A PHYLOGENETIC REVISION OF THE MEDICINAL LEECHES OF THE WORLD (HIRUDINIDAE, MACROBDELLIDAE, PRAOBDELLIDAE)

By

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ABSTRACT

A PHYLOGENETIC REVISION OF THE MEDICINAL LEECHES OF THE WORLD (HIRUDINIDAE, MACROBDELLIDAE, PRAOBDELLIDAE)

By Anna Jane Phillips

Advisor: Dr. Mark E. Siddall

The term "medicinal leech" refers to more species than just *Hirudo medicinalis*, the preferred species for bloodletting in 19th century Europe. In the past, freshwater, leeches with similar morphological characterstics to *H. medicinalis* have been divided into two families: the bloodfeeding Hirudinidae and the non-bloodfeeding Haemopidae. With a broader taxon sampling than in previous analyses, an analysis of multiple nuclear (18S rDNA and 28S rDNA) and mitochondrial (12S rDNA and cytochrome *c* oxidase I) gene fragments found Hirudinidae not to be monophyletic, instead placing in two independently originated lineages separated by the two families of terrestrial leeches, Haemadipsidae and Xerobdellidae. Members of Haemopidae were scattered among members of Hirudinidae of both lineages, thus rendering Haemopidae polyphyletic. The lineage containing *H. medicinalis* retained the name Hirudinidae, while the other lineage was shown to consist of three families: Macrobdellidae (North and South American bloodfeeders), Semiscolecidae (South American non-bloodfeeders), and Praobdellidae (a biogeographically diverse clade of species that feed primarily from mammalian mucous membranes). With the familial relationships within these two lineages established, attention was given to revising the intra-familial and generic relationships. Two new genera and two new species resulted from these investigations: *Tyrannobdella rex* n. gen. n. sp., a leech found feeding inside the nasal passages of humans placed within Praobdellidae, *Mesobdella lineata* was re-described as *Parapraobdella lineata* n. gen. within Praobdellidae, and *Hirudinaria bpling*

n. sp. placed within the Hirudinidae. Endosymbiotic bacteria from the digestive tracts of members of the Hirudinidae, Macrobdellidae, and Praobdellidae were detected and determined to be *Aeromonas* species as well as an unculturable Bacteroidetes. The *Aeromonas* isolates did not show a predictable association based on the phylogeny of the leech hosts or geography, while the Bacteroidetes isolates did show a correlation with leech taxonomy. An analysis with the most thorough taxon sampling to date of the families of Hirudiniformes and Erpobdelliformes was performed. Gastrostomobdellidae, a group of macrophagous leeches hypothesized to be similar to Erpobdelliformes or Hirudiniformes, was strongly supported within Erpobdelliformes. The establishment of the relationships provides a basis for further systematic study, as well as a investigations into the evolution of these charismatic worms.

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CHAPTER 1

Historical Overview

Leeches are most well known for their utility in bloodletting in both past and modern medicine. Beyond their medical relevance, the subclass Hirudinida provides a diversity of morphological structures, habitats, and feeding preferences for study in an evolutionary context. With an upper estimate of 1,000 described species within the subclass Hirudinea, the family Hirudinidae has traditionally contained the European medicinal leech, *Hirudo medicinalis* Linneaus, 1758*,* and its closest allies, comprising less than 200 species in all. The external and internal morphology has been characterized for many of these species, but numerous species remain with vague descriptions and few specimens in good condition. The advent of DNA sequencing revolutionized leech systematics and altered many of the major relationships of the subclass established by over 100 years of morphological study. With a phylogeny of leeches in place, more complicated questions about gene evolution can be addressed. For example, with the large amounts of data generated using Expressed Sequence Tag libraries and pyrosequencing technologies we are gaining a better understanding of leech evolution (Min *et al.,* 2010; Siddall *et al*., 2011).

Taxonomy based on morphology

Prior to the late 19th century, descriptive work of leeches examined only external characteristics such as differences in annulation patterns, locations of eyespots, nephropores, and gonopores, the formation of the oral and caudal sucker, and presence of salivary papillae on the surface of the jaws (Whitman, 1884, 1886, 1889; Blanchard, 1893, 1896a, 1896b). Considering only external characters, the variation of those structures was easily organized into a single family, Hirudinidae (Blanchard, 1893; Richardson, 1969). Whitman (1889) and Blanchard (1893) both placed taxonomic value on these external characters, but Blanchard further divided

Hirudinidae into two groups depending on whether the teeth were arranged in a single row (monostichodont) or two rows (distichodont). Just before the turn of the 20th century internal morphological characteristics accessed through dissections were included in descriptive work of leeches with the most taxonomically informative structures being the male and female reproductive systems and those of the pharynx (Moore, 1898, 1901a; Richardson, 1969). As dissections became routine in the taxonomic study of leeches, the documentation of morphological diversity increased, as did the need to classify that diversity in more than a single family. Hirudinid taxonomy entered a period of extensive cataloguing of these previously overlooked characters and categorizing the species into various existing taxa and creating new taxa as were warranted (Harding and Moore, 1927; Moore, 1939; Sciacchitano, 1952).

J. Percy Moore of the National Academy of Sciences in Philadelphia and active in leech systematics from 1898-1958 and he demonstrated a level of detail in descriptive work previously unprecedented in leech taxonomy. His efforts provided an excellent example to other researchers in using both internal and external morphology for taxonomic comparisons (Moore, 1901a, 1939, 1958). Moore's expertise began with the North American leech fauna and quickly expanded, but this handful of North American species always served as his morphological reference point. Moore frequently used the terms "haemopisine" and "*hirudo*-like" to describe structures similar to the North American macrophagous Haemopidae and the European *Hirudo* species, respectively (Richardson, 1969). As the scope of taxa he studied expanded to include leeches from other geographic regions, he continued to use these vague terms and, as a result, their meanings became increasingly broad and confusing. As these terms were applied in different ways to ever-more taxa, their systematic value decreased until they were as uninformative as Blanchard's jaw dentition characters that do not differentiate beyond a single family. This is not

to detract from the numerous other characters he described fully and in detail that were taxonomically informative, often as a part of characterizations of taxa that were originally much less detailed. Several of Moore's publications (Harding and Moore, 1927; Moore, 1939, 1958) are still some of the most detailed morphological accounts of well-studied species, such as *Macrobdella decora* (Moore, 1901a).

Many other researchers have also contributed to leech taxonomic literature, often focusing on the leech fauna of a particular geographic region. Oka and Whitman documented much of the diversity of hirudinids in Asia, mostly from Japan (Oka, 1895, 1925; Whitman, 1886). Caballero was principal in characterizing taxonomy of the Mexican hirudinids (Caballero, 1932, 1933, 1934, 1941, 1957), a group endemic to the region for which the taxonomy has since remained relatively unchanged. Ringuelet published extensively on the leech fauna of South America (Ringuelet, 1945, 1976a, 1976b, 1985) and, with respect to hirudinids, placed high systematic value on the location of the gonopores and other distinctive reproductive structures.

Even though the methods of leech taxonomy had progressed to include dissections and detailed examinations of internal as well as external morphological characters, workers did not uniformly adopt these practices. Sciacchitano was active in Italy for almost 30 years (1935-1963) describing African leeches from European museum collections (Sciacchitano, 1935, 1936, 1952, 1959). Much of his early descriptions were often dependent upon external characters known to be intraspecifically variable or taxonomically uninformative in preserved specimens, such as color pattern or size (Moore, 1958). Later, he did incorporate internal morphology into his descriptions but during his career he described at least eight genera and almost 60 species from preserved specimens with dubious characteristics. This number was excessive to other

experienced leech researchers at the time who considered the variation described in Sciacchitano's work as uninformative for delimiting species or higher taxa (Meyer, 1951). Many of these species were declared *species inquerindae* (Soós, 1969) as the type specimens were in poor condition or were the only representatives of their species studied.

The application of systematic principles

Richardson (1969) transformed hirudinid taxonomy with a revision of Hirudinidae that took documentation of leech biodiversity from a strictly taxonomic process based on cataloguing variation to a systematic process based on comparative morphology. Richardson's (1969) revision was just on the tails of the New Synthesis, a movement in taxonomy that emphasized capturing the maximum amount of evolutionary information within taxonomic classifications that do not necessarily match information contained within phylogenies (Schuh, 2000). In this study, Richardson examined specimens representing every genus of Hirudinidae. The detailed work by J. P. Moore and others was enough to apply "systematic principles. . . [resulting from] an organogeny derived from comparative morphology" (Richardson, 1969). He attempted to standardize the terminology used to describe morphological structures in leeches (e.g., male atrium vs. prostate gland) and refined the description of characters formerly considered "haemopisine" and "*hirudo*-like" by Moore. In his revision (1969) of Hirudinidae and related taxa, Richardson described characters that are derived from or change according to their "function or functional efficiency" and comparative morphology, such as pharynx characters and internal reproductive structures. He discussed the progression of muscularization in the male reproductive structures that was supported by embryology and would explain variation between species and even genera (Richardson, 1969). While Richardson was the first to apply systematic

theory to the taxonomy of leeches, his proposed groupings reflect the similarities in morphology between taxa rather than the evolutionary histories driving the variation. Richardson did not think this revision (1969) was complete, but he knew it demonstrated a new approach to leech systematics that would lead to a more organized classification scheme based on thorough morphological examinations. He was still hesitant to make zoogeographical conclusions until the mechanisms of dispersal and the distributions of leech species were better documented.

Concurrent with the submission of Richardson's (1969) revision for publication, Soós (1969) had submitted an identification key of hirudinids to the same journal. Upon seeing the thoroughness of Richardson's morphological observations and how his use of systematic principles would transform leech systematics, Soós revised his manuscript (Soós, 1969). He wrote that Richardson's (1969) work was exceptional in that it was based upon examinations of type material for almost all genera (the two exceptions being *Asiaticobdella* and *Hirudobdella*), and resolved many classification and terminology inconsistencies in the literature on leech taxonomy. Soós (1969) did offer a few criticisms of the revision (Richardson, 1969), one being that only type material was examined and the diversity within genera cannot be captured in a single species or specimen. Also, many species are only known by their external characters because they were either originally described vaguely or before the thorough methods exemplified by Moore had become routine practice. This was a contributing factor for Soós's key (1969) to focus on external features of leech species instead of Richardson's (1969) emphasis on the internal morphology of reproductive systems and the pharynx (Soós, 1969).

Leech taxonomy remained stable and relatively unchanged after Sawyer's (1986) revision of all leeches based on systematic literature. Within the Hirudiniformes, Sawyer (1986) made many new combinations of genera and species based upon the literature and the examination of

relatively few specimens. Sawyer valued internal reproductive structures, number of teeth, zoogeography, and feeding habits as characters for distinguishing and/or uniting taxa. According to Sawyer (1986), Hirudiniformes is within the order Arhynchobdellida (jawed leeches) and contains five families that are diverse in diet, habitat preference, and geographic distribution: Americobdellidae Caballero, 1956, Cylicobdellidae Ringuelet, 1972, Haemopidae Richardson, 1969, Haemadipsidae Blanchard 1893, and Hirudinidae Whitman, 1886. Haemopidae and Hirudinidae were the most widely distributed families with each having species on all continents except both families lacking representatives on Antarctica, and Haemopidae lacking members on Australia. These two families encompass relatively large, vermiform, swimming, freshwater leeches. Taxa that exhibited a macrophagous feeding behavior were grouped into the family Haemopidae, regardless of geographic distribution, while sanguivorous taxa were retained in Hirudinidae. Americobdellidae, Cylicobdellidae, and Haemopidae contain carnivorous taxa, while Haemadipsidae and Hirudinidae include only sanguivorous taxa. Americobdellidae, Cylicobdellidae, and Haemadipsidae contain terrestrial taxa, while species of Hirudinidae and Haemopidae are aquatic. Geographically, hirudiniformes are found worldwide with some families restricted to certain areas. Cylicobdellidae was endemic to South America, and Americobdellidae has only been recorded from Chile and Argentina. Members of Haemadipsidae (sensu Sawyer, 1986) were restricted to the IndoPacific.

The addition of molecular evidence

The advent of DNA sequencing technology has both reaffirmed taxonomic groupings as well as shown many to be unnatural. Siddall and Burreson (1998) was the first paper to use molecular data in a phylogeny of leeches that explored the relationships among the major

families as well as the evolution of bloodfeeding. While Siddall and Burreson (1998) used a single gene, Apakupakul *et al.* (1999) and Trontelj *et al*. (1999) both used multi-gene datasets (Apakupakul *et al*.: 18S rDNA, CO1 and Trontelj *et al*.: 18S rDNA and 12S rDNA) to establish the backbone of leech relationships at a higher taxonomic level. In a phylogeny of seven families of arhynchobdellid leeches with morphological and molecular data, Borda and Siddall (2004b) used four genes (18S rDNA, 28S rDNA, 12S rDNA, and CO1) to evaluate not only the relationships of the families, but also the origins of terrestrialism and bloodfeeding. With a limited taxon sampling, the family Hirudinidae was found to be polyphyletic, Haemopidae was not found to be monphyletic. Semiscolecidae was established as a separate family and Macrobdellidae was resurrected as a family to accommodate the North and South American members of the traditional Hirudinidae. The placement of *Limnatis nilotica* Savigny, 1822, an abundant species from the Nile in Africa, complicated the distinction between the North and South American clades Macrobdellidae and Semiscolecidae by placing basal to both of these families yet separate from other African species in the tree (Borda and Siddall, 2004b). An expansion of Semiscolecidae was mentioned as a resolution although the authors did not propose further taxonomic changes until the taxonomic sampling of these families was increased (Borda and Siddall, 2004b).

This dissertation explored the evolutionary history of Hirudinidae *sensu* Sawyer, 1986, as well as the major groups within the family, using molecular and morphological data. Changes in taxonomy are made when appropriate to attain agreement between the evolutionary histories of the taxa and their classifications. The diversity of intestinal bacteria within representative species has also been assessed. The goals of this dissertation were:

1. To determine if the family Hirudinidae is monophyletic, and if not, to revise the family level taxonomy on the basis of phylogenetic results,
2. To more generally compare these results to the classification schemes of previous revisions (Richardson, 1969; Soós, 1969; Sawyer, 1986) to assess the validity of subfamilies, genera and species,
3. To assess biogeographic patterns of diversification of the clade(s),
4. To detect any correlation between hirudinid diversity and the diversity of bacterial fauna contained within the digestive tracts of representatives of the resulting clades of leeches.

The work included in this dissertation has been featured in several publications (Phillips and Siddall, 2009; Phillips *et al.*, 2010, 2011; Oceguera-Figueroa *et al*., 2011; Siddall *et al.*, in press). In Oceguera-Figueroa *et al*. (2011), I assisted in the collection of data, data analyses, as well as preparation of the manuscript and provided expertise in relation to the placement of the family Gastrostomobdellidae. Siddall *et al*. (in press) is a larger review paper with three parts: 1) a review of recent work in leech systematics, 2) an examination of the diversity of bacterial endosymbionts of the digestive tracts of members of several families of Hirudiniformes, and 3) new information about the salivary transcriptomes from across the lineages of medicinal leeches. My role in the project was with the examination of the bacterial endosymbiont diversity and I have included only that portion of the manuscript adapted for this dissertation.

CHAPTER 2

Poly-paraphyly of Hirdinidae: many lineages of medicinal leeches

(Adapted from Phillips AJ and Siddall ME. 2009. Poly-paraphyly of Hirdinidae: many lineages of medicinal leeches. *BMC Evolutionary Biology* 9: 246).

INTRODUCTION

"Medicinal leech" is a common name that describes bloodfeeding clitellate annelids in the family Hirudinidae of the order Hirudinida. The use of leeches for bloodletting has been a part of Western medicine since Galen (Sternback *et al*., 2001). Indeed, the word "leech" is actually derived from the Old English word, *læce,* for physician (Merriam-Webster Dictionary). Their utility has also been recorded in several Eastern traditions, having been documented in the Charaka Samhita (Maurya period, roughly 3rd century BCE) as one of five treatments for an imbalance of humors and by Wang Ch'ung (27-100 A.D) (Schwartz, 2005). François-Joseph-Victor Broussais, physician to Napoleon and his troops, was the major proponent of leeching in Europe, particularly in the early 1800s, during which he was infamous for using copious numbers of leeches during Napoleon's campaign through Europe (Porter, 1999). As little as five and up to 50 leeches at a time were used for patients suffering from various conditions until Pierre Charles Alexander Louis and contemporaries finally questioned the effectiveness of phlebotomy as a cure-all; the practice was not curbed until approximately 100 years later (Gross and Apesos, 1992; Morabia, 1996).

As a result of their great medical popularity during the 18th and 19th centuries, European leech populations were over-harvested and leeches became increasingly scarce in parts of Western Europe. Consequently, various countries, such as Italy, Hungary, and Poland, with seemingly abundant sources, began exporting large numbers in order to satisfy the high demand. As early as 1823, restrictions were put in place to manage the number of leeches being exported through Hannover, Germany, and collecting seasons were instituted in Russia; these represent some of the first measures in history meant to conserve an animal species (Wells and Combes, 1987).

The clinical use of leeches was revived by Derganc and Zdravic (1960) to relieve post-operative venous congestion in patients recovering from tissue flap and replantation surgery. Their application in this regard proved so successful that European medicinal leeches were approved by the US Food and Drug Administration in June, 2004 as a medical device due to their mechanically relieving venous congestion and delivering anti-coagulants (Rados, 2004). The powerful anti-coagulants in leech salivary secretions have been of interest since the anti-thrombin, hirudin, was purified (Pirkle and Markland, 1985). The first human dialysis treatment accomplished by Haas (1924) was only possible in light of the newly available purified hirudin, though it would later be supplanted by widely available and less expensive heparin.

The namesake of the family Hirudinidae, *Hirudo medicinalis* Linnaeus, 1758 (European Medicinal Leech), is the species most commonly referenced for its use in medicine, though a recent study (Siddall *et al*., 2007b?; Kutschera, 2006) found the commercially distributed leech used in most Western hospitals is *Hirudo verbana* Carena 1820, not *H. medicinalis*. In fact, within the family Hirudinidae, approximately 200 species have been described from all continents, save for Antarctica. Some of these species are used in medical practices in place of *Hirudo* species where they are abundant (e.g., *Richardsonianus australis* (Bosisto, 1859), *Hirudinaria manillensis* (Lesson, 1842), and *Hirudo nipponia* Whitman 1886; Hong *et al*., 1999).

Traditionally, the family Hirudinidae included any sanguivorous, swimming, freshwater leech with three jaws (one dorsal and two ventrolateral) and a distinctively caecate crop. Richardson (1969) separated the Hirudinidae into five families, which Sawyer (1986) made into new combinations and subfamilies of the family Hirudinidae based on sexual morphology and geographic distributions (Figure 1). Apakupakul *et al.* (1999) suggested that the Hirudinidae is

FIGURE 1.

Classification schemes of the suborder Hirudiniformes

Figure 1.

Previous classification scheme (Richardson, 1969)

- Richardsonianidae
- Ornithobdellidae
- Haemopidae
- Macrobdellidae
- Hirudinidae

Current classification scheme (Sawyer, 1986; Borda et al., 2008)

- Americobdellidae
  - 1 genus; South America
- Cylicobdellidae
  - 6 genera; South America, Japan, Borneo, Hawaii
- Haemopidae
  - Haemopinae
    - 3 genera; Northern Hemisphere
  - Semiscolescinae
    - 4 genera; South America
- Haemadipsidae (see Borda *et al*., 2008)
- Xerobdellidae (see Borda *et al*., 2008)
- Hirudinidae
  - Ornithobdellinae
    - 3 genera; Australasia
  - Praobdellinae
    - 3 genera; Africa, Southeast Asia
  - Macrobdellidae
    - 5 genera; North and South America
  - Hirudinariinae
    - 3 genera; Southeast Asia
  - Richardsonianinae
    - 4 genera; Australasia
  - Hirudininae
    - 4 genera; Eurasia and Africa

Revised classification scheme (this study)

- Cylicobdellidae
- Haemadipsidae (see Borda *et al*., 2008)
- Xerobdellidae (see Borda *et al.,* 2008)
- Hirudinidae
  - *Aliolimnatis, Asiaticobdella, Dinobdella, Hirudinaria, Hirudo, Poecilobdella, Whitmania,* etc.
- Semiscolescidae (*sensu lato*)
  - *Semiscolex, Macrobdella, Limnatis, Limnobdella,* etc.

polyphyletic, finding the North American medicinal leech *Macrobdella decora* (Say, 1824) to be only distantly related to *H. medicinalis*. Borda and Siddall's (2004b) analyses found the family Hirudinidae to be split into two major clades with the terrestrial leeches and the non-bloodfeeding Haemopidae falling in between. All taxonomic revisions of the family until now have been performed only with morphological characters [e.g., (Richardson, 1969; Sawyer, 1986; Soós, 1969)]. Here, we revisit the phylogenetic relationships and systematics of the family Hirudinidae while testing the monophyly of the family, and for the first time utilizing an expanded taxon sampling from each continent with representatives of most previously proposed subfamilies.

MATERIALS AND METHODS

Taxon selection

A total of 48 species composing 61 terminal taxa were used in the analyses (Table 1). Taxa new to phylogenetic analyses include: *Motobdella montezuma* Davies, 1985, *Limnobdella mexicana* Blanchard, 1893 from several localities, *Limnatis* cf. *nilotica*, *Limnatis paluda* (Tennent, 1859), *Semiscolex intermedius* Ringuelet, 1942, *Semiscolex lamothei* Oceguera-Figueroa, 2005*, Asiaticobdella fenestrata* (Moore, 1939), and *Goddardobdella elegans* (Grube, 1867). Species involved in previous analyses, but in this study with new material, include: *Aliolimnatis michaelseni* (Augener, 1936)*, Haemopis sanguisuga* (Linnaeus, 1758), *Hirudinaria javanica* (Wahlberg, 1856), *Hirudinaria manillensis* (Lesson, 1842) from several localities*, Hirudo troctina* Johnson, 1816*,* and *Whitmania laevis* (Baird, 1869).

Table 1: Taxa used for the phylogenetic analyses of the family Hirudinidae along with collection localities and GenBank accession numbers. Asterisks indicate type species for the genera of the Ingroup.

Taxon	Locality	GenBank Accession Numbers			
		18S	28S	12S	CO1
Ingroup					
Aliolimnatis africana	Ctr. African Republic	AY425469	AY425387	AY425428	AY425451
Aliolimnatis michaelseni	Guinea Bissau	GQ368780	GQ368761	GQ368803	GQ368738
Aliolimnatis michaelseni	Congo	AF116010	AY425388	AY425429	AF116029
Aliolimnatis oligodonta	Tanzania	GQ368781	GQ368762	_____	GQ368739
Aliolimnatis buntonensis	South Africa	GQ368782	_____	_____	GQ368740
Asiaticobdella fenestrata	Zambia	GQ368783	GQ368763	GQ368804	GQ368741
Chtonobdella bilineata	Australia	AF116006	AY425361	_____	AF003267
Chtonobdella whitmani	Australia	EU100065	EU100074	_____	EU100087
Diestecostoma magnum	Mexico	EU100067	EU100076	_____	EU100088

Table 1 continued

Diestecostoma mexicana	Mexico	EU100068	EU100077	_____	EU100089
Diestecostoma trujillensis	Mexico	EU100066	EU100075	_____	EU100090
Goddardobdella elegans 1*	Australia	GQ368784	GQ368764	GQ368805	GQ368742
Goddardobdella elegans 2*	Australia	GQ368785	GQ368765	GQ368806	GQ368743
Goddardobdella elegans R*	Australia	GQ368786	GQ368766	GQ368807	GQ368744
Haemadipsa interrupta	Thailand	EU100069	EU100078	_____	EU100091
Haemadipsa sylvestris	Vietnam	AF116005	AY425373	AY425416	AF003266
Haemadipsa sumatrana	Borneo	AY425464	AY425372	AY425415	AY425446
Haemopis grandis	Manitoba	AY425465	AY425377	AY425420	AY425447
Haemopis kingi	Manitoba	AY425466	AY425378	AY425421	AY425448
*Haemopis sanguisuga**	Sweden	AF099941	AY425381	AF099960	AF462021
Haemopis terrestris	OH, USA	AY786465	EU100080	_____	EU100092
*Hirudinaria javanica**	Vietnam	GQ368787	GQ368767	GQ368808	GQ368745
Hirudinaria manillensis	Dominican Republic	GQ368788	GQ368768	GQ368809	_____
Hirudinaria manillensis	Puerto Rico	AY425467	AY425384	AY425426	AY425449

Table 1 continued

Hirudinaria manillensis	Thailand	GQ368789	GQ368769	_____	GQ368746
Hirudinaria manillensis 11	Vietnam	GQ368791	GQ368771	GU045561	GQ368748
Hirudinaria manillensis 24	Vietnam	GQ368790	GQ368770	GQ368810	GQ368747
*Hirudo medicinalis**	BioPharm, UK	AF116011	AY425385	AF099961	AF003272
Hirudo nipponia	Korea	AY425468	AY425386	AY425427	GQ368749
Hirudo orientalis	Azerbaijan	GQ368792	_____	GQ368811	GQ368750
Hirudo troctina	Morocco	GQ368793	GQ368772	GQ368812	GQ368751
Hirudo verbana	Leeches USA	GQ368794	GQ368773	GQ368813	GQ368752
Idiobdella seychellensis	Seychelles	EU100070	EU100081	_____	EU100094
*Limnatis nilotica**	Bosnia	_____	_____	AY763161	AY763152
Limnatis cf. *nilotica*	Namibia	GQ368795	GQ368774	GQ368815	GQ368754
Limnatis paluda	Afghanistan	GQ368796	GQ368775	_____	GQ368755
Limnatis paluda	Israel	AY425470	AY425389	AY425430	AY425452
*Limnobdella mexicana 1**	Mexico	GQ368797	GQ368776	GQ368818	GQ368758
*Limnobdella mexicana 2**	Mexico	_____	_____	GQ368819	GQ368759

Table 1 continued

*Limnobdella mexicana 3**	Mexico	GQ368798	GQ368777	GQ368816	GQ368756
*Limnobdella mexicana 4**	Mexico	GQ368799	GQ368778	GQ368817	GQ368757
*Macobdella decora**	MI, USA	AF116007	AY425390	AY425431	AF003271
Macrobdella diplotertia	MO, USA	DQ097214	DQ097205	_____	DQ097223
Macrobdella ditetra	GA, USA	AY425471	AY425391	AY425432	AY425453
Malagadbdella fallax	Madagascar	EU100071	EU100083	_____	EU100096
Mesobdella gemmata	Chile	AY425472	EU100084	_____	EU100097
Nesophilaemon skottsbergi	Juan Fernandez Island	EU100072	EU100085	_____	EU100098
Oxyptychus brasiliensis	Brazil	AY425473	AY425398	AY425436	AY425455
*Oxyptychus striatus**	Argentina	AY425474	AY425399	_____	_____
Patagoniobdella fraterna	Chile	AY425477	AY425405	AY425441	AY425459
*Patagoniobdella variabilis**	Chile	AY425476	_____	_____	AY425458
*Philobdella floridana**	SC, USA	DQ097210-13	DQ097201-14	DQ097226	DQ097219-22
Philobdella gracilis	LA, USA	DQ097209	DQ097200	DQ097225	DQ097218

Table 1 continued

Semiscolex intermedius	Argentina	GQ368800	_____	_____	GU045562
Semiscolex lamothei	Mexico	GQ368801	_____	_____	GU045563
Semiscolex similis	Bolivia	AY425475	AY425402	AY42543	AY425475
Whitmania laevis	Taiwan	AY786467	AY786454	AY786447	_____
Xerobdella lecomtei	Slovenia	AF099947	EU100086	_____	EU100099
Outgroup					
Americobdella valdiviana	Chile	AY425461	AY425358	AY425407	AY425443
Cylicobdella coccinea	Bolivia	AY425462	AY425362	AY425411	AY425444
Erpobdella montezuma	AZ, USA	GQ368802	GQ368779	GQ368820	GQ368760

Three arhynchobdellid outgroup taxa were included in the analyses: *Americobdella valdiviana* (Philippi, 1872) of the family Americobdellidae, *Cylicobdella coccinea* Kennel, 1886 of the family Cylicobdellidae, and *Motobdella montezuma* of the family Erpobdellidae. An additional 17 hirudiniform taxa from the families Haemadipsidae and Xerobdellidae were used for comparative purposes. The three outgroup taxa were selected based on prior phylogenetic work (Apakupakul *et al.*, 1999). Locality data and GenBank Accession Numbers are listed in Table 1.

Specimens were identified using morphological characters. These included examination of arrangement of eyespots, number of annuli separating the gonopores, number of gastic caecae, and the size and shape of internal reproductive organs such as the penis, vagina, testisacs, ovaries, and common oviduct if present. During this process, it was determined that a specimen used in earlier studies previously identified as *L. nilotica* (18S: AY425470, 28S: AY425389, 12S: AY425430, CO1: AY425452) collected in Israel used in Borda and Siddall (2004b) was actually *L. paluda*. The morphological differences between the two species were verified by the examination of the morphology of the *L. paluda* specimen from Afghanistan.

