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Development of new marker methods—an example from oil palm

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Abstract

This paper reviews marker methods based on retrotransposons and illustrates examples from oil palm. Prior to this study, very little had been known about the repetitive DNA present in oil palm and no marker systems based on retrotransposons had been developed. Firstly, *copia*-like retrotransposons of *Elaeis oleifera*, *Elaeis guineensis* and *Cocos nucifera* were characterized by performing phylogenetic analyses on a portion of the reverse transcriptase (RT) gene of *copia*-like retrotransposons. The results identified three classes of *copia*-like retrotransposons in the three species studied. While the C-class RT sequences seemed to have amplified preferentially only in *Elaeis guineensis*, the classes A and B were present in *Elaeis guineensis* and *Cocos nucifera* as well. Secondly, long terminal repeats (LTRs) from these retrotransposon classes were isolated as a prelude to developing a marker system in palm based on retrotransposons. The B- and C-class LTRs were isolated by primer walking from the RT region and the B-class from *Elaeis oleifera* and *Cocos nucifera* with the use of oil palm-specific RnaseH primers. The method has been developed and applied for breeding purposes in oil palm, hybrids between *E. oleifera* and *E. guineensis* and within the tribe Cocoeae. There is scope for the method to be used in the isolation of new retrotransposon families endogenous for each palm species and as an alternative to amplified fragment length polymorphism (AFLP) in diversity studies within the Palmae.

Keywords: *Elaeis guineensis*; *Elaeis oleifera*; *Cocos nucifera*; *copia*-like retrotransposons; diversity

Introduction

The genetic variation inherent in most species provides both the basis for breeding and natural selection and the means to track these processes. Genetic variation is associated with polymorphisms in DNA sequences, which can be studied by using molecular markers. Molecular marker technology is playing a vital role in plant biology, including DNA fingerprinting, genetic linkage mapping and phylogenetic studies to reveal ancestry. Retrotransposons (RTNs) can be used as a source of informative markers because of their ability to integrate into a multitude of loci in the genome and thereby generate insertional polymorphisms between individuals. RTNs encode the proteins needed for their own propagation, and through cycles of replication have come to represent major fractions of the genome in many eukaryotes. While in this way they are both 'selfish' and parasitic, RTNs nevertheless can be of potential benefit to their host.

Marker methods based on retrotransposons

Long terminal repeat (LTR) RTNs are ubiquitous in plant genomes (Flavell *et al.*, 1992; Kubis *et al.*, 1998) and often

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present in high copy number. They are widely dispersed on chromosomes and widely distributed within the euchromatin domains of chromosomes in plants (Pearce *et al.*, 1997; Castilho *et al.*, 2000) whereas their distribution in heterochromatin may differ between plant species (Pearce *et al.*, 1996). Ty3-*gypsy* elements show a preference for centromeric regions and may co-localize with rRNA genes (Presting *et al.*, 1998; Balint-Kurti *et al.*, 2000). Sequence heterogeneity is high among retroelements, associated with the comparatively low fidelity of both transcription and reverse transcription, resulting in accelerated sequence divergence (Gabriel and Mules, 1999). Because of their ubiquity and diversity, RTNs have great potential as genetic markers for plant genome and biodiversity analysis (Lee *et al.*, 1990; Kumar *et al.*, 1997; Waugh *et al.*, 1997; Ellis *et al.*, 1998; Kalendar *et al.*, 1999; Pearce *et al.*, 2000).

There have been several marker systems based on LTR RTNs, including sequence-specific amplified polymorphism (SSAP), inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP), developed for plants (Waugh *et al.*, 1997; Ellis *et al.*, 1998; Kalendar *et al.*, 1999). In the case of RTN-based markers, polymorphisms are generated by retrotransposition, which is a unidirectional process resulting in insertions of RTN daughter copies into new sites without removal of the parental copies from their sites. The consequence of retrotransposition is the alteration of a target site by integration of a few hundred base pairs to a few kilobases. By contrast, marker systems based on simple sequence repeats (SSRs) detect random, small-scale (i.e. from one up to a few tens of nucleotides) and bi-directional changes at a particular locus between individuals of the same species. Genomic locations of MITE (miniature inverted transposable element) transposons are also variable within species. These polymorphisms have been successfully exploited as genetic markers in maize and rice (Casa *et al.*, 2000; Chang *et al.*, 2001). The fact that MITEs reside close to genes and the high sequence identity within some families such as *Heartbreaker* (*Hbr*) makes them useful genetic markers (Casa *et al.*, 2000; Chang *et al.*, 2001), although they may not stay stably integrated.

The SSAP (Fig. 1) method is a modified version of amplified fragment length polymorphism (AFLP; Vos *et al.*, 1995), in which one AFLP primer is replaced with an LTR primer. REMAP (Fig. 2) exploits polymorphisms among amplicons produced between anchored microsatellite sequences and RTNs. IRAP (Fig. 3) amplifies segments lying between two LTR sequences in any orientation. Retrotransposon-based insertional polymorphism (RBIP) involves polymerase chain reaction (PCR) with primers derived from the RTN and flanking sequences to codominantly score for the presence or absence of an insertion at specific loci. Retrotransposon internal variation polymorphism (RIVP; Fig. 4) exploits heterogeneity of an internal structure of a RTN in a PCR-based SSAP method (Vershinin and Ellis, 1999).

