

Research Paper

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Gathering morphological and molecular data for identifying two species of trematodes from the neotropical Vaillant's frog, *Lithobates vaillanti* (Amphibia: Ranidae) in the highlands of Chiapas, south-eastern Mexico

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Abstract

Mexico possesses a large diversity of amphibians partly due to its complex topography and transitional position between the Nearctic and Neotropical biogeographical regions. However, its helminth parasite fauna has been relatively poorly studied. Specimens of the Vaillant's frog, *Lithobates vaillanti* (Brocchi) were sampled in the tropical rain forest of Nahá, in the Chiapas Highlands, and examined for parasites. Two trematode species were collected from their hosts; morphologically, specimens were allocated to the genera *Langeronia* Caballero and Bravo-Hollis, 1949 and *Haematoloechus* Looss, 1899, respectively. Individuals were sequenced for two molecular markers (the mitochondrial cytochrome *c* oxidase gene, and the ribosomal gene 28S), and processed for morphological analyses, including scanning electron microscopy. The new evidence was not enough to accomplish the identification at species level of *Langeronia* sp. due to the lack of sequence data from the type localities of *Langeronia parva* Christian, 1970 and *Langeronia macrocirra* Caballero and Bravo-Hollis, 1949. Likewise, the newly generated data were useful to properly identify the adult specimens of lung flukes as *Haematoloechus complexus* Seely, 1906.

Introduction

Mexico possesses a large diversity of amphibians partly due to its transitional position between the Nearctic and Neotropical biogeographical regions, where the complex topography of the territory has promoted numerous events of isolation and speciation on the different mountain systems; this has resulted in a highly diverse fauna of amphibians, and other groups occurring within its territory. Mexico contains more than 420 amphibians' species (Frost, 2021) of which 61% are endemic. Mexico holds the seventh place in amphibians' world biodiversity (Quintero-Vallejo & Ochoa-Ochoa, 2022). Irrespective of their high diversity, amphibians are one of the less studied vertebrates in Mexico from the parasitological perspective. According to Cabrera-Guzmán *et al.* (2021), about 139 nominal species of parasites have been reported parasitizing 66 amphibian species in Mexico; of these, 68 species are Platyhelminthes. These species are unevenly distributed among biogeographical provinces which may be an artefact of sampling effort bias, although Cabrera-Guzmán *et al.* (2021) demonstrated a significant correlation between helminth and host richness by biogeographical province. One of them is the Chiapas Highlands biogeographical province in southern Mexico.

During a survey of the amphibians distributed across the Selva Lacandona, in Chiapas, southern Mexico, specimens of the Vaillant's frog, *Lithobates vaillanti* were sampled in the vicinities of Nahá and examined for parasites. The area of Nahá encompasses several vegetation types such as tropical rain forest, montane cloud forests and pine-oak forest (Ochoa-Ochoa & Whittaker, 2014). The Vaillant's frog is a Neotropical species distributed from Veracruz, Mexico southwards to Colombia, along the Gulf of Mexico and Caribbean slopes, and from Oaxaca, Mexico southwards to Ecuador, along the Pacific Ocean slope (AmphibiaWeb, 2022)

The examination of the frog specimens revealed the presence of two species of trematodes parasitizing either the intestine or the lungs of their hosts. These specimens were morphologically determined as belonging to the genera *Haematoloechus* and *Langeronia*, respectively. In Mexico, two species of the genus *Langeronia* have been previously recorded, namely

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Langeronia macrocirra Caballero and Bravo-Hollis, 1949, and *Langeronia jimenezi* Iruegas-Buentello & Salinas-López, 1989. For the genus *Haematoloechus*, 16 species have been reported, 12 of which are endemic (Pérez-Ponce de León *et al.*, 2000; León-Règagnon, 2010; León-Règagnon & Topan, 2018; Velázquez-Urrieta *et al.*, 2019; Velázquez-Urrieta & Pérez-Ponce de León, 2022). Two of these have been recorded in Chiapas, *Haematoloechus floedae* Harwood, 1932 and *Haematoloechus* sp. parasitizing *Lithobates* cf. *forreri* Boulenger and *Lithobates brownorum* Sanders (Velázquez-Urrieta & León-Règagnon, 2018). The main objective of this study was to identify both species of trematodes recovered from the Vaillant's frog trematodes up to species level using molecular and morphological evidence (including observations through scanning electron microscopy (SEM)), and to discuss the finding in terms of host association and biogeography.

