BELONOLAIMUS MALUCEROI SP. N. (TYLENCHIDA: BELONOLAIMIDAE) FROM A TROPICAL FOREST IN MEXICO AND KEY TO THE SPECIES OF *BELONOLAIMUS*

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ABSTRACT

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

A new species of the genus *Belonolaimus* was collected in a tropical forest at La Mancha, Experimental Ecological Station of the Ecology Institute, Veracruz State, Mexico. *Belonolaimus maluceroi* sp. n. females are characterized by body length less of than 2 mm; head set off and divided by longitudinal grooves into four separated sectors; stylet length less than 103 µm; excretory pore less than 195 µm from the anterior end; vulval lips not protruding; vagina without sclerotized pieces; tail hemispherical and 50-96 µm in length. Male head similar to female; spicules slightly curved, less than 34 µm long; gubernaculum 11-15 µm. Lateral field with one lateral line, which starts after the cephalic constriction and can reach tenth or 24th annulus from the posterior body end in females or males, respectively. Phylogenetic and sequence analysis of the D2-D3 expansion fragments of 28S rRNA and ITS rRNA gene sequences of *Belonolaimus* confirmed a unique status for *B. maluceroi* sp. n.. Sequences of rRNA genes for *B maluceroi* sp. n. are related to those of *B. longicaudatus* "D" (DQ494800) from Texas and *B. longicaudatus* "D" (DQ672357) from Oklahoma. Phylogenetic relationships between the new species and other representatives of the genus are shown and discussed.

Key words: Belonolaimus, D2-D3 of 28S rRNA, ITS rRNA, new species, phylogeny, sting nematode, taxonomy, tropical forest.

RESUMO

Cid Del Prado Vera, I., and S. A. Subbotin, 2012. *Belonolaimus maluceroi* sp. n. (Tylenchida: Belonolaimidae) presente en un bosque tropical en México y clave para la identificacion de las especies de *Belonolaimus*. Nematropica 42:201-210.

Una nueva especie del género *Belonolaimus* fue colectada en un bosque tropical en La Mancha, en la Estación Experimental del Instituto de Ecología, Estado de Veracruz, México. Las hembras de *Belonolaimus maluceroi* sp. n. se caracterizan porque su cuerpo es menor a 2 mm de largo; la cabeza está separada del contorno del cuerpo y se divide en cuatro sectores por incisuras longitudinales; estilete largo y flexible, menor a 103 µm de largo; poro excretor a menos de 195 µm del extremo anterior; labios vulvares no sobresalientes del contorno del cuerpo y la vagina sin piezas esclerotizadas; cola hemisférica, de 50-96 µm de largo. La cabeza del macho es similar a la de las hembras; espículas ligeramente curvadas, menores a 34 µm de largo, gubernáculo de11-15 µm de largo, su parte curvada anterior de 3-4 µm de largo. Campo lateral con una incisura iniciando posterior a la región cefálica y terminando aproximadamente 10 anillos antes del término de la cola en las hembras y 24 anillos en los machos. Los análisis filogenéticos de las secuencias de los fragmentos D2-D3 de 28S rRNA y de los genes ITS rRNA confirman un nuevo estado especifico de *Belonolaimus maluceroi* sp. n. Se muestra y se discute la relación filogenética de esta nueva especie con otros representantes del.

Palabras clave: Belonolaimus, D2-D3 de 28S rRNA, ITS rRNA, especie nueva, filogenia, taxonomía, bosque tropical.

INTRODUCTION

The genus *Belonolaimus* was established with the type species *B. gracilis* by Steiner (1949). Later, Rau (1958, 1961 and 1963) described four new species, from the southeastern United States, found around the roots of different species of *Pinus* (*P. elliottii* Engelm., *P. caribea* Morelet., *P. clausa* Chapm., *P. rigida serotina* Michx., *P. taeda* L., *P. palustris* Mill.), *Quercus nigra* L. and other hosts, such as *Lobelia cardinalis* L., *Carya cordiformis* Wangenh, sea oats (*Uniola paniculata* L.) and a corn (*Zea mays* L.) field in Texas. Presently, the genus contains five species including *B. longicaudatus,* which is widely distributed in the Atlantic coastal plains and considered as a serious nematode pest of many agricultural crops. *Belonolaimus* spp. have been reported from sixteen states of USA, Mexico and Puerto Rico (Siddiqi, 2000; Anon, 2009).

During a nematology field trip to the Experimental Ecology Station of the Ecology Institute, La Mancha, State of Veracruz, Mexico, abundant numbers of females, a few males and juveniles of *Belonolaimus* were found around the roots of banyan trees (*Ficus benghalensis* L.) growing in sandy soil near the sea. Morphological and molecular analyses revealed that this nematode belongs to a new species. The purpose of this paper is to characterize and describe this species from Mexico.

MATERIALS AND METHODS

Soil samples were collected from La Mancha, Veracruz, Mexico on March 5, 2011, from sandy soil around relatively undisturbed *Ficus benghalensis* L. Nematodes were extracted from samples of 200 cm^3 of soil by decanting and sieving followed by the centrifugation-sugar flotation technique (Jenkins, 1964). Sub-samples of the extract were examined for live nematodes to distinguish relevant specimens on the basis of stylet length, morphology and activity. Specimens in bulk samples of the nematode extract were killed by heating (to 40 ºC) in about 7 ml water in a 5 cm diam. Petri dish, until movement ceased. An equal volume of 8% formalin was added to the suspension to achieve a final fixative concentration of 4% formalin; the covered dish was stored at room temperature for 10 days. The volume of the fixative was then carefully reduced to approximately 4 ml using a Pasteur pipette without disturbing the nematodes. The covered Petri dish was placed in a small desiccator over 95% ethanol and incubated at 40ºC. After 3 days, when the odor of formalin was no longer detectable, the volume of liquid in the dish was reduced to half, without disturbing the nematodes, by removing liquid with a pipette.

