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A new genus and species of Microphalloidea (Digenea), parasite of *Peropteryx* spp. (Chiroptera: Emballonuridae) from the Neotropical region of Mexico revealed by morphological and phylogenetic analyses

N.G. Ruiz-Torres[1,2,3], D.I. Hernandez-Mena[4], L. García-Prieto[4], L.I. Falcón[5], J.A. Panti-May[6] and O. Rico-Chávez[3]

[1]Posgrado en Ciencias Biológicas, Ciudad Universitaria, Universidad Nacional Autónoma de México 04510, Mexico City, Mexico.; [2]Facultad de Ciencias, Universidad Nacional Autónoma de México 04510, Mexico City, Mexico; [3]Laboratorio de Ecología de Enfermedades y Una Salud, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México 04510, Mexico City, Mexico; [4]Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria 04510, Mexico City, Mexico; [5]Instituto de Ecología, Unidad Mérida, Universidad, Nacional Autónoma de México 97357, Ucú, Yucatán, Mexico and [6]Centro de Investigaciones Regionales 'Dr. Hideyo Noguchi', Universidad Autónoma de Yucatán, Av. Itzáes, Centro, 97000, Mérida, Yucatán, Mexico.

Research Paper

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Corresponding author:
O. Rico-Chávez;
Email: orich@unam.mx

Abstract

The present study aims to describe a new genus and species of microphalloid digenean parasite of two species of bats of the genus *Peropteryx* from the Mexican Neotropics (in the states of Chiapas and Yucatán). Morphological and molecular data (28s rDNA ribosomal gene sequences) were used to study Digeneans. *Sagittatrema zutzi* gen. nov. sp., nov., is diagnosed morphologically by having a sagittiform body, a genital pore in the midline of the body, posterior to the ventral sucker, and a cirrus sac running through much of the diameter of the ventral sucker. The nine sequences generated from the 28S rDNA gene were used to examine the phylogenetic affinities of this new taxon within the superfamily Microphalloidea Ward, 1901, using Maximum Likelihood and Bayesian Inference analyses. Both analyses resulted in trees with similar topologies and formed a well-supported clade (Bt = 100; pp = 1) with the *Sagittatrema* sequences. Because of the new genus's phylogenetic position and that some sister families to the proposed taxa, like Pleurogenidae and Prosthogonimidae, are polyphyletic, we prefer to consider *Sagittatrema* as a genus *incertae sedis* within Microphalloidea. A full systematic review of microphalloids is needed to confirm their phylogenetic position.

Introduction

Bats (Mammalia: Chiroptera) are the second-largest group of mammals on earth (surpassed only by Rodentia), with approximately 1460 species recorded worldwide (Burgin *et al.*, 2018). One of the areas with the greatest diversity of bats is the Neotropical Region; particularly for Mexico, bats constitute about 15% of the total mammals distributed in the country (Ceballos *et al.*, 2005).

This group is an excellent environment to study symbiotic (host-parasite) interactions because they host many distinct kinds of eukaryotic macrosymbionts with parasitic strategies, such as helminths (Dick & Patterson, 2006; Morand et al., 2007; Dittmar *et al.*, 2015; Jiménez *et al.*, 2017). The helminths linked to this group have been the subject of irregular, local, and asymmetrical research from a helminthological perspective for Mexico and Latin America (Caspeta *et al.*, 2017).

According to data from the Colección Nacional de Helmintos (UNAM, 2024, Data not published), there are 58 species of helminths in Mexico. These comprise 26 digeneans, five cestodes, and 27 nematodes. These numbers come from just 29 of the country's 140 species of bats that have been recorded. From this perspective, the group's knowledge has advanced by only 20.71%. Seven of the eight families of bats distributed in Mexico have reported helminth species; the most represented is Phyllostomidae, with 11 species examined, and the least studied is Emballonuridae, with only one species. Because of this difference, more in-depth studies are needed to finish the helminthological record of bats, especially in places with a great deal endemism and/or human-caused disturbance (Jiménez *et al.*, 2017). As part of a larger project to analyze the relationship between helminth richness and microbial composition in Neotropical bats, we collected a species of digenean belonging to the superfamily Microphalloidea. The aim of the present work is to describe a new genus and species of this superfamily, a parasite of two species of chiropterans of the genus *Peropteryx* (Emballonuridae) from southeast Mexico, and to determine its phylogenetic position within this superfamily based on morphological and molecular characteristics.

Material and Methods

Collection of hosts and helminths

During December 2021, three males and three females of *Peropteryx kappleri* Peters, 1867 collected in Chajul, Chiapas, and one male specimen of the Lesser dog-like bat *Peropteryx macrotis* Wagner, 1863, collected in August 2022, from Grutas las Sartenejas, Tekax, Yucatán, were examined for trematodes (Figure 1). The sampling followed the protocols established by the American Society of Mammalogists (Sikes *et al.*, 2019) and the Food and Agriculture Organization (El Masry *et al.*, 2020), under the scientific collector's permit DGVS/04214 /19 issued to ORC by the Secretaría del Medio Ambiente y Recursos Naturales de México. Each bat was kept individually in cotton bags and transferred to the laboratory. Its taxonomic identification was made with the help of a field guide (Medellín *et al.*, 2008). The hosts were euthanized via inhaled isoflurane overdose, and an examination was performed on all internal organs to detect trematodes *in situ.* For morphological analysis, worms were fixed in hot 4% formalin and stored in 70% ethanol; for molecular analysis, they were preserved in 96% ethanol. Infection parameters follow Bush *et al.* (1997).

