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Recent Advances in the Development of Molecular Markers for Hevea *Studies*

F.C. LOW*#, SAFIAH ATAN*, HAFSAH JAAFAR* AND H.TAN*

Four DNA molecular marker techniques were developed and applied to Hevea *studies. These included the conventional Southern-based marker technique called restriction fragment length polymorphisms (RFLPs) and three polymerase chain reaction amplification-based techniques,* viz. *random amplified polymorphic DNA, microsatellites and DNA amplification fingerprinting (DAF). These molecular markers were able to elicit interspecific as well as intraspecific polymorphisms. They were also able to assist in several identification studies. Cultivars which share two common parents (siblings), such as PR 255 and PR 261; RRIM 901 and RRIM 905; and RRIM 937 and RRIM 938, were distinguished by their DNA polymorphisms. Similarly, molecular markers had assisted the identification of 'rogues' in a sample of monoclonal PB 5/51 seedlings.The genetic diversity of genotypes from the 1981 IRRDB* Hevea *germplasm collection from Brazil was displayed and confirmed by their RFLPs. For the first time, microsatellites had been detected in the* Hevea *genome. Three microsatellite sequences were detected within one gene (hydroxylmethyl-glutaryl coenzyme A reductase-1). Preliminary evidence suggested that one of these microsatellite sequences,* $(GA)_9$, *was polymorphic. A reduction in the number of ribosomal DNA (rDNA) genes during cell culture was suggested when pTa71, a wheat rDNA probe, was hybridised with digested gDNA obtained from* in vitro *culture plants. Somaclonal variations were detected among a population of GL 1 plants derived from anther and ovule culture by a recently developed DNA fingerprinting technique called DAF. A genetic linkage map comprising of RFLPs and other markers is being constructed. The advantages and disadvantages of these marker techniques were compared.*

In plant breeding, the generation of improved genotypes is dependent on the genetic recombination and segregation of traits among the progenies of a genetic cross between two parents. The identification of the desired recombinant trait among the segregating population can be an arduous task for the breeder. The efficiency and precision of plant breeding programmes in certain crops such as tomato and corn have been markedly increased by the development of molecular markers[1–2]. Through DNA marker technology, a particular locus on a chromsomal segment which is linked to a desirable trait can be identified with a selectable DNA marker and the segregation of that particular chromosomal segment (desirable trait) can be monitored in subsequent crossings. As tools to assist the plant breeder, DNA markers are more useful and powerful than

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*Rubber Research Institute of Malaysia, P.O. Box 10150, 50908 Kuala Lumpur, Malaysia

#Corresponding author

morphological markers for genetic analysis of higher plants[3]. Since DNA markers are direct characters of the genotype and are independent of the environment and phenotype for their evaluation, they provide more accurate identification at a much earlier age. As a source for genetic fingerprints, DNA molecular markers can assist in phylogeny (parentage identification), pedigree analysis, assessment of genetic stability and genotype validation. They can also be exploited for marker-assisted selection in breeding.

DNA molecular marker technology can be classified under two groups. These are the conventional hybridisation-based (Southern) techniques such as restriction fragment length polymorphisms (RFLPs)[4] and the more recent *in vitro* amplification-based techniques. *In vitro* DNA amplification is achieved by the thermostable *Taq* DNA polymerase enzyme *via* the DNA polymerase chain reaction (PCR)[5,6]. This technology has generated several DNA molecular markers such as random amplified polymorphic DNA (RAPD)[7,8], microsatellites or simple sequence repeats (SSRs)[9,10], DNA amplified fingerprinting (DAF)[11] and amplified fragment length polymorphisms (AFLPs)[12]. In comparision to RFLPs, these latter PCR-based marker techniques are faster, more efficient and economical on DNA usage. The use of radioisotopes can also be avoided without losss of sensitivity of detection by these techniques. Hence, these markers have become increasingly popular and have superceeded the use of RFLPs in many crop plants.

