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Morphological and Molecular Description of *Haematoloechus meridionalis* n. sp. (Digenea: Plagiorchioidea: Haematoloechidae) from *Rana vaillanti* Brocchi of Guanacaste, Costa Rica

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MORPHOLOGICAL AND MOLECULAR DESCRIPTION OF *HAEMATOLOECHUS MERIDIONALIS* N. SP. (DIGenea: PLAGIORCHIOIDEA: HAEMATOLOECHIDAE) FROM *RANA VAILLANTI* BROCCHI OF GUANACASTE, COSTA RICA

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ABSTRACT: An undescribed species of *Haematoleechus* inhabits the lungs of *Rana vaillanti* in northwestern Costa Rica. The new species is most similar morphologically to *H. medioplexus*, having a very small, but well-developed, ventral sucker and lacking extracecal uterine loops, and apparently was mis-identified previously as *H. medioplexus* in *Rana palmipes* from Colombia. It differs from *H. medioplexus*, notably by (1) the shape of the oral sucker, which is elliptical in *H. meridionalis* and spherical in *H. medioplexus*; (2) the posterior extent of the vitelline fields, which terminate at the same level in *H. medioplexus*, but always at different levels in *H. meridionalis*; (3) the position of the testes, which are immediately posterior to the seminal receptacle and are close together in the new species and far from the seminal receptacle and separated from each other in *H. medioplexus*; and (4) the location of the genital pore, which is ventral to the cecal bifurcation in the new species and ventral to the pharynx in *H. medioplexus*. In addition, the new species is unique among members of *Haematoleechus* by having flaps of tissue on the interior margins of the ventral sucker. The new species shows almost 5% sequence divergence from *H. medioplexus* in the variable D1 region of the 28S rDNA, complementing the morphological differences.

As part of an inventory of eukaryotic parasites of vertebrates in the Area de Conservación de Guanacaste (ACG) in Costa Rica, specimens of a previously undescribed species of digenean inhabiting the lungs of *Rana vaillanti* Brocchi were collected. In this paper, the morphological description of a new species of *Haematoleechus* is given, and the sequence of a variable region of the large subunit of the rDNA is provided in order to construct a molecular data base that reinforces the morphological features in an effort to clarify the systematics of the group.

MATERIALS AND METHODS

Worms collected from freshly killed amphibians initially were placed in saline (0.65%) for 5–10 min. For the morphological study, they were fixed by sudden immersion in hot 4% formaldehyde and preserved in 70% alcohol. Specimens were stained with Mayer's paracarmine or Gomori's trichrome, dehydrated, cleared in methyl salicylate, and mounted in Canada balsam. Some specimens were mounted permanently between cover slips and held in Cobb slides. Measurements are presented as the range with means in parentheses and expressed in micrometers (μm), unless otherwise stated. Figures were drawn with the aid of a drawing tube. The following abbreviations are used: CNHE, Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México; USNPC, United States National Parasite Collection; BMNH, British Museum of Natural History, London. Specimens for molecular work were identified in vivo and preserved in 100% ethanol. Standard phenol extraction methods were used to recover DNA from individual specimens. Laboratory protocols follow Palumbi (1996) and Hillis et al. (1996). Polymerase chain reaction (PCR) was used for amplifying the 5' end of the 28S ribosomal RNA gene (884 bp, including the D1 variable region). Amplification and sequencing was performed using the primers 28Sy 5'cta acc agg att ccc tca gta acg gcg agt 3' (forward) and 28Sz 5'aga ctc ctt ggt ccg tgt ttc aag ac 3' (reverse) (Hillis and Dixon, 1991). Amplification program consisted of 1 min at 94 C followed by 35 cycles of 30 sec at 92 C, 30 sec at 50 C, and 1 min at 72 C, followed by 4 min at 72 C for final elongation. PCR products were sequenced directly on an ABI Prism 310 automated DNA sequencer or using Thermo Sequenase radiolabeled terminator cycle sequencing kits (Amersham Life Science, Cleveland, Ohio). Sequences

were aligned visually with *H. medioplexus* (Genbank AF133112), *H. complexus* (AF133104), *H. coloradensis* (AF133108), and *H. illinis* (AF13313109) sequences using the computer program ESEE (version 3, Cabot and Beckenbach, 1989). A pairwise distance matrix was constructed using PAUP (version 4.0b4a Swofford, 1998) and a phenogram was constructed using UPGMA (Krebs, 1989).

