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Inheritance study and linkage mapping of resistance loci to *Hemileia vastatrix* in Híbrido de Timor UFV 443-03

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Abstract Coffee leaf rust (CLR) caused by *Hemileia vastatrix* Berk. et Br. is one of the major *Coffea arabica* diseases worldwide. CLR resistance has been attributed to at least nine dominant genes, as single or in combination. We present an inheritance study and mapping loci involved in the Híbrido de Timor (HDT) UFV 443-03 resistance to race I, race II, and pathotype 001 of *H. vastatrix*. Molecular markers were used to ascertain the phenotypic results and to map the putative resistance loci. For all tree isolates, the inheritance study indicated that the resistance of HDT UFV 443-03 is conditioned by two independent dominant loci or by three independent loci (two dominant and one recessive). Molecular marker analyses ascertained that the resistance of HDT UFV 443-03 to race II is conditioned by at least two independent dominant loci, while the resistance to race I and pathotype 001 is conditioned by at least four independent dominant loci. Gene pyramiding might result in a cultivar with durable resistance; however, it is difficult to distinguish between plants with one or more resistance genes due to epistatic effects. Molecular markers linked to these genes were indicated for marker-assisted selection, as it is an efficient breeding alternative for CLR resistance with no such epistatic effects.

Keywords Coffee · Leaf rust · Breeding · MAS · Linkage map · QTL

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Introduction

Two out of 104 *Coffea* species are economically important: *Coffea arabica* L. (arabica coffee) and *Coffea canephora* Pierre ex A. Froehner (robusta coffee). *C. arabica* presents higher cup quality than *C. canephora*. *C. arabica* is autogamous and allotetraploid ($2n=44$), while *C. canephora* is allogamous and diploid ($2n=22$). *C. canephora* presents higher general genetic variability than *C. arabica* (Lashermes et al. 1999; de Kochko et al. 2010).

Coffee leaf rust caused by *Hemileia vastatrix* Berk. et Br. is one of the major *C. arabica* diseases worldwide. This fungus attacks leaves, causing severe yield losses. Coffee tree resistance to *H. vastatrix* has been attributed to at least nine dominant genes (S_H1 to S_H9), as single or in combination. *C. arabica* is a source of S_H1, S_H2, S_H4, and S_H5; *Coffea liberica* of S_H3; and *C. canephora* of S_H6, S_H7, S_H8, and S_H9 (Wagner and Bettencourt 1965; Noronha-Wagner and Bettencourt 1967; Bettencourt and Carvalho 1968; Bettencourt and Noronha-Wagner 1971; Bettencourt et al.

1980; Bettencourt and Rodrigues 1988). Other major and minor genes of *C. canephora* may condition coffee leaf rust (CLR) resistance (Rodrigues et al. 1975; Eskes and Da Costa 1983; Bettencourt and Rodrigues 1988; Eskes et al. 1990; Várzea and Marques 2005; Herrera et al. 2009; Romero et al. 2010, 2014).

The use of CLR cultivars is efficient, cheap, and environmentally safe. However, host resistance may be overcome by new races of *H. vastatrix*, as a consequence of high variability of this biotrophic fungus and selection pressure (Zambolim et al. 1999; Várzea and Marques 2005; Cabral et al. 2009; Maia et al. 2013). S_H1, S_H2, S_H4, and S_H5 genes of *C. arabica*, for instance, were already overcome by existing rust races (Owuor and Van der Vossen 1981; Berthaud and Charrier 1988). S_H6, S_H7, S_H8, and S_H9 identified in a Híbrido de Timor (HDT) population, derived from a *C. arabica × C. canephora* spontaneous cross (Bettencourt et al. 1980, 1992), have been successfully used to breed *C. arabica* for *H. vastatrix* resistance. Many introductions of HDT selections were made in Brazil, by vegetative propagation or by means of samples of seeds collected in coffee trees selected in the Centro de Investigações das Ferrugens do Cafeeiro (CIFC), Portugal, and experimental stations of Angola (e.g., UFV 443-03 introduced by seeds from Estação Regional de Uige (ERU), Angola). This germplasm contain different numbers of rust-resistant genes that are not characterized yet. Despite the importance of these germplasms, the cultivars derived from HDT are gradually losing the resistance to rust in Brazil and other countries, due to the development of new virulent races (Várzea and Marques 2005).

Gene stacking or pyramiding might result in cultivars with a more durable resistance compared to cultivars with single race-specific resistance gene. In conventional breeding, a segregating coffee population inoculated with physiologic race of *H. vastatrix* can be easily screened for resistance. However, it is difficult to distinguish between plants with one or more resistance genes due to epistatic effects. Since molecular markers show no such epistatic effects, marker-assisted selection (MAS) can be an efficient alternative. MAS requires no phenotyping but requires previous development of molecular markers linked to each gene under selection (Caixeta et al. 2009).

Saturated genetic linkage maps of *C. arabica* should be useful for mapping resistance genes and to identify markers linked to those genes. However, they are not yet available due to low molecular polymorphism and the polyploidy nature of this species. Despite these constraints, few studies have attempted to map molecular markers linked to *H. vastatrix* resistance genes. In a F_2 population, introgression analysis of S_H3 from *C. liberica* into *C. arabica* resulted in 21 amplified fragment length polymorphism (AFLP)-linked markers (Prakash et al. 2004). Additional analysis resulted in two sequence-characterized genetic markers that co-segregated perfectly with S_H3, thus suitable for MAS and gene pyramiding (Mahé et al. 2008). A resistance gene of HDT UFV 427-15 to race II of *H. vastatrix* was mapped with two AFLP markers (Brito et al. 2010). Subsequently, the same gene was mapped with six sequence-characterized amplified region (SCAR) markers delimiting a chromosome region of 9.4 cM and flanking the resistance gene by 0.7 and 0.9 cM (Diola et al. 2011). The analysis of a F_2 population resulted in five AFLP and two single sequence repeat (SSR) markers associated with partial resistance of a HDT-derived line (Herrera et al. 2009). Additional analysis of the same population resulted in a quantitative trait locus (QTL) mapped between two SSR markers within the interval of 2.5 cM (Romero et al. 2014).

We now present an inheritance study of HDT UFV 443-03 resistance to races I and II and to pathotype 001 described below of *H. vastatrix*. Molecular markers were used to ascertain the phenotypic results and to map the putative resistance loci.

