Natural Rubber Research, **25**(1): 21-30, 2012

EFFICIENT SCREENING OF AFLP PRIMER COMBINATIONS FOR EVALUATING GENETIC DIVERSITY AMONG CULTIVATED RUBBER (*HEVEA BRASILIENSIS*) CLONES

C. Bindu Roy, Minimol Ravindran and Thakurdas Saha

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

Received : 20 October 2011 Accepted : 26 December 2011

Roy, C.B., Ravindran, M. and Saha, T. (2012). Efficient screening of AFLP primer combinations for evaluating genetic diversity among cultivated rubber (*Hevea brasiliensis*) clones. *Natural Rubber Research*, **25(**1): 21-30.

Multilocus amplified fragment length polymorphism (AFLP) markers are used in genetic mapping, diversity analysis and genotyping. Success of the technique mainly depends on the identification of the primer combinations for selective amplification. In this study a simple method was demonstrated to identify the potential primer combinations using a mini polyacrylamide gel electrophoresis (PAGE) followed by silver staining. A modified silver staining protocol was developed to save time without compromising on the sensitivity. Further, the utility of the protocol in developing AFLP markers in rubber (*Hevea brasiliensis*) for assessment of genetic diversity among cultivated clones from different South East Asian rubber growing countries was demonstrated.

Keywords: AFLP, Denaturing mini PAGE, Genetic diversity, *Hevea brasiliensis*, Silver staining

INTRODUCTION

Molecular markers are a pre-requisite for genome analysis of any organism. During the last two decades, several techniques *viz*. RFLP, RAPD, AFLP, microsatellite and SNP have been developed for polymorphism studies. AFLP marker system is widely used for genomic characterization, genome mapping and also to develop diagnostic markers that are linked to various agronomic traits. This technique is PCR-based and produces dominant markers having a high multiplex ratio because of the random placement of restriction sites between different genomes combined with nucleotide sequence variability within a short stretch of DNA directly flanking these restriction sites (Vos *et al*., 1995).

Although the AFLP technique is tedious and time consuming, this appeared to be the marker of choice for its multi-allelic properties. Success in developing AFLP markers depends on several important steps: (1) complete digestion of DNA samples with two restriction enzymes; (2) adaptor ligation of the restricted DNA; (3) pre-amplification of the adaptor ligated DNA sample using complementary oligonucleotides as primers; (4) selective amplification of the pre-amplified product using different primer combinations with a change at their 3′ ends

Correspondence: Thakurdas Saha (Email: saha@rubberboard.org.in)

and (5) visualization of polymorphic bands through denaturing polyacrylamide gel electrophoresis (PAGE). One of the primers may be radiolabelled that facilitates detection of bands through exposure to X-ray film after running the samples on a denaturing PAGE. Alternatively, the amplified products may be detected through silver staining.

Monitoring of each and every step is essential for successful development of AFLP markers in any organism. The most important step is the screening of primer combinations for selective amplification of the template DNA as all the primer combinations may not be useful for studying the polymorphisms in a given species. Therefore, it is necessary to identify efficient primer-pairs that give greater polymorphism, which is essential for genomic characterisation. Here a simple and rapid methodology for screening of several primer combinations using a mini-PAGE followed by silver staining is described. This methodology is not only ideal for screening of primer combinations for AFLP studies, but is also efficient in identifying the incomplete restricted DNA samples and the degraded samples that lead to erroneous results. This methodology has successfully been used for AFLP marker development in *H. brasiliensis*, a tree crop producing latex of commercial utility.

MATERIALS AND METHODS

Plant material and DNA extraction

Twenty seven cultivated clones of *H. brasiliensis* were chosen for the study (Table 1). Of these, 13 were primary Wickham clones (Wycherley, 1968; Dean, 1987) and the rest were derived from hybridization of primary clones in South-East Asian rubber-growing countries. Total genomic DNA was extracted from one gram young leaves of each clone following the CTAB (cetyl trimethyl ammonium bromide) protocol (Doyle and Doyle, 1990).