DESCRIPTION

Haematoleechus meridionalis n. sp.

(Figs. 1–3)

Description (based on 16 mature specimens): Body elongate, 5.85–11.08 (8.25) mm long, 1.05–2.57 (1.69) mm wide at midhindbody. Tegument spinose; spines small, and delicate. Oral sucker subterminal, elongate, 1.1–1.38 (1.21) times longer than wide, 479–618 (535) long, 409–518 (447) wide; opening directed ventrally. Pharynx globular, 159–279 (223) long, 35.1–49.2% (41.5%) of oral sucker length, 219–289 (246) wide, 47.7–60.9% (55.4%) of oral sucker width. Gland cells surrounding anterior region of pharynx. Esophagus bulbous, surrounded anteriorly by gland cells. Ceca bifurcating at 700–1,086 (833) (8.1–11.7% [10.3%] of body length [BL]) from anterior extremity. Ceca narrow, terminating blindly, sometimes asymmetrical; right cecum ending at 169–798 (395) (2.1–7.7% [4.73%] of BL) from posterior extremity; left cecum at 169–568 (336) (3.2–5.7% [4.1%] of BL). Ventral sucker well developed, 74–123 (105) long, 57–148 (101) wide, at 2.6–4.2 (3.25) mm (31–52% [40.4%] of BL) from anterior extremity, with several flaps on internal border. Ventral sucker sometimes obscured by ascending uterus. Sucker length ratio 1:5.2, sucker width ratio 1:4.4. Testes 2, oval, tandem, slightly oblique, immediately posterior to seminal receptacle. Anterior testis opposite ovary, 379–800 (574) long, 598–1,125 (747) wide. Posterior testis 279–825 (547) long, 550–1,900 (889) wide. Cirrus sac 2.66–4.67 (3.12) mm long (26.2–47.1% [38.7%] of BL). Internal seminal vesicle elongate, coiled, 1,495–2,821 (2,120) long, occupying 55.3–75.4% (65.8%) of cirrus sac. Ejaculatory duct weakly muscular, surrounded by prostatic gland cells; 648–1,854 (1,094) long. Ovary dextral or sinistral, oval to triangular, 459–817 (644) long, 259–600 (438) wide; at 3,250–4,680 (3,862) (34.9–58.9% [47.9%] of BL) from anterior extremity. Seminal receptacle ventrally overlapping posterior part of ovary; 369–875 (587) long, 349–1,000 (645) wide. Mehli's gland dorsal to seminal receptacle. Laurer's canal absent. Vitellaria forming clusters, anterior limit of distribution 1,650–2,900 (2,110) (19.8–36.7% [26.3%] of BL) from anterior end; posterior limit differing on each side of body. On ovarian side, 1 pretesticular group of 7–8 extracecal plus 1–3 intracecal clusters of acini, reaching the level of anterior testis. On opposite side of body, 2 groups of clusters, 1 preovarian formed by 5–7 extracecal plus 1–2 intracecal clusters, second posttesticular of 3–4 extracecal plus 1–2 intracecal clusters, reaching midway between posterior testis, posterior end of body. Descending uterus forming short transverse loops on ovarian side of body, not overlapping testes, filling intraextracecal space toward posterior end

