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Genetic variability of *Meloidogyne paranaensis* populations and their aggressiveness to susceptible coffee genotypes

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Meloidogyne paranaensis is one of the most destructive root-knot nematode (RKN) species parasitizing coffee in Brazil and in the Americas generally. The objectives of this study were to assess the genetic variability, aggressiveness and virulence of seven different *M. paranaensis* populations on susceptible and resistant *Coffea* spp. All seven RKN populations were identified by biochemical and molecular methods. Coffee seedlings were inoculated in the greenhouse, and the nematode reproduction factor was used to infer their reproduction on coffee genotypes. Phylogenetic studies showed a low genetic variability in *M. paranaensis* populations, regardless of the existence of three esterase phenotypes (Est P1, P2 and P2a), except for the population Est P2a from Guatemala, which is genetically different from other *M. paranaensis* populations from Brazil. The Est P2a and Est P2 (Herculândia, SP, Brazil) populations were the most aggressive on two susceptible *C. arabica* cultivars under greenhouse conditions. None of the *M. paranaensis* populations were virulent on resistant coffee genotypes, confirming their resistance to the seven *M. paranaensis* populations tested. The resistant coffee cultivars, namely Clone 14 INCAPER, Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III), Apoatã IAC 2258, Timor Hybrid UFV 408-01 (E1 6-6 II) and IPR 100, exhibited segregation for resistance in the ratio of 0%, 2.4%, 12%, 26% and 29%, respectively. These are promising results, because they validate resistance against several *M. paranaensis* populations in different *Coffea* spp. genetic resources, which can be used in breeding programmes or as rootstocks, such as Apoatã IAC 2258 and Clone 14 INCAPER.

Keywords: *Coffea* spp., esterase phenotypes, molecular markers, resistance, root-knot nematodes, virulence

Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are the most economically important plant-parasitic nematodes infecting coffee (*Coffea* spp.) in the world (Campos & Villain, 2005). Studies of intraspecific variability and aggressiveness of *Meloidogyne* spp. populations are essential, because genetic resistance is considered one of the most recommended control strategies (Bertrand & Anthony, 2008).

Meloidogyne paranaensis is a major RKN species on coffee due to its worldwide economic importance (Carneiro *et al.*, 1996). It is commonly found in major regions of coffee production in Brazil (i.e. Minas Gerais, São Paulo and Paraná States; Gonçalves & Silvarolla, 2007; Ferraz, 2008), in the Americas, including Guatemala and Hawaii (Carneiro & Cofcewicz, 2008), and more recently in Mexico (Lopez-Lima *et al.*, 2015). This nematode species is highly aggressive to *Coffea arabica* genotypes, which results in limited growth and reduced yield of plants cultivated in infected fields (Ferraz, 2008).

Sources of resistance to RKN have been identified in *C. canephora* and in some progenies of interspecific hybrids, i.e. Timor Hybrid IAPAR 59 (*C. arabica* × *C. canephora*) (Gonçalves & Silvarolla, 2007; Bertrand & Anthony, 2008; Ferraz, 2008), a hybrid with the major *Mex-1* locus derived from *C. canephora* with resistance to *M. exigua* (Noir *et al.*, 2003). The rootstocks Apoatã IAC 2258 and Nemaya (*C. canephora*) derived from the CATIE germplasm collection are also resistant to *Meloidogyne* spp. (Bertrand & Anthony, 2008; Ferraz, 2008). More recently, resistance to *Meloidogyne* spp. was confirmed in *C. canephora* 'Conilon' clonal cultivars, and Clone 14 INCAPER stood out by showing multiple resistance to RKN and drought tolerance (Lima *et al.*, 2015). In Brazil, Sera *et al.* (2009) confirmed that IPR 100 is a new source of resistance to *M. paranaensis*; this is a cultivar with resistance genes inherited from *C. liberica*, thus being recommended for planting in areas infested by this nematode.

In *C. arabica*, all commercial cultivars are considered susceptible to *M. paranaensis* (Anzueto *et al.*, 2001), while some wild arabica coffee trees from Ethiopia are considered

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resistant to this species (Anzueto *et al.*, 2001; Boisseau *et al.*, 2009). Recently, it was shown that some crosses of Catuaí Vermelho × Amphillo MR2161 are highly resistant to *M. paranaensis* under greenhouse/field conditions and showed good yield. This is the only genuine cross material of *C. arabica* × *C. arabica* that is resistant to both *M. paranaensis* and *M. incognita* (Peres *et al.*, 2017).

Resistance genes can be introgressed from wild coffee into cultivated genotypes at either intraspecific or interspecific levels (Bertrand & Anthony, 2008). Studies showed that crossing between *C. arabica* × *C. canephora* and *C. arabica* × *C. liberica* resulted in progenies with resistance to *M. paranaensis*; however, they have segregating phenotypes for this trait (Gonçalves *et al.*, 1996; Sera *et al.*, 2009).

Recently, three different *M. paranaensis* populations (esterase phenotypes: Est P1, P2 and P2a) were detected in coffee plantations in Brazil and Guatemala (Carneiro *et al.*, 2004; R. M. D. G. Carneiro, unpublished data).

