

Identification of a major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches' broom disease

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Abstract

The witches' broom disease caused by the fungus *Crinipellis perniciosa* is the main limiting factor for cocoa production in South America and the Caribbean. In Brazil, this disease affects almost all cocoa-growing regions, causing serious economic, social and ecological damage. The aim of this study was to map genomic regions associated with resistance to *C. perniciosa* using an F₂ population derived from a cross between 'Scavina-6' (resistant) and 'ICS-1' (susceptible). The phenotypic index was determined as the average number of vegetative witches' brooms per canopy area of each plant, the witches' brooms were counted and eliminated during six field evaluations between May 1998 and August 1999. A total of 124 random amplified polymorphic DNA (RAPD) and 69 amplified fragment length polymorphism (AFLP) markers were mapped along 25 linkage groups covering 1713 cM of cocoa genome. After employing single factor and composite interval mapping analyses, a major quantitative trait loci (QTL) flanked by the marker AV14.940 was identified in the linkage group 11, explaining almost 35% of the resistance to witches' broom. The present result suggests that this QTL acts as a major dominant component of resistance to this pathogen, with great potential for use in marker-assisted selection procedures in cocoa breeding programmes.

Key words: *Crinipellis perniciosa* — *Theobroma cacao* — mapping — molecular markers — QTL — witches' broom

Cocoa is a neotropical perennial plant cultivated in the lowlands of South and Central America, Africa and Asia for the production of beans mainly used in the chocolate and cosmetics industries. The cocoa area of the American continent, responsible for 20% of the world production (Zadoks 1997), is threatened by the witches' broom, a devastating disease caused by the fungus *Crinipellis perniciosa* (Stahel) Singer. This pathogen infects vegetative meristematic tissues, flower cushions and young pods, reducing crop yields by up to 80%, and causing plant death after successive cycles, mainly when associated with abiotic stresses (Andebrhan 1984). A recent outbreak of the witches' broom disease in the south of Bahia state, Brazil, one of the largest cocoa-producing areas in the world, drastically reduced the yield of this crop, causing many social, economic and environmental problems (Andebrhan et al. 1999).

Integrated approaches for the control of the witches' broom disease usually have little effect and are highly expensive. Therefore, genetic resistance is the most efficient way of

controlling this disease in zones with high inoculum pressure (Andebrhan 1984). Different sources of resistance to *C. perniciosa* have been identified (Pires et al. 1999), among which the 'Scavina' selections are the most important. Although field observations have suggested that the resistance to *C. perniciosa* in Scavina is controlled by few genes, no conclusive study is available with respect to the inheritance of this trait (Ahnert and Pires 2000).

Molecular markers have been used to facilitate studies of quantitatively inherited traits, particularly for perennial crops that have long selection cycles. Cocoa is a diploid species, to which medium to high-density linkage maps have been reported (Lanaud et al. 1995, Cruzillat et al. 1996, Risterucci et al. 2000). Resistance to the 'black pod' disease in cocoa has been studied using molecular markers, with one quantitative trait loci (QTL) appearing to be a major component of resistance in two related cocoa populations, explaining around 48% of the phenotypic variance in an F₁ population (Cruzillat et al. 2000, Flament et al. 2001). For the witches' broom disease, however, little information is available about genomic regions potentially associated with resistance to this disease.

The objective of the present study was to employ random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) techniques to search for markers explaining a significant proportion of the phenotypic variation for resistance to *C. perniciosa* in an F₂ population derived from a cross between 'Scavina-6' (resistant) and 'ICS-1' (susceptible) cultivars. The potential use of this information in marker-assisted selection in cocoa breeding programmes is discussed.

Materials and Methods

Plant materials: The population of 82 F₂ individuals of cocoa, *Theobroma cacao* L., used in this study was generated by self-pollination of 'TSH 516', an F₁ cocoa clone derived from a cross between 'Scavina-6' and 'ICS-1', two contrasting genotypes for susceptibility to witches' broom. 'Scavina-6' is an upper Amazonian selection belonging to the 'Forastero' group that has been widely used as source of resistance in commercial hybrid production in Brazil, and 'ICS-1' is a susceptible clone belonging to the 'Trinitario' group, chosen because of its high agronomic performance (Pires et al. 1999). To overcome the 'TSH 516' self-incompatibility, self-pollination was

attained by using the mentor pollen technique (Pereira and Yamada 1999), with the mentor pollen obtained from the related genus *Herrania* spp. Cocoa flowers pollinated with *Herrania* pollen showed normal development but the resulting seeds were abnormal, thus facilitating the identification of the self-pollinated seeds (Bartley 1969).

