Journal of Nematology 49(1):103–113. 2017.

Occurrence of *Belonolaimus* in Sinaloa, Northwestern Mexico: A New Report on Distribution and Host Range

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Abstract: The present study reports the occurrence of the genus *Belonolaimus* in the state of Sinaloa, Mexico, associated with native plants (i.e., *Ziziphus amole* and *Stenocereus alamosensis*) in a natural coastal ecosystem. Both morphological and molecular approaches were employed to characterize the Sinaloa population. Notwithstanding of some morphological and morphometric variation between *Belonolaimus* from Sinaloa and other valid species, the characterization indicates that this population might belong to the *Belonolaimus longicaudatus* species complex. Molecular analyses based on the 28S gene and ITS1-5.8S-ITS2 regions of the ribosomal RNA (rRNA) identified four major clades within *Belonolaimus*; however, none of the species including *B. longicaudatus*, *B. gracilis*, and *B. euthychilus* were supported as monophyletic; yet monophyly is argued to be a basic requirement of species status. Sequence divergence among different *Belonolaimus* populations and species varied according to the rRNA dataset (i.e., ITS1-5.8S-ITS2 > 28S > 18S) used, thus showing the importance of using genes with different rates of evolution to estimate species relationships. The fact that *Belonolaimus* has not been found in other cultivated (including on suitable hosts) areas in Sinaloa and that this population is relatively distant from the common *B. longicaudatus* groups (i.e., clades A and B) suggests that its appearance was not due to a recent introduction associated with the local agriculture.

Key words: Belonolaimus, *Belonolaimus longicaudatus*, host–parasite relationships, Mexico, morphology, phylogeny, Sinaloa, sting nematode.

The subfamily Belonolaiminae Whitehead, 1960, is represented by four genera: *Carphodorus* Colbran, 1965, characterized by having two incisures in the lateral field (LF) and a cephalic region divided into six sectors, *Ibipora* Monteiro & Lordello, 1977, characterized by having four incisures in the LF and a cephalic region divided into four sectors, *Morulaimus* Sauer, 1966, also with four incisures in the LF, however not with a well-defined cephalic region, and *Belonolaimus* Steiner, 1949, distinct from the above genera by having a single incisure in the LF (Siddiqi, 2000; Geraert, 2011). Currently, the genus *Belonolaimus* comprises six species including *B. 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 and Subbotin, 2012.

Belonolaimus species are native and widespread throughout the Southeast and Midwest of United States (Gozel et al., 2006). Within the genus, *B. longicaudatus* is the most economically important species causing extensive damage to a large number of agronomic and horticultural plants such as maize, citrus, cotton, peanut, potato, soybean, and strawberry (Jenkins and Taylor, 1967; Duncan et al., 1996; Koenning et al., 1999). In the Southeastern United States, it is currently recognized among the 10 most serious plant pests (Kutsuwa et al., 2015). It is also highly pathogenic on turf grasses, including hybrid Bermuda/couch grasses (crosses between *Cynodon dactylon* (L.) Pers. and *C. transvaalensis* Burtt Davy, creeping bentgrass *Agrostis palustris* Huds.) and other grasses that are grown in home lawns, golf courses, and other recreational areas (Mundo-Ocampo et al., 1994; Bekal and Becker, 2000; Zeng et al., 2012; Crow et al., 2013).

Belonolaimus longicaudatus is, even in low population densities, a highly destructive plant pathogen, reaching and damaging the plant meristematic root tip tissues with its long stylet (Huang and Becker, 1997). All mobile stages of *B. longicaudatus* are strictly ectoparasitic and major effects on plant hosts might include decreasing water and nutrient uptake, stunted growth, premature wilting, leaf chlorosis, and in some cases plant death (Giblin-Davis et al., 1992; Huang and Becker, 1999). Occurrence of *B. longicaudatus* and its damage capacity on plants is particularly high in sandy (i.e., sand content >80%) soils (Robbins and Barker, 1974; Crow and Han, 2005).

The discovery of *Belonolaimus* in Sinaloa, Mexico, generates an interesting query in regard to the origin of this population as well as on the distribution of the genus. Furthermore, the association with native host plants not previously reported launches new insight into the host–parasitic relationships of *Belonolaimus*. In the present study, the *Belonolaimus* population from Sinaloa is characterized using morphological and molecular approaches. In addition, we discuss the implications on the dispersal and the ecological significance of *Belonolaimus* for the Pacific Northwest region of the Sinaloa State and the coastal region of the Gulf of California, Mexico.

Materials and Methods

Sampling: A survey to determine the soil nematode biodiversity in the northern region of Sinaloa State, Mexico, was accomplished during 2011 to 2013. Samples, including natural and disturbed agricultural sites,

Received for publication November 14, 2016.
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This work was supported by UC-MEXUS-CONACyT, Collaborative Research Grants Program, and partially by the National Science Foundation grants DEB 0731516 and DEB 1257331 to S. A. Nadler and JGB. Thanks to CIIDIR-IPN, Unidad Sinaloa. TJP also thanks CAPES Foundation (Ministry of Education, Brazil) for his doctoral fellowship.
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This paper was edited by Zafar A. Handoo.

were collected alongside the Guasave River (a transect of about 80 km), from the coast to the slope of the mountain range of the Sierra of Sinaloa (Fig. 1). Soil samples of about 1 kg were taken from approximately 30 cm depth. Roots of various native and cultivated plants were also collected. Samples were transported to the Nematology Laboratory at the CIIDIR-IPN Sinaloa and nematodes extracted from soil with a modified Baermann funnel method. Additionally, subsamples were processed at the Plant Nutrition Laboratory at the CIIDIR-IPN Sinaloa to determine the soil texture and pH.

Morphological characterization: Nematode specimens were killed by gentle heating (55–60°C) and fixed in 4% formaldehyde. After 48 hr, specimens were processed and mounted in glycerin on glass slides by a modification of Seinhorst's (1959) method. Specimens were measured (×40 to ×1000 magnifications) with an ocular micrometer on a Zeiss Axioskop compound