DNA extraction and purification

Specimens were stored at either -20°C or at ambient temperature in 95-100% ethanol. Tissue was collected from the caudal sucker rather than from gastric or intestinal regions to avoid contamination of the host/prey DNA. A DNeasy Tissue Kit (Qiagen Valencia, CA) was used for tissue lysis and DNA purification.

DNA amplification

Primers used in Borda and Siddall (2004b) were used for the PCR amplification of nuclear 18S rDNA (18S) and 28S rDNA (28S) and mitochondrial 12S rDNA (12S) gene fragments. PCR amplification of mitochondrial cytochrome *c* oxidase I (COI) gene fragments was accomplished using the primers COI-A and COI-B [33] or LCO1490 and HCO2198 (Borda and Siddall, 2004b). All amplification reactions of gene fragments were made using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) with 0.5 μl of each 10μM primer, 1 μl DNA template, and 23 μl RNase-free H2O (total volume 25 μl) and were performed in an Eppendorf® Mastercycler®. The following amplification protocols were used: for 18S, 94°C for 1 min, followed by 35 cycles of 94°C (30 sec), 49°C (30 sec), 68°C (2 min 30 sec) and a final extension at 68°C for 1 minute; for 28S and 12S, 94°C for 5 min, followed by 39 cycles of 95°C (1 min), 52°C (1 min), 70°C (1 min) and a final extension of 72° for 7 minutes; for COI, 94°C for 1 min, followed by 30 cycles of 94°C (30 sec), 48°C (30 sec), 68°C (45 sec), 68°C (1 min) and a final extension of 68°C for 1 min. PCR amplification products were purified with AMPure™ (Agencourt Bioscience Corporation).

DNA sequencing and alignment

Cycle sequence reactions were performed with an Eppendorf® Mastercycler® using one of two different strategies: 7 μl Rnase-free H_2O, 1 μl ABI Big Dye™ Terminator (v1.1 or v3.1), 1 μl Big Dye™ Extender Buffer (v1.1 or v3.1), 1 μl of 1 μM primer and 3 μl of cleaned PCR template (13 μl total volume) or 0.5 μl ABI Big Dye™ Terminator (v1.1 or v3.1), 0.5 μl Big Dye™ Extender Buffer (v1.1 or v3.1), 1 μl of 1 μM primer and 3 μl of cleaned PCR template (5 μl total volume). Sequences were purified by 70% isopropanol/70% ethanol precipitation and

analyzed with an ABI PRISM® 3730 sequencer (Applied Biosystems). CodonCode Aligner (CodonCode Corporation) was used to edit and reconcile sequences. Alignments of all genes were accomplished using the European Bioinformatics Institute server for MUSCLE applying default settings (MUltiple Sequence Comparison by Log-Expectation) v. 3.7 (Edgar, 2004).

Phylogenetic analyses

Parsimony analyses of the genes (18S, 28S, COI, and 12S) individually and in combination were performed using PAUP* v. 4.02b (Swofford, 2002). Heuristic searches used 500 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All characters were left unweighted and non-additive. Gaps were treated as missing data. Parsimony jackknife values for combined analyses were obtained using random taxon addition and tree-bisection-reconnection branch swapping with 36% deletion and 100 heuristic pseudoreplicates (Farris *et al.*, 1996).

Bayesian Inference was performed on the combined dataset using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The data were partitioned by gene for 18S, 28S, 12S, and by codon position for COI (three partition; 3p). A GTR+Γ+I model was assumed for each unlinked data partition based on the AIC via ModelTest v. 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). 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 2,600,000 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 three million generations, leaving 7,000 trees sampled for estimation of posterior probabilities (pp).

Maximum Likelihood analyses were performed on the combined dataset using Treefinder v. 1.1 (Jobb, 2008) with the GTR+Γ+I model applied for each unlinked data partition with default settings.

Monophyly of the presumed monophyletic family Hirudinidae was tested with the Templeton test (Templeton, 1983) as implemented in PAUP* v. 4.02b. Bayes Factors were calculated using the equation 2[ln(harmonic mean of constraint) – ln(harmonic mean of original analysis)] in which strongly negative values (below –10) indicate rejection of the constrained analysis (Brown and Lemmon, 2007). In addition, topological tests were conducted under the likelihood criterion with Treefinder (Jobb, 2008) in which independent (unlinked) models were employed for the locus and codon partitions defined as above. Constraints that were compared to the optimal solution included 1) all traditional Hirudinidae taxa as monophyletic but excluding the non-bloodfeeding haemopids, and 2) all traditional Hirudinidae taxa and traditional Haemopidae taxa as monophyletic but not constraining either of these two subgroups to individually be monophyletic.

RESULTS

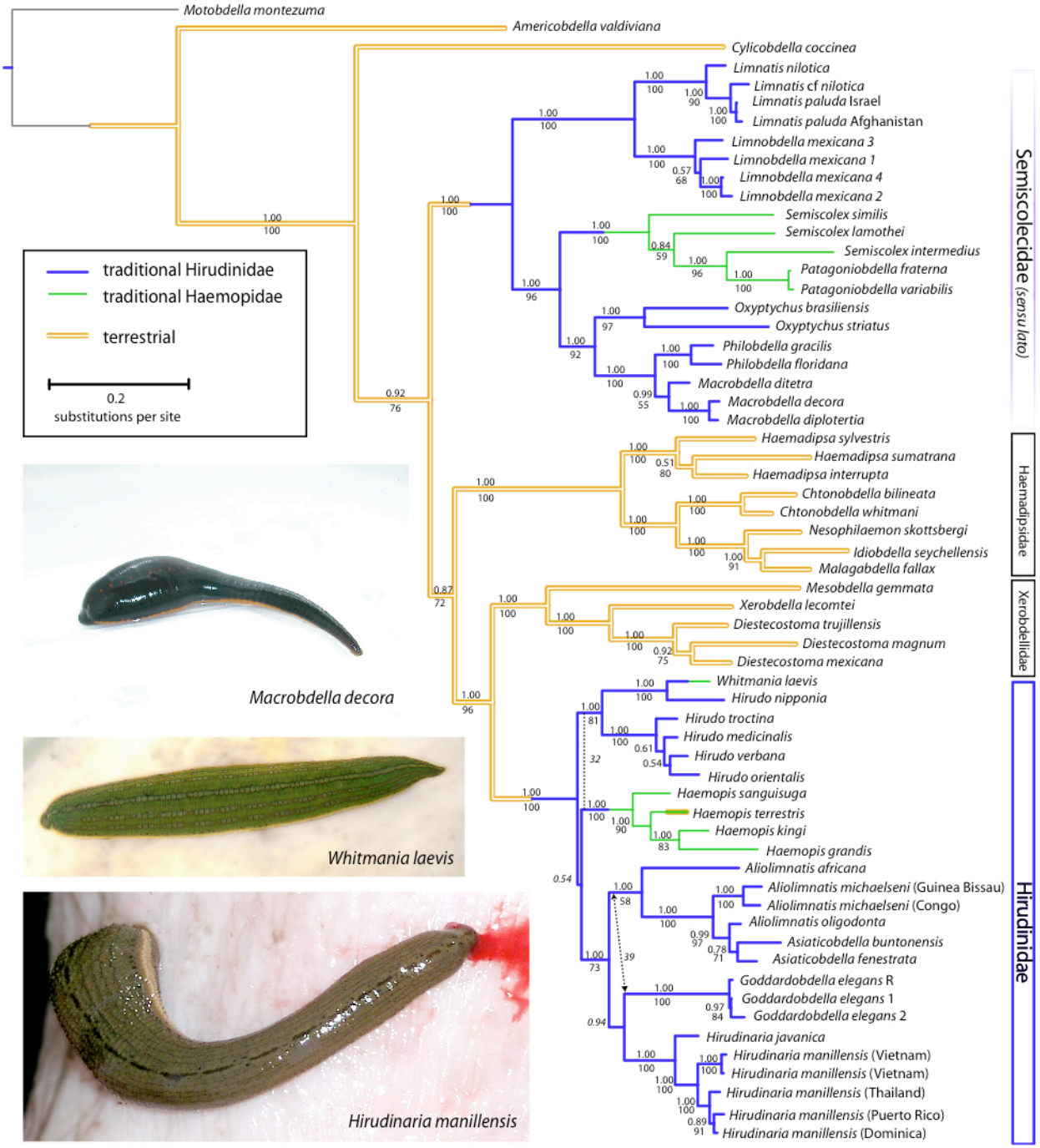
The combined dataset had a total of 6086 characters (18S: 2034 characters, 28S: 2162 characters, 12S: 575 characters, CO1: 1315 characters). The parsimony analysis produced 9 equally parsimonious trees with 8266 steps while the harmonic mean of log-likelihood values from two runs of the Bayesian (BI) analysis averaged –44555.69. The log-likelihood of the topology produced by the Maximum Likelihood analysis was -43311.984.

Parsimony and BI methods largely agreed in terms of the tree topology, including that the family Hirudinidae was not monophyletic (Figure 2). In parsimony, monophyly of an

FIGURE 2.

Bayesian Inference tree topology based on 18S rDNA, 28S rDNA, 12S rDNA, and COI datasets combined. Posterior probabilities are above the node and jackknife values as a result of parsimony analyses are below the node. The black arrow with a dashed line indicates alternate tree topologies. Branch lengths in orange correspond to terrestrialism, branch lengths in blue correspond to traditional members of the family Hirudinidae, and branch lengths in green correspond to traditional members of the family Haemopidae. Boxes to the right provide the family designation following the revised classification.

Figure 2.


Motobdella montezuma
Americobdella valdiviana
Cylicobdella coccinea
Limnatis nilotica
Limnatis cf nilotica
Limnatis paluda Israel
Limnatis paluda Afghanistan
Limnobdella mexicana 3
Limnobdella mexicana 1
Limnobdella mexicana 4
Limnobdella mexicana 2
Semiscolex similis
Semiscolex lamothei
Semiscolex intermedius
Patagoniobdella fraterna
Patagoniobdella variabilis
Oxyptychus brasiliensis
Oxyptychus striatus
Philobdella gracilis
Philobdella floridana
Macrobdella ditetra
Macrobdella decora
Macrobdella diplotertia
Haemadipsa sylvestris
Haemadipsa sumatrana
Haemadipsa interrupta
Chtonobdella bilineata
Chtonobdella whitmani
Nesophilaemon skottsbergi
Idiobdella seychellensis
Malagabdella fallax
Mesobdella gemmata
Xerobdella lecomtei
Diestecostoma trujillensis
Diestecostoma magnum
Diestecostoma mexicana
Whitmania laevis
Hirudo nipponia
Hirudo troctina
Hirudo medicinalis
Hirudo verbana
Hirudo orientalis
Haemopis sanguisuga
Haemopis terrestris
Haemopis kingi
Haemopis grandis
Aliolimnatis africana
Aliolimnatis michaelseni (Guinea Bissau)
Aliolimnatis michaelseni (Congo)
Aliolimnatis oligodonta
Asiaticobdella buntonensis
Asiaticobdella fenestrata
Goddardobdella elegans R
Goddardobdella elegans 1
Goddardobdella elegans 2
Hirudinaria javanica
Hirudinaria manillensis (Vietnam)
Hirudinaria manillensis (Vietnam)
Hirudinaria manillensis (Thailand)
Hirudinaria manillensis (Puerto Rico)
Hirudinaria manillensis (Dominica)
traditional Hirudinidae
traditional Haemopidae
terrestrial
0.2
substitutions per site
Macrobdella decora
Whitmania laevis
Hirudinaria manillensis
Semiscolecidae (sensu lato)
Haemadipsidae
Xerobdellidae
Hirudinidae

a priori presumed-monophyletic Hirudinidae would require 179 extra steps (Templeton test: z=-8.299, p>0.0001). The harmonic mean of log-likelihood values constraining traditional hirudinids to be monophyletic was –45054.72 (yielding a Bayes Factor of –998.06). Similarly, with this constraint under the likelihood criterion, monophyly of Hirudinidae was rejected with Treefinder (Jobb, 2008), in that *p*-values were highly significant (Shimodiara-Hasegawa < 0.000001, approximately unbiased test < 0.000001). The harmonic mean of log-likelihood values constraining traditional hirudinids and traditional haemopids together to be a monophyletic group was –44589.01 (yielding a Bayes Factor of –66.64). Similarly, with this constraint under the likelihood criterion, monophyly of Hirudinidae+Haemopidae was rejected with Treefinder (Jobb, 2004), in that *p*-values, while not as profound as in the simple case of constraining Hirudinidae to be monophyletic, still were significant at the 5% level (Shimodiara-Hasegawa = 0.0195, approximately unbiased test = 0.0164).

Hirudinid taxa placed among two strongly supported clades (Figure 2). One clade contained the genera *Macrobdella, Philobdella*, *Oxyptychus*, *Semiscolex*, *Patagoniobdella*, *Limnobdella*, and *Limnatis*. A second clade contained the genera *Aliolimnatis*, *Asiaticobdella*, *Hirudinaria*, *Goddardobdella*, *Hirudo*, *Whitmania*, and *Haemopis.* The precise placement of the genus *Haemopis* varied among analyses and received little support in each of Parsimony (jackknife=32) and BI (pp=0.54) analyses. Between the two principal hirudinid clades was a paraphyletic assemblage of terrestrial leeches in the families Haemadipsidae and Xerobdellidae. The parsimony analysis found the genus *Haemopis* to be sister to the *Hirudo* clade (including *Whitmania laevis* (Baird, 1869)), whereas the BI analyses found the genus *Haemopis* sister to a clade comprised of the genera *Aliolimnatis, Asiaticobdella, Goddardobdella,* and *Hirudinaria,* exclusive of the genus *Hirudo*. Species-level disagreements were apparent between the

parsimony analysis and the BI analyses involving species of *Hirudo* as well as species of *Aliolimnatis* and *Asiaticobdella*. Regardless of optimality criterion, within the *Hirudo* clade were the various European *Hirudo* species along with the Asian *H. nipponia,* which itself was sister to the Asian non-sanguivorous *W. laevis* (traditionally Haemopidae; Figure 2). Within its own clade, *H. manillensis* individuals were clustered by locality with Caribbean individuals closely related to those from Thailand. Representatives of the genus *Asiaticobdella* fell within, and rendered paraphyletic, the genus *Aliolimnatis*. Regardless of optimality criterion, the genera *Macrobdella, Philobdella,* and *Oxyptychus* each were monophyletic and together formed a clade that was sister to the non-sanguivorous Semiscolescidae (also traditionally Haemopidae) as opposed to the bloodfeeding genera, *Limnatis* and *Limnobdella*. Mexican leeches of the genus *Limnobdella* formed a monophyletic group sister to the monophyletic genus *Limnatis* with high support values (jackknife=100; pp=1.00).

DISCUSSION

The family Hirudinidae, long taken for granted to be monophyletic, is not. Hirudinid leeches, characterized as relatively large, vermiform, swimming leeches that feed on blood by making an incision with three armed jaws, fall into two separate clades: one typified by the North American *M. decora* and the other by the European *H. medicinalis*. The Hirudinidae is represented by two independent origins of aquatic medicinal leeches, each from a terrestrial ancestor. Both groups create spongy cocoons that are deposited on shore, leaving the hatchlings to search for the water in a manner similar to newly hatched sea turtles. Also, both groups have internal insemination, a behavior common to terrestrial organisms to prevent sperm desiccation, unlike the aquatic leech families Glossiphoniidae and Piscicolidae that exhibit external traumatic

insemination. The clade containing *M. decora* includes additional New World genera, such as the South American *Oxyptychus*, *Semiscolex*, and *Patagoniobdella,* as well as the North American *Macrobdella*, *Philobdella*, and *Limnobdella*. Unexpectedly, within this otherwise New World clade is the Old World bloodfeeding genus *Limnatis* distributed from Eastern Europe, throughout Africa, and eastward to the Indian subcontinent. The second hirudinid clade contains *H. medicinalis* and related genera found only in the Old World including Africa (*Aliolimnatis* and *Asiaticobdella* spp.), Asia (*Hirudinaria* spp., some *Hirudo* spp., and *Whitmania* spp.), Australia (*Goddardobdella* spp.), and Europe (*Hirudo* spp.). This polyphyly of the family Hirudinidae is further complicated by each of the two clades' inclusion of non-bloodfeeding taxa heretofore assigned to the family Haemopidae (Sawyer, 1986).

The deep divergence between the two hirudinid clades was hinted at by Borda and Siddall (2004b) in their findings that the Old World *Limnatis nilotica* (Savigny, 1822) placed closer to the North American *M. decora* than to other African species of the genus *Aliolimnatis*. With our addition of members of the genus *Limnobdella* that group sister to *Limnatis* species, the nature of this relationship is more precise. Prior work regarding the anticoagulant profiles of various medicinal leeches may have been prescient regarding polyphyly of the so-called "medicinal leeches". A variety of anticoagulants have been characterized from hirudinid leeches, with each compound targeting a different point in the clotting process (Baskova and Zavalova, 2001; Salzet, 2002). It is generally held that the major protease inhibitors employed by *Hirudo* species and their allies block thrombin, whereas that for *M. decora* targets platelet aggregation as opposed to the clotting cascade itself [e.g., (Munro *et al.*, 1992)]. Regarding the close association of Old World *Limnatis* species and New World *Limnobdella* species, generalized morphological similarities have previously been noted. Richardson and Oosthuizen

lamented in personal letters (in the possession of MES) their inability to find a synapomorphy for the two genera that might allow them to erect a new family.

As noted above, in addition to the polyphyletic origin of the medicinal leeches, both hirudinid clades are paraphyletic in light of members of the family Haemopidae placing within each group. Previously, non-bloodfeeding, relatively large, vermiform, swimming leeches were grouped together on the basis of their macrophagous feeding behavior, regardless of geographic distribution. The family Haemopidae, among other non-bloodfeeding taxa, included the genera *Haemopis*, *Whitmania*, *Semiscolex*, and *Patagoniobdella* (Sawyer, 1986). Our analyses demonstrate that this family is not phylogenetically corroborated because haemopid genera fall variously within the two independent hirudinid clades, thus rendering each of the clades paraphyletic. *Whitmania laevis* is sister to a bloodfeeding species within the genus *Hirudo*, and not even monophyletic with the other nearby non-bloodfeeding species of *Haemopis*. The macrophagous genera *Semiscolex* and *Patagoniobdella,* while monophyletic, are sister to a clade containing the sanguivorous taxa, *Oxyptychus*, *Macrobdella* and *Philobdella*. Though the ancestral hirudinid was clearly a bloodfeeder (Borda and Siddall, 2004b), what is remarkable is the number of times that sanguivory has been abandoned by this group of annelids otherwise notorious for its ectoparasitic dependence on vertebrate blood. Already the loss of sanguivory has been inferred for other groups of leeches such as Erpobdellidae, with a predilection for chironomid larvae, and the glossiphoniid genera *Helobdella*, *Glossiphonia,* and *Alboglossiphonia* that prefer the hemolymph of gastropods or other annelids. Even the terrestrial haemadipsid, *Idiobdella seychellensis* Harding, 1913 shifted away from feeding on blood on remote islands where terrestrial gastropods are more plentiful (and often larger) than resident anurans (Richardson, 1978).

To reflect the phylogeny, the family Hirudinidae *sensu stricto* must hereafter exclude those bloodfeeding taxa unrelated to *H. medicinalis* and minimally includes those more closely related sanguivores [e.g., *Hirudo, Goddardobdella, Hirudinaria, Aliolimnatis, Asiaticobdella* included here], but must also include the non-sanguivorous genera *Haemopis* and *Whitmania* if leech taxonomy is to avoid both polyphyly and paraphyly of this family. The remaining genera previously included in the family Hirudinidae are in want of a unifying taxonomic name. Macrobdellidae (Richardson, 1969) could include the genera *Macrobdella, Philobdella,* and *Oxyptychus* so as to reflexively retain a family for the non-bloodfeeding Semiscolescidae (Sciban & Autrum, 1934), their sister taxon. Yet, this would leave the genera *Limnatis* and *Limnobdella* without a synapomorphy for any family that would be required to include them. Conveniently, the Hirudinidae *sensu stricto* are easily differentiated from the hirudinid clade typified by *M. decora* by virtue of their profoundly muscular ejaculatory bulbs in the median male reproductive apparatus that are efferent to the epididymes; a characteristic Hirudinidae shares with the Haemadipsidae. In the absence of a clear morphological synapomorphy for the *Limnobdella/Limnatis* clade, we acknoweldge that the genera *Macrobdella, Philobdella, Oxyptychus, Limnobdella, Limnatis,* and *Semiscolex* could presently be considered genera in the family Semiscolescidae (*sensu lato*), in that this family has taxonomic priority over the alternatives. Ironically, such a revision would leave the characteristically bloodfeeding Hirudinidae encompassing some non-bloodfeeding taxa and the traditionally non-bloodfeeding family Semiscolescidae (*sensu lato*) including notable bloodfeeders.

The genus *Patagoniobdella* is, by virtue of its relationships, merely a junior synonym of *Semiscolex*. *Asiaticobdella fenestrata* (Moore, 1939) falls within the genus *Aliolimnatis*. It is likely that these two genera will have to be synonymized, though we are presently reluctant in

the absence of either of the type species for the genera. Similarly, though *W. laevis* falls within the genus *Hirudo*, formal revision should require the inclusion of the type species, *Whitmania pigra* (Whitman, 1884).

Both *H. nipponia* and *L. nilotica* are known to include multiple morphological variants (Moore, 1924) (Oosthuizen notes in the possession of MES) over a wide distribution (the latter from Eastern Europe through the entire continent of Africa and parts of India, and the former throughout much of East Asia) and so most likely these each represent multiple lineages. Notably, our determinations of the identity of leeches matching the description of *L. nilotica* represent a paraphyletic assemblage relative to *L. paluda*. More sampling across the range of these taxa is needed in order to better define lineages and distinguish potentially cryptic species.

While there are no fossil data for correlation in historical interpretations of the Hirudinidae, geologic events can be used as a rough estimate when considering the current distributions of leech taxa. Assuming a vicariance-dominated explanation, both clades would have had to originate on Pangea with significant diversification in all groups prior to the supercontinent's breakup. The Semiscolecidae-related group seems to have originated in South America with diversification into the clades containing *Oxyptychus, Semiscolex,* and *Patagoniobdella* on that continent before approximately three million years ago (Mya) when North and South America became proximal. Thereafter, the lineage leading to *Macrobdella* and *Philobdella* could have dispersed north, a pattern mirrored in other leech groups, such as *Helobdella* and *Haementeria* (Siddall *et al.*, 2005). Some diversification would have had to occur prior to the breakup of Pangea in order to explain the presence of the genus *Limnobdella* in the New World and the genus *Limnatis* in Old World locales. Long distance dispersal of some

ancestral *Limnatis* or *Limnobdella* species should be considered, though presently this is only known for terrestrial leeches in the family Haemadipsidae feeding on birds.

The clade containing *H. medicinalis* also seems to have undergone an intense period of diversification around the time of the breakup of Pangea. The node joining the *Aliolimnatis*/*Asiaticobdella*, *Hirudinaria*, and *Goddardobdella* clades is short and unstable suggesting a rapid diversification associated with the continental breakup of Pangea during the Cretaceous. Closely related taxa from Africa, Australia, and Southeast Asia follow a Gondwanan vicariance distribution, distinctly separate from the Laurasian *Haemopis*/*Hirudo* sector of the Hirudinidae *sensu stricto*. The sister group relationship of *H. nipponia* and *W. laevis* reflects the geologic history of Asia with their northerly origin in Laurasia and a later dispersal of the non-bloodfeeder into southern regions following a period of isolation from the remaining Hirudinidae by the presence of the Turgai Sea (93 - 89 Mya; Baraboshkin *et al.*, 2003). The unusual recent distribution of *H. manillensis* in the Caribbean closely related to the others from (for example) Thailand can only be explained by *H. manillensis* having been introduced to the Caribbean in the 1800s by physicians using leeches on board galleons transporting goods and persons between Spanish holdings in the Pacific and the New World (Sawyer *et al*., 1998; Kutschera and Roth, 2006). Clarity regarding this potentially invasive species might be better assessed through haplotype analyses involving individuals from the Philippines and Northern Taiwan, which were under Spanish influence when leech phlebotomy was heavily practiced by European surgeons.

Despite extensive collection efforts, the type species of several genera in the family Hirudinidae have not been included in this analysis. These include *Aliolimnatis diversa* Richardson, 1972, *Asiaticobdella birmanica* (Blanchard, 1894), *Semiscolex juvenilis* Kinberg,

1866, and *Whitmania pigra* (Whitman, 1884). As such, definitive segregation of genera, and even their proper familial designations remain underdetermined. Approximately 15 genera, an inordinate number of which are monotypic taxa from Australia described by Richardson (1969), are not yet included in phylogenetic analyses. We anticipate that the addition of these and the multitudinous, however poorly distinguished, species described by Sciacchitano from Africa [e.g., (Sciacchitano, 1935, 1936, 1939, 1941, 1952, 1959, 1960)], might yet provide better support for some nodes, and further our understanding of the interrelationships of these medically important annelids.

CONCLUSIONS

The finding that the two groups of medicinal leeches have independent evolutionary origins is not surprising because the two clades do have subtle morphological and behavioral differences. *Hirudo* species when swimming form a complete sine wave with their bodies, while *M. decora* forms a sine wave and a half. Also, each group produces different anticoagulants (Salzet, 2002). This division, now supported by molecular data, calls for an extensive revision of all hirudinid-like taxa. Each taxon will have to be carefully evaluated as some are not placing as would be expected; a prime example being members of the genus *Limnatis*. This brings a large majority of leech systematics into question, and has far reaching implications. The distinctions are critical to researchers who use members of the Hirudinidae in their work, such as neurobiologists who use *H. medicinalis* as a model organism. These findings will have a greater impact upon those interested in characterizing the anticoagulants isolated from the members of the two clades, making knowledge of the proper evolutionary history of the group essential to giving context to future results.

AUTHORS' CONTRIBUTIONS

AJP and MES contributed equally to each stage of project conception and design, data collection, data analysis, and preparation of the manuscript. AJP and MES each contributed reagents, materials, and analytical tools to the study.

CHAPTER 3

Tyrannobdella rex n. gen. n. sp. and the evolutionary origins of mucosal leech infestations

(Adapted from Phillips AJ, Arauco-Brown R, Oceguera-Figueroa A, Gomez GP, Beltrán M, Yi-Te L, and Siddall ME. 2010. *Tyrannobdella rex* n. gen. n. sp. and the evolutionary origins of mucosal leech infestations. *PLoS One* 5(4): e10057).

INTRODUCTION

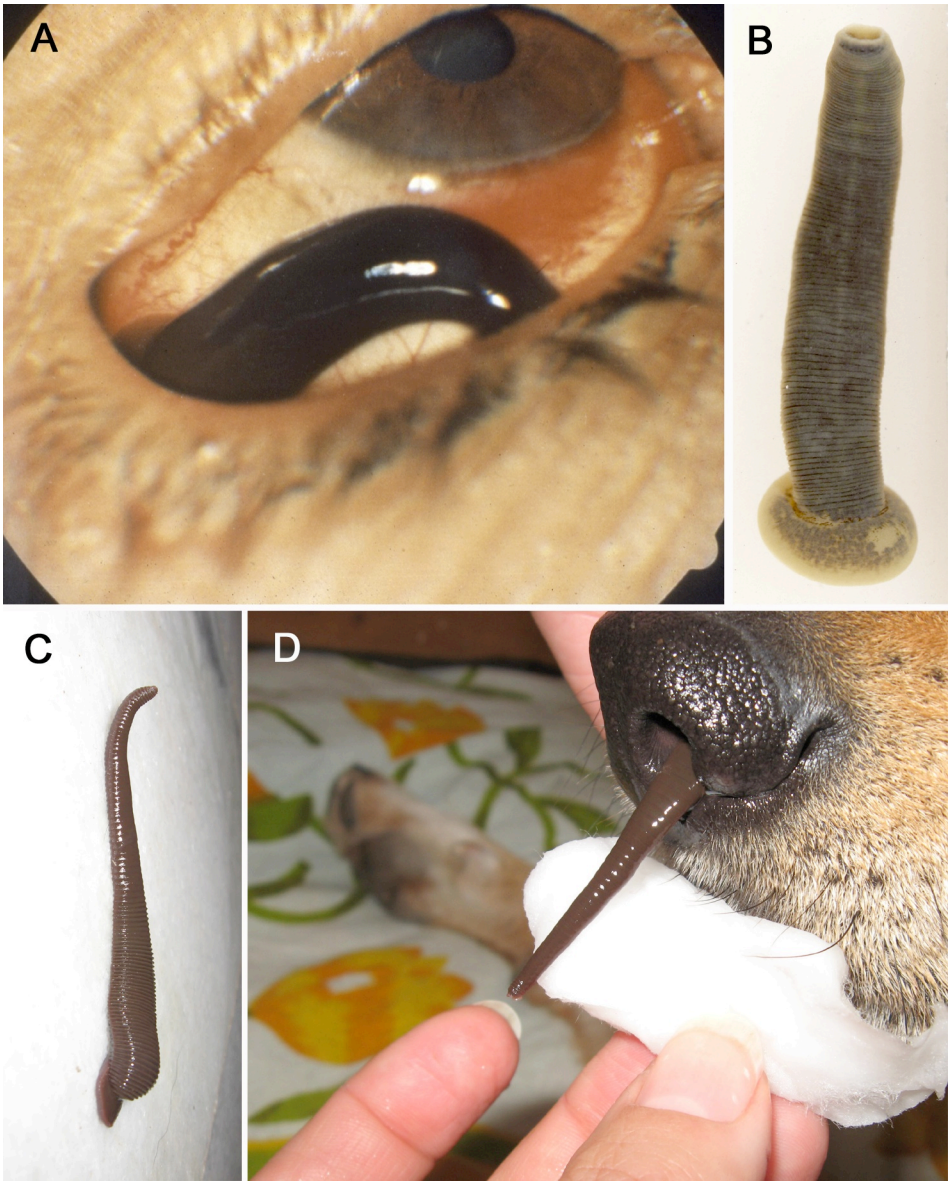
Most people realize that they are being parasitized by a leech upon finding the worm attached to their skin. Disturbingly, leeches occasionally enter human orifices – a condition known as mucosal, orifical, vesical, or internal hirudiniasis depending on the localization of the leech (Figure 3). While most bloodfeeding leeches feed as ecto-parasites 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, etal., 2008). Underlying conditions, such as coagulation disorders or secondary bacterial infections can cause a patient's condition to escalate from relatively minor to life-threatening 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 for orificial hirudiniasis in relation to the amount of time such animals spend at

FIGURE 3.

