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MOLECULAR BIOLOGY AND PHYSIOLOGY

18S and ITS1 Genomic Sequence Variations in *Rotylenchulus reniformis* Isolates from Alabama

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ABSTRACT

Upland cotton, *Gossypium hirsutum* L., is highly susceptible to infection by reniform nematode (*Rotylenchulus reniformis*), which can cause over 10% reduction of cotton yields in Alabama. Detection of reniform nematode (RN) and analysis for molecular variation within its population is important for understanding its interactions with cotton and other host plant species. Restriction analysis of PCR products of ITS1 regions was achieved using four restriction enzymes, *Hae*III, *Hha*I, *Msp*I, and *Rsa*I. These showed similar banding patterns for both male and female populations. However, *Msp*I digestion of ITS1 amplification products showed variants within the combined sex and location effects primarily attributed to a 500 bp fragment that was absent in other restriction digestions. Intra-nematodal variations in 18S and ITS1 rDNA were studied in detail by sequencing a minimum of ten clones in each individual male and female RN isolates in both directions. Multiple sequence alignment of the 18S rDNA sequences showed two major types of sequences within this gene for both male and female RN clones, which could be distinguished at 27 specific sites. Two distinct ITS1 fragments of lengths (550 bp and 720 bp) were observed; referred to as ITS1S and ITS1L respectively. Neighbor-joining analysis was used in revealing the relationships and grouping characteristics between male and female RN clones, with clones grouping together irrespective of sex and isolate. Sequencing of one-third of the 18S and ITS1 rDNA regions provided clear evidence of intra-and inter-nematode variability, in addition to gene conversion events in the 18S rDNA of individual male and female RN clones.

The reniform nematode *(Rotylenchulus reniformis)* is widely distributed in many tropical and subtropical regions of the world, where it parasitizes about 300 plant species (Heald and Inserra, 1988; Inserra and Dunn, 1992; Davis et al., 2003) in more than 38 countries in Africa, the Americas, the Middle East, East Asia, and Australia (Luc et al., 2005; Siddiqi, 1972). Within the United States, the reniform nematode (RN) is established in at least ten southern states, from North Carolina to New Mexico (Weaver et al., 2007). The wide distribution of this nematode is a result of its diverse host range, and its ability to endure prolonged periods in dehydrated conditions while being dispersed to distant locations through dust storms (Gaur, 1988). Monoculture and the lack of genetic resistance in cotton further enhance population increases of the RN (Gazaway and McLean, 2003).

In most populations reproduction is amphimictic with 40-60% males; however, there are also parthenogenetic populations with few to no males (Nakasono, 1977, 1983; Sivakumar and Seshadri, 1971). Only the female RN parasitizes plant roots. An immature female imbeds its head into root tissue while the tail end remains in the soil interspace. As it feeds and grows, the portion of the body inside the root enlarges. Young infective adult females mate after establishing a feeding site and eggs are laid in a gelatinous matrix (Birchfield, 1962; Linford and Oliviera, 1940; Nakasono, 1977; Peacock, 1956; Sivakumar and Seshadri, 1971).

Significant damage to cotton yields by RN occurs in Alabama (Gazaway et al., 2001), Louisiana (Overstreet, 1999), and Mississippi (Lawrence and McLean, 1999). In 2012, 105,402 bales of cotton were estimated to have been lost in Alabama,

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Louisiana, and Mississippi through RN infestation (Blasingame and Patel, 2013). Monoculture and the lack of genetic resistance in cotton, aid in the maintenance of high population densities of the RN (Gazaway and McLean, 2003). Over the past decade an average of 7% loss in yield was experienced in Alabama, resulting in an annual loss of $126 million (Blasingame et al., 2009).

Molecular characterization for nematode species and populations have been achieved using mitochondrial DNA (mtDNA), nuclear high copy DNA, nuclear low copy DNA (Blouin, 2002; Carpenter et al., 1992), simple sequence repeat (SSR) markers (Arias et al., 2009) and ribosomal DNA. The first ribosomal transcribed spacer (ITS1) is part of the eukaryotic cistron of ribosomal DNA located between the genes coding for 18S and 5.8S rRNA. Due to its non-coding structure, ITS1 shows a high evolutionary rate and has been used for phylogenetic studies of closely related species of animals, plants, and fungi at the population and species level (Cherry et al., 1997; Chrisanfova et al., 2008). Evidence exists suggesting that populations of nematodes can be analyzed through PCR-RFLP digestion of the ITS1 region (Powers et al., 1997). Variations in the ITS1 region in *R. reniformis* populations from Alabama show the appearance of two main clusters with individual branches, confirming the presence of variability in the ITS1 rDNA region (Tilahun et al., 2008).

