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PHYLOGENETIC RELATIONSHIP OF *HEVEA* SPECIES AS REVEALED THROUGH MOLECULAR MARKERS

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Phylogenetic relationship was studied among three species of rubber available in India, *Hevea brasiliensis, H. benthamiana* and *H. spruceana* by employing different molecular marker techniques namely, RAPD, chloroplast DNA (cpDNA) PCR-RFLP and heterologous chloroplast microsatellites. RAPD analysis clearly indicated a high degree of polymorphism among the three species. Using twenty-five arbitrary primers, a total of 305 bands were amplified in the clones RRII 105 and GT 1 belonging to *H. brasiliensis, H. benthamiana, H. spruceana* and FX 516, an interspecific hybrid of *H. benthamiana* and *H. brasiliensis*, out of which 256 bands were polymorphic. Analysis of the interrelationships among the species clearly revealed that the clones of *H. brasiliensis* (>50% genetic dissimilarity) are closer to *H. benthamiana* than to *H. spruceana* (>70% genetic dissimilarity). Species-specific RAPD markers were identified for each species and their locus specificity was proved through hybridization. RFLP analysis using three hyper variable intergenic spacers, *rbcL-ORF106, trnM-rbcL* and *trnC-trnD* could not detect variability. Among different primer-pairs for heterologous chloroplast microsatellites tested, five could successfully be amplified, of which one (ccmp6) was highly polymorphic and could detect both intra- and inter-species polymorphism to prove the maternal mode of inheritance of the chloroplast genome in *Hevea*.

Key words: Chloroplast microsatellite, cpDNA polymorphism, DNA marker, *Hevea brasiliensis, Hevea benthamiana, Hevea spruceana*, Phylogenetic relationship, RAPD.

INTRODUCTION

The genus *Hevea* (family: Euphorbiaceae), to which the commercially cultivated rubber tree *Hevea brasiliensis* belongs consists of ten species originating in the Amazonian region of South America (Schultes, 1970; Wycherley, 1992; George and Panikkar, 2000). Rubber is now commercially cultivated in the tropical regions of Asia, Africa and America of which the former contributes the major share of the total global production. Among the other nine species, *H. benthamiana* also produces latex of comparable quality but has rarely been used in breeding programme (Lespinasse *et al.*, 2000). Although *H. brasiliensis* is a highly heterogeneous outbreeder, several years of directional selection for yield and vegetative method of propagation has narrowed down the genetic base of cultivated rubber. The exploitation of genetic resources available in wild species in breeding may be worthwhile in improving resistance to biotic and abiotic stress in *H. brasiliensis*. As only three species of *Hevea* are available in India namely, *H. brasiliensis, H. benthamiana* and *H. spruceana* (Varghese and Mydin, 2000), this study is restricted to these species.

Although genetic characterization of *H. brasiliensis* clones and wild germplasm have been attempted (Besse *et al.*, 1993, 1994; Shoucai *et al.*, 1994; Luo *et al.*, 1995; Varghese *et al.*, 1997; Lespinasse *et al.*, 2000; Venkatachalam *et al.*, 2002; Saha *et al.*, 2005), interspecific genetic relationship has

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not been studied. In the present study, an attempt is made to use three polymerase chain reaction based genetic molecular approaches namely, random amplified polymorphic DNA (RAPD), PCR-RFLP of intergenic spacers of chloroplast genome and heterologous chloroplast microsatellites, to identify interspecific and intraspecific variation. RAPD (Williams *et al.*, 1990; Demeke *et al.*, 1992; Lashermes *et al.*, 1993; Jarret and Austin, 1994), restriction site analysis of the non-coding intergenic spacer regions of chloroplast genome (Arnold *et al.*, 1991; Ogihara *et al.*, 1991; Parfitt and Badenes, 1997; Aradhya *et al.*, 1999) and chloroplast microsatellites (Provan *et al.*, 1997; Weising and Gardner, 1999; Kaundun and Matsumoto, 2002) have been used to study the interspecific variability in nuclear and chloroplast genomes of the three selected species of *Hevea* for establishing the phylogenetic relationship among them. The scope for using species-specific DNA markers for screening inter-specific hybrids has also been discussed.

