An evaluation of multiple techniques for the creation of a diagnostic tool for *Meloidogyne* species identification in Alabama

by

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Abstract

Species identification of *Meloidogyne spp*. (root-knot nematode, RKN) is an important tool to offer growers in the state of Alabama because it is beneficial for planning and implementing a crop rotation to reduce the impact of these yield-limiting nematodes. RKN has a very wide host range, but these host ranges are species dependent. By implementing a crop rotation, a grower can potentially lower RKN levels by planting a nonhost if species is known and economical. Species analysis also allows a grower to know RKN species levels, and determine if there is a need for resistant crop varieties, if they are available. The goal of this project was to evaluate multiple species identification techniques and determine the best combination of methods for implementing a practical and efficient assay for RKN species identification.

To do this, three different techniques were evaluated for their ability to quickly and accurately identify RKN species. The techniques used in this study were morphological measurements, differential-host test, and molecular analysis. Each of these techniques was used on multiple RKN populations, starting with a known *M. incognita* race 3 population. This greenhouse population was previously identified via the differential-host test. Initial results showed a confirmation of species with the differential-host test and PCR amplification, but morphological measurements of juveniles did not distinguish our test population from *M. arenaria* and *M. javanica*.

Soil and root samples were then collected from throughout Alabama for RKN species identification. Overall, 75 samples from 14 counties in Alabama were collected from grower fields

for species analysis. Crops sampled during collection included cotton, soybean, corn, peanut, sweet potato, squash, pepper, kiwi, turmeric, and turf. Both molecular analysis (PCR) and the differential-host test were used for species identification. Primers used for PCR include those that identify commonly found RKN species: *M. incognita, M. arenaria, M. javanica, M. hapla, M. fallax, M. chitwoodi,* and *M. enterolobii*. Of these samples, 73 were identified as *M. incognita* (97%), and two were identified as *M. arenaria* (3%). These species were identified through the differential-host test and PCR using primer sets IncK-14F/IncK-14R (*M. incognita*) and Far/Rar (*M. arenaria*). Overall, *M. incognita* is the most prevalent species of root-knot nematode that has been found on cropping systems in Alabama during this project.

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Chapter 1: Introduction and a Review of Literature

Introduction and Problem Statement

This project centers on the plant-parasitic nematode *Meloidogyne* spp. (root-knot nematode, RKN). Reverend Miles Joseph Berkeley first reported symptoms of the root-knot nematode in 1855 by noting galls on cucumber roots in a greenhouse. These galls were later attributed to the root-knot nematode by Maxime Cornu in 1879 and placed into the genus *Anguillula* (Hunt and Handoo, 2009). Carl Muller also is attributed to identifying a different nematode with similar galling type symptoms that he classified as *Heterodera* in 1884. The genus name of *Meloidogyne* was not proposed until 1887 by Emilio Goldi (Hunt and Handoo, 2009). In 1949, the genus *Meloidogyne* was revised with a defining list of multiple species and an overarching description of the root-knot nematode, removing certain species from *Heterodera* and *Anguillula* and creating a larger *Meloidogyne* genus (Hunt and Handoo, 2009).

The genus *Meloidogyne* is now comprised of hundreds of species found worldwide, with several that are commonly found plant-parasitic species in the United States. These species include *M. incognita* (southern root-knot nematode), *M. arenaria* (peanut root-knot nematode), *M. javanica* (soybean root-knot nematode), *M. hapla* (northern root-knot nematode), *M. chitwoodi* (Columbia root-knot nematode), *M. enterolobii, M. nassi, M. marylandi* and *M. fallax* (Elling, 2013). In the southeastern region of the United States, the most common species are *M. incognita, M. arenaria,* and *M. javanica* (Walters and Barker, 1994). While these species are very similar in biology and appearance, they have a very different host range preference. Each of these nematodes

has a different host range, and if the species is known, rotating to a nonhost is a relatively easy and cost effective management strategy (Taylor and Sasser, 1978). Recent first reports of *M. enterolobii* on cotton and soybean in North Carolina in 2013 raise the question of whether this species could potentially be present in Alabama and other southern soybean producing states (Ye et al. 2013). Thus, determining distribution and refining known occurrences for RKN species is of utmost importance.

While it can be difficult to assess the total impact that RKN accounts for on a global level, it has been estimated at approximately 14.6% of crop loss in tropical and sub-tropical climates, and 8.8% in developed countries (Nicol et al. 2011). These estimates occur because it is hard to assess total impact of RKN. Many growers may not be aware of RKN presence in a field, and yield loss can often go unidentified. In the state of Alabama alone, an estimated 2% of the total cotton yield was lost during the 2016 season to RKN. A 2% yield loss is equivalent to 14,200 bales, which is currently valued at 5.2 million dollars (Lawrence et al. 2016). This does not include all other crops in the state that RKN can attack. With such a large economic impact to the agricultural industry in Alabama, much research has been done on various control strategies for root-knot nematode management. The main strategy that this research project focuses on is the implementation of a crop rotation. The principles of crop rotation rely on growing a sequence of various crop types in the same area over multiple growing seasons. This helps with nutrient management, soil fertility, reducing soil erosion, and increasing crop yields (Havlin et. al. 1989). A crop rotation also helps control pests and pathogens by changing the host in the field and breaking pest life cycles and the in-field host (Govaerts et al. 2007). All species of root-knot nematodes are host dependent, meaning that they are obligate parasites. By knowing what species is present, a grower can rotate to a nonhost crop to help lower the root-knot nematode population

density. This project aims to investigate the current available methods for species identification of RKN and implement a reliable and efficient method or combination of methods to differentiate species and improve nematode management. Species identification will also allow for the construction of a database of sampled fields throughout the state and RKN species distribution in Alabama.

Meloidogyne spp. Biology and Ecology

Meloidogyne spp. is endoparasitic in its feeding habits, and is symptomized by the galling or knots that form on the host roots as the nematode feeds (Faske and Starr, 2006). Galls were the symptoms originally observed by Reverend Miles Joseph Berkeley in 1855 on cucumber roots (Hunt and Handoo, 2009). *Meloidogyne* spp. thrive in coarse, sandy, and light soils where it can reach a high population density (Robinson et al, 1987; Starr et al. 1993). Root-knot nematode numbers tend to decline in heavier soil types with high concentrations of silt and clay (Robinson et al. 1987; Starr et al. 1993). Threshold levels for RKN also are dependent upon host crop. When sampling in the late summer and fall, recommendations in Georgia have corn and cotton thresholds at 50 J2's per 100cc soil, peanut at 10 J2's per 100cc soil, and soybean at 60 J2's per 100cc soil (Jagdale, 2013).

The life cycle of RKN contains an egg stage, four juvenile stages, and an adult stage (Taylor and Sasser, 1978). Moens et al. (2009) summarized the life cycle of the root-knot nematode, and it is as follows: (1) the nematode initially begins embryonic development inside the egg, where it molts to a first-stage juvenile, then to a second-stage juvenile. The second-stage juvenile is considered the infective stage of the nematode. (2) Once the J2 hatches from the egg, it begins searching and seeking for a host to establish a feeding site (Taylor and Sasser, 1978). (3) After

the J2 locates a root system, it penetrates just above the root cap. The nematode moves intracellularly and intercellularly through the root cortex until reaching the region of cell elongation, where the J2 then uses its stylet to pierce a cell wall (Taylor and Sasser, 1978). (4) The juvenile begins to feed on the root cells, causing the cells to differentiate into specialized nurse cells referred to as giant cells (Moens et al. 2009). Giant cell formation is typically accompanied by intense cell multiplication, also referred to as hyperplasia (Taylor and Sasser, 1978). These two events are what leads to the formation of galls on the roots around the feeding nematode. (5) The nematode becomes sedentary and enlarges into a swollen shape, and continues to feed on the vascular tissue of the roots where it goes through addition molts (Moens et al. 2009). These molts are the third-stage and fourth-stage juvenile where the body retains mainly feeding muscles when it molts, and the preadult molt (fourth) where sexually dimporhic individuals are present (Moens et al. 2009). Some species do not form males, and reproduce through parthenogenesis (Elling, 2013). (6) Prior to adult stage, if males are present, the male nematodes quit feeding, molt into a vermiform stage, exit the root, attempt to mate with females, and die. (7) The female stays in the root system, where she begins to produce eggs. (Moens et. al. 2009; Taylor and Sasser, 1978). (8) The female's body continues to enlarge as the root gall continues to build around her. (9) She then begins to produce eggs both inside and outside her body in an egg sac that is part of a gelatinous matrix (Taylor and Sasser, 1978). These eggs eventually hatch and the life cycle starts over.

With several common RKN species present, it is important to understand which species are in the field, because each species reacts differently to management. For example, a common current crop rotation in the southeastern United States is cotton and peanuts. Cotton growers can often have infestations of *M. incognita*, and to reduce numbers of nematodes they rotate to peanut. However, peanut is an excellent host of *M. arenaria*, which is often found in the same field as *M.*

incognita (Davis and Timper, 2000). This can be problematic, because if more than one species is in the field, setting up a crop rotation for lowering population density can be difficult. While planting one crop will lower a specific species population density, it may not lower the population density of all the species present in the field. This is a major issue because the nematode population density is usually not completely controlled with the implementation of a crop rotation; densities are often only lowered. A host may act as a poor host and inhibit a species reproduction for a year or two, but they can successfully survive for multiple years at low numbers and bounce back once a crop that the species is a host of is planted again. This is possible because while the crop may act as a lesser host, the population will still reproduce, but at very low numbers that do not actively inhibit growth of the plant. Weed hosts can also act as alternative hosts for species in a field if left uncontrolled, thus allowing the RKN to survive even though a nonhost was planted that year (Tedford and Fortnum, 1988). For reasons such as this, the identification of RKN is important for crop protection.

Morphological Identification

One of the first methods for identification of RKN is through morphological or physical characteristics of the individual nematodes. Using morphological measurements for species differentiation in the genus *Meloidogyne* can be traced back to Chitwood (1949) when he used these measurements to determine that there were multiple size differences between species that compromised the genus. There are multiple ways to use morphological measurements of RKN species for identification. These include various structures of both male and female root-knot nematodes (Eisenback et al. 1980; Eisenback and Hirschmann, 1981). Morphological identification can be beneficial because it is reliable, rapid and practical if differentiating characteristics can be observed over the various life stages (Eisenback and Hirschmann, 1981).

Characteristics that have been used in the past include stylet morphology and head shape of males and females, perineal patterns of females, and overall body sizes of juveniles, males, and females (Taylor and Sasser, 1978). Freshly infected roots could be used for juvenile measurements, but mature females are needed for perineal pattern analysis (Eisenback et al. 1980). Since perineal patterns are most definitive, recovery of females from root gall samples is very important for morphological analysis. Identification via perineal patterns is done by cutting the female body and removing the posture portion including the vulva and anus, thus isolating the perineal area. The perineal area is be placed upon a microscope slide and viewed under a microscope at 1000-x magnification. Each RKN species has a specific pattern, and if the procedure is done correctly, species identification can be done. Juveniles and males, if present, are found in the surrounding soil or in the roots.

