MORPHOLOGICAL AND MOLECULAR BIOLOGICAL CHARACTERIZATION OF *BELONOLAIMUS LONGICAUDATUS* [1]

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ABSTRACT

Han, H-R., A. Jeyaprakash, D. P. Weingartner, and D. W. Dickson, 2006. Morphological and Molecular Biological Characterization of *Belonolaimus longicaudatus*. Nematropica 36:37-52.

Belonolaimus longicaudatus has been reported as a complex species because of its variations in morphology and pathogenicity among different isolates. However there is no information about genetic characteristics of *B. longicaudatus* among different populations coupled with detailed morphological characteristics. Our objectives were to determine the intraspecific variation based on morphology and ITS1 DNA sequence among isolates collected from different geographical locations and host crops. Five isolates compared by all criteria were HA—potato, Hastings, FL; GV—bermudagrass, Gainesville, FL; LA—citrus, Lake Alfred, FL; GA—cotton, Tifton, GA; and NC—corn, Scotland County, NC. Two additional isolates compared with these five isolates with respect to ITS1 DNA sequence were NE—corn, Columbus, NE, and TX—bermudagrass, Poteet, TX. The ITS1 DNA sequence previously reported for South Carolina (SC) isolate (GenBank accession #U89696) was included for sequence comparison. Females of the LA and NC isolates were longer in body length and the LA isolate had a longer stylet compared with the other isolates ($P \leq 0.05$). The stylet knobs of the NC and LA isolates were typically teardrop or kidney-shaped, whereas the GA isolate was typically oval. The vaginal pieces of the LA isolate were the most prominent and clearly recognized among all isolates, but that of NC isolate was weakly developed and not clearly recognized. The lengths of the ITS1 region were similar, 468 bp for all isolates, excepting the TX isolate which had a length of 428 bp. For phylogenetic analysis, maximum parsimony, neighbor-joining, and maximum likelihood methods were used and phylogenetic trees were constructed. All three trees supported that the NE, SC, and TX isolates were clearly different from the FL (GV, HA, LA), GA, and NC isolates. The TX isolate seemed to have diverged from the group of FL, GA, and NC isolates, which was supported by a 100 bootstrap value.

Key words: Belonolaimus longicaudatus, intraspecific variation, morphology, ITS1, phylogeny, molecular systematics.

RESUMEN

Han, H-R., A. Jeyaprakash, D. P. Weingartner, y D. W. Dickson, 2006. Caracterización Morfológica y Molecular de *Belonolaimus longicaudatus*. Nematropica 36:37-52.

Belonolaimus longicaudatus ha sido considerada una especie compleja debido a las variaciones en morfología y patogenicidad observadas en diferentes aislamientos. Sin embargo, no existe información acerca de las características genéticas de las diferentes poblaciones de *B. longicaudatus* analizadas en relación con características morfológicas detalladas. Nuestros objetivos fueron determinar la variación intraespecífica basada en morfología y secuencia de ITS1 en aislamientos colectados en diferentes lugares y hospedantes. Los cinco aislamientos comparados fueron HA-papa, Hastings, FL; GV – pasto bermuda, Gainesville, FL; LA—cítricos, Lake Alfred, FL; GA—algodón, Tifton, GA; y NC—

maíz, Scotland County, NC. Se comparó la secuencia de ITS1 de dos aislamientos adicionales, NE—maíz, Columbus, NE, y TX – pasto bermuda, Poteet, TX. También se incluyó la secuencia de ITS1 previamente registrada para un aislamiento de Carolina del Sur (SC) (GenBank accession #U89696) en la comparación. Las hembras de los aislamientos LA y NC poseían mayor longitud del cuerpo, y el aislamiento LA tenía estilete más largo que los demás aislamientos ($P \leq 0.05$). Los aislamientos NC y LA poseían bulbos del estilete típicamente reniformes o con forma de gotera, mientras que en el aislamiento GA la forma era típicamente ovalada. La estructura vaginal del aislamiento LA fue la más prominente y distintiva de todos los aislamientos, pero la del aislamiento NC fue la de más débil desarrollo y no distinguible claramente. La longitud de la región ITS1 fue similar en todos los aislamientos, 468 bp, excepto en el caso de TX que tuvo una longitud de 428 bp. Se construyeron dendrogramas para el análisis filogenético, utilizando los métodos de máxima parsimonia, distancia y máxima verosimilitud. Los tres dendrogramas sustentan la diferenciación de los aislamientos NE, SC y TX de los aislamientos de FL (GV, HA, LA), GA y NC. El aislamiento de TX parece haberse separado del grupo de FL, GA, y NC, sustentado con un valor de bootstrap de 100.

Palabras clave: Belonolaimus longicaudatus, variación intraespecífica, morfología, ITS1, filogenia, sistemática molecular.

INTRODUCTION

Belonolaimus longicaudatus Rau, 1958 is an economically important ectoparasitic nematode that is virulent on a wide range of host plant taxa including vegetables, grains, fruits, forage crops, turfgrasses, ornamentals, and forest trees (Christie *et al.*, 1953; Esser, 1976; Holdeman, 1955; Robbins and Barker, 1973). Its geographical distribution is limited primarily to sandy soils along the eastern seaboard of United States (Brodie, 1976), however, this species was recently reported in California (Mundo-Ocampo *et al.*, 1994). There are also known sites infested in some areas of the mid-western United States.

