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# Identification of Root-knot Nematodes (Meloidogyne spp.) of Arkansas using Molecular Diagnostics

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**Abstract**

Root-knot nematodes (*Meloidogyne* spp.) are highly-adaptable, obligate plant parasites distributed worldwide. In addition, root-knot nematodes are an economically important genus of plant-parasitic nematodes. *Meloidogyne incognita, M. arenaria, M. javanica, M. hapla* and *M. graminis* have been reported from Arkansas during 1964 to 1994. Previous identifications were based primarily on morphological characters and host differentials. In this study, identification using molecular diagnostics methods was performed to identify *Meloidogyne* species present in Arkansas. A total of 106 soil and root samples from 36 of the 75 counties were collected and processed to obtain root-knot nematodes. Polymerase chain reaction (PCR) was performed to amplify a region between cytochrome oxidase II and 16s ribosomal mitochondrial DNA (mtDNA) genes of root-knot nematodes. Of the species identified in the present survey, *M. incognita* was the most abundant followed by a few isolated samples of *M. marylandi, M. haplanaria, M. hapla, M. arenaria* and *M. partityla*. As a result of the sequencing analysis, a new set of PCR primers that amplifies the same region of mtDNA was designed to identify *M. incognita*. Furthermore, a species specific PCR protocol was developed using mitochondrial marker for routine nematode identifications of the most common species, *M. incognita*. Additionally, cultures of different species identified were established and maintained in the greenhouse on a suitable host to facilitate future research. Since correct identification of species in the field is imperative for effective control of any pathogen, findings from this research will be useful in developing suitable crop management strategies in Arkansas.

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## Dedication

This thesis is dedicated to my parents.

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# 1 Literature review

## 1.1 Root-knot nematodes on different crops

Root-knot nematodes attack a large number of crops ranging from woody ornamentals to annual and perennial flowers, field crops, fruit and nut crops, vegetable crops and grasses. These soil inhabiting plant root parasites are responsible for 12.3% loss of most major cash crops of the world (Sasser, 1987).

Different attempts have been made over different time periods to identify *Meloidogyne* spp. present in Arkansas using classical method. *Meloidogyne graminis* Sledge and Golden, 1964 was first found in 1967 by R. D. Riggs on *Zoysia* spp. of Arkansas (Grisham et al. 1974). Taylor et al. (1982) reported *M. hapla* Chitwood, 1949 on black locust (*Robinia pseudoacacia*) near the Mississippi river in Arkansas. 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. Survey on *Meloidogyne* spp. of the United States by Walters and Barker (1994) revealed the presence of *M. hapla*, *M. incognita, M. arenaria* and *M. javanica* (Treub, 1885) Chitwood, 1949 in Arkansas. Monfort et al. (2007) studied damage potential of *M. incognita* on cotton (*Gossypium hirsitum*) of Southwestern Arkansas.

Turfgrass is a major industry in the United States (McClure et al., 2012). Root-knot nematodes are one of the most common pests of turf grass (Crow et al., 2009) and regarded as one of the principal agents responsible for turf decline. Root-knot nematode (*M. nassi* Franklin, 1965) is one of the nine common genera of plant-parasitic nematodes that are found on *Agrostis* spp. (Sikora et al., 1972). Indirect loss from turf decline occurs due to reduced ability of

turfgrasses to absorb applied water and nutrients (Crow, 2005). Starr et al. (2007) reported *M. marylandi* Jepson & Golden *in* Jepson, 1987 as a pest of bermudagrass (*Cynodon* spp.) which is one of the major turfgrasses. McClure et al. (2012) conducted a survey on 238 golf courses in 10 states of the western United States and found *M. naasi*, *M. marylandi* Jepson and Golden, *M. graminis*, *M. chitwoodi* Golden, O' Bannon, Santo and Finley, 1980, *M.* minor Karssen, Bolk, van Aelst, van den Beld, Kox, Korthals, Molendijk, Zijlstra,van Hoof and Cook, 2004 and *M. fallax* Karssen, 1996.

*Meloidogyne enterlobii* Yang & Eisenback, 1983 which was reported from Florida in late 2001 is a highly virulent species infecting cover crops, fruit trees, weeds, ornamental and agronomic plants (Brito et al. 2004; Cetintas et al., 2008). This was the first report of *M. enterlobii* in the United States. Ye et al. (2013) reported *Meloidogyne enterolobii* on *Gossipium hirsutum* and *Glycine max* in North Carolina.

Pecan (*Carya illinoinensis*) is a valuable nut tree crop grown in the United States. Nyczepir and Wood (2008) contend that Root-knot species that have been reported to attack pecan are *Meloidogyne partityla* Kleynhans, 1986, *M. incognita* and *M. arenaria*. Of these, *M. partityla* was reported to be found in Texas (Starr et al., 1996), which was the first occurrence of this species in the United States on pecan. In the United States *M. partityla* was reported from pecan trees in Texas, New Mexico, Georgia, Florida, Arizona and Oklahoma (Brito et al., 2006). Other than pecan, *M. partityla* has been reported on hickory (*Carya ovata*) and walnut (*Juglans hindsii* and *J. regia*) indicating it is restricted to the plants under family Juglandaceae (Starr et al., 1996). Interestingly, Brito et al. (2013) reported laurel oak (*Quercus longifolia*) as a newly identified host of *M. partityla* in Florida. There are no reports of occurrence of *M. partityla* on pecan trees of Arkansas.

*Meloidogyne hapla* has been reported as a pest of *Daucus carota*, *Apium graveolens*, Allium cepa and *Lactuca sativa* (Mitkowski, 2002). Nyoike et al. (2011) reported *M. hapla* infecting Florida strawberries (*Fragaria* spp.) that were transplants imported from Ontario, Canada. *Solanum lycopersicum* is attacked by many *Meloidogyne* species such as *M. arenaria, M. incognita, M. javanica. M. mayaguensis* and *M. floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar and Higgins, 2004 (Cetintas et al., 2007). Brito et al. (2004) reported *M. mayaguensis* from Florida making first report of this species in the United States. A greenhouse test conducted by Kokalis-Burelle and Rosskopf (2012) reported that American jointvetch (*Aeschynomene americana*) and common purslane (*Portulaca oleracea*) are good hosts for *M. arenaria, M. incognita,* and *M. javanica.* Bendezu et al. (2004) described *Arachis hypogaea*, *Phaseolus vulgaris*, *Pisum sativum*, *Raphanus sativus*, *Glycine max, Vigna unguiculata* and *Solanum melongena* as host of *M. haplanaria* Eisenback, Bernard, Starr, Lee & Tomaszewski, 2004. *Meloidogyne javanica* is an important pest of *Phaseolus vulgaris* (Vito et al., 2007). Rich and Green (1981) described *M. javanica* as a pest of *Helianthus annuus*.

*Meloidogyne hapla, M. incognita* and *M. javanica* are major pests of flue-cured tobacco (*Nicotiana tabacum*) in the southwestern USA (Rich and Zimet, 1996; Garcia and Rich (1983). Villain et al. (2013) stated that *M. exigua* Goeldi, 1887, *M. arenaria, M. hapla, M. enterlobii and M. incognita* attack coffee (*Coffea* spp.) plants. Other species attacking *Coffeea* spp. are *Meloidogyne arabicida* Lopez and Salazar, 1989; *Meloidogyne izalcoensis* Carneiro, Almeida, Gomes & Hernandez, 2005; *Meloidogyne paranaensis* Carneiro, Carneiro, Abrantes, Santos and Almeida, 1996; and *Meloidogyne morocciensis* Rammah & Hirschmann, 1990 (Villain et al., 2013).

Several ornamental plants have been reported to be host for *M. incognita* (Wang & McSorley, 2005). Brito et al. (2010) reported that *M. arenaria, M. floridensis, M. graminis, M. incognita, M. javanica* and *M. mayaguensis* are six common root-knot species attacking ornamental plants in Florida. Similarly, Dale (1973) from New Zealand considered *M. hapla* as a serious pest of roses (*Rosa* spp.).

Rich et al. (2008) enunciated that a large number of weeds serve as major reservoir for many root-knot nematodes. *Meloidogyne incognita, M. javanica, M. arenaria, M. hapla, M. graminicola* Golden and Birchfield, 1965*, M. mayaguensis, M. chitwoodi*, and *M. floridensis* are among the root-knot species that reproduce or survive on weeds during presence or absence of an economic crop (Rich et al., 2008). This implies that care should be taken while adopting any root-knot nematode management practices if the field contains suitable weed hosts.

**1.2 Identification approaches of root-knot nematodes**

Morphometry has long been used to study variation within and among root-knot nematode populations. Until the 1970's, morphological study was only the method to distinguish different species of nematodes. Species differentiation was basically performed by looking at morphological features of second stage juveniles (J2), males and females and sometimes host specificity (Esbenshade and Triantaphyllou, 1990). Some common parameters used to identify nematodes using morphometric include body length, body width, anal body width, stylet length, dorsal gland opening, head end to excretory pore, head end to metacorpous valve, oesophagus length, tail length and hyaline tail length.

Eisenback et al. (1980) distinguished *M. hapla, M. arenaria, M. incognita* and *M. javanica* based on the head structure, perineal pattern and stylet of females using scanning electron microscopy (SEM) and light microscopy (LM). Eisenback and Hirschmann (1981)

identified *M. arenaria, M. hapla, M. incognita* and *M. javanica* based on head shape and stylet morphology of the males using SEM and LM. Using the morphological features Eisenback et al. (1981) developed a pictorial key to distinguish *M. incognita, M. javanica, M. arenaria* and *M. hapla*. Jepson (1983) used tail morphology to successfully distinguish second stage juveniles of *M. graminicola, M. nassi and M. javanica*. Moreover, Jepson (1987) described identification of many *Meloidogyne* spp. using more detailed morphology. Nematode identification using morphometric data is relatively easy up to the genus level, but it becomes a laborious job to distinguish further to species level. Overlapping of key descriptive characters between species is not uncommon, this overlap can lead to misidentification.

Since proper identification of *Meloidogyne* spp. is an important aspect of crop management, a more accurate method of identification was necessary. To overcome this problem biochemical method was soon developed as a complement to morphological method of identification (Hyman, 1990).

Dickson et al. (1971) did allozyme analysis of *Meloidogyne* species by studying enzyme profiles of malate dehydrogenase (MDH), α-glycerophosphate dehydrogenase (GDH) and esterase. They proposed that the enzyme phenotype can be used to distinguish different *Meloidogyne* species. This was probably the first reported biochemical analysis intended to identify *Meloidogyne* species. Dalmasso and Berge (1978) used esterase isozyme analysis to distinguish *M. arenaria, M. javanica, M. incognita, M. hapla* and *M. nassi*. Dalmasso and Berge (1978) described biochemical method as more reliable method of species discrimination when compared with morphological method. Esbenshade and Triantaphyllou (1985) found esterase to be most useful enzyme to identify major *Meloidogyne* species. Venkatachari et al. (1991) revealed that esterase and phosphoglucomutase analysis is useful in differentiating five

*Meloidogyne* species namely *M. incognita, M. javanica, M. arenaria, M. hapla* and an undescribed *Meloidogyne* species. Since cellular expression of any enzyme or protein is affected by environment and developmental stages of an organism, readily detectable differences between closely related species/races may not be found all the time (Hyman, 1990). Additionally, biochemical method is feasible only for adult females but not for most commonly available second stage juveniles, eggs or males which renders this method not applicable for them (Tigano et al., 2005; Esbenshade and Triantaphyllou, 1990).

Polymerase chain reaction (PCR) has been used to speciate many fungi, bacteria and plant parasitic nematodes (Martin et al., 2000). PCR is a technique where a specific region of the genome is amplified using a set of primers. Combined with Restriction Fragment Length Pplymorphism (RFLP) or sequencing PCR can be used to compare genetic similarity or variability between and among different organisms. Powers (2004) explained the usefulness of studying DNA that codes for genes with an example as a protein coding 600 nucleotide segment of DNA could be enough to identify ten million species based on the variability present on that segment. As a region of the genome is amplified with PCR, this example suggests how useful a PCR could be in distinguishing specimens. PCR is completed in repetition of three steps: denaturation, annealing and extension. Denaturation of double stranded DNA is obtained at high temperature (90°C-95°C) to allow the primers to bind at a specific site of single stranded DNA. Binding of oligonucleotide primers to the target region, known as annealing, is brought about at relatively lower temperature (45-60°C). As the primers bind to the target site, temperature is slightly increased (70-74°C) to allow the primer to extend on the template DNA with the help of DNA polymerase, a step known as extension. This process is usually repeated for 30 to 40 times to obtain approximately a million fold amplification of target site.

