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Diversity of *Rhabdochona mexicana* (Nematoda: Rhabdochonidae), a parasite of *Astyanax* spp. (Characidae) in Mexico and Guatemala, using mitochondrial and nuclear genes, with the description of a new species

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Abstract

Among fish parasitic nematodes *Rhabdochona* is one of the most speciose genera, with *c.* 100 species. Twelve congeneric species occur in Mexican freshwater fishes, in a region located between the Nearctic and Neotropical biogeographical regions. Host association and biogeographical history have determined the high species richness of *Rhabdochona* in Mexico. One of these species, *Rhabdochona mexicana*, is highly specific to the characid genus *Astyanax*. Characids are a group of freshwater fish with Neotropical affinity. In this paper, we explore the genetic diversity of *R. mexicana* through samples obtained from populations of *Astyanax* spp. across river basins of Mexico and Guatemala. Sequences of one mitochondrial and two ribosomal genes were obtained from 38 individuals and analysed using Maximum Likelihood and Bayesian Inference analysis. Phylogenetic analyses using *cox*1, and a concatenated alignment of 18S + 28S + *cox*1 recovered two genetic lineages. One of them corresponded with *R. mexicana sensu stricto*; this lineage included three reciprocally monophyletic subgroups; the other lineage was highly divergent and represented a putative candidate species. A detailed morphological study was conducted to corroborate the molecular findings. We describe a new species herein and discuss the implications of using molecular tools to increase our knowledge about the diversity of a speciose genus such as *Rhabdochona*.

Introduction

The nematode genus *Rhabdochona* Railliet, 1916 comprises *c.* 100 species of intestinal parasites of freshwater fish in all zoogeographical regions except Antarctica (Moravec, 2010). In the Americas, the genus contains 21 species, although they do not comprise a monophyletic assemblage, and their presence in a wide variety of fish species suggests an evolutionary history with extensive ecological host extension and host-switching events (Mejía-Madrid *et al.*, 2007a, b). Most of the congeneric species are highly host specific, usually at the level of host family (Moravec *et al.*, 2012). In Mexico, 12 species of *Rhabdochona* have been reported as parasites of freshwater fishes, i.e. *R. acuminata* Molin, 1860, *R. ahuehuellensis* Mejía-Madrid and Pérez-Ponce de León, 2003, *R. canadensis* Moravec and Arai, 1971, *R. cascadilla* Wigdor, 1918, *R. guerreroensis* Caspeta-Mandujano, Aguilar-Aguilar and Salgado-Maldonado, 2002, *R. ictaluri* Aguilar-Aguilar, Rosas-Valdez and Pérez-Ponce de León, 2010, *R. kidderi* Pearse, 1936, *R. lichtenfelsi* Sánchez-Álvarez, García-Prieto and Pérez-Ponce de León, 1998, *R. mexicana* Caspeta-Mandujano, Moravec and Salgado-Maldonado, 2000, *R. ovifilamenta* Weller, 1938, *R. salgadoi* Caspeta-Mandujano and Moravec, 2000, and *R. xiphophori* Caspeta-Mandujano, Moravec and Salgado-Maldonado, 2001 (Garrido-Olvera *et al.*, 2006; Pérez-Ponce de León *et al.*, 2009, 2010; Aguilar-Aguilar *et al.*, 2010; Moravec *et al.*, 2012). Seven of the 12 species (58%) are endemic and specific to a particular group of freshwater fish. The presence of the remaining five species of *Rhabdochona* in freshwater fish of Mexico reflects the transitional nature of the area, as the country lies between the Nearctic and Neotropical biogeographical regions (Pérez-Ponce de León, 2003; Pérez-Ponce de León and Choudhury, 2005). For instance, *R. canadensis*, *R. ovifilamenta* and *R. cascadilla* are typically associated with Nearctic freshwater fish groups such as catostomids and cyprinids, and a few others (Hoffman, 1999; Arai and Smith, 2016); meanwhile, *R. kidderi* and *R. acuminata* are usually found in Neotropical fishes such as cichlids, heptapterids and characids (Moravec, 1998).

Rhabdochona mexicana is an endemic and highly host-specific species and is morphologically characterized by having 10 anteriorly directed teeth in the prostom, large spicule

possessing a small bifurcation at its distal tip, short spicule without a barb at its distal tip, eggs with an irregular flocculent coating lacking filaments, bifurcate deirids, and a conical tail lacking a cuticular spike (Caspeta-Mandujano *et al.*, 2000). *Rhabdochona mexicana* was originally described from the intestine of two species of characids, the Mexican tetra, *Astyanax mexicanus* (De Filippi), and the banded tetra, *A. fasciatus* (Cuvier) [= *A. aeneus* (Günther)], in four localities of the Panuco River drainage and four localities of the Balsas River drainage, in the Atlantic and Pacific Ocean slopes of Mexico, respectively (Caspeta-Mandujano *et al.*, 2000). Currently, the species is widely distributed, having been found in at least 20 localities across Mexico (Garrido-Olvera *et al.*, 2006; Moravec *et al.*, 2012). The presence of this host-specific nematode in separate river basins across Mexico, in close association with *Astyanax* spp., has resulted in allopatric distribution patterns, raising the possibility of genetic differentiation among populations irrespective of the fact that morphological characters seem to be conserved, with the concomitant possibility of uncovering a complex of cryptic species (Pérez-Ponce de León and Nadler, 2010; Nadler and Pérez-Ponce de León, 2011). In this study, we provide for the first time molecular data for individuals of *R. mexicana* from different populations across its distributional range; one mitochondrial and two nuclear genes were sequenced to search for the existence of cryptic species through a molecular prospecting approach (Blouin, 2002). The main objectives of this paper were (1) to explore the genetic diversity among specimens of *R. mexicana* obtained from different hydrological systems across Mexico and Guatemala, (2) to determine if *R. mexicana* represents a species complex, and (3) to combine information from molecular data and morphology to describe a new species of *Rhabdochona*.

