Identification of microsatellite markers in coffee associated with resistance to *Meloidogyne exigua*

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ABSTRACT. *Meloidogyne* species are destructive phytonematodes that result in reduced yields of coffee. The classic test for resistance to *Meloidogyne exigua* in coffee progenies is both expensive and time-consuming. The use of molecular marker techniques can assist the selection process when it is difficult to measure the phenotype, such as in cases of resistance to nematode infestation. The objective of this study was to identify microsatellite markers associated with resistance to *M. exigua* in F_5 progenies of coffee derived from a cross between Híbrido de Timor 440-10 and Catuaí Amarelo IAC 86. Of the 44 simple sequence repeat (SSR) markers evaluated, 11 showed a polymorphic pattern with a mean number of 4.5 alleles per marker. Clustering analysis classified 82 progenies into three groups related to

the response to nematodes and parental genotypes allocated to different groups (resistant and susceptible). SSRCafé 40 allele 2, SSRCafé 15 allele 3, SSRCafé 20 allele 3, and SSRCafé 13 allele 1 were negatively correlated with reproduction factor. In addition, SSRCafé 13 allele 2, SSRCafé 19 allele 3, SSRCafé 40 allele 2, SSRCafé 15 allele 3, and SSRCafé 20 allele 3 were correlated with the root gall index of *M. exigua*. These SSR markers, which have been validated in this population, represent a potential method to select progenies resistant to nematodes in coffee-breeding programs.

Key words: Genetic diversity; SSR; Stepwise regression; Nematodes; *Coffea arabica*

INTRODUCTION

Brazil is the major producer and exporter of coffee in the world. In 2014, a total of 32 million bags of *Coffea arabica* and 13 million bags of *Coffea canephora* were collected from a 2,267,577.8 ha area of coffee plantations (Companhia Nacional de Abastecimento - CONAB, 2014).

Meloidogyne exigua Goeldi 1887 is probably the most widespread species parasitizing the coffee tree in the Americas, which results in yield losses that vary depending upon the cultivar, soil, and environmental conditions involved. The presence of phytonematodes is thought to be the principal factor that limits the growth and productivity of the coffee tree (Barbosa et al., 2008). This microscopic organism is found in the root and affects the development of the whole plant. *M. exigua* is one of the most important parasitic species predominant in all coffee-producing states of Brazil. This pathogen is also widely distributed in all coffee-growing countries of the world, and results in large losses in productivity (Sasser, 1977). The diverse dissemination and adaptive capability of *Meloidogyne* species in various regions have resulted in this species being reported in 95% of the municipality of South of Minas Gerais State (Barbosa et al., 2008). This species causes nutritional changes and reduces the yield of coffee plants due to deficient absorption and translocation of water and nutrients (Barbosa et al., 2008).

Due to the damage caused to coffee plants, farmers continuously seek methods to control nematode. The use of resistant cultivars is the most common and economical viable method of controlling nematode infestation (Alpizar et al., 2007). In this regard, Híbrido de Timor presents resistance to *M. exigua* and has contributed to the development of coffee cultivars with high productivity (Pereira et al., 2012). Progenies derived from these hybrids possess genes that can modify the phenotype and provide resistance to nematodes (Anthony et al., 2005). Based on this strategy, various cultivars of *C. arabica* have been developed for production in Brazil and provide considerable economic gain for the farmers in these regions (Setotaw et al., 2013).

Study on the inheritance of root-knot nematode resistance in coffee using the F_2 population revealed that resistance to *M. exigua* is controlled by the simple inheritance of major genes, whereas the gall index exhibited incomplete dominant expression (Noir et al., 2003). Complete dominance of the *Meloidogyne* species has also been reported in *Capsicum annuum* (de Souza-Sobrinho et al., 2002) and lettuce (Maluf et al., 2002).

Molecular markers, such as microsatellites or simple sequence repeats (SSR), can be used to select progenies that are resistant to *M. exigua* as an alternative to the classic method

of evaluation, which is both costly and time-consuming (Jenkins et al., 2012). If these markers are evaluated and validated in a population of coffee plants infested with nematodes, and their association with nematode resistance is established, they can be used to evaluate a high number of progenies in a short period. In addition, unlike morphological markers, molecular markers are not influenced by the environment (Machado et al., 2013).

