

Research Paper

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Multilocus phylogenetic analysis and morphological data reveal a new species composition of the genus *Drepanocephalus* Dietz, 1909 (Digenea: Echinostomatidae), parasites of fish-eating birds in the Americas

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Abstract

Members of the genus *Drepanocephalus* are endoparasites of fish-eating birds of the families Phalacrocoracidae and Sulidae distributed across the Americas. Currently, *Drepanocephalus* contains three species, i.e. *D. spathans* (type species), *D. olivaceus* and *D. auritus*. Two additional species, *D. parvicephalus* and *D. mexicanus* were transferred to the genus *Petasiger*. In the current study, available DNA sequences of *D. spathans*, *D. auritus* and *Drepanocephalus* sp., were aligned with newly generated sequences of *D. spathans* and *Petasiger mexicanus*. Phylogenetic analyses inferred with three nuclear (LSU, SSU and ITS1, 5.8S, ITS2) and two mitochondrial (*cox1*, *nad1*) molecular markers showed that the sequences of *D. spathans* and *D. auritus* are nested together in a single clade with very low genetic divergence, with *Petasiger mexicanus* as its sister species. Additionally, *P. mexicanus* was not a close relative of other members of the genus *Petasiger*, showing that *P. mexicanus* actually belongs to the genus *Drepanocephalus*, suggesting the need to re-allocate *Petasiger mexicanus* back into the genus *Drepanocephalus*, as *D. mexicanus*. Morphological observations of the newly sampled individuals of *D. spathans* showed that the position of the testes is variable and testes might be contiguous or widely separated, which is one of the main diagnostic traits for *D. auritus*. Our results suggest that *D. auritus* might be considered a synonym of *D. spathans* and, as a result, the latter represents a species with a wide geographic range across the Americas, parasitizing both the Neotropical and the double-crested cormorant in Argentina, Brazil, Paraguay, Venezuela, Colombia, Mexico, USA and Canada.

Introduction

Members of the genus *Drepanocephalus* Dietz, 1909 (Digenea: Echinostomatidae) use fish-eating birds of the genera *Nannopterum* Brisson and *Sula* Brisson as definitive hosts (Fedynich *et al.*, 1997; Kostadinova *et al.*, 2002; Flowers *et al.*, 2004; Robinson *et al.*, 2008; Monteiro *et al.*, 2011; Violante-González *et al.*, 2011; Griffin *et al.*, 2014). Freshwater fish such as siluriforms (*Ictalurus punctatus* Rafinesque) and cichlids (e.g. *Astatheros robertsoni* Allen, *Paratheraps bifasciatus* Steindachner, *P. fenestratus* Günther, *Paraneetroplus synspilus* Hubbs, *Thorhichthys helleri* Steindachner, *Cichlasoma pearsei* Hubbs, *Mayaheros urophthalmus* Günther and *Oreochromis niloticus* L.) are used as second intermediate hosts (Jimenez-García, 1993; Vidal-Martínez *et al.*, 2002; Griffin *et al.*, 2012; Pinto *et al.*, 2014). Planorbid snails (*Planorbella trivolvis* Say and *Biomphalaria straminea* Dunker) are used as the first intermediate hosts (Griffin *et al.*, 2012).

Currently, *Drepanocephalus* comprises three species, i.e. *D. spathans* Dietz, 1909 (type species), *D. olivaceus* Nasir & Marval, 1968 and *D. auritus* Kudlai, Kostadinova, Pulis & Tkach, 2015, all distributed across the Americas (Dietz, 1910; Nasir & Marval, 1968; Kostadinova *et al.*, 2002; Kostadinova, 2005; Kudlai *et al.*, 2015a). However, the taxonomic status of some species has been controversial and, in the first taxonomic revision of the genus, two species, *D. parvicephalus* Rietschel & Werding, 1978 and *D. mexicanus* Lamothe-Argumedo & Pérez Ponce de León, 1989, were transferred to the genus *Paryphostomum* Dietz 1909 by Kostadinova *et al.* (2002), and more recently Tkach *et al.* (2016) transferred these species from the genus *Paryphostomum* to *Petasiger* Dietz, 1909, using a phylogenetic framework, inferred with partial sequences from the large subunit of the ribosomal DNA.

Over the past several years, two species of cormorants (*Nannopterum brasilianus* Gmelin and *Nannopterum auritus* Lesson), as well as one species of water turkey (*Anhinga anhinga* L.) have been sampled in several localities across Mexico. Gravid echinostomatids belonging to the genera *Drepanocephalus*, *Petasiger* and *Euparyphium* Dietz, 1909 were collected from the intestines of these fish-eating birds. Fresh samples of these trematodes allowed us to analyse their morphology using light and scanning electron microscopy, and to obtain DNA sequences; host details and geographic distribution of *Drepanocephalus* species across the Americas were also retrieved from several bibliographical sources. In some cases, the literature indicates confusion about the taxonomic status of *D. spathans* / *D. auritus* (see Drago *et al.*, 2011; Sheehan *et al.*, 2016). In this study, we conducted a multilocus phylogenetic analysis of species of *Drepanocephalus* and *Petasiger*, using newly generated as well as available sequences from GenBank to assess their evolutionary relationships within the Echinostomatidae. Our phylogenetic and morphological analyses clearly suggested that the species composition of the genus *Drepanocephalus* should be re-evaluated.

Materials and methods

Specimen collection

A total of 63 birds including 47 Neotropical cormorants (*N. brasilianus*), 14 double-crested cormorants (*N. auritus*) and 2 water turkeys (*A. anhinga*) were collected between June 2005 and March 2015 in 25 localities across Mexico (table 1, fig. 1). Bird nomenclature follows Kennedy & Spencer (2014). Birds were dissected within 2 h after capture; their viscera were placed in separate Petri dishes containing a 0.75% saline solution and examined under a dissecting microscope. Trematodes were washed in 0.75% saline solution and fixed with hot 4% formalin for morphological studies and in 100% ethanol for DNA analyses.

Morphological study

Unflattened digeneans preserved in formalin were stained with Mayer's paracarmine or with iron acetocarmine, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted as permanent slides in Canada balsam. Drawings were made with the aid of a drawing tube. Measurements are given in micrometres (μm) followed by the range.

Abbreviations were used to refer to each morphological character, following the terminology of Kudlai *et al.* (2015a): BL, body length; BWVS, body width at level of ventral sucker; BWAT, body width at level of anterior testis; CL, collar length; CW, collar width; OSL, oral sucker length; OSW, oral sucker width; PL, prepharynx length; PHL, pharynx length; PHW, pharynx width; OL, oesophagus length; CSL, cirrus sac length; CSW, maximum cirrus sac width; SV1L, length of anterior portion of seminal vesicle; SV1W, width of anterior portion of seminal vesicle; SV2L, length of posterior portion of seminal vesicle; SV2W, width of posterior portion of seminal vesicle; VSL, ventral sucker length; VSW, ventral sucker width; OVL, ovary length; OVW, ovary width; MEL, Mehlis' gland length; MEW, Mehlis' gland width; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; EL, egg length; EW, egg width; FORE, forebody length; OVAR, distance between ovary and posterior margin of ventral sucker; TEND, distance between

posterior margin of posterior testis and posterior extremity of body. In addition to the standard measurements, the following relative proportions were calculated according to Kostadinova (2005): BW (%), maximum body width as a proportion of body length; FO (%), length of forebody as a proportion of body length; U (%), length of uterine field posterior to ventral sucker (used as an approximation for uterine length) as a proportion of body length; T (%), length of post-testicular field as a proportion of body length.

Voucher specimens (paragenophore) were deposited in the Colección Nacional de Helminthos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, Mexico. The species identification was conducted following the most recent revision of the genus *Drepanocephalus* by Kostadinova *et al.* (2002) and from original descriptions (Lamothe-Argumedo & Pérez-Ponce de León, 1989; Kudlai *et al.*, 2015a). Newly collected specimens (paragenophore) were compared with type and voucher material of *Drepanocephalus* and *Petasiger*, deposited in the CNHE: *P. mexicanus* (syn. *D. mexicanus*), holotype (CNHE 1279), three paratypes (CNHE 1280.2–4); *D. olivaceus* (syn. *D. spathans*), seven vouchers (CHNE 1411, three slides; CNHE 4998–9, two slides; CNHE 7577–8, two slides).

Six specimens, three of *D. spathans* and three of *P. mexicanus*, fixed in 4% hot formalin were examined using 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 scanning electron microscope (Hitachi Ltd, Tokyo, Japan) at the Instituto de Biología, UNAM, Mexico.

Specimens and DNA isolation

A total of 60 specimens, 38 identified as *D. spathans*, 20 as *P. mexicanus* and 2 as *Euparyphium capitaneum* Dietz, 1909 (see table 2 for details), were placed individually in tubes and digested overnight at 56°C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na₂EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using the DNAzol reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturer's instructions.

Amplification and sequencing of DNA

Three regions of nuclear ribosomal DNA (rDNA) were amplified using the polymerase chain reaction (PCR). The D1–D3 domains of the large subunit (LSU) ribosomal DNA (rDNA) were amplified using the forward primer 5'-GAA CAT CGA CAT CTT GAA CG-3' (Hernández-Mena *et al.*, 2014) and reverse primer 5'-CAG CTA TCC TGA GGG AAA C-3' (García-Varela & Nadler, 2005). The near-complete small subunit (SSU) rDNA (~1800 bp) was amplified in two overlapping PCR fragments of 1000–1200 bp. Primers used for SSU amplicon 1 were forward primer 5'-GGC GAT CGA AAA GAT TAA GCC ATGC A-3' and reverse primer 5'-CGA AGT CCT ATT CCA TTA TTC-3'; amplicon 2, forward 5'-GCA GCC GCG GTA ATT CCA GCT C-3' and reverse 5'-CGA AGT CCT ATT CCA TTA TTC-3' (García-Varela & Nadler, 2005). The internal transcribed spacer (ITS) region, including ITS1, 5.8S and ITS2, was amplified using the forward primer 5'-GTC GTA ACA AGG TTT CCG TA-3' and the reverse primer 5'-ATC TAG ACC GGA CTA GGC TGT G-3' (Bowles & McManus, 1993). Two partial regions

Table 1. Sampling details of the fish-eating birds examined for the presence of echinostomatids in Mexico.

Host	State	Locality (legend in fig. 1)	Geographic coordinates	<i>n</i>	Date of collection	
Genus <i>Nannopterum</i> Sharpe						
<i>N. auritus</i> (Lasson)	Baja California	Guerrero Negro (2)	27°57'32"N, 114°03'22"W	1	6 November 2006	
	Sonora	Ciénaga de Santa Clara (1)	32°21'12"N, 114°54'07"W	3	12 December 2007	
	Tamaulipas	Altamira (13)	22°21'05"N, 97°59'24"W	1	11 December 2008	
		Punta de Piedra (12)	24°29'0"N, 97°45'0"W	1	8 December 2008	
	Yucatán	Celestún (23)	24°54'16"N, 90°20'34"W	8	3 July 2006	
<i>N. brasiliensis</i> (Gmelin)	Campeche	Ulumal (22)	18°16'43"N, 90°37'26"W	4	10 October 2009	
	Chiapas	Presa La Angostura (11)	16°11'31"N, 92°59'52"W	3	25 March 2014	
	Durango	Laguna de Santiaguillo (5)	24°52'24"N, 104°54'38"W	1	26 November 2009	
		Río Guatimape (4)	24°52'24"N, 104°54'38"W	4	26 November 2009	
	Nayarit	La Tovar, San Blas (7)	21°32'00"N, 105°17'22"W	1	30 October 2006	
	Oaxaca	Lagunas de Manialtepec (9)	15°56'45"N, 97°11'40"W	2	3 December 2013	
		Presa Río Verde (8)	19°06'35"N, 97°43'58"W	3	1 December 2013	
		Río Tehuantepec (10)	16°23'55"N, 95°18'30"W	1	26 March 2015	
		Temascal, Tuxtepec (15)	18°14'22"N, 96°24'11"W	1	24 November 2010	
				3	6 December 2011	
	Quintana Roo	Río Hondo (25)	18°28'35"N, 89°09'52"W	1	15 August 2013	
	Sinaloa	Huizache (6)	23°06'47"N, 106°13'45"W	2	30 November 2009	
		Topolobampo (3)	25°36'10"N, 109°01'13"W	1	28 April 2011	
	Tabasco	El Espino (21)	18°14'47"N, 92°49'57"W	1	15 December 2011	
		Teapa (20)	17°34'59"N, 92°53'30"W	5	12 October 2009	
	Veracruz	Catemaco (18)	18°25'00"N, 95°07'00"W	1	9 December 2011	
		Coatzacoalcos (19)	18°06'31"N, 94°27'14"W	2	12 December 2011	
		Los Chivos (16)	18°56'00"N, 95°58'00"W	1	23 February 2008	
		Tecolutla (14)	20°27'09"N, 97°01'13"W	1	11 December 2010	
		Tlacotalpan (17)	18°36'0"N, 95°39'0"W	2	26 June 2005	
					3	21 March 2014
					2	21 March 2015
Yucatán	Chuburná, Puerto Progreso (24)	21°13'56"N, 89°49'44"W	1	5 October 2009		
			1	19 August 2013		
Genus <i>Anhinga</i> Brisson						
<i>A. anhinga</i> (L.)	Nayarit	La Tovar, San Blas (7)	21°32'00"N, 105°17'22"W	1	31 October 2006	
	Veracruz	Tecolutla (14)	20°27'09"N, 97°01'13"W	1	11 December 2010	

