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P. L. Jack · T. A. F. Dimitrijevic · S. Mayes

Assessment of nuclear, mitochondrial and chloroplast RFLP markers in oil palm (*Elaeis guineensis* Jacq.)

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Abstract A variety of DNA probes was used to screen a diverse set of oil palm accessions in order to identify markers with a utility in genotype discrimination. This survey included samples of the commercial oil palm native to Africa (*Elaeis guineensis* Jacq.), the closely-related South American species [*E.oleifera* (HBK) Cortes] and inter-specific hybrids of the two. Of 106 major chloroplast bands none showed differences between *E. guineensis* and *E. oleifera*. Mitochondrial and ribosomal probes were more informative inter-specifically (the former allowing identification of the maternal inheritance of mitochondria) and may be useful in hybrid breeding programmes; however, they were unable to identify polymorphism within *E. guineensis*. In contrast, low-copy nuclear genomic clones were able to identify intra-specific variation, though in most cases they revealed a relatively small number of allelic variants. One DNA probe showed a much larger number of band variants, revealing ten patterns amongst 13 *E. guineensis* accessions, and should prove useful in genetic fingerprinting and evaluation of oil-palm germplasm collections.

Key words Oil palm · *Elaeis guineensis* *Elaeis oleifera* · RFLP · Genetic fingerprinting

Introduction

The oil palm (*Elaeis guineensis* Jacq.) represents one of the most important sources of vegetable oil and is used in a wide range of edible products. Worldwide commercial production in 1992 amounted to 16.6 million tonnes of palm and kernel oil, second only in importance to soyabean (USDA 1993). Yields of 6 tonnes of oil per hectare per year are possible under favourable conditions, considerably exceeding that of the annual oil crop production of around 1 tonne per hectare (Jones 1983). Although originating from West Africa, commercial plantings have been established throughout the equatorial regions of the world and now cover some 3 million hectares. Breeding and selection began in the early 1920s and since then considerable improvements have been achieved both in yield and quality characters (Rosenquist 1985; Hartley 1988). Nevertheless, the long breeding cycle (over 10 years) and the extensive variation still encountered, suggests that there exists considerable scope for improvement in yield (Corley 1983), disease resistance (Flood et al. 1989), and oil composition.

The oil palm, like maize, is monoecious and is naturally cross-pollinated. Artificial selfing has, in some populations, been reported to result in considerable yield depression, though in others, yields of inbred progeny have been reported to exceed selected palm crosses (Hartley 1988). Such differences may arise through contrasting levels of heterozygosity, but lack of conventional genetic markers has made this difficult to confirm. The recent availability of DNA markers, such as those based upon restriction fragment length polymorphism (RFLP), facilitates such comparisons through direct genome analysis; indeed work with maize has shown that RFLP analysis provides the best predictor of grain-yield heterosis, even exceeding that of pedigree information (Smith et al. 1990). At a simpler level, RFLPs also provide valuable information concerning breeding history, can be used to identify illegitimate outcrossing events in elite progenies, and provides a means of characterising germplasm collections. The latter is of particular relevance to oil-palm improvement since many of the major commercial plantings are derived from materials with a narrow genetic base. For example, Malaysia, the world's largest producer, has an industry based upon the Deli dura population derived from only four ancestral palms of uncertain origin, introduced into an Indonesian botanic garden in 1848 (Hartley 1988).

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P. L. Jack (✉) · S. Mayes
Plant Breeding International, Maris Lane, Cambridge, CB2 2LQ, UK

T. A. F. Dimitrijevic
Unilever Research, Colworth House, Sharnbrook, Bedford, MK44 1LQ, UK

The most effective way of utilising RFLP markers is through the development of an ordered linkage map, as has been achieved for most of the major temperate crop species (Chang and Myerowitz 1991). Such a map allows analysis of the entire genome using the minimum number of probes and would, in oil palm, allow identification of markers for important simply-inherited characters, such as shell thickness (Jones 1983) and resistance to Fusarium wilt (de Franqueville and de Greef 1988), and potentially may also facilitate dissection of complex quantitative traits, such as yield and oil quality, into individual genetic components or quantitative trait loci (QTLs), as described by Lander and Botstein (1989).