Harris et al. (1990) used PCR to identify *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* by amplifying the mitochondrial DNA (mtDNA). Powers and Harris (1993) identified *M. incognita, M. javanica, M. arenaria, M. hapla* and *M. chitwoodi* by amplifying the region between cytochrome oxidase II and 16s ribosomal RNA gene of mitochondrial DNA using PCR and restriction enzymes. They also were able to amplify the same region of *M. marylandi, M. nassi* and *M. nataliei* Golden, Rose and Bird, 1981 suggesting the primer they developed could be useful in identification of other *Meloidogyne* species. Powers et al. (1997) described internal transcribed spacer region (ITS) of ribosomal DNA (rDNA) as a taxonomic marker for different nematode genera including the following *Meloidogyne* spp.: *M. incognita, M. arenaria, M. javanica, M. konaensis* Eisenback, Bernard & Schmitt, 1995, *M hapla, M. mayaguensis, M chitwoodi* and *M. graminicola.* In 2005, Powers et al. described mitochondrial marker as a better target for identification compared with the 18S marker. In addition, *M. chitwoodi, M. fallax, M. graminicola, M. graminis, M. hapla, M. haplanaria, M. incognita, M. mayaguensis*, *M. arenaria* and *M. partityla* were identified using PCR primers C2F3/1108 targeting mitochondrial marker. Moreover, the PCR-RFLP distinguished *M. javanica* and *M. chitwoodi*. Adam et al. (2007) developed a molecular diagnostic key to distinguish seven *Meloidogyne* species; *M. incognita, M. javanica, M. arenaria, M. mayaguensis, M. hapla, M. chitwoodi* and *M. fallax* using combination of PCR, sequence characterized amplified region (SCAR) and random amplified polymorphic DNA (RAPD) primers. Powers (2004) illustrated that ribosomal RNA specifically the nuclear 18S ribosomal subunit, internal transcribed spacer (ITS) region, intergenic spacer region (IGS), 28S ribosomal subunit, D3 expansion region and 5S ribosomal subunit; mitochondrial genome such as cytochrome oxidase I (COI), cytochrome oxidase II (COII) and 16S ribosomal DNA are most common genetic regions being used to identify nematode taxa.

Powers further emphasized that the region between COII and 16S ribosomal DNA is a good target for the identification of *Meloidogyne* spp.

Morphometric data when combined with molecular data give a better understanding of any species. Karssen and Moens (2006) contended that the best approach of identification would be a combination and synthesis of data regarding morphological, isozyme, molecular, host, reproduction, chromosome number and distribution. Skantar et al. (2008) used both the morphological and molecular method to identify an unusual population *M. arenaria* found on roots of Traveler's tree (*Ravenala madagascariensis)* roots.

## 2 Introduction

### 2.1 Nematodes

Nematodes are the most abundant multicellular animals on earth (Lorenzen, 1994). These metazoans have a triploblastic, pesudocoelomate, unsegmented bilaterally symmetrical body and may have or lack a distinct oral stylet. More than 25,000 species of nematodes have been described, about 3,000 species are considered to be parasitic to plants (Castagnone-Sereno, 2002; Abad et al. 2008). The distinguishing feature of plant-parasitic nematodes is the presence of a distinct oral stylet. Based on nematode population density, plant-parasitic nematodes can cause crop losses ranging from minor injury to plant death. Additionally, crop losses depend upon several factors such as nematode species, soil type, host, season and crop rotation practices (Moens et al., 2009). It is estimated that total annual crop losses of 14.6% is caused by plant-parasitic nematodes in tropical and subtropical climates (Nicol et al., 2011). Annual worldwide agricultural losses caused by plant-parasitic nematodes are estimated to be $173 billion with $13 billion in the United States (Elling, 2013).

## 2.2 Root-knot nematodes

Root-knot nematodes (*Meloidogyne* Goldi (Tylenchida: Meloidogynidae), are highly-adaptable, obligate plant parasites that are distributed worldwide parasitizing almost every species of vascular plants (Garcia and Sanchez-Puerta, 2012; Jones et al. 2013). Jones et al. (2013) described root-knot nematodes as the most economically important plant-parasitic nematodes in the world. Root-knot nematode was first reported by Berkeley (1855) attacking *Cucumis spp*. Root-knot nematodes are vermiform at the early stages of their life cycles and females have an enlarged body. These soil borne plant parasites got their colloquial name because they form galls (root-knots) in host roots. The term '*Meloidogyne*' was derived from Greek words meaning apple-shaped female (Karssen and Moens, 2006; Moens et al., 2009). Worldwide crop yield losses attributed to *Meloidogyne* spp. only is reported to be 5% (Sasser and Carter, 1985). Based on the report of Sasser and Carter (1985) it seems reasonable to estimate that present economic losses by *Meloidogyne* spp. alone are several billion dollars (Elling, 2013). The genus has 98 species (Jones et al., 2013); however, only 31 species have been reported from the United States (John Eisenback, personal communication). Jepson (1987) reported that economically important root-knot nematodes are based on their cosmopolitan distribution and broad host ranges, which include; *Meloidogyne incognita, M. arenaria*, *Meloidogyne javanica*, and *Meloidogyne hapla*. Additionally, *Meloidogyne mayaguensis* Rammah & Hirschmann, 1988 (=*Meloidogyne enterlobii*) is a highly virulent species with a wide host range including cover crops, fruit trees, weeds, ornamental and agronomic plants (Cetintas et al., 2008). Other important species with relatively narrow host range are *Meloidogyne graminicola, Meloidogyne nassi, Meloidogyne exigua, Meloidogyne acronea* Coetzee, 1956, *Meloidogyne chitwoodi*, *Meloidogyne artiellia* Franklin, 1961, *Meloidogyne decalineata*

Whitehead, 1968, *Meloidogyne africana* Whitehead, 1960, *Meloidogyne coffeicola* Lordello and Zamith, 1960, *Meloidogyne oryzae* Maas, Sanders and Dede 1978, and *Meloidogyne thamesi* Chitwood, 1952 (Jepson, 1987).

Some root-knot species such as *M. incognita* attack up to 3,000 plant species (Castagnone-Sereno, 2002; Fuller et al. 2008) whereas *M. hapla* has been reported on some 550 wild and cultivated hosts (Jepson, 1987). Similarly some species of root-knot nematodes such as *M. chitwoodi* are important from the view point of quarantine. Given the broad host range of the most important row crop species of *Meloidogyne* weed species serve as good hosts, which can sustain a population of nematodes even with proper crop rotation practices.

Eisenback (1982) emphasized that different *Meloidogyne* spp. attack different plants and resistance is not effective against all the species/cultivars, thus correct identification of species in the field is imperative for effective control. Based on accurate identification suitable management practices can be adopted such as resistance, crop rotation or chemical to suppress root-knot nematodes in agricultural cropping systems. Furthermore, accurate identification of root-knot nematodes is critical in breeding programs as well as in regulatory programs such as certification and quarantine (Brito et al., 2010). An example of root-knot nematode management decision regarding the host as given by Jepson (1987) is that if a soil contains *M. exigua*, a resistant robusta coffee plant should be planted, but not a susceptible arabica coffee. At the same time Jepson (1987) also mentioned that both arabica and robusta coffee should not be planted if the same soil contains *M. incognita* and/or *M. coffeicola*.

Accurate identification of root-knot species using classical methods is often a difficult task and requires personnel with a high level of expertise. Classical methods of identification were based on use of morphology and differential tests. Since these methods are time consuming,

require morphometric data of all the life stages of the specimen and not useful for cryptic (ambiguous) and degraded specimen, attempts were directed towards complementing classical methods by molecular methods. In the 1980's and 1990's isozyme analysis that required adult root-knot females was performed as molecular method of species differentiation (Hyman, 1990). Later on polymerase chain reaction (PCR) followed by restriction digestion method was used to distinguish species of root-knot nematodes (Adam et al., 2007). First study to identify *M. incognita, M. hapla, M. javanica* and *M. arenaria* using PCR-RFLP method was done by Harris et al. (1990). Molecular diagnostics i.e. PCR facilitates nematode identification without regard to different life stages and allows personnel lacking nematode taxonomic ability to identify root-knot nematodes

**2.3 Symptoms in plants infected by root-knot nematodes**

Galls are induced by root-knot nematodes on the infected root system of a susceptible host. Galls induced by root-knot nematodes disrupt the vascular system of plants making them grow poorly or sometimes leading to death under heavy infestation (Dale, 1973). Brandon et al. (2011) further explain that this disruption of vascular system is responsible for the plants to develop symptoms such as reduced growth, chlorotic leaves, premature death and increased susceptibility to stress such as drought and other pathogens.

**2.4 Life cycle of root-knot nematodes**

The life cycle of root-knot nematodes is completed in three basic steps: egg, juvenile and adult stage. A female can lay up to 300-500 eggs on a susceptible host. The eggs are deposited on the surface of the root covered in a gelatinous mass. Gelatinous mass released from rectal glands protects eggs from environmental extremes and attack by microbes (Moens et al., 2009). When conditions are favorable embryogenesis takes place and first stage juvenile is formed. The

first stage juvenile (J1) develops inside the egg and molts to become the infective second stage juvenile (J2) that hatches from the egg. According to Curtis et al. (2009) it is believed that hatching of J2 from egg and movement towards a host root tip is dependent on temperature, moisture, oxygen availability, physiological barriers (such as diapause), root exudates and other unknown factors. Once a J2 finds a suitable host root it usually penetrates behind the root cap (Karssen and Moens, 2006; Abad et al., 2009). Penetration takes place by thrusting plant cell continuously with protractible stylet and releasing secretions containing cell wall degrading cellulytic and pectolytic enzymes (Karssen and Moens, 2006) produced possibly in dorsal pharyngeal glands (Abad et al, 2009). However, exact mechanism of host pathogen interaction at molecular level is still unknown (Abad et al., 2003). Once J2 penetrates the host, it migrates intercellularly in the cortical zone to the vascular tissue where it establishes a special type of feeding site called giant cells (Abad et al., 2003). Giant cells are formed by nematode secretions that redifferentiate a single cell into a large multinucleate cell (Abad et al., 2003; Abad et al., 2008). Abad et al. (2003) further elucidated that usually a single juvenile stimulates five to seven normal parenchymatous cells into metabolically active giant cells. Each giant cell has dense cytoplasm, numerous mitochondria, plastids, ribosomes, golgi apparatus, endoplasmic reticulum, vacuoles and most significantly up to 100 nuclei per cell (Abad et al., 2003). Hyperplasia of surrounding cells occurs leading to the formation of visible root galls (Abad et al., 2009). The J2 molts into third stage juvenile (J3) which molts again to become the fourth stage juvenile (J4) then female (Perry et al., 2009). The J3 and J4 do not have a functional stylet and cannot feed. Male nematodes after J2 stage, on the other hand, stop feeding, become vermiform rather than enlarging their body diameter (Perry et al., 2009). Males usually emigrate from the root into the soil (Abad et al. 2003). Males are commonly observed in parthenogenetic species when

conditions are unfavorable for female development such as lack of sufficient food (Perry et al., 2009). The life cycle of root-knot nematode is completed when mature female begins to reproduce eggs. The number of days to complete life cycle is dependent on species, host and environmental condition. However, in general, it takes 21 to 25 days to complete one life cycle at 80°F and 87 days at 67°F (Anonymous, 1993). A tropical root-knot species, *M. graminicola* completes its life cycle in less than 20 days (Luc et al., 2005). In a typical cropping season multiple life cycles are common thus population densities can increase to damaging levels by season end.

