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The Internal Transcribed Spacer Region of *Belonolaimus* (Nemata: Belonolaimidae)[1]

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Abstract: *Belonolaimus* isolates from six U.S. states were compared by restriction endonuclease digestion of amplified first internal transcribed spacer region (ITS1) of the nuclear ribosomal genes. Seven restriction enzymes were selected for evaluation based on restriction sites inferred from the nucleotide sequence of a South Carolina *Belonolaimus* isolate. Amplified product size from individuals of each isolate was approximately 700 bp. All Midwestern isolates gave distinct restriction digestion patterns. Isolates identified morphologically as *Belonolaimus longicaudatus* from Florida, South Carolina, and Palm Springs, California, were identical for ITS1 restriction patterns. The correlation between ITS1 restriction patterns and the distribution of *B. longicaudatus* isolates suggest that the California isolate is a relatively recent introduction into the state.

Key words: *Belonolaimus,* ITS, molecular diagnostics, ribosomal DNA, sting nematode, systematics, taxonomy.

Belonolaimus (sensu Siddiqi, 1986) is a genus of economically important ectoparasitic nematodes located exclusively in North America. On agronomic and native hosts, they are found only in extremely sandy soils, including coastal beach sands. Corn and turfgrass are among the cultivated hosts severely affected by *Belonolaimus* feeding (Smart and Nguyen, 1991; Todd, 1989), and while *Belonolaimus* species are typically considered common pests of graminaceous plants, their host range extends from monocotyledonous plants and gymnosperms to dicots. *Belonolaimus gracilis* was first reported in 1949 as a parasite of pine seedlings in Florida and northern Georgia (Steiner, 1949). Currently, five described species comprise the genus, although crossbreeding, host range, and morphological evidence suggest that additional species remain to be described in the southeast United States (Robbins and Hirschmann, 1974). In 1979, a *Belonolaimus* species was found associated with corn in north-central Nebraska (Kerr and Wysong, 1979). This represented the northern and westernmost distribution of the genus until the recent discovery of *Belonolaimus* in California (Mundo-Ocampo et al., 1994). The Nebraskan species was identified as *B. nortoni,* but it was noted that these nematodes differed somewhat from descriptions of type specimens found in DeWitt County, Texas. Populations from Nebraska tend to have larger stylets and longer tails (Table 1) (Rau, 1961, 1963). Morphologically similar populations are generally distributed in sandy soils throughout Arkansas, Oklahoma, and Kansas (Todd, 1989). In 1994, *Belonolaimus longicaudatus* was reported infecting bermudagrass on residential estates and golf greens in Palm Springs, California (Mundo-Ocampo et al., 1994). This was the first report of *Belonolaimus* species from Californian soils and could represent a recent introduction into this arid desert location.

To improve our understanding of *Belonolaimus* systematics and distribution, we analyzed the internal transcribed spacer 1 (ITS1) region of representative *Belonolaimus* populations. Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), individual nematodes were compared at a molecular level to assess genetic identity. The ITS1 region is particularly well-suited for species and population level analyses because of appreciable nucleotide polymorphism (Campbell et al., 1995; Chilton et al., 1995; Ferris et al., 1993, 1994,

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TABLE 1. Average stylet lengths of *Belonolaimus* populations.

Locality	Species	Females N/mean ± SD (range)	Males N/mean (range)	Reference
Daytona Beach, FL	*B. maritimus*	194/149 ± 7 (119–171)		(Rau, 1963)
Fort Lauderdale, FL	*B. longicaudatus*	12/119 (112–125)	4/115 (112–116)	(TOP)[a]
Ocala Nat. Forest, FL	*B. euthychilus*	130/154 ± 7 (131–168)		(Rau, 1963)
Ocala Nat. Forest, FL	*B. gracilis*	61/152 (130–168)	58/137 (99–154)	(Rau, 1963)
Sanford, FL	*B. longicaudatus*	31/127 (116–140)	28/120 (111–132)	(Rau, 1958)
Fulwood, GA	*B. longicaudatus*	24/129	24/123	(R&H)[b]
Tifton, GA (site 1)	*B. longicaudatus*	24/128	24/120	
Tifton, GA (site 2)	*B. longicaudatus*	24/130	24/123	(R&H)
Garden City, KS (site 1)	*B.* spp.	10/104 (98–108)	5/94 (84–96)	(TCT)[c]
Garden City, KS (site 2)	*B.* spp.	6/105 (97–115)		(TCT)
Hutchinson, KS	*B.* spp.	4/100 (95–110)		(TCT)
Wichita, KS	*B.* spp.	5/102 (97–107)		(TCT)
Sterling, KS	*B.* spp.	5/105		(TCT)
Battle Creek, NE	*B.* spp.	10/101 (98–108)		(TOP)
Dudley, NC	*B. longicaudatus*	24/110		(R&H)
Ocracoke Island, NC	*B. maritimus*	24/137	24/134	(R&H)
Severn, NC	*B. longicaudatus*	24/114	24/101	(R&H)
Tarboro, NC	*B. longicaudatus*	24/107	24/101	(R&H)
Hocheim, TX	*B. nortoni*	53/90 (78–98)		(Rau, 1963)