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

Figure 3.



leech-inhabited watering holes (Harding and Moore, 1928). 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 [e.g., (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

Leeches 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 this study. Specimens of *Pintobdella chiapasensis* were collected from forest streams leading to the lakes of Montebello, State of Chiapas, Mexico between 6 and 18 July, 2008. One *Myxobdella annandalei* was received in December, 2008 from Dharamsala, India. Tissue samples of *D. ferox* were collected on 13 April, 2008 in Taiwan and provided for sequencing. 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 SPOT-RT digital camera. Drawings were made by superposition of vector art over images placed in Adobe Illustrator® 10 and Adobe Photoshop® 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; Predini, 2005; Whiting, 2002; Simon *et al.,* 1994; Folmer *et al*., 1994) and are listed in Table X. Amplification reactions of gene fragments were conducted using either Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ) with 0.5 μl of each 10μM primer, 1 μl DNA template, and 23 μl Rnase-free H2O (total volume 25 μl), or homemade Taq with 1.0 μl Taq, 2.5 μl MgCl, 2.5 μl 10x Buffer A, 1.0 μl dNTPs, 0.5 μl of each 10 μM primer, 2.0 μl template, and 15 μl H_2O (total volume 25 μl). PCR reactions were performed in an Eppendorf Mastercycler. The following amplification protocols were used: for 18S, 94°C (1 min) followed by 35 cycles of 94°C (30 sec), 49°C (30 sec), 68°C (2 min 30 sec) and final extension at 68°C (1 min); for 28S and 12S, 94°C (5 min), followed by 39 cycles of 95°C (1 min), 52°C (1 min), 70°C (1 min) and final extension of 72° (7 min); for COI, 94°C (1 min), followed by 30 cycles of 94°C (30 sec), 48°C (30 sec), 68°C (45 sec), 68°C (1 min) and final extension of 68°C (1 min). PCR amplification products were purified with AMPure™ (Agencourt Bioscience Corporation). Cycle sequencing reactions were performed with an Eppendorf Mastercycler® using 1 μl Big Dye™ Extender

Table 2: Primers used for gene fragment amplification and sequencing.

Gene	**Primer Name**	**Primer Sequence**	**Citation**
Nuclear gene fragment		5' to 3'	
18S rDNA			
	A	AACCTGGTTGATCCTGCCAGT	Apakupakul *et al.*, 1999
	L	CCAACTACGAGCTTTTTAACTG	Apakupakul *et al.,* 1999
	C	CGGTAATTCCAGCTCCAATAG	Apakupakul *et al*., 1999
	Y	CAGACAAATCGCTCCACCAAC	Apakupakul *et al*., 1999
	O	AAGGGCACCACCAGGAGTGGAG	Apakupakul *et al.*, 1999
	B	TGATCCTTCCGCAGGTTCACCT	Apakupakul *et al*., 1999
28S rDNA			
	28srD1a	CCCSCGTAAYTTAAGCATAT	Prendini *et al*., 2005
	28sB	TCGGAAGGAACCAGCTAC	Whitming, 2002
	28sA	GACCCGTCTTGAAGCACG	Whitming, 2002

Table 2 continued

	28sBout	CCCACAGCGCCAGTTCTGCTTACC	Prendini *et al*., 2005
	28srD5a	GGYGTTGGTTGAAGCACG	Whitming, 2002
	28srd7b1	GACTTCCCTTACCTACAT	Whitming, 2002
Mitochondrial gene fragments			
Cytochrome *c* oxidase I (COI)	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.*, 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.,* 1994
12S rDNA	12Sa	AACIIGGATTAGATACCC	Simon *et al*., 1994
	12Sb	GAGAGTGACGGGCGATGTGT	Simon *et al*., 1994

Buffer v3.1, 1 μl of 1 μM primer and 3 μl of cleaned PCR template (13 μl total volume). Sequences were purified by 70% isopropanol/70% ethanol precipitation and analyzed with an ABI PRISM® 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 1. Alignments of all genes were accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004) applying default settings.

Phylogenetic analyses

A total of 17 species comprising 19 terminals were used in the analyses with *Hirudo medicinalis* specified as the outgroup in the analyses (Table 2). Phylogenetic analyses were conducted using two approaches: Parsimony and Bayesian Inference (BI). Parsimony analyses were conducted in TNT v 1.1 (Goloboff *et al.*, 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 Huelsenbeck, 2003). The data were partitioned by gene for 18S, 28S, 12S, and by codon position for COI (three partition; 3p). A GTR+I+Γ model was applied to each unlinked data partition based on the Akaike Information Criterion via ModelTest v. 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). 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. Split frequencies of the standard deviation of simultaneous BI analyses were well below 0.01.

Table 3: Taxa, collection localities, and GenBank accession numbers used for the phylogenetic analysesof the family Praobdellidae. Type species of the genera are indicated with an asterisk.

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

Table 3 continued

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

The BI analyses burned in before 100,000 generations. 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: 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 Martín, 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 45 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 Junín, 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 Junín, 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 five annulate. Cephalic eyespots, 5 pair in parabolic arc. Anus between last annulus and caudal sucker. Caudal sucker wider than the posterior of body. Reproductive organs micromorphic. Feeds from mucosal surfaces of mammals.

TYPE SPECIES: *Tyrannobdella rex* n. sp.

Tyrannobdella rex n. sp.

HOLOTYPE: Preserved body length 445 mm, maximal width 9.5 mm, fixed and stored in 90% ethanol. Collected at La Merced Chanchamayo Junín, 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 Martín and Ayacucho, Perú in 1997 by Dra. María Beltrán; 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 teeth forming a single (i.e., monostichodont) row (Figure 4a,c). Two of eight teeth may be sub-cuticular and observable only with compound microscopy (Figure 4c). Pharynx muscular and tubular. Crop from IX to XXV, first nine cecal pairs in IX through XIX, post-caecae 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. Papillae absent. Oral sucker small and velar (Figure 4b). Oral opening central and dorsoventrally oval. Posterior sucker large, wider than posterior of body (Figure 5a). 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 uniannulate 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 5b). Male gonopore on XI b6, female gonopore on XII b6, gonopores separated by 1/2 + 4 + 1/2 annuli. Nephropores 17 pairs on posterior margins of annulus b2 in each somite on the ventral surface, from VIII-XXV.

Male and female reproductive organs extremely micromorphic, same size as or smaller than ventral ganglia (Figure 5c). Penis sheath U-shaped, with initial posterior disposition and subsequent anterior procurrent portion leading to small epidydimis. Ejaculatory bulbs absent. Vagina present, U-shaped, no common oviduct and oviducts half the size of vagina. Ovaries simple, bulbous.

ETYMOLOGY: *Tyrannobdella*: tyrannos (G.) - "tyrant" + bdella (G.) - "leech"; *rex* (G.) - "king"

FIGURE 4.

Compartive Jaw Morphology. (A) Stereomicrograph of *Tyrannobdella rex* jaw with large teeth. (B) *Tyrannobdella rex* anterior sucker with velar mouth. (C) Compound micrograph in lateral view of eight large teeth of *T. rex*. (D) Lateral view of jaw of *Limnatis paluda* illustrating typical size of hirudinoid teeth.

Figure 4.

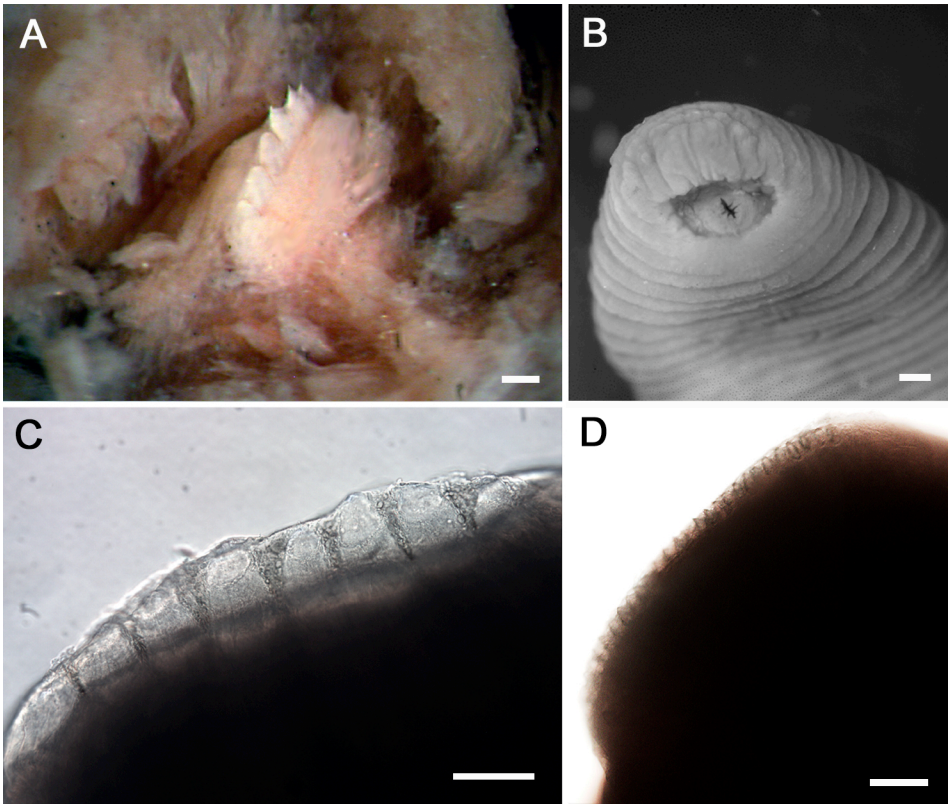
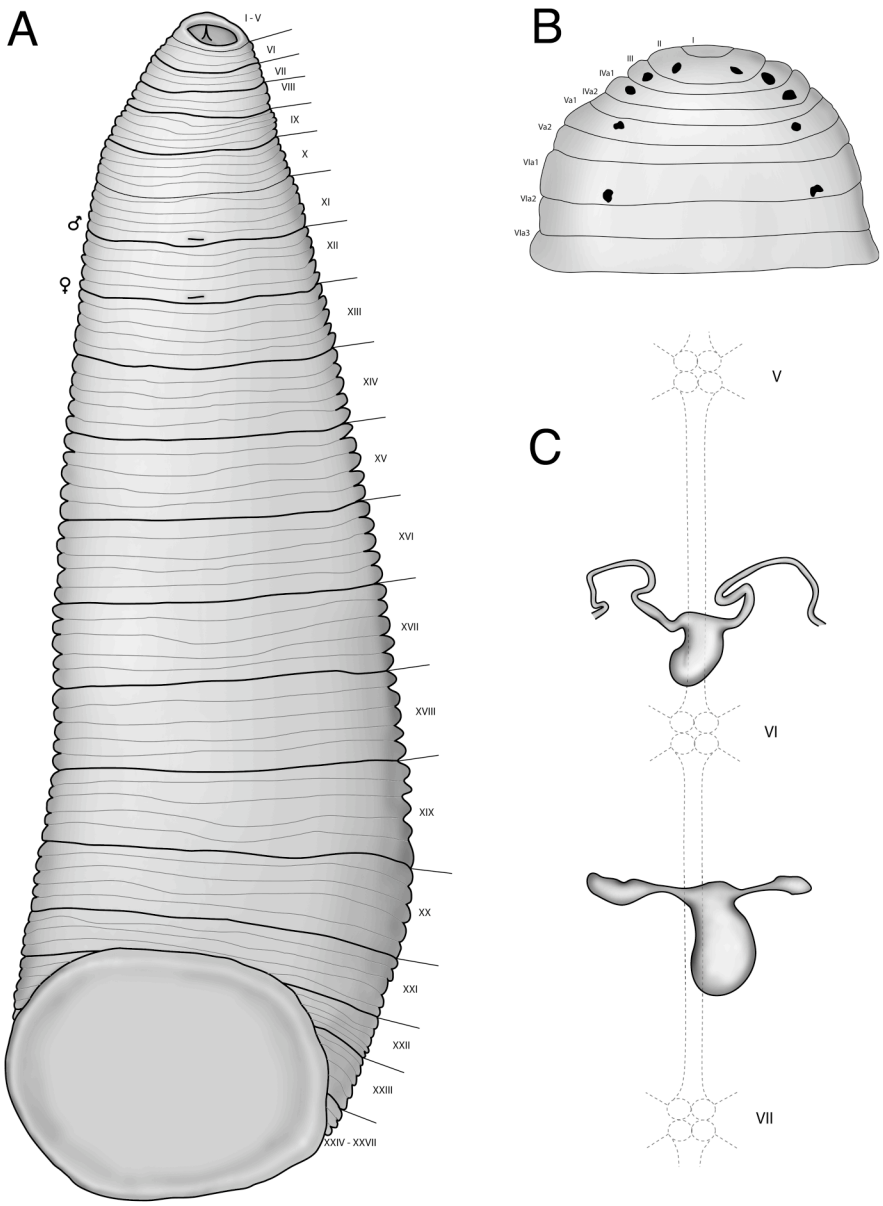


FIGURE 5.

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.

Figure 5.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VI
VII

REMARKS: No other leech species is known to have but a single armed jaw. The reduced number of teeth, a caudal sucker wider than the width 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, Limantis,* and *Limobdella*. *Pintobdella chiapasensis* (Caballero, 1957) similarly has few (six) teeth per jaw, albeit for each of three jaws (Caballero, 1957). *Dinobdella ferox* also possesses three small jaws entirely without teeth (Harding and Moore, 1927), leaving *T. rex* n. sp. unique in possessing only one jaw with eight large teeth.

Members of the genus *Limnobdella* have two pairs of equal crop caeca per somite and an extended female reproductive structure (Caballero, 1932, 1933, 1934, 1941). In comparison, *T. rex* has one pair of crop caeca per somite except in the first two chambers of the crop, which have two unequal crop caeca per somite, but overall the relatively simple structure of the reproductive system resembles that of *Limnobdella* species, although with 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* have two equal pairs of crop caeca 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, 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 five-annulate somites with only the three most posterior somites having partially subdivided annuli. Species in the genus *Myxobdella* have gonopores separated by 5 or 5+1/2 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 7 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 be –19830.23. Phylogenetic analyses recovered identical topologies regardless of phylogenetic method (Figure 6).

A clade of hirudinoid leeches (including genera *Limnobdella*, *Limnatis*, *Dinobdella*, *Myxobdella*, *Pintobdella* and *Tyrannobdella*) distinguished by their propensity for feeding on mammalian mucous membranes was recovered as monophyletic with good 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 also closely related (bs=100; pp=1.00). Representatives of the Old World genus *Limnatis* formed a monophyletic clade sister to the New World 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

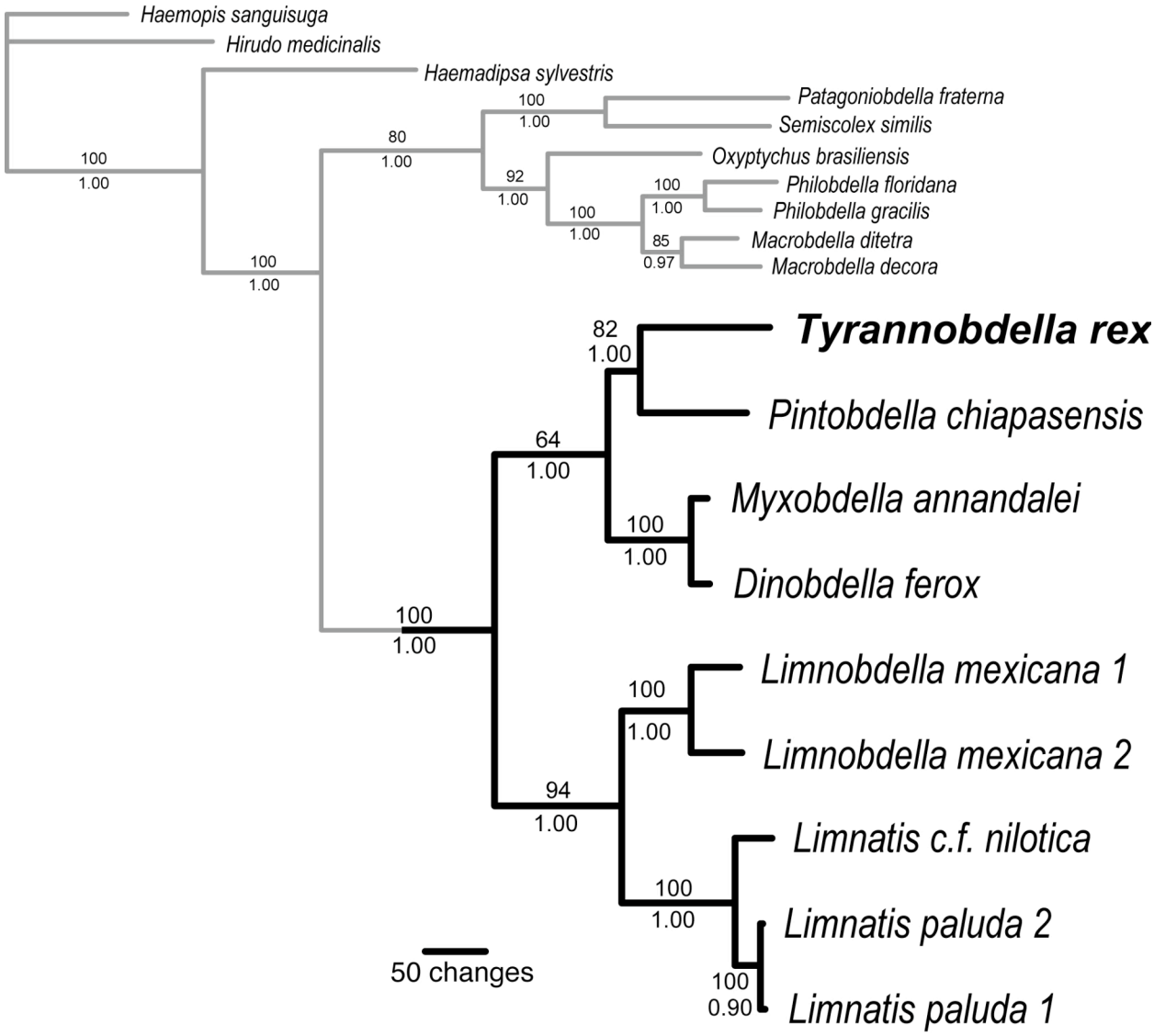
Hirudinoid leeches that show a preference for mammalian mucosal surfaces all appear to have descended from a single common ancestor millions of years ago. Among these, the new species *T. rex* n. sp. is the first from South America and one with a particularly unpleasant habit of infesting humans (Beltran *et al.*, 1997). Another New World orifice-invading leech known from southern Mexico, *P. chiapasensis*, which is the sister taxon of *T. rex* n. sp., 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, 1932). The consistency with which pain was reported by its victims may relate to the relatively enormous teeth *T. rex* n.sp. has on its one 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 (Hamid and Mohd Nar, 1996). 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 medicinal 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 would be indicated in all cases of orificial hirudiniasis.

FIGURE 6.

Single most parsimonious tree (CI= 0.633, RI= 0.625) based on combined 18S rDNA, 28s rDNA, 12s rDNA, and COI datasets. The family Praobdellidae (in black) forms a well supported monophyletic group of leeches that exhibits a predilection for mammalian mucosa. Branches are drawn proportional to amount of change. Bootstrap values are above each node. Posterior probabilities are below each node.

Figure 6.


Haemopis sanguisuga
Hirudo medicinalis
Haemadipsa sylvestris
Patagoniobdella fraterna
Semiscolex similis
Oxyptychus brasiliensis
Philobdella floridana
Philobdella gracilis
Macrobdella ditetra
Macrobdella decora
Tyrannobdella rex
Pintobdella chiapasensis
Myxobdella annandalei
Dinobdella ferox
Limnobdella mexicana 1
Limnobdella mexicana 2
Limnatis c.f. nilotica
Limnatis paluda 2
Limnatis paluda 1
50 changes

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 *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: 1) reduced number of teeth– less than 12 in *Myxobdella, Dinobdella, Praobdella, Tyrannobdella,* and *Pintobdella*, and less than 40 in *Limnatis* and *Limnobdella*, 2) the caudal sucker is wider than the width of the body, 3) no post-anal annuli, and 4) 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). In spite of their obvious preference for mammalian mucosa, at least one species of praobdellid has been reported feeding opportunistically on amphibians when mammals are not available (Lukin, 1976).

The systematics of the family Praobdellidae (*sensu* Sawyer, 1986) has been plagued by ill-defined groups and 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 conundrums 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* as suggested in Borda and Siddall (2004b).

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, 1896a). Only external morphology was mentioned in Blanchard's (1896a) description and the type specimens have 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 *Myxobdella 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.

AUTHORS' CONTRIBUTIONS

AJP, AOF, and MES contributed to the conception and design of the experiments, data collection, and data analyses. AJP, RAB, GPG, MB, YTL, and MES contributed reagents, materials, or analytical tools to the study. AJP, RAB, AOF, and MES contributed to the preparation of the manuscript.

CHAPTER 4

Redescription, phylogenetic placement, and taxonomic reassignment of *Mesobdella lineata* (Sciacchitano, 1959) (Hirudinida: Arhynchobdellida)

(Adapted from Phillips AJ, Oosthuizen JH, and Siddall ME. 2011. Redescription, phylogenetic placement, and taxonomic reassignment of *Mesobdella lineata* (Sciacchitano, 1959) (Hirudinida: Arhynchobdellida) *American Museum Novitates*).

INTRODUCTION

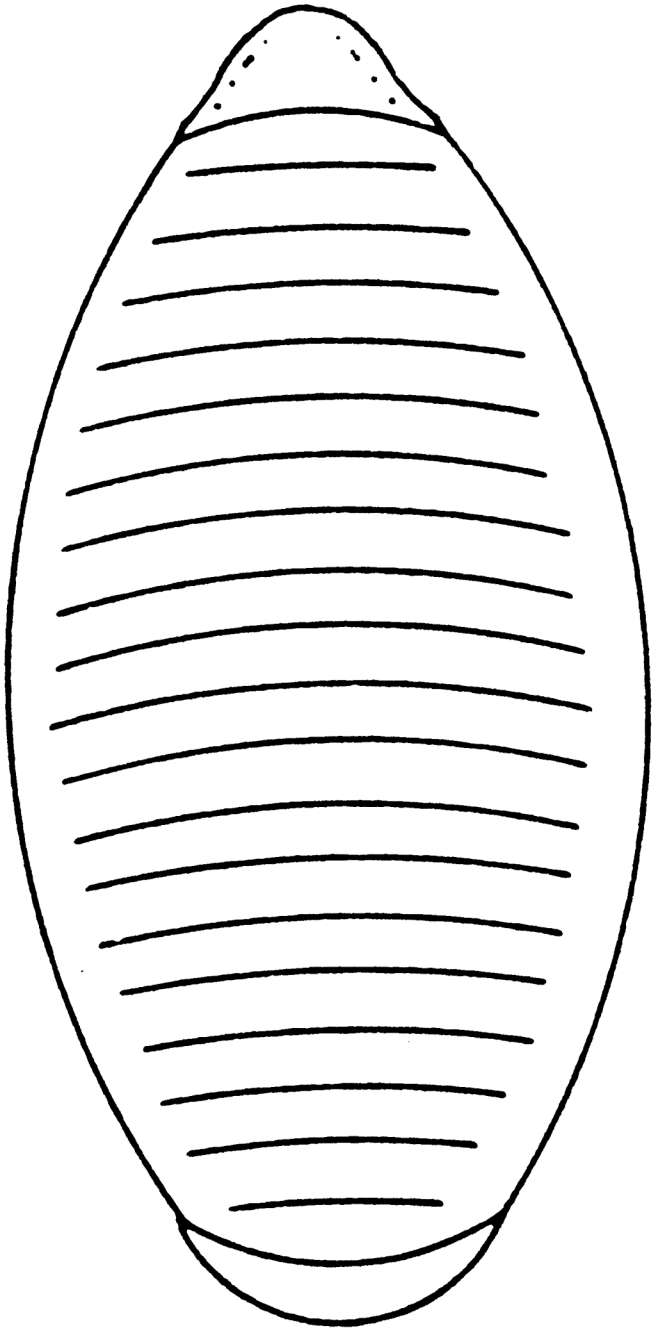
Terrestrial leeches have been recorded on all continents except Antarctica (Borda *et al.*, 2008; Sawyer, 1986), with only one trignathous species described from Africa: *Mesobella lineata* Sciacchitano, 1959. *Mesobdella lineata* was placed in the genus *Mesobdella*, and in the family Haemadipsidae, with consideration of its terrestrial habit and Sciacchitano's (1959) observation of tri-annulate mid-body somites. Leech taxonomists active at the time and since have repudiated much of Sciacchitano's work (Moore, 1939, 1958; Oosthuizen, 1978, 1979, 1982, 1989; Sawyer, 1986), in which he described numerous species and genera without sufficient diagnostic characters (Sciacchitano, 1935, 1936, 1941, 1952, 1960). Many of Sciacchitano's available names, particularly of the family Hirudinidae, have subsequently been synonomized with other taxa or are considered *species inquerindae* (Soós, 1969; Sawyer, 1986). Sciacchitanos' (1959) description of *M. lineata* (1959) was based upon mostly external morphological characters observed in specimens at the Museum of Zoology at Lund University, Lund, Sweden that were collected during a university sponsored expedition in 1950-1951. Aside from a trivial diagram of the external characters (Figure 7; redrawn here for clarification), very little was known about the morphology and natural history of this leech when described.

Since its description, *M. lineata* has been passively carried along through taxonomic recategorizations of the genus, yet without morphological examination (Ringuelet, 1972; Sawyer, 1986; Borda *et al.*, 2008). Even though it was an exception in geographic distribution and several morphological characters, Blanchard (1849) placed *Mesobdella* within the family Haemadipsidae. Moore (1946) examined *Mesobdella gemmata* (Blanchard, 1849), type species of the genus, and found it to be morphologically similar to *Xerobdella lecomtei* (Frauenfeld,

FIGURE 7.

Sciacchitano's (1959) illustration of *Mesobdella lineata* redrawn from the original description.

Figure 7.



1868) and *Diestecostoma* species, but the report of haemadipsine reproductive structures by Ringuelet (1943) caused Moore to hesitate in suggesting the subfamily Xerobdellinae as including *Xerobdella* and *Diestecostoma,* but not *Mesobdella*. Ringuelet (1972) placed *Mesobdella* into its own family, Mesobdellidae. Disregarding previous taxonomy, Sawyer (1986) returned all bloodfeeding terrestrial leeches to Haemadipsidae and separated the taxa into duognathous and trignathous series, the later of which included *Mesobdella*.

The systematics of both haemadipsoid and hirudinoid leeches has been substantially altered in consideration of molecular phylogenetic work and the recognition of various morphological synapomorphies (Apakupakul *et al.*, 1999; Borda and Siddall, 2004b; Phillips and Siddall, 2005; Borda *et al*., 2008). With the addition of molecular data, Trontelj *et al*., (1999) and Borda and Siddall (2004b) found *X. lecomtei* and *M. gemmata* to not be sister to the bloodfeeding terrestrial leeches of the IndoPacific (i.e. Haemadipsidae). *Mesobdella* was returned to the subfamily Xerobdellinae, which was at the same time elevated to family Xerobdellidae, with additional morphological and molecular examinations of more taxa by Borda *et al*., (2008).

