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M.C. Moretzsohn · C.D.M. Nunes · M.E. Ferreira
D. Grattapaglia

RAPD linkage mapping of the shell thickness locus in oil palm (*Elaeis guineensis* Jacq.)

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Abstract Shell thickness is an important trait in oil palm breeding programs and is the basis for the classification of the varieties of oil palm into the types dura, tenera and pisifera. This trait seems to be controlled by a single locus, with two alleles (sh^+ and sh^-) showing co-dominant expression. Two single-tree linkage maps were constructed for a maternal tenera (sh^+ sh^-) palm and for a paternal pisifera (sh^- sh^-) palm using the pseudo-testcross mapping strategy in combination with RAPD markers through the analysis of an F_1 tenera×pisifera progeny. A total of 308 arbitrary primers were screened in a sample of eight F_1 plants and 121 markers were detected in a testcross configuration. An average of 1.66 polymorphic marker per selected primer were identified in this cross. At LOD 5.0 (with some few exceptions) and θ=0.25 the maternal tenera map included a total of 48 markers distributed in 12 linkage groups or pairs of markers (449.3 cM) while the paternal pisifera map included 42 markers distributed in 15 linkage groups or pairs of markers (399.7 cM). We used RAPD and bulked segregant analysis (BSA) to identify markers more tightly linked to the sh^+ locus. A total of 174 new primers not previously used in the linkage analysis were screened using bulks of DNA extracted from plants selected for the contrasting shell-thickness phenotypes. Two RAPD markers (R11–1282 and T19–1046) were identified to be linked on both sides of the sh^+ locus on linkage group 4. The estimated map distances from sh^+ to R11–1282 and to T19–1046 were 17.5 cM and 23.9 cM, respectively. The results demonstrate the usefulness of RAPD markers and the pseudo-testcross mapping strategy for developing genetic linkage information, and constitute an important step towards early marker-assisted selection for shell thickness in oil palm.

Key words *Elaeis guineensis* · RAPD · Pseudo-testcross · Genetic linkage map · bulked segregant analysis · Shell thickness

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M.C. Moretzsohn (✉) · M.E. Ferreira · D. Grattapaglia
Laboratório de Genética de Plantas,
Embrapa Recursos Genéticos e Biotecnologia C.P.02372,
70849–970 Brasília DF, Brazil
e-mail: marciocm@cenargen.embrapa.br
Tel.: +55 61 348-4780, Fax: +55 61 340-3624

C.D.M. Nunes
Embrapa Amazônia Ocidental C.P.319 Manaus AM 69011–970, Brazil

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is currently the second major source of vegetable oil. World production in 1996 amounted to 19.0 million tons of palm and kernel oils. In 1995 palm oils accounted for 19.7% of world oil production and 39.2% of main oil exports (FAO 1997). The culture of oil palm is in great expansion, especially in countries of South-East Asia, Africa and Central and South Americas. Factors such as high yield (average of 6 tons per hectare per year under favorable conditions), price competitivity, high quality and nutritional content of the oil, coupled with adaptation to humid tropical climate and perennial habit, make this crop of great interest both to government and private projects. Oil palm is used in a wide range of products and has the potential to substitute diesel as a fuel.

Oil palm trees can be classified according to fruit traits into three types: dura, homozygous for the major gene (sh^+sh^+), characterized by the production of large fruits with thick shell, and a small proportion of oil-bearing mesocarp; tenera (sh^+sh^-) that produces smaller fruits with a thinner shell, smaller kernels and a larger proportion of oil-bearing mesocarp as compared to dura; and pisifera (sh^-sh^-), shell-less but essentially female-sterile palms and, for this reason, not grown as a commercial crop. Occasionally, the kernel may be absent in pisifera fruits, with the oil-bearing mesocarp constituting the entire fruit. The monofactorial inheritance of fruit type was established by Beirnaert and Vanderweyen (1941). Within each oil palm type there is still considerable variation in

shell thickness, under apparent polygenic control. Tenera is the material commercially used for oil extraction due to its higher yields than dura. It is produced by controlled crosses of dura×pisifera palms. Pisifera is always used as the male progenitor due to its female sterility.

