Quantitative field testing *Heterodera glycines* from metagenomic DNA samples isolated directly from soil

By

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Molecular diagnostic assays have been developed and utilized to diagnose and to confirm the diagnoses of many plant-parasitic nematodes. We screened several gene sequences of soybean cyst nematode (SCN) [*Heterodera glycines,* Ichinohen] for their use as molecular markers. A methodology then was developed to use them to detect and quantify the number of *H. glycines* directly from Mississippi soil. A novel procedure utilizing multiple databases containing nematode DNA and EST sequences was developed to assist in the selection of SCN primers used in the PCR and qPCR assays. In vitro testing demonstrated that the DNA primers and probes developed from the novel procedure for the qPCR assays could accurately detect the presence of SCN. Subsequent testing resulted in a trend of increasing observed numbers of SCN contributing to increasing estimates by qPCR.

DEDICATION

I would like to dedicate this research to my grandmother, Yinxiu Wu, my parents, Xueqi Li and Jiuxiang Yao and my sister Xia Li.

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CHAPTER I

INTRODUCTION

Importance of Soybeans (*Glycine max* L. Merr.)

Soybeans (*Glycine max*), the second-largest crop in the U.S., contribute $18 billion annually to farm sales. The world soybean production increased by 4.6% annually from 1961 to 2007 and reached average annual production of 217.6 million tons in 2005-2007 (Masuda and Goldsmith, 2009). Soybeans serve as an oil seed crop and feed for livestock and aquaculture and also provide a good source of protein for the human diet (tofu, soybean cooking oil, soy milk, soy sauce), as well as bio-fuel feedstock. Bio-energy (biodiesel), primarily from soybean, accounted for an estimated 250 million gallons of fuel in 2006 (Carriquiry 2007). According to the National Biodiesel Board, there are 105 biodiesel manufacturing plants in operation as of early 2007 with an annual production capacity of 864 million gallons (Carriquiry 2007). Therefore, soybean is an important plant for the economy and the future of bio-fuel.

The host range of *H. glycines* has been updated to over 399 species (Niblack et al., 2002). Several plnat-parasitic nematodes are important pathogens of soybean, including the lesion nematode (*Pratylenchus* spp.), reniform nematode (*Rotylenchulus reniformis*), root-knot nematode (*Meloidogyne incognita*), lance nematode (*Hoplolaimus* spp.), and soybean cyst nematode (*Heterodera glycines*) (Fourie, McDonald et al 2001; Niblack et al., 2002). The most significant pathogen affecting soybean cultivation and

production is its major pathogen, *H. glycines*, the soybean cyst nematode. Each year soybean growers lose $ 1 billion in yield to this pathogen (Lawton, 2010).

Pathogenicity and distribution of soybean cyst nematode (*Heterodera glycines*)

Nematodes are microscopic worm like animals. They may be free-living, predacious, or parasitic. *H. glycines* is the major economic pathogen of soybean plants. This nematode can reproduce on at least but not limited to 97 legume hosts and 63 non-legume hosts (Epps and Chambers, 1958; Riggs and Hamblen, 1962, 1966a, b). In general, SCN has a wide host range, mainly on weed species, of at least 23 families, including *Boraginaceae*, *Capparaceae*, *Caryophyllaceae*, *Chenopodiaceae*, *Brassicaceae*, *Lamiaceae*, *Fabaceae*, *Scrophulariaceae* and *Solanaceae*. Typical weed hosts are *Cerastium holosteoides*, *Lamium amplexicaule* and *Stellaria media*. Some cultivated hosts, mainly *Fabaceae*, are *Lespedeza* spp., *Lupinus albus*, *Penstemon* spp., *Phaseolus vulga*ris, *Vicia villosa*, *Vigna angularis* and *V. radiate*, Sugarbeet (*Beta vulgaris*) and tomatoes (*Lycopersicon esculentum*) have been found to be experimental hosts (Epps and Chambers, 1958; Riggs and Hamblen, 1962, 1966a, b). The host range of *Heteradera glycines* has been updated to over 399 species (Niblack et al., 2002). However, soybean is the major economic host of SCN.

SCN infection of soybean is responsible for an estimated $ 1 billion in production losses, annually, in the U.S. (Lawton 2010) and about $15 billion worldwide. Earlier observations of nematodes that were probably SCN date back to 1881 (Noel 1992). In 1938 the nematode was reported from Manchuria (then an independent state, followed by China) and then from several other parts of Asia, including the Amur District in Russia (Ichinohe, 1961). SCN was first detected in North Carolina in 1954 (Winstead, 1955).

Unfortunately, SCN is readily transmissible as evidenced by its identification in localities as far away as Mississippi only a few years later in 1957 (Spears, 1957). The nematode subsequently has been identified in 26 states (Chen and MacDonald, 2001). SCN is not restricted to any particular soil type and spread anywhere under favorable conditions for them. Recent reports rank SCN infection as causing more agronomic loss of *G. max* production than the rest of its pathogens combined (Wrather et al. 2006). Management of SCN is accomplished partly by the use of resistant genotypes. Although the use of resistant genotypes is a component of recommended control measures (Riggs and Niblack, 1999), consistent use of soybean cultivars with the same sources of resistance can lead to adaptation of the existing SCN population to cultivars with that source of resistance (Luedders and Anand, 1989). Along with host resistance, crop rotation and application of nematicides are also greatly considered as good practices in the management of soybean cyst nematode (Niblack, 2005).

Life stage development of *Heterodera glycines*

Heterodera glycines is a bisexual cyst-forming species of plant-parasitic nematodes (Ichinohe, 1952). The timing of stages presented here is adapted from Lauritis et al. (1983) (Figure 1). Like all nematodes, *Heterodera glycines* has six life stages - egg, four juvenile stages (J1-J4), and the adult stage (Lauritis et al., 1983; Davis, Tylka, 2000). The duration of the SCN life cycle runs from 3-4 weeks, but this may be influenced by environmental conditions (mainly adequate temperature and moisture) (Lauritis et al., 1983; Davis, Tylka, 2000). Depending upon the environment, several generations of SCN can be completed in a typical soybean growing season. After embryonic development within the egg (Figure 2), first-stage juveniles (J1) molt into second-stage juveniles (J2)

within the eggs (Ichinohe, 1952) (Figure 2). The eggs hatch and the second stage juveniles (J2) (Figure 3) then migrate toward the root (Ichinohe, 1952). J2 become infective J2 when they invade the root (Lauritis et al. 1983). During this process, the infective J2 burrows through the epidermal and cortex cells, ultimately reaching the root stele (Lauritis et al. 1983). By one day post infection (dpi) the infective J2 pierce a cell, typically a pericycle cell, with its tubular mouthpiece known as a stylet (Lauritis et al. 1983). The infective J2 presumably injects substances into the pericycle cell, changing the physiology of that cell. Soon after, the cell walls of the feeding site initial (FSi) begin to dissolve, which allows the cell to merge with neighboring cells. Since the cells are now multinucleate with a common cytoplasm, the feeding site is known as a syncytium. The syncytium is a multinucleate transfer cell, meaning that it differentiates into a cell that is modified for short distance import of materials (Jones, 1981). The J2 nematodes then molt 2 additional times (J3, J4) (Lauritis et al. 1983). At the end of J3 stage, the mature mobile vermiform males will burrow out of the root (Lauritis et al. 1983). In contrast, the female nematode remains at this feeding site as it develops through the vermiform juvenile stages into the swollen adult form (Lauritis et al. 1983). The swelling of the female disrupts the tissues of the host root, and the body of the nematode finally protrudes from the surface (Lauritis et al. 1983). The vermiform males are attracted toward the female by sex hormones (Perry and Aumann, 1998), such as vanillic acid (Jeffe et al. 1989), where copulation takes place (Lauritis et al. 1983). Eggs are formed within the female and some are laid into an egg sac or gelatinous matrix. Males may sometimes be found in the gelatinous matrix. When the female dies, the body becomes a hardened protective cyst (Figure 4) enclosing the eggs (Lauritis et al. 1983). In the

absence of a host, eggs within the cysts may remain viable in soil for 9 years (Inagaki and Tsutsumi, 1971), making SCN infestation problem more difficult to manage.

Management practices for *Heterodera glycines*

Soybean cyst nematode in a production field is not always possible to eliminate once it has become established. Therefore, the nematode must be managed using all available agronomic practices. The primary objective of soybean management is to reduce nematode numbers to a level that is not injurious to soybean plant health and yield potential. This goal requires an integrated approach which combines the use of resistant varieties, crop rotation, and the use of nematicides when necessary.

Resistant soybean varieties

Unlike susceptible varieties, resistant soybean varieties reduce the ability of SCN to develop and complete its life cycle on soybean plants. An HG Type is a description or profile of a *Heterodera glycines* SCN population based on the nematodes' ability to develop on resistant soybean lines (Niblack, 2005). The HG Type test is similar to a race test (a test that determines a group of nematodes with certain hosts), but is more informative and easier to understand. The number or numbers in the HG Type designation correspond directly to sources of resistance used in available SCN-resistant soybean varieties (Table 1).

Using resistant varieties is one of the most efficient and economical methods of SCN control. However, resistant varieties vary in their levels of resistance. In general, SCN reproduction on a resistant soybean variety will be less than 10 percent of what occurs on a susceptible variety. The use of resistant varieties (e.g. *G. max*[Peking/PI 548402]

and *G. max*[PI 88788]) allows the farmers to grow soybeans profitably, while managing SCN numbers so that future soybean crops may be profitably grown. In the recent past, farmers may have been reluctant to use resistant varieties because there was a yield gap between resistant and susceptible varieties in fields that were not infested with SCN (Niblack, 2005). Because of the joint efforts of soybean breeders and nematologists, high yielding SCN-resistant varieties are now available.

Several different sources of SCN resistance have been used to develop resistant soybean varieties (Table 1). The accessions are denoted by a plant introduction (PI) number. The lines in Table 1 are the original soybean lines from China or Russia that are the ancestors of every SCN-resistant variety today.

