*Developments in Plantation Crops Research*, 1998, pp. 40-45.

# APPLICATION OF MOLECULAR MARKERS WITH SPECIAL REFERENCE TO RAPDS FOR GENETIC ANALYSIS IN *HEVEA*

Y. ANNAMMA VARGHESE, C. KNAAK*, W. ECKE*,
C.K. SARASWATHYAMMA and M.R. SETHURAJ

Rubber Research Institute of India, Kottayam - 686 009, Kerala

An evaluation of RAPD markers for genetic analysis in *Hevea* was made and protocols for extraction of DNA as well as RAPD assay were standardized. Total genomic DNA was extracted from 48 clones in the breeding pool and RAPD reactions run. The final amplification products was visualized by agarose gel electrophoresis after staining with ethidium bromide. A total of 126 decamer oligonucleotide primers were screened with 6 test clones. From these 42 informative primers were further tested with 24 selected clones which resulted in 111 polymorphic fragments, revealing the suitability of RAPD markers for genetic analysis in *Hevea*. Genetic distances estimated among the tested clones using 80 RAPD makers revealed a range of 0.05 to 0.75 with a mean of 0.5 indicating the presence of a fairly large variability among the tested clones. Estimation of genetic distances among the clones facilitated identification of genetically divergent parents for breeding. Possible application of molecular markers in *Hevea* breeding is discussed.

## INTRODUCTION

Conventional genetic analysis is, in general, elaborate, time consuming and also influenced by environmental interactions. This is especially true in perennial plantation crops like *Hevea* due to their long breeding and selection cycle and also because of the difficultly in generating segregating F2 progeny. On the other hand, molecular markers, which deal directly with DNA are reliable, free from environmental interactions and can be assayed at any stage of plant growth and practically with all types of tissues.

RAPDs are novel DNA polymorphisms described by Williams *et al.* (1990). RAPD is an *in vitro* technique to detect polymorphism or genetic differences in the DNA from different individuals. It is an enzymatic DNA amplification based assay using a random primer of arbitrary nucleotide sequence. Attempts were made to develop molecular markers for *Hevea* using RAPD technique.

Reports on molecular markers in *Hevea* are rather scanty. The available reports include development of molecular markers for *Hevea* breeding (Low *et al.*, 1995; Varghese *et al*, 1996), assessment of genetic variability in Wickham and wild material using RFLPs of ribosomal (Besse *et al.*, 1993) and nuclear DNA (Besse *et al.*, 1994), and the estimation of phylogenetic relationships from mitochondrial DNA RFLPs (Luo *et al.*, 1995).

The objectives of the present study were: (1) Standardize protocols for (a) extraction of DNA and (b) RAPD assay in *Hevea*, (2) Examine the feasibility of using RAPD makers in

* Institute of Plant Breeding, University of Gottingen, Federal Republic of Germany.

*Hevea* and (3) Evaluate the extent of the genetic variability among different clones in the breeding pool.

## MATERIALS AND METHODS

### Materials

Fresh as well as lyophilized leaves from a total of 48 clones developed in different South East Asian countries were used for the extraction of DNA. From among these 48 clones, 24 were selected for the final RAPD assay (Table 1).

**Table 1. Clones used for RAPD analysis**

| Clone | Parentage | Country of origin |
|---|---|---|
| RRII 5 | Primary clone | India |
| RRII 105 | Tjir x Gl 1 | India |
| RRII 208 | Mil 3/2 x Av 255 | India |
| RRII 308 | PB 5/60 x Gl 1 | India |
| RRIM 600 | Tjir 1 x PB 86 | Malaysia |
| PB 5/51 | PB 86 x PB24 | Malaysia |
| PB 28/59 | Primary clone | Malaysia |
| PB 86 | Primary clone | Malaysia |
| PB 217 | PB 5/51 x PB 6/9 | Malaysia |
| PB 235 | PB 5/51 x PB S.78 | Malaysia |
| PB 260 | PB 5/51 x PB 49 | Malaysia |
| PB 280 | Primary clone | Malaysia |
| PB 312 | RRIM 600 x PB 235 | Malaysia |
| PCK 1 | Tjir 1 x PR 107 | India |
| Tjir 1 | Primary clone | Indonesia |
| Gl 1 | Primary clone | Malaysia |
| GT 1 | Primary clone | Indonesia |
| SCATC 88-13 | RRIM 600 x Pil B 84 | China |
| KRS 25 | Primary clone | Thailand |
| HP 82/14 | RRII 105 x RRII 100 | India |
| HP 86/120 | RRII 105 x RRII 118 | India |
| RRIC 100 | RRIC 52 x PB 83 | Sri Lanka |
| HP 86/602 | RRII 203 x PB 5/51 | India |
| HP 86/68 | PB 5/51 x RRII 208 | India |