Morphological analysis

For permanent mounts, the material was stained with Mayer's paracarmine and mounted in Canada balsam. All measurements are given in micrometres (μm) unless stated otherwise. They are shown as the mean followed by the standard deviation (± SD), and the range and number of specimens measured (n) in parentheses. The drawings were made with the aid of a drawing tube. The individuals studied under scanning electron microscopy (SEM) were dehydrated in alcohol graduated to 100% ethanol. Subsequently, they were critically dried with CO_2 and mounted to be coated with a mixture of gold and palladium. The observation of samples was made under a Hitachi S2460N microscope in the Laboratory of Microscopy and Photography of Biodiversity I, of the Institute of Biology, Universidad Nacional Autónoma de México (IBUNAM), Mexico City. Type specimens were deposited in the Colección Nacional de Helmintos, Instituto de Biología, IBUNAM.

DNA extraction and 28S rRNA gene amplification and sequencing

Total genomic DNA from eight adult worms was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) modifying slightly the manufacturer's protocol: To concentrate DNA, this was eluted with 300 μL of AE buffer and precipitated with absolute ethanol, 0.1 volumes of 3M sodium acetate, and 2 μL of glycoblue. DNA was re-suspended in 30 μL of molecular grade water and stored at –20 °C until PCR amplification (Gaona *et al.*, 2020). An approximately 1467-bp-long region at the 5′ end of the nuclear of the 28S ribosomal RNA gene region (D1-D3) was amplified using primers forward 391 5′ AGCGGAGGAAAAAGAAACTAA-3′ (Nadler & Hudspeth, 1998) and reverse 536 5′-CAGCTATCCT GAGGGAAAC-3′ (Stock *et al.*, 2001). Each PCR reaction was made using DNA MyTaq Mix polymerase (Bioline); annealing temperature during the thermal cycling was 50 °C. PCR products were purified with ExoSAP-IT (Applied Biosystems, Inc.), following the manufacturer's instructions. Sequencing reactions were performed with the two amplification primers and the internal primers 503 and 504 (see in Mendoza-Garfías *et al.*, 2022) using BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Inc.),

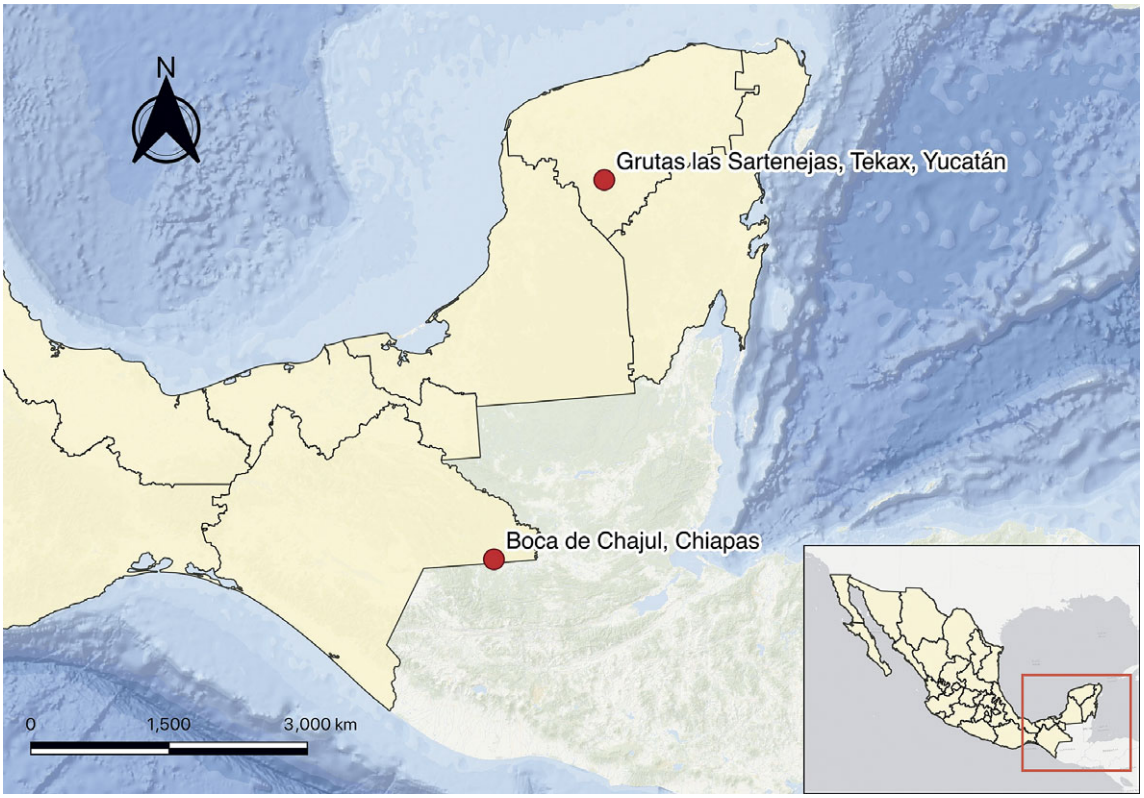

Figure 1. Map of Mexico showing the sampled sites for chiropterans.

