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A PHYLOGENY OF *BELONOLAIMUS* POPULATIONS IN FLORIDA INFERRED FROM DNA SEQUENCES

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ABSTRACT

Gozel, U., B. J. Adams, K. B. Nguyen, R. N. Inserra, R. M. Giblin-Davis, and L. W. Duncan. 2006. A phylogeny of *Belonolaimus* populations in Florida inferred from DNA sequences. Nematropica 36:155-171.

The D2-D3 and ITS regions of rDNA from 33 Florida populations of *Belonolaimus* spp. were sequenced and subjected to phylogenetic analysis. Our objective was to derive a theoretical evolutionary framework for interpreting phenotypic differences as they relate to the taxonomy of the genus. The most striking aspect of the phylogenetic analysis is that none of the three nominal species (*B. longicaudatus, B. euthychilus*, and *B. gracilis*) are monophyletic. Additionally, two taxa appear to have discordant ITS and LSU sequences. Three major clades of *B. longicaudatus* exhibited discernible, overlapping, geographic foci from east to west across the peninsula. Morphological character states considered important for species identification (stylet:tail ratio > or < unity; absence or presence of lip constriction) were paraphyletic; however, these characters may be valid if different quantitative states are used (e.g., stylet:tail ratio > or < a value somewhat greater than 1.0; degree of lip constriction). We were unable to identify suites of morphological/morphometric character states that discriminated between the molecular-derived clades of *B. longicaudatus*. The large number of autapomorphies for the relatively conserved D2-D3 region both between and within the nominal species reinforces previous observations that the genus *Belonolaimus* is far more complex than currently recognized.

Key words: D2-D3, ITS, phylogenetics, rDNA, sting nematode, taxonomy.

RESUMEN

Gozel, U., B. J. Adams, K. B. Nguyen, R. N. Inserra, R. M. Giblin-Davis, y L. W. Duncan. 2006. Filogenia de poblaciones de *Belonolaimus* en Florida inferida a partir de secuencias de ADN. Nematropica 36:155-171.

Se secuenciaron las regiones D2-D3 e ITS de ADN ribosomal (rDNA) de 33 poblaciones de *Belonolaimus* spp. y se sometieron las secuencias a análisis filogenético. Nuestro objetivo era construir un marco evolucionario teórico para interpretar las diferencias fenotípicas asociadas a la taxonomía del género. El hallazgo más impactante en el análisis filogenético fue que ninguna de las tres especies nominales (*B. longicaudatus*, *B. euthychilus* y *B. gracilis*) es monofilética. Se identificaron tres clados mayores de *B. longicaudatus* con focos geográficos discernibles y traslapados de este a oeste a través de la península. Los caracteres morfológicos considerados importantes en la identificación de especies (relación estilete:cola > ó < unidad; ausencia o presencia de constricción labial) fueron parafiléticos. Sin embargo, estos caracteres pueden ser válidos si se utilizan estados cuantitativos diferentes (por ejemplo, relación estilete:cola > ó < un valor algo mayor que 1.0; grado de constricción labial).

No pudimos identificar caracteres morfológicos/morfométricos que discriminaran entre clados de *B. longicaudatus* derivados molecularmente. La gran cantidad de autapomorfismos en la región D2-D3 relativamente conservada, tanto entre especies nominales como al interior de ellas, refuerza previas observaciones acerca de la complejidad del género *Belonolaimus*.

Palabras clave: Belonolaimus, D2-D3, ITS, filogenética, rDNA, taxonomía.

INTRODUCTION

Belonolaimus (sting nematode) is a genus of economically important ectoparasitic nematodes in which nine species are currently recognized (Fortuner and Luc, 1987): *Belonolaimus gracilis* Steiner, 1949; *B. longicaudatus* Rau, 1958; *B. euthychilus* Rau, 1963; *B. maritimus* Rau, 1963; *B. nortoni* Rau, 1963; *B. lineatus* Roman, 1964; *B. anama (*Monteiro and Lordello, 1977) Fortuner and Luc, 1987; *B. jara* (Monteiro and Lordello, 1977) Fortuner and Luc, 1987; and *B. lolii* Siviour, 1978. *Belonolaimus* species with a single lateral line occur only in the USA where they are widespread in the Southeast and Midwest and occur sporadically in other regions. *Belonolaimus* species with four lateral lines are known to occur in Australia, Puerto Rico, Venezuela, and Brazil and are considered by some authorities (Siddiqi, 2000) to constitute a separate genus, *Ibipora* (Monteiro and Lordello, 1977).

Belonolaimus longicaudatus, a serious pest of numerous agronomic and horticultural crops, primarily in the southeastern USA, is the sting nematode most frequently encountered in agriculture (Owens, 1951; Christie *et al.*, 1952; Sasser and Cooper, 1961; Perry and Norden, 1963; Ducharme, 1954; Standifer and Perry, 1960; Duncan *et al.*, 1996; Koenning *et al.*, 1999; Crow *et al.*, 2000). The existence of *B. longicaudatus* pathotypes or physiological races was inferred from early field observations (Perry and Norden, 1963) and subsequently supported by controlled studies of populations collected from different crops and localities (Abu-Garbieh and Perry 1970; Han *et al.*, 2006a, b). Robbins and Hirschmann (1974) studied six populations of *B. longicaudatus* from North Carolina and Georgia because sting nematode damage to peanut was evident in North Carolina, but not in Georgia. They identified several morphological characters that discriminated populations from each state and demonstrated that mating between individuals in a Georgia and a North Carolina population produced only a few nonfertile offspring. Robbins and Hirschmann (1974) also reported pronounced intraspecific morphological variation among the populations within both states, noting that these populations differed significantly from the type population described by Rau (1958). Although none of these populations were subsequently described as new species, Robbins and Hirschmann (1974) provided clear evidence that *B. longicaudatus* is a species complex. More recent reports of marked regional variation, including molecular, among populations of *B. longicaudatus*, support the need to further investigate the evolution and taxonomy of the species (Duncan *et al.*, 1996; Cherry *et al.*, 1997; Han *et al.*, 2006a, b).

In this paper, we analyze relationships between DNA sequences of 33 populations of *Belonolaimus* spp. collected throughout Florida and a population from Oklahoma. We also report morphometric relationships between a subset of the populations. Our objective was to infer the evolutionary relationships between these populations as a first step in interpreting the importance

of morphological differences and reproductive compatibility to the taxonomy of the genus (e.g., Duncan *et al.*, 1999; Inserra *et al.*, 2001).

