BIODIVERSITY, TAXONOMY AND SYSTEMATICS OF NEW WORLD FRESHWATER LEECHES (ANNELIDA: HIRUDINEA) WITH PARTICULAR EMPHASIS ON GLOSSIPHONIID LEECHES AND THEIR BACTERIAL ENDOSYMBIONTS

BY

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ABSTRACT

BIODIVERSITY, TAXONOMY AND SYSTEMATICS OF NEW WORLD FRESHWATER LEECHES (ANNELIDA: HIRUDINEA) WITH PARTICULAR EMPHASIS ON GLOSSIPHONIID LEECHES AND THEIR BACTERIAL ENDOSYMBIONTS.

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The phylum Annelida Lamarck, 1809 includes segmented worms such as leeches, earthworms, lugworms, sandworms and clamworms that inhabit almost all possible environments and places of the world. Leeches (Class Hirudinea) represents only one group of around 680 species out of the approximately 16,500 described species of Annelida. The class Hirudinea has been divided in two groups based on their mouthparts. The order Rhynchobdellida, a paraphyletic assemblage, includes species with a large and eversible proboscis and the order Arhynchobdellida that includes species with a muscular pharynx with or without jaws. Both orders include organisms specialized to feed on vertebrate blood. This study includes the description of eigth species of leeches new to science that belong to three families (Glossiphoniidae, Macrobdellidae and Praobdellidae). The phylogenetic relationships of species of three families (Glossiphoniidae, Macrobdellidae and Praobdellidae) and one suborder (Erpobdelliformes) were investigated using molecular and morphological data and a suite of phylogenetic methods (Parsimony, Maximum Likelihood and Bayesain Inference). The description of the new species *Tyrannobdella rex* (Praobdellidae) and *Oxyptychus bora* (Macrobdellidae) are discussed in the context of their placement in phylogenies.

The phylogenetic study of Erpobdelliformes includes the comparison of alternative classification schemes. Based on the results, the phylogenetic position of the terrestrial and macrophagous *Orobdella octonaria* (Gastrostomobdellidae) within the Erpobdelliformes is established for the first time. The phylogenetic relationships of the proboscis-bearing species of the genera *Haementeria*, *Helobdella* and *Placobdella* were investigated using a combination of nuclear and mitochondrial markers and Parsimony and Bayesian Inference methods. In addition to the monophyly of *Haementeria*, *Helobdella* and *Placobdella*, the 3 genera formed a monophyletic group notwithstanding their different feeding preferences. The correlation with phylogeny and some morphological traits is shown. These include, eyespot morphology, annulation patterns, shape of the ovisacs, sensory organs on the dorsal surface and presence of bacteriomes. Species of *Haementeria* and *Placobdella* have specialized organs called bacteriomes associated with their salivary complex that harbor symbiotic proteobacteria. Using DNA bacterial sequences (16S rRNA), the exclusive association of *Haementeria* spp. with gammaproteobacteria and *Placobdella* spp. with alphaproteobacteria is shown. Using pyrosequencing technology, the nucleotide sequences of a DNA sample extracted from the bacteriomes of *Placobdella parasitica* were analyzed. A total of 1,053,345 DNA fragments were obtained and assembled. Leech and symbiont DNA fragments were separated using Blast tools and 50 bacterial and *Helobdella robusta* genomes for reference. Finally, the so-called DNA barcoding protocol is discussed and some recommendations were given to increase the information content of the database (Bold system). In addition, DNA barcoding protocol was used to estimate the diversity of species of *Helobdella* from Mexico.

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«Yo soy el concienzudo del espíritu, respondió el interrogado, y en las cosas del espíritu difícilmente hay alguien que las tome con mayor rigor, severidad y dureza que yo, excepto aquel de quien yo he aprendido eso, Zaratustra mismo.

¡Es preferible no saber nada que saber mucho a medias! ¡Es preferible ser un necio por propia cuenta que un sabio con arreglo a pareceres ajenos! Yo - voy al fondo: - ¿qué importa que éste sea grande o pequeño? ¿Que se llame pantano o cielo? Un palmo de fondo me basta: ¡con tal que sea verdaderamente fondo y suelo!

- un palmo de fondo: sobre él puede uno estar de pie. En la verdadera ciencia concienzuda no hay nada grande ni nada pequeño.»

«¿Entonces tú eres acaso el conocedor de la sanguijuela?, preguntó Zaratustra; ¿y estudias la sanguijuela hasta sus últimos fondos, tú concienzudo?»

«Oh Zaratustra, respondió el pisado, eso sería una enormidad, ¡cómo iba a serme lícito atreverme a tal cosa!

En lo que yo soy un maestro y un conocedor es en el cerebro de la sanguijuela: - ¡ése es mi mundo!

Así habló Zaratustra, F. Nietzsche

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GENERAL CHARACTERISTICS OF THE GROUP

GENERAL CHARACTERISTICS OF THE GROUP

The phylum Annelida Lamarck, 1809 (from Latin "anellus" and Greek "eidos" form) includes approximately 16 500 described species, such as leeches, earthworms and tubeworms. Annelids are bilaterial organisms, segmented, schizocelic, protostomes, with complete digestive system, closed circulatory system with pigments such as hemoglobin, clorocruorina and hemerythrin, well-developed nervous system with a dorsal ganglion and longitudinal nerve cords, with metanephridial excretory apparatus, hermaphrodites and several species have trochophore larvae, which is lost secondarily in some groups. Most of the species of the phylum are marine, however some species inhabit terrestrial and freshwater environments. The phylum Annelida includes two classes: Polychaeta and Clitellata, the latter is composed by the subclass Oligochaeta (paraphyletic) containing more than 6 000 species of terrestrial, freshwater and some marine forms, arranged in approximately 25 families and the subclass Hirudinoidea, which includes species with a fixed number of somites annulated superficially, without setae or parapodia or if present, in small number. Species of the group present a clitellum and one or two suckers. The posterior sucker works as an attachment organ and is present in all members of the group. Anterior sucker abscent in one group. Almost all species live in freshwater or marine environments, some are semi-terrestrial, ectoparasites, predators or macrophagous (Brusca and Brusca, 2003). The Subclass Hirudinoidea includes 3 orders:

Order Acanthobdellida: Maximum body length 3 cm, freshwater habitat, ectoparasites of fish, body with 30 somites, posterior sucker present, setae restricted to anterior somites, partially reduced coelom, with one species: *Acanthobdella pelledina*, parasite of salmon and grayling.

Order Branchiobdellida: Usually, less than 1 cm long. Ectocommensals or ectoparasites of freshwater crustaceans, the body is composed of 15 somites, with a posterior sucker, without setae, small but spacious coelom.

Order Hirudinida: The members of this group are considered "true leeches" (for Sawyer, 1986 this group correspond to the Subclass Euhirudinea). Most of the species are freshwater and marine, some are semi-terrestrial or amphibious, hematophagous ectoparasites or free-living predators or scavengers. Some parasitic forms act as vectors pathogenic protozoa, treamtodes and cestodes. Setae absent, coelom reduced to a complex system of channels (lacuna). Body with 34 somites, of which only 27 are observed externally. The simplest complete somite (midd-body somite) consists of three primary annulli called a1, a2 and a3, the middle ring is the a2, which carries the sensilla or sensory organs and reflects the location of the ganglion. In some species, primary rings are split forming secundary annuli. Most leeches are dorsoventrally flattened (Davies, 1991). Besides gonopores and nefridiopores, leeches may have tubercules, papillae and another sensory organs. The digestive system is complete with mouth and anus at the ends. In members of the families Ozobranchidae, Piscicolidae and Glossiphoniidae, the pharynx is modified to form a muscular and eversible proboscis. Other species have might have a muscular pharynx with or without jaws. Continuing from the pharynx, the crop might be tubular or with 6 to 11 pairs of blind caeca. From there it continues to the intestine, which is a simple tube in Hirudiniforms, but in the species of the family Glossiphoniidae has four pairs of blind caeca. The anus opens dorsally in the somite XXVII. Leeches are hermaphrodites with complex reproductive systems, sexual reproduction and direct development. The male and female gonopores are observable

externally on the ventral surfice of somites XI and XII. Male gonopore always anterior. Leeches present ovisacs and testisacs. Testisacs may be spherical or foliaceous. Vas deferens are anteriorly directed and join the epididymis, then the atrial cornua and finally, reaching the common atrium. The female reproductive system consists of a single pair of small and spherical ovisacs confined to somite XII, or might elongated tubes. During egg laying, the clitellum secretes an ootheca that protects embryos. The circulatory system consists of dorsal and ventral vessels interconnected through lateral vassels. Gas exchange occurs through the body wall. The excretory system is metanephridial and consists of a maximum of 17 pairs of metanephridia.

BACKGROUND

Human knowledge of leeches dates back to antiquity, evidence of this can be found in Greek and Roman writings. Most modern and accurate records are found between 1500 to 1750 (Moquin-Tandon, 1846), but nevertheless it is not until the work of Linnaeus (1758) that the study of this group formally started. Linnaeus divided all the animals into six groups, of which only two were invertebrates: Insecta and Vermes. Leeches were included in group of Vermes. In 1809, Lamarck subdivided Vermes into four independent groups: mollusks, echinoderms and polyps and what remained of the group Vermes. Subsequently, Lamarck splited Vermes again and recognized two large groups with completely different internal morphology. On of the groups included relatively simple worms without internal organs, most of them parasites of vertebrates. The other group included annelids, or segmented worms with complex internal morphological traits. Lamarck recognized the affinities between polychaetes and oligochaetes, but

surprisingly, leeches remained grouped with trematodes or flipping between the annelids and trematodes until 1851 when Vogt finally established the relationships between leeches and annelids (Gould, 1999, Brusca and Brusca, 2003). During the first decades of the nineteenth century in France, the taxonomic school of comparative morphology of hirudinea developed. This tradition based their observations on the analysis of annulation patterns. This school continued mainly by Whitman, Oka, Harding, Weber, Pinto and Caballero. Later, J. Percy Moore studied anatomy of both internal and external characters and proposed various terms to refer to types or kinds of structures. Particularly important is the detailed study of the reproduct structures, which constitute the basis of his taxonomic practise (Richardson, 1969).

The taxonomic arrangement of the groups has been the subject of recent work. Siddall *et al*. (2001) concluded that the sister group of the hirudinea are branchiobdellids, and together, they form the sister group of acanthobdellids. With the development of phylogenetic systematics and the implementation molecular biology techniques, a large number of groups and hypotheses of evolutionary relationships have been tested (Apakupakul *et al*., 1999; Borda and Siddall, 2003; Borda *et al*., 2005; Light and Siddall, 1999; Oceguera-Figueroa *et al*., 2005; Phillips and Siddall, 2005; Siddall, 2002; Siddall and Burreson, 1995, 1998; Trontelj and Sket, 2000) suggesting that some groups recognized by taxonomists are artificial. It is clear that a lot of aspects on the taxonomy and phylogenetic analyses of leeches have been investigated, however several groups remain understudied and the phylogenetic position of several groups remains unresolved.

General Objective

The main objective of this work is the study of the biodiversity of freshwater leeches from the New World with emphasis on the members of the family Glossiphoniidae. The first approach to the study of biodiversity included the morphological characterization and taxonomic description of new species. Secondarily, the phylogenetic relationships of several groups were analyzed using a suite of methods, including distance methods and character based methods such as Maximum Parsimony, Maximum Likelihood and Bayesian Inference. Finally, the last approach to understand biodiversity of leeches was the understanding of their symbiotic relationships with bacteria using two approaches: phylogenetics and genomics.

CHAPTER 2

Barcoding

2.1 Barcoding, types and the *Hirudo* files: Using information content to critically evaluate the identity of DNA barcodes

(Adapted from: Kvist, S., Oceguera-Figueroa A., Siddall, M. E. and Erséus, C. 2010. Barcoding, types and the *Hirudo* files: Using information content to critically evaluate the identity of DNA barcodes. Mitochondrial DNA, 21: 198–205.)

INTRODUCTION

The growth and increased frequency of DNA barcoding campaigns emphasizes the importance of validating the databases upon which species identifications are based. In terms of actual practice, relatively little attention is given by submitters and users of barcodes to the layers of validation that would ensure proper identifications. One conspicuous aspect of this shortcoming is that the two main repositories of barcodes [GenBank/NCBI and the Barcode of Life Data System (BOLD)] do not fully recognize the distinction between the reference database entries and subsequently generated barcodes. The former should be predicated on authoritative entries providing a baseline for association with the latter. In other words, the taxonomic identities of reference barcodes need to be disambiguated through information content and critical evaluation prior to any sensible identity inference of query barcodes. This scenario is often overlooked in the growing barcode databases (although somewhat alleviated in BOLD; see below) such that all barcodes are collectively lumped into a single bin upon which subsequent identifications are based. In addition, there is a lack of information annotated in the barcode submissions that, if present, would allow for discrimination of the validity of the taxonomic labels of the barcodes in the bin. This has led to uncritical majority rules in actual barcoding practice; the determination of a result from barcoding is based on the number of high scoring hits that are encountered in the database. This is even more disconcerting given that taxonomic labels from consistently wrongly identified specimens are free to spread throughout the public sequence repositories (Nilsson *et al.,* 2006; Ross and Murugan, 2006). Had holotypes for all species ever described been sequenced for the barcode region, or at least been provided with a DNA voucher, subsequent barcoding

would be greatly improved. Yet, several issues such as multiple copies of CO1 in the mitochondrion, nuclear pseudogenes, or the fact that different species have almost identical CO1 sequences (Williams and Knowlton, 2001; Wiemers and Fiedler, 2007) would still be problematic. A sensible approach to ameliorating the problem of non-sequenceable type specimens would be major barcode repositories retaining and providing information about barcoded non-types regarding georeferenced locality data, collector, identifier of record, dates of collection and identification, as well as morphological and genetic voucher catalogue numbers. Insofar as CO1 sequences (i.e. barcodes) are available for rather few type specimens, considerable care must be taken to provide maximal information content relating to specimens actually being used to generate the reference barcode database. The barcoding process is intended to more rapidly allow specimen identification through comparisons of short DNA sequences without the laborious process of morphological identifications (Hebert and Gregory, 2005). To the extent that reference barcodes are useful for such a rapid procedure, they must actually be predicated on just such a laborious process. It has been rightly argued that DNA barcoding itself is a poor tool for species discovery and delimitation (Will and Rubinoff, 2004; DeSalle *et al.,* 2005; Ebach and Holdredge, 2005; Will *et al.,* 2005). Standard delimitation of species requires rigorous morphological analyses and, in some cases, genetic investigations. Typically, this is achieved by comparisons with type material or voucher specimens as well as specialized literature such as original descriptions, monographs, and/or taxonomic keys. Instead, barcoding should be used as a tool for identifying specimens belonging to a species already represented in the database or as an initial, crude way of "flagging" a potentially new species that needs further

investigation (DeSalle, 2006). However, the decreased attention to morphological attributes of specimens from which query barcodes are generated strongly increases the need for taxonomic validity of specimens from which the reference sequences were obtained. Furthermore, it increases the need for a connection between the barcode sequence and morphology or other descriptive characteristics (like geography) that are common in standard taxonomy. We recognize the initial need for momentum to generate as many DNA barcodes for as many species as possible for the idea of barcoding to gain traction. For the approach to mature, however, it is now more critical that authoritative barcode reference sequences be created for each species, with specifications as to which pre-existing barcode(s) have the highest informati on content relative to its connection to type material. Here, we contemplate this issue by discussing the scientific value added by the use of information-rich voucher specimens and the inherent need to create barcode reference databases that allow for critical evaluation of their validity.

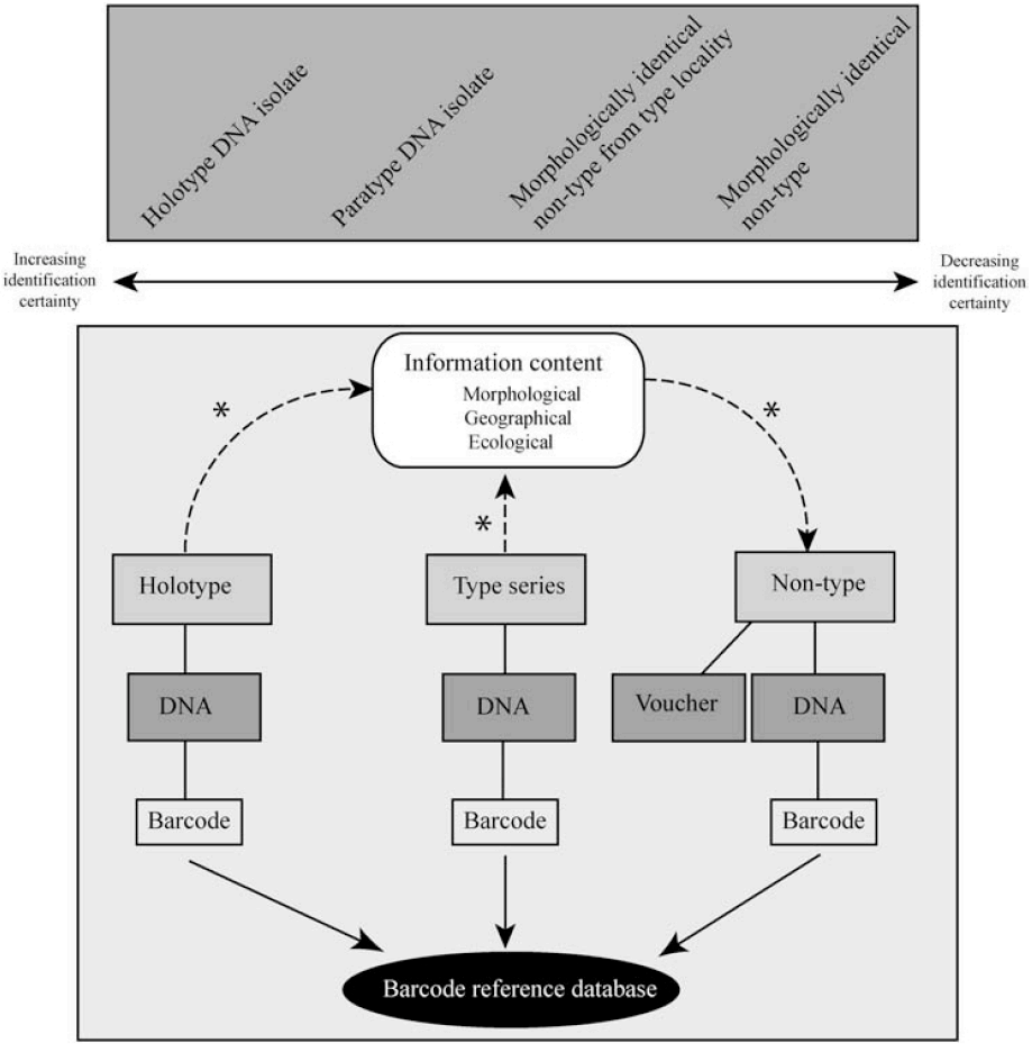
LOCATION, LOCATION, LOCATION

Unlike GenBank, which is not designed to aid barcoding initiatives, BOLD does distinguish between "validated" and "unvalidated" barcodes. This distinction might readily give the user a sense of security that some barcoding-based identification of a query sequence carries the weight of scientific authority if only made in reference to the "validated" subset. Such complacency is misplaced as the criteria for inclusion in BOLD's "validated" barcode database are only that three or more minimum length representatives with the same taxonomic label are included in a cluster and are collectively less than 2% different (Ratnasingham and Hebert, 2007). Given these criteria

for validity, the taxonomic label of any consistently misidentified species will remain faulty (e.g. Siddall *et al*., 2009). Barcode databases are not heavily policed, nor do any submissions necessarily have the benefit of peer review. Such procedures, while they would allow for an approach to validation, might also severely hamper the progress of barcoding initiatives. One possible counterbalance to the presently unknowable taxonomic validity of even the "validated" BOLD sequences is to ameliorate the scarcity of information content regarding geographical location of a specimen's collection site, its defining morphological characteristics, as well as collection dates, collector, identifier and the like. In particular, too little attention has been paid to the significance of type localities. A schematic layout of the information content establishment for reference barcodes is presented in Figure 1. The best approach to DNA barcoding would be that a single yardstick for each species would be based on the highest possible information content; that is a barcode from the holotype (or, when applicable, the lectotype or neotype) that would forever represent a species as the reference barcode. Any identity of subsequent sequences, on the other hand, would only be determined based on their similarity to that reference barcode. However, much of the type material in museums or other scientific collections is too old, too degenerate, or stored in media not allowing for DNA sequencing. As such, the second highest level of certainty when creating a reference barcode would come from sequencing specimens from the remainder of the type series (e.g. paratypes, and paralectotypes). If these also prove refractor y to sequencing, it is reasonable to suggest that the third highest level of certainty would be brought through an established morphological and geographical relatedness between the specimen from which the reference barcode is to be obtained and the holotype of the

FIGURE 1.

Schematics of the process of establishing information content for reference barcodes. The largest certainty in the taxonomic identity of a specimen comes from following the left path (using the holotype), the second largest from following the middle path (using remainder of type series), and the third largest from following the right path (using nontype specimens from the type locality with the addition of voucher specimens). *Information carry-over (morphological, geographic and ecological) from the type series to nontype specimens.


Holotype DNA isolate
Paratype DNA isolate
Morphologically identical non-type from type locality
Morphologically identical non-type
Increasing identification certainty
Decreasing identification certainty
Information content
Morphological
Geographical
Ecological
*
*
*
Holotype
Type series
Non-type
DNA
DNA
Voucher
DNA
Barcode
Barcode
Barcode
Barcode reference database

species. As with any taxonomic survey program, this should include information regarding some correspondence to the holotype itself or its description. That is, beyond being morphologically compatible, information content would be increased if there were some geographic correspondence between specimens from which the reference sequence is obtained and the type specimen. Collecting specimens from the type locality (the collection site of the holotype) has two main benefits. To the extent that both biotic and abiotic factors have shaped species distributions, these factors are information embodied by the type locality. A link between species, their geographic distribution, and a reference sequence would greatly improve the argument that the reference barcode obtained from a specimen from the type locality belongs to the same species as the holotype. Furthermore, in light of the many morphologically cryptic species being uncovered (Bickford *et al.,* 2007), in large part eventuated by barcoding initiatives themselves, geography can assist in determinations. Present DNA databases largely consist of specimens for which identifications have either not been accurately validated in these ways, or for which such critical evaluation is not possible. This is not to ignore the possibility of several morphologically cryptic species co-occurring at a type locality (Hebert *et al.*. 2004; Bely and Weisblat, 2006) although, in such cases, if viable holotype DNA is absent, neither genetics nor morphology can solve the ambiguity. With a more information-rich database, different levels of certainty (or actual validity) would be discernable from the annotations of individual barcodes. Even without the benefit of peer review for multitudinous reference sequences, users would be empowered to evaluate identifications in more rigorous ways. If no morphological information is available for a barcode submission or if it came from other than the type locality, one need not

completely disregard a submission's identification, putative as it may be. However, any vague association between such a putative reference sequence and the type series should be readily indicated in some way. This more careful strategy for establishing a reference barcode library mirrors the best practices of taxonomy, wherein a holotype has precedence over the remainder of the type series and in which neotypes are best designated from the type locality (International Commission on Zoological Nomenclature 1999, Article 75.3.6). This practice is also followed in comprehensive phylogenetic revisions where the authors spend considerable time and resources collecting specimens of type species of type genera from type localities (e.g. Oceguera-Figueroa *et al.,* 2005; Borda *et al.,* 2008). Even in cases where a type locality has been destroyed, geographic information allows for making the best of a bad situation: a reference sequence should be taken from a specimen with inferred biogeographic affinity to the former type locality. This information content must take precedence over the current "validation" system provided by BOLD. To the extent that we have focused on type localities as a proxy for creating reference barcodes, this should be expanded to include several other inherent biological realities; for example, host–parasite or plant-associated insect systems. For example, any reference barcode sequence for a spiral valve tapeworm taken from a thorny skate (*Amblyraja radiata* Donovan, 1808) would lack the taxonomic authority inherent in a morphologically indistinguishable cestode originally described from the winter skate typehost (*Leucoraja ocellata* Mitchell, 1815), even if the former was collected from the type locality where both skates cooccur. The study of these conjoined systems is highly dependent on accurate identifications of both associates (Besansky *et al.,* 2003). It is reasonable to suggest that both a parasite and its host should be subjected

to simultaneous DNA barcoding, applying the same guidelines concerning the validation of reference barcodes as detailed above. These added efforts in turn provide for enhanced scientific inquiry to the extent that they will allow barcoding databases to provide proper identifications of juveniles or of various life history stages in intermediate hosts.

THE USE OF VOUCHER SPECIMENS

Voucher specimens necessarily are stored in natural history collections at museums or universities and can be any part of an organism that is needed to disambiguate taxonomic identity (e.g. whole specimens, microscope slides, herbarium sheets, and digital photographs; see Miller, 2007; Pleijel *et al.,* 2008). Our appeal for the use of voucher specimens in barcoding is that they allow for subsequent re-evaluation. Concerns about the taxonomic identity of specimens become increasingly urgent with the growing use of molecular data (Goldblatt *et al.,* 1992; Schander and Willassen, 2005; Nilsson *et al.,* 2006; Pleijel *et al*., 2008). A voucher specimen of any barcoded organism will enable later cross-referencing between genetics and morphology as well as provide for critical re-evaluation of the identity of the specimen. That is, voucher specimens are at their best when anyone can use them to control the labels associated with a DNA sequence. Pleijel *et al.,* (2008) argued that some organisms are easily recognized but deciphering the taxonomic identity of others still requires the attention of a small number of taxon-specific experts. The growing body of taxonomic dilettantes further underscores the need for enabling subsequent re-evaluation of the identity of specimens used both for creating the reference library and for subsequent queries. In an attempt to quantify the incidence of voucher use, Pleijel *et al.,* (2008) found that only 46% of the DNA sequence

submissions they examined include references to voucher depositions. Although a formatted field for voucher catalogue numbers is present in both GenBank and BOLD, it seems as though voucher deposition is far from standard protocol for sequence submission to GenBank. While the vast majority of barcode submissions in BOLD are annotated with a storing institution, considerably fewer include catalogue numbers. We acknowledge that there are several cases where the deposition of voucher specimens becomes impractical or even impossible (environmental samples or destructive extraction protocols). These issues are rather frequently discussed (e.g. Neigel *et al.,* 2007; Rowley *et al.,* 2007; Hunter *et al.,* 2008) and we are confident that progress is being made towards ameliorating them. Regardless, in the event that barcode submissions cannot provide information-rich background data, none of them ought to be considered authoritative, much less validated.

CASE STUDY AND THE USE OF REFERENCE BARCODES

To underscore the importance of the morphological and geographic components to specimen identification, we provide an empirical example that illustrate how barcoding can connect to name-bearing types or type localities of species. The notorious European medicinal leech, *Hirudo medicinalis* Linnaeus, 1758, is frequently employed in, for example, leech therapy (Whitaker *et al.,* 2004), developmental biology (Fernández and Stent, 1982) and neurobiology (Muller *et al.,* 1981). Accurate interpretations of both bioactive compounds, such as anticoagulants, and developmental and neurophysiologic characteristics presuppose accurate specimen determinations (Siddall *et al.,* 2007a). Siddall *et al.,* (2007a) showed that commercially available medicinal leeches are in fact

not *H. medicinalis* but rather a close relative, *Hirudo verbana* Carena, 1820. While this result is less than astonishing owing to the similarity of morphological features between these two leech species, it can largely affect their usage in medicine because of their putatively different repertoire of anticoagulants. Linnaeus (1758) did not leave any type material for *H. medicinalis*, which makes subsequent identifications more complicated. In addition to a morphological predicament, specimens from which publicly available CO1 sequences have been attained were collected in areas that are largely separate, both geographically and ecologically, from what was, no doubt, a Swedish type locality. Linnaeus did not specify the type locality in his description but, because he was very active in his native area in and around the province of Uppland in Sweden (Reid, 2009), we assume that the specimens used to describe the species were collected in that area. For the present study, we collected specimens from both Uppland and Gotland (the Swedish island in the Baltic Sea) (Table 1). The geographic localities for *H. medicinalis* specimens used for genetic studies include Slovenia, Ukraine, Germany, France, and Sweden (Jördens *et al.,* 2004; Trontelj and Utevsky, 2005; Siddall *et al.,* 2007a; present study). DNA was extracted and the CO1 region amplified and sequenced following standard protocols described elsewhere (De Wit *et al.,* 2009). All publicly available CO1 sequences (n = 41) for *H. medicinalis, H. verbana*, and *Hirudo orientalis* Trontelj and Utevsky, 2005 were downloaded from GenBank and aligned with the newly generated sequences using the ClustalW2 algorithm (Larkin *et al.,* 2007) on the European Bioinformatics Institute web server applying default settings; three CO1 sequences annotated as *H. medicinalis* (EU100093, AF003272, AY364862) were excluded as these have been shown to not be *Hirudo* species (Trontelj and Utevsky, 2005; personal

Table 1. List of specimens used in the *H. medicinalis* case study

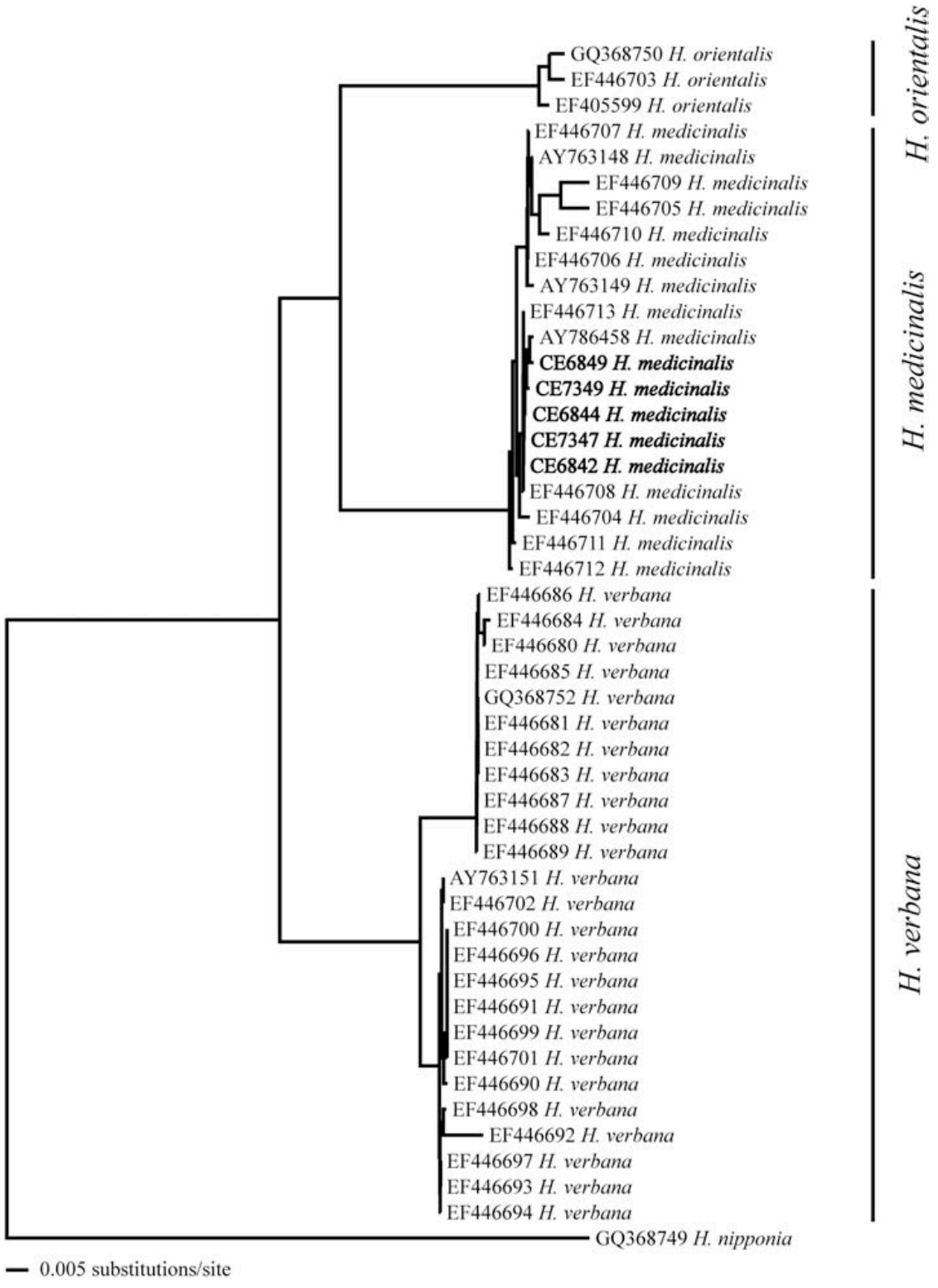
Voucher	DNA isolate	GenBank accession number	Collector	Collection locality in Sweden	Latitude/longitude	Collection date
SMNH111543	CE6842	HQ333519	S. Eliasson	Gotland, Gåsavätar Swamp	N 57°40′52′ E 18°35′35′	13 July 2009
SMNH111544	CE6844	HQ333518	S. Eliasson	Gotland, Gåsavätar Swamp	N 57°40′52′ E 18°35′35′	13 July 2009
SMNH111545	CE6849	HQ333517	S. Eliasson	Gotland, Gåsavätar Swamp	N 57°40′52′ E 18°35′35′	13 July 2009
Type-8027	**CE7347**	**HQ333516**	**S. Lundberg**	**Uppland, Fräkensjön Lake**	**N 60°34′13′ E 17°52′57′**	**24 July 2008**
SMNH111546	CE7349	HQ333515	S. Lundberg	Uppland, Fräkensjön Lake	N 60°34′13′ E 17°52′57′	24 July 2008

Notes: Bold denotes the neotype. Linnaeus (1758) is the authority for all specimens. Specimens were identified by C. Erséus and were not feeding on a host.

observation). In addition, a CO1 sequence of *Hirudo nipponia* Whitman, 1886 was used to root the tree. PAUP* 4.0b10 (Swofford, 2002) was used to construct a neighbor-joining tree using the Kimura two-parameter correction model (Kimura, 1980). As an aside, the Kimura two-parameter model is frequently employed by DNA barcoders without explicit justification. Here, it is used to minimize the disparity between this and other barcoding studies, but we note that a formal justification for using the Kimura two-parameter model in barcoding studies is greatly needed. The resulting neighbor-joining tree is presented in Figure 2. The CO1 sequences down- loaded from GenBank and labeled "*H. medicinalis*" are identical to or extremely similar to the newly acquired specimens, suggesting that these also conform to *H. medicinalis* Linnaeus, 1758. Importantly, sequences of the specimens collected in the more southern part of Sweden (Gotland) are also very similar or identical to those of the Uppland specimens, suggesting that both localities are inhabited by the same population. As such, the geographical affinity between our specimens and those of Linnaeus increases the information content annotated in the barcodes. Below, we assign a neotype of *H. medicinalis* based on morphological characteristics and underscored by our understanding of the locality at which Linnaeus' original material was collected. Also, we associate the neotype with a barcode generated from the specimen, and this barcode will enjoy authoritative power with respect to subsequent identifications.

FIGURE 2.

Neighbor-joining tree of the CO1 locus showing three distinct clades representing *H. orientalis, H. medicinalis* and *H. verbana* respectively. Specimens in bold were collected near the assumed type locality of the species. CE7347 denotes the neotype.


GQ368750 H. orientalis
EF446703 H. orientalis
EF405599 H. orientalis
EF446707 H. medicinalis
AY763148 H. medicinalis
EF446709 H. medicinalis
EF446705 H. medicinalis
EF446710 H. medicinalis
EF446706 H. medicinalis
AY763149 H. medicinalis
EF446713 H. medicinalis
AY786458 H. medicinalis
CE6849 H. medicinalis
CE7349 H. medicinalis
CE6844 H. medicinalis
CE7347 H. medicinalis
CE6842 H. medicinalis
EF446708 H. medicinalis
EF446704 H. medicinalis
EF446711 H. medicinalis
EF446712 H. medicinalis
EF446686 H. verbana
EF446684 H. verbana
EF446680 H. verbana
EF446685 H. verbana
GQ368752 H. verbana
EF446681 H. verbana
EF446682 H. verbana
EF446683 H. verbana
EF446687 H. verbana
EF446688 H. verbana
EF446689 H. verbana
AY763151 H. verbana
EF446702 H. verbana
EF446700 H. verbana
EF446696 H. verbana
EF446695 H. verbana
EF446691 H. verbana
EF446699 H. verbana
EF446701 H. verbana
EF446690 H. verbana
EF446698 H. verbana
EF446692 H. verbana
EF446697 H. verbana
EF446693 H. verbana
EF446694 H. verbana
GQ368749 H. nipponia
H. orientalis
H. medicinalis
H. verbana
0.005 substitutions/site

ARYNCHOBDELLIDA Blanchard, 1894

HIRUDINIDAE Whitman, 1886

Hirudo medicinalis Linnaeus, 1758

Material examined: Two specimens collected 24 July 2008 in Fräkensjön Lake, Hallnäshalvön Peninsula, Uppland, Sweden by Stefan Lundberg. An additional three specimens collected 13 July 2009 in Gasavätar Swamp, Tingstäde, Gotland, Sweden by Sara Eliasson. All specimens are lodged at the Swedish Museum of Natural History (SMNH), Stockholm.

Neotype: Whole leech fixed in 80% ethanol. Collected by Stefan Lundberg on 24 July 2008 (SMNH Type-8027; GenBank accession number HQ333516).

Neotype locality: Fräkensjön Lake NW of Hallnäs, Hallnäshalvön Peninsula, Tierp, Uppland, Sweden N 60°34´13´´ E 17° 52´57´´. Other barcoded specimens from Sweden: One whole leech specimen fixed in 80% ethanol; collected by Stefan Lundberg at neotype locality on 24 July 2008 (SMNH 111546). Three whole leech specimens fixed in 80% ethanol; collected at Gasavätar Swamp, Tingstäde, Gotland, Sweden (N 57°40´52´ E 18°35´35´) by Sara Eliasson on 13 July 2009 (SMNH 111543-111545).

Description: See Linnaeus (1758) as redescribed by Moquin-Tandon (1827) and verified by Siddall *et al.,* (2007a).

Remarks: Siddall *et al,*. (2007a) showed that the dorsal color patterns associated with each of *H. medicinalis*, *H. verbana*, and *H. orientalis* are species specific. Furthermore, the dorsal pattern of the *H. medicinalis* neotype corresponds to both the description by Linnaeus (1758) and that of Moquin-Tandon (1827). The latter did distinguish between *H. medicinalis* and *H. verbana* based on the dorsal color pattern. The consensus between

these three investigations regarding the species specific color patterns of *H. medicinalis* corresponds to our observations of the specimens collected in or close to the assumed type locality.

Author contribution

The ideas contained in this section regarding the importance of the importance of the type specimens and the barcoding initiative resulted from the intense discussions with Sebastian Kvist. During fieldwork in Peru, I discussed with Mark Siddall about the importance of type localities in taxonomy in general and its implications for barcoding. This issue was particularly important since we were trying to collect leeches in their type localities, that is, the localities were the specimens that were used to describe the leeches were collected. Some of those type localities were highly modified by human activities, particularly mining resulting in the complete disappearance of the original ecosystem. This fact has implications both for taxonomy and for barcoders.

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

(Adapted from: Oceguera-Figueroa, A., León-Règagnon, V. and Siddall M. E. 2011. barcoding reveals Mexican diversity within the freshwater leech genus *Helobdella* (Annelida: Glossiphoniidae). Mitochondrial DNA, 21 (S1): 24–29.)

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 (See Oceguera-Figueroa, 2007 and Oceguera-Figueroa and León- Règagnon, 2005). Two belong to the *"stagnalis"* series: *Helobdella atli* Oceguera-Figueroa & León- Règagnon, 2005 and *Helobdella stagnalis*. Three species are in the *"triserialis"* series: *Helobdella virginiae* Oceguera-Figueroa, 2007, *Helobdella conchata* (Caballero, 1941) and *Helobdella 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 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 2). Specimens belonging to 7 nominal species of *Helobdella* were collected from 11 states in Mexico, one sample was collected in 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 gradual addition of 70% ethanol and fixed in 96% ethanol. Voucher specimens were deposited in the "Colección Nacional de Helmintos" (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México.

Sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI), of 20 specimens from Mexico, 2 from the USA and one from South Africa were newly generated for the present study. Methods of leech DNA extraction, COI amplification and sequencing were described elsewhere (Apakupakul *et al*., 1999; Borda and Siddall, 2004a). Sequences of 42 species of *Helobdella* from previous studies were included in the present analyses. COI sequences of *Haementeria ghilianii* de Filippi, 1849 and

Table 2. Taxa, localities, and GenBank accession numbers for the CO1 sequences/ catalogue number (Colección Nacional de Helmintos, UNAM, México) of leeches of *Helobdella* spp. used in the Neighbor-Joining analyses. (*) indicate new COI sequences.

Taxon	Locality	GenBank/CNHE Catalogue #
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, Ohio, USA	AF329040
Helobdella modesta HW1	Washington, USA	HQ179853*
Helobdella modesta HW2	Washington, USA	HQ179854*
Helobdella moorei	Guanajuato, Mexico	HQ179870*/5569
Helobdella nununununojensis	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

Table 2. Continued

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, Texas, USA	DQ995306
Helobdella "robusta"	Sacramento, California, USA	DQ995301
Helobdella "robusta" CASA 1	Sacramento, California, 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
Heobdella virginiae	Catemaco, Veracruz, Mexico	HQ179864*/5474-76

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 ATRIBUTES

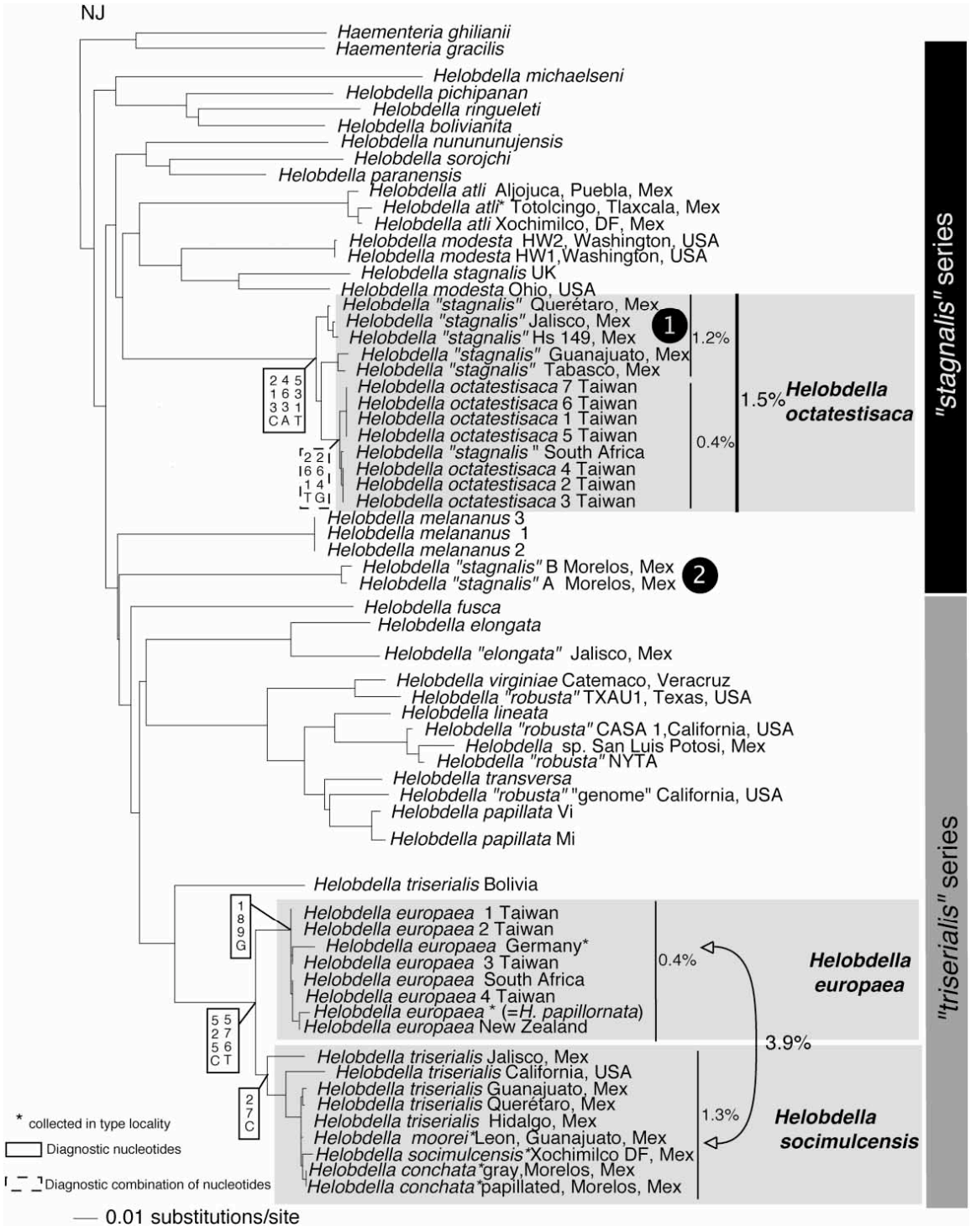
All of the COI sequences obtained for this study, as well as sequences obtained from GenBank were aligned with MUSCLE (Edgar, 2004) on the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/muscle/index.html), applying default settings. A Neighbor-Joining (NJ) tree was calculated in PAUP* (Swofford, 2002) using the Kimura 2-parameter (K2P) model of base substitution following previous barcoding studies (Hebert *et al*., 2004). All of the distance values among COI sequences were calculated in PAUP* (Swofford, 2002) using the K2P model. Diagnostic nucleotide positions for particular clusters (molecular synapomorphies) were determined through the implementation of the Characteristic Atribute Organization System (CAOS) software (Sarkar *et al*., 2002a, 2002b).