Materials and methods

Sample collection and molecular methods

Nematodes were recovered from 15 populations of *Astyanax* spp. in 13 locations of Mexico and two of Guatemala (table 1, fig. 1). In addition, specimens of three other species of *Rhabdochona* and one species of *Spinitectus* were sampled from their freshwater fish hosts (table 1). Hosts were collected using seine nets and maintained alive until parasitological analyses. Individual fish were euthanized by putting them in ice water (4°C), dissected, internal organs obtained, placed in Petri dishes with 0.65% saline, and examined using a stereomicroscope. Nematodes were recovered from the intestine of their hosts; some were fixed in 100% ethanol for molecular analysis, and a few others in hot 4% formalin and stored in 70% ethanol for morphological studies. Genomic DNA was extracted from the mid-section of individual nematodes. Each worm was incubated for 8 h in a solution containing 10 mM Tris–HCl (pH 7.6), 20 mM NaCl, 100 mM Na_2EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was extracted with DNAzol reagent (Invitrogen) following the manufacturer's instructions. The anterior and posterior ends of each individual nematode (hologenophores *sensu* Pleijel *et al.*, 2008) were used to contrast the molecular data with the morphology of the paragenophores that were used for the species description. The mitochondrial gene cytochrome *c* oxidase subunit 1 (*cox*1) and two nuclear genes, 28S and 18S rRNA genes, were amplified for partial sequences using polymerase chain reaction (PCR) and the primers listed in table 2. The PCR reactions (25 µl) consisted of 2 µl of genomic DNA, 1 µl of each primer (10 pmol), 0.125 µl Taq DNA Polymerase (Vivantis), 2.5 µl dNTP (2 mM), 1.5 µl $MgCl_2$, and 14.375 µl ddH_2O. The PCR conditions were as follows: 94°C for 2 minutes; 30 cycles of 94°C for 1 minute (denaturalization), annealing temperature varied according to the primer combination (table 2), 72°C for 2 minutes (extension) and 72°C for 7 minutes (final extension). The amplicons were purified using ExoSAP-IT (Affymetrix). Sequencing reactions were performed using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Samples were sequenced on an ABI 3730 capillary DNA sequencer.

Phylogenetic analyses and genetic divergence

The forward and reverse sequences were assembled using Geneious v7 (Kearse *et al.*, 2012), aligned with the algorithm implemented in Clustal Omega, with gaps treated as missing data (McWilliam *et al.*, 2013), and edited in Mesquite v3.10 (Maddison and Maddison, 2016). For the *cox*1 dataset, sequences obtained for the same individual with several primer combinations were assembled to obtain the sequence for each individual. New sequences for the three molecular markers were generated for species of *Rhabdochona*, i.e. *R. acuminata*, *R. salgadoi* and *R. canadensis*. Also, a sequence of *Spinitectus mexicanus* Caspeta-Mandujano, Moravec and Salgado Maldonado, 2000 was generated and used as outgroup for rooting the trees, based on previous phylogenetic analyses that showed that *Spinitectus* and *Rhabdochona* were sister groups (Černotíková *et al.*, 2011; Choudhury and Nadler, 2018). Sequences of other species of *Rhabdochona* available in GenBank were downloaded and used in phylogenetic analyses, particularly for the 18S rRNA gene (supplementary table S1). The *cox*1 dataset was trimmed after alignment. The best substitution model for the aligned datasets was obtained using jModelTest v2.1.7 (Posada, 2008) and selected under Akaike Information Criterion (AIC). The model GTR+ G+I was used for Maximum Likelihood (ML) and Bayesian Inference (BI) analysis. The phylogenetic reconstructions were performed for each molecular marker separately, and for a dataset containing all genes (*cox*1 + 28S + 18S rRNA). ML analyses were performed using RaxML v1.5 (Stamatakis, 2014) with 10,000 bootstrap replicates. Bayesian inference analyses were performed in MrBayes v3.2.5 (Ronquist and Huelsenbeck, 2003), including two parallel runs for 10 million generations; the trees in each chain were sampled every 1000 generations, with a burn-in of 25%. The generated trees were visualized in FigTree v1.4.2 and edited in Illustrator. To assess the level of variation among isolates of each lineage, the uncorrected (*p*) pairwise genetic distance was calculated in MEGA v7 (Kumar *et al.*, 2016) for the *cox*1 and the 28S rRNA datasets.

Morphological analysis

Photomicrographs and measurements of nematode specimens were obtained with an Olympus BX51 inverted microscope. The anterior and posterior regions of hologenophores preserved in ethanol, and complete individuals fixed in formalin, were mounted and cleared in glycerol-alcohol, and examined using differential interference contrast (DIC). Some individuals were prepared for scanning electron microscopy (SEM) observations; specimens were dehydrated in a graded series of ethanol, dried, and mounted on a strip of carbon conductive tape. Samples were sputter coated with gold and observed in a Hitachi Stereoscan Model SU1510 (Hitachi Ltd, Tokyo, Japan).

Table 1. Sampling localities, host species and nematode species. Locality code corresponds with those in fig. 1.

Country	State /Department	Locality	Code	Latitude	Longitude	Host species	Nematode species
Mexico	Chiapas	Metzabok	ME	17°07′03.41″N	91°37′54.61″W	*A. aeneus*	*R. mexicana* (6)*
		Rio Pichucalco	RPI	17°30′35.56″N	93°06′58.94″W	*Brycon guatemalensis*	*R. acuminata* (2)
	Jalisco	Rio La Rosa, San Vicente, Tamazula	RRT	19°38′56.04″N	103°15′24.59″W	*A. aeneus*	*R. mexicana* (2)
	Michoacan	Manantial Rico	MR	19°49′51.85″N	102°30′07.98″W	*Allotoca regalis*	*R. lichtenfelsi* (1)
	Morelos	Real de Coacalco, Yautepec	RCY	18°51′35.56″N	99°04′33.23″W	*A. aeneus*	*R. mexicana* (1)
		Rio Amacuzac, at El Chisco	RAM	18.45°80′73″N	99.19°04′02″W	*A. aeneus*	*R. mexicana* (1)
	Oaxaca	Nacimiento del Rio Tehuantepec	NCR	16°53′57.68″N	96°09′57.33″W	*A. aeneus*	*R. mexicana* (2)
						Profundulus sp.	*R. salgadoi* (2)
	Queretaro	La Vereda	VE	21°02′35.34″N	99°50′30.50″W	*A. mexicanus*	*R. mexicana* (1)
		Rio Jalpan, Santa Maria, Jalpan	RJ	21°12′14.76″N	99°28′28.2″W	*A. mexicanus*	*R. mexicana* (5)
		Rio Estorax, at El Oasis	OA	20°59′59.98″N	99°42′30.37″W	*A. mexicanus*	*R. mexicana* (2)
	San Luis Potosi	Axtla de Terrazas	ATS	21°28′02.7″N	98°57′11.3″W	*Heterandria bimaculata*	*S. mexicanus* (1)
		Rio Gallinas, at Arroyo Canoas	CA	22°09′79″N	99°32′36.54″W	*A. mexicanus*	*R. mexicana* (1)
	Tabasco	El Mangal, Tenosique	MT	17°38′46.99″N	91°22′58″W	*Brycon guatemalensis*	*R. acuminata* (1)
	Tamaulipas	Plan de Ayala	PA	23°34′02.2″N	99°24″W	*A. mexicanus*	*R. mexicana* (6)
	Veracruz	Afluente Rio Actopan, at La Esperanza	RAE	19°28′53.95″N	96°33′16.33″W	*A. aeneus*	*R. mexicana* (6)
		Atoyac, Paso de Macho	PM	18°58′18.80″N	96°43′53.32″W	*A. aeneus*	*R. mexicana* (1)
		Rio Paso de Ovejas, Pueblo el Crucero	RPO	19°19′01.35″N	96°32′11.59″W	*A. aeneus*	*R. mexicana* (18)
	Zacatecas	Sain Alto	SZ	23°34′40″N	103°20′48″W	*G. conspersa*	*R. canadensis* (2)
Guatemala	Escuintla	Rio Achihuate	RAG	14°04′16″N	90°53′59″W	*A.* cf. *aeneus*	*R. mexicana* (2)
	El Progreso	Rio las Cabezas, Saranate	RCG	14°44′23″N	90°04′52″W	*A.* cf. *aeneus*	*R. mexicana* (1)