While morphological analysis can lead to species identification, problems may arise with morphological characteristics. This technique can be a tedious process that requires a steady hand and much practice to perfect. Thus, leading to a skill that requires time to develop to successfully, consistently, and correctly identify the species. Physical characteristics may also vary with in a species. An example of this is that the stylet might have more than one form in *M. javanica,* resulting in a false species identification by an untrained eye (Eisenback and Hirschmann, 1981). For these reasons, other methods of species identification in RKN likely need to be used in conjunction with morphological identification.

Differential Host Test

	Cotton	Tobacco	Pepper	Watermelon	Peanut	Tomato
***Meloidogyne incognita* race 1**	-	-	+	+	-	+
***Meloidogyne incognita* race 2**	-	+	+	+	-	+
***Meloidogyne incognita* race 3**	+	-	+	+	-	+
***Meloidogyne incognita* race 4**	+	+	+	+	-	+
***Meloidogyne arenaria* race 1**	-	+	+	+	+	+
***Meloidogyne arenaria* race 2**	-	+	-	+	-	+
Meloidogyne javanica	+	+	-	+	-	+
Meloidogyne hapla	-	+	+	-	+	+

Table 1: +/- represents if the *Meloidogyne* population reproduces on the corresponding host based upon the 0-5 rating for egg mass numbers on host (Sasser, 1972)

The differential host test is one of the methods for root-knot nematode species identification. Sasser (1972) developed the test to determine the species and race of four commonly found plant-parasitic root-knot nematodes: *M. incognita* (race 1-4), *M. arenaria* (race 1 and 2), *M. javanica,* and *M. hapla.* In host-differential identification, the unknown nematode population is added as eggs at initial planting to cotton (Deltapine 16), tobacco (NC 95), pepper (California Wonder), watermelon (Charleston Grey), peanut (Flor-runner), and tomato (Rutgers). These host plants are replicated five times, and the nematode population is inoculated evenly over all plants in the test. After 45 days, the plants are harvested and rated for root galling and number of eggs. The +/- represents if the RKN population reproduces on the corresponding host based upon the 0-5 rating for egg mass numbers per host. The scale is defined as follows: 0 = 0 egg masses per plant, 1 = 1-2 egg masses, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = more than 100.

Traditionally, plants with an average egg mass rating of 2 or less are considered resistant, and ratings greater than 2 are considered susceptible (Hartman and Sasser, 1985). Host range can also be determined based upon the reproductive factor of the population. Reproductive factor is calculated by the following formula: Rf = final egg density ÷ initial egg density as described by Oostenbrink (1966). The Rf values are then grouped into four categories and are as follows: Rf = 0 – 0.09, nonhost; Rf = 0.1 – 0.9, poor host; Rf = 1 – 2, moderate host; Rf >2, suitable host (Oostenbrink, 1966). The layout of this test can be seen in Table 1. While this is a common and reliable practice, it is not the most practical approach for modern research. It takes from 45 to 60 days to completely allow the nematode population to complete its life cycle, reproduce, and reach levels of infection in host plants. This process can take longer if plants such as tobacco have to be planted before the test is begun. This is a reliable method for species and race identification, but it does not fit in a practical timeline for growers who want to know which species is in a field as soon as possible. This test also requires a greenhouse or other type of growth area for the plants to grow and develop. It can also become problematic if multiple tests need to be run at once, requiring a large amount of greenhouse space. However, this test does have the ability to differentiate race, which can only be done through host range analysis (Elling, 2013). That, along with its reliability makes it an invaluable asset in species identification. However, issues can arise if there are multiple RKN species present in a field. If the population on the differential-host test contains multiple species, the test will not give accurate results.

Isozyme Analysis

Isozyme analysis was first used to distinguish RKN species by Esbenshade and Triantaphyllou (1985). They successfully differentiated protein patterns from multiple RKN species that seemed to correlate to specific species. Isozymes are defined as enzymes that convey

a specific amino acid sequence. It was found that certain species of RKN carry specific isozymes (Blok and Powers, 2009). By determining which isozyme is present in the root-knot nematode, a species identification can be performed. Multiple isozyme systems have been used since its initial discovery, including carboxylesterase/esterase (EST), malate dehydrogenase (MDH), superoxide dismutase (SOD), and glutamate-oxaloacetate transaminase (GOT) (Blok and Powers, 2009). These techniques are run to differentiate multiple species. For example, MDH is known to separate *M. hapla* from *M. incognita, M. arenaria,* and *M. javanica.* GOT can separate *M. incognita* from *M. javanica, M. arenaria,* and *M. hapla* (Esbenshade and Triantaphyllou, 1985). A major advantage of this technique is that with the correct equipment, results can be obtained in as little time as a couple days.

While this technique has been found to be a successful technique and a reliable option for species identification of RKN, it does have some drawbacks. Isozyme analysis on a population requires the mature female-egg laying stage for the assay to show differentiation (Devran and Sogut, 2009). Since a majority of the nematodes found in soil and root samples are second-stage infective juveniles, this can be problematic. Egg laying females can be hard to come by in these samples, and often require greenhouse increases of the nematode to obtain the correct life stage. Previous work has shown variable results in isozyme phenotypes. Examples include atypical esterase phenotypes reported from *M. incognita* isolates in Libya and atypical patterns reported by Molinari et al. (2005) during a survey of India, Venezuela, Cuba and Egypt. Atypical esterase patterns have also been reported from Spain of *M. incognita* (Hernandez et al. 2004). Reasons for these atypical results remains unclear. Thus, for this system to be successfully implemented, multiple enzymes need to be analyzed over a given population for an overall comparison to limit

false readings. This also needs to be paired with other identification techniques so an overall diagnosis of species can be confidently confirmed.

Molecular Identification

The current and most commonly explored way for identification of root-knot nematodes is through the use of molecular techniques to investigate the DNA of a given population. This technique was originally implemented and used diagnostically by Powers and Harris (1993). They used PCR (Polymerase Chain Reaction) to amplify distinct genetic regions specific to known RKN species in various regions. The primer set used (C2F3, 5'GGTCAATGTTCAGAAATTTGTGG'3 and 1108 5'TACCTTTGACCAATCACGCT'3) was distinct to several common *Meloidogyne* species: *M. incognita, M. arenaria, M. hapla, M. javanica,* and *M. chitwoodi* (Powers and Harris, 1993). This technique, when successful, can allow for species differentiation based upon PCR band size when run through an electrophoresis agarose gel. Another type of primers used for identification are species-specific primers. These primers amplify specific DNA fragments on only certain RKN species (Adam et al. 2006; Naz et al. 2012). Available species-specific primers for species that may be found in Alabama include *M. incognita, M. arenaria, M. javanica, M. hapla, M. enterollobi* and *M. chitwoodi.*

Another method of molecular identification besides PCR band fragment analysis is DNA sequencing of genetic regions of RKN. These regions include 18S, ITS, and 28S (Ye et al. 2015; Zeng et al. 2014). In this research, populations collected in North Carolina were analyzed using primers that amplify certain areas of the ribosomal DNA of RKN. These amplified regions can then be run through the NCBI GenBank for comparison to known RKN species sequences for these regions. Both methods of primers for band comparison and DNA sequencing for species

differentiation are key identification methods that will be analyzed in this study. However, there are some limitations and drawbacks to these techniques. Reports indicate some of these primers are not very robust, and amplification does not always occur even though the primer correlates to the correct species (Khanal et al. 2016; Ye et al. 2015). Thus, false negatives are possible.

Management and Application of Meloidogyne spp. Identification

In most modern cropping systems, it is very easy for plant-parasitic nematodes, especially the root-knot nematode, to go from low population density levels to above threshold levels and become a major issue (i.e. 40-50 J2 per 100cc of soil in the fall on cotton). As previously stated, these thresholds vary depending upon crop and geographic region. RKN can easily be introduced to a field by equipment that has been in a field that has a high population density or by environmental conditions such as rain washing the nematodes from higher elevated levels of a field to lower levels (Planchon, 2000). Once in a field with an adequate host, root-knot nematode levels can grow exponentially in just one season (Seinhorst, 1965). Thus, successful management and long-term control plans are very important. Control of plant-parasitic nematodes includes many different aspects of agriculture, and is best achieved through the application of various management methods. These range from cultural, to chemical and biological practices (Taylor and Sasser, 1978). There are several crops grown in the United States that have resistance genes to the root-knot nematode. These include soybean, tomato and cotton. These resistant varieties work well for management, but emergence of virulent biotypes in some root-knot nematode populations shows that other management practices need to be used as well (Jacquet et al. 2005). Biological and chemical control methods are also common practices that have had success in nematode control, but can sometimes be expensive and/or unreliable (Dube and Smart, 1987; Kiewnick and Sikora, 2006; Sharon et al. 2001; Faske and Hurd, 2015). The implementation of a

crop rotation is another tactic for management of root-knot nematodes. This can be applied to almost all major row crops that can be infested with root-knot nematodes, because these systems typically have the ability to rotate crops each year. Management tactics in the past have demonstrated that lowering nematode population density can be created by using non-host crops and resistant cultivars (Weaver et al. 1995; Kratochvil et al. 2004). Understanding what species is present in a grower's field is a good first step in building an integrated pest management system that will keep the root-knot nematode population density at lower levels. This allows a crop rotation plan to be implemented along with other techniques to combat root-knot infestations.

The overall hypothesis for this study is that through the implementation of PCR, isozyme, and/or morphological techniques along with the differential host test, a quick and reliable root-knot nematode species identification assay will be developed for the diagnostic identification of RKN species. The supporting objectives for this hypothesis are to: 1) establish base level root-knot nematode populations for building a diagnostic assay; 2) design a diagnostic assay that can identify root-knot nematodes to a species level as quickly, efficiently, and accurately as possible; and 3) create a species distribution map of RKN as it is found throughout the state of Alabama.

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Chapter 2: *Meloidogyne spp.* Identification and Distribution in Alabama via Morphological Features, the Differential-Host Test, and Molecular Analysis

ABSTRACT

Three different techniques were performed to determine their efficacy for species identification of *Meloidogyne* spp. (root-knot nematode, RKN). These techniques were morphological measurements, the differential-host test, and molecular analysis. These were initially used on a known greenhouse population of *Meloidogyne incognita* race 3 that had previously been identified by the differential-host test. Initial results showed a confirmation of species with the differential-host test and PCR amplification, but morphological measurements of juveniles did not distinguish the test population from *M. arenaria* and *M. javanica*. With the success of species identification, statewide collection began for RKN species identification in Alabama. Seventy-five samples from 14 counties in Alabama were collected from growers fields. Molecular analysis (PCR) and the differential-host test were used for species identification. Primer sets IncK-14F/IncK-14R (*M. incognita* specific) and Far/Rar (*M. arenaria* specific) were the most robust primer sets used, and were able to identify all 75 samples to species level. Of these samples, 73 were identified as *M. incognita* (97%), and two were identified as *M. arenaria* (3%). Overall, *M. incognita* is the most prevalent species of RKN that has been found on cropping systems in Alabama during this project.

