

# Mapping QTLs for Resistance to Frosty Pod and Black Pod Diseases and Horticultural Traits in *Theobroma cacao* L.

J. Steven Brown,\* Wilbert Phillips-Mora, Emilio J. Power, Cheryl Krol, Cuauhtemoc Cervantes-Martinez, Juan Carlos Motamayor, and Raymond J. Schnell

## ABSTRACT

A heterozygous  $F_1$  mapping population of cacao (*Theobroma cacao* L.) was created and evaluated for resistance to frosty pod [caused by *Moniliophthora roreri* (Cif. and Par.) Evans et al.], and black pod [caused by *Phytophthora palmivora* (Butl.) Butl.] and for five horticultural traits at CATIE in Turrialba, Costa Rica. The population consisted of 256  $F_1$  progeny from the cross 'Pound 7' × 'UF 273'. Progeny were used to form a linkage map using 180 markers. The linkage map contained 10 linkage groups (LGs), numbered as the LG in the cacao reference map, and was used to locate putative quantitative trait loci (QTL) for resistance to the aforementioned diseases and five horticultural traits. Resistance to frosty pod was measured by internal and external pod resistance. Five QTLs for frosty pod resistance were found on three LGs, 2, 7, and 8, with UF 273 appearing to be the source of resistance. These alleles are being used for scoring progeny in ongoing cooperative marker-assisted selection projects, and constitute the first QTLs identified for frosty pod resistance. Three QTLs for black pod resistance were found on LG 4, 8, and 10, with the most favorable alleles coming from Pound 7. One QTL was found on LG 4 for average trunk growth rate, and two QTLs for height of first jorquette were identified on LGs 4 and 6. One QTL each for average trunk diameter growth and pod color was found on LG 4.

J.S. Brown, E.J. Power, C. Krol, C. Cervantes-Martinez, and R.J. Schnell, USDA-ARS, Subtropical Horticulture Research Station, 13601 Old Cutler Rd., Miami, FL 33158; W. Phillips-Mora, Tropical Agricultural Research and Higher Education Center, 7170 Turrialba, Costa Rica; J.C. Motamayor, M&M Mars, Inc., c/o USDA-ARS, Subtropical Horticulture Research Station, 13601 Old Cutler Rd., Miami, FL 33158. Received 30 Nov. 2006. \*Corresponding author (miajb@ars-grin.gov).

**Abbreviations:** AFLP, amplified fragment length polymorphism; CATIE, Tropical Agricultural Research and Higher Education Center; LG, linkage group; LOD, log likelihood score; MQM, multiple QTL mapping; PCR, polymerase chain reaction; QTL, quantitative trait locus (loci); RGH, resistance gene homolog; SHRS, Subtropical Horticulture Research Station; SSCP, single strand conformation polymorphism; SSR, single sequence repeat.

**C**ACAO (*Theobroma cacao* L.) is an understory tree originally from the Amazon basin that is well suited for mixed stands of sustainable forestry grown by small landholders. Other species of the genus have economic value, especially *T. grandiflorum* (Willd ex Spreng) Schum., known locally in Brazil mostly as “cupuaçu” and its powder, similar to cocoa powder, as “cupulate.” Though it is a potential source of variability for cacao breeding, heretofore seldom used, it has become a promising new crop in the production of juices, ice cream, candies, desserts, and liquors (Alves and Figueira, 2002). Cacao production in South and Central America has been especially affected by two fungal pathogens: *Moniliophthora perniciosa* (Stahel) Aime and Phillips-Mora comb. nov. (Aime and Phillips-Mora, 2005), causing witches' broom disease, and *M. roreri* (Cif. and Par.) Evans et al. causing frosty pod rot. Black

Published in Crop Sci. 47:1851–1858 (2007).

doi: 10.2135/cropsci2006.11.0753

© Crop Science Society of America

677 S. Segoe Rd., Madison, WI 53711 USA

All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher.

pod (*Phytophthora* spp.) is also a significant disease in South America, especially the species *P. palmivora* (Butl.) Butl., and is the most important cacao disease worldwide, being especially important in Africa, where the most aggressive species, *P. megakarya* Brasier and Griffen, is reported to predominate in at least one country (Cameroon) (Ndoumbé et al., 2001). The most important region of chocolate production of Brazil, the state of Bahia, has *P. capsici* Leonian as the predominant species, with lesser presence of *P. citrophthora* (R.E. Sm. and E.H. Sm.) Leonian and *P. palmivora*, though *P. citrophthora* has been found to be the most virulent (Bowers et al., 2001).

Witches' broom has spread northward into Panama and southward as far as Bahia, Brazil, causing major outbreaks in some of the most important cacao producing regions in South America (Andebrhan et al., 1999; Queiroz et al., 2003), especially Bahia. Frosty pod is a very severe disease, and fortunately has not yet spread extensively; however, the disease is continuously spreading from the northwest of South America into Central America, toward the South of Mexico, with confirmed reports of its presence in Nicaragua, Honduras, and Guatemala (Pereira, 1996; Evans et al., 2003). Its spread southward involves areas as far as Peru and eastward as far as Venezuela. Chemical treatment of these diseases is very expensive and involves year-round application, which also raises environmental concerns (Bowers et al., 2001); therefore, interest in resistance breeding has increased despite the long generation time involved. Research is also being conducted for biological disease control in the ARS and in CABI Bioscience of the United Kingdom, mainly using fungi that have a certain degree of action against cacao pathogens (Crozier et al., 2006).

