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FISH PARASITOLOGY - ORIGINAL PAPER

Taxonomic status of *Rhabdochona ictaluri* (Nematoda: Rhabdochonidae) based on molecular and morphological evidence

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Abstract

The genus *Rhabdochona* includes more than 100 species infecting freshwater fishes in all zoogeographical regions of the world. In Mexico, 12 nominal species of *Rhabdochona* have been recorded. Of these, *Rhabdochona ictaluri* was originally described as a parasite of endemic catfishes of the family Ictaluridae; however, the species was later considered on morphological grounds as a junior synonym of *Rhabdochona kidderi*. In this study, newly sampled specimens of *R. ictaluri* were obtained from the type host and type locality and were used to perform a detailed morphological analysis and molecular phylogenetic inferences through one mitochondrial and two nuclear genes; data were used in an integrative taxonomy context to test the taxonomic status of *R. ictaluri*. This approach proved to be very useful to confirm the validity of this species, and robust species limits were established between these two putative species considering morphology, molecular data, host association, and biogeography.

Keywords Nematoda · Integrative taxonomy · Species limits · Morphology · COX1

Introduction

Species are the basic units of biological studies, although delimiting species is a highly complex subject (Sites and Sites and Marshall 2003; Mckay et al. 2013). Currently, most parasite species are delimited based on morphological characters; nevertheless, such practice turns problematic when distinguishing characters are subjectively stated, or erroneously evaluated (Hawkins 2000; McKay et al. 2013).

The use of different sources of information contributes to a better understanding of the intraspecific variation that may exist among individuals of the same species; also, it might be useful for discovering species and for testing hypotheses of species limits (Padial et al. 2010; Meik et al. 2018). The conceptual framework of integrative taxonomy is useful for delimiting units of biodiversity by using several sources of information (Dayrat 2005; De Queiroz 2007). In this study, we explored several sources of information to accomplish a robust species delimitation of the nematode *Rhabdochona ictaluri*, and to test its taxonomic validity as one of the species of the genus *Rhabdochona*, a widely diverse group of nematodes parasitic in freshwater fishes around the world (Moravec et al. 2013; Moravec and Adlard 2016). Up to date, 12 nominal species of *Rhabdochona* have been recorded in Mexico (see Garrido-Olvera et al. 2006; Caspeta-Mandujano 2010; Aguilar-Aguilar et al. 2010). The high levels of species-richness and endemism of *Rhabdochona* in the Mexican freshwater fishes have been attributed to two main factors: a complex evolutionary history consisting of extensive ecological host extensions accompanied by host-switching events (Mejia-Madrid et al. 2007b), and the fact that Mexico lies between the Nearctic and Neotropical biogeographical regions, making this country an interesting area from the zoogeographical point of view (see Moravec et al. 2012). *Rhabdochona ictaluri* was described from the Yaqui catfish, *Ictalurus pricei*, and the Lerma catfish, *I. dugesii*, both endemic species of ictalurid catfishes in northern Mexico,

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corresponding with the Nearctic biogeographical region (Aguilar-Aguilar et al. 2010). However, the species was later considered as a junior synonym of *Rhabdochona kidderi* (Moravec et al. 2012). According to these authors: "The general morphology of *R. ictaluri* corresponds with that of *R. kidderi*, and the latter species is also reported from congeneric hosts (*Ictalurus* spp.), [thus…] *R. ictaluri* should be considered a junior synonym of *R. kidderi kidderi*". In our opinion, the argument is not satisfactory because *R. ictaluri* was synonymized with *R. kidderi* on the basis of an apparent similarity of measurements of some structures, and the fact that the parasite was found in the same host group (siluriform catfishes), disregarding other morphological data, host association particularities related with their biogeographical differences (ictalurids have a Nearctic affinity, whereas heptapterids are Neotropical fishes [Miller et al. 2005; Helfman et al. 2009; De Borba et al. 2012; Pérez-Rodríguez et al. 2015]), and lacking any comparison with the type material of *R. ictaluri.* Here, we obtained sequence data through one mitochondrial and two nuclear genes of newly sampled specimens of *R. ictaluri* and an integrative taxonomy approach was followed to test the validity of *R. ictaluri*.

Material and methods

Sample collection

Nematodes belonging to six species of the genus *Rhabdochona* were collected between 2015 and 2016 from the intestine of freshwater fishes in eight localities across Mexico (Table 1). Hosts were sampled using minnow traps, spoon nets, or seine nets, and transported alive to the laboratory. Individual fish were necropsied a few hours after their capture; all internal organs were placed individually in Petri dishes with saline solution (0.65%) and observed under a stereomicroscope. Collected nematodes were fixed by sudden immersion in hot 4% formalin for morphological procedures, or in 100% ethanol for DNA extraction and sequencing.

Morphological study

All the collected nematodes were identified at species level following standard procedures. Newly sampled individuals were cleared with a solution of 1:1 glycerin-distilled water (Lamothe-Argumedo 1997). Specimens were then compared with those deposited at the Colección Nacional de Helmintos, Mexico City (CNHE): *R. ictaluri* ex *Ictalurus pricei*, Río Tunal at Nombre de Dios, Durango (CNHE 6551, Holotype); *R. kidderi* ex *Rhamdia guatemalensis*, Cenote Xnmcuy, Yucatán (CNHE 2698); ex *Typhliasina pearsei*, Cueva Nahech (CNHE 3286); ex *Theraps irregularis*, Río Lacantún, Chiapas (CNHE 8065). Morphometric study of each nematode included the diagnostic features used for species of the genus *Rhabdochona*. Measurements were obtained using the software Image-Pro Plus 7.0 (Media-Cybernetics, Maryland, USA). In addition, the ultrastructure of the body surface of some specimens of *R. kidderi* and *R. ictaluri* was studied through scanning electron microscopy (SEM). Specimens were dehydrated through an ethanol series, mounted on a metal stub with carbon adhesive tabs, then gold coated, and examined at 15 kV in a Hitachi Stereoscan Model SU1510 (Hitachi Ltd., Tokyo, Japan). Voucher specimens of *R. ictaluri* and *R. kidderi* were deposited at the CNHE (accession numbers 10808 and 10865, respectively).

