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RESEARCH PAPER

DNA barcoding reveals Mexican diversity within the freshwater leech genus *Helobdella* (Annelida: Glossiphoniidae)

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Abstract
We investigated the genetic distances and taxonomic status among species of *Helobdella*, a genus of non-blood-feeding leeches, based on mitochondrial cytochrome *c* oxidase subunit I sequences. Sampling included 20 specimens representing nine nominal species collected in 11 states in Mexico as well as previously published sequences of different species of *Helobdella* from several places. A neighbor-joining tree, as well as identification of diagnostic nucleotides, was used to suggest the presence of seven species of *Helobdella* in Mexico including potentially two undescribed forms.

Keywords: COX1, *DNA barcoding*, Helobdella, *Mexico*

Introduction

The non-blood-feeding genus *Helobdella* Blanchard, 1896 (Annelida: Glossiphoniidae) may be the most diverse genus of leeches including more than 50 species. Even though some species have been recorded and described in all continents (with the exception of Antarctica), South America is where the highest diversity of species is found (Ringuelet 1985; Sawyer 1986). Based on phylogenetic analyses, it has been proposed that leeches in the genus *Helobdella* evolved from a blood-feeding ancestor that shifted to feed on the hemolymph of mollusks and other freshwater invertebrates (Siddall and Borda 2003; Siddall et al. 2005). Sawyer (1986) subdivided the genus into two main groups or series of species: a "*stagnalis*" series defined by the presence of a chitinous nuchal scute, including the type species for the genus *Helobdella stagnalis* (Linnaeus 1758); and a "*triserialis*" series for leeches having longitudinal stripes on the dorsal surface including *Helobdella triserialis* Blanchard, 1849 and related forms. Phylogenetic analyses of the group recognized the monophyly of both series (Siddall and Borda 2003; Siddall et al. 2005). However, nested within those groups were species of the genera *Gloiobdella* and *Adaetobdella* that were, subsequently, synonymized with *Helobdella*. In Mexico, six species of *Helobdella* are currently recognized (Oceguera-Figueroa and León-Règagnon 2005; Oceguera-Figueroa 2007). Two belong to the "*stagnalis*" series: *Helobdella atli* (Oceguera-Figueroa & León-Règagnon 2005) and *H. stagnalis*. Three species are in the "*triserialis*" series: *Helobdella virginiae* (Oceguera-Figueroa 2007), *Helobdella conchata* Caballero, 1941 and *H. triserialis*. Two additional species now considered junior synonyms of *H. triserialis* have been described for Mexico: *Helobdella socimulcensis* Caballero, 1932 and *Helobdella moorei* Caballero, 1933 (see Ringuelet 1981). The sixth valid species is *Helobdella* (*Gloiobdella*) *elongata* Caste, 1900.

Here, we reanalyze the taxonomic status of the Mexican species of *Helobdella* including specimens representing all of the species names recorded and

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described from Mexico, notwithstanding their nomenclatural validity, as well as other leech representatives from several parts of the world.

Materials and methods

Specimen collection

Twenty specimens of *Helobdella* were collected from 2002 to 2008, primarily from Mexico (Table I). Specimens belonging to seven nominal species of *Helobdella* were collected from 11 states in Mexico, one sample was collected near Hoedspruit, South Africa and two in Washington State, USA. Specimens were collected under the scientific collecting license FAUT0056 issued to Virginia León-Règagnon. Leeches were hand-collected from submerged rocks and plants. All specimens were relaxed with the gradual addition of 70% ethanol and fixed in 96% ethanol. Voucher specimens were deposited in the Colección Nacional de Helmintos, Instituto de Biología, Universidad Nacional Autónoma de México.

Sequences of the mitochondrial cytochrome *c* oxidase subunit I (*COX1*) of 20 specimens from Mexico, two from the USA, and one from South Africa were newly generated for the present study. Methods of leech DNA extraction, *COX1* amplification and sequencing have been described elsewhere (Apakupakul et al. 1999; Borda and Siddall 2004). Sequences of 42 species of *Helobdella* from previous studies were also included in the present analyses for comparative purposes. *COX1* sequences of *Haementeria ghilianii* de Filippi, 1849 and *Haementeria gracilis* Cordero, 1941 were used to root the analysis since they constitute the sister group of *Helobdella* (Siddall and Borda 2003; Siddall et al. 2005).