Samples were transferred to glycerin using a modified Seinhorst (1959) method. An equal volume of the Seinhorst solution A (glycerin : 95% ethanol : water = 1 : 20 : 79) was added to the dish, which was then incubated at 40ºC with the cover slightly open. When the solution level dropped to 2 mm, solution B (95% ethanol : glycerin = 95 : 5) was added and the dish again incubated at 40ºC. When the solution level dropped to 1 mm, the solution C (95% ethanol : glycerin = 80 : 20) was added. Three days later, 1 ml of pure glycerin was added to the dish. Selected nematodes were hand-picked from the dish for mounting on Cobb and glass slides using the paraffin wax ring method (de Maeseneer & d'Herde, 1963). Measurements and drawings were made using a drawing tube mounted on an American Optical compound microscope.

For molecular studies, several nematode specimens were put into a drop of water and cut with a scalpel under a binocular microscope. Nematode remains were transferred into an Eppendorf tube containing 16 µl ddH_2O, 2 µl of 10X PCR buffer (Qiagen), 2 µl proteinase K (600 µg/ml) (Promega). The tube was then incubated at 65ºC (1 h) and 95ºC (10 min). The methods used for PCR amplification, cloning and sequencing were similar to those described by Tanha Maafi *et al*. (2003). The forward primer TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and the reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') amplifying the ITS1-5.8S-ITS2 of rRNA and forward D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and reverse D3B (5'-TCGGAAGGAACCAGCTACTA-3') amplifying the D2-D3 expansion segments of 28S rRNA were used in PCR. New sequences obtained in the present study were submitted to the GenBank database under the accession numbers JN967751 to JN967754.

The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson *et al*., 1997) with default parameters with corresponding gene sequences for *Belonolaimus* published in the GenBank (Cherry *et al*., 1997; Gozel *et al*., 2006; Han *et al*., 2006; Subbotin *et al*., 2006). Outgroup taxa for each dataset were chosen according to the results of previously published data (Subbotin *et al*., 2006). Sequence datasets for each gene fragment were analysed separately with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). BI analysis under the GTR + I + G model for each gene was initiated with a random starting tree and was run with four chains for 1.0 x 10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 10^3 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Sequence analyses of alignments were performed with PAUP* 4b10 (Swofford, 2003). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data.

Belonolaimus maluceroi sp.n.

DESCRIPTION

(Measurements: See Table 1)

Female Body C-shaped when relaxed by gentle heat, cylindroid, tapering anteriorly and posterior to the anus. Conspicuous annulations in the anterior and mid-body, reducing in width on the tail. Lateral field with one incisure starting posterior to the lip region and ending almost 10 annuli to the end of the tail. Lateral field with one incisure starting posterior to the lip region and ending almost at the end of the tail. Head rounded, set off from the body by a deep constriction, 7-10 µm high and 9-15 µm wide, bearing 8-9 annuli; labial disc with hexagonal shape surrounded by cuticular ring with external irregular border. Head divided by longitudinal grooves into four separate sectors. Stylet long with the dorsal knob slight projecting anteriorly and subventral knobs rounded. Median oesophageal bulb round, isthmus short. Oesophageal glands overlapping dorso-ventrally, 45-60 µm long. Hemizonid located 3 or 4 annuli anterior to the excretory pore. Ovaries outstretched, spermathecae well developed, the anterior one 15-25 µm long by 12-20 µm wide and the posterior 15-25 µm long by 11-19 µm wide, containing sperm. Vagina without sclerotized pieces and vulval lips not protruding. Tail hemispherical with crenate terminus. Phasmids 17-35 µm from the anus.

Male Body C-shaped when relaxed by gentle heat, tapering anteriorly and posterior to the anus. Head rounded, set off from the body by a constriction, 8-9 µm high and 11-13 µm wide, bearing 8-9 annuli which are less visible than in the females. Stylet long and flexible, the knobs somewhat less developed than in the female. Median oesophageal bulb oval, 15-20 µm long by 11-15 µm wide. Oesophageal glands overlapping dorso-ventrally, 42-70 µm long. Hemizonid anterior to excretory pore by 10-15 µm. Lateral field with one lateral line start after the cephalic constriction and finish almost 24 annuli before the end of the tail. The single testis is outstretched but in one specimen had a flexure. Spicules slight curved, distal end pointed; gubernaculum well developed with anterior flexure and acute distal end. Bursa peloderan, enveloping the tail. Phasmids 16-30 µm from the anus.

Diagnosis and relationships. *Belonolaimus maluceroi* sp. n. is characterized by small female body 1.4-1.8 (1.6) µm long, short tail 50-96 (83) µm long, stylet 85-103 (96) µm long, non-protruding vulval lips and position of phasmids relative to tail end at 42-70 (59) µm.

