IDENTIFICATION AND HAPLOTYPE DESIGNATION OF *MELOIDOGYN*E SPP. OF ARKANSAS USING MOLECULAR DIAGNOSTICS

Churamani Khanal*[1], Robert T. Robbins[1], Travis R. Faske[2], Allen L. Szalanski[3], Edward C. McGawley[4], and Charles Overstreet[4]

[1]Department of Plant Pathology, University of Arkansas, Fayetteville, AR 72701; [2]University of Arkansas, Division of Agriculture, Lonoke Research and Extension Center, Lonoke, AR 72086; [3]Department of Entomology, University of Arkansas, Fayetteville, AR 72701; [4]Department of Plant Pathology and Crop Physiology, 302 Life Science Building, Louisiana State University, Baton Rouge, LA 70803. *Corresponding author: ckhana1@lsu.edu

ABSTRACT

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In this study, polymerase chain reaction (PCR) and DNA sequencing analysis were performed to identify *Meloidogyne* species present in Arkansas. A total of 106 soil and root samples from 36 of the 75 counties were collected, of which 79 contained root-knot nematodes. To identify species, PCR was performed using primers C2F3/1108 to amplify a region of mitochondrial DNA (mtDNA) of root-knot nematodes. Additionally, *M. incognita* specific primers were designed to confirm speciation, as *M. incognita* was the most abundant species that was identified in 54 of the 79 samples. Other species found in this survey were *M. marylandi*, *M. haplanaria*, *M. hapla*, *M. arenaria*, and *M. partityla*. Haplotype designation was performed for each species based on nucleotide variation. With a limited number of samples, this study designated distinct mtDNA haplotypes of *Meloidogyne* spp. endemic in Arkansas. Unlike previous reports, *M. javanica* and *M. graminis* were not detected from any of the samples collected during this study.

Key words: Arkansas, haplotype, *Meloidogyne* spp., mtDNA.

RESUMEN

Khanal, C., R. T. Robbins, T. R. Faske, A. L. Szalanski, E. C. McGawley, and C. Overstreet. 2016. Identificación y designacion de haplotipos de *Meloidogyne* spp. en Arkansas usando diagnóstico molecular. Nematropica 46:261-270.

En este estudio, se realizó la reacción en cadena de la polimerasa (PCR) y un análisis de secuenciación del ADN para identificar las especies de *Meloidogyne* presentes en Arkansas. Un total de 106 muestras de suelo y raíces de 36 de los 75 condados fueron recolectados, de los cuales 79 contenían nemátodos de nudos de raíz. Para identificar las especies, se realizó PCR utilizando los primers C2F3/1108 para amplificar una región de ADN mitocondrial (ADNmt) de nematodos de nudos de raíz. Además, se diseñaron primers específicos para *Meloidogyne incognita* para confirmar la especie, ya que *M. incognita* fue la especie más abundante que se identificó en 54 de las 79 muestras. Otras especies encontradas en este muestreo fueron *Meloidogyne marylandi*, *M. haplanaria*, *M. hapla*, *M. arenaria*, y *M. partityla*. La designación del haplotipo se realizó para cada especie basada en la variación de nucleótidos. Con un número limitado de muestras, este estudio designó distintos haplotipos mtDNA de *Meloidogyn*e spp. endémico de Arkansas. A diferencia de los informes anteriores, *M. javanica* y *M. graminis* no fueron detectados en ninguna de las muestras durante este estudio.

Palavras chave: Arkansas, haplotipo, *Meloidogyne* spp., mtDNA.

INTRODUCTION

Root-knot nematodes [*Meloidogyne* spp. Goldi (Tylenchida: Meloidogynidae)], are highly-adaptable, obligate plant parasites that are distributed worldwide parasitizing almost every species of vascular plant (Garcia and Sanchez-Puerta, 2012; Jones *et al*., 2013) with 98 species in the genus (Jones *et al*., 2013); however, only a few species have been reported from the United States.

Previous surveys of *Meloidogyne* spp. in Arkansas were conducted by using classical morphological methods. *Meloidogyne graminis* Sledge and Golden, 1964 was first found in 1967 by R. D. Riggs on *Zoysia* spp. in Arkansas (Grisham *et al*., 1974). *Meloidogyne hapla* Chitwood, 1949 was reported on black locust (*Robinia pseudoacacia*) near the Mississippi River in Arkansas (Taylor *et al*., 1982). Norton *et al.* (1984) mentioned the occurrence of *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. hapla*, and *M. incognita* (Kofoid & White, 1919) Chitwood, 1949 in Arkansas. Wehunt *et al.* (1989) reported *M. incognita*, *M. hapla*, *M. arenaria*, *M. graminis*, and *M. javanica* from soybean fields near the Mississippi river in Arkansas and more recently, Walters and Barker (1994) reported *M. hapla*, *M. incognita*, *M. arenaria*, and *M. javanica* (Treub, 1885) Chitwood, 1949 in Arkansas.