Until now, morphological and molecular investigations of *Mesobdella* have primarily focused on *M. gemmata* whereas *M. lineata* has had little attention. *Mesobdella lineata* stands apart as the sole African species in Xerobdellidae, yet inhabiting similar temperate latitudes as its South American counterpart (*M. gemmate*). Herin, re-examinations of internal and external morphological characters of the holotype as well as additional specimens of *M. lineata* were performed. Phylogenetic methods were used to analyze morphological characters and taxonomic reassignment is made.

MATERIALS AND METHODS

Specimens examined include the holotype (Museum of Zoology, Lund University, Lund, Sweden; catalogue number L951/4421) and a paratype specimen (Museum of Zoology, Lund University, Lund, Sweden; catalogue number L951/3643). On 7 March 1975 three specimens of *M. lineata* were collected from moist vegetation near streams at Dap Naude Dam (23˚ 48' 30.84"S 29˚ 58' 12.18"E), Limpopo Province, South Africa, 6.7 km West of the type locality and an additional five specimens were collected between 27 December and 31 December 1975 from the same locality (American Museum of Natural History, New York, NY, USA; 4502, 4503, 4726, 4728, 4729, 4730, 4731, 4733). Leeches were either relaxed in 30% ethanol, fixed and preserved in 70% ethanol or fixed in 10% formalin and preserved in 5% formalin. Examination of external and internal morphology was accomplished with a Nikon® SMZ645 stereomicroscope on whole and dissected specimens. Roman numerals in all cases indicate body somites (e.g., prostomium and peristomium are I and II) and annuli receive alphanumeric designations as per Sawyer (1986). Photographs were taken with a Nikon® Coolpix® 5000 digital camera and drawings were made by hand. Illustrations were digitized using Adobe® Illustrator® 10.

Representative taxa (Table 3) of the families Haemadipsidae, Xerobdellidae, Semiscolecidae, Macrobdellidae, Hirudinidae, and Praobdellidae were included with *M. lineata* in a morphological matrix of 32 characters (Table 4) adapted from Borda and Siddall (2004b). Parsimony analyses of the morphological matrix employed PAUP* 4.0b10 (Swofford, 2002). Heuristic searches used 500 replicates of the data set with random taxon addition and tree-bisection-reconnection branch swapping. Parsimony jackknife (jac) values were obtained using

Table 4: Taxa included in the phylogenetic analyses of *Mesobdella lineata*.

Americobdellidae	*Americobdella valdiviana* (outgroup)
Cylicobdellidae	*Cylicobdella coccinea*
Haemadipsidae	*Haemadipsa sylvestris*
Hirudinidae	*Haemopis terrestris*
	Aliolimnatis michaelseni
	Hirudo medicinalis
	Hirudinaria manillensis
Macrobdellidae	*Oxyptypchus striatus*
	Macrobdella decora
	Philobdella floridana
Praobdellidae	*Limnatis paluda*
	Tyrannobdella rex
	Myxobdella annandalei
	Dinobdella ferox

Table 4 continued

	Limnobdella mexicana
Semiscolecidae	*Semiscolex similes*
	Patagoniobdella variabilis
Xerobdellidae	*Xerobdella lecomtei*
	Mesobdella gemmata

Table 5: Morphological characters adapted from Borda and Siddall (2004b) with eight additional characters.

Character 1	Muscular jaws (0) absent, (1) present
Character 2	Teeth arrangement (0) astichodont, (1) monostichiodont, (2) distichodont
Character 3	Number of jaws (0) agnathous, (1) monognathous, (2) duognathous, (3) trignathous
Character 4	Feeding habit (0) macrophagous, (1) haematophagous/sanguivorous, (2) omnivorous
Character 5	Salivary papillae (0) absent, (1) present
Character 6	Number of annuli with eyespots (0) none, (1) five
Character 7	Vaginal duct (0) absent, (1) present
Character 8	Vaginal caecum (0) absent, (1) present
Character 9	Ovisac shape (0) absent, (1) present
Character 10	Common oviduct (0) absent, (1) present
Character 11	Male atrium extended into elongated penis and sheath (0) absent, (1) present
Character 12	Penis shape (0) straight, (1) recurved

Table 5 continued

Character 13	Ejaculatory ducts (0) U-shaped, (1) S-shaped
Character 14	Accessory glands (0) absent, (1) present
Character 15	Intergonadal conducting tissue (0) absent, (1) present
Character 16	Testisacs per body somite (0) one pair, (1) two pairs
Character 17	Cocoons (0) brooded, (1) cemented, (2) spongy and deposited on land
Character 18	Mid-body nephropore(s) (0) ventromedial (1) ventrolateral
Character 19	Nephridia (0) single funnel apparatus, (1) multiple funnels in a ciliated organ
Character 20	Friction rays on caudal sucker (0) absent, (1) present
Character 21	Respiratory auricles (0) absent, (1) present
Character 22	Muscular ejaculatory bulbs (0) absent, (1) present
Character 23	Number of teeth per jaw (0) none, (1) 1-12, (2) 13-50, (3) 50 or more
Character 24	Number of post-anal annuli (0) none, (1) one
Character 25	Number of crop caeca per mid-body somite (0) none, (1) one, (2) two

Table 5 continued

Character 26	Number of annuli between third and fourth pairs of eyespots (0) none, (1) one, (2) two
Character 27	Number of annuli between fourth and fifth pairs of eyespots (0) none, (1) one, (2) two
Character 28	Number of annuli between gonopores (0) none, (1) one, (2) two, (3) three, (4) four, (5) five, (6) six [species with gonopores falling on the annulus rather than in the furrow resulting in non-whole numbers of annuli between gonopores are scored as the next highest whole number]
Character 29	Longitudinal furrow in upper lip (0) absent, (1) present
Character 30	Velar mouth (0) absent (1) present
Character 31	17^{th} pair of nephropores (0) fused into mid-ventral pore (1) two pores situated bilaterally on venter
Character 32	Caudal sucker (0) narrower than the width of the four most posterior body somites (1) wider than the width of the four most posterior body somites

random taxon addition and tree-bisection-reconnection branch swapping with 36% deletion and 100 psuedoreplicates. All characters initially were weighted equally and were non-additive. Additional searches were performed with implied weighting (Goloboff, 1993) varying values of *k* from 1 to 20. Despite several attempts, DNA was unable to be extracted from the specimens therefore limiting the analyses to include only morphological data.

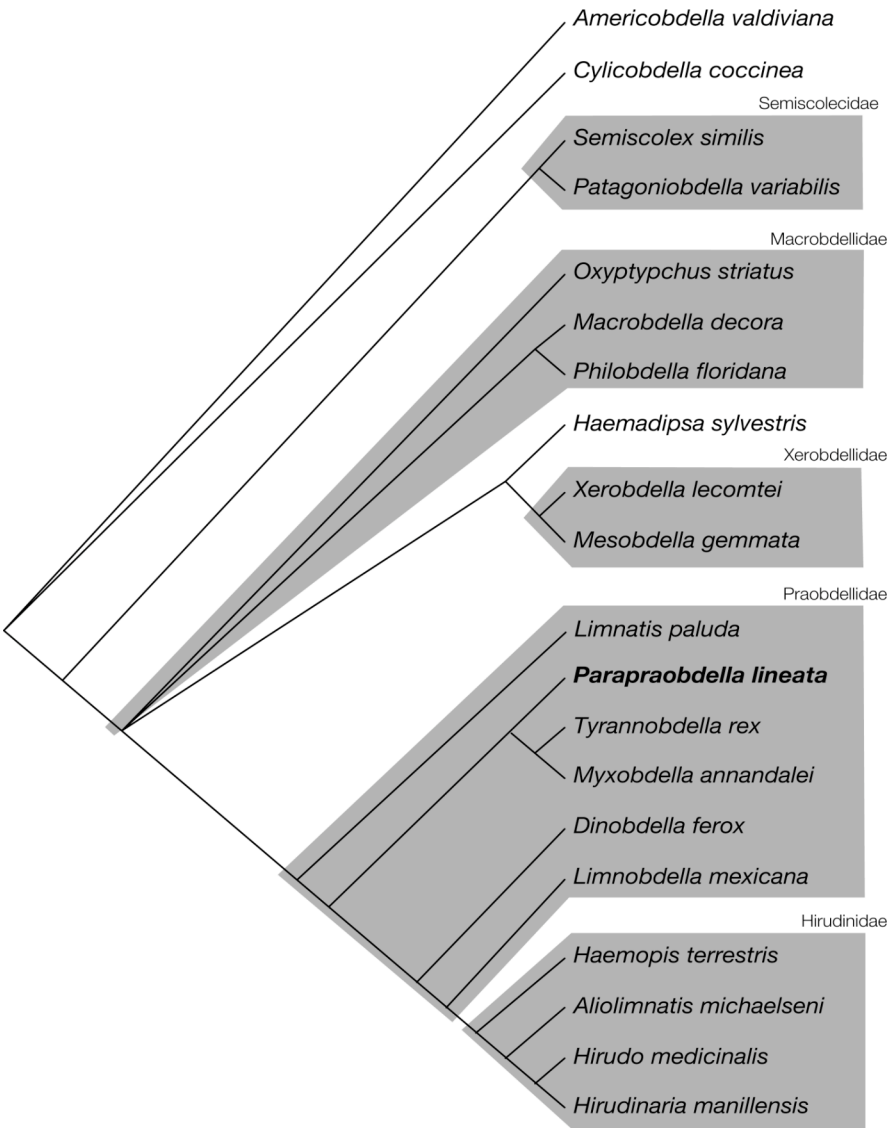
RESULTS

The morphological dataset analyzed under implied weighting yielded 12 equally parsimonious trees with a best score of –23.59329. The strict consensus tree (Figure 8) remained unchanged for values of *k* ranging from 1 to 20. *Parapraobdella lineata* was in a clade with *T. rex* and *Myxobdella annandalei* (Oka, 1917) among a paraphyletic Praobdellidae. The lack of resolution in parsimony analyses with unweighted characters is similar to previous analyses with unweighted morphological characters (Borda and Siddall, 2004b; Phillips and Siddall, 2005). *Mesobdella gemmate* clearly places with *Xerobdella* within Xerobdellidae although *M. lineata* places within Praobdellidae. This would render the genus *Mesobdella* polyphyletic. The presence of several morphological characers in *M. lineata* does not agree with the defining characteristics of Xerobdellidae and warrant a new genus within Praobdellidae to accommodate *M. lineata*.

FIGURE 8.

Strict consensus of 12 equally parsimonious trees obtained from 32 morphological characters with implied weighting.

Figure 8.


Americobdella valdiviana
Cylicobdella coccinea
Semiscolecidae
Semiscolex similis
Patagoniobdella variabilis
Macrobdellidae
Oxyptypchus striatus
Macrobdella decora
Philobdella floridana
Haemadipsa sylvestris
Xerobdellidae
Xerobdella lecomtei
Mesobdella gemmata
Praobdellidae
Limnatis paluda
Parapraobdella lineata
Tyrannobdella rex
Myxobdella annandalei
Dinobdella ferox
Limnobdella mexicana
Hirudinidae
Haemopis terrestris
Aliolimnatis michaelseni
Hirudo medicinalis
Hirudinaria manillensis

SUBORDER ARHYNCHOBDELLIDA BLANCHARD, 1894

FAMILY PRAOBDELLIDAE (SAWYER, 1986)

Parapraobdella, new genus, Phillips *et al.*, 2011

Figure 9 - 10

DESCRIPTION: Trignathous, monostichodont. Longitudinal furrow in dorsal lip of oral sucker. Complete somite five-annulate. Eyespots dorsal, five pair in broad arch. Crop caeca simple with one pair of caecaeper somite, post-caeca extending bilaterally. Intestine simple, acecate. Anus between last annulus and caudal sucker. Caudal sucker wider than four most posterior somites of body. Reproductive organs micromorphic. Nephridia ventral and bilateral in all cases.

TYPE SPECIES: *Parapraobdella lineata* (Sciacchitano, 1959)

ETYMOLOGY: *Para* – alongside, close to the genus *Praobdella.*

Parapraobdella lineata (Sciacchitano, 1959)

Figure 9 - 10

RE-DESCRIPTION: Holotype, body length 6.4 mm, maximal width 3.7 mm; dissected. Body muscular. Dorsal surface with transversal bands of light olive green on two annuli and dark olive green on the following three annuli, repeated along the length of the body, although color pattern faded in preserved specimens (Figure 9, Figure 10a). Fifteen five-annulate mid-body somites from somite X to XXIV; b1 and b6 diminutive falsely leading toward a triannulate

FIGURE 9.

Whole body photograph including the color pattern in a preserved specimen.

Figure 9.

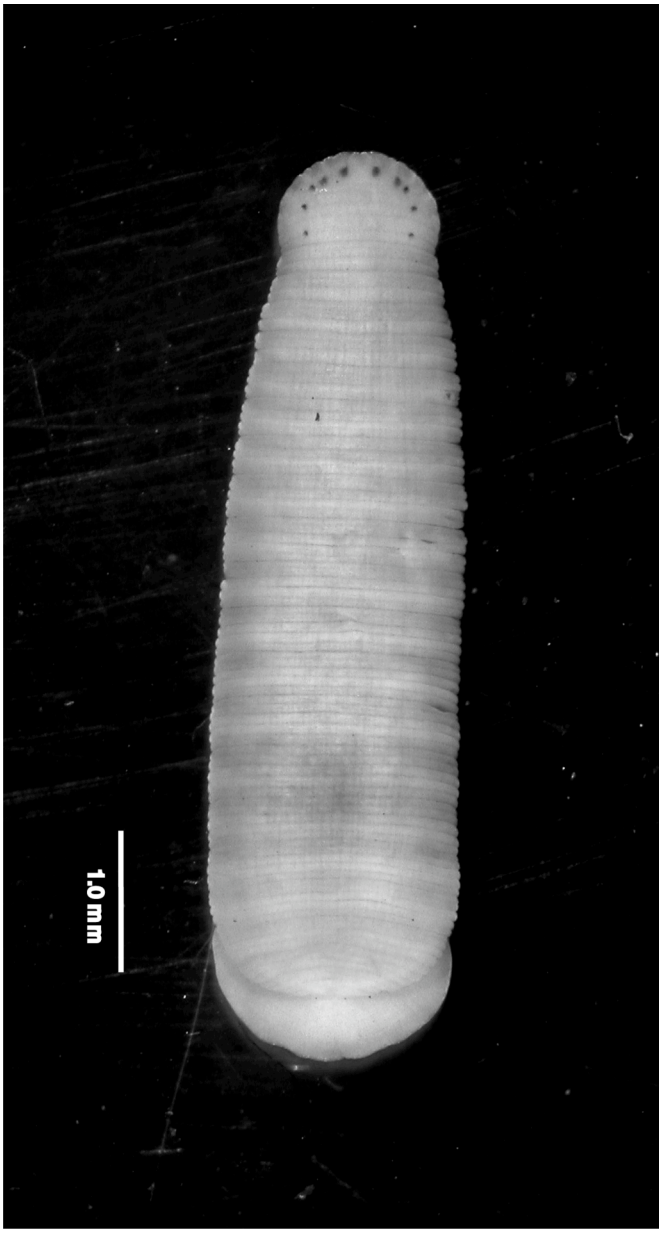
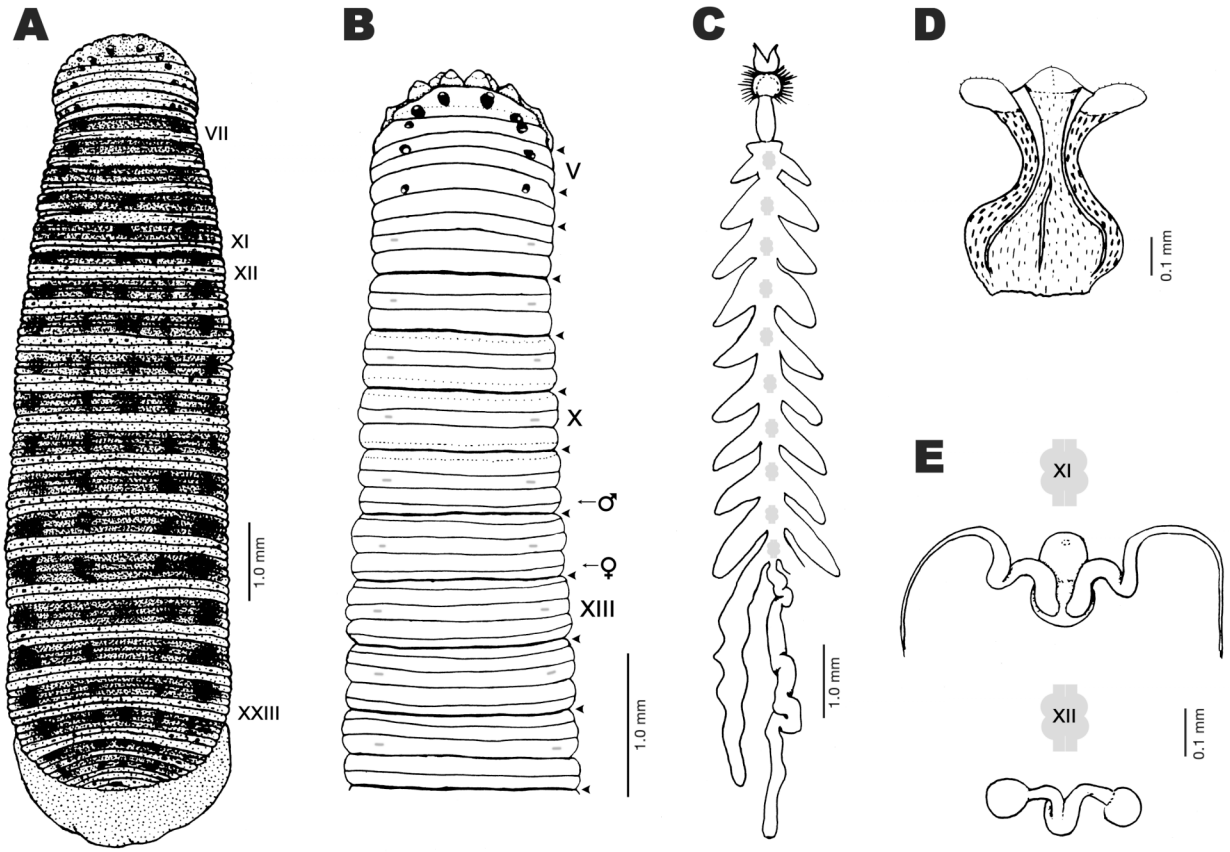


FIGURE 10.

Parapraobdella lineata n. gen. A) Dorsal surface of body with somites indicated by Roman numerals. B) Dorsal view of the head showing five pairs of eyespots, position of the first nine pairs of nephridia, sensillae around the oral opening, and location of male and female gonopores on the ventral side. Intersegmental furrows indicated with small black arrows. C) Internal gastric system. D) Jaws with eight small teeth and pharynx. E) Internal male and female reproductive organs relative to somatic ganglia XI through XII.

Figure 10.



appearance. Somites I – III uniannulate, somite IV and V biannulate, somite VI biannulate with anterior annulus subdivided dorsally, somite VII triannulate with annulus b6 subdivided ventrally, somite VIII quadrannulate, somite IX quadrannulate with annulus a3 subdivided dorsally, somites X – XXIV five-annulate, somite XXV triannulate, somite XXVI biannulate, and somite XXVII uniannulate. Eyespots arranged dorsally in five pairs in a broad arch. Pair 1 anterior on somite III, remaining eyespots situated laterally on contiguous annuli of a pair on each annulus of somite IV and anterior annulus of somite V. Pairs 4 and 5 separated by one annulus (IVa3; Figure 10b). First two pairs of eyespots with pigment concentrated anterolaterally in eyespot, last three pairs with pigment concentrated posterolaterally in eyespot. Dorsal lip of oral sucker with medial longitudinal furrow and nine sensillae, which are more pronounced in some specimens (Figure 10b). Oral velum absent. Sensillae obvious in live condition, mostly obscure in preserved specimens. Nephropores 17pairs on ventral surface situated bilaterally from somite VII to somite XXIII; in complete somites, just posterior to b2/a2 furrow and 17th pair in same position as others. Caudal sucker round, wider than the four most posterior somites without friction rays. Male gonopore at XI b5/b6 in furrow, female gonopore at XII b5/b6 in furrow, five annuli between gonopores.

Jaws trignathous, monostichodont, no salivary papillae (Figure 10d). Jaws moderate in size, low, rounded. Teeth located in shallow open grooves, eight teeth per jaw. Pharynx short, terminating in somite VIII (Figure 10d). Internal muscular ridges, six arranged in dorsomedial and ventrolateral pairs, each pair fusing at base of each jaw. Esophagus narrow, lumen wide, tubular, tapering posteriorly to junction with crop. Pharynx terminating in somite VIII followed by an acaecate compartment, which reaches up to the middle of somite IX. Crop, from somite IX to somite XX, first ten pairs crop caeca simple and equal size, post-caeca not folaceous

extending bilaterally posterior to somite XVI. Intestine tubular, acaecate, joining rectum at somite XXIV (Figure 10c). Rectum thin-walled, tubular. Anus in furrow between last annulus and caudal sucker.

Internal male and female reproductive organs micromorphic. Male atrium bulbous, not extended into elongated penis or sheath (Figure 10e). Epididymis and ejaculatory bulbs absent. Vasa deferentia insert to dorsal median of male atrium. Vasa deferentia pass anterolaterally with two bends in an S-like shape before descending to testisacs. Vagina short. Common oviduct absent. Ovaries simple, bulbous.

HABITAT: Terrestrial. Associated with moist surroundings, such as vegetation by streams (Oosthuizen, pers. obs.).

TYPE LOCALITY: Debegeni Falls (23˚ 48' 49.71"S 30˚ 01' 44.87"E), Limpopo Province, South Africa (previously Transvaal).

REMARKS: *Mesobdella lineata* is not consistent with any other described genus and warrants the creation of the new genus, *Parapraobdella*. The species does not belong in the genus *Mesobdella* within Xerobdellidae: it is five-annulate, not three-annulate; it lacks the xerobdellid mid-ventral nephridial pore; it does not have friction rays on the caudal sucker; the male atrium does not extended into a penis or sheath; it lacks epididymes (Sawyer, 1986; Borda *et al.*, 2008). *Parapraobdella lineata* differs from *X. lecomtei*: it does not have epididymes or defined ejaculatory bulbs; it lacks a seminal receptacle and the associated accessory pore (Borda *et al*., 2008). The species does not belong in the family Haemadipsidae: it lacks respiratory auricles; the eyespots are not arranged in the "haemadipsine" ocular arch; it has a caudal sucker that is round and lacks friction rays; the median reproductive apparatus is small compared to the highly

robust apparatus typical of haemadipsids (Sawyer, 1986; Borda *et al*., 2008). The species is also not of Hirudinidae: it does not have muscular ejaculatory bulbs adjacent to the epididymes; the male atrium is not extended into an elongated penis and sheath; it has eight teeth compared to 60-150 teeth seen in Hirudinidae (Harding and Moore, 1927; Moore, 1939; Richardson, 1969). The species is not a macrobdellid: it does not have accessory glands, muscular ejaculatory bulbs, or annuli between the anus and the caudal sucker; it has one rather than two crop caeca per somite; it has eight teeth compared to the 30-60 teeth observed in macrobdellids (Richardson, 1969; Phillips and Siddall, 2005).

The caudal sucker being wider than the four most posterior somites of the body, possessing only eight teeth, micromorphic reproductive structures, and no post-anal annuli all point to a placement of this genus and species within the family Praobdellidae along with, yet distinct from, the genera *Praobdella, Myxobdella, Dinobdella, Limnatis, Limnobdella, Pintobdella,* and *Tyrannobdella* (Phillips *et al.*, 2010). Members of the genus *Praobdella* have more than five annuli between the gonopores, while *P. lineata* has exactly five annuli between the gonopores (Sawyer, 1986). Whereas *P. lineata* has three jaws armed with eight small teeth and does not have a velar mouth, it is unlike *Pintobdella chiapasensis* (Caballero, 1958) which has three jaws with six large teeth without a velum and unlike *Tyrannobdella rex* (Phillips *et al*., 2010) which has only a single jaw with eight large teeth and a velum (Caballero, 1958; Phillips *et al*., 2010). The new genus *Parapraobdella* is distinct from *Dinobdella ferox* (Moore, 1927) that does not possess teeth and from the distichodont genera *Myxobdella* and *Praobdella* (Moore, 1927, 1939; Sawyer, 1986).

In Sciacchitano's (1959) original description, he noted the presence of red papillae along the dorsal surface of the body. No such coloration of the papillae was observed in this study and

although the specimens examined here were preserved, several have retained their pigmentation. Also, Sciacchitano (1959) described 40 black longitudinal lines on the ventral surface of the specimens he examined, but it seems that he was describing the internal longitudinal muscles of the body wall.

DISCUSSION

This redescription based on morphological characters indicates that the species formerly known as *Mesobdella lineata* requires a new genus, *Parapraobdella* (Phillips *et al.*, 2011). Sciacchitano (1959) placed this species in the genus *Mesobdella* on the basis of its terrestrial habitat and apparent possession of three annuli per mid-body somite. Examination of Sciacchitanos' (1959) types and the Dap Naude Dam material revealed five annuli per mid-body somite, not three. In complete somites, b1 and b6 annuli are smaller than annuli b2, a2, and b5 leading to a tri-annulate appearance.

Praobdellidae is the best family to accommodate this genus and species when considering the wide caudal sucker, eight teeth per jaw, internal reproductive morphology, and lack of post-anal annuli of *P. lineata*. When first proposed by Sawyer (1986) as a subfamily within the distichodont series, subfamily Praobdellinae was distinguished as having a velar mouth, mesomorphic reproductive structures, most species with a large caudal sucker, typically parasitic in nasal passages in mammals, and distributed in Africa and southeast Asia. The subfamily was elevated to family Praobdellidae and expanded by Phillips *et al.* (2010) to include *Praobdella, Myxobdella,* and *Dinobdella* (*sensu* Sawyer, 1986) with the addition of *Limnobdella, Limnatis, Pintobdella*, and *Tyrannobdella* strongly supported by molecular data. This expansion was also supported by morphological and behavioral observations: 0-12 teeth per jaw in most members of

the group but 30-40 teeth per jaw in *Limnatis* and *Limnobdella*, the caudal sucker being wider than the four most posterior somites of the body, a lack of post-anal annuli, and primarily feeding from mammalian mucous membranes (Phillips *et al.*, 2010). This unique combination of morphological and behavioral characters encompasses the diversity of morphologies seen across these eight genera as well as defines the family.

Phylogenetic analyses of the morphological data while inconclusive when left unweighted, are shown to carry more information in favor of *P. lineata*'s place among the Praobdellidae than against it. While the family is not supported as monophyletic here, the clade has been well supported with DNA sequence data in previous studies (Phillips *et al.,* 2010). The feeding preference of *P. lineata* has not yet been observed, yet the crop is caecate and several specimens were filled with what appears to be blood. The teeth and jaws are also equipped to allow bloodfeeding although host preference cannot be determined from these structures. It is more similar in teeth morphology, lacking a velar mouth, the number of annuli between gonopores, and micromorphic reproductive structures to the Neotropical members of Praobdellidae (*Pintobdella* and *Tyrannobdella*) than those with geographic distributions in the Old World.

CHAPTER 5

A new species of Asian medicinal leech of the genus *Hirudinaria*

(Hirudinida: Hirudinidae) from Thailand

INTRODUCTION

Asian medicinal leeches or buffalo leeches, also known as members of the genera *Hirudinaria* or *Poecilobdella* of the family Hirudinidae and the subfamily Hirudinariinae, are some of the most aggressive leeches that feed on mammals and are found in India and Southeast Asia. These leeches are abundant in rice fields, water tanks, and other disturbed water bodies and are frequently encountered by humans, cattle, or water buffalo working nearby or swimming in leech-infested water (Harding and Moore, 1927; Bhatia, 1941). These strictly sanguivorous leeches will aggressively attack a variety of vertebrates including turtles, frogs, and snakes, as well as mammals (Harding and Moore, 1927). Several species have been used in folk and Ayurvedic medicine, having been a source of trade throughout India and parts of Southeast Asia since 200 B.C. (Bhatia, 1941; Sawyer, 1998), and have served as the model leech in university classrooms throughout India and Southeast Asia for decades (Bhatia, 1941).