Most individual resistant varieties carry resistance from only one source. This may allow the farmer to rotate sources of SCN resistance to help prevent the development of more damaging HG Types. Unfortunately, SCN-resistant varieties which yield comparably do not necessarily control the nematode equally (Niblack, 2005). Furthermore, even the best SCN-resistant varieties can vary considerably in how well they control nematode population densities. Greater SCN reproduction will result in a higher SCN population in the soil the next time subsequent susceptible crops are grown in that field. Consequently, growers must consider how SCN resistant soybean varieties affect SCN populations in addition to how well the varieties yield to maintain the long-term productivity of the field for soybean production. Selecting SCN-resistant varieties based solely on yield data is short-sighted and risky because some relatively high-yielding soybean varieties allow substantial amounts of SCN reproduction (Niblack, 2005).

Crop rotation

Crop rotation produces many benefits and should be part of the management program whether the field has SCN or not. If it has SCN, crop rotation should include nonhost crops and resistant soybean varieties. A good SCN management plan should not include other hosts for SCN since SCN has a wide host range (Niblack et al., 2002). SCN population numbers have been increasing in edible bean production areas of the United States and Canada, and their inclusion in a rotation system will only increase SCN populations.

Nonhost crops (corn, cotton, rice, peanut and sweet potato, etc.) cannot be used as a food source by SCN. In a field planted to a nonhost, SCN numbers will not increase and should decrease. When nonhosts are grown, juveniles will hatch from a percentage of the eggs and will starve or be destroyed by natural enemies. The amount of decrease of SCN varies in relation to geographical area. SCN numbers may decrease by as much as 90 percent in the southern United States but only 10 to 40 percent in the north (some of the difference is due to poor winter survival in the South due to higher soil temperatures, which allows hatching) (Niblack et al., 2005).

Rotation design depends on conditions specific to the farm and individual fields as well as commodity prices and input costs. Success at reducing SCN numbers is clearly related to geographical region. Farmers in the northern Soybean Belt will observe slow reduction in SCN regardless of rotation design. In these areas, more frequent use of nonhost crops is appropriate. Several rotation sequences may be required before an appreciable drop in SCN is observed. Farmers in the southern United States usually observe a more rapid reduction in SCN numbers. A southern rotation may consist of

alternating years of nonhosts and resistant soybean varieties. Double-cropped soybean after wheat should be considered after a full year of soybean. SCN buildup in double-crop soybean may be less than in full season soybean, but a significant increase is just as likely to occur. Rotation designs have been thoroughly tested in many locations in the United States. The slower that SCN numbers decrease, the more often it is needed to grow non-host crops. Monitoring the population of SCN is essential for determination of the effectiveness of the rotation. If SCN numbers increase on resistant varieties, their source of resistance may no longer be effective and they should choose a variety with a different source of resistance or plant a nonhost crop.

Chemical control

An SCN control program using a combination of resistant varieties and rotation is generally more effective than nematicides in reducing SCN umbers and is more economically feasible in most instances (Niblack et al., 2005). Usually a susceptible variety with a nematicide does not yield as well as a resistant variety without a nematicide (Niblack et al., 2005). Circumstances such as development of new races of the SCN, poorly adapted resistant varieties, infeasibility of growing nonhost crops or occurrence of nematodes other than SCN may lead some farmers to use nematicides with well-adapted susceptible varieties. A fair to good yield increase because of a nematicide depends on the SCN population level and the susceptibility of the variety grown (Niblack et al., 2005).

The soil from infested fields or those suspected of having an SCN problem should be analyzed for the problem of the nematode before investing in nematicides or application equipment. Usually, nematicides do not generate season-long control of SCN

but if applied at planting will last long enough to provide an economic return. By the end of a growing season, the number of cysts and juveniles present in the soil may be almost the same or even higher than at planting. No nematicide will kill all SCN in the soil. Therefore, producers may need to use a nematicide every year where susceptible varieties are planted annually in cyst infested soils.

Nematicides currently available for control of SCN are classified as fumigants or non-fumigants. Fumigant nematicides (e.g. Telone II, Vapam, etc.) spread through the soil as gases and kill nematodes directly. The non-fumigant nematicides (e.g. Temik, Vydate, etc.) may kill the nematodes by contact in the soil but usually have some systemic properties and are absorbed by the soybean's root system.

Biological control

Biological control is the use of natural enemies to reduce a pest population. Some fungi, bacteria, predacious nematodes and mites are natural enemies of nematodes. SCN eggs can be parasitized by several fungi (Niblack, 2005). Naturally occurring populations of these fungi may reduce SCN populations over many years. These fungi are widely distributed in soybean growing areas. The buildup of these natural enemies depends on the types already present in a field and conditions favorable for their growth and development. Biological control can reduce SCN populations, synergistically; it can improve the efficiency of other control methods such as nematicides or resistant varieties.

The development of molecular diagnostic tools for nematodes

Molecular diagnostic tests have been developed and utilized to diagnose as well as confirm diagnoses of many plant-parasitic nematodes (Block et al. 2005; Gasser et al.

2006; Showmaker et al. 2011). The first molecular technique developed for nematode species identification was isozyme analysis of the *Meloidogyne* genus, with the markers developed in the 1970's (Esbenshade and Triantaphyllou, 1990). After the development of DNA amplification technology through polymerase chain reaction (PCR), the potential for molecular diagnostics increased dramatically. Nucleic acids analysis of plant-parasitic nematodes began with the study of genomic and mitochondrial DNA (Harris et al., 1990). The PCR analysis was applied to develop simple-sequence length polymorphisms (SSLPs), restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphisms (AFLPs) from *Meloidogyne* spp. (Gasser and Monti, 1997; Powers and Harris, 1993; Cenis, 1993; Semblat et al., 1998). The methodology was used to collect specific nematode species, extract DNA, conduct PCR, employ restriction enzymes where applicable, and analyze the products on an agarose gel. This method provided a DNA fingerprint that was unique to the taxon. A similar approach was used to develop markers for *R. reniformis* in Mississippi (Showmaker et al., 2011).

Molecular diagnostics involving PCR assays can obtain several types of data that are useful for diagnostic study (Showmaker et al., 2011). These types of data include present-absent outcome, where a specific piece of DNA can be amplified specifically in one taxon but not another. The type of PCR reaction that produces a single unique amplicon specific to a taxon is easily utilized in diagnostic screening. Analysis of PCR assays that produce a specific amplicon is quickly conducted by viewing the presence or absence of a specific band on an agarose gel. A second type of information is obtained by the banding patterns of the DNA when they are run out on an agarose gel (Showmaker et

al., 2011). These fingerprinting methods in which every band must be compared with other bands in different taxa are more cumbersome to work with and may involve interpretation (Showmaker et al., 2011). This would be unfavorable if the goal of the molecular diagnostic was not having highly experienced and highly remunerated workers perform the work. Diagnostic PCR assays that produce a single confirmative amplicon, thus, have many characteristics that make them advantageous over fingerprinting diagnostic PCR assays that use specific banding patterns to identify species or different taxa (Showmaker et al., 2011).

Single amplicon diagnostic PCR assays have been designed utilizing different methodologies which produce a species specific primer pair or a genus specific primer pair. These methods include sequence characterized amplified region (SCAR) and RAPD analyses. Other methods include those involving primer design from sequenced genes such as heat shock protein 70 (HSP70), glucuronidase, and the ribosomal DNA (rDNA) genes 28S 18S, 5.8S, D2D3 and IGS, intertranscribed spacer 1 (ITS1), intertranscribed spacer 2 (ITS2). In these cases, taxon specific primer pairs are designed from known gene sequences (e.g. HSP70, glucuronidase and ribosomal DNA genes and spacers) by obtaining the homologous sequences of the genes for the target organism and off target organisms and then aligning their primary sequence (Blok 2005, Berry et al. 2008, Showmaker et al. 2011). Subsequently, primer pairs are selected in DNA regions where they differ in sequence. The SCAR and RAPD species specific primer pair generation is conducted by running a RAPD PCR reaction which is conducted with shorter primer pairs than those that randomly amplify DNA from the target and off target organisms (Blok 2005, Berry et al. 2008, Showmaker et al. 2011). After the PCR, the randomly

amplified DNA is subject to electrophoresis on an agarose gel. The banding pattern on the gel that represents the amplified DNA fragments is analyzed. The bands are identified that are only present in the target taxon that is the focus of the particular analysis. The bands unique to the target taxon are excised, and their DNA is isolated, cloned and sequenced. The produced sequence is used to generate new primers either by extending the original rapid primers used in the original PCR reaction or by generating new primers from the sequence within the RAPD amplicon. This procedure is performed just as in the species specific primer pair generating process from the gene sequences under study. Importantly, the procedure is simple and reliable. For example, Agudelo et al (2005) reported a sequence for the first ITS1 region of the ribosomal DNA segment of *Rotylenchulus reniformis*. The sequence was shown to be present in 27 populations of the *R. reniformis* that were isolated from around the world. The regions included populations from Honduras, Columbia, United States, and Japan. The ITS1 sequence reported only differed from a pathogenic strain of *R. reniformis* found in Japan, which is not known to occur in the continental United States (Agudelo et al., 2005).

Quantitative PCR

The PCR-based strategy can provide usable information on the presence of a particular organism in an environmental sample. An extension of the PCR method ushers in quantitative capability, allowing the researcher to determine the exact number of individuals in a population (Showmaker et al., 2011). This method, known as real-time PCR or quantitative PCR, specifically assays the PCR reaction by incorporating a molecular tag whose amplification products are measured during the PCR reaction. The ability to measure PCR during the reaction provides a quantifiable measure of relative

amounts of DNA that are synthesized from a known standard template and unknown samples. Comparative analyses between the known sample and the unknown provide a way to analytically determine the amount of a taxon in the unknown as compared to the known sample (Livak and Schmittgen, 2001). Currently, little research has been conducted on the potential of real-time PCR for diagnostic nematology and its suitability has yet to be determined. Population estimates for species such as, *Meloidogyne javanica*, *Pratylenchus zeae*, and *Xiphinema elongatum* have been determined with real time PCR on native soil samples from trial plots (Berry et al., 2008). As with single amplicon, diagnostic PCR assays such as real-time PCR have been designed from relatively few loci of rDNA and major sperm protein 1 (Mspl) satellite DNA, specifically.