Results

The NJ tree (Figure 3) resulting from the analyses of COI 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 one, forming a paraphyletic group (*Helobdella "stagnalis* 1") presents 1.2% of genetic variation within its members

FIGURE 3.

Neighbor-joining tree based on the Kimura two-parameter substitution model of the CO1 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.


NJ
Haementeria ghilianii
Haementeria gracilis
Helobdella michaelseni
Helobdella pichipanan
Helobdella ringueleti
Helobdella bolivianita
Helobdella nununununujensis
Helobdella sorojchi
Helobdella paranensis
Helobdella atli Aljojuca, Puebla, Mex
Helobdella atli* Totolcingo, Tlaxcala, Mex
Helobdella atli Xochimilco, DF, Mex
Helobdella modesta HW2, Washington, USA
Helobdella modesta HW1,Washington, USA
Helobdella stagnalis UK
Helobdella modesta Ohio, USA
Helobdella "stagnalis" Querétaro, Mex
Helobdella "stagnalis" Jalisco, Mex
Helobdella "stagnalis" Hs 149, Mex
Helobdella "stagnalis" Guanajuato, Mex
Helobdella "stagnalis" Tabasco, Mex
Helobdella octatestisaca 7 Taiwan
Helobdella octatestisaca 6 Taiwan
Helobdella octatestisaca 1 Taiwan
Helobdella octatestisaca 5 Taiwan
Helobdella "stagnalis " South Africa
Helobdella octatestisaca 4 Taiwan
Helobdella octatestisaca 2 Taiwan
Helobdella octatestisaca 3 Taiwan
1
1.2%
0.4%
1.5%
Helobdella octatestisaca
245 163 331 CAT
226 614 TG
Helobdella melananus 3
Helobdella melananus 1
Helobdella melananus 2
Helobdella "stagnalis" B Morelos, Mex
Helobdella "stagnalis" A Morelos, Mex
2
Helobdella fusca
Helobdella elongata
Helobdella "elongata" Jalisco, Mex
Helobdella virginiae Catemaco, Veracruz
Helobdella "robusta" TXAU1, Texas, USA
Helobdella lineata
Helobdella "robusta" CASA 1,California, USA
Helobdella sp. San Luis Potosi, Mex
Helobdella "robusta" NYTA
Helobdella transversa
Helobdella "robusta" "genome" California, USA
Helobdella papillata Vi
Helobdella papillata Mi
Helobdella triserialis Bolivia
189G
Helobdella europaea 1 Taiwan
Helobdella europaea 2 Taiwan
Helobdella europaea Germany*
Helobdella europaea 3 Taiwan
Helobdella europaea South Africa
Helobdella europaea 4 Taiwan
Helobdella europaea * (=H. papillornata)
Helobdella europaea New Zealand
0.4%
Helobdella europaea
3.9%
557 256 CT
Helobdella triserialis Jalisco, Mex
Helobdella triserialis California, USA
Helobdella triserialis Guanajuato, Mex
Helobdella triserialis Querétaro, Mex
Helobdella triserialis Hidalgo, Mex
Helobdella moorei*Leon, Guanajuato, Mex
Helobdella socimulcensis*Xochimilco DF, Mex
Helobdella conchata*gray,Morelos, Mex
Helobdella conchata*papillated, Morelos, Mex
27C
1.3%
Helobdella socimulcensis
"stagnalis" series
"triserialis" series
* collected in type locality
Diagnostic nucleotides
Diagnostic combination of nucleotides
0.01 substitutions/site

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" genetic distance averaged 2.2%. The second cluster: *Helobdella "stagnalis* 2" was found nestled between the "*stagnalis*" and "*triserialis"* series. Within the *"triseriaslis"* series, *Helobdella virginiae* and *Helobdella* sp. from San Luis Potosi, Mexico, appeared grouped with various forms of *Helobdella "robusta", Helobdella lineata* and *Helobdella transversa* from the USA. *Helobdella elongata* from Mexico appeared in the same cluster with *H. elongata* from the USA, these two specimens having a genetic distance of 7%. *Helobdella 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 out in 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 to be 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 *Helobdella stagnalis* collected from the United Kingdom relative to those from Ohio, USA and reestablished Verrill's name *H. modesta* for North American species (see also Madill and Hovingh, 2007; Oceguera-Figueroa *et al.,* 2010). Mexican scutiferous samples were found in three different parts of the tree as putatively distinct species: *Helobdella atli, H. "stagnalis* 1*"* and as-yet underscribed species from Temixco, Morelos here designated *H. "stagnalis* 2*"*. All three samples of *Helobdella 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 *Helobdella modesta* and European *Helobdella stagnalis*.

*Helobdella "stagnalis*1*"* from several localities in Mexico clustered with the recently described *Helobdella 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 field-work nor thorough examinations of scientific collections had previously uncovered this species. The extremely low genetic variation (0.4%) within the samples of *Helobdella octatestisaca* and the 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 *Helobdella europaea* (Siddall and Budinoff 2005, 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, *Helobdella 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 caeca) are unreliable indicators of recent diversification.

Helobdella 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 COI lineages of *H. robusta* are found in the type locality in Sacramento, California ("CASA 1 "and "genome" in Figure 3). Absent more detailed morphological analysis of each of the

different lineages in comparison to 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. *Helobdella 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 COI 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. *Helobdella socimulcensis* Caballero, 1931 from Xochimilco, D. F. and *Helobdella 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 *Helobdella 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 suggests 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 *Helobdella socimulcensis* Caballero 1931 would be used for this group, which appears to be closely related to *H. europaea*.

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. *Helobdella 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 New World distribution. In both cases, Mexican samples appeared next to the putative invasive species clusters, but in any case identical COI 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: position 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 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 samples that, in agreement with their genetic similarity, should be renamed as *H. socimulcensis*.

The use of DNA barcoding to identify species relies on the assumption that COI variation between species (i.e. interspecific) exceeds in 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: *Helobdella atli, H. octatestisaca, H. virginiae, H. elongata, H. socimulcensis,* and two forms diagnosed only with molecular data: *Helobdella* sp. from San Luis Potosi and *Helobdella* "*stagnalis"* from Temixco, Morelos.

3.3 Phylogeny of macrophagous leeches of the suborder Erpobdelliformes (Hirudinea, Clitellata) based on molecular data and evaluation of the barcoding locus.

(Adapted from: Oceguera-Figueroa, A., Phillips, A. J., Pacheco-Chaves, B., Reeves, W. K. and Siddall, M. E. 2011. Phylogeny of macrophagous leeches of the suborder Erpobdelliformes (Hirudinea, Clitellata) based on molecular data and evaluation of the barcoding locus. Zoologica Scripta, 40: 194–203.)

INTRODUCTION

Freshwater leeches of the suborder Erpobdelliformes Sawyer, 1986 (=Pharyngobdelliformes Caballero, 1952) are macrophagous and feed exclusively on invertebrates such as mollusks, arthropods and annelids, even cannibalizing members of their own species (Young and Ironmonger, 1979; Toman and Dall, 1997). Based on previous phylogenetic studies, Erpobdelliformes were proposed to have evolved from a bloodfeeding ancestor that modified its feeding preferences (Siddall and Burreson, 1998; Apakupakul *et al*., 1999; Trontelj *et al*., 1999; Borda and Siddall, 2004a). Sawyer (1986) recognized two families of Erpobdelliformes: Erpobdellidae (Blanchard, 1894) for leeches with multiple testisacs, and lacking pharyngeal stylets, gastropores and postcephalic eyespots, and having a geographical distribution restricted to the Northern hemisphere; and Salifidae (Johansson, 1910) for species with postcephalic eyespots, pharyngeal stylets, with or without gastropores, and having a geographical distribution restricted to Africa, India, Australia and islands in the South Pacific. Several species of terrestrial macrophagous leeches with gastropores previously had been included in the family Erpobdellidae (Oka, 1895; Moore, 1929; Soós, 1966) until Richardson (1971) moved them to a distinct family, Gastrostomobdellidae including the genera *Gastrostomobdella, Mimobdella,* and *Orobdella*. Sawyer (1986) revised these to a subfamily within the family Cylicobdellidae (Hirudiniformes).

Species of Erpobdelliformes have been used to study various ecological processes including population ecology, competition, niche partitioning, predation and life history strategies (Maltby and Callow, 1986; Young, 1988). In addition, leeches of this group

have been studied as biological indicators of polluted environments (e.g., Wicklum and Davies, 1996; Zaranko *et al*., 1997).

Phylogenetic studies focusing on erpobdelliform species are less common than those concerning hirudiniforms and glossiphoniid leeches (Borda and Siddall, 2004a; Borda *et al*. 2008; Phillips and Siddall, 2005, 2009). Moreover, previous analyses typically have evaluated only a limited number of species, most of them of the family Erpobdellidae (Govedich *et al*., 1998; Siddall, 2002; Oceguera-Figueroa *et al*., 2005; Grosser and Trontelj, 2008). Siddall's (2002) examination of the family was performed through a parsimony analysis of a combined data set of morphological and molecular information and found that no genus with more than one species in his analysis (with the exception of *Trocheta*) was monophyletic. For this reason, he proposed the suppression of the generic names *Mooreobdella* Pawlowski, 1955, *Dina* Blanchard, 1892, *Trocheta* Dutrochet, 1817 and *Nephelopsis* Verrill, 1872 with *Erpobdella* (Blainville, 1918) remaining the sole valid name for all species in the family. Later treatments did not adopt the nomenclatural changes proposed by Siddall (2002) and yet without comment (e.g. Grosser and Kutschera, 2004; Pfeiffer *et al*., 2005). Others have described new species as members of the junior synonym genus *Dina* (e.g. Grosser and Eiseler, 2008; Grosser and Pesic, 2008) continuing to rely on the very morphological characters that Siddall (2002) found to be poor predictors of their phylogenetic relationships. The fact that Siddall's (2002) phylogenetic nomenclatural changes were not incorporated by others might relate to the fact that only parsimony methods were used to investigate the relationships of the group, or perhaps to having been a limited representation of Erpobdellidae along with even fewer species of Salifidae in the analyses.

Here we include newly collected taxa such as the type species of Salifidae, *Salifa perspicax* Blanchard, 1897. Also included in the analyses is *Orobdella octonaria* Oka, 1895; an elusive, burrowing, macrophagous leech from Japan with a gastropore, a structure also present in the genus *Barbronia* as well as a classical erpobdellid eyespots arrangement. This study represents the most intensive phylogenetic analyses of the family to date both in terms of the species and molecular data included, as well of the methods used to investigate and test phylogenetic hypotheses.

Materials and methods

Several DNA sequences included in this study have been reported in previous work (Apakupakul *et al*. 1999; Borda and Siddall 2004a; Grosser and Trontelj 2008; Oceguera-Figueroa *et al*. 2005; Pfeiffer *et al*. 2005; Siddall 2002; Siddall *et al*. 2001; Siddall and Burreson 1998; Sket *et al*. 2001; Trontelj and Sket 2000; Trontelj *et al*. 1999). Sequences newly generated for this study include those for newly included taxa: *Barbronia weberi* collected in Costa Rica, 5 samples of *Salifa perspicax* (Blanchard, 1897), *Erpobdella* cf. *octoculata* from Uzbekistan, *Motobdella montezuma* (Davis, Singhal and Blinn, 1985) from Montezuma's Well in Arizona, *Erpobdella costata* (Moore, 1901) from Texas, *Erpobdella punctata* (Leidy, 1870) from Ontario, *Erpobdella* cf. *punctata* from Washington State, *Erpobdella triannulata* Moore, 1908 from Chiapas, *O. octonaria* from Japan as well as new 28S sequences for *Barbronia arcana*, *Erpobdella mexicana, Erpobdella ochoterenai* and *Erpobdella triannulata* (Table 3). Leeches were collected from freshwater ponds, lakes and rivers. Specimens were found attached to submerged

Table 3. Taxa used for the phylogenetic analyses of the suborder Erpobdelliformes with collection localities and GenBank accession numbers. *Type species of Salifidae and Erpobdellidae

Taxon	Locality	COI	12S	28S	18S
Outgroup					
Americobdella valdiviana	Chile	AY425443	AY425407	EU100073	AY425461
Cylicobdella coccinea	Bolivia	AY425444	AY425462	AY425362	AY425362
Gastrostomobdellidae					
Orobdella octonaria	Tokio, Japan	HQ336338	HQ336348	HQ336355	HQ336372
Salifidae					
Barbronia gwalagwalensis	Hoedspruit, South Africa	AY786455	---	AY786449	AY786462
Barbronia arcana	Morelos, Mexico	DQ235598	DQ235588	HQ336356	DQ235608
Barbronia weberi formosana	Taiwan	AY786456	---	AY786448	AY786461
Barbronia sp.	South Africa	AY786457	---	AY786450	AY786463
Barbronia weberi	San Jose, Costa Rica	HQ336339	---	---	---
Barbronia wuttkei	Germany	DQ009666	---	---	---
Linta be	Tolagnaro, Madagascar	AY786460	---	AY786453	AY786466
Salifa perspicax 009*	Lake Kivu Kibuye Rwanda	HQ336340	---	HQ336358	HQ336373
Salifa perspicax 005*	Lake Ihema, Rwanda	---	---	HQ336357	HQ336374
Salifa perspicax 012*	Lake Ihema, Rwanda	HQ336341	HQ336349	HQ336359	HQ336375
Salifa perspicax 013*	Lake Ihema, Rwanda	HQ336342	HQ336350	---	HQ336376
Salifa perspicax 014*	Lake Ihema, Rwanda	HQ336343	HQ336351	HQ336360	HQ336377
Erpobdellidae					
Erpobdella lineata	Denmark Fakse/Falster	---	AF099952	AY425367	AF099950
Erpobdella mestrovi	Croatia	---	---	---	AF272842
Erpobdella johanssoni	Koper, Slovenia	---	AF169370	EF417047	---
Erpobdella krasensis	Vrhnika, Slovenia	---	AF169373	---	---

Table 3. Continued

Erpobdella bychowskii	Ljubljana, Slovenia	DQ009667	AF169372	---	---
Erpobdella subviridis	Cavtat, Croatia	---	AF169374	---	---
Erpobdella haskonis	Germany	DQ009668	---	---	---
Erpobdella dubia	Michigan, USA	AF116023	AF462022	---	AF115997
Erpobdella obscura	Ontario, Canada	AF003276	AF462028	AY425396	AF116004
Erpobdella testacea	France	AF116027	AF462025	AY425370	AF116003
Erpobdella japonica	Korea	AF116026	AF462023	AY425366	AF116000
*Erpobdella octoculata**	France	AF003274	AF099954	EF417048	AF116001
Erpobdella cf. *octoculata**	Uzbekistan	HQ336344	---	HQ336361	HQ336378
Erpobdella vilnesis	Germany	DQ009663	---	EF417049	---
Erpobdella monostriata	Germany	DQ009665	---	---	---
Erpobdella nigricolis	Germany	DQ009664	---	---	---
Erpobdella melanostoma	Michigan, USA	AF116025	AF462027	AY425395	AF115999
Erpobdella cf. *punctata*	Washington, USA	HQ336345	----	HQ336362	HQ336379
Erpobdella punctata 1	Ontario, Canada	AF003275	AF462024	AY425369	AF116002
Erpobdella punctata 2	Ontario, Canada	HQ336346	HQ336352	HQ336363	HQ336380
Erpobdella bucera	Michigan, USA	AF116024	AF462026	AY425394	AF115998
Erpobdella mexicana	Tlaxcala, Mexico	DQ235601	DQ235591	HQ336364	DQ235611
Erpobdella mexicana	Guanajuato, Mexico	DQ235597	DQ235587	HQ336365	DQ235607
Erpobdella mexicana	Mexico City, Mexico	DQ235595	DQ235585	---	DQ235605
Motobdella montezuma	Arizona, USA	GQ368760	GQ368820	GQ368779	GQ368802
Erpobdella triannulata	Tabasco, Mexico	DQ235604	DQ235594	HQ336366	DQ235614
Erpobdella triannulata	Veracruz, Mexico	DQ235602	DQ235592	HQ336367	DQ235612
Erpobdella triannulata	Chiapas, Mexico	HQ336347	HQ336353	HQ336368	---
Erpobdella costata	Georgia, USA	AY425460	AY425442	AY425406	AY425478
Erpobdella costata	Texas, USA	---	HQ336354	HQ336369	HQ336381
Erpobdella ochoterenai	Mexico City, Mexico	DQ235593	DQ235586	---	DQ235606
Erpobdella ochoterenai	Tlaxcala, Mexico	DQ235603	DQ235593	HQ336370	DQ235613
Erpobdella ochoterenai	Ameca, Jalisco, Mexico	DQ235599	DQ235589	HQ336371	DQ235609
Erpobdella ochoterenai	Vega, Jalisco, Mexico	DQ235600	DQ235590	---	DQ235610

rocks and plants with the exception of *Orobdella octonaria, which was* found crawling on grass after a heavy rainfall. Once collected, each specimen was fixed through the gradual addition of 96% ethanol and preserved in 100% ethanol.

Morphology

Leech identifications were conducted using available taxonomic keys (Klemm, 1982; Sawyer, 1986). Examination and dissections were done using a Nikon SMZ-U stereomicroscope and photodocumentation of leeches was accomplished using a Sony α330 digital camera. Illustrations were facilitated by Adobe Illustrator 10 and Adobe Photoshop 7. Illustrations of the different eyespots arrangements of *Erpobdella* spp. were adapted from Klemm (1982).

Molecular Techniques

The DNeasy Tissue Kit (QIAGEN Inc., Valencia, CA) was used to extract total DNA from a tissue sample of the caudal sucker. PCR amplification of nuclear 18S rDNA (18S) and 28S rDNA (28S), as well as mitochondrial cytochrome *c* oxidase subunit I (COI), and 12S rDNA (12S) gene fragments were accomplished using the primers listed in Table 4. Amplification reaction mixtures used Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) with: 23 µl of RNase-free H_2O, 0.5 µl of each 10 µM primer, and 1 µl DNA template (total volume, 25 µl). All amplification reactions were performed in an Eppendorf Master Cycler. Gene fragments were amplified using the following protocol: 94° C (1 min), followed by 35 cycles of 94° C (30 sec), 48 – 50° C (30 sec), and 68° or 72° C (45 sec) and then 68 or 72° C (7 min). PCR amplification products were purified with AMPure (Agencourt Bioscience Corporation). Samples were cycle sequenced on an Eppendorf Mastercycler using 1µl ABI Big Dye Terminator (v1.1

Table 4. Primers used for gene amplification and sequencing

Gene	Primer Name	Primer Sequence	
NUCLEAR			
18S rDNA		5' → 3'	
1	A	AACCTGGTTGATCCTGCCAGT	Apakupakul *et al.* 1999
	L	CCAACTACGAGCTTTTTAACTG	Apakupakul *et al.* 1999
2	C	CGGTAATTCCAGCTCCAATAG	Apakupakul *et al.* 1999
	Y	CAGACAAATCGCTCCACCAAC	Apakupakul *et al.* 1999
3	O	AAGGGCACCACCAG GAGTGGAG	Apakupakul *et al.* 1999
	B	TGATCCTTCCGCAGGTTCACCT	Apakupakul *et al.* 1999
28S rDNA			
1	28srD1a	CCCSCGTAAYTTAAGCATAT	Prendini *et al.* 2005
	28sB	TCGGAAGGAACCAGCTAC	Whiting, 2002
2	28sA	GACCCGTCTTGAAGCACG	Whiting, 2002
	28SBout	CCCACAGCGCCAGTTCTGCTTACC	Prendini *et al.* 2005
3	28srD5a	GGYGTTGGTTGCTTAAGACAG	Whiting, 2002
	28srD7b1	GACTTCCCTTACCTACAT	Whiting, 2002
MITOCHONDRIAL			
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* 1994
12S	12Sa	AACIIGGATTA GATACCC	Simon *et al.* 1994
	12Sb	GAGAGTGACGGGCGATGTGT	Simon *et al.* 1994

or v3.1), 1 µl Big Dye Extender Buffer (v. 1.1 or v. 3.1), 1 µl of 1 µM primer and 3 µl of cleaned PCR template (Total 6µl). Sequences were purified with CleanSeq (Agencourt Bioscience Corporation) and analyzed with an ABI PRISM 3730 sequencer.

Sequences were edited and reconciled using CodonCode Aligner (CodonCode Corporation). Alignment of the two nuclear gene fragments and mitochondrial 12S were accomplished using MUSCLE (Edgar, 2004) with the default gap open and extension values. Alignment of COI was trivial as there were no insertions or deletions. Kimura 2 Parameters (K2P) distances of DNA sequences were calculated in PAUP* (Swofford, 2002).

PHYLOGENETIC ANALYSES

Maximum Parsimony (MP) analyses of the combined 18S, 28S, 12S and COI data were performed using New Search technology with Ratchet and Tree fusing algorithms in TNT (ver 1.1) performing 100 repetitions (Goloboff *et al*., 2008). Resulting trees were used as starting trees for a traditional search using tree-bisection-reconnection (TBR) branch swapping. All characters were equally weighted and non-additive. Gaps were treated as missing data. Bootstrap values for combined analyses were obtained in TNT with 1000 heuristic pseudoreplicates, using random taxon addition and TBR branch swapping. To evaluate alternative taxonomic classification within Erpobdellidae, constraint trees were constructed in MacClade ver. 4.06 (Maddison and Maddison, 2003) and then exported to TNT (Goloboff *et al*. 2008) forcing the analysis to find the shortest tree with a predefined group.

Maximum likelihood (ML) analysis was conducted in TREEFINDER (Jobb, 2008). Data were partitioned and analyzed considering each gene, as well as 1st, 2nd and 3rd codon positions of the COI sequence as independent (6 partitions in total). Models of evolution for each partition were selected based on the Akaike information criterion (AIC) as implemented in FindModel (Tao *et al.,* 2008). For 1stand 3rd position of COI, HKY+G model was selected. For 2nd position of COI, HKY model was selected. For 12S, TVM+G and for 18S and 28S, GTR+G model was selected. Bootstrap support values were calculated in TREEFINDER performing 1000 replicates.

Constraint analyses required monophyly of species previously recognized as members of the genera *Mooreobdella*, *Dina*, and *Erpobdella* (3 independent analyses), as well as a topology requiring all of the genera simultaneously to be monophyletic. Results of parsimony analyses were evaluated in terms of extra steps and statistical significance was evaluated using Templeton test (Templeton 1983) as implemented in PAUP* (Swofford, 2002). In addition, Shimodaira-Hasegawa and approximately unbiased topological tests were conducted under the likelihood criterion with TREEFINDER using the same data partitions as described before.

Results

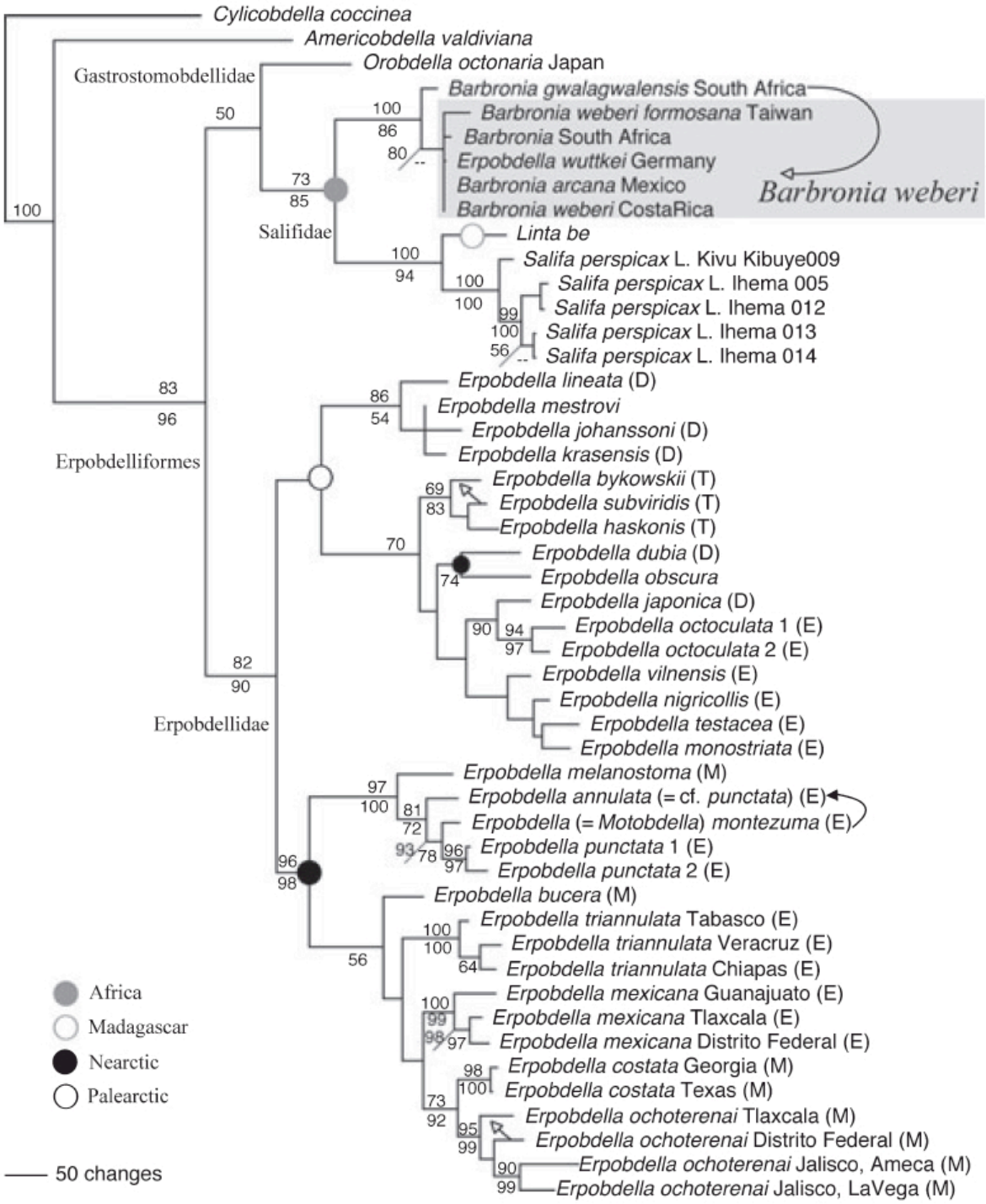
The complete dataset analyzed in this study included 49 terminals, 713 bp of COI; 805 bp of 12S; 2066 bp of 28S and 1956 bp of 18S (a total of 5540 aligned characters). K2P distance within all the samples of Erpobdelliformes (excluding outgroup) showed the highest average values in 12S (22%), followed by COI (18%), 28S (8%) and lastly 18S

(3.7%). Comparison of COI sequences between *B. weberi formosana* from Taiwan, *B. weberi* from Costa Rica, *B. arcana* from Mexico, *Barbronia* sp. from South Africa, and *B. wuttkei* from Germany showed extremely low genetic variation (between 0-0.8%). This result contrasts with the average 7% genetic distance between any of the above mentioned *Barbronia* sample to *B. gwalagwalensis,* their congener, and also with the elevated intraspecific COI variation for individual erpobdelliform species, exemplified by the 2.4% among Rwandan samples of *S. perspicax* and the 11% among Mexican samples of *E. ochoterenai*. The average divergence in 28 intraspecific comparisons is 4.2%, and is 18.5% in 713 interspecific comparisons, however, some overlap occurs between the range of 4-13%

Maximum Parsimony analysis of the complete data set resulted in 30 most parsimonious trees of 4063 steps. The strict consensus tree is shown in figure 4., CI= 0.52 and RI= 0.65. The log-likelihood of the tree resulting from the ML analysis was –24320.99. Both analyses resulted in highly congruent topologies. Monophyly of major groups (i.e. Erpobdelliformes, Salifidae and Erpobdellidae) were recovered by both methods and each with substantial bootstrap support values. Gastrostomobdellidae, represented by *O. octonaria*, falls within the suborder Erpobdelliformes with good support. However, its sister relationship with Salifidae is not well supported. Within Salifidae, the genera *Salifa* and *Barbronia* were recovered as monophyletic regardless of the optimality criterion. Some disagreement between MP and ML was detected in the position of *Barbronia gwalagwalensis* Westergren and Siddall, 2004, *Erpobdella subviridis, Motobdella montezuma* and *Erpobdella ochoterenai* from Distrito Federal, however, none of these alternative grouping achieve meaningful bootstrap support.

FIGURE 4.

Strict consensus tree resulting from parsimony analysis of Erpobdelliformes based on 28S, 18S, 12S and COI sequences. Branch lengths are proportional to amount of change. Numbers above and under nodes indicate bootstrap values for MP and ML, respectively. Letters in parentheses indicate the previous classification: (E) *Erpobdella*, (M) *Mooreobdella*, (D) *Dina* and (T) *Trocheta*. Arrows with straight lines indicate that those species appear as sister groups in ML analyses. Black arrow with curved line indicates that species are switched in ML analysis and empty arrow with curved line indicate the alternative placement of *B. gwalagwalensis* in a polytomy of *Barbronia* spp.


Cylicobdella coccinea
Americobdella valdiviana
Orobdella octonaria Japan
Gastrostomobdellidae
Barbronia gwalagwalensis South Africa
Barbronia weberi formosana Taiwan
Barbronia South Africa
Erpobdella wuttkei Germany
Barbronia arcana Mexico
Barbronia weberi CostaRica
Barbronia weberi
Salifidae
Linta be
Salifa perspicax L. Kivu Kibuye009
Salifa perspicax L. Ihema 005
Salifa perspicax L. Ihema 012
Salifa perspicax L. Ihema 013
Salifa perspicax L. Ihema 014
Erpobdella lineata (D)
Erpobdella mestrovi
Erpobdella johanssoni (D)
Erpobdella krasensis (D)
Erpobdelliformes
Erpobdella bykowskii (T)
Erpobdella subviridis (T)
Erpobdella haskonis (T)
Erpobdella dubia (D)
Erpobdella obscura
Erpobdella japonica (D)
Erpobdella octoculata 1 (E)
Erpobdella octoculata 2 (E)
Erpobdella vilnensis (E)
Erpobdella nigricollis (E)
Erpobdellidae
Erpobdella testacea (E)
Erpobdella monostriata (E)
Erpobdella melanostoma (M)
Erpobdella annulata (= cf. punctata) (E)
Erpobdella (= Motobdella) montezuma (E)
Erpobdella punctata 1 (E)
Erpobdella punctata 2 (E)
Erpobdella bucera (M)
Erpobdella triannulata Tabasco (E)
Erpobdella triannulata Veracruz (E)
Erpobdella triannulata Chiapas (E)
Erpobdella mexicana Guanajuato (E)
Erpobdella mexicana Tlaxcala (E)
Erpobdella mexicana Distrito Federal (E)
Erpobdella costata Georgia (M)
Erpobdella costata Texas (M)
Erpobdella ochoterenai Tlaxcala (M)
Erpobdella ochoterenai Distrito Federal (M)
Erpobdella ochoterenai Jalisco, Ameca (M)
Erpobdella ochoterenai Jalisco, LaVega (M)
Africa
Madagascar
Nearctic
Palearctic
50 changes

Previously recognized genera, including *Mooreobdella, Dina* and *Erpobdella* were not found to be monophyletic.

In the parsimony analyses, constraint trees forcing species formerly recognized as members of the genus *Mooreobdella* to be monophyletic required 24 extra steps (Templeton test: z = -10.005088; p <0.0001). Forcing *Dina* to be monophyletic required 89 extra steps (Templeton test: z = -8.811452; p <0.0001). Forcing *Erpobdella* to be monophyletic required 177 extra steps (Templeton test: z = -10.792393; p < 0.0001). Finally, constraint trees forcing the three genera to be simultaneously monophyletic requires 243 extra steps (Templeton test: z = -11.192299; p < 0.0001). Under likelihood criteria, p -values were highly significant in all tree topology comparisons rejecting monophyly of these groups. (Shimodaira-Hasegawa <0.001, approximately unbiased test <0.001).

DISCUSSION

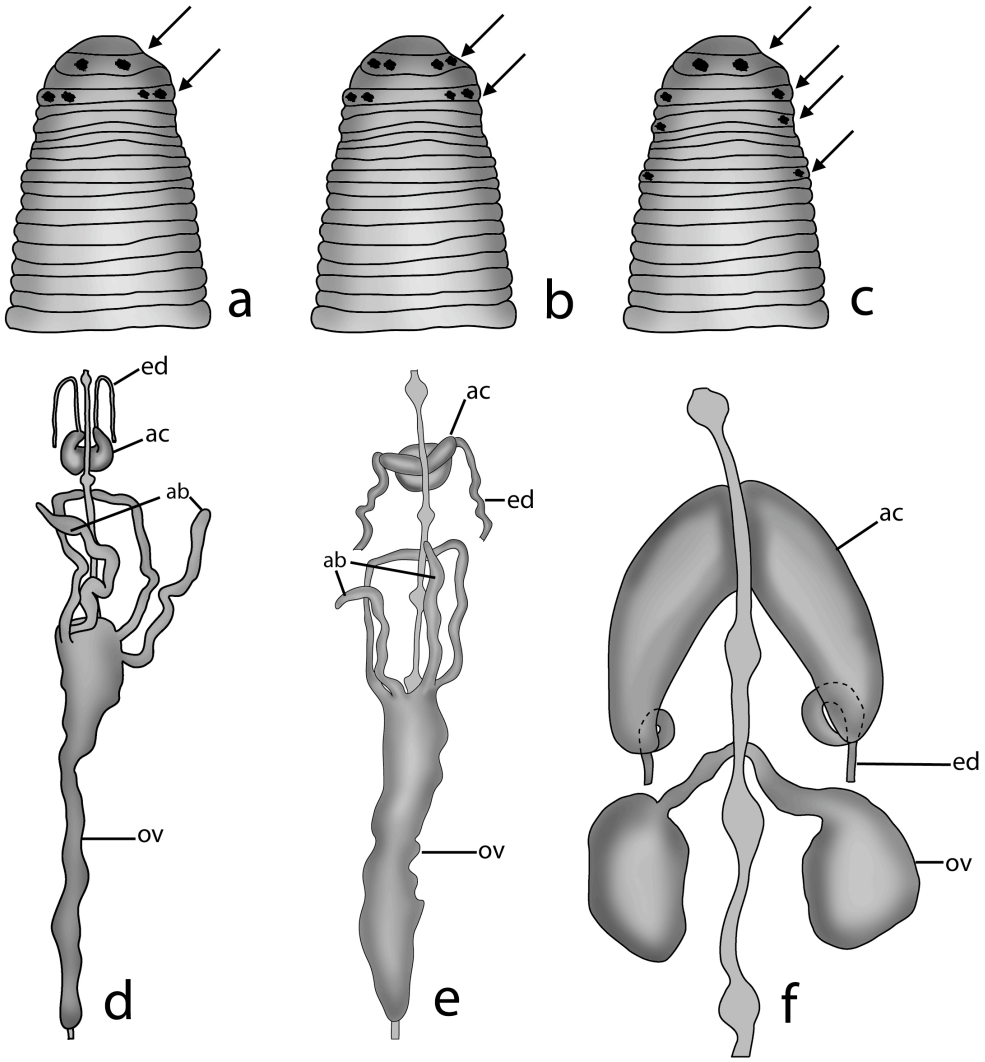
The use of morphological traits to predict phylogenetic relationships or their validation as secondary homologies varies even among closely related groups. Whereas morphology seems to be a good predictor of generic relationships in Salifidae, such is not the case in Erpobdellidae. Cases in which COI intraspecific diversity is greater than interspecific variation appears to be typical for Erpobdelliformes, particularly in the range between 4-13% of divergence. This contrasts dramatically with the absence of genetic variation among samples of *Barbronia* from 5 disjunct geographical areas and then reinforcing their conspecificity.

In the present analysis, which for the first time includes *E. octoculata* and *S. perspicax,* the type species of the two currently recognized families within Erpobdelliformes (i.e. Erpobdellidae and Salifidae), the traditional classification is partially corroborated. All of the species of the family Salifidae included in our analyses exhibit three "muscular jaws" (myognaths), one dorsal and two ventrolateral, each of which is armed with a pair of small stylets. In addition to this remarkable character, the shape of the ovaries is clearly distinct between the two families. Whereas species of Salifidae have simple ovaries (Figure 5 f) like those of some glossiphoniid leeches (i.e., *Helobdella, Glossiphonia*), species in Erpobdellidae have more complex ovaries, with anteriorly directed branches and a median joint on the distal portion of posteriorly directed branches (Figure 5 d, e). The shape of the male atrial cornua among species of Erpobdellidae is highly variable, but they are always directed anteriorly or laterally (Figure 5 d,e). In contrast, the atrial cornuae of salifids are consistently directed posteriorly (Figure 5 f). The number of testisacs per somite is another clear difference between Salifidae and Erpobdellidae. Whereas salifids have typical arhynchobdellid testisacs, species in Erpobdellidae present multiple testisacs per segment.

Species of Salifidae included in the present phylogenetic analysis were found forming two well-supported clades, one of them formed by species of the genus *Barbronia* united by having gastropores anterior and posterior to the gonopores. This character has been considered of taxonomic importance by several authors, with El-Shimy (1969) even recognizing its family status (Barbronidae). The classical erpobdellid eyespots arrangement, represented by labial and bucal eyespots (Figure 5 a,b) is also characteristic of *Barbronia* spp. and contrasts with the arc-shape distribution of the eyespots of species

FIGURE 5.

Morphology of selected erpobdelliformes. Arrangement of eyespots: (a) *Erpobdella punctata,* (b) *Erpobdella octoculata,* (c) *Salifa perspicax*. Arrows indicate eyespots. Dorsal view of male and female genitalia, (d) *Erpobdella punctata,* (e) *Erpobdella costata,* (f) *Salifa perspicax*. (ac) atrial cornua, (ab) anteriorly directed ovarian branches. (ed) ejaculatory duct. (ov) ovaries.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of *Linta* and *Salifa* (Figure 5c). The four samples of *S. perspicax* from Lake Ihema, Rwanda form a monophyletic group, sister to *S. perspicax* from Lake Kibuye, Rwanda. The COI genetic distance among samples from Lake Ihema was considerably less (at 1%) relative to the 3 % average distance they exhibit relative to the sample from Lake Kibuye. In the absence of morphological and/or ecological differences there is no reason to suspect cryptic speciation for *Salifa perspicax*, particularly given levels of intraspecific genetic variation seen among other erpobdelliformes. The placement of *O. octonaria* in previous classifications has been controversial. The presence of erpobdellid features such as eyespot arrangement, absence of stylets, and anteriorly directed male atrial cornua together with Salifid features such as simple ovaries, few testisacs per segment and gastropores lead some authors to propose a close affinity between *O. octonaria* with Erpobdelliformes (Moore, 1929). However, given the importance that former authors conferred to ecological attributes of the leeches to erect taxonomic groups, *Orobdella* spp. and related terrestral forms with gastropores were considered to be a subfamily of the terrestrial-leech family Cylicobdellidae *sensu* Sawyer, 1986. Based on the position of *O. octonaria* within the Erpobdelliformes, it is clear that most of its morphological characteristics are plesiomorphies: the absence of stylets and the presence of male atrial cornua anteriorly directed of *O. octanaria* are also the condition of Erpobdellidae species and the outgroups. Furthermorethe eyespot arrangement represented by labial and buccal eyespots seems to be restricted to the Erpobdelliformes including *O. octanaria*, but this condition is transformed again in *Linta*/*Salifa* species. On the other hand, the presence of few tetisacs per segment and simple ovaries characteristics of *O. octonaria* and Salifidae species are also present in the outgroups. The presence of gastropores, an structure of

unknown function that has been speculated to function as an alternative way to dispose non-digested wastes from the crop, seems to be restricted to the clade of Salifidae+*O octonaria*, however, if this character was lost in *Linta*/*Salifa* or gained independently in *O. octonaria* and *Barbronia* spp. is not fully understood.

For years, erpobdellid taxonomy was based on two sets of characters: subdivision of annuli and shape of the male reproductive system, particularly the arrangement of the ejaculatory ducts. The basic structure of the erpobdellid somite is five-annulate, however annulus b5 can be subdivided, forming the annuli c11 and c12, characteristic of "*Dina"* species. Further subdivision of annulus c12 into d23 and d24 together with the subdivision of b1 into c1 and c2 results in an octoannulate complete somite characteristic of "*Trocheta"* species. Uncertainty in the determination of annulation pattern in leeches is high and may depend on factors like age of the specimens or method of fixation. Even though species traditionally considered members of *Trocheta* were found to be monophyletic here, Trontelj and Sket (2000) found *Trocheta bychowskii krasense*, nested within European "*Dina"* species, notwithstanding their octoannulate annulation pattern. Instead of concurring with Siddall's (2002) suppressing the names *Dina* and *Trocheta,* and notwithstanding their own evidence that such morphological traits are of poor systematic value, Trontelj and Sket (2000) merely transferred *T. b. krasense* to the genus *Dina* (i.e. *Dina krasensis*) leaving the definition of each genus for future studies.

Siddall (2002) adopted a phylogenetic point of view, suppressing not only the generic names *Dina* and *Trocheta*, but also those of all other genera of Erpobdellidae save for *Erpobdella* Blainville, 1918 (i.e. *Nephelopsis, Mooreobdella*, and *Croatobranchus*) in

light of their neither being monophyletic nor placing in a manner that would render *Erpobdella* paraphyletic. The expanded dataset analyzed in the present paper continues to corroborate Siddall´s (2002) perspective. Recognition of each erpobdellid genus would leave us having to justify the adoption of a tree 243 steps longer than the most parsimonious hypothesis. Furthermore, all the taxonomic arrangements within Erpobdellidae were discarded as valid hypotheses by all statistical tests under parsimony and likelihood frameworks (Templeton, Shimodaira-Hasegawa and approximately unbiased test). Like the suppressed monotypic genera *Nephelopsis* and *Croatobranchus*, our finding of *Motobdella montezuma* Davis and Singhal, 1985 nested within *Erpobdella* clade, necessitates the new combination *Erpobdella montezuma*. The clade including *M. montezuma,* and *E. punctata* from two localities in Canada, and *Erpobdella* cf. *punctata* from Washington State share the presence of three pairs of eyespots (Figure 5a), preatrial loops (Figure 5d) and gonopores separated by two annuli. However, the genetic distance of the COI sequences between them (9 to10% between *Erpobdella punctata* to either *Erpobdella* cf. *punctata* or *E. montezuma*) and more importantly, the relative position of *Erpobdella* cf. *punctata* as sister species of the clade formed by *M. montezuma* + *E. punctata* from Canada necessitates the resurrection of Moore's 1922 epithet "annulata" for specimens with strongly pigmented dorsal transverse stripes (one on each annulus), a common form on the west coast of Washington State. Since Moore (1922) recognized "*annulata*" as a subspecies of *Erpobdella punctata,* a name that was not recognized in later studies of the group (Klemm, 1982; Sawyer, 1986), here we formally elevate the name *Erpobdella annulata* Moore, 1922 for the species from Washington.

Whereas the exclusive use of COI sequence to identify species and/or to conduct

biodiversity inventories remains controversial, the barcoding approach allows evaluation of the taxonomic status of widely distributed species, like *Barbronia weberi* (R. Blanchard, 1897). The fact that the various samples of *B. weberi* were collected on a global scale, together with the previous recognition that *B. weberi* is an invasive species (Genoni and Fazzone, 2008) is sufficient reason to consider all the samples in the cluster (*B. arcana*, *B. w. formosana*, *Barbronia* sp. and *B. wuttkei*) to be *B. weberi*.

The invasive leech *B. weberi* has been documented from several regions of the world in the last 34 years. Mason (1976) recorded its presence in New Zealand; Sawyer (1986) in England; Nesseman and Neubert (1999) in Germany; Pamplin and Rocha (2000) in Brazil; Rutter and Klemm (2001) in USA; Govedich *et al*. (2002) in Australia; Oceguera-Figueroa *et al*. (2005) in Mexico; and Genoni and Fazzone (2008) in Italy. According to the Fauna Europea database, *B. weberi* is also present in Austria. Here, we add Costa Rica to the list of countries with records of this invasive leech. Govedich *et al*. (2003) studied some ecological characteristics of *B. weberi*, demonstrating the ability of adult organisms and cocoons to be transported by aquatic plants and the high rates of growth and feeding activity of this leech. The ecological characteristics of *B. weberi* facilitate its accidental dispersion in freshwater environments, for this reason the potential negative impact of this leech has to be evaluated in order to propose management plans.

One of the most striking results of our analysis is the obvious determination that *B. wuttkei* is yet another case of the invasive *B. weberi*. Kutschera (2004) described *Erpobdella wuttkei* based on specimens collected from an aquarium in Germany. The original description of this species relied only on broad external characters. Grosser and Trontelj (2008) more carefully examined the morphological characters of *E. wuttkei* and

correctly determined this to be the first ever European species of *Barbronia*. These authors, however, decided to retain the specific name as valid (i.e., *B. wuttkei*). The absence of any molecular or morphological differences between *B. wuttkei* and the globally invasive *B. weberi* demonstrates that *B. wuttkei* is a junior synonym of *B. weberi*. As such, determinations made by Pfeiffer *et al*. (2005) regarding the evolution of terrestrialism and body size should be understood in this context.

Sawyer (1986) noted a clear geographic structure for the taxonomic arrangements in the suborder Erpobdelliformes. That pattern remains evident in the phylogenetic tree (Figure 4) in that the genus *Erpobdella* has two clades: one comprising only Nearctic species, the other being mostly Palearctic but with a recent clade of two North American species. With the more distantly related Salifidae being excluded from what was Laurasia, it is tempting to consider the diversification of the Erpondelliformes as having been driven first by Tethyan and then Atlantic vicariant events with the breakup of Pangea. Absent any fossils for these groups (or any other leech family) molecular calibration of dates of origins for these clades remains elusive as does any corroboration of the apparent vicariance.