MATERIALS AND METHODS

Populations of *Belonolaimus* spp. were obtained from soil samples collected for pest management diagnosis and research (citrus, strawberry, turf, corn, and sugarcane) and for a survey of *Xiphinema americanum* in Florida (oak and pine) (Table 1). The type localities for *B. longicaudatus*, *B. gracilis*, and *B. euthychilus* were included among the sample sites. Nematodes were extracted from soil using a density centrifugation method (Niblack and Hussey, 1995). Populations were assigned tentative species designations based on the standard morphological and morphometric characters of diagnostic value for the genus (Smart and Nguyen, 1990). Some populations were maintained on sorghum in a glasshouse. For morphological studies, specimens were killed by gentle heating, fixed in 4% formaldehyde, and processed and mounted in glycerin on glass slides by a modification of Seinhorst's (1959) method. Several *B. longicaudatus* populations from each of the major sister clades of a phylogenetic analysis were selected for morphological comparison. Morphological and morphometric characters from 20 females from each of 11 populations were observed and measured using a light microscope. The populations were compared by principal components analysis (PCA) of all morphometric characters and ratios and of arbitrary subsets of those data (Minitab Software, State College, PA).

For molecular analysis, DNA was extracted from individual female nematodes for each population using DNeasy tissue extraction kits (QIAGEN, Inc., Santa Clarita, CA). Each female was first examined to insure that major morphological characters conformed to the putative identity of the population. Ribosomal DNA of the internal transcribed region was PCR amplified using the 18S (forward) and 26S (reverse) primers designed by Vrain (1993) which bind to the posterior 3' portion of the 18S small ribosomal subunit (forward), and the 5' end of the 28S subunit region (reverse). Also the D2-D3 rDNA was amplified using two sets of primers D3A (5'-GAC CCG TCT TGA AAC ACG GA-3') (forward) and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') (reverse) (Baldwin *et al.*, 1997), and 502 (5'-CAA GTA CCG TGA GGG AAA GTT GC 3') (forward) and 536 (5'-CAG CTA TCC TGA GGG AAA C 3') (reverse) (Stock *et al.*, 2001). Polymerase chain reactions were carried out in 25 μl volumes. PCR mix was added to each tube: 2.5 μl 10 * PCR buffer, 1.5 μl $MgCl_2$, 1 μl dNTP mixture (10 mM each), 1 μl of 10 pM forward primer, 1 μl of 10 pM reverse primer, 0.25 μl of Taq polymerase (Continental Lab Products, San Diego, CA), 19.55 μl of distilled water and 5 μl of DNA. All PCR reactions were run in a PTC-100 Thermocycler (MJ Research, Inc., Waltham, MA) with the cycling profile: 1 cycle of 94°C for 7 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min (for ITS region 55°C for 1 min), 72°C for 1 min. The last step was 72°C for 10 min. The resultant PCR products were cloned into the vector PCR 2.1 using Topo-TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA was purified from bacterial cultures using QIAprep Spin Miniprep kit (QIAGEN, Inc., Santa Clarita, CA). For direct sequencing, PCR products were purified using QIAquick PCR purification kit (QIAGEN, Inc., Santa Clarita, CA). All products were sequenced at the University of Florida ICBR sequencing core facility on Perkin Elmer/Applied Biosystems automated DNA sequencers. The primers used

Table 1. Species identification, sample location and plant species associated with populations of *Belonolaimus* spp. used in this study. Topotype populations of *B. longicaudatus*, *B. euthychilus*, and *B. gracilis* are BlCo1, BePi1 and BgPi1, respectively.

Putative species based on morphology	Sample location (county)	Associated plant	Survey code	Analysis code
B. longicaudatus	Sanford (Seminole)	Corn	BLT	BlCo1
	Trenton (Gilchrist)	Corn	TR	BlCo2
	Polk City (Polk)	Citrus	IF	BlCi3
	Lake Alfred (Polk)	Citrus	CREC	BlCi4
	Lake Alfred (Polk)	Citrus	ALBRIGO	BlCi5
	Haines City (Polk)	Citrus	PRATT	BlCi6
	Lake Alfred (Polk)	Citrus	LA	BlCi7
	Ft. Pierce (St. Lucie)	Citrus	TH	BlCi8
	Frostproof (Polk)	Citrus	B10612	BlCi9
	Frostproof (Polk)	Citrus	B10613	BlCi10
	Fort Pierce (St. Lucie)	Citrus	B10739	BlCi11
	Fort Pierce (St. Lucie)	Citrus	KIR	BlCi12
	Plant City (Hillsborough)	Strawberry	PL	BlSt13
	Dover (Hillsborough)	Strawberry	JNA	BlSt14
	Valrico (Hillsborough)	Strawberry	JNB	BlSt15
	Valrico (Hillsborough)	Strawberry	JNC	BlSt16
	Sanford (Seminole)	Grass	HR	BlGr17
	Ft. Lauderdale (Broward)	Grass	FLREC	BlGr18
	Belleview (Marion)	Bermuda grass	OB	BlGr19
	Palatka (Putnam)	Grass	WCR	BlGr20
	Villages (Sumter)	Grass	WCJAY	BlGr21
	Ridge Manor (Hernando)	Pine	LD1	BlPi22
	Panhandle (Santa Rosa)	Pine	GRAY	BlPi23
	Jay (Santa Rosa)	Pine	JAY	BlPi24
	Polk city (Polk)	Pine	456	BlPi25
	Fort Pierce (St. Lucie)	Pine	434	BlPi26
	Indiantown (Martin)	Sugarcane	IT	BlSu27
	Winter Haven (Polk)	Oak	429	BlOa28
	Oklahoma (Unknown)	Unknown	OK	BlGr29
B. euthychilus	Ocala (Marion)	Pine	LD9	BePi1
	Ocala (Marion)	Pine	LD17	BePi2
	Gainesville (Alachua)	Pine	BE	BePi3
B. gracilis	Ocala (Marion)	Pine	BG	BgPi1
	Gainesville (Alachua)	Pine	DPI	BgPi2

for sequencing were the same used for PCR amplification. The ITS rDNA and D2-D3 region sequences of sting nematode isolates were deposited in GenBank (Accessions DQ672343-DQ672386).

Sequences were edited using Sequencher (4.1.2 Gene Codes Corporation). Restriction maps were generated using Webcutter 2.0 (firstmarket.com/cutter/cut2.html). The sequences obtained in this study were aligned to each other and the outgroup taxon *Pratylenchus coffeae* (Zimmermann) Filipjev & Schuurmans Stekhoven (LSU GenBank accession #AF170443) using the default parameters of Clustal X 1.83 (Thompson *et al.*, 1997). Alignments were made of the ITS region only, the LSU region only, and a concatenated matrix of the two. The alignments were adjusted manually in MacClade 4.0 (Maddison and Maddison, 2002). The high degree of variation between the ingroup and outgroup taxa *B. gracilis* and *P. coffeae* made it difficult to optimize several regions of the multiple sequence alignments. In order to reduce the number of alignment ambiguous regions in the datasets, we initially aligned only the ingroup taxa to each other, and then aligned the outgroup taxon to the ingroup using the profile alignment mode in ClustalX. Further, to explore character state changes rendered ambiguous by character polarization established by the outgroup taxa *B. gracilis* and *P. coffeae*, we performed preliminary phylogenetic analyses to establish the ingroup taxon that was sister to the rest of the ingroup, removed the outgroup taxa and columns of gaps only from the alignments, and then interpreted character state changes using the former to root the trees and establish character polarity.