Phenotypic evaluation: The phenotypic index used to assess the levels of resistance and susceptibility to *C. perniciosa* was the number of vegetative brooms per canopy area of each cocoa plant. The vegetative witches' brooms were counted and then eliminated every 3 months from May 1998 to August 1999 during six periodic evaluations. The number of brooms was divided by the canopy area of each plant to standardize the plant vigour and the availability of infection points. The area of each plant was calculated using the plant height (h) and the canopy radius (r) according to the formula $(\pi \times h \times r)/2$. Five-year-old F_2 trees were evaluated under field conditions in a 3 m \times 3 m arrangement, shaded by *Erithrina glauca* at the Experimental Station of CEPEC/CEPLAC (Centro de Pesquisas do Cacau/Comissão Executiva da Lavoura Cacaueira), located in Itabuna, BA, an area largely affected by this disease in Brazil. The parental clones and the F_1 hybrid were concomitantly observed during the F_2 data collection period.

Molecular markers: Cocoa genomic DNA was extracted from leaves according to Doyle and Doyle (1990) and RAPD markers were generated following the procedures published by Lercetou et al. (1997). Amplified DNA fragments were separated by 1.2% agarose-gel electrophoresis, stained with ethidium bromide (10 mg.ml⁻¹), and photo-documented by Eagle Eye II system (Stratagene, La Jolla, CA, USA) under UV light.

Amplified fragment length polymorphisms (Vos et al. 1995) were evaluated by simultaneously digesting 250 ng of genomic DNA from each individual with *EcoRI* and *MseI*, and ligating specific adapters to each of these restriction sites using the AFLP Analysis System I (Gibco Invitrogen Corp., Carlsbad, CA, USA) as recommended by the supplier. Digested-ligated DNA fragments were diluted 1:10, and pre-amplified using AFLP core primers with 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Pre-amplification products were diluted 1:50 and used as template for selective amplification using the combinations of *MseI*- and *EcoRI*-specific primers. The selective amplifications were performed with fluorescently labelled primers supplied by the AFLP Selective Primers kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. A sample of each of the three selective amplifications was mixed with size standard GeneScan 500-ROX (Applied Biosystems, Foster City, CA), formamide and blue dextran mix, denatured at 95°C for 5 min before loading on the gel. AFLP fragments were resolved in a denaturing 4% polyacrylamide gel and fluorescence peaks were detected and analysed by ABI Prism 377 DNA sequencer, using the GeneScan software (Applied Biosystems).

Random amplified polymorphic DNA markers were named using the Operon's primer identification, and their size in base pairs was calculated by the software OnedScan (Stratagene), using lambda DNA digested with *EcoRI*, *HindIII* and *BamHI* as the size standard. AFLP markers were coded by the selective bases of the *EcoRI* and *MseI* primers, respectively, with their molecular size estimated by GeneScan software, using GeneScan 500-ROX as standard.

Linkage analysis: The RAPD and AFLP polymorphisms were scored as 'present' or 'absent', and the segregation ratios were tested by chi-square analysis ($P = 0.05$). Only the markers without a distorted segregation ratio were assigned to linkage groups using the MapMaker EXP/2.0 (Lander et al. 1987) with a minimum LOD score of 4.0 and a maximum recombination frequency of 0.3. Distances in centimorgans (cM) were calculated using the mapping function described by Kosambi (1944).

QTL mapping: Associations between molecular markers and resistance to witches' broom were initially evaluated by single factor analysis

using QGene software (Nelson 1997), with the complete-missing data option. Composite interval mapping (Jansen and Stam 1994, Zeng 1994) was performed using a backward and forward regression model with a window size of 10 and 1 cM precision interval by the software QTL Cartographer (Basten et al. 1998). Significance threshold for the QTL mapping was calculated by 1000 random permutations of the phenotypic data at $P = 0.05$ according to Churchill and Doerge (1994) and Doerge and Rebaï (1996).

