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A new aspect of genetic diversity of Indonesian oil palm (*Elaeis guineensis* Jacq.) revealed by isoenzyme and AFLP markers and its consequences for breeding

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Abstract Oil palm (*Elaeis guineensis* Jacq.) plays an important economic role in some countries of Southeast Asia like Indonesia, which is the world's second producer of palm and palm kernel oil. The quality improvement of planting material needs a better understanding of the genetic relationships between genotypes from different populations used in the breeding programmes. In this study, 48 parents, representative of four populations used in Indonesian Oil Palm Research Institute (IOPRI) breeding programmes, were analysed with five selected AFLP primer pairs and four isoenzymatic systems. One hundred and fifty eight scorable band levels were generated of which 96 (61%) were polymorphic. AFLP allowed us to identify off–type descendants which were excluded from analysis. The use of unbiased Rogers distance clearly separated the four studied populations. The Neighbor-Joining method re-groups two African populations which are known as originating from different regions. Nevertheless, the variability revealed is in accordance with oil palm breeders' knowledge. The results obtained with AFLP showed that the crosses among the African sub-population, which is excluded in oil palm reciprocal recurrent selection (RRS) breeding programmes, may be more interesting than the crosses between the African and the Deli populations.

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Introduction

Oil palm is a crop plant originating from West and Central Africa (Hartley 1988). Cultivated for palm oil and palm kernel oil, it occupies the second rank in the world for oil and fat production. The approximate share of 19.6% in 1998 is slightly behind soybean (22.1%) (Mielke 1998). Due to favourable climatic conditions in Southeast Asia, oil palm is at present the primary industrial crop in both Malaysia and Indonesia. In Indonesia, oil palm plays an important economic role for millions of planters and plantation workers.

Four seedlings were introduced into Indonesia in 1848. Reports, cited by Hartley (1988), indicated that these palms show homogeneous vegetative characteristics and bunch performance. Nevertheless, several differentiations resulted from breeding programmes developed independently by some private companies. Currently, 13 Deli sub-populations are used in world-wide breeding programmes (Rosenquist 1986).

In the twenties/thirties and seventies, the government research centre and the plantation companies introduced selected materials respectively from Africa and the Institut de Recherche pour les Huiles et Oléagineux (IRHO) (Lubis 1989). This introduction was the starting point of the organized breeding programme carried out in Marihat Research Station (now the Indonesian Oil Palm Research Institute/IOPRI). The reciprocal recurrent selection (RRS) used by IRHO was largely adapted. This selection scheme is based on the genetic complementarity of two groups: "A" (Deli, Angola) and "B" (La Mé, Yangambi, Yocoboué, Nigeria, Cameroon) (Meunier and Gascon 1972).

Isoenzyme characterisation of the breeding material has been performed (Ghesquière 1984). New types of markers are now available for oil palm. Random amplified polymorphic DNA (RAPD) and restriction fragments

length polymorphism (RFLP) were developed for, respectively, the evaluation of genetic polymorphism in oil palm germplasm (Shah et al. 1994) and genotype identification (Jack et al. 1995). Although simpler and cheaper, RAPD analysis is inadequate due to low reproducibility and reliability (Karp and Edwards 1997); on the other hand, RFLP is expensive and time consuming.

The amplified fragment length polymorphism (AFLP) reaction seems more adequate (Vos et al. 1995). It generates more polymorphism than RFLP or RAPD and is reliable (Maughan et al. 1996; Ajmone Marsan et al. 1998). AFLP was used: (1) to measure the within and between variability of cultivated and wild soybeans (Maughan et al. 1996), (2) to optimize the genetic relationship in *Lactuca* spp, (Hill et al. 1996), (3) to suggest a new heterotic group in sunflowers (Hongtrakul et al. 1997), and (4) to assay the level of variability existing in the Sri Lanka coconut germplasm (Perera et al. 1998).