[a] Measured by T.O. Powers.
[b] Robbins and Hirschmann (1974).
[c] Measured by T.C. Todd.

1995; Hoste et al., 1995; Nasmith et al., 1996; Stevenson et al., 1995; Vrain et al., 1992; Wendt et al., 1995; Wesson et al., 1992; Zijlstra et al., 1995). Furthermore, as a genetic marker in taxonomic studies, it has the added advantage of a sizable bibliographic database representing studies of plants, fungi, protozoa, invertebrates, and vertebrates ⟨http://ianrwww.unl.edu/ianr/plntpath/nematode/nemabib.htm⟩. This study presents a comparative analysis, using restriction endonuclease digestion and high resolution agarose gels, of the ITS1 region from *Belonolaimus* populations from six U.S. states.

MATERIALS AND METHODS

Nematodes were received from source populations as living specimens in water. Single nematodes were processed for PCR by placing them in a 15-µl drop of distilled water on a glass cover slip and manually disrupting them as previously described (Powers and Harris, 1993). Typically, 10 to 14 individual nematodes were assayed per population. The two PCR amplification primers, rDNA2 and rDNA1.58S, are 21 and 20 nucleotides in length, respectively, used at a concentration of 0.3 µM each, in a reaction buffer containing a 1.5-mM concentration of $MgCl_2$. The rDNA2 primer (5′-TTGATTACGTCCCTGCCCTTT-3′) has been described by Vrain et al. (1992), and rDNA1.58S (3′-GCCACCTAGTGAGCCGAGCA-5′) was designed by comparative sequence alignments of various nematode species. Samples were amplified in 50-µl volumes, using Perkin-Elmer TC-1 and Techne Gene-E thermal cyclers. Standard profiles included initial denaturation at 94 °C for 2 minutes, followed by 40 cycles of a 1-minute, 94 °C denaturation; a 1-minute, 57 °C annealing; and a 2-minute, 72 °C extension. After PCR amplification the samples remained at 25 °C until removal and were stored at −20 °C prior to digestion.

Amplified ITS1 DNA was digested accord-

ing to manufacturer's recommendations (Promega) using the enzymes Cla I, Hae III, Hha I, Hinc II, Hinf I, Sau 3A, and Taq I. The digestion reaction included 6.0 µl sterile double-distilled H_2O, 6.0 µl amplified ITS1 DNA, 1.4 µl 10× digestion buffer, and 10 units of restriction enzyme. Reactions were incubated at 37 °C for 8 hours except Taq I digestions, which were conducted at 65 °C.

For analysis of the restriction digest, the digested ITS1 DNA was loaded into a 2.5% agarose gel (MetaPhor agarose FMC) buffered with 0.5× TBE (Sambrook et al., 1989). Electrophoresis was normally at a constant voltage of 100 V or less.

