SYSTEMATICS AND BIODIVERSITY OF ARHYNCHOBDELLIDA (OLIGOCHAETA: HIRUDINIDA) WITH A FOCUS ON THE EVOLUTIONARY HISTORY OF BLOODFEEDING TERRESTRIAL LEECHES

BY

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ABSTRACT

SYSTEMATICS AND BIODIVERSITY OF ARHYNCHOBDELLIDA (OLIGOCHAETA: HIRUDINIDA) WITH A FOCUS ON THE EVOLUTIONARY HISTORY OF BLOODFEEDING TERRESTRIAL LEECHES

ADVISER: Dr. Mark E. Siddall

Arhynchobdellida are a group of specialized annelids characterized by the possession of trignathous (tripartite) muscular jaws used for feeding. Arhynchobdellid leeches are found globally in both freshwater and terrestrial environments and include some of the most widely recognized bloodfeeding species. Arhynchobdellida are remarkably diverse in life history strategies and morphology and were traditionally divided into two major groups: the carnivorous Erpobdelliformes and the diverse Hirudiniformes. This study included an expanded sampling of all major arhynchobdellid families in order to evaluate the phylogenetic relationships and the evolution of life history strategies for Hirudinida. Phylogenetic analyses included two nuclear (18S rDNA and 28S rDNA) and one mitochondrial (mt) *cox1* gene data for forty-seven species and six clitellate species in the outgroup. The resulting hypotheses supported the monophyly of Erpobdelliformes and Hirudiniformes, yet conflicted with traditional classification schemes for Arhynchobdellida, particularly for Hirudiniformes. The phylogenetic hypothesis provides new insights towards our understanding of the evolution of bloodfeeding, terrestrialism and cocoon deposition in Hirudinida and a framework by which to re-evaluate traditional classification of Arhynchobdellida.

With respect to Hirudiniformes, the hypothesis suggested that Haemadipsidae was found in two clades – one associated with the IndoPacific and a second with the New

Word and Europe. Haemadipsidae traditionally include species that are adapted to damp terrestrial environments and are ectoparasites of vertebrate blood. The phylogenetic and biogeographic relationships of the haemadipsids were assessed based on a broader taxonomic sampling from across their range and from the combined analyses of nuclear rDNA 18S and 28S and mt *cox1* and *cox3* data. The elevation of the subfamily Xerobdellinae to family for the non-IndoPacific land leeches was formally proposed for non-IndoPacific terrestrial leeches. IndoPacific leeches were monophyletic and found within two subclades clades roughly divided by geography and morphology. Three-jawed leeches were found to be paraphyletic, with the two-jawed leeches nested within the trignathous clade. Haemadipsidae is formally proposed for bloodfeeding terrestrial leeches from the IndoPacific. The phylogeny provides a framework for the revision of Haemadipsidae and provided new insights towards our understanding of the evolution and biogeography of this group.

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Historical Overview

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Origin of Hirudinida

The origin of leeches and their phylogenetic placement within Clitellata has been controversial and a topic of considerable debate among annelid systematists. The phylogenetic affinities between leeches and branchiobdellidans were proposed as early as 1823, when Odier described branchiobdellidans, better known as the crayfish worms, under the umbrella of leeches. Branchiobdellidans are leech-like freshwater ectoparasites of crustaceans, in particular, crayfish (Hobbs et al., 1967). Some species are grazers of the detritus or epizoic flora found on the surfaces of theirs hosts, while others are parasitic in habit, inhabiting the gill chambers and feeding on dermal tissue, apparently ingesting haemolymph (Holt, 1965: Sawyer, 1986). In Livanow's (1906; 1931) descriptions of *Acanthobdella peledina*, he considered this ectoparasite of salmonid fish to be reminiscent of an ancient hirudinean. Acanthobdellidans have a morphology that appears transitional between oligochaetes and leeches, including the possession of setae restricted to the cephalic region, and an oligochaete-like seminal funnel, yet they possess leech-like fused male gonopores, oblique musculature and a caudal sucker for attachment to their host.

Leeches, and leech-like worms, are a group of specialized clitellate annelids. Members of Clitellata are identified on the basis of the possession of a clitellum – a swollen gland located one-third of the way down the body from the anterior end, and associated with cocoon secretion and deposition; it is most prominent in the common earthworm (e.g. Oligochaeta: Lumbricidae). Clitellates are also hermaphrodites. Unlike the typical earthworm, which has a variable number of body somites (segments), leeches, branchiobdellidans and acanthobdellidans are characterized by a fixed number of body somites (i.e. 34, 15 and 27, respectively). Additionally, they are also distinguish from other clitellates in their possession of a caudal (posterior) muscular sucker, a reduced coelomic space, the reduced (acanthobdellidans) or complete loss (leeches and branchiobdellidans) of setae, and adaptation to an ectocommensalistic or ectoparasitic way of life. In addition to the posterior sucker, leeches are distinct from branchiobdellidans and acanthobdellidans in also possessing a well-developed muscular oral (anterior) sucker that is used to aid in the attachment to their hosts and for locomotion on land or in water.

Before the late 1990's, hypotheses on the evolution of leeches were limited to the subjective interpretation and identification of plesiomorphic morphological characters and corresponding homologies. Cladistic analyses of morphological data supported leeches, branchiobdellidans and *Acanthobdella peledina* having a common origin (Brinkhurst and Gelder, 1989; Purschke et al., 1993; Brinkhurst 1994; Siddall and Burreson, 1995) and the subsequent inclusion of molecular data further corroborated this hypothesis (Siddall and Burreson, 1998; Apakupakul et al., 1999; Trontelj et al., 1999). Others, however, explained these relationships based on the convergent adaptation to an ectocommensalistic lifestyle and not due to shared ancestry (Holt 1989; Brinkhust and Gelder, 1989; Purschke et al., 1993; Brinkhurst, 1994;1999). The lack of exact correspondence of several morphological characters was central to this debate. Siddall et al. (2001) reexamined the higher-level relationships within the Clitellata using an expanded taxonomic sampling, with over 100 annelids and using molecular data alone (i.e. nuclear 18S rDNA and mitochondrial cytochrome c oxidase subunit I gene sequences). These results, once again, supported the notion that leeches, branchiobdellidans and *Acanthobdella peledina* are a monophyletic group, each in their own respective clades and together are a derived group of oligochaetes, with lumbriculids (Lumbriculida) as their sister taxon. As such, Siddall et al. (2001) proposed that each (i.e. leeches, branchiobdellidans and *Acanthobdella peledina*) be considered orders of equal ranking: Hirudinida, Branchiobdellida and Acanthobdellida, respectively and that Clitellata be synonymized with Oligochaeta.

Classification of Hirudinida

There are approximately 650 described species of leeches worldwide (Sawyer 1986), an underestimation with many more species yet to be discovered. Leeches have been classified into two major groups: the Rhynchobdellida Blanchard, 1894 and the Arhynchobdellida Blanchard, 1894 (see Sawyer, 1986). Several monographs have described the diversity of leeches of the world, including descriptions of the Neotropical and Subantarctic taxa (e.g. Weber, 1915, Cordero, 1937, Ringuelet, 1944, 1976,1985; Caballero, 1956; Soós 1966; Siddall, 2001a, b; Siddall and Borda, 2004), North American leeches (e.g. Davies, 1971; Sawyer et al., 1976; Klemm, 1982), African (Augener, 1936; Oothuizen, 1980), European (e.g. Autrum, 1958; Minelli, 1979; Mann, 1961, 1967) and IndoPacific (e.g. Blanchard, 1917; Moore, 1924b; Harding and Moore, 1927; Keegan et al., 1968; Soós, 1967; Richardson, 1975, 1978; Borda, 2006) and many more. The most comprehensive taxonomic contribution to date is Roy Sawyer's (1986) 3-volume, *Leech Biology and Behaviour.* At the time it was, and for the most part still is, the most up to date classification for leeches, based on traditional methods in taxonomy and classification. However, many of Sawyer's (1986) recommended taxonomic revisions for leeches were not formally described or justified.

The advent of molecular data acquisition (used alone or in combination with behavioral and morphological data) for phylogenetic work brought new light into leech systematics in the late 1990's. Several studies have collectively established the framework for the higher-level evolutionary relationships of leeches (Siddall and Burreson, 1995, 1998; Apakupakul et al., 1999; Trontelj et al., 1999) and the subsequent inclusion of a broader taxonomic sampling of leech species has been instrumental in understanding the interfamilial, intergeneric and intrageneric relationships (Siddall 2002; Siddall and Borda, 2003; Utevsky and Trontelj, 2004; Pfeiffer et al., 2004; Williams and Burreson, 2006).

"Rhynchobdellida"

Rhynchobdellid leeches are characterized by the possession of a muscular proboscis that is used to penetrate deep vascularized tissue for bloodfeeding (e.g. *Haementeria ghilianii*) or sucking the coelomic fluid of their invertebrate prey (e.g. *Glossiphonia complanata*). There are three rhynchobdellid families: the dorso-ventrally flattened Glossiphoniidae Vaillant, 1890, the freshwater and marine fish leeches in the Piscicolidae Johnston, 1865 and the turtle leeches of the Ozobranchidae Pinto, 1921. Sawyer (1986) divided the Glossiphoniidae into subfamilies: Glossiphoniinae, Haementeriinae, and Theromyzinae based on characters related to mode of reproduction, cocoon deposition and parental care (see Cocoons and Parental Care; below). Except for the Theromyzinae, the subfamilies of the Glossiphoniidae were rendered paraphyletic in an analysis by Light and Siddall (1999). Theromyzinae (i.e. *Theromyzon*) is monophyletic, but was found nested within a clade containing members from the other two subfamilies. Moreover, reproductive behaviors and mode of parental care overlap across glossiphoniid taxa and were considered not to be consistent characters for delimiting groups (see also Siddall et al., 2005).

The family Piscicolidae were divided into the Platybdellinae Epshtein, 1970, Pontobdellinae Llewellyn, 1966, and Piscicolinae Caballero, 1940 based on the specialization of coelomic space, the presence or absence of pulsatile vesicles and reproductive morphologies. Like Glossiphoniidae, results from recent phylogenetic investigation of the Piscicolidae (Utevsky and Trontelj 2004; Williams and Burreson, 2006), suggest that the traditional groupings into subfamilies, on morphology alone are not supported.

Lastly, the Ozobranchidae, including species that are parasitic on sea turtles and one freshwater species, are anomalous in terms of its phylogenetic placement within the Rhynchobdellida. Previous phylogenetic work has found Ozobranchidae (represented by *Ozobranchus margoi*) as sister to Piscicolidae (Siddall and Burreson, 1998; Apakupakul et al., 1999) or as sister to Glossiphoniidae (Utevsky and Trontelj 2004; Williams and Burreson, 2006). The inclusion of only a single species as the sole representative for the family and limited molecular data (i.e. 18S rDNA, mt COI, mt NDI) underscores the need for addition taxonomic representation for the family, as well as the exploration of new genes.

Phylogenetic assessments including members of the Rhynchobdellida have indicated that the group is not monophyletic (Apakupakul et al., 1999; Trontelj et al.

1999; Borda and Siddall 2004). Save for the presence of a proboscis, the Glossiphoniidae and Piscicolidae appear to have independent origins (Apakupakul et al., 1999; Trontelj et al., 1999; Borda and Siddall 2004). The term rhynchobdellid leech will still be used here in reference to leeches that possess a proboscis, but continued use of Rhynchobdellida would imply accepting the paraphyletic assemblage for the group.

Arhynchobdellida

Arhynchobdellid leeches lack a proboscis and are characterized by a pharynx that has been modified into muscular "jaws" for feeding. The monophyletic Arhynchobdellida Blanchard, 1894 are further subdivided into the Erpobdelliformes Caballero, 1952 and the diverse Hirudiniformes Caballero, 1952 (Siddall and Burreson 1995, 1996 and 1998; Apakupakul et al. 1999; Trontelj et al. 1999; Borda and Siddall 2004). The former are strictly carnivorous and traditionally include the families Salifidae Johansson, 1910 and Erpobdellidae Blanchard, 1894. The Erpobdellidae are widely distributed throughout the Northern Hemisphere/Palaearctic region, while the Salifidae are found throughout the Southern Hemisphere, except South America (Sawyer 1986); both are found in freshwater systems.

Traditionally, hirudiniform leeches were divided into five families: Americobdellidae Caballero, 1956, Cylicobdellidae Ringuelet, 1972, Haemadipsidae Blanchard, 1893, Haemopidae Richardson, 1969, and Hirudinidae Whitman, 1886 (see Sawyer, 1986). The first two families and the Haemopidae are predaceous carnivores, and the remaining sanguivorous Hirudiniformes were principally divided based on habitat preference (i.e. the semi-aquatic Hirudinidae and the terrestrial Haemadipsidae).

Hirudiniform leeches are found in all continents, except Antarctica, in freshwater and terrestrial communities. Some of the families are strictly endemic to certain regions, such as Cylicobdellidae known only from South America, Americobdellidae found in Chile and Haemadipsidae from the IndoPacific.

Previous phylogenetic works including members of Arhynchobdellida have found that the majority of the traditional families in Hirudiniformes were not true monophyletic assemblages, namely the families Hirudinidae, Haemopidae and Haemadipsidae (Siddall and Burreson, 1995; Apakupakul et al., 1999; Trontelj et al., 1999). In this study, the phylogenetic relationships and evolution of life history strategies of Hirudinida will be examined, with a focus on the arhynchobdellid leeches. Subsequently, the evolutionary history and biogeographic relationships of bloodfeeding terrestrial leeches of the world are assessed.

CHAPTER 2

Evolution of Hirudinida, with a Focus on the Arhynchobdellid Leeches (Annelida: Oligochaeta: Hirudinida)

(Adapted from: Borda, E. and Siddall, M. E. 2004a. Arhynchobdellida (Annelida: Oligochaeta: Hirudinida): Phylogenetic Relationships and Evolution. *Molecular Phylogenetics and Evolution* 30: 213 – 225; Borda, E. and Siddall, M. E. 2004b. Review of the evolution of life history strategies and phylogeny of Hirudinida (Annelida: Oligochaeta) *Lauterbornia* 52: 5–25).

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INTRODUCTION

Arhynchobdellida are characterized by their possession of muscular jaws for feeding and the lack of a proboscis. Arhynchobdellid leeches are remarkably diverse in morphology and in life-history strategies, and are found globally in freshwater and terrestrial habitats. Some widely-recognized species include the sanguivorous 'medicinal' leeches found in freshwater lakes and ponds, and terrestrial leeches found in tropical jungles where "they are among the most dominant and self-assertive elements" (Moore, 1927: p. 224). Arhynchobdellid leeches are well known for their bloodletting capacity and have been frequently encountered in historical and contemporary medical applications, with some of the earliest accounts dating as far back as 200 A. D. (Kunja Lal Sharma's *Sus'ruta Samhitá*). The 19th century saw increased exploitation of the European medicinal leech, *Hirudo medicinalis*. Overharvesting for therapeutic phlebotomy throughout Europe (Shipley, 1927; Sawyer, 1986), rendered wild populations either threatened or endangered in over 15 European countries (Wells et al., 1983; Elliott and Tullett, 1984, 1992; IUCN, 1993; Council of Europe, 1998). This traditional use led to medical and biochemical uses for post-operative haematoma treatment (Walsmann and Markwardt, 1985; Lent, 1986), as well as to the discovery of anti-thrombin and anti-platelet activities in their salivary secretions (Baskova et al., 1987; Munro et al., 1992a, 1992b; Vindigni et al., 1994; Yang et al., 1997; Hong et al., 1999).

In addition to the sanguivorous aquatic and terrestrial jungle leeches, other arhynchobdellid groups occupy various niches as non-sanguivorous predators of oligochaetes or of aquatic invertebrate larvae. Arhynchobdellida also includes temperate terrestrial leeches and even 'eyeless' cave-dwelling species. Exhibiting a marked scope of morphology and life history strategies, as well as a diversity of feeding and reproductive behaviors, arhynchobdellid species have been used as model organisms in several fields of biology. These include neurobiology and development (Munro et al., 1992b; Aisemberg et al., 2001; Burrell et al., 2003), phenology (Demirsoy et al., 2001), ecology (Dall, 1987; Schalk et al., 2002), environmental science (Shapkarev and Vagner, 1989) and conservation (Sawyer, 1979, 1981; Council of Europe, 1998; Kasparek et al., 2000).

The evolutionary relationships of leeches and their relationship to other annelids, have included work based on morphology (Brinkhurst and Gelder, 1989; Holt, 1989; Siddall and Burreson, 1995), life history (Siddall and Burreson, 1996), nuclear and mitochondrial gene sequences (Siddall and Burreson, 1998; Siddall et al., 2001), combined morphology and molecular data at the familial level (Apakupakul et al., 1999; Light and Siddall, 1999; Siddall, 2002) and at the generic level (Siddall and Borda, 2003). Recent phylogenetic assessments have suggested and accepted that many traditional groupings of leeches are artificial and not monophyletic (Sawyer, 1986; Siddall and Burreson, 1995, 1998; Apakupakul et al., 1999; Trontelj et al., 1999).

In a continued effort towards resolving the ingroup relationships of Hirudinida, the evolutionary relationships and divergence patterns of the seven families of Arhynchobdellida, as recognized by Sawyer (1986), are investigated here.

MATERIALS AND METHODS

Taxa

Forty-six species were chosen to represent the seven arhynchobdellid families, as well as seventeen rhynchobdellid species for phylogenetic analyses (Table 1). Collectively these cover an extensive global distribution (Table 2). Species not included in previous phylogenetic work are *Aliolimnatis africana*, *Americobdella valdiviana*, *Barbronia gwalagwalensis*, *Barbronia weberi formosana*, *Barbronia* sp., *Cylicobdella coccinea*, *Erpobdella costata*, *Haemadipsa picta*, *Haemadipsa sumatrana*, *Haemopis kingi*, *Haemopis grandis, Haemopis terrestris, Hirudinaria manillensis, Hirudo medicinalis* (sensu stricto), *Hirudo nipponia, Limnatis nilotica, Linta be, Macrobdella ditetra*, *Mesobdella gemmata*, *Oxyptychus braziliensis*, *Oxyptychus striatus*, *Patagoniobdella variabilis*, *Patagoniobdella fraterna*, *Semiscolex similis*, *Whitmania laevis*. The following outgroup taxa were selected based on prior phylogenetic work (Siddall et al., 2001): *Lumbriculus variegates*, *Eclipidrilus frigidus*, *Cambarincola gracilis*, *Branchiobdella pentadonta*, *Branchiobdella parasitica*, *Acanthobdella peledina*. All taxa included in this study, their sampling localities and GenBank accession numbers for new sequences, as well as sequences reported in other work are listed in Table 2.

DNA Extraction and Purification

Leeches were stored in 95 – 100% ethanol at –20° C or at ambient temperature until used for DNA extraction. Tissue from the caudal sucker was used in order to minimize the possibility of contamination from host/prey DNA found in the

TABLE 1.
Current classification of Hirudinida (Sawyer, 1986; Siddall et al., 2001), with representative arhynchobdellid families and genera included in this study.

- **PHYLUM** Annelida
  - **CLASS** Oligochaeta (= Clitellata)
    - **ORDER** Hirudinida
      - **“RHYCHOBDELLIDA”**
        - **FAMILY** Glossiphoniidae (~13 genera)
          - *Glossiphonia*
          - *Helobdella*
          - *Theromyzon*
          - *Haementeria*
          - *Desserobdella*
          - *Placobdella*
        - Ozobranchidae (2 genera)
          - *Ozobranchus*
        - Piscicolidae (~21 genera)
          - *Piscicola*
          - *Stribarobdella*
          - *Pontobdella*
      - **ARHYNCHOBDELLIDA**
        - **Hirudiniformes** (5 families)
          - **FAMILY** Cylicobdellidae (6 genera)
            - *Cylicobdella*
          - Haemopidae (4 genera)
            - *Haemopis*
            - *Patagoniobdella*
            - *Semiscolex*
            - *Whitmania*
          - Hirudinidae (22 genera)
            - *Aliolimnatis*
            - *Hirudinaria*
            - *Hirudo*
            - *Limnatis*
            - *Macrobdella*
            - *Oxyptychus*
          - Haemadipsidae (~17 genera)
            - *Chtonobdella*
            - *Haemadipsa*
            - *Mesobdella*
            - *Xerobdella*
          - Americobdellidae (1 genus)
            - *Americobdella*
        - **Erpobdelliformes** (2 families)
          - **FAMILY** Erpobdellidae (3 genera)
            - *Erpobdella*
          - Salifidae (5 genera)
            - *Barbronia*
            - *Linta*

TABLE 2.
Collection localities and GenBank accession numbers for taxa used for the phylogenetic analyses of Hirudinida.

		GenBank Accession Number			
TAXON	**LOCALITY**	**12S**	**18S**	**28S**	**COI**
INGROUP					
ARHYNCHOBDELLIDA					
Aliolimnatis africana	Central African Republic	AY425428	AY425469	AY425387	AY425451
Aliolimnatis michaelseni	Republic of Congo	AY425429	AF116010	AY425388	AF116029
Americobdella valdiviana	Chile	AY425407	AY425461	AY425358	AY425443
Barbronia sp.	South Africa	–	AY786463	AY786450	AY786457
Barbronia gwalagwalensis	South Africa	–	AY786462	AY786449	AY786455
Barbronia weberi	Austria	–	AF099951	–	–
Barbronia weberi formosana	Hawaii, USA	–	AY786461	AY786448	AY786456
Chtonobdella bilineata	Australia	AY425410	AF116006	AY425361	AF003267
Cylicobdella coccinea	Bolivia	AY425411	AY425462	AY425362	AY425444
Erpobdella lineata	Denmark	AF099952	AF099950	AY425367	–
Erpobdella bucera	Michigan, USA	AF462026	AF115998	AY425394	AF116024
Erpobdella costata	Georgia, USA	AY425442	AY425478	AY425406	AY425460
Erpobdella dubia	Michigan, USA	AF462022	AF115997	AY425365	AF116023
Erpobdella japonica	Korea	AF462023	AF116000	AY425366	AF116026
Erpobdella melanostoma	Michigan, USA	AF462027	AF115999	AY425395	AF116025
Erpobdella mestrovi	Croatia	–	AF272842	–	–

TABLE 2. Continued

Erpobdella obscura	Ontario, Canada	AF462028	AF116004	AY425396	AF003273
Erpobdella octoculata	France	AF099954	AF116001	AY425368	AF003274
Erpobdella punctata	Ontario, Canada	AF462024	AF116002	AY425369	AF003275
Erpobdella testacea	France	AF462025	AF116003	AY425370	AF116027
Haemadipsa picta	Borneo	–	AY425463	–	AY425445
Haemadipsa sumatrana	Borneo	AY425415	AY425464	AY425372	AY425446
Haemadipsa sylvestris	Vietnam	AY425416	AF116005	AY425373	AF003266
Haemopis caeca	Romania	AY425419	AY040687	AY425376	AY040702
Haemopis grandis	Manitoba, Canada	AY425420	AY425465	AY425377	AY425447
Haemopis kingi	Manitoba, Canada	AY425421	AY425466	AY425378	AY425448
Haemopis lateromaculata	Michigan, USA	AY425422	AF116009	AY425379	AF116028
Haemopis marmorata	Michigan, USA	AY425423	AF116008	AY425380	AF003270
Haemopis sanguisuga	Sweden	AF099960	AF099941	AY425381	AF462021
Haemopis terrestris	Ohio, USA	AY786446	AY786465	AY786452	AY786459
Hirudinaria manillensis	Puerto Rico	AY425426	AY425467	AY425384	AY425449
Hirudo medicinalis	United Kingdom	AF099961	AF116011	AY425385	AF003272
Hirudo medicinalis	France	–	AY786464	AY786451	AY786458
Hirudo nipponia	Korea	AY425427	AY425468	AY425386	AY425450
Limnatis nilotica	Israel	AY425430	AY425470	AY425389	AY425452
Linta be	South Africa	–	AY786466	AY786453	AY786460
Macrobdella decora	Michigan, USA	AY425431	AF116007	AY425390	AF003271
Macrobdella ditetra	Georgia, USA	AY425432	AY425471	AY425391	AY425453

TABLE 2. Continued

Mesobdella gemmata	Chile	AY425434	AY425472	AY425393	AY425454
Oxyptychus braziliensis	Brazil	AY425436	AY425473	AY425398	AY425455
Oxyptychus striatus	Argentina	–	AY425474	AY425399	AY425456
Patagoniobdella fraterna	Chile	AY425441	AY425477	AY425405	AY425459
Patagoniobdella variabilis	Chile	–	AY425476	–	AY425458
Semiscolex similis	Bolivia	AY425439	AY425475	AY425402	AY425457
*Whitmania laevis**	Taiwan	AY786447	AY786467	AY786454	–
Xerobdella lecomtei	Slovenia	–	AF099947	–	–
"RHYNCHOBDELLIDA"					
Branchellion torpedinis	South Carolina, USA	AY425408	AF115993	AY425359	AF003265
Calliobdella vivida	Virginia, USA	AY425409	AF115992	AY425360	AF003260
Desserobdella picta	Ontario, Canada	AY425413	AF115988	AY425364	AF116020
Glossiphonia complanata	United Kingdom	AY425414	AF115982	AY425371	AF003277
Haementeria ghilianii	French Guyana	AY425417	AF115985	AY425374	AF329035
Haementeria gracilis	Uruguay	AY425418	AF115984	AY425375	AF329034
Helobdella paranensis	Uruguay	AY425412	AF115987	AY425363	AF329037
Helobdella stagnalis	France	AY425424	AF115986	AY425382	AF116018
Hemiclepsis marginata	France	AY425425	AF115981	AY425383	AF003259
Marsupiobdella africana	South Africa	AY425433	AF115979	AY425392	AF116015
Oligobdella biannulata	North Carolina, USA	AY425435	AF115989	AY425397	AF116021

TABLE 2. Continued

Ozobranchus margoi	Virginia, USA	–	AF115991	–	AF003268
Piscicola geometra	France	AY425437	AF115995	AY425400	AF003280
Placobdella parasitica	Ontario, Canada	AY425438	AF115990	AY425401	AF003261
Pontobdella muricata	Slovenia	AF099958	AF099945	–	–
Stibarobdella macrothela	Virginia	AY425440	AF115996	AY425403	AF116022
Theromyzon tessulatum	France	AF099957	AF115980	AY425404	–
OUTGROUP	–				
Acanthobdella peledina	–	–	–	–	AF003264
Branchiobdellida parasitica	–	–	AF310690	–	AF310707
Branchiobdellida pentadonta	–	–	AF310691	–	AF310708
Cambarincola gracilis	–	–	AF310692	–	AF310709
Eclipidrilus frigidus	–	–	AY040692	–	AY040706
Lumbriculus variegates	–	–	AY040693	–	AY519464

gastric and intestinal regions. DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA) was used for tissue lysis and DNA purification.

Nuclear and Mitochondrial DNA Sequence Amplification

PCR amplification of nuclear 18S and 28S rDNA, and mitochondrial (mt) 12S rDNA and cytochrome *c* oxidase subunit I (COI) gene fragments was accomplished with the primers in Table 3. To obtain 18S rDNA fragments, the primer pairs “AL,” “CY” and “BO” were used, yielding three overlapping shorter double stranded DNA fragments of approximately 600 base pair (bp) each in length (Apakupakul et al., 1999). Amplifications of 28S, 12S and COI yielded fragments of approximately 365, 400 and 665 bp, respectively. Amplification reaction mixtures for gene fragments used Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ), 1 μl of each 10 μM primer, 2 μl DNA template and 21 μl RNase-free H_2O (total volume, 25 μl). All amplification reactions were performed in a GeneAmp PCR System 9700 (P E Applied Biosystems).

The following amplification protocols were used: 18S — heated to 94° C for 5 minutes, followed by 35 cycles of 94° C (15 sec), 44° C (20 sec), and 70° C (90 sec) and a final extension at 72° C (7 min); 28S — heated to 94° C for 5 minutes, followed by 30 cycles of 95° C (1 min), 52° C (1 min), and 70° C (1 min) and a final extension at 72° C (7 min); 12S — heated to 94° C for 5 minutes, followed by 30 cycles of 95° C (1 min), 52° C (1 min), and 70° C (1 min) and a final extension at 72° C (7 min); and COI — heated to 94 C for 5 minutes followed by 15 cycles of 94° C (45 sec), 47° C (45 sec), and 72° C (45 sec), then 25 cycles of 94° C (20 sec), 45° C (20 sec) and 72° C (30 sec) and a

TABLE 3.
Primers used for gene amplification and sequencing.

GENE	**PRIMER NAME**	**PRIMER SEQUENCE** 5' → 3'	**REFERENCE**
NUCLEAR			
18S rDNA	A	AACCTGGTTGATCCTGCCAGT	Apakupakul et. al., 1999
	L	CCAACTACGAGCTTTT	Apakupakul et. al., 1999
	C	CGGTAATTCCAGCTC	Apakupakul et. al., 1999
	Y	CAGACAAATCGCTCC	Apakupakul et. al., 1999
	B	TGATCCTTCCGCAGGTTCACCT	Apakupakul et. al., 1999
	O	AAGGGCACCACCAG	Apakupakul et. al., 1999
28S rDNA	28S-A	GACCCGTCTTGAAGCACG	Whiting, 2002
	28S-B	TCGGAAGGAACAGCTACTA	Whiting, 2002
12S rDNA	12S-AI	AAACTAGGATTAGATACCCTATTAT	Palumbi, 1996
	12S-BI	AAGAGCGACGGGCGATGTGT	Simon et al, 1990
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994

final extension at 72° C (6 min). The QIAquick PCR Purification Kit protocol (QIAGEN, Inc.) was employed to purify amplification products.

DNA Sequencing

Amplification products were sequenced in both directions. Each 8 μl sequencing reaction mixture included 2 μl BigDye™ (Applied Biosystems, Perkin-Elmer Corporation), 2 μl of dye 'extender' buffer (1 M Tris, pH 9; 25 mM $MgCl_2$), 2 μl of 1 μM primer and 2 μl of gene amplification product. Samples were sequenced in a GeneAmp PCR System 9700 for 30 cycles at 96° C (10 sec), 50° C (10 sec) and 60° C (4 min). Sequences were purified by 70% isopropanol / 70% ethanol precipitation to remove primers and unincorporated dyes and were electrophoresed in an ABI Prism™ 3700 sequencer (Applied Biosystems).

DNA Sequence Alignment

Sequences of complimentary strands were edited and reconciled with Sequence Navigator (Applied Biosystems). Alignments for the two nuclear sequences and mt 12S sequences were accomplished using Clustal W in MacVector™ 7.0 (Symantec Corporation) software package under the following alignment parameters: gap cost = 6; open gap = 3 (6:3) or gap cost = 10; open gap = 3 (10:3) and alignment of COI fragments was done by eye across all taxa because there were no insertions or deletions. The alignment that produced the shortest number of steps, regardless of number of trees, was favored for this study.

Phylogenetic analyses

Parsimony analyses were performed using PAUP* 4.0b10 (Swofford, 2000). Analyses used 20 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All characters were left unweighted and non-additive. Parsimony jackknife (jac) values were obtained with 1000 pseudoreplicates and tree-bisection-reconnection branch swapping with 37% deletion (Farris, 1999). TreeRot.v2b (Sorrenson, 1999) was used to calculate support values (b, see Bremer, 1988). Retention indices (RI) and consistency indices (CI) were calculated with PAUP* (Swofford, 2000).

RESULTS

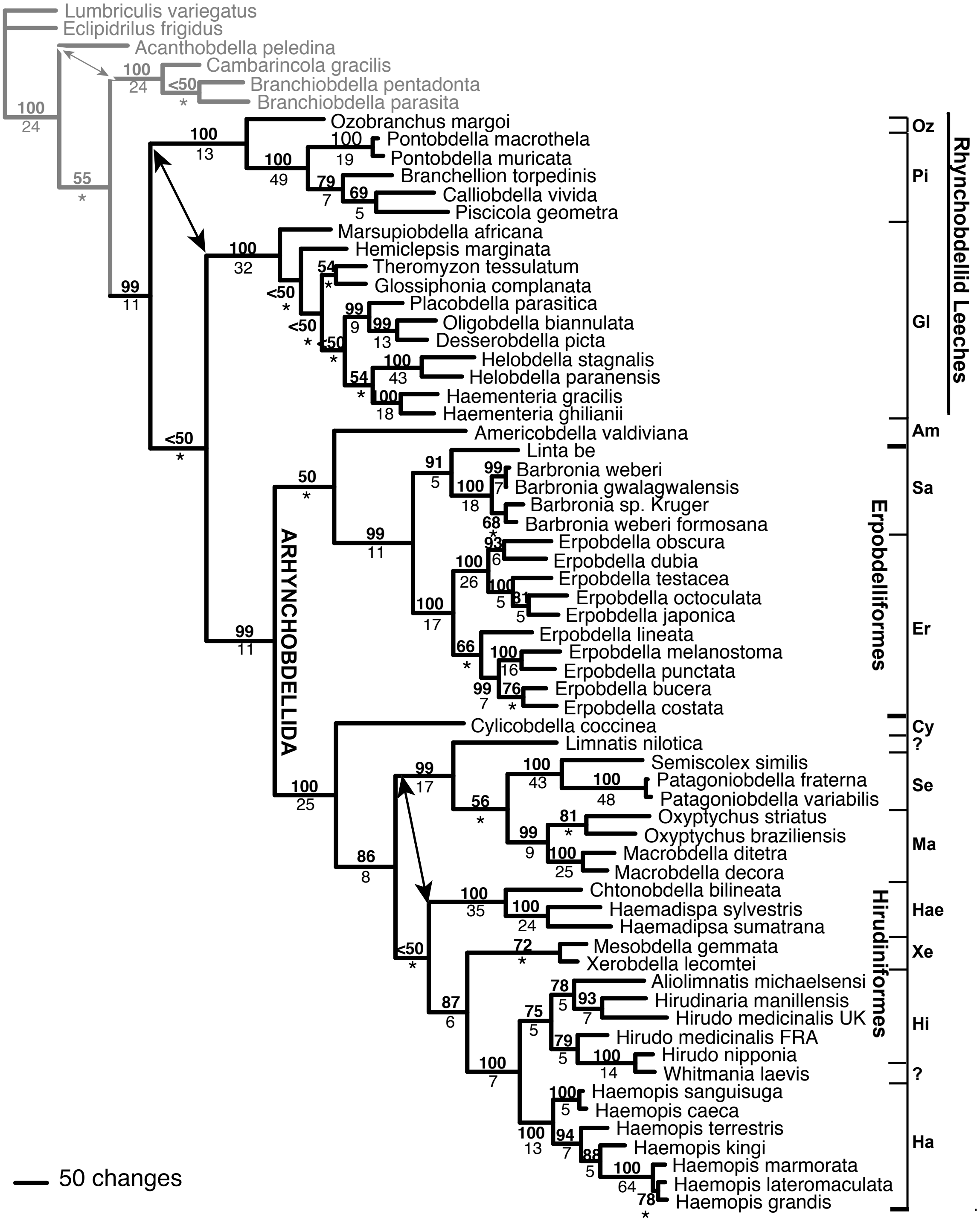
The phylogenetic analyses of two nuclear genes (18S rDNA, 28S rDNA) and two mitochondrial genes (12S rDNA, COI) combined (3118 characters total, of which 1190 were parsimony-informative) for 67 taxa resulted in two equally parsimonious trees with a tree length of 8167 steps (CI=0.329; RI=0.642; Fig. 1), under alignment parameter set 6:3. The analysis of the data under alignment parameter set 10:3 produced a single tree that was 190 steps longer (L=8357; CI=0.325; RI=0.641). Therefore, the hypotheses in this study are based upon the results from alignment parameter set 6:3.

The consensus of the two trees (not shown) strongly supported the monophyly (jac = 100) of Acanthobdellida + Branchiobdellida + Hirudinida, with the Branchiobdellida (jac = 100) sister to Hirudinida (jac = 99), though poorly supported (jac=55). The phylogenetic position of *Acanthobdella peledina* and branchiobdellidans with respect to leeches has varied in previous studies and is also equivocal here (Siddall and Burreson 1995, 1996, 1998; Apakupakul 1999; Trontelj et al. 1999; Siddall et al.

FIGURE 1.

One of two most parsimonious trees resulting from the analysis of combined 18S rDNA, 28S rDNA, 12S rDNA and COI sequence data. The topology is based on the alignment parameters 6:3, which produced the shortest trees. Arrows at the nodes indicate positions that are considered to be equivocal. Jackknife values and Bremer support indices are located above (bold) and below the node, respectively. The asterisks (*) below the node indicate Bremer support values under 5.

Figure 1.