In contrast to the dearth of studies in using real-time PCR for analyses involving plant parasitic nematodes, real-time PCR's ability to quantify the PCR amplification process is a highly utilized tool in pathogen detection. It has been used to identify and quantify various bacteria, fungi, and viruses (Atallah and Stevenson, 2006; Gao et al., 2004; Okubara et al., 2008; Finetti-Sialer and Ciancio, 2005). In those studies, the samples were taken from the soil, plant tissues, or nematode samples. Real-time PCR works quantitatively because it detects pathogens by the using the amount of DNA present in a sample to obtain a cycle threshold (Ct) value which corresponds to the level of infection (Livak and Schmittgen, 2001). Real-time PCR is advantageous over regular PCR because it provides a quick gel-free way to screen PCR reactions which can increase work output relative to the time invested by the investigator to conduct the reaction. The work is gel free because the analyses are done during the PCR reaction by the thermocycling machine. By accurately and effectively detecting the different levels of

template DNA present from a desired genome(s), estimates of the amount of the species (e.g. number of individuals or number of colony forming units) can be generated from a designed model.

Advent of genomics in molecular diagnostics

The availability of genome data from the nematode *Caenorhabditis elegans* has simplified molecular diagnostics for parasitic nematodes (The *Caenorhabditis elegans* Sequencing Consortium, 1998). That work was followed by its relative *C. briggsae* (Stein et al., 2003). The availability of plant-parasitic nematode genomes for *Meloidogyne incognita* (Abad et al., 2008) and *Meloidogyne hapla* (Opperman et al., 2008) have allowed for a variety of comparative studies. The *C. elegans* genome has been a useful tool for cellular and developmental biologists and possesses the highest quality annotation genome of any nematode because of the extensive molecular work done on it. The *C. elegans* research community has established a data warehouse at the website http://www.wormbase.org/ that houses the genome as well as genetic and bioinformatics-based information. Reverse genetic information of *C. elegans* development received a substantial boost by the identification of RNA interference (Fire et al., 1998). In classical genetic analyses, mutants are created in screens, and mutant phenotypes are observed, scored, studied and their cellular and developmental defects are studied as the genes underlying the process are cloned. In contrast to classical genetic analyses, RNAi works when double stranded RNA for a particular gene is ingested by the nematode, injected by the researcher or imbibed through cellular pores in a process called soaking. Typically, the ensuing development or cellular process(es) are altered in a manner that copies the phenotype observed in mutants for that gene. Hence, RNAi defects are called

phenocopies (Fire et al., 1998). Extensive RNAi work has been conducted on *C. elegans* to identify phenotypes lacking specific genes which have been knocked out through the RNAi pathway (Fire et al., 1998; Piano et al., 2000; Kamath et al., 2003; Sonnichsen et al., 2005). Subsequent studies determined that the process also works in plant-parasitic nematodes (Urwin et al., 2001; Alkharouf et al., 2007; Klink et al., 2009).

In 2007, Alkharouf et al. (2007) made a comparative analysis between approximately 24,000 expressed sequence tags (ESTs) of SCN and both the genome and EST sequences of *C. elegans*. The purpose of the analysis was to identify genes that with a very high probability would be essential for the viability of SCN. The analysis of the 24,000 SCN ESTs resulted in them filtering down into 8,334 unigenes. Those unigenes were then compared to the *C. elegans* sequences and parsed into six groups based on the conservation level of their primary DNA sequences. The group with the most highly conserved gene sequences had 1,508 genes. Of those 1,508 Group 1 genes that were the most highly conserved between SCN and *C. elegans*, 266 were also shown to have lethal RNAi phenocopies when silenced or mutated in *C. elegans* (Alkharouf et al., 2007). The genes, while clearly being homologs, were also shown to function in a similar manner between SCN and *C. elegans* in yielding lethal phenocopies in SCN (Alkharouf et al., 2007; Klink et al., 2009; Li et al., 2010). The availability of the data simplified the selection of genes for the development of molecular markers.

Thesis objectives

Previously, from research work presented by Alkharouf et al. (2007), markers in the form of genus-level-specific amplicons were generated with available genomic and cDNA sequences from SCN. The sequences provided a framework on which

discrimination against other nematode sequences (e.g. *Meloidogyne incognita* and *Rotylenchulus reniformis*) were built. *M. incognita* and *R. reniformis* were examined since they represent other significant endoparasitic plant nematodes (EPNs) in the soil microfauna. It is noted that they do not represent the nearest sister group to the genus *Heterodera*. The work presented by Alkharouf et al., (2007) that the genes identified in soybean cyst nematode and additional analyses presented herein permitted the generation of taxon-specific primers because those DNA sequences provided enough information for a basis for discrimination. The analyses determined the homology between the genes from different taxa while also being unique enough so that a genus-level primer pair and Taqman® probe were created from them. The genus-specific primers (designed from the sequences identified by Alkharouf et al., 2007) generated in this study were screened through traditional PCR to determine their specificity. Once a primer pair has shown to be specific to SCN, Taqman® real-time PCR probes were created. Genus-level real-time PCR assay was developed for its potential to diagnose and quantify the SCN from field samples. Therefore, the specific objectives of this study are, 1) to identify SCN and isolate metagenomic (SCN) DNA directly from soil; 2) to screen and generate molecular markers for PCR; 3) to develop quantitative PCR-based SCN diagnostic methodology.

Table 1 The seven soybean plant introductions used in HG type tests. (Niblack and Tylka, 2005)

HG type index number	HG type indicator line
1	**PI 548402(Peking)**
2	**PI 88788**
3	**PI 90763**
4	**PI 437654**
5	**PI 209332**
6	**PI 89772**
7	**PI 548316 (Cloud)**

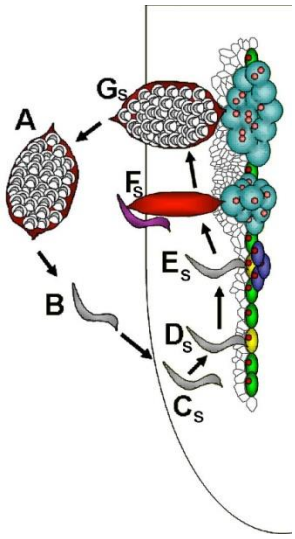

Figure 1 Life stage development of SCN

Notes: A, cyst. B, pre-infective-J2 (gray) hatch and migrate toward the root of soybean plants. C_S, infective-J2 nematodes burrow into the root and migrate toward the pericycle (green). D_S, infective-J2 select a cell (yellow) for feeding site establishment. E_S, infective-J2 nematodes have molted into J3. F_S, The J3 undergo a subsequent molt into J4 nematodes. Meanwhile, the female continues to grow circumferentially as it feeds. The male discontinues feeding at the end of its J3 stage. Male and female J4 nematodes become adults. The vermiform male (blue) burrows outside the root and subsequently copulates with the female. G_S, After ~30 days, the female with eggs is clearly visible and emerging from the root. Figure adapted from Lauritis et al. (1983) and Klink et al. (2009a)