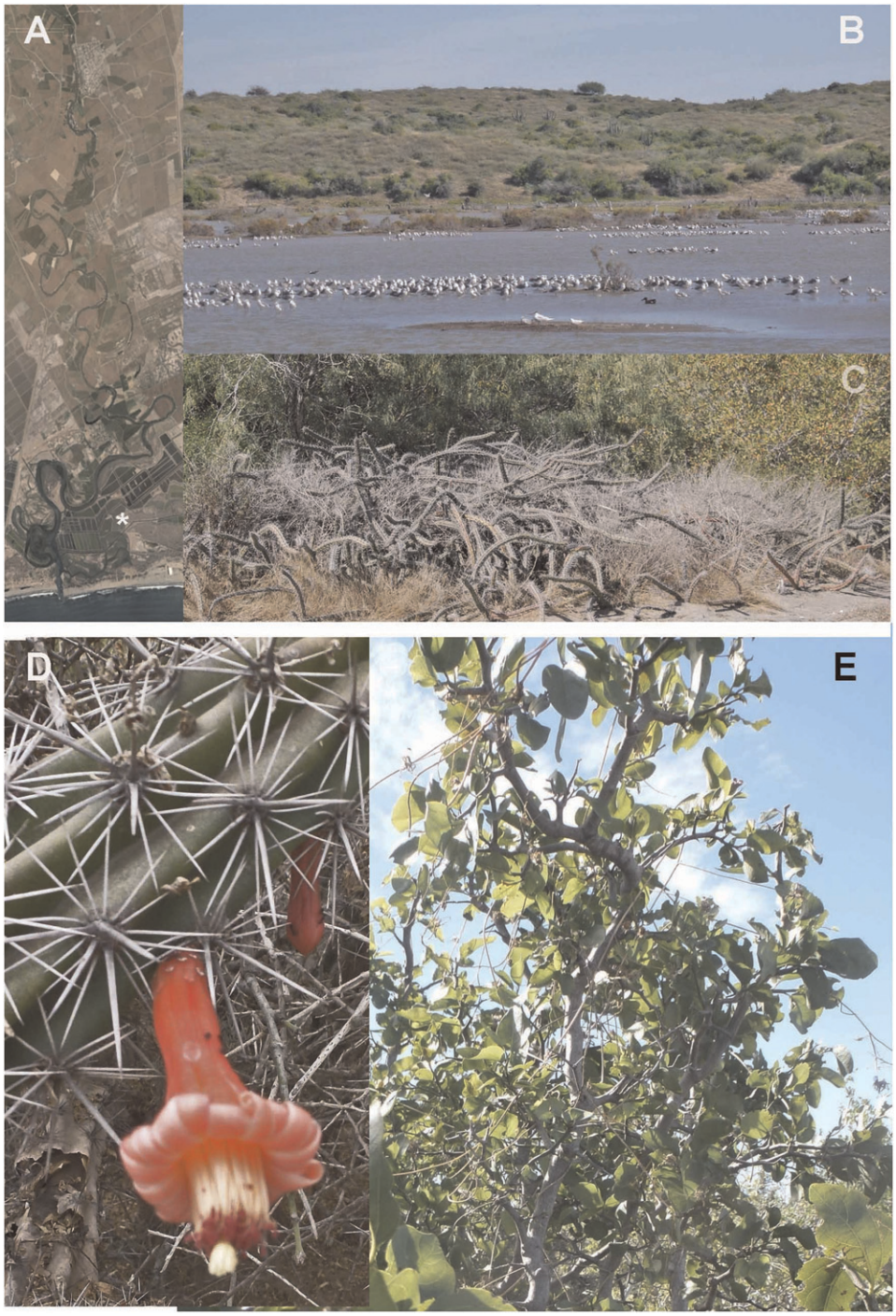

Fig. 1. A. Aerial view of the Sinaloa river, asterisk (*) indicates the collecting site. B. View of a pristine environment showing native host plants. C, D. Close up of cactus "Nacido" (*Stenocereus alamosensis*). E. Close view of "Saituna or Nanchi" (*Ziziphus amole*).

microscope. Morphological diagnostic characters considered to be important (total body length, stylet, stylet cone and stylet shaft lengths, head height, tail length, tail width, anterior end to excretory pore distance, posterior end to phasmid distance, body width, spicule and gubernaculum lengths, ratios a [body length/body width], b [body length/pharynx length], c [body length/tail length], tail/body width, stylet/tail length, and V% [vulva position as percentage of body length]) for the identification of *Belonolaimus* species (Robbins and Hirschmann, 1974; Geraert, 2011; Kutsuwa et al., 2015) were measured from 20 females and 10 males, and they were compared to the original descriptions of *Belonolaimus* species. In addition, observations of qualitative characters such as the cephalic region, tail, spicule, gubernaculum, and bursa shape were recorded. Photomicroscopy of selected specimens, including females and males, was carried out using Openlab (version 5.0) software and a digital camera (RT-Color Spot; Diagnostic Instruments, Inc., Sterling Heights, MI) coupled to a Nikon Eclipse E600 compound microscope. Photographs were saved as tiff files and later developed as plates using Adobe Photoshop CS4 (version 11). Additional specimens were prepared for scanning electron microscopy (SEM) observations following Mundo-Ocampo et al. (2003).

Molecular procedures: Genomic DNA was extracted from single individuals as described in Atighi et al. (2013). PCR amplification included the 18S and 28S genes of the ribosomal RNA (rRNA) as well as the 5.8S rRNA and its flanking regions (i.e., ITS1 and ITS2). PCR reactions of 25 µl were made with 5 µl of DNA template, 0.2 µl of primers (20 µM stock), and 19.6 µl of PCR purified water in combination with PuReTaq Ready-to-Go kit (GE Healthcare). PCR primers for the 18S rRNA gene were G18S4 and 18P (Blaxter et al., 1998), for the 5.8S rRNA gene and its flanking regions were N93 and N94 (Nadler et al., 2000), and for the 28S rRNA gene (specifically the D2-D3 domains) were D2Ab and D3B (De Ley et al., 1999). Amplification success was evaluated electrophoretically on 1% agarose gel. PCR products were purified for sequencing using the QIAquick PCR purification kit (Qiagen Inc., Germantown, MD) following the manufacturer's protocol. Sequencing was performed in both directions with PCR primers using ABI-PRISM Dye- Deoxy-Terminator Big DyeTM v3.1 (Applied Biosystems, Foster City, CA) with an automatic sequencer Gene Analyzer ABI 3100 (Applied Biosystems) at the Genomics Center, University of California, Riverside (UCR). For the 18S rRNA gene, internal primers (4R, 22F, 13R, and 4F) with overlapping regions were also used (Bert et al., 2008).

Phylogenetic analysis: Newly obtained sequences were manually checked, edited, and assembled using CodonCode Aligner v. 4.2.7 (LI-COR, Inc.). In order to evaluate the phylogenetic position of *Belonolaimus* from Sinaloa in relation to other *Belonolaimus* species, DNA sequences from additional taxa were downloaded from GenBank. Outgroup taxa for phylogenetic analyses included sequences from *Ibipora lolii* Monteiro & Lordello, 1977 (i.e., a sister outgroup) and from species of the genus *Tylenchorhynchus* Cobb, 1913 (i.e., a more distant outgroup) in accordance with previous studies (Cid del Prado and Subbotin, 2012; Stirling et al., 2013). Sequences representing the different datasets were separately aligned on Mafft v7.0 using the iterative refinement method G-INS-i (http://mafft.cbrc.jp/alignment/server, Katoh and Standley [2013]). Sequences representing the outgroup taxa were added into the multiple alignment using the option mafft-add. Sequence divergences between major clades were estimated using p-distance and raw distance (bp differences) measures on MEGA 6 (Tamura et al., 2013).

Phylogenetic relationships among sequences (18S, ITS1-5.8S-ITS2, and 28S datasets) were estimated with maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference (BI). MP analyses were performed in PAUP* 4.0b10 using heuristic searches and TBR branch swapping to seek the most parsimonious trees (maximum tree number = 1,000). Gaps in the alignment were treated as missing data. Nonparametric bootstrap analysis (BS), 1,000 pseudoreplicates, was used to assess branch support (Swofford, 2002). ML and BI analyses were performed on the CIPRES Science Gateway (http://www.phylo.org/). The best-fitting substitution model for the different datasets was estimated using jModelTest 2.1.2 (Darriba et al., 2012) based on the Akaike information criterion. ML analyses were performed using RAxML-HPC 8.2.4 under the GTR GAMMA model, and the GTR CAT approximation was used for ML bootstrapping. Gamma parameters were estimated from log-likelihood units and bootstrap support (1,000 pseudoreplicates) was automatically calculated for the best-scoring ML tree (Stamatakis, 2014, 2006). BI analyses were performed on MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) under the GTR + I + G model, but the model-based estimates of gamma shape and proportion of invariable sites were not fixed. The settings for BI analyses were as it follows: random starting tree, two independent runs with four chains (1.0×10^6 generations). Markov chains were sampled at intervals of 1,000 generations. After assessing chain convergence using the standard deviation of split frequencies (less than 0.01) and Potential Scale Reduction Factors, close to 1.0, burn-in phase was set at 25% of the results. A 50% majority rule consensus tree was generated and posterior probabilities were calculated for each clade.