CHAPTER 3

Biodiversity and species descriptions

3.1 Leech Collections from Washington State, with the Description of Two New Species of *Placobdella* (Annelida: Glossiphoniidae)

(Adapted from: Oceguera-Figueroa A., Kvist, S., Watson, S. C., Sankar, D. F. Overstreet, R. M., Siddall, M. E. 2010. Leech Collections from Washington State, with the Description of Two New Species of *Placobdella* (Annelida: Glossiphoniidae). American Museum Novitates, 3701: 1–14.

INTRODUCTION

Washington State displays a wide variety of climates, environments, and ecological niches (e.g., Kruckeberg, 1991; Peterson *et al.,* 1997). The Cascade Mountain Range divides the state from north to south and the climate varies greatly from one side of the range to the other: West of the Cascades is a marine west coast climate with substantial parts covered by conifer forests and temperate rainforests. East of the range is warmer and more arid with steppe as well as true desert. The Cascades themselves are a series of dormant and active volcanoes, which, coupled with Pleistocene glaciations, have given rise to an almost unique geomorphological complexity of the landscape (e.g., Armstrong *et al.,* 1965; Crandell, 1971). This complexity, in turn, provides the fauna with several microhabitats such as isolated ponds, small rock formations, and patches of bushy growth (e.g., McCune *et al.,* 2000; Peterson *et al.,* 1997).

Accounts of the leech fauna of the Pacific Northwest are scarce, especially when compared to the numerous accounts of the fauna east of the Rocky Mountains (e.g., Moser *et al.,* 2008; Phillips and Siddall, 2005; Sawyer, 1967; Sawyer *et al.,* 1975). Klemm (1982) produced what is probably the most comprehensive list of morphological attributes and species distributions of North American leeches, which he based on published literature and collection records of the time. Therein, *Actinobdella inequiannulata* Moore, 1901, *Glossiphonia elegans* (Verrill, 1872)*, Helobdella elongata* (Castle, 1900)*, Helobdella modesta* (Verrill, 1872)*, Placobdella montifera* Moore, 1906*, Myzobdella lugubris* Leidy, 1851, *Erpobdella punctata* (Leidy, 1870)*, Erpobdella obscura* (Verrill, 1872) and the marine leech *Piscicola salmositica* Meyer, 1946, are all reported as present in Washington State (Klemm, 1982). Pursuant to our recent collection

efforts across Washington, we here present an assessment of the distribution of 11 species of leeches and, furthermore, describe two new species of the genus *Placobdella*, both collected in the state.

MATERIALS AND METHODS

Leeches were collected from 13 localities in 10 counties in Washington state (Figure. 6; table 5) in September 2008 and October–November 2009. Leeches were found on the undersides of rocks, wood, and debris in freshwater environments. Alternatively, blood-feeding leeches were collected by immersing legs into the water at the edges of the lakes, waiting for approximately 1 min, and then examining for leeches attached to skin. Specimens were relaxed with gradual addition of ethanol and fixed with 96% ethanol. The posterior sucker of representative specimens was fixed in 100% ethanol. Dissections of leeches were accomplished using stereoscopic microscopy. Photographs of whole specimens were taken using either a Nikon Coolpix P5000 or a P5100 digital camera attached to a microscope. Drawings were made by superposition of vector art on placed images in Adobe Illustrator (Adobe Systems, San Jose, California). All measurements mentioned herein are in millimeters. The type series and voucher specimens were deposited in the American Museum of Natural History (AMNH), New York.

FIGURE 6.

Map of Washington showing the 13 collection sites in solid circles. Grey areas indicate bodies of water.

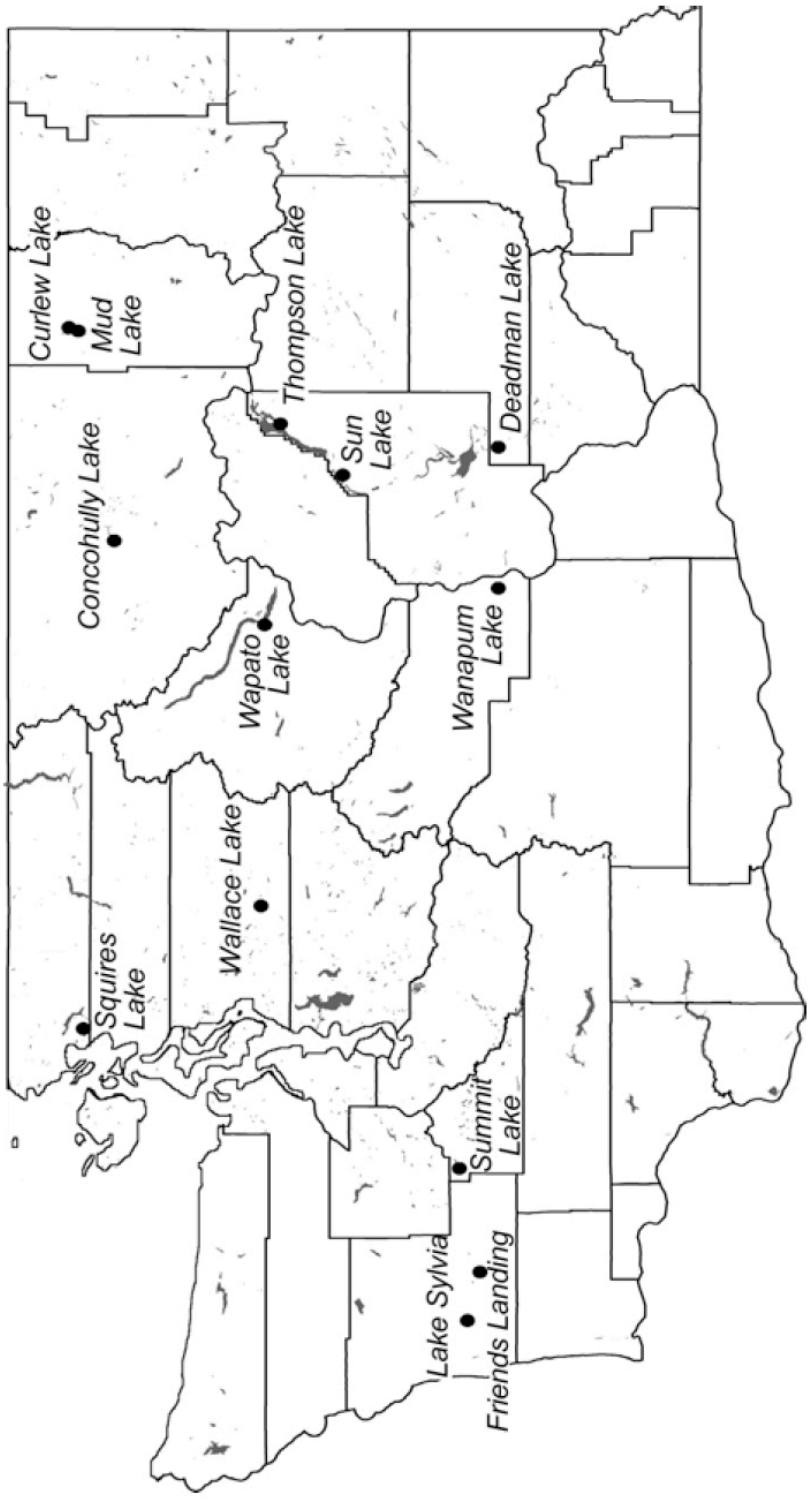

Curlew Lake
Mud Lake
Thompson Lake
Deadman Lake
Sun Lake
Concohully Lake
Wapato Lake
Wanapum Lake
Wallace Lake
Squires Lake
Summit Lake
Lake Sylvia
Friends Landing

TABLE 5. Species and collection locality information for for leech specimens encountered in this study

Site	Taxa	County	Georeference	Date	AMNH catalog no.
Conconully	*Helobdella modesta* (G) $n = 19$	Okanogan	48°33′52.72″N 119°43′45.12″W	26 Sept 2009	5513
Curlew Lake	*Erpobdella obscura* (E) $n = 11$	Ferry	48°43′37.57″N 118°39′32.38″W	25 Sept 2009	5514
	Erpobdella punctata (E) $n = 3$				5515
	Glossiphonia elegans (G) $n = 17$				5516
	Helobdella modesta (G) $n = 1$				5517
Friends Landing	*Placobdella montifera* (G) $n = 1$	Grays Harbor	46°56′52.21″N 123°38′19.54″W	3 Sept 2008	5518
Mud Lake	*Helobdella modesta* (G) $n = 24$	Ferry	48°40′16.35″N 118°45′59.54″W	25 Sept 2009	5519
	Glossiphonia elegans (G) $n = 3$				5520
	Theromyzon cf. *rude* (G) $n = 1$				5521
Summit Lake	*Erpobdella annulata* (E) $n = 8$	Thurston	47°02′56.84″N 123°06′58.42″W	29 Sept 2009	5522
	Placobdella montifera (G) $n = 1$				5523
	Helobdella modesta (G) $n = 21$				5524
	Helobdella elongata (G) $n = 1$				5525
Sun Lake	*Erpobdella punctata* (E) $n = 8$	Grant	47°36′20.20″N 119°21′17.35″W	26 Sept 2009	5526
Squires Lake	*Placobdella kwetlumye*, n. sp. (G) $n = 2$	Whatcom	48°39′36.98″N 122°20′02.76″W	30 Sept 2009	5527, 5528
	Placobdella sophieae, n. sp. (G) $n = 8$				5529, 5530
	Theromyzon cf. *rude* (G) $n = 5$				5531
	Helobdella modesta (G) $n = 63$				5532
	Helobdella elongata (G) $n = 1$				5533
	Erpobdella annulata (E) $n = 5$				5534
Lake Sylvia	*Erpobdella annulata* (E) $n = 2$	Grays Harbor	46°59′38.68″N 123°38′36.92″W	29 Sept 2009	5535
	Helobdella modesta (G) $n = 48$				5536
Thompson Lake	*Helobdella modesta* (G) $n = 5$	Grant	47°50′59.07″N 119°08′09.81″W	26 Sept 2009	5537
	Helobdella papillata (G) $n = 39$				5538
Wallace Lake	*Helobdella modesta* (G) $n = 23$	Snohomish	47°54′06.87″N 121°40′30.55″W	28 Sept 2009	5539
Wanapum Lake	*Helobdella modesta* (G) $n = 2$	Kittitas	46°53′58.48″N 119°59′10.08″W	27 Sept 2009	5540
	Erpobdella punctata (E) $n = 5$				5541
Wapato Lake	*Helobdella modesta* (G) $n = 32$	Chelan	47°55′11.12″N 120°09′43.49″W	26 Sept 2009	5542
	Erpobdella punctata (E) $n = 1$				5543
Deadman Lake	*Helobdella modesta* (G) $n = 18$	Adams	46°53′02.69″N 119°14′35.54″W	27 Sept 2009	5544

SYSTEMATICS

Rhynchobdellida Blanchard, 1894

Glossiphoniidae Vaillant, 1890

Placobdella kwetlumye, new species

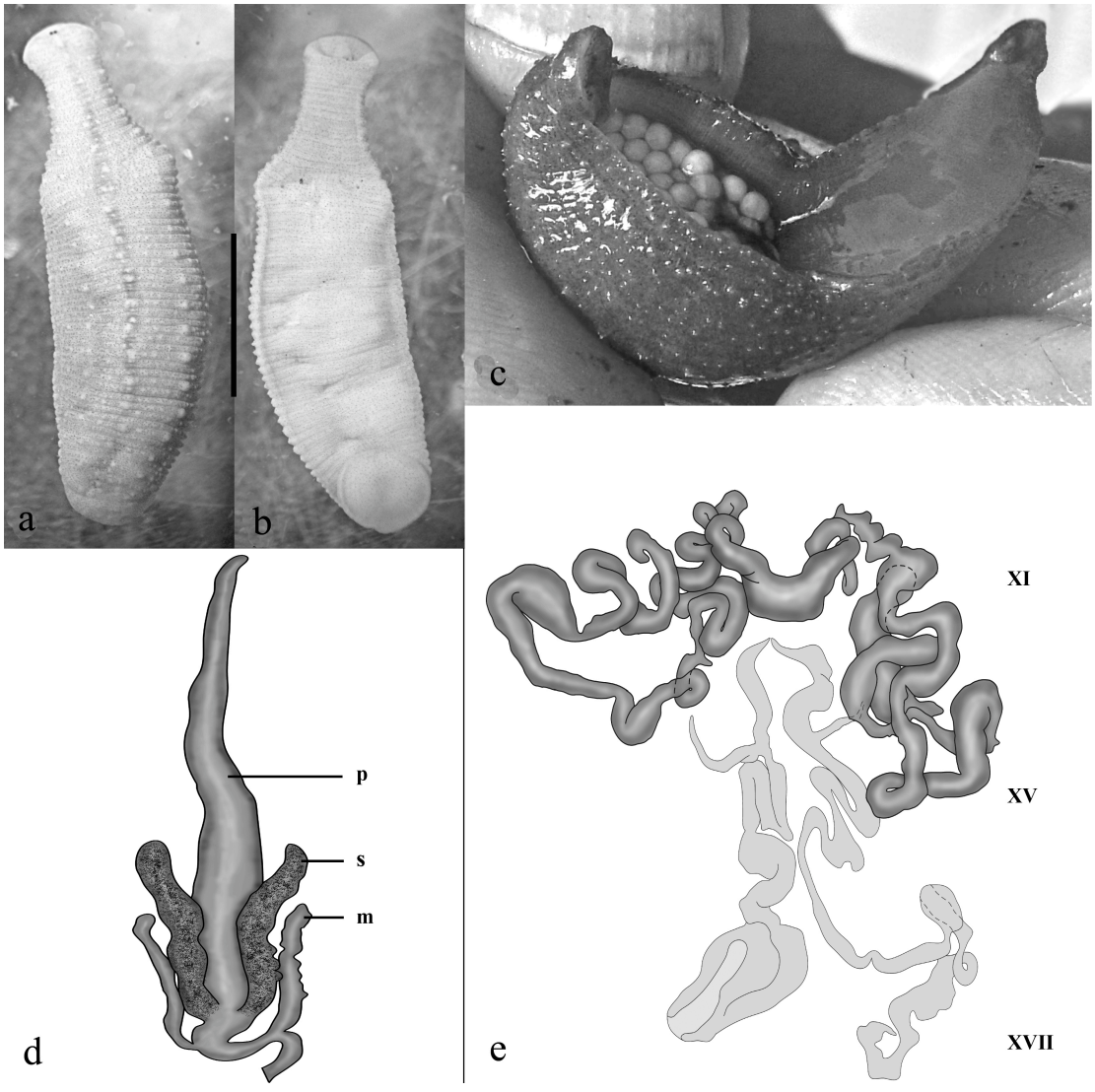
Figure 7

Material Examined: Two specimens collected in Squires Lake, Washington on September 30, 2009, by Alejandro Oceguera-Figueroa and Sebastian Kvist. Collected from the legs of AOF and on the underside of submerged rocks and wood.

Description: External morphology based on two specimens. Body dorsoventrally flattened, lanceolate, brownish tegument. Average body length 17.75 (14.60–20.90), average body width 4.70 (4.60–4.80). Complete somite triannulate, partially subdivided in somites at middle of body. Somites I and II uniannulate; III–IV biannulate; V–XXIV triannulate; XXV–XXVI biannulate; XXVII uniannulate. Two pair eyespots on III in "placobdellid" arrangement (i.e., in which the anterior most eyespots are coalesced medially to each other and posteriorly to the second pair). Oral sucker small, mouth pore on anterior border. Posterior sucker circular with papillae on dorsal surface. Anus located on dorsal surface of XXVII. Solid longitudinal stripe in midline of dorsal surface from Vto XXIV, partially interrupted by large papillae and replaced by unpigmented medial stripe in most anterior somites. Dorsum with seven rows of papillae. Large papillae forming five longitudinal rows, prominent papillae on a2. Medial and lateral rows more conspicuous than marginal rows. Minor paramedial rows formed of medium-sized papillae bilaterally in the area between medial and lateral rows. From XXV–XXVII minor paramedial rows only are prominent. Dorsal surface not occupied by large and

FIGURE 7.

Morphological characteristics of *Placobdella kwetlumye,* n. sp. **a.** Paratype, dorsal view. **b.** Paratype, ventral view. **c.** Eggs attached to the venter, ventral view. **d.** Illustration of the proboscis (p), compact salivary glands (s), and mycetomes (m), in dorsal view. **e.** Illustration of the male and female reproductive systems, dorsal view; testisacs not shown. Scale bars = 5 mm.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XVII
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medium papillae, covered with punctiform, evenly distributed papillae (Figure 7a). Ventral surface smooth and brownish, without metameric stripes, papillae or spots (Figures 7b, c). Male gonopore between XI and XII. Female gonopore at XII a2/a3; two annuli between gonopores.

Internal morphology based on one dissected specimen. Proboscis large, in membranous sheath extending posteriorly to XI when retracted, unlooped. One pair well-developed anteromedial compact salivary glands extending from IX a3 to XI, discharging into base of proboscis. Posterolateral salivary glands absent. Esophagus short, folded, with one pair elongated mycetomes extending anteriorly from XI/XII to X a2 (Figure 7d). Crop with seven pairs foliaceous caeca, last pair forming well-developed postcaeca (diverticula) extending posteriorly to XXV. Intestine with four pairs simple caeca in XX–XXIII. Male reproductive system with well-developed atrial cornua and highly coiled ejaculatory ducts. Six pairs intersegmen tal testisacs from XIII/XIV to XVIII/XIX. Ovisacs without common oviduct, anteriorly bilobed, extending posteriorly to XVII, anterior ovisac bifurcation at XIII/XIV. Anterior lobe extending anteriorly to XIII (Figure 7e).

Holotype: Dissected, fixed in ethanol. 20.9 length, 5.0 maximum width. Collected by Alejandro Oceguera-Figueroa on September 30, 2009 (AMNH 5527).

Type Locality: Squires Lake, Whatcom County, Washington, 48°39’36.98’’N; 122°20’02.76’’W.

Paratype: One undissected specimen fixed in ethanol, collected by Sebastian Kvist, Squires Lake, Whatcom County, Washington, 48°39’36.98’’N; 122°20’02.76’’W, on September 30, 2009 (AMNH 5528).

Additional Material: Thirteen specimens fixed in ethanol collected by Robin M. Overstreet. Some specimens found free living, some feeding from the cloaca of a gadwall (*Anas strepera*) in Summer Lake Wildlife Area, Lake County, Oregon, on August 14, 2002.

Remarks: This species stands apart from all other species of *Placobdella* by its possessing a single pair of compact salivary glands. Siddall *et al.* (2005) and Siddall and Bowerman (2006) redefined the genus *Placobdella* to include species provided with two pairs of eyespots (with the anterior pair smaller and coalesced), with one pair of cecate mycetomes connected to the esophagus and with bilobate ovaries. The morphological characteristics found in *P. kwetlumye,* n. sp., are consistent with that definition. *Placobdella kwetlumye,* n. sp., resembles other papillated members of the genus described for North America: *Placobdella burresonae* Siddall and Bowerman, 2006*, Placobdella multilineata* Moore, 1953*, Placobdella ali* Hughes and Siddall, 2007*, Placobdella ornata* (Verrill, 1872), and *Placobdella papillifera* (Verrill, 1872). However, the new species is easily distinguished from the last three of these owing, among other things, to the pattern of papillation. *Placobdella ali, P. ornata,* and *P. papillifera* present highly papillated dorsal surfaces, not forming well-structured rows like those present in *P. burresonae*, *P. multilineata,* and *P. kwetlumye,* n. sp. In addition, those highly papillated species exhibit two pairs of well-developed compact salivary glands connecting to the base of the proboscis; *P. kwetlumye,* n. sp., is the only species of the genus lacking the posterior pair. Furthermore, *P. ali* and *P. papillifera* present ventral pigmentation patterns, completely absent in *P. kwetlumye,* n. sp.

Placobdella burresonae and *P. multilineata* are superficially the most

morphologically similar species to *P. kwetlumye,* n. sp. However, some differences in the external as well as in the internal morphology can be recognized. Medial and paramedial dorsal rows in *P. burresonae* and *P. multilineata* are formed by papillae of different size in every single annulus (see fig. 1 in Siddall and Bowerman, 2006), whereas in *P. kwetlumye,* n. sp., rows are formed by prominent papillae only on a2. Moreover, no marginal furrows are present in *P. burresonae* in contrast with *P. kwetlumye,* n. sp. (which has marginal papillae instead).

Etymology: The specific epithet is based on the Nlaka'pamux (an Interior Salish "Thompson group language) word for "leech" or "bloodsucker," k'wét_'um'ye. The species name should be pronounced "kwaitle-oom-yay." The type locality of *P. kwetlumye,* n. sp., corresponds to a region inhabited by the Coast Salish, but because no word for "leech" could be found in the Coast Salish lexicon, we opted for the lexicon of the geographically closest group.

Placobdella sophieae, new species

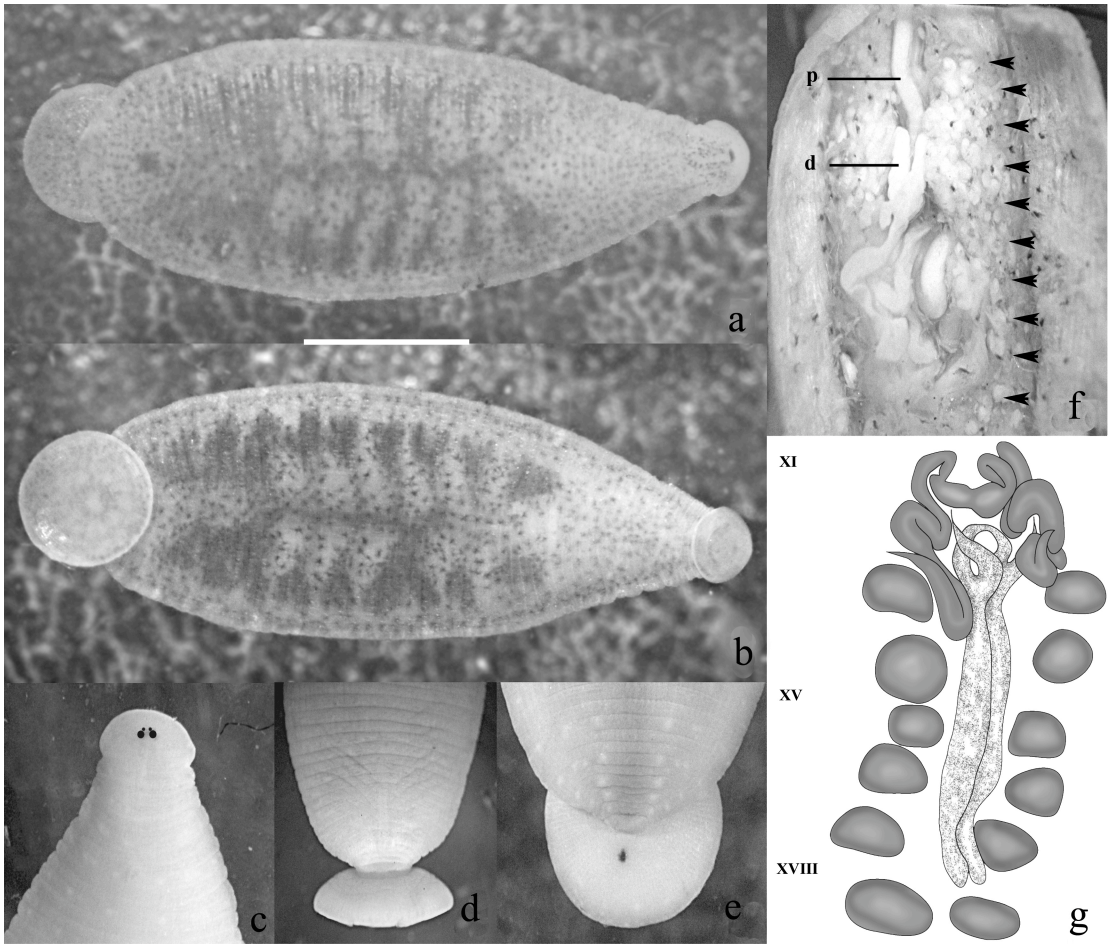
Figure 8

Material Examined: Eight specimens collected in Squires Lake, Washington, September 30, 2009, by Alejandro Oceguera-Figueroa and Sebastian Kvist. Collected from the legs of A.O.-F. and from the underside of submerged rocks and wood.

Description: External morphology based on eight specimens. Body dorsoventrally flattened, ovate-lanceolate, semitransparent tegument when alive, with three pairs of dorsal rows of brownish-pigmented spots on a2 of each somite, otherwise chromatophores randomly arranged on ventral and dorsal surfaces (Figures 8a,b). Body

FIGURE 8.

Morphological characteristics of *Placobdella sophieae,* n. sp. **a.** Holotype, dorsal view. **b.** Holotype, ventral view. **c.** Eyespots showing characteristic "placobdellid" arrangement. **d.** Posterior sucker showing the pedicel (peduncle). **e.** Posterior sucker showing dorsal papilla. **f.** Proboscis (p) and bundle of ductules (d), dorsal view; mycetomes not shown. Diffuse salivary glands indicated by arrows. **g.** Dorsal view of the male and female reproductive systems. Scale bars = 3 mm.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c
d
e
p
d
f
XI
XV
XVIII
g

brownish when fixed. Average body length 12.6 (11.0–14.1), average body width 4.8 (4.0–6.4). Complete somite triannulate. Somites I and II uniannulate; III–V biannulate; VI–XXIII triannulate; XXIV biannulate; XXV–XXVII uniannulate. Two pair eyespots on III in "placobdellid" arrangement (Figure 8c). Oral sucker small, mouth pore on anterior border. Posterior sucker circular, separated from body by obvious constriction or pedicel (Figure 8d). Anus on dorsal surface of XXVII (Figure 8e). Male gonopore between XIand XII. Female gonopore at XIIa2/a3; 2 annuli between gonopores.

Internal morphology based on three dissected specimens. Proboscis short, in membranous sheath extending posteriorly to IX, unlooped when retracted. Diffuse salivary glands dispersed dorsolaterally from VII to XIII, connecting to base of proboscis through well-developed bundles of ductules (Figure 8f). Esophagus large, folded, with one pair cecate mycetomes at X. Crop with seven pairs foliaceous caeca, last pair forming well-developed postcaeca (diverticula) extending posteriorly to XXV. Intestine with four pairs simple caeca in XX–XXIII. Male reproductive system with well-developed atria and highly coiled ejaculatory ducts. Six pair intersegmental testisacs from XIII/XIV to XVIII/XIX. Ovisacs without common oviduct, anteriorly bilobed, extending posteriorly to XVIII, ovisac bifurcation at XIII. Anterior lobe very short extending only to XII (Figure 8g).

Holotype: Undissected, fixed in ethanol. 15.0 length, 5.8 maximum width. Collected by Alejandro Oceguera-Figueroa on September 30, 2009 (AMNH 5529).

Type Locality: Squires Lake, Whatcom County, Washington, 48°39'36.98''N; 122°20'02.76''W.

Paratypes: Seven specimens (3 dissected and 4 undissected), fixed in ethanol. Collected

by Alejandro Oceguera-Figueroa and Sebastian Kvist, Squires Lake, Whatcom County, Washington, on September 30, 2009 (AMNH 5530).

Etymology: The species is named after Sophie Alice Burgess in honor of her birth.

Remarks: The morphological characteristics found in *P. sophieae,* n. sp., are consistent with synapomorphies of the genus (Siddall et al., 2005; Siddall and Bowerman, 2006). The presence of a small pedicel (peduncle) separating the posterior sucker from the rest of the body, present in *P. sophieae,* n. sp., has been recorded in at least two other *Placobdella* species, *Placobdella pediculata* Hemingway, 1908, and *Placobdella cryptobranchii* (Johnson and Klemm, 1977), as well as in *Actinobdella inequiannulata* Moore, 1901, and *Actinobdella annectens* Moore, 1906. *Placobdella sophieae,* n. sp., is distinguished from species of *Actinobdella,* in that the latter have several retractile digitate processes on the rim of the caudal sucker; absent in the new species. In addition to the presence of a small pedicel, *P. pediculata* and *P. cryptobranchii* also have diffuse salivary glands like those of *P. sophieae,* n. sp. However, *P. sophieae,* n. sp., is distinguished principally by its external morphology, which contrasts with the absence of prominent papillae, white patches or colored dots in *P. pediculata* and *P. cryptobranchii.* Moser *et al.* (2008) described two paramarginal rows of metameric spots on the dorsal surface as well as white patches (typically 3) between white cephalic and caudal somites for *P. cryptobranchii*, in any case, the pattern corresponds with that of *P. sophieae,* n. sp. Furthermore, Hemingway (1908) recorded the anus at XXIII/XXIV in *P. pediculata,* whereas in *P. sophieae,* n. sp., the same structure is situated in XXVII. The diffuse salivary glands of *Placobdella sophieae,* n. sp., connect to the base of the proboscis through well-developed common bundles. These structures were not recorded by Johnson

and Klemm (1977) for *P. cryptobranchii*. *Placobdella pediculata* and *P. cryptobranchii* were described as permanent parasites of the fresh-water sheepshead (*Aplodinotus grunniens*) and the Ozark hellbender (*Cryptobranchus alleganiensis*) respectively. Hemingway (1908) and Johnson and Klemm (1977) reported that the leeches were always found attached to their host, never free living, which contrasts with our findings of *Placobdella sophieae,* n. sp. In addition to *P. pediculata* and *P. cryptobranchii*, discussed above, *Placobdella phalera* (Graff, 1899)*, Placobdella michiganensis* (Sawyer, 1972), and *Placobdella picta* (Verrill, 1872) also possess diffuse salivary glands, yet are easily distinguished from *P. sophieae,* n. sp., based on external pigmentation. *Placobdella phalera* and *P. michiganensis* have white patches together with a white nuchal ring on the dorsal surface. *Placobdella picta* has a dark greenish-brown dorsum and a thin median line. These characters contrast with the semitransparent tegument of *P. sophieae,* n. sp.

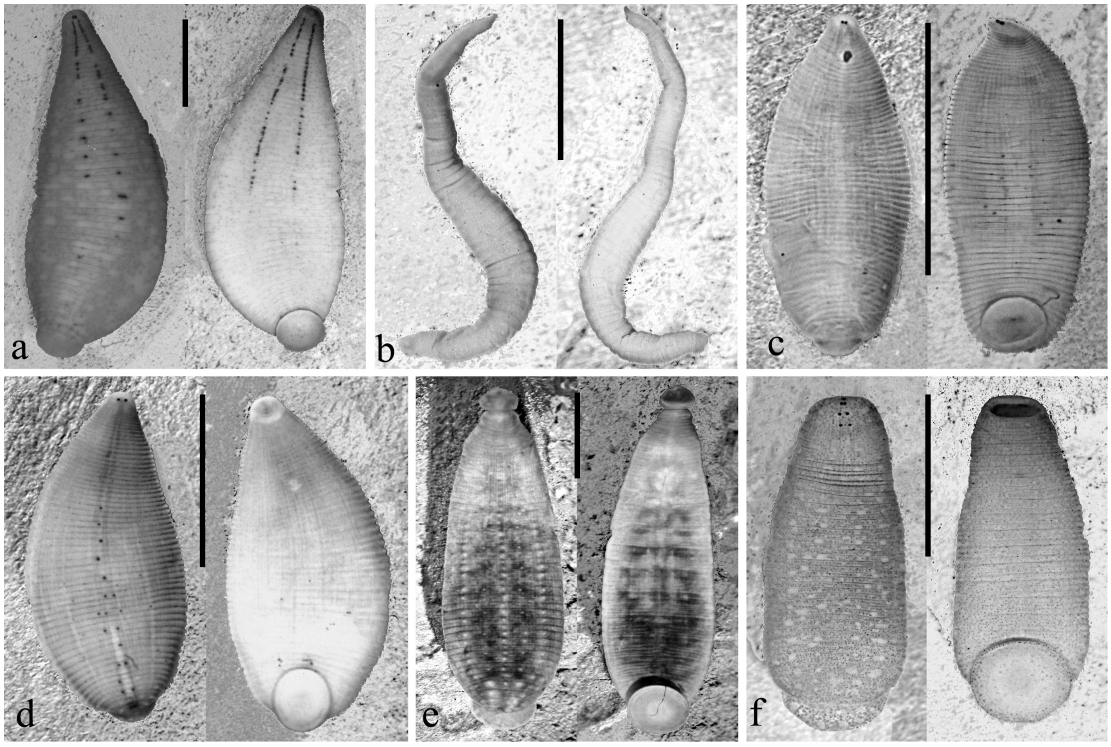
Glossiphonia elegans (Verrill, 1872) Castle, 1900

Figure 9a

The presence of three pairs of eyespots, dorsal papillation and pigmentation pattern, two paramedial longitudinal stripes in the ventral surface, two annuli between gonopores, and a relatively large size correspond with the description of *Glossiphonia elegans*. North American and European forms have very similar morphological traits, to the extent that previous taxonomists (i.e., Klemm, 1982; Sawyer, 1986) considered them to be the same species: *Glossiphonia complanata* (Linnaeus, 1758). However, Siddall et al. (2005), using phylogenetic criteria, found that they represent different evolutionary lineages and

FIGURE 9.

Glossiphoniids found in Washington in this study; dorsal and ventral views, left and right respectively. **a.** *Glossiphonia elegans*. **b.** *Helobdella elongata*. **c.** *H. modesta*. **d.** *Helobdella papillata*. **e.** *Placobdella montifera*. **f.** *Theromyzon* cf. *rude*. Scale bars = 5 mm.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resurrected Verrill's 1872 name *Glossiphonia elegans* for the North American species (see also Madill and Hovingh, 2007). Found on the underside of submerged rocks and wood.

Helobdella elongata (Castle, 1900)

Figure 9b

The absence of a chitinous nuchal scute as well as the presence of an unpigmented, nonpapillated, and subcylindrical body all correspond to the description of *H. elongata*. Washington specimens lacked obvious eyespots, a characteristic noticed for some individuals of this species elsewhere (Klemm, 1982). Found on the underside of submerged rocks and wood.

Helobdella modesta (Verrill, 1872) Siddall *et al.,* 2005

Figure 9c

Specimens all correspond to the description of *H. modesta* due to the presence of an obvious chitinous nuchal scute on the dorsal surface of VIII, one pair of eyespots, and the absence of dorsal or ventral papillation or pigmentation. The presence of a nuchal scute had led previous taxonomists to synonymize almost every previously described species exhibiting this characteristic under the name *Helobdella stagnalis* Linnaeus, 1758. Siddall *et al.,* (2005), using a phylogenetic perspective, resurrected *Helobdella modesta* (Verrill, 1872) for the North American species. Found on the underside of submerged rocks and wood.

Helobdella papillata (Verrill, 1872) Siddall and Borda, 2003

Figure 9d

The presence of one pair of eyespots, longitudinal dorsal pigmented stripes, and rows of black-tipped papillae as well as one annulus between gonopores and diffuse salivary glands match the description of *Helobdella papillata*. This species belongs to the "*triserialis*" complex of species defined by Sawyer, 1986. *Helobdella triserialis* (Blanchard, 1894) was originally described based on specimens collected in Chile. However, because of the high degree of variation in the various forms in the New World, Ringuelet (1943) lumped them all under "*H. triserialis.*" Siddall and Borda (2003) found that at least North and South American forms each constitute distinct evolutionary lineages and resurrected Verrill's name, *Helobdella papillata,* for North American forms, all of which are genetically similar regardless of pigmentation. Specimens were found on the underside of submerged rocks and wood.

Placobdella montifera (Moore, 1906)

Figure 9e

The presence of three longitudinal ridges on the dorsal surface (one median and two lateral) and the distinctively narrow neck constriction, together with the "placobdellid" eyespot arrangement are in agreement with the description of *P. montifera* (see Klemm, 1982). Found on the undersides of submerged rocks and wood.

Theromyzon, cf. *rude* (Baird, 1869)

Figure 9f

The presence of four pairs of eyespots arranged in parallel, two annuli between gonopores, cylindrical male atrium and weakly developed atrial cornua agree with the description of *Theromyzon rude* (see Oosthuizen and Davies, 1993). The type locality of *T. rude* is in the Northwest at Great Bear Lake, Canada. Sawyer (1986) considered this species common in the western United States and Canada. A major revision of the genus, including their phylogenetic relationships has yet to be attempted. Found on the underside of submerged rocks and wood.

Arhynchobdellida Blanchard, 1894

Erpobdellidae Blanchard, 1894

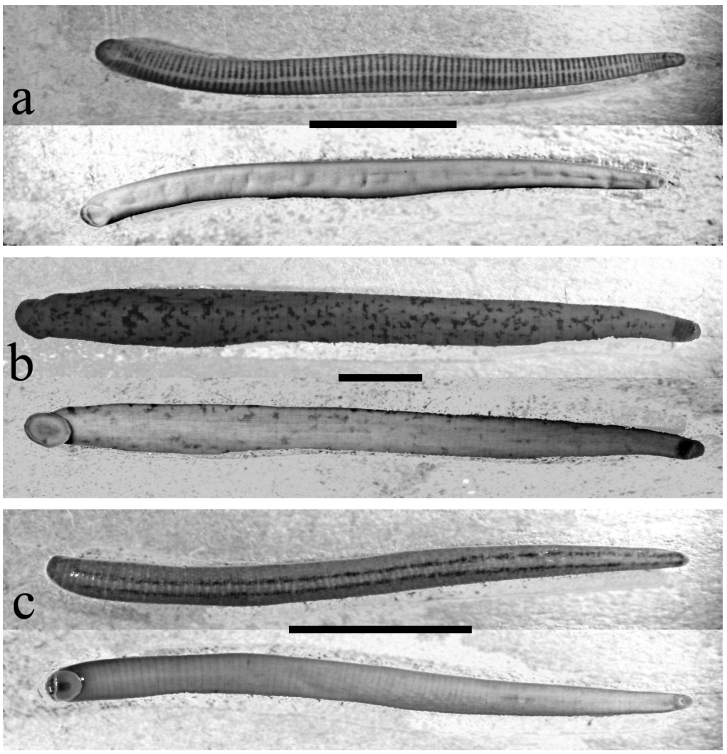
Erpobdella annulata (Moore, 1922) Oceguera-Figueroa et al. (2011)

Figure 10a

Moore (1922) described *Erpobdella punctata annulata* from the vicinity of Vancouver, Canada, and noticed that in the humid Pacific regions of Washington, Oregon, and British Columbia this subspecies replaces the more common *E. punctata punctata*. The main morphological characteristic that distinguishes *E. annulata* from *E. punctata* is that the former has strongly pigmented dorsal transverse stripes (one on each annulus), unlike the longitudinal pigmentation of *E. punctata* sensu stricto or any of its variants (see Madill and Hovingh, 2007). Oceguera-Figueroa *et al.,* (2011) suggested that the name *E. annulata* should be used for the transversally striped form from the Pacific Northwest. All other morphological characters, including three pairs of eyespots, two annuli between gonopores, and ejaculatory ducts not forming preatrial loops, are consistent with those of *E. annulata*. Found on the underside of submerged rocks and wood.

FIGURE 10.

Erpobdellids found in Washington in this study; dorsal and ventral views, top and bottom respectively. **a.** *Erpobdella annulata*. **b.** *E. obscura*. **c.** *Erpobdella punctata*. Scale bars = 5 mm.


a
b
c

Erpobdella obscura (Verrill, 1872) Siddall, 2002

Figure 10b

The presence of four pairs of eyespots, colored patches dispersed on the dorsal and ventral surface, two annuli between gonopores, spirally coiled atrial cornua, ejaculatory ducts forming preatrial loops, and a relatively large size correspond to the description of *Erpobdella obscura* (see Klemm, 1982). Found on the underside of submerged rocks and wood.

Erpobdella punctata (Leidy, 1870)

Figure 10c

Erpobdella punctata is widely distributed in North America with highly variable pigmentation patterns (see diagrams in Klemm, 1982; Madill and Hovingh, 2007). The presence of two longitudinal paramedial stripes on the dorsal surface, as well as three pairs of eyespots, two annuli between gonopores and ejaculatory ducts not forming preatrial loops are consistent with the characteristics of *E. punctata*. Found on the underside of submerged rocks and wood.

CONCLUSIONS

This paper represents a first broad attempt to investigate the hirudinifauna from Washington. We found a relatively rich diversity of leeches with 11 species of two families (Glossiphoniidae and Erpobdellidae) including two new glossiphoniids: *Placobdella kwetlumye,* n. sp., and *Placobdella sophieae,* n. sp. Altogether, 14 species are now known for the state of Washington, including those identified by Klemm (1982).

Our discovery of heretofore undescribed species of *Placobdella* continues a recent pattern of increasing clarity regarding the diversity of this principally North American genus of blood-feeding leeches (Hughes and Siddall, 2007; López-Jiménez and Oceguera-Figueroa, 2009; Oceguera-Figueroa and Siddall, 2008; Siddall and Bowerman, 2006). Additional faunistic surveys of neighboring freshwater habitats in Idaho, Wyoming, northern California, and southwestern Canada may yet reveal species in addition to those already found in Oregon and Washington.

Previously, glossiphoniid leeches known to feed on birds were exclusively in the genus *Theromyzon*. *Placobdella kwetlumye,* n. sp., is the first species of the genus found feeding from waterfowl, though not from their narines, as is typical for species of *Theromyzon*. Whether this represents a specificity for waterfowl by *P. kwetlumye,* n. sp., or is merely reflective of very general tastes is not yet clear.

Morphology of the salivary complex has long been used in the taxonomy of the genus and some species formerly considered as belonging to *Placobdella* were transferred to the genus *Desserobdella* Barta and Sawyer, 1990, because of their possession of diffuse salivary glands (Barta and Sawyer, 1990; Jones and Woo, 1990). However, Siddall *et al.,* (2005) recovered *Desserobdella* as polyphyletic showing the poor phylogenetic value of this character and considered *Desserobdella* a junior synonym of *Placobdella*. Regardless of this, the character is useful in distinguishing between species since no evidence of intraspecific (within a particular species) variation of the salivary glands in *Placobdella* species is known to occur. *Placobdella kwetlumye,* n. sp., is unique among the species of *Placobdella* insofar as it possesses a single pair of compact salivary glands; its inclusion in the genus *Placobdella* is guaranteed by its possession of diagnostic characters but

provide evidence for the extremely variable condition of the salivary cells. Similar levels of salivary gland variation occur in the genus *Helobdella*, which includes non–blood-feeding leeches with compact, partially compact and diffuse salivary glands (Siddall and Borda, 2003) but in sharp contrast with the unique salivary gland morphology present in all the species of *Haementeria* (e.g., Oceguera-Figueroa, 2008).

3.2 Especie nueva de sanguijuela del género *Helobdella* (Rhynchobdellida: Glossiphoniidae) del Lago de Catemaco, Veracruz, México

(Adapted from: Oceguera-Figueroa A., 2008. Especie nueva de sanguijuela del género *Helobdella* (Rhynchobdellida: Glossiphoniidae) del Lago de Catemaco, Veracruz, México. Acta Zoologica Mexicana (Nueva Serie) 23, 15–22)

Introducción

El género *Helobdella* incluye especies de sanguijuelas dulceacuícolas que se caracterizan por presentar en la mayoría de los casos el cuerpo aplanado dorsoventralmente, un par de manchas oculares cefálicas, un anillo separando los gonoporos y por carecer tanto de micetomas como de órganos esofágicos (Siddall y Borda, 2003). La mayoría de las especies del género se alimentan de invertebrados acuáticos como moluscos y oligoquetos y presentan una distribución mundial con excepción de la antártica con la mayor riqueza de especies en América del Sur. Sawyer (1986) dividió al género *Helobdella* en dos series: La serie *stagnalis* que incluye a especies con placa quitinoide dorsal y a la serie *triserialis* que carecen de dicha placa. Recientes estudios sobre las relaciones filogenéticas del grupo han puesto en duda la validez de dicho carácter para reconocer grupos naturales (Siddall y Borda 2003), y han demostrado que varias características morfológicas (i.e. Glándulas salivales compactas y número de ciegos en el buche) empleadas por Ringuelet (1978) para subdividir el género *Helobdella* en cinco géneros (*Dacnobdella, Acritobdella, Adaetobdella, Gloiobdella* y *Helobdella*) no definen grupos naturales y por lo tanto no sustentan la existencia de dichos géneros, por lo cual se considera a *Helobdella* como el único género válido (Siddall y Borda 2003).

En México se han registrado dos especies de *Helobdella* con palca quitinoide dorsal: *Helobdella stagnalis* y *Helobdella atli* (Oceguera-Figueroa y León-Règagnon 2005) y tres especies sin placa quitinoide dorsal: *Helobdella elongata*, *Helobdella conchata* y *Helobdella triserialis*. Las especies *Helobdella socimulcensis*, *Helobdella moorei* y el registro de *Helobdella fusca* realizado por Oka (1932) y Caballero (1935, 1940a,b) son consideradas como *H. triserialis* por Ringuelet (1985) y Sawyer (1986).

Recientemente se ha sugerido, con base en hipótesis filogenéticas, que las formas norteamericanas (EUA) de *Helobdella triserialis* y *Helobdella stagnalis* deben ser consideradas como *Helobdella papillata* y *Helobdella modesta* respectivamente (Siddall y Borda, 2003; Siddall *et al.,* 2005), sin embargo, al no haber incluido ejemplares recolectado es en México, se consideran a *H. triserialis* y *H. stagnalis* especies valida para nuestro país hasta que se realice un estudio formal del grupo para las especies mexicanas.

