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Molecular phylogeny of the New World bloodfeeding leeches of the genus *Haementeria* and reconsideration of the biannulate genus *Oligobdella*

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ABSTRACT

The phylogenetic relationships of species of the New World bloodfeeding genus *Haementeria* were investigated for the first time. The analysis included five molecular markers. The mitochondrial COI, 12S and ND1 as well as the nuclear 28S and ITS. The evolutionary history of the group was investigated through Maximum Parsimony and Bayesian Inference. Both phylogenetic methods resulted in highly congruent hypotheses. The correlation between the phylogeny and morphological traits such as eyespot number, annulation, Lang's organs, salivary glands, bacteriomes and reproductive organs is discussed. Restricted to *Haementeria* are Lang's organs, spherical bacteriomes and ovaries forming an anterior ring around the ventral nerve cord. In addition, *Oligobdella brasilensis* was formally transferred to *Haementeria*, providing additional arguments for the disposal of the genus *Oligobdella*. *Haementeria gracilis* is shown to be just a junior synonym of *Haementeria depressa* as suggested by previous authors. Finally, the geographical distribution of species of *Haementeria* was compared with that of other non-leech and leech taxa. Multiple events of South–North American interchange were proposed to explain the current geographical distribution of the species of *Haementeria*.

1. Introduction

The genus *Haementeria* (*haemo* = blood; *enterium* = intestine) was erected to accommodate a large and voracious bloodfeeding leech from South America: *Haementeria ghilianii* (de Filippi, 1849). The so-called "Giant Amazonian leech" is the type species of the genus and is the largest recorded leech species reaching almost 500 mm in length (Sawyer et al., 1981). Scientific interest in species of *Haementeria* has a history dating to when leeches were commonly used to bleed people in order to heal several kinds of afflictions. The use of, what was probably *Haementeria officinalis* de Filippi, 1849 in México as a medicinal leech and its deleterious effect were documented by Jiménez (1865, 1866). More recently, *Haementeria* species have been the focus of several studies, ranging from the characterization of anticoagulant proteins (Holt et al., 1989; Salzet, 2001; Tuszynski et al., 1987), gene expression of the salivary glands (Faria et al., 2005), neuron physiology and regeneration (De-Miguel et al., 2001) to cophylogenetic analyses of *Haementeria* spp. and their endosymbiotic gammaproteobacteria (Perkins et al., 2005).

Since its establishment, at least 12 hematophagous species of *Haementeria* have been described. Several of them were previously members of different genera that subsequently were lumped into *Haementeria*, such as *Blenobdella* E. Blanchard, 1849, *Hybobdella* Weyenbergh, 1879 and *Liostomum* Blanchard, 1899. All species of the genus *Haementeria* are distributed in the New World, from Patagonia to the Mesa Central of México (Oceguera-Figueroa, 2008; Ringuelet, 1985; Sawyer, 1986). In a taxonomic revision of Hirudinea, Autrum (1936) synonymized the Palearctic genus *Placobdella* Blanchard, 1893 with *Haementeria* based on the somite annulation pattern, the presence of compact salivary glands and their bloodfeeding habit. Autrum's (1936) proposal was disregarded by most leech taxonomists who still considered *Placobdella* and *Haementeria* as separate genera. Caballero (1956, 1959), and more recently Sawyer (1986), even considered them to belong to different subfamilies (Glossiphoniinae and Haementeriinae respectively). Siddall et al. (2005) investigated the phylogenetic relationships of a variety of members of the family Glossiphoniidae using molecular data; finding that *Placobdella*, *Haementeria* and *Helobdella* constitute a moderately well supported clade notwithstanding the preference for hemolymph and soft tissues of invertebrates by *Helobdella* species.

Based on glossiphoniid specimens that were found feeding on desmognathine salamanders in North America, *Oligobdella biannulata* (Moore, 1900) was described. The most remarkable characteristic of this species was the presence of only two annuli per somite, in contrast to the three-annulate condition that is characteristic of

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the family. Later, at least three additional biannulate species were described from South America and included in the genus *Oligobdella* (see Ringuelet, 1985; Sawyer, 1986). Phylogenetic analysis found *O. biannulata* nested well within a clade of *Placobdella* spp. and was transferred to the genus *Placobdella* (Siddall et al., 2005). Accordingly, South American species initially placed in *Oligobdella* could have been transferred to *Placobdella*, but this issue was not discussed by Siddall et al. (2005) nor has it been analyzed in a phylogenetic context.

In this study, we present a phylogenetic analyses of the genus *Haementeria* including a broad taxonomic sample of recently collected and described species as well as an expanded set of nuclear and mitochondrial molecular markers. In order to investigate if biannulate species are each others closest relatives, *Oligobdella brasilensis* (Cordero, 1937), from Brazil and *Placobdella biannulata* from USA were included in this analysis. The phylogenetic relationships of the group are investigated using two phylogenetic methods; Maximum Parsimony and Bayesian Inference. Internal and external morphological characteristics of *Haementeria*, *Helobdella* and *Placobdella* species are discussed in light of their phylogenetic relationships. Finally, the geographical distribution of the group is analyzed and compared with other taxa.