AFLP fingerprinting

Restriction enzyme digestion and ligation of adapters

AFLP was performed according to the protocol of Vos *et al*. (1995) with minor

Table 1. ***H. brasiliensis*** **clones used in the study**

Clone	Origin	Country
RRII 105	Tjir 1 x Gl 1	India
RRII 118	Mil 3/2 x Hil 28	India
RRII 33	Primary clone	India
RRII 5	Primary clone	India
RRIC 52	Primary clone	Sri Lanka
RRIC 100	RRIC 52 x PB 86	Sri Lanka
RRIC 104	RRIC 52 x Tjir 1	Sri Lanka
RRIM 703	RRIM 600 x RRIM 500	Malaysia
RRIM 701	44/553 x RRIM 501	Malaysia
BD 10	Primary clone	Indonesia
PB 86	Primary clone	Malaysia
PB 314	RRIM 600 x PB 235	Malaysia
PB 235	PB 5/51 x PB S/78	Malaysia
PB 217	PB 5/51 x PB 6/9	Malaysia
PB 5/51	PB 56 x PB 24	Malaysia
PB 28/59	Primary clone	Malaysia
PR 107	Primary clone	Indonesia
PR 255	Tjir 1 x PR 107	Indonesia
PR 261	Tjir 1 x PR 107	Indonesia
Tjir 1	Primary clone	Indonesia
Tjir 16	Primary clone	Indonesia
SCATC 93/114	TR 31-45 x Heck 3-11	China
SCATC 88/13	RRIM 600 x PilB 84	China
GT 1	Primary clone	Indonesia
Gl 1	Primary clone	Malaysia
KRS 25	Primary clone	Thailand
Haiken 1	Primary clone	China

Table 2. **Sequence details of adapters and primers used in the AFLP analysis**

Adapters	Sequence (5′-3′)
Eco RI adapter	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTC
Mse I adapter	GACGATGAGTCCTGAG
	TACTCAGGACTCAT
Pre-selective primers	
E-A	GACTGCGTACCAATTCA
M-A	GATGAGTCCTGAGTAAA
Selective primers	
E-AGA	GACTGCGTACCAATTCAGA
E-AGC	GACTGCGTACCAATTCAGC
E-AGT	GACTGCGTACCAATTCAGT
E-AGG	GACTGCGTACCAATTCAGG
E-ACA	GACTGCGTACCAATTCACA
M-AAC	GATGAGTCCTGAGTAAAAC
M-ACA	GATGAGTCCTGAGTAAACA
M-AGA	GATGAGTCCTGAGTAAAGA
M-ACT	GATGAGTCCTGAGTAAACT
M-AGT	GATGAGTCCTGAGTAAAGT

modifications. Oligonucleotides used as primers and for the preparation of adapters (Table 2) were synthesized at Sigma Aldrich, Bangalore. Genomic DNA (500 ng) was digested with 10 U *Eco* RI and 10 U *Mse* I restriction enzymes (New England Biolabs). Reactions were performed in 1x One-Phor-All buffer (Amersham Biosciences) in a final volume of 50 µl with 0.5 µl BSA (10 mg/ml) and incubated at 37°C for 3 hours. Reaction was heat inactivated at 70°C for 15 min. Digested samples were purified through ethanol precipitation and dissolved in 10 µl water. Both *Eco* RI (5 pmol) and *Mse* I (50 pmol) adaptors were ligated onto the restricted fragments at 16°C overnight using 2 U of T4 DNA ligase (Amersham Biosciences) in a final volume of 20 µl. Ligations were diluted 10-fold in TE [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA] and used as templates in the pre-amplification PCR reaction.

PCR amplification

Pre-amplification was performed in 25 µl volume using 5 µl of the diluted ligation mixture, 0.2 µM each of *Eco* RI and *Mse* I primer (Table 2) and 1 U of *Taq* DNA polymerase (Roche Applied Sciences). PCR was carried out for 20 cycles using following conditions: denaturation at 94°C for 30 sec., annealing at 56°C for 30 sec. and extension at 72°C for 1 min. Samples were diluted 10-fold with sterile water and used as templates in selective amplification. PCR reaction was carried out using *Mse* I primer and *Eco* RI primer having three selective nucleotides at their 3′ ends. The final reaction volume was 25 µl containing 5 µl of 10-fold diluted pre-amplified template DNA, 2 mM of $MgCl_2$, 200 mM of each dNTP, 1.2 µM of *Mse* I primer, 0.2 µM of *Eco* RI primer and 1 U of Taq DNA polymerase (Promega). Amplification reactions were performed in GeneAmp PCR system 9600 (Perkin Elmer). The temperature cycle profile involving an initial touch down program was as follows: 94°C for 30 sec., 65°C for 1 min., $\Delta\downarrow$ 0.7°C for 12 cycles, 72°C for 1 min. This was followed by a normal cycling of 94°C for 30 sec., 56°C for 1 min., 72°C for 1 min. for 23 cycles and a final extension at 72°C for 10 min. After the completion of PCR reactions, equal volume of formamide loading buffer [0.1% each of bromophenol blue and xylene cyanol FF, 10 mM EDTA (pH 7.5) and 98% deionized formamide] was added immediately to stop the reaction and stored at -20°C. Amplification products were run either on a mini-PAGE (Bio-Rad) or on a large sequencing gel (Appligene).