Whitman (1886) described the genus *Hirudinaria* with *Hirudinaria javanica* (Wahlberg, 1856) as the type species. Later Blanchard (1893) transferred this species and *Hirudinaria granulosa* (Savigny, 1822) to *Poecilobdella*, a subgenus of *Limnatis*, with the type species being *Poecilobdella granulosa*. Moore (1901b) closely examined the internal reproductive structures of *H. javanica* through dissections but found few similarities with *Limnatis* species. Blanchard's (1893) definition of *Limnatis* included two groups: one typified by *Limantis nilotica* from Egypt, and the other by *H. javanica* inclusive of *Poecilobdella* species. From this, Moore (1901b, 1924) suggested that species of *Hirudinaria* and *Poecilobdella* are either two separate genera or belong in a single genus and in that case the name *Hirudinaria* (Whitman, 1886) has priority. Through 108 dissections during the examination of over 900 specimens of *Hirudinaria*, Harding and Moore (1927) determined *Hirudinaria* to be distinct from *Limnatis* and deserving of

its own genus with *Poecilobdella* included as a subgenus of the latter. Within the same volume, Harding and Moore (1927) determined the type lots of *P. granulosa* to include specimens of several species, *H. granulosa, Hirudinaria manillensis*, and *Hirudinaria viridis*, of which Blanchard (1893) had only examined external morphological characters that do not delimit these species. Despite Moore's findings (1901b, 1927) that the type lots of *P. granulosa* were a mixture of other related species and the description of *Hirudinaria* by Whitman (1886) predated Blanchard's (1893) use of *Poecilobdella*, Soós (1969) incorrectly listed *Hirudinaria* as a junior synonym of *Poecilobdella* and the type species as *P. granulosa*. In a revision of all leeches, Sawyer (1986) listed both *Hirudinaria* and *Poecilobdella* within the subfamily Hirudinariinae along with *Illebdella*, a genus distributed in Southeast Asia and the Pacific Islands. In this revision (Sawyer, 1986), *Hirudinaria* species were characterized as lacking a vaginal stalk or duct, a structure "well developed, equalling or exceeding [the length of the] vaginal caecum" in *Poecilobdella* species.

Whitman's use of *Hirudinaria* has priority over *Poecilobdella* if these species are to be included within a single genus, but until detailed morphological examinations can be paired with DNA sequence data of a broader sampling of both genera, Sawyer's (1986) distinction between the two genera is morphologically and taxonomically sound. A new species of *Hirudinaria* is described from specimens collected in Phang Nga province, Thailand that is morphologically as well as molecularly a separate species than those previously described within the genus.

MATERIALS AND METHODS

Leeches were relaxed in 30% ethanol, fixed and preserved in at least 90% ethanol. Examination of external and internal morphology was accomplished with a Nikon® SMZ645

stereomicroscope on whole and dissected specimens. Photographs were taken with a Nikon Coolpix® 5000 digital camera. Illustrations were made using Adobe Illustrator®10. Roman numerals in all cases indicate body somites (e.g., prostomium is I) and annuli receive alphanumeric designations as per Sawyer (1986).

Representative taxa (Table 5) including 13 species with 18 individuals of the family Hirudinidae were included with *Hirudinaria* n. sp. in a molecular matrix. *Xerobdella lecomtei* served as the outgroup. Locality data and GenBank accession numbers are also listed in Table 1. Specimens were stored at either -20°C or at ambient temperature in 90-100% ethanol. Tissue was collected from the caudal sucker rather than from gastric or intestinal regions to avoid contamination by the host/prey DNA. DNeasy Tissue Kits (Qiagen Valencia, CA) were used for tissue lysis and DNA purification.

Primers used in Phillips *et al.* (2010) were used for the PCR amplification of nuclear 18S rDNA (18S) and 28S rDNA (28S) and mitochondrial 12S rDNA (12S) and cytochrome *c* oxidase I (COI) gene fragments (Table X). All amplification reactions of gene fragments were made using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) with 0.5 μl of each 10μM primer, 1 μl DNA template, and 23 μl RNase-free H2O (total volume 25 μl) and were performed in an Eppendorf Mastercycler®. The following amplification protocols were used: for 18S, 94°C for 1 min, followed by 35 cycles of 94°C (30 sec), 49°C (30 sec), 68°C (2 min 30 sec) and a final extension at 68°C for 1 minute; for 28S and 12S, 94°C for 5 min, followed by 39 cycles of 95°C (1 min), 52°C (1 min), 70°C (1 min) and a final extension of 72° for 7 minutes; for COI, 94°C for 1 min, followed by 30 cycles of 94°C (30 sec), 48°C (30 sec), 68°C (45 sec), 68°C (1 min) and a final extension of 68°C for 1 min. PCR amplification products were purified with AMPure™ (Agencourt Bioscience Corporation).

Table 6: Taxa used for the phylogenetic analyses of *Hirudinaria* n. sp. along with collection localities and GenBank accession numbers. Type species of the genus indicated by *.

Taxon		**Locality**	**GenBank Accession Numbers**			
			18S	**28S**	**12S**	**CO1**
Ingroup						
Family Hirudinidae	*Aliolimnatis michaelseni*	Congo	AF116010	AY425388	AY425429	AF116029
	Aliolimnatis oligodonta	Tanzania	GQ368781	GQ368762	_____	GQ368739
	Asiaticobdella fenestrata	Zambia	GQ368783	GQ368763	GQ368804	GQ368741
	Goddardobdella elegans *	Australia	GQ368784	GQ368764	GQ368805	GQ368742
	Haemopis elegans	Germany	XXXXXX	XXXXXX	XXXXXX	EF125042
	Haemopis grandis	Manitoba	AY425465	AY425377	AY425420	AY425447
	Haemopis kingi	Manitoba	AY425466	AY425378	AY425421	AY425448
	*Haemopis sanguisuga**	Sweden	AF099941	AY425381	AF099960	AF462021

Table 6 continued

	Haemopis terrestris	OH, USA	AY786465	EU100080	_____	EU100092
	*Hirudinaria javanica**	Vietnam	GQ368787	GQ368767	GQ368808	GQ368745
	Hirudinaria manillensis	Dominica	GQ368788	GQ368768	GQ368809	_____
	Hirudinaria manillensis	Puerto Rico	AY425467	AY425384	AY425426	AY425449
	Hirudinaria manillensis	Thailand	GQ368789	GQ368769	_____	GQ368746
	Hirudinaria manillensis 24	Vietnam	GQ368790	GQ368770	GQ368810	GQ368747
	Hirudinaria bpling n. sp. 1	Thailand	XXXXXX	XXXXXX	XXXXXX	XXXXXX
	Hirudinaria bpling n. sp. 2	Thailand	XXXXXX	XXXXXX	XXXXXX	XXXXXX
	*Hirudo medicinalis**	BioPharm, UK	AF116011	AY425385	AF099961	AF003272
	Hirudo verbana	Leeches USA	GQ368794	GQ368773	GQ368813	GQ368752
Outgroup						
Family Xerobdellidae	*Xerobdella lecomtei**	Slovenia	AF099947	EU100086	_____	EU100099

Cycle sequence reactions were performed with an Eppendorf Mastercycler® using one of two different strategies: 7 μl Rnase-free H_2O, 1 μl ABI Big Dye™ Terminator v3.1, 1 μl Big Dye™ Extender Buffer v3.1, 1 μl of 1 μM primer and 3 μl of cleaned PCR template (13 μl total volume) or 0.5 μl ABI Big Dye™ Terminator v3.1, 0.5 μl Big Dye™ Extender Buffer v3.1, 1 μl of 1 μM primer and 3 μl of cleaned PCR template (5 μl total volume). Sequences were purified by 70% isopropanol/70% ethanol precipitation and analyzed with an ABI PRISM® 3730 sequencer (Applied Biosystems). CodonCode Aligner (CodonCode Corporation) was used to edit and reconcile sequences. Alignments of all genes were accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004) applying default settings.

Parsimony analyses of the genes (18S, 28S, 12S, and COI) separately and in combination employed PAUP* 4.0b10 (Swofford, 2002). All characters initially were weighted equally and were non-additive. Gaps were treated as missing data. Heuristic searches used 500 replicates of the data set with random taxon addition and tree-bisection-reconnection branch swapping. Parsimony jackknife (jac) values were obtained using random taxon addition and tree-bisection-reconnection branch swapping with 36% deletion, 100 psuedoreplicates and 50 random addition sequences. Pairwise (uncorrected "p") sequence distances were calculated using PAUP* 4.0b10 (Swofford, 2002) for the species of *Hirudinaria*. Maximum Likelihood (ML) analyses of the combined dataset were performed with Treefinder v. 12.7.0 (Jobb, 2008) with default settings. A GTR+G model was assumed for each unlinked partition based on the AIC (via FindModel, an online independent implementation of ModelTest; Posada and Crandall, 1998). The data were partitioned by gene for 18S, 28S, 12S, and by codon position for COI (three partition; 3p). Bootstap (bs) analyses were performed with 1000 replicates.

RESULTS

The combined molecular dataset included 3477 aligned characters (18S: 1860 characters, 28S: 537 characters, 12S: 422 characters, COI: 658 characters). The parsimony analysis recovered a single tree with 1473 steps (CI=0.6361 and RI=0.6144), while the log likelihood of the topology produced by the Maximum Likelihood analysis was -10947.08 (Figure 11). Regardless of phylogenetic method, *Hirudinaria* n. sp. was found to be monophyletic with strong support (bs=100, jac=100) and distinct from individuals of *H. manillensis*, including one individual from Thailand thus supporting the validity of *Hirudinaria* as a genus. The genus *Hirudinaria*, including *H. manillensis, H. javanica,* and *Hirudinaria* n. sp., was also found to be monophyletic with strong support (bs=100, jac=100). A clade of *Hirudinaria manillensis* represented from several localities in Asia and the Caribbean was also monophyletic (bs=100, jac=98). Individuals of *H. manillensis* from Dominica and Puerto Rico were sister to each other (bs=85, jac=91) and placed well within the clade of *H. manillensis* individuals from Asian localities. Phylogenetic analyses recovered highly congruent tree topologies with the jackknife tree producing two polytomies: one for the placement of *H. javanica* within *Hirudinaria*, and the second for *Aliolimnatis*+*Asiaticobdella*.

Within COI sequences, pairwise sequence distances (Table X) between *H. manillensis* and *Hirudinaria* n. sp. averaged 11.83% (high value: 12.87% between *Hirudinaria* n. sp. 1 and *H. manillensis* from Thailand; low value: 11.41% between *Hirudinaria* n. sp. 1 and *H. manillensis* from Vietnam), and pairwise sequence distances between *H. javanica* and *Hirudinaria* n. sp. averaged 11.83% (11.7% and 11.95%). Within 12S sequences, pairwise distances between *H. manillensis* and *Hirudinaria* n. sp. averaged 5.42% (high value: 6.74% between *Hirudinaria* n. sp. 2 and *H. manillensis* from Puerto Rico; low value: 3.85% between *Hirudinaria* n. sp. 1 and

FIGURE 11.

Topology resulting from Maximum Likelihood analyses (log likelihood = -10947.08). Numbers above and below the nodes represent bootstrap and jackknife values, respectively.

Figure 11.

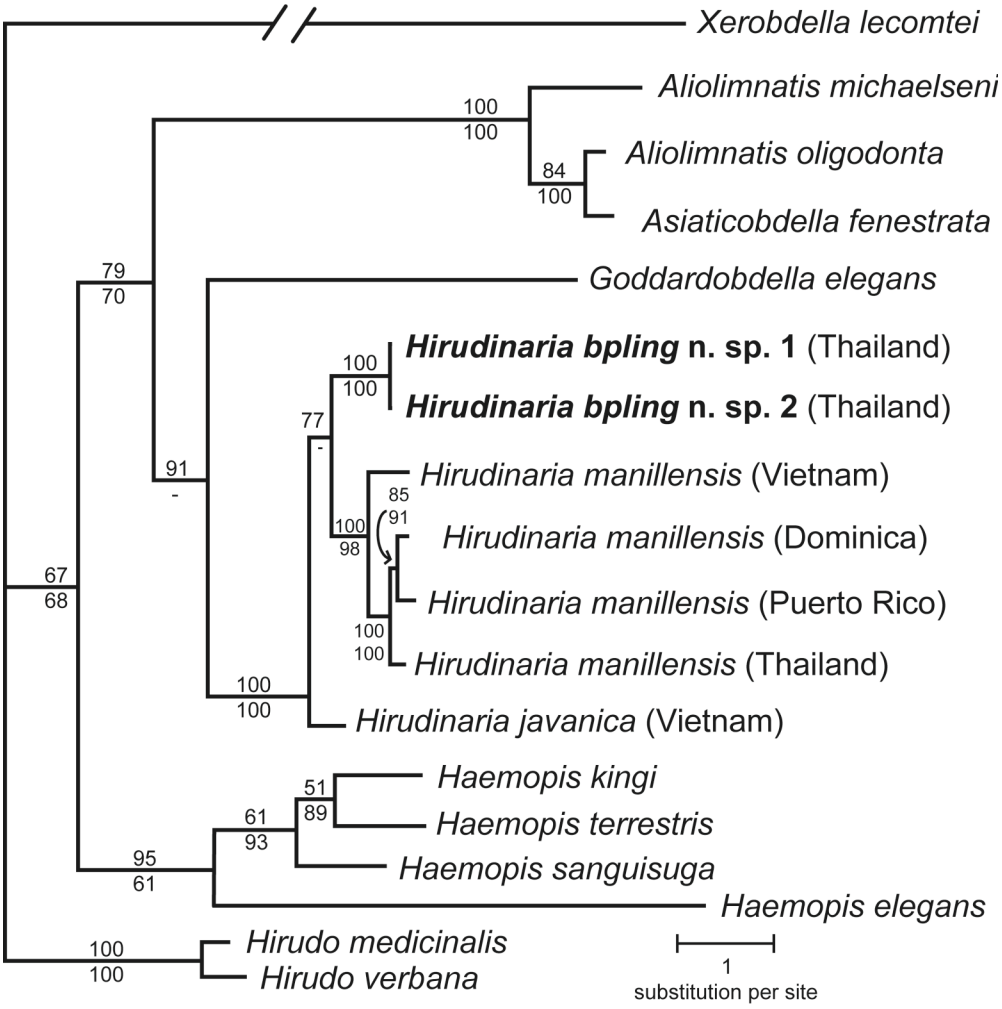

Xerobdella lecomtei
Aliolimnatis michaelseni
Aliolimnatis oligodonta
Asiaticobdella fenestrata
Goddardobdella elegans
Hirudinaria bpling n. sp. 1 (Thailand)
Hirudinaria bpling n. sp. 2 (Thailand)
Hirudinaria manillensis (Vietnam)
Hirudinaria manillensis (Dominica)
Hirudinaria manillensis (Puerto Rico)
Hirudinaria manillensis (Thailand)
Hirudinaria javanica (Vietnam)
Haemopis kingi
Haemopis terrestris
Haemopis sanguisuga
Haemopis elegans
Hirudo medicinalis
Hirudo verbana
100
100
84
100
79
70
91
-
100
100
77
-
85
91
100
98
100
100
67
68
100
100
51
89
61
93
95
61
100
100
1
substitution per site

Table 7: Pairwise sequence distances between *Hirudinaria bpling* n. sp. and *Hirudinaria manillensis* and *Hirudinaria javanica*.

		Hirudinaria manillensis	*Hirudinaria javanica*
COI	*Hirudinaria bpling*	11.83%	11.83%
12S	*Hirudinaria bpling*	5.42%	6.13%
28S	*Hirudinaria bpling*	0.38%	0.21%
18S	*Hirudinaria bpling*	0.22%	0.00%

H. manillensis from Vietnam) and pairwise distances between *H. javanica* and *Hirudinaria* n. sp. averaged 6.13% (5.14% and 7.11%). Within 28S sequences, pairwise distances between *H. manillensis* and *Hirudinaria* n. sp. averaged 0.38% (high value: 0.45% between *Hirudinaria* n. sp. 2 and *H. manillensis* from Dominica; low value: 0.28% between *Hirudinaria* n. sp. and *H. manillensis* from Vietnam) and pairwise distances between *H. javanica* and *Hirudinaria* n. sp. averaged 0.21% (0.20% and 0.22%). Within 18S sequences, pairwise distances between *H. manillensis* and *Hirudinaria* n. sp. averaged 0.22% (high value: 0.40% between *Hirudinaria* n. sp. and *H. manillensis* from Puerto Rico; low value: 0% between *Hirudinaria* n. sp. and *H. manillensis* from Thailand) and 18S sequences between *H. javanica* and *Hirudinaria* n. sp. were identical.

SUBORDER ARHYNCHOBDELLIDA BLANCHARD, 1894

Family Hirudinidae Whitman, 1886

Genus *Hirudinaria* Whitman, 1886

Hirudinaria bpling, new species

Figure 12

HOLOTYPE: Preserved body length 152 mm, maximal 15.5 mm wide; dissected (AMNH XXXX). Collected 24 October 2005 from Bang Lae, Phang Nga Province, Thailand (N 08° 45.928’ E 98° 26.351’, elevation 31 meters).

PARATYPES: (AMNH XXXX), collected 24 October 2005 from Bang Lae, Phang Nga Province, Thailand (N 08° 46.557’ E 98° 26.310’, elevation 36 meters).

ADDITIONAL MATERIAL: AMNH XXXX (N 08° 45.928’ E 98° 26.351’, elevation 31 meters). AMNH XXXX (N 11° 27’ 32.38” E 107° 20’ 34.46”, elevation 127 meters). AMNH XXXX (N 11° 25’ 31.13” E 107° 25’ 44.97”, elevation 125 meters).

DESCRIPTION: Body muscular (Figure 12 A, B). In complete somites, dorsal surface with brown to yellow background field, marked by median dark brown stripe, sometimes broken, lateral black spots on b2 and b5, two broken lateral black stripes on each side of midline, annuli b1 and b6 with mottled black spotting without distinct pattern (Figure 12 C). Ventral surface dark green with narrow marginal pale yellow stripe. Fifteen five-annulate mid-body somites from somite IX to XXVIII. Somites I-III uniannulate, somite IV and V biannulate, somites VI and VII triannulate, somite VIII quadrannulate, somites IX-XXIII five-annulate, somite XXIV quadrannulate, somite XXV triannulate, somite XXVI biannulate, somite XXVII biannulate.

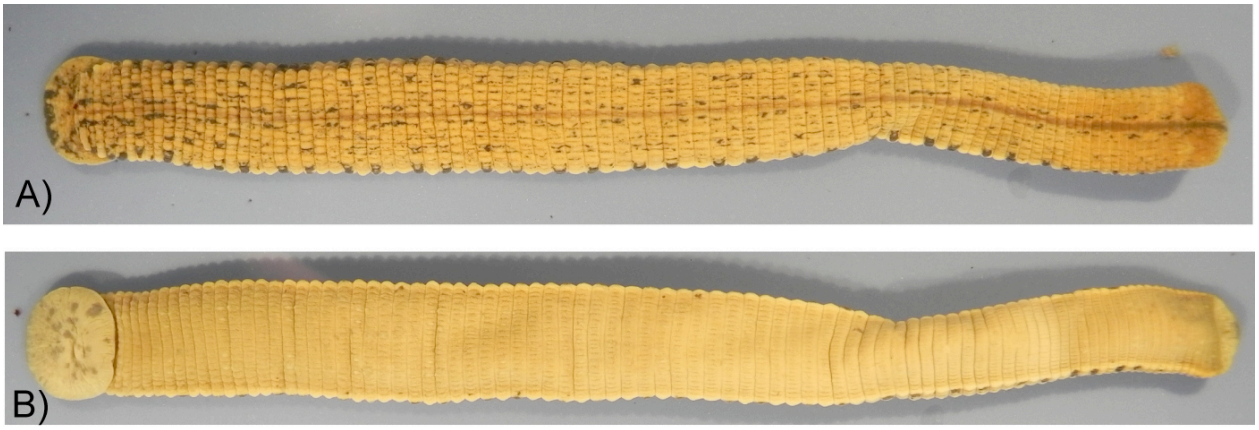
Eyespots arranged dorsally in five pairs in a broad arch. Pair 1 anterior on somite II, remaining eyespots situated laterally. Pair 2 on somite III and both pairs 1 and 2 with pigment concentrated posteriomedialy in eyespot. Pair 3 on somite IV a2. Pair 4 on somite V a2. Pair 5 on somite VI a2. Sensillae present on annuli a2 of complete somites, two on each side of midline and a pair laterally on each side. Nephropores 17 pairs on ventral surface situated bilaterally from somite VIII to XXIV; in complete somites, just posterior to b2/a2 furrow. Caudal sucker round with eight rays of sensillae on the dorsal surface radiating out from center of sucker. Male gonopore at XI b5/b6 in furrow, female gonopore at XII b5/b6 in furrow, five annuli between gonopores.

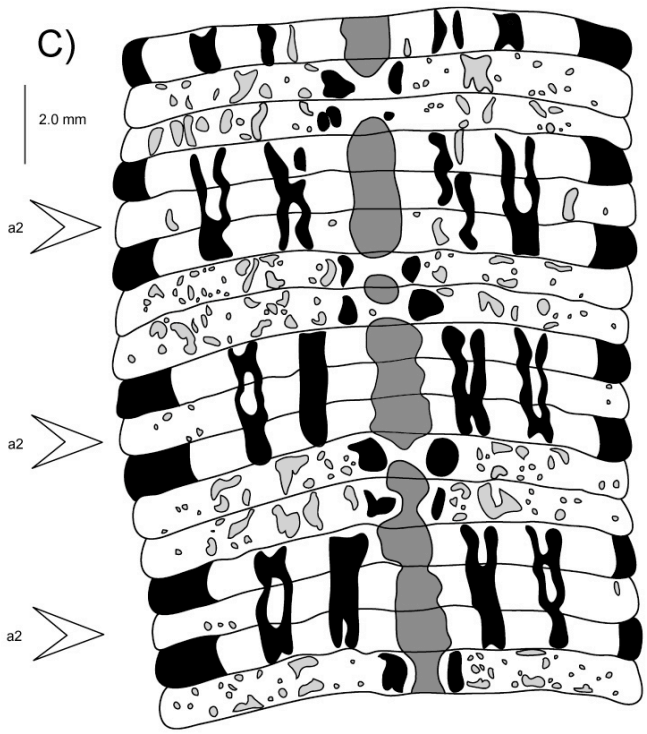
Oral sucker with prominent dorsal lip with deep medial longitudinal furrow on ventral side of lip that extends from margin of lip to entrance to pharynx and several shallow furrows on

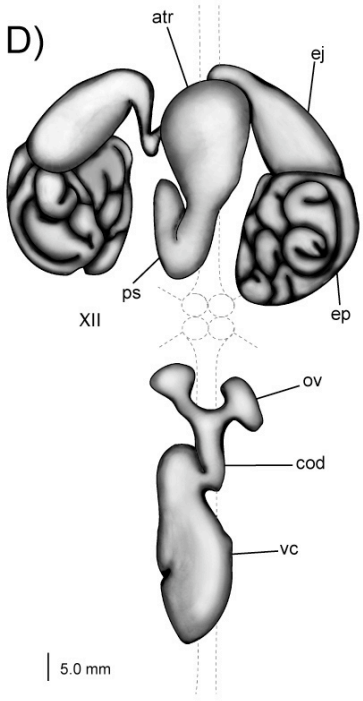
FIGURE 12.

External and internal morphology of *Hirudinaria bpling* n. sp.: (A) Dorsal view, (B) Ventral view, (C) Color pattern of dorsal surface, (D) Dorsal view of male and female reproductive organs. Arrows to left of (C) indicate annulus a2 of each somite. atr = male atrium, cod = common oviduct, ej = ejaculatory bulbs, ep = epididymes, ps = penis sheath, ov = ovaries, vc = vaginal caecum.

Figure 12.







each side. Ventral side of sucker with fleshy flap of tissue below margin of sucker covering opening to mouth, approaching a velum. Jaws trignathous, large with strong salivary papillae. Teeth monostichodont, small in size with more than 100 teeth per jaw. Pharynx muscular and tubular. Crop, from somite VIII to somite XVII, first nine cecal pairs simple and equal size with small lobe anterior to the main caecal chamber, post-caeca extending bilaterally posterior to somite XIII. Caeca two per somite. Intestine tubular, acaecate from somite XIX - XXII. Rectum thin walled, tubular from somite XXII-XXVII. Anus on somite XXVII a3.

Median male reproductive system found in XI/XII (Figure 12 D). Penis sheath recurves anteriorly to larger bulbous atrium. Vasa deferentia insert to atrium anteroventrally. Ejaculatory bulbs present and connect to anterior portion of epididymes. Mass of epididymes twice as wide as ejaculatory bulbs. Median female reproductive system found in XII/XIII (Figure 12 D). Ovaries small, globular with narrow short oviducts that join into short, narrow common oviduct with albumin gland at point of intersection. Common oviduct connects anteriodorsally to large vaginal caecum well above insertion point to female bursa. Vaginal caecum two times as long as is wide at its widest point with anterioventral end connecting to female bursa.

HABITAT: Freshwater in muddy shallow areas with abundant aquatic vegetation, such as rice fields.

FEEDING PREFERENCE: Strictly sanguivorous, feeds readily on humans and cattle.

TYPE LOCALITY: Bang Lae, Phang Nga Province, Thailand (N 08° 45.928’ E 98° 26.351’)

ETYMOLOGY: *bpling*: bpling (G.) – phonetic spelling of “leech” in Thai.

REMARKS: The specimens examined here cannot be placed in either of the two existing species of *Hirudinaria* or the four species of *Poecilobdella*. It is clearly a member of *Hirudinaria* and not *Poecilobdella* because it does not have a vaginal duct, which is well developed and extended in *Poecilobdella* species (Harding and Moore, 1927; Sawyer, 1986; Yang, 1996). The specimens also possess characteristics common among both *Hirudinaria* and *Poecilobdella*: a precise color pattern similar to, yet distinct from, other members of the genera, a very large vaginal caecum, salivary papillae, a median longitudinal furrow in the upper lip, a flap of tissue on the ventral internal surface of the oral sucker similar to a velum, and a very large size (often times over 50 mm). *Hirudinaria javanica* (Wahlberg, 1856) has seven annuli between the gonopores, with the female gonopore in the furrow at somite XIII b1/b2, and the common oviduct and vaginal caecum opening together into the female bursa (Harding and Moore, 1927). This differs from *H. bpling* n. sp. that has five annuli between the gonopores, the female gonopore at somite XII b5/b6, and the common oviduct opening to the dorsal portion of the vaginal caecum, not the female bursa. *Hirudinaria bpling* n. sp. is distinct from *Hirudinaria manillensis* (Lesson, 1842) in possessing a unique color pattern dominated by yellow and brown instead of red and green, epididymes that only connect to the ejaculatory bulbs at the insertion point, a smooth, extended, and recurved penis sheath without glandular tissue, and a common oviduct that opens into the dorsal side of the vaginal caecum (Harding and Moore, 1927; Moore, 1938). *Poecilobdella* species have a well-developed and extended vaginal duct leading to the vaginal caecum that is of equal length or longer than the vaginal caecum *(*Sawyer, 1986) that is not observed in *H. bpling*. In addition to the female reproductive organs, *Poecilobdella granulosa* (Savigny, 1922) does not possess ejaculatory bulbs (Harding and Moore, 1927; Bhatia, 1941), whereas *H. bpling* has large muscular ejaculatory bulbs. The reproductive organs of *Poecilobdella viridis* Moore, 1927

include a penis sheath that is three or four times longer than the male atrium, only slightly enlarged ejaculatory bulbs, and a vaginal duct that is at least twice the length of the vaginal caecum (Harding and Moore, 1927). *Poecilobdella hubeiensis* Yang, 1980 does not have ejaculatory bulbs and it has a vaginal duct that is of equal length to the vaginal caecum. *Poecilobdella nanjingensis* Yang, 1996 has a narrow vaginal caecum and vaginal duct, the later of which is equal in length to the vaginal caecum. The morphology of *H. bpling* is most similar to *H. javanica*.

DISCUSSION

Hirudinaria bpling is a new species supported by morphological as well as molecular evidence. The pairwise distances of the mitochondrial gene fragments (Table X) are strongly supportive of *H. bpling* being a distinct and separate species from *H. manillensis* and *H. javanica*. Uncharacteristic of the family Hirudinidae, *H. bpling* has a large caudal sucker and a velum-like structure below the margin of the ventral lip of the oral sucker similar to the velum of some members of Praobdellidae. Some reports indicate that occassionally *Hirudinaria* species invade the orifices of cattle (Jesus, 1934) but others insist that *Hirudinaria/Poecilobdella* species do not exhibit such behavior (Harding and Moore, 1927). Although some superficial similarities between *H. bpling* and members of the Praobdellidae exist, *H. bpling* possesses large jaws with over 100 teeth and one post-anal annulus, characters typical of *Hirudinaria* species within Hirudinidae but none of which have been observed in Praobdellidae (Phillips *et al.*, 2010).

The tropical climate of India and Southeast Asia and the constant source of blood of cattle and agricultural workers in and around rice fields (Bhatia, 1941) create a prime habitat for hirudinids. While *Hirudo* species prefer natural, undisturbed freshwater habitats, typical of most

members of the family, *Hirudinaria* and *Poecilobdella* species are most commonly found in highly disturbed areas such as rice fields, flooded grasslands with cattle, and water tanks (Harding and Moore, 1927; Chandra, 1983). The affinity of these leeches for such habitats makes the chance of invasiveness high considering the current rate of degradation of pristine freshwater habitats throughout the tropical regions of the world.