The new species is similar to *B. nortoni* Rau, 1963 in small body size, position of vulva and absence of vulval sclerotized plates, but differs in longer stylet [85-103 (96) *vs* 78-98 (90) µm], shorter tail [50-96 (83) *vs* 84-130 (108) µm], presence of rounded stylet knobs vs subventral knobs weakly rounded and dorsal knob slightly projecting anteriorly and c and c' [16.3-23.4 (19.5) *vs* 15-18 (23) and 2.6-4.2 (3.3) *vs* 3.4-4.7(4.1), respectively].

Belonolaimus maluceroi sp. n. resembles *B. longicaudatus* Rau, 1958, *B. maritimus* Rau, 1963 and *B. gracilis* Steiner, 1949 in the lip region set off by a shallow constriction, presence of a spermathecae, peloderan bursa, shape of the gubernaculum, but differ from all these species by shorter lengths of body, stylet and tail.

Type locality and habitat. *Belonolaimus maluceroi* sp. n. was collected from soil around roots of (*Ficus benghalensis* L.), in a tropical forest in La Mancha, Municipio de Actopan, Estado de Veracruz, Mexico (N 19°35.820; W 96°22.550), 2 m above sea level.

Type specimens. Accession numbers of type specimens deposited in the Laboratorio de Helmintología del Instituto de Biología, UNAM, Mexico, are holotype CNHE 8200 female, allotype CNHE 8201 male, paratype females CNHE 8202. Other paratype materials are deposited in the University of California at Riverside Nematode Collection (UCRNC), Wageningen University Nematode Collection (WUNC), the Colegio de Postgraduados Nematode Collection (CPNC) and the United States Department of Agriculture Nematode Collection, Beltsville, MD, USA.

Etymology. The specific epithet is in memory of the enthusiastic and promising nematologist, Miguel Angel Lucero, who was a student of Dr. I. Cid Del Prado Vera.

Molecular characterization and phylogenetic relationships within Belonolaimus. PCR with TW81 and AB28 primers yielded a product of approximately 910 bp in length. The ITS1-5.8S-ITS2 alignment included 44 sequences and had 1170 positions in length. The ITS sequences of *B maluceroi* sp. n. obtained from three clones differed in one to 7 nucleotides (up to 0.8%) from each other and in 10 nucleotides of ITS1 (2%) from closely related *B. longicaudatus* type "D" (DQ494800) from Texas. Sequences of *B. maluceroi* sp. n. and *B. longicaudatus* "D" shared same long deletion in the ITS1 sequences. Phylogenetic analysis of the ITS sequence alignment revealed several major clades on 50% majority consensus BI tree (Fig. 5): (i) *B. longicaudatus* "A" (PP = 76%); (ii) *B. longicaudatus* "B" (93); (iii) *B. euthychilus* + *Belonolaimus* sp. + *B. gracilis* "A" (100); (iv) *B. longicaudatus* "C"; (v) *B. gracilis* "B"; (vi) *B. maluceroi* sp. n. + *B. longicaudatus* "D" from Texas (72); (vii) *B. longicaudatus* "E" and "F" (100).

PCR with D2A and D3B primers yielded a product of approximately 760 bp in length. The D2-D3 alignment included 27 sequences and had 732 positions in length. The D2-D3 of 28S rRNA gene sequence of *B. maluceroi* sp. n. differed in 10 nucleotides (1.4%) from closely related *B. longicaudatus* "D" (DQ672357) from Oklahoma. Phylogenetic analysis of the D2-D3 sequence alignment revealed several major clades on 50% majority consensus BI tree (Fig. 6): (i) *B. longicaudatus* "A" + *B. longicaudatus* "B" (PP =

Table 1. Measurements and ratios for characters of females and males of *Belonolaimus maluceroi* sp. n. (measurements in µm, except for L in mm).

Females	Holotype female	Paratype females	Allotype male	Paratype males
n	1	18	1	7
L	1.8	1.4-1.8 (1.6)	1.3	1.2-1.5 (1.3)
a	57.8	47-59 (54)	57	50-58 (54)
b	9.8	7.2-11.3 (9.6)	8.5	7.8-9.4 (8.3)
b’	7.6	4.9-8.7 (7.6)	6.0	5.6-6.5 (6.0)
c	17.6	16.3-23.4 (19)	16.1	14-22 (17)
c'	3.4	2.6-4.2 (3.3)	4.1	3.3-4.8 (4.4)
V%	52	46-53 (51)	-	-
Stylet	103	85-103 (96)	89	83-98 (90)
Stylet cone	75	63-78 (70)	65	59-72
Stylet shaft	25	23-28 (25)	22	22-24 (23)
Stylet knobs wide	4	3-5(4)	4	3-4 (4)
Tail length	89	50-96 (83)	81	60-98 (80)
Tail/Body width ratio	2.9	2.3-3.4 (2.8)	-	0.9-1.5 (1.2)
Tail width	26	22-28 (24)	20	17-20 (18)
Anterior end to hemizonid	180	148-180 (164)	157	135-160 (150)
Anterior end to excretory pore	195	146-195	172	145-172 (162)
Posterior end to phasmids	62	42-70 (59)	65	42-68 (56)
Style/Tail ratio	1.6	1.1-1.8 (1.2)	1.1	0.9-1.5 (0.2)
Spicules	-	-	31	25-34 (30)
Gubernaculum	-	-	15	11.0-15 (13)
Body width	31	27-31(29)	23	23-26 (24)

100%), where *B. longicaudatus* “B” formed a separate subclade (75), (ii) *B. euthychilus* + *B. gracilis* “A” (100); (iii) *B. gracilis* “B”; (iv) *B. longicaudatus* “C”; (v) *B. maluceroi* sp. n. + *B. longicaudatus* “D” from Oklahoma (85).

