African Journal of Biotechnology Vol. 7 (18), pp. 3217-3224, 17 September, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Nuclear ribosomal DNA diversity of a cotton pest (*Rotylenchulus reniformis*) in the United States

Yonathan Tilahun[1]*, Khairy Soliman[1], Kathy S. Lawrence [3], Leland J. Cseke[2] and Joel W. Ochieng[4]

[1]Natural Resources and Environmental Sciences, Alabama A and M University, P.O. Box 1927 Normal, AL 35762, U.S.A.
[2]Department of Biological Sciences, University of Alabama in Huntsville, Huntsville, AL 35899, U.S.A.
[3]Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36049, U.S.A.
[4]Centre for Plant Conservation Genetics, Southern Cross University, Lismore, NSW 2480 Australia.

Accepted 1 August, 2008

The reniform nematode (*Rotylenchulus reniformis*) has emerged as a major cotton pest in the United States. A recent analysis of over 20 amphimictic populations of this pest from the US and three other countries has shown no sequence variation at the nuclear ribosomal internal transcribed spacer (ITS) despite the region's usual variability. We investigated this unexpected outcome by amplifying, cloning and sequencing two regions of the nuclear ribosomal DNA (18S, ITS1) to ascertain whether any variation occurred within and among populations of reniform nematodes in Alabama, US. Both the nrITS1 and the relatively conserved 18S region showed a fairly substantial amount of variation among populations. The identity among ITS sequences ranged from 1.00 to 0.86, while sequence identity at the 18S ranged from 1.00 to 0.948. We conclude that variation does exist in these sequences in reniform nematodes, and the earlier report showing no ribosomal ITS variation in this pest might have been caused by preferential amplification of a conserved ITS paralog. Current and future application towards resistance in cotton varieties to this pest requires reliable information on the molecular variability of the nematode in cotton-growing areas.

Key words: Ribosomal DNA, ITS, 18S, reniform nematode.

INTRODUCTION

The reniform nematode (*Rotylenchulus reniformis*) has become a major pest of cotton in the United States (Koenning et al., 2004). Yield losses due to reniform nematode in 2004 were estimated to be US $166 M or 425,000 bales (Hollis, 2005). The reniform nematode is believed to cause the greatest amount of damage to cotton (Hollis, 2005). Traditional pest management strategies such as nematicides are harmful to the environment and costly (Davis et al., 2003). Host plant resistance has been advocated as a preferred control method, and research efforts to develop commercial cotton cultivars resistant to nematodes are underway (Agudelo et al., 2005; Koenning et al., 2004; Robinson et al., 2004). A major drawback to these efforts is the absence of reliable information on the variability of this nematode in cotton-growing areas (Agudelo et al., 2005). Several studies have reported phenotypic variability within and among populations (Agudelo et al., 2005; Lehman and Insera, 1989), however, phenotypic variation is not necessarily genetic. Identification of genetic diversity in nematodes has been a reliable form of nematode pest management strategies (Agudelo et al., 2005). However, genes for resistance to reniform nematode have not yet been discovered, especially in cultivated tetraploid cotton. Sequence information provided by three research groups including the present report has provided important insight toward a more accurate method of determining

*Corresponding author. E-mail: yontil@gmail.com. Tel: + 1-256-372-4547. Fax: + 1-256-372-8404.

genetic variability in nematodes such as reniform nematode.

In a recent effort to quantify the level of both morphometric and molecular genetic diversity within and among populations of reniform nematodes in cotton-growing areas of the United States, Agudelo et al. (2005) analyzed morphometric variability as well as variation at the first internal transcribed spacer of the nuclear ribosomal DNA (ITS1). The authors used a total of 27 populations (20 from the US, two each from Brazil and Colombia, one population from Honduras and two populations from Japan). Twenty six populations comprised both sexes (amphimictic), while the population from Japan contained no males and was considered parthenogenic. For simplicity, the abbreviation AEA will be used to represent Agudelo et al. (2005) in subsequent citations. AEA amplified DNA from two to five individual samples per population made up of 40 - 100 individuals, and sequenced multiple clones per individual. This work was very important, being the first published evaluation of molecular genetic diversity of *R. reniformis* in the United States.