Phylogenetic Analyses

Maximum parsimony (MP), maximum likelihood (ML) and minimum evolution of log determinant transformed distances (ME) analyses were performed on ITS, LSU, and concatenated matrices using PAUP* (Swofford, 2002). Combinability of the LSU and ITS datasets was evaluated using the incongruence length difference test (Farris *et al.*, 1994, 1995). For MP analyses, in separate runs, indels in the matrix were treated either as missing data, or as a fifth character state. All characters were considered unordered, and of equal weight. MP tree search parameters were heuristic, with starting trees obtained by stepwise addition with random addition sequence of 10 replicates.

The ME tree search based on LogDet transformed distances proceeded via heuristic search with negative branch lengths allowed but set to zero for tree comparison calculations. The starting tree was obtained via neighbor-joining, and proceeded via TBR branch-swapping.

The model of DNA sequence evolution for maximum likelihood analyses was selected based on hierarchical likelihood ratio tests (hLRT; ModelTest 3.5; Posada and Crandall, 1998). These were performed on the ITS and LSU regions separately, as well as the combined matrix. Although there are advantages to choosing the Akaike information criterion (AIC; Posada and Buckley, 2004), we chose the hLRT because it makes fewer assumptions about the coding nature of the sequences. The model selected for the ITS and the combined dataset was a variant of the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985) (two classes of substitution with a gamma distribution of rate substitution at variable sites). The model assumed a transition/transversion ratio of 1.86 (kappa = 3.77) and use of empirical nucleotide frequencies (A = 0.22, C = 0.22, G = 0.29, T = 0.27). No sites were considered invariable, and the distribution of rates at variable sites approximated a discrete gamma distribution (four rate categories,

alpha = 0.218). The best fit model for the LSU data alone was the Tamura-Nei model (Tamura and Nei, 1993) with a gamma distributed rate heterogeneity. The search for the ML tree of each dataset proceeded heuristically, starting with the ME tree of the previous analyses followed by TBR branch-swapping.

All trees were compared by MP optimality criteria using the Kishino-Hasegawa test (Kishino and Hasegawa, 1989) and the Templeton non parametric test (Templeton, 1983), and by ML using the K-H and Shimodaira-Hasegawa tests (Shimodaira and Hasegawa, 1999). The latter tests utilized a RELL test distribution (1000 bootstrap replicates) to obtain two-tailed P values.

RESULTS

Our observation of morphological and morphometric characters revealed three species, *B. longicaudatus*, *B. gracilis*, and *B. euthychilus*, among the 34 populations studied (Tables 1 and 2). Several attempts to recover *B. maritimus* from the rhizosphere of *Uniola paniculata* (sea oats) on the eastern coastal shoreline were unsuccessful. Twenty-three of the populations listed in Table 1 were identified as *B. longicaudatus* for having females with an offset lip region, a stylet longer that 100 microns and tail length > stylet length. Five additional populations (BlCi4-7 and BlSu27) with offset lip regions were also designated as *B. longicaudatus* despite having stylets longer than tails, characteristics normally associated with *B. gracilis*. However, the stylet and tail lengths of these populations were closer to those of *B. longicaudatus* than those of *B. gracilis*. Moreover, these five populations had sclerotized pieces in the vagina that are lacking in *B. gracilis*. The population BgPi1 (from the type locale) exhibited all of the morphological and morphometric characters reported by Rau for *B. gracilis*. The population BgPi2 was identified as *B. gracilis* for having a stylet length > tail length, a pronounced lip constriction and sub-hemispherical or hemispherical tail terminus (Table 2). However, this population lacked males, females had an obscure spermatheca, and the tail integument ranged from 3.8-4.9 μm thick, which is lower than that reported (8.5-15.4 μm) by Rau (1963). Three populations (BePi1-3) were considered *B. euthychilus* for having stylet length > tail length, lip region continuous with the body, a functional spermatheca full of sperm and numerous males with partially degenerated esophagus. Tail integument thickness for the BePi3 population (5.8-8.0 μm) was considerably thicker than that of BgPi2 and within the range reported for the species (7.0-9.8). Additional morphometrics of population BePI3 females are shown in Table 2. Morphometrics (mean ± sd, range in parentheses; all μm) of 14 males from this population are: body length 1525 ± 82 (1372-1659); body width 31.5 ± 1.0 (29.4-33.3); width at anus 21.5 ± 1.3 (19.5-23.5); pharynx length 172.7 ± 9.7 (156.5-186.0); tail length 81.6 ± 5.8 (70.5-91.0); stylet length 95.4 ± 4.9 (86.0-104.0); distance of phasmid from tail terminus 46.2 ± 3.7 (37.0-50.5); distance of excretory pore from anterior 188.9 ± 13.1 (172.0-225.0); spicule length 32.5 ± 1.5 (30.0-35.0); gubernaculum 15.2 ± 0.4 (14.7-16); pharyngeal overlap 41.5 ± 4.1 (37.0-49.0).

A comparison of nucleotide usage among the taxa in the analysis showed no apparent deviation from stationarity (p = 1.0) and there was little variation in base frequencies among terminal taxa. Acknowledging limitations concerning the interpretation of the incongruence length difference test (Farris *et al.*, 1995; Dolphin *et al.*, 2000; Yoder *et al.*, 2001; Darlu and Lecointre, 2002) we found no evidence to reject the combinability of the LSU and

Table 2. Morphometric measurements and derived variables for females from populations of *Belonolaimus* spp. described in Table 1. Statistics include mean (n = 20) ± standard deviation with range shown in parentheses.