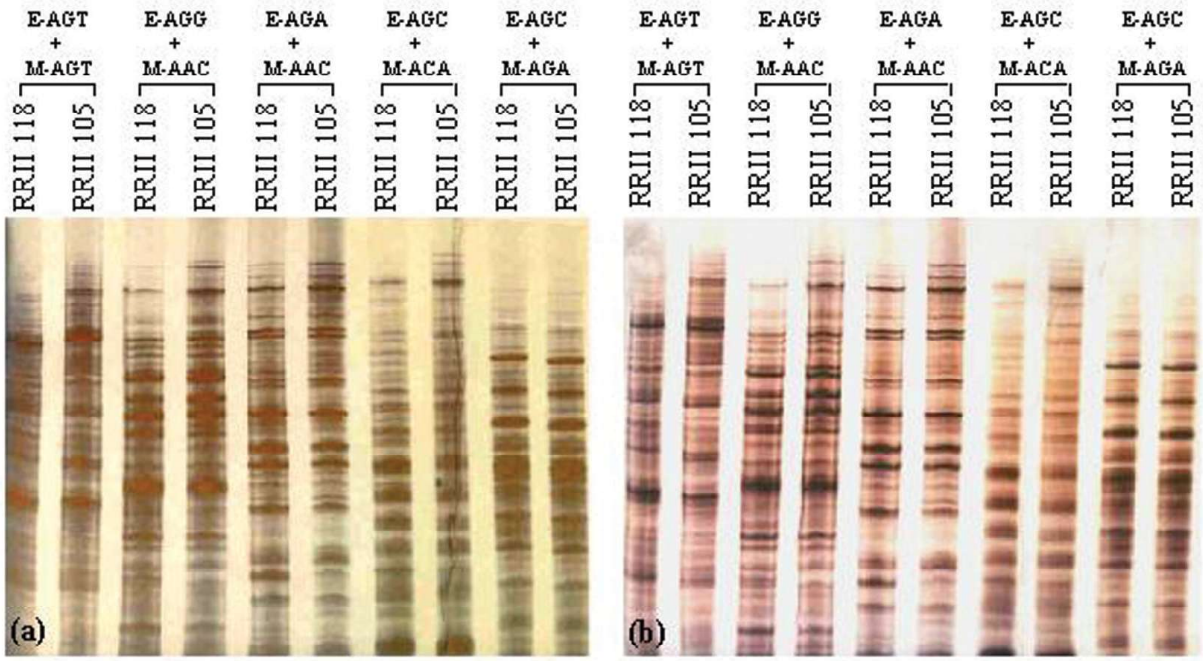

Fig. 1. Silver stained AFLP profiles of two genotypes, RRII 118 and RRII 105, with different primer combinations on denaturing mini-PAGE. (a) silver stainer gel and (b) staining protocol adopted with minor modifications showing lesser or no background

Silver staining for detection of bands on a mini-PAGE

Amplified products were separated on a denaturing 6% polyacrylamide gel in 1×TBE buffer (Sambrook *et al*., 1989) and silver stained to visualize the AFLP profiles. Two commonly used silver stain protocols (Bassam *et al*., 1991; Creste *et al*., 2001) were compared for their sensitivity to detect AFLP pattern on a mini-gel (Bio-Rad). A summary of both the silver staining methods is described in Table 3. Silver staining protocol of Creste *et al*. (2001) was adopted with minor modifications and described as 'modified protocol' (Table 3). The run was stopped after xylene cyanol dye had migrated out of the gel. Mini-gels were briefly washed in ddH_2O to remove surface urea and buffer. The gels were treated for oxidation by dipping in 1% nitric acid for 3 min. and rinsed thrice with double distilled water H_2O. This was followed by impregnating with 0.1% silver nitrate solution for 20 min. and rinsed with ddH_2O. Ice-cold developing solution was added to the gel and agitated until the bands appeared. The reaction was stopped by adding sufficient water just before the desired degree of development had been achieved. The gel was rinsed in water, blotted on to a 3 MM Chr. Whatman paper and dried using a Gel drier (Bio-Rad).

