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Nematology

A High-Throughput Molecular Pipeline Reveals the Diversity in Prevalence and Abundance of *Pratylenchus* and *Meloidogyne* Species in Coffee Plantations

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ABSTRACT

Coffee yields are adversely affected by plant-parasitic nematodes and the pathogens are largely underreported because a simple and reliable identification method is not available. We describe a polymerase chain reaction-based approach to rapidly detect and quantify the major *Pratylenchus* and *Meloidogyne* nematode species that are capable of parasitizing coffee. The procedure was applied to soil samples obtained from a number of coffee farms in Brazil, Vietnam, and Indonesia to assess the prevalence of these species associated both with coffee (*Coffea arabica* and *C. canephora*) and its intercropped species *Musa acuminata* (banana) and *Piper nigrum* (black pepper). *Pratylenchus coffeae* and *P. brachyurus* were associated with coffee in all three countries but there were distinct profiles of *Meloidogyne* spp. *Meloidogyne incognita, M. exigua*, and *M. paranaensis* were identified in samples from Brazil and *M. incognita* and *M. hapla* were detected around the roots of coffee in Vietnam. No *Meloidogyne* spp. were detected in samples from Indonesia. There was a high abundance of *Meloidogyne* spp. in soil samples in which *Pratylenchus* spp. were low or not detected, suggesting that the success of one genus may deter another. *Meloidogyne* spp. in Vietnam and *Pratylenchus* spp. in Indonesia were more numerous around intercropped plants than in association with coffee. The data suggest a widespread but differential nematode problem associated with coffee production across the regions studied. The issue is compounded by the current choice of intercrops that support large nematode populations. Wider application of the approach would elucidate the true global scale of the nematode problem and the cost to coffee production.

Nematodes are very abundant in soils and many are pathogens of belowground plant tissue (Groombridge 1992; Hunt et al. 2005). Their pathogenicity affects many physiological processes of plants that can result in the reduction of aboveground yields, such as in *Coffea* spp. (Barbosa et al. 2004). Nematodes reduce global production of the economically important *Coffea arabica* and *C. canephora* species by approximately 15% (Campos et al. 1990). Several *Meloidogyne* and *Pratylenchus* spp. cause damage throughout the main coffee-producing regions of Brazil, Central America, Columbia, Vietnam, and Indonesia (Carneiro et al. 2004; Gaitan and Cortina 2008; Trinh et al. 2009; Villain et al. 2013; Wiryadiputra 2008).

Pratylenchus and *Meloidogyne* are two of the most economically important plant-parasitic nematode genera of global agriculture (Jones et al. 2013). All juvenile and adult *Pratylenchus* nematodes are migratory and can enter and leave roots. They feed on plant cytosol during intracellular migration, leading to destruction of root tissue and promotion of secondary infection by other pathogens. Females lay eggs inside the root or in adjacent soil (Jones and Fosu-Nyarko 2014). Second-stage juveniles of *Meloidogyne* invade host roots, establish a feeding site, and become sedentary. Most species are parthenogenetic and females become committed to a feeding site from which they utilize host resources. Species of both genera parasitize many crops globally (Trudgill and Blok 2001). Virulence of *Meloidogyne* spp. to coffee plants varies and thereby modulates the extent of yield loss and can sometimes result in plant death (Bertrand 2008). *Meloidogyne* and *Pratylenchus* spp. can coexist on the same coffee plant, possibly increasing the damage potential and the economic impact on the plantation (Herve et al. 2005).

Intercropping systems are common throughout coffee production and have many benefits to the grower (Jassogne et al. 2013). Two commonly grown intercrop plants are banana and black pepper but both are susceptible to nematode species that damage coffee (Gowen et al. 2005; Thuy et al. 2012). The impact of these hosts on nematode diversity in coffee fields is previously unexplored and increases the complexity of coffee nematode management. Accurate diagnosis of nematode prevalence and distribution in the soil of a plantation is important for pest management and will underpin future control efforts.

Identification of economic nematodes in a coffee plantation involves detection and estimating population numbers relative to damage thresholds. The latter requirement is made more complex by the aggregated distribution of *Pratylenchus* and *Meloidogyne* spp. in soil around coffee plants (Herve et al. 2005). Nematode identification commonly requires examination of many individual nematodes under a microscope and considerable taxonomic expertise is needed to identify and quantify the economic species of either *Pratylenchus* or *Meloidogyne* (Correa et al. 2013). This has been applied to identify multiple species of these two genera from coffee fields in several countries (Avelino et al. 2009; Carneiro et al. 1996, 2004; Mekete et al. 2008; Trinh et al. 2009). Polymerase chain reaction (PCR)-based identification has been proposed to overcome these difficulties (Berry et al. 2008; Correa et al. 2013, 2014; Machado et al. 2007; Toyota et al. 2008). Very little assessment of these molecular methods for diagnosis of *Pratylenchus* or *Meloidogyne* spp. in coffee fields has been reported (Carneiro et al. 2004, 2005; Sirias and Cristina 2011). A rapid, reliable, and sensitive method for assessing

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the plant-parasitic nematode community would enable greater understanding of the soil fauna and highlight the major species of concern, not just for coffee but for the entire farm.

We report the development of a molecular pipeline that combines novel with previously reported primer sets to identify and quantify nematodes extracted from soil surrounding coffee plants and its intercrops. A main aim was to identify the complex of species of *Pratylenchus* and *Meloidogyne* present in the soil rather than a single species. The procedures were designed to require neither specialized equipment for nematode extraction nor taxonomic expertise, thus underpinning the potential for widespread adoption by extension workers. The approach was then evaluated for exemplar fields in Brazil, Vietnam, and Indonesia. We report the occurrence and abundance of each species detected on coffee, banana, and black pepper and discuss the implications of the intercropping strategy.

