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Evidence of new species of *Haematoloechus* (Platyhelminthes: Digenea) using partial *cox1* sequences

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Abstract

Background and aims. *Haematoloechus*, digeneans parasites of amphibians, is a species-rich genus with more than 50 species around the globe. Establishing an accurate taxonomy for this group has been difficult due to high intraspecific variability. Nuclear DNA sequences have given independent information about species validity and phylogeny of the group.

Materials and methods. In this paper, I test the performance of partial sequences of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene in the differentiation of recognized species of the genus and in the detection of potential new taxa. Samples from 13 nominal species were sequenced, plus four samples that could not be assigned to any described species based on morphology.

Results. Parsimony analysis of the amplified 360 bp fragment resulted in six most parsimonious trees showing the same grouping of samples, differing in the samples' arrangement within those groups. All 13 species were recovered on the trees, and five potential new species are shown.

Conclusion. Additional sampling and sequencing is necessary to support this hypothesis, but with this preliminary information the search for diagnostic characters that allow the description of the new taxa is less difficult.

Keywords: *Digenea,* Haematoloechus*, Mexico,* cox1 *sequences*

Introduction

Species delimitation of soft-bodied animals, like helminths, can be a particularly controversial issue, especially in those cases where morphological variability can confound the taxonomy of a group (León-Règagnon et al. 2005), or in which morphological characters are so conservative that independent lineages cannot be differentiated (Razo-Mendivil et al. 2010). In these cases, DNA sequence data provide an independent source of information to differentiate diverging lineages, and to evaluate morphological characters that have been traditionally used in the taxonomy of the groups (Nadler 2002).

Haematoloechus, the frog lung flukes, is a species-rich genus with more than 50 described species around the globe. Morphological variability within species of this genus has caused controversy regarding species validity (Odening 1960; Prokopic and Krivanek 1974; Kennedy 1981). Kennedy (1981), e.g. considered that only 6 out of the 15 previously known species from the USA and Canada were valid. This author concluded that morphological characters, such as the shape of the ovary and testes, arrangement of uterine loops, presence or absence of spines on body surface, sucker ratio, and egg size, exhibited intraspecific variation as the result of several factors (i.e. developmental stage, host species, crowding effect), and, therefore, they are not useful to differentiate species of *Haematoloechus*. Years later, using ribosomal DNA sequences (ITS2 and partial 28S), it was demonstrated that most of those morphological characters were actually useful to differentiate species, and some of them had phylogenetic value (León-Règagnon et al. 1999, 2001;

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León-Règagnon and Paredes-Calderón 2002; León-Règagnon and Brooks 2003). Twelve species have been reported in Mexico, six of which were named as distinct endemic species in the central region (*Haematoloechus danbrooksi*, *Haematoloechus elongatus*, *Haematoloechus illimis*, *Haematoloechus macrorchis*, *Haematoloechus parcivitelarius*, *Haematoloechus pulcher*, and *Haematoloechus varioplexus*), with the remaining six commonly found in southern Canada and the USA (*Haematoloechus coloradensis*, *Haematoloechus complexus*, *Haematoloechus floedae*, *Haematoloechus longiplexus*, and *Haematoloechus medioplexus*) (Goldberg and Bursey 2002; León-Règagnon 2003; León-Règagnon et al. 2005). The validity of five out of the six endemic species has been corroborated using molecular data, while morphological characters still support the validity of *H. parcivitellarius* (León-Règagnon 2003). The presence of three out of the six nearctic species in Mexico is currently recognized (*H. coloradensis*, *H. floedae*, and *H. longiplexus*), while records of *H. medioplexus* correspond to *H. danbrooksi*, records of *Haematoloechus varioplexus* are doubtful because of morphological differences observed in Mexican specimens, and records of *H. complexus* apparently correspond to several undescribed species according to the large genetic divergence observed in preliminary studies of Mexican populations (León-Règagnon 2003).