*Nematode sample size

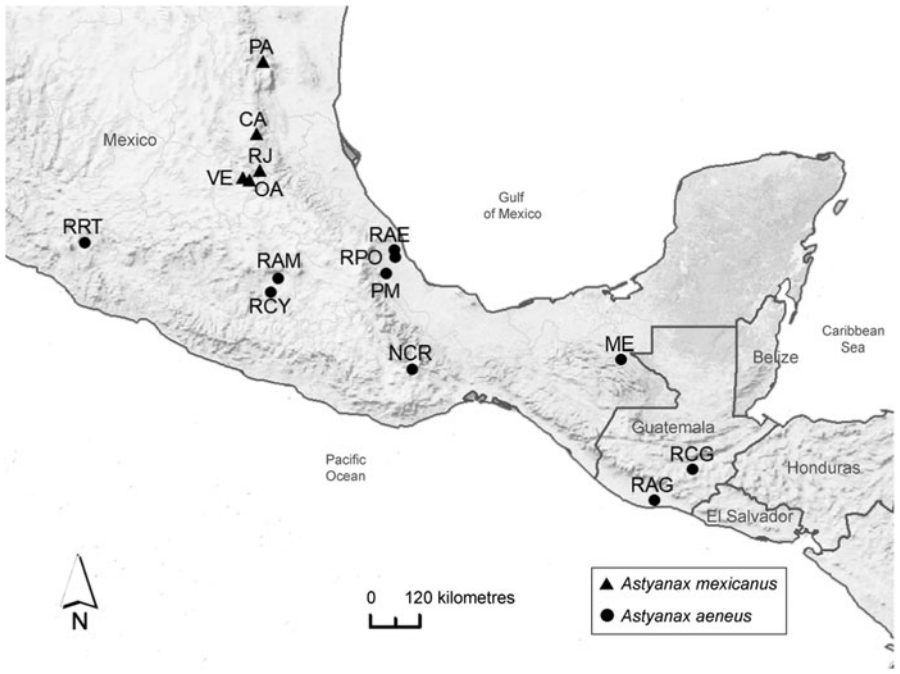

Fig. 1. Collecting sites of *Rhabdochona* from *Astyanax mexicanus* and *A. aeneus*. The locality code corresponds with table 1.

Table 2. Sequences of primers used in this study.

Gene	Primer name	Sequence (5′→3′)	Annealing temperature	Reference
*Cox*1	COI int F	F: TGATTGGTGGTTTTGGTAA	52°C	Casiraghi *et al.* (2001)
	COI int R	R: ATAAGTACGAGTATCAATATC		Casiraghi *et al.* (2001)
	pr-a	F: TGGTTTTTTGTGCATCCTGAGGTTTA	40°C	Bessho *et al.* (1992)
	pr-b	R: AGAAAGAACGTAATGAAAATGAGCAAC		Bessho *et al.* (1992)
	HCO2198/588	R: TAAACTTCAGGGTGACCAAAAAATCA	48°C	Folmer *et al.* (1994)
	507	F: AGTTCTAATCATAARGATATYGG		Nadler *et al.* (2006)
28S rRNA	391	F: AGCGGAGGAAAAGAAACTAA	52°C	Nadler and Hudspeth (1998)
	502*	F: CAAGTACCGTGAGGGAAAGTTGC		García-Varela and Nadler (2005)
	536	R: CAGCTATCCTGAGGGAAAC		García-Varela and Nadler (2005)
18S rRNA	G18S4	F: GCTTGTCTCAAAGATTAAGCC	54°C	Blaxter *et al.* (1998)
	136*	R: TGATCCTTCTGCAGGTTCACCTAC		Nadler and Hudspeth (1998)
	649	R: TAAGAACGGCCATGCACCAC		Nadler *et al.* (2007)

F: forward; R: reverse; Asterisk indicates primers used only in sequencing reaction

Drawings were made with the aid of a drawing tube attached to a light microscope (Olympus BX51). Measurements are presented in micrometers, unless specified otherwise. Observations and data in this study were compared with the original description by Caspeta-Mandujano *et al.* (2000). Voucher specimens were deposited in Colección Nacional de Helmintos (CNHE), Universidad Nacional Autónoma de México, Mexico City, with the accession numbers CNHE 10866–10869 and CNHE 10966.

Results

Phylogenetic analyses

Thirty-eight individuals of *R. mexicana* were sequenced from 15 localities. In addition, we sequenced eight individuals from four other species of *Rhabdochona* and one individual of *S. mexicanus* used as outgroups. The datasets generated in our study were analysed separately for each molecular marker. A subsample of individuals was sequenced for the 18S rRNA gene, the less informative, and analysed through BI and ML (supplementary fig. S1). The 10 species of *Rhabdochona* available for the analysis appeared as a monophyletic assemblage with high posterior probability support (although bootstrap support value was moderate), and specimens of *R. mexicana* sequenced in our study were recovered forming two independent lineages, one of them including the type and host locality of *R. mexicana*. Furthermore, 28S rDNA sequences were analysed through BI and ML (supplementary fig. S2). As expected, the 28S rRNA tree seems to possess a higher phylogenetic signal than the 18S rRNA tree; sequenced individuals also formed two well-supported and reciprocally