Results

Habitat characterization: Belonolaimus was recovered from soil samples associated with native plants locally

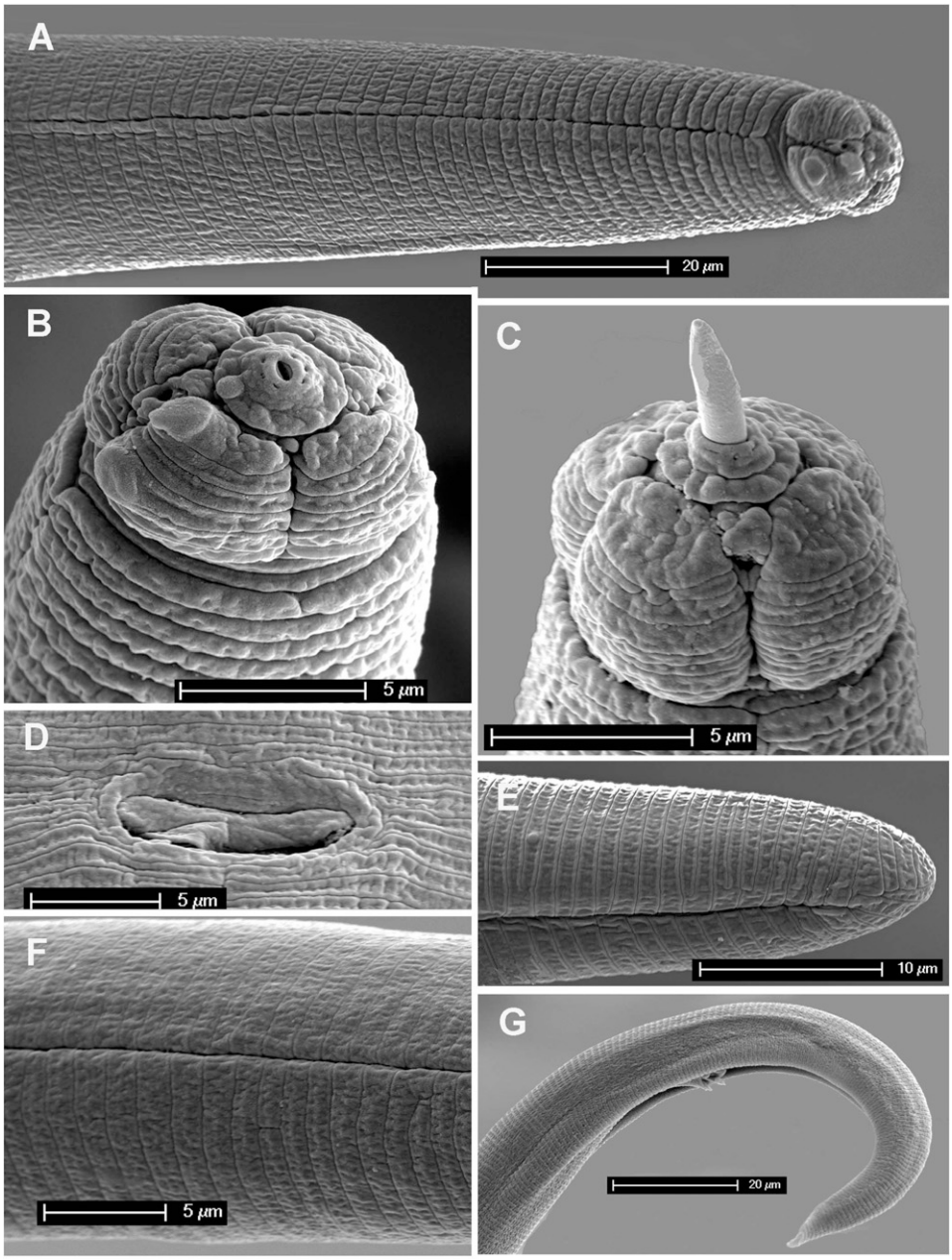

Fig. 2. Scanning electron micrographs. A. Anterior-lateral view of female. B. Anterior view of female. C. Anterior view of male. D–F. Vulva, tail, and lateral field of female, respectively. G. Posterior region of male showing spicules and bursa.

known as "Saituna" or "Nanchi" (*Ziziphus amole* (Sessé & Moc.) M.C. Johnst.) and the cactus "Nacido" (*Stenocereus alamosensis* (Coulter) Gibson & Horak) at a site near the Gulf of California (N 25° 17′11. 94″ and W 108° 28′ 08.78″, Fig. 1). These plants are found in a coastal plain habitat near the ocean, in sandy soil. The coastal plain is a narrow strip of land that extends the length of Sinaloa State and the Gulf of California, with a warm subtropical climate. Temperatures on coastal plains range from 22°C to 43°C, with rain and storms during the summer months and dry conditions throughout most of the year. Soil property analyses characterized the site as sandy (90.0% sand, 6.0% silt, and 4.0% clay), with a pH of 6.2 and a salinity of 1,600 ppm. Preliminary field observations of population densities of *Belonolaimus* suggest that highest abundance of females and juveniles occur around the end of September. During the months of October and November, the number of males increases in the population. During late November to mid-December, the population density drops below detection level only to reappear during May of the next year. These observations, however, will be further investigated and tested. Pathogenicity tests under greenhouse conditions using tomato (*Solanum lycopersicum* L.) and St. Augustine grass (*Stenotaphrum secundatum* (Walt.) Kuntze) were attempted but unsuccessful.