Isotopic detection of bands on a sequencing PAGE

The primer combinations that produced clear amplification of bands having similar intensities throughout the lane, as visualized through silver staining, were chosen. Initially, two distantly related *Hevea* clones (RRII 105 and RRII 118) were chosen for conducting a primer trial with 25

Table 3. **Steps involved in two widely used silver staining methods and the modified protocol followed in the study**

No.	Step	Bassam *et al*. (1991)	Creste *et al*. (2001)	Modified protocol
1.	Fix	10% acetic acid - 20 min	10% ethanol and 1% acetic acid - 10 min.	-
2.	Wash	-	H_2O - 1 min.	H_2O, 1 min., 2 times
3.	Oxidize	-	1.5% HNO_3 - 3 min.	1% HNO_3, 3 min.
4.	Rinse	H_2O - 2 min, 3 times	H_2O - 1 min.	H_2O - 1 min., 3 times
5.	Impregnate	0.1% $AgNO_3$, 1.5 ml 37% formaldehyde liter^{-1} - 30 min	0.2% $AgNO_3$ - 20 min.	0.1% $AgNO_3$ -20 min.
6.	Rinse	H_2O - 20 sec. (optional)	H_2O -30 sec., 2 times	H_2O -30 sec., 2 times
7.	Develop	3% Na_2CO_3, 1.5 ml 37% formaldehyde liter^{-1}, 2 mg $Na_2S_2O_3.5H_2O$ liter^{-1}, 2-5 min	3% Na_2CO_3, 0.54 ml 37% formaldehyde liter^{-1}, 4-7 min.	3% Na_2CO_3, 0.54 ml 37% formaldehyde liter^{-1}, 4-7 min.
8.	Stop	10% acetic acid - 5 min	5% acetic acid -5 min.	2-5 min. -wash in H_2O

Fixation in 10% acetic acid for 10 – 15 min is essential for large sequencing PAGE and same solution can be used further to stop developing reactions.

combinations (5 each of *Eco* RI and *Mse* I primers with three selective nucleotides) to identify informative primers. Nine primer-pairs (E-AGA/M-AAC, E-AGC/M-AAC, E-AGT/M-ACA, E-AGT/M-AGA, E-AGT/M-AAC, E-AGG/M-ACA, E-AGA/M-ACT, E-AGC/M-AGA, and E-AGT/M-ACA) were finally selected based on the AFLP profiles generated on mini-PAGE for their further use in characterization of 27 cultivated *Hevea* clones. *Eco* RI primers (with 3 additional bases at the 3′ end) of all the nine selected primer combinations were end-labelled using γ^{33}P-ATP prior to selective amplification. Selective amplification was carried out in a lesser volume (10 µl) containing 2 µl of 10-fold diluted pre-amplified DNA. The temperature cycle profile was the same as mentioned earlier. Amplification products were run on a 6% denaturing polyacrylamide gel containing 7 M urea using 0.6x TBE buffer at a constant power of 55 W. The gels were dried and then autoradiographed on X-ray film using standard procedures.

Data analysis

The presence or absence of bands on the autoradiogram was scored manually for all the 27 *Hevea* clones used in the study. Observed differences in band position were considered to represent different loci, each potentially having two allele states (presence or absence of bands) and variation in the presence of bands was recorded as polymorphic AFLP loci. Unambiguously scored loci were only used to quantify genotypic variation. Polymorphism information content (PIC), which determine the efficiency of each primer-pair in revealing polymorphism, was calculated as