There continues to be interest in the nematode's taxonomic status because of reported variations in morphology and host specificity among different isolates (Abu-Gharbieh and Perry, 1970; Duncan *et al.*, 1996; Good, 1968; Owens, 1951; Perry and Norden, 1963; Rau and Fassuliotis, 1970; Robbins and Barker, 1973; Robbins and Hirschmann, 1974). Reports of differences in morphology of *B. longicaudatus* populations from Florida vary. No differences were found among three Florida *B. longicaudatus* isolates collected from different locations and hosts (Abu-Ghabieh and Perry, 1970); however, recently morphological variations among *B. longicaudatus* isolates collected from citrus groves on the central Florida ridge were reported (Duncan *et al.*, 1996). The stylet length of *B. longicaudatus* populations sampled in northeastern Polk County, Florida, unlike all the other populations studied, exceeded tail lengths.

Molecular methods provide the potential to elucidate the relatedness among morphologically similar isolates of nematodes (Adams *et al.*, 1998; Ferris *et al.*, 1995; Gasse and Hoste, 1995; Zijlstra, 1997; Wendt *et al.*, 1995; Zhu *et al.*, 2000; Zijlstra *et al.*, 1995). A comparative analysis of the internal transcribed spacer (ITS1) region located between 18S and 5.8S in ribosomal DNA was made of several populations of *B. longicaudatus* collected from southern, mid-western, and western United States (Cherry *et al.*, 1997). All mid-western isolates revealed unique restriction profiles, whereas the southern isolates and the western (California) isolate were identical. Such heterogeneity of the ITS1 region has been observed in other nematodes (Pow-

ers *et al.*, 1997); however, the structural nature of heterogeneity of ITS1 in *Belonolaimus* spp. is still unclear and questions remain regarding the amount of variation within individuals and among populations.

The objectives of these studies were to (i) compare morphological characteristics among different isolates of *B. longicaudatus*, (ii) examine ITS1 sequence variations in *B. longicaudatus*, (iii) construct phylogenetic trees using ITS1 sequence data, and (iv) evaluate the use of ITS1 as a genetic marker to estimate the taxonomic status of isolates of *B. longicaudatus*.

MATERIALS AND METHODS

Nematode Isolates

Five isolates of *B. longicaudatus* were collected from different geographical locations and different hosts. The isolate designation, previous host crops, and locations were: HA isolate from potato grown at the Yelvington farm, Research and Education Center, University of Florida, Hastings; GV isolate from bermudagrass grown at the Gainesville Golf and Country Club, Gainesville, FL; LA isolate from citrus grown in block four, Citrus Research and Education Center, University of Florida, Lake Alfred; GA isolate from cotton grown at the Rural Developmental Center, University of Georgia, Tifton; and NC isolate from corn grown in Scotland County, NC.

Two additional isolates from Nebraska (NE isolate from corn grown in Columbus, NE) and Texas (TX isolate from bermudagrass grown in Poteet, TX) (supplied by Dr. G. E. Dappen, Nebraska Wesleyan University, Lincoln) were included for ITS1 sequence analysis. For out-group comparisons an unidentified *Belonolaimus* sp. (designated UN) collected from *Daphne* sp. in the Elizabeth garden located in Manteo, North Carolina (supplied by Dr. K. R. Barker, North Carolina State University, Raleigh), and *Belonolaimus euthychilus* Rau (designated BU), which was collected from roots of a pine tree located in The Natural Teaching Laboratory located directly west of the Entomology and Nematology Building, Natural Area Drive, University of Florida, Gainesville were also included. The sequence of ITS1 of a South Carolina isolate of *B. longicaudatus* (designated SC) was downloaded from GenBank (accession no. U89696), and included in the data set for comparison.

Artificial Culture of Nematodes

The procedures and methods were as reported previously (Han *et al.*, 2006).

Morphometric Characterization

The second-stage juveniles (J2) and females (immediately following the 4th molt) were selected manually, and prepared for measurements. Thirty J2 were measured after being relaxed by gentle-heat treatment, while 35 females were measured following several steps of fixation. For fixation, the females were placed in hot (60°C) triethanol amine formaldehyde (TAF) (Courtney *et al.*, 1955) and left at room temperature for 24 hours. The TAF was replaced with ethanol-glycerin (20% ethanol: 1% glycerin: 79% water) for 12 hours and ethanol-glycerin (95% ethanol: 5% glycerin) for 5 to 7 days.

Measurements were made with a compound microscope fitted with a drawing tube. Ratios were calculated using de Man indicies (Jones, 1965). The morphological data from both female and J2 were subjected to statistical analysis. In addition, all morphological data were compared to the original description of *B. longicaudatus*. The mean, standard deviation, and range were calculated, and compared among different isolates by Duncan's multiple-range test.

Preparation of Nematode Sample (ITS1 Characterization)

Specimens of the Florida, Georgia, and North Carolina isolates were obtained from greenhouse cultures, whereas specimens of the Nebraska, Texas, and unknown *Belonolaimus* sp. were obtained from the original soil samples. *B. euthychilus* was extracted from soil samples taken from a pine tree located in the Natural Area Teaching Laboratory each time specimens were required. Nematodes from each sample were extracted by the Baermann funnel method, and selected manually. Nematodes were placed in 1.5 ml microcentrifuge tubes filled with sterile distilled water and washed by centrifuging at 10,000*g* for 2 minutes, three separate times.

DNA Extraction

Single female nematodes were picked manually, and placed on a glass cover slip in 10-µl-sterile water for the Standard PCR, or in 25-µl-sterile water for Long PCR. Nematodes were cut into five or seven pieces with a sterile knife, and this material was mixed directly with PCR buffer, dNTP, primers, and sterile water for PCR amplification as described below. Usually, four to five individual nematodes from each isolate were used for PCR amplification and ITS1 products from a single female nematode were selected for further investigations.

