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Mitochondrial Haplotype-based Identification of Root-knot Nematodes (*Meloidogyne* spp.) on Cut Foliage Crops in Florida

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Abstract: Florida accounts for more than 75% of the national cut foliage production. Unfortunately, root-knot nematodes (RKN) (*Meloidogyne* spp.) are a serious problem on these crops, rendering many farms unproductive. Currently, information on the *Meloidogyne* spp. occurring on most commonly cultivated cut foliage crops in Florida, and tools for their rapid identification are lacking. The objectives of this study were to (i) identify specific RKN infecting common ornamental cut foliage crops in Florida and (ii) evaluate the feasibility of using the mtDNA haplotype as a molecular diagnostic tool for rapid identification of large samples of RKN. A total of 200 *Meloidogyne* females were collected from cut foliage plant roots. *Meloidogyne* spp. were identified by PCR and RFLP of mitochondrial DNA. PCR and RFLP of mitochondrial DNA were effective in discriminating the *Meloidogyne* spp. present. *Meloidogyne incognita* is the most dominant RKN on cut foliage crops in Florida and must be a high target for making management decisions. Other *Meloidogyne* spp. identified include *M. javanica*, *M. hapla*, *Meloidogyne* sp. 1, and *Meloidogyne* sp. 2. The results for this study demonstrate the usefulness of the mtDNA haplotype-based designation as a valuable molecular tool for identification of *Meloidogyne* spp.

Key words: cut foliage, endonuclease, identification, mitochondrial haplotype, MORF/MTHIS, mtDNA, PCR-RFLP, restriction enzymes, TRNAH/MRH106.

Florida is the largest producer of cut foliage in the United States and accounts for over 76% of the total cut foliage production in the country (Stamps, 1999; USDA/NASS, 2014). However, many reports suggest that RKN, *Meloidogyne* spp., is a major pest of a wide range of ornamental plants grown in Florida (McSorley and Marlatt, 1983; Benson and Barker, 1985; McSorley and Dunn, 1990; McSorley and Frederick, 1994, 2001; McSorley et al., 2004; Brito et al., 2004, 2010). Nonetheless, the *Meloidogyne* spp. associated with specific cut foliage plants in Florida is currently unknown. Identification of these species is critical for effective nematode management and certification or quarantine regulatory programs.

Traditionally, *Meloidogyne* species are identified based on morphology, morphometrics, isozyme phenotype, or differential host tests (Taylor and Sasser, 1978; Jepson, 1987; Esbenshade and Triantaphyllou, 1985, 1987, 1990; Carneiro et al., 2000; Brito et al., 2004, 2008, 2010; Handoo et al., 2004; Hunt and Handoo, 2009; Moens et al., 2009). However, morphological characters are variable under different environmental conditions and different hosts. Moreover, the most widespread, economically important species including the tropical group of *Meloidogyne* spp. that reproduce by mitotic obligatory parthenogenesis are recently suggested to have reticulate (hybrid) origin (Lunt, 2008; Lunt et al., 2014; Fargette et al., 2010; Janssen et al., 2016). These species have a wide host range, intraspecific variation, interspecific similarities, and indistinct species boundaries or species complexes, which make their identification by the traditional methods more challenging. The use of isozyme phenotype is limited by the requirement of young females, whereas differential host test is useful for only certain *Meloidogyne* spp. that are important on some important row crops. It is suggested that host specificity may be under epigenetic control (Robertson et al., 2009; Perfus-Barbeoch et al., 2014). Consequently, DNA-based identification methods have become an attractive alternative because they are rapid, more reliable, and are independent of the state or the life stage of the nematode (Powers, 2004; Powers et al., 2005; Jeyaprakash et al., 2006; Qiu et al., 2006; Adam et al., 2007). Several species-specific primers have been developed from genomic DNA sequences for several *Meloidogyne* species (Zijlstra et al., 2000; Wishart et al., 2002; Meng et al., 2004; Hu et al., 2011). However, a major limitation for using species-specific primers is that, for a given RKN sample it is often difficult to determine the appropriate species-specific primer to use, which often calls for a random selection of primers or combination of primers in a multiplex PCR. This makes the identification process cumbersome and time-consuming, especially for a large number of samples from crops where the common nematode species complex is unknown. Consequently, a universal genetic marker that can be used to identify a spectrum of *Meloidogyne* spp. in a relatively short time with a few number of PCRs is desirable.

Recent studies have shown that the clade I RKN species have reticulate origin, i.e., they arose by hybridization of two sexual species with a common parent, followed by loss of ability to reproduce sexually (Lunt, 2008; Lunt et al., 2014; Janssen et al., 2016). The

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current diagnostic strategies including morphological and molecular techniques now appear to be unreliable to resolve clade I species due to their hybrid origin. The molecular diagnostic strategies based on the DNA sequence of ribosomal RNA (rDNA) genes is unable to resolve the clade I species, particularly *M. incognita*, *M. arenaria*, and *M. javanica* due to the presence of high sequence variation in rDNA copies within an individual nematode than between species (Lunt et al., 2008; Pagan et al., 2015).