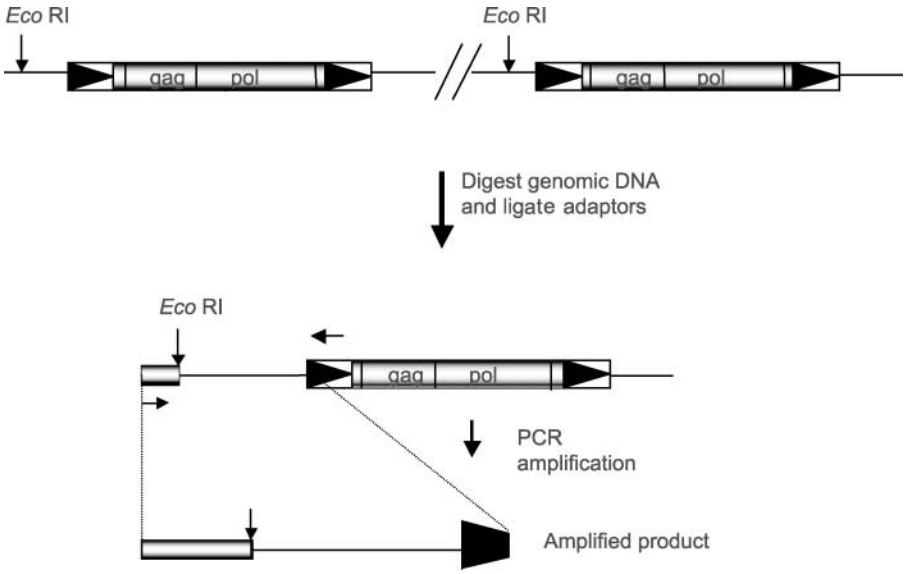

Fig. 1. SSAP (sequence-specific amplified polymorphism). DNA is digested with one or two enzymes, adapters are ligated to all cut fragments, and the product is used for PCR. The choice of enzyme is dependent on the fact that it should cut in the flanking DNA but not within the LTR between the priming site and the terminus. PCR is performed using primers complementary, respectively, to the adapter and to ends of LTRs (arrows). Insertional polymorphisms are visualized as dominant bands on a sequencing gel. The SSAP method has been applied in barley (Kumar *et al.*, 1997; Waugh *et al.*, 1997), peas (Ellis *et al.*, 1998; Pearce *et al.*, 2000), wheat (Gribbon *et al.*, 1999), *Medicago* (Porceddu *et al.*, 2002), tobacco (Melayah *et al.*, 2001) and grapevine (Pelcy and Merdinoglu, 2002).

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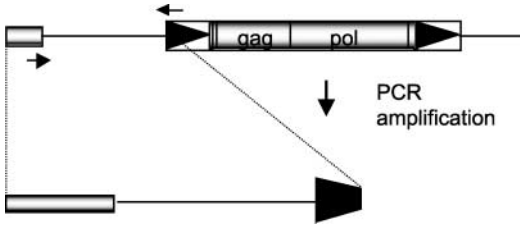

Fig. 2. REMAP (retrotransposon microsatellite amplified polymorphism). PCR primers (black arrows) face each other respectively from the end an LTR and the proximal end of a microsatellite. The microsatellite is anchored at its flank. Microsatellites and retrotransposons appear to be often associated (Ramsay *et al.,* 1999; Castilho *et al.,* 2000). This method was used in barley (Kalendar *et al.,* 1999; Manninen *et al.,* 2000; Vicient *et al.,* 2001); in producing a high-density cytogenetic map of *Aegilops tauschii* (Boyko *et al.,* 2002); and in studying the genomic stability of an allopolyploid *Spartina anglica* (Baumel *et al.,* 2002).

The LTR sequences for developing marker methods based on RTNs can be isolated either by primer walking from the reverse transcriptase gene or can be mined from databases for some crops. However, the development of a rapid technique for isolating LTRs made this process faster and easier (Pearce *et al.*, 1999). Marker systems based on LTR RTNs such as SSAP (Waugh *et al.*, 1997), IRAP and REMAP (Kalendar *et al.*, 1999) and RBIP (Ellis *et al.*, 1998) provide useful genetic markers for saturating existing genetic maps, for assessing biodiversity within crop plants, managing genetic resources in genebanks, for fingerprinting of existing or new crop plant varieties and for mapping agronomically important traits and resistance to diseases. Some of the recent applications are described below.

Identifying new insertions

RTNs increase their copy number by replication followed by insertion of a new copy into the genome. It is possible to visualize those insertions with RTN-based marker methods. Melayah *et al.* (2001) showed that 38 out of 41 new RTN marker bands corresponded to *de novo* insertions of *Tnt*1 in stressed tobacco plants. The IRAP method was also used in identifying new insertions of *BARE*-1 element in an interspecific hybrid in *Spartina* (cordgrass; Baumel *et al.*, 2002). *BARE*-1 is an active, *copia*-like RTN (Jääskeläinen *et al.*, 1999), which shows remarkable variation among species in the genus *Hordeum* and among populations of the wild barley species *Hordeum spontaneum* (Vicient *et al.*, 1999; Kalendar *et al.*, 2000). Approximately 100 years after its formation, the parental marker bands were still additive and few new insertions were observed in the interspecific *Spartina* hybrid (Baumel *et al.*, 2002). Novel *BARE*-1 insertions were observed by Yu and Wise (2000) in