Material and methods

Plant material

Three segregating populations derived from crossing Catuaí Amarelo IAC 64 (UFV 2148-57) (susceptible parent) and Híbrido de Timor UFV 443-03 (resistant parent) were used in this study. The HDT UFV 443-03 is an important rust-resistant source used in breeding programs, although their genes have not yet been characterized. UFV 443-03 was introduced in Brazil by seeds from U209/7 plant of ERU, Angola, which was derived by seeds from CIFC 2570 plant of CIFC, Portugal. The F_1 plant, designated H-511-1, was self-crossed under controlled conditions to obtain the segregating F_2 population (247 individuals). In addition, the F_1 plant was backcrossed with HDT UFV 443-03 and with Catuaí Amarelo IAC 64, generating 87 backcrossed plants with the resistant parent (BCr) and 115 backcrossed plants with the susceptible parent (BCs).

Phenotypic data

Races I and II and pathotype 001 of *H. vastatrix*, which show contrasting reactions between Catuaí Amarelo IAC 64 (susceptible) and HDT UFV 443-03 (resistant), were used in this study. Races I and II were collected from commercial fields of Catuaí Vermelho and the pathotype 001 from Oeiras cultivar. Races I and II were characterized in a series of differentiating coffee clones, as described by Capucho et al. (2012). The pathotype 001 breaks down the resistance of Oeiras (HDT CIFC 832/1 × Caturra CIFC 19/1) released as a rust-resistant cultivar. It also breaks down the resistance of Icatu Vermelho (*C. arabica × C. canephora*). Pathotype 001

was characterized in a series of differentiating coffee clones as race II. Since all known isolates of race II do not infect Oeiras and Icatu Vermelho, pathotype 001 should carry additional virulence genes not detected by the differentiating clones (Capucho et al. 2009).

Uredospores of the fungus were multiplied and used as an inoculum according to methodology described by Capucho et al. (2009). Parents, F_1, and all 449 segregating plants (247 F_2, 87 BCr, and 115 BCs) were inoculated using a leaf disc method described by Eskes (1982), with three replications.

The symptom evaluation (phenotyping) was performed according to Capucho et al. (2009), using a scale (scores 1 to 6) described by Tamayo et al. (1995), based on the absence or presence of uredospores. The phenotypes were scored as follows: no symptoms (score 1), small chlorotic injuries (score 2), large chlorotic injuries without sporulation (score 3), large chlorotic injuries with small sporulation occupying less than 25 % of the area (score 4), injuries with sporulation occupying from 25 to 50 % of the area (score 5), and injuries with sporulation occupying more than 50 % of the area (score 6). The maximum score of three replications was used to represent the symptom of each plant.

Inheritance study

For inheritance study, resistant plants were scored 1 to 3 and susceptible plants were scored 4 to 6, according to the scale described by Tamayo et al. (1995).

Phenotypic segregation ratios of F_2 and BCs populations were chi-square (χ^2) tested, using GENES software (Cruz 2013). Estimated P value >0.05 does not reject the hypothesis. Expected F_2 ratios (resistant/susceptible) were tested for one locus (3:1 and 1:3), two loci (15:1, 13:3, 7:9, 9:7, 3:13, and 1:15), and three loci (63:1, 61:3, 55:9, 37:27, 27:37, 9:55, 3:61, 1:63, 57:7, 51:13, 49:15, 43:21, 25:39, and 19:45). Expected BCs ratios were tested for one locus (1:1) and two or three loci (3:1).

Molecular marker study

A linkage map constructed with molecular markers allowed us to use the QTL mapping technique to ascertain the previous inheritance study results and to map putative resistance loci.

DNA extraction

DNA extraction of parents (Catuaí Amarelo IAC 64 and HDT UFV 443-03), F_1 (H 511-1), and 247 F_2 plants was performed according to Diniz et al. (2005). DNA was quality controlled on agarose gel (1 %) and quantified with Thermo Scientific NanoDrop 2000 Spectrophotometer. Final DNA concentration was adjusted to 25 ng/μL and freezer stored at −20 °C.

SSR marker data

A preliminary polymorphism survey of SSR markers was performed with DNA samples over the parents and 10 F_2 plants using 373 coffee SSR primers (Combes et al. 2000; Rovelli et al. 2000; Baruah et al. 2003; Coulibaly et al. 2003; Moncada and McCouch 2004; Poncet et al. 2004; Bhat et al. 2005; Hendre et al. 2008; Missio et al. 2009b, 2011; Ferrão et al. 2015). Polymorphic SSR markers were then selected and surveyed with the entire F_2 population. Polymerase chain reaction (PCR) was performed according to Missio et al. (2009a), and SSR polymorphisms were observed in 6 % polyacrylamide denaturing gel stained with silver nitrate, as described by Brito et al. (2010). The polymorphisms were alternatively observed by DNA sequencing with an ABI 3130xl Genetic Analyzer (Applied Biosystems). Each SSR locus was first amplified by PCR, using a forward primer labeled with a specific fluorophore [6-FAM (blue) or NED (yellow) or HEX (green)]. PCR reactions with each primer pair were performed separately, and the reactions of the three specific fluorophores were mixed, characterizing a pseudo-multiplex. Each PCR product solution was diluted at a 1:10 ratio, and 1 μL of each diluted reaction was transferred to a special sequencing plate containing 6.8 μL of formamide HI-DI (Applied Biosystems) and 0.2 μL of Rox 500 (standard molecular weight labeled with RED fluorophore). The DNA was denatured in a thermocycler at 95 °C for 3 min. The samples were electro-injected into the sequencer, into a 50-cm-length silica capillary with 50 μm in diameter filled with POP 7 polymer, following the manufacturer's recommendation. The DNA fragments were analyzed using GeneMapper V4.1 software (Applied Biosystems).

AFLP marker data

AFLP markers were generated as described by Brito et al. (2010), with modifications. DNA samples of parents (HDT UFV 443-03 and Catuaí Amarelo IAC 64) and F_2 individuals were digested with *Eco*RI and *Mse*I restriction enzymes for 8 h at 37 °C. DNA fragments were linked to specific adaptors, using *T4 DNA* ligase for 14 h at 4 °C. The solution was diluted 1:10 in TE (pH 8.0) and stored at −20 °C. Pre-selective PCR amplification was performed with primers complementary to the adaptors that contained an additional nucleotide (N) at the 3′ terminus. The pre-amplified product was diluted 1:40 in TE (pH 8.0) and stored at −20 °C. Seventeen combinations of selective primers resulting from *Eco*RI and *Mse*I were used for PCR amplification: E-CAG/M-TGC, E-CAT/M-AGT, E-CCT/M-ATA, E-CCC/M-AGC, E-CCC/M-AGA, E-CGA/M-ACA, E-CGG/M-ACG, E-CGT/M-TCT, E-CGA/M-TCC, E-CGC/M-ATA, E-CGT/M-ATC, E-CTG/M-AAT, E-CTG/M-TAC, E-CTT/M-TGC, E-CTC/M-AGT, E-CTC/M-AAG, and E-CTT/MTCG. Eight microliters of formamide (98 %

formamide, 10 mM EDTA (pH 8.0), 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue) was added to the amplified product solutions. AFLP markers were observed in denaturing polyacrylamide gels (6 %), stained with silver nitrate.