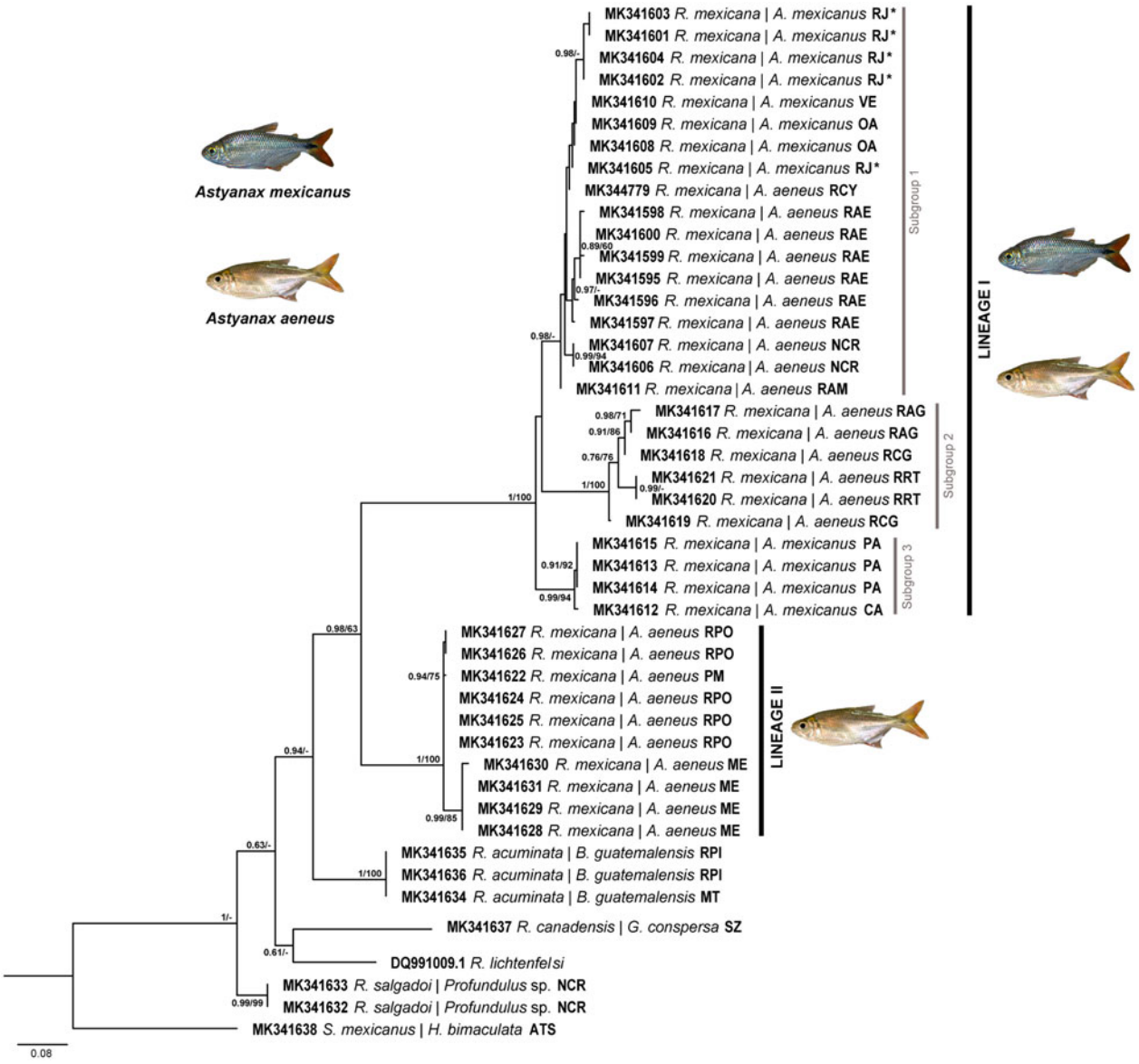

Fig. 2. Maximum likelihood phylogenetic reconstruction recovered from sequences of *cox*1. Posterior probability support value from the BI analysis and bootstrap support value (above 60%) are shown in the internodes. The scale bar refers to branch length. An asterisk indicates the type locality.

monophyletic lineages. Both ribosomal genes yielded a phylogenetic tree showing *R. mexicana* as paraphyletic.

The topologies derived from *cox*1 were the most well resolved and informative, contained the largest number of individuals, and were analysed through ML and BI inference methods; both recovered similar topologies with high bootstrap and posterior probability support values, respectively (fig. 2). This tree showed that *R. mexicana* consisted of two well-supported and reciprocally monophyletic lineages; Lineage I comprised three subgroups, each with high nodal support. One of them (subgroup 1) contained samples from seven localities, including some from the type host and locality (*Astyanax mexicanus*; Río Jalpan, Querétaro [RJ]) (see fig. 1); subgroup 2 included individuals from two geographically disjunct populations found in *A. aeneus* in Jalisco, Mexico (RRT) and in *A.* cf *aeneus* from Guatemala (RAG and RCG), in river basins draining to the Pacific Ocean slope. Individuals from subgroup 3 were collected only from the Mexican tetra, *A. mexicanus* in two localities of San Luis Potosí and Tamaulipas, in northern Mexico. As samples from the type locality were included in Lineage I, this was considered as *R. mexicana sensu stricto*. Lineage II was highly divergent and reciprocally monophyletic, was collected from *A. aeneus* in two localities of the state of Veracruz (PM and RPO) and one of Chiapas (ME), and was recovered as the sister group of Lineage I. In this phylogenetic tree, *R. acuminata* was recovered as the sister species of the two lineages of *R. mexicana* (fig. 2).

The concatenated tree from the three molecular markers (18S + 28S + *cox*1) recovered the same topology as the *cox*1 tree showing two reciprocally monophyletic lineages for *R. mexicana*, suggesting one of them as a separate species; relationships were supported by high bootstrap and posterior probability values (supplementary fig. S3). Also, in the concatenated tree, the sister group of the species complex of *Rhabdochona* in *Astyanax* was *R. acuminata*, a species recovered from the Macabi tetra, *Brycon guatemalensis* Regan.

Table 3. Genetic divergence among species of *Rhabdochona* and *S. mexicana* (outgroup).

	Species	1	2	3	4	5	6	7	8	9
1	*R. mexicana* Lineage I – Subgroup 1	**1.09**	0.34	0.23	6.93	5.11	5.87	6.42	5.73	21.92
2	*R. mexicana* Lineage I – Subgroup 2	5.45	**1.53**	0.33	6.99	5.24	5.92	6.44	5.82	22.18
3	*R. mexicana* Lineage I – Subgroup 3	4.51	6.24	**0.24**	6.69	4.96	5.52	6.14	5.41	21.12
4	*R. mexicana* Lineage II	10.94	11.33	11.51	**0.92**	5.16	5.06	6.08	5.47	23.3
5	*R. acuminata*	12.01	12.67	12.73	8.32	**0**	3.82	5.00	4.61	21.95
6	*R. canadensis*	13.83	14.43	14.29	11.83	11.5	**0**	2.76	2.47	19.54
7	*R. lichtenfelsi*	11.71	13.81	13.48	11.56	9.21	10.31	–	2.57	20.21
8	*R. salgadoi*	11.44	12.27	12.45	9.69	7.42	10.22	8.99	**0**	19.33
9	*S. mexicana*	15.27	16.29	16.43	13.65	13.22	14.82	13.82	12.44	–

The numbers on the diagonal are the intraspecific genetic variation of each taxon. The numbers below the diagonal indicate the genetic distance calculated with the *cox*1 matrix, and those above the diagonal correspond to the genetic distance from the 28SrDNA matrix. The distances are shown as percentages.