The development of molecular techniques has opened up new prospects for *Meloidogyne* species identification and for the study of intraspecific variability of RKNs. Neutral molecular markers, such as RAPD and AFLP, have been used to analyse the genetic diversity of *Meloidogyne* species from coffee (Randig *et al.*, 2002; Carneiro *et al.*, 2004; Muniz *et al.*, 2008; Santos *et al.*, 2012). Based on PCR-RAPD analysis, some *M. paranaensis* species showed low intraspecific variability (Carneiro *et al.*, 2004), and the RAPD fragments were transformed into sequence characterized amplified region (SCAR) markers (par-C09-F/R; Randig *et al.*, 2002). This specific marker was validated for *M. paranaensis*, using a few populations of this species (Carneiro *et al.*, 2004).

Variation in pathogenesis observed among populations of RKN can be expressed at three levels: (i) (non)-host status, (ii) aggressiveness, and (iii) virulence. Aggressiveness reflects the ability of nematodes to reproduce on a susceptible host, as measured by the nematode reproduction factor, whereas virulence is their ability to reproduce on resistant genotypes (Hussey & Janssen, 2002).

Research on genetic variability, aggressiveness and virulence among RKN populations affecting coffee is still scarce; to the authors' knowledge no reports are available on these topics regarding different populations of *M. paranaensis* in coffee. To date the variability seen in some populations of *M. paranaensis* showing different esterase phenotypes (Est P1, P2 and P2a) has not been correlated to their aggressiveness on different *Coffea* spp. The objectives of this study were to assess the genetic variability, aggressiveness and virulence of seven *M. paranaensis* populations on susceptible and resistant *Coffea* spp. genotypes and to clarify the genetic segregation phenotype for these genetic materials.

Materials and methods

Identification of nematode species and races

Six populations of *M. paranaensis* originating from Brazil and one from Guatemala were used in this study (Table 1). They were identified using esterase (Est) phenotyping according to Carneiro & Almeida (2001) and confirmed with SCAR-PCR (Randig *et al.*, 2002). Races of *M. paranaensis* were determined according to Hartman & Sasser (1985). A population of *M. enterolobii* (esterase phenotype E2) was used as outgroup in the diversity analysis (Table 1).

Table 1 List of *Meloidogyne* spp. isolates used in this study

Population	Species	Origin	Original host species	Est[a]
Par 1	*M. paranaensis*	Londrina, PR, Brazil	*Coffea arabica*	P2
Par 2		Herculândia, SP, Brazil	*C. arabica*	P2
Par 3		Guatemala	*C. arabica*	P2a
Par 4		Rolândia, PR, Brazil	*C. arabica*	P1
Par 5		Piumbí, MG, Brazil	*C. arabica*	P1
Par 6		São Paulo, SP, Brazil	*C. arabica*	P1
Par 7		Franca, SP, Brazil	*C. arabica*	P1
Ent 1	*M. enterolobii*	Petrolina, PE, Brazil	*Psidium guajava*	E2

[a]Esterase isozyme phenotype.

Eggs, second-stage juveniles and DNA extractions

Egg extraction was done according to Carneiro *et al.* (2004), and second-stage juveniles (J2) were extracted from egg masses, handpicked and placed on a modified Baermann funnel for hatching.

Total genomic DNA was extracted from *c.* 200 to 300 µL of nematode eggs using a regular phenol-chloroform extraction method as described by Randig *et al.* (2002). Genomic DNA was also extracted from single J2 using the Quick gDNA Mini-Prep extraction kit (Zymo Research) according to the manufacturer's instructions.

Random amplified polymorphic DNA (RAPD) analysis

The RAPD reactions were performed in a volume of 13 µL containing 9 ng genomic DNA, using the PCR conditions described by Randig *et al.* (2002). The following 30 random 10-mer oligonucleotide primers (Operon Technologies) were used in the analysis: A4, A10, A12, A14, AB03, AB04, AQ12, AS08, B6, B12, D13, E15, E18, G4, G5, G13, H01, K1, K16, K19, K20, L08, M10, M20, N7, P1, P5, R4, R7 and R8. Amplification products were separated by agarose gel electrophoresis as previously described (Randig *et al.*, 2002).

Amplified fragment length polymorphism (AFLP) analysis

For each sample, approximately 1 µg genomic DNA was digested with *Eco*RI and ligated to *Eco*RI adaptors in a 20 µL volume and incubated overnight at 37 °C (Suazo & Hall, 1999). The digestion-ligation reactions were diluted with Tris-EDTA buffer to a final volume of 200 µL and stored at −20 °C. A series of thirteen 13-mer primers (Integrated DNA Technologies)

were used, consisting of the *Eco*RI adapter core sequence 5′-GACTGCGTACCAATTCAGT-3′ plus the 3′ selective nucleotides AGT, ACT, ATT, GGC, CAG, TGG, CCT, ACC, GCC, CGA, CTC, CAT and CCG. Amplification products were separated by electrophoresis as previously described (Semblat *et al.*, 1998).

Phylogenetic analysis

Amplified bands were scored as present or absent from the digitized gels, and DNA fingerprints of the populations were converted into a binary matrix. Phylogenetic analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm implemented in PAUP* v. 4b10 (Swofford, 2002). The dataset was assumed to be unordered without weighting. For all analyses, 1000 bootstrap replicates were performed to test the node support of the generated trees. Moreover, as the RAPD and AFLP markers amplified here could reasonably be considered as independent characters, the datasets that exhibited polymorphism between populations were combined to run a global UPGMA analysis, according to the total-evidence approach (Huelsenbeck *et al.*, 1996), using the same computer settings as in the two individual analyses. A population of *M. enterolobii* was used as outgroup.

