RESEARCH/INVESTIGACIÓN

PHYLOGENETIC RELATIONSHIPS OF *BELONOLAIMUS LONGICAUDATUS* POPULATIONS ASSOCIATED WITH TURFGRASSES IN THE SOUTHEASTERN USA

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ABSTRACT

Habteweld, A., M. L. Mendes, R. N. Inserra, and W. T. Crow. 2021. Phylogenetic relationships of *Belonolaimus longicaudatus* populations associated with turfgrasses in the southeastern USA. Nematropica 51:41-55.

The D2-D3 and ITS regions of rDNA from 31 populations of *Belonolaimus longicaudatus* associated with turfgrasses in different locations of southeastern USA were sequenced and subjected to phylogenetic analysis. The result showed that there were variations in DNA sequences among populations and morphology and morphometric characters as well. The phylogenetic analysis using D2-D3 LSU and ITS1-5.8S-ITS2 rDNA sequences grouped the south Florida populations together with 91 and 81% support, separated from a subclade containing other *B. longicaudatus* populations from other localities. The south Florida populations as a group tended to have smaller mean stylet knob width and stylet/tail length and shorter mean lip, stylet, and esophagus lengths. Principal components analysis of seven selected morphometric characters also showed correlations among populations related to the subclade to which they belong. Overall, results suggest that the populations from turfgrasses in south Florida fit morphologically with the paratypes of *B. longicaudatus* described by Rau (1958) from central Florida, which had a stylet and tail length ratio value ≤ 1. Conversely, the populations from other localities grouping in a different subclade in the phylogenetic tree had the highest range of stylet and tail length values >1. Although parasitism to turfgrasses by the *B. longicaudatus* populations grouping in the two different subclades results in serious damage, more studies are needed to define many biological, ecological and genetic aspects of these populations from turfgrasses.

Key words: *Belonolaimus longicaudatus*, D2-D3, ITS, phylogenetics, south Florida, sting nematode, turfgrass

RESUMEN

Habteweld, A., M. L. Mendes, R. N. Inserra, y W. T. Crow. 2021. Relaciones filogenéticas de algunas poblaciones de *Belonolaimus longicaudatus* asociadas a césped en el Sureste de los Estados Unidos. Nematropica 51:41-55.

Las regiones D2-D3 e ITS de ADNr de 31 poblaciones de *B. longicaudatus* asociadas a césped en

diferentes localidades del Sureste de los Estados Unidos fueron secuenciadas y utilizadas en un análisis filogenético. El resulto mostró variaciones en las secuencias de ADN entre poblaciones, así como en los caracteres morfológicos y morfométricos. El análisis filogenético basado en secuencias de ADNr de D2-D3 LSU y ITS1-5.8S-ITS2 agrupó las poblaciones del Sur de Florida, con un soporte de 91 y 81%, separado de un subclado formado por otras poblaciones de *B. longicaudatus* de otras localidades. Las poblaciones del Sur de la Florida como grupo tendieron a poseer una menor media en el ancho de los nódulos del estilete y estilete/cola; menor media en labio, estilete y longitud del esófago. Un análisis de componentes principales de siete caracteres morfométricos seleccionados también mostró correlaciones entre poblaciones relacionadas al subclado que pertenecen. Los resultados generales sugieren que las poblaciones de césped en el Sur de Florida se ajustan morfológicamente a los paratipos de *B. longicaudatus* descritos por Rau (1958) del Centro de Florida, el cual posee un radio St/T con valor ≤ 1. Al contrario, las poblaciones de otras localidades agrupadas en diferente subclado en el árbol filogenético posee el rango más alto de valores St/T >1. Aunque el parasitismo del césped por poblaciones de *B. longicaudatus* que se agrupan en dos subclados diferentes da como resultado un daño grave, se necesitan más estudios para definir muchos aspectos biológicos, ecológicos y genéticos de estas poblaciones en césped.

Palabras clave: *Belonolaimus longicaudatus*, D2-D3, ITS, filogenética, Sur de Florida, nematodo del aguijón, césped

INTRODUCTION

The genus *Belonolaimus* was established with the type species *B. gracilis* by Steiner (1949). Presently, the genus contains six species namely *Belonolaimus gracilis* Steiner, 1949; *B. longicaudatus* Rau, 1958; *B. euthychilus* Rau, 1963; *B. maritimus* Rau, 1963; *B. nortoni* Rau, 1963; and *B. maluceroi* Cid del Prado Vera and Subbotin, 2012. *Belonolaimus longicaudatus* is among the most destructive plant-parasitic nematodes to a wide range of plants including turfgrasses, ornamentals, forages, vegetables, agronomic crops, and trees (Duncan *et al.*, 1996; Cid del Prado Vera and Subbotin, 2012; Crow and Duncan, 2018). In addition to direct root damage, *B. longicaudatus* predisposes plants to stress caused by adverse conditions like drought and heat that could lead to a poor yield and quality (Lucas, 1982).

The University of Florida Nematode Assay Laboratory (NAL) receives thousands of samples containing soil, plugs, and roots from turfgrasses annually from Florida and other states for diagnosis purpose. Turfgrasses represent over 90% of the samples received by NAL annually (Habteweld and Crow, 2018). Results of our diagnoses indicated that *B. longicaudatus* is one of the top plant-parasitic nematodes associated with high and moderate nematode damage in turfgrasses (Habteweld and Crow, 2018).

Belonolaimus longicaudatus is found predominately in sandy coastal areas of the southeastern United States where soils consist of >80% sand (Robbins and Barker, 1974; Crow, 2018). It is also common in sandy regions along the Gulf of Mexico and Atlantic coasts from Texas to Virginia and also sandy areas inland (Crow, 2018). Florida is considered to be the point-of-origin for *B. longicaudatus* where it exhibits a great deal of diversity in morphology, host preference, and genetics as noted in the original description by Rau (1958; 1961).

Robbins and Hirschmann (1974) reported pronounced intra- and inter-population morphology and host preference variations including mating incompatibility among six populations form North Carolina and Georgia. The existence of *B. longicaudatus* pathotypes was shown in early field observations (Perry and Norden, 1963) and subsequently supported by greenhouse studies of populations collected from different crops and localities (Abu-Gharbieh and Perry 1970; Han *et al.*, 2006a,b).