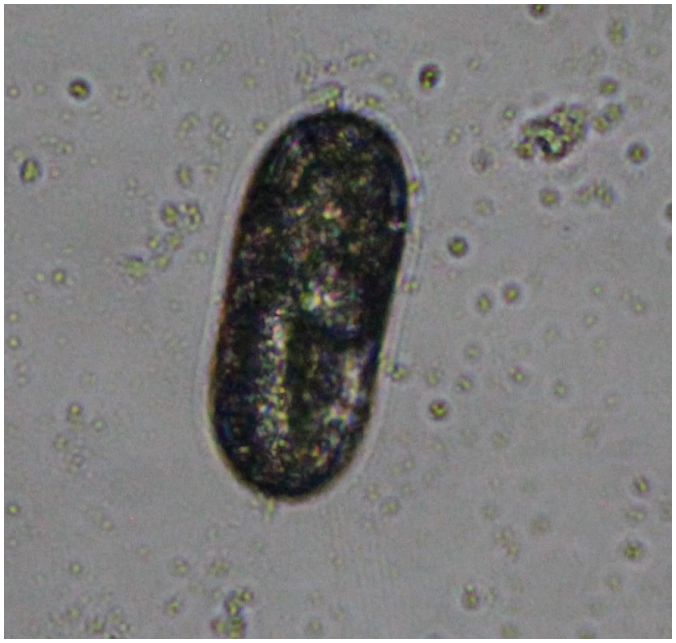

Figure 2 Egg stage of SCN, 1300x

Figure 3 Second-stage juvenile (J2) of SCN, 1,000x

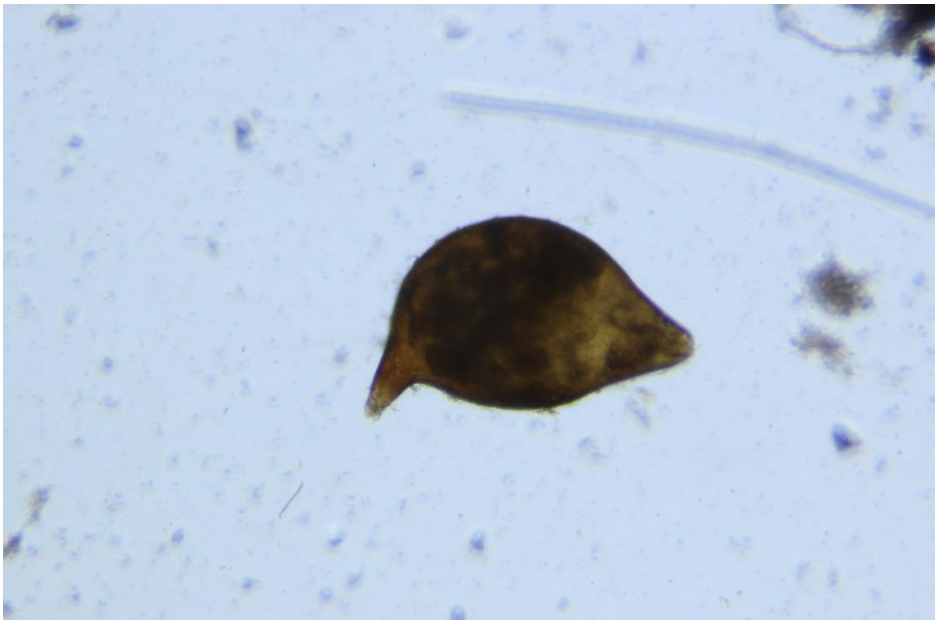

Figure 4 Cyst stage of SCN, 10x

CHAPTER II

MATERIALS AND METHODS

Plant and nematode procurement

The target nematode *H. glycines* and off-target nematodes *M. incognita* and *R. reniformis* from Mississippi and Alabama were cultured under ambient conditions in a greenhouse at the Mississippi Agriculture and Forestry Experiment Station (MAFES), RR Foil Plant Science Research Center at North Farm, Mississippi State University. Supplemental fluorescent light was provided to bring the day length to a 16 hour day/8 hour night cycle. Temperatures were kept in a constant temperature range between 28.9-34.4^{o}C (84.0-94.0^{o}F). Nematodes were cultured in 500 cm^{3} diameter clay pots for a period of 2-6 months in a 50:50 mixture of a fine sandy loam (46.25 % sand, 46.50 % silt, and 7.25 % clay) and sand (93.00 % sand, 5.75 % silt, and 1.25 % clay). Harvesting cysts was accomplished by massaging the infected roots in water. Massaging was achieved by placing the infected root mass between the index finger and thumb. The index finger and thumb were gently rubbed together with the root mass between them. This gentle rubbing activity dislodged the cysts so that they could be collected on a 150 μm pore sieve under a constant water steam. The cysts are not damaged because of their protective hardened nature. Cysts were collected by rinsing them through nested 850 μm pore sieve (debris) and a 150 μm sieve (cyst). Then cysts were rinsed out of the 150 μm

pore sieve into a beaker with 100 ml of water. Juveniles were collected by rinsing them through 250 μm pore sieve (debris) and 45 μm pore sieve (J2) and transferred to a beaker with 100 ml water in it for counting. Cysts were crushed by using Janke & Kunkel IKA-WERK crushing machine (IKA-WORKS, INC), for 30 seconds to 1 minute with a timer. Removal of debris smaller than the eggs was done by washing the slurry though a 25 μm pore sieve. The eggs were transferred to a beaker with 100 ml water in it for counting.

Plant inoculation

Prior to nematode inoculation, Hutcheson[PI 518664] seedlings of *G. max* were grown in sterilized sand: soil mix (1:1) in clay pots for a period of 7 to 14 days. The nematodes of second stage juveniles were counted, concentrated and diluted to a final concentration of 2,000 J2/3 ml which were then added to each root. This meant that 3 ml of inoculums that contained 2,000 nematodes were added to each root system on each plant by inoculating on two spots evenly.

Soybean cyst nematode DNA isolation from individual cyst

The first objective was to isolate DNA from a single cyst. To accomplish this, a single cyst was picked up under a stero-microscope and placed into a 1.5 ml micro-centrifuge tube, with 14 μl sterile distilled water. The tube was placed in -20^{o}C for 1 hour to freeze the cyst (or liquid nitrogen for 1 min). A glass rod sterilized with 75% ethanol was used to grind the ice with the frozen cyst until it was thawed. After grinding, 3 μl 10x PCR buffer and 3μl proteinase K was then added to the tube. The material was incubated at -20^{o}C for at least 2 hours. The material was incubated for 90 min at 65^{o}C to denature the protein, and 15 min at 9^{o}C to unwind the DNA. The material was

centrifuged for 1 min at 8,000 rpm with the supernatant transferred to a new 1.5 ml microcentrifuge tube. The DNA was stored at -20^{o}C for later PCR analysis.

Primer pairs screening

Research presented by Alkharouf et al. (2007) was used as a guide to identify SCN genes that would serve for primer design. The methodology was adapted from Showmaker et al. (2011) who used the identical methodology for quantitative molecular detection of *Rotylenchulus reniformis* DNA from metagenomic samples isolated from soil. Alkharouf et al. (2007) had divided genes into six groups based on their homology to SCN unigenes. The first group (Group 1) had 266 genes with *E*-values (Expect value, a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size) between 0 and 1*E*-100. The list was used as a guide to screen genes of which can amplify products from metagenomic DNA from soil. Using the single cyst DNA to test 11 primers (Table 2) made from the gene list, the primer pairs that showed amplification products well on agarose were chosen to be sequenced. These 11 pairs of primers (Table 2) (Alkharouf et al. 2007) were designed at 100 bp amplification of the products. Tests on these 11 pairs of primers were run on pure single nematode cyst DNA, the PCR and 0.1% agarose gel showed that Hg-unc-78, Hg-dys-1, Hg-unc-89 and Hg-unc-9 worked on pure single SCN cyst DNA. From later PCR tests of screening, Hg-unc-78 primarily showed the ability to amplify the gene and was able to detect the different amount of DNA from certain numbers of second stage juveniles (1, 10, 100, and 1000). Detailed information of the primers amplifying products of 100 bp and 300 bp are listed in Table 2 and Table 3. The amplification product that matched its DNA sequence was used for further analysis.

Metagenomic soybean cyst nematode DNA isolation

Metagenomic DNA from soil isolations were conducted by using the Powersoil DNA extraction kit (MO BIO Laboratories, Inc; Carlsbad, CA). The manufacturer's protocol was followed with modifications. The modifications included using 0.3 grams of soil instead of 0.25 grams of soil in step for developing the standard curve and adding 0.1 ml of nematode suspension (water containing the numbers of J2 extracted from greenhouse cultured soil samples). In its place, 0.1 ml of the nematode suspension, extracted from either greenhouse pots or field soil, was pipetted into the bead beating tube. Secondly, in the step when first instructed to remove the supernatant, a standard volume of 550 ml of supernatant was removed from each tube. This ensured success for downstream applications. The DNA was eluted from the spin column in 50 µl of nuclease free water (Promega; Madison, WI) and stored at –20°C.

Standare PCR conditions

For PCR, a 50 µl PCR reaction consisting of 2 µl DNA template, 5.0 µl 10x PCR buffer, 1.0 µl dNTP, 3.0 µl $MgCl_2$, 0.25 µl recombinant Taq Polymerase, 1.25 µl of 100 nM forward and reverse primers each, 36 µl nuclease free water (Ambion) (Promega®) was used. The reaction conditions, as reported by Agudelo et al. (2005) were modified to include a 2 minute pre-denaturation step at 94^{o}C. The procedure then followed the Agudelo et al. (2005) protocol that included a denaturation at 94^{o}C for 45 sec, annealing at 59^{o}C for 45 sec and primer extension at 72^{o}C for 60 sec for 40 cycles. The PCR reaction products were run out by gel electrophoresis on a 1% agarose gel with 0.01% SYBR-Green incorporated into the gel. The DNA amplification products were visualized

and recorded with digital imagery using a FOTO/Analyst Apprentice System® (FOTODYNE® Inc.; Hartland, WI).

Gel extraction, D-Topo cloning and sequencing

After using accuprime polymerase for the PCR, the bright bands were extracted on the gel by using the gel extraction kit (QIAQuick Gel Extraction Kit), mixing 4 μl of the gel purified DNA with 1 μl of salt solution and 1 μl of TOPO vector for the TOPO cloning reaction (pENTR ^{TM} Directional TOPO Cloning Kits, Invitrogen) in a 1.5 ml microcentrifuge tube. The tube was incubated at room temperature for 5 min and then placed on ice. Chemically competent *E. coli* cells stored at -80^{o}C were thawed on ice. Transformation began by distributing the *E. coli* cells in a separate 1.5 ml microcentrifuge tube, at a volume of 20 μl each, which is proceeded on ice. Then, 3 μl of TOPO cloning reaction was added to the *E. coli* cells, tapping the tube gently. The *E. coli* solution was incubated on ice for 15 min followed by heat shocking the *E. coli* cells at 42^{o}C for 30 seconds on the heat block. The *E. coli* solution then was placed back on ice immediately. Subsequently, 100 μl of SOC media was added to the tube and shaken at 37^{o} C for 1 hour. After incubation, 70 μl of the solution was plated out on LB-kanamycin plates. The plates were incubated at 37^{o}C overnight for 12-24 hours. The next day, single colonies were picked and placed in a 50 ml centrifuge tube containing LB-kanamycin culture agar. The tubes were cultured for 37^{o}C for no more than 16 hours, usually around 15 hours. The culture was spun down and followed by a plasmid prep according to manufacturer's suggestions (QIAPrep, Miniprep, QIAGEN).

Quantitative PCR conditions

Quantitative PCR (qPCR) Taqman® 6-carboxyfluorescein (6-FAM) probes (MWG Operon; Birmingham, AL) were used. First, I designed the forward and reverse primers for approximately 100 bp products through Primer 3 Output, with the Tm values about 63 ºC. And then I chose the center of the product sequence for about 20 bp with a Tm 70 ºC. The 6-FAM probes have a maximum excitation at 495 nm and maximum emission at 520 nm. The quencher used in the qPCR reactions was the Black Hole Quencher (BHQ1) (MWG Operon®), with the maximum excitation at 534 nm that was used for the analyses. Assays were conducted for primers that produced a single amplicon and had no off target amplification that were determined during the previous screening procedure. The qPCR reaction conditions included a 20 µl Taqman Gene Expression Master Mix (Applied Biosystems®; Foster City, CA), 0.9 µl 100 uM forward primer, 0.9 µl 100 uM reverse primer, 2 µl 2.5 µM 6-FAM (MWG Operon®) probe and 4.4 µl metagenomic template DNA. The conditions were a denaturation at 94ºC for 45 sec, annealing at 54ºC for 45 sec and primer extension at 72ºC for 60 sec for 40 cycles. The qPCR reactions were performed on an ABI 7300 (Applied Biosystems®).