As mentioned, the study was composed of a wide geographic scale covering 10 states of the US as well as four countries outside of the US. Although reasonable phenotypic polymorphisms were recorded, AEA reported observing no polymorphic nucleotide site for 26 populations, except one population from Japan, which showed 11.78% divergence from the rest (Agudelo et al., 2005). In the words of AEA, "All amphimictic populations were identical for their ITS1 sequence, but several are distinguishable by their morphometrics and (or) host preferences." AEA suggested that the lack of ITS1 diversity in this pest could result from a lack of geographic barriers or a recent very rapid radiation from a common origin. The latter case, they argued, could impose a genetic bottleneck effect resulting from a limited number of introductions, such as passive transport by human activities.

While the explanation given by AEA was the most parsimonious and convincing in the context of the data obtained, we believed this outcome with reluctance, because of our experience with molecular markers (including ITS) and theoretical expectations. The ITS region is generally variable in most life forms, even within populations (and sometimes records intra-individual variability). This gene has been shown to be highly variable (Amiri et al., 2002; Floyd et al., 2002). Indeed, AEA were aware of this expectation and cited cases (De Ley et al., 1999; Hugall et al., 1999) in which "this marker has been considered too variable to be used for determining the relationships among nematode populations" (Agudelo et al., 2005). We have embarked on a large project to study the pattern of variation among populations of reniform nematodes in the US. We found it prudent to use a cheaper and efficient method to first survey the prospect of finding any variation among populations, which happened to coincide with the report by AEA. We sequenced the usually variable ITS1 region as well as the more conserved 18S region of the nuclear ribosomal DNA (rDNA) in several nematode populations from cotton-growing areas of Alabama. To obtain rapid results, we combined nematodes from the same population into DNA pools. Our results show a definitive explanation for the AEA report in addition to a more clear description of what is occurring in the regions used for molecular, phylogenetic identification of the reniform nematode.

We are aware that the nuclear rDNA may occur in multiple copies in some genomes. The existence of paralogs would thus make it difficult to distinguish between intra- and inter-individual variations. In this particular study, however, our aim was not to precisely quantify this variation. Rather, as indicated earlier, we utilized DNA pools as a much simpler and faster preliminary method in order to evaluate an earlier report showing that no variation existed at this locus in amphimictic reniform nematodes. The assumption was that variation in the reinform isolates (especially with respect to the 18S rDNA sequence), would correspond to geographic or edaphic properties. An additional advantage of using pooled DNA is the capacity to identify allelic variants, if any were present, in a mixed population.

MATERIALS AND METHODS

Sample locations

Soil samples from nematode-infested cotton fields were obtained from seven locations in five counties in Alabama through two repositories: the Alabama Agricultural Research Station at Belle Mina and the Plant Diagnostics Laboratory, Auburn University. Soil samples obtained from Belle Mina were from counties in Northern Alabama and were designated Limestone county Anderson farm (AL), Lawrence county Haney farm (LH), Lawrence county Posey farm (LP), Morgan county farm unknown (LC), and Morgan county Collins farm (MC), while those from Auburn University were from the counties in southern Alabama and were designated Autauga county farm unknown (AC) and Escambia county farm unknown (EC) (Figure 1). Soil samples were collected from the root zones of infected cotton plants using an established protocol found at http://www.aces.edu/dept/plantdiagnosticlab/form2.htm. A population was defined as a cotton field for this analysis. Nematodes were extracted from 100 cm^3 soil samples using gravity screens and 40% w/w sucrose centrifugal flotation (Jenkins, 1964). *R. reniformis* was identified by microscopic examination and distinguished by stylet length, vulva position, head shape, tail shape, and the presence of males with distinct spicules on tail ends (Robinson et al., 1997). After morphometric identification of each nematode and species confirmation, eight adult nematodes were placed in 25 µL of sterile distilled water, manually ruptured with a Shepherd's dental hook, and 10 aliquots of a given population were pooled (250 µL total).