The 18S rDNA is approximately 1700 base pairs (bp) in length and 50% of the nucleotide variability is observed in the third of the sequence closest to the 5' region. According to Hillis and Dixon (1991), intra-specific variation in this region is influenced by the availability of many copies of 18S rDNA per genome, and its homogenization through evolution. Intra-individual variation occurrence within the *18S* rDNA of animal genomes is rare. However, 18S rDNA variation within a metazoan was first noted in *Dugesia* (*Schmidtea*) *mediterranea*, a free-living platyhelminth. This gene is made of two types of rDNA (type I and II) and 8% of nucleotides distinguished the variants (Carranza et al., 1996). Within the near full 18S rDNA of an individual RN, sequence variants (RN_VAR1 and RN_VAR2) exist, and these can be distinguished using 96 specific base sites (Nyaku et al., 2013). The 18S rDNA is a slow evolving region in living organisms, and thus forms the basis for inference of phylogenetic history across taxa (Fitch et al., 1995; Nadler and Hudspeth, 1998).

The aim of this study was to identify variation among and within five populations from Alabama using restriction digestion of ITS1, and to determine within nematode variation for the nuclear 18S and ITS1 rDNA regions in single male and female RN isolates from Alabama.

MATERIALS AND METHODS

Soil Sample Collection and Establishment of Reniform Nematode Populations. Samples of RN infested soils were obtained from five cotton farms located in three counties in Alabama (Table 1). Each soil sample was thoroughly mixed and kept at 4°C; a 150 cm^3 subsample was used for extraction of the nematodes. These populations were further maintained on cotton cultivar 'Delta and Pineland 425 BG/RR' (DPL 425) together with a mixed-Alabama population in the greenhouse.

Reniform Nematode Extraction from Soil Samples. The nematodes were extracted from the soil samples as described by Deng et al. (2008). The suspension containing the nematodes was transferred into a Petri dish using a pipette, and placed on an Olympus SZH-ILLD dissection microscope (Olympus optical Co. Ltd. Japan) at 10X magnification. Individual nematodes were picked using a sterilized hook, and then placed in a drop of water on a glass slide for morphological identification, under an Olympus IMT-2 compound microscope (Olympus optical Co. Ltd. Japan). DNA was then extracted from individual male and female RN isolates using two methods of isolation.

Table 1. GIS information for five Alabama (AL) cotton farm sites infested with RN sampled for this study.

Abbreviation	Location	County/State	Latitude	Longitude	Infested since
B	Belle Mina	Limestone, AL	86.89 W	34.66 N	Early 1990s
S	Shaw	Limestone, AL	86.94 W	34.64 N	Early 1990s
R	Murphy	Limestone, AL	86.75 W	34.59 N	Late 1980s
T	Thornton	Lawrence, AL	87.37 W	34.73 N	Early 1980s
W	Whitehead	Fayette, AL	87.73 W	33.84 N	Late 1980s

DNA Extraction (Method 1). This set of extractions were performed solely on the Belle Mina isolates. DNA was extracted separately from ten individual male and female RN isolates using a DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol.

Polymerase chain reaction (Method 1). Two micro-liters of extracted DNA (approximately 1.0 ng/µl) from a single male and female RN was transferred to PCR tubes containing 2.5 µl 10x high fidelity PCR buffer, 1.0 µl $MgCl_2$ (50 mM), 0.5 µl dNTPs (10 mM), 0.5 µl of forward and reverse primers each (10 µm) (synthesized by MWG-Biotech AG, USA), 0.2 µl of high fidelity platinum taq (Invitrogen, Carlsbad, CA, USA.) and sterile DNase-free water added to a final volume of 25 µl. Primer pairs Nem_18S_F (5'-GGCGATCAGATACCGCCCTAGTT-3') and Nem_18S_R (5'-TACAAAGGGCAGGGACGTATT-3') were used to amplify a 600 bp region of the 18S rRNA gene of the RN. The second pair of primers (Powers et al., 1997) for amplifying the first transcribed spacer region (ITS1) of the nematodes were Nem_ITS1_F (5'-TTGATTACGTCCCTGCCCTTT-3') and Nem_ITS1_R (5'-ACGAGCCGAGTGATCCACCG-3'). Polymerase Chain Reaction (PCR) was performed in a Peltier Thermal Cycler (PTC) tetrad 2 DNA engine (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: 94°C for 2 min, then 30 cycles of: 94°C for 30 s, 60°C for 30 s, and 68°C for 1 min. A final extension phase of 72°C for 7 min concluded the amplifications.