Three different protocols were tried for the extraction of DNA of which a modified method described by Rogers and Bendich (1988) was found to be the best. Test gels were run using samples from all clones to check the quality of DNA. The concentration of DNA samples was measured and each sample was diluted to a uniform concentration of 10 ng/µl.

### RAPD assay

A modified protocol from Quiros *et al.* (1991) was found suitable for RAPD assay in *Hevea*. Each reaction mixture of 25 µl comprised 0.1 mM dNTPs, 1.9 mM $MgCl_2$, 1x buffer, 0.2 µM primer, 1.4 U Taq DNA polymerase and 50 ng of genomic DNA. Amplifications were done in a Perkin Elmer Cetus Thermal Cycler TC 480 with an initial cycle of 30 sec. at 94°C, 45 cycles of 1 min at 92°C, 1 min at 35°C and 2 min at 72°C and a final cycle of 5 m in at 72°C. After amplification the reaction products were separated by electrophoresis at 90 V for 5 hrs on 1.8 per cent agarose gels in 1x TAE buffer with 0.1 µg/ml ethidium bromide. A 1 kb DNA ladder was used as molecular weight standard. The gels were visualized in a trans-illuminater under UV light and photographed using a video camera attached to a micro computer. For comparison of RAPD pattern of fresh and lyophilized samples, DNA from four clones each were subjected to RAPD assay using three primers.

A total of 126 random decamer oligonucleotide primers (Operon Technology, USA) were first screened using six test clones of apparently different pedigree. From among these 42 informative primers from kits A, AK, AL, AM, AN, Q, R, S and T were further used to screen 24 of the selected clones. The nucleotide sequence, distribution of total and RAPD fragments varied from primer to primer (Table 2).

### Statistical analysis

Data were scored as discrete variables using 1 to indicate the presence and 0 the absence of a band. Eighty polymorphic fragments which were reproducible in repeated amplifications were considered for estimation of genetic distance (GD). GD was estimated as a complement of Jaccard (1908) coefficient of similarity according to the following equation :

**Table 2. Details of informative primers used**

| Primer | Nucl. seq. 5 to 3 | Total fragments | RAPD fragments |
|---|---|---|---|
| OPA -01 | CAGGCCCTTC | 4 | 1 |
| OPAK -02 | CCATCGGAGG | 5 | 2 |
| OPAK -05 | GATGGCAGTC | 7 | 4 |
| OPAK -07 | CTTGGGGGAC | 6 | 3 |
| OPAK -08 | CCGAAGGGTG | 4 | 2 |
| OPAK -09 | AGGTCGGCGT | 5 | 2 |
| OPAK -11 | CAGTGTGCTC | 6 | 1 |
| OPAK -16 | CTGCGTGTCC | 5 | 2 |
| OPAK -18 | ACCCGGAAAC | 5 | 2 |
| OPAK -20 | TGATGGCGTC | 6 | 1 |
| OPAL -05 | GACTGCGCCA | 5 | 2 |
| OPAL -06 | AAGCGTCCTC | 4 | 1 |
| OPAL -07 | CCGTCCATCC | 6 | 3 |
| OPAL -11 | GTCACGTCCT | 6 | 1 |
| OPAL -12 | CCCAGGCTAC | 8 | 1 |
| OPAM -03 | CTTCCCTGTG | 6 | 1 |
| OPAM -10 | CAGACCGACC | 7 | 2 |
| OPAM -11 | AGATGCGCGG | 6 | 3 |
| OPAM -13 | CACGGCACAA | 6 | 2 |
| OPAM -20 | ACCAACCAGG | 5 | 2 |
| OPAN -02 | CACCGCAGTT | 4 | 2 |
| OPAN -06 | GGGAACCCGT | 4 | 2 |
| OPAN -07 | TCGCTGCGGA | 4 | 3 |
| OPAN -10 | CTGTGTGCTC | 4 | 3 |
| OPAN 15 | TGATGCCGCT | 6 | 1 |
| OPAN -16 | GTGTCGAGTC | 3 | 3 |
| OPAN -18 | TGTCCTGCGT | 3 | 1 |
| OPAN -20 | GAGTCCTCAC | 3 | 2 |
| OPQ -04 | AGTGCGCTGA | 6 | 3 |
| OPQ -06 | GAGCGCCTTG | 7 | 1 |
| OPQ -18 | AGGCTGGGTG | 5 | 1 |
| OPR -01 | TGCGGGTCCT | 5 | 3 |
| OPR -05 | GACCTAGTGG | 4 | 2 |
| OPR -06 | GTCTACGGCA | 8 | 3 |
| OPR -07 | ACTGGCCTGA | 3 | 1 |
| OPR -10 | CCATTCCCCA | 6 | 1 |
| OPR -15 | GGACAACGAG | 5 | 2 |
| OPR -19 | CCTCCTCATC | 6 | 1 |
| OPS -01 | CTACTGCGCT | 5 | 2 |
| OPS -11 | AGTCGGGTGG | 4 | 1 |
| OPT -03 | TCCACTCCTG | 9 | 1 |
| OPT -08 | AACGGCGACA | 4 | 3 |