To generate a standard curve for the amount of SCN in a soil sample, estimates of approximately 1,000 nematodes in 0.1 ml of water were placed into the Powersoil® DNA isolation kit® bead beating tubes and extracted as described previously, and this process was replicated 2 more times. A 1:10 serial dilution series of DNA extracted from approximately 1,000 nematodes was created and used for generation of the standard curve by qPCR. To evaluate the accuracy of the standard curve, samples containing 100, 10, 1, and 0 SCN nematodes were generated by carefully hand collecting them under a

stereoscope, 3 replicates of each, and isolated the DNA by the Powersoil® DNA isolation kit® as described previously. The qPCR methodology works quantitatively because it detects pathogens by using the amount of DNA present in a sample to obtain a cycle threshold (Ct) value which corresponds to the amount of target DNA (Livak and Schmittgen, 2001). The lower the Ct, the greater the amount of the corresponding DNA (target organism) is present in a sample.

Confirmation of PCR and qPCR amplification products

To confirm that the DNA amplicons in both PCR and qPCR reactions were products of SCN DNA and not spurious amplification of off-target DNA, DNA amplification products were run out on and then isolated from the 1% agarose gels. The DNA was purified using the Qiaquick Gel Extaction Kit (Qiagen®; Valencia, CA) according to the manufacturer's specifications. The isolated DNA was ligated into the pGEM®-T Vector System II (Promega®). The ligation reaction was shuttled into competent JM109 *E. coli* cells and selected using 50 μg/ml ampicillin on LB-agar plates. Colonies were selected and grown in liquid culture in LB media containing 50 ug/ml ampicillin. Plasmid DNA was isolated from the bacteria using the Qiaprep kit (Qiagen®). The DNA from the plasmid preps was sequenced to determine if the DNA amplification product was correctly amplifying the proper target. The DNA sequence was trimmed using Crimson Editing freeware (http://www.crimsoneditor.com/). In this procedure, the pGEM®-T Vector DNA sequence was trimmed leaving the qPCR-generated sequence. The trimmed sequence was blasted in GenBank using the blastn query option. This additional quality control step demonstrated the accuracy of the qPCR reaction conditions.

Table 2 PCR primer pairs made to amplify a product of approximately 100 bp

H. glycines gene	*H. glycines* est	Product Size	Primer	TM
Hg-unc-9	CB281382	124	F-CCATGGTGCGCTATTTGTCA	62.95
			R-CTGCCCCCAAATTGTTTGAA	63.00
Hg-unc-22	CB378705	120	F-GACGAAATTGTGGCCGAGTC	62.91
			R-AAATTGTCCCGCGTCCTCTT	63.11
Hg-unc-31	CB378080	110	F-CTCCGATGGTTGTCCGCTAC	62.91
			R-GGTTGAGCAACCGTCTTTGC	63.05
Hg-unc-52	CK350534	114	F-ACCGCAGGTGTACGATGGTT	62.69
			R-CCGTAGGCGGTCACTTTGTC	62.92
Hg-unc-101	CB379764	94	F-CATGCAAGGCAACAGATTCG	62.70
			R-TAACAGCGCACATCCAAACG	63.09
Hg-unc-115	CK350435	103	F-ACGGAAGTCGCGCTATTCAA	63.06
			R-GTCGTTGTCCACGGAAGAGG	63.01
Hg-dys-1	CB934909	124	F-GCTATTTGCCGGTCGAACAA	63.28
			R-TTGTCCAATCTCGCGGCTAT	62.92
Hg-nep-1	CB824545	114	F-TATTCGGGCGTCAAAAATGC	63.04
			R-GCCAATCACTGCTCCAATCC	62.9
Hg-unc-89	CB379143	120	F-GCGCGGTACTGACGAAAGTC	63.19
			R-GCAGGACAGTTTCCGCATTC	63.04
Hg-unc-78	CB238521	97	F-CGTTTTTGGGACACCACACA	62.79
			R-TGCTGTCCTCAGACCACGAA	63.04
		qPCR probe	GAAGTCGGAGTTCGCTCTTCTTTCG	70.98

Table 3 Additional PCR primer pairs made to amplify a product of approximately 300 bp

H. glycines gene	*H. glycines* est	Product size	Primer	TM
Hg-unc-9	CB281382	253	F-GCGCGAAAGGACGATGATTTTTC	66.5
			R-CTTACGGCCGGACGAATACCTCTC	66.6
Hg-dys-1	CB934909	303	F-GTTTCCGATCGTTGGACTTCG	66.5
			R-GCTGGTGCATTGCCTCTGTTTC	66.4
Hg-unc-89	CB379143	289	F-CCCGTACACACATTTCCGCAGTC	66.3
			R-CAGCCGACCATCGAGTTCCATAC	66.5
Hg-unc-78	CB238521	313	F-GTGGAGACCAATCGGGCAAAATC	66.4
			R-GAAAGGAGGGCCTTCGAAAATGG	66.5

CHAPTER III

RESULTS

Assessment of primer pairs

From the uncoordinated genes that were identified in Alkharouf et al. (2007), 11 genes (Table 2) were evaluated for their utility for molecular marker development. Several primer pairs were able to amplify DNA from the extracted genomic DNA that was isolated from a single cyst of SCN. These pairs were Hg-unc 9, Hg-unc 115, Hg-unc 89, Hg-unc 78, Hg-dys-1, and Hg-nep-1, which produced bands of the predicted size (100 bp) on the gel (Figure 5). This initial assessment of the primer pairs provided proof of concept that the approach would work and allowed for more focused tests on using the primers on controlled numbers of nematodes.

Evaluating primers on controlled numbers of SCN

The primer pairs that provided positive amplification as shown in Figure 5 were then used in tests with SCN DNA isolated from different numbers of nematodes. For these tests, genomic DNA was isolated from single nematode, 10, 100 and samples containing approximately 1,000 SCN J2s. PCR experiments were performed using the primer pairs evaluated in figure 5 to determine the threshold whereby the primer pairs could reliably amplify the target DNA. From those analyses, it was determined that the Hg-unc 78 primer pair worked the best as determined by well-focused amplification

products. The experiments were repeated only using the Hg-unc 78 primer pair (Figure 6). By testing pure soybean cyst nematode juveniles with 4 different counts, it was found out that 1 individual did not appear to amplify DNA as revealed on the agarose gel (Figure 6), however, the 10, 100 and 1000 nematode count samples all produced bands (Figure 6). As expected, with increasing amounts of DNA from increasing amounts of nematodes, band brightness (intensity) increased. The samples from 100 (lane 13-15) and 1,000 (lane 16-18) nematodes were tested three times because they always amplify the correct target (Figure 6), while the samples from 1 (lane 1-6) and 10 (lane 7-12) nematodes were tested 6 times due to the potential amplification problems encountered with low DNA amount. The specificity of the reactions was demonstrated in controls where the reaction contents either lacked primers (lane 19) or lacked DNA (lane 20) (Figure 6). The DNA was isolated from gel and sequenced (Figure 7), revealing that the band was actually the target DNA (Figure 8). The experiments from Figure 6 determined that the Hg-unc 78 primer pair worked for samples having genomic DNA extracted from 10 nematodes but not one nematode. To determine the threshold where the primer pairs would successfully amplify DNA, further experiments were conducted in using SCN genomic DNA isolated from 1, 2, 4, 6, 8, 10 soybean cyst nematode juveniles (Figure 9). The gel started showing bands at 4 SCN J2 from lane 15-17 with the arrows pointing out in figure 9. The experiments were replicated individually at least 3 times each, demonstrating the specificity of the PCR primers.

Isolation DNA directly from soil

The experiments proceeded to determine if SCN DNA could be isolated directly from soil samples. To accomplish this, the Powersoil® DNA Isolation kit was used to

isolate DNA from known numbers of nematodes that were added to the soil. The initial experiment revealed that the Hg-unc 78 band could be detected in samples where as few as 10 J2 SCN were added to the Powersoil column (Figure 6). This experiment demonstrated that Powersoil® kit is able to isolate the DNA of soybean cyst nematode from soil samples.

Standardization of PCR conditions

Magnesium chloride is an essential cofactor for the DNA polymerase in PCR. Its concentration must be optimized for every primer: template system. Many components of the reaction bind magnesium ion, including primers, template, PCR products and dNTPs. The main 1:1 binding agent for magnesium ion is the high concentration of dNTPs in the reaction. Because it is necessary for free magnesium ions to serve as an enzyme cofactor in PCR, the total magnesium ion concentration must exceed the total dNTP concentration. To improve the amplification results, PCR reaction condition was modified. By doubling the concentration of 50mM Mg^{+2} cations from 1.5 ul to 3.0 ul and increasing cycles from 30 to 40 in the PCR reaction resulted in an increase in specificity of the reaction and enhanced amplification whereby the Hg-unc 78 could be detected in soil samples where a single SCN was added (Figure 11). Afterwards, this modification was always conducted in later PCR experiments.

Specificity of Hg-unc 78

From the assessment of designing the primers, 11 pairs of primers were designed from the expression sequence tags (ESTs) which were identified in Alkharouf et al. and Klink et al (2007) and should be highly specific for SCN. To further confirm the

specificity of Hg-Unc 78, experiments on agriculturally significant plant-parasitic reniform nematodes (*Rotylenchulus reniformis*) (Yik., et al. 1982) and root knot nematodes (RKN, *Meloidogyne incognita*) along with SCN DNA were used in PCR experiments. As expected, no amplification was achieved on reniform nor RKN DNA, but amplifications were successful on soybean cyst nematode DNA, which indicates that Hg-unc 78 is specific to soybean cyst nematode among these 3 types of plant-parasitic nematodes. Also, to further assure the specificity, metagenomic DNA from soil without soybean cyst nematode added (which contains all DNA; except SCN DNA) and metagenomic DNA from soil with 10 soybean cyst nematodes added (which contains all DNA along with SCN DNA) was isolated and tested on PCR and agarose gel. No amplification was obtained if there was no SCN DNA in the reactions (lane 4 and lane 5, Figure 14). The absence of amplification product, while not absolute proof of specificity, demonstrates that the Hg-unc 78 primers are more capable of amplifying from its designed SCN target than off target DNA.