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and products were purified by filtering through hydrated Sephadex (GE Healthcare) columns. DNA Sequencer ABI PRISM 3730 (Applied Biosystems, Carlsbad, CA) was used to generate DNA sequences at the Laboratorio de Secuenciación Genómica de la Biodiversidad, IBUNAM. The consensus DNA sequences for each specimen were assembled using Geneious Pro 4.8.4 software (Biomatters; http://www.geneious.com/). DNA sequences were subjected to a BLAST search using the NCBI website (http://www.ncbi.nlm.nih.gov) to discard possible contamination or amplification of host DNA. All DNA sequences generated in the present study were submitted to GenBank under accession numbers: PQ069219-PQ069227.

DNA sequence alignment and phylogenetic analyses

A data set for the 28S rRNA gene was built in Mesquite 3.62 (http://www.mesquiteproject.org/), with the new sequences generated in this study and deposited in Genbank. *Macroderoides spiniferus* (EU850400) was used to give a root to the trees obtained from the Microphalloidea superfamily. DNA sequences were aligned using MAFFT (Katoh *et al*., 2018) through the web site https://mafft.cbrc.jp/alignment/server/index.html with the default settings for opening and gap extension. The substitution models and parameters were selected in jModelTest v.2.1.6 (Posada, 2008), based on the Akaike Information Criterion (Akaike, 1973). Phylogenetic inference was performed with the data set of the 28S rRNA gene, following two different approaches. The tree obtained from Maximum Likelihood (ML) was implemented using RAxMLGUI v 1.3 (Stamatakis, 2006); to obtain node support, a bootstrap analysis (Bt) was performed with 1,000 replicates using the GTR+I+G nucleotide evolution model. Bayesian inference (BI) was performed in Mr. Bayes 3.2. (Huelsenbeck & Ronquist, 2001) using four independent Markov chain Monte Carlo runs, each comprising four chains (one cold, three heated), and a random starting tree; each chain was run for 20,000,000 generations, with 10% discarded as burn-in. Trees were sampled every 1,000 generations. After the burn-in, the remaining samples from the four runs were combined to estimate the topology, branch lengths, and posterior probabilities (PP). Finally, convergence of the Markov chain Monte Carlo runs was assessed by visual inspection of trace plots for all parameters using Tracer v. 1.7 (Rambaut *et al*., 2018). Both RaxML and MrBayes were used online on the CIPRES Science Gateway (Miller *et al*., 2010). The phylogenetic trees obtained from both analyses were visualized in FigTree v. 1.4.2.

Results

Morphological descriptions

Superfamily: Microphalloidea Ward, 1901
Family: *Incertae sedis*
Genus: ***Sagittatrema* gen. nov.** (Figures 2, 3)

Diagnosis. Digenea, Microphalloidea. Body elongated, arrow-shaped forebody, narrowing posteriorly or to level of ventral sucker. Body completely covered by spines,. Oral sucker spherical and terminal, larger than ventral sucker. Preequatorial and intercecal ventral sucker. Prepharynx absent. Oval pharynx. Relatively short esophagus; cecal bifurcation anterior to vitelline follicles, cirrus sac, and ventral sucker. Intestinal caeca end at level of the posterior end of ovary. Testes symmetrical, entire, separated, postequatorial. Proximal part of cirrus-sac anterior; cirrus-sac oriented posteriorly from this point, very long, forming several loops, lateral to ventral sucker (dextral), containing elongate internal seminal vesicle, pars prostatic (bulb shaped), and wide muscular ejaculatory duct. Genital pore ventral, median, posterior to ventral sucker. Ovary lateral (sinistral), slightly posterior to ventral sucker. Ootype overlapping ovary. Seminal receptacle lateral (sinistral), pre-ovarian, slightly anterior to ventral sucker. Laurer's canal not observed. Mehlis' gland posterior to ventral sucker. Vitelline follicles confluent in forebody, beginning posteriorly to intestinal bifurcation, extending at anterior end of ovary. Vitelline reservoir ventral to Mehlis' gland frequently posterior to ventral sucker. Uterus coiled, occupying most of hind-body; metraterm not observed. Eggs operculated, not embryonated. Excretory vesicle I-shaped, reaching posterior margin of posterior testis. Excretory pore terminal. Parasites of bats in Neotropical region.

***Type and single species*: *Sagittatrema zutzi* n. sp.**

Etymology: The generic name comes from the characteristic arrow-shaped morphology of the body of this digenea.