Lumbriculis variegatus
Eclipidrilus frigidus
Acanthobdella peledina
Cambarincola gracilis
Branchiobdella pentadonta
Branchiobdella parasita
Ozobranchus margoi
Pontobdella macrothela
Pontobdella muricata
Branchellion torpedinis
Calliobdella vivida
Piscicola geometra
Marsupiobdella africana
Hemiclepsis marginata
Theromyzon tessulatum
Glossiphonia complanata
Placobdella parasitica
Oligobdella biannulata
Desserobdella picta
Helobdella stagnalis
Helobdella paranensis
Haementeria gracilis
Haementeria ghilianii
Americobdella valdiviana
Linta be
Barbronia weberi
Barbronia gwalagwalensis
Barbronia sp. Kruger
Barbronia weberi formosana
Erpobdella obscura
Erpobdella dubia
Erpobdella testacea
Erpobdella octoculata
Erpobdella japonica
Erpobdella lineata
Erpobdella melanostoma
Erpobdella punctata
Erpobdella bucera
Erpobdella costata
Cylicobdella coccinea
Limnatis nilotica
Semiscolex similis
Patagoniobdella fraterna
Patagoniobdella variabilis
Oxyptychus striatus
Oxyptychus braziliensis
Macrobdella ditetra
Macrobdella decora
Chtonobdella bilineata
Haemadispa sylvestris
Haemadipsa sumatrana
Mesobdella gemmata
Xerobdella lecomtei
Aliolimnatis michaelsensi
Hirudinaria manillensis
Hirudo medicinalis UK
Hirudo medicinalis FRA
Hirudo nipponia
Whitmania laevis
Haemopis sanguisuga
Haemopis caeca
Haemopis terrestris
Haemopis kingi
Haemopis marmorata
Haemopis lateromaculata
Haemopis grandis
ARHYNCHOBDELLIDA
Rhynchobdellid Leeches
Erpobdelliformes
Hirudiniformes
Oz
Pi
Gl
Am
Sa
Er
Cy
?
Se
Ma
Hae
Xe
Hi
?
Ha
50 changes

2001). The topology also weakly suggests that Piscolidae+Ozobranchidae (jac = 100) are the most basal lineage of Hirudinida. This position was poorly supported and equivocal with Glossiphoniidae, whose monophyly with Arhynchobdellida was not supported above 50 % in the jackknife analysis. The two parsimony topologies were also incongruent in the positions of the Haemadipsidae and the clade including *Limnatis nilotica +Semiscolecidae+Macrobdellidae* within the Hirudiniformes.

Arhynchobdellida was strongly supported to include a monophyletic erpobdelliform (jac = 99) and hirudiniform clade (jac = 100). Within Erpobdelliformes (jac = 99), the Erpobdellidae (jac = 100) was monophyletic with respect to the family Salifidae (jac = 91). Within Salifidae, *Linta be* was found basal (jac = 91) to a monophyletic *Barbronia* clade (jac = 100) (see also Westergren and Siddall 2004). The Austrian *Barbronia weberi* was found to be sister to the South African *Barbronia gwalagwalensis* (jac = 99) and *Barbronia* sp., also from South Africa, was sister to the Hawaiian *Barbronia weberi formosana* (jac = 68). Americobdellidae was poorly supported as sister to the erpobdelliform clade (jac=50).

Americobdellidae was not found to group with other hirudiniform leeches. *Americobdella valdiviana* was poorly supported as sister to the erpobdelliform clade (jac=50), however, under alternate alignment schemes and analyses under alternate optimality criteria (i.e. Maximum Likelihood and Bayesian Inference; not shown), *A. valdiviana* was also weekly supported as sister to the Hirudiniformes or basal to Arhynchobdellida. Cylicobdellidae was basal to Hirudiniformes (jac=98) and the families Haemadipsidae, Haemopidae and Hirudinidae, and the genera *Hirudo* and *Aliolimnatis* were not found to be monophyletic.

Members traditionally recognized in the family Hirudinidae were roughly divided into two independent New World and Old World clades. The New World hirudinid clade (jac=99) included the South American 'haemopids' (*Semiscolex similis* + *Patagoniobdella* species; jac = 100) together in a low supported monophyletic group (jac = 56) with North American *Macrobdella* species + South American *Oxyptychus* species (jac = 99). The African *Limnatis nilotica* was basal to this clade. The Old World hirudinids (jac = 75) had a Bremer support index of six and overall, Old World and New World clades combined, there was low support for most of the internal clades. *Hirudo medicinalis* from the UK and France were not monophyletic. Instead *Hirudo medicinalis* (UK) was sister to *Hirudinaria manillensis* (see also Borda and Siddall, 2004a), while *H. medicinalis* (France) was sister to the other *Hirudo* species, *Hirudo nipponia* and the Asian haemopid, *Whitmania laevis*. Haemopidae was polyphyletic and, as already mentioned, the South American 'haemopids' were sister to the New World "Hirudinidae" and *Whitmania laevis* nested within the Old World Hirudinidae. The genus *Haemopis* is monophyletic (jac=100) and this group is sister to the Old World hirudinids (jac=100). The Northern American *Haemopis* species (jac = 94) were monophyletic with respect to the European *Haemopis sanguisuga* and *Haemopis caeca*. Lastly, within Haemadipsidae, the tropical terrestrial *Chtonobdella bilineata* from Australia and the Asian *Haemadipsa* species were monophyletic (jac=100), to the exclusion of the terrestrial *Mesobdella gemmata* from Chile and *Xerobdella lecomtei* from Slovenia. Together *Mesobdella gemmata* and *Xerobdella lecomtei* (jac = 72) were sister to the Old World Hirudinidae + Haemopidae (jac = 87).

DISCUSSION

The resulting hypothesis from the combined analysis of two nuclear genes and two mitochondrial genes yielded a hypothesis which conflicts with traditional classifications of Arhynchobdellida (e.g. Sawyer, 1986). With respect to higher taxonomic subdivisions, monophyly of an erpobdelliform and hirudiniform clade was well supported. Current classification (Table 1) divides arhynchobdellid leeches into seven families (Sawyer, 1986), however, the resulting phylogenetic hypothesis suggests the need for taxonomic revision of Arhynchobdellida in which polyphyly of Haemopidae, Hirudinidae and Haemadipsidae would be ameliorated. Arhynchobdellid leech systematics has its origins in European faunal descriptions (E. Blanchard, 1849; Johansson, 1913; Ringuelet, 1954; Richardson, 1969; Sawyer, 1986) where groups are distinct and conveniently classified in either Erpobdelliformes or in Hirudiniformes. The discovery of South American species which deviated from that strict classification and exhibiting both “erpobdellid” and “hirudinid” characters confounded taxonomists (Moore, 1946; Ringuelet, 1954; Soós, 1966; Richardson, 1969). Ringuelet (1954) and Richardson (1969) recognized that the diverse life history strategies and morphological attributes exhibited by arhynchobdellid leeches merited subdivisions at the family level. Our results resolve the phylogenetic positions for aberrant taxonomic groups including Americobdellidae, Cylicobdellidae, the genera *Semiscolex*, *Patagoniobdella*, *Mesobdella*, and others traditionally classified under Hirudinidae.

Americobdellidae

The taxonomic status of the large and anatomically unusual *Americobdella valdiviana* has eluded systematists since its discovery (Philippi, 1872; Pinto, 1923; Moore, 1924). Endemic to the Valdivian temperate forest of southern Chile, this cryptic predator of lumbricid oligochaetes measures up to 20 cm. It lacks eyes and possesses a mix of hirudinid gross morphological (i.e. large worm-like body size) and rhynchobdellid reproductive characters. In particular, *Americobdella valdiviana* has a pair of ducts connecting the male atrium and the female ovarian ducts more typical of piscicolid leeches (Moore, 1924; Caballero, 1940; Siddall and Burreson, 1995). Reflecting this mixture of traits, *Americobdella valdiviana* has been classified as a member of the Erpobdellidae (=Herpobdellidae) (Weber, 1915; R. Blanchard, 1917; Harant, 1929), a subfamily within Hirudinidae (Moore, 1924; Ringuelet, 1944), or has been placed in its own family within the Hirudiniformes (Caballero, 1956; Ringuelet, 1976, 1985; Sawyer 1986). The first phylogenetic analysis of the relationships of leeches based on morphological and life history data (Siddall and Burreson, 1995, 1996) suggested that *Americobdella valdiviana* was sister to Arhynchobdellida in its own lineage corroborating Ringuelet's (1954) belief that *Americobdella valdiviana* originated from ancient arhynchobdellids before the evolution of the other families. Our analysis further substantiates the notion that *Americobdella valdiviana* originated from an ancient stock of arhynchobdellid leeches (Moore, 1924; Ringuelet, 1976; Sawyer, 1986).

Cylicobdellidae

In contrast to the strictly macrophagous Erpobdelliformes, there are both predaceous and sanguivorous Hirudiniformes that are typically characterized by the presence of five pairs of eyes and jaws armed with rows of teeth. However, conflicting with this classification is the South American Cylicobdellidae. *Cylicobdella coccinea* is a terrestrial predator of oligochaetes, lacking eyes and having unarmed agnaths (rudimentary jaws). Cylicobdellidae have erpobdelloid (i.e. bilobed) atria, a simple acaecate gastric tube and rudimentary jaws, but possess a hirudinoid body type, and deposit spongy cocoons (Ringuelet, 1972 a, b). Cylicobdellid species were first classified as erpobdellids (R. Blanchard, 1896; Harant, 1929; Cordero, 1937; Ringuelet, 1944, 1954), but later were afforded their own family as predaceous hirudinids allied to the Erpobdelliformes (Ringuelet, 1972 a, b; Sawyer, 1986). Siddall and Burreson's (1995) phylogentic analysis found that Cylicobdellidae nested within the Erpobdelliformes. Their incorrect coding of 'cocoons: cemented to substrate' (an erpobdellid mode of cocoon deposition) determined this placement in their tree (Siddall and Burreson, 1995). With the revised coding of spongy cocoon deposition on land, *Cylicobdella coccinea* is sister to the Hirudiniformes (Fig. 1). Morphologically, this group retains plesiomorphic similarities to erpobdellid leeches, thus explaining the historical difficulties with its systematic position. It is noteworthy that if Cylicobdellidae is forced to group with the Erpobdelliformes, an extra 219 steps are required.

Haemadipsidae

Rafael Blanchard (1893, 1917) grouped together species with a terrestrial habit, possessing a broad ocular arch with 5-pairs of eyes, where pairs 1-4 are in contiguous annuli and 4-5 are separated by two annuli, and having lateral Nephridiopores (the last of which are concealed within respiratory auricles) in the family Haemadipsidae. This family, traditionally includes sanguivorous terrestrial leeches found throughout the tropical rainforests of the Indian sub-continent, Southeast Asia, Wallacea, Australia, Melanesia and Madagascar. Deviating from this narrow global distribution of terrestrial leeches are a few zoogeographic terrestrial anomalies, like *Mesobdella gemmata* and *Xerobdella lecomtei*. First described by Emile Blanchard (1849), *Mesobdella gemmata* is a temperate terrestrial blood-feeder from southern Chile that has been of particular interest biogeographically and because of difficulties associated with its placement and inclusion within the Haemadipsidae (Blanchard, 1893, 1917; Ringuelet, 1943; Caballero, 1940; Richardson, 1971). The presence of caudal (respiratory) auricles is a synapomorphy for haemadipsid leeches (Siddall and Burreson, 1996); however, *Mesobdella gemmata* does not have auricles. Although the species exhibits 'haemadipsine' characteristics (i.e. stout body, ocular cephalic arch pattern), Richardson (1971) believed these to be convergent adaptations to a terrestrial jawed sanguivorous habit acquired independent of the tropical Old World counterparts.

Trontelj et al. (1999) found with mitochondrial data that *Xerobdella lecomtei*, a European sanguivorous terrestrial leech, is more closely related to the genera *Hirudo* and *Haemopis*, than to *Haemadipsa* species. Similarly, our results suggest that *Mesobdella gemmata* does not group with the other Gondwanan terrestrial tropical leeches, but with the temperate Palearctic *Xerobdella lecomtei*, together forming a sister group to the Old World hirudinids and *Haemopis* species. Several workers had anticipated this relationship separate from haemadipsids (Ringuelet, 1943, 1972b; Moore, 1946; Richardson, 1971) and it seems reasonable, therefore, to recognize the family Xerobdellidae, elevated from the subfamilial rank described by Moore (1946). Further analysis of this group should include the other New World non-auriculate sanguivorous terrestrial leech genus *Diestecostoma*, for which greater morphological affinities with the genera *Mesobdella* and *Xerobdella* have been suggested (Moore, 1946; Ringuelet, 1954; Richardson, 1971).

Hirudinidae and *Haemopidae*

Because the terrestrial bloodfeeders are not each other's closest relatives (Fig. 2), the classification of leeches based on ecological habit and gross external morphology is not a universally reliable means for delimiting groups. Similarly, Apakupakul et al. (1999) found Hirudinidae to be polyphyletic — *Macrobdella decora* was not found in a sister-group relationship with *Hirudo medicinalis* and *Aliolimnatis michaelseni*. Blanchard (1896) characterized leeches in Hirudinidae as having 10 eyespots arranged in a parabolic arch and being 5-annulate. He further grouped them based on the number of rows of teeth (i.e. monostichodont or distichodont), effectively including the haemopids. The South American "haemopids" (Semiscolecinae, *sensu* R. Blanchard, 1896), *Semiscolex* species and *Patagoniobdella* species are exclusively macrophagous in habit, but are monophyletic with the macrobdellids (i.e. *Macrobdella* and *Oxyptychus* species). An analogous split between sanguivores and predators exists at the divergence between the hirudinids and *Haemopis* species. Richardson (1969, 1971) noted that *Semiscolex* and *Patagoniobdella* seem to share greater morphological affinities (i.e. acaecate vagina, straight penis and an amyomeric male median apparatus) with the freshwater sanguivorous genera *Oxyptychus* and *Macrobdella* than with *Haemopis* species (Richardson 1969, 1971). Ignoring these differences, Richardson (1969) classified them as Haemopidae because they were predators. *Macrobdella* and *Oxyptychus* species, forming a monophyletic group, supports resurrection of the family Macrobdellidae (Richardson, 1969), with Semiscolecidae (R. Blanchard, 1896; Scriban and Autrum, 1934) as its sister taxon. However, the basal position of *Limnatis nilotica* complicates its inclusion in either family. Morphologically, *Limnatis nilotica* grouped near haemadipsid leeches (Fig. 1) — only molecular synapomorphies place this genus with the macrobdellids and semiscolecids. An expanded family Semiscolecidae would have priority, but was not intended to be inclusive of sanguivorous taxa.

Although there is some precedence for separating the New World macrobdellids from the Old World hirudinids (Apakupakul et al., 1999), an unexpected finding in this phylogenetic hypothesis is the complete lack of monophyly for any of the genera in the latter. The genera *Aliolimnatis* and *Limnatis* were differentiated principally based on somatic annulation patterns (Richardson, 1972). Their separation (Fig. 1) appears to corroborate this distinction. Borda and Siddall (2004a) found the genus *Hirudo* to be paraphyletic with two species as representatives (*Hirudo medicinalis* and *Hirudo nipponia*). However, DNA extraction and sequences of *Hirudo medicinalis* included here and in previous phylogenetic work (Siddall and Burreson 1998; Apakupakul et al. 1999, Borda and Siddall 2004) were obtained from specimens bred in captivity (i.e. BioPharm). Therefore the inclusion of a specimen of *Hirudo medicinalis* collected from Rennes, France in this analysis reveals a possible divergence between captive bred individuals and individuals that are found in the wild. Morphologically, both specimens fit the descriptions of *Hirudo medicinalis*, but show considerable variation in the mitochondrial

COI gene. Alternatively, the independent analyses of 18S and 28S recover *Hirudo medicinalis* as monophyletic, but COI suggests that they are not.

Richardson's (1972) intent clearly was to distinguish *Aliolimnatis* from *Limnatis*, not from *Hirudo*, which was not considered in the articulation of the new genus *Aliolimnatis*. Richardson's (1969) wholesale reorganization of the systematics of Hirudiniformes, establishing five new genera for species previously in *Hirudo* and three new genera for species previously in *Haemopis* (six were monotypic), appears completely unjustified. The genus *Haemopis* is monophyletic and several genera may eventually have to be returned to junior synonymy with *Hirudo*. The latter cannot be properly determined without a broader inclusion of the type species of *Aliolimnatis* and the many South and East Asian aquatic leeches.

Evolution of Bloodfeeding

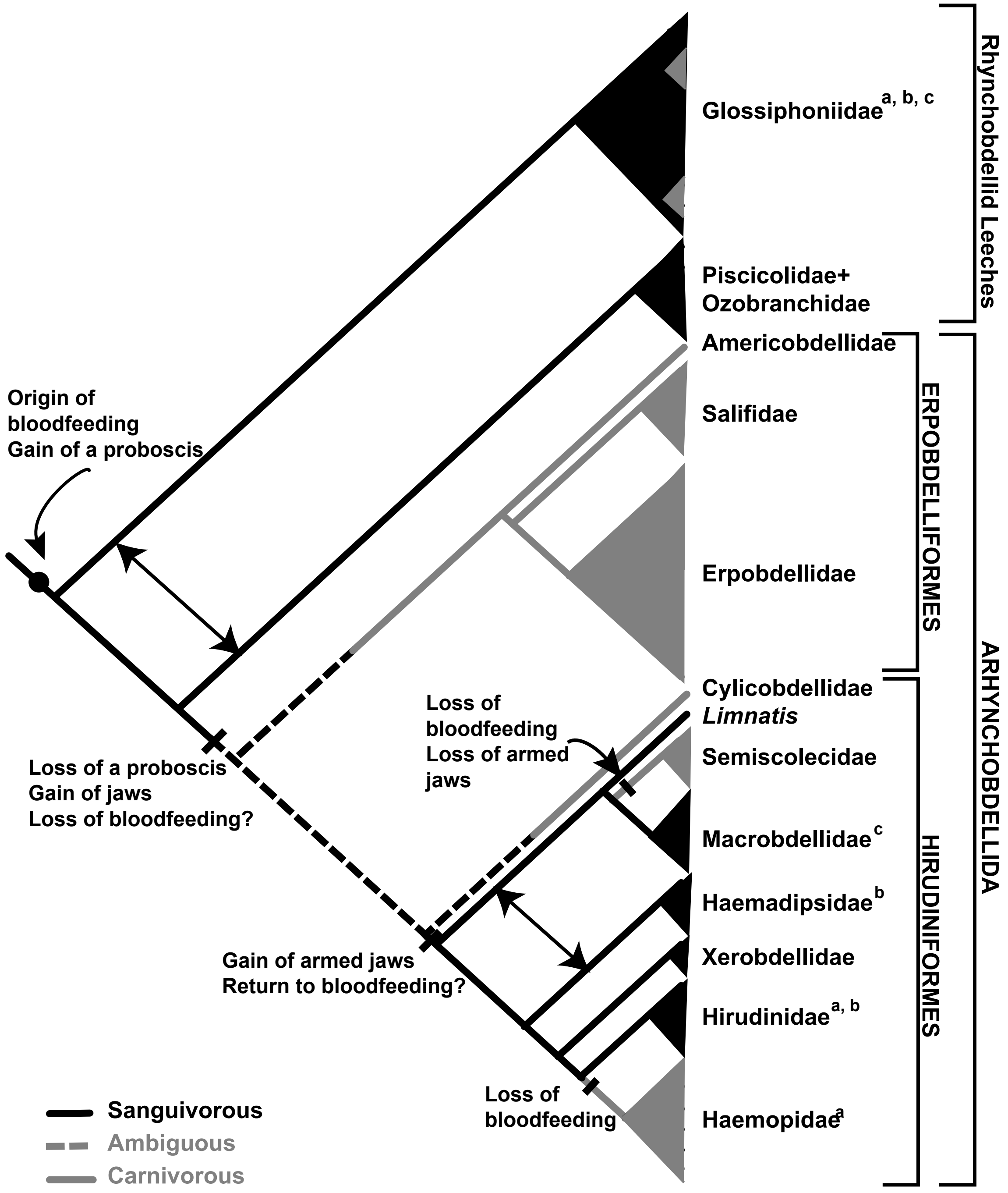
Although leeches are notorious for their bloodfeeding habits, sanguivorous species are far outnumbered by their predatory and carnivorous counterparts (Sawyer 1986). For example, there are over 40 species in the non-bloodfeeding genus *Helobdella* (Siddall and Borda 2003), approximately 40 species in the genus Erpobdella (Siddall, 2002), and countless other carnivorous taxa (e.g. *Barbronia*, *Cylicobdella*, *Glossiphonia*, *Haemopis*, *Semiscolex*). An optimized reconstruction of the feeding habits of leeches is shown in Figure. 2.

The positions of branchiobdellidans and *Acanthobdella peledina* as the most closely related groups to leeches provide a gradation of feeding behaviors towards strict sanguivory (see Fig. 1). Parasitic branchiobdellidans and *Acanthobdella peledina* feed on

FIGURE 2.

The optimized reconstruction of the evolution of feeding preferences for Hirudinida. Arrows indicate equivocal position of taxonomic groups. Known anticoagulants present in groups of leeches: a) inhibitor of factor Xa; b) thrombin inhibitors; c) inhibitor of glycoprotein IIb/IIIa (adapted from Siddall 2003); black = sangivorous, grey = carnivorous, dashed black = ambiguous.

Figure 2.


Glossiphoniidae[a, b, c]
Piscicolidae+
Ozobranchidae
Americobdellidae
Salifidae
Erpobdellidae
Cylicobdellidae
Limnatis
Semiscolecidae
Macrobdellidae[c]
Haemadipsidae[b]
Xerobdellidae
Hirudinidae[a, b]
Haemopidae[a]
Rhynchobdellid Leeches
ERPOBDELLIFORMES
HIRUDINIFORMES
ARHYNCHOBDELLIDA
Origin of
bloodfeeding
Gain of a proboscis
Loss of a proboscis
Gain of jaws
Loss of bloodfeeding?
Loss of
bloodfeeding
Loss of armed
jaws
Gain of armed jaws
Return to bloodfeeding?
Loss of
bloodfeeding
Sanguivorous
Ambiguous
Carnivorous

the dermal tissue of their hosts and as a consequence ingest blood (Holt 1965 and 1989, Sawyer 1986). The related branchiobdellidans and *Acanthobdella peledina* either are ectocommensalistic or ectoparasites of crustaceans and salmonid fish, respectively, and similarly, the Piscicolidae, Ozobranchidae and some glossiphoniid species (e.g. *Placobdella parasitica*, *Placobdella ornata*) are ectoparasites of blood specific to fish (in the former) or turtles (in the latter). Morphologically, the possession of a proboscis appears to be pleisiomorphic, as it is present in the basal most groups (i.e. Glossiphoniidae, Ozobranchidae, Piscicolidae), which is corroborated by the presence of a rudimentary proboscis in *Acanthobdella peledina* (Sawyer 1986: Siddall and Burreson 1995: Trontelj et al. 1999).

If this hypothesis accurately represents the evolution of leeches, there is an indication of a common origin in a sanguivorous ancestry, with multiple independent losses of the behavior throughout the evolutionary history of leeches (Siddall and Burreson 1996; Apakupakul et al. 1999; Light and Siddall 1999; Trontelj et al. 1999; Borda and Siddall 2004). Within the Glossiphoniidae there have been at least two convergent losses of bloodfeeding, for example in *Helobdella* species and *Glossiphonia* species (Light and Siddall 1999; Fig. 2). Losses of sanguivory also are well represented among arhynchobdellid taxa, with over half of the families having abandoned bloodfeeding for a predatory and carnivorous lifestyle (Borda and Siddall 2004a; Fig 2).

The biomedical interests and identification of coagulation inhibitors in a wide range of species have uncovered that anticoagulants are not restricted to sanguivorous species, but also are found in some non-bloodfeeding species (Siddall, 2003; see Fig. 2). This not only supports a shared ancestry in a sanguivore, but also the retention of pleisiotypic characteristics from the leech ancestor in derived groups, regardless of feeding preference. *Hirudin*, a potent thrombin inhibitor, which remains concentrated in the wound for hours after the leech has stopped feeding, was the first anticoagulant to be isolated from *Hirudo medicinalis*. Thrombin inhibitors have also been isolated from haemadipsids (i.e. *haemadin*) and from *Theromyzon* species (i.e. *theromin*) (Sawyer 1986). The giant Amazonian leech, *Haementeria ghilianii* produces another medically important platelet inhibitor (Sawyer 1986, Baskova et al. 1987, Munro et al. 1992a and 1992b, Vindigni et al. 1994, Yang et al. 1997, Hong et al. 1999). Protease inhibitors (anti-factor Xa) are shared by multiple sanguivorous species (e.g. *Theromyzon* species, *Haementeria* species, Hirudinidae) and in the carnivorous genus *Whitmania* (Siddall, 2003).

The morphological characteristics and feeding preferences of the arhynchobdellid ancestor remains unresolved based on the phylogeny. It is possible that the arhynchobdellid ancestor did not possess a proboscis or armed jaws, but instead had a pharynx that was modified into rudimentary jaws as seen in the Americobdellidae and Cylicobdellidae. If there were two origins of bloodfeeding in leeches, the evolution of the armed jaws of sanguivorous hirudiniform species may have been coincident with the reacquisition of a bloodfeeding habit. The absence of sanguivory is apparent in the majority of the basal-most groups of Arhynchobdellida (i.e. Americobdellidae, Erpobdelliformes and Cylicobdellidae). Complicating this picture, members of the Semiscolecidae and some species of the Haemopidae (e.g. Haemopis caeca) have independently lost a bloodfeeding habit. As such, there are two possible scenarios based on the current phylogenetic hypothesis (Fig. 2): either the arhynchobdellid ancestor was a bloodfeeder and there have been at least four losses of bloodfeeding within the group or the arhynchobdellid ancestor had abandoned sanguivory and there was a secondary return to bloodfeeding in the Hirudiniformes. Either hypothesis requires subsequent losses of bloodfeeding in groups such as the Semiscolecidae and Haemopidae

Evolution of Habit Preference

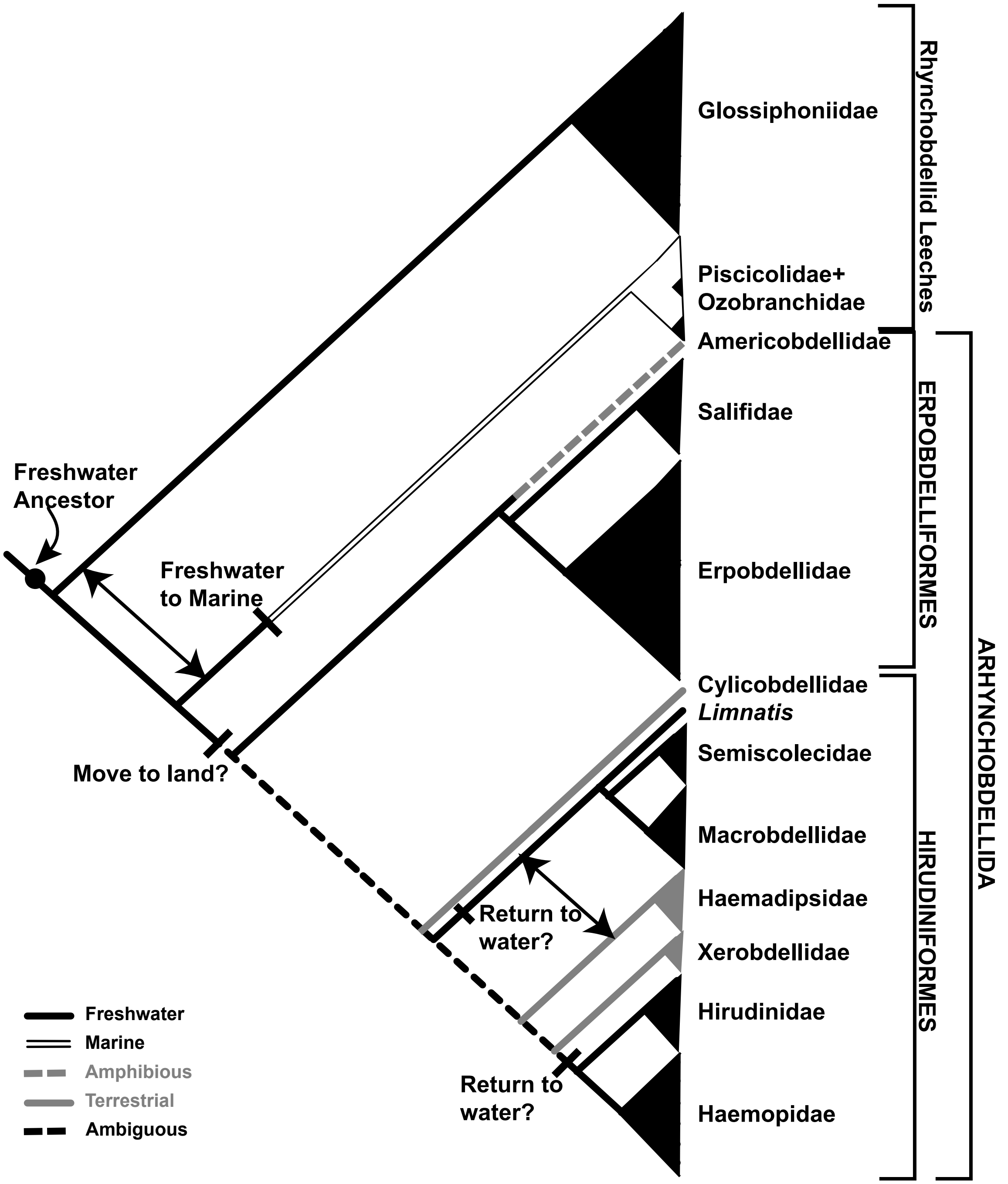
Leeches are better known for being aquatic in habit as is exemplified by the prevalence of freshwater taxa in the tree (Fig. 3). All rhynchobdellid leeches are aquatic, as are members of the Erpobdelliformes. While some hirudiniform leeches (i.e. Hirudinidae; Haemopidae; Macrobdellidae; Semiscolecidae) spend most of their lives in water, they nonetheless exhibit behaviors, such as foraging and cocoon deposition, on land. Furthermore, within the Hirudiniformes, species in the families Xerobdellidae and Haemadipsidae are specifically adapted to a terrestrial lifestyle, albeit a damp one. The reconstruction of the evolution of habitat preferences depicted in Fig. 3 indicates that the ancestral leech was adapted to a freshwater environment (see also Siddall & Burreson 1986). The shared ancestry of leeches with the freshwater Acanthobdellida, Branchiobdellida and Lumbriculida further corroborates this notion. With the exception of some Piscicolidae (and Ozobranchidae), which are found in environments ranging from freshwater to marine (Sawyer 1986, Siddall & Burreson 1996, Utevsky & Trontelj 2004), all other aquatic leeches are restricted to or associated with freshwater systems.

Somewhere in the ancestry of the Arhynchobdellida, there must have been a movement out of the water and onto land. Clues to the origin of terrestrialism may lie with *Americobdella valdiviana*, a leech that several have suggested may represent a

FIGURE 3.

The optimization of the evolution of habitat preference. Arrows indicate equivocal position of taxonomic groups. black = freshwater; white = marine; gray = terrestrial; dashed gray = amphibious; dashed black = ambiguous.

Figure 3.


Glossiphoniidae
Piscicolidae+
Ozobranchidae
Americobdellidae
Salifidae
Erpobdellidae
Cylicobdellidae
Limnatis
Semiscolecidae
Macrobdellidae
Haemadipsidae
Xerobdellidae
Hirudinidae
Haemopidae
Rhynchobdellid Leeches
ERPOBDELLIFORMES
HIRUDINIFORMES
ARHYNCHOBDELLIDA
Freshwater Ancestor
Freshwater to Marine
Move to land?
Return to water?
Return to water?
Freshwater
Marine
Amphibious
Terrestrial
Ambiguous

relictual taxon (Ringuelet 1954, Siddall & Burreson 1996, Borda & Siddall 2004, Siddall & Borda 2004). This robust leech resembles a hirudinid externally, but internally has reproductive morphologies that deviate from most arhynchobdellid leeches. *Americobdella valdiviana* is the only arhynchobdellid leech retaining the pleisiomorphic intergonadal conducting tissue, a feature known in the piscicolid leeches and *Acanthobdella peledina* (Sawyer 1986, Siddall & Burreson 1995). *Americobdella valdiviana* seems to be more amphibious than terrestrial in habit, with reports of the species found in habitats ranging from damp earth to ditches and streams (Moore 1924a). It is possible that *Americobdella valdiviana* spends most of its life in the damp soil, as it is a predator of terrestrial earthworms (Moore 1924a, Ringuelet 1985, Siddall & Borda 2004), retreating to water to incubate or to digest its meal. Our own collections of A. valdiviana from Valdivia, Chile resulted in specimens collected both from damp soil in a forest and from under a rock of a shallow stream (Siddall & Borda 2004). The analysis weakly supports *Americobdella valdiviana* as an erpobdelliform leech (jac =50), such that the ancestral state (aquatic or terrestrial) for the Arhynchobdellida cannot be definitively deduced. Nevertheless, terrestrialism appears be the original state for hirudiniform leeches. Cylicobdellids are terrestrial predators of oligochaetes and place as the basal most hirudiniform lineage. The evolution of the remaining groups have radiated into strictly terrestrial species (Haemadipsidae and Xerobdellidae) or those of semi-aquatic and amphibious habit (Hirudinidae, Haemopidae, Macrobdellidae and Semiscolecidae). Regardless of habitat preference, all hirudiniform leeches share a common mode of cocoon deposition (see below), which corroborates terrestrialism as the ancestral state of the group.

Evolution of Cocoon Deposition and Parental Care

The diversity of habitat preferences in leeches also is reflected in behaviors associated with parental care. Ultimately the type of parental care exhibited by leeches determines the fate of the cocoon and offspring after deposition. For most leeches, cocoons are secreted from the clitellum, which is slipped off the head, and is encased in a protective cover that is resistant to environmental fluctuations and is abandoned, with no parental care exhibited by the leech (Sawyer 1986, Siddall & Burreson 1996, Apakupakul 1999, Kutschera & Wirtz 1986 and 2001). Piscicolids and erpobdelliform leeches deposit hardened proteinaceous cocoons onto smooth substrate. Members of the Hirudiniformes leave the freshwater environment to deposit and abandon their cocoons on moist shorelines of lakes, ponds and streams. Members of the Glossiphoniidae produce and deposit a fragile membranous sac that is invariably guarded by the parent. The leech remains protecting the soft cocoon until the embryos emerge, after which the hatchings will attach to the venter of leech (Kutschera and Wirtz 1986 and 2001, Sawyer 1986). In the case of sanguivorous species hatchlings remain on the venter until they are carried to their first blood meal (Kutschera & Wirtz 1986 and 2001, Sawyer 1986).

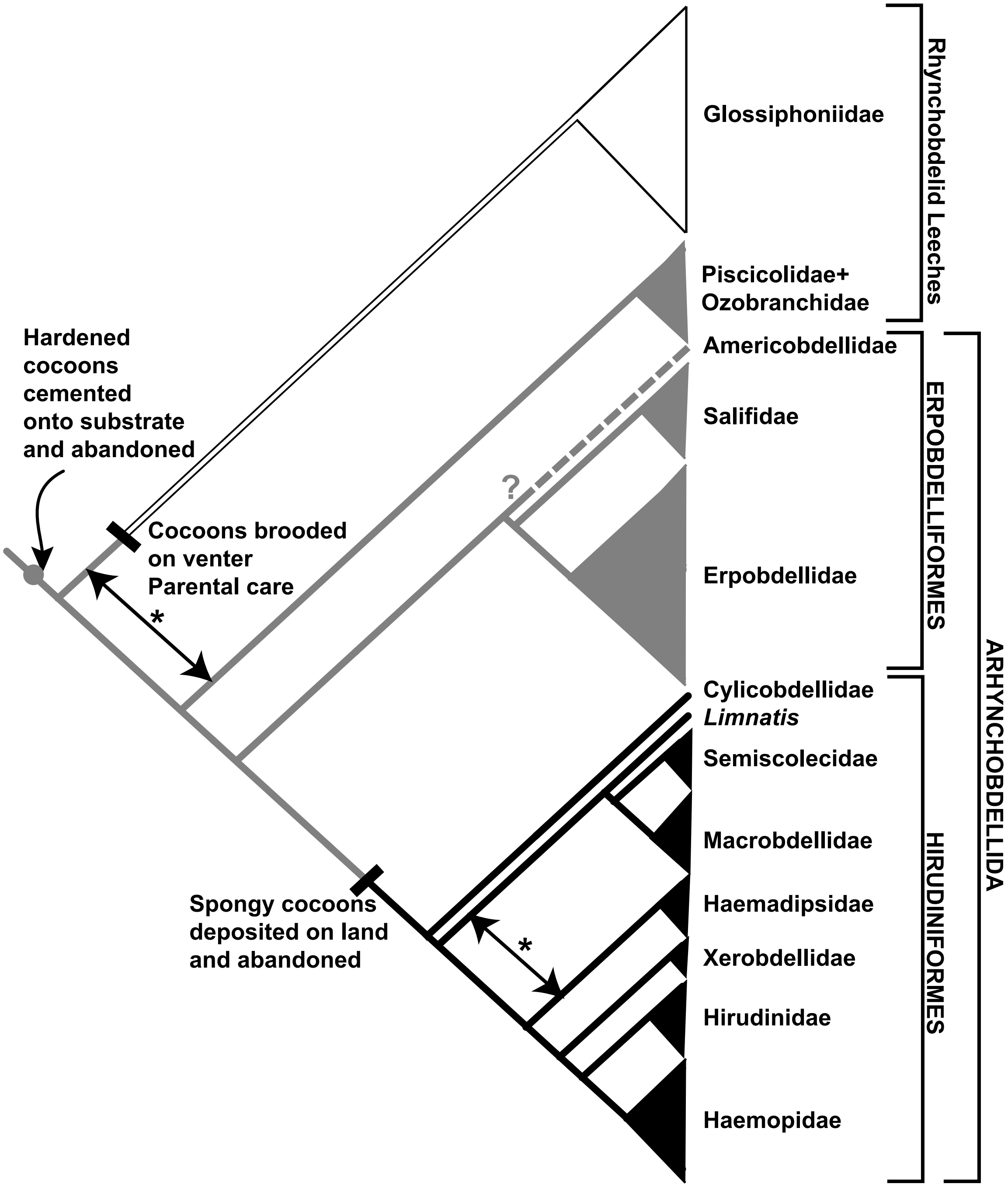
According to the current phylogenetic estimate (Fig. 4), and corroborated in other works (Siddall & Burreson 1996, Apakupakul & al. 1999, Kutschera & Wirtz 2001), deposition of a hardened protective cocoon onto a smooth substrate appears to be the ancestral condition in the Hirudinida. This behavior is apparent in the Piscicolidae and the Erpobdelliformes and is exhibited in the related *Acanthobdella peledina* and branchiobdellidans (Fig. 4; Sawyer 1986, Siddall & Burreson 1995 and 1996, Kutschera & Wirtz 1986 and 2001), all of which are accomplished in their aquatic environments.