Morphological and molecular studies of different populations of *B. longicaudatus* from wide geographical locations associated with a specific crop would provide relevant information on their phylogenetic relationships, and the inter- and intra-population variabilities. There is no information on the morphological and genetic variability of *B. longicaudatus* populations from turfgrasses. A study was conducted with the objectives: i) to characterize the D2-D3 expansion

segment of the large subunit of nuclear rDNA, ITS1-5.8S-ITS2 rDNA region and morphometric characteristics of a subset of populations from turfgrasses in the southeastern of United States; and ii) to estimate phylogenetic relationships among *B. longicaudatus* populations associated with turfgrasses from Florida and other states.

MATERIALS AND METHODS

Nematode populations

Nematode populations used in this study were obtained from 31 soil and root samples received by the NAL in 2019 from turfgrass fields including golf courses and residential lawns from Florida and other southeastern states of the US (Table 1). At these sites, nematode populations may have been introduced with sod from turfgrass production fields located largely in Florida, Georgia, and Texas. These populations were at first tentatively assigned to *B. longicaudatus* based on standard morphological and morphometric characters of diagnostic value for the species (Cid del Prado Vera and Subbotin, 2012). Then some specimens were kept in a refrigerator in a tap water suspension, and others were used for molecular and phylogenetic analyses. Subsequently, refrigerated specimens from four populations that clustered in two different main subclades in the phylogenetic analysis were selected for detailed morphological and morphometric comparisons.

Nematode extraction

Nematodes were extracted from 100 cm³ of soil using centrifugation and sugar-floatation techniques (Harrison and Green, 1976). Nematodes were also extracted from four 3.8-cm-diam. and 5 cm-deep turf plugs which were incubated for 72 hr in a mist chamber (Crow *et al.*, 2020).

Molecular characterization

Total genomic DNA was extracted following the proteinase K method. A single female was transferred to a 200 µl PCR tube containing 20 µl of DNA extraction buffer consisted of 18 µl of TE buffer, 1 µl proteinase K (20 mg/ml) and 1 µl of 2% triton X. To facilitate penetration of the extraction buffer into the nematodes, the tubes containing the nematodes were frozen and thawed

five times. The genomic DNA was extracted by incubating the samples at 56°C for 1 hr for optimum proteinase K activity and proteinase K was deactivated by incubating at 95°C for 15 min using T100TM thermocycler (Bio-Rad Laboratories Inc., Hercules, CA). DNA amplification was performed in 25 µl of reaction mix containing 12.5 µl Apex 2x Hot Start Master mix (Genessee Scientific, San Diego, CA), 0.75 µl forward and reverse primers, 9.5 µl sterile nuclease free water (Thermo Fisher Scientific, Gainesville, FL) and 1.5 µl template DNA. For amplification of the D2-D3 expansion segment of the large subunit (LSU) rDNA, the primers sets D2A (5′-CAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) were used. The ribosomal region spanning the internal transcribed spacer (ITS)1, 5.8S gene, and ITS2 was amplified using the primers TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′) and AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′). The thermocycling was carried out using a T100™ thermo cycler (Bio-Rad Laboratories, Inc., CA). The thermocycling reaction for both DNA regions was as follows: 95°C for 15 min; followed by 30 cycles of 94°C for 45 sec, 56°C for 45 sec and 72°C for 90 sec; and a final extension step of 72°C for 7 min.

DNA fragments were separated by electrophoresis in 1X TBE buffer and 1.2% agarose gel, stained with SYBR safe DNA Gel Stain (Invitrogen, Thermo Scientific Inc.), and visualized under UV light using the ChemiDOC XRS One 4.5.2 program (Bio-Rad Laboratories Inc.). The PCR products were sent to Genewiz LLC (South Plainfield, NJ) for PCR product purification and sequencing. To identify the species, sequences were checked and edited manually using Geneiuos Prime 2020.1.2 (Auckland, New Zealand). Consensus sequences obtained were compared with those deposited in the GenBank database using BLAST engine search for sequence homology. The newly obtained consensus sequences were submitted to the GenBank database under accession numbers MZ045436 to MZ045466 and MZ045467 to MZ045497 for D2-D3 and ITS rRNA, respectively (Table 1).

Phylogenetic analysis

To perform the phylogenetic analysis, D2-D3 LSU, ITS1-5.8S-ITS2 rDNA sequences obtained

Table 1. *Belonolaimus longicaudatus* populations characterized in this study.

No.	Sample location	Lab number	Grass	Sample source	Analysis code	D2-D3 accession #	ITS accession #
1	Ft. Lauderdale, Broward, FL	Experimental site	Bermudagrass Latitude36	Golf course	Bl Broward2 FL	MZ045463	MZ045495
2	Naples, Collier, FL	2019-1202	Bermudagrass 'Tifdwarf'	Golf course	Bl Collier1 FL	MZ045439	MZ045470
3	Plantation, Broward, FL	2019-1358	Bermudagrass 'Tifeagle'	Golf course	Bl-Broward1-FL	MZ045438	MZ045469
4	Tequesta, Palm Beach, FL	2019-368	Bermudagrass 'TifEagle'	Golf course	Bl PalmBeach1 FL	MZ045449	MZ045481
5	Vero Beach, Indian River, FL	2019-1377	Bermudagrass 'Celebration'	Golf course	Bl Indian River1 FL	MZ045441	MZ045472
6	Sanibel, Lee, FL	2019-1088	Seashore Paspalum 'Sealsle 1'	Golf course	Bl Lee1 FL	MZ045445	MZ045476
7	Citra, Marion, FL	Experimental site	Bermudagrass 'Latitude 36'	Golf course	Bl Marion1 FL	MZ045465	MZ045497
8	Sun City, Hillsborough, FL	Lab culture	Bermudagrass 'Tifway'	Golf course	Bl Hillsborough1 FL	MZ045464	MZ045494
9	The Villages, Lake, FL	2019-497	Bermudagrass 'Tifdwarf'	Golf course	Bl Lake1 FL	MZ045442	MZ045473
10	Houston, Harris, TX	2019-530	Bermudagrass 'Miniverde'	Golf course	Bl Harris1 TX	MZ045459	MZ045491
11	Houston, Harris, TX	2019-531	Bermudagrass 'Miniverde'	Golf course	Bl Harris2 TX	MZ045460	MZ045492
12	Key Largo, Monroe, FL	2019-718	Bermudagrass 'Miniverde'	Golf course	Bl Monroe1 FL	MZ045448	MZ045479
13	Bradenton, Manatee, FL	2019-734	Bermudagrass 'Tifway'	Golf course	Bl Manatee1 FL	MZ045462	MZ045478
14	My. Dora, Lake, FL	2019-1051	Bermudagrass 'Tifdwarf'	Golf course	Bl Lake2 FL	MZ045443	MZ045474
15	Birmingham, Jefferson, AL	2019-1243	Bermudagrass 'Tifdwarf'	Golf course	Bl Jefferson1 AL	MZ045436	MZ045467
16	Orlando, Orange, FL	2019-1343	Bermudagrass 'Tifeagle'	Golf course	Bl Orange1 FL	MZ045466	MZ045480
17	Gainesville, Alachua, FL	2019-1345	St. Augustinegrass 'Unknown'	Lawn	Bl Alachua1 FL	MZ045437	MZ045468

Table 1. Continued.