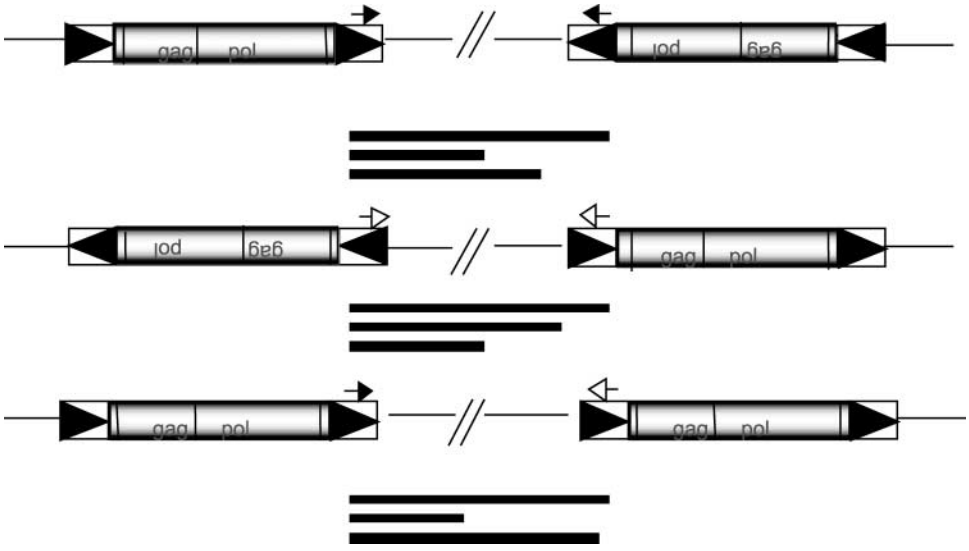

Fig. 3. IRAP (inter-retrotransposon amplified polymorphism). IRAP amplifies polymorphism between two LTR sequences in any orientation. There is no need to digest genomic DNA with enzymes. PCR is performed with one or two LTR primers. The figure illustrates bands that might be observed when either or both of two primers (black and white arrows) matching the LTR and oriented in either the sense (black) or antisense (white) direction with respect to the coding region are used. The retrotransposons can be in the three possible orientations shown. Insertional polymorphisms are visualized as polymorphic, dominant bands on either sequencing or on agarose gels. This method was first implemented in *Hordeum* by Kalendar *et al.* (1999) and since then has used by Kalendar *et al.* (2000) and Vicient *et al.* (2000) for fingerprinting and biodiversity analysis, and for mapping of agronomically important traits by Manninen *et al.* (2000) and Boyko *et al.* (2002). Baumel *et al.* (2002) investigated genomic stability of an allopolyploid (*Spartina anglica*) with the use of this method. Price *et al.* (2002a) reported development and application of IRAP for oil palm.

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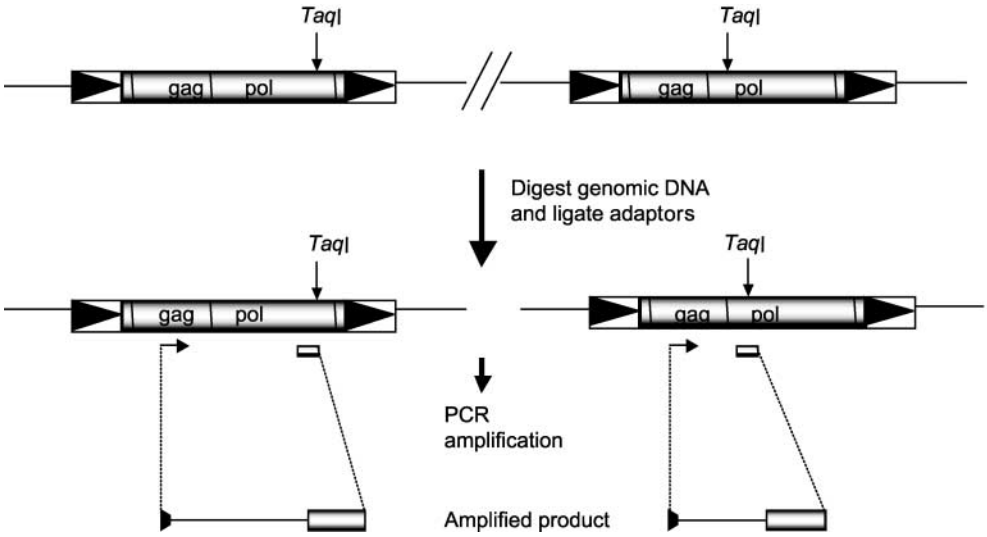

Fig. 4. RIVP (retrotransposon internal variation polymorphism). DNA is digested with one or two enzymes, adapters are ligated to all cut fragments and the mixture is used for PCR. PCR is performed using primers complementary to the adapter and primers that are complementary to GAG (arrows). Insertional polymorphism appears as polymorphic, dominant bands on a sequencing gel. The profile of amplified products is visualized on a sequencing (PAGE) gel. This method has been used for studying genetic diversity in 56 *Pisum* accessions (Vershinin and Ellis, 1999).

recombinant inbred (RI) lines of *Avena*. Over a broad range of grasses, *BARE*-1 is actively expressed (Vicient *et al.*, 2001).