Cacao is an out-crossing diploid ( $n = x = 10$ ) with a very small genome (ca. 447 Mb). There is thought to be a rather large amount of diversity within cacao, however most commercial cultivars have a narrow genetic base. Due to disease problems and interest in increasing the variability within cultivated germplasm, there have been rather recent collections within Brazil by national groups and within Peru by our project and by Peruvian groups. The Subtropical Horticulture Research Station (SHRS) is consolidating collections made by Allen and Chalmers in the mid-20th century, as some clones of which have become scattered and some lost in certain collections (Schnell et al., 2007). The traditional primary types of cacao have been recognized by breeders for several centuries to be Forastero, Trinitario, and Criollo. There is a small number of national, proprietary groups with distinctive quality traits in smaller populations, such as Nacional in Ecuador and certain Criollos in Venezuela, among others. Criollo types are generally considered to have the highest quality, while Forastero, divided into upper Amazon and lower Amazon types, has traditionally been thought to

provide vigor and disease resistance for world production. Upper Amazon types are likely to contain most of the remaining exploitable variability. Trinitarios are hybrids that have been developed during the last few centuries by crossing lower Amazon Forastero and Criollo. During the last decade, more intense visual inspection of germplasm collections and the application of molecular markers have revealed a serious problem of mislabeling that can be as high as 20 to 38% (Motilial and Butler, 2003; Turnbull et al., 2004). This can happen whether plant materials are a single cross population for breeding and mapping, such as used in this research; a set of structured crosses, such as those made at CATIE and analyzed with single sequence repeat markers (SSRs) for off-types by Takrama et al. (2005) and by Cervantes-Martinez et al. (2006) for general and specific combining ability; or a regional germplasm collection, such as in Puerto Rico (Zhang et al., 2006). Cocoa breeders have generally attributed the lack of progress in cacao breeding to the narrow genetic base of cultivated cacao; however this rather recent discovery of the prevalence of off-types has awakened many researchers to the need to verify genotypes with molecular markers (Takrama et al., 2005).

The Plant Genetics group of the USDA-ARS, SHRS in Miami has undertaken the creation of a series of cooperative breeding projects with national agricultural agencies and other international organizations, that are carrying out research to avoid the spread of these two fungal diseases, given their seriousness, to currently unaffected areas (Schnell et al., 2007). Cacao requires usually approximately 18 mo to 2 yr to yield at a sufficiently high level to enable clone comparisons, and a minimum of 3 yr of data is considered necessary for accurate yield comparisons. In commercial plantings, trees often remain in production for long periods, from 10 to 50 yr. Therefore the ability to select resistant seedlings or seedlings desirable for other horticultural traits in breeding programs is quite advantageous. In the project reported here, the staff of the Cacao Breeding Program at CATIE, Turrialba, Costa Rica, created a cross of the two clones, 'Pound 7' and 'UF 273', producing 256 progeny, with the two parental clones being reciprocally resistant, respectively, to black pod and frosty pod. The population and data were then analyzed with molecular markers to produce a genomic map, and to search for quantitative trait loci (QTLs) of interest.

## MATERIALS AND METHODS

### Generation of Plant Materials and Collection and Basic Analysis of Phenotypic Data

A population of 256  $F_1$  cacao progeny from the cross Pound 7  $\times$  UF 273 was generated at CATIE, Turrialba, Costa Rica, for the production of a genomic map, to generate phenotypic data for QTL analysis, as well as for the production of clones for potential future agricultural and breeding uses. Trees were

planted during the month of May 1998, with spacing between trees of 3 by 3 m at La Montaña Farm of CATIE. Temporary shade was provided by plantain (*Musa* sp.) (AAB-triploid), and permanent shade was provided by the tree, Immortelle [*Erythrina poeppigian* (Walp.) O.F. Cook], at spacings of 6 by 6 m. Soil type of the first 15 cm was classified by the soils lab at CATIE as a “Franc” type with a slightly higher proportion of sand, and the profile from 15 to 30 cm was classified as a sandy-clayey Franc type. The pH was slightly low, typical for tropical soils, being pH 4.96 as measured in H<sub>2</sub>O. During the growth and production and inoculation periods, biennial maintenance of the shade species was performed (pruning, etc.), and weed control was practiced by manual and mechanical methods thrice yearly. Fertilizer was applied to maintain fertility (200 g per tree of 18–5–15) in March, July, and November.

Prior pathological testing at CATIE showed Pound 7 to have tolerance to black pod and high susceptibility to frosty pod, and UF 273 to show good tolerance to frosty pod and moderate susceptibility to black pod. Hence, the creation of the population was done for two purposes: (i) to make a genomic map and subsequently to attempt mapping of QTL for resistance to frosty pod and black pod, as well as horticultural traits of interest, and (ii) to combine two clones with complementary resistance and horticultural properties in a large population, hopefully combining some or all of the desired traits through transgressive segregation into superior clones and breeding material. Pound 7 was used as the maternal parent and UF 273 as the paternal parent.