Key to the species of *Belonolaimus*

1. Cephalic region not separated from the body .... *B. euthychilus* Rau, 1963
   Cephalic region separated from the body 2

2. Average female stylet less than 103 µm, male stylet less than 100 µm 3
   Average female stylet equal or more than 103 µm, male stylet equal or more than 100 µm 4

3. Female tail= 84-130 (108) µm; c’= 3.4-4.7 (4.1); stylet/ tail ratio= 0.7-0.9 ..... *B nortoni* Rau, 1963
   Female tail = 50-96 (83) µm; c’ = 2.6-4.2 (3.3); stylet/tail ratio = 1.1-1.8 ..... *B. maluceroi* sp. n.

4. Large sclerotized vulval pieces present; cephalic region separated from rest of body by shallow constriction..... *B. maritimus* Rau, 1963
   Vulva sclerotized pieces weak or absent; cephalic region separated from rest of body by a deep constriction..... 5

5. Female tail = 115-200 µm; sclerotized vulval pieces present; tail end hemispherical; median bulb nearly spherical ..... *B. longicaudatus* Rau, 1958
   Female tail = 53-134 µm; sclerotized vulval pieces absent; tail end convex-conoid; median bulb elongated..... *B. gracilis* Steiner, 1949

DISCUSSION

Scanning electron microscopy analysis revealed that the head of *B. maluceroi* sp. n. had two large subdorsal and two large subventral lobes and two small

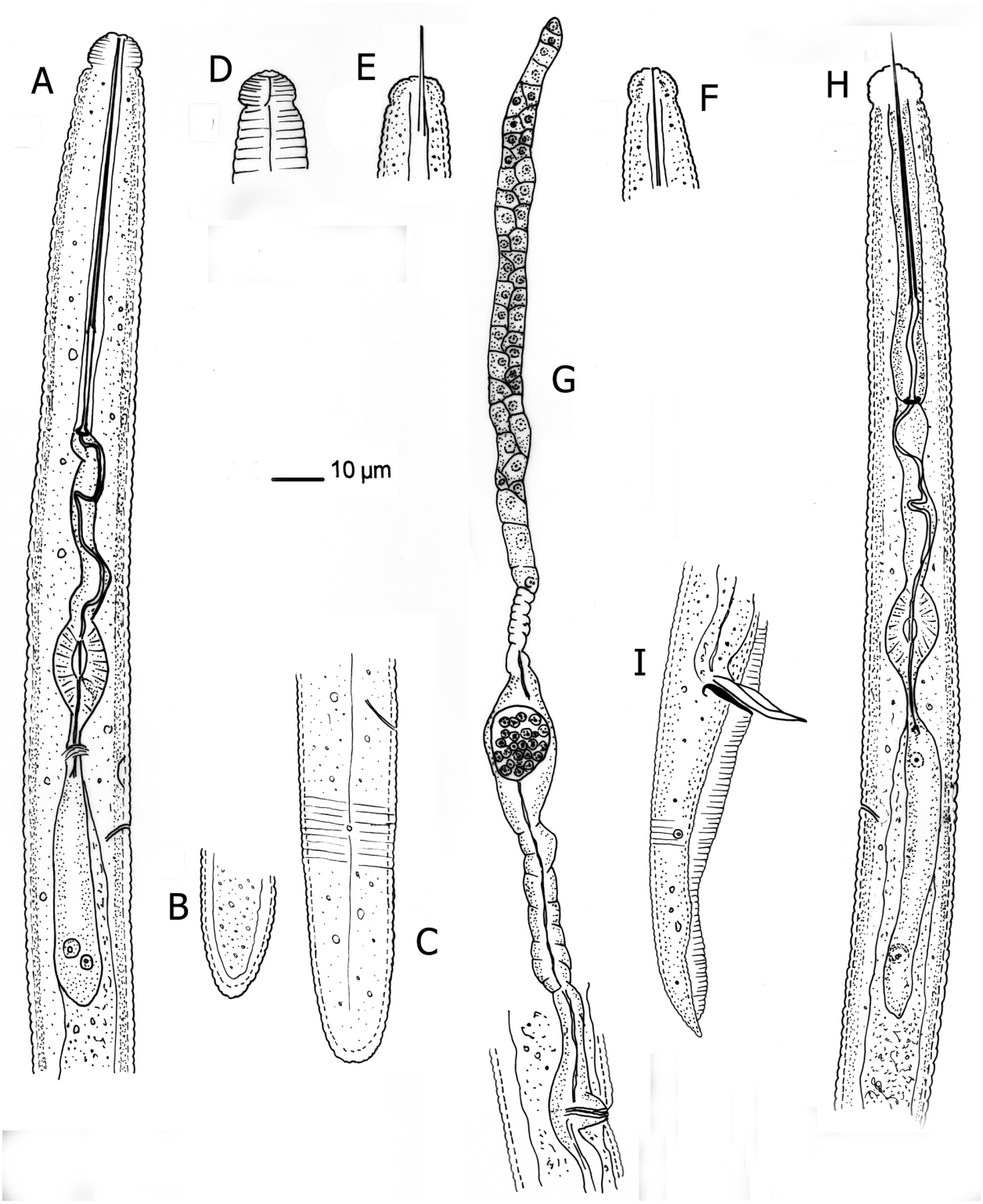

Fig.1. *Belonolaimus maluceroi* sp. n. Female: A-G. A. Anterior end; B, C. Posterior end; D.E. Head; G. Anterior gonad; Male: H, I; H. anterior end; I. Tail.