MATERIALS AND METHODS

Plant material

Four genotypes belonging to three species of *Hevea*, *viz. H. brasiliensis* (cultivated clones RRII 105 and GT 1), *H. benthamiana* (clone F4542), *H. spruceana* and FX 516 (an interspecific hybrid between *H. benthamiana* and *H. brasiliensis* 'AVROS 363' as the male parent) constituted the experimental material. Besides the clones RRII 105 and GT 1 of *H. brasiliensis*, 26 other popular clones of the same species, were also included in the study to confirm the species-specific nature of a RAPD marker identified in *H. brasiliensis* clone GT 1.

Genomic DNA extraction and purification

Genomic DNA was isolated from one-gram fresh leaf samples of the five genotypes including the hybrid FX 516. Leaf samples were washed three times with sterile distilled water, blotted dry and immediately ground in liquid nitrogen. Extraction and purification of the total genomic DNA were carried out following a CTAB (Hexadecyl trimethyl ammonium bromide, Sigma Co., St. Louis, USA) method of Doyle and Doyle (1990) with minor modifications (Saha *et al.*, 2002).

RAPD analysis

Pre-selected 25 informative arbitrary decamer primers (Operon technology Inc. USA; Table 1) were used for assays. RAPD amplification was performed as described by Saha *et al.* (2002). The amplification of the samples was reproduced twice. Intense bands were scored on the basis of their presence or absence in the gel. Pair-wise comparison of the RAPD profiles of the isolates, based on both unique and shared amplification products, were employed to calculate genetic distance (Link *et al.*, 1995) using the Treecon programme (van de Peer and De Wachter, 1994). The data were subsequently used for cluster analysis to construct a dendrogram.

Preparation of RAPD blots

The RAPD bands resolved on a 1.4% agarose gel were transferred onto a positively charged nylon membrane (Hybond N+, Amersham Biosciences) using 0.4 N NaOH as transfer solution in a vacuum blotter (TransVac TE 80, Hoefer Scientific Instruments). The membrane was then neutralized with neutralizing buffer (0.5 M Tris pH 7.2, 1.5 M NaCl) for 30 min and washed in 2x SSC and the blots cross-linked using UV

irradiation (1200 x 100 J/cm^2) in a UV crosslinker (Hoefer Pharmacia Biotech Inc.) and stored at 4°C until further use.

Probe preparation, hybridization and detection

Desired RAPD fragments were excised from the gel and purified using GFX PCR DNA and gel band purification kit (Amersham Biosciences). The extracted DNA fragment was denatured by heating for 10 min in a boiling water bath and chilled quickly on ice. Non-radioactive random primed DNA labelling with digoxigenin-dUTP was carried out using DIG DNA Labelling and Detection Kit (Boehringer Mannheim).

Hybridization of the RAPD blots with DIG-labelled DNA probe was carried out in an appropriate volume of standard hybridization buffer (5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) at 65°C for 16-18 h in a hybridization oven (Personal Hyb, Stratagene). The hybridized membranes were washed twice in sufficient volume of 2x SSC, 0.1% SDS for 15 min each at room temperature followed by two washes with 0.1x SSC, 0.1% SDS at 68°C for 15 min each under constant agitation.

Immunological detection of the hybridized DNA fragments was carried out using the same DIG DNA Labelling and Detection Kit following the manufacturer's instructions. Results were documented by scanning the wet membrane. All the techniques adopted were essentially similar to that of Sambrook *et al.* (1989).

PCR amplification of hyper-variable, non-coding regions of chloroplast genome

Three pairs of chloroplast DNA primers (Table 2), which are homologous to the most conserved coding regions of cpDNA and capable of amplifying the more variable non-coding regions (Demesure *et al.*, 1995; Dumolin-Lapegue *et al.*, 1997), synthesized at M/s. Sigma-Genosys, UK, were used to amplify three non-coding spacer regions of cpDNA. PCR amplification was performed in a total volume of 50 µl containing 100 ng of template DNA with 0.5 mM of each primer, 0.2 mM of each dNTP, 2 units of Taq DNA polymerase (Promega, USA), 5 µl of 10x DNA polymerase buffer (100 mM Tris-HCl (pH 9.0), 500 mM KCl, 20 mM $MgCl_2$). Amplifications were performed in a thermal cycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, USA) with an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 30 sec at 94°C, 1 min at 58°C and 2 min at 72°C with a final extension of 10 min at 72°C and subsequent cooling at 4°C. Amplified products were separated on 1% agarose gel in 1x TAE buffer. The gels were stained and viewed using a gel documentation system (Eagle Eye II, Stratagene).