The abandonment of cocoons after deposition is a behavior that is retained in the Hirudiniformes, but the development of a protective casing that prevents desiccation is unique and associated to the modes of terrestrialism in the hirudiniform ancestor. The secretion of a membranous sac and exhibition of parental care is unique to glossiphoniids. The reproductive behaviors of *Americobdella valdiviana* are unknown. Such knowledge could provide clues for the missing link between rhynchobdellid leeches and the Arhynchobdellida.

FIGURE 4.

The optimization of the evolution of cocoon deposition in the Hirudinida. Black = cocoon deposition on land (no parental care), white = cocoon brooded on venter of leech (parental care), gray = cocoon cemented to substrate in aquatic habitats (no parental care), black = cocoons deposited on land (no parental care), dashed gray = unknown.

Figure 4.


Glossiphoniidae
Piscicolidae+
Ozobranchidae
Americobdellidae
Salifidae
Erpobdellidae
Cylicobdellidae
Limnatis
Semiscolecidae
Macrobdellidae
Haemadipsidae
Xerobdellidae
Hirudinidae
Haemopidae
Rhynchobdelid Leeches
ERPOBDELLIFORMES
HIRUDINIFORMES
ARHYNCHOBDELLIDA
Hardened cocoons cemented onto substrate and abandoned
Cocoons brooded on venter Parental care
*
?
Spongy cocoons deposited on land and abandoned
*

CHAPTER 3

Leech collections from Chile including two new species of *Helobdella* (Annelida: Hirudinida)

(From: Siddall, M. E. and Borda, E. 2004. Leech collections from Chile including two new species of *Helobdella* (Annelida: Hirudinida) *American Museum Novitates* 3457: 1–18)

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INTRODUCTION

Southern Chile comprises one of the world's most unusual temperate ecozones beginning at 39º S, with the Araucaria forests at the northern edge of Region X (Región de los Lagos) through the Valdivian coastal rainforests and South to Gondwanan forests dominating in Tierra del Fuego. Consequently, Chile is home to one-third of the world's remaining temperate primary forest. Region X in the vicinity of Valdivia, is marked by a geological history of mega thrust subduction, volcanism and Pleistocene glaciations, which together have sculpted a complex, landscape with the greatest concentration of freshwater west of the Andes. More than 90% of the biodiversity in this region is endemic and constitutes the highest species diversity among the world's temperate forests.

Chilean leeches have received an unusual amount of attention in the last 150 years. The first published record of leeches in South America was Emile Blanchard's (1849) treatise included in the third volume of Claudio Gay's *Historia Fisica y Politica de Chile* in which four species were described: *Blenobdella depressa*, *Glossiphonia triserialis*, *Hirudo cylindrica* and *Hirudo gemmata* (now these are *Haementeria depressa*, *Helobdella triserialis* and the latter two are synonyms of *Mesobdella gemmata*). Major contributions that followed include Phillippi's (1872) discovery of the anatomically unusual *Americobdella valdiviana* and its later frequent scrutiny (Blanchard, 1917; Moore, 1924; Cabellero, 1956), the rediscovery and reassessment of *Mesobdella gemmata*, one of few New World haemadipsids (Grube, 1871; Blanchard, 1893; Weber, 1915), and a series of contributions by Raphaël Blanchard. The latter (no relation to Emile) had his interest in South American leeches piqued when he received (anonymously) a leech apparently taken from a nutria (*Myocastor coypus*: Rodentia) and which proved to be a species of *Theromyzon* (Blanchard, 1892b; Ringuelet, 1947). Additional material received by him, particularly from Wilhelm Michaelsen's expedition to Chile in 1892 and 1893, contributed to several new species (Blanchard, 1893, 1896, 1900, 1917) and a growing realization that the temperate latitudes of Chile harbored an unusually high diversity of leeches, particularly those of the non-sanguivorous genus *Helobdella* (see also Moore, 1911).

Beginning in 1944, Raul Ringuelet turned his attention from branchiuran fish parasites to the freshwater leech diversity of South America, which in part concerned the trans-Andean relationships of Chilean and Argentinian taxa. Two seminal contributions summarized contemporary knowledge of that hirudifauna. Ringuelet (1944a) catalogued 360 leech specimens in the Museo de La Plata, and provided detailed internal anatomical descriptions for species that had not previously been dissected (Ringuelet, 1944b). After detailing Argentinian spider fauna and ichthyofauna into the 1960s, Ringuelet continued to make major contributions to knowledge of leeches in the last 10 years of his life, culminating in a trip to Santiago in 1979 and one of his last publications: a complete synopsis of Chile's known leech diversity (Ringuelet, 1985a).

In the southern summer of 2001 we engaged in an expedition to the Región de los Lagos with an aim to collect the two unusual Valdivian leeches, *Americobdella valdiviana* and *Mesobdella gemmata*, and incorporate them in ongoing molecular phylogenetic work (Borda and Siddall, 2004). Our collection efforts also focused on inland freshwater drainages closer to the cordillera and not previously investigated (or not readily accessible) by Michaelsen in an effort to more fully characterize the endemic diversity of this region in advance of its steady disappearance. Moreover, this constitutes the first photographic documentation of Chilean hirudifauna.

MATERIALS AND METHODS

Leeches were collected from 17 localities in Chile. Collections were made in the Reserva Nacional Yerba Loca, east of Santiago on 18 February 2001, as well as in the Región de la Araucania and Región de los Lagos between 39º07'30"S and 42º47'23"S from 19 February 2001 to 6 March 2001. Collection methods included examination of the underside of rocks and submerged debris along shorelines of lakes and streams, wandering bare-legged in humid forests, as well as overturning logs and digging into the topsoil in those forests.

Leeches were relaxed with the gradual addition of ethanol and were either fixed in 10% formalin buffered with $CaCl_2$, and later transferred and stored in 70% ethanol or fixed in 95 - 100% ethanol until used for DNA extraction. Specimens were photographed alive with a Nikon 990 digital camera. Preserved animals were examined and photographed using a Nikon SMZ-U stereomicroscope with a SPOT-RT digital camera.

To assess the genetic variation of color morph varieties in *Americobdella valdiviana*, the mitochondrial cytochrome *c* oxidase subunit I (COI) was amplified and sequenced both for a terrestrial (dark maroon) specimen and for an aquatic (light gray) specimen. Tissue from the caudal sucker was used in order to minimize the possibility of contamination from prey DNA found in the gastric and intestinal regions. The DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA) was used for tissue lysis and DNA purification.

The PCR amplification and sequencing of mitochondrial COI followed protocols in Borda and Siddall (2004a)

RESULTS

Twelve species of leeches were collected of which two are new to science. Half were non-sanguivorous species of *Helobdella* and two were principally terrestrial in habit. The only species found at only one collection locality was the new species of *Helobdella* from Santuario de la Naturaleza Yerba Loca.

ORDER ARHYNCHOBDELLIDA BLANCHARD, 1894

SUBORDER ERPOBDELLIFORMES CABALLERO, 1952

FAMILY AMERICOBDELLIDAE (CABALLERO, 1956)

Americobdella valdiviana (Philippi, 1872)

Figure 5

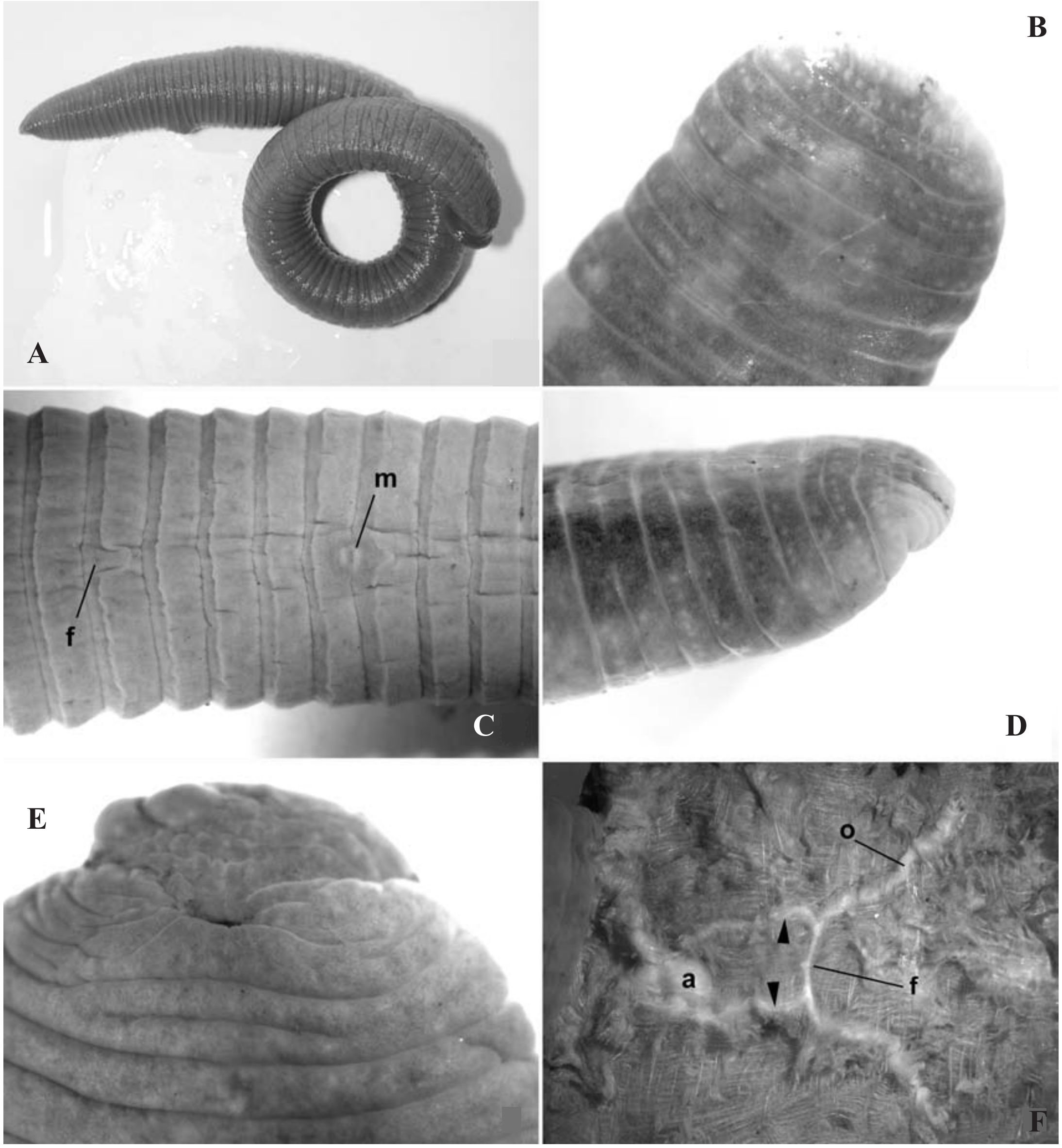
One partial specimen (AMNH 4301, Annelida) free-living from Parque Nacional Oncol (CV01-15), 495 m, 39º42'27.1"S, 78º18'28.2"W, 28 February 2001, dug-up by shovel in two pieces, with earthworm in gut, dark gray ventrally to maroon dorsally when alive, fixed and stored in 100% ethanol. One intact specimen (AMNH 4302, Annelida) 137 mm in length, free-living at Fundo Buenaventura near Hueyelhue (CV01-16), approximately 39º45'S, 73º08'W, 2 March 2001, from under rock in stream, slate grey ventrally and dorsally with faint yellow mid-dorsal line when alive which disappeared upon fixation in 100% ethanol. Additional material examined include a large pale grey specimen and a smaller dark specimen preserved in formalin at the Universidad Austral in Valdivia.

This species, though rare, is large and robust (Fig. 5A), and easily recognizable. Male gonopore in furrow at XI b5/b6 and female gonopore in furrow at XII b5/b6 (Fig. 5B). Though reported by Moore (1924), no eyespots were observed in the live leech (Figs. 5D, E) or following fixation. Nephridiopores in ventral annulus b5, midbody annuli with an irregular tendency towards subdivision at margins (Fig. 5B). There are no post-anal annuli (Fig. 5C). Although the parenchymal spaces are difficult to dissect due to the extensive connective tissue, we observed the bilobed male atrium, caecate ovisacs and the longitudinal connecting conduit between male and female median reproductive apparati (Fig. 5F). Mitochondrial COI sequence fragments were identical for leeches from these two localities (unpublished).

FIGURE 5.

Americobdella valdiviana. **A.** Whole specimen approximately 20 cm in length from the Universidad Austral. **B.** Ventral view of female (f) and male (m) gonopores. **C.** Dorsal view of caudal somites in which there are no annuli between the anus and the sucker. **D.** Dorsal view of cephalic somites in live specimen from Hueyelhue. **E.** Lateral view of cephalic somites in live specimen from Hueyelhue. **F.** Dissection of clitellar somites revealing the atrial region (a), ovisacs (o), female gonopore (f) and the conducting tissue (arrowheads) between the oviducts and male median reproductive apparatus.

Figure 5.



SUBORDER HIRUDINIFORMES CABALLERO, 1952

FAMILY XEROBDELLIDAE MOORE, 1946

Mesobdella gemmata (E. Blanchard, 1849)

Figure 6

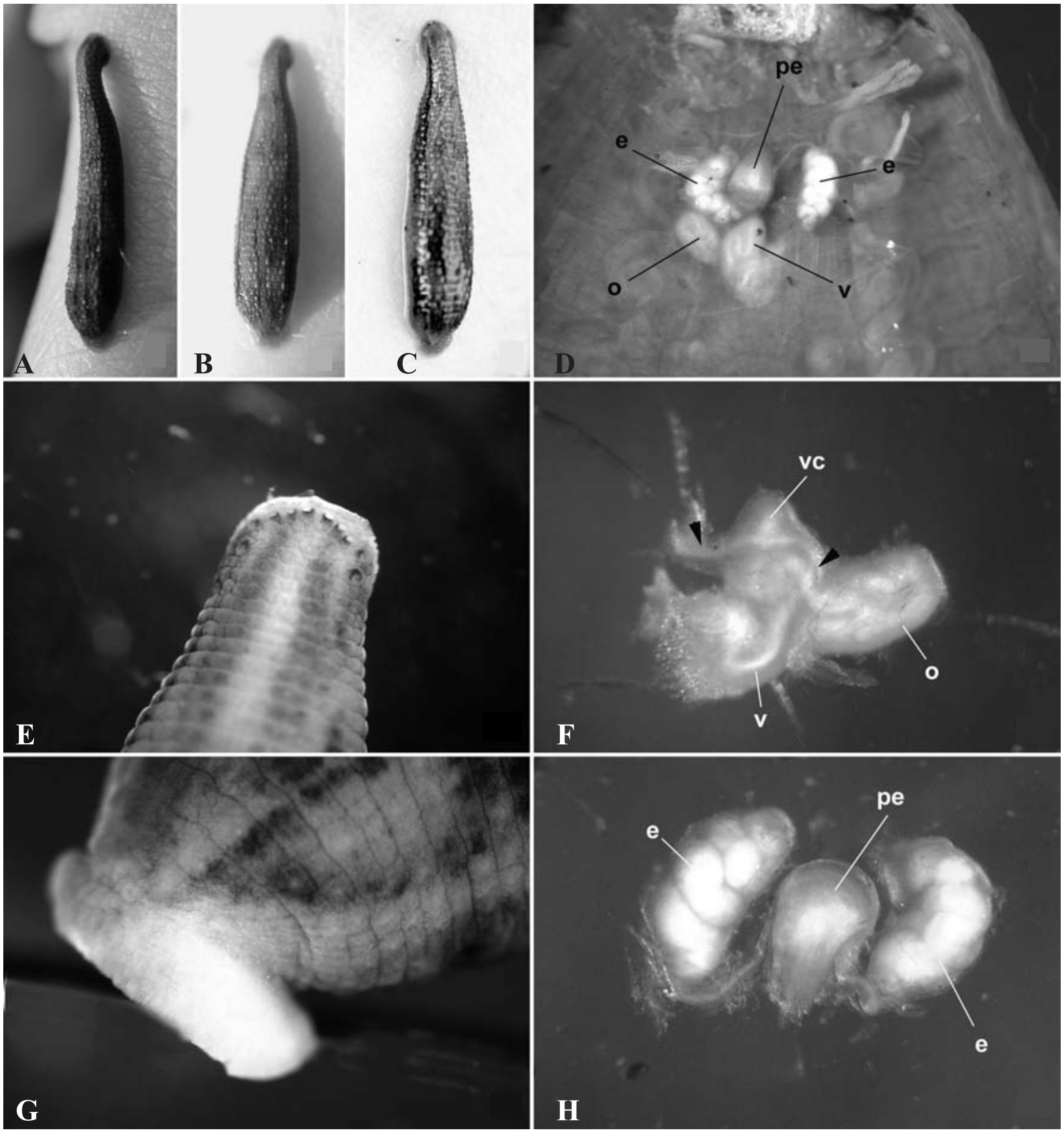
Three specimens (AMNH 4303, Annelida) fixed in 100% ethanol and three specimens (AMNH 4304, Annelida), fixed in 10% formalin, collected from exposed skin prior to or while bloodfeeding, 528 m, Chan-Chan Alto (CV01-13), 39º33'49.7"S, 73º12'23.9"W, 26 February 2001, in humid forest and near roadside pools of water where frogs were calling and mating. One specimen (AMNH 4305, Annelida) fixed in 100% ethanol, at Fundo Buenaventura near Hueyelhue (CV01-16), approximately 39º45'S, 73º08'W, 2 March 2001.

Dorsum variable tawny brown to deep brown field when alive with darker longitudinal pigment patterns following paramedial and marginal ridges especially posteriorly (Fig. 6A – C). Cephalic somites slightly widened at V with five pairs of eyespots, one on each of II, III, IV, V and VI (Fig. 6D). Midbody somites triannulate. Posteriolateral respiratory auricles absent (Fig. 6E). Male gonopore on annulus XI a3 very near furrow of XI a2/a3, female gonopore in furrow at XII/XIII. Median reproductive apparatus micromorphic (Fig. 6F) with male penis sheath in XII a1 and a2, entirely anterior to female organs (Fig. 6F). No common oviduct. Paired oviducts short, inserting ventrally into vagina in XII a3. Ovisacs ovoid in XII a3. Vagina initially in XIII a1 but recurved anteriad well into XII a3 and with short caecum anterior to oviductal insertion point (Fig. 6G). Ejaculatory ducts thin with short preatrial loop between base of penis sheath and epididymes (Fig. 6H). Epididymes in XII a1 and a2.

FIGURE 6.

Mesobdella gemmata. **A– C.** Live specimens from Chan-Chan Alto while feeding on the second author. **D.** Dorsal view of cephalic somites. **E.** Lateral view of caudal somites in which no respiratory auricle is visible. **F.** Dissection of clitellar somites revealing the median reproductive apparatus including the penis sheath (pe), epididymes (e), vaginal sac (v) and an ovary (o). **G.** Female median reproductive apparatus exhibiting independent oviducts (arrowheads) and a small vaginal caecum (vc). **H.** Male median reproductive apparatus showing the stout penis and sheath (pe) and associated epididymes (e).

Figure 6.



FAMILY SEMISCOLESCIDAE SCRIBAN AND AUTRUM, 1934

Patagoniobdella variabilis (Blanchard, 1900)

Figure 7 (A – E, H – J)

Two specimens (AMNH 4308, Annelida) fixed in 100% ethanol and one dissected specimen (AMNH 4309, Annelida) fixed in 10% formalin, free living, collected from underside of rocks, 137 m, Lago Villarica (CV01-06), 39º16'39.6"S, 71º58'47.4"W, 21 February 2001. One free-living specimen collected from under submerged branch on shoreline of Lago Huerquehue (CV01-09), 1411 m, 39º07'36.9"S, 71º41'45.4"W, 22 February 2001, fixed in 100% ethanol (AMNH 4310, Annelida) and dissected. The latter specimen was unusual in that the dorsum and dorsal surface of the caudal sucker were centrally black and with broad marginal longitudinal zones of emerald-green pigment when alive (Fig. 7A).

Dorsum typically dark reddish-brown with medial and paramarginal longitudinal zones of light brown pigment patches when alive (Fig. 7B). Cephalic somites with five pairs of pyriform eyespots arranged in parabolic arc on uniannulate somites II, III, IV, and on first annulus of biannulate somites V and VI (Figs. 7C, D). Gonopores separated by 1/2+2+1/2 annuli, male gonopore on annulus XII b1, female gonopore on annulus XII b5 (Fig. 7E). Testisacs at interganglionic intervals. Anteriormost testisacs at XIV/XV are unpaired (i.e., two testisacs at interval), whereas those at XV/XVI through XXIII/XXIV appear as doublets on each side (Figs. 7H, J). Ascending male sperm ducts (i.e., "vasa deferentia") lateral to testisacs, thin and fragile in specimens from Lago Villarica but robust in specimen from Lago Heurquehue (Figs. 7H, J). Tightly coiled proximal sperm ducts (i.e., "epididymes") in XIV and XV, both sets arranged to the left of penis sheath with ejaculatory ducts entering "prostate" glandular region of penis sheath at XVI without procurrent posterior loop (Fig. 7H). Base of penis sheath at XIX. Female reproductive anatomy arranged principally to the right of midline (Fig. 7I). Vaginal sac ends at XIII, common oviduct without procurrent loop and returning anteriad along vaginal sac to a single globular ovisac in XII. Gastric tissue and intestines unbranched. Gastric/intestinal sphincter in XIX. Two annuli between anus and caudal sucker.

Patagoniobdella fraterna Ringuelet, 1976

Figures 7 (F, G)

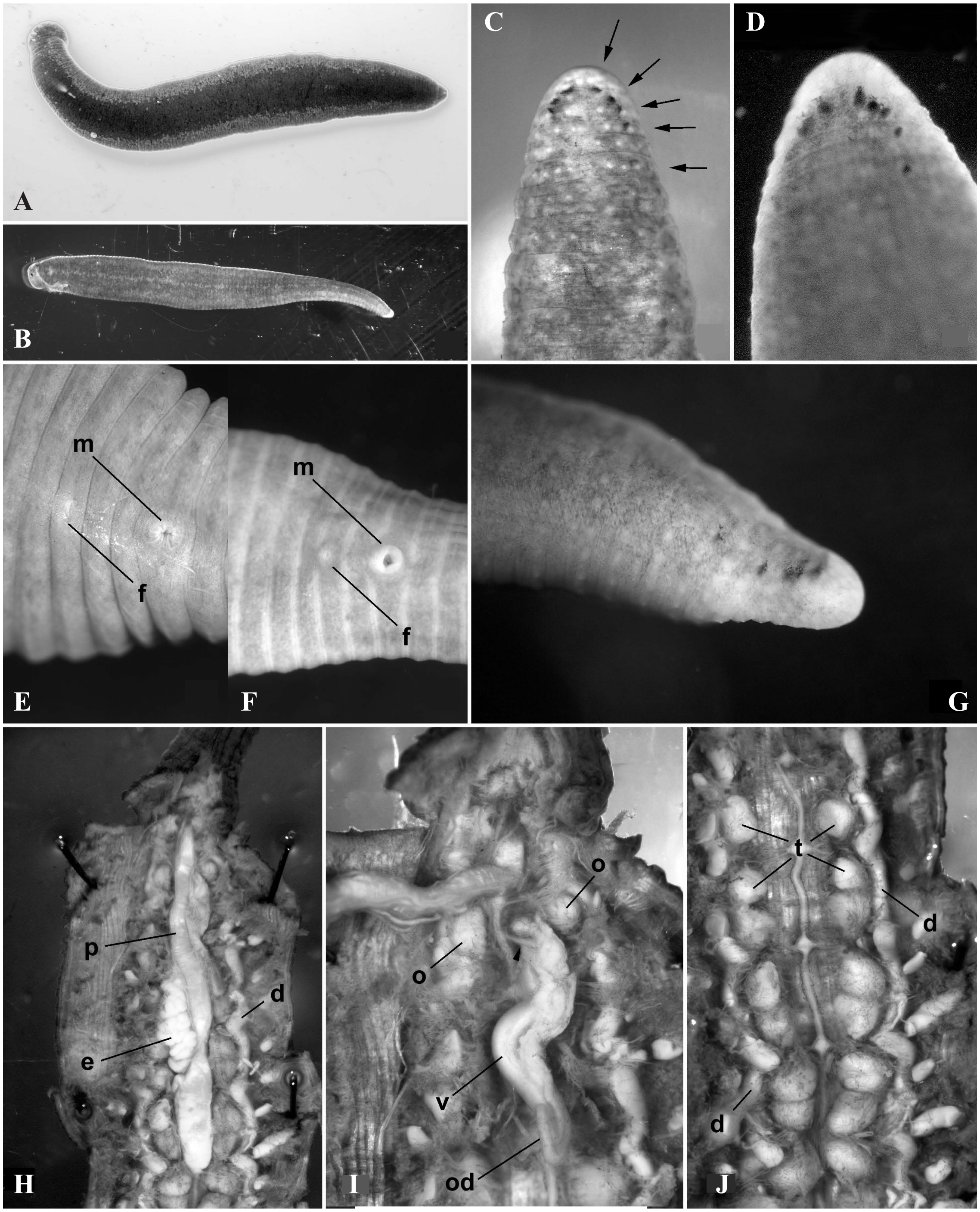
One specimen (AMNH 4306, Annelida) fixed in 100% ethanol and one specimen (AMNH 4307, Annelida) fixed in 10% formalin, free living, collected from underside of submerged branches at, 214 m, Lago Neltume (CV01-03), 39º46'32.3"S, 71º57'27.7"W, 20 February 2001.

Dorsum similar to *Patagoniobdella variabilis*, with dark reddish-brown with medial and paramedial longitudinal zones of light brown pigment patches when alive. Cephalic somites with five pairs of irregular eyespots arranged in parabolic arc on uniannulate somites II, III, IV, and on first annulus of biannulate somites V and VI (Fig. 7G). Male gonopore on annulus XII b1, female gonopore on annulus XII a2 (i.e., separated by 1/2+1+1/2 annuli) (Fig. 7F). Testisacs at interganglionic intervals. Anteriormost testisacs at XIV/XV are paired (i.e., four testisacs at interval), as are those at XV/XVI through XXIII/XXIV which appear as doublets on each side. Ejaculatory duct between epididymes and penis sheath with posterior procurrent loop.

FIGURE 7.

Patagoniobdella species. **A.** Live specimen of *Patagoniobdella variabilis* from Lago Huerquehue; the lighter pigment zones were emerald-green. **B.** *Patagoniobdella fraterna* from Lago Neltume; the darker pigment zones were brick-red. **C.** Dorsal view of cephalic somites of *Patagoniobdella variabilis* from Lago Huerquehue showing the 5 pairs of eyespots (arrows). **D.** Dorsal view of cephalic somites of *Patagoniobdella variabilis* from Lago Villarica. **E.** Ventral view of clitellar somites of *Patagoniobdella variabilis* from Lago Huerquehue. **F.** Ventral view of clitellar somites of *Patagoniobdella fraterna* from Lago Neltume. **G.** Lateral view of cephalic somites of *Patagoniobdella fraterna* from Lago Neltume. **H.** Mid-body dissection of *Patagoniobdella variabilis* from Lago Huerquehue showing the long penis and sheath (pe) with a single mass of associated epididymes (e) and the lateral robust ascending sperm ducts (d). **I.** Ovisacs (o), common oviduct (od) and vagina (v) of *Patagoniobdella variabilis*. **J.** Ascending sperm ducts (d) lateral to the testisacs (t) of *Patagoniobdella variabilis*.

Figure 7.



ORDER RHYNCHOBDELLIDA BLANCHARD 1894

FAMILY GLOSSIPHONIIDAE VAILLANT, 1890

Haementeria gracilis (Weyenberg, 1833)

Figure 8A

One specimen (AMNH 4311, Annelida) fixed in 100% ethanol collected from the first author's left ankle at Laguna de los Lotos, Isla Teja, Valdivia (CV01-12), 13 m, 39º47'58.6"S, 73º15'45.7"W, 24 February 2001.

One pair of eyespots. Gonopores separated by two annuli. Midbody annuli dorsally subdivided. Two pairs of longitudinal rows of major papillae on dorsum on a1 associated with circular unpigmented zones.

Theromyzon propinquium Ringuelet, 1947

Figure 8B

Three specimens (AMNH 4312, Annelida) fixed 10% formalin and three specimens (AMNH 4336, Annelida) fixed in 100% ethanol, collected from overturned rocks at 214 m, Lago Neltume (CV01-03), 39º46'32.3"S, 71º57'27.7"W, 20 February 2001. One specimen (AMNH 4313, Annelida) fixed in 10% formalin, collected from overturned rock at Lago Panguipuilli (CV01-04), 39º38'39. 2"S, 72º19'31.4"W, 20 February 2001. One specimen (AMNH 4314, Annelida) fixed in 70% formalin, collected from overturned rock at Lago Villarica (CVO1-06), 39º16'39. 6"S, 71º58'47. 4"W, 21 February 2001. One specimen (AMNH 4315, Annelida) fixed in 100% ethanol collected from overturned rock at Lago Tinquilco (CV01-07), 41º47'20.0"S, 71º50'52.8"W, 21 February 2001. One specimen (AMNH 4316, Annelida) fixed in 100% ethanol collected from overturned rock at Miraflores, Chiloé (CV01-18) 42º47'23.5"S, 73º51'12.3"W, 6 March 2001.

Four pairs of eyespots. Gonopores separated by three annuli. When alive, dorsum with olive green chromatophores and numerous irregular patches of orange pigment that appear to mostly follow four longitudinal rows repeating in each somite. Caudal sucker very clear with scattered olive green chromatophores and marginal arc of orange pigmented spots.

Remarks: Several weeks after detailing the anatomy of the European *Theromyzon tessulatum* (see Blanchard, 1892a), Blanchard (1892b) received a leech that appeared to be identical to the European duck leech from an anonymous donor in Chile. Believing the Chilean specimens to be the same species, Blanchard (1892b) considered several hypotheses regarding this unusually disjunct distribution: that it was transported on migratory birds, that it was introduced via domesticated ducks or that it was introduced via moist soils on aquatic plants. Later Ringuelet (1978) determined that early accounts of this species in Chile and Argentina were attributable to *Theromyzon propinquium* Ringuelet, 1947 which is distinct from the European species in terms of live color patterns and internal anatomy (Ringuelet, 1947).

Helobdella duplicata Moore, 1911

Figure 8C

Nine specimens (AMNH 4317, Annelida) fixed in 100% ethanol and 15 specimens (AMNH 4318, Annelida) fixed in 10% formalin collected from underside of rocks at Lago Panguipuilli (CV01-04), 39º38'39.2"S, 72º19'31.4"W, 20 February 2001.

Twelve specimens (AMNH 4319, Annelida) fixed in 100% ethanol and 16 specimens (AMNH 4320, Annelida) fixed in 10% formalin collected from underside of rocks at Lago Panguipuilli (CV01-05), 39º38'38.3"S, 72º19'24.0"W, 20 February 2001. Three specimens (AMNH 4321, Annelida) fixed in 10% formalin, collected from under rocks at 137 m, Lago Villarica (CV01-06), 39º16'39.6"S, 71º58'47.4"W, 21 February 2001.

One pair of eyes. Gonopores separated by one annulus. Nuchal glands without scute on VIII a1. Midbody annuli subdivided with transverse metameric darker coloration on a1. A pair of broad paramedial zones of approximately six fine longitudinal lines.

Helobdella simplex Moore, 1911

Figure 8D

Fifty specimens (AMNH 4322, Annelida) fixed in 100% ethanol and 15 specimens (AMNH 4323, Annelida) fixed in 10% formalin collected from underside of rocks at Lago Panguipuilli (CV01-04), 39º38'39.2"S, 72º19'31.4"W, 20 February 2001. Six specimens (AMNH 4324, Annelida) fixed in 100% ethanol and six specimens (AMNH 4345, Annelida) fixed in 10% formalin collected from underside of rocks at Lago Panguipuilli (CV01-05), 39º38'38.3"S, 72º19'24.0"W, 20 February 2001. Eleven specimens (AMNH 4325, Annelida) fixed in 100% ethanol and 19 specimens (AMNH 4326, Annelida) fixed in 10% formalin, collected at 137 m, Lago Villarica (CV01-06), 39º16'39.6"S, 71º58'47.4"W, 21 February 2001. Two specimens (AMNH Annelida 4327) fixed, collected from under rocks at Lago Tinquilco (CV01-07), 41º47'20.0"S, 71º50'52.8"W, 21 February 2001.

One pair of eyes. Gonopores separated by one annulus. Margins of mid-body annuli rendering serrated appearance. One row of medial papillae on each annulus of postgenital somites.

Helobdella michaelseni Blanchard, 1900
Figure 8E

Six specimens (AMNH 5253, Annelida) fixed in 100% ethanol and two specimens (AMNH 5249, Annelida) fixed in 10% formalin collected from underside of rocks at Lago Calafquen (CV01-02), 39º29'26.2"S, 72º08'50.1"W, 19 February 2001. Seven specimens (AMNH 5256, Annelida) fixed in 100% ethanol and six specimens (AMNH 5252, Annelida) fixed in 10% formalin collected from underside of rocks at Lago Neltume (CV01-03), 39º46'32.3"S, 71º57'27.7"W, 20 February 2001. One specimens (AMNH 5254, Annelida) and 29 specimens (AMNH 5255, Annelida) fixed in 100% ethanol and four specimens (AMNH 5250, Annelida) and six specimens (AMNH 5251, Annelida) fixed in 10% formalin, collected at Lago Panguipuilli (CV01-06), 39º38'39. 2"S, 72º19'31.4"W, 20 February 2001. One specimen (AMNH 5257, Annelida) fixed in 100% ethanol, collected from under rocks at 137 m, Lago Villarica (CV01-06), 39º16'39.6"S, 71º58'47.4"W, 21 February 2001.

Body thin and elongate. Caudal sucker terminal. Atria piriform with strongly curved anterior cornua. Male sperm ducts very thick and recurving at XVI. Ovaries short and lobate, to XIII only. No papillae. No nuchal glands. Gastric tube without postcaeca. Pigmentation is highly variable from colorless to irregular fine brown speckling. This species appears to have a remarkable variation in Chile from the very stout to extremely thick and vermiform representatives.

Helobdella cordobensis Ringuelet, 1943
Figure 8F

Three specimens fixed in 10% formalin (AMNH 4342, Annelida) from 207 m, Lago Calafquen (CV01-02), 39º29'26.2"S, 72º08'50.1"W, 19 February 2001. Three specimens fixed in 10% formalin (AMNH 4343, Annelida) and two fixed in 100% ethanol (AMNH 4344, Annelida) from 214 m, Lago Neltume (CV01-03), 39º46'32.3"S, 71º57'27.7"W, 20 February 2001.