Genetic divergence

The genetic divergence (uncorrected *p*-distance) among the three subgroups that comprise Lineage I varied from 4.51 to 6.24%. However, the genetic divergence between the three subgroups of Lineage I, and Lineage II varied between 10.94 and 11.51%. Furthermore, the divergence between *Rhabdochona acuminata* and the two lineages of *R. mexicana* varied between 8.32 and 12.73%. With respect to the remaining species of *Rhabdochona* depicted in fig. 2, the lineages of *R. mexicana* diverged by 8.32–14.43%. The *cox*1 intraspecific variation among individuals of each of the lineages and subgroups uncovered in the present study varied between 0.24 and 1.53% (table 3). Additionally, the genetic divergence for the 28S rRNA gene among the three subgroups that form Lineage I, and Lineage II varied from 0.23 to 6.99%, with the highest divergence also exhibited by Lineage II (table 3). Intraspecific divergence among individuals of the lineages of *R. mexicana* for the 28S rRNA gene was nil.

Morphological analyses

The three subgroups of *R. mexicana* lineage I are reciprocally monophyletic and reached some level of genetic divergence; however, the three subgroups recovered within Lineage I are morphologically similar in that all possess 10 teeth in the prostom, bifurcate deirids, and eggs without filaments (see supplementary fig. S4). They represent a cryptic species complex. Instead, a detailed observation of the specimens of *R. mexicana* Lineage II allowed us to corroborate that they represented a different species, characterized by having a prostom with 14 teeth instead of 10, a simple deirid that is not bifurcate, and a large number of preanal papillae. The morphological description of Lineage II is presented next.

Family Rhabdochonidae Skrjabin, 1946
Genus *Rhabdochona* Railliet, 1916
***Rhabdochona osorioi* n. sp. (figs 3–5)**

Description

(Based on the measurements of the holotype (CNHE 10866), the allotype (CNHE 10966), and 13 paratypes (CNHE 10867–10869); measurements taken from adult worms.) Medium-sized nematodes with smooth cuticle. Oval oral aperture surrounded by four cephalic papillae and two amphids; prostom with 14 teeth, four dorsal, four ventral and three lateral on either side (figs 3C and 4A). Simple and large deirids, sometimes asymmetrically disposed near cephalic end (figs 3D and 4C), usually not exceeding the end of prostom. Conical tail in both sexes without cuticular extensions (figs 3J, 4E and 5F).

Male (based on eight individuals). Body length 7.8–10.5 (9.5) mm, maximum width 163–200 (181). Prostom 23–34 (29) long, 15–23 (19) wide; length of vestibule including prostom 112–128 (117). Muscular oesophagus 221–0.342 (0.305) long; glandular oesophagus 1.8–3.2 (2.5) mm long. Nerve ring surrounding muscular oesophagous, 125–169 (150) from anterior end. Deirids and excretory pore 39–49 (42) and 202–249 (220) from anterior end, respectively (fig. 5A–D). Area rugosa absent. Fifteen pairs of preanal papillae. Subventral postcloacal papillae present, 7 + 7 and occasionally 7 + 6, plus one pair of lateral papillae between subventral papillae one and two (figs 3L and 4F). Terminal end of left spicule scoop-shaped, 414–451 (430) long (fig. 3G, H); shaft 251–273 (258) long, representing 60.2–60.9% of entire spicule length; right spicule 117–138 (125) long (fig. 3F). Tail 151–347 (272) long.

Female (based on seven individuals). Body length 11.6–27.3 (16.9) mm. Maximum width 154–327 (218). Prostom 24–41 (34) long, 21–28 (24) wide; length of vestibule including prostom 112–128 (117). Muscular oesophagus 173–436 (300) long. Nerve ring 147–227 (185) from anterior end; deirids and excretory pore 39–45 (42) and 173–240 (204) from the anterior end, respectively. Deirids large and simple. Vulva 4.6–14.1 (7.9) mm from anterior end (figs 3K and 5E). Eggs numerous, with no filaments (fig. 4D). Tail 218–364 (311) long (fig. 4E). Fourth-stage larva (based on two individuals) (fig. 3E). Body length 3.2–3.7 mm, maximum width 81–83. Prostom 15 long, 8–10 wide: length of vestibule including prostom 84–85. Muscular oesophagous 180–206 long, maximum width 23. Glandular oesophagous 1.1–1.3 mm long, 49–54 wide. Deirids, nerve ring and excretory pore 17, 115–121 and 154, respectively from anterior end. Tail conical 112–121 long (figs 3I and 5H). Vulva opening not observed.

Taxonomic summary

Type host. Astyanax aeneus (Günther, 1860).

Type locality. Río Paso de Ovejas, Pueblo el Crucero, Veracruz, Mexico (19°19′1.35″N, 96°32′11.59″W).