As described by Perry et al. (2009), reproduction in root-knot nematodes takes place in different ways: sexual or amphimixis (e.g. *M. carolinensis* Eisenback, 1982; *M. microtyla* Mulvey, Townshend and Potter, 1975; and *M. pini* Eisenback, Yang and Hartman, 1985), meiotic parthenogenetic or automixis (e.g. *M. exigua, M. chitwoodi* and *M. graminicola*) and mitotic parthenogenetic or apomictic (e.g. *M. incognita, M. enterlobii* and *M. oryzae*). In general, parthenogenetic species are characterized by short life cycle, wide host range, morphological similarity to other parthenogenetic species, tropical or sub-tropical distribution, production of large galls, chromosome number (n) more than 18 and having no or few males (Jepson, 1987.) In contrast amphimictic species, in general, are characterized by longer life cycle, more host specific, distinct morphology, temperate or subtropical distribution, small to no gall production, chromosome number (n) 18 and having more or less equal number of males and females (Jepson, 1987).

**2.5 Classification of root-knot nematodes**

Different classification systems for nematodes have been described in different times by different taxonomists. There is no single classification system proposed so far which is agreed by

all taxonomists. Since molecular phylogenies are becoming increasingly important, classification efforts these days have been directed to develop a system which combines both the classical and phylogenetical schemes. Decraemer and Hunt (2006) describe the following classification scheme of plant-parasitic nematodes by combining a classification scheme based on phylogenetic relationships as proposed by De Ley and Blaxter (2002) at family level and above; and a scheme of Siddiqi (2000) and Hunt (1993) at subfamily and generic level.

Phylum Nematoda Potts, 1932

Class Chromadorea Inglis, 1983

Subclass Chromadoria Pearse, 1942

Order Rhabditida Chitwood, 1933

Suborder Tylenchina Thorne, 1949

Infraorder Tylenchomorpha De Ley & Blaxter, 2002

Superfamily Tylenchoidea Örley, 1880

Family Meloidogynidae Skarbilovich, 1959

Subfamily Meloidogyninae Skarbilovich, 1959

Genus *Meloidogyne* Goeldi, 1892

**2.6 Phylogenetic tree**

Phylogenetic tree, also called a phylogeny, is a leaf -labeled tree or diagram illustrating evolution of a taxa from their most recent common ancestor (Moret et al., 2004; Baum, 2008). It is an incredibly important tool to study and analyze the evolution and diversity of any biological entity. As defined by Hoang and Sung (2011), a phylogenetic tree is an unordered distinctly leaf-labeled tree. Huelsenbeck and Ronquist (2001) explained that the most common methods to infer phylogenetic trees are parsimony method, distance methods, maximum likelihood method and

Bayesian method. They further accentuate that Bayesian inferences of phylogenies are based upon the posterior probabilities of phylogenetic trees. Some advantages of Bayesian inference over other methods of phylogenetic inferences are easy interpretation of results, the ability to incorporate available prior information and some computational advantages (Huelsenbeck and Ronquist, 2001). With help of phylogenetic tree, relationship between different root-knot species of Arkansas can be analyzed.

## 3 Objectives

The use of molecular diagnostics has proven to be a beneficial tool in the speciation of *Meloidogyne* spp. and given a statewide survey of root-knot nematode was a priority among University of Arkansas extension plant pathologist our objectives were:

i. To identify species of *Meloidogyne* present in Arkansas using molecular diagnostics.
ii. To develop a species specific polymerase chain reaction method for quick and cost efficient identification of *M. incognita* in Arkansas.
iii. To generate pure cultures of different root-knot nematode species obtained.

## 4 Methodology

### 4.1 Sampling

Nematode samples were collected across various cropping system and horticultural gardens in Arkansas during May to October in 2013 and 2014 (Table 1). Attempts were made to collect samples from as many crop plants and locations as possible. Samples were taken from areas where plants looked stressed, diseased or exhibiting some growth and development problems. A foot powered conical core sampler or shovel was used to take the soil and root samples. Approximately 500 cubic centimeters of soil and roots was collected, mixed thoroughly, labeled and kept in an insulated container during transit from field to nematode

extraction lab. Samples collected were stored (4°C) until extraction at the Cralley/Warren Research Laboratory at Arkansas Agricultural Research and Extension Center (AAREC).

Farmers, master gardeners and extension County agents were asked to provide samples from nematode suspected or diseased plants. Personal contact with home gardeners was made using email while mass contact with other people was made by publishing a request for the samples in Plant Health Clinic News, University of Arkansas. Other sources for sample collection were University of Arkansas extension plant pathologists, Plant Health Clinic, Fayetteville and Arkansas Nematode Testing Service (Hope). Hosts included were turf grasses, hardwood trees, field crops, vegetable crops and ornamental crops. Detailed information about the samples such as location (GPS information, if possible), date and host was recorded (Table 1).

**4.2 Nematode extraction**

Root-knot juveniles (J2) were extracted from the soil samples or egg masses from root systems. Nematodes from each sample were extracted using a rapid centrifugal-flotation technique as described by Jenkins (1964) with slight modifications as indicated below. About 250 cubic centimeters of soil was kept in a 10 liter bucket and filled more than half with water. Soil was thoroughly mixed and stirred by hand. The water was then passed through a 20-mesh and 60-mesh sieves to remove any debris. Samples were collected in 400-mesh sieve and the soil transferred into a 50 ml centrifuge tubes. Tubes were spun at 1400 rpm for five minutes in a centrifuge. Supernatant was carefully discarded and 71.4% sugar solution (prepared by mixing 2000 grams of sugar and 100 ml bleach in 2700 ml of water) was added to the soil and mixed thoroughly. The mix was spun at 1400 rpm for three minutes in a centrifuge and nematodes were collected in a 500-mesh sieve, rinsed with clean water and transferred into a beaker. This sample

was examined using a stereoscopic microscope for the presence of root-knot juveniles (Figure 2). Root-knot J2 were distinguished to genus from ring, dagger, sting, lance and other free living nematodes by looking at stylet, tail and movements. Root-knot J2 were hand-picked and transferred into a separate vial containing clean water to reduce contamination. The extraction and separation process was completed for each nematode sample. The handpicked nematodes were stored at 4°C in a refrigerator or the cool room until molecular analysis.

Root-knot females (Figure 3) were excised from root galls (Figure 1) using a sterilized needle, scalpel and a stereoscopic microscope. Individual females excised from root were kept in a vial containing water. Root-knot females obtained were ready to use for DNA extraction.

**4.3 DNA extraction**

DNA from root-knot nematode juveniles (J2) was extracted by using the smash method as described by Powers and Harris (1993) with slight modifications as indicated below. A single root-knot specimen was hand-picked using a cactus spine looking through a stereoscopic microscope. Individual J2 was placed in 2.5 µl of double distilled water on a glass slide and cut into at least two pieces using a sterilized 10 µl micropipette tip. The solution was stirred five to ten times with pipette on glass slide. The squashed solution was immediately transferred to 0.2 ml PCR tube and kept on ice to prevent possible degradation of DNA. The squashed solution containing pieces of nematode served as template for the Polymerase Chain Reaction (PCR).

DNA from root-knot nematode females were extracted using puregene technique, a protocol based on rapid isolation of mammalian DNA (Sambrook and Russel, 2007a). An individual female was grounded in a 1.5 ml eppendorf tube containing 300 µl of cell lysis buffer (prepared by mixing 100 mL of 20 mM Tris-HCl pH 7.8, 0.61 gram ethylenediaminetetraacetic acid and 1 gram sodium dodecyl sulphate) using a 1 ml micropipette tip. The buffer solution

containing grounded female was mixed and kept in a freezer (-80°C) for at least two hours. After freezing, the solution was thawed and incubated for five minutes at 65°C in a water bath. After incubation, 100 µl of protein precipitation solution (7.5M ammonium acetate which was prepared by mixing 57.8 grams ammonium acetate and adding water to make final volume of 100 ml) was added and vortexed for 20 seconds. Sample was centrifuged for three minutes at 13,000 rpm and 300 µl of supernatant was transferred into a 1.5 µl Eppendorf tube. For DNA precipitation, 300 µl of 100% isopropanol was added and centrifuged for four minutes at 13,000 rpm. Supernatant was discarded, tube was blotted on clean paper towel and 300 µl of 70% ethanol was added then centrifuged for four minutes at 13,000 rpm. The solution was discarded followed by blotting tube on clean paper towel. The tube was incubated uncapped in a heat block for five minutes at 65°C to evaporate any remaining ethanol. After complete evaporation of ethanol 12 µl of double distilled water was added and mixed gently. Finally, capped tube was incubated at room temperature overnight. This solution was used as a DNA template for PCR throughout this study. DNA samples were stored at -20°C until further use.

**4.4 Polymerase Chain Reaction (PCR)**

Mitochondrial DNA (mtDNA) was selected as a marker to distinguish different species of root-knot nematodes. The primer set used was C2F3 and 1108 (Table 3) which amplifies the region between COII and 16S ribosomal mitochondrial genes of root-knot nematodes (Powers and Harris, 1993; Powers et al., 2005). Number of J2 obtained per extraction was 2 to 20. Of the samples with root-knot nematodes, 2 to 10 individuals were randomly selected to identify by PCR. *Meloidogyne incognita* collected from cultures maintained in the greenhouse was used as positive control. PCR reaction master mix was prepared in such a way that each reaction gets 2.5 µl of 10X CL buffer (Qiagen, mat no. 1032517), 17.5 µl of PCR water, 1 µl of 25 mM $MgCl_2$

(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 from J2 or female described above was added and well mixed. PCR was performed in a PTC-100® Peltier Thermal Cycler. Amplification conditions included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 90 seconds. A final extension step was conducted for 10 minutes at 72°C.

Agarose gel (2%) was prepared by mixing 2 g agarose in 100 ml 1X TBE solution and stained with 3.5 µl of Gel Red Nucleic Acid (Biotium, cat: 41003). A 5 µl sample from the PCR product was loaded in one well while 5 µl of 100bp DNA ladder (Promega, ref: G210A) was loaded in another well. Agarose gel was run at 168V for 50 minutes. Separated DNA bands were visualized using a UV transilluminator (UVP BioDoc-It™ System) (Figure 4).

**4.5 Purification and quantification of PCR product**

Each PCR product remained after running a gel was purified using "standard ethanol precipitation of DNA in microfuge tubes" method (Sambrook and Russell, 2001b) with slight modifications as described below. PCR product was transferred into a sterilized 1.5ml tube. Double distilled water was added to it to make a total volume of 100 µl and mixed with pipet tip. 100 µl of 100% isopropanol was added and mixed at least 50 times with a pipette followed by centrifugation at 13,000 rpm for seven minutes which precipitates the DNA. The supernatant was discarded and the tube was blotted on clean paper towel. 300 µl of 70% ethanol was added to the tube to wash salts and impurity in the tube followed by centrifugation at 13,000 rpm for four minutes. The supernatant was discarded and blotted again on clean paper towel. Now the tube was kept in a heat block at 65°C for five minutes to allow the remaining ethanol to evaporate.

Finally, 10 µl of double distilled water was added to the tube. This whole process gives purified DNA. Concentration of purified DNA was measured by loading 1 µl DNA solution into a spectrophotometer (Nanodrop 1000 v 3.6.0).

**4.6 DNA sequencing**

The purified PCR products were prepared according to sample preparation guidelines provided by the DNA Resource Center at the University of Arkansas, Fayetteville. A portion of the purified DNA (10ng to 40ng) from each sample was transferred into two 1.5 ml Eppendorf tubes and 1 µl of forward or reverse primer was added per tube. PCR water was added to bring final volume of 13 µl in each tube. DNA samples were sequenced in both directions by the DNA Resource Center (University of Arkansas, Fayetteville) 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 specimen was determined looking at the highest matches (maximum score, total score, query cover and E value) with a threshold of 97% identity.