of the mitochondrial DNA were also amplified. Primers for the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) were forward 5'-AGA TTC GTA AGG GGC CTA ATA-3' (Morgan & Blair, 1998) and reverse 5'-CTT CAG CCT CAG CAT AAT-3' (Kostadinova *et al.*, 2003) and, finally, to amplify the gene cytochrome *c* oxidase subunit I (*cox1*) the primers were forward 5'-TCT TTR GAT CAT AAG CG-3' and reverse 5'-CCA AAA AAC CAA AAC ATA TGC TG-3' (Kudlai *et al.*, 2015a).

PCR reactions (25 µl) consisted of 10 µM of each primer, 2.5 µl of 10× buffer, 2 mM MgCl₂ (1.5 µl), 10 µM of deoxynucleoside triphosphates (dNTPs) (0.5 µl), 2 µl of the genomic DNA and 1 U of *Taq* DNA polymerase (0.125 µl) (Platinum *Taq*, Invitrogen

Corporation, São Paulo, Brazil). PCR cycling parameters for amplifications included denaturation at 94°C for 3 min; followed by 35 cycles of 94°C for 1 min, annealing at 45°C for *cox1* and *nad1* and 50°C for LSU, SSU and ITS for 1 min, and extension at 72°C for 1 min; followed by a post-amplification incubation at 72°C for 10 min. Sequencing reactions were performed using ABI Big Dye (Applied Biosystems, Boston, Massachusetts, 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 Codoncode Aligner version 5.0.2 (Codoncode Corporation, Dedham, Massachusetts, USA). Sequences were deposited in the GenBank dataset (table 2). All sequences were aligned using the

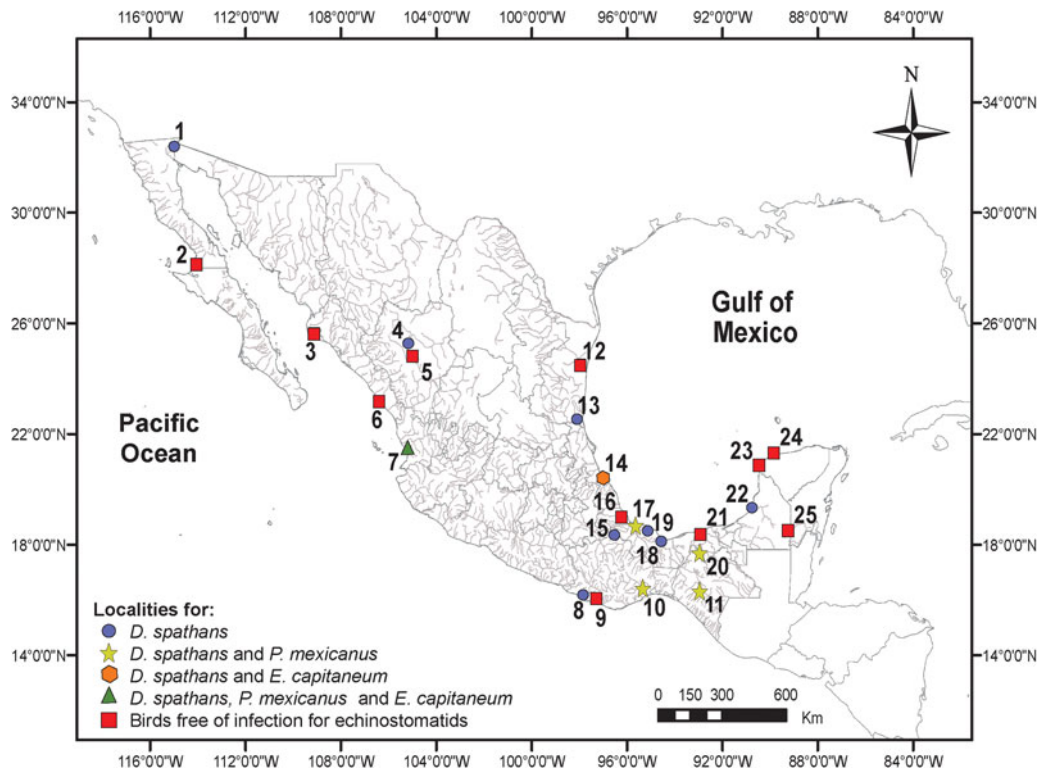


Fig. 1. Map of Mexico showing sampling sites for cormorants and water turkeys. Legends for localities are indicated in table 1.

software Clustal W (Thompson *et al.*, 1997). Maximum likelihood (ML) and Bayesian inference analyses (BI) were performed for each dataset. The ML tree was inferred using RAxML 7.0.4. (Stamatakis, 2006). jModelTest v0.1.1 was used to get the best model of nucleotide substitution (Posada, 2008). Tree searches were performed using 1000 (ML) random taxon addition heuristic searches. Clade support was assessed by bootstrap resampling with 10,000 replicates. Bayesian analyses were performed with MrBayes version 3.2.2 (Ronquist *et al.*, 2012). Settings were two simultaneous runs of the Markov chain (MCMC) for 10 million generations, sampling every 1000 generations, a heating parameter value of 0.2 and a 'burn-in' of 25%. Trees were drawn using FigTree version 1.3.1 (Rambaut, 2012). The genetic divergence among taxa was estimated using uncorrected 'p' distances with the program MEGA version 6 (Tamura *et al.*, 2013).

Phylogenetic analyses

Our strategy was to obtain sequence data of the same molecular markers (LSU, SSU, ITS, *cox 1* and *nad 1*) as those available for other species of *Drepanocephalus* (e.g. Griffin *et al.*, 2012; O'Hear *et al.*, 2014; Kudlai *et al.*, 2015a; Van Steenkiste *et al.*, 2015; Pinto *et al.*, 2016), as well as other echinostomatids, and *Fasciola hepatica* was used as outgroup to root the trees. We constructed five alignments, one for each of the three nuclear and the two mitochondrial genes.

Results

Morphological identification

Following the original description of Lamothe-Argumedo & Pérez-Ponce de León (1989) and Kostadinova *et al.* (2002), our specimens collected in cormorants from Mexico were identified

as *P. mexicanus* and *D. spathans*; however, wide morphological variation between testes was found and therefore some specimens were similar to *D. auritus* (see Kudlai *et al.*, 2015a).

Nuclear genes

The LSU dataset included 1230 characters and the best evolution model obtained was TIM3 + I + G. The alignment includes four sequences of *D. spathans*, five of *D. auritus*, a sample identified as *Drepanocephalus* sp. from Brazil, and three specimens of *P. mexicanus* (including a specimen from the type host and type locality), and two other species of the genus *Neopetasiger*, plus *E. capitaneum* and other echinostomatids (see table 2). The phylogenetic analysis inferred with ML and BI shows that the sequences of *P. mexicanus* and a single sequence of a cercaria identified as *Drepanocephalus* sp. (KP053261) from *B. straminea* from Brazil formed a clade, which is sister species to a clade containing sequences of *D. spathans* and *D. auritus*. Our analysis revealed that the new sequences of *P. mexicanus* are not nested with other species of the genus *Petasiger* (fig. 2a). The SSU dataset included 1783 characters and the best evolution model obtained was TIM3 + I + G. This alignment includes 16 sequences of *D. spathans*, and eight of *P. mexicanus* (including specimens from the type host and type locality), plus two species of the genus *Petasiger* and two specimens of *E. capitaneum*, other species of echinostomatids, and *F. hepatica* (outgroup) (see table 2). The phylogenetic analysis inferred with ML and BI also showed that *P. mexicanus* is not nested with the other members of the genus *Petasiger*. In addition, the eight sequences of *P. mexicanus* plus 16 sequences of *D. spathans* are nested in a clade forming a basal polytomy (see fig. 2b). The topologies inferred with LSU and SSU datasets are similar to the phylogenetic tree inferred with ITS, although the latter includes a larger number of

Table 2. Sequences used in the phylogenetic analyses. LSU, large subunit rDNA; SSU, small subunit rDNA; ITS, internal transcribed spacer; *nad1*, nicotinamide adenine dinucleotide dehydrogenase subunit 1 gene; *cox1*, cytochrome c oxidase subunit I gene.

Species	Life-cycle stage	Host	Locality	GenBank accession no.					Source
				LSU	SSU	ITS	<i>nad1</i>	<i>cox1</i>	
Genus <i>Drepanocephalus</i> Dietz, 1909									
<i>D. auritus</i> Kudlai, Kostadinova, Pulis & Tkach, 2015	C	<i>Biomphalaria straminea</i> (Dunker)	Belo Horizonte, Minas Gerais (Brazil)	KP053260	–	KP053260	KP053263	–	Pinto <i>et al.</i> , 2014
						–	KP053255		
			<i>Planorbella trivolvis</i> (Say)	USA	KP053259	–	KP053259	KP053262	
			Canada	–	–	–	–	KR259644	
	A	<i>Nannopterum auritus</i> (Lasson)	Nelson County, North Dakota (USA)	–	–	KP683117	–	–	Gordy <i>et al.</i> , 2016
			Beltrami County, Minnesota (USA)	KP683122	–	–	–	–	Kudlai <i>et al.</i> , 2015a
			George County, Mississippi (USA)	KP683123 KP683124	–	–	–	–	
					–	KP683122	–	–	
					–	–	–	KP683125	
					–	–	–	KP683126	
					–	–	–	KP683127	
					–	–	–	KP683128	
<i>D. mexicanus</i> Lamothe-Argumedo & Pérez-Ponce de León, 1989	A	<i>Nannopterum brasilianus</i> (Gmelin)	La Tovar, San Blas, Nayarit (Mexico)	–	–	KY636265	KY636282	KY636199	Present study
					–	KY636266	KY636283	KY636198	
					–	KY636267	–	KY636197	
					–	–	–	KY636196	
					–	–	–	KY636195	
						Presa La Angostura, Chiapas (Mexico)	MF351542	KY636319	
				–	KY636315	KY636263	–	KY636202	
					KY636322	KY636277	–	KY636185	

(Continued)

Table 2. (Continued.)