DNA Extraction (Method 2). DNA was extracted from five individual male and female RN isolates from five Alabama populations, and from four individual female RN isolates from the mixed-Alabama population soil samples, according to Floyd et al. (2002), and maintained separately. The individual nematodes were picked and placed directly into 20 µl of 0.25M NaOH in 0.2 ml tubes, and then kept at room temperature for 3-16 h (Stanton et al., 1998). The lysate was then heated for 3 min at 95°C. Four µl of 1M HCl and 10 µl of 0.5 M Tris-HCl buffer at pH 8.0 were added to neutralize the base. Five µl of 2% Triton X-100 was added, and the lysate was heated again for 3 min at 95°C and stored at -20°C.

Polymerase Chain Reaction (Method 2). Extracted DNA (1 µl) of approximately 1.0 ng/µl was transferred to PCR tubes containing 2.5 µl 10x buffer (Promega, Madison, WI), 2 µl of 25 mM $MgCl_2$ (Promega, Madison, WI), 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM primer (synthesized at MWG-Biotech AG, USA), 0.3 µl of taq DNA polymerase (Promega, Madison, WI, USA) and sterile PCR-grade water added to a final volume of 25 µl. The Nem_ITS1_F and Nem_ITS1_R primers were used to amplify the ITS1 region (Powers et al., 1997). To amplify the 5' one-third of the 18S rRNA gene, the 18S_F (5'-GCTTGTCTCAAAGATTAAGCC-3') and18S_R (5'-TGATCCWKCYGCAGGTTCAC-3') primers were used. PCR reactions were performed in a Peltier Thermal Cycler (PTC) tetrad 2 DNA engine (Bio-Rad, Hercules, CA, USA). PCR conditions were: 95°C for 5 min, then 30 cycles of the following: 95°C for 30 s, 57°C for 30 s, and 72°C for 45 s. The final extension phase was 72°C for 5 min. The quality of PCR products were checked by electrophoresis of 6 µl of PCR reaction in 1% agarose gel with ethidium bromide staining. The bands were visualized and photographed under ultraviolet light. The size of each PCR product was determined by comparison with a 100 bp DNA marker.

PCR-RFLP Analysis. Four restriction enzymes were used in the digestion of ITS1 amplicons of the ribosomal DNA from five individual male and female RN isolates from the five Alabama populations. The restriction enzymes used were four-base cutters, *Hae*III, *Hha*I, *Msp*I, and *Rsa*I. Amplicons digested with these enzymes were separated on a 6% polyacrylamide gel using the CDASG-400-50: Dual Adjustable Mega Gel Kit System (CBS Scientific, CA) at 250V for 2 h and 40 min.

Cloning of PCR Products. The 18S and ITS1 amplicons from ten individual male and ten female RN isolates, from the Belle Mina population, together with four female RN isolates from the mixed-Alabama populations, were purified before cloning using a QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacture's protocol. The purified fragments were then cloned into a plasmid vector using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The ligation reaction was made up of 4.0 µl of PCR product, 1.0 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), and 1.0 µl of TOPO vector. Several clones were picked for verification of inserts from PCR amplifications for each selected clone through colony PCR. This was performed by amplification of the clonal DNA using M13 forward (5'-TGTAAAACGACGGCCAGT-3') and reverse (5'-AGCGGATAACAATTTCACAC-3') primers. PCR conditions were as follows: 94°C for 5 min, then 40 cycles of the following: 94°C for 30s, 55°C for 1 min, and 72°C for 1 min.

The final extension phase was 72°C for 10 min. Individual bacterial colonies with inserts were picked and placed into 96 well blocks with 1.3 mL of liquid Luria- Bertani (LB) media containing 100 µg/mL ampicillin and shaken at 37°C for 24 h at 300 rpm in an Innova 4300 rotary incubator shaker (New Brunswick Scientific, Edison, NJ, USA). The 96 well blocks containing the bacterial cells were then centrifuged for 12 min at 2,000 x g in a an Eppendorf 5804R centrifuge (Brinkmann Instruments Inc., Westbury, NY, USA) to obtain cell pellets. Plasmid DNA from the bacterial cells was isolated using a QIAprep Mini-prep kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol.

Sequencing. Plasmid inserts from at least ten colonies originating from each individual nematode for the 18S and ITS1 rDNA regions were sequenced in both directions with the vector primers M13 F and M13R, and T7 (5'-TAATACGACTCACTATAGGG-3') and T3 (5'-ATTAACCCTCACTAAAGGGA-3') primers for the mixed-Alabama and Belle Mina populations, respectively. Sequencing was performed using the ABI PRISM Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) in an ABI 3100 nucleotide sequencer.