Isolate Code	BlCi3	BlGr17	BlGr29	B1St16	BlCi4	BlCo1	BlSt13	BlCi12	BlGr20	BlGr21	BlCi6	BgPi2	BePi3
L	2619 ± 51.2	2243 ± 47.4	2228 ± 47.2	2846 ± 53	2636 ± 51.3	2674 ± 51.7	2730 ± 52.2	2705 ± 52	2710 ± 52	2782 ± 52.7	2477 ± 49.7	1836 ± 151	1812 ± 109
	(2434-2752)	(2078-2560)	(1938-2421)	(2639-3132)	(2391-2777)	(2812-2946)	(2572-3198)	(2427-3006)	(2483-2892)	(2379-3331)	(2114-2740)	(1538-2181)	(1612-1972)
Body width	41.2 ± 6.4	44.1 ± 6.6	37.1 ± 6.1	39.3 ± 6.3	45.7 ± 6.7	41.5 ± 6.4	44.7 ± 6.6	46.9 ± 6.8	39.9 ± 6.3	42.9 ± 6.5	47.1 ± 6.8	43.2 ± 2.2	40.4 ± 2.1
	(39-43)	(41-48)	(33-40)	(36-45)	(41.8-48)	(39-45)	(42-50)	(45-48)	(38-43)	(39-47)	(44-48.4)	(39.2-47.5)	(37.2-44.1)
a	63.7 ± 7.9	53.4 ± 7.3	60.1 ± 7.7	72.5 ± 8.5	57.7 ± 7.5	64.3 ± 8.1	60.9 ± 7.8	57.8 ± 7.6	67.8 ± 8.2	64.8 ± 8.1	52.6 ± 7.2	42.5 ± 2.2	44.9 ± 2.2
	(58-68.6)	(50.4-58)	(51.5-66.1)	(64-81.5)	(53.3-57.2)	(57.6-70.5)	(56.8-68.9)	(52.7-62.1)	(64.9-77.3)	(52.9-74.7)	(44.7-59.5)	(39.2-47.8)	(41.1-49.4)
b	8.7 ± 2.9	7.9 ± 2.8	8.5 ± 2.9	9.1 ± 3.1	8.2 ± 2.8	8.5 ± 2.9	8.6 ± 2.9	8.5 ± 2.9	8.9 ± 2.9	9.1 ± 3	7.7 ± 2.7	8.2 ± 0.5	8.8 ± 0.5
	(8.3-9.4)	(7.2-8.6)	(7.9-9.4)	(8.7-9.7)	(7.7-8.5)	(7.7-8.6)	(8.2-9.5)	(7.7-9.2)	(8-9.7)	(8.4-10.3)	(7-8.4)	(7.2-9.6)	(8.0-9.8)
c	16.2 ± 4.1	16.1 ± 4.1	17.5 ± 4.2	17.7 ± 4.2	18.9 ± 4.3	15.8 ± 3.9	17.4 ± 4.1	14.8 ± 3.8	16.8 ± 4.1	16.9 ± 4.1	19 ± 4.3	18.8 ± 0.9	20.4 ± 1.5
	(14.3-17.8)	(14.8-17.5)	(14.8-20.3)	(15.4-19.5)	(17.8-19.9)	(14-17.1)	(15.5-19.1)	(13.5-16.2)	(15.4-19.1)	(15.1-18.7)	(17.1-20.9)	(17.2-20.9)	(18.2-24.5)
Vulva %	49.6 ± 7.1	51.5 ± 7.2	50.1 ± 7.1	49.7 ± 7.1	50.9 ± 7.1	50.2 ± 7.1	50.5 ± 7.1	50.1 ± 7.1	49.3 ± 7.1	49.1 ± 7.1	51.8 ± 7.1	53.8 ± 1.7	53.0 ± 1.6
	(48.4-53)	(50.4-54)	(48.5-51.9)	(48.5-52.3)	(50.1-52.1)	(48.3-52.3)	(49-53.1)	(48.6-52)	(46.5-52.6)	(47.1-51.5)	(49.6-54.1)	(50.8-56.7)	(50.7-56.5)
Lip length	10.6 ± 3.2	10.9 ± 3.3	10.3 ± 3.2	10.6 ± 3.2	10.3 ± 3.2	10.4 ± 3.2	11.2 ± 3.3	11.1 ± 3.3	10.3 ± 3.2	10.3 ± 3.2	10.3 ± 3.2		
	(10-12)	(10-12)	(10-11)	(10-12)	(10-11)	(10-11)	(10.6-12)	(10.6-12.1)	(10-11)	(10-11)	(10-11)		
Stylet cone	90.2 ± 9.4	90 ± 9.5	75.1 ± 8.7	94.2 ± 9.7	101 ± 10.1	88.1 ± 9.3	101.5 ± 10.1	96.1 ± 9.8	93.9 ± 9.6	96.5 ± 9.8	97.4 ± 9.8		
	(83-98)	(85-96)	(68-80)	(85-100)	(94-110)	(70-98)	(95-108)	(91-109)	(86-107)	(89-104)	(89-104)		
Stylet length	127.5 ± 11.2	124.8 ± 11.1	106.5 ± 10.2	130.7 ± 11.4	141 ± 11.9	123.1 ± 11.1	137.8 ± 11.7	133.4 ± 11.5	128.9 ± 11.3	134.7 ± 11.6	137.3 ± 11.7	145.6 ± 5.1	134.2 ± 5.8
	(119-134)	(114-136)	(98-112)	(124-136)	(133-147)	(109-132)	(129-144)	(127-146)	(103-145)	(127-144)	(129-143)	(135.2-156.5)	(124.0-145.0)
DEGO	130.2 ± 11.5	128.4 ± 11.3	110.1 ± 10.5	134.8 ± 11.6	145 ± 12.1	127.2 ± 11.2	141.6 ± 11.9	137.3 ± 11.7	133.1 ± 11.5	139.1 ± 11.7	141 ± 11.8	5.6 ± 0.4	4.8 ± 0.4
	(123-138)	(120-141)	(103-118)	(131-140)	(136-153)	(112-136)	(133-148)	(131-149)	(107-149)	(131-150)	(133-147)	(4.9-6.3)	(4.0-5.3)
Nerve ring	219.5 ± 14.8	210 ± 14.4	191.4 ± 13.8	212.6 ± 14.6	230 ± 15.1	212.8 ± 14.5	228.9 ± 15.1	232.8 ± 15.2	219.5 ± 14.8	223 ± 14.9	233.3 ± 15.2		
	(206-245)	(195-227)	(176-200)	(191-230)	(207-247)	(191-233)	(212-250)	(208-254)	(195-245)	(209-244)	(215-251)		
Excretory pore	251.4 ± 15.8	250.3 ± 15.8	217.4 ± 14.7	254.3 ± 15.9	264 ± 16.2	255.5 ± 15.9	268.8 ± 16.3	273.2 ± 16.5	252.4 ± 15.8	247.5 ± 15.7	270.1 ± 16.4	238.6 ± 11.7	205.5 ± 18.1
	(230-272)	(236-280)	(207-227)	(236-276)	(242-278)	(211-277)	(236-289)	(251-289)	(223-277)	(227-264)	(242-298)	(219.5-255.5)	(163.0-232.0)

Table 2. (Continued) Morphometric measurements and derived variables for females from populations of *Belonolaimus* spp. described in Table 1. Statistics include mean (n = 20) ± standard deviation with range shown in parentheses.