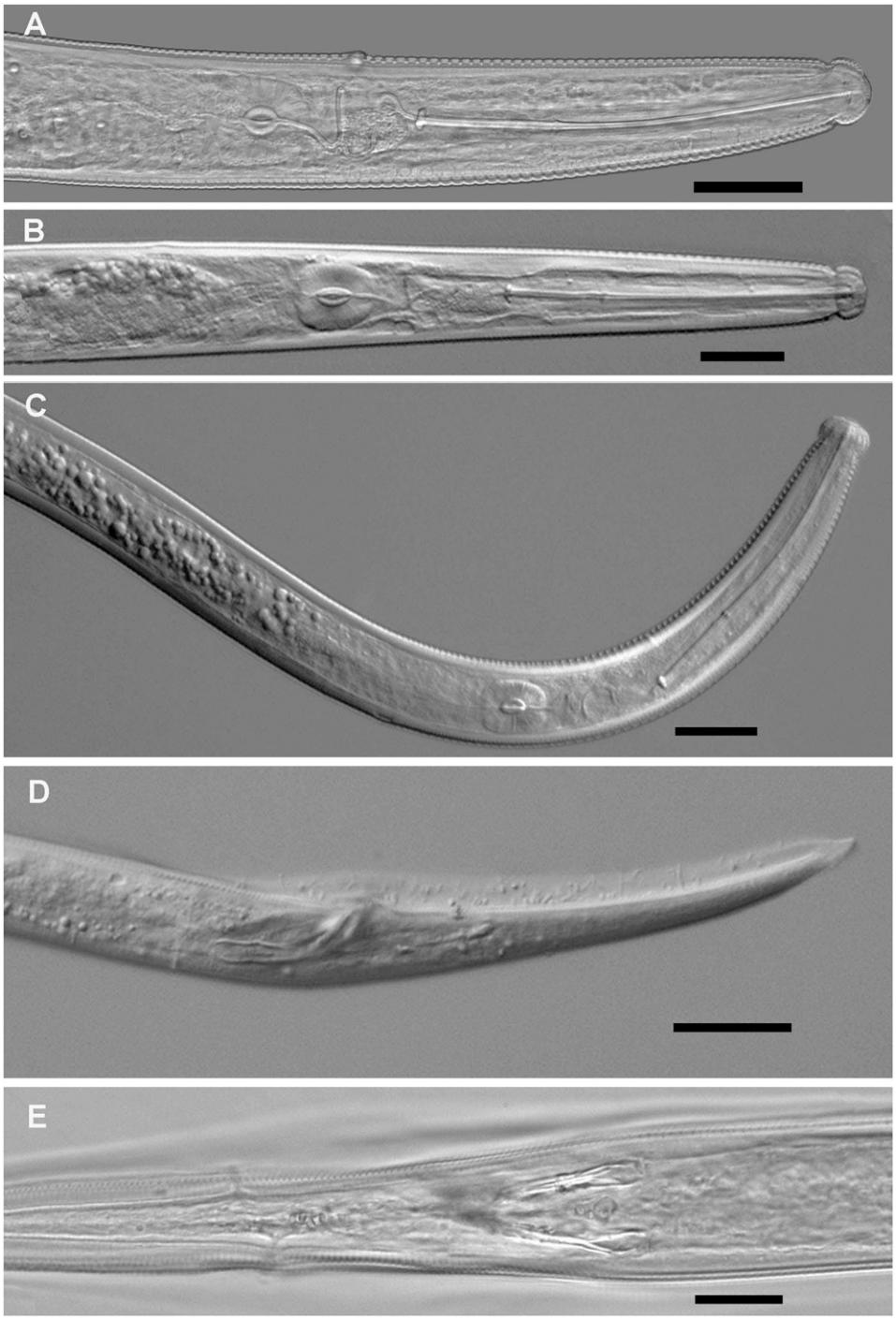

FIG. 3. Light microscopy photographs. A, B. Lateral view of female. C. Anterior view of male. D, E. Lateral and ventral views, respectively, of the posterior region of male. (Bars = 25 μm).

The identity of Belonolaimus from Sinaloa: Selected morphological differences could be used to separate the Sinaloa population from other populations and extant species of *Belonolaimus*. For example, the population from Sinaloa differs from *B. gracilis* by the absence of sclerotized plates in the vagina, a spherical metacorpus, and a hemispherical convex conoid tail shape. In addition, *B. gracilis* presents sexual dimorphism, including degenerate stylet and pharynx in males and by having a constriction between the cephalic region and the rest of the body.

The morphology of *Belonolaimus* from the Sinaloa resembles, in several respects, that of *B. longicaudatus* (*sensu lato*). For example, the en face views from SEM (Fig. 2) show two large subventral, two large subdorsal, and two small lateral lobes. The amphids are located on the outer margins of those lateral lobes (Fig. 2B,C). Both females and males exhibit a constriction between

the cephalic region and the rest of the body (Fig. 3A–C). However, a slight variation in the morphometrics including body and stylet lengths (i.e., shorter in males) was observed in the *Belonolaimus* from Sinaloa (Table 1). In addition, morphological variations of the cephalic region and with respect to differences of the labial disc between males and females of this population are noticeable (Fig. 3). The bursa in males envelops the entire tail (peloderan type; Figs. 2G; 3D) as reported for other *B. longicaudatus* populations. The Sinaloa population differs from the type descriptions of *B. longicaudatus* by having a smaller body length, a shorter stylet, and larger stylet/tail ratio. With respect to *B. maluceroi*, the Sinaloa population differs in tail length (89.0 μm vs. 112.3 μm) and in the stylet/tail ratio (1.2 vs. 0.92) of females. In spite of these morphological differences, at this point, the specific identity of the Sinaloa population remains unresolved, and until new information is obtained, we consider this population as a morphotype belonging to the *B. longicaudatus* species complex (see phylogenetic results).

Molecular characterization of Belonolaimus from Sinaloa: Sequences produced in the present study have been deposited on GenBank (18S: KY272116-KY272117, 28S: KY272118-KY272120, and ITS1-5.8S-ITS2: KY272121-KY272123). Phylogenetic relationships between *Belonolaimus* from Sinaloa and other *Belonolaimus* populations and species were inferred based on three datasets (Figs. 4–6). Overall, the tree topologies based on the 28S and ITS1-5.8S-ITS2 datasets, which included the larger number of sequences, were highly congruent with respect to the formation of clades. In both phylogenies, two (clades A and B) major clades containing only sequences of *B. longicaudatus* are identified (Figs. 4,5). However, variation within and between these two clades is low as depicted by the short branches in the 28S and ITS1-5.8S-ITS2 phylogenetic trees (Figs. 4,5; Table 2). In the 28S phylogeny, clades A and B are strongly supported as sister groups by all phylogenetic methods (BI = 1.0, ML = 98, MP = 99, Fig. 4). On the other hand, in the ITS1-5.8S-ITS2 phylogeny, the sister relationship of clades A and B is only recovered by ML (89% bootstrap support, data not shown), while unresolved with BI and MP methods (Fig. 5). Overall, the resolution and support for all four clades (i.e., A–D) was consistent between 28S and ITS1-5.8S-ITS2 datasets as well as among inference methods (Figs. 4,5).

In addition to clades A and B, the phylogenetic analyses based on the abovementioned datasets revealed two slightly more divergent clades: clade C contains sequences from different *Belonolaimus* species including *B. euthychilus*, *B. gracilis*, and *B. longicaudatus*, which are not reciprocally monophyletic in both 28S and ITS1-5.8S-ITS2 phylogenies (Figs. 4,5). Yet, clade D in the 28S phylogeny (Fig. 4), which is weakly supported by BI = 0.7 and ML = 51, harbors sequences of *B. maluceroi* from Mexico, *B. longicaudatus* (Oklahoma), and *Belonolaimus* from Sinaloa. A similar result is observed in the ITS1-5.8S-ITS2 phylogeny (Fig. 5) where sequences representing different populations (South Carolina, Nebraska, and Texas) of *B. longicaudatus*, *B. maluceroi*, and *Belonolaimus* from Sinaloa also formed a clade (only well supported with BI = 1.0). The sister taxon of *Belonolaimus* from Sinaloa slightly differs depending on the dataset used. For example, in the 28S phylogeny (Fig. 4), all methods recovered *Belonolaimus* from Sinaloa as sister to *B. longicaudatus* + *B. maluceroi*, whereas the ITS1-5.8S-ITS2 dataset (Fig. 5) strongly supports *Belonolaimus* from Sinaloa as sister to all other species in clade D (although weakly supported by ML and MP, Fig. 5).

