Four new species of *Tripylina* Brzeski, 1963 (Enoplida: Tripylidae) from México, with an emended diagnosis of the genus

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Summary.- Four new species of *Tripylina* collected in México are described and illustrated. *Tripylina bravoae* sp. n. has a slender body, asymmetric lip region, dorsal tooth posterior to smaller subventral teeth, two ventromedian setae in the cervical region of males but absent in females, indiscernible excretory pore, a post-uterine sac, vulval lips slightly protruding and sclerotized pieces present around the vagina. *Tripylina ixayocensis* sp. n. is characterized by the presence of sparse somatic setae, conoid and asymmetric lip region, long outer labial setae and shorter cephalic setae in a single whorl, dorsal wall of stoma thickened, dorsal tooth small and anterior to subventral teeth, excretory pore conspicuous, vulva with protruding lips, and vagina without sclerotized structures. *Tripylina tlamincasensis* sp. n. has sparse somatic setae, asymmetric lip region, outer labial setae and cephalic setae in two whorls, dorsal wall of stoma not thickened, dorsal tooth posterior to subventral teeth, indiscernible excretory pore, vulva with non-protruding lips, and vagina with small sclerotized pieces. *Tripylina montecilloensis* sp. n. is distinguished by its sparse somatic setae, conoid and asymmetric lip region, outer labial setae slightly anterior to cephalic setae, dorsal wall of stoma not thickened and dorsal tooth posterior to subventral teeth, two ventromedian cervical setae, excretory pore indiscernible, vulval lips not protruding and vagina without sclerotized pieces. Phylogenetic analysis of SSU rDNA sequence data supports monophyly of *Tripylina* species, and depicts *T. bravoae* as sister to other analyzed *Tripylina* species. The diagnosis of the genus is emended based on newly recognized characteristics.

Keywords: Emended diagnosis, México, new species, predatory nematodes, phylogeny, SEM, SSU rDNA, systematics, taxonomy, Tripylidae, *Tripylina*.

Resumen.- Se describen e ilustran cuatro nuevas especies de *Tripylina* colectadas en México. *Tripylina bravoae* sp. n. presenta un cuerpo alargado, región labial asimétrica, diente dorsal posterior a los pequeños dientes subdorsales, dos setas ventromedianas en la región cervical en los machos y ausentes en las hembras, poro excretor no visible, presencia de saco postuterino, labios vulvares ligeramente sobresaliendo del contorno del cuerpo y piezas esclerotizadas en la vagina. *Tripylina ixayocensis* sp. n. se caracteriza por tener setas somáticas esparcidas en el cuerpo, región labial conoidea y asimétrica, setas labiales externas largas y setas cefálicas cortas en un solo círculo, pared dorsal del estoma gruesa, diente dorsal pequeño y anterior a los dientes subventrales, poro excretor conspicuo, vulva con labios sobresaliendo del contorno del cuerpo y vagina sin estructuras esclerotizadas. *Tripylina tlamincasensis* sp. n. se distingue por sus setas somáticas esparcidas, región labial asimétrica, las setas externas y las cefálicas en dos círculos, la pared dorsal del estoma no engrosada, el diente dorsal posterior a los dientes subventrales, poro excretor no visible, vulva con los labios no proyectados, y vagina con pequeñas piezas esclerotizadas. *Tripylina montecilloensis* sp. n. tiene setas somáticas esparcidas, cabeza conoidea y asimétrica, setas externas ligeramente anteriores a las cefálicas, pared dorsal del estoma no engrosada y diente dorsal posterior a los dientes subventrales, dos setas cervicales ventromedianas, poro excretor no visible, labios vulvares no proyectados, y vagina sin piezas esclerotizadas. El análisis filogenético derivado de las secuencias de SSU rDNA apoya la monofilia de las especies de *Tripylina* y muestra que *T. bravoe* es la especie hermana del otro grupo de especies de *Tripylina* analizado. La diagnosis del género es corregida a partir del reconocimiento de nuevos rasgos.

Palabras clave: Diagnosis enmendada, filogenia, México, nematodos predadores, nuevas especies, SEM, SSU rDNA, sistemática, taxonomía, Tripylidae, *Tripylina*.

Introduction

Nematodes of the family Tripylidae de Man, 1876 are generalist predators of small aquatic and soil organisms. Many authors have commented on the freshwater and wet soil in which these nematodes are found, and on the nature of their prey based on observation of intestinal contents or from behavior. Among the recorded prey, as reviewed and collated by Small (1987), are nematodes, rotifers and protozoa. Andrássy (2007) recognized three subfamilies within the Tripylidae: Tripylinae de Man, 1876, which includes the genera *Tripyla* Bastian, 1865, *Tripylina* Brzeski, 1963 and *Tripylella* Brzeski & Winiszewska-Ślipińska, 1993; Trischistomatinae Andrássy, 2007, only with the genus *Trischistoma* Cobb, 1913; and

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Tobriliinae Andrássy, 2007, only with the genus *Tobrilia* Andrássy, 1967. Tripylidae and Tripylinae lack monophyly as revealed by molecular phylogenies (Cid del Prado-Vera *et al.*, 2010), indicating that taxonomical revision of these taxa is needed.

The genus *Tripylina* is characterized by, among other features, its outer labial and cephalic whorls of setae located close together, dorsal tooth either anterior or posterior to the two subventral teeth in the stoma, and a single gonad in females (Tsalolikhin, 1983; Zullini, 2006). Its taxonomical position has been matter of discussion since it was placed under Triplonchida (De Ley & Blaxter, 2004) or Enoplida (Andrássy, 2007; Zhao *et al.*, 2012). And molecular phylogenetic analysis of SSU sequences strongly supports the relationship between *Tripylina* and Enoplida (Zhao & Buckley, 2009; Cid del Prado-Vera *et al.*, 2010).

Abundant material of *Tripylina* was collected from four locations in Mexico. Its studied revealed it consisted of four unknown species, whose description is presented in the following.

Material and Methods

The nematodes were killed by heat, fixed in 4% formalin solution, and dehydrated slowly and mounted in anhydrous glycerine according to the protocol by Seinhorst (1959) modified after Cid del Prado-Vera *et al.* (2010). Selected nematodes were hand-picked for mounting on 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. The specimens for scanning electron microscope studies were fixed in 4% formalin and, after 10 days, were placed in a pH 7.2 buffer for 20 minutes and dehydrated through a series of alcohol concentrations (20% to 100%) in 15 minute time steps before critical dry point and coating with gold palladium. Specimens were not post-fixed in glutaraldehyde or osmium tetroxide. A Jeol JSM-6390 microscope at 10 KV acceleration voltage was used for SEM.

Nucleic acids used for polymerase chain reaction (PCR) amplifications were extracted from individual or pooled adults hand-picked from water and transferred to 95% ethanol. Nucleic acids were prepared either by the sodium hydroxide method (Floyd *et al.*, 2002) or by a Chelex protocol as follows: nematodes were placed in 20 µL of a 5% aqueous solution of Chelex 100 (Sigma-Aldrich Corporation) in a 500 µl microcentrifuge tube, 1 µL of proteinase K solution (25 mg/ml) was added, and the tube was incubated at 56 °C for 1 hour; the tube was then boiled at 100 °C for 8 minutes to denature the proteinase K. For both the sodium hydroxide and Chelex methods, 2 mL of nucleic acid extract was used to amplify and sequence a region of the SSU ribosomal DNA (3'-end) as described in Cid del Prado-Vera *et al.* (2010).

A SSU dataset was prepared using sequences from the new taxa, species analyzed in a previous investigation (see Cid del Prado-Vera *et al.*, 2010) and related taxa obtained from GenBank. Outgroup taxa were chosen and trees rooted based on Cid del Prado-Vera *et al.* (*op. cit.*). Sequences were aligned using ProAlign Version 0.5 (Loytynoja & Milinkovitch, 2003). The GTR+I+G substitution model was used for Bayesian phylogenetic inference because it was previously found to be the best-fit model for these taxa (Cid del Prado-Vera *et al.*, *op. cit.*). The posterior probability distribution of trees was determined using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003)r as executed using the Cipres Portal (Miller *et al.*, 2010). The dataset, which consisted of 72 taxa and 658 aligned SSU sites, was run for 5 million generations, and chains were sampled every 5000 generations. Burn-in was estimated empirically as 20% by assessment of the convergence in the log likelihood values of the chains.

Descriptions

Tripylina bravoae sp. n.
(Figs 1A-G, 2A-G & 3A-D)

Measurements: See Table I.

General description: Body long and slender, spiraling ventrally in the posterior region after fixation in both females and males. Cuticle 1-2 µm thick, bearing very fine transverse striation indiscernible under LM but visible under SEM, and with sparse somatic setae (Fig. 4A,D). Lip region asymmetric, 20-26 (23±0.7) µm wide, separated from the body contour by a slight constriction; three triangular lips; inner labial papillae small, conical, 2 µm long, two at the base of each lip; six outer labial setae strongly developed, 13-20 (17±1.0) µm long or 50-87 (76)% of the corresponding lip region width; four cephalic setae 5-8 (6.8±0.6) µm long, in a single whorl with the outer labial setae 3 µm behind them. Amphid apertures oval, at 19-27 µm from the anterior end. Stoma opening shifted dorsally, with its wall thickened dorsally; strong dorsal tooth directed posteriad, 2 µm long, located at 18-35 µm from the anterior end and posterior to the two minute subventral teeth. Pharynx heavily muscular in the posterior part, separated from the intestine by a large cardia with two pericardiac cells. No excretory pore. Two ventromedian setae in the cervical region in males, the first at 52-85 µm and the second at 187-208 µm from the anterior end, but absent in females. Rectum 0.8-1.3 times as long as anal body width. Tail curved in a ventral spiral, the first third wide but then tapering to a slender cylindrical shape (Fig. 4C). Caudal glands occupying almost the full width of the tail, with small spinneret. Pseudocoelomocytes large and ovoid, with a distinct nucleus.