**4.7 New primers design**

The primer set C2F3/1108 has been used by many diagnosticians to identify different species of root-knot nematode. This primer set amplify the mtDNA with amplicon size ranging from approximately 500 to 1700 bp. However, longer amplification products (for *Meloidogyne incognita* in this research) were usually obtained with some non-specific bands. The purified PCR products when sent for sequencing were returned with some non-reading sequences making analysis difficult. To overcome this shortcoming, a separate primer set was designed. For this,

DNA sequences obtained from *Meloidogyne* spp. during this study were aligned together with complete mitochondrial genome (Humphreys-Pereira and Elling, 2014) available in GenBank using BioEdit 7.1 (Hall, 1999). A forward primer and a reverse primer were designed (Table 3). PCR reaction master mix using these primers was prepared in the same way as described for primer set C2F3/1108. PCR was performed in a PTC-100® Peltier Thermal Cycler. *Meloidogyne incognita* collected from cultures maintained in the greenhouse was used as positive control. Amplification conditions optimized for this primer included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 43°C for 60 seconds, and extention at 72°C for 90 seconds. A final extension step was conducted for 10 minutes at 72°C.

**4.8 Phylogenetic tree construction**

*Meloidogyne* sequences of Arkansas along with other *Meloidogyne* sequences available in GenBank were used to construct phylogenetic tree based on Bayesian analysis (Figure 8). Sequences were aligned using Geneious (version 6.1.8). Bayesian tree was constructed 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 pallid*a (GenBank accession number HQ670401) was used as outgroup.

**4.9 *Meloigodyne incognita* specific PCR**

DNA sequences amplified by COF475 and COR999 and complete *M. incognita* mitochondrial genome (Humphreys-Pereira and Elling, 2014) available in GenBank were aligned

using BioEdit 7.1 and a primer COF779 was designed (Table 3). COF475, COF779 and COR999 were used as *M. incognita* specific primers. PCR reaction master mix was prepared in such a way that each reaction gets 2.5 µl of 10X CL buffer (Qiagen, mat no. 1032517), 16.5 µl of PCR water, 1 µl of 25 mM $MgCl_2$ (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 tubes 22 µl PCR reaction master mix aliquot was kept and 2.5 µl DNA template was added. *Meloidogyne incognita* collected from cultures maintained in the greenhouse was used as positive control. PCR reaction master mix was stirred well with pipette tip before putting in a thermal cycler. PCR was performed in a PTC-100® Peltier Thermal Cycler. Amplification conditions optimized included an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 60 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 90 seconds. A final extension step was conducted for 10 minutes at 72°C. A 7 µl of PCR product was loaded in 2% agarose gel and visualized using a UV transilluminator (UVP BioDoc-It™ System) (Figure 4). Annealing temperature was screened over a range of 42-49°C.

**4.10 Maintenance of root-knot nematodes**

Part of each soil sample was used as nematode inoculum on four-week old *Solanum lycopersicum* (Rutgers), *Arachis hypogaea* (Georgia 06 G), *Gossypium hirsutum* (Stoneville ST4288B2F), *Carya illinoinensis* or *Cynodon dactylon* in greenhouse to collect an isolate for future studies. The inoculum was mixed with enough sterilized sandy soil to fill four inch clean clay pots. In case of root sample, it was cut into small pieces, mixed with sterilized sandy soil and inoculated on each host. Greenhouse temperature was set to 90°F and minimal watering was done twice a day.

### 4.11 Pure Culture

Host plants maintained in greenhouse were examined for root-knot nematode reproductions 45 days post inoculation. Roots were washed free of soil and debris. Roots were examined for the presence of galls and egg masses using a dissecting microscope. Individual egg masses with females from root system were picked and placed into small vials containing water. Egg masses were from females that were used for identifications in this study. Individual egg mass were kept at room temperature for J2 to hatch. Four-week-old seedlings grown in six ounces Styrofoam cups were used for J2 inoculation. Greenhouse temperature was kept at about 90°F and watered twice a day. Inoculated plants were inspected for the presence of root-knot nematodes 90 days after inoculation.

## 5 Results

A total of 106 soil and root samples from 36 of 75 Arkansas counties were collected and processed in this survey. Samples include 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 crops (some not shown in Table 1). Of 106 samples examined, 79 were positive for root-knot nematodes (Table 2).

A total of 576 PCR reactions were performed to identify root-knot nematodes obtained during this study. PCR amplification was obtained from 401 reactions while 175 reactions did not amplify probably because of low amount of DNA template available from individual nematodes. Furthermore difficulty occurred in PCR amplification of target DNA from juvenile root-knot nematodes, probably due to low amounts of template DNA. DNA recovered from mature root-knot females was normally sufficient for PCR. Minimum concentration for

sequencing was obtained from 217 reactions of which 29 reactions failed to identify species. A total of 188 reactions were sequenced that include 61 from second stage juveniles and 127 from mature females.

*Meloidogyne incognita* was identifed from 54 samples including 25 soybeans and 11 tomatoes (table 2). *Meloidogyne partityla* was found in one of six pecan groves sampled (Table 2). *Meloidogyne marylandi* was found in 5 bermudagrass samples (Table 2). Similarly, *M. haplanaria, M. hapla* and *M. arenaria* were found in 13, 5 and 2 samples respectively (Table 2). Distribution of root-knot species collected during this study is shown in Figure 7.

Phylogenetic tree constructed using Bayesian analysis produced distinct clades of *M. incognita, M. haplanaria, M. arenaria, M. hapla, M. marylandi* and *M. partityla* of Arkansas (Figure 8). Formation of sub-clades for *M. incognita, M. haplanaria, M. marylandi* and *M. hapla* indicates that variation was present between root-knot species of Arkansas. Additionally, phylogenetic tree indicates that *M. partityla* and *M. hapla* are closely related but clearly distinct species.

Intraspecific variation for each different species of Arkansas was analyzed by aligning the sequences using ClustalW (Thompson et al., 1994). Nucleotide substitution, insertion or deletion was detected in sequecces from *M. incognita, M. haplanaria, M. arenaria, M. marylandi* and *M. hapla*. Comparision of *M. incognita* sequences were made with sequences of *M. incognita* from sample no. 21. A single nucleotide insertion was observed in *M. incognita* from sample no. 10, 14, 20, 17 and 18 (Figure 10). A single nucleotide deletion was observed in *M. incognita* from sample no. 12, 13, 38, 52, 70 and 77 (Figure 10). Two nucleotide substitutions were observed for *M. incognita* from sample no. 14, 20, 17, 18 and 19 (Figure 10). A single nucleotide substitution was found in *M. incognita* from sample no. 7, 16, and 75 (Figure 10).

Comparisons of *M. haplanaria* sequences were made with *M. haplanaria* from sample no 47 (Figure 11). A nucleotide insertion was detected in *M. haplanaria* from sample no. 15, 39, 40 and 41 (Figure 11). *Meloidogyne haplanaria* from sample no. 15 showed 4 nucleotide deletions (Figure 11). Comparisons of M. marylandi sequences were made with *M. marylandi* from sample no. 11 (Figure 12). *Meloidogyne marylandi* from sample no. 6 was observed with 2 nucleotide variations while from sample no. 69 a single nucleotide variation was observed (Figure 12). *Meloidogyne arenaria* from Arkansas show variations in 12 nucleotide positions (Figure 13). Comparison of *M. hapla* sequences were made with *M. hapla* from sample no. 48. *Meloidogyne hapla* from sample no. 37, 44 and 51 show variation in 9, 2 and 2 nucleotides respectively (Figure 14). Moreover, 13 nucleotide variations including a nucleotide deletion was detected in *M. hapla* from sample no. 6 (Figure 14).

Any two sequences of a species completely identical to each other are considered as same haplotype. Five haplotypes (*Mi* $A_1$, *Mi* $B_1$, *Mi* $C_1$, *Mi* $D_1$, and *Mi* $E_1$) were found for *M. incognita* identified using primers C2F3/1108 (Figure 9, Table 2). Number of nucleotide substitutions found in haplotypes *Mi*$B_1$, *Mi*$C_1$, *Mi*$D_1$ and *Mi*$E_1$ when compared with haplotype *Mi*$A_1$ were 2, 1, 2 and 3 respectively. Ten haplotypes were found for *M. incognita* identified using primers COF475/COR999 (Figure 10). A total of 32 *M. incognita* sequences grouped to haplotype *Mi* A; 2 of each grouped to haplotype *Mi* B, *Mi* H and *Mi* J; 3 were grouped to haplotype *Mi* G; and 1 of each formed haplotype *Mi* C, *Mi* D and *Mi* F (Table 2). *Meloidogyne haplanaria* sequences were grouped into eight different haplotypes (Figure 11). Five *M. haplanaria* sequences grouped to haplotype *Mhr* A, 2 grouped to haplotype *Mhr* E and one of each formed haplotype *Mhr* B, *Mhr* C, *Mhr* D, *Mhr* F, *Mhr* G and *Mhr* H (Table 2). *Meloidogyne marylandi* sequences were grouped into 3 different haplotypes (Figure 12). Four *M. marylandi* sequences formed haplotype

*Mm* A while one of each *M. marylandi sequences* formed haplotype *Mm* B and *Mm* C (Table 2). *Meloidogyne hapla* sequences grouped into 4 different haplotypes (Figure 14). Two *M. hapla* sequences formed haplotype *Mha* A and one of each *M. hapla* sequences formed haplotypes *Mha* A, *Mha* C and *Mha* D (Table 2).

*Meloidogyne incognita* specific primers (COF475, COF779 and COR999) produced two distinct bands of approximately 550 and 250bp for *M. incognita* obtained during this study (figure 6). These primers did not produce two distinct bands for other root-knot species collected during this study.

Individual egg mass inoculation to suitable hosts was done to obtain pure culture of identified specimen. *Meloidogyne incognita, M. arenaria, M. marylandi* and *M. partityla* were inoculated on cotton, peanut, bermudagrass and pecan respectively. *Meloidogyne haplanaria* and *M. hapla* were inoculated on tomato. Cultures of *M. incognita, M. marylandi, M. haplanaria, M. hapla*, *M. partityla* and *M. arenaria* have been obtained and maintained in the greenhouse to facilitate future research.

**6 Discussion**

The primer set C2F3/1108 distinguished *M. incognita* and *M. arenaria* by producing amplicon sizes of 1.5 and 1.1 kb, respectively (Figure 4). *Meloidogyne haplanaria, M. marylandi*, *M. hapla* and *M. partityla* produced amplicon sizes of 0.5 kb (Figure 4). Although primer set C2F3/1108 was reported to be good enough 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. This made it difficult to analyze the sequencing results as they were obtained with some non-reading sequences. Thus, it was necessary to design a separate primer set for *M. incognita*. New designed primer set

COF475/COR999 produced approximately 550 bp amplicon for *M. incognita* of Arkansas (Figure 5). Although primer BLAST shows primers COF475/COR99 could amplify *M. incognita*, *M. arenaria* and *M. javanica*, the primers did not amplify any species other than *M. incognita* obtained during this study. Furthermore, all the species (*M. incognita*) identified using COF475/COR999 were reconfirmed by running PCR using C2F3/1108 and looking at the DNA band in 2% agarose gel. Positive control was always used to confirm *M. incognita* bands.

Low concentration of template DNA extracted from individual nematode was always a problem. Low PCR yield was probably due to less amount of DNA template available. Therefore other DNA extraction methods that yield enough template for PCR should be used for future nematode identifications.

*Meloidogyne incognita* is a widespread root-knot nematode in Arkansas (Figure 7) and was the only root-knot species identified from 25 soybean samples collected across the state. Cotton is a good host for *M. incognita* and according to United States Department of Agriculture (USDA), in 1930 cotton was a major crop that was planted in 3,446,485 acres. The present cotton acreage is about 300,000 while soybean is estimated at 3, 500, 000 acreage from a high of 5,000,000 acreage in the late 1970's (Robert Robbins, personal communication). Much of the cotton acreage was gradually replaced by soybean in Arkansas, which is also a good host for *M. incognita* thus contributing the high frequency of this nematode species on soybean in Arkansas (Robert Robbins, personal communication).

*Meloidogyne partityla* was first reported in the United States from pecan of Texas (Starr et al., 1996). *Meloidogyne partityla* was also reported from New Mexico, Georgia, Florida, Arizona and Oklahoma (Brito et al., 2006). As a result of this study, *M. partityla* was identified in Arkansas, which is the first report of this species in the state, specifically in Locan County

near the Arkansas river. Pecan trees infected with this nematode species had a few small galls. These pecan trees expressed upper branches that appeared to be dying or having small leaves, which indicates stress, probably from root-knot nematodes. Now that *M. partityla* has been identified further studies are needed to determine its range of distribution on pecan orchards in the state, especially in orchards that may be replanted in pecan due to poor pecan production or where this species was detected in this study.