No.	Sample location	Lab number	Grass	Sample source	Analysis code	D2-D3 accession #	ITS accession #
19	Hilton Head, Beaufort, SC	2019-1309	Bermudagrass 'Tifdwarf'	Golf course	Bl Beaufort2 SC	MZ045453	MZ045485
20	Lady Lake, Lake, FL	2019-1348	Bermudagrass 'Tifway'	Golf course	Bl Lake4 FL	MZ045444	MZ045475
21	Palm Beach Gardens, Palm Beach, FL	2019-1351	Bermudagrass 'Latitude 36'	Municipality	Bl PalmBeach2 FL	MZ045450	MZ045482
22	Myrtle Beach, Horry, SC	2019-1381	Bermudagrass 'Tifway'	Golf course	Bl Horry1 SC	MZ045454	MZ045486
23	Myrtle Beach, Horry, SC	2019-1384	Bermudagrass 'Tifway'	Golf course	Bl Horry2 SC	MZ045455	MZ045487
24	Crescent City, Putnam, FL	2019-1500	Bermudagrass 'Tifeagle'	Golf course	Bl Putnam1 FL	MZ045451	MZ045483
25	Anyor, Horry, SC	2019-2195	Centipedegrass	Lawn	Bl Horry3 SC	MZ045456	MZ045488
26	Young Harris, Towns, GA	2019-2157	Bentgrass Mix '1020/1019'	Golf course	Bl Towns1 GA	MZ045440	MZ045471
27	Luling, St. Charles Parish, LA	2019-2249	Bermuda 'Tifdwarf'	Golf course	Bl St.CharlesParish1 LA	MZ045446	MZ045477
28	Spring Branch, Comal, TX	2019-2375	Bermudagrass	Golf course	Bl Comal1 TX	MZ045457	MZ045489
29	Spring Branch, Comal, TX	2019-2376	'Miniverde'	Golf course	Bl Comal2 TX	MZ045458	MZ045490
30	Austin, Travis, TX	2019-3799	Bermudagrass 'Miniverde'	Golf course	Bl Travis1 TX	MZ045461	MZ045493
31	Diamondhead, Hancock, MS	2019-3800	Bermudagrass	Golf course	Bl Hancock1 MS	MZ045447	MZ045496

from 31 populations of *B. longicaudatus*: 17 sequences from Florida, and 5, 5, 1, 1, 1, 1 from South Carolina, Texas, Alabama, Georgia, Lousiana and Mississippi, respectively (Table 1), sequences from *B. longicaudatus* (specified in the following sections) and other *Belonolaimus* species (*B. euthychilus*, *B. gracilis* and *B. maluceroi*) retrieved from NCBI databases were aligned over the same length in MUSCLE using MEGA v. X (Kumar *et al.*, 2018). The alignment was analysed to obtain the base substitution model for these sequences using MEGA X (Kumar *et al.*, 2018). For D2-D3 LSU and ITS1-5.8S-ITS2 rDNA sequences, the evolutionary history was inferred by using Tamura-Nie model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The phylogram was generated using a base substitution model selected for each alignment and running Maximum Likelihood Model with 1000 bootstrap replicates using MEGA X (Kumar *et al.*, 2018) to assess the degree of support for each branch on the tree (Landa *et al.*, 2008). The D2-D3 sequence from *Tylenchorynchus leviterminalis* (EU368591) and *T. dubius* (DQ328707) were used as outgroups for constructing the phylogenetic tree based on the sequences from the D2-D3 expansion segment of 28S rRNA. *Tylenchorynchus zeae* (EF519711) and *T. annulatus* (EF030983) were used as outgroups in for the phylogenetic tree based on ITS1-5.8S-ITS r DNA sequence.

Morphometric characterization

Twenty females were used for morphological and morphometric characterization to supplement the molecular data. Nematodes were picked and transferred to a PCR tube and killed with hot (95°C) 4% formalin. The PCR tube with heat-killed nematodes was immediately placed in a thermos-cycler: 95°C for 2 min, 65°C for 10 min, 75°C for 10 min, 85°C for 10 min, 95°C for 10 min. After the tube reached room temperature, the tube was rinsed with distilled water, and the content was transferred to a staining dish. Then, nematodes were picked out and transferred to a glass slide with a cavity filled with a mixture of glycerol and distilled water in proportion of 1:20, placed on a hot plate at 70°C for 15-20 min and mounted on glass slides following the method described by De Grisse (1969) and Habteweld *et al.* (2019).

Measurements of specimens were taken using the ZEN lite software on ZEISS Axiocam ERc5s digital camera installed on an Olympus BH-2 microscope (Olympus, Japan). Morphological and morphometric characteristics were compared with identification keys for *Belonolaimus* spp. (Cid del Prado Vera and Subbotin, 2012). Morphometeric characteristics of different populations were statistically analyzed using SAS vr. 9.4 (SAS Institute, Cary, NC). Morphometric characters of the populations were first analyzed by one-way analysis of variance (ANOVA) using the General Linear Model (GLM). Then, the morphometric characters of the two *B. logicaudatus* subclades were analyzed using two-tail t-test ($\alpha = 0.05$). To study the correlation among populations, principal components of analysis of seven morphometric characters which differentiated the two subclades of *B. longicaudatus* was conducted using a Proc Princom procedures in SAS vr 9.4.

