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One hundred microsatellite markers from *Hevea* genome for characterization of cultivated clones of rubber (*Hevea brasiliensis*)

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INTRODUCTION

Detection and quantification of genetic variation is central to our understanding of many biological phenomena. Such studies require polymorphic assay procedures that are independent of environmental influence. Polymorphism in DNA sequences provides the basis for genetic analysis. Microsatellites, also known as simple sequence repeats (SSR), are arrays of short tandem repeat motifs of 1 to 6 base pairs in length and occur frequently and randomly in eukaryotic genomes. Because of their hypervariability, co-dominance and high reproducibility, microsatellites became the ideal markers for constructing high-resolution genetic maps and identifying loci controlling traits of interest. Direct isolation of clones containing SSR from genomic library of *Hevea brasiliensis*, an important tree crop producing latex of commercial utility, was the method of choice for developing microsatellite markers in the present study. The microsatellite markers developed were used for genomic characterization and phylogenetic analysis of cultivated rubber clones.

MATERIALS AND METHODS

A small insert *Hevea* genomic library of the clone GT1 was constructed in lambda vector (ZAP Express; Stratagene, USA). Recombinant phage was plated after incubation with *E. coli* XL-1 Blue cells. Plaques were transferred onto positively charged Nylon membranes and screened through plaque hybridization with radiolabelled (γ^{32}P-dATP) synthetic dinucleotide repeat probes $(AC)_{20}$, $(CT)_{20}$ and $(AT)_{20}$. Positive plaques were recovered individually and were subjected to a second round of screening following the same procedure. Recombinant lambda vector from each of the positive plaque was converted into pBK-CMV phagemids by *in vivo* excision. All the positive clones were subjected to PCR amplification using vector directed T3 and T7 promoter primer-pair to determine the size of the insert. The excised phagemids were double digested with *Xba* I and *Bam*H I to identify the duplicates for their elimination prior to sequencing. Positive clones were sequenced from both ends of the inserts using a BigDye Terminator Cycle Sequencing kit and the product was run with an Applied Biosystems ABI 3700 Sequencer at Macrogen Inc., South Korea to identify presence of microsatellites/SSRs in the genomic clones of *Hevea*.

For developing microsatellite markers, oligonucleotide primer pairs flanking the microsatellite motifs were designed that had an approximately 60% GC content to yield an expected amplicon size of around 200 bp using the PRIMER3 software package. Forty-five random microsatellites were amplified for their evaluation as markers in five RRII 400 series hybrid clones along with their parents (RRIC 100 and RRII

105). The forward primers were end labelled with ^{33}P γATP. PCR reaction was carried out in a 10 µl final volume containing 20 ng of genomic DNA, 0.2 µM each of the forward and reverse primers, 200 µM dNTPs and 0.7 units of AmpliTaq Gold polymerase along with the buffer supplied by Applied Biosystems. The temperature cycle profile involved an initial denaturation step of 5 minutes at 95°C followed by a touch down PCR program. Temperature profiles of the touch down PCR for 7 cycles were as follows: 94°C for 30 seconds, 63°C for 1 minute, Δ↓ 1°C for 7 cycles, 72°C for 1 minute. This was followed by a normal cycling of 94°C for 30 seconds, 56°C for 1 minute, 72°C for 1 minute for 23 cycles and a final extension at 72°C for 10 minutes. After completion of PCR, the reactions were stopped by the addition of 10 µl formamide loading buffer and stored at -20°C. 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 then dried and autoradiographed on X-ray film using standard procedures. Out of forty-five microsatellite markers, sixteen were selected based on their informativeness and evaluated against 35 popular *H. brasiliensis* clones to determine the level of polymorphism for genetic relationship studies. Phylogenetic relationship was established by constructing a dendrogram based on distance matrix data through cluster analysis using Treecon software.

RESULTS AND CONCLUSION

Synthetic oligonucleotides comprising of $(AC)_{20}$, $(CT)_{20}$ and $(AT)_{20}$ were used as probes in screening the *Hevea* genomic library as these repeats are reported to be prevalent in many plant species. Among the 15,000 clones/plaques (approx.) screened, 204 were found to be positive for CT/GA and AC/TG repeats, which were recovered individually for second round of screening confirming 154 positive clones for the repeats. Finally, 114 clones were selected through PCR amplification. Six clones (5.3%) appeared to be duplicates and were thus eliminated. Sequencing of 108 clones revealed various kinds of repeat motifs and existence of more than one repeat motifs within a single clone were noticed with nine clones. Primers could be designed for hundred repeat motifs including simple, compound perfect and compound imperfect repeats. Out of 100 repeats, 77 were CT repeats and 23 were AC repeats and the loci were designated as *'Hmct'* and *'Hmac'* respectively suffixing the number. This revealed prevalence of CT repeats followed by AC repeats in *Hevea* genome. Minimum occurrence of AT repeats in association with other repeats was observed within the genomic DNA of *Hevea*. Primer-pairs for 45 randomly selected loci were tested using touch down PCR as touch down protocol eliminated stuttering and artifact bands. Sixteen primer-pairs were selected based on their simple allelic profile and informativeness for phylogenetic analysis of popular *H. brasiliensis* clones. Allele designation of the loci was comparatively easy because of their screening in RRII 400 series hybrid clones along with their parents, as it facilitated studying inheritance pattern of the alleles. The number of alleles ranged from 1 to 6.

Genetic distance was calculated among the clones using the genotyping/allelic data of the microsatellite loci. Phylogenetic relationship revealed narrow genetic diversity existing among the rubber clones, which may be due to loss of genetic variation resulting from a directional selection for latex yield for several years. Geographical origin of the clones did not reveal any correlation with their clustering pattern except for the Sri Lankan clones and some of the 'PB' clones of *H. brasiliensis* from Malaysia. 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. An assessment of the extent of genetic variability among the cultivated rubber clones is fundamental for *Hevea* breeding and is particularly useful as a general guide in the choice of parents for breeding hybrids.

The microsatellite markers generated here provides a reliable marker system for evaluating genetic diversity among *Hevea* clones and their wild relatives. These co-dominant markers are highly essential especially for perennial tree crops like rubber, in generating a genetic linkage map for identification of QTLs and marker assisted selections for desirable traits.