Alignment, neighbor-joining analysis, and recognition of molecular characteristic attributes

All of the *COX1* sequences obtained for this study, as well as sequences obtained from GenBank, were aligned with MUSCLE (Edgar 2004) on the European Bioinformatics Institute webserver (http://www.ebi.ac.uk/Tools/muscle/index.html), applying default settings. A neighbor-joining tree was calculated in PAUP* (Swofford 2002) using the Kimura two-parameter model of nucleotide substitution (Kimura 1980) following previous barcoding studies (Hebert et al. 2004). All of the distance values among *COX1* sequences were calculated in PAUP* using the Kimura two-parameter model. Diagnostic nucleotide positions for particular clusters (molecular synapomorphies) were determined through the implementation of the Characteristic Attribute Organization System software (Sarkar et al. 2002a,b).

Results

The neighbor-joining tree (Figure 1) resulting from the analyses of *COX1* sequences of 63 samples of *Helobdella* rooted with two species of *Haementeria* recovers as a cluster all of the species of *Helobdella*. The "*stagnalis*" series was represented as a paraphyletic assemblage relative to a monophyletic "*triserialis*" series. Samples of *Helobdella* "*stagnalis*" from Mexico were found in two separate parts of the tree. The first forming a paraphyletic group (*Helobdella* "*stagnalis* 1") presents 1.2% of genetic variation within its members and appears closely related to *Helobdella octatestisaca* from Taiwan and one sample from South Africa. The genetic variation within the latter group averaged 0.4%, and between those and *H.* "*stagnalis* 1" the genetic distance averaged 2.2%. The second cluster (*Helobdella* "*stagnalis* 2") was found nestled between the "*stagnalis*" and "*triserialis*" series. Within the "*triserialis*" series, *H. virginiae* and *Helobdella* sp. from San Luis Potosi, Mexico, appeared grouped with various forms of *Helobdella* "*robusta*", *Helobdella lineata*, *Helobdella papillata*, and *Helobdella transversa* from the USA. *H. elongata* from Mexico appeared in the same cluster with *H. elongata* from the USA, these two specimens having a genetic distance of 7%. *H. moorei*, *H. conchata*, *H. socimulcensis* and forms of *H. triserialis* from several localities of Mexico, and an unidentified leech from San Francisco, California grouped in a single cluster with an average of 1.3% genetic distance among them. This cluster appeared most closely related (average distance of 3.9%) to the genetically homogeneous ($<0.4\%$ within-group genetic distance) and globally invasive *Helobdella europaea* Kutschera, 1985.

Discussion

The "stagnalis" series

There has historically been considerable taxonomic confusion surrounding the name *H. stagnalis*, and the fact that specimens identified as *H. stagnalis* fall into three different parts of the tree may partly reflect this confusion. This species was described by Linnaeus in 1758 based on common European specimens. Historically, the presence of a conspicuous chitinous scute on the dorsal surface, as seen on leeches collected in several areas of the world, would lead to diagnosis as *H. stagnalis* such that this has been considered a cosmopolitan species (Sawyer 1986). For example, a nearly indistinguishable leech described as *Helobdella modesta* Verrill, 1872 was later synonymized under *H. stagnalis* (Klemm 1972, 1982; Sawyer 1986). Siddall et al. (2005) found a high degree of genetic variation between *H. stagnalis* collected from the UK relative to those from Ohio, USA and reestablished Verrill's name *H. modesta* for North American species (see also Madill and Hovingh 2007; Oceguera-

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Table I. Taxa, localities, and GenBank accession numbers for the *COX1* sequences/catalog number (Colección Nacional de Helmintos [CNHE], UNAM, México) of leeches of *Helobdella* spp. used in the neighbor-joining analyses.