RESULTS

Molecular characterization and phylogenetic relationships

D2-D3 of the 28S rDNA: PCR with D2A and D3B primers yielded a product of approximately 751-772 bp in length. D2-D3 expansion segment of 28S rDNA of the populations showed 96-99% similarity with *B. longicaudatus* sequences from NCBI GenBank. The D2-D3 alignment included 42 sequences and had 671 positions in length. Out of the 42 sequences, 31 of them were obtained from *B. longicaudatus* populations isolated in the present study, 11 sequences were from *B. longicaudatus* (KF963100, GQ896548, AB6026050), *B. maluceroi*, *B. euthychilus*, *B. gracilis* and two out-group sequences (*T. leviterminalis* and *T. dubius*) retrieved from NCBI GenBank. Phylogenetic analysis of the D2-D3 sequences alignment revealed three major clades among *Belonolaimus* populations on 50% majority consensus (Fig. 1). Clade I contained *B. euthychilus* (DQ672359, DQ672360, DQ672361) + *B. gracilis* (DQ672362, DQ672363) (88%), clade

II *B. maluceroi* (JN967754) (54%), and clade III all *B. longicaudatus* populations including sequences retrieved from GenBank (KF963100, GQ896548, AB6026050) (100%). In this clade, the south Florida *B. longicaudatus* in the present study and the sequence retrieved from GenBank (AB602650) grouped in a subclade IIIA (91%), whereas the remaining populations from Florida and other states including the retrieved sequences (KF963100, GQ896548) grouped in a subclade IIIB (94%). The subclade IIIA populations defined by eight unique autapomorphies in the alignment of the D2-D3 region. Within each of these subclades there were 0-9 nucleotide (0-1.3%) differences in D2-D3 of 28S rRNA gene sequences among *B. longicaudatus* populations (Fig. 1). However, the south Florida *B. longicaudatus* populations (subclade IIIA) had differences of 10-18 nucleotides (1.5-2.7%) from those of the other *B. longicaudatus* populations (subclade IIIB).

ITS1-5.8S-ITS2 rDNA: PCR with TW81 and AB28 primers yielded a product of approximately 887-941 bp in length. Based on BLAST analysis, ITS1-5.8S-ITS2 of the populations showed 95-99% similarity with *B. longicaudatus* sequences from NCBI GenBank. The ITS1-5.8S-ITS2 alignment included 44 sequences and had 1092 positions in length. Out of the 44 sequences, 31 of them were obtained from *B. longicaudatus* populations isolated in the present study, 13 sequences were obtained from *B. longicaudatus* (KF963098, GQ896549, AB602614), *B. maluceroi*, *B. euthychilus*, *B. gracilis* and the out-group sequences (*T. zeae* and *T. annulatus*) from NCBI GenBank. Phylogenetic analysis of the ITS sequences alignment generated three major clades on 50% majority consensus (Fig. 2). Clade I contained *B. maluceroi* (JN967751, JN967752, JN967753), *B. longicaudatus* sequences retrieved from the GenBank (KF963098, GQ896549, AB602614) and *B. gracilis* (DQ672386) (60%), Clade II contained *B. euthychilus* (DQ672381, DQ672382, DQ672383) + *B. gracilis* (DQ672385) (100%), and clade III contained all *B. longicaudatus* populations in the present study (100%). In this clade, most of the south Florida populations formed a separate subclade IIIA (81%), whereas the remaining *B. longicaudatus* populations grouped in the subclade IIIB with 100% consensus support. The subclade containing most of the south Florida populations is defined by 29 autapomorphies in the ITS region. There were 5-84 (0.5-7.7%) nucleotide differencesin the ITS1-5.8S-ITS2 gene sequences within *B. longicaudatus* populations in subclade IIIB and 5-19 (0.5-1.7%) nucleotide differences within those in subclade IIIA (Fig. 2). Within some populations in both subclades IIIA and IIIB there were nucleotide differences with variable consensus support (Fig. 2). The populations in subclade IIIA differed from those in subclade IIIB by 31-179 (2.8%-16.4%) nucleotides. Based on DNA sequence data, populations in the *B. longicaudatus* group were broadly related to geographic locality (Figs. 1, 2). The eight populations in the subclade IIIA were collected mainly from south Florida except a population from northcentral Florida (Marion County). All the rest of the populations in subclade IIIB were from various localities including some from south Florida (Monroe, Palm Beach, and Manatee Counties).

Morphometric characterization

Observation of morphological and morphometric characters of females revealed that all populations were representatives of *B. longicaudatus* with an offset lip region, a stylet longer than 100 microns and tail length > stylet length. The morphometric characters of these populations were in the range reported for the species (Table 2). However, there have been intra- and inter-population variations in morphology and morphometric characters such as degree of lip constriction, lip length, distance of excretory pore from anterior end, esophagus length, median bulb shape, tail shape, stylet/tail ratio and integument thickness. The range in values of the ratio between stylet and tail length were ≤ 1 in the populations in the subclade IIIA. On the contrary, the highest range value of this ratio was > 1 in populations from the subclade IIIB (Table 2).