Meloidogyne spp. have a broad host range and genetic resistance is not always effective against all the species; thus, accurate identification of species is imperative for effective use of host-plant resistance (Eisenback, 1982). Accurate identification of root-knot species using classical morphological methods is often a difficult task and requires personnel with a high level of expertise. Molecular techniques such as polymerase chain reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), and DNA sequencing have been widely used in species identification and genetic studies, because they are fast, less laborious, more sensitive, specific, efficient, and applicable to all life stages of a specimen compared to the conventional methods (Magistrado *et al*., 2001; Keramas *et al*., 2004; Powers, 2004, Adzitey *et al*., 2013;). In recent years, mitochondrial DNA (mtDNA) sequence has been used as a tool for the study of phylogenetic relationships among Metazoa (Gissi *et al*., 2008) and the analysis of haplotypes derived from mtDNA sequences has recently been suggested to be efficient in diagnostics and study of evolutionary relationships of root-knot nematodes (Pagan *et al.*, 2015; Janssen, *et al*., 2016) and entomopathogenic nematodes (Tang and Hyman, 2007; Hyman *et al*., 2011).

The objective of this research was to identify endemic species of root-knot nematodes (RKN) of Arkansas based on molecular diagnostics and to analyze variation of haplotypes among root-knot nematode species.

MATERIALS AND METHODS

Sample collection and nematode extraction

A request to submit plant roots showing gall symptoms was made through plant health clinic news, the University of Arkansas, Fayetteville, Arkansas. Samples were submitted by farmers, county agents, and master gardeners to the Arkansas Nematode Diagnostic Laboratory, Hope, AR, and the Arkansas Plant Health Clinic, University of Arkansas, Fayetteville, AR. Samples submitted constituted many of samples collected for this study. Furthermore, plant root and/or soil samples were collected by the authors across various cropping systems, horticultural gardens, golf courses, sod farms, and some of the non-agricultural lands in Arkansas. A foot-powered conical core sampler or shovel was used to take soil and root samples up to a depth of about 20 cm. Approximately 500 cm^3 of soil and roots was collected, mixed thoroughly, and stored for less than 24 hr prior to extraction or processed immediately.

Second-stage juveniles (J2) were extracted from the soil samples using a rapid centrifugal-flotation technique (Jenkins, 1964) and identified to genus level by using a stereoscopic microscope. Two to twenty J2 were obtained per soil sample extraction. Individual J2 were hand-picked and transferred into a separate vial containing distilled water to reduce contamination and stored at 4°C until DNA was extracted.

Root-knot females were excised from root galls using a sterilized needle, scalpel and a stereoscopic microscope. Individual females excised from roots were kept in a vial containing distilled water and stored at 4°C until DNA was extracted.

Processing of nematode specimens for PCR

Root-knot nematode J2 obtained from each sample (2 to 10 individuals) were randomly selected for DNA extraction using the smash method (Powers and Harris, 1993). An individual J2 was placed in 2.5 µl of PCR water on a glass slide and cut into at least two pieces using a sterilized 10 µl micropipette tip. The solution containing smashed J2 was used immediately as a template for PCR.

Up to 10 individuals of RKN females were randomly selected and excised for DNA extraction using rapid isolation of mammalian DNA technique

(Sambrook and Russel, 2001a). An individual female was ground in a 1.5 ml Eppendorf tube using a 1 ml micropipette tip. As a final step, 12 µl of PCR water was added to the tube and mixed. This solution was used as a DNA template to run PCR. DNA samples were stored at -20°C until further use.

The primer set C2F3 (5'GGTCAATGTTCAGAAATTTGTGG3') and 1108 (5'TACCTTTGACCAATCACGCT3') was used to amplify the region between COII and 16S ribosomal mitochondrial genes of root-knot nematodes (Powers and Harris, 1993; Powers *et al*., 2005). *Meloidogyne incognita* previously identified and maintained on tomatoes in the greenhouse served as a positive control throughout this study. For this process, the PCR master mix was prepared so that each reaction had 2.5 µl of 10X CL buffer (Qiagen, mat no. 1032517), 17.5 µl of PCR water, 1 µl of 25 mM MgCl2 (Qiagen, mat no. 1005482), 0.5 µl of dNTP (10 mM each) (Qiagen, mat no. 1005631), 0.5 µl of 10 µM of each primer (Operon), and 0.25 µl Taq DNA polymerase (5 units/µl) (Qiagen, mat no. 1005476). To the PCR reaction master mix, 2.5 µl of DNA template from J2 or female was added and mixed. PCR was performed in a PTC-100® Peltier thermal cycler (MJ Research, Waltham, MA). Amplification conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 58°C for 45 sec, and extension at 72°C for 90 sec. A final extension step was conducted for 10 mins at 72°C.