Due to a limited number of 18S sequences representing the genus *Belonolaimus* in GenBank, molecular phylogenetic analyses were restricted to a few populations of *B. longicaudatus*, *Belonolaimus* from Sinaloa, and outgroup taxa. In the 18S phylogeny, *Belonolaimus* from Sinaloa was sister to a clade containing *B. longicaudatus* sequences from different populations sampled in the United States and this relationship is strongly supported by all three inference methods (Fig. 6).

Sequence divergence between clades A and B (i.e., 28S and ITS1-5.8S-ITS2 phylogenies) calculated as p-distance and bp difference showed little variation within and between these two clades (Table 2). For example, sequence divergence within clades A or B was no greater than 0.5% or 1.5%, for the 28S and ITS1-5.8S-ITS2 datasets, respectively. Yet, the variation between clades A and B was 1.6% and 6.1% for the 28S and ITS1-5.8S-ITS2 datasets, respectively. On the other hand, sequence divergence within clades C and D are relatively higher (up

TABLE 1. Measurements in micrometers of the *Belonolaimus* population from Sinaloa, Mexico.

Characters	Mean	Minimum	Maximum	SD
L	1,917	1,780	2,025	98.51
a	70.48	61.79	79.32	5.65
b	8.32	7.73	8.80	0.42
c	17.07	15.47	21.32	1.77
V (%)	51.88	47.80	55.80	2.61
Stylet	103.8	98.6	110	3.99
Stylet cone	73.5	67.6	82.5	4.37
Stylet shaft	30.37	28.1	32.5	2.03
Stylet knobs width	5.35	4.90	6.00	0.51
Anterior end to excretory pore	186.1	122.0	231.3	30.73
Excretory glands length	230.40	220.00	240.00	7.36
Anterior end to hemizonid	189.6	175.0	225.0	21.27
Anterior end to vulva	965.0	890.0	1,020.0	55.0
Vulva to posterior end	962.0	890.0	1,025.0	59.85
Tail length	112.3	95	125.3	10.14
Tail/body width	4.14	3.78	4.32	8.22
Anal body width	27.13	25.1	29.0	1.23
Ovary length	277.4	274.0	281.0	3.20
Posterior end to phasmids	38.03	32.5	45.0	5.75
Stylet/tail ratio	.920	0.79	1.15	.095
Spicules	40.05	39.0	42.0	1.68
Gubernaculum	17	16.5	17.5	0.42

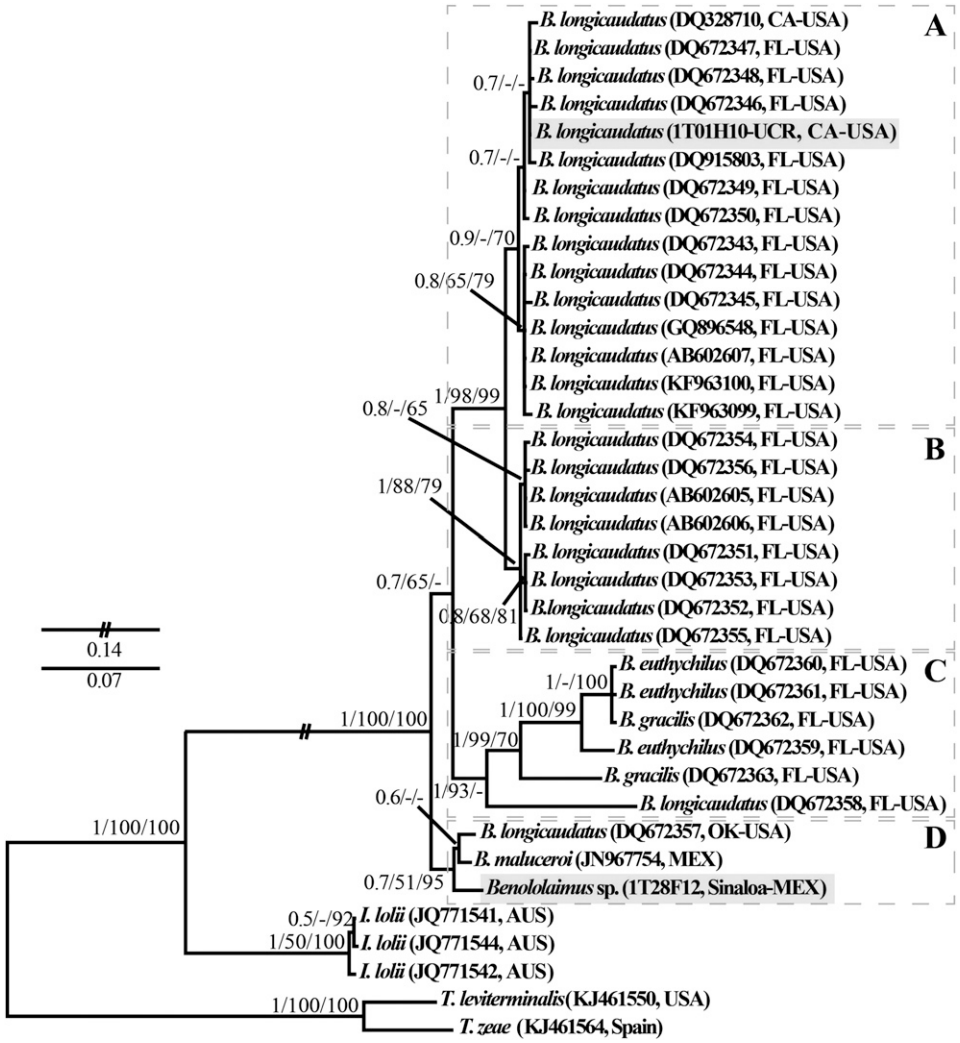

FIG. 4. Molecular phylogeny of *Belonolaimus* species based on the 28S rRNA gene. The 50% majority rule consensus tree from the Bayesian analysis is presented. Four clades (A-D) are identified among *Belonolaimus* sequences. Branch support (only above 50%) is shown on branches as Bayesian inference (BI)/maximum likelihood (ML)/maximum parsimony (MP). A dash (-) indicates branch support below 50% or incongruence between BI and ML/MP analyses. Tree is rooted to *Tylenchorhynchus* species. Sequences produced in this study are highlighted in gray.

5.1% and 8.8% for the 28S and ITS1-5.8S-ITS2 datasets, respectively). Moreover, values of sequence divergence for clades C and D with respect to clades A and B were higher as well, particularly so between clades A and C or B and C for the ITS1-5.8S-ITS2 dataset, which for instance approached values of sequence divergence compared to those shown between the same clades (i.e., A or B) and the sister outgroup taxon (i.e., with respect to *I. lolii*, Table 2). For the 18S rRNA gene, sequence variation within *B. longicaudatus* clade was only 0.5% (8 bp difference) and between the former clade and *Belonolaimus* from Sinaloa was 0.9% (15 bp difference, data not shown).

DISCUSSION

Based on the morphological characterization, the Sinaloa population fits the diagnostic characters for *Belonolaimus* (*sensu lato*), and there is a significant resemblance of this population with *B. longicaudatus*. In spite of this, morphometric differences, host preference, and geographical region are indications of a unique origin for this population, which makes difficult unequivocally establishing its specific identity. Most importantly, and basic to species status, phylogenetic analyses suggest that the population cannot be established as a unique linage within *Belonolaimus* and particularly relative to *B. longicaudatus*, a putative species that has been shown to not be monophyletic; yet monophyly is argued to be a basic requirement of species status (Adams, 1998; Nadler, 2002).