Random amplified polymorphic DNA marker data

Forty-six primers from Operon Technologies were used to generate random amplified polymorphic DNA (RAPD) markers by PCR, as described by Ferrão et al. (2013). PCR was performed with a 25 μL solution containing 25 ng genomic DNA, 1 U *Taq* DNA polymerase, 0.1 mM of each dNTP, 0.2 μM of primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl$_2$, and Milli-Q sterile water. RAPD markers were observed under UV light in 1.2 % agarose gels stained with ethidium bromide.

CARF 005 marker

CARF 005 marker was used as describes by Alvarenga et al. (2011). This marker was developed from mined sequences of the Brazilian Coffee Genome Project to differentiate the presence/absence of a band between coffee plants that were resistant or susceptible to *H. vastatrix*. It amplifies a sequence that corresponds to a resistance gene fragment with annotation gi|24459841|emb|CAC82597.1| "disease resistance-like protein" of *C. arabica*.

Linkage map

The linkage map was constructed with 247 F$_2$ plants using SSR, AFLP, RAPD, and CARF 005 polymorphic markers.

Marker data were encoded and analyzed using GQMOL software (Cruz 2008). The line graphs of linkage groups were constructed using MapChart software (Voorrips 2002). Mendelian segregation (3:1) for each marker was evaluated using a chi-square test (χ^2) ($P=0.05$, linkage group (LG) 1) using Bonferroni correction. SSRs were scored as dominant markers. Markers with segregation distortion were included in the map if they did not change the linkage order of markers without distortion.

Linkage groups were formed with a minimum logarithm of likelihood ratio (LOD score) of 3.0 and maximum recombination of 30 %. Genetic distance in centiMorgans (cM) was calculated based on estimated recombination frequencies and a Morgan map function.

Mendelian factor location

The QTL mapping technique was originally designed for mapping Mendelian factors underlying quantitative traits (Lander and Botstein 1989). The same technique was used for mapping Mendelian factors underlying a qualitative trait,

CLR resistance, which allowed us to ascertain the previous inheritance study of this trait and to develop molecular markers linked to the putative loci. We assumed that QTLs corresponded to large effect loci.

For QTL mapping, plants were scored 1 to 6 according to the scale described by Tamayo et al. (1995). The statistical analyses were performed as described by Lander and Botstein (1989), using GQMOL software (Cruz 2008). Simple interval method was used with regression analysis. A QTL determination coefficient (R^2) corresponded to the peak of large QTL significance, adopting a LOD score value higher than 3.0. R^2 expressed the percentage of phenotypic variation explained using a QTL.

To ascertain the previous inheritance study results, the following data were analyzed for each QTL: additive effect (*a*), dominance effect (*d*), average degree of dominance (*k*), allelic interaction, average of disease severity, and mean severity reduction.

Average degree of dominance (*k*) was estimated according to the formula $k=|d|/|a|$, indicating the following allelic interaction possibilities for a marker associated with QTL: absence of dominance (AD) if $k=0$, partial dominance (PD) if $0<k<1.0$, complete dominance (CD) if $k=1.0$, and overdominance (OD) if $k>1.0$.

Average disease severity and mean severity reduction were calculated to estimate the effect of each QTL in the resistance. The average disease severity (based on scores 1 to 6 of leaf rust symptoms) was calculated for a group of plants presenting a marker linked to a QTL and compared with the average disease severity of a group of plants without the same marker. The comparison method of simple marker was performed using F test ($P=0.05$), with GQMOL software (Cruz 2008). Mean severity reduction associated with the presence of a marker linked to a QTL was calculated in percentage.

Results

Inheritance study

HDT UFV 443-03, F$_1$, and BCr plants were all resistant (score 1) to races I and II and to pathotype 001 of *H. vastatrix*, while Catuaí Amarelo IAC 64 was susceptible (score 6). The graphic distribution of F$_2$ plants according to the symptom (scores 1, 2, 3, 4, 5, or 6) showed the predominance of resistant plants (score 1, 2, and 3) in the population (Fig. 1). These results indicated that the resistance of HDT UFV 443-03 to all three pathogen isolates presents dominance effect.

F$_2$ and BCs populations showed phenotypic segregation for resistance (scores 1 to 3) and susceptibility (scores 4 to 6) to all three isolates of *H. vastatrix* (Table 1). F$_2$ population inoculated with race I of the pathogen presented 226 resistant
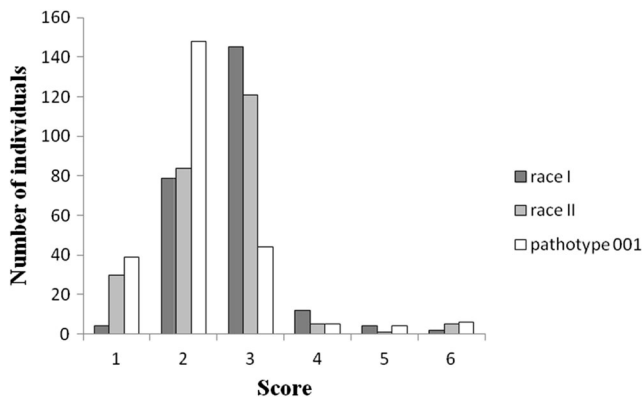
Fig. 1 Frequency of F_2 coffee plants, following the scale of Tamayo et al. (1995). Resistant individuals (1–3) and susceptible (4–6) are shown. 1 absence of symptoms, 2 small chlorotic injuries, 3 large chlorotic injuries without sporulation, 4 large chlorotic injuries with few uredospores occupying less than 25 % of the injury area, 5 injuries with sporulation occupying from 25 to 50 % of the area, 6 injuries with sporulation occupying more than 50 % of the area with uredospores

and 17 susceptible plants, and BCs population presented 84 resistant and 30 susceptible plants. With race II inoculation, F_2 population presented 235 resistant and 11 susceptible plants and BCs population presented 80 resistant and 35 susceptible plants. With pathotype 001 inoculation, F_2 population presented 231 resistant and 15 susceptible plants and BCs population presented 85 resistant and 30 susceptible plants. For all the three isolates of *H. vastatrix*, the following expected phenotypic segregation ratios were confirmed using χ^2 test ($P > 0.05$): 15:1 (two dominant independent loci) and 61:3 (two dominant loci and one recessive independent locus) for F_2 populations and 3:1 (two dominant independent loci or two dominant loci and one recessive independent locus) for BCs populations.