Five cloned trees of UF 273 were used as parents, and one tree was discovered out of the five UF 273 trees, henceforth referred to as UF 273 Type II, to have differing alleles for 22 markers out of the 180 (12%) used for the map. This UF 273 Type II tree gave rise to 71 progeny, which differed usually for only one allele of the two per locus. Type I trees were parental to 185 progeny.

The following horticultural traits were measured from 1998 to 2005 as appropriate for each trait: (i) height of the first jorquette (first point at which trunk breaks into lateral branches, usually held to one jorquette in commercial plantings); (ii) trunk diameter, measured semi-annually; (iii) and (iv) months from planting in the field to flowering and fruiting; and (v) pod color, evaluated as red or green only, disregarding intermediate shades. Cacao can take between 18 mo to 3 yr to begin to flower and fruit, depending on genetics and management of the crop. It was our desire to minimize time to flowering and fruiting genetically in this experiment. Trunk diameter was converted into average rate of trunk growth by taking the difference between successive measurements and subsequently forming the average rate of growth per month.

Groups of trees in moderate numbers (ca. 25–35) were inoculated by the method of Phillips-Mora (1996) to measure frosty pod reaction, starting in 2000 and continuing until 2004, scoring for both internal and external infection of pods. Each tree was inoculated several times (6–10 times usually), using as many fruits as were available at that time (usually one to three). Pod inoculation for black pod began in January 2000 and continued until approximately August 2004. Data for black pod were obtained using the method described in Crouzillat et al. (2000), scoring 10 d post-inoculation. Several pods from each

tree were sampled at each inoculation date, and each tree was inoculated several times, similar to the method used for frosty pod. A mixed linear model was run on the data for disease lesions using Proc Mixed of SAS V9 (SAS Institute, 2002–2003), to remove the effect of number of inoculations, number of pods per inoculation per tree (random effects), to adjust and to test for effects of UF 273 in the cross (Type I or Type II), and for the effect of each tree (fixed effects) nested within type. Least-square means were produced for each tree, to be subsequently used for QTL analysis. This model converged easily on each run, no problems of estimability were experienced in obtaining least square means, and the error of the least-square means was thereby reduced. Therefore, the mixed model accomplished removal of error from raw values of the random variables and sufficient adherence to normality to obviate transformation of disease ratings, as further explained below.

Agreement of each variable with normality was checked using Proc Univariate of SAS V9. No extreme deviations from normality were found, except for height of the first jorquette, and for internal and external disease rating for frosty pod and black pod, for which running the mixed model was sufficient for adjustment. Plotting residuals versus predicted values from the mixed models gave a mound-shaped histogram, as one would expect with normality. Log-normal plots conformed to a straight line sufficiently closely that we were quite comfortable in using least square means for QTL searching. Conformation of height of the first jorquette to the normal distribution was achieved using a simple log transformation.

## Fragment Analysis, Map Construction, and QTL Mapping

One hundred eighty markers were analyzed on 256 individual trees, after DNA extraction using the FAST DNA Kit (Bio101, Carlsbad, CA) with 200-mg tissue samples taken from semi-adult leaves. DNA extraction, polymerase chain reaction (PCR) amplification, and capillary electrophoresis for SSR markers or single strand conformation polymorphism (SSCP) of resistance gene homolog (RGH) and “WRKY” markers were performed as described in Kuhn et al. (2003). Markers used in this study included: 165 SSR markers with the prefix “mTcCIR” produced at CIRAD, Montpellier, France; 12 SSR markers with the prefix “SHRS” produced at the Miami ARS station; RGH markers (Kuhn et al., 2003) and one “WRKY” stress-related marker (Borrone et al., 2004). All markers met the requirement for this type of analysis, being heterozygous for one parent or the other.

A SAS macro program was written in SASV9 to convert genotype data to JoinMap V4 format, otherwise a tedious, laborious, and error-prone task. JoinMap V4 (Kyazma, Wageningen, the Netherlands) was used to accomplish the genomic mapping of the cross, while MapQTL V4 (Kyazma) was used to perform QTL mapping.

The method of Churchill and Doerge (1994) was used (1000 permutations) to determine the genome-wide threshold for each phenotypic trait. The method of restricted multiple QTL mapping (MQM) with cofactors determined by backward elimination ( $\alpha = 0.05$ ) (Table 1) consistently gave the most informative and accurate LOD curves, though the results of the other two possible methods in MapQTL, simple interval mapping, and

**Table 1. Cofactors found for all traits with quantitative trait loci (QTLs) and their corresponding linkage group.**

QTL	Linkage Group									
	1	2	3	4	5	6	7	8	9	10
Fros_PodExt1	-	mTcCIR252	-	-	mTcCIR10	-	mTcCIR46	mTcCIR236	mTcCIR145	-
Fros_PodInt1	-	mTcCIR176, mTcCIR252	-	mTcCIR168	mTcCIR10	-	mTcCIR179	mTcCIR225	-	-
Phytoph	-	mTcCIR73	-	mTcCIR168	mTcCIR111, mTcCIR248	-	-	mTcCIR236	-	mTcCIR37, mTcCIR91
Ave_gr_rt1	-	mTcCIR253	-	mTcCIR168	-	mTcCIR255	mTcCIR179	mTcCIR225, mTcCIR236	-	-
Jor1	mTcCIR29, mTcCIR273	-	-	mTcCIR18, mTcCIR168	mTcCIR127	mTcCIR255	-	-	mTcCIR145, mTcCIR283	-
Pod_Color	Categorical trait-Proc BTL used, based on a different method using the chi-squared distribution.									

