Plant Mol Biol Rep (2009) 27:177–183
DOI 10.1007/s11105-008-0069-9

Mapping of Quantitative Trait Loci for Butter Content and Hardness in Cocoa Beans (*Theobroma cacao* L.)

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Published online: 2 December 2008

Abstract Cocoa butter is an important raw material for the chocolate, pharmaceutical, and cosmetic industries. The butter content and quality in cocoa beans are genetically controlled characteristics, and affect its commercial value and industrial applicability. In the present work, an F_2 population derived from the cross between the ICS-1 and Scavina-6 cocoa clones was used for molecular mapping. A linkage map was constructed based on amplified fragment length polymorphism, random amplified polymorphic DNA, and simple sequence repeat markers, resulting in a total of 273 markers, distributed in 14 linkage groups (LGs). Phenotyping of butter content was performed after ether extraction and butter hardness was determined by sweeping differential calorimetry. One quantitative trait locus (QTL) associated to butter content was mapped at linkage group 9 (LG9) and two QTLs for butter hardness were identified at linkage groups 9 and 7 (LG9 and LG7). The two QTLs mapped at the LG9 explained 51.0% and 28.8% of the phenotypic variation for butter content and hardness, respectively. These QTLs were concentrated in the same map region, suggesting a close genetic linkage or pleiotropic effect. The QTLs identified may be useful in further marker-assisted selection breeding programs aimed at cocoa butter quality improvement.

Keywords Butter content · Butter hardness · QTL · *Theobroma cacao*

Abbreviations

QTL	Quantitative trait loci
LG	Linkage group
AFLP	Amplified fragment length polymorphism

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RAPD Random amplified polymorphic DNA
SSR Simple sequence repeat
SCAR Sequence characterized amplified region

Introduction

Butter produced from cocoa beans has unique physicochemical characteristics among the vegetable fats. It is an important raw material for the chocolate, pharmaceutical, and cosmetic industries due to its functional properties (Tucci et al. 1996). In chocolate production, cocoa butter is the main substrate for suspension of cocoa powder, sugar, and other ingredients, being also responsible for soft texture, viscosity, and easy flavor diffusion (Liendo et al. 1997).

Cocoa butter is the fatty component of the cocoa bean and constitutes approximately 45% of the whole bean. Although the butter content represents an important aspect, the quality of this fat is also an interesting trait. The butter quality is associated with its melting point (hardness), which is a consequence of the proportion of unsaturated fatty acids (Lambert et al. 1996).

Cocoa breeding aiming to increase the fat content in beans has been effective (Pires et al. 1998). However, direct selection is hindered by the complexity of this trait, the late evaluation, and the high costs of identifying superior genotypes. Thus, characteristics related to cocoa industrial quality (butter content, hardness, and chocolate flavor) present great potential for the use of indirect selection, via molecular markers (Figueira and Cascardo 2001).

The majority of economic traits in crop plants are quantitative in nature, each controlled by many genes or gene complexes that are described as quantitative trait loci (QTL). Genetic mapping of these QTL has been greatly facilitated in recent years due to two important developments, the availability of molecular markers, and the development of a variety of powerful and improved statistical methods (Kulwal et al. 2003). Consequently, the identification of the genetic loci contributing to variation in traits has great importance in plant breeding (Peighambari et al. 2005).

Several genetic maps have been built for the cocoa tree from data generated by molecular markers, and most of them have been used to aid the identification of QTLs related to different traits of agronomic interest (Lanaud et al. 1995, 1997; Crouzillat et al. 1996, 2000a, b; Risterucci et al. 2000; Clement et al. 2000; Motilal et al. 2000; Flament et al. 2001; Queiroz et al. 2003; Clement et al. 2003a, b; Risterucci et al. 2003; Pugh et al. 2004; Faleiro et al. 2006). However, QTLs related to cocoa butter physicochemical characteristics have not been mapped.

Recently, a cocoa genetic linkage map was constructed (Queiroz et al. 2003) using an F_2 population derived from the cross between ICS-1 and Scavina-6 clones. Later, this map was saturated by Faleiro et al. (2006), which had identified QTL of bigger effect that controls resistance to witches' broom disease. The objective of the present study was to saturate the previous linkage map with additional amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and microsatellite markers and to identify QTLs related to cocoa butter content and hardness.