Taxon	Locality	GenBank/CNHE catalog number
Haementeria ghilianii	Biopharm (French Guiana)	AF329035
Haementeria gracilis	Arroyo Espinas, Uruguay	AF329034
Helobdella atli	Totolcingo, Tlaxcala, Mexico	HQ179851*/5208-10
Helobdella atli	Aljojuca, Puebla, Mexico	HQ179850*/5531
Helobdella atli	Xochimilco, D. F., Mexico	HQ179852*/5532
Helobdella bolivianita	Laguna Volcan, Bolivia	AF329053
Helobdella conchata gray	Cuautla, Morelos, Mexico	HQ179871*
Helobdella conchata papillated	Cuautla, Morelos, Mexico	HQ179872*
Helobdella elongata	Michigan, USA	AF3229045
Helobdella "elongata"	Jalisco, Mexico	HQ179863*/5538
Helobdella europaea 1	Taiwan	FJ000350
Helobdella europaea 2	Taiwan	FJ000352
Helobdella europaea 3	Taiwan	FJ000351
Helobdella europaea 4	Taiwan	FJ000349
Helobdella europaea NZ	New Zealand	AY856049
Helobdella europaea SA	South Africa	AY856048
Helobdella europaea (=*H. papillornata*)	Aura Vale Lake, Australia	AY856047
Helobdella europaea	Germany	AY576008
Helobdella fusca	Wild Goose Lake, Michigan, USA	AF329038
Helobdella lineata	Michigan, USA	AF329039
Helobdella melananus 1	Taiwan	FJ000353
Helobdella melananus 2	Taiwan	FJ000354
Helobdella melananus 3	Taiwan	FJ000355
Helobdella michaelseni	Lago Calafquen, Chile	AF536824
Helobdella modesta	Columbus, OH, USA	AF329040
Helobdella modesta HW1	Washington, USA	HQ179853*
Helobdella modesta HW2	Washington, USA	HQ179854*
Helobdella moorei	Guanajuato, Mexico	HQ179870*/5569
Helobdella nunununununojensis	Madidi, Bolivia	AF329048
Helobdella octatestisaca 1	Taiwan	FJ000342
Helobdella octatestisaca 2	Taiwan	FJ000343
Helobdella octatestisaca 3	Taiwan	FJ000344
Helobdella octatestisaca 4	Taiwan	FJ000345
Helobdella octatestisaca 5	Taiwan	FJ000346
Helobdella octatestisaca 6	Taiwan	FJ000347
Helobdella octatestisaca 7	Taiwan	FJ000348
Helobdella papillata Mi	Michigan, USA	AF329042
Helobdella papillata Vi	Virginia, USA	AF329046
Helobdella paranensis	Arroyo Espinas, Uruguay	AF329037
Helobdella pichipanan	Lago Chico, Chile	AY962456
Helobdella ringueleti	Madidi, Bolivia	AF329051
Helobdella "robusta" TXAU1	Austin, TX, USA	DQ995306
Helobdella "robusta"	Sacramento, CA, USA	DQ995301
Helobdella "robusta" CASA 1	Sacramento, CA, USA	DQ995299
Helobdella "robusta" NYTA	New York, Valhalla College, USA	DQ995305
Helobdella socimulcensis	Xochimilco, Mexico	DQ995311
Helobdella sorojchi	Madidi, Bolivia	AF329050
Helobdella sp.	San Luis Potosí, Mexico	HQ179865*/5565
Helobdella "stagnalis"	South Africa	HQ179860*
Helobdella "stagnalis 1"	Guanajuato, Mexico	HQ179858*/5546
Helobdella "stagnalis 1"	Mexico, Hidalgo149	HQ179857*
Helobdella "stagnalis 1"	Queretaro, Mexico	HQ179855*/5549
Helobdella "stagnalis 1"	Tabasco, Mexico	HQ179859*/5545
Helobdella "stagnalis 1"	Ameca, Jalisco H001	HQ179856*/5548
Helobdella stagnalis	Costwolds, UK	AF329041
Helobdella "stagnalis A2"	Temixco, Morelos, Mexico	HQ179862*/5552
Helobdella "stagnalis B2"	Temixco, Morelos, Mexico	HQ179861*/5552
Helobdella transversa	Michigan, USA	AF329044
Helobdella triserialis	Laguna Volcán, Bolivia	AF329054
Helobdella triserialis	California, USA	DQ995303
Helobdella triserialis	Querétaro, Mexico	HQ179868*/5563
Helobdella triserialis	Guanajuato, Mexico	HQ179867*/5561
Helobdella triserialis	Hidalgo, Mexico	HQ179869*/5560
Helobdella triserialis	Jalisco, Mexico	HQ179866*/5562
Helobdella virginiae	Catemaco, Veracruz, Mexico	HQ179864*/5474-76