Remarks: The new genus clearly pertains to Microphalloidea following Bray (2008). However, it cannot be included in one of the 17 families contained in this superfamily by the position of genital pore in hindbody, in the midline of body, posterior to ventral sucker. *Collyryclum*, another microphallid genus also has a genital pore posterior to the ventral sucker; notwithstanding, their genital ducts open on a small nipple. In addition, the sagittiform body of the new genus is an exclusive trait within the superfamily. Furthermore, it can be distinguished from the members of the seven phylogenetically closest families (Figure 4) by the following morphological features: (1) distribution of the vitelline glands: in the genera of Collyriclidae exceed the cecal bifurcation and those of Prosthogonimidae are arranged in lateral fields not confluent, that reach the posterior end, whereas in *Sagittatrema*, they constitute a single group in the anterior part of the body, confluent at the level of the cecal bifurcation. (2) The extension of the ceca: genera of Colyrichlidae and Stomylotrematidae, they reach the posterior end of the body; in the members of Microphallidae and Lecithodendriidae, they generally do not extend beyond the ventral sucker and in the genera of the families Phaneropsolidae, Pleurogenidae, and Prosthogonimidae, their extent is variable compared to those presented by *Sagittatrema*, where ceca reach into the hindbody but not as far testes. Finally, (3) by the site of infection: *Sagittatrema* parasitizes the gallbladder and intestine, whereas the genera of five of the seven families parasitize only the intestine (Microphallidae, Phaneropsolidae, Lecithodendriidae, Prosthogonimidae, and Stomylotrematidae) or are encyst in the intestinal wall (Collyriclidae) or in the hepatic ducts (Pleurogenidae) (Table 1).

***Sagittatrema zutzi* sp. nov.** (Figures 2, 3,4)

Description based on 11 specimens mounted in Canada balsam and 2 studied under SEM. Body large, arrow-shaped forebody, narrowing posteriorly at level of ventral sucker, 1.49 mm ± 0.40 (1.07–2.12 mm; n = 11) long, 0.61 ± 0.14 mm (0.40–0.85 mm; n = 11) maximum width. Body completely covered by spines. Length of spines at anterior end of body 8.75 ± 0.89 (5.5–10; n = 20); at midbody 9.43 ± 1.11 (7.36–11.6; n = 20); at posterior end 8.77 ± 0.98 (7.05–10.3; n = 17). Oral sucker terminal, muscular, oval, 0.16 ± 0.03 (0.11–0.21, n = 11) long by 0.15 ± 0.02 (0.11–0.18; n = 11) wide. Ventral sucker muscular, rounded 0.13 ± 0.02 (0.09–0.17; n = 11) long, 0.14 ± 0.02 (0.10–0.18; n = 11) wide, at midline of body, preequatorial. Ratio oral sucker length/ventral sucker length 1:1.18 (1.06-1.27; n = 8). Prepharynx absent. Pharynx muscular, oval-shaped, 0.06 ± 0.007 (0.04–0.07; n = 11) long, 0.9 ± 0.006 (0.05–

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Table 1. Main morphological characters of families of Microphalloidea* included in the phylogenetic analyses; traits of the new genus are highlighted

Family	Body	Spines	Ventral sucker	Vitelline glands	Genital pore	Caeca	Site of infection	Host
Collyriclidae	Small; spheric and convex	All body covered	Pre-equatorial	Overpass cecal bifurcation	Midline and equatorial; open in a nipple-like structure	Reach posterior end of body	Intestine wall	Encysted in birds and mammals
Microphallidae	Small, slender, or pyriform	All body covered; squamous	Post-equatorial	Most post-cecal that pre-cecal	Sinistral or dextral to ventral sucker	Generally, not posterior to ventral sucker	Intestine	Vertebrates
Phaneropsolidae	Small and ovoid	Spinose	Pre o post-equatorial	Hindbody, rarely forebody	Sublateral, at ventral sucker level	Short or large	Intestine	Mammals, birds and rarely reptiles
Pleurogenidae	Small-medium; pyriform to elongated	All body covered; spines well developed	Pre-equatorial or equatorial	Variable in extend	Marginal, lateral or sublateral	Short, medium, or long	Intestine or encysted in hepatic system; oral cavity	Amphibian, rarely reptiles
Lecithodendriidae	Small and ovoid	Typical, with spines	Pre-equatorial	Anterior o at mid-body	Midline in forebody	Short, anterior to mid-body	Intestine	Bats; occasionally Birds
Prosthogonimidae	Small to median, rounded posteriorly	Typical, with spines	Pre-equatorial	Lateral bunches reaching posterior end	Anterior end of body	Usually not reach posterior end	Intestine or body cavity in mammals or bursa Fabrici, oviduct or cloaca in birds	Mammals and Birds
Stomylotrematidae	Small to medium, broadly oval	Spinous or smooth	Pre-equatorial or equatorial	Fore and hidbody	Marginal, at level of ventral sucker	Reaching posterior end	Digestive tract	Birds
Sagittatrema	**Large, arrow-shaped**	**All body covered; spines well developed**	**Pre-equatorial**	**Forebody**	**Midline, posterior to ventral sucker**	**Pretesticular in forebody**	**Gall bladder**	**Bats**

*Based on Bray *et al.* (2008).