Multivariate statistical analysis

A matrix of 11 metrical variables for males and seven variables for females was built with the morphological data obtained from specimens of *R. ictaluri* and *R. kidderi*. Equality of variances between groups was examined using the *F* test. Values of the morphometric variables were standardized and used to perform a standard discriminant function analysis to evidence the significant differences between the two populations analyzed. In addition, a Principal Component Analysis (PCA) was performed to explore and describe patterns of variation among the data, with the aim of visualizing the clustering of specimens in the morphometric space (Agustí et al. 2005). For the discriminant function and PCA analyses, 40 individuals per species (20 of each sex) were selected. Analyses were performed with STATISTICA software version 7.1 (StatSoft Inc 2005).

Extraction, amplification, and sequencing of DNA

Specimens were placed individually in Eppendorf tubes containing 150 μl ultrapure water for 10 min to remove the excess of alcohol. Water was replaced with 25 μl of the *Extraction solution* and 6.25 μl of the *Tissue Preparation Solution* included in the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich Corporation, St. Louis, USA). Samples were incubated at 55 °C for 1 h, and posteriorly digested in the Thermal Cycler MJ Research PTC-100 (Global Medical Instrumentation Inc., Ramsey, USA) at 95 °C for 3 min. The reaction was neutralized by 25 μl of *Neutralization Solution B*. The cytochrome *c* oxidase subunit I (*COXI*), 18S ribosomal RNA (18S), and 28s ribosomal RNA (28S) were amplified using the polymerase chain reaction (PCR). Primers COIint-F (TGATTGGT GGTTTTGGTAA) and COIint-R (ATAAGTACGAGTAT CAATATC) were used to amplify the *COXI* (Casiraghi et al. 2001); while primers used for amplifying 18S and 28S were 502-F (CAAGTACCGTGAGGGAAAGTTGC) and 536-R (CAGCTATCCTGAGGGAAAC) (García-Varela and Nadler 2005), and G18s-F (GCTTGTCTCAAAGATTAAGCC) and 136-R TGATCCTTCTGCAGGTTCACCTAC (Nadler et al.

Table 1 Nematode species analyzed in this study, including host species, localities, GenBank accession numbers, sequence code, and number of sequences per molecular marker studied in this work

Parasite/host (Family)	Localities (code in Figs. 3 and 4) (Geographical coordinates)	GenBank accession number (Sequence code in Figs. 3 and 4)	Molecular marker (number of sequences)	Reference
Rhabdochona ahuehuellensis				
Ilyodon whitei (Goodeidae)	Huaquechula, Puebla (RAP) (18° 46′ 1″ N, 98° 32′ 09″ W)	MK353475-77 (Om20–22)	*COX1* (3)	Present study
Rhabdochona canadensis				
Gila conspersa (Cyprinidae)	Sain Alto, Zacatecas (SZ) (23° 34′ 40″ N, 103° 20′ 48″ W)	MK353484-86 (Om10–12)	18s (1), 28s (2), *COX1* (3)	Present study
Rhabdochona ictaluri				
Ictalurus pricei (Ictaluridae)	Nombre de Dios, Durango (NDD) (23° 47′ 22″ N, 104° 18′ 09″ W)	MK353478-82 (Om25, 27–28, 31–32)	18s (1), 28s (2), *COX1* (5)	Present study
Rhabdochona kidderi				
Rhamdia guatemalensis (Heptapteridae)	Cenote Choo-ha, Quintana Roo (CCC) (20° 28′ 09″ N, 87° 46′ 03″ W)	MK353474 (131A)	18s (1), 28s (1), *COX1* (1)	Present study
	Cenote Punta Laguna, Quintana Roo (CPC) (20° 38′ 56″ N, 87° 38′ 11″ W)	MK353472-73 (132-133A)	28s (1), *COX1* (2)	Present study
Rhabdochona lichtenfelsi				
Goodea atripinnis (Goodeidae)	Tocumbo, Michoacán (TM) (19° 42″ 07″ N, 102° 30′ 58″ W)	DQ990995	*COX1* (1)	Mejia-Madrid et al. 2007a
	La Minzita, Michoacán (MM) (19° 38′ 40″ N, 101°16′ 28″ W)	DQ990987	*COX1* (1)	Mejia-Madrid et al. 2007a
	Pátzcuaro, Michoacán (PM) (19° 36′ 05″ N, 101° 39′ 13″ W)	DQ990983	*COX1* (1)	Mejia-Madrid et al. 2007a
Rhabdochona mexicana				
Astyanax mexicanus (Characidae)	Río Jalpan, Querétaro (RJ) (21° 13′ 25″ N, 99° 28′ 21″ W)	MK353488-89 (14-15A)	18s (1), 28s (1), *COX1* (2)	Present study
	Plan de Ayala, Tamaulipas (PA) (22° 34′ 09″ N, 98° 44′ 02″ W)	Not deposited (135A)	18s (1), 28s (1), *COX1* (1)	Present study
Rhabdochona xiphophori				
Poeciliopsis gracilis (Poeciliidae)	Camino a Hierve el Agua, Oaxaca (HAO) (16° 54′ 23″ N, 96° 19′ 55″ W)	MK353483 (Om5)	18s (1), 28s (1), *COX1* (1)	Present study
Spinitectus mexicanus				
Heterandria bimaculata (Poeciliidae)	Axtla de Terrazas, San Luis Potosí (SLP) (21° 26′ 12″ N, 98° 52′ 02″ W)	MK353487 (201A)	18s (1), 28s (1), *COX1* (1)	Present study

2000), respectively. PCR reactions (25 μl) consisted of 1 μl of each primer 10 mM, 2.5 μl of 10XA buffer, 1.5 μl of 50 mM $MgCl_2$ (1.5 μl), 2.5 μl of 2 mM dNTP'S, 2 μl of the genomic DNA, 0.125 μl of Taq DNA polymerase (Vivantis, Subang Jaya, Malaysia), and 14.375 μl of ultrapure water. PCR cycling was performed in a Thermal Cycler MJ Research PTC-100. Parameters of amplification included the following: (1) denaturation at 94 °C for 2 min, (2) preparing annealing at 94 °C for 30 s, (3) annealing at 45 °C for 1 min, and (4) extension at 72 °C for 2 min (repeating 2–4 by 35 cycles) followed by a post-amplification incubation at 72 °C for 7 min for *COXI*. The same parameters were used for 18S and 28S with a variation in the temperature and time in the annealing step (54 °C for 30 s for 18 s and 50 °C for 1 min for 28 s). Sequencing reactions were performed using ABI Big Dye (Applied Biosystems, Boston, USA) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Geneious version 5.0.4. (Drummond et al. 2010). Sequences were deposited in the GenBank data (accession numbers MK353470 to MK353487).