*New *COX1* sequences.

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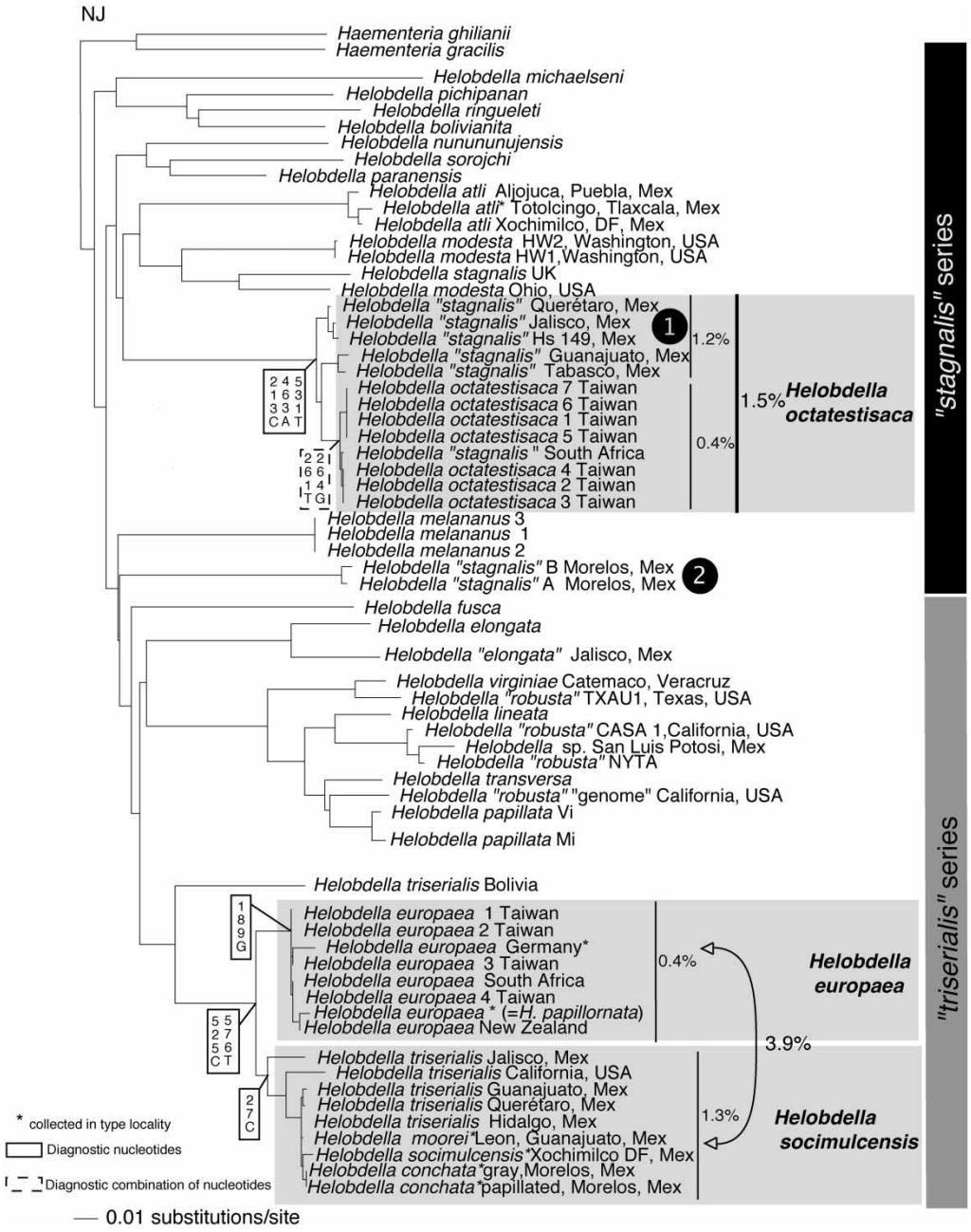