The stable climate of the tropics provides conditions that allow not only the genus with the largest body size in Hirudinidae, rivaled only by some species of *Haemopis* (Richaradson, 1973), that are able to reproduce only 102 days after hatching. Young individuals of *Hirudinaria* are almost immediately capable of biting through the tough skin of mammals (Sawyer, 1986; Keegan etal, 1968; Khan 1912; Matthai 1920), such as water buffalo or even elephants (Blanchard 1897b; Moore 1932b; Acharya, Acharya, and Patnaik, 1974). Species of *Hirudinaria* are strictly sanguivorous (Kutschera, 2006; Zhang *et al.*, 2008) with jaws that are larger and armed with more teeth than other members of Hirudinidae, even the quintessential medicinal leech, *Hirudo medicinalis* (Sawyer, 1986).

Hirudinaria bpling has been collected in Thailand at low elevations both in coastal and interior regions of the country and is more broadly distributed into Vietnam and Maylasia (pers. comm. Phillips). Similarly, the geographic distribution of *H. manillensis* has consistently been reported from coastal lowland areas of India and Southeast Asia at elevations below 500 meters (Harding and Moore, 1927; Soós, 1969), although Sawyer (1986) also reported *H. manillensis* commonly found in coastal China and Hong Kong. Chandra (1983) expanded the distribution of *H. manillensis* to include more inland areas, such as Pakistan and China, and nearby islands, such as Sri Lanka, Borneo, and Philippines. Given its preference and ability to thrive in regions with warm, humid climates, *H. manillensis* and species with similar environmental tolerances

would not be expected to establish populations in regions with significant temperature fluctuations, although have established populations in regions with similar climates (i.e. Caribbean; Sawyer, 1998; Kutschera, 2006; Phillips and Siddall, 2009).

Hirudinids have been exported from India for medicinal purposes that have established populations in Mauritius, Martinique, St. Lucia, Dominica, and Puerto Rico (Moore, 1901b; Sawyer, 1998; Kutschera, 2006). Pondicherry, India was a major port for the exportation of leeches in Asia and nearby regions in the nineteeth century when leeches were a popular tool for bloodletting (Sawyer, 1998). Moore examined specimens of *Hirudinaria* from Puerto Rico and described them as *Hirudinaria blanchardi* (Moore, 1901b), which Sawyer (1986) included in *Poecilobdella*, although recent records of Asian hirudinid populations in the Caribbean have been identified as *H. manillensis* (Sawyer, 1998; Kutschera, 2006; Phillips and Siddall, 2009). The individuals of *H. manillensis* included in this study from Dominica and Puerto Rico, while most closely related to each other, are strongly supported within the clade of *H. manillensis* and not as a separate species, as would be expected of populations that had undergone long distance dispersal in recent times (Phillips and Siddall, 2009). This confirms Sawyer (1998) and Kutschera (2006) attributing these populations of *H. manillensis* in the Caribbean to humans transporting leeches for medicinal purposes from India and Southeast Asia to meet the demand of people emigrating from India and Southeast Asia to the West Indies. These leeches were subsequently released into the freshwater streams and ponds after their utility as a medical treatment was realized, only to establish self-sustaining populations. Further studies of the haplotype diversity present in the Asian populations and the representation of that diversity in residence in the Caribbean will detail the origins of this human-mediated invasion.

CHAPTER 6

Phylogenetic diversity of microbial symbionts in medicinal leeches

(Adapted from Siddall, M.E., Min, G-S., Fontanella, F.M., Phillips, A.J., and Watson, S.C. (in press) Bacterial symbiont and salivary peptide evolution in the context of leech phylogeny. *Parasitology*).

INTRODUCTION

Like many other bloodfeeding animals, leeches harbour select prokaryotic flora in association with their digestive tracts. Proboscis-bearing sanguivorous species possess mycetomal organs specific to this task with intracellular alphaproteobacteria or gammaproteobacteria (Kikuchi and Fukatsu, 2002; Siddall *et al.,* 2004; Perkins *et al.,* 2005). In contrast, jaw bearing medicinal leeches in the Hirudinidae and Macrobdellidae host a limited flora in the intraluminal fluid of the crop (Graf, 1999; Siddall *et al*., 2007a; Laufer *et al*., 2008). To date, only a single culturable bacterial species has been detected in any individual medicinal leech: *Aeromonas veronii* in the European *Hirudo verbana* (Graf, 1999, 2002), which was often mistakenly reported as *Hirudo medicinalis* (Siddall *et al*., 2007b), *Aeromonas jandaei* in the North American *Macrobdella decora* (Siddall *et al*., 2007a), and either of these two *Aeromonas* species (but never both) in the crop of the European *Hirudo orientalis* (Laufer *et al*., 2008). In addition to these individual culturable gammaproteobacteria, Worthen et al. (2006) demonstrated the co-presence of an unculturable Bacteroidetes microbe closely related to *Rikenella* species in *H. verbana*.

The crop, or gastric caeca, occupies approximately one-third of a leech's body somites allowing the annelid to expand more than six times its unfed body weight during feeding, permitting extended periods between feeding events (Munro *et al.*, 1992). The role of the resident microbial flora is not yet well elucidated. Functions could range from the provision of essential nutrients not readily available in a diet that is limited exclusively to blood (e.g., Nogge, 1981) to antimicrobial activities inhibiting putrefaction of the blood meal (Rio *et al*., 2007).

Species of *Aeromonas*, including *A. veronii* and *A. jandaei*, are ubiquitous in circumglobal freshwater habitats raising questions regarding the historical maintenance of a

single species of the genus in any given leech. Graf (2000) was first to suggest that oral vertical transmission is responsible insofar as all leeches must withdraw their oral anterior through the egg-bearing cocoon after it is secreted by the clitellum. Corroborating this, the medicinal use of leeches has repeatedly demonstrated their propensity for introducing *Aeromonas* infections at a bite wound (Whitaker *et al.,* 2009). Recent work confirms that *Aeromonas veronii* is present as soon as *H. verbana* cocoons are deposited (Rio *et al*., 2009). The *Rikenella*-like symbiont is detectable later (Rio *et al*., 2008). While not a prerequisite, such vertical transmission of associated microbes hints at emergent coevolutionary histories (Moran, 2001).

The revision of medicinal leeches into several families (Phillips *et al.,* 2010) demonstrates that the two genera examined thus far for their intraluminal microbial crop symbionts are distantly related representatives of Macrobdellidae and the revised Hirudinidae (Figure 13). Here we investigate the crop flora of a broader range of leech genera and families, and evaluate historical patterns of this tripartite symbiotic system.

MATERIALS AND METHODS

Intraluminal blood-meal of leech species of the families Hirudinidae, Macrobdellidae, and Praobdellidae (Table X) was removed following transverse bisection of leeches at the region of the gastric tissue and well anterior of the intestinal tract. DNeasy Tissue Kit (Qiagen Valencia, CA) was used for tissue lysis and DNA purification. *Aeromonas*-specific primers for DNA gyrase B (*gyr*B) were AerogyrBf TGTTGCTGACCATTCGTCGTAAC and AerogyrBr TTGGCATCGCTCGGGTTTTC with a predicted optimal annealing temperature of 59.4 C. Amplification reactions employed Taq Gold (Applied Biosystems) and 50 cycles of 94 C (45 sec), 55 C (45 sec) and 72 C (60 sec) following a 10 min pre-melt at 94 C. Bacteroidetes-

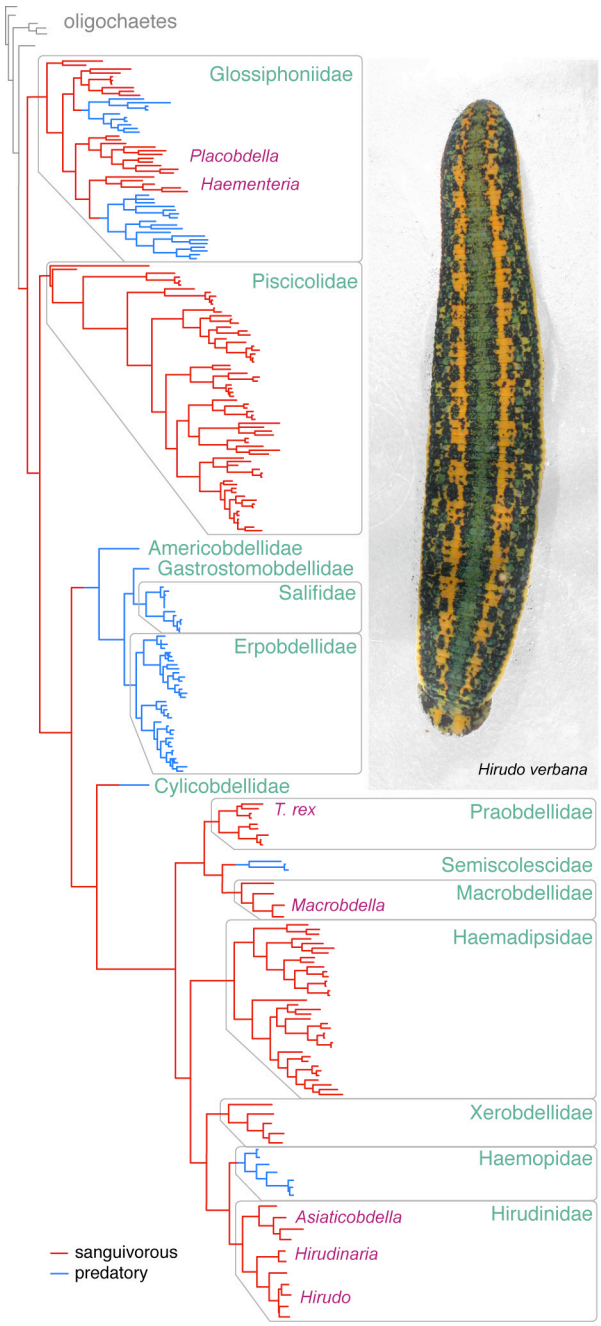
Table 8: List of leech species with the bloodmeal examined for presence of bacterial endosymbionts and resulting amplified bacteria.

Leech species with bloodmeal examined	**Bacterial isolated amplified and sequenced from leech bloodmeal**
Asiaticobdella buntonensis	Bacteroidetes = "Pedobacter"
Asiaticobdella fenestrata	*Aeromonas hydrophila* Bacteroidetes = "Pedobacter"
Goddardobdella elegans	Bacteroidetes = "Pedobacter"
Hirudinaria manillensis	*Aeromonas veronii*
Hirudo medicinalis	*Aeromonas hydrophila* Bacteroidetes = cf. *Rikinella*
Hirudo nipponia	Bacteroidetes = cf. *Rikinella*
Hirudo orientalis	*Aeromonas jandaei* *Aeromonas veronii* Bacteroidetes = cf. *Rikinella*
Hirudo verbena	*Aeromonas veronii* Bacteroidetes = cf. *Rikinella*
Limnatis paluda	Bacteroidetes = "Flexibacter"
Limnobdella mexicana	*Aeromonas veronii* Bacteroidetes = "Flexibacter"

FIGURE 13.

Composite metaphylogeny of the order Hirudinida based on a collection of prior work illustrating current knowledge of the relationships of most leech families. The relationships of the Hirudindiformes (below *Hirudo verbana*) indicate the complex evolutionary history of the "medicinal" leech families Hirudinidae, Praobdellidae and Macrobdellidae. Each terminal represents a species in a molecular phylogenetic analysis. Branches are proportional to change within families. Backbone phylogeny based on Apakupakul *et al.*, (1999), Siddall *et al.,* (2001) and Phillips and Siddall (2009). Blood-feeding lineages in red; non-sanguivorous lineages in blue.

Figure 13.


oligochaetes
Glossiphoniidae
Placobdella
Haementeria
Piscicolidae
Americobdellidae
Gastrostomobdellidae
Salifidae
Erpobdellidae
Cylicobdellidae
T. rex
Praobdellidae
Semiscolescidae
Macrobdellidae
Macrobdella
Haemadipsidae
Xerobdellidae
Haemopidae
Hirudinidae
Asiaticobdella
Hirudinaria
Hirudo
sanguivorous
predatory
Hirudo verbana

specific primers employed for amplification of 16S rDNA from the co-symbiont and to avoid co-amplification of the gammaproteobacterium were SSUrik416F GCAGGAAGACGGCTCTATGAGTTG and SSUrik781 RATCGTTTACGGCGTGGACTACC with a predicted optimal annealing temperature of 56.7 C. Amplification reactions employed Ready-To-Go PCR Beads (GE Healthcare) and 35 cycles of 94 C (15 sec), 50 C (15 sec) and 72 C (40 sec) following a 4 min pre-melt at 94 C. PCR amplification products were purified with AMPure™ (Agencourt Bioscience Corporation). Cycle sequencing reactions were performed with an Eppendorf Mastercycler® using 1 μl Big Dye™ Extender Buffer v3.1, 1 μl of 1 μM primer and 3 μl of cleaned PCR template (13 μl total volume) and analyzed with an ABI PRISM® 3730 sequencer (Applied Biosystems). CodonCode Aligner (CodonCode Corporation) was used to edit and reconcile sequences. Sequences employed for comparative purposes were downloaded from NCBI. Alignments were accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004). Parsimony analyses were conducted in TNT v 1.1 (Goloboff *et al.*, 2008) using ten replicates of random taxon addition, sectorial searching, the Ratchet (Nixon, 1999), and tree fusing algorithms, with a requirement that the minimum length be found at least three times. Trees resulting from these new technology searches were submitted to tree-bisection-reconnection branch swapping retaining up to 10,000 trees. Resampling in TNT employed the parsimony jackknife (Farris *et al*., 1996), with five replicates of random taxon addition, sectorial searching, the Ratchet (Nixon, 1999), and tree fusing, 36% deletion, and with no requirement that the minimum length be found multiple times.

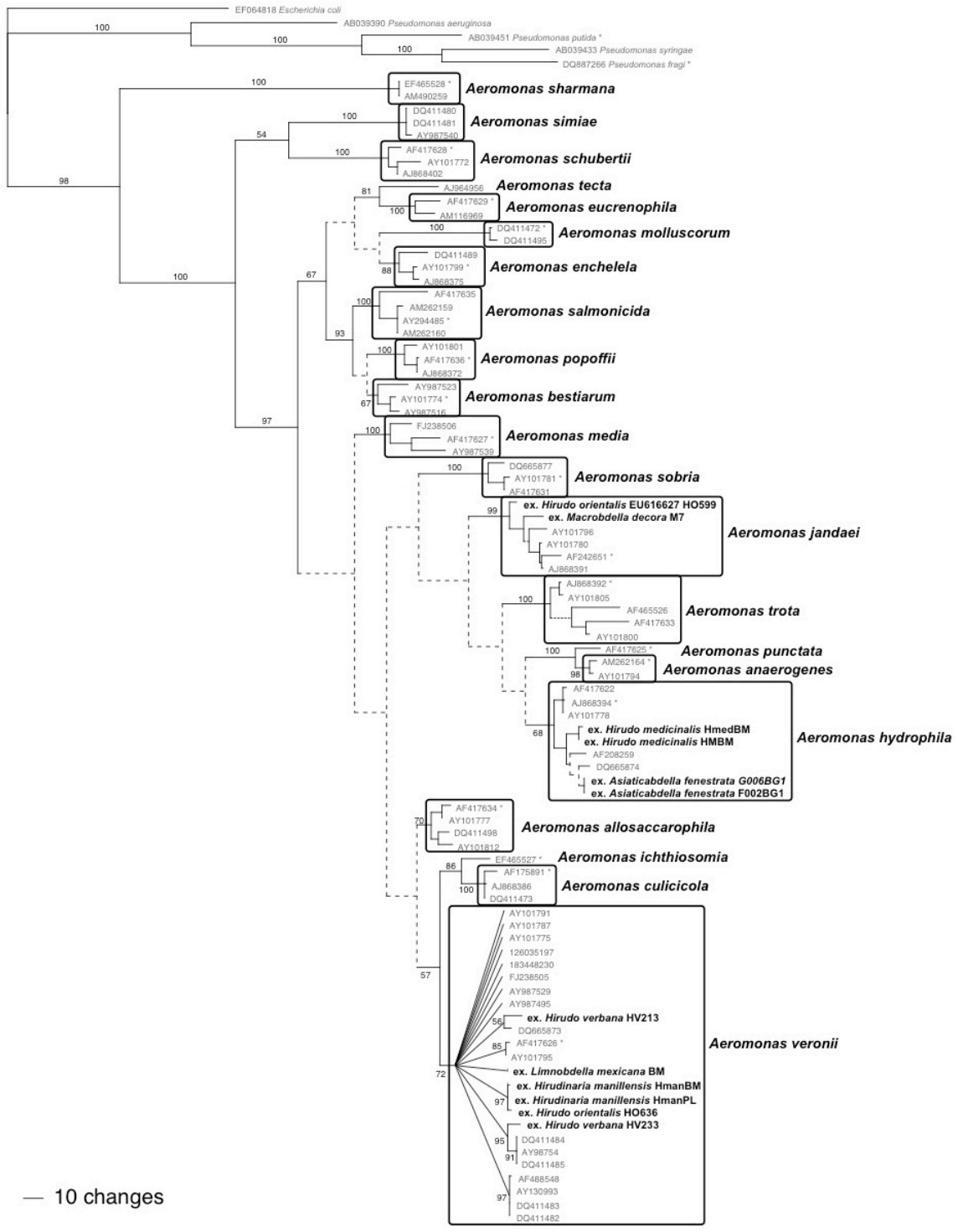
RESULTS

Parsimony analysis of *gyr*B sequences (Figure 14) for species of *Aeromonas* resulted in

FIGURE 14.

Consensus of 100 equally parsimonious trees resulting from analysis of *gyr*B sequences of species of *Aeromonas* species as well as those isolated from the crop of hirudiniform leeches (bold). Asterisks denote sequences obtained from type-strains for species. Numbers at nodes are jackknife frequencies (not shown within species). Relationships supported in fewer than 50 jackknife replicates are represented by interrupted lines.

Figure 14.


EF064818 Escherichia coli
AB039390 Pseudomonas aeruginosa
AB039451 Pseudomonas putida *
AB039433 Pseudomonas syringae
DQ887266 Pseudomonas fragi *
EF465528 *
AM490259
Aeromonas sharmana
DQ411480
DQ411481
AY987540
Aeromonas simiae
AF417628 *
AY101772
AJ868402
Aeromonas schubertii
AJ964956
Aeromonas tecta
AF417629 *
AM116369
Aeromonas eucrenophila
DQ411472 *
DQ411495
Aeromonas molluscorum
DQ411489
AY101799 *
AJ868375
Aeromonas enchelela
AF417635
AM262159
AY294485 *
AM262160
Aeromonas salmonicida
AY101801
AF417636 *
AJ868372
Aeromonas popoffii
AY987523
AY101774 *
AY987516
Aeromonas bestiarum
FJ238506
AF417627 *
AY987539
Aeromonas media
DQ665877
AY101781 *
AF417631
Aeromonas sobria
ex. Hirudo orientalis EU616627 HO599
ex. Macrobdella decora M7
AY101796
AY101780
AF242651 *
AJ868391
Aeromonas jandaei
AJ868392 *
AY101805
AF465526
AF417633
AY101800
Aeromonas trota
AF417625 *
AM262164 *
AY101794
Aeromonas punctata
Aeromonas anaerogenes
AF417622
AJ868394 *
AY101778
ex. Hirudo medicinalis HmedBM
ex. Hirudo medicinalis HMBM
AF208259
DQ665874
ex. Asiaticabdella fenestrata G006BG1
ex. Asiaticabdella fenestrata F002BG1
Aeromonas hydrophila
AF417634 *
AY101777
DQ411498
AY101812
Aeromonas allosaccarophila
EF465527 *
Aeromonas ichthiosomia
AF175891 *
AJ868386
DQ411473
Aeromonas culicicola
AY101791
AY101787
AY101775
126035197
183448230
FJ238505
AY987529
AY987495
ex. Hirudo verbana HV213
DQ665873
AF417626 *
AY101795
ex. Limnobdella mexicana BM
ex. Hirudinaria manillensis HmanBM
ex. Hirudinaria manillensis HmanPL
ex. Hirudo orientalis HO636
ex. Hirudo verbana HV233
DQ411484
AY98754
DQ411485
AF488548
AY130993
DQ411483
DQ411482
Aeromonas veronii
— 10 changes

100 equally parsimonious trees with 2232 steps for 504 informative characters and a retention index of 0.75. Each species of *Aeromonas* for which multiple sequences were available was resolved as monophyletic with jackknife frequency values ranging from 67% for *Aeromonas bestiarum* towards 100% for most species. Isolates from European *H. verbana* and *H. orientalis*, Mexican *Limnobdella mexicana* and Southeast Asian *Hirudinaria manillensis* grouped in the *A. veronii* clade. Isolates from European *H. medicinalis* and African *Asiaticobdella fenestrata* grouped in the *Aeromonas hydrophila* clade. Isolates from North American *M. decora* and European *H. orientalis* grouped in the *A. jandaei* clade. Amplification reactions of g*yr*B were not successful with Asian *Limnatis paluda* and *Hirudo nipponia*, African *Asiaticobdella buntonensis*, or Australian *Goddardobdella elegans*.

Parsimony analyses of the 16S rDNA sequences obtained from Bacteroidetes symbionts only of hirudiniform leeches (Figure 15) resulted in 105 trees of length 321 for 115 informative characters and a retention index of 0.92. The resulting consensus grouped isolates in a manner that was highly congruent with the phylogenetic history of "medicinal" leeches. That is, six of eight vertices of the 16S rDNA tree map to nodes on the leech tree without conflict (Figure 15). The two conflicting vertices resulted from non-monophyly of the isolates from *Asiaticobdella* species and from the preëxisiting isolate from *H. verbana* not grouping with other European *Hirudo* species.

Parsimony analysis of 16S rDNA sequences for a broader sampling of Bacteroidetes (Figure 16) resulted in 36 equally parsimonious trees with 10,560 steps for 870 informative characters and a retention index of 0.67. Isolates from European and Asian species of *Hirudo* formed a clade sister to a fish gut symbiont, which together were more closely related to species of *Alistipes* than to *Rikenella microfusus*. The isolate from North American *M. decora* clustered

FIGURE 15.

Consensus of 105 equally parsimonious trees resulting from analysis of Bacteroidetes-specific 16S rDNA amplicons from hirudiniform leeches. Closed circles at nodes correspond to divergences that are consistent with leech phylogeny in Figure 13.

Figure 15.

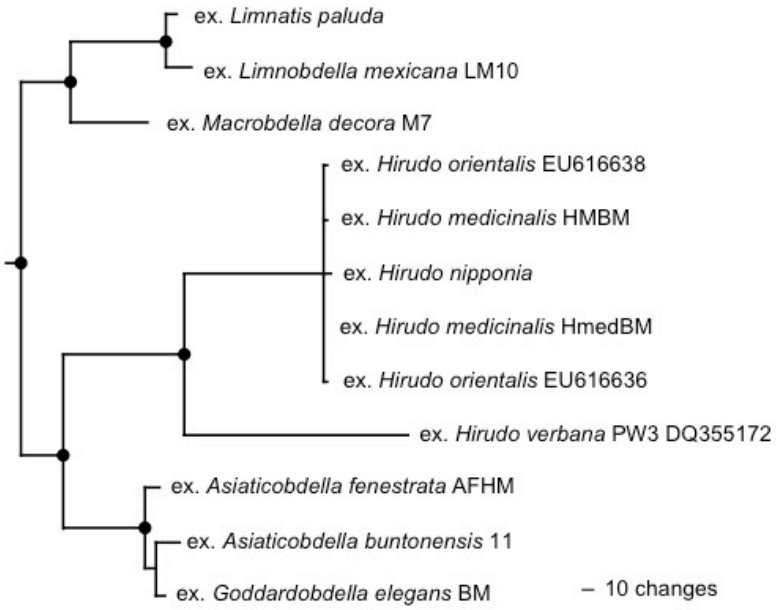
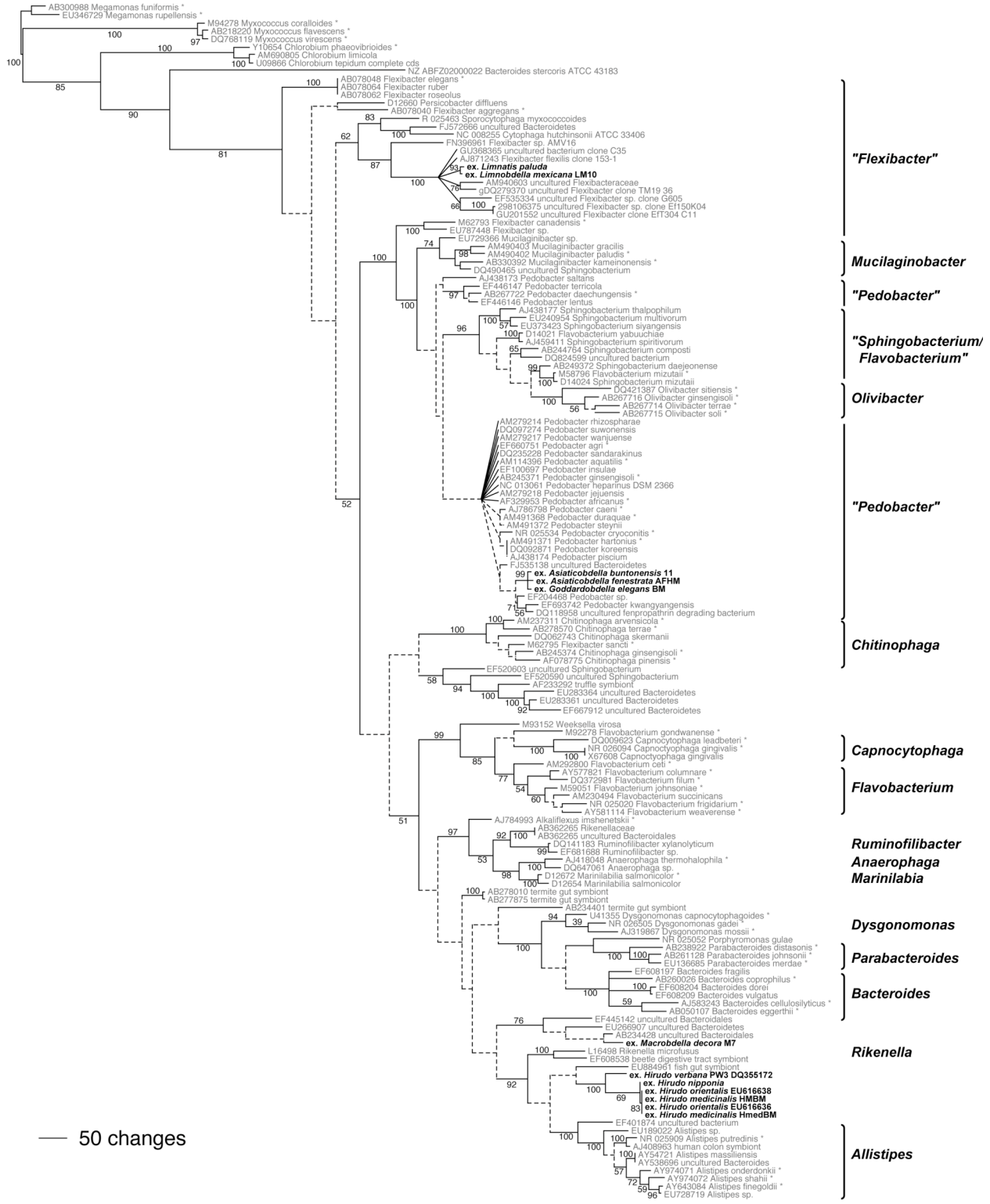

ex. Limnatis paluda
ex. Limnobdella mexicana LM10
ex. Macrobdella decora M7
ex. Hirudo orientalis EU616638
ex. Hirudo medicinalis HMBM
ex. Hirudo nipponia
ex. Hirudo medicinalis HmedBM
ex. Hirudo orientalis EU616636
ex. Hirudo verbana PW3 DQ355172
ex. Asiaticobdella fenestrata AFHM
ex. Asiaticobdella buntonensis 11
ex. Goddardobdella elegans BM
– 10 changes

FIGURE 16.

Consensus of 36 equally parsimonious trees resulting from analysis of 16S rDNA sequences of a variety of species and strains of Bacteroidetes, as well as those isolated from the crop of hirudiniform leeches (bold). Asterisks denote sequences obtained from type-strains for species. Bacteroidetes genera and close relatives are indicated to the right of the tree. Numbers at nodes are jackknife frequencies. Relationships supported in fewer than 50 jackknife replicates are represented by interrupted lines.

Figure 16.