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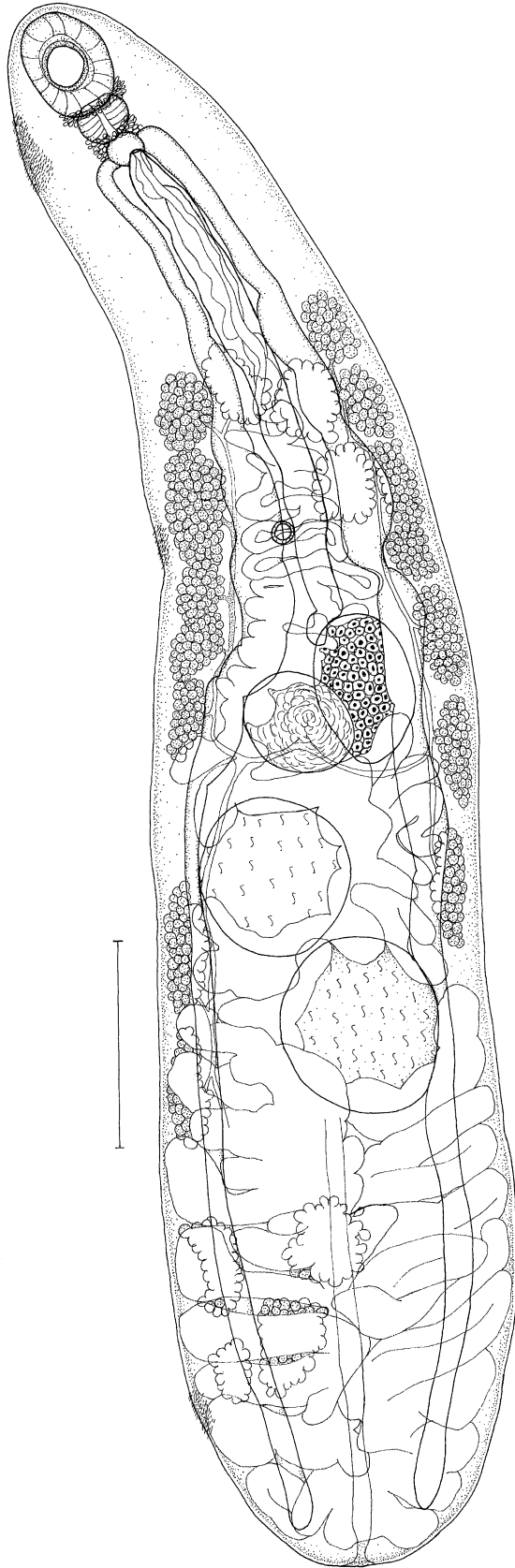


FIGURE 1. *Haematoloechus meridionalis* n. sp. ventral view.

