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Diagnostics of the peach root-knot nematode, *Meloidogyne floridensis* using multiplex real-time PCR

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Abstract The peach root-knot nematode, *Meloidogyne floridensis* is an emerging pest of peach and other crops that is currently known to occur only in California, Florida, Georgia and South Carolina, USA. Morphological and morphometric differentiation of this species from other related species is difficult and requires taxonomic expertise. Molecular methods can provide rapid and reliable diagnostics of *M. floridensis*. Currently, molecular identification of *M. floridensis* is based on the analysis of the intergenic mitochondrial *COII*-16S region and the *nad5* gene sequence polymorphism using PCR-RFLP and sequencing. However, these techniques are time-consuming and not appropriate for the analysis of large numbers of samples. In this study, RAPD and whole genome sequence datasets were used to reveal species-specific DNA fragments for *M. floridensis* to develop a new diagnostic method. A rapid method of *M. floridensis* detection using multiplex Real-time PCR with TaqMan probes of three different fluorescent reporter dyes was developed in this study. Multiplex Real-time PCR simultaneously observed amplifications of three fragments with the following primer/probe sets: set 1 – species-specific for *M. floridensis/ M. hispanica*; set 2- specific for the tropical group of the root-knot nematodes and set 3 - specific for the tropical group of the root-knot nematodes excluding *M. floridensis*. Positive signals from primer/probe sets 1 and 2 and a negative signal from primer/probe set 3 indicated the presence of *M. floridensis* in a sample. These results showed that Real-time PCR with specific primers and probes, was sensitive enough to detect the *M. floridensis* DNA extracted from a single second-stage juvenile specimen per a reaction tube alone or in mixture with other nematodes. Our results also showed that widely used diagnostics for the southern root-knot nematode, *M. incognita* based on PCR with species-specific primers can generate amplicons of similar lengths with *M. floridensis* samples and therefore, compromise these methods that were specifically developed for the detection of *M. incognita.* The Seville root-knot nematode, *M. hispanica* was also detected in Florida and Mexico for the first time as a results of our field sample testing.

Keywords Intergenic *COII* -16S region, *Meloidogyne floridensis* · *Meloidogyne hispanica* · Multiplex real-time PCR, root-knot nematodes

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Introduction

The peach root-knot nematode, *Meloidogyne floridensis* was first discovered in Florida, USA and was initially identified as *M. incognita* race 3 (Handoo et al., 2004). In Florida, *M. floridensis* has infected peach, several economically important crops and weeds. It has been reported in sixteen counties and extends from Miami-Dade County northward to Alachua County (Brito et al., 2015; Maquilan et al., 2018) This nematode species can parasitize *Prunus* rootstocks 'Flordaguard', 'Guardian', and 'Nemared', all of which are resistant to *M. incognita* and *M. javanica* (Brito et al., 2015). Prior to 2018, *M. floridensis* had not been reported outside of Florida. In 2018, samples of rhizosphere soil and roots with galls symptomatic for root-knot nematode infection were collected from almond orchards in Merced and Kern Counties, California, USA. Almond trees grafted on the root-knot nematode resistant peach-almond hybrid, 'Hansen 536' rootstock also showed strong symptoms of growth decline. This associated root-knot nematode was identified as *M. floridensis*, by both morphological and molecular methods. With its resistance-breaking ability and its potential for further dispersal from infected areas, this nematode species can cause negative economic impact on almond production in California (Westphal et al., 2019). Recently, the peach root-knot nematode was also found in roots of 'Guardian' peach rootstock in South Carolina (Reighard et al., 2019) and in a tomato field in Georgia (Marquez et al., 2021).

The peach root-knot nematode and several other species including three major tropical root-knot nematode species, *M. incognita*, *M. javanica* and *M. arenaria*, are commonly referred to as the tropical root-knot nematode (RKN) group or complex. This group does not include *M. enterolobii* and *M. haplanaria,* which are also with the tropical RKN group belonging to the RKN Clade I (Álvarez-Ortega et al., 2019).

Accurate and timely diagnostics are an imperative first step in the developing pest management strategies. The diagnosis of *M. floridensis* can be challenging due to the high morphological and morphometrical similarities to several other root-knot nematodes including *M. javanica, M. incognita,* and *M. arenaria.* More recently, several biochemical and molecular methods were applied for *M. floridensis* identification. Esterase isozyme phenotypes were used to distinguish this nematode species from other species (Handoo et al., 2004). Nucleotide polymorphisms in two gene fragments have also been used for identification of *M. floridensis*. One method includes characterization of the mitochondrial (mtDNA) gene fragment between *COII* and 16S rRNA using sequencing or PCR-Restriction fragment length polymorphism (RFLP). The mtDNA region between *COII* and 16S rRNA genes amplified by the C2F3 and 1108 primer set can be sequenced or digested with *Hinf*I and *Ssp*I restriction enzymes. Digestion of the PCR product with *Hinf*I yields two unique fragments of approximately 770 bp and 370 bp for *M. floridensis*, which consistently allows distinction of *M. floridensis* from *M. arenaria* and certain other species (Jeyaprakash et al., 2006; Smith et al., 2015). The second method is based on analysing a short sequence of the mitochondrial *nad5* gene, which has several unique nucleotides for this species. Other genes, including ribosomal RNA genes (Handoo et al., 2004; Tigano et al., 2005) are too conserved to differentiate *M. floridensis* reliably from other RKN species. All these techniques are time-consuming and not appropriate for the high throughput analysis of large sample numbers. However, PCR with species-specific primers can have high accuracy, sensitivity, and convenience (Blok & Powers, 2009; Subbotin et al., 2021) and could be applied for *M. floridensis* diagnostics.

The main goal of this study was to develop a rapid and reliable method of *M. floridensis* detection using Real-time PCR with species-specific primers. The following tasks had to be fulfilled: *i*) evaluate some published species-specific *Meloidogyne* primers against *M. floridensis*; *ii*) identify DNA genome fragments containing sufficient nucleotide polymorphism to differentiate *M. floridensis* from other *Meloidogyne* species; *iii*) design and test the species-specific and group-specific primer/probe for detection of *M. floridensis* and other root-knot nematodes from the tropical group; *iv*) optimize and evaluate multiplex Real-time PCR for detection of *M. floridensis.*

Historically *M. floridensis* was morphologically and molecularly grouped with *M. incognita,* therefore it was necessary to re-examine several published *M. incognita* specific primers (Dong et al., 2001; Randig et al., 2002; El-Ghore et al.,2004; Meng et al., 2004; Kiewnick et al., 2013) against *M. floridensis* to test their specificity.

The following two approaches were applied to identify genome fragments containing sufficient nucleotide polymorphism to differentiate *M. floridensis* from other

species. The first included applying Random Amplified Polymorphic DNA (RAPD) analysis for *M. floridensis* and other RKN species to find a sequence-characterized amplified region (SCAR) to design species-specific primers. The second approach included comparative in silico analysis of *M. floridensis* and other RKN contigs obtained from the genome projects (Lunt et al., 2014; Szitenberg et al., 2017). Unknown DNA genome fragments containing unique polymorphism were identified using comparative in silico analysis and several putative species-specific primers to *M. floridensis* were designed. Further primer testing with PCR revealed that most of them were not species-specific, and only one primer set was selected as species-specific for *M. floridensis*. However, during Real-time PCR assay validation of field samples, it has been found that this primer set can also give a positive reaction with the Seville RKN, *M. hispanica*, which was unexpectedly found among the RKN samples from Florida and Mexico.