Morphological analyses of *B. longicaudatus* isolates from different locations of the eastern and southern regions of the United States indicate a high range of variation. For example, characters such as body length, head shape, number of annuli in the lip region,

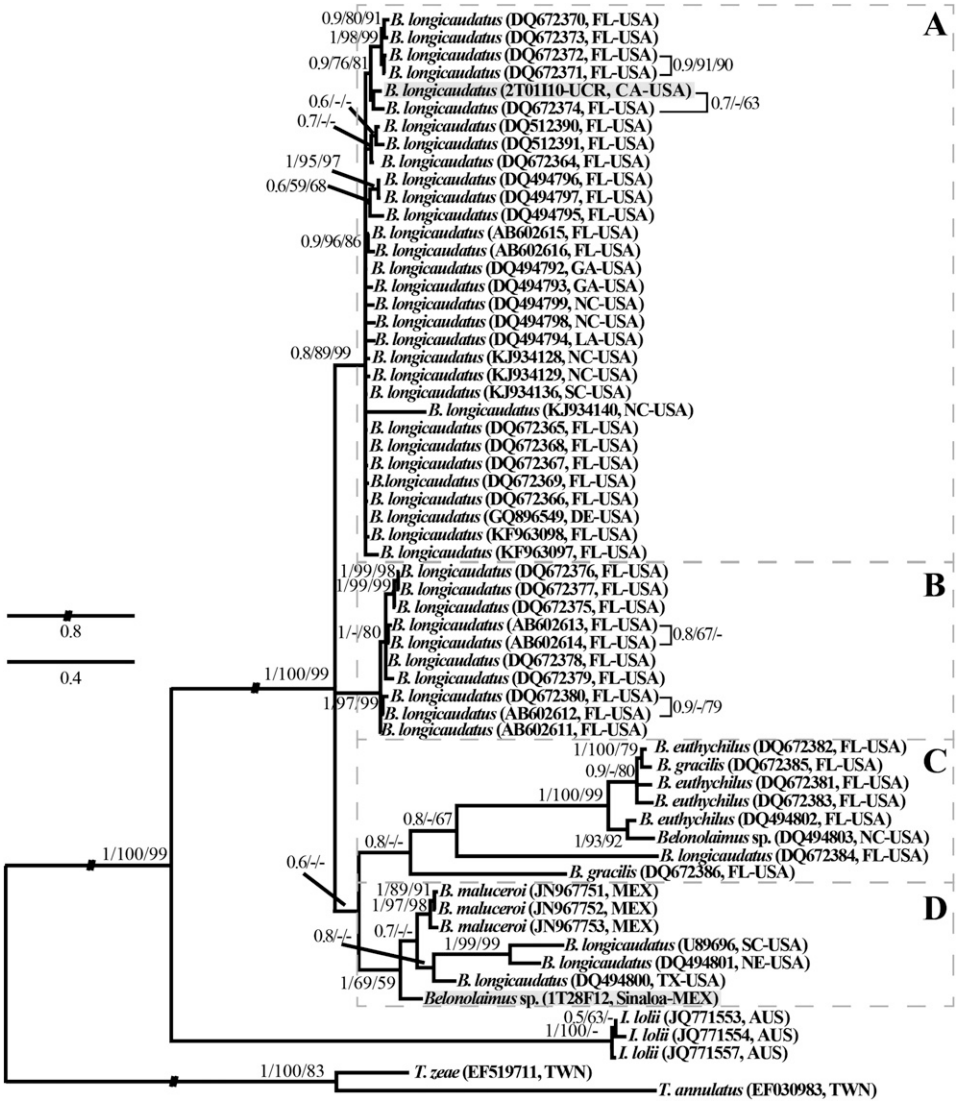

FIG. 5. Molecular phylogeny of *Belonolaimus* species based on the IT1-5.8S-ITS2 region. The 50% majority rule consensus tree from the Bayesian analysis is presented. Four clades (A-D) are identified among *Belonolaimus* sequences. Branch support (only above 50%) is shown on branches as Bayesian inference (BI)/maximum likelihood (ML)/maximum parsimony (MP). A dash (-) indicates branch support below 50% or incongruence between BI and ML/MP analyses. Tree is rooted to *Tylenchorhynchus* species. Sequences produced in this study are highlighted in gray.

variation with respect to lip constriction, stylet length, among others vary considerably and might not be suitable to differentiate populations or even species (Miller, 1962; Abu-Gharbieh and Perry, 1970, Rau and Fassuliotis, 1970; Robbins and Hirschmann, 1974). In addition, morphological variation has been related to reproductive incompatibility, host range, pathogenicity, environmental conditions (i.e., nutrient availability), and age structure of the population, suggesting that *B. longicaudatus* is highly polymorphic and its taxonomic status should be further evaluated (Robbins and Hirschmann, 1974; Duncan et al., 1998).

Analyses of morphological variability, geographical distribution, and presence of pathotypes or physiological races in conjunction with molecular phylogenies of *Belonolaimus* species have been controversial. For example, Han et al. (2006) compared populations of *B. longicaudatus* from different plant hosts and regions of the southern United States and reported major morphological differences among populations; however, these were not supported by the ITS-1 rRNA phylogeny. Similarly, Gozel et al. (2006) observed morphological differences when comparing multiple populations of *B. longicaudatus*, *B. gracilis*, and *B. euthychilus* collected throughout Florida. However, their molecular phylogenetic analyses based on the D2-D3 expansion segments of the 28S gene and the ITS1-5.8S-ITS2 regions showed that all three *Belonolaimus* species are not reciprocally monophyletic.

Molecular phylogenetic analyses performed in the present study show that *Belonolaimus* from Sinaloa is relatively distant from populations (including

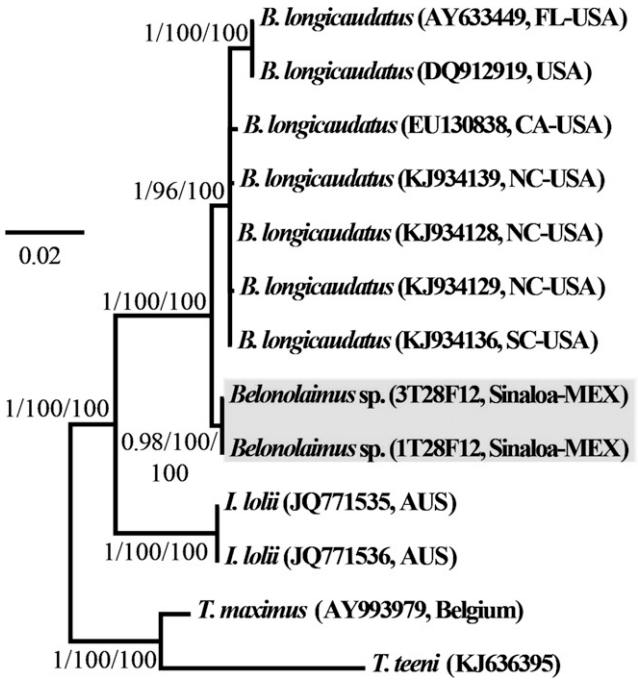

FIG. 6. Molecular phylogeny of *Belonolaimus* species based on the 18S rRNA gene. The 50% majority rule consensus tree from the Bayesian analysis is presented. Branch support (only above 50%) is shown on branches as Bayesian inference (BI)/maximum likelihood (ML)/maximum parsimony (MP). A dash (-) indicates branch support below 50% or incongruence between BI and ML/MP analyses. Tree is rooted to *Tylenchorhynchus* species. Sequences produced in this study are highlighted in gray.

populations from the type locality) representing the typical *B. longicaudatus* (clades A and B). Accordingly, four major clades of *Belonolaimus*, an outcome consistent between the 28S and ITS1-5.8S-ITS2 phylogenies, are identified (Figs. 4,5). These findings are also in agreement with previous studies focusing on the relationships among *Belonolaimus* species (Gozel et al., 2006; Cid Del Prado and Subbotin, 2012; Kutsuwa et al., 2015).