Both hybridisation-based and PCR-based DNA molecular markers had been developed for *Hevea*. Polymorphisms between *Hevea* species as well as within species had been successfully revealed by RFLPs[13,14]. Similarly, the genetic diversity of wild and cultivated *Hevea* [15], as well as their polymorphisms in mitochondrial[16] and ribosomal[17] DNA were assessed by RFLPs. Notwithstanding that RFLPs are powerful for studying genetic diversity and mapping, the technology is slow, labour-intensive, requires large DNA samples and often uses radio-isotopes. It also has a low marker index value (expressed as the number of polymorphic products per sample) of 0.10 , compared to other PCR-based marker systems such as RAPDs (0.23), SSRs (0.60) and AFLPs (6.08)[18]. In order to augment the mapping project and to increase the efficiency of other studies, several PCR-based marker techniques such as RAPD, sequence tagged microsatellite sites (STMS) and DAF were therefore developed for *Hevea*[19–21]. The advantages and disadvantages of these markers will be discussed and their general utility will be compared.

MATERIALS AND METHODS

Plant Materials

Seven *Hevea* species (*H. brasiliensis, H. benthamiana, H. carmagoana, H. guianensis, H. nitida, H. pauciflora* and *H. spruceana*) and 20 *H. brasiliensis* clones consisting of four primary clones (GT 1, Tjir 1, PB 86, PR 107) and 16 secondary clones were studied. These included clones in the RRIM 600, 700 and 900 series as well as those from PB and PR origins. *In vitro* culture plants were derived from *H. brasiliensis* clone GL 1. Two types of control plants were used. These were plants belonging to the same clone, but were propagated vegetatively by either cutting or budding.

DNA and RFLP Methods

DNA samples were isolated as described[13]. DNA was digested overnight at 37°C with various restriction enzymes according to the manufacturer's instructions. Restriction enzyme digestion was carried out at a concentration of 10 units of restriction enzyme per µg of DNA, in the presence of 5mM spermidine and 100 µg/ml casein. Other RFLPs methods were similar to those described earlier[14] .

Computer Search in the Databank

Published *Hevea* gene sequences recorded in the European Molecular Biology Library (EMBL) database was searched for the presence of microsatellites. Primers flanking the identified microsatellite regions were designed with the PRIMER computer program from Whitehead Institute for Biomedical Research, USA, and synthesised at the Cambridge Laboratory, Norwich, United Kingdom.

Sequence-tagged Microsatellite Sites

Amplification of microsatellite regions by STS was carried out according to Low *et al.* [20] The reaction containing 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.2 µM dNTPs, 0.2 µM of each primer (forward and reverse), 0.5% Tween 20, 0.5% Nonidet P-40, 0.8 units Taq DNA polymerase and 20 ng DNA template, in a final reaction volume of 50 µl was subjected to *in vitro* amplification by the PCR reaction. PCR conditions included an initial denaturation at 95°C for 5 min, followed by 60 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min with a final extension at 72°C for 7 min. Amplified products were analysed by electrophoresis in non-denaturing 6%–8% polyacrylamide gels after ethidium bromide staining.

Random Amplified Polymorphic DNA (RAPD)

Genomic DNA from *in vitro* culture and control plants were amplified with random 10-mer primers (Operon Technologies, USA) by the modified method of Koller *et al.*[22] Amplification products were analysed in 1.2% agarose gels in 1XTAE buffer.

DNA Amplification Fingerprinting (DAF)

Pairs of short oligonucleotide arbitrary primers (10-mers from Operon Technologies, USA) were sequentially tested for amplification of gDNA. Amplification reaction consisted of 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.125mM dNTP, 0.25 µM primer, 1.25 units Taq polymerase, 0.125% each of Nonidet NP-40 and Tween 20 and 10 ng DNA template. Cycling conditions were carried out as described[11]. Amplification was achieved after 45 cycles of 1 sec at 96°C, 10 sec at 30°C and 10 sec at 72°C, followed by storage at 4°C, until samples were anaylsed. Amplification products were separated by vertical electrophoresis in denaturing polyacrylamide gels and viewed after staining with silver nitrate.

