Genetic variability and phylogenetic characterization of different populations of Meloidogyne izalcoensis and reaction of coffee genotypes to this new species detected in Brazil

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Sheila Almeida, Marcilene Santos, Daniela Stefanelo, Vanessa Mattos, Paolo Rodrigues-Silva, et al.. Genetic variability and phylogenetic characterization of different populations of Meloidogyne izalcoensis and reaction of coffee genotypes to this new species detected in Brazil. Plant Pathology, 2023, 72 (3), pp.636-650. 10.1111/ppa.13686 . hal-04169764

HAL Id: hal-04169764
https://hal.inrae.fr/hal-04169764

Submitted on 24 Jul 2023

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1 **Genetic variability and phylogenetic characterization of different populations of**
2 ***Meloidogyne izalcoensis* and reaction of coffee genotypes to this new species detected**
3 **in Brazil.**
4
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26 **Abstract:** A new root-knot nematode (RKN) *Meloidogyne izalcoensis* was detected on
27 coffee in Brazil. This species was first described on coffee from El Salvador and later
28 detected in Africa. There are no reports on the genetic diversity of this species. The
29 genetic resistance of coffee to RKN is considered one of the main nematode management
30 strategies and the resistance to *M. izalcoensis* has not yet been studied in coffee cultivars.
31 The objectives of this study were to assess the genetic variability of five *M. izalcoensis*
32 populations from different geographic locations and their phylogenetic relationships
33 based on distinct regions of ribosomal DNA (rDNA), mitochondrial gene cytochrome *c*
34 oxidase II (COII) and nuclear protein coding gene HSP90, and to evaluate the reaction of
35 different coffee genotypes resistant to other RKN species to the Brazilian population of
36 *M. izalcoensis*. All populations were identified by esterase phenotype and SCAR specific
37 markers. Based on RAPD and AFLP markers, a low intraspecific variability was detected
38 among *M. izalcoensis* populations from Africa, Vietnam and Brazil, except for the
39 population from El Salvador that showed fewer genetic differences from the other
40 populations. Phylogenetically, all populations of *M. izalcoensis* from different locations
41 (El Salvador, Kenya, Tanzania, Vietnam and Brazil) grouped with 90% and 69% bootstrap
42 support for COII and HSP90 regions, respectively, indicating that these markers are
43 highly conserved for the species. In addition, both markers allowed the separation
44 between *M. izalcoensis* populations and other important coffee *Meloidogyne* species,
45 including *M. exigua*, *M. paranaensis*, *M. incognita*, *M. arabicida* and *M. lopezi*.
46 Considering the resistance studies, four separated assays were performed with the
47 objective of evaluating the reaction of fifteen coffee genotypes to *M. izalcoensis*, using
48 the same inoculum concentration and arranged in a completely randomized design with
49 eight replications. Overall, it was observed susceptibility of the genotypes tested with
50 resistance to other *Meloidogyne* spp. (Amphillo x Catuaí, Hybrid of Timor, IAPAR 59,

IPR 99, IPR 100, IPR 102, IPR 103, IPR 105, IPR 106, IPR 107 and IPR 108), except for the rootstock cv. Apoatã IAC 2258 which proved to be moderately resistant, with a genetic segregation for this character of 43.8%.

K E Y W O R D S

AFLP, COII, *Coffea*, genetic variability, RAPD, resistance, root-knot nematode.

57 **INTRODUCTION**

58 Root-knot nematodes (RKN) *Meloidogyne* spp., are amongst the most
59 economically important plant parasitic nematodes infecting coffee (*Coffea* spp.) in several
60 countries in the Americas, including Brazil, El Salvador, Guatemala, Costa Rica and
61 Hawaii, in Africa and in other continents (Campos and Villain, 2005; Villain et al., 2018).
62 Recently, a new species *Meloidogyne izalcoensis* Carneiro, Almeida, Gomes and
63 Hernandez, 2005 was detected on coffee in Brazil (Stefanelo et al., 2018). This species
64 was first described from a population collected in El Salvador where it causes substantial
65 plant damage (Carneiro et al., 2005a; Villain et al., 2018) and detected in Africa in Kenya
66 (Kabete) and Tanzania (Mufindi) without damage information (Jorge Júnior et al., 2016).
67 *Meloidogyne izalcoensis* causes small galls with external egg masses and necrosis at root
68 ends (Figure 1), showing different root symptoms when compared with other
69 *Meloidogyne* spp. from coffee in Brazil (Carneiro et al., 2005a; Stefanelo et al., 2018).

70 The morphological diagnosis of *M. izalcoensis* is difficult due to the similarity of
71 its perennial pattern to that of *M. incognita* and *M. paranaensis* Carneiro, Carneiro,
72 Abrantes, Santos and Almeida, 1996 (Carneiro et al., 2005a). Biochemically, the esterase
73 (Est I4) phenotype is unique and can be used to differentiate *M. izalcoensis* from other
74 RKN species of coffee (Carneiro and Cofcewicz, 2008).