Isolation of metagenomic DNA directly form soil

Since it appeared possible to amplify target DNA from soil containing as little as a single SCN J2 nematode, experiments proceeded whereby DNA was isolated from field samples containing numerous parasitic and free-living nematodes as well as other living organisms (e.g. fungi and bacteria). Soil samples were collected in triplicate and an assessment of the nematodes within the soil sample was determined (Table 4). This work demonstrated that the soil indeed contained other species of nematodes. Known numbers of SCN were added to the soil samples and the metagenomic DNA was isolated as

previously described. The amplification profile by using the Hg-unc 78 primers is shown (Figure 13).

Quantitative PCR estimates standard curve using pure greenhouse SCN J2 without soil

The qPCR experiments started with greenhouse cultured SCN second stage juveniles without using soil in the DNA isolation step (Figure 6) and the DNA was used in the previously described method for qPCR experiments (Table 5). In the table, each count had triplicates and the yellow shadowed value is the average of the 3 experimented CT-values. By using the average data in Table 5, a standard curve using pure greenhouse SCN J2 samples was established in Figure 16. From this curve, it demonstrated that the Hg-unc 78 primers are able to detect the different amount of SCN DNA, which can be a guide to correlate the DNA amount to the estimated number of juveniles.

Quantitative PCR estimates standard curve using soil samples with SCN J2 added

The final objective was to estimate number of SCN juveniles directly from soil samples. The qPCR experiments were conducted using metagenomic DNA isolated from microplots soil with predefined number of SCN J2 added in the DNA isolation step. The PCR amplification result was resolved on agarose gel electrophoresis (Figure 16). The qPCR resulted in 12 CT values for 4 different counts of J2 done in triplicate, each (Table 6). The yellow shadowed value is the average of the 3 experimental CT-values, which produced a standard curve by using soil samples with SCN added (Figure 17) along with a trend line, predictable formula, and R^2 value. R^2 is most often seen as a number between 0 and 1.0, used to describe how well a regression line fits a set of data. An R^2 close to 1.0 indicates that a regression line fits the data well, while an R^2 closer to

0 implies a regression line does not fit the data very well. Comparing Figure 16 and 17, the R^2 in Figure 17 is less than the one in Figure 16, which implies that the soil compounds make a difference in the DNA isolation step by decreasing the DNA isolation efficiency. Thus the figure demonstrated this method using Hg-unc 78 can possibly predict the DNA amount, furthermore predicting the number of nematodes in the soil sample from the isolation step.

Primary correlation of DNA among individual cyst, egg, and second stage juvenile

An experiment of an individual cyst, egg and J2, 46 replicates, each, was conducted. The experiment was done to investigate the relations among the cyst, egg, and J2 DNA from the DNA isolation. qPCR CT values were obtained successfully in the experiment. By averaging the experimental values (Table 7), the standard curve of using soil samples (Figure 17) was used to calculate the DNA amount compared the cyst, egg and J2 DNA to the one J2 DNA established in Figure 17. The table shows that from a cyst, DNA amount could be 69.2 times higher than the value established in Figure 17. From an egg, DNA amount could be 5.7 times higher. Even the J2 in the PCR study from Table 7 generated 9.8 times more DNA than the one achieved in standard curve (Figure 17). These results indicate that DNA isolation efficiency can be modified, but two of the studies are within an order of magnitude of being accurate.

Table 4 Off target soil dwelling nematodes identified from microplot soil

Microplots	*Hoplolaimus* spp	*Mononchus* spp	Free living
1	1287.5	772.5	772.5
2	772.5	2317.5	257.5
3	257.5	0	515
4	257.5	257.5	515

Table 5 CT Values obtained from DNA isolated from different number of pure SCN J2 from greenhouse samples

SCN J2 #, no soil	CT Value
1AP	35.68
1BP	34.27
1CP	34.66
1P Ave	34.87
10AP	28.15
10BP	26.38
10CP	30.00
10P Ave	28.18
100AP	22.98
100BP	23.08
100CP	23.27
100P Ave	23.11
1,000AP	20.86
1,000BP	20.33
1,000CP	20.93
1,000P Ave	20.71

Note: A, B, C: equivalent copy of each count of nematodes, P: Pure SCN suspension, yellow highlighted numbers are the average of each count.

Table 6 CT Values from metagenomic DNA isolated from soil with different SCN J2 added

SCN J2 added in Soil	CT Value
1AS	32.07
1BS	33.94
1CS	33.14
1S Ave	33.05
10AS	27.51
10BS	27.06
10CS	27.61
10S Ave	27.39
100AS	24.84
100BS	25.68
100CS	24.40
100S Ave	24.97
1,000AS	23.78
1,000BS	22.79
1,000CS	23.39
1,000S Ave	23.32

Note: A, B, C: equivalent copy of each count of nematodes, S: Soil samples with SCN added, yellow highlighted numbers are the average of each count.

Table 7 Correlations in individual cyst, egg, J2 DNA, based on the standard curve from Figure 17 compared to the one nematode

DNA Type	Mean CT Value	Estimate of J2 based on Figure 17
Cyst	25.86	69.22
Egg	29.47	5.69
J2	28.64	9.80

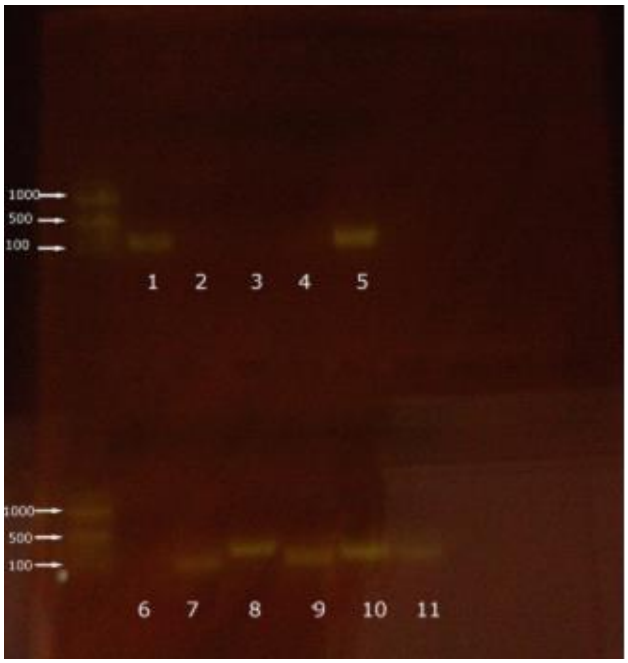

Figure 5 Screening of 11 Hg-unc primer pairs

Notes: Lane 1: Hg-unc 9, Lane 2: Hg-unc 22, Lane 3: Hg-unc 31, Lane 4: Hg-unc 52, Lane 5: Hg-unc 115, Lane 6: Hg-unc 101, Lane 7: Hg-dys-1, Lane 8: Hg-nep-1, Lane 9: Hg-unc 89, Lane 10: Hg-unc 78, Lane 11: control gene.

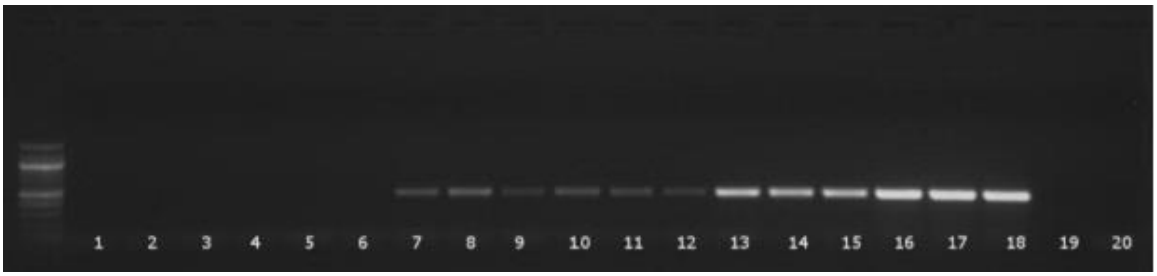

Figure 6 Amplification of Hg-unc-78 from different numbers of SCN J2 cultured in greenhouse

Notes: Lanes 1-6: 1 SCN, Lanes 7-12: 10 SCN, Lanes 13-15: 100 SCN, Lanes16-18: 1,000 SCN, Lane 19: without primer, Lane 20: without DNA.

