Integration of microsatellite (SSR) markers in Hevea *spp. reference genetic map: a powerful tool for genetic analysis of qualitative and quantitative agronomic traits.*

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In 2000, was published (Lespinasse et al. 2000) the 1st genetic map of rubber tree (Hevea spp.). This saturated map encompassed 717 loci, allowing an efficient coverage of the 18 chromosomes of the Hevea genome, and it can be considered as the reference map for the H. brasiliensis species. Nevertheless, the molecular markers used were manly RFLPs and AFLPs. RFLP markers proved to be very efficient in terms of genetic polymorphism and are sparsely distributed on rubber tree genome, but their applications to genotyping of large progenies are time and cost consuming. AFLPs were very useful PCR based markers, allowing rapid identification and mapping of hundreds of markers. But they are less informative than RFLPs as segregating AFLP bands are usually not locus specific and, in consequence, do not allow comparative alignment between independent maps. We thus developed Hevea specific microsatellite (alias SSRs for Simple Sequence Repeat) markers which potentially combine the advantages of RFLPs (polymorphism, abundance in plant genomes and locus specificity) and of AFLPs (high output genotyping thanks to PCR technique). Integration of SSRs in the Hevea reference map confirmed the usefulness of such molecular markers in terms of cost, rapidity, genome coverage and locus specificity. This last property means that selecting a set of SSR markers from the reference map allows obtaining a colinear map, whatever the Hevea clones used as progenitors of the mapping populations. Thus, the creation of a dense SSR map of rubber tree is very useful for genetic mapping and comparison of chromosome locations of Quantitative Traits Loci (QTLs) obtained from different mapping projects.

Keywords: genome mapping; *Microcyclus ulei*; SSR; AFLP; RFLP; QTL

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Genome mapping using molecular markers proved to be very useful for rubber tree genetic and breeding, providing both knowledge on genome organization, and efficient tools for genetic improvement of traits of interest. It allowed for the first time establishing genetic determinism of agronomic traits such as SALB resistance (Seguin et al., 1996b; Lespinasse et al., 2000a; Le Guen et al., 2007) or latex yield and growth (Clément-Demange et al., 2006; Clément-Demange et al., 2008) providing in the same time, linked genetic markers for future marker aided selection (MAS).

But it also provided precise information on the genome organization of *Hevea* spp. Indeed, the first *Hevea* genetic map published (Seguin et al., 1996a; Lespinasse et al., 2000b) demonstrated that the genome of the two studied species - *Hevea brasiliensis* and *H. benthamiana* – are diploid with a limited number of duplicated chromosome segments. This work and further analyses of other mapping populations (Prapan et al., 2006) revealed that, for all the *Hevea* accessions studied, loci / markers order was conserved (complete colinearity of the genomes) even between clones from different *Hevea* species. At the time, no

chromosome rearrangements such as translocations or inversions have been observed in at least the 6 clones used as progenitors in mapping experiments. These results mean that rubber tree genetic and breeding are relatively easier than for species with polyploidy genome and/or with chromosome structure variability, as it is the case for many cultivated crops (banana, sugarcane, yam…).
In addition, the complete colinearity of the chromosomes implies that the saturated genetic map published by Lespinasse et al (2000a and b), based on the segregation analysis of the PB260 x RO38 progeny, can be considered as a reference genetic map for the cultivated *H. brasiliensis* species and, may be for the whole *Hevea* genus. Selection of markers from this map allows speeding up new mapping projects, facilitating rationale of marker selection for genotyping. Nevertheless, the transferability of markers from one clone to another is possible only in the case of locus specific markers. Today, different types of genotyping techniques of molecular genetic markers are available for genome mapping. The *Hevea* reference published map encompassed mainly two kinds of markers called RFLPs (Restriction Fragment Length Polymorphism) and AFLPs (Amplified Fragment Length Polymorphism). AFLP are useful PCR based, allowing high output genotyping, and rapid identification and mapping of markers but they are not locus specific: following exactly the same protocol (same PCR primers) do not allow identifying the same locus in different accessions (clones). RFLP markers are locus specific and, in addition, appeared more sparsely distributed on rubber tree genome, leading to a more efficient coverage of the genome. But RFLP being not a PCR based technique is more cost and time consuming.
For those reasons, we developed microsatellite (or SSR) markers for *Hevea* genome mapping (Seguin et al., 2001): this kind of molecular genetic markers combines technical and genomic properties of AFLPs (high output PCR markers) and of RFLPs (abundance and distribution in plant genomes, level of genetic polymorphism and heterozygosity). The published map encompassed only 18 SSR loci (Lespinasse et al., 2000a), and we report here the current state of SSR markers integration in the *Hevea* reference map.

EXPERIMENTAL

Development of SSR markers.

We developed *Hevea* SSR markers from 4 different sources: 1- genic SSRs from published gDNA or cDNA sequences of *Hevea* expressed genes available in public databases; 2- a *H. brasiliensis* PstI genomic library used for RFLP marker development in our laboratory (Besse et al., 1994); 3- specific *H. brasiliensis* genomic libraries, enriched for di-nucleotide (GA)n and (CA)n repeats (Gay et al., 1999; Seguin et al., 2001); and 4- BAC ends sequences (BES) from the *Hevea* spp. BAC-library built bye our team (Piffanelli et al., 2003; Seguin et al., 2006). All the corresponding DNA sequences are registered in international public databases (EMBL/Genebank/NCBS, accession numbers: G73376 & G73377, AF221696 to AF221711, AF383928 to AF383944, AY486558 to AY486910, Q115593 to DQ115640).
PCR amplification and genetic polymorphism of the SSR markers were tested on a reduced set of 6 rubber tree clones and on additional clones from non brasiliensis species (*H. benthamiana, spruceana, pauciflora, camargoana* and *guianensis*; 1 clone per species). All SSR markers can be efficiently amplified using standard PCR conditions on all the *Hevea* species tested and can thus also be used for mapping projects implying interspecific crosses. Among the 660 SSR sequences identified and tested for PCR amplification, 420 provided polymorphic SSR markers. Eighty five percent of the markers come from the SSR enriched

libraries. Complementary information on laboratory protocols and map locations of the SSR markers can be requested by e-mail to the authors (marc.seguin@cirad.fr).

RESULTS

Integration of SSR markers in *Hevea* spp. reference map

Among the 420 SSR markers, 391 appeared polymorphic in the PB260xRO38 mapping population, *i.e.* are heterozygous in at least one of the PB260 or RO38 parents, and could consequently be integrated in the reference map. At the time, 234 of them have been effectively integrated by genotyping the 197 progenies of the PB260xRO38 cross (figure 1). The progenies are the same than the ones used by Lespinasse *et al*. (2000a and b) for the first published version of the map. Genotyping were performed using either ^{33}P radio-labelling or fluorescent labelling (for LiCOR sequencer) of PCR products. The map was built using JoinMap® 3.0 software (Van Ooijen and Voorrips, 2001) at a grouping LOD of 5.

SSR loci appear well distributed on the *Hevea* genome and form less clusters of tightly linked markers than AFLPs. Clusters of AFLP markers are frequently observed in many plant species, and the main clusters in each linkage group are usually supposed to correspond to the centromeric regions of the chromosomes.

For rubber tree, SSR markers appeared highly locus specific as, in the present mapping work, none of them revealed duplicated loci or mapped on different locations between the parental maps.

The selection of sparsely distributed SSR markers on the reference map helped to rapidly identify and mapped markers for new mapping projects as it is illustrated on the figure 2, in the case of a PB260xFX2784 mapping population (unpublished data). This strategy was also efficiently applied for SSR mapping of the RRIM600xPB217 population (Prapan et al., 2006) Thanks to their locus /chromosome specificity, all SSRs mapped in the vicinity of their expected map locations. In the few cases of chromosome location discrepancy, it was always due to experimental errors. The use of makers in common with the reference map, on each of the linkage group, allowed unambiguous assigning of the linkage groups of the new maps to their homologous referenced one and to the corresponding linkage group number (from g1 to g18).

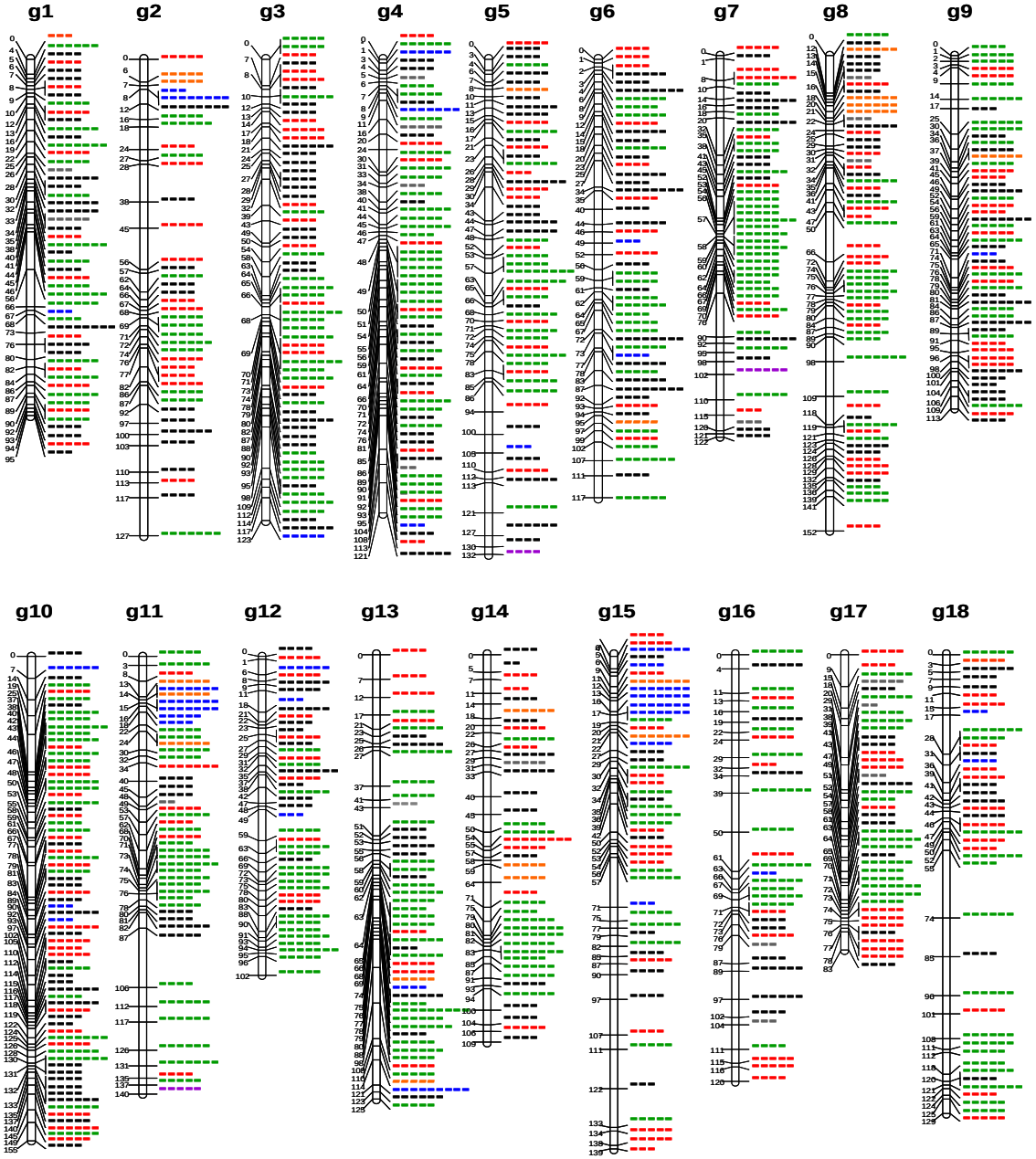

*Figure 1.*Hevea *spp. reference genetic map. The map was built by genotyping the same 197 F1 individuals, from the PB260 x RO38 cross, used for the first published map (Lespinasse et al. 2000b). Expressed genes including isozymes are in blue; RFLPs in black; AFLPs in green and genic SSRs in purple and other (genomic) SSRs in red.*

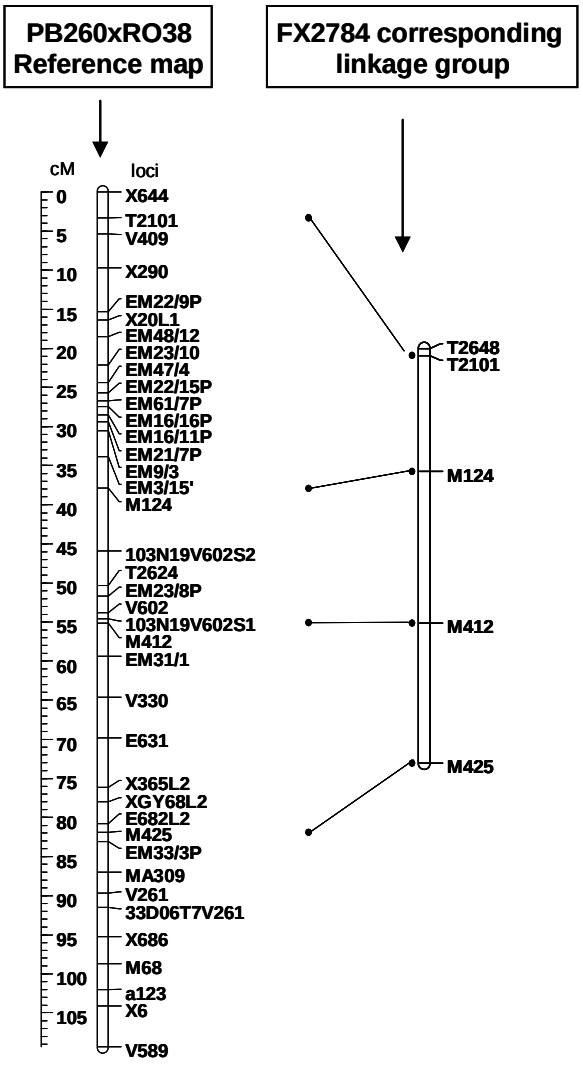

Figure 2.Transferability of SSR markers. Four SSR markers were selected from one linkage group (g14) in the reference map and used for genotyping a progeny of 115 F1 from the PB260 x FX2784 cross. Only the FX2784 parental map of g14 is shown here. A sixth SSR marker (T2648), heterozygous in FX2784 but of previously unknown location, was later added without a priori knowledge of its linkage group assignment.

CONCLUSION

Microsatellite / SSR markers represent at the time the best ones for accurate genome mapping in rubber tree for several reasons: high level of polymorphism, abundance and regular distribution in the nuclear genome, reduced laboratory costs and time and, last but not least, high locus specificity allowing map integration and linkage group alignments. This last property is particularly important for the comparison of chromosome location of QTL and genes identified in independent mapping projects. SSR mapping can be efficiently completed using AFLP technique, for instance, in order to increase the markers density. Using a frame of SSR loci anchored on the reference map, it is then possible to assign any additional linked marker to the homologous region (figure 2).

Consequently, for any new genome mapping project in rubber tree, it would be recommended to include a framework of SSR markers from the reference map, the choice of the SSRs to be mapped being based firstly on their map position (exact information can be obtained from the authors, pending final publication in international revues), and secondly on their heterozygosity in the parents of the mapping population used which as to be assessed for each new mapping population. We will continue to enrich the PB260xRO38 reference map with additional SSR markers. When the density of SSR markers will be sufficient in this map, it will be possible to directly integrate mapping results from other progenies and to constitute a SSR synthetic map of reference for rubber tree genome mapping.

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