Materials and methods

Sample collection and morphological study

Specimens of the Vaillant's frog, *L. vaillanti* were sampled in Nahá, Chiapas (16° 58' 03" N, 91° 34' 35" W). Frogs were captured with dip nets and killed by an overdose of sodium pentobarbital in the brain; the helminthological examination was completed within 24 h of capture. Trematodes were collected either from the intestine or the lungs of their hosts and washed in saline solution (0.65%) for 4–5 min. Specimens for morphology were fixed in hot (near boiling) 4% formalin, and preserved in 70% ethanol; thereafter, individuals were stained with Gomori's trichrome, dehydrated through a graded ethanol series, cleared in methyl salicylate and mounted in Canada balsam between coverslips. For SEM, specimens were dehydrated through a graded ethanol series, critical point dried with K850 Critical Point Drier (Emitech, Ashford, England), sputter-coated with gold with Q150R Modular Coating System (Quorum, Ashford, England) and examined with a Hitachi SU1510 SEM (Hitachi, Tokyo, Japan). Voucher specimens were deposited in the Colección Nacional de Helmintos, (CNHE), Biology Institute, whereas hosts were deposited in the Museo de Zoología 'Alfonso L. Herrera', Facultad de Ciencias, both at the National Autonomous University of Mexico. Host species nomenclature follows 'Amphibian species of the world' (AmphibiaWeb, 2022). Additionally, specimens of *L. macrocirra* deposited at the CNHE (accession numbers: 1385, 4093, 4874–4877, 7276 – 7279, 1525–1527 and 3307) were examined for comparison.

Molecular analyses

For molecular analyses, specimens were preserved in 100% ethanol; total DNA was extracted from individuals following the DNAzol protocol (Molecular Center Inc., Cincinnati, Ohio). The primers used for amplification and sequencing were JB3 5'-TTT TTT GGG CAT CCT GAG GTT TAT-3'/JB4.5 5'-TAA AGA AAG AAC ATA ATG AAA ATG-3' (Bowles *et al.*, 1995) for the partial cytochrome *c* oxidase (COI) gene sequence, and 28sl 5'-AAC AGT GCG TGA AAC CGC CTC-3' (Palumbi, 1996)/LO 5'-GCT ATC CTG AGR GAA ACT TCG-3' (Tkach *et al.*, 2000) for the 28S rDNA fragment. The MyTaq DNA Polymerase (Bioline) was used for amplification of the two regions. The thermal cycling conditions used were the same for both primers: 95°C for 5 min, followed by 35 cycles of 92°C–95°C

for 30 s; annealing was of 50°C for 45 s, and extension at 72 °C for 1 min. Sequences were revised and assembled in Geneious 5.1.7 (Biomatters Ltd., Auckland, New Zealand). DNA sequences were aligned with sequences downloaded from GenBank, using Multiple Alignment using Fast Fourier Transform (Katoh *et al.*, 2005; Katoh & Standley, 2013) with default parameters. Alignments were trimmed to the shortest sequence. Phylogenetic analyses were built through Bayesian inference, using Markov chain Monte Carlo in MrBayes V 3.1.2 (Ronquist *et al.*, 2012). The appropriate model of evolution (GTR + 1 + 1) was determined by jModelTest 0.1.1 (Posada, 2008). The chains were run for 1,500,000 generations, sampling trees every 1,000 generations; the first 25% of the trees sampled were discarded according to Tracer V 1.5 (<http://beast.bio.ed.ac.uk/tracer>); consensus topology and posterior probability values were calculated from the remaining 75% of the trees. Genetic distance was estimated through uncorrected *p* distance using MEGA-X (Kumar *et al.*, 2018).

Results

Two species of trematodes were sampled. Specimens of *Langeronia* were obtained from the intestine of their hosts, and were not identified up to species level, whereas specimens of the lung fluke of the genus *Haematoloechus* corresponded with *Haematoloechus complexus*.

Langeronia sp. (fig. 1)

Morphological data

Morphologically, specimens collected from the intestine of the Vaillant's frog corresponded to *Langeronia*. They are characterized by having a relatively short but discernable pre-pharynx, a long oesophagus, vitellaria extending anteriorly to mid-level between pharynx and caecal bifurcation, testes oval to round, and a Y-shaped excretory bladder (fig. 1a).

Ultrastructure of the body surface through SEM

Specimens of *Langeronia* possess a tegument covered entirely with pectinate spines heterogeneously distributed along the body (fig. 1b, c). In the anterior and equatorial regions, spines are robust and abundant, with 10–16 distal projections; in the posterior region spines are small, with 3–6 distal projections, and less abundant. The tegument also possesses numerous papillae of variable form arranged randomly (fig. 1d, e). The oral sucker is rounded, not covered with spines, and possesses smooth edges; one pair of ciliate papillae, and one pair of simple papillae are localized on the outer surface of sucker (fig. 1f). The ventral sucker is pre-equatorial, rounded, and possesses nine simple papillae on the outer surface, and three pairs on the inner surface (fig. 1g). The genital pore is round, pre-acetabular, lack spines, and presents a muscular edge and radial corrugations.

