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From the Worm in a Bottle of Mezcal: iDNA Confirmation of a Leech Parasitizing the Antillean Manatee

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ABSTRACT: Invertebrate-derived ingested DNA (iDNA) is quickly proving to be a valuable, non-invasive tool for monitoring vertebrate species of conservation concern. Using the DNA barcoding locus, we successfully identified both the blood-feeding leech *Haementeria acuecuyetzin* and its blood meal—the latter is shown to be derived from the Caribbean manatee, *Trichechus manatus*. DNA amplification was successful despite the fact that the specimen was fixed in Mezcal (a beverage distilled from agave). We report the first confirmed case of a leech feeding on a manatee, the first record of *H. acuecuyetzin* for the State of Chiapas and, to our knowledge, the first case of successful DNA amplification of a biological sample fixed in Mezcal other than the caterpillar “worms” more commonly found in that beverage.

Invertebrate-derived ingested DNA (iDNA) is quickly proving to be a valuable, non-invasive tool for monitoring vertebrate species of conservation concern (Schnell et al., 2015). Various blood-feeding ectoparasites can be leveraged for these purposes, among the earliest of which were the marine fish hosts from blood meals in gnathiid isopods on Australia’s Great Barrier Reef identified by Nagel and Loughheed (2006). Terrestrial leeches have generated considerable attention in light of their large blood meal size and the relative stability of host mitochondrial DNA in haemadipsid leech crops (Schnell et al., 2012, 2015), provided that the leeches are placed in an appropriate fixative when collected. Species in the family Haemadipsidae are not unique among leeches in terms of consuming more than their own body weight in blood from a host. Those in the sanguivorous freshwater leech genera *Haementeria* de Filippi, 1849 and *Placobdella* Blanchard, 1893 typically exhibit foliaceous crop ceca, permitting considerable expansion during blood feeding and implying equivalently durable environments for recovery of host DNA even in sub-optimal preservation regimes.

A large leech specimen was found attached to the head of a captive female Antillean manatee, *Trichechus manatus* Linnaeus, 1758, during a veterinary examination in March of 2015. The host is 1 of 3 manatees in a 6,824-m² artificial lagoon located in Palenque, Chiapas, Mexico (17°29’10”N, 92°01’10”W). In light of a lack of alternatives at the time, the leech was placed in commercially available Mezcal (a beverage distilled from agave), stored at 4 C, and later deposited at the Colección Nacional de Helmintos of the Instituto de Biología, Universidad Nacional Autónoma de México (catalog number 5670). DNA was extracted from

a small portion of the posterior sucker using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, Wisconsin). Two iDNA samples were recovered, 1 each from the anterior crop and the posterior crop, using the same purification kit as for the leech. Amplicons of the barcoding locus *cox1* and sequencing reactions were obtained using universal *cox1* primers (Folmer et al., 1994) under the same protocols and conditions as described by Phillips et al., (2010). Sequences were reconciled and edited in Geneious Pro v.5.1.7 (Biomatters Ltd., Auckland, New Zealand).

Preliminary barcoding identifications employed remote BLASTn (Altschul et al., 1990) comparisons to the non-redundant (nr) database of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Confirmatory identifications proceeded through phylogenetic grouping of sequenced isolates with comparable *cox1* records retrieved from the nr database. These included representatives of the genus *Haementeria* for the leech sucker isolate using species of *Helobdella* Blanchard, 1896 as a root and, for the blood meal sample, members of the order Sirenia Illiger, 1811 (*T. manatus* and *Dugong dugon* [Müller, 1776]) using *Meriones tamariscinus* (Pallas, 1773) as a root. Sequences were aligned using MUSCLE v3.7 (Edgar, 2004) on the European Bioinformatics Institute server (<http://www.ebi.ac.uk/Tools/msa/muscle/>) and analyzed with TNT v.1.1 (Goloboff et al., 2008). Genetic distances using the Kimura two-parameter (K2P) substitution model were calculated in PAUP* v.4.0 (Swofford, 2002). GenBank accession codes are indicated after taxon names in Figure 1.

Notwithstanding the successful preservation of DNA by fixation in Mezcal, comparison of distorted morphological information of the leech to relevant literature (Ringuelet, 1985; Ocegüera-Figueroa, 2006, 2008) allowed determination only to the level of genus: *Haementeria*. Parsimony analysis of the leech barcoding dataset resulted in 2 equally parsimonious trees of 900 steps each (Fig. 1A), placing the leech from the manatee with samples of *Haementeria acuecuyetzin* Ocegüera-Figueroa, 2008 previously available from Veracruz and Oaxaca, Mexico. Intraspecific K2P distances between this leech and other *H. acuecuyetzin* were less than 0.1%. The blood meal barcoding dataset resulted in a single tree of 182 steps (Fig. 1B), confirming the presence of manatee DNA in the crop of the leech. Intraspecific K2P distances between crop isolates and *cox1* from the mitochondrial genome sequence of *T. manatus* were also less than 0.1%.

Barcoding data unequivocally demonstrate the first known instance of leech ectoparasitism of a manatee. Previously, Hartman (1979) noted that a few small leeches were occasionally encountered in the diversity of invertebrates found among blue-green algal mats on sirenians. With the exception of a copepod (Humes, 1964), the wide variety of epibionts recorded from sirenians, including copepods, ostracods, barnacles, tanaids, isopods, and small gastropods (Nico et al., 2009), are understood to be ectophoretic, not parasitic (Hartman, 1979). Similar phoresis by non-sanguivorous species of *Helobdella* are well documented (Reyes-Prieto et al., 2013). In this instance, however, parasitism was demonstrable by the presence of amplifiable DNA of *T. manatus* throughout the crop of the leech.