MQM with cofactors, were also observed for complementary information. Pod color, being a categorical variable, was analyzed using a beta release of Proc BTL, a new procedure of SAS, capable of analyzing categorical data for QTL in a mixed model context, using Proc Mixed. Two methods were used to find a significance threshold for Proc BTL: (i) a standard Bonferroni correction and (ii) a permutation method based on the chi-squared statistic produced by Proc BTL. Little difference was found between the results of two methods for pod color, being 15.08 for the permutation threshold, which we choose to be the final threshold.

## RESULTS

### Map Construction

Minimal LOD scores of 2.0 to maximal scores of 6.0 sufficed for determining linkage groups, corresponding to the 10 known cacao chromosomes as numbered in Lanaud et al. (1995) (LG 1–10). For ordering markers within linkage groups, a maximum recombination coefficient of 0.45 was used with a maximum LOD of 1.0.

All parent trees used in making the cross were also analyzed with all markers, and no parent trees from the four potential Pound 7 parental trees appeared to differ. One parental tree out of the five trees used to make the cross from UF 273, differed at 22 marker loci, as mentioned above. Any locus possessing any allele for any of the 22 loci in Type II not existing in Type I was replaced by missing data for both alleles, enabling the use of both groups of progeny for creating the map. The map constructed after removing both alleles at loci with unique Type II alleles gave the most acceptable map, with the correct number of linkage groups and with genes ordered most similar to the consensus map of Lanaud et al. (1995), the subsequent map made with only codominant markers by Pugh et al. (2004), and other maps. This map also appeared to be more acceptable than an earlier map created with all markers and all alleles, and with a map formed after removing all markers differing for Type II completely from the map, as well the map from only UF 273 Type I data. Before determining

the existence of Type I and Type II for UF 273, there were very few markers showing deviation from expected segregation ratios when statistically tested, however patterns could be seen by visual inspection of the segregation data. After recoding both alleles at loci containing Type II alleles, an increased number of markers deviating from the expected segregation ratios was seen, and markers differing in the Type II population appeared to be grouped. Four loci deviated clearly due to missing data, and a total of 38 markers out of 180 (21.1%) had significant chi-squared values due to deviation from expected ratios, many (22) of them due to recoding the differing Type II alleles. Markers showing only patterns of deviation before recoding became significant after replacing Type II alleles. It should be remembered, however, that only informative markers are being considered here, and that the percentage of total markers (informative and noninformative), and hence of the total genome, would be lower. The final percentage would depend on the total number of markers tested for informativeness, in our case, 212 markers, or 10%. Ultimately, such markers with deviation are not usually thought to seriously disturb the map (Lorieux et al., 1995; Hackett and Broadfoot, 2003), nor were these markers associated with the locations of QTLs subsequently found. The tests of effects between Type I and Type II progeny for internal and external infection was nonsignificant.

The entire genome map, LOD curves, and QTLs with 1- and 2-LOD confidence intervals (van Ooijen, 1992) are shown in Fig. 1. Linkage groups ranged from 42.8 to 125.2 cM, with a total genome length of 884.8 cM, compared to 671.9 cM for the F<sub>2</sub> map from Brazil used for mapping witches' broom (Brown et al., 2005) and 782.8 cM for the map by Pugh et al. (2004), which was made with the original population used by Lanaud et al. (1995) to construct the cacao reference population, but using only codominant markers. The length of the new map is very close to that of the high-density cacao map (885.4 cM) of Risterucci et al. (2000). The final version of the map contains 170 markers, making the average space