Linkage analyses and mapping of agronomic traits

Kenward *et al.* (1998) cloned and sequenced a randomly amplified polymorphic DNA (RAPD) maker associated with black root resistance in tobacco and found it to be a part of the retrotransposon Tnd-1. The Tnd-1 RTN is present in multiple copies in *Nicotiana debneyi*, a wild progenitor of *Nicotiana tabacum*, and it is believed to have been introgressed into cultivated tobacco in association with the black root resistance gene. A *BARE*-1 RTN was found to be located 0.28 cM from the *Mla* locus, a member of a multigene family that confers resistance to powdery mildew (*Erysiphe graminis*). Analysis of nested complexes of LTR RTNs adjacent to the *Mla* locus showed that the present *Mla* locus evolved over a period of more than seven million years via duplication, inversion and transposon-insertion events and that meiotic recombination was not favoured as a means of diversification of this locus (Wei *et al.*, 1999, 2002). Bhattacharyya *et al.* (1997) isolated and characterized the low copy *copia*-like RTN Tgmr, tightly linked to the *Rps1-k* allele that confers race-specific resistance to *Phytophtora sojae* in soybean. Schneider *et al.* (1999) identified a *PDR*-1-based marker closely associated (~0.5 cM) with the gene *Sym19* which is likely to be involved in the early stages of nodule formation in peas.

SSAP analysis was used for constructing linkage maps in barley (Waugh *et al.*, 1997), peas (Ellis *et al.*, 1998), oat (Yu and Wise, 2000), and IRAP was used for oil palm (Price *et al.*, 2002a). Manninen *et al.* (2000) identified 10 REMAP markers linked to seedling resistance to net blotch in barley and suggested the possibility of applying RTN markers in mapped-based cloning. RTN markers are often clustered as observed by Manninen *et al.* (2000) and some clustering of *BARE*-1-based SSAP markers is obvious in Yu and Wise's (2000) map of *Avena* as well. Boyko *et al.* (2002) mapped 80 RTN (IRAP and REMAP) markers on to a high-density cytogenetic map of *Aegilops tauschii*. Although most of the RTN loci were clustered in pericentromeric regions, some mapped into gene islands. RTN clusters coincided with the areas of lower recombination frequency and thus clustering most likely reflects the structure of the grass genome in which 'gene islands' are surrounded by 'repeat seas' (Panstruga *et al.*, 1998). Similarly in *Arabidopsis*, higher RTN density coincided with regions of lower recombination (Arabidopsis Genome Initiative, 2000).

Biodiversity, phylogeny and varietal classification

Phylogenetic analysis of a portion of the RT gene revealed a complex relationship between RTNs within the Triticeae (Gribbon *et al.*, 1999; Matsuoka and Tsunewaki, 1999). These studies showed that many groups were shared though some were species-specific. The lowest organizational level consists of a subgroup that is shared among closely related species but not shared among distant relatives. The subgroups cluster

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into supergroups (such as *BARE*1 and *Tta*15) that are present across the entire extent of the Gramineae. The cladistic topology of RTNs in the Triticeae is consistent with a long history of replication and integration, combined with the more recent emergence of new variants.

The insertional polymorphism of *BARE*-1 RTNs in the Triticeae was examined by SSAP analysis (Gribbon *et al.*, 1999). The analysis revealed common bands shared by members of the Triticeae. However, various RTNs exhibit different degrees of insertional polymorphism within the same species (Gribbon *et al.*, 1999). SSAP markers cannot be attributed solely to retroelement mobility because of the contribution of variability in the occurrence of restriction sites to the overall observed polymorphism. Vicient *et al.* (2001) used IRAP to examine insertional polymorphism of the *BARE*-1 retroelement in the grass family. The authors reported amplification of bands not only in the tribes Triticeae (*Triticum* spp.) and Erhathoideae (*Oryza* spp.) but also for the cordgrass tribe Chloroideae and for maize (tribe Panicoideae). Furthermore, IRAP polymorphisms revealed that *Aegilops tauschii*, the D-genome donor to the bread wheat (*Triticum aestivum*), shares with bread wheat monomorphic bands that may represent *BARE*-1 copies integrated prior to the advent of *T. aestivum*. The prevalence of RTNs in IRAP is consistent with the observed retroelement clusters in grasses (San Miguel *et al.*, 1996; Panstruga *et al.*, 1998; Shirasu *et al.*, 2000).

Iwamoto *et al.* (1999) used intron-2 of the *Oryza sativa Cat*A catalase gene, which shows similarity to the retroposon *p-SINE1*, for phylogenetic studies within genus *Oryza*. His studies revealed that *Oryza* species with genomes other than AA do not possess the *Cat*A homologue with an intron-2. Tatout *et al.* (1999) applied *SINE1* insertions (the presence or absence of *SINE1* at a given genomic site) and a microsatellite-like variation of *S1* 3′ poly(A) tails as a classification criterion for evaluating phylogenetic relationships within *Brassica oleareacea* complex. RTN insertional polymorphism was exploited in varietal classification of barley (Gribbon *et al.*, 1999; Kalendar *et al.*, 1999), *Hibiscus* (Lee *et al.*, 2002), *Citrus clementina* (Breto *et al.*, 2001) and grapevine (Pelcy and Merdinoglu, 2002).