In this paper, our objectives, using AFLP and isoenzyme polymorphism, were to: (1) estimate the genetic variability present in IOPRI's oil palm breeding populations coming from several countries of origin, and (2) organize the genetic structure of the breeding materials.

Materials and methods

Plant materials

Forty eight parents were chosen in the IOPRI's breeding programme according to their representativeness and their descendants use in the second breeding cycle (Table 1). Nine parents were represented by themselves and 39 parents were represented by five descendants (only one parent was represented by four descendants) previously obtained by self-pollination. Our sample, analysed using AFLPs and isoenzymes, comprised 203 individuals. The number of parents chosen in each population was unbalanced because the main objective of IOPRI was to test local populations used as genetic resources by several plantations and research centres in the colonial era.

AFLP assays

Total DNA was extracted from 300 mg of freeze-dried spear leaves using the CARLSON solution as a buffer for materials ground in liquid nitrogen. This buffer contained 100 mM Tris HCl pH 8, 1.4 M NaCl, 20 mM EDTA pH 8, 2% MATAB, 1% PEG 6000 and 0.5% sodium bisulphite. The solution of grounded leaves was then incubated for 30 min in a water bath at 74°C. After addition of 5 ml of CIAA (chloroform: isoamyl alcool=24:1 v/v), the mixture was centrifuged at 4000 g/min for 15 min. The aqueous phase was transferred to a tube and the DNA was precipitated by adding an 0.7 vol of isopropanol. After dilution in a new buffer containing 50 mM Tris HCl, 0.7 M NaCl and 10 mM EDTA at pH 7, DNA was purified by passing through a QIAGEN-Tip 20 micro-column.

AFLP analyses were performed according to the procedure provided with the ”AFLP Core Reagent Kit” and the ”AFLP Starter Primer Kit” of Life Technologies. The primers used in this study were those generating most polymorphic bands in a previous oil palm experiment (Billotte 1997). The extensions of these *Eco*RI/*Mse*I primer pairs are: E-ACC/M-CAG, E-ACC/M-CAC, E-ACA/M-CAG, E-ACC/M-CAA and E-ACC/M-CTT.

The amplification products were analysed in pre-warmed 5% acrylamide electrophoresis gels. Gels were run at 55 W for approximately 2 h and then dried for 20 min under vacuum (BioRad Model 583). AFLP products were revealed by exposure to X-ray films (X-OMAT) for 4–5 days.

Isoenzyme assays

The same genotypes were then assayed for isoenzyme polymorphism. About 150 mg of freeze-dried leaves were ground at 4°C, adding 1.5 ml of a buffer containing 0.1 M Tris, 0.2 M sucrose, 0.012 M cysteine, 0.025 M ascorbic acid, 0.02 M sodium sulphate and 1% ß-mercaptoethanol of the final volume. Eleven isoenzyme patterns were revealed according to procedures described by Glaszmann (1987). Only four enzymatic systems out of 11 were retained for analysis, due to monomorphic banding patterns: phosphoglucose isomerase (PGI), acid phosphatase (ACP), isocitrate dehydogenase (IDH) and shikimate dehydrogenase (SDH). Each system is assumed to represent one locus.

Data analysis

The banding patterns, obtained from AFLP autoradiographs, were scored as levels and coded as present (1) or absent (0). The levels of those bands were numbered according to their relative position to the bands of tomato DNA, assayed with the primer pair E-AAC/M-CAG, used as a control in each assay as described by Billotte (1997). The isoenzyme bands were scored as (1,1) for the homozygoxus genotype of allele 1; (2,2) for the homozygoxus genotype of allele 2; (1,2) for the heterozygoxus genotype.