*Meloidogyne marylandi* was detected in bermudagrass samples of Arkansas. Up to 15 root-knot J2 per 250 cubic centimeters of soil were present in samples from a sod farm in Hempstead County and a golf course in Drew County. A golf course in Drew County was in rough shape showing a big light yellowish spot (Terry Spurlock, personal communication). Since number of *M. marylandi* juveniles detected was low, the symptom was possibly a disease complex caused by root-knot nematode and other pathogens. Practices directed towards managing *M. marylandi* in golf coursees and sod farms would help prevent their dispersal in Arkansas. This nematode species was detected in bermudagrass samples processed whereas *M. marylandi* was not detected in this study. Given only five samples were processed it is unlikely *M. marylandi* is the most dominant species; however, further samples of golf courses and sod farms are needed to determine the most prominent grass root-knot species in Arkansas.

*Meloidogyne arenaria* was detected in soil sample from Hempstead County and on a tomato root (♀) from Sebastian County. Only one *M. arenaria* specimen was obtained from each sample suggesting that a good host was not present, sampling missed the main population, the soil was not favorable for reproduction, that it was present in very low numbers etc.

More variation was observed for *M. haplanaria* than any other root-knot species of Arkansas. *Meloidogyne haplanaria* from Arkansas show 96-99% identity with the *M. haplanaria*

GenBank accession numbers AY757905 and AY757906. *Meloidogyne marylandi* of Arkansas were highly similar (98-99% identity) to sequences submitted by McClure et al. (2012) from Arizona (GenBank accession numbers JN241917 and JN241918) indicating they were probably introduced from a common source. *Meloidogyne hapla* was found in samples from Washington, Logan and Craighead counties suggesting distribution of this species in northern regions of Arkansas (Figure 7). *Meloidogyne partityla* of Arkansas shows 95-99% sequences identity with *M. partityla* GenBank accession numbers AY672412 and AY672413. Intraspecific variation was not detected in root-knot species from the same isolates. Since the variation was observed in species from different locations and hosts, it would be reasonable to mention the variation was due to host range and geography.

Major processes controlling haplotype diversity of an organism are mutation, recombination, marker ascertainment and demography (Stumpf, 2004). Five haplotypes (*Mi* $A_1$, *Mi* $B_1$, *Mi* $C_1$, *Mi* $D_1$, and *Mi* $E_1$) were found for *M. incognita* identified using primers C2F3/1108 (Figure 9, Table 2). Haplotypes *Mi* $B_1$, *Mi* $C_1$ and *Mi* $D_1$ and *Mi* $E_1$ had 2, 1, 2 and 3 nucleotide substitutions respectively when compared with *Mi* $A_1$ (Figure 9). Haplotypes *Mi* $B_1$, *Mi* $C_1$ and *Mi* $D_1$ and *Mi* $E_1$ were from soybean of Pulaski, Pope, Ashley and Woodruf counties respectively (Table 2). Ten haplotypes were found for *M. incognita* identified using primers COF475 and COR999 (Figure 10). *Meloidogyne incognita* from 17 soybean, 1cotton and 6 tomato samples were *Mi* A haplotypes indicating the haplotypes were possibly distributed from a common source, possibly old cotton fields. Haplotypes *Mi* C, *Mi* H and *Mi* I were also from soybean samples. A single nucleotide deletion was found in haplotypes *Mi* B, *Mi* G and *Mi* I while a single nucleotide insertion was found in haplotype *Mi* C and *Mi* H (Figure 10) showing close affinity to haplotype *Mi* A.

*Meloidogyne haplanaria* sequences were grouped into eight different haplotypes (Figure 11). Of the five *Mhr* A haplotypes, four *M. haplanaria* were from Washington County (hosts: *Quercus sp*., *Prunus sevotina*, *Fraxinus* sp. and *Agoseris heterophylla*) and one was from Faulkner County (Host: *Crataegus* sp.). This difference in haplotypes based on location and host suggests location and host have an influence on genetic diversity. Haplotypes *Mhr* B, *Mhr* C, *Mhr* D, *Mhr* E, *Mhr* F, *Mhr* G and *Mhr* H had 2, 6, 16, 15, 9, 12 and 11 substitutions respectively (Figure 11). Haplotypes *Mhr* C had 4 nucleotides deletion while haplotypes *Mhr* F, *Mhr* G and *Mhr* H had single nucleotide deletion (Figure 11).

*Meloidogyne marylandi* were grouped into 3 different haplotypes (Figure 12). Haplotypes *Mm* B and *Mm* C had 2 and 1 nucleotide substitutions respectively when compared with *Mm* A (Figure 12). *Mm* A haplotypes were found in *Cynodon dactylon* from Perry, Craighead and Hempstead counties indicating the haplotype is possibly host specific.

Two different haplotypes were found for *M. arenaria* (Figure 13). Haplotype *Ma* B had 11 nucleotide substitutions when compared with *Ma* A (Figure 13). Haplotype *Ma* A was from Hempstead County and *Ma* B was from Sebastian County indicating the haplotypes are possibly geographically influenced.

*Meloidogyne hapla* sequences grouped into 4 different haplotypes (Figure 14). Haplotypes *Ma* B, *Ma* C and *Ma* D had 1, 10 and 12 nucleotide substitutions respectively when compared with *Mha* A (Figure 14). Additionally, single nucleotide deletion was found in haplotype *Mha* D (Figure 14). Haplotype *Mha* A was found in *Phacelia popei* and *Ulmus* spp. from Washington County indicating haplotypes are probably geographically influenced.

As *M. incognita* was a most common species in Arkansas, development of a species specific identification technique was needed for routine identifications. Species specific PCR is

useful in quickly identifying a specimen without sequencing. Species specific PCR was performed using two forward primers COF475 and COF779, and one reverse primer COR999. This produced two distinct bands of approximately 550 and 250 bp (Figure 6). These primers did not produce those two distinct bands when used for amplifying *M. marylandi, M. haplanaria, M. hapla, M. arenaria* or *M. partityla* showing their specificity to *M. incognita* of Arkansas. However, using the primers with *M. arenaria, M. javanica* and other species from other states would be useful to determine specificity of the primers.

This research identifies *M. haplanria, M. marylandi* and *M. partityla* new species to Arkansas. Finding *M. incognita* in the majority of the sampled counties indicates that this root-knot nematode species is the most commonly occurring species in Arkansas. *Meloidogyne incognita* is widespread across the state and a major issue in nearly all Arkansas counties (Terry Kirkpatrick, personal communication). Although *M. 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), but it is likely with more samples these species would be detected. It is possible for disappreance of an existing species or introduction of new species. As an example soybean cysts (*Heterodora glycines*) were prevalent in Louisiana 30 years ago, but soybean cysts are seldom detected currently (Charles Overstreet, personal communication). Also, misidentification of a species is possible when identification methods are based on host differentials. An extensive survey should be made to attempt corroboration or rebuttal of the presence of *M. javanica* and *M. graminis* in Arkansas. Presence of pecan root-knot nematode (*M. partityla*) poses a threat to the pecan industry of Arkansas. Similarly, detection of *M. marylandi* in golf course and sod farm of Arkansas requires approaches directed towards management and prevention of dispersal. Additionally, cultures of *M. incognita, M. haplanaria, M. marylandi, M. partityla, M. hapla and*

*M. arenaria* were maintained in greenhouse to facilitate future research. This survey provides an indication of the diversity of root-knot nematode species present in Arkansas.

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Table 1. Sample number, County, host/soil, collection date and location of nematode samples collected in Arkansas. Only samples that contained root-knot nematodes are listed in the table. Samples are listed according to County for species distribution.

| **SN** | **County** | **Host/soil** | **Date** | **Location** |
|---|---|---|---|---|
| 1 | Lafayette | *Zea mays* | 4/22/2013 | Gin City, AR |
| 2 | Washington | *Salix* spp. | 7/12/2013 | 36°06.18'N 94°09.743'W |
| 3 | Ashley | *Glycine max* | 8/5/2013 | Portland, AR |
| 4 | Pulaski | *Glycine max* | 8/12/2013 | Scott, AR |
| 5 | Pope | *Glycine max* | 8/17/2013 | 35°09'27.9"N 93°06'12.6"W |
| 6 | Logan | *Carya illinoinensis*/soil | 8/25/2013 | 35°20'58"N 93°40'52"W |
| 7 | Philips | *Cucurbita* sp. | 8/28/2013 | unknown |
| 8 | Woodruf | *Glycine max* | 8/29/2013 | McCrory, AR |
| 9 | Crawford | *Cucurbita* sp. | 9/5/2013 | Mountainburg, AR |
| 10 | Crawford | *Solanum lycopersicum* | 9/8/2013 | Mountainburg, AR |
| 11 | Hempstead | *Cynodon dactylon* | 9/9/2013 | 33°32'20"N 93°46'10"W |
| 12 | Lawrence | *Glycine max* | 9/17/2013 | Hoxie, AR |
| 13 | Pulaski | *Begonia* sp. | 9/26/2013 | Little Rock, AR |
| 14 | Jefferson | *Solanum lycopersicum* (Rutgers) | 10/7/2013 | White Hall, AR |
| 15 | Baxter | *Solanum lycopersicum* | 10/14/2013 | unknown |
| 16 | Pope | *Solanum lycopersicum* | 10/17/2013 | Russellville, AR |
| 17 | Montgomery | *Ocimum tenuiflorum* | 11/6/2013 | Mt. Ida, AR |

| SN | County | Host/soil | Date | Location |
|---|---|---|---|---|
| 18 | Garland | *Abelmoschus esculentus* | 11/6/2013 | Hot springs, AR |
| 19 | Drew | *Glycine max* | 11/6/2013 | Dermott, AR |
| 20 | Jefferson | *Glycine max* | 11/6/2013 | Altheimer, AR |
| 21 | Lonoke | *Glycine max* | 3/20/2014 | 34°43'59.9"N 92°00'00.0"W |
| 22 | Logan | *Glycine max* | 3/20/2014 | 35°20'56.4"N 93°40'51.6"W |
| 23 | Desha | *Gossypium hirsutum* | 3/20/2014 | Tiller, AR |
| 24 | Lonoke | *Glycine max* (Edamame) | 3/20/2014 | 34°44'54.6"N 92°00'36.0"W |
| 25 | Lonoke | *Glycine max* (Edamame) | 3/20/2014 | 34°44'44.9"N 92°00'36.0"W |
| 26 | Randolph | *Glycine max* | 3/20/2014 | unknown |
| 27 | Lonoke | *Glycine max* | 3/20/2014 | Scott, AR |
| 28 | Yell | *Glycine max* (Edamame) | 3/20/2014 | 35°09'25.2"N 93°06'10.8"W |
| 29 | Lonoke | *Glycine max* | 3/20/2014 | Scott, AR |
| 30 | Lonoke | *Glycine max* (Edamame) | 3/20/2014 | 34°43'58.8"N 92°00'00.0"W |
| 31 | Johnson | *Glycine max* (Edamame) | 3/20/2014 | 35°24'34.6"N 93°39'00.0"W |
| 32 | Lonoke | *Glycine max* (Edamame) | 3/20/2014 | 34°44'44.9"N 92°00'36.0"W |
| 33 | Faulkner | *Pisum sativum* | 3/20/2014 | Guy, AR |
| 34 | Washington | *Daucus carota* | 4/4/2014 | Fayetteville, AR |
| 35 | Washington | *Coriandrum sativum* | 4/4/2014 | Fayetteville, AR |
| 36 | Faulkner | *Crataegus* sp. | 3/26/2014 | Conway, AR |
| 37 | Craighead | *Rosa* sp. | 3/31/2014 | Jonesboro, AR |
| 38 | Faulkner | garden soil | 5/2/2014 | Guy, AR |
| 39 | Washington | *Cynodon dactylon* | 5/2/2014 | 36°6'11"N 94°20'35"W |