Two group specific primer sets were also designed and tested. One set was specific for the tropical RKN group species and was used to confirm successfulness of DNA extraction and PCR. The second set was specific for all tropical RKN group species excluding *M. floridensis*.

All candidate primers were initially screened for specificity using conventional PCR and subsequently with Real-time PCR and TaqMan probes. First, the sensitivity and efficiency of the Real-time PCR primers and probes were evaluated using different concentrations of DNA template. Finally, the Real-time PCR primers and probes were tested in singleplex and multiplex reactions against target and non-target nematodes. In the multiplex Real-time PCR experiments with specific and universal primer-probe sets, the signals were simultaneously observed for several target genes.

Materials and methods

Nematode samples

Second-stage juveniles (J2), males and females of the root-knot nematodes from root and soil samples were extracted using the centrifugal-flotation method (Coolen, 1979) or root dissection method. This study used two isolates of *M. floridensis* from California (Atwater, Merced County and Bakersfield, Kern County) and several isolates of this species collected from Florida (Table 1). Several RKN species from the tropical group, *M. arenaria, M. incognita, M. javanica, M. hispanica* and other groups, *M. enterolobii*, *M. haplanaria, M. baetica, M. hapla* and *M. graminis* (Table 1) obtained from the CDFA collection and other sources were also used for the specificity experiment and field validation.

DNA extraction

Nematode specimens were cut using a dental stainless-steel needle and placed in 0.5 ml Eppendorf tubes. DNA was extracted from J2 of each of the RKN species using the proteinase K protocol with 3 μl proteinase K (200 μg/μl), 2 μl 10X PCR buffer (Qiagen, USA), and 15 μl of water. Samples were then incubated at 65 °C for 1 hour followed by 15 min at 95 °C (Subbotin, 2021). Several DNA extracts were prepared: *i*) J2s; *ii*) J2s of *M. floridensis* with other non-target soil nematodes; *iii*) *M. floridensis* females with egg-masses. DNA extracted from 100 J2s of *M. floridensis* was used to construct a six-point, 5-fold serial dilution standard curve. DNA concentration was measured with a NanoVue spectrophotometer (GE Healthcare).

Molecular identification of RKN

RKN were identified using the analysis of the *nad5* gene and the intergenic *COII*-16S mitochondrial gene region sequences. The *nad5* gene was amplified with NAD5F2 (5′ - TAT TTT TTG TTT GAG ATA TAT TAG - 3′) and NAD5R1 (5′ - CGT GAA TCT TGA TTT TCC ATT TTT - 3′) primer set (Janssen et al., 2016) and the intergenic *COII*-16S mitochondrial gene region was amplified with C2F3 (5′ - GGT CAA TGT TCA GAA ATTT GTG G - 3′) (Powers & Harris, 1993) and R-time-MeluR2 (5′ - AAA TCT TYT CCC TAA TAA TTT TTC GTA-3′) (this study) primer set. The PCR reaction was performed in a total volume of 22 μl containing 10 μl Dream Taq Green PCR Master Mix (Thermo Fisher Scientific, USA), 10 μl nuclease - free water, 0.15 μl of each primer, and 1.7 μl DNA. The following thermal cycler conditions were used: an initial denaturation for 4 min at 94 °C, followed by 40 cycles of 60 sec at 94 °C, 60 sec at 45 °C, 90 sec at 72 °C and finally an extension for 10 min at 72 °C. PCR product was run on 1% agarose gel in TAE buffer (Promega, Madison, USA) at 100 V for 55 min. Amplicons were stained with GelGreen nucleic acid stain

Table 1 List of *Meloidogyne floridensis* samples and other root-knot nematode species used in the Real-time PCR assay

Species	Sample code	Origin	Plant-host	Real-time PCR assay, Primer/Probe Set		
				Set 1	Set 2	Set 3
M. floridensis	CD2993	USA, California, Merced County	*Prunus dulcis**	+	+	–
M. floridensis	CD3063	USA, California, Kern County	*P. dulcis*	+	n/a**	n/a
M. floridensis	CD3064, CD3104	USA, California, Kern County	*P. dulcis*	+	n/a	n/a
M. floridensis	CD3324	USA, California, Kern County	*P. dulcis*	+	+	–
M. floridensis	CD2219; N16–688	USA, Florida, Alachua County	*Beta vulgaris*	+	n/a	n/a
M. floridensis	CD2224; N16–687	USA, Florida, Alachua County	*B. vulgaris*	+	n/a	n/a
M. floridensis	CD2420; N16–116	USA, Florida, Charlotte County	*P. persica*	+	n/a	n/a
M. floridensis	CD2422; N16–1651	USA, Florida, Marin County	Mixed roots	+	n/a	n/a
M. floridensis	CD2424; N17–137	USA, Florida, Citrus County	*P. persica*	+	n/a	n/a
M. floridensis	CD2425; N16–1564	USA, Florida, Polk County	*P. persica*	+	n/a	n/a
M. floridensis	CD2426; N16–1562	USA, Florida, Polk County	*P. persica*	+	n/a	n/a
M. floridensis	CD2445; N17–212	USA, Florida, Citrus County	*P. persica*	+	n/a	n/a
M. floridensis	CD2446; N17–219	USA, Florida, Citrus County	*P. persica*	+	n/a	n/a
M. floridensis	CD2447; N17–217	USA, Florida, Citrus County	*P. persica*	+	+	–
M. floridensis	CD2452; N17–214	USA, Florida, Citrus County	*P. persica*	+	+	–
M. floridensis	CD3045; N16–1081-2	USA, Florida, Alachua County	*P. persica*	+	n/a	n/a
M. floridensis	CD3047; N16–1081-3	USA, Florida, Alachua County	*P. persica*	+	n/a	n/a
M. floridensis	CD3048; N16–597-2	USA, Florida, Alachua County	*P. persica*	+	n/a	n/a
M. floridensis	CD3053; N16–697-1	USA, Florida, Dixie County, Suwannee	Mixed roots	+	n/a	n/a
M. floridensis	CD3095; N19–287	USA, Florida, St. Lucie County	*P. persica*	+	n/a	n/a
M. floridensis	CD3106; N16–696-1	USA, Florida, Alachua County	*Antirrhinum* sp.	+	+	–
M. arenaria	CD3040	University of California, Riverside, Collection	*Solanum lycopersicum*	–	n/a	n/a
M. arenaria	CD3342; N20–00865-1	USA, Florida, Alachua County	*Quercus nigra*	–	+	+
M. arenaria	CD3343; N20–00676-1	USA, Florida, Polk County	*Murraya koenigii*	–	+	+
M. arenaria	CD3344; N20–00678-6	USA, Florida, Polk County	*Amyris baisamifera*	–	+	+
M. arenaria	CD3043; Isolate 46	University of California, Riverside, Collection	*S. lycopersicum*	–	+	+
M. arenaria	CD3086; 410P50001351	USA, Florida, Orange County	*Dizygotheca* sp.	–	+	+
M. arenaria	CD3227b, c; A401	USA, California	*Vitis vinifera*	–	n/a	n/a
M. arenaria	CD3338; N20–794-13	USA, Florida, Alachua County	Mixed roots	–	+	+
M. arenaria	CD3352; N20–579-4	USA, Florida, Dade County	Mixed roots	–	+	+
M. arenaria	CD3100; MA0101-UF	USA, Florida, Levy County	*Arachis hypogaea*	–	+	+
M. arenaria	CD2410; N16–1552	USA, Florida, Polk County	*P. persica*	–	+	+
M. arenaria	CD2413; N16–1555	USA, Florida, Polk County	*P. persica*	–	+	+
M. arenaria	CD2442; N17–305	USA, Florida, Palm Beach County	*Eupatorium capillifolium*	–	+	+
M. arenaria	CD2444; N16–1565	USA, Florida, Polk County	*P. persica*	–	+	+
M. arenaria	CD3093; N16–01563-3B	USA, Florida, Polk County	*P. persica*	–	+	+
M. arenaria	CD3228; B101	USA, California, Merced County	*V. vinifera*	–	+	+
M. arenaria	CD3230a, c; C401	USA, California	*V. vinifera*	–	+	+