A 5 µl sub-sample from each PCR reaction was loaded into a well of a 2% agarose gel. The agarose gel (2%) was prepared by mixing 2 g agarose in 100 ml 1X Tris:Borate EDTA (TBE) solution and stained with 3.5 µl of Gel Red Nucleic Acid (Biotium, cat: 41003). Some 5 µl of 100 bp DNA ladder (Promega, ref: G210A) was used to determine size of PCR products and were separated with electrophoresis set at 168V for 50 min. Separated DNA bands were visualized using a UV transilluminator (UVP BioDoc-ItTM, Upland, CA).

Purification and sequencing

Each PCR product was purified using the "standard ethanol precipitation of DNA in microfuge tubes" method (Sambrook and Russell, 2001b). Purified DNA was quantified by loading 1 µl DNA solution into a spectrophotometer (Nanodrop 1000 v 3.6.0).

DNA samples meeting minimum concentration requirement set by DNA Resource Center at the University of Arkansas, Fayetteville, AR, were selected to submit for sequencing. Samples were prepared according to guidelines provided by the sequencing facility. DNA samples were sequenced in both directions using an ABI 3130xl analyzer BigDye 3.1 chemistry to provide automated DNA sequencing. Pairwise alignment of forward and reverse sequences was performed using ClustalW (Thompson *et al*., 1994) to get consensus sequences. Consensus sequences obtained were compared with non-redundant sequences available in GenBank through Nucleotide BLAST (Basic Local Alignment Search Tool) and species was determined using the highest matches (maximum score, total score, query cover, and E value).

Meloidogyne incognita *specific primer design*

Based on preliminary experiments, C2F3/1108 primers were sometimes inconsistent when it came to identifying *M. incognita*. Therefore, to ensure PCR samples were identified correctly a second set of species-specific primers were developed. DNA sequences obtained from *Meloidogyne* spp. during this study were aligned together with the complete mitochondrial genome (Humphreys-Pereira and Elling, 2014) available in GenBank using BioEdit 7.1 (Hall, 1999). Based on conserved regions, new primers COF475 (5'CTTTATTAGATCGGGGTTTAAT3'), COF779 (5'TAATAGATTTAGTTCATCTG3'), and COR999 (5'TGATTTAATTCATTATGATA3') were designed. Root-knot nematode identification was first performed with primers C2F3/1108 and gel electrophoresis. If bands in agarose gel matched with *M. incognita*, another PCR reaction was performed with primers COF475/COR999. PCR product amplified with primers COF475/COR999 was purified, sequenced, and confirmed as *M. incognita* based on minimum of 99% homology to specimens in GenBank. Preparation of PCR master mix, gel electrophoresis and sequencing were performed as described above. Amplification conditions optimized for the primer COF475/COR999 included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 43°C for 60 sec, and extension at 72°C for 90 sec. A final extension step was conducted for 10 min at 72°C. Purification and sequencing of PCR product was performed as described above.

In order to design a PCR technique for routine identifications of *M. incognita* without sequencing, DNA templates of *M. incognita* obtained from this study were run with three primers COF475/COF779/COR999. The PCR master mix was prepared so that each reaction had 2.5 µl of 10X CL buffer (Qiagen, mat no. 1032517), 16.5 µl of PCR water, 1 µl of 25 mM MgCl2 (Qiagen, mat no. 1005482), 0.5 µl of

dNTP (10 mM each) (Qiagen, mat no. 1005631), 0.5 µl of 10 µM of each primer (Operon), and 0.25 µl Taq DNA polymerase (5 units/µl) (Qiagen, mat no. 1005476). In each PCR tube 22 µl PCR reaction master mix aliquot was kept and 2.5 µl DNA template was added. Optimized amplification conditions included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 60 sec, annealing at 48°C for 30 sec, and extension at 72°C for 90 sec. A final extension step was conducted for 10 min at 72°C. Negative controls used were *M. haplanaria*, *M. marylandi*, *M. hapla*, *M. arenaria*, and *M. partityla* that were identified during this study. Gel electrophoresis was performed as described above except 7 µl of the PCR product was used.

Phylogenetic analysis

Bayesian molecular phylogenetic analysis was done with the best-fitting nucleotide substitution model chosen in accordance with the general time reversible gamma (GTR+G) model among 64 different models using the ModelTest v 3.7 (Posada and Crandall, 1998) and PAUP 4.0b10 (Swofford, 2001) programs. Phylogenetic trees were obtained using Bayesian inference with the GTR+G model using BEAUti and the BEAST 1.7 software (Drummond *et al*., 2012). *Globodera pallida* (GenBank accession number DQ631911) was used as the outgroup taxon. Additionally, intraspecific variation for each different species was analyzed by aligning the sequences using ClustalW (Thompson *et al*., 1994).