Alignments, phylogenetic analyses, and genetic pairwise difference

Newly generated sequences were aligned and compared with other sequences of members of the genus *Rhabdochona* available in GenBank. Nucleotide sequences were aligned using Clustal W (Thompson et al. 1994) implemented in the web version of the software at http://www.genome.jp/tools/clustalw/ with the option slow/accurate pairwise alignment. Datasets were analyzed separately for each molecular marker and were later concatenated. Tree searches were run under the maximum likelihood (ML) criterion and Bayesian inference (BI) employing a GTR+GAMMA+I model as suggested by jModelTest ver. 2 (Darriba et al. 2012). Likelihood inference (100 replicates), model parameters, and bootstrap (BS) support (1000 repetitions) were estimated with RaxML GUI 1.5 beta (Silvestro and Michalak 2012). The software MrBayes v. 3.2.1 (Ronquist et al. 2012) was used to perform BI analysis, running four independent MCMC of two chains each run for 20 million generations with a heating parameter value of 0.05. Sampling tree topologies every 1000 generations and burn-in periods were set to the first 1500 generations. A 50% majority-rule consensus tree plus posterior probability values of nodal support were calculated from the remaining trees. The phylogenetic trees were visualized with the software FigTree ver. 1.4.2 (Rambaut 2012). The genetic pairwise difference among samples was estimated using uncorrected "p" distances with the program MEGA version 6 (Tamura et al. 2013). The species *Spinitectus mexicanus*, sampled from the poeciliid *Heterandria bimaculata* from one locality of Mexico (Table 1), was used as outgroups following recent phylogenetic analyses that place this genus and *Rhabdochona* as sister groups (Choudhury and Nadler 2018).

Results

In total, 230 adult nematodes of the genus *Rhabdochona* were collected from the intestine of seven species of freshwater fishes in 10 locations of Mexico comprising river basins in both, Nearctic and Neotropical biogeographical regions.

Morphological study

Measurements of the main morphological traits of *Rhabdochona ictaluri* and *R. kidderi* are shown in Table 2. The range of most of the morphological traits is overlapped, although overall, *R. kidderi* is smaller than *R. ictaluri* and females possess a lower number of eggs in the uterus (Fig. 1); both species possess a prostom armed with 14 teeth and small bifurcated deirids, and the major diagnostic trait distinguishing both species is the presence of six teeth in the base of the prostom in *R. ictaluri*, and their consistent lack in *R. kidderi* (Fig. 1).

Discriminant function analysis and PCA

Discriminant function analysis showed a significant separation between the specimens of *R. ictaluri* and *R. kidderi.* Values of Mahalonobis distance, Wilks' lambda and *F* for males and females are shown in Table 3. Morphometric variables that significantly contributed to the discriminant function were the body width and the length of left spicule in males, and body length and body width in females (Table 3). Principal Component Analyses were performed for males and females separately. Both analyses showed two separate clusters (Fig. 2), one corresponding with *R. ictaluri*, and the other with specimens of *R. kidderi*. In males, body length and glandular esophagus length had a greater contribution to component 1, while vestibule, prostom, and muscular esophagus length contributed more to component 2. In females, body length and width as well as the position of the vulva contributed more to component 1, meanwhile the vestibule, prostom, and muscular esophagus length contributed to component 2 (see Table 3).

Phylogenetic analyses and genetic pairwise difference

The alignments of the three molecular markers were analyzed separately; *COX1* alignment consisted of 21 terminals (plus *Spinitectus mexicanus* as outgroup) and was 629 bp long; alignments of the nuclear genes represented a subset of the *COX1* samples and included six and 10 terminals for 18S and 28S rDNA with a length of 1797 bp and 1257 bp, respectively. In all datasets analyzed separately, phylogenetic trees showed that

Table 2 Measurements of the diagnostic characters of the species *Rhabdochona ictaluri* and *R. kidderi*. For comparative purposes, measurements from previous studies are included. When available, the measurements are shown as intervals and/or mean value. Abbreviations in parentheses correspond to abbreviations in Fig. 2