Alignment and Phylogenetic Analysis. The SeqMan Pro program within the DNASTAR Lasergene v8.0 software (DNASTAR Inc., Madison, WI) was used in generating consensus sequences from both forward and reverse sequences and any extraneous sequences outside the respective amplification fragments trimmed-off. Multiple sequence alignment and phylogenetic analysis were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software version 5.0 (Tamura et al., 2011). Identical sequences for both 18S and ITS1 regions were grouped into contigs and screened for homology to nematode sequences using the standard nucleotide-nucleotide BLAST [blastn] on the NCBI website (http://www.ncbi.nlm.nih.gov/blast).

The mixed-Alabama and Belle Mina population 18S gene sequences have been deposited in GenBank with accession numbers JN695066-JN695091, and KF019952- KF020136 respectively. The ITS sequences for the mixed-Alabama and Belle Mina populations have also been deposited in GenBank with accession numbers JN695092-JN695137, and KF020137- KF020277 respectively.

Sequence examination for chimera presence. Potential production of chimeric DNA in our 18S and ITS1 rDNA clones was examined. The analysis was carried out using Ballerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) with default settings (300 bp window). The Huber-Hugenholtz correction was used with Clustalw alignment (Huber et al., 2004). No chimeras were observed in the sequences utilized in this study.

Evolutionary relationships of taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length was 0.52. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) were shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura and Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 217 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 496 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

RESULTS AND DISCUSSION

Restriction Digestion of the ITS1 rDNA Region of female and male reniform nematodes. The undigested amplicons of the ITS1 region showed two fragment sizes of approximately 550 bp and 750 bp, respectively. Restriction digestion of the ITS1 region for female and male RN isolates using *Hae*III produced fragments that ranged from 40 bp to 500 bp. Fragments of digested PCR products ranged from 30 bp to 550 bp for *Hha*I, 230 bp to 550 bp for *Msp*I and 120 bp to 550 bp for *Rsa*I. Banding patterns for all the restriction enzymes were similar in both male and female RN isolates in the populations. The restriction enzyme, *Msp*I produced a 500 bp band in the individual nematodes for female Murphy, male WhiteHead and in both female and male Belle Mina RN isolates (Fig. 1). Our study involved the amplification of the ITS1 rRNA region of five individual male and female RN isolates across populations for a faster diagnosis for this pest, and to determine if sex-based differences existed. Banding patterns analyzed showed that of the four restriction enzymes used in digestion of the ITS1 region, *Msp*I was the most-informative enzyme because a 500 bp band was consistently produced in female, male; and in both female and male nematodes from Murphy,

Whitehead and Belle Mina locations, respectively. The other restriction digestion enzymes produced similar banding profiles and therefore did not differentiate nematode populations. Associations among populations of three species of the stem nematode *Ditylenchus destructor, D. myceliophagus*, and seven host races of *D. dipsaci* from different locations have been verified using this method (Wendt et al., 1993). They could differentiate among *D. dipsaci* host races using southern blot hybridization with a 7.5 kb ribosomal cistron probe. This technique has also been used in differentiating root-knot nematode species (Zijlstra et al., 1997). *Xiphidorus* species have also been deetermined using the restriction enzymes (*Taq*I, *Rsa*I, and *Hinf*I) after digestion of the ITS1 region (Oliveira et al., 2004).

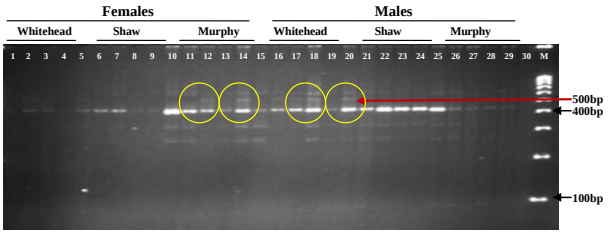

Figure 1. Restriction Fragment Length Polymorphism from the digestion of ITS1 region of 5 female and five male reniform nematodes in three locations - WhiteHead (W), Shaw (S),and Murphy (R) digested using the restriction enzyme *Msp*I and separated on 6% Polyacrylamide Gel (PAGE). Circled regions show presence of 500 bp bands among the amplicons. Lane M=100bp marker.

Sequencing Analysis for 18S rRNA gene. New rDNA sequences (211) were generated from individual male and female RN isolates. These sequences comprised of 26, 95, and 90 sequences from four female, ten male and ten female RN isolates from the mixed-Alabama and Belle Mina populations, respectively. The 18S data generated after performing MSA showed 689 characters made up of a number of sites. Sites within the aligned sequences were conserved and parsimony-informative sites, representing 615 (89.3%), and 74 (10.7%) sites, respectively. These regions showed single base change, no base change, two or more base changes and a combination of singletons and parsimony-informative sites, respectively. The 211 sequences exhibited average nucleotide frequencies of 24.2%, 24.5%, 23.3%, and 28.0% for thymine (T), cytosine (C), adenine (A) and guanine (G), respectively. Estimate of the evolutionary divergence among the 18S rDNA sequences showed an overall average pairwise distance of 0.02 among the sequences.