DNA barcoding (Hebert et al. 2003) has proven to be a useful tool to differentiate species of various taxa (e.g. Smith et al. 2006, 2008), including some groups of helminths (Ferri et al. 2009; Moszczynska et al. 2009). The aim of this study is to test the performance of partial sequences of *cox1* in the discrimination of

Table I. Hosts and collection localities of *Haematoloechus* species, and accession numbers of voucher specimens (in CNHE) and DNA sequences (in GenBank).

Haematoloechus species	Host species	Collection locality	Voucher number	Isolate (GenBank accession number)
H. coloradensis	*Lithobates dunni*	Michoacán, Mexico	3892	Ha19 (HQ141685)
H. complexus	*L. pipiens*	Georgia, USA		Ha58 (HQ141702)
H. cf. complexus	*L. vaillanti*	Veracruz, Mexico	4086	Ha15 (HQ141684)
				Ha51 (HQ141701)
H. cf. complexus	*Ambystoma* sp.	Puebla, Mexico		Ha86 (HQ141710)
H. danbrooksi	*L. vaillanti*	Veracruz, Mexico	4112, 4151	Ha49 (AY672116)
				Ha14 (HQ141683)
				Ha34 (HQ141693)
				Ha36 (HQ141694)
				Ha37 (HQ141695)
				Ha48 (HQ141699)
				Ha50 (HQ141700)
H. exoterorchis	*Hoplobatrachus occipitalis*	Sierra Leone	4662	Ha47 (HQ141698)
H. floedae	*L. catesbeiana*	California, USA	4664	Ha74 (HQ141709)
		Georgia, USA	4663	Ha75 (HQ141708)
				Ha70 (AY672119)
	L. cf. *forreri*	Guanacaste, Costa Rica	5065	Ha78 (AY672120)
				Ha79 (AY672121)
	Lithobates sp.	Chiapas, Mexico		Ha61 (HQ141703)
	L. vaillanti	Yucatán, Mexico	5067	Ha55 (AY672117)
				Ha57 (AY672118)
H. illimis	*L. montezumae*	Estado de Mexico	3381–3383	Ha20 (AY672122)
				Ha21 (HQ141686)
H. longiplexus	*L. catesbeiana*	Nebraska, USA	4664	Ha11 (HQ141682)
				Ha24 (HQ141688)
				Ha25 (HQ141689)
				Ha26 (HQ141690)
H. macrorchis	*L. montezumae*	Estado de Mexico	3394	Ha22 (HQ141687)
H. medioplexus	*L. pipiens*	Neligh, Nebraska, USA	4665	Ha17 (AY672123)
H. meridionalis	*L. vaillanti*	Guanacaste, Costa Rica	4199–4203	Ha9 (HQ141680)
				Ha10 (HQ141681)
		Veracruz, Mexico		Ha46 (HQ141697)
H. micrurus	*H. occipitalis*	Sierra Leone	4666	Ha44 (HQ141696)
H. parviplexus	*L. catesbeiana*	Nebraska, USA	4405	Ha16 (AY672124)
				Ha27 (HQ141691)
				Ha28 (HQ141692)
Haematoloechus sp.	*L. psilonota*	Jalisco, Mexico		Ha62 (HQ141704)
	Lithobates sp.	Oaxaca, Mexico		Ha64 (HQ141706)
Haematoloechus sp.	*L. zweifeli*	Oaxaca, Mexico		Ha65 (HQ141707)
Haematoloechus sp.	*L.* cf. *forreri*	Colima, Mexico		Ha63 (HQ141705)
H. varioplexus	*L. blairi*	Nebraska, USA		Ha12 (AY672125)

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species of *Haematoloechus* recognized with morphology, and the detection of potential new species.