The mitochondrial genome has emerged as a useful genetic material to identify many closely related *Meloidogyne* species (Powers and Harris, 1993; Hugall et al., 1997; Blok et al., 2002; Xu et al., 2004). Mitochondrial genome due to its uniparental inheritance can be a suitable diagnostic marker to distinguish RKN species with hybrid origin (Pagan et al., 2015). The amplification of the mitochondrial DNA (mtDNA) followed by digestion with specific restriction enzymes resulted in haplotype patterns specific to most of the tropical RKN, and importantly, these patterns corresponded with specific isozyme phenotypes (Powers and Harris, 1993; Hugall et al., 1994; Powers, 2004). Stanton et al. (1997) amplified two small mitochondrial DNA regions, that, together, span the intergenic spacer between the cytochrome oxidase II (COII) and large subunit of rDNA and part of the adjacent large subunit (16S) rRNA and then digested the PCR product with *Hinf*I or *Mnl*I to discriminate many *Meloidogyne* spp. Subsequently, Pagan et al. (2015) not only identified more mtDNA haplotypes in these regions to further separate more *Meloidogyne* spp., but also, utilized this procedure to identify isolates of *Meloidogyne* species from Africa. They also confirmed the identities of characterized isolates of *Meloidogyne* species from a wide geographical locations. Following these developments, Janssen et al. (2016) demonstrated that mitochondrial haplotypes are strongly linked and are consistent with traditional esterase isozyme patterns.

The goal of this study was to utilize the mtDNA haplotype designation for identification of *Meloidogyne* spp. in a survey of cut foliage farms. The objectives of this study were to (i) identify the *Meloidogyne* spp. infecting commonly cultivated ornamental cut foliage crops in Florida and (ii) evaluate the feasibility of using the mtDNA haplotype as a molecular diagnostic tool for rapid identification of large samples of RKN.

Materials and Methods

Root and nematode sampling: A total of 270 root samples were collected from seven cut foliage plant species from six farms in two major cut foliage producing counties (Volusia and Putnam) in central Florida. The plant species sampled include *Pittosporum tobira* (Japanese pittosporum), *Liriope gigantea* (lily turf), *Ruscus hypophyllum* (Israeli ruscus), *Aspidistra elatior* (cast iron plant), *Rumohra adiantiformis* (leatherleaf fern), *Asparagus virgatus* (tree fern), and *Asparagus setaceus* (lace fern) (Table 1). From each farm (measuring an average of 1.5 ha), 10 root samples were systematically sampled per available plant species and a single RKN female was taken per root sample for genomic DNA extraction.

Table 1. Farms and plant species from which root samples were collected for identification of *Meloidogyne* spp. during this study.

Farm	Plant species	Sample ID	Number of samples	Specimen ID	Geographical location
A	*Aspidistra elatior*	A.a.e	10	A.a.e (1 – 10)	29.1249620° N, 81.2841950° W
	Pittosporum tobira	A.p.t	10	A.p.t (1 – 10)	
	Liriope gigantea	A.l.g	10	A.I.g (1 – 10)	
	Ruscus hypophyllum	A.r.h	10	A.r.h (1 – 10)	
	Asparagus setaceus	A.a.v	10	A.a.v (1 – 10)	
B	*Aspidistra elatior*	B.a.e	10[a]	B.a.e (1 – 20)	29.129525° N, 81.303441° W
	Pittosporum tobira	B.p.t	10	B.p.t (1 – 10)	
	Liriope gigantea	B.l.s.	10	B.l.s (1 – 10)	
	Ruscus hypophyllum	B.r.a	10	B.r.a (1 – 10)	
C	*Aspidistra elatior*	C.a.e	10	C.a.e (1 – 10)	29.338379° N, 81.498607° W
	Pittosporum tobira	C.p.t	10	C.p.t (1 – 10)	
	Liriope gigantea	C.l.g	10	C.l.g (1 – 10)	
	Ruscus hypophyllum	C.r.h	10	C.r.h (1 – 10)	
D	*Pittosporum tobira*	D.p.t	10	D.p.t (1 – 10)	29.053947° N, 81.348838° W
	Liriope gigantea	D.l.g	10	D.l.g (1 – 10)	
	Aspidistra elatior	D.a.e	10	D.a.e (1 – 10)	
	Rumohra adiantiformis	D.r.a	10	D.r.a (1 – 10)	
E	*Aspidistra elatior*	E.a.e	10[a]	E.a.e (1 – 20)	29.295009° N, 81.457539° W
	Pittosporum tobira	E.p.t	10	E.p.t (1 – 10)	
	Liriope gigantea	E.l.g	10	E.l.g (1 – 10)	
	Rumohra adiantiformis	E.r.a	10	E.r.a (1 – 10)	
F	*Rumohra adiantiformis*	P.r.a	10[a]	P.r.a (1 – 20)	29.355883 N, 81.475014 W
	Asparagus setaceus	P.a.s	10	P.a.s (1 – 10)	
	Asparagus virgatus	P.a.v	10	P.a.v (1 – 10)	

A = Select Growers, B = Quality Growers; C = FernTrust; D = Donaldson Farm; E = Deans Farm; F = Prevatts Farm.
[a] These sites were sampled twice.

Genomic DNA extraction: In total, 200 female RKN were recovered from the plant roots that had RKN infection using a pair of forceps and scalpel under a dissecting microscope. DNA was extracted from each individual female using the NaOH method (Stanton et al., 1998; Hübschen et al., 2004). Individual females were washed in sterile distilled water and directly transferred into 0.2-ml PCR tubes containing 20 µl of 0.25 M NaOH solution and incubated for 16 hr (overnight) at 25°C. The tubes were heated at 99°C for 3 min and cooled to room temperature (25°C). Then, 5 µl of 1 M HCl; 10 µl of 0.5 M tris-HCl (pH 8.0) and 5 µl of 2% Triton X-100 were added to the lysate, mixed briefly and heated at 99°C for 3 min using the Eppendorf Mastercycler® Pro (Eppendorf, Hamburg, Germany). The digest was cooled to room temperature and used immediately for PCR or stored at −20°C.