Clearly long-lived perennial crops with long breeding cycles, poorly characterised genotypes, and high individual value, offer one of the most productive opportunities for DNA marker-assisted breeding (Jack and Mayes 1993). As a first step towards such RFLP applications in oil palm, we have assessed DNA polymorphism using a variety of genetic elements, including those from mitochondrial, chloroplast, ribosomal and low-copy DNA and have identified probes of high utility in genotype characterisation.

Materials and methods

Germplasm

Identification of interspecific informative probes was achieved using a range of genotypes including ten of *E. guineensis*, seven of *E. oleifera*, and one *E. guineensis* × *E.oleifera* hybrid (Fig. 1).

For identification of intra-specific polymorphism, a larger set of *E. guineensis* genotypes was examined. This latter comprised two samples of *E. oleifera* originating from Brazil and Central America (numbers 1 and 2 respectively in Table 1), two inter-specific hybrids (numbers 3 and 4) and 15 samples (13 genotypes) of *E. guineensis* derived from plantations throughout Africa and South East Asia, some of which are related by pedigree as indicated in Table 1. Most DNA preparations were made from ramets (tissue culture-derived clonal lines, Jones 1983) and in two cases were also obtained from the original explanted palms (ortets) for comparison (numbers 9, 10 and 11, 12 of Table 1). One DNA sample (number 6) was also prepared from pollen, stored at ambient for 4 years.

Probes

Oil-palm mitochondrial probes (designated pOPm...) were selected from an *Eco*R1/pUC13 library constructed from DNA of purified mitochondria, prepared by differential centrifugation of meristem tissue-homogenates. Low-copy-number DNA probes (designated pOPg...) were selected from an *Eco*R1/pUC13 library of total spear leaf DNA by hybridisation to total oil-palm DNA (Helentjaris et al. 1986). The wheat ribosomal probe pTA71, isolated by Gerlach and Bedbrook (1979), was kindly supplied by R. B. Flavell (John Innes Centre, Norwich), whilst the wheat chloroplast probes pTacB(8,9), pTacB(10–18), pTacB(2–15), pTacP3, pTacP4 and pTacP7 (Bowman and Dyer 1986) were gifts from C. M. Bowman (John Innes Centre, Norwich) and E. A. Kellog (Harvard University).

Southern analysis

Total oil-palm DNA was purified from all tissues using a modified form of the method of Dellaporta et al. (1983). The modifications involved inclusion of 1% polyvinyl-pyrrolidone 40 in the extraction buffer, omission of the 65°C incubation step, and removal of RNA by digestion with 50 µg/ml of pre-boiled pancreatic ribonuclease (Maniatis et al. 1982). DNA (2 µg) was digested with restriction enzymes, fragments were separated on 1.0% agarose gels at 30 V for 24 h, capillary blotted (Southern 1975) to nylon membranes (Hybond-N, Amersham International) and UV-fixed (Khandjian 1987). Filters were pre-hybridised at 65°C for at least 5 h in 6 × SSPE, 1 × Denhardt's, 100 µg/ml of boiled salmon sperm DNA (Maniatis et al. 1982). Alkali-denatured probes (12 ng), labelled with 20 µCi of 3000 Ci/mmole ^{32}P-dCTP (Feinberg and Vogelstein 1983) and purified by gel filtration, were then added and hybridisation continued overnight. Filters were washed once with 2 × SSC/0.1% SDS and twice with 0.1 × SSC/0.1%SDS, each for 20 min at room temperature. Autoradiography was performed with X-Omat AR film (Kodak) at –70°C with intensifying screens. Filters were stripped by immersion in 0.1% SDS at 95°C for 10 min.

Results

Ribosomal RNA genes (rDNA)

Spacer-length variation between individual rDNA repeats has been identified in many plant species and has been used to distinguish related species (e.g., *Brassica oleracea* and *B. campestris*, Bennett and Smith 1991), to identify introgressed chromosomal regions (e.g., *Lycopersicon hirsutum* in *L. esculentum*, Levesque et al. 1990), and to distinguish between cultivars (e.g., *Hordeum vulgare*, Saghai-Maroof et al. 1984). Figure 1a shows the oil-palm rDNA spacer-length polymorphism identified with *Hin*dIII. This enzyme cuts once within each rDNA repeat in *E. guineensis* to give a single band at 18 kb. *E. oleifera* samples reveal a much-higher-molecular-weight band running at the upper gel-mobility limit (over 30 kb), due presumably to absence of the *Hin*dIII site. As expected, both bands are present in the inter-specific hybrid. Analysis of the larger set of *E. guineensis* samples described in Table 1 confirmed that this variation was solely inter-specific (data not shown).