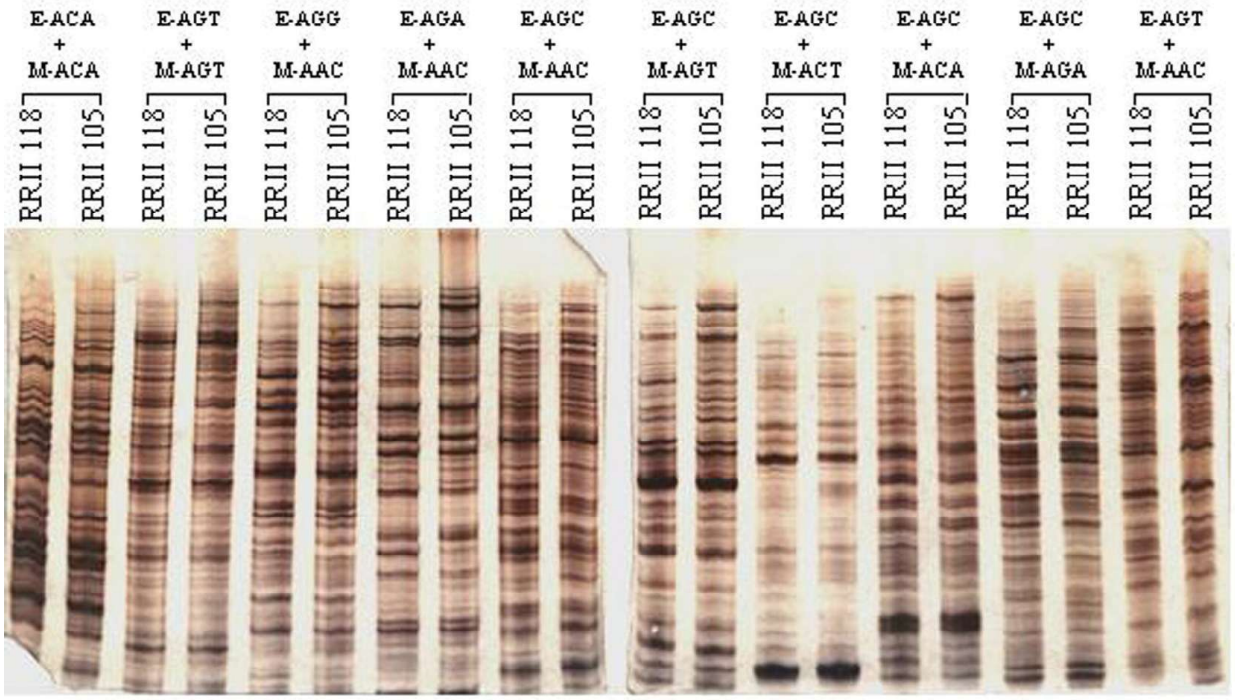

Fig. 2. Representative silver stained mini-PAGE showing AFLP profiles of two genotypes with ten primer combinations

PIC = 1-[f^2 + (1-f)^2], where 'f' is the frequency of the marker in the data set as described by De Riek *et al*. (2001) for dominant marker. PIC calculated for each band/ locus was averaged over all bands produced by a primer-pair. Pair-wise comparisons of the AFLP profiles, based on both unique and shared amplification products/bands, were employed to calculate genetic distance (Link *et al*., 1995). The data was subsequently used to construct a dendrogram using the UPGMA clustering algorithm. All calculations were made using the TREECON program version 1.3b (Van de Peer and De Wachter, 1994).

RESULTS AND DISCUSSION

AFLP reactions followed by visualization through mini-PAGE stained with silver showed clear fingerprints of the tested *Hevea* clones. Clear banding patterns could be visible with several primer combinations. Some of the primer combinations gave uneven banding pattern showing greater intensity localized to the upper half of the gel and thus eliminated from final AFLP assay. The other primer combinations that gave only few intense bands were eliminated as indicated earlier. This might have resulted from the amplifications of the repeat regions of the genome. Finally the primer combinations were selected based on intensity of the fingerprints as well as their polymorphic pattern as viewed on mini-PAGE followed by silver staining.

In the present study the two different silver staining protocols adopted for the optimization of conditions for detection of AFLP profiles on denaturing mini-PAGE displayed identical banding patterns. However, both methods had similar