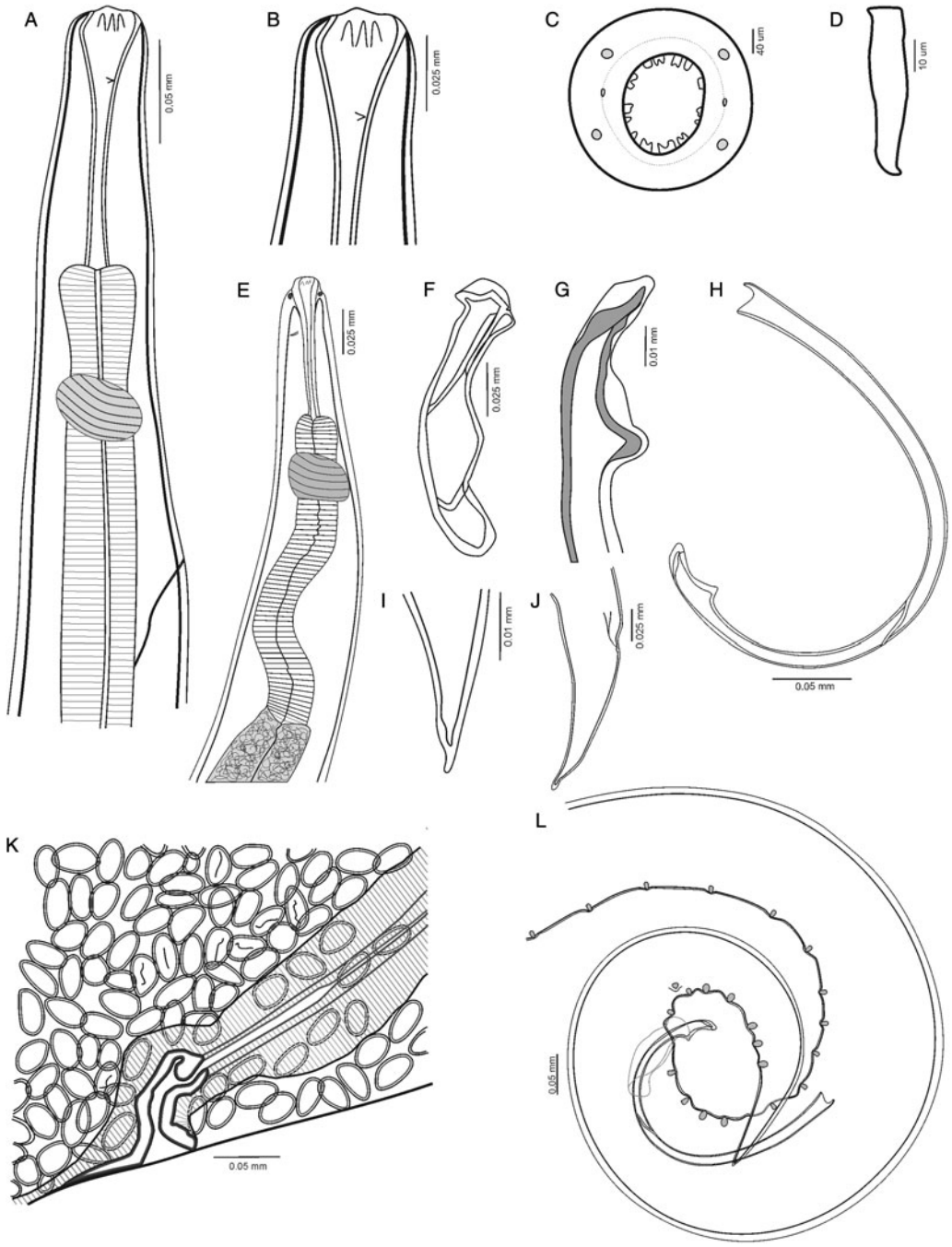

Fig. 3. Line drawings of *Rhabdochona osorioi* n. sp. (A) Lateral view from anterior end, (B) lateral view of prostom from an adult, (C) apical view of the anterior end, (D) deirid, (E) anterior end of a fourth-stage larva, lateral view, (F) right spicule, (G) distal termination of left spicule, (H) complete left spicule, (I) tail of fourth-stage larva, (J) tail of female, lateral view, (K) vulvar opening of gravid female, lateral view, and (L) posterior end of male, lateral view.

Other localities. Atoyac, Paso de Macho, Veracruz (18° 58′18.80″N, 96°43′53.32″W); and Metzabok, Chiapas (17° 7′3.41″N, 91°37′54.61″W).

Type material. CNHE 10866 (holotype); CNHE 10966 (allotype); CNHE 10867–10869 (paratypes).

ZooBank registration. To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Rhabdochona osorioi* n. sp. is urn:lsid:zoobank.org:act: 1D9F9E2A-371E-4028-A9EF-00272EF15059.

Etymology. The epithet *osorioi* is for Professor David Osorio in recognition of his long contribution (40 years!) in describing the nematode fauna of Mexican vertebrate wildlife, and training students in nematode taxonomy.

Representative DNA sequences in GenBank. MK341595–MK341638, MK344779 (*cox*1), MK341639–MK341652 (18S rRNA), MK341653–MK341687 (28S rRNA).

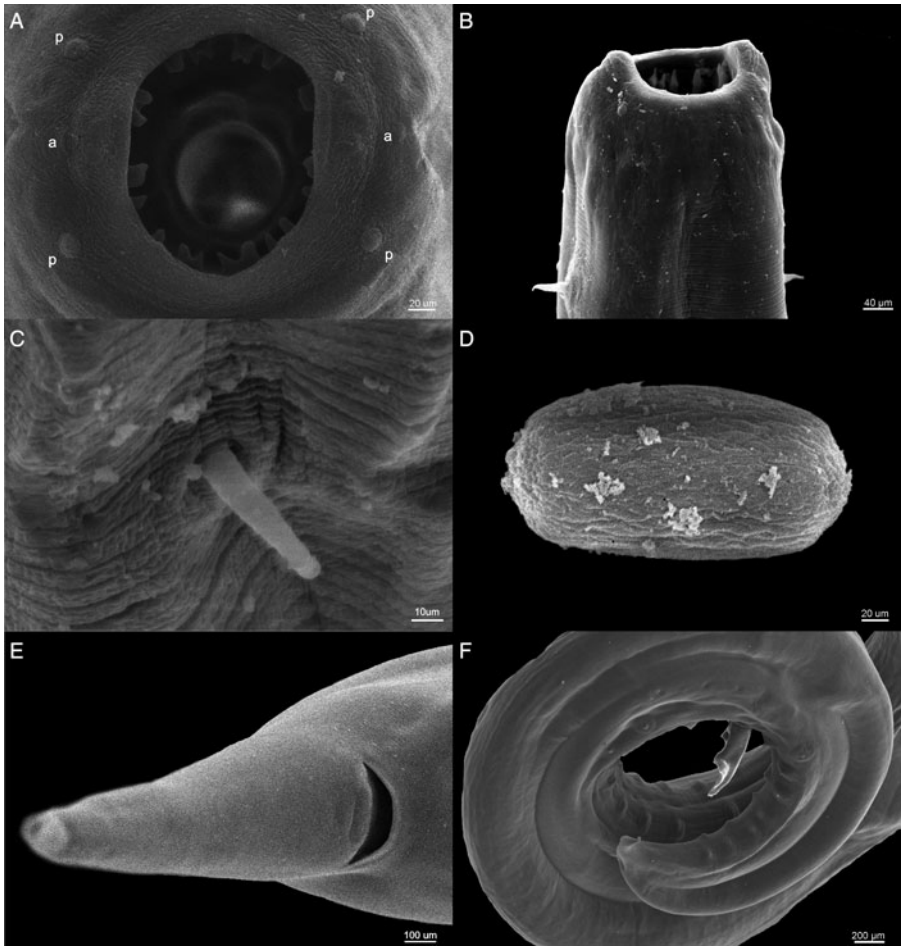

Fig. 4. SEM microphotographs of *Rhabdochona osorioi* n. sp. (A) Apical view from anterior extremity, (B) lateral view from anterior end, (C) deirid, (D) egg, (E) posterior end of female, and (F) posterior end of body, lateral view. Abbreviations: p, papillae; a, amphid.