Despite its commercial importance, little molecular-marker research has been developed for oil palm. Isozymes have been used for the study of genetic diversity in African and Brazilian natural populations of *Elaeis* spp. (Ghesquiere 1985; Ghesquiere et al. 1987; Santos 1991; Moretzsohn 1995). In recent years, RFLP technology has been developed for oil palm (Jack et al. 1995) and applied to oil palm linkage mapping (Mayes et al. 1997) and clone fingerprinting (Mayes et al. 1996).

RAPD (random amplified polymorphic DNA) markers have allowed a significant advance in the ability to carry out genetic analysis in crops where limited genetic information was available. In *Elaeis*, RAPD markers have been employed for the analysis of genetic variation among African germplasm accessions (Shah et al. 1994) and unsuccessfully used for the detection of somaclonal variants among regenerant populations (Rival et al. 1998).

Genetic linkage information is potentially a very powerful tool for accelerating oil palm breeding through marker-assisted selection. Selection of palms with desirable fruit types in oil palm breeding programs is possible only after 3–4 years, when fruiting begins. A simple marker-based early selection method for oil palm fruit type could prove extremely valuable. At the nursery stage only desirable types would be selected, significantly optimizing resource allocation and reducing planting costs. The combined use of RAPD and bulked segregant analysis (BSA) (Michelmore et al. 1991) has proved to be a highly efficient strategy for the identification of molecular markers linked to genes controlling traits of interest (Paran and Michelmore 1993; Grattapaglia et al. 1996, Scholten et al. 1997).

The objectives of the present study were to: (1) generate linkage mapping information for tenera and pisifera types using the pseudo-testcross mapping strategy and RAPD markers, and (2) identify RAPD markers linked to the locus controlling fruit types in oil palm, as a first step to develop a marker-assisted selection protocol for this trait.

Materials and methods

Plant material

The experimental material consisted of a single controlled cross between a tenera and a pisifera palm made in 1992 at the Centro de Pesquisa Agroflorestal da Amazônia Ocidental (CPAA) – EMBRAPA, Brazil. The tenera palm (RU36T) is derived from an elite palm from the Ivory Coast and the pisifera pollen progenitor (P687P) was introduced from Zaire. Leaflet samples were collected from 95 F_1 individuals as well as the female tenera progenitor.

DNA extraction and quantification

Total genomic DNA was extracted from freeze-dried adult leaf tissue of the 96 plants using a modified CTAB protocol (Grattapaglia and Sederoff 1994). DNA was quantified comparing the fluorescence intensities of the samples to those of λ DNA standards in ethidium bromide-stained 1% agarose gels under UV light.

RAPD analysis and primer screening

RAPD analysis was carried out as described earlier (Grattapaglia and Sederoff 1994). A total of 308 10-base random primers (Operon Technologies Inc.) were screened for polymorphism using a sample of eight random oil palm F_1 individuals. Of these, 73 polymorphic primers were selected, based on the RAPD marker quality, and used on the entire mapping population for linkage analysis. Repeatability of RAPD reactions was estimated by additional assays of the 96 individuals with 12 randomly selected primers. Segregating RAPD markers were coded according to primer denomination of the Operon kit, followed by a number indicating the fragment size in base pairs. A quality score ranging from 1 to 3 was given, 3 being the best markers (Grattapaglia and Sederoff 1994).

Linkage analysis of RAPD markers and fruit-type locus

The presence of a segregating band was scored as 1 (heterozygous) while absence was scored as 2 (homozygous). In the "pseudo-testcross" configuration, markers present in one parent and absent in the other are expected to segregate 1:1 in the F_1 progeny. Conformity to this expectation for each marker was tested by a χ^2 test. Markers with a P value >0.05 for a 1:1 ratio were included in the linkage analysis using MAPMAKER (Lander et al. 1987). The 95 F_1 individuals were scored for fruit type as 1, if tenera, or 2, if pisifera. The MAPMAKER backcross model assumes that all markers are in coupling. To allow the detection of linkage of RAPD markers in repulsion phase, the data set was duplicated and recoded, as described by Grattapaglia (1997).