Neighbor-Joining analysis showed two major groupings (A and B) (Figs. 2A and 2B, respectively) for the 18S rRNA gene in both the mixed-Alabama and Belle Mina populations. Within each of these groups A and B, clone sequences from different nematodes irrespective of the sex of nematode isolate clustered together. Twenty-seven parsimony informative sites were used in distinguishing the two major groups, with signs of inter-locus gene conversions among the mixed-Alabama isolates (Table 2). Similarly, 27 sites could distinctly separate the Belle Mina population sequences into two types in both male and female RN isolates (Supplementary Tables 1 and 2). Within group 1, of the mixed-Alabama isolates, RN18S4A, RN-18S4B, RN18S4E, RN18S11B, and RN18S12G clones had a total of six, six, ten, five, and seven inter-locus gene conversion sites respectively, and in group B, five, five, five, and eleven inter-locus gene conversion sites were observed for RN18S4F, RN18S5H, RN18S12H, and RN18S12D clones, respectively. The presence of indels at specific loci were observed, these were at positions 68, 406, and 407, respectively. Phylogenetic relationships among nematodes using DNA sequence data from the 18S rDNA have been reported (Blaxter et al., 1998). Neighbor-joining analysis on the 18S rDNA of the RN clones showed the presence of intra- and inter-nematode variation within this gene from single male and female RN isolates. This variation was confirmed through grouping of clones from the same nematode with those from other nematodes irrespective of the sex. Tilahun et al. (2008) also found variation within the 18S rDNA region among seven populations from Alabama. However, intra-individual nematode variation could not be determined from a particular sex because sequence data was generated from a pooled population of eight RN isolates. In our study, intra-individual variation was clearly determined because, single male and female RN isolates were used for DNA isolation, amplification, cloning, and sequencing of selected rDNA regions. The six clone sequences from Tilahun et al. (2008) included in our analysis also showed these sequences clustering with other RN clones in both groups confirming the presence of variation within this gene. Eukaryotes exhibiting intra-individual variation in 18S rRNA gene include *Plasmodium* (Rogers et al., 1995), *Acanthamoeba* (Ledee et al., 1998), and *Trypano-*

soma cruzi (Stothard et al., 2000). Powers et al., (2005) detected significant variation within the 18S gene of *Meloidogyne* spp, however, this gene could not be used in distinguishing *M. arenaria, M. incognita*, and *M. javanica*. Explanations for the variation within the 18S rRNA gene have been through models relating to concerted evolution occurring within the rDNA locus due to unequal crossovers and gene conversions. Unequal crossover between sister chromatids occurs more often compared to exchanges between homologous chromosomes (Eickbush and Eickbush , 2007). Another possible explanation of two main types of sequences within an individual male or female RN 18S gene is the presence of two nucleolar organizing regions which undergo crossing-over occasionally producing small gene conversion tracts in this gene. Studies in our laboratory have shown the presence of two variants (RN_VAR1 and RN_VAR2) for the 18S RNA gene in the RN, with a 5% difference distinguishing among these variants (Nyaku et al., 2013).

Table 2. Parsimony-informative and inter-locus gene conversion sites within the 18S rRNA clones of female reniform nematode isolates from mixed-Alabama population.

Group 1 variations at specific base locations in 18S rRNA clones (14)																											
	61	63	64	68	73	78	390	392	407	438	439	442	519	521	524	530	537	549	551	552	562	563	569	601	624	633	655
RN18S4A	G	C	G	T	T	C	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S4B	G	C	G	T	T	C	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S4E	G	C	G	T	T	C	G	A	T	-	-	G	C	G	A	C	G	A	C	G	C	C	T	C	T	G	T
RN18S4G	A	T	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S5A	A	T	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S5B	A	T	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S5C	A	T	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S5D	A	T	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S5E	A	T	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S11B	A	T	C	C	T	T	G	A	T	-	-	G	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S11C	A	C	C	C	-	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S12B	A	T	C	C	T	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S12F	A	T	C	C	T	T	A	G	C	T	T	G	C	G	A	C	G	A	C	G	C	C	T	C	T	G	C
RN18S12G	A	T	C	C	T	T	A	G	C	T	T	A	C	G	A	C	G	A	C	G	G	A	A	T	C	A	T
Group 2 variations at specific base locations in 18S rRNA clones (12)																											
RN18S12H	A	T	C	C	T	T	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S4C	G	C	G	T	T	C	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S4D	G	C	G	T	T	C	G	A	C	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S12D	A	T	C	C	T	T	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S12E	A	T	C	C	T	T	A	G	C	T	T	A	A	T	G	T	C	G	T	T	G	A	A	T	C	A	C
RN18S5F	G	C	G	T	-	C	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S5G	A	C	G	T	T	C	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S11A	G	C	G	T	-	C	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S12A	G	C	G	T	-	C	G	A	A	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S12C	G	C	G	T	-	C	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S5H	A	T	C	C	T	T	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T
RN18S4F	A	T	C	C	T	T	G	A	T	-	-	G	A	T	G	T	C	G	T	T	G	A	A	T	C	A	T

Highlighted regions in yellow show gene conversion sites and positions with gaps are 73, 438, and 439 shown in green color.