Mitochondrial and chloroplast probes

Organellar probes have also been used for identification purposes, as in the discrimination of specific *Brassica* cytoplasms in sexual or somatic hybrids (Kemble 1987). In addition, since mitochondrial and chloroplast genomes are usually uni-parentally inherited (from the maternal parent in most angiosperms and the paternal parent in gymnosperms, Neale et al. 1986), such probes can be used to confirm the direction of a cross. In order to establish the inheritance of mitochondrial genes in oil palm, a DNA library was prepared using DNA from purified oil-palm mitochondria and a number of recombinant clones examined. Figure 1b shows typical results where probe pOPm3 identifies two bands common to both *E. guineensis* and *E. oleifera*, as well as 4.4 kb *E. oleifera*-specific and 2.5-kb *E. guineensis*-specific bands (arrowed). The hybrid possesses

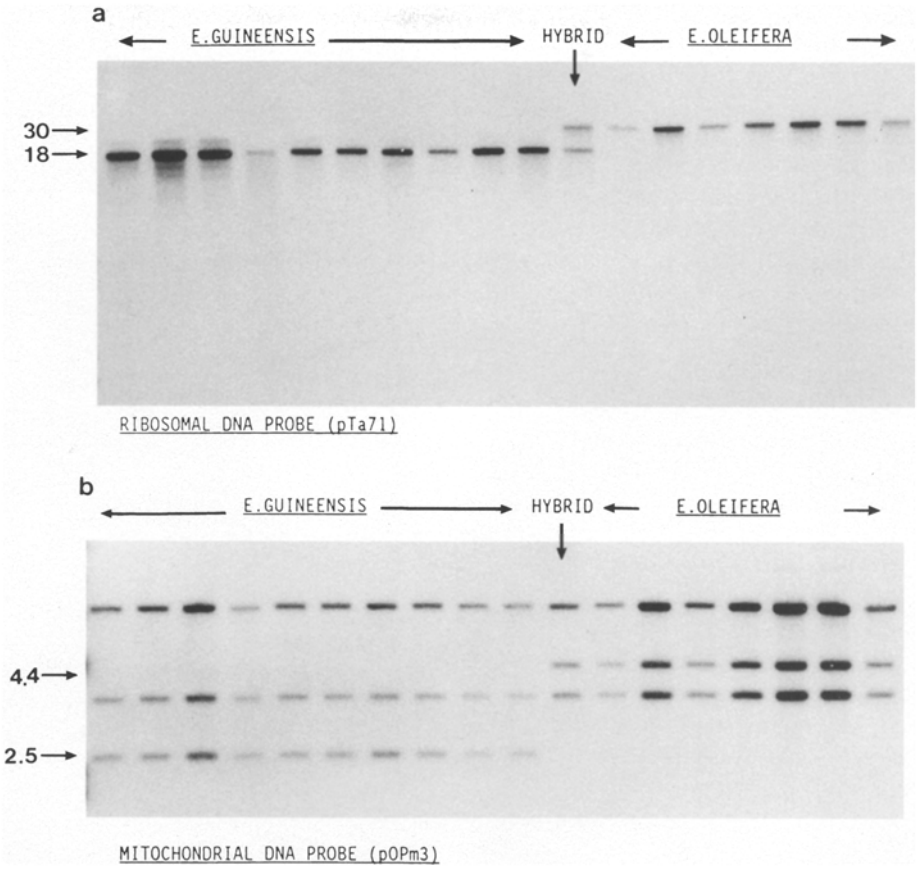

Fig. 1a, b Examples of interspecific polymorphism in oil palm. Southern analysis using: **a** the ribosomal (pTa71) probe on *Hin*dIII-digested DNA, identifying 18-kb and >30-kb bands (*arrowed*) in *E. guineensis* and *E. oleifera* respectively and **b** the mitochondrial (pOPm3) probe on the same samples digested with *Bam*H1, identifying 2.5-kb and 4.4-kb bands (*arrowed*) in *E. guineensis* and *E. oleifera* respectively, though only the maternal band in the hybrid