Haementeria acuecuyetzin, as do other species in the genus, is believed to feed from a variety of vertebrate hosts, including mammals if they are available (Ocegüera-Figueroa, 2008). While not previously known from the state of Chiapas, *H. acuecuyetzin* has been

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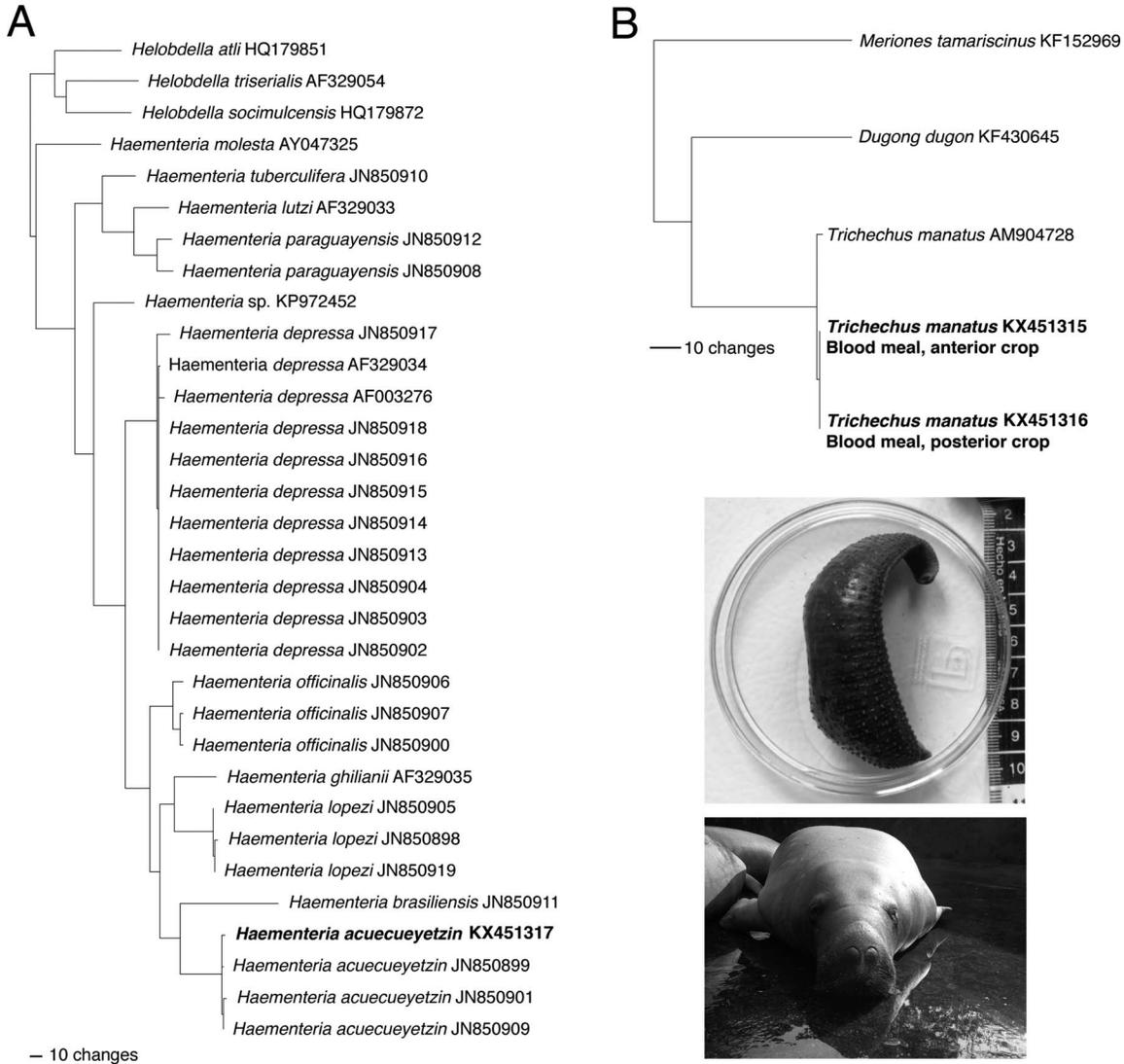


FIGURE 1. Phylogenetic trees derived from parsimony analyses of the COI datasets. (A) Analysis of leech sequences. (B) Analysis of manatee sequences and closely related organisms. Branch lengths are drawn proportional to the amount of change. Codes after terminal names indicate GenBank accession numbers. Taxa in bold were sequenced for the present study.

documented from the neighboring states of Veracruz and Oaxaca (Oceguera-Figueroa and León Rêgagnon, 2014). *Trichechus manatus* inhabits the coastal and inland waterways of Southeast Mexico in the States of Veracruz, Tabasco, Chiapas, Campeche, Yucatán, and Quintana Roo (Rodas-Trejo et al., 2008; Flores-Cascante, 2010). It is not presently possible to determine whether this observed association is restricted to the captive situation or is also a naturally occurring phenomenon. That manatees are euryhaline, inhabiting marine, fresh, and brackish water (Hartman, 1979; Reynolds and Odell, 1991), may effectively minimize overall exposure to ectoparasites that are incapable of managing osmotic stress. Moreover, although glossiphoniid leeches do vector haemogregarines (Siddall and Desser, 1991), flagellates (Siddall and Desser, 1992; Fermiño et al., 2015), and viruses (Greenblatt et al., 2004), there is no documented evidence that they serve as vectors of any blood parasites of mammals.

We believe this is the first instance of successful amplification of DNA extracted from animal tissue fixed with Mezcal, save for that of the caterpillar “worms” more commonly found in that beverage (Shokralla et al., 2010).

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