RESULTS AND DISCUSSION

Restriction Fragment Length Polymorphisms (RFLPS)

In general, RFLPs between *Hevea* species (*Figure 1*) were easy to obtain. Polymorphisms detected between *H. brasiliensis* and *H. pauciflora* suggested that the RFLPs were a consequence of insertion/deletion as well as base-substitution events. On the other hand, RFLPs within the same species, particularly among widely cultivated clones of *H. brasiliensis*, were more difficult to detect. Monomorphic DNA patterns between popular

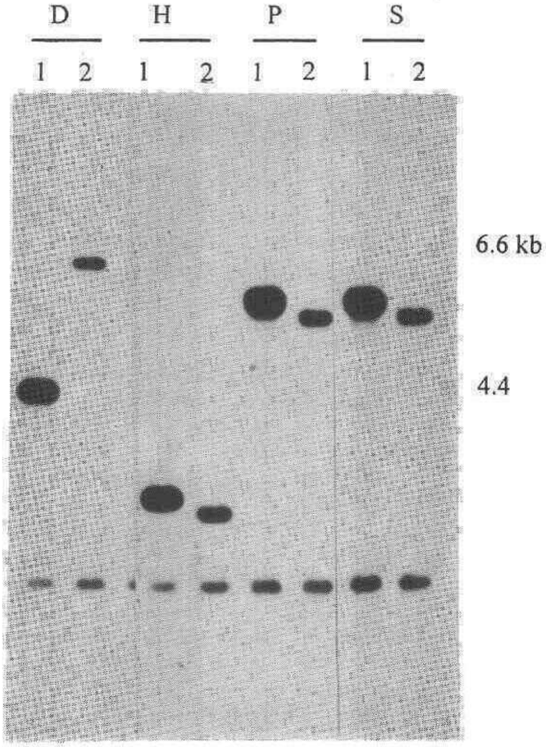

Figure 1. Interspecific RFLPs between Hevea brasiliensis *and* Hevea pauciflora, *revealed after gDNA digested by* DraI *(D)*, HindIII *(H)*, PstI *(P) and* SstI *(S) was hybridised with anonymous gDNA probe, pJ4D1.*
1 = H. brasiliensis; 2 = H. pauciflora

cultivars were often obtained. This is mainly because of the narrow genetic base from which the cultivated *Hevea* clones were derived. Most of the clones bred to date in Malaysia and other South-East Asian countries originate from a limited number of dominant parents or 'primary clones'. These are, as pointed by Tan[23], to be Tjir 1, Pil A 44, Pil B 84, PB 24, PB 49, PB 56 and PB 86. Notwithstanding this, by the judicious use of probe/enzyme combination, RFLPs within the same species could be observed[13]. The ability to detect RFLPs within the same species was applied to several studies of genetic variability, identification and validation.

By a combination of appropriate probes and enzymes, some of the popular clones could be identified by their RFLPs. Besides identification, RFLPs had been sufficiently powerful to distinguish between some siblings, *i.e.* cultivars which share two common parents. Siblings pairs such as PR 255 and PR 261; RRIM 937 and RRIM 938 which are progenies of cross between Tjir 1 × PR 107 and between PB 5/51 × RRIM 703, respectively, were distinguishable by their RFLPs. Similarly, the progenies of the genetic cross between PB 5/51 and RRIM 600, *viz.* RRIM 901 and RRIM 905 could be differentiated by their fingerprints when hybridised with a DNA probe derived from the bacteriophage M13 (*Figure 2*).

Although the above pairs of siblings could be identified by an experienced *Hevea* Clone inspector, the RFLP technique is nonetheless useful and more precise because the identification can be accomplished with any tissue of the plant at an early age whereas clonal identification by phenotypic characters can only occur at a more advanced stage of tree development. Earlier identification can lead to earlier selection and culling, thereby, reducing costs, labour and land utilisation.