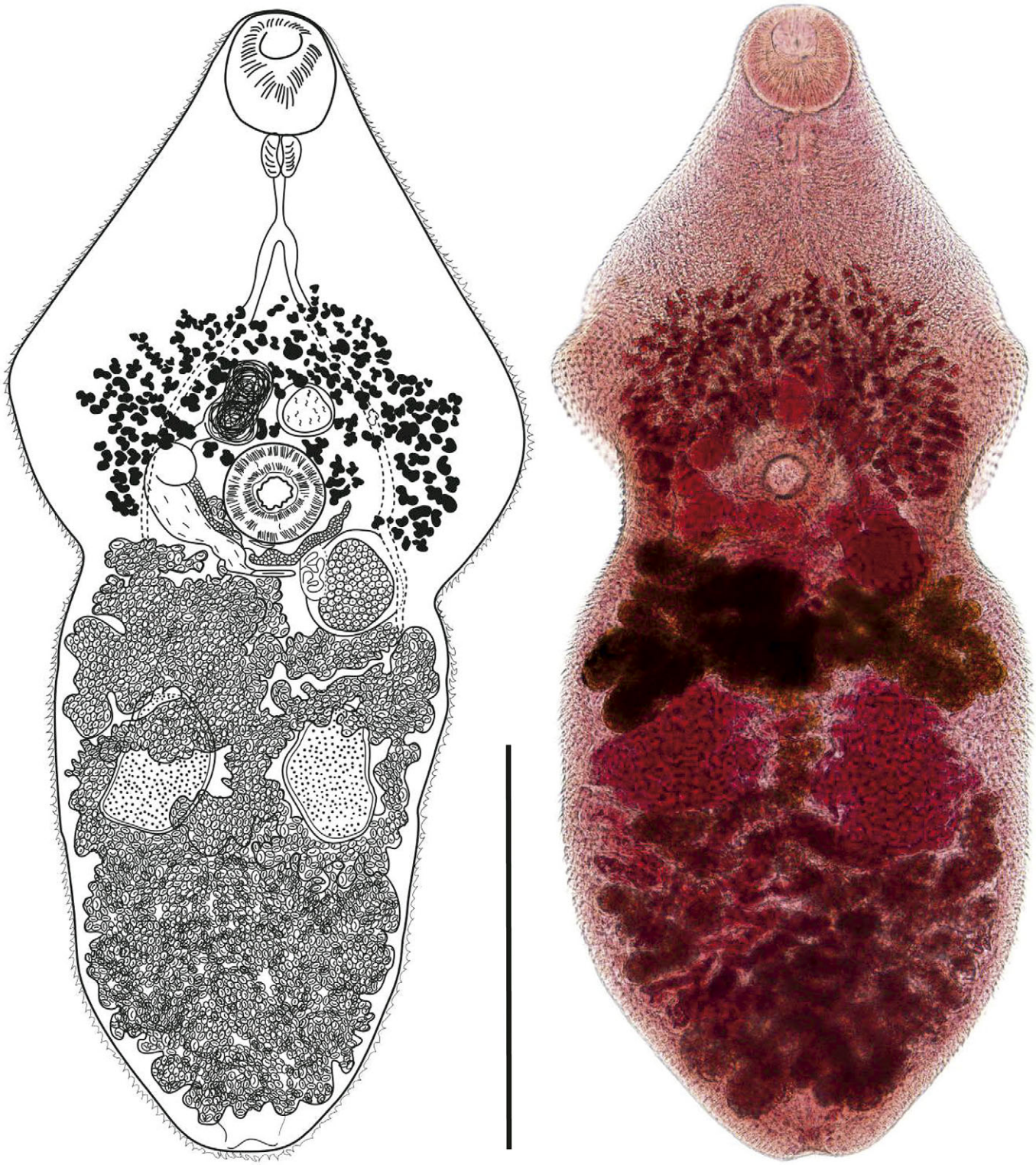

Figure 2. *Sagittatrema zutzi* n. sp. (entire adult worm, ventral view) from *Peropteryx kappleri.* a) line drawings, ventral view, and b) optical microscopy photograph. Scale bars: a) and b) 500 μm.

0.07, n = 11) wide. Esophagus 0.085 ± 0.27 (0.055–0.12; n = 5) long. Intestine bifurcates anterior to vitelline follicles. Intestinal caeca thin, ending between ovary and testes. Testes two, oblong, symmetrical, separated, at beginning of last third of body: right testis 0.23 ± 0.05 (0.18–0.33, n = 9) long, 0.16 ± 0.03 (0.13–0.21, n = 9) wide; left testis 0.22 ± 0.03 (0.18–0.29, n = 9) long, 0.17 ± 0.04 (0.09–0.23, n = 9) wide. Cirrus sac oriented longitudinally, very long, forming several loops, passes dextrally to ventral sucker, containing elongate internal seminal vesicle, bulb-shaped pars prostatica, and wide muscular ejaculatory duct. Genital pore opening on ventral surface,

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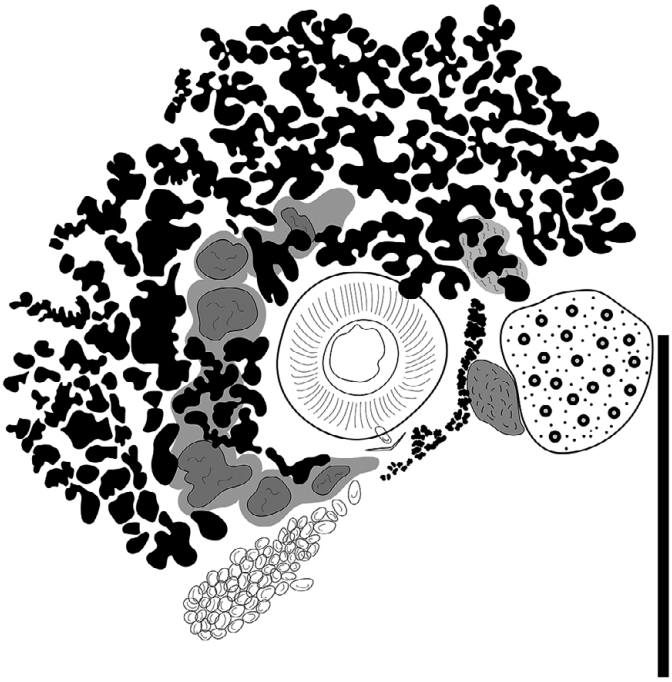

Figure 3. Detail of male and female reproductive systems. Scale bar: 8 μm.