Table 1 (continued)

Species	Sample code	Origin	Plant-host	Real-time PCR assay, Primer/Probe Set		
				Set 1	Set 2	Set 3
M. arenaria	CD3231; B201	USA, California, Kern County	*V. vinifera*	–	n/a	n/a
M. arenaria	CD3233; B301	USA, California, Fresno County	*V. vinifera*	–	n/a	n/a
M. arenaria	CD3618, CD3619	USA, California, Merced County	*P. persica,* cv. Butte	–	+	+
M. graminis	CD3424, N20–821-5	USA, Florida	Mixed roots	–	–	–
M. incognita	CD3336; N20–00859-6	USA, Florida, Palm Beach County	*Sansevieria* sp.	–	+	+
M. incognita	CD3337; N20–00588-13	USA, Florida, Palm Beach County	Mixed roots	–	+	+
M. incognita	CD3031, Isolate 20	University of California, Riverside, Collection	*S. lycopersicum*	–	n/a	n/a
M. incognita	CD3033, Isolate 33	University of California, Riverside, Collection	*S. lycopersicum*	–	n/a	n/a
M. incognita	CD3034, Isolate 47	University of California, Riverside, Collection	*S. lycopersicum*	–	+	+
M. incognita	CD3036, Isolate 48	University of California, Riverside, Collection	*S. lycopersicum*	–	+	+
M. incognita	CD3037, Isolate 49	University of California, Riverside, Collection	*S. lycopersicum*	–	+	+
M. incognita	CD3038, CD3041, Isolate 18	University of California, Riverside, Collection	*S. lycopersicum*	–	+	+
M. incognita	CD3042, Isolate 45	University of California, Riverside, Collection	*S. lycopersicum*	–	+	+
M. incognita	CD3101b, c	USA, California	*S. lycopersicum*	–	n/a	n/a
M. incognita	CD3229; A201	USA, California, Kern County	*V. vinifera*	–	n/a	n/a
M. incognita	CD3334; N20–650-1	USA, Florida	*Ligustrum japonicum*	–	+	+
M. incognita	CD3353; N20–873-11	USA, Florida, Sumter County	Mixed roots	–	+	+
M. incognita	CD3414; N20–815-2	USA, Florida, Alachua County	*Abelmoschus esculentus*	–	+	+
M. incognita	CD3426; N20–815-1	USA, Florida, Alachua County	*A. esculentus*	–	+	+
M. incognita	CD3437; N20–613-42	USA, Florida, Dade County	Mixed roots	–	+	+
M. incognita	CD3438; N20–859-10	USA, Florida, Palm Beach County	*Sansevieria* sp.	–	+	+
M. incognita	CD3232; A301	USA, California, Fresno County	*V. vinifera*	–	n/a	n/a
M. incognita	CD3378	USA, California, Kern County	*V. vinifera*	–	+	+
M. incognita	CD3219	USA, California, Madera County	*Ficus carica*	–	+	+
M. incognita	CD3451	USA, California	*S. lycopersicum*	–	+	+
M. javanica	CD3332; N20–640-19	USA, Florida, Polk County	*Casimiroa tetrameria*	–	+	+
M. javanica	CD3350; N20–797-1	USA, Florida, Levy County	Mixed roots	–	+	+
M. javanica	CD3406; N20–1132-1	USA, Florida, St. Lucie County	Mixed roots	–	+	+
M. javanica	CD2355	USA, Florida, Hillsborough County	*Humulus lupulus*	–	+	+
M. javanica	CD2417; N16–00081	USA, Florida, Pasco County	*P. persica*	–	+	+
M. javanica	CD2427; N16–1282	USA, Florida, Polk County	*P. persica*	–	+	+
M. javanica	CD2430; N16–1288	USA, Florida, Polk County	*P. persica*	–	+	+
M. javanica	CD2428; N16–1289	USA, Florida, Polk County	*P. persica*	–	+	+
M. javanica	CD2421; N16–00075	USA, Florida, Pasco County	*P. persica*	–	+	+
M. javanica	CD3049; N16–84-A	USA, Florida, Pasco County	*P. persica*	–	n/a	n/a

Table 1 (continued)

Species	Sample code	Origin	Plant-host	Real-time PCR assay, Primer/Probe Set		
				Set 1	Set 2	Set 3
M. javanica	CD3050; N16–84-B	USA, Florida, Pasco County	*P. persica*	–	+	+
M. javanica	CD3103; N16–1091-3	USA, Florida, Alachua County	*P. persica*	–	+	+
M. javanica	CD3110	USA, California, Kern County	*S. lycopersicum*	–	+	+
M. javanica	CD2433; N16–01283	USA, Florida, Polk County	*P. persica*	–	+	+
M. javanica	CD2434; N16–01280	USA, Florida, Polk County	*P. persica*	–	+	+
M. javanica	CD2443; N16–01268	USA, Florida, Polk County	*P. persica*	–	+	+
M. javanica	CD2451; N17–184	USA, Florida, Citrus County	*P. persica*	–	+	+
M. hispanica	CD2435; N17–304	USA, Florida, Palm Beach County	*Urena lobata*	+	+	+
M. hispanica	CD2436; N17–305	USA, Florida, Palm Beach County	*E. capillifolium*	+	+	+
M. hispanica	CD2439; N17–303	USA, Florida, Palm Beach County	*U. lobata*	+	+	+
M. hispanica	CD3416; N20–668-9	USA, Florida, Highlands County	*Caladium* × *hortulanum*	+	+	+
M. hispanica	M309MpEb; M309MVEa; M309MVEb	Mexico, Puebla State, Acatzingo	*Allium ampeloprasum*	+	n/a	n/a
M. baetica	CD3382	Spain	*Olea europaea*	–	–	–
M. hapla	C44	USA, California	*S. lycopersicum*	–	–	–
M. haplanaria	CD3446	USA, California	*Sarracenia* sp.	–	–	–
M. enterolobii	CD3415; N20–962-5	USA, Florida, Lee County	Mixed roots	–	–	–