Species	Life-cycle stage	Host	Locality	GenBank accession no.					Source
				LSU	SSU	ITS	<i>nad1</i>	<i>cox1</i>	
					–	KY636274	KY636296	KY636187	
					–	KY636275	KY636297	–	
				–	KY636320	KY636273	–	KY636188	
				MF351543	KY636321	KY636276	KY636298	KY636186	
					KY636314	KY636262	–	–	
			Río Tehuantepec, Oaxaca (Mexico)		–	KY636270	–	KY636182	
			Teapa, Tabasco (Mexico)	MF351544	KY636302	KY636242	–	KY636226	
					KY636301	KY636241	–	KY636225	
					–	KY636244	KY636281	KY636228	
					–	KY636243	KY636280	KY636227	
					–	KY636245	–	KY636229	
			Tlacotalpan, Veracruz (Mexico)		–	–	–	KY636184	
<i>D. spathans</i> (Dietz, 1909)	C	<i>P. trivolvis</i>	Mississippi (USA)	JN993270	JN993268	–	–	–	Griffin et al., 2012
	A	<i>N. auritus</i>	Mississippi (USA)	JN993269	JN993271	–	–	–	
			Mississippi (USA)	–	–	–	–	JX468067	O'Hear et al., 2014
			Ontario, Lake Erie (Canada)		–	–	–	KM538090	Van Steenkiste et al., 2015
			Altamira, Tamaulipas (Mexico)	–	–	KY636240	KY636290	KY636223	Present study
	A	<i>N. brasiliensis</i>	Catemaco, Veracruz (Mexico)	–	KY636308	KY636256	KY636293	KY636209	
					–	–	–	KY636210	
				–	KY636309	–	–	KY636208	
	A	<i>N. auritus</i>	Ciénaga de Santa Clara, Sonora (Mexico)		KY636299	–	–	KY636231	
					–	–	–	KY636232	
				–	KY636300	–	–	KY636219	
					–	KY636238	–	KY636221	
					–	KY636239	–	KY636222	
					–	KY636237	KY636289	KY636220	

A	<i>N. brasiliensis</i>	Coatzacoalcos, Veracruz (Mexico)	-	KY636310	KY636257	KY636294	KY636207
			-	KY636311	KY636258	-	KY636206
				-	KY636259	-	-
				-	-	-	KY636205
				-	-	-	KY636204
		La Tovar San Blas, Nayarit (Mexico)		-	KY636264	KY636286	-
				-	-	-	KY636200
		Presa La angostura, Chiapas (Mexico)	-	KY636316	-	-	KY636201
		Presa Río Verde, Oaxaca (Mexico)	MF351545	KY636313	KY636261	KY636285	KY636203
			-	KY636312	KY636260	-	KY636234
		Río Guatimape, Durango (Mexico)		-	-	-	KY636233
		Teapa, Tabasco (Mexico)		-	KY636248	-	KY636217
				-	KY636249	-	-
				-	KY636247	-	KY636218
				-	KY636246	KY636284	KY636230
		Tecolutla, Veracruz (Mexico)	-	KY636303	KY636251	-	KY636215
				KY636304	KY636252	-	KY636214
				KY636305	KY636250	KY636291	KY636216
A	<i>N. brasiliensis</i>	Temascal, Oaxaca (Mexico)	-	KY636307	KY636254	-	KY636212
				-	KY636255	-	KY636211
			MF351546	KY636306	KY636253	KY636292	KY636213
		Tlacotalpan, Veracruz (Mexico)		-	KY636269	-	KY636192
				-	-	-	KY636191
				-	KY636268	KY636287	KY636194
				-	-	-	KY636193
		Río Tehuantepec, Oaxaca (Mexico)	-	-	KY636271	KY636288	KY636190
				-	-	-	KY636189
A	<i>N. auritus</i>	Ulumal, Campeche (Mexico)		-	-	-	KY636224

(Continued)

Table 2. (Continued.)

Species	Life-cycle stage	Host	Locality	GenBank accession no.					Source
				LSU	SSU	ITS	<i>nad1</i>	<i>cox1</i>	
<i>Drepanocephalus</i> sp.	C	<i>B. straminea</i>	Belo Horizonte, Minas Gerais (Brazil)	KP053261	–	KP053261	KP053264	KP053256	Pinto et al., 2014
Genus <i>Echinostoma</i> Rudolphi, 1809									
<i>E. caproni</i> Richard, 1964	A	<i>Mus musculus</i> L.	Egypt	–	–	GQ463131	–	–	Detwiler et al., 2010
<i>E. miyagawai</i> Ishii, 1932	A	<i>Aythya fuligula</i> L.	Tovacov (Czech Republic)	–	–	–	KP065640	–	Georgieva et al., 2014
<i>E. revolutum</i> (Fröhlich, 1802)	C	<i>Lymnaea stagnalis</i> (L.)	South Bohemia (Czech Republic)	–	–	–	KP065658	–	
	A	<i>Mesocricetus auratus</i> (Waterhouse)	UK		AY222132	–	–	–	Olson et al., 2003
	A	<i>Columba livia</i> (Gmelin)	Bulgaria	–	–	AY168930	–	–	Kostadinova et al., 2003
<i>E. trivolvis</i> (Cort, 1914)	A	<i>Ondatra zibethicus</i> (L.)	Ontario, Lake Erie (Canada)	–	–	–	–	KM538091	Van Steenkiste et al., 2015
Genus <i>Euparyphium</i> Dietz, 1909									
<i>E. capitaneum</i> Dietz, 1909	A	<i>Anhinga anhinga</i> (L.)	Mississippi (USA)	KP009620	–	KP009616	–	–	Kudlai et al., 2015b
			La Tovar, Nayarit (Mexico)	–	KY636317	KY636278	–	KY636236	
			Tecolutla, Veracruz (Mexico)	–	KY636318	KY636279	–	KY636235	Present study
Genus <i>Echinoparyphium</i> Dietz, 1909									
<i>E. aconiatum</i> Dietz, 1909	R & C	<i>L. stagnalis</i>	Lake Pyykosjarvi (Finland)	–	–	–	AY168947	–	Kostadinova et al., 2003
<i>E. recurvatum</i> (Linstow, 1873)	C	<i>Radix peregra</i> (Müller)	Lake Frongoch, Wales (UK)	–	–	AY168931	–	–	
				–	–	–	AY168944	–	
	A	<i>Anas clypeata</i> L.	Lago de Patzcuaro, Michoacan (Mexico)	–	–	KJ435271	–	–	Sereno-Uribe et al., 2015
	A	<i>Gallus gallus</i> (L.)	Lago de Patzcuaro, Michoacan (Mexico)	–	–	KJ435272	–	–	

Genus <i>Hypoderaeum</i> Dietz, 1909									
<i>H. conoideum</i> (Bloch, 1782)	C	<i>R. peregra</i>	Grigorevo (Bulgaria)	-	-	-	AY168949	-	Kostadinova <i>et al.</i> , 2003
			Lake Manitoba (Canada)	-	-	-		KM538101	Van Steenkiste <i>et al.</i> , 2015
Genus <i>Isthmiophora</i> Lühe, 1909									
<i>I. hortensis</i> (Asada, 1926)	A	<i>Procyon lotor</i> (L.)	Wakayama (Japan)		AB189982	-	-	-	Sato & Suzuki, 2006
<i>I. melis</i> (Schrank, 1788)	R & C	<i>Planorbis</i> sp.	Wales (UK)	AF151941	-	AY168932	-	-	Kostadinova <i>et al.</i> , 2003
			Llyn Mawr, Wales (UK)	-	-	-	AY168948	-	
	A	<i>Nyctereutes procyonoides</i> (Gray)	Ukraine	-	AY222131	-	-	-	Olson <i>et al.</i> , 2003
Genus <i>Petasiger</i> Dietz, 1909									
<i>P. radiatus</i> (Dujardin, 1845)	A	<i>Phalacrocorax carbo</i> (L.)	Israel	KT956927	AY245708	-	-	-	Dzikowski <i>et al.</i> , 2004
						AY245708	-	-	
			Hungary			KM973000	-	-	Van Steenkiste <i>et al.</i> , 2015
<i>P. phalacrocoracis</i> (Yamaguti, 1939)	A	<i>P. carbo</i>	Israel	KT956926	AY245709	-	-	-	Dzikowski <i>et al.</i> , 2004
	M	<i>Rutilus rutilus</i> L.	Hungary			KM972991	-	-	Molnar <i>et al.</i> , 2015
<i>P. exaeretus</i> Dietz, 1909	A	<i>Phalacrocorax carbo</i>	Ukraine	KT956923	-	-	-	-	Tkach <i>et al.</i> , 2016
Genus <i>Neopetasiger</i> Dietz, 1909									
<i>Neopetasiger</i> sp.	A	<i>Podiceps grisegena</i>	USA	KT956925	-	-	-	-	
<i>N. islandicus</i> Kostadinova & Skirnisson, 2007	A	<i>Aechmophorus occidentalis</i>	USA	KT956924	-	-	-	-	
	R	<i>Gyraulax laevis</i> (Alder)	Iceland				JQ425590	-	Georgieva <i>et al.</i> , 2012
Genus <i>Ropalias</i> Stiles & Hassall, 1898									
<i>R. macracanthus</i> Chandler, 1932	A	<i>Didelphis virginiana</i>	USA	KT956959	-	-	-	-	Tkach <i>et al.</i> , 2016
Genus <i>Cathaemasia</i> Loosse, 1899									

(Continued)

Table 2. (Continued.)

Species	Life-cycle stage	Host	Locality	GenBank accession no.						
				LSU	SSU	ITS	<i>nad1</i>	<i>cox1</i>	Source	
<i>C. hians</i> Rudolphi, 1809	C	<i>Planorbis planorbis</i>	Czech republic	KT956947	-	-	-	-	-	-
Genus <i>Ribeiroia</i> Travassos, 1939										
<i>R. ondatrae</i> Price, 1932	A	<i>Pelecanus erythrorhynchos</i>	USA	KT956956	-	-	-	-	-	-
Outgroup taxa										
<i>Fasciola hepatica</i> (L.)	A	<i>Bos primigenius</i> (Bojanus)	Spain	-	AJ004969	-	-	-	-	Feranadez <i>et al.</i> , 1998
		<i>Ovis aries</i> (L.)	Tehran (Iran)	-	-	-	EF612493	-	-	Lotfy <i>et al.</i> , 2008
	A	<i>Bubalus bubalis</i> (L.)	Tanta (Egypt)	-	-	-	-	AB553825	-	Amer <i>et al.</i> , 2011
<i>Fasciola gigantica</i> Cobbold, 1855	A		Guangxi (China)	-	-	-	-	-	-	Liu, <i>et al.</i> , 2014

Life-cycle stage: adult (A), metacercariae (M), cercariae (C), rediae (R).

terminals (a larger number of newly generated sequences, and other species of echinostomatids for which ITS sequences are available). The ITS dataset consists of 1146 characters, with 59 terminals and the best evolution model for this dataset was TVM + G. The ITS tree also showed that the species *P. mexicanus* is nested within the genus *Drepanocephalus*. In addition, four sequences identified as *D. auritus*, three of them from either adults or cercariae in the USA, and one from Brazil (see table 2), plus 24 newly generated sequences of *D. spathans*, formed a clade with relatively high bootstrap and posterior probability support values (see fig. 2c). Interrelationships among species of *Drepanocephalus* are not well resolved with this nuclear gene.

Mitochondrial genes

The *cox1* dataset included 658 characters with 68 terminals and the best selected model was GTR + I + G. This tree supported the monophyly of *Drepanocephalus* with very high bootstrap and posterior probability support values. The samples of 18 specimens of *P. mexicanus* and a single sequence for a cercaria identified as *Drepanocephalus* sp. from *B. straminea* from Brazil (Pinto *et al.*, 2016), formed a monophyletic clade that is nested within the genus *Drepanocephalus*. *cox1* sequences from specimens identified by us as *D. spathans* from different localities across Mexico, as well as those available in the GenBank dataset from Mississippi, USA, and Canada (O'Hear *et al.*, 2014; Gordy *et al.*, 2016) are nested within a monophyletic clade with those identified as *D. auritus* from Brazil, Canada and the USA (Kudlai *et al.*, 2015a; Gordy *et al.*, 2016; Pinto *et al.*, 2016) (see fig. 3a, table 2). Finally, the *nad1* dataset included 510 characters with 30 terminals. The best model for this dataset was GTR + G. This tree showed similar topology to that of the *cox1* tree, with the genus *Drepanocephalus* as a monophyletic assemblage with very high nodal support values. For this molecular marker, a basal polytomy is also formed, although two well-defined clades are recovered, one containing *nad1* sequences of eight specimens identified as *P. mexicanus* plus a single specimen identified as *Drepanocephalus* sp. from Brazil (KP053264) (Pinto *et al.*, 2016). Another clade includes 11 specimens identified as *D. spathans*, whose sequences were generated in this study, plus a specimen identified as *D. auritus* from Brazil (KP053263) (Pinto *et al.*, 2016). Interestingly, a single sequence of *D. auritus* from USA (KP053262) is not nested within this clade and forms a basal polytomy (fig. 3b).