Molecular markers associated with parasitism of the genus *Meloidogyne* have been used in different crop species. In soybean, Silva et al. (2001) and Fuganti et al. (2004) demonstrated an association between the microsatellite marker SOYHS 176 and the number of galls in *Meloidogyne javanica*, whereas Jenkins et al. (2012) verified the association of microsatellite markers with the reaction to *Meloidogyne incognita* in cotton. However, in coffee, only an association between AFLP markers and resistance to the nematode species *M. exigua* has been reported (Noir et al., 2003; Diniz et al., 2005). This highlights the need for more research to identify molecular markers associated with resistance to root-knot nematode in coffee in order to explore the advantage of this technology in the development of coffee cultivars that are resistant to nematodes. In addition, such research would benefit coffee researchers working in different parts of the world.

One of the methods used to identify associations between molecular markers and phenotypic characteristics is stepwise multiple-regression analysis. In this method, all markers evaluated enter the model and stay in the model when conditions set by the researcher are satisfied. Stepwise multiple regression is frequently used to identify molecular markers associated with disease resistance and other agronomic ,traits in different crop species (Pereira et al., 2008; Ruan et al., 2009).

Research leading to the development of *M. exigua*-resistant cultivars by combining conventional breeding with biotechnology are rare in coffee-breeding programs; therefore, the present study represents the first to investigate the association of microsatellite markers with resistance to *Meloidogyne* species. Here, a strong correlation was observed between molecular markers and resistance to nematodes, highlighting the potential use of these markers to select progenies resistant to nematodes in breeding programs. Therefore, these results may contribute significantly to the advancement of genetic improvement in coffee, especially in the production of cultivars that are resistant to nematodes in Brazil as well as in other coffee-growing countries.

Therefore, the objective of this study was to identify microsatellite markers associated with resistance to the nematode *M. exigua* in the F_5 progenies of coffee seedlings derived from the hybridization of Híbrido de Timor 440-10 and Catuaí Amarelo IAC 86.

MATERIAL AND METHODS

Progenies

Molecular analyses were performed in the Plant Biotechnology Laboratory of Agricultural Research Company of Minas Gerais localized in the EPAMIG, Caldas, Minas Gerais. Phenotypic analyses were performed in the EPAMIG/UFLA in Lavras-Minas Gerais in a greenhouse and in the nematology laboratory.

Eighty-two F_5 progenies of coffee seedlings derived from a cross between Híbrido de Timor 440-10 (resistant progeny of *C. canephora* var. *robusta* x *C. arabica*) and Catuaí Amarelo 86 (susceptible progeny of *C. arabica* cultivar) belonging to the coffee-breeding program of EPAMIG in Minas Gerais (Rezende et al., 2013) were used in this study (Table 1).

Table 1. Identification of standard *Coffea arabica* cultivars and F_5 progenies selected based on the response to nematodes in fields naturally infected with *Meloidogyne exigua*.