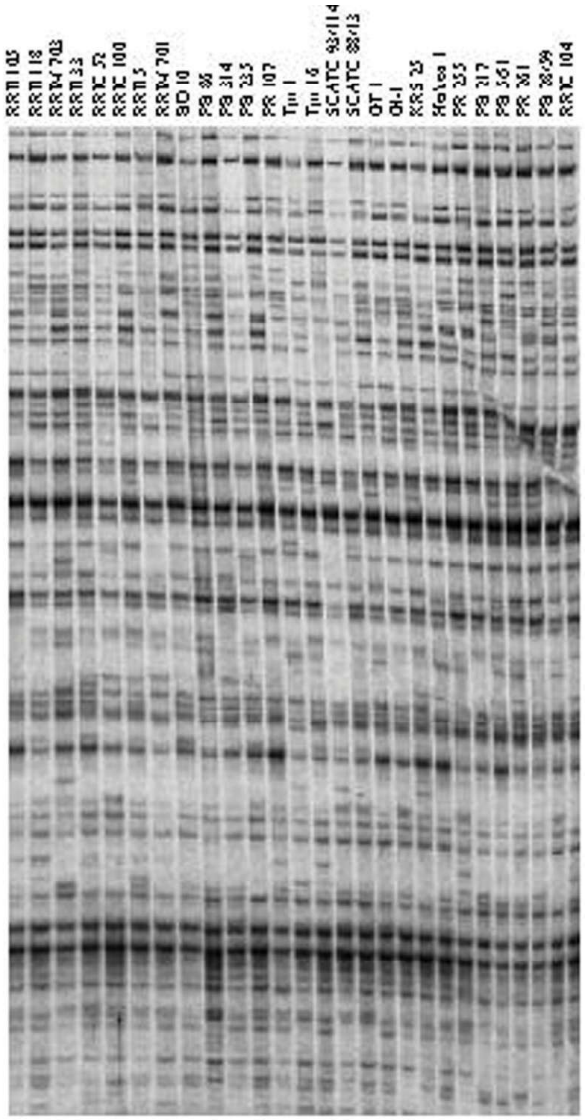

Fig. 3. Representative autoradiogram showing AFLP profiles of 27 cultivated rubber clones from South East Asian rubber growing countries using primer combination E-AGA + M-AAC

sensitivity but differed in contrast. Although the bands were clear with the protocol proposed by Bassam *et al*. (1991), the background staining could not be avoided, which may be due to the presence of formaldehyde in the staining solution and also its higher concentration (~ three-fold) in the developing solution, compared to the protocol developed by Creste *et al.* (2001). It was noticed that sometimes the background staining even obscured some of the minor bands. With the protocol of Creste *et al*. (2001) staining was more perfect as there was no detectable background staining. Therefore, this protocol was adopted for rest of the study with minor modifications. No detectable variation in staining efficiency was observed when the fixation of mini gels with 10% ethanol and 1% acetic acid was omitted. Instead the gel was washed briefly with distilled water prior to oxidation with 1% nitric acid. The modified protocol worked well with mini-PAGE even when the silver concentration was reduced to 0.1%. The impregnation of silver was uniform as the gel was freed from the glass plate during the initial stage of staining itself. With the large format gel (sequencing gel), both fixation step and higher concentration of silver nitrate may be essential as the gel is bound to the glass plate throughout the staining procedure. Further, the duration of treatment specially at staining and developing steps need to be extended. However, problems may be encountered with staining if the same tray is used both for staining and developing, even after washing the gel with sufficient water in between the two processes. This may be avoided by transferring the impregnated gel to a clean tray containing the cold developing solution. All the solutions except the developing solution could be reused at least twice without any detectable loss of sensitivity in staining.

AFLP analysis was employed in the present study to obtain necessary data about the level of genetic diversity existing among cultivated rubber clones. Based on AFLP profiles viewed on silver stained mini-PAGE, nine primer combinations were selected for polymorphism assay with 27 cultivated *Hevea* clones. All primer

Table 4. **Scores of polymorphic bands/loci amplified with nine primer combinations and their respective polymorphism information content (PIC) values**

Primer combination	Number of bands amplified	Number of polymorphic bands	Percentage of polymorphism	Polymorphism information content (PIC)
E-AGA/M-AAC	83	49	59.04	0.169
E-AGC/M-AAC	27	19	70.37	0.235
E-AGT/M-ACA	17	9	52.94	0.148
E-AGT/M-AGA	34	18	52.29	0.102
E-AGT/M-AAC	40	22	55.00	0.159
E-AGG/M-ACA	49	35	71.43	0.223
E-AGA/M-ACT	57	32	56.14	0.177
E-AGC/M-AGA	64	43	67.19	0.192
E-AGT/M-ACA	63	35	55.55	0.142
Total	434	262	60.37	

combinations were successfully used to determine the extent of genetic variation among these clones. The number of AFLP loci scored was 434 with an average of 48.3 loci generated with each primer combination (Table 4). The primer combination E-AGA/M-AAC showed maximum number of amplified loci (83) whereas the primer combination E-AGT/M-ACA showed least number of amplified loci (17). Out of 434 loci, 262 were polymorphic (60.4%). With all the selected primer combinations more than 50% polymorphism could be detected and two primer combinations: E-AGG/M-ACA and E-AGC/M-AAC generated over 70% polymorphism among the clones used in the study.