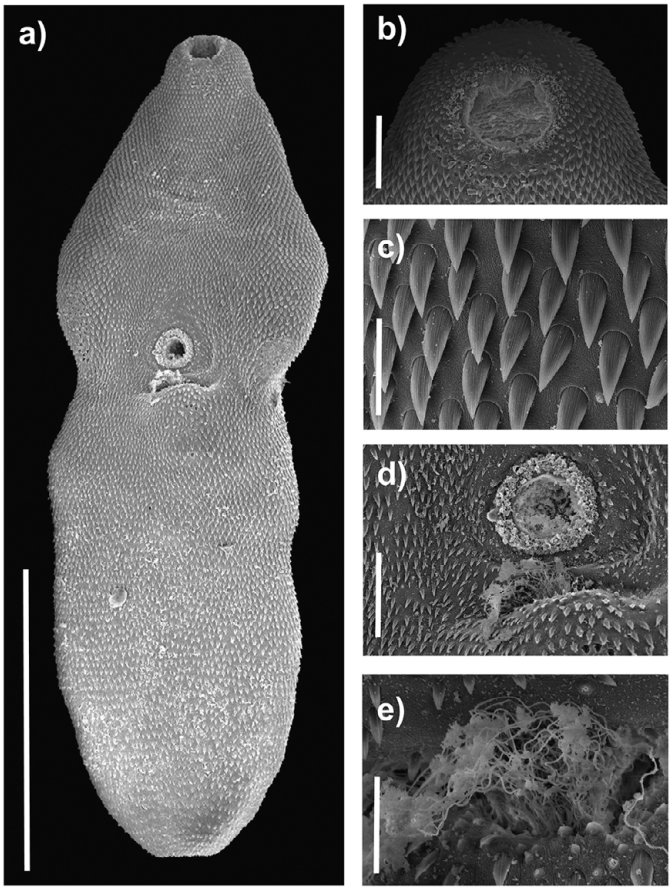

Figure 4. Scanning electron micrographs of *Sagittatrema zutzi* n. sp., from *Peropteryx kappleri*. a) adult, ventral view, b) anterior region, oral sucker, c) spines, equatorial region, d) ventral sucker, and e) excretory pore. Scale bars: a) 500 μm b) 50 μm; c) 20 μm; d) 50 μm, and e) 20 μm.

posterior to ventral sucker, on median line of body. Ovary ellipsoidal, posterior to ventral sucker, pre-testicular, 0.14 ± 0.03 (0.10–0.17, n = 9) long, 0.10 ± 0.02 (0.07–0.14, n = 9) wide. Ootype cylindrical, overlapping ovary dextrally, 0.082 ± 0.002 (0.0115–0.055, n = 6) long, 0.041 ± 0.010 (0.024–0.052, n = 6). Seminal receptacle sinistral-lateral, pre-ovarian, slightly anterior to ventral sucker. Laurer's canal not observed. Mehlis' gland posterior to ventral sucker. Vitelline follicles confluent posteriorly to intestinal bifurcation, extending to level of posterior margin of ventral sucker. Vitelline reservoir ventral to Mehlis' gland, surrounding posterior area of ventral sucker. Uterus long, occupying almost all hindbody. Eggs ovate, numerous, operculated, not embryonated, 0.024 ± 0.011 (0.019–0.055, n = 100) long, 0.0110 ± 0.0007 (0.009–0.0119, n = 100) wide. Excretory vesicle I-shaped.

ZooBank Life Science Identifier: urn:lsid:zoobank.org:act:DBCDAC35-B1A0-4F83-B082-E4312E591693

Taxonomic summary

Type Host: Peropteryx kappleri Peters, 1867, Greater dog-like bat (Chiroptera: Emballonuridae).

Site of infection: Gallbladder and intestine.
Type locality: Boca de Chajul (16°6'58" N; 90°55'25"W), Chiapas, Mexico.
Other host: *Peropteryx macrotis* Wagner, 1863, lesser dog-like bat (Chiroptera: Emballonuridae).
Other locality: Grutas las Sartenejas, Tekax, Yucatán (20°12'8.3" N; 89° 17'16.1"W).
Infection parameters: Chiapas: prevalence 4/6 (66%); mean abundance 4.16; mean intensity 6.25; intensity range: 1-11. Yucatán: 1/1 (100%) and intensity 2/1(2).
Specimens deposited: Holotype (CNHE 12854); 10 paratypes from *P. kappleri* (CNHE 12855) and 1 paratype from *P. macrotis* (CNHE 12856).
Etymology: The species name refers to the host group of the new species. Zutzi means bat in Mayan, the language of the region where this species was found.