MATERIALS AND METHODS

Field sampling, nematode extraction, and DNA preparation. Soil samples were taken from 6 coffee fields in Minas Gerais, Brazil; 14 coffee fields in Dak Lak, Vietnam; and 8 coffee fields in Lampung, Sumatra, Indonesia. In Brazil, 71 samples were obtained from soil surrounding the roots of coffee plants in monoculture plantations. In Vietnam, samples were obtained from soil around the upper roots of coffee ($n = 82$) plus those of two intercrops: black pepper ($n = 40$) and banana ($n = 10$). Similarly, in Indonesia, soil samples were taken from around coffee ($n = 42$), black pepper ($n = 7$), and banana ($n = 23$). Each individual sample consisted of three soil cores (30 cm deep by 2.5 cm in diameter) that were taken from halfway between the stem and edge of the canopy of a single plant and pooled into one bag (Manzanilla-Lopez 2012). The main aim was to detect nematode presence; therefore, plants that showed signs of damage from some cause were selected where available (Manzanilla-Lopez 2012). Nematodes were extracted from 100 g of the soil sample using the method of Whitehead and Hemming (1965), as used before for populations of *Pratylenchus* and *Meloidogyne* from field soils (Bell and Watson 2001; Rodriguez-Kabana and Pope 1981; Wang and McSorley 2008; Yan et al. 2012). Soil was spread across a single layer of paper tissue that was supported in a wire basket. This was placed inside a plastic tray and water was added until the soil was damp but not waterlogged. Water containing nematodes was collected from the tray after 24 h. Nematodes were recovered using a 25-µm sieve and concentrated by centrifugation at 3,000 × *g* for 3 min or by leaving the sample to settle overnight. The nematode pellet was resuspended in 100 µl of lysis buffer (100 mM NaCl, 10 mM Tris [pH 8], 10 mM EDTA, 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and proteinase K at 100 µg ml^{-1}), then incubated at –20°C for at least 30 min, 60°C for 1 h, and 90°C for 10 min to lyse cells and release DNA (Adam et al. 2007).

Specificity testing of diagnostic PCR primer sets and application to field samples. Primer sets for identification of several *Pratylenchus* and *Meloidogyne* spp. were obtained from

TABLE 1. Primer sequences used for the identification (ID) and quantification (Q) of *Pratylenchus* and *Meloidogyne* spp., alongside the annealing temperatures, product sizes, and sources

Target	Purpose	Primer set	Ta	Product size	Forward and reverse sequence (5′–3′)	Reference
Nematode sp.	ID	SSU18A	52	1,000	AAAGATTAAGCCATGCATG	Blaxter et al. (1998)
…	…	SSU26R	…	…	CATTCTTGGCAAATGCTTTCG	…
Pratylenchus sp.	ID	P18sF	62	800–1,500	TTGATTACGTCCCTGCCCTTT	Waeyenberge et al. (2000)
…	…	P18sR	…	…	GGAATCATTGCCGCTCACTTT	…
Pratylenchus brachyurus	ID	18sF	57	267	TTGATTACGTCCCTGCCCTTT	Machado et al. (2007)
…	…	ACM7R	V	…	GCWCCATCCAAACAAYGAG	…
P. coffeae	ID	PC28sF	62	530	CCGTGAGGGAAAGTTGAAAA	This study
…	…	PC28sR	…	…	GCTCCTAACGGAAACGTTCA	…
P. zeae	ID	18sF	57	250	TTGATTACGTCCCTGCCCTTT	Berry et al. (2008)
…	…	Praty-R	…	…	CTGCATTGGAAGCGCGCTTG	…
P. loosi	ID	PL1	65	668	CAGTCAGCTAGCTGCTGGAT	Uehara et al. (1998)
…	…	PL2	…	…	TGAGAGCATAGTCGCTGTG	…
P. vulnus	ID	D3B	67	287	TCGGAAGGAACCAGCTACTA	Al-Banna et al. (2004)
…	…	PVULF	…	…	GAAAGTGAACGCATCCGCAA	…
P. thornei	ID	D3B	67	288	TCGGAAGGAACCAGCTACTA	Al-Banna et al. (2004)
…	…	PTHO	…	…	GAAAGTGAAGGTATCCCTCG	…
P. brachyurus	Q	PbqF	60	106	CTGTGTGATAGATTATGGGCGAC	This study
…	…	PbqR	…	…	ACATCGTCTTTGATCAACATCAAC	…
P. coffeae	Q	Pce1qF	61	73	TGCCCAAAACCACAAAGCC	This study
…	…	Pce1qR	…	…	GTTCGGATTGGAGCCATATTGC	…
Meloidogyne sp.	ID	194	50	700/720	TTAACTTGCCAGATCGGACG	Blok et al. (1997)
…	…	195	…	…	TCTAATGAGCCGTACGC	…
Meloidogyne javanica	ID	Fjav	64	720	CAGGCCCTTCAGTGGAACTATAC	Zijlstra et al. (2000)
…	…	Rjav	…	…	CTCTGCCCAATGAGCTGTCC	…
M. incognita	ID	MI-F	62	999	GTGAGGATTCAGCTCCCCAG	Meng et al. (2004)
…	…	MI-R	…	…	ACGAGGAACATACTTCTCCGTCC	…
M. hapla	ID	JMV1	50	440	GGATGGCGTGCTTTCAAC	Wishart et al. (2002)
…	…	JMVHapla	…	…	AAAAATCCCCTCGAAAAATCCACC	…
M. paranaensis	ID	Par-C09F	63	208	GCCCGACTCCATTTGACGGA	Randig et al. (2002)
…	…	Par-C09R	…	…	CCGTCCAGATCCATCGAAGTC	…
M. exigua	ID	Ex-D15-F	63	562	CATCCGTGCTGTAGCTGCGAG	Randig et al. (2002)
…	…	Ex-D15-R	…	…	CTCCGTGGGAAGAAAGACTG	…
M. incognita	Q	RKNf	61	185	GCTGGTGTCTAAGTGTTGCTGATAC	Toyota et al. (2008)
…	…	RKNr	…	…	GAGCCTAGTGATCCACCGATAAG	…
M. hapla	Q	Mh-f	61	87	ATGTTGGTACGCAGCGATTTGTA	Watanabe et al. (2013)
…	…	Mh-r	…	…	CAGCGGGTGATCTCGACTGA	…
M. paranaensis	Q	MpqF	61	85	AGACCGTGAGGGAAAGTTGC	This study
…	…	MpqR	…	…	CCGACTCTATCCGTTTCCACC	…
M. exigua	Q	MexqF	62	129	GTGGGGATTTCTGAGACAGAT	This study
…	…	MexqR	…	…	CAATCTATCTGACGCACGTAGG	…
Radopholus similis	ID	RsimF	54	398	GATTCCGTCCTTTGGTGGGCA	Ravindran et al. (2011)
…	…	RsimR	…	…	GAACCAGGCGTGCCAGAGG	…