Female: Cardia 11-19 (15±1.8) µm long and 17-25 (21±1.7) µm wide. Distance from posterior end of

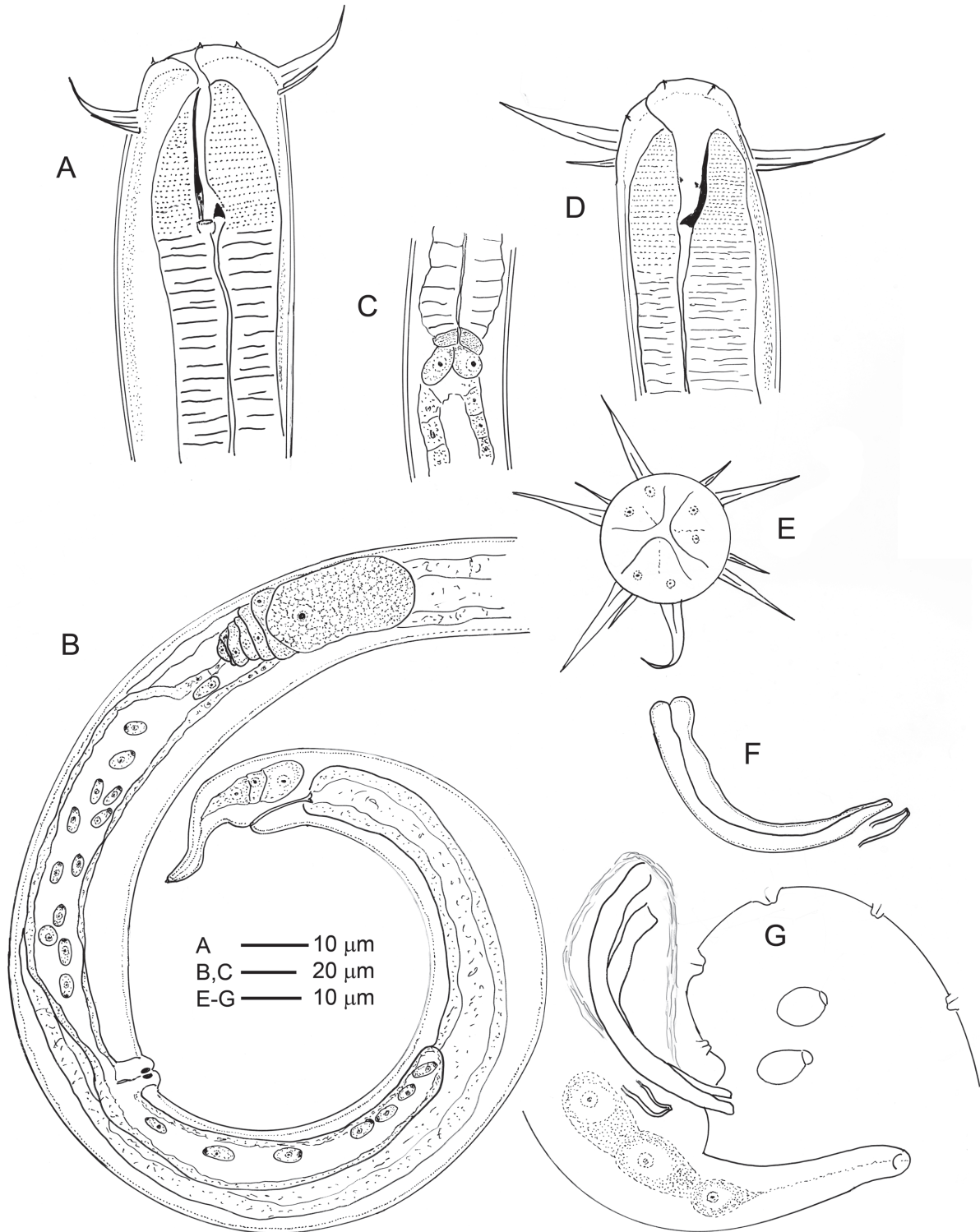*J. Nematode Morphol. Syst.*, 15 (2): 71-86 (2012)

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FIGURE 1. ***Tripylina bravoae* sp. n.** A-C: Female. D-G: Male. A: Anterior region. B: Posterior region. C: Cardia. D: Anterior end. E: Face view. F: Spicules and gubernaculum. G: Posterior region lateral and spermatozoa.
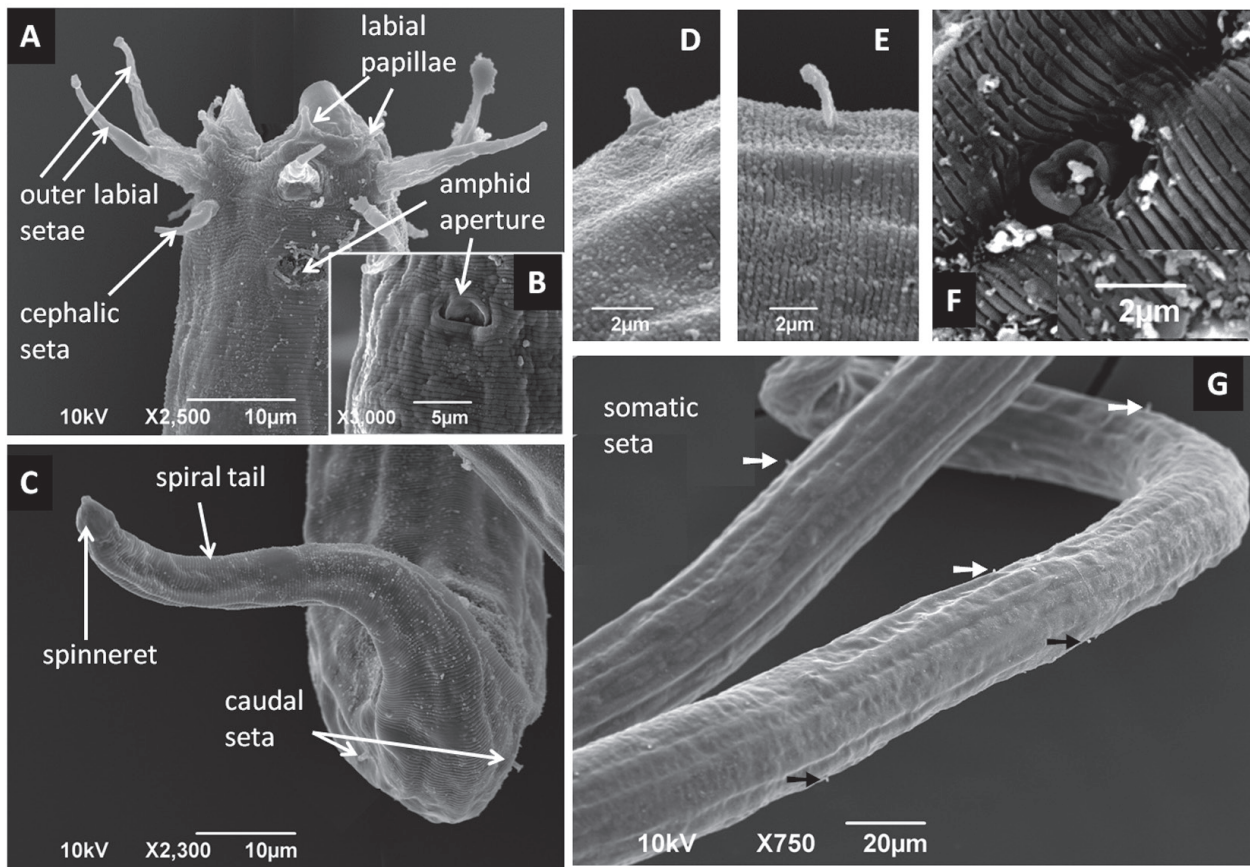
Figure 2. *Tripylina bravoae* sp. n. (SEM). A: Female anterior. B: Amphid aperture. C: Characteristic spiral of tail with two small caudal setae. D: Somatic seta, female. E: Cervical seta, female. F: Precloacal supplement, male. G: Entire body with scattered somatic setae.

pharynx to vulva 763-1244 (1056) µm and from pharynx base to anus 1133-1398 (1265) µm. Reproductive system monodelphic-prodelphic: ovary short and reflexed; uterus 80-109 µm (97.4±39.5) long or 5.1-6.7% of body length, occasionally filled with sperm cells; vagina short, with two oval sclerotized pieces (Fig. 6B); vulval lips slightly protruding; post-uterine sac 130-175 (149±5.1) µm long or 44-73% of the vulva-anus distance, containing amoeboid sperms (Fig. 6C). Vulva-anus distance 141-325 (264±20) µm. One pair of small latero-dorsal setae is present behind the anus.