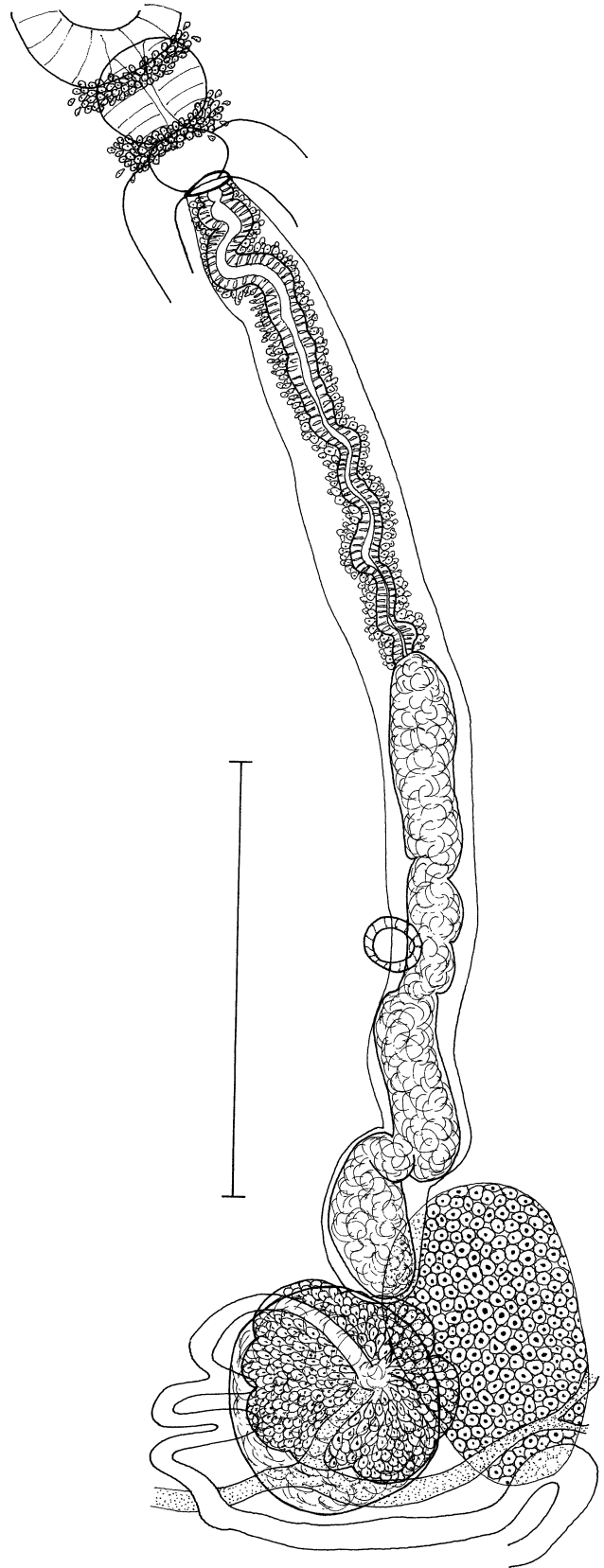


FIGURE 2. *Haematoloechus meridionalis* n. sp. terminal genitalia.

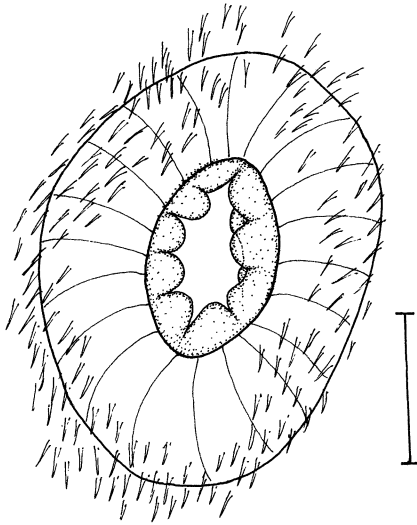


FIGURE 3. *Haematoloechus meridionalis* n. sp. acetabulum.

of body. Ascending uterus forming short transversal loops filling opposite side of body, not overlapping testes or ovary, becoming medial anterior to ovary. In larger specimens, preovarian uterus may form short transverse loops. Metraterm poorly developed, surrounded by gland cells. Genital pore 788–113 (922) from anterior end (9.4–15.5% [11.4%] of BL). Eggs amber, 17–22 (20) long, 12–16 (15) wide. Excretory vesicle I-shaped. Excretory pore terminal.

Taxonomic summary

Type host: *R. vaillanti* Brocchi, 1887.

Site of infection: Lungs.

Type locality: Rio Pizote, Area de Conservación Guanacaste, Costa Rica.

Other localities: Cali, Colombia in *R. vaillanti* (reported as *H. medioplexus* in *R. palmipes*, Uribe-Piedrahita, 1939). Record by León-Règagnon et al. (1999) of *H. iturbei* in *Rana palmipes* Spix from Costa Rica corresponds to *H. meridionalis* in *R. vaillanti*.

Holotype: CNHE 4199.

Paratypes: CNHE 4200–4201, USNPC 091036.00–09037.00, BMNH 2001.3.19.1–3.

Vouchers: CNHE 4202–4203.

Etymology: The name of the species refers to its geographical distribution.