Species (or subspecies)	*Rhabdochona kidderi*								*Rhabdochona ictaluri*	
Source	Pearse 1936		Moravec et al. (1995)		Moravec and Huffman (1988) (as *R. k. texensis)*		Present study		Present study	
	Male	Female	Male	Female	Male ($n = 10$)	Female ($n = 10$)	Male ($n = 20$)	Female ($n = 20$)	Male ($n = 20$)	Female ($n = 20$)
Body length (BL)	7.4	7.4–10.8 (8.9)	5.64–8.7	6.42–9.26	5.88–8.19	9.0–15.83	5.74–7.66 (6.34 ± 0.6)	3.22–7.83 (5.96 ± 1.19)	5.75–10.18 (7.53 ± 2.76)	5.73–18.49 (11.51 ± 4.35)
Body width (BW)	0.5	0.8	0.015–0.021	0.082–0.095	0.082–0.095	0.122–0.218	0.05–0.095 (0.07 ± 0.01)	0.043–0.081 (0.06 ± 0.011)	0.08–0.16 (0.14 ± 0.02)	0.12–0.26 (0.17 ± 0.04)
Vestibule length, including prostom (VppL)	–	0.135	0.135–0.168	0.15–0.18	0.126–0.144	0.135–0.159	0.09–0.13 (0.016 ± 0.01)	0.10–0.12 (0.11 ± 0.0078)	0.08–0.10 (0.11 ± 0.6)	0.09–0.14 (0.11 ± 0.017)
Length of muscular esophagus (MEL)	–	–	0.3–0.36	0.3–0.36	0.258–0.36	0.330–0.405	0.19–0.32 (0.25 ± 0.04)	0.17–0.38 (0.29 ± 0.05)	0.17–0.33 (0.25 ± 0.04)	0.23–0.38 (0.3 ± 0.05)
Length of glandular esophagus (GEL)	–	–	1.27–2.1	1.14–1.66	1.59–2.05	1.93–2.49	1.01–1.56 (1.27 ± 0.05)	0.90–1.59 (1.28 ± 0.25)	1.15–2.81 (6.34 ± 0.6)	1.48–3.72 (2.1 ± 0.54)
Nerve ring distance, from anterior extremity (DNR)	–	–	0.168–0.225	0.71–0.2	0.189–0.195	0.186–0.225	0.13–0.15 (0.14 ± 0.008)	0.12–0.16 (0.14 ± 0.01)	0.12–0.22 (0.2 ± 0.43)	0.11–0.23 (0.18 ± 0.03)
Deirids, distance from anterior extremity	–	–	0.066–0.078	0.189–0.291	0.063–0.066	0.072–0.087	0.57–0.64 (0.12 ± 0.008)	0.175–0.184 (0.35 ± 0.006)		
Total length of left spicule (LEL)	1.0	–	0.651–1.167	–	0.67–1.98	–	0.29–0.53 (0.36 ± 0.07)	–	0.28–0.56 (0.5 ± 0.68)	–
Length of shaft (FL)	–	–	0.30–0.54	–	0.525–0.684	–	46.51–84.51 (57.94 ± 10.68)	–	0.16–0.24 (0.22 ± 0.25)	–
Percent of shaft (FP)	–	–	32–52%	–	31–55%	–	37.83–57.69%	–	37.83–57.69 (45.33 ± 4.82)	–
Total length of right spicule (RSL)	0.08	–	0.075–0.087	–	0.084–0.102	–	0.09–0.12 (0.11 ± 0.009)	–	0.06–0.17 (0.14 ± 0.03)	–
Ratio between spicule length (RBSL)	–	–	1:7.9–13.9	–	1:18.73–22.50 (1:22.50	–	1:1.5–1:7.2 (1:4.16 ± 1.43)	–	1:4.0–12.18(1:5.82 ± 1.73)	–
Length of tail	0.2	0.2	0.15–0.22	0.11–0.17	0.252–0.264	0.147–0.225	0.005–0.013 (0.008 ± 0.002)	0.0053–0.0142 (0.0114 ± 0.0056)	0.007–0.016 (0.09 ± 0.13)	0.007–0.039 (0.16 ± 0.017)
Vulva, distance from posterior extremity (BLDV)	–	–	–	2.52–3.63	–	3.32–6.39	–	1.79–3.38 (2.47 ± 0.54)	–	1.79–8.63 (7.81 ± 1.3)
Eggs length	–	0.14	–	0.045–0.05	–	0.036–0.042	–	0.031–0.035 (0.03 ± 0.0013)	–	0.024–0.034 (0.03 ± 0.02)
Eggs wide	–	0.022	–	0.02–0.024	–	0.021–0.024	–	0.019–0.022 (0.0195 ± 0.0009)	–	0.013–0.019 (0.017 ± 0.019)