RESULTS

Seventy-five percent of the 106 soil and root samples collected from 36 of the 75 counties in Arkansas were confirmed to contain at least one of six species of root-knot nematodes (Table 1). Root-knot nematode samples collected in this survey included 27 from soybean (*Glycine max*), 6 from pecan (*Carya illinoinensis*), 13 from tomato (*Solanum lycopersicum*), 5 from bermudagrass (*Cynodon dactylon*), 2 from cotton (*Gossypium hirsutum*), 3 from okra (*Abelmoschus esculentus*), and 50 from other plants.

The use of molecular diagnostics was helpful to identify RKN, but there were challenges to DNA collection. A total of 576 PCR reactions were performed to identify root-knot nematodes. PCR amplification was obtained from 401 reactions while 175 reactions did not amplify. Minimum template

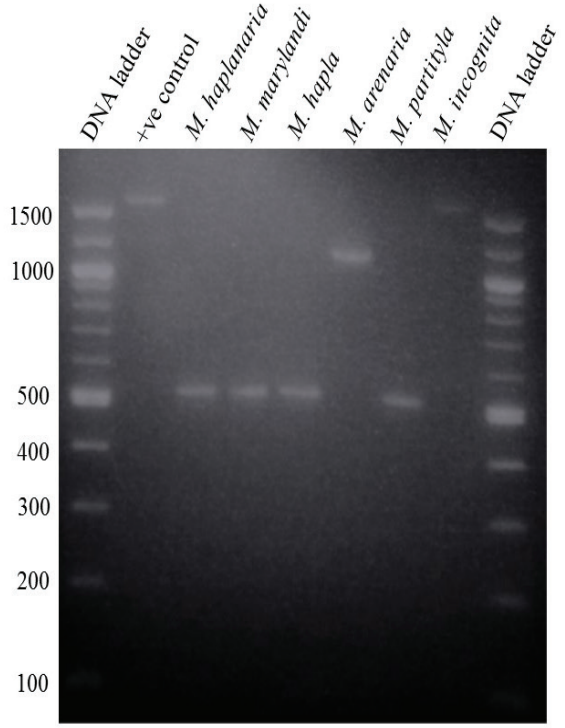

Fig. 1. Visualization of PCR products from different *Meloidogyne* spp. with primer set C2F3/1108.

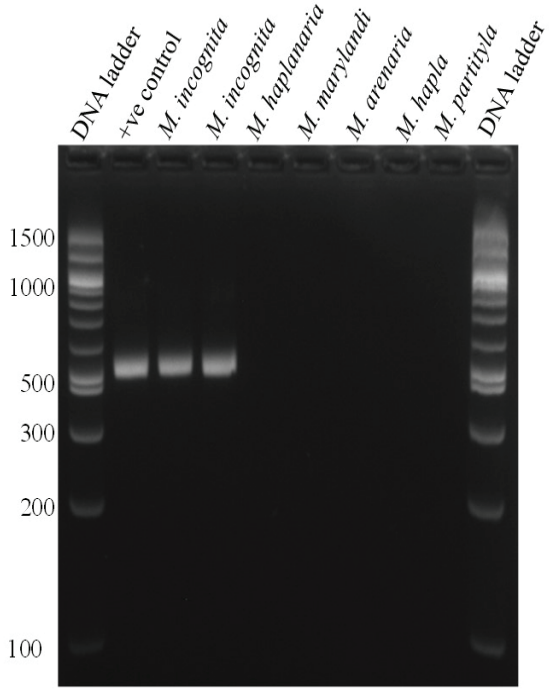

Fig. 2. Visualization of PCR products from different *Meloidogyne* spp. with primer set COF475/COR999.

Table 1. List of *Meloidogyne* spp. collected during this study with their source of origin (soil or likely host), samples positive for root-knot nematodes (RKN), number of counties each species was obtained from, and number of assigned haplotypes.

Meloidogyne spp.	Samples positive for RKN	No. of Counties[w]	Hosts/soil	No. of haplotypes
M. incognita	54	30	14	5[x], 10[y]
M. haplanaria	13	3	11	8[x]
M. marylandi	5	5	2	3[x]
M. hapla	5[z]	3	5	4[x]
M. partityla	1	1	1	1[x]
M. arenaria	2	2	2	2[x]

[w]A county may represent more than one root-knot nematode positive sample.
[x]Species identification was done with primers C2F3/1108.
[y]Species identification was done with primers COF475/COR999.
[z]One of the soil samples positive for *M. partityla* was also positive for *M. hapla*.