In summary, all phylogenetic analyses are congruent in that *P. mexicanus* is nested within *Drepanocephalus* and, as a result, it should be transferred to *Drepanocephalus*, i.e. to the genus in which the species was placed when it was described by Lamothe-Argumedo & Pérez-Ponce de León (1989) as *D. mexicanus*. Sequences identified as *Drepanocephalus* sp., from cercariae collected from snails in Brazil, correspond with this species. Additionally, the synonymy between *D. auritus* and *D. spathans* is confirmed based on the multilocus phylogenetic analyses. These nomenclatural acts require the re-description of the two species that we are validating in this study, *D. spathans* and *D. mexicanus*, as follows.

Drepanocephalus spathans Dietz, 1909

Taxonomic summary

Synonym. *Drepanocephalus auritus* Kudlai, Kostadinova, Pulis & Tkach, 2015.

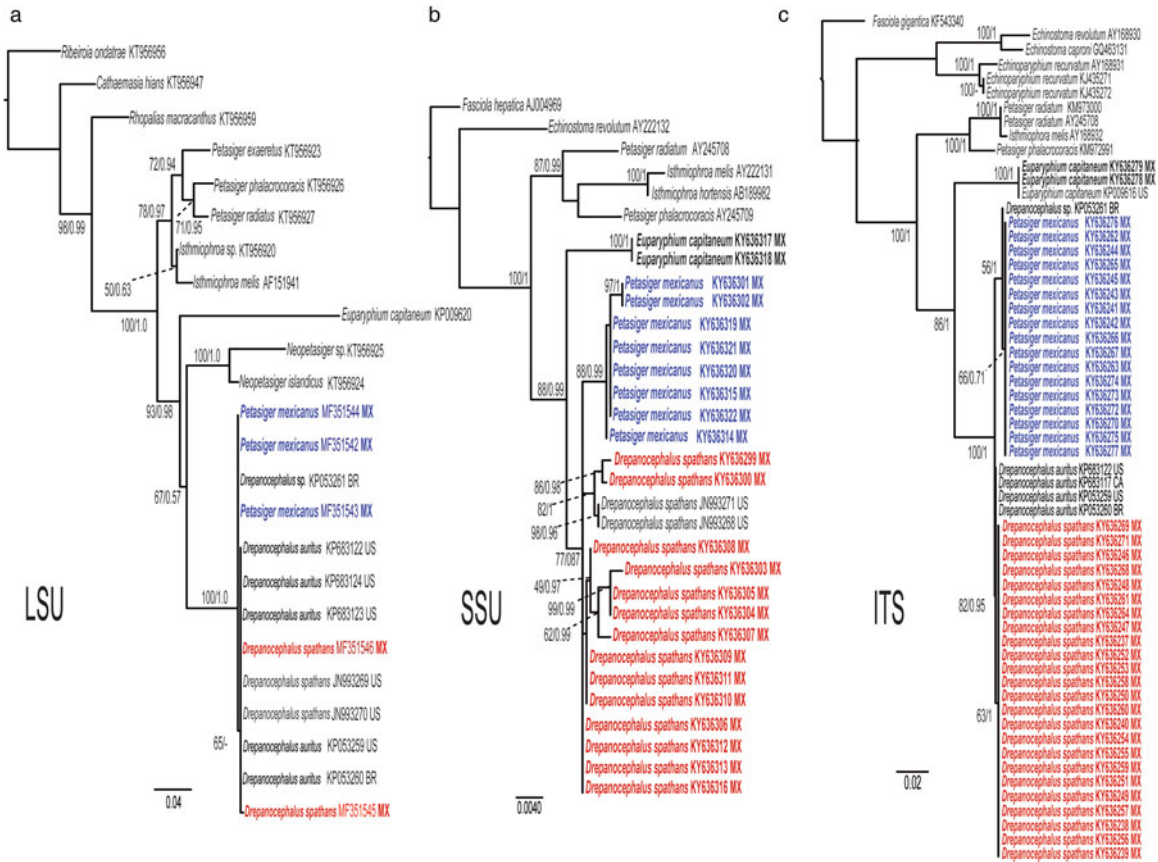


Fig. 2. Maximum likelihood tree and consensus Bayesian inference trees inferred for nuclear ribosomal DNA markers. (a) LSU dataset; (b) SSU dataset; (c) ITS dataset. Numbers near the internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). Abbreviations of each country: MX, Mexico; US, USA; CA, Canada; BR, Brazil.

Type host. *Nannopterum brasilianus* Gmelin (Pelecaniformes: Phalacrocoracidae).

Other hosts. *Nannopterum auritus* Lassel (Pelecaniformes: Phalacrocoracidae), *Sula leucogaster* Brisson (Pelecaniformes: Sulidae).

Type-locality. Brazil.

Localities in Mexico. Ulumal (18°16'43"N, 90°37'26"W), Campeche; Presa La Angostura (16°11'31"N, 92°59'52"W), Chiapas; Río Guatimape (24°52'24"N, 104°54'38"W) Río Guatimape (24°52'24"N, 104°54'38"W), Durango; La Tovar, San Blas (21°32'00"N, 105°17'22"W), Nayarit; Presa Río Verde (19°06'35"N, 97°43'58"W), Río Tehuantepec (16°23'55"N, 95°18'30"W), Temascal (18°14'22"N, 96°24'11"W), Oaxaca; Ciénaga de Santa Clara (32°21'12"N, 114°54'07"W), Sonora; Teapa (17°34'59"N, 92°53'30"W), Tabasco; Altamira (22° 21'05"N, 97°59'24"W), Tamaulipas; Catemaco (18°25'00"N, 95°07'00"W), Coatzacoalcos (18°06'31"N, 94°27'14"W), Tecolutla (20°27'09"N, 97°01'13"W), Tlacotalpan (18°36'0"N, 95°39'0"W), Veracruz.

Localities in other countries. Colombia (Rietschel & Werding, 1978), Venezuela (Lutz, 1928; Caballero Caballero & Díaz Ungria, 1958), Argentina (Ostrowski de Núñez, 1968; Drago et al., 2011), USA (Threlfall, 1982; Fedynich et al., 1997; Flowers et al., 2004; Griffin et al., 2014; O'Hear et al., 2014; Sheehan et al., 2016), Paraguay (Kostadinova et al., 2002) and Canada (Robinson et al., 2010; Wagner et al., 2012) (see table 2).

Site in host. Intestine.

Voucher specimens. CNHE 10310–10318.

Description

Based on 15 gravid specimens from *N. brasilianus*. Body (figs 4a–c, 5) elongate, with maximum width at level of head collar (fig. 5a). Body 4459–9181 long, 917–1439 wide at level of ventral sucker and 734–1,512 at level of anterior testis. BW = 3.6–8.5%. Forebody 1031–1627 long (FO = 3.4–6.7%); lateral area between head collar and ventral sucker covered with small spines (fig. 4a–c). Head collar falciform, strongly muscular, 646–995 long, 1044–1794 wide; ventral lappets possessing two invaginations. Collar spines 27, large, in single row. Eight angle spines, four on each edge of ventral lappet (pair 1: ventral 172–312 × 49–74, dorsal 200–338 × 48–74; pair 2: ventral 152–293 × 40–70, dorsal 153–324 × 40–77). Four lateral spines on each lappet, in single row, first 83–170 × 28–44 and remaining 60–176 × 29–48. Dorsal spines 11 in single row, smaller than lateral, diminishing in size towards the middle of the collar (59–165 × 20–47). Oral sucker subterminal, subspherical, 232–362 long, 256–403 wide. Ventral sucker muscular, cup-shaped, located in anterior half of body, 814–1118 long, 611–1066 wide. Prepharynx visible, 33–74 long. Pharynx muscular, elongate, 206–363 long, 149–240 wide (fig. 5a). Oesophagus 522–742 long, leading to an intestinal bifurcation anterior to ventral sucker. Caeca extended laterally to the posterior end of body. Testes in posterior half of body, deeply

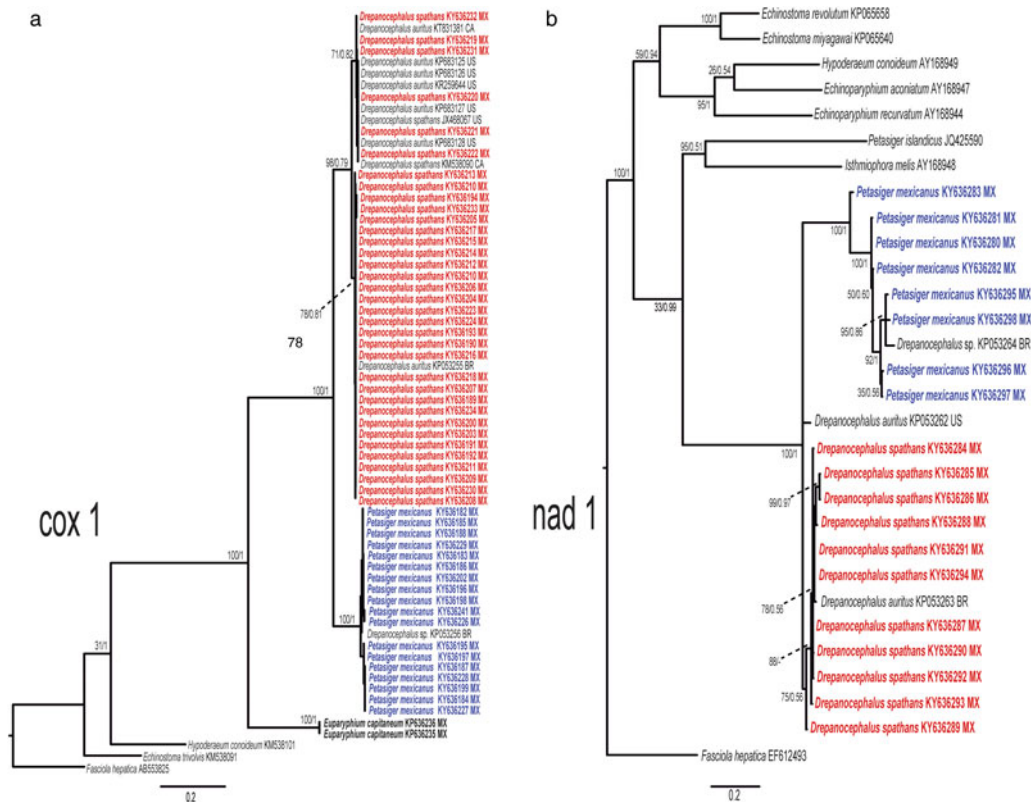


Fig. 3. Maximum likelihood tree and consensus Bayesian inference trees inferred for mitochondrial DNA markers. (a) *cox1* dataset; (b) *nad1* dataset. Numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). Abbreviations of each country: MX, Mexico; US, USA; CA, Canada; BR, Brazil.

lobated, contiguous or widely separated, in tandem; anterior testis with lobes, 396–626 long, 425–911 wide; posterior testis with 4–7 lobes, 385–717 wide, 425–894 long (fig. 5b, c). Distance between posterior testis and posterior margin of body 1178–3751. Cirrus sac relatively small, oval, 646–995 long, 1044–1794 wide, anterodorsal to ventral sucker. Cirrus tubular, coiled, unspined. Internal seminal vesicle bipartite. Anterior portion smaller than posterior portion. Pars prostatica weakly developed; prostatic cells surround pars prostatica. Genital pore median, between ventral sucker and intestinal bifurcation (fig. 5d). Ovary small, slightly dextral, entire, oval, 176–308 long, 182–270 wide, anterior to Mehli's gland. Mehli's gland median. Laurer's canal not observed. Metratrem muscular, slightly larger than cirrus sac (fig. 5d). Eggs numerous, 65–91 long, 37–61 wide. Vitellarium in two lateral fields consisting of small follicles, between posterior margin of ventral sucker and level of the end of caeca, confluent in the post-testicular region; excretory pore terminal.