Durante la recolecta de ejemplares de hirudíneos en la laguna de Catemaco, Veracruz, México, se detectó una especie nueva del género *Helobdella*, cuya descripción morfológica se realiza en el presente trabajo.

MATERIALES Y MÉTODO

Las sanguijuelas se encontraron fijadas en raíces y bajo piedras a las orillas de la laguna de Catemaco, Veracruz, México. (Permiso de colector científico FAUT0056 para Virginia León Règagnon). Los ejemplares se recolectaron con la mano y se transportaron a laboratorio en recipientes de plástico con agua del medio, donde se procedió a anestesiarlos mediante la adición gradual de alcohol al 70% y posteriormente se fijaron en formol al 4% o en alcohol 100%. Las observaciones, disecciones y esquemas se realizaron con un microscopio estereoscopico Zeiss 475052-9901. Las microfotografias de los ejemplares fueron tomadas con una camara digital Olympus (Camedia C-3040 Zoom) adaptada a un microscopio. Los ejemplares de referencia se depositaron en la Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional

Autónoma de México y en la colección de anélidos del American Museum of Natural History, Nueva York, EUA. (AMNH).

RESULTADOS

Helobdella virginiae sp. nov.

Figura 11

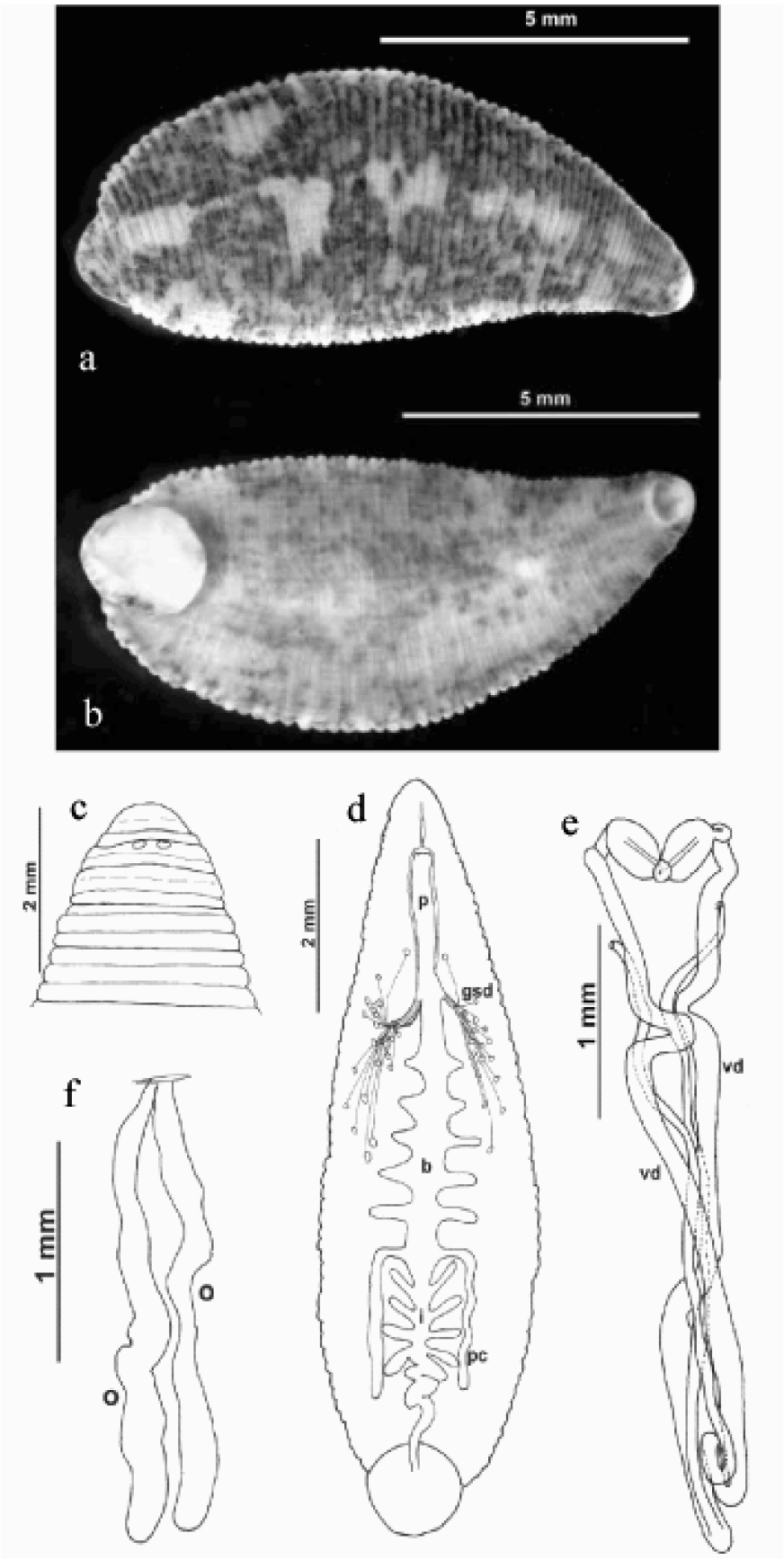
La descripción se realizó con base en 23 ejemplares completos (dos preparaciones permanents, dos disecciones y 19 ejemplares completos).

MORFOLOGÍA EXTERNA

Sanguijuelas pequeñas, los ejemplares miden de 5.7-9.6 mm de largo por 2.7- 4.9 mm de ancho. Los organismos adultos presentan la superficie dorsal obscura con manchas blancas de número y disposición muy variable (Figura 11a). En la mayoría de los ejemplares la superficie ventral es blanca o marrón con pigmentos obscuros mas evidentes hacia los márgenes laterales del cuerpo (Figura 11b). Superficie dorsal con 3-5 hileras longitudinales de papilas en a2 de cada somita, más evidentes en la región media y posterior del cuerpo, una de las hileras es media y dos a cada lado, algunos ejemplares carecen de papilas evidentes. Superficie ventral sin papilas. Gonoporo masculino en XII a1/a2 y gonoporo femenino en XII a2/a3. Gonoporos separados por un anillo. Un par de manchas oculares en IV, bien separadas entre sí (Figura 11c). Carece de placa quitinoide dorsal. Ano localizado en la superficie dorsal en entre las somitas XXVI y XXVII. Somitas I-III unianuladas, Somitas IV y V bianuladas. Somita VI- XXIV trianuladas. Somita XXV y XXVI bianuladas. Somita XXVII unianulada. Ventosa anterior más o

FIGURE 11.

Morphological characteristics of *Helobdella virginiae,* n. sp. **a.** Holotype, dorsal view. **b.** Holotype, ventral view. **c.** Eyespots. **d.** Internal morphology. **e.** Male reproductive system. **f.** Female reproductive system. Proboscis (p), diffuse salivary glands (gsd), crop (b), posterior ceaca (pc), intestine (i), vas deferens (vd), ovisacs (o)**.**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menos triangular. Boca en la región anterior de la ventosa, subterminal. Ventosa posterior circular.

Morfología interna

Proboscis envuelta en una cubierta membranosa. Células salivales dispersas en el parénquima entre XI-XVI. Las conductos de las células salivales se unen formando un conducto común que se inserta en la base de la proboscis en la somita XI. Esófago simple, no recurvado. Micetomas ausentes. Buche con seis pares de ciegos de XIV a XIX, el último de ellos con recorrido descendente, formando un par de post-ciegos o divertículos que alcanzan la somita XXIV. Intestino con cuatro pares de ciegos de XX a XXIII (Figura 11d). Seis pares de testisacos intersegmentales de XIII/XIV-XVIII/XIX, el primer par poco desarrollado. Vasos deferentes simples muy largos, sin recorrido ascendente, con recorrido descendente hasta la somita somita XX (Figura 11e). Ovisacos simples que alcanzan la somita XVII (Figura 11f).

Material examinado

Holotipo. (CNHE 5474) Fijado en Formol 4%, Conservado en una mezcla de glicerina, alcohol y formol. Xmm largo, XXmm ancho. MÉXICO. Lago de Catemaco, Colonia "La Victoria", Veracruz. 18.65^{o} N 96.43 ^{o} W. Recolectado por A. Oceguera-Figueroa, E. Martínez, L. Romero, R. Bautista y A. Espinoza. 9 Septiembre 2005.

Paratipos. (CNHE 5475, 5476) 19 ejemplares, (AMNH 3526) 4 ejemplares. Misma localidad, recolectores y fecha que el holotipo.

Etimologia: El epíteto especifico "*virginiae"* hace referencia la Dra. Virginia León Règagnon, en reconocimiento a sus investigaciones en el campo de la helmintología.

DISCUSIÓN

De acuerdo con la diagnosis del género elaborada por Siddall & Borda (2003), los miembros del género *Helobdella* presentan los gonoporos separados por un solo anillo, un par de manchas oculares cefálicas, sin órganos esofágicos ni micetomas. Sin representantes hematófagos. Estos caracteres son consistentes con los de la especie que aquí se describe, por lo cual es claro que se trata de una especie del género *Helobdella*.

En México se consideran como validas a tres especies del género *Helobdella* sin placa quitinoide dorsal: *Helobdella elongata* que carece de ciegos en el buche a excepción de postciegos o diverticulos y el cuerpo es largo, angosto y carece papilas y pigmentos mientras que *H. virginiae* presenta cinco pares de ciegos en el buche, cuerpo aplanado dorsoventralmente así como pigmentos y papilas. *Helobdella triserialis* presenta cuatro o seis franjas longitudinales de pigmentos obscuros en el dorso formando un patrón completamente distinto al de *H. virginiae* que presenta una superficie dorsal obscura con grandes manchas blancas de disposición muy variable. Por ultimo, *H. virginiae* es fácilmente diferenciable de *H. conchata* ya que esta ultima presenta los anillos subdivididos mientras que *H. virginiae* presenta tres anillos no subdivididos por cada somita completa.

En Estados Unidos y Canadá se han registrado cinco especies del género *Helobdella* sin placa quitinoide dorsal (Klemm 1982; Sawyer 1986; Siddall & Borda 2003): *Helobdella elongata* de la cual ya se discutió sus diferencias con respecto a la nueva especie aquí descrita. *Helobdella robusta* y *Helobdella lineata*, se diferencian de *H. virginiae,* ya que esta ultima presenta la región dorsal obscura con manchas blancas mientras que las primeras dos presentan numerosas hileras longitudinales. *Helobdella*

transversa tiene franjas transversales de manchas blancas, estas se encuentran en el anillo a2 de cada somita, formando un patrón completamente distinto al de *H. virginiae*, que carece de franjas transversales. *Helobdella fusca* es una especie que presenta una gran variación en cuanto a los patrones de pigmentacion de la superficie dorsal (Ver Klemm 1982), *Helobdella virginiae* es muy parecida a la forma señalada por Klemm (1982) como "scattered pigmented form", sin embargo, es fácilmente diferenciable de *H. virginiae*, ya que *H. fusca* carece de cualquier tipo de papilas en la superficie dorsal, además de presentar manchas blancas la región anal (anal patches) mientras que *H. virginiae* presenta tres o cinco hileras longitudinales de papilas en la superficie dorsal y carece de manchas blancas en la región anal.

En América del Sur se encuentra la mayor diversidad de especies del género *Helobdella* (Ringuelet 1986, Siddall & Borda 2003). Hasta la fecha se han registrado aproximadamente 25 especies sin placa quitinoide dorsal en esa región del continente.

Helobdella virginiae se puede diferenciar de *Helobdella michaelseni*, *Helobdella obscura, Helobdella wodzickiorum* y *Helobdella similis*, ya que *H. virginiae* presenta cinco pares de ciegos simples en el buche y un par de postciegos o diverticulos, mientras que *H. michaelseni* y *Helobdella obscura* carecen de ciegos en el buche y *H. similis* y *H. wodzickiorum* únicamente presentan un par de postciegos o diverticulos.

Las especies *Helobdella cryptica, Helobdella chaquensis, Helobdella dubia, Helobdella longicolis, Helobdella xenoica, Helobdella titicacensis*, *Helobdella malvinensis* y *Helobdella sorojchi* presentan glándulas salivales compactas, mientras que *H. virginiae* tiene glándulas salivales difusas en el parénquima.

Helobdella villarsi presenta los gonoporos en los anillos, mientras que *H. virginiae* presenta los gonoporos el los surcos interanulares. *Helobdella paraguayensis, Helobdella chilensis, Helobdella peruvensis* y *Helobdella huaroni* presentan dos anillos entre los gonoporos, por el contrario *H. virginiae* tiene un solo anillo entre los gonoporos. La presencia de dos anillos entre los gonoporos en las especies señaladas no corresponde con la definición del género propuesta por Siddall & Borda (2003) sin embargo, es probable que se trate de observaciones no muy precisas que deben ser tomadas con precaución. Independientemente de este carácter dudoso, *H. paraguayensis* y *H chilensis* presentan la faringe recurvada y no recta como *H. virginiae*. *Helobdella peruvensis* presenta siete líneas dobles longitudinales obscuras y *H. huaroni* el dorso gris claro uniforme o pardo obscuro, características que no corresponden con la superficie obscura con manchas blancas de *H. virginiae*.

Helobdella ampullariae, Helobdella festai, Helobdella hyalina, Helobdella cordobensis y *Helobdella araucana* carecen de papilas en la superficie dorsal, mientras que *H. virginiae* presenta tres o cinco hileras longitudinales dorsales de papilas. En *Helobdella simplex* y *Helobdella brasilensis* hay papilas dorsales en los tres anillos de cada somita, formando hileras longitudinales continuas, mientras que en *H. virginiae*, las papilas dorsales se encuentran en el anillo a2 de cada somita, formando hileras longitudinales con las papilas separadas entre sí por dos anillos. *Helobdella striata* y *Helobdella triserialis* presentan un número variable de hileras longitudinales pigmentadas y de papilas, características que no concuerdan con las descritas para *H. virginiae*.

3.3 A new glossiphoniid leech from Catemaco Lake, Veracruz, México.

(Adapted from: Oceguera-Figueroa A., 2008. A new glossiphoniid leech from Catemaco Lake, Veracruz, México. Journal of Parasitology, 94, 375–380)

INTRODUCTION

Genus *Haementeria* de Filippi 1849 includes approximately 10 species of blood-feeding on vertebrates leeches common in South America with only two representatives in North America: *Haementeria officinalis* de Filippi 1849 and *Haementeria lopezi* Oceguera-Figueroa 2006. This genus comprises species with 1 or 2 compact salivary glands connected to the base of the proboscis and two pairs of spheroidal symbiont-bearing organs called mycetomes (Ringuelet, 1976a,1985). Members of this group have been used in studies of neurophysiology (De-Miguel *et al.,* 2001), salivary anticoagulant properties (Salzet, 2001) and co-evolutionary patterns with associated bacteria (Perkins *et al.,* 2005).

MATERIALS AND METHODS

One specimen of the species described herein was sent to the author in March, 2005. This leech was removed from the leg of a high school student in Catemaco Lake, Veracruz, Mexico. A scientific expedition to the same locality was conducted in September, 2005 in order to obtain additional specimens. Leeches were collected by immersing legs into the water in the edges of the lake, waiting 1 minute and then, examining for leeches attached to skin. Six leeches were obtained prior to blood ingestion, placed in plastic containers and transported to the laboratory. Specimens were relaxed with gradual addition of alcohol and fixed with 4% formalin. The posterior sucker of one specimen was fixed in 100% alcohol. One leech was compressed between two glass-slides, stained with a mixture of Elrhich Hematoxylin-Mayer´s Paracarmin and mounted on a slide. Dissections were accomplished under a stereoscopic microscope. Photographs of whole specimens

were taken using a Sony Cyber-shot DSC-H5. Photographs of internal structures were taken using a Nikon SMZ-U stereo-microscope with a SPOT-RT (Diagnostic Instruments, Inc.) digital camera. Drawings were made by superposition of vector-art on placed images in Adobe Illustrator (Adobe Systems, San Jose California). Specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México and in the American Museum of Natural History, New York, USA. (AMNH).

DESCRIPTION

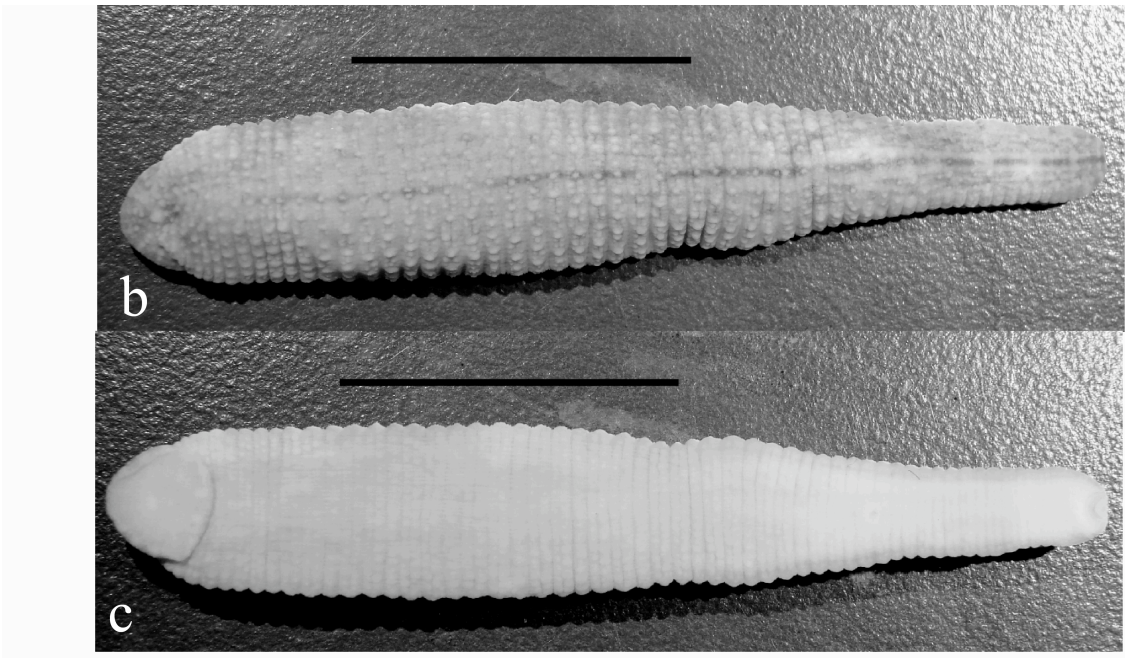
Haementeria acuecueyetzin n. sp.

Figure 12

External morphology: Brownish, largest specimen 58 mm long, 10.7 mm wide. Complete somite triannulate, a1, a2 and a3 dorsally subdivided but only a1 and a3 subdivided ventrally. Somites I-II uniannulate. Somites III-IV biannulate. Somites V-XXIV triannulate. Somites XXV-XXVII biannulate. Dorsum rough with 6 longitudinal rows white smooth papillae along the body, 1 pair paramedial, 1 pair lateral and 1 pair submarginal. This kind of white papillae occurs on a2 of each somite, are typically roundshaped but in some cases bar-shaped. All annuli with numerous (more than 20) tubercles not forming obvious longitudinal rows. Each tubercle covered with minute papillae. Additionally, small papillae in space not occupied by tubercles and smooth papillae (Figure 12a). Well-defined obscure longitudinal mid-dorsal line starts immediately posterior to eyespots continuing to end of body, partially interrupted on a2 of each annuli anteriorly. Some specimens with 2 indistinct longitudinal rows, 1 on each

Figure 12

Morphological characteristics of *Haementeria acuecueyetzin,* n. sp. **a**. Dorsal view of the posterior sucker. (smp) smooth papillae; (t) tubercles; Small papillae not shown. **b** Dorsal view. **c**. Ventral view. Bar = 20 mm. **d**. Base of the proboscis from the dorsal perspective. **e**. Male and female reproductive systems from the dorsal perspective. Somites are numbered with roman numbers and correspond with individual ganglia. pr, proboscis; bpr, base of the proboscis ov, ovisacs; ac, atrial cornua; ed, ejaculatory ducts. my, mycetomes; csg, compact salivary glands; es, esophagus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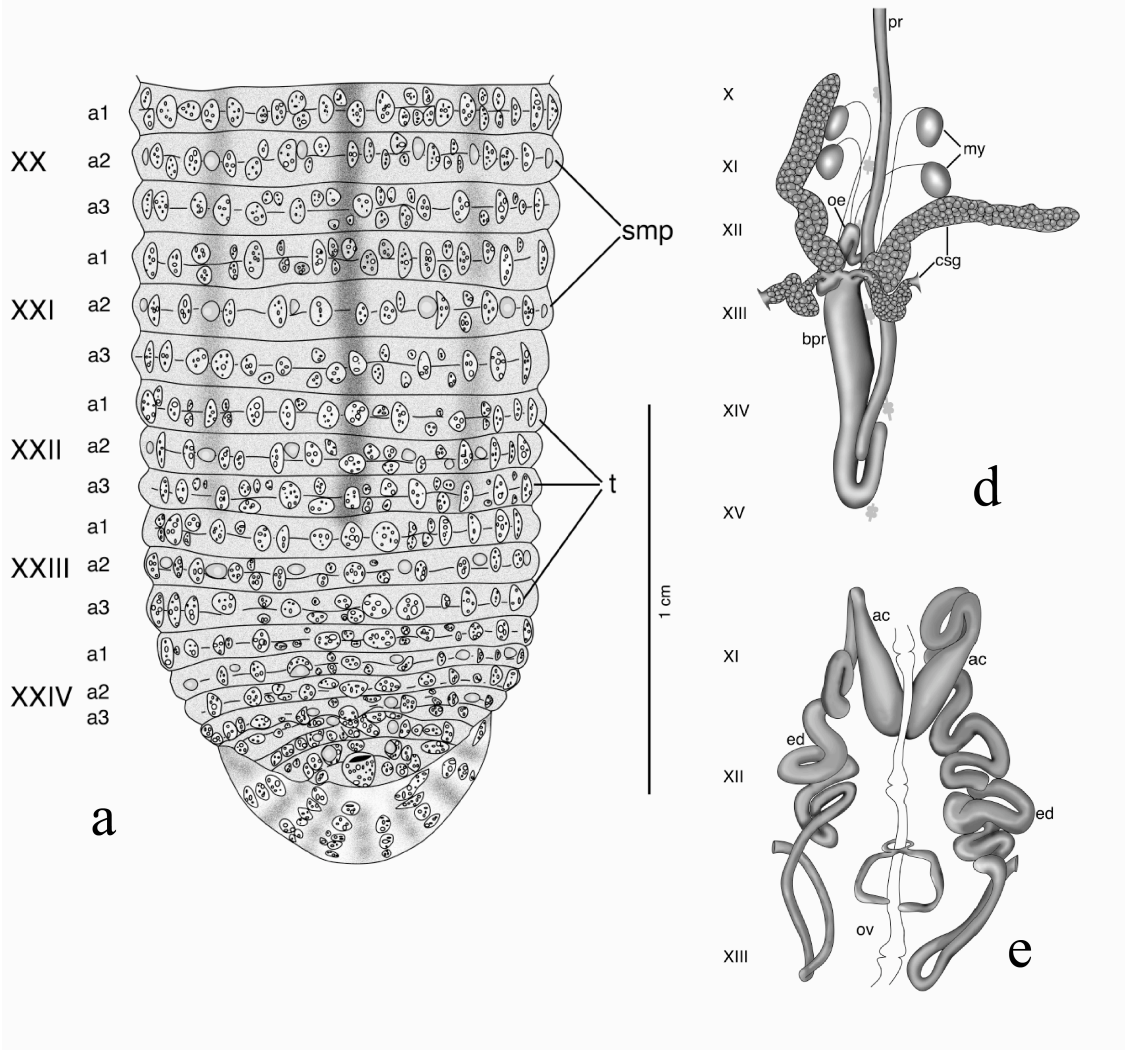
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side (Figure 12b). Cephalic somites with 4-6 white spots on a2 arranged metamerically. One pair of eyespots separated by less than diameter of an eyespot, located on first annulus of biannulate somite III. Ventral surface lighter than dorsum, fully covered with small tubercles (Figure 12c). Male gonopore conspicuous, between XI/XII. Female gonopore on XII between a2/(b5+b6). Two annuli between gonopores. Oral sucker somewhat triangular, mouth pore subterminal in the anterior border. Anus located on dorsal surface, at XXVII. Caudal sucker directed ventrally in relaxed specimens, thinner than posterior part of body, with small papillae and radial white lines on dorsal surface. *Internal morphology:* Proboscis in membranous sheath extending posteriorly to somite XIV/XV, forming loop, returning anteriorly and connecting with oesophagus at XII/XIII. Two pairs compact salivary glands discharge into base of proboscis; biggest pair is located from somite X to XII/XIII connecting anteriorly to base of proboscis via strong ducts; second pair of compact salivary glands folded in somite XIII, each provided with a peduncle connecting the distal region with parenchyma. Oesphagus extending anteriorly to somite XI/XII, forming loop, returning posteriorly to crop. Two pairs spheroidal mycetomes located latero-ventrally in somite XI. Each mycetome connecting independently via thin ductules to oesophagus (Figure 12d). Crop with 7 pairs branched caeca from XIII to XIX; last pair forming postcaeca. Intestine with 4 simple caeca in somites XX-XXIII. Atrial cornua well-developed. Highly coiled ejaculatory ducts immediately posterior to atrial cornua. Six pairs intersegmental testisacs from XIII/XIV to XVIII/XIX. Ovisacs short and simple, forming a ring around the ventral nerv cord and reaching somite XIII b6 (Figure 12f).

Taxonomic summary

Type host: Unknown. This species has been observed on cattle and horses, and sometimes on humans, but a spectrum of vertebrate hosts is likely.

Type locality: Catemaco Lake, Veracruz State, Mexico. Near "Colonia La Victoria" 18º 22' 33.4" N, 95º 06' 34.4" W. Altitude 335 m.

Type material: Holotype, CNHE 6036; Paratypes CNHE 6037, 6038, AMNH 5428 annelida. Collected by A. Oceguera-Figueroa. September 9, 2005. All specimens collected in the same locality.

Etymology: ACUECUEYETZIN means "leech" in the Nahuatl language, and derives from the conjunction of the words a=atl= water; cuecueya= cueyatl in plural=frogs and tzintli=diminutive. Small water frogs.

Remarks

According to the diagnosis of *Haementeria* elaborated by Ringuelet (1976a, 1985), members of this group have 1 pair of eyespots, 1 or 2 pairs of compact salivary glands, 2 pairs of spheroidal mycetomes and triannulate somites with annulus a1 and a3 subdivided ventrally, giving the appearance of quinquannulate somites in the ventral surface. These characteristics are consistent with those of the new species. Two species of the genus *Haementeria* have been reported in Mexico: *H. officinalis* (Caballero, 1930, 1932b, 1940a, 1941; Oka, 1932; Ringuelet, 1976a, 1981, 1985; Sawyer, 1986; Lamothe-Argumedo *et al.,* 1997) and *H. lopezi* (see Oceguera-Figueroa, 2006a). *Haementeria acuecueyetzin* n. sp. can be easily distinguished from *H. officinalis* because the former has 6 longitudinal dorsal rows of white smooth papillae which *H. officinalis* lacks. Additionally, *H. officinalis* lacks tubercles on the ventral surface. *Haementeria lopezi* has

undivided dorsal annuli with almost flat white papillae in contrast to the subdivided dorsal annuli and conspicuous papillae and tubercles of *H. acuecueyetzin* n. sp.. The greatest diversity of *Haementeria* is found in South America, with 8 species of the genus (Ringuelet, 1985). Unlike *H. acuecueyetzin* n. sp., *Haementeria ghilianii* de Filippi, 1849 from Brazil and French Guyana lacks subdivided dorsal annuli, and may reach almost 200 mm. *Haementeria molesta* (Cordero, 1934), from Brazil and Uruguay, has only one annulus between gonopores and five longitudinal rows of dorsal papillae. Like *H. acuecueyetzin* n. sp., *Haementeria paraguayensis* (Weber, 1915) exhibits ventral tubercles, but differs in their being arranged in 4 to 6 longitudinal rows. Moreover, *H. paraguayensis* is unusual among species of the genus in having only a single pair of compact salivary glands discharging into the base of the proboscis. Adult *Haementeria eichhorniae* Ringuelet, 1978 measure 15 mm, much smaller than *H. acuecueyetzin* n. sp., and have 5 longitudinal rows of dorsal papillae, whereas *H. acuecueyetzin* n. sp. exhibits 3 pairs and lacks a mid-dorsal row. Other species in the genus differing from *H. acuecueyetzin* n. sp. by having a middorsal row of papillae are *Haementeria lutzi* (Pinto, 1920) with 7 longitudinal rows and *Haementeria vizottoi* (Castro, 1971) with 5 rows. *Haementeria depressa* (Blanchard, 1849) is reported to have 4 or 5 rows, either of which is fewer than those described here for *H. acuecueyetzin* n. sp. None of the foregoing has tubercles in the ventral surface. Lacking a mid-dorsal row of papillae, *Haementeria tuberculifera*. (Grube, 1871) has 6 longitudinal rows of dorsal papillae like *H. acuecueyetzin* n. sp. but clearly lacks tubercles on the ventral surface. Early studies on the geographic distribution of *Haementeria*, considered that a unique taxon dispersed from South America to North America after the formation of Panamanian Isthmus. With the

recognition of *Haementeria acuecueyetzin* n. sp., the third species of the genus described for Northern Hemisphere, a more complicated pattern is emerging. Whether Mexican species form a monophyletic group with post-dispersal speciation, or that each species represents an individual dispersal event from the Southern Hemisphere needs to be clarified in order to improve our understanding of biogeographic history of the Mesoamerican transitional zone.

3.4 *Placobdella lamothei* n. sp. (Hirudinea: Glossiphoniidae), a new leech parasite of freshwater turtles from Estado de México, Mexico

(Adapted from: Oceguera-Figueroa A., and Siddall, M. E. 2008. *Placobdella lamothei* n. sp. (Hirudinea: Glossiphoniidae), a new leech parasite of freshwater turtles from Estado de México, Mexico. Revista Mexicana de Biodiversidad, 79, 135S–139S)

INTRODUCTION

Placobdellid leeches are well known and relatively common in North America where they occur feeding on amphibians, turtles and fish. According to Sawyer (1986), the genus *Placobdella* includes species defined by possessing triannulate somites, placobdellid ocular morphology (1 obvious pair of ocelli plus another smaller coalesced pair), 2 pairs of compact salivary glands and 1 pair of sac-like symbiont-bearing structures in the oesophagus called mycetomes. In a recent phylogenetic analysis of Glossiphoniidae, Siddall et al., (2005) found that members having placobdellid ocular morphology like those of *Desserobdella* spp. and *Oligobdella biannulata* Moore, 1900 all appear well-nested within *Placobdella,* notwithstanding their possession of diffuse salivary glands in the first case and biannulate somites in the later. As such, *Desserobdella* and *Oligobdella* should be considered subjective junior synonyms of *Placobdella.* The number of species of the genus is not well established and it is clear that a major revision is needed in order to clarify this issue. Tentativelly, 16 species of *Placobdella* have been described in North America, 1 in Europe: *Placobdella costata* (Müller 1864), and 3 poorly characterized species from South America (Ringuelet, 1985) that may well be species of *Haementeria* typical of that area. Contrasting with the substantial number of species from USA and Canada, only 2 species of placobdellids have been recorded in Mexico (Sawyer, 1986): *Placobdella moorei* Autrum, 1936 and *Placobdella multilineata* Moore 1953.

MATERIALS AND METHODS

In the course of ongoing research on the reproductive ecology of freshwater turtles

conducted by Rodrigo Macip- Ríos in Tonatico, Estado de México, Mexico, placobdellid leeches were found attached to the carapace of *Kinosternon integrum* Le Conte, 1854. Leech specimens were collected and sent to the first author in July, 2004 in order to identify them. Following morphological inspection of the leeches, additional collections were made so as to preserve specimens in 100% ethanol for further studies and in order to record the colors of live specimens. Freshwater turtles were collected using a beach seine and examined for leeches attached to the plastron, the carapace, and to soft body regions. All the specimens were collected under the Scientific Collecting License FAUT0056, issued to Virginia León-Règagnon. Leeches were detached from their host, transported to the laboratory and relaxed with gradual addition of 96% ethanol followed by fixation in 70% ethanol. The posterior sucker of 1 specimen was stored in 100% ethanol. Examination of external morphology and dissections were accomplished with a Nikon SMZ-U stereomicroscope. Photographs were taken using a Sony Cyber-shot DSC-H5 and a SPOT-RT (Diagnostic Instruments, Inc.) digital camera attached to a stereomicroscope. Drawings were made by superposition of vector-art on placed images in Adobe Illustrator (Adobe Systems, San Jose California). Leeches were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico and in the American Museum of Natural History, New York, New York, USA (AMNH). For comparative purposes, *Placobdella rugosa* (Verrill, 1874) (CNHE 1691, 1695) and *Placobdella mexicana* (Moore, 1898) (CNHE 1646, 1683) also were examined.

DESCRIPTION

Placobdella lamothei n. sp.

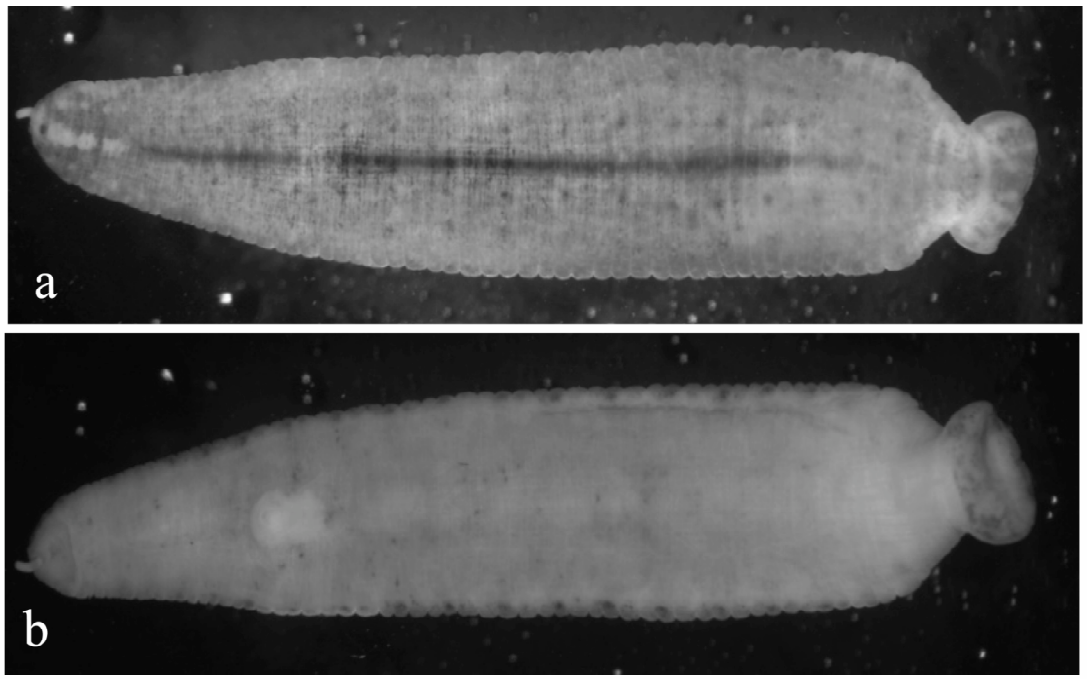
Figure 13

External morphology. Brownish, largest specimen 19 mm long, 4.8 mm wide. Complete somite triannulate, Somites I-II uniannulate. Somites III-IV biannulate. Somites V-XXIV triannulate. Somites XXV-XXVII biannulate. Dorsum with 3 inconspicuous longitudinal rows of papillae from VII through XXV, 1 medial row with papillae in a1, a2 and a3, and 2 paramedial rows with papillae only on a2. Small papillae irregularly dispersed in the space not occupied by rows of papillae. One heavily pigmented longitudinal strip from VII to XXIV. Metameric paramedial and marginal pigmented patches on a2 from IX to XIV. Unpigmented medial zone on dorsum in somites I-VIII partially interrupted in VI and VII a3. Posterior sucker brownish without radial pigmentation or papillae (Figure 13a). Ventral surface unpigmented save for rare chromatophores and without papillae (Figure 13b). Two pair eyespots in «placobdellid» arrangement, inconspicuous confluent pair on II and larger, conspicuous pair on III, also confluent. Mouth pore on the anterior margin of the oral sucker. Male gonopore between XI and XII. Female gonopore on XII between a2/a3, thus 2 annuli between gonopores. Anus between (a1,a2) and a3 of the biannulate somite XXVII.

Alimentary tract. Short and robust proboscis in membranous sheath reaching posteriorly to IX connecting to a conspicuously long and recurved oesophagus. Two pairs of well developed compact salivary glands connecting into base of proboscis; anterior (medial) pair is located in VIII connecting to base of proboscis via strong ducts in IX; posterior (lateral) pair of compact salivary glands in IX. Oesophagus unusually long for the genus,

FIGURE 13

Morphological characteristics of *Placobdella lamothei,* n. sp. **a**. Holotype, dorsal view. **b.** Holotype, ventral view. **c.** Proboscis from the dorsal perspective. **d.** Dorsal view of the male and female reproductive systems. Pr= proboscis, csg= compact salivary glands. Bpr.= base of the proboscis. E=esophagous. My=mycetomes. Ac=atrial cornua, mg=male gonopore. Ed=ejaculatory duct. Fg=female gonopore. Ov=ovaries.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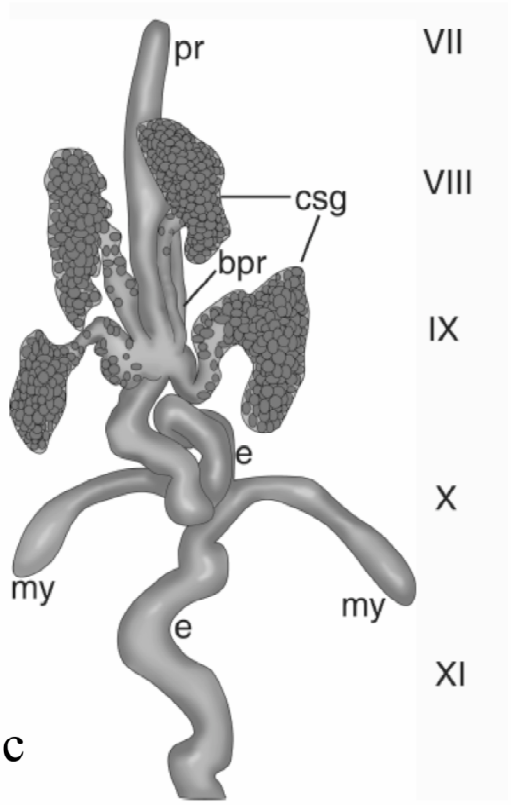
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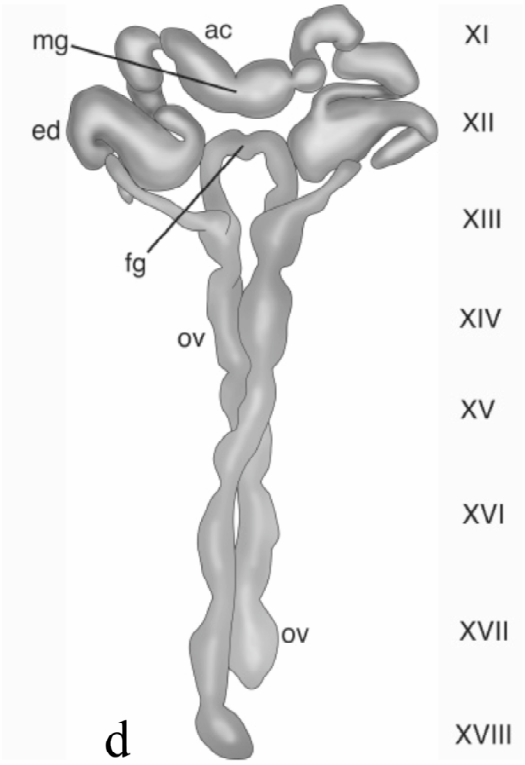
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thick, connected anteriorly to base of proboscis and posteriorly with crop. One pair of sac-like mycetomes in X connected with the oesophagus. Length of oesophagus from base of proboscis to mycetomes is approximately 2 times the length of the proboscis (Figure 13c). Crop with 7 pairs branched caeca from XIII to XIX; last pair forming postcaeca. Intestine with 4 simple caeca in somites XIX-XXIII. *Reproductive anatomy.* Atrial cornua well-developed anterolaterally directed. Highly coiled ejaculatory ducts immediately posterior to atrial cornua on somites XI-XIII. Male median reproductive anatomy entirely anterior to XIII/a2. Five pairs intersegmental testisacs from XIII/XIV to XVII/XVIII. Ovisacs without common oviduct. Ovarian lobes from XII to XVIII, accessory anterior lobe arising from bifurcation at XIII, extending anteriorly to XII a3 (Figure 13d).

TAXONOMIC SUMMARY

Type host: freshwater turtle *Kinosternon integrum* Le Conte, 1854. *Type locality*: La Puerta de Santiago, Municipio de Tonatico, Estado de México. Mexico. 18°45´00´´N 99°37´31´´W. Altitude 1 630 m *Type material*: holotype, CNHE 6030, August, 11 2007. Paratypes CNHE 6031, AMNH 5510 annelida, July, 2004. Specimens collected by Rodrigo Macip-Ríos, Noemí Matías-Ferrer and Alejandro Oceguera-Figueroa in the same locality and on the same host. *Etymology*: this species is named in honor of Rafael Lamothe Argumedo, in recognition of his prolifi c scientifi c production and for being professor of invertebrate zoology for many years in the Facultad de Ciencias, UNAM, inspiring and sharing with young biologists his love and passion for invertebrates.

REMARKS

According to the most recent account of World leeches (Sawyer, 1986), 2 species of *Placobdella* have been recorded from Mexico: *P. moorei* Autrum, 1936 and *P. multilineata* Moore, 1953. Autrum (1936) considered *Placobdella* a junior synonym of *Haementeria*. According to this, the name *Placobdella mexicana* Moore, 1898 was considered preoccupied by *Haementeria mexicana* de Fillipi, 1849. For this reason, *Placobdella moorei* Autrum, 1936 was erected in order to solve this apparently subjective synonymy. Interestingly, *Haementeria mexicana* was previously synonymized with *Haementeria officinalis* by Blanchard (1893) and presently *Placobdella* is no longer considered a synonym of *Haementeria*. This confusion was well understood and clarified in Ringuelet (1981, 1982b) but not fully recovered in Sawyer's (1986) treatment, wherein he recognized *Placobdella* and *Haementeria* as distinct genera, yet still used the name *Placobdella moorei* instead of the correct ressurection of the available name *Placobdella mexicana*. As such, herein we formally resurrect *Placobdella mexicana* and consider *Placobdella moorei* to be a junior synonym. *Placobdella mexicana* has been recorded only twice: in the original description, which was based on 3 specimens from an unspecified locality in Mexico (Moore, 1898) and 34 years later, by Oka (1932) who recorded this species from Chapala, Jalisco. Caballero (1940a, 1941) recorded *Placobdella rugosa* Verrill, 1874 from Xochimilco, México City and Cacahuamilpa, Guerrero. This species was considered a junior synonym of *Placobdella ornata* (Verrill, 1872) by Soós (1969), to the extent that Klemm (1982) recorded *P. ornata* in Mexico based solely on this synonymy. The existence of multiple species of papillated leeches in North America has recently become clearer (Huges and Siddall, 2007; Siddall and

Bowerman, 2006). Moore (1960) noted that the leeches depicted in Caballero's (1940a; 1941) figures resemble *Placobdella multistriata* (Johansson, 1909) more closely than they do *P. rugosa* (i. e*., P. ornata*). This is an apparently mistake of Moore, 1960, who actually was referring to *P. multilineata* and not to *P. multistriata* as was made clear previously by Moore (1953). Alas, Sawyer (1986) considered the Mexican records of this leech to be *P. multilineata*. Based on the revision of the material deposited in the CNHE labeled with the name *Placobdella rugosa*, collected in Xochimilco, México City (CNHE 1695) that contributed to the confusion described above, we determined that these specimens, illustrated in Caballero (1940a) actually correspond to 2 different species of *Helobdella*: 4 specimens of *Helobdella socimulcensis* (Caballero, 1931) and 1 specimen of *Helobdella atli* Oceguera-Figueroa and León-Règagnon, 2005. Specimens collected in Cacahuamilpa, Guerrero (CNHE 1691) which were noted by Caballero (1941), correspond to the new species described in this work: *Placobdella lamothei* n. sp. *Placobdella lamothei* n. sp. can be easily differentiated from the other placobdellid leech distributed in Mexico, *P. mexicana* (as noted above, previously *P. moorei* sensu Autrum, 1936 and Sawyer, 1986) based on the dorsal coloration pattern. *Placobdella lamothei* has a single, continuous longitudinal stripe in the dorsal surface, whereas in *P. mexicana* is interrupted. In addition to this character, is the unusual length of the oesophagus of *Placobdella lamothei* n. sp. that can be used to distinguish between both species. The length of the portion of the oesophagus between the base of the proboscis and the mycetomes is approximately 2 times the length of the proboscis in *Placobdella lamothei* n. sp., whereas in *P. mexicana*, it is shorter than the proboscis. In terms of the development of dorsal papillae, the weak rows of papillae on *Placobdella lamothei* n. sp.

strongly contrast with those of *Placobdella ali* Hughes and Siddall, 2007, *Placobdella papillifera* (Verrill, 1872), *Placobdella ornata*, *Placobdella multilineata* Yang and Davies, 1984 and *Placobdella burresonae* Siddall and Bowerman, 2006. *Placobdella biannulata* (Moore, 1900) presents biannulate somites clearly distinguishing it from *Placobdella lamothei* n. sp. *Placobdella phalera* (Graff, 1899), *Placobdella michiganensis* (Sawyer, 1972), *Placobdella cryptobranchii* (Johnson and Klemm, 1977), *Placobdella transluscens* (Sawyer and Shelley, 1976) and *Placobdella picta* (Verrill, 1872) lack compact salivary glands clearly distinguishing these species from *Placobdella lamothei* n. sp. *Placobdella montifera* Moore, 1906, which also has 3 rows of pronounced papillae, and *Placobdella nuchalis* Sawyer and Shelley, 1976 regularly present a constriction in the neck region (VII), resulting in a discoid head, in contrast to *Placobdella lamothei* n. sp. that lacks that characteristic. *Placobdella parasitica* (Say, 1824) lacks papillae altogether, and has 8-12 bluish, greenish or brownish ventral longitudinal stripes. *Placobdella translucens* (Sawyer and Shelley, 1976) presents an indistinct medial interrupted longitudinal dorsal stripe and is weakly muscularized. Any of these characteristics are as observed in *Placobdella lamothei* n. sp. In conclusion, 2 species of placobdellid leeches parasites of freshwater turtles occur in Mexico, *Placobdella lamothei* n. sp. and *P. mexicana*. Previous record of *Placobdella ornata* by Caballero (1941) should be considered erroneous, actually, this record corresponds to *Placobdella lamothei* n. sp.

