Identification of simple sequence repeats in rubber (*Hevea brasiliensis*)

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The present study was undertaken to identify simple sequence repeats (SSRs) or microsatellites in *Hevea*, an important tree crop producing latex of commercial utility. A small-insert genomic library of *Hevea*, derived from a clone GT1, was screened for the presence of SSRs (AC/TG and CT/GA motifs) and finally 154 clones were recovered. PCR amplification with vector-directed primers as well as with the repeat sequences confirmed 114 positive clones. Restriction analyses of the clones identified six duplicates and were thus eliminated. Fifty positive clones out of 108 were sequenced. Similarly, a large-insert genomic library, derived from a popular clone RRII105, was also screened and 24 positive clones were sequenced to validate the presence of microsatellites in the *Hevea* genome. Sequence data revealed the presence of 67 microsatellites having characteristic simple and compound repeats, out of which 59 were from GT1 and eight from RRII105. Different types of repeat motifs comprising dinucleotides (TG/AC, AG/TC, TA/AT), trinucleotides (AAG, AGG, ATT), tetranucleotides (GAAA, AAGG, ATCC, TAAA, AAAT) and one pentanucleotide (GAAAT) were detected. The abundance of CT/GA repeats as observed in *Hevea* genome was akin to those reported in other crops.

MICROSATELLITES or simple sequence repeats (SSRs) are among the most efficient class of molecular markers due to their hyper-variable and co-dominant nature, with relatively high abundance and random distribution in the genome[1]. Such repeats display high levels of polymorphism because of variation in repeat length and can be rapidly analysed through PCR and gel electrophoresis. Microsatellites also allow relatively simple interpretation and genetic analysis of a single-locus and are easily accessible to other laboratories through published primer sequences[2,3]. In plants, SSR markers have been successfully applied to a variety of questions, including the construction of genetic maps[4], assessment of genetic diversity[5], cultivar identification and pedigree studies[6]. Recently, microsatellites have been isolated and characterized in several tree species, including apple[7], grapevine[8] and peach[9].

The rubber tree *Hevea brasiliensis* (Wild. Ex. Adr. de. Juss. Muell. Arg), produces about 98% of the world's natural rubber. Conventional genetic analysis in *Hevea* is difficult because of its perennial nature, long breeding and selection cycles, and difficulties in raising F2 progenies. With the advent of PCR technology in the nineties[10], there has been a remarkable progress in the development of an array of potential molecular markers, including RAPD, AFLP, microsatellites, etc. for characterization of plant genome. Available reports on *Hevea* described the successful application of RAPD or RFLP markers, including assessment of genetic variability in cultivated clones and wild accessions using RFLPs[11], clonal identification and evaluation of genetic diversity in popular clones using RAPDs[12,13], estimation of phylogenetic relationships from mitochondrial DNA RFLPs[14] and identification of mildew resistance genes by RAPD techniques[15]. Low *et al.*[16] detected for the first time, microsatellites in the *Hevea* genome through the database search of some *Hevea* gene sequences. DNA fingerprints in *H. brasiliensis* using heterologous minisatellite probes from humans were reported by Besse *et al.*[17] and the construction of a microsatellite-enriched library in *H. brasiliensis* was reported by Atan *et al.*[18]. Lespinasse *et al.*[19] also described the construction of a genetic linkage map of rubber tree using different molecular markers. There are only two published reports of microsatellites in *Hevea*[19,20]. Therefore, it was felt necessary to develop this potential marker system for its exploitation in the characterization of the *Hevea* genome. With an aim of producing microsatellite markers in *Hevea*, an attempt has been made towards isolation of microsatellites from *H. brasiliensis*. The present work reports the successful identification and characterization of microsatellite-bearing genomic clones, which could be considered as a major step towards the development of microsatellite markers in *Hevea*.