Restriction digestion of the amplified cpDNA fragments

In the absence of any sequence data, restriction enzymes were chosen randomly for the digestion of the PCR products. PCR amplified products were purified and digested with the restriction enzymes *Alu*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hae*III, *Hin*dIII, *Kpn*I, *Rsa*I, *Sau*3AI and *Taq*I using the buffers and conditions recommended by the suppliers (Promega). The digested fragments were separated on 2% agarose gel, stained and visualized as described above.

Analysis of chloroplast microsatellite markers

Ten consensus primer pairs, ccmp1 to ccmp10, flanking chloroplast microsatellites developed in *Nicotiana* (Weising and Gardner, 1999) were tried for the amplification. PCR was carried out in a 20 µL final volume containing 20 ng of genomic DNA, 0.2 mM of each of the forward and reverse primers, 200 mM dNTPs and 0.7 units of AmpliTaq Gold polymerase along with the buffer (supplied by Applied Biosystems). The temperature cycling profile involved an initial denaturation step of 5 min at 95°C followed by a touch down PCR. Temperature profiles of the touch down PCR for 7 cycles were as follows: 94°C for 30 sec, 63°C for 1 min, $\Delta\downarrow$1°C for 7 cycles and 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 and an extension of 72°C for 10 min. The touch down protocol was used to eliminate stutter and artifact bands. Radioactively labelled (one of the primers was labelled with ^{33}P) amplification products were run on acrylamide gel and visualized by exposure on X-ray film.

RESULTS

The three molecular marker techniques, RAPD, PCR-RFLP and chloroplast microsatellites were useful for characterizing both nuclear and chloroplast genomes in *Hevea*.

RAPD

With the 25 selected primers (Figs. 1a-1e) a total 305 RAPD loci were amplified in the five genotypes of *Hevea* with an average amplification rate of 12.2 loci per primer. Out of the amplified loci, 256 (84%) were polymorphic. Maximum number of polymorphic bands was noticed with OPH-03. However, the polymorphism index was higher (1.00) with the primers OPAA-19, OPAC-06 and OPC-05, as all the amplified bands were found to be polymorphic (Table 1). High levels of polymorphisms observed using different primers indicate the presence of remarkable interspecific genetic variation among these species. Specific amplification of parental bands in the hybrid clone FX 516 could be observed with some of the primers, proving its hybrid nature.

Assessment of genetic diversity

Genetic relationships among the genotypes were analyzed using genetic distance data based on pair-wise comparison of the RAPD profiles. All the 305 amplified

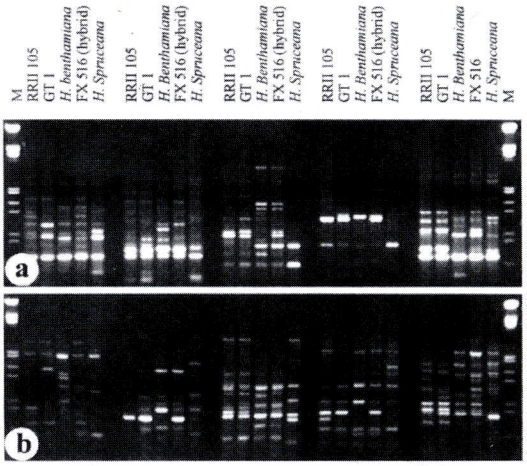

Fig. 1. Representative RAPD profiles generated with the Operon primers OPAA-15, OPAC-04, OPAC-05, OPAC-06, OPAC-12 (a) and OPAA-07, OPC-05, OPD-08, OPG-10, OPH-03 (b) for three *Hevea* species including two popular clones belonging to *H. brasiliensis*, RRII 105 and GT 1. Lane M: molecular weight marker (λ–DNA/*Eco* RI + *Hin*d III).