Results

Cocoa response to witches' broom disease

The number of vegetative brooms per canopy area, a phenotypic index that allowed the proper distinction between resistant and susceptible individuals, ranged from 0 to 17.68, with an average of 1.32 and a standard deviation of 2.81 (Fig. 1). Among the 82 F_2 individuals, 60 had less than one witches' broom per canopy area, and were considered as resistant plants. Using this index to classify the resistant and susceptible individuals, the segregation displayed by the F_2 population fitted the 3:1 ratio ($\chi^2 = 0.1463$; $P = 0.702\%$), thus fitting the inheritance pattern of a single dominant gene.

Polymorphism analysis

Of 450 RAPD primers tested in the parents, 110 (24.4%) revealed polymorphisms and were evaluated in the F_2 population, yielding 201 polymorphic fragments with an average of 1.83 polymorphisms per primer. All 64 commercial AFLP primer combinations revealed polymorphisms between the progenitors. The 30 most informative combinations were employed to the whole population, yielding 90 polymorphisms. A substantial proportion of the RAPD (28.9%) and AFLP (18.9%) markers deviated from the expected 3:1 segregation ratio, in agreement with previous observations (Ronning et al. 1995, Spielmeier et al. 1998). These markers were not used in the map construction.

Cocoa linkage map

All polymorphisms were scored as dominant markers, yielding 193 DNA markers (124 RAPD and 69 AFLP) that were mapped along 25 cocoa linkage groups, covering a distance of 1713 cM (Fig. 2). The polymorphism generated by the RAPD technique was similar to previous results (Ronning et al. 1995,

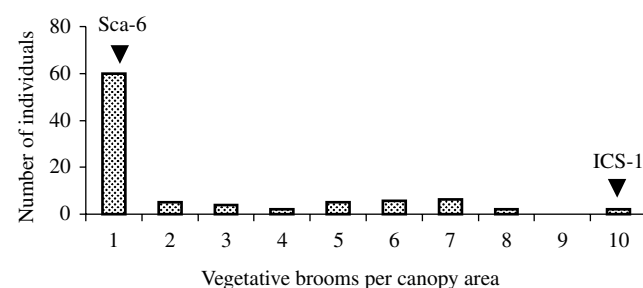


Fig. 1: Frequency distribution of the ratio between the mean number of vegetative brooms per canopy area of each cocoa plant, used as a phenotypic index to evaluate the resistance to *C. perniciosa* in the 82 F_2 progeny. Each class includes all individuals with a smaller ratio than the respective number, while class 10 includes all plants with a ratio higher than nine. The arrows indicate the phenotypic index displayed by the parental clones, where 'Scavina-6' (Sca-6) showed 0.59 and 'ICS-1' showed 11.35