Male: Slightly larger than female. Cardia 12-17 (15±0.6) µm long and 23-25 (24±0.4) µm wide. Distance from posterior end of pharynx to cloaca 1513-1724 (1414) µm. Genital system monorchic, with 74.5-105.7 (89±3.3) µm long testis, which occupies 4.7-5.9 (5.1±0.1)% of body length. Sperm cells oval, 8-12 (11.8±0.5) µm long and 6-8 (7.5±0.4) µm wide. Five pre-cloacal supplements (Fig. 4C), located at 8-12, 19-29, 38-61, 63-97 and 84-131 µm from cloacal aperture. Spicules sickle-shaped, 38-60 (49±2.3) µm long, with bifid terminus and completely surrounded by a muscular sheath. Gubernaculum

U-shaped, 10-17 (12±0.8) µm long and 3-4 (3.2±0.1) µm wide (Fig. 6D). One pair of small, latero-dorsal setae behind the cloacal aperture.

Diagnosis: *Tripylina bravoae* sp. n. is characterized by its long and slender body of both females and males, asymmetric lip region, thin cuticle with fine striation only perceptible under SEM and sparse somatic setae, indiscernible excretory pore, presence of post-uterine sac, one pair of latero-dorsal setae behind the cloacal aperture in males and the anus in females, males with a single testis and five pre-cloacal supplements, spicules incompletely surrounded by a muscular sheath, and a distinct U-shaped gubernaculum.

Relationships: The new species is similar to *T. longa* Brzeski & Winiszewska-Ślipińska, 1993 in the thickness of the dorsal stoma wall, the presence of a post-uterine sac, the shape of the spicules, the presence of five ventromedian supplements in males, and the presence of a pair of latero-dorsal setae on tail of both females and males. Nevertheless, it can be separated from this in the absence
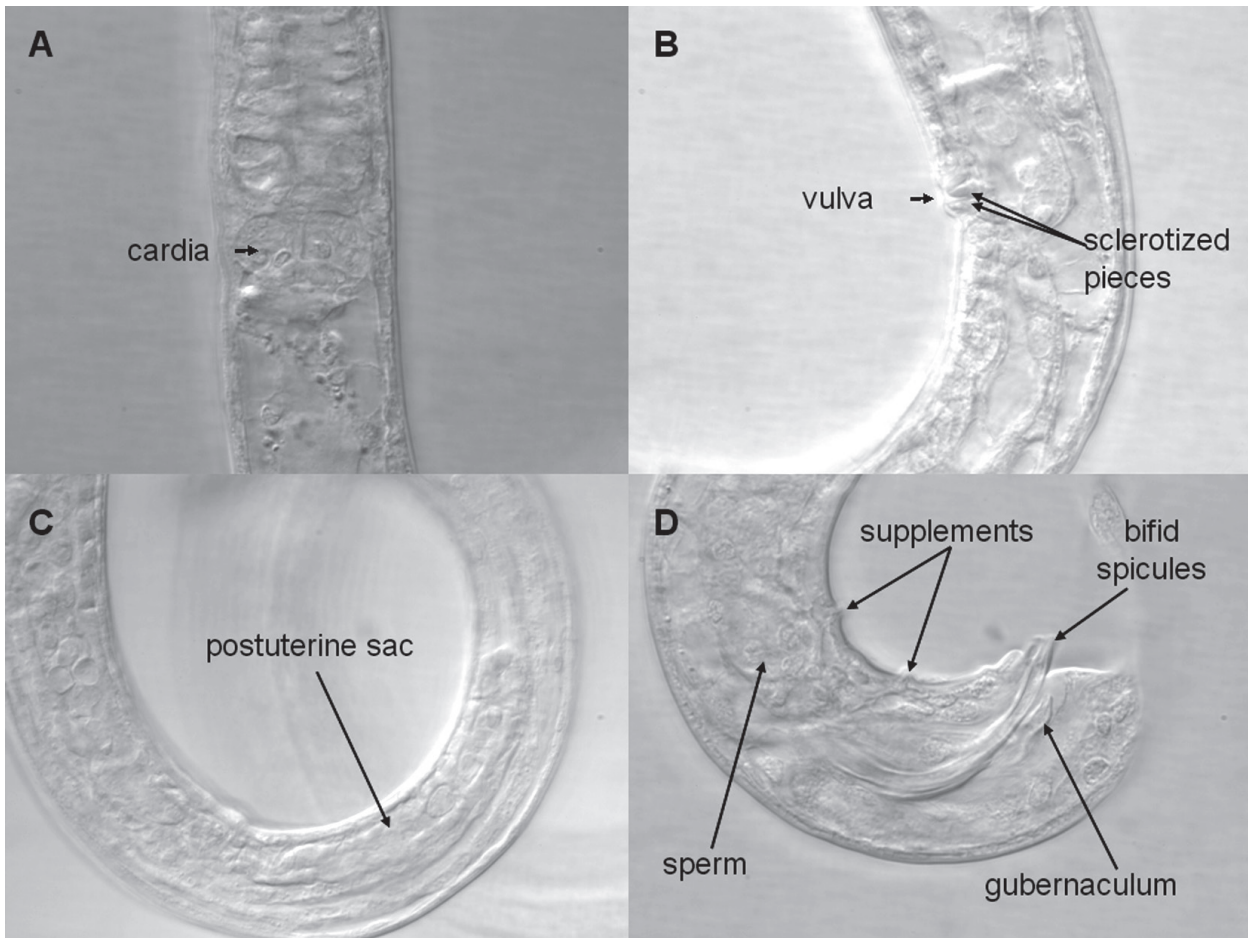
FIGURE 3. *Tripylina bravoae* **sp. n.** (LM). A: Cardia at base of pharynx. B: Vulva with sclerotized pieces. C: Post-uterine sac. D: Spicules and gubernaculum.

of the two ventromedian setae in the cervical region of females, the absence of body pores, the presence of sparse somatic setae in both females and males, the U-shaped gubernaculum, the shape of the sclerotized pieces around the vagina, and in the indiscernible excretory pore.

Type locality and habitat: México, Morelos State, Zacatepec, N18° 39.47', W99° 11.52', altitude 920 m a.s.l., in association with sugar cane field.

Type specimens: Female holotype on slide CNHE 8191, male paratype on slide CNHE 8192, and female and male paratypes on slides CNHE 8193 (3♀♀, 3♂♂), deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Laboratorio de Helmintología, Universidad Nacional Autónoma de México. Other paratypes distributed as follows: Colección Nematologica del Colegio de Postgraduados (CNCP) (5♀♀, 5♂♂), University of California Riverside Nematode Collection (UCRNC) (2♀♀, 1♂♂), and Wageningen Nematode Collection (WaNeCo, WT3565) (1♀).

Etymology: This new species is named in honor of Dr. Margarita Bravo Hollis, for her outstanding activity as a professor of Zoology and her contributions to Helminthology in México and in the world.

Tripylina ixayocensis sp. n.
(Figs 4A-D & 5A-E)

Measurements: See Table I.

Female: Body C-shaped in relaxed specimens. Cuticle 1.0-2.0 µm thick, with fine transverse striation visible under SEM; a few somatic setae. Body pores absent. Lip region continuous with body contour, asymmetric, conoid and 22-25 µm wide; inner labial papillae conical, outer labial setae 13-17 (15.4±0.3) µm long, 50-94 (72)% of lip region width at their base; cephalic setae 5-10 (8.0±0.6) µm long, slightly posterior to the whorl of outer labial setae; oral aperture surrounded by three small lips. Stoma with thickened dorsal wall: dorsal tooth

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Table I. Morphometric characteristics of *Tripylina bravoae* sp. n., *T. ixayocensis* sp. n., *T. tlamincasensis* sp. n. *and T. montecilloensis* sp. n. In µm except L in mm and in the form average ± standard deviation (range).