Coffee genotypes

Coffea genotypes used in this study (Table 2) were obtained from the breeding programmes of Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG), Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper) and Instituto Agronômico (IAC). These genotypes had been previously tested and were shown to be resistant to *Meloidogyne* spp. (Ferraz, 2008; Lima *et al.*, 2015; Peres *et al.*, 2017).

Nematode inoculum

Six populations of *M. paranaensis* originating from Brazil and one from Guatemala maintained in *C. arabica* 'Mundo Novo' were used in this study (Table 1). Prior to inoculation, the populations were multiplied on tomato (*Solanum lycopersicum* 'Santa Clara') for 3 months under greenhouse conditions. Eggs were extracted from infected roots using 0.5% NaOCl according to Hussey & Barker (1973), using a blender instead of manual agitation. Counting was done by using a light microscope and Peter's slides.

Table 2 Description of *Coffea* spp. genotypes used in this study

Assay[a]	Species	Cultivar	Phenotype[b]
1	*Coffea arabica*	Catuaí IAC 81	S
	C. canephora	Clone 14 INCAPER	R
2	*C. arabica*	Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III)	R
	C. canephora	Apoatã IAC 2258	R
	C. arabica	Timor Hybrid UFV 408-01 (E1 6-6 II)	R
	C. arabica	IPR 100	R
	C. arabica	Mundo Novo 379-19	S

[a]Genotypes tested in assay 1 (Table 3) and assay 2 (Table 4).
[b]S, susceptible; R, resistant to *Meloidogyne* spp.

Evaluation of *M. paranaensis* aggressiveness on coffee cultivars

Two assays were carried out to study the aggressiveness/virulence among *M. paranaensis* populations against *Coffea* genotypes (Table 3). Eight plants of each genotype were grown in pots (20 × 15 cm) filled with a mixture (1:1) of autoclaved soil and Bioplant compost and maintained at 25–30 °C under greenhouse conditions. Seedlings of about 10–20 cm height were inoculated with 10 000 eggs of *M. paranaensis* by pipetting the nematode suspension into 2–3 cm holes in the soil around the stem base. Plants were arranged in a randomized block design with six replications, and were watered and fertilized as needed. Eight months after inoculation, the root systems were rinsed under tap water and weighed. Eggs were extracted using a modified extraction method according to Hussey & Barker (1973), using 1% NaOCl. The reproduction factor (RF) was calculated as RF = FP/IP, where FP = final nematode population and IP = initial nematode population (IP = 10 000). The average RF was transformed as $\log(x + 1)$, submitted to analysis of variance and the means separated using Scott–Knott's test ($P < 0.05$). A highly resistant genotype supports little RKN reproduction (RF < 10% of the susceptible genotypes), whereas a partially resistant genotype supports an intermediate level of reproduction (Hussey & Janssen, 2002). Based on this concept, genotypes were classified as susceptible (RF > 2), resistant (1 < RF < 2) or highly resistant (RF < 1). The percentage of segregating plants was calculated based on 56 (eight replicates × seven *M. paranaensis* isolates) plants per genotype: replications that presented RF > 2 were considered segregating for resistance.

Results

Characterization of nematode populations

Three phenotypes for EST activity were recognized among the seven *M. paranaensis* populations from coffee (Table 1). The phenotype P1 (relative electrophoretic mobility compared to marker dye, Rm, 1.32) was detected in the populations Par 4, Par 5, Par 6 and Par 7 (Fig. 1a), while the phenotype P2, with a major band (Rm 1.32) and a minor band (Rm 1.0), was detected in populations Par 1 and Par 2 (Fig. 1b). The EST phenotype P2a (Rm 0.9 and 1.32) was only detected in the population Par 3 originating from Guatemala (Fig. 1c).

Three of the seven populations of *M. paranaensis* were tested in the differential host test as representatives of the three esterase phenotypes (Par 1: Est P2, Par 3: Est P2a and Par 7: Est P1). The three populations reproduced on tomato cv. Rutgers, tobacco cv. NC95 and watermelon cv. Charleston Gray (RF = 88.9), and no reproduction occurred on pepper cv. Early California Wonder, cotton cv. Deltapine 61 and peanut cv. Florunner.

Molecular analysis

Meloidogyne paranaensis populations were tested with specific markers developed for major RKN species infecting coffee, i.e. *M. incognita*, *M. exigua* and *M. paranaensis*. A single fragment of 208 bp was obtained for all

Table 3 Reaction of *Coffea arabica* 'Catuaí IAC 81' (susceptible control) and *C. canephora* 'Clone 14 INCAPER', 8 months after inoculation with seven *Meloidogyne paranaensis* populations under controlled conditions

M. paranaensis population	Cultivar	Root weight (g)[a]	Eggs		RF[d]	Reaction
			No. per g roots[b]	Total[c]		
Par 1	Catuaí IAC 81	203.58	1431.86 a	291 500	29.15 a	S
	Clone 14	191.33	12.19 b	2 333	0.23 b	HR
Par 2	Catuaí IAC 81	143.08	4633.07 a	662 900	66.29 a	S
	Clone 14	329.08	0.33 b	111	0.01 b	HR
Par 3	Catuaí IAC 81	146.08	3660.32 a	534 701	53.47 a	S
	Clone 14	150.33	14.63 b	2 200	0.22 b	HR
Par 4	Catuaí IAC 81	189.83	1935.65 a	367 444	36.74 a	S
	Clone 14	149.83	3.35 b	472	0.05 b	HR
Par 5	Catuaí IAC 81	128.17	2774.73 a	355 167	35.52 a	S
	Clone 14	190.42	2.63 b	500	0.05 b	HR
Par 6	Catuaí IAC 81	183.58	751.04 a	137 877	13.79 a	S
	Clone 14	319.15	1.04 b	333	0.03 b	HR
Par 7	Catuaí IAC 81	130.42	667.93 a	87 111	8.71 a	S
	Clone 14	138.42	6.82 b	944	0.09 b	HR