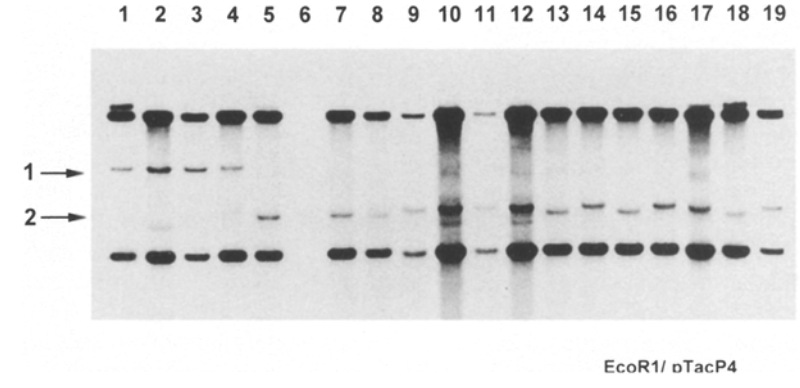

Fig. 2 Autoradograph showing minor band variation (*arrowed*) encountered with the chloroplast probe P4 on *Eco*R1-digested DNA from the genotype set described in Table 1

the 4.4-kb but not the 2.5-kb band and, since the maternal parent was *E. oleifera*, indicates maternal inheritance of mitochondria in oil palm (confirmation that this sample was indeed hybrid can be seen with the rDNA probe). Similar levels of variation were obtained with known-function mitochondrial probes, such as the *atpA* gene (Small et al. 1987).

Chloroplast DNA has also been used to characterise germplasm (Terauchi et al. 1992), though lower levels of RFLP-accessed polymorphism generally restrict it to inter- rather than intra-specific studies. Wilson et al. (1990) have described chloroplast variation in 22 palm species (though not in *E. guineensis*) and reported a much reduced rate of sequence change relative to annual plants. In order to establish chloroplast RFLP variation in oil palm, a set of six wheat chloroplast probes, encompassing 67 kb (50%) of the wheat chloroplast genome, were used to examine three genotypes (one *E. oleifera* and two *E. guineensis* samples), following digestion with eight restriction enzymes. This set of 40 probe/enzyme combinations generated 106 major scorable bands, none of which differed. Variation was, however, frequently encountered with minor chloroplast-hybridising bands. An example is shown in Fig. 2 of an autoradiographic over-exposure of probe pTacP4 on *Eco*R1-digested DNA. In this figure, the minor bands arrowed 1 and 2 appear to be allelic and species-spe-