Identification	Progenies	Identification	Progenies
1	Híbrido de Timor 440-10*	43	514-7-16-C208-B1P3
2	Catuaí Amarelo IAC 86*	44	514-7-16-C208-B1P4
3	514-7-4-C130-B1P1	45	514-7-16-C208-B1P6
4	514-7-4-C130-B1P4	46	514-7-16-C208-B1P7
5	514-7-4-C130-B1P6	47	514-5-2-C494-B1P7
6	514-7-16-C208-B1P3	48	514-7-14-C73-B2P2
7	514-7-16-C208-B1P4	49	514-7-4-C130-B2P3
8	514-7-16-C208-B1P6	50	514-7-4-C130-B2P4
9	514-7-16-C208-B1P7	51	514-7-4-C130-B2P5
10	514-5-2-C494-B1P7	52	514-7-4-C130-B2P6
11	514-7-14-C73-B2P2	53	514-7-16-C208-B2P2
12	514-7-4-C130-B2P3	54	514-7-16-C208-B2P3
13	514-7-4-C130-B2P4	55	514-7-16-C208-B2P4
14	514-7-4-C130-B2P5	56	514-7-16-C208-B2P5
15	514-7-4-C130-B2P6	57	514-5-2-C494-B2P6
16	514-7-16-C208-B2P2	58	514-5-2-C494-B2P7
17	514-7-16-C208-B2P3	59	514-5-2-C494-B2P8
18	514-7-16-C208-B2P4	60	514-7-14-C73-B3P1
19	514-7-16-C208-B2P5	61	514-7-14-C73-B3P5
20	514-5-2-C494-B2P6	62	514-7-4-C130-B3P2
21	514-5-2-C494-B2P7	63	514-7-4-C130-B3P3
22	514-5-2-C494-B2P8	64	514-7-4-C130-B3P5
23	514-7-14-C73-B3P1	65	514-7-16-C208-B3P1
24	514-7-14-C73-B3P5	66	514-7-16-C208-B3P3
25	514-7-4-C130-B3P2	67	514-7-16-C208-B3P4
26	514-7-4-C130-B3P3	68	514-7-8-C364-B3P2
27	514-7-4-C130-B3P5	69	514-7-8-C364-B3P4
28	514-7-16-C208-B3P1	70	514-5-2-C494-B3P3
29	514-7-16-C208-B3P3	71	514-5-2-C494-B3P6
30	514-7-16-C208-B3P4	72	514-7-4-C130-B4P2
31	514-7-8-C364-B3P2	73	514-7-16-C208-B4P1
32	514-7-8-C364-B3P4	74	514-7-16-C208-B4P6
33	514-5-2-C494-B3P3	75	514-5-2-C494-B4P4
34	514-5-2-C494- B3P6	76	514-5-2-C494-B4P5
35	514-7-4-C130-B4P2	77	514-7-4-C130-B4P4
36	514-7-16-C208-B4P1	78	514-7-6-C208-B1P6
37	514-7-16-C208-B4P6	79	514-7-16-C208-B1P7
38	514-5-2-C494-B4P4	80	514-5-2-C494-B1P7
39	514-5-2-C494-B4P5	81	514-7-4-C130-B2P3
40	514-7-4-C130-B1P1	82	514-5-2-C494-B2P7
41	514-7-4-C130-B1P4	83	514-7-14-C73-B3P1
42	514-7-4-C130-B1P6	84	514-7-4-C13-B3P5

*Parents used as control. C, pit; B, block; P, plant.

Evaluation of resistance to *M. exigua*

Progenies derived from a cross between Híbrido de Timor 440-10 and Catuaí Amarelo 86 (Table 1) were planted in a greenhouse and seedlings were established in 850-cm^3 plastic sacks containing soil and manure at a 3:1 ratio and disinfected with dazomet (Basamid®) (BASF Corporation, Florham Park, NJ, USA). In each plastic sack, one F_5 plant was planted and used to evaluate the response to nematodes and to collect leaf samples for molecular analyses.

The inoculum was obtained from the roots of coffee plants from a naturally infested farm in the municipality of Campos Altos. Eggs were extracted from the root according to the technique described by Hussey and Barker (1973) and subsequently used to prepare the inoculum. The egg suspension was calibrated with the aid of a light microscope using Peters' slides. To confirm the identity of the nematode species, females isolated from the galls

were identified using isoenzyme analysis (Carneiro and Almeida, 2001) with *M. exigua* E1 phenotype (Rm = 1.5). When the seedling had 4-6 pairs of leaves, they were inoculated with 5000 eggs of *M. exigua* around the root.

To evaluate the response to nematodes after 320 days of inoculation, eggs and juvenile *M. exigua* were extracted from the roots using 0.5% NaOCl, according to the method described by Hussey and Barker (1973). The size of the nematode population was estimated under a light microscope using Peters' slides. Then, the reproduction factor was estimated from the ratio of the final population density and initial density (Seinhorst, 1967), by account of three replicates.

The index of galls was recorded using a scale of 0 to 5 based on the number of galls in three replicates (0 = absence of galls, 1 = 1-2 galls, 2 = 3-10, 3 = 11-30, 4 = 31-100, 5 = >100 galls) according to the method recommended by Hartman and Sasser (1985).

DNA extraction and quantification

Completely expanded leaves were collected from F_5 progenies and control seedlings, and transported to the laboratory where they were stored in a deep freezer (-80°C) until subsequent lyophilization and DNA extraction. DNA was extracted according to the methodology described by Nunes et al. (2011).

To verify the quality of DNA, samples were visualized under UV light after electrophoresis on 0.7% agarose gel in the presence of ethidium bromide. DNA was quantified at 260 nm using an NIR spectrophotometer. The quality of DNA was estimated using a 260/280 nm ratio, and samples with a ratio of 1.5-2.0 were considered adequate for further analysis. Finally, samples were standardized to 50 ng/μL for further analysis.