Genomic DNA of *H. brasiliensis* (GT1 clone) was extracted from tender green leaves following the CTAB (hexadecyl trimethyl ammonium bromide, Sigma Co, St. Louis, USA) method of Doyle and Doyle[21]. A small insert *Hevea* genomic library of the clone GT1 was constructed in lambda vector (ZAP Express, Stratagene, USA). Genomic DNA was completely digested with the restriction enzymes *Eco*RI and *Xho*I, then size fractionated on an agarose gel to isolate the fragments in the range of 200–800 bp, which were purified through gel extraction using the GFX column (Amersham Biosciences, USA), then subsequently cloned into ZAP Express vector and packaged in Gigapack III gold packaging extract following the protocol (Stratagene, USA). This phage was plated after incubation with *E. coli* XL-1 Blue cells. Plaques were transferred onto positively charged Nylon membranes (Amersham Biosciences, UK) according to standard procedures[22] and screened through plaque hybridization and probing with a mixture of radiolabelled (γ^{32}P-dATP) synthetic dinucleotide repeat probes $(AC)_{20}$, $(CT)_{20}$ and $(AT)_{20}$. The positive plaques were recovered individually and were subjected to a second round of screening following the same procedure. The recombinant lambda vector from each of the positive plaques was converted into pBK-CMV phagemids by *in vivo* excision that allows insert characterization in the plasmid system. All the positive clones were subjected to PCR amplifica-

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tion using vector-directed T3 and T7 promoter primer-pair to determine the size of the insert. The same primer was also used in combination with repeat primers $(AC)_8$ and $(CT)_8$ to identify the presence of sufficient flanking regions around the repeats, which was essential for effective primer designing. The excised phagemids were double-digested with *Xba*I and *Bam*HI to identify the duplicates for their elimination prior to sequencing. Positive clones were sequenced from both the ends of the inserts using a BigDye Terminator Cycle Sequencing kit and the product was run with an Applied Biosystems ABI 3700 Sequencer at the Microsynth GmbH, Switzerland to validate the presence of microsatellites/SSRs in the genomic libraries of *Hevea*. Similarly, another large insert *Hevea* genomic library, constructed with the popular clone RRII105 was also screened for the presence of SSRs.

Synthetic oligonucleotides comprising $(AC)_{20}$, $(CT)_{20}$ and $(AT)_{20}$ were used as probes in screening the *Hevea* genomic library as these repeats are most prevalent in many plant species[23–26]. Among the approximately 15,000 plaques screened, 204 clones/plaques were found to be positive for CT/GA and AC/TG repeats. All these putative positive clones were recovered individually for a second round of screening with the same probe and 154 clones were confirmed to be positive for the repeats. Finally, 114 clones were selected through PCR amplification using vector-directed T3 and T7 promoter primers (Figure 1) and also with the repeat sequences $(GT)_8$, $(AC)_8$, $(AG)_8$ and $(CT)_8$ as one of the primers, which facilitated the identification of sufficient flanking sequences around the repeats. Six clones (5.3%) appeared to be duplicates following digestion of the clones with *Xba*I and *Bam*HI, and were thus eliminated. Fifty positive clones out of 108 were subjected to sequencing and various kinds of repeat motifs were identified as described in Table 1.

Regarding the screening of large-insert genomic library developed from the *Hevea* clone RRII105, 24 purified phagemid clones having an insert size ranging from 4 to 6 kb (comparatively smaller inserts from the large-insert library) were subjected to single-pass sequencing reactions from both the ends. Eight clones were identified to possess microsatellite repeats (both simple and compound) in them. As the size of the inserts was more than 4 kb, sequencing of the entire insert was difficult and tedious. The observed frequency of the repeat sequences was low from this library, possibly due to the presence of these repeats beyond the sequenced area subjected to a single-pass reaction. Owing to this problem, further screening of the clones for the presence of the repeats was performed making use of the small insert library alone, developed from the GT1 clone of *H. brasiliensis*. Screening was also performed with the dinucleotide repeat probe AT/TA repeat and no positive signal was obtained. However, the AT repeats were found to be associated with other dinucleotide repeats (Table 1), as simple or compound, when probed with AC and CT repeats. This observation indicates that AT repeats are indeed present in the *Hevea* genomic clones but could not be identified when hybridized with the same repeats as probe, possibly due to their self-annealing nature which has to be carefully evaluated. As there are reports about the prevalence of AT repeats in some crop species[24,25], an attempt was made for screening of genomic library through hybridization with freshly denatured AT repeats as probe. However, the situation could not be improved, which led to the conclusion of the minimum occurrence of AT repeats within the genomic DNA of *Hevea*.