$$GD_{ij} = 1 - (Nij)/(N_{ij} + N_i + N_j)$$

where

$N_i$ = Number of fragments in clone i and not in clone j

$N_j$ = Number of fragments in clone j and not in clone i

$N_{ij}$ = Number of fragments in clone i and j

Thus $GD_{ij}$ reflects the proportion of DNA fragments (RAPD markers) distinguishing the two clones compared relative to the total number of fragments occurring in this pair. GD values may range from 0 to 1. All computations were performed using the computer programme SYNTAX IV.

## RESULTS AND DISCUSSION

Protocols could be standardized for extraction of DNA as well as RAPD assay. Only milligram quantities of leaf samples were required for DNA extraction. Test gels run with DNA samples from all 48 clones revealed that the quality of DNA was good.

RAPD patterns of fresh and lyophilized samples of four clones using three primers were comparable (Fig. 1). This allows storage of samples by lyophilization when immediate extraction is not possible.

RAPD patterns produced by two primers AN16 and R15 (Fig. 2) clearly demonstrate the feasibility of using this technique in *Hevea*. From a total of 126 primers screened using six test clones, 57 were found to be polymorphic. Fourty two of these informative primers were further used to screen 24 clones (Table 2). These amplified 111 polymorphic fragments of which 80 (72%) were repeatable and clearly scorable across all 24 clones (Table 3). All clones tested could be distinguished by these 80 DNA polymorphisms, revealing the feasibility of RAPD markers for clone identification in *Hevea*. Since morphological variations among clones are not very distinct in immaturity period in *Hevea*, identification based on phenotypes is rather difficult. RAPD makers have been used for cultivar iden-

tification in species like cocoa (Wilde *et al.*, 1992), grapevine (Collins and Symore 1993); celery (Yang and Quiros 1993), apple (Tancred et al., 1994) and mango (Schnell *et al.*, 1995).

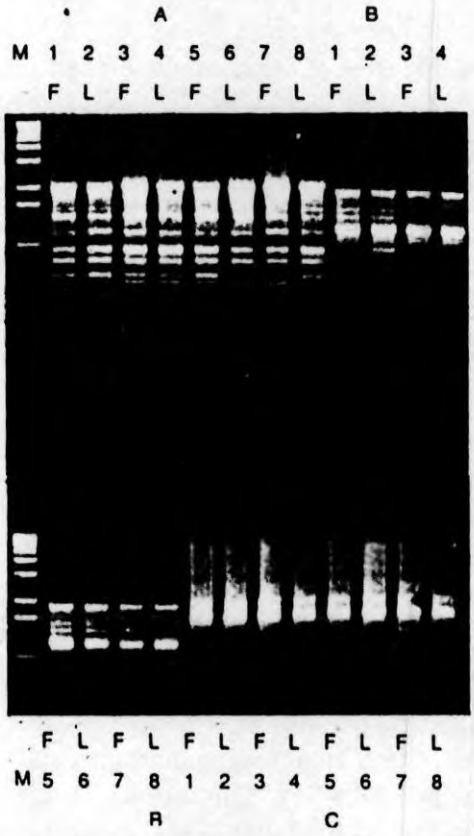


Fig.1. Comparison of RAPD pattern of fresh (F) and lyophilised (L) leaf samples using four clones (1, 2 : RRII 105; 3, 4 : PB 5/51; 5, 6 : PB 86 and 7, 8 : KRS 163) and three primers (A : AC 12; B : AK 11 and C : AN 12) M: Molecular size marker