published sources (Table 1) and tested for specificity through PCR screening with target and nontarget DNA. Template DNA was extracted as above from a range of reference nematode populations: *Pratylenchus coffeae* (populations from Ghana, Guatemala, Japan, and Uganda), *P. brachyurus* (United States), *P. zeae, P. vulnus* (United States), *P. loosi* (Japan), *P. thornei* (United States), *Meloidogyne exigua* (Brazil), *M. paranaensis* (Brazil), *M. incognita* (Brazil), *M. hapla, M. javanica* (Turkey), and *Radopholus similis* (Uganda). This DNA was used in PCR with MyTaq polymerase (Bioline) according to the manufacturer's instructions and under the following conditions: 94°C for 60 s and 40 cycles of 94°C for 10 s, X°C* for 30 s, 72°C for 30 s and 72°C for 5 min (X°C* indicates specific annealing temperature for each primer pair) (Table 1). The results prompted the design of new species-specific primers for *P. coffeae*, PC28sF/PC28sR (Table 1), based upon 28S ribosomal DNA sequence data obtained from GenBank (accession number KY424281).

Generic nematode-specific primers (SSU18A and SSU26R) (Blaxter et al. 1998) (Table 1) were utilized in PCR as above with 1 µl of each field sample DNA, and reaction products were visualized on a 1% agarose gel to determine the success of nematode DNA extraction from soil samples. Genus-specific primer sets (Blok et al. 1997; Waeyenberge et al. 2000) were then used to identify the presence of *Pratylenchus* or *Meloidogyne* spp. within a sample. Species-specific primer sets then detected the presence of *P. brachyurus* (Machado et al. 2007), *P. coffeae, P. zeae* (Berry et al. 2008), *P. loosi* (Uehara et al. 1998), *P. vulnus* (Al-Banna et al. 2004), *P. thornei* (Al-Banna et al. 2004), *M. javanica* (Zijlstra et al. 2000), *M. incognita* (Meng 2004; Meng et al. 2004), *M. hapla* (Wishart et al. 2002), *M. paranaensis* (Randig et al. 2002), *M. exigua* (Randig et al. 2002), or *R. similis* (Ravindran et al. 2011), another economically important nematode for coffee.

Specificity testing of quantitative PCR primer sets and quantification of nematodes. Quantitative PCR (qPCR) primer pairs RKNf/r (Toyota et al. 2008) and Mh-f/r (Watanabe et al. 2013) were confirmed to be specific for *M. incognita* and *M. hapla*, respectively. Primer pairs were designed to be specific for *P. brachyurus* (PbqF/R), *P. coffeae* (Pce1qF/R), *M. exigua* (MeqF/R), and *M. paranaensis* (MpqF/R) based upon sequences present in GenBank (accession numbers KF537388.1, EU176871, AF435796, and AF435798, respectively). DNA was extracted from triplicate batches of 200, 100, 50, 20, and 5 individuals from each nematode species, as described above. These were used as standards in qPCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) to construct calibration curves of nematode number versus cycle threshold (C_t) values. qPCR with species-specific primer sets was then applied to each field sample DNA with three technical replicates to obtain sample C_t values. These C_t values were aligned to the calibration curve to obtain an estimate of nematode number per sample.

Data analysis. Data were analyzed using a *t* test and one-way analysis of variance (ANOVA) for comparison of two and three means, respectively, in SPSS (SPSS v24; IBM Corporation).