| SN | County | Host/soil | Date | Location |
|---|---|---|---|---|
| 40 | Washington | *Betula* sp. | 5/2/2014 | 36°6'11"N 94°20'35"W |
| 41 | Washington | *Agoseris heterophylla* | 5/2/2014 | 36°6'11"N 94°20'35"W |
| 42 | Washington | *Arundinaria* sp. | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 43 | Washington | *Fraxinus* sp. | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 44 | Washington | *Phacelia popei* | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 45 | Washington | *Quercus* sp. | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 46 | Washington | *Agoseris heterophylla* | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 47 | Washington | *Prunus sevonita* | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 48 | Washington | *Quercus* sp. | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 49 | Washington | *Agoseris heterophylla* | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 50 | Washington | *Ulmus* sp. | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 51 | Washington | *Ulmus* sp. | 5/5/2014 | 36°6'20"N 94°0'42"W |
| 52 | Bradley | *Solanum lycopersicum* (Mt. Glory) | 5/6/2014 | unknown |
| 53 | Craighead | *Cynodon dactylon* | 5/21/2014 | Jonesboro, AR |
| 54 | Columbia | *Solanum lycopersicum* | 6/11/2014 | Magnolia, AR |
| 55 | Perry | *Cynodon dactylon* | 7/1/2014 | Toad Suck Park |
| 56 | Conway | *Glycine max* | 7/2/2014 | Plumerville, AR |
| 57 | Hempstead | *Phaseolus vulgaris* | 7/9/2014 | Old Washington State Park, AR |
| 58 | Lincoln | *Glycine max*(Armor4744) | 7/9/2014 | Star City, AR |
| 59 | Craighead | *Cynodon dactylon* | 7/15/2014 | Caraway, AR |
| 60 | Hempstead | garden soil | 7/17/2014 | Old Washington State Park, AR |

| SN | County | Host/soil | Date | Location |
|---|---|---|---|---|
| 61 | Cleburne | *Cucurbita* sp. | 7/23/2014 | Quitman, AR |
| 62 | Desha | *Glycine max* | 8/4/2014 | Rison, AR |
| 63 | Jackson | *Glycine max* | 8/15/2014 | Newport, AR |
| 64 | Pope | *Glycine max* | 8/18/2014 | 35°11'36.4"N 93°05'33.3"W |
| 65 | Pulaski | *Abelmoschus esculentus* | 8/29/2014 | Little Rock, AR |
| 66 | Sebastian | *Solanum lycopersicum* | 9/4/2014 | Fort Smith, AR |
| 67 | Faulkner | *Solanum lycopersicum* | 9/4/2014 | Conway, AR |
| 68 | Baxter | *Solanum lycopersicum* (Amish Paste) | 9/4/2014 | Cotter, AR |
| 69 | Drew | *Cynodon dactylon* (Mini verde) | 9/4/2014 | 33°39'21.99" N 91°48'46.17" W |
| 70 | Pulaski | *Cucumis* sp. | 9/4/2014 | Little Rock, AR |
| 71 | Van Buren | *Abelmoschus esculentus* (Glemson spineless) | 9/11/2014 | Bee Branch, AR |
| 72 | Pulaski | *Solanum lycopersicum* | 9/11/2014 | Little Rock, AR |
| 73 | Pulaski | *Cucumis* sp. | 9/11/2014 | Little Rock, AR |
| 74 | Pulaski | *Solanum lycopersicum* | 9/11/2014 | Little Rock, AR |
| 75 | Pulaski | *Cucumis* sp. | 9/11/2014 | Little Rock, AR |
| 76 | Sebastian | *Solanum lycopersicum* | 9/18/2014 | Greenwood, AR |
| 77 | Pulaski | *Solanum lycopersicum* (Bradley) | 9/18/14 | Little Rock, AR |
| 78 | Lawrence | *Glycine max* (Croplan 4752) | 9/24/2014 | 36°04'02.8"N 91°05'56.5"W |

| SN | County | Host/soil | Date | Location |
|---|---|---|---|---|
| 79 | Lawrence | *Glycine max* | 10/1/14 | 36°06'02.4"N 90°59'34.0"W |

Table 2. Sample number, County, host/soil, *Meloidogyne* spp., number of sequenced individuals and haplotypes collected in Arkansas. 'X' and 'Y' in parenthesis after species indicate primer set C2F3/1108 and COF475/COR999 respectively was used to identify the species.

| **SN** | **County** | **Host/soil** | ***Meloidogyne. spp.*** | **Sequenced** | **Haplotype** |
|---|---|---|---|---|---|
| 1 | Lafayette | *Zea mays* | *M. incognita* (X) | 1 J2 | *Mi* $A_1$ |
| 2 | Washington | *Salix* spp. | *M. haplanaria* (X) | 4 J2 | *Mhr* D |
| 3 | Ashley | *Glycine max* | *M. incognita* (X) | 1 ♀ | *Mi* $D_1$ |
| 4 | Pulaski | *Glycine max* | *M. incognita* (X) | 1 ♀ | *Mi* $B_1$ |
| 5 | Pope | *Glycine max* | *M. incognita* (X) | 1 ♀ | *Mi* $C_1$ |
| 6 | Logan | *Carya illinoinensis* | *M. partityla* (X) | 3 ♀ | *Mpa* A |
| 6 | Logan | pecan grove soil[a] | *M. hapla* (X) | 1 ♀ | *Mha* D |
| 6 | Logan | pecan grove soil[b] | *M. marylandi* (X) | 2 ♀ | *Mm* B |
| 7 | Philips | *Cucurbita* sp. | *M. incognita* (Y) | 2 ♀ | *Mi* F |
| 8 | Woodruff | *Glycine max* | *M. incognita* (X) | 1 ♀ | *Mi* $E_1$ |
| 9 | Crawford | *Cucurbita* sp. | *M. incognita* (Y) | 3 ♀ | *Mi* B |
| 10 | Crawford | *Solanum lycopersicum* | *M. incognita* (Y) | 3 ♀ | *Mi* D |
| 11 | Hempstead | *Cynodon dactylon* | *M. marylandi* (X) | 4 J2 | *Mm* A |
| 12 | Lawrence | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* I |
| 13 | Pulaski | *Begonia* sp. | *M. incognita* (Y) | 2 ♀ | *Mi* G |
| 14 | Jefferson | *Solanum lycopersicum* (Rutgers) | *M. incognita* (Y) | 2 ♀ | *Mi* H |
| 15 | Baxter | *Solanum lycopersicum* | *M. haplanaria* (X) | 2 ♀ | *Mhr* C |

| SN | County | Host/soil | *Meloidogyne. spp.* | Sequenced | Haplotype |
|---|---|---|---|---|---|
| 16 | Pope | *Solanum lycopersicum* | *M. incognita* (Y) | 3 ♀ | *Mi* E |
| 17 | Montgomery | *Ocimum tenuiflorum* | *M. incognita* (Y) | 2 ♀ | *Mi* J |
| 18 | Garland | *Abelmoschus esculentus* | *M. incognita* (Y) | 2 ♀ | *Mi* J |
| 19 | Drew | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* C |
| 20 | Jefferson | *Glycine max* | *M. incognita* (Y) | 2 ♀ | *Mi* H |
| 21 | Lonoke | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 22 | Logan | *Glycine max* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 23 | Desha | *Gossypium hirsutum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 24 | Lonoke | *Glycine max* (Edamame) | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 25 | Lonoke | *Glycine max* (Edamame) | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 26 | Randolph | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 27 | Lonoke | *Glycine max* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 28 | Yell | *Glycine max* (Edamame) | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 29 | Lonoke | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 30 | Lonoke | *Glycine max* (Edamame) | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 31 | Johnson | *Glycine max* (Edamame) | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 32 | Lonoke | *Glycine max* (Edamame) | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 33 | Faulkner | *Pisum sativum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 34 | Washington | *Daucus carota* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 35 | Washington | *Coriandrum sativum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 36 | Faulkner | *Crataegus* sp. | *M. haplanaria* (X) | 3 J2 | *Mhr* A |
| 37 | Craighead | *Rosa* sp. | *M. hapla* (X) | 3 J2 | *Mha* C |

| SN | County | Host/soil | *Meloidogyne. spp.* | Sequenced | Haplotype |
|---|---|---|---|---|---|
| 38 | Faulkner | garden soil | *M. incognita* (Y) | 2 ♀ | *Mi* G |
| 39 | Washington | *Cynodon dactylon* | *M. haplanaria* (X) | 2 J2 | *Mhr* G |
| 40 | Washington | *Betula* sp. | *M. haplanaria*(X) | 3 J2 | *Mhr* H |
| 41 | Washington | *Agoseris heterophylla* | *M. haplanaria* (X) | 2 J2 | *Mhr* F |
| 42 | Washington | *Arundinaria* sp. | *M. haplanaria* (X) | 3 J2 | *Mhr* E |
| 43 | Washington | *Fraxinus* sp. | *M. haplanaria* (X) | 3 J2 | *Mhr* A |
| 44 | Washington | *Phacelia popei* | *M. hapla* (X) | 2 J2 | *Mha* B |
| 45 | Washington | *Quercus* sp. | *M. haplanaria* (X) | 2 J2 | *Mhr* A |
| 46 | Washington | *Agoseris heterophylla* | *M. haplanaria* (X) | 4 J2 | *Mhr* B |
| 47 | Washington | *Prunus sevonita* | *M. haplanaria* (X) | 2 J2 | *Mhr* A |
| 48 | Washington | *Quercus* sp. | *M. hapla* (X) | 2 J2 | *Mha* A |
| 49 | Washington | *Agoseris heterophylla* | *M. haplanaria* (X) | 4 J2 | *Mhr* A |
| 50 | Washington | *Ulmus* sp. | *M. haplanaria* (X) | 2 J2 | *Mhr* E |
| 51 | Washington | *Ulmus* sp. | *M. hapla* (X) | 2 J2 | *Mha* B |
| 52 | Bradley | *Solanum lycopersicum* (Mt. Glory) | *M. incognita* (Y) | 3 ♀ | *Mi* I |
| 53 | Craighead | *Cynodon dactylon* | *M. marylandi* (X) | 3 J2 | *Mm* A |
| 54 | Columbia | *Solanum lycopersicum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 55 | Perry | *Cynodon dactylon* | *M. marylandi* (X) | 3 J2 | *Mm* A |
| 56 | Conway | *Glycine max* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 57 | Hempstead | *Phaseolus vulgaris* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 58 | Lincoln | *Glycine max*(Armor4744) | *M. incognita* (Y) | 2 ♀ | *Mi* A |

| SN | County | Host/soil | *Meloidogyne. spp.* | Sequenced | Haplotype |
|---|---|---|---|---|---|
| 59 | Craighead | *Cynodon dactylon* | *M. marylandi* (X) | 3 J2 | *Mm* A |
| 60 | Hempstead | garden soil | *M. arenaria* (X) | 1 J2 | *Ma* A |
| 61 | Cleburne | *Cucurbita* sp. | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 62 | Desha | *Glycine max* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 63 | Jackson | *Glycine max* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 64 | Pope | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 65 | Pulaski | *Abelmoschus esculentus* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 66 | Sebastian | *Solanum lycopersicum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 67 | Faulkner | *Solanum lycopersicum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 68 | Baxter | *Solanum lycopersicum* (Amish Paste) | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 69 | Drew | *Cynodon dactylon* (Mini verde) | *M. marylandi* (X) | 3 J2 | *Mm* C |
| 70 | Pulaski | *Cucumis* sp. | *M. incognita* (Y) | 2 ♀ | *Mi* G |
| 71 | Van Buren | *Abelmoschus esculentus* (Glemson spineless) | *M. incognita* (Y) | 3 ♀ | *Mi* A |
| 72 | Pulaski | *Solanum lycopersicum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 73 | Pulaski | *Cucumis* sp. | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 74 | Pulaski | *Solanum lycopersicum* | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 75 | Pulaski | *Cucumis* sp. | *M. incognita* (Y) | 2 ♀ | *Mi* E |
| 76 | Sebastian | *Solanum lycopersicum* | *M. arenaria* (X) | 1 ♀ | *Ma* B |
| 77 | Pulaski | *Solanum lycopersicum* (Bradley) | *M. incognita* (Y) | 3 ♀ | *Mi* I |

| SN | County | Host/soil | *Meloidogyne. spp.* | Sequenced | Haplotype |
|---|---|---|---|---|---|
| 78 | Lawrence | *Glycine max* (Croplan 4752) | *M. incognita* (Y) | 2 ♀ | *Mi* A |
| 79 | Lawrence | *Glycine max* | *M. incognita* (Y) | 3 ♀ | *Mi* B |

♀ = female, sequenced indicates number of individual nematodes sequenced. *Mi= Meloidogyne incognita, Mhr = Meloidogyne haplanaria, Mha = Meloidogyne hapla, Mm = Meloidogyne marylandi, Ma = meloidogyne arenaria, Mp = Meloidogyne partityla.* Subscript (e.g. *Mi* $A_1$) after haplotype name indicates the species was identified using primers C2F3/1108. Pecan grove soil[a] and pecan grove soil[b] indicate soil from pecan grove was inoculated on tomato and bermudagrass respectively in greenhouse.