Meloidogyne paranaensis populations and *Coffea* cultivars are described in Tables 1 and 2, respectively. Data were transformed as $\log(x + 1)$. Means with different letters within columns are significantly different according to Scott–Knott's test ($P < 0.05$). Coefficient of variation 18.05% (no segregation) and 21.41% (mean). Coffee cultivars were rated as susceptible (S) and highly resistant (HR) (Hussey & Janssen, 2002).

[a]Mean values of root weight (g) ($n = 8$).

[b]Mean values of eggs per g roots ($n = 8$).

[c]Mean values of total number of eggs ($n = 8$).

[d]Mean values of nematode reproduction factor ($n = 8$).

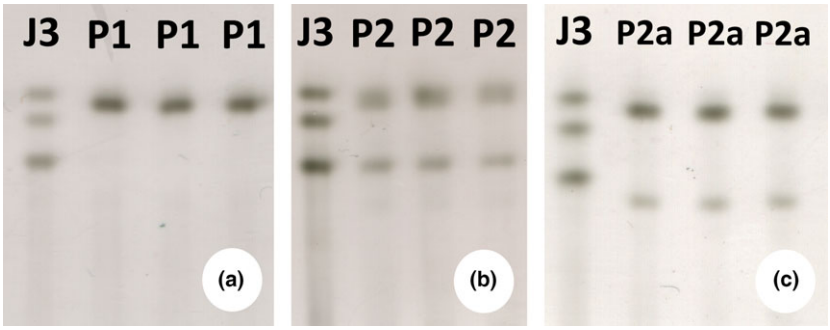

Figure 1 Esterase phenotypes of *Meloidogyne paranaensis*. (a) Est P1; (b) Est P2; (c) Est P2a. *M. javanica* (Est J3) is included as a reference. [Colour figure can be viewed at wileyonlinelibrary.com]

seven *M. paranaensis* populations from Brazil and Guatemala, showing different esterase phenotypes (Est P1, P2 and P2a), and no amplification products were seen in other control species tested, confirming their identification (Fig. 2).

In the genetic diversity, a total of 13 AFLP and 30 RAPD primers were used. The sizes of amplified fragments ranged from 200 to 4500 bp (Fig. 3a,b), and the number of reproducible amplified fragments was 925 for both markers. Overall, only 404 fragments (44%) were polymorphic in this study. All scorable amplified bands were recorded to build a 0–1 matrix, on which cluster analysis was done using UPGMA.

The dendrogram resulting from a concatenated dataset of RAPD and AFLP markers is shown in Figure 4. Overall, the diversity among different populations of *M. paranaensis* was low: the six *M. paranaensis* populations (Par 1, Par 2, Par 4, Par 5, Par 6 and Par 7) clustered together with 99% bootstrap support; however, the Par 3 population (Est P2a; Guatemala) clustered separate from other populations (Brazil). *Meloidogyne paranaensis* populations from Brazil clustered according to their esterase phenotypes, the Est P1 and P2 populations with 99% and 100% bootstrap support, respectively (Fig. 4).

Aggressiveness of *M. paranaensis* populations on coffee genotypes

In the first assay using different *M. paranaensis* populations against *C. arabica* cultivars (cv. Catuaí IAC 81, positive control) and *C. canephora* (cv. Clone 14 INCAPER), the high susceptibility of cv. Catuaí IAC 81 with

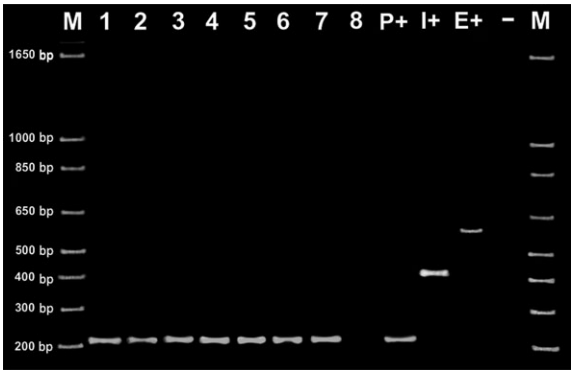

Figure 2 PCR amplification patterns for 11 populations of *Meloidogyne* spp. generated with specific SCAR primers inc-K14-F/R (Randig *et al.*, 2002). (1–7): *M. paranaensis*, (8): *M. enterolobii*, (P+, I+, E+): positive controls, *M. paranaensis*, *M. incognita* and *M. exigua*, respectively. (–) DNA: negative control. M: 1 kb Plus DNA ladder (Invitrogen). Amplifications were done using bulk DNA purified from juveniles.

reproduction factor RF > 30 was confirmed, as well as the high resistance of the clonal variety Clone 14 INCAPER with RF < 0.2 (Table 3). Similar results were observed regarding the number of eggs per gram of roots, a variable that is directly related to the reproduction factors observed for both cultivars. Considering the weight of roots, both cultivars showed good root development, on average 160.7 and 209.8 g for the susceptible and resistant, respectively (Table 3).