INTRODUCTION

The root-knot nematode (*Meloidogyne spp*.; RKN) is one of the most damaging plant-parasitic nematodes in the world. RKN was first reported by Reverend Miles Joseph Berkeley in 1855, when he noted galling on cucumber roots in a greenhouse that was eventually attributed to the root-knot nematode (Hunt and Handoo, 2009). These galls or swellings that form are a key symptom for determination of RKN infection (Taylor and Sasser, 1978). Since all species of RKN are host dependent, a grower can sometimes rotate to a nonhost crop if the nematode is identified to species level. For example, cotton (*Gossypium hirsutum* L.) growers often have infestations of *M. incognita* (Kofoid and White) Chitwood. To help lower this RKN populations density, a common crop rotation from cotton is peanut (*Arachis hypogaea* L.). However, peanut is an excellent host of *M. arenaria* (Neal) Chitwood, which can be found in the same field as *M. incognita* (Davis and Timper, 2000). Thus, knowing which species are present in a field is very important, and there are multiple techniques for RKN species identification.

We selected three methods for RKN species identification and examined various nematode populations to determine their efficacy in implementing a diagnostic tool for this identification at Auburn University. These techniques were morphological measurements, differential-host tests, and molecular analysis including PCR band comparison and genetic sequencing for species comparison.

For species identification of RKN, morphological measurements were one of the original methods used. This can be traced back to Chitwood when he used morphology of *Heterodera marioni* to establish the genus *Meloidogyne* containing multiple species (Chitwood, 1949) Schmidt. Morphological measurements have been used to differentiate *Meloidogyne* species based upon structures of males, females, and juveniles (Eisenback et al. 1980; Eisenback and

Hirschmann, 1981). These structures include stylet morphology and head shape of males and females, perineal patterns of females, and overall body sizes of juveniles, males, and females (Taylor and Sasser, 1978).

Another technique for species identification is the differential-host test. This test was used to determine the species and race of four commonly found plant-parasitic root-knot nematodes: *M. incognita* (race 1-4), *M. arenaria* (race 1 and 2), *M. javanica* Treub, and *M. hapla* Chitwood (Taylor and Sasser, 1978). This test uses specific cultivars of cotton, tobacco (*Nicotiana tabacum* L.), tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annuum* L.), watermelon (*Citrullus lanatus)* Matsum and Nakai, and peanut to determine species and race of an unknown RKN population. Species and race identification can be made based upon which of the plants the RKN population reproduces. Being the only current technique for RKN species identification that can also differentiate race, the differential-host test is an extremely valuable method for identification.

Another common method for RKN species identification that most research groups have begun to focus on in recent years is through molecular techniques that focus on genetic diversity of RKN. Powers and Harris (1993) were the first group to implement this as a diagnostic tool when they used PCR to amplify genetic regions specific to known RKN species. They were able to differentiate *M. arenaria, M. chitwoodi* Golden, O'Bannon, Santo and Finley, *M. hapla, M. incognita, M. javanica,* and (Powers and Harris, 1993). Species-specific primers are also used for identification. These primers amplify specific DNA fragments only on certain RKN species (Adam et al. 2006; Naz et al. 2012). Available species-specific primers for species that may be in Alabama include *M. arenaria, M. chitwoodi, M. enterolobii, M. fallax* Karssen*, M. hapla, M. incognita,* and *M. javanica,* (Yang and Eisenback).

The objectives for this research are to 1) establish base level RKN populations for building a diagnostic assay; 2) design a diagnostic assay that can identify RKN to a species level; and 3) create a species distribution map of RKN throughout Alabama. The overall goal of this project is to design a tool for Auburn University to provide growers in Alabama and the surrounding states accurate species identification of RKN populations for improved management strategies.

MATERIALS AND METHODS

Nematode collection, extraction, and processing

Laboratory testing

All greenhouse tests and inoculum preparation were conducted at the Plant Science Research Center (PSRC) greenhouse located at Auburn University in Auburn, AL. For initial experiments, nematodes were collected from RKN stock cultures maintained on corn in the greenhouse. Cotton plants (Fibermax 1944 GLB2; Bayer CropScience, Research Triangle Park, NC) in 500 cm^3 polystyrene pots containing RKN were grown for 60 days to increase egg levels. Soil used in the greenhouse was a Kalmia loamy sand (80% sand, 10% silt, and 10% clay) collected from the Plant Breeding Unit (PBU; 32°29'43.2"N 85°53'26.3"W) located at the E.V. Smith Research Center of Auburn University near Tallassee, AL. This soil was steam pasteurized at 180 °C for 90 minutes, cooled for 24 hours, and the entire process was repeated again prior to use. Fertilizer and lime was added to the pasteurized soil as recommended by the Auburn University Soil Lab. Soil was mixed with sand at a rate of 60:40 soil to sand, and this mixture used for each pot. Four cotton seeds were planted per pot, and RKN inoculum (described below) was added at planting. Cotton plants were watered as needed to maintain soil moisture between 40% and 60% of the field capacity, and adequate lighting was supplied via 1000 watt halide bulbs producing

110,00 lumens at a rate of 14 hours per day. Temperatures in the greenhouse ranged from 24°C to 35°C.

At the end of the 60 days, shoots were removed from the cotton roots, and the roots were gently washed to remove surrounding soil. The RKN eggs were extracted from the cotton roots following a modified version of the methodology of Hussey and Barker (1973). The root mass was placed in a 0.625% NaOCl solution and shaken for 4 minutes at 1 g-force on a Barnstead Lab Line Max Q 5000 E Class shaker (Conquer Scientific, San Diego, CA). Roots were then scrubbed by hand, and the eggs were collected on a 25-µm pore sieve and washed into a 50 mL centrifuge tube. The contents were centrifuged at 427 g-forces for 1 minute in a 1.14 specific gravity sucrose solution based on Jenkins (1964) methodology. Eggs, now located in the supernatant of the sucrose solution, were recollected on a 25-µm pore sieve, rinsed with water to remove sucrose from eggs, and their presence confirmed via a Nikon TSX 100 inverted microscope at 40x magnification. Nematode inoculum egg numbers were enumerated via the Nikon TSX 100, and nematode eggs extracted for PCR amplification or morphological measurements were hatched to the juvenile stage by being placed in a modified Baermann funnel for 48 hours as described by Vilglierchio and Schmitt (1983). The second-stage juveniles were then used for DNA amplification or morphological analysis.

Field studies

Locations in the state were identified containing RKN populations by taking samples of both the soil and plant roots for nematode extraction. Potential RKN infested fields were found through talking with Alabama extension agents and specialists, private agronomists, and farm managers of Auburn University research stations. Samples were taken via a conical soil probe (2.54 cm wide and 20 cm deep) and shovel to obtain both soil and root samples. A field was

sampled in a zigzag walking pattern over the entire field. Soil cores were combined and bulked in a plastic bag for processing. The crops sampled for this were soybean, cotton, corn, and peanut. After the samples were taken, extraction and processing occurred as previously described. RKN extraction from soil is similar to root extraction, but varies slightly. While root extraction is for obtaining RKN eggs, soil extraction is done to obtain juvenile and adult male nematodes. Soil extraction was completed by gravity sieving followed by sucrose centrifugation (Barker, 1985). These samples of RKN eggs and J2's were used for morphological measurements, differential-host tests, and molecular analysis.

Morphological identification

The first technique implemented for RKN species identification was morphological analysis. This was done on four of the populations sampled. Second-stage juveniles (J2) were used for morphological comparison. When a root or soil sample was obtained, extraction occurred as previously described. The nematode suspension was then placed on a gridded petri dish (Fisher Scientific; Hampton, NH) and observed under a Nikon Eclipse TS100 at 40-x magnification to confirm RKN juvenile presence. After confirmation, the sample was transferred to a Nikon SMZ 1500 and observed at 10-x magnification. Temporary mounts were then created for analysis as described by Eisenback (1986). Individual juveniles were transferred to a 5 µL droplet of water on a microscope slide. A coverslip was then placed on the water droplet containing the RKN juvenile, and was heat fixed with a small flame to the slide. The slide was then placed under an Axiovert 200 Inverted Flourescence/Live Cell Imaging microscope and images were taken at 100-x magnification. Based upon these images, measurements in µM were taken of 20 J2's per population. Measurements taken were body length, body width, body width at anus, stylet length, stylet base to head end, and tail length. Body length ÷ body width (a), body length ÷ esophagus

length (b), body length ÷ tail length (c), and body length ÷ body width at anus (c') were also calculated and included (Eisenback et al., 1981; Cliff and Hirschmann, 1985).

Differential-host test

For the differential-host test (DHT), six crops were inoculated with an unknown population of RKN and grown over 60 days to determine the host range of the population. This allows for species and race determination of the nematode population. The test was set up as described by Taylor and Sasser (1978) (Table 1). The crops used in this test were cotton (FM 1944), tobacco (NC 95), pepper (California Wonder), watermelon (Charleston Grey), peanut (Flor-runner), and tomato (Rutgers) (Sasser, 1972). The original publication of this by Sasser (1972) uses cotton variety Deltapine 16. We were unsuccessful in finding this cotton variety, so FM 1944 was used. FM 1944 is listed as susceptible to RKN, and provides a suitable alternative to Deltapine 16. Each crop was planted in a 500 cm^3 polystyrene pot and allowed to grow for 7-10 days until germination had occurred. Tobacco was germinated previously in potting soil, and transplanted two plants per pot at the time of RKN inoculation. Planting rates depended upon crop and stand was thinned to two plants per pot immediately after seedling establishment. Soil for this was obtained from the same method as stock cultures in the greenhouse as explained previously. The pots were inoculated with the test RKN population at a rate of 1,000 eggs per pot. Nematode inoculum for the test was extracted from plant roots as described previously after allowing for the population to grow on the original host crop under greenhouse conditions. Each DHT was arranged in a randomized complete block design with five replications. Temperatures in the greenhouse ranged from 24 to 35°C with an average of 30°C.

The target date for test termination was 60 days post inoculation of RKN. Plant shoots were cut from roots and roots were gently washed under a faucet to remove loose soil. Root and

shoot weights were recorded and RKN was extracted from roots as previously described. Eggs were enumerated via a Nikon TSX 100 inverted microscope at 40-x magnification. These egg numbers were then used to determine the reproductive factor (Rf) of each host. Reproductive factor was calculated by the following formula: Rf = final egg density at 45 days ÷ initial egg density as described by Oostenbrink (1966). The Rf values were then grouped into four categories and are as follows: Rf = 0 – 0.09, nonhost; Rf = 0.1 – 0.9, poor host; Rf = 1-2, moderate host; R > 2, suitable host (Oostenbrink, 1966).