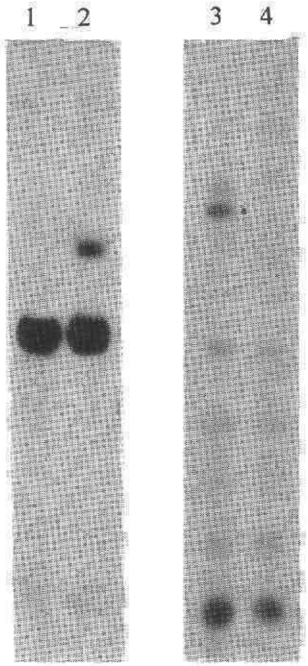

Figure 2. Intraspecific RFLPs between clones which share two common parents (siblings).
1 = PR 255; 2 = PR 261;
3 = RRIM 901; 4 = RRIM 905.

RFLPs had been successfully applied to a study on cultivar validation. Examination of a population of 27 purported monoclonal seedlings of clone PB5/51 by RFLPs suggested the presence of some 'rogues' in the population. Several of these 27 seedlings were shown to be 'rogues'. Since a block of monoclonal GT 1 stand was next to the PB 5/51 monoclonal block under study, some monoclonal GT 1 seeds might have been collected inadvertently, thereby resulting in these 'rogues'. Their RFLPs suggested that these 'rogues' might have resulted from selfing of GT 1 as well as from crossing with PB5/51 (*Figure 3*).

The feasibilty of assessing genetic variability using RFLPs was examined with the IRRDB 1981 *Hevea* germplasm collection. The polymorphisms elicited were able to indicate the genetic similarity as well as diversity of the germplasm collection (*Figure 4*).

The sensitivity of the RFLP technique to detect DNA changes during cell culture was investigated. Comparision of hybridisation patterns of pTa71, (a wheat ribosomal DNA fragment), between *Alu*I-digested DNA from a random sample of five GL 1 *in vitro* culture plants with similarly digested DNA from six control plants revealed a reduction in the number of copies of rDNA genes in the *in vitro* culture plants (*Figure 5*). The result is consistent with the results of Leitch *et al.*[24] who observed a loss in the number of rDNA sites and genes in wheat derived from a long term suspension culture. A similar deficiency in rDNA genes was reported in somaclonal variants of potato[25]. The detection of changes