PCR amplification of mtDNA: Species identification was performed using mitochondrial genes and species-specific primers. The *mtDNA* region between the COII subunit and the large ribosomal RNA (l-rRNA) was amplified using two pairs of primers TRNAH/MRH106 (Stanton et al., 1997) and MORF/MTHIS (Hugall et al., 1994). The PCR was carried out using the Eppendorf Mastercycler® Pro in a 25 µl reaction volume consisting of 1 µl of DNA extract, 1.25 µl of 0.5 µM each primer, 9 µl of sterile water, and 12.5 µl of 2× Apex™ Taq DNA Polymerase Master Mix (Genesee Scientific, San Diego, CA). The amplification conditions consisted of an initial denaturation at 95°C for 15 min followed by 35 cycles of 94°C for 30 sec (denaturation), 50°C for 30 sec (annealing), and 68°C for 60 sec (extension), and with a final extension cycle of 68°C for 7 min. The amplicons were separated by gel electrophoresis using a 1.5% Apex general purpose agarose (Genesee Scientific) gel in 1× tris-borate-EDTA buffer (Apex TBE Buffer, Genesee Scientific) for 30 min at 150 V and visualized under UV light using the ChemiDoc XRS Quantity One 4.5.2 program (Bio-Rad Laboratories, Life Science Group, Hercules, CA) after staining in ethidium bromide (100 ppm) for 20 min.

The PCR product for TRNAH/MRH106 was subjected to endonuclease digestion using *Mnl*I and *Hinf*I restriction enzymes (Thermo Fisher Scientific Inc., Waltham, MA) as recommended by the manufacturer. Briefly, 9.5 µl PCR products were mixed with 3 µl of 10× Buffer G (*Mnl*I) or Buffer R (*Hinf*I), 18 µl of nuclease free water, and 1 µl of *Mnl*I or *Hinf*I restriction enzyme. The digestion reaction was gently mixed and centrifuged for 10 sec and incubated at 37°C for 2 hr followed by thermal inactivation of both enzymes at 65°C for 20 min. The restriction products were separated on 1.8% Apex general purpose agarose in 1× Apex TBE buffer for 30 min at 150 V. The gel was then stained in ethidium bromide (100 ppm) for 20 min and visualized under UV light.

Species-specific primers for most commonly known *Meloidogyne* spp. namely: Mi2F4/Mi1R1 (Kiewnick et al., 2013) for *M. incognita*, Fjav/Rjav (Zijlstra et al., 2000) for *M. javanica*, Far/Rar (Zijlstra et al., 2000) for *M. arenaria,* JMV/JMV1 (Wishart et al., 2002) for *M. hapla*, and Me-F/Me-R (Hu et al., 2011) for *M. enterolobii* were used to verify the mtDNA identification. Other mitochondrial regions such as the cytochrome oxidase subunit I (COI) and the 63 bp variable number tandem repeats region were amplified for some isolates using the Co12R5/JB3 (Kiewnick et al., 2014) and 63VNL/63VTH (Stanton et al., 1997) primer pairs, respectively. All the primer pairs used in this study are listed in Table 2. Florida isolates of *M. arenaria* (MaA71-1), *M. incognita* (Race1), *M. javanica*, *M. hapla*, and *M. enterolobii* were obtained from the Entomology and Nematology Department, University of Florida, Gainesville, for use as reference species. These RKN species were single egg mass lines maintained separately on tomato plants (*Solanum esculentum* cv. Rutgers) in a greenhouse at a temperature range of 25°C to 28°C and 14-hr daylight.

TABLE 2. Primer sets used for the identification of *Meloidogyne* spp. collected from major cut foliage plant species in Florida.

Code	*Meloidogyne* spp.	Primer sequence 5′-3′	Source
TRNAH	Nonspecific	TGAATTTTTTATTGTGATTAA	Stanton et al. (1997)
MHR106	Nonspecific	AATTTCTAAAGACTTTTCTTAGT	Stanton et al. (1997)
MORF	Nonspecific	ATC GGGGTTTAATAATGGG	Hugall et al. (1994)
MTHIS	Nonspecific	AAATTCAATTGAAATTAATAGC	Stanton et al. (1997)
Far	*M. arenaria*	TCGGCGATAGAGGTAAATGAC	Zijlstra et al. (2000)
Rar	*M. arenaria*	TCGGCGATAGACACTACAAACT	Zijlstra et al. (2000)
Fjav	*M. javanica*	GGTGCGCGATTGAACTGAGC	Zijlstra et al. (2000)
Rjav	*M. javanica*	CAGGCCCTTCAGTGGAACTATAC	Zijlstra et al. (2000)
Mi2F4	*M. incognita*	ATG AAG CTA AGA CTT TGG GCT	Kiewnick et al. (2013)
Mi1R1	*M. incognita*	TCC CGC TAC ACC CTC AAC TTC	Kiewnick et al. (2013)
JMV	*M. hapla*	GGATGGCGTGCTTTCAAC	Wishart et al. (2002)
JMV2	*M. hapla*	TTTCCCCTTATGATGTTTACCC	Wishart et al. (2002)
63 VNL	Nonspecific	GAAATTGCTTTATTGTTACTAAG	Stanton et al. (1997)
63VTH	Nonspecific	TAGCCACAGCAAAATAGTTTTC	Stanton et al. (1997)
CoI2R5	Nonspecific	YTRWYCTTAAATCTAAATKMGTATG	Kiewnick et al. (2014)
JB3	Nonspecific	TTTTTTGGGCATCCTGAGGTTTAT	Kiewnick et al. (2014)
Me-F	*M. enterolobii*	AACTTTTGTGAAAGTGCCGCTG	Hu et al. (2011)
Me-R	*M. enterolobii*	TCAGTTCAGGCAGGATCAACC	Hu et al. (2011)