*Almond trees grafted on the root-knot nematode resistant peach-almond hybrid

**n/a - not applicable

(Biotium, USA) and visualized under a UV light on the Gel Doc XR imaging system (Bio-Rad, USA). The PCR amplicon was purified by using QIAquick PCR Purification Kit (Qiagen, USA) according to the manufacturer's instructions. PCR products were directly sequenced by Quintara Bioscience (CA, USA). Identification of nematodes was based on comparison with the reference sequences for each species (Janssen et al., 2016). The new sequences for *nad5* and *COII*-16S genes were deposited in the GenBank with accession numbers: OM418720-OM418761.

RAPD analysis

RAPD-PCR was performed using the Extract-N-Amp PCR Ready Mix (Sigma-Aldrich, USA) in a final volume of 20 µl containing 10 µl 2X N-Amp PCR Mix, 0.15 µl of primer, 0.5 µl $MgCl_2$ (25 mM), 7.35 µl nuclease-free water, and 2 µl DNA. The following thermal cycler conditions were used: an initial denaturation at 94 °C for 4 min, 39 cycles of denaturation at 95 °C for 1 min, annealing at 36 °C for 1 min, extension at 72 °C for 1 min, and a final incubation at 72 °C for 10 min. The RAPD-PCR amplicons were loaded in a 1.2% agarose gel in TAE buffer for separation using electrophoresis (100 V for 3 hours) and the bands were visualized as described above. Thirteen primers were screened in the study. Sequences of these 10-mer oligonucleotide RAPD primers (J10, J20, K06, K04, K07, K09, K14, K16, K19, K20, M10, P02, P05) are given by Handoo et al. (2004).

The band of interest identified in the RAPD analysis was selected, excised from gel, and purified using the QIAquick PCR Purification Kit (Qiagen, USA). RAPD-PCR amplicons were ligated to the pGEM-T vector system and transformed into *Escherichia coli* JM109 Competent Cells (Promega, USA). PCR was done on three independent clones to confirm the presence of the

inserted DNA fragment. The amplified products were sequenced in both directions by Quintara Bioscience (CA, USA). Blastn was used to determine whether this sequence had some identity with any known sequences in the GenBank database.

Species-specific primer and probe set design

Primers were developed based on sequence comparison of randomly selected contigs from the whole genome sequence projects of *M. floridensis* (CCDZ00000000, Lunt et al., 2014; RCFN00000000, Szitenberg et al., 2017), with contigs of other root-knot nematode species from other genome projects. The contigs of *M. floridensis* were aligned using ClustalX 1.83 (Thompson et al., 1997) with contigs of other species selected based on the highest Blastn similarities. Several species-specific primers and probes for *M. floridensis* were manually designed based on sequence polymorphism.

Group-specific primer and probe set design

Two group-specific primer and probe sets were developed to detect the tropical RKN group species. The first group set was designed based on nucleotide polymorphism of the intergenic 5S–18S ribosomal RNA spacer region (Blok et al., 1997). This set was specific for all tropical group nematode species and was used to confirm the successful extraction and PCR amplification of nematode DNA. The second group set was designed based on nucleotide polymorphism of the intergenic *COII*-16S rRNA region with an insertion/deletion location (Humphreys-Pereira et al., 2014; Powers & Harris, 1993). This set was specific for the tropical RKN group excluding *M. floridensis*, which has a unique deletion in this genome region (Table 3).

Conventional PCR with species-specific and group-specific primers

Validation of new *M. floridensis* and published *M. incognita* species-specific primer pairs and group-specific primers for the tropical group were carried out using BIO-RAD (C1000 Touch) thermo cycler. The reaction was performed in a total volume of 22 µl containing 2.5 µl 10X PCR buffer, 0.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTPs, 17.1 µl nuclease - free water, 0.1 µl of Taq polymerase, 0.15 µl of each primer and 1 µl DNA. The PCR conditions used were as follows: an initial denaturation at 94 °C for 4 min, followed by 34 cycles of 60 sec at 94 °C, 90 sec at 55 °C, 2 min at 72 °C and finally an extension for 10 min at 72 °C. In the present study five *M. incognita* species-specific primer pair sets were used: *i*) Inc-K14-F and Inc-K14-R (Randig et al., 2002); *ii*) Mi2F4 and Mi1R1 (Kiewnick et al., 2013); *iii*) MI-F and MI-R (Meng et al., 2004); *iv*) MiF and MiR (Dong et al., 2001); *v*) MIE-for and MIE-rev (El-Ghore et al., 2004).

Multiplex real-time PCR assay with species-specific and group-specific primers

Real-time PCR experiments were performed using the SensiFast Probe Lo-ROX Kit (Bioline, USA). The reaction for a singleplex format was performed in a total volume of 20 µl containing 10 µl 2X SensiFast Probe Lo-ROX, 1 µl of each primer (400 nM), 0.4 µl of Taqman probe (200 nM), 5.6 µl nuclease-free water, and 2 µl of DNA. For duplex and triplex formats, the amount of water was reduced to account for the additional primer and probe volumes. The reactions were carried out in Applied Biosystem QuantStudio 7 Flex Real-Time PCR system in singleplex, duplex and triplex formats. Annealing temperature optimization for primers was conducted on different temperatures ranges (58, 60, and 62 °C). Following annealing temperature optimization, the thermal condition used for all subsequent Real-time PCR runs were as follows: an initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 sec, annealing/extension and plate read at 60 °C for 60 sec. Two or three replicates of each variant including no template control, NTC (negative control without DNA) were performed for sensitivity and specificity experiments across several runs. The specificity of the primer-probe set was tested in singleplex, duplex and triplex reactions where the signals were detected simultaneously against target and non-target genes. PCR amplicons were also run on agarose gels as above for band verification.

Real-time PCR assay of field samples

To evaluate and validate the practical application of the Real-time PCR assays with species-specific and group-specific primers in singleplex or multiplex formats, samples of root-knot nematodes obtained from nematology field surveys, commercial samples shipped to the CDFA (Table 1), and a control sample containing

M. floridensis DNA were tested. Presence of root-knot nematodes in field samples were also determined by microscopic examination of soil extracts. Species of the RKN in the samples were identified using *nad5* and *COII*-16S gene sequences.