Remarks

Specimens collected in 14 localities of Mexico, from two species of cormorants, *N. brasiliensis* and *N. auritus*, possess features that are consistent with the diagnosis of the genus *Drepanocephalus*: an elongate body, forebody short, tegument armed with small spines, head collar wide with 27 spines in a single row, angle spines 2 × 4, oral sucker spherical, ventral sucker cup-shaped, prepharynx short, pharynx similar in size to oral sucker, oesophagus short, intestinal bifurcation just anterior or dorsal to ventral sucker, testes in tandem, contiguous and widely separated, lobed; cirrus sac elongated, ovary small, dextral, pre-equatorial; uterus short, excretory vesicle Y-shaped and excretory pore

subterminal (Kostadinova *et al.*, 2002; Kostadinova, 2005; Kudlai *et al.*, 2015a). In particular, our specimens were identified as *D. spathans* by possessing a falciform head collar, with the maximum width at head-collar level, and tegument with small and wide spines laterally placed between head collar and ventral sucker (see fig. 4a–c). Our specimens identified as *D. spathans* showed some level of morphological intraspecific variation (see fig. 5b, c, e). For instance, some meristic data of newly collected material with respect to previous descriptions possess lower limits for the following characters: CW (1044–1794), PHL (206–363), CSL (250–555), SV1W (58–161), SV2W (82–183), MEW (206–423), TEND (1178–3751), BW (%) (3.6–8.5), FO (%) (3.4–6.7) and T (%) (2.3–3.8). Likewise, newly collected material provides higher limits for the following characters BWAT (734–1512), CSW (210–460), ATW (425–911), PTW (425–894), EW (37–61), OVAR (101–655), FO (%) (3.4–6.7), U (%) (8.2–19.7) (see table 3). Our morphological observations and measurements of *D. spathans* (table 3), indicate some level of intraspecific morphological variability and, apparently, the morphological characters used by Kudlai *et al.* (2015a) to distinguish *D. auritus* from *D. spathans*, i.e. shape of the testes, position and distance between the testes (contiguous and widely separated see fig. 5e), position of the ovary and Mehli's gland, size of head collar, prepharynx, pharynx and seminal vesicle, fall into the range variation of *D. spathans*, and these observations are corroborated by molecular data, since sequences are nested together in a monophyletic clade. Morphological and molecular data obtained in the present study then suggest that these species are indistinguishable from each other. Therefore we consider *D. auritus* as a synonym of *D. spathans*.

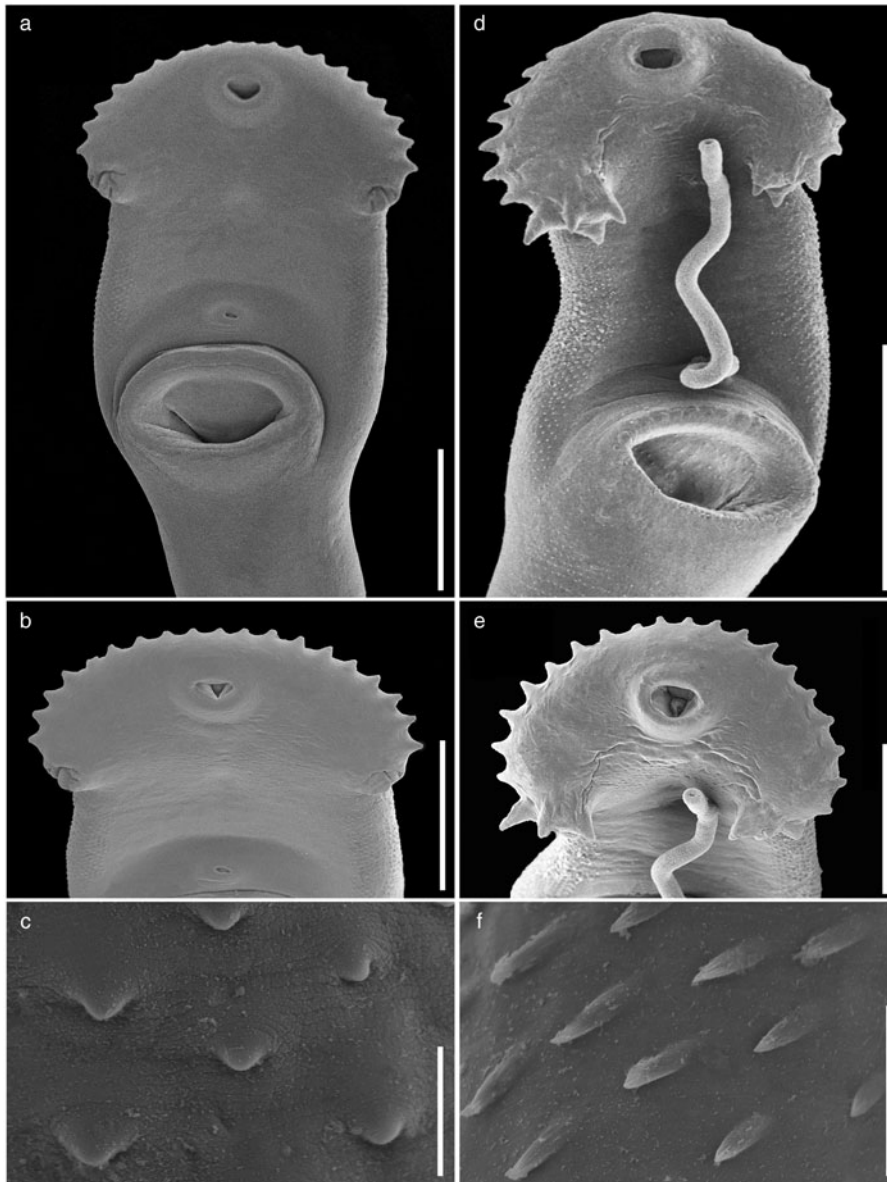


Fig. 4. Scanning electron micrographs of the forebody of adults of *Drepanocephalus* spp. from Mexico. *Drepanocephalus spathans* from *N. brasiliensis*: (a) forebody; (b) head collar; (c) tegumental spines. *Drepanocephalus mexicanus* from *N. brasiliensis*: (d) forebody; (e) head collar; (f) tegumental spines. Scale bars: (a, b, d) 500 μ m; (c, f) 20 μ m; (e) 300 μ m.

***Drepanocephalus mexicanus* Lamothe-Argumedo & Pérez-Ponce de León, 1989**

Taxonomic summary

Synonyms. *Paryphostomum mexicanum* (Kostadinova *et al.*, 2002); *Patasiger mexicanus* (Tkach *et al.*, 2016).

Type host. *Nannopterum brasiliensis* Gmelin (Pelecaniformes: Phalacrocoracidae).

Type locality. Teapa (17°34'59"N, 92°53'30"W), Tabasco.

Other localities. Presa La Angostura (16°11'31"N, 92°59'52"W), Chiapas; La Tovar, San Blas (21°32'00"N, 105°17'22"W), Nayarit; Río Tehuantepec (16°23'55"N, 95°18'30"W), Oaxaca; Tlacotalpan (18°36'0"N, 95°39'0"W), Veracruz.

Localities in other countries. Brazil (Pinto *et al.*, 2016, as *Drepanocephalus* sp., cercariae from *Biomphalaria straminea*).

Site in host. Intestine.

Voucher specimens. CNHE 10307–10309.

Description

Based on 21 gravid specimens. Body (figs 4d–f, 6) elongate (BW = 5.2–7.9%) with maximum width (598–946) at level of ventral sucker. Body length 3060–6450; width at level of anterior testis 390–826. Forebody 569–1342 long (FO = 4.1–6.9%). Tegument with small spines distributed laterally, from head collar, to reach the posterior end of ventral sucker (fig. 4d–f). Head collar reniform, strongly muscular, 282–560 long, 402–836 wide, ventral lappets well developed, inclined towards the ventral region. Collar spines 27, large, in single row. Four angle spines in two pairs with ventral and dorsal spines (pair 1: ventral 102–157 long, 24–46 wide; dorsal 104–161 long, 26–44 wide; pair 2: ventral 94–151 long, 26–35 wide; dorsal 105–190 long, 29–39 wide). Four lateral spines on each lappet, in single row, first 69–106 long, 22–36 wide and remaining 44–113 long, 20–44 wide. Dorsal spines 11 in single row, of the same size (33–68 long, 16–43 wide). Oral sucker subterminal, subspherical, 106–219 long, 123–233 wide. Ventral sucker strongly muscular, cup-shaped, located in first quarter of body, 480–835 long, 363–657 wide. Prepharynx visible,

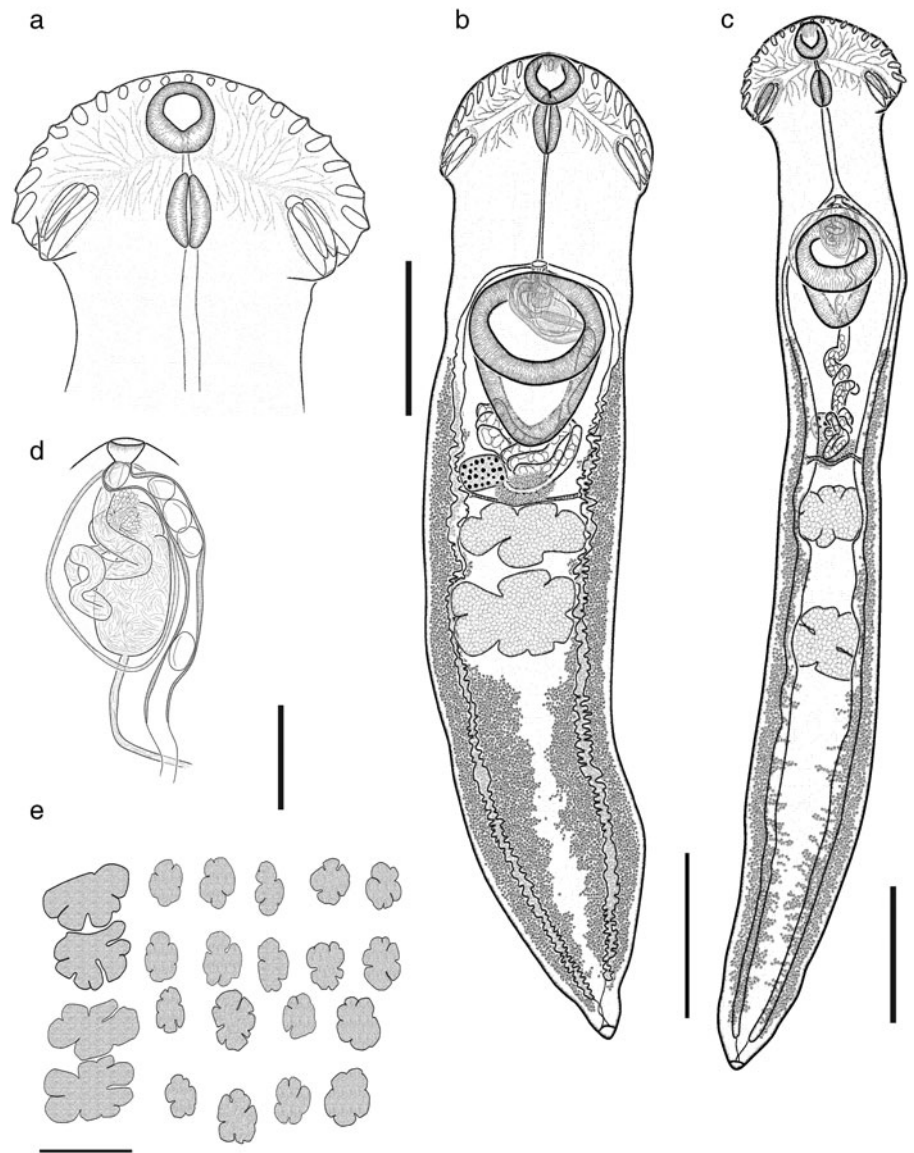


Fig. 5. *Drepanocephalus spathans* from *N. brasiliensis*: (a) head collar; (b, c) whole worm with contiguous and widely separated testes, respectively; (d) terminal genitalia; (e) variation in shape of testes. Scale bars: (a) 500 µm; (b, c) 1000 µm; (d) 200 µm; (e) 1000 µm.