Two separate parental maps were constructed. A minimum LOD score of 5.0 and a maximum recombination fraction (θ) of 0.25 were used as thresholds for detecting linkages and for multipoint ordering of markers. After the establishment of a reliable framework, LOD threshold was lowered to 3.0 to allow linkage of some unlinked markers. The orders of the markers for each linkage group were then tested by comparing the log-likelihood of the possible orders. Recombination fractions were converted into map distances using Kosambi's mapping function. The expected total length of the genomes was estimated using "method 3" of Chakravarti et al. (1991): G=2MX/K, where G is the estimated genome length, M the number of locus pairs, K the observed number of locus pairs with a LOD score Z or greater (we chose Z≥3), and X the largest estimated map distance value among the K pairs.

Bulked segregant analysis

Following the establishment of the linkage groups, a bulked segregant analysis (BSA) was performed to identify markers more tightly linked to the fruit-type locus. Two replicate bulks were constructed by adding equal amounts of DNA from ten tenera or ten pisifera individuals per bulk. A total of 174 additional RAPD primers different from the 308 previously used for linkage mapping were used. RAPD markers showing polymorphism between the bulks were selected and scored in all 95 F_1 tenera x pisifera individuals to determine map distances between them and fruit-type locus.

Results

Primer screening and amplification characteristics

Of the 308 arbitrary primers screened, 107 (34.7%) uncovered at least one polymorphic fragment, 116 (37.7%)

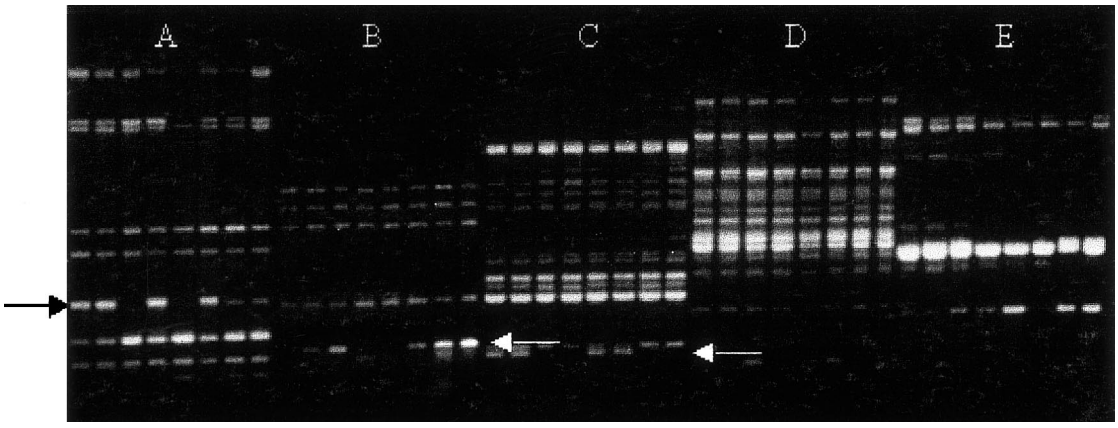

Fig. 1 Screening of arbitrary 10-base primers (Operon Technologies Inc.) for DNA polymorphism among eight random F_1 oil palm plants. Primers are Y02 (*A*), Y01 (*B*), Y04 (*C*), Y06 (*D*) and Y07 (*E*). *Arrows* indicate the segregating informative RAPD markers (Y02-796, Y01-486 and Y04-437) used for linkage analysis