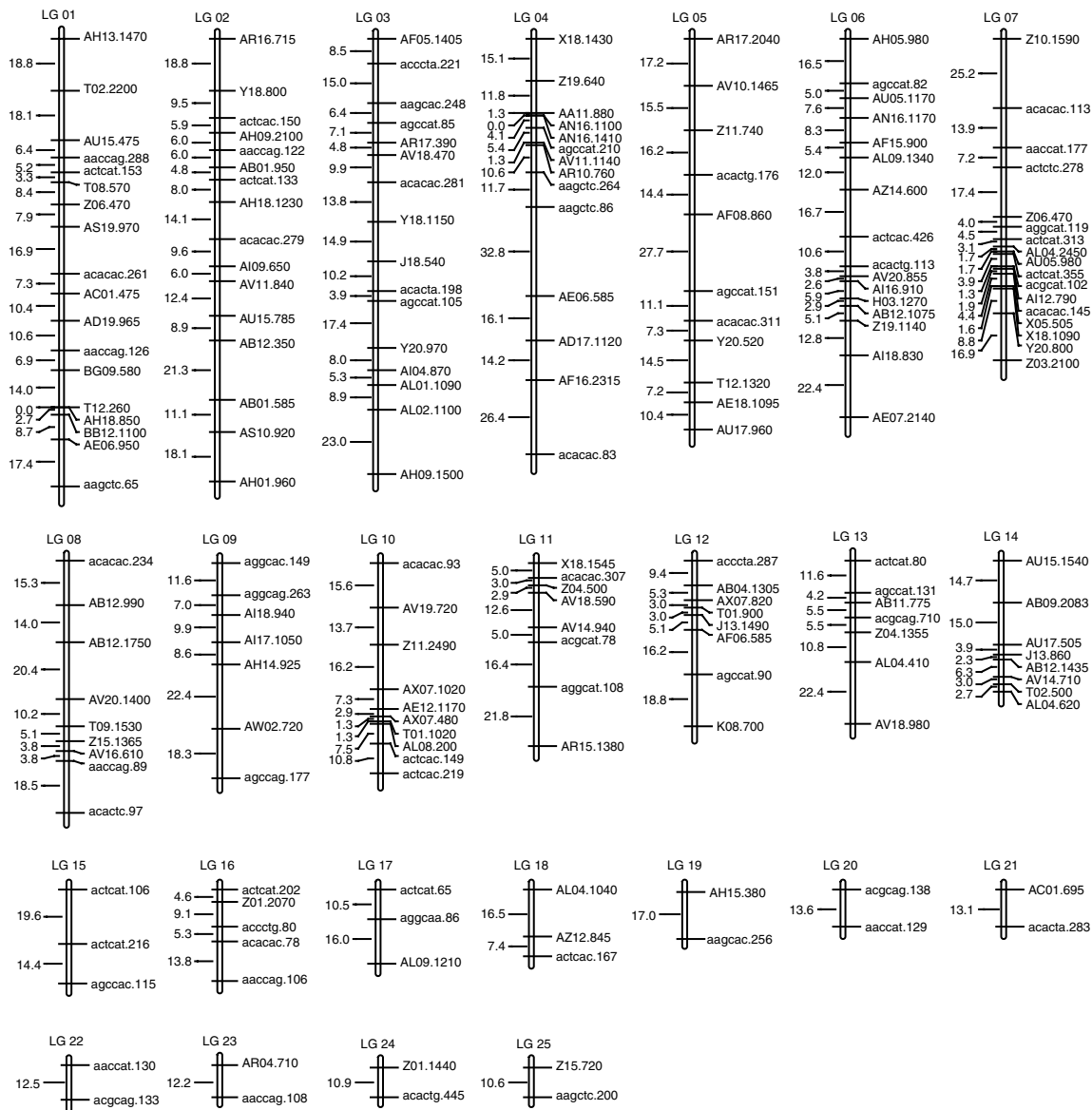


Fig. 2: A cocoa genetic linkage map showing 25 linkage groups comprising 124 RAPD and 69 AFLP markers covering 1713 cM of the cocoa genome. All Mendelian markers were grouped with LOD > 4.0 and recombination frequency < 0.3. The numbers on the left of each linkage group indicate the distance in centimorgan estimated by Kosambi's mapping function

Crouzillat et al. 1996). However, the AFLP showed a reduced average of polymorphisms per primer combination in comparison with Risterucci et al. (2000), probably because of the genetic differences between the cocoa progenitors.

The sizes of the linkage groups ranged from 163 to 10.8 cM and were highly correlated with the number of markers per map unit ($R^2 = 0.92$), clearly indicating a random distribution of the markers within the linkage groups. The map distance between adjacent markers was 8.87 cM on average, where 93.5% of the intervals were smaller than 20 cM, confirming a suitable coverage of the present map for QTL analyses. Nevertheless, the number of linkage groups was higher than the number of chromosomes in cocoa (Glicenstein and Fritz 1989).

Mapping the witches' broom resistance

Single factor analysis detected all the markers in linkage group 11 significantly associated with resistance to *C. perniciosa* in

cocoa ($P < 0.001$). However, after the multiple regression analysis only the marker AV14.940 was maintained in the model explaining 34.8% of the phenotypic variation that also flanked a QTL explaining 36.1% of the total trait variance using composite interval mapping (Fig. 3). This RAPD-based marker was in coupling phase to the resistance, and Table 1 gives the number of resistant and susceptible individuals for both marker classes.

Discussion

The number of vegetative brooms per canopy area appeared to be an adequate phenotypic index to assess the response of cocoa to *C. perniciosa*, as it allowed standardization of the vegetative area and, consequently, the frequency of infection points among the individuals. The 3 : 1 segregation ratio based on the phenotypic classification index employed, which suggested an inheritance model involving a single dominant