**Table 3. Summary of detection of RAPD markers in *Hevea***

| | | |
|---|---|---|
| Total number of primers screened | : | 126 |
| Number of polymorphic primers (Monomorphic : 40; weak : 14; no amplification : 15) | : | 57 |
| Total number of fragments amplified by 42 informative primers | : | 220 |
| Size range of amplified products | : | 0.5-3.5 kb |
| Average number of fragments per primer | : | 5.2 |
| Total number of RAPDs identified | : | 111 |
| Average number of RAPDs per polymorphic primer | : | 2.6 |
| Range | : | 1-5 |
| Total number of repeatable RAPDs | : | 80 |
| Percentage over total RAPDs | : | 72 |

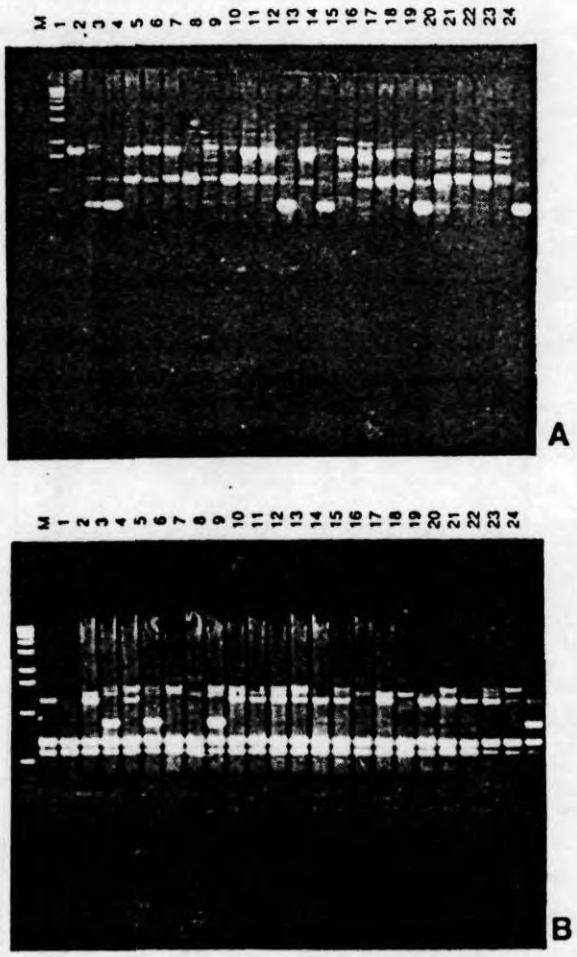


Fig.2. RAPD pattern of 24 *Hevea* clones generated by two primers OP-AN 16(A) and OP-R(B) Numbers 1-24 represent the clones listed in table 1. M indicates molecular size marker

Among the different clones tested RRIC 100 displayed the highest mean genetic distance of 0.616. Other clones with genetic distance above the general mean include RRII 5, RRII 105, RRII 208, HP 86-120, PB 28/59, PB 86, PB 217, PB 280, SCATC 88-13, and KRS 25 (Table 4). Frequency distribution of genetic distance shows that a majority of values are towards a higher range (Fig. 3). An average genetic distance of 0.5 is a rather high value indicating the presence of a fairly large amount of polymorphism among the clones tested and thereby the possibility of further genetic improvement. Similar results have been reported by Markose (1984) and Mydin *et al.*, (1992) using conventional genetic analysis and by Chevallier (1988) using isozymes.