3.5 New species of Rhynchobdellid leech (Hirudinea: Glossiphoniidae): A parasite of Turtles from Chiapas, Mexico.

(Adapted from: López-Jiménez, S. and Oceguera-Figueroa A. 2009. New species of Rhynchobdellid leech (Hirudinea: Glossiphoniidae): A parasite of Turtles from Chiapas, Mexico. Journal of Parasitology, 95: 1356–1359.)

INTRODUCTION

The phylogenetic relationships of species in the genus *Placobdella* Blanchard, 1893 (Annelida: Glossiphoniidae) were partially studied by Light and Siddall (1999) and Siddall et al., (2005). These studies clearly showed species of *Desserobdella* Barta and Sawyer, 1990, and *Oligobdella biannulata* Moore, 1900, to be well nested among species of *Placobdella*, notwithstanding their possession of clear distinct morphological traits (diffuse salivary glands and biannulate somites vs. compact salivary glands and triannulate somites characteristics in most species of *Placobdella*). The results in 2 of the previously cited studies led those authors to establish the defining morphological traits for species of *Placobdella*: (1) the presence of compound eyespots, including of 1 large pair and 1 small pair of eyespots; (2) the presence of 1 pair of mycetomes; and (3) the presence of a bilobated ovaries. Tentatively, 17 species of *Placobdella* have been described in North America, 1 in Europe (*Placobdella costata*), and 3 not very well characterized species from South America (Klemm, 1982; Ringuelet, 1985; Sawyer, 1986). In contrast with the substantial number of species from North America, in Mexico only 2 species of *Placobdella* have been recorded, i.e., *Placobdella mexicana* Moore, 1898, and *Placobdella lamothei* Oceguera- Figueroa and Siddall, 2008 (Lamothe-Argumedo *et al.,* 1997; Oceguera-Figueroa and Siddall, 2008). In the present paper, we describe a new species of *Placobdella* possessing the characteristic ocular morphology of the group, as well as 2 pairs of compact salivary glands, 1 pair of mycetomes, triannulate somites, and bilobate ovaries. The new species was found feeding on blood of freshwater turtles in Chiapas, Mexico.

MATERIALS AND METHODS

The leech species described herein was formerly recognized and named by Serapio López-Jiménez in his undergraduate dissertation (López Jiménez, 1985). However, that publication did not comply with Articles 8.1 and 8.5 of the International Code of Zoological Nomenclature (ICZN, 1999) regarding the characteristics that a scientific publication must meet in order that the published scientific name be considered valid. In his dissertation López-Jiménez also mentioned that his description was a transcription of a formal publication that, nevertheless, has not been published. For these reasons, we present the description of this species, which was earlier recognized, but not formally described. Leech specimens were collected from skin of the freshwater turtle *Kinosternon leucostomum* Duméril and Bibron, 1851, in Laguna Bélgica, Municipio de Ocozocuautla (‘‘El Ocote’’ Biosphere Reserve) in June 1981, from *Dermatemys mawii* Gray, 1847 in Mal Paso Dam in June 1981, and from *Staurotypus triporcatus* (Wiegmann, 1828) in the Zoológico Miguel Alvarez del Toro, Tuxtla Gutierrez, in December 2007. All of the localities are in the Estado de Chiapas, Mexico. New collections were made under the Scientific Collecting License FAUT0056, issued to Virginia León-Règagnon. Leech specimens were relaxed with gradual addition of alcohol and fixed with 4% formalin. Eight leeches were compressed between glass slides, stained with a mixture of Ehrlich’s hematoxylin-Mayer’s paracarmin, and mounted on slides. Seventeen specimens were stored in 96% ethanol for external morphology and internal anatomy characterization. Dissections were accomplished using a stereoscopic microscope. Photographs of whole specimens were taken using a Sony Cyber-shot DSC-H5. Drawings of internal structures were accomplished using a camera lucida and edited by superposition of vector-art on

placed images in Adobe Illustrator and Adobe Photoshop (Adobe Systems, San Jose California). Measurements are given in mm with sample size (n=5) and range in parentheses. Voucher specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico, and in the American Museum of Natural History, New York, New York.

DESCRIPTION

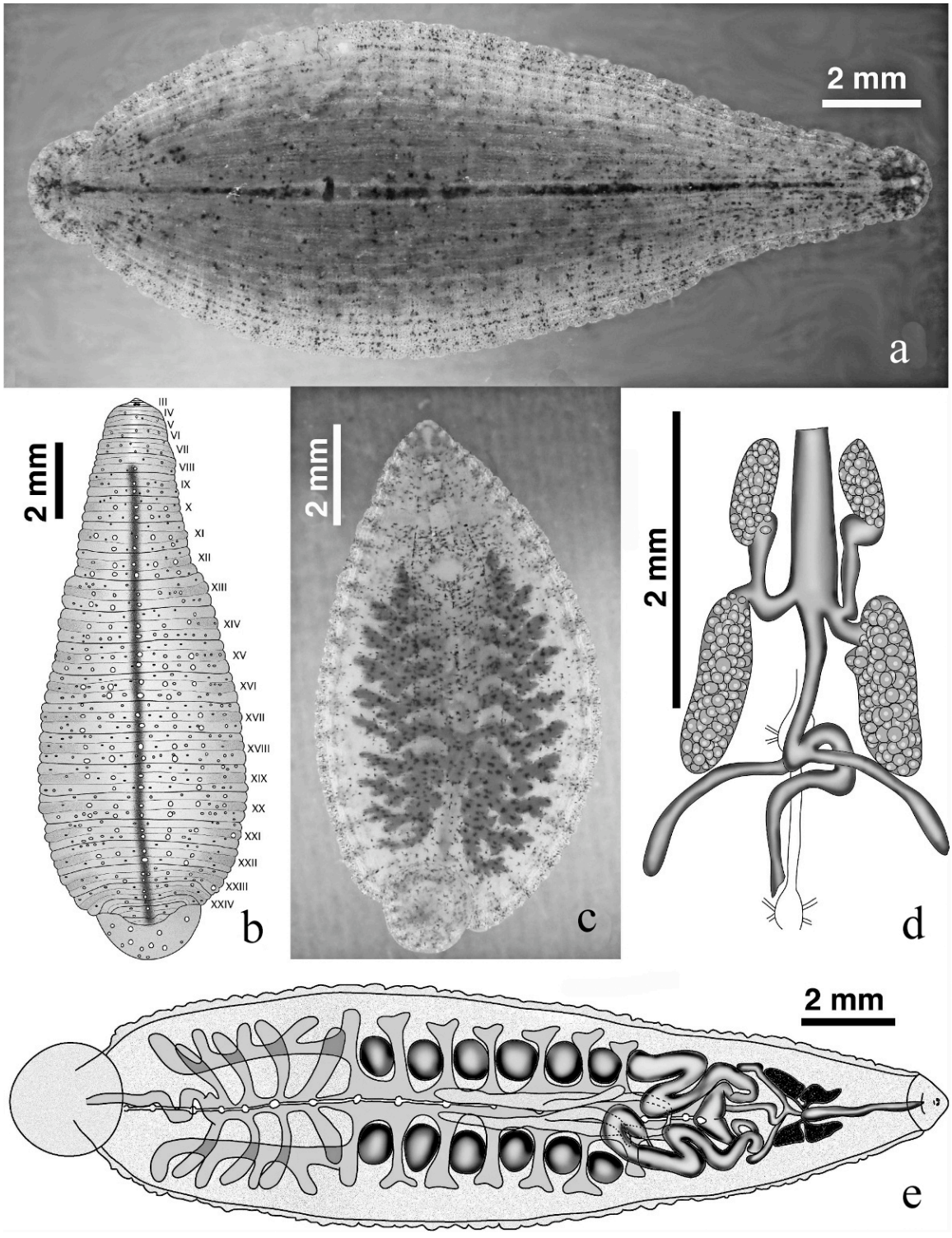
Placobdella ringueleti n. sp.

Figure 14

External morphology (based on 25 specimens): Body dorso-ventral flattened, ovate-lanceolate, semi-transparent teguments when alive, brownish once fixed, light green in posterior part, light metameric transverse lines of black/green pigments on 2 complete somites at borders of body. Average body length 21.18 (n 5 17) (16–27), average body width 7.31 (n 5 17) (6.4–8.6). Complete somite triannulate. Somites I and II uniannulate. Somites III–V biannulate. Somites VI–XXIII triannulate. Somite XXIV biannulate. XXV–XXVII uniannulate. Two pairs of eyespots on III. Oral sucker small, mouth on anterior border. Posterior sucker circular. Anus located on dorsal surface of XXVII. Solid, uninterrupted longitudinal stripe in midline of dorsal surface, from VIII to XXVII, this stripe replaced by white stripe in most anterior somites. Dorsum covered with regular size papillae forming 5 longitudinal rows. Medial row more conspicuous than others, presenting papillae on a2 and a3 of each segment from head region to anus region, more evident in last 2/3 of the body. Paramedial and paramarginal rows always with papillae on a2 and sometimes on a3. Space not occupied by regular size papillae covered with

FIGURE 14

Morphological characteristics of *Placobdella ringueleti,* n. sp. **a**. Dorsal view. Live specimen. **b**. Dorsal view showing the papillation pattern. Somites are numbered with Roman numbers. **c**. Ventral view. Live specimen. **d** Base of the proboscis, 2 pairs of compact salivary glands and mycetomes. e Internal anatomy, showing reproductive and digestive systems.


2 mm
a
2 mm
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
b
2 mm
c
2 mm
d
2 mm
e

small evenly distributed papillae (Figure 14 a,b). Ventral surface brownish, without metameric stripes, papillae, or spots (Figure 14c). Male gonopore between XI and XII. Female gonopore at XII a2/a3; 2 annuli between gonopores. Internal morphology: Proboscis short, 3.06 (n=5) (2.6–3.5), in membranous sheath extending posteriorly to IX, unlooped when retracted. Two pairs well-developed compact salivary glands, medial glands extending anteriorly to VII–VIII, lateral pair at X. Ductle bundles of medial and anterior glands of each side of body converge in common ductle discharging into proboscis base at IX. Esophagus short, folded, with 1 pair elongated mycetomes at X (Figure 14d). Crop with 7 pairs foliaceous caeca, last pair forming well-developed post-caeca or diverticula extending posteriorly to XXV. Intestine with 4 pairs simple caeca at XX–XXIII. Male reproductive system presents a well-developed atrial cornua with highly coiled ejaculatory ducts. Six pairs intersegmental testisacs from XIII/XIV to XVIII/XIX. Ovisacs without common oviduct, bilobed, extending posteriorly to XVII, ovisac biffurcation at XIV/XV. Anterior lobe extending anteriorly to XII/XIII (Figure 14e).

TAXONOMIC SUMMARY

Type host*: Kinosternon leucostomum* Duméril and Bibron, 1851.

Type locality: Laguna Belgica, ‘‘El Ocote’’ Biosphere Reserve. Municipio de Ocozocoautla, Chiapas. 17°08 ´N, 93°19 W. Altitude 320 m. Site of infection: Body appendages.

Type material: Holotype: CNHE 1681; Collected by Manuel Lemus and Susana López. June 1981. Paratypes: CNHE 1716; 7 slides, same host, collectors and date as holotype.

CNHE 1645; 2 specimens collected by Manuel Lemus and Susana López on *Dermatemis mawii,* in Presa Mal Paso June 1981; CNHE 6803, 10 specimens and AMNH 5511, 5 specimens collected by Alejandro-Oceguera-Figueroa, Ricardo Paredes- León, and Diego Contreras Medrano, on *Staurotypus triporcatus*, Zoológico Miguel Alvarez del Toro, Tuxtla Gutierrez, Chiapas, Mexico, December 2007.

Etymology: The new species is named for the Argentinean biologist Dr. Raul Adolfo Ringuelet, for his impressive contributions to our understanding of South American leeches. Dr. Ringuelet visited Mexico in May and June 1981. His analysis of the collection of leeches at the Instituto de Biología, UNAM, is the last and most complete revision of Mexican leeches (Ringuelet, 1981, 1982b). His enthusiasm about this group strongly influenced Serapio López-Jiménez during the time they spent together collecting leeches in central Mexico.

REMARKS

The presence of 2 pairs of eyespots arranged as typically found in the species of Placobdella, 1 pair of elongated mycetomes connected to the esophagus, and bilobate ovaries in the species described herein is consistent with the description of Placobdella spp. provided by Siddall et al., (2005) and Siddall and Bowerman (2006). The new taxon described herein clearly must, therefore, be included in this genus. There are 2 species of *Placobdella* known in Mexico to feed on the blood of freshwater turtles, i.e., *P. mexicana* and *P. lamothei* (see Oceguera- Figueroa and Siddall, 2008). Externally, *P. ringueleti* n. sp. is similar to *P. lamothei* in the ovate-lanceolate body shape, brownish color, and presence of a single continuous middle stripe on the dorsal surface. However, there are substantial differences in the degree of the development of the dorsal papillae. In *P.*

ringueleti, dorsal papillae are more prominent than in *P. lamothei*. This is particularly clear in the dorsal surface of the posterior sucker in which *P. lamothei* lacks any kind of papillae, whereas the new species is replete with these sensory structures. Internally, the length of the portion of the esophagus between the base of the proboscis and the mycetomes is approximately 2 times the length of the proboscis in *P. lamothei*, whereas in *P. ringueleti* this section is shorter than the proboscis. The other Mexican species in *Placobdella, P. mexicana*, as well as the more northern species, i.e., *Placobdella ornata* (Verrill, 1872), *Placobdella multilineata* Moore, 1953, *Placobdella translucens* Sawyer and Shelley, 1976, and *Placobdella ali* Hughes and Siddall, 2007, all present an interrupted dorsal medial stripe, contrasting with the uninterrupted stripe of *P. ringueleti*. This characteristic is also important in separating the new species from *Placobdella parasitica* (Say, 1824) because the latter lacks the distinctive medial dorsal stripe. Characteristic of *Placobdella papillifera* (Verrill, 1872) is the independent insertion of each of the 4 compact salivary glands into the base of the proboscis, as well as a vague, median, dorsal longitudinal stripe. These characteristics are clearly distinct from those found in *P. ringueleti.* Four species of *Placobdella* possess diffuse salivary glands, i.e., *Placobdella phalera* (Graf, 1899), *Placobdella michiganensis* (Sawyer, 1972), *Placobdella picta* (Verrill, 1872), and *Placobdella cryptobranchii* (Johnson and Klemm, 1977). In addition, *Placobdella burresonae* Siddall and Bowerman, 2006, presents weakly developed salivary glands (particularly the anterior pair) and a bilobated lateral pair. These characteristics contrast with the well-developed compact salivary glands of *P. ringueleti*. A distinctive body constraint in the neck region (VII) forming an obvious anterior discoid head occurs in *Placobdella montifera* Moore, 1906, and *Placobdella*

nuchalis (Sawyer and Shelley, 1976); this characteristic is clearly absent in *P. ringueleti*. The amphibian leech parasite, *Placobdella biannulata* (Moore, 1898), in addition to being less than 10 mm in length (Moore, 1900), possesses a complete somite formed by 2 annuli, clearly distinct from the 3-annulate *P. ringueleti. Placobdella hollenisi* (Whitman, 1892) is unique among the species of the genus because of the presence of accessory eyespots; this characteristic is plainly distinct from *P. ringueleti*.

DISCUSSION

Ringuelet (1985) validated the presence of *Placobdella* spp. in South America based on 3 isolated records from Brazil, i.e., *Placobdella bistriata* (Pinto 1920), *Placobdella striata* Oka, 1932, and *P. maculata* Weber, 1915. Little is known, however, about these species, and no new records have been established since their original description. Ringuelet's (1985) paper indicates that he studied only specimens of *P. bistriata* deposited in the Colecciones del Museo de Historia Natural, Montevideo, Uruguay. Most of the information about this leech is related to the external morphology. However, he did describe a few internal characters that are more representative of species of *Haementeria* (the more common genus of blood-feeding glossiphoniid in South America), e.g., the loop-shaped proboscis and spherical esophageal glands. Records of the other 2 placobdellids are based on the original descriptions that were made, which ignored the internal morphology. Species of *Placobdella* and *Haementeria* are difficult to distinguish solely on external characters, and, for this reason, they were considered to belong to a single genus (*Haementeria*) by Autrum (1936). However, clear morphological differences between these genera are apparent once dissection of specimens is conducted. This practice, obligatory today, was not common for some leech systematists of previous

generations. Because detailed analysis of the internal characteristics of any of the placobdellids from South America has not been done, and no new records of this group exist, we consider placobdellids from Brazil as *species inquirendae*. These arguments leave *P. ringueleti* n. sp. as the sole Neotropical species of the otherwise Nearctic genus *Placobdella*. This geographical pattern is unusual among hirudineans, because most of the examples include Neotropical groups with representatives that disperse to the Nearctic, i.e., *Helobdella* spp., *Haementeria* spp., and *Semiscolex* spp. Further investigations are needed to establish the southern range limit of this species. This issue has clear biogeographical implications for understanding the species composition of southern Mexico and Central America. The type locality of *P. ringueleti* is part of the El Ocote Biosphere Reserve. This reserve was established in 2000 (Diario Oficial de la Federación, 2000) and has been the subject of several programs of conservation and management. Several studies have been conducted to characterize the plant and vertebrate composition (Riechers-Pérez, 2004; Luna-Reyes et al., 2005; Escobar-Ocampo and Ochoa-Gaona, 2007), yet little attention has been paid to invertebrates. *Placobdella ringueleti* represents the first record of leeches for the area, providing potentially useful information for more comprehensive management and conservation programs for the region.

Author contribution

This species was originally detected by Serapio López in 1986 but it was not formally described. For this section, I collected more specimens, expanded the morphological characterization and the geographical records. I wrote the whole manuscript and I took the pictures. I adapted the drawings done by López Jiménez, 1986.

CHAPTER 4

Phylogeny of leeches

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

(Adapted from: Oceguera-Figueroa A. Molecular phylogeny of the New World bloodfeeding leeches of the genus *Haementeria* and reconsideration of the biannulate genus *Oligobdella*. Molecular Phylogenetics and Evolution (in preparation)

INTRODUCTION

The genus *Haementeria* (*haemo*=blood; *enterium*=intestine) was erected to accommodate a large and voracious blood-feeding 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* in Mexico 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; Tuszynsky *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 gammaproteobacteria (Perkins *et al.,* 2005).

Since its establishment, at least 12 haematophagous species of *Haementeria* have been described. Several 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 Mexico (Oceguera-Figueroa, 2008; Ringuelet, 1985; Sawyer, 1986). In a taxonomic revision of Hirudinea, Autrum (1936) lumped the Palearctic genus *Placobdella* into *Haementeria* based on the somite annulation pattern, the presence of compact salivary glands and their bloodfeeding habit. Autrum's (1936) proposal was not followed 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 (Glossiphoninae and Haementeriinae respectively). Siddall *et al*., (2005) investigated the phylogenetic relationships of a variety of members of Glossiphoniidae using molecular data; finding that *Placobdella*, *Haementeria* and *Helobdella* form a moderately well supported clade notwithstanding the preference for haemolymph 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 the species was the presence of only two annulli per somite, in contrast to the three-annulate condition that is characteristic of the family. Several 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). Acordingly, South American species initially placed in *Oligobdella* could have be 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, including *Oligobdella brasilensis* (Cordero, 1937) and an expanded set of nuclear and mitochondrial molecular markers. 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* sp. are discussed in light of their phylogenetic relationships. Finally, the geographical distribution of the group is analyzed and compared with other taxa.

MATERIALS AND METHODS

Taxa

Newly collected samples of *Haementeria* spp. included in this study represent a broad geographical distribution including Argentina, Brazil, Mexico and Perú. Information of newly collected material including locality and GenBank accession numbers is presented in Table 6. Unless otherwise indicated, leeches were collected by immersing legs into water at the edges of lakes or ponds, waiting for 5 minutes 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.

Morphology

Leech specimens were identified using taxonomic keys and descriptions (Ringuelet, 1981, 1976b, 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, Mexico 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.

Molecular Techniques

Leech tissue samples were obtained from the posterior sucker of the leech in order to

Table 6. Taxa, localities and GenBank accession numbers for the phylogenetic analyses of *Haementeria* spp

	Locality	COI	12S	NADH	ITS	28S
Outgroup						
Placobdella rnateate		AF116021	AY425435	AY047343		AY425397
Placobdella costata	Italy	AY962461	XXXX	AY962448	XXXX	
Placobdella rnate		AY047326	XXXX	AY047352	XXXX	
Placobdella parasitica		AF003261	AY425438	AY047348		AY425401
Helobdella bolivianita	Bolivia	AF329053	----	AF329076		
Helobdella elongata		AF329045	XXXX	AF329068		
Helobdella triserialis	Bolivia	AF329054	XXXX	AF329077		
Ingroup						
Oligobdella brasilensis	Brazil		----	XXXX		XXXX
Haementeria acuecueyetzin Haeo	Mexico	XXXX	XXXX	XXXX	XXXX	
Haementeria acuecueyetzin HaOfl	Veracruz, Mexico	XXXX	XXXX	XXXX	XXXXX	XXXX
Haementeria acuecueyetzin Hacu	Catemaco, Veracruz, Mexico	XXXX	XXXX	----	XXXXX	XXXX
Haementeria depressa 12A	Uruguay	XXXX	XXXX	XXXX	X	
Haementeria depressa 12B	Uruguay	XXXX	XXXX	XXXX		
Haementeria depressa 38A	Uruguay	XXXX	XXXX	XXXX	X	XXXX
Haementeria depressa 38B	Uruguay	XXXX	XXXX	XXXX		
Haementeria depressa 38C	Uruguay	XXXX	XXXX	XXXX		

Table 6. Continued

Haementeria depressa 42A	Uruguay	XXXX	XXXX	XXXX	X	XXXX
Haementeria depressa 42B	Uruguay	XXXX	XXXX	XXXX		
Haementeria depressa EST	Uruguay	CN807309.1	CN807801.1	HDAH04B07		
Haementeria depressa UR23	Uruguay	XXXX	XXXX	XXXX		
Haementeria depressa UR23B	Uruguay	XXXX	XXXX	XXXX		
Haementeria ghilianii GB	BioPharm, French Guiana	AF329035	AY425417	AF329058	XXXX	AY425374
Haementeria gracilis	Chile	AF003276	----	----		
Haementeria gracilis	Arroyo Espinas, Uruguay	AF329034	AY425418	AF329057		AY425375
Haementeria lopezi	Colima, Mexico Ex. *Smilisca boudinii*	XXXX	XXXX	XXXX	XXXX	XXXX
Haementeria lopezi HoBm	Jalisco, Mexico. Ex *Rhinella marinus*	XXXX	XXXX	XXXX	XXXX	XXXX
Haementeria lopezi HoSb	Jalisco, Mexico	XXXX	XXXX	XXXX		XXXX
Haementeria lutzi	Rio Pastaza, Ecuador	AF329033	----	AF329056		
Haementeria officinalis HR	Michoacan, Mexico	XXXX	XXXX	XXXX	XXXX	XXXX
Haementeria officinalis Hdur	Durango, Mexico	XXXX	----	XXXX	XXXXX	XXXX
Haementeria officinalis HFDM	Queretaro, Mexico	XXXX	XXXX	XXXX	XXXX	XXXX
Haementeria paraguayensis Pe16	Iquitos, Peru	XXXX	----	----		XXXX
Haementeria paraguayensis UR46	Argentina	XXXX	XXXX	----		XXXX
Haementeria tuberculifera Pe15	Iquitos, Peru	XXXX	----	XXXX	XXXXX	XXXX
Haementeria vizzotoi	Brazil	XXXX	XXXX	XXXX	XXXX	XXXX

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 nuclear 18S rDNA (18S), and the ITS region (with the Internal Transcribed Spacers 1 and 2 flanking the 5.8S ribosomal gene) were obtained using the primers listed in Table 7 and used to investigate the internal relationships of the genus. Amplification reactions of each gene fragment were conducted using Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ) with 0.5 μl of each 10 μM primer, 1 μl DNA template, and 23 μl Rnase-free H_2O (total volume 25 μl). PCR reactions were performed with an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The following amplification protocols were used: for COI, 94°C (5 min), followed by 30 cycles of 94°C (45 sec), 48°C (45 sec), 72°C (1 min) and final extension of 72°C (7 min); for 18S, 94°C (5 min) followed by 35 cycles of 94°C (20 sec), 47°C (20 sec), 68°C (50 sec) and final extension at 70°C (7 min); for 12S and 28S, 94°C (5 min), followed by 44 cycles of 95°C (1 min), 52°C (1 min), 70°C (1 min) and final extension of 72° (7 min) and finally for ITS (using primers ITS-5 and ITS-4), 95°C (5 min) followed by 35 cycles of 95°C (30 sec), 50°C (30 sec), 72°C (1.5 min) and final extension at 72°C (8 min). PCR products were purified with AMPure (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA.). Cycle sequencing reactions were performed with an Eppendorf Mastercycler using 1 μl ABI Big Dye Terminator V 3.1 (Applied Biosystems, Carlsbad, California, USA), 1 μl of 1 μM primer, 3 μl of cleaned PCR product and 1 μl Rnase-free H_2O (total volume 6 μl). Sequencing reactions were purified by 70% isopropanol/70% ethanol

Table 7. Primers used for gene amplification and sequencing for the phylogenetic analyses of *Haementeria* spp

Gene	Primer Name	Primer Sequence	
NUCLEAR			
28S rDNA	28sA	GACCCGTCTTGAAGCACG	Whiting (2002)
	28SBout	CCCACAGCGCCAGTTCTGCTTACC	Prendini et al., (2005)
ITS	ITS4	TCCTCCGCTTATTGATATGC	White et al., (1990)
	ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al., (1990)
	5.8MussR	GATGTCGATGTTCAATGTGTCCTGC	Källersjö et al., (2005)
	5.8MussF	CGCAGCCAGCTGCGTGAATTAATGT	Källersjö et al., (2005)
MITOCHONDRIAL			
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., (1994)
12S	12Sa	AACIIGGATTA GATACCC	Simon et al., (1994)
	12Sb	GAGAGTGACGGGCGATGTGT	Simon et al., (1994)
ND1	LND300	TGGCAGAGTAGTGCATTAGG	Light & Siddall (1999)
	HND1932	CCTCAGCAAAATCAAATGG	Light & Siddall (1999)

precipitation and sequenced with an ABI PRISM 3730 sequencer (Applied Biosystems, Carlsbad, California, USA). CodonCode Aligner (CodonCode Corporation, Dedham, Massachusetts, USA) was used to edit and reconcile sequences.

Taxon names and GenBank accession numbers of taxa included in the present analyses are presented in Table 7. Representative species of *Helobdella* and *Placobdella,* including *Placobdella biannulate,* were included in the analyses in order to test the position of the biannulate *Oligobdella brasilensis*. Alignment of all gene sequences was accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004). Alignments were obtained applying the default settings for gap opening and extension penalties.

Phylogenetic analyses

Maximum Parsimony (MP) analyses of the combined 18S, 28S, 12S and COI data were performed using New Technology Search with the Ratchet and tree fusing algorithms in TNT ver 1.1 (Goloboff *et al*., 2008) performing 100 repetitions. Resulting trees were used as starting trees for a traditional search using tree-bisection-reconnection (TBR) branch swapping. All characters were equally weighted and non-additive. Gaps were treated as missing data. Bootstrap values for combined analyses were obtained in TNT with 1000 pseudoreplicates, using random taxon addition and TBR branch swapping.

Bayesian inference (BI) was conducted in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) through the Computational Biology Service Unit computing interface at Cornell University (http://cbsuapps.tc.cornell.edu/). The data were partitioned by gene for COI, 12S, ND1, 28S and ITS. A GTR+I+G model of evolution was applied to each unlinked data partition based on the Akaike Information Criterion via FindModel

(Tao et al., 2008). For the Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analyses, default prior distributions of parameters were used for two runs, each with one cold and three hot chains for 20 million generations and trees were sampled every 1000th generation. Using the cumulative function, the web version of AWTY (Wilgenbusch et al., 2004) was used to verify that post burn-in generations had reached stationarity in all data sets.

RESULTS

Phylogenetic analyses

The complete dataset analyzed in this study included 35 terminals; 658 bp of COI, 401 bp of 12S, 631 bp of ND1, 338 bp of 28S and 1009 bp of ITS, for a total of 3037 aligned characters. Maximum Parsimony analyses resulted in 9 most parsimonious trees of 3138 steps, CI = 0.54 and RI = 0.65. Disagreement between the most parsimonious trees was restricted to alternative arrangements within *H. depressa*/*H. gracilis* samples. The strict consensus tree of the MP analyses is shown in Figure 15. The BI analyses appeared to burned-in before 100,000 generations. Split frequencies of the standard deviation of simultaneous BI analyses were well below 0.01. Nevertheless, the burn-in was set to discard the first 5,000,000 generations, leaving 15,001 trees sampled for estimation of posterior probabilities. The majority rule consensus is shown in Figure 16.

The phylogenetic hypotheses resulting from both analyses (MP and BI) resulted in highly compatible trees (Figures 15, 16). Both analyses recovered *Haementeria*+ *Oligobdella brasilensis* as monophyletic with good support (98 for MP and 1.00 for BI). The difference between both trees is the position of *Haementeria lutzi,* which appeared

FIGURE 15.

Strict consensus tree resulting from parsimony analysis of *Haementeria* based on the combined dataset (COI, 12S, ND1, 28S and ITS sequences). Branch lengths are proportional to amount of change. Numbers next to nodes indicate bootstrap values. Black lines indicate North American lineages. Gray lines indicate South American lineages.

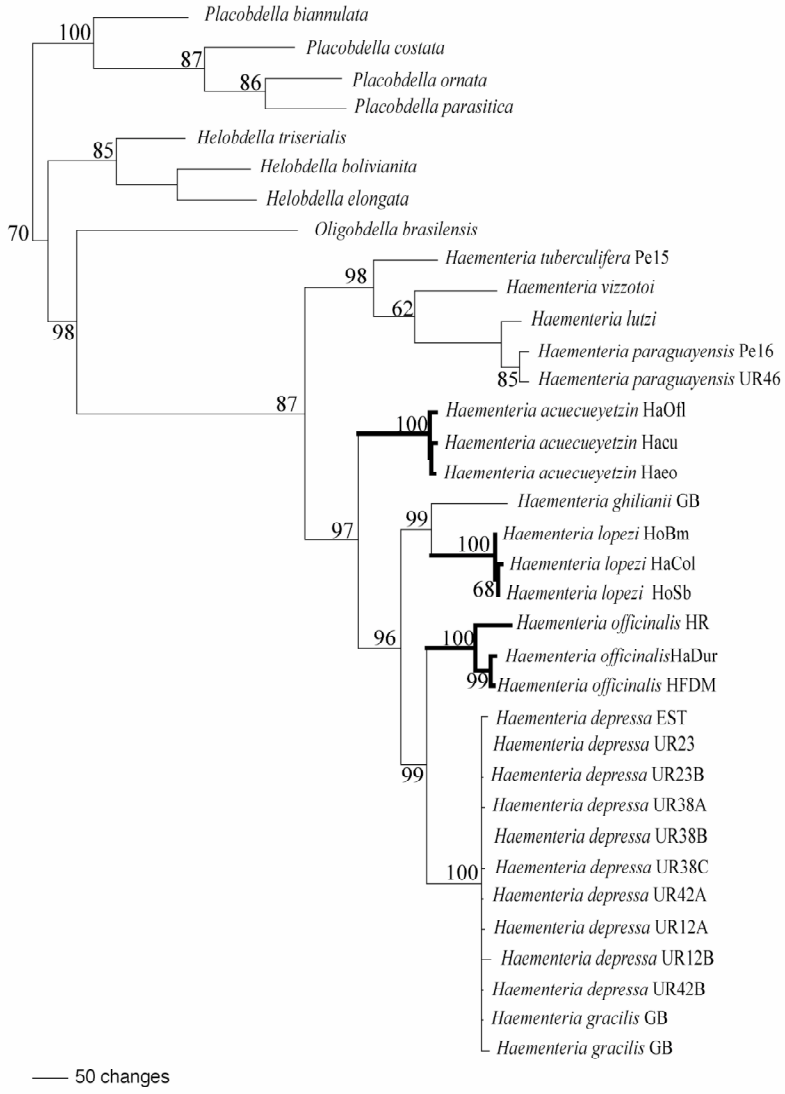
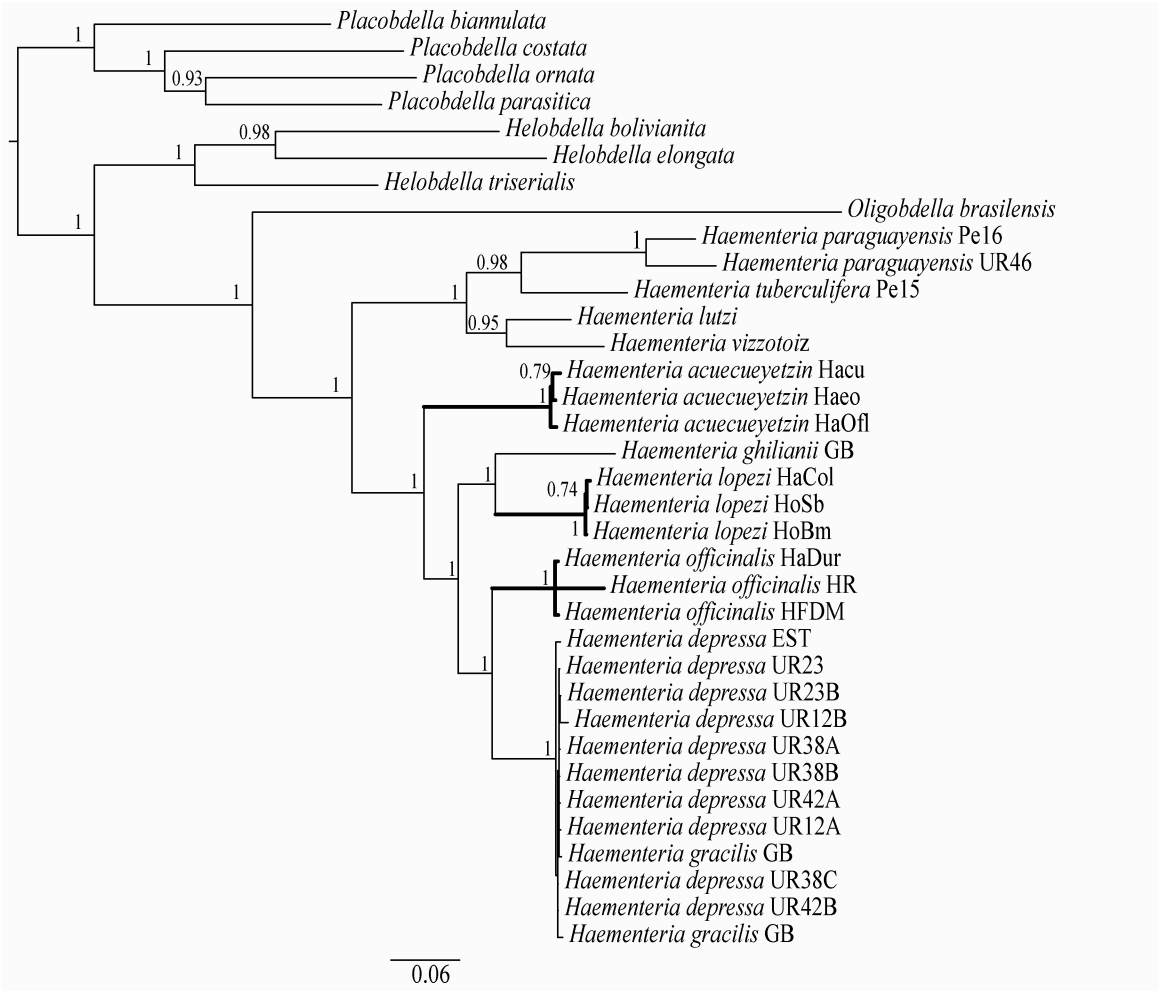

Placobdella biannulata
Placobdella costata
Placobdella ornata
Placobdella parasitica
Helobdella triserialis
Helobdella bolivianita
Helobdella elongata
Oligobdella brasilensis
Haementeria tuberculifera Pe15
Haementeria vizzotoi
Haementeria lutzi
Haementeria paraguayensis Pe16
Haementeria paraguayensis UR46
Haementeria acuecueyetzin HaOfl
Haementeria acuecueyetzin Hacu
Haementeria acuecueyetzin Haeo
Haementeria ghilianii GB
Haementeria lopezi HoBm
Haementeria lopezi HaCol
Haementeria lopezi HoSb
Haementeria officinalis HR
Haementeria officinalisHaDur
Haementeria officinalis HFDM
Haementeria depressa EST
Haementeria depressa UR23
Haementeria depressa UR23B
Haementeria depressa UR38A
Haementeria depressa UR38B
Haementeria depressa UR38C
Haementeria depressa UR42A
Haementeria depressa UR12A
Haementeria depressa UR12B
Haementeria depressa UR42B
Haementeria gracilis GB
Haementeria gracilis GB
50 changes

FIGURE 16.

Bayesian Inference tree topology resulting from analysis of *Haementeria* based on the combined dataset (COI, 12S, ND1, 28S and ITS sequences). Posterior probabilities are indicated next to nodes. Black lines indicate North American lineages. Gray lines indicate South American lineages.


Placobdella biannulata
Placobdella costata
Placobdella ornata
Placobdella parasitica
Helobdella bolivianita
Helobdella elongata
Helobdella triserialis
Oligobdella brasilensis
Haementeria paraguayensis Pe16
Haementeria paraguayensis UR46
Haementeria tuberculifera Pe15
Haementeria lutzi
Haementeria vizzotoiz
Haementeria acuecueyetzin Hacu
Haementeria acuecueyetzin Haeo
Haementeria acuecueyetzin HaOfl
Haementeria ghilianii GB
Haementeria lopezi HaCol
Haementeria lopezi HoSb
Haementeria lopezi HoBm
Haementeria officinalis HaDur
Haementeria officinalis HR
Haementeria officinalis HFDM
Haementeria depressa EST
Haementeria depressa UR23
Haementeria depressa UR23B
Haementeria depressa UR12B
Haementeria depressa UR38A
Haementeria depressa UR38B
Haementeria depressa UR42A
Haementeria depressa UR12A
Haementeria gracilis GB
Haementeria depressa UR38C
Haementeria depressa UR42B
Haementeria gracilis GB
0.06

sister to *H. paraguayensis* in the MP tree contrary to the sister relationship with *H. vizzotoi* in the BI analyses. Both methods recovered a multitude of samples of *H. depressa* and *H. gracilis* from different localities of three countries forming a group without any internal resolution and minimum branch lengths.

DISCUSSION

Taxonomy

Notwithstanding the taxonomic treatment of the group (Ringuelet, 1985; Sawyer, 1986), most of the species considered valid for the genus, including two new species described recently (Oceguera-Figueroa, 2007, 2008) are studied for the first time in a phylogenetic analysis. The monophyly of *Haementeria* including *Olibobdella brasilensis* with good node support is the most notable result of the present analyses and provides additional support to the sister relationship of *Haementeria* and *Helobdella,* as previously proposed (Light and Siddall, 1999; Siddall *et al.,* 2005).

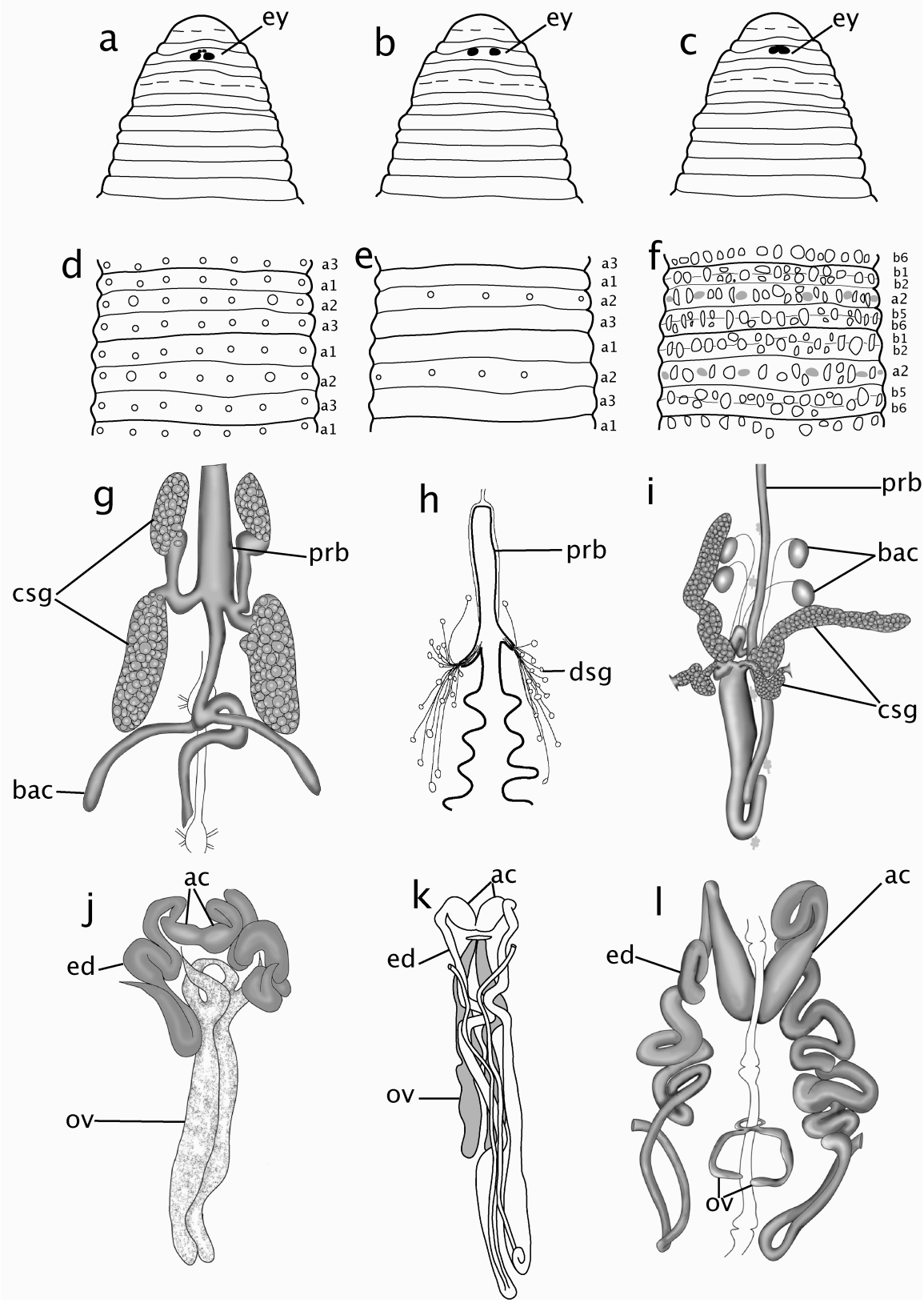
The placement of *Oligobdella brasilensis* within the mostly South American genus *Haementeria,* but not sister to *Placobdella biannulata* (the type species of the former *Oligobdella*) inidicates that biannulate somites arose independently. Sawyer's (1986) definition of *Oligobdella* highlighted the presence of one pair of eyespots and bi-annulate somites as diagnostic for the genus. In his treatment of the group, Sawyer (1986) grouped Asian bi-annulate species of the genus *Torix* (which have species with more than one pair of eyespots) thus recognizing the fact that the bi-annulate condition by itself is not sufficient to justify a genus. Nevertheless, Sawyer (1986) retained *Oligobdella* for biannulate species of the New World. All previously described species of *Oligobdella* are

small (less than 20 mm) relative to the species of *Haementeria* and *Placobdella*. As a result, many characteristics of the genera *Placobdella* and *Haementeria* could have easily been overlooked. This seems to have played a role in Sawyer´s (1986) characterization of the genus *Oligobdella* who admitted even then that the placement of the genus into the subfamily Haementeriinae was doubtful. Like the disposal of *Oligobdella* as a junior synonym of *Placobdella* by Siddall *et al.,* 2005, here *Oligobdella brasilensis* is formally transferred to *Haementeria brasilensis* comb. nov.