Statistical analysis of morphometric characters between populations in subclades IIIA and IIIB showed significant differences in some of the morphometric characters. Overall, populations in subclade IIIA (south Florida) had smaller mean lip length, stylet knob width, stylet/tail and tail integument; shorter mean stylet length, excretory pore distance from anterior end, and esophagus length (Table 3). The principal component analysis of seven morphometric characters that were significantly different between the subclades showed that the populations in each subclade IIIA
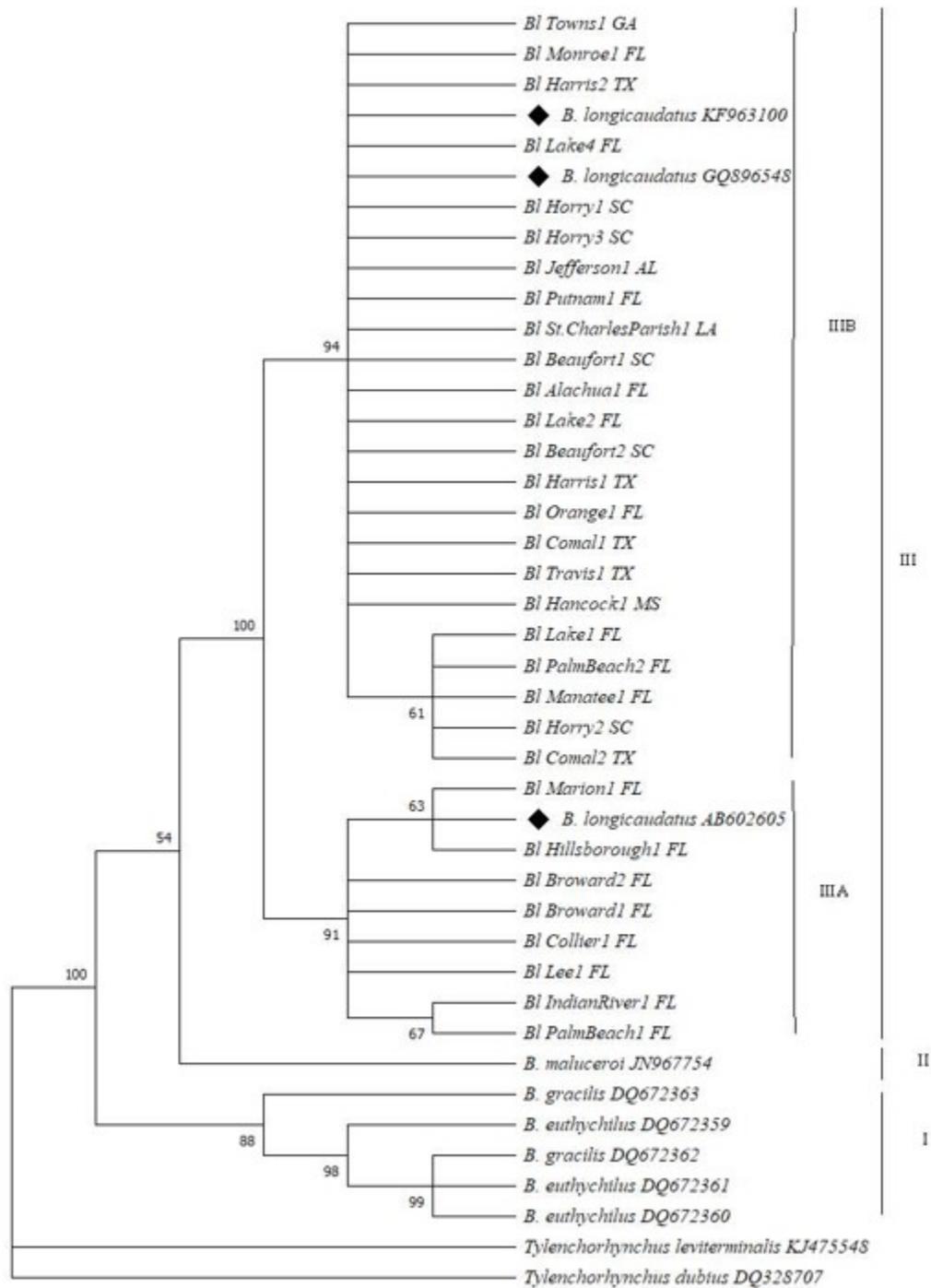
Figure 1. Phylogenetic relationship based on D2-D3 expansion segments of 28S rDNA gene sequences within the genus *Belonolaimus*. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-2519.68) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 42 nucleotide sequences. There were a total of 671 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Accessions preceded by ♦ are *B. longicaudatus* sequences retrieved from the GenBank®.
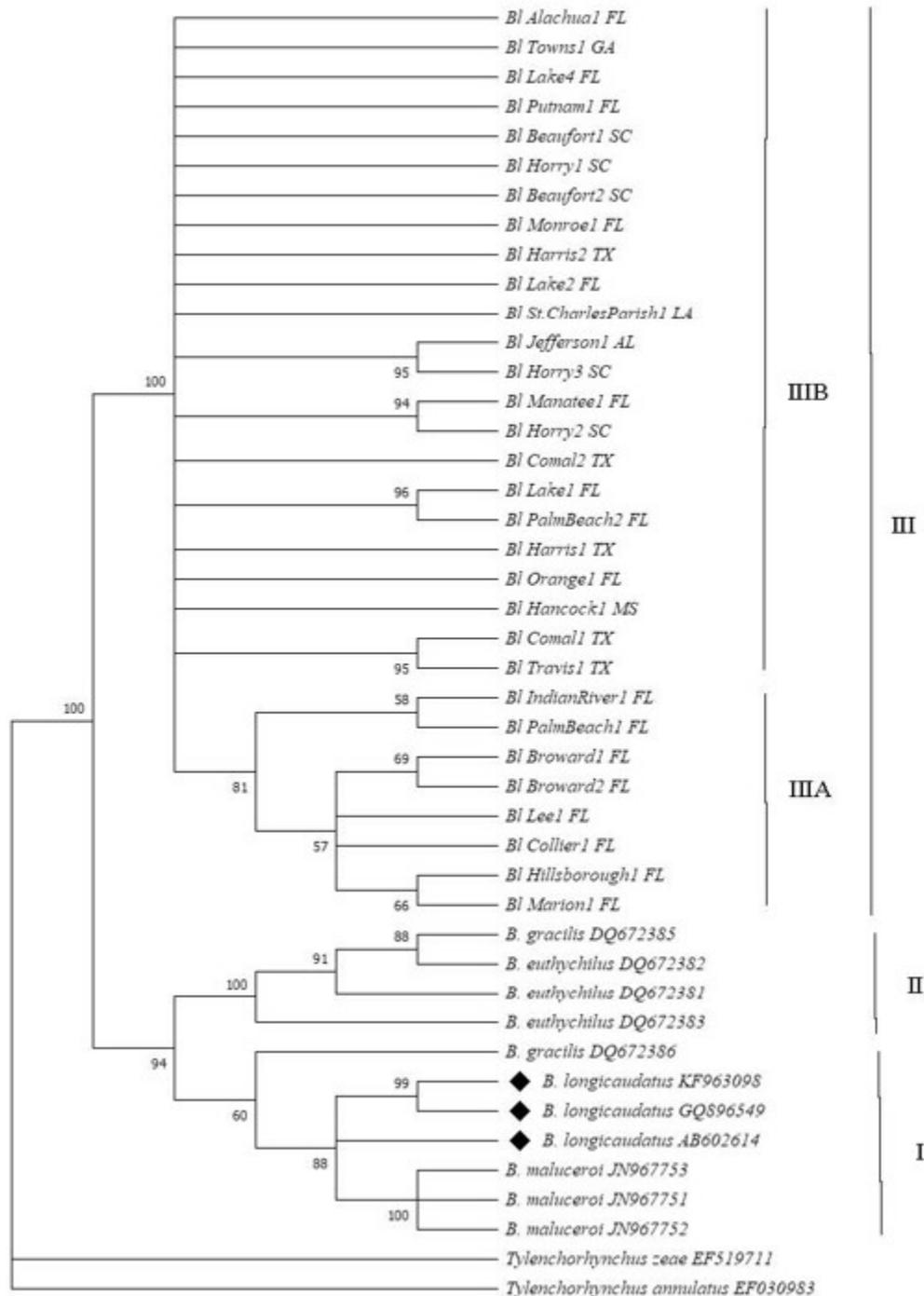
Figure 2. Phylogenetic relationship based on ITS rDNA sequences within the genus Belonolaimus. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (-7123.47) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This analysis involved 44 nucleotide sequences. There were a total of 1092 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Accessions preceded by ♦ are *B. longicaudatus* sequences retrieved from the GenBank®.