Sequence Analysis of ITS1 ribosomal DNA region. New ITS1 sequences (185) generated from individual male and female RN isolates consisted of 145 and 40 new ITS1 sequences made up of two types of fragments with average lengths, 549.3 and 720.8 bp and hereafter, called ITS1S and ITS1L, respectively. These ITS1S sequences consisted of 29, 39, and 77 sequences from four female, ten female, and ten male RN isolates from the mixed-Alabama and Belle Mina populations respectively. Similarly, the ITS1L sequences consisted of much smaller sequence numbers made up of 16, 12, and 12 sequences for mixed-Alabama and Belle Mina populations, respectively. The ITS1 fragments were made up of conserved and parsimony-informative sites representing 406 (69.2%), and180 (30.8%), respectively for ITS1S, and 516 (69.7%) and 224 (30.3%), respectively for ITS1L. Within the ITS1S sequences, the average nucleotide frequencies were 27.1%, 24.7%, 24.4%, 23.9% for T, C, A, and G, respectively. The larger ITS fragment (ITS1L) had average nucleotide frequencies of 25.7%, 25.9%, 22.2%, and 26.2%, for T, C, A, and G, respectively.

Neighbor Joining analysis on ITS1S and ITS1L sequences produced consensus trees showing clone sequences from disparate nematodes clustering together (Figs. 3 and 4). However, two clones EC07 and EC10 did not cluster with any of our sequences. The overall average pairwise distance among the ITS1S and ITS1L sequences was 0.03 and 0.05, respectively. Sequencing of the ITS1 region of rDNA showed considerable intra- and inter-nematodal variation within individual male and female RN isolates. This variation might have resulted from differences in the rates of ITS1 evolution occurring within the nematodes. Variation within this region was also observed by Tilahun et al. (2008), although individual nematodal variation could not be accessed because amplifications were from pooled RN DNA. In another study, similar variations within the ITS1 region were observed for the amphimictic populations from cotton-growing regions in mid-land U.S., and those from Brazil, Colombia, Hawaii, and Honduras (Agudelo et al., 2005). However, a parthenogenetic population from Japan could be distinguished from the other populations using the ITS1 region. Indels and substitutions were observed in our ITS1 sequences after MSA was performed. Although mutation rates occur at higher rates in loops and stems of rRNA (Dixon and Hillis, 1993), mechanisms involved may be different (Tusher et al., 2004). Identification of short diallelic indels is facilitated by examining aligned sequences of individuals of a population, for the presence of gaps within the sequence. The identification and characterization of diallelic insertion-deletion polymorphisms for example in the human genome has been observed, this constituted 2,303 indels within 330 candidate genes (Tusher et al., 2004). Intra-nematodal variation has been observed within the ITS-2 region of the swim bladder nematodes, *Cystidicola* spp. (Miscampbell et al., 2004). Two ITS-2 variants differed at four nucleotide positions which were all from different populations.

Findings from this study and from eukaryotic species for transcribed and non-transcribed regions of 18S gene for RN are not exceptions to the concept of concerted evolution. This is believed to homogenize multiple copies of rDNA within species and promote optimal translation efficiency, and reduce intra-specific variance. Estimate of evolutionary divergence among the sequences showed the more divergence in ITS1 sequences, than 18S sequences, with ITS1L sequences having the greatest divergence. Sequence similarities were more prevalent in ITS1S than in ITS1L, thus implying a more recent origin of ITS1S than more diverse ITS1L. A higher degree of size variation was noted in ITS1L, and the rates of variation in ITS1 regions differed between clones compared to the 18S sequences. Factors associated with extreme size variation include high substitution rates and duplication of repeat sequences. Reports on size variation between closely related taxa indicate lengths not exceeding 400 bp, except for the *Schistosoma japonica* (Platyhelminthes) and the *Anopheles gambiae* (Insecta) species, with 900 bp and 3,000 bp sizes respectively (von der Schulenburg et al., 2001). Ladybird beetles (Coleoptera: Coccinellidae) have an ITS1 region ranging from 791 bp up to 2,572 bp, which shows increased level of variability.