Materials and Methods

Plant Material

The map population consisted of 82 F_2 plants, which were developed in 1992 by the Executive Commission of the Cocoa Tree Planting Plan (CEPLAC), Ilhéus, Bahia, Brazil, as described by Faleiro et al. (2006). In 1994, the F_2 plants were cloned in three new areas, representing four replications in the field. The parental clones were ICS-1 (female parent) and Scavina-6. ICS-1 belongs to the Trinitario group, has large beans, high yield, high general combining ability, and susceptibility to *Crinipellis perniciosa*. Scavina-6 belongs to the Forastero group (Upper Amazon) having small beans and green fruits. Scavina-6 has greater butter content and hardness when compared to the ICS series. The cross between the clones ICS-1 and Scavina-6 produced the TSH-516 clone (F_1), which was selfed to obtain the F2 population used for mapping.

Determination of Cocoa Butter Content and Hardness

The characteristics under study were determined in the samples of seeds from the parental clones (ICS-1 and Scavina-6) and the 82 F_2 individuals (with two replications for each plant). The method described by Pires et al. (1995) was used to determine the butter content. Five seeds per sample had its coat removed and were ground to a fine powder. The powder was dried at 100°C for 16 h and 5 g of each sample was resuspended in 100 ml of a previously heated (60°C) solution of 4 M HCl, and filtered through filter paper. The solid retained by the filter paper was transferred to soxhlet cartridge and dried in a chamber at 60°C for approximately 16 h. The fat was extracted in a soxhlet extractor set with 150 ml petroleum ether for 4 h. After this period, the solvent was recovered and the balloons were kept on a hot plate until the residual petroleum ether had evaporated completely. The remaining fat was dried in a chamber at 105°C for 12 h and, after cooling and weighing, the fat percentage of the dry matter was calculated. Butter hardness was determined by sweeping differential calorimetry (DSC) using a differential scanning calorimeter (Model DSC-7, Perkin-Elmer, Nor-

walk, CT, USA) according to the protocol described by Tucci et al. (1996). The DSC unit represents the solid cocoa butter fraction at 16°C (integrated area).

DNA Extraction

Leaves from the parents, the F_1 hybrid (TSH-516), and from each F_2 plant were collected and stored at −80°C for DNA extraction. Leaf genomic DNA was extracted by method described by Doyle and Doyle (1990) with modifications suggested by Motilal et al. (2000). After quantification and analysis in 0.8% agarose gel, the DNA samples were diluted to 10 ng/μl.

Molecular Markers

AFLP analyses were carried out according to the procedures described by Vos et al. (1995) and the modifications suggested by Queiroz et al. (2003) using the AFLP™ Core Reagent Kit (Gibco BRL–Life Technologies). The AFLP kit (Applied Biosystems, Foster City, CA, USA) was used for the pre-selective amplification with the following reaction conditions: 20 cycles at 94°C for 20 s, 56°C for 1 min and 72°C for 1 min, and a final step of 60°C for 30 min. The selective amplification reactions were performed with 64 combinations of the *Mse*I primers containing three selective nucleotides (N+3) and *Eco*RI fluorescent primers containing two (N+2) and three (N+3) selective nucleotides (Applied Biosystems). PCR reactions were carried out under the following conditions: 13 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min (on each cycle the annealing temperature was decreased by 1°C) plus 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The samples were mixed to the size standard 500-ROX (Applied Biosystems) and analyzed in a DNA automatic sequencer ABI Prism 377 (Applied Biosystems) under electrophoresis at 50 W for 4 h, in a 4% polyacrylamide gel and 1× TBE (90 mM Tris–Borato, 1 mM EDTA).

RAPD reactions were obtained by reactions performed according with Williams et al. (1990) with modifications described by Queiroz et al. (2003). The DNA was amplified in a total volume of 25 μl with the following reaction conditions: 40 cycles of 94°C for 15 s, 35°C for 30 s, 72°C for 90 s; followed by one step of 72°C for 7 min. After amplification, the fragments were resolved in 1.2% agarose gel in TBE 1× buffer and detected under ultraviolet light in the presence of ethidium bromide.