Table 2. Morphometrics and derived variables for females from a subset of populations of *Belonolaimus longicaudatus* listed in Table 1. All measurements are in micrometers and the form: mean (n = 20) ± standard deviation (range).

Population	Subclade IIIB				Subclade IIIA			
	Bl Manatee1 FL	Bl Lake4 FL	Bl Harris1 TX	Bl Horry1 SC	Bl Hillsborough1 FL	Bl Lee1 FL	Bl Broward2 FL	Bl Marion1 FL
L	2083 ± 124	2238 ± 127	2102 ± 132	2235 ± 135	2137 ± 154	2115 ± 108	2166 ± 65	1993 ± 146
	(1880-2224)	(2059-2520)	(1913-2326)	(2089-2550)	(1889-2397)	(1897-2222)	(2077-2273)	(1811-2267)
Body width	36 ± 2.2	40.1 ± 3.3	38.5 ± 3.0	41.0 ± 2.3	38.4 ± 1.8	36.9 ± 1.7	37.6 ± 1.3	36.4 ± 2.1
	(31.0-38.0)	(34-44)	(33.3-42.0)	(37.4-44.0)	(36.2-41.4)	(35.0-40.2)	(36.0-40.3)	(33.0-39.5)
a	58.7 ± 4.7	56 ± 4.2	54.8 ± 5.2	54.5 ± 2.8	55.6 ± 2.4	57.4 ± 4.0	57.7 ± 3.0	54.9 ± 4.2
	(50.7-67.4)	(51.3-65.4)	(45.6-66.5)	(49.2-57.9)	(52.0-59.0)	(51.6 - 62.4)	(51.6-61.4)	(49.5-61.8)
b	8.5 ± 1.1	8.1 ± 0.35	7.9 ± 0.8	7.9 ± 0.4	8.7 ± 0.6	8.6 ± 0.7	7.7 ± 0.6	8.0 ± 0.8
	(7.3-10.5)	(7.0-8.6)	(6.6-9.0)	(7.1-8.2)	(7.9-9.8)	(7.7-10.5)	(6.7-8.5)	(7.3-9.2)
c	15.8 ± 1.3	16.0 ± 0.55	15.7 ± 0.9	16.7 ± 1.6	16.1 ± 1.4	15.9 ± 1.4	15.2 ± 0.5	14.9 ± 1.7
	(14.1-18.0)	(15.3-17.1)	(14.3-17.1)	(15.1-20.2)	(13.7-17.7)	(13.9-18.1)	(14.0-15.9)	(11.9-17.1)
V%	50.1 ± 1.6	50.0 ± 1.0	51.2 ± 2.1	50.0 ± 1.8	50.4 ± 1.7	49.1 ± 2.0	51.1 ± 2.0	51.9 ± 3.0
	(49.0-53.0)	(48.4-51.5)	(47.5-54.6)	(48.1-53.9)	(48.8-54.8)	(44.8-51.5)	(46.0-54.0)	(48.8-57.3)
Lip length	9.1 ± 0.9	11.0 ± 1.0	10.3 ± 1.2	10.9 ± 5.5	9.4 ± 1.2	9.6 ± 0.6	10.5 ± 0.7	8.9 ± 1.2
	(7.8-10.2)	(9.5-12.9)	(9.1-12.3)	(10.0-11.7)	(7.3-11.1)	(8.4-10.2)	(9.4-12.2)	(6.7-11.0)
Stylet cone	86.9 ± 9.6	98.0 ± 5.9	91.7 ± 4.3	89.8 ± 3.7	83.5 ± 4.4	82.7 ± 5.2	85.3 ± 4.5	83.4 ± 5.5
	(64.1-95.0)	(88.0-107.0)	(85.2-97.3)	(85.0-97.0)	(78.4-89.9)	(73.4-88.7)	(80.0-92.8)	(73.1-90.2)
Stylet shaft	34.1 ± 2.0	35.5 ± 2.4	33.5 ± 4.0	35.0 ± 2.2	35.2 ± 2.6	30.8 ± 2.7	36.4 ± 2.9	33.8 ± 1.7
	(29.2-35.3)	(30.4-39.3)	(27.3-38.4)	(32.0-38.6)	(30.6-39.6)	(27.1-34.8)	(32.0-40.7)	(31.6-37.3)
Stylet length (St)	121 ± 10	133.0 ± 6.4	125.2 ± 5.2	124.7 ± 5.8	118.7 ± 5.8	113.5 ± 6.9	121.7 ± 5.7	117.2 ± 5.9
	(99.4-130)	(122.0-141.6)	(116.4-131.4)	(117.0 ± 135.2)	(112.5-129.5)	(102.0-120.2)	(112.1-128.5)	(108.2-127.5)
Stylet Knob width	5.7 ± 0.4	6.0 ± 0.6	5.6 ± 0.5	5.6 ± 0.6	5.3 ± 0.3	5.6 ± 0.5	5.6 ± 0.4	5.2 ± 0.6
	(5.0-6.2)	(5.0-6.7)	(5.0-6.6)	(5.0-6.7)	(5.0-5.8)	(4.6-6.1)	(5.0-6.3)	(4.5-6.4)
DEGO	6.1 ± 0.6	6.0 ± 0.7	6.0 ± 0.9	5.7 ± 0.3	5.1 ± 1.1	5.8 ± 0.4	6.2 ± 0.6	5.4 ± 1.1
	(5.2-7.0)	(5.0-7.1)	(5.0-7.8)	(5.4-6.2)	(4.1-6.5)	(5.3-6.2)	(5.2-7.6)	(4.1-7.3)

Table 2. Continued.