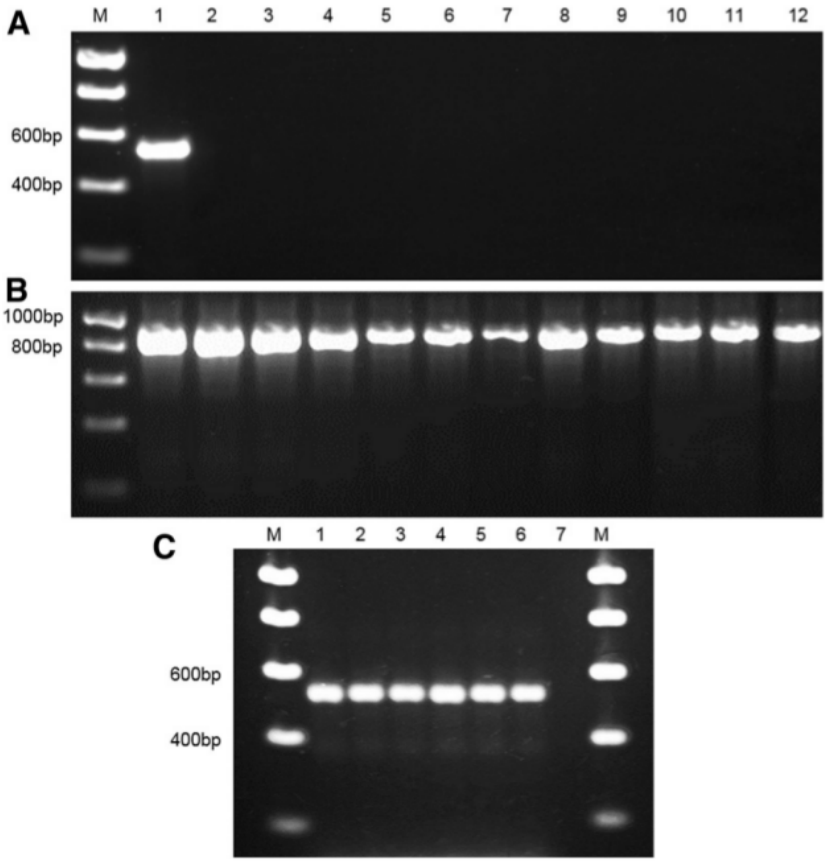

Fig. 1. Testing of primers specific to *Pratylenchus coffeae*. **A,** Specificity test of primers PC28sF and PC28sR on DNA derived from different nematode species: *P. coffeae* (lane 1), *P. brachyurus* (lane 2), *P. zeae* (lane 3), *P. vulnus* (lane 4), *P. loosi* (lane 5), *P. thornei* (lane 6), *Meloidogyne exigua* (lane 7), *M. paranaensis* (lane 8), *M. incognita* (lane 9), *M. hapla* (lane 10), *M. javanica* (lane 11), and *Radopholus similis* (lane 12). **B,** Quality of DNA from these species was confirmed through polymerase chain reaction with nematode 18S primers (same lane labels as in A). **C,** PC28sF and PC28sR were tested on DNA from six populations of *P. coffeae* that were recovered from different hosts and locations: taro, Japan (lane 1); sweet potato, Japan (lane 2); coffee, Guatemala (lane 3); coffee, Guatemala (lane 4); coffee, Uganda (lane 5); and unknown host, Ghana (lane 6). Lane 7 is the no-template control and lane M signifies HyperLadder (1 kb).

Correlations were determined using correspondence analysis in SPSS.

RESULTS

Extraction and primer specificity tests. The Whitehead and Hemming (1965) tray extraction method yielded vermiform nematodes from soil samples in 24 h. Following lysis steps, these samples were then used immediately for identification. The specificity of new primers to *P. coffeae* (PC28sF and PC28sR) (Table 1) was confirmed when tested with template DNA from reference populations of *P. coffeae, P. brachyurus, P. zeae, P. vulnus, P. loosi, P. thornei, M. exigua, M. paranaensis, M. incognita, M. hapla, M. javanica*, and *R. similis*. This primer set amplified a 530-bp PCR product from *P. coffeae* DNA with no amplification for any other species tested (Fig. 1A). The quality of the DNA from the nontarget species was confirmed through PCR with generic nematode 18S primers (Fig. 1B). The broad utility of the primer set was verified following amplification of the species-specific product from DNA of six different *P. coffeae* populations from diverse geographic locations and multiple hosts (Fig. 1C).

Nematode detection in field samples. Presence of a species-specific PCR product established the detection of the plant-parasitic nematode species within the samples. The following results should not be interpreted as reflecting nationwide status of each nematode although, for simplicity, values are identified below by their country

TABLE 2. *Pratylenchus* and *Meloidogyne* spp. detected in soil surrounding coffee (C), black pepper (BP), and banana (B) crops in the 28 fields sampled

		Proportion (%)[a]												
		Pratylenchus brachyurus			*P. coffeae*			*Meloidogyne exigua*	*M. paranaensis*	*M. incognita*			*M. hapla*	
Area, field	Crop[b]	C	BP	B	C	BP	B	C	C	C	BP	B	C	BP
Minas Gerais, Brazil														
1	C	67	…	…	33	…	…	…	…	…	…	…	…	…
2	C	42	…	…	…	…	…	58	…	…	…	…	…	…
3	C	25	…	…	…	…	…	63	…	…	…	…	…	…
4	C	57	…	…	…	…	…	…	57	…	…	…	…	…
5	C	21	…	…	…	…	…	…	79	76	…	…	…	…
6	C	14	…	…	…	…	…	71	15	…	…	…	…	…
Buon Ma Thuot, Vietnam														
7	C	…	…	…	40	…	…	…	…	50	…	…	10	…
8	C	…	…	…	40	…	…	…	…	70	…	…	10	…
9	C	…	…	…	60	…	…	…	…	50	…	…	20	…
10	C, BP	…	…	…	67	50	…	…	…	33	75	…	16	25
11	C, BP	…	…	…	100	100	…	…	…	80	60	…	20	40
12	C, BP	…	…	…	40	40	…	…	…	80	60	…	…	60
13	C, BP	…	…	…	40	40	…	…	…	60	60	…	…	…
14	C, BP	40	…	…	…	20	…	…	…	40	60	…	…	20
15	C, BP	…	…	…	…	…	…	…	…	…	40	…	…	20
16	C, BP	…	…	…	20	20	…	…	…	40	20	…	…	…
17	C, B	…	…	…	…	…	…	…	…	100	…	67	…	…
18	C, BP	…	…	…	…	…	…	…	…	60	80	…	…	…
19	C, B	…	…	…	…	…	…	…	…	60	…	40	…	…
20	C	…	…	…	…	…	…	…	…	70	…	…	…	…
Lampung, Indonesia														
21	C, B	60	…	50	…	…	…	…	…	…	…	…	…	…
22	C, B	60	…	50	20	…	50	…	…	…	…	…	…	…
23	C, BP	40	…	…	100	50	…	…	…	…	…	…	…	…
24	C, B	40	…	75	40	…	25	…	…	…	…	…	…	…
25	C, B	40	…	50	20	…	25	…	…	…	…	…	…	…
26	C, B	20	…	25	40	…	…	…	…	…	…	…	…	…
27	C, B	20	…	…	80	…	75	…	…	…	…	…	…	…
28	C, BP, B	40	…	25	60	25	25	…	…	…	…	…	…	…

[a] Percentage values indicate the proportion of field crop samples that yielded positive detection for the species.
[b] Types of crops sampled.