Standard PCR Amplification

The DNA sample (10 µl) was suspended in a 25 µl reaction volume containing PCR buffer (10 mM Tris pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl), 200 µM dNTPs, 400 pM primers, and 0.8 units of *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN) (Saiki, 1989). The upstream primer used was 5'-TTGATTACGTCCCTGCCCTTT-3' (Vrain *et al.*, 1992), located to include nucleotide positions from 199 to 178 upstream from the start of ITS1. The downstream primer was 5'-ACGAGCCGAGTGATCCACCG-3' (Cherry *et al.*, 1997), which corresponds to the 3' end of 5.8S rDNA gene from the junction of ITS1. The DNA was amplified using 35 cycles. Each cycle consisted of a denaturation at 94°C for 30 seconds, annealing at 56°C for 1 minute, and extension at 72°C for 1.5 minutes. PCR products (10 µl) were subjected to 1% agarose gel electrophoresis and 1 µl of fresh PCR product was used for cloning. The ITS1 of GV, HA, LA, GA, NC, and TX isolates were amplified by the Standard PCR method. A DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) was used for all PCR assays.

Long PCR Amplification

DNA samples (25 µl) were mixed with 25 µl containing 50 mM Tris (pH 9.2), 16 mM ammonium sulfate, 1.75 mM $MgCl_2$, 350 µM dNTPs, 800 pM of primers, 1 unit of *Pwo* and five units of *Taq* DNA polymerases (Barnes, 1994). Long PCR was performed using three linked profiles: 1) 1 cycle of denaturation at 94°C for 2 minutes, 2) 10 cycles of denaturation at 94°C for 10 seconds, annealing at 65°C for 30 seconds, an extension at 68°C for 1 minute, and 3) 25 cycles of denaturation at 94°C for 10 seconds, annealing at 65°C for 30 seconds, an extension at 68°C for 1 minute plus an additional 20 seconds for every consecutive cycle. The primers used were 5'-TTGATTACGTCCCTGCCCTTTG-TACACACC-3' (upstream) and 5'-ACGAGCCGAGTGATCCACCGATGAGAC TTG-3' (downstream). The primers were designed based on the sequences of 18S and 5.8S of *B. longicaudatus* isolates amplified by the Standard PCR method (Cherry *et al.*, 1997; Vrain *et al.*, 1992). Each 10-µl PCR sample was subjected to 1% agarose

gel electrophoresis and 1 µl of fresh PCR product was used for cloning. The ITS1 of the NE isolate, *B. euthychilus*, and the unidentified *Belonolaimus* sp. were amplified by the Long PCR method because this method was five to six times more efficient than Standard PCR for amplifying DNA (Jeyaparakash and Hoy, 2000). A DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) was used for all PCR assays.

Recombinant DNA Technique

Recombinant DNA techniques, including restriction enzyme digestion, ligation, bacterial transformation, and plasmid DNA preparation were performed by standard methods (Sambrook *et al.*, 1989). For the cloning of ITS1, both Standard and Long PCR products were ligated into the plasmid pCR2.1-TOPO and used to transform *E. coli* following the protocol provided by the manufacturer (Invitrogen Corporation, Carlsbad, CA). The transformed colonies were identified by their clear color and 10 transformed colonies were picked arbitrarily for analysis. For the analysis of positive clones, the selected colonies were cultured in 100 ml LB medium containing 50 µg/ml of ampicillin. After 16 hours of incubation, plasmids were extracted using Plasmid Midi-prep Kit (Qiagen, Valencia, CA). The extracted pure plasmids were digested with *Eco*RI and subjected to restriction analysis. After analyses of positive clones, both strands of inserted DNA, from two independent clones, were sequenced using Applied Biosystem Abi Prism Automated DNA Sequencer (Perkin-Elmer, Norwalk, CT) at the University of Florida Interdisciplinary Center for Biotechnology Research Core Facility.

Sequence Alignment and Phylogenetic Analysis

All amplified DNA sequences were aligned after excluding the primer sequences using CLUSTAL W (Thompson *et al.*, 1994), then manually improved in the PAUP program (Swofford, 1999). All phylogenetic analyses were performed using both distance and character-based methods. The Kimura 2-parameter was selected for distance method and maximum parsimony (MP) and maximum likelihood (ML) were used for character-based methods. Through the heuristic search in PAUP 4.0 program (Swofford, 1999) both MP and ML trees were constructed. The sequence variability within individuals and between isolates was determined by pairwise comparisons in the PAUP program.

RESULTS

Comparisons of J2 Morphometrics

The juveniles of the five isolates varied in body length and width, stylet morphology, and head height (Table 1). The LA isolate had the longest stylet, and the GV isolate had the shortest stylet compared with the other isolates ($P \leq 0.05$). The HA isolate was distinctive with the lowest head height ($P \leq 0.05$) (Table 1). The morphological variation among J2 was much greater than that of females, especially in head height and stylet length.

Comparisons of Female Morphometrics

Females of the LA and NC isolates had longer body length, greater body width and greater head to excretory pore distance than those of other isolates, and the GV isolate had the shortest body and stylet shaft and knob length ($P \leq 0.05$) (Table 2). The LA isolate was distinctive in having the longest stylet, longest stylet cone length, and a greater head height than all other isolates ($P \leq 0.05$) (Table 2). The stylet lengths of the GA and NC isolates were intermediate among all isolates however, the GA isolate had a larger stylet cone and shorter stylet

Table 1. Measurements of second-stage juveniles (n = 30) of five *Belonolaimus longicaudatus* isolates[z] taken from excised corn roots cultured in Gamborg B-5 medium at 28°C.