**Table 4. Mean genetic distance of 24 clones**

| Clone | Genetic distance | | |
|---|---|---|---|
| | Mean | Min. | Max. |
| RRII 5 | 0.535 | 0.404 | 0.661 |
| RRII 105 | 0.514 | 0.143 | 0.614 |
| RRII 208 | 0.533 | 0.327 | 0.617 |
| RRII 308 | 0.458 | 0.050 | 0.655 |
| PCK 1 | 0.486 | 0.400 | 0.656 |
| HP 82/14 | 0.498 | 0.392 | 0.661 |
| HP 86/120 | 0.515 | 0.396 | 0.689 |
| HP 86/602 | 0.444 | 0.304 | 0.609 |
| HP 86/68 | 0.486 | 0.365 | 0.610 |
| RRIM 600 | 0.496 | 0.265 | 0.683 |
| PB 5/51 | 0.454 | 0.050 | 0.655 |
| PB 28/59 | 0.548 | 0.491 | 0.672 |
| PB 86 | 0.529 | 0.143 | 0.625 |
| PB 217 | 0.502 | 0.333 | 0.729 |
| PB 235 | 0.432 | 0.250 | 0.552 |
| PB 260 | 0.468 | 0.339 | 0.613 |
| PB 280 | 0.535 | 0.327 | 0.636 |
| PB 312 | 0.450 | 0.625 | 0.623 |
| GL 1 | 0.446 | 0.255 | 0.571 |
| Tjir 1 | 0.491 | 0.345 | 0.593 |
| GT 1 | 0.476 | 0.339 | 0.613 |
| SC 88/13 | 0.539 | 0.375 | 0.746 |
| RRIC 100 | 0.616 | 0.453 | 0.746 |
| KRS 25 | 0.550 | 0.458 | 0.621 |

Genotypes with medium to high levels of genetic distances have a high probability of producing heterotic hybrids (Thakur and Zarger 1989). Hence, for the improvement of a complex quantitative trait like rubber yields, it is suggested to select parents with desirable yield components and medium to high genetic distances. Similar should be the strategy adopted for selection of component clones for polyclonal seed gardens for production of promising polycross progeny. Clones recommended for commercial planting as clonal composites should also be genetically divergent so as to maintain genetic heterogeneity in the population.

## ACKNOWLEDGEMENT

This investigation was carried out at the Institute of Plant Breeding, Georg-August University, Gottingen, FRG and supported by a World Bank aided training programme. The senior author is grateful to Chairman, Rubber Board, Ministry of Commerce, Govt., of India for granting deputation for this work. The authors are grateful to Prof. Dr. G. Robbelen and Prof. Dr. H. Becker of the Institute of Plant

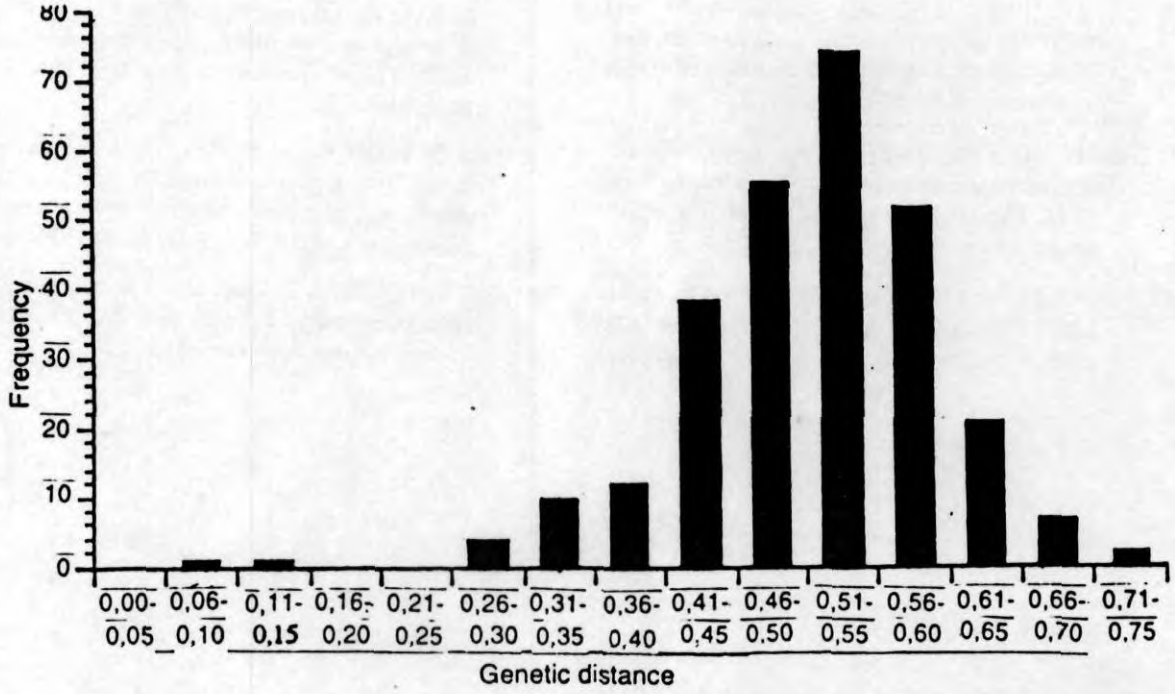


Fig. 3 Frequency distribution of genetic distance values of 24 *Hevea* clones (data of 80 scorable RAPD fragments)

Breeding, Gottingen, FRG, for making the facilities of the Institute available for the investigation.