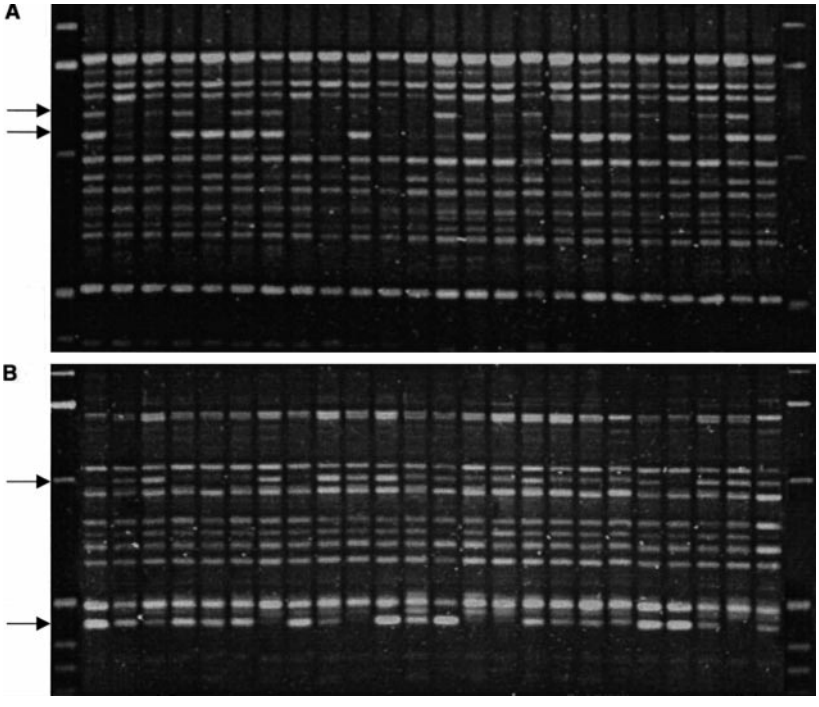

Fig. 2 Segregation of RAPD markers in 24 F_1 plants using primers Y20 (panel *A*) and R08 (panel *B*). In both panels the *first and the last lanes* are 1-kb DNA ladder size-standards. Segregating RAPD markers are indicated by *arrows*

produced only monomorphic bands, and 85 (27.6%) did not yield any visible or scorable fragment (Fig. 1). Out of the 107 polymorphic primers only 73 were selected for linkage analysis based on the intensity and quality of amplification products. The 73 selected primers amplified a total of 121 polymorphic fragments which were used for mapping purposes (Fig. 2). An average of 1.66 markers per selected primer or 0.39 markers per any arbitrary primer was observed. None of these fragments segregated in a codominant fashion. Forty six primers produced only one polymorphic fragment, 18 produced two, nine produced three, and three produced four. The size of the amplification products ranged from approximately 270 to 2260 bp.

All 12 replicate amplifications of the whole population produced consistently identical patterns for the selected bands. Moreover, all primers produced identical patterns in the primer screening step and the mapping step.

Genetic linkage map

Chi-square analysis indicated that 17 of the 121 polymorphic RAPD fragments departed from the Mendelian expectation of 1:1 at α=0.05: seven from tenera, three from pisifera and seven unlinked markers. Only one departure was detected at α=0.01. This proportion of distorted markers (14.8%) is higher than those reported for some linkage RAPD marker studies and is higher than expected by chance alone (approximately 6 markers at α=0.05). Twenty one markers, produced by 17 primers,