75 Application of molecular tools, particularly neutral molecular markers, e.g.,
76 random amplified polymorphic DNA – RAPD, amplified fragment length polymorphisms
77 – AFLP have been extensively used to analyze the genetic diversity within *Meloidogyne*
78 spp. from coffee, *i.e.*, *M. exigua* Göldi, 1887, *M. incognita* (Kofoid and White, 1919)
79 Chitwood, 1949 and *M. paranaensis* (Carneiro et al., 2004, 2008; Muniz et al., 2008;
80 Randig et al., 2002; Santos et al., 2012, 2018). Phylogenetic analyzes of coffee RKN
81 using molecular data such as rDNA and mt-DNA sequences have been used as

82 characterization methods for different species, and allowed solving evolutionary
83 processes at deep levels of divergence (Humphrey-Pereira et al., 2014; Janssen et al.,
84 2017; Santos et al., 2019). However, until now there are practically no reports on the
85 genetic variability and phylogenetic diversity of *M. izalcoensis*. Among genomic
86 sequences of interest, species-specific SCAR markers were developed for the
87 identification of Brazilian *Meloidogyne* spp. from coffee: *M. exigua*, *M. incognita* and *M.*
88 *paranaensis* (Randig et al., 2002) and more recently for *M. izalcoensis* and *M. arabicida*
89 Lopez and Salazar, 1989 (Correa et al., 2013), extending the molecular markers for all
90 RKN species from the Americas, except for *M. lopezi* Humphreys-Pereira, Flores-
91 Chavez, Gomez, Salazar, Gomez-Alpizar and Elling, 2014 described later. Also, the gene
92 encoding heat shock protein 90 (Hsp90) has been increasingly used in phylogenetic
93 studies of plant-parasitic nematodes (Mundo-Ocampo et al., 2008; Skantar and Carta,
94 2004; Skantar et al., 2008) or for species identification (Skantar et al., 2008), although
95 sequence divergence among nematode species is generally lower than mitochondrial gene
96 differences reported for the same taxa (Skantar and Carta, 2004).
97 The genetic resistance of coffee is considered one of the main nematode
98 management strategies, since it enables the reduction of nematode population density in
99 the soil and acceptable economic maintenance of crop yield in infested areas (Villain et
100 al., 2018). The main focus to produce nematode resistant rootstocks was in *C. canephora*
101 Pierre ex A. Froehner, *C. liberica* Hiern, *C. dewevrei* De Wild. and T. Durand, and *C.*
102 *congensis* A. Froehner, since these species present abundant root systems and resistance
103 to pathogens as well. However, resistance genes found in wild or semi-wild lines of *C.*
104 *arabica* L. from Ethiopia (Fatobene et al., 2017; Holderbaum et al., 2020), as well as
105 accessions of Amphillo could be used in intraspecific hybridization with other *C. arabica*
106 with good agronomic characteristics (Peres et al., 2017).

107 Most sources of resistance to RKN have been identified in *C. canephora* (Bertrand
108 et al., 2000; Sera et al., 2006). Three cultivars of *C. canephora* are commercially available
109 to be used as rootstocks for resistance to *M. exigua*, *M. incognita* and *M. paranaensis*:
110 'Apoatã' IAC 2258, 'Nemaya' and 'Goytacá' (Clone 14) and can receive grafts of *C.*
111 *arabica* and *C. canephora*. These robusta cultivars are highly recommended for planting
112 in extensive *Meloidogyne* spp. infested areas (Ferraz 2008; Fonseca et al., 2008;
113 Gonçalvez et al., 2021; Lima et al., 2015,). Timor hybrid, which is phenotypically an
114 arabica coffee is probably a natural hybrid between *C. arabica* and *C. canephora* that has
115 frequently been used in the Brazilian genetic breeding programs as a source of resistance
116 to *Meloidogyne* spp. Among its derivates are the cultivars Obatã IAC 1669-20, 'Tupi'
117 IAC 1669-33, IAC 125 RN and IAPAR -59, which are resistant to *M. exigua* and to coffee
118 leaf rust caused by the fungus *Hemileia vastatrix* Berk. and Broome (Fazuoli et al., 2018;
119 Ferraz, 2008; Muniz et al., 2009; Salgado et al., 2005). Progenies of 'Icatu Vermelho IAC
120 4160'resulted from crosses between *C. arabica* and *C. canephora* have been rated as
121 resistant to *M. paranaensis* under greenhouse and field conditions (Ferraz, 2008). An
122 important source of resistance to *M. paranaensis* used by some breeding programs in
123 Brazil was Icatu IAC 925 (Shigueoka et al., 2016), which was used to develop the cultivar
124 (IPR 106) (Sera et al., 2020). For *C. arabica*, most commercial cultivars are highly
125 susceptible to coffee RKN, with the exception of cultivars selected by coffee breeders
126 Acauã, Catucaí 785-15, IAPAR 59, IAC 125 RN, IPR 100, IPR 106, MGS Catiguá, MGS
127 Guaiçara and MGS Vereda, which are available in Brazil to be recommended for areas
128 infested with RKN, depending on the identified nematode species in the planting area.
129 These genotypes have different sources of resistance (Salgado et al., 2021; Villain et al.,
130 2018).

131 The first objective of this study was to assess the genetic variability of populations
132 of *M. izalcoensis* originating from different geographic locations in the world (El
133 Salvador, Kenya, Tanzania, Vietnam and Brazil) using neutral DNA markers, and their
134 phylogenetic relationships using distinct regions of ribosomal DNA (rDNA), the
135 mitochondrial cytochrome *c* oxidase II (COII) gene and the nuclear protein coding gene
136 HSP90. The second aim of this study was to evaluate the reaction to the Brazilian
137 population of *M. izalcoensis* of different coffee cultivars harbouring resistance genes to
138 other *Meloidogyne* spp.
139
140 **MATERIALS AND METHODS**
141 **Nematode populations**
142 Five *M. izalcoensis* populations from different geographic locations in the world
143 (El Salvador, Kenya, Tanzania, Vietnam and Brazil) were maintained by periodic
144 subculturing on tomato plants (*Solanum lycopersicum* L. cv. Santa Clara) under
145 greenhouse conditions (Table 1). All genetic and phylogenetic studies were performed
146 using these populations. They were identified using esterase (Est) phenotyping, according
147 to Carneiro and Almeida (2001) and confirmed with SCAR-PCR (Correa et al., 2013).
148 One population of *M. enterolobii* Yang and Eisenback, 1983 was included in the study as
149 an outgroup.
150 **Genomic DNA preparation**
151 For each population, total genomic DNA was extracted and purified from 100 µl
152 aliquots of eggs following the method described by Randig et al. (2002). Isolated DNA
153 was quantified in a 1% agarose gel.
154 **RAPD analysis**