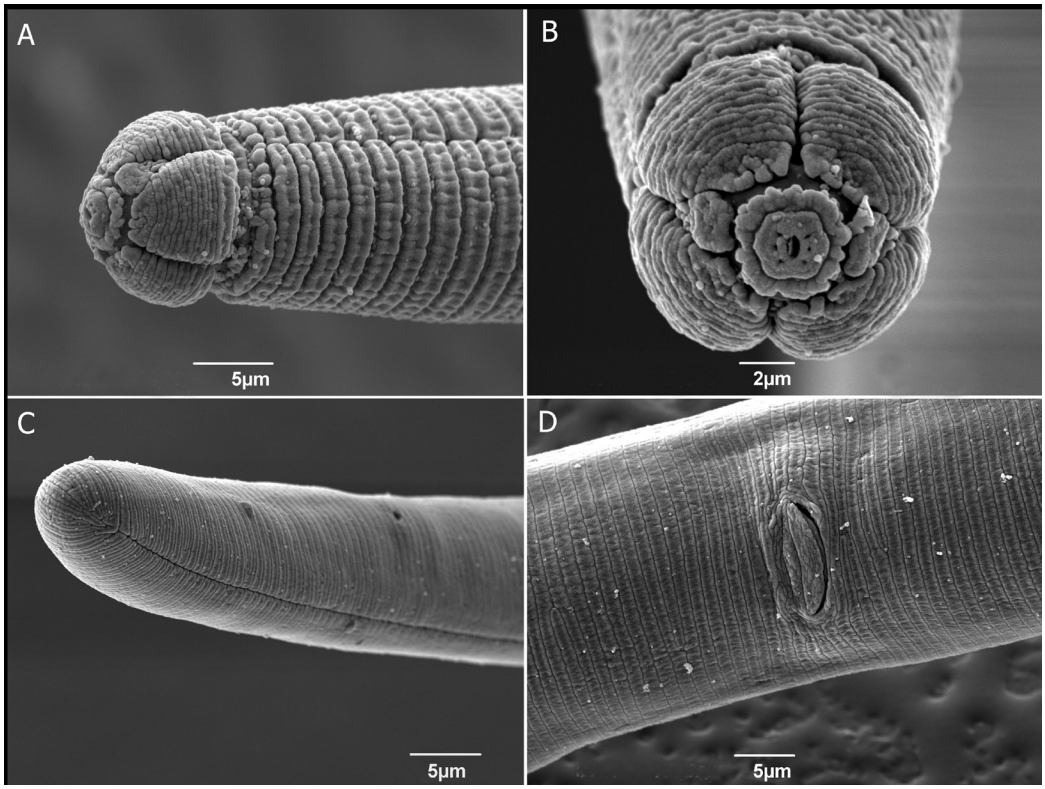

Fig. 2. *Belonolaimus maluceroi* sp. n. SEM. Female. A: Anterior end, latero-ventral view; B: Face view; C: Tail; D: Vulva, ventral view.

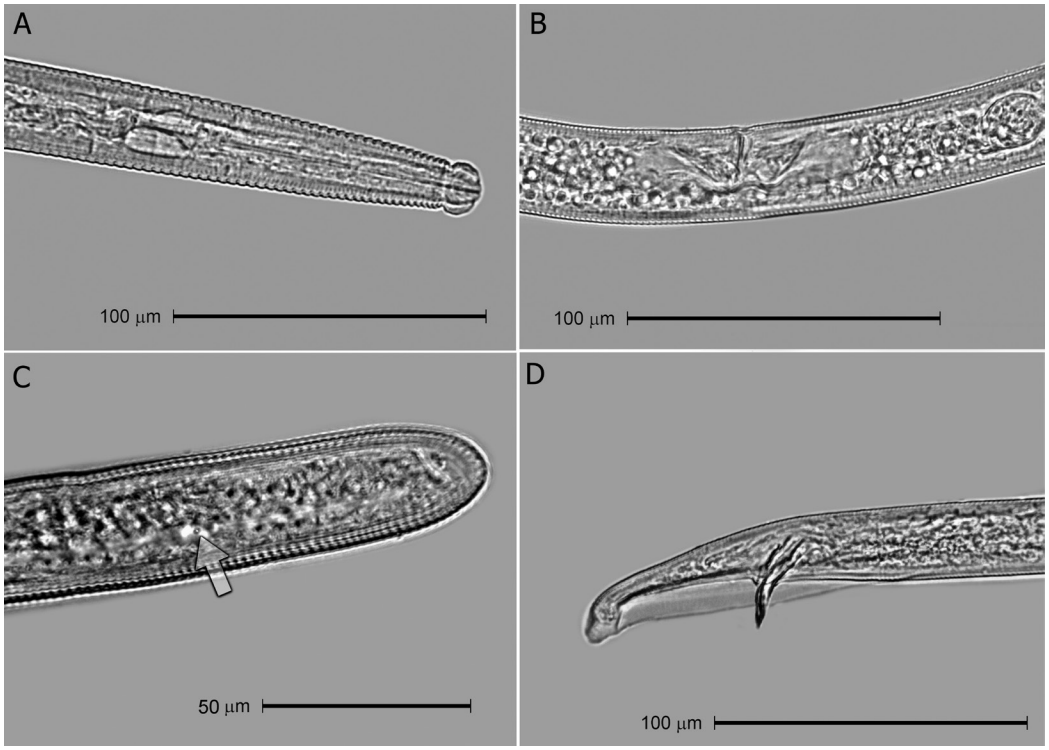

Fig. 3. *Belonolaimus maluceroi* sp. n. LM. Female: A-C; Male: D; A: Anterior end; B: Vulva and spermatheca, lateral view; C: Posterior end; D: Tail.