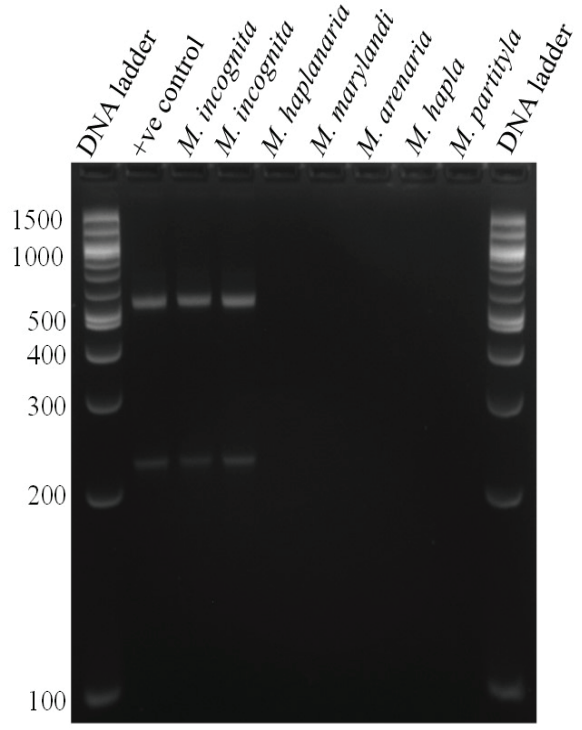

Fig. 3. Visualization of PCR products from different *Meloidogyne* spp. of Arkansas with primers COF475, COF779, and COR999.

concentration for sequencing was obtained from 217 reactions of which 29 reactions failed to identify a species. A total of 188 PCR reactions were sequenced that include 61 from J2 and 127 from females.

The primer set C2F3/1108 detected *M. incognita* and *M. arenaria* by producing amplicons of 1.5 and 1.1 kb, respectively, and *Meloidogyne haplanaria*, *M. marylandi*, *M. hapla*, and *M. partityla* produced an amplicon size of 0.5 kb (Fig.1). Although primer set C2F3/1108 was reported to be sufficient to identify many root-knot species (Powers and Harris, 1993; Powers *et al*., 2005), this primer set generally produced some faint and non-specific bands for *M. incognita* collected during this study. *Meloidogyne incognita* specific primers (COF475/COR999) designed in this study produced approximately 550 bp amplicon (Fig. 2). Furthermore, a combination of three *M. incognita* specific primers (COF475/COF779/COR999) produced two distinct bands of 550 bp and 250 bp (Fig. 3). The newly designed primer set only amplified *M. incognita* collected form this study.

Meloidogyne incognita was the most frequently identified species as it was confirmed in 54 of the 106 samples. It was predominantely associated with 25 soybeans and 11 tomato samples and root or soil obtained from corn, cotton, garden pea, carrot, cilantro, tomato, cowpea, cucumber, okra, squash, begonia, and holy basil. The identification of *M. partityla* from pecan mentioned in this study has already been published (Khanal *et al*., 2016). Therefore, discussion on *M. partityla* will not be provided in this article.

Other populations of RKN found were *Meloidogyne marylandi*, *M. haplanaria*, *M. hapla*, and *M. arenaria*. One of the samples was positive for both *M. partityla*, and *M. hapla*. *Meloidogyne marylandi* was found in soil samples from bermudagrass; *Meloidogyne haplanaria* was identified in root or soil samples from Indian hawthorn, ash, oak, cherry laurel, maple, tomato, willow, rivercane, elm, bermudagrass, and birch; *Meloidogyne hapla* was found in root or soil samples from tomato, oak, pope's phacelia, elm, and rose;

Table 2. List of *Meloidogyne* spp. haplotypes designated in current study with GenBank accession number, origin of sample, and host.