Esophagus	299.1 ± 17.2	296.4 ± 17.2	261.1 ± 16.1	298.6 ± 17.2	320 ± 17.8	310.1 ± 17.6	314.7 ± 17.7	316 ± 17.7	303.3 ± 17.4	308 ± 17.5	319.8 ± 17.8	223.9 ± 12.2	205.9 ± 9.1
	(282-315)	(284-324)	(242-274)	(282-324)	(292-346)	(262-335)	(279-362)	(289-345)	(269-336)	(280-330)	(294-340)	(204.5-248.0)	(194.0-228.0)
Median bulb length	30.8 ± 5.5	33.7 ± 5.8	29.4 ± 5.4	29.1 ± 5.3	32 ± 5.7	30.1 ± 5.4	32.1 ± 5.6	34.4 ± 5.8	30.1 ± 5.4	30.2 ± 5.5	32.8 ± 5.7	24.2 ± 1.9	23.1 ± 1.9
	(28-34)	(30-39)	(27-32)	(27.2-30.3)	(30.9-34.8)	(27-36)	(30.3-36.3)	(31.8-37.1)	(27-33.3)	(28-33)	(30.3-37.8)	(21.5-28.1)	(16.6-25.4)
Median bulb width	23.7 ± 4.8	24.8 ± 4.9	21.5 ± 4.6	21.8 ± 4.7	25 ± 5	22.7 ± 4.7	24.9 ± 4.9	25.1 ± 5	22.7 ± 4.7	23.9 ± 4.8	27.4 ± 5.2	22.2 ± 1.6	21.1 ± 1.3
	(22-26)	(23-27)	(18-24)	(19-24)	(22.7-26.2)	(20-28)	(21.5-25.7)	(22.5-27.2)	(19.6-25)	(21-26)	(25.7-28.7)	(19.6-24.5)	(17.6-23.0)
Anal body width	35.5 ± 5.9	36 ± 6	32.2 ± 5.7	33.1 ± 5.7	38 ± 6.1	33.8 ± 5.8	37.3 ± 6.1	43.1 ± 6.5	32.5 ± 5.7	35.3 ± 5.9	38.4 ± 6.1	34.9 ± 2.2	33.2 ± 1.5
	(30-42)	(32-39)	(29-35)	(30-38)	(35.1-39.3)	(30-37)	(31.8-43.9)	(39.4-45.4)	(30.3-34.8)	(32-39)	(35.8-40.1)	(31.3-38.2)	(31.3-37.2)
Tail length	160.8 ± 12.7	147.2 ± 12.1	127.9 ± 11.3	159.6 ± 12.6	138 ± 11.7	168.2 ± 12.9	157.1 ± 12.5	182.4 ± 13.5	161.3 ± 12.7	164.3 ± 12.8	130.2 ± 11.4	97.8 ± 6.2	89.4 ± 7.0
	(145-175)	(135-163)	(106-159)	(132-188)	(130-144)	(155-184)	(138-200)	(159-198)	(144-179)	(144-193)	(121-142)	(85.2-109.5)	(75.4-98.0)
Stylet/tail	0.81 ± 0.88	0.86 ± 0.92	0.82 ± 0.91	0.82 ± 0.9	1.02 ± 0.9	0.73 ± 0.8	0.88 ± 0.8	0.73 ± 0.8	0.81 ± 0.8	0.82 ± 0.7	1.05 ± 1.02	1.49 ± 0.07	1.51 ± 0.17
	(0.71-0.85)	(0.75-0.95)	(0.76-0.95)	(0.68-0.97)	(1-1.08)	(0.65-0.77)	(0.72-0.98)	(0.68-0.83)	(0.69-0.88)	(0.72-0.96)	(0.98-1.12)	(1.36-1.62)	(1.28-1.92)

ITS datasets (p = 0.07) for total evidence analyses (Kluge, 1998, 2004) and thus the two datasets were concatenated. MP analyses treating gaps as missing data or a fifth base produced 3 (592 steps) and 12 (919 steps) equally parsimonious trees, respectively. The ME search (minimum evolution optimality criterion with LogDet/paralinear distances) yielded one tree, as did the ML search (Ln = -5939.51). ML and MP comparisons of the trees when indels were treated as missing data revealed no significant differences among the topological solutions. However, under MP criterion, when gaps were treated as a fifth base, both the ME and ML solutions were significantly worse than the most parsimonious solution (p = 0.02 and p = 0.03, respectively). The bootstrap trees of both MP (indels treated as missing data) and ML solutions are topologically identical and very similar in terms of support values (Fig. 1).

Phylogenetic analysis of the ITS and D2-D3 region of rDNA resulted in clades that do not conform precisely with the species designations that are based on morphology (Fig. 1). The largest clade (*B. longicaudatus* group) contains 28 isolates all identified as *B. longicaudatus*, whereas a second clade (*B. euthychilus* group) contains three isolates identified as *B. euthychilus*, and one isolate identified as *B. gracilis*. Unresolved is the relationship of *B. gracilis* population BgPi1, collected from the type locality. The BlPi24 isolate appears as sister taxon to the entire clade, as established by the outgroup taxon *Pratylenchus coffeae*. This (BlPi24) and another isolate (BlPi23) are from the Florida Panhandle. To the exclusion of the BlPi24 isolate, isolate BlGr29 (from Oklahoma) forms a clade with the remaining *B. longicaudatus* taxa.

All terminal taxa have evolved one or more diagnostic, autapomorphic characters except for BlPi22, BlCo1, and BlPi26. Autapomorphies for terminal taxa, as well as more inclusive clades are displayed in Fig. 1, as is their distribution from the sampled ITS and LSU regions (synapomorphies that unite these clades are not presented). The nucleotide sequence diversity between these clades ranges from 10-17 autapomorphies for the D2-D3 region and 26-51 autapomorphies for the ITS region. Both the *B. longicaudatus* and *B. euthychilus* groups also contain populations that can be discriminated by numerous autapomorphies in ITS sequences as well as in the more conserved D2-D3 sequences.

Two major clades within the *B. longicaudatus* group are based on 10 autapomorphies each for ITS and two and four autapomorphies, respectively, for D2-D3. The largest of these clades is comprised of 20 populations. Within this clade, the populations BlCo2, BlCi3, BlCi10, BlSt13, BlSt14, BlSt15, BlSt16, BlGr19, BlGr20, BlGr21, and BlOa28 have tail length > stylet length, whereas populations BlCi4, BlCi5, BlCi6, BlCi7, and BlSu27 have stylet length > tail length. DNA sequences were identical for all but one population among the latter group (stylet > tail), but varied greatly among the former group (tail > stylet). The second large clade of the *B. longicaudatus* group contains a topotype population (BlCo1) among six other populations, all with tail length > stylet length. Principal components analysis of all measured morphometric characters for selected populations of the *B. longicaudatus* group (Table 2) did not discriminate the DNA-derived clades within the group (Fig. 2a). Selected characters (stylet length and the ratio c) discriminated the clades significantly better, although a discrepancy in the relationship of BlCi3 to the other populations indicates that the morphometric characters used are unreliable for estimating the relatedness of populations (Fig. 2b).