Microsatellite markers

Forty-four microsatellite markers believed to be polymorphic for *C. arabica* species were tested in this study (**Table S1**).

PCR was performed in a volume of 25 μL, containing 50 ng DNA, 6 μL 5X reaction buffer, 1 mM $MgCl_2$, 150 μM each dNTP, 0.1 μM primers, and 0.6 U Taq DNA polymerase (Go Taq Flexi, Promega, Madison, WI, USA). Reactions were carried out in a gradient thermocycler (Multigene Gradient, Labnet International, USA) using touchdown PCR with the following program: 94°C for a 2-min initial denaturing, followed by 13 cycles at 94°C for 30 s; annealing temperature 67°-55°C for 30 s, reducing 1°C in each cycle, extension at 72°C for 30 s. This was followed by 30 cycles of denaturation at 94°C for 30 s, 55°C for 30 s, and extension at 72°C for 30 s. A final extension occurred at 72°C for 8 min.

The amplification products were subjected to denatured gel electrophoresis on 6% polyacrylamide gel at 60 W power for variable times according to the size of the alleles. Next, bands were visualized using silver nitrate according to the method described by Setotaw et al. (2010). The gel was then dried overnight at room temperature and photographed.

Statistical analysis

For each microsatellite marker, each amplified fragment was coded 1 for the presence or 0 for the absence of each band. To study genetic diversity among progenies of coffee, the Jaccard coefficient of similarity (Jaccard, 1908) was estimated using the GENES statistical

programs (Cruz, 2013). A dendrogram was produced using MEGA 6.06 (Tamura et al., 2013) based on the Jaccard genetic distance (1-Jaccard coefficient of similarity), using the unweighted pair group method with arithmetic average (UPGMA) method with the objective of clustering the progenies in their respective groups. Principal coordinate analysis (PCoA) was performed using GenAlex 6.2 (Peakall and Smouse, 2006) based on the Jaccard genetic distance. In this analysis, the genetic distance matrix was used to produce the coordinate of the graphs where the progenies were represented as points in the Cartesian plane.

Identification of microsatellite markers associated with resistance to *M. exigua*

Polymorphic microsatellite markers were grouped as being positively or negatively associated, based on the Pearson correlation, with the nematode reaction (reproduction factor and gall index). The Pearson correlation coefficient was estimated using the GENES statistical analysis program (Cruz, 2013).

Thereafter, associations between microsatellite markers and parameters related to parasitism were determined using stepwise multiple-regression analysis with the objective of identifying microsatellite markers related to the response to nematodes in the progenies. The quantitative index of parasitism was considered as the dependent variable and the positive and negative markers as independent variables. A significance level of 0.15 was used as the criteria for the marker to enter and remain in the model. Stepwise regression analysis was carried out using the PROCGLM program of Statistical Analysis System (SAS Institute, 2004) to identify the markers associated with phenotypic characteristics.

RESULTS

Among the primers used in the F_5 progenies, only 11 showed a polymorphic pattern among the parental genotypes, these were as follows: SSRCafé 4, SSRCafé 13, SSRCafé 14, SSRCafé 15, SSRCafé 19, SSRCafé 20, SSRCafé32, SSRCafé 37, SSRCafé 39, SSRCafé 40, and SSRCafé 41. The primers produced 2-8 polymorphic alleles among progenies with an average 4.5 alleles per primer (Table 2).

Table 2. Polymorphic microsatellite markers, number of polymorphic alleles, and percentage polymorphism among the *Coffea arabica* progenies.