Remarks

Two genera belonging to different families within Microphalloidea are the most similar to the new genus described here: *Parabascus* (Pleurogenidae see Lotz & Font, 2008) by the distribution of vitelline follicles mainly in the hindbody (Kirillova *et al.*, 2022), and the monospecific genus *Collyriclum* (Collyriclidae) by the position of the genital pore almost in the mid-line of the body (Blair & Barton, 2008). *Sagittatrema sutzi* n. sp. can be differentiated from the only species of *Parabascus* distributed in Mexico (*Parabascus yucatanensis* [Stunkard, 1938] Odening, 1969]) because the ovary in *S. sutzi* n. sp. is dextral to the ventral sucker, whereas in *P. yucatanensis* it is sinistral to this structure. In addition, in the species of Stunkard, the intestinal caeca reach the posterior end of the testes, whereas in *S. sutzi* n. sp., they only overpass the ventral sucker. Other differences between *P. yucatanensis* and the new species are the oval-shaped body and the lateral genital pore with separated sexes (Stunkard, 1938), versus the body-arrow-shaped and common genital pore located in the mid-body line, respectively. On the other hand, *Collyriclum faba* (Bremser in Schmalz, 1831) Kossack, 1911, has separate genital pores, almost adjacent to one another on a small nipple (traits absent in the new species), sets of spaced spines, lacks a ventral sucker, and has clusters of non-confluent vitelline follicles, inhabiting subcutaneous cysts (Blair & Barton, 2008), whereas in *S. sutzi* n. sp., the body is covered completely with spines, has a

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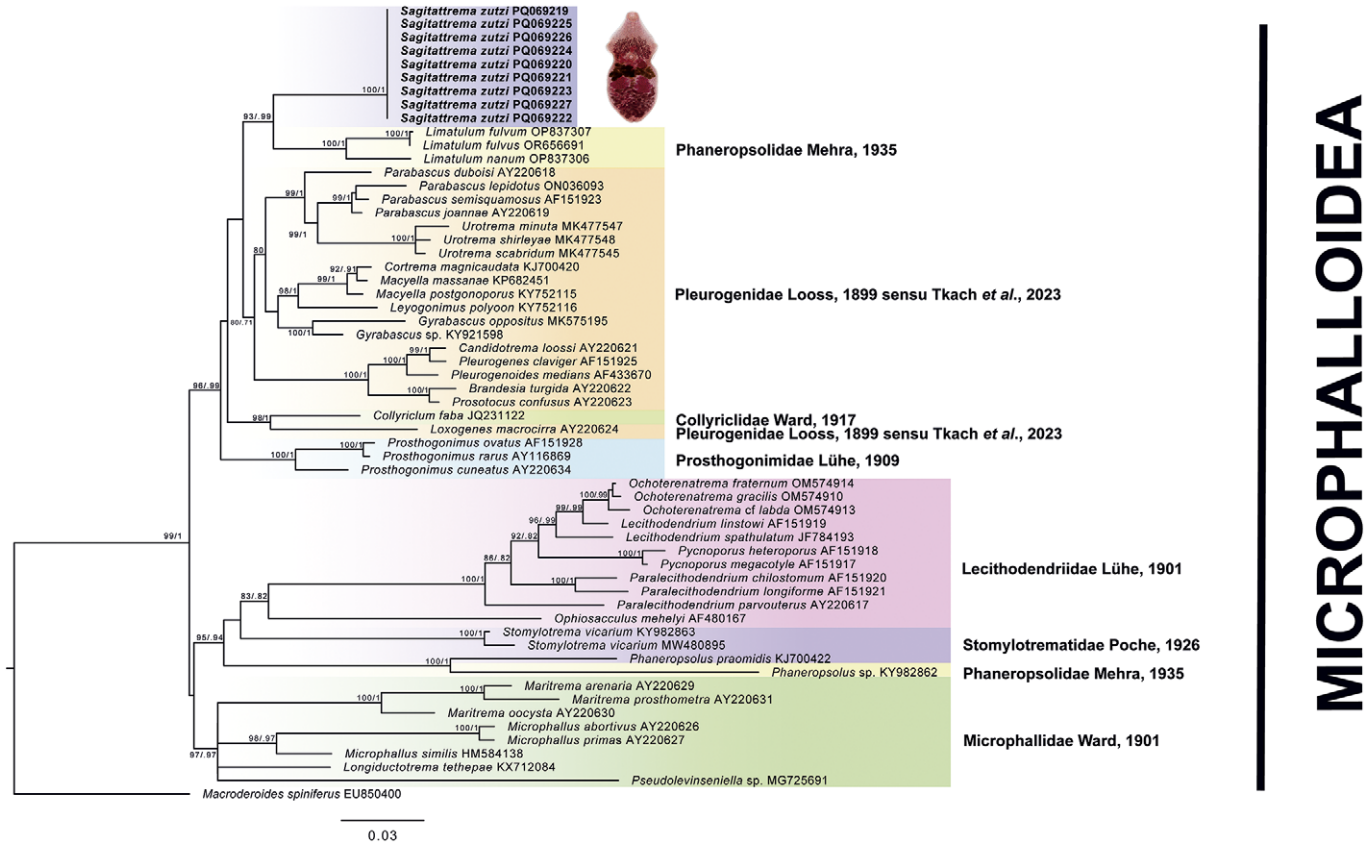

Figure 5. Phylogenetic hypothesis of the superfamily Microphalloidea. Trees inferred with Maximum Likelihood (ML) and consensus Bayesian Inference (BI), based on 28s rDNA gene sequences. Numbers near internal nodes show ML bootstrap percentage (BP) values and Bayesian posterior probabilities (BPP). The clade highlighted in bold indicates the position of the new genus and species studied in this work. Scale bars represent the branch length.

ventral sucker, its vitelline follicles are confluent, and it infects the gallbladder and intestine.