Table 1. **Details of the primers used and the fragments amplified in RAPD assay**

Operon primer	Sequence 5'-3'	No. of amplified fragments	Fragment size range (bp)	No. of polymorphic fragments	Polymorphism index
OPAA-01	AGACGGCTCC	13	546 - 2427	9	0.69
OPAA-02	GAGACCAGAC	16	745 - 2329	15	0.94
OPAA-07	CTACGCTCAC	14	563 - 2508	13	0.93
OPAA-10	TGGTCGGGTG	17	457 - 1965	16	0.94
OPAA-15	ACGGAAGCCC	12	492 - 2208	8	0.67
OPAA-17	GAGCCCGACT	16	430 - 3073	12	0.75
OPAA-19	TGAGGCGTGT	9	638 - 1809	9	1.00
OPAB-06	GTGGCTTGGA	13	455 - 2465	12	0.92
OPAB-07	GTAAACCGCC	7	493 - 1466	6	0.86
OPAB-11	GTGCGCAATG	8	440 - 1253	6	0.75
OPAB-14	AAGTGCGACC	8	402 - 1411	6	0.75
OPAB-19	ACACCGATGG	5	700 - 1225	4	0.80
OPAC-04	ACGGGACCTG	15	434 - 2439	13	0.87
OPAC-05	GTTAGTGCGG	12	557 - 3062	11	0.92
OPAC-06	CCAGAACGGA	3	777 - 1289	3	1.00
OPAC-12	GGCGAGTGTG	13	462 - 2656	12	0.92
OPAD-10	AAGAGGCCAG	9	428 - 2456	3	0.33
OPAD-12	AAGAGGGCGT	19	290 - 2767	16	0.84
OPAD-14	GAACGAGGGT	17	332 - 2586	16	0.94
OPAE-14	GAGAGGCTCC	14	373 - 1627	10	0.71
OPAE-17	GGCAGGTTCA	7	527 - 1659	5	0.71
OPC-05	GATGACCGCC	10	532 - 1670	10	1.00
OPD-08	GTGTGCCCCA	17	502 - 2638	15	0.88
OPG-10	AGGGCCGTCT	12	485 - 2358	8	0.67
OPH-03	AGACGTCCAC	19	700 - 2452	18	0.95
TOTAL		305		256	

loci were considered for the analysis. A dendogram showing the interspecific (among *H. brasiliensis*, *H. benthamiana* and *H. spruceana*) genetic distances (Fig. 2), was constructed using the UPGMA clustering method. The five genotypes were clustered into three groups. In one group, RRII 105 and GT 1 were grouped together with a minimum distance coefficient of 0.28. *H. benthamiana* and FX 516 were clustered to-

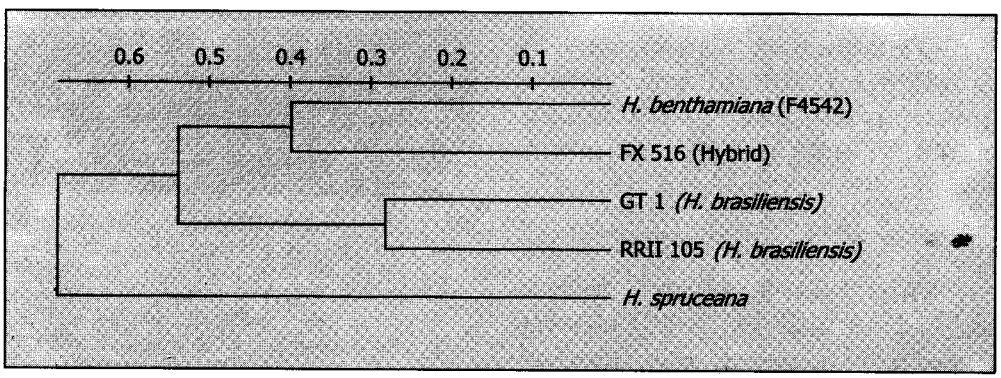

Fig. 2. Dendrogram showing intra- and inter-species genetic relationships in *Hevea*.

gether to form another group with a distance coefficient of 0.39. *H. spruceana* was placed in a separate group indicating its greater genetic distance from the other two groups. Clustering of RRII 105 with GT 1 in one group and *H. benthamiana* with FX 516 in another is logically expected as RRII 105 and GT 1 belong to *H. brasiliensis*, while FX 516 is a derivative of an interspecific hybridization where the maternal parent is *H. benthamiana*. Grouping of FX 516 with *H. benthamiana* revealed more genetic contribution in the form of organelle genomes from maternal parent compared to its paternal parent 'AVROS 363' (*H. brasiliensis*) as noticed in most of the angiosperms. More than 50% genetic dissimilarity was recorded between the two groups. The third species, *H. spruceana* was placed alone in another group with distance coefficients of 0.66 and 0.71 with *H. benthamiana* and *H. brasiliensis* respectively indicating comparative closeness to the former.