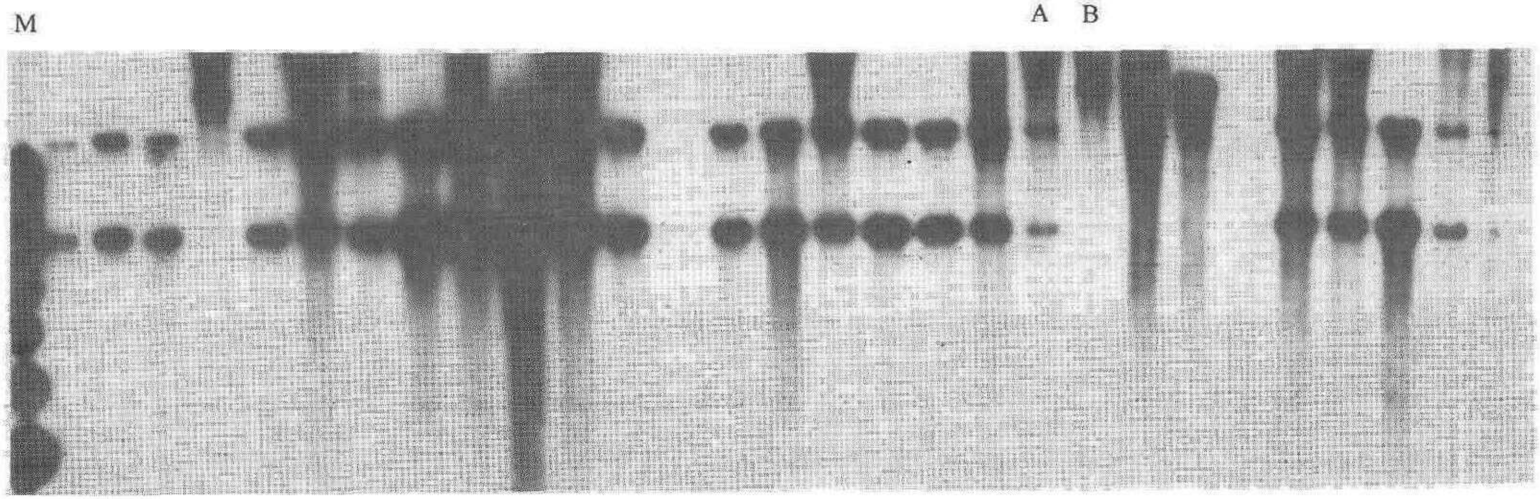

Figure 3. Detection of 'rogues' in a sample of PB 5/51 monoclonal seedlings after digestion with ApaI *and hybridisation with gDNA probe pJ32.*
A = PB 5/51; B = GT 1; M = Mol. wt. marker.

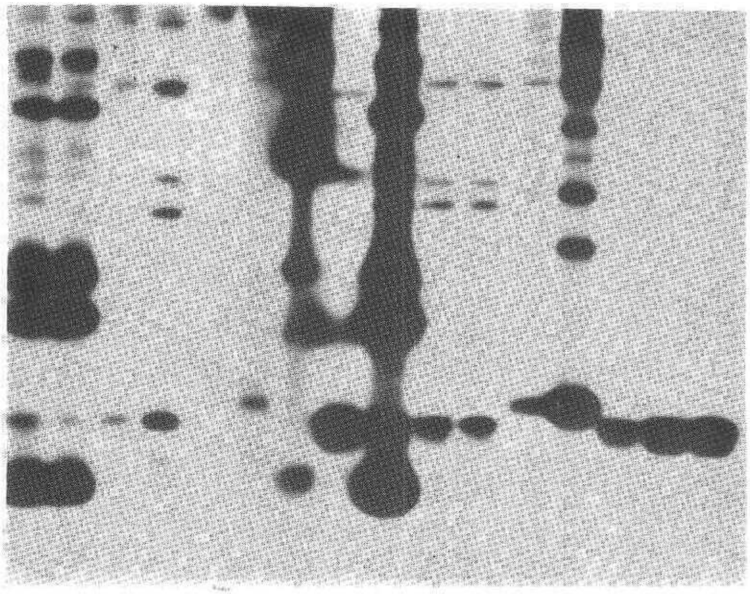

Figure 4. The genetic variability and similarity among a sample of genotypes from the IRRDB 1981 Hevea *Germplasm collection, as revealed by RFLPs.*

in rDNA during *in vitro* culture has not been reported previously in *Hevea*. Its significance, though not apparent at present, may be important for other research which encompasses *in vitro* culture.

The application of RFLPs for the construction of a genetic map has been hampered by the lack of polymorphic probes. Although a fairly large genomic DNA (gDNA) library of 700 clones was generated as described[13] and that screening with total gDNA suggested that these clones consisted of mainly low-copy sequences, their utility, however, as polymorphic probes to monitor segregation in the mapping population had been poor. A total of 60 probes in combinations with ten enzymes initially, but finally with four enzymes, were tested. Less than 10% of these were shown to be polymorphic for a sample of RRIM 600 monoclonal seedlings tested. It would appear that for genetic mapping studies, appropriate genetic crosses with divergent parents, coupled with a more extensive pool of hybridisation probes would be needed.