TABLE 3. Percentage of total samples obtained from soil around the roots of coffee, black pepper, and banana in Minas Gerais (Brazil), Buon Ma Thuot (Vietnam), and Lampung (Indonesia) that contained each nematode species

		Samples with nematode detected (%)						
Country, crop sampled	Total[a]	*Pratylenchus brachyurus*	*P. coffeae*	*Meloidogyne exigua*	*M. paranaensis*	*M. incognita*	*M. hapla*	Any[b]
Brazil								
Coffee	71	37	7	35	27	17	0	75
Vietnam								
Coffee	82	2	23	0	0	53	8	56
Black pepper	40	0	35	0	0	53	18	61
Banana	10	0	0	0	0	60	0	60
Indonesia								
Coffee	43	32	34	0	0	0	0	63
Black pepper	7	0	29	0	0	0	0	29
Banana	23	26	35	0	0	0	0	65

[a] Number of total crops sampled.
[b] Any of the six species detected.

of origin. In total, two *Pratylenchus* spp. and four *Meloidogyne* spp. were detected in soil samples from Brazil, Vietnam, and Indonesia (Table 2). *P. brachyurus* and *P. coffeae* were detected in all three countries from soil surrounding coffee as well as the intercrops of black pepper and banana. *Pratylenchus* spp. were detected in six fields in Brazil which were monocultures of coffee plants. *M. exigua, M. paranaensis*, and *M. incognita* were detected in samples from the coffee plantations in Brazil whereas *M. incognita* and *M. hapla* were identified in Vietnam from soil surrounding the roots of coffee and black pepper. Nematodes of both genera occurred in 5 of the 6 Brazilian fields and 9 of the 14 Vietnamese fields. No *Meloidogyne* spp. were detected in samples from the Indonesian coffee fields.

Plant-parasitic nematodes from the genera *Meloidogyne* and *Pratylenchus* were detected in 75% of the total samples obtained from coffee fields in Minas Gerais, Brazil (Table 3). *P. brachyurus* was present in more samples than *P. coffeae* (37 and 7%, respectively). The frequency of detection for *M. exigua*, *M. paranaensis*, and *M. incognita* was 35, 27, and 17% of samples, respectively. *P. coffeae* was the more frequently detected species of that genus in Buon Ma Thuot, Vietnam and detected in 23 and 35% of samples taken from soil around coffee and black pepper, respectively. *M. incognita* was detected in 53, 53, and 60% of soil samples associated with coffee, black pepper, and banana, respectively (Table 3). *P. brachyurus* and *P. coffeae* were present in 32 and 34% of samples from coffee in Indonesia and 26 and 35% of samples from banana, respectively. For black pepper, *P. coffeae* occurred in 29% of soil samples but *P. brachyurus* was not detected. Another economic nematode, *R. similis*, was not detected in any sample from the three countries.

Evaluation of quantification primers. All primer sets were confirmed to be specific to the reference populations of the target species by qPCR using DNA from target and nontarget nematodes. The latter were the same species used in previous primer testing. Calibration curves were generated from the C_t values of standards made from known numbers of nematodes (Fig. 2). These displayed the expected negative correlation between C_t value and the number of nematodes ($R^2 > 0.97$ for all primer sets). This provided the linear calibration from which the numbers of nematodes are established below.

Quantification of plant-parasitic nematode species in field samples. *Pratylenchus* spp. were detected in only 44% of 71 samples obtained in Brazil but in the range of 54 to 482 individuals per 100 g of soil per sample when present (Fig. 3A). In fields in Vietnam, the nematode was detected in only 25% of the 82 samples from soil around coffee roots but, when present, ranged from 15 to 102 individuals per 100 g of soil. In Indonesia, *Pratylenchus* spp. were detected in 63% of the 43 samples from soil around coffee roots in the range of 19 to 493 individuals per 100 g of soil. One or more *Meloidogyne* spp. were present in 69% of samples for the five Brazilian fields in which the genus was recorded at a range of 11 to 529 *Meloidogyne* per 100 g of soil (Fig. 3B). Only *M. incognita* and *M. hapla* were detected in Vietnam, with a prevalence of 58% of samples obtained from coffee in the range of 14 to 274 *Meloidogyne* per 100 g of soil.

In samples from the three countries, the mean population densities of detected species in soil around coffee plants were considerably greater than previously suggested damage thresholds for *P. brachyurus* ($n = 0$), *P. coffeae* ($n = 0$), *M. exigua* ($n = 25$), and *M. incognita* ($n = 200$) on coffee (Oliveira et al. 1999; Rodrigues and Crozzoli 1995; Trinh et al. 2011; Vovlas and Di Vito 1991) (Fig. 4). The densities of both *Pratylenchus* spp. were higher on coffee in Brazil than Vietnam or Indonesia ($P < 0.05$, *t* test). Both *Pratylenchus* spp. were similarly abundant in the sampled fields in Brazil and Vietnam; however, *P. coffeae* was more numerous than *P. brachyurus* in Indonesian fields ($P < 0.05$, one-way ANOVA). *M. incognita* was more abundant than *M. hapla* in samples obtained from coffee ($P < 0.01$, *t* test). Nematode populations were at higher densities in soil associated with black pepper and banana plants than coffee in both Vietnam and Indonesia (Fig. 4) ($P < 0.05$, *t* test and one-way ANOVA for comparison of two and three means, respectively).

The relationship between *Pratylenchus* and *Meloidogyne* spp. abundance in soil samples. There was a significant negative correlation between numbers of *Pratylenchus* and *Meloidogyne* spp. for 96 samples associated with coffee or black pepper for fields in which both genera were detected ($R = -0.225$; $P < 0.05$, two-tailed test). The relationship was examined further using correspondence analysis and a significant summary χ^2 (23.5, $P < 0.001$) was obtained. The resultant biplot represents 24% of the variation in the data with most of that value (21%) represented by the horizontal axis of the graph. Therefore, the plot suggests that, in fields where both genera were detected, there is an association of high numbers of *Meloidogyne* spp. where *Pratylenchus* spp. were not detected. This is indicated by the proximity of the relevant data points in the horizontal axis (M_h and P_{nd}) (Fig. 5).