155 RAPD-PCR reactions were performed in a 13 µl final volume containing 1.3 µl
156 10× PCR reaction buffer (Invitrogen®), 0.4 µl 10 µM primer, 2 µl 1.25 mM dNTPs
157 (Invitrogen®), 0.2 µl5U µl −1 Taq DNA polymerase (Invitrogen®) and 3 µl total genomic
158 DNA (3 ng µl −1) of each population. The following 30 random 10-mer oligonucleotide
159 primers (Operon Technologies) were used in the analysis: OPA-12 (TCGGCGATAG),
160 OPAB-06 (GTGGCTTGGA), OPAU-13 (CCAAGCACAC), OPC-07
161 (GTCCCGACGA), OPC-09 (CTCACCGTCC), OPF-06 (GGGAATTCGG), OPG-04
162 (AGC GTG TCT G), OPG-13 (CTCTCCGCCA), OPJ-19 (GGACACCACT), OPK-01
163 (CATTCGAGCC), OPK-01 (CATTCGAGCC), OPK-19 (CACAGGCGGA), OPL-19
164 (GAGTGGTGAC), OPM-20 (AGGTCTTGGG), OPN-07 (CAGCCCAGAG), OPN-10
165 (ACAACTGGGG), OPP-01 (GTAGCACTCC), OPP-06 (GTGGGCTGAC), OPR-03
166 (ACACAGAGGG), OPR-07 (ACTGGCCTGA), OPT-06 (CAAGGGCAGA), OPU-05
167 (TTGGCGGCCT), OPV-07 (GAAGCCAGCC), OPW-05 (GGCGGATAAG), OPW-06
168 (AGGCCCGATG), OPW-15 (ACACCGGAAC), OPX-20 (CCCAGCTAGA), OPY-05
169 (GGCTGCGACA), OPY-16 (GGGCCAATGT), OPZ-04 (AGGCTGTGCT) and OPZ-17
170 (CCTTCCCACT). Amplifications were performed on a PTC-100 thermocycler, using the
171 following settings: 5 min at 94°C; 40 cycles of 30 s at 94°C, 45 s at 36°C, 2 min at 70°C;
172 and a final extension of 10 min at 70°C (Randig et al., 2002). PCR products were
173 separated by electrophoresis in a 1.5% (w/v) agarose gel, stained with ethidium bromide
174 (0.3 µg ml−1) and visualized under UV light. All RAPD analyses were repeated at least
175 twice.

176 **AFLP analysis**

177 For each isolate, 1 µg of total genomic DNA was digested overnight at 37°C with
178 EcoRI (15 U µl $^{-1}$; Invitrogen®) and ligated to the specific adapters following the method
179 of Suazo and Hall (1999). A series of seven random 22-mer primers (Integrated DNA

180 Technologies) were used, consisting of the EcoRI adapter core sequence 5 -
181 GACTGCGTACCAATTCAGT-3 plus the 3 selective nucleotides (AGT, ACT, ATT,
182 GGC, CAG, CCT, and TCG). PCR reactions were performed in a 25 μl final volume
183 containing 1 μl (50 ng μl^{-1}) digested DNA, 2.5 μl 10× PCR buffer without magnesium
184 chloride (Invitrogen), 1 μl 50 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 1 μl 10 μM primer and
185 0.3 μl Taq DNA polymerase (5 U μl^{-1}; Invitrogen). DNA was amplified using the PT-
186 C100 thermocycler (MJ Research) under the following cycling parameters: 1 min at 95°C;
187 37 cycles of 1 min at 94°C, 1 min at 56°C, 2 min and 30 s at 72°C; and a final extension
188 of 10 min at 72°C (Suazo and Hall, 1999). PCR products were separated by
189 electrophoresis in a 1.5% (w/v) agarose-synergel (0.7% agarose, 0.4% synergel;
190 Diversified Biotech), stained with ethidium bromide (0.3 μg ml^{-1}) and photographed
191 under UV light. The analysis was repeated at least twice.

192 **Genetic diversity of *Meloidogyne izalcoensis***

193 DNA fingerprints obtained with RAPD and AFLP markers were used to infer the
194 genetic diversity of the five populations of *M. izalcoensis* plus one population of *M.*
195 *enterolobii* used as an outgroup. For each marker, amplified bands were scored as present
196 or absent from the digitized photographs of the gels and converted into a 0-1 binary
197 matrix. Phylogenetic reconstruction was performed using the Neighbour-Joining (NJ)
198 algorithm (Saitou and Nei, 1987) in PAUP∗ version 4b10 (Swofford, 2002), considering
199 the data as unordered with no weighting. Testing of node support for the resulting trees
200 was performed on 1000 bootstrap replicates with a cut-off value of 50%. Since the two
201 types of markers could be considered independent from one another, the two datasets
202 were analyzed into a global NJ analysis using the total evidence approach proposed by
203 Huelsenbeck et al. (1996) with the same settings as for the individual NJ analyses.