Notwithstanding a taxonomic history of more than two hundred years, a clear picture of the evolution of the morphological traits within species of *Haementeria*, *Helobdella* and *Placobdella* has remained elusive. Explicit phylogenetic analyses of the group are relatively recent (Siddall *et al.,* 2005; Siddall and Burreson, 1998). Correlation between some morphological traits and the phylogeny is worth exploring. The presence of two pairs of eyespots, with the anterior pair smaller and coalesced (Figure 17a) is a characteristic of *Placobdella* spp. and contrasts with the single pair eyespots of *Helobdella* and *Haementeria* species (Figure 17b,c), a plesiomorphic character present in some other glossiphonides (Siddall *et al.,* 2005). The number of annuli per complete somite (mid-body somite) is plesiomorphically three-annulate (Figure 17 d,e). Most species of *Haementeria* have annuli a1 and a3 subdivided, giving the appearance of penta-annulate somites (Figure 17f). Other than the biannulate *H. brasilensis* comb. nov. exceptions include *H. ghilianii* and *H. lopezi* these grouped together in the phylogenetic hypotheses. Both of which lack subdivisions dorsally while still being penta-annulate ventrally.

FIGURE 17.

External and internal morphology of glossiphoniid leeches. Eyespot morphology: (a) *Placobdella* sp. (b) *Helobdella* sp. (c) *Haementeria* sp. Dorsal view of complete somites: (d) *Placobdella* sp. (e) *Helobdella* sp. (f) *Haementeria* sp. Lang's organs in gray. Salivary complex: (g) *Placobdella* sp. (h) *Helobdella* sp. (i) *Haementeria* sp. Reproductive organs: (j) *Placobdella* sp. (k) *Helobdella* sp. (l) *Haementeria* sp. ey=eyespots, csg=compact salivary glands, prb=proboscis, dsg=diffused salivary glands, bac=bacteriomes, ac=atrial cornua, ed=ejaculatory ducts, ov=ovaries. (b,h,k redrawn from Oceguera-Figueroa, 2007; f,i,l redrawn from Oceguera-Figueroa, 2008; g redrawn from López-Jiménez and Oceguera-Figueroa, 2009; j redrawn from Oceguera-Figueroa et al., 2010).


a
ey
b
ey
c
ey
d
a3
a1
a2
a3
a1
a2
a3
a1
e
a3
a1
a2
a3
a1
a2
a3
a1
f
b6
b1
b2
a2
b5
b6
b1
b2
a2
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b6
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prb
csg
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h
prb
dsg
i
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bac
csg
j
ac
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ov
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ed
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ed
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Dorsal sensory structures such as papillae and tubercles are often used by taxonomists to distinguish among species (Oceguera-Figueroa, 2006a, 2008; Siddall and Bowerman, 2006). The presence of Lang's organs or sensitive papillae forming 3 pairs of dorsal longitudinal rows on each a2 is unique to species of *Haementeria* (Lang, 1890). Lang's organs have a transversally arranged bar-shape, but may also be circular. These sensory structures are smooth and flat, easily distinguishable from tubercles, which have minute papillae giving a stellate appearance. Number and arrangement of tubercles on the body surface are important for distinguishing among the species of *Haementeria* whereas Lang's organs are always in the same position (Figure 17 f).

Characteristic of glossiphiniid leeches is variation in salivary cell structures that discharge through the base of the proboscis. Both *Placobdella* and *Helobdella* include species with compact and species with diffuse salivary glands (Figure 17 g-h). In contrast, all *Haementeria* species described have two pairs of compact salivary glands (Figure 17 i). Also associated with the digestive system of the haematophagous glossiphoniids, are structures specialized in harboring symbiotic bacteria. Species of *Placobdella* have one pair of sac-like bacteriomes (referred as mycetomes in leech literature) attached to the esophagus (Figure 17 g) whereas *Haementeria* spp. have two pairs of spherical bacteriomes connected to the esophagus through tiny ductules (Figure 17 i). Different bacteriome structures have different bacterial counterparts. *Placobdella* species harbor alphaproteobacteria whereas *Haementeria* species are host of gammaproteobacteria (Perkins *et al.,* 2005). *Helobdella* species lack bacteriomes, a fact that seems to be related to their dietary preference for haemolymph and soft tissues of invertebrates (liquidosomatophagia).

The structure of the male reproductive system is highly conserved all across the family Glossiphoniidae being represented by a pair of atrial cornua anterolaterally directed and coiled ejaculatory ducts that reach the testisacs on either side of the body (Figure 17 j-l). Some variation exists in relation to the female reproductive system. In species of *Placobdella*, the ovisacs lack a common oviduct but each lateral branch has a bifurcation forming anterior and posterior lobes (Figure 17 j). *Helobdella* spp. have simple ovisacs without common oviducts or other complex arrangement (Figure 17 k). This structure might represent the plesiomorphic state of the family. Unique to species of *Haementeria*, the ovisacs form an anterior ring around the ventral nerve cord (Figure 17 l).

Molecular evidence to support the monophyly of the group formed by *Placobdella*, *Helobdella* and *Haementeria* is solid (Oceguera-Figueroa. 2005; Siddall *et al.,* 2005). To date, no morphological trait (putative synapomorphy) has been found to corroborate this group. It is possible that structures such as sperm, spermatophore and proboscis ultrastructure are potential sources of morphological information which might provide information with potential impact in our understanding of the evolution of the group.

Biogeography

The South American land mass remained isolated for 65-80 million years until recently (3-3.5 MYA) with the closing of the Panamanian Isthmus (Flynn and Wyss, 1998). This isolation allowed for the evolution of several groups of organisms, some of which eventually colonized North America. The extent of such colonization varies among taxonomic groups, with some such as anteaters and New World Monkeys reaching

Neotropical lowlands areas of Mexico. Others such as didelphids and armadillos reached Northern latitudes.

Based on their basal placement on the tree, the high number of species and the geographical distribution of its sister group, a South American origin of *Haementeria* is proposed. The only three exceptions of the otherwise exclusively South American group inhabit Mexico. The disjunct geographical distribution within Mexican representatives (*H. acuecueyetzin, H. lopezi* and *H. officinalis*), would suggest that they are the result of vicariant speciation events after a single colonization by an ancestral South American stock. This hypothesis would require Mexican species to be monophyletic. The fact that *H. lopezi* and *H. officinalis* have South American sister species indicate a more complicate faunal interchange between both landmasses than previously thought. Given the tree topology, two alternative explanations can be suggested. One requires the colonization of North America by a single lineage, speciation events and two secondarily colonizations of South America by *H. ghilianii* and *H. depressa*. The alternative explanation would require that the tree Mexican species represent three independent events of colonization from South America.

The geographical distribution of *Haementeria* is not unique among leeches. The origin of the liquidosomatophagous genus *Helobdella* was hypothetisized to be South American from where two lineages colonized North America and one of these also colonized Europe (Siddall *et al.,* 2005). The origin of the macrophagous leeches of *Semiscolex* is also South American with a single species, *Semiscolex lamothei* from the Mexican Neotropics (Oceguera-Figueroa, 2006b; Phillips and Siddall, 2009). The fact that these leeches have similar biogeographical patterns regardless of feeding preferences

would imply that the distribution of *Haementeria* spp. is not restricted to the distribution of their hosts. Species of *Haementeria* are well-known for their ability to feed on a variety of vertebrate hosts, including teleosts, amphibians, reptiles, birds and mammals (Ringuelet, 1986). Evidence of this wide host spectrum is *H. lopezi* which was found feeding on *Smilisca baudinii* (Hylidae) and in *Rhinella marinus* (Bufonidae) in the same locality (Oceguera-Figueroa, 2006a). Furthermore, individuals of the same leech species were collected with my own legs as bait.

Since the description of *Haementeria depressa* from Chile by E. Blanchard (1849), records of this species feeding on a wide variety of vertebrates has been documented in several localities from Chile, Uruguay, Brazil and Paraguay. Weyenberg (1883) described *Haementeria gracilis* based on specimens from Uruguay and recently it was recorded in Valdivia, Chile and Maldonado, Uruguay (Light and Siddall, 1999; Siddall and Borda, 2004). Ringuelet (1986), considered that *H. gracilis* is merely a junior synonym of *H. depressa*, an opinion that is supported in the present analyses since no molecular differences between *H. gracilis* and *H. depressa* were found even in COI sequences, a molecule that has been used extensively as a reliable marker to distinguish between closely related species (Hebert *et al.,* 2004; Oceguera *et al.,* 2010). The extensive geographical distribution of what is the only *Haementeria* species with trans-Andean distribution is striking. Several records are from highly disturbed areas such as farms and acequias (water reservoirs) were cattle normally drink and get parasitized with this leech. Given the close contact between this leech and cattle, it is not difficult to consider that this leech has been dispersed inadvertently by humans to several areas in South America were they formed stable colonies.

4.2 Jaw morphology evolution of hirudiniform leeches with the description of a new blood-feeding species of *Oxyptychus* (Annelida: Hirudiniformes) from the Peruvian Amazon

(Adapted from: Oceguera-Figueroa, A., Barrio, A. K., Aldea-Guevara, M. I. and Siddall, M. E. Jaw morphology evolution of hirudiniform leeches with the description of a new blood-feeding species of *Oxyptychus* (Annelida: Hirudiniformes) from the Peruvian Amazon. Invertebrate Systematics (In review))

INTRODUCTION

Commonly believed to be characteristic of all leeches, the preference for vertebrate blood seems to be more labile than originally thought. Based on phylogenetic analyses, it has been inferred that the last common ancestor of all leeches was a blood-feeder, a condition that has been lost at least seven times throughout the evolution of the group (Phillips and Siddall, 2009; Siddall *et al*., 2004). In general, there are two major forms of vertebrate blood intake by leeches: Rhynchobdellid leeches (e.g. *Placobdella, Placobdelloides, Haementeria, Piscicola*) penetrate the hosts' tissues using a large and eversible proboscis. Alternatively, arhynchobdellid leeches bear jaws and use those structures to rip up the skin of their host (Sawyer, 1986). The number of jaws, ranging from none to three, as well as the number of teeth or denticles and its arrangement has long been used in leech taxomony (Ringuelet, 1953, 1982b), Richardson, 1975, 1978; Sawyer 1986). As it can be expected from the plasticity of feeding preferences among Hirudiniformes, jaw morphology might be also a poor predictor of natural groups (Borda *et al*., 2008; Phillips *et al*., 2010). However, this issue has not been analyzed in a phylogenetic context.

According to Ringuelet (1985), South American freshwater blood-feeding leeches belong to two genera: *Haementeria* De Fillipi 1849 for Glossiphoniid species with an eversible proboscis, and *Oxyptychus* (Grube, 1851) for three-jawed hirudiniform species. Recently, a novel leech possessing a single jaw armed with enormous teeth was characterized: *Tyrannobdella rex* Phillips, *et al.,* 2010, parasite of the respiratory tract of humans from the East side of the Peruvian Andes (Phillips *et al*., 2010). Finding a new species of leech in the upper Amazon is not surprising given that the leech fauna native to Perú has been only partially studied; most records are from the watersheds in the Andes

and the western side (Ringuelet, 1976b, 1985). Shain *et al.* (2007) conducted the first study of Peruvian Amazonian freshwater leeches recording six species: *Semiscolex similis* (Weyenbergh, 1879), *Haementeria depressa* (Blanchard, 1849), *Haementeria maculata* (Weber, 1915), *Helobdella striata* (Ringuelet, 1942), *Helobdella* sp. in addition to what was then considered to be a new ozobranchid leech (*Bogobdella* sp.), which might be *Unoculobranchobdella expansa* Solano Lobo Peralta *et al*., 1998, a leech parasite of freshwater turtles from Brazil (Solano Lobo Peralta *et al*., 1998).

In this study, a new species of the blood-feeding genus *Oxyptychus* from the Peruvian Amazonia is described. In addition to the investigation of its phylogenetic affinities using molecular and morphological data, a broad analysis of the evolution of jaws and teeth morphology of leech members of the recently redefined families Macrobdellidae, Semiscolecidae and Praobdellidae (Phillips and Siddall, 2009; Phillips *et al*., 2010) is also provided.

MATERIALS AND METHODS

Taxa

One specimen was found lying over a floating tree trunk in the upper Amazonian river, Jenaro Herrera District, Loreto, Perú. For comparative purposes, DNA sequences obtained from GenBank and morphological data of several representatives of three recently redefined families: Praobdellidae, Semicolecidae and Macrobdellidae (Phillips and Siddall, 2005; 2009; Phillips *et al.,* 2010) were selected and used to investigate the phylogenetic position of the new species of *Oxyptychus* described here. Taxon names, collection localities and GenBank accession numbers are presented in Table 8. In

Table 8. Species of Hirudiniformes used for the phylogenetic analyses with species names, collection localities and GenBank accession numbers.

Species	Locality	COI	12S	18S	28S
Outgroup					
Haemopis sanguisuga	Sweden	AF426202	AF099960	AF099941	AY425381
Hirudo medicinalis	France	AY786458	DQ097197	AY786464	AY786451
Ingroup					
Dinobdella ferox	Taiwan	GU394006	GU394010	GU394002	-----
Haemadipsa sumatrana	Borneo	AY425446	AY425415	AY425464	AY425372
Limnatis paluda	Afghanistan	GQ368796	GQ368775	-----	GQ368755
Limnobdella mexicana	Mexico	GQ368797	GQ368776	GQ3268818	GQ368758
Macrobdella decora	MI, USA	AF003271	AY425431	AF116007	AY425390
Macrobdella diplotertia	MO, USA	DQ097223	-----	DQ097214	DQ97205
Macrobdella ditetra	LA, USA	DQ097215	DQ097224	DQ097206	DQ097198-
Mesobdella gemmata	Chile	AY425454	AY425434	AY425472	AY425393
Myxobdella annandalei	India	GU394007	GU394013	GU394005	GU394014
Oxyptychus brasilensis	Brazil	AY425455	AY425436	AY425473	AT425398
Oxyptychus striatus	Argentina	AY425456	-----	AY425474	AY425399
Oxyptychus bora	Peru	XXXX	XXXX	XXXX	XXXX
Patagoniobdella fraternal	Chile	AY425459	AY425441	AY425477	AY425405
Patagoniobdella variabilis	Chile	AY425458	-----	AY425476	-----
Philobdella floridana	SC, USA	DQ097219	DQ097226	DQ097210	DQ09201
Philobdella gracilis	LA, USA	DQ097218	DQ097225	DQ097209	DQ097200
Pintobdella chiapasensis	Chiapas, Mexico	GU394008	GU394012	GU394004	GU394015
Semiscolex similis	Bolivia	AY425475	AY425402	AY425443	AY425475
Tyrannobdella rex	Peru	GU394009	GU394013	GU394005	GU394016

addition, 16 morphological characters adapted from Phillips and Siddall (2005) were codified for the expanded taxon sampling. All measurements are in millimeters.

DNA extraction, amplification and sequencing

The tissue sample was 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 and total DNA extraction and purification. The mitochondrial cytochrome *c* oxidase subunit I (COI) and 12S rDNA (12S), as well as the nuclear 18S rDNA (18S), and 28S rDNA (28S) were obtained and used to investigate the phyolgenetic relationships of the group. Amplification reactions of gene fragments were conducted using Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ) with 0.5 µl of each 10 µM primer, 1 µl DNA template, and 23 µl Rnase-free H_2O (total volume 25 µl). PCR reactions were performed with an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The following amplification protocols were used: for COI, 94°C (5 min), followed by 30 cycles of 94°C (45 sec), 48°C (45 sec), 72°C (1 min) and final extension of 72°C (7 min); 18S, 94°C (5 min) followed by 35 cycles of 94°C (20 sec), 47°C (20 sec), 68°C (50 sec) and final extension at 70°C (7 min); for 12S and 28S, 94°C (5 min), followed by 44 cycles of 95°C (1 min), 52°C (1 min), 70°C (1 min) and final extension of 72° (7 min). PCR products were purified with AMPure (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA.). Cycle sequencing reactions were performed with an Eppendorf Mastercycler using 1 µl ABI Big Dye Terminator V 3.1 (Applied Biosystems, Carlsbad, California, USA), 1 µl of 1 µM primer, 3 µl of cleaned PCR product and 1 µl Rnase-free H_2O (total volume 6 µl). Sequencing reactions were purified by 70% isopropanol/70% ethanol precipitation and analyzed with an ABI PRISM 3730 sequencer (Applied

Biosystems, Carlsbad, California, USA). CodonCode Aligner (CodonCode Corporation, Dedham, Massachusetts, USA) was used to edit and reconcile sequences.

Morphological data

The newly collected leech was relaxed with gradual addition of 70% ethanol, fixed in 100% ethanol, and stored in 90% ethanol. Observation of external characteristics and dissection was accomplished with the use of a stereoscopic microscope. Photographs of the whole specimen were taken with a SONY α-330 digital camera. Photographs of internal structures were taken using NIKON Coolpix P5000 digital camera attached to a stereoscopic microscope. All leech species were scored for sixteen internal and external characters (Table 9).

1 Pharynx: (0) absent, (1) present.

2 Rows of teeth: (0) monostichodont, (1) distichodont, (2) astichodont.

3 Number of jaws: (0) agnathous, (1) trignathus, (2) monoagnathus.

4 Feeding habit: (0) macrophagous, (1) haematophagous/sanguivorous, (2) omnivorous.

5 Salivary papillae: (0) absent, (1) present.

6 Number of annuli with eyespots: (0) none, (1) one, (2) two, (3) three, (4) four, (5) five.

7 Eyespots per annulus: (0) one pair, (1) two or more pairs.

8 Vaginal duct: (0) absent, (1) present.

9 Vaginal caecum: (0) absent, (1) present.

10 Ovisac shape: (0) tubular, (1) spheroid.

11 Common oviduct: (0) absent, (1) present.

12 Male atrium extension into elongated penis and sheath: (0) absent, (1) present.

13 Ejaculatory epididymis: (0) U-shaped, (1) S-shaped.

14 Copulatory gland pores: (0) absent, (1) present.

15 Testisacs per body somite: (0) one pair, (1) two pairs, (2) four pairs.

16 Total testisacs: (0) less than 10 pairs, (1) 10 pairs, (2) more than 10 pairs.

Sequence alignments and phylogenetic analyses

Alignment of all gene sequences was accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004). Alignments were obtained applying the default settings for gap opening and extension penalties. The final matrix included 21 terminals and 4208 aligned characters: 650 bp of COI; 360 bp of 12S; 2019 bp of 18S; 1164 bp of 28S and 16 morphological characters. Maximum parsimony (MP) analyses of morphological, molecular data sets individually and the combined data set (total evidence) were conducted in PAUP* 4.0b10 (Swofford 2002). A heuristic search used 1000 replicates of random taxon addition and tree bisection-reconnection (TBR) algorithm branch swapping. All characters were equally weighted and considered as non-additive. Gaps were considered as missing data. Bootstrap values (performing 1000 pseudoreplicates) as well as consistency and retention indices were calculated with PAUP* 4.0b10 (Swofford 2002).

Maximum likelihood (ML) analysis of the molecular data set was conducted in TREEFINDER (Jobb 2008). Data were partitioned and analyzed considering each gene independently (4 partitions in total). Models of evolution for each partition were selected based on the Akaike information criterion (AIC) as implemented in FindModel (Tao *et al*. 2008). For COI and 12S, TVM+G model was selected, for 18S, GTR+G and for 28S,

Table 9. Morphological matrix of Hirudiniformes used in the phylogenetic analyses.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Haemopis sanguisuga	1	1	1	0	0	5	0	1	1	1	1	1	1	0	0	0
Hirudo medicinalis	1	0	1	1	0	5	0	1	1	1	1	1	1	0	0	0
Dinobdella ferox	1	2	1	1	?	5	0	1	0	1	1	1	0	0	0	0
Haemadipsa sumatrana	1	0	1	1	1	5	0	1	1	1	1	0	0	0	0	0
Limnatis paluda	1	0	1	1	1	5	0	1	0	1	1	1	0	0	0	0
Limnobdella mexicana	1	0	1	1	1	5	0	1	0	1	1	1	0	0	1	0
Macrobdella decora	1	0	1	1	0	5	0	1	0	1	1	0	0	1	0	0
Macrobdella diplotertia	1	0	1	2	1	5	0	1	0	1	1	0	0	1	0	0
Macrobdella ditetra	1	0	1	1	0	5	0	1	0	1	1	0	0	1	0	0
Mesobdella gemmata	1	0	1	1	0	5	0	1	1	1	0	0	0	0	0	0
Myxobdella annandalei	1	1	1	1	?	5	0	1	1	1	1	1	0	0	0	0
Oxyptychus bora sp. nov.	1	0	1	1	?	5	0	1	0	1	0	0	0	0	0	0
Oxyptychus brasiliensis	1	0	1	1	0	5	0	1	0	1	0	0	0	0	0	0
Oxyptychus striatus	1	0	1	1	0	5	0	1	0	1	0	0	0	0	0	0
Patagoniobdella fraterna	1	2	0	0	0	5	0	1	0	1	1	1	0	0	1	1
Patagoniobdella variabilis	1	2	0	0	0	5	0	1	0	1	1	1	0	0	1	1
Philobdella floridana	1	0	1	2	0	5	0	1	1	1	0	0	0	1	0	0
Philobdella gracilis	1	0	1	2	0	5	0	1	1	1	0	0	0	1	0	0
Pintobdella chiapasensis	1	0	1	1	?	5	0	1	0	1	0	0	0	0	0	0
Semiscolex similis	1	2	0	0	0	5	0	1	0	1	1	1	0	0	0	0
Tyrannobdella rex	1	0	2	1	0	5	0	1	0	1	0	0	0	0	0	0

TIM+G models were selected. Bootstrap support values were calculated in TREEFINDER performing 1000 pseudoreplicates.

Squared-change parsimony method (Maddison, 1991) to reconstruct ancestral states was used to infer the number of teeth or denticles per jaw in hypothetical ancestors. Square-change parsimony was designed to minimize the sum of squared changes of continuous characters on the branches of a given topology, in this case in the MP tree. The analysis was implemented in MacClade (Maddison and Maddison, 2003) using empirical data (i.e. number of teeth per jaw) obtained from our own observations and reported by previous studies (Sawyer, 1986; Phillips and Siddall, 2006). Due the inability of the program to distinguish between absence of particular traits (e.g. *Dinobdella ferox* completely lacks teeth on their jaws) and the non-applicability of a trait (i.e. species of *Semiscolex* and *Patagoniobdella* lack jaws, therefore, number of teeth is not applicable), Semiscolecids were pruned before the squared-change parsimony analysis. In the cases where a single value for the number of teeth for terminals was known, it was used directly in the analysis. In those cases where a range in the number of teeth is known, minimum and maximum values were used in separate analyses. Estimates of the values of ancestral nodes resulting from both analyses are plotted next to their respective nodes.

RESULTS

Species description

Oxyptychus bora sp. nov.

Figure 18

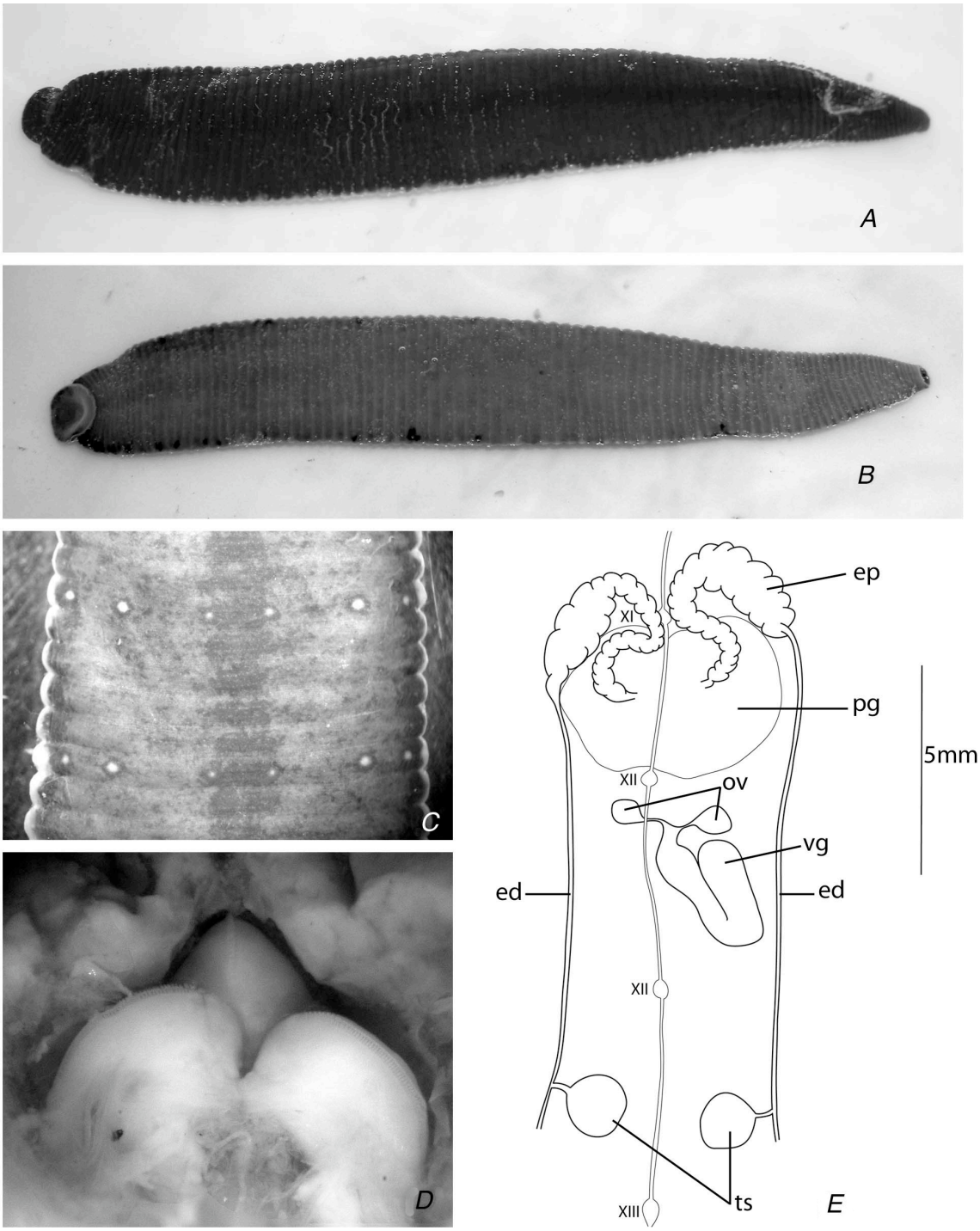
Description based on a single specimen. Large, 77.5 length, 14.4 maximum width. Complete somite pentaännulate. Somites I-III uniannulate. Somites IV-V biannulate. Somites VI-VII triannulate. Somite VIII tetraänulate. Somites IX-XXIV pentaännulate. Somite XXV tetraännulate. Somites XXVI-XXVII biannulate. Sixteen complete somites Dorsum olive green with a wide medial row of black pigments (Figure 18a). Three pairs of sensilla on a2 forming longitudinal rows: one pair paramedial, one paramarginal internal and one paramarginal external (Figure 18c). Ventral surface light orange to pink (Figure 18b). Five pairs eyespots in parabolic arc in somites II, III, IV a2, V a2 and VI a2. Male gonopore XI b5/b6. Female gonopore XII b5/b6. Five annuli between gonopores. Eighteen pairs of nephropores. Anus in XXVII. Posterior sucker oval, 6.5 width, 4.4 length.

Three jaws, one dorsal and two ventrolateral. Monostichodont. Each jaw armed with 50-60 diminute teeth or denticles (Figure 18d). Pharynx short. Crop from IX to XIX. Crop in somites IX and X with a single pair caeca. Somites XI and XII with two pairs of equal caeca, XIII and XIV with anterior pair shorter, posterior pair slightly bilobated, XV to XVII two pairs equal size, XVIII anterior pair larger and posteriorly directed, posterior pair reduced. Somite XIX with single pair of large postcaeca or diverticula extending to XXIV. Intestine from XX to XXVII formed by a simple acecate tube.

Male reproductive system with nine pairs testisacs, one pair per segment, from XIII to XXI. Vasa deferentia lateral connecting to each testisac through a short vas efferens. Each vas deferens connects laterally to a highly convoluted and S-shaped epididymus on X discharging into the male gonopore. No ejaculatory bulbs. Prostate

FIGURE 18.

External and internal morphology of *Oxyptychus bora* sp. nov.: **a.** Dorsal view; **b** Ventral view; **c** Detailed view of the dorsal surface of mid-body somites showing 3 pairs rows of sensilla; **d** Dorsal view of jaws, showing teeth forming a single row. **e** Dorsal view of the male and female reproductive apparatus. ov= ovaries; ej= ejaculatory ducts; pg= prostatic gland.


A
B
C
D
ep
XI
pg
5mm
XII
ov
vg
ed
ed
XII
ts
XIII
E

(prostatic gland) large laying on the ventral body surface. Female reproductive system forming a U-shaped vagina. Ovaries simple, spheroidal. No common oviduct (Figure. 18e).

Holotype: Stored in 90% ethanol; dissected; deposited in the Museo de Historia Natural (MHNC). Universidad Nacional de San Antonio Abad del Cusco, Perú.

Type locality: Distrito Jenaro Herrera, Provincia de Requena, Departamento de Loreto, Perú. 4.916355^{o} S; 73.73677^{o} W.

Etymology: Named after the Bora, an indigenous group of about 2,000 people that inhabit the upper Amazonia, Perú.

Collector: Alejandro Oceguera Figueroa.

Diagnosis: Macrobdellid in general aspect. Monostichodont. No copulatory glands. Male and female reproductive systems mesomorphic. Five annuli between gonopores.

Remarks:

Oxyptychus bora sp. nov. is the only species of the genus with five annuli between gonopores. The number of annuli between gonopores typically has been used to distinguish between the eight species currently recognized for the genus (Ringuelet 1985). *Oxyptychus antelarum* (Moore 1901) from Puerto Rico, Dominicana and Panama, like *Oxyptychus bora*, has a large and strong vagina, a character atypical of the genus. Whether or not *O. antelarum* belongs to this otherwise South American genus (Figure 19), it can easily be distinguished from *Oxyptychus bora* sp. nov. based on the number of annuli between gonopores, which is 3+1/2 in *O. antellarum*.

Oxyptychus inexpectatus (Ringuelet 1945) from Argentina and *Oxyptychus striatus* (Grube 1850) from Argentina, Brazil, Uruguay and Venezuela resemble each other by having a conspicuous median longitudinal yellowish strip on the dorsal surface

FIGURE 19.

Map of South America, plotting known locality records of the species of *Oxyptychus*.


O. antelarum
O. brasilensis
O. festai
O. inexpectatus
O. ornatus
O. riopretensis
O. strennus
O. striatus
O. bora n. sp.

accompanied by marginal strips of the same color. This patterns contrasts with the single dorsal longitudinal black strip of *O. bora* sp. nov. In addition, *O. inexpectatus* have the male and female gonopores on XIIb2 and XIIa2 respectively (i.e. on consecutive annuli) and *O. striatus* have 1+1/2 annuli between gonopores, clearly different to *Oxyptychus bora* sp. nov.

Oxyptychus brasilensis Pinto, 1920, from Brazil and Argentina has gonopores separated by 4 annuli and a solid longitudinal dorsal line similar to *Oxyptychus bora* sp. nov. however, in addition of having a different number of annuli between gonopores, *O. brasilensis* has an additional pair of lateral and longitudinal lines in the dorsal surface and fewer denticles per jaw (34-43) than *Oxyptychus bora* sp. nov.

Oxyptychus ornatus Weyenberg, 1883 from Argentina, Brazil and Uruguay has gonopores separated by 1/2+2+1/2 annuli and around 40 denticles per jaw, contrasting with the five annuli between gonopores and the more than 50 denticles per jaw characteristic of *Oxyptychus bora* sp. nov. *Oxyptychus festai* Dequal, 1916 from western Ecuador and *Oxyptychus riporetensis* Castro, 1971 from Sao Paulo, Brazil have gonopores separated by 4 annuli and 10 pairs of testisacs contrary to 5 annuli separating gonopores and the 9 pairs of testisacs of *Oxyptychus bora* sp. nov. In addition, *O. riopretensis* has 5 longitudinal lines in the dorsal surface, contrasting with the unique one of *Oxyptychus bora* sp. nov. *Oxyptychus strennus* Ringuelet, 1948 from the Chaco (Formosa, Argentina and Guaira, Paraguay) have 3+1/2 annuli between gonopores and 38-41 denticles per jaw which contrast with the characteristics of *Oxyptychus bora* n. sp

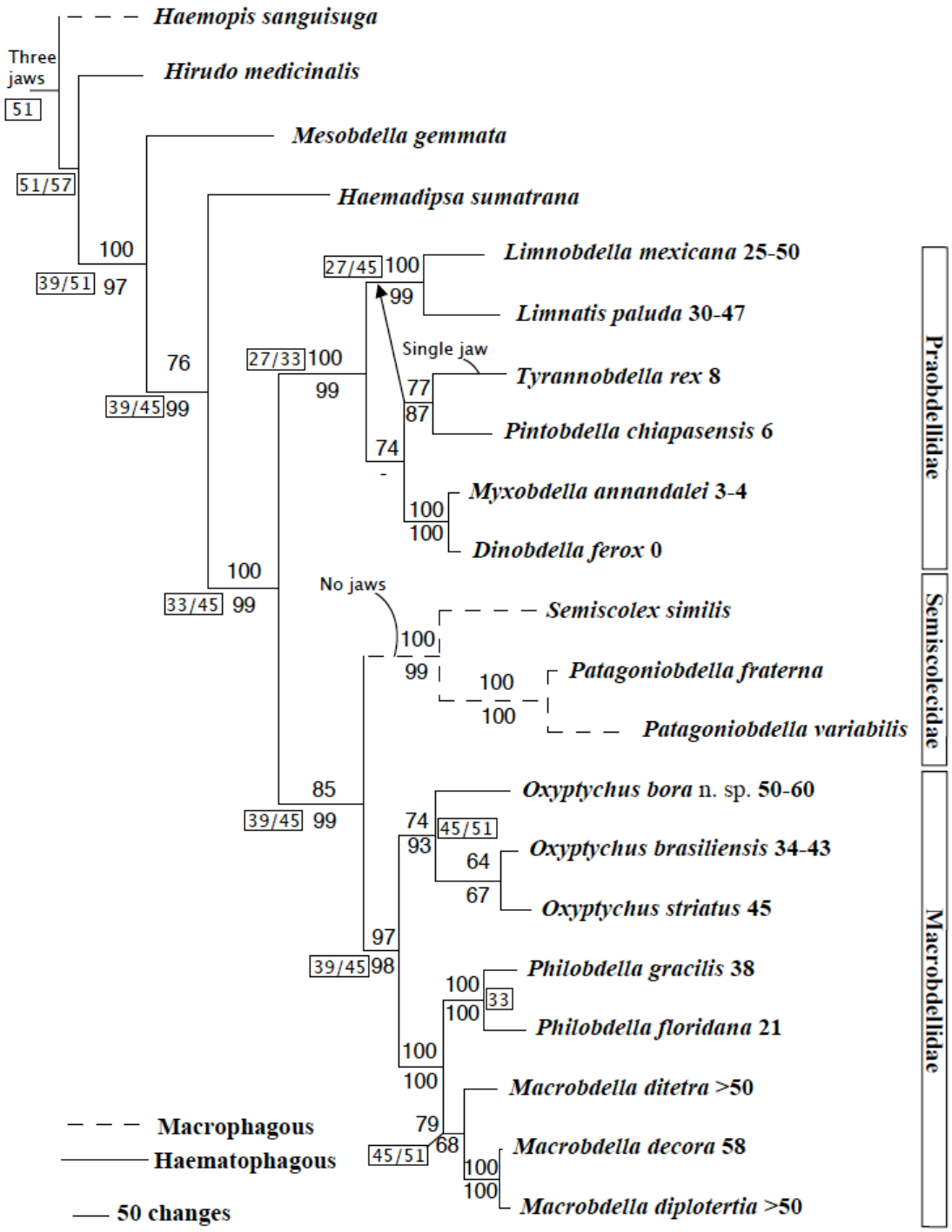
Phylogenetic analyses

Maximum Parsimony (MP) analyses of the 16 morphological characters yielded 37 equally parsimonious trees with 27 steps (CI=0.59; CI=0.78). The strict consensus tree did not provide any resolution regarding the internal relationships of the group with the exception of the genus *Philobdella, Macrobdella,* and the family Semiscolecidae (*Semiscolex similis*, *Patagoniobdella fraterna* and *Patagoniobdella variabilis*) that were found to be monophyletic. Analysis of molecular data (4193 aligned characters) resulted in a single most parsimonious tree with 2974 steps (CI=0.59; RI-=0.54) which is identical to the single most parsimonious tree resulted from the total evidence analysis (3008 steps, CI= 0.59 and RI= 0.54) which is shown in Figure 20. The log-likelihood of the tree resulting from the Maximum Likelihood (ML) analysis was –21322.65. ML and MP resulted in highly congruent trees with only one difference; whereas in the ML tree, *Myxobdella annandalei* and *Dinobdella ferox* appeared as sister group of *Limnobdella mexicana* and *Limnatis paluda*, in MP they appear sister to *Tyrannobdella rex* and *Pintobdella chiapasensis*.

The resulting phylogenetic trees support the monophyly of the South American genus *Oxyptychus,* including *O. bora,* the new species described herein, with good bootstrap supports under the two optimality criteria (74/93 for MP and ML respectively). The *Philobdella* and *Macrobdella* clade, which includes the well-known North American medicinal leeches, were also found to be monophyletic and forming the sister group of *Oxyptychus* with elevated bootstrap values (MP=97; ML=98). Semiscolecidae (*sensu stricto*) was found forming a monophyetic and well supported group. Taken together, species of *Oxyptychus, Macrobdella, Philobdella* and the Semiscolecidae formed a well-

FIGURE 20.

Maximum parsimony tree of species of Hirudiniformes based on morphological and molecular data. Numbers next to nodes indicate bootstrap values of Maximum parsimony/Maximum likelihood analyses respectively. Numbers inside squares indicate the number of teeth per jaw inferred using square-change parsimony method calculated based on minimum and maximum values of teeth number.


Haemopis sanguisuga
Three jaws
51
Hirudo medicinalis
Mesobdella gemmata
51/57
Haemadipsa sumatrana
100
39/51 97
27/45 100
99
Limnobdella mexicana 25-50
Limnatis paluda 30-47
Single jaw
27/33 100
99
76
39/45 99
Tyrannobdella rex 8
77
87
Pintobdella chiapasensis 6
74
-
Myxobdella annandalei 3-4
100
100
Dinobdella ferox 0
100
33/45 99
No jaws
Semiscolex similis
100
99
Patagoniobdella fraterna
100
100
Patagoniobdella variabilis
85
39/45 99
Oxyptychus bora n. sp. 50-60
74
93
45/51
Oxyptychus brasiliensis 34-43
64
67
Oxyptychus striatus 45
97
39/45 98
Philobdella gracilis 38
100
100
33
Philobdella floridana 21
100
100
Macrobdella ditetra >50
79
45/51
68
Macrobdella decora 58
100
100
Macrobdella diplotertia >50
Macrophagous
Haematophagous
50 changes
Praobdellidae
Semiscolecidae
Macrobdellidae

supported group of exclusively New World' species with relatively good support (MP=85; ML=99) and is sister to the recently redefined family Praobdellidae (Phillips *et al*., 2010) which exclusively includes haematophagous leeches adapted to feed on the mucous membranes of a diversity of mammals, including humans.

DISCUSSION

Jaw evolution

The poor value of the bloodfeeding preference to predict the phylogenetic relationships of leeches, shown in previous analyses (Borda and Siddall, 2004a,b; Siddall *et al*., 2006; Phillips and Siddall, 2005, 2009; Phillips *et al*., 2010) is corroborated here and is considered as one of the major findings in reference to leech systematics. The presence of jaws, sometimes obviated as a "byproduct" character of the bloodfeeding habit, seems to have a better fit on the phylogenetic hypotheses than the preference for blood itself. Jaws appeared once as a novelty in the evolution of leeches and have disappeared just one time, in the clade of Semiscolecidae. This scenario of appearance/disappearance contrasts with the many transformations of feeding preferences amongst the leeches.

The plesiomorphic three-jawed condition, which is the most common state in the group, has transformed on at least in three independent occasions: In the duognathus clade of Haemadipsid leeches (not presented here but discussed in Borda *et al*. 2008), in the single-jawed *Tyrannobdella rex* and finally in Semicolecidae where those structures are completely absent (Figure 20). Interestingly, changes in jaw number do not seem to have an impact on food preference as single-jawed and duognathus leeches still feeding

on blood. However, the completely absence of jaws is correlated with the macrophagous/carnivorous preference of Semiscolecidae species.

Species of the family Praobdellidae, contrasting with their particularly prominent posterior sucker, present a reduction in their mouth morphology. Jaws of the species of this group are weakly developed so extensively that even *T. rex* has only one jaw. Interestingly, the number of denticles per jaw of the species of the complete cade is also reduced, ranging from none to ten when *T. rex, Pintobdella chiapasensis, Myxobdella annandalei* and *Dinobdella ferox* are considered, and to no more than 50 if *L. mexicana* and *Limnatis paluda* are considered. The reduced number of denticles per jaw seems to be correlated with their predilection for feeding from the mouth and respiratory tracts of vertebrates, organs characterized by their soft and highly vascularized tissues.

Species of Macrobdellidae, in constrast to species of Praobdellidae, present well-developed jaws armed with multiple denticles forming a single raw, a condition known as monostichodontia (*Philobdella gracilis* has been reported as partially distichodont). The number of denticles seems to be correlated with the evolutionary history of the group. Species of *Oxyptychus* have no more than 50 denticles per jaw, with the only exception of *O. bora* sp. nov. which has between 50 and 60. This fact is true not only for the 3 species included in the present analysis but for all the species described for the genus (see Ringuelet 1986). In *Philobdella* species, the reduction in the number of denticles is even more dramatic than in the other groups*, Philobdella gracilis* has 38 denticles per jaw and *Philobdella floridiana* only 21. The number of denticles in species of *Macrobdella,* on the contrary, is always more than 50, a condition that resembles a variety of species of

Hirudinidae including the infamous European medicinal leech *Hirudo medicinalis*, which has 65 denticles per jaw (ranging from 60-100).

Squared-change parsimony analyses suggest that the number of teeth per jaw in the most basal node on the tree (Figure 20) was higher than 50. That value decreased reaching values of 33/45 in more derived nodes, including the last common ancestor of Macrobdellidae, Semiscolecidae and Praobdellidae. The number of teeth seems to increase independently 2 times; the first one includes the clade of species of *Oxyptychus* for which a value of 45/51 was estimated and finally, the group including the species of *Macrobdella* spp with the same values. The reduced number of teeth per jaw of several species in the analyses, as well as in the inferred ancestors, might be related with their predilection for amphibians (particularly anurans and salamanders), and for the respiratory and buccal tracts of mammals (Brandt 1936; Graham and Borda 2010; Phillips *et al*. 2010; Schalk *et al*. 2002; Ringuelet 1985; Turberville and Briggler 2003). Interestingly and correlated with the elevated number of teeth per jaws, *Macrobdella* spp. are the only representatives of the group that are able to feed on the external surface of mammals, which represents a barrier more difficult to penetrate than amphibians skin or mucous membranes of mammals.

Biogeography

The biogeographical pattern observed in the analysis regarding the species of Macrobdellidae, suggests a connection between South and North American, particularly with Eastern USA. A similar pattern has been detected before in another group of leeches, in the proboscis bearing species of *Placobdella* (Siddall *et al*., 2005). Of particular interest is the fact that the genus *Philobdella* and *Macrobdella* as well as

Placobdella are particularly diverse in Eastern USA and Canada, but not in Mexico and Central America where *Philobdella* and *Macrobdella* are virtually absent. In addition, both groups have their respective closest relatives in South America. This pattern suggests that the colonization of North America is not related with the closing of the Panamanian Isthmus (3 MYA), an explanations that seems more plausible to justify the sister relationship of the Peruvian *T. rex* and *P. chiapasensis* from Southern Mexico. The same geological event has been used to explain the current distribution of species of *Semiscolex*, *Haementeria* and *Helobdella* (Oceguera-Figueroa, 2005; Oceguera-Figueroa, 2006b; Oceguera-Figueroa, 2008; Siddall *et al*., 2005) that have South and North American representatives, including several species in Mexico and Central America. Whether the distribution of the species of Macrobdellidae supports the idea of land connections between North and South America other/older than the Panamanian Isthmus, or if it is merely a reflect of poor taxonomic sampling/multiple extinctions should be investigated in future analyses.

The position of *Oxyptychus bora* sp. nov. on the tree, in a sister relationship with *O. striatus* and *O. brasilensis* (Figure 20) confirms its inclusion in the group. The monophyly of *Oxyptychus* validates the diagnostic morphological characteristics of the genus (*i.e*., no accessory glands; one pair of testisacs per somite; prostate strongly developed; five or fewer annuli between gonopores) as phylogenetically informative.