Code	Marker name	Reference	No. of alleles	Polymorphism (%)
SSRCafé 4	SSRCa087	Missio et al. (2009)	5	80.0
SSRCafé 13	CFGA792b	Cristancho and Gaitan (2008)	7	85.7
SSRCafé 14	CFCA281	Cristancho and Gaitan (2008)	4	100.0
SSRCafé 15	CFGA627	Cristancho and Gaitan (2008)	6	50.0
SSRCafé 19	CFCA360	Cristancho and Gaitan (2008)	3	66.0
SSRCafé 20	AJ250254	Combes et al. (2000)	4	100.0
SSRCafé 32	AJ308819	Rovelli et al. (2000)	4	100.0
SSRCafé 37	BQ448809	Rovelli et al. (2000)	4	75.0
SSRCafé 39	EU597602	López-Gartner et al. (2009)	2	100.0
SSRCafé 40	EU597603	López-Gartner et al. (2009)	7	100.0
SSRCafé 41	EU597604	López-Gartner et al. (2009)	8	100.0

Jaccard similarity coefficient compares the number of common alleles present and the total number of alleles involved without considering the number of common alleles that are absent (Jaccard, 1908). The progenies evaluated in this study were grouped into three

clusters using the UPGMA clustering method based on the Jaccard genetic distance (1-Jaccard similarity coefficient) (Figure 1). The result revealed high genetic variability among the progenies and verified the efficiency of microsatellite markers for their classification.

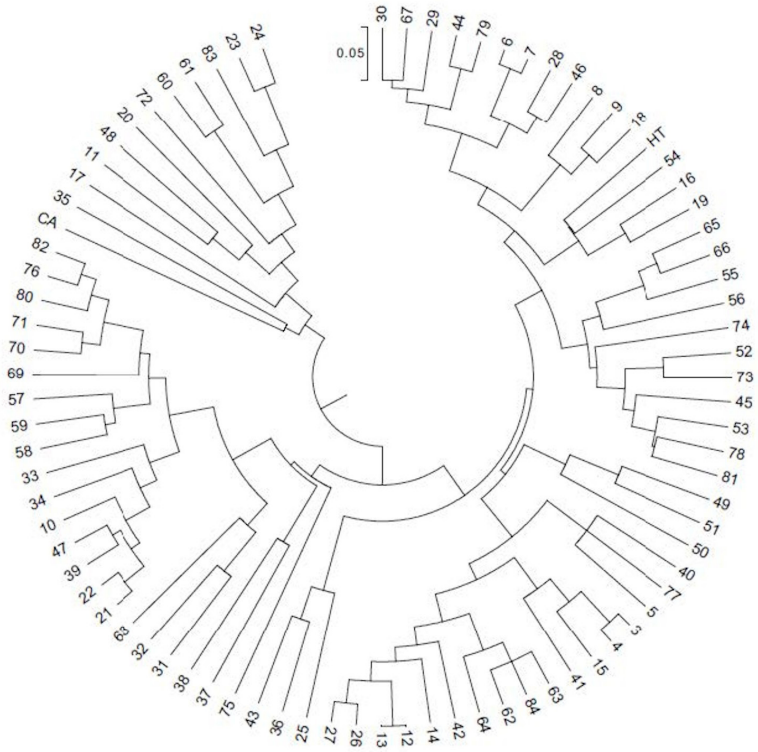

Figure 1. Dendrogram produced using the unweighted pair group method with arithmetic average (UPGMA) clustering method based on the Jaccard genetic dissimilarity coefficient obtained using microsatellite markers.

The progenies with identification numbers 12 and 13 showed a high level of similarity and a high number of common alleles. Both progenies were obtained from the same plant through selfing. In addition, these genotypes were immune to the effects of *M. exigua* and presented reproduction factors of 0.

The cluster group with the highest number of progenies included the cultivar Híbrido de Timor. Progenies included in this group showed resistant or displayed low reproduction factor for *M. exigua*, similar to Híbrido de Timor. Group III was also of interest as approximately 90% of the progenies presented a score of 0 for the gall index and exhibited an immune reaction and resistance to the reproduction factor of *M. exigua*. This observation is of interest since microsatellite markers were able to group a large number of resistant genotypes into the same cluster.

The resistant (Híbrido de Timor 440-10) and susceptible (Catuaí IAC 86) genotype standards presented resistant and susceptible responses, respectively, and were classified in distinct groups. Of note, the F_5 progenies are derived from the same cross, which demonstrates high genetic diversity among the parental lines.

The information obtained from the PCoA, is consistent with the data observed on the dendrogram, and showed the genetic relationship of the populations (Figure 2). In general, the majority of genotypes is more similar to Híbrido de Timor and lay below coordinate 2. Conversely, cultivar Catuaí Amarelo IAC 86 was isolated and separated from the other progenies. The progeny with identification number 72 was positioned near to Catuaí and presented a similarly high level of susceptibility to nematodes.