	GV	HA	LA	GA	NC
	Mean, standard deviation, and range (µm)				
Body length	535 ± 69 ab	492 ± 35 c	515 ± 37 bc	535 ± 30 ab	543 ± 42 a
	(410-693)	(422-545)	(449-615)	(476-590)	(452-633)
Body width	24.8 ± 3.4 b	23.1 ± 2.3 c	22.9 ± 0.9 c	23.2 ± 1.7 c	27.8 ± 2.5 a
	(16.7-30.3)	(19.7-28.8)	(21.2-25.0)	(20.5-28.0)	(21.2-30.3)
Stylet length	63.1 ± 4.8 d	65.7 ± 2.5 c	707 ± 1.6 a	67.8 ± 2.4 b	65.2 ± 2.9 c
	(50.0-68.9)	(62.1-71.2)	(68.2-74.2)	(62.9-72.0)	(56.8-69.7)
Stylet cone length	44.5 ± 3.8 d	46.5 ± 2.5 c	50.7 ± 1.6 a	48.5 ± 2.1 b	45.8 ± 2.3 cd
	(34.9-49.2)	(42.4-51.5)	(48.5-54.6)	(43.9-52.3)	(39.4-50.0)
Stylet shaft and knobs length	18.5 ± 1.5 c	19.1 ± 1.0 b	20.0 ± 0.8 a	19.3 ± 0.9 b	19.4 ± 1.3 b
	(14.4-20.5)	(16.7-21.2)	(18.2-21.2)	(18.2-21.2)	(17.4-22.7)
Head height	7.3 ± 1.2 ab	6.8 ± 0.9 d	7.5 ± 0.9 a	7.0 ± 0.7 bc	7.2 ± 0.8 ab
	(6.1-8.3)	(6.1-7.6)	(6.8-8.3)	(6.1-7.6)	(6.1-9.1)

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).
[z]The isolates of *B. longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), corn in Scotland County, NC (NC).

Table 2. Measurements of females (n = 35) of five *Belonolaimus longicaudatus* isolates[x] taken from excised corn roots cultured at 28°C on Gamborg B-5 medium.

	GV	HA	LA	GA	NC
	Mean, standard deviation, and range				
			Linear (µm)		
Body length	2,047.2 ± 148.2 c	2,189.8 ± 178.6 b	2,277.9 ± 163.4 a	2,135.5 ± 88.3 b	2,320.8 ± 133.6 a
	(1,757.6-2,424.2)	(1,878.8-2,606.1)	(1,909.1-2,681.8)	(2,000.0-2,348.5)	(2,030.3-2,515.2)
Body width	40.9 ± 3.5 b	39.0 ± 2.5 c	42.8 ± 3.0 a	40.8 ± 2.2 b	42.5 ± 4.3 a
	(36.4-51.5)	(34.9-45.5)	(34.9-48.5)	(36.4-45.5)	(36.4-53.0)
Stylet length	115.5 ± 7.0 c	118.1 ± 6.4 c	129.4 ± 9.4 a	124.5 ± 7.6 b	123.5 ± 6.7 b
	(103.0-132.6)	(107.6-129.6)	(114.4-171.2)	(106.1-145.5)	(109.1-139.4)
Stylet cone length	84.6 ± 5.8 d	85.7 ± 5.3 cd	94.9 ± 7.5 a	91.4 ± 5.6 b	88.2 ± 5.9 c
	(72.7-98.5)	(75.8-95.5)	(83.3-128.8)	(75.8-104.6)	(75.8-103.1)
Stylet shaft and knobs length	31.0 ± 2.1 d	32.5 ± 2.7 c	34.5 ± 3.1 ab	33.1 ± 4.5 bc	35.3 ± 1.8 a
	(27.3-34.9)	(25.8-37.9)	(28.8-42.4)	(27.3-54.6)	(31.1-39.4)
Excretory pore to head end	201.1 ± 16.1 c	208.7 ± 20.0 bc	226.9 ± 16.5 a	209.2 ± 13.3 bc	228.5 ± 19.5 a
	(165.2-247.0)	(174.2-243.2)	(180.3-257.6)	(180.3-228.8)	(154.6-257.6)
Tail length	115.0 ± 17.7 b	134.7 ± 13.2 a	127.4 ± 12.6 b	124.0 ± 14.8 b	142.1 ± 12.9 a
	(75.8-150.0)	(106.1-168.2)	(99.2-159.1)	(101.5-156.1)	(119.7-165.2)
Esophagus length	264.1 ± 18.9 d	270.0 ± 20.5 b	286.3 ± 17.6 a	270.4 ± 16.1 b	283.8 ± 20.1 a
	(228.8-309.1)	(222.7-310.6)	(233.3-321.2)	(237.9-297.0)	(221.2-319.7)

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

[x]The isolates of *B. longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC).

[y]a = Total body length per body width, b = Total body length per length of esophagus, and c = Total body length per tail length.

[z]Distance from vulva to anterior body end as a percentage of total body length.

Table 2. (Continued) Measurements of females (n = 35) of five *Belonolaimus longicaudatus* isolates[x] taken from excised corn roots cultured at 28°C on Gamborg B-5 medium.