DNA extraction and PCR amplification

Nematode DNA extraction method of Harris et al. (2001)

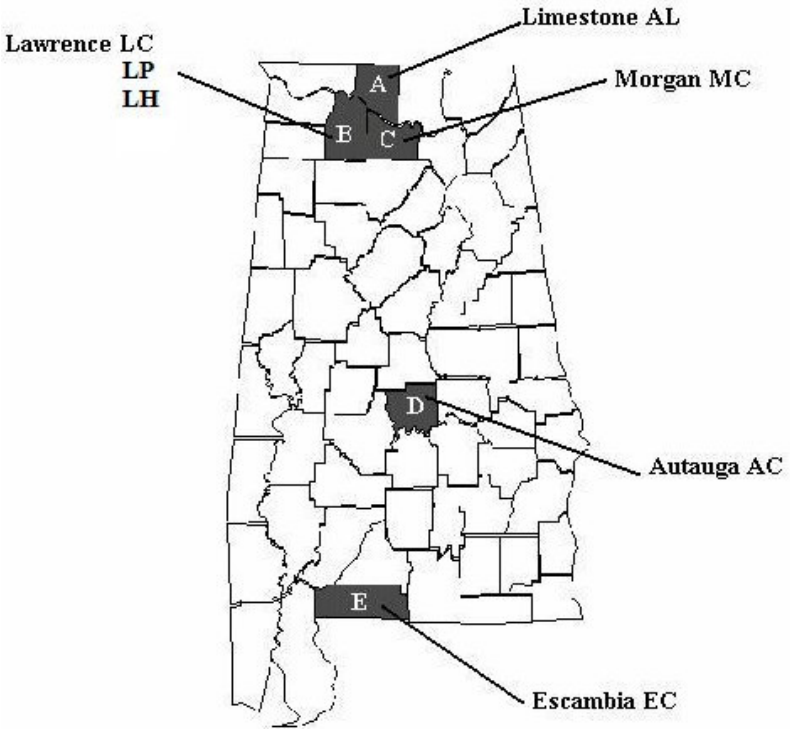

Figure 1. Map of Alabama showing the areas where samples were obtained.

was adopted. Amplifications of the ITS1 regions were carried out using ITS1F primer located in the 3' portion of the 18S rRNA gene approximately 190 bp from its junction with the first internally transcribed spacer (ITS1), and ITS1R primer located in the first 20 bp of the 5.8S (Powers et al., 1997). Amplifications of the 18S regions were carried out using 18S primer pairs (Cherry et al., 1997), both located in the 3' portion of the 18S gene, about 630 bases apart.

A PCR reaction for each population was performed using a 10 µL aliquot of the pooled nematode DNA in a 50 µL reaction containing 100 µM dNTPs (Promega, Madison, WI), 2.5 U Platinum High Fidelity Taq Polymerase, PCR 1X buffer, 2.5 mM $MgSO_4$ (Invitrogen-Life Technologies Carlsbad, CA) using either the 18S or the ITS1 primer-pairs while including a negative control containing no DNA. Amplifications were carried out in an MJ Research PTC-200 PCR engine (MJ Research, Inc., Watertown, MA) using the touchdown parameters of Harris et al. (2001). Thermal cycles consisted of 2 min and 45 s at 94˚C; 9 cycles of 1 min at 94˚C, 45 s at 64˚C decreasing 1˚C for each cycle, and 2 min at 72˚C; followed by 29 cycles of 1 min at 94˚C, 45 s at 55˚C, 2 min at 72˚C; and a final elongation step of 10 min at 72˚C. Successful amplifications were confirmed by electrophoresis of an 8 µL aliquot of PCR product on a 1% agarose gel. Successful PCRs showing an amplified product of expected size were stored at -20˚C.

DNA cloning and sequencing

PCR products were cloned using a one-shot TOPO TA Cloning Kit for Sequencing (Invitrogen-Life Technologies, Carlsbad, CA, USA) and plasmid DNA was isolated using a Qiaquick Spin Miniprep Kit (Qiagen, Valencia, CA). Five clones showing a positive insert were selected for sequencing. Samples were prepared with BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions for forward and reverse sequences of each clone were carried out using M13 forward and M13 reverse primers provided in the TOPO TA Cloning Kit for Sequencing (Invitrogen-Life Technologies, Carlsbad, CA, USA). Sequencing reac-tions were carried out as follows: 24 cycles of 1˚C/s to 96˚C, 96˚C for 10 s, 1˚C/s to 50˚C, 50˚C for 5 s; 1 ˚C/s to 60˚C for 4 min; 1˚C/s to 4˚C. Sequence products were separated on an ABI 3100 DNA Sequencer (Applied Biosystems, Foster City, CA).