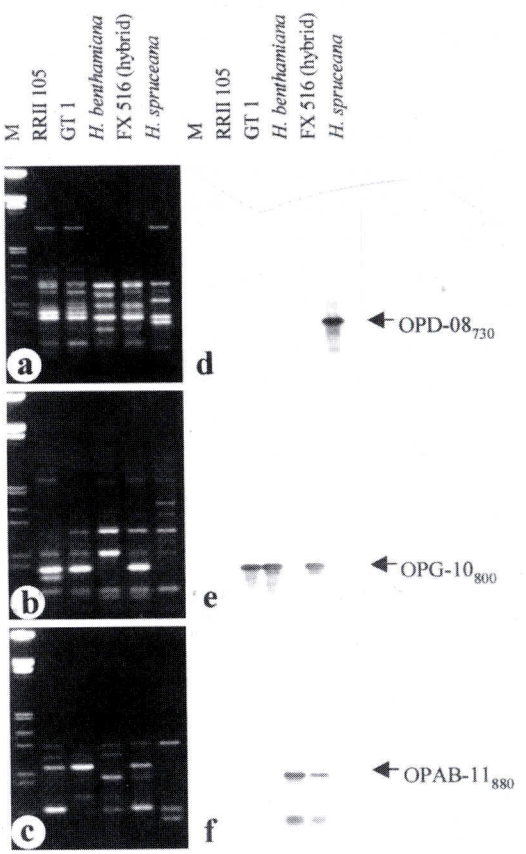

Fig. 3. Identification of species-specific RAPD markers in *Hevea*. RAPD fragments OPD-08_{730} (a), OPG-10_{800} (b) and OPAB-11_{880} (c) show species-specific amplifications for *H. spruceana*, *H. brasiliensis* and *H. benthamiana* respectively (arrow). Hybridization of the RAPD blots with the DIG labelled species-specific fragments clearly show locus specificity for all the three species (d, e and f). Lane M: molecular weight marker (λ-DNA/ *Eco* RI + *Hind* III).

Species-specific RAPD markers

Species-specific RAPD profiles were detected with several Operon primers used in this study. RAPD profiles with OPD-08, OPG-10 and OPAB-11 were considered for developing species-specific markers. These three primers amplified unique fragments of 730 bp in *H. spruceana* (Fig. 3a), 800 bp in *H. brasiliensis* (Fig. 3b) and 880 bp in *H. benthamiana* (Fig. 3c) respectively. To confirm their species-specific nature, these fragments were excised out of the gel and used as probes for hybridization against the RAPD profiles of all the species with the respective primers. This was essential in order to check whether sequence homology existed among the amplified products from different species, which would indicate the locus specificity of the selected marker fragment. The respective marker fragments (*H. spruceana* – OPD-08_{730}; *H. brasiliensis* – OPG-10_{800} and *H. benthamiana* – OPAB-11_{880}) clearly showed their species-specificity when subjected to hybridization against the RAPD blots with respective primers and

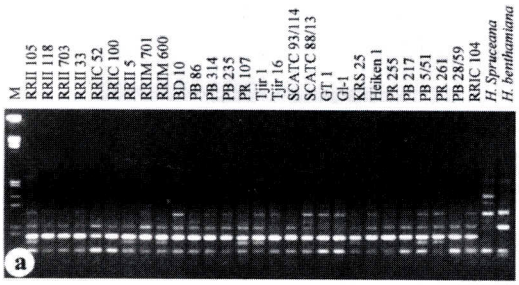

Fig. 4. Gel photograph showing RAPD profiles of 28 popular clones of *H. brasiliensis* and two other species (a), when hybridized against a *H. brasiliensis* specific marker OPG-10$_{800}$, consistent locus specific hybridization is noticed across all the *H. brasiliensis* clones (b). Clones are indicated along the lanes. Lane M: molecular weight marker (λ-DNA/*Eco* RI + *Hind* III).

detection using non-radioactive probes (Figs. 3d-3f). Locus specific hybridization was noticed in the interspecific hybrid FX 516 with the species-specific RAPD marker for *H. brasiliensis* (Fig. 3e) as well as for the marker specific to *H. benthamiana* (Fig. 3f), indicating genetic introgression from both the parents through hybridization. Species specificity of the marker fragment for *H. brasiliensis* (OPG-10$_{800}$) was confirmed again by the consistent locus specific hybridization across the 28 popular clones of *H. brasiliensis* (Fig. 4).