	Species n	*T. bravoae* sp. n.			*T. ixayocensis* sp. n.		*T. tlamincasensis* sp. n.		*T. montecilloensis* sp. n.	
Character		Holotype ♀	Paratypes 11♀♀	Paratypes 10♂♂	Holotype ♀	Paratypes 10♀♀	Holotype ♀	Paratypes 19♀♀	Holotype ♀	Paratypes 12♀♀
L		1.60	1.6±0.6 (1.2-1.9)	1.7± 0.9 (1.5-2.0)	1.6	1.4±0.5 (1.1-1.6)	0.81	0.80±0.02 (0.67-0.91)	1.0	0.94±0.38 (0.8-1.2)
a		43	44±5.4 (36-53)	49±1.6 (42-58)	35.1	33±1.2 (25-38)	23	22±0.01 (18-26)	26	24±1.2 (19-33)
b		5.4	5.7±0.6 (4.9-6.9)	5.4±0.2 (5.3-7.3)	6.2	5.9± 0.09 (5.4-6.3)	5.1	4.8±0.1 (3.8-5.9)	5.8	6.0±0.5 (5.2-6.2)
c		36.2	30±5.1 (22-36)	31±1.8 (23-39)	17.5	20±1.3	21.5	18±0.5 (15-23)	11.1	13±0.1 (9.8-15.5)
c'		1.7	2.1±0.4 (1.7-3.1)	1.9±0.1 (1.5-2.8)	2.8	2.6±0.2 (1.8-3.5)	1.5	2.0±0.08 (1.5-2.8)	3.1	2.7±0.1 (2.1-3.5)
V		82	80±1.6 (78-83)	-	64	66±1.1 (59-71)	67	67±0.6 (63-73)	65	64±0.6 (62-69)
Body diameter		37	36±2.3 (23-44)	36±1.0 (30-40)	47	43± 1.1 (38-47)	35	24±2.6 (12-42)	40	24±2.6 (12-42)
Anal body diameter		26	26±0.7 (23-30)	30±1.1 (25-37)	34	29± 0.9 (23-34)	25	22±0.5 (18-25)	30	40±1.8 (32-58)
Dorsal tooth to ant. buccal cavity		25	15.8±0.5 (18-35)	24±1.1 (20-27)	18	17±0.5 (15-20)	13	16±0.51 (10-19)	16	16±0.5 (14-20)
Nerve ring		123	110±72 (90-123)	100±6.3 (94-113)	123	109± 5.5 (91-123)	92	75±3.7 (55-92)	87	81±3.7 (60-96)
Pharynx length		297	280±8.0 (236-320)	247±5.0 (236-302)	264	241±6.4 (198-264)	160	166±6.3 (113-207)	179	167±6.2 (141-207)
Pharynx (% body length)		18.5	17.6±0.6 (14-21)	16±0.6 (14-19)	16.0	17±0.3 (15.8-18.6)	19.8	21±0.58 (17-26)	17.3	18±0.32 (16-20)
Tail length		44	53±1.6 (44-56)	58±2.8 (46-72)	94	74±6.0 (42-94)	37.7	45±1.3 (34-57)	93	74±3.5 (57-93)
Tail (% body length)		2.8	3.3±0.2 (2.8-4.5)	5.2±0.3 (2.5-4.2)	5.7	5.2±0.3 (3.9-6.4)	4.7	5.7±0.2 (4.4-6.8)	9.0	7.9±0.3 (6.4-10)
Spinneret length		2	1-2	1.8±0.2 (1-2)	2	2	1-2	1-2	2	1-2

small, located at 15-22 (18.5±0.8) µm from the anterior end; subventral teeth 4-7 (5.3±0.4) µm behind the dorsal tooth. Amphid aperture circular, slightly posterior to the level of dorsal tooth. Two cervical setae, located 23-85 (68±4.8) µm and 68-132 (94±4.6) µm from the anterior end. Cardia 15-20 (17±0.7) µm long and 28-35 (31±0.8) µm wide, with two pericardiac cells; distance from pharyngeal base to vulva 518-792 (702±30.4) µm, and 820-1291 (1105±41.0) µm to the anus. A large portion of a prey nematode was observed within the intestine of one specimen (Fig. 5E). Nerve ring at 91-122 (108±5.6) µm, excretory pore at 120-170 (138±5.7) µm from anterior end. Genital system monodelphic-prodelphic, 141-264 (213±13.5) µm long or 9.8-19 (14.5±1.2)% of body length: ovary reflexed, vagina lacking sclerotized structures, and vulva with protruding lips and ornamented

with fine grooves (Fig. 4B,D). Tail curved ventrad in a spiral, with a pair of latero-dorsal setae, terminating in a small spinneret. Pseudocoelomocytes ovoid, bearing a distinct nucleus each.

Male: Unknown.

Diagnosis: *Tripylina ixayocensis* sp. n. is characterized by the absence of body pores, presence of a few somatic setae, presence of two cervical setae, subventral teeth located behind the small dorsal tooth, and absence of sclerotized structures in the vaginal lips (Tables I & II).

Relationships: The new species is similar to *T. arenicola* (de Man, 1880) Brzeski, 1963, in the position of the subventral teeth and the location of vulva, but it differs from this in its generally larger size (body length 1.1-1.65

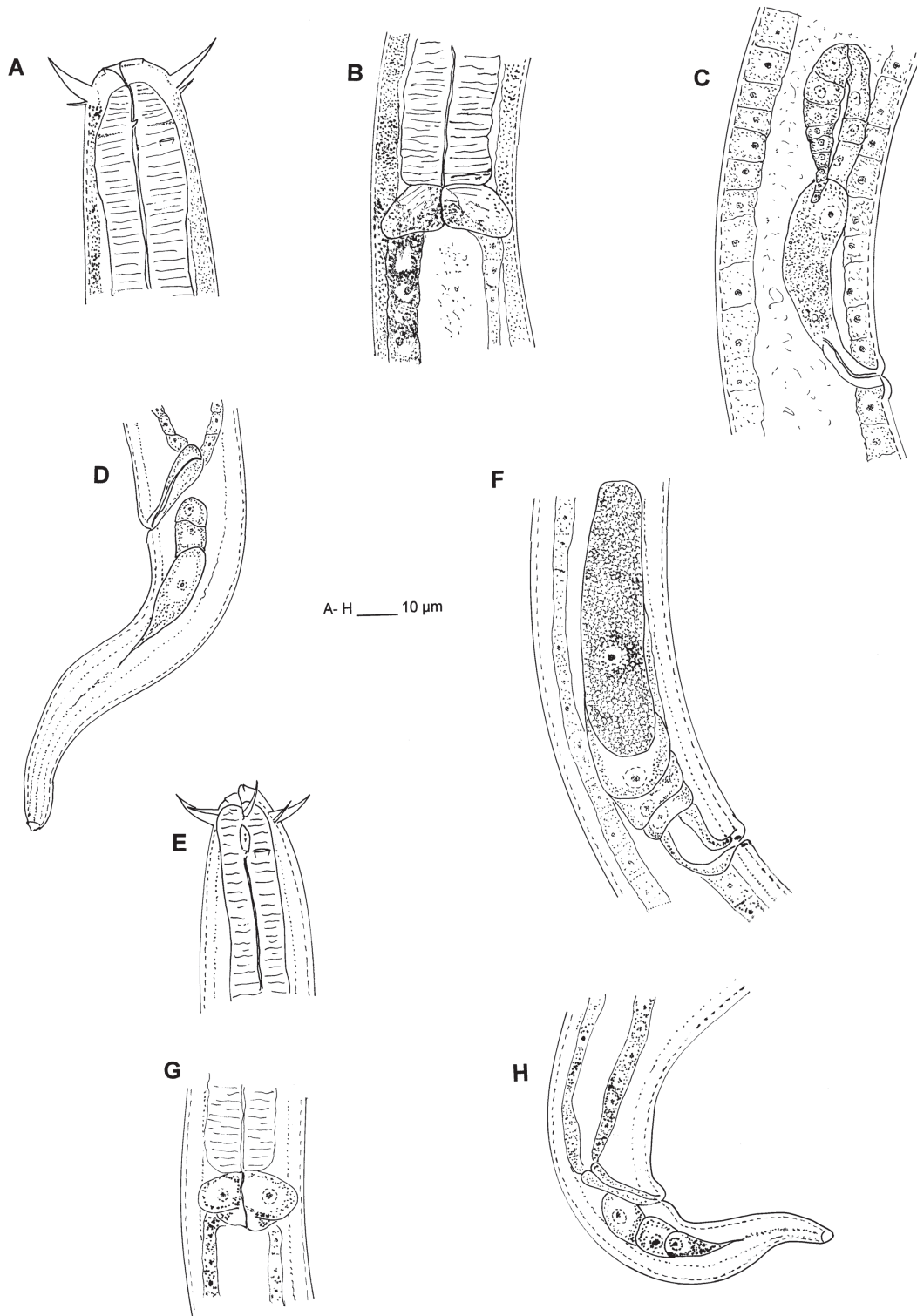*J. Nematode Morphol. Syst.*, 15 (2): 71-86 (2012)