Sequencing analysis and multiple sequence alignment

A total of 59 clones were sequenced in both the 5' and 3' directions. The resulting 118 sequences were assembled by clone identity using CAP3 program (Huang and Madan,

Table 1. Pairwise sequence identity among *Rotylenchulus reniformis* sequences at the nrITS1 region.

Sequence	EC09	EC07	Amph	EC02	LH01	EC03	EC06	AY191a	AY192a	AC01	EC18	Agu-p	AY190-p
Escambia(SA)EC10	1.000	0.989	0.989	0.989	0.989	0.986	0.989	0.989	0.989	0.959	0.915	0.870	0.877
Escambia(SA)EC09		0.989	0.989	0.989	0.989	0.986	0.989	0.989	0.989	0.959	0.915	0.870	0.877
Escambia(SA)EC07			1.000	1.000	1.000	0.996	1.000	1.000	1.000	0.969	0.925	0.867	0.873
Agudelo-Amph				1.000	1.000	0.996	1.000	1.000	1.000	0.969	0.925	0.867	0.873
Escambia(SA)EC02					1.000	0.996	1.000	1.000	1.000	0.969	0.925	0.867	0.873
Lawrence(NA)LH01						0.996	1.000	1.000	1.000	0.969	0.925	0.867	0.873
Escambia(SA)EC03							0.996	0.996	0.996	0.966	0.922	0.863	0.870
Escambia(SA)EC06								1.000	1.000	0.969	0.925	0.867	0.873
AY335191-Amph									1.000	0.969	0.925	0.867	0.873
AY335192-Amph										0.969	0.925	0.867	0.873
Autauga(SA)AC01											0.942	0.877	0.883
Escambia(SA)EC18												0.860	0.867
Agudelo-Parth													0.993

Agudelo (Agu-)- Agudelo et al. (2005); Amp- Amphimictic; Parth- parthogenic; A or P- amphimictic or parthogenic according to Agudelo et al. (2005). Sequences in boldface are identital (1.00) and have a GC content lower than 72%.

1999). The assembled sequences were trimmed for vector sequence by comparison to vector sequences in GenBank using the VecScreen tool (http://www.ncbi.nlm.nih.gov/VecScreen/). Any bacterial contamination that may have occurred in the early stages of DNA isolation and that may have been carried through was identified during the BLAST search. Bacterial contamination was subsequently removed. A total of fifty-two 18S sequences and sixteen ITS1 sequences with good quality were chosen for further analysis. Ribosomal 18S and ITS1 sequences were screened by standard nucleotide-nucleotide BLAST against all sequences in GenBank on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) in order to detect homology to archived nematode 18S and ITS1 sequences, respectively. Further, all sequences were compared against themselves using local BLAST server for determining the similarity of sequences within the dataset. A total of 52 sequences from seven nematode pools were obtained, aligned, and deposited to GenBank under accession numbers AY373658 through AY373537. In addition to sequences obtained in this study, we included the two ITS sequences shared by all amphimictic and parthenogenic *R. reniformis* respectively, published by AEA. For comparison, three *R. reniformis* ITS sequences in GenBank (AY335190; AY335191; AY335192) were included. Sequences were aligned (18S and ITS1 independently) using the CLUSTALW applica-tion embedded in the BioEdit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/BioEdit/). Multiple sequence alignment was further edited manually using Jalview (http://www.ebi.ac.uk/jalview/). Incomplete sequences or those comprising less than 90% of the targeted length (as a result of read-length problems rather than indels) were excluded from the analysis. Sequence identity, as well as neighbour-joining clustering was computed using BioEdit analysis software (http://www.mbio.ncsu.edu/BioEdit/).

This study reports genetic variation at the nuclear ribosomal ITS1 in *R. reniformis* from a narrow geographic range (Alabama) as would be expected from theory and from previous work on other nematode species. The nrITS1 region showed a substantial amount of variation among populations analyzed. The new sequences were also polymorphic when compared to the sequences of AEA, and to the GenBank sequences of Iwahori and Sano (unpublished). The identity among sequences ranged from 1.00 to 0.86 (Table 1). Comparisons showed that all sequences recovered in this study were more homologous to amphimictic groups identified by AEA. Sequence identity among amphimictic types ranged from 0.915 to 1.00; however, the two parthenogenic sequences (AY335190 and the

Table 2. Pairwise sequence identity among *Rotylenchulus reniformis* sequences at the nr18S region.