Meloidogyne spp.	Haplotype	Accession number	County	Host[z]
M. incognita	Mi A1	KU948011	Lafayette	corn
M. incognita	Mi B1	KU948012	Pulaski	soybean
M. incognita	Mi C1	KU948013	Pope	Soybean
M. incognita	Mi D1	KU948014	Ashley	soybean
M. incognita	Mi E1	KU948015	Woodruff	soybean
M. incognita	Mi A	KU948016	Lonoke, Logan, Desha, Randolf, Johnson, Faulkner, Washington, Columbia, Conway, Hempstead, Lincoln, Cleburne, Desha, Jackson, Pope, Pulaski, Sebastian, Baxter, Van Buren, Lawrence	soybean, cotton, garden pea, tomato, carrot, cilantro, cowpea, squash, okra
M. incognita	Mi B	KU948017	Crawford, Lawrence	squash, soybean
M. incognita	Mi C	KU948018	Drew	soybean
M. incognita	Mi D	KU948019	Crawford	tomato
M. incognita	Mi E	KU948020	Pope, Pulaski	tomato, melon
M. incognita	Mi F	KU948021	Philips	squash
M. incognita	Mi G	KU948022	Pulaski, Faulkner	begonia, melon, soil sample
M. incognita	Mi H	KU948023	Jefferson	tomato, Soybean
M. incognita	Mi I	KU948024	Lawrence, Bradley, Pulaski	soybean, tomato
M. incognita	Mi J	KU948025	Montgomery, Garland	holy basil, okra
M. arenaria	Ma A	KU948037	Hempstead	garden soil
M. arenaria	Ma B	KU948038	Sebastian	tomato
M. haplanaria	Mhr A	KU948026	Faulkner, Washington	Indian hawthorn, ash, oak, cherry, maple
M. haplanaria	Mhr B	KU948027	Washington	maple
M. haplanaria	Mhr C	KU948028	Baxter	tomato
M. haplanaria	Mhr D	KU948029	Washington	willow
M. haplanaria	Mhr E	KU948030	Washington	rivercane, elm
M. haplanaria	Mhr F	KU948031	Washington	maple
M. haplanaria	Mhr G	KU948032	Washington	Bermuda grass
M. haplanaria	Mhr H	KU948033	Washington	birch
M. marylandi	Mm A	KU948034	Hempstead, Craighead, Perry	Bermuda grass
M. marylandi	Mm B	KU948035	Logan	pecan grove soil
M. marylandi	Mm C	KU948036	Drew	Bermuda grass
M. hapla	Mha A	KU948039	Washington	oak
M. hapla	Mha B	KU948040	Washington	pope weed, elm
M. hapla	Mha C	KU948041	Craighead	rose
M. hapla	Mha D	KU948042	Logan	pecan grove soil
M. partityla	Mpa A	KP975420	Logan	pecan

[z]Host indicates the sample (root or soil) was collected from around the root zone of respective plant.