Results

Testing of *Meloidogyne incognita* species-specific primers with *M. floridensis* samples

Five *M. incognita* species-specific primer sets were tested for possible nonspecific amplification with *M. floridensis* using conventional PCR. The *M. incognita* species-specific primer sequences and the expected amplified fragment length are listed in Table 2 Four of the primer sets produced amplicons with expected lengths for *M. incognita* sample: *i*) Inc-K14-F and Inc-K14-R – a fragment of ~399 bp; *ii*) Mi2F4 and Mi1R – a fragment of ~300 bp;1 *iii*) MI-F and MI-R – a fragment of ~955 bp; *iv*) MiF and MiR – a main fragment of ~1350 bp (Fig. 1). However, multiple amplicons were observed on the gel with the MIE-for and MIE-rev primers for both *M. incognita* and *M. floridensis* samples. *Meloidogyne floridensis* samples also produced visible amplicons of expected length as specific amplicons of *M. incognita,* when tested with three species-specific primer sets: Inc-K14-F and Inc-K14-R, Mi2F4 and Mi1R and MiF and MiR (Fig. 1).

RAPD analysis of *Meloidogyne floridensis* samples

Six *M. floridensis* samples and three samples for each species, *M. incognita*, *M. arenaria*, and *M. javanica* were used in this study. Two primers K20 (Fig. 2a) and P05 (Fig. 2b) of the thirteen primers (data not shown), generated maximum numbers of polymorphic bands ranging between 200 bp and 1500 bp. Primer P05 resulted in a unique RAPD band of approximately 380 bp for all six *M. floridensis* samples but was absent in other root-knot nematodes (Fig. 2b). This band was gel purified, cloned and sequenced. The SCAR fragment sequences (OM427503-OM427506) showed 99–100% similarity with GenBank sequences of *M. floridensis* (RCFN01001898), but also with *M. luci* (CACSLI010000154) and therefore were excluded from further study.

Species-specific primers for detection of *Meloidogyne floridensis/M. hispanica*

Fourteen putative species-specific *M. floridensis* primer sets were designed based on polymorphic sites of aligned genomic nucleotide contigs from the genome RKN projects and screened by conventional PCR with DNA templates of different *Meloidogyne* species. After preliminary testing primer set 1 (FlorF7/ FlorR7) was found to be most promising for *M. floridensis* producing a unique band of about 200 bp (Fig. 3). However, further testing showed that similar length bands were also observed for several RKN samples from Florida and Mexico. Molecular identification of these samples revealed that they belonged to *M. hispanica.* Thus, the Flor7 primer set was identified to be specific for two species: *M. floridensis* and *M. hispanica*. No amplifications were obtained for the other RKN species, including *M. arenaria*, *M. incognita*, and *M. javanica* (data not shown).

Table 2 *Meloidogyne incognita* species-specific primers used in this study

Primer	Sequence (5′ – 3′)	Amplicon size (bp)	Reference
MiF MiR	TAG GCA GTA GGT TGT CGG G CAG ATA TCT CTG CAT TGG TGC	1350	Dong et al. (2001)
Inc-K14-F Inc-K14-R	GGG ATG TGT AAA TGC TCC TG CCC GCT ACA CCC TCA ACT TC	399	Randig et al. (2002)
MIE-for MIE-rev	TCC GTG CTG TAG CTT GCC C CAC CAT CCG TTA TAA GCT CTG	900	El-Ghore et al. (2004)
MI-F MI-R	GTG AGG ATT CAG CTC CCC AG ACG AGG AAC ATA CTT CTC CGT CC	955	Meng et al. (2004)
Mi2F4 Mi1R1	ATG AAG CTA AGA CTT TGG GCT TCC CGC TAC ACC CTC AAC TTC	300	Kiewnick et al. (2013)

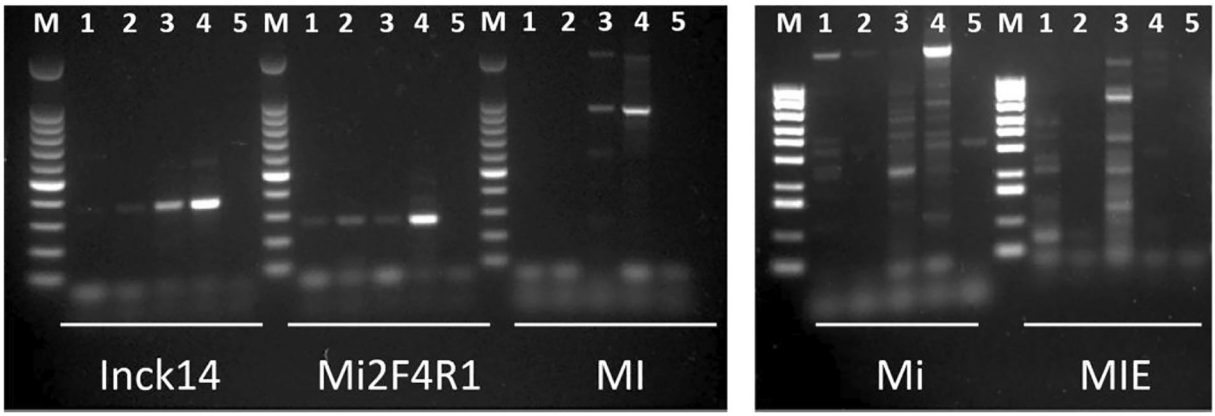

Fig. 1 Results of PCR amplification with the species-specific *Meloidogyne incognita* primers: Inc-K14-F and Inc-K14-R; Mi2F4 and Mi1R; MI-F and MI-R; MiF and MiR; MIE-for MIE-rev. Lanes: 1 and 2: *M. floridensis* CD3053, CD3095); 3 and 4: *M. incognita* (CD3031, CD3036); 5: control without DNA (water); M: DNA marker (100 bp and 1 kb)

Group-specific primers for detection of the tropical RKN species

The group-specific primer set 2 (MtropF2-Real/MeloidR)(Table 3) with conventional PCR showed high specificity to all tested species from the tropical RKN group, producing a PCR product of 238 bp and did not amplify DNA from any other species (data not shown). The group-specific primer set 3 (R-time-MeluF1/R-time-MeluR2)(Table 3) showed only specificity to the tropical RKN group species, except for *M. floridensis*, and produced a PCR product of 146 bp (data not shown).

Development of real-time PCR assay

Annealing temperature optimization Annealing temperature optimization for Real-time PCR with the primer/probe set 1 (FlorF7/FlorR7/Flor7-probe) was performed at a temperature range of 58, 60, and 62 °C. All Real-time PCR assays carried out using the different primer annealing temperatures resulted in positive signal for *M. floridensis* samples but not for negative control (water) and non-*M. floridensis* samples. The primer annealing temperature of 60 °C resulted in the lowest average Ct-value (28) compared to the annealing temperature of 58 °C, which resulted in an average Ct-value of 30. The annealing temperature of 62 °C yielded no amplification. As a result, the annealing temperature of 60 °C was adopted for all subsequent real-time PCR runs.