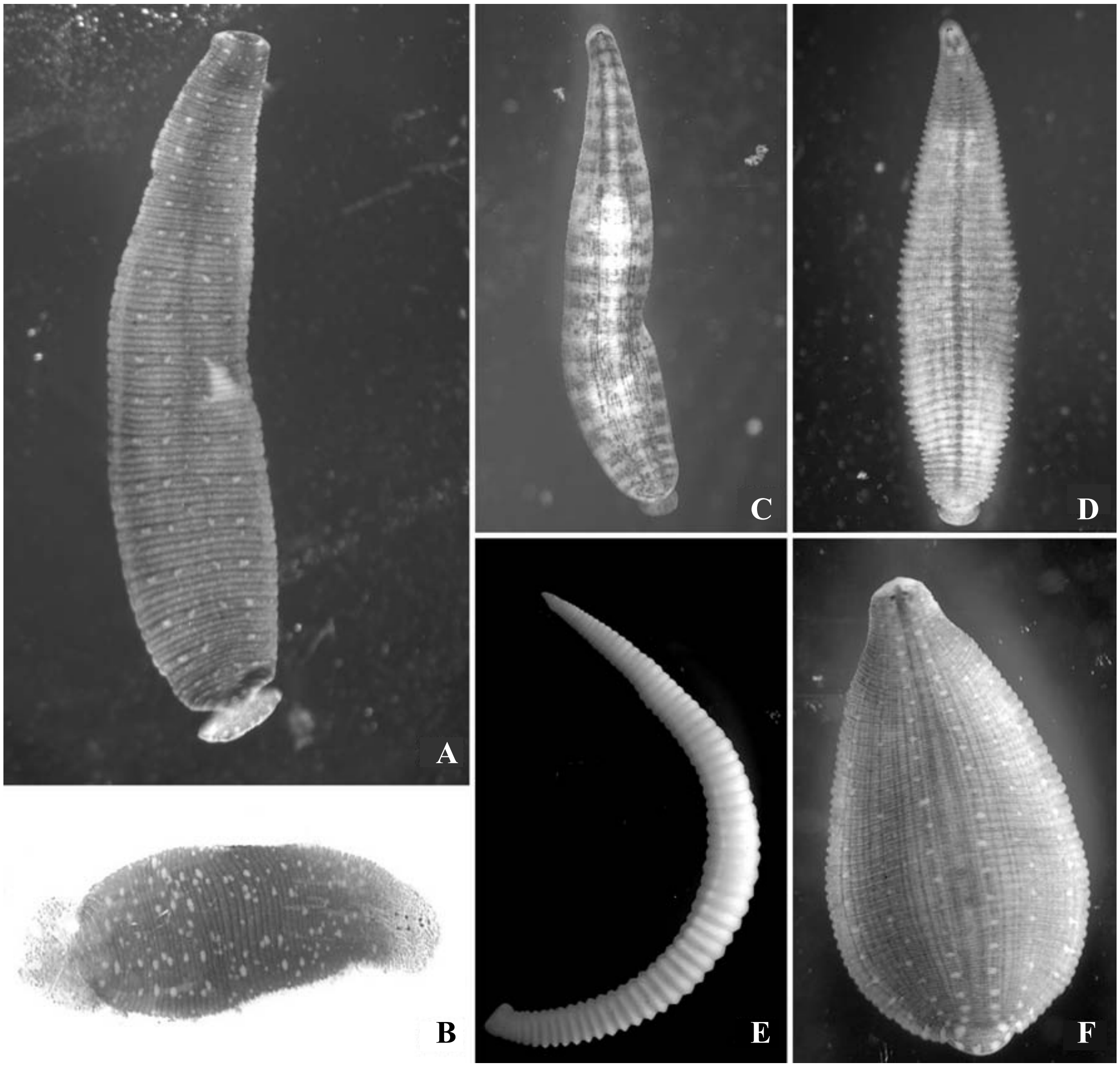
Dorsum with three pairs of rows of white spots. Three pairs of inconspicuous rows of papillae between white spots. Fourteen pairs of longitudinal lines of pigment. Venter has major pair of paramedial stripes, with minor lateral longitudinal striping. Sucker is ventrally directed. Proboscis extends to XIII, with thick salivary ducts at base and diffuse salivary cells in parenchyma extending from X to XVI/XVII. Oesophagus very short. Four pairs of digitate gastric caeca, plus a pair of postcaeca. Four pairs of corrugated intestinal caeca. Atria weakly developed. Ejaculatory ducts recurved in XVII. Ovaries broad and long, extending to XIX.

Remarks: *Helobdella cordobensis,* which may previously have been confused with *Batracobdella gemmata*, as they look remarkably similar dorsally (Blanchard, 1900), was originally a subspecies in the *Helobdella triserialis* group (Ringuelet, 1943a). Like other members of this group, it has four digitate gastric caeca differing from the typical 5 caeca found for other members of the genus. The Chilean representatives of the species differ somewhat from those at the type locality in terms of external pigmentation and their slightly longer ejaculatory ducts (to XVII instead of XVI).

FIGURE 8.

Several glossiphoniid leeches. **A.** *Haementeria gracilis*. **B.** *Theromyzon propinquium* (live). **C.** *Helobdella duplicata*. **D.** *Helobdella simplex*. **E.** *Helobdella michaelseni*. **F.** *Helobdella cordobensis*.

Figure 8.



Helobdella wodzickiorum, new species

Figure 9

Holotype: Collected from underside of rocks in streams Santuario de la Naturaleza Yerba Loca (CV01-01), 2259 m, 33°18'S, 70°17'W, 18 February 2001, fixed in 10% formalin (AMNH 4338, Annelida). Body 12 mm in length and 1 mm wide.

Paratypes: Fifteen specimens fixed in 100% ethanol (AMNH 4339, Annelida) and fourteen specimens fixed in 10% formalin (AMNH 4340, Annelida) collected at Santuario de la Naturaleza Yerba Loca (CV01-01), 2068 to 2259 m, 33°18'S, 70°17'W, 18 February 2001.

Description: Dorsum irregularly mottled pale tawny brown to white field (Fig. 9A). Venter irregularly mottled as in dorsum (Fig. 9B). Annuli not subdivided. Sucker terminal (Figs. 9A, B). Neither nuchal scute nor glands present in VIII. One pair of eyespots on III. Base of proboscis at XIII/XIV (Fig. 9C). Salivary glands at base of proboscis in XIV and with diffuse parenchymal salivary cells (Fig. 9C). Gastric tract tubular and aceacate. Postcaeca to XXII (Fig. 9E). Four intestinal caeca. Ejaculatory ducts insert into atria dorsolaterally (Fig. 9F); recurve at XXII. Descending and ascending portions with distinct transition in XXII (Fig. 9F inset). Atria bulbous and directed laterally (Fig. 9F, G). Long, tubular ovisacs extending to XIX (Fig 9G).

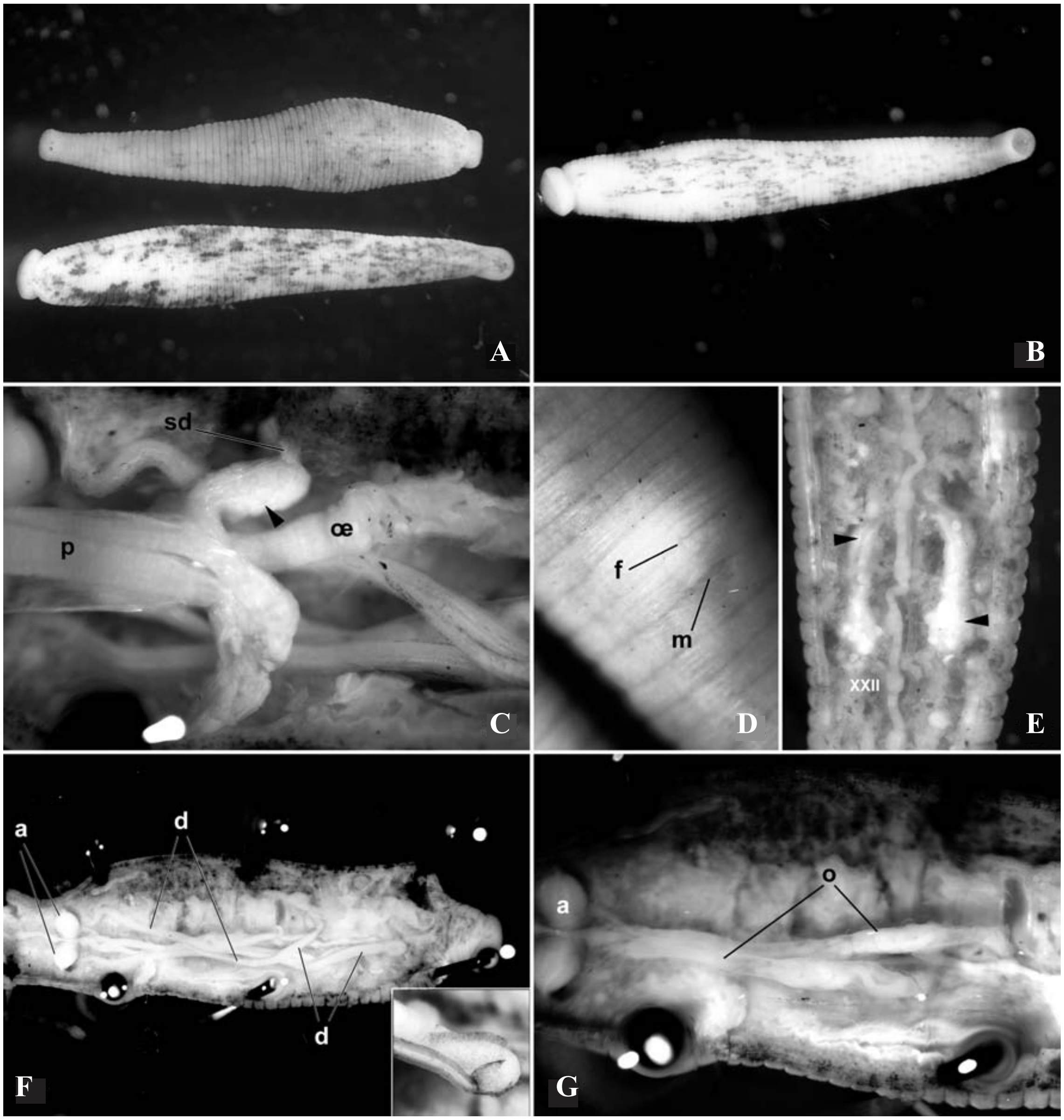
Remarks: Species of *Helobdella* with irregularly mottled dorsal pigmentation include *Helobdella paranensis* (Oka, 1930), *Helobdella similis* Ringuelet, 1942 (see Ringuelet, 1942a, 1985b), and *Helobdella nununununojensis* Siddall, 2001. Both *H. similis* and *H. nununununojensis* also share several internal features with *H. wodzickiorum*, like the tubular crop lacking caeca, and short post-caeca (Ringuelet, 1985b; Siddall, 2001b). Each of *Helobdella bolivianita* Siddall, 2001, *H. nunununununojensis*, *Helobdella cryptica* (Ringuelet, 1978) and *H. paranensis* have the unusual glandular salivary tissue around the salivary ducts that drain cells in the parenchyma. *Helobdella bolivianita*, however, has a scute at VIIIa1/a2 (Siddall, 2001a) and *H. nunununununojensis* has a very small caudal sucker (Siddall, 2001b) and would not be readily confused with *H. wodzickiorum* even on external anatomy. Whereas several species of *Helobdella* have ovaries as long as those seen in *H. wodzickiorum*, like *H. cordobensis* (Fig. 8F), no other species in the genus has a larger male reproductive anatomy with the large prominent atria and male sperm ducts extending all the way to somite XXIII.

Etymology: The species is named for Wojtek Wodzicki and Kathryn Gregory-Wodzicki who conveyed us to the Yerba Loca Reserve for what should have been only a pleasant afternoon hike in the Andes but also unintentionally became our first collection locality.

FIGURE 9.

Helobdella wodzickiorum n. sp. **A.** Two specimens demonstrating dorsal appearance and variability. **B.** Ventral appearance. **C.** Base of proboscis (p) at junction with esophagus (œ) where small salivary glands (arrowheads) surround the salivary ductule bundle (sd) draining parenchymal salivary cells. **D.** Female (f) and male (m) gonopores on venter. **E.** Gastric postcaeca (arrowheads) viewed ventrally. **F.** Dorsal view of male median reproductive apparatus showing the prominent atria (a) and extremely long sperm ducts (d) for which the ascending and descending portions are markedly different in diameter (inset). **G.** Elongate ovisacs (o).

Figure 9.



Helobdella pichipanan, new species

Figures 10

Holotype: Collected from underside of submerged branches on shoreline at Lago Chico (CV01-08), 1239 m, 39º08'33.3"S, 71º42'44.1"W, 22 February 2001, fixed in 10% formalin (AMNH 4328, Annelida). 39 mm in length and 7.0 mm in width. Dorsum dark grey to brown with pale beige longitudinal middorsal zone; venter pale with 2 pairs of black paramedial lines.

Paratypes: One specimen at Lago Tinquilco (CV01-07), 41º47'20.0"S, 71º50'52.8"W, 21 February 2001, fixed in 100% ethanol (AMNH 4329, Annelida). Three specimens fixed in 100% ethanol (AMNH 4330, Annelida) and five fixed in 10% formalin (AMNH 4331, Annelida) from Lago Chico (CV01-08), 1239 m, 39º08'33.3"S, 71º42'44.1"W, 22 February 2001. Eleven specimens fixed in 100% ethanol (AMNH 4332, Annelida) and eleven specimens fixed in 10% formalin (AMNH 4333, Annelida) from Lago Huerquehue (CV01-09), 1411 m, 39º07'36.9"S, 71º41'45.4"W, 22 February 2001. One specimen fixed in 100% ethanol (AMNH 4334, Annelida) and three fixed in 10% formalin (AMNH 4335, Annelida) from Lago Patos (CV01-010), 1467 m, 39º07'30.0"S, 71º42'14.6"W, 22 February 2001. Two specimens fixed in 100% ethanol (AMNH 4336, Annelida) and one with brood fixed in 10% formalin (AMNH 4337, Annelida) from Lago Verde (CV01-011), 1285 m, 39º08'07.2"S, 71º42'31.0"W, 22 February 2001.

Description: Dorsum dark gray median to light brown/beige paramedially with pale beige broad middorsal longitudinal zone (Fig. 10A). One pair of dorsal continuous paramedial faded black stripes. Two to three pairs of discontinuous dark and light rows of pigment within the continuous stripes and six or more pairs of discontinuous dark and light rows of marginal to continuous stripes. Two to three pairs of dark transverse lines per annulus. Some dorsal papillation towards posterior end (Fig. 10A). Venter pale beige with 2 pairs paramedial faded black stripes (Fig. 10A). Anterior and posterior sucker of same pale beige color; caudal sucker subterminal. Marginal longitudinal black stripes ventrally. In juveniles, dorsal paramedial stripes conspicuously dark and continuous. Pale, dorsal longitudinal zone, not conspicuous. No dorsal posterior papillation. Inner pair of ventral paramedial stripes prominent and continuous; outer pair discontinuous.] Annuli weakly subdivided (Figs, 10B, C). Nuchal gland present on VIII a1/a2 (Fig. 10B). One pair of eyes on III. Base of proboscis at XIII. Thick salivary ducts at base of proboscis with parenchymal salivary cells; diffuse (Fig. 10E). Gastric tract tubular with weak caecal bulges mid-somite; one pair of postcaeca present and four pairs of intestinal caeca (Fig. 10F). Gonads micromorphic. Male atria very weakly developed, nearly absent (Fig. 10D). Ejaculatory ducts extend only to XII/XIII. Ovisacs extend only to XII/XIII (Fig. 10D).

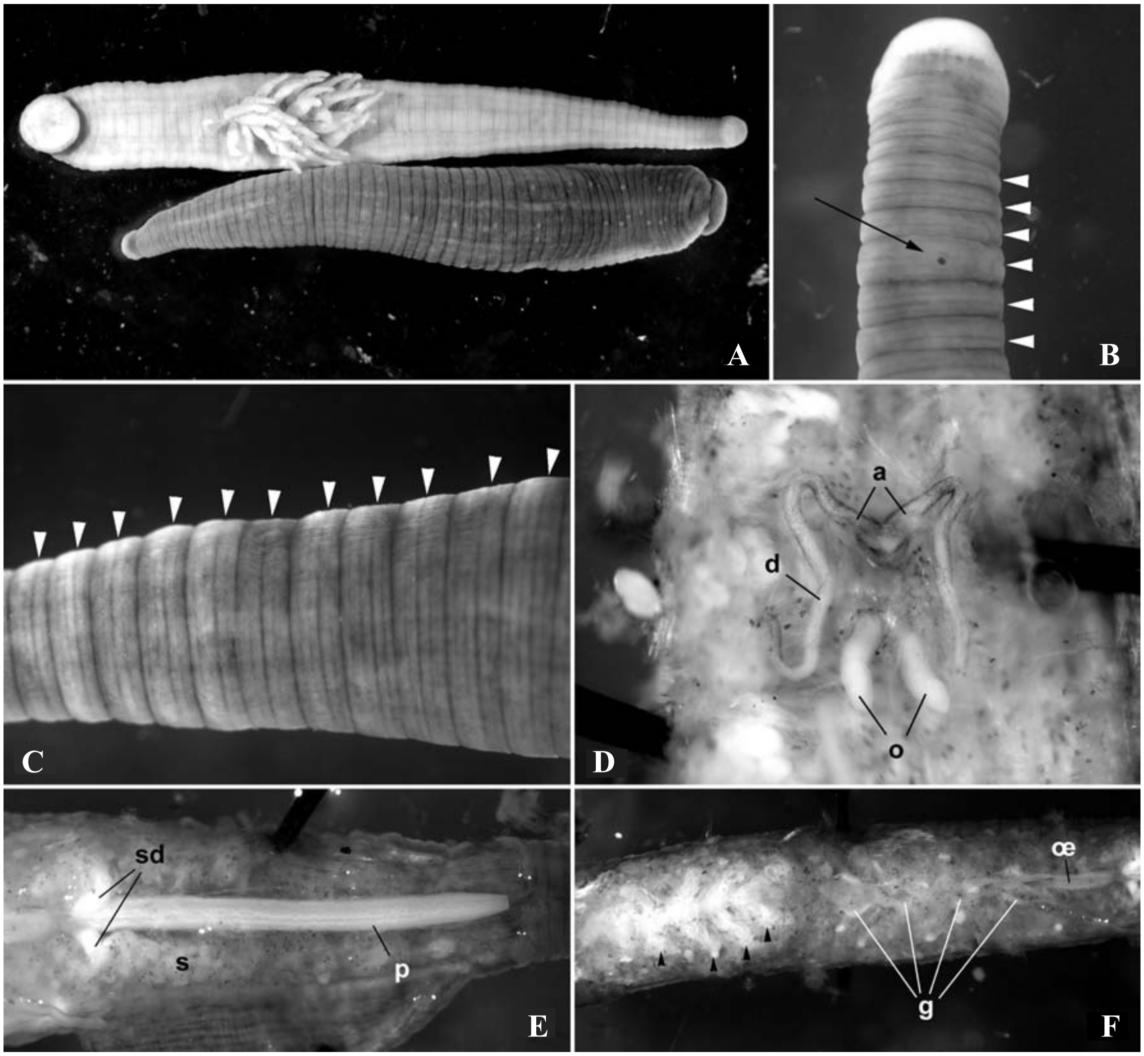
Remarks: Several leeches in South America possess a glandular region at VIIIa1/a2, the same position at which *Helobdella stagnalis* (among others) exhibits a hardened scute. Each of these, *H. duplicata*, *H. diploides*, *H. simplex*, *Helobdella ringueleti* Siddall, 2001, and *H. nununununojensis*, is readily distinguished from *H. pichipanan*. *Helobdella duplicata* has pronounced dorsal metameric banding (Fig. 8C) and *H. simplex* (Fig. 8D) has the obvious mid-dorsal row of papillae on all annuli (Moore, 1911). The caudal sucker of *H. nununununojensis* is much smaller than the body width (Siddall, 2001b). Like *H. ringueleti*, differences from *H. diploides* could be difficult to discern on the basis of external anatomy alone. Whereas *H. diploides* also has subdivided annuli, it does not have a dorsal pigment pattern (Ringuelet, 1948). The dorsal and ventral pigmentation of *H. ringueleti* and *H. pichipanan* is nearly identical, and both species have subdivided annuli. Even on dissection the two species similarly lack prominent gastric ceaca save for the postcaeca, and have small ovaries residing in XII only (Siddall, 2001b). However, these two differ markedly in the male reproductive anatomy. In fact, no other species of *Helobdella*, has male genitalia that are smaller, an atrium that is as weakly developed or sperm ducts that are less extensive.

Etymology: The name (pron. pee chee pah nahn) is Mapuche for small male genitalia.

FIGURE 10.

Helobdella pichipanan n. sp. **A.** Ventral (top) surface of adult with 33 attached juveniles, and dorsal surface (bottom) of another adult. **B.** Dorsal view of cephalic somites showing the position of the nuchal gland (arrow) and the subdivision of annuli (arrowheads). **C.** Subdivision of annuli (arrowheads) in clitellar and mid-body somites. **D.** Median reproductive apparatus with diminutive atria (a), sperm ducts (d) and ovisacs (o). **E.** Dissection of anterior somites revealing the long proboscis (p) and salivary ductule bundle (sd) draining parenchymal salivary cells (s). **F.** Alimentary canal comprising the esophagus (œ), weakly defined gastric caeca (g) and intestinal caeca (arrowheads).

Figure 10



DISCUSSION

The diversity and endemism of neotropical hirudifauna, attributed to the limited dispersal range of the majority of its species, is well known (Weber, 1915; Moore, 1911; 1924; Pinto 1923; Richardson, 1969; Soós, 1966; Ringuelet, 1944a, 1985a,b; Sawyer, 1986; Siddall, 2001a, b), and continues to be reflected in the collections made during this expedition. There are over 30 described *Helobdella* species from South America alone, in particular from Argentina, Bolivia, Brazil, Chile, Paraguay, Peru and Uruguay. The recent synonymy of other glossiphoniid genera with *Helobdella* (i.e., *Adaetobdella*, *Acritobdella*, *Desmobdella* and *Gloiobdella*; Siddall and Borda, 2003) increases that number to approximately 40 species, including recent descriptions of new *Helobdella* species from Bolivia (Siddall, 2001a,b).

These collections and the resulting genetic sequence and morphological data from each of *Americobdella valdiviana*, *Mesobdella gemmata* and *Patagoniobdella* species have been essential in resolving key phylogenetic questions about relationships among arhynchobdellid groups (Borda and Siddall, 2004). From a systematic standpoint, the phylogenetic placement and classification of each of these species had been problematic. This is a consequence of each having unusual life-history strategies, and possessing morphological characteristics that apparently deviate from other members of traditionally established arhynchobdellid families and genera (Blanchard, 1893, 1917; Forbes, 1890; Moore, 1911, 1924; Cordero, 1937; Ringuelet, 1945, 1954, 1985a,b; Richardson, 1969, 1971, 1976; Sawyer, 1986). The combination of a new phylogenetic framework (Borda and Siddall, 2004) and the morphological details above permits clarification of their systematic position among the Hirudinida.

Americobdella

In the original description (Phillippi, 1872), the monotypic *Americobdella valdiviana* was classified as an erpobdellid (Phillippi, 1872) because it was a predator and had rudimentary jaws, typical of erpobdellid leeches (Blanchard, 1917; Harant, 1929; Weber, 1915). Phillippi (1867) remarked on its resemblance 'to the European *Trocheta* in both appearance and habit'. Moore (1924: 43) argued that this comment caused 'all subsequent writers to be misled', and he recommended reclassifying *Americobdella* in the distichodont series of Hirudinidae, because several external characteristics pointed directly to an affinity with the Hirudinidae. Caballero (1956) placed *A. valdiviana* in its own family, but still classified it with the Hirudiniformes, where it remained (Ringuelet, 1985a, b; Sawyer, 1986). The unstable classification of *A. valdiviana* can be attributed to its being reminiscent of an ancestral arhynchobdellid leech, retaining morphological traits and habitat preferences seemingly transitory between rhynchobdellid and arhynchobdellid leeches. Most significant, is the presence of the intergonadal conducting tubules, connecting the male atrium and the female ovarian ducts, typically found in rhynchobdellid piscicolid leeches. As a result, Moore (1924; see also Ringuelet, 1954; Soós; 1966) proposed that *A. valdiviana* belonged to an ancestral lineage that originated before other arhynchobdellid groups. Likewise, Siddall and Burreson's (1995, 1996) phylogenetic analyses based on morphology and life-history data found that *A. valdiviana* was in a transitional position between the two major groups of leeches (i.e. Rhynchobdellida and Arhynchobdellida). Recent phylogenetic work by Borda and Siddall (2004), using combined morphological and molecular data, indicates that *A. valdiviana* is more closely related to the erpobdelliforms (i.e. *Barbronia* species, *Erpobdella* species), and not the hirudiniforms as previously suggested (Caballero, 1956; Moore, 1924; Ringuelet, 1944a, 1976, 1985a,b; Sawyer, 1986). As the most basal lineage of the erpobdellid leeches and it should be formally removed from the Hirudiniformes and placed under the Erpobdelliformes, together with the families Erpobdellidae and Salifidae.

Phillippi (1872) provided conflicting information regarding the habitat preference of *A. valdiviana*. In his description he noted that *A. valdiviana* was a species living in damp earth and feeding on earthworms. However, the specimens he examined were collected in “ditches of mill races” and from a brook. Corroborating this were descriptions of the species suggesting external color variation to be associated with habitat preference (Moore, 1924; Ringuelet, 1985a, b; Weber, 1915). Weber (1915) described terrestrial specimens collected from Corral as being dorsally dark grey, with a uniform yellowish-grey venter. Moore (1924) received specimens “collected in damp earth in Valdivia” (p. 30) and a letter from a Professor Montealegre Randolph describing an aquatic *Americobdella* (=*Phillippia*) that was “blue-slate [in color] and bears only one red stripe on the back” (p. 29; footnote). On our expedition, a terrestrial dark grey to maroon specimen (found in the midst of consuming an oligochaete) and an aquatic slate-grey specimen with a yellow mid dorsal line were collected. These collections are at variance with color types associated with a specific habitat. Regardless, the fact that 18S rDNA sequences were found to be identical for both specimens suggests that *A. valdiviana* is neither exclusively terrestrial nor aquatic, but rather amphibious in habit. The variation in color pattern and habitat preference could be age specific or developmental; darker specimens appear to be smaller in size than their lighter counterparts.

The presence or absence of eyes in *A. valdiviana* has been a matter of speculation (Blanchard, 1917; Moore, 1924; Pinto, 1923; Ringuelet, 1985a, b; Sawyer, 1986; Weber, 1915). *Americobdella valdiviana* was described by Phillippi (1872) as lacking eyes; a diagnostic character for the species in subsequent reports (Blanchard, 1917; Weber, 1915; Pinto, 1923). The lack of eyes was recognized as being associated to a partial subterranean habitat preference similarly found in other burrowing, blind oligochaete predators in the genera *Trocheta* and *Cylicobdella* (Phillippi, 1872; Weber, 1915). To the contrary, Moore (1924: 33) suggested that "this leech has always been considered to be eyeless, and my first examination led to the same conclusion. However, under intense lighting…there is no difficulty in making out…several whitish areas within small deeply pigmented fields which are evidently eyes". Consequently, other authors followed this revision and even illustrated the presence of six pairs of eyes or "eye-like organs" (Ringuelet, 1944a, 1985a, b; Sawyer, 1986). However, examination of the live specimen, suggests once again that *A. valdiviana* in fact does not have eyes.

Mesobdella

The terrestrial sanguivore, *M. gemmata*, was traditionally classified a haemadipsid (Blanchard, 1893, 1900; Ringuelet, 1942b, 1943b, 1944a, 1985a; Sawyer, 1986) based on gross morphological characters (i.e. ocular arch; wide dorsal median field; position of 1st nephripores (Richardson, 1971)) and life history strategies (i.e. terrestrial and sanguivorous). These apparent affinities suggested that *M. gemmata* shared a common ancestry with the terrestrial IndoPacific leeches (R. Blanchard, 1893, 1917; Ringuelet, 1943b, 1944c; Caballero, 1940; Sawyer, 1986), but its geographic distribution, in Chile, and apparent shared reproductive homologies with South American hirudinids (e.g. *Oxyptychus* species) suggested otherwise (Richardson, 1969; Ringuelet, 1972).

Moore (1946) proposed a subfamily Xerobdellinae to include *M. gemmata* with members of other terrestrial genera, such as *Xerobdella* Frauenfeld, 1868 from Europe, and the Mexican *Diestecostoma* Vaillant, 1890. He believed these shared a resemblance in the position of nephripores and the absence of auricles (Moore, 1946). Xerobdellinae was synonymized with Dietecostomatidae (Ringuelet, 1954), but later Ringuelet (1972) established the family Mesobdellidae to the exclusion of the genera *Xerobdella* and *Diestecostoma*. Ringuelet (1972) created the family Mesobdellidae in light of Richardson's (1969, 1971) comparative work showing affinities with Neotropical aquatic sanguivores (i.e. *Oxyptychus* species) and included only the South American taxa, *Mesobdella* species and *Nesophilaemon skottsbergii*, the latter from the Juan Fernandez archipelago. Richardson (1971: 218) suggested the external "haemadipsine-like features" to be a secondary adaptation to terrestrialism, whereas the "systems which have not come under the influence of this habit, the female median region and the simple form and paramedial position of the anterior region of the male paired duct, [are] unlike the auriculate land-leeches".

Recent phylogenetic work (Trontelj et al., 1999; Borda and Siddall, 2004) supports the divergence of a second terrestrial sanguivorous lineage separate from the IndoPacific haemadipsids. Further studies including additional representative taxa from the genera *Mesobdella* and *Xerobdella* (i.e. *Mesobdella lineata*, *Mesobdella notohilica*, *Xerobdella anulata*, and *Xerobdella prealpina*), as well other New World terrestrial genera, *Diestecostoma* and *Nesophilaemon*, could resolve their phylogenetic relationships and therefore lead to the redefinition of previously proposed families (Ringuelet, 1953; 1972; 1982; Harant and Grassé, 1959; Soós, 1966).

Patagoniobdella

Ringuelet (1972) emended the family Semiscolecidae Scriban & Autrum 1934 to include members of the genera *Semiscolex* Kingberg 1867 and *Patagoniobdella*. Currently (Ringuelet, 1985b), the genus *Semiscolex* includes five species (*S. intermedius* Ringuelet 1942; *S. juveniles* Kingberg 1867; *S. notatus* Cordero 1937; *S. similis* Weyenbergh 1879; *S. zonatus* Oka 1930) and the genus *Patagoniobdella* includes three species (*P. adomonia* Ringuelet 1976, *P. fraterna* and *P. variabilis*). Ringuelet (1972) established the genus *Patagoniobdella* for *Semiscolex variabilis*, which has two pairs of testisacs per interganglionic space and thick ejaculatory ducts. Otherwise, external morphological characters, digestive track and reproductive organs are much like in *Semiscolex* species.

Members of Semiscolecidae variously have been classified among the erpobdelliforms or the hirudiniforms (Blanchard, 1896; Ringuelet, 1954; Weyenberg, 1879), but have mostly been associated with the family Haemopidae (Moore, 1911; Richardson, 1969; Ringuelet, 1944a, b, 1954; Sawyer, 1986). Blanchard (1896) anticipated that these South American predators were not closely related to other hirudinids and had them in their own subfamily (Semiscolecinae). Ringuelet (1954: 5; see also Blanchard, 1896; Moore, 1911; Richardson, 1969) pointed to the apparent mixture of erpobdellid characteristics, with hirudinid eyes and anatomy and a haemopid alimentary tract. Richardson (1969) suggested there were differences in reproductive morphology (i.e. acaecate vagina) in the semiscolecids compared to haemopid leeches, but he still tentatively retained them in the Haemopidae. In the most recent classification, Sawyer (1986) placed the semiscolecids under Haemopidae, but under the subfamily Semiscolecinae, to distinguish the South American genera *Cyclobdella*, *Orchibdella*, *Patagoniobdella* and *Semiscolex*, from the North American haemopids. On the other hand, Ringuelet (1985a, b), in his final contributions adhered to his earlier classification (Ringuelet, 1972). The inclusion of representative semiscolecids, *Semiscolex similis* from Bolivia and the two *Patagoniobdella* species described here, in Borda and Siddall's (2004) phylogenetic analysis has shed some light on their phylogenetic and systematic position within Arhynchobdellida. They appear to share common ancestry with the South American *Oxyptychus* species and North American *Macrobdella* species and not with the haemopids at all. The latter, were found in a separate lineage together with the medicinal leeches, typified by the genus *Hirudo* and allies. Therefore, we formally resurrect the family Semiscolecidae as defined by Ringuelet (1972).

CHAPTER 4

A Revision of the Malagabdellinae (Arhynchobdellida: Domanibdellidae), with a Description of a New Species of *Malagabdella* from Ranomafana National Park, Madagascar

(From: Borda, E. A revision of the Malagabdellinae (Arhynchobdellida: Domanibdellidae), with a description of a new species, *Malagabdella niarchosorum*, from Ranomafana National Park, Madagascar. *American Museum Novitates* 3531: 1–13.)

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INTRODUCTION

Other than being known as tiny vampires of the eastern rainforests, little is known about the natural and evolutionary history of the terrestrial leeches of Madagascar. There have been three major contributions toward the classification of Malagasy leeches. In his *Monographie des Hémadipsines*, Blanchard (1917) described four new species from Madagascar, assigning three to the only five-annulate genus at the time, *Haemadipsa* Tennet, 1859: *Haemadipsa fallax* Blanchard, 1917, *Haemadipsa vagans* Blanchard, 1917, and *Haemadipsa morsitans* Blanchard, 1917, and one species to the four-annulate genus *Philaemon* Lambert, 1898: *Philaemon grandidieri* Blanchard, 1917.

Richardson (1975) revised previous classifications (e.g., Blanchard, 1917; Moore, 1927; Augner, 1931; Soós, 1967, 1970) that placed all terrestrial leeches under a single subfamily, Haemadipsinae Blanchard, 1893, or family Haemadipsidae Blanchard, 1893, by roughly dividing the group based on geographic region and jaw morphology. He also elevated the land leeches to superfamily Haemadipsoidea Richardson, 1975. Richardson (1975) redefined and restricted the Haemadipsidae for the trignathous leeches of India, East Asia, and Southeast Asia and erected two families: Domanibdellidae Richardson, 1975 for the duognathous leeches from the Australasian region (including Oceana, with limited extensions into Wallacea) and Idiobdellidae Richardson, 1975 for the duognathous leeches found in the Seychelles and Madagascar. Richardson (1975) also erected the genera *Malagabdella* Richardson, 1975 and *Tristabdella* Richardson, 1975 for the five-annulate and four-annulate Malagasy leeches, respectively.

In his erratum, Richardson (1978) admitted to having been premature in his previous classification of the Malagasy leeches within the Idiobdellidae (see Richardson, 1975). His original classification (Richardson, 1975) was based only on the dissection of one specimen of *Idiobdella seychellensis* Harding, 1913 from the Seychelles, wherein due to his extensive experience with the apparent uniformity of the reproductive morphology of leeches, he assumed that the morphology of the Malagasy species would be similar to the species from the Seychelles Islands, leading him to place them in Idiobdellidae (Richardson, 1978). Richardson (1978) amended his initial conclusions based on differences found in the median male reproductive region resulting from his examination of *Malagabdella vagans* (Blanchard, 1917). As a result, Richardson (1978) restricted Idiobdellidae to include only the leeches from the Seychelles and erected the subfamily Malagabdellinae Richardson, 1978, and placed it under the Domanibdellidae.

In February and March of 2002, as part of an American Museum of Natural History expedition team, I attempted to rediscover the blood-feeding terrestrial leeches of Madagascar, even though these were well known by locals, tourists, and visiting field biologists as mere nuisances. The collection of these leeches would allow their incorporation into ongoing phylogenetic work and permit photo-documentation of the Malagasy leeches for the first time. During this expedition, efforts were focused in humid rainforests in three principal localities throughout the east coast of Madagascar: Fort Dauphin (southeastern Toliara Province), Ranomafana National Park (Fianarantsoa Province), and Andasibe (Taomasina Province). These collections allowed a better insight into the diversity of yet another group of endemic organisms from Madagascar in the face of a changing and disappearing environment.