Population	Subclade IIIB				Subclade IIIA			
	Bl Manatee1 FL	Bl Lake4 FL	Bl Harris1 TX	Bl Horry1 SC	Bl Hillsborough1 FL	Bl Lee1 FL	Bl Broward2 FL	Bl Marion1 FL
Nerve ring	189.5 ± 16.5	214.0 ± 22.0	187.4 ± 39.8	210.7 ± 10.5	193.5 ± 11.1	187.0 ± 23.6	209.4 ± 8.0	191.2 ± 7.3
	(159-212)	(175.0-238.1)	(116.0-225.6)	(199.0-229.6)	(181.4-212.0)	(133.6-225.1)	(195.0-217.8)	(180.0-200.5)
Excretory pore	207.4 ± 23.5	238.5 ± 22.8	220.0 ± 24.6	237.3 ± 12.3	209.2 ± 11.3	198.0 ± 22.9	222.6 ± 9.0	211.6 ± 16.1
	(170.7-246.5)	(200.0-263.5)	(186.0-263.5)	(218.0-251.0)	(199.5-230.3)	(143.6-228.0)	(208.5-233.0)	(183.5-228.0)
Esophagus	247.9 ± 27.3	275.5 ± 20.9	269.8±25.7	282.9 ± 16.8	245.7 ± 11.0	248.9 ± 24.2	283.9 ± 16.6	249.0 ± 9.0
	(210.8-290.0)	(240.0-304.5)	(233.5-309.2)	(265.0-315.7)	(236.0-272.0)	(199.0-288.0)	(257.0-310.6)	(228.1-259.5)
Median bulb length	24.3 ± 2.0	27.2 ± 2.3	26.5 ± 3.3	27.8 ± 2.7	29.4 ± 1.9	27.0 ± 2.0	27.2 ± 1.0	26.0 ± 2.6
	(22.0-27.3)	(24.0-30.0)	(23.0-33.3)	(25.0-32.5)	(25.7-31.3)	(23.9-30.0)	(25.3-28.4)	(23.3-31.4)
Median bulb width	18.5 ± 1.4	20.9 ± 2.0	20.7 ± 3.5	20.8 ± 2.0	19.4 ± 2.2	19.8 ± 1.7	20.2 ± 1.5	19.7 ± 1.2
	(15.7-20.0)	(18.0-24.2)	(16.5-27.0)	(17.0-25.0)	(16.8-22.6)	(17.0-23.2)	(17.6-23.5)	(18.5-22.1)
Anal body width	29.8 ± 1.4	33.0 ± 3.3	35.6 ± 8.5	32.8 ± 2.6	32.1 ± 2.9	30.5 ± 1.4	31.6-1.2	32.1 ± 1.7
	(28-32)	(25.6-37.8)	(26.0-52.7)	(28.7-35.1)	(29.0-37.7)	(27.3-32.9)	(30.2-33.8)	(29.8-35.0)
Tail length (T)	132.5 ± 9.7	139.7 ± 7.7	134.3 ± 9.1	134.3 ± 10.7	133.5-14.6	133.5 ± 12.5	142.4 ± 6.5	134.8 ± 13.0
	(118-145)	(128.0-153.0)	(117.3-146.0)	(107.5-148.0)	(115.0-157.8)	(115.6-147.8)	(137.0-155.4)	(117.3-155.9)
Stylet/tail (St/T)	0.92 ± 0.08	0.96 ± 0.05	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
	(0.78-1.06)	(0.87-1.01)	(0.8-1.1)	(0.86-1.2)	(0.8-1.0)	(0.7-1.0)	(0.8-0.9)	(0.8-1.0)
Tail integument	7.2 ± 1.1	7.1 ± 0.7	7.1±1.1	7.6 ± 0.6	6.1 ± 0.8	6.5 ± 0.8	7.8 ± 1.3	6.5 ± 1.3
	(5.4-8.6)	(6.4-8.5)	(6.0-9.1)	(6.7-8.4)	(4.8-7.6)	(5.3-8.4)	(6.3-10.1)	(5.0-8.4)

Table 3. Statistically significant morphometric characters of females from populations of *Belonolaimus longicaudatus* in subclade IIIA (south Florida) and subclade IIIB (other localities) in D2-D3 and ITS phylogenetic trees shown in Figs. 1, 2. All measurements are in micrometers and in the form: mean (n = 20) ± standard deviation (range).

	Subclade IIIA	Subclade IIIB
Lip length	9.6 ± 1.1 b	10.3 ± 1.2 a
	(6.7-12.2)	(7.8-12.9)
Stylet length	118 ± 7 b	126 ± 8 a
	(102-129)	(99-142)
Stylet knob width	5.4 ± 0.5 b	5.7 ± 0.5 a
	(4.5-6.4)	(5.0-6.7)
Excretory pore distance	210 ± 18 b	226 ± 26 a
	(144-233)	(171-264)
Esophagus length	257 ± 22 b	269 ± 26 a
	(199-311)	(211-316)
Stylet/tail	0.87 ± 0.07 b	0.94 ± 0.08 a
	(0.72-1.0)	(0.78-1.2)
Tail integument	6.7 ± 1.2 b	7.2 ± 0.9 a
	(4.8-10.1)	(5.4-9.1)

and III B were closely related, with exception of one population from each subclade (Fig. 3).

DISCUSSION

This study showed that there were variations in DNA sequences among the populations of *B. longicaudatus* from turfgrasses. Differences in sequences of rDNA genes between *B. longicaudatus* populations were observed in previous studies (Cherry *et al.*, 1997; Gozel *et al.*, 2006; Han *et al.*, 2006b; Cid del Prado Vera and Subbotin, 2012). In the phylogenetic trees using D2-D3 of the 28S rDNA and ITS1-5.8S-ITS2 rDNA gene sequences, the populations from turfgrasses in the present study clustered in two different moderately supported subclades (IIIA and IIIB), which reflected their geographical locations. Subclade IIIA contained populations mainly from south Florida while subclade IIIB contained populations from Florida and other states. Subclade IIIA showed unique autapomorphies for the group which shared 8 and 29 autapomorphies for D2-D3 and ITS regions, respectively. The number of autapomorphies are in the range reported for *B. longicaudatus* consisting of 10-17 and 26-51

autapomorphies for D2-D3 and ITS regions, respectively (Gozel *et al.*, 2006).