Reconstructions of the missing parental genotypes were based on AFLP data from its five selfing descendants. A band was assumed to be present in a parent when that corresponding level was present in at least one of its selfed descendants. For each band level i, the deviation $x_i – \bar{x}$ has been calculated, where x_i is the value of the band present (1) or absent (0) of one individual, and $\bar{x}$ is the average value of the band level i of all descendants representing the same parent. The individuals who have a value of $\sum (x_i – \bar{x})^2$ in excess of 10 (arbitrarily chosen) were considered to be susceptible off-types and were not taken into account in the reconstruction of the parent's genotype.

The genetic similarities were calculated using the Simple-Matching index, which is the ratio between the sum of double presence plus double absence divided by the total number of

Table 1 Origin of the genotypes used for AFLP and isoenzymes assays

Population	No. of parents	Parents represented by		No. of genotypes assayed
		Themselves	Their descendants	
Cameroon	6	3	3	18
Deli	28	0	28	140
Ivory Coast	6	3	3	17
Zaire	8	3	5	28
Total	48	9	39	203

bands. This index was suggested to be an appropriate estimator of relatedness using binary data obtained from dominant markers like AFLPs (Perrier et al. 1999). All the band levels (polymorphs and monomorphs) were taken into account in distance calculations to avoid over-estimation of the distance (Ghérardi et al 1998). Genotype relationships were constructed using the Neighbor-Joining Trees (NJ-Trees) procedure (Saitou and Nei 1987). The similarity matrices were constructed with the NTSYS-software pc version 1.80 (Rolf 1995) and used to build the trees with Darwin software [an uncommercialized software of CIRAD, Perrier et al. (1999)]. The distance separating two populations was calculated from unbiased estimators of Roger's distance as described in Ghérardi et al. (1998). The re-sampling procedure with 1000 permutations was used to test the null value of this distance.

The expected within-populations/observed heterozygote frequencies within-populations were estimated from isoenzyme data. The method proposed by Rousset and Raymond (1995) was used to test the heterozygote excess or deficiency using the GENEPOP software version 3.1b (an updated GENEPOP version 1.2 described in Raymond and Rousset 1995).

Results

Using AFLP markers with five combinations of primer pairs, 158 banding levels were identified (Table 2) of which 96 (61%) were polymorphic. The number of primer-generated polymorphic band levels varied from 5 for E-ACC/M-CTT to 36 for E-ACA/M-CAG, with an average of 19.2 polymorphic markers per primer pair. The numbers of polymorphic band levels, generated by each primer pair, were slightly different according to the population. Markers found only in Ivory Coast and Zaire populations have been identified (Fig. 1). E-ACC/M-CAA_9.5, E-ACC/M-CAA_19.1 and E-ACC/M-CTT_6.2 for the Ivory Coast population, and E-ACC/M-CAG_25.0 for the Zaire population, were present in more than half of the total number of genotypes of these groups.

AFLP data have enabled us to detect several potential off-type genotypes. Eight out of thirty nine parents have been reconstructed using four, instead of five, of their selfing descendants, and one of them with only three descendants. In most cases, the excluded individuals have more than 3% of bands present or/and absent alone. This was not the case in the isoenzyme assays where the off-type individuals detected by AFLP did not modify the reconstruction of parental genotypes; thus, all the selfing descendants were used in the analysis.

The Simple-Matching similarity indices ranged from 0.042 to 0.406. The highest distance was observed between one parent from the Deli and one parent from the Ivory Coast population. Data clustering, using the NJ algorithm,

Table 2 Number of polymorphic fragments obtained from five AFLP primer pairs

Primer combination	Total no. of fragments	No. of polymorphic bands	Polymorphic bands (%)	No. of polymorphic bands			
				Deli	Cameroon	Ivory Coast	Zaire
E-ACC/M-CAC	16	9	56	7	6	4	6
E-ACC/M-CAG	32	20	63	14	14	12	11
E-ACC/M-CTT	16	5	31	2	2	5	2
E-ACA/M-CAG	52	36	69	26	22	22	24
E-ACC/M-CAA	42	26	62	19	15	17	13
Total	158	96		68	59	60	56
Average	32	19.2	60.8	14	12	12	11