Specificity assay The TaqMan fluorescent probe-based real-time PCR system was used for the detection and amplification of all assays. Figures 4 and 5 represent examples of amplification plots for three primers and

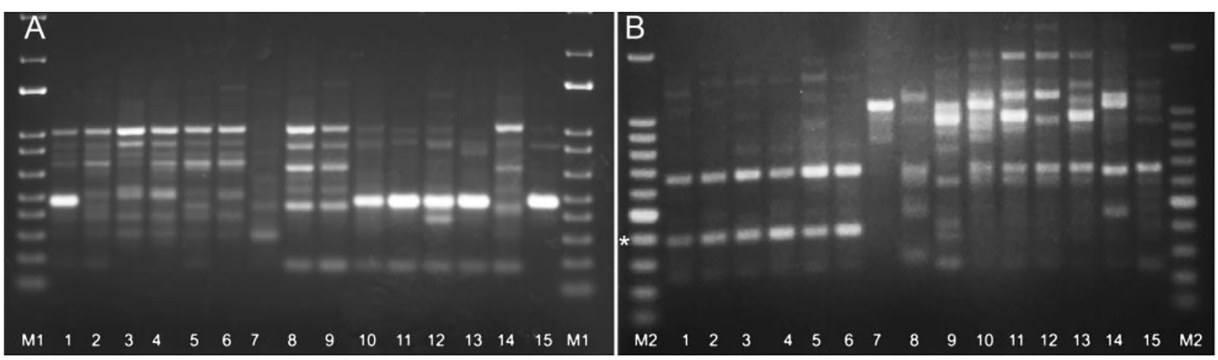

Fig. 2 RAPD fingerprinting generated from *M. floridensis, M. incognita*, *M. arenaria*, and *M. javanica* DNA using primers K20 (5′- GTG TCG CGA G - 3′) (**a**) and PO5 (5′- CCC CGG TAA C - 3′) (**b**). Lanes: 1–6: *M. floridensis* (CD3048, CD3053, CD3064, CD5095, CD3063, CD3104); 7–9: *M. incognita* (CD3031, CD3037, CD3042); 10–12: *M. arenaria* (CD3040, CD3043, CD3086); 13–15: *M. javanica* (CD3110, CD3050, CD3049). M: DNA marker (1 kb)

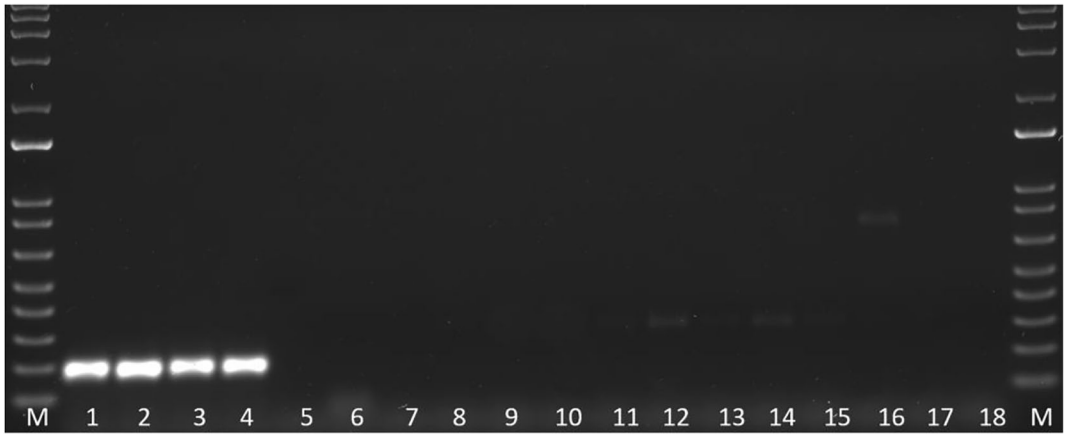

Fig. 3 Specificity of *M. floridensis* specific primers (Flor7F/FlorR7) tested with DNA templates of four *Meloidogyne* species. Depiction of Real-time PCR result on agarose gel. Lanes: M: DNA marker (1 kb); 1–4: *M. floridensis*; 5–8: *M. incognita*; 9–12: *M. javanica*; 13–16: *M. arenaria*; 17–18: control without DNA (water)

probe sets run in multiplex with *M. floridensis, M. hispanica, M. javanica* and other species, the normalized fluorescence signal (ΔR) was plotted against the cycle number. Based on analysis of amplification plots with target, no-target samples, and NTC from all experiments with singleplex and multiplex formats, the threshold level for *M. floridensis* detection was estimated with a Ct-value of 34. Samples producing an exponential amplification curve above the threshold value were considered positive, whereas amplification below the threshold level were considered negative.

The primer/probe set 1 showed a high detection rate with all *M. floridensis* samples used in Real-time PCR, and none with *M. arenaria*, *M. incognita*, and *M. javanica* samples (Figs. 3, 4 and 5). However, additional testing with a wider range of samples revealed that the primer/probe set 1 gave a positive signal for the RKN samples identified in our study as *M. hispanica* (Fig. 5a). Multiplex Real-time PCR using the three primer/probe sets detected all *M. floridensis* samples and was able to differentiate this species from *M. hispanica*. Multiplex Real-time PCR simultaneously

Table 3 Primers and probes used for the Real-time PCR assays

Primer/probe set	Primer or probe code	Genome region	Sequence (5′ – 3′)
Set 1 (species-specific for *M. floridensis* and *M. hispanica*)	FlorF7	Unknown genome fragment	TTT TCC TCT GAA AGG GAA TGG
	FlorR7		TGG TCT GAT AAA TAT TTC TAG
	Flor7-probe		[FAM]* TTG CCA GCA CTT CTC CTT AGG T [BHQ-1]**
Set 2 (group-specific for the tropical RKN species)	MtropF2-Real	intergenic 5S–18S ribosomal RNA spacer region, nuclear	TAT AAC TTT TGT GAA TTT ATA ATT
	MeloidR		ACA TCA GTT CAG GCA GGA TCA
	Mtrop - probe2		[JOE]* ACC AGC AGT CTC GGT AAT TCA AGC T [BHQ-1]**
Set 3 (group specific for the tropical RKN species except for *M. floridensis*)	R-time-MeluF1	intergenic *COII*-16S rRNA region, mitochondrial	CAA TTG GTT GTG TTA ATC ATT CT
	R-time-MeluR2		AAA TCT TYT CCC TAA TAA TTT TTC GTA
	R-time-Melu-probe		[Texas Red]* TAT TAG ATC GGG GTT TAA TAA TGG GTT C [BHQ-2]**