The second assay confirmed the resistance of four coffee genotypes against seven *M. paranaensis* populations: Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III), *C. canephora* rootstock Apoatã IAC 2258, Timor Hybrid UFV 408-01 (E1 6-6 II) and IPR 100 (*C. arabica*) (Table 4), showing RFs < 2.0. Similar results were observed regarding the number of eggs per gram of roots. Considering root weight, all cultivars showed a smaller root development compared with the first assay (Table 4). On average, the roots in the second assay

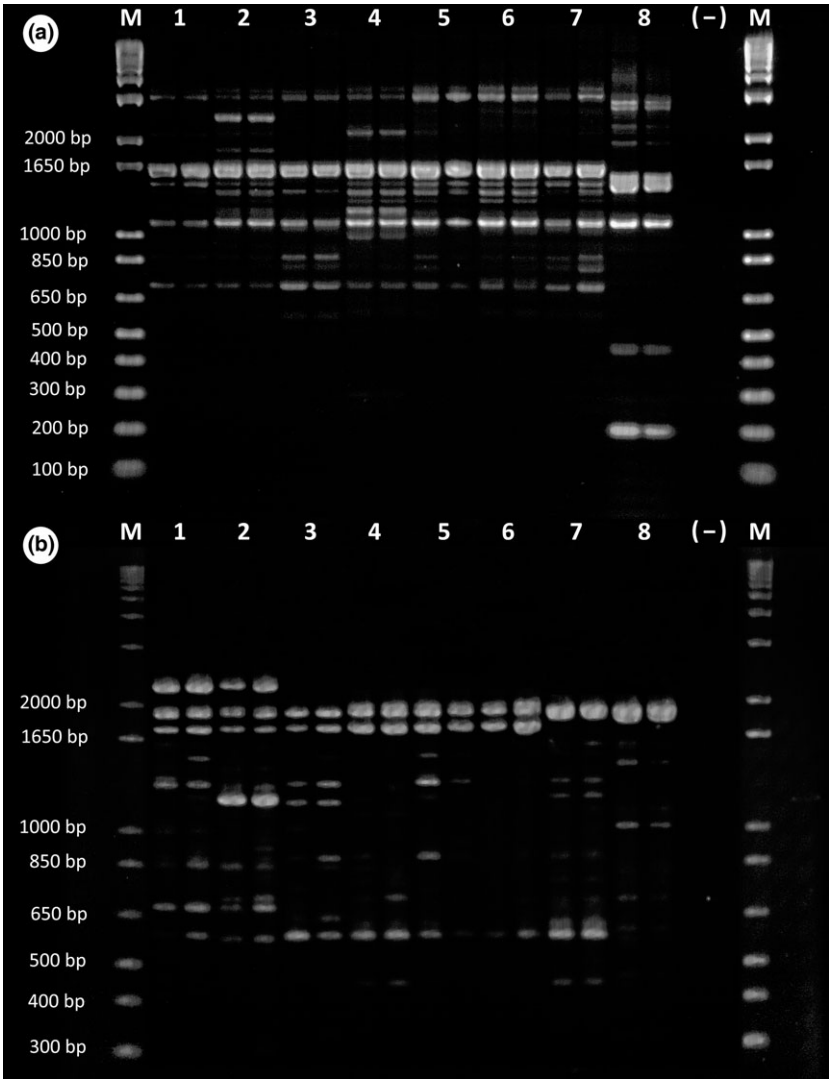

Figure 3 Genetic diversity of *Meloidogyne paranaensis* analysed with primers RAPD AQ12 (a) and AFLP 06 (b). (1–7): *M. paranaensis* and (8): *M. enterolobii* (outgroup). (–): DNA negative control. M: 1 kb Plus DNA Ladder (Invitrogen); bp: base pairs. For each population, two duplicate amplifications were loaded side by side on the gel.

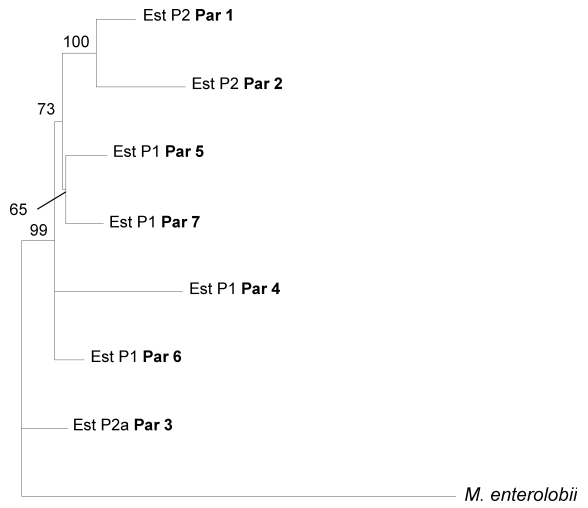

Figure 4 Dendrogram showing the relationships of *Meloidogyne paranaensis* populations using a concatenated dataset of RAPD and AFLP. Bootstrap values (>50%) based on 1000 replicates are shown. Isolate codes are described in Table 1.

were smaller for the susceptible control Mundo Novo 379-19 (56.9 g) and the resistant coffees IPR 100 (81.2 g), Catuaí Vermelho × Amphillo MR2161 (23.3 g), Timor Hybrid UFV 408-01 (22.7 g) and Apoatã IAC 2258 (10.1 g), considering that coffee seedlings were inoculated at smaller sizes (10 cm), compared to seedlings of 20 cm height in the first assay.