mtDNA sequencing and in silico analysis: To identify the *Meloidogyne* isolates, a preliminary mtDNA haplotype analysis was carried out. The primer set TRNAH/MRH106 was used to obtain mtDNA product for 10 random samples. Based on the sizes of the *Mnl*I and *Hinf*I digestion fragments of the TRNAH/MRH106 product and MORF/MTHIS product, we tentatively assigned haplotype groupings to all the 10 isolates. We further confirmed the identities of these isolates by sequencing TRNAH/MRH106 regions of three isolates, which gave unique patterns. Restriction mapping analysis was conducted for these sequences (using Restriction Mapper V.3) in silico for *Hinf*I and *Mnl*I restriction enzymes and the expected restriction fragment sizes compared with fragment sizes on gel.

The *Meloidogyne* isolates that deviated from this pattern including isolates from specimen B.r.a5, B.a.e1, and A.p.t5 were also sequenced in their TRNAH/MRH106 region and deposited in the GenBank with accession numbers B.r.a5 (KX452370), B.a.e1 (KX452368), and A.p.t5 (KX452369). Also, the COI gene for the isolates from B.a.e1 and A.p.t5 were also sequenced and deposited in the GenBank with accession numbers B.a.e1 (KX452372) and A.p.t5 (KX452371). The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol and sent for sequencing at the DNA Sequencing Core (Interdisciplinary Center for Biotechnology Research [ICBR], University of Florida Gainesville, FL).

The mtDNA sequence from each of the isolate was searched against the homologous sequences at the NCBI data base using the BLAST (Altschul et al., 1990) to identify the most similar sequence. Unique sequences obtained for COI and COII genes in this study and those retrieved from the NCBI databases were aligned over the same length in CLUSTALW using MEGA v. 6 (Tamura et al., 2013). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted in MEGA v.6. Bootstrap analysis with 1,000 replicates was conducted to assess the degree of support for each branch on the tree.

Results

RKN species identification by mtDNA analysis: PCR amplification products using the TRNAH/MRH106 and MORF/MTHIS primer sets were obtained for all the samples. Based on the analysis of the mtDNA regions, five haplotype groups were obtained (Table 3). The restriction pattern observed on the gel (Fig. 1) was confirmed by in silico restriction analysis of TRNA/MRH106 product sequence from each haplotype group, and species-specific primers (Fig. 2). The first and most frequently encountered haplotype pattern generated product of ≈742 bp with MORF/MTHIS primer set and ≈557 bp products for TRNAH/MRH106. Digestion of the 557 bp amplicon with *Hin*f1 enzyme produced three fragments of ≈396, ≈112, and ≈49 bp, whereas *Mnl*1 generated two fragments of ≈340 and ≈217 bp corresponding to haplotype B, which is previously reported for *M. incognita* (Pagan et al., 2015). The PCR amplification using *M. incognita* species-specific primers and in silico sequence analysis of COII fragment further confirmed the identity of the nematode as *M. incognita*.

For the second haplotype group, the amplification products for MORF/MTHIS or for TRNAH/MRH106 primers did not differ significantly from the first pattern. However, *Hinf*1 digestion of the 558 bp produced no cleavage, but *Mnl*1 produced three cleavage fragments of ≈341, ≈140, and ≈77 bp, corresponding to haplotype D, which is previously reported for *M. javanica*. The identity of the nematode was further confirmed by PCR analysis using *M. javanica* species-specific primers and in silico sequence analysis of TRNAH/MRH106 fragments. The third haplotype produced no product for MORF/MTHIS region, but a ≈556 bp product for TRNAH/MRH106. *Hinf*1 endonuclease digestion of the TRNAH/MRH106 product generated ≈446 and ≈110 bp fragments, but without any products for *Mnl*1 as previously reported in the case of *M. hapla* (Pagan et al., 2015). We further confirmed the identity of the nematode

Table 3. PCR products using TRNAH/MRH106 and MORF/MTHIS primer sets and restriction fragment sizes obtained from *Meloidogyne* spp. collected from roots of four cut foliage plant species growing in five commercial cut foliage farms in Florida.

Meloidogyne spp.	TRNAH/MRH106 amplicon (bp)	*Mnl*1/TRNAH/MRH106 amplicon (bp)	*Hinf*1/TRNAH/MRH106 amplicon (bp)	MORF/MTHIS Amplicon (bp)
M. incognita	557	340, 217	396, 112, 49	742
M. javanica	558	341, 140, 77	558	743
M. hapla	556	556	446, 110	NP
Meloidogyne sp. 1	559	342, 140, 77	447, 112	744
Meloidogyne sp. 2	557	417, 140	557	NP

NP = no product, or weak nonspecific bands.