Genetic distance was calculated among the clones using the AFLP data generated. Phylogenetic relationship was established by constructing a dendrogram through cluster analysis using the distance matrix data. Although an average of 60% polymorphism was noticed, narrow genetic diversity (30%) was detected among the clones, which may be due to loss of genetic variation resulting from a directional selection for latex yield for several years. The above inference could be drawn based on our study involving wild *Hevea* accessions, where the genetic diversity detected among them was much higher than in popular clones (Saha *et al*., 2007). Geographical origin of the clones did not reveal any correlation with their clustering pattern except for the Sri Lankan clones. This is in agreement with the breeding history of *Hevea* as all the South East Asian *Hevea* clones had their origin from the Wickham collections (Wycherley, 1968; Dean, 1987). However, genetic relationship among some of the *Hevea* clones could be correlated with their pedigree. For example three Sri Lankan clones RRIC 52, RRIC 100 and RRIC 104 are grouped together at a distance co-oefficient of 0.23. The clone RRIC 52 is a primary clone, whereas RRIC 100 and RRIC 104 are hybrid clones derived from crosses where RRIC 52 was used as maternal parent. Similarly two Indonesian clones PR 255 and PR 261 were grouped together as expected as they were same origin (Tjir 1 x PR 107). However, an

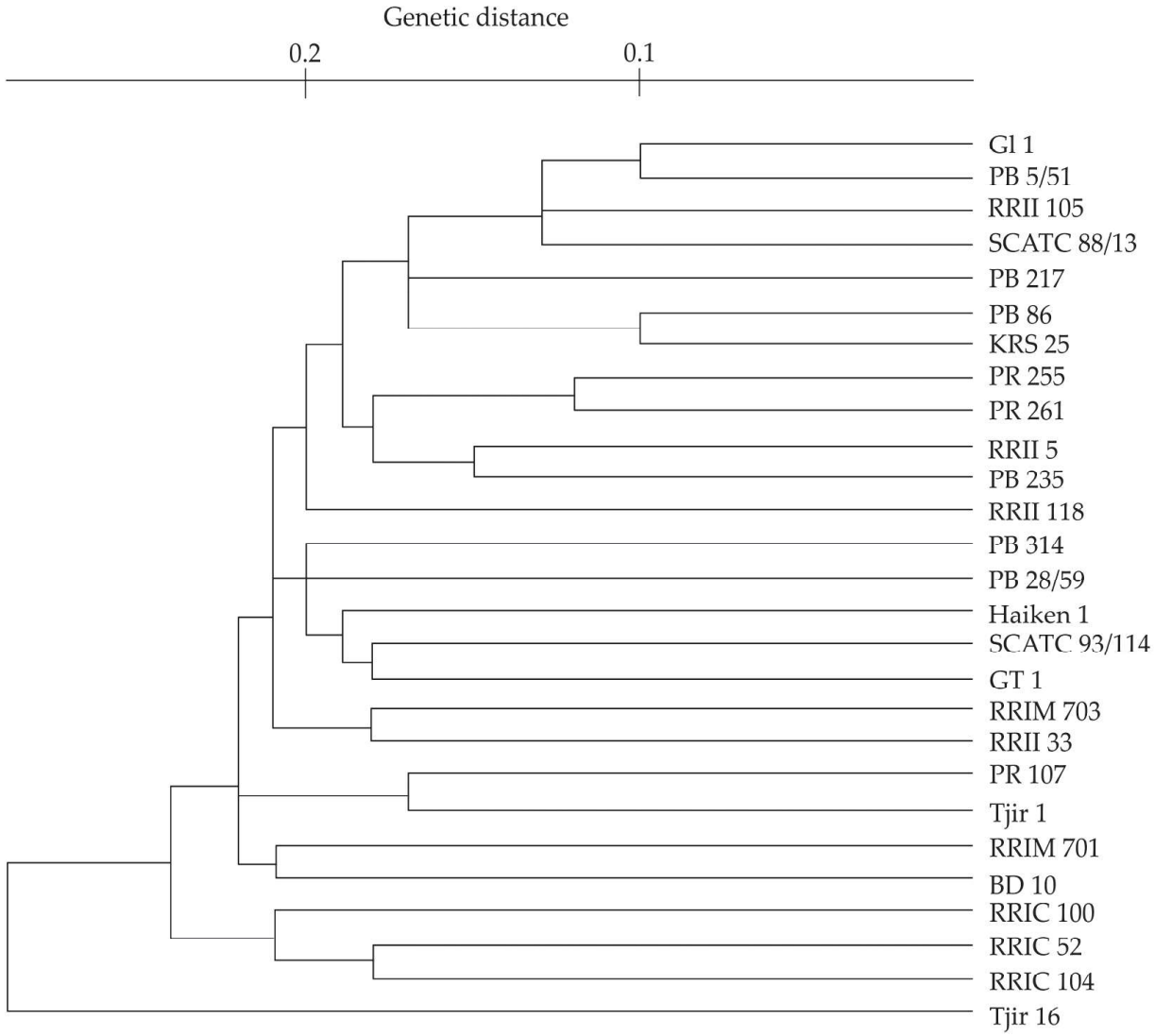

Fig. 4. Dendrogram showing inter-relationship among 27 clones of *H. brasiliensis* based on AFLP marker data

assessment of the extent of genetic variability among the cultivated rubber clones is essential for *Hevea* breeding and is particularly useful in choosing the right parental combination for hybridization program.

AFLP techniques are generally considered more powerful compared to other commonly used marker systems, such as RFLPs, RAPDs and microsatellites (Powell *et al*., 1996; Milbourne *et al*., 1997; Russell *et al*., 1997). Selection of AFLP primers based on silver-stained mini-PAGE certainly provides advantages over the direct use of primers for AFLP on large sequencing PAGE. This methodology appeared to be very cost effective, as to run sequencing PAGE only selected primer combinations are required. Adopting this technique radiation exposure can be