Considering the aggressiveness of seven different populations of *M. paranaensis* against two susceptible coffee cultivars (controls), namely Catuaí IAC 81 and Mundo Novo 379-19 (Table 5), the population Par 2 from Herculândia (Est P2) (RF = 66.3, 76.0, respectively) and Par 3 (Est P2a) from Guatemala (RF = 53.5, 60.6) were the most aggressive, followed by Par 5 (Est P1) from Piumbí (RF = 35.5, 56.9) and Par 4 (Est P1) from Rolândia (RF = 36.7, 47.3). The least aggressive isolates were Par 1 (RF = 29.1, 19.3), Par 6 (RF = 13.8, 28.2) and Par 7 (RF = 8.7, 19.3; Table 5). No *M. paranaensis* populations were virulent to all five resistant cultivars: Clone 14 INCAPER, Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III), Apoatã IAC 2258, IPR 100 and Timor Hybrid UFV 408-01 (E1 6-6 II). These coffee genotypes exhibited segregation for resistance at ratios of 0.0% (clonal genotype), 2.4%, 12%, 26% and 29%, respectively, under greenhouse conditions (Tables 3 & 4).

Discussion

This study evaluated the genetic variability and aggressiveness/virulence of seven populations of *M. paranaensis* from Brazil and one from Guatemala in different coffee cultivars that harbour resistance genes to RKNs. Similar results have not been reported for *M. paranaensis* species in coffee. For instance, there are no reports about the extent of genetic and pathogenic variability for this species.

Despite the existence of three esterase profiles (P1, P2 and P2a), a low genetic variability was observed among *M. paranaensis* populations in coffee. Similar results were reported for *M. incognita*, also with different esterase phenotypes (Santos *et al.*, 2012). Phylogenetically, all Brazilian populations of *M. paranaensis* clustered together with 99% bootstrap support (Est P1 and P2), except the Guatemalan population (Est P2a), which was separated from other populations from Brazil and was highly aggressive to susceptible coffee genotypes. The Brazilian populations of *M. paranaensis* clustered according to the esterase phenotypes (Est P1 and P2). Similar results were observed, partially, for *M. incognita* Est I1, I2 and S2 (Santos *et al.*, 2012). The phenotype Est P2a has not been detected in Brazil, suggesting a genetic divergence of the *M. paranaensis* population from Guatemala (Carneiro *et al.*, 2004). No correlation was observed between enzyme esterase phenotypes and aggressiveness. The population Est P2 from Herculândia presented high aggressiveness, whereas the population from Londrina with the same esterase phenotype presented intermediate aggressiveness.

SCAR markers have been developed for RKNs often associated with coffee in the Americas (Randig *et al.*, 2002; Correa *et al.*, 2013). In the present study, the SCAR primers inc-K14-F/R, ex-D15-F/R and par-C09-F/R, which were developed for the Brazilian RKN species damaging coffee, i.e. *M. exigua*, *M. paranaensis* and *M. incognita* (Randig *et al.*, 2002), were further validated by analysing seven populations of *M. paranaensis* from different geographic locations in Brazil and Guatemala with different esterase phenotypes (P1, P2 and P2a), whereas previous studies have been carried out with few populations of this species (Carneiro *et al.*, 2004). While esterase isozyme phenotypes are restricted in characterizing RKN females (Carneiro & Cofcewicz, 2008), PCR-based methods are more suitable for routine diagnosis, especially in coffee roots infested with *Meloidogyne* spp., where females collected from fields are frequently in bad conditions of preservation, making the identification difficult when using esterase isozyme only. PCR is fast, can be used in a large number of samples and can detect single J2 from field soil samples and roots. It also does not require nematode multiplication on the host plant until they reach female adult stages.

Molecular markers have been shown to be valuable in discriminating *Meloidogyne* species and in studying the intraspecific variability for different crops (Castagnone-Sereno *et al.*, 1994; Randig *et al.*, 2002; Carneiro *et al.*, 2004; Santos *et al.*, 2012). Considering the RKN from coffee, Santos *et al.* (2012) observed small variability among populations of *M. incognita*, but aggressiveness was not studied. The opposite was observed among populations of *M. exigua*. All populations presented high genetic variability (Muniz *et al.*, 2008) and differences in aggressiveness (Muniz *et al.*, 2009). One population

Table 4 Reaction of five *Coffea arabica* cultivars 8 months after inoculation with seven *Meloidogyne paranaensis* populations under greenhouse conditions