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FIGURE **4. A-D:** *Tripylina ixayocensis* **sp. n.** (female). A: Anterior end. B: Pharyngeo-intestinal junction. C: Vulva region. D: Tail. **E-H:** *Tripylina tlamincasensis* **n. sp.** (female). E: Anterior end. F: Vulva region. G: Oesophago-intestinal junction. H: Tail.
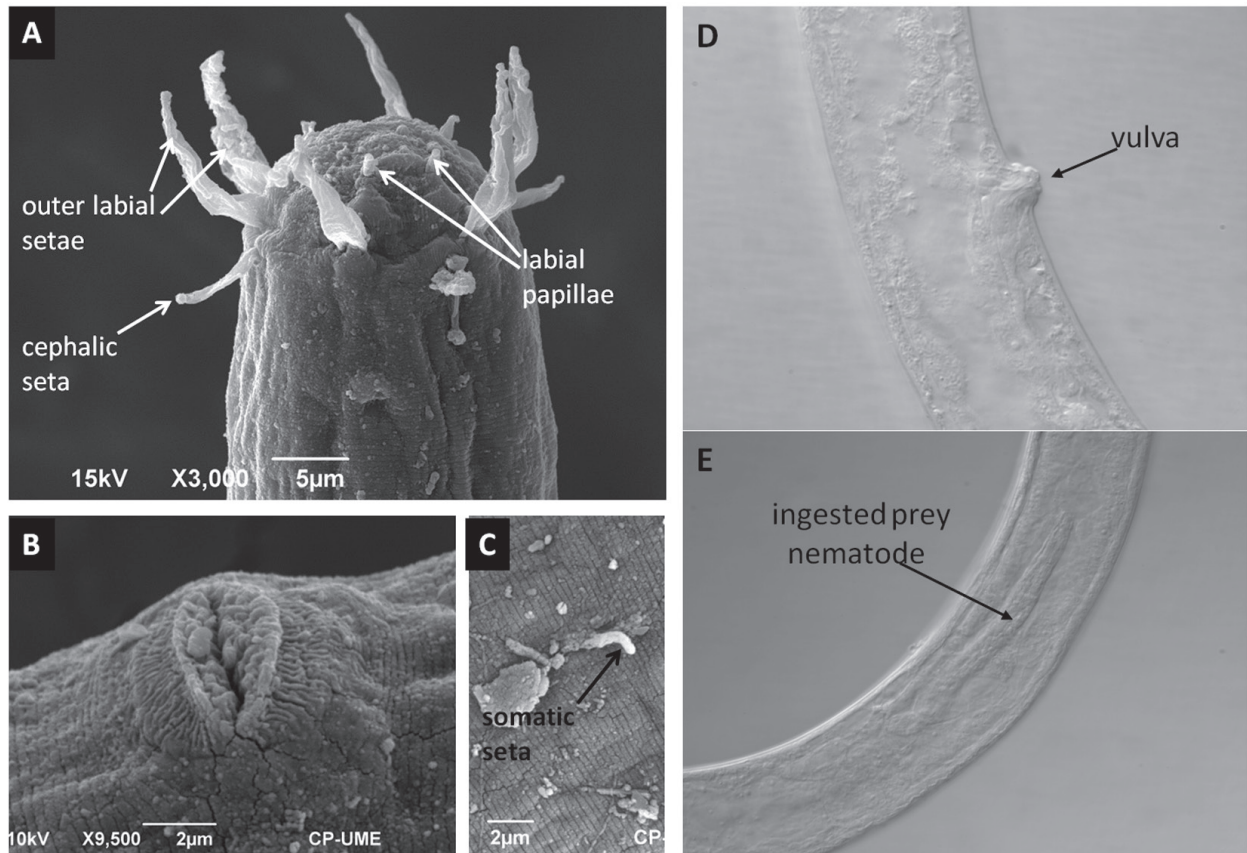
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FIGURE 5. A-C: *Tripylina ixayocensis* **sp. n.** (female, SEM). A: Anterior end. B: Vulva latero-ventral view. C: Somatic seta. **D-E:** *Tripylina ixayocensis* **sp. n.** (female, SEM). D: Protruding vulva. E: Ingested prey nematode in intestine.

vs 0.81-1.25 mm), longer outer labial setae (13-17 *vs* 10-14 μm), absence (*vs* presence) of vaginal sclerotization, and presence (*vs* absence) of a pair of cervical setae. It is also similar to *T. ymyensis* Tahseen & Nusrat, 2010 in the posterior position of the subventral teeth, the length of labial outer setae, the location of vulva and in the absence of body pores, but it can be distinguished from this in having larger general size (body length 1.3-1.6 *vs* 1.0-1.1 mm), generally longer cephalic setae (5.0-10.0 *vs* 4.0-6.0 μm), larger vulva-anus distance (325-499 *vs* 278-290 μm), and in having a conspicuous excretory pore.

Type locality and habitat: México, México state, Texcoco, forested area of San Pablo Ixayoc, altitude 2588 meters a.s.l. (N19° 28.114', W98° 47.254'), in moss on trunk of oak tree *Quercus peduncularis*.

Type specimens: Female holotype on slide CNHE 8194 and female paratypes on slide CNHE 8196 (2♀♀), deposited in CNHE; other female paratypes distributed as follows: CNCP (5♀♀), UCRNC (1♀) and WaNeCo WT3567 (1♀).

Etymology: The specific epithet refers to geographical origin of the type specimens, San Pablo Ixayoc, Texcoco, México State, México.

Tripylina tlamincasensis sp. n.
(Figs 4E-H, 7A-C)

Measurements: See Table I.

Female: Body C-shaped in relaxed specimens, with tail curved ventrad. Cuticle 1.0-3.0 μm thick, bearing fine transverse striation under SEM. Body pores and somatic setae absent. Lip region asymmetric, conoid and 12-18 μm wide; oral aperture with three small triangular lips, each with a pair of conical inner labial papillae at its base; outer labial setae 10-15 (12.5±0.36) μm long or 71-100 (84.6)% of lip region width at its base; cephalic setae 4-6 (5.0±0.09) μm long, in a whorl 1 μm behind the outer labial setae. Amphid apertures small, elliptical, close to the level of the dorsal tooth and located at 10-20 (15.1±0.5) μm or 0.6-1.2 times the lip region width from

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the anterior end. Two cervical setae were observed in 10 out of 19 specimens, the anteriormost of which situated at 23-85 (49) µm and the posteriormost one at 82-132 (94) µm from the anterior end; presence or absence could not be determined on the remaining specimens due to their positions on the slide. Excretory pore indiscernible. Dorsal wall of stoma not thickened; dorsal tooth small, located at 10-19 (15.6±0.5) µm from the anterior end and at 3-5 (3.9±0.2) µm behind the subventral teeth. Cardia relatively small, 8.8-10.1 (9.5±0.3) µm long and 20-27 (24±0.4) µm wide, with two pericardiac cells. Distance from base of pharynx to vulva 306-467 (371±9.2) µm and to anus 504-664 (586±11.8) µm. Nerve ring at 55-92 (75±3.7) µm from the anterior end. Genital system monodelphic-prodelphic, 64-160 (104±6.2) µm long or 7.5-22 (12.9±0.9)% of the body length: ovary reflexed, vagina with very small sclerotized pieces, and vulva radially grooved, with non-protruding lips (Fig. 7C). Tail curved ventrad, with twisted terminus, ending in a small spinneret; one pair of latero-dorsal setae present. Pseudocoelomocytes ovoid, with a distinct nuclei.

Male: Unknown.

Diagnosis: *Tripylina tlamincasensis* sp. n. is characterized by having two cervical setae, stoma with non-thickened dorsal wall, dorsal tooth located behind the subventral teeth, absence of a discernible excretory pore, very small sclerotized pieces around the vagina, and one pair of latero-dorsal setae on tail (Tables I & II).

Relationships: The new species is similar to *T. longa* Brzeski & Winiszewska-Ślipińska, 1993 in having two cervical setae and the position of the dorsal tooth behind the subventral teeth, but it differs from this in its smaller general size (body length 0.67-0.91 *vs* 1.48-1.72 mm), absence (*vs* presence) of a discernible excretory pore, shorter outer labial setae (10-15 *vs* 15-18 µm), shorter pharynx (113-207 *vs* 216-242 µm), absence (*vs* presence) of post-uterine sac, very small (*vs* large) sclerotized pieces around the vagina, and more anterior vulva (V = 63-73 *vs* V = 76-80). It is also similar to *T. sheri* Brzeski, 1963 in having asymmetric lip region, anterior position of the subventral teeth, length of the cephalic setae, pharynx length and vulva position, but it can be separated from this by its smaller general size (body length 0.67-0.91 *vs* 0.91-1.38 mm), stoma with non-thickened dorsal wall, length of the outer labial setae, and absence of a discernible excretory pore. *Tripylina tlamincasensis* sp. n. is close to *T. valiathani* Tahseen & Nusrat, 2010 in the lengths of rectum and caudal region as well as the absence of body pores and excretory pore, but it can be distinguished from this in having smaller general size (body length 0.67-0.91 *vs* 0.9-1.0 mm), more stout body (*a* = 18-26 *vs a* = 31-39), and in the absence of body setae. Finally, the new species is also similar to *T. tearoha* Zhao, 2009 in the length of the outer labial setae, the position of the vulva and length of the rectum and the presence of one pair of short post-anal setae, but it differs from this in its smaller general size (body length 0.67-0.91 *vs* 0.96-1.1 mm), absence of body

pores, and shorter pharynx (113-207 *vs* 610-716 µm) and caudal region (34-57 *vs* 62-77 µm).

Type locality and habitat: México, México State, Texcoco, Tlamincas, archeological site at Cerro Tetzcuitzinco, altitude 2448 m a.s.l. (N19° 29.874', W98° 49.072'), in moss on rock.

Type specimens: Female holotype on slide CNHE 8203 and female paratypes on slide CNHE 8205 (6♀♀), deposited in CNHE. Other female paratypes distributed as follows: CNCP (6♀♀), UCRNC (5♀♀) and WaNeCo WT3568 (1♀).

Etymology: The specific epithet refers to geographical origin of the type specimens, Tlamincas, Texcoco, México State, México.

Tripylina montecilloensis sp. n.
(Figs 6A-H & 7D-G)

Measurements: See Table I.