Restriction analysis of amplified CpDNA

Three non-coding intergenic spacer regions of chloroplast DNA amplified approximately 3.2, 3.0 and 3.1 kb fragments with the primer pairs *rbcL-ORF106*, *trnM-rbcL* and *trnC-trnD* (Table 2) respectively, without showing any length polymorphisms among the species (Fig. 5). There was no detectable variation in the restriction profiles among the species, including the interspecific hybrid, with each of the restriction enzymes tested (Figs. 6a-6c). Hence, informative data could not be generated for phylogenetic relationship studies.

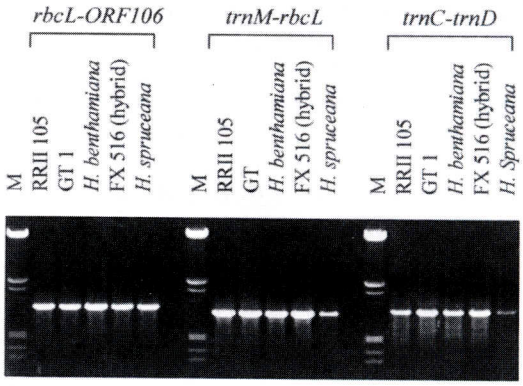

Fig. 5. PCR amplification of non-coding intergenic spacer regions of chloroplast genome in *Hevea*. Genotypes and the primer pairs used for the amplifications of the chloroplast DNA regions are indicated. Length polymorphisms of the amplified products are not detected among the species. Lane M: molecular weight marker (λ-DNA/*Eco* RI + *Hind* III).

Table 2. **Primers used for amplification of hyper-variable intergenic spacer sequences from *Hevea* chloroplast genome**

cpDNA intergenic spacer	Universal primer-pair	Nucleotide sequence(5′-3′)	Amplified product size (kb)
rbcL-ORF106	*rbcL ORF106*	ATGTCACCACAAACAGAAACTAAAGCAAGT ACTACAGATCTCATACTACCCC	3.2
trnM-rbcL	*trnMrbcL*	CCAGTTCAAATCTGGGTGTC GGGATTGTAGTTCAATTGGT	3.0
trnC-trnD	*trnCtrnD*	ACGGAGAGTTTGATCCTG AAAGGAGGTGATCCAGCCGCACCTTC	3.1

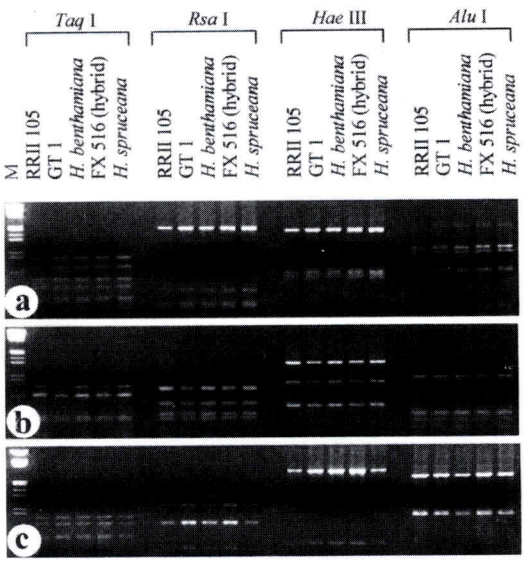

Fig. 6. Gel photographs of the restriction digestion profiles of the PCR amplified chloroplast intergenic spacers with the primer pairs *rbcL – ORF106* (a), *trnM – rbcL* (b) and *trnC – trnD* (c). Genotypes and the restriction enzymes are indicated. Lane M: molecular weight marker (λ-DNA/*Eco* RI + *Hin*d III).