Phylogenetic analyses

The analysed data set, based on 61 taxa from seven families of Microphalloidea (including nine sequences of *Sagittatrema zutzi* n. sp.) had a length of 1325 pair of bases (bp). The nucleotide substitution model that best fit this set was GTR+GAMMA+I. The nucleotide frequencies were A = 0.21, C = 0.22, G = 0.32 and T = 0.24. The trees obtained from ML and BI practically had the same topology and showed the same phylogenetic relationships, although in the node support, the Bt values were generally lower than the posterior probabilities. The ML tree had a value of 12007.1461. Both phylogenies recovered *S. zutzi* as a monophyletic group, with Bt = 100; PP = 1, support values. The Microphalloidea families were distributed into seven clades (Figure 5). However, the specimens sequenced of this study nested within the clade formed by the families: Collyriclidae Ward, 1917, Phaneropsolidae Mehra, 1935, Prosthogonimidae Lühe, 1909 and Pleurogenidae Looss, 1899 (Bt = 96; PP = 0.99). It is important to emphasize that in this tree, both Pleurogenidae (*sensu* Tkach *et al.* 2023) and Phaneropsolidae are not monophyletic, because *Collyriclum* (Bremser in Schmalz, 1831) (Collyriclidae) was nested within Pleurogenidae, and *Phaneropsolus* Looss, 1899 and *Limatulum* Macy, 1931 (Phaneropsolidae) are in separated groups. More specifically, the new genus grouped with Prosthogonimidae and Collyriclidae + Pleurogenidae. The Pleurogenidae (*sensu* Tkach *et al.*, 2023) + Collyriclidae clade was the sister group to the *Sagittatrema* + *Limatulum* clade (Bt = 96; PP = 0.99). Finally, *Sagittatrema* and *Limatulum* clustered together with high bootstrap and posterior probability values (Bt = 93; PP = 0.99).

Discussion

Sagittarema sutzi n. sp., represents the first record for the *Peropteryx* bat genus in the Mexican territory; previously, only digenean flatworms of the genera *Castroia*, *Limatulum*, *Ochoterenatrema* and *Urotrema* had been recorded in Colombia and Brazil for these bats (Santos & Gibson, 2015). Morphologically, the new genus distinguishes itself from other members of the superfamily Microphalloidea with a sagittiform body, an excretory pore in the midline that does not protrude into a nipple and is posterior to the ventral sucker, and a very large cirrus sac that surrounds almost the entire ventral sucker. Additionally, the phylogenetic position of *Sagittatrema* using the 28s rRNA ribosomal gene places it in a monophyletic clade well supported in the two analyses performed (Bt =100; PP = 1). In our study, we obtained phylogenetic relationships within Microphalloidea consistent with previous proposals (Pérez-Ponce de León & Hernández-Mena, 2019; Tkach *et al.*, 2003; 2019; 2023). However, in this case of the new genus, although it is related to Pleurogenidae (sensu Tkach *et al.*, 2023), it is not clear that it is part of this family. Based on these results, we considered that *Sagittatrema* n. gen. is not part of Pleurogenidae for several reasons: (1) Pleurogenidae is not monophyletic because of the

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inclusion of *Collyriclum* in our analysis as a sister group to the pleurogenid *Loxogenes*, suggesting that the position of Collyriclidae and the relationships between the internal clades of Pleurogenidae are not yet resolved; this coincides with previous phylogenetic hypotheses (Shchenkov *et al*., 2020; Sokolov *et al*., 2020; Kirillova *et al*., 2022; Vlasenkov *et al*., 2023); however, we agree with Vlasenkov *et al*., (2023) who pointed out that elimination of Collyriclidae and the inclusion of *Collyriclum* in Pleurogeidade are not possible at the moment because of the lack of morphological evidence to support it; this is also due to the constant eliminations and inclusions of genera in this family and (2) the nesting of *Sagittatrema* n. gen., with *Limatulum* making Phaneropsolidae apparently paraphyletic, which is consistent with the previous phylogenetic analysis of Moguel-Chin *et al*., (2024). Furthermore, *Limatulum* is a genus whose morphology does not exhibit all the diagnostic characters of Pleurogenidae. According to a more detailed review, the clade comprising *Sagittatrema* + *Limatulum* suggests the eventual creation of a new family within Microphalloidea, a possibility currently under analysis. However, the establishment of a new family requires the acquisition of a larger number of additional morphological and molecular data, so in the present work we prefer to adopt a more conservative approach and maintain *Sagittatrema* as a genus *incertae sedis*.

As mentioned by Tkach *et al*., (2019), this publication, along with other recent ones that preceded it on the phylogenetic systematics of Microphalloidea, "it is yet another step toward the re-organization and improvement of the systematics of this highly diverse, derived lineage of digeneans".

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

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Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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