Female: Body C-shaped upon fixation. Cuticle 1.0-3.0 µm thick, with fine transverse striation visible under SEM; body pores absent but somatic setae, each surrounded by a cuticular ring at the base, sparsely distributed along the body (Fig. 7G). Lip region asymmetric, conoid, continuous with body contour, 14-23 (18.4±0.7) µm wide at the base of the outer labial setae; oral aperture surrounded by three lips; two conical inner labial papillae at the base of each lip; outer labial setae 10-15 (13±0.5) µm long or 55-83 (73)% of lip region width at their base; cephalic setae conical, 5-10 (6.5±0.5) µm long, 1.5 µm behind the outer labial setae. Amphid apertures small, oval, at level of the dorsal tooth, 15-26 (18.1±0.9) µm or 0.7-1.3 times the lip region width from the anterior end. Two ventromedian cervical setae, 22 µm apart, at 60-100 µm behind the lip region. Dorsal wall of stoma not thickened; dorsal tooth small, located at 14-20 µm (16±0.5) from the anterior end and 3-6 µm behind the subventral teeth. Cardia relatively small, 10-16 (12.7±0.6) µm long and 10-29 (23.6±1.6) µm wide, with two pericardiac cells. Distance from pharynx base to vulva 349-556 (442±21) µm and to anus 514-1034 (723±42) µm. Nerve ring at 60-96 (81.0±3.8) µm from the anterior end. Excretory pore indiscernible. Genital system monodelphic-prodelphic, 75-174 µm long or 9-19% of body length: ovary reflexed, vagina lacking sclerotized pieces, and vulval lips not protruding. Tail curved ventrad, ending in a small spinneret; latero-dorsal setae lacking. Pseudocoelomocytes spindle-shaped, acute at the poles, with distinct nuclei.

Male: Unknown.

Diagnosis: Tripylina montecilloensis sp. n. is characterized by the absence of body pores, indiscernible excretory pore, anterior position of the subventral teeth relative to the small dorsal tooth, presence of two cervical

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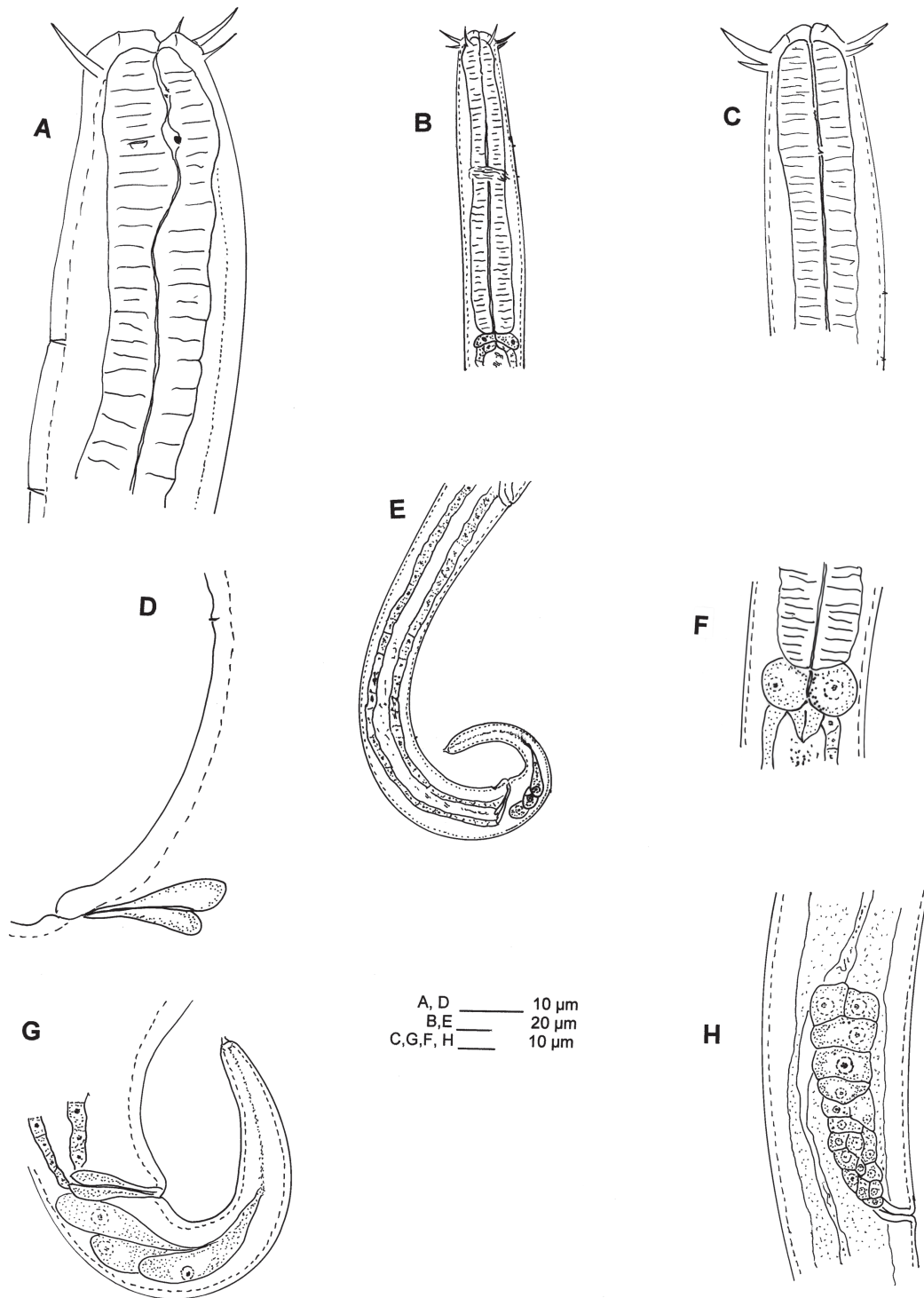*J. Nematode Morphol. Syst.,* 15 (2): 71-86 (2012)

FIGURE 6. *Tripylina montecilloensis* **sp. n.** (female). A,C: Anterior end. B: Pharynx. D: Preanal papillae. E: Posterior end. F: Pharyngeo-intestinal junction. G: Tail. H: Vulva region.
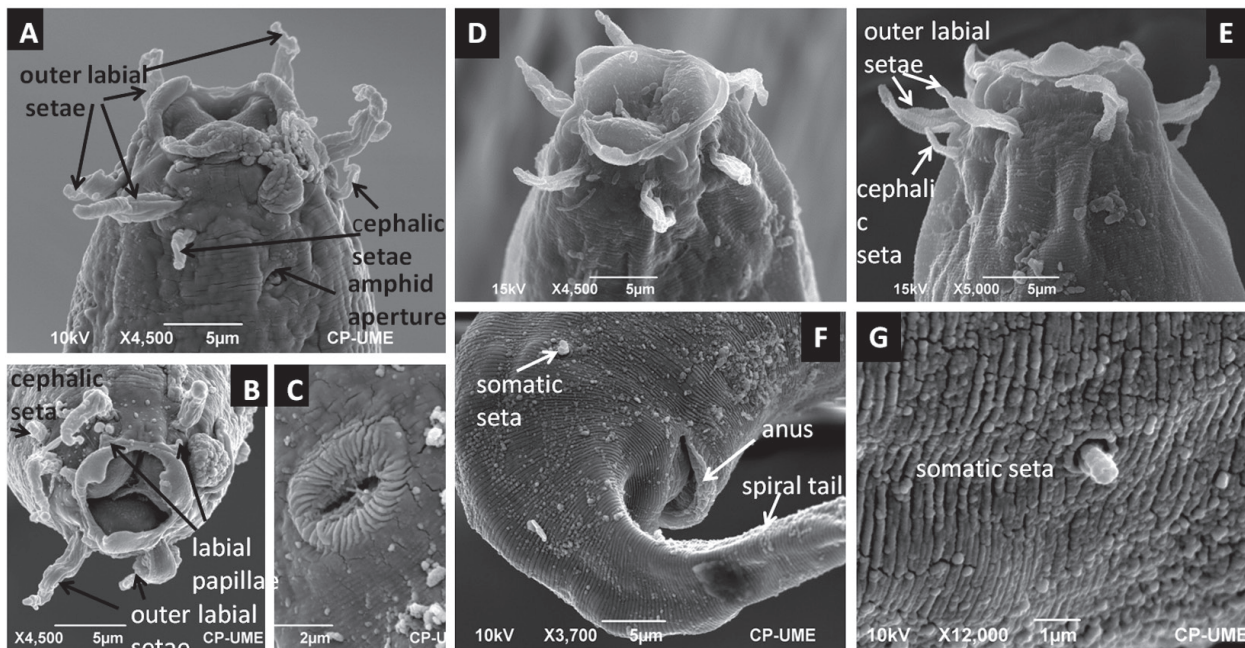
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FIGURE 7. **A-C:** *Tripylina tlamincasensis* **sp. n.** (female, SEM). A: Anterior end. B: Face view. C: Vulva ventral view. **D-G:** *Tripylina montecilloensis* **sp. n.** (female, SEM). D: Face view. E: Anterior end. F: Posterior region. G: Somatic seta.

setae, non-protruding vulval lips, absence of vaginal sclerotized pieces, and absence of latero-dorsal setae on tail (Tables I & II).