The DNA and morphometric characteristics of populations in the *B. longicauda-*

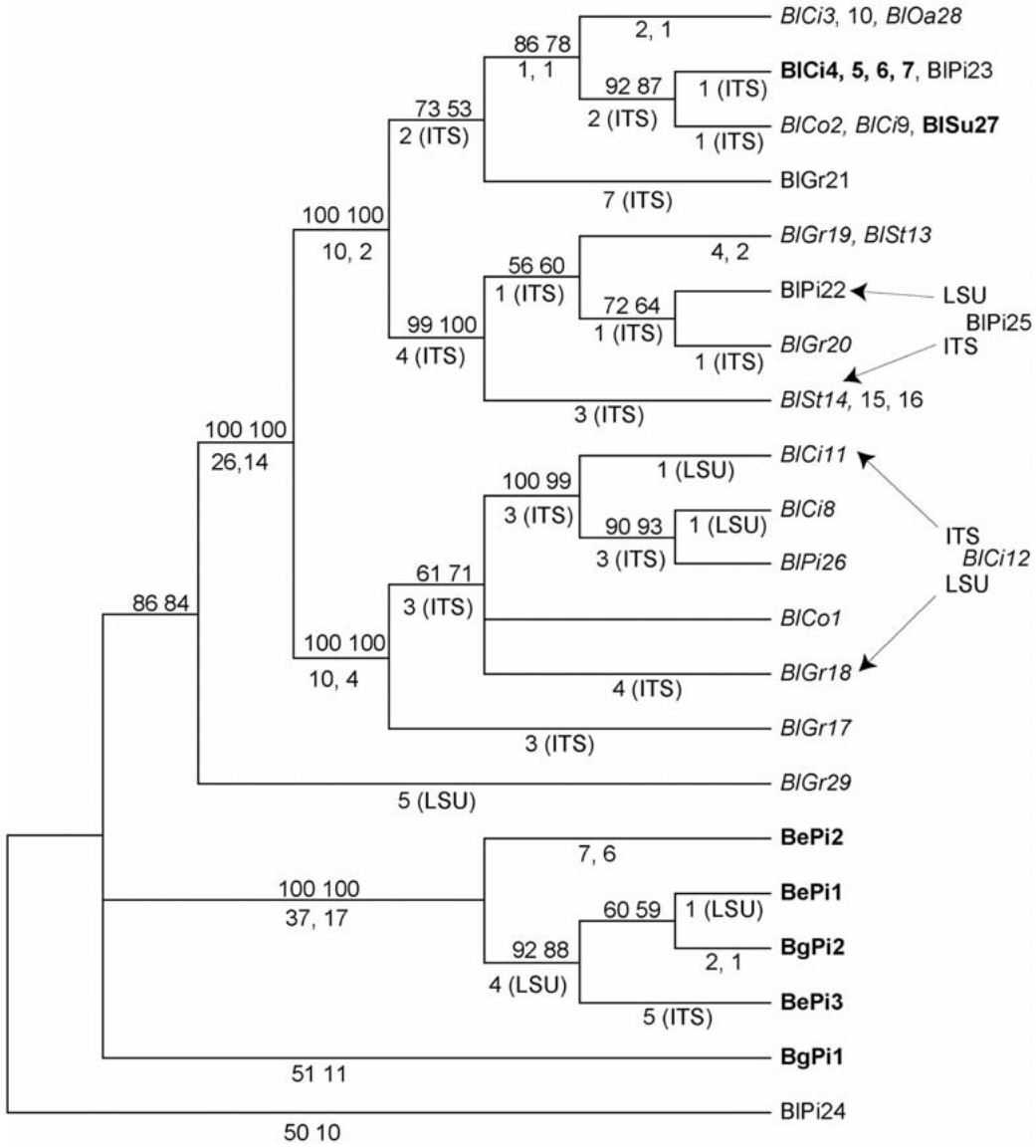

Fig. 1. Phylogenetic distribution of isolates of *Belonolaimus longicaudatus*, *B. euthychilus* and *B. gracilis*. Topology is from ML and MP bootstrap analyses, which obtain congruent resolution. Values at nodes above branches represent MP and ML bootstrap values (100 reps) respectively. Values below branches represent autapomorphic characters for the ITS and LSU regions, respectively, as polarized by outgroup comparison (outgroup taxon *Pratylenchus coffeae* not shown). Taxa having tail lengths greater than stylet lengths are italicized. Taxa having stylet lengths greater than tail lengths are in bold. Arrows indicate that isolate BlPi25 has an LSU sequence that is identical to BlPi22 and an ITS sequence that is identical to BlSt14, 15, 16; BlCi12 has an ITS sequence that is identical to BlCi11 and a LSU sequence that is identical to BlGr18. Bl = *Belonolaimus longicaudatus*, Be = *B. euthychilus*, Bg = *B. gracilis*. BlCo1, BgPi1, and BePi1 are topotype populations for *B. longicaudatus*, *B. gracilis*, and *B. euthychilus*, respectively. Location and host plant species for each taxon is presented in Table 1.

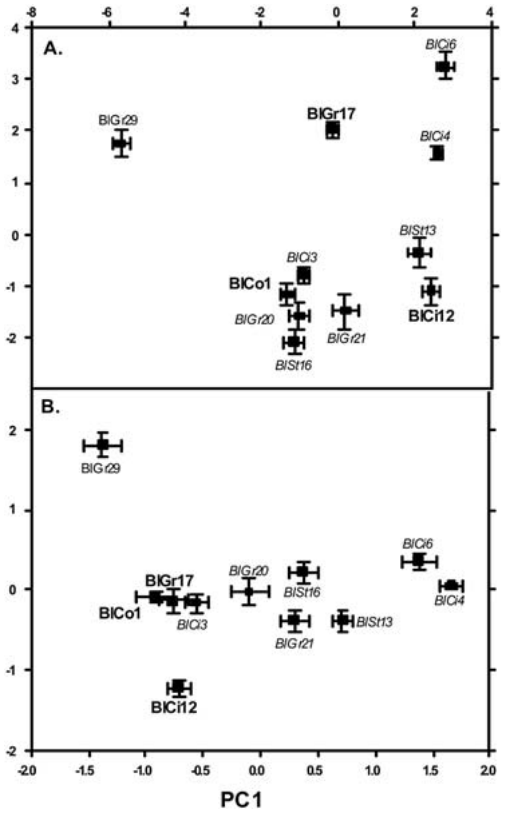

Fig. 2. Principal components analyses of the means of (A) 18 morphometric characters and (B) two arbitrary characters (stylet length and c) from 11 populations of *Belonolaimus longicaudatus*. Populations in the same clade (Fig. 1) as the type population (BlCo1) are shown in large bold type. Populations in the sister clade to that of the type population are shown in italics. Population BlGr29 is a unique population in a sister clade to those of all other *B. longicaudatus* populations.