Seq->	LC09	LH09	EC07	LP06	LH10	LC05	AC05	AC01	LC04	EC04	AL01	AC06	MC02	LP03	EC02	MC03	LH06	LC08	AL04	MC06	EC10
LP08	0.995	0.997	0.997	0.987	0.995	0.955	0.953	0.955	0.955	0.955	0.955	0.955	0.965	0.955	0.953	0.953	0.955	0.962	0.995	0.990	0.995
LC09		0.997	0.997	0.992	1.000	0.955	0.953	0.955	0.955	0.955	0.955	0.955	0.965	0.955	0.953	0.953	0.955	0.967	1.000	0.995	0.995
LH09			1.000	0.990	0.997	0.958	0.955	0.958	0.958	0.958	0.958	0.958	0.967	0.958	0.955	0.955	0.958	0.965	0.997	0.992	0.997
EC07				0.990	0.997	0.958	0.955	0.958	0.958	0.958	0.958	0.958	0.967	0.958	0.955	0.955	0.958	0.965	0.997	0.992	0.997
LP06					0.992	0.953	0.950	0.953	0.953	0.953	0.953	0.953	0.962	0.953	0.950	0.950	0.953	0.965	0.992	0.987	0.987
LH10						0.955	0.953	0.955	0.955	0.955	0.955	0.955	0.965	0.955	0.953	0.953	0.955	0.967	1.000	0.995	0.995
LC05							0.997	1.000	1.000	1.000	1.000	1.000	0.985	1.000	0.997	0.995	1.000	0.982	0.955	0.950	0.955
AC05								0.997	0.997	0.997	0.997	0.997	0.982	0.997	0.995	0.992	0.997	0.980	0.953	0.948	0.953
AC01									1.000	1.000	1.000	1.000	0.985	1.000	0.997	0.995	1.000	0.982	0.955	0.950	0.955
LC04										1.000	1.000	1.000	0.985	1.000	0.997	0.995	1.000	0.982	0.955	0.950	0.955
EC04											1.000	1.000	0.985	1.000	0.997	0.995	1.000	0.982	0.955	0.950	0.955
AL01												1.000	0.985	1.000	0.997	0.995	1.000	0.982	0.955	0.950	0.955
AC06													0.985	1.000	0.997	0.995	1.000	0.982	0.955	0.950	0.955
MC02														0.985	0.982	0.980	0.985	0.997	0.965	0.960	0.965
LP03															0.997	0.995	1.000	0.982	0.955	0.950	0.955
EC02																0.992	0.997	0.980	0.953	0.948	0.953
MC03																	0.995	0.977	0.953	0.948	0.953
LH06																		0.982	0.955	0.950	0.955
LC08																			0.967	0.962	0.962
AL04																				0.995	0.995
MC06																					0.990

sequence P of Agudelo et al., 2005) were less homologous to the amphimictic group (sequence identity 0.877 to 0.860; Table 1). Computation of the GC content (percentage of G and C nucleotides; data not shown) within the ITS1 region corresponded to two main groupings: one group comprised sequences with GC content less than 72%, while the other group was characterized with greater than 72% GC. The two groups are indicated in Figure 2. Members of the first group are indicated in bold face in Table 1. While the second group comprised sequences with considerable variation as evidenced by a low sequence identity, the group characterized by a lower GC content were conserved (sequence identity; Table 1). This group includes the invariable amphimictic sequence of AEA.