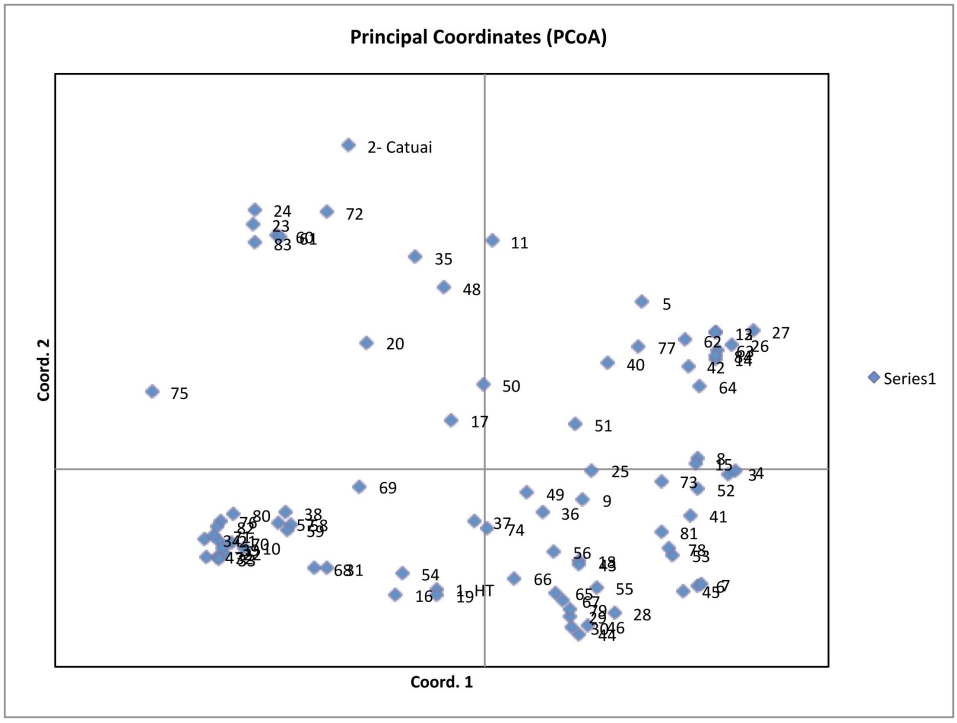

Figure 2. Principal coordinate analysis of 82 F_5 progenies and parental genotypes (Catuaí Amarelo IAC 86 and Híbrido de Timor 440-10) using microsatellite markers.

The first two principal coordinates explained 37.03% of the total variation whereby the first and second explained 21.11 and 15.92%, respectively. This showed that the first principal coordinates explained a low percentage of variability.

Based on the results obtained from the molecular and statistical analyses, it is possible to conclude that the progenies presented high genetic diversity and were clustered in different groups, especially in relation to their response to nematodes. This suggests that it might be possible to identify molecular markers that correlate with resistance to *M. exigua*.

Pearson correlation coefficient revealed associations between SSR markers and nematode parasitism in F_5 progenies. Based on the Pearson correlation coefficient, the markers were classified as being positively or negatively correlated with nematode resistance. The markers significantly correlated with the nematode response were subjected to stepwise regression analysis in order to select the microsatellite markers related to nematode resistance.

Stepwise regression revealed a significant relationship among the SSR markers and the

response to nematode infestation among the progenies (Table 3). The microsatellite markers SSRCafé 32 allele 2 (96 bp), SSRCafé13 allele 5 (125 bp), SSRCafé 41 allele 5 (90 bp), and SSRCafé 20 allele 1 (130 bp) were positively correlated with reproduction factor and shown to have a significant effect on resistance to *M. exigua* among the F_5 progenies. Therefore, the alleles amplified by these markers were associated with high levels of reproduction factor or high susceptibility of the plant. These markers explained 28.43% of the total phenotypic variation (Table 3). The SSRCafé 32 allele 2 (96 bp) explained a high phenotypic proportion (R^2) of both parameters related to nematode parasitism. The markers positively correlated with the phenotype can be used in breeding programs for marker-assisted selection (MAS) with the objective of culling progenies presenting specific alleles.

Table 3. Microsatellite markers associated with the reproduction factor of *Meloidogyne exigua* in F_5 progenies of *Coffea arabica*.