tus group were broadly related to geographic locality (Fig. 3). The seven populations in the clade containing the topotype population were collected from sites in eastern Florida extending from Broward County in the south to Volusia County in the North. The clade containing the remaining 20 populations is further defined by two clades distinguished by two and four autapomorphies in the ITS region. One of these clades contained populations collected in western and central Florida from strawberry fields in Hillsborough County (BlSt13, 14, 15, 16), from pine stands in Polk and Hernando counties (BlPi22, 25), and from turf in Marion and Putnum counties (BlGr19, 20). The other clade contained populations collected primarily in central Florida. The five populations with identical sequence for ITS and D2-D3, with stylet > tail were collected from citrus orchards in Polk County and from pine in Santa Rosa County in the Panhandle. The remaining populations in the clade, all with tail > stylet, were collected from Martin County in the southeast to Santa Rosa County in the northwest.

The three populations contained in the *B. euthychilus* group exhibited as much genetic variability as those in the *B. longicaudatus* group. Indeed, populations BePi1 and BePi2, both from the Ocala National Forest, differ from one another by 11 autapomorphies in the D2-D3 region. It is also noteworthy that while the population BgPi2 conforms to the concept of *B. gracilis* by having stylet > tail, and an offset lip region, the latter character is paraphyletic according to the phylogeny derived from rDNA. Although just three populations of *B. euthychilus* were selected for study, more than a third of 35 samples collected from pine and oak trees in the Ocala National Forest contained specimens of *Belonolaimus* without an offset lip region. The topotype locality and host for *B. euthychilus* are the same as the neotype locality and host for *B. gracilis* (Rau, 1963). Interestingly, we also recovered both species from this site, the only one among the 35 sites at which *B. gracilis* was detected.

DISCUSSION

The most striking feature of Fig. 1 is the fact that, although rooted by the longest branch, at present it cannot be rooted

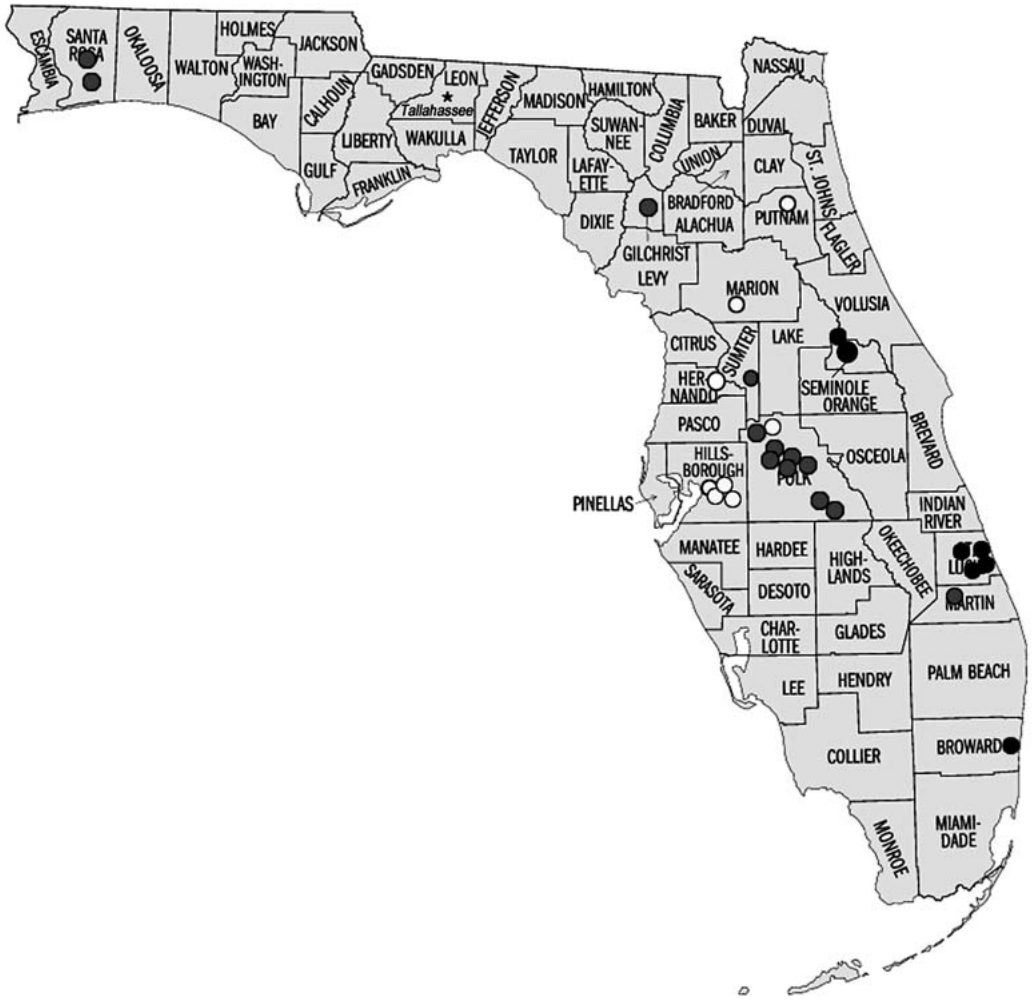

Fig. 3. Spatial patterns of populations of *Belonolaimus longicaudatus* classified according to phylogeny as given in Fig. 1. Clades include that containing the topotype and 6 other populations (black circles), another containing BlSt13-16, BlPi22, 25 and BlGr19-20 (white circles) and one containing BlCi 3-7 & 9, BlOa28, BlPi23, BlCo2, BlSu27, and BlGr21 (gray circles).

more logically or even arbitrarily so that any of the nominal species are monophyletic. The second most striking feature is the incidence of two taxa (BlPi25 and BlCi12) that have discordant ITS and LSU sequences. BlPi25 has an LSU sequence that is identical to BlPi22 but its ITS sequence is identical to BlSt14, 15, and 16. Similarly, BlCi12 has an ITS sequence that is identical to BlCi11, but its LSU sequence is identical to BlGr18. The most parsimonious explanation for this pattern is that within one of these lineages one gene did not change while the other gene did. Therefore, the mode and frequency of nucleotide substitutions in the LSU clearly occur independently of those in the ITS region. This is most apparent in the different rates of

change between the two gene regions. In the case of BlPi25, it appears that the ITS gene region remained static while the LSU gene underwent three substitutions. Conversely, for BlCi12, it was the LSU gene that remained unchanged while the ITS gene region underwent four substitutions.