M. paranaensis population	Cultivar[a]	Root weight (g)[b]	Eggs per g roots[c]		RF[d]		Reaction[e]
			Segregation	Nonsegregation	Segregation	Nonsegregation	
Par 1	MN 379-19	47.71	3988.68	3988.68 a	19.03	19.03 a	S
	Apoatã IAC 2258	6.08	592.10	592.10 b	0.36	0.36 b	HR
	TH (UFV 408-01)	18.83	3138.61	706.32 b	5.91	1.33 b	R
	CV × Am MR2161	19.17	57.38	57.38 b	0.11	0.11 b	HR
	IPR 100	71.17	865.53	85.71 b	6.16	0.61 b	HR
Par 2	MN 379-19	58.87	12 926.78	12 926.78 a	76.10	76.10 a	S
	Apoatã IAC 2258	6.08	1710.50	1710.50 b	1.04	1.04 b	R
	TH (UFV 408-01)	19.08	1368.69	1006.29 b	2.71	1.92 b	R
	CV × Am MR2161	25.66	374.12	374.12 c	0.96	0.96 b	HR
	IPR 100	68.9	1695.21	143.69 c	11.68	0.99 b	HR
Par 3	MN 379-19	71.00	8535.21	8535.21 a	60.60	60.60 a	S
	Apoatã IAC 2258	15.75	114.28	114.28 b	0.18	0.18 b	HR
	TH (UFV 408-01)	16.25	1956.92	160.30 b	3.18	0.26 b	HR
	CV × Am MR2161	15.80	126.58	126.58 b	0.20	0.20 b	HR
	IPR 100	70.00	1602.86	52.86 c	11.22	0.37 b	HR
Par 4	MN 379-19	72.30	6543.56	6543.56 a	47.31	47.31 a	S
	Apoatã IAC 2258	8.40	1178.57	1178.57 b	0.99	0.99 b	HR
	TH (UFV 408-01)	34.60	309.24	49.13 c	1.07	0.17 b	HR
	CV × Am MR2161	25.90	73.36	73.36 c	0.19	0.19 b	HR
	IPR 100	121.20	438.12	6.60 d	5.31	0.08 b	HR
Par 5	MN 379-19	43.71	13 017.61	13 017.61 a	56.90	56.90 a	S
	Apoatã IAC 2258	11.16	241.94	241.94 b	0.27	0.27 b	HR
	TH (UFV 408-01)	25.75	100.97	100.97 b	0.26	0.26 b	HR
	CV × Am MR2161	29.00	93.10	93.10 b	0.27	0.27 b	HR
	IPR 100	70.42	568.02	52.54 c	4.00	0.37 b	HR
Par 6	MN 379-19	70.92	3983.36	3983.36 a	28.25	28.25 a	S
	Apoatã IAC 2258	15.33	45.66	45.66 b	0.07	0.07 b	HR
	TH (UFV 408-01)	23.88	46.06	46.06 b	0.11	0.11 b	HR
	CV × Am MR2161	24.91	0.00	0.00 c	0.00	0.00 b	HR
	IPR 100	45.20	789.82	0.00 c	3.57	0.00 b	HR
Par 7	MN 379-19	44.64	6442.65	6442.65 a	28.76	28.76 a	S
	Apoatã IAC 2258	8.08	1856.43	1856.43 b	1.50	1.50 b	R
	TH (UFV 408-01)	21.66	2987.07	457.06 b	6.47	0.99 b	HR
	CV × Am MR2161	22.83	70.08	70.08 c	0.16	0.16 b	HR
	IPR 100	121.70	875.10	67.38 c	10.65	0.82 b	HR

[a]Mundo Novo 379-19 (susceptible), *C. canephora* 'Apoatã IAC 2258' (resistant), Timor Hybrid UFV 408-01 (E1 6-6 II) (resistant), Catuaí Vermelho × Amphilo MR2161 (E1 16-5 III) (resistant), IPR 100 (resistant) (Hussey & Janssen, 2002).

[b]Mean values of root weight ($n = 8$).

[c]Mean values of eggs per g roots ($n = 8$).

[d]Mean values of nematode reproduction factor (RF) ($n = 8$). Data were transformed as $\log(x + 1)$. Means with different letters within columns are significantly different according to Scott–Knott's test ($P < 0.05$). Coefficient of variation: 49.05% (eggs per g root) and 56.54% (RF).

[e]Reaction of *Coffea* cultivars: susceptible (S), resistant (R) and highly resistant (HR).

from Rio de Janeiro State clustered together with other populations of *M. exigua* from Brazil and other countries, showing high variability and aggressiveness. This population has overcome the *Mex-1* resistance gene of cultivars IAPAR 59 and Paraíso (H419-5-4-5-2) (Muniz *et al.*, 2009). Thus, this population is considered a virulent isolate against resistant coffee cultivars (Muniz *et al.*, 2009). In this study, the seven *M. paranaensis* populations from coffee showed low genetic variability, but with different degrees of aggressiveness observed in susceptible cultivars. However, virulence against resistant cultivars (Clone 14 INCAPER, Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III), Apoatã IAC 2258, IPR 100 and Timor Hybrid UFV 408-01 (E1 6-6 II) was not detected.

To date, few resistant materials have been found in *C. arabica* cultivars (Campos & Villain, 2005), which contrast with some wild coffee trees from Ethiopia that exhibited resistance to *M. paranaensis* (Boisseau *et al.*, 2009). The present study confirmed the high susceptibility of *C. arabica* cultivars Catuaí IAC 81 and Mundo Novo 379-19 tested against seven populations of *M. paranaensis*, and observed that populations Par 2, Par 3 (Est P2 and P2a) and Par 5 (Est P1) were highly aggressive when compared with others, on both susceptible coffee cultivars.