MATERIALS AND METHODS

Terrestrial leeches were collected from exposed skin prior to or while they were bloodfeeding. Collection strategies involved walking along forest trails and through the damp undergrowth, especially after rain, to attract leeches. Other methods included walking barefoot through the forest to increase skin exposure for leeches found in the undergrowth. Coordinates for localities were estimated using a Garmin eTrex GPS unit, when signal was available. After collection, leeches were relaxed using an ethanol gradient, starting with an ~10% ethanol solution and gradually adding 95% ethanol until the leeches were relaxed (and dead). Leeches were fixed either in 10% buffered ($CaCl_2$) formalin to use for dissection and/or scanning electron microscopy or in 95% ethanol to use for DNA extraction. Formalin-fixed specimens were later transferred to and stored in 70% ethanol. Examination and dissection of specimens were done using a Nikon SMZ-U stereomicroscope. Photo-documentation of leeches was done using a SPOT-RT (Diagnostic Instruments, Inc.) digital camera.

In the most recent classification of leeches, Sawyer (1986) placed all blood-feeding terrestrial leeches in the Haemadipsidae and divided the leeches based on jaw and pharynx morphology into series (i.e., *duognathous* series and *trignathous* series). Therefore, the Malagasy leeches were classified under the *duognathous* series of the Haemadipsidae. Until a more complete revision of the systematics of blood-feeding terrestrial leeches is available, Richardson’s (1975, 1978) classification for the leeches of Madagascar will be followed, in part. Species redescriptions are based on the somital limits and morphology used by Richardson (1975, 1978), which were adapted from Moore (1927). Richardson’s (1975, 1978) classification scheme represents the diversity of the leeches of the IndoPacific, which, otherwise, is lost in the more recent treatments of this group (i.e., Sawyer, 1986). The superfamily Haemadipsoidea proposed by Richardson (1975) for the land leeches will not be considered here based on the results from Borda and Siddall (2004) and unpublished data showing that leeches from the IndoPacific are monophyletic and are a derived group within a monophyletic hirudiniform clade. Therefore, the IndoPacific land leeches are considered to be of equal family ranking to other groups of hirudiniform leeches (Borda and Siddall, 2004).

Type specimens from the Muséum National d'Histoire Naturelle (Paris, France) were also examined and include: *Haemadipsa dussumieri* Blanchard, 1917 (MNHN 802; no. 198), *Haemadipsa fallax* Blanchard, 1917 (MNHN 751; no. 143 and no. 146A), *Haemadipsa moristans* Blanchard, 1917 (MNHN 802; no. 198) and *Haemadipsa vagans* Blanchard, 1917 (MNHN 751; no. 145). MNHN numbers are lot numbers, not catalog numbers. The Hirudinea collection is stored in lots, with type and non-type specimens stored together. Therefore, the scientific name is used to locate the specimen within a lot. Richardson (1975) designated *Haemadipsa fallax* as the type species for the genus *Malagabdella* based on page priority in Blanchard (1917).

RESULTS

Three endemic species from the terrestrial blood-feeding genus *Malagabdella* were collected from the east coast of Madagascar; one species is new to science. These include the species described below.

ORDER HIRUDINIDA

SUBORDER ARHYNCHBODELLIDA BLANCHARD, 1894

HIRUDINIFORMES CABALLERO, 1952

FAMILY DOMANIBDELLIDAE RICHARDSON, 1975

SUBFAMILY MALAGABDELLINAE RICHARDSON, 1978

GENUS MALAGABDELLA RICHARDSON, 1975

(REVISED AND RESTRICTED)

TYPE SPECIES: *Haemadipsa fallax* Blanchard, 1917.

DIAGNOSIS: Duognathous; Cephalic somites II, III, and IV uni-annulate; V two-annulate, VI and VII three-annulate; VIII to XXIII five-annulate; XXIV two-annulate (incomplete three-annulate or incomplete four-annulate); XXV to XXVII uni-annulate; Male gonopore in XI b5/b6 (or XI/XII); female gonopore in XII b5/b6 (anterior in b5 or anterior in XIII); 57 (or 58) friction rays, ventral, on caudal sucker; respiratory auricles bilobed.

REMARKS: Richardson (1978) recognized four species in the genus *Malagabdella*: *Malagabdella fallax*, *Malagabdella dussumieri*, *Malagabdella morsitans*, and *Malagabdella vagans.* After the type specimens of *Malagabdella* species were examined, it became questionable whether *Malagabdella dussumieri* was actually a leech from Madagascar.

First, Blanchard's (1917) description of *Haemadipsa dussumieri* was based on a single specimen (length: 36 mm; MNHN 802; no. 198; $N = 1$) collected by Dussumier from an unknown type locality. Blanchard (1917) never suggested that *Haemadipsa* *dussumieri* was from Madagascar and actually proposed that this leech was from the south of Hindustan (Madras). When Richardson (1975) transferred *Haemadipsa dussumieri* into the genus *Malagabdella*, he referred to having read Dussumier's letter (1827) written to the administrators of the Muséum National d'Histoire Naturelle. In it he (Dussumier) said that most of his collections were from the Malabar Coast, with some additional collections of vertebrates in the Seychelles Islands and Madagascar. There is no mention of leeches having been collected specifically from Madagascar. Based on the latter and the fact that the somital annulation patterns appeared to deviate from *Haemadipsa* species, Richardson (1975) accepted this as evidence and merely guessed that *Haemadipsa dussumieri* was from Madagascar. Second, domanibdellid leeches are duognathous. When the type specimen of *Malagabdella dussumieri* was examined, widening of the oral cavity exposed a dorso-medial slit for the presence of a jaw, indicating that this leech is trignathous. Third, Sawyer (1986) showed the systematic utility of counting the number of friction rays found on the caudal sucker in *Haemadipsa* species. According to Sawyer (1986), most species of *Haemadipsa* are described as having more than 80 friction rays on the ventral side of the sucker. The total number of friction rays for *Malagabdella dussumieri* is 86, which suggests that this leech actually may have been from India, as originally speculated by Blanchard (1917). *Malagabdella* species only have 57 (or 58) friction rays on the ventral side of the caudal sucker.

Malagabdella dussumieri should be removed from the genus *Malagabdella*, as well as from the family Domanibdellidae, and returned to the Haemadipsidae sensu stricto under its original name, *Haemadipsa dussumieri*, until a more thorough evaluation of the Haemadipsidae is available.

Malagabdella fallax (Blanchard, 1917)

Figure 11

Haemadipsa fallax Blanchard, 1917: 664.

Chtonobdella fallax — Augener, 1931: 9; Soós, 1967: 423.

Malagabdella fallax — Richardson, 1975: 142; Richardson, 1978: 859–860; Sawyer, 1986: 762.

TYPE MATERIAL: *Syntype*, Madagascar—east coast forests, 1882, Lantz (MNHN 751; no. 143; *N* =1). *Syntype*, Madagascar—east coast forests, 1890, Dr. Catat (MNHN 751; no. 146A; *N* =1).

ADDITIONAL MATERIAL (*N* = 134): Eight specimens (AMNH 5277, Annelida) fixed in 95% ethanol and three specimens (AMNH 5278, Annelida) fixed in formalin, 196 m, Domaine de la Cascade, Taolagnaro (Fort Dauphin) (MG02-01), 24°59.34′S, 46°55.43′E, February 21, 2002, southeast rainforest, 10 km west from Hotel Dauphin. Ten specimens (AMNH 5279, Annelida), fixed in 95% ethanol and seven specimens (AMNH 5280, Annelida) fixed in formalin, Parc National d'Andohahela (MG02-03) in "Manangotry forest", February 27, 2002. Twenty-eight specimens (AMNH 5281, Annelida) fixed in 95% ethanol and thirty-two specimens (AMNH 5282, Annelida) fixed in formalin, Talatakely (selectively logged, low montane forest), Parc National Ranomafana (MG02-05), March 5, 2002. Eighteen specimens (AMNH 5283, Annelida) fixed in 95% ethanol, on trail between Talatakely and Vatoharanana, Parc National Ranomafana (MG02-06), March 5, 2002. Twenty-eight specimens (AMNH 5284, Annelida) fixed in 95% ethanol, near campsite in Vatoharanana (undisturbed, low montane forest) ~4 km south of Talatakely, Parc National Ranomafana (MG02-07), March 5, 2002. All specimens collected by Mark Siddall, Elizabeth Borda, Evon Hekkala, Clara Imboule, and Raina Rakotondriany.

DESCRIPTION: Dorsum with beige background and a wide dorsal band, beige, with light brown mid-dorsal line. Paramedial dark mottling with "chain-link" pattern, dark brown to black on borders of each "link" of chain; beige color within "link". Venter with beige background and a pair of dark brown to black paramedial lines, each thick anteriorly, becoming thinner or less pigmented posteriorly (Fig. 11A).

Duognathous, monostichodont jaws. Cephalic somites with five pairs of eyespots, one on each of II, III, IV, V, and VI (Fig. 11B). Somites II, III, and IV uni-annulate, V and VI two-annulate (incomplete three-annulate, ventrally) and three-annulate, respectively, VII three-annulate. Midbody somites, VIII–XXIII, five-annulate. Nephripores lateral in furrow of b2/a2 of each somite (Fig. 11B), with first nephripore observed in VIII b2/a2. Posterior series, XXIV two-annulate, XXV–XXVII uni-annulate (Fig. 11C). Seven annuli between XXIII a2 and anus (Fig. 11C). Posterio-lateral respiratory auricles bilobed and formed along the lateral margins of XXIV a2 to XXVII (Fig. 11C). Caudal sucker, ventral, with 57 friction rays ventrally on sucker. Prehensile lobe absent (Fig. 11D).

General organization and regional morphology of male and female reproductive systems haemadipsoid (Figs. 11E – G). Male genital pore opens at furrow of XI b5/b6 and female genital pore opens at furrow of XII b5/b6 (or anteriorly in b6) (Fig. 11F). Gonopores separated by five complete annuli. Median male reproductive system in XI and XII (Fig. 11E). Male atrium found in XI, micromorphic, bulbous, and conical.

Ejaculatory ducts exit male atrium anteriorly at XI. Ejaculatory ducts run lateral to atrium, posteriorly directed, giving rise to large ejaculatory bulbs at XI/XII. Ejaculatory bulbs approximately twice the size of male atrium and observed within XI and XII. Small tightly coiled epididymes in XII and XIII, each recurving anteriad at XIII. Single pair of globular ovaries in XII, each with thin oviducts that converge at XII/XIII into a common oviduct (Fig. 11F). The long, thin, common oviduct enters anterior face of oviducal glandular sac at XIII. Oviducal glandular sac extends posteriorly to XVI.

REMARKS: The posterior annulation series of this species does not match that designated by Richardson (1975, 1978) for *Malagabdella fallax*. According to Richardson (1975, 1978), XXIV is three-annulate, with eight annuli between XXIII a2 and the anus. The posterior series for these specimens collected for this study is consistent, in part, with that described for *Malagabdella morsitans* (Richardson, 1978) (i.e., seven annuli between XXIIIa2 and the anus). However, the position of the gonopores appears to be consistent with Blanchard (1917) and Richardson (1975, 1978) for *Malagabdella fallax*. Richardson (1975) noted that the only differences between *Malagabdella fallax* and *Malagabdella morsitans* were limited to the latter having somite XXIII being four-annulate and with genital pores being found in the furrows of XI/XII and XII/XIII. Some have proposed that these two species be synonymized (Augener, 1931).

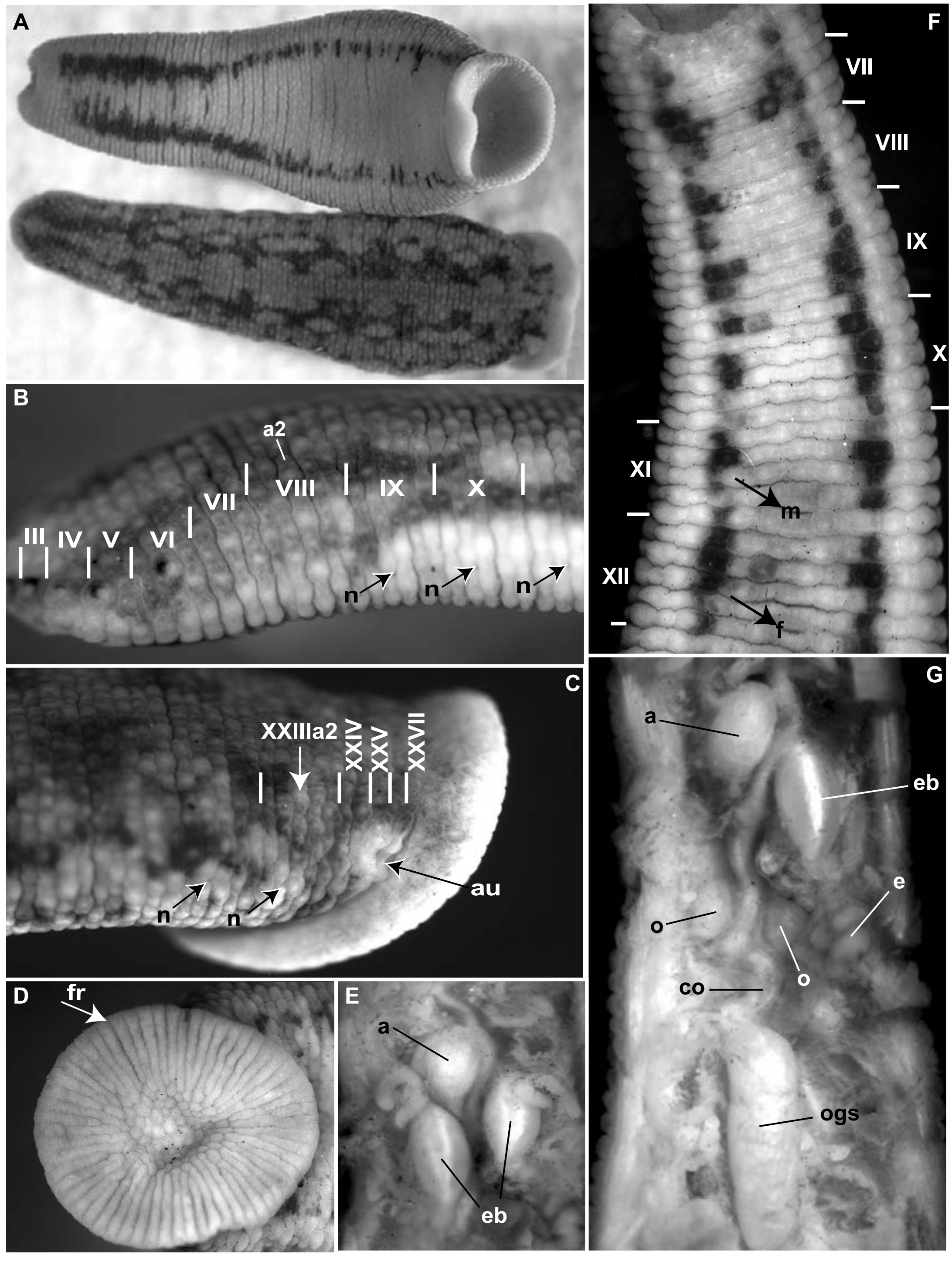
In describing *Haemadipsa fallax*, Blanchard (1917) examined two specimens from Le Muséum de Paris: no. 143 (MNHN 751; Lantz, 1882) and no. 146A (MNHN 751; Catat, 1890). With the opportunity to examine these specimens, I found that both were in very poor condition, and this prohibited the verification of the annulation pattern described by Blanchard (1917) and Richardson (1975, 1978). Of the two, Blanchard's specimen no. 146A was intact, but engorged with blood. The somites are visible on the specimen, but the annuli within somites are not, making it impossible to corroborate Richardson's (1978) claim regarding the posterior annulation series. The gonopores on the type specimen are also visible and are found within somites, confirming the gonopore position of *Malagabdella fallax* and for the specimens detailed here.

Richardson's (1978) redescription of *Malagabdella morsitans* was based on a specimen that was identified by Moore (U.S. Nat. Mus. 37970, Montagne d'Amor, Madagascar, Shickenburg Coll.—Acc. No. 259718), not based on Blanchard's specimen (MNHN 802; No. 198). Richardson (1978) reported that the gonopores of *Malagabdella morsitans* were located at XI/XII and XII/XIII and illustrated the posterior series with XXIII complete four-annulate and XXIV incomplete three-annulate. The intersomital position of gonopores and the posterior annulation series are evident from the types (MNHN 802; *N* = 7), rendering them distinct from *Malagabdella fallax* and the specimens detailed here.

FIGURE 11.

Malagabdella fallax. **A.** Dorsal and ventral views of color patterns showing “chain-link” patterns on the dorsum. **B.** View of the cephalic somites and anterior somital series, showing the position of the nephropores (n) at b2/a2 of each somite. **C.** Dorso-lateral view of the posterior somital annulation series, the nephropores (n) and respiratory auricle (au). **D.** Ventral view of caudal sucker showing 57 friction rays (fr). **E.** Median male reproductive system showing micromorphic atrium (a) and large ejaculatory bulbs (eb). **F.** Ventral view of the anterior somital annulation series and positions of the male (m) and female (f) gonopores, showing five complete annuli between gonopores. **G.** Male and female reproductive systems, showing the male atrium (a), the right ejaculatory bulb (eb) and the right epididymes (e); left male ejaculatory bulb moved to expose paired female ovaries (o), the common oviduct (co) and the haemadipsoid oviducal glandular sac (ogs).

Figure 11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Malagabdella morsitans (Blanchard, 1917)

Haemadipsa morsitans Blanchard, 1917: 664, Fig. 13.

Malagabdella morsitans – Richardson, 1975: 142; Richardson, 1978: 864, Fig. 4E; Sawyer, 1986: 761.

TYPE MATERIAL: *Syntypes*, Madagascar—Moramonga, east coast forests, 1893, Sikora? (MNHN 802; *N* = 7).

DESCRIPTION: Duognathous, monostichodont jaws. Cephalic somites with five pairs eyespots, one on each of II, III, IV, V, and VI. Somites II, III, and IV uni-annulate, V and VI two-annulate and three-annulate, respectively, VII three-annulate. Midbody somites VIII–XXII five-annulate. Nephropores lateral in furrow of b2/a2 in each somite. XXIII incomplete five-annulate (four annuli dorsally). XXIV incomplete three-annulate, XXV–XXVII uni-annulate. Seven (eight) annuli between XXIII a2 and anus. Male genital pore at furrow of XI/XII and the female genital pore at furrow of XII/XIII. Gonopores separated by five complete annuli.

REMARKS: Because I did not have access to fresh material of *Malagabdella morsitans* for dissection, the characteristics of the reproductive systems remain a mystery. Richardson's (1978) redescription of this species was solely based on the somital annulation patterns and based on the position of the gonopores. The specimen Richardson (1978) examined (USNM 37970) was dried out and with a strongly compressed body. He also mentioned that the male pore was not obvious, but that the female pore was found in XII/XIII (i.e., "two full annuli to a weakly defined end of the clitellum"; p. 864). The position of the gonopores has been confirmed based on the examination of the type specimen (see also *Malagabdella fallax*: REMARKS). Based on the latter and based on the annulation patterns renders *Malagabdella morsitans* distinct from other *Malagabdella* species, although the comparison of the reproductive systems to other species of *Malagabdella* is pending for a redescription of this species.

Malagabdella niarchosorum, new species

Figures 12

TYPE MATERIAL: *Holotype*, specimen fixed in 95% ethanol, Madagascar—Parc National Ranomafana in Talatakely (selectively logged, low montane forest; MG02-05), March 5, 2002. Collected by Mark Siddall, Elizabeth Borda, Evon Hekkala, Clara Imboule, and Raina Rakotondriany (AMNH 5285, Annelida). *Paratypes* (*N* = 2), one specimen fixed in formalin, dissected, same collection data as holotype (AMNH 5287, Annelida). One specimen fixed in 95% ethanol, Parc National Ranomafana, on trail between Talatakely and Vatoharanana (MG02-06), March 5, 2002. Collected by Mark Siddall and Elizabeth Borda, (AMNH 5286, Annelida).

DIAGNOSIS: Male pore in XI b5/b6; Female pore XII b5 (anterior); four complete annuli between gonopores; XXIV incomplete four-annulate; nine annuli between XXIII a2 and anus; oviducal glandular sac absent; muscular vaginal tube present. Prehensile lobe present.

DESCRIPTION: Dorsum with dark brown to black background field, wide beige medial chain-link band, each link with darker field at center, approximately five annuli per link. Medial chain becomes continuous light field bordered with dark brown to black paramedial lines in posterior series. Paramedial dorsal light brown to white diamond-shaped patches at approximately the same position of every other chain-link of medial band. Dorsolateral light spots on sensillae of a2. Dark spots found along lateral margins, against light background that continues ventrally. Venter, light background with dark brown to black mottling. Annular papillae, present (Figs. 12A, B).

Duognathous, monostichodont jaws. Cephalic somites with five pairs eyespots, one on each of II, III, IV, V, and VI. Somites II, III, and IV uni-annulate, V and VI two-annulate and three-annulate, respectively, VII three-annulate. Midbody somites, VIII–XXIII, five-annulate. Nephropores, lateral, in furrow of b2/a2 of each somite, first nephropore observed at IX b2/a2. Posterior somites, XXIV incomplete four-annulate (b6 converges with b5 along the anterio-lateral margin of the respiratory auricle) and XXV–XXVII uni-annulate (Fig. 12C). Nine annuli (dorsally) between XXIII a2 and anus. Posteriolateral respiratory auricles, bilobed, and formed along the lateral margins of XXIV b5 to XXII (Fig. 12C). Caudal sucker, ventral, with 57 friction rays ventral on sucker. Prehensile lobe, present (Fig. 12D).

General organization and regional morphology of male and female reproductive systems, not typical haemadipsoid (Figs. E–H). Male genital pore opens at the furrow of XI b5/b6 and female genital pore opens anteriorly in XII b5 (or a2/b5) (Fig. 12E). Gonopores separated by four complete annuli. Median reproductive system micromorphic (Fig. 12F). Median male reproductive system found entirely in XI (Fig. 12G). Male atrium, narrow, recurves anteriorly at XII. Short ejaculatory ducts exit the male atrium laterally in XI and give rise to small, globular ejaculatory bulbs in XI. Tightly coiled epididymes arise from ejaculatory bulbs, each twice the size of an ejaculatory bulb and recurved anteriad at XII. Median female reproductive system found entirely in XII (Fig. 12H). Single pair of globular ovaries, posteriorly directed in XII, with thin-walled oviducts that converge into common oviduct. Common oviduct is tightly coiled ventrally and inserts into a simple, muscular, vaginal tube. Vaginal tube recurves anteriad at approximately XIII, with tube widening anteriorly and terminating in bulb at the insertion point of the gonopore at XII b5. Haemadipsoid oviducal glandular sac absent.

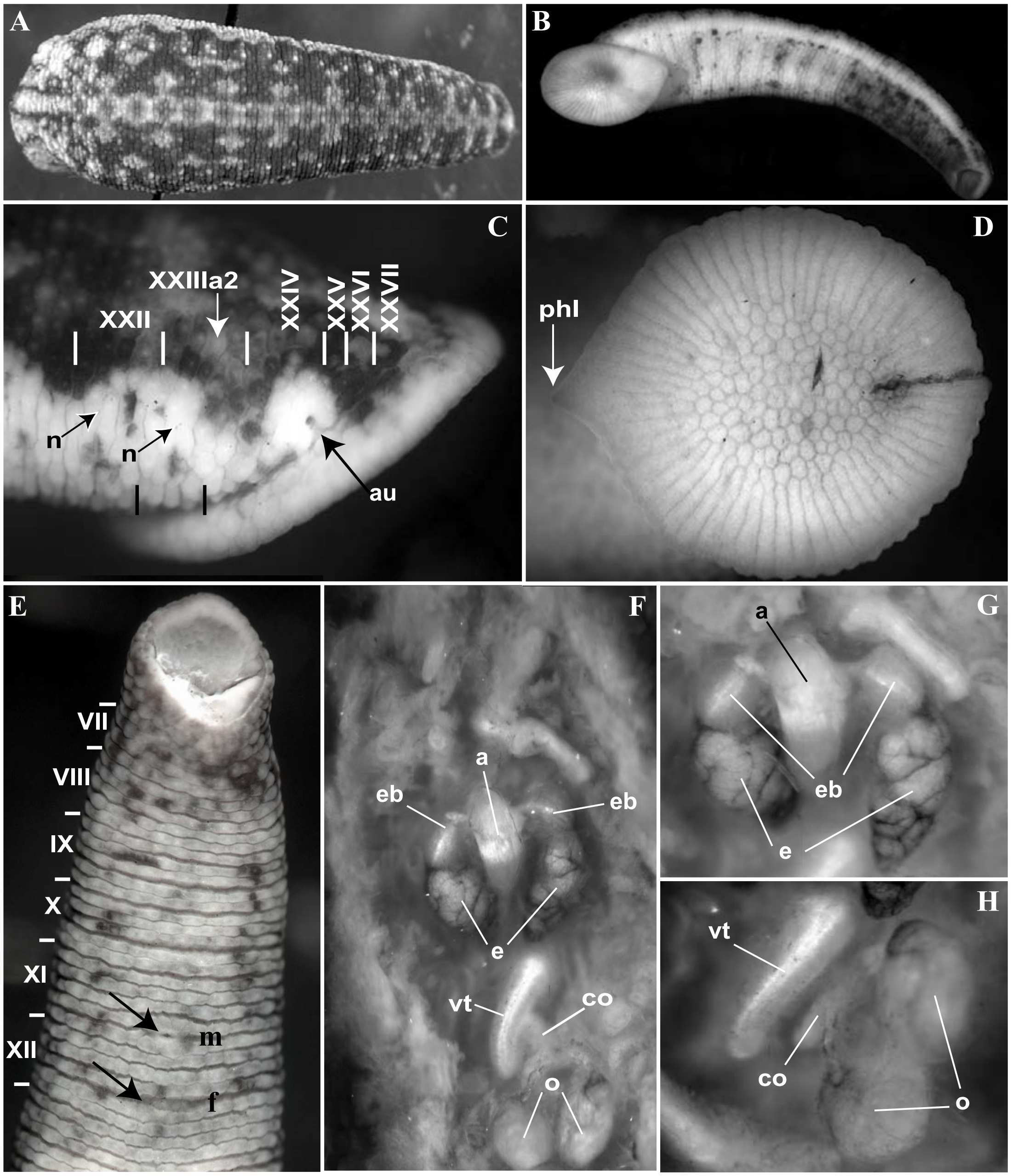
REMARKS: When Richardson (1975, 1978) included *Haemadipsa dussumieri* as a member of the genus *Malagabdella*, it was the only species that possessed four complete annuli between the gonopores. This was initially consistent with *Malagabdella niarchosorum*, n.sp. However, after the examination of the type specimen, it became obvious that *Malagabdella dussumieri* was distinct from *Malagabdella niarchosorum*, n.sp. (and from the *Malagabdella*). For example, the position of the female pore for the type specimen of *Haemadipsa dussumieri* is on an obviously ventrally subdivided annulus of XII b5, whereas in *Malagabdella niarchosorum* it is not subdivided. In addition, as detailed above, *Malagabdella dussumieri* possesses characters that deviate from domanibdellid leeches. Therefore, with the exclusion of *Malagabdella dussumieri* from the Malagabdellinae, *Malagabdella niarchosorum*, n.sp. is distinct from all other members of the genus based on external coloration and patterns, the characteristics of the male and female reproductive systems, the position of the gonopores, and the posterior annulation series, but inclusive in the Malagabdellinae based on somital annulation pattern and the possession of 57 friction rays ventrally on the caudal sucker.

ETYMOLOGY: This species is named after the Niarchos family in light of their generosity in funding this expedition. Without their support, the rediscovery of known species from Madagascar and the opportunity to describe another endemic species would.

FIGURE 12.

Malagabdella niarchosorum, n.sp. **A.** Dorsal view of color patterns showing mid-dorsal “chain-link” band and dark brown to black background field with light brown to white diamond-shaped patches (paratype). **B.** Ventral view showing light background field with dark brown to black mottling, especially in the anterior region. **C.** Dorso-lateral view of the posterior somital annulation series, position of the nephropores (n) and the respiratory auricle (au). **D.** Ventral view of the caudal sucker showing 57 friction rays and prehensile lobe (phl). **E.** Ventral view of the anterior somital annulation series and the positions of the male (m) and female (f) gonopores, with four complete annuli between gonopores. **F.** Male and female reproductive systems showing the male atrium (a), ejaculatory bulbs (eb), epididymes (e), female paired ovaries (o), common oviduct and vaginal tube (vt). **G.** Male reproductive system exhibiting a narrow male atrium (a), small bulbous ejaculatory bulbs (eb) and coiled epididymes (e). **H.** Female reproductive system showing paired globular ovaries, each with independent oviducts that converge into the common oviduct (co) that inserts into a simple vaginal tube (vt).

Figure 12.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Malagabdella vagans (Blanchard, 1917)

Figure 13

Haemadipsa vagans Blanchard, 1917: 665, Fig. 14.

Haemadipsa vagans – Soós, 1967: 426.

Malagabdella vagans – Richardson, 1975: 142; Richardson, 1978: 860–863, Fig. 4 A–D); Sawyer, 1986: 762.

TYPE MATERIAL: *Syntype*, Madagascar—east coast forests, 1882, Lantz (MNHN 751; no. 143; *N* =1). *Syntype*, Madagascar—Montagne d'Ambre, Diego-Suarez, 1890, Dr. Catat? (MNHN 802; *N* =3).

ADDITIONAL MATERIAL: Six specimens (AMNH 5288, Annelida) fixed in 95% ethanol and six specimens (AMNH 5289, Annelida) fixed in formalin (two specimens used for SEM), in forest adjacent to town of Andasibe, ~3 km from entrance of Analamazoatra (Perinet Special Reserve) (MG02-04), near the Antananarivo–Taomasina railroad, March 3, 2002.

DESCRIPTION: Brilliant green or orange when alive. Pale in color, almost neutral when fixed. Only markings found dorsally are paramedial wavy lines that become faded posteriorly. Dorsal and ventral papillae present (Fig. 13A).

Duognathous, monostichodont jaws armed with serrated edge of minute teeth (Fig. 13B). Cephalic somites with five pairs of eyespots, one on each of II, III, IV, V, and VI. Somites II, III, and IV uni-annulate, V and VI two-annulate and three-annulate, respectively, VII three-annulate. Midbody somites VIII–XXIII five-annulate.

Nephropores lateral in furrow of b2/a2 in each somite, with first nephropore observed in IX b2/a2. XXIV– XXVII uni-annulate (Fig. 13C). Six annuli between XXIII a2 and anus. Respiratory auricles present, bilobed, and formed along the lateral margins of XXIV a2 to XXVII. Caudal sucker, ventral, with 58 friction rays ventral on sucker. Small prehensile lobe present (Fig. 13D).

General organization and regional morphology of male and female reproductive systems not typical haemadipsoid (Figs. 13E – F). Male genital pore opens at the furrow of XI b5/b6 and the female genital pore opens at the furrow of XIII b1/b2 (Fig. E). Gonopores separated by seven complete annuli. Median reproductive system micromorphic and entirely in XI (Figs. 13F, G). Male atrium bulbous. Short ejaculatory ducts exit the atrium ventrally in anterior XI and run laterally, posteriorly directed, giving rise to small ovoid ejaculatory bulbs. Epididymes, small, approximately the size of the ejaculatory bulbs, tightly coiled, and recurve anteriad at XII. Median female reproductive system micromorphic (Fig. 13H). Single pair of globular ovaries in XII, with short thin walled oviducts that coil and converge into the common oviduct. The common oviduct is tightly coiled in the median and exits in XIII. Oviducal glandular sac and muscular vaginal tube absent.

REMARKS: Richardson (1975) noted a resemblance in the somital annulation pattern of *Haemadipsa vagans* to *Idiobdella seychellensis* and, therefore, originally included *Haemadipsa vagans* as a member of the genus *Idiobdella*. Richardson (1978) revised his previous classification of the leeches of Madagascar based on a specimen from the collection of the National Museum of Natural History that he identified as *Malagabdella vagans* (U.S. Nat. Mus. Cat. No. 55386; Acc. 242082; 2 km east of

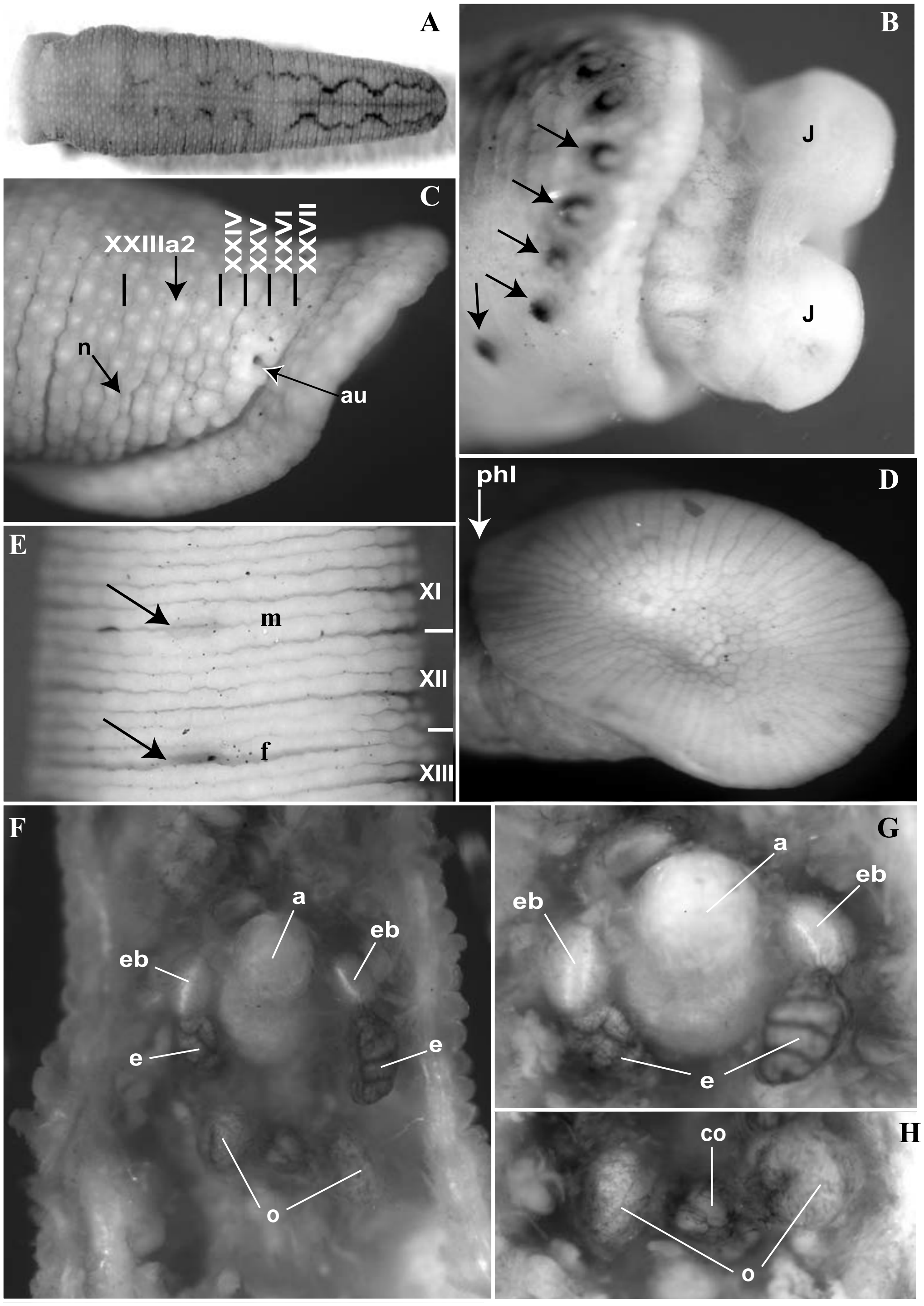
Perinet, Tamatave Province; off human skin). This resulted in the revision of the genus *Malagabdella*, the removal of the genus from Idiobdellidae, and the establishment of the Malagabdellinae (Richardson, 1978).

Interestingly, Richardson (1978) reported that the specimen of *Malagabdella vagans* was engorged with blood and with the “annulation obliterated”. Furthermore, it does not appear that he examined any type material for this species. Therefore, it is difficult to imagine that the annulation patterns he described and illustrated for this species came from this specimen (see also *Malagabdella fallax* REMARKS), but rather a conversion of the annulation patterns described by Blanchard (1917). In his revision, Richardson (1978) described the male reproductive system for Malagasy leeches based on this USNM specimen of *Malagabdella vagans*. This resulted in an overgeneralization for the characteristics of male reproductive systems for the *Malagabdella* species. It should be mentioned that the observation of the male system was facilitated by the USNM specimen having been “split through transversely at XII/XIII providing access to the anterior portion of the male paired system and the male median region” (Richardson, 1978: 863), yet he illustrates the majority of male system (i.e., atrium and ejaculatory bulbs and epididymes) found within XI.

FIGURE 13.