Vershinin and Ellis (1999), Ellis *et al.* (1998) and Pearce *et al.* (2000) used RBIP and SSAP analysis for assessing patterns of genetic diversity and phylogeny within 56 accessions of the genus *Pisum*. Vershinin and Ellis (1999) estimated a phylogenetic tree based on the *gag* gene of the *PDR*1 RTN and Pearce *et al.* (2000) combined data from four different pea RTNs with contrasting integrational histories (i.e. the time and rate of integration). The data indicated that each element shows a high level of polymorphism but a different level of fixation within the species studied as revealed by F_{st} (level of fixation) and Nei's genetic distance. The finding that each element provides a high resolution for the *Pisum* lineages in which it has been active suggests that individual elements may proliferate differently as each diversifies through evolution. Both the *gag* and the combined phylogenetic trees resolved the *Pisum* accessions into four main clusters corresponding to different *Pisum* species lineages.

Genomic diversity

Kalendar *et al.* (1999) examined genomic diversity within a natural population of wild barley (*Hordeum spontaneum*) with the use of a REMAP marker system. They described patterns of RTN accumulation on a local spatial scale. The barley plants studied represented six natural populations growing under different conditions distributed across a 300 m transect of a single canyon in Israel. They observed variation in genome size which spanned across an environmental gradient and noted that plants from stressed sites (dry and south facing) had larger genome sizes. Using the REMAP marker system they examined transpositional activity of *BARE*-1 RTN and were able to distinguish clearly populations from south-facing slopes and populations from north-facing slopes. An important implication of this study is that the positive correlation between the height of the canyon and the *BARE*-1 copy number and observed *BARE*-1 transpositional activity might represent co-adaptation to environmental conditions on a local ecological scale (i.e. plants from stressed sites have the highest ratio of full-length to solo LTRs).

Gene tagging

The ability of some LTR RTNs to insert into low-copy regions (*Tos17*, Yamazaki *et al.*, 2001; *Tto1*, Okamoto and Hirochika, 2000) can be used for gene tagging (reviewed by Kumar and Hirochika, 2001). The ability of *Tos17* to produce mutations has been exploited by Hirochika (2001) in generating mutant lines. The insertion into a particular gene can be identified by PCR with gene-specific and RTN-specific primers using DNA samples from a pool of mutant lines. The insertional polymorphisms are identified within a DNA pool by using a two- or three-dimensional pooling system. Several genes isolated from the tagged mutants by PCR screening for pooled DNAs of *Tos17* transposed lines have been reported (a mutant of the homeobox gene *OSH15*, Sato *et al.*, 1999; a mutant of the phytochrome A (*phyA*) photoreceptors in plants, Takano *et al.*, 2001).

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Okamoto and Hirochika (2000) reported that *Tto*1, the tobacco LTR RTN, is activated in *Arabidopsis* during tissue culture. The analysis of sequences flanking the *Tto*1 insertion showed that many of the amplified products exhibit homology to known genes or hypothetical proteins. The authors suggested that *Tto*1 could be used as a tool for efficient insertion mutagenesis of *Arabidopsis*.

Oil palm

Palms are woody monocotyledons in the family *Arecaceae*, which is placed in the order *Arecales* (Jones, 1994). Oil palm, *Elaeis guineensis*, plays a vital role in the economy of many developing countries in South-East Asia, such as Malaysia and Indonesia. The major advantages of oil palm are firstly its high yield, and secondly the fact that two distinct oils (which can be used for different purposes) are produced. In addition, palm oil also has potential in development of alternative technologies, such as bio-fuel.

Copia-like RTNs of *Elaeis guineensis*, *Elaeis oleifera* and *Cocos nucifera* were characterized by performing phylogenetic analyses on a portion of the RT gene of *copia*-like RTNs. In addition to this, the aim of this work was to develop a marker system based on *copia*-like RTNs in oil palm. A prerequisite for such a marker system is the isolation of LTRs. The LTRs, a characteristic feature of LTR RTNs, are oriented as direct repeats at termini of members of this class of elements. Although LTRs do not encode proteins, they contain promoters and terminators necessary for transcription of the RTNs. In addtion, the LTRs are recognized by the RTN-encoded integrase that inserts the element into the genome. The LTRs are divided into three regions: the U3 region, the R region and the U5 region (reviewed in Kumar and Bennetzen, 1999).

Materials and methods

A PCR reaction was carried out using degenerate primers as described in Flavell *et al.* (1992) and Price *et al.* (2002b) in order to amplify a band of approximately 280 base pairs corresponding to part of the RT domain of *copia*-like RTNs. The primers correspond to domains conserved on the protein level within RTs. Multiple sequence alignments were made with Clustal W (Thompson *et al.*, 1994), using the default gap insertion/extension penalties. Phylogenetic reconstruction of the aligned sequences was performed by using maximum likelihood, with PAUP* 4.0b10 (Swofford, 2001). Model for maximum likelihood (ML) (HKY + I + G) was calculated by Modeltest3-04 (Posada and Crandall, 1998).