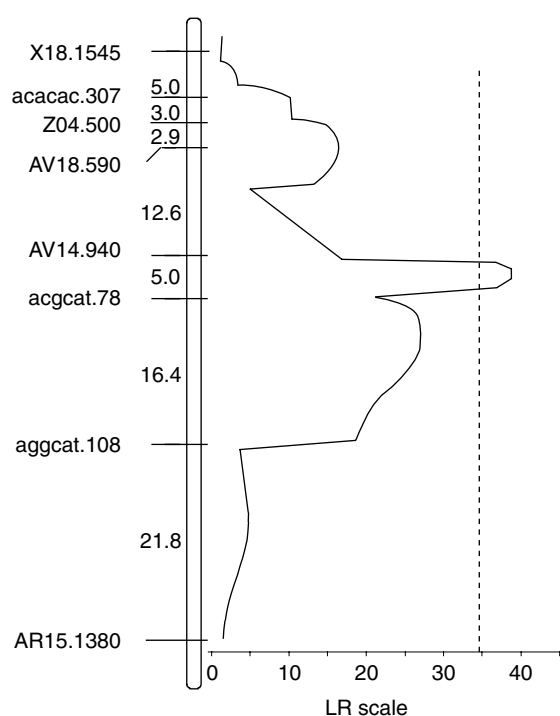


Fig. 3: QTL mapping for resistance to *Crinipellis pernicioso* in the cocoa linkage group 11. Likelihood ratio scope peaks were calculated by composite interval mapping using QTL Cartographer software. Genetic distance between markers were in centimorgan and the vertical line indicates the significance threshold (34.96), calculated by a 1000 permutation at $P = 0.05$

Table 1: Association between marker AV14.940 and resistance to *Crinipellis pernicioso*

Marker	Resistant	Susceptible	Sum
Presence	56	4	60
Absence	4	18	22
Sum	60	22	82

gene, probably oversimplifies the genetic mechanisms underlying cocoa's resistance to *C. pernicioso*, because the quantitative phenotypic distribution was reduced to only two classes. However, the possibility of this trait being controlled by major genes, probably in a cluster configuration that segregates as a single locus, would be in agreement with the other resistance genes that have already been mapped (Ming et al. 1997, Crouzillat et al. 2000). The results obtained in this work add strong support to this contention.

The QTL flanked by the marker AV14.940 confirmed the phenotypic 3:1 (resistance : susceptibility) segregation ratio displayed by the F_2 population, acting as a major dominant gene for this trait. This would seem to be the first report of a single major QTL controlling resistance to *C. pernicioso* in cocoa, a proposal fully endorsed by the close agreement found between the phenotypic and molecular data. A similar situation has been reported for *Phytophthora palmivora* in cocoa, in which a major QTL associated with disease resistance, explained around 48% of the phenotypic variance (Crouzillat et al. 2000). Therefore, further studies are certainly warranted to provide a better understanding of the genomic organization of QTLs associated with the resistance to this disease.

The introgression of witches' broom resistance in cocoa is a complex, labour-intensive and long-term breeding process that depends on the cultivation and inoculation of the fungal pathogen, on the proper environmental conditions for disease development and on large numbers of field evaluations. The development of methodology allowing early identification of resistant genotypes without artificial inoculations or waiting for natural infections will certainly be of great value. Assuming that the selection based on the marker AV14.940 was equivalent (92.2%) to the six phenotypic evaluations under field conditions, the use of this marker promises to be a successful strategy to facilitate and speed up the selection of resistant cocoa clones.