204 **Phylogenetic analysis of DNA regions**

205 For the phylogenetic analyses, DNA of the five previously characterized
206 populations of *M. izalcoensis* was amplified and investigated using the primers for ITS1-
207 5.8S-ITS2 region of rRNA (primer set: forward 5-TTGATTACGTCCCTGCCCTTT-3
208 and reverse 5-TCCTCCGCTAAATGATATG-3; Schmitz et al., 1998); the D2-D3
209 fragment of the 28S rRNA gene (primer set: forward 5-
210 ACAAAGTACCGTGAGGGAAAGTTG-3 and reverse 5-
211 TCGGAAGGAACCAGCTACTA-3; De Ley et al., 1999); mtDNA cytochrome *c* oxidase
212 II (COII) gene (forward 5'-GGTCAATGTTCAGAAATTTGTGG-3' and reverse 5'-
213 TACCTTTGACCAATCACGCT-3'; Powers and Harris, 1993) using the PCR conditions
214 described by Subbotin et al. (2000) and HSP90 (primer set: forward 5'-
215 GCYGATCTTGTYAACAACCYTGGAAC-3' and reverse 5'-
216 TCGAACATGTCAAAAGGAGC-3' PCR conditions according to Nischwitz et al.
217 (2013). PCR products were cleaned using the Wizard® SV Gel/PCR Clean Up System
218 (Promega) and cloned into the pGem-T® Easy Vector (Promega), following the
219 manufacturer's instructions. Sequencing of the insert was carried out on two independent
220 clones by Macrogen.

221 *Meloidogyne izalcoensis* populations cloned sequences were aligned using
222 ClustalW in Mega 5.0.3 (Tamura et al., 2011) and compared with other retrieved
223 sequences from the NCBI database of important coffee *Meloidogyne* species, *i.e. M.*
224 *exigua*, *M. paranaensis*, *M. incognita*, *M. arabicida* and *M. lopezi*. The program
225 MrModeltest (Nylander, 2004) implemented in PAUP* was used to identify the best-fit
226 models of each analysis. The phylogenetic trees were generated based on Maximum
227 Likelihood (ML) analysis in IQtree (Trifinopoulos et al., 2016). The phylogram was
228 bootstrapped 1,000 times, and only support values above 50% were considered.

229 **Resistance assays**

230 The inoculum of *M. izalcoensis* used in the experiments was multiplied in tomato
231 and coffee plants for 3 and 8 months, respectively. The eggs of *M. izalcoensis* were
232 extracted from infected roots according to the protocol described by Hussey and Barker
233 (1973), using a blender for 40 seconds instead of hand shaking. Plants of coffee were
234 sown in trays and later transplanted to polyethylene pots (20x40x0,015 cm and capacity
235 for 5 L), filled with a mixture of autoclaved soil and Bioplant® compost (1:1). When they
236 hit 4 to 6 pairs of leaves they were inoculated with 10,000 eggs of *M. izalcoensis*/plant.

237 Fifteen coffee genotypes were tested: Catuaí Amarelo IAC 62 and Catuaí
238 Vermelho IAC 99 (susceptible controls), Catuaí Vermelho x Amphillo 2-161 (28-2-II),
239 Catuaí Vermelho x Amphillo 2-161 (16-5-III), Hybrid of Timor UFV 408-01 MG (6-I-
240 III), IAPAR 59, IPR 99, IPR100, IPR 102, IPR 103, IPR 105, IPR 106, IPR 107, IPR 108
241 and Apoatã IAC 2258 (*C. canephora*) (Table 2). The plants were arranged in a completely
242 randomized design with eight replications for each experiment. Four experiments were
243 conducted under greenhouse conditions at Embrapa Genetic Resources and
244 Biotechnology (Cenargen, Brasilia, Brazil): the first one was implanted on 3/27/2018, the
245 second on 4/11/2019 (repetition), the third and fourth on 3/18/2020 and 6/12/2020,
246 respectively.

247 The evaluation of the four experiments was performed at 240-350 days after
248 inoculation (DAI). The following variables were determined: fresh root weight, gall index
249 (GI), total number of eggs per gram of root, total number of eggs, reproduction factor
250 (RF) and global reaction. Before evaluating the trials, some supplementary control plants
251 were always analyzed to determine whether the reproduction factor was high enough to
252 evaluate the bioassays (Hussey and Jansen, 2002).

253 To determine fresh root weight, roots were carefully washed to remove adhered
254 soil and, after excess water was removed, they were weighed. The root systems were

255 stained with Phloxine B at 0.0015 % (15 mg/L) for 15-20 minutes and evaluated for gall
256 index. The gall index (GI) was determined based on a 0 to 5 scale proposed by de Hartman
257 and Sasser (1985), as follows: 0 = absence; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to
258 100, and 5 = more than 100 galls per plant. The extraction of eggs was performed
259 according to the methods mentioned above, using 1% NaOCl. The total number of eggs
260 per plant was determined under a light microscope using Peters' slides in triple count.
261 The reproduction factor (RF) was calculated as RF = FP/IP, where FP corresponds
262 to the final nematode population and IP refers to the initial nematode population (IP =
263 10,000), according to Oostenbrink (1966). Genotypes for which RF < 1 were considered
264 resistant (R), while those for which RF > 1 were considered susceptible (S), according to
265 Sasser et al. (1984). Genotypes were also scored using intermediate criteria as: low
266 susceptibility (LS), susceptibility (S), high susceptibility (HS) and moderate resistance
267 (MR), using statistical analysis. Data were transformed in log (x+1) prior to analysis of
268 variance, and treatments were compared using Scott-Knott test (P<0,05), using the
269 software SISVAR (Ferreira, 2011).
270
271 **RESULTS**
272 **Characterization of nematode populations**
273 The esterase phenotype (Est I4, Rm: 0.86, 0.96, 1.24, 1.30) allowed the
274 identification for all *M. izalcoensis* populations (Figure 2), as reported in the description
275 of Carneiro et al. (2005a). To confirm the identification, the specific SCAR marker
276 developed for *M. izalcoensis*, i.e., a single amplicon of 670 bp, was obtained for all the
277 *M. izalcoensis* populations (Iza 1-5) used in this study (Figure 3).
278 **Genetic diversity of *Meloidogyne izalcoensis***