Molecular data

The COI alignment including the new sequences and those available in GenBank, consisted of 21 sequences of 380 base pairs (bp) long for *Langeronia*. The phylogenetic tree revealed four reciprocally monophyletic and well-supported clades for species of *Langeronia* (fig. 2). The two newly sequenced individuals from Chiapas, identified morphologically as *Langeronia* sp., appeared as the sister clade of *L. macrocirra* from Veracruz. Both monophyletic groups were highly supported by posterior probability support values. The genetic divergence between specimens from

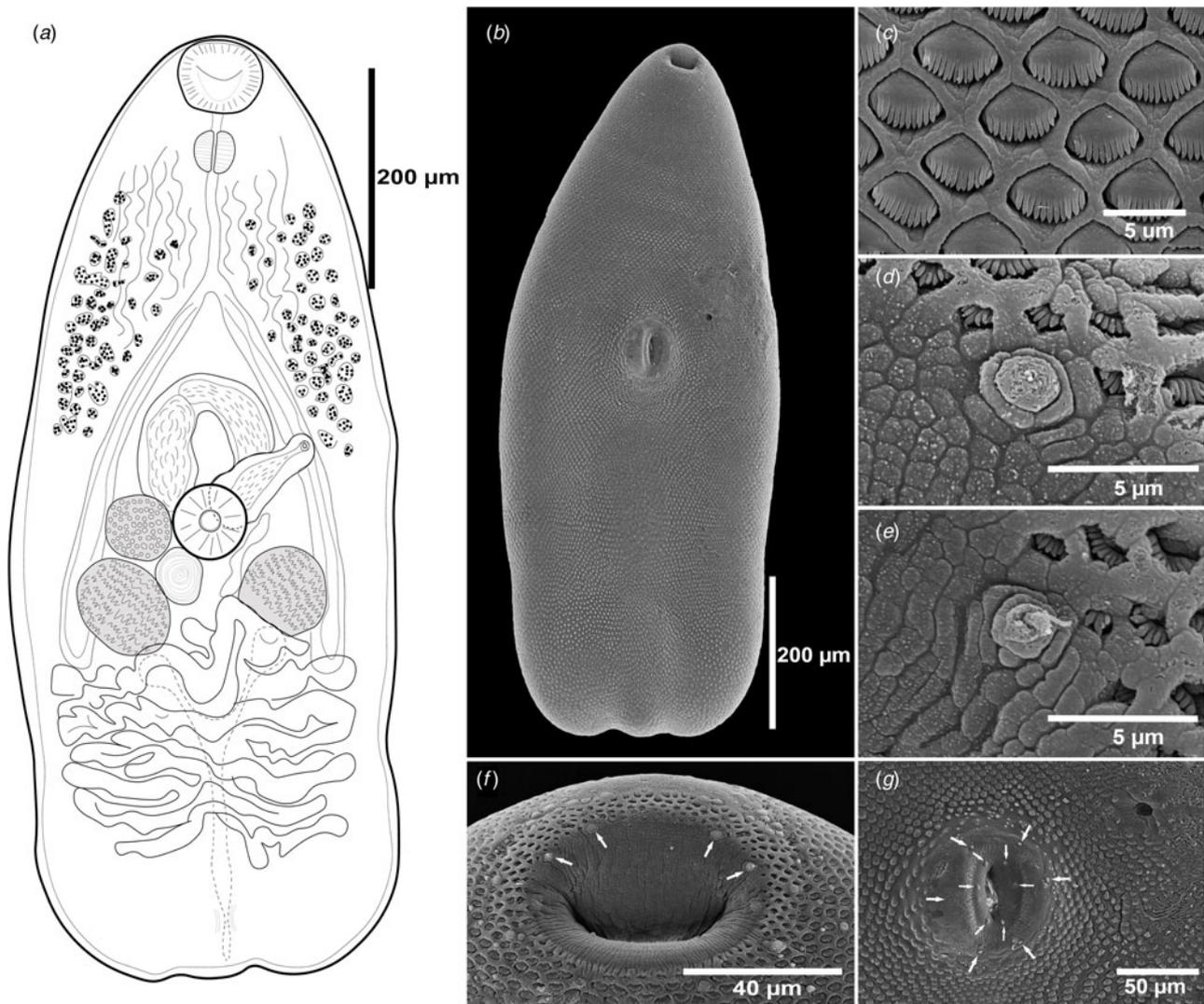


Fig. 1. Line drawings and scanning electron microscopy (SEM) photomicrographs of *Langeronia* sp. From Nahá, Chiapas: (a) line drawings, ventral view; (b) SEM, ventral view; (c) spines; (d) simple papillae; (e) ciliate papillae; (f) oral sucker; and (g) ventral sucker.