These results indicated, for all the three isolates, two inheritance possibilities: (1) the resistance of HDT UFV 443-03 is conditioned by at least two independent dominant loci, or (2)

Table 1 Expected and observed ratios for two and three loci responsible for resistance, in progenitors, F_1 and F_2 population, resistant backcrossing (BCr), and susceptible backcrossing (BCs)

Population	Expected ratio		Observed number		Expected number		χ^2	Probability (%)
	R	S	R	S	R	S		
Race I								
UFV 443-03	1	0	1	0	1	0	–	–
UFV 2148-57	0	1	0	1	0	1	–	–
F_1	1	0	1	0	1	0	–	–
BCr[a]	1	0	87	0	87	0	–	–
F_2	15[b]	1	226	17	227.8	15.2	0.23	63.1
F_2	61[c]	3	226	17	231.6	11.4	2.90	8.9
BCs[d]	3[e]	1	84	30	85.5	28.5	0.10	74.6
Race II								
UFV 443-03	1	0	1	0	1	0	–	–
UFV 2148-57	0	1	0	1	0	1	–	–
F_1	1	0	1	0	1	0	–	–
BCr[a]	1	0	87	0	87	0	–	–
F_2	15[b]	1	235	11	230.6	15.4	1.33	24.9
F_2	61[c]	3	235	11	234.5	11.5	0.02	87.3
BCs[d]	3[e]	1	80	35	86.25	28.75	1.81	17.8
Pathotype 001								
UFV 443-03	1	0	1	0	1	0	–	–
UFV 2148-57	0	1	0	1	0	1	–	–
F_1	1	0	1	0	1	0	–	–
BCr[a]	1	0	87	0	87	0	–	–
F_2	15[b]	1	231	15	230.6	15.4	0.009	92.1
F_2	61[c]	3	231	15	234.5	11.5	1.09	29.5
BCs[d]	3[e]	1	85	30	86.25	28.75	0.07	78.8

R resistant plants, S susceptible plants, χ^2 chi-square

[a] Resistant backcrossing

[b] Expected ratio for two loci (15:1, in generation F_2)

[c] Expected ratio for three loci (61:3, in generation F_2)

[d] Susceptible backcrossing

[e] Expected ratio for two and three loci in susceptible backcrossing

the resistance is conditioned by at least three independent loci (two dominant loci and one recessive locus).

Molecular marker study

SSR, AFLP, RAPD, and CARF 005 data

SSR markers were tested for polymorphism between Catuaí Amarelo IAC 64 (susceptible parent) and HDT UFV 443-03 (resistant parent) with 373 pairs of primers. There were 58 (15.5 %) polymorphic and 271 monomorphic SSR markers (72.7 %), whereas 44 (11.8 %) failed to amplify. Those polymorphic SSR markers were tested with F_2 population.

Seventeen combinations of selective AFLP primers generated 74 polymorphic markers when tested with parents and F_2 population. AFLP markers presented an average of 4.3 polymorphic bands per pair of primers.

Forty-six RAPD primers generated four polymorphic markers when tested with parents and F_2 population, with one polymorphic band/primer.

CARF 005 marker was polymorphic for parents and F_2 population.
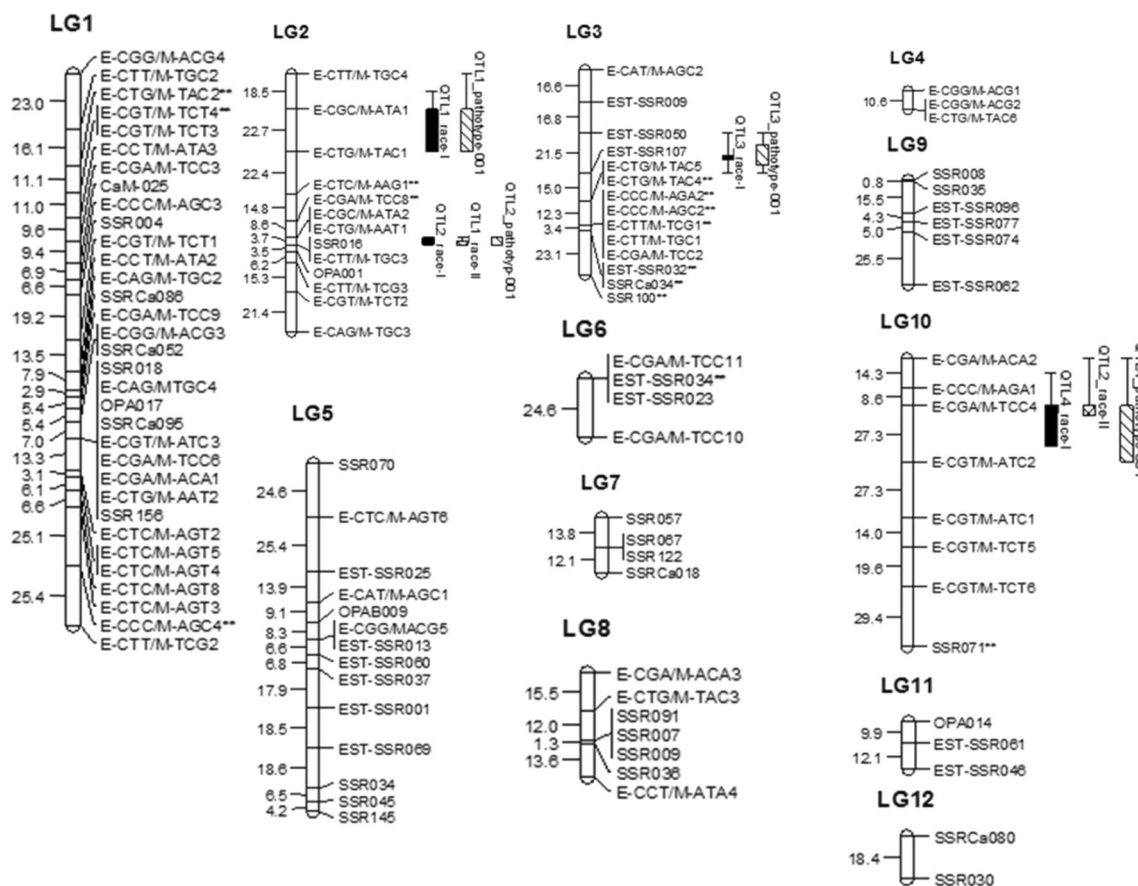
Linkage map

A set of 137 polymorphic markers (58 SSR, 74 AFLP, 4 RAPD, and 1 CARF 005) was used to assay a segregant population of 247 F_2 plants. Twenty-one markers (12 AFLP, 8 SSR, and 1 CARF 005) presented segregation ratio distortion (15.3 % distortion).

A linkage map was constructed with 111 markers distributed in 12 LGs (Fig. 2, Table 2), while 26 markers (12 AFLP, 13 SSR, and 1 CARF 005) did not link to any of the groups. The SSR markers mapped are presented in Table 3.