* - *reporter,* ** - quencher

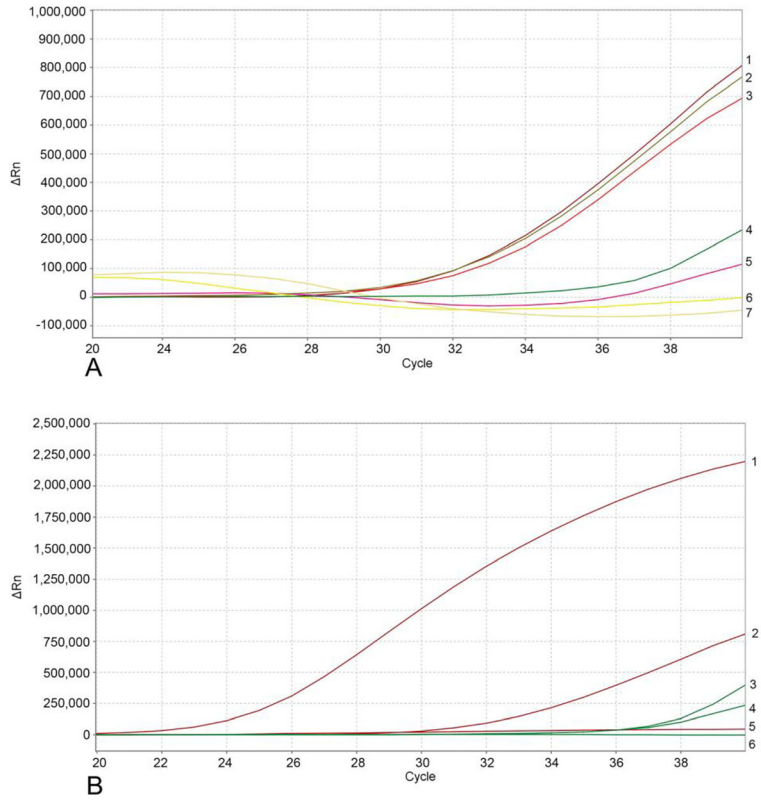

Fig. 4 Real-time PCR assay with examples of amplification plots. **A** Specificity assay with DNA samples of *Meloidogyne floridensis* and other *Meloidogyne* species using the primer/probe set 1. Line: 1, 2, 3: *M. floridensis* (CD3324, CD2447, CD2993); 4: control without DNA (water); 5: *M. arenaria* (CD3093); 6: *M. incognita* (CD3038); 7: *M. javanica* (CD2451); **B** Amplification of DNA sample of *M. floridensis* (CD3324) in a triplex format (the primer/probe sets 1, 2, 3). Line: 1: *M. floridensis* (the primer/probe set 2); 2: *M. floridensis* (the primer/probe set 1); 3: control without DNA (the primer/probe set 3); 4: control without DNA (the primer/probe set 1); 5: *M. floridensis* (the primer/probe set 3); 6: control without DNA (the primer/probe set 2)

amplifies three specific fragments with the following primers/probe sets: set 1 - designed based on an unknown genome fragment and species-specific for *M. floridensis/M. hispanica*; set 2 - designed based on nuclear intergenic 5S–18S ribosomal RNA gene spacer region and specific for the tropical group of the root-knot nematodes and set 3 – designed based on mitochondrial intergenic *COII*-16S rRNA region and specific for the tropical group of the root-knot nematodes excluding *M. floridensis*. Positive signals ($Ct \leq 34$) from the primer/probe sets 1 and 2 and a negative signal from primer/probe set 3 indicated the presence of *M. floridensis* in a sample (Fig. 4B).

Sensitivity assay Detection sensitivity for primer/probe set 1 was tested on a set of three DNA samples, each extracted from a single J2 of *M. floridensis*. DNA concentration was 24.0 ng/μl corresponding to 1 J2/μl. The Real-time PCR result confers high detection sensitivity, with as low as one J2 per reaction tube being successfully detected in all runs. Similar results were obtained using a serial dilution experiment with a reliable detection limit of a single J2 per a reaction. The Real-time PCR carried out using DNA extracted from a single J2 of *M. floridensis* (primers/probe set 1: Ct = 32.2 ± 0.1; primers/probe set 2: Ct = 25.5 ± 0.3) mixed with 30 specimens of other soil nematodes (set 1: Ct = 32.1 ± 0.2; set 2: Ct = 25.8 ± 0.03) resulted in a positive signal for *M. floridensis.* This assay was also able to detect at least 1/20 of a female in a reaction (set 1: Ct = 29.1 ± 0.8; set 2: Ct = 22.3 ± 0.5).

Standard curve analysis Amplification plots were observed for the target gene with the primer/probe set 1 with serial five-fold dilutions of the *M. floridensis* DNA template. Ct values were proportional to the log-transformed numbers of nematodes, showing that the relative numbers of *M. floridensis* juveniles could be quantified. A standard curve was constructed from Real-time PCR performed with this serial dilution of DNA (Fig. 6). There was a significant linear correlation ($R^2 = 0.99$) between the Ct values and nematode number or DNA concentration for the primers/probe sets 1 and 2. The Ct values of 20, 10, 5, 1 and 0.1 J2 per a reaction tube were 28.0 ± 0.5, 29.0 ± 0.6, 30.0 ± 0.1, 32.1 ± 0.1, 36.8 ± 3.2 for the primers/probe set 1 with amplification efficiency = 85%.

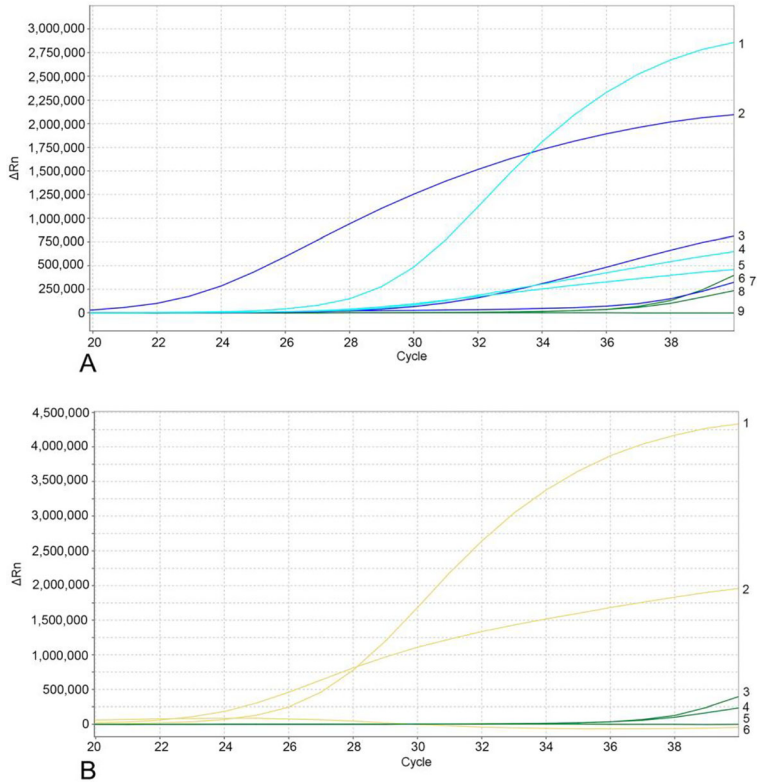

Fig. 5 Real-time PCR assay with examples of amplification plots. **A** Amplification of DNA sample of *M. hispanica* (CD2436) in a triplex format (the primer/probe sets 1, 2, 3). Line: 1: *M. hispanica* (the primer/probe set 3); 2: *M. floridensis* (CD2993)(the primer/probe set 2); 3: *M. floridensis* (CD2993)(the primer/probe set 1); 4: *M. hispanica* (the primer/probe set 1); 5: *M. hispanica* (the primer/probe set 2); 6: control without DNA (the primer/probe set 3); 7: *M. floridensis* (CD2993)(the primer/probe set 3) 8: control without DNA (the primer/probe set 1); 9: control without DNA (the primer/probe set 2); **B** Amplification of DNA sample of *M. javanica* (CD2451) in a triplex format (the primer/probe sets 1, 2, 3). Line: 1: *M. javanica* (the primer/probe set 3); 2: *M. javanica* (the primer/probe set 2); 3: control without DNA (the primer/probe set 3); 4: control without DNA (the primer/probe set 1); 5: control without DNA (the primer/probe set 2); 6: *M. javanica* (the primer/probe set 1)