4.3 *Tyrannobdella rex* N. Gen. N. Sp. and the Evolutionary Origins of Mucosal Leech Infestations

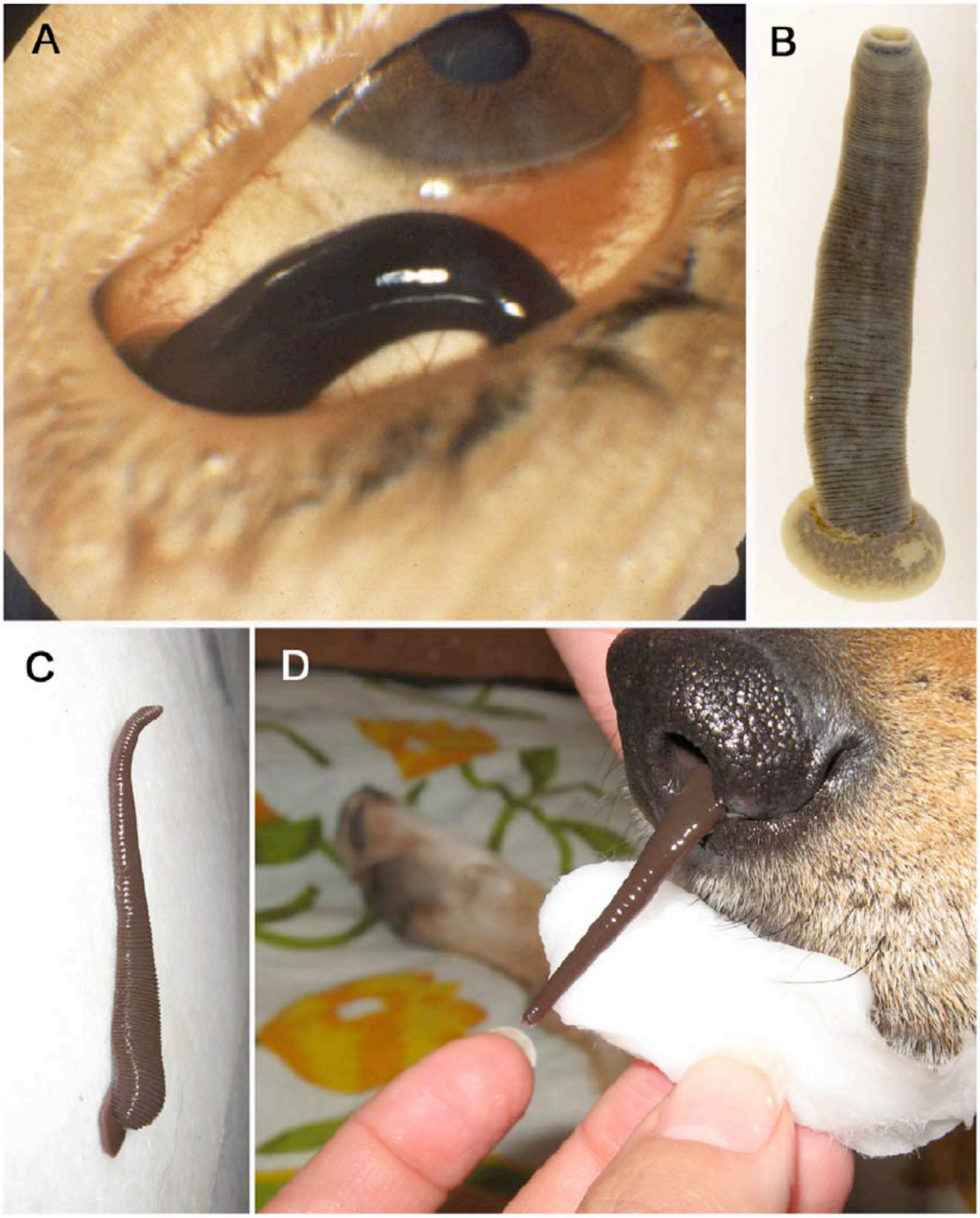
(Adapted from: Phillips, A. J., Arauco-Brown, R., Oceguera-Figueroa, A., Gomez, G. P. Beltran, M. Lai, Y. T., and Siddall, M. E. 2010. *Tyrannobdella rex* N. Gen. N. Sp. and the Evolutionary Origins of Mucosal Leech Infestations. PloS ONE 5(4): e10057.doi:10.1371/journal.pone.0010057)

INTRODUCTION

Most people realize that they are being parasitized by a leech upon finding the worm attached to their skin. Disturbingly, leeches occasionally enter human orifices, a condition known as mucosal, orifical, vesical, or internal hirudiniasis depending on the localization of the leech (Figure 21). Whereas most bloodfeeding leeches feed as ectoparasites for short periods of time, those that feed on mucous membranes have been known to stay in an orifice for days or weeks on end (Harding and Moore, 1927; Cundall *et al.,* 1986). Cases of hirudiniasis are underreported because patients suffering from orificial hirudiniasis may only resort to medical attention if they are personally unsuccessful in extracting the leech (Montazeri *et al.,* 2008). Whereas invasive leeches are usually found in the nasopharyngeal region, there are many cases of leeches infesting various body orifices such as the eyes, urethra, vagina, or rectum (Almallah, 1968). Depending on the exact site of the bite in the nasopharyngeal region, symptoms may include hemorrhaging, hemoptysis, dysphonia, coughing, a tickling sensation, dyspnea or, in extreme cases, severe anemia and death (Masterman, 1908; Turner, 1969). Hemorrhaging from leeches in the urethra, or even in the bladder, also poses a particular problem in that clot formation is inhibited by urine flow *Alam et al*., 2008). Underlying conditions, such as coagulation disorders or secondary bacterial infections can cause a patient's condition to escalate from relatively minor to lifethreatening very quickly (Cundall *et al.,* 1986; Kose, 2008; Alam *et al.,* 2008). Reported cases of human orificial hirudiniasis are most common in rural areas of the Middle East, Africa, and Asia, however cases have been recorded from almost all continents (Cundall *et al.,* 1986). Domestic and wild mammals in these regions, especially livestock, are at the greatest risk

Figure 21.

Mucosally invasive hirudinoid leeches. Known from a wide variety of anatomical sites including eyes (A) as in this case involving *Dinobdella ferox* (B), mucosal leech species, as in a case involving *Myxobdella annandalei* (C), more frequently feed from the nasopharyngeal surfaces of mammals (D).


A
B
C
D

for orificial hirudiniasis in relation to the amount of time such animals spend at leech-inhabited watering holes (Harding and Moore, 1927). Some species are more likely to afflict humans, such as *Dinobdella ferox* (Blanchard, 1896; literally translated to ‘‘terrible ferocious leech’’), or species of *Limnatis, Praobdella*, and *Myxobdella* (Sawyer, 1986). Leech systematics has been transformed with the addition of comparative sequence data and most of the groups historically recognized by taxonomists have been redefined or eliminated (Phillips and Siddall, 2009; Borda *et al.,* 2008). That said, Phillips and Siddall (2009) expressed reluctance to fully reorganize the systematics of New World hirudinoid leeches under Semiscolescidae in light of the unexpected finding of several interrelated Old World species in that group. Our discovery of a species that is new to science, found feeding from the upper respiratory tract of humans in Perú, leads to a reanalysis of the phylogeny and classification of one clade of hirudinoid leeches, clarifying a single evolutionary origin of a group that specializes on mucous membranes and poses a threat to human health.

MATERIALS AND METHODS

Specimens of *T. rex* were collected from two states of Perú in 1997; one from a health center in Lamas province, department of San Martin, Perú, and one from a local heath center in Yochegua province, San Francisco district. Both of these specimens were preserved in formalin. A third specimen collected from a clinic in La Merced Chanchamayo Junin, Perú in 2007, was preserved in ethanol, and was the specimen chosen both for the holotype and for sequencing in these analyses. Specimens of *P. chiapasensis* were collected from forest streams leading to the lakes of Montebello, State

of Chiapas, Mexico between 6 and 18 July, 2008. One *M. annandalei* was received in December, 2008 from Dharamsala, India. Tissue samples of *D. ferox* were collected on 13 April, 2008 in Taiwan. Examination of external and internal morphology was accomplished with a Nikon SMZ-U stereo microscope on whole and dissected specimens. Photographs were taken with a SPOTRT digital camera. Drawings were made by superposition of vector art over images placed in Adobe IllustratorH 10 and Adobe PhotoshopH 7.

DNA sequencing and alignment Tissue was collected from the caudal sucker in order to avoid contamination from host DNA in gastric or intestinal regions of the leech. DNeasy Tissue Kit (Qiagen Valencia, CA) was used for tissue lysis and DNA purification. Primers for the PCR amplification of nuclear 18S rDNA and 28S rDNA and mitochondrial cytochrome oxidase I (COI) and 12S rDNA gene fragments were adapted from published protocols (Apakupakul *et al.,* 1999; Prendini *et al.,* 2005b; Whithing, 2002; Simon *et al.,* 1994; Folmer *et al.,* 1994). Amplification reactions of gene fragments were conducted using either Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ) with 0.5 ml of each 10 mM primer, 1 ml DNA template, and 23 ml Rnase-free H2O (total volume 25 ml), or homemade Taq with 1.0 ml Taq, 2.5 ml MgCl, 2.5 ml 10x Buffer A, 1.0 ml dNTPs, 0.5 ml of each 10 mM primer, 2.0 ml template, and 15 ml H2O) (total volume 25 ml). PCR reactions were performed in an Eppendorf Mastercycler. The following amplification protocols were used: for 18S, 94uC (1 min) followed by 35 cycles of 94uC (30 sec), 49uC (30 sec), 68uC (2 min 30 sec) and final extension at 68uC (1 min); for 28S and 12S, 94uC (5 min), followed by 39 cycles of 95uC (1 min), 52uC (1 min), 70uC (1 min) and final extension of 72u (7 min); for COI, 94uC (1 min), followed

by 30 cycles of 94uC (30 sec), 48uC (30 sec), 68uC (45 sec), 68uC (1 min) and final extension of 68uC (1 min). PCR amplification products were purified with AMPureTM (Agencourt Bioscience Corporation). Cycle sequencing reactions were performed with an Eppendorf MastercyclerH using 1 ml Big DyeTM Extender Buffer v3.1, 1 ml of 1 mM primer and 3 ml of cleaned PCR template (13 ml total volume). Sequences were purified by 70% isopropanol/70% ethanol precipitation and analyzed with an ABI PRISMH 3730 sequencer (Applied Biosystems). CodonCode Aligner (CodonCode Corporation) was used to edit and reconcile sequences. GenBank accession numbers are listed for sequences derived from each taxon in Table 2. Alignments of all genes were accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 applying default settings (Edgar, 2004). Phylogenetic analyses A total of 17 species comprising 19 terminals were used in the analyses with *Hirudo medicinalis* specified as the outgroup (Table 10). Phylogenetic analyses were conducted using two approaches: Parsimony and Bayesian Inference (BI). Parsimony analyses were conducted in TNT v 1.1 Goloboff, 2008) using 10 replicates of random taxon addition, sectorial searching, the Ratchet (Nixon, 1999), and tree-bisection reconnection branch swapping for each gene as well as for the combined dataset (18S, 28S, 12S, COI). Bootstrap values for combined analyses were obtained using 10 heuristic pseudoreplicates and the same analytical settings. Bayesian analyses were conducted in MrBayes v. 3.1.2 (Ronquist and Hulsenbeck, 2003). The data were partitioned by gene for 18S, 28S, 12S, and by codon position for COI (three partition; 3p). A GTR+I+C model was applied to each unlinked data partition based on the Akaike Information Criterion [via ModelTest v. 3.7(Posada and Crandall, 1988; Posada and Buckley, 2004)].

Table 10.

Taxa used for the phylogenetic analyses of the family Hirudinidae along with collection localities and GenBank accession numbers * indicates type species for the genera of the Ingroup

Taxon	Locality	GenBank Accession Numbers			
		18S	28S	12S	CO1
Ingroup					
Dinobdella ferox	Taiwan	GU394006	GU394010	GU394002	_____
Limnatis cf. *nilotica*	Namibia	GQ368795	GQ368774	GQ368815	GQ368754
Limnatis paluda 1	Afghanistan	GQ368796	GQ368775	_____	GQ368755
Limnatis paluda 2	Israel	AY425470	AY425389	AY425430	AY425452
Limnobdella mexicana 1*	Mexico	GQ368798	GQ368777	GQ368816	GQ368756
Limnobdella mexicana 2*	Mexico	GQ368799	GQ368778	GQ368817	GQ368757
Myxobdella annandalei	India	GU394007	GU394011	GU394003	GU39414
Pintobdella chiapasensis	Chiapas, Mexico	GU394008	GU394012	GU394004	GU394015
Tyrannobdella rex n.sp.	Perú	GU394009	GU394013	GU394005	GU394016
Outgroup					
Haemadipsa sylvestris	Vietnam	AF116005	AY425373	AY425416	AF003266
*Haemopis sanguisuga**	Sweden	AF099941	AY425381	AF099960	AF462021
*Hirudo medicinalis**	BioPharm, UK	AF116011	AY425385	AF099961	AF003272
*Macobdella decora**	MI, USA	AF116007	AY425390	AY425431	AF003271
Macrobdella ditetra	GA, USA	AY425471	AY425391	AY425432	AY425453
Oxyptychus brasiliensis	Brazil	AY425473	AY425398	AY425436	AY425455
Patagoniobdella fraterna	Chile	AY425477	AY425405	AY425441	AY425459
*Philobdella floridana**	SC, USA	DQ097210-13	DQ097201-14	DQ097226	DQ097219-22
Philobdella gracilis	LA, USA	DQ097209	DQ097200	DQ097225	DQ097218
Semiscolex similis	Bolivia	AY425475	AY425402	AY42543	AY425475

For the Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analyses, default prior distributions of parameters were used twice with one cold chain and three hot chains for 10 million generations and sampled every 1000th generation. The BI analyses burned-in before 100,000 generations. Split frequencies of the standard deviation of simultaneous BI analyses were well below 0.01. As such, the burn-in was set to discard the first 100,000 generations, leaving 9,900 trees sampled for estimation of posterior probabilities. Nomenclatural Acts The electronic version of this document does not represent a published work according to the International Code of Zoological Nomenclature (ICZN), and hence the nomenclatural acts contained in the electronic version are not available under that Code from the electronic edition. Therefore, a separate edition of this document was produced by a method that assures numerous identical and durable copies, and those copies were simultaneously obtainable (from the publication date noted on the first page of this article) for the purpose of providing a public and permanent scientific record, in accordance with Article 8.1 of the Code. The separate print-only edition is available on request from PLoS by sending a request to PLoS ONE, 185 Berry Street, Suite 3100, San Francisco, CA 94107, USA along with a check for $10 (to cover printing and postage) payable to ‘‘Public Library of Science’’. In addition, this published work and the nomenclatural acts it contains have been registered in ZooBank, the proposed online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved, and the associated information viewed, through any standard web browser by appending the LSID to the prefix ‘‘http://zoobank.org/’’. The ZooBank LSID for this publication is: urn:lsid:zoobank.org:pub:8D431ED1-B837- 4781-A591-D3886285283A.

RESULTS

Clinical Presentations

In 1997, a previously healthy six-year-old boy was admitted to a health center in Lamas province, department of San Martın, Perú complaining of frontal cephalgia. The patient's history revealed that, prior to admission, he frequently bathed in local lakes and natural streams. The patient reported neither bleeding nor respiratory distress. A 25 mm long leech was removed from the right nostril and preserved in formalin. Again, in 1997, a 16-month-old boy was admitted to a local heath center in Yochegua province, San Francisco district, department of Ayacucho, Perú also complaining of frontal cephalgia and also without respiratory symptoms. It was ascertained that prior to admission the boy had bathed in small local lakes. A 60 mm leech was removed from the patient's nasal cavity, washed with saline solution and preserved in 10% formalin. Nasal bleeding continued for two days. In 2007, a nine-year-old girl was admitted to La Merced hospital in Chanchamayo province, department of Junın, Perú following a two-week history of frontal cephalgia and a ''sliding'' sensation inside her nose. The patient's parents noticed a black worm moving inside her right nostril and sought medical attention. No other respiratory symptoms presented. The patient volunteered that she had been traveling in Satipo province, department of Junın, Perú where she frequently bathed in lakes, rivers and streams. Physical examination was remarkable only for nasal pain with hand pressure and a black mass inside the right nasal cavity. With some effort, a 65–70 mm black leech was removed without significant bleeding from the patient's nasal cavity, and was preserved in ethanol.

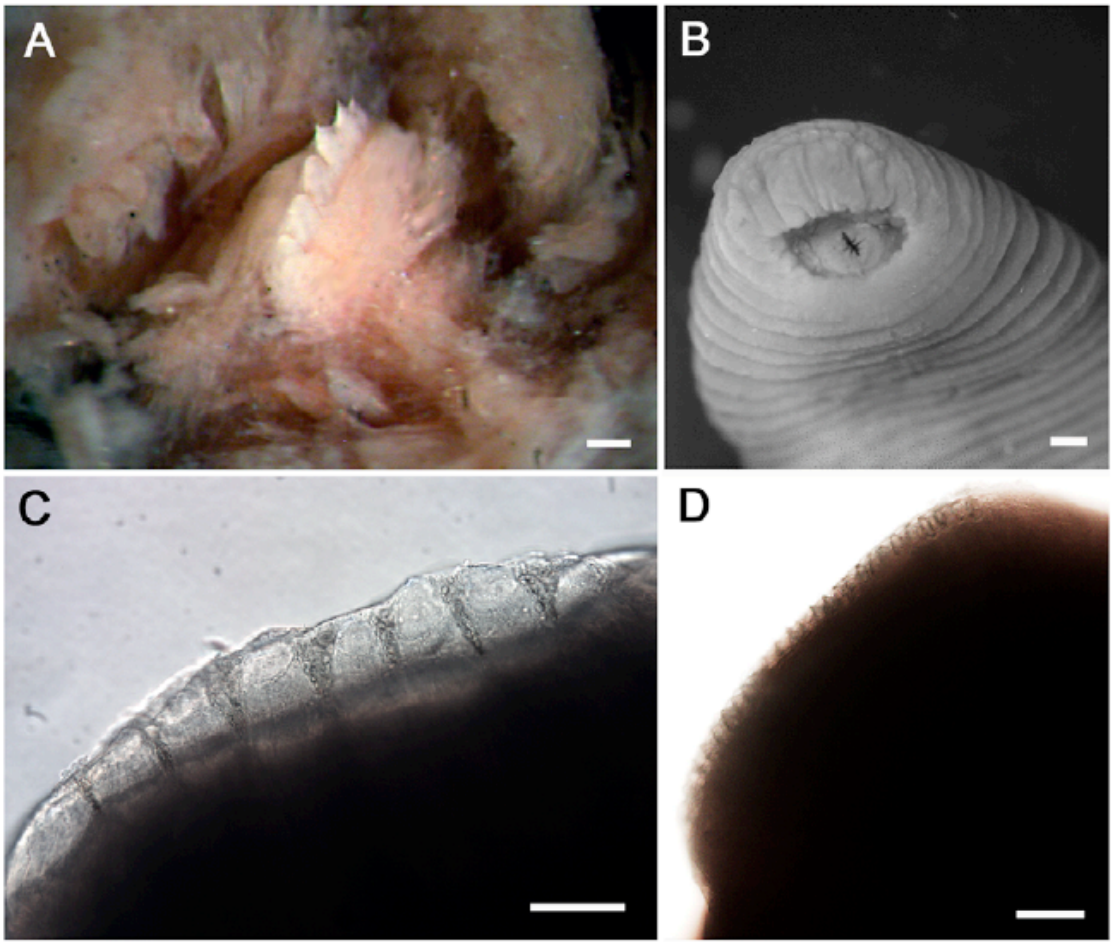
DESCRIPTION

Tyrannobdella n. gen. One dorsal monostichodont jaw armed with few, large denticular teeth. Mouth velar with single slot for jaw. Ventrolateral jaws absent. Complete somite fiveannulate. Cephalic eyespots, five pair in parabolic arc. Anus between last annulus and caudal sucker. Caudal sucker wider than posterior of body. Reproductive organs micromorphic. Feeds from mucosal surfaces of mammals. ZooBank LSID for the genus *Tyrannobdella* is urn:lsid:zoobank.org:act:43D55B49-C888-4D6BAF6F-61238EC1339B. Type species: *Tyrannobdella rex* n. sp. *Tyrannobdella rex* n. sp.

Holotype: Preserved body length 44.5 mm, maximal width 0.95 mm, fixed and stored in 90% ethanol; dissected. Collected at La Merced Chanchamayo Junın, Perú in 2007 by Dr. Renzo Arauco-Brown; deposited in the Museum of Natural History of San Marcos University, Perú (catalogue number 2841). Paratypes: Two mature specimens fixed in formalin and stored in 90% ethanol. Collected in departments San Martın and Ayacucho, Perú in 1997 by Dra. Marıa Beltran; one specimen deposited in the Enteroparasitology laboratory at the Peruvian Health Institute and another at the Museum of Natural History of San Marcos University, Perú (catalogue number 2842). One dorsal jaw armed with eight large (up to 130 mm high) teeth forming a single (i.e., monostichodont) row (Figure 22a,c). Two of eight teeth may be sub-cuticular and observable only with compound microscopy (Figure 22c). Pharynx muscular and tubular. Crop from IX to XXV, first nine cecal pairs in IX through XIX, post-ceca extend blitaterally to XXV. First and second cecal chambers subdivided into two unequal sub-ceca with the larger being posterior, otherwise one cecal pair per somite. Intestine tubular, acecate. Body muscular, uniformly pigmented brown to grey without stripes or other ornamentation after preservation.

FIGURE 22.

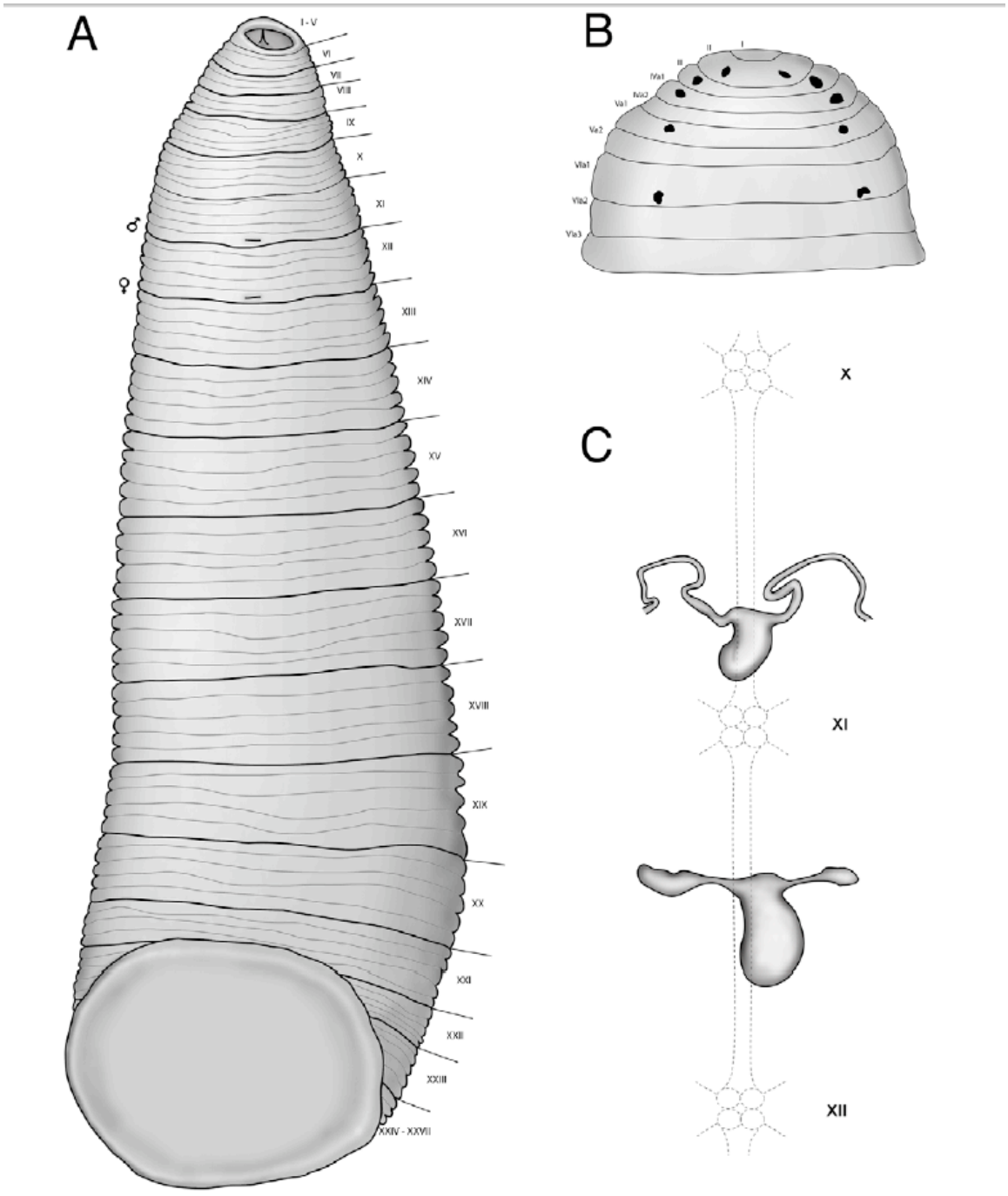
Comparative jaw morphology of *Tyrannobdella rex*. **a.** Stereomicrograph of the single dorsal jaw of *T. rex* with large teeth. Scale bar is 100 mm. **b.** *Tyrannobdella rex* anterior sucker exhibiting velar mouth and longitudinal slit through which the dorsal jaw protrudes when feeding. Scale bar is 1 mm. **c.** Compound micrograph in lateral view of eight large teeth of *T. rex*. Scale bar is 100 mm. **d.** Lateral view of jaw of *Limnatis paluda* illustrating typical size of hirudinoid teeth. Scale bar is 100 mm.


A
B
C
D

Papillae absent. Oral sucker small and velar (Figure 22b). Oral opening central and dorsoventrally oval. Posterior sucker large, wider than posterior of body (Figure. 23a). Somites I - III uniannulate, somites IV – V biannulate, somites VI - VIII triannulate, somites IX - XXIV quinqueannulate, somites XXV triannulate with annulus a1 dorsally subdivided, somite XXVI biannulate with annulus a1 dorsally subdivided, and somite XXVII unianulate with a faint dorsal furrow visible. Anus between last annulus and caudal sucker. Eyespots, five pairs on II, III, IV a1, V a1 and VI a2, forming a parabolic arc (Figure 23b). Male gonopore on XI b6, female gonopore on XII b6, gonopores separated by 1/2+4+1/2 annuli. Nephropores 17 pairs from VIII-XXV, each pair ventral on posterior margins of annulus b2 of somite. Male and female reproductive organs extremely micromorphic, same size as or smaller than ventral ganglia (Figure 23c). Penis sheath U-shaped, with initial posterior disposition and subsequent anterior procurrent portion leading to small epidydimis. Ejaculatory bulbs absent. Glandular prostate absent. Vagina present, Ushaped, no common oviduct and oviducts half the size of vagina. Vaginal cecum absent. Ovaries simple, bulbous. The ZooBank LSID for the species *T. rex* is urn:lsid:zoobank. org:act:F8C0E97B-F525-4EB3-B11B-B8CBA1CB8F5F. Etymology: *Tyrannobdella*: *tyrannos* (G.) – ‘‘tyrant’’ + *bdella* (G.) – ‘‘leech’’; rex: rex (G.) – ‘‘king’’. Remarks: No other leech species is known to have but a single armed jaw with such large teeth. The reduced number of teeth, a caudal sucker wider than the posterior of the body, and preference for feeding on mucous membranes of mammals all indicate the placement of this new taxon within the family *Praobdellidae* among the genera *Praobdella, Myxobdella, Dinobdella, Limnatis,* and *Limnobdella. Pintobdella*

FIGURE 23.

Comparative internal and external anatomy of *Tyrannobdella rex*. **a.** Whole body ventral view illustrating annulation, relative size of the caudal sucker and relative position of gonopores. **b.** Eyespot arrangement illustrated dorsally. **c.** Male and female median reproductive anatomy.


A
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XXII
XXIII
XXIV - XXVII
♂
♀
B
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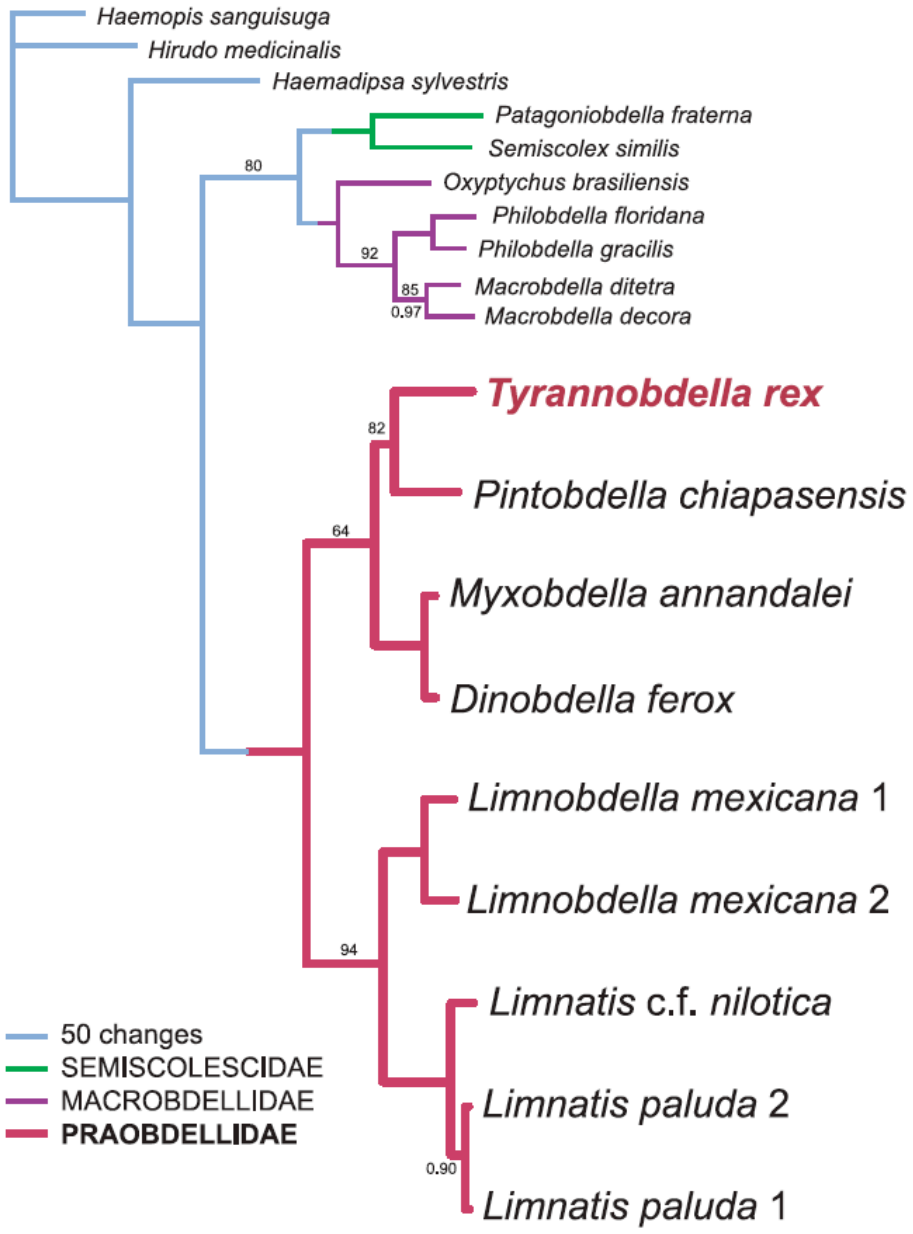
III
IVa1
IVa2
Va1
Va2
VIa1
VIa2
VIa3
C
X
XI
XII

chiapasensis (Caballero, 1957) similarly has few (six) teeth per jaw, albeit for each of three jaws. *Tyrannobdella rex* n.sp. unique in possessing only one jaw with eight large teeth (e.g., ,five times the height of those in the genus *Limnatis*). Members of the genus *Limnobdella* have two pairs of equal crop ceca in each gastric somite and an extended female reproductive structure. In comparison, *T. rex* has one pair of crop ceca per somite except in the first two chambers of the crop, which have two unequal crop ceca per somite. Overall, the relatively simple structure of the reproductive system in *T. rex* resembles that of *Limnobdella* species, but with considerable differences in size. *Tyrannobdella rex* is easily distinguished from members of the genus *Limnatis* by the possession of smooth jaws without salivary papillae, having a velar mouth without a longitudinal furrow in the upper lip, and by the simple minute reproductive structures. Also, species of *Limnatis*, like *Limnobdella*, have two equal pairs of crop ceca per somite, whereas *T. rex* has a single pair per mid-body somite. Species of *Myxobdella* and *Praobdella* are morphologically similar to *T. rex* in possessing a velar mouth, a reduced number of teeth, and micromorphic reproductive structures. Unlike *Myxobdella* and *Praobdella*, each possessing two rows of teeth (i.e. distichodont) and three jaws, *T. rex* only possesses a single row (i.e. monostichodont) and one jaw. *Myxobdella* species are distributed throughout Southeast Asia and Africa, whereas *Praobdella* species are restricted to Africa. Besides differences in jaw armature, the genus *Myxobdella* is characterized by imperfect annulation and annulation furrows of unequal depth. In contrast, *T. rex* demonstrates 15 complete fiveannulate mid-body somites with only the three most posterior somites having partially subdivided annuli. Species in the genus *Myxobdella* have gonopores separated by five or five and a half annuli, whereas *T. rex*

has gonopores separated by 1/2+4+1/2 annuli. Species in the genus *Praobdella* lack the velar mouth and have at least seven annuli between gonopores. Phylogenetic analyses The combined dataset included a total of 5256 molecular characters (18S rDNA: 2041 characters, 28S rDNA: 2189 characters, 12S mt rDNA: 367 characters, COI: 657 characters). Parsimony recovered a single tree with 2725 steps and the two runs of the Bayesian analysis had a harmonic mean of log likelihood values that averaged to–19830.23. Phylogenetic analyses recovered identical topologies regardless of method (Figure 24). A clade of hirudinoid leeches (including the genera *Limnobdella, Limnatis, Dinobdella, Myxobdella, Pintobdella* and *Tyrannobdella*), distinguished by their propensity for feeding on mammalian mucous membranes, was recovered as monophyletic with strong support (bs= 100; pp = 1.00). Sister to this was a strictly New World clade (bs= 80; pp = 1.00) comprising both the families Semiscolescidae (*Semiscolex* + *Patagoniobdella*) and Macrobdellidae (*Macrobdella* + *Philobdella* + *Oxyptychus*). The new Peruvian species, *T. rex*, was sister to the Mexican *P. chiapasensis* (bs= 82; pp = 1.00). *Dinobdella ferox* and *Myxobdella annandalei* Oka, 1917 were most closely related (bs= 100; pp = 1.00). Representatives of the Old World genus *Limnatis* formed a monophyletic clade sister to the Mexican genus *Limnobdella* (bs =94; pp = 1.00). The clades (*T. rex* + *P. chiapasensis*) and (*D. ferox* + *M. annandalei*) also form their own clade sister to (*Limnatis* spp. + *Limnobdella* spp.). Discussion Hirudinoid leeches that show a preference for mammalian mucosal surfaces all appear to have descended from a common ancestor millions of years ago. Among these, the new species *Tyrannobdella rex* is the first from South America and one with a particularly unpleasant habit of infesting humans (Beltran *et al.,* 1997). Another New World orifice-invading

FIGURE 24.

Single most parsimonious tree based on combined 18S rDNA, 28s rDNA, 12s rDNA, and COI datasets. The family Praobdellidae formed a well-supported monophyletic group of leeches that exhibits a predilection for mammalian mucosa. All groups received 100 percent bootstrap support and posterior probabilities of 1.00 except as noted on the tree. Branches are drawn proportional to amount of change.


Haemopis sanguisuga
Hirudo medicinalis
Haemadipsa sylvestris
Patagoniobdella fraterna
Semiscolex similis
80
Oxyptychus brasiliensis
Philobdella floridana
92
Philobdella gracilis
85
Macrobdella ditetra
0.97
Macrobdella decora
Tyrannobdella rex
82
Pintobdella chiapasensis
64
Myxobdella annandalei
Dinobdella ferox
Limnobdella mexicana 1
Limnobdella mexicana 2
94
Limnatis c.f. nilotica
Limnatis paluda 2
0.90
Limnatis paluda 1
50 changes
SEMISCOLESCIDAE
MACROBDELLIDAE
PRAOBDELLIDAE

leech known from southern Mexico, *P. chiapasensis*, and sister taxon to *T. rex*, has only been found to parasitize the nasal passages of tapirs (Caballero, 1957). *Limnobdella* species from central and northern Mexico are known to be pests of livestock (Caballero, 1932a). The consistency with which pain was reported by its victims may relate to the relatively enormous teeth *T. rex* has on its jaw. Most of the documented cases of leech infestation are in tropical regions. Such cases are closely related to unsafe drinking water habits and people swimming in natural sources. It is in these situations that these worms enter the rectum, vagina or upper airway and attach to the mucosa (Caballero, 1932a). A recent study revealed that the nose is the most common site of infestation (71%), followed by the hypopharynx (14%) (Raza, *et al.,* 2006). Less often, leech infestations affect the lower airways causing haemoptysis, haematemesis, severe anaemia, airway obstruction or death (Singh and Naim, 1979). While little is known of the symbiotic fauna for praobdellids, species of *Aeromonas* are known to inhabit the gastric ceca of various hirudinoid leeches (Siddall, *et al*., 2007; Laufer, *et al.,* 2008). Insofar as praobdellids have been reported to remain attached for prolonged periods (Harding and Moore, 1927), there may also be a serious risk of bacterial infection to the extent that prophylactic antibiotic treatments is indicated in all cases of orificial hirudiniasis. Several species of leech are known to invade human orifices, most notably various Old World species in the genera *Myxobdella, Praobdella*, and *Dinobdella*. Until now, the family Praobdellidae (sensu Sawyer, 1986) included only those three genera, two representatives of which were monophyletic in our analyses: *Myxobdella annandalei* and *Dinobdella ferox*. We found strong support for monophyly of that pair in a broader clade that also includes species of *Limnatis, Limnobdella, T. rex* and *P. chiapasensis*. This clade is

defined not only by our molecular evidence, but also by three morphological and behavioral synapomorphies: reduced number of teeth–less than 12 in *Myxobdella, Dinobdella, Praobdella, Tyrannobdella*, and *Pintobdella*, and less than 40 in *Limnatis* and *Limnobdella*; the caudal sucker is wider than the posterior of the body; and a preference for feeding primarily on mammalian mucous membranes. The enlarged caudal sucker seen throughout this family may well be an adaptation that mediates attachment to moist mucous membranes (Cundall, *et al.,* 1986). Only once has a praobdellid been reported feeding opportunistically on amphibians when mammals were not available (Lukin, 1976). The systematics of the family Praobdellidae (sensu Sawyer, 1986) has been plagued by ill-defined groups and by substandard type specimens being the sole representatives for some species (Harding and Moore, 1927; Cundall, *et al.,* 1986). The characteristics of the oral sucker, the color pattern, and the location of the gonopores seem to hold the most phylogenetic information among species, but organizing these species within genera has been confused. The Terrible Ferocious Leech, *Dinobdella ferox*, for example, was initially described as a species of *Whitmania*, a genus of non-bloodfeeding leeches more closely related to *Hirudo* (Phillips and Siddall, 2009). Several morphological similarities have been noted (Moore, 1958) between *Praobdella radiata* Moore, 1958 and *Myxobdella africana* Moore, 1939, while *Praobdella guineensis* Blanchard, 1896, *Praobdella buettneri* Blanchard, 1896, and *Praobdella maculata* (Moore, 1939) have each been considered potential synonyms of *D. ferox* (Moore, 1958). It has generally been agreed that these taxonomic conundra will only be resolved with the addition of fresh specimens (Cundall, *et al.,* 1986; Moore, 1958). Nonetheless, the monophyly in our phylogenetic analyses of the genera *Myxobdella, Dinobdella, Limnatis,*

Limnobdella, Tyrannobdella, and *Pintobdella* agree with the morphological and behavioral synapomorphies observed throughout the clade suggesting that the family Praobdellidae should be expanded to include them all. In turn, this settles the problem faced by Phillips and Siddall (2009), and allows Semiscolecidae Scriban and Autrum, 1934 to retain its traditional scope comprising non-bloodfeeding South American taxa and allows Macrobdellidae Richardson, 1969 to encompass the bloodfeeding genera *Macrobdella, Philobdella* and *Oxyptychus*. Representatives of the genus *Praobdella*, preferably the type species *P. buettneri*, are sorely needed to definitively establish the relationships of members of the family Praobdellidae. *Praobdella buettneri* has not been collected since its description in 1896 (from Bismarksburg, Togoland, now the Togolese Republic) along with *P. guineensis*, which shares the same type locality (Blanchard, 1896). Only external morphology was mentioned in Blanchard's (1896) description and the type specimens are long-since dried out making it difficult to relate them to newly collected material not found at the type locality. Additional species of this family that warrant scrutiny are *M. africana, Myxobdella sinanensis* Oka, 1925, *Myxobdella weberi* (Blanchard, 1897), *Myxobdella nepalica* Nesemann and Sharma, 2001*, P. maculata*, and *P. radiata*. Further collection efforts in Africa and Asia may yet successfully provide the required material, though our standard methods of attracting leeches to our exposed selves may prove awkward given their established propensity for particular anatomical feeding sites.

Author Contributions

I participated in the dissection of the first specimen of *Tyrannobdella rex*. The description of the species was done in collaboration with Anna J. Phillips. In order to clarify the phylogenetic relationships of the new species we had to include several New World's bloodfeeding leeches. We conducted fieldwork to Mexico to collect *Pintobdella chiapasensis* that turned out to be the sister species of *T. rex* as we suspected based on the morphological data. I also participated in the discussion of each one of the sections of the manuscript.

CHAPTER 5

BACTERIAL ENDOSYMBIONTS

5.1 Genes and phylogeny of *Reichenowia parasitica*, an alphaproteobacterial endosymbiont of the freshwater leech *Placobdella parasitica*: insights from genomic data.

(Adapted from: Kvist, S., Narechania, A., Oceguera-Figueroa A., Siddall, M. E. Genes and phylogeny of *Reichenowia parasitica*, an alphaproteobacterial endosymbiont of the freshwater leech *Placobdella parasitica*: insights from genomic data. (In preparation)

INTRODUCTION

Hematophagous leeches of the family Glossiphoniidae posses specialized organs related to the esophagous whose only known function is to house intracellular bacterial symbionts (Graf *et al.,* 2006; Reichenow, 1921, 1922). These structures, known as bacteriomes (stemming from the early misconception that the endosymbionts were fungi these structures are referred as mycetomes in leech literature), show high morphological plasticity across the family ranging from granular tube-like structures circumscribing the esophagous in the genus *Placobdelloides* to distinct spheroid structures in the genus *Haementeria* (Graf *et al.,* 2006). In the genus *Placobdella*, the mycetomes are arranged as a pair of blind caeca connected to the esophagous (Graf *et al.,* 2006; Siddall *et al.,* 2004). Because of the retention of these organs in hematophagous glossiphoniid leeches, it is likely that the bacterial symbionts play an important role for the hosts. It has been hypothesized that the lack of essential nutrients (such as vitamins and enzymes), brought by the leeches' restricted diet (vertebrate blood), is ameliorated by the provision of nutrients by bacterial symbionts housed in the bacteriomes (Perkins *et al.,* 2005). Commonly, obligate bacterial symbionts (primary symbionts) are housed in a distinct set of host-cells, known as bacteriocytes, and are strongly associated with these cells, to the point that they cannot invade unspecialized tissues (Moran *et al.,* 2008). Notably, bacteriomes and associated symbionts are completely absent from those leeches in Glossiphoniidae that have given up blood-feeding entirely (e.g. species of *Glossiphonia* and *Helobdella*). The importance of the bacterial symbionts is putatively evidenced by the vertical transovarial transmission of the symbionts (Siddall *et al.,* 2004).

Although symbiotic associations between bacteria and leeches are well-documented (e.g. Graf *et al*., 2006; Perkins *et al*., 2005; Siddall *et al*., 2004; Kikutchi and Fukatsu, 2002), several questions concerning the details of the symbioses still remain. In particular, neither the function of the bacterial symbionts nor their putative "symbiont syndrome" has been clearly determined. The symbiont syndrome is a collective term for a set of features that are characteristic of intracellular bacterial symbionts (Andersson and Kurland, 1998; Moran and Wernegreen, 2000). These include a reduction in genome size, A-T bias, rapid rates of mutation and infrequent gene rearrangements.

Siddall *et al*. (2004) characterized the alphaproteobacterium *Reichenowia parasitica* from the bacteriomes of its freshwater leech host, *Placobdella parasitica*. Other commensal alphaproteobacteria inhabit plants (e.g. *Rhizobium, Agrobacterium*) and those that do infect animals (e.g. *Brucella*) are exclusively parasitic (Moreno, 1998 and references therein). Among other things, bacterial plant-symbionts aid in nitrogen fixation and nodulation in the plants, allowing for more effective nutrient uptake and rapid growth (Fischer, 1994). The nitrogen fixation ability of prokaryotes has been highly studied because of its large impact on ecosystem health (Townsend *et al*., 2003; Carpenter *et al*., 1998; Howarth *et al*., 1988).

Using phylogenetic analysis, Siddall *et al*. (2004) recovered *R. parasitica* within the family Rhizobiaceae but with low resolution concerning the internal placement of the species within this group. Moreover, for Siddall *et al*. (2004), all attempts at cultivating the bacteria, using various media, were unsuccessful, suggesting that the symbiont has a reciprocally obligate relationship with the host.

Advances in sequencing technology allows for high-throughput and high-coverage sequencing of bacterial symbionts without the need to cultivate the bacteria. Here, we sought to investigate the genome of *R. parasitica* to understand the presence and function of genes, how these may affect the host and to assess the phylogenetic position of the symbiont among a wide range of bacteria, with greater taxonomic and much greater genetic coverage than that of previous phylogenetic hypotheses.

MATERIAL AND METHODS

A total of 39 specimens of *Placobdella parasitica* were collected in Algonquin Park, Ontario, Canada in July 2009. Most specimens were found attached to and feeding on hosts, specifically painted turtles (*Chrysemys picta*) and snapping turtles (*Chelydra serpentina*). A total of 72 mycetomes (36 pairs) were removed from the leeches and directly transferred to Buffer AL (Qiagen Ltd.).