Nahá and its sister species, *L. macrocirra* from Veracruz and Oaxaca varied from 3.6 to 4% and from 4 to 4.3%, respectively (table 1). The genetic divergence of COI of the newly sequenced individuals exhibited a higher genetic divergence with respect to the clades of *Langeronia* from Guatemala and Costa Rica, and those from Colima, Mexico (table 1); these individuals were identified as *L. macrocirra* but molecular data reveal they represent separate species, as discussed previously in other studies. DNA sequences of other congeneric species are not available for comparison. For the 28S rDNA fragment (1100 bp long), the newly sequenced individuals of *Langeronia* diverged 0.35% from *L. macrocirra* from Veracruz, and 0.5%, from those from Guatemala (tree not shown).

Haematoloechus complexus (fig. 3)

Morphological data

The specimens collected from the lungs of *L. vaillanti* were morphologically very similar to three congeneric species, that is, *H. complexus*, *Haematoloechus caballeroi* (Skrjabin & Antipin, 1962) and *Haematoloechus veracruzanus* León-Règagnon and

Topan (2018). Our specimens share some traits with these species, such as the oral/ventral sucker ratio, ovary and testes shape, and the diagonal arrangement of uterine loops. However, our specimens clearly correspond with the description of *H. complexus* in that they possess unevenly distributed vitellaria, and the uterine loops in the post-acetabular region are transversally distributed (fig. 3a).

Ultrastructure of the body surface through SEM

The SEM study of *H. complexus* revealed that the tegument is covered entirely with short and delicate sensorial bristles with homogeneous distribution along the body (fig. 3b, c). Numerous small and simple papillae with random arrangement distributed along the body surface (fig. 3c). The oral sucker is oval to spherical, covered by sensorial bristles, and lack papillae in their inner borders (fig. 3d). The genital pore is rounded and simple and located near to oral sucker. Ventral sucker slightly smaller than oral sucker, pre-equatorial, covered by sensorial bristles, without papillae in their inner borders, and with numerous papillae of variable form arranged randomly on the outer surface of sucker (fig. 3e).