The 18S region is usually conserved in most life forms, and primers to amplify the spacer region (ITS) are often designed within the 18S by multiple alignment (Ochieng et al., 2007; Powers et al., 1997). Despite this, a fair amount of sequence difference was observed at this locus among populations, albeit generally less than that observed at the ITS1 locus. Sequence identity at the 18S ranged from 1.00 to 0.948 (Table 2). As with the ITS1, the 18S was characterized by a conserved group of sequences as well as a variable one. Owing to the analysis approach in which nematodes from each population were pooled before DNA extraction (intra- and inter-individual variants are expected), neighbour-joining (NJ) rather than parsimony was used to visualize the diversity as a network of similarity. The NJ trees (Figures 2 and 3) therefore do not imply anything about the nature of the evolution of the ITS variants, rather, they show the similarity or dissimilarity among sequences. Consequently, sequences that diverge from the same node with

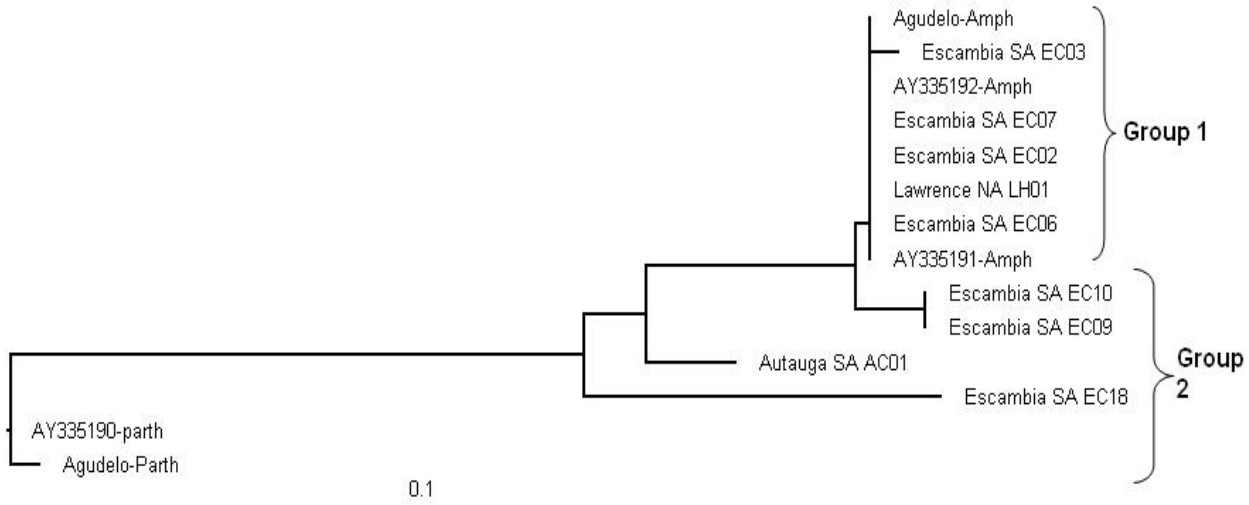

Figure 2. Neighbour-joining analysis showing the clustering of nrITS sequences.