AB300988 Megamonas funiformis *
EU346729 Megamonas rupellensis *
M94278 Myxococcus coralloides *
AB218220 Myxococcus flavescens *
DQ768119 Myxococcus virescens *
Y10654 Chlorobium phaeovibrioides *
AM690805 Chlorobium limicola
U09866 Chlorobium tepidum complete cds
NZ ABFZ02000022 Bacteroides stercoris ATCC 43183
AB078048 Flexibacter elegans *
AB078064 Flexibacter ruber
AB078062 Flexibacter roseolus
D12660 Persicobacter diffluens
AB078040 Flexibacter aggregans *
R 025463 Sporocytophaga myxococcoides
FJ572666 uncultured Bacteroidetes
NC 008255 Cytophaga hutchinsonii ATCC 33406
FN396961 Flexibacter sp. AMV16
GU368365 uncultured bacterium clone C35
AJ871243 Flexibacter flexilis clone 153-1
ex. Limnatis paluda
ex. Limnobdella mexicana LM10
AM940603 uncultured Flexibacteraceae
gDQ279370 uncultured Flexibacter clone TM19 36
EF535334 uncultured Flexibacter sp. clone G605
298106375 uncultured Flexibacter sp. clone Ef150K04
GU201552 uncultured Flexibacter clone ETT304 C11
M62793 Flexibacter canadensis *
EU787448 Flexibacter sp.
EU729366 Mucilaginibacter sp.
AM490403 Mucilaginibacter gracilis
AM490402 Mucilaginibacter paludis *
AB330392 Mucilaginibacter kameinonensis *
DQ490465 uncultured Sphingobacterium
AJ438173 Pedobacter saltans
EF446147 Pedobacter terricola
AB267722 Pedobacter daechungensis *
EF446146 Pedobacter lentus
AJ438177 Sphingobacterium thalpophilum
EU240954 Sphingobacterium multivorum
EU373423 Sphingobacterium siyangensis
D14021 Flavobacterium yabuuchiae
AJ459411 Sphingobacterium spiritivorum
AB244764 Sphingobacterium composti
DQ824599 uncultured bacterium
AB249372 Sphingobacterium daejeonense
M58796 Flavobacterium mizutaii *
D14024 Sphingobacterium mizutaii
DQ421387 Olivibacter sitiensis *
AB267716 Olivibacter ginsengisoli *
AB267714 Olivibacter terrae *
AB267715 Olivibacter soli *
AM279214 Pedobacter rhizospharae
DQ097274 Pedobacter suwonensis
AM279217 Pedobacter wanjuense
EF660751 Pedobacter agri *
DQ235228 Pedobacter sandarakinus
AM114396 Pedobacter aquatilis *
EF100697 Pedobacter insulae
AB245371 Pedobacter ginsengisoli *
NC 013061 Pedobacter heparinus DSM 2366
AM279218 Pedobacter jejuensis *
AF329953 Pedobacter africanus *
AJ786798 Pedobacter caeni *
AM491368 Pedobacter duraquae *
AM491372 Pedobacter steynii
NR 025534 Pedobacter cryoconitis *
AM491371 Pedobacter hartonius *
DQ092871 Pedobacter koreensis
AJ438174 Pedobacter piscium
FJ535138 uncultured Bacteroidetes
ex. Asiaticobdella buntonensis 11
ex. Asiaticobdella fenestrata AFHM
ex. Goddardobdella elegans BM
EF204468 Pedobacter sp.
EF693742 Pedobacter kwangyangensis
DQ118958 uncultured fenpropathrin degrading bacterium
AM237311 Chitinophaga arvensicola *
AB278570 Chitinophaga terrae *
DQ062743 Chitinophaga skermanii
M62795 Flexibacter sancti *
AB245374 Chitinophaga ginsengisoli *
AF078775 Chitinophaga pinensis *
EF520603 uncultured Sphingobacterium
EF520590 uncultured Sphingobacterium
AF233292 truffle symbiont
EU283364 uncultured Bacteroidetes
EU283361 uncultured Bacteroidetes
EF667912 uncultured Bacteroidetes
M93152 Weeksella virosa
M92278 Flavobacterium gondwanense *
DQ009623 Capnocytophaga leadbeteri *
NR 026094 Capnocytophaga gingivalis *
X67608 Capnocytophaga gingivalis
AM292800 Flavobacterium cell *
AY577821 Flavobacterium columnare *
DQ372981 Flavobacterium filum *
M59051 Flavobacterium johnsoniae *
AM230494 Flavobacterium succinicans
NR 025020 Flavobacterium frigidarium *
AY581114 Flavobacterium weaverense *
AJ784993 Alkaliflexus imshenetskii *
AB362265 Rikenellaceae
AB362265 uncultured Bacteroidales
DQ141183 Ruminofilibacter xylanolyticum
EF681688 Ruminofilibacter sp.
AJ418048 Anaerophaga thermohalophila *
DQ647061 Anaerophaga sp.
D12672 Marinilabilia salmonicolor *
D12654 Marinilabilia salmonicolor
AB278010 termite gut symbiont
AB277875 termite gut symbiont
AB234401 termite gut symbiont
U41355 Dysgonomonas capnocytophagoides *
NR 026505 Dysgonomonas gadei *
AJ319867 Dysgonomonas mossii *
NR 025052 Porphyromonas gulae
AB238922 Parabacteroides distasonis *
AB261128 Parabacteroides johnsonii *
EU136685 Parabacteroides merdae *
EF608197 Bacteroides fragilis
AB260026 Bacteroides coprophilus *
EF608204 Bacteroides dorei
EF608209 Bacteroides vulgatus
AJ583243 Bacteroides cellulosilyticus *
AB050107 Bacteroides eggerthii *
EF445142 uncultured Bacteroidales
EU266907 uncultured Bacteroidetes
AB234428 uncultured Bacteroidales
ex. Macrobdella decora M7
L16498 Rikenella microfusus
EF608538 beetle digestive tract symbiont
EU884961 fish gut symbiont
ex. Hirudo verbana PW3 DQ355172
ex. Hirudo nipponia
ex. Hirudo orientalis EU616638
ex. Hirudo medicinalis HMBM
ex. Hirudo orientalis EU616636
ex. Hirudo medicinalis HmedBM
EF401874 uncultured bacterium
EU189022 Alistipes sp.
NR 025909 Alistipes putredinis *
AJ408963 human colon symbiont
AY54721 Alistipes massiliensis
AY538696 uncultured Bacteroides
AY974071 Alistipes onderdonkii *
AY974072 Alistipes shahii *
AY643084 Alistipes finegoldii *
EU728719 Alistipes sp.
"Flexibacter"
Mucilaginobacter
"Pedobacter"
"Sphingobacterium/ Flavobacterium"
Olivibacter
"Pedobacter"
Chitinophaga
Capnocytophaga
Flavobacterium
Ruminofilibacter
Anaerophaga
Marinilabia
Dysgonomonas
Parabacteroides
Bacteroides
Rikenella
Allistipes
50 changes

nearby among a variety of uncultured and unidentified isolates, but closest to a termite gut symbiont. Isolates from African species of *Asiaticobdella* and the Australian *G. elegans* formed a clade deriving from among *Pedobacter* species. Isolates from the Asian *L. paluda* and the Mexican *L. mexicana* formed a clade deriving from among a paraphyletic assemblage of *Flexibacter* species.

DISCUSSION

The two distinct microbial groups resident in the intraluminal crop fluid of hirudiniform leeches exhibit markedly different patterns of historical conservation with respect to their leech hosts. In terms of the genus *Aeromonas*, and even though there appears to be marked host specificity and vertical transmission (Rio *et al.*, 2009), leech phylogenetic relationships are entirely non-predictive of the associations. Nor is geography. That is, the North American *M. decora* (Macrobdellidae) and European *H. orientalis* (Hirudinidae) each harbour *A. jandaei*. Similarly, European *H. medicinalis* harbors the same symbiont, *A. hydrophila*, as the African *A. fenestrata* and is thus distinct from its European congeners *H. verbana* and *H. orientalis*. *Aeromonas veronii* proved to be the most ubiquitous culturable symbiont, inhabiting the crop of leeches in three distinct genera from two families across three continents. Whether or not species of *Aeromonas* are involved in the origins or maintenance of species-level cohesion where recently derived leech congeners are sympatric is an intriguing possibility.

The culturable gut flora of the European medicinal leech, originally named *Pseudomonas hirudinis* Busing, 1953, was widely considered to be a strain of *A. hydrophila* and has been a matter of some concern in the post-operative use of commercially available leeches (Whitaker *et al.*, 2009). Graf (1999) demonstrated that all isolates from commercially available leeches

actually were *A. veronii* and that previous identifications as *A. hydrophila* were misled by the vagaries of chemotaxonomy. Since then, Siddall *et al.*, (2007a) have demonstrated that commercially available European medicinal leeches actually are *H. verbana*, not *H. medicinalis*. It is as ironic to discover *H. medicinalis* harboring *A. hydrophila,* as it is accidentally fortuitous that *H. verbana* has been the leech commercially available for clinical use; it harbors a considerably less-pathogenic *A. veronii* (Silver *et al.,* 2007).

Bacteroidetes symbionts, unlike species of *Aeromonas*, exhibit a considerably tighter historical association with their respective hosts, and one that is more obviously phylogenetically than geographically constrained. The phylogenetic results of Bacteroidetes leech symbionts alone (Figure 15), while remarkably topologically similar to historical expectations from hirudiniform phylogeny (Figure 13) prove illusory when reconsidered in the context of Bacteriodetes more fully (Figure 16). Half of the apparent coevolutionary pattern depicted in the leech-symbiont-only tree evaporates under broader phylogenetic consideration in a manner that should serve as a caution to other investigations of host-symbiont cospeciation. This problem of scale in cospeciation studies has a precendent in Nishiguchi *et al*.'s (1998) work concerning light-emitting symbiotic vibrionids in sepiolid squid. That is, while a preliminary analysis based on only seven species evidenced tight coevolutionary patterns between *Vibrio fischeri* strains and squid hosts, an error of scale that is frequently taken for granted (Kimbell *et al*., 2002; Kimbell and McFall-Ngai, 2003; Nishiguchi, 2002, 2004; Soto, 2009), that cospeciation pattern was erased under fuller consideration of squid and fish associated strains of bioluminescent vibrionids (Dunlap *et al.*, 2007; Keading *et al.*, 2007).

In the broader evaluation of Bacteroidetes, however, the symbiont-leech associations retain more phylogenetic than geographic constraint (Figure 16). All symbionts from species of

Hirudo, whether European or Asian (*H. nipponia*), form a clade of apparently indistinguishable *Alistipes* species. Reflecting leech genus-level diversification (Figure 13), and notwithstanding their inhabiting well-separated continents, Australian species of *Goddardobdella* are host to a symbiont that is closely related to (and barely distinguishable from) a *Pedobacter* species found in two African *Asiaticobdella* species. Similarly, symbionts of leeches in the Praobdellidae form a clade of *Flexibacter*-like species despite the obvious geographic separation of Mexico and Afghanistan. Taken together these results are suggestive of recent, but not ancient, tight historical association between leech hosts and their unculturable Bacteroidetes symbionts. A similar recent historical pattern has been noted in relation to glossiphoniid leeches and their mycetomal symbionts in which three clades of leeches, the genera *Placobdella*, *Placobdelloides* and *Haementeria*, are host to three distinct clades of endosymbiotic bacteria each occupying three distinct mycetomal morphological types (Perkins *et al.*, 2005).

Hints regarding a physiological role that the unculturable symbionts may play in the gut of their leech hosts come from other Bacteroidetes symbionts of invertebrates. Flavobacteriaceae symbionts of termites, for example, are involved both in synthesizing essential amino acids and in recycling nitrogen from uric acid (Bourtzis and Miller, 2006). Whereas we were unable to amplify a Bacteroidetes 16S rDNA from the Asian *H. manillensis*, given its belonging to the family Hirudinidae (Figure 13), we would anticipate such an isolate to group with others in *Pedobacter*. Likewise, others in the mammalophilic clade of mucous membrane feeders (i.e., the Praobdellidae) should prove to be relatively closely related to the marine *Flexibacter flexis*. The inconsistency with which we were able to amplify *Aeromonas* species relative to Bacteroidetes reflects the crop dynamics reported for these two symbionts; both flourish in response to a

bloodmeal but the unculturable Bacteroidetes symbiont persists at higher levels for considerably longer periods after feeding (Kikuchi and Graf, 2007).

AUTHORS' CONTRIBUTIONS

AJP, SCW, and MES contributed to the conception and design of these experiments and data collection. AJP and MES performed the data analyses, contributed reagents, materials, or analytical tools to the study, as well as contributed to the preparation of this portion of the manuscript.

CHAPTER 7

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 AJ, Pacheco-Chaves B, Reeves WK, and Siddall ME. 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 & Ironmonger, 1979; Toman & Dall, 1997). Species of Erpobdelliformes have been used to study various ecological processes including population ecology, competition, niche partitioning, predation, and life history strategies (Maltby & Callow, 1986; Young, 1988). In addition, leeches of this group have been studied as biological indicators of polluted environments (e.g., Wicklum & Davies, 1996; Zaranko *et al.*, 1997). Based on previous phylogenetic studies, Erpobdelliformes were proposed to have evolved from a bloodfeeding ancestor that modified its feeding preferences (Siddall & Burreson, 1998; Apakupakul *et al.*, 1999; Trontelj *et al.,* 1999; Borda & Siddall, 2004b). Sawyer (1986) recognized two families of Erpobdelliformes: 1) 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 2) 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 (Gastrostomobdellinae) within the family Cylicobdellidae.

Phylogenetic studies focusing on erpobdelliform species are less common than those concerning hirudiniforms and glossiphoniid leeches (Borda & Siddall, 2004b; Borda *et al.*, 2008; Phillips & 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 & Trontelj, 2008). Siddall's (2002) examination of the family was performed trough a parsimony analyses of a combined data set of morphological and molecular datasets and found that no genus with more than one species in his analysis (with the exception of *Trocheta*) to be 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 & Kutschera, 2004; Pfeiffer *et al.*, 2005). Others have described new species as members of the junior synonym genus *Dina* (e.g. Grosser & Eiseler, 2008; Grosser & 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 be related 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 a representative of the family Gastrostomobdellidae: *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 (i.e. *E. punctata*). This study represents the most intensive phylogenetic analyses of the family to date in terms of both species and molecular data included as well as because 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 & Siddall 2004b; Grosser & Trontelj 2008; Oceguera-Figueroa *et al*., 2005; Pfeiffer *et al*., 2005; Siddall 2002; Siddall *et al.,* 2001; Siddall & Burreson 1998; Sket *et al*., 2001; Trontelj & 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 & Blinn, 1985) from Montezuma's Well in Arizona, *Erpobdella costata* (Moore, 1901a) 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 6). Leeches were collected from freshwater ponds, lakes and rivers. Specimens were found attached to submerged 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.

Table 9: Taxa used for the phylogenetic analyses of the suborder Erpobdelliformes with collection localities and GenBank accession numbers. *Type species of the families 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	Tokyo, 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

Table 9 continued

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, 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	---

Table 9 continued

Erpobdella krasensis	Vrhnika, Slovenia	---	AF169373	---	---
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 1*	France	AF003274	AF099954	EF417048	AF116001
Erpobdella octoculata 2*	Uzbekistan	HQ336344	---	HQ336361	HQ336378
Erpobdella vilnensis	Germany	DQ009663	---	EF417049	---
Erpobdella monostriata	Germany	DQ009665	---	---	---
Erpobdella nigricollis	Germany	DQ009664	---	---	---

Table 9 continued

Erpobdella melanostoma	Michigan, USA	AF116025	AF462027	AY425395	AF115999
Erpobdella annulata (=cf. *punctate*)	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	Distrito Federal, Mexico City, Mexico	DQ235595	DQ235585	---	DQ235605
Erpobdella (=*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

Table 9 continued

Erpobdella costata	Texas, USA	---	HQ336354	HQ336369	HQ336381
Erpobdella ochoterenai	Distrito Federal, 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	La Vega, Jalisco, Mexico	DQ235600	DQ235590	---	DQ235610

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 2. 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 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 individual gene fragments and the combined 18S, 28S, 12S and COI data were performed using New Search technology with the Ratchet (Nixon, 1999) 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 & 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 v. 1.1 (Jobb, 2008) performing 1000 replicates.

Constraint analyses included on the one hand species recognized previously as members of the genera *Mooreobdella*, *Dina*, and *Erpobdella* (3 independent analyses) as well as 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 likelihood criterion with TREEFINDER using the same data partitions described above.

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 with a range of 4-13%.

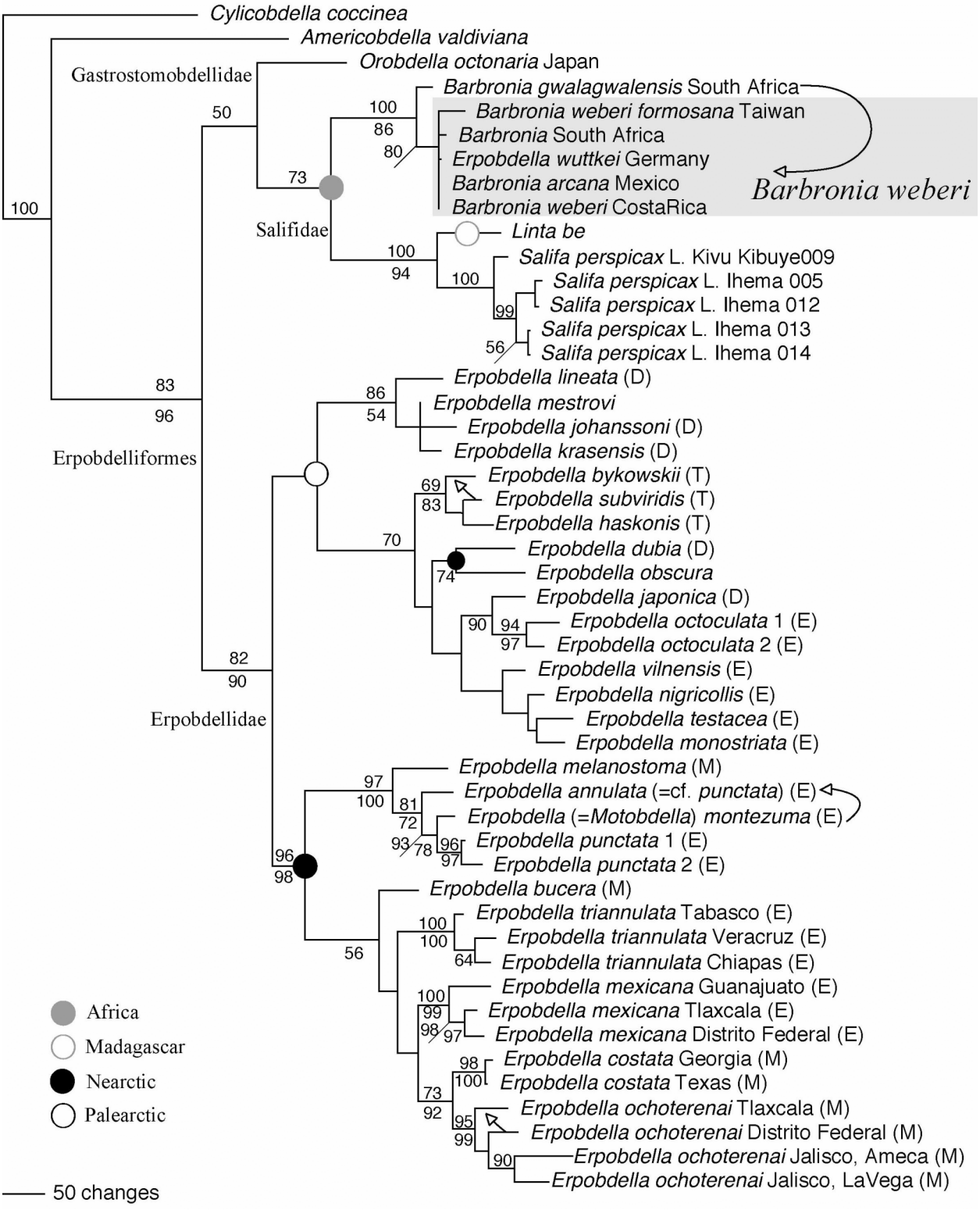
Maximum Parsimony analysis of the complete dataset resulted in 30 most parsimonious trees of 4063 steps, CI= 0.52 and RI= 0.65. The strict consensus tree is shown in Figure 17. The log-likelihood of the tree resulting from the ML analysis was –24320.99. Both analyses resulted in highly congruent topologies. Monophyly of major groups (i.e. Erpobdelliformes, Salifidae and Erpobdellidae) was 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* each were recovered as monophyletic regardless of the optimality criterion. Some disagreement between MP and ML was detected in the position of *Barbronia gwalagwalensis* Westergren & Siddall, 2004, *Erpobdella subviridis, Motobdella montezuma* and *Erpobdella ochoterenai* from Distrito Federal, however, none of their respective alternative grouping achieve meaningful bootstrap support. 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

FIGURE 17.

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 species that appear as sister groups in ML analyses. Arrows with curved lines indicate that species are switched in ML analyses and open arrows with curved lines indicate the alternative placement of *B. gwalagwalensis* into a polytomy of Barbronia spp.

Figure 17.


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)
Erpobdella testacea (E)
Erpobdella monostriata (E)
Erpobdellidae
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

topology comparisons rejecting monophyly of these groups. (Shimodaira-Hasegawa <0.001, approximately unbiased test <0.001).

DISCUSSION

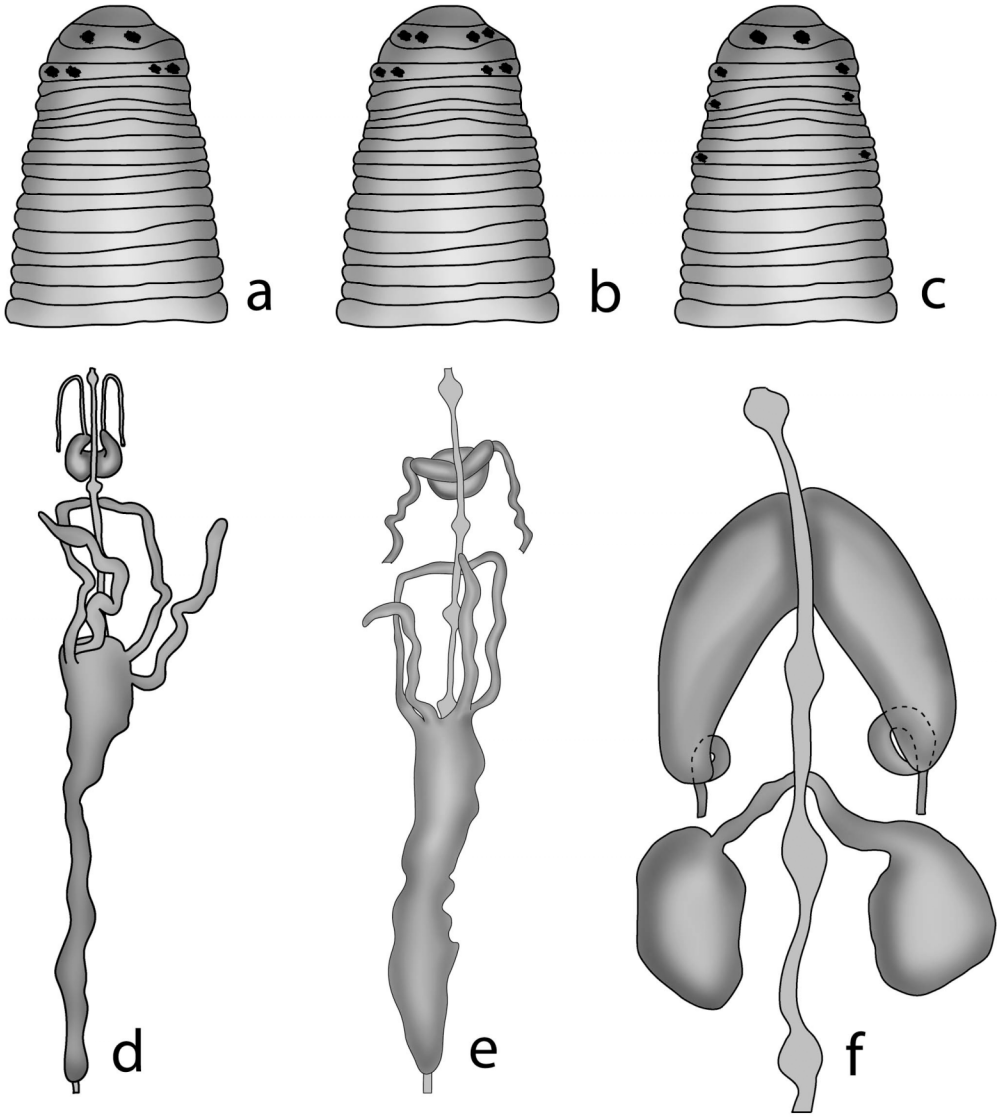
The use of morphological traits to predict phylogenetic relationships or their validation as secondary homologies varies even among closely related groups. Whereas morphology seems to be a good predictor of generic relationships in Salifidae, such is not the case in Erpobdellidae. Cases in which COI 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, 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 18f) 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 18d, e). The shape of the male atrial cornua among species of Erpobdellidae is highly variable, but they are always directed anteriorly or laterally (Figure 18d, e). In contrast, the atrial cornuae of salifids are consistently directed

FIGURE 18.

Morphology of selected erpobdelliformes. Arrangement of eyespots. (a) *Erpobdella punctata*, (b) *Erpobdella octoculata*, (c) *Salifa perspicax*. Dorsal view of male and female genitalia, (d) *Erpobdella punctata,* (e) *Erpobdella costata,* (f) *Salifa perspicax* (f). ac = atrial cornua; ed = eyaculatory duct; ov = ovaries.

Figure 18.



posteriorly (Figure 18f). 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 formed by species of the genus *Barbronia* united by gastropores anterior and posterior to the gonopores. This character has been considered of taxonomic importance by several authors, with El-Shimy (1969) even proposing elevation of the genus to the family Barbronidae. The classical erpobdellid eyespots arrangement, represented by labial and bucal eyespots (Figure 18a,b) is also characteristic of *Barbronia* spp., and contrasts with the arc-shape distribution of the eyespots of species of *Linta* and *Salifa* (Figure 18c). 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 led 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 terrestrial 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 in these analyses, 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. Furthermore, the eyespot arrangement represented by labial and buccal eyespots seems to be restricted to the Erpobdelliformes including *O. octanaria*. However, this condition is transformed again in *Linta*/*Salifa* species. The presence of few testisacs per segment and simple ovaries, characteristics of *O. octonaria* and Salifidae species, are also present in the outgroups. The presence of gastropores, a 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+*Orobdella octonaria*. However, whether this character was lost in *Linta*/*Salifa* or gained independently in *Orobdella 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 (Figure 17), Trontelj & 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 despite their own evidence that such morphological traits are of poor systematic value, Trontelj & 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 & 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 2a), preatrial loops (Figure 2d) 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". *Erpobdella annulata* should be

applied to 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 & 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 & Neubert (1999) in Germany; Pamplin & Rocha (2000) in Brazil; Rutter & Klemm (2001) in USA; Govedich *et al*., (2002) in Australia; Oceguera-Figueroa *et al.,* (2005) in Mexico; and Genoni & 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 & 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 1) 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.

AUTHORS' CONTRIBUTIONS

AOF, AJP, and MES contributed to the conception and design of the experiments, data collection, data analysis, and wrote the paper. AOF, AJP, BPC, WKR, and MES contributed reagents, materials, or analytical tools to the study.

CHAPTER 8

Summary

This dissertation focused on the relationships of the families Hirudinidae, Macrobdellidae, Semiscolecidae, and Praobdellidae. Traditionally, *Hirudo medicinalis* and other freshwater, bloodfeeding arhynchobdellid leeches composed a single family, Hirudinidae, and freshwater, non-bloodfeeding species with a similar body form contained within Haemopidae (Sawyer, 1986). Previous investigations into higher-level relationships included members of Hirudinidae and Haemopidae, but none had focused on the internal relationships within each family (Apakupakul *et al.*, 1999; Trontelj, *et al.*, 1999; Borda and Siddall, 2004b). The intent of this study was to: 1) determine if Hirudinidae is monophyletic, and if not, to revise the family level taxonomy based on phylogenetic results, 2) generally compare the results of phylogenetic analyses to previous classification schemes (Richardson, 1969; Soós, 1969; Sawyer, 1986) to assess the validity of subfamilies, genera, and species, 3) determine if biogeographic patterns of diversification are detectable within and among the traditional members of Hirudinidae, and 4) detect any correlation between hirudinid diversity and bacteria fauna contained within the digestive tracts of various hirudinids.