Figure 1. Neighbor-joining tree based on the Kimura two-parameter substitution model of the *COX1* locus of representative species of *Helobdella* showing the "*stagnalis*" and "*triserialis*" series. Numbers next to straight lines indicate average genetic distance within samples of a particular cluster. Number next to arrows indicates average genetic distance of pairwise comparisons between members of the two different clusters (*H. europea* and *H. socimulcensis*). Asterisks indicate specimens collected in the type locality. Numbers next to vertical lines indicate average genetic distances.

Figueroa et al. in press). Mexican scutiferous samples were found in three different parts of the tree as putatively distinct species: *H. atli*, *H.* "*stagnalis* 1", and as-yet undescribed species from Temixco, Morelos here designated as *H.* "*stagnalis* 2". All three samples of *H. atli*, including that collected in Totolcingo, Tlaxcala, the type locality for the species, were found forming a single cluster next to the lineage that includes *H. modesta* and European *H. stagnalis*.

Helobdella "*stagnalis*" from several localities in Mexico clustered with the recently described *H. octatestisaca* Lai & Chang, 2009 from Taiwan, a lineage that also included a South African specimen. Lai et al. (2009) suggested that *H. octatestisaca* might be a recently introduced species in Taiwan, because neither exhaustive fieldwork nor thorough examinations of scientific collections had previously uncovered this species. The extremely low genetic variation (0.4%) within the samples of *H. octatestisaca* and the

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sample from South Africa contrasts with the 1.5% among the whole cluster when also including the Mexican samples. This fact is in agreement with previous studies in a variety of organisms, including *H. europaea* (Siddall and Budinoff 2005; see Figure 1), predicting that invasive species show relatively low genetic variation compared with that of their source population (Tsutsui et al. 2000; Suarez and Tsutsui 2008).

The "triserialis" series

Helobdella triserialis was originally described based on specimens collected in Chile. However, because of the high degree of pigment variation throughout its presumed range, Ringuelet (1943) recognized at least four subspecies. Siddall and Borda (2003) found that North and South American forms constitute distinct evolutionary lineages and expanded Verrill's (1872) name *Helobdella papillata* for North American representatives. Surprisingly, *H. elongata* is included in this cluster notwithstanding its unusual morphology and is only distantly related (>2.5% genetic distance) to the morphologically similar (and formerly congeneric under *Gloiobdella*) South American counterpart *Helobdella michaelseni*. This suggests that several morphological attributes (i.e. cylindrical body, unpigmented teguments, and absence of gastric ceca) are unreliable indicators of recent diversification.

H. robusta is perhaps the best-known lophotrochozoan model organism. Efforts to understand the complex developmental mechanisms of this species culminated with the sequencing of its full genome (Weisblat and Kuo 2009). Bely and Weisblat (2006) have demonstrated that at least three different lineages of leeches previously considered to be *H. robusta* have been independently employed in developmental biology research. Complicating this issue was that two distinct and unrelated *COX1* lineages of *H. robusta* are found in the type locality in Sacramento, CA, USA ("CASA 1" and "genome" in Figure 1). In the absence of a more detailed morphological analysis of each of the different lineages in comparison with the holotype, the problem of which lineage is the real *H. robusta* remains unresolved. Indeed, the full genome that was sequenced may well belong to an undescribed species. *H. virginiae* and *Helobdella* sp. from San Luis Potosí, Mexico also appeared to be closely related to specimens of *H.* "*robusta*" from Texas and New York, respectively. In both cases, branch lengths suggest that they might represent independent species. Rather than quickly multiplying the number of species representing *H. robusta* on the basis of a single locus, we should also consider the possibility that *H. virginiae*, *H. robusta*, *H. lineata*, *H. transversa*, and *H. papillata* are capable of limited introgression to the extent that *COX1* may not provide a reliable indication of species groups for this particular cluster.