The map encompassed 976.8 cM of the genome. Linkage group size ranged from 18.4 cM (LG 12) to 234.6 cM (LG 1). The distance between two adjacent markers (interval) varied from 0 cM (i.e., two completely linked markers) to 29.4 cM, with the average distance of 9.9 cM. Most intervals (75.7 %) did not exceed 20 cM. The mean distance between markers within each linkage group varied from 5.3 cM (LG 5) to 20.1 cM (LG 10).

Mendelian factor location

As described in the previous section, the inheritance study indicated that resistance of HDT UFV 443-03 is conditioned

Fig. 2 Linkage map for *C. arabica* containing 111 markers. *On the right of bar* are names of molecular markers and *on the left* distances in centiMorgans. *Bars beside marker name* indicate QTL positions for race I, race II, and pathotype 001

Table 2 Summary of linkage map for the F$_2$ population of *C. arabica*

LG	Length (cM)	Number[a]	AFLP	SSR	RAPD	RGA	Mean distance[b] (cM)	Minimum and maximum distance[c] (cM)
1	234.6	33	25	7	1	–	7.3	0.0–25.4
2	137.1	13	11	1	1	–	11.4	0.0–22.7
3	108.7	14	8	6	–	–	8.4	0.0–23.1
4	10.6	3	3	–	–	–	5.3	0.0–10.6
5	160.4	14	3	10	1	–	13.4	0.0–25.4
6	24.6	4	2	2	–	–	8.2	0.0–24.6
7	25.9	4	–	4	–	–	8.6	0.0–13.8
8	42.4	7	3	4	–	–	7.1	0.0–15.5
9	51.1	6	–	6	–	–	10.2	0.8–25.6
10	140.5	8	7	1	–	–	20.1	8.6–29.4
11	22.0	3	–	2	1	–	11.0	9.9–12.1
12	18.4	2	–	2	–	–	18.4	18.4
General	976.8	111	62	45	4	–	9.9	0.0–29.4
NL[d]		26	12	13	–	1		
Distor.[e]		21	12	8	–	1		

LG linkage group, *cM* unit in centiMorgans

[a] Number of markers

[b] Mean distance between markers

[c] Minimum and maximum distance between markers

[d] Not linked markers

[e] Distorted markers

by at least two independent dominant loci or by at least three independent loci (two dominant loci and one recessive locus). Molecular data below were used to ascertain these results.

Four independent QTLs were associated with CLR resistance to race I and pathotype 001. Two independent QTLs were associated with CLR resistance to race II (Fig. 2, Table 4).

For every QTL, the average disease severity of a group of plants presenting a marker linked to the QTL was lower than that of the group without the same marker. Every marker linked to a QTL was a HDT UFV 443-03 (resistant parent)-derived marker. The mean severity reduction associated with the presence of a marker linked to a QTL ranged from 4.1 to 31.0 % (Table 4).

All allelic interactions (Table 4) showed dominance effect (CD, PD, or OD), confirming the inheritance study of earlier section, which indicated that the resistance is conditioned by dominant genes. No allelic interaction showing absence of dominance (AD) was observed, indicating no recessive gene action evidence conditioning the resistance. Consequently, the second hypothesis of inheritance study was rejected.

Therefore, the molecular marker analyses ascertained that the resistance of HDT UFV 443-03 to race II of *H. vastatrix* is conditioned by two independent dominant loci (mapped as two independent QTLs). However, the molecular analyses also indicated that the resistance to race I and pathotype 001 is conditioned by four independent dominant loci (mapped as four independent QTLs), while the inheritance study indicated that the resistance is conditioned by two independent dominant loci. These results are discussed in the next section.

Four independent QTLs were associated with CLR resistance to race I (Fig. 2, Table 4): QTL1_raça-1 mapped in LG 2, at 22.7 cM from marker E-CGC/M-ATA 1, explaining 9.1 % of the phenotypic variation; QTL2_raça-I mapped in LG 2, at 3.0 cM from marker E-CTG/M-AAT 1, explaining 8.9 % of the phenotypic variation; QTL3_raça-I mapped in LG 3, at 13.0 cM from marker EST-SSR 050, explaining 5.7 % of phenotypic variation; and QTL4_raça-I mapped in LG 10, at 12.0 cM from E-CGA/M-TCC 4, explaining 7.8 % of phenotypic variation.

Two independent QTLs were associated with CLR resistance to race II (Fig. 2, Table 4): QTL1_raça-II mapped in LG 2, at 3.7 cM from marker E-CTG/M-AAT 1, explaining 5.8 % of the phenotypic variation, and QTL2_raça-II mapped in LG 10, at 8.0 cM from marker E-CCC/M-AGA1, explaining 8.1 % of the phenotypic variation.

Four independent QTLs were associated with CLR resistance to pathotype 001 (Fig. 2, Table 4): QTL1_pathotype-001 mapped in LG 2, at 17.0 cM from marker E-CGC/M-ATA 1, explaining 13.4 % of the phenotypic variation; QTL2_pathotype-001 mapped in LG 2, at 0 cM from marker

Table 3 List of mapped SSR primers and their melting temperature (T_m) and amplicon size in base pairs (bp)