34–200 long. Pharynx muscular, elongate, 127–228 long, 97–209 wide (fig. 6a). Oesophagus 218–637 long, leading to an intestinal bifurcation anterior to ventral sucker. Caeca reach close to posterior extremity of body. Testes tandem, lobed, widely separated; anterior testis just post-equatorial, with 3–6 lobes, 300–602 long, 180–534 wide; posterior testis with 3–7 lobes, 248–655 long, 199–546 wide. Post-testicular region 1168–2120 long (fig. 6b). Cirrus sac small, oval 172–377 long, 111–253 wide, antero-dorsal to ventral sucker. Cirrus tubular, coiled, unspined. Internal seminal vesicle bipartite. Anterior portion smaller than posterior portion. Pars prostatica weakly developed; prostatic cells surround pars prostatic. Genital pore median just post-bifurcal (fig. 6c). Ovary small, dextral, entire, rounded, 122–248 long 113–201 wide anterior to Mehlis' gland. Distance from ventral sucker to ovary ($U = 5.6\text{--}33.4\%$). Mehlis' gland median. Uterus relatively long, metraterm muscular, slightly larger than cirrus sac (fig. 6c). Eggs 62–86 long, 33–57 wide. Vitellarium in two lateral fields of small follicles, between posterior margin of ventral sucker and posterior extremity. Follicles tend to overlap in the post-testicular space (fig. 6b).

Remarks

Specimens of this species were collected in four localities of Mexico as parasites of *N. brasiliensis*. In particular, our specimens were identified as *D. mexicanus* by having a reniform head collar, tegument with long and thin spines placed laterally between head collar and posterior part of ventral sucker (see fig. 4d–f). Our specimens of *D. mexicanus* showed some level of morphological variability. For instance, meristic data of newly collected material provide lower limits for the following characters: BL (3060–6450), BWVS (598–946), BWAT (390–826), CL (282–560), CW (402–836), OSL (106–219), OSW (123–233), PHL (127–228), PHW (97–209), OL (218–637), CSL (172–377), SV1L (30–94), VSW (363–657), MEL (130–222), ATW (180–534), PTW (199–546), EL (62–86), EW (33–57), FORE (569–1342), OVAR (185–510), BW (%) (5.2–7.9), FO (%) (4.1–6.9), U (%) (5.6–33.4) and T (%) (2.5–3.4) (table 4). Likewise, newly collected material provides higher upper limits for the size of the body, head collar, cirrus sac, posterior portion of seminal vesicle, Mehlis' gland, testes and eggs (table 4). Additionally, our study reveals higher limits

Table 3a. Comparative measurements of *Drepanocephalus spathans* Dietz, 1909.

Source	Present study	Dietz, 1909, 1910	Ostrowski de Núñez, 1968	Rietschel & Werding, 1978	Ramos-Ramos, 1995	
Identified as	<i>Drepanocephalus spathans</i> Dietz, 1909	<i>D. spathans</i>	<i>D. spathans</i>	<i>D. spathans</i>	<i>D. olivaceus</i>	
Hosts	<i>Nannopterum brasilianus</i> (Gmelin)	<i>N. brasilianus</i>	<i>N. brasilianus</i>	<i>N. brasilianus Sula leucogaster</i> (Boddaert)	<i>N. brasilianus</i>	
Localities	Temascal, Oaxaca; Teapa, Tabasco; Catemaco, Tecolutla, Veracruz (Mexico)	Brazil	Buenos Aires (Argentina)	Magdalena (Colombia)	Oaxaca (Mexico)	
	Range	Mean ± SD	Range	Range	Range	
BL	4459–9181	6692 ± 1591	7250	5106–9990	4800–6660	8210–11,109
BWVS	917–1439	1109 ± 136	340–1400	925–1369	990–1110	1368–1626
BWAT	734–1512	1009 ± 243	–	1360 ^a	1000 ^a	1111 ^a
CL	646–995	769 ± 102	–	773 ^a	580 ^a	800 ^a
CW	1044–1794	1383 ± 170	–	1110–1739	1260–1470	1481–1819
OSL	232–362	279 ± 34	–	221–351	160–212	273–418
OSW	256–403	315 ± 42	350	221–390	235–310	322–354
PL	33–74	49 ± 15	460	0–91	23–103	0–144
PHL	206–363	300 ± 43	354	249–390	259–263	483–490
PHW	149–240	187 ± 25	261	143–221	164–226	241–273
OL	522–742	637 ± 64	430	351–715	684–780	611–885
CSL	250–555	376 ± 85	–	286–455	500 ^a	434–563
CSW	210–460	264 ± 62	–	182–325	350 ^a	354–450
SV1L	82–134	101 ± 14	–	–	–	–
SV1W	58–161	101 ± 30	–	–	–	–
SV2L	101–265	197 ± 47	–	–	–	–
SV2W	82–183	131 ± 30	–	–	–	–
VSL	814–1118	940 ± 110	950	845–1235	684–912	1046–1223
VSW	611–1066	768 ± 130	770	611–884	780–864	1040–1223
OVL	176–308	252 ± 43	308	130–312	180–216	225–257
OVW	182–270	222 ± 23	231	234–273	132–264	241–338
MEL	142–302	209 ± 54	–	130–416	150 ^a	342 ^a
MEW	206–423	297 ± 74	280–350	221–325	300 ^a	575 ^a
ATL	396–626	510 ± 69	740	325–728	300–456	627–805
ATW	425–911	594 ± 141	690	312–715	552–600	547–692
PTL	385–717	549 ± 100	–	299–884	384–540	644–901
PTW	425–894	590 ± 129	–	364–715	540–684	623–756
EL	65–91	80 ± 7	62–65	70–95	61–80	80 (mean)
EW	37–61	48 ± 7	50–53	36–56	43–56	48 (mean)
FORE	1031–1627	1325 ± 186	–	1200 ^a	1300 ^a	1631 ^a
OVAR	101–655	408 ± 203	–	200 ^a	100 ^a	1111 ^a
TEND	1178–3751	2504 ± 809	–	1840 ^a	2650 ^a	4044 ^a
BW (%)	3.6–8.5	6.1 ± 1.8	–	5.0 ^a	6.4 ^a	7.1 ^a
FO (%)	3.4–6.7	5.1 ± 1.1	–	4.3 ^a	5.2 ^a	5.6 ^a
U (%)	8.2–19.7	13.2 ± 4.2	–	14.6 ^a	9.6 ^a	8.9 ^a
T (%)	2.3–3.8	2.8 ± 0.4	–	2.8 ^a	2.5 ^a	2.7 ^a

^aMeasurements taken from the published figure according to the scale presented in the figure.

Table 3b. Comparative measurements of *Drepanocephalus spathans* Dietz, 1909, continued.

Source	Ramos-Ramos, 1995 Present study (CNHE 1411.1-3)		Kostadinova et al., 2002		Violante-González et al., 2011 Present study (CNHE 7577-8)		Kudlai et al., 2015a
Identified as	<i>D. spathans</i>		<i>D. spathans</i>		<i>D. spathans</i>		<i>D. auritus</i>
Hosts	<i>N. brasiliensis</i>		<i>N. brasiliensis</i>		<i>N. brasiliensis</i>		<i>N. auritus</i>
Locality(ies)	Oaxaca (Mexico)		Paraguay		Guerrero (Mexico)		USA
	Range	Mean ± SD	Range	Range	Mean ± SD	Range	Mean
BL	8132–11,004	9389 ± 1469	4384–4494	6826–7543	7185 ± 507	6584–9129	8123
BWVS	1338–1592	1492 ± 135	1000 ^a	764–1150	957 ± 273	779–1186	944
BWAT	1138–1184	1162 ± 23	932–1284	687–1233	960 ± 386	531–974	719
CL	808–1012	907 ± 102	884–963	531–909	720 ± 267	558–797	638
CW	1511–1579	1545 ± 48	1210–1482	1062–1509	1286 ± 316	991–1416	1165
OSL	296–421	355 ± 63	247–301	191–266	229 ± 53	195–336	274
OSW	281–451	360 ± 86	235–333	233–299	266 ± 47	212–319	264
PL	0–101	–	0–7	29–45	37 ± 11	17–106	73
PHL	359–412	391 ± 28	247–281	229–289	259 ± 42	212–372	287
PHW	256–295	274 ± 20	161–198	171–199	185 ± 20	124–212	171
OL	601–888	702 ± 161	411–685	669–745	707 ± 54	513–761	599
CSL	480–567	524 ± 62	408	255	–	320–578	440
CSW	354–435	395 ± 57	231–272	216	–	212–331	257
SV1L	329	–	197	65	–	88–159	109
SV1W	234	–	163	79	–	66–154	104
SV2L	92	–	291	129	–	278–498	361
SV2W	204	–	202	129	–	146–265	193
VSL	1011–1218	1115 ± 146	766–1110	729–991	860 ± 185	797–1115	914
VSW	765–857	823 ± 51	671–973	525–896	711 ± 262	566–867	692
OVL	257–271	264 ± 10	116–171	153–312	233 ± 112	150–283	225
OVW	247–266	257 ± 13	192–253	136–261	199 ± 88	124–248	186
MEL	270–344	307 ± 52	86–164	199–327	263 ± 91	106–306	182
MEW	302–346	324 ± 31	222–288	216–366	291 ± 106	195–372	252
ATL	639–757	712 ± 64	247–363	108–600	354 ± 348	389–708	560
ATW	585–701	661 ± 66	593–685	107–637	372 ± 375	283–549	421
PTL	652–804	746 ± 82	358–582	124–609	367 ± 343	443–689	579
PTW	626–679	647 ± 28	480–685	107–649	378 ± 383	319–549	410
EL	– ^b	– ^b	73–91	59–80	73 ± 12	74–99	88
EW	– ^b	– ^b	35–54	36–59	49 ± 12	42–61	52
FORE	1645–2061	1793 ± 233	980–1028	1360–1468	1414 ± 76	1239–1628	1459
OVAR	615	–	–	354–518	436 ± 116	531–1530	809
TEND	2839–4000	3582 ± 645	1507–1987	2500–2975	2738 ± 336	2372–3264	2857
BW (%)	5.9–6.9	6.2 ± 0.6	18.9–30.7	6.6–8.9	7.8 ± 1.7	10.6–12.1	11.6
FO (%)	4.9–5.5	5.2 ± 0.3	4.3 ^a	5.0–5.2	5.1 ± 0.1	15.6–20.0	18.1
U (%)	4.1–8.4	6.9 ± 2.4	0–0.9	10.0	–	8.1–16.8	10.0
T (%)	2.3–2.9	2.7 ± 0.3	32.2–40.2	2.5–2.7	2.6 ± 0.1	34.0–36.0	35.2

^aMeasurements taken from the published figure.^bEggs hydrated in all specimens examined.