Sequencing of the positive clones revealed 31 simple repeats harbouring dinucleotide repeats like CT/GA, TG/AC, TA/AT; trinucleotide repeats such as AAG, AGG, AAT; tetranucleotide repeats like AAAT, GAAA, AAGG, ATCC, TAAA and also a pentanucleotide repeat GAAAT motif. The microsatellites frequently contained more than one SSR motif (compound microsatellites), whereas some were simple, perfect and long (Table 1). Majority of the dinucleotide

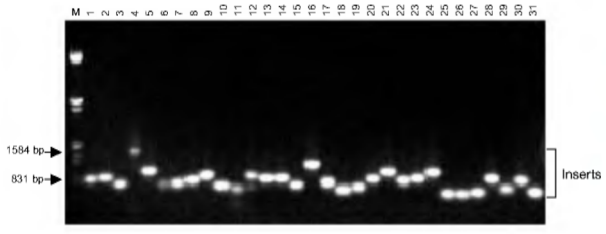

Figure 1. Representative photograph of a gel showing genomic inserts of various sizes from SSR-bearing *Hevea* clones, amplified using vector-directed T3 and T7 promoter primers. Lanes 1–31, Positive genomic clones for AC and CT repeats; lane M, molecular weight marker (λ-DNA marker/*Eco*RI + *Hin*dIII).

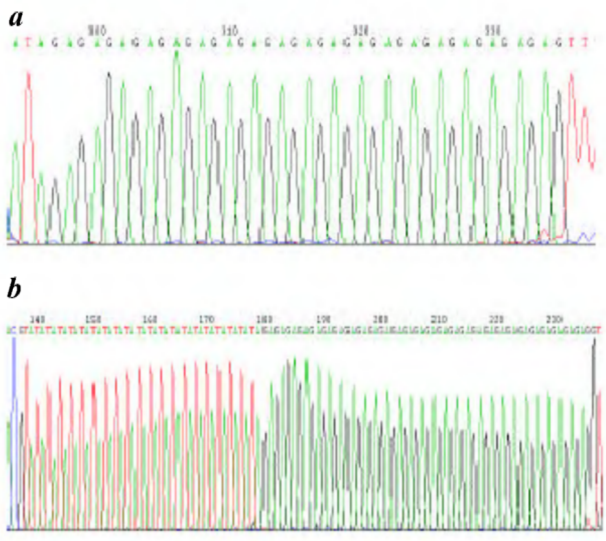

Figure 2. Chromatograms of repeat regions in *Hevea* genomic clones. ***a***, Simple dinucleotide repeat comprising $(AG)_{20}$; ***b***, Long stretches of compound perfect repeat characterized by $(AT)_{20}(AG)_{29}$.

Table 1. Characteristics and frequency of occurrence of SSRs isolated from *Hevea* clones