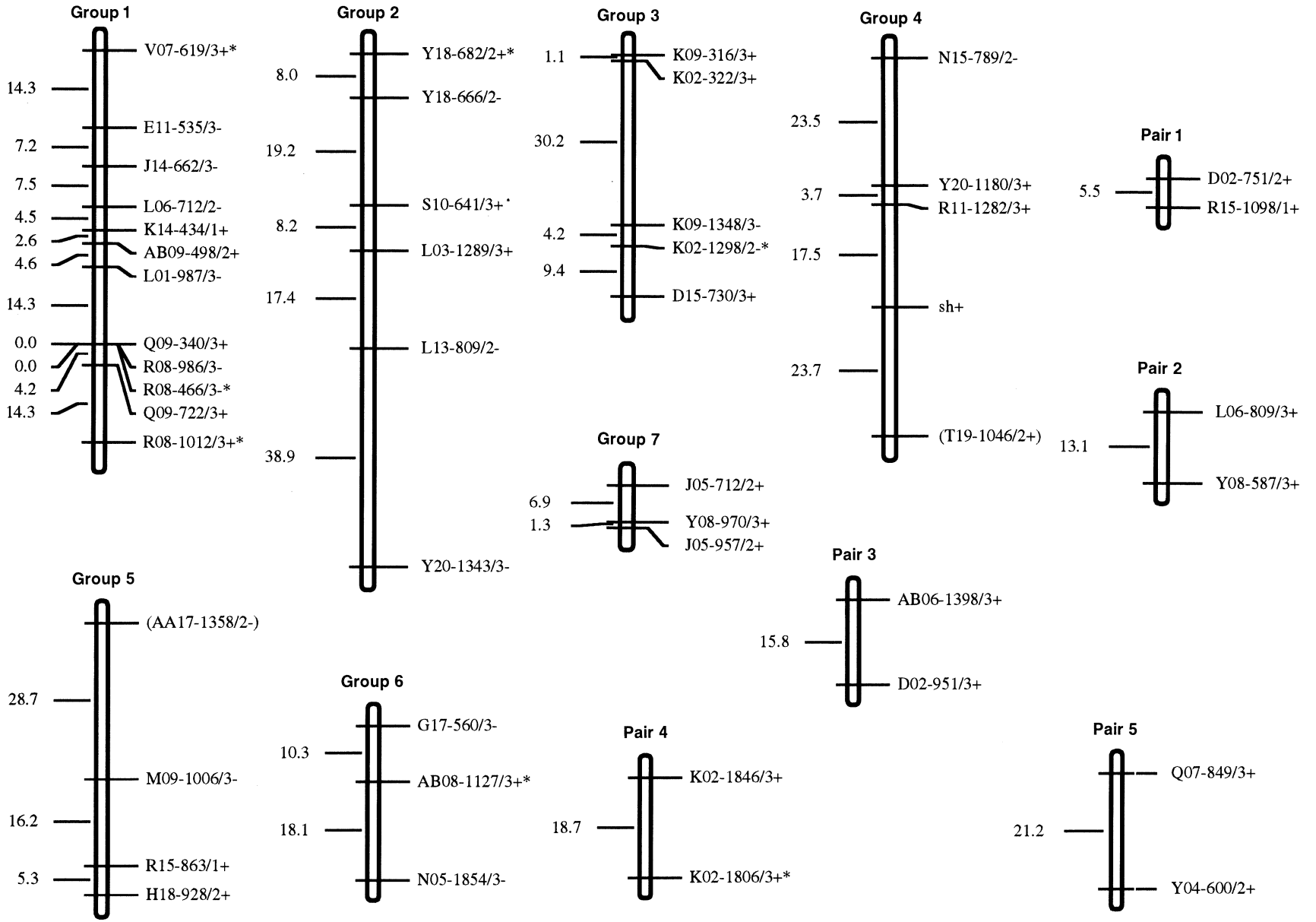

Fig. 3 Genetic linkage map of the tenera female parent RU36T. All markers were mapped with a minimum LOD score of 5.0, except for the two markers enclosed in *parenthesis* (groups 4 and 5). Distances on the left side of the linkage groups are in Kosambi centimorgans (cM). RAPD markers are identified by the Operon primer code, fragment size in base pairs, fragment amplification intensity and linkage phase (+ or –). Distorted markers (α=0.05) are identified by an *asterisk*