CONCLUSIONS

Our results show the presence of variants of nematodes within Alabama populations based on the following: (1) the presence of the 500 bp band observed in some of the PCR-RFLP amplifications in the ITS1 digests, and (2) the presence of 27 parsimony-informative bases distinctly separating the 18S rDNA clones in these populations into two major types of sequences. Phylogenetic analysis of the multiple sequence alignments revealed the existence of two putative 18S rRNA gene sequence types and many ITS1 sequence variants within individual male and female RN isolates. Sequencing of 18S and ITS1 rDNA regions was critical in detecting differences between individual clones.

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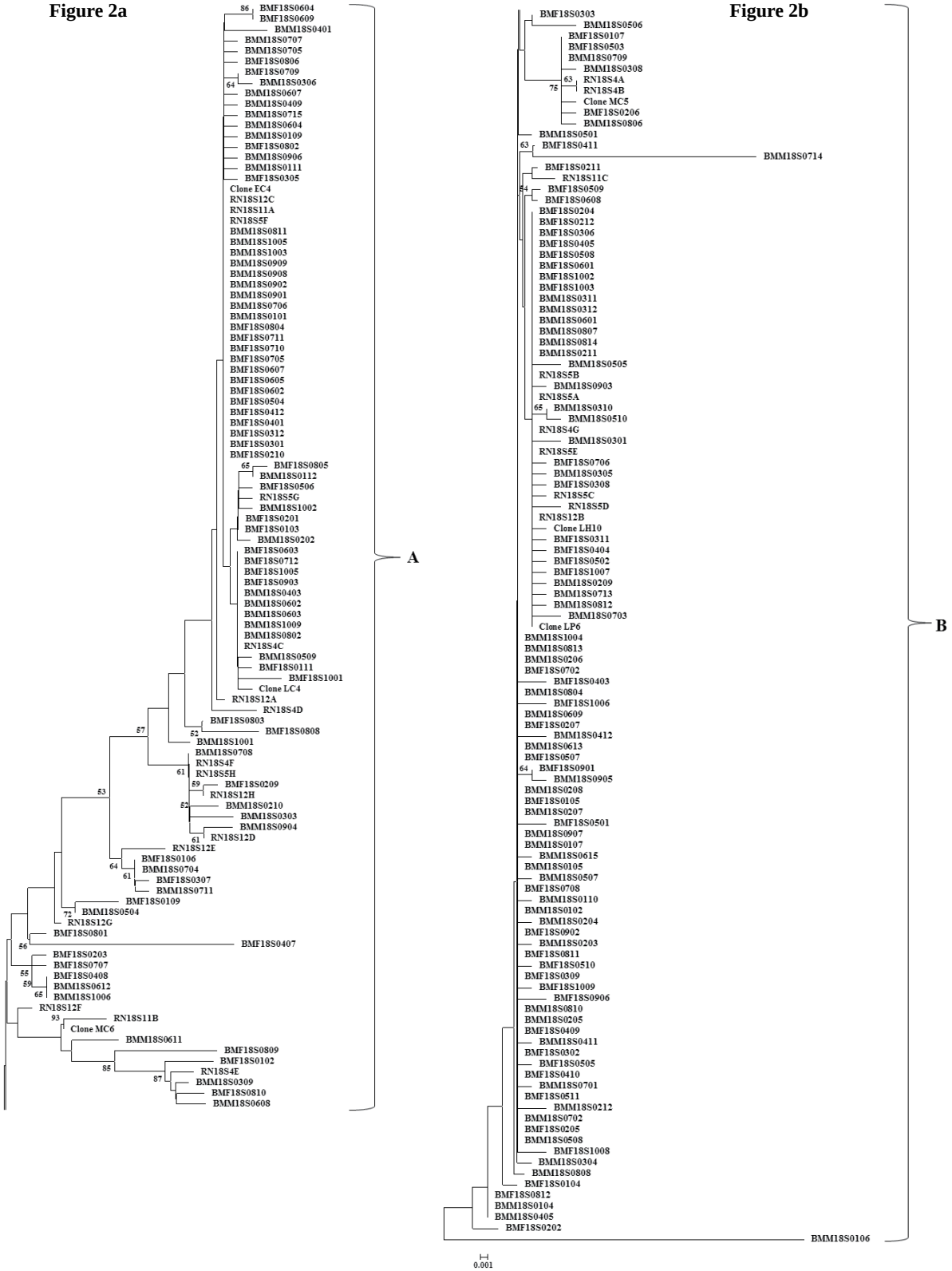

Figure 2. Phylogram generated from Neighbor-Joining analysis for 18S clone sequences of female and male reniform nematodes from mixed-Alabama and Belle Mina populations. The percentages of bootstrap replicates supporting the clades are indicated at the branch points. Bootstrap values greater than 50% are shown beside nodes. RN= Reniform nematode; 18S =18S rRNA gene; 01-10 = reniform nematode isolate IDs; A, B, C, D, E, F, G, H/01-15= clone IDs, BM=Belle Mina, F=Female, M=Male.