Remarks

The new species differs from other *Haematoloechus* species and resembles *H. medioplexus* Stafford, 1902, in having a very small (less than one-fourth the diameter of the oral sucker) but well-developed acetabulum. *Haematoloechus meridionalis* further resembles *H. medioplexus* in lacking extracecal longitudinal uterine loops. The new species differs from *H. medioplexus* notably by (1) the shape of the oral sucker (elliptical in *H. meridionalis* and spherical in *H. medioplexus*), (2) the posterior extent of the vitelline fields (terminating at the same level in *H. medioplexus*, at different levels in *H. meridionalis*), (3) the position of the testes (immediately posterior to the seminal receptacle and close together in the new species, far from the seminal receptacle and separated from each other in *H. medioplexus*), and (4) location of the genital pore (ventral to the cecal bifurcation in the new species, ventral to the pharynx in *H. medioplexus*). Finally, *H. meridionalis* is unique among reported members of *Haematoloechus* by virtue of having several flaps of tissue on the internal margins of the ventral sucker (Fig. 3).

Uribe-Piedrahita (1948) reported *Haematoloechus medioplexus* in *Rana palmipes* from Cali, Colombia. Later, Dobbin (1957b) considered those specimens to be *H. iturbei*. Uribe-Piedrahita (1948) did not include a description of the specimens, and they are not available for examination, but according to his illustration, it appears that he had

specimens of *H. meridionalis*. This suggestion is supported by information about the anuran host. In their revision and phylogenetic analysis of the *R. palmipes* clade, Hillis and De Sá (1988) showed that the member of the *R. palmipes* clade living in Cali is *R. vaillanti* and not *R. palmipes*. Two nominal species of *Haematoloechus* have been reported in *R. palmipes* (sister species of *R. vaillanti* according to Hillis and De Sá, 1988). *Haematoloechus iturbei* (Cordero and Vogelsang, 1939) Walton, 1949, from Venezuela (Cordero and Vogelsang, 1939) and Brazil (Dobbin, 1957a, 1957b) is larger than *H. meridionalis* and differs from the new species in having a spherical rather than elongate oral sucker, lacking a ventral sucker, having elongate testes located far behind the ovary, and having extracecal uterine loops. *Haematoloechus lutzi* Freitas and Lent, 1939 (Syn: *H. tejeræ* (Cordero and Vogelsang, 1939) Walton, 1949; see Dobbin, 1957b) differs from the new species in having an acetabulum the same size as the oral sucker and in having extracecal uterine loops.

Molecular data: A 875-bp-long fragment (partial 28s sequence, including the D1 variable domain) was amplified. Sequences of *H. meridionalis* isolates from different individual hosts of the type locality are identical; sequence available from Genbank (accession number AY026897). The DNA sequences of the new species and *H. medioplexus* are compared to evaluate the information given by morphological characters; included in the comparison are other species that are morphologically clearly different from them (Figs. 4, 5).

DISCUSSION

The taxonomy of *Haematoloechus* has been controversial because many nominal species have been proposed based on a small number of morphological characters, at least some of which are variable and difficult to characterize (Prokopic and Krivanec, 1974; Kennedy, 1980a, 1980b, 1981). In a previous paper, León-Règagnon et al. (1999) used comparisons of rDNA sequence data (ITS1–5.8S–ITS2 and 28s regions) to provide additional support for some nominal species, thereby validating some of the morphological traits used in the systematics of the group. We therefore compared the partial 28s sequences of the new species and other American representatives of the genus. The genetic differences between the specimens of *H. meridionalis* and *H. medioplexus* (4.91%) are larger than those observed with other clearly distinguishable species, such as *H. illimis* Caballero, 1942 (1.85%). *Haematoloechus illimis* differs from *H. meridionalis* in having an acetabulum that is almost the same size as the oral sucker; in the general shape of the body, which is shorter and wider in *H. illimis*; in having a spherical oral sucker; in the distribution of the uterus (which is not arranged in 2 lateral fields and possesses short longitudinal loops), and in the shape of the testes and ovary, which are elongate and lobate. Whereas the geographical distribution of *H. illimis* has not been well studied, it apparently is endemic to the central part of Mexico (Caballero, 1942; León-Règagnon et al., 1999; Pérez-Ponce de León et al., 2000) and is more commonly found in leopard frogs (members of the *Rana pipiens* clade), but little is known about the southeastern region of this country and others in Central America. Of the species examined by León-Règagnon et al. (1999), *H. illimis* was most closely related to *H. medioplexus* and *H. coloradensis* Cort, 1915. *Haematoloechus meridionalis* appears to be part of this group of species, possibly sister species of *H. illimis*.