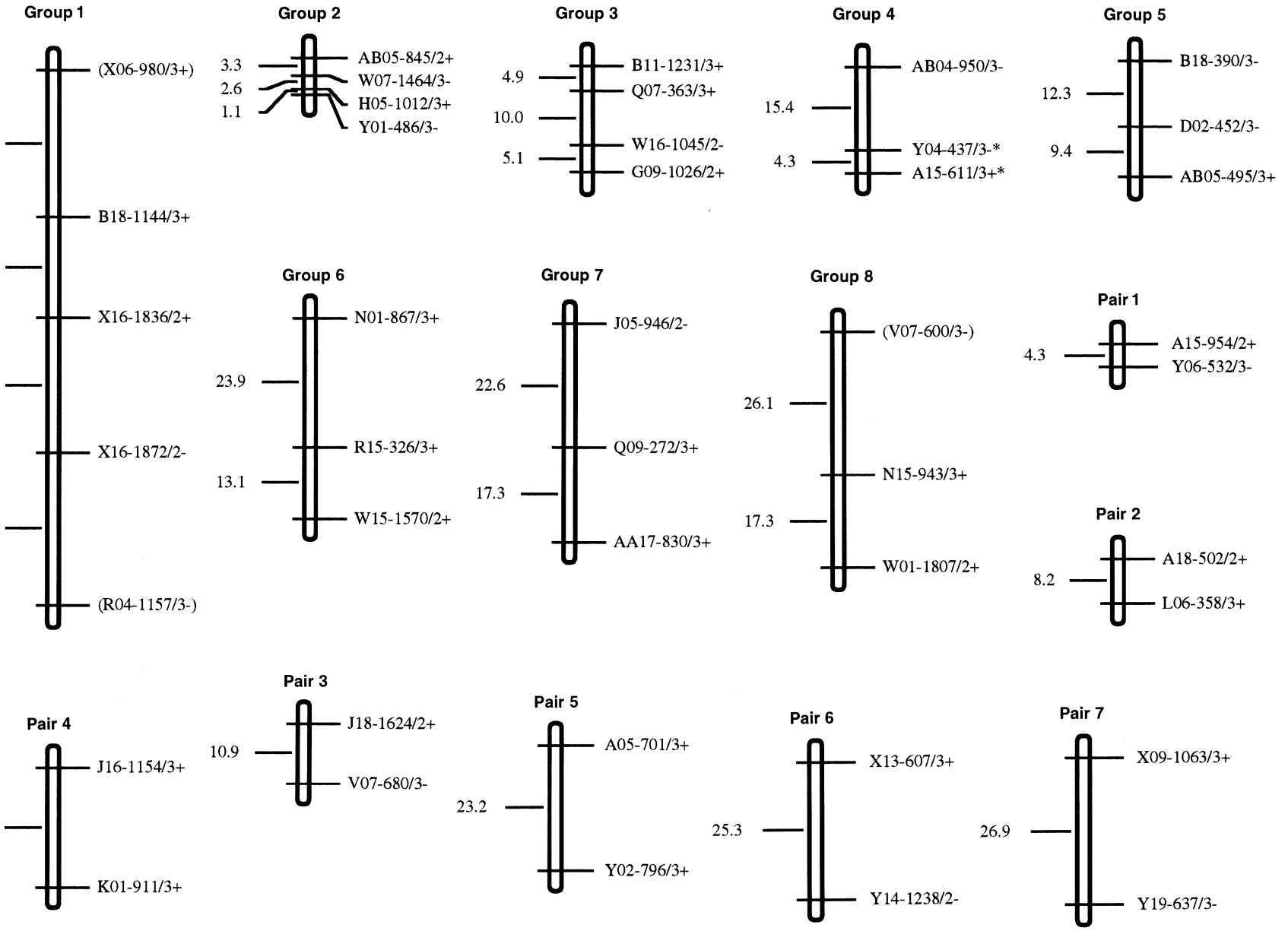

Fig. 4 Genetic linkage map of the pisifera male parent P687P. All markers were mapped with a minimum LOD score of 5.0, except for the three markers enclosed in *parenthesis* (groups 1 and 8). Distances on the left side of the linkage groups are in Kosambi centimorgans (cM). RAPD markers are identified by the Operon primer code, fragment size in base pairs, fragment amplification intensity and linkage phase (+ or –). Distorted markers (α=0.05) are identified by an *asterisk*

segregated in a 3:1 ratio and were not included in the analysis. Distorted markers are scattered on groups 1, 2, 3, 6 and pair 4 of the tenera map and on groups 4 and 5 of the pisifera map. These markers are identified with an asterisk on the maps.