GTGGCCAATCGGGCAAAATCCGTTTTTGGGACACCACACAATCAGTCCGTTTCGGTTGACATTA
ATTGCAATTGGCGTTAAATTAACACCAGTGTAGACGCACATTCTGAAGTCGGAGTTCGCGCTTC
TTTCGGGCGCAATTCGCGACATTTCGTGGTCTGAGGACAGCAAAAGAGTCGCGATAGTCGGCG
AAGGCCATGAAAGGTTGGCTATTTTAGTGTCCTTTTCTTAACTTTTTAAAAAATTAACACTTTTTC
CAGGTTTGGCCATGTCTTCCTCTTTGACACGGGTACGAGCAACGGCAATTTGTCGGGACAAAGT
CGGCCGATGAATGTATTCCAAAACAGCAGTAATTCCTTGGTTTTTCTACCTTACCCCCCTTTCAAA
GAGCATTGATTTTCGTCCGACAC
GGCCTTATCGTTTGGTCAGTGGTTCGGAGGATAACACTGTCGCCATTTTCGAAGGCCCTCCTTTC

Figure 7 Hg-unc 78 gene sequence from gel purified amplification product

Notes: Red highlighted regions are the PCR primer sequences. Green font sequences section is Fragment 1 from Figure 8. Blue font sequences section is Fragment 2 from Figure 8. Orange font sequences section is Fragment 3 from Figure 8. Yellow font sequences section is Fragment 4 from Figure 8. The gaps between four fragments could be introns that cannot be aligned with the original Hg-unc 78 sequences which is cDNA sequences.

```
FRAGMENT 1:

Score = 75.0 bits (40),  Expect = 1e-13
 Identities = 40/40 (100%), Gaps = 0/40 (0%)
 Strand=Plus/Plus

Query  5    CCAATCGGGCAAAATCCGTTTTTGGGACACCACACAATCA  44
            ||||||||||||||||||||||||||||||||||||||||
Sbjct  267  CCAATCGGGCAAAATCCGTTTTTGGGACACCACACAATCA  306


FRAGMENT 2:

Score =  187 bits (101),  Expect = 2e-47
 Identities = 110/114 (96%), Gaps = 1/114 (1%)
 Strand=Plus/Plus

Query  98   ACGCACATTCTGAAGTCGGAGTTCGCGCTTCTTTCGGGCGCAATTCGCGACATTTCGTGG  157
            ||||| ||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  307  ACGCATATTCTGAAGTCGGAGTTCGCTCTTCTTTCGGGCGCAATTCGCGACATTTCGTGG  366

Query  158  TCTGAGGACAGCAAAAGAGTCGCGATAGTCGGCGAAGGCCATGAAAGGTT-GGC  210
            |||||||||||||||||||||| |||||||||||||||||||||||||| |||
Sbjct  367  TCTGAGGACAGCAAAAGAGTCGCAATAGTCGGCGAAGGCCATGAAAGGTTTGGC  420


FRAGMENT 3:

 Score =  139 bits (75),  Expect = 5e-33
 Identities = 75/75 (100%), Gaps = 0/75 (0%)
 Strand=Plus/Plus

Query  259  AGGTTTGGCCATGTCTTCCTCTTTGACACGGGTACGAGCAACGGCAATTTGTCGGGACAA  318
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  412  AGGTTTGGCCATGTCTTCCTCTTTGACACGGGTACGAGCAACGGCAATTTGTCGGGACAA  471

Query  319  AGTCGGCCGATGAAT  333
            |||||||||||||||
Sbjct  472  AGTCGGCCGATGAAT  486


FRAGMENT 4:

 Score = 89.8 bits (48),  Expect = 5e-18
 Identities = 48/48 (100%), Gaps = 0/48 (0%)
 Strand=Plus/Plus

Query  389  AGCATTGATTTTCGTCCGACACGGCCTTATCGTTTGGTCAGTGGTTCG  436
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  AGCATTGATTTTCGTCCGACACGGCCTTATCGTTTGGTCAGTGGTTCG  534
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Figure 8 The HG-unc-78 DNA sequence of the amplification product from SCN genomic DNA isolated from 100 individual SCN

Notes : Shown are 4 fragments that align to the original Hg-unc 78 sequence that is deposited in Genbank. They are overlapping regions that compose the sequence found in Figure 9. The gaps between four fragments could be introns that cannot be aligned with the original Hg-unc 78 sequences which is cDNA sequences.

Figure 9 DNA amplification from low threshold numbers of SCN

Notes: Lanes 1-6: 1 SCN, Lanes 7-12: 2 SCN, Lanes 13-18: 4 SCN, Lane 19: without primer, Lane 20: without DNA, Lanes 21-26: 6 SCN, Lanes 27-32: 8 SCN, Lanes 33-38: 10 SCN, Lane 39: without primer, Lane 40: without DNA. Three pointed arrows are light gel bands showed on lanes 15-17 under the UV light.

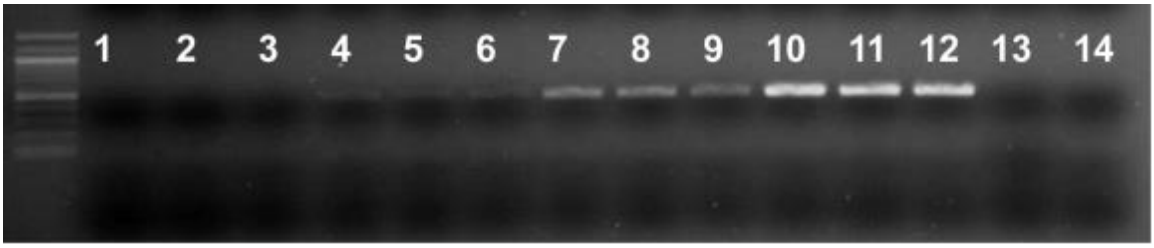

Figure 10 DNA amplification from soil containing SCN using lower Mg^{+2} concentrations

Notes: Lanes 1-3: 1 SCN, Lanes 4-6: 10 SCN, Lanes 7-9: 100 SCN, Lanes 10-12: 1,000 SCN, Lane 13: control without primers. Lane 14: control without DNA.

Figure 11 DNA amplification from soil containing SCN using higher Mg^{+2} concentrations

Notes: Lanes 1-3: 1 SCN, Lanes 4-6: 10 SCN, Lanes 7-9: 100 SCN, Lanes 10-12: 1,000 SCN.

Figure 12 The specificity of the PCR reaction as compared to off target nematodes

Notes: Lanes 1-3: 100 Rr, Lanes 4-6: 1,000 Rr, Lanes 7-9: 100 RKN, Lanes 10-12: 1,000 RKN, Lanes 13-15: 100 SCN, Lanes 16-18: 1,000 SCN, Lane 19: negative control without primers in PCR reaction, Lane 20: negative control without DNA in PCR reaction.

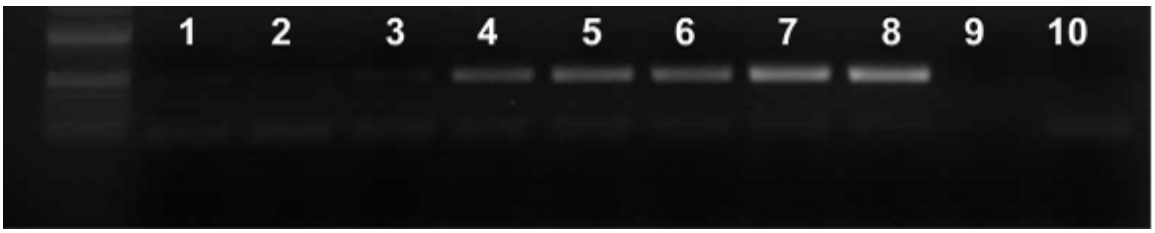

Figure 13 DNA amplification from metagenomic DNA samples

Notes: Lanes 1-3, metagenomic DNA including 1 SCN J2; lanes 4-6, metagenomic DNA including 10 SCN J2; lanes 7 and 8, metagenomic DNA including 100 SCN J2. Lane 9, PCR reaction minus primers; Lane 10, PCR reaction minus DNA. Amplification product is Hg-unc 78.

Figure 14 Amplification of Metagenomic DNA from Microplots soil with or without the additional of known number of SCN J2 added

Notes: Lanes 1-3: DNA from microplots soil with 10 SCN J2 added; Lanes 4-5: Microplots soil without any SCN J2 added; Lane 6: Green house soil heavily infested with SCN J2; Lane 7: negative control without primers in PCR reaction; Lane 8: negative control without DNA PCR reaction; Lane 9: positive control with DNA (PlasmidPrep for sequencing) and primers.

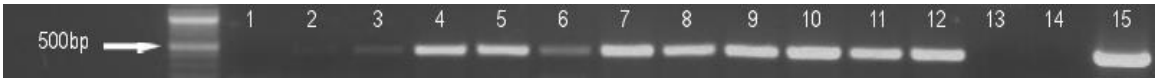

Figure 15 DNA amplification from metagenomic DNA samples

Notes: Lanes 1-3, metagenomic DNA including 1 SCN J2; lanes 4-6, metagenomic DNA including 10 SCN J2; lanes 7-9, metagenomic DNA including 100 SCN J2; lanes 10-12, metagenomic DNA including 1000 SCN J2 Lane 13, PCR reaction minus primers; Lane 14, PCR reaction minus DNA; Lane 15, positive control. Amplification product is Hg-unc 78.

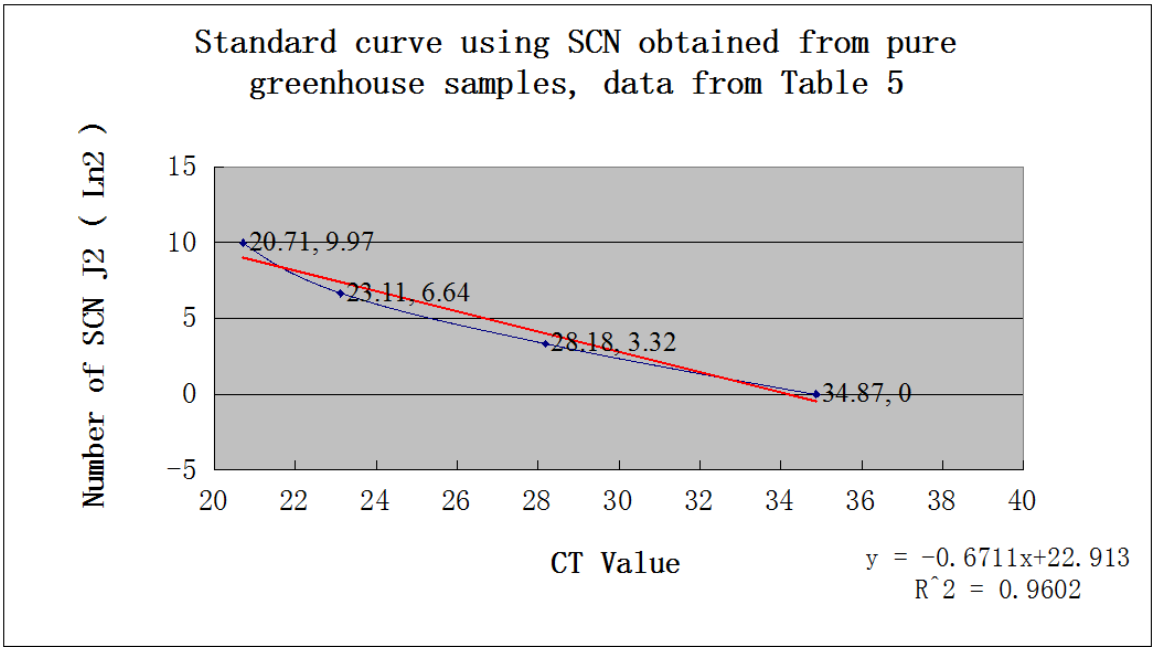

Figure 16 Standard curve using pure greenhouse samples, data from Table 5

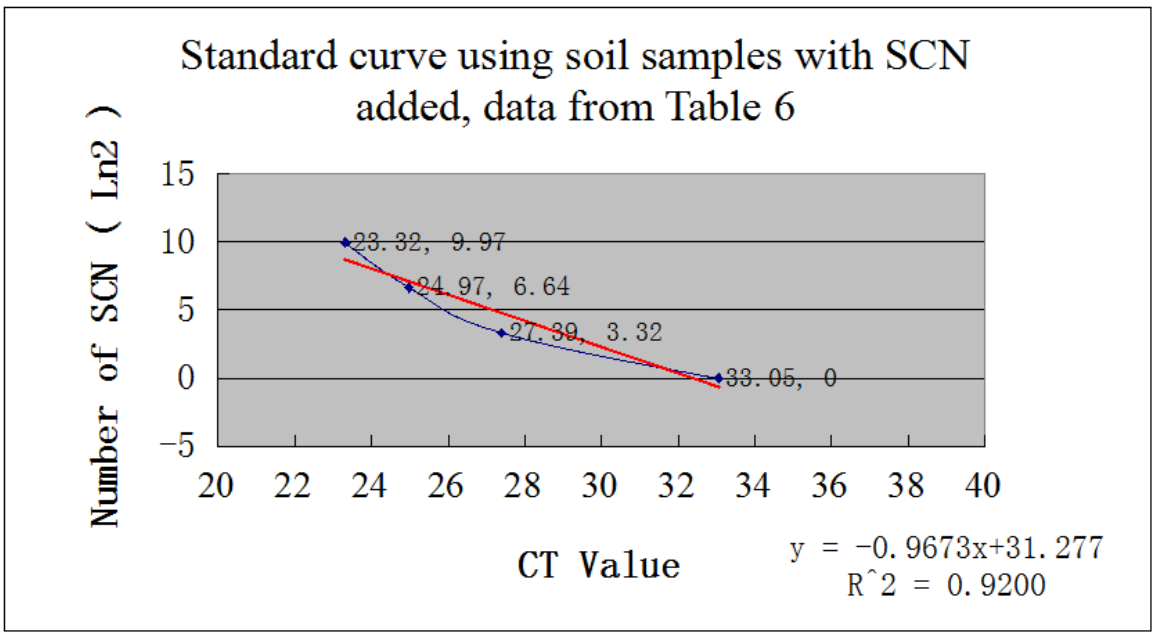

Figure 17 Standard curve using soil samples with SCN added, data from Table 6

CHAPTER IV

DISCUSSION

The determination of the numbers of nematodes in the soil is important in agriculture for understanding threshold populations that could detrimentally affect plant productivity. The analysis presented here was done in order to develop a simple molecular diagnostic technique that could determine the number of SCN from metagenomic DNA isolated directly from soil. The research began by identifying genes that could be used to develop reliable PCR primers. Those primers then were used to amplify SCN DNA from metagenomic DNA isolated directly from soil. From those genes, quantitative PCR primers were designed and used on actual soil samples from real agricultural sites obtained from within the state of Mississippi. The outcome is the beginning of a molecular diagnostic technique that can determine low thresholds of SCN from DNA isolated directly from soil. It is expected that intermediate and high numbers of nematodes could be determined as well using these procedures.

DNA isolation efficiency

In Figure 17, a standard curve was built for this research primarily. Metagenomic SCN DNA was successfully isolated from soils. This curve could have been used for the field sample test. However, before applying this to field sample test, the result from Table 7 requires us to rethink and reconsider to establish a new standard curve. Table 7 was