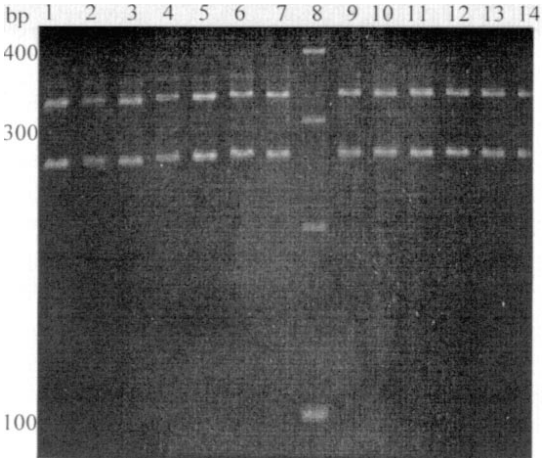
DNA sequence of ITS1 was obtained from an individual *Belonolaimus* adult female from the South Carolina population. The amplification product was purified with Geneclean II (Bio 101, Vista, CA) and resuspended in 30 µl of TE (pH 7.5). DNA was end-repaired with T4 DNA Polymerase (Stratagene, La Jolla, CA), digested with Sma I, ligated to dephosphorylated, Sma I digested, Bluescript SK+ plasmid vector (T4 DNA ligase, Stratagene). Transformations used 10–20 µl XL-1 Blue *E. coli* cells (Stratagene). Clones generating anticipated PCR products were converted to single-stranded molecules and sequenced in both directions using two vector primers that target adjacent T3 promotor and T7 promotor regions (Gibco BRL, Gaithersburg, MD). Sequencing was performed on a LI-COR Model 4000 DNA Sequencer (LI-COR, Lincoln, NE) in the University of Nebraska-Lincoln DNA Sequencing Lab, Lincoln, Nebraska.

```
1                                                                  50
TTGATTACGT CCCTGCCCTT TGTACACACC GCCCGTCGCT GCCCGGGACT

51                                                                100
GAGCCATTTC GAGAAACTTG GGGATTGTTG GTTTAGCGGT TTTCGGATCG
         Taq I                                          Sau3A I
101    Taq I                                                     150
CTTCATCGAT GAGAACCAAT TTAATCGCAG TGGCTTGAAC CGGGCAAAAG
      Cla I
151                                                               200
TCGTAACAAG GTGGCTGTAG GTGAACCTGC TGCCGGATCA TTACTCATGT
                                          Sau3A I
201                                                               250
ATTGATCACA TACACACTTT TGTGTGCATC CATGCAGACC AATCGCTATT
   Sau3A I
251                                                               300
GTCTGGTCAG CGATGGTCCG ATTAAGTAGC GTCCAGCTGG CTCGTCTGAT

301                                                               350
GGGTTTGATG CTGGCCACTG TCTGATGTGG TTAGTGATCA CCCGCTGGTT
              Hae III                     Sau3A I
351          Taq I HincII                                         400
GTATGCTATG GAGTCGACGC TTGTCTGATA GCGTTACATG GACCGTGCCC
             Hinf I
401                                                               450
GTGATCTGGT CATTGTCTGA TTGACTCGTT GTGTTGGATG GTGTTGTCTG
  Sau3A I                   Hinf I
451                                                               500
TTTACAGCGC CGATCCAGCG TGACGTTTAC AGGGCCTAAC GGCTTTGCTG
      Hha I  Sau3A I                   Hae III
501                                                               550
GCGTCTATGC GTGGTTGAGT AGTTGTTGTC TCTCCGTCCG TGACTGTGAT

551                                                               600
GAGACCGCGC GTTAGGGCCC TTGCCTTGCC TCTGGCATGT TGGCTTAAGA
      Hha I      Hae III
601                                                               650
CTTGATGAGC GCGATGCCTT GCGCCGCCAG CACCCACTTT ATTCACTACA
         Hha I            Hha I
651                                                               700
TTTATATGTT CGAAGCGAAG AATTCAAGTC TCATCGGTGG ATCACTCGGC
           T aq I                                Sa u3A I
701
TCGT
```

FIG. 1. Nucleotide sequence of the amplified ITS1 region of a *Belonolaimus* spp. from South Carolina including portions of the 18 S genes (nucleotide 1–194) and 5.8S gene (675–704). Restriction sites confirmed by PCR-RFLP are in bold. Ribosomal gene sequences are in italics, and primer sequences are underlined.

RESULTS

Amplification of *Belonolaimus* spp. resulted in a 704-bp product, as determined by nucleotide sequencing. The amplification product included 194 bp of the 18 S ribosomal gene and 30 bp of the 5.8 S ribosomal gene (Fig. 1) as well as the ITS1 region. A restriction map of the ITS1 sequence from a South Carolina isolate was generated with Cutter Software (http://firstmarket.com/firstmarket/cutter/), revealing several restriction sites for commercially available enzymes. When individuals from the same South Carolina isolate were examined by restriction analysis, patterns were usually identical among all individuals. Figure 2 displays a Taq I digestion of ITS1 from 13 individuals from a Palm Springs, California population. Although small sample sizes prevent a definitive statement on intra-population variation, it appeared that patterns were fixed within the populations but differed among certain populations (Figs. 3–5). Restriction digestion of the amplified product, however, did not always result in the simple digestion