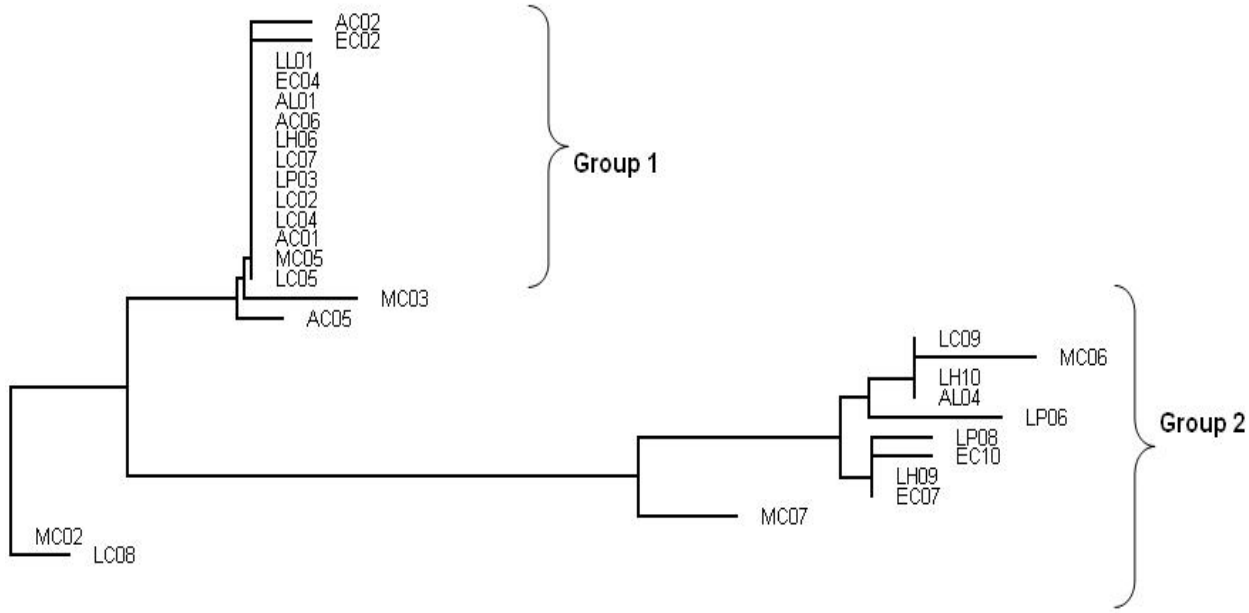

Figure 3. Neighbour-joining analysis showing the clustering of 18S sequence.

no branch lengths are inferred to be identical. Some of the populations analyzed for 18S were not analyzed at ITS1; therefore variation at the two regions cannot be compared for the same set of sequences.

Current and future application towards resistance in cotton varieties to this pathogen requires reliable information on the molecular variability of this nematode in cotton-growing areas. This study reports genetic variation at the nuclear ribosomal ITS1 and 18S rDNA in *R. reniformis* from a narrow geographic range (Alabama) as would be expected from theory and from previous work on other nematode species (Amiri et al., 2002; Stack et al., 2000; Bendezu et al., 1998). The study by AEA covered a wider range than this study- as stated earlier- yet the authors detected no polymorphism at the ITS1 among amphimictic populations. The authorship of AEA comprised a team of considerable experience in nematology and molecular biology. Their work was conducted competently. It is, thus, most parsimonious to believe that the discrepancy between the two datasets is a result of

technical aspects in the amplification process. The results would appear to suggest that AEA amplified one paralog of the ITS in their study. Sequences that have evolved from a single most recent common ancestor (MRCA) at the root of a clade are said to be orthologous. In contrast, DNA sequences that are the result of gene duplication events are paralogs. The ITS region has exhibited paralogy in the studies of many life forms, including nematodes (Hugall et al., 1999). As stated earlier, our study recovered two sets of sequences: a conserved group and a more variable one. The conserved sequence group was characterized by a lower GC content and a sequence identity of 1.00, showing that the ITS1 was invariable among members of this group. The ITS1 sequence of amphimictic nematodes reported by AEA as showing no variation clustered with this invariable group. It is reasonable to believe that these two sequence sets are paralogs of ITS. Results of 18S, which also showed a conserved group as well as a variable group, strengthen the argument that this ribosomal DNA could be characterized by paralogy. If only members showing no variation were amplified in this work, we would easily conclude that no variation existed at the ITS region. This might explain why AEA found no variation at the ITS1. The more variable set had a higher GC content, perhaps suggesting some structural stability. However, the identification of pseudogenes is beyond the scope of this manuscript.

PCR problems in DNA sequencing can be twofold: small inevitable variations in PCR conditions can influence which of the paralogs are amplified. AEA designed amplification primers from the genome of an amphimictic *R. reniformis* nematode, which was then used to amplify other members of the same group. The primers used in the present study were designed from the genome of a related genus, *Belonolaimus* (Cherry et al., 1997). The expected nucleotide variation between the genomes amplified in this study and that of *Belonolaimus* might have influenced the amplification of a more variable copy of the nrITS in this study. Despite the high fidelity of the DNA polymerases, there is a variable error rate in the synthesized DNA due to nucleotide mis-incorporations (Ochieng et al., 2007), and by many factors in the reaction mixture, such as concentrations of dNTPs, magnesium ions, and pH (Ling et al.,1991), as well as the kind and amount of DNA being amplified. The possibility that PCR and gel recovery artefacts might have caused variation in our work can be ruled out because in this study, all the large indels occurred at the same nucleotide position for each population and were widespread. There were defined segments of rDNA sequence that showed variation shared between and among groups of the nematodes, authenticating that real variation existed among populations. We conclude that *R. reniformis* is diverse at the nuclear ribosomal DNA. An earlier report (Agudelo et al., 2005) showing no variation of this molecule in the reniform nematode in the US might have been caused by the amplification of a conserved ITS paralog identified in this study.