using the standard curve in Figure 17. The previously established standard curve using soil samples to obtain metagenomic DNA estimated that the average of individual second stage juvenile is 9.8 times more than 1 which it was supposed to be according to the curve in Figure 17, which indicates the efficiency of the metagenomic SCN DNA has varied during more practices of DNA isolation and it was improved by increasing the number of each biological replicate, compared to the standard curve which only had three of each biological replicate. Therefore the efficiency of DNA isolation can be enhanced or standardized, possibly by modifying the procedure.

Competence of Hg-unc 78

The guide used to identify SCN genes that would serve for primer design was from work presented by Alkharouf et al. (2007). These genes were the focus of our analysis because we knew that no other agriculturally important Heterodera species have been identified in Mississippi soils. Thus, the gene sequence could actually be designed to specifically bind to its target in a manner previously demonstrated by Showmaker et al. (2011). Analyses done by Klink et al. (2007) identified genes that were specifically expressed at high levels during the mobile stages of SCN development. Many of these genes including the uncoordinated class were first described in C. elegans. The genetically-defined uncoordinated (unc) genes perform many functions in C. elegans. The protein products of the unc genes are involved in muscle focal adhesion, architecture and stimulation (via neuromuscular connections). The unc mutants all display uncoordinated motion, slow movement, or paralysis ((Zengel and Epstein, 1980). The unc family of mutants contains 114 different members (Zengel and Epstein, 1980; Johnsen and Baillie

1997). The Alkharouf et al. (2007) gene list was used to identify SCN homologs of unc genes**.**

The presented experiments show that it is possible to use highly conserved gene sequences from SCN and use them to specifically amplify gene fragments from as few as 1 J2 SCN. A number of genes from the *uncoordinated* (*unc*) group of *C. elegans* genes were used. Sequence analyses of the DNA products from these PCR reactions have shown that the amplification product perfectly matches its target DNA from SCN. Using the same reaction conditions on off-target, but plant-parasitic nematodes (*Rotylenchulus reniformis* and *Meloidogyne incognita*) of significant economic importance showed that the off target DNA failed to amplify a product, nor did it achieve a product from metagenomic DNA without any SCN presence. I have been able to easily identify amplification from as low as 1 SCN J2. Lastly, using the same primers to amplify target DNA from metagenomic DNA isolated directly from soil samples reveals that it is possible to amplify the target as shown by sequencing reactions of that amplification product. The objective of this study was to be able to determine the number of target nematodes from metagenomic DNA isolated directly from soil samples. To accomplish this experiment, the metagenomic DNA samples contained different concentrations of known SCN, 1, 10, 100 and 1,000. This was accomplished by adding the counted number of SCN directly into soil samples as originally performed by Showmaker et al. (2011) for *R. reniformis*. The planned quantitative PCR experiments allowed me to compare the counted number of nematodes directly to the amount of nematodes as quantified by qPCR according to Showmaker et al. (2011). Once I have been able to faithfully determine the amount of amplification that correlates to a known amount of SCN, I was

then able to determine the number of nematodes in a sample with an unknown amount of SCN. However, before these experiments were conducted, I planned on partial extraction of all nematodes from these samples according to the procedures of Showmaker et al (2011). This will allow me to know the numbers and genera/species of nematodes in the samples. Once those estimates have been made, the qPCR procedure will be performed to reveal how well the qPCR data correlate to the numbers obtained by counting the extracted nematodes.

Soil compounds and their influence on DNA isolation efficiency

I have found out that SCN DNA can be successfully isolated by using the PowerSoil DNA isolation kit. However, I was using soil samples to directly isolate the meta-genomic DNA. A key question remained here is whether different compositions of soil will affect the DNA isolation. If different types of soil make a difference, the standard curve of this study will need to be justified for each soil type. There are 5 different soil types out of 12 that exist in the state of Mississippi, varying among alfisols, entisols, inceptisols, ultisols and vertisols due to different areas (Ritter, 2009). It is possible that these different soil compositions could affect the efficiency of the DNA isolation. I have not observed a major influence on amplification from the different sample types used in the various analyses. However, a thorough analysis on different soil types from the obtained unknown samples from around the state of Mississippi would address it. An experiment using different soil type but with other conditions remaining the same will need to be conducted to investigate this problem.

DNA correlation between individual cyst, individual egg and individual second stage juvenile

Soybean cyst nematodes go through several life stages including cyst stage, second stage juvenile, third stage juvenile, fourth stage juvenile, adult stage, and egg stage. The stages present in soil are mainly cyst and second stage juvenile. The egg stage might be found due to a broken cyst, but normally not. Only examining the number of second stage juveniles will not be a thorough research method. However, SCN cysts are very small and usually clustered in the soil, making SCN soil sample results notoriously variable (Niblack et al., 2005). With a typical 1-inch-diameter soil probe, random placement of the probe into the soil can have a tremendous effect on how many egg-filled SCN cysts are recovered. The ability of isolating DNA from cyst in the soil (Table 7) demonstrated that cyst DNA from the soil needs to be considered in this study. An average of 200 eggs is usually contained in the cyst shell (Lauritis. 1983). The different texture of the cyst shell with the egg shell and the juvenile cuticle may affect the isolation efficiency of DNA. Table 7, the cyst DNA amount calculated by the standard curve established in Figure 17 is 69 times more than an individual juvenile DNA, which not only implies that DNA isolation procedure can apply to cyst stages in the soil, but also demonstrated that the efficiency is lower than it was supposed to be (around 200 times), possibly because of the cyst shell and egg shell (instead of being around 200 times more). The value I observed, 69 times more, is much less than it was supposed to be, but it can be improved. The table showed Powersoil kit is able to isolate DNA out of the eggs. The development of the correlation among these three stage type of DNA will assist in determining the possible equivalent number of juveniles in the soil, which can finally help to achieve the objective in a manner of efficiency and accuracy of this study.

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