279 For the study on genetic diversity, a total of 7 AFLP and 30 RAPD primers were
280 used. The sizes of amplified fragments ranged from 200 to 4500 bp and the cumulative
281 number of reproducible amplified fragments was 306 for the two markers. Overall, there
282 was a low level of polymorphism in the *M. izalcoensis* populations (22%) (Figure 4). The
283 0-1 binary matrix (absence/presence of fragments) obtained from the entire set of markers
284 was used to infer the genetic relationships among the isolates. The results from the NJ
285 dendrogram confirmed a low intraspecific variability among *M. izalcoensis* populations
286 from Africa (Kenya and Tanzania), Vietnam and Brazil, with 91% bootstrap, while the
287 population from El Salvador exhibited a few genetic differences from the other
288 populations (Figure 4).

289 **Phylogenetic analysis of DNA regions**

290 In the phylogenetic analysis obtained from the intergenic ITS1-5.8S-ITS2 gene
291 and the D2D3 region of the 28S gene, respectively, *M. izalcoensis* populations did not
292 form cohesive clusters (Figure 5a and 5b), suggesting a poor phylogenetic resolution of
293 these sequences.

294 However, for the two other sequences, all populations of *M. izalcoensis* from
295 different origins grouped together with 90% and 69% bootstrap support for COII and
296 Hsp90 regions, respectively (Figures 6 and 7), indicating that these markers are highly
297 conserved for this species. In addition, both markers allowed the separation between *M.*
298 *izalcoensis* populations and other important RKN coffee species, including *M. exigua*, *M.*
299 *paranaensis*, *M. incognita*, *M. arabicida* and *M. lopezi*, showing to be excellent specific
300 markers for these species.

301 **Resistance and susceptibility assays**

302 In the four assays (Tables 3, 4, 5 and 6), the differences of fresh root weight
303 between all cultivars, especially the Apoatã rootstock and cv IPR 103 were evidenced by

304 statistical analysis, these differences are not very important since plants genetically very
305 different were compared.
306 In the first experiment (Table 3), regarding the number of eggs per gram of root,
307 only the genotype Catuaí Vermelho x Amphillo 2-161 (16-5-III) and the rootstock Apoatã
308 IAC 2258 differ statistically from the control. When considering the reproduction factor
309 (RF), the genotypes IPR 100, Hybrid of Timor and Catuaí Vermelho x Amphillo 2-161
310 (28-2-II) did not differ statistically from the control and were considered highly
311 susceptible (HS) to *M. izalcoensis*. In contrast, the genotype Catuaí Vermelho x Amphillo
312 2-161 (16-5-III) and Cv. Apoatã IAC 2258 differed statistically from the control and are
313 considered susceptible and moderately resistant with a RF reduction of 83 %,
314 respectively.
315 In the repetition of the first experiment (Table 4), the results were similar to those
316 of the first trial, with no differences in the reaction between cultivars. Again, the ‘Apoatã
317 rootstock’ was the only one that proved to be moderately resistant. Analyzing the 16
318 individual replications of cv Apoatã, a genetic segregation of 43.8% occurred, with RFs
319 values ranging from 9.8 to 0, showing susceptible and resistant plants in the same
320 treatments (data not shown).
321 In the third and fourth experiments (Tables 5 and 6), the genotypes IAPAR 59,
322 IPR 102, IPR 105, IPR 106, IPR 107, IPR 108 and IPR 99 showed a higher number of
323 eggs per gram of root when compared to the control. In the third experiment, there was
324 no statistical difference between the cultivars IPR 100, IPR 103 and the control treatment
325 regarding the number of eggs per gram of root (Table5). As the reproduction factor (RF)
326 is concerned, the genotypes IAPAR 59, IPR 100, IPR 102, IPR 105, IPR 106, IPR 108
327 and IPR 99 did not differ statistically from the control and were considered susceptible to
328 *M. izalcoensis*. In both experiments, the IPR 103 genotype had the lower RF when

329 compared to all other treatments and was considered with low susceptibility (LS).
330 Probably due to the smaller size of the roots (1/5 of the control), the reproduction factor
331 was smaller, since this parameter depends on the total number of eggs/plant, which
332 depends on the root size.

333 The gall index was the same (GI = 5) for all genotypes in the four experiments,
334 except for the rootstock cv. Apoatã IAC 2258, which presented GI=4 in experiment 1
335 (Table 3) and GI=3 in experiment 2 (Table 4). All coffee cultivars and genotypes tested
336 showed characteristic symptoms of *M. izalcoensis*: presence of small rounded galls,
337 usually in greater numbers on younger roots, necrosis at the root tip and external egg
338 masses. Nevertheless, Apoatã IAC 2258 showed typical symptoms of *M. izalcoensis* in
339 some plants evaluated, whereas in other plants these symptoms were not observed (data
340 not shown). This behavior is probably related to the segregation present in this cultivar as
341 commented above.