Random Amplified Polymorphic DNA (RAPD)

Initial attempts at the development of this marker for *Hevea,* following the procedure of Williams *et al.*[8] were fruitful in yielding polymorphisms. But, reproducibility of these results was difficult. This problem is not

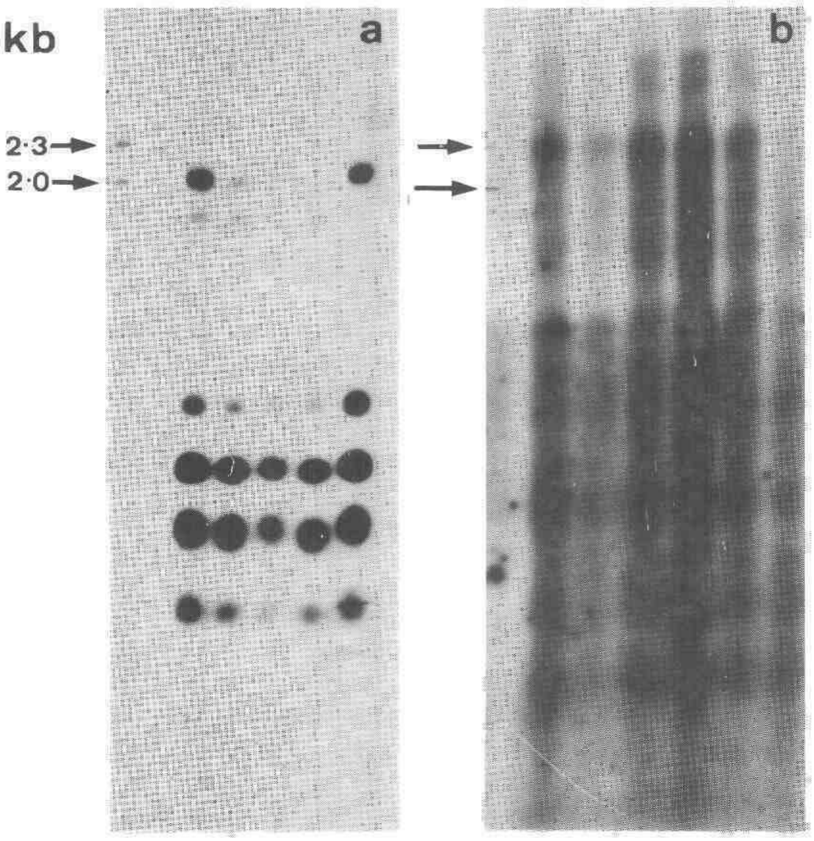

Figure 5. Changes in rDNA during cell culture as demonstrated by hybridisation of pTa71 on Alu*I-digested gDNA.*
A = In vitro culture plants; B = Control plants.

confined to our laboratory, but is also faced by others[26]. However, after modifications in the experimental procedures of Koller[22], reproducible polymorphisms were obtained. As demonstrated in *Figure 6a*, samples of the same DNA obtained from different dates of extractions were amplified reproducibly by the RAPD technique. A total of 60 random primers were screened and only three of these were able to elicit polymorphism repeatedly.

The applicability of RAPD markers to genotype and cultivar identification in *Hevea* was investigated. Polymorphisms between several cultivars of *H. brasiliensis* were observed. Similarly, polymorphisms between four lots of DNA, each pooled from 9 different individual *in vitro* culture plants were observed (*Figure 6b*). The detection of somaclonal variants in *in vitro* culture plants, though not unknown to some crops[24,25,27,28], is new to *Hevea*.