Primer	Forward primer	Reverse primer	Estimated fragment size (pb)	Linkage group mapped	Reference
EST-SSR 001	GAAGACCAAGCACCCTCAAC	ACACCAACTACGGGCAGACA	151	LG 5	Ferrão et al. (2015)
EST-SSR 009	TCCGAGTCACATCCCATAAA	GGAGGAAAGTGAAGGAAGAAGA	160	LG 3	Ferrão et al. (2015)
EST-SSR 013	GCCTTGCTCATATCTGCTGTCT	GATCCTTCAACTGAGCCAAA	141	LG 5	Ferrão et al. (2015)
EST-SSR 023	TGTCAGTCGTCGGTCAAATC	GGTTGGACTCGCTTCTTCTTCT	218	LG 6	Ferrão et al. (2015)
EST-SSR 025	TCCGTTCCGGGCTTATGAT	AAACAGACGCAGATCCCAGA	224	LG 5	Ferrão et al. (2015)
EST-SSR 032	AGTCCTTGGCACTTGCTTT	CAGACAACGATCAATACCTTCC	200	LG 3	Ferrão et al. (2015)
EST-SSR 034	GATACTTCCATGAGGATCGTTG	GATCATACAATGTGGGAAGG	139	LG 6	Ferrão et al. (2015)
EST-SSR 037	CCTTTGAGGTGGTCAGCCTA	CCCTGATGACCTAGAGAACC	212	LG 5	Ferrão et al. (2015)
EST-SSR 046	CCCTCCCTCCTACTTGTCCTAA	ATCCGGCATCATCATCAGAG	234	LG 11	Ferrão et al. (2015)
EST-SSR 050	AAGGAGGAAGAGCGACCAAA	GCGTCAATGTTGGAAGGAAA	162	LG 3	Ferrão et al. (2015)
EST-SSR 060	TCCTCCTCGTGCTTCTCAAC	GGCAGCATTCTCCTGATCCT	126	LG 5	Ferrão et al. (2015)
EST-SSR 061	GAGCATGAGACACTCTCTGGAA	CATTGGGTGTTGATGGAGGA	142	LG 11	Ferrão et al. (2015)
EST-SSR 062	CACTTAGTTCCAAAGTGCTACC	TTCCTCAACTTCCCAGGAGAT	129	LG 9	Ferrão et al. (2015)
EST-SSR 069	TTGTGTTCTTTCTCCACCTC	CAGGAGTCGTATAACGCTGAA	132	LG 5	Ferrão et al. (2015)
EST-SSR 074	ACAAGCCAAGGCGACTGAA	CCGTAGGGAATAGAGGAGCA	140	LG 9	Ferrão et al. (2015)
EST-SSR 077	GCACCTGGAGGGAGACAAAT	CGCACTACCAAAAGCAGGAA	216	LG 9	Ferrão et al. (2015)
EST-SSR 096	AGACAGCTTTGGTGGTCCTG	TGAATGTGTGGCCCTTTAGC	223	LG 9	Ferrão et al. (2015)
EST-SSR 107	AACAAGAGTTTCTGCCTGTG	TCTCTCGAAGTAGATTGCTCTG	159	LG 3	Ferrão et al. (2015)
SSRCa 018	GTCTCGTTTCACGCTCTCTC	ATTTTTGGCACGGTATGTTC	115	LG 7	Missio et al. (2009a, b)
SSRCa 034	TGGACAAGAAATTGAAGTGG	GGGTTTAAATTATCGGGTGT	257	LG 3	Missio et al. (2009a, b)
SSRCa 052	GATGGAAACCCAGAAAGTTG	TAGAAGGGCTTTGACTGGAC	129	LG 1	Missio et al. (2009a, b)
SSRCa 080	GTTCTTTCCGCCGTCAAT	GAGAAGAGAGAGGAAGGGAAA	250	LG 12	Missio et al. (2009a, b)
SSRCa 086	AGAGAGAAGCCATGATTTGA	TCAGTCCCAGAGAATAAGGA	105	LG 1	Missio et al. (2009a, b)
SSRCa 095	GAGAGAGCCGAGTGAAGAGA	GAGAGAGAAGCCATGATTTGA	185	LG 1	Missio et al. (2009a, b)
SSR 004	AGGCCTTCATCTCAAAAACC	AGCGTTACTTGAGGCAAAGA	217	LG 1	Rovelli et al. (2000)
SSR 007	TGACATAGGGGGCTAAATTG	TTAATGGTGACGCTTTGATG	215	LG 8	Rovelli et al. (2000)
SSR 008	CACTGGCATTAGAAAGCACC	GGCAAAGTCAATGATGACTC	198	LG 9	Rovelli et al. (2000)
SSR 009	ATGCCAAGTCGGAAAAGAA	GGCAAGCTCTAGCCTTTGA	136	LG 8	Rovelli et al. (2000)
SSR 016	ACCCGAAAGAAAGAACCAAG	CCACACAACTCTCCTCATTC	140–146	LG 2	Combes et al. (2000)
SSR 018	GGCTCGAGATATCTGTTTAG	TTTAATGGGCATAGGGTCC	132–166	LG 1	Combes et al. (2000)
SSR 030	ATGGGGCCAACTTGAATATG	CAGGGCATCTATCTACTTCTCTTT	220	LG 12	Poncet et al. (2004)
SSR 034	GGAGACGCAGGTGGTAGAAG	TCGAGAAGTCTTGGGGTGTT	294	LG 5	Poncet et al. (2004)
SSR 035	CTGGCATTAGAAAGCACCTTG	GCTTGGCTCACTGTAGGACTG	158	LG 9	Poncet et al. (2004)
SSR 036	ATGCCAAGTCGGAAAAGAA	CACTAGCATAATTGCGTGGA	157	LG 8	Poncet et al. (2004)
SSR 045	CTGAGCGCATGGAAGGAGTA	GGAGACGCAGGTGGTAGAAG	194	LG 5	Poncet et al. (2004)
SSR 057	CTCGCTTTCACGCTCTCTCT	CGGTATGTTCCTCGTTCCTC	102	LG 7	Coulibaly et al. (2003)
SSR 067	CGTCTCGTTTCACGCTCTCT	GATCTGCATGTACTGGTGCTTC	237	LG 7	Poncet et al. (2004)
SSR 070	GTAACCACCACCTCCTCTGC	TGGAGGTAACGGAAGCTCTG	167–230	LG 5	Baruah et al. (2003)
SSR 071	GCTAAGTTCAATTGCCCCTGT	GGGTTAATTTGATTGCGTGA	210–232	LG 10	Baruah et al. (2003)
SSR 091	TTGCTTCGTTTTAAATGTGAGG	AATTGTCTATAAAAAAGAGAGAGA	135	LG 8	Moncada and McCouch (2004)
SSR 100	ACCCTTTACTACTTATTTACTCTC	ACATCCCCTTGCCATTTCTTC	157	LG 3	Moncada and McCouch (2004)
SSR 145	CTGCGAGGAGGAGTTAAAGATACCAC	GCCGGGAGTCTAGGGTTCTGTG	138–150	LG 5	Bhat et al. (2005)
SSR 156	CGGCTCAAAGGTGCTCAAT	TCCCAATTCTTTCTTTCTTTCTGT	135	LG 1	Moncada and McCouch (2004)
CaM-25	TCCATCTTCCTTCATTTCTGCTGCTAA	CCTTCACCCCCTTTGCACTTCCTTA	186	LG 1	Hendre et al. (2008)

E-CTG/M-AAT 1, explaining 16.2 % of the phenotypic variation; QTL3_pathotype-001 mapped in LG 3, at 12.0 cM from marker EST-SSR 050, explaining 7.5 % of phenotypic variation; and QTL4_pathotype-001 mapped in LG 10, at 0 cM from E-CGA/M-TCC 4, explaining 13.8 % of phenotypic variation.