Microsatellite markers were obtained after the PCR reaction, 15 μl final volume, containing 30 ng DNA 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.4 mM $MgCl_2$, 150 μM of each of one of the deoxynucleotides (dATP, dTTP, dGTP, and dCTP), 3 pM of each primer, and 1 U *Taq* DNA polymerase. The sequences of the primers used were obtained from Lanaud et al. (1999). The amplification conditions were the following: 94°C for 4 min; 10 cycles of 94°C for 30 s, 60°C for 1 min (on each cycle the annealing temperature was decreased by 1°C), and 72°C for 90 s; plus 30 cycles of 94°C for 30 s, 48°C for 1 min, 72°C for 90 s; one step of 72°C for 6 min. Two types of electrophoresis were used to resolve the amplified fragments. In the first one, a 3% agarose gel was run at 90 V for 4 h in 1× TBE and then stained with ethidium bromide. In the second method, separation was performed in a 6% polyacrylamide gel, which was run at 55 W for 2 h, in TBE 1×. In this case, the primers were fluorescently labeled and the fragments were analyzed in an ABI 377 automatic DNA sequencer.

AFLP and RAPD markers were named according to the presence of fragments in the ICS-1 (i) or Scavina-6 clones (s), followed by the name of the primer combination and the size of the fragment in base pairs. Microsatellite markers were named based on their identification according to Lanaud et al. (1999).

Statistical Analyses and Linkage Map Construction

The linkage map was constructed using the Mendelian markers with a 5% of probability. The expected segregating ratio of the markers in an F_2 population, 3:1 for dominant markers (RAPD and AFLP), and 1:2:1 for co-dominant markers (microsatellites), was compared with the observed ratios by the chi square test.

The cocoa linkage map was constructed using the software Mapmaker/EXP version 3.0 (Lander et al. 1987). Markers were clustered with a minimum LOD value of 4.0 and a maximum recombination frequency of 0.40. The recombination frequencies were converted to centiMorgans (cM) using the Kosambi mapping function (Kosambi 1944). QTLs were detected by composite interval mapping (CIM) model 6 (Zeng 1993, 1994; Jansen 1993) with a window size of 10 cM using the software QTL Cartographer version 2.0 (Basten et al. 1994, 1999). The presence of QTLs between markers within each interval was ascertained by the likelihood ratio test performed at every cM along the linkage group, using the significance threshold of LOD 2.5 (α=5%). The position of each QTL was estimated, along with its additive and dominance effects, the mean degree of dominance, and the percentage of total variance of the trait explained by the QTL.

Results and Discussion

Characteristics Associated to Butter Quality

The phenotypic characterization of the butter quality was performed for the parents (ICS-1, Scavina-6), the F_1 (TSH-

516), and the 82 F_2 individuals. The value observed for butter content in the parental ICS-1 was 52.1% while Scavina-6 presented 54.8%. The F_1 value was 53.5%. The phenotypic values obtained for butter hardness were 74.3 for ICS-1 and 68.8 the Scavina-6. The F_1 value was 67.5. According to the traditional classification of the *Theobroma cacao* L. species, three racial groups are established: Forastero, Criollo, and Trinitario (hybrid group derived from the cross between Criollo and Forastero). The first group was further classified into Upper Amazon ('forest') and Lower Amazon subgroups (Dias 2001). Pires et al. (1998) reported that the diversity assessment for butter content and yield showed that the genotypes originally collected under forest conditions in the Upper Amazon show a high level of fat in the seeds, while the domesticated genotypes tend to present lower values. Figueira et al. (1999) observed that a number of cocoa populations originating from the forest presented a composition of fatty