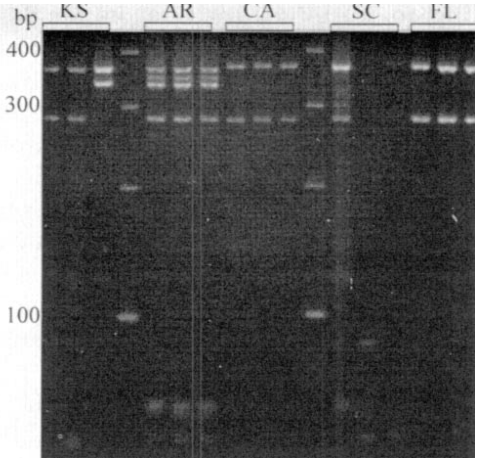
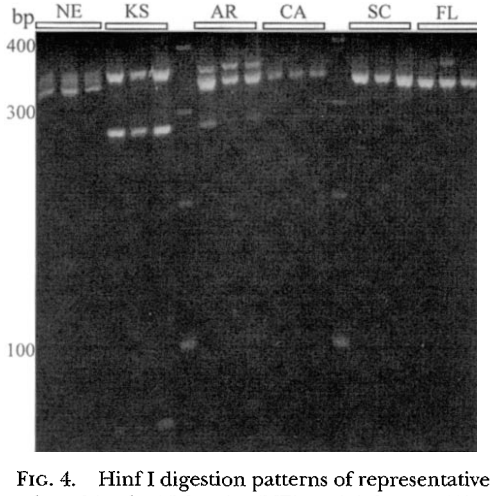


FIG. 2. Taq I digestion patterns of 13 *Belonolaimus longicaudatus* individuals from the Palm Springs, California isolate.

FIG. 3. Hinc II digestion patterns of representative *Belonolaimus* individuals from different isolates. KS = Kansas, AR = Arkansas, CA = California, SC = South Carolina, FL = Florida. Note the three fragments between 300 bp and 400 bp mentioned in RESULTS.

patterns predicted by the nucleotide sequence.

Restriction patterns of the *Belonolaimus* ITS1 region suggest that this region varies within an individual nematode. Individual ITS1 heterogeneity was inferred from the consistent observation of digestion patterns in which the sum of the products exceeded the size of the original, undigested amplification product. For example, Hinc II digestion of ITS1 from the Arkansas population (Fig. 3) displayed three fragments between 300 and 400 bp. Fragment size estimates of 360-, 370-, 380-, and 290-bp Hinc II digestion resulted in fragments totaling approximately 1.4 kb, about twice the size of the undigested amplification product. Hinf I digestion of the Clearwater, Nebraska, isolate (Fig. 4) displayed three fragments of approximately 360, 370, and 380 bp, and Hae



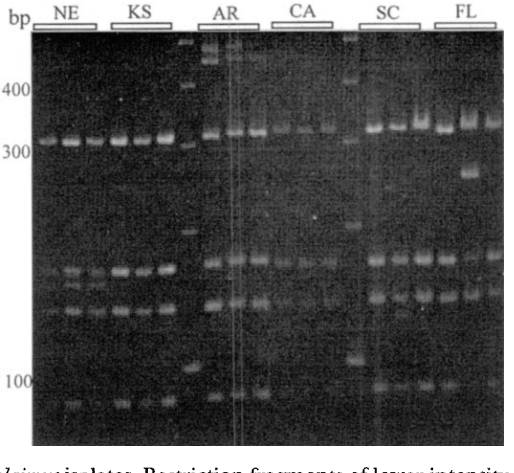


FIG. 4. Hinf I digestion patterns of representative *Belonolaimus* isolates. Restriction fragments of lower intensity are found in the Nebraska (NE) and Arkansas (AR) isolates.

FIG. 5. Hae III digestion patterns of representative *Belonolaimus* isolates. Hae III patterns are similar among geographically distant isolates with the exception of less intense fragments of approximately 580 bp and 560 bp in AR isolates, a unique fragment of approximately 260 in one of the FL individuals, and the additional fragment in NE isolates discussed in RESULTS.

III digestion of the same DNA (Fig. 5) exhibited an additional 170-bp fragment compared to patterns of other populations. These "extra" fragments were produced regardless of duration of digestion, were consistent among individuals from the same population, and persisted in mixing experiments in which nematode DNA was combined with plasmid DNA to evaluate the completeness of digestion. Furthermore, the "extra" fragments were consistently observed in some, but not all, populations.