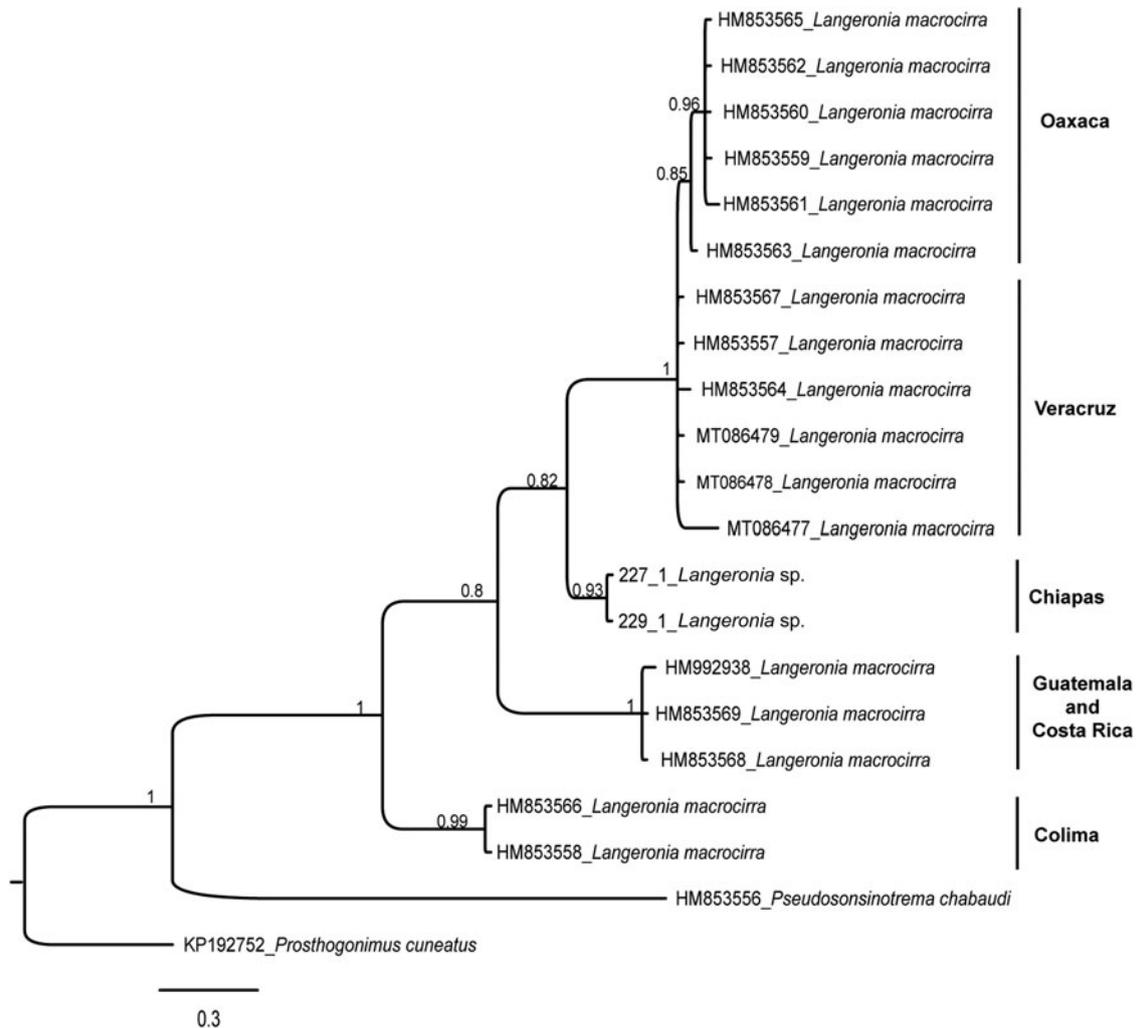


Fig. 2. Bayesian phylogenetic trees of the cytochrome c oxidase gene, showing the phylogenetic positions of *Langeronia* sp. from Nahá, Chiapas, and locality sequences used in the analyses.

Molecular data

For COI phylogenetic analyses for species of *Haematoloechus*, the alignment including the new sequences and those available in GenBank, consisted of 30 sequences with 360 bp long. In the

COI phylogenetic tree, the newly generated sequences appear nested together with *H. complexus* (specimens from the United States), although the relationships among these two clades are supported with a low to high value (0.72) (fig. 4). The newly

Table 1. Genetic divergence (%) of cytochrome c oxidase gene sequences among species of *Langeronia* from Mexico and Central America used for phylogenetic analyses.

Species	1	2	3	4	5	6
1 <i>Langeronia macrocirra</i> Costa Rica	0.7					
2 <i>L. macrocirra</i> Guatemala	0.27	0				
3 <i>L. macrocirra</i> Colima, Mexico	8.1–7.8	8.–7.8	0			
4 <i>L. macrocirra</i> Veracruz, Mexico	7	7	8.15–8.42	0		
5 <i>L. macrocirra</i> Oaxaca, Mexico	6.7–7.3	7.3	8.42–8.69	0.54–0.8	0.2	
6 <i>Langeronia parva</i> Chiapas, Mexico	6.25	6.25	6.7	3.6–4	4–4.3	0