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References

- Ahnert, D., and J. L. Pires, 2000: Use of the available genetic variability of cocoa in Brazil. In: Technical Meeting: State of the Knowledge on Mass Production of Genetically Improved Propagules of Cocoa, 104–113. Ilhéus, Brazil.
- Andebrhan, T., 1984: Studies on the Epidemiology and Control of Witches' Broom Disease of Cocoa in the Brazilian Amazon, International Cocoa Research Conference Lome, Togo, 395–402.
- Andebrhan, T., A. Figueira, M. M. Yamada, J. Cascardo, and D. B. Furtak, 1999: Molecular fingerprinting suggests two primary outbreaks of witches' broom disease (*Crinipellis pernicioso*) of *Theobroma cacao* in Bahia, Brazil. *Eur. J. Plant Pathol.* **105**, 167–175.
- Bartley, B. G., 1969: Selfing on self-incompatible trees. *Ann. Rep. Cacao Res.* **1968**, 22–23.
- Basten, C. J., B. S. Weir, and Z.-B. Zeng, 1998: QTL Cartographer: a Reference Manual and Tutorial for QTL Mapping. North Carolina State University, Raleigh.
- Churchill, G. A., and R. W. Doerge, 1994: Empirical threshold values for quantitative trait mapping. *Genetics* **138**, 963–971.
- Crouzillat, D., E. Lerceteau, V. Petiard, J. Morera, H. Rodriguez, D. Walker, W. Phillips, C. Ronning, R. Schnell, J. Osei, and P. Fritz, 1996: *Theobroma cacao* L.: a genetic linkage map and quantitative trait loci analysis. *Theor. Appl. Genet.* **93**, 205–214.
- Crouzillat, D., W. Phillips, P. J. Fritz, and V. Petiard, 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.
- Doerge, R. W., and A. Rebaï, 1996: Significance thresholds for QTL interval mapping tests. *Heredity* **76**, 459–464.
- Doyle, J. J., and J. L. Doyle, 1990: Isolation of plant DNA from fresh tissue. *Focus* **12**, 13–15.
- Flament, M. H., I. Kebe, D. Citment, I. Pieretti, A. M. Risterucci, J. A. K. N'Goran, C. Cilas, D. Despreaux, and C. Lanaud, 2001: Genetic mapping of resistance factors to *Phytophthora palmivora* in cocoa. *Genome* **44**, 79–85.
- Glicenstein, L., and P. Fritz, 1989: Ploidy level in *Theobroma cacao*. *J. Hered.* **80**, 464–467.
- Jansen, R. C., and P. Stam, 1994: High resolution of quantitative traits into multiple loci via interval mapping. *Genetics* **136**, 1447–1455.
- Kosambi, D. D., 1944: The estimation of map distances from recombination values. *Ann. Eugen.* **12**, 173–175.

- Lanaud, C., A. M. Risterucci, A. K. J. N'Goran, D. Clement, M. H. Flament, V. Laurent, and M. Falque, 1995: A genetic linkage map of *Theobroma cacao* L. *Theor. Appl. Genet.* **91**, 987—993.
- Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly, and S. E. Lincoln, 1987: Mapmaker: an interactive computer package for constructing genetic linkage maps of experimental and natural populations. *Genomics* **1**, 174—181.
- Lerceteu, E., T. Robert, V. Pétiard, and D. Crouzillat, 1997: Evaluation of the extent of genetic variability among *Theobroma cacao* accessions using RAPD and RFLP markers. *Theor. Appl. Genet.* **95**, 10—19.
- Ming, R., J. L. Brewbaker, R. C. Pratt, T. A. Musket, M. D. McMullen, 1997: Molecular mapping of a major gene conferring resistance to maize mosaic virus. *Theor. Appl. Genet.* **95**, 271—275.
- Nelson, J. C., 1997: QGene: software for marker-based genomic analysis and breeding. *Mol. Breeding* **3**, 239—245.
- Pereira, T. N. S., and M. M. Yamada, 1999: Hibridação em Cacau. In: A. Borém (ed.), *Hibridação Artificial de Plantas*, 153—174. Editora UFV, Viçosa.
- Pires, J. L., W. R. Monteiro, E. D. M. N. Luz, S. D. V. M. Silva, L. R. M. Pinto, A. Figueira, K. P. Gramacho, U. V. Lopes, P. S. Beviláquia Albuquerque, M. M. Yamada, D. Ahnert, and M. I. B. Brugnerotto, 1999: Cocoa breeding for witches' broom resistance at CEPEC, Bahia, Brazil, International Workshop on the Contribution of Disease Resistance to Cocoa Variety Improvement, Salvador, Brazil, 91—101.
- 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*. *Theor. Appl. Genet.* **101**, 948—955.
- Ronning, C. M., R. J. Schnell, and D. N. Kuhn, 1995: Inheritance of random amplified polymorphic DNA (RAPD) markers in *Theobroma cacao*. *J. Amer. Soc. Hort. Sci.* **120**, 681—686.
- Spielmeyer, W., A. G. Green, D. Bittisnich, N. Mendham, and E. S. Lagudah, 1998: Identification of quantitative trait loci contributing to Fusarium wilt resistance on an AFLP linkage map of flax (*Linum usitatissimum*). *Theor. Appl. Genet.* **97**, 633—641.
- Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, and M. Zabeau, 1995: AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**, 4407—4414.
- Zadoks, J. C., 1997: Disease Resistance Testing in Cocoa, International Workshop on the Contribution of Disease Resistance to Cocoa Variety Improvement, Salvador, Brazil, 57.
- Zeng, Z.-B., 1994: Precision mapping of quantitative trait loci. *Genetics* **136**, 1457—1468.