For example, Cid Del Prado and Subbotin (2012) also identified four major clades within *Belonolaimus*. Based on some morphological differences as well as sequence divergence from the closest species (2% and 1.4% for ITS-1 region and 28S gene, respectively), the authors defined *B. maluceroi* as a new species, however, without commenting on the lack of monophyly of *B. longicaudatus*, which is polyphyletic in their analyses (and also in the present study). Sequence divergence between *Belonolaimus* from Sinaloa and other representatives from clade D ranged from 1.4% to 2.2% for the 28S gene and 0.4% to 1.9% for ITS1-5.8S-ITS2 region, that is, in the same range as those used by Cid Del Prado and Subbotin (2012). However, the more conserved 18S rRNA gene showed that variation between *Belonolaimus* from Sinaloa and the main clade of *B. longicaudatus* (Fig. 6) was only about 1%. In this sense, the use of a more conserved region such as the 18S gene shows that the Sinaloa population might indeed be part of the *B. longicaudatus* species complex as previously stated.

The discovery of *Belonolaimus* in association with endemic plants in a natural ecosystem in the Pacific Southwest region of North America provides new insight on the biology and distribution of the genus. Moreover, its association to hosts never previously reported suggests a unique origin event for this population, instead of an introduction from other sites, where it has been reported. In fact, extensive surveys conducted in the surrounding agricultural areas support this hypothesis since *Belonolaimus* has not been found in any locally cultivated crops (Mundo-Ocampo et al., unpubl. data), including common hosts for *B. longicaudatus* (e.g., corn, tomato).

Existing reports on distribution of *Belonolaimus* species include the coastal plains of the Southeastern United States, along the Atlantic Coast from New Jersey to the Gulf of Mexico (Gozel et al., 2006; Cid Del Prado

TABLE 2. Sequence divergence (p-distance % and bp difference) between and within clades recovered in the 28S and ITS (ITS1-5.8S-ITS2) phylogenies.

	Divergence between and within clades					
	Clade A	Clade B	Clade C	Clade D	*Ibipora*	*Tylenchorhynchus*
Clade A	1.5% (8 bp) 0.5% (4 bp)	6.1% (32 bp)	14.2% (64 bp)	8.6% (43 bp)	19.6% (34 bp)	32.2% (156 bp)
Clade B	1.6% (11 bp)	0.7% (4 bp) 0.3% (2 bp)	13.7% (62 bp)	8.5% (43 bp)	18.2% (32 bp)	33.1% (161 bp)
Clade C	7.5% (53 bp)	7.4% (54 bp)	8.8% (38 bp) 5.1% (37 bp)	14.2% (63 bp)	20.9% (37 bp)	32.5% (141 bp)
Clade D	5.0% (35 bp)	4.9% (35 bp)	6.9% (49 bp)	4.5% (22 bp) 1.8% (13 bp)	19.1% (33 bp)	33.8% (158 bp)
Ibipora	17.2% (118 bp)	17% (119 bp)	18.5% (129 bp)	16.7% (116 bp)	1.1% (2 bp) 0.3% (2 bp)	25.4% (45 bp)
Tylenchorhynchus	20.5% (140 bp)	20.4% (141 bp)	21.1% (146 bp)	19.8% (137 bp)	15.5% (107 bp)	18.6% (98 bp) 6.3% (44 bp)

Below and above the diagonal are the values (between clade comparisons) calculated from the 28S and ITS1-5.8S-ITS2ITS datasets, respectively. Sequence divergence within clades is given off diagonal (highlighted in gray, first line: ITS1-5.8S-ITS2ITS; second line: 28S).

and Subbotin, 2012). *Belonolaimus longicaudatus* has also been reported from the Coachella Valley in California, possibly due to an introduction of infested turf materials imported from Florida (Mundo-Ocampo et al., 1994). This population from California has been cultured in greenhouse conditions at UCR and is included in the 28S and ITS1-5.8S-ITS2 phylogenies. In both analyses, *B. longicaudatus* (UCR) falls in the clade A (i.e., only *B. longicaudatus* sequences), evidence that *Belonolaimus* from Sinaloa could not be an introduction from this site. *Belonolaimus* was also reported from the Pacific coast of Costa Rica associated with citrus; however, no details on the morphometrics and species identity were given (López-Chavez, 1978).

Soil physical characteristics, particularly soil texture, play an important role in the geographical distribution and population structure of *Belonolaimus*. For example, rates of reproduction for *B. longicaudatus* are usually reduced in fine soils (i.e., clay content >10%, Crow and Han, 2005). Conversely, *Belonolaimus* from Sinaloa was found in sandy soil and its population structure at that location seems to be mostly affected by seasonal temperature changes (Mundo-Ocampo et al., unpubl. data).

Further evaluation on the taxonomic status of *Belonolaimus* species, especially *B. longicaudatus*, is much needed. In that sense, the use of faster evolving genes of the mitochondrial genome (e.g., cytochrome oxidase *c* subunit) I might be more suitable for resolving recent speciation events as well as population structure when compared to rRNA genes (Blouin, 2002). Certainly, this will provide robust information to understand the complexity of this economic important genus/species. At the same time, regardless of the taxonomic status, additional studies are necessary to fully understand the biology and host specificity of the *Belonolaimus* from Sinaloa, so that its potential economic impact in the regional agriculture can be defined.

Literature Cited

Abu-Gharbieh, W. I., and Perry, V. G. 1970. Host differences among Florida populations of *Belonolaimus longicaudatus* Rau. Journal of Nematology 2:209–216.

Adams, B. J. 1998. Species concepts and the evolutionary paradigm in modem nematology. Journal of Nematology 30:1–21.

Adams, B. J., Burnell, A. M., and Powers, T. O. 1998. A phylogenetic analysis of *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. Journal of Nematology 30:22–39.

Atighi, M. R., Pourajam, E., Pereira, T. J., Okhovvat, S. M., Alizada, B. A., Mundo-Ocampo, M., and Baldwin, J. G. 2013. Redescription of *Filenchus annulatus* (Siddiqui & Khan, 1983) Siddiqi, 1986 based on specimens from Iran with contributions to the molecular phylogeny of the Tylenchidae. Nematology 15:129–141.

Bekal, S., and Becker, J. O. 2000. Population dynamics of the sting nematode in California turfgrass. Plant Disease 84:1081–1084.

Bert, W., Leliaert, F., Vierstraete, A. R., Vanfleteren, J. R., and Borgonie, G. 2008. Molecular phylogeny of the Tylenchina and evolution of the female gonoduct (Nematoda: Rhabditida). Molecular Phylogenetics and Evolution 48:728–744.