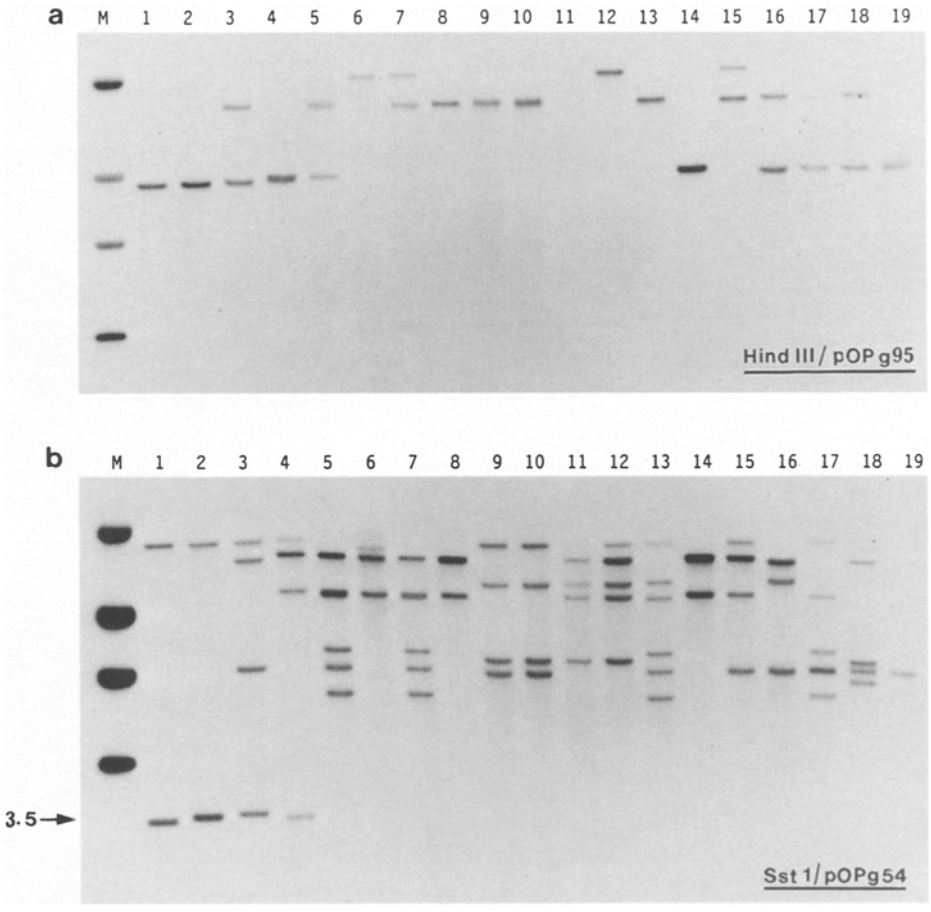

Fig. 3a, b Southern analysis using low-copy nuclear probes. The Table 1 genotypes probed with either **a** pOPg95 on *Hin*dIII-digested DNA, or **b** pOPg54 on *Sst*1-digested DNA, revealing substantial intra-specific polymorphism

cific. However, we were reluctant to score such variants as chloroplast polymorphisms, partly because they are minor bands but also because their presence does not always correlate with the major chloroplast species. This is particularly evident in pollen DNA where major chloroplast bands are absent (Fig. 2, lane 6), but where the minor band can still be detected on the original autoradiogram. Such bands may arise from subgenomic species which are not subject to the same regulatory mechanisms as the major plastid genome.

Three other palms were also included in this analysis, coconut (*Cocos nucifera*), date (*Phoenix dactylifera*) and sago (*Metroxylon sagu*), as well as a wheat control. These species were found to have 65%, 62%, 49% and 8% band identity, respectively, with *E. guineensis,* in contrast to the near 100% identity of *E. oleifera* and *E. guineensis*. These results demonstrate the very close similarity of chloroplast genomes in the two *Elaeis* species, despite their presumed separation of 84–106 million years through the African and South American continental divide (Pitman et al. 1993).

Low-copy probes

The above ribosomal and organellar probes revealed little variation between the relatively diverse set of *E. guineensis* genotypes examined. In order to identify probes capable of identifying such differences, random low-copy-number inserts from an *Eco*R1 library were examined. Figure 3a shows the result obtained with one such clone, pOPg95, on *Hin*dIII-digested DNA. In this case polymorphism is evident within *E. guineensis* (three bands, present in all combinations), though unlike the ribosomal and organellar probes above, no band combinations are species-specific. Most probes examined were of this type; however, one DNA clone (pOPg54) revealed substantially more polymorphism. This is shown in Fig. 3b where pOPg54 has been used to probe *Sst*1-digested DNA. In this case a 3.5-kb band can be identified in the two *E. oleifera* accessions, both of the hybrids (as well as a further five *E. oleifera* accessions examined, data not shown), but not in any of the *E. guineensis* samples, thus providing a third type of inter-specific diagnostic marker. This particular probe generates between two and six bands for each genotype, with the set of 17 genotypes as a whole yielding at least ten clearly distinct bands in 13 different combinations i.e., most genotypes can be distinguished from each other. In fact, using a combination of pOPg54/*Sst*1 and pOPg95/*Hin*dIII, all of the 17 *E. guineensis* genotypes can be unambiguously distinguished from each other (Table 1). Note that the pairs of ortet (palm) and ramet (clonal line) samples (9 and 10, 11 and 12) cannot be separated, as expected for the same genotype.