Chloroplast microsatellite and interspecific polymorphism

Out of 10 primer-pairs for chloroplast microsatellites: ccmp1 to ccmp10, 5 primer-pairs gave amplification in *Hevea* (ccmp2, ccmp3, ccmp5, ccmp6 and ccmp7). Intra- and inter-species polymorphisms were detected only with the primer pair ccmp6 (Fig. 7) having the following sequences 5'-CGATGCATATGTAGAAAGCC-3' (forward primer) and 5'-CATTACGT GCGACTATCTCC-3' (reverse primer). Other four were monomorphic. Three unique cpDNA haplotypes with the size variants of 95, 97 and 98 bp at the locus ccmp6 were detected among the *Hevea* species under study. The haplotype 95 bp, present in *H. benthamiana*, was also detected in the hybrid FX 516 proving *H. benthamiana* as the

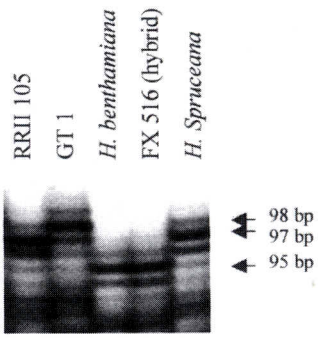

Fig. 7. Autoradiogram showing the amplification of heterologous chloroplast microsatellite marker ccmp6 in *Hevea*. Three different haplotypes are detected among the *Hevea* species. Both *H. brasiliensis* (clone RRII 105) and *H. spruceana* share a common haplotype (97 bp), but the clone GT 1 shows the presence of a haplotype (98 bp) although it belongs to *H. brasiliensis*. *H. benthamiana* and the interspecific hybrid FX 516 share the common haplotype (95 bp).

maternal parent of the hybrid. An interesting observation was made with *H. brasiliensis* revealing the presence of two different haplotypes in two popular *Hevea* clones RRII 105 and GT 1, although they belong to the same species (Fig. 7). Clone GT 1 was characterized by the presence of a haplotype of 98 bp, whereas RRII 105 had 97 bp, shared by another species *H. spruceana*. Consistency of the haplotype in other clones of *H. brasiliensis* was also checked and it was found that all the experimental clones had the same haplotype *i.e.*, 97 bp detected in RRII 105 (results not shown). Presence of a similar haplotype both in *H. spruceana* and *H. brasiliensis* may be indicative of their common maternal ancestry.

DISCUSSION

In this study, RAPD markers have successfully been used to understand the genetic diversity existing among three *Hevea* species, which is not possible using morpho-

logical traits due to their high degree of plasticity. RAPD clearly revealed a high degree of polymorphism among the three species and a phylogenetic relationship was established. Species-specific markers developed for each species provide an additional tool to discriminate the species as well as to identify inter-specific hybrids at an early stage of growth in breeding experiments. A large number of unique RAPDs detected in *H. benthamiana*, which is a source of resistance to many fungal diseases, could be very useful in marker-assisted selection if they are carefully linked to the desirable traits.

Chloroplast genome analysis provides complementary information to that obtained with nuclear markers. cpDNA has been frequently utilized for phylogenetic analysis in plants because many cpDNA regions appear to evolve at rates appropriate for resolving relationships at the interspecific and higher levels (Palmer *et al.* 1988; Liston, 1992; Petersen and Doebley, 1993; Huang and Sun, 2000). However, PCR-RFLP of chloroplast DNA in *Hevea* could not detect any polymorphism, which is possibly indicative of their conserved nature across the species. Of the ten universal chloroplast microsatellites developed in *Nicotiana*, only five loci were successfully amplified in *Hevea* indicating substantial differences in the flanking regions of the microsatellite motifs between these two genera belonging to two different families, which can be attributed to their distant phylogenetic relationship. Polymorphisms were detected only with one locus ccmp6 identifying three haplotypes among the experimental *Hevea* genotypes. Use of chloroplast microsatellites for polymorphism study was thus more effective than the PCR-RFLP of intergenic spacer regions of cpDNA in *Hevea*. Analysis of a single microsatellite locus with only three *Hevea* species constrains us from arriving at a conclusion regarding the phylogenetic relationships in *Hevea*. However, this single chloroplast microsatellite locus clearly indicated the maternal mode of inheritance of the chloroplast genome in *Hevea* as evidenced by the shared haplotype between the maternal parent *H. benthamiana* and the interspecific hybrid FX 516. Such maternal inheritance is observed in majority of angiosperms although with exceptions (Harris and Ingram, 1991; Testolin and Cipriani, 1997; Yang *et al.*, 2000).

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