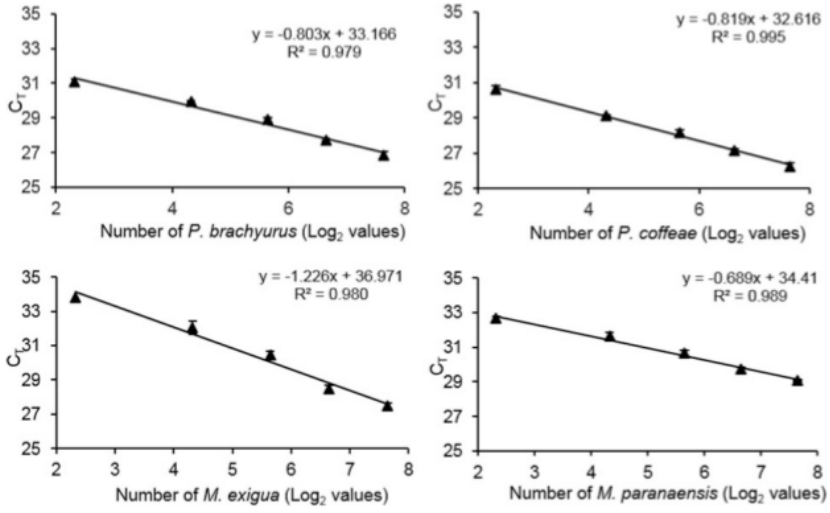

Fig. 2. Calibration curve of cycle threshold (C_t) values versus $\log_2$ number of nematodes for quantification of *Pratylenchus brachyurus*, *P. coffeae*, *Meloidogyne exigua*, and *M. paranaensis*. Error bars represent standard error of the mean for three biological replicates. Data points are larger than error bars in some instances.

DISCUSSION

Here, we demonstrate a molecular pipeline for rapid identification and estimation of soil populations of *Pratylenchus* and *Meloidogyne* nematode species that commonly damage coffee crops. This is the first molecular-based study to assess the plant-parasitic nematode community within coffee fields by sampling multiple crop plants in three major coffee-producing countries.

This study confirmed the ability of the Whitehead and Hemming tray method to extract high numbers of mobile *Pratylenchus* and *Meloidogyne* nematodes from soil (Rodriguez-Kabana and Pope 1981). Nematode extraction required minimal preparation time and equipment and the lysis method for DNA extraction provided sufficient extract for approximately 100 reactions and avoided the time-consuming DNA preparations of much previous work (Adam et al. 2007; Castagnone-Sereno et al. 1995; Stanton et al. 1998). The assay revealed that *P. brachyurus* and *P. coffeae* were similarly abundant in Brazil whereas *P. coffeae* was the more numerous species of the genus in Indonesia. Both of these species are known pathogens of coffee in these countries and can cause great damage to coffee roots and result in reduced plant growth (Oliveira et al. 1999; Trinh et al. 2011; Wiryadiputra 2008). This study detected *M. exigua* and *M. paranaensis* in Brazil, where they are widespread parasites of coffee plants (Barbosa et al. 2004; Carneiro et al. 1996; Muniz et al. 2008; Salgado et al. 2015). *M. incognita* was detected in both Brazil and Vietnam, where it is prevalent in coffee plantations (Carneiro et al. 2004; Trinh et al. 2009). This study reports the first detection of *M. hapla* in Vietnam, where it may have been previously identified as a different species or introduced through movement of cultivars. Although it has been reported in Hawaii, Guatemala, El Salvador, Brazil, India, and several countries in Africa, the species is not widespread in coffee plantations (Campos and Villain 2005; Handoo et al. 2005; Lordello 1982; Villain et al. 2013). Although the presence of any parasitic-nematode species capable of damaging coffee is of interest, *M. hapla* has not been reported as widespread and it results in relatively low damage to coffee roots (Villain et al. 2013). Consequently it may not represent as major a concern for the growers as some other species of this genus.

Genetically distinct populations of *M. incognita, M. paranaensis*, and *M. exigua* are present in Central and South America, suggesting