342 Most genotypes evaluated in the four experiments (Catuaí Amarelo 62, Catuaí
343 Vermelho IAC 99, Hybrid of Timor HT UFV 408-01 MG 0294 pl.1 R1 (6-I-III), Catuaí
344 Vermelho x Amphillo 2-161 MG 0179 pl3 R1 (28-2-II), Catuaí Vermelho x Amphillo 2-
345 161 MG 0179 pl1 R1 (16-5-III), IAPAR 59, IPR 99, IPR 100, IPR 102, IPR 105, IPR
346 106, IPR 107 and IPR 108) showed susceptibility to *M. izalcoensis*. Only the rootstock
347 cv. Apoatã IAC 2258 can be considered as moderately resistant with some plants
348 segregating for this character (data not shown).

349

350 **DISCUSSION**

351 Intraspecific variation and genetic structure are not well characterized for *M.*
352 *izalcoensis* populations, but such information is crucial to avoid misidentification and to
353 optimize effective control strategies (Monteiro et al., 2016). Genetic studies along with

354 traditional taxonomic methods integrated with enzymatic and molecular approaches
355 proved to be useful in solving problems of nematode identification (Santos et al., 2012;
356 2018). This is the first study on genetic and phylogenetic characterization of different
357 populations of *M. izalcoensis,* a species from coffee recently detected in Brazil (Stefanelo
358 et al., 2018).

359 The development of molecular techniques has opened new prospects for
360 identification of *Meloidogyne* species and for studies on intraspecific variability and
361 phylogenetic relationships in distinct regions of ribosomal DNA (rDNA), mitochondrial
362 gene cytochrome *c* oxidase II (COII) and nuclear protein coding gene (Hsp90) for RKNs
363 (Carneiro et al., 2004; Leite et al., 2020; Randig et al., 2002; Santos et al., 2012; 2018).
364 SCAR markers have been developed for RKNs often associated with coffee in Brazil and
365 the Americas (Correa et al., 2013; Randig et al., 2002), *i.e. M. exigua*, *M. paranaensis*
366 and *M. incognita* (Randig et al., 2002) and for the new species *M. izalcoensis* and *M.*
367 *arabicida* (Correa et al., 2013). In the present study, this approach was validated by
368 analyzing five *M. izalcoensis* populations from different geographic locations in the world
369 and exhibiting the same esterase phenotype (Est I4). While esterase isozyme phenotypes
370 are restricted to the characterization of RKN females (Carneiro and Cofcewicz, 2008),
371 PCR-based methods are more suitable for routine diagnosis, especially, in coffee roots
372 infected with *Meloidogyne* spp., where females collected from fields are frequently in bad
373 conditions of preservation, making the identification difficult when using esterase
374 isozyme only (Muniz et al., 2008). PCR is fast, can be used on a large number of samples
375 and can be applied to eggs and J2 from field soil samples and roots. It also does not require
376 nematode multiplication on the host plant until they reach female adult stages (Carneiro
377 et al., 2000).

378 Neutral molecular markers, such as RAPD and AFLP, have been used to analyze
379 the genetic diversity of *Meloidogyne* species from coffee (Carneiro et al., 2004; Muniz et
380 al., 2008; Randig et al., 2002; Santos et al., 2012; 2018). Here, using these markers, a low
381 genetic variability was found among five *M. izalcoensis* populations from different
382 geographic locations in the world. This low genetic variability was also observed for other
383 *Meloidogyne* spp. related to coffee, *i.e.*, *M. incognita* (Santos et al., 2012) and *M.*
384 *paranaensis* (Santos et al., 2018). However, the opposite was observed among
385 populations of *M. exigua*, among which a high level of genetic variability was observed
386 (Muniz et al., 2008). This result is to be linked to the mode of reproduction of the
387 nematodes, i.e., mitotic pathenogenesis for *M. incognita*, *M. paranaensis* and M.
388 izalcoensis, and meiotic parthenogenesis for *M. exigua.*

389 Sequences of ITS1-5.8S-ITS2, D2-D3 (28S) rRNA, mitochondrial COII and
390 HSP90 genes have been used to infer phylogenetic relationships in RKN species and may
391 allow evolutionary processes to be solved at deep levels of divergence (Humphreys-
392 Pereira et al., 2014; Janssen et al., 2017; Santos et al., 2019). However, in the present
393 study, an overall lack of polymorphic sequences was noticed for *M. izalcoensis*
394 populations.

395 The ribosomal DNA is relatively conserved with high similarity among the three
396 most common tropical root-knot nematode species, *M. incognita*, *M. arenaria* (Neal,
397 1889) Chitwood, 1949 and *M. javanica* (Treub, 1885) Chitwood, 1949, and is therefore
398 not useful as a barcode region for identifying these species (Janssen et al., 2017; Ye et al.,
399 2015). Despite this limitation, it can be an useful tool for identification of other species
400 such as *M. chitwoodi* Golden, O'Bannon, Santo and Finley, 1980 and *M. fallax* Karssen,
401 1996 (Onkendi and Moleleki, 2013; Ye et al., 2015). The combined analyses of 18S and
402 28S nuclear rDNA sequences also distinguished haplotypes of *Meloidogyne* species on

403 coffee (Herrera et al., 2011). The multi-copy basis of rDNA provides a sample target for
404 PCR amplification, and it allows the intraspecific variation to be detected in *Meloidogyne*
405 spp. populations (Adam et al., 2007; Zijlstra et al., 1995). However, in the analyzes
406 obtained from the D2D3 regions of the 28S and intergenic ITS1-5.8S-ITS2 gene from the
407 rDNA, *M. izalcoensis* populations did not form cohesive clusters, demonstrating a
408 phylogenetic limitation of these markers.