Two-point linkage analysis resulted in the assignment of 85 markers to linkage groups using a minimum LOD score of 5.0 and maximum θ of 0.25. Five markers were assigned to four groups with a LOD score between 3.5 and 3.8. The maternal tenera map has a total of 48 markers included in 12 linkage groups or pairs of markers (Fig. 3), and the paternal pisifera map included 42 markers in 15 linkage groups or pairs of markers (Fig. 4). The average size of the 12 linkage groups of the tenera map was 37.4 cM, ranging from 5.5 to 91.7 cM. The 15 linkage groups on the pisifera map had an average size of 26.6 cM, ranging from 4.3 to 98.3 cM. The average distance between two adjacent markers was 9.36 cM for tenera and 9.52 cM for pisifera. Fifteen tenera markers (24.2%) and 17 pisifera markers (28.8%) remained unassigned to any linkage group. Decreasing the LOD score (below 3.0) or increasing θ resulted in the agglomeration of some linkage groups and in the linkage of some previously unassigned markers. But the use of these parameters probably resulted in many spurious linkages. Comparisons of the log-likelihood ratios indicated that the most-likely order is only slightly more probable than the next-best order for some linkage groups (data not shown).

Identification of markers linked to *sh*$^+$

Of the 174 extra primers screened by BSA, 28 (16.1%) showed no amplification products or produced smears and six primers amplified seven fragments polymorphic between the bulks. These seven polymorphic bands were analyzed on the whole population and two of them were shown to be linked to the *sh*$^+$ gene (R11–1282, closer than Y20–1180 previously grouped before BSA, and T19–1046, on the other side). The combined use of primers R11 and T19 allowed the identification of tenera and pisifera plants. By using both markers to select plants from the population used in the present study, only 1 out of 95 plants would be wrongly classified. This plant is abortive (and, thus, supposed to be pisifera), but showed the presence of the band with both primers. Sixty six plants would be accurately classified, i.e., 36 tenera plants showed both bands (R11–1282 and T19–1046) and 30 pisifera plants showed the absence of the two bands. The other 28 plants showed the presence of the fragment with one primer and the absence with the other, and, thus, would not be precisely identified.

Genome length and map coverage

The 12 linkage groups or pairs of markers of tenera covered a total of 449.3 cM, while the 15 linkage groups or pairs of markers of pisifera covered 399.7 cM. By using "method 3" of Chakravarti et al. (1991), total genome sizes were estimated to be 1604 cM for the maternal parent tenera and 1561 cM for the paternal parent pisifera. Considering these estimates, the maps represented 28.0% and 25.6% of the tenera and pisifera genomes, respectively. The estimate of the genome size of *E. guineensis* using flow cytometry was found to be 3.4×10^9 bp/2 C (Rival et al. 1997). Thus, given the estimated total map distances, the average physical equivalent of 1 cM would correspond to 1.06 and 1.09 Mbp, respectively, for tenera and pisifera.

Discussion

Polymorphism level and segregation of RAPD markers

In the primer screening step, a total of 308 primers were analyzed in a sample of eight random F_1 plants, since the DNA sample from the paternal parent pisifera was not available. By using this strategy, the probability of missing a polymorphic marker segregating 1:1 was 0.008. The screening step made it possible to increase the time efficiency and to minimize the final cost of the analysis, since only 34.7% of the screened primers were selected to be used on the entire population. Similar or smaller ratios of polymorphic primers have been reported for many other intraspecific studies (Binelli and Bucci 1994; Chaparro et al. 1994; Lefebvre et al. 1995; Paillard et al. 1996; Stockinger et al. 1996). Thus, this step is very important for intraspecific genetic mapping. At least two other practical advantages justify this approach: (1) it provides an estimate of the reproducibility of RAPD patterns and, (2) it makes possible the selection of primers that amplify a higher number of polymorphic markers (higher multiplex ratio).