Malagabdella vagans. **A.** Dorsal view showing beige background field with paramedial wavy lines that fade posteriorly. **B.** Anterior view of cephalic somites, with partial view of the left five eyespots (arrows) and a set of producible duognathous jaws (j). **C.** Lateral view of the posterior somital annulation series and the respiratory auricle (au). **D.** Ventral view of the caudal sucker showing 58 friction rays and a small prehensile lobe (phl). **E.** Ventral view of the positions of the male (m) and female (f) gonopores, with seven complete annuli between gonopores. **F.** Medial male and female reproductive systems, showing the male atrium (a), ejaculatory bulbs (eb), epididymes (e), and paired ovaries (o). **G.** Male reproductive system showing a bulbous male atrium (a), small ejaculatory bulbs (eb), and small epididymes (e). **H.** Female reproductive systems showing a pair globular ovaries (o) with short independent oviducts that converge into a coiled common oviduct (co) before inserting into gonopore.

Figure 13.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DISCUSSION

Richardson (1969a) showed that the male and female reproductive systems had important diagnostic characters for hirudinid leeches, from the family level down to the species level. However, Richardson (1969b, 1975, 1978) repeatedly referred to the characteristics of the reproductive systems for land leeches as being “monotonous” and seemed to overvalue the somital annulation patterns, above all else.

Before this study, description of the male and female reproductive systems for Malagasy leeches was incomplete, other than what was described for the male anatomy for *Malagabdella vagans* (Richardson, 1978). Resulting from this study was the opportunity to rediscover some of the known five-annulate species from Madagascar and to include a new species of terrestrial leech. This study has shed some light toward the classification of Malagasy leeches, but more specifically, it has shown that the “monotony” of male and female reproductive systems may have been overestimated by Richardson (1978). Of these species, only *Malagabdella fallax* appears to possess the typical haemadipsoid reproductive system—micromorphic male median region and the female region with a large oviducal glandular sac—whereas the remaining species observed here deviate from this form. Therefore, a better characterization of the reproductive morphologies across all land leeches is still warranted, as there may be a greater diversity and taxonomic value in these systems than previously thought (Richardson 1975, 1978). In addition, the position of the gonopores and the posterior annulation series appear to be good diagnostic characters for species, as they were distinct among the species examined here.

The endemism of continental landmasses associated with the breakup of Gondwanaland has been well documented across various leech taxa (Moore, 1927; Soós, 1967; Richardson, 1975, 1978; Sawyer, 1986; Westergren and Siddall, 2004), an example being Madagascar, the world's fourth largest island, having been isolated from Africa and India for approximately 160 million years and 84–88 million years, respectively (Storey et al., 1995; Briggs, 2003). This may suggest that the hirudifauna of Madagascar, which are found nowhere else, may have originated ~84 mya or earlier.

There are five described species of terrestrial blood-feeding leeches from Madagascar and a single record of a freshwater leech (i.e., *Linta be* Westergren and Siddall, 2004). Our collection efforts were limited to three localities along the east coast forests of Madagascar, possibly resulting in an underestimation of the species diversity of leeches, although successful in the discovery of two new species (i.e., *L. be* and *Malagabdella niarchosorum*). The lack of knowledge of the Malagasy hirudifauna is attributed to our limited collection and that of previous workers. Additionally, because terrestrial leeches are seasonal (Sawyer, 1986), former collections may have been made during times of the year when leeches were least abundant (i.e., the dry season). Finally, such practices as slash-and-burn agriculture and deforestation of the eastern rainforests of Madagascar threaten the habitats of many of its endemic species. Therefore, expeditions such as this one are key to better assess species diversity of invertebrates, whose loss may go unnoticed in an ever-changing landscape.

PROVISIONAL KEY TO THE TERRESTRIAL BLOODFEEDING LEECHES OF THE MALAGABDELLINAE (revised)

1. Cephalic somites, II–IV uni-annulate, V two-annulate, VI three-annulate, XXV–XXVII uni-annulate 2

2. VII three-annulate; somites of middle series, VIII–XXIII complete five-annulate; XXV–XXVII uni-annulate . . . . .g. *Malagabdella*. 3

— VII four-annulate; somites of middle series, VIII–XXIII complete four-annulate; XXIV two-annulate . . . . g. *Tristabdella* Richardson, 1975 9

3. Male pore XI b5/b6 4

— Male pore XI/XII 8

4. Prehensile lobe absent 5

— Prehensile lobe present 6

5. Female pore XII b5/b6; five complete annuli between gonopores; XXIV two-annulate; seven annuli between XXIII a2 and anus; XXIV two-annulate; 57 friction rays; oviducal glandular sac present to XV . . . . *Malagabdella fallax*

6. Female pore XII b5; four complete annuli between gonopores; XXIV incomplete four-annulate; nine annuli between XXIII a2 and anus; 57 friction rays; oviducal glandular sac absent; muscular vaginal tube present; . . . . *Malagabdella niarchosurum*

7. Female pore XIII b1/b2; seven complete annuli between gonopores; XXIV uni-annulate; six dorsal annuli between XXIII a2 and anus; 58 friction rays; simple

coiled common oviduct inserts into gonopore; oviducal glandular sac absent; muscular tube absent . . . . *Malagabdella vagans*

8. Female pore XII/XIII; five complete annuli between gonopores; XXIII incomplete five-annulate (four annuli dorsally); XXIV incomplete three-annulate; seven (eight) annuli between XXIII a2 and anus . . . . *Malagabdella morsitans*

9. Seven annuli between XXIII a2 and anus; male pore XI b5, female pore XII b5/b6; four complete annuli between gonopores . . . . *Tristabdella grandidieri* (Blanchard, 1917)

CHAPTER 5

On the classification, evolution and biogeography of terrestrial haemadipsoid leeches (Hirudinida: Arhynchobdellida: Hirudiniformes)

(Adapted from: Borda, E., Oceguera-Figueroa, Alejandro and Siddall, M. E. In Press. On the classification, evolution and biogeography of terrestrial haemadipsoid leeches (Hirudinida: Arhynchobdellida: Hirudiniformes). *Molecular Phylogenetics and Evolution*)

INTRODUCTION

Bloodfeeding terrestrial leeches have fascinated (and have been loathed by) "travelers, sportsman and missionaries, whose quest have carried them into the [leech infested] humid valleys and jungles" (Haeckel, 1883: 188) of the IndoPacific. Although these leeches are adapted to a terrestrial way of life, they are restricted to damp forests that are subject to seasonal rainfall (Sawyer, 1986). As such, the majority of bloodfeeding terrestrial leech species is distributed throughout tropical and subtropical IndoPacific landmasses. There are, however, 11 unusual and lesser-known terrestrial species found in isolated pockets of subtropical or temperate zones in South America, Meso America and Europe (Sawyer, 1986).

The classification of bloodfeeding terrestrial leeches has a tortuous history. The placement of the genera *Xerobdella*, *Diestecostoma*, *Mesobdella* and *Nesophilaemon* within Haemadipsidae (and among Hirudiniformes) has been especially problematic. Blanchard (1896) established Haemadipsinae (=Haemadipsidae *sensu* Soós, 1967) to distinguish bloodfeeding terrestrial leeches from their aquatic sanguivorous (i.e. bloodfeeding) and carnivorous counterparts in Hirudininae. Blanchard's (1896, 1917) classification included those species that:

1) were terrestrial and bloodfeeding in habit;
2) had a unique "haemadipsine" ocular arch (Fig. 14A)- eyespot pairs 1 through 4 typically arranged on uni-annulate somites II – V, with eyespots 4 and 5 (tri-annulate) separated by two complete annuli. Variations to this arrangement includes the presence of a partial or complete annulus between eyespots 3 and

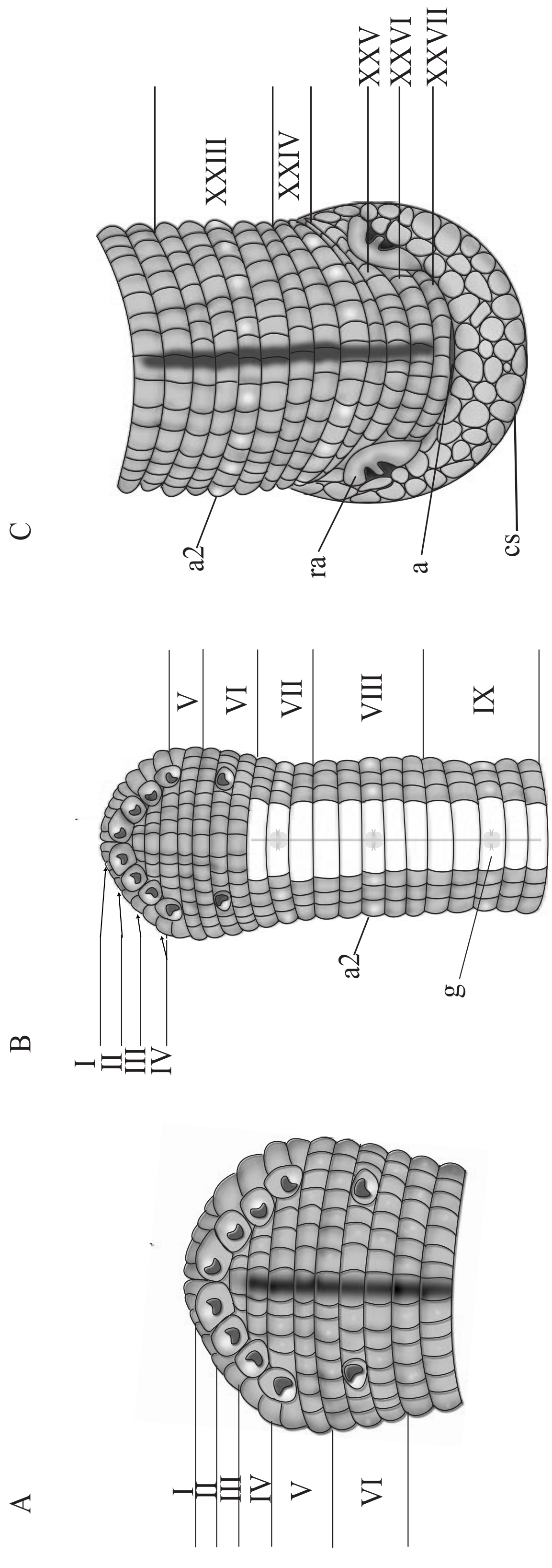
4 (bi-annulate), with two annuli between 4 and 5 (e.g. *Haemadipsa sylvestris*) or the presence of three annuli between eyespots 4 and 5 (e.g. *Planobdella*)

3) possessed a unique annulation pattern:
   a. Anterior series (Fig. 14B) – VII and VIII, typically three-annulate and four-annulate, respectively. VIII variable by genus
   b. Midbody somites (Fig. 14B) – the distance (in annuli) between one neural ganglion and the next, with the neural annulus (a2) typically with sensory papillae. Alternatively, the number of annuli between nephropores (always on annulus before a2) also corresponds to midbody somital patterns. Somites variable by genus:
      i. Three-annulate – *Mesobdella*: a1, a2, a3
      ii. Four annulate – *Philaemon*: a1, a2, b5, b6
      iii. Five annulate – *Haemadipsa*: b1, b2, a2, b5, b6
      iv. Six annulate - *Phytobdella*: c1, c2, b2, a2, b5, b6
      v. Seven-annulate – c1, c2, b2, a2, b5, c11, c12
   c. Posterior series (Fig. 14C) –the number of annuli between XXIII a2 and the anus, with variations in number of somites with XXIV, XXV – XXVII typically uni-annulate.
4) had lateral nephridia, with the last pair concealed beneath respiratory auricles (Fig. 14C)
5) had a distribution throughout the Indian Ocean and the Pacific Ocean – there were some geographic exceptions.

FIGURE 14.

Diagnostic characters for haemadipsid leeches. **A.** "Haemadipsine" ocular arch of *Haemadipsa zeylanica*, showing fourth and fifth eyespot pairs separated by two annuli (=segment); **B.** Anterior annulation series of *Haemadipsa zeylanica*; **C.** Posterior annulation series of *Haemadipsa zeylanica.* Roman numbers represent somites; g=neural ganglion, a2=neural annulus; ra=respiratory auricle; a=anus; cs=caudal sucker

Figure 14.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Blanchard (1917) tentatively included the Chilean *Mesobdella gemmata* (E. Blanchard, 1849) and the European *Xerobdella lecomtei* Frauenfeld, 1868, yet recognized that they deviated from other members of the group due to their isolated distributions, the eyespot arrangement, the ventral position (rather than lateral) of the nephridia and the lack of the respiratory auricles. Not surprisingly, others proposed these species were only distantly related to the true haemadipsids from the IndoPacific because they were geographically displaced and/or deviated from the morphological characters normally used to define members of Haemadipsidae (Moore, 1946; Richardson, 1969, 1971; Ringuelet, 1972). In additional to the external morphologies several workers identified the systematic utility of the following morphological characters to distinguish haemadipsoid leeches (Harding, 1913; Moore,1946; Richardson,1969l 1971; 1975):

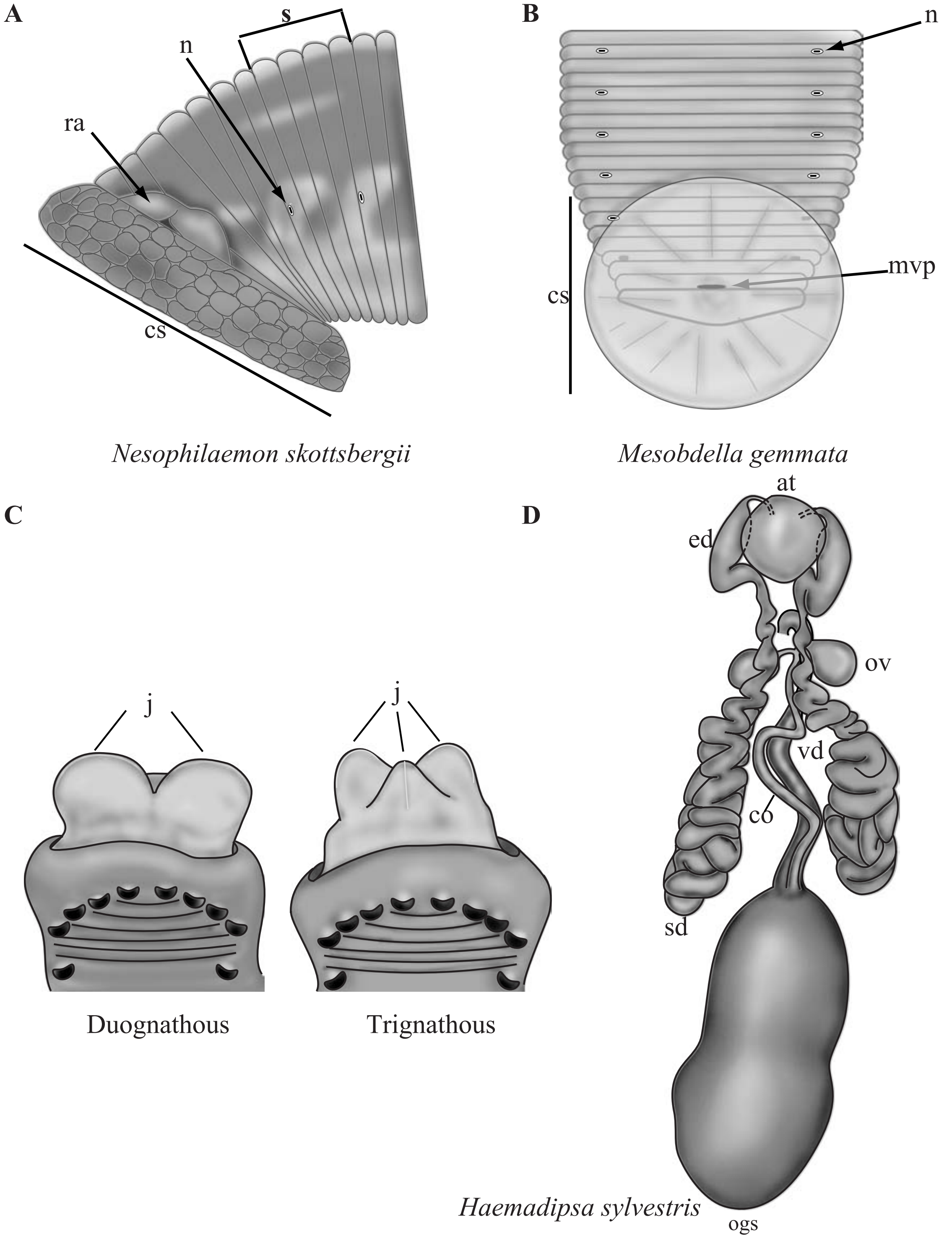
1) Nephropore position (Figs. 15A, B) – lateral (*e.g. Nesophilaemon*) or ventral (*Mesobdella*)
2) Jaw morphology (Fig. 15 C) – trignathous, with a set of tripartite muscular jaws or duognathous, paired jaws; absence of the dorsomedial jaw.
3) Reproductive morphology – Figure 15D is the typical haemadipsoid form. Variation from this form found among the leeches from Madagascar *(Malagabdella*), Papua New Guinea (*Domanibdella* and *Planobdella*), the New World and Europe (*Mesobdella*, *Diestecostoma* and *Xerobdella*) and Juan Fernandez Islands (*Nesophilaemon*) (see DISCUSSION).

In spite of being duognathous (two-jawed) and sharing characters with other duognathous IndoPacific haemadipsid leeches (e.g. lateral nephridia, respiratory auricles; Fig. 14B), Richardson (1971) proposed that *Nesophilaemon skottsbergii* shared a greater

FIGURE 15.

Additional diagnostic characters for haemadipsoid leeches. **A.** Lateral view of *Nesophilaemon skottsbergii* showing respiratory auricles and lateral nephridia; **B.** Ventral view of *Mesobdella gemmata* showing ventral nephridia and the medioventral pore (through the caudal sucker); **C.** Jaw morphology, duognathous (two jawed) and trignathous (three jaws); **D.** Typical haemadipsoid form of the male and female reproductive system. a=annulus (annuli; pl.); cs=caudal sucker; gp=gonopore; mvp= medioventral pore; n=nephropore; ra=respiratory auricles; at=male atrium; ed=ejaculatory ducts; sd=sperm ducts; ov=ovaries; vd=vaginal duct; co=common ovaries; ogs=oviducal glandular sac

Figure 15.



Nesophilaemon skottsbergii

Mesobdella gemmata

Duognathous

Trignathous

Haemadipsa sylvestris

affinity to *Mesobdella gemmata*. This was in light of its proximity to the New World, approximately 700 km off the east coast of Chile in the Juan Fernandez Archipelago. Unlike *Nesophilaemon skottsbergii*, the remaining non-IndoPacific genera were trignathous (three-jawed) and lack respiratory auricles normally found in the IndoPacific leeches. Ultimately, debates and disagreement regarding the affinities of non-IndoPacific leeches to Haemadipsidae led to each taxon being placed into its own respective subfamily or family: Xerobdellinae *sensu* Moore, 1946, Diestecostomatidae Ringuelet, 1953, Mesobdellidae Ringuelet, 1972 and Nesophilaemonidae Ringuelet, 1982. In contrast, more recent treatments of bloodfeeding terrestrial leeches placed them all in a single family, Haemadipsidae, subdivided into the duognathous series and trignathous series (Sawyer, 1986; see also Table 4)

Phylogenetic Relationships of Bloodfeeding Terrestrial Leeches

Trontelj et al. (1999) showed that *Xerobdella lecomtei* was distantly related to the haemadipsid leeches based on nuclear 18S rDNA. With an expanded taxonomic sampling of Arhynchobdellida, Borda and Siddall (2004a) corroborated previous work (i.e. Trontelj et al., 1999) by showing Haemadipsidae (*sensu* Sawyer, 1986) not to be monophyletic. Specifically, *Xerobdella lecomtei* and *Mesobdella gemmata* were found not to be sister to the monophyletic IndoPacific leeches (Trontelj et al., 1999; Borda and Siddall, 2004a). Borda and Siddall (2004a) proposed returning the subfamily Xerobdellinae Moore 1946 to family status (*sensu* Harant and Grassé, 1959) for the non-IndoPacific clade. However, without the inclusion of other non-IndoPacific genera (i.e. *Diestecostoma*, *Nesophilaemon*) a formal revision of the group was not possible.

TABLE 4.
Classification schemes for bloodfeeding terrestrial leeches: (A) *sensu* Sawyer (1986) and (B) adapted from Ringuelet (1953[1]; 1982[2]), Borda and Siddall (2004a[3]) and Richardson (1975[4]; 1978[5]). Listed genera are those included in this study.

A.

- ANNELIDA
- Oligochaeta
  - Hirudinida
    - Arhynchobdellida
      - Hiruddiniformes
        - **Haemadipsidae**
          - **Trignathous series** [5 Genera]
            - *Haemadipsa*
            - *Diestecostoma*
            - *Mesobdella*
            - *Xerobdella*
          - **Duognathous series** [12 Genera]
            - Group A
              - *Idiobdella*
              - *Nesophilaemon*
            - Group B
              - *Chtonobdella*
              - *Malagabdella*

B.

- ANNELIDA
- Oligochaeta
  - Hirudinida
    - Arhynchobdellida
      - Hirudiniformes
          - **Diestecostomatidae[1]** [1 Genus]
            - *Diestecostoma*
          - **Xerobdellidae[3]** [2 Genera]
            - *Diestecostoma*?
            - *Mesobdella*
            - *Xerobdella*
        - **Haemadipsoidea**
          - **Haemadipsidae[4]** [2 Genera]
            - *Haemadipsa*
          - **Domanibdellidae[4]** [21 Genera]
            - *Chtonobdella*
            - *Malagabdella*
          - **Idiobdellidae[5]** [2 Genera]
            - *Idiobdella*
          - **Nesophilaemonidae[2]** [1 Genus]
            - *Nesophilaemon*

In this study, we have expanded the taxonomic representation of Haemadipsidae (*sensu* Sawyer, 1986) and also include the type species of each non-IndoPacific putative family (*sensu* Moore, 1946; Ringuelet, 1953, 1972, 1982) in order to better understand their phylogenetic placement among hirudiniform leeches relative to traditional classification schemes (Moore, 1946; Sawyer, 1986; Caballero, 1956, 1959; Soós, 1966, 1967; Richardson, 1971, 1975, 1978; Ringuelet, 1972, 1982). In doing so, we reexamine the morphological characters used to define Haemadipsidae (Blanchard, 1896, 1917; Richardson, 1975), as well as examine the evolution of feeding preferences and the biogeographic patterns of haemadipsoid terrestrial leeches in a phylogenetic framework.

MATERIALS AND METHODS

Taxa

Sampling localities and GenBank accession numbers are listed in Table 5. Species new to this study include: *Chtonobdella whitmani* (Lambert, 1899) from Australia, *Diestecostoma mexicana* (Baird, 1869), *Diestecostoma magna* Moore 1945 and *Diestecostoma trujillensis* Ringuelet 1976, all from Mexico, *Haemadipsa interrupta* Moore 1935 from Malaysia, *Idiobdella seychellensis* Harding 1913 from the Seychelles Archipelago, *Malagabdella fallax* (Blanchard 1917) from Madagascar and *Nesophilaemon skottsbergii* (Johansson, 1924) from the Juan Fernandez Archipelago. Representatives from the major families of Hirudiniformes were included as outgroup taxa based on previous phylogenetic work (Borda and Siddall, 2004a). Terrestrial leeches were collected from exposed skin or from under rotting logs. Collection strategies involved walking along forest trails and searching through damp undergrowth,

TABLE 5.
Collection localities and GenBank accession numbers for taxa used for the phylogenetic analyses of bloodfeeding terrestrial leeches. *Type species of family

		GenBank Accession Number		
TAXON	**LOCALITY**	**18S**	**28S**	**COI**
INGROUP				
Chtonobdella bilineata	Australia	AF116006[1]	AY425361[3]	AF003267[4]
Chtonobdella whitmani	Australia	–	–	–
Diestecostoma magna	Mexico	–	–	–
*Diestecostoma mexicana**	Mexico	–	–	–
Diestecostoma trujillensis	Mexico	–	–	–
Haemadipsa interrupta	Thailand	–	–	–
Haemadipsa sumatrana	Borneo	AY425464[3]	AY425372[3]	AY425446[3]
Haemadipsa sylvestris	Vietnam	AF116005[1]	AY425373[3]	AF003266[4]
*Idiobdella seychellensis**	Seychelles Islands	–	–	–
*Malagabdella fallax**	Madagascar	–	–	–
*Mesobdella gemmata**	Chile	AY425472[3]	–	AY425454[3]
*Nesophilaemon skottsbergii**	Juan Fernandez Islands	–	–	–
*Xerobdella lecomtei**	Slovenia	AF099947[2]	–	–

TABLE 5. Continued

OUTGROUP				
Aliolimnatis michaelseni	Congo	AF116010[1]	AY425388[3]	AF116029[1]
Americobdella valdiviana	Chile	AY425461[3]	–	AY425443[3]
Cylicobdella coccinea	Bolivia	AY425462[3]	AY425362[3]	AY425444[3]
Haemopis grandis	Manitoba, Canada	AY425465	AY425377	AY425447
Haemopis kingi	Manitoba, Canada	AY425466	AY425378	AY4254848
Haemopis terrestris	Ohio, USA	AY786465[5]	–	–
Hirudo medicinalis	France	AY786464[5]	–	AY786458[5]
Limnatis nilotica	Israel	AY425470	AY425389	AY425452
Macrobdella decora	Michigan, USA	AF116007[1]	–	–
Semiscolex similis	Bolivia	AY425475[3]	AY425402[3]	AY425457[3]

especially after rain, to attract leeches. Other methods included walking barefoot (i.e. third author) through the forest to increase skin exposure for leeches found in the undergrowth. After collection, leeches were relaxed using an ethanol gradient, starting with an ~10% ethanol solution and gradually adding 95% ethanol until the leeches were relaxed (and dead). Leeches collected from the field were stored in 95 – 100% ethanol at –20° C or at –4 ° C until used for DNA extraction.

Morphology

Examination and dissection of Diestecostoma mexicana, Idiobdella seychellensis, *Malagabdella fallax*, *Mesobdella gemmata* (from Siddall and Borda, 2004), *Nesophilaemon skottsbergii* and *Xerobdella lecomtei* were done with a Nikon SMZ-U stereomicroscope and photo-documentation of leeches was accomplished using a SPOT-RT (Diagnostic Instruments, Inc.) digital camera. Illustrations were facilitated in Adobe® Illustrator® 10 and Adobe® Photoshop® 7. Illustration for *Haemadipsa sylvestris* was adapted from Keegan et al. (1968).

Molecular Techniques

Tissue from the caudal sucker was used for DNA extraction in order to minimize the possibility of contamination from host/prey DNA found in the gastric and intestinal regions. The DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA) was used for tissue lysis and DNA purification. PCR amplification of nuclear 18S rDNA (18S) and 28S rDNA (28S), and mitochondrial cytochrome *c* oxidase subunit I (COI) gene fragments were accomplished using the primers listed in Table 6. Amplification reaction mixtures used Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) with: 23 µl

TABLE 6.
Primers used for gene amplification and sequencing.

GENE	PRIMER NAME	PRIMER SEQUENCE 5' → 3'	REFERENCE
NUCLEAR			
18S rDNA			
1	A	AACCTGGTTGATCCTGCCAGT	Apakupakul et. al., 1999
	L	CCAACTACGAGCTTTT	Apakupakul et. al., 1999
2	C	CGGTAATTCCAGCTC	Apakupakul et. al., 1999
	Y	CAGACAAATCGCTCC	Apakupakul et. al., 1999
3	O	AAGGGCACCACCAG	Apakupakul et. al., 1999
	B	TGATCCTTCCGCAGGTTCACCT	Apakupakul et. al., 1999
28S rDNA			
1	28srD1a	CCCSCGTAAYTTAAGCATAT	Prendini et al., 2005
	28sB	TCGGAAGGAACCAGCTAC	Whiting, 2002
2	28sA	GACCCGTCTTGAAGCACG	Whiting, 2002
	28SBout	CCCACAGCGCCAGTTCTGCTTACC	Prendini et al., 2005
3	28srd4.5a	AAGTTTCCCTCAGGATAGCTG	Whiting, 2002
	28srD7b1	GACTTCCCTTACCTACAT	Whiting, 2002
MITOCHONDRIAL			
COI			
1	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994
2	COI-A	CCTGTTCTTGCTGGTGCTATTACIAT	Bely and Wray, 2004
	COI-B	TAGTCAGAATATCGCCGAGGTATICC	Bely and Wray, 2004

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: heated to 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 a final extension at 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. For alternative amplification and sequencing protocols see Oceguera-Figueroa et al. (2005). Sequences were edited and reconciled using CodonCode Aligner (CodonCode Corporation). Alignment of the two nuclear gene fragments were accomplished using Clustal X software under a range of alignment parameters, gap cost: gap extension = 6:3; 10:5; 15:6.66 (default). Alignments for *COI* were done by eye, as there were no ambiguous insertions or deletions. Individual rDNA datasets were selected for inclusion in the combined analyses based on those alignment parameters that produced optimal trees.

Phylogenetic analyses

Maximum Parsimony (MP) analyses of the combined 18S, 28S and COI data, as well as for each gene alone were performed using PAUP* v. 4.06b10 (Swofford, 2000). Heuristic searches used 100 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All characters were equally weighted and non-additive. Gaps were treated as missing data. TreeRot.v2c (Sorenson, 1999) was used to calculate Bremer support indices (b; Bremer, 1988). The retention (RI) and consistency (CI) indices were calculated in PAUP*. Parsimony jackknife (jac) values for combined analyses were obtained with 1000 heuristic pseudoreplicates, using random taxon addition and tree-bisection-reconnection branch swapping with 37% deletion (Farris, 1999).

Maximum Likelihood (ML) analyses were performed for each gene, and across alignment parameters for nuclear genes, using PHYML v. 2.4.4.1 (Guindon and Gascuel, 2003). Modeltest 3.06 (Posada and Crandall, 1998) was used to select the appropriate model of evolution for each gene under the Akaike Information Criterion (AIC; Posada and Buckley, 2004). The general time reversible (GTR) model of evolution, with proportion of invariable sites (I) and gamma distribution (Γ) was selected for each gene. ML analyses were performed under GTR+I+Γ, with I and Γ fixed as specified by the AIC, and base frequencies were estimated from the data.

Combined data analyses using Bayesian Inference (BI) were performed in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). The data were partitioned in two ways: 1) partitioned by gene, for 18S and 28S, and by codon position for *COI* (5p); and 2) partitioned by gene, 18S, 28S and *COI* (3p). BI analyses assumed a decoupled GTR+I+Γ model for each data partition, based on the AIC (via ModelTest). The default prior distribution of parameters were used for Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analyses , with one cold chain and three heated chains for 10 million generations and sampled every 1000th generation. The BI analyses burned-in almost instantly (<25,000 generations). Split frequencies of the standard deviation of simultaneous BI analyses were well below 0.01 and the R-statistic (Gelman and Rubin, 1992) for each parameter partition approached 1, ensuring convergence of chains, log likelihoods and model parameters. The burn-in was set to discard the first million generations, leaving 18002 trees sampled total (from the two simultaneous runs) for estimation of posterior probabilities (pp). Bayes factors were used to assess the best partitioning scheme for the GTR+I+Γ model for combined data BI analyses, *a posteriori* (Nylander et al., 2004).

RESULTS

Morphology of Terrestrial Leeches

With respect to the general classification of land leeches, the external and reproductive morphologies of select taxa were evaluated (Figs. 14 and 15). Species belonging to the genera *Xerobdella*, *Diestecostoma* and *Mesobdella* all possessed ventral paired nephridia, but lacked the 17th nephridial pair. The latter were "replaced" by a medioventral common pore at the base of the sucker (Fig. 15B). Annulation patterns of mid-body somites across these genera were not consistent: *Xerobdella lecomtei* was five-annulate, *Mesobdella gemmata* was three-annulate and *Diestecostoma* species were 10-annulate (*D. mexicana*, *D. trujillensis*) or 12-annulate (*D. magna*). In contrast to *Mesobdella gemmata*, which had only one annulus between the 4th and 5th pair of eyespots, *Xerobdella lecomtei* and *Diestecostoma* species each possessed a "haemadipsine" ocular arch (*sensu* Blanchard, 1917), with the last two pairs of eyespots being separated by two annuli (e.g. Fig. 14A)

All non-IndoPacific genera shared the position of the insertion points (or ventral gonopores) of the male and female reproductive aparati in somites XI (male) and XIII (female) regardless of number of annuli separating the gonopores. *Xerobdella lecomtei* also possessed an accessory pore in XII. Both *Mesobdella gemmata* and *Xerobdella lecomtei* had micromorphic male and female apparati (Figs. 16A, B), with the male reproductive system (excluding testisacs) found almost entirely in XI, with partial extension into X and the female systems in XII and XIII. The male system of *Mesobdella gemmata* included a pear-shaped penis sheath, with thin sperm duct that inserted into sac-like epididymes, whereas *Xerobdella lecomtei* possessed a spherical atrium, with defined ejaculatory bulbs that insert into small epididymes. The female reproductive system of *Mesobdella gemmata* consisted of a vaginal duct that inserted into a simple vaginal sac and a pair of globular ovisacs. The reproductive system of *Diestecostoma mexicana* was robust and elongated (Fig. 16C). The male system had a slender penis sheath that elongated posteriorly into XIV. At the base of the penis was a pair of ejaculatory bulbs that inserted into coiled epididymes that terminated with thin sperm ducts that traveled anteriorly beneath the penis sheath before turning posteriorly at the insertion point in XI. The vaginal duct in *Diestecostoma mexicana* was also long, extending from XIII to XVI, and slightly widened into and elongated sac. At the base was a pair of fused ovisacs.

In contrast to the other non-IndoPacific genera, the four-annulate *Nesophilaemon skottsbergii* possessed a lateral position of the paired nephridia, respiratory auricles (Fig. 15B) and had a "haemadipsine" ocular arch. The male system of *Nesophilaemon skottsbergii* (Fig. 16D) consisted of a spherical atrium, with a pair of ejaculatory bulbs that inserted into thick sperm ducts extending posteriorly into XIX, before curving anteriorly. The female system includes a vaginal duct with an insertion point at XII. The vaginal duct extended posteriorly, then coiled beneath the oviducal glandular sac that extended to XV, inserting into the anterior portion of the sac. Paired globular ovaries inserted independently into the glandular sac, via oviducts that joined into a common oviduct that coiled along with the vaginal duct beneath the sac. The reproductive systems of the five-annulate *Haemadipsa sylvestris* (Fig. 16 E) and *Idiobdella seychellensis* (Fig. 16F) were similar in basic form in having the atrium present in XI, with sperm ducts that extended posteriorly and coiled at XV and XIII, respectively. The female systems include an elongated vaginal duct that either inserted directly (*Haemadipsa sylvestris*), or coiled beneath before inserting (*Idiobdella seychellensis*) into the oviducal glandular sac. The female insertion point of the vaginal duct was found in XII (*Haemadipsa sylvestris*) and XIII (*Idiobdella seychellensis*). Like *Nesophilaemon skottsbergii*, both haemadipsid species also possessed globular ovisacs that inserted independently into a common oviduct before inserting into the oviducal glandular sac. Both the vaginal duct and the common oviduct of *Idiobdella seychellensis* coiled together beneath the sac. *Idiobdella seychellensis* lacked obvious respiratory auricles, unlike *Nesophilaemon skottsbergii* and exhibited the ventral position of the nephridia and the haemadipsine ocular arch.