Table 3: List of PCR primers used to amplify regions of the mtDNA genome of *Meloidogyne* spp. sampled during this study.

| Primer | Nucleotides (5'-3') | Melting temp. (Tm) | References |
|---|---|---|---|
| C2F3 | GGTCAATGTTCAGAAATTTGTGG | 59.2°C | Powers and Harris (1993) |
| 1108 | TACCTTTGACCAATCACGCT | 58.4°C | Powers and Harris (1993) |
| COF475 | CTTTATTAGATCGGGGTTTAAT | 55.2°C | Current study |
| COF779 | TAATAGATTTAGTTCATCTG | 50.2°C | Current study |
| COR999 | TGATTTAATTCATTATGATA | 46°C | Current study |

Figure 1. Typical galls induced by root-knot nematodes on soybean roots

Figure 2. A root-knot second stage juvenile (J2)

Figure 3. A root-knot female on pecan root

Figure 4. Visualization of PCR products from different *Meloidogyne* spp. with primer set C2F3/1108

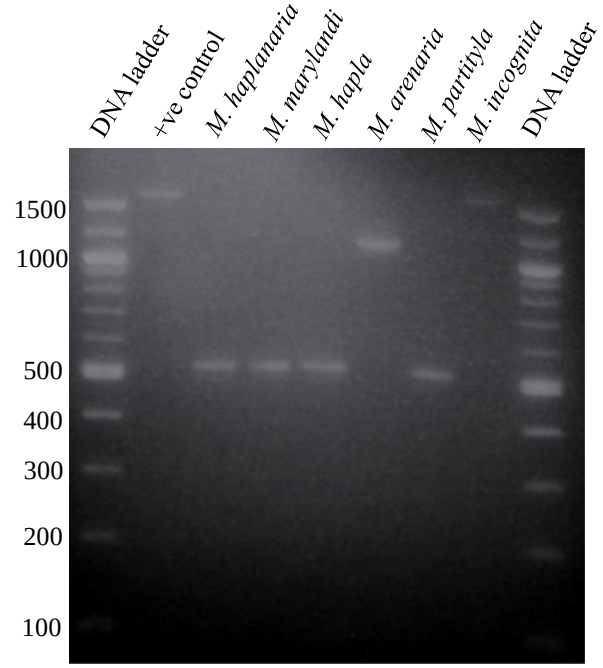


Figure 5. Visualization of PCR products from different *Meloidogyne* spp. with primer set COF475/COR999

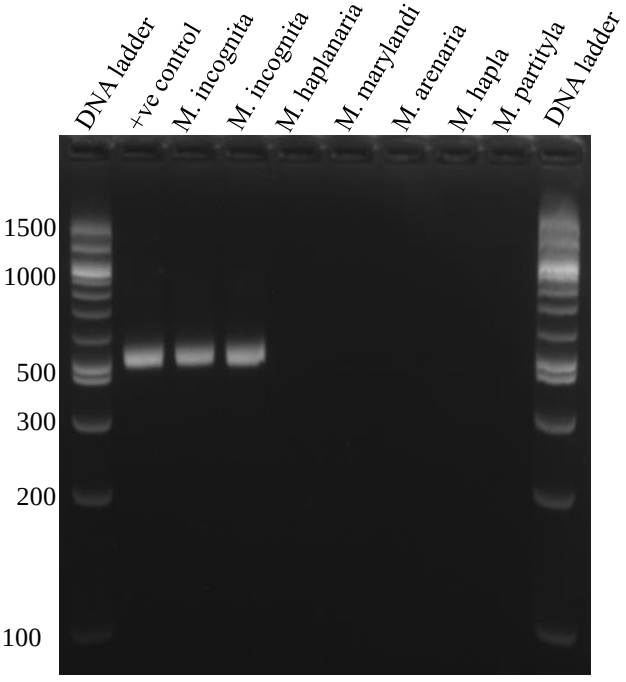

Figure 6. Visualization of PCR products from different *Meloidogyne* spp. with primers COF475, COF779 and COR999

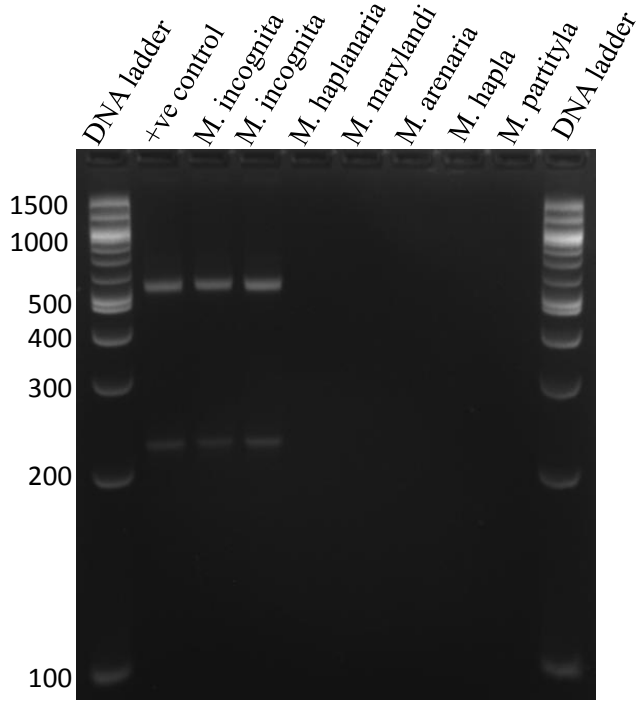

Figure 7. Map showing collection sites of *Meloidogyne* spp. in Arkansas. A single dot may represent multiple samples from a County.

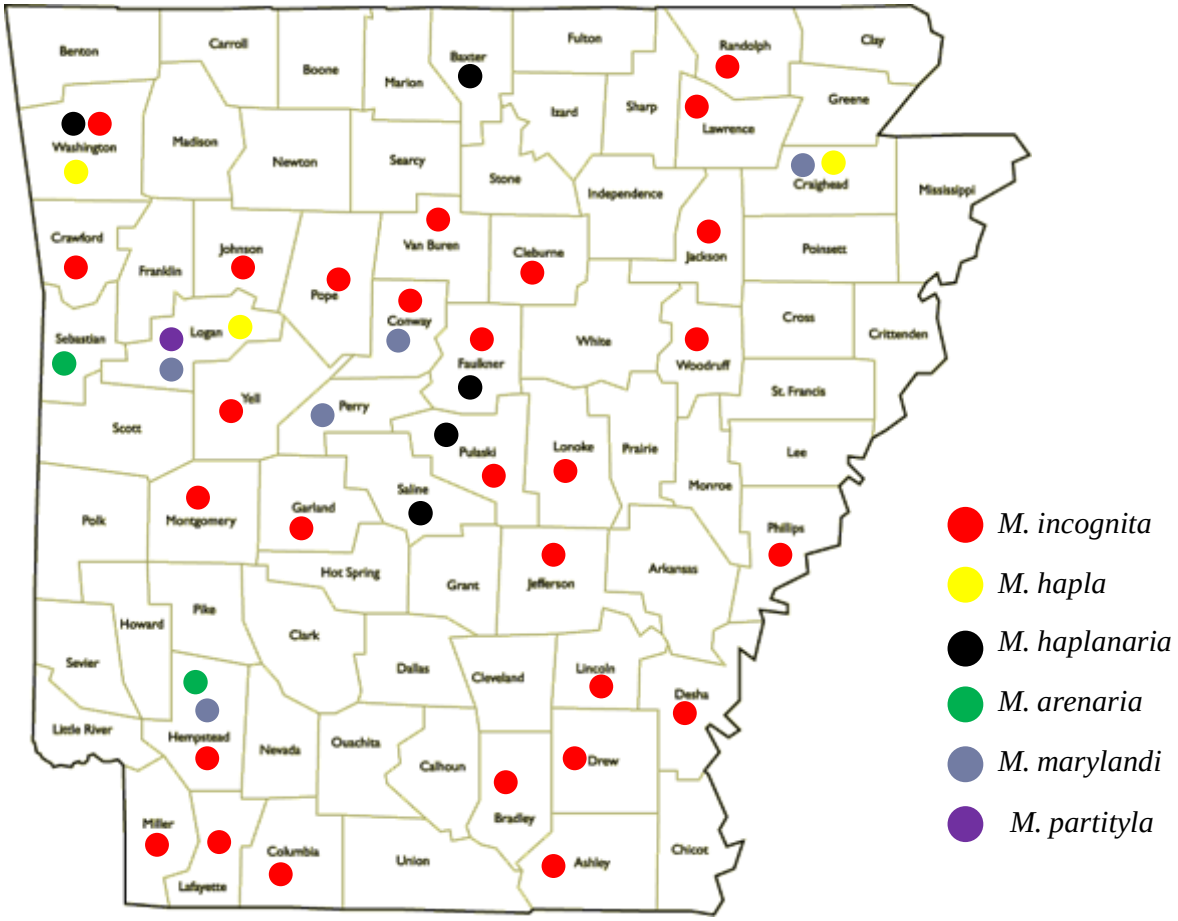

Figure 8: Bayesian tree inferred from mtDNA (region between COII and 16s rRNA) sequences of *Meloidogyne* spp. of Arkansas. Posterior probability values are provided at each node. Numerical values after species represent sample number from which the species was obtained.