In the Area de Conservación Guanacaste, *Rana vaillanti* is the ecological equivalent of the bullfrog (*Rana catesbeiana*) or green frog (*Rana clamitans*) of North America. They are large, territorial, actively predatory frogs that are more aquatic than the leopard frogs (members of the *Rana pipiens* clade) with which they often are sympatric. Bullfrogs and green frogs are

compl	????GGGAT	AAGCCAGCA	CCGAAGCCTG	TAGCCATTG	GTTACTAGGC	AATGTGGTGT	TTAGTCGTT	CCGTGGATAT	TCTGCTCCAC	CCTAAGTCCA	95
color	?????			.NN-				.C..GG.			
illim	?????	T		--NN			.C	.C..GG.			
merid	GAACA			.GCG.			.C	.C..GG.			
medio	GAACA			.GCG.				.C..GG.			
compl	TCAATGAGTA	CGGTATTATG	GACATGGCCC	ATAGAGGGTG	AAAGGCCCGT	GGGGGTGGAG	ATTCGGTAGG	ACAGAATATT	CTTGGGTAGA	CCTTGGAGTC	195
color				.G.				C....CT.C	.C		
illim				.C				C....CT.C	.C		
merid				.C				C....CT.C	TC		
medio				.C				C....CT.C	.C		
compl	GGGTTGTTTG	TGAATGCAGC	CCAAAGTGGG	TGG-TAAACT	CCATCCAAGG	CTAAATACTT	GCACGAGTCC	GATAGCGAAC	AAGTACCGTG	AGGAAAGTTG	294
color				-							
illim	A			-							
merid				-							
medio				.G.							
compl	AAAAGTACTT	TGAAGAGAGA	GTAACAGTG	CGTGAAACCG	CTCAGAGGTA	AACGGGTGGA	GTTGAACTGC	AAGCTCTGGG	AATTCAACTG	GTGAGTGTGG	394
color											
illim											
merid											
medio											
compl	TTTTAGCTTG	GTA AAATTGG	TGGACATTGG	GGTCTGCGTA	GTAGCAGGTC	TTCGCCTTCG	GGTGGGGATG	CGCGATGCAC	TTATCAAGTG	TTGTGCTCCT	494
color	.G.	.C.T	.G.C.		.A					.G.	
illim	.G.	.C.T	.G.C.							.G.	
merid	.G.	.C.T	.G.C.		--CG					.G.	
medio	.G.	.C.T	.G.C.		.A					.G.	
compl	CAGTGGTCAT	ACCGACCAAC	TCGCTAGTGC	ACTTTCTCAG	AGTGTTCACC	ACGACCGGCG	CCGCTGTCTG	GCCTCTATAG	TTAAACCGGT	TTTGCATAGT	594
color	.G..T.--T.	C..G.			.G.		.T.	.A..G.G.	.AG		
illim	.G..T.--T.	C..G.			.G.			.G..G.G.	.AG		
merid	.G..T.--T.	C..G.			.G.	.A.	.G.	.G..G.G.	.AA	.C.	
medio	.G..T.--T.	C..G.			.G.	.A	.T.	.A..G.G.	.AG		
compl	CCTTGTGGCT	TTGCTTAGTC	GGGACGGCAG	GTAGCTCGTT	GACTTGCTTG	TGTTTGCCTG	C-AAGCGTGG	TTTTCGAGTG	TAATCAGCTG	ACTGTAGTTG	693
color	.GA.	.TAAG.	.T.	.C.	.CT.	.T.GCC	AT...T..T.	.C..A..C	.C.	.C..G.	
illim	.C.		.T.			.CG.C.	.G.-	.A	.C.	.C..G.	
merid	.C.		.T.	.T.	.C.	.CG.	.G.-	.A	.C.	.C..G..G.	
medio	.GA.	.TAAG.	.T.	.C.	.CT.	.T.GCC	AT...T..T.	.C..A..C	.C.	.C..G..G.	
compl	TTCTGTGCAG	TGTGTCGGAG	ACGGCGGCTT	GAGGTGTGTG	CATGCGTAGT	TGTTTTGCTG	ACTGGTTCGA	GTTTGGTTAT	TTGTT-GCCT	GTTTCATGCAG	792
color		.G.	.C		.T.C	.C...T.	.C.		.TG.A	.AT.	
illim		.G.	.C		.T.C		.C.		.TG.A	.AT.	
merid	.A.	.G.	.C		.T.C		.C.		.TG.A	.AT.	
medio		.G.	.C		.T.C	.G.	.C.	.G.	.TG.A	.AT.	
compl	GTCTGGTAGT	AGCTCGAATT	TGTTCCGGCTG	GCGACTGCGT	GTGTGGCATT	TTACCAAGGG	CCAATAGTCT	GTGGTGTAGT	GGT		875
color		.G.		.G.G.		.G.T	.C.				
illim		.G.		.GTG.		.G.T..G.	.C.				
merid		.G.		.GTG.	.C.	.G.T	.C.				
medio	.T.	G.	.G.	.CG.	.A..CTG.	.C.	.G.T	.C.			