Taxonomic remarks

Rhabdochona osorioi n. sp. is diagnosed by having simple and large deirids, 14 anterior prostomal teeth and by the number and arrangement of papillae in males. This combination of characters clearly sets it apart from *R. mexicana*. In the Americas, nine *Rhabdochona* species possesses 14 teeth in the prostom: seven in the Nearctic biogeographical region (i.e. *R. cascadilla* Wigdor, 1918; *R. cotti* Gustafson, 1949; *R. decaturensis* Gustafson, 1949; *R. milleri* Choquette, 1951; *R. canadensis* Moravec et Arai, 1971; *R. catostomi* Kayton, Kritsky et Tobias, 1979; and *R. ictaluri* Aguilar-Aguilar, Rosas-Valdez and Pérez-Ponce de León, 2010) and two in the Neotropical biogeographical region (i.e. *R. acuminata* Molin, 1860 and *R. kidderi* Pearse, 1936). *Rhabdochona osorioi* n. sp. is the 10th species in the Americas with 14 teeth. Even though Mejía-Madrid *et al.* (2007a) argued that the species of *Rhabdochona* could not be grouped consistently by number of teeth in the prostom, we compared the new species with all the species of the Americas with the same number of teeth. *Rhabdochona osorioi* n. sp. differs from four of the Nearctic species (i.e. *R. canadensis, R. milleri, R. catostomi* and *R. cotti*) in having eggs with no filaments or any other ornamentation. The new species differs also from *R. decaturensis*, a parasite of the freshwater drum, *Aplodinotus gruniens* Rafinesque, 1819, in the USA and Canada, and from *R. cascadilla*, a parasite of several fish species across the USA and Canada, in that the eggs of these two species are covered with a thin gelatinous layer, although this character might be overlooked during processing; additionally, the new species differ from these two species by having a larger number of preanal papillae (see Gustafson, 1949). From *R. ictaluri*, a species described from ictalurid catfishes in northern Mexico (Aguilar-Aguilar *et al.*, 2010), the new species differs by having a simple deirid, and a prostom without basal teeth. In *R. ictaluri* deirids are bifurcated and the prostom possesses six basal teeth.

Rhabdochona osorioi n. sp. most closely resembles the two Neotropical *Rhabdochona* species (*R. kidderi* and *R. acuminata*) in having 14 teeth in the prostom and eggs with smooth surface; however, it can easily be differentiated from *R. kidderi* because in this species deirids are bifurcate and males possess 5–7 pairs of preanal papillae. Instead, the new species possesses simple deirids, and 15 pairs of preanal papillae in males. Even though *R. kidderi* and the new species are sympatrically distributed in southern Mexico, host associations are different; the new species is found only in the characid *Astyanax aeneus*, whereas adults of *R. kidderi* are found in different species of cichlids and in heptapterids (Garrido-Olvera *et al.*, 2006). *Rhabdochona acuminata* was recovered in our analyses as the sister species of the *R. mexicana* species complex and *R. osorioi* n. sp. (fig. 1; supplementary fig. S3); *R. acuminata* is morphologically very similar to the new species and is also found in characiforms. A morphometric comparison between the new species and *R. acuminata* is presented in supplementary table S2. Both species possess 14 teeth in the prostom and single deirids. However, the new species can be readily distinguished from *R. acuminata* by the number of preanal papillae in

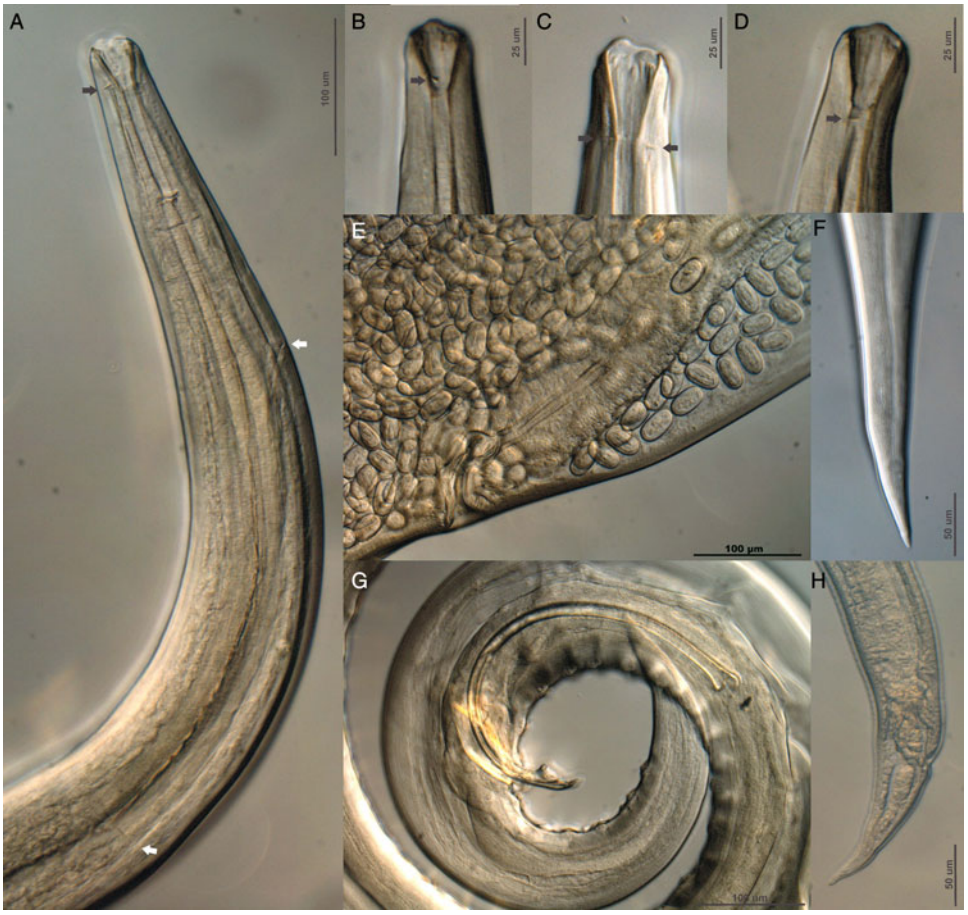

Fig. 5. Differential interference contrast microphotographs of *Rhabdochona osorioi* n. sp. (A) Lateral view of anterior end (black arrow indicates the position of the deirids, white arrow indicates the division of the muscular and glandular oesophagous), (B, C and D) lateral view of anterior end of different male specimens (black arrow indicates the position of deirids), (E) vulvar opening, lateral view, (F) posterior end of female, (G) male posterior end, lateral view, and (H) tail of fourth-stage larva, lateral view.