	GV	HA	LA	GA	NC
	Mean, standard deviation, and range				
Head height	10.9 ± 1.19 b	10.7 ± 0.9 b	11.8 ± 0.9 a	10.9 ± 0.7 b	10.6 ± 0.8 b
	(9.1-15.9)	(9.1-2.9)	(9.9-13.6)	(9.9-13.6)	(9.1-13.6)
			Ratio[y]		
a	50.3 ± 4.7 d	56.3 ± 5.3 a	53.4 ± 4.5 bc	52.5 ± 3.2 cd	55.2 ± 6.3 ab
	(40.0-59.3)	(47.3-67.4)	(42.0-65.2)	(45.4-59.2)	(41.7-69.2)
b	7.8 ± 0.6 b	8.1 ± 0.6 a	8.0 ± 0.8 ab	7.9 ± 0.5 ab	8.2 ± 0.5 a
	(6.5-9.1)	(7.1-9.2)	(6.8-11.4)	(7.0-9.1)	(7.2-9.9)
c	18.1 ± 2.6 a	16.3 ± 1.2 b	18.0 ± 1.4 a	17.4 ± 1.9 a	16.4 ± 1.5 b
	(14.0-25.2)	(14.2-18.3)	(14.6-20.6)	(13.7-20.9)	(13.9-19.5)
Stylet/tail	1.01 ± 0.2 a	0.88 ± 0.1 b	1.03 ± 0.1 a	1.01 ± 0.1 a	0.88 ± 0.1 b
	(0.8-1.5)	(0.7-1.1)	(0.8-1.5)	(0.8-1.2)	(0.7-1.1)
Body length/stylet	17.6 ± 0.2 b	18.5 ± 1.2 a	17.7 ± 1.4 b	17.2 ± 1.0 b	18.8 ± 1.3 a
	(15.7-20.6)	(16.6-21.1)	(14.9-19.8)	(13.8-21.9)	(16.1-21.9)
			Proportion[z]		
V	49.6 ± 2.2 ab	49.2 ± 1.9 b	50.6 ± 1.7 a	50.6 ± 2.5 a	50.5 ± 1.8 a
	(44.7-54.3)	(45.2-54.4)	(44.7-54.0)	(44.9-59.7)	(46.0-54.6)

Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

[x]The isolates of *B. longicaudatus* were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC).

[y]a = Total body length per body width, b = Total body length per length of esophagus, and c = Total body length per tail length.

[z]Distance from vulva to anterior body end as a percentage of total body length.

shaft and knobs than the NC isolate. The LA, GA, and GV isolates had longer stylets, and tail ratios greater than 1.0. The longer tail lengths and ratio c of the HA and NC isolates differentiated them from other isolates. The GA, LA, and GV isolates had a greater stylet-tail ratio than those of the NC and HA isolates ($P \leq 0.05$). The HA isolate was the thinnest nematode isolate having the smallest body width ($P \leq 0.05$). Regarding the morphological characters, the largest variations among isolates were stylet cone lengths, stylet shaft and knob lengths, esophagus lengths, and a ratios, whereas the vulva position (%) had the least variation.

Comparison of Female Stylet Knobs

Two different types of stylet knobs were observed among the five isolates of *B. longicaudatus*. The stylet knobs of the NC and LA isolates were typically teardrop or kidney-shaped, whereas the GA isolate was typically oval (Fig. 1). The HA and GV isolates were variable in that specimens from each had both round and kidney-shaped stylet knobs.

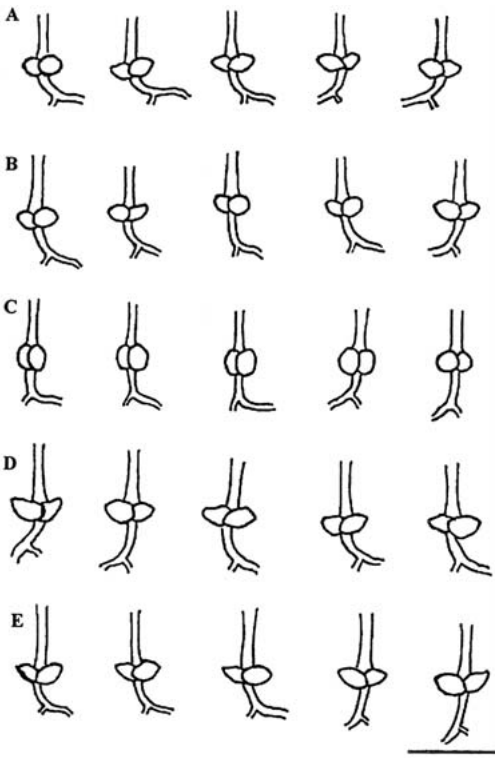

Fig. 1. Morphological variation of stylet knobs among five isolates of *Belonolaimus longicaudatus*. A. Gainesville isolate, B. Hastings isolate, C. Georgia isolate, D. Lake Alfred isolate, E. North Carolina isolate. Scale bar indicates 10 µm.

Comparison of Female Reproductive Organ

Opposed vaginal pieces were observed in all isolates of *B. longicaudatus* except for the GA isolate (Fig. 2). There were no vaginal pieces in the GA isolate, whereas morphological variation of the vaginal pieces occurred in other isolates. The vaginal pieces of the LA isolate were the most prominent and clearly recognized among all isolates. The HA and GV isolates possessed smaller vaginal pieces than the others, and that of NC isolate was weakly developed and not clearly recognized.

Length of ITS1 in Belonolaimus Isolates

The sequenced ITS1 with partial 18S and 5.8S segments showed some length variations among different isolates of *B. longicaudatus*. The length of total amplified DNA from all the Florida (GV, HA, LA), Georgia (GA), North Carolina (NC) and South Carolina (SC) isolates were identical at 665 bp (GenBank accession no. DQ494792 to DQ494799, DQ512390 and DQ592391) (Table 3). However, the length of the analogous region in the Nebraska (NE) isolate was 664 bp (DQ494801) (Table 3). At 624 bp, the Texas (TX) isolate contained the smallest amplified region, which was the result of 40 bp deletion from the middle of ITS1 (428 bp) (DQ494800). Except for the TX isolate, all ITS1 for the other isolates were 468 bp.