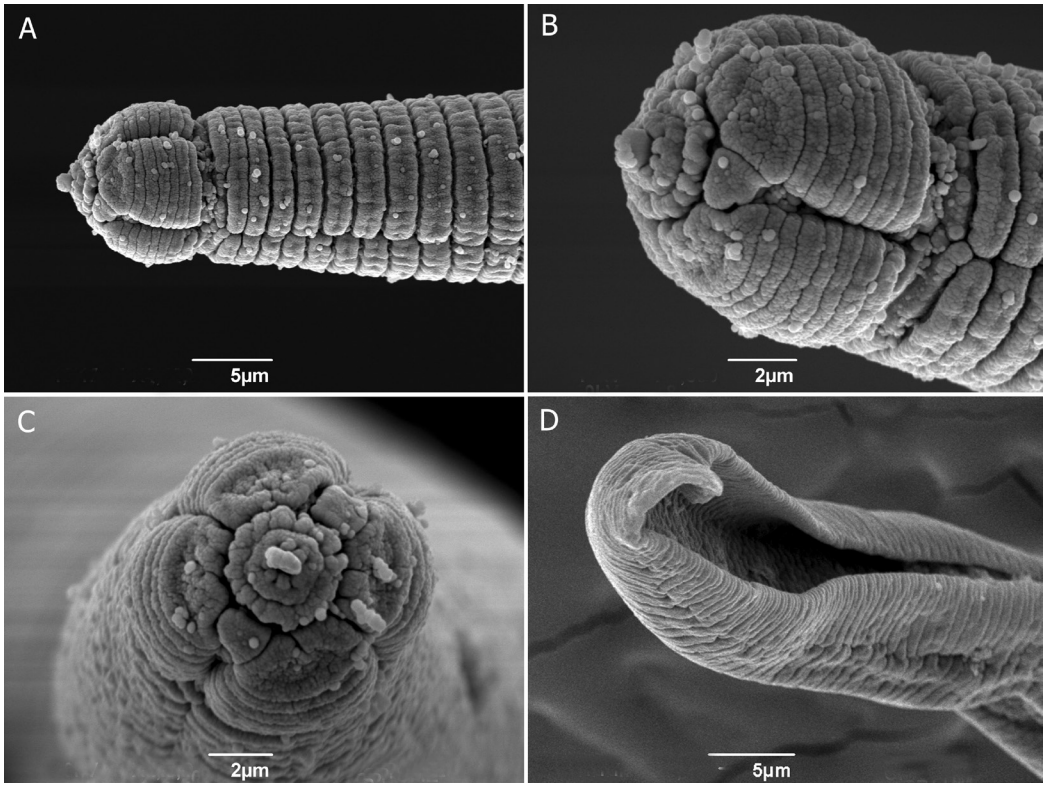

Fig. 4. *Belonolaimus maluceroi* sp. n. SEM. Male. A: Anterior end, latero-vientral view; B: Head, lateral view; C: Face view; D: Bursa, ventral view.

lateral lobes, as previously described by Smart *et al.* (1972). The labial disc in females had a hexagonal shape and it was almost quadrangular in males; in both sexes it was surrounded by a cuticular ring with external irregular border; six labial papillae are clearly present on the lateral side of the mouth. Amphids were not observed on the lateral lobes as illustrated by Smart *et al.* (1972), but located within deep lateral clefts as described by Thorne (1961). Thus, *B. maluceroi* sp. n. differs from *B. longicaudatus* by the presence of the labial papillae on the labial disc versus into the buccal aperture and the absence of the amphids on the lateral lobes.

Belonolaimus longicaudatus has been considered as a species complex by several authors because of differences in morphological and morphometrical characters (Robbins and Hirschmann, 1974; Duncan *et al.*, 1996; Gozel *et al*., 2006; Han *et al*., 2006) and in pathogenicity (Abu-Gharbieh and Perry, 1970; Perry and Rhoades, 1982) among populations. Molecular analyses made by Cherry *et al.*, (1997), Gozel *et al.* (2006) and Han *et al.* (2006) also revealed significant differences in sequences of ribosomal RNA genes between populations. Cherry *et al.* (1997) was the first who showed distinct groupings of *B. longicaudatus* from different states based on PCR-RFLP of ITS1 and concluded that each of the Midwestern populations differed in ITS1 and produced a unique RFLP profile, whereas the ITS1 of southern USA and Californian isolates were identical, that may confirm a hypothesis on introduction of *B. longicaudatus* into California. Gozel *et al*. (2006) and Han *et al.* (2006) used the D2-D3 and ITS sequences to reconstruct relationships between *Belonolaimus* species and populations. The results of our molecular analysis are generally similar to those previously published. Phylogenetic analyses of the ITS and the D2-D23 of 28S rRNA gene sequences resulted in congruent trees with more clear separations of clades in the ITS tree. Several major clades containing isolates morphologically identified as *B. longicaudatus* could be recognized on these trees. They are mainly grouped according to their geographical locations: (i) *B. longicaudatus* type "A" collected mainly in the Atlantic coastal plains: western and central Florida (Gozel *et al.*, 2006), Delaware (Handoo *et al.*, 2010), North Carolina and Georgia (Han *et al*., 2006) with possible introduction of this type in California (Mundo-Ocampo *et al*., 1994; Cherry *et al.,* 1997); (ii) *B. longicaudatus* type "B" containing the topotype population only from eastern Florida (Gozel *et al*., 2006); (iii) *B.*