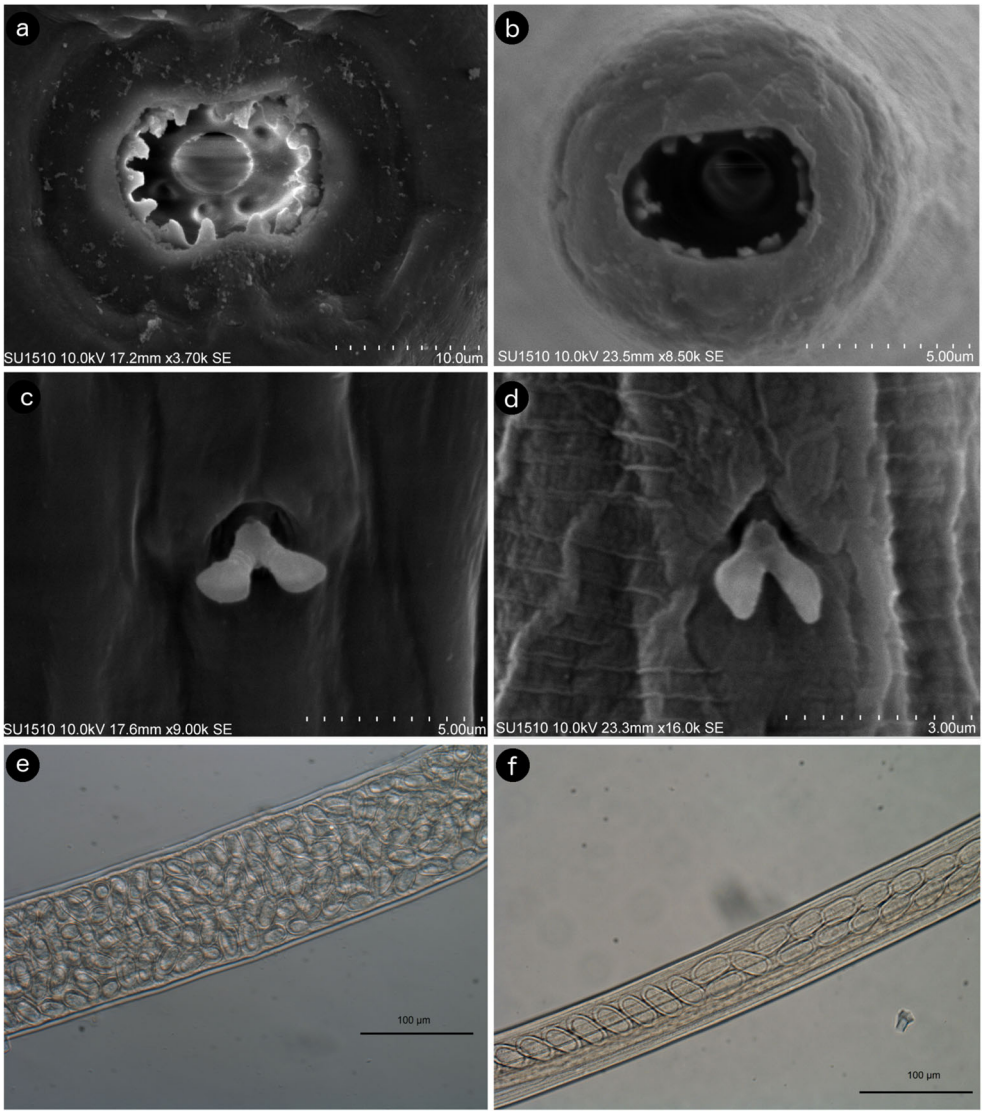

Fig. 1 Microphotographs of adults of *Rhabdochona ictaluri* (**a**, **c**, and **e**) and *R. kidderi* (**b**, **d**, and **f**) with scanning electron microscopy (**a**–**d**) and light microscopy (**e**–**f**). **a**–**b** Detail of the prostom, note the presence of six teeth at the base of the vestibule in *R. ictaluri* and their absence in *R. kidderi*; **c**–**d** detail of deiridium; **e** middle-body portion of the female of *R. ictaluri*, scale bar 100 μm; **f** middle-body portion of the *R. kidderi* female, scale bars 100 μm

Rhabdochona is a monophyletic assemblage. The 18S tree showed very low resolution; *R. kidderi* and *R. ictaluri* are sister taxa and are nested with *R. canadensis* (tree not shown). The 28S tree showed the two isolates of *R. ictaluri* as sister taxa of two isolates of *R. kidderi* forming a well-supported monophyletic clade (tree not shown). Figure 3 depicts the resulting *COX1* ML tree (-ln = 8244.039539) that includes five isolates of *R. ictaluri* and three isolates of *R. kidderi.* The tree yielded seven reciprocally monophyletic clades for *Rhabdochona* species, showing *R. ictaluri* and *R. kidderi* as independent and closely related species each highly supported by bootstrap and posterior probability support values, although relationships between them were supported by low bootstrap and posterior probability values. The Bayesian tree retrieved the same topology as the ML tree. These trees showed that the two species of *Rhabdochona* are the sister taxa of a group consisting of *R. canadensis* and *R. ahuehuellensis* + *R. xiphophori*. The concatenated analysis of the three molecular markers (18S, 28S, and *COXI*) analyzed through ML and BI, uncovered the same sister group relationships and higher nodal support for the relationships between *R. ictaluri* and *R. kidderi*; however, these two species nest as the sister taxa of *R. mexicana* (Fig. 4). The mean intraspecific genetic divergence for *COX1* among isolates of each *Rhabdochona* species varied from 0 to 4.8%. Variation of this value within *R. kidderi* was 2.6%, while within *R. ictaluri* was 0.07%. The interspecific divergence values for *COX1* are shown in Table 4. The *COXI* interspecific genetic divergence between the species *R. ictaluri* and *R. kidderi* was very high, with a mean value of 13.14%.

Discussion

The taxonomic identification in nematodes is a difficult task because the characters used for the description of the species require the use of high-resolution microscopy techniques and

Table 3 Summary of the discriminant function analysis for 40 individuals of *R. ictaluri* ($n = 20$) and *R. kidderi* ($n = 20$). Values contributing to the discriminant function are indicated in italic (p value = 0.05)

	Lambda de Wilks	Lambda parcial	F-remove (1, 28)	p level	Tolerancia	1-Tolerancia(R-Sqr.)
Male						
Body length	0.096	0.955	1.313	0.261	0.428	0.571
Body width	*0.143*	0.641	*15.670*	*0.000*	0.325	0.674
Length of vestibule including prostom	0.094	0.970	0.865	0.360	0.117	0.882
Length of muscular esophagus	0.096	0.954	1.323	0.259	0.307	0.692
Length of glandular esophagus	0.095	0.961	1.133	0.296	0.476	0.523
Nerve ring position from anterior extremity	0.091	0.999	0.016	0.898	0.078	0.921
Total length of left spicule	*0.110*	0.833	*5.597*	*0.025*	0.062	0.937
Length of shaft	0.096	0.954	1.335	0.257	0.023	0.976
Percent of shaft	0.097	0.944	1.633	0.211	0.028	0.971
Total length of right spicule	0.093	0.982	0.498	0.486	0.348	0.651
Length of tail	0.100	0.910	2.741	0.108	0.256	0.743
Female						
Length of body	*0.131*	0.750	*10.617*	*0.002*	0.040	0.959
Maximum body width	*0.330*	0.298	*75.234*	*0.000*	0.121	0.878
Length of vestibule including prostom	0.102	0.961	1.271	0.267	0.223	0.776
Length of muscular esophagus	0.113	0.867	*4.897*	0.034	0.348	0.651
Length of glandular esophagus	0.109	0.901	3.482	0.071	0.446	0.553
Nerve ring position from anterior extremity	0.109	0.897	3.653	0.064	0.336	0.663
Vulva position from posterior extremity	0.098	0.999	0.001	0.965	0.187	0.812

a relatively specialized knowledge of the morphology of the group (De Ley et al. 2005; Chaudhary et al. 2017). In addition, factors such as the lack of robust diagnostic characters in the descriptions, intraspecific variation, samples of individuals at different developmental stages or from different sex sometimes impede an objective comparison and contribute to the lack of accuracy in the taxonomic differentiation between taxa (De Ley et al. 1999, 2005; Pereira et al. 2010; Archidona-Yuste et al. 2016; Chaudhary et al. 2017; Janssen et al. 2017). Therefore, the use of several sources of information through an integrative taxonomy approach (Dayrat 2005; Schlick-Steiner et al. 2009) is necessary; data from morphology, DNA, ecology (host association), and biogeography have proven to be very useful to accomplish a robust species delimitation and even to recognize the existence of cryptic species (Nadler and Pérez-Ponce de León 2011).