## REFERENCES

BESSE, P., SEGUIN, M., LEBRUN, P. and LANAUD, C. 1993. Ribosomal DNA variations in wild and cultivated rubber tree (*Hevea brasiliensis*). *Genome*, **36**: 1049-1057.

BESSE, P., SEGUIN, M., LEBRUN, P. CHEVALLIER, M.H. and NICHOLAS, D. 1994. Genetic diversity among wild and cultivated populations of *Hevea brasiliensis* assessed by nuclear RFLP analysis. *Theoretical and Applied Genetics*, **88**: 199-207.

CHEVALLIER, M.H. 1988. Genetic variability of *Hevea brasiliensis* germplasm using isozyme markers. *Journal of Natural Rubber Research*, 3(1): 42-53.

COLLINS, G.G. and SYMORE, R.H. 1993. Polymorphisms in grapevine DNA detected by the RAPD PCR technique. *Plant Molecular Biology Reporter*, 11(2) : 105-112.

JACCARD, P. 1908. Nouvelles recherches sur la distribution florale *Bull. Soc. Vand. Sci. Nat.*, 44:223-270.

LOW, F.C., ATAN, S., JAAFAR, H. and TAN, H. 1995. The development of molecular markers for *Hevea* breeding. *Proceedings IRRDB Symposium*, 1995, China, pp. 8-18.

LUO, H., COPPENOLLE, B.V., SEGUIN, M. and BOUTRY, M. 1995. Mitochondrial DNA polymorphism and phylogenetic relationships in *Hevea brasiliensis*. *Molecular Breeding*, **1**: 51-63.

MARKOSE, V.C. 1984. *Biometric analysis of yield and certain yield attributes in the para rubber tree*. Ph.D. Thesis, Kerala Agricultural University, Kerala, 119 p.

MYDIN, K.K., NAIR, V.G., SETHURAJ, M.R., SARASWATHY, P. and PANICKAR, A.O.N. 1992. Genetic divergence in *Hevea brasiliensis*. *Indian Journal of Natural Rubber Research*, 5 (1&2) : 120-126.

QUIROS, C.F., HU, J., CHEVRE, A.M. and DELSENY, M. 1991. Development and chromosomal localization of genome-specific makers by polymerase chain reaction in *Brassica*. *Theoretical and Applied Genetics*, **82**: 627-632.

ROGERS, S.O. and BENDICH, A.J. 1988. Extraction of DNA from plant tissues. *Plant Molecular Biology Manual*, **A6**: 1-10.

SCHENELL R.J. and KNIGHT, R.J. 1993. Genetic relationships among *Mangifera* spp. based on RAPD makers. *Acta Hortic.*, **341**: 86-92

TANCRED, S.J., ZEPPA, A.G. and GRAHAM, G.C. 1994. The use of PCR-RAPD technique in improving the plant variety rights description of a new Queensland apple (*Malus domestica*) cultivar. *Australian Journal of Experimental Agriculture*, **34**: 665-667.

THAKUR, H.L. and ZARGER, M.A. 1989. Heterosis in relation to genetic divergence and specific combining ability in Indian mustard *Brassica juncea* L. Czern *Indian Journal of Genetics and Plant Breeding*, 49(2) : 223-226.

VARGHESE, Y.A., KNAAK, C., ECKE, W. and SETHURAJ, M.R. 1996. Evaluation of RAPD makers in *Hevea* breeding. *Second International Crop Science Congress*, 1996, New Delhi, India.

WILLIAMS, J.G.K., KUBELIK, A.R. LIVAK, K.J., RAFALSKI, J.A. and TINGEY, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18**: 6531-6535.

WILDE, J., WAUGH, R. and POWELL, W. 1992. Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. *Theoretical and Applied Genetics*, **83**: 871-877.

YANG, X. and QUIROS, C. 1993. Identification and classification of celery cultivars with RAPD markers. *Theoretical and Applied Genetics*, **86**: 205-212.