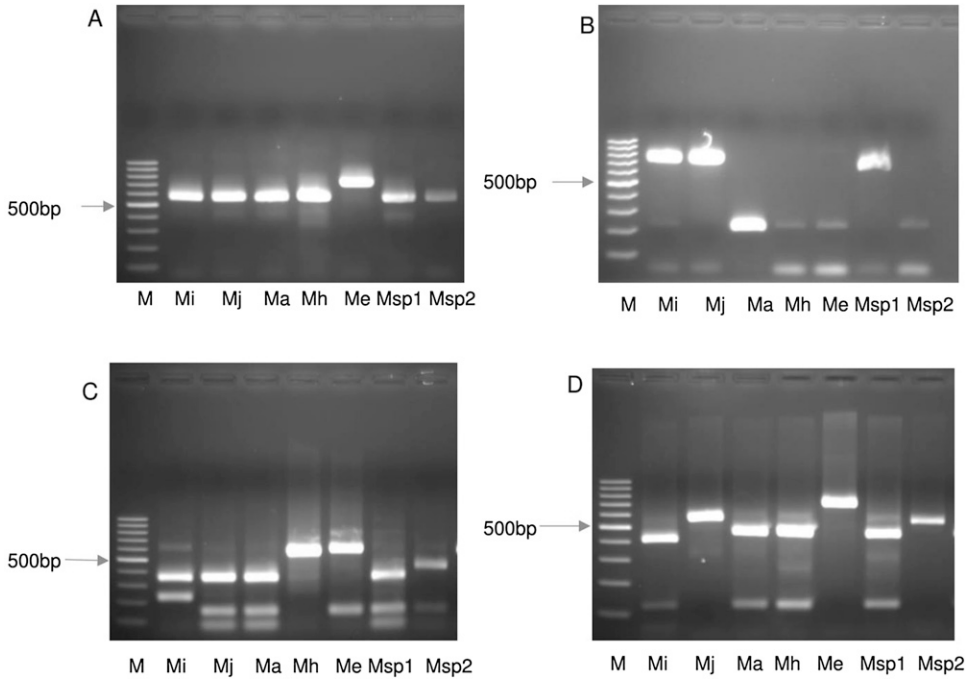

FIG. 1. A model diagnostic pattern from mitochondrial DNA of *Meloidogyne* spp. A. Amplification products from root-knot nematode species obtained using TRNAH/MRH106 primer set. B. Amplification products obtained with MORF/MTHIS primer set. Fragments obtained after cleavage of TRNAH/MRH106 products with C. *Mnl*I, or D. *Hinf*I. Mi = *Meloidogyne incognita*, Mj = *M. javanica*, Ma = *M. arenaria*, Mh = *M. hapla*, Me = *M. enterolobii*, Msp1 = *Meloidogyne* sp.1, Msp2 = *Meloidogyne* sp. 2. Lanes labeled M contain 100-bp marker ladder (Fermentas), with the position of the 500-bp band indicated by an arrow.

by additional PCR using *M. hapla*-specific primers as well as sequence analysis of COII fragment. BLAST (Altschul et al., 1990) analysis of TRNAH/MRH106 fragment (KX452370) from this nematode showed 98% similarity (100% query cover) with the sequence of *M. hapla* (L76262.1) retrieved from the GenBank.

The PCR products for MORF/MTHIS and TRNAH/MRH106 from fourth haplotype group were not much different from the first and second groups, but *Mnl*1 endonuclease digestion of the TRNAH/MRH106 fragment produced three fragments of ≈342, ≈140, and ≈77 bp, whereas the *Hinf*1 produced two fragments of ≈447 and ≈112 bp. This isolate was named *Meloidogyne* sp. 1; however, this pattern was very similar to *M. arenaria* V2 haplotype. To confirm the species identity, further PCR analysis using the species-specific primers for *M. arenaria* was performed on this nematode. The FAR/RAR primer set specific for *M. arenaria* could amplify genomic DNA of *M. arenaria* control but not genomic DNA of *Meloidogyne* sp. 1. An alignment of *Meloidogyne* sp. 1 COII gene sequence generated with the TRNAH/MRH106 primer set (KX452369) produces 99% similarities (100% query cover) to COII sequences of *M. javanica* (KP202352.1), *M. arenaria* (KP202350.1), *M. ethiopica* (KM042847.1), *M. incognita* (KJ476151.1), and *M. hispanica* (JN673274) in the GenBank. Similarly, the COI gene sequence generated with the Co12R5/JB3 primer set (accession no. KX452371) produces 90% similarities (99% query cover) to the COI gene sequences of *M. arenaria* (KP202350.1), *M. javanica* (KP202352.1), and *M. incognita* (KJ476151.1), 88% similarity (99% query cover) to that of *M. haplanaria* (KU174206.1), 87% similarity (99% query cover) to *M. enterolobii*. Moreover, both COII and COI gene sequences clustered *Meloidogyne* sp. 1 with clade I species including *M. arenaria* and *M. hispanica*, which also have haplotype G pattern. The results with COI are shown in Fig. 3.