Using two different approaches, oil palm LTRs, the basis for a marker system, were isolated. Firstly, primer-walking from the RT region of the C- and B-class to the LTR of the element was performed. Secondly, the LTRs from oil palm (D-class), *Elaeis oleifera* (B-class) and *Cocos nucifera* (B-class) were identified by using a modified method of Pearce *et al.* (1999, 2000) and by using RnaseH primers specific to oil palm LTRs isolated originally by primer walking (Fig. 5). The IRAP (Fig. 3) PCR amplification was performed in a Hybaid PCR Express machine using the protocol of Kalendar *et al.* (1999). Following the PCR reaction, 20 μl of gel loading buffer containing formamide were added to each sample. Products were analysed by electrophoresis on a denaturing 4.5% PAGE sequencing gel (Sambrook and Russell, 2001) and visualized by autoradiography using Kodak MR film. The reproducibility of each primer combination was tested in two separate amplifications and on two different Hybaid PCR Express machines. Clones that did not identify a match to any known RTN sequence in the databases were not used as the basis for developing the marker system.

Results and discussion

The results confirmed previous results of Price *et al.* (2002b) and demonstrated that no lineages that include RT sequences from just *Cocos nucifera* and *Elaeis oleifera* were detected. The C-class RT sequences seem to have amplified preferentially only in *Elaeis guineensis* (Fig. 6). The unrooted phylogram of RT sequences exhibits a similar pattern to that revealed in the oil palm tree (Price *et al.*, 2002b). It shows that: class C1 sequences are present only in oil palm, and that class C2 includes one coconut and two *E. oleifera* sequences. In addition to this it also illustrates that class B is present in all the three species and divides into two subclasses and that class A consists of many subclasses. The phylogenetic topologies of retroelements are often interpreted at three levels: the length of the main branches indicate heterogeneity of RT sequences; clustering of related elements defines groups and the highly unresolved terminal branches indicate the explosive proliferation associated with the transposition process (Feschotte *et al.*, 2002). The C1-class is associated with highly unresolved, 'star'-like trees which might be an indication that the C1-class RT sequences diverged rapidly at the same time.

Futhermore, we have developed a method based on a medium copy number (< 10,000 copies) family of *copia*-like RTNs in oil palm. This PCR-based approach detects individual RTN insertions using primers derived from the RTN. The insertions can be seen as bands on a sequencing gel; the polymorphic bands (polymorphic markers) are then detected as an absence or presence

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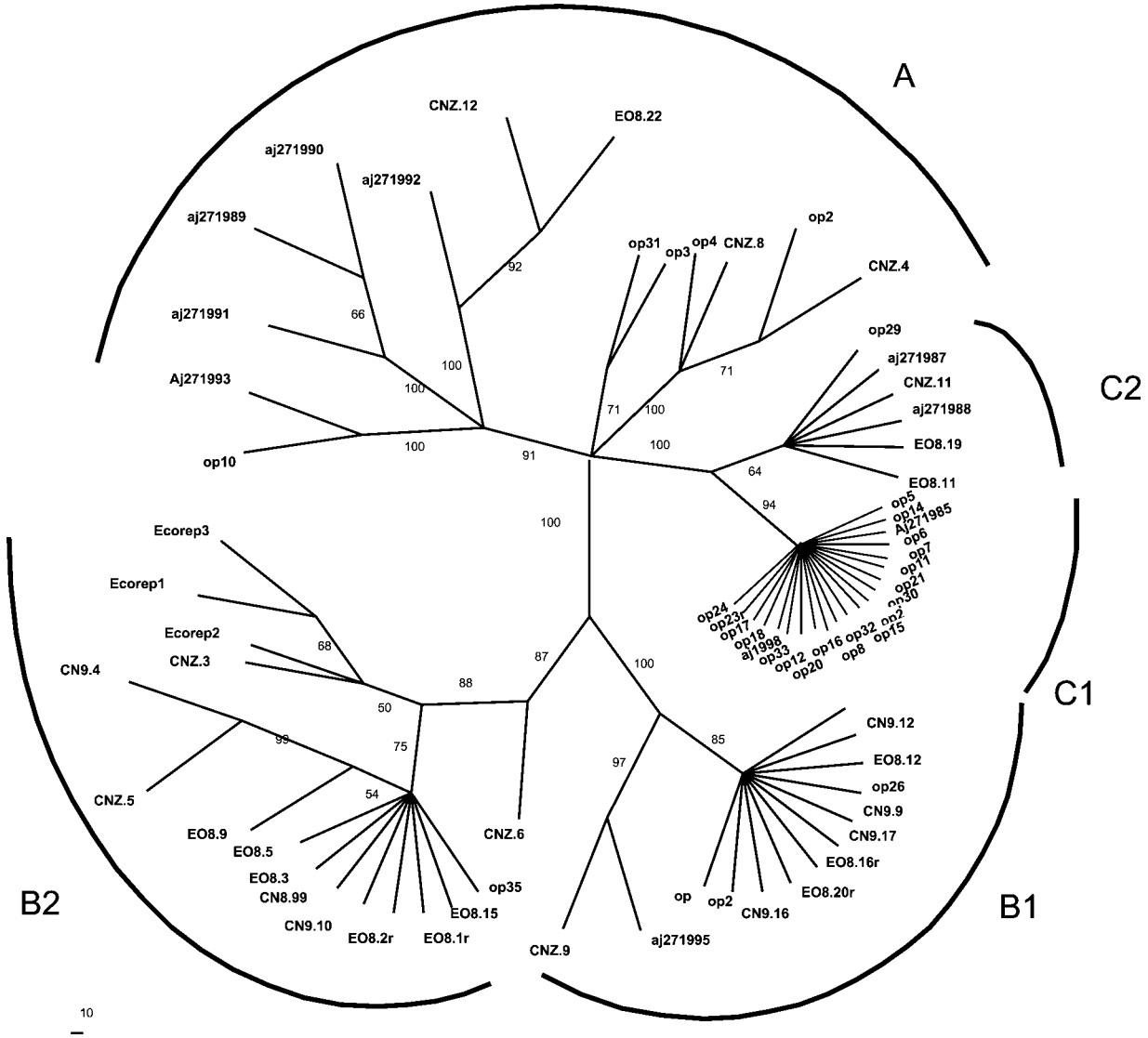