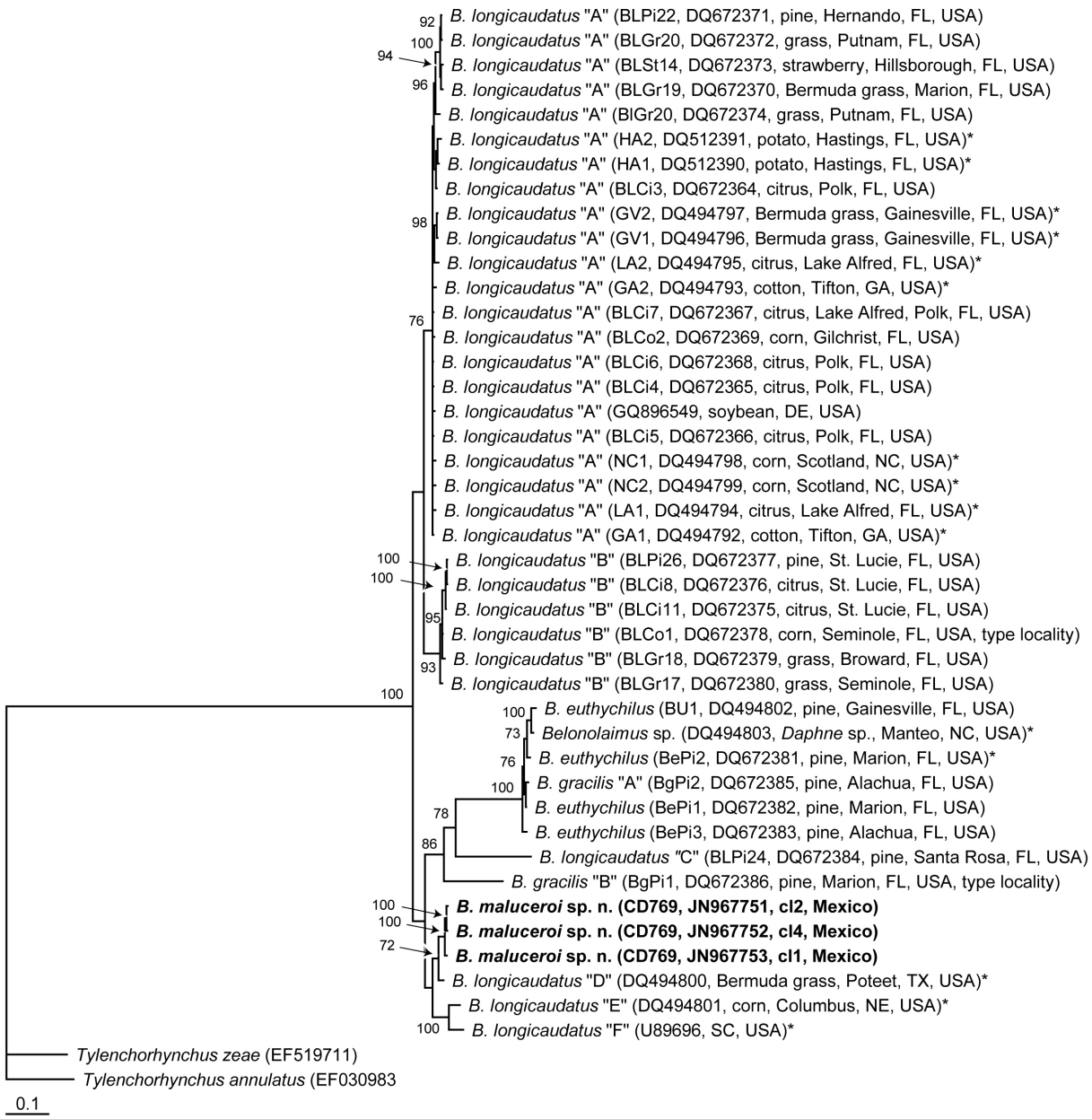

Fig. 5. Phylogenetic relationships within the genus *Belonolaimus* as inferred from the Bayesian analysis of the ITS rRNA gene sequences. * - only ITS1 sequence.