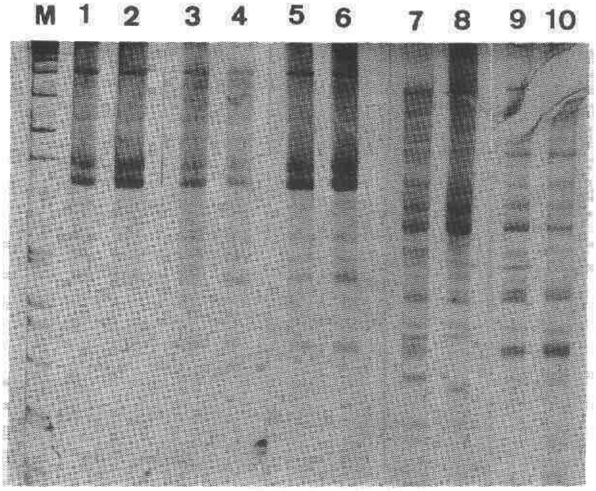

Figure 6a: Reproducibility of the RAPD technique. Amplification of duplicate samples of gDNA obtained from separate extractions at different times by primer OpE19.

Lanes 1–2: Tjir 1
Lanes 3–4: PB 86
Lanes 5–6: RRIM 600
Lanes 7–8: PB 5/51
Lanes 9–10: PB 49
M: Mol. wt. marker, kb ladder

Microsatellite or Simple sequence Repeats (SSRs)

A computer search in the European Molecular Biology Laboratory (EMBL) database enabled *Hevea* gene sequences which contained simple sequence repeats to be identified. The search revealed that of the *Hevea* gene sequences which had been recorded in the database, only hydroxymethyl-glutaryl coenzyme A reductase-1 (HMGR1) was shown to contain microsatellites. These were $(GA)_9$, $(TA)_{16}$ and $(TTA)_8$. Even though $(GA)_9$ was less than ten dinucleotide repeats to qualify as microsatellite, as defined by Morgante and Olivieri[29], these were nevertheless included for studies. Amplification of the $(GA)_9$ region with appropriate primers thus converted these regions into sequence-tagged microsatellite sites (STMS). Polymorphisms in STMS were revealed between all five *Hevea* species examined (*Figure 7*). Similar polymorphisms between clones within the *H. brasiliensis* species were also detected. Two types of polymorphisms were observed. These were differences in the number of amplified bands and variation in the length of the amplified bands. Polymorphisms between clones were mainly in the number of bands, whilst polymorphisms between species comprised of both types of differences. Hence STS polymorphisms in the $(GA)_9$ microsatellite regions between clones within the *H. brasiliensis* species appeared to be due to

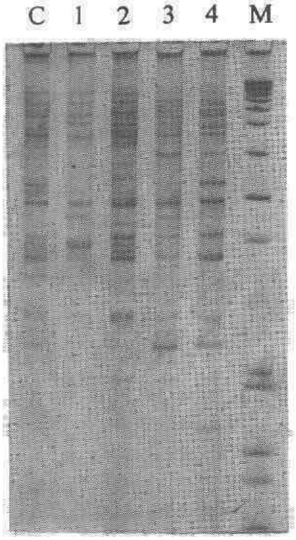

Figure 6b. RAPD polymorphisms in vitro *culture plants. 1–4 = Pooled gDNA from nine different* in vitro *culture plants.*
C = Control plant (cutting); M = Mol. wt. marker, kb ladder.

allelic differences in that particular locus arising from genetic recombination. On the other hand, polymorphisms between species may be the result of genetic events such as DNA insertion/ deletion as well as DNA point mutations which occurred during the evolution of the *Hevea* species.

DNA Amplified Fingerprinting (DAF)

The most recently developed DNA marker for *Hevea* studies is DAF. Since DAF appeared to be more robust than RAPD and is as efficient, its application to *Hevea* polymorphism studies was initiated. A total of 20 primers in 190 combinations as primer pairs were screened to identify the most useful pairs of primer which would yield polymorphisms. Three pairs of such primer pairs had been identified. When the DAF technique was applied to six *Hevea* species, all were found to display distinctly different DAF profiles. Except for one DNA band which appeared to be shared by all species, all the other bands differed both in size, number and intensities, when viewed after silver nitrate staining (*Figure 8*). The DNA profiles as elicited by DAF were in confirmation with earlier results of interspecific polymorphisms, as seen by other DNA markers. The potential of DAF markers in the construction of a genetic map is being evaluated.