409 In complement, the region encoding the HSP90 gene has also been used to
410 distinguish different *Meloidogyne* species (Blouin, 2002; Onkendi and Moleleki, 2013;
411 Skantar et al., 2008; Tigano et al., 2005). Here, a low diversity was reported for HSP90
412 sequences among *M. izalcoensis* populations.

413 From the perspective of identification, the mitochondrial genome provides a rich
414 source of genetic markers for identification (Hu and Gasser, 2006). This region has a
415 faster rate of evolution than the corresponding nuclear genes (Blok and Powers, 2009). In
416 fact, the mitochondrial coding region is already considered a better potential barcode
417 region for some *Meloidogyne* spp. groups, since intergenic regions have been shown to
418 contain AT repeats that appear not to correlate with speciation events (Pagan et al., 2015).
419 This region has been used for diagnosis of *Meloidogyne* spp. (Blok et al., 2002; Brito et
420 al., 2004; Powers and Harris, 1993). In the present study, *M. izalcoensis* COII sequences
421 proved to be useful for both species identification and phylogenetic studies, as observed
422 for some other RKN coffee species, *i.e.*, *M. paranaensis* (Santos et al., 2019). Indeed, this
423 marker allowed the separation between *M. izalcoensis* populations and other important
424 *Meloidogyne* species of coffee, including *M. exigua*, *M. paranaensis*, *M. incognita*, *M.*
425 *arabicida* and *M. lopezi*.

426 Considering coffee resistance to *M. izalcoensis,* our study both confirmed
427 previous results and provided new information. Similar results to those obtained here for

428 the Timor Hybrid UFV 408-01 have been reported by Carneiro et al. (2008), testing the
429 H419-5-4-5 progeny obtained from artificial crossing between the cultivar Catuaí
430 Amarelo IAC 30 X Timor UV 445-46, which was susceptible to *M. izalcoensis* but
431 resistant to *M. exigua*. Hernandez *et al.* (2004) did not observe resistance to *M. izalcoensis*
432 in cultivars of *C. arabica* from Ethiopia, but resistance was observed to *M. paranaensis*
433 (Boisseau et al., 2009). The same occurred with the genotypes whose source of resistance
434 are 'Amphillo' (*C. arabica* from Ethiopia) (Peres et al., 2017). Probably, resistance from
435 'Amphillo' is not efficient in controlling *M. izalcoensis*, altough it controlled *M.*
436 *paranaensis* in greenhouse (Peres et al., 2017) and under field conditions (Salgado et al.,
437 2014).

438 Some of the genotypes or crosses evaluated in this study with the Hybrid of Timor
439 as resistance source, like IAPAR 59, have already been tested for other *Meloidogyne*
440 species, in greenhouse conditions and were shown resistant to *M. exigua* and susceptible
441 to *M. incognita* and *M. paranaensis* (Muniz et al., 2009). Unfortunately, in this study the
442 five genetic materials tested with this resistance source mentioned in Table 2 were
443 susceptible to *M. izalcoensis.*

444 Other studies had shown that the rootstock cultivar Apoatã IAC 2258 is resistant
445 to *M. paranaensis* (Fonseca et al., 2008; Santos et al., 2018), *M. exigua* (Salgado et al.,
446 2005) and *M. incognita* (Fonseca et al., 2008; Sera et al., 2006). Since 1987, the adoption
447 of such multi-resistant rootstock cultivars has been recommended for nematode
448 management in infested coffee-growing areas, being justified in field conditions because
449 more than one species of *Meloidogyne* may occur simultaneously (Carneiro et al., 2005b;
450 Sera et al., 2017). The resistance of this rootstock originates from *C. canephora* (clone
451 T3561) belonging to the CATIE germplasm collection from Costa Rica (Bertrand and
452 Anthony, 2008). In this study, we observed only moderate resistance of the cultivar

453 Apoatã IAC 2258 to *M. izalcoensis*, with confirmed segregation. The same was observed
454 for different populations of *M. paranaensis* in greenhouse conditions (12% segregation,
455 Santos et al., 2018). Although the use of resistant cultivars or rootstocks is an
456 environmentally correct and efficient control method, it shows limitations, such as
457 segregation for susceptibility to nematodes (10 to 15%), breakage in the grafting region
458 and need of replanting (about 10 to 15%) (Gonçalves and Silvarolla, 2007).

459 All the cultivars developed by IDR-Paraná evaluated in this study are considered
460 susceptible to *M. izalcoensis*, although, in other studies, some of these cultivars showed
461 resistance to other *Meloidogyne* species, and represent an important tool, as they enable
462 the reduction of nematode population density in an economically acceptable way
463 (Barrantes et al., 2020). The Arabica cultivar IPR 100 carrying genes from *C. liberica*
464 (Sera et al., 2017) is resistant to *M. exigua* (Rezende et al., 2017), *M. paranaensis*
465 (Andreazi et al., 2015; Santos et al., 2018; Sera et al., 2017) and *M. incognita* populations
466 (Sera et al., 2017). The resistance of cultivar IAPAR 59 to different populations of *M.*
467 *exigua* was observed by Muniz et al. (2009) and Salgado et al. (2005), but this cultivar is
468 susceptible to *M. paranaensis*, *M. incognita* (Muniz et al., 2009) and *M. izalcoensis* (this
469 study). There is evidence that IAPAR 59 resistance to *M. exigua* originates from the Timor
470 Hybrid, since the resistance to this nematode has been shown to be controlled by one major
471 gene in this genotype (Noir et al., 2003) with incomplete dominance (Alpizar et al., 2007).
472 Barrantes et al. (2020) showed that the cultivars IPR 99, IPR 100, IPR 102, IPR 103 and
473 IPR 107 also showed resistance to *M. exigua* from Costa Rica. Despite the cultivar IPR
474 106 has not shown resistance to *M. izalcoensis*, the resistance of this cultivar has already
475 been demonstrated to *M. paranaensis* and *M. incognita* (Sera et al., 2020). It is believed
476 that the cultivars IPR 98, IPR 99 and IPR 107 inherited the *Mex-1* gene from the Timor
477 Hybrid CIFC 832/2, as demonstrated for the 'IAPAR 59', while cultivars IPR 102 and