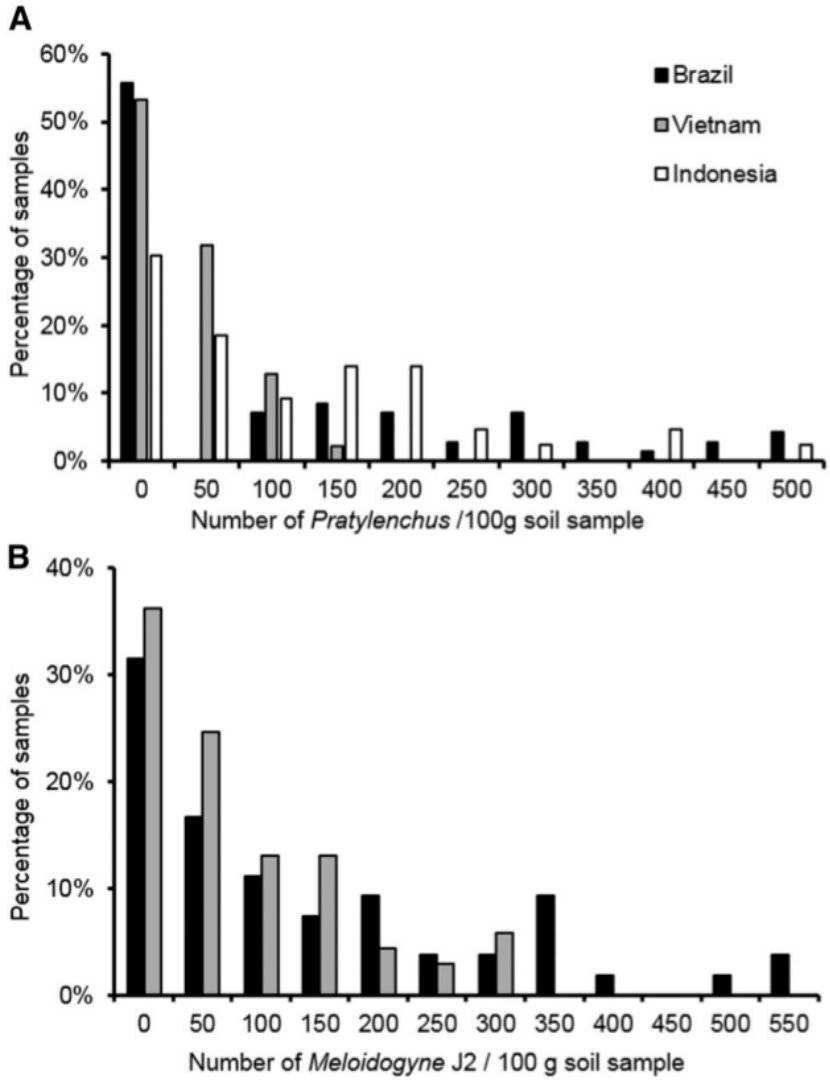

Fig. 3. Percentage of samples and the range of densities per 100 g of soil sample for fields in Brazil, Vietnam, and Indonesia where **A,** *Pratylenchus* or **B,** *Meloidogyne* nematodes were detected. Bar charts are based on 160 samples for *Pratylenchus* and 123 for *Meloidogyne*.

that genetic divergence has resulted in multiple species types (Carneiro et al. 2004; Randig et al. 2002). The chances of false negatives due to genetic divergence is reduced because several primer sets used in this report have previously been applied to samples from widespread locations (Devran and Sogut 2009; Hu et al. 2011; Randig et al. 2002). Further molecular analysis could help elucidate the origins of populations detected in this study, map their distributions, and assist in developing management strategies based on resistant cultivars. No *Meloidogyne* spp. were detected for the Indonesian samples, which was unexpected, given the abundance of the genus in Brazilian and Vietnamese samples and its known wide international distribution and polyphagy. Presence of *M. incognita* has been reported on other crops in the country; therefore, it is of utmost importance that coffee fields are protected from introduction of the pathogen (Tuminem et al. 2015). The lack of *Meloidogyne* spp. may also be due to the less structured farming system observed in Indonesia, compared with fields sampled in Brazil and Vietnam (unpublished data). The irregular nature of planting may have had a negative impact on the establishment and spread of *Meloidogyne* spp. throughout a field, whereas this may not prove to be as important for *Pratylenchus* spp. that can migrate through soil at a greater rate than *Meloidogyne* second-stage juveniles (Nježić et al. 2014), thereby possibly allowing them to locate hosts at a greater distance.

The occurrence of the target nematodes in soils associated with intercrops at similar or higher densities than around coffee roots justifies reconsideration of appropriate crops to favor marginal or nonhosts. Furthermore, the greater abundance of several nematode species on the intercrop compared with the main crop raises the concern that an intercrop may not only be a suitable host but also

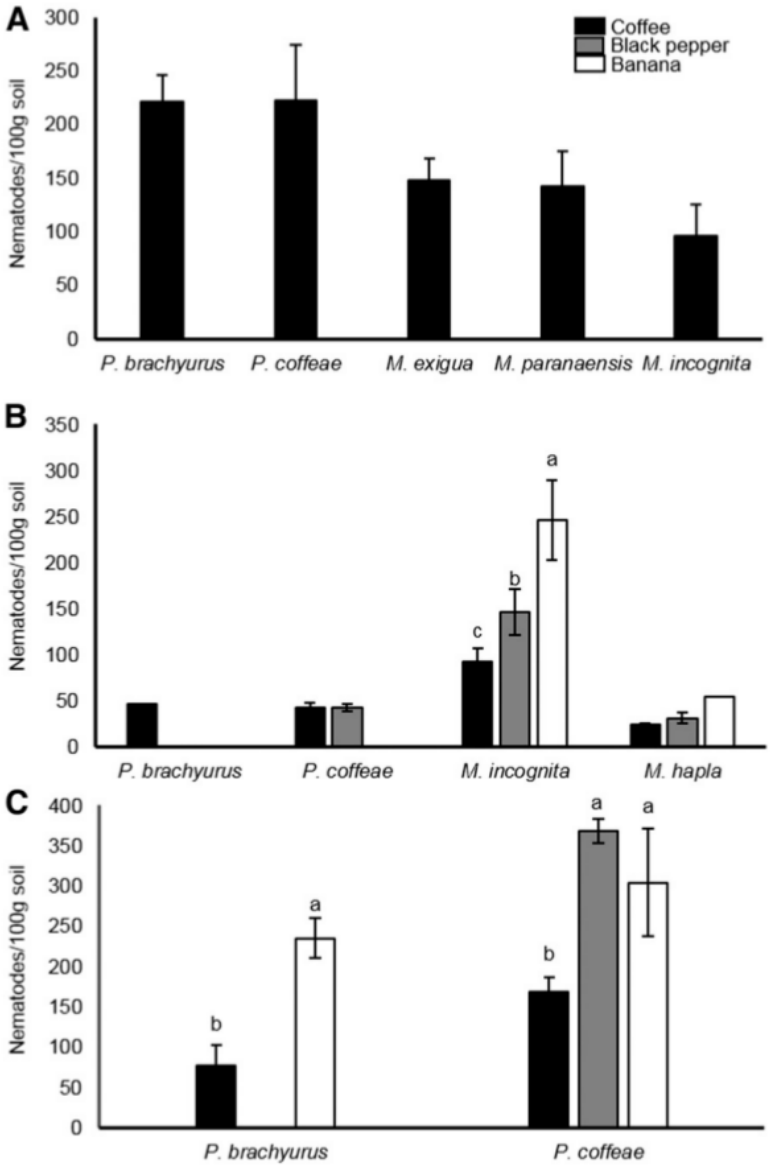

Fig. 4. Mean species abundance per 100 g of soil obtained from each crop in **A,** Minas Gerais, Brazil; **B,** Buon Ma Thuot, Vietnam; and **C,** Lampung, Indonesia. Letters note significant difference for a species between crops. Error bars represent standard error of the mean (absence indicates that <3 samples contained the nematode species).

provide tissue for nematode populations to increase in the field relative to densities associated with coffee plants. Predominance of *P. coffeae* in Vietnam may arise from conversion of fields from banana to coffee because both crops are hosts (Trinh et al. 2009). Currently, there is an increase in the cropping of black pepper within Brazilian coffee fields due to its drought tolerance and high value (Terazono 2017). This will influence the relative abundance of different nematodes present in the plantations, particularly because black pepper is not a known host for *P. brachyurus* and was also not indicated as such in this study.