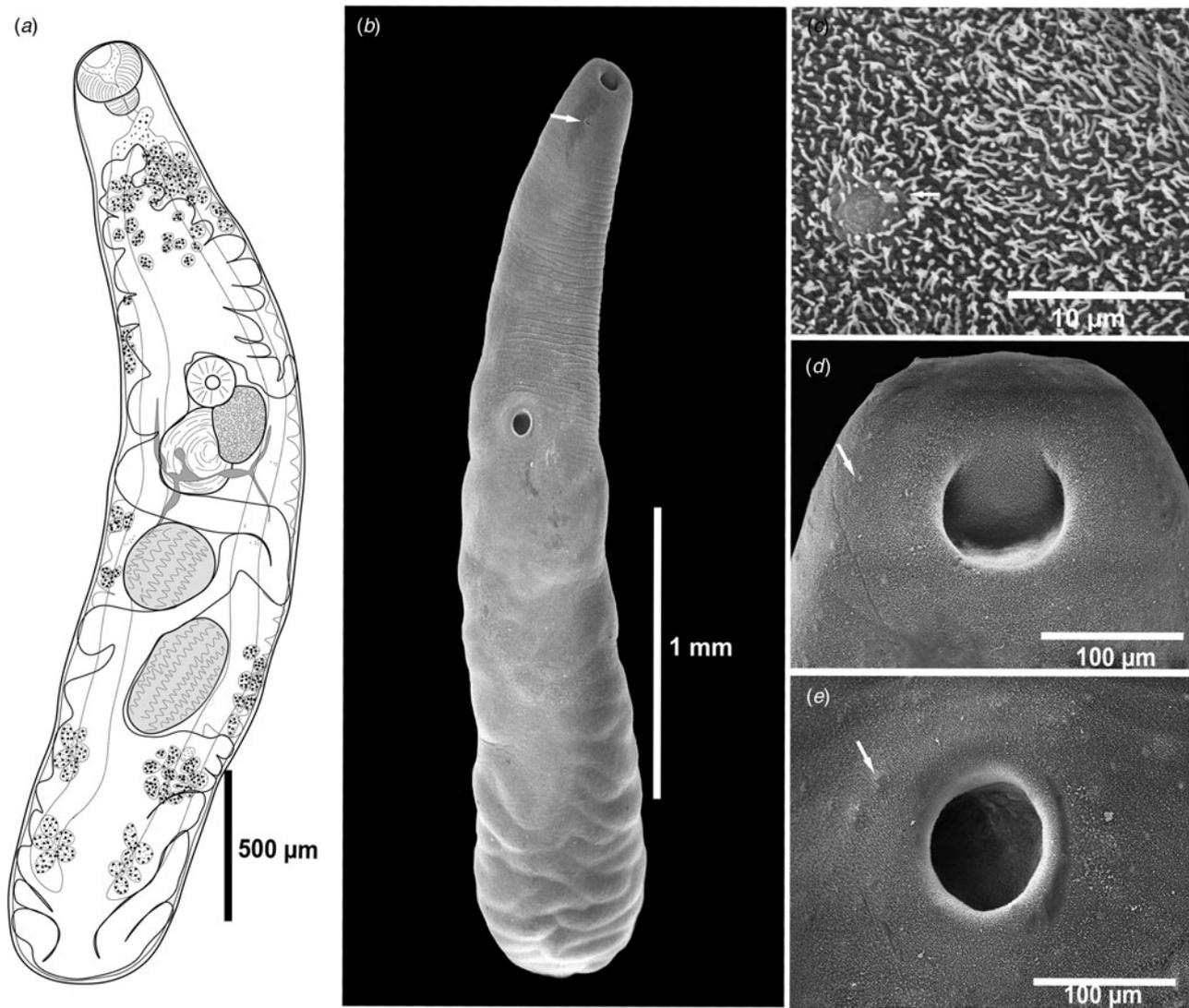


Fig. 3. Line drawings and scanning electron microscopy (SEM) photomicrographs of *Haematoloechus complexus* from Nahá, Chiapas: (a) line drawings, ventral view; (b) SEM, ventral view; (c) sensorial bristles and simple papilla; (d) oral sucker; and (e) ventral sucker.

obtained COI sequences of *Haematoloechus* from the Vaillant's frog showed a very low genetic variation with respect to those of *H. complexus* (0.59%) from Illinois, showing conspecificity; and high genetic variation with respect to *H. veracruzanus*, *Haematoloechus celestunensis* and *Haematoloechus occidentalis* (table 2). Unfortunately, COI sequences of *H. caballeroi* are not available for comparison. Instead, the 28S sequences (990 bp long) of our specimens diverge from *H. complexus* in 0–0.32%, *H. veracruzanus* in 1.4%, *H. celestunensis* in 1.5%, *H. occidentalis* in 1.2%, and *H. caballeroi* in 1.8% (tree not shown).

Discussion

The taxonomic history and species composition of the genus *Langeronia* has been controversial (Velazquez-Urrieta & Pérez-Ponce de León, 2020); in Mexican specimens that have been studied in detail, some morphological characters are quite variable, and it is possible that many of these are not reliable for species differentiation. For instance, specimens morphologically assigned to *Langeronia* cf. *parva* by Martínez-Salazar & León Rêgagnon (2010) are not recovered in the same clade in the

phylogenetic tree; sequences of these specimens appear in two clades, nested together with *L. macrocirra*; therefore, there is no correlation between morphology and molecular data (Martínez-Salazar & León Rêgagnon, 2010). This suggests that the presence of pre-pharynx, and the ovary and testes shape are probably not informative characters. The fact that the fixation procedure of the specimens altered their morphology cannot be ruled out either. The specimens of *Langeronia* we studied herein did not show any signs of intraspecific morphological variability; all specimens showed the same morphological characteristics (presence of pre-pharynx, ovary and testes oval to round shaped, and vitelline follicles distributed between the mid-level of ventral sucker and pharynx). We studied museum specimens deposited at the CNHE and noticed that *L. macrocirra* from Veracruz and Oaxaca possess a pre-pharynx, whereas specimens from Central America and Colima, on the Pacific slope of Mexico lack pre-pharynx. Additionally, we also noticed that characters such as the ovary and testes shape are variable among individuals, perhaps due to the fixation procedure (see Online Supplementary fig. S1).