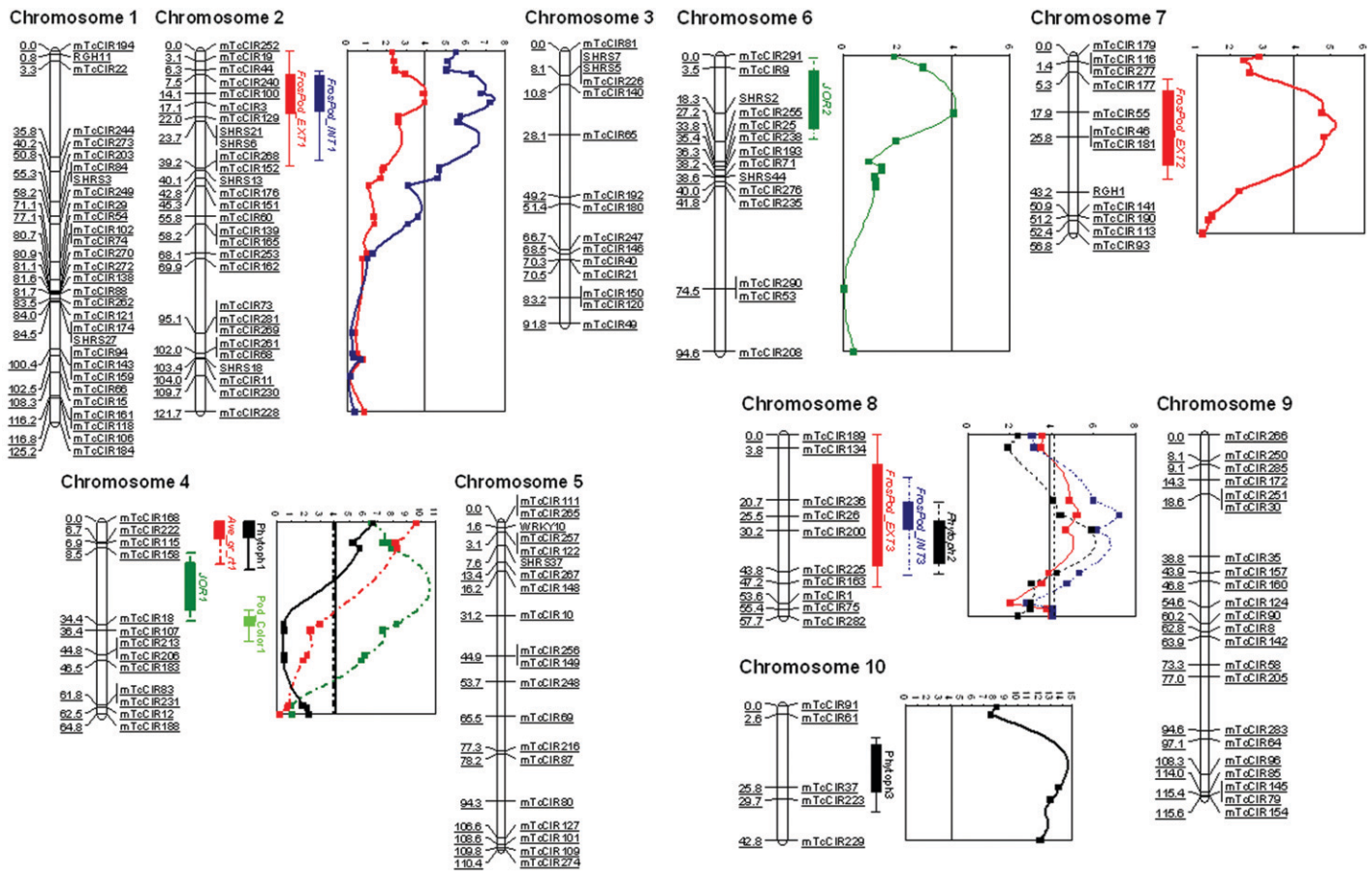


Figure 1. The genomic linkage map for the ‘Pound 7’ × ‘UF 273’ F<sub>1</sub> cacao population, the position of each marker on each chromosome, and charts showing the LOD curve for each trait, with significant quantitative trait loci (QTLs) and location on each respective LG. The peak of the LOD curve lies within the area denoted by the horizontal line in the thicker bar of the two bars (thick and thin) of the QTL confidence interval, corresponding to 1-LOD and 2-LOD intervals in each direction. QTLs are labeled as follows: FrosPod\_EXT1, FrosPod\_INT1; frosty pod external resistance 1, frosty pod internal resistance 1, and similarly for those ending in 2 and 3. Phytoph1; black pod resistance1, and similarly for those ending in 2 and 3. Jor1; First QTL for centimorgan to formation of first jorquette, and similarly for second QTL for formation of first jorquette. Pod Color1 is the first QTL for pod color; Ave\_gr\_rt1 is the first QTL for average growth rate of trunk diameter. Each linkage group is marked with the number from the reference map (LG 1, etc.). Pod color has no corresponding curve, being a categorical variable and based on a chi-squared statistic, instead of a LOD score.

between markers approximately 5.2 cM. Ten markers were not able to be placed within their respective linkage groups in the more conservative map used by MapQTL and were discarded.

Twelve QTLs were found in this experiment; five for frosty pod (internal and external), three for black pod, two for height of first jorquette (cm), one for pod color, and one for average trunk growth rate. Associated data, as would be needed by a breeder to use these QTLs based on this map, are listed in Table 2. Five QTLs were found for frosty pod, however two QTL locations (first and third frosty pod QTLs) contain two QTLs for correlated measurements (internal and external lesions), and could be considered to be the same QTLs. UF 273 was the source of all QTLs for frosty pod resistance. Figure 1 also contains LOD curves; the first and third frosty pod curves on LG 2 and LG 8 take very similar shapes, with the internal measurements’ curve being approximately 2 to 3 LOD higher than that of the external measurements. Linkage

group 7 contains the second frosty pod QTL, with the peak lying clearly between mTcCIR55 and mTcCIR46. All three of these peaks are well above the genome-wide threshold, if not for both measurements, then for measurements of internal lesions. The first QTL for black pod resistance, on LG 4, mapped on the proximal end of the chromosome. Clément et al. (2003b) also found a QTL on LG 4 for black pod resistance in ‘DR1’ and ‘IMC78’ based on the percentage of rotten pods of the total pods produced, using composite interval mapping, very similar to restricted MQM, with cofactors selected using forward-backward stepwise regression, also similar to the method used by MapQTL<sup>®</sup>. The biggest differences between the methods of Clément et al. (2003b) and our methods consist of the types of markers used by Clément et al. (2003a, 2003b), in that they used restriction fragment length polymorphisms, microsatellites (SSRs), and amplified fragment length polymorphisms (AFLPs). They also observed the results of three heterozygous clones ‘DR1’,