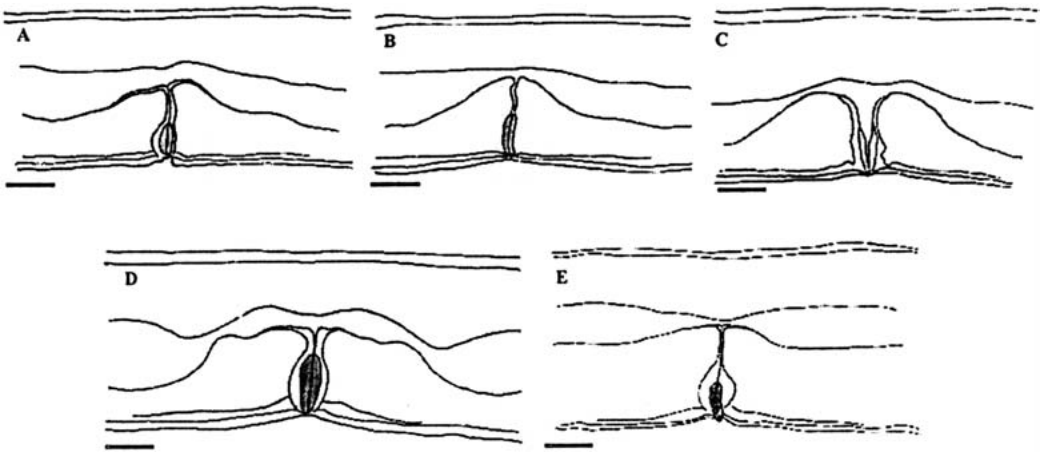

Fig. 2. Morphological variation of opposed vaginal pieces among five isolates of *Belonolaimus longicaudatus*. A. Gainesville isolate, B. Hastings isolate, C. Georgia isolate, D. Lake Alfred isolate, E. North Carolina isolate. Scale bar indicates 10 µm.

Belonolaimus euthychilus and the unknown *Belonolaimus* sp. displayed different length variations in the amplified sequences (563 to 570 bp) than was found in *B. longicaudatus* isolates (624 to 665 bp) (Table 3). There also was variation in the ITS1 length between two clones from each individual from *B. euthychilus* (364 and 366 bp) and the unknown *Belonolaimus* sp. (366 and 371 bp). However, no similar length variation of ITS1 was found between any two clones of the *B. longicaudatus* isolates (Table 3). The heterogeneity in the length of ITS1 occurred only in the out-group taxa and not within the in-group taxon of *B. longicaudatus*. Hence, one clone each from *B. euthychilus* (565 bp) (DQ494802) and the unknown *Belonolaimus* sp. (570 bp) (DQ494803) were selected for further analysis.

Heterogeneity of ITS1 Nucleotides

Variations in nucleotides were observed in the ITS1 of all isolates of *B. longicaudatus* except for the TX, GV, and NE isolates, in which the two independent clones were identical. The highest degree of dissimilarity was found in the LA and NC isolates which displayed 8 and 6 nucleotide differences, respectively, at various locations. The two ITS clones from the HA isolate differed at four different locations. The GA clones differed at two different locations. The point mutation between ITS1 copies was a common phenomenon in *B. longicaudatus* even though they were identical in size. Since high fidelity PCR was used to amplify the genomic DNA, the base pair changes detected are likely to be real rather than a *Taq* generated error.

Characterization of the Sequence of ITS1

All the sequences from *B. longicaudatus* isolates (GA, LA, GV, HA, NC, TX, NE and SC) and one each from the two out groups; *B. euthychilus* (BU) and unknown *Belonolaimus* sp. (UN) were aligned, adjusted and deposited in the European Molecular Biology Nucleotide Sequence Alignment Database (ALIGN_001019). A total of 697 aligned characters were obtained.

The character numbers 1 to 177 is the terminal end of 18S, and 675 to 697 is the beginning part of 5.8S, whereas the remain-

Table 3. The length variations of total amplified DNA, and ITS1 from different isolates of *Belonolaimus longicaudatus*, *Belonolaimus euthychilus*, and an unknown *Belonolaimus* sp.

Code	Geographical origin	Host plant	Species	ITS1 length (bp)	Total amplified DNA (bp)
pHR 1	Tifton, GA	Cotton	*B. longicaudatus*	468	665
pHR 2	Tifton, GA	Cotton	*B. longicaudatus*	468	665
pHR 3	Scotland Co., NC	Corn	*B. longicaudatus*	468	665
pHR 4	Scotland Co., NC	Corn	*B. longicaudatus*	468	665
pHR 5	Hastings, FL	Potato	*B. longicaudatus*	468	665
pHR 6	Hastings, FL	Potato	*B. longicaudatus*	468	665
pHR 7	Lake Alfred, FL	Citrus	*B. longicaudatus*	468	665
pHR 8	Lake Alfred, FL	Citrus	*B. longicaudatus*	468	665
pHR 11	Gainesville, FL	Bermudagrass	*B. longicaudatus*	468	665
pHR 12	Gainesville, FL	Bermudagrass	*B. longicaudatus*	468	665
pHR 22	Columbus, NE	Corn	*B. longicaudatus*	468	664
U89696[y]	South Carolina	Unknown[z]	*B. longicaudatus*	468	663
pHR 13	Poteet, TX	Bermudagrass	*B. longicaudatus*	428	624
pHR 17	Gainesville, FL	Pine tree	*B. euthychilus*	366	565
pHR 18	Gainesville, FL	Pine tree	*B. euthychilus*	364	563
pHR 19	Manteo, NC	*Daphne* sp.	*Belonolaimus* sp.	371	570
pHR 20	Manteo, NC	*Daphne* sp.	*Belonolaimus* sp.	366	565

[y]The GenBank accession number for South Carolina isolate of *B. longicaudatus*.
[z]The original host for the South Carolina isolate was not reported.