Langeronia parva is considered a Nearctic species, since the species was previously reported from Vermont, United States

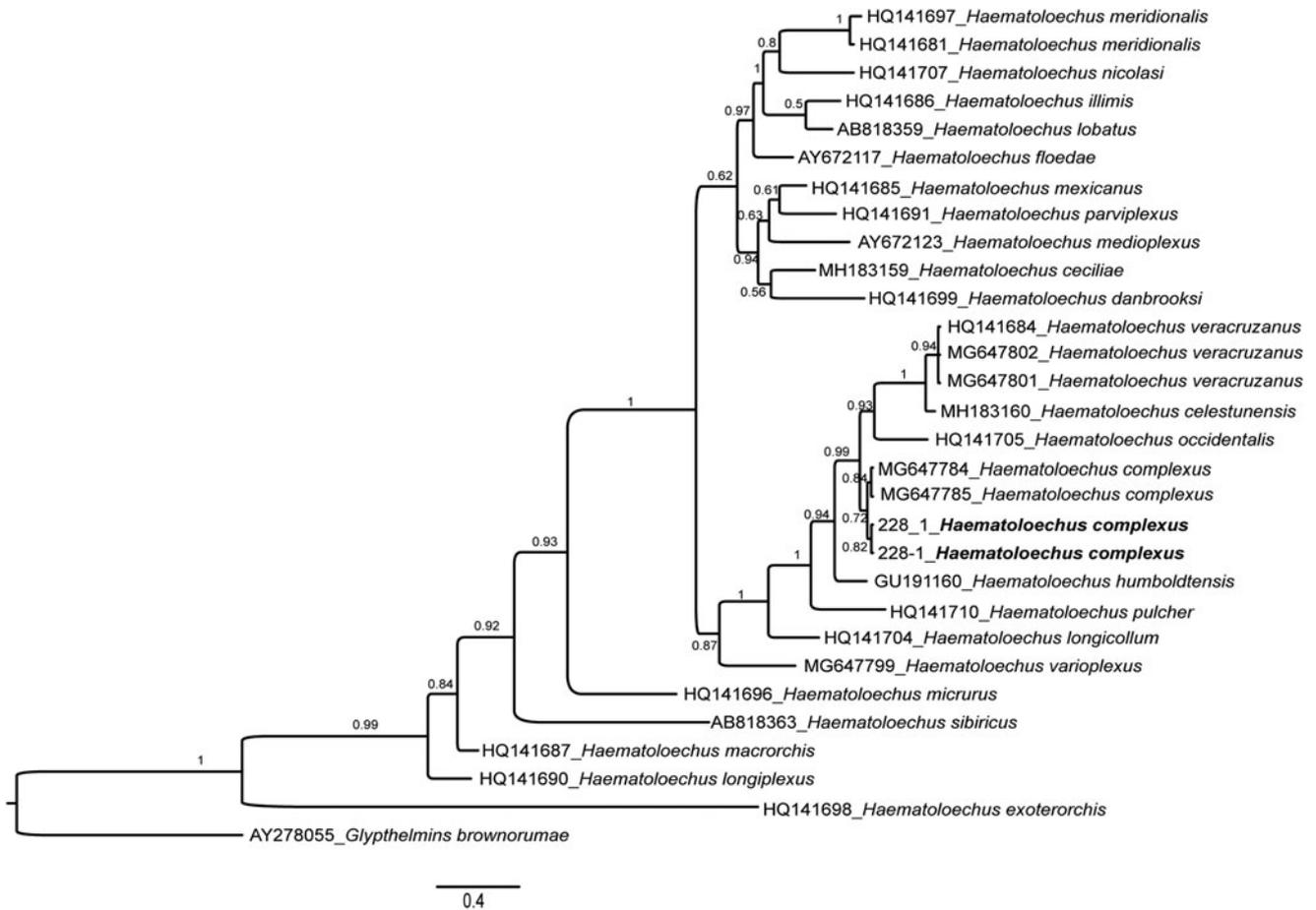


Fig. 4. Bayesian phylogenetic trees of the cytochrome c oxidase gene, showing the phylogenetic position of *Haematoloechus complexus* from Nahá, Chiapas.