**Table 2. Quantitative trait loci (QTLs) found and descriptive information for use in selecting progeny.**

QTL name	LG <sup>†</sup> (reference map no.)	Significance threshold	LOD peak	% Variance explained	First flanking markers	Second flanking marker	Most desirable allele set	Second most desirable allele set	More desirable parent
Fros_Pod EXT1	2	3.9	3.9	4.5	mTcCIR240	mTcCIR100	ad <sup>‡</sup>	bd <sup>‡</sup>	UF 273
Fros_Pod INT1	2	3.9	7.0	9.4	mTcCIR240	mTcCIR100	ad	bd	UF 273
Fros_Pod EXT2	7	3.9	5.1	9.6	mTcCIR55	mTcCIR46	ac	bc	UF 273
Fros_Pod EXT3	8	3.9	5.2	8.6	mTcCIR236	mTcCIR26	bd	bc	UF 273
Fros_Pod INT3	8	3.9	7.2	9.8	mTcCIR26	mTcCIR26	bd	bc	UF 273
Phytoph1	4	4.1	6.7	8.7	mTcCIR168	mTcCIR168	ac	ad	Pound 7
Phytoph2	8	4.1	6.1	7.3	mTcCIR200	mTcCIR225	bd	ad	UF 273
Phytoph3	10	4.1	14.6	23.0	mTcCIR61	mTcCIR37	ac	ad	Pound 7
Ave_gr_rt1	4	3.9	9.6	13.1	mTcCIR168	mTcCIR222	ac	bc	Pound 7
Jor_1	4	4.0	10.7	19.8	mTcCIR158	mTcCIR18	bc	ac	both <sup>§</sup>
Jor_2	6	3.9	4.5	3.5	mTcCIR9	SHRS2	bd	ad	both <sup>§</sup>
Pod_Color	4	15.08	253.08 <sup>¶</sup>	N/A	mTcCIR158	mTcCIR107	–	–	–

<sup>†</sup>LG, linkage group.

<sup>‡</sup>Chi-squared value rather than LOD.

<sup>§</sup>Both parents had desirable alleles. UF 273 could have slightly better effect.

<sup>¶</sup>Complete explanation for calculation of this estimate is given in MapQTL Ver.4 manual: alleles a and b are from the first parent (Pound 7), alleles c and d are from the second parent (UF 273). Estimates are deviations of that allele combination with the from the grand mean of that QTL in the (0,0) linkage phase state (both alleles in coupling in both parents).

‘S52’, and ‘IMC78’, when crossed onto the homozygous line, ‘Catongo’. Clément et al. (2003a, 2003b) measured trunk circumference only once, 1 m from the ground 12 yr after planting, as opposed to the method described above for our experiment and they estimated canopy size in the ninth year after planting. Their QTL on LG 4 for black pod resistance seemed to be shifted downward compared to ours; however, with all AFLPs above mTcCIR18, the closest SSR in common with our map in DR1, where the QTL aligns in DR1, but with a much lower LOD score. The LOD score for this QTL on the IMC 78 map was 5.0, compared to ours of 6.51. Though a definitive conclusion is impossible to establish as to the identity of these QTL over the two maps given the difference in markers, nonetheless it is likely that they are the same QTL. A QTL for average growth rate of trunk diameter was found also in this area of LG 4 in our cross and for trunk circumference by Clément et al. (2003b), essentially in the same area as the black pod resistance QTL in both maps. Slightly farther down the chromosome at approximately 10.66 cM from the proximal end, the first of two QTL was found for height of the first jorquette. Farther yet down the chromosome, a QTL was located using Proc BTL for pod color. Since this analysis was done by Proc BTL and is based on a chi-squared statistic, no curve is included in the graph for this QTL.

Linkage group 6 contains the second QTL for jorquette formation at 3.9 cM from the proximal end. As both QTLs for jorquette formation have desirable alleles from both parents, UF 273 perhaps being slightly better, the source of alleles were referred to as “both.” Linkage group 8 has two resistance QTLs interestingly closely together, assuming the curve for the third frosty pod QTL is being

reflected by both the internal and external LOD curves, as seems clearly to be the case. The one cofactor for internal frosty pod resistance is mTcCIR225, while the one cofactor for external resistance is mTcCIR236. Therefore the dip in the frosty pod LOD curve is not caused by a cofactor, as can happen (Brown et al., 2005). The last resistance QTL comprising this cluster is the second QTL for resistance to black pod. This QTL is quite near the pair for resistance to frosty pod. Its cofactor is mTcCIR236, between the two QTLs. With cofactors lying within and nearby this cluster of markers, and given the high LOD peaks, there is little doubt that both QTL exist, and that one is not an artifact of the other.

Finally, the third QTL for black pod resistance was found on LG 10. The entire LOD curve is well above the threshold, which is very conservative, as all the genome-wide thresholds are. The peak of this QTL is 15.08, probably the largest LOD peak we have seen for black pod. Measuring each tree multiple times with artificial inoculation and removing the error due to other effects from the mean was clearly helpful in finding the QTL more definitively.