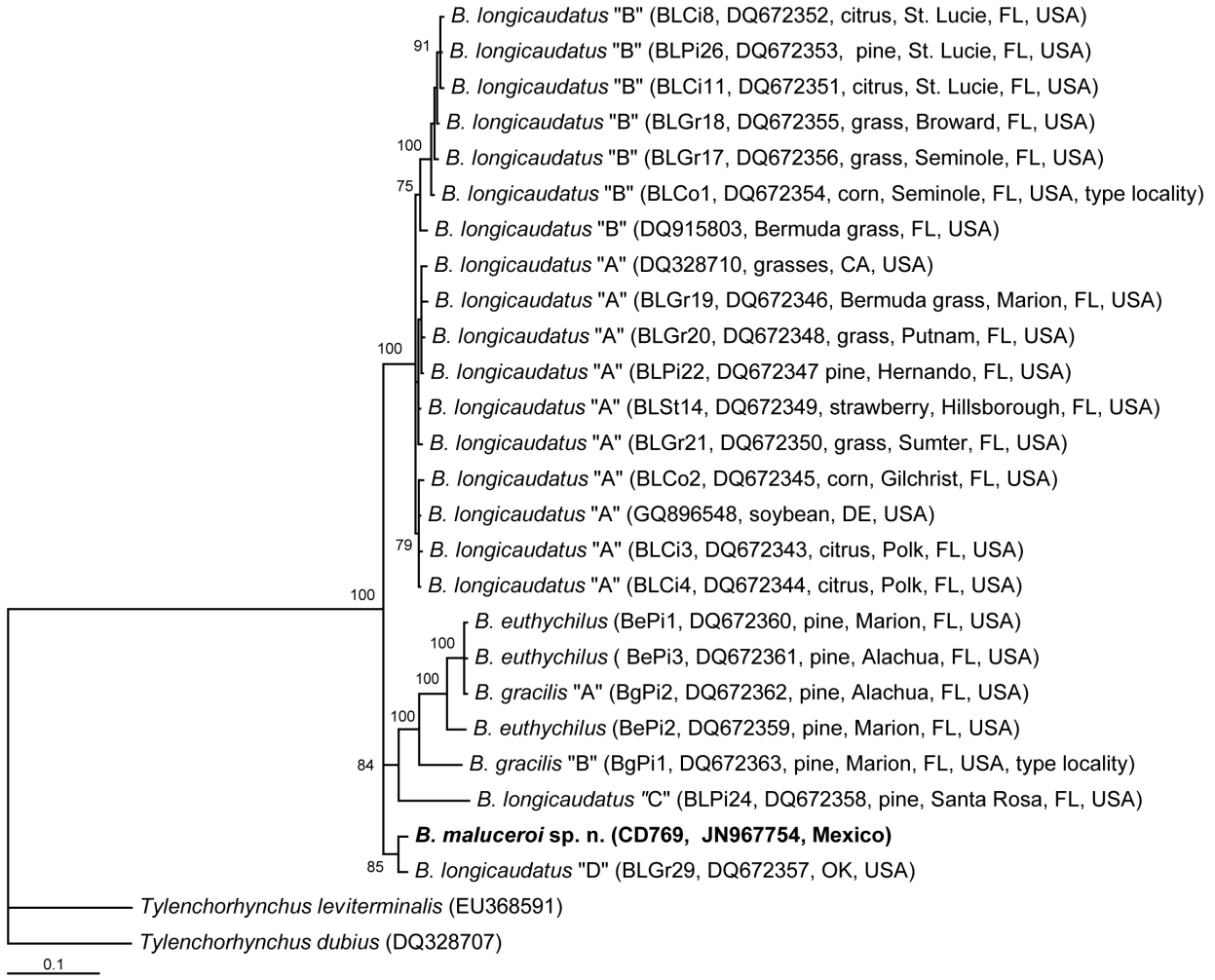

Fig. 6. Phylogenetic relationships within the genus Belonolaimus as inferred from the Bayesian analysis of the D2-D3 of 28S rRNA gene sequences.

longicaudatus type "C" from northwest Florida (Gozel *et al.*, 2006); (iv) *B. longicaudatus* type "D" from Texas and Oklahoma (Gozel *et al*., 2006; Han *et al*., 2006); (v) *B. longicaudatus* type "E" from Nebraska (Han *et al.*, 2006) and *B. longicaudatus* type "F" from South Carolina (Cherry *et al*., 1997). Morphological and morphometrical analyses conducted by Gozel *et al*. (2006) and Han *et al.* (2006) did not allow distinct discrimination of two groups of *B. longicaudatus*, which in our study correspond to type "A" and "B". Gozel *et al.* (2006) noticed that select characters: stylet length and the c ratio discriminated the clades significantly better. Shorter stylet length was reported for specimens of *Belonolaimus* populations from Arkansas, Oklahoma, Kansas and Nebraska (Cherry *et al.,* 1997; Gozel *et al*., 2006), which might belong to our types "D – F". It is remarkable that *B maluceroi* sp. n. is characterized by a short stylet, which makes this species more similar with these types.

Belonolaimus maluceroi sp. n. in our ITS and D2-D3 trees formed clades with samples identified as *B. longicaudatus* from Texas and Oklahoma, respectively, and named here as the type "D". Although *B. longicaudatus* from Oklahoma is clearly distinguished from other *B. longicaudatus* populations by several characters: shorter body length, 1938-2421 (2228) µm long; shorter stylet length, 98-112 (107) µm long; shorter tail length, 106-159 (128) µm long; shorter body width, 33-40 (37) µm (Gozel *et al.*, 2006), all these morphometric characters have higher values than those for *Belonolaimus maluceroi* sp. n. Thus, *B. maluceroi* sp. n. does not seem to be co-specific with *B. longicaudatus* type "D" from Oklahoma. Additional molecular phylogenetic studies need to clarify the relationships of a new species with *B. longicaudatus* type "D" from Texas and Oklahoma.

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