Relationships: The new species is similar to *T. macroseta* (Vinciguerra & La Fauci, 1978) Tsalolikhin, 1983 in the size of the outer labial setae and the position of both the subventral teeth and the vulva, but it differs from this in the absence of body pores, the presence of two cervical setae, and the absence of vaginal sclerotized pieces. *Tripylina montecilloensis* sp. n. is also close to *T. tlamincasensis* sp. n. in the absence of body pores, length of the outer labial setae, position of vulva and absence of a discernible excretory pore, but it can be distinguished from this in the absence of vaginal sclerotized pieces and tail length (57-93 *vs* 34-57 μm).

Type locality and habitat: México, México State, Texcoco, Campus Montecillo, altitude 2220 m a.s.l. (N 19° 27' 48.042", W 98° 54' 15.451"), in soil from an area of nursery trees at the Colegio de Postgraduados.

Type specimens: Female holotype on slide CNHE 8197 and females paratypes on slide CNHE 8199 (4♀♀), deposited in CNHE; other female paratypes distributed as follows: CNCP (4♀♀), UCRNC (2♀♀) and WaNeCo WT3577 (1♀).

Etymology: The specific epithet refers to geographical origin of the type specimens, Montecillo, Texcoco, México State, México.

Molecular characterisation

Sequences of the four new species herein described were obtained from DNA extracts of pooled (3-5) individuals. Direct sequencing of the PCR products yielded high-quality electrophorograms with no sequence polymorphisms or differences between individuals (*T. bravoae*). New SSU sequences are deposited in GenBank (accession numbers in Fig. 8).

Tripylina bravoae sp. n. was distinct in nucleotide sequence when compared to all other congeners, including sequences from Genbank (*i.e., T. tamaki* Zhao, 2009, *T. tearoha, T. manurewa* Zhao, 2009, *T. arenicola*, and *Tripylina* sp. *cf. affinis*), with 5-14 sequence (pairwise) differences when compared to these taxa. In contrast, pairwise comparisons of the other *Tripylina* species showed 0-2 sequence differences. The Bayesian posterior probability (BPP) for the monophyly of *T. bravoae* sp. n. individuals was 100% (Fig. 8). The other sampled *Tripylina* species formed the sister group to *T. bravoae* sp. n. with high (95%) posterior probability. There was no resolution of species relationships within this clade consisting of *T. ixayocensis* sp. n., *T. tlamincasensis* sp. n., *T. montecilloensis* sp. n., *T. tamaki, T. tearoha, T. manurewa, T. arenicola*, and

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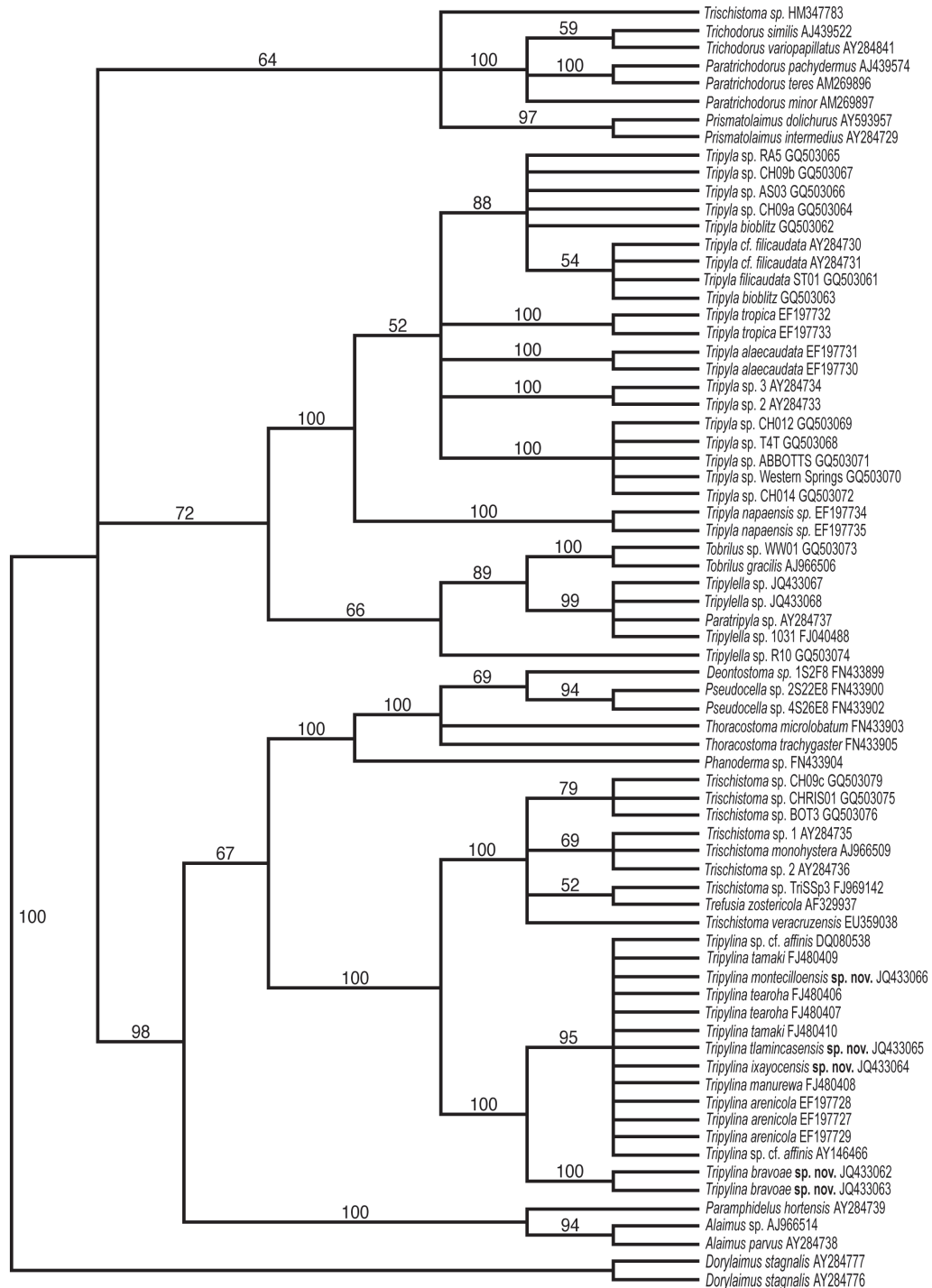
FIGURE 8. Bayesian posterior probability consensus tree inferred from the SSU rDNA sequence data. MCMC posterior clade probabilities are shown above branches. GenBank accession numbers are listed for each taxon and isolate designations are given when provided in the original publication or accession. New species are indicated in boldface. Tripylina sp. cf. affinis was originally deposited in GenBank (AY146466) as Tripyla sp. cf. affinis, but both phylogenetic analysis and reexamination of the archived microscopic images (by the GenBank authors, P. Mullin and T. Powers) confirm that this is a species of Tripylina.

Tripylina sp. *cf. affinis. Tripylina* sp. *cf. affinis* was originally deposited in GenBank (AY146466) as *Tripyla* sp. *cf. affinis*, but both phylogenetic analysis and reexamination of the archived microscopic images (by the GenBank authors, P. Mullin and T. Powers) confirm that this is a species of *Tripylina* and not *Tripyla affinis* de Man, 1880.

The clade representing all *Tripylina* sequences was monophyletic (100% BPP) and was the sister-group to a clade including nine representatives of *Trischistoma* Cobb, 1913 plus *Trefusia* de Man, 1893 (100% BPP). Support for this more comprehensive clade (*Tripylina, Trefusia, Trischistoma*) was also high (100% BPP, Fig. 8). One *Trischistoma* sequence obtained from GenBank (HM347783) was placed in an unexpected position (with *Trichodorus* Cobb, 1913, *Paratrichodorus* Siddiqi, 1974 and *Prismatolaimus* de Man, 1880) as judged from the strong support for monophyly of eight other taxa from this genus. The phylogenetic analysis indicated that the clade represented by *Tripylina*, *Trischistoma*, and *Trefusia* (Tripyloidea and Trefusiida) was strongly supported as nested within a more comprehensive group of Enoplea including members of Oxystominoidea and Enoploidea (Fig. 8).

Discussion

Based on descriptions by Brzeski and Winiszewska-Ślipińska (1993), Zullini (2006), Andrássy (2007) and Zhao (2009), features characterizing the genus *Tripylina* include the arrangement of the six outer labial and four cephalic setae in a single whorl, the thin, non-striated cuticle, and differences among species in the relative positions of dorsal tooth and subventral teeth. The tail is 1.5 to 4.5 anal body diameters long and S-shaped, terminated in a spinneret. Females are monodelphic-prodelphic, lacking a post-uterine sac and with vulva at 59-83% of body length. Males, which are rare, have supplements confined to the precloacal region. While indiscernible by light microscopy, somatic setae and fine cuticular striation are visible in previously published SEM images (Tahseen & Nusrat, 2010).