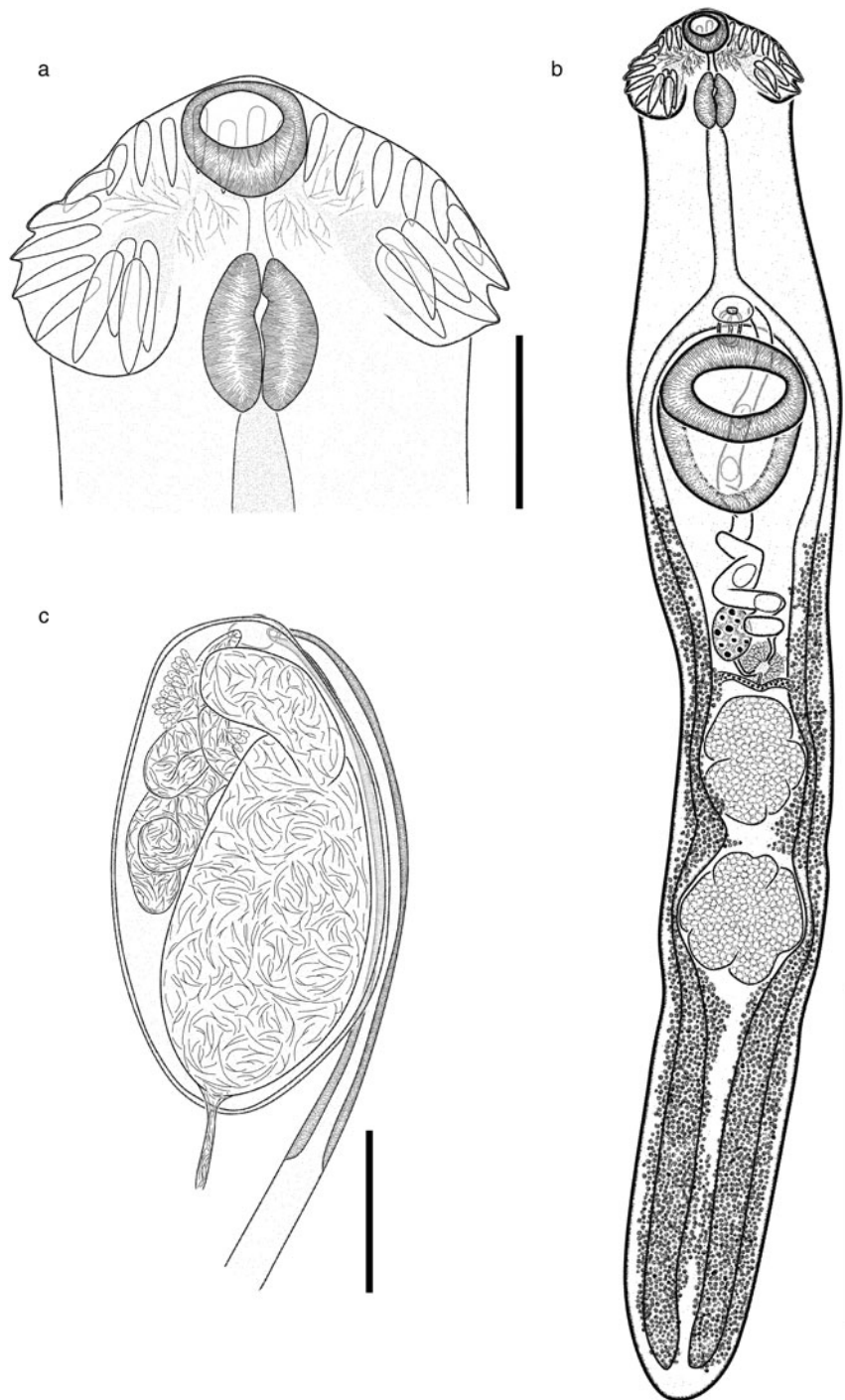


Fig. 6. *Drepanocephalus mexicanus* from *N. brasiliensis*: (a) head collar; (b) whole worm; (c) terminal genitalia. Scale bars: (a) 200 μm ; (b) 1000 μm ; (c) 100 μm .

for the length of the oral and ventral suckers, prepharynx, oesophagus, ovary, forebody and uterus, and for the width of the pharynx and the anterior portion of the seminal vesicle (table 4).

Discussion

The genus *Drepanocephalus* was erected to include *D. spathans*, a parasite of the Neotropical cormorant *N. brasiliensis* from Brazil. This species was described from a single specimen (Dietz, 1909). Kostadinova *et al.* (2002) re-described the species from specimens collected from the type host in Paraguay. The species composition

of the genus has been controversial ever since. Until now, the genus contained three valid species, i.e. *D. spathans*, *D. olivaceus* and *D. auritus*. *Drepanocephalus spathans* is distributed mostly in the Neotropical region, parasitizing *N. brasiliensis* in localities of Argentina, Brazil, Colombia, Paraguay and Venezuela (Caballero Caballero & Díaz Ungria, 1958, and references therein; Kostadinova *et al.*, 2002; Drago *et al.*, 2011), but also in *S. leucogaster* in Colombia (Rietschel & Werding, 1978). In addition, this species was recorded as a parasite of the double-crested cormorant, *N. auritus*, in some localities of the USA and Canada (e.g. Flowers *et al.*, 2004; Wagner *et al.*, 2012; O'Hear *et al.*, 2014). The second species, *D. olivaceus*, is a parasite of *N. brasiliensis*

Table 4. Comparative measurements of *Drepanocephalus mexicanus* Lamothe-Argumedo & Pérez-Ponce de León, 1989.

Source	Present study	Lamothe-Argumedo & Pérez-Ponce de León, 1989	Present study (CNHE 1279, 1280; type material of Lamothe-Argumedo & Pérez-Ponce de León, 1989)	
Hosts	<i>Nannopterum brasiliensis</i>	<i>N. brasiliensis</i>	<i>N. brasiliensis</i>	
Locality(ies)	La Tovar San Blas, Nayarit; Tehuantepec, Oaxaca; Teapa, Tabasco (Mexico)	Teapa, Tabasco (Mexico)	Teapa, Tabasco (Mexico)	
	Range	Mean ± SD	Range	Range (holotype)
BL	3060–6450	4569 ± 811	3171–4749	3702–4771 (4771)
BWVS	598–946	717 ± 91	644–837	627–869 (869)
BWAT	390–826	565 ± 109	–	576–844 (844)
CL	282–560	385 ± 74	–	324–502 (502)
CW	402–836	579 ± 109	483–805	479–786 (786)
OSL	106–219	148 ± 28	161	155–187 (187)
OSW	123–233	160 ± 30	193	150–241 (241)
PL	34–200	85 ± 45	–	0–80 (0)
PHL	127–228	171 ± 23	144–241	137–209 (209)
PHW	97–209	121 ± 30	96–161	113–161 (161)
OL	218–637	443 ± 120	402–483	406–511 (511)
CSL	172–377	275 ± 54	243	240–316 (316)
CSW	111–253	179 ± 44	–	95–184 (184)
SV1L	30–94	65 ± 19	–	62–135 (135)
SV1W	48–167	101 ± 40	–	34–85 (85)
SV2L	54–267	186 ± 76	–	52–109 (87)
SV2W	44–182	139 ± 47	–	37–76 (71)
VSL	480–835	591 ± 92	438–805	488–793 (793)
VSW	363–657	482 ± 77	402–692	395–698 (698)
OVL	122–248	176 ± 32	96–161	153–187 (187)
OVW	113–201	153 ± 25	96–193	87–207 (207)
MEL	130–222	168 ± 28	–	153–220 (220)
MEW	125–283	198 ± 54	–	112–253 (253)
ATL	300–602	414 ± 78	241–434	292–449 (449)
ATW	180–534	341 ± 73	241–499	279–514 (514)
PTL	248–655	447 ± 90	225–483	292–426 (426)
PTW	199–546	360 ± 82	209–466	330–506 (506)
EL	62–86	76 ± 8	64–80	– (-) ^a
EW	33–57	43 ± 6	40–48	– (-) ^a
FORE	569–1342	924 ± 226	–	823–944 (944)
OVAR	185–510	331 ± 101	–	252–366 (366)
TEND	1168–2120	1553 ± 278	–	718–1534 (1534)
BW (%)	5.2–7.9	6.4 ± 0.8	–	5.5–5.7 (5.5)
FO (%)	4.1–6.9	5.1 ± 0.9	–	4.5–5.1 (5.1)
U (%)	5.6–33.4	12.1 ± 8.7	–	9.5–11.7 (11.7)
T (%)	2.5–3.4	3.1 ± 0.3	–	3.1–3.5 (3.1)

^aEggs hydrated in all specimens examined.

in Venezuela (Nasir & Marval, 1968), although it was later reported in the same host in Mexico (Ramos-Ramos, 1995) and Brazil (Monteiro *et al.*, 2011). The third species, *D. auritus*, was recently described as a parasite of the double-crested cormorant *N. auritus* in the USA and, apparently, is also found in Brazil, based on the comparison of sequence DNA data from cercariae obtained from the snail *B. straminea* (Pinto *et al.*, 2016). The generation of molecular data for several molecular markers in recent years for individuals of *Drepanocephalus*, either as larval forms (mostly cercariae from snails) or adults from cormorants, in localities of Brazil, Canada and the USA has resulted in a re-interpretation of distribution range of the aforementioned species. For instance, in the most recent assessment of the taxonomic identity of some species of *Drepanocephalus*, Pinto *et al.* (2016) concluded that one of the two morphotypes of the cercariae released by *B. straminea* in Brazil corresponded, according to molecular markers, with *D. auritus*. Meanwhile, a second cercarial morphotype was smaller and differed significantly from *D. auritus* for two mitochondrial markers (*nad1* and *cox1*). These authors suggested that this species may be conspecific with *D. spathans* or, alternatively, with *D. olivaceus*.

The results obtained in the present study are very useful for re-evaluation of the current species composition of the genus *Drepanocephalus*. The large number of adult individuals belonging to the genus *Drepanocephalus* from a wide geographic range, comprising both the Nearctic and Neotropical regions of Mexico, filled a gap in our knowledge of the genetic make-up of species allocated to that genus, because only sequences from Canada, USA and Brazil had been documented (Griffin *et al.*, 2012; O'Hear *et al.*, 2014; Kudlai *et al.*, 2015a; Pinto *et al.*, 2016). Based solely on morphology, our specimens were identified as *D. spathans*, and that included specimens with contiguous and separate testes, a morphological character used by Kudlai *et al.* (2015a) to distinguish *D. auritus* from *D. spathans*. Our molecular and morphological results revealed the need to conduct some nomenclatural changes, and to revise the current species composition of the genus *Drepanocephalus*.

First, the ML and Bayesian trees obtained in the current study, inferred with five molecular markers, consistently showed that the species *P. mexicanus* is not closely related to other members of the genus *Petasiger* and, in consequence, we propose to re-allocate it to its original denomination as *D. mexicanus* (Lamothe-Argumedo & Pérez-Ponce de León, 1989). Interestingly, in our phylogenetic analyses inferred with LSU, ITS, *cox1* and *nad1*, the sequences identified as *Drepanocephalus* sp. by Pinto *et al.* (2016) from Brazil are nested within all newly generated sequences of *D. mexicanus*. Accordingly, it seems that the distribution range of this species extends from Mexico to Brazil, infecting the Neotropical cormorant *N. brasiliensis* as the definitive host, and the snail *B. straminea* as the first intermediate host. According to Thompson (2011), at least 11 species of *Biomphalaria* occur in Mexico, although the species *B. straminea* is restricted to other areas of the Neotropical region. Even though the snail has enlarged its distribution over the past decades and can be considered as the most invasive planorbid species (introduced in the Caribbean area and islands of the Lesser Antilles, and even outside of the neotropics (Pointier *et al.*, 2005, and references therein)), it has not been introduced into Mexico. However, we can expect to find the cercariae of *D. mexicanus* parasitizing one or more species of planorbid snails in Mexico, but this needs to be confirmed in future studies using a molecular approach to confirm conspecificity. In the USA, the cercariae of *D. spathans* have been reported in the planorbid snail *P.*

trivolis (Griffin *et al.*, 2012). This shows that other species of planorbid snails may act as the first intermediate host of this group of trematodes.

Second, the phylogenetic position of the newly generated sequences with respect to all those available in the GenBank corroborates that *D. spathans* and *D. auritus* nest in the same monophyletic lineage. The genetic divergence among specimens was very low for the molecular markers (0% for ITS, SSU, LSU; from 0 to 1.8% for *nad1*; between 2.65 and 4.41% for *cox1* and between 0 and 3% among specimens that showed both morphotypes that shared the same clade for *cox1*). According to Kudlai *et al.* (2015a), the most characteristic morphological traits that differentiate the species *D. auritus* from *D. spathans* are the shape of the testes and their position with respect to each other, i.e. the inter-testicular space. Some published reports on the presence of *D. spathans* from South America (e.g. Drago *et al.*, 2011, Fig. 10 on p. 874) clearly show that specimens may possess a wide inter-testicular space. Our observations of the morphology and measurements of *D. spathans* (table 3), show a wide range of morphological variation in the diagnostic characters used by Kudlai *et al.* (2015a) to differentiate *D. auritus* from *D. spathans* (fig. 5e). In addition, the morphometric data provided in our study for specimens collected from cormorants across Nearctic and Neotropical areas of Mexico (table 3), show an overlap in the range of the measurements used by Kudlai *et al.* (2015a) to further distinguish between *D. auritus* and *D. spathans*, including collar length, hindbody width, forebody length, post-testicular space, prepharynx length and length of the posterior part of the seminal vesicle. Overall, our results show that all the specimens distributed between Canada in North America and Argentina in South America are conspecific, corresponding with the species *D. spathans*. In contrast with the decision taken by Pinto *et al.* (2016) to consider one of the species of *Drepanocephalus* from Brazil to be conspecific with *D. auritus*, we follow the principle of priority of the International Code of Zoological Nomenclature (Art. 23) and the valid name for the species should be *D. spathans* Dietz, 1909, and we propose that the species *D. auritus* is a synonym of *D. spathans*. It is our hope that our results contribute to solving the unsettled taxonomic history of this species, and help avoid future confusion about the identification of this species. For instance, considering the current controversy and status of *D. auritus*, Sheehan *et al.* (2016) actually identified adult specimens in cormorants from 11 locations in Alabama, Minnesota, Mississippi and Vermont, USA as *D. (auritus) spathans*.

Phylogenetic analysis also showed that both *D. spathans* and *D. mexicanus* form well-supported monophyletic clades, particularly when mtDNA genes are used for phylogenetic inference. They are sister species, although no sequences of the other allegedly valid species in the genus are yet available. The genetic divergence between *D. mexicanus* and *D. spathans* was relatively high, particularly for the two mtDNA genes (13.45–15.90% for *nad1*, and 12.40–14.40% for *cox1*). These high levels of genetic divergence of mitochondrial genes among species are similar to those exhibited between species pairs of echinostomatids. For instance, the genetic divergence among species of *Echinostoma* Rudolphi, 1809 varied from 9.6 to 30.8% for *nad1* (Bowles & McManus, 1993). In a study aimed at comparing between mitochondrial genes and ribosomal internal transcribed regions in prospecting for cryptic species of plathyhelminths, Vilas *et al.* (2005) found genetic divergence levels between 11.9 and 16.6% for *nad1* between four species pairs of echinostomatids. More recently, divergence levels from 16 to 32% for *cox1* were reported

Table 5a. Measurements of head-collar spines of *Drepanocephalus spathans* Dietz, 1909 and *D. mexicanus* Lamothe-Argumedo & Pérez-Ponce de León, 1989.

Source		Present study				Present study (CNHE 1411; voucher material of Ramos-Ramos, 1995)			Kostadinova et al., 2002	
Identified as		<i>Drepanocephalus spathans</i> Dietz, 1909				<i>D. spathans</i>			<i>D. spathans</i>	
Host		<i>Nannopterum brasiliensis</i> (Gmelin)				<i>N. brasiliensis</i>			<i>N. brasiliensis</i>	
Locality(ies)		Temascal, Oaxaca; Teapa, Tabasco; Catemaco, Tecolutla, Veracruz (Mexico)				Oaxaca (Mexico)			Paraguay	
		<i>n</i>	Range	Mean ± SD	<i>n</i>	Range	Mean ± SD	<i>n</i>	Range	Mean ± SD
Angle spines										
First pair										
Ventral	Length	27	172–312	265 ± 42	6	241–291	261 ± 21	12	219–320	274 ± 28
	Width	27	49–74	59 ± 6	6	54–91	64 ± 14	12	49–66	55 ± 6
Dorsal	Length	27	200–338	268 ± 38	5	209–288	253 ± 28	12	246–384	324 ± 32
	Width	27	48–74	59 ± 7	5	47–56	52 ± 22	12	58–72	64 ± 5
Second pair										
Ventral	Length	29	152–293	241 ± 35	5	212–309	266 ± 48	11	205–297	247 ± 25
	Width	29	40–70	52 ± 8	5	46–64	54 ± 7	11	39–51	44 ± 4
Dorsal	Length	27	153–324	244 ± 42	4	202–239	218 ± 17	12	256–402	302 ± 47
	Width	27	40–77	55 ± 10	4	39–50	44 ± 5	12	43–78	55 ± 11
Lateral spines										
First	Length	15	83–170	124 ± 26	3	110–143	132 ± 19	11	142–197	175 ± 19
	Width	15	28–44	36 ± 6	3	37–39	38 ± 1	11	31–47	37 ± 5
Remaining	Length	44	60–176	122 ± 31	8	87–145	110 ± 19	27	148–243	190 ± 27
	Width	44	29–48	40 ± 4	8	34–49	42 ± 6	27	22–47	37 ± 5
Dorsal spines										
	Length	41	59–165	95 ± 24	–	–	–	15	135–225	171 ± 27
	Width	41	20–47	37 ± 7	–	–	–	15	25–40	35 ± 5

Table 5b. Measurements of head-collar spines of *Drepanocephalus spathans* Dietz, 1909 and *D. mexicanus* Lamothe-Argumedo & Pérez-Ponce de León, 1989, continued.

Source	Present study (CNHE 7577-8; voucher material of Violante-González <i>et al.</i> , 2011)			Present study			Present study (CNHE 1279, 1280; type-material of Lamothe-Argumedo & Pérez-Ponce de León, 1989)			
Identified as	<i>D. spathans</i>			<i>Drepanocephalus mexicanus</i> Lamothe-Argumedo & Pérez-Ponce de León, 1989			<i>D. mexicanus</i>			
Host	<i>N. brasiliensis</i>			<i>N. brasiliensis</i>			<i>N. brasiliensis</i>			
Locality(ies)	Guerrero (Mexico)			La Tovar San Blas, Nayarit; Tehuantepec, Oaxaca; Teapa, Tabasco (Mexico)			Teapa, Tabasco (Mexico)			
	<i>n</i>	Range	Mean ± SD	<i>n</i>	Range	Mean ± SD	<i>n</i>	Range	Mean ± SD	
Angle spines										
First pair										
Ventral	Length	4	238–276	258 ± 20	22	102–167	134 ± 19	6	102–157	123 ± 25
	Width	4	51–68	60 ± 8	22	24–46	33 ± 7	6	26–42	32 ± 6
Dorsal	Length	4	207–243	227 ± 15	23	100–170	142 ± 20	6	104–161	130 ± 24
	Width	4	48–64	56 ± 7	23	24–44	35 ± 6	6	26–44	34 ± 7
Second pair										
Ventral	Length	4	202–224	209 ± 10	22	107–192	134 ± 19	6	94–151	115 ± 24
	Width	4	45–51	48 ± 3	22	21–40	31 ± 6	6	26–35	30 ± 4
Dorsal	Length	4	178–312	243 ± 72	22	84–188	143 ± 27	6	105–190	138 ± 35
	Width	4	40–65	52 ± 13	22	21–46	34 ± 8	6	29–39	35 ± 4
Lateral spines										
First	Length	4	97–121	108 ± 10	22	68–116	97 ± 15	5	69–106	86 ± 18
	Width	4	34–46	40 ± 5	22	16–32	24 ± 5	5	22–36	28 ± 5
Remaining	Length	12	84–154	113 ± 26	42	71–126	102 ± 15	14	44–113	79 ± 22
	Width	12	32–48	41 ± 6	42	14–36	25 ± 5	14	20–44	31 ± 9
Dorsal spines										
	Length	14	58–124	88 ± 21	24	41–117	80 ± 19	21	33–68	48 ± 12
	Width	14	31–49	37 ± 5	24	15–35	23 ± 5	21	16–43	23 ± 7

between two species of *Echinostoma* (*E. revolutum* Fröhlich, 1802 and *E. malayanum* Leiper, 1911) (Saijuntha et al., 2011).

Morphologically, *D. mexicanus* differs from *D. spathans* in: (1) the head-collar shape (reniform vs. falciform, see fig. 4); (2) a shorter first pair of ventral (102–167 vs. 172–320) and dorsal (100–171 vs. 200–384) angle spines (see table 5); (3) well-developed ventral lappets vs. head collar with an invagination formed by the two lappets (fig. 4b, e); (4) tegumental spines distributed between head collar and posterior margin of ventral sucker vs. spines distributed between head collar and anterior margin of ventral sucker (fig. 4a, d); and (5) shape and size of the tegumental spines (large–sharp (11.6–16.1 × 2.9–7.) vs. short–wider (4.2–12.9 × 3.3–13.9)) (see fig. 4c, f).

With the re-allocation of *D. mexicanus* and the synonymy of *D. auritus* with *D. spathans* proposed in this study, the genus *Drepanocephalus* now contains three nominal species: (1) *D. spathans*, associated with the Neotropical cormorant *N. brasiliensis*, the double-crested cormorant *N. auritus* and the brown booby *S. leucogaster*, distributed widely across the Americas, in Argentina, Brazil, Paraguay, Venezuela, Colombia, Mexico, USA and Canada (Ramos-Ramos, 1995; Kostadinova et al., 2002; Wagner et al., 2012; Griffin et al., 2014; O’Hear et al., 2014; Pinto et al., 2016; Sheehan et al., 2016); (2) *D. mexicanus*, only found parasitizing the Neotropical cormorant *N. brasiliensis* distributed in Mexico and Brazil; and, finally, (3) *D. olivaceus* from *N. brasiliensis* in Venezuela and Brazil.

The species *Petasiger parvicephalum* was originally described as a member of *Drepanocephalus* and transferred to *Paryphosomum* by Kostadinova et al. (2002), and more recently to the genus *Petasiger* (Tkach et al., 2016). Considering the morphology of the species, it may belong in *Drepanocephalus* but further molecular data from the type locality in Colombia are necessary to confirm its taxonomic status. As we show in this study, the records of *D. olivaceus* in Mexico (Pineda-López et al., 1985; Ramos-Ramos, 1995; Violante-González et al., 2011) are incorrect, and in fact correspond with *D. spathans*. Our observations of the morphological characters of museum specimens from the studies of Ramos-Ramos (1995) (CNHE 1141) and Violante-González et al. (2011) (CNHE 7577-7578) corroborate the conclusion that these specimens belong to *D. spathans*.

In addition, sequence data were generated from specimens of *Drepanocephalus* collected in Temascal, Oaxaca – the same host and locality as in the study by Ramos-Ramos (1995), demonstrating that these worms belong to *D. spathans*. Our results also show that both species of *Drepanocephalus* (*D. spathans* and *D. mexicanus*) occur in sympatry in the Neotropical cormorant in at least five of the sampled localities (see localities 7, 10, 11, 17, 20 in fig. 1), corresponding to the Neotropical biogeographical region. Still, the study of the first and second intermediate hosts in the life cycle of these parasites is necessary to establish a link among life-cycle stages, especially in species such as *D. spathans*, whose extensive distributional range across the Americas (from Canada to Argentina) is determined primarily by the vagility of their bird hosts, although it is possible that the first and second intermediate hosts are different across the distributional range of the species. We have demonstrated here that, contrary to what could be expected in finding a complex of cryptic species, considering the wide distributional range of *D. spathans* (Blouin, 2002), they actually represent a single species, genetically very similar but showing some level of intraspecific morphological variability. This seems to represent a case of a widely distributed

species across the Americas, whose distributional range is mainly determined by the bird definitive host, although, surely, first and second intermediate hosts must also play an important role in its geographical distribution. Other trematode parasites of both species of cormorants (*N. brasiliensis* and *N. auritus*), i.e. *Austrodiplostomum* and *Hysteromorpha*, may follow the same biogeographical pattern in cormorants across the Americas, from Argentina to Canada (see Drago et al., 2011; Locke et al., 2011; García-Varela et al., 2016); however, more detailed taxonomic work is required. The case of the status of *Austrodiplostomum ostrowskiae* Dronen, 2009 in relation to the species from South America, *A. mordax* Szidat & Nani, 1951 and *A. compactum* (Lutz, 1928) Dubois, 1936, may result in a single and widely distributed species in cormorants across the Americas (pending molecular analysis including samples from the southern part of the continent), as is the case for *Hysteromorpha triloba* Rudolphi, 1919.

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