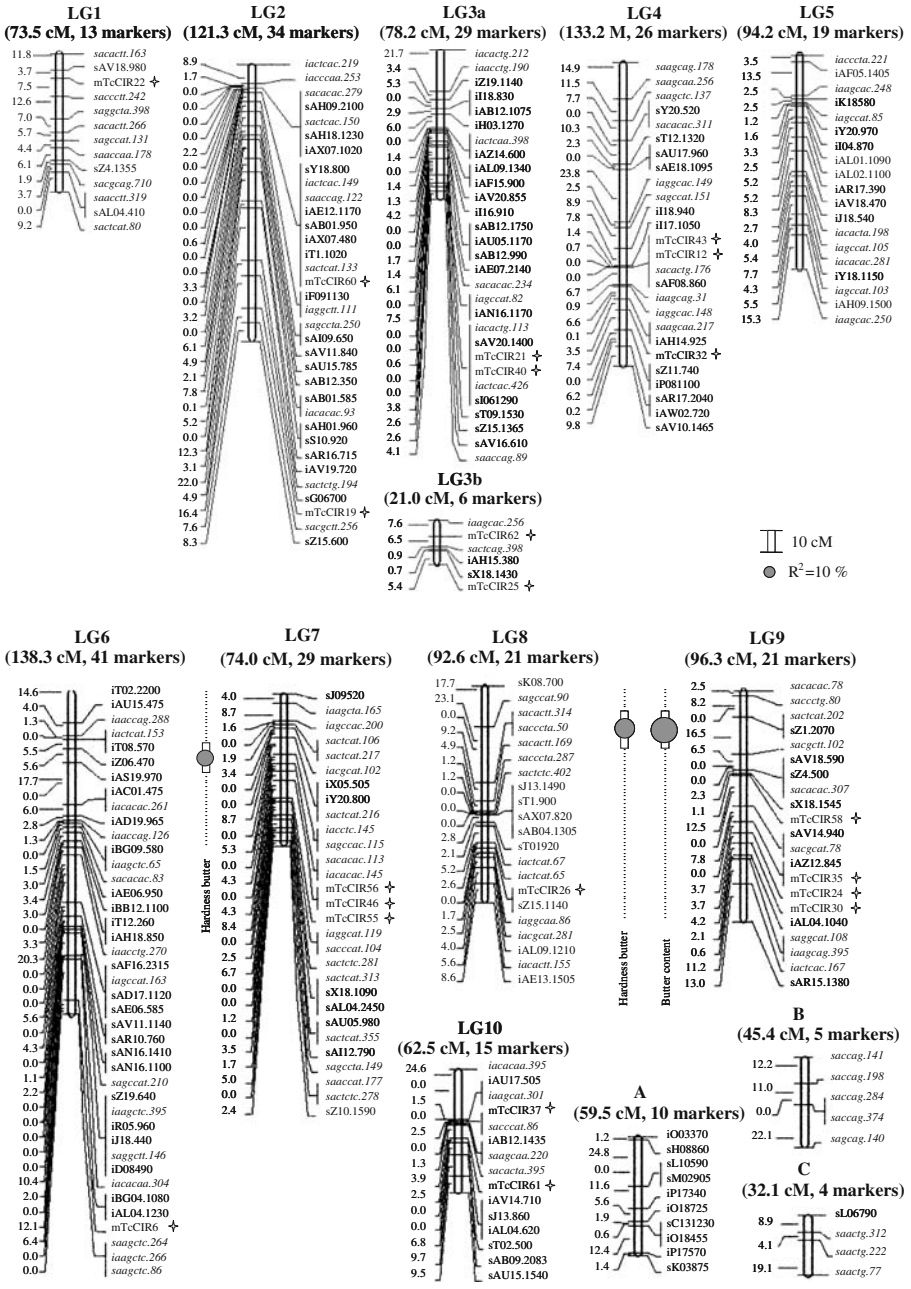

Fig. 1 Genetic linkage map of the cocoa (*Theobroma cacao*) based on segregation analysis of 273 molecular markers, using a LOD of 4.0 and maximum recombination frequency of 40 cM. The regions marked with *circles* correspond to the QTLs identified for butter content and hardness. The SSR markers are indicated by (✣), AFLP markers are indicated in *italic*, and RAPD markers are indicated by *capital letters*

acids, triacylglycerols, and butter hardness with great potential for use in breeding programs. These data corroborate with the results presented here, in which the parental Scavina-6 (Forastero from the Upper Amazon) presented a superior phenotypic value for butter content compared to ICS-1 (Trinitario group).

The butter contents observed in ICS1 and SCA6 differ by 2.7% (52.1% and 54.8%, respectively). These values are close to the average obtained by Pires et al. (1998) that analyzed the fat content for 490 accessions of the Centro de Pesquisa do Cacau, and found 53.2%. Although there are accessions with higher differences on butter content, the selected parent presents additional characteristics essential for breeding programs. Among those, we can highlight the disease resistance (*Crinipellis perniciosa*) and productivity.

Linkage Analyses and Map Construction

Among the 279 markers generated, 273 (21 microsatellites, 115 AFLP, and 137 RAPD) were mapped along the 14 linkage groups (LGs) (Fig. 1). The total coverage of the genome was of 1,122 cM. This genome coverage was 31% (1,713 cM) smaller than that obtained by Queiroz et al. (2003) and 60% (670 cM) bigger than obtained by Faleiro et al. (2006), which mapped 193 loci (124 RAPD and 69 AFLP) along 25 LG and 342 loci (33 SSR, 77 AFLP, and 232 RAPD) along 16 LG, respectively, for the same population.