In Mexico, three species morphologically similar to *H. triserialis* have been described. *H. socimulcensis* Caballero, 1931 from Xochimilco, D. F. and *H. moorei* Caballero, 1933 from León, Guanajuato, each were considered to be junior synonyms of *Helobdella triserialis lineata* by Ringuelet (1981). The third species in this series is *H. conchata* Caballero, 1941 from Cuautla, Morelos, Mexico. Our results, including several samples for each name and including samples from the respective type localities, failed to recognize significant differences among them and strongly suggest that this entire group should be considered a single species. This cluster forms a lineage independent of *H. triserialis sensu stricto* (Bolivia) and, given the lack of morphological differences, the name *H. socimulcensis* Caballero, 1931 would be used for this group, which appears to be closely related to *H. europaea*.

Invasive species

The pattern of an invasive species with low genetic variation next to samples collected in their inferred natural habitat displaying high levels of genetic variation was found in two independent parts of the tree. *H. octatestisaca* and *H. europaea* were originally described from Taiwan and Germany, respectively. Both are geographic areas well removed from what appears to be their otherwise Helobdella robusta New World distribution. In both cases, Mexican samples appeared next to the putative invasive species clusters, but in any case identical *COX1* sequences were found across them. Even though the general pattern in both parts of the tree seems similar, a closer analysis of each case would give different results. In both cases, the genetic distance between the Mexican populations and the invasive species averages more than 2%, a number that seems high enough to suggest the presence of multiple species (Hebert et al. 2004). Furthermore, in both cases, each group taken as a whole presents diagnostic nucleotides; in the case of the *H. octatestisaca* cluster, position 213 of the alignment presents a cytosine (C), 463 an adenine (A), and 531 a thymine (T), while the cluster *H. europaea* + *H. socimulcensis* presents two diagnostic nucleotides at positions 525 (C) and 576 (T). The difference between the two cases is that the cluster of *H. europaea* has an exclusive guanine (G) at position 189, whereas *H.* "*stagnalis* 1" samples form a paraphyletic assemblage. In addition, *H. socimulcensis* presents a diagnostic C at position 27, but on the contrary *H. octatestisaca* lacks a diagnostic nucleotide, but exhibits a diagnostic combination of T and G at positions 261 and 264. With this collective information in mind, it seems reasonable to suggest the renaming of the Mexican samples of *H.* "*stagnalis* 1" as *H. octatestisaca*, but keeping different names for the invasive species *H. europaea* and for the Mexican

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samples that, in agreement with their genetic similarity, should be renamed *H. socimulcensis*.

The use of DNA barcoding to identify species relies on the assumption that *COX1* variation between species (i.e. interspecific) exceeds by a considerable amount the variation present within species (i.e. intraspecific). Although the straight use of genetic distances (>2%) as a criterion to differentiate species would lead to considering *H.* "*stagnalis* 1" as a species independent of *H. octatestisaca*, we think this could be an overestimation of species-level biodiversity. Using a discrete, fixed character-based approach represents, in our opinion, a better option, because it is in agreement both with the philosophical approaches of modern methods of phylogenetic analyses and with the need for diagnosis in classical taxonomy.

In conclusion, at least seven species of *Helobdella* occur in Mexico: *H. atli*, *H. octatestisaca*, *H. virginiae*, *H. elongata*, *H. socimulcensis*, and two forms diagnosed only with molecular data—*Helobdella* sp. from San Luis Potosi and *H.* "*stagnalis*" from Temixco, Morelos.

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