Nematode preparation for PCR

Identification of RKN via molecular techniques consisted of three steps: nematode preparation, DNA amplification (PCR), and amplification analysis. Individual second-stage root-knot nematodes were obtained from each sample for molecular analysis (Figure 1). RKN was extracted from soil and roots as described previously and viewed with the Nikon TSX 100 inverted microscope at 40-x magnification to determine RKN presence and life cycle stage. If no juveniles were present in the sample, eggs were hatched to the J2 stage by a modified Baermann funnel for 48 hours as described by Vilglierchio and Schmitt (1983). Samples were then placed under a Nikon SMZ800 microscope at 10-x magnification. Individual juveniles were picked out by a Kerr Style D dental pick (Kerr Manufacturing Co.; Detroit, Michigan) and placed into a 20-µL droplet of sterile water on a microscope coverslip. Each individual juvenile was then ruptured into several pieces using a 200-µL pipette tip (VWR North America, Radnor, PA) via the smashing method as described by Powers and Harris (1993). The water solution containing the ruptured J2 was then pipetted into a 0.5 mL PCR tube and stored at -20°C until used for PCR amplification.

Table 2: Primers used for polymerase chain reaction and DNA sequencing of *Meloidogyne spp.*

Primer	Marker	Sequence (5' to 3')	Reference
JB3	COI	TTTTTTGGGCATCCTGAGGTTTAT	Kiewnick et al. 2014
JB5	COI	AGCACCTAAACTTAAAACATAATGAAAATG	Kiewnick et al. 2014
D2a	28S D2/D3	ACAAGTACCGTGAGGGAAAGT	Nunn, 1992
D3b	28S D2/D3	TGCGAAGGAACCAGCTACTA	Nunn, 1992
rDNA1.58S	ITS	ACGAGCCGAGTGATCCACCG	Cherry et al. 1997
rDNA2	ITS	TTGATTACGTTCCCTGCCCTTT	Vrain et al. 1992
SSUF07	18S	AAAGATTAAGCCATGCATG	Floyd et al. 2002
SSUR26	18S	CATTCTTGGCAAATGCTTTCG	Floyd et al. 2002
18S965	18S	GGCGATCAGATACCGCCCTAGTT	Mullin et al. 2005
18S1573	18S	TACAAAGGGCAGGGACGTAAT	Mullin et al. 2005
C2F3	COII	GGTCAATGTTCAGAAATTTGTGG	Powers and Harris, 1993
MRH106	COII	AATTTCTAAAGACTTTTCTTAGT	Powers and Harris, 1993
1108	COII	TACCTTTGACCAATCACGCT	Powers and Harris, 1993
Fjav	*M. javanica* specific	GGTGCGCGATTGAACTGAGC	Zijlstra et al. 2000
Rjav	*M. javanica* specific	CAGGCCCTTCAGTGGAACTTATAC	Zijlstra et al. 2000
Me-F	*M. enterolobi* specific	AACTTTGTGAAAGTGCCGCTG	Zijlstra et al. 2000
Me-R	*M. enterolobi* specific	TCAGTTCAGGCAGGATCAACC	Zijlstra et al. 2000
JMV1	*M. chitwoodi M. hapla* specific	GGATGGCGTGCTTTCAAC	Wishart et al. 2002
JMV2	*M. chitwoodi* specific	TTTCCCTTATGATGTTTACCC	Wishart et al. 2002
JMV hapla	*M. hapla* specific	AAAAATCCCTCGAAAAATCCACC	Wishart et al. 2002
Inc-K14F	*M. incognita* specific	CCCGCTACACCCTCAACTTC	Randig et al. 2002
Inc-K14R	*M. incognita* specific	GGGATGTGTAAATGCTCCTG	Randig et al. 2002
Far	*M. arenaria* specific	TCGGCGATAGAGGTAAATGAC	Zijlstra et al. 2000
Rar	*M. arenaria* specific	TCGGCGATAGACACTACAAACT	Zijlstra et al. 2000
MF5	5S-18S	GGGGATGTTTGAGGCAGATTTG	Hu et al. 2011
MR5	5S-18S	AACCGCTTCGGACTTCCACCAG	Hu et al. 2011
MI-F	*M. incognita* specific	GTGAGGATTCAGCTCCCCAG	Meng et al. 2004
MI-R	*M. incognita* specific	ACGAGGAACATACTTCTCCGTCC	Meng et al. 2004
194	5S-18S	TTAACTTGCCAGATCGGACG	Blok et al. 1997
195	5S-18S	TCTAATGAGCCGTACGC	Blok et al. 1997
SEC-1F	*M. incognita* specific	GGGCAAGTAAGGATGCTCTG	Teserova et al. 2003
SEC-1R	*M. incognita* specific	GCACCTCTTTCATAGCCACG	Teserova et al. 2003
Finc	*M. incognita* specific	CTCTGCCCAATGAGCTGTCC	Zijlstra et al. 2000
Rinc	*M. incognita* specific	CTCTGCCCTCACATTAGG	Zijlstra et al. 2000

DNA amplification

After preparation, the ruptured J2 DNA mix was removed from -20°C and thawed to room temperature. PCR amplification was run in 30 µL reactions. Each PCR reaction contained 9 µL of J2 DNA, 1.8 µL of each 20 µM forward and reverse primer, 15 µL of 2x JumpStart REDTaq ReadyMix (Sigma-Aldrich; St. Louis, MO), and 2.4 µL of ddH_2O. The mixture was placed in a MultiGene DNA thermal cycler (Labnet International; Edison, NJ). PCR amplification protocol was dependent upon which primer set was used (Table 2, Table 3). Primers used were obtained from Invitrogen (ThermoFisher Scientific; Waltham, MA). Each RKN population was screened against all primers that would amplify if a certain species was present. After amplification, samples were run on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Biotium; Fremont, CA) and visualized on a midrange UV box. Typically, 7-10 µL of PCR product were sufficient for showing amplified gel patterns.

Table 3: PCR amplification profiles for *Meloidogyne* with different SCAR primers used in species identification with annealing temperature varying depending upon primer

	35 cycles					
Initial Denaturation	Denaturation	Annealing		Extension	Final Extension	
			Primer:			
		62°C	MI-F/MI-R			
		55°C	IncK14F/IncK14R			
		50°C	JMV1/JMV2/JMVhapla			
		64°C	Me-F/Me-R			
		64°C	Fjav/Rjav			
94°C	94°C	61°C	Far/Rar	72°C	72°C	4°C
2 min.	30 sec.	30 sec.		2 min.	7 min.	∞

PCR for sequencing was run using multiple primer sets that amplify various genetic regions of RKN. These primers are D2a/D3b (28S), rDNA1.58S/rDNA2 (ITS1), SSUF07/SSUR26 (18S), and 18S965/18S1573 (18S) (Floyd et al. 2002; Mullin et al. 2005; Vrain et al. 1992; Cherry et al 1997). The PCR mixture was combined using the same concentrations as the previous methodology listed: 9 µL of J2 DNA, 1.8 µL of each 20 µM forward and reverse primer, 15 µL of 2x JumpStart REDTaq ReadyMix (Sigma-Aldrich; St. Louis, MO), and 2.4 µL ddH_2O. Amplification was carried out in MultiGene DNA thermal cycler, and temperatures were based upon primers sets as presented in Table 4. After PCR, samples were confirmed by running on a 1% agarose gel for band presence. The PCR products were then sent to Eurofin Genomics (Huntsville, AL) for sequencing. Sequence results were aligned using BioEdit Sequence Alignment Editor, and compared to previously published sequences in the National Center for Biotechnology Information (NCBI) GenBank for species analysis (Zeng et al. 2015).

Table 4: PCR amplification profile for *Meloidogyne* primers used for sequencing with annealing temperature varying depending upon primer

	40 cycles					
Initial Denaturation	Denaturation	Annealing		Extension	Final Extension	
95°C 6 min.	95°C 30 sec.	50°C 30 sec.	Primer: D2a/D3b rDNA1.58S/rDNA2 SSUF07/SSUR26 18S965/18S1573	72°C 1 min.	72°C 10 min.	4°C ∞

Alabama diagnostic map and database

Once the PCR diagnostic tool was successful at consistently identifying RKN species in a population, extension agents and specialists, private consultants, agronomists, and growers were contacted in search of fields that could potentially be infested with RKN. Specifically, there were 31 samples taken from cotton (*Gossypium hirsutum* L.), 19 from soybean (*Glycine max* L.), 15 from corn (*Zea mays* L.), 2 from peanut (*Arachis hypogaea* L.), 1 from sweet potato (*Ipomoea batatas* L.), 2 from squash (*Cucurbita* L.), 1 from pepper (*Capsicum annuum* L.), 1 from kiwi (*Actinidia deliciosa* L.), 1 from turmeric (*Curcuma longa* L.) and 2 from turf (*Eremochloa ophiuroides* L.). Soil samples with plant roots were taken by a hand held conical soil probe and a shovel as previously described. Typically, a liter size bag was filled for soil samples, and 4-5 root systems were taken when possible for root sampling. Samples were immediately stored in a shaded cooler and kept at cool temperatures as they were transported to Auburn University.

A database containing samples processed, accession number, original host, county location, genus and species information, and method(s) used for ID was created as identification occurred (Table 8). A map of Alabama with county borders was then used to color code each county as different species were identified within the state (Figure 5).

RESULTS

Seventy-five of approximately 100 soil and root samples collected contained RKN. These populations were used for RKN species identification. Of these 75 populations, four were used for morphological identification, 17 were used for the differential-host test, and all populations were used for molecular analysis.

Morphological identification

Morphological measurements were taken of J2's from four different populations found in Alabama (Figure 1). These populations were AU1 (Lee County), AU33 (Autauga County), AU24 (Henry County), and AU39 (Henry County). Average RKN body lengths and widths for AU1, AU33, AU24, and AU39 were 380.2 x 16.7, 372.6 x 15.4, 404.2 x 19.3, and 420.1 x 21.4 respectively. Stylet lengths also varied. Average stylet lengths were 13.7, 14.5, 12.3, and 15.6 µM respectively (Table 5). Body length, body width, body width at anus, and tale length were all on average higher from the two populations found on peanut (AU24 and AU39) than the two populations extracted from cotton (AU1 and AU33).

Measurements of J2's did not provide enough differences to allow for species identification. These measurements fall into the ranges of what *M. incognita* and *M. arenaria* previously reported (Cliff and Hirschmann, 1985; Eisenback et al. 1981).

Figure 1: Micrographs of various stages of *M. incognita* (AU1, Lee County) population. 1: *M. incognita* egg before hatching at 40-x magnification. 2: entire body of *M. incognita* J2 at 40-x magnification. 3: Tail end of J2 at 100-x magnification. 4: Esophageal region of *M. incognita* head showing stylet at 100-x magnification.