Table 1 Genotypes used in Figs. 2, 3 and 4, and identification of polymorphic bands (letters A–J from top of gel) generated with ribosomal (pTa71), mitochondrial (pOPm3) and low-copy anonymous clones (pOPg54, pOPg95). E.o.=*E. oleifera*, E.g.=*E. guineensis*

Lane no.	Species	Accession (comments)	Polymorphic bands present			
			Ta71	pOPm3	pOPg95	pOPg54
1	E.o.	–	A	B	C	A, J
2	E.o.	90/6/7	A	B	C	A, J
3	E.o.×E.g.	–	A, B	B	B, C	A, B, G, J
4	E.o.×E.g.	5417 (4/3312 × 5006)	A, B	B	C	A, B, D, J
5	E.g.	5006	B	A	B, C	B, D, E, G, I
6	E.g.	– (2/2311 self)	B	A	A	B, D
7	E.g.	5015 (2/2311 × 5006)	B	A	A, B	B, D, E, G, I
8	E.g.	7001 (2/2311 × 5006)	B	A	B	B, D
9	E.g.	7037	B	A	B	A, C, F, G
10	E.g.	7037	B	A	B	A, C, F, G
11	E.g.	7083	B	A	A	A, B, C, D, F
12	E.g.	7083	B	A	A	A, B, C, D, F
13	E.g	5049 (sib 5045)	B	A	B	A, C, D, E, G, I
14	E.g.	5045 (sib 5049)	B	A	C	B, D
15	E.g.	5056	B	A	A, B	A, B, D, G
16	E.g.	5082	B	A	B, C	B, C, G
17	E.g.	5062 (sib 5063)	B	A	B, C	A, D, E, G, I
18	E.g.	5063 (sib 5062)	B	A	B, C	B, F, G, H
19	E.g.	115E	B	A	C	B, G

Digestion of oil-palm DNA with the same enzyme as used to prepare the probe library (*Eco*R1) gives further information concerning these markers. In particular it will allow selection of those probes displaying length polymorphism on PCR amplification. Probe pOPg95 (Fig. 4a) yields two band alternatives, present either singly or in combination, suggesting a single locus with two alleles. Comparison of Figs. 4a and 3a, however, shows no correspondence between polymorphisms generated by *Hin*dIII and *Eco*RI, indicating that at least one further polymorphic event is involved. In contrast, the highly polymorphic pOPg54 probe yields a very uniform pattern, with two major bands at 1.15 kb and 1.05 kb present in all samples (Fig. 4b). Additional minor bands are found in four genotypes, presumably representing occassional loss of *Eco*R1 sites (Fig. 4b). The lack of variation in the major bands in Fig. 4b demonstrates that the hypervariable nature of this locus is conferred by a region outwith the cloned element.

Discussion

We have used a range of oil-palm genotypes to assess DNA polymorphism at a number of distinct loci, including ribosomal RNA genes, mitochondrial, chloroplast and low-copy-number nuclear DNA sequences. Different levels of RFLP are encountered within each of these classes, providing an array of probes of complementary utility. Those markers which show polymorphism between, rather than within, species, such as the ribosomal and mitochondrial probes (Fig. 1), are of value in inter-specific hybridisation programmes. For example, although the major plantation species is *E. guineensis*, originating from West Africa (Hartley 1988) and now grown throughout the equatorial world, there is interest in the South American relative *E. oleifera* (formerly known as *E. melanococca*), with which it can form fertile hybrids (Meunier et al. 1976). The South American species possesses a more liquid and less saturated oil, resistance to certain diseases, and a procumbent growth habit, all of which are of commercial interest, but it also has a much lower yield than *E. guineensis*, as do hybrids between the two. Identification of high-yielding progeny in back-crossing programmes and rapid expansion through tissue-culture clonal propagation may provide a route for introgressing desirable *E. oleifera* characters into a high-yielding *E. guineensis* background. However, it would be advantageous to be able to confirm the hybrid status of original crosses and screen out the non-hybrid rogues, which frequently arise through errors in pollination and during seed treatment.