The existence of post-uterine sac in females and the presence of males were observed in *T. bravoae* sp. n. Specimens were examined by Dr. I. Andrássy who concurred that the new species is close to *T. longa* Brzeski & Winiszewska-Ślipińska, 1993 except for the differences we indicate in the differential diagnosis and the presence of a post-uterine sac, which is usually absent in *Tripylina* (Andrássy, pers. comm.). Our male specimens of *T. bravoae* sp. n. have only a single testis. Andrássy (2007) indicates two testes in the diagnostic characters of the Tripylidae; however, in a recent description of new species of *Trischistoma* by Andrássy (2011), males are described as monorchic. At our request, Dr. Grazyna Winiszewska re-examined the type specimens of *T. longa* and confirmed that all the females have a more or less visible post-uterine sac and that males appear to have a single testis

(Winiszewska, pers. comm.). It is clear that the original description of the genus by Brzeski and Winiszewska-Ślipińska (1993) was incomplete. Our molecular data independently confirms that *T. bravoae* sp. n. belongs to the genus *Tripylina*. Thus, a post-uterine sac and single testis are confirmed for both *T. bravoae* and *T. longa* and the possible occurrence of these characters for species of *Tripylina* should now be included in the diagnosis of the genus. Other recently described discriminatory morphological characters of *Tripylina* are the presence of lateral cervical setae (Zhao, 2009), scattered somatic setae and fine cuticular striation (Tahseen & Nusrat, 2010) (Table II).

Our purpose in analyzing sequence data was to compare the new *Tripylina* species with species already represented in GenBank, and to determine whether these and previously identified *Tripylina* were monophyletic. As previously reported (Zullini, 2006; Zhao & Buckley, 2009; Cid del Prado-Vera *et al.*, 2010), SSU phylogenies do not support monophyly of Tripylidae in the traditional sense, and strongly support a close relationship between *Tripylina* and Enoplida (Zhao & Buckley, *op. cit.*; Cid del Prado-Vera *et al.*, *op. cit.*). Similarly, shared substitutions in loops of SSU rDNA hairpin structures support this relationship between *Tripylina* and members of Enoplida (Zhao *et al.*, 2012). There is strong molecular support for most recent common ancestry of *Trischistoma*, *Tripylina*, *Trefusia* and a more comprehensive clade including representatives of other enoplean families (Alaimidae, Leptosomatidae, Phanodermatidae) not traditionally considered closely related to Tripylidae. The monophyly of *Trischistoma* and *Trefusia* was previously recovered based on near full-length SSU data (Holterman *et al.*, 2006), and previous analysis of partial SSU sequences added *Tripylina* to this clade, as sister to *Trefusia* and *Trischistoma* (Cid del Prado-Vera *et al.*, *op. cit.*). Phylogenetic analyses of these additional partial SSU sequences resolved the four new species herein described as part of the *Tripylina* clade, with *T. bravoae* sp. n., also characterized by the presence of males and a post-uterine sac in females, as a distinct lineage. Resolution of *T. bravoae* sp. n. as the sister group to the other *Tripylina* species provides molecular support for its recognition as a new species. In contrast, partial SSU sequences did not resolve branching relationships among the other *Tripylina* species, consistent with previous analyses (Cid del Prado-Vera *et al.*, *op. cit.*), whereas partial LSU sequences showed greater phylogenetic resolution among certain *Tripylina* species (Zhao, 2009). The lack of resolution among most *Tripylina* species based on partial SSU sequences reflects low levels of sequence difference rather than character conflict. With the exception of comparisons involving *T. bravoae* sp. n., pairwise comparisons of SSU sequences from the new *Tripylina* species show only two or fewer nucleotide differences. The lack of independent molecular phylogenetic support for delimiting several species as distinct is likely due to the relatively conservative rate of change for this SSU region, and cannot by itself be interpreted as falsifying the observed morphological differences with respect to species diagnosis.

Table II. Morphometric, morphological and anatomical characters useful in differentiating species of *Tripylina*.

Species / Character	L	a	b	c	c'	V	exc. pore	sclerot. pieces	subvent. vs. dorsal	Cervical setae	Somatic setae	Post. uter. sac	Body pores
arenicola	0.8-1.2	21-27	4.7-5.6	13-18.7	1.9-2.8	63-70	Yes	Yes	Post.	Yes	?	No	Many
bravoae	1.2-1.9	36-53	4.9-6.9	22-36	1.7-3.1	78-83	No	Yes	Ant.	Yes	Yes	Yes	No
ixayocensis	1.3-1.6	30-38	5.5-6.2	15-26	2.0-3.5	59-71	Yes	No	Post.	Yes	Yes	No	No
kaikoura	1.5-1.6	22-23	6-6.2	14-15.8	2.7-3.1	65-66	Yes	No	Ant.	No	?	No	No
longa	1.5-1.7	33-40	6.3-7.4	25-30	1.7-2.6	76-80	Yes	Yes	Ant.	Yes	?	Yes	Many
macroseta	0.8-0.9	20-24	4.8-5.3	12-13	3.2	62-65	Yes	Yes	Ant.	No	?	No	?
manureva	1-1.2	24-29	5.4-6.1	10.7-17.8	2.3-3.6	64-67	Yes	Yes	Ant.	Yes	?	No	Many
montecilloensis	0.8-1.2	19-33	5.2-6.2	9.8-15.5	2.1-3.5	62-69	No	No	Ant.	Yes	No	No	No
sheri	0.9-1.4	25-38	5.2-6.4	13-22	2.2-4.5	64-70	Yes	Yes	Ant.	Yes	?	No	?
stramenti	1.5-1.7	30-36	5.7-6.2	13-16.8	3.3-3.5	60-64	No	No	Ant.	Yes	No	No	No
tamaki	0.9-1.2	20-22	5.8-6.5	14.5-15	2.9-3.2	64-67	Yes	Yes	Ant.	Yes	No	No	No
tearoha	1.0-1.1	25-30	5.3-5.9	13.6-17.7	2.9-3.3	65	Yes	No	Ant.	Yes	?	No	Many
tlamincaensis	0.7-0.9	18-26	3.8-5.9	15-23	1.5-2.8	63-73	No	Yes	Ant.	Yes	No	No	No
ursulae	0.8-1.2	22-26	4.8-5.7	13.6-27	3.5	61-67	No	No	Post.	Yes	No	No	No
valiathani	0.9-1.0	31-39	4.8-5.5	21-25.8	s1.5-1.9	66-68	No	Yes	Ant.	Yes	Yes	No	No
yeatesi	1.5-1.6	29-30	5.9-6.0	18-26	2.0-2.6	67-68	Yes	Yes	Ant.	No	?	No	No
ymyensis	1.0-1.1	26-29	4.8-5.5	16-19	14.7-5.2	66-67	No	No	Post.	No	Yes	No	No

Exc. pore = excretory pore; Sclerot. pieces = sclerotized pieces surrounding vagina; Subvent. vs. Dorsal = position of subventral teeth in relation to dorsal tooth; Cervical setae = somatic setae in pharyngeal region; Somatic setae = setae scattered along body posterior to pharyngeal region; Post. ut. sac = presence of a post-uterine sac

Emended diagnosis of the genus *Tripylina*

Tripylidae, Tripylinae. Cuticle thin, smooth under LM but bearing fine transverse striation under SEM, which also reveals minute somatic setae sparsely scattered over the body in some species. Lip region asymmetric, not set off from body contour, with three lips, each with two labial papillae at the base; outer labial setae strongly developed; the six outer labial and the four cephalic setae may be in a single whorl or slightly separated. Stoma narrow, with three small teeth. Pharyngo-intestinal junction with cardia and two pericardiac cells. Female genital system monodelphic-prodelphic, ovary reflexed, with or without post-uterine sac, vagina with or without sclerotized pieces, and vulva at 59-83% of body length. Males present or absent, with a single testis; spicules may or may not be enclosed in a muscular pouch; five pre-cloacal supplements. Caudal region a short spiral in both sexes, with well-developed caudal glands and spinneret.

Key to the species of *Tripylina:*

1a - V > 75, post-uterine sac present 2
1b - V < 75, post-uterine sac absent 3
2a - Excretory pore present; body pores numerous ... *longa*
2b - Excretory pore absent; no body pores .... *bravoae* sp. n.
3 – Vagina without sclerotized pieces 4
3b – Vagina with sclerotized pieces 10
4a - Excretory pore indiscernible 5
4b - Excretory pore prominent 8
5a - Subventral teeth in front of dorsal tooth 6
5b - Subventral teeth behind the dorsal tooth 7
6a - L ≥ 1.5 .... *stramenti*
6b - L < 1.5 .... *montecilloensis* sp. n.
7a - a ≥ 26; c' ≥ 4.7.. *ymyensis*
7b - a ≤ 26; c' < 4.7 .... *ursulae*
8a - Subventral teeth behind the dorsal tooth ... *ixayocensis*
8b - Subventral teeth in front of dorsal tooth 9
9a - L > 1.4; body pores absent .... *kaikoura*
9b - L < 1.4; body pores numerous .... *tearoha*
10a - Subventral teeth in front of dorsal tooth 11
10a - Subventral teeth posterior to dorsal tooth .... *arenicola*
11a - Excretory pore indiscernible 12
11b - Excretory pore prominent 13
12a - a < 28; somatic setae absent .... *tlamincasensis*
12b - a > 28; somatic setae present .... *valiathani*
13a - L > 1.4, a >= 29 .... *yeatesi*
13b - L < 1.4, a <= 29 14
14a - L ≤ 0.9; cervical setae absent .... *macroseta*
14b - L ≥ 0.9; cervical setae present 15
15a - Body pores numerous .... *manurewa*
15b - No or few body pores 16
16a - a > 24 .... *sheri*
16b - a < 24 .... *tamaki*

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