Subclade IIIA contained south Florida populations except one population from northcentral Florida, but not populations from other states. The sod for the northcentral Florida (Marion County) site was received from south Florida (Sarasota County), which may explain the similarities of this population with those from south Florida. Morphological and genetic differences that were related to different geographical localities were reported for *B. longicaudatus* populations (Gozel *et al.* 2006; Cid del Prado Vera and Subbotin, 2012). However, these differences may have been induced by the different hosts of these populations. In our study, turfgrasses were the only host of the populations indicating that the variability among our populations was mainly related to the geographical localities. Our principal components analysis supported that populations were correlated to each other based on the *B. longicaudatus* subclades to which they belong. This suggests populations of same subclades are correlated to their geographical locations.

The three D2-D3 *B. longicaudatus* sequences retrieved from GenBank fit into the respective

subclades corresponding to their localities. AB602605, which was obtained from Hillsborough County in south Florida, clustered together in the south Florida subclade IIIA whereas KF963100 and GQ896548, which were obtained from Levy County in north-central Florida and Delaware, respectively, clustered together in subclade IIIB. However, the three ITS *B. longicaudatus* sequences clustered together in clade I with *B. maluceroi*. The possible explanations for the discordance between D2-D3 and ITS trees could be caused by independent nucleotide substitutions and/or occurrence of polymorphism in the D2-D3 and ITS regions (Gozel *et al.*, 2006; Han *et al.*, 2006b; Kutsuwa *et al.*, 2015). Cid del Prado Vera and Subbotin (2012) and Kutsuwa *et al.* (2015) reported results consistent with those of the present study. In their phylogenetic analysis based on the ITS region, some *B. longicaudatus* populations grouped together with *B. maluceroi* in the same clade. Furthermore, none of the nominal *Belonolaimus* species they studied were monophyletic based on this gene. These findings may indicate that *B. longicaudatus* is a species complex.

The morphometrics of the populations from south Florida in subclade IIIA fit those of the paratypes of this species from central Florida reported by Rau (1958), which have values of the stylet and tail length ratio ranging from 0.68-1.0. Subsequently, Rau (1961) reported stylet and tail length ratio values ranging from 0.67-1.14 for another population collected from the same host corn (*Zea mays*) and type locality (Sanford, FL) and pointed out the presence of many "ecotypes' of this species. Our results are in line with this statement and the results of previous studies reporting intra- and inter-population variations in morphology and morphometric characters among populations from different localities and host plants
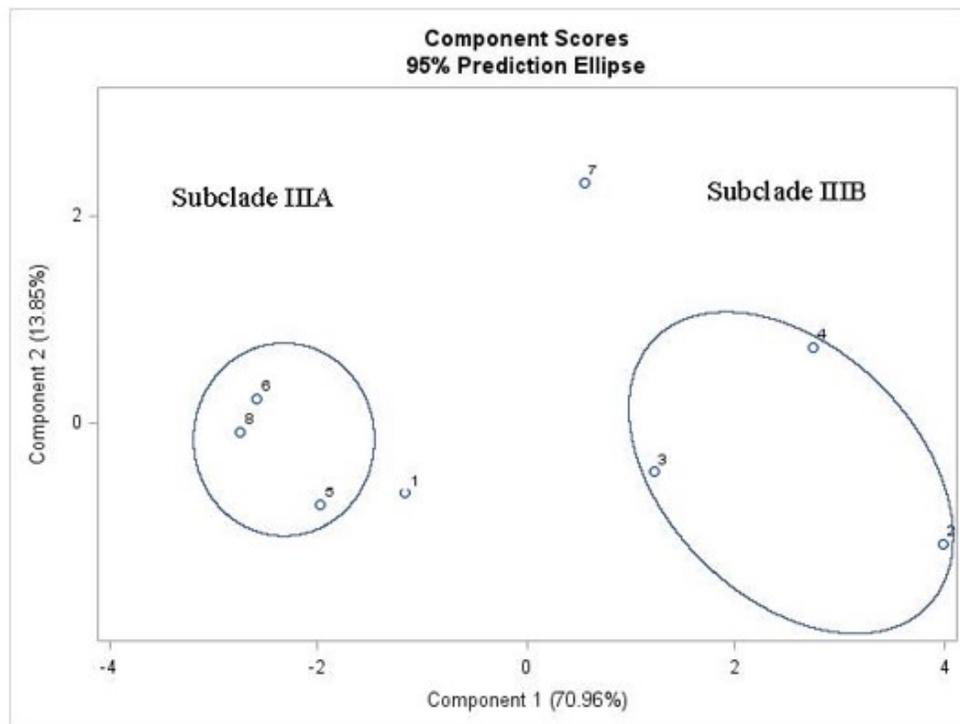
Figure 3. Principal component analysis of the means of seven morphometric characters shown in Table 3 from eight populations of *Belonolaimus longicaudatus*. Populations in the same subclade of *B. longicaudatus* (Figs. 1 and 2) generally grouped together except one population from each subclade. Numbers represent: 1= Bl-Manatee1-FL, 2= Bl-Lake1-FL, 3= Bl-Harris1-TX, 4= Bl-Horry1-SC, 5=Bl-Hillsborough-FL, 6=Bl-Lee1-FL, 7=Bl-Broward2-FL, 8=Bl-Marion-FL.

(Robbins and Hirschmann, 1974; Duncan *et al.*, 1996; Gozel *et al.*, 2006; Han *et al.*, 2006b).

The morphological similarities between the populations from turfgrasses in south Florida and paratypes of *B. longicaudatus* prevent any speculation about the origin of this species in Florida. The *B. longicaudatus* populations with stylet and tail length ratios ≥ 1 may have evolved on slash pine that is native to central and south Florida or turfgrasses such as St. Augustinegrass (*Stenotaphrum secundatum*) that is native to regions bordering the Gulf of Mexico. Our results confirm that *B. longicaudatus* is a species complex that deserves further studies to verify differences in the biology, parasitic habit, and damaging effect on turfgrasses of populations with stylet and tail length ratios of ≤ 1 compared with those with ratios ≥ 1.

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