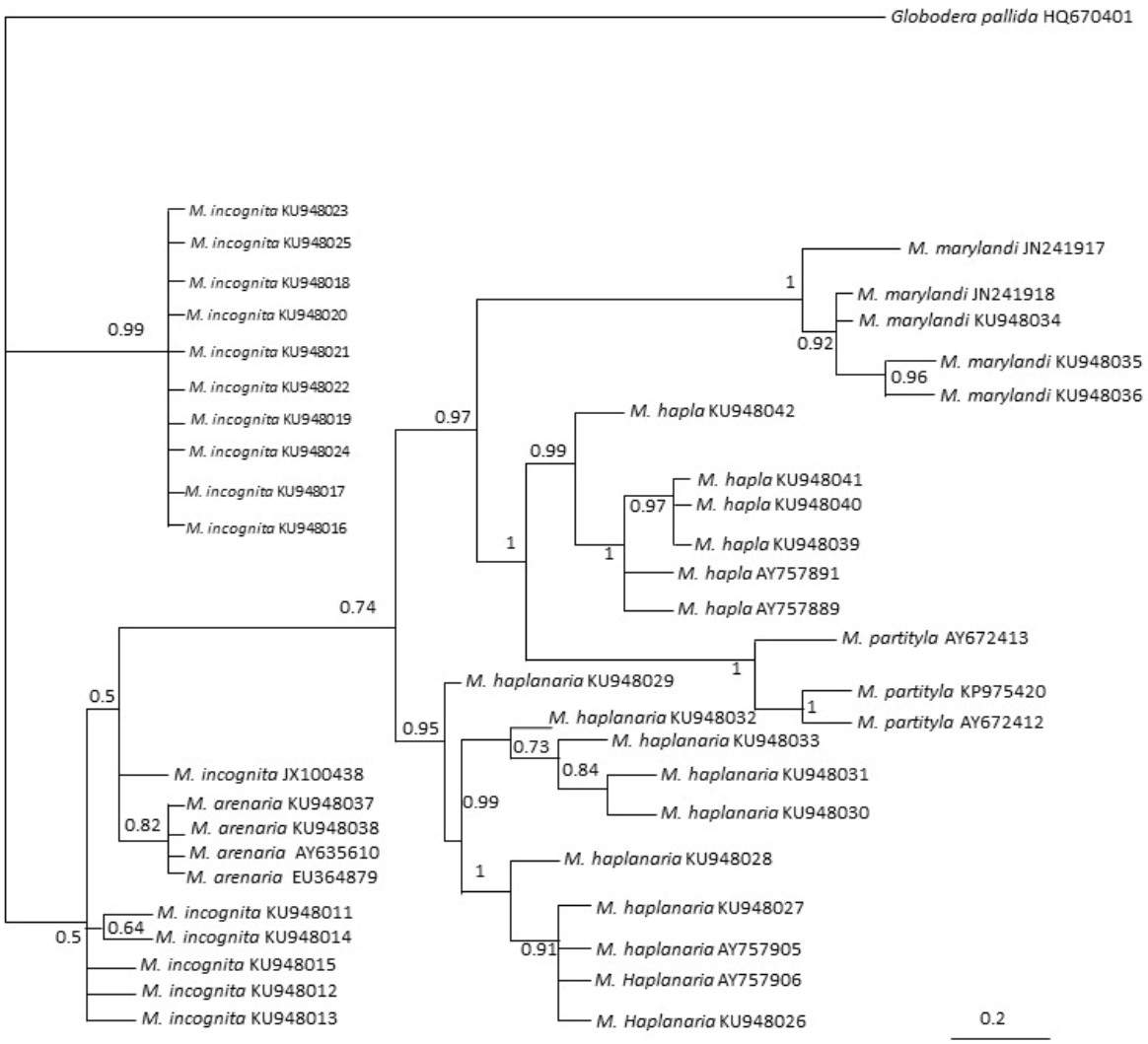

Fig. 4. Bayesian tree inferred from mitochondrial DNA sequence under GTR+I+G model (-INL=2438.149; freqA=0.3074; freqC=0.0659; freqG=0.1252; freqT=0.5014; R(a)=1.27417; R(b)=3.35613; R(c)=1.37264; R(d)=1.16204; R(e)=1.32528; R(f)=1. Numbers at clade branch points represent posterior probability values. GenBank accessions KU948011-KU948042, and KP975420 represent the haplotypes obtained from this study. Rest of the accessions were pulled from GenBank database as references.

Meloidogyne arenaria was obtained from a garden soil sample and a tomato root (Table 1-2).

Molecular phylogenetic analysis produced distinct clades of *M. incognita*, *M. haplanaria*, *M. arenaria*, *M. hapla*, *M. marylandi*, and *M. partityla* and formation of sub-clades for *M. incognita*, *M. haplanaria*, *M. marylandi*, and *M. hapla* indicates that variation is present within root-knot species of Arkansas (Fig. 4).

Any two sequences of a species completely identical to each other were considered as the same haplotype. Haplotype designation was based on reference sequences for each species which are one of the mtDNA sequences obtained from respective species identified in this study. The amount of genetic variation in each haplotype was determined based on number of nucleotide insertions, deletions, or substitutions in contrast to a reference haplotype (Table 1-2). *Meloidogyne incognita* haplotypes (GenBank accessions KU948011-KU948025) had up to 3 nucleotide insertions, deletions, or substitutions. *Meloidogyne arenaria* haplotypes (GenBank accessions KU948037-KU948038) differed by 11 nucleotide substitutions. *Meloidogyne haplanaria* haplotype sequences (GenBank accessions KU948026-KU948033) had highest genetic variation of up to 4 nucleotide deletions and up to 16 nucleotide substitutions. *Meloidogyne marylandi* haplotypes

(GenBank accessions KU948034-KU948036) had up to 2 nucleotide substitutions. Similarly, one of the *M. hapla* haplotypes (GenBank accessions KU948039-KU948042) had 1 nucleotide deletion while others had 1 to 12 nucleotide substitutions. DNA sequences of each haplotype were submitted to GenBank.

DISCUSSION

In this study six species of root-knot nematodes occurring in Arkansas were identified: *M. incognita*, *M. haplanaria*, *M. marylandi*, *M. hapla*, *M. arenaria*, and *M. partityla*. Similar studies using molecular analysis have been conducted in various regions of the United States. A regional survey conducted in the central United States identified *M. incognita*, *M. arenaria*, *M. hapla*, *M. graminis*, *M. javanica*, *M. chitwoodi*, *M. fallax*, *M. enterlobii*, *M. partityla*, and *M. konaensis* using mitochondrial primers (C2F3/1108) and restriction digestion enzymes (Powers *et al*., 2005). A survey of golf courses of the western United States identified *M. nassi*, *M. graminis*, *M. marylandi*, *M. minor*, and *M. chitwoodi* (McClure *et al*., 2012). Unlike the reports by Powers *et al*. (2005) and McClure *et al.* (2012), *M. graminis*, *M. javanica*, and any regulatory species were not detected in this study.