ing portion (178 to 674) of ITS1 ranged in length from 428 to 468 bp. Nucleotide mutation was common in the non-coding region of ITS1, but was rare in the coding regions of the 18S or 5.8S. The out-group taxa, *B. euthychilus* and the unknown *Belonolaimus* sp., showed nucleotide variations in the same locations in the sequence encoding the 5' region of 18S rDNA, which includes two transitions (G→A, and C→T) at 60 and 83, and one transversion (A→T) at 64. However, *B. euthychilus* had an additional transversion (A→T) at 134. The in-group taxa, NE and GA isolates also showed transitions (G→A, and T→C) at 68 and 174. These nucleotide mutations occurred in the functional gene. Comparatively higher variations of the ITS1 sequences were observed in the isolates of *B. longicaudatus*, whereas conservative nucleotide sequences were observed in both the 18S and 5.8S regions. The deletion of nucleotides was observed in both the TX isolate and out-group taxa. These were formed between the positions 216 to 382 in the ITS1. Upstream from the beginning of the 5.8S gene within ITS1, a unique repeated thymine (T) pattern was observed.

Phylogenetic Analysis

Three different phylogenetic trees were constructed by using both distance and character-based molecular phylogenetic algorithms. Bootstrap analysis for all

Belonolaimus lineages determined by neighbor-joining (NJ) (Fig. 3), maximum parsimony (MP) (Fig. 4), and maximum likelihood (ML) (Fig. 5) methods provided support for all of these trees. In addition, the general branch divergence patterns among the three trees were similar. The NE, SC, and TX isolates were clearly differentiated from the GA, HA, NC, LA, and GV isolates. In the NJ tree, the relationship between the NE and SC isolates was supported by a high bootstrap value of 99, which related to other *B. longicaudatus* isolates with an 84 bootstrap value. The TX isolate seems to have diverged more recently from the FL, GA, and NC isolates than the NE and SC isolates, which was supported by the bootstrap value of 100. This pattern was consistent in both ML and MP trees.

Two separate copies of ITS1 were sequenced from FL, GA, and NC isolates to determine the level of heterogeneity present within individuals. In the NJ tree (Fig. 3), two clones of ITS1 from each isolate were supported as a monophyletic group with a bootstrap value of more than 50, but the LA isolate was an exception. In the MP (Fig. 4) and ML (Fig. 5) trees, both the LA and GA isolates were not supported as a monophyletic group. The heterogeneity of ITS1 in the LA isolate was large and each copy of ITS1 showed a different branch divergence in all three different trees. On the other hand, the GV isolate showed high similarity of nucleotide sequence between ITS1 clones, which was supported by high bootstrap values in all three (ML, MP, and NJ) analyses.

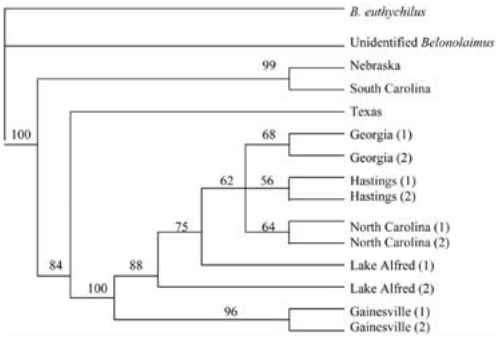

Fig. 3. Neighbor-joining tree based on ITS1 sequence data of *Belonolaimus longicaudatus* isolates, *Belonolaimus euthychilis*, and an unknown *Belonolaimus* sp. The tree was constructed by using the distance method of Kimura's 2-parameter. Bootstrap values in each branch line represent the supported numbers per 100 replications.

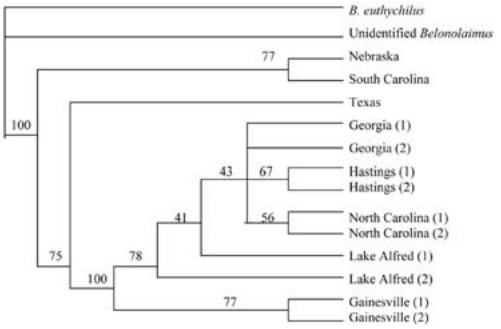

Fig. 4. Parsimony tree based on ITS1 sequence data of *Belonolaimus longicaudatus* isolates, *Belonolaimus euthychilis*, and an unknown *Belonolaimus* sp. The tree was constructed by using the optimality criterion of maximum parsimony method. Bootstrap values in each branch line represent the supported numbers per 100 replications.

The relationship among FL, GA, and NC isolates also was different between character-based and distance-based methods because of different bootstrap values. In both ML and MP, the relationship among the GA, HA, and NC isolates were poorly represented in tree topologies; however, based on the NJ tree, the GA, HA, and NC isolates were more closely related to each other than the LA and GV isolates. The GV isolate was assumed to be an ancestor group that diverged much earlier than the HA, LA, GA, and NC isolates, which was consistently implied in all three analysis methods.

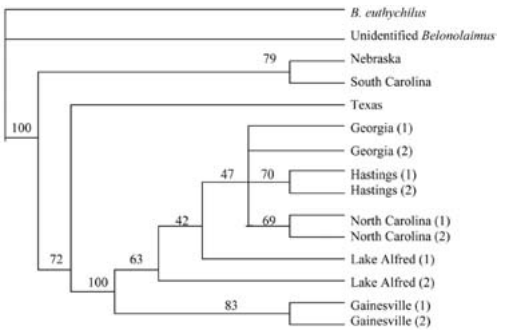

Fig. 5. Maximum likelihood tree based on ITS1 sequence data of *Belonolaimus longicaudatus* isolates, *Belonolaimus euthychilis*, and an unknown *Belonolaimus* sp. The tree was constructed by using the optimality criterion of maximum likelihood method.