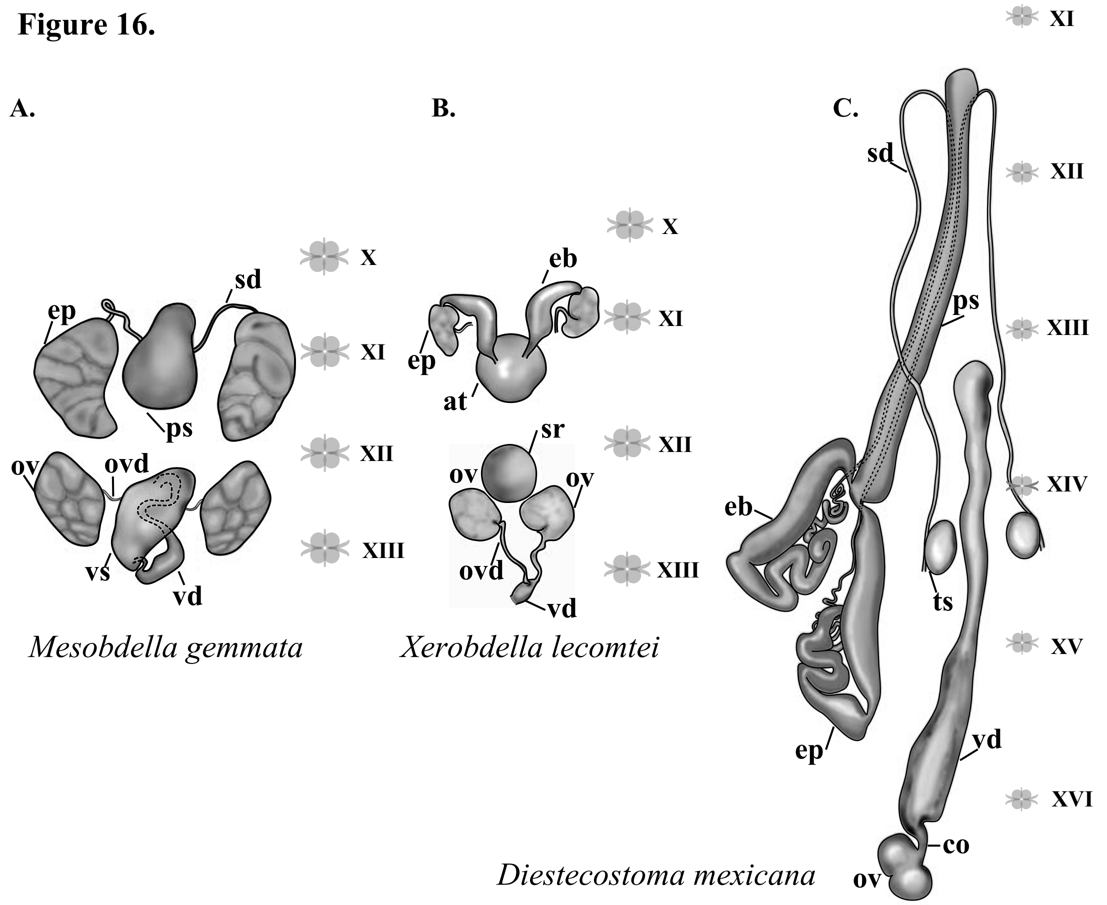
Molecular Data and Alignments

With respect to the alignment schemes for nuclear genes, the general trend was that as gap opening cost: gap extension cost parameters increased, tree length (MP) and log likelihood scores (ML) worsened. With the exception of 28S (15:6.66), the results of

FIGURE 16.

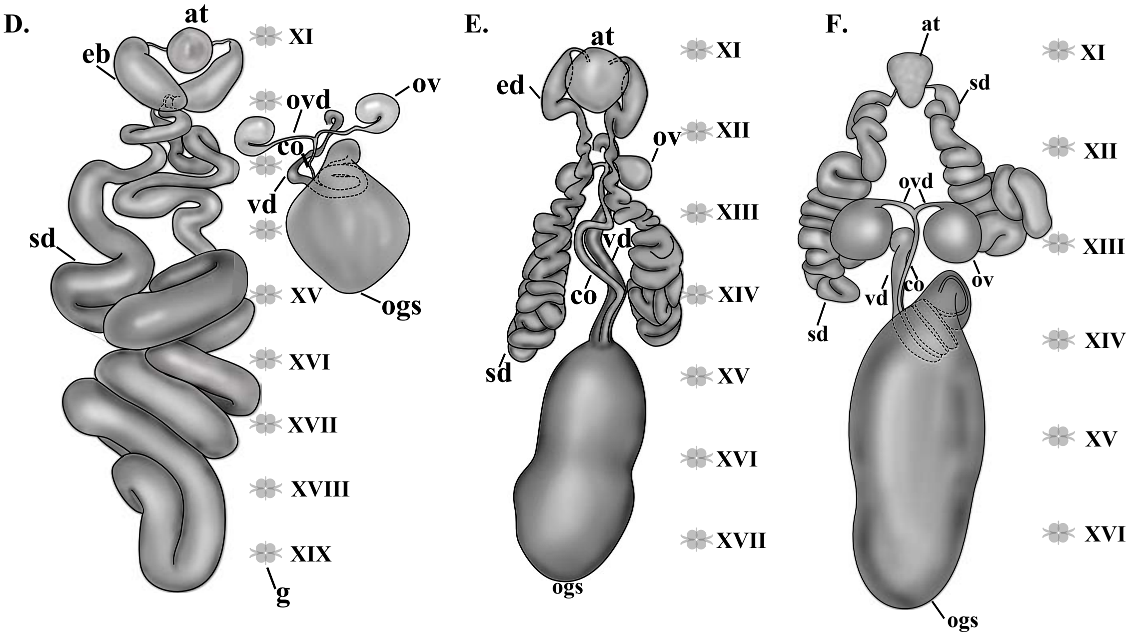
Comparison of the reproductive morphology of haemadipsoid leeches. Xerobdellidae: **A.** *Mesobdella gemmata*, **B.** *Xerobdella lecomtei*, **C.** *Diestecostoma mexicana*. Haemadipsidae: **D.** *Nesophilaemon skottsbergii*, **E.** *Haemadipsa sylvestris*, **F.** *Idiobdella seychellensis*. at= atrium; co=common oviduct; eb=ejaculatory bulb; ep=epididymes; g=ganglion; ogs=oviducal glandular sac; ov=ovaries; ovd=oviduct; ps=penis sheath; sd=sperm ducts; sr=female seminal receptacle; v=vagina; vd=vaginal duct.

Figure 16.



Mesobdella gemmata *Xerobdella lecomtei*

Diestecostoma mexicana



Nesophilaemon skottsbergii *Haemadipsa sylvestris* *Idiobdella seychellensis* the analyses of 18S and 28S under different alignment schemes were merely nearest-neighbor interchange of each other. Based on the alignments that produced trees with the shortest number of steps and the highest likelihood scores, the alignment parameters 6:3 and 10:5 were selected for 18S and 28S respectively, for all combined data analyses. For BI analyses, Bayes Factors favored the more parameter rich model over the three-partitioned (3p) GTR+I+Γ model. The results from BI analyses are based on the topology and posterior probabilities of the 5p GTR+I+Γ model.

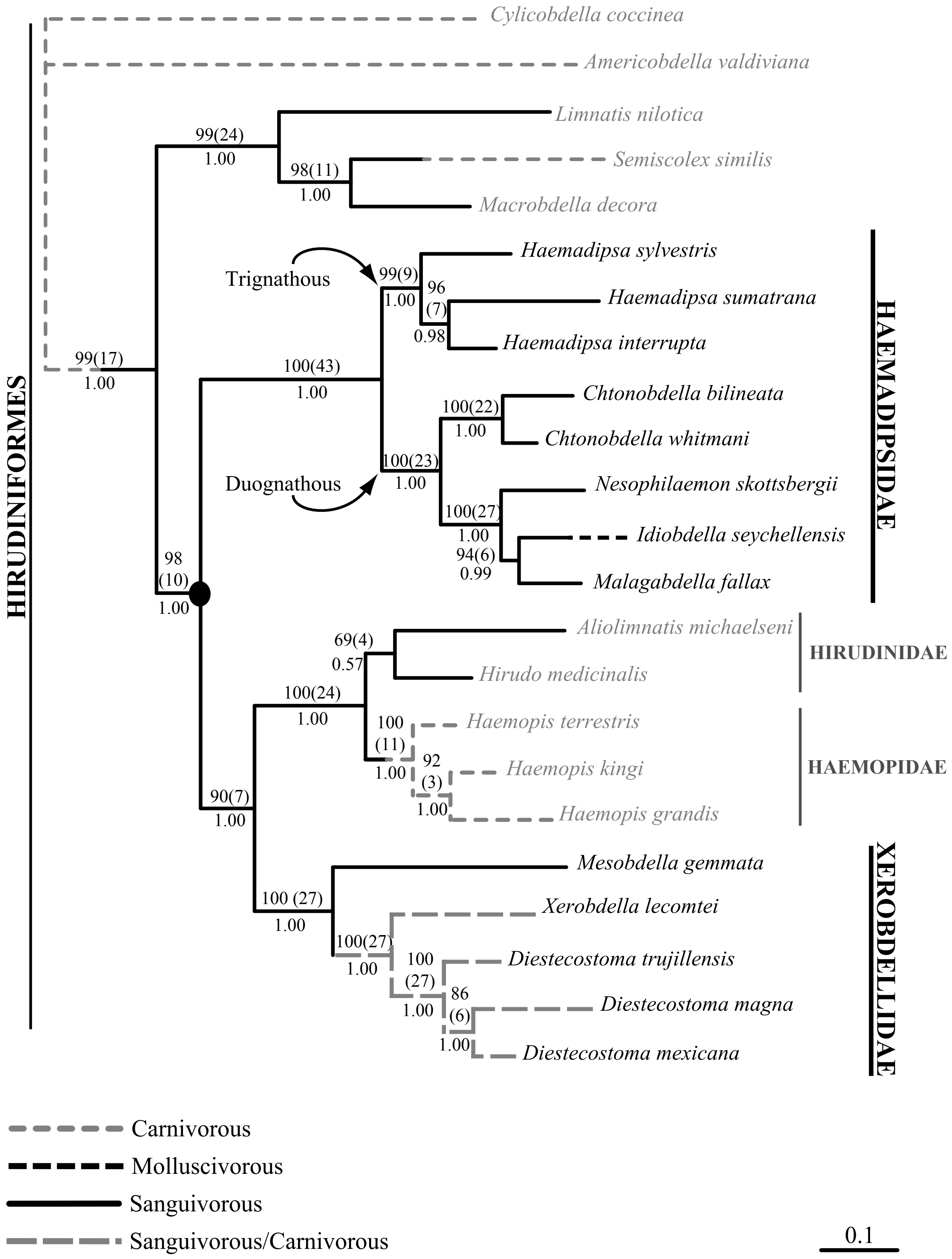
Phylogenetic Relationships

The analyses of the combined 18S, 28S and *COI* data yielded identical topologies with all major clades strongly supported, regardless of optimality criterion (Fig. 4). The combined data analyses supported two lineages of "haemadipsoid" terrestrial leeches, which can be divided into: an IndoPacific clade and a non-IndoPacific clade, which together were not each other's closest relatives. Constraining the IndoPacific and non-IndoPacific clades to be monophyletic would require only an additional 7 steps. The non-IndoPacific clade included *Mesobdella gemmata* as the sister taxon (jac=100; b=27; pp=1.00) to *Xerobdella lecomtei* + a monophyletic *Diestecostoma* (MEX; jac=100; b=27; pp=1.00). *Diestecostoma trujillensis* was sister (jac=100; b=27; pp=1.00) to *Diestecostoma mexicana* + *Diestecostoma magna* (jac=86; b=6; pp=1.00). Together the non-IndoPacific clade was well supported as the sister group (jac=90; b=7; pp=1.00) to members of the families Hirudinidae and Haemopidae (jac=100; b=24; pp=1.00).

FIGURE 17.

BI tree topology based on combined data analysis for 18S rDNA, 28S rDNA and COI (5p). MP analysis resulted in a single most parsimonious tree (L=4136; CI=0.556; RI=0.513). Numbers above the nodes refer to MP jackknife values and Bremer support, in parentheses. BI posterior probabilities are indicated below the nodes. Dotted lines denote non-bloodfeeding lineages.

Figure 17.


Cylicobdella coccinea
Americobdella valdiviana
Limnatis nilotica
99(24)
1.00
98(11)
1.00
Semiscolex similis
Macrobdella decora
Haemadipsa sylvestris
Trignathous
99(9)
1.00
96
(7)
0.98
Haemadipsa sumatrana
Haemadipsa interrupta
99(17)
1.00
100(43)
1.00
Chtonobdella bilineata
100(22)
1.00
Chtonobdella whitmani
100(23)
1.00
Duognathous
Nesophilaemon skottsbergii
100(27)
1.00
94(6)
0.99
Idiobdella seychellensis
Malagabdella fallax
98
(10)
1.00
HAEMADIPSIDAE
HIRUDINIFORMES
69(4)
0.57
Aliolimnatis michaelseni
Hirudo medicinalis
HIRUDINIDAE
100(24)
1.00
100
(11)
1.00
Haemopis terrestris
92
(3)
1.00
Haemopis kingi
Haemopis grandis
HAEMOPIDAE
90(7)
1.00
Mesobdella gemmata
100 (27)
1.00
Xerobdella lecomtei
100(27)
1.00
100
(27)
1.00
Diestecostoma trujillensis
86
(6)
1.00
Diestecostoma magna
Diestecostoma mexicana
XEROBDELLIDAE
Carnivorous
Molluscivorous
Sanguivorous
Sanguivorous/Carnivorous
0.1

The southeastern Pacific *Nesophilaemon skottsbergii* was found nested within the duognathous IndoPacific clade (jac=100; b=23; pp=1.00) and strongly supported as sister (jac=100; b=27; pp=1.00) to Western Indian Ocean *Malagabdella fallax* + *Idiobdella seychellensis* (jac=96; b=6; pp=0.99). Constraining *Nesophilaemon skottsbergii* to be monophyletic with *Mesobdella gemmata*, its geographically most proximal species, would require an additional 197 steps. The *Nesophilaemon* + *Malagabdella* + *Idiobdella* clade is sister to *Chtonobdella* species from Australia (jac=100; b=22; pp=1.00). The monophyletic, trignathous *Haemadipsa* clade (jac=99; b=9; pp=1.00), including the Malaysian *Haemadipsa interrupta* + the Bornean *Haemadipsa sumatrana* (jac=96; b=7; pp=0.98) were sister to *Haemadipsa sylvestris* from Vietnam. The IndoPacific (and south Pacific) leeches (jac=100; b=43; pp=1.00) were reciprocally monophyletic (jac=98; b=10; pp=1.00) with the non-IndoPacific clade + hirudinid clade (jac=93; b=7; pp=1.00).

DISCUSSION

Our results corroborate previous findings (Trontelj et al. 1999; Borda and Siddall, 2004a) and confirm what others (Richardson, 1969, 1971, 1975; Moore, 1946; Ringuelet, 1972; 1982) had suspected regarding the relationships of non-IndoPacific bloodfeeding terrestrial leeches. The presence of two haemadipsoid terrestrial lineages among Hirudiniformes is well supported here and these results have shed light on the taxonomic status of the Xerobdellidae, as well as on the placement of proposed associated genera. The expanded sampling of all genera in question confirms that "haemadipsoid" leeches from Europe and the New World, to the exclusion of *Nesophilaemon skottsbergii*, are in fact only distantly related to the IndoPacific haemadipsid leeches. This group also appears to have greater affinities to the principally Old World and North American hirudinid leeches than with any other group (Trontelj et al., 1999; Borda and Siddall, 2004). Borda and Siddall (2004a) proposed the resurrection of Xerobdellidae (*sensu* Moore, 1946 and Harant and Grassé, 1959) for bloodfeeding terrestrial leeches found in the New World (i.e. *Mesobdella*) and "displaced" in Europe. Our phylogenetic hypothesis confirms that Xerobdellidae should also include *Diestecostoma* species.

When Moore (1946) described *Diestecostoma octannulata* from Guatemala he addressed Caballero's (1940) proposed relationship of *Hygrobdella palaezi* (=*Diestecostoma mexicana* (Baird, 1969) to Haemadipsinae (=Haemadipsidae). Moore (1946) noted that although *Diestecostoma mexicana* and *Diestecostoma octannulata* shared a number of external and behavioral affinities with Haemadipsidae, they also deviated from haemadipsids in several respects (i.e. lack of auricles, ventral nephridia, characteristics of the reproductive system and lack of friction rays on the caudal sucker). Moore (1946) believed that the differences outweighed the similarities (e.g. habit, "haemadipsine" ocular arch) and suggested *Diestecostoma* species were closely allied to *Xerobdella lecomtei* found in the Southern European mountains. He based this on the position of the jaws, the ventral nephridia, and each possessing a medioventral common pore at the base of the sucker, which appeared to replace the usual 17th nephridial pair. On this basis, Moore (1946) also anticipated affinities to *Mesobdella gemmata*. Moore (1946), thus, concluded that "it may become necessary to erect the subfamily Xerobdellinae for them" (Moore, 1946: 190), but acknowledged that a formal revision was premature without a better understanding of the South American leeches. Remarkably, Moore (1946) withdrew his initial conclusions in a footnote (in that same paper) after reviewing Ringuelet's (1943) manuscript containing a description of the reproductive anatomy of *Mesobdella gemmata* as being "typically haemadipsine" in nature. From our phylogenetic results it is clear that the apparent resemblance of *Mesobdella gemmata*, *Diestecostoma* species and *Xerobdella lecomtei* to haemadipsid leeches was superficial.

The taxonomic framework established by Blanchard (1896, 1917) was reinforced by others (Caballero, 1940, 1956, 1959; Ringuelet, 1943,1953, 1955) and continued with Soós's (1967) treatment of Haemadipsidae. Soós (1967) included in Haemadipsidae genera that were distributed throughout Asia (*Haemadipsa*), Madagascar (in the genus *Chtonobdella*), the Seychelles (*Idiobdella*), Australia (*Philaemon*, *Chtonobdella*), Papua New Guinea (*Phytobdella*), and the Pacific Islands (*Phytobdella*, including *Nesophilaemon*). Soós (1967) also included *Mesobdella gemmata* in the family. Soós (1966), however, also followed Moore's (1946) recommendation in treating *Diestecostoma* species and *Xerobdella* species as a separate taxonomic group from haemadipsids in Diestecostomatidae (=Xerobdellidae, here), as proposed by Ringuelet (1953).

Richardson (1969, 1971), however, questioned the inclusion of *Nesophilaemon skottsbergii* (and *Mesobdella gemmata*) in Haemadipsidae (*sensu* Soós, 1967). He found that the reproductive morphology as illustrated and described by Ringuelet (1955) not only set *Nesophilaemon* apart from the four-annulate Australian genus *Philaemon* (as it was originally described), but also from haemadipsid leeches in general (Richardson, 1971). He concluded that *Nesophilaemon skottsbergii* (and *Mesobdella gemmata*) were haemadipsine only on the basis of annulation patterns, in the ocular somites and having a secondary adaptation for terrestrial and sanguivorous habits (Richardson, 1969). This resulted in their removal from Haemadipsidae and their placement in a new family, Mesobdellidae (Moore, 1972).

Richardson (1975) later revised Soós's (1967) classification of land leeches, to the exclusion of the non-IndoPacific genera (*Mesobdella* and *Nesophilaemon*), by raising IndoPacific haemadipsids to superfamily Haemadipsoidea and dividing the group on the basis of jaw and reproductive morphologies, as well as annulation patterns and geography. Richardson (1975; 1978) restricted Haemadipsidae to the trignathous leeches from India, East Asia and Southeast Asia and erected two new families: Domanibdellidae, for the duognathous leeches found in Australia, Papua New Guinea, Madagascar and Oceana (Domanibdellidae) and another for those found in the Seychelles (Idiobdellidae).

IndoPacific Haemadipsidae (*sensu stricto*) was supported as a monophyletic group, however, our hypothesis was in strong conflict with Sawyer's (1986) revision of Haemadipsidae (see Table 4A). Sawyer's (1986) classification removed all notion of zoogeography for haemadipsoid leeches, removed the monogeneric family ranks for each of *Mesobdella*, *Diestecostoma, Xerobdella* and *Nesophilaemonidae* and ignored the taxonomic recommendations by Moore (1946) and Richardson (1975). Haemadipsidae was simply divided into two groups, the trignathous series (e.g. *Diestecostoma, Haemadipsa*, *Mesobdella* and *Xerobdella*) and the duognathous series (e.g. *Chtonobdella, Idiobdella, Malagabdella, Nesophilaemon*). Although he was aware that some of the genera he assigned to the trignathous series tended to be "isolated groups which sometimes, but unnecessarily, [were] recognized as monotypic families in their own right" (Sawyer, 1986: 689), they were nevertheless included in Haemadipsidae. To Richardson's (1975) credit, our phylogeny supports a monophyletic IndoPacific haemadipsid clade, divided into a monophyletic Asian trignathous clade (*Haemadipsa*) and a monophyletic non-Asian duognathous clade, resembling Richardson's Haemadipsidae and Domanibdellidae, respectively. Our results indicate that *Nesophilaemon skottsbergii* is unequivocally a member of Haemadipsidae and associated with Richardson's (1975) duognathous Domanibdellidae, based on morphology and molecular data. Puzzlingly, this southeastern Pacific species appears to be more closely allied to the isolated *Malagabdella fallax* and *Idiobdella seychellensis* from the western Indian Ocean than to duognathous *Chtonobdella* species from eastern Australia.

Insofar as each of the genera *Mesobdella*, *Xerobdella* and *Diestecostoma* are found to form a clade, only one family group name is required. Xerobdellinae Moore, 1946 has precedence, in spite of Moore's (1946) own ambivalence, and has inclusiveness insofar as he anticipated the group to include "the last two of these genera and possibly the first" (Moore, 1946: 190). Ringuelet (1953, 1976) clearly did not intend for Xerobdellinae Moore, 1946 to be a subfamily of Diestecostomatidae Ringuelet, 1953 insofar as Diestecostomatidae was intended to explicitly exclude *Mesobdella* (see also Soós, 1966). Xerobdellinae Moore, 1946 also satisfies the ICZN descriptive requirements in which the family group name is to include species in genera that are the same in "the position of the nephropores, the first pair buccal, the last median beneath the sucker, and the remaining pairs ventral, in the absence of auricles, the structure of the caudal sucker" (Moore, 1946: 190). Thus, we formally recognize Xerobdellidae Moore, 1946 as valid, leaving each of Diestecostomatidae Ringuelet, 1953 and Mesobdellidae Ringuelet, 1972 as junior synonyms.

With respect to the IndoPacific haemadipsid leeches, although Richardson (1975) was correct to divide haemadipsids by zoogeographic region, the status of some groups at the family level (i.e. Domanibdellidae, Nesophilaemonidae and Idiobdellidae) will likely need revision. We recommend that they be recognized in Haemadipsidae *sensu stricto*, however, a broader sampling of IndoPacific species is warranted to properly address the phylogenetic and taxonomic status of species within this group.

Morphology of Haemadipsoid Leeches

Unique external morphological characters (e.g. annulation, ocular patterns and position of nephridia) and life history strategies have defined bloodfeeding terrestrial leeches apart from other groups of leeches (Blanchard, 1917; Harding and Moore, 1924; Soós, 1966; Richardson, 1975; Sawyer, 1986); in particular, the presence of lateral nephridia and obvious respiratory auricles. However, despite lacking these precise diagnostic characteristics, the superficial similarities in general form and adaptation for terrestrial sanguivory led workers to include non-auriculate, non-IndoPacific haemadipsoid species in Haemadipsidae. The lateral position of nephridia in haemadipsid leeches has been speculated to aid in keeping the leech moist (Sawyer, 1986), particularly when actively feeding and during dry seasons. This does not explain why this would not be essential to other terrestrial leech groups (e.g. Cylicobdellidae, Xerobdellidae).

The physiological function of the respiratory auricles is also not well understood, but is thought to act as a damp brush to form a watery seal along the margins of the caudal sucker (Sawyer, 1986). It is present in virtually all Haemadipsidae *sensu stricto*, with the exception of *Idiobdella seychellensis* and all other groups of leeches. Contrasting with the respiratory auricles in Haemadipsidae is the opening of the 17th nephridial pair via a medioventral common pore in Xerobdellidae. This character is a synapomorphy for Xerobdellidae and appears to be a modification that might serve a similar function in purporting a watery seal along the margins of the caudal sucker, which seems necessary for life on land.

The reproductive systems of xerobdellid leeches not only drastically deviate in form from haemadipsids, they share little in common with each other. *Xerobdella lecomtei* and *Mesobdella gemmata* share the simple and micromorphic nature of the male and female systems (Fig. 16A, B). An accessory female seminal receptacle is exclusive to *Xerobdella* species (Minelli, 1971) and is unusual for arhynchobdellid leeches (Sawyer, 1986). The micromorphic reproductive systems seen in *Mesobdella gemmata* and *Xerobdella lecomtei*, considerably contrasts with that of *Diestecostoma mexicana*. Based on the internal anatomy alone, it would be easy to believe that these species would not have a shared ancestry (contrary to Moore, 1946). However, external morphological characters include several synapomorphies for Xerobdellidae (e.g. medioventral pore). The considerable internal morphological variation between the genera of Xerobdellidae might be explained by long-term isolation from each other, with origins that date back to Pangaea.

In contrast to Xerobdellidae, the reproductive systems of haemadipsids appear to share a basic form with a micromorphic male atrium, globular ovaries that insert independently into a common oviduct, a long vaginal duct (either straight or coiled) that inserts into an oviducal glandular sac (Fig. 16D-F). Richardson (1969; 1975) revised the classification of haemadipsids in recognition of the geographic distribution of species and of the systematic utility of the reproductive morphology of land leeches. In his evaluation, he may have underestimated the diversity of form in the reproductive systems of haemadipsids in light of his previous experience with the "monotony of the internal morphology in land leeches" (Richardson, 1978; p. 838). However, recent assessments of terrestrial leeches from Madagascar reveal a great diversity of characteristics in the reproductive anatomy (see Borda, 2006 for an overview), which deviate from the basic form seen in other Haemadipsidae (Fig. 16D-F).

The availability of fresh samples of *Nesophilaemon skottsbergii* facilitated the reexamination of its reproductive morphologies. The last published description and illustration of the reproductive system of *Nesophilaemon skottsbergii* was in 1955 by Ringuelet. Thereafter, Richardson (1969) held that *Nesophilaemon skottsbergii* was closer to *Mesobdella gemmata*, despite the presence of a "haemadipsine" ocular arch, being four-annulate and being a duognathous leech. The duognathous condition of the jaws is known only in Haemadipsidae. Our dissections reveal that *Nesophilaemon skottsbergii* is haemadipsid in all respects. The reproductive anatomy as illustrated by Ringuelet (1955) does not resemble fresh specimens whatsoever. Even among the haemadipsids, the reproductive anatomy of *Nesophilaemon skottsbergii* is distinct with its stout female oviducal glandular sac and long and thick sperm ducts, not typically seen in this group. As already mentioned, the reproductive anatomy of some Malagasy leeches appears to diverge from other Haemadipsidae. Therefore, it might be of no surprise that *Nesophilaemon skottsbergii* would be closely allied to this clade. The extent of the diversity of the reproductive anatomy across IndoPacific haemadipsid leeches is still needed to gain more insight.

*Feeding Habits of Land Leeche*s

Already a phylogenetic framework has clarified the diversity and evolution of life history strategies for leeches (e.g. Siddall and Burreson, 1996; Apakupakul et al., 1999; Trontelj, et al., 1999; Utevsky and Trontelj, 2003; Borda and Siddall, 2004a, b). In this framework the preference for bloodfeeding is now understood to have been lost or have modified multiple times throughout the evolutionary history of leeches (Borda and Siddall, 2004a, b). Historically, haemadipsoid leeches have been associated with being uniformly sanguivorous. Our phylogenetic hypothesis challenges this notion with the support of two distantly related terrestrial and bloodfeeding lineages, one of which includes species that have a more varied diet.

When Frauenfeld (1868) described *Xerobdella lecomtei* from the Austrian Alps and placed it in Haemadipsidae, he did so based on its terrestrial habit and being parasitic on salamanders. Subsequent work indicated that *Xerobdella lecomtei* has a more diverse diet and actually feeds on invertebrates, including earthworms, gastropods and dipteran larvae (Reisinger 1951; Moosbrugger and Reisinger, 1971; Sawyer, 1986). Its closest relatives, *Diestecostoma* species similarly have also been found on salamanders (e.g. *Ambystoma* species), as well as on oligochaetes (Caballero, 1940). Of all members of

Xerobdellidae, species of *Mesobdella* are alone in being exclusively sanguivorous, readily observed feeding on humans (Siddall and Borda, 2004), but likely feeding on boreal frogs (Ringuelet 1953). Haemadipsidae, on the other hand, are infamous for their voracious appetite for vertebrate blood, *Idiobdella* species appear to have lost this habit in favor of being a parasite of mollusks (Richardson, 1978) and other soft-bodied invertebrates (Harding, 1913). This may reflect the Seychelles Islands having initially been devoid of any vertebrates (Hume, 2004), prior to more recent introductions (e.g. Racey and Nicoll, 1984; Austin et al., 2003).

Biogeographic Patterns

The diversification of Annelida has been attributed to the Cambrian explosion (Ayala, et al., 1998; Rousset et al., 2006) with the oldest know fossils dating back to the Silurian (>400 mya; Briggs et al., 2003). Unequivocal fossils are not known for leeches (Siddall et al., 2006), as these soft-bodied annelids lack structures that would mineralize in order to leave their mark in the fossil record. However, the phylogenetic patterns observed in Figure 17 appear to be consistent with geological events that might have contributed to the diversification of haemadipsoid leeches (Fig. 18).

The rarified endemicity and current distribution of Xerobdellidae may indicate a Pangaean origin. The presence of *Mesobdella gemmata* in temperate regions of Valdivia (Chile) is consistent with the warm temperate zone of southwestern South America persisting since the Permian (> 250 mya; Rees, et al., 2002). While, *Diestecostoma* species are distributed throughout northern South America and Meso America, with no records above Mexico, any relationship with *Xerobdella lecomtei* would have been present through a Laurasian connection (~170 mya; Scotese, 2004) absent of any mechanism for intercontinental dispersal. That the distribution of the sister group of Xerobdellidae, the hirudinids, includes each of North America, Europe and Africa, further suggests a Pangaean relationship.

The distribution of Haemadipsidae appears to have diversified via vacariant events related to the breakup of Gondwana. The relationships of duognathous leeches represented here from Australia, Madagascar, the granitic Seychelles Islands and the Juan Fernandez archipelago, would need to have had an ancestor that was present before the Indo-Malagasy landmass began to separate from Gondwana (>150mya; Briggs, 2003), and after Africa separated from Madagascar (~165 mya; Rabinowitz et al., 1983). The relationship of *Idiobdella seychellensis* and *Malagabdella fallax* reflects that their common ancestor was at least present on the IndoMalagasy landmass. After the separation from Madagascar (~85 mya; Storey, 1995), the Indian landmass left behind remnants, which later became known as the Seychelles Islands (~65 mya; Briggs, 2003) as India moved northward towards Asia (Scotese, 1988). This is confounded by the absence of a close relationship between these and Asian species. Although, the sampling of Asian *Haemadipsa* species is limited, the high species diversity of *Haemadipsa* species throughout India, East Asia and Southeast Asia is well documented (Moore, 1924), possibly pointing towards an eastward diversification of the genus as a dispersal out of India, that is, after India's collision into Asia (~50 mya; Briggs, 2003).

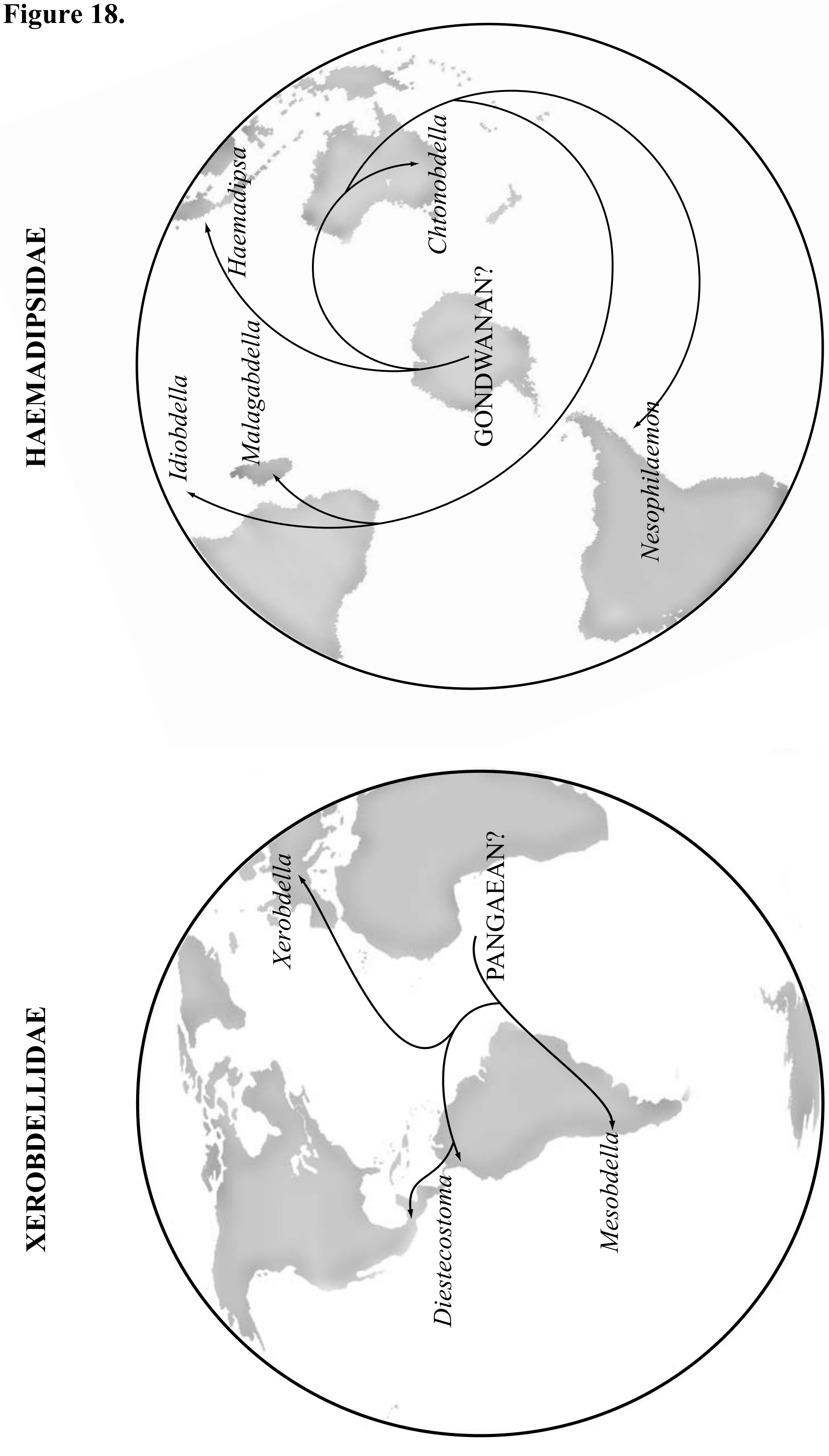
Among the Haemadipsidae, *Nesophilaemon skottsbergii* has one of the most peculiar distributions and has been found only on Isla Mas Afuera (or Alejandro Selkirk Island). Ringuelet (1955) proposed that the presence of a *Nesophilaemon skottsbergii* in the archipelago was best explained by long distance, trans-oceanic dispersal on *Pterodroma* species (Procellariiformes) from the Australasian region given the endemic *Pterodroma externa* and *Pterodroma longirostis* on Isla Mas Afuera (Brooke, 1987). Though passive dispersal on birds is a reasonable hypothesis for *Nesophilaemon skottsbergii* – the volcanic Juan Fernandez Islands are young, with Isla Mas Afuera having formed ~1–2.4 mya (Baker et al., 1987) – an Australian origin is problematic given the close relationship between the East Pacific *Nesophilaemon skottsbergii* and species from the West Indian Ocean.

Few deep historical biogeographic studies exist for soft-bodied invertebrates leaving no fossil trace. Our robust phylogeny for the terrestrial bloodfeeding leeches, leave perhaps as many questions unanswered as answered, which beckons more extensive consideration of these notorious annelids.

FIGURE 18.

Biogeographic correlates to portions of the phylogenetic hypothesis for Haemadipsidae and Xerobdellidae. A) Relationships among xerobdellid leeches appear to correspond to patterns expected for terrestrial taxa initially occupying Southern Pangaea, with eventual dispersal northward, then following the eventual split of Laurasia (~180 mya) and subsequently Laurentia and Eurasia (~100 mya). B) Antarctic view of the earth, with a schematic representation of the relationships of Haemadipsidae (arrows).

Figure 18.


HAEMADIPSIDAE
Haemadipsa
Chtonobdella
GONDWANAN?
Malagabdella
Idiobdella
Nesophildemon
XEROBDELLIDAE
Xerobdella
PANGAEAN?
Diestecostoma
Mesobdella

CHAPTER 6

Old World Haemadipsidae (Arhynchobdellida: Hirudiniformes: Haemadipsidae): Diversity, Evolution and Biogeography of IndoPacific Bloodfeeding Terrestrial Leeches

INTRODUCTION

Haemadipsidae include the notorious terrestrial annelids found throughout the tropical IndoPacific. Despite their infamy, the diversity, ecology and the evolutionary history of haemadipsid leeches are still poorly understood. A comprehensive phylogenetic hypothesis for Haemadipsidae is currently lacking and the available taxonomic contributions are dated (Blanchard, 1917; Moore, 1924; Soós, 1966; Richardson 1975; Sawyer, 1986). Several studies have focused on the ecological and behavioral aspects of leeches (Sawyer, 1986; see references within), but few have emphasized on the bloodfeeding terrestrial leeches. The studies that are available are based on members of the Indian and Asian genus *Haemadipsa* (e.g. Chen et al., 1998; Yoshiba; 1996; Asada et al., 1995; Tan et al., 1994; Gladstone, 1989; Fogden and Proctor, 1985; Bhatia and Bora, 1973; Keegan et al., 1968; Stammers, 1950; Oka, 1930) and one study on *Chtonobdella limbata* from Australia (Richardson, 1968).