Materials and methods

Samples

For this study, we included samples of nine of the species listed above to be present in Mexico (including the doubtful *H. complexus*), samples of four species from other geographical regions, and four Mexican samples that could not be assigned to any described species based on morphology. Collecting localities and hosts are presented in Table I. 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% formalin and preserved in 70% ethanol. Specimens were stained with Mayer's paracarmine or Gomori's trichrome, dehydrated, cleared in methyl salicylate, and mounted in Canada balsam. Specimens were mounted permanently between cover slips and held in Cobb slides. Voucher specimen accession numbers are listed in Table I. The following abbreviations are used: CNHE, Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de Mexico; USNPC, US National Parasite Collection, Beltsville, MD; and HWML, Harold W. Manter Laboratory, Lincoln, NE, USA. The following specimens were examined for comparison: *H. danbrooksi* León-Règagnon and Paredes-Calderón 2002 CNHE 4112,4151, USNPC 92220; *H. floedae* USNPC 30879, 84804, 091507; *Haematoloechus parviplexus* Irwin, 1929 USNPC 75445, 81467, HWML 20142-43, 20753, 21660, CNHE 4405; *H. varioplexus* Stafford, 1902 USNPC 75447, 81915, HWML 20151-20160, 38396. Specimens were assigned to a putative species *in vivo* using morphology and were preserved in 100% ethanol.

Laboratory methods

Standard phenol extraction methods were used to recover DNA from individual specimens. Laboratory protocols follow Palumbi (1996) and Hillis et al. (1996). PCR was used for amplifying a fragment of approximately 360 bp of the *cox1* mitochondrial gene, including the 5′ end of the standard barcode (~100 bp). Amplification and sequencing were performed using the primers JB3 5′-TTTTT-TGGG CATCCTGAGGTTTAT-3′ (forward) and JB4.5 5′-TAAAGAAAGAACATAATG AAAATG-3′ (reverse) (Bowles et al. 1993). The amplification program consisted of 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 45 s at 50°C, and 1 min 30 s at 72°C, followed by 10 min at 72°C for final elongation. PCR products were sequenced directly on an ABI Prism 310 Genetic Analyzer (Applied Biosystems,

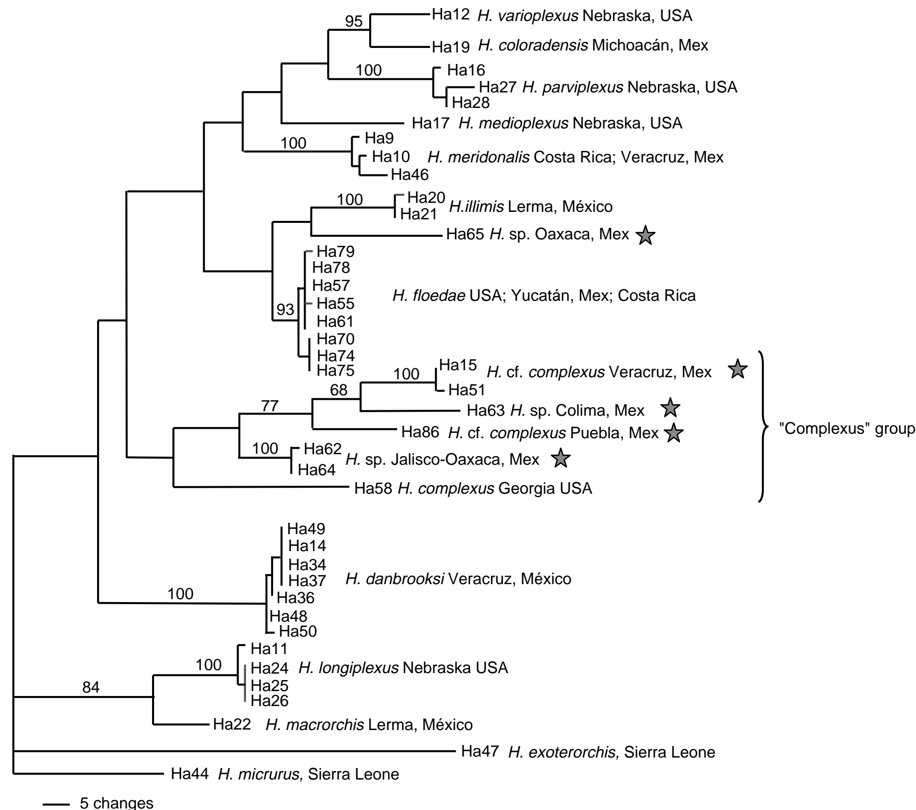

Figure 1. One of six most parsimonious trees (tree length = 496; CI = 0.548) obtained from partial *cox1* sequences of *Haematoloechus* spp. Numbers above the internode branches denote bootstrap support percentages. ★, potential new species.