The synonymy of *Rhabdochona ictaluri* with *R. kidderi* as proposed by Moravec et al. (2012) solely on morphological grounds provided an opportunity for the scrutiny of the taxonomical act by following an integrative taxonomy approach.

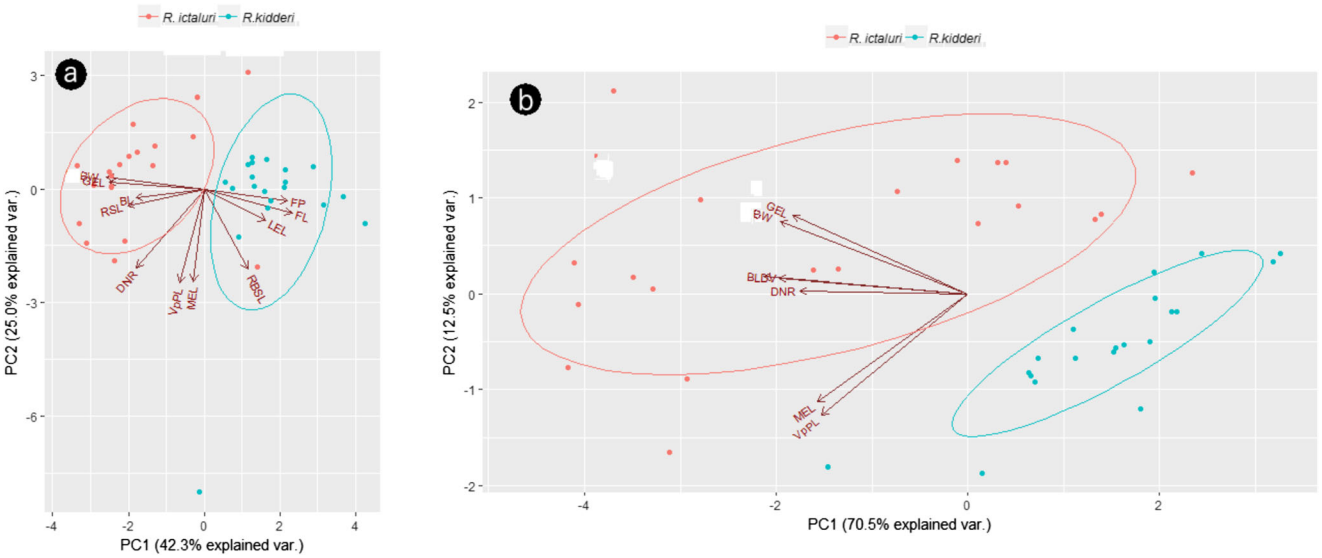

Fig. 2 PCA of the males (**a**) and females (**b**) of *Rhabdochona ictaluri* and *R. kidderi* in the morphometric space. Vector codes correspond to the abbreviations in parentheses in Table 2