(Christian, 1970). Likewise, *L. macrocirra* has been recorded in central Mexico (in the states of Colima, Estado de Mexico and Veracruz), but also in Central America, in locations of Guatemala and Costa Rica (Martínez-Salazar & León Rêgagnon, 2010); this suggests that *L. macrocirra* is a widely distributed species. Molecular phylogenetic analyses show that *L. macrocirra* may represent a species complex across its distributional range. We cannot ascertain whether our specimens of *Langeronia* from Nahá correspond to either *L. parva* or even *L. macrocirra*. The

issue remains unresolved since at the present time no sequences are available from the type locality where *L. macrocirra* and *L. parva* were described (see Caballero & Bravo-Hollis, 1949; Christian, 1970).

In the case of *Haematoloechus* spp., the morphological and molecular evidence unequivocally suggest that specimens of *Haematoloechus* from Nahá belong to *H. complexus*; therefore, this is the first record of *H. complexus* for Chiapas state, and the second for Mexico since the species was reported in Los

Table 2. Genetic divergence (%) of cytochrome c oxidase gene sequences among species of *Haematoloechus* closely related to *Haematoloechus complexus*.

Species	1	2	3	4	5	6
1 <i>Haematoloechus celestunensis</i> Yucatan, Mexico	–					
2 <i>H. complexus</i> Illinois, United States	6.58	–				
3 <i>Haematoloechus humboldtensis</i> California, United States	9.06	6.01	–			
4 <i>Haematoloechus occidentalis</i> Colima, Mexico	8.77	6.55	9.41	–		
5 <i>Haematoloechus veracruzanus</i> Veracruz, Mexico	4.04	7.12–7.18	8.58	9.69	–	
6 <i>H. complexus</i> Chiapas, Mexico	6.64	0.56–0.59	6.7	7.67	6–74	–

Tuxtlas tropical rain forest in Veracruz, a record based on the finding of the metacercariae obtained from the dragonfly *Agriogomphus tumens* (Velázquez-Urrieta & Pérez-Ponce de León, 2022). Previously, *H. complexus* had been reported in frogs distributed in Mexico, particularly in *Lithobates tlaloci* from Xochimilco, *Lithobates montezumae* from Estado de México, *Lithobates forreri* from Colima, and *Lithobates vaillanti* from Veracruz; however, in the study by León-Régagnon & Topan (2018), these authors argued that the species identification was incorrect, and they actually described these specimens as new species, namely *H. caballeroi*, *H. occidentalis* and *H. veracruzanus*, respectively. The taxonomic history and species composition of the genus *Haematoloechus* has been controversial because the reliable characters for species differentiation are difficult to observe and characterize; also, some species are morphologically like *H. complexus*, and in most of the cases it is very difficult to achieve the species delimitation only based on morphological grounds. Furthermore, the molecular data have corroborated the reliability of some characters for the differentiation of species, such as the length and width suckers' ratio, shape of ovary and testes, and the distribution of vitellaria and uterus (León Regágnon & Topan, 2018). *Haematoloechus complexus* has a Nearctic distribution. In North America the species has been reported in eight species of anurans across eastern United States (Bolek & Janovy, 2007), and in Mexico it has been reported only from Veracruz (Velázquez-Urrieta & Pérez-Ponce de León, 2022). Therefore, this species is distributed between the United States (Wisconsin) and Mexico (Chiapas).

Lithobates vaillanti is a Neotropical species whose distributional range extends from Veracruz and Oaxaca, Mexico, southwards to Costa Rica (AmphibiaWeb, 2022). Interestingly, in Mexico, its area of distribution overlaps in Veracruz with the leopard frog, *Lithobates berlandieri*, a Nearctic species mainly distributed from western Texas, southern New Mexico, United States, through eastern Chihuahua and Durango south to central Veracruz, Mexico. Therefore, it might be plausible in this case to postulate that the wide distribution of *H. complexus* is probably due to host switching events between the two frog species (*L. vaillanti* and *L. berlandieri*).

The combination of genetic and morphological data provides the best estimate for the identification and recognition of species. In this case, both sources of information were very useful to accomplish the identification of *H. complexus* for which sequence data are available from their native range in other regions of North America; however, the approach was not sufficient in the case of *Langeronia* spp. Here, additional molecular data are needed to fully understand the species composition as well as the evolutionary and biogeographical history of the species included in the genus. Still, our study provides further evidence to support the idea that the species delimitation of trematodes must be based on the combination of morphological and molecular evidence.

Supplementary material. To view supplementary material for this article, please visit <https://doi.org/10.1017/S0022149X22000906>.

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Conflicts of interest. None.

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