Fig. 5. Phylogram of oil palm, coconut and *E. oleifera* RT sequences. Phylogenetic reconstruction of the aligned sequences was performed by using maximum likelihood, with PAUP* 4.0b10 (Swofford, 2001). The numbers at the ends of the branches indicate individual RT DNA sequences (op—oil palm, CN—coconut, EO—*E. oleifera*, aj series—oil palm and Ecorep—coconut RT sequences from NCBI). The numbers on the branches indicate bootstrap values for the support of the branches. The branches with bootstrap values under 50 have been collapsed. A, B and C indicate RT groups and B1, B2, C1 and C2 subgroups.

of bands. The method was successfully used to detect polymorphisms in 67 samples of the DM635 cross (Fig. 7). This is a dura × dura cross (Dami dura × Ulu Remis) of relatively narrow genetic base central to the NBPOL dura improvement programme. The method is easily transferable between different populations and allows objective assessment of polymorphisms (Price, 2003). The study of Price (2003) indicated that AFLP and RTN-based markers in oil palm had similar numbers of polymorphic bands per primer combination but the RTN-based marker system showed a far higher proportion of polymorphic bands per band generated (approximately eight times higher; Price, 2003). A range of breeding populations of restricted origin (BPRO) has been tested, such as POBE, LAME, Yangambi, Deli dura, Avros and others, documented by Rosenquist (1985), as well as hybrids between *E. oleifera* and *E. guineensis*. The number of polymorphic bands per primer combination in the DM635 cross ranged from one to 16 with an average of six. The primer combinations between B- and C-, and B- and D-classes were the most polymorphic ones, producing on average seven polymorphic bands per primer combination (Table 1).

Preliminary results demonstrated the possibility of applying the method to not only breeding purposes in oil palm but also within closely related species such as

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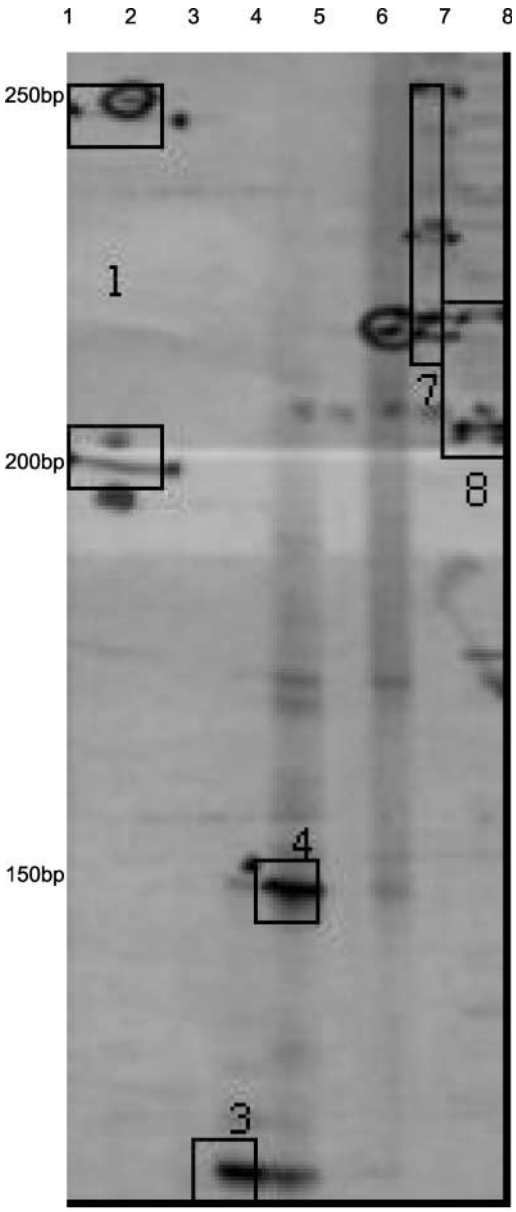
coconut (Price, 2003). However, the primers isolated for the tribe Cocoeae have not been applied to other palm subfamilies. The authors believe that there is scope for applying the approach in botanical research within the family Palmae and for assessing diversity



1. *E. oleifera* (D5) *Eco*RI
2. *E. oleifera* (D6) *Eco*RI
3. *C. nucifera* (D8) *Eco*RI
4. *E. guineensis* DM742.207 *Msp*I
5. *E. guineensis* DM742.207 *Sau3*AI
6. *E. guineensis* DM742.207 *Eco*RI
7. *E. guineensis* (B89) *Sau3*AI
8. *E. guineensis* DM742.207 *Mse*I