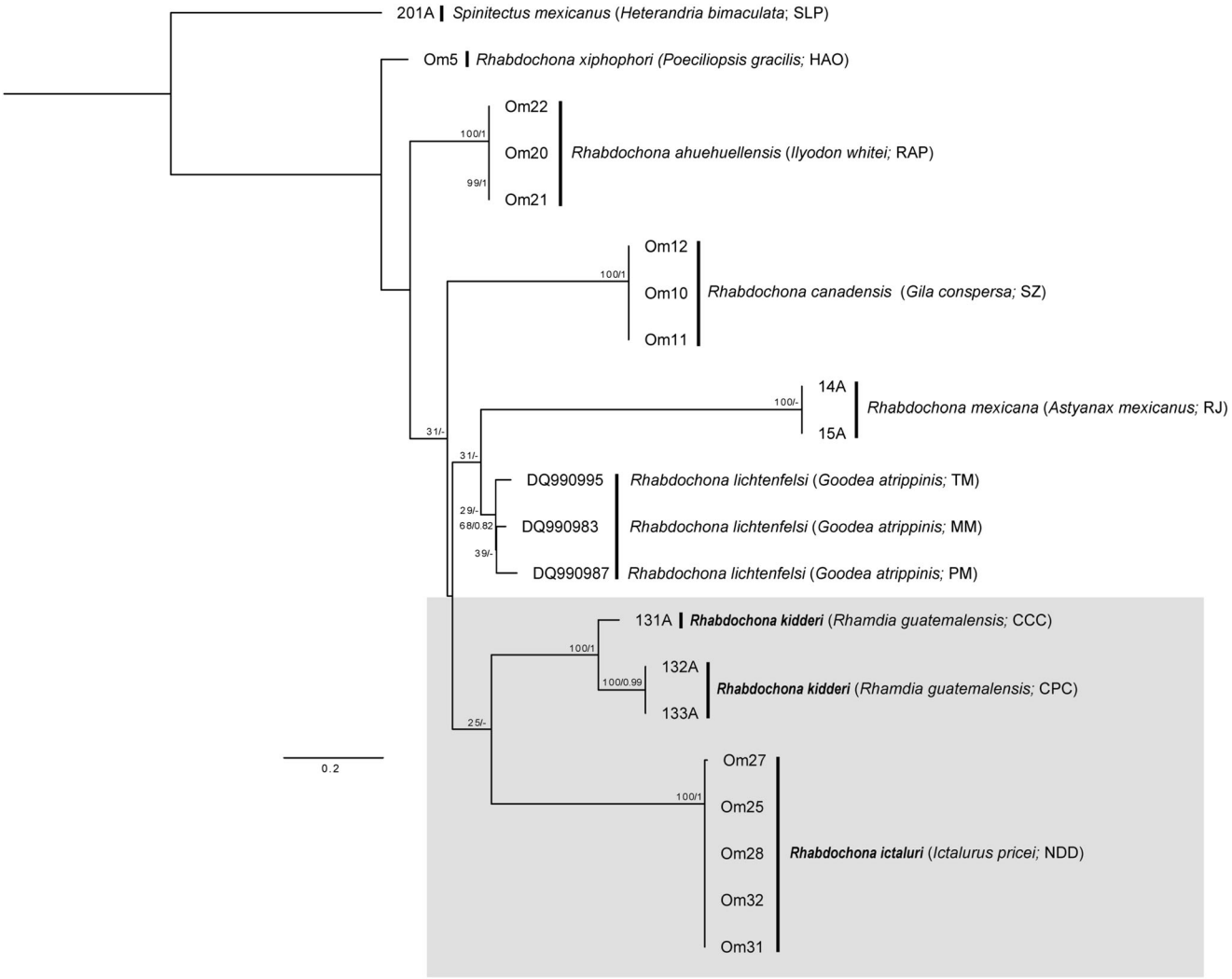

Fig. 3 *COX1* phylogenetic tree of *Rhabdochona* spp. through Maximum likelihood and Bayesian Inference. Bootstrap and the posterior probability values next to the nodes. The scale bar represents the number of nucleotide substitutions per site. The identification codes and abbreviations of the localities correspond to those presented in Table 1

First of all, the results of our morphological analyses confirmed that the most notorious difference between both species is the presence of six basal teeth at the prostom in *R. ictaluri*, a character that is absent in *R. kidderi*, as pointed out by Aguilar-Aguilar et al. (2010). The specimens of *R. kidderi* deposited at the CNHE were studied, and the consistent lack of such teeth was corroborated. We acknowledge that the comparison of morphometric traits indicated a high level of overlap between the two species, but overall, *R. kidderi* is smaller than *R. ictaluri* and possesses a lower number of eggs in the uterus in females. Likewise, such morphological differences might be considered not reliable to establish the separation among the two species of *Rhabdochona.* To further corroborate the species distinction, we performed multivariate statistical analyses with the aim of reducing the potential subjectivity of the morphometric differences; these methods have also proven to be useful to distinguish species in several taxonomic groups (e.g., Albrecht 1980; Cheng et al. 2005; Mazur et al. 2010; Egan 2015). In the present study, discriminant analysis and principal components allowed us to recognize that both species are clearly separated, and that separation is statistically significant. In principle, our morphological analyses suggested that *R. ictaluri* is a valid species and does not corroborate the proposed synonymy with *R. kidderi* suggested by Moravec et al. (2012), more likely to be a result of a misinterpretation of the intraspecific variation of the species.

However, to further corroborate the species distinction, we used other sources of information following an integrative approach (Dayrat 2005; Will et al. 2005; Schlick-Steiner et al. 2009; Pante et al. 2015). Molecular data provide information about species limits by detecting genetic variation among species and populations (see Pante et al. 2015 and references therein), and it is not subjected to phenotypic