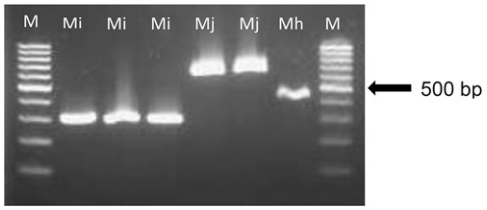

FIG. 2. PCR amplification product generated using species-specific primer sets. Mi = *Meloidogyne incognita* genomic DNA amplified using MI-F/MI-R primer set; Mj = *M. javanica* genomic DNA amplified using Fjav/Rjav primer set; Mh = *M. hapla* genomic DNA amplified using JMV1/JMV2 primer set; M = 100-bp DNA ladder.

The fifth haplotype observed in this study produced no product for MORF/MTHIS, but a ≈557 bp product for TRNAH/MRH106 region. *Hinf*1 endonuclease had no cleavage site in this ≈557 bp product, but *Mnl*1 generated two fragments of ≈417 and ≈140 bp. We named this isolate *Meloidogyne* sp. 2. This nematode specifically infected *A. elatior* in all the farms. The

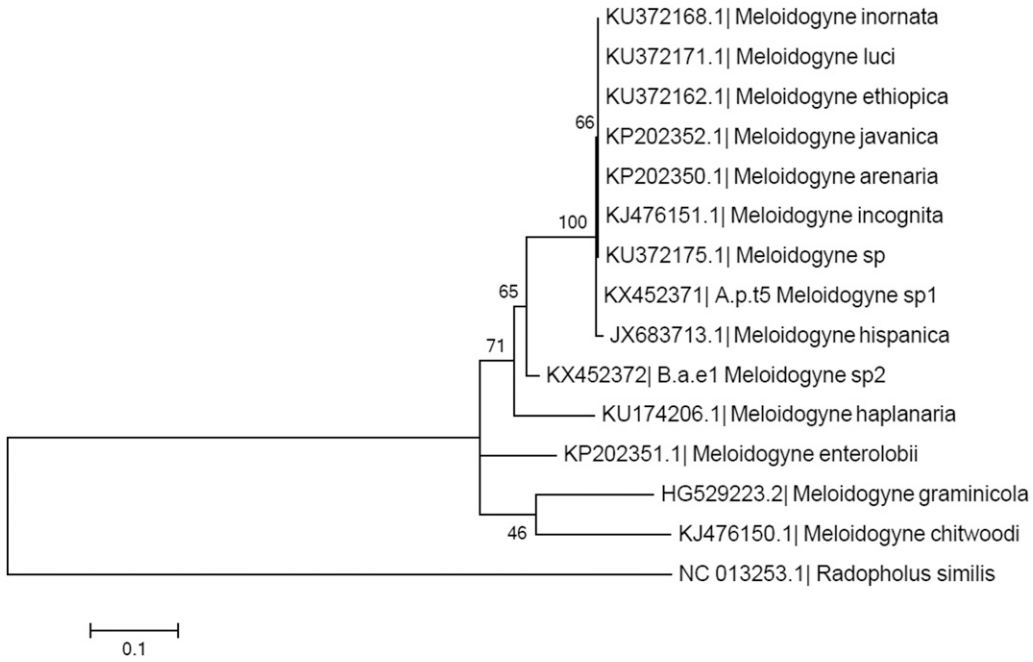

Fig. 3. Maximum likelihood tree after an alignment of COI gene of the mitochondrial genome of *Meloidogyne* populations. Bootstrap support for each clade is indicated at the nodes.

mtDNA pattern of this isolate was very different from known haplotypes. We tested different species-specific primers on this nematode for further characterization. Only *Meloidogyne enterolobii* species-specific primers, Me-F/Me-R (Hu et al., 2011) were able to amplify ≈200 bp from *Meloidogyne* sp. 2 as in *M. enterolobii* (data not shown). However, this nematode had haplotype pattern different from *M. enterolobii*. An alignment of *Meloidogyne* sp. 2 COII gene sequence generated with the TRNAH/MRH106 primer set (KX452368) produces 89% similarity to COII regions of *M. javanica* (L76261.1) (query cover 100%) and 88% similarities (query cover 100%) to that of *M. arenaria* (KP202350.1) and *M. incognita* (KJ476151.1). The COI gene, however, produces 99% similarities (99% query cover) to that of *M. javanica* (KP202352.1), *M. incognita* (KJ476151.1), and *M. arenaria* (KP202350.1). However, the phylogenetic analysis of COII and COI fragments revealed a unique position of *Meloidogyne* sp. 2 with other RKN species. The results with COI are shown in Fig. 3.

For the total of 270 plant root samples collected from the six cut foliage farms, 200 roots had *Meloidogyne* infections while the rest had no detectable infection. The plant species from which *Meloidogyne* females were isolated include *P. tobira*, *L. gigantea*, *R. hypophyllum*, and *A. elatior*, whereas *R. adiantiformis*, *A. virgatus*, and *A. setaceus* had no detectable infection (Table 4). Based on the mtDNA haplotype patterns and species-specific primer analysis, three known *Meloidogyne* spp. including *M. incognita*, *M. javanica*, and *M. hapla* and two unknown *Meloidogyne* sp., named *Meloidogyne* sp. 1 and *Meloidogyne* sp. 2, were identified from the 200 root samples. *Meloidogyne incognita* was the most dominant RKN species in all the farms, except farm F. It also had the highest incidence on *P. tobira*, *L. gigantea*, and *R. hypophyllum*, and constituted nearly 58% of the total number of *Meloidogyne* spp. identified. The incidence of *M. hapla* or *Meloidogyne* sp. 1 was about 1% of the total number of female specimens analyzed (Table 5).