ACKNOWLEDGMENTS

This manuscript is dedicated to the memory of the late Allan Zipf. Sequences published by Paula Agudelo and co-workers, and those submitted to GenBank by Iwahori and Sano have been used for comparison. The authors would like to express additional appreciation to R. Kantety, L. Carter, T. O. Powers, M. Burns, R. Robbins, C. Burmester, K. Siggers, D. Marsic, and Z. Wu for their assistance on this project. This study was supported by a grant from the Applied Genomics for Cotton Improvement (State Grant ALAX-2280143164-0188544), a portion of a Master's thesis by Yonathan Tilahun, Contributed by the Agricultural Experiment Station, Alabama A&M University, publication No.529. Mention of trade name, warranty, proprietary product, or vendor does not constitute an endorsement of a product and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

REFERENCES

Agudelo PR, Robbins T, Stewart J, Szalanski A (2005). Intraspecific variability of *Rotylenchulus reniformis* from cotton-growing regions in the United States. J. Nematol. 37(1): 105-114.

Amiri S, Subbotin SA, Moens M (2002). Identification of the beet cyst nematode *Heteroders schachtii* by PCR. Eur. J. Plant Pathol. 10: 497-506.

Bendezu IF, Evens K, Burrows PR, De Pomerai D, Canto-Saenz M (1998). Inter- and Intra-specific genomic variability of the potato cyst nematodes *Globodera pallida* and *G. rostochiensis* from Europe and South America using RAPD-PCR. Nematologica, 44: 49-61.

Cherry T, Szalanski AL, Todd TC, Powers TO (1997). The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonoaimidae). J. Nematol. 29: 23-29.

Davis RF, Koenning SR, Kemerait RC, Cummings TD, Shurley WD (2003). *Rotylenchulus reniformis* management in cotton with crop rotation. J. Nematol. 35: 58-64.

De Ley IT, Karssen G, De Ley P, Vierstraete A, Waeyenberge L, Moens M, Vanfleteren J (1999). Phylogenetic analyses of internal transcribed spacer region sequences within *Meloidogyne*. J. Nematol. 31: 530-531.

Harris TH, Szalanski AL, Powers TO (2001). Molecular identification of nematodes manual. Manual 1. http://nematode.unl.edu/IS_ID/lts_id.htm.

Hugall A, Stanton J, Moritz C (1999). Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. Mol. Biol. Evol. 16: 157-164.

Jenkins WR (1964). A rapid centrifugal floatation technique for separating nematodes from soil. Plant Dis. Rep. 48: 692.

Koenning SR, Kirkpatric TL, Starr JL, Wrather JA, Walker NR, Mueller JD (2004). Plant-parasitic nematodes attacking cotton in the United States: Old and emerging production challenges. Plant Dis. 88: 100-113.

Lehman PS, Inserra RN (1989). Morphometric variation of *Rotylenchulus parvus* and *Rotylenchulus reniformis* populations in the southern United States. Proc. Soil Crop Sci. Soc. Florida 49: 220-226.

Ling LL, Keohavong P, Dias C, Thilly WG (1991). Optimization of the

polymerase chain reaction with regard to fidelity: Modified T7, Taq, and Vent DNA polymerases. PCR Methods Appl. 1: 63-69.
Ochieng JW, Henry RJ, Baverstock PR, Steane DA, Shepherd M (2007). Nuclear Ribosomal Pseudogenes resolve a corroborated Monophyly of the eucalypt genus *Corymbia* despite misleading hypotheses at functional ITS paralogs. Mol. Phylogenet. Evol. 44: 752-764.
Powers TO, Todd TD, Burnell AM, PC, Murray B, Fleming CC, Szalanski AL, Adams BA, Harris TS (1997). The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. J. Nematol. 29: 441-450.
Robinson AF, Inserra RN, Caswell-Chen EP, Vovlas N, Troccoli A (1997). *Rotylenchulus* species: identification, distribution, host ranges, and crop plant resistance. Nematropica, 27(2): 127-180.
Stack CM, Easwaramoorthy SG, Metha M, Downes J, Griffin CT, Burnell AM (2000). Molecular characterization of *Heterorhabditis indica* isolates from India, Kenya, Indonesia, and Cuba. Nematology, 2(5): 477-487.