Fig. 6. LTR isolation method with the use of RnaseH primer RnaseHOP4 (B-class). The figure shows an autoradiograph of a 6% PAGE (polyacrylamide) sequencing gel. Templates for the PCR reaction were partially digested oil palm DNAs (samples 1–8) ligated into T-GEM easy vector (Promega). The template DNAs (lanes 1–8) were digested with the enzymes *Eco*RI, *Msp*I, *Mse*I and *Sau*3AI (Invitrogen) as indicated. The PCR reaction was performed by using the oil palm RnaseH primer labelled with γ^{33}P (B-class, RnaseHOP4) and M13 universal primers. The samples 1, 3, 4, 7 and 8 were excised, re-amplified and sequenced (for details see Pearce *et al.*, 2000).

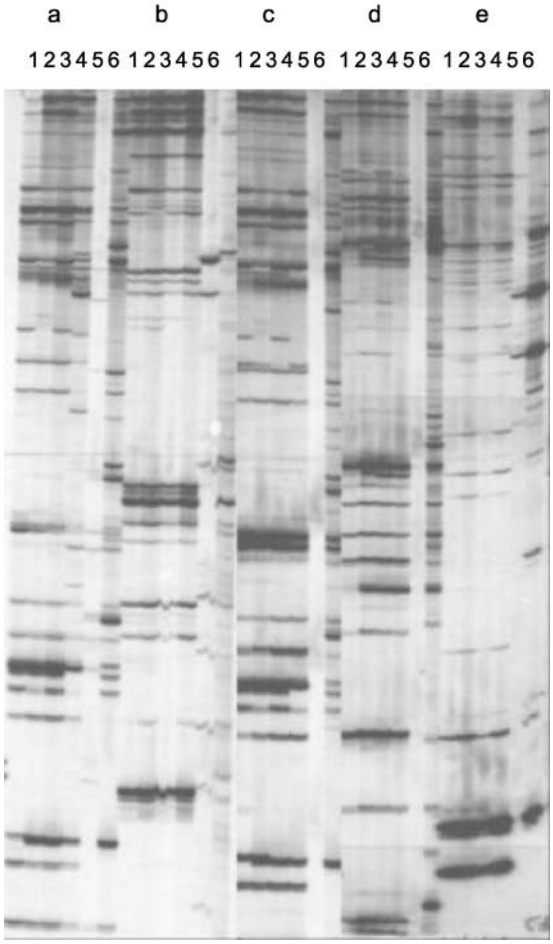


Fig. 7. Application of an IRAP marker system in oil palm (*Elaeis guineensis* Jacq.), mapping population DM635. The autoradiograph above of a 4.5% PAGE gel shows bands generated by primer combinations between LTR5r (labelled with γ^{33}P) and LTR2.7r (a), LTR5r and LTRole7r (b), LTR5r and LTR4.1r (c), LTR5r and LTR15r (d), LTR 5r and LTRole6r (e). The samples used were of DM635 (lanes 1–4), one of *Elaeis oleifera* (lane 5) and one of *Elaeis guineensis* and *Elaeis oleifera* hybrid (lane 6). The primers LTRole6r and LTRole7r were based on *Elaeis oleifera* LTR sequence (note the stronger signal in the *E. oleifera* lane).

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Table 1. PCR primer used in the IRAP marker system

Primer combination	LTR combination	Polymorphic bands
LTR5r + LTR7r	BD	9
LTR5r +LTR3f	BB	2
LTR5r + LTR6r	BD	5
LTR5r + LTR5r	BB	2
LTR5r + LTR1f	BB	2
LTR6f + LTR7r	BD	2
LTR5r + LTR14br	BC	7
LTR5r + LTR2.7r	BD	8
LTR5.3r + LTR5r	BD	7
LTR5r + LTR15r	BC	12
LTR4.1r + LTR5r	BD	9
LTR6f + LTR5.3r	BD	16
LTRole1f + LTR5r	BoB	8
LTR5r + LTR14r	BC	4
LTRole6r + LTR5r	BoB	2
LTR7r1 +LTR5r	BD	1

The DNA sequences for each primer are detailed in Price (2003). LTRole1f, LTRole6r, LTRole6f and LTRole7r were isolated from *Elaeis oleifera*.

within endangered palm species and the palm species stored in genebanks. For this purpose the method can also be used for isolation of new RTN families endogenous for each palm species as suggested by Mhiri and Grandbastien (2001) for Solanaceae.

There is a great need for easily developed, new marker systems not requiring extensive sequencing, as do single nucleotide polymorphism (SNP) or microsatellite-based markers. Many plants will not be subjects of major cDNA or genomic sequencing projects. The intriguing aspect about repetitive DNA, mostly RTNs and up to 80% of many genomes, is that it can itself be used as a genetic tool to analyse plant genomes and thereby improve our knowledge about the biology of plants. There are significant applications for RTNs in determining phylogeny (ancestry), for assessing diversity of wild, cultivated or endangered species, and in functional analyses of plant genes. For the future, the methods and analyses presented here could be used as tools for understanding one of the underlying mechanisms of plant diversity that has shaped and is still shaping plant genomes.

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