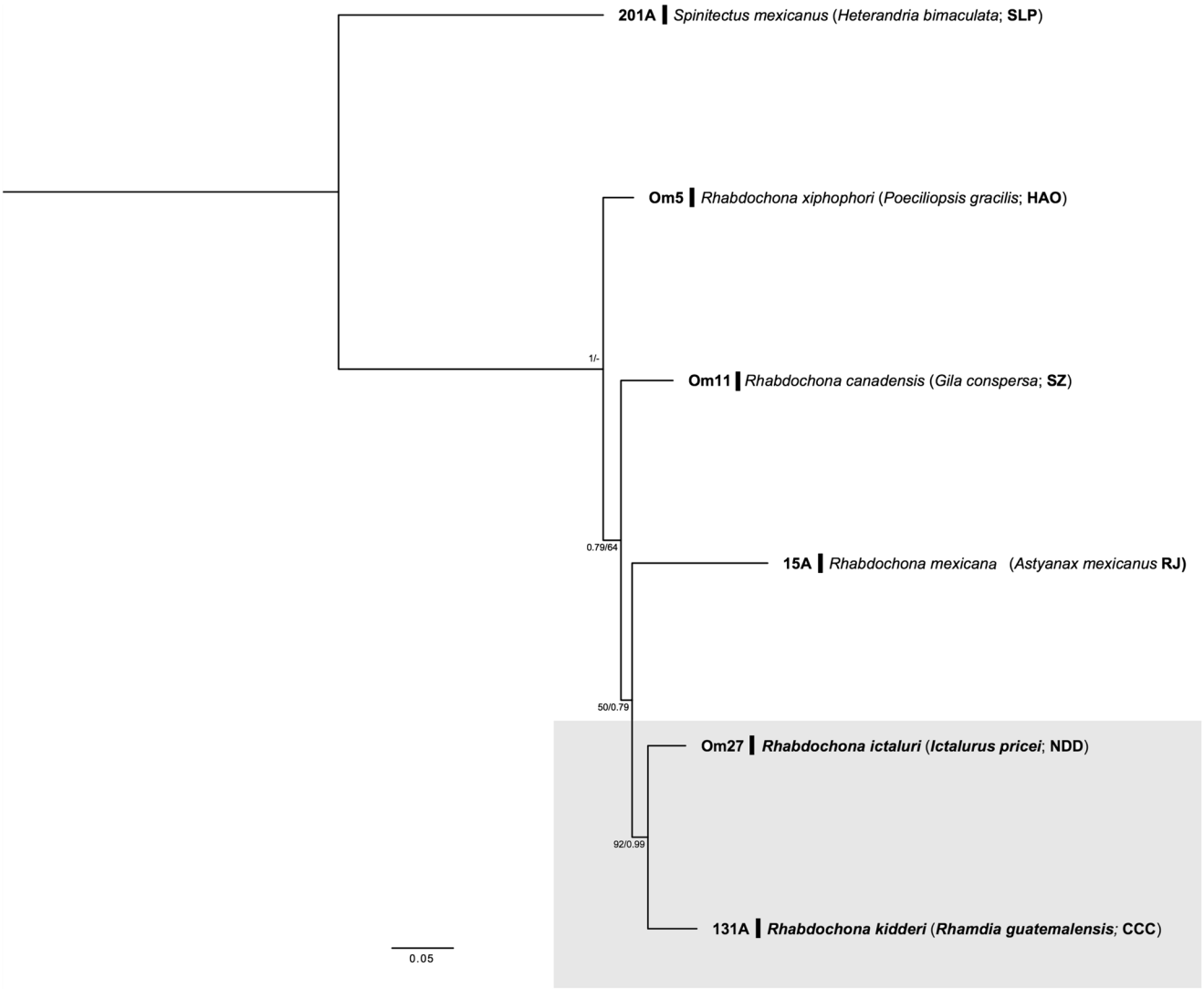

Fig. 4 Phylogenetic tree derived from the concatenated matrix of sequences of 18S, 28S, and *COX1* for *Rhabdochona* spp. through Maximum likelihood and Bayesian Inference. Bootstrap and the posterior probability values next to the nodes. The scale bar represents the number of nucleotide substitutions per site. The identification codes and abbreviations of the localities correspond to those presented in Table 1

variation and the potential overlap on the measurements of morphological traits. We used molecular data through one mitochondrial and two nuclear genes to explore the genetic divergence among the sampled individuals of the two species of *Rhabdochona*, to test for reciprocal monophyly and to assess the phylogenetic interrelationships with respect to other congeneric species occurring in Mexican freshwater fishes. The phylogenetic analyses of independent data sets, as well as the concatenated analysis of the three molecular markers (Fig. 4) showed that isolates of *R. ictaluri* and *R. kidderi* represented reciprocally monophyletic lineages. The nuclear DNA sequences of 18S and 28S were less informative than *COX1* which possess a higher substitution rate. The cytochrome oxidase *c* subunit 1 of the mitochondrial gene (*COX1*) has been proposed as the preferred marker for the molecular differentiation among species (e.g., Huang et al. 2008; Eberhardt 2010; Satler et al. 2013); particularly in nematodes, *COX1* also represents the preferred molecular marker for species delimitation studies (see Blouin 2002; Singh et al. 2015). Our study revealed high levels of interspecific genetic divergence of *COX1* among species of *Rhabdochona* with overall values varying from 6.14 to 14.09%. The average genetic divergence between *R. ictaluri* and *R. kidderi* was 13.14%. The use of a genetic yardstick has been highly debated (see discussion in Nadler and Pérez-Ponce de León 2011), and even though there is not a well-defined threshold to differentiate among parasite species, in most studies on parasitic nematodes, species limits are considered when *COX1* values reach about 6% (see Blouin 1998, 2002; Nakano et al. 2006; Solórzano-García et al. 2016; Singh et al. 2015; Chaudhary et al. 2017). The molecular study further corroborated that *R. ictaluri* is a valid species.

Table 4 *COX1* interspecific divergence percentages among species of genus *Rhabdochona* and *Spinitectus mexicanus*. Value in italic denotes the divergence between the species *R. ictaluri* and *R. kidderi*

	1	2	3	4	5	6	7	8
1 *R. ahuehuellensis*	–							
2 *R. canadensis*	9.87%	–						
3 *R. kidderi*	10.01%	12.87%	–					
4 *R. ictaluri*	11.89%	12.5%	*13.14%*	–				
5 *R. lichtenfels*	6.80%	9.28%	10.31%	10.61%	–			
6 *R. mexicana*	11.18%	14.25%	14.33%	14.09%	11.48%	–		
7 *R. xiphophori*	6.14%	9.87%	10.53%	9.47%	7.38%	12.06%	–	
8 *S. mexicanus*	12.28%	14.25%	13.74%	14.4%	12.94%	16.01%	12.06%	–

The phenotypic and genetic cohesion found that among the species compared in the present study seems to respond to the genetic isolation derived from ecological and biogeographical barriers (Templeton 1989). The diversification of helminths in Mexican freshwater fishes has been correlated with the geographical and ecological isolation of their hosts (Pérez-Ponce de León and Choudhury 2005, 2010; Mejia-Madrid et al. 2007a, b; Alcántar-Escalera et al. 2013; Martínez-Aquino et al. 2014; Sereno-Uribe et al. 2013; Pérez-Ponce de León et al. 2016). The species *R. ictaluri* has been found only in freshwater fish hosts with Nearctic affinity, such as ictalurid catfishes (Mayden 1992; Miller et al. 2005); instead, *R. kidderi* has been registered predominantly in fish hosts with a marked Neotropical affinity, particularly in species of the families Cichlidae and Heptapteridae (Miller et al. 2005), or in Nearctic fishes of the genus *Ictalurus* inhabiting Neotropical localities (Caspeta-Mandujano 2010). Several studies (e.g. Rego 2000; Rosas-Valdez and Pérez-Ponce de León 2008; Quiroz-Martínez and Salgado-Maldonado 2013; Choudhury et al. 2017) have actually suggested a very limited or null faunal exchange of helminth parasites among freshwater fishes with different biogeographical origin, as in this case, ictalurids (Nearctic origin) vs heptapterids and cichlids (Neotropical origin) (Pérez-Ponce de León and Choudhury 2005; Rosas-Valdez and Pérez-Ponce de León 2008). In this context, empirical geographical evidence seems to reinforce the differentiation between the species *R. ictaluri* and *R. kidderi*, being the first a Nearctic species, and a Neotropical species the latter. The catfishes of the genus *Ictalurus* are not the preferred host of *R. kidderi* and the sporadic records in these fishes (see Pérez-Ponce de León and Choudhury 2002; Caspeta-Mandujano 2010) resulted from an ecological host-extension, although the very low prevalence values and the immature nature of specimens of *R. kidderi* indicate that they represent accidental infections. Host association and biogeography provided another piece of information to recognize the validity of *R. ictaluri* as separate species. The results of our study show the convenience of gathering evidence from different sources of information to attain a more robust species delimitation, contributing to resolve taxonomical controversies that result in the synonymy of species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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