males (15 pairs *vs* 9–11 pairs), by the size and position of deirids (large deirids near cephalic end, usually not exceeding the end of prostom in the new species *vs* small deirids in the first third of vestibular length in *R. acuminata*), in the arrangement of the 14 teeth in the prostom (4 dorsal, 4 ventral and 3 lateral on either side in the new species *vs* 3 dorsal, 3 ventral and 4 on each lateral side in *R. acuminata*), and by the fact that teeth in *R. acuminata* are entire, whereas in the new species teeth are bifurcated. In terms of host association, even though *R. acuminata* was originally described as a parasite of the bryconid *Brycon falcatus* Müller and Troschel in the Paraná River in Brazil (Molin, 1860), it has been additionally recorded in a wide array of distantly related hosts, including five species of characiforms, three species of pimelodids and one species of cichlid in Brazil, but also from one species of diplomystid (Siluriformes), one species of anablepid and one species of percychthid in Argentina, and one species of cichlid, one of characid and one of pimelodid in Ecuador (Cremonte *et al.*, 2002; Ramallo, 2005; Pinto *et al.*, 2010). Based on host association pattern, it seems likely that most of these records need to be further verified, and whether or not *R. acuminata* in South America represents a species complex needs to be determined by further molecular phylogenetic analyses. In Mexico, *R. acuminata* has been reported only as a parasite of the bryconid *Brycon guatemalensis* Regan in the Usumacinta River basin (Caspeta-Mandujano *et al.*, 2005), in southern Mexico. *Rhabdochona osorioi* n. sp. is also found in the same geographical area, occurring in water bodies of Chiapas and Veracruz. However, they are clearly differentiated, and both are valid species. *Rhabdochona osorioi* n. sp. is the fifth species of *Rhabdochona* known to parasitize characiform freshwater fishes in the Neotropical biogeographical region. The other three species are found in South America, *R. acuminata* and *R. fabianae* Ramallo, 2005 from *Bryconamericus iheringi* Boulanger, 1887 (in Argentina), and *R. uruyeni* Díaz-Ungría, 1968 from the characiform *Piabucina* sp. (and also from the freshwater scianid *Pachiurus squamipennis* Agassiz, 1831) (Cremonte *et al.*, 2002; Ramallo, 2005; Pinto *et al.*, 2010).

Discussion

Our analyses uncovered two genetic lineages within *R. mexicana*. Two lines of evidence allowed us to recognize the existence of these two genetic lineages. Firstly, genetic divergence for the mitochondrial cytochrome *c* oxidase subunit I (*cox*1) gene was high and allowed us to recognize them as independent evolutionary entities, with divergence levels between 10.94 and 11.51%. Secondly, sequenced individuals belonging to both lineages were reciprocally monophyletic (fig. 2). The molecular results prompted us to conduct a detailed morphological examination of the specimens and, as a result, *Rhabdochona mexicana*

Lineage II was described as a new species, for which the name *Rhabdochona osorioi* n. sp. was coined; however, the lack of morphological differences, genetic divergence values of *cox*1, and the tree topology led us to further investigate a potential case of cryptic species for the subgroups formed within *Rhabdochona mexicana* Lineage I.

Lineage I was composed of three genetic subgroups and was considered as a cryptic species complex based on the fact that they represent reciprocally monophyletic groups, with moderate genetic divergence for the mitochondrial gene *cox*1, and with no clear morphological differentiation. To shed light on the genetic diversity of *R. mexicana* Lineage I, our study followed a molecular prospecting approach, as suggested by Blouin (2002); we used sequence data and a genetic yardstick to search for populations that could represent a cryptic species, assuming the null hypothesis that individuals represented a single species, i.e. *R. mexicana* (see Pérez-Ponce de León and Nadler, 2010). By comparing species-pairs of different genera of nematodes, Blouin (2002) suggested that if two groups show genetic divergence values around 10% (with a range between 6.9 and 13%) for the *cox*1 gene, we might consider that they are not conspecific. Overall, cryptic species of nematodes have been detected using nuclear and mitochondrial molecular markers (e.g. Derycke *et al.*, 2005; Ristau *et al.*, 2013; Jorge *et al.*, 2013; Chilton *et al.*, 2016), although mitochondrial genes have been shown to be more informative because of the higher substitution rate (Blouin, 2002).

The three subgroups within *R. mexicana* Lineage I exhibited a genetic divergence varying from 4.41 to 6.24%; it seems plausible then to postulate they may represent a recent diversification event (see Fišer *et al.*, 2018). Nadler and Pérez-Ponce de León (2011) discussed the use of pair-wise distance threshold for initial cryptic species prospecting as a reasonable exploratory approach; however, whether or not 6.9% is the minimum distance threshold to evaluate if two putative taxa are different enough to merit recognition as separate species in nematodes is debatable, given the heterogeneity of the evolutionary patterns in different groups. For instance, Miranda *et al.* (2008) reported a genetic divergence of 7% among populations of *Ancylostoma caninum* Ercolani, 1859 in Brazil, suggesting the existence of a cryptic species; St-Onge *et al.* (2008) reported *cox*1 divergence between 12.9 and 15.7% in the mermithid *Mesomermis flumenalis* Welch, 1962 in Canada, and considered that the species represented four clearly distinguished species. In contrast, the genetic divergence of the congeneric species *Rhabdochona lichtenfelsi*, a parasite of endemic freshwater fishes in central Mexico, was evaluated by Mejía-Madrid *et al.* (2007b). In that study, genetic divergence values for *cox*1 were lower than 4.9% among populations from different localities and no genetic structure associated either with host species or geographical distribution was found; in this case, the genetic divergence pattern was not in agreement with the intricate evolutionary history of their host, which experienced a large diversification process in central Mexico resulting from the complex geological and hydrographical history. Our results also showed that the three subgroups of the *Rhabdochona mexicana* cryptic species complex exhibit a well-defined geographical distribution pattern, and currently represent allopatric populations. Subgroup 1 is distributed in river basins of central Mexico; subgroup 2 exhibits a wider geographical distribution range, although restricted along the Pacific Ocean slope, between Jalisco, in western Mexico and Guatemala; and subgroup 3 is found only in locations of northern Mexico (San Luis Potosí and Tamaulipas), and exclusively infects the intestine of the Mexican tetra, *A. mexicanus*, the species of characiform that reach the most northern distributional range in the Americas (see Ornelas-García *et al.*, 2008).

The taxonomic identification of *Rhabdochona* species in Mexican freshwater fishes has been sometimes based on the host association pattern, without a rigorous analysis of the morphological traits; this practice might have resulted in inaccuracies in species identification, even though we acknowledge that most species are host-specific and are part of the biogeographical core helminth fauna of freshwater fishes, especially at family level (see Pérez-Ponce de León and Choudhury, 2005). Our study also showed that in addition to host association, a detailed morphological study is necessary to establish the species' identity, and that an accurate estimate of diversification in the genus *Rhabdochona* requires scrutiny through DNA sequences. The generation of DNA sequences for representative species of *Rhabdochona* from across the world will increase our understanding of the current diversity within the genus and will help to elucidate their evolutionary and biogeographical history. We also suggest that an integrative taxonomy approach must be followed to fully understand the diversity of this speciose group of nematodes, adding as many sources of information as possible, as recently discussed by De Sousa *et al.* (2018) for the study of nematode taxonomy.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19000014

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