Correlation	Marker	F value	R^2 (%)	R^2 accumulated (%)	Molecular weight (bp)
Positive	SSRCafé 32 allele 2	14.90*	15.87	15.87	96
	SSRCafé 13 allele 5	10.86**	5.92	21.78	125
	SSRCafé 41 allele 5	8.83**	3.81	25.59	90
	SSRCafé 20 allele 1	7.55**	2.83	28.43	130
Negative	SSRCafé 13 allele 1	10.13**	11.36	11.36	155
	SSRCafé 20 allele 3	8.14**	5.90	17.26	100
	SSRCafé 40 allele 2	7.47*	5.30	22.55	250
	SSRCafé 15 allele 3	7.10*	4.60	27.20	190

*,**P < 0.001 and P < 0.0001, respectively; R^2, coefficient of determination.

For the gall index, besides SSRCafé 32 (96 bp), markers SSRCafé 19 allele 2 (130 bp), SSRCafé 20 allele 1 (130 bp), SSRCafé 37 allele 2 (260 bp), SSRCafé 39 allele 1 (100 bp), and SSRCafé 13 allele 5 (125 bp) were positively correlated with parasitism (Table 4) with a combined coefficient of determination of 38.92%. Microsatellite markers SSRCafé 20 allele 3 (100 bp), SSRCafé 40 allele 2 (250 bp), and SSRCafé 15 allele 3 (190 bp) presented significant negative correlations with both of the characters evaluated related to *M. exigua*. SSRCafé 20 allele 3 and SSRCafé 19 allele 3 demonstrated negative correlations with gall index and explained a high percentage of phenotypic variation (Table 4).

Table 4. Microsatellite markers associated with gall index of *Meloidogyne exigua* in F_5 progenies of *Coffea arabica*.

Correlation	Marker	F value	R^2 (%)	Accumulated R^2 (%)	Molecular weight (bp)
Positive	SSRCafé 32 allele 2	11.22**	12.44	12.44	96
	SSRCafé 19 allele 2	9.70*	7.47	19.91	130
	SSRCafé 20 allele 1	9.62*	7.35	27.26	130
	SSRCafé 37 allele 2	9.24**	5.46	32.73	260
	SSRCafé 39 allele 1	8.82**	4.30	37.02	100
	SSRCafé 13 allele 5	7.86**	1.90	38.92	125
Negative	SSRCafé 13 allele 2	10.53*	11.76	11.76	150
	SSRCafé 20 allele 3	11.85**	11.76	23.31	100
	SSRCafé 19 allele 3	12.35**	9.18	32.48	118
	SSRCafé 40 allele 2	10.86**	3.89	36.38	250
	SSRCafé 15 allele 3	9.97**	3.55	39.93	190

*,**P < 0.001 and P < 0.0001, respectively; R^2, coefficient of determination.

DISCUSSION

Low polymorphism of molecular markers among progenies of *C. arabica* is well documented (Lashermes et al., 1999; Bertrand et al., 2001), since this species has a narrow

genetic base, is autogamous in nature, tetraploid (Cristancho and Gaitan, 2008), and has a low frequency of microsatellite regions in the genome (Cristancho and Gaitan, 2008; Al-Murish et al., 2013). Furthermore, in this case, all progenies originated from the crossing of two genotypes. Low polymorphism was also reported by Combes et al. (2000), who only identified five polymorphic microsatellite markers among 11 SSR primers tested in *C. arabica*. Furthermore, Cristancho and Gaitan (2008) also identified five polymorphic markers among 12 SSR markers tested in tetraploid *Coffea*.

In this study, 4.5 polymorphic alleles per marker were observed among the F_5 coffee progenies. The number of polymorphic alleles in a population depends on the population size, its genetic constitution, and the different microsatellite markers used. Missio et al. (2009) reported similar results (with 5.1 polymorphic alleles per marker) among coffee cultivars. They also verified the existence of high levels of genetic diversity among genotypes evaluated using PCoA, which is an important and desirable characteristic for breeding programs, as it is needed to increase the genetic base of the breeding population. Even if their occurrence is low in *C. arabica*, the efficiency of microsatellite markers for the study of genetic diversity in this species has been reported (Missio et al., 2009; Setotaw et al., 2010), consistent with the results of the present study.