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Table II. Percentage of sequence divergence among clades of *Haematoloechus* species recovered in this study.

1. *H. varioplexus*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2. *H. coloradensis*	2.4	–															
3. *H. parviplexus*	6.4–8.8	6.3–7.4	–														
4. *H. medioplexus*	8.0	9.1	8.6–10.2	–													
5. *H. meridonalis*	10.8–11.1	10.2–10.5	11.6–12.6	10.5–11.7	–												
6. *H. illimis*	10.5	10.5–11.1	11.9–13.3	10.5	11.1–12.8	–											
7. *Haematoloechus* sp. O	13.3	13.3	13.3–14.1	14.4	11.6–12.6	11.4–11.9	–										
8. *H. floedae*	7.2–8.6	7.2–8.6	8.9–10.6	9.4–11.3	8.3–10.8	8.3–10.3	9.9–11.9	–									
9. *H. cf. complexus V*	13.8–14.1	14.9–15.2	15.7–17.2	14.7–14.9	14.5–14.9	15.8–16.1	16.6–16.8	12.7–14.8	–								
10. *Haematoloechus* sp. C	13.8	14.7	14.4–15.2	14.1	14.1–14.5	15.2	16.3	12.2–13.6	9.7–9.9	–							
11. *H.* cf. *complexus P*	14.9	15.2	14.4–15.2	13.8	15.5–15.8	15.2–15.5	12.2	12.3–13.6	10.5–10.8	12.2	–						
12. *Haematoloechus* sp. JO	14.1	13.3	13.8–15.2	13.6	13.0–13.6	13.6–14.1	14.1–14.7	10.2–12.5	11.1–11.9	12.5–13.0	10.8	–					
13. *H. complexus*	13.2	13.8	15.5–16.3	14.9	11.9–12.5	13.8	14.0	9.9–11.9	13.8–14.1	14.1	15.5	11.91	–				
14. *H. danbrooksi*	9.4–9.7	8.6–9.4	10.9–12.5	11.1–11.4	11.4–13.0	10.3–12.5	13.9–14.4	9.4–10.2	13.8–14.7	12.8–13.3	14.4–15.2	13.3–13.6	13–13.6	–			
15. *H. longiplexus*	16.9–17.1	16.2–16.9	18.0–19.1	18.3	17.2–18.1	17.4–17.7	20.2	14.4–15.2	17.4–18.0	17.7–18.0	19.1	16.6–18.0	16.3	15.2–16.1	–		
16. *H. macrorchis*	16.9	17.2	18.8–19.7	17.7	16.3–16.6	18.0–18.3	19.1	14.6–15.2	17.4	18.0	19.7	17.1–17.7	14.9	14.6–15.2	7.8	–	
17. *H. exoterorchis*	26.3	25.8	29.1–29.6	27.1	27.8–28.2	26.0–26.6	28.2	24.1–24.9	27.7–27.9	27.9	29.3	26.3–26.8	25.5	24.0–24.4	23.3	23.3	–
18. *H. micrurus*	17.2	17.7	18.6–19.4	17.7	17.4–17.7	18.3–18.6	19.9	17.4–18.0	18.0–18.3	19.9	20.5	17.7–18.2	17.2	16.9–17.1	15.8	14.4	27.1

Note: Minimum and maximum values are shown for groups with multiple sequences and intraspecific variation. O, Oaxaca; V, Veracruz; C, Colima; P, Puebla; and JO, Jalisco-Oaxaca.

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Inc., Carlsbad, California; Amersham Life Science, Inc., Buckinghamshire, England), using Thermo Sequenase radiolabeled terminator cycle sequencing kits (Amersham Life Science, Inc.). GenBank accession numbers are listed in Table I.