Meloidogyne incognita was identified as the most widespread root-knot nematode in Arkansas and was the only species of root-knot nematode identified from 25 soybean samples collected in the state. Most of the cotton acreage in the late 1970's was gradually replaced by soybean in Arkansas, which likely contributed to the high frequency of this nematode species on soybean (Robert Robbins, personal communication). Interestingly, *M. incognita* from samples of cotton, soybeans, and tomato samples were Mi A haplotypes, suggesting the haplotypes were distributed from old cotton fields as a common source. *Meloidogyne incognita* collected and amplified with primers COF475/COR999 had 99 to 100% genetic identity with *M. incognita* sequences available in GenBank indicating a high level of intraspecific variation in the species.

Only *M. marylandi* was detected on Poaceae plants as *Cynodon dactylon* whereas *M. graminis* was undetected. Given that only five samples were processed and all turned out to be positive for *M. marylandi*, this root-knot nematode is probably a dominant species from golf courses in Arkansas. However, further samples of golf courses and sod farms are needed to determine the most common species of RKN on grass in Arkansas. *Meloidogyne marylandi* collected in this study had 98-99% genetic identity to sequences submitted by McClure *et al*. (2012) from Arizona (GenBank accession numbers JN241917 and JN241918) suggesting the existence of variability in geographic isolates of the same species.

Meloidogyne arenaria was only detected from two soil samples, a residential garden and a tomato root sample (Table 2). Only one *M. arenaria* specimen was obtained from each of the samples suggesting many possibilities: a good host was not present, sampling missed the main population, the soil was not favorable for reproduction, or it was just present in very low numbers in the sample. *Meloidogyne arenaria* shared 99% identity with *M. arenaria* GenBank accession numbers AY635610 and KF993637.

More genetic variation was observed for *M. haplanaria* than any other root-knot species. This genetic variation was also reflected in the Bayesian phylogenetic tree (Fig. 4). *Meloidogyne haplanaria* from Arkansas shared 96-99% identity with the *M. haplanaria* from Texas (GenBank accession numbers AY757905 and AY757906).

Meloidogyne hapla was found in samples from northern regions of Arkansas, which suggests their distribution in this region. However, performing an extensive sampling from southern regions of Arkansas would be useful to confirm their exact distribution. *Meloidogyne hapla* from Arkansas shared 98-99% identity with *M. hapla* GenBank accession numbers KF993633 and AY757899.

Phylogenetic tree (Fig. 4) was useful to study the relationships among the specimens collected. Each species formed distinct clade with known nucleotide sequences pulled form GenBank. Formation of distinct clade for each species provided further evidence on identification of accurate species despite the identity score of 96% to 100% in nucleotide BLAST.

Haplotype variation provides an insight on extent of genetic variation in a species, and a few studies have been conducted to study mtDNA haplotype variation of nematodes. With a limited number of samples, our study identified and designated several mtDNA haplotypes of *Meloidogyne* spp. endemic to Arkansas (Table 1 and 2). A study with larger number of samples from Arkansas and other states would reveal the presence of other mtDNA haplotypes in RKN and it will provide more insight on haplotypes distribution.

Low level of PCR amplification was obtained with the primers C2F3/1108. The possible reasons for not getting any amplification with some of the specimens include a low amount of DNA template was recovered, DNA was degraded, and/or the specimen picked was not a root-knot nematode. Unlike C2F3/1108 primers, *M. incognita* specific primers designed in this study produced sufficient

DNA template for sequencing. *Meloidogyne incognita* primers have previously been designed based on ribosomal DNA sequences (Saeki *et al.,* 2003; Qiu *et al*., 2006; Adam *et al*., 2007), however, availability of the *Meloidogyne incognita* specific primers targeting mtDNA genes were lacking. Additionally, use of multiple primers can be more effective in species determination, as false identification is minimized when diagnostics is based on multiple bands. Specificity of the primers designed in this study were tested only for the species obtained from this survey. However, validation of newly designed primers with other species or the species from outside Arkansas would provide more evidence on the limits of their specificity. Additionally, specificity of the primers could not be tested with *M. javanica* due to unavailability of the specimen during study period. However, the region segment that the primers amplified are not completely identical in *M. incognita* and *M. javanica*. Because *M. incognita* and *M. javanica* share some common nucleotide sequences in their genome, it is suggested that the primers designed in this study should not be used for diagnostic purposes before their specificity test with *M. javanica*.

Meloidogyne graminis and *M. javanica* were not detected in this survey as in previous reports (Grisham *et al*., 1974; Wehunt *et al*., 1989; Walters *et al*., 1994) because of lack of information on exact location. However, it is likely that with more extensive surveys these species could be detected.

Results from this study provide a basis for study of variations and distribution of root-knot nematodes and their mtDNA haplotypes across various geographic locations. Once information on existing haplotypes in different states are available, it will open an opportunity to study the distribution, variations, and co-evolution of *Meloidogyne* spp. across the country.

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