Of the 308 primers screened, 107 (34.7%) amplified at least one polymorphic fragment and only 73 (23.7%) showed clear and scorable bands. The 73 primers produced a total of 121 polymorphic fragments (1.66 marker/selected primer). Similar ratios have been reported for intraspecific RAPD polymorphisms in *Pinus elliottii* (Nelson et al. 1993), *Eucalypthus nitens* (Byrne et al. 1995), cacao (Crouzillat et al. 1996), and papaya (Sondur et al. 1996). Considerably higher polymorphism levels have been detected both in interspecific and other intraspecific studies, and up to five RAPD polymorphic markers per selected primer have been detected in a intraspecific cross of apple (Hemmat et al. 1994). This low polymorphism level of oil palm was expected, since the genetic base of most of the oil palm breeding programs is known to be narrow (Meunier 1976; Hardon et al. 1985). Wider interspecific crosses (*E. guineensis*×*E. oleifera*) would probably allow the detection of higher polymorphism levels. We chose a cross that would be more relevant and could immediately generate useful information to the EMBRAPA oil palm breeding program.

Genetic linkage maps and genome length estimates

We used the pseudo-testcross mapping strategy in combination with the RAPD assay to construct genetic linkage maps of *Elaeis*. These maps are individual-specific and cannot be consolidated into a single map. By using this approach, individual-specific maps can be quickly constructed to elite palms on breeding programs, aiming at a marker-assisted selection. The utility of individual-specific maps and the possibility to integrate linkage maps had been thoroughly discussed by Grattapaglia and Sederoff (1994).

Given the estimated total map distances and genome sizes of *Elaeis*, the relationships between physical and genetic distances could be established (1.06 Mbp/1 cM and 1.09 Mbp/1 cM, for tenera and pisifera, respectively). A limited genome coverage was obtained with the two maps (28.0%, for tenera and 25.6%, for pisifera). Lin and Ritland (1996), using a bootstrap approach, showed that only approximately 40 markers are needed to give an accurate estimate of genome length. Therefore, the estimates in the present study should not be affected by the relatively low number of markers mapped (48 for tenera and 42 for pisifera).

Marker-assisted selection for shell thickness

Results obtained in the present study confirm the utility of bulked segregant analysis for the identification of markers in specific chromosomal regions. Although the genome coverages were limited, it was possible to identify two markers linked on both sides of the sh^+ locus (R11–1282 at 17.5 cM and T19–1046 at 23.9 cM). The identification of markers linked to the sh^+ gene confirmed the existence of a major locus controlling fruit types in oil palm, corroborating the study of Beirnaert and Vanderweyen (1941).

In oil palm breeding programs tenera and pisifera palms are often selected from progenies that include all three types of fruits (tenera×tenera crosses) or at least two types (dura×tenera, tenera×pisifera). By using the flanking marker-based assay developed in this study, tenera and pisifera palms should be correctly identified in 96% of the cases, i.e., in the selection of tenera or pisifera palms resulting from this cross, wrong identification is expected to occur only for 4% of the plants (0.175×0.239=0.042), the approximate frequency of double recombinants. A smaller value was found in the present study, where only 1 out of 95 plants (less than 1%) were wrongly classified. The ability to select plants very early in plant development using these primers would allow one to separate desirable oil palm types prior to field transplanting. In the production of commercial tenera hybrids, pollen is always collected from pisifera plants of different crosses and, thus, spread out in different areas. The possibility of grouping pisifera plants in the same area would considerably increase the time efficiency and decrease the cost of pollen collection and maintenance of plants. Moreover, these markers would make possible a more precise and fast identification of supposed pisifera plants. These plants show a drastic female sterility, but this trait can also appear in tenera and dura plants. Therefore, some plants maintained in the field for more than 10 years could not be classified, because of the absence of fruits. Obviously, these associations must be confirmed on other crosses and this map region must be saturated with other molecular markers. Mapping of markers closer to the sh^+ locus than T19–1046 will make possible more efficient selections, since it was classified as type 1 (low-intensity bands) and was linked with a LOD score below 5.0 (3.64).

Associations of markers to genes controlling other characters of interest to oil palm breeding programs are being pursued in this project. Such characters include pisifera female sterility, disease resistance, oil composition and yield components. Linkage to a QTL (fruit production) was attempted with this population using MAPMAKER-QTL (Lander and Botstein 1989). No significant associations were found, probably due to the low genome coverage of the maps and to the population size. Map saturation with more markers is currently under development.

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