FIGURE 4. Alignment of partial 28s sequences of *Haematoloechus meridionalis* and other American representatives of the genus.

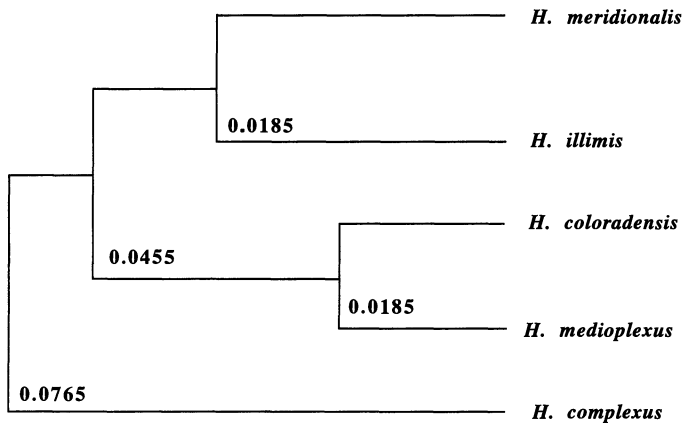


FIGURE 5. Phenogram representing genetic distances of *Haematoloechus meridionalis* and other American representatives of the genus.

hosts for multiple species of *Haematoloechus*, not all of which are the closest relatives. It is, therefore, not surprising to find that *H. meridionalis* does not appear to be the closest relative of *H. medioplexus* living in *Rana palmipes*, the sister species of *R. vaillanti* (Hillis and DeSa, 1988).

More than 50 species of *Haematoloechus* have been described worldwide, 28 of which are restricted to North and South America. This is the first record of species of the genus in Central America. If the report by Uribe-Piedrahita (1948) of *H. medioplexus* actually is *H. meridionalis*, the species' range extends to South America. If it is most closely related to the *H. illimis* (*H. coloradensis*, *H. medioplexus*) group (León-Régagnon et al., 1999), which is limited to the Nearctic region (see also Pérez-Ponce de León et al., 2000), these species might represent an ancient speciation event associated with the original separation of North and South America resulting from the break-up of Pangaea. In that case, the occurrence of *H. meridionalis* in Costa Rica represents a northward dispersal that has occurred since the emergence of the Panamanian Isthmus 3.5–5.7 million yr ago (Lessios, 1979). The biological richness of Costa Rica is due in great part to its biotic influences from both the north and the south (Janzen, 1983). Only by including information of more species and more characters in a phylogenetic analysis will we be able to test the phylogenetic, biogeographic and coevolutionary affiliations of *H. meridionalis*.

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