Category of repeat	Repeat motif	Number of SSRs obtained	Repeat length (n)	Average repeat length ± SD
Simple repeat				
	$(CT)_n/(GA)_n$	13	4 to 20	11.78 ± 6.47
	$(TG)_n/(AC)_n$	4	4 to 18	11.50 ± 5.97
	$(TA)_n/(AT)_n$	2	4 to 5	4.50 ± 0.71
	$(AAG)_n$	3	3 to 5	4.00 ± 1.00
	$(AAT)_n$	1	4	–
	$(AGG)_n$	1	3	–
	$(AAAT)_n$[1]	1	5	–
	$(TAAA)_n$	2	3 to 5	4.00 ± 1.41
	$(GAAA)_n$	1	3	–
	$(AAGG)_n$	1	3	–
	$(ATCC)_n$	1	4	–
	$(GAAAT)_n$	1	3	–
Compound perfect repeat				
	$(AT)_{19}(AG)_{23}$	1	–	–
	$(AT)_{20}(AG)_{29}$	1	–	–
	$(CT)_{27}(GT)_2$	1	–	–
	$(CT)_9(CA)_3$[1]	1	–	–
	$(CT)_2(GT)_{12}(AT)_5$	1	–	–
	$(CT)_{19}(AT)_{14}(CT)_1$	1	–	–
	$(CT)_{14}(AT)_7(CT)_2$	1	–	–
	$(CG)_3(TG)_5(AG)_{11}$[1]	1	–	–
	$(TA)_4(TG)_{13}(AG)_7$	1	–	–
	$(CT)_{17}(CA)_3(CT)_2(GT)_{16}$[1]	1	–	–
Compound imperfect repeat				
	$(AC)_2AA(AC)_8$	1	–	–
	$(AG)_{14}A(AG)_2$	1	–	–
	$(AG)_3AC(AG)_8$	1	–	–
	$(AG)_1GA(AG)_{10}$	1	–	–
	$(AG)_{11}TG(AG)_1$	1	–	–
	$(AG)_{13}ATGA(AG)_2$	1	–	–
	$(AT)_2GT(AT)_4$	1	–	–
	$(CT)_{10}GT(CT)_{11}$	1	–	–
	$(CT)_{12}AG(GT)_8$[1]	1	–	–
	$(CT)_2CATT(CT)_{10}$	1	–	–
	$(TG)_5GG(TG)_5A(TG)_2$	1	–	–
	$(TG)_{11}TT(TG)_2$	1	–	–
	$(AG)_3AA(AG)_5G(AG)_3$	1	–	–
	$(AG)_9TG(AG)_2TG(AG)_1$	1	–	–
	$(AT)_{12}(AC)_1A(AC)_{22}$	1	–	–
	$(AG)_2C(TG)_4AG(TG)_6TA(TG)_2$	1	–	–
	$(CT)_3(T)_3(CT)_{12}(T)_{15}$	1	–	–
	$(CT)_2CC(CT)_2C(CT)_7C(CT)_1$	1	–	–
	$(CT)_6N_8(CT)_7C(CT)_1CC(CT)_2TT(CT)_3$	1	–	–
	$(TG)_{15}AG(TG)_1AG(TG)_3G(TG)_2$	1	–	–
	$(TG)_3(CT)_2(CA)_5(TG)_2AAA(AG)_{13}$	1	–	–
	$(TCA)_6TCT(TCA)_4$	1	–	–
	$(AAG)_1AGGAG(AAG)_3$	1	–	–
	$(AAG)_1A(AAG)_3AATGA(AAG)_2$	1	–	–
	$(GAT)_4(GTT)_3(GA)_5AA(GA)_9$[1]	1	–	–
	$(TTTCC)_1TCCG(TTTCC)_1$-TTTCG$(TTTCC)_2$TTTCG$(TTTCC)_1$	1	–	–
Total number of different types of repeats identified		67		

[1]Compound microsatellites identified from a large-insert genomic library derived from the clone RRII105 of *H. brasiliensis*. Besides, two more simple repeats $(TG)_{14}$ and $(CA)_{18}$ were also identified and included in calculating the total number of dinucleotide repeats.