From the mycetomes, total DNA from both the host and the bacterial associates was extracted using a DNeasy Blood and Tissue Kit (Qiagen Ltd.) following the manufacturer's protocol with the addition of 1 μl of ribonuclease in order to increase the DNA/RNA ratio (i.e., 260/280 ratio). Extracted DNA was subjected to whole-genome amplification using a REPLI-G UltraFast Mini Kit (Qiagen Ltd.). A GS Titanium Shotgun sequence library was prepared and massively parallel pyrosequencing of the amplicon was performed on the GS/FLX Titanium Shotgun XLR sequencing platform at SUNY Buffalo's Center for Excellence in Bioinformatics and Life Sciences.

The combined pool of host and symbiont DNA from the FLX run were jointly assembled into contigs using Newbler ver. 2.3 (454 Life Sciences).

To separate the host and symbiont DNA, contigs were subsequently used as queries against 50 selected bacterial target genomes as well as against the only sequenced leech genome, *Helobdella robusta*, Included in the 50 bacterial genomes are 40 alphaproteobacteria and 10 non-alphaproteobacteria (Beta-, Gamma-, Delta-, Epsilonproteobacteria, Firmicutes, Aquificae, Bacteroidetes, Cyanobacteria), both from endosymbiotic and free-living bacteria, and with largely varying genome sizes (Table 11). The leech genome is available at the DOE Joint Genome Institute portal website (http://genome.jgi-psf.org/Helro1/Helro1.home.html). Two local searches were performed using the BLASTn protocol applying default settings, one with a cut-off expectation value of $1E^{-5}$ and the other with $1E^{-2}$. All contigs simultaneously matching both associates were excluded from the analyses. Sequences matching leech using the $1E^{-2}$ cut-off rate were also deleted from the $1E^{-5}$ bacterial data set. This was done both to ensure that the retained sequences stemmed from bacterial, as opposed to leech DNA and as an attempt to purge the data set of putative chimeric sequences resulting from the nested assembly of both associates as all retained bacterial hits necessarily had a three orders of magnitude lower e-value for the bacterial match than for any putative leech match. Annotations of the *R. parasitica* sequences follow the GenBank annotations of the 50 bacterial genomes and inferences of molecular function follow information from UniProt and appropriate references.

Retained bacterial contigs were also subjected to gene prediction using GeneMark Ver. 2.4 (Lukashin & Borodovsky, 1998), using *Sinorhizobium meliloti* as a scaffold genome. Resulting nucleotide sequences of putative genes were translated into stop-codon-free amino acid sequences by GeneMark and these were then queried against the

Table 11. List of species used for subtractive scaffolding, orthologue recovery and phylogenetic analysis.

Species	Class	Family	GenBank RefSeq	Estimated Genome Size (Mbp)
Acetobacter pasteurianus	Alphaproteobacteria	Acetobacteraceae	NC_013209.1	3.328
Agrobacterium radiobacter	Alphaproteobacteria	Rhizobiaceae	NC_011985.1	7.26491
Agrobacterium tumefaciens	Alphaproteobacteria	Rhizobiaceae	NC_00306.2	5.66716
Agrobacterium vitis	Alphaproteobacteria	Rhizobiaceae	NC_011989.1	6.33537
Aquifex aeolicus	Aquificae	Aquificaceae	NC_000918.1	1.59079
Azorhizobium caulinodans	Alphaproteobacteria	Xanthobacteraceae	NC_009937.1	5.36977
Bacillus anthracis	Firmicutes	Bacillaceae	NC_007530.2	5.50242
Bartonella grahamii	Alphaproteobacteria	Bartonellaceae	NC_012846.1	2.36933
Bartonella henselae	Alphaproteobacteria	Bartonellaceae	NC_005956.1	1.93105
Bartonella quintana	Alphaproteobacteria	Bartonellaceae	NC_005955.1	1.58138
Bdellovibrio bacteriovorus	Deltaproteobacteria	Bdellovibrionaceae	NC_005363.1	3.8
Beijerinickia indica	Alphaproteobacteria	Beijerinickiaceae	NC_01058.1	4.41715
Bradyrhizobium japonicum	Alphaproteobacteria	Bradyrhizobiaceae	NC_004463.1	9.1
Brucella abortus	Alphaproteobacteria	Brucellaceae	NC_006932.1	3.28645
Brucella canis	Alphaproteobacteria	Brucellaceae	NC_010103.1	3.3
Brucella melitensis	Alphaproteobacteria	Brucellaceae	NC_003317.1	3.27779
Brucella suis	Alphaproteobacteria	Brucellaceae	NC_004310.3	3.3
Buchnera aphidicola	Gammaproteobacteria	Enterobacteriaceae	NC_002528.1	0.655086
Campylobacter concisus	Epsilonproteobacteria	Campylobacteraceae	NC_009802.1	2.09901
Candidatus Carsonella rudii	Gammaproteobacteria	-	NC_008512.1	0.16
Candidatus Sulcia muelleri	Bacteroidetes	-	NC_014004.1	0.24
Caulobacter crescentus	Alphaproteobacteria	Caulobacteraceae	NC_002696.2	4
Chromobacterium violaceum	Betaproteobacteria	Neisseriaceae	NC_005085.1	4.75108
Ehrlichia canis	Alphaproteobacteria	Anaplasmataceae	NC_007354.1	1.3
Ehrlichia chaffeensis	Alphaproteobacteria	Anaplasmataceae	NC_007799.1	1.17625
Erythrobacter litoralis	Alphaproteobacteria	Erythrobacteraceae	NC_007722.1	3.0524
Gluconobacter oxydans	Alphaproteobacteria	Acetobacteraceae	NC_006677.1	2.92021
Jannaschia sp.	Alphaproteobacteria	Rhodobacteraceae	NC_007802.1	4.386
Mesorhizobium loti	Alphaproteobacteria	Phyllobacteriaceae	NC_002678.1	7.5963
Methylobacterium chloromethanicum	Alphaproteobacteria	Methylobacteriaceae	NC_011757.1	6.18091
Methylobacterium extorquens	Alphaproteobacteria	Methylobacteriaceae	NC_012808.1	6.86846
Nitrobacter hamburgensis	Alphaproteobacteria	Bradyrhizobiaceae	NC_007964.1	5
Paracoccus denitrificans	Alphaproteobacteria	Rhodobacteraceae	NC_008686.1	5.23238
Prochlorococcus marinus	Cyanobacteria	Prochlorococcaceae	NC_009091.1	1.64188
Rhizobium etli	Alphaproteobacteria	Rhizobiaceae	NC_010994.1	6.44
Rhizobium leguminosarum	Alphaproteobacteria	Rhizobiaceae	NC_008380.1	7.74714
Rhodobacter capsulatus	Alphaproteobacteria	Rhodobacteraceae	NC_014034.1	3.83
Rhodobacter sphaeroides	Alphaproteobacteria	Rhodobacteraceae	NC_007494.1	4.607
Rhodopseudomonas palustris	Alphaproteobacteria	Bradyrhizobiaceae	NC_008435.1	5.5
Rickettsia conorii	Alphaproteobacteria	Rickettsiaceae	NC_003103.1	1.26876
Rickettsiaprowazekii	Alphaproteobacteria	Rickettsiaceae	NC_000963.1	1.1
Rickettsia ricketsii	Alphaproteobacteria	Rickettsiaceae	NC_009882.1	1.25771
Rickettsia typhi	Alphaproteobacteria	Rickettsiaceae	NC_006142.1	1.1115
Ruegeria pomeroyi	Alphaproteobacteria	Rhodobacteraceae	NC_003911.11	4.59
Sinorhizobium fredii	Alphaproteobacteria	Rhizobiaceae	NC_012587.1	6.89574
Sinorhizobium medicae	Alphaproteobacteria	Rhizobiaceae	NC_009636.1	6.83636
Sinorhizobium meliloti	Alphaproteobacteria	Rhizobiaceae	NC_003047.1	6.70836
Wigglesworthia glossinida	Gammaproteobacteria	Enterobacteriaceae	NC_004344.2	0.7053

50 bacterial genomes, now translated into proteomes, for orthologue recovery employing a character-based approach as implemented in OrthologID (Chiu et al., 2006). A 70% similarity cut-off rate and a lower e-value limit of $1E^{-10}$ were employed. OrthologID was also used to align the amino acid sequences using multiple sets of alignment parameters and employing the MAFFT L-INS-I algorithm (Katoh et al., 2005).

Phylogenetic analysis

The matrix of the aligned amino acid orthologues recovered by OrthologID was subjected to parsimony analysis using TNT (Goloboff et al., 2008). A New Technology search was conducted employing sectorial searching, with the tree fusing and ratcheting algorithms turned on. Trees were retrieved by a driven search using 100 initial addition sequences and requiring that the minimum length tree be found a total of 10 times. Gaps were treated as missing data. Support values for nodes were also calculated in TNT through both standard bootstrap re-sampling and partition bootstrapping (Siddall, 2010) using the *blockboot.run* script available at the TNT Wiki site (http://tnt.insectmuseum.org/index.php/Manual) for the latter. Both of the bootstrap analyses employed 100 iterations, each subjected to ten iterations of ratcheting and three rounds of tree fusing after an initial five rounds of Wagner tree building. The tree was rooted with *Chromobacterium violaceum* (betaproteobacteria).

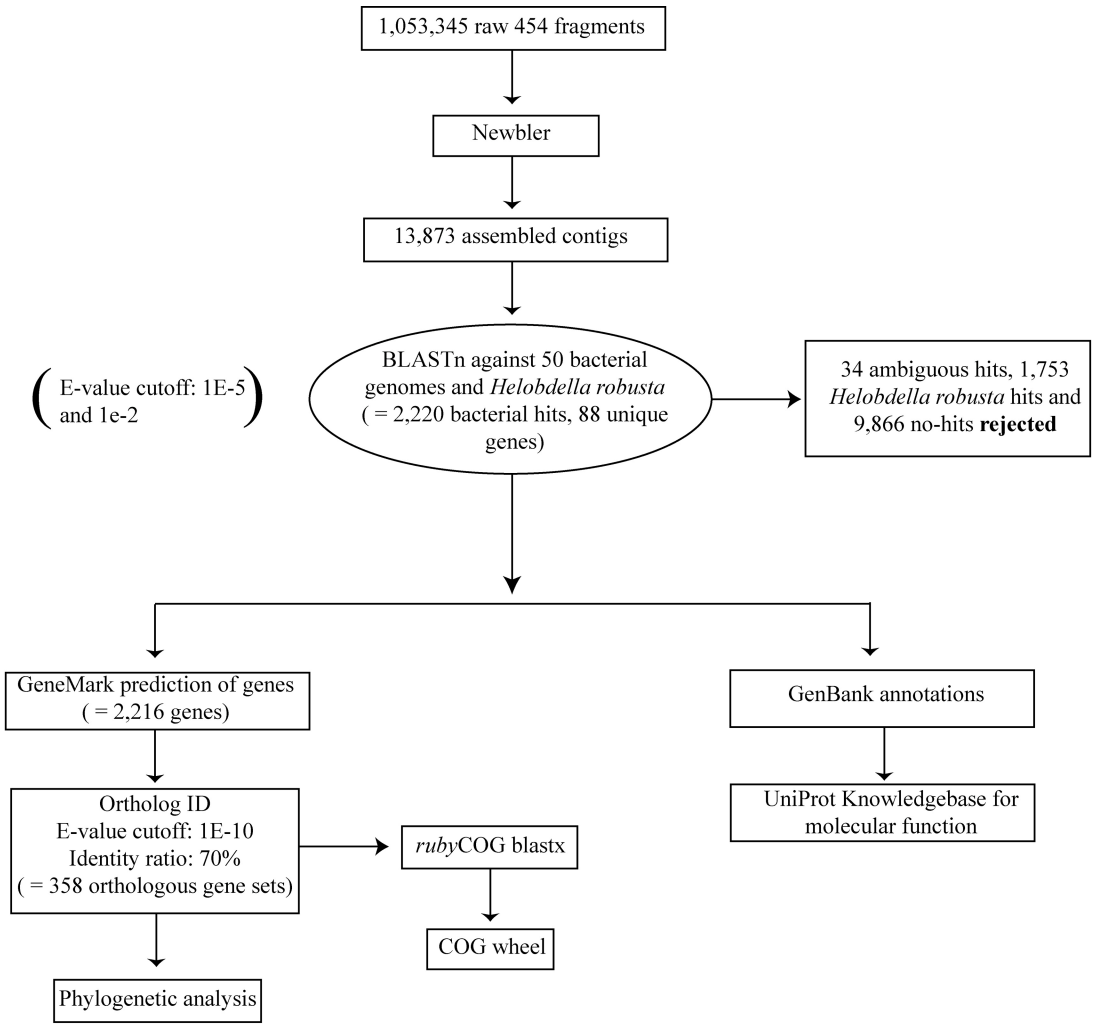
Results

Sequence analysis

The main workflow of this study is presented as a chart in Figure 18. The pyrosequencing returned 1,053,345 fragments of mixed host and symbiont DNA and these were assembled into 13,873 contigs by Newbler. The BLASTn search using a cutoff value of $1E^{-5}$ showed that 2,247 of the contigs hit bacteria alone, 1,753 contigs hit leech alone, 7 contigs hit both the 50 bacterial genomes and the leech and 9,866 contigs did not hit either of these. Among the 7 ambiguous contigs, 4 hit bacteria with very high e-values ($1E^{-37}$-$1E^{-175}$) while, at the same time, showing low e-values for the leech hit ($1E^{-6}$-$1E^{-10}$). The remaining 3 hits showed the reverse with high e-values for leech hits and low hits for bacterial hits, implying that these are not contigs shared by the leech and bacteria genomes but rather artifacts of the BLAST protocol. The second BLASTn search ($1E^{-2}$) showed that 2,611 of the contigs hit bacteria alone, 4,553 contigs hit leech alone, 207 contigs hit both bacteria and leech and 6,502 contigs hit neither bacteria nor leech. From the 2,247 leech contigs from the $1E^{-5}$ Blast, the 207 contigs matching both associates at $1E^{-2}$ were removed, as mentioned above. Descriptions of all hits with hit counts are presented in Table 1. Most of the 207 contigs that matched both associates at $1E^{-2}$ were predicted leech hits at $1E^{-5}$ and also hit bacteria with marginal e-values; only 27 hits were removed, which resulted in 2,220 retained bacterial contigs after the pruning of $1E^{-2}$-hits for leech. These contigs, in turn, pertained to 88 uniquely annotated genes among the 50 bacterial genomes and 39 of these were hit with a perfect e-vaule (0). As was expected, most of the bacterial contigs hit multiple times for the same annotated loci but with differing e-values and starting/stopping points for a total of 42,025 hits

FIGURE 25.

Main workflow followed in this study


1,053,345 raw 454 fragments
Newbler
13,873 assembled contigs
BLASTn against 50 bacterial genomes and *Helobdella robusta* (= 2,220 bacterial hits, 88 unique genes)
E-value cutoff: 1E-5 and 1e-2
34 ambiguous hits, 1,753 *Helobdella robusta* hits and 9,866 no-hits **rejected**
GeneMark prediction of genes (= 2,216 genes)
GenBank annotations
Ortholog ID
E-value cutoff: 1E-10
Identity ratio: 70%
(= 358 orthologous gene sets)
*ruby*COG blastx
UniProt Knowledgebase for molecular function
COG wheel
Phylogenetic analysis

stemming from the 2,220 *R. parasitica* contigs. The most frequently found annotations of the *R. parasitica* contigs, in terms of representation, seem to relate to two biological processes: transportation (5,308 hits out of the total 42,025) and catalytic activity (6,977 hits out of the total 42,025) of various components. Other rather highly represented biological processes among the contigs conform to DNA transcription and metabolic processes, and for several of the hit-descriptions of our contig matches there is little or no information in the Protein Knowledgebase, UniProtKB (e.g. polyhydroxyalkonate synthesis repressor; 1975 hits).

Gene prediction and phylogeny

Among the 2,220 *R. parasitica* contigs, GeneMark predicted 2,916 genes for a total of 1,785,377 basepairs. The G+C content pertaining to these was slightly elevated at 62.78%.

OrthologID identified a total of 9,135 orthologous genes among the 51 (including *R. parasitica*) genomes, 358 of which included an *R. parasitica* orthologue (3.9% of the total gene-groups). That is, among the 2,916 *R. parasitica* genes predicted, only 358 were found orthologous to any of the genes in the 50 bacterial genomes. These orthologues accounted for 181,848 aligned amino acids sites, and these were jointly submitted to TNT for phylogenetic analysis. The percentage of missing data amounted to ~55% within the total data set, due to numerous instances of gene loss, common in bacterial genomes and even more so in endosymbionts (Ochman and Moran, 2001; Casjens, 1998).

Out of the 181,848 aligned amino acid sites, 58,887 were parsimony informative. Each of the retained gene groups containing an *R. parasitica* orthologue (n=358) was

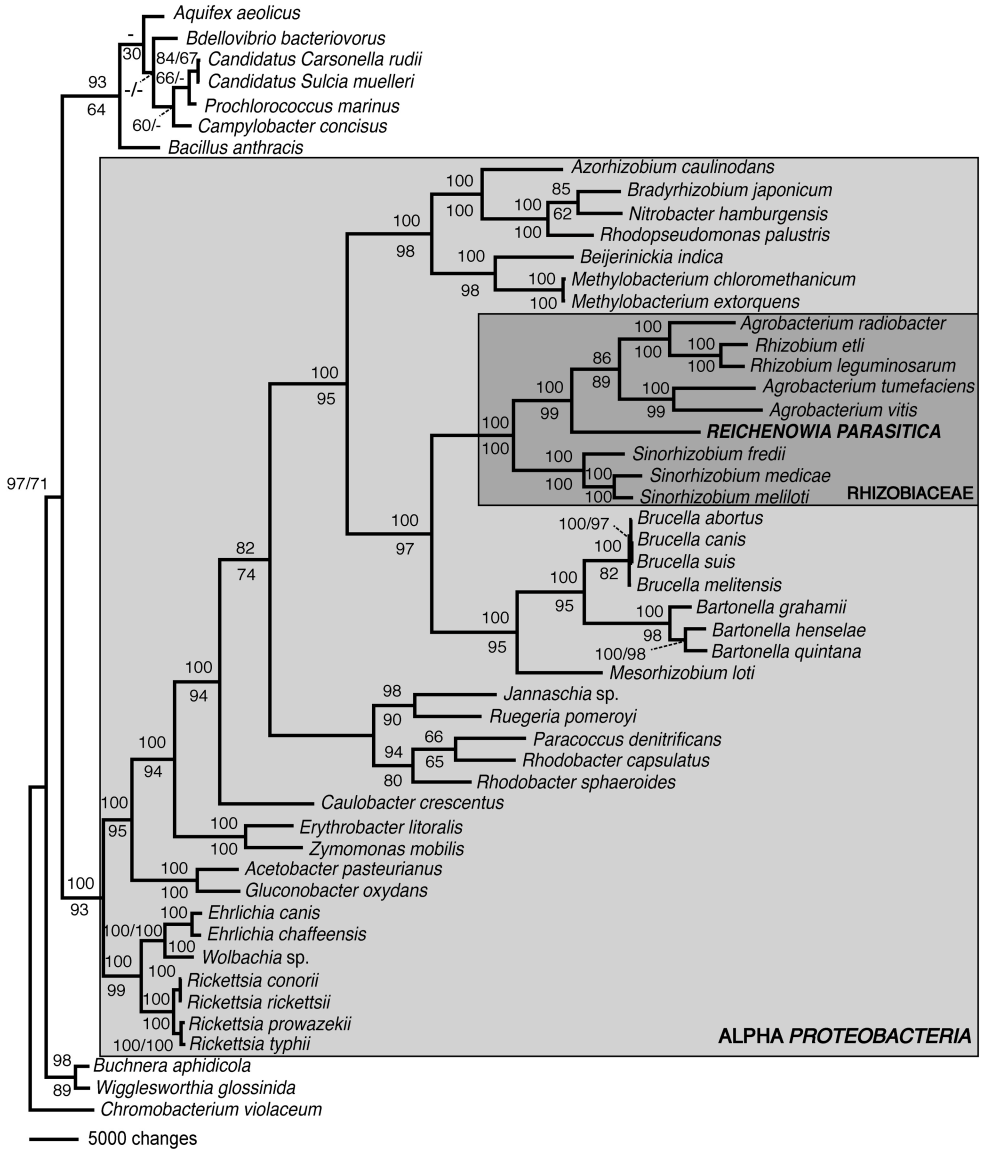
used as an independent block for the partition bootstrapping. The TNT run returned a single most parsimonious tree with a length of 408192 steps. In the tree (Figure 26), the alphaproteobacterial species, as well as each of the families contained therein was recovered as monophyletic, and most of the nodes show high support (>95% bootstrap support: bs; partitioned bootstrap support: pbs). *Reichenowia parasitica* was recovered nested within the Rhizobiaceae (100% bs; 100% pbs), as sister taxon to a monophyletic cluster consisting of *Agrobacterium* and *Rhizobium* species (86% bs; 89% pbs), which in turn placed as sister to the *Sinorhizobium* species (100%bs; 100% pbs). Rhizobiaceae (the genera mentioned above) was recovered as sister to a larger assemblage containing species of the families Brucellaceae, Bartonellaceae and Phyllobacteriaceae (100% bs; 97% pbs).

Discussion

Beyond greatly strengthening the hypothesis that *Reichenowia parasitica*, a bacterial symbiont of the fresh-water leech *Placobdella parasitica*, places among the alphaproteobacterial Rhizobiaceae, the present study also revealed several interesting features of the genomic makeup of the bacterium. Some of its BLAST-based orthologues, e.g. histidine ammonia-lyase (1100 hits among the *R. parasitica*) are fairly common across prokaryotes and eukaryotes alike (Röther et al., 2001) while others are more elusive, making them of special interest based on our, albeit limited, knowledge of the biology of the symbiont. Some of these orthologues are discussed below and a broad phylogenetic discussion is presented. In the discussion below, one must take into

FIGURE 26.

Single most parsimonious tree (length=408192, CI=0.64, RI=0.64) recovered from the phylogenetic analysis of the 358 partitions across 51 taxa. Values above the nodes are standard bootstrap re-sampling values and values below the nodes are partition bootstrap values. See text for further discussion.


Aquifex aeolicus
Bdellovibrio bacteriovorus
Candidatus Carsonella rudii
Candidatus Sulcia muelleri
Prochlorococcus marinus
Campylobacter concisus
Bacillus anthracis
Azorhizobium caulinodans
Bradyrhizobium japonicum
Nitrobacter hamburgensis
Rhodopseudomonas palustris
Beijerinickia indica
Methylobacterium chloromethanicum
Methylobacterium extorquens
Agrobacterium radiobacter
Rhizobium etli
Rhizobium leguminosarum
Agrobacterium tumefaciens
Agrobacterium vitis
REICHENOWIA PARASITICA
Sinorhizobium fredii
Sinorhizobium medicae
Sinorhizobium meliloti
RHIZOBIACEAE
Brucella abortus
Brucella canis
Brucella suis
Brucella melitensis
Bartonella grahamii
Bartonella henselae
Bartonella quintana
Mesorhizobium loti
Jannaschia sp.
Ruegeria pomeroyi
Paracoccus denitrificans
Rhodobacter capsulatus
Rhodobacter sphaeroides
Caulobacter crescentus
Erythrobacter litoralis
Zymomonas mobilis
Acetobacter pasteurianus
Gluconobacter oxydans
Ehrlichia canis
Ehrlichia chaffeensis
Wolbachia sp.
Rickettsia conorii
Rickettsia rickettsii
Rickettsia prowazekii
Rickettsia typhii
ALPHA PROTEOBACTERIA
Buchnera aphidicola
Wigglesworthia glossinida
Chromobacterium violaceum
5000 changes

consideration that the *R. parasitica* genome was only partially sequenced in the present study and thus no examination on the functional consequences of lack of genes can be performed.

Phylogeny

Based on both parsimony and likelihood algorithms, Siddall *et al*. (2004) performed a phylogenetic analysis of three *Reichenowia* species using 16S and 23S ribosomal RNA. That study, in unison with the present study recovered *R. parasitica* among the Rhizobiaceae as sister to a group including the *Rhizobium* and *Agrobacterium* species. Later, Perkins *et al*. (2005) recovered the same three species as sister to a group containing *Sinorhizobium meliloti* (with an unresolved position), *Brucella melitensis* and *Brucella henselae*. In the analysis performed by Perkins *et al*. (2005), the *Agrobacterium* species and the *Rhizobium* species were recovered as consecutive sister-groups to this larger group. From a biological standpoint, and because contemporary bacterial taxonomy and phylogenetics focuses largely on 16S and 23S (e.g. Bouchon *et al.,* 1998; Burnett and McKenzie, 1997; Manz *et al.,* 1996), it is comforting to know that concordant phylogenetic signal is present in 16S or 23S alone as in the 358 genes used here.

The well-supported plant-symbiont affiliation of *R. parasitica* raises some interesting questions concerning the evolutionary history of the organism. Because of the basal position of the *Sinorhizobium* species in the phylogenetic hypothesis presented here, the ancestral life history trait of the Rhizobiaceae seems to be plant parasitism, with

R. parasitica showing a host switch from plant to leech. This is further supported by the finding of several plant-associated genes, such as phosphatase, in the genome of *R. parasitica*. Out of the 358 orthologues detected among the *R. parasitica* contigs, several were private to *Rhizobium, Agrobacterium, Sinorhizobium* and *Reichenowia*, indicating common ancestry among these genera. However, it is also possible that the ancestor of the *R. parasitica* was free-living by virtue of the current rod-shape of the bacterium, common in several other free-living taxa (van Brussel *et al.,* 1977), and it is possible that the same free-living ancestor also evolved into the plant-parasitic bacteria that we see today. A more taxonomically rich study of the alphaproteobacteria as a whole will likely shed light on the ancestral life-history strategy of the Rhizobiaceae.

The phylogenetic hypothesis enables some inferences regarding the genome size of *R. parasitica*. Among other things, an understanding of the genome size of this symbiont may aid in guiding future sequencing efforts of its entire genome. The size of the chromosomal genomes of the *Agrobacetrium* and *Rhizobium* species used here range between 5.66-7.42 megabasepairs (Mbp), whereas the *Sinorhizobium* species (basal to *Reichenowia*) posses chromosomal genomes in the range of 6.71-6.89 Mbp. By extension, it is probable that the genome size of *Reichenowia parasitica* is somewhere in the vicinity of that of its closest relatives, between 5.66-7.42 Mbp. However, in an early evaluation of the pyrosequencing data, we performed a genome-size calculation based on statistical inferences. We examined the trend in average (not total) contig length (then assembled using EGassembler; Masoudi-Nejad et al., 2006) for 16.5%, 33%, 66% and 100% of the total pyrosequencing fragment pool with the asymptotic end-point being predictive of full-genome size using Newton-Rhapson estimation on a non-linear general

logistic equation [GENOME*(1-(1/e$^{(obs*CONSTANT)}$))]. The resulting predicted genome size of *R. parasitica* was 2.84 Mbp (Fig. 5). This value corresponds better to the reduced genomes evident in several other animal endosymbionts and would imply that *R. parasitica* displays at least one feature of the symbiont syndrome. To this end, we can only speculate as to the genome size of the symbiont, the entire genome of *R. parasitica* would have to be sequenced in order to securely establish it.

Sequencing the entire genome should be the focus of future studies as it would also allow for insights into the full genomic makeup of the symbiont, including the consequences of the absence of genes, and the potential finding of more genes related to the endosymbiotic lifestyle of this non-parasitic, animal-inhabiting alphaproteobacterium.

5.2 Closely related hosts leeches associated with distantly related proteobacteria.

Bacterial symbionts

Symbiotic relationships between bacteria and invertebrates are common for those animals presenting a restricted diet that is poor in several vitamins and some essential amino acids, like blood or sap. These kinds of relationships have been demonstrated several times, particularly between proteobacteria and invertebrates. Well known examples have been studied, such as tsetse flies (*Glossina* spp) and *Wigglesworthia glossinida* (Askoy, 1995) and aphids and *Buchnera* spp. (Clark *et al.,* 2000). Showing the importance of this kind of symbiotic association is the fact that bacteria are vertically transmitted from parents to offspring. Furthermore, attempts to eliminate bacteria from such associations affects host fitness (Hill and Campbell, 1973). Straight symbiotic associations are supposed to be ideal systems to study several biological problems; one of them is coevolution (analysis of the evolutionary relationships of two or more groups of organisms with a closed ecological interaction). Coevolution can be studied principally from 2 points of view: coadaptation (including arms-race scenarios) and cospeciation. These 2 approximations differ, among other things, in the scale of the study; coadaptation normally involves the reciprocal adaptations of a couple of species on an ecological scale (few generations), whereas cospeciation analyze the evolution of associated lineages over a large scale of time and space, normally including more than 3 species in each lineage. Even coadaptation can occur without cospeciation, some degree of coadaptation is expected in cases of cospeciation. The study of cospeciation is based on a phylogenetic hypothesis of the interacting groups, in this sense, is strongly based in our ability to generate phylogenetic hypotheses. If cospeciation is the unique process involved in the evolution of a pair of associated lineages, the phylogeny of one of the lineages would be

the exact mirror of the other and vice versa (Co-phylogeny). This is rarely the case because other biological processes can occur like speciation of one but not the other of the interacting species, extinction of one of the lineages or change of partner (called host switching). Co-phylogenetic studies are a special case of the broader problem referred to the analysis of historical associations. The parallels between host/parasite, biota and their areas of distribution (Biogeography) and tree genes/species trees, all those examples of historical associations, have been intrigued biologists for a long time (Page, 2003). In general, the reconstruction of the historical association of these three levels of organization faces the same kind of problems and could be analyzed using common methods.

Proboscic bearing leech species (Annelida: Glossiphoniidae), such as the members of the genus *Haementeria* and *Placobdella* present a restricted diet consisting of blood of amphibians, reptiles and in some cases, even mammals. Contrasting with the similarity between their ecological functions, species of *Haementeria* and *Placobdella* inhabit completely different geographical areas. *Placobdella* spp. are specially abundant in Canada and USA, with one species from Europe and only three species in Mexico. Displaying the opposite biogeographical pattern, species of *Haementeria* are abundant in South America and reach their Northern distribution in Mexico. The geographical distribution of species of *Placobdella* and *Haementeria* overlaps in the Mesa Central of Mexico and in the low lands of Veracruz, Oaxaca and Chiapas were they can coexist even in the same pond. Contrary to the widely accepted classification of leeches elaborated by Sawyer (1986) that considers *Placobdella* and *Haementeria* to belong to different subfamilies (Glossiphoniinae and Haementeriinae respectively), recent

phylogenetic hypotheses have shown that the two genera are actually closely related (Siddall *et al,* 2005). The most parsimonious interpretation of the evolution of feeding preferences within Glossiphoniidae suggests that the last common ancestor of *Placobdella* and *Haementeria* was a bloodfeeder (Siddall *et al.,* 2005). This means that both groups inherit the bloodfeeding preference from a common ancestor and is not an independent acquisition. Based on this, it should be expected that, in addition of displaying the same feeding preferences of their common ancestor, species of *Haementeria* and *Placobdella* also would inherit the same kind of bacterial endosymbionts, especially those that are intracellular and live in specialized organs such as bacteriomes. Supporting this idea is the fact that *Placobdella* and *Haementeria* species have bacteriomes that connect into the esophagus. The morphology of such bacteriomes are completely different (Graf *et al*., 2006; Siddall *et al*., 2004, Oceguera-Figueroa in review) but based on the arguments presented before, it might be suspected that both groups of leeches would be associated with a single clade of bacteria, inherited from their last common ancestor.

Perkins *et al.* (2005) demonstrated, based on a phylogenetyc analysis (using 16S rRNA) of the symbiotic bacteria, that *Placobdella* and *Haementeria* species are associated with members of different sections of Proteobacteria: Alphaproteobacteria and Gammaproteobacteria respectively.

In this study, we present an expanded dataset of 16S rRNA of symbiotic bacteria of both, *Haementeria* and *Placobdella* species from several localities of the New World, including geographical areas were members of both groups coexist.

MATERIALS AND METHODS

Specimens of *Haementeria depressa* were collected in Uruguay. Specimens of *Haementeria lopezi, Haementeria officinalis* and *Haementeria acuecueyetzin* were collected in Mexico (See Oceguera-Figueroa, in review for details). Specimens of *Placobdella costata* were collected in Spain, *Placobdella sophieae* from Idaho, *Placobdella montifera* from Washington, *Placobdella parasitica* and *Placobdella papillifera* from New York, *Placobdella kwetlumye* from Oregon. All these localities in the USA. *Placobdella lamothei* and *Placobdella ringueleti* were collected in Mexico. In total, 8 new samples of *Haementeria* spp. symbionts and 12 new samples of *Placobdella* spp. symbionts were included in the analyses.

To perform symbiont DNA isolation, bacteriomes of leeches were dissected. Leeches were narcotized through gradual addition of ethanol. Dissections were accomplished under a Nikon SMZ-U stereomicroscope. For each *Haementeria*, the 4 spheroideal mycetomes were extracted and placed in the same tube. For each *Placobdella*, the two sac-lice bacteriomes were extracted and placed in the same tube. Total DNA extraction (including leech and bacterial DNA) was extracted with the DNeasy Extraction kit (QIAGEN, Valencia, Calif.), following the protocol for animal tissues. Bacterial rRNA sequences were amplified using bacterial universal primers 4 BSF8 with BSR1541 following Perkins *et al*., (2005) protocols of amplification and sequencing.

Bacterial sequences of representative sections of Proteobacteria obtained from GenBank, together with the sequences generated in this study were aligned using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004). Alignments were obtained applying the default settings for gap opening and extension penalties. *Clostridium tetani* was used as an outgroup to root the tree.

PHYLOGENETIC ANALYSES

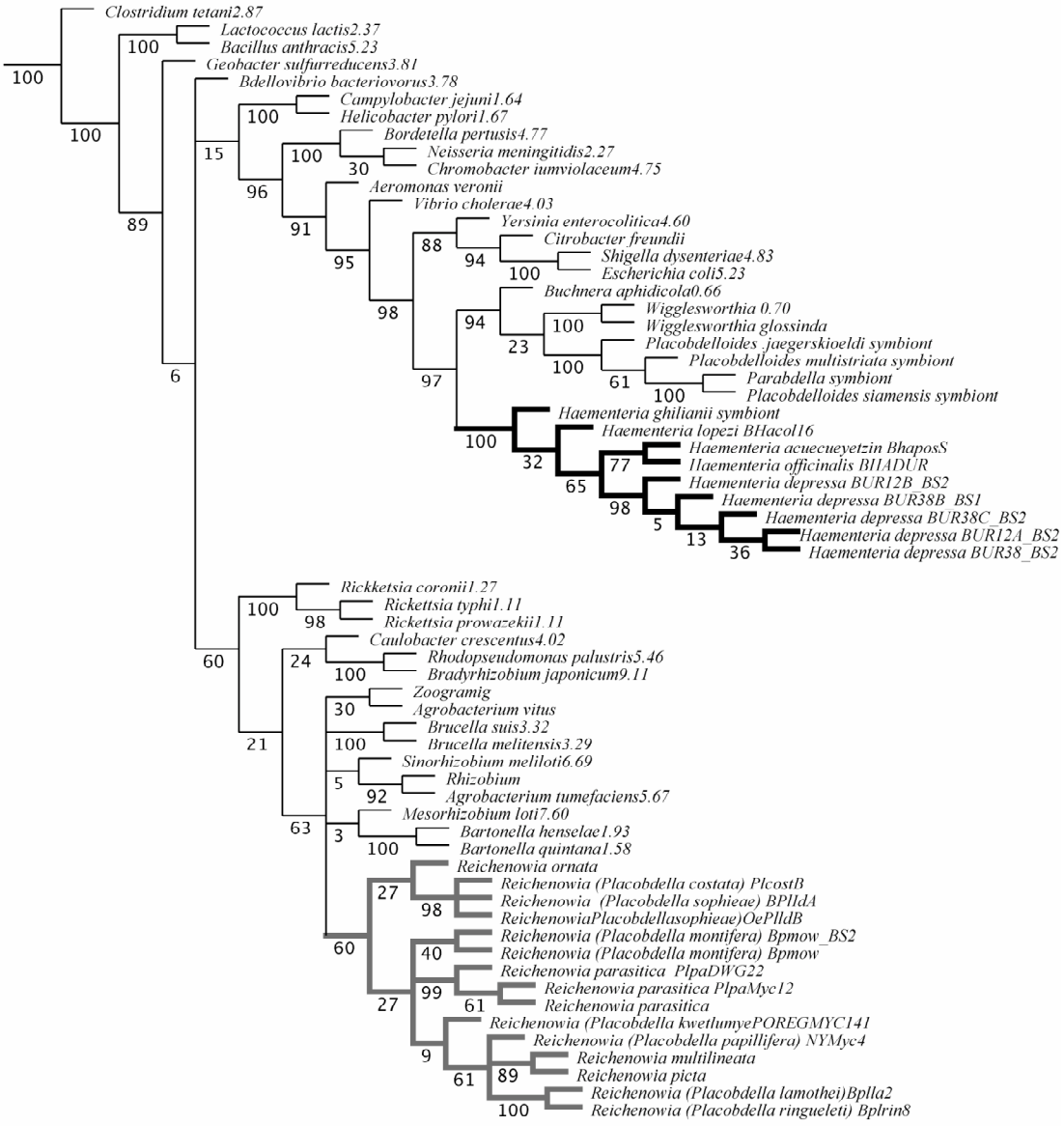
Maximum Parsimony (MP) analyses of the data were performed using New Technology Search with the Ratchet and tree fusing algorithms in TNT ver 1.1 (Goloboff *et al*., 2008) performing 100 repetitions. All characters were equally weighted and non-additive. Gaps were treated as missing data. Bootstrap values were obtained in TNT with 1000 pseudoreplicates, using random taxon addition and TBR branch swapping.

RESULTS

Maximum Parsimony analyses of the dataset resulted in 4 equally parsimonious trees of 3897 steps. The strict consensus of the tree is shown in Figure 27. In this tree, the bacterial symbionts of both groups of leeches studied here were found forming two monophyletic and non-closely related groups. The clade of symbiotic bacteria of *Haementeria* was found well nested within the Gammaproteobacteria, whereas the symbionts of *Placobdella* were found within Alphaproteobacteria. *Haementeria* symbionts were found forming a well-supported clade (Bootstrap = 100), however, *Reichenowia* (*Placobdella* spp symbionts) are just moderately supported (Bootstrap = 60). In Both cases, the node support for the internal nodes is low, with only few exceptions, particularly when more than one bacterial sample was extracted from different leeches of the same species.

FIGURE 27

Phylogenetic relationships of leech bacterial symbionts


Clostridium tetani2.87
Lactococcus lactis2.37
Bacillus anthracis5.23
Geobacter sulfurreducens3.81
Bdellovibrio bacteriovorus3.78
Campylobacter jejuni1.64
Helicobacter pylori1.67
Bordetella pertusis4.77
Neisseria meningitidis2.27
Chromobacter iumviolaceum4.75
Aeromonas veronii
Vibrio cholerae4.03
Yersinia enterocolitica4.60
Citrobacter freundii
Shigella dysenteriae4.83
Escherichia coli5.23
Buchnera aphidicola0.66
Wigglesworthia 0.70
Wigglesworthia glossinda
Placobdelloides jaegerskioeldi symbiont
Placobdelloides multistriata symbiont
Parabdella symbiont
Placobdelloides siamensis symbiont
Haementeria ghilianii symbiont
Haementeria lopezi BHacol16
Haementeria acuecueyetzin BhaposS
Haementeria officinalis BIIADUR
Haementeria depressa BUR12B_BS2
Haementeria depressa BUR38B_BS1
Haementeria depressa BUR38C_BS2
Haementeria depressa BUR12A_BS2
Haementeria depressa BUR38_BS2
Rickketsia coronii1.27
Rickettsia typhi1.11
Rickettsia prowazekii1.11
Caulobacter crescentus4.02
Rhodopseudomonas palustris5.46
Bradyrhizobium japonicum9.11
Zoogramig
Agrobacterium vitus
Brucella suis3.32
Brucella melitensis3.29
Sinorhizobium meliloti6.69
Rhizobium
Agrobacterium tumefaciens5.67
Mesorhizobium loti7.60
Bartonella henselae1.93
Bartonella quintana1.58
Reichenowia ornata
Reichenowia (Placobdella costata) PlcostB
Reichenowia (Placobdella sophieae) BPlldA
ReichenowiaPlacobdellasophieae)OePlldB
Reichenowia (Placobdella montifera) Bpmow_BS2
Reichenowia (Placobdella montifera) Bpmow
Reichenowia parasitica PlpaDWG22
Reichenowia parasitica PlpaMyc12
Reichenowia parasitica
Reichenowia (Placobdella kwetlumyePOREGMYC141
Reichenowia (Placobdella papillifera) NYMyc4
Reichenowia multilineata
Reichenowia picta
Reichenowia (Placobdella lamothei)Bplla2
Reichenowia (Placobdella ringueleti) Bplrin8
Gammaproteobacteria associated with Haementeria spp.
Alphaproteobacteria associated with Placobdella spp.

DISCUSSION

Based on the results presented here, it is clear that, despite being closely related, species of *Haementeria* and *Placobdella* are associated with completely different kinds of bacteria. With the inclusion of several bacterial symbionts from *Haementeria* and *Placobdella*, we support the same pattern obtained by Perkins *et al*., (2005) which was obtained, admittedly, with a limited sample size, particularly of *Haementeria* (n=1)

The fact that symbionts from all *Haementeria* species were found forming a monophyletic group, within the Gammaproteobacteria (A group which comprises several insect symbionts) suggest a strong association between this group of leeches and their Gammaproteobacteria. This is more interesting if we consider that this analysis included samples that were collected in completely different areas. For example; *Haementeria depressa* was collected in Uruguay and their bacterial symbionts are closely related with those of any of Mexican species of *Haementeria*. The same pattern but in the opposite geographical direction is observed in the bacterial symbionts from *Placobdella*.

The fact that *Placobdella lamothei* and *Placobdella ringueleti* share the same geographical distribution with *Haementeria officinalis* and *Haementeria acuecueyetzin* respectively, provides an interesting natural experiment in which the two groups of leeches (*Placobdella* and *Haementeria*) can, at least in terms of their overlapping geographical distribution, interchange their bacterial symbionts. However, even the ecological conditions are appropriate for this, our data supports the hypothesis of vertical transmission the bacteria in their respective group of leeches.

Unfortunately, the low resolution of the internal topology (in terms low values of bootstrap) within *Reichenowia* and symbionts from *Haementeria* do not allow for the analyses of cophylogenetic patterns. However, all the evidence suggest that, with the analyses of appropriate molecular markers we should be able to obtain the same phylogenetic trees of both members of this symbiotic association.

GENERAL CONCLUSIONS

SPECIES DESCRIPTIONS

In total, 8 new species of freshwater leeches are described based on morphology, including internal and external characteristics. All of them are known from the New World exclusively.

Rhynchobdellida

Glossiphoniidae:

Haementeria acuecueyetzin Veracruz, Mexico.

Helobdella virginiae Veracruz, Mexico.

Placobdella kwetlumye Washington, US.

Placobdella lamothei Estado de Mexico, Mexico

Placobdella ringuelti Chiapas, Mexico.

Placobdella sophieae Washington, US

Arhynchobdellida

Praobdellidae

Tyrannobdella rex Peru.

Macrobdellidae

Oxyptychus bora Peru

One potentially new species of *Helobdella* were detected in Mexico based on COI (barcoding) sequences. The morphological traits of these leeches do not allow their differentiation from *Helobdella modesta*. Based on molecular characteristic attributes, the

reestablishment of *Helobdella socimulcensis* and *Helobdella octatestisaca* is recommended.

PHYLOGENY BASED NOMENCLATURAL CHANGES

Based on phylogenies, some nomenclatural changes are proposed and some groups are redefined.

Haementeria brasilensis Cordero, 1937 was member of *Placobdella*, but it was found forming a monophyletic group with the South Amerincan *Haementeria*. For this reason it was transferred to *Haementeria*.

Haementeria gracilis Cordero, 1941 is confirmed as a junior synonym of *Haementeria depressa* Blanchard,1849.

Erpobdella annulata Moore 1922 is reestablished. It was considered a subspecies of *Erpobdella punctata* but its position on the phylogeny, guarantees the use of *E. annulata*.

Motobdella montezuma Davis, Singhal and Blinn, 1985 is transfered to *Erpobdella* based on its position on the phylogenetic tree. In addition, the genus *Motobdella* is now considered just annother synonym of *Erpobdella*.

Orobdella octonaria Oka, 1895 is a member of the family Gastrostomobdellida. The position of this groups has been debated in leech literature since it description. The results presented here, unambyguosly places *O. octanaria* within the suborden Erpobdelliformes.

ENDOSYMBIOTIC BACTERIA

The analysis of the sequences generated through the use of Pyrosequencing technology resulted in a better understanding of the phylogenetic placement of *Reichenowia parasitica* within the Alphaproteobacteria since for the firs time we use a multi locus approach to solve this problem, which was studied before using only 16s rRNA sequences. The phylogenetic position of *Reichenowia parasitica* within a group of bacteria known for their ability to fixate nitrogen in plants is intriguing and possibly, Reichenowia parasitica would be displaying this function in *Placobdella parasitica*. Finally, we provide additional information to characterize the joint evolution of *Haementeria* and *Placobdella* with their respective Gamma and Proteobacteria endosyombionts since both monophyletic groups of leeches were found associated with monophyletic groups of bacteria.

DISCLAIMER

New species and new taxonomic names presented in this dissertation are not intended to formally establish new names

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