Discussion

We have successfully used the mtDNA haplotype-based designations in our survey to identify the *Meloidogyne* species infecting cut foliage crops in Florida. The mtDNA primer pairs produced products from a spectrum of RKN species which is useful for rapid and reliable identification of a large number of samples without prior knowledge especially in survey studies. Importantly, the mitochondrial haplotypes are strongly linked with isozyme phenotypes (Hugall et al., 1994; Pagan et al., 2015, Janssen et al., 2016) making mtDNA-based identification preferable over nuclear gene-based or morphological identification. This work therefore expands the utility of the mtDNA haplotype-based identification of *Meloidogyne* spp.

The majority of the haplotype groups identified matched with haplotypes B and D, corresponding to *M. incognita* and *M. javanica*, respectively. These *Meloidogyne* spp. produced consistent fragment sizes and cleavage patterns that uniquely identified them compared with previous reports (Pagan et al., 2015; Janssen et al., 2016). The MORF/MTHIS segment, which spans the intergenic spacer between the COII and l-rRNA genes, neither had enough nucleotide polymorphism to confer variability nor size differences, but the presence or

TABLE 4. The number and incidence of *Meloidogyne* spp. identified from roots of cut foliage plant species growing in six commercial cut foliage farms in Florida.

Farm	*Meloidogyne* spp.	Haplotype group	Number of nematodes	Incidence (%)
A	*Meloidogyne incognita*	B	31	78
	M. javanica	D	4	10
	Meloidogyne sp.1	-	1	2
	Meloidogyne sp. 2	-	4	10
B	*M. incognita*	B	29	58
	M. javanica	D	8	16
	M. hapla	-	2	4
	Meloidogyne sp. 2	-	11	22
C	*M. incognita*	B	24	60
	M. javanica	D	6	15
	Meloidogyne sp. 2	-	10	25
D	*M. incognita*	B	17	57
	M. javanica	D	6	20
	Meloidogyne sp. 2	-	7	23
E	*M. incognita*	B	26	65
	M. javanica	D	5	13
	Meloidogyne sp. 2	-	9	22
F	None	N/A	N/A	N/A

A = Select Growers; B = Quality Growers; C = FernTrust; D = Donaldson Farm; E = Deans Farm; F = Prevatts Farm; N/A = not applicable; - = cannot be assigned a known haplotype group.

absence of MORF/MTHIS product helped separate *M. hapla* or *Meloidogyne* sp. 2 from *M. incognita*, *M. javanica*, and *Meloidogyne* sp. 1. Similarly, the TRNAH/MRH106 region (which spans $tRNA^{His}$ and part of l-rRNA genes) had few size polymorphisms among the *Meloidogyne* spp., but harbored nucleotide polymorphisms sufficient to separate most *Meloidogyne* spp. through endonuclease digestion. The *Hinf*I recognition sites are absent in *M. javanica* and the *Meloidogyne* sp. 2 but present in *M. hapla*, *M. incognita*, and *Meloidogyne* sp. 1, whereas the *Mnl*I restriction site is absent in *M. hapla* but present in *M. incognita*, *M. javanica*, *Meloidogyne* sp. 1, and *Meloidogyne* sp. 2. These results are consistent with previous reports (Stanton et al., 1997; Pagan et al., 2015). Insertion and deletion within the COII gene and l-rRNA generate size polymorphisms and has been used to differentiate several *Meloidogyne* species (Hugall et al., 1994, 1997; Jeyaprakash et al., 2006; Moens et al., 2009). As compared to *Meloidogyne javanica*, *Meloidogyne incognita*, *M. arenaria*, and *M. paranaensis* have accumulated deletions, whereas *M. izalcoensis* and *M. arabicida* have gained insertions (Pagan et al., 2015). Consequently, the length of the mtDNA region between the COII and the l-rRNA amplified by MORF/MTHIS primer set is large in species such as *M. incognita* and *M. javanica*.

TABLE 5. The number and incidence of different *Meloidogyne* spp. identified using 200 *Meloidogyne* females from roots of major cut foliage plant species in in Florida.

Plant species	*Meloidogyne* species	Haplotype group	Number of nematodes	Incidence (%)
Pittosporum tobira	*M. incognita*	B	47	94
	M. javanica	D	2	4
	Meloidogyne sp. 1	-	1	2
Liriope gigantea	*M. incognita*	B	39	78
	M. javanica	D	11	22
Ruscus hypophyllum	*M. incognita*	B	18	60
	M. javanica	D	10	33
	M. hapla	-	2	7
Aspidistra elatior	*M. incognita*	B	12	17
	M. javanica	D	6	9
	Meloidogyne sp. 2	-	52	74
Rumohra adiantiformis	N/A	N/A	N/A	N/A
Asparagus setaceus	N/A	N/A	N/A	N/A
Asparagus virgatus	N/A	N/A	N/A	N/A

N/A = not applicable; - = cannot be assigned a known haplotype group.