Of note, the use of PCoA in the present study revealed high divergence between the Catuaí cultivar and other progenies studied. This may be explained by previous selection pressures on the coffee progenies in areas infested with *M. exigua*, in which highly susceptible progenies were possibly eliminated.

The results of the PCoA verified that the first two principal coordinates explained a low percentage of variability among the progenies studied. Similar results were reported by Souza et al. (2013) using microsatellite markers that explained 24.9% of the total variation among the germplasm of *C. canephora* found in Brazil. In contrast to our findings, Setotaw et al. (2010) were able to explain 67% of the total variation from the first two principal coordinates obtained using amplified fragment length polymorphism (AFLP) and random-amplified polymorphic DNA (RAPD) molecular markers. These results can be explained by the high levels of polymorphism identified by RAPD and AFLP. In addition, the population used consisted of Híbrido de Timor, which contains higher genetic variability than the population used in the present study.

The aim of the stepwise regression analysis was to select SSR markers that had high percentage of contribution and were associated with the nematode reaction in F_5 progenies in this study. Stepwise regression analysis was used to identify molecular markers associated with resistance and agronomic characteristics in different crops (Silva and Vencovsky, 2002). Ruan et al. (2009) successfully applied stepwise regression to study the association between molecular markers and dryness caused by disease in *Hippophae* L. Pereira et al. (2008) used stepwise regression analysis to identify quantitative trait loci linked to grain productivity in common bean. Missio et al. (2011) used stepwise regression to associate microsatellite markers with the response of different genotypes of coffee to leaf rust. This method also proved to be efficient when used to identity microsatellite markers associated with nematode resistance in the present study.

The negative correlation found between some markers and the evaluated characters is of interest, since this demonstrates the presence of an allele that is associated with the low value of the parameter related to the disease, or, in other words the progenies resistant to *M. exigua*. The microsatellite markers negatively correlated with both characteristics associated

with nematode resistance were SSRCafé 20 allele 3, SSRCafé 15 allele 3, and SSRCafé 40 allele 2. Fuganti et al. (2004) identified two microsatellite markers, named Satt 144 and SOYHSP 176, which were correlated with the gall number and infestation scores of *M. javanica* in soybean. Ruan et al. (2009) identified inter-simple sequence repeat markers associated with disease resistance in *Hippophae* L. Chen et al. (2012) used multiplex PCR to identify molecular markers linked to root-knot nematode resistance in tomato, and identified a specific marker (*Mi*).

The coefficient of determination of each marker could have been affected by the low number of primers used. Another difficulty when studying nematode resistance is the high morphological and chromosomal variations present among individuals within the same species, with the possibility of pathotypes and races that can disrupt the genetic resistance of the plant (Medina Filho et al., 2008). This makes it necessary to evaluate the resistance of progenies infested with different nematode populations. In our study, the SSR markers SSRCafé 20 allele 3, SSRCafé 15 allele 3, and SSRCafé 40 allele 2 were significantly negatively correlated with reproduction factor and gall index, and produced a reasonable coefficient of determination. Therefore, they may be used as indirect criteria for the selection of genotypes tolerant to root-knot nematode in *Coffee arabica*.

This study also showed that Híbrido de Timor is an important source of resistance genes to diseases and pests, including nematode, since we were able to identify various progenies with resistance to nematodes, which can be released as cultivars in the near future. Setotaw et al. (2013) reported the development of *C. arabica* cultivars using Híbrido de Timor as a source of genes for resistance to pests and diseases, and their release for commercial production in Brazil.

The combined use of the markers selected in this study can lead to the successful selection of genotypes that carry genes conferring resistance to *M. exigua* in coffee, since the combined coefficient of determination from these markers is satisfactory. We recommend that the markers selected in this study are validated in other coffee progenies and nematode populations for potential future use in breeding programs as MAS. The validation of these markers should be performed in a population with a variable response to nematodes. In addition, the results obtained in this study will facilitate the selection process in breeding programs of coffee related to nematode resistance, which require labor and time.

In addition, the research outputs presented in this report not only serve the breeding program in Brazil, but also the international coffee breeder working to develop nematode-resistant coffee cultivars. The results of this study show that it is possible to identify SSR markers associated with nematode resistance, which should encourage others to undertake similar research and obtain further information on this subject, which is important for coffee-breeding programs throughout the world.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Sequence and expected allele size (bp) of the microsatellite markers used in this study.