reduced, which can cause DNA damage and mutations. In this study, two commonly used silver staining protocols were compared and we developed a modified one, which technically more straightforward. By adopting this modified technique, potentially informative primer combinations can be screened for use in genetic diversity analysis among the popular clones of *H. brasiliensis*.

REFERENCES

Bassam, B.J., Caetano-Anollés, G. and Gresshoff, P.M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Annals of Biochemistry*, **196**: 80-83.

Creste, S., Neto, T.A. and Figueira, A. (2001). Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biology Reporter*, **19**: 299-306.

Dean, W. (1987). Brazil and the struggle for rubber: A study in environmental history, Cambridge University Press, Cambridge.

De Riek, J., Calsyn, E., Everaert, I., Van Bockstaele, E. and De Loose, M. (2001). AFLP band alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. *Theoretical and Applied Genetics*, **103**: 1254-1265.

Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, **12:** 13-15.

Link, W., Dixkens, C., Singh, M., Schwall, M. and Melchinger, A.E. (1995). Genetic diversity in European and Mediterranean faba bean germplasm revealed by RAPD markers. *Theoretical and Applied Genetics*, **90**: 27-32.

Milbourne, D., Meyer, R., Bradshaw, J.E., Baird, E., Bonar, N., Provan, J., Powell, W. and Waugh, R. (1997). Comparison of PCR based marker system for the analysis of genetic relationships in cultivated potato. Molecular Breeding, 3: 127-136.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S. and Rafalski, A. (1996). The comparison of RAPD, RFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding*, **2**: 225-238.

Russell, J.R., Fuller, J.D., Macaulay, M., Hatz, B.G., Jahoor, A., Powell, W. and Waugh, R. (1997). Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theoretical and Applied Genetics*, **95**: 714-722.

Saha, T., Roy, C.B., Ravindran, M., Bini, K. and Nazeer, M.A. (2007). Allelic diversity revealed through SSR polymorphisms at the locus encoding HMG-CoA reductase in rubber (*Hevea brasiliensis*). *Silvae Genetica*, **56**: 58-65.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Van de Peer, Y. and De Wachter, R. (1994). TREECON in Windows: A software package for the construction and drawing of evolutionary trees for Microsoft Windows. *Computer Applications in the Biosciences*, **10**: 569-570.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, L., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, **23**: 4407-4414.

Wycherley, P.R. (1968). Introduction of *Hevea* to the Orient. *The Planter*, **44**: 127-137.