## CONCLUSIONS

In the progeny of this cross, the first three QTLs for frosty pod resistance have been found with very solid supporting statistics. Simultaneously, in this cross three QTLs for black pod (*P. palmivora*) resistance have also been located with extremely high supporting statistics. Phillips-Mora (1996) reported a strong correlation between internal and external lesions, with internal lesions giving slightly higher infection responses. Prior plant pathological testing at CATIE was confirmed by the parental sources of

the resistance in the QTLs except in one case, when the source of black pod resistance appeared to be UF 273 rather than Pound 7; however, it is not unusual for the “poor” line to have a small number of desirable QTLs. Taking multiple fruits as often as possible over multiple sampling times allowed removal of error from random sources by using Proc Mixed of SAS. Of course, if certain traits or components are to be studied at certain times in the life span of the trees, only these times can be included in the model. This was not the case with these two traits, with the artificial inoculation method used. If natural infection were used for black pod, it would be more desirable to use trees in their latter years (Clément et al., 2003b) when the infection rate would be higher, and any subsequent models would require appropriate construction. Now that more QTL projects are being accumulated in cacao, we are beginning to get commonality among results. For example, Clément et al. (2003b) found a resistance gene on LG 4 for two clones, DR1 and IMC 78 for black pod that is very likely the same QTL that we found on the same chromosome. Lanaud also found a putative QTL for black pod resistance on the same chromosome (Lanaud et al., 2000). Clément et al. (2003b) also found a QTL nearby on the same chromosome for pod weight, interestingly, with a negative effect, and a QTL for trunk circumference, as we did for average growth of the trunk. It seems that these QTL and their effects are beginning to support one another, especially if clones are related. This is becoming even clearer with the emergence of databases, both in-house and on-line, such as CocoaGen-DB (<http://cocoagendb.cirad.fr/>), produced by CIRAD, Montpellier, France, and the University of Reading, UK, with support by the USDA.

Molecular markers are being used in cacao selection in our marker-assisted selection project to accelerate selection for disease traits, allowing undesirable progeny to be eliminated more quickly, and the nursery to contain more desirable progeny. This allows selection for yield and other less-heritable traits to be done on more individuals. Self-compatibility, an important trait in cacao production, began to be assessed in 2005 in this population, and continues. Yield measurement commenced in January 2006 and will continue until sufficient data has accumulated, to enable searching for more QTLs.

### Acknowledgments

We wish to acknowledge Robert Sautter for his helpful contributions with graphical presentations, and Cecile Olano for significant editorial contribution. We also wish to thank José Castillo and Antonio Alfaro for help with inoculation and data collection, and M & M Mars, Inc., for financial support.

### References

Aime, M.C., and W. Phillips-Mora. 2005. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) from a new lineage of Marasmiaceae. *Mycologia* 97:1012–1022.

- Alves, R.M., and A. Figueira. 2002. CUPUASSU (*Theobroma grandiflorum*) genetic resources and breeding in the Brazilian Amazon. *Ingenic Newsl.* 7:25–30.
- Andebrhan, T., A. Figueira, M.M. Yamada, J. Cascardo, and D.B. Furtek. 1999. Molecular fingerprinting suggests two primary outbreaks of witches broom disease (*Crinipellis perniciosa*) of *Theobroma cacao* in Bahia, Brazil. *Eur. J. Plant Pathol.* 105:167–175.
- Borrone, J.W., D.N. Kuhn, and R.J. Schnell. 2004. Isolation, characterization, and development of WRKY genes as useful genetic markers in *Theobroma cacao*. *Theor. Appl. Genet.* 109(3):495–507.
- Bowers, J.H., B.A. Bailey, P.K. Hebbar, S. Sanogo, and R.D. Lumsden. 2001. The impact of plant diseases on world chocolate production. Available at [www.plantmanagementnetwork.org/pub/php/review/cacao/](http://www.plantmanagementnetwork.org/pub/php/review/cacao/) (verified 18 June 2007). *Plant Health Prog.* DOI 10.1094/PHP-2001-0709-01-RV.
- Brown, J.S., R.J. Schnell, J.C. Motamayor, U. Lopes, D.N. Kuhn, and J.W. Borrone. 2005. Resistance gene mapping for witches' broom disease in *Theobroma cacao* L. in a F<sub>2</sub> population using SSR markers and candidate genes. *J. Am. Soc. Hortic. Sci.* 130(3):366–373.
- Cervantes-Martinez, C., J.S. Brown, R.J. Schnell, W. Phillips, J.F. Takrama, and J.C. Motamayor. 2006. Combining ability for disease resistance, yield, and horticultural traits of cacao (*Theobroma cacao* L.) clones. *J. Am. Soc. Hortic. Sci.* 131(2):231–241.
- Churchill, G.A., and R.W. Doerge. 1994. Empirical threshold values for quantitative trait mapping. *Genetics* 138:963–971.
- Clément, D., A.M. Risterucci, J.C. Motamayor, J. N'Goran, and C. Lanaud. 2003a. Mapping quantitative trait loci for bean traits and ovule number in *Theobroma cacao* L. *Genome* 46:103–111.
- Clément, D., A.M. Risterucci, J.C. Motamayor, J. N'Goran, and C. Lanaud. 2003b. Mapping QTL for yield components, vigor, and resistance to *Phytophthora palmivora* in *Theobroma cacao* L. *Genome* 46:204–212.
- Crouzillat, D., W. Phillips, P.J. Fritz, and V. Pétiard. 2000. Quantitative trait loci analysis in *Theobroma cacao* using molecular markers. Inheritance of polygenic resistance to *Phytophthora palmivora* in two related cacao populations. *Euphytica* 114:25–36.
- Crozier, J., S.E. Thomas, M.C. Aime, H.C. Evans, and K.A. Holmes. 2006. Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. *Plant Pathol.* 55:783–791.
- Evans, H.C., K.A. Holmes, and A.P. Reid. 2003. Phylogeny of the frosty pod rot pathogen of cocoa. *Plant Pathol.* 52:476–485.
- Hackett, C.A., and L.B. Broadfoot. 2003. Effect of genotyping errors, missing values, and segregation distortion in molecular marker data on the construction of linkage maps. *Heredity* 90:33–38.
- Kuhn, D.N., M. Heath, R.J. Wissler, A. Meerow, J.S. Brown, U. Lopes, and R.J. Schnell. 2003. Resistance gene homologues in *Theobroma cacao* as useful genetic markers. *Theor. Appl. Genet.* 107:191–202.
- Lanaud, C., M.-H. Flament, S. Nyassé, A.M. Risterucci, M. Fargeas, I. Kébé, L. Motilial, J.-M. Thévenin, D. Paulin, M. Ducamp, D. Clément, J.A.K. N'Goran, and C. Cilas. 2000. An account of the genetic basis of cocoa tree resistance to *Phytophthora* using molecular markers. p. 127–134. *In Proc. 13th Int. Conf. on Cocoa Research*, Kota Kinabalu, Malasia. 9–14 Oct. 2000. Cocoa Producer's Alliance, Lagos, Nigeria.
- Lanaud, C., A.M. Risterucci, J.A.K. Goran, D. Clement, M.H. Flament, V. Laurent, and M. Falque. 1995. A genetic linkage