DISCUSSION

Previous reports indicate a wide range of morphological variability for isolates of *B. longicaudatus*. Morphological variation, such as size, in a species is influenced by environmental conditions such as nutrient availability and age structure of the population (Duncan *et al.*, 1998). *Belonolaimus longicaudatus* is a polymorphic species. To eliminate some of these factors, all isolates of *B. longicaudatus* tested were cultured in vitro with the same host and under the same temperature. Variation in *B. longicaudatus* morphology was detected, which is in agreement with earlier reports (Abu-Gharbieh and Perry, 1970; Duncan *et al.*, 1996; Robbins and Hirshmann, 1974). Compared to the five isolates of *B. longicaudatus* in this study, there are some similarities and dissimilarities in morphometric data. The stylet length of the LA isolate in this study and the central Florida isolates (Duncan *et al.*, 1996) was longer than other Florida isolates. However, mean values of the body length of the NC isolate and stylet length of the GA isolate in this study were not as close as those of the North Carolina and Georgia isolates (Robbins and Hirshmann, 1974).

Comparisons have been reported on the morphological variation in the stylet knobs and female vulva area between the Georgia and North Carolina isolates (Robbins and Hirshmann, 1974). Based on their report, the stylet knobs of the North Carolina nematodes are typically teardrop or kidney-shaped, whereas those of the Georgia isolate are typically rounded or oval. When the isolates reported herein were compared the stylet knobs of the GV, HA, LA, and NC isolates were kidney-shaped, and the stylet knobs of the GA isolate were oval-shaped, which is consistent with Robbins and Hirshmann (1974). The NC isolate in these studies had opposed sclerotized vaginal pieces that were lacking or very faint in the GA isolate. This result is contrary to what was reported by Robbins and Hirshmann (1974). The GV, HA, LA, and NC isolates possessed opposed vaginal pieces lacking sclerotization and there were morphological variations in vaginal pieces among them. Even when isolates of *B. longicaudatus* were tested under the same host and temperature, morphological variation was observed in stylet and body length among the five isolates of *B. longicaudatus* and in the stylet knobs and vaginal pieces of the GA isolate. A great number of measurements from many different isolates of *B. longicaudatus* will be required to make clear the range of variation within and among isolates of *B. longicaudatus*.

The genus *Belonolaimus* had been categorized into a group of nematodes that have little or no size variation in their ITS1 (Powers *et al.*, 1997); however, ca. a 100 bp difference between *B. euthychilus* and *B. longicudatus* was determined in this study. The TX isolate of *B. longicaudatus* showed a 41 bp deletion in ITS1, and exhibited a unique profile among the various isolates of *B. longicaudatus*. Further, the ITS1 size polymorphism was not only limited among species or isolates, but also

within individual nematodes. For instance, both *B. euthychilus* and the unknown *Belonolaimus* sp. showed variations in size of ITS1 within each individual nematode. However, no variations were detected in size of ITS1 in any of the isolates of *B. longicaudatus*. Thus, size heterogeneity appears to be a characteristic limited to certain species of *Belonolaimus*. Based on nucleotide variation of the ITS1, the putative ITS1 heterogeneity of *Belonolaimus* was already inferred by the profiles of the PCR-RFLP (Cherry *et al.*, 1997). However, the detailed information in this study provides a more rigorous comparison with which to evaluate phylogeny of *Belonolaimus* species. The unique thymine pattern we observed also has been observed in the genera *Meloidogyne* (Zijlstra *et al.*, 1995, 1997) and *Heterodera* (Szalanski *et al.*, 1997).

The heterogeneity of ITS1 has been reported for free-living nematodes (Joyce *et al.*, 1994), plant-parasitic nematodes (Szalanski *et al.*, 1997; Zijlstra *et al.*, 1995, 1997), certain insect species (Rich *et al.*, 1997; Onyabe and Conn, 1999), and even in angiosperms (Baldwin *et al.*, 1995). In plant nematodes, however, the characteristics of ITS1 are seen as being more species specific. For instance, there was no heterogeneity in the size of ITS1 in *Heterorhabditis* (Joyce *et al.*, 1994), *Heterodera*, *Globodera*, *Meloidogyne*, *Hoplolaimus*, *Trichodorus*, and *Xiphinema* (Powers *et al.*, 1997), whereas size variation was detected in *Aphelenchoides* (Ibrahim *et al.*, 1994), *Tylenchorhynchus* (Powers *et al.*, 1997), and *Pratylenchus* (Orui, 1996). The heterogeneity of nucleotide variation in ITS is commonly observed in most sequences from different species of plant-parasitic nematodes, which includes *Meloidogyne* (Zijlastra *et al.*, 1995, 1997), *Heterodera* (Szalanski *et al.*, 1997), *Belonolaimus* (Cherry *et al.*, 1997), and *Globodera* (Thiery *et al.*, 1997). The significance of ITS1 heterogeneity is being considered because of questions concerning the suitability of sequences for phylogenetic analyses, and the problems related to the application of sequence data from ITS1 (Rich *et al.*, 1997; Onyabe and Conn, 1999).

In this study the tree topologies given by different phylogenetic analyses supported our understanding of the relationship among *B. longicaudatus* isolates. Most isolates from the southeastern United States including Florida and Georgia were closely related to each other but clearly separated from those from Nebraska and Texas. Even the morphology, developmental time, and host specificity of *B. longicaudatus* from the southeastern United States varied from those from the Midwestern United States. The ITS1 of ribosomal gene did not reflect these variations in phylogenetic relationship. Nevertheless, ITS1 information itself is useful in the characterization of species of plant-parasitic nematodes. Sequencing the mitochondrial and nuclear genes from *Belonolaimus longicaudatus* could help to establish the relationships of different population in this complex species.

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Received: *9/XI/2005*

Recibido:

Accepted for Publication: *5/I/2006*

Aceptado para Publicación: