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Morphological and molecular evidence reveals a new species of *Lyperosomum* Looss, 1899 (Digenea: Dicrocoeliidae) from *Melanerpes aurifrons* (Wagler, 1829) from northern Mexico

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Research Paper

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Abstract

A new species of the genus *Lyperosomum* Looss, 1899, from the intestine of the golden-fronted woodpecker (*Melanerpes aurifrons*) from northern Mexico is described. *Lyperosomum cuauhxinqui* sp. n. is morphologically distinguished from other congeneric species from the Americas by a higher oral/ventral sucker ratio and its body length and width. The sequences of domains D1–D3 of the large subunit (LSU) of nuclear ribosomal DNA and cytochrome *c* oxidase subunit 1 (*cox 1*) from the mitochondrial DNA of the new species were obtained and compared with available sequences from GenBank. The genetic divergence estimated between the new species and other congeneric species ranged from 2 to 6% and 13.4 to 17.3% for LSU and *cox 1*, respectively. Phylogenetic analyses based on the two (LSU and *cox 1*) molecular markers consistently showed that *L. cuauhxinqui* sp. n. was nested within the genus *Lyperosomum*, with strong bootstrap support (100%) and Bayesian posterior probabilities (1.0). In particular, the LSU tree indicated that the sequence of the new species is closely related to sequences from *Zonorchis alveyi*, *Zonorchis delectans* and *Zonorchis* sp. from Central America, suggesting that these sequences should be transferred to the genus *Lyperosomum*. The new species represents the first record from Mexico and the fifth species identified in the Americas. Our study also revealed that the taxonomy of the genus *Lyperosomum* should be re-examined by combining molecular, morphological and ecological characteristics.

Introduction

Dicrocoeliidae Looss, 1899 is a family of digenean parasites from the bile ducts, gallbladder and intestines of birds and, rarely, mammals distributed around the world, including approximately 400 species classified into 46 genera (Hildebrand *et al*., 2016, 2019; Tkach *et al*., 2018). The genus *Lyperosomum* Looss, 1899 is among the most diverse genera in this family, with approximately 33 recognized species, mostly parasitizing passerine birds (Hildebrand *et al*., 2019). The species of *Lyperosomum* are characterized by the following traits: oral sucker smaller than the ventral sucker, testes positioned closely to the ventral sucker, ovary posterior and distant from the posterior testis, genital pore located anterior to the intestinal bifurcation and vitellarium forming two relatively long lateral bands of follicles, beginning at the level of the testes and not reaching the caecal ends (Pojmańska, 2008). Based on these morphological traits, the history of the taxonomy and species composition of the genus *Lyperosomum* has been complex and unstable due to the phenotypic plasticity of some diagnostic characteristics that define the species. Recently, Hildebrand *et al*. (2019) conducted one of the most extensive studies of the genus *Lyperosomum*, combining morphological and molecular data. Their analyses also included species representing the genera *Skrjabinus* Bhalerao, 1936 and *Zonorchis* Travassos, 1944 from Dicrocoeliidae. These authors found that the species of *Lyperosomum* analysed were paraphyletic because some species of *Zonorchis* were nested in the genus *Lyperosomum*.

In the Americas, four species of the genus *Lyperosomum* have been recorded. *Lyperosomum intermedium* Denton & Kinsella, 1972 was described from the pancreas of rice rats, *Oryzomys palustris* Harlan, 1837, from Georgia and Florida in the US (Denton & Kinsella, 1972). *Lyperosomum petiolatum* (Railliet, 1900) Hildebrand, Pyrka, Sitko, Jeżewski, Zaleśny, Tkach & Laskowski, 2019 was isolated from the gall bladder of blue jays, *Cyanocitta cristata* (Linnaeus, 1758), from Texas, Mississippi and Nebraska in the USA (Denton & Byrd,

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1951). *Lyperosomum oswaldoi* Travassos, 1919 was described from the liver and gall bladder of brown thrashers, *Toxostoma rufum* (Linnaeus, 1758), from Georgia, Mississippi and Texas in the USA (Denton & Byrd, 1951) and in the bile duct of great antshrike, *Taraba major* (Vieillot, 1816) from Argentina (Travassos, 1944; Lunaschi & Drago, 2013). Finally, *Lyperosomum byrdi* Denton & Krissinger, 1975 was described from the liver and gall bladder of rufous-sided towhees, *Pipilo erythrophthalmus* (Linnaeus, 1758), from Florida and Georgia in the USA (Denton & Krissinger, 1975).

During a helminthological expedition in northern Mexico, adult digeneans were recovered from the intestine of the golden-fronted woodpecker, *Melanerpes aurifrons* (Wagler, 1829). The examination of this material revealed the presence of an undescribed species of the genus *Lyperosomum*. Therefore, the aim of this study was to (1) provide a morphological description of the new species and (2) test the systematic position of the new species within *Lyperosomum* using molecular data from the large subunit (LSU) of nuclear ribosomal DNA and cytochrome *c* oxidase subunit 1 (*cox 1*) from mitochondrial DNA.

Materials and methods

Specimen collection

A total of 12 specimens of *Lyperosomum* sp. were obtained from a single *M. aurifrons* individual from northern Mexico. The host was examined for parasites under a dissecting microscope a few hours after its capture. The collected digeneans were preserved either in 100% ethanol for DNA extraction or in hot (steaming) 4% formalin for morphological examination. The avian definitive host was identified using the field guide of Howell & Webb (1995) and the American Ornithologists' Union (1998) guidelines, and the nomenclature follows the Avibase database (http://avibase.bsc-eoc.org).

Amplification and sequencing of DNA

Two specimens were placed individually in tubes and digested overnight at 56°C in a solution containing 10 mM Tris-hydrochloride (pH 7.6), 20 mM sodium chloride, 100 mM Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2 EDTA) (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, OH, USA) according to the manufacturer's instructions. The cytochrome *cox 1* of mitochondrial DNA and D1–D3 domains of the LSU of nuclear ribosomal DNA were amplified using polymerase chain reaction (PCR). A fragment of *cox 1* was amplified using the forward JB3 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ and reverse JB4.5 5′-TAAAGAAAGAACATAATGAAAATG-3′ primers (Bowles *et al.*, 1993).

Domains D1–D3 from LSU were amplified using forward primer 391, 5′-AGCGGAGGAAAAGAAACTAA-3′ (Nadler *et al.*, 2000), and reverse primer 536, 5′-CAGCTATCCTGAGGGAAAC-3′ (Garcia-Varela & Nadler, 2005). The amplification reactions (25 µl) consisted of 1 µl of each primer (10 µM), 2.5 µl of 10× buffer, 1.5 µl of 2 mM magnesium chloride, 0.5 µl of Ethylenediaminetetraacetic acid disodium salt dihydrate (dNTPs) (10 mM), 16.37 µl of water, 2 µl of genomic DNA and 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). The PCR cycling conditions for amplification included denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, annealing at 48°C for *cox 1* and 50°C for LSU for 1 min, and extension at 72°C for 1 min, with a final postamplification incubation at 72°C for 10 min. The sequencing reactions were performed using the initial primers for *cox 1* and LSU plus two internal primers 503, 5′-CCTTGGTC CGTGTTTCAAGACG-3′ and 504, 5′-CGTCTTGAAACACGG ACTAAGG-3′ (Garcia-Varela & Nadler, 2005) for LSU with ABI Big Dye (Applied Biosystems, Boston, MA, USA) terminator sequencing chemistry, and the reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled, and base-calling differences were resolved using CodonCode Aligner 5.1.5 (CodonCode Corporation, Dedham, MA, USA). Sequences were deposited in the GenBank database (table 1).

Alignments and phylogenetic analyses

The new sequences were aligned using the software SeaView version 4 (Gouy *et al.*, 2010) and adjusted with the Mesquite program (Maddison & Maddison, 2011). The LSU alignment included the new sequences plus 21 sequences from *Lyperosomum* spp. and seven sequences that were used as an outgroup (table 1). Two new *cox 1* sequences were aligned with 17 other sequences of *Lyperosomum* spp., plus seven other sequences that were used as an outgroup (table 1). The phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI) methods. The ML analyses were carried out with RAxML version 7.0.4 (Silvestro & Michalak, 2011), and BI analyses were inferred with MrBayes version 3.2.7 (Ronquist *et al.*, 2012) using the online interface Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway version 3.3 (Miller *et al.*, 2010). The best model was estimated with the Akaike information criterion using the jModel Test version 0.1.1 program (Posada, 2008). The best model for each dataset was GTR + I + G for the LSU dataset and TPM3uf + I + G for the *cox 1* dataset. ML analyses were inferred with models previously estimated for each molecular marker. To support each node, 10,000 bootstrap replicates were run. The BI analyses included Markov Chain Monte Carlo searches of two simultaneous runs for ten million generations, with sampling every 1000 generations, a heating parameter value of 0.2 and a 'burn-in' of 25%. Trees were drawn using FigureTree program 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).

Morphological study

For taxonomic identification, seven specimens were stained with Mayer's paracarmine, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted on permanent slides with Canada balsam for deposition in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Mexico City. Whole-mount specimens were examined using a Leica DM1000 LED (Leica Microsystems GmbH, Wetzlar, Germany) compound microscope. Measures are given in micrometres (µm). Illustrations of internal morphological features were produced using a drawing tube attached to a Leica MC120HD. For scanning electron microscopy (SEM), three specimens were dehydrated in a graded ethanol series, critical-point dried, sputter-coated with gold and examined with a Hitachi Stereoscan Model S-2469 N scanning

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Table 1. Specimens analysed in this study; host name, localities and GenBank accession numbers of each molecular marker.

Taxa	Host	Locality	GenBank number		Source
			LSU	*cox 1*	
Brachylecithum lobatum	*Corvus corone* (B)	Czech Republic	KU212200	KU212199	Hildebrand *et al.*, 2016
Brachydistomum ventricosum	*Erithacus rubecula* (B)	Czech Republic	KU563713		Unpublished
	Delichon urbicum (B)	Czech Republic		MK445283	Hildebrand *et al.*, 2019
Dicrocoelium dendriticum	*Marmota bobak* (M)	Ukraine	AF151939		Tkach *et al.*, 2000
	Capra aegagrus hircus (M)	China		NC025280	Liu *et al.*, 2014
Dicrocoelium chinensis		China		LC333988	Unpublished
Lutztrema attenuatum	*Turdus merula* (B)	Poland	KT387687		Unpublished
	Turdus philomelos (B)	Czech Republic		KU563721	Unpublished
Stromitrema koshewnikowi	*Hirundo rustica* (B)	Czech Republic	MK474483	MK445284	Hildebrand *et al.*, 2019
Lyperosomum cuauhxinqui sp. n.	*Melanerpes aurifrons* (B)	México	**MT340826**	**MT348379–80**	This study
Lyperosomum intermedium	*Oryzomys palustris* (M)	USA	MH158563		Tkach *et al.*, 2018
Lyperosomum transcarpathicus	*Sorex minutus* (M)	Ukraine	AF151943		Tkach *et al.*, 2001
Lyperosomum clathratum	*Apus apus* (B)	Czech Republic	MK478493–94	MK445289 MK445290 MK445287	Hildebrand *et al.*, 2019
Lyperosomum cf. *turdia*	*Turdus philomelos* (B)	Czech Republic	MK478496	MK445292	
	Turdus merula (B)	Czech Republic	MK478497	MK445293–95	
	Turdus merula (B)	Poland	MK478486	MK445291	
Lyperosomum petiolatum	*Motacilla alba* (B)	Czech Republic	MK626684		
	Prunella modularis (B)	Czech Republic		MK445300	
	Pica pica (B)	Czech Republic	MK626683 MK478480	MK445302	
	Garrulus glandarius (B)	Poland		MK445296	
	Sylvia atricapilla (B)	Czech Republic	AY222259		Olson *et al.*, 2003
	Sylvia atricapilla (B)	Czech Republic	KU212193	KU212192	Hildebrand *et al.*, 2016
	Sylvia atricapilla (B)	Czech Republic		MK445303 MK621923	Hildebrand *et al.*, 2019
	Alauda arvensis (B)	Czech Republic	MK478485	MK445315	
	Emberiza schoeniclus (B)	Czech Republic	MK478475	MK445304	
	Cyanistes caeruleus (B)	Czech Republic	MK478476		
	Parus major (B)	Czech Republic	MK478483		
Lyperosomum sarothrurae	*Sarothrura pulchra* (B)	Uganda	KP765767		Hildebrand *et al.*, 2015
Lyperosomum sp.	*Pogoniulus scolopaceus* (B)	Uganda	MK480326		Hildebrand *et al.*, 2019
	Acrocephalus arundinaceus (B)	Czech Republic		MK445285	
	Turdus merula (B)	Czech Republic	MG560864		Aldhoun *et al.*, 2018
	Acrocephalus arundinaceus (B)	Czech Republic	MK496656		Hildebrand *et al.*, 2019
	Delichon urbicum (B)	Czech Republic	MK496657		

(*Continued*)

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Table 1. (*Continued.*)

Taxa	Host	Locality	GenBank number		Source
			LSU	*cox 1*	
Skrjabinus kalmikensis	*Delichon urbicum* (B)	Czech Republic	MK478495	MK445286	
Zonorchis alveyi	*Zonotrichia albicollis* (B)	USA	MK480327		
Zonorchis delectans	*Caryothraustes poliogaster* (B)	Costa Rica	MK480329		
Zonorchis sp.	*Phaenostictus mcleannani* (B)	Costa Rica	MK480328		

Sequences in bold were generated in this study. B, bird; M, mammal.

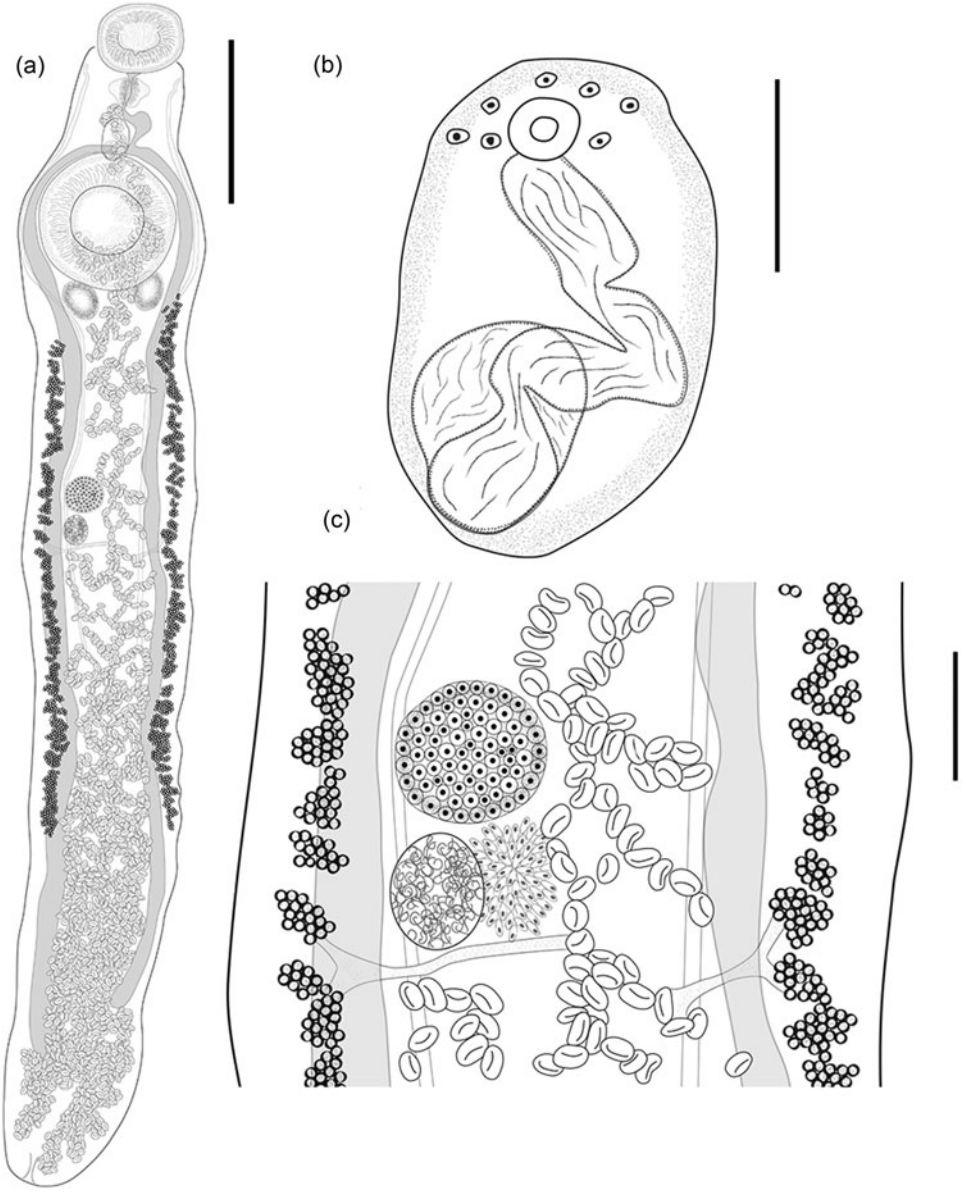

Fig. 1. *Lyperosomum cuauhxinqui* sp. n. from *Melanerpes aurifrons*: (a) whole worm holotype, ventral view; (b) enlarged view of cirrus sac; (c) ovarian region showing relative position of ovary, seminal receptacle and Mehlis' gland. Scale bars: (a) 500 μm; (b) 100 μm; (c) 50 μm.

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electron microscope operating at 15 kV from the Instituto de Biología, UNAM.

Results

Lyperosomum cuauhxinqui sp. n.

Morphological description

Description (based on seven mounted adult specimens and three analysed by SEM). Measurements of holotype are provided in the description. Measurements of paratypes are provided in table 2.

Body elongate, slender, 3682 long. The maximum width in the ventral sucker region was 527 (figs 1a and 2a). Tegument thin with no spines, rough, with sensory conical papillae in the forebody (fig. 2c). Forebody short, 903. Forebody-to-body-length ratio 1:4. Suckers close to each other. Oral sucker terminal, round, 252 long, 218 wide, bearing three pairs of internal dome-like papillae (fig. 2a, b). Ventral sucker well developed, larger than the oral sucker, very muscular, situated at the end of the first third of the body, 431 long, 419 wide (figs 1a and 2c). Oral-to-ventral-sucker-length ratio 1:1.7. Oral-to-ventral-sucker-width ratio 1:1.9. (figs 1a and 2b). Prepharynx present, 15 long. Small pharynx 92 long, 119 wide. Oesophagus curved, 170 long. Caeca long, unequal in length, right caeca longer than the left (3111 and 2811, respectively), terminating in the posterior of the body (fig. 1a). Testes spherical, intercaecal, situated symmetrically just posterior to ventral sucker (fig. 1a), right testis 127 long, 94 wide, left testis 121 long, 91 wide. Cirrus sac small, oval, containing a sinuous seminal vesicle and cirrus, 136 long, 82 wide (fig. 1b). Cirrus sac situated posterior to the pharynx and anterior to the ventral sucker. Genital pore situated posterior to the pharynx. Ovary spherical, 119 long, 116 wide, distant from the testes, separated by numerous uterine coils (fig. 1c). Distance between testes and ovary, 481. Ovary submedial, located anterior to the middle of the body, dextral ($n = 4$) or sinestral ($n = 3$) position. Mehlis' gland situated post-ovary, contiguous with the seminal receptacle. Seminal receptacle, oval, 81 long, 68 wide (fig. 1c). Laurer's canal not observed. Vitellarium conformed to numerous small follicles arranged in two lateral narrow rows, mostly in extracaecal fields. Vitelline fields asymmetrical, beginning at the posterior level of the testes, extending anteriorly to the end of the caeca. Right and left vitelline fields are 1547 and 1685 long, respectively. Vitelline-field-length-to-body-length ratio 1:2.1. Uterus convoluted, filling most of the hindbody, occupying the intertesticular region, dorsal to the ventral sucker. Mature eggs numerous, thick-walled, 20–32 long, 16–22 wide ($n = 20$). The excretory canals with thin walls originate on the excretory vesicle and extend to the anterior region at the level of the pharynx. Excretory vesicle I-shaped. Excretory pore terminal.

Taxonomic summary

Type host. Golden-fronted woodpecker, *M. aurifrons* (Wagler, 1829) (Aves: Piciformes: Picidae).

Type locality. Río Purificación (24°05′21.4″N, 99°09′54″W), Tamaulipas, México.

Site of infection. Intestine.

Date of collection. 17, February, 2016.

Type material. Holotype, CNHE 11245, and six paratypes, CNHE 11246.

Representative DNA sequences deposited. MT348379-80 (*cox 1*), MT340826 (LSU).

Etymology. The new species is named in reference to its definitive host, the golden-fronted woodpecker. The specific epithet derives from the Náhuatl language. *Cuauhxinqui* = woodpecker ('carpintero' in Spanish).

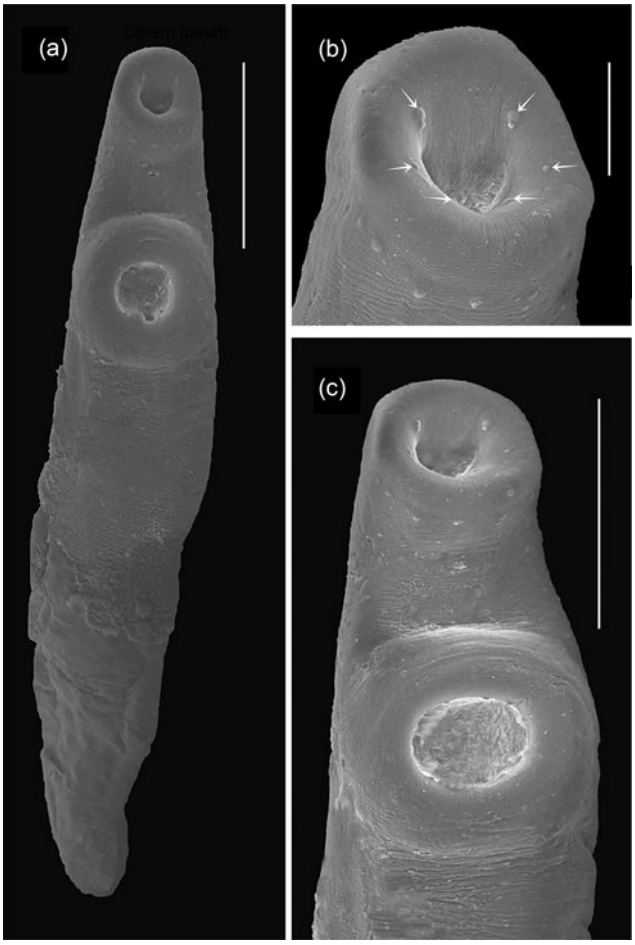

Fig. 2. Scanning electron micrographs of the whole worm adults of *Lyperosomum cuauhxinqui* sp. n. from *Melanerpes aurifrons*: (a) whole worm; (b) oral sucker showing the arrangement of three pairs of papillae (arrows); (c) forebody. Scale bars: (a) 500 μm; (b) 100 μm; (c) 400 μm.

Note. All the measures presented in the description were recorded before the forebody of the type specimen was slightly damaged during the drawing process.

Remarks

The new species possesses features that are consistent with the diagnosis of the genus *Lyperosomum*: testes positioned closely to the ventral sucker, genital pore located anterior to the intestinal bifurcation, ovary positioned posterior to the testis, vitellarium forming two lateral bands of follicles extending anteriorly past the level of the ovary and a ventral sucker larger than the oral sucker (see Pojmańska, 2008). In the Americas, four species of *Lyperosomum* have been found parasitizing birds and mammals. The new species can be differentiated from *L. oswaldoi* by a shorter body length (2263–3682 vs. 4300–10,650 in *L. oswaldoi*). The new species is also differentiated from *L. byrdi* by a greater body width (528–702 vs. 300–441 in *L. byrdi*). *Lyperosomum cuauhxinqui* sp. n. is differentiated from *L. intermedium* by a greater body width (528–702 vs. 335–420 in *L. intermedium*). In addition, the new species can be differentiated from *L. oswaldoi*, *L. byrdi* and *L. intermedium* by exhibiting symmetrical rather than oblique testes. *Lyperosomum cuauhxinqui* sp. n. is differentiated from the type species, *L. petiolatum*, by an oral sucker that is larger than that of *L. petiolatum*

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Table 2. Comparative measurements of *Lyperosomum* Looss, 1899 from the Americas.

Species	*L. cuauhxinqui* sp. n.	*L. petiolatum*	*L. byrdi*	*L. oswaldoi*		*L. intermedium*
Type locality	Tamaulipas, Mexico		Florida and Georgia, USA			Florida and Georgia, USA
Other localities		Texas, Mississippi and Nebraska, USA		Texas, USA	Formosa, Argentina	
Source	Present study	Denton & Byrd, 1951	Denton & Krissinger, 1975	Travassos, 1944; Denton & Byrd, 1951	Lunaschi & Drago, 2013	Denton & Kinsella, 1972
Habitat	Intestine	Liver	Liver and gallbladder	Liver and gallbladder	Bile ducts	Pancreas
Type host	*Melanerpes aurifrons*	*Garrulus glandarius*	*Pipilo erythrophthalmus*	*Toxostoma rufum*		*Oryzomys palustris*
Other hosts		*Cyanocitta cristata*, *Cardinalis*, *Pheucticus ludovicianus* and *Melanerpes erythrocephalus*		*Cyanocitta cristata*	*Taraba major*	
Body length	2263–3682 (2910)	2260–4180	2098–3745	4350–10650	4300	1650–4200
Body width	528–702 (591)	340–920	300–441	390–770	680	335–420
Forebody/body length ratio	1:2.7–4.0 (3.2)	1:6.4–11.8[a]	1:5.05–9.3[a]	1:8.43–20.6[a]	1:7.8[a]	1:4.2–10.8[a]
Forebody	817–999 (900)	352[a]	403[a]	516[a]	548[a]	388[a]
Hindbody	1429–2810 (2015)	2963[a]	2805[a]	7290[a]	3468[a]	1592[a]
Forebody/hindbody ratio	1:3.1–5.7 (4.9)	1:5.1[a]	1:7.9[a]	1:13[a]	1:7.5	1:4.1
Oral sucker length	213–315 (270)	130–200	188–230	150–300	238	132–150
Oral sucker width	219–285 (258)	130–200	222[a]	160–310	217	125–145
Ventral sucker length	340–437 (392)	250–400	308–400	370–660	386	160–215
Ventral sucker width	316–432 (393)	250–400	375[a]	548[a]	430	160–215
Oral/ventral sucker width ratio	1:1.16–2.05 (1.47)	1:1.7–2.19	1:1.6–1.9	1:2.3[a]	2	1:1.3–1.5
Pharynx length	90–116 (100)	50–100	63–78	60–150	88	60–74
Pharynx width	83–128 (110)	50–100	76–96	–	114	60–74
Ovary length	82–128 (106)	80–200	50–128	70–270	193	63–145
Ovary width	99–117 (108)	80–200	60–144	130–310	226	70–150
Right testis length	114–168 (147)	90–200	60–125	110–300	119	111–240
Right testis width	94–115 (126)	90–200	111[a]	130–360	179	111–240
Left testis length	122–165 (145)	90–200	60–125	226[a]	133	111–240
Left testis width	92–145 (128)	90–200	111[a]	290[a]	190	111–240
Eggs length	19–34 (28)	30–36	30–35	26–33	26–29	46–51
Eggs width	14–24 (20)	20–24	18–21	18–22	19–21	23–27
Distance between posterior testis and ovary	145–482 (267)	204[a]	333[a]	323[a]	81[a]	143[a]

Right vitelline field length	991–1547 (1257)	1148[a]	800–1110	3548[a]	1548[a]	468–668	
Left vitelline field length	880–1686 (1234)	1093[a]	1222[a]	3000[a]	2000[a]	468–668	
Cirrus sac length	115–175 (145)	140–210	132–152	200[a]	143	111–150	
Cirrus sac width	65–83 (74)	50–60	60–69	83[a]	53	50–60	
Seminal receptacle length	60–81 (69)	–	111[a]	–	–	61[a]	
Seminal receptacle width	43–69 (52)	–	69[a]	–	–	92[a]	

Measurements in micrometres. Mean in brackets.
[a]Estimated from the published drawing.

(213–315 × 219–285 vs. 130–200 × 130–200). Finally, the new species occurs in the intestine of its definitive host, whereas the other congeneric species occur in the liver, gallbladder, pancreas and bile ducts (see table 2).

Phylogenetic analysis

Nuclear marker

The LSU dataset included 1373 characters, and the best evolution model obtained was GTR + I + G. The alignment included 17 sequences representing six species of *Lyperosomum* and four other sequences belonging to *Lyperosomum* sp., in addition to sequences from seven other genera from Dicrocoeliidae that were used as an outgroup (see table 1). The phylogenetic tree inferred with the ML and BI methods suggests that the species of *Lyperosomum*, including the new species, form a monophyletic assemblage with strong bootstrap support (100%) and a high Bayesian posterior probability (1.0). The phylogenetic trees (fig. 3) showed that the species *L. intermedium* and *Lyperosomum transcarpathicus* (Bychovskaja-Pavlovskaja, Vysotzkaja & Kulakova, 1970), which are parasites isolated from mammals from the New World and Old World, respectively, formed two independent clades of *Lyperosomum*. The phylogenetic trees inferred with other species of parasites from birds were divided into two major clades. The first clade included two isolates of *Lyperosomum clathratum* (Deslongchamps, 1824) from the Czech Republic + *Lyperosomum* sp. (MK480326), in addition to three isolates of *Lyperosomum turdia* (Ku, 1938) from Poland and the Czech Republic + *Lyperosomum* sp. (MG560864) and a subclade that contained nine isolates of *L. petiolatum* from Poland and the Czech Republic. The second major clade included the species *Skrjabinus kalmikensis* (Skrjabin & Issaitschikow, 1927) (MK478495) plus two isolates of *Lyperosomum* sp. (MK496656, MK496657) from the Czech Republic. One subclade contained an isolate of *Lyperosomum sarothrurae* (Baer, 1959) from Africa and was sister to *L. cuauhxinqui* sp. n. plus *Zonorchis alveyi* (Martin & Gee, 1949) (MK480327), *Zonorchis delectans* (Travassos, 1944) (MK480329) and *Zonorchis* sp. (MK480328). All of these phylogenetic relationships received strong bootstrap support and showed very good Bayesian posterior probabilities (see fig. 3). The genetic divergence estimated among the species of *Lyperosomum* ranged from 2 to 6%, whereas the lowest genetic divergence found was 2%, between *L. cuauhxinqui* sp. n. and *L. sarothrurae* from Africa, and the greatest genetic divergence was 6%, between *L. cuauhxinqui* sp. n. and *L. intermedium* from the USA (table 3). Finally, the genetic divergence among isolates of *L. clathratum*, *L. turdia* and *L. petiolatum* ranged from 0 to 0.1% (table 3).

Mitochondrial marker

The *cox 1* dataset included 411 characters with 26 terminals, and the best selected model was TPM3uf + I + G. This alignment included 16 specimens representing three species of *Lyperosomum* and a single sequence identified as *Lyperosomum* sp., plus six other genera from Dicrocoeliidae that were used as an outgroup. The phylogenetic tree inferred with the ML and BI methods suggested that the species of *Lyperosomum* form a monophyletic group (fig. 4) that is subdivided into two major clades. The first clade included an isolate of *Lyperosomum* sp. (MK445285) from the Czech Republic + *S. kalmikensis* (MK445286) and three isolates of *L. clathratum* from the Czech Republic. The second clade included two isolates of *L.*

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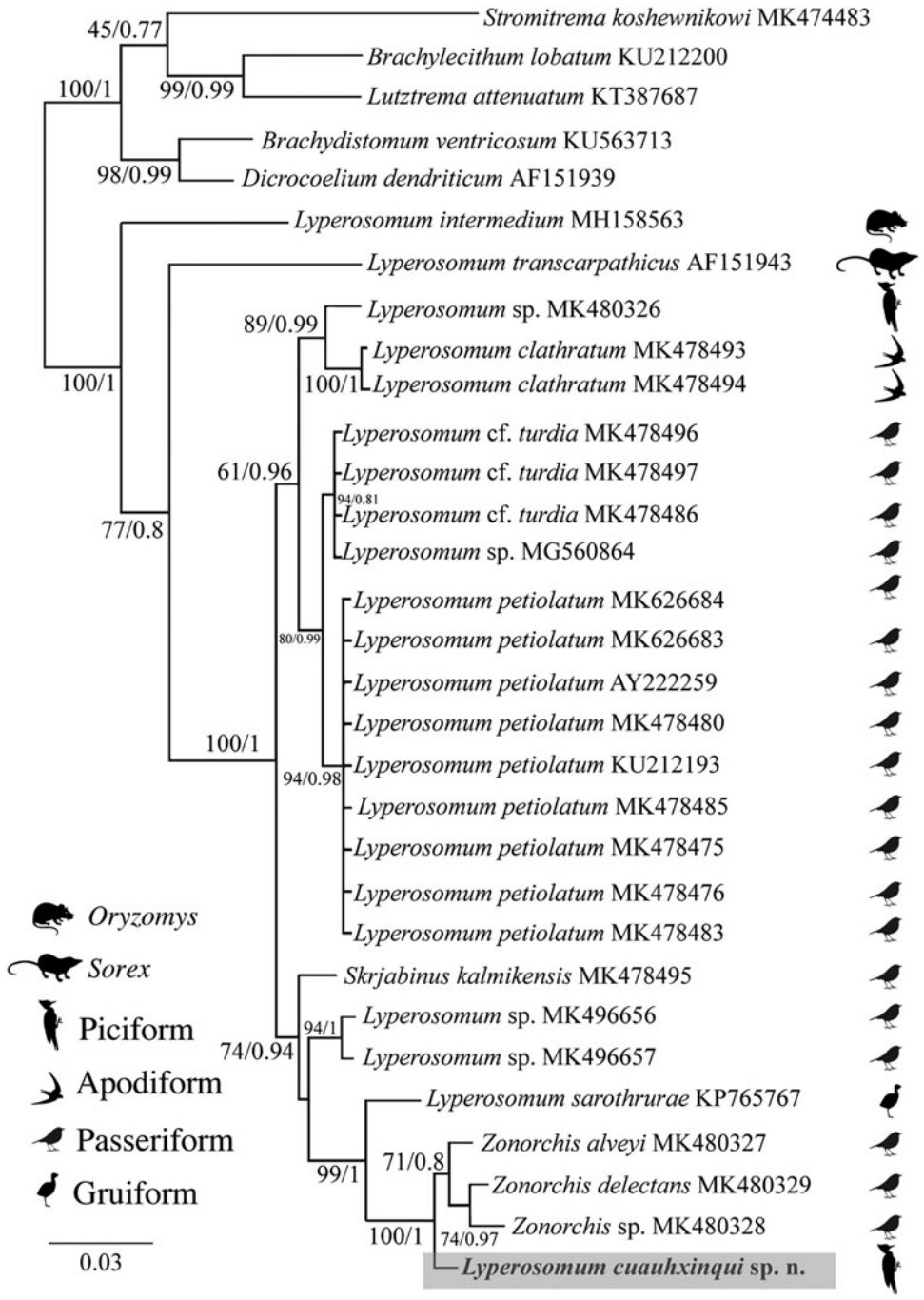

Fig. 3. Maximum likelihood (ML) tree and consensus Bayesian inference (BI) tree inferred from the large subunit from nuclear ribosomal DNA. Numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). Scale bar shows the number of substitutions per site.

cuauhxinqui sp. n. from northern Mexico and was sister to another subclade formed by eight isolates of *L. petiolatum* plus five isolates of *L.* cf. *turdia* from the Czech Republic and Poland. The genetic divergence among the species of *Lyperosomum* ranged from 5.9 to 13.8%. The lowest genetic divergence value was 13.4%, between *L. cuauhxinqui* sp. n. and the isolates of *L. petiolatum* from the Czech Republic and Poland, and the greatest value was 17.3%, for *L. clathratum* from the Czech Republic. The genetic divergence among the isolates of *L. clathratum*, *L. turdia* and *L. petiolatum* ranged from 0 to 0.32%, and that between the two isolates of *L. cuauhxinqui* sp. n. was 0.2% (table 3).

In summary, the phylogenetic analyses inferred with the LSU and *cox 1* datasets are congruent in that *L. cuauhxinqui* sp. n. is nested within *Lyperosomum*. As a second result derived from the phylogenetic trees, the sequences identified as *Z. alveyi* (MK480327), *Z. delectans* (MK480329), *Zonorchis* sp. (MK480328) plus *S. kalmikensus* (MK478495 and MK445286) should be transferred to *Lyperosomum*.

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Table 3. Pairwise nucleotide sequence comparisons among taxa for the LSU sequences ($N = 1373$ nt) (below the diagonal) and for the *cox 1* sequences ($N = 411$ nt) (above the diagonal).

Taxon name	*Lyperosomum cuauhxinqui* sp. n.	*Zonorchis* sp.	*Zonorchis delectans*	*Zonorchis alveyi*	*Lyperosomum sarothrurae*	*Lyperosomum transcarpathicus*	*Lyperosomum intermedium*	*Skrjabinus kalmikensis*	*Lyperosomum petiolatum*	*Lyperosomum turdia*	*Lyperosomum clathratum*	*Dicrocoelium dentriticum*	*Brachydistomum ventricosum*	*Lutztrema attenuatum*	*Brachylecithum lobatum*	*Stromitrema koshewnikowi*
Lyperosomum cuauhxinqui sp. n	0 / 0–0.2	–	–	–	–	–	–	16.8–17.0	13.4–14.9	15.4–16.2	16.3–17.3	24.2–24.5	21.9–22.1	22.2–22.5	22.9–23.2	22.3 22.6
Zonorchis sp.	1.06	0	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Zonorchis delectans	0.98	0.71	0	–	–	–	–	–	–	–	–	–	–	–	–	–
Zonorchis alveyi	0.71	1.06	0.71	0	–	–	–	–	–	–	–	–	–	–	–	–
Lyperosomum sarothrurae	2.0	2.4	2.3	2.1	0	–	–	–	–	–	–	–	–	–	–	–
Lyperosomum transcarpathicus	5.8	6.0	6.0	5.8	5.2	0	–	–	–	–	–	–	–	–	–	–
Lyperosomum intermedium	6.1	6.1	6.1	5.9	5.3	5.6	0	–	–	–	–	–	–	–	–	–
Skrjabinus kalmikensis	2.5	2.7	2.7	2.5	2.1	5.7	5.0	0	15.4–16.5	15.7–16.2	13.4–13.8	24.2	22.6	22.2	23.7	19.7
Lyperosomum petiolatum	2.4–2.6	2.8–3.1	2.7–3.1	2.5–3.0	2.2–2.7	4.7–5.7	5.2–5.9	1.8–2.1	0–0.1 / 0–1.5	5.9–8	10.7–13.8	22.2–23.7	21.3–21.8	23.2–24.7	20.7–21.8	18.3–18.9
Lyperosomum turdia	2.4	2.8	2.7	2.5	2.2	5.2	5.4	1.6	0.41–0.56	0 / 0–1.3	10.8–13.3	21.6–21.8	21.3–22.1	24.4	22.4–23.2	16.3–17.0
Lyperosomum clathratum	3.2–3.3	3.3–3.6	3.3–3.5	2.9–3.3	2.9–3.0	4.9–5.2	5.1–5.3	1.7–1.9	1.2–1.5	1.2–1.3	0 / 0–0.32	20.9–21.8	20.3–20.5	23.2–23.6	23.2–23.9	18.7 –19.0
Dicrocoelium dentriticum	6.7	6.9	7.0	6.7	6.0	6.6	5.9	5.9	5.5–6.6	5.8	5.7 –5.9	0	14.6	20.2	17.06	19.3
Brachydistomum ventricosum	7.2	7.6	7.5	7.1	6.5	7.1	6.5	6.2	6.3–7.4	6.6	6.5–6.8	1.9	0	19.2	17.3	18.0
Lutztrema attenuatum	8.2	8.5	8.5	8.2	7.8	7.5	7.4	7.1	7.5–9.0	7.6	7.1–7.4	4.9	4.6	0	16.6	22.3
Brachylecithum lobatum	8.6	8.9	8.9	8.5	8.0	8.4	7.6	7.7	7.8–9.4	8.0	7.7–7.8	4.5	4.6	3.7	0	20.9
Stromitrema koshewnikowi	8.5	8.6	8.6	8.3	7.8	8.7	8.2	7.3	7.7–9.2	7.5	7.3–7.6	6.6	6.6	6.7	7.2	0

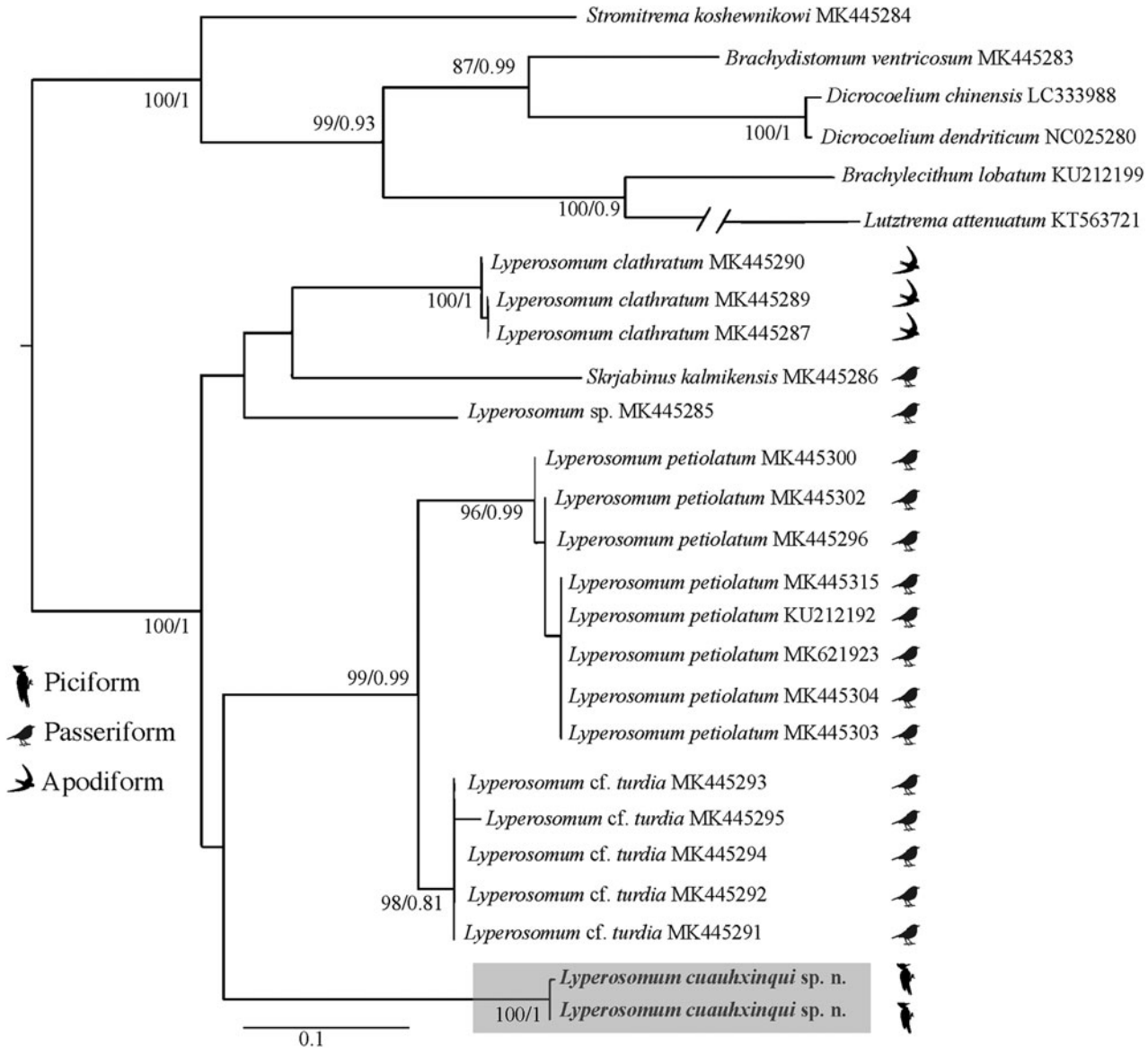

Fig. 4. Maximum likelihood (ML) tree and consensus Bayesian inference (BI) tree inferred from the *cox 1* of the mitochondrial DNA. Numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

Discussion

The genus *Lyperosomum* was erected to include four species, with *Lyperosomum longicaudata* (Rudolphi, 1809) as the type species. The species composition of the genus has been unsettled ever since, and, prior to the present study, it contained 33 valid species (see Hildebrand *et al.*, 2015, 2019). Recently, Hildebrand *et al.* (2019) performed a comprehensive study that included representative samples of *Lyperosomum* from the Americas and other regions of Europe and Africa; the authors of that study recognized several species on the basis of *cox 1* and LSU sequences, which represents the starting point for further studies within the genus *Lyperosomum*. Therefore, the results obtained in the current study add new evidence that allows us to better understand the taxonomy and systematics of the genus *Lyperosomum*. Our phylogenetic trees inferred with the LSU dataset placed *L. cuauhxinqui* sp. n. in a subclade together with two other species of the genus *Zonorchis* (*Z. alveyi* (MK480327), *Z. delectans* (MK480329) and *Zonorchis* sp. (MK480328)) from the Americas (see fig. 3). The phylogenetic trees exhibited the same branching order as the phylogenetic tree inferred previously by Hildebrand *et al.* (2019), with the exception of the branches belonging to the new species. The genus *Zonorchis* includes species from the Americas and differs morphologically from *Lyperosomum* in the position of the testes (oblique in *Lyperosomum* and symmetrical in *Zonorchis*). However, the inconsistency in the position of the testes has been the major reason for the taxonomic confusion between the species of the genera *Lyperosomum* and *Zonorchis* (see Denton & Byrd, 1951; Odening, 1964; Hildebrand *et al.*, 2019). For example, specimens of *L. clathratum* and *L. petiolatum* and the new species exhibit symmetrical testes, and based on this morphological trait, the three species should be placed in the genus *Zonorchis*. However, in the phylogenetic trees inferred with the LSU dataset, the species *L. clathratum*, *L. petiolatum*

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and *L. cuauhxinqui* sp. n. were nested within the genus *Lyperosomum*. Therefore, the position of the testes alone is not a suitable character for distinguishing between the two genera (see Hildebrand *et al*., 2019).

The four species of *Lyperosomum* found in the Americas associated with birds exhibit a wide spectrum of definitive hosts: *L. petiolatum*, *L. oswaldoi* and *L. byrdi* are associated with passerines of the families Corvidae, Mimidae and Passerellidae, respectively, whereas *L. cuauhxinqui* sp. n. is associated with the golden-fronted woodpecker (Picidae) distributed from Texas, USA, to Guatemala in Central America (Howell & Webb, 1995). The genetic divergence estimated among the congeneric species of *Lyperosomum* ranged from 2 to 6% for LSU. The genetic divergence between *L. cuauhxinqui* sp. n. and its sister taxon species, such as *Z. alveyi* (MK480327), *Z. delectans* (MK480329) and *Zonorchis* sp. (MK480328), ranged from 0.7 to 1.6%; between two recognized species of *Lyperosomum*, *L. turdia* and *L. petiolatum*, the genetic divergence ranged from 0.4 to 0.56%; and between *L. intermedium* and *L. transcarpathicus*, it was 5.6%. These ranges of genetic divergence for LSU are wider than those previously described for congeneric species of *Brachylecitum*, ranging from 0.1 to 0.7% (see Hildebrand *et al*., 2016). With respect to the *cox 1* gene, the genetic divergence estimated among the five congeneric species of *Lyperosomum* ranged from 5.9 to 17.3%. The genetic divergence among *L. cuauhxinqui* sp. n. and its sister taxon species, such as *L. turdia* and *L. petiolatum*, ranged from 13.4 to 16.2%. The high level of genetic divergence among these species in the *cox 1* gene confirms that the *cox 1* gene evolves faster than LSU and that it is highly informative for distinguishing closely related species of digeneans.

Our phylogenetic trees inferred with LSU and *cox 1* agree with the phylogenies previously inferred by Hildebrand *et al.* (2019). The sequences identified as *Z. alveyi*, *Z. delectans*, *Zonorchis* sp. and *S. kalmikensus* were nested within *Lyperosomum*; therefore, these sequences should be considered to belong the genus *Lyperosomum*. It is imperative to review the taxonomy of the genus *Lyperosomum* by using a combination of ecological, morphological and molecular characteristics, as suggested previously by Hildebrand *et al.* (2019), with the aim of building a more robust classification of the genus that allows us to better understand the evolution of this group of digeneans.

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