Pagan et al. (2015) reported two haplotypes of *M. arenaria* based on MORF/MTHIS product sizes. The first haplotype was found on peanut in United States with 214 bp MORF/MTHIS product and the second on soybean in Brazil with 743 bp. The latter shared resemblance to *Meloidogyne* sp. 1 in both MORF/THIS or TRNAH/MRH106 regions and in *Mnl*I endonuclease digestion fragments of TRNAH/MRH106. In order to separate the *Meloidogyne* sp. 1 found in this study from the *M. arenaria* haplotype from Brazil, a *M. arenaria* species-specific FAR/RAR primer set (Adam et al., 2007) was used to amplify the mtDNA of both species using *M. incognita* as negative control. The species-specific SCAR primers only amplified the *M. arenaria* but not *Meloidogyne* sp. 1 found in this study or the negative control. Nevertheless, *M. arenaria* was recently demonstrated to be a molecular diverse species cloud (Janssen et al., 2016) and it is currently unclear if species-specific primers are able to distinguish *M. arenaria* from closely related species. There are reports of high genetic diversity within *M. arenaria*, which does not group as a single haplotype and represents a molecular diverse species cloud stemming from its suggested hybrid

origin (Lunt, 2008; Fargette et al., 2010; Lunt et al., 2014; Janssen et al., 2016). Moreover, sequence analysis and a phylogenetic analysis based on the COII and COI fragments suggest *Meloidogyne* sp. 1 is closely related to that of haplotype G; and both the COII and COI sequences of *Meloidogyne* sp. 1 showed close homologies to that of *M. javanica*, *M. arenaria*, *M. ethiopica*, and *M. hispanica*. Pagan et al. (2015) state that *M. hispanica, M. arabicida, M. ethiopica*, *M. arenaria* V2, *M. inornata*, *M. morocciensis*, *M. petunia*, and *M. paranaensis* produce the same mitochondrial haplotype and are hard to differentiate. Also, based on the MORF/MTHIS fragment, *M. hispanica, M. arenaria* V2, *M. ethiopica*, *M. inornata*, and *M. petunia* are indistinguishable. The relatively closely related lineages of haplotype G members could be attributed to the mostly parthenogenetic nature and the suggested hybrid origin of *Meloidogyne* lineages in clade I (Lunt et al., 2014). Consequently, based on the available data, we only could conclude that the *Meloidogyne* sp. 1 haplotype was similar to haplotype G, but could be either of another species or a new species. Additional genetic analysis in combination with isozyme electrophoresis and morphological data are required to confirm the identity of *Meloidogyne* sp. 1.

The second unidentified *Meloidogyne* sp. (*Meloidogyne* sp. 2) found in this study had a unique mtDNA haplotype pattern. It was differentiated from other *Meloidogyne* spp. based on the TRNAH/MRH106 and MORF/MTHIS regions. Based on the available data, we could confirm that *Meloidogyne* sp. 2 could neither be *M. incognita*, *M. javanica, M. hapla*, nor *M. enterolobii* since these species are easily distinguishable from other tropical RKN species due to their unique mitochondrial haplotypes. Although, PCR amplification with Me-F/Me-R primers (Hu et al., 2011) in *Meloidogyne* sp. 2 yielded expected amplicon size as in *M. enterolobii*, but the haplotype pattern as well as the COI and COII sequences of *Meloidogyne* sp. 2 is entirely different from that *M. enterolobii*. This indicates that these primers should be used with much caution to distinguish *M. enterolobii* from other RKN species.

The majority of the haplotypes identified in this study matched that of B and D corresponding to *M. incognita* and *M. javanica*. Other work reports similar results owing to the wide spread and economic importance of these nematodes (Stanton et al., 1997; Onkendi et al., 2014; Pagan et al., 2015). The study also revealed that *M. incognita* dominated RKN species found on most of these cut foliage crops suggesting a probably economic importance in the production of these crops. The high occurrence of *M. incognita* also suggests a polyphagous, high adaption to warm, subtropical climates of the southern United States, as has been previously reported (Taylor and Sasser, 1978). *Meloidogyne incognita* is reported to be damaging on many plant species, including woody ornamentals, annual and perennial flowers in Florida (McSorley and Frederick, 2001, 2004; Brito et al., 2010; Baidoo et al., 2014). On the other hand, *M. hapla* is regarded as a temperate RKN and its occurrence is limited by warmer temperatures above 24°C (Taylor and Sasser, 1978). Hence, the occurrence of this nematode in subtropical Florida is unusual; this nematode might have been transported on their host transplant materials into Florida. This dispersal pattern of *M. hapla* has been observed in other crops in and outside of the United States. Nyoike et al. (2012) observed the same dispersal pattern of *M. hapla* in strawberry in Florida where the nematode was believed to have been transported with the transplants from Canada. Owing to its rare occurrence in cut foliage crops according to our survey, *M. hapla* may not pose significant threat to Florida's cut foliage industry.

In this survey, we have demonstrated that mtDNA haplotype designation coupled with species-specific primers was sufficient to identify most economically important *Meloidogyne* spp. in a large number of samples in a fast, rapid, and reliable way useful for management, regulatory and quarantine purposes. There were only limited differences between the mtDNA regions analyzed in this study, which support the theory of a recent separation from a common or highly related ancestral mother. Nonetheless, these small differences, especially for the TRNAH/MRH106 region present sufficient nucleotide polymorphisms to separate several *Meloidogyne* lineages. However, the examination of more than one molecular characteristic will be a suitable approach for identification and evolutionary studies of a particular species. Nonetheless, this study demonstrates the usefulness of the mtDNA haplotype-based designation as a valuable molecular tool for identification of *Meloidogyne* spp. The study also revealed that *M. incognita* is the most dominant RKN on cut foliage crops in Florida and must be a high target for making management decisions.

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