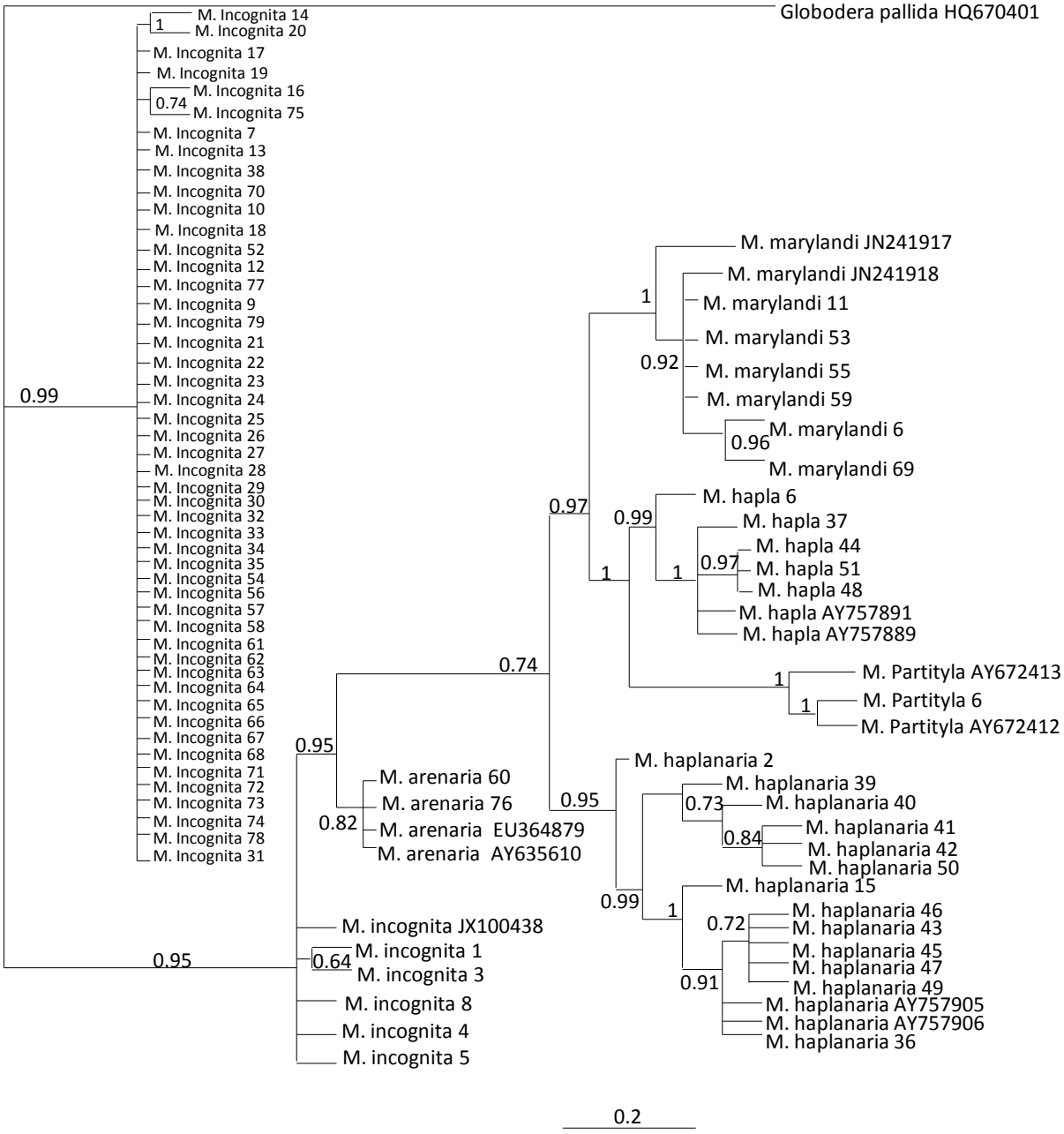

Figure 9. Alignment of *M. incognita* sequences (with primers C2F3/1108) showing haplotypes collected from Arkansas, USA. Sample number indicates nematode was obtained from that particular sample. Comparision was made with Haplotype A (Sample No. 1): sequence identity is indicated by '*' and deletions by '-'. Full or part of the sequences not shown are identical to haplotype A.

```
Sample/Hap. 60                                                        120
1-A1        ACTTTATTTGATTTTTTTAAGTTAAATTTATTAACTAATTGATTATTTTATTTTTGTTGA
4-B1        ****************************T*******************************
8-E1        *******A*************************************************

            1081                                                      1140
1-A1        TTTGGAATTTACCAAGGTAGAATTATACGTTAAATTTAGAAGAATTGTTGAAAAGAATGA
3-D1        ************************C************************************
8-E1        ************************C************************************

            1141                                                      1200
1-A1        ATTCTTAATGGAAACAGTAAGGATATTTTTATATAATTATTTTTAATAATATTAAAAATA
5-C1        *******************************A****************************
3-D1        ************************************************T***********
8-E1        ************************************************T***********

            1500                                                      1560
1-A1        TAATAATTAAATATAAAATTTTTTATTAAATAAATTTAATAATAAATGTTTTTTAAATTC
4-B1        ********************A***************************************
```

Figure 10. Alignment of *M. incognita* sequences (with primers COF475/COR999) showing haplotypes collected from Arkansas, USA. Sample number indicates nematode was obtained from that particular sample. Comparision was made with haplotype A (sample no. 21): sequence identity is indicated by '*' and deletions by '-'. Full or part of the sequences not shown are identical haplotype A.

```
Sample/Hap. 1                                                          60
21-A        CTTTATTAGATCGGGGTTTAATAATGGGTTCTTTATTGTGTTAAT-TATTAAAATATTAT
10-D        **************************************************A***************
17-I        **************************************************A******T********
18-I        **************************************************A******T********

            61                                                        120
21-A        TTTGGTTTTTTAGTGAAATTTTTTATTTAATTTATTTTTATACGAAAA-ATTATTAGGGA
7-F         **************************************************T**************
14-H        **************************************************T**************
20-H        **************************************************T**************
12-I        **-************************************************************
52-I        **-************************************************************
77-I        **-************************************************************

            181                                                       240
21-A        TTTTTAGTATTCTATTGAAATAGAAGAATTATAAATTAGTTAATTCTAATTTTATTTATT
9-B         -************************************************************
19-C        C************************************************************
79-B        -************************************************************

            241
21-A        TTATATGGATTATATAAAATTTTAAATTTTTTGATTTTAGTTTATTTACGTATTTAGTTG
19-C        **************************************************A**********

            361                                                       420
21-A        ACCTGAAGTTAAAAATGGTCAATTATATGATATAACTTTTGGTTATCATCAAGAATATTT
16-E        **************************A*********************************
75-E        **************************A*********************************

            421                                                       480
21-A        TAAGGAAATTATGGTGCTCATCCTGATAAAGATCGTAATTTTTATAGTTGTGATATTGTT
13-G        ************************************************************_**********
38-G        ************************************************************_**********
70-G        ************************************************************_**********

            481                                                 525
21-A        ATACGTCAATCACAAGCTTTAGAAGAATATCATAATGAATTAAATCA
14-H        **********************T*************************
20-H        **********************T*************************
```

Figure 11. Alignment of *M. haplanaria* sequences showing haplotypes collected from Arkansas, USA. Sample number indicates nematode was obtained from that particular sample. Comparision was made with haplotype A (sample no. 47): sequence identity is indicated by '*' and deletions by '-'. Sequences not shown are identical to haplotype A.

```
Sample/Hap. 1                                                          60
47-A        GGTCAATGTTCAGAAATTTGTGGGGTTAATCATTCGTTTATACCAATTTTAGTGGAGGTT
15-C        ************************************************************
2-D         ************************************************************
42-E        ********************************C*********C**C**************GC
50-E        ********************************C*********C**C**************GC
41-F        ********************************C************C***************
39-G        ************************************************************AA
40-H        ************************************************************A

            61                                                         120
47-A        ACAT-TATTTGATTTTTTTAAATTAAATTTATTAACTAATTAATTAGCTATTAATTTCTA
15-C        ********************************G********----**************
2-D         ****G***************************************G*A**************
42-E        ********************************G***********************
50-E        ********************************G***********************

            121                                                        180
47-A        TTGAATTTTTTATTGTGATTAAAAGAGTTTTT-AGCTAATTTTATTTTAAGATTATTTTT
15-C        ************************A*******T*****************************
2-D         ************************A************************************
42-E        ************************A************************************
50-E        ************************A************************************
41-F        ************************A************************************
39-G        ************************A************************************
40-H        ************************A************************************

            181                                                        240
47-A        TATTGTTGAAAAAATTAAAAATAAATTGTTAAAAACAAAAATTAAAATTTATAATATTTC
46-B        ********************************T*****************************
2-D         ********************************TTT*****************************
42-E        ********************************T*****************************
50-E        ********************************T*****************************
41-F        ********************************T*****************************
39-G        ************************G*****T*****************************
40-H        ********************************T*****************************

            241                                                        300
47-A        TGTTTTTTATTTTT-AGTTAAAAATAAAAATAATAAATATGAAAAATAGAAAAATTTTGT
15-C        ************************************************************A***
41-F        **************T*********************************************
39-G        **************T*******************************T*************
40-H        **************T*********************************************
```

Figure 11 continued

Figure 12. Alignment of *M. marylandi* sequences showing haplotypes collected from Arkansas, USA. Sample number indicates nematode was obtained from that particular sample. Comparision was made with haplotype A (sample no. 11): sequence identity is indicated by '*' and deletions by '-'. Full or part of the sequences not shown are identical to haplotype A.

```
Sample/Hap. 181                                                    240
11-A        AATTAATAAATTGTTGAATAATTCCTGAATTAAAAATTATGCTAAAAAATATATTTTTTT
6-B         **********************************************T***************

            421                                                    480
11-A        TAAATTAAATTTTATAAAAAATGTTTATTAAAATCTTTTGTAATTTTTTTTTTAATTATT
69-C        **********************************************A***************
6-B         **********************************************A***************
```

Figure 13. Alignment of *M. arenaria* sequences showing haplotypes collected from Arkansas, USA. Sample number indicates nematode was obtained from that particular sample. Comparision was made with haplotype A (sample no. 60): sequence identity is indicated by '*' and deletions by '-'. Full or part of the sequences not shown are identical to haplotype A.

```
Sample/Hap. 1                                                          60
60-A        GGTCAATGTTCAGAAATTTGTGGTATTAATCATTCATTTATGCCATTTTTGGTTGAAATT
76-B        ************************************************A***************

            61                                                        120
60-A        ACTTTATTTGATTTTTTAAAGTTAAATTTATTAACTAATTGATTAATTTATTTTTGTTGA
76-B        ******************T*****************************T***************

            421                                                       480
60-A        TTAATATCATTTTTATTAATTATTAGGATATTTTCAATTGGTTGTGTTAATCATTCTTAA
76-B        ******************T***********************************************

            481                                                       540
60-A        TTAGAACGGGGTTTAATAATGGGTTCTTTATTGTGTTAATTATTAAAATATTATTTTGGT
76-B        *****T*****T*******************************************************

            661                                                       720
60-A        TATTATTAAAAATAAAGCTATTAATTTCAATTGAATTTTTTATTGTGATTAATAAAGTTT
76-B        ************************************************************A*******

            721                                                       780
60-A        TTGGCTAAATTATTTTTTTAGATTATTTTTTATTGTTGAAAAATTTAAAAACAAATTGTT
76-B        ********************************************A*****************

            841                                                       900
60-A        TACAAATATGAAAAATAAAAAAATTTTGTTATAAATTAAATTAATTTATTTATATTTAAA
76-B        ************************************************************A***

            1021                                                     1080
60-A        TGTTTTTTAAATTCTTTGAGGTTTTGAATTTTGAATTTTTGTTTCTGCTCATTGTTAAAG
76-B        ****************************T******T************************
```

Figure 14. Alignment of *M. hapla* sequences showing haplotypes collected from Arkansas, USA. Sample number indicates nematode was obtained from that particular sample. Comparision was made with haplotype A (sample no. 48): sequence identity is indicated by '*' and deletions by '-'. Full or part of the sequences not shown are identical to haplotype A.

```
Sample/Hap. 1                                                          60
48-A        GGTCAATGTTCAGAAATTTGTGGAGTTAATCATTCTTTTATACCTATTATAATTGAAGTA
37-C        ****************************CC*************C***G**************

            61                                                         120
48-A        GTTTTATTTGATTTTTTTAAGAATTATGTTTAAGCTATTAATTTCTAGTGAATTTTTTAT
6-D         ***********************T****A*********************************

            121                                                        180
48-A        TGTGATTAAAAAAGTTAATTAGCTATTTTATTTTGATATTATTTTTTATTGTTGAAAAAA
44-B        ****************T***********************************************
51-B        ****************T***********************************************

            241                                                        300
48-A        AGTTTTAAATAAAAAAAATAAAATTAATATATATAAAAATTTTGTTATAAATTATAATAG
6-D         ***************T*********T************************************

            301                                                        360
48-A        TTTATTTATTTATTTTTTTATAATAAATAATAGTATTTTTTTTATTATTTTATAAAAAAA
6-D         *******G**********-************************T*****************

            361                                                        420
48-A        AATAATTTAATTATAATTATTAAAAGTAAATTTGTTATTTAGAATAATATATTAATATAA
6-D         **********************G*******T*******************************

            421                                                        480
48-A        ATAACAAAAATATTAAATATTTTTTAAATTTTTTGGGGATTTAAGTTTTAATTTTTTGTG
37-C        ****************T*****************CG************A*************
6-D         ****A*********************************A***A******************

            481                                                    429
48-A        TCTGCTCAGTGAATTTAAATAGCACTTTTAGCGTGATTGGTCAAAGGTA
37-C        **G******G*****************************************
6-D         ************T*****************************************
```