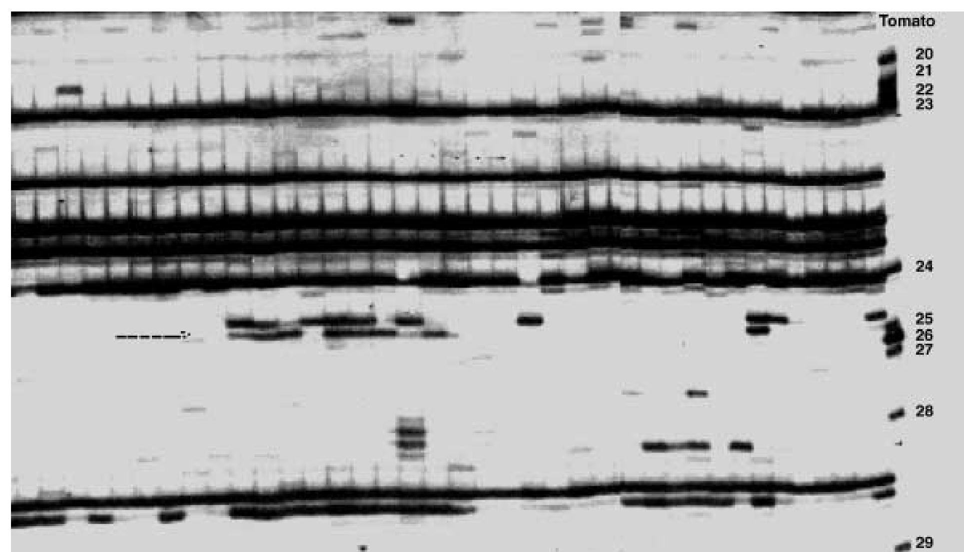

Fig. 1 The oil palm AFLP profile with the E-ACC/M-CAG primer pair. Tomato DNA analyzed with the E-AAC/M-CAG primer pair has been used as a control in each assay. The *arrow* indicates a band present in the Zaire population but absent in the others