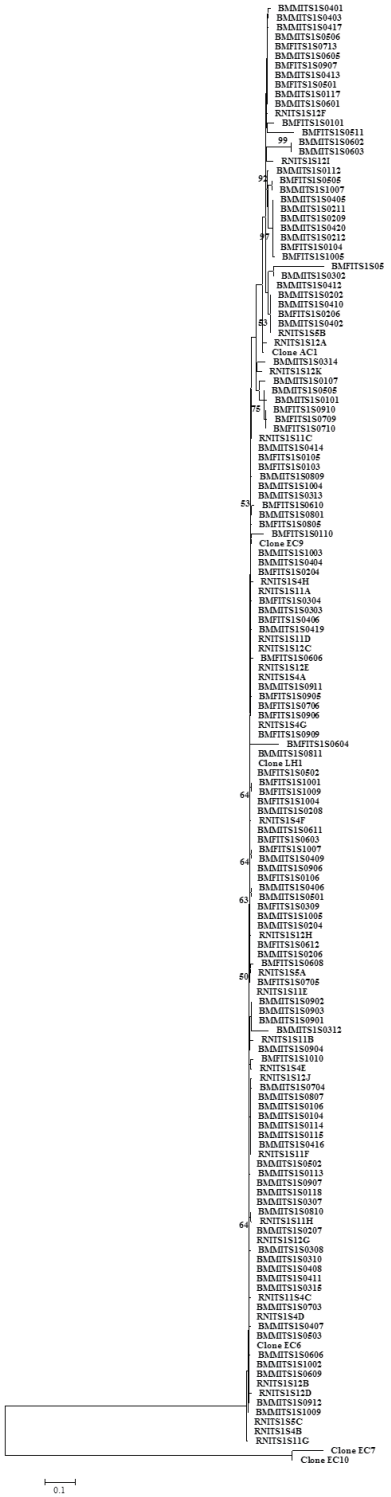

Figure 3. Phylogram generated from Neighbor-Joining analysis for ITS1-550 sequences for female and male reniform nematodes from mixed-Alabama and Belle Mina populations. The percentages of bootstrap replicates supporting the clades are indicated at the branch points. Bootstrap values greater than 50% are shown beside nodes. RN= Reniform nematode; ITS1S = Small ITS1 rDNA; 01-10 = reniform nematode isolate IDs; A, B, C, D, E, F, G, H/01-20= clone IDs, BM=Belle Mina, F=Female, M=Male.

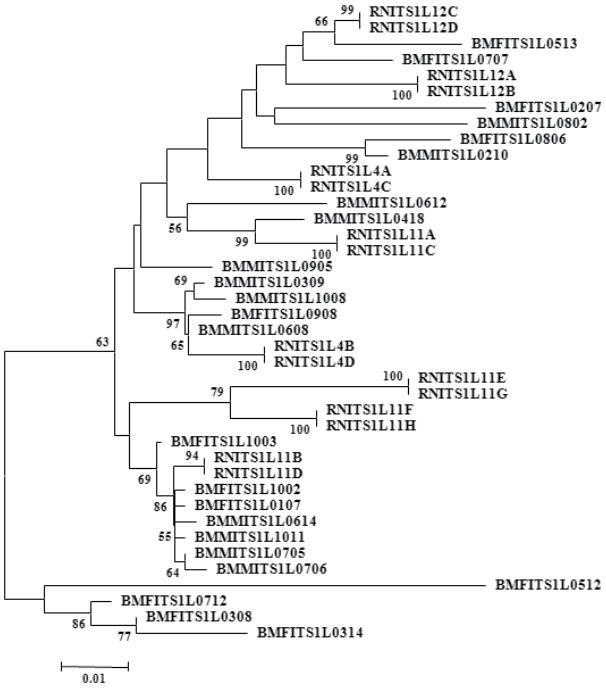

Figure 4. Phylogram generated from Neighbor-Joining analysis for ITS1-720 sequences for female and male reniform nematodes from mixed-Alabama and Belle Mina populations. The percentages of bootstrap replicates supporting the clades are indicated at the branch points. Bootstrap values greater than 50% are shown beside nodes. RN= Reniform nematode; ITS1L= Large ITS1 rDNA; 01-10 = reniform nematode isolate IDs; A, B, C, D, E, F, G, H/01-18= clone IDs, BM=Belle Mina, F=Female, M=Male.