Table 5: Morphometric comparison (mean, standard deviation, and range) of second-stage juveniles of four *Meloidogyne* populations collected in Alabama: AU1, AU33, AU24, AU49

County Location	Lee	Autauga	Escambia	Henry		
Host Crop	Cotton	Cotton	Peanut	Peanut	Published Average	Published Average
Population	AU1	AU33	AU24	AU39	*M. incognita*	*M. arenaria*
n[a]	20	20	20	20	Average[f]	Average
Body Length	380.2 ± 19.4 (367.2 - 405.4)	372.6 ± 22.5 (345.1 - 404.3)	404.2 ± 24.4 (356.4 - 458.3)	420.1 ± 21.7 (387.1 - 498.4)	405 (346-463)	521 (398-605)
Body Width	16.7 ± 0.9 (14.33 - 18.4)	15.4 ± 0.7 (13.1 - 18.4)	19.3 ± 0.87 (17.2 - 23.4)	21.4 ± 3.3 (14.2 - 26.4)	-	-
Body Width at Anus	11.5 ± 0.4 (11.2 - 12.2)	9.7 ± 0.3 (9.1 - 10.1)	14.3 ± 0.4 (13.2 - 15.8)	15.4 ± 0.8 (13.3 - 17.1)	-	-
Stylet Length	13.7 ± 0.3 (13.3 - 14.4)	14.5 ± 0.4 (13.4 - 15.2)	12.3 ± 0.6 (11.4 - 15.6)	14.9 ± 0.6 (13.3 - 17.2)	16 (14-16)	15 (13-17)
Stylet Base to Head End	15.7 ± 0.3 (15.1 - 16.2)	14.7 ± 0.5 (13.8 - 15.9)	14.6 ± 0.5 (12.2 - 17.2)	15.6 ± 0.7 (13.8 - 16.4)	16 (15-17)	15 (14-16)_
Tail Length	48.6 ± 2.4 (42.8 - 53.4)	45.5 ± 3.1 (40.2 - 51.7)	52.7 ± 5.2 (42.3 - 60.1)	54.7 ± 4.9 (48.7 - 59.6)	52 (42-62)	58 (44-69)
a[b]	22.7 ± 1.3 (20.2 - 24.3)	24.4 ± 1.5 (21.2 - 28.1)	20.9 ± 1.4 (18.4 - 22.9)	19.6 ± 1.8 (16.6 - 25.7)	-	-
b[c]	5.9 ± 0.3 (5.1 - 6.7)	4.8 ± 0.3 (4.3 - 5.4)	4.4 ± 0.4 (3.6 - 5.1)	5.2 ± 0.6 (4.2 - 5.8)	-	-
c[d]	7.8 ± 0.4 (7.1 - 8.3)	8.2 ± 0.3 (7.6 - 8.9)	7.7 ± 0.6 (7.0 - 8.9)	7.7 ± 0.5 (6.8 - 8.4)	-	-
c'[e]	33.0 ± 3.1 (29.4 - 38.8)	38.5 ± 4.2 (32.4 - 45.1)	28.2 ± 4.1 (21.7 - 38.4)	33.1 ± 3.7 (27.4 - 39.4)	-	-

[a] Measurements (µM) were taken using 20 juveniles from each population

[b] Value determined by body length ÷ body width

[c] Value determined by body length ÷ esophagus length

[d] Value determined by body length ÷ tail length

[e] Value determined by body length ÷ body width at anus

[f] Averages from Eisenback et al. 1981

Differential-host test

The DHT was employed for species identification on 17 different populations of RKN (Figure 2, Table 6). With such a large number of RKN samples being processed, a 45-day DHT test per population along with a limited amount of greenhouse space was not feasible for all 75 populations. The DHT was successful for species identification on 15 of the 17 populations tested. Of these populations, 13 were identified as *M. incognita* race 3, and 2 as *M. arenaria* race 1 (Table

6). Two populations showed inconsistent results by reproducing on tobacco and not on pepper when run on the differential-host test (AU2 and AU3).

Table 6: Greenhouse differential-host test results for 17 populations across 4 different crops and 7 counties in Alabama based upon reproductive factors for species and race identification

			Reproductive Factor							
Assession Population[c]	**Original Host**	**Location**	**Cotton**	**Tobacco**	**Pepper**	**Watermelon**	**Peanut**	**Tomato**	**Species ID**
AU1	Cotton	Lee County	12.5 (+)[a]	0.7 (-)	1.9 (+)	1.9 (+)	0 (-)	3.3 (+)	*M. incognita* race 3
AU2[b]	Cotton	Lee County	2.7 (+)	1.5 (+)	0.1 (-)	1.6 (+)	0.1 (-)	1.7 (+)	Unknown
AU3[b]	Cotton	Lee County	2.2 (+)	2.1 (+)	0.2 (-)	2.1 (+)	0 (-)	1.5 (+)	Unknown
AU4	Cotton	Macon County	2.6 (+)	0 (+)	1.6 (+)	2.5 (+)	0.1 (-)	3.8 (+)	*M. incognita* race 3
AU5	Cotton	Macon County	5.5 (+)	0 (-)	1.4 (+)	1.1 (+)	0 (-)	4.8 (+)	*M. incognita* race 3
AU6	Cotton	Macon County	9.7 (+)	0.1 (-)	1.1 (+)	1.8 (+)	0.2 (-)	5.5 (+)	*M. incognita* race 3
AU7	Cotton	Elmore County	4.1 (+)	0.5 (-)	2.2 (+)	1.7 (+)	0 (-)	4.5 (+)	*M. incognita* race 3
AU8	Soybean	Elmore County	6.8 (+)	0.1 (-)	2.6 (+)	6.6 (+)	0.1 (-)	8.4 (+)	*M. incognita* race 3
AU12	Soybean	Elmore County	5.7 (+)	0.4 (-)	1.4 (+)	4.2 (+)	0.1 (-)	10.1 (+)	*M. incognita* race 3
AU24	Peanut	Escambia County	0.7 (-)	1.4 (+)	6.9 (+)	1.4 (+)	6.8 (+)	4.8 (+)	*M. arenaria* race 1
AU25	Corn	Escambia County	5.3 (+)	0 (-)	10.1 (+)	1.6 (+)	0 (-)	4.1 (+)	*M. incognita* race 3
AU26	Corn	Escambia County	4.7 (+)	0.6 (-)	1.8 (+)	1.8 (+)	0.1 (-)	6.2 (+)	*M. incognita* race 3
AU32	Cotton	Autauga County	23.1 (+)	0.8 (-)	1.6 (+)	7.2 (+)	0 (-)	4.7 (+)	*M. incognita* race 3
AU33	Cotton	Autauga County	10.8 (+)	0.7 (-)	0.8 (-)	1.5 (+)	0 (-)	2.2 (+)	*M. incognita* race 3
AU39	Peanut	Henry County	0 (-)	4.2 (+)	2.4 (+)	1.1 (+)	10.5 (+)	3.7 (+)	*M. arenaria* race 1
AU40	Cotton	Baldwin County	7.4 (+)	0 (-)	1.1 (+)	2.8 (+)	0 (-)	3.4 (+)	*M. incognita* race 3
AU41	Soybean	Baldwin County	3.3 (+)	0.4 (-)	1.9 (+)	2.1 (+)	0 (-)	6.3 (+)	*M. incognita* race 3

[a] Reproductive factor determines host based upon following scale: 0 – 0.09, nonhost; 0.1 – 0.9, poor host; 1.0 – 2.0, moderate host; > 2, host
[b] Populations AU2 and AU3 did not reproduce on pepper
[c] Eggs were inoculated at 1,000 eggs per pot; test was run for 45 days post inoculation with five reps per test; test was repeated once

Figure 2: The differential-host test run on two populations 21 days after inoculation: AU1 (left) and AU 32 (right)

<u>*Molecular analysis*</u>

Primers used for PCR included those that identify commonly found RKN species: *M. incognita, M. arenaria, M. javanica, M. hapla, M. fallax, M. chitwoodi,* and *M. enterolobii* (Table 2). Successful species identification occurred using primer sets IncK-14F/IncK-14R (*M. incognita*), MI-F/MI-R (*M. incognita*), Finc/Rinc (*M. incognita*), and Far/Rar (*M. arenaria*). With IncK-14F/IncK14-R being the most readily available primer set, it was used for *M. incognita* amplification on all populations. Upon amplification, the IncK-14F/IncK14-R set produced a 399 base pair fragment, and the Far/Rar set produced a 420 base pair fragment. Figure 3 indicates PCR amplification from single RKN J2's with lanes B (AU 3, Lee County) and C (AU32, Autauga County) populations identified as *M. incognita*, and lane D (AU24, Henry County) identified as not *M. incognita* with the IncK-14F/IncK-14R primer set. Lane E was a negative control with the Inc-K14 primers. Lanes F (AU3, Lee County) and G (AU32, Autauga County) were indicated to not be *M. arenaria* with the Far/Rar primers. The lane H population (AU24, Henry County) was identified as *M. arenaria* with the Far/Rar primers. Lane I was a negative control with the Far/Rar primers. All 75 populations screened were identified with these two primer sets. IncK-14F/IncK-

14R identified 73 populations as *M. incognita*, and Far/Rar identified 2 of the populations as *M. arenaria*. This gave a 97% identification of *M. incognita* and 3% identification of *M. arenaria. Meloidogne incognita* was the most prevalent species found by a very wide margin.

Sequencing did not differentiate between the *M. incognita* and *M. arenaria* populations. Three of the populations (AU1, *M. incognita* race 3; AU32, *M. incognita* race 3; AU39, *M. arenaria* race 1) were sequenced using primers that amplify sections of the COI (JB3/JB5), 18S (18S965/18S1573), and 28S (D2a/D3b) genetic regions. With high costs involved in sequencing, only these three populations were selected for sequencing because they had previously been identified via the greenhouse differential-host tests and PCR. The JB3/JB5 primers amplified a ~400bp fragment from the COI region (Figure 4), the 18S965/18S1573 primers amplified an ~600bp fragment from the 18S region, and the D2a/D3b primers amplified an ~700bp fragment from the 28S region. Each sequence was cross referenced with NCBI Genbank. The Genbank Megablast search of the AU1, AU32, and AU39 18S regions had a 98% shared identity to *M. arenaria* (KF112872) and *M. incognita* (U81578), 97% shared identity to *M. javanica* (JX100422), and a 99% shared identity to *M. hapla* (AY268119) (Table 7). The blast search of the AU1, AU32, and AU39 COI regions yielded a 99% shared identity to *M. incognita* (JX683696), *M. arenaria* (KU360143), *M. javanica* (JX683706), and an 88% shared identity to *M. hapla* (KU517171). The blast search of the 28S regions for AU1, AU32, and AU39 yielded a 97% shared identity for *M. incognita* (KP901081), 98% shared identity for *M. arenaria* (AF435803), 96% shared identity for *M. javanica* (JQ317915), and a 97% shared identity for KJ645428). The NCBI database comparisons did confirm these accessions are RKN, but it was not sufficient to differentiate these closely related species.