- map of *Theobroma cacao* L. *Theor. Appl. Genet.* 9:987–993.
- Lorieux, M., X. Perrier, B. Goffinet, and C. Lanaud. 1995. Maximum likelihood models for mapping genetic markers showing segregation distortion: 2. F2 populations. *Theor. Appl. Genet.* 90:81–89.
- Motilial, L., and D. Butler. 2003. Verification of identities in global cacao germplasm collections. *Genet. Resour. Crop Evol.* 50:799–807.
- Ndombé, M., D. Bieysse, and C. Cilas. 2001. Multi-trait selection in a diallel crossing scheme of cocoa. *Plant Breed.* 120:365–367.
- Pereira, J.L. 1996. Prospects for effective control of cocoa diseases. p. 81–86. *In Proc. 12th Int. Cocoa Research Conf.*, Bahia, Brazil. 17–23 Nov. 1996. Cocoa Producer's Alliance, Lagos, Nigeria.
- Phillips-Mora, W. 1996. Studies at CATIE on moniliasis resistance [*Moniliophthora roreri* (Cif. & Par.) Evans et al.]. p. 111–117. *In Proc. Int. Workshop on the Contribution of Disease Resistance to Cocoa Variety Improvement*, Bahía, Brazil. 24–26 Nov. 1996. Ingenic, Bahía, Brazil.
- Pugh, T., A.M. Fouet, P. Brottier, M. Abouladze, C. Deletrez, B. Courtois, D. Clement, P. Larmande, J.A.K. N'Goran, and C. Lanaud. 2004. A new cacao linkage map based on codominant markers: Development and integration of 201 new microsatellite markers. *Theor. Appl. Genet.* 108:1151–1161.
- Queiroz, V.T., C.T. Guimarães, D. Anhert, I. Schuster, R.T. Daher, M.G. Pereira, V.R.M. Miranda, L.L. Loguercio, E.G. Barros, and M.A. Moreira. 2003. Identification of a major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches' broom disease. *Plant Breed.* 122:268–272.
- Risterucci, A.M., L. Grivet, J.A.K. N'Goran, I. Pieretti, M.H. Flament, and C. Lanaud. 2000. A high-density linkage map of *Theobroma cacao* L. *Theor. Appl. Genet.* 101:948–955.
- SAS Institute. 2002–2003. SAS procedures guide, ver. 9. SAS Inst., Cary, NC.
- Schnell, R.J., J.S. Brown, D.N. Kuhn, C. Cervantes-Martinez, J.W. Borrone, C.T. Olano, J.C. Motamayor, W. Phillips, E. Johnson, E. Monteverde-Penso, F. Amores, and U. Lopes. 2007. Current challenges of tropical tree crop improvement: Integrating genomics into an applied cacao breeding program. *Acta Hort.* 738:129–144.
- Takrama, J.F., C. Cervantes-Martinez, W. Phillips-Mora, J.S. Brown, J.C. Motamayor, and R.J. Schnell. 2005. Determination of off-types in a cacao breeding programme using microsatellites. *Ingenic Newsl.* 10:2–8.
- Turnbull, C.J., D.R. Butler, N.C. Cryer, D. Zhang, C. Lanaud, A.J. Daymond, C.S. Ford, M.J. Wilkinson, and P. Hadley. 2004. Tackling mislabeling in cacao germplasm collections. *Ingenic Newsl.* 9:8–11.
- van Ooijen, J. 1992. Accuracy of mapping quantitative trait loci in autogamous species. *Theor. Appl. Genet.* 84:803–811.
- Zhang, D., S. Mischke, R. Goenaga, A.A. Hemeida, and J.A. Saunders. 2006. Accuracy and reliability of high-throughput microsatellite genotyping for cacao clone identification. *Crop Sci.* 46:2084–2092.