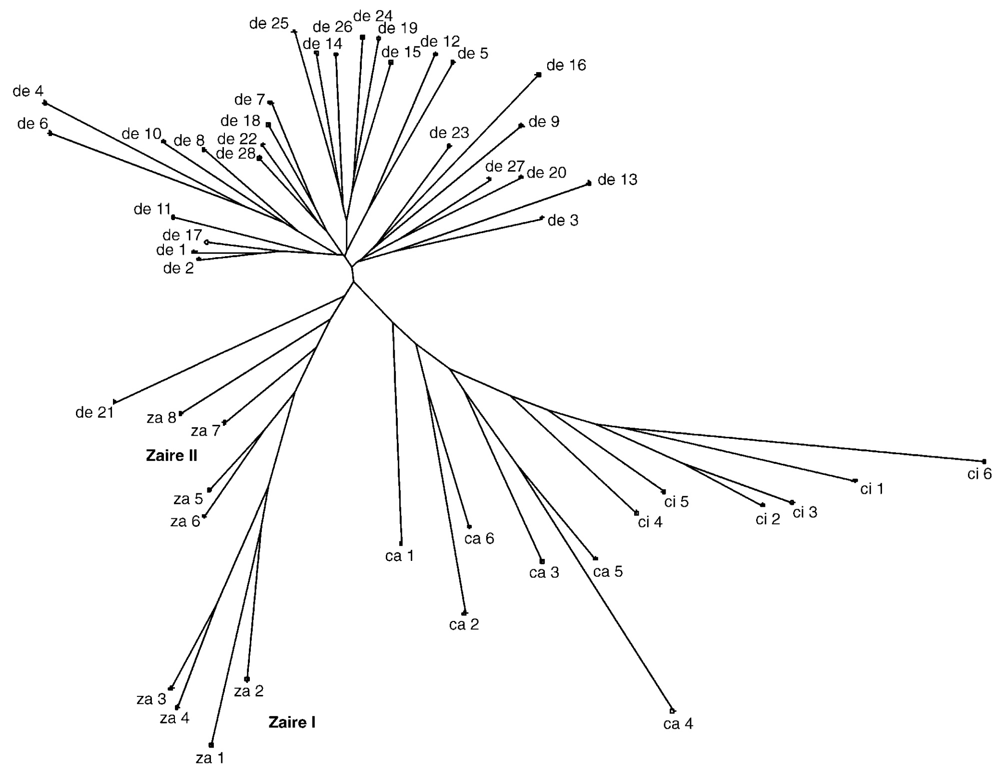

Fig. 2 Tree obtained by the NJ method for 48 parents with 158 AFLP markers (*ca*=Cameroon, *de*=Deli, *ci*=Ivory Coast, *za*=Zaire)

were performed from the matrix of calculated distances. The resulting tree (Fig. 2) showed three distinct groups in the studied populations: Deli, Ivory Coast/"Cameroon" and Zaire. Except for one case (de 21), all the Deli parents were well-separated from the African populations (Ivory Coast, "Cameroon" and Zaire populations).

The re-sampling test, with 1000 permutations, indicated that all population distances calculated by the Roger's method are significantly different from zero (Table 3). Deli and "Cameroon" were closest (4.5), whereas Deli and Ivory Coast were most distant (9.6). Zaire appears at the same distance from the "Cameroon" and the Ivory Coast populations.

With isoenzymes, deviations from the Hardy-Weinberg expectation were detected in all populations (Table 4). The main deviation, except for the Ivory Coast population, is related to a heterozygote excess, whereas the Deli population only exhibited a significant excess based on the test at P=0.05.

Table 3 Estimated Rogers distance ($\times 10^{-2}$) between populations using 158 AFLP bands

Population	Cameroon	Deli	Ivory Coast
Deli	4.5		
Ivory Coast	4.8	9.6	
Zaire	7.1	5.2	7.2

Discussion

A high level of polymorphism and a heterozygote excess were identified in the studied populations. The detection

Table 4 Genetic diversity within four oil palm populations revealed by four enzymatic systems

Population	Number of indiv.	Total no. of alleles	alleles/ locus	H_o[a]	H_e[b]	F_{is}[c]	Deviation from HW	
							Deficiency	Excess
Cameroon	6	5	1.25	0.125	0.102	–0.222	ns	ns
Deli	29	8	2	0.431	0.35	–0.232	ns	**
Ivory Coast	6	8	2	0.208	0.25	0.167	ns	ns
Zaire	8	8	2	0.563	0.496	–0.134	ns	ns

[a] H_0=frequency of observed heterozygotes

[b] H_e=frequency of estimated heterozygotes, calculated as $H_e^l = (2n_l-1)\left[1-\sum_{al=1}^{A_l} P_{al}^2\right]$ where P_{al} is the frequency of allele a at locus l, n_l is the number of individuals characterized at locus l

[c] F_{is}=deviation from Hardy-Weinberg, calculated as follows: $(F_{is})_s = 1-\frac{(\overline{H}_o)_s}{(\overline{H}_e)_s}$, where $(H_o)_s$ and $(H_e)_s$ are the mean observed and expected heterozygote frequencies within the population s, respectively

** P<0.01; ns=P>0.05

of several potential off-type genotypes by AFLP analysis resulted in some parents that have been reconstructed using only four or three of their selfing descendants. The clustering analysis showed three distinct groups, even though all four populations are distantly different from zero when calculated by the Roger's method.