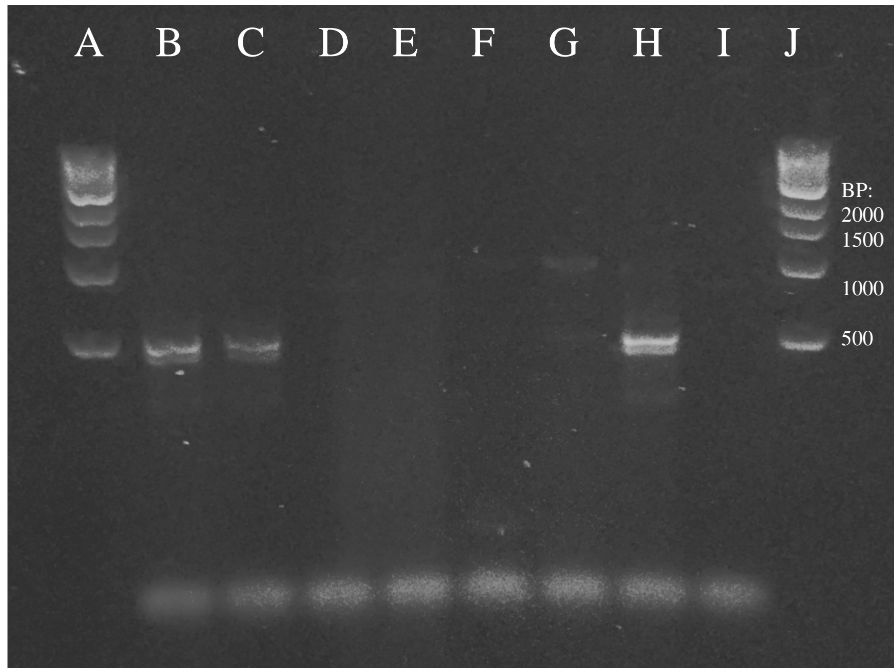

Figure 3: Typical separation on a 1.0% agarose gel of products from PCR amplification from single *Meloidogyne* second-stage juveniles. Lanes A and J: 1 kb ladder. Lane B: AU3 (Lee County) population identified as *M. incognita* with Inc-K14 primers. Lane C: AU32 (Autauga County) population identified as *M. incognita* with Inc-K14 primers. Lane D: AU24 (Henry County) population indicated to not be *M. incognita* with Inc-K14 primers. Lane E: Negative control with water and Inc-K14 primers. Lane F: AU3 population indicated not to be *M. arenaria* with Far/Rar primers. Lane G: AU32 population indicated not to be *M. arenaria* with Far/Rar primers. Lane H: AU24 population identified as *M. arenaria* with Far/Rar primers. Lane I: Negative control with water and Far/Rar primers.

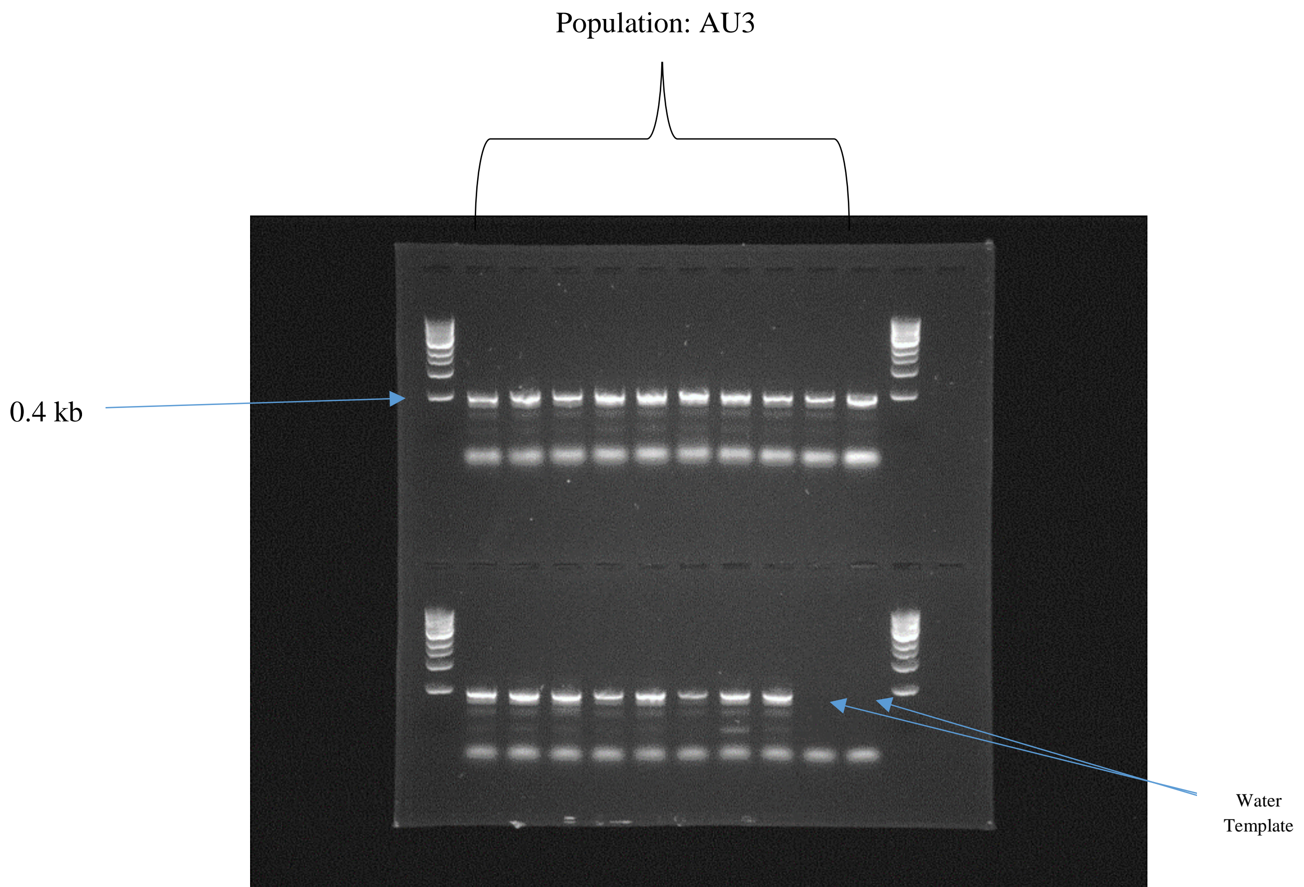

Figure 4: Typical separation on a 1.0% agarose gel of JB3/JB5 primer amplification with single *Meloidogyne* second-stage juvenile. The 400 bp (0.4 kb) product is characteristic of *Meloidogyne spp.,* but does not differentiate the different species. Four lanes of 1 Kb DNA ladder (Quick-Load 1Kb DNA ladder, New England Biolabs, Inc., Ipswich, MA). Empty lanes indicate water template as negative control.

Table 7: Percent matches of three populations of *Meloidogyne* to the four major RKN species based upon the NCBI Genome Database

AU1/Lee County (*M. incognita*)								
Primer:	*M. incognita*	Accession	*M. arenaria*	Accession	*M. javanica*	Accession	*M. hapla*	Accession
JB3/JB5	99%	JX683696	99%	KU360143	99%	JX683706	88%	KU517171
D2a/D3b	97%	KP901081	98%	AF435803	96%	JQ317915	97%	KJ645428
18S965/18S1573	98%	U81578	98%	KF112872	97%	JX100422	99%	AY268119
AU32/Autauga County (*M. incognita*)								
Primer:	*M. incognita*	Accession	*M. arenaria*	Accession	*M. javanica*	Accession	*M. hapla*	Accession
JB3/JB5	99%	JX683696	99%	KU360143	99%	JX683706	88%	KU517171
D2a/D3b	97%	KP901081	98%	AF435803	96%	JQ317915	97%	KJ645428
18S965/18S1573	98%	U81578	98%	KF112872	97%	JX100422	99%	AY268119
AU39/Henry County (*M. arenaria*)								
Primer:	*M. incognita*	Accession	*M. arenaria*	Accession	*M. javanica*	Accession	*M. hapla*	Accession
JB3/JB5	99%	JX683696	99%	KU360143	99%	JX683706	88%	KU517171
D2a/D3b	97%	KP901081	98%	AF435803	96%	JQ317915	97%	KJ645428
18S965/18S1573	98%	U81578	98%	KF112872	97%	JX100422	99%	AY268119

Alabama map of species present

With the success of species identification, a map and database were created for populations as they were identified (Table 8, Figure 5). Overall, 75 samples from 14 counties in Alabama were collected from grower fields for species analysis. All 75 RKN populations were screened with PCR, 17 with DHT, and 4 with morphological measurements. DNA sequencing was also performed on 3 of the RKN populations. Of these 75 samples, 73 were identified as *M. incognita* race 3, and 2 were identified as *M. arenaria* race 1 (Table 8). The *M. arenaria* populations were both found on peanut.

Table 8: Alabama *Meloidogyne* spp. (RKN) isolates, source, location and characterization based upon multiple ID methods

Accession Number*	Original Host	County	Species ID	Method Used for ID
AU1	Cotton	Lee	*M. incognita* race 3	DHT, Morphology, PCR
AU2	Cotton	Lee	*M. incognita* race *3*	DHT, PCR
AU3	Cotton	Lee	*M. incognita* race *3*	DHT, PCR
AU4	Cotton	Macon	*M. incognita* race 3	DHT, PCR
AU5	Cotton	Macon	*M. incognita* race 3	DHT, PCR
AU6	Cotton	Macon	*M. incognita* race 3	DHT, PCR
AU7	Cotton	Elmore	*M. incognita* race 3	DHT, PCR
AU8	Soybean	Elmore	*M. incognita* race 3	DHT, PCR
AU9	Cotton	Elmore	*M. incognita*	PCR
AU10	Cotton	Elmore	*M. incognita*	PCR
AU11	Cotton	Elmore	*M. incognita*	PCR
AU12	Soybean	Elmore	*M. incognita* race 3	DHT, PCR
AU13	Corn	Elmore	*M. incognita*	PCR
AU14	Corn	Butler	*M. incognita*	PCR
AU15	Cotton	Butler	*M. incognita*	PCR
AU16	Corn	Lee	*M. incognita*	PCR
AU17	Corn	Escambia	*M. incognita*	PCR
AU18	Corn	Escambia	*M. incognita*	PCR
AU19	Cotton	Escambia	*M. incognita*	PCR
AU20	Soybean	Escambia	*M. incognita*	PCR
AU21	Cotton	Lowndes	*M. incognita*	PCR
AU22	Soybean	Lowndes	*M. incognita*	PCR
AU23	Soybean	Escambia	*M. incognita*	PCR
AU24	Peanut	Henry	*M. arenaria* race 1	DHT, Morphology, PCR
AU25	Corn	Escambia	*M. incognita* race 3	DHT, PCR
AU26	Corn	Escambia	*M. incognita* race 3	DHT, PCR
AU27	Soybean	Covington	*M. incognita*	PCR
AU28	Soybean	Covington	*M. incognita*	PCR
AU29	Sweet Potato	Escambia	*M. incognita*	PCR
AU30	Corn	Montgomery	*M. incognita*	PCR
AU31	Soybean	Montgomery	*M. incognita*	PCR
AU32	Cotton	Autauga	*M. incognita* race 3	DHT, PCR
AU33	Cotton	Autauga	*M. incognita* race 3	DHT, Morphology, PCR
AU34	Soybean	Butler	*M. incognita*	PCR
AU35	Soybean	Butler	*M. incognita*	PCR