478 IPR 103 would have inherited the resistance of *C. canephora* from Icatu (Barrantes et al., 479 2020).

480 Sera et al. (2020) evaluated the performance of Arabica coffee cultivars in area 481 infested by the nematode *M. paranaensis* and *M. incognita* and confirmed that the cultivar 482 IPR 100 and IPR 106 showed the best performance with a high vegetative vigor and yield, 483 while the cultivars IPR 103, IAPAR 59, and Catuaí Vermelho IAC 81 showed reduction 484 in yield production and poor vegetative development of plants with up to 45% of dead 485 plants after seven years of planting.

486 In studies of genetic diversity of coffee root-knot nematodes, *M. izalcoensis* was 487 separated phylogenetically from all other species (*M. exigua*, *M. paranaensis*, *M.* 488 *incognita*, *M. arenaria, M. enterolobii* and *M. lopezi*). In this way, it is expected that the 489 sources of genetic resistance to this species are also different. The present study has 490 demonstrated that the sources of resistance of the coffee plants to *M. izalcoensis* are not 491 the same as those previously reported for *M. paranaensis*, *M. incognita* and *M. exigua*. 492 Although some available sources of resistance as the Hybrid of Timor, Amphillo, Icatu 493 IAC 925 and IPR 100 (*C. liberica*) showed susceptibility to *M. izalcoensis*, cultivars as 494 Apoatã IAC 2258, whose source of resistance originates from a pure *C. canephora*, are 495 promising for studying resistance to *M. izalcoensis.* Other pure *C. canephora* genotypes 496 should be studied, such as the cultivar 'Goytacá' (=Clone 14) recently registered by 497 INCAPER, and the polyclonal cultivars from IAC, composed of clones CcK1, CcR2, 498 CcR8 and CcR10, which according to Gonçalves et al. (2021) are resistant to *M. exigua*, 499 *M. paranaensis* and *M. incognita*. Seed fields for commercial production of IAC clones 500 will be established after registration and protection of the clones.

501

502 **ACKNOWLEDGEMENTS**

503 We gratefully acknowledge the financial support of the Brazilian Consortium of Coffee
504 to EMBRAPA Recursos Genéticos e Biotecnologia.
505

506 **CONFLICT OF INTEREST**

507 The authors have no conflict of interest to declare.
508

509 **DATA AVAILABILITY STATEMENT**

510 The data that support the findings of this study are available from the corresponding
511 author upon reasonable request.
512

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869 **FIGURES LEGENDS**

870 **FIGURE 1.** Symptoms caused by the root-knot nematode *Meloidogye izalcoensis* on
871 coffee (*Coffea arabica* cv. Mundo Novo) roots stained with Phoxina B . A) Small round

872 galls induced by the nematode, egg-mass outside the gall tissue and necrosis at the end of
873 roots. B) Whole root view. Photos: Cláudio Bezerra.
874 **FIGURE 2.** Esterase phenotypes of *Meloidogyne izalcoensis* (Est I4). *M. javanica* (Est
875 J3) is included as a reference.
876 **FIGURE 3.** PCR amplification patterns for five populations of *Meloidogyne izalcoensis*
877 generated with specific SCAR primer izAB02F/R (Correa et al., 2013). (1-5): *M.*
878 *izalcoensis,* (-) DNA: negative control. M: 1kb Plus DNA ladder (Invitrogen).
879 **FIGURE 4.** Concatenated Neighbor-Joining (NJ) tree showing the analysis of genetic
880 variability (by RAPD and AFLP) of *Meloidogyne izalcoensis* populations. Numbers to
881 the left of the arms are the bootstrap values of 1000 replicates. Population codes detailed
882 in Table 1.
883 **FIGURE 5.** Maximum Likehood analysis showing the phylogenetic relationships of
884 *Meloidogyne izalcoensis* populations with related species based on the ITS1-5.8S-ITS2
885 rRNA sequences (a), and on the D2-D3 fragment of 28S rRNA sequences (b),
886 respectively. Numbers to the left of the branches are bootstrap values for 1000
887 replications. Populations codes and accession numbers for gene sequences retrieved from
888 Genbank are listed following species names.
889 **FIGURE 6.** Maximum Likehood analysis showing the phylogenetic relationships of
890 *Meloidogyne izalcoensis* populations with related species based on the COII-mtDNA
891 sequences. Numbers to the left of the branches are bootstrap values for 1000 replications.
892 Populations codes and accession numbers for gene sequences retrieved from Genbank are
893 listed following species names.
894 **FIGURE 7.** Maximum Likehood analysis showing the phylogenetic relationships of
895 *Meloidogyne izalcoensis* populations with related species based on the Hsp90 gene
896 sequences. Numbers to the left of the branches are bootstrap values for 1000 replications.

897 Populations codes and accession numbers for gene sequences retrieved from Genbank are

898 listed following species names.

899