Table 5 Reproduction factors of *Meloidogyne paranaensis* populations used to evaluate their aggressiveness against susceptible *Coffea arabica* genotypes: first (Catuaí IAC 81) and second (Mundo Novo) assays

		Genotype	
Population	Esterase	Catuaí IAC 81	Mundo Novo
Par 1	P2	29.15 b	19.03 c
Par 2	P2	66.29 a	76.01 a
Par 3	P2a	53.47 a	60.61 a
Par 4	P1	36.74 b	47.31 b
Par 5	P1	35.52 b	56.90 a
Par 6	P1	13.79 c	28.25 c
Par 7	P1	8.71 c	28.76 c

Mean values of nematode reproduction factors ($n = 8$). Data were transformed as $\log(x + 1)$. Means with different letters within columns are significantly different according to Scott–Knott's test ($P < 0.05$).

Nevertheless, this study confirmed a new source of resistance in *C. arabica*. For instance, the accession from crossing Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III) was highly resistant to seven *M. paranaensis* populations with a low segregation ratio (2.4%), confirming previous studies carried out on the same accession, but with only a single population of *M. paranaensis* from Piumbí, MG state (Peres *et al.*, 2017). This accession has the potential to advance to the next generations and to become a new *C. arabica* variety with increased resistance to *M. paranaensis* and good agronomic traits.

The data here corroborate those of Peres *et al.* (2017) who also reported resistance to *M. incognita* and *M. paranaensis* in the Timor Hybrid UFV 408-01 (E1 6-6 II) accession. A segregation ratio for resistance of 29% was observed for this accession, a range that has been reported by other authors (Gonçalves & Silvarolla, 2007; Peres *et al.*, 2017). Overall, this accession makes a valuable source of resistance to *Meloidogyne* spp., being commonly used in breeding programmes seeking resistance to RKN (Fazuoli, 2004).

It was also confirmed that the IPR 100 cultivar is highly resistant to seven *M. paranaensis* populations as indicated by a low RF, although it showed a segregation ratio of 26%. Similar results were reported by Sera *et al.* (2007). Several studies have reported segregation for resistance to RKN nematodes and other agronomic traits in coffee progenies, even with advanced generations (Gonçalves *et al.*, 1996).

The identification of *C. canephora* clones combining drought tolerance with other agronomic traits (e.g. efficient flowering, productivity, vigour) and resistance to RKN is of particular interest in generating new varieties better adapted to climate changes and biotic stress (Ferrão *et al.*, 2010). For example, the multiresistant reactions of coffee Clone 14 INCAPER were shown for three highly aggressive RKN species (*M. exigua*, *M. incognita* and *M. paranaensis*; Lima *et al.*, 2015). In this study, the resistance of this material was confirmed for seven *M. paranaensis* populations. The authors also suggested that resistance to *Meloidogyne* spp. in Clone 14 INCAPER is probably controlled by more than one resistance gene (Lima *et al.*, 2015).

Planting *C. canephora* in all coffee regions is not recommended because there are climate restrictions. In this case, the use of this coffee species as rootstock for *C. arabica* cultivars is advised, e.g. rootstock Apoatã IAC 2258 and Nemaya which presented resistance to *M. paranaensis* under field conditions (Bertrand & Anthony, 2008; Ferraz, 2008). This study confirmed the resistance of the rootstock Apoatã IAC 2258 for seven different *M. paranaensis* populations, with a segregation ratio of 12%. However, there are some drawbacks in using rootstocks compared to *C. arabica* cultivars. The segregation phenotype for susceptibility to nematodes, the need for replanting (about 10–15%) and a higher cost of seedlings are some examples (Gonçalves & Silvarolla, 2007). However, the use of rootstocks in Brazil has allowed survival and competitiveness of coffee growing in areas infested by *Meloidogyne* spp. (Campos & Villain, 2005).

Resistance based on a few genes may impose a selection pressure on nematode populations and hasten the selection of virulent isolates (Hussey & Janssen, 2002), as has been observed in tomato with the *Mi* resistance gene in wild potato with the *Rmc-2* gene (Hussey & Janssen, 2002) or in coffee with the *Mex-1* gene (Muniz *et al.*, 2009). Parthenogenic RKN species exhibit a high capacity to respond to environmental selection, and their ability to overcome plant resistance genes has been demonstrated for some crops (Castagnone-Sereno, 2002; Castagnone-Sereno *et al.*, 2007). Fortunately, in this study no *M. paranaensis* population overcame the resistance in four resistant cultivars studied here.

Resistance to single RKN species has been reported in some coffee genotypes (Campos & Villain, 2005; Bertrand & Anthony, 2008). The present study confirmed the resistance of five cultivars, namely Clone 14 INCAPER, Apoatã IAC 2258, Catuaí Vermelho × Amphillo MR2161 (E1 16-5 III), Timor Hybrid UFV 408-01 (E1 6-6 II) and IPR 100 (*C. arabica*), to seven *M. paranaensis* populations with different esterase phenotypes (Est P1, P2 and P2a) and small genetic variability. These resistant cultivars show great potential for breeding programmes because they are promising in obtaining advanced generations of resistant genotypes. Furthermore, the results suggested that coffee breeding programmes should consider the low genetic variability and no virulence of different populations of *M. paranaensis*, confirming what happens in field conditions: segregation of resistant genotypes. In addition, it was shown that populations Par 2, Par 3 and Par 5 showed the highest aggressiveness and should be recommended for use in further screenings to select new sources of resistance in coffee germplasm. Proper description and characterization of highly aggressive *M. paranaensis* populations are important not only for future breeding programmes but also for designing other appropriate management strategies.

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