Sequence analysis

Sequences were aligned manually using the computer program BioEdit (Hall 1999). Uncorrected distance matrices were obtained for the pairs of examined sequences and phylogenetic trees were constructed using PAUP* 4b10 (Swofford 2002). Unweighted parsimony analyses using branch and bound searches were performed considering character states as unordered and gaps as missing data. Nonparametric bootstrap (Felsenstein 1985) with 1000 pseudoreplicates was applied to evaluate the stability of nodes in the resulting topologies.

Results

A total of 37 samples from 13 recognized species were sequenced, plus four samples that could not be assigned to any described species based on morphology (Table I). The analysis of the final alignment of 361 bp (no internal gaps) produced six most parsimonious trees (123 parsimony-informative sites; tree length = 496 steps; CI = 0.548). All of them showed the same grouping of samples with high bootstrap support. They differed in the internal arrangement of sequences within those groups, and in the position of *Haematoloechus meridionalis* (as sister species to *H. medioplexus* in four trees, Figure 1; or as sister species to *H. floedae* + *H. illimis* + *Haematoloechus* spp. in two trees). All 13 morphological species were differentiated on the phylogenetic trees.

Sequence divergence did not exceed 2.2% within the same species (0.5–1.9% in *H. meridionalis*, 0–1.1% in *H. floedae*, 0–0.8% in *H. danbrooksi*, 0–0.5% in *H. longiplexus*, 0.8–2.2% in *H. parviplexus*, 0.5% in *H. illimis*), while species differed with each other by at least 6.6% (Table II), except for *H. varioplexus* and *H. coloradensis* that differed by 2.4%. In this case, additional sequencing and reexamination of morphology are needed to clarify the specific identity of samples. The putative *H. complexus* was separated into three different clades (Figure 1). The sample from *Lithobates pipiens* from Georgia, USA (collected from the type host, within the original geographical range of *H. complexus* [Eastern USA]) appears as an independent clade from Mexican samples, showing that *H. complexus sensu stricto* is not present in Mexico. *Haematoloechus* spp. samples Ha62, Ha63, Ha64, and Ha65 were thought to be one single species in the preliminary morphological study, but according to *cox1* sequences they might belong to three different species: two of them included in the '*complexus* group' and another that appears as sister species of *H. illimis*. Overall, five potentially new species of *Haematoloechus* were detected.

Discussion

cox1 Sequences have been widely used to define species limits and phylogenetic affinities in the platyhelminthes (Bowles et al. 1993, 1995; Bowles and McManus 1994; Iwagami et al. 2000, 2003; Morgan et al. 2003; Razo-Mendivil et al. 2004, 2008); nevertheless, most of those studies used a region of the *cox1* that overlaps only in a short fragment at the 5′ end of the standard barcode. The generation of primers that amplify the standard barcode region for a wide variety of platyhelminth groups has been a challenge (Moszczynska et al. 2009), and is still at the experimental stage. Although the *cox1* fragment traditionally used in platyhelminths is shorter than the standard barcodes, it has given valuable information about the taxonomy and phylogeny of different groups, most of the time supported by other genes and morphology (Iwagami et al. 2000, 2003; Morgan et al. 2003; Razo-Mendivil et al. 2004). This is apparently also true for *Haematoloechus* spp., since *H. complexus*, which was split in several clades in our analysis, was already proposed to be a complex of 'cryptic species' based on ribosomal DNA sequences (León-Règagnon et al. 1999; León-Règagnon 2003; León-Règagnon and Brooks 2003). This split is also supported by differences in the size of the pharynx related to the size of the suckers, and the arrangement of the posterior uterine loops (personal observation). Additional sampling effort, sequencing of the complete barcode region, and combined analyses with other genes are needed to support these results. Nonetheless, even with the strongest molecular evidence for the existence of different evolutionary lineages, no formal description can be done without morphological evidence. Intraspecific morphological variability in this group renders the diagnosis of different species more difficult, but with the aid of DNA evidence we are able to identify morphological traits that can be useful to differentiate species and allow the formal description of these taxa.

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