Those probes displaying high levels of variability within *E. guineensis*, for example pOPg54 (Fig. 4b), have a number of applications. One such use is in confirming

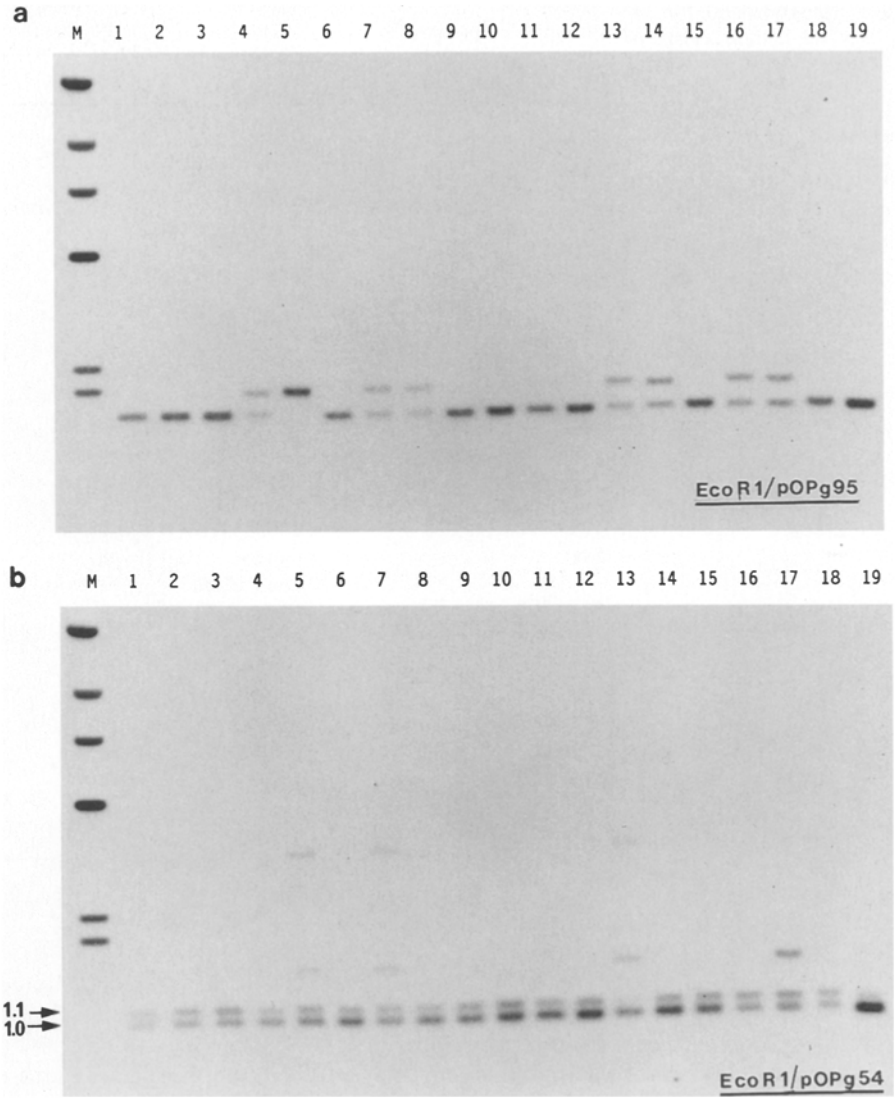

Fig. 4a, b Southern analysis of RFLP variation within the probe domain. DNA from the genotypes of Table 1 were digested with *Eco*R1 (the enzyme used for library preparation) and probed to **a** pOPg95 or **b** pOPg54

the identity of tissue culture-derived clones to the original selected elite palms. This is particularly important for oil palm given that it is grown in widely contrasting geographic locations and is unlikely to perform equally across all of those environments. For example, Malaysian progenies are highly susceptible to the devastating and uncontrollable vascular wilt caused by *Fusarium oxysporum* f. sp. *elaeidis* – a major African disease, totally absent from Malaysia (Ho et al. 1985). In addition, Africa suffers more extreme droughts than S.E. Asia. Such regional differences mean that it is essential to avoid inadvertant mixing of clones selected from different environments. We show here that only a small number of DNA probes are required to confirm the identity of genotypes.

Such probes can also be used to assess genetic diversity within breeding populations and to assist in parental selection; for example, to avoid inbreeding depression and maximise genetic contrast. It is likely that they will also have considerable merit in characterising wild prospections (e.g., those described by Rajanaidu 1986) in order to identify potentially novel sources of genic variation, as well as assisting in the maintenance of such collections, and in confirming that they represent truly wild material, rather than material released from existing breeding programmes.

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