Accession Number	Original Host	Location	Species ID	Method Used for ID
AU36	Cotton	Covington	*M. incognita*	PCR
AU37	Cotton	Covington	*M. incognita*	PCR
AU38	Cotton	Covington	*M. incognita*	PCR
AU39	Peanut	Henry	*M. arenaria* race 1	DHT, Morphology, PCR
AU40	Cotton	Baldwin	*M. incognita* race 3	DHT, PCR
AU41	Soybean	Baldwin	*M. incognita* race 3	DHT, PCR
AU42	Corn	Baldwin	*M. incognita*	PCR
AU43	Corn	Baldwin	*M. incognita*	PCR
AU44	Corn	Covington	*M. incognita*	PCR
AU45	Cotton	Covington	*M. incognita*	PCR
AU46	Cotton	Covington	*M. incognita*	PCR
AU47	Tumeric	Escambia	*M. incognita*	PCR
AU48	Soybean	Escambia	*M. incognita*	PCR
AU49	Corn	Escambia	*M. incognita*	PCR
AU50	Corn	Escambia	*M. incognita*	PCR
AU51	Cotton	Escambia	*M. incognita*	PCR
AU52	Cotton	Escambia	*M. incognita*	PCR
AU53	Turfgrass	Escambia	*M. incognita*	PCR
AU54	Pepper	Escambia	*M. incognita*	PCR
AU55	Soybean	Macon	*M. incognita*	PCR
AU56	Soybean	Macon	*M. incognita*	PCR
AU57	Squash	Escambia	*M. incognita*	PCR
AU58	Corn	Crenshaw	*M. incognita*	PCR
AU59	Cotton	Crenshaw	*M. incognita*	PCR
AU60	Cotton	Crenshaw	*M. incognita*	PCR
AU61	Cotton	Crenshaw	*M. incognita*	PCR
AU62	Soybean	Crenshaw	*M. incognita*	PCR
AU63	Soybean	Crenshaw	*M. incognita*	PCR
AU64	Cotton	Crenshaw	*M. incognita*	PCR
AU65	Soybean	Crenshaw	*M. incognita*	PCR
AU66	Turfgrass	Lee	*M. incognita*	PCR
AU67	Squash	Escambia	*M. incognita*	PCR
AU68	Cotton	Butler	*M. incognita*	PCR
AU69	Cotton	Butler	*M. incognita*	PCR
AU70	Cotton	Butler	*M. incognita*	PCR
AU71	Cotton	Elmore	*M. incognita*	PCR
AU72	Soybean	Elmore	*M. incognita*	PCR
AU73	Corn	Elmore	*M. incognita*	PCR
AU74	Kiwi	Lee	*M. incognita*	PCR
AU75	Soybean	Escambia	*M. incognita*	PCR

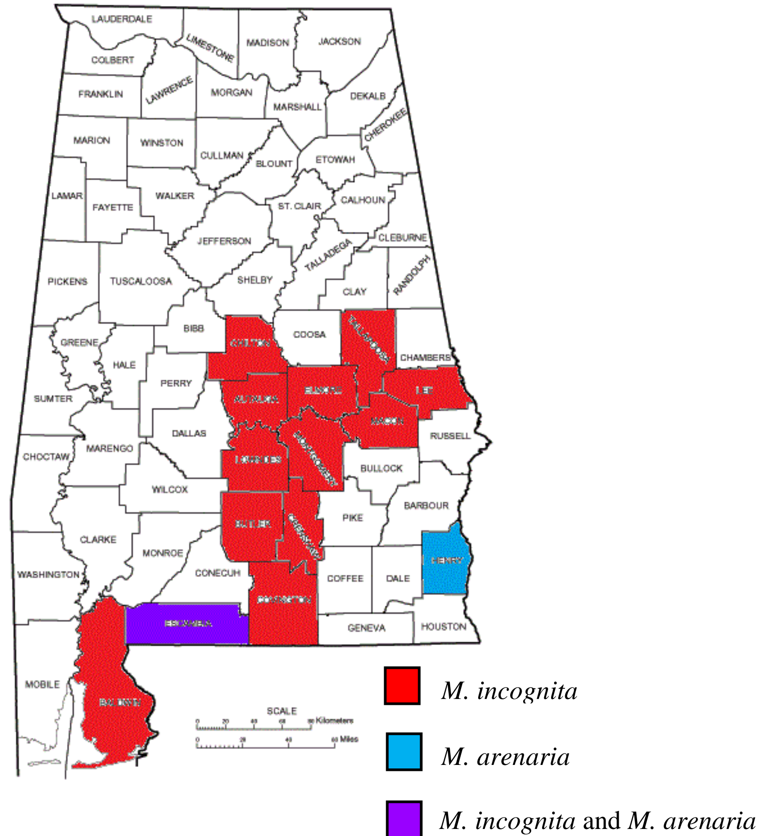

Figure 5: Statewide county map of Alabama showing distribution of species found in Alabama in as of June 2017. Red counties indicate *M. incognita* presence, blue counties indicate *M. arenaria* presence, and purple counties indicate both *M. incognita* and *M. arenaria* presence.

DISCUSSION

Morphological Identification

Morphological measurements of second-stage juveniles were unable to successfully identify the RKN populations AU1, AU33, AU24, and AU39 to species level. All species were within the average size of the RKN species *M. incognita* and *M. arenaria* (Eisenback et al. 1981). The *M. arenaria* populations were on average larger than the *M. incognita* populations, but both species fell into ranges of additional species, including *M. incognita, M. arenaria*, *M. javanica*, and *M. hapla* (Table 5) (Eisenback et al. 1981). This made identification based solely upon J2

measurements challenging. Previous studies have shown that this trend is typical (Eisenback, 1985; Chitwood, 1949; Cliff and Hirschmann, 1985). Thus, morphological measurements of J2's does not provide enough information for species identification. Perineal patterns were attempted, as they are the most important physical characteristic for species identification via morphological measurements. However, no clear perineal patterns were obtained for analysis.

Differential-host Test

The differential-host test proved to be a solid technique for RKN species identification. Seventeen different populations were evaluated with the differential-host test, and 15 were successful in identifying species and race of the unknown RKN population. The two populations that gave atypical results (AU2, AU3) did so because they reported as being a host of tobacco and not pepper, despite later being identified as *M. incognita* via several primer sets (MI-F/MI-R, IncK-14F/IncK-14R, Finc/Rinc). There are several possibilities as to why these results occurred. It is possible that these field populations were mixed with both *M. incognita* and *M. arenaria* populations. However, no *M. arenaria* was identified through molecular work at the conclusion of the DHT's for these populations. The more likely result is simply that an atypical response by the populations occurred. Previous studies have found that the NC differential-host test can have issues with reliability (Stanton and O'Donnell, 1998). In that study, it was found that approximately 14% of populations induced host reactions that were considered atypical, meaning no identity could be determined from the test alone. The results of this survey gave an atypical response 11% of the time, which is similar to that study. Thus, 15 of 17 populations evaluated were identified with the differential-host test at an 88% success rate for species identification.

Molecular Diagnostics

Molecular diagnostics also provided insight into species identification in Alabama. Of the 75 samples collected containing RKN for the survey, all were successfully identified through PCR using species-specific primers. These species were *M. incognita, M. arenaria, M. javanica, M. hapla, M. enterollobii, M. fallax,* and *M. chitwoodi*. Initially, low levels of PCR amplification occurred with a majority of primers screened. There are multiple possibilities for why this occurred. These include possible DNA degradation from improper storage, too low levels of DNA template for amplification, and initial misidentification of root-knot nematode and the sample being another type of nematode. Each are likely, and as the project continued the number of failed amplifications decreased by a large margin. Similar issues have been found and reported during similar studies (Khanal et al. 2016). The primer sets IncK-14F/IncK14-R (*M. incognita*) and Far/Rar (*M. arenaria*) were found to be the most robust in that all samples were identified with them. Other reports have found similar success with the use of these primers for PCR identification (Adam et al. 2006, Ye et al. 2015).

Sequencing of the 18S, 28S, and COI regions of populations AU1, AU32, and AU39 yielded identical results across all populations despite AU1 and AU32 being *M. incognita* and AU39 being *M. arenaria* (Table 7). In the 18S region, all populations had at least a 97% shared identity with *M. incognita, M. arenaria, M. javanica,* and *M. hapla.* This is a major conserved genetic region, and seems not to be specific enough for species differentiation among these RKN populations. In the 28S region there was more variability, but not enough to differentiate RKN species. Of the three regions, the COI region indicated the most similarity between *M. incognita, M. arenaria,* and *M. javanica.* All populations had a 99% shared identity to these species in this genetic region, but *M. hapla* only had an 88% shared identity. Previous work has shown that *M.*

incognita, M. arenaria, and *M. javanica* have high levels of genetic similarity (Powers and Harris, 1993; Powers, 2004). Based upon these results, the parts of the 18S, 28S, and COI regions that were sequenced are too similar between *M. incognita* and *M. arenaria* and did not provide RKN species identification. Going forward, more analysis needs to be done on other genetic regions that may provide stronger differentiation between *M. incognita, M. arenaria,* and *M. javanica.*

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Chapter 3: Root-knot nematode distribution based upon the differential-host test among multiple samples across different crops in a singular field

ABSTRACT

Three large soil samples were taken from a root-knot nematode (*Meloidogyne,* RKN) infested field in central Alabama at the Auburn University Plant Breeding Unit (PBU) near Tallassee, Alabama in October of 2016. A soil sample was taken from each crop: cotton, soybean, and corn. A differential-host test was then run on each of the samples for RKN species and race identification and for host range and reproductive analysis. The reproductive factor was calculated for each population on eight different crops, and all three samples were determined to be *Meloidogyne incognita* race 3. There was some variability in the reproductive factors for each population across the different hosts, but all three populations were the same RKN species and race. Thus, management for each section of the field remains the same in regards to crop rotation and nematicide application. However, this does suggest that there may be some variability of the reproductive factor between original host crops despite being the same species and race of RKN.

INTRODUCTION

The root-knot nematode (RKN, *Meloidogyne* spp.) is one of the most damaging plant parasitic nematodes in the world. It has been estimated to account for 14.6% of crop loss in tropical and sub-tropical climates, and 8.8% in developed countries (Nicol et al. 2011). With such a high potential for crop damage, detection and management of RKN populations in a field are very important. Since all species of RKN are host dependent, a grower can sometimes rotate to a nonhost crop if the nematode is identified to species level. For example, cotton growers often have infestations of *M. incognita*. To help lower this RKN populations density, a common crop rotation from cotton is peanut. However, peanut is an excellent host of *M. arenaria*, which can be found in the same field as *M. incognita* (Davis and Timper, 2000). Thus, knowing what species is present in a field is key for RKN management.