The distribution of nematode field populations can frequently be described by negative binomial expression (Herve et al. 2005; Nyczepir 2009). A balance is needed between the time and cost of taking many samples against the risk of not detecting a population that is present. The negative binomial suggests that 10 samples per field, as in this study, will reliably record a population if extensive sampling established that 40% or more samples are expected to contain the target species. Therefore, our sampling strategy should reliably record the occurrence of a target species from a plantation. Sampling close to coffee plants that look damaged reduces the likelihood of reaching a false-negative conclusion and more extensive sampling could be considered if the population is not detected; however, confidence in that outcome is important.

Although several nematode species were detected within fields, there was an association of high numbers of *Meloidogyne* spp. in soil samples in which *Pratylenchus* spp. were low or not detected. Soil type, topography, and climatic factors have a differential effect on *P. coffeae* and *M. exigua* on coffee roots in Costa Rica (Avelino et al. 2009). That work also indicated competition between the two species in the roots of coffee plants, resulting in negative correlation between their populations. An association of high *Meloidogyne* spp. populations and low density of *P. coffeae* was also reported previously in the roots of coffee in Costa Rica using correspondence analysis (Herve et al. 2005). Previous work on peach, onion, mung bean, sugarcane, and barley roots has demonstrated competition between both genera of nematode with the effect and scale of effect differing with the host (BieYun 2008; Fontana et al. 2015; Nyczepir 2009; Pang et al. 2009; Umesh et al. 1994). It is of interest that the competition is observed in samples from both Brazil and Vietnam in this study, indicating its importance in global coffee-farming systems. Intergeneric competition will affect the success of each species and, therefore, impact the entire nematode community with consequences on field output. Further analysis is required to determine the effects of coffee cultivars on the scale of competition.

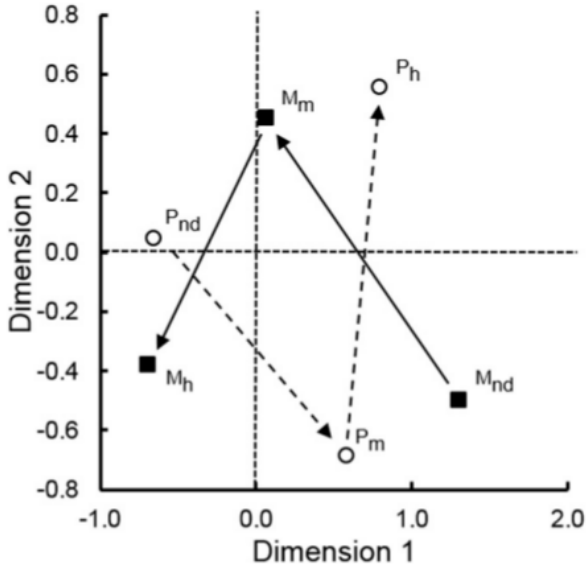

Fig. 5. Correspondence analysis showing association between *Pratylenchus* and *Meloidogyne* spp. on coffee or black pepper for 96 samples from fields in Brazil and Vietnam, in which both genera were detected. Association is indicated by proximity of data points for the three categories. Account should be taken of the relative contributions to the overall variation in the data that is explained in dimension 1 (21.0%) and dimension 2 (3.7%) of *Pratylenchus* (P) and *Meloidogyne* (M) nematodes per 100 g of soil that were (i) not detected in a sample (P_{nd} and M_{nd}), (ii) moderate (P_m 12-51 and M_m 11-111), and (iii) high (P_h 52-486 and M_h 112-529).

The status of nematodes on coffee is often assessed by sampling roots, thereby disturbing the plants. However, assessments are also made on soil populations (Barros et al. 2014; Herrera et al. 2011; Trinh et al. 2012). Additionally, the presence of *Pratylenchus* spp. known to damage coffee is reason enough to initiate a management program without considering its population level (Villain 2008). Coffee growers should also be advised to remain vigilant for the presence of root-knot nematodes in their plantations (Campos and Silva 2008). The nondestructive approach taken in this work is more favorable to growers and, thus, may gain wide acceptance for routine screening for management decisions, particularly where crops with sensitive roots such as black pepper are grown.

Our soil sampling of established coffee plants indicated similar mean soil densities for *P. brachyurus* and *P. coffeae* on coffee in all three countries. Alarmingly, these densities are above the damage thresholds for these species on coffee, indicating that the crop is negatively affected by this genus. There is evidence that *M. exigua* in Brazil is severely damaging to coffee and the densities found in our samples reveal the frequent severity of infestations (Rodrigues and Crozzoli 1995). More work is required in Vietnam to determine how often populations of *M. incognita* and *M. hapla* damage coffee and the damage thresholds of species complexes in different environments. The current work provides a rapid approach that can be applied with support from any competent molecular laboratory and, therefore, enables inexpensive widespread evaluation for many coffee plantations. It indicates that *Pratylenchus* spp. may be widely damaging to coffee plantations whereas species of *Meloidogyne* have more limited distributions. Work is planned to relate results from the rapid estimation of soil populations to damage severity and thresholds for each nematode in a range of growing conditions. Additional primers could be designed to detect further species that occur in these countries but not in the regions sampled in this study, such as *P. jaehni* (Inserra et al. 2001). Alongside the work studied here, this will underpin global efforts to manage populations and support selection of appropriate resistant cultivars for different localities.

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