Discussion

Inheritance study

Although the inheritance study was performed by phenotyping plants in an appropriate genetic design (contrasting parents, F₁,

Table 4 Molecular markers associated with QTLs for coffee plant resistance to rust, identified by simple interval and regression analysis (LOD>3)

	LG	Marker/QTL[a]	bp[b]	Pos. (cM)	LOD[c]	R^{2d} (%)	a^e	d^f	k^g	Allelic interaction	Average of disease severity		Mean severity reduction (%)
											A_1-	A_2A_2	
Race I													
QTL 1	2	E-CGC/M-ATA 1	145	22.7	5.516	9.1	−0.49	−0.51	1.03	CD	2.7828	3.2500	14.4
QTL 2	2	E-CTG/M-AAT 1	670	3.0	4.941	8.9	0.39	−1.57	−4.11	OD	2.7850	3.2955	15.5
QTL 3	3	EST-SSR 050	129	13.0	3.127	5.7	0.45	−1.87	−4.19	OD	2.8529	2.9762	4.2
QTL 4	10	E-CGA/M-TCC 4	800	12.0	4.345	7.8	−0.11	−0.75	6.98	OD	2.7717	3.1774	12.8
Race II													
QTL 1	2	E-CTG/M-AAT 1	670	3.7	3.147	5.8	−0.21	−0.42	2.00	OD	2.4500	2.8864	15.1
QTL 2	10	E-CCC/M-AGA 1	220	8.0	4.505	8.1	−0.96	1.16	−1.21	OD	2.3846	3.0169	21.0
Pathotype 001													
QTL 1	2	E-CGC/M-ATA 1	145	17.0	8.071	13.4	−0.71	−0.56	0.79	PD	2.0859	2.8333	26.4
QTL 2	2	E-CTG/M-AAT 1	670	0.0	9.968	16.2	−0.90	0.29	−0.33	PD	2.0700	3.0000	31.0
QTL 3	3	EST-SSR 050	129	12.0	4.229	7.5	0.40	−2.38	−3.46	OD	2.1520	2.6190	17.8
QTL 4	10	E-CGA/M-TCC 4	800	0.0	8.351	13.8	−0.80	0.39	−0.48	PD	2.0054	2.9032	30.9

LG linkage group, *CD* complete dominance, *AD* absence of dominance, *PD* partial dominance, *OD* overdominance

[a] Identified markers associated whit QTLs of races I and II and pathotype 001

[b] Base pairs of molecular marker

[c] Base logarithm 10

[d] Coefficient of determination, which corresponds to the percentage of phenotypic variation explained by QTL

[e] Additive effect corresponding to homozygote genotypes, which assumes values −1 and 1 for qq and QQ, respectively

[f] Dominance effect corresponding to heterozygote genotype, Qq

[g] Average degree of dominance, indicating incidence of different types of allelic interactions

F_2, BCr, and BCs), it was not possible to conclude whether resistance of HDT UFV 443-03 to race I, race II, and pathotype 001 of *H. vastatrix* is controlled by two independent dominant loci or three independent dominant loci (two dominant and one recessive). The population sizes did not allow us to test (χ^2 test) if the resistance is controlled by four loci. For these reasons, QTL mapping was useful to ascertain the inheritance study.

Previous inheritance studies of HDT resistance to *H. vastatrix* reported that it was conditioned by major dominant genes. Pereira (1995), working with seven accessions of HDT and races II and XXV of *H. vastatrix*, reported that the resistance was conditioned by three independent dominant genes, in six accessions, and in one accession, the resistance was conditioned by one dominant gene. Brito et al. (2010) reported one dominant gene conditioning the resistance of HDT UFV427-15 to race II. Romero et al. (2014) reported one QTL region corresponding to a new and genetically independent S_H gene of DI200, a line derived from Caturra×HDT CIFC1343, using a mix of uredospores of *H. vastatrix* field collected from Caturra cultivar.

Linkage map

SSR markers are widely used for constructing linkage maps in many species, because they are highly polymorphic, locus specific, and useful as anchors for map integration, and require a small DNA quantity for PCR (Chistiakov et al. 2006; Ball et al. 2010). SSRs can also be used as fluorescent in situ hybridization (FISH) probes for physical mapping. However, construction of a *C. arabica* genetic linkage map was hampered by low polymorphism level of SSR markers. Only 58 out of 373 SSR markers were polymorphic. Such low polymorphism in *C. arabica* was also reported by others, indicating the narrow genetic base of the species. Vieira et al. (2010) reported that 22 out of 127 SSR markers were polymorphic. Herrera et al. (2009) reported 3 out of 13 markers, and Geleta et al. (2012) reported 8 out of 12 markers.

Low polymorphism of SSR markers in *C. arabica* leads us to add other types of molecular markers to construct a map. Seventeen combinations of selective AFLP primers generated 74 polymorphic markers with an average of 4.3 polymorphic bands per pair of primers. Such low polymorphism was also reported by Ky et al. (2000), Pearl et al. (2004), and Brito et al. (2010), who identified 1.1, 6.5, and 1.4 polymorphic bands per primer, respectively. Forty-six RAPD primers generated only four polymorphic markers, with one polymorphic band/primer. Such low polymorphism was also reported by Teixeira-Cabral et al. (2004) and Oliveira et al. (2007), who identified 1.2 and 1.3 polymorphic bands per primer,

respectively. Despite having low polymorphism, these markers, especially the multilocus AFLP markers, helped to construct a map encompassing 976.8 cM of the genome.

We observed 21 markers (12 AFLP, 8 SSR, and 1 CARF 005) with segregation ratio distortion (15.3 % distortion). The segregation ratio distortion may vary among species, types of markers, and types of populations. Constructing RAPD-based map for a HDT-derived population (backcross with *C. arabica* recurrent parent), Oliveira et al. (2007) and Teixeira-Cabral et al. (2004) reported 24.5 and 5.5 % distortion, respectively. Paillard et al. (1996) reported 20 % distortion of RAPD marker in *C. canephora*. With AFLP markers and different *Coffea* species, Ky et al. (2000) reported approximately 30 % distortion. Pearl et al. (2004) reported 25 % distortion of AFLP markers in *C. arabica*.

Geng et al. (2007) demonstrated that markers with segregation distortion affect genetic distance in the linkage map, causing an increase in distance between adjacent markers. However, even assuming that segregation distortion affects Mendelian segregation, Luo et al. (2005) do not recommend the exclusion of markers with segregation distortion in genetic linkage maps with the purpose of mapping QTLs. According to the authors, the exclusion of these markers might cause a loss of information if the marker with segregation distortion is associated with any QTL. Some authors do not use markers with segregation distortion for QTL mapping; nevertheless, these markers have been routinely used for QTL mapping in several studies, without important effects (Truco et al. 2007; Couto et al. 2010; Guo et al. 2013). With simulated data, Zhang et al. (2010) and Wen and Zhang (2013) observed that if the distorted marker is intimately linked with any QTL, distortion will not have a significant impact on QTL mapping.

Linkage map was constructed with 111 markers, including some with segregation distortion, which increased the map saturation. However, in the present study, markers with segregation distortion were not associated to QTLs.