The discordance of the ITS and LSU DNA sequences in these taxa implicates several interesting phenomena relevant to the evolution of *Belonolaimus* spp. Firstly, although both of the gene regions used for phylogenetic inference are part of the ribosomal RNA cistron, the evolution of these gene regions is not correlated. While this is generally considered a positive aspect, it can lead to gene-tree species-tree discordance, as is evidenced by the apparent paraphyly of BlPi25 and BlCi12 individuals. The discordance between the two gene trees also provides a clear example of the dangers of inferring phylogenetic relationships from a single genetic marker. Secondly, as with phylogenetic reconstruction, species delimitation is also influenced by the distribution of DNA sequence substitution patterns among evolving lineages. For example, while ample autapomorphies appear to delimit the BlCi12 and BlPi25 lineages, the failure of these lineages to obtain reciprocal monophyly argues against the notion that the differences are phylogenetic, but are instead tokogenetic. BlCi12 and BlPi25 are unique in that the combination of their two gene sequences is found nowhere else in the tree. However, the gene sequences themselves are not unique, as an identical copy of each is found in one or more other lineages. Because most methods of phylogenetic reconstruction will find an optimal bifurcating solution, even when it does not exist (McDade, 1995), this condition also illustrates the shortcomings of certain variations of the phylogenetic species concept whereby combinations of characters are used to delimit species (Wheeler, 1999; Wheeler and Platnick, 2000). The distribution of characters for BLCi12 and BlPi25 clearly illustrates the suboptimal properties of combinations of characters as evidence of speciation. In this case, combinations of characters behave as privative evidence, whereby they delimit lineages based on the illogical notion of the "absence of nothing" (Adams, 2001). By using autapomorphies to identify lineage independence, the pattern of variation among many of the sister lineages (terminal tips of the tree in Fig. 1) appears to be more of what is expected from outcrossing populations rather than species.

The selected morphometric characteristics of 11 populations of *B. longicaudatus* were less informative about phylogenetic relationships inferred from DNA sequences than has been reported for some other species. Principal components analysis of arbitrarily selected morphological or morphometric characters for five species of the *X. americanum* group (26 populations) and seven closely related species in *P. coffeae* group (31 populations) resolved groups of populations that were congruent with the molecular based (LSU and/or ITS) phylogenetic species (Duncan *et al.*, 1999; Gozel *et al.*, 2006). Because the number of uniquely derived groups (based on DNA sequence) as a percent of the populations studied was 63% for *B. longicaudatus* compared to just 35% and 21% for the *P. coffeae* and *X. americanum* groups, respectively, it may prove more difficult to identify character suites that are diagnostic for phylogenetic species within *B. longicaudatus*. The restriction map predicted from ITS sequences (not shown) could be used to develop diagnostic tools. A single enzyme (SaII) can distinguish the two major clades of *B. longicaudatus*, and suites of enzymes distinguish all the sister clades except that of BlGr17.

Belonolaimus longicaudatus is distinct from *B. gracilis* for having sclerotized plates in the vagina, an elongated rather than spherical metacorpus, and a hemispherical rather than convex-conoid tail shape. *B. euthychilus* is very similar to *B. gracilis*, but exhibits sexual dimorphism (degenerate stylet and pharynx in males) and lacks a constriction between the labial region and the body (Rau, 1963). Two additional character traits given importance for species diagnosis by Rau (1961, 1963) and others (Smart and Nguyen, 1990) appear to be polyphyletic. The ratio 'stylet length:tail length' has been considered to be a morphometric relationship that distinguishes *B. longicaudatus* from both other species, with 83-100% of *B. longicaudatus* specimens from 15 populations ranging from Florida to New Jersey having stylets shorter than tails (ratio < 1.0) and all observed specimens of *B. gracilis* and *B. euthychilus* having stylets longer than tails (ratio > 1.0) (Rau, 1961, 1963). However, four of five *B. longicaudatus* populations obtained from citrus orchards in Polk County and one population from sugarcane in Martin County had stylets that were on average longer than tails (Fig. 1; Duncan *et al.*, 1999). In fact, the ratios given in Table 2 for two of these four populations (means = 1.03 and 1.05; ranges = 0.98-1.12; 83% > 1.0) are intermediate between those reported by Rau (1961) for *B. longicaudatus* (mean 0.83; range 0.67-1.14; 6% > 1.0) and *B. gracilis* (mean 1.76; range 1.33-2.31). Rau did not report mean stylet:tail ratios for *B. euthychilus*, noting only that the ratios for 100% of 192 specimens exceeded unity. Nevertheless, stylet:tail ratios for *B. euthychilus* are likely to be very similar to those of *B. gracilis* because the mean lengths of stylets (154 ± 7 and 151 ± 11, respectively) and tails (88 ± 10, 89 ± 14, respectively) of the two species are very similar. Therefore, the character can likely discriminate *B. gracilis* and *B. euthychilus* from *B. longicaudatus* so long as unity is not used as an arbitrary criterion. It is suggestive of a more recent evolution that all *B. longicaudatus* populations with a ratio > 1.0 have sequences that differ by no more than two base pairs for ITS, whereas most *B. longicaudatus* populations with a ratio < 1.0 exhibit wide variation in both D2-D3 and ITS nucleotide sequences.

A second character that discriminates *B. gracilis* from *B. euthychilus*, the presence or absence of an offset head, appears invalid based on the morphology of population BgPi2. The population was identified as *B. gracilis*, but is much closer to *B. euthychilus* for the gene sequences we characterized. Again, however, the character appears to be intermediate between the two species, being somewhat less distinctly offset than in *B. gracilis*. Although Rau reported variation in the degree of head offset in *B. maritimus*, this is the first such report for *B. euthychilus*. A more profound difference between population BgPi2 and other populations in the *B. euthychilus* clade was its absence of sperm in the spermatheca in accordance with an absence of males. Rau (1963) reported a similar occurrence in a population collected on St. John's Island, SC. The integument thickness at the tail tip was also well outside the ranges reported for either *B. euthychilus* or *B. gracilis*.

The phylogeny inferred from the DNA sequences of the sting nematode populations in this study supports the likelihood that *B. longicaudatus* and *B. euthychilus* are species complexes (Adams, 1998). The numbers of derived characters for D2-D3 that are uniquely shared by some lineages are similar to numbers found in populations of the *P. coffeae* 'complex' that were subsequently described as independent species (Duncan *et al.*, 1996; Inserra *et al.*, 2001). There is evidence that populations of *B. longicaudatus* outside of Florida are

reproductively isolated (Robbins and Hirschmann, 1974) and there are several studies in addition to the present one that report differing host range and wide morphometric variation among *B. longicaudatus* populations (Abu-Gharbieh and Perry, 1970; Robbins and Hirschmann, 1974; Duncan *et al.*, 1996). Our data provide a basis for additional studies of reproductive compatibility, behavior, and morphology of specific genotypes that are likely to reveal a genus that is far more complex than currently understood.

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