Among the 14 linkage groups obtained in this study, ten (LGs 1, 2, 3a, 4, 6, 7, 8, 9, 10, and LG3b) were numbered in accordance with nine groups established by Pugh et al. (2004) and the chromosome numbers listed by Saunders et al. (2004) and Borrone et al. (2004). LG3b, although separated from the LG3a in the present work, contains SSRs that allow its association with such linkage groups by

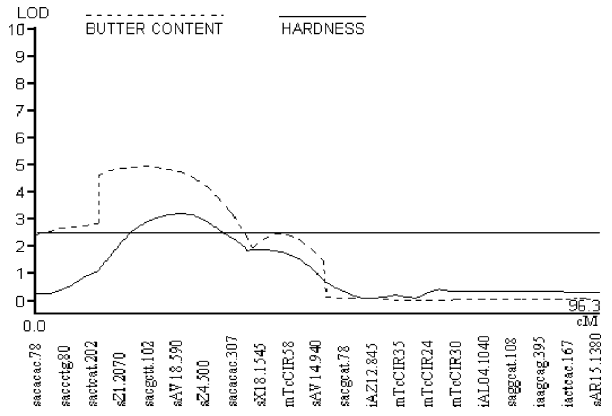

Fig. 2 QTLs identified for cocoa butter content and hardness located in linkage group 9. The level of significance of each peak was calculated by composite interval mapping using the Cartographer QTL program. The genetic distances among the markers are in centiMorgans and the *horizontal line* indicates the LOD cutting point (2.5)

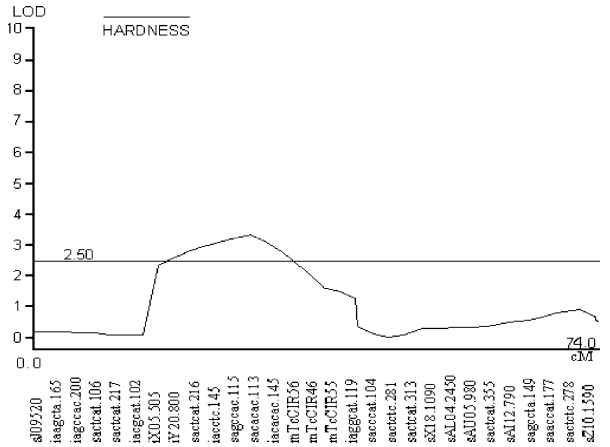

Fig. 3 QTL identified for cocoa butter hardness located in linkage group 7. The level of significance of the peak was calculated by composite interval mapping using the Cartographer QTL program. The genetic distances among the markers are in centiMorgans and a *horizontal line* indicates the LOD cutting point (2.5)

overlapping the maps published by Pugh et al. (2004) and Borrone et al. (2004). The linkage groups 5, A, B, and C do not contain SSR markers, and were arbitrarily numbered. When compared with the map developed by Faleiro et al. (2006), ten of the 14 linkage groups were equivalents (LGs 1, 2, 3a, 4, 5, 6, 7, 8, 9, 10, and LGA).

The coverage of the present map was similar to the one published by Lanaud et al. (1995), covering 759 cM of the cocoa genome, with a progeny of 100 individuals, derived from the cross between UPA 402 and UF 676. This cocoa map was further saturated by Risterucci et al. (2000), covering 885.4 cM. Other cocoa maps showed similar coverage range: 772 cM (Crouzillat et al. 2000a, b), 793 cM (Flament et al. 2001), 682 cM (Risterucci et al. 2000, 2003), and 782 cM (Pugh et al. 2004). This variation may be explained by the differences among the populations, crosses, numbers, and types of molecular markers used for mapping.

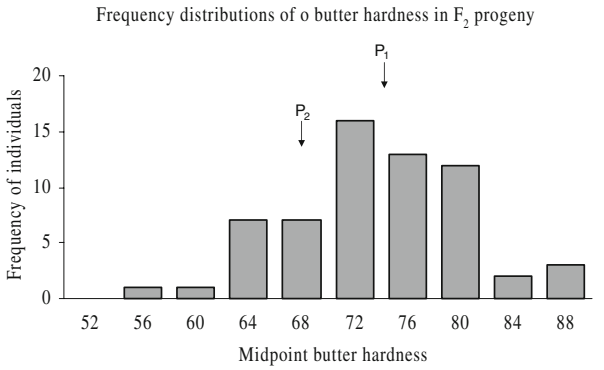

Fig. 4 Frequency distributions of butter hardness in the F_2 population of cacao. The mean phenotypic values of the parents ICS-1 (P_1) and Scavina-6 (P_2) are shown by *arrows*

