollen isolation could not be discounted. Furthermore, pollen contamination has been reported in other controlled crosses, such as in *Betulla allenghaniensis* (Roy *et al.*, 1992) and conifers (Adams *et* al., 1988), and is also known to occur frequently in oil palm (Chin, 1995).

Since pollen contamination could be the main reason for the appearance of new bands in the progeny not found in either parent, such individuals are very likely 'illegitimates' or contaminants. These contaminants need to be eliminated from the mapping population to avoid distortion of data. In the mapping population studied, we observed one such individual (*Figure 3*). This contaminant was detected by all 10 primer pairs used and was eliminated from the mapping population.

The possibility of having contaminating individuals even in a controlled cross shows the importance of making available molecular or biochemical markers to monitor the identity of pedigree material within the framework of a tree-improvement programme.

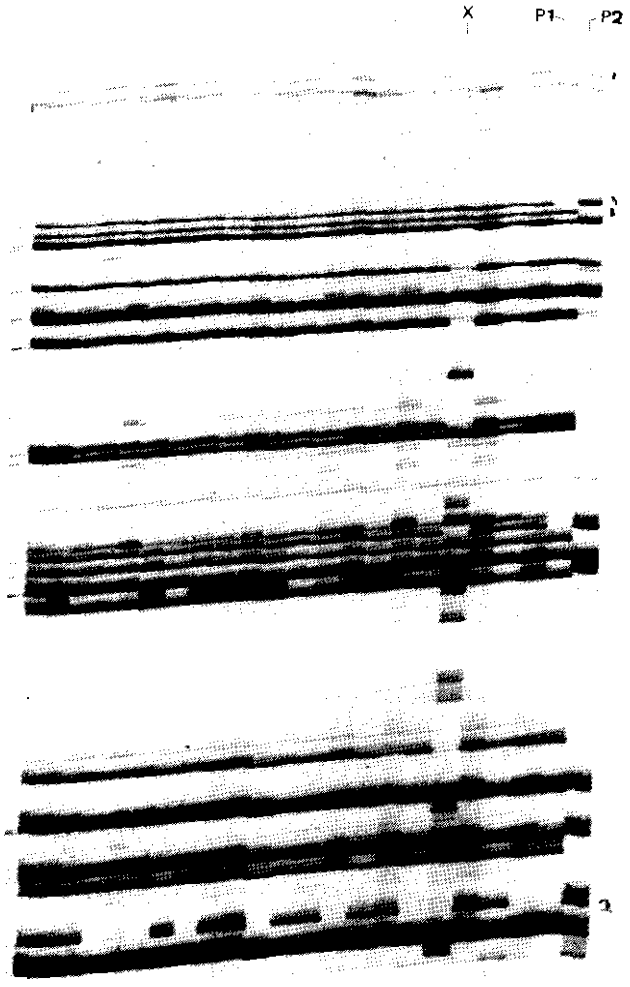

Figure 3. Contaminating individual (marked X) detected in a segregating population with primer pair E-ACC/M-CAC. P1 and P2 refer to parents UP1026 and T128 respectively.

CONCLUSION

The AFLP technique proved to be useful in the generation of molecular markers in the oil palm. The strength of this technique is in the high multiplex ratio, that is, the number of different genetic loci that can be simultaneously analysed per experiment. The number of different bands observed in a single reaction ranged from 46 to 99. This clearly shows that AFLP has the capacity to detect a much larger number of loci for polymorphism in the oil palm than other currently available marker systems. This also indicates that the enzymes used (*Eco*RI and *Mse*I) have a great many restriction sites in the oil palm genome. There was also a positive correlation between the total number of bands amplified with the number of segregating markers observed. This shows that with a judicious selection of primer combinations, increased but readable pattern complexities can be obtained, enabling rapid generation of molecular markers in the oil palm.

In this study, using only 10 primer pair combinations, we were able to amplify 674 loci to generate 91 segregating markers. Eighty percent of these markers met the, goodness of tit to the 1:1 segregation ratio expected in a testcross. This shows that a high proportion of AFLP markers in the oil palm is stably inherited from parents to offspring following the rules of Mendelian inheritance. The high frequency of AFLP bands showing normal Mendelian inheritance indicates that the interspecific

cross is useful for future studies aimed at producing a high density map with AFLP markers.

In this experiment, it was also observed that a majority of these markers (90%) was contributed by the male parent, T128. This is a clear indication that the male guineensis parent was more heterozygous than the female *oleifera* parent, UP1026. The AFLP technique *also* proved useful in assessing the fidelity of progeny palms derived from controlled crosses in the oil palm. Contaminants could be easily detected by the appearance of bands not present in either of the parents. Since there is a high possibility that the contaminants could have arisen from pollen contamination, they need to be rapidly identified and removed from the mapping population. The results here show that AFLP markers, in addition to generating segregating markers, could also be useful in assessing the fidelity of controlled crosses or in conducting paternity tests in the oil palm.

The AFLP technique in this experiment has proven to be useful for analysis of the oil palm genome. It permits rapid generation of numerous reproducible dominant markers and holds great promise for map saturation, even with large genomes like in the oil palm. This technique is currently being used in combination with RFLP to develop a linkage map for the oil palm.

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