Blaxter, M. L., De Ley, P., Garey, J. R., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R., Mackey, L. Y., Dorris, M., Frisse, L. M., Vida, J. T., and Thomas, W. K. 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392:71–75.

Blouin, M. S. 2002. Molecular prospecting for cryptic species of nematodes: Mitochondrial DNA versus internal transcribed spacer. International Journal for Parasitology 32:527–531.

Cherry, T., Szalanski, A. L., Todd, T. C., and Powers, T. O. 1997. The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonolaiminae). Journal of Nematology 29:23–29.

Cid Del Prado Vera, I., and Subbotin, S. A. 2012. *Belonolaimus maluceroi* sp. n. (Tylenchida: Belonolaimidae) from a tropical forest in Mexico and key to the species of *Belonolaimus*. Nematropica 42:201–210.

Crow, W. T., Luc, J. E., Sekora, N. S., and Pang, W. 2013. Interaction between *Belonolaimus longicaudatus* and *Helicotylenchus pseudorobustus* on bermudagrass and seashore paspalum hosts. Journal of Nematology 45:17–20.

De Ley, P., Felix, M. A., Frisse, L. M., Nadler, S. A., Sternberg, P. W., and Thomas, W. K. 1999. Molecular and morphological characterisation of two reproductively isolated species with mirror-image anatomy (Nematoda: Cephalobidae). Nematology 1:591–612.

Duncan, L. W., Noling, J. W., Inserra, R. N., and Dunn, D. 1996. Spatial patterns of *Belonolaimus* spp. among and within citrus orchards on Florida's central ridge. Journal of Nematology 28:352–359.

Ferris, V. R., Miller, L. I., Faghihi, J., and Ferris, J. M. 1995. Ribosomal DNA comparison of *Globodera* from two continents. Journal of Nematology 27:273–283.

Geraert, E. 2008. The Tylenchidae of the world: Identification of the family Tylenchidae (Nematoda). Ghent, Belgium: Academia Press.

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

Han, H. R., Jeyaprakash, A., Weingartner, D. P., and Dickson, D. W. 2006. Morphological and molecular biological characterization of *Belonolaimus longicaudatus*. Nematropica 36:37–52.

Huang, X., and Becker, J. O. 1997. In vitro culture and feeding behavior of *Belonolaimus longicaudatus* on excised corn (*Zea mays* L.) roots. Journal of Nematology 29:411–415.

Huang, X., and Becker, J. O. 1999. Life cycle and mating behavior of *Belonolaimus longicaudatus* in gnotobiotic culture. Journal of Nematology 31:70–74.

Giblin-Davis, R. M., Cisar, J. L., Bilz, F. G., and Williams, K. E. 1992. Host status of different bermudagrasses (*Cynodon* spp.) for the sting nematode, *Belonolaimus longicaudatus*. Journal of Nematology 24:749–756.

Hogan, C. M. 2009. Elephant tree: *Bursera microphylla*, GlobalTwitcher.com, ed. N. Stromberg.

Huelsenbeck, J. P., and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755.

Katoh, K., and Standley, D. M. 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Molecular Biology and Evolution 30:772–780.

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O., and Fortnum, B. A. 1999. Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. Journal of Nematology 31:587–618.

Kutsuwa, K., Dickson, D. W., Brito, J. A., Jeyaprakash, A., and Drew, A. 2015. *Belonolaimus longicaudatus*: An emerging pathogen of peanut in Florida. Journal of Nematology 47:87–96.

López-Chaves, R. 1978. *Belonolaimus*, un nuevo integrante de la nematofauna de Costa Rica. Agronomía Costarricense 2:83–85.

Mundo-Ocampo, M., Becker, J. O., and Baldwin, J. G. 1994. Occurrence of *Belonolaimus longicaudatus* on bermudagrass in the Coachella Valley. Plant Disease 78:529.

Mundo-Ocampo, M., Baldwin, J. G., Dorado-Ramirez, O., and Morales-Ruiz, M. D. C. 2003. *Acrobeles zapatai* n. sp. (Rhabditida: Cephalobidae) from the Biosphere Reserve "Sierra de Huautla" (Mexico) with a discussion of the taxonomic limits of the genus. Journal of Nematode Morphology and Systematics 5:21–32.

Nadler, S. A., Hoberg, E. P., Hudspeth, D. S. S., and Rickard, L. G. 2000. Relationships of *Nematodirus* species and *Nematodirus battus* isolates (Nematoda: Trichostrongyloidea) based on nuclear ribosomal DNA sequences. Journal of Parasitology 86:588–601.

Nadler, S. A. 2002. Species delimitation and nematode biodiversity: Phylogenies rule. Nematology 4:615–625.

Perry, V. G., and Norden, A. J. 1963. Some effects of cropping sequence on populations of certain plant nematodes. Proceedings of the Soil Science Society of Florida 23:116–120.

Rau, G. J. 1963. Three new species of *Belonolaimus* (Nematoda: Tylenchida) with additional data on *Belonolaimus longicaudatus* and *B. gracilis*. Proceedings of the Helminthological Society of Washington 30:119–128.

Rau, G. J., and Fassuliotis, G. 1970. Equal-frequency tolerance ellipses for population studies of *Belonolaimus longicaudatus*. Journal of Nematology 2:84–92.

Robbins, R. T., and Barker, K. R. 1974. The effect of soil type, particle size, temperature, and moisture on reproduction of *Belonolaimus longicaudatus*. Journal of Nematology 6:1–6.

Robbins, R. T., and Hirschmann, H. 1974. Variation among populations of *Belonolaimus longicaudatus*. Journal of Nematology 6:87–94.

Seinhorst, J. W. 1959. A rapid method for the transfer of nematodes from fixative to anhydrous glycerin. Nematologica 4:67–69.

Siddiqi, M. R. 2000. Tylenchida: Parasites of plants and insects. 2nd ed. Wallingford, UK: CABI Publishing.

Smart, G. C. Jr., and Nguyen, K. B. 1990. Sting and awl nematodes: *Belonolaimus* spp. and *Dolichodorus* spp. Pp. 627–667 *in* W. R. Nickle, ed. Manual of agricultural nematology. New York: Marcel Dekker.

Stamatakis, A. 2006. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313.

Stirling, G. R., Stirling, A. M., Giblin-Davis, R. M., Ye, W., Porazinska, D. L., Nobbs, J. M., and Johnston, K. J. 2013. Distribution of southern sting nematode, *Ibipora lolii* (Nematoda: Belonolaimidae), on turfgrass in Australia and its taxonomic relationship to other belonolaimids. Nematology 15:401–415.

Swofford, D. L. 2002. PAUP*. Phylogenetic analysis using parsimony (*and Other Methods). Version 4. Sunderland: Sinauer Associates.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30:2725–2729.

Zeng, Y., Ye, W., Martin, S. B., Martin, M., and Tredway, L. 2012. Diversity and occurrence of plant-parasitic nematodes associated with golf course turfgrasses in North and South Carolina, USA. Journal of Nematology 44:337–347.

Zijlstra, C., Uenk, B. J., and Van Silfhout, C. H. 1997. A reliable, precise method to differentiate species of root-knot nematodes in mixtures on the basis of ITS-RFLPs. Fundamental and Applied Nematology 20:59–63.