The sizes of the 14 linkage groups ranged from 21 cM, LG14, to 138.3 cM, LG6, which clustered six and 41 markers, respectively. The marker-to-marker distance varied from 0.0 to 24.8 cM, with an average distance of 4.1 cM, with less than seven marker regions exceeding 20 cM. This average marker-to-marker distance is considerably shorter than observed for the map built by Queiroz et al. (2003), 8.8 cM. However it, is closer to the values determined in the maps developed by Faleiro et al. (2006), Risterucci et al. (2000), Crouzillat et al. (2000a, b), Flament et al. (2001), Risterucci et al. (2003), and Pugh et al. (2004) that were 2.7, 2.1, 4.8, 3.7, 3.2, and 1.7, respectively.

QTL Mapping

The analyses revealed QTLs associated to both the analyzed traits. For butter hardness, one QTL was located at the LG9, linked to the marker sZ1.2070 (Fig. 2), and another QTL was detected at LG7, linked to the iacctc.145 marker (Fig. 3). These QTLs explained 22.8% and 18.6% of the phenotypic variance, respectively. One major QTL present in LG9, linked to the RAPD marker sZ1.2070, was related to butter content, explaining 51.0% of the phenotypic variance.

The phenotypic values for butter hardness within the F_2 population ranged from 55.39 to 88.20, average value 73.54, while the parents ICS-1 and Scavina-6 presented values of 74.25 and 68.72, respectively (Fig. 4). The butter hardness values in the population surpassed the observed in the parent samples, suggesting a transgressive segregation for this trait. Transgressive segregation is the phenomenon in which some individuals in a segregating population outperform the parents (Xu 1997). So, alleles with positive and negative effects (increasing or decreasing trait values) were dispersed between the two parents. The transgression could be attributed to the association of alleles of similar (positive or negative) effects at the QTLs in the same individual of the progeny. Additionally, 45% of the individuals of the F_2 population presented hardness higher than observed values for the parent with the highest phenotypic value (ICS-1). These observations suggest the presence of favorable QTLs in both parents, which probably explains the transgressive segregation effect observed.

The phenotypic average value for butter content within the F_2 population was 48.9. Different from the result observed for butter hardness, the individual maximum value for butter content within F_2 population (52.3) was inferior to the observed for Scavina-6 (54.8) (genitor of higher phenotypic value for this characteristic).

Two QTLs associated with the both different butter-related traits were mapped close to each other at LG9, linked to the sZ1.2070 marker. The presence of QTLs related to different traits in the same genomic region might suggest the existence of a gene, or gene block, with pleiotropic effect (Fig. 1). However, the maximum likelihood positions were not the same, detected at 17.12 and 22.75 cM, respectively (Fig. 2). Thus, the proximity of these QTLs may be due to the existence of linked genes, each one controlling a characteristic separately. Similar results were reported by Clement et al. (2003b) who detected proximity among QTLs related to seed characteristics and ovule number in cocoa fruit.

Little is known or studied about the inheritance of the components of production and other characters of importance for the improvement of the cocoa (Dias and Resende 2001). So, the use of molecular markers as a tool for indirect selection is especially useful. Additionally, marker-assisted selection is even more attractive in perennial species, such as cocoa, and for traits whose measurement is complex, such as bean butter content. The identified sZ1.2070 marker explains 51% of the phenotypic variation for butter content. Thus, its conversion into a sequence characterized amplified region (SCAR) marker will be carried through and its use in the early selection of potentially superior clones may significantly contribute to further breeding programs. Furthermore, this approach allows a best exploitation of the wide variability of cocoa bean quality traits (Are and Atanda 1972; Atanda and Jacob 1973) and the potentially useful identification of new alleles.

Acknowledgements The authors wish to thank the funding from the International Cocoa Organization (ICCO) and the Common Fund for Commodities (CFC) through project CFC/ICCO/BIOMOL/FUNPAB and the collaboration of Dr. Antonio Augusto Franco Garcia from the Department of Genetics, ESALQ/USOP and Dr. Ivan Schuster from the Central Agricultural Research Cooperative (COODETEC) who helped with the analyses.

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