microsatellites isolated in the *Hevea* genome were CT/GA repeats followed by TG/CA repeats. GA repeat was found to be the longest simple repeat having a length of 20 repeat units (Figure 2 *a*) and the average repeat length was found to be 11.78. Our observation on the frequency of GA repeats in the *Hevea* genome appeared to be similar to most of the published reports on SSR isolation from many plant species, where the GA repeats were consistently more abundant than the CA repeats[1,27,28]. Two types of compound repeats, i.e. compound perfect repeats and compound imperfect repeats were detected. Out of 36 compound repeats identified, 10 were perfect repeats and 26 were imperfect repeats. Most of the compound perfect repeats were characterized by the presence of 2 to 3 dinucleotide repeat motifs with varying repeat length. A relatively long stretch of compound perfect repeat containing AT/TA and GA/CT motifs with a repeat length of 20 and 29 respectively, was identified (Figure 2 *b*). Among the compound imperfect repeats, AG motifs seemed to occur at a higher frequency, which were interrupted by 1 to 4 non-repeat nucleotides. Besides the AG motifs, different motifs comprising di-, tri- and pentanucleotide also existed as part of compound imperfect repeats. High percentage of compound microsatellites detected in the *Hevea* genomic clones is unusual compared to other plant species, where majority of the microsatellites harbour a single repeat type and a lower number of repeating units. Long stretches of poly(T) or poly(A) were also noticed along with the other repeats (data not included in Table 1). Generally, such mononucleotide stretches are found in the chloroplast genome of higher plants, which may be polymorphic among different species and accessions[29–33]. As the total genomic DNA was used as the source for the *Hevea* genomic library construction, these long poly(T) or poly(A) tracts possibly could have originated from the chloroplast genome.

We have isolated a representative number of *Hevea* microsatellites from a genomic library for their ultimate use in the development of microsatellite markers. Out of 4×10^9 bp, estimated to be the haploid genome size of *Hevea* (an allotetraploid)[34,35], we sequenced approximately 48×10^3 bp (32×10^3 bp in GT1 and 16×10^3 bp in RRII105) based on an average insert size of 650 bp per clone and identified various repeat motifs. Until now, there are only two reports[19,20] available on the isolation and characterization of *Hevea* microsatellites. In many crop species, microsatellites were predominantly isolated by screening the sequence database in addition to their development through conventional techniques involving construction of a genomic library, screening with repeat oligonucleotide probes for the identification of positive clones etc., which is more laborious and time-consuming. In *Hevea,* there is a limitation in finding microsatellites from the database, as only a few full-length gene sequences are reported in the database. There are three *Hevea* gene sequences in the database, i.e. HMG-CoA reductase, Mn-SOD and thioredoxin h, detected with repeat sequences at their untranslated regions (UTRs) of mRNA or in introns in genomic sequences. Dinucleotide repeats AG and CT, present at the 3′ UTR of HMG-CoA reductase and the intron of Mn-SOD respectively, have already been used as markers for genetic relationship studies[19,20]. However, markers could not be developed from the thioredoxin h sequence having trinucleotide repeats AAG at its 5′ UTR, due to insufficient flanking regions for primer designing.

The present work would be useful towards the development of a reliable marker system for evaluation of genetic diversity among *Hevea* clones and their wild relatives. Further work is in progress on designing oligonucleotide primers based on the flanking sequences both upstream and downstream of the microsatellite sequences for their evaluation as DNA markers in *Hevea*, which will ultimately be used to develop a linkage map.

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On the relation between magnitude and liquefaction dimension at the epicentral zone of 2001 Bhuj earthquake

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The 26 January 2001 Bhuj earthquake (*Mw* 8.7) is one of the largest seismic events of its kind in India and also in the intra-plate zones in the world. Complete documentation of the liquefaction-related deformation features are difficult in the hostile salt-playa areas of Kachchh, however we attempted shallow trenching study at previously identified liquefaction sites that partially fills the gap in the liquefaction database of 2001 Bhuj earthquake. Liquefaction dimension was found to be a function of epicentral distance, magnitude of the earthquake, depth of the hypocenter, availability of the source material and also proximity to the major lineaments. We relate the above factors for 2001 Bhuj earthquake using shallow trenches at Umedpur, Chobari, Baniyari and Amarsar villages and reveal comparative picture of the dimension of liquefaction at the epicentral zone. Proximity to the active fault system, availability of shallow groundwater and liquefiable sand source has played major role in the size and dimension of the liquefaction in 2001 Bhuj earthquake. This study discusses the scope and limitations relating the sizes of sand blows and their relation to epicentral distance and the magnitude of the causative earthquake.

THE 2001 Bhuj earthquake (*Mw* 8.7) is one of the largest seismic events amongst the intra-plate earthquakes in the post-instrumental era. The energy was released on a 90 km long E–W trending and 55° dipping fault plane.

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