Our linkage map had a mean genetic distance of 9.7 cM, which was similar to other coffee studies. Pearl et al. (2004), Teixeira-Cabral et al. (2004), and Oliveira et al. (2007) reported mean genetic distance of 10.2, 7.3, and 7.5 cM, respectively. Despite presenting few linkage gaps, our map was suitable for QTL mapping.

In this study, 247 F_2 individuals were used to a construct linkage map. This population was larger than the map populations used by Paillard et al. (1996), Ky et al. (2000), Lashermes et al. (2001), Pearl et al. (2004), Teixeira-Cabral et al. (2004), and Oliveira et al. (2007). Increased accuracy in constructing genetic linkage maps can be obtained with large populations. With simulated data of F_2, recombinant-inbred lines, and backcross populations, Ferreira et al. (2006) reported that populations with 200 individuals are considered to be sufficient for the construction of reasonably precise genetic maps.

Mendelian factor location

Molecular markers linked to QTLs as explained from 5.7 to 16.2 % of the phenotypic variation. Similar values were identified for QTLs associated with resistance to other diseases in different species. Siviero et al. (2006) identified QTLs, explaining a phenotypic variation from 14 to 24 % for citrus gummosis. Risterucci et al. (2003) identified a phenotypic variation explained by QTLs associated with resistance to *Phytophthora* spp. from 7.5 to 12.4 %. In chickpea, Sabbavarapu et al. (2013) identified two QTLs for resistance to *Fusarium oxysporum* f. sp. *ciceris*, explaining 10.4 and 18.8 % of the phenotypic variation and six QTLs for *Ascochyta rabiei*, with the highest phenotypic variation explained by one of the QTLs at 31.9 %. The authors reported that resistance to *F. oxysporum* is an oligogenic trait and that the two identified QTLs correspond to two large effect genes. However, for *A. rabiei*, the only QTL that explained 31.9 % of the phenotypic variation corresponded to a QTL of large effect.

The phenotypic variation explained by QTL (coefficient of determination, R^2) can be affected by the distance of the marker to the QTL and the trait heritability. Therefore, it is possible that R^2 of markers associated with QTLs in this study was underestimated, as the marker distance from QTLs ranged from 0.0 to 22.7 cM. The refinement of QTL mapping would help to identify the putative loci. Romero et al. (2014) identified one QTL for resistance of *C. arabica* to *H. vastatrix*, explaining 52.1 to 70.3 % of the phenotypic variation. They evaluated adult plants resistance using an inoculum composed of a mix of uredospores of *H. vastatrix* collected in the field and scored area of lesion, area under disease curve, and field rust data. In the other hand, we evaluated coffee leaf discs using three monopustule isolates of *H. vastatrix* and scored the race-specific resistance using a score scale based on the absence or presence of uredospores. These experimental differences may explain the different results.

It was possible to notice that the QTL located in LG 2 associated with marker E-CTG/M-AAT 1 as well as the QTL located in LG 10 associated with marker E-CCC/M-AGA 1 conferred resistance to race I, race II, and pathotype 001. The QTL in LG 2 associated with marker E-CGC/M-ATA 1, as well as the QTL located in LG 3 between markers EST-SSR 050 and EST-SSR 107 resulted in resistance to race I and pathotype 001. Hence, the same genes might be involved in resistance to race I and pathotype 001 or the region contains different and closely linked genes that confer resistance to them. In fact, resistance genes are frequently clustered. Clustering can be a result of duplication and recombination of one or more parental genes. Recombination between closely linked or very close genes in the chromosome may generate new pathogen recognition specificities, such as other

resistance genes (Keen 1990). This mechanism is important for plant-pathogen co-evolution.

From the QTLs identified in the linkage map, none was recessive, showing that no recessive gene contributes to the resistance of the HDT UFV 443-03. This result rejects the hypothesis suggested in the inheritance study of the three loci (two dominant and one recessive) conferring resistance to this coffee plant. By the combination of phenotypic and genotypic data, it was concluded that at least two dominant and independent loci control the resistance of HDT UFV 443-03. For race II, two QTLs confirmed the presence of two dominant loci, conferring resistance to this race of *H. vastatrix*. However, for race I and pathotype 001, four QTLs were identified, implying that resistance of HDT UFV 443-03 to these two isolates can be controlled by four resistance loci. In the inheritance study, the population sizes did not allow us to test (χ^2 test) if the CLR resistance is controlled by four loci.

According to the QTL mapping results, HDT UFV 443-03 has four important putative loci conditioning CLR resistance located in four genomic regions: two regions (LG 2 and LG 10) for races I and II and pathotype 001 and two other regions (LG 2 and LG 3) for race I and pathotype 001. Thus, marker-assisted selection (MAS) should allow introgression of these loci into new cultivars with durable resistance to at least the three isolates (race I, race II, and pathotype 001) of *H. vastatrix*. Additional markers for CLR resistance identified by other authors (Romero et al. 2014; Prakash et al. 2004; Mahé et al. 2008; Brito et al. 2010; Diola et al. 2011) can be simultaneously used with MAS, allowing rapid and efficient identification of resistance genes in progenies without needing to evaluate the phenotype (Caixeta et al. 2009).

A high-resolution mapping of regions where the QTLs were detected should allow cloning of these CLR resistance loci. Moreover, SSR markers included in the map are useful to integrate partial coffee plant maps as an alternative to obtain a saturated map. It should aid breeding resistant and productive new cultivars.

Conclusion

The present study indicated that CLR resistance of HDT UFV 443-03 to race II of *H. vastatrix* is conditioned by at least two independent dominant loci. One locus is located in the LG 2 and the other in the LG 10. The CLR resistance of HDT UFV 443-03 to race I and pathotype 001 is conditioned by at least four independent dominant loci. Two loci are located in the LG 2, one in the LG 3, and one in the LG 10. The same four independent loci condition the resistance to race I and pathotype 001. Two of these four independent loci also condition the resistance to race II. The best markers identified, so far, for MAS of CLR resistance loci derived from HDT UFV 443-03 were the following: E-CTG/M-AAT 1 (LG 2) and E-

CCC/M-AGA1 (LG 10) for race II and E-CGC/M-ATA 1 (LG 2), E-CTG/M-AAT 1 (LG 2), EST-SSR 050 (LG 3), and E-CGA/M-TCC 4 (LG 10) for race I and pathotype 001.

Data Archiving Statement   All oligonucleotides used in this work have been published previously (Combes et al. 2000; Rovelli et al. 2000; Baruah et al. 2003; Coulibaly et al. 2003; Moncada and McCouch 2004; Poncet et al. 2004; Bhat et al. 2005; Hendre et al. 2008; Missio et al. 2009b, 2011). Thus, their sequences are available in the respective literatures.

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