Borda and Siddall (2004b) established the higher-order relationships among the major groups of Arhynchobdellida providing the phylogenetic backbone for a focused study of Hirudinidae with an expanded taxon sampling. In an analysis of nuclear and mitochondrial data for over 45 species, the traditional Hirudinidae was confirmed to actually contain two independent lineages of medicinal leeches separated by the two families of terrestrial leeches, Haemadipsidae and Xerobdellidae (Phillips and Siddall, 2009), a distinction suggested by previous analyses including molecular data (Borda and Siddall, 2004b; Phillips and Siddall, 2005). Members of the family Haemopidae were found scattered throughout both clades of traditional hirudinid taxa, demonstrating the non-bloodfeeding family Haemopidae to be an

unnatural group. The broader sampling of species of the traditional Hirudinidae revealed the surprising placement of the African genus *Limnatis* and the Mexican genus *Limnobdella* in a clade outside of the monophyletic North and South American leeches in a lineage without any other taxon from the Old World. The lack of a morphological synapomorphy uniting *Limnatis* and *Limnobdella* prevented the clade from being declared a family. A potential resolution would be to expand the family Semiscolecidae, which has priority over Macrobdellidae, to include members of the Macrobdellidae, *Limnatis,* and *Limnobdella.* Phillips and Siddall (2009) only suggested this drastic change in taxonomy pending the addition of more taxa closely related to *Limnatis* and *Limnobdella*.

A new species from Peru more closely related to individuals of *Limnatis* from Africa than other South American taxa brought new information to the taxonomic conundrum of the clade of *Limnatis/Limnobdella. Tyrannobdella rex* is a species unique among leeches because it has only a single jaw with very large teeth. It is also different than most leeches in that it feeds primarily from mucous membranes of mammals, a behavior exhibited by members of the Praobdellidae (Sawyer, 1986). This family originally consisted of three genera (*Praobdella*, *Dinobdella*, and *Myxobdella*) with species distributions restricted to regions of Africa, Asia, and the Middle East. Prompted by the description of the new genus and species, *T. rex*, a closer investigation of the phylogenetic relationships of members of the Praobdellidae was made. With molecular data from fresh specimens, Praobdellidae was determined to be a much more diverse group than previously thought, containing not only the original three genera assigned by Sawyer (1986), but also the genera *Limnatis* and *Limnobdella.* The synapomorphy unique to *Limnatis* and *Limnobdella* preventing their status of a separate family was found to be the behavior of feeding

primarily from mammalian mucous membranes, which is exhibited in all members of the family (Phillips *et al.*, 2010).

The investigations of these families has provided the foundation for further revision of genera and previously described species of hirudiniformes. *Mesobdella lineata* Sciacchitano, 1959 was originally placed within the genus *Mesobdella* because it appeared to be three-annulate. Upon the observation that the mid-body somites are not three-annulate, but actually five-annulate (a common condition of many species of hirudiniformes) it was re-described as *Parapraobdella lineata* within Praobdellidae supported by both external and internal morphological characters (Phillips *et al*., 2011). A new species of Asian medicinal leech, or buffalo leech because of the large size and aggressiveness towards humans, is *Hirudinaria bpling* n. sp. from Thailand that placed within Hirudinidae supported by morphological and molecular evidence. These are just two examples of the ongoing process of taxonomic re-description at the generic and species level within hirudiniformes as a result of the phylogenetic investigations of the familial relationships.

The knowledge of the family relationships has provided the opportunity to explore relationships of bacteria within the digestive tracts of several families of hirudiniformes. Species of *Aeromonas* and an unculturable Bacteroidetes symbiont were detected in the intraluminal crop fluid of members of the families Macrobdellidae, Hirudinidae, and Praobdellidae with different patterns of historical conservation within their hosts. *Aeromonas* species do not directly correlate with host phylogeny or geographic distribution, while the unculturable Bacteroidetes symbionts are more tightly correlated with the taxonomy of their leech hosts than the geographic distribution or phylogeny of those hosts. Within the Bacteroidetes symbionts, this suggests a recent association between bacteria and host, a pattern similar to that observed between the

bacterial symbionts in the mycetomes of species of *Placobdella, Placobdelloides,* and *Haementeria* (Siddall *et al.*, in press; Perkins *et al*., 2005).

Currently, Hirudiniformes includes the freshwater families Hirudinidae, Macrobdellidae, Praobdellidae, and Semiscolecidae, the terrestrial families Haemadipsidae and Xerobdellidae, and terrestrial, microphagous Americobdellidae and Cylicobdellidae. During the course of this study, a broader taxon sampling was acquired for the microphagous Erpobdelliformes, which occur in many of the same habitats as the families of interest in this study. For the first time phylogenetic analyses of the families Salifidae and Erpobdellidae included the type species of each family and morphological characters of the internal reproductive structures, eyespot arrangement, and annulation patterns supported the resulting relationships. The position of the Gastrostomobdellidae, represented by *Orobdella octonaria*, as a member ofErpobdelliformes was supported by molecular data. An evaluation of the barcoding locus for *Barbronia weberi* from several continents adds evidence for regarding this species as invasive (Oceguera-Figueroa *et al.*, 2011).

An analysis of Hirudiniformes and Erpobdelliformes

An analysis of 96 taxa and 159 individuals across the families Macrobdellidae, Semiscolecidae, Praobdellidae, Hirudinidae, Salifidae, Erpobdellidae, and Gastrostomobdellidae (Table X) was performed with datasets adapted from Phillips and Siddall (2009) and Oceguera-Figueroa *et al.* (2011) and the addition of 35 specimens never before included in phylogenetic analyses (Table X): *Odontobdella* sp. from South Korea, *Orobdella whitmani*, *Barbronia weberi* from China and South Korea, *Erpobdella* sp. from Iraq, *Erpobdella japonica* from South Korea, *Semiscolex notatus*, *Orchibdella peruviensis, Orchibdella diaguita*, *Limnatis* sp. from Rwanda,

Table 10: Taxa included in the phylogenetic analyses of Hirudiniformes and Erpobdelliformes. Asterisks indicate type species of the genus.

Family	**Taxon**	**Locality**
Americobdellidae	*Americobdella valdiviana*	Chile
Cylicobdellidae	*Cylicobdella coccinea*	Bolivia
Gastrostomobdellidae	*Orobdella octonaria*	Tokyo, Japan
Glossiphoniidae	*Haementeria ghilianii*	
Erpobdellidae	*Erpobdella lineata*	Denmark Fakse/Falster
	Erpobdella mestrovi	Croatia
	Erpobdella johanssoni	Koper, Slovenia
	Erpobdella krasensis	Vrhnika, Slovenia
	Erpobdella bychowskii	Ljubljana, Slovenia
	Erpobdella subviridis	Cavtat, Croatia
	Erpobdella haskonis	Germany
	Erpobdella dubia	Michigan, USA
	Erpobdella obscura	Ontario, Canada

Table 10 continued

	Erpobdella testacea	France
	Erpobdella japonica	Korea
	*Erpobdella octoculata**	France
	Erpobdella cf. *octoculata**	Uzbekistan
	Erpobdella vilnesis	Germany
	Erpobdella monostriata	Germany
	Erpobdella nigricolis	Germany
	Erpobdella melanostoma	Michigan, USA
	Erpobdella cf. *punctata*	Washington, USA
	Erpobdella punctata 1	Ontario, Canada
	Erpobdella punctata 2	Ontario, Canada
	Erpobdella bucera	Michigan, USA
	Erpobdella mexicana	Tlaxcala, Mexico
	Erpobdella mexicana	Guanajuato, Mexico
	Erpobdella mexicana	Mexico City, Mexico
	Erpobdella montezuma	Arizona, USA
	Erpobdella triannulata	Tabasco, Mexico
	Erpobdella triannulata	Veracruz, Mexico

Table 10 continued

	Erpobdella triannulata	Chiapas, Mexico
	Erpobdella costata *Erpobdella costata*	Georgia, USA Texas, USA
	Erpobdella ochoterenai	Mexico City, Mexico
	Erpobdella ochoterenai	Tlaxcala, Mexico
	Erpobdella ochoterenai	Ameca, Jalisco, Mexico
	Erpobdella ochoterenai	Vega, Jalisco, Mexico
Haemadipsidae		
	Chtonobdella bilineata	Australia
	Chtonobdella whitmani	Australia
	Haemadipsa interrupta	Thailand
	Haemadipsa sylvestris	Vietnam
	Haemadipsa sumatrana	Borneo
	*Idiobdella seychellensis**	Seychelles
	*Malagadbdella fallax**	Madagascar
Hirudinidae	*Aliolimnatis africana*	Central African Republic

Table 10 continued

	Aliolimnatis michaelseni	Guinea Bissau
	Aliolimnatis michaelseni	Congo
	Aliolimnatis oligodonta	Tanzania
	Aliolimnatis buntonensis	South Africa
	Asiaticobdella fenestrata	Zambia
	Goddardobdella elegans 1*	Australia
	Goddardobdella elegans 2*	Australia
	Goddardobdella elegans R*	Australia
	Haemopis grandis	Manitoba
	Haemopis kingi	Manitoba
	*Haemopis sanguisuga**	Sweden
	Haemopis terrestris	OH, USA
	*Hirudinaria javanica**	Vietnam
	Hirudinaria manillensis	Dominican Republic
	Hirudinaria manillensis	Puerto Rico
	Hirudinaria manillensis	Thailand
	Hirudinaria manillensis 11	Vietnam
	Hirudinaria manillensis 24	Vietnam

Table 10 continued

	*Hirudo medicinalis**	BioPharm, UK
	Hirudo nipponia	Korea
	Hirudo orientalis	Azerbaijan
	Hirudo troctina	Morocco
	Hirudo verbana	Leeches USA
	*Limnatis nilotica**	Bosnia
	Limnatis cf. *nilotica*	Namibia
	Limnatis paluda	Afghanistan
	Limnatis paluda	Israel
	*Limnobdella mexicana 1**	Mexico
	*Limnobdella mexicana 2**	Mexico
	*Limnobdella mexicana 3**	Mexico
	*Limnobdella mexicana 4**	Mexico
	*Macobdella decora**	MI, USA
	Macrobdella diplotertia	MO, USA
	Macrobdella ditetra	GA, USA
	Oxyptychus brasiliensis	Brazil
	*Oxyptychus striatus**	Argentina

Table 10 continued

	Patagoniobdella fraterna	Chile
	*Patagoniobdella variabilis**	Chile
	*Philobdella floridana**	SC, USA
	Philobdella gracilis	LA, USA
	Semiscolex intermedius	Argentina
	Semiscolex lamothei	Mexico
	Semiscolex similis	Bolivia
	Whitmania laevis	Taiwan
Salifidae	*Barbronia gwalagwalensis*	Hoedspruit, South Africa
	Barbronia arcana	Morelos, Mexico
	Barbronia weberi formosana	Taiwan
	Barbronia sp.	South Africa
	Barbronia weberi	San Jose, Costa Rica
	Barbronia wuttkei	Germany
	*Linta be**	Tolagnaro, Madagascar
	Salifa perspicax 009*	Lake Kivu, Rwanda
	Salifa perspicax 005*	Lake Ihema, Rwanda

Table 10 continued

	Salifa perspicax 012*	Lake Ihema, Rwanda
	Salifa perspicax 013*	Lake Ihema, Rwanda
	Salifa perspicax 014*	Lake Ihema, Rwanda
Xerobdellidae	*Diestecostoma magnum*	Mexico
	*Diestecostoma mexicana**	Mexico
	Diestecostoma trujillensis	Mexico
	*Xerobdella lecomtei**	Slovenia
	*Mesobdella gemmate**	Chile

Table 11: Taxa or specimens new to phylogenetic analyses.

Erpobdellidae	*Erpobdella* sp.	Iraq
	Erpobdella japonica	South Korea
Gastrostomobdellidae	*Orobdella whitmani* *	
Hirudinidae	*Aliolimnatis* cf *fenestrata 14*	Rwanda
	Aliolimnatis cf *fenestrata* 06	Rwanda
	Aliolimnatis cf *michaelseni* Bama Lrg	Burkina Faso
	Aliolimnatis cf *michaelseni* 07	Rwanda
	Aliolimnatis cf *michaelseni*	Guinea
	Aliolimnatis cf *michaelseni* Bama	Burkina Faso
	Aliolimnatis cf *michaelseni* BFora	Burkina Faso
	Bassianobdella fusca	Tasmania, Australia
	Bassianobdella "bundabergi"	Queensland, Australia
	Goddardobdella elegans	Kimberely desert, Western Australia
	Hirudinaria bpling VN	Vietnam

Table 11 continued

	Hirudinaria bpling WA	Vietnam
	Hirudinaria manillensis	St. Lucia
	Hirudinaria manillensis VN	Vietnam
	Hirudinaria manillensis 11	Vietnam
	Hirudinaria manillensis 24	Vietnam
	Hirudinaria manillensis 25	Vietnam
	Hirudinaria manillensis 28	Vietnam
	Orchibdella diaguita	Uraguay
	Orchibdella peruviensis	Peru
	Richardsonianus stagni 1	Perth, Western Australia
	Richardsonianus stagni 2	Perth, Western Australia
	Richardsonianus stagni	South Australia
	Semiscolex notatus	
	*Whitmania pigra**	South Korea
	Whitmania edentula 1	South Korea
	Whitmania edentula 2	South Korea
Praobdellidae	*Limnatis* cf *nilotica*	Rwanda

Table 11 continued

	Limnobdella mexicana	Baja California Sur, Mexico
	Limnobdella mexicana AG	Mexico
	Limnobdella mexicana	Guanajuato, Mexico
	Limnobdella mexicana	Hidalgo, Mexico
	Limnobdella mexicana	Colima, Mexico
Salifidae	*Odontobdella* sp.	South Korea
	Barbronia weberi	China
	Barbronia sp.	South Korea

Limnobdella Mexicana from several localities in Mexico, *Whitmania pigra*, *Whitmania edentula*, *Haemopis elegans, Priscabdella hickmani* from Tasmania*, Habeobdella stagni* from two localities in Australia, *Goddardobdella elegans* from the Kimberly, Australia, *Hirudinaria bpling* from Vietnam, *Hirudinaria manillensis* specimens from Vietnam and St. Lucia, *Aliolimnatis diversa,* and *Aliolimnatis* cf. *michaelseni* from Rwanda, Guinea, Guinea Bissau, and Burkina Faso. Species identification of newly acquired specimens was made by morphological examination using a Nikon-SMZ645 stereomicroscope. Specimens were stored at -20°C or room temperature in 95-100% ethanol.

Molecular and analytical techniques

Methods of DNA extraction, amplification, cycle sequence reactions, and sequence purification followed procedures previously mentioned in this dissertation (see Chapters 2, 3, 5, and 7). Primers from Phillips *et al*., (2010) were used for the PCR amplification of the nuclear genes 18S rDNA (18S) and 28S rDNA (28S) and the mitochondrial genes 12S rDNA (12S) and cytochrome *c* oxidase I (COI). CodonCode Aligner (CodonCode Corporation) was used to edit and reconcile sequences. Alignments of all genes were accomplished using the European Bioinformatics Institute server for MUSCLE v. 3.7 (Edgar, 2004) applying default settings.

Parsimony analyses of the genes (18S, 28S, 12S, and COI) were performed individually and in combination using TNT v. 1.1 (Goloboff *et al.,* 2008). A new technology search using the Ratchet (Nixon, 1999) for 100 random sequence additions and TBR branch swapping was performed. All characters were left un-weighted and non-additive and gaps were treated as a fifth state. Jackknife values for combined analyses were obtained using random taxon addition and TBR branch swapping with 36% deletion and 100 pseudoreplicates.

Results of phylogenetic analyses

The combined dataset had a total of 3527 characters (18S: 1975 characters, 28S: 386 characters, 12S: 510 characters, and COI: 656 characters). The parsimony analysis produced 4320 equally parsimonious trees with 11974 steps (Figure 19). The relationships between the major families were recovered as in previous analyses of select families (Phillips and Siddall, 2005; Phillips and Siddall, 2009; Phillips *et al.*, 2010; Oceguera-Figueroa *et al*., 2011). The clade of Gastrostomobdellidae species was monophyletic with strong support (jac=100) and placed basal to Erpobdelliformes rather than basal to Salifidae in Oceguera-Figueroa *et al*., (2011). Of the taxa new to phylogenetic analyses, all individuals were most similar to conspecific taxa, or in the case of new specimens being the only representatives of a species, placed within clades of congeneric taxa. It is especially encouraging that the familial relationships of the taxa do not change when gaps are treated as missing data or as a fifth state, although the support values of several nodes that create the backbone of the tree increase by more than 20% with gaps treated as a fifth state.

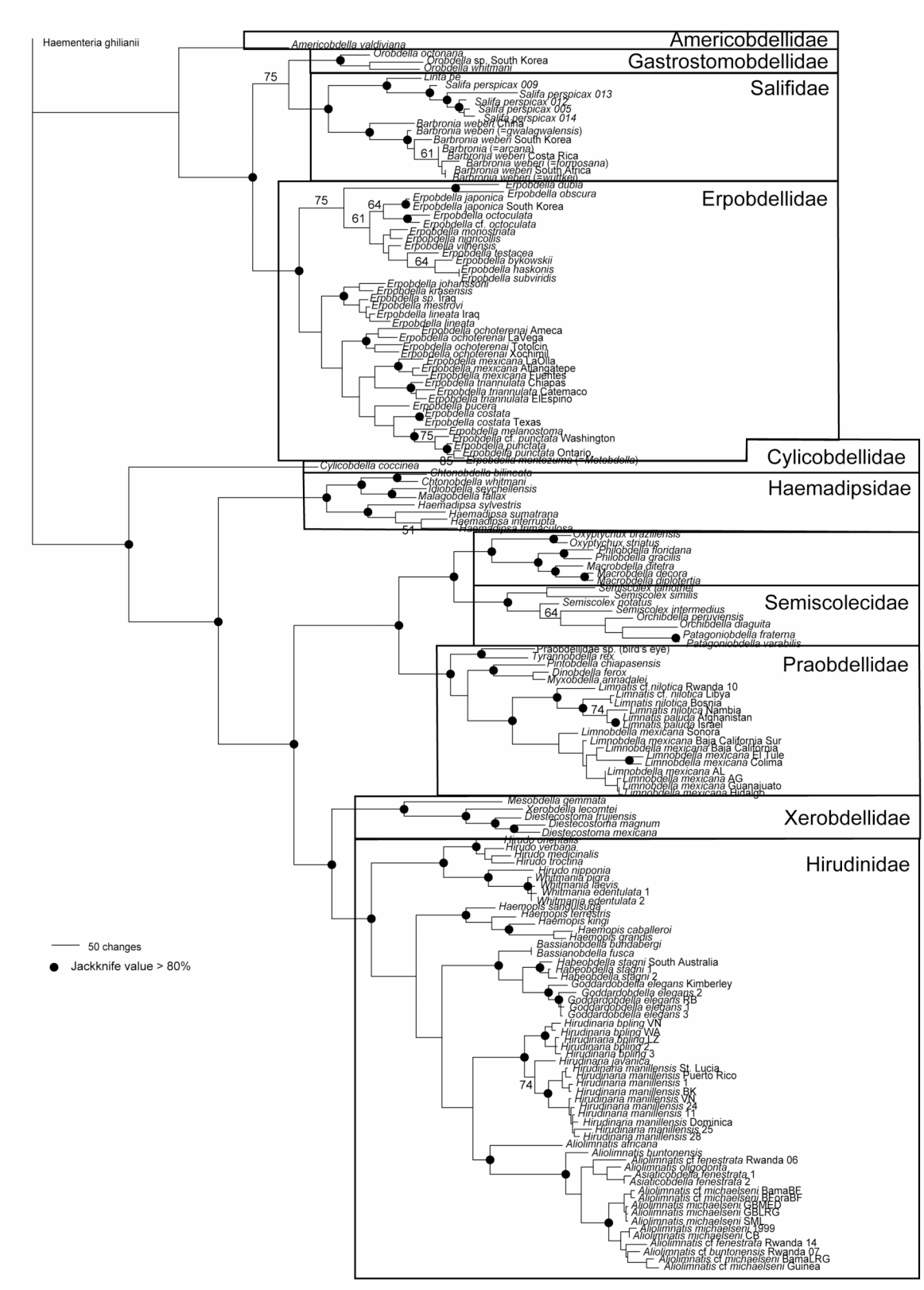
Conclusions

At this point, the lack of fossil evidence as a calibration point for Hirudinea prevents the estimation of dates of origins of any of these groups. Also, the highly diverse geographic distributions of some families (e.g. Praobdellidae) make determining the geographic point of origin of the family very difficult. This is complicated by the fact that the host data for most bloodfeeding species is unknown because they were collected either as freeliving, attached to the

FIGURE 19.

Strict consensus tree resulting from parsimony analysis of members of the families Erpobdellidae, Gastrostomobdellidae, Haemadipsidae, Hirudinidae, Macrobdellidae, Praobdellidae, Salifidae, Semiscolecidae, and Xerobdellidae based on 18S, 28S, 12S and COI sequences. Branch lengths are proportional to the amount of change. Black dots at the nodes indicate greater than 80% jackknife support. Numbers below the nodes indicate jackknife support values. Taxa in bold indicate taxa and specimens never before included in phylogenetic analyses.

Figure 19.


Haementeria ghilianii
Americobdellidae
Gastrostomobdellidae
Salifidae
Erpobdellidae
Cylicobdellidae
Haemadipsidae
Semiscolecidae
Praobdellidae
Xerobdellidae
Hirudinidae
Americobdella valdiviana
Orobdella octonana
Orobdella sp. South Korea
Orobdella whitmani
Linta pe
Salifa perspicax 009
Salifa perspicax 013
Salifa perspicax 012
Salifa perspicax 005
Salifa perspicax 014
Barbronia weberi China
Barbronia weberi (=gwalagwalensis)
Barbronia weberi South Korea
Barbronia (=arcana)
Barbronia weberi Costa Rica
Barbronia weberi (=formosana)
Barbronia weberi South Africa
Barbronia weberi (=wuhlkei)
Erpobdella dubia
Erpobdella obscura
Erpobdella japonica
Erpobdella japonica South Korea
Erpobdella octoculata
Erpobdella cf. octoculata
Erpobdella monostriata
Erpobdella nigricollis
Erpobdella vilnensis
Erpobdella testacea
Erpobdella bykowskii
Erpobdella haskonis
Erpobdella subviridis
Erpobdella johanssoni
Erpobdella kraesnisis
Erpobdella sp. Iraq
Erpobdella mestrovi
Erpobdella lineata Iraq
Erpobdella lineata
Erpobdella ochoterenai Ameca
Erpobdella ochoterenai LaVega
Erpobdella ochoterenai Totolcin
Erpobdella ochoterenai Xochimilc
Erpobdella mexicana LaOlla
Erpobdella mexicana Atlangatepe
Erpobdella mexicana Fuentes
Erpobdella triannulata Chiapas
Erpobdella triannulata Catemaco
Erpobdella triannulata ElEspino
Erpobdella pucera
Erpobdella costata
Erpobdella costata Texas
Erpobdella melanostoma
Erpobdella cf. punctata Washington
Erpobdella punctata
Erpobdella punctata Ontario
Erpobdella montezuma (=Motobdella)
Cylicobdella coccinea
Chtonobdella bilineata
Chtonobdella whitmani
Idiobdella seychellensis
Malagobdella fallax
Haemadipsa sylvestris
Haemadipsa sumatrana
Haemadipsa interrupta
Haemadipsa trimaculosa
Oxyptychus brazilensis
Oxyptychus striatus
Philobdella floridana
Philobdella gracilis
Macrobdella ditetra
Macrobdella decora
Macrobdella diplotertia
Semiscolex armorel
Semiscolex similis
Semiscolex gotatus
Semiscolex intermedius
Orchibdella peruviensis
Orchibdella diaguita
Patagoniobdella fraterna
Patagoniobdella varabilis
Praobdellidae sp. (bird's eye)
Tyrannobdella rex
Pintobdella chiapasensis
Dinobdella ferox
Myxobdella annadalei
Limnatis cf nilotica Rwanda 10
Limnatis cf. nilotica Libya
Limnatis nilotica Bosnia
Limnatis nilotica Namibia
Limnatis paluda Afghanistan
Limnatis paluda Israel
Limnobdella mexicana Sonora
Limnobdella mexicana Baja California Sur
Limnobdella mexicana Baja California
Limnobdella mexicana El Tule
Limnobdella mexicana Colima
Limnobdella mexicana AL
Limnobdella mexicana AG
Limnobdella mexicana Guanajuato
Limnobdella mexicana Hidalgo
Mesobdella gemmata
Xerobdella lecomtei
Diestecostoma trujiensis
Diestecostoma magnum
Diestecostoma mexicana
Hirudo orientalis
Hirudo verbana
Hirudo medicinalis
Hirudo troctina
Hirudo nipponia
Whitmania pigra
Whitmania laevis
Whitmania edentulata 1
Whitmania edentulata 2
Haemopis sanguisuga
Haemopis terrestris
Haemopis kingi
Haemopis caballeroi
Haemopis grandis
Bassianobdella buhdabergi
Bassianobdella fusca
Habeobdella stagni South Australia
Habeobdella stagni 1
Habeobdella stagni 2
Goddardobdella elegans Kimberley
Goddardobdella elegans 2
Goddardobdella elegans RB
Goddardobdella elegans 1
Goddardobdella elegans 3
Hirudinaria bpling VN
Hirudinaria bpling WA
Hirudinaria bpling LZ
Hirudinaria bpling 2
Hirudinaria bpling 3
Hirudinaria javanica
Hirudinaria manillensis St. Lucia
Hirudinaria manillensis Puerto Rico
Hirudinaria manillensis 1
Hirudinaria manillensis BK
Hirudinaria manillensis VN
Hirudinaria manillensis 24
Hirudinaria manillensis 11
Hirudinaria manillensis Dominica
Hirudinaria manillensis 25
Hirudinaria manillensis 28
Aliolimnatis africana
Aliolimnatis buntonensis
Aliolimnatis cf tenestrata Rwanda 06
Aliolimnatis oligodonta
Asiaticobdella tenestrata 1
Asiaticobdella tenestrata 2
Aliolimnatis cf michaelseni BamaBF
Aliolimnatis cf michaelseni BEnraBF
Aliolimnatis michaelseni GBMED
Aliolimnatis michaelseni GBLRG
Aliolimnatis michaelseni SML
Aliolimnatis michaelseni 1999
Aliolimnatis michaelseni CB
Aliolimnatis cf tenestrata Rwanda 14
Aliolimnatis cf buntonensis Rwanda 07
Aliolimnatis cf michaelseni BamaLRG
Aliolimnatis cf michaelseni Guinea
75
61
64
74
51
50 changes
Jackknife value > 80%

collector's body, or do not have a preference more specific than vertebrate blood. The dispersal mechanism of these leeches is also poorly understood and could be a significant factor in defining the extent of the geographic distributions of a single species of leech. For example, members of the Praobdellidae have an affinity for mammalian orifices and have been reported as remaining in the orifice for days or weeks at a time (Harding and Moore, 1927; Cundal *et al.*, 1986). Many of the species of Praobdellidae are found in Africa where mammals that migrate vast distances in short periods of time are in abundance, thereby providing a terrestrial dispersal mechanism for an aquatic organism. This is the most extreme example of the lifestyle of an aquatic member of Hirudiniformes extending the geographic distribution beyond when it can swim (Phillips *et al*., 2010). Species of Hirudinidae and Macrobdellidae have also been recorded feeding on amphibians (Graham and Borda, 2010), which presents the possibility of the leech being transported between watersheds. Establishing a foundation of phylogenetic relationships between families and genera is the first step to providing the context for delimiting species and detecting the boundaries of geographic distributions. This will lead to closer examinations of various aspects of the biology of hirudiniformes as well as provide context for comparison with other groups of annelids or even freshwater invertebrates. A clearer idea of the phylogenetic relationships of hirudiniformes will allow explorations into the potential for invasiveness of select species (e.g. *Hirudinaria*) and conservation methods both to manage overharvested populations (e.g. *Hirudo* medicinalis) or to guard against the introduction of species (e.g. *Barbronia weberi*) into fragile ecosystems.

DISCLAIMER

The new species name presented in Chapter 5 is not intended to formally establish a new taxonomic name.

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AUTOBIOGRAPHICAL STATEMENT

Anna J. Phillips was born in Whiteville, NC and spent her teenage years in Mount Airy, NC. She first came to the American Museum of Natural History (AMNH) in 2004 as undergraduate intern in the National Science Foundation Research Experience for Undergraduates (REU) program. Her summer research project focused on the systematics of the family Macrobdellidae, the North American medicinal leeches. After earning a B.S. in biology from Appalachian State University in Boone, NC, she returned to the AMNH in 2006 as a PhD student at The City University of New York working with Mark Siddall. Anna has always been active in scientific education in public settings through a variety of AMNH sponsored programs as well as summer research programs, such as North Carolina's Summer Ventures in Science and Mathematics program and the AMNH REU program. Anna has engaged students at The Spence School as a substitute science teacher and at the collegiate level as a lab instructor at The City College of New York. In the future, Anna will continue her research with leech systematics while maintaining her involvement in scientific education both inside and outside the classroom.