Table 2 summarizes digestion patterns from the seven enzymes. Key features in these digestion patterns included: (i) individuals from Florida, South Carolina, and Palm Springs, California, produced identical restriction patterns in all cases; (ii) each of the Midwestern *Belonolaimus* populations (Arkansas; Garden City, KS; Hutchinson, KS; Clearwater, NE) produced a unique pattern; (iii) within all populations tested, PCR-RFLP patterns were nearly identical.

Discussion

Restriction digestion of the ITS1 region has revealed an unexpected level of variation among midwestern populations of *Belonolaimus*. Each of the four midwestern isolates gave a unique restriction profile in spite of very similar morphologies. These genetic differences may reflect evolutionary divergence that has occurred in allopatry, since *Belonolaimus* populations are confined to sandy soils that are often geographically isolated. Phylogenetic analysis is necessary to determine population structure and to explain present-day distribution. The restriction analyses do suggest that the California populations may have been established recently, perhaps through introduction of contaminated soil from the southeastern United States. It might be argued that the California population on turfgrass could be derived from native hosts in that desert region; however, the similarity with restriction patterns from the southeast U.S. isolates, and the observed diversity among midwestern isolates, suggests that Californian and southeastern *B. longicaudatus* populations share a relatively recent ancestry.

Table 2. Nucleotide base pair fragment length patterns of various digested *Belonolaimus* isolates.

	Isolate					
Fragment	FL/CA/SC		AR	KS I	KS II	NE
Hinc II						
(fig. 3)	*obs.*	*pred.*	370	370	370	370
	380	365	360	350	290	290
	290	339	350		70	60
			290			
			60			
			50			
Hae III						
(fig. 5)	*obs.*	*pred.*	310	310	310	310
	310	314	180	180	180	180
	180	170	150	150	150	(170)
	150	137	80	80	80	150
	80	83				80
Taq I						
(fig. 2)	*obs.*	*pred.*	300	300	300	300
	300	296	280	280	280	280
	280	258	240	60	60	270
	60	59	(200)	40	40	60
	40	47	60			40
		44	40			
Hinf I						
(fig. 4)	*obs.*	*pred.*	(350)	350	350	(350)
	330	361	330	290	290	(345)
	330	281	(290)		70	330
		62				
Sau 3A						
	obs.	*pred.*	500	300	300	300
	(490)	228	300	250	250	250
	460	132	200	150	150	240
	(440)	95	100	100	100	110
	100	90	90	90	90	90
	90	67		20		
		59				
		20				
Cla I						
	obs.	*pred.*	600	600	600	600
	600	598	110	110	110	110
	110	106				
Hha I						
	obs.	*pred.*	450	450	450	450
	550	459	(440)	100	100	100
	80	100	100	80	80	80
	40	52	80	50	50	50
		12	50			40
			40			

() = bands that are of lesser intensity.
FL = Florida; Ca = California; SC = South Carolina; Ar = Arkansas.
KS I = Hutchinson, Kansas; KS II = Garden City, Kansas; NE = Nebraska.
Predicted patterns (pred.) based upon DNA sequence, observed (obs.) inferred from agarose gels.

ITS1 heterogeneity within individuals has been observed in *Meloidogyne* (Zijlstra et al., 1995), *Trichostrongylus* (Hoste et al., 1995),

and other invertebrates (Wesson et al., 1992), but the structural nature of this heterogeneity in *Belonolaimus* is unclear. In the mitotically parthenogenetic, polyploid *Meloidogyne* species, heterogeneity might be expected, particularly if these species had a recent hybridogenetic origin (Hyman and Powers, 1991). The genome of *M. arenaria* has been shown to possess a complex array of ribosomal variants (Vahidi and Honda, 1991; Vahidi et al., 1991). *Belonolaimus,* however, is a taxon of diploid, amphimictic species. The experiments reported here cannot distinguish between variation among copies derived from a single locus of the ribosomal repeating array of genes, the presence of a second variant ribosomal locus, or two ribosomal alleles constituting a ribosomally heterozygous individual. If the heterogeneity results from more than one locus on the genome, then both loci have preserved the identical ITS1 size, and all individuals in the population maintain the same variant ITS1 sequences. Regardless of the underlying structural basis for the polymorphism among populations, it is apparent that these genetic differences can distinguish among populations and are convenient diagnostic markers.

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