APPLICATION OF QTL MAPPING FOR EARLY SELECTION ON GROWTH AND LATEX YIELD TRAITS IN RUBBER BREEDING

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ABSTRACT

The objective of this study was to apply the QTL mapping by Markers-Assisted Selection approach for early selection on growth and latex yield in rubber breeding. The plant material consisted of 196 progenies derived from the F_1 family RRIM 600 x PB 217. A genetic linkage map was built for this family with 229 SSR markers (microsatellites) and 198 AFLP markers. Phenotyping was carried out over a 6 years period on a field trial of 5 hectares, with around 2,400 trees measured individual. The two major QTLs were detected repeatedly. The QTL g16-6 was associated with latex yield near the position of marker a131. This QTL explained up to 66% of the phenotypic variance. It was also associated to other traits that were strongly correlated to production such as inorganic phosphorus and dry rubber content (latex diagnostic), as well as plugging index. This finding indicates the existence of one major gene (or a cluster of genes) located on linkage group g16 and involved in the intensity of metabolic activity of latex cell. A second important QTL associated with girth of the trunk (growth) was detected (QTL g3-60) at the position of marker a312. It explained up to 31% of the phenotypic variance. The discovery of the two major QTLs g3-60 and g16-6 suggest that two major genes act as limiting factors in the genetic determinism of the growth in girth during favorable condition and of rubber production in low-intensive tapping systems. These loci should become privileged targets for the identification of key-genes in rubber. The possible use of their neighboring markers for developing Markers-Assisted Selection (MAS) in the short run appears very reasonable. It should contribute to improve the accuracy of the first selection stage which is the weakest point of the rubber clonal selection scheme.

Keywords: QTL-mapping, Markers-assisted Selection (MAS), growth, latex yield, early selection, rubber breeding.

INTRODUCTION

This genetics research developed the first QTL-mapping approach on the domesticated Wickham population of the rubber tree (*Hevea brasiliensis*) for application to rubber breeding by Marker-Assisted Selection (MAS). It was based on a F_1 family issued from two widely cultivated rubber clones (RRIM 600 x PB 217). Genetic mapping (Lekawipat, 2005; Prapan *et al.*, 2006) was carried out by use of 427 PCR-based molecular genetic markers (229 SSR and 198 AFLP). Phenotyping was carried out on 196 progenies in East-Thailand over period from 2002 to 2009. The target traits were related with ecophysiology (growth), latex cell physiology (rubber production) and rubber quality (monomodal or bimodal distributions of the molar masses of the rubber chains). The choice of a F_1 family was adapted to the rubber tree, a tree crop and an outbred species, with vegetative propagated varieties in the form of heterozygous clones budded onto seedling rootstocks. This

project was one of the French-Thai HRPP projects (*Hevea* Research Programme in Partnership), associating RRIT-DOA, Kasetsart University, and CIRAD. Thailand is the largest rubber producing country, with a cultivated area of more than 2.7 million hectares, and a production of 3.16 million tons of rubber in 2009, as compared to a total world production of 6.88 MT. Therefore, improving the efficiency of rubber breeding and delivering performance and adapted clones to the planters is an important economical objective for Thailand.

Currently, rubber varieties are highly heterozygous clones multiplied by budding onto seedling rootstocks. Recombination between parental clones by hand pollination generates full-sib families of seedlings that are submitted to initial screening in a Seedling Evaluation Trial (SET). The selected genotypes are then budded and passed to two successive selection trials: the Small Scale Clonal Trial (SSCT) and the Large Scale Clonal Trial (LSCT). The first selection stage (SET) is considered as the weak point of the process, as far as it is applied to a very large population of genotypes with poor information available. New tools able to provide more accurate genetic information would improve the efficiency of that initial selection stage (Clément-Demange *et al*., 2007).

Following the traditional agromorphological markers, proteic and DNA genetic markers have known fast development since 30 years, with isozymes, RFLP (Restriction Fragment Length Polymorphism), and then with targeted PCR techniques (Polymerase Chain Reaction), especially SSR markers (Single Sequence Repeats also called microsatellites) that have a high level of polymorphism. Those genetic markers have been used, in rubber as in many other plants, for the description of the neutral genetic diversity of genetic resources, and the identification of cultivars and of their parentages (Seguin *et al*., 2003). With the identification of an increasing number of molecular genetic markers, mapping of loci involved in quantitative variation, i.e., quantitative trait loci (QTL; Geldermann, 1975) has progressively become a central method in quantitative genetics.

QTL detection is typically based on joint genotyping and phenotyping of a segregating population. Due to the diversity of its alleles (polymorphism), each marker makes possible the partition of a population of progenies in genotypic classes. For any measured quantitative trait, it is possible to assess the significance of the differences between those classes and to identify genetic associations due to linkage disequilibrium between the marker and the genetic factor (genes or clusters of genes) underlying the expression of the trait. Moreover mapping of the markers makes possible the approximate localization of the involved genetic factors on the genetic map. Although molecular genetic markers are non-expressed DNA fragments (neutral or anonymous markers), as opposed to ESTs (Expressed Sequence Tags) or full-length gene sequences, QTL detection is seen as a way to investigate the number of genes that control quantitative traits, the magnitude and the distribution of their effects. As a result, the markers associated to the QTLs can become new sources of genetic information in the framework of Markers-Assisted Selection (MAS).

The opportunity to select varieties based on the complementary genotypic information brought by molecular markers at very early stage, added to phenotypic measurements, is very attractive to plant breeders. For traits where a low heritability makes phenotypic evaluation costly, molecular genetic information, independent from the environment, would be very useful.

In autogamous plant, MAS can be used to facilitate selection when developing inbred lines by the pedigree method. After QTL identification, the favorable alleles can be selected in an enlarged F_2 populations before developing the next generations by selfing. MAS can be used also to introgress favorable alleles by recurrent backcrossing, which is probably the most important application of MAS today. This method can be enlarged to the pyramiding of different genes in one same genotype used as the back-crossed parent. For

outbred species with vegetative propagation, selection can be applied to immature individuals, even before they develop the character on which the adults are selected.

Lande and Thompson (1990) presented the general methodology for integrating molecular genetics and conventional selection on phenotypes (MAS), based on selection indices. MAS was considered for the improvement of a single character by individual selection, and was restricted to only the additive genetic effects of the QTLs. The efficiency of selection could be increased substantially by using MAS after the hybridization of selected cultivars and initial QTL detection. The additive effects can be estimated by multiple regression of individual phenotypic values on marker genotypes. The proportion of the additive genetic variance explained by the QTLs is related with the heritability of the trait (h^2) and the number of individual genotypes included in the detection study. Consequently, for traits with moderate or low h^2, the chances of QTL detection with small sample sizes are low, unless the QTL explains a substantial proportion of the genetic variance. The authors also underline that marker-QTL associations are continually eroded by recombination.

Strauss *et al*. (1992) expected that the potential of MAS in forest tree breeding would be limited in non-hybrid populations (populations not issued from controlled crossing). But « Marker-aided selection within individually mapped full-sib families can substantially aid phenotypic selection, but only where large restrictions of genetic base are tolerated, trait heritabilities are low, markers are able to explain much of the additive variance, selection intensities within families are high compared with that among families, and very large numbers of progeny are examined. Actually this scenario is very close to that which one can consider for rubber. The authors continue: Consideration of trait characteristics suggests that marker-aided selection will be most efficient in direct selection with high-value, low-heritability traits such as height and diameter growth. These traits, however, often show genotype-by-environment interactions and unfavorable genetic correlations with other desirable traits, and are likely to be controlled by a large number of minor genes rather than relatively few major ones. Traits with the most potential for marker-aided selection in non-hybrid tree populations will therefore be strongly inherited ones for which phenotypic assay is difficult.

Stability of the QTLs over generations is a concern. Experimental populations, in which QTLs were detected, usually stay at linkage disequilibrium (LD) during the first mating generations. LD is necessary for searching favorable alleles among a wide population, and more precisely for MAS selection of genotypes in such a population. This is the case when parental genotypes (genitors) are to be selected from progeny testing (inter-family selection). The situation is different when the candidates to selection are the progenies themselves (intra-family selection). In this case, there is only one generation from the parents to the progenies, and QTL detection does not require LD in the population. From one family to the other, the known QTLs can be re-estimated without any new genetic mapping, by targeting the genome areas where the QTLs were first detected. This situation, much more favorable to MAS application, is met in the case of the rubber tree.

MATERIAL AND METHODS

Plant material

The two heterozygous parents RRIM 600 and PB 217 were chosen for their contrasted positions in the physiological classification of rubber clones. As most clone, RRIM 600 has a rather intensive metabolism whereas PB 217 latex cells have a low initial metabolism but a high sucrose content fitted to the intensification of the tapping and stimulation system. (Gohet *et* al., 2003)

The F_1 family RRIM 600 x PB 217 was created by hand pollination at Chachoengsao Rubber Research Center (CRRC) in 2000. The genetic linkage map (genotyping) was developed over 18 working months for one person. A field trial was planted in June 2002 with 196 progenies in the form of budded trees and measurements were carried out since then.

The Genetic linkage map

Hand pollination generated more than 600 progenies in the family RRIM600 x PB217. For a number of 427 molecular genetic markers (229 SSR and 198 AFLP markers), genotyping was achieved for the 196 progenies that were planted in the field trial. The genetic linkage map was built by the double pseudo-test cross strategy. The total length of the map, distributed over a number of 18 linkage groups corresponding to the 18 chromosomes of the haploid genome of rubber, was of 2075 cM. The average length between two successive markers varied from 3.14 to 7.12 cM depending on the linkage group, with a global average of 4.86. The largest length between two successive markers was of 38 cM (Lekawipat, 2005; Prapan, 2006). The genotypic data and the positions of the markers on the linkage groups make the basic genetic information to be used jointly with the field phenotypic data for QTL detection.

Experimental design of the field trial

A flat field of 6.32 ha was planted with a planting density of 625 trees per hectare (4 x 4 m), slightly higher than the usual density in rubber cropping (500 t/ha). For each progeny there were 16 budded trees distributed in 4 plots with 4 trees per plot. For controlling the variations of the environment between plots, an α (0, 1)-design (Patterson and Williams, 1976) was set with 4 full replications, and 25 incomplete blocks of 8 plots per replication (4 x 25 x 8 = 800 plots). Every full replication included all the 196 progenies in 196 plots (+ 2 plots for each parent), i.e. 200 levels of treatment. Every block included 8 genotypes (progenies or parents). Randomization of the design was carried out with a CIRAD software (J.P. Jacquemoud, unpublished.) so that every progeny (4 plots in 4 different blocks) might be compared with 4 x 7 = 28 other progenies or parents) in similar conditions. As a result, among the 19,900 possible pairs of varieties, 17,100 were in no block, and 2,800 were in one block. A total of 3200 trees contributed to the experiment.

Traits measurements

Growth

Growth was studied by measuring the girth of the trunk and the height of the trees twice a year, at the beginning of the rainy season and at the beginning of the dry season.

Girth was measured at 1 m high as well as at 1.7 m high on around 2,400 trees.

Latex yield

A first period of tapping was carried out from June to October 2007 on around 2,350 trees with girth at 1 m high higher than 25 cm. Tapping was in S/2 d3 with no ethyphon stimulation. A second period of tapping was carried out from June to September 2008 with the same tapping system, and with one stimulation applied in the middle of July (20 mg of ethyphon per tree). A third period of tapping was carried out from May to October 2009. Tapping was in S/2 d2 with three stimulations applied in June, July and September (25 mg per tree).

The cumulated latex yield of each month was weighed for each individual tree.

QTL detection

MapQTL5 software (Van Ooijen, 2004) was used for QTL detection. For traits with normal distributions, the analyses were carried out first by the “Interval Mapping” method, and then by the MQM method. The LOD score threshold was estimated for many different

traits by permutation test. In all cases, this threshold was found included in the interval [4.4, 4.6]. Therefore one same LOD significance threshold, « LOD threshold = 4.5 », was used for all the traits. For the die-back index (non-normal distribution), the method of Kruskal-Wallis was used.

RESULTS AND DISCUSSIONS

The two major QTLs were detected over both of the studied traits. The QTL g16-6 was detected for latex yield and g3-60 was detected for growth.

Latex yield

The discovery of the QTL Hbg16a131 was the most important result from this research (table 1). This major QTL was associated with latex production (Lp) and many other related traits (Inorganic phosphorus, Drc, Sucrose, W2, W1, Plugging index, PI, Growth during tapping). It was also detected for traits of the macromolecular structure of rubber (M_n, I_p, Gel), and for traits of bark thickness. This QTL showed its maximum effect for the girth-adjusted production trait Lp81a, with %exp = 66 % and LOD score = 49.2. The LOD support interval, estimated with the « Interval Mapping » method, was from 4.8 to 7.8 cM, and therefore the precision of the localization was of only 3 cM. The nearest marker a131 was at the position g16-5, and therefore at 1 cM from the peak position of the QTL.

Table 1 Characterization of the QTL g16-6 as detected for the production traits.

Trait	Group	Position	LOD	%exp	ac	ad	bc	bd
Lp71a	16	6	35.6	47	100	96	108	103
Lp72a	16	6	25.7	40	100	96	106	102
Lp73a	16	7	39.2	46	100	97	106	101
Lp74a	16	6	38.2	50	100	96	106	102
Lp75a	16	7	27.9	39	100	97	104	101
Lp81a	16	6	49.2	66	100	95	110	101
Lp82a	16	7	20.3	36	100	97	104	100
Lp83a	16	7	26.4	45	100	96	104	101
Lp91a	16	6	28.7	51	100	98	105	100
Lp92a	16	3	10.6	18	100	99	101	101
Lp93a	-	-	-	-	-	-	-	-
Lp94a	16	6	6.8	13	100	99	102	100
Lp95a	16	7	11.6	23	100	99	103	101
Lp96a	16	6	11.7	23	100	98	102	101

The effect of the QTL was important from production trait Lp71 to Lp91, and much lower thereafter. It was not detected for Lp93 and Lp94 (for traits non-adjusted to the girth). In the same time, the effect of the QTL g3-60 on the non-adjusted production traits became higher since Lp92, thus indicating a higher dependence of the production on the size of the trees (figure 1).

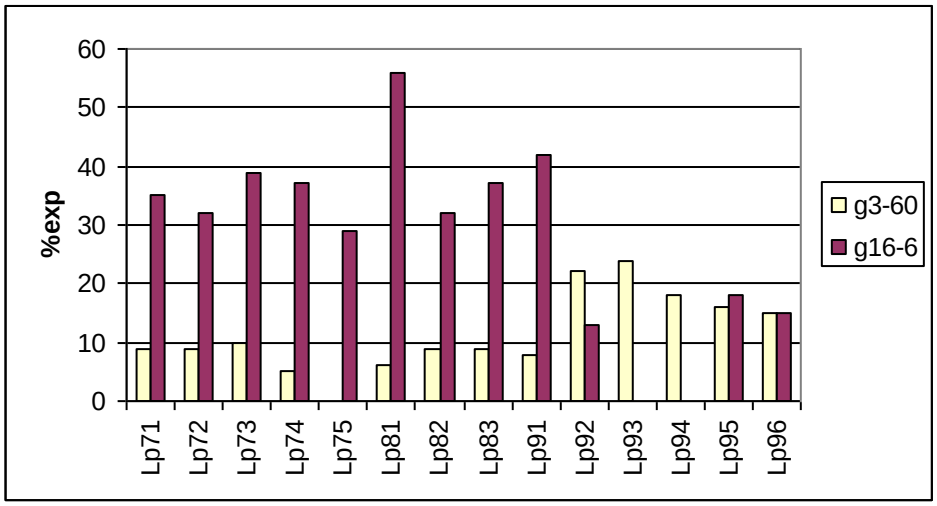

Figure 1 Percentages of explanation (%exp) of the 14 non-adjusted production traits by the two QTLs g3-60 and g16-6.

Table 2 shows the levels of cumulated girth-adjusted production (from P71 to P96) of the four genotypic classes of the QTL g16-6 (or of the four genotypic classes of the marker a131).

Table 2 Values of the cumulated rubber production (g) from P71a to P96a (girth-adjusted traits) for the genotypic classes of the QTL g16-6, for RRIM600 and the extreme genotypes n° 169 and n° 32, allelic classes of the nearest marker (a131).

QTL g16-6	Nb	Marker a131	Lp7196a	Index
ac	51	ad	24.87	84
ad	52	ac	21.09	72
bc	42	bd	33.04	112
bd	51	bc	26.48	90
RRIM600	1	ab	29.47	100
n° 169 (max)	1	bd	44.07	150
n° 32 (min)	1	ac	11.8	40

Growth

A second important QTL g3-60 was associated with the growth in girth, with maximum effect during the most favorable growth periods (table 3). Its maximum effect was observed for girth at 59 months (G59) with 31 percent of explanation and LOD score 15.8. For this trait, the LOD support interval of g3-60, estimated with the "Interval Mapping" method, was from 58.1 to 62.4 cM, thus indicating a precision of the localization of less than 5 cM. The nearest marker, a312 was located exactly at the peak position of the QTL (60 cM). The 3 alleles of the nearest marker a312 segregated according to the pattern "ef x eg" and generated the 4 classes "ee", "ef", "eg" and "fg". For all the girth measurements, the class "ee" was found smaller than the three other classes.

The main effect of the QTL was due to a lower level of girth for the QTL-genotype "bc" corresponding to the marker-genotype "ee" of the marker a312 (table 4). Although this effect is moderate, this QTL might be used in MAS too.

Table 3 Characteristics of the QTL g3-60 for growth traits before tapping, position varying from 59 to 68 cM, index 100 for the genotypic class "ac" taken as reference, LOD was not much below significance level for height increment during 17 to 23 months (Hi1723).

Trait	Group	Position	LOD	%exp	ac	ad	bc	bd
Hi1723	3	56	4.3	10	100	97	121	99
H53	3	59	6	14	100	100	97	98
Ga18	3	60	9.1	18	100	100	95	98
G18	3	60	6.9	15	100	100	95	99
Ga23	3	60	9.5	20	100	100	95	99
G23	3	60	7.3	16	100	100	96	99
G31	3	60	8.0	17	100	100	97	99
G36	3	60	6.5	14	100	100	98	99
G43	3	60	9.1	19	100	100	98	99
G47	3	60	12.4	26	100	100	97	99
G53	3	60	13.4	27	100	100	96	99
G59	3	60	15.8	31	100	101	96	99
Gai1218	3	60	9.4	18	100	102	92	98
Gi4347	3	60	5.3	12	100	97	51	85
Gi4753	3	60	8.4	18	100	100	93	98
Gi5359	3	70	9.7	18	100	104	88	97
Biom2331	3	50	7.2	16	100	99	93	96

Table 4 Value of G59 (cm) for the genotypic classes of the QTL g3-60, for RRIM 600, PB 217 and genotypes n° 83 and n° 1 (allelic classes of the nearest markers a312)

QTL g3-60	Nb	Marker a312	G59
ac	48	ef	31.77
ad	50	fg	31.99
bc	40	ee	30.40
bd	57	eg	31.38
RRIM 600	1	ef	30.84
n° 83 (max)	1	fg	34.35
n° 1 (min)	1	ee	29.28

Towards validation of the result

The present QTL results were obtained on the one set of 196 genotypes of family RRIM 600 x PB 217, in one ecological site, and with an accurate experimental design. It was shown that MAS would be more efficient for within-family selection by focusing on a limited number of QTLs with important effects for improving the accuracy of estimation of traits with low heritability or difficult to measure at very early stage. The two QTLs g3-60 g16-6 concern two important selection criteria (growth in girth, and latex yield) and they appear well-adapted for a first application of MAS to rubber selection.

As a matter of fact, it can be waited that the relationships between QTL-alleles and marker-alleles will change from one family to the other, and that the positions of the markers and of the QTLs will not be exactly the same. This makes necessary to re-estimate the QTLs in each family, but it can be done with simplified phenotyping and targeted genotyping based on a limited number of markers. We can also assume that the QTL positions are known with enough accuracy for efficient use in selection.

One first validation would be to assess the results with the same set of genotypes and a similar experimental design but in another ecological site. Another validation would be to use the same ecological site and the same family, but another set of genotypes and a less accurate experimental design adapted to the usual initial selection conditions.

CONCLUSIONS

Application of this research should be extended to other families and other ecological site for validating the results and detecting new QTLs. We think that those results make possible, as soon as now, first attempts of Markers-Assisted Selection for validating its potential efficiency. Moreover, the detection of 2 important QTLs now suggests that we should try to identify and clone the underlying genes. This could be greatly facilitated by genome sequencing in the genome areas of the 2 QTLs. But another way would be to search for polymorphic markers associated with candidate genes issued from functional genomics. We could make genetic mapping of the candidate genes and maybe identify co-localization of some candidate genes with already known QTLs. Moreover, the functional genomics and association genetics are two important branches of plant molecular biology. Functional genomics appears as an analytical method directly focused on some genes. By contrast, association genetics of the genome in some specific environment and detect the most important genetic effects involved in this expression and that can be detected. This is a synthetic way, more targeted towards breeding and selection application.

Towards Markers-Assisted Selection (MAS) for early selection in rubber

From these results, the most simple application of MAS would be to make selection on a large set of genotypes in the progeny of the family RRIM 600 x PB 217. DNA extraction can be carried out from the leaflets of the genotypes before planting them in field trials, and genotype the marker a131 and a312 for direct screening of high yielding and vigorous genotypes. Only those genotypes would be planted in field trials. But this approach might strongly reduce genetic diversity and select only one type of clones, those with very active metabolism.

Another strategy would be to genotype all the genotypes for markers a131 and a312, to plant all of them in field trials and to use molecular information as added information for a more diversified and more accurate selection.

Genmap research can also be developed on another family in the same way, with detection of QTL on 200 genotypes and then molecular breeding on 3,000 genotypes. With a new family, we could also genotype only the 2 markers a131 and a312 and set the whole

progeny to field trial in order to assess the effect of the alleles at those 2 QTLs for this family. After that, molecular breeding can be applied to the whole family.
It would be attractive to apply initial molecular screening among a large number of genotypes prior to the testing of the selected genotypes at field level. This would be possible in the family RRIM600 x PB217, in which the QTLs were detected and characterized. In other families, a phenotyping phase is necessary, even at early stage and with limited samples of the families, mainly for the identification of the favorable bi-allelic combinations of the markers linked to the QTLs. Whatsoever, combining molecular and phenotypic information, although more costly, may show many advantages such as a better balance between selection on the QTLs and on other loci, and a more accurate estimation of genetic values.

Normally, a rubber breeding program should include both the selection of progenies for the release of new varieties, and the selection of parents for recombination and the creation of new genetic variability. In rubber, selection of the parents was mainly based on their own value. Building mating designs for assessing the parents based on their progenies was limited by the low female fertility of most of the clones. However many families were created, but little efforts were made for comparing them with enough accuracy. In the Wickham population, due to its limited genetic variability, within-family variance was found to be larger than between-family variance, thus encouraging the breeders to focus on within-family selection. This was reinforced by the possibility of cloning the best trees at very early stage. Moreover, this simple approach was adapted to many rubber breeding programs which benefited from limited funding, and the importance of within-family clonal selection, if not fully determined by the biology of the species, can be considered as more or less intrinsic to rubber breeding. This aspect, and the necessity of early selection, can be seen as a favorable context for the success of MAS application at the initial stage of selection. The creation of full-sib families by hand pollination will continue to generate important and unpredictable variation within each family, due to the highly heterozygous nature of rubber tree. Therefore, a new approach focusing more on selection within large-sized families rather than on the comparison of many small-sized families might be efficient.

It was shown that the first selection stage, the Seedling Evaluation Trial (SET) was critical for rubber selection. A high heritability was found for rubber production in the specific trial used for QTL mapping. This explains why it was possible to select for production in SET. Therefore, MAS combined with SET evaluation could increase the accuracy of production estimations and selection efficiency for this trait, with a global benefit taking into account the genotyping costs.

As an alternative, it is suggested to substitute another design to the usual SET by budding, a small number of copies of each progeny (say, 3 copies) in a so-called Clonal Evaluation Trial (CET). Genotyping would be targeted to the two QTLs g3-60 and g16-6. Phenotyping of this trial would include the measurement of girth, production and sucrose content. Such a scheme would allow early selection on an index of the three traits, combining genotypic and phenotypic information.

If really efficient, this scheme, combined with a higher selection rate, might open new opportunities such as merging the two early selection stages (SET and SSCT). In such a case, the LSCT design (the third stage) would probably require some adaptation to the evaluation of a larger number of clones (20-40 clones).

ACKNOWLEDGEMENTS

This QTL mapping research is part of the project "Towards the improvement of rubber tree productivity" developed in Thailand by RRIT-DOA, Kasetsart University, Prince of Songkhla University, and CIRAD, under the cover of the *Hevea* Research Platform in Partnership (HRPP). We wish to thank the RRIT, French-Thai Committee for Research and Higher Education, as well as Agropolis for funding this research. We also wish to thank the technical staff of the breeding team of Chachoengsao Rubber Research Center (CRRC, RRIT-DOA) for the collection of the very large quantity of data that were necessary for achieving the results presented here.

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