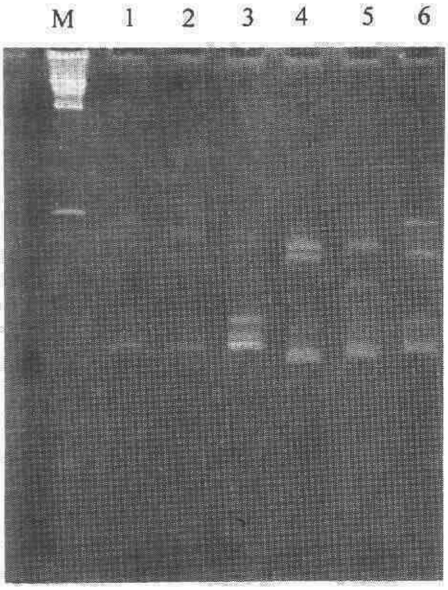

Figure 7. Polymorphisms in the (GA)$_9$ region of the hydroxymethyl-glutaryl coenzyme A reductase-1 (HMGR-1) gene of H. brasiliensis *between several species.*
1 = H. pauciflora; 2 = H. pauciflora;
3 = H. guianensis; 4 = H. camargoana;
5 = H. benthamiana; 6 = H. brasiliensis;
M = Mol. wt. marker, kb ladder.

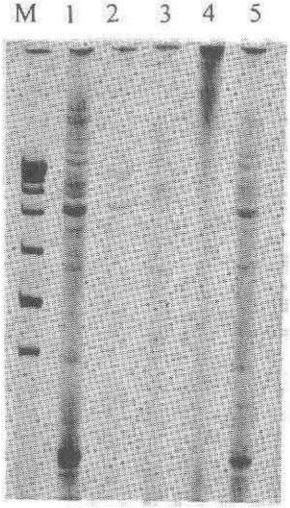

Figure 8. Polymorphisms in DAF between several Hevea *species.*
1 = H. brasiliensis; 2 = H. benthamiana;
3 = H. camargoana; 4 = H. nitida;
5 = H. pauciflora;
M = Mol. wt. marker, kb ladder.

Besides interspecific polymorphisms, intraspecific differences in DNA profiles were observed in all ten *H. brasiliensis* clones examined. Because of the close genetic relationship between these clones, a greater similarity in band size and number were demonstrated between these clones than between species.

The utility of DAF in the detection of somaclonal variation was also investigated. Comparision of DNA profiles of 48 plants produced by *in vitro* culture revealed a heterogenous DNA pattern, suggesting that these were not genetically homogenous. Although most of these plants were very similar in their DNA profiles, a few were distinctly different. The significance of these differences is not apparent at present, but these DNA differences will be monitored together with other marker techniques.

CONCLUSION

Several DNA markers such as RFLPs, RAPD, STMS and DAF had been developed for *Hevea* and their applications had been described. All these markers could be applied for identification and genetic variability studies. Though RFLPs were the first marker to be developed, they are gradually being replaced by more recently developed markers which are mediated through PCR. Unlike RFLPs where polymorphic hybridisation probes are imperative for eliciting DNA variations, polymorphisms from amplification of DNA by PCR are independent of probes but dependent on primers and amplification conditions. Among the three PCR-based markers which had been developed for *Hevea*, DAF appeared to be the most promising. Though RAPD and DAF markers are both based on the amplification of multiple unknown sites in the genome with short (5–10 nucleotides) primers of arbitrary sequences, the DAF technique is more robust and reproducible. In comparison with DAF, the STMS technique requires DNA sequence data for the design of primers flanking the regions targetted for amplification. This limits amplification to only regions with known sequence information and only one region can be studied at a time, rendering the method less efficient. Additionally, the need to design longer (17 or more nucleotides) primers for STMS makes the technique more expensive than those where random decamer primers are used.

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