One of the most common ways to identify RKN species is through the differential-host test. This test was originally developed by Sasser (1972) to determine the species and race of four commonly found plant-parasitic root-knot nematodes in the southeastern United States (Table 1). These are *M. incognita* (race 1-4), *M. arenaria* (race 1-2), *M. javanica,* and *M. hapla.* For the differential-host test, an unknown nematode population is added to cotton (Deltapine 16), tobacco (NC 95), pepper (California Wonder), watermelon (Charleston Grey), peanut (Flor-runner), and tomato (Rutgers). After 45 days, the plants are harvested and host determination is made of each crop for the population, allowing for species and race identification to be made based upon the RKN populations reproductive host range. The overall objective of this study was to determine if reproductive factors calculated from the DHT of a RKN population differ based on the host crop in the field.

MATERIALS AND METHODS

Soil samples were taken from a 3.25-acre area section of a root-knot nematode infested field at the Plant Breeding Unit (PBU) located at the E.V. Smith Research Center of Auburn University near Tallassee, AL in October of 2016. The field has continually been used for root-knot nematode research for 10 years, and the crops planted in the field for the 2016 growing season were cotton, corn, and soybean. The field's soil is a Kalamia loamy sand (80% sand, 10% silt, 10% clay). Three independent soil samples were taken and sections were divided up based upon the previously planted crop (Figure 6). Soil samples were taken with a hand held conical soil probe (2.54 cm wide and 20 cm deep), and were evenly taken over the entire area of the field by walking a zigzag pattern over each area in order to fill up an entire 5-gallon bucket per sample. Once the soil samples were obtained, 100cc of soil was used for RKN extraction to determine RKN population density. Nematodes were extracted by gravity sieving followed by sucrose centrifugation following the methodology of Barker (1985). After extraction, nematode presence was confirmed and enumerated via a Nikon TSX 100 inverted microscope at 40-x magnification. Based upon the density of 100cc of soil, total population density was determined of the 5-gallon sample taken for initial population density at planting of the differential-host test.

Figure 6: Aerial image of the field at the Plant Breeding Unit (PBU) of Auburn University near Tallassee, Alabama showing three distinct zones of cotton, corn, and soybean crops from the 2016 growing season

Once initial density was known, the soil taken from PBU was used for three different differential-host tests (DHT) based upon crop sampled. For the DHT, six crops were planted in the RKN infested soil and grown over a 45-day period to determine the host range and reproductive factor of the unknown RKN population. The test was set up as described by Taylor and Sasser (1978). This test traditionally uses eggs to inoculate at planting of the DHT, but field soil with infective juveniles of a known density was used for the test, so no inoculation occurred. Crops used in the test were cotton (Fibermax 1944), tobacco (NC 95), pepper (California Wonder), watermelon (Charleston Grey), peanut (Flor-runner), and tomato (Rutgers) (Sasser 1972). For this test, corn (Mycogen 2R042; Dow AgroSciences, Indianapolis, IN) and soybean (Asgrow 5935; Monsanto Company, St. Louis, MO) were also included in the differential-host test. While these crops are not traditionally included in the DHT, they were included for reference as they were the original host of two of the three samples screened. The original publication of this by Sasser (1972) uses cotton variety Deltapine 16. We were unsuccessful in finding an adequate supply of this cotton seed, so Fibermax 1944 was used. FM 1944 is listed as susceptible to RKN, and

provides a suitable alternative to Deltapine 16. Each crop was planted in a 500 cm^3 polystyrene pot containing some of the soil samples taken from PBU. Planting rates depended upon crop and stand was thinned to two plants per pot immediately after seedling establishment. Each DHT was arranged in a randomized complete block design with five replications. Temperatures in the greenhouse ranged from 24 to 35°C with an average of 30°C.

The target date for test termination was 45 days after emergence. Plant shoots were cut from roots and roots were gently washed under a faucet to remove loose soil. Root and shoot weights were recorded and RKN was extracted from roots and soil. The RKN eggs were extracted from the plant roots by placing the root mass in a 0.625% NaOCl solution and shaken for 4 minutes at 1 g-force on a Barnstead Lab Line Max Q 5000 E Class shaker (Conquer Scientific, San Diego, CA). This was immediately followed by a rinse with water and physical scrubbing of the root system following the methodology of Hussey and Barker (1973). After scrubbing the roots, the eggs were collected on a 25-µm pore sieve and washed into a 50 mL centrifuge tube. RKN were run through a sucrose centrifugation as previously described. Eggs were enumerated via a Nikon TSX 100 inverted microscope at 40-x magnification. Juveniles were extracted from soil as previously described for calculating final density. These numbers were then used to determine the reproductive factor (Rf) of each host. Reproductive factor was calculated by the following formula: Rf = final nematode density of eggs and juveniles at 45 days ÷ initial nematode density of eggs and juveniles as described by Oostenbrink (1966). The Rf values were then grouped into four categories and are as follows: Rf = 0 – 0.09, nonhost; Rf = 0.1 – 0.9, poor host; Rf = 1-2, moderate host; R > 2, suitable host (Oostenbrink, 1966).

RESULTS

All three samples (AU76, AU77, AU78) were identified as *Meloidogyne incognita* race 3 through the use of the differential-host test (Table 9). The reproductive factor was highest on the crop that was the original host of the population in all three samples. AU76 had a Rf of 8.7 on cotton, AU77 had a Rf of 4.1 on corn, and AU78 had a Rf 6.6 on soybean. None of the three samples were a host of tobacco or peanut. All samples were a host of cotton, pepper, watermelon, tomato, corn and soybean.

Table 9: Greenhouse differential-host test results for the three samples across cotton, soybean, and corn taken from a 3.25 acre area of a field at the Auburn University Plant Breeding Unit in Tallassee, AL (32°29'43.2"N 85°53'26.3"W)

			Reproductive Factor								
Sample ID	**Original Host**	**Location**	**Cotton**	**Tobacco**	**Pepper**	**Watermelon**	**Peanut**	**Tomato**	**Corn**	**Soybean**	**Species ID**
AU76	Cotton	Elmore County, PBU	8.7 (+)	0.2 (-)	2.2 (+)	1.8 (+)	0.1 (-)	3.5 (+)	1.7 (+)	2.2 (+)	*M. incognita* race 3
AU77	Corn	Elmore County, PBU	2.7 (+)	0.4 (-)	1.9 (+)	2.3 (+)	0 (-)	2.8 (+)	4.1 (+)	1.7 (+)	*M. incognita* race 3
AU78	Soybean	Elmore County, PBU	3.2 (+)	0.1 (-)	3.4 (+)	1.7 (+)	0 (-)	4.5 (+)	2.1 (+)	6.6 (+)	*M. incognita* race 3

DISCUSSION

This trial successfully identified each population as *M. incognita* race 3. Thus, the management implications for this field are the same despite coming off three different host crops. The results indicate that there is only one species of RKN present in the field, and that a similar management approach can be used for all sections. It was interesting, however, that the reproductive factor for each population was highest on the original host crop of the population. AU76 and AU78 were very noticeable with this. AU76 had a Rf of 8.7 on cotton, and AU78 had a Rf of 6.6 on soybean. These numbers were much higher than the other crops across the DHT. In general, AU77 seemed to have a lower Rf across most crops that it was a host of compared to AU76 and AU78. These numbers seem to demonstrate that multiple populations of the same RKN

species may respond in a slightly different manner if taken from different host crops of a field. However, the differences are not significant enough to cause differences in management of an RKN population if the population is of the same species.

Overall, this study shows that there was minimal benefit of collecting RKN from different crops in the same field for species analysis even though the RKN population was present on all crops. However, the results of a differential-host test can vary among RKN populations sampled from a single field even when the results show that it is the same RKN species present.

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OVERALL CONCLUSION

This study's primary focus was on evaluating multiple techniques to develop an accurate and efficient assay that can differentiate RKN species. By using the differential-host test and PCR primers, this was successful for identifying two species of root-knot nematodes (*M. incognita* and *M. arenaria*) in Alabama. This is somewhat unusual based upon multiple previous surveys that found a wider variety of species in other regions of the United States. For example, a study in Arkansas identified *M. incognita, M. haplanaria, M. marylandi, M. hapla, M. arenaria,* and *M. partityla* (Khanal et al. 2016.). Another study identified *M. incognita, M. arenaria, M. hapla, M. graminis, M. javanica, M. chitwoodi, M. fallax, M. enterlobii, M. partityla,* and *M. konaensis* in a large regional survey of potato fields in seven states of the central United States (Powers et al. 2005). Thus, while only *M. incognita* and *M. arenaria* were found, it is likely that there are more species present in Alabama than have been found at this point.

Of the two species found during the survey, *M. incognita* was by far the most prevalent species found. Seventy-three of the 75 samples with RKN from throughout Alabama were identified as *M. incognita*, and the other 2 were identified as *M. arenaria.* This gave a 97% *M. incognita* identification and a 3% *M. arenaria* identification. *Meloidogyne incognita* was found on a wide range of crops: cotton, corn, soybean, sweet potato, squash, pepper, kiwi, turmeric, and turf, showing the wide host range and impact that *M. incognita* can have on a large scale (Taylor and Sasser, 1978; Moens et al. 2009). These 73 samples were found over 13 different counties, mostly in the central and southern parts of Alabama (Figure 5). Two of the 75 populations were identified as *M. arenaria*. Both of these populations were found on peanut fields in southern Alabama (Henry County and Escambia County) and identification was done through PCR and

confirmed with a differential-host test. These populations were also confirmed to have a 99% match with the *M. arenaria* GenBank accession number KP202350.1.

The goals of this project were to 1) establish base level root-knot nematode populations for building a diagnostic assay; 2) design a diagnostic assay to identify root-knot nematodes to a species level; and 3) begin building a species distribution map of RKN throughout Alabama. Base level RKN populations were easily obtained for beginning building the diagnostic assay. While morphological measurements of second-stage juveniles were not different enough between species for identification to be determined, they were beneficial for building an overall picture of the species found in Alabama. Both molecular techniques and the differential-host test proved to be the most reliable methods for RKN species identification. Of 17 Alabama populations analyzed via the DHT, 15 were successfully identified to species level. Thirteen of these were found to be *Meloidogyne incognita* race 3, and two were identified as *Meloidogyne arenaria* race 1. Two of the populations used in a differential-host test failed to produce reliable numbers for species and race identification. All 75 RKN populations that were found in Alabama were successfully identified through PCR. Primers IncK14-F/IncK14-R (*M. incognita* specific) and Far/Rar (*M. arenaria* specific) were the most reliable sets used, and consistently produced bands for species identification. A RKN species distribution map for Alabama has been started, and is continually being added to as new species are found. As of 2017, this research group has identified 73 populations of *M. incognita* in 13 different counties, and 2 populations of *M. arenaria* in 2 different counties.