2. Materials and methods

2.1. Taxa

Newly collected samples of *Haementeria* spp. included in this study represent a broad geographical distribution including Argentina, Brazil, México and Perú. Information of newly collected material as well as additional material included in this study, with the locality and GenBank accession numbers is presented in Table 1. Unless otherwise indicated, leeches were collected by immersing legs into water at the edges of lakes or ponds, waiting for 5 min and examining for leeches attached to skin. Leeches were narcotized using an ethanol gradient, adding a few drops of 96% ethanol to a plastic container with water covering the leeches until the leeches were relaxed. Specimens then were transferred to 96% ethanol for storage.

2.2. Morphology

Leech specimens were identified using taxonomic keys and descriptions (Ringuelet, 1976, 1981, 1985; Sawyer, 1986) and through the comparison with voucher specimens deposited in the Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México and in the Invertebrate Collection, National Museum of Natural History, USA. Examination and dissections of selected specimens were done with a Nikon SMZ-U stereomicroscope.

2.3. Molecular techniques

Leech tissue samples were obtained from the posterior sucker of the leech in order to minimize the possibility of contamination from host DNA that could be present in the gastro-intestinal tract. DNeasy Tissue Kit (Qiagen, Valencia, CA) was used for tissue lysis, total DNA extraction and purification. The mitochondrial cytochrome *c* oxidase subunit I (COI), nicotinamide adenine dinucleotide dehydrogenase subunit I (ND1), 12S rDNA (12S), as well as

Table 1
Collection localities and GenBank accession numbers for species used for the phylogenetic analyses of the species of the genus *Haementeria*.

	Locality	COI	12S	NADH	ITS	28S
Outgroup						
Placobdella biannulata	North Carolina, USA	AF116021	AY425435	AY047343	–	AY425397
Placobdella costata	Italy	AY962461	JN850873	AY962448	JN850935	–
Placobdella ornata	Ontario, Canada	AY047326	JN850872	AY047352	JN850934	–
Placobdella parasitica	Ontario, Canada	AF003261	AY425438	AY047348	–	AY425401
Helobdella bolivianita	Santa Cruz, Bolivia	AF329053	–	AF329076	–	–
Helobdella elongata	Michigan, USA	AF329045	JN850882	AF329068	–	–
Helobdella triserialis	Santa Cruz, Bolivia	AF329054	JN850883	AF329077	–	–
Ingroup						
Oligobdella brasilensis	Brazil	JN850911	JN850874	JN850936	–	JN850895
Haementeria acuecueyetzin Haeo	Oaxaca, Mexico	JN850909	JN850871	JN850939	JN850931	–
Haementeria acuecueyetzin HaOfl	Veracruz, Mexico	JN850901	JN850863	JN850938	JN850923	JN850887
Haementeria acuecueyetzin Hacu	Catemaco, Veracruz, Mexico	JN850899	JN850862	–	JN850921	JN850885
Haementeria depressa 12A	Colonia, Uruguay	JN850904	JN850866	JN850945	JN850926	–
Haementeria depressa 12B	Colonia, Uruguay	JN850917	JN850879	JN850949	–	–
Haementeria depressa 38A	Maldonado, Uruguay	JN850902	JN850864	JN850943	JN850924	JN850888
Haementeria depressa 38B	Maldonado, Uruguay	JN850915	JN850877	JN850947	–	–
Haementeria depressa 38C	Maldonado, Uruguay	JN850916	JN850878	JN850950	–	–
Haementeria depressa 42A	Jaureguiberry, Uruguay	JN850903	JN850865	JN850944	JN850925	JN850889
Haementeria depressa 42B	Jaureguiberry, Uruguay	JN850918	JN850880	JN850951	–	–
Haementeria depressa EST	Brazil	CN807309.1	CN807801.1	HDAH04B07	–	–
Haementeria depressa UR23	La Trinidad, Uruguay	JN850913	JN850875	JN850946	–	–
Haementeria depressa UR23B	La Trinidad, Uruguay	JN850914	JN850876	JN850948	–	–
Haementeria ghilianii GB	BioPharm, French Guiana	AF329035	AY425417	AF329058	JN850932	AY425374
Haementeria gracilis	Valdivia, Chile	AF003276	–	–	–	–
Haementeria gracilis	Arroyo Espinas, Uruguay	AF329034	AY425418	AF329057	–	AY425375
Haementeria lopezi HaCol	Colima, Mexico	JN850898	JN850861	JN850940	JN850920	JN850884
Haementeria lopezi HoBm	Jalisco, Mexico. Ex *Rhinella marinus*	JN850905	JN850867	JN850941	JN850927	JN850890
Haementeria lopezi HoSb	Jalisco, Mexico Ex. *Smilisca boudinii*	JN850919	JN850881	JN850942	–	JN850897
Haementeria lutzi	Rio Pastaza, Ecuador	AF329033	–	AF329056	–	–
Haementeria officinalis HR	Michoacan, Mexico	JN850906	JN850868	JN850952	JN850928	JN850891
Haementeria officinalis HDur	Durango, Mexico	JN850900	–	JN850953	JN850922	JN850886
Haementeria officinalis HFDM	Queretaro, Mexico	JN850907	JN850869	JN850954	JN850929	JN850892
Haementeria paraguayensis Pe16	Iquitos, Peru	JN850912	–	–	–	JN850896
Haementeria paraguayensis UR46	Chaco, Argentina	JN850908	JN850870	–	JN850930	JN850893
Haementeria tuberculifera Pe15	Iquitos, Peru	JN850910	–	JN850937	JN850933	JN850894