Using five AFLP primer pair combinations we surveyed 158 bands of which 96 (61%) were polymorphic. This level of polymorphism is similar to frequencies reported in coconut, and higher than that reported in soybean (36%), sunflower (48%) and rice (22%) (Maughan et al. 1996; Hongtrakul et al. 1997; Maheswaran et al. 1997; Perera et al. 1998). All tested primer pairs revealed polymorphism between the four populations. This was not the case with RFLPs and RAPDs where only 2.5% of RFLP enzyme/probe combinations and nine out of 20 RAPD primers showed polymorphism within the *E. guineensis* species (Cheah et al. 1991; Shah et al. 1994), although these authors used a somewhat broader oil palm diversity in their studies. This could perhaps be explained by the previous selection of the five primer-pair combinations. Billotte (1997) tested all 96 commercial primer-pair combinations in order to identify AFLP primers with a high potential in revealing polymorphic loci in the oil palm species.

Isoenzyme data show that only the Deli population exhibits a significant excess of heterozygotes. Oil palm breeders, by using negative assortative mating among individuals, increase the heterozygote frequency within the Deli population. In practice, Cochard et al. (1993) showed that this type of mating is frequently used within Deli where the within-population intercrossing with the complementary partners seems more effective empirically.

Using a simple method, such as deviation analysis, the AFLP data identified nine palms that were dissimilar from their other full-sibs. Consequently, for the RRS scheme of oil palm breeding, where the genotype of the parent per se is more important than the population itself, this information will be very useful in selecting only a ”true-to-type” parent for the recombination programme. These off-type palms might result from a technical or a human error. The AFLP technique has been proven to be stable and repeatable as long as the DNA template has been properly digested (Vos et al. 1995). In our case, ambiguity in reading the bands has been reduced by the proof-reading of another person. So, possibilities could be a mixing-up of pollen preparations or an error in controlled pollination which produce an individual presenting the ”paternal” bands which do not exist in other selfing descendants. Finally, confusing the etiquettes during nursery or plantation phases is most likely the cause of these illegitimate individuals.

The inclusion of Yocoboué genotypes (ci 5 in the Fig. 2), in order to increase the effectives as well as to enlarge the genetic variability of the Ivory Coast population (Meunier 1969), is confirmed as a reasonable approach since this genotype is very close to La Mé. Our data analysis showed a separation between Zaire and the other populations. In addition, it seems that there are two sub-populations within the Zaire. Sub-population Zaire I, which is known as Yangambi, and the descendants of SP 540 T material, originating from ”Djongo” palm from the Eala Botanical Garden, could be separated from the sub-population Zaire II, imported by a private company earlier in this century from other parts of Zaire.

The parents within the Deli population were tightly clustered, reflecting its narrow genetic basis. The phenotypical divergence among parents within this population is probably due to a high heterozygosity of their four ancestors. Ghesquière (1984) supported this hypothesis with isoenzymes showing a high heterozygosity level in Deli compared to a large sample of African populations.

The similarity between the “Cameroon” and the Deli populations is surprising, since Deli has been believed to originate from a different part of Africa. The ”Cameroon” material, introduced into Indonesia by a German plantation company in the thirties, was supposed to come from Cameroon because this country was under German colonialization during that period. Barcelos (1998) used different parents originating from Cameroon and found that they were grouped with Deli in the same axis of a factorial analysis of correspondence (FAC). Thus, another origin from Cameroon of our ”Cameroon” material is possible.

The computation of AFLP data with the unbiased Rogers distance showed that the four populations were significantly differentiated from each other. The “classical” grouping of the Cameroon, Ivory Coast and Zaire populations in the “B” group *vis a vis* the Deli population in the “A” group of the modified reciprocal recurrent selection (RRS) scheme (Meunier and Gascon 1972) merits discussion. Crosses between Zaire and Ivory Coast or Cameroon, which were excluded in the modified RRS scheme since they were supposed to belong to the same group, may now be more interesting than crosses between Cameroon and Deli. Further studies using agronomic data are being conducted to test this proposition.

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