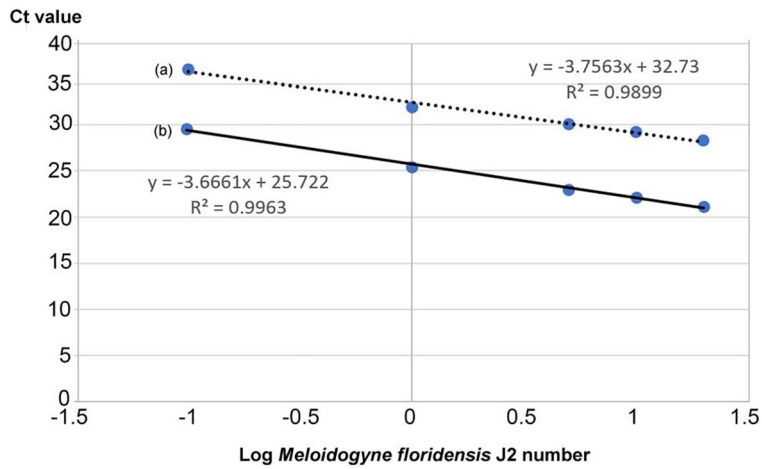

Fig. 6 Standard curves calculated with the log starting quantity and threshold cycle of the five-fold serially diluted DNA from *M. floridensis* J2 with the specific primers/probe set 1 (**a**) and the primers/probe set 2 (**b**) in multiplex format

Real-time PCR assay of field samples

This assay was validated using DNA extracts from field samples containing *M. floridensis* and other RKN species. The Real-time PCR assay detected *M. floridensis* DNA in all expected samples and showed signals below the threshold level (Ct ≤ 34) in all samples with *M. floridensis* DNA (Table 1) (primers/probe set 1: Ct = 23.5–33.1). Samples with *M. floridensis* DNA did not produce fluorescence or give a Ct value with primer/probe set 3. The DNA from other nematodes, except for *M. hispanica*, did not produce fluorescence or give a Ct value with primer/probe set 1. Positive signals for samples containing other species from the tropical RKN resulted in Ct values ranging from 19.7–32.9 and 23.5–31.8 for sets 2 and 3 primer/probe, respectively.

Discussion

In the past three decades, many SCAR-based species-specific primers have been developed for use in nematode diagnostics. These primers are designed based on the characterization and sequencing of polymorphic bands from RAPD analysis. To date, many primer pairs are also available for detection of RKN species, including *M. arabicida, M. arenaria, M. enterolobii, M. incognita, M. javanica, M. paranaensis, M. exigua, M. chitwoodi, M. graminicola, M. hapla,* and others (Subbotin et al., 2021). In this study, we tested several *M. incognita* specific primers and found that at least three pairs (Inc-K14-F and Inc-K14-R, Mi2F4 and Mi1R and MiF and MiR) produced a positive reaction with *M. floridensis*. Thus, our results show that PCR with species-specific SCAR primers should be used carefully and that the researcher must be aware of the peculiarities of this method, especially, if the primer set was not tested with a wide range of RKN species.

A draft genome of *M. floridensis* was recently released and analysed (Lunt et al., 2014; Szitenberg et al., 2017). It has been suggested that *M. floridensis* is one of the parental species in the hybrid origins of *M. incognita* because very many loci were found to be nearly identical between *M. incognita* and *M. floridensis*. Overall, the genome structure and content of tropical *Meloidogyne* had complex origins. It is likely that hybridization, ploidy change, and subsequent aneuploidy have all played a role in the evolution of the diversity in this genus (Lunt et al., 2014). However, if some *Meloidogyne* species are in fact hybrids, then this would present problems for finding species-specific markers for molecular diagnostics, as was encountered in this project. The SCAR and genome fragments obtained in this study were identical to those from *M. luci* and *M. hispanica.* Because the selection of a unique DNA fragment and the design of species-specific primers was difficult, a multiplex Real-time PCR assay with three fluorescent reporter dyes was developed for this study. Multiplex Real-time PCR simultaneously tracked amplifications of three fragments with the following primer/probe sets: set 1 – species-specific for *M. floridensis/ M. hispanica*; set 2 - specific for the tropical group of the root-knot nematodes and set 3 - specific for the tropical group of the RKN excluding *M. floridensis*. Positive signals from sets 1 and 2 and a negative signal from set 3 indicates the presence of *M. floridensis* in a sample.

Real-time PCR assays are presently developed as a diagnostic tool for detecting and quantifying of several root-knot nematode species: *M. arenaria, M. chitwoodi, M. enterolobii, M. fallax, M. graminicola, M. hapla, M. incognita, M. javanica* and *M. minor* (Subbotin et al., 2021). Sensitivity is an important parameter for Real-time PCR assay evaluation. Sensitivity performance is limited by many factors including quality of the primers and probes, and the targeted genome fragments. In the present assay, the reliable detection of one J2 per reaction seems to be relatively low compared to results of other Real-time PCR assays of RKN (Katsuta et al., 2016; Zijlstra & Van Hoof, 2006). Observed low sensitivity levels in our assay are likely the result by of the targeted genome fragment being in low copy number in the *M. floridensis/M. hispanica* genomes. A comparison of amplification plots generated for *M. floridensis/ M. hispanica* species-specific primers/probes with those produced with primers/probes designed based on multicopy ribosomal RNA and mitochondrial genes, where the latter one showed significantly higher amplification rates, confirms this suggestion. If higher sensitivity for *M. floridensis* samples is needed, investigation of new target genome fragments will be required.

One interesting and unexpected result of our study was that the Seville RKN, *M. hispanica* was detected during the field validation assay. To our knowledge this is only the second record of this nematode species in the USA following a recent report in South Carolina (Skantar et al., 2021) and it is the first report of this

nematode species in Florida and Mexico. The Real-time PCR assay developed here is able to detect *M. hispanica*. Positive signals obtained from all three primer/probe sets indicates the presence of *M. hispanica*, however, additional testing with other RKN species is needed to confirm the utility of this assay for detection of *M. hispanica*. Nevertheless, multiplex Real-time PCR assay for *M. floridensis* developed in this study could be implemented in survey and plant certification programs for root-knot nematode identification in diagnostic laboratories. It provides a timely and reliable detection of this important pest that is necessary to minimise the risk of its spread.

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Declarations The research did not involve any studies with human participants or animals performed by any of the authors, and all authors informed consent.

Conflict of interest All authors herewith declare that there is no conflict of interests. All authors are informed and agree on the publication of the manuscript.

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