There are less than 70 haemadipsid species described from the IndoPacific, however this is likely an underestimate of their diversity (Keegan et al., 1968; Richardson, 1975; Sawyer, 1986; Nessemann and Sharma, 1996; 2001). Table 7 represents a general list of major genera (*sensu* Sawyer, 1986), their associated jaw morphologies, annulation patterns and proposed zoogeographic distributions. IndoPacific haemadipsid leeches have been traditionally divided into two major groups based on jaw morphology: trignathous (three-jawed) and duognathous (two-jawed). Approximately 24 species have been classified in the genus *Haemadipsa*, while the remaining species have been placed in 30 genera (Nybelin, 1943; Richardson, 1975, 1978) or restricted to thirteen genera (Sawyer, 1986; Table 7). All bloodfeeding terrestrial leeches were

TABLE 7.
Major representative genera of Haemadipsidae, *sensu* Sawyer (1986).

Genus	# Jaws	Annulation Pattern	# Species Described	Approximate Distribution
Haemadipsa	3	5-annulate	24	Indian subcontnent, E. & S. E. Asia
Titetrabdella	3	4-annulate	2	E. Asia, S. E. Asia
Chtonobdella	2	5-annulate	5	Australia
Domanibdella	2	5-annulate	5	Papua, Indonesia, Oceana
Idiobdella	2	5-annulate	2	Seychelles Islands
Leiobdella	2	4-annulate	4	Papua New Guinea
Mahebdella	2	5-annulate	1	Seychelles Islands
Malagabdella	2	5-annulate	4	Madagascar
Neoterrabdella	2	4-annulate	1	Australia (Northern Territory)
Nesophilaemon	2	4-annulate	1	Juan Fernandez Islands
Philaemon	2	4-annulate	5	Australia, Tasmania, Lord Howe Is.
Phytobdella	2	6-annulate	5	Philippines
Planobdella	2	7-annulate	3	Papua New Guinea, Oceana
Tristabdella	2	4-annulate	1	Madagascar

initially classified under a single family Haemadipsidae (Blanchard, 1893; Sawyer, 1986), but were later separated into three families: Haemadipsidae, Domanibdellidae and Idiobdellidae (*sensu* Richardson, 1975, 1978), with Domanibdellidae further subdivided into four subfamilies: Chtonobdellinae Richardson 1975, Domanibdellinae Richardson 1975, Leiobdellinae Richardson 1975, Philaemoninae Richardson 1975, and Malagabdellinae Richardson 1978.

The strict adaptation to damp terrestrial environments makes haemadipsid leeches unique among bloodfeeding leeches, however the most compelling aspect of this group is their distribution. Absent from most continental landmasses including North America, South America, Africa and Europe, they are specifically adapted to tropical regions throughout the IndoPacific, which are characterized by periods of cyclical rainfall (Sawyer, 1986). As a result of their limited distribution, workers (Whitman, 1886; Moore, 1924; Richardson, 1975) have speculated on the origins of land leeches in reference to Old World geological patterns throughout the region (e.g. break-up of Gondwana; tectonic evolution of Southeast Asia; collision of India into Asia, etc.). Two centers of origins for haemadipsid leeches had been proposed: a) the southern slopes of the Himalayan mountains, with subsequent dispersal throughout the region on birds (Whitman, 1886; Moore, 1924) and b) throughout a former landmass now fragmented into Papuan and Oceanic islands (Richardson, 1975).

Recent phylogenetic work suggests that hirudiniform leeches (Arhynchobdellida: Hirudiniformes) share a common origin within an amphibious ancestor with a tendency towards terrestrialism, which also corresponds to mode of fertilization and cocoon deposition (Borda and Siddall, 2004a, b). Throughout the evolutionary history of

Hirudiniformes, strict terrestrialism has evolved at least twice among bloodfeeding leeches. Previous phylogenetic hypotheses (Borda and Siddall, 2004; Borda et al., In Press) have suggested that the IndoPacific terrestrial leeches are monophyletic, though these studies were based on a limited taxonomic representation. Therefore the goals of this study were to: 1) provide a comprehensive phylogenetic estimation for Haemadipsidae; 2) evaluate the concordance between evolutionary history and traditional classification schemes, with special considerations to those proposed by Moore (1924), Richardson (1975) and Sawyer (1986); and 3) to provide a phylogenetic framework from which to better explore the biogeographic history of Haemadipsidae.

MATERIAL AND METHODS

Taxa

Taxa included in this study, their sampling localities and GenBank accession numbers for taxa used in previous work are listed in Table 8. Species new to this study are indicated by an asterisk in Table 8. Representatives from the major families of Hirudiniformes were included as outgroup taxa based on previous phylogenetic work (Borda and Siddall, 2004a). Collection strategies involved walking along forest trails and searching through damp undergrowth, especially after rain, to attract leeches. Leeches were collected from the ground and exposed skin prior to, or while, feeding. After collection, leeches were relaxed using an ethanol gradient, starting with a ~10% ethanol solution and gradually adding 95% ethanol until the leeches were relaxed (and dead). Leeches collected from

TABLE 8.
Classification of haemadipsoid leeches (*sensu* Richardson, 1975), with taxa, collection localities and genes used for phylogenetic analyses. Genus names with superscript indicate those names that were synonymized by Sawyer (1986).

		GenBank Accession Number			
TAXA	**COLLECTION LOCALITY**	**18S**	**28S**	**COI**	**COIII**
ARHYNCHOBDELLIDA					
HIRUDINIFORMES					
HAEMADIPSIDAE					
Haemadipsa zeylanica zeylanica	Sri Lanka	–	–	–	–
Haemadipsa sylvestris	Vietnam	AF116005	AY425373	AF003266	–
Haemadipsa limuna	Guanxi Province, China	–	–	–	–
Haemadipsa japonica ryukyuana	Taipei City, Taiwan	–	–	–	–
Haemadipsa trimaculosa	Thailand	–	–	–	–
Haemadipsa trimaculosa	Vietnam	–	–	–	–
Haemadipsa sumatrana	Borneo	AY425464	AY425372	AY425446	–
Haemadipsa c.f. zeylanica (09)	Philippines	–	–	–	–
Haemadipsa c.f. zeylanica (HAG)	Philippines	–	–	–	–
Haemadipsa c.f. zeylanica (04)	Philippines	–	–	–	–
Haemadipsa c.f. sylvestris SO1	Khao Sok, Thailand	–	–	–	–
Haemadipsa c.f. sylvestris SO2	Khao Sok, Thailand	–	–	–	–
Haemadipsa zeylanica montivindicus	Nepal	–	–	–	–
Haemadipsa zeylanica agilis	Nepal	–	–	–	–
Haemadipsa picta	Malaysia	–	–	AY425445	–

TABLE 8. Continued

Haemadipsa crenata	Huai Thong Cho, Thailand				
Haemadipsa montana	Nepal	–	–	–	–
Haemadipsa zeylanica japonica	Chiba Province, Japan	–	–	–	–
Haemadipsa zeylanica subagilis NO3	Thailand	–	–	–	–
Haemadipsa zeylanica subagilis NO4	Thailand	–	–	–	–
Haemadipsa interrupta 52	Khao Sok, Thailand	–	–	–	–
Haemadipsa interrupta 89	Thailand	–	–	–	–
Haemadipsa interrupta 1	Malaysia	–	–	–	–
Haemadipsa interrupta 65	Jahor, Malaysia	–	–	–	–
Haemadipsa interrupta 44	Malaysia	–	–	–	–
Haemadipsa interrupta 50	Tioman Island, Malaysia	–	–	–	–
Titetrabdella scandens 47	Khao Sok, Thailand	–	–	–	–
Titetrabdella scandens 49	Sanklaburi, Thailand	–	–	–	–
Titetrabdella scandens	Guanxi Province, China	–	–	–	–
DOMANIBDELLIDAE					
CHTONOBDELLINAE					
Amicibdella[1] *nigra*	Mt. Baldy, Atherton, Australia	–	–	–	–
Chtonobdella whitmani	Davies Creek, Australia	–	–	–	–
Chtonobdella whitmani	Davies Creek, Australia	–	–	–	–
Chtonobdella bilineata	Lamington NP, Australia	AF116006	AY425361	AF003267	AF003267
Chtonobdella bilineata	Gibraltar Range NP, Australia	–	–	–	–

TABLE 8. Continued

DOMANIBDELLINAE					
(Group A)					
Domanibdella n. sp.	Sanduan Province, PNG				
Neoterrabdella australensis	Northern Territory, Australia	–	–	–	–
Elocobdella[2] *novabritanniae*	Sanduan Province, PNG	–	–	–	–
(Group B)					
Phytobdella catenifera	Philippines	–	–	–	–
Phytobdella meyeri	Philippines	–	–	–	–
PHILAEMONINAE					
Philaemon pungens	NSW, Australia	–	–	–	–
Castrabdella[3] *c.f. nymboidae*	NSW, Australia	–	–	–	–
Philaemon n. sp. 79	Queensland, Australia	–	–	–	–
Philaemon n. sp. DC1	Cairns, Queensland Australia	–	–	–	–
Philaemon n. sp. MH	Queensland, Australia	–	–	–	–
Philaemon c.f. grandis	Tasmania	–	–	–	–
Micobdella[4] *gloriosi*	Queensland, Australia	–	–	–	–
Xenobdella[5] *mediarubra*	Lord Howe Island	–	–	–	–
LEIOBDELLINAE					
Leiobdella sp.	Sanduan Province, PNG	–	–	–	–
MALAGABDELLINAE					
Malagabdella fallax	Ranomafana, Madagascar	–	–	–	–
Malagabdella morsitans	Sorota Mts. Madagascar				

TABLE 8. Continued

Malagabdella niarchosorum	Ranomafana, Madagascar	–	–	–	–
Malagabdella vagans	Perinet, Madagascar	–	–	–	–
IDIOBDELLIDAE					
Idiobdella seychellensis	Seychelles Islands	–	–	–	–
NESOPHILAEMONIDAE					
Nesophilaemon skottsbergii	Juan Fernandez Islands	–	–	–	–
UNKNOWN PLACEMENT					
Cave Leech	Laos	–	–	–	–
OUTGROUP					
Blanchardiella camelliae	Venezuela	–	–	–	–
Cylicobdella sp.	Brazil	–	–	–	–
Cylicobdella joseiensis	Brazil	–	–	–	–
Diestecostoma magna	Mexico	–	–	–	–
Diestecostoma mexicana	Mexico	–	–	–	–
Diestecostoma trujillensis	Mexico	–	–	–	–
Haemopis terrestris	Ohio, USA	AY786465	AY786452	AY786459	–
Hirudinaria manillensis	Puerto Rico	AY425467	AY425384	AY425449	–
Hirudinaria sp.	Thailand	–	–	–	–
Hirudo medicinalis	France	AY786464	AY786451	AY786458	–
Lumbricobdella schaefferi	Dominica	–	–	–	–
Macrobdella decora	Michigan, USA	AF116007	AY425390	AF003271	–

TABLE 8. Continued

Mesobdella gemmata	Chile	AY425472	AY425393	AY425454	–
Xerobdella lecomtei	Slovenia	AF099947	–	–	–

[1] *Amicibdella* = *Chtonobdella*

[2] *Elocobdella* = *Planobdella*

[3] *Castrabdella* = *Philaemon*

[4] *Micobdella* = *Philaemon*

[5] *Xenobdella* = *Philaemon*

the field were stored in 95 – 100% ethanol at –20° C or at 4 ° C until used for DNA extraction.

Molecular Techniques

Genomic DNA was extracted from the caudal sucker to minimize the possibility of contamination from host/prey DNA found in the gastric and intestinal regions with DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA). PCR amplification of nuclear 18S rDNA (18S) and 28S rDNA (28S), mitochondrial cytochrome *c* oxidase subunit I (COI) and cytochrome c oxidase subunit 3 (COIII) gene fragments were accomplished using the primers listed in Table 3. Amplification reactions consisted of 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: heated to 94° C (1 min), followed by 35 cycles of 94° C (30 sec), 47 – 50° C (30 sec), and 68° or 72° C (45 sec) and a final extension at 68 or 72° C (7 min). PCR 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) or using isopropanol/ethanol precipitation protocol and analyzed using an ABI PRISM® 3730 sequencer.

TABLE 9.
Primers used for amplification and sequencing.

GENE	PRIMER NAME	PRIMER SEQUENCE 5' → 3'	REFERENCE
18S rDNA	A	AACCTGGTTGATCCTGCCAGT	Apakupakul et. al., 1999
	L	CCAACTACGAGCTTTT	Apakupakul et. al., 1999
	C	CGGTAATTCCAGCTC	Apakupakul et. al., 1999
	Y	CAGACAAATCGCTCC	Apakupakul et. al., 1999
	O	AAGGGCACCACCAG	Apakupakul et. al., 1999
	B	TGATCCTTCCGCAGGTTCACCT	Apakupakul et. al., 1999
28S rDNA	28srD1a	CCCSCGTAAYTTAAGCATAT	Prendini et al., 2005
	28sB	TCGGAAGGAACCAGCTAC	Whiting, 2002
	28sA	GACCCGTCTTGAAGCACG	Whiting, 2002
	28SBout	CCCACAGCGCCAGTTCTGCTTACC	Prendini et al., 2005
	28srd4.5a	AAGTTTCCCTCAGGATAGCTG	Whiting, 2002
	28srD7b1	GACTTCCCTTACCTACAT	Whiting, 2002
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994
	COI-A	CCTGTTCTTGCTGGTGCTATTACIAT	Bely and Wray, 2004
	COI-B	TAGTCAGAATATCGCCGAGGTATICC	Bely and Wray, 2004
COIII	COIII-F	TGGTGGCGAGATGTKKTNCGNGA	Boore and Brown, 2000
	COIII-R	ACWACGTCKACGAAGTGTCARTATCA	Boore and Brown, 2000

Sequences were edited and reconciled using CodonCode Aligner (CodonCode Corporation). Alignment of the two nuclear gene fragments were accomplished using Clustal X software under a range of alignment parameters: gap cost: gap extension = 6:3 or 10:5. Alignments for COI and COIII were done by eye, as there were no ambiguous insertions or deletions. The concatenated dataset that produced the most parsimonious trees(s) was selected for combined data analyses under the following alignment parameters combinations for 18S and 28S: 1) 18S (6:3), 28S (6:3), COI; 2)18S (6:3), 28S (10:5), COI; 3) 18S (10:5), 28S (6:3), COI; and 4)18S (10:5), 28S (10:5), *COI*.
Due to a large amount of missing data for COIII, only three datasets were assembled: 1) all taxa (n=74) with 18S, 28S and COI; 2) all taxa with 18S, 28S, COI and COIII; and 3) limited taxa (n=44) with 18S, 28S, COI and COIII.

Phylogenetic analyses

Maximum Parsimony (MP) analyses were performed in PAUP* v. 4.06b10 (Swofford, 2000) on all three datasets. Heuristic searches used 100 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All characters were equally weighted and non-additive. Gaps were treated as missing data. Parsimony jackknife (jac) values for combined dataset were obtained with 1000 heuristic pseudoreplicates, using random taxon addition and tree-bisection-reconnection branch swapping with 37% deletion (Farris, 1999).

Combined data analyses of data sets 1 and 3 using Bayesian Inference (BI) were performed in Parallel MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) via the Cornell University Computational Biology Service Unit (http://cbsuapps.tc.cornell.edu/mrbayes

.aspx). Modeltest 3.06 (Posada and Crandall, 1998) was used to select the appropriate model of evolution for each gene under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). Each dataset was partitioned in two ways: 1) by gene (Dataset 1 – 18S, 28S and *COI* (3p) and Dataset 3 – 18S, 28S, COI and COIII (4p)) and 2) partitioned by gene for 18S and 28S and by codon position for COI and COIII (i.e. 18S, 28S, COI (5p) and 18S, 28S, COI and COIII (8p)). BI analyses assumed a decoupled GTR+I+Γ model for each data partition, based on the AIC (via ModelTest). The default prior distribution of parameters were used for Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC) analyses, with one cold chain and three heated chains for 10 million and 8 million generations (datasets 1 and 3, respectively) and sampled every 1000th generation. Split frequencies of the standard deviation from the simultaneous BI analyses were well below 0.001 and the R-statistic (Gelman and Rubin, 1992) for each parameter partition approached 1, ensuring convergence of chains, log likelihoods and model parameters. In order to estimate the posterior probabilities (pp), the burn-in was set to discard the first million generations. Bayes factors were used to assess the best partitioning scheme for the GTR+I+Γ model for combined BI analyses, *a posteriori* (Nylander et al., 2004).

RESULTS

With respect to the alignment schemes for the two nuclear genes, the general trend was that increasing the gap opening: gap extension cost parameters resulted in an increased tree length (MP). Based on the alignments that produced trees with the shortest length for loci analyzed separately (not shown), the alignment parameters 6:3 and 6:3 were selected for 18S rDNA and 28S rDNA respectively, for all combined analyses.

For BI analyses, Bayes Factors favored those datasets partitioned by gene for nuclear genes and codon position for mt genes: 5p (Harmonic mean (HM) of lnL= -46786.62) and 8p (HM = lnL= -47040.81) over 3p (HM lnL=-47364.06) and 4p (lnL= -47672.87). Therefore the results from BI analyses are based on the topology and posterior probabilities of the 5p and 8p partitioned GTR+I+Γ datasets.

Phylogenetic Relationships

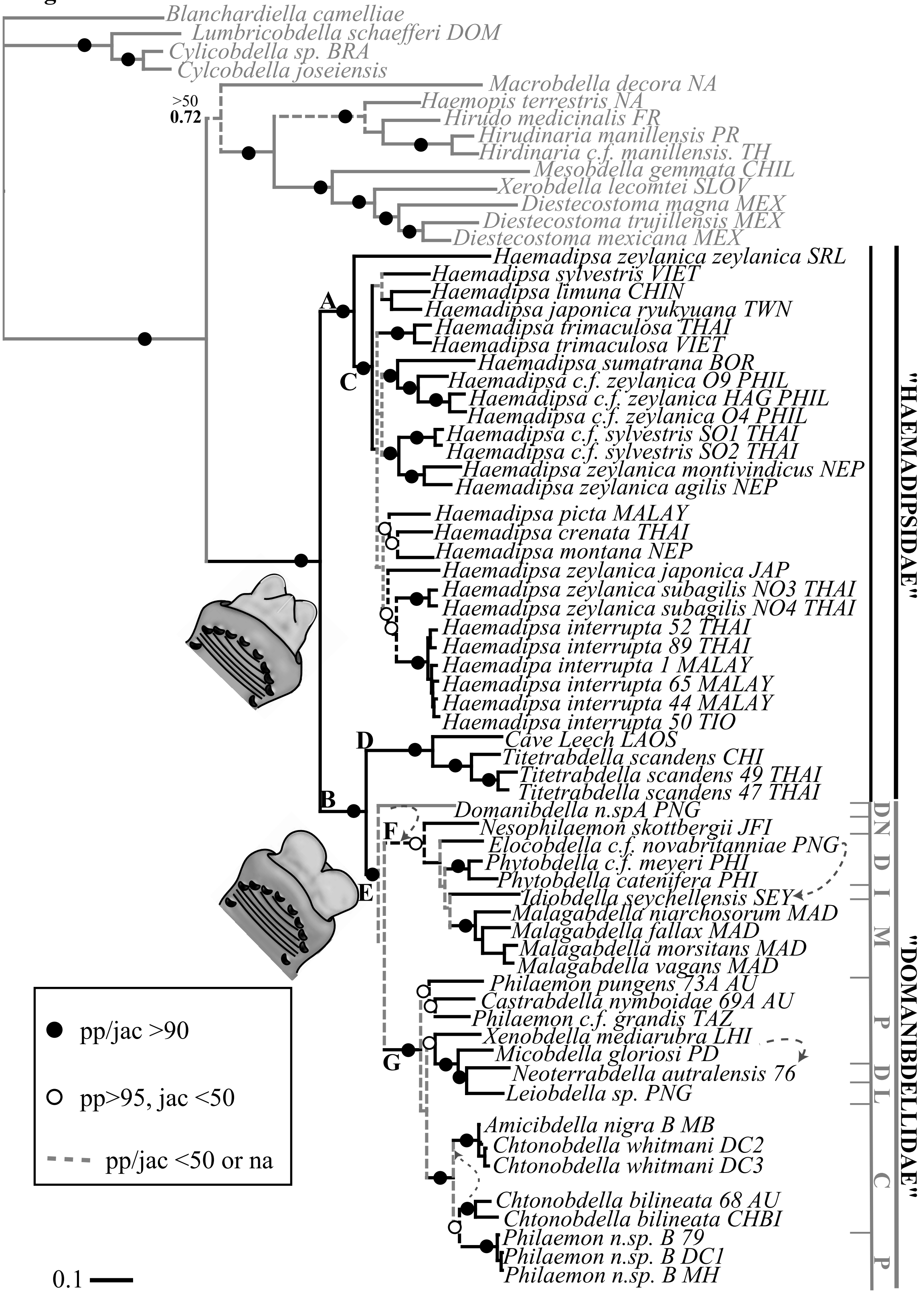
MP analysis of the combined 18S rDNA, 28S rDNA and *COI* data yielded a single tree (5423 characters of which 1360 were parsimony informative; L=8716) with a topology that was nearly identical to the BI tree (Fig. 19). With respect to traditional classification schemes (*sensu* Richardson, 1975; 1978; and Sawyer 1986), the majority of the proposed taxonomic groupings (*sensu* Richardson, 1975) were not recovered in the phylogenetic reconstruction and the following were rendered paraphyletic: Haemadipsidae, Chtonobdellinae, Domanibdellinae and Philaemoninae. Richardson's (1975) and Sawyer's (1986) divisions of haemadipsid leeches on the basis of jaw morphology were not mutually exclusive. That is, duognathous leeches (jac=100; pp=1.00) were not independent, but rather were nested within a trignathous haemadipsid clade. Overall, haemadipsid leeches were strongly supported as a monophyletic clade (jac=100; pp=1.00) and subdivided into two monophyletic clades (Clades **A** and **B**).

The five-annulate, trignathous *Haemadipsa* clade (Clade **A**; jac=100; pp=1.00) included all species of the genus distributed throughout India, East Asia and South East Asia. *Haemadipsa zeylanica zeylanica* from Sri Lanka was consistently supported as the sister taxon (jac=100; pp=1.00) to all remaining *Haemadipsa* species (Clade **B**; jac=93;

FIGURE 19.

BI tree topology based on combined data analysis for 18S rDNA, 28S rDNA and COI (5p). Grey arrows indicate variable node or taxon position resulting from the MP analyses. C=Chtonobdellinae; D=Domanibdellinae; I=Idiobdellidae; L=Leiobdellinae; M=Malagabdellinae; N=Nesophilaemonidae; P=Philaemoninae.

Figure 19.


Blanchardiella camelliae
Lumbricobdella schaefferi DOM
Cylicobdella sp. BRA
Cylcobdella joseiensis
>50
0.72
Macrobdella decora NA
Haemopis terrestris NA
Hirudo medicinalis FR
Hirudinaria manillensis PR
Hirdinaria c.f. manillensis. TH
Mesobdella gemmata CHIL
Xerobdella lecomtei SLOV
Diestecostoma magna MEX
Diestecostoma trujillensis MEX
Diestecostoma mexicana MEX
A
C
Haemadipsa zeylanica zeylanica SRL
Haemadipsa sylvestris VIET
Haemadipsa limuna CHIN
Haemadipsa japonica ryukyuana TWN
Haemadipsa trimaculosa THAI
Haemadipsa trimaculosa VIET
Haemadipsa sumatrana BOR
Haemadipsa c.f. zeylanica O9 PHIL
Haemadipsa c.f. zeylanica HAG PHIL
Haemadipsa c.f. zeylanica O4 PHIL
Haemadipsa c.f. sylvestris SO1 THAI
Haemadipsa c.f. sylvestris SO2 THAI
Haemadipsa zeylanica montivindicus NEP
Haemadipsa zeylanica agilis NEP
Haemadipsa picta MALAY
Haemadipsa crenata THAI
Haemadipsa montana NEP
Haemadipsa zeylanica japonica JAP
Haemadipsa zeylanica subagilis NO3 THAI
Haemadipsa zeylanica subagilis NO4 THAI
Haemadipsa interrupta 52 THAI
Haemadipsa interrupta 89 THAI
Haemadipa interrupta 1 MALAY
Haemadipsa interrupta 65 MALAY
Haemadipsa interrupta 44 MALAY
Haemadipsa interrupta 50 TIO
D
Cave Leech LAOS
Titetrabdella scandens CHI
Titetrabdella scandens 49 THAI
Titetrabdella scandens 47 THAI
B
E
F
Domanibdella n.spA PNG
Nesophilaemon skottbergii JFI
Elocobdella c.f. novabritanniae PNG
Phytobdella c.f. meyeri PHI
Phytobdella catenifera PHI
Idiobdella seychellensis SEY
Malagabdella niarchosorum MAD
Malagabdella fallax MAD
Malagabdella morsitans MAD
Malagabdella vagans MAD
Philaemon pungens 73A AU
Castrabdella nymboidae 69A AU
Philaemon c.f. grandis TAZ
G
Xenobdella mediarubra LHI
Micobdella gloriosi PD
Neoterrabdella autralensis 76
Leiobdella sp. PNG
Amicibdella nigra B MB
Chtonobdella whitmani DC2
Chtonobdella whitmani DC3
Chtonobdella bilineata 68 AU
Chtonobdella bilineata CHBI
Philaemon n.sp. B 79
Philaemon n.sp. B DC1
Philaemon n.sp. B MH
"HAEMADIPSIDAE"
"DOMANIBDELLIDAE"
DN
D
I
M
P
DL
C
P
● pp/jac >90
○ pp>95, jac <50
- - - pp/jac <50 or na
0.1

pp=1.00). The basal relationships within Clade **B** were poorly supported in both analyses; though there was some resolution for derived clades. The Vietnamese *Haemadipsa sylvestris* was found to be poorly supported (jac=no support (<50); pp=0.90) as sister to the Chinese *Haemadipsa limuna* + Taiwanese *Haemadipsa japonica ryukyuana* (jac=99; pp=0.66). The Bornean *Haemadipsa sumatrana* was consistently found as the sister taxon (jac=71; pp=1.00) to *Haemadipsa c.f. zeylanica* from the Philippines, which was also found to be monophyletic (jac=95; pp=1.00). *Haemadipsa c.f. sylvestris* (SO1 / SO2) from Southern Thailand was sister (pp=1.00) to the Nepalese *Haemadipsa montivindicus* + *Haemadipsa agilis*. Although the relationship of the Nepalese *Haemadipsa montana* was strongly supported in the BI analyses (pp=1.00) as sister to *Haemadipsa crenata* from Northern Thailand + *Haemadipsa picta* from Malaysia (pp=0.96); there was no jackknife support for this relationship (<50). The placement of *Haemadipsa japonica* from Japan was not well supported in either analysis, however it placed as either sister to the *Haemadipsa interrupta* clade (jac=<50) or sister to *Haemadipsa interrupta* + *Haemadipsa zeylanica subagilis* from Thailand (pp=0.98).

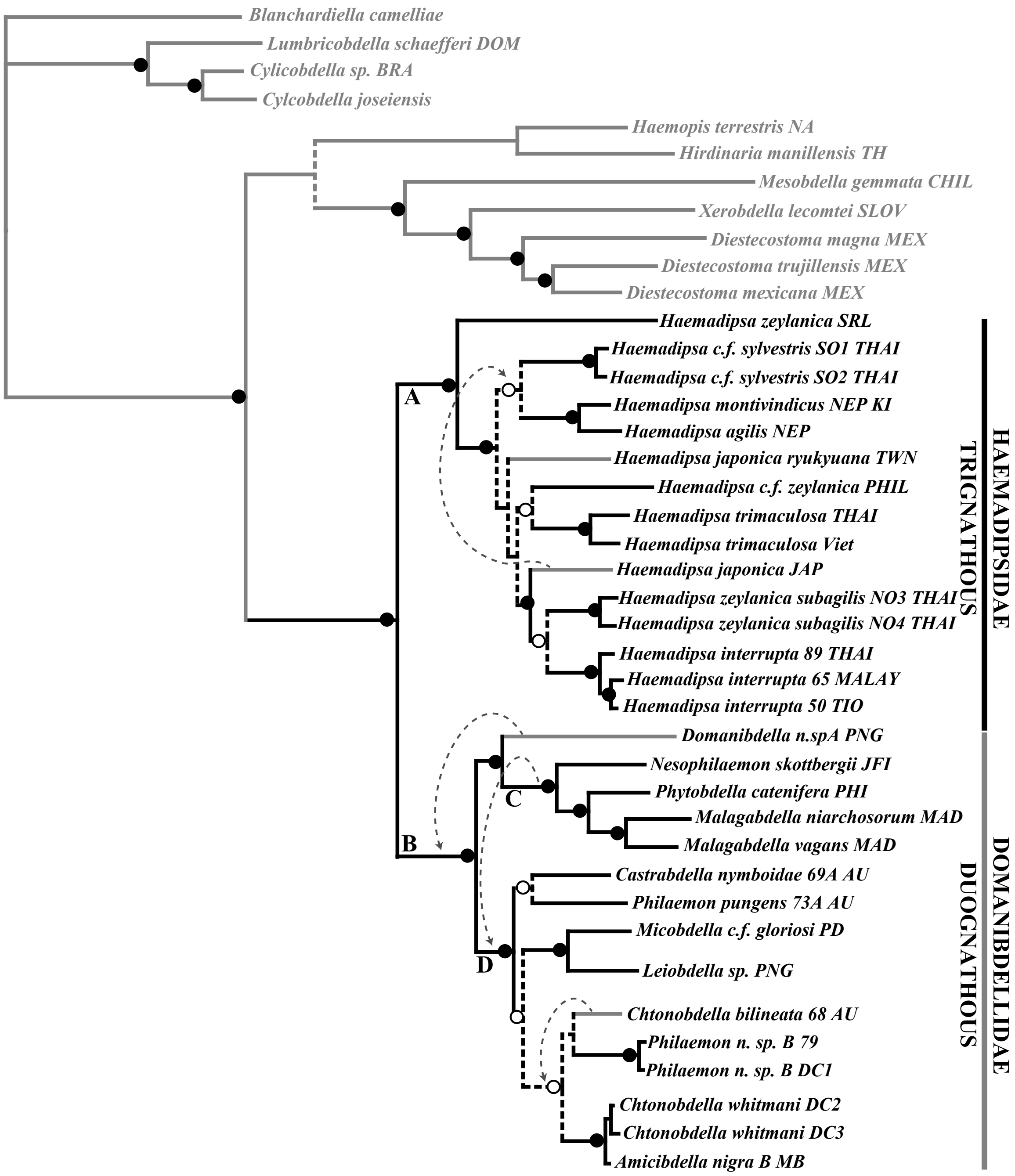
The remaining representatives of Haemadipsidae were placed in Clade **B**; jac=100; pp=1.00), with two major subclades. The first, Clade **D**, included the Southeast Asian four-annulate leeches from the genus *Titetrabdella* (jac=100; pp=1.00) from China and Thailand and sister to those was a five-annulate cave leech from Laos (jac=100; pp=1.00). The position of Clade **D** differed in the MP analysis where it was inferred as sister to Clade **G**, but was poorly supported (jac=64). Clade **E** was much more complex. *Domanibdella* n. sp. from Papua New Guinea (PNG) was poorly supported as either sister to the remaining duognathous leeches (pp=<.50) or as sister to *Nesophilaemon* *skottsbergii* from the Juan Fernandez Islands (jac=<50). Clade **F** included a monophyletic *Malagabdella* clade (jac=98; pp=1.00), with the position of the remaining members being unresolved: the seven-annulate *Planobdella* species from PNG, the six-annulate *Phytobdella* species from Philippines (jac=100; pp=1.00), five-annulate *Idiobdella seychellensis* from the Seychelles Islands and four-annulate *Nesophilaemon skottsbergii* from the Juan Fernandez Islands. Node **G** (jac=100; pp=1.00) included three clades that were strongly supported, however the relationship between these clades was not. The leeches assigned to the Australian four-annulate genera were found in two clades. *Castrabdella nymboidae* + *Philaemon* c. f. *grandis* from Tasmania (pp=0.82) was sister to *Philaemon pungens* (pp=1.00) and were found together in the BI topology, however, this relationship was not recovered in the MP topology. *Philaemon* n. sp. "brown" was sister to *Chtonobdella bilineata* (pp=0.95), which together were sister to *Amicibdella nigra* + *Chtonobdella whitmani*. In the MP topology the *Amicibdella* clade was switched with *Chtonobdella bilineata* (jac=<50). *Xenobdella mediarubra* (Philaemoninae) from Lord Howe Island (pp=1.00) was sister to a clade including the Papuan *Leiobdella* sp. + *Neoterrabdella australensis* (pp=1.00), which together were sister to *Micobdella c.f. gloriosi* (pp=1.00)

The inclusion of COIII to the all taxon dataset increased the number of trees to 20, with a tree score of 10832 (6015 characters, of which 1664 were parsimony informative), and producing trees with similar overall topologies to the combined 18S, 28S and COI analyses and which did not improve the support values of various clades (not shown). The restricted analysis (n=44) with all available data produced two trees with a length of 8241 steps (6137 characters of which 1569 were parsimony informative). Figure 20

FIGURE 20.

BI tree topology based on combined data analysis for 18S rDNA, 28S rDNA and COI and *COIII* (8p). Grey arrows indicate variable node or taxon position resulting from the MP analyses.

Figure 20.


Blanchardiella camelliae
Lumbricobdella schaefferi DOM
Cylicobdella sp. BRA
Cylcobdella joseiensis
Haemopis terrestris NA
Hirdinaria manillensis TH
Mesobdella gemmata CHIL
Xerobdella lecomtei SLOV
Diestecostoma magna MEX
Diestecostoma trujillensis MEX
Diestecostoma mexicana MEX
Haemadipsa zeylanica SRL
Haemadipsa c.f. sylvestris SO1 THAI
Haemadipsa c.f. sylvestris SO2 THAI
Haemadipsa montivindicus NEP KI
Haemadipsa agilis NEP
Haemadipsa japonica ryukyuana TWN
Haemadipsa c.f. zeylanica PHIL
Haemadipsa trimaculosa THAI
Haemadipsa trimaculosa Viet
Haemadipsa japonica JAP
Haemadipsa zeylanica subagilis NO3 THAI
Haemadipsa zeylanica subagilis NO4 THAI
Haemadipsa interrupta 89 THAI
Haemadipsa interrupta 65 MALAY
Haemadipsa interrupta 50 TIO
Domanibdella n.spA PNG
Nesophilaemon skottbergii JFI
Phytobdella catenifera PHI
Malagabdella niarchosorum MAD
Malagabdella vagans MAD
Castrabdella nymboidae 69A AU
Philaemon pungens 73A AU
Micobdella c.f. gloriosi PD
Leiobdella sp. PNG
Chtonobdella bilineata 68 AU
Philaemon n. sp. B 79
Philaemon n. sp. B DC1
Chtonobdella whitmani DC2
Chtonobdella whitmani DC3
Amicibdella nigra B MB
A
B
C
D
HAEMADIPSIDAE
TRIGNATHOUS
DOMANIBDELLIDAE
DUOGNATHOUS

shows the BI tree with alternate position of clades and taxa relative to the MP tree (not otherwise shown). Overall a similar structure to Figure 19 was found including: 1) *Haemadipsa zeylanica zeylanica* as sister to all *Haemadipsa* species, with the within group relationships not well resolved (Clade **H**); 2) a monophyletic duognathous clade (Clade **I**); 3) *Domanibdella* n. sp. was either sister to the remaining duognathous clade or sister to Clade **J**; 4) *Malagabdella* species are derived within Clade **J**; 5) *Philaemon* n. sp. "brown" are sister to *Chtonobdella* species.

DISCUSSION

Since the late 1990's, traditional views of the classification of leeches have been brought to new light, particularly for bloodfeeding terrestrial leeches. Trontelj et al. (1999) was the first to show that the European *Xerobdella lecomtei* was not sister to IndoPacific haemadipsids, based on molecular data (18S rDNA), contrary to traditional classification schemes (e.g. Blanchard, 1917; Sawyer, 1986). Borda and Siddall (2004) substantiated this with the inclusion of the Chilean *Mesobdella gemmata*, which was found to be the sister taxon of *Xerobdella lecomtei* (based on morphology and molecular data). Borda et al. (Chapter 5) expanded the taxonomic sampling of non-IndoPacific haemadipsoid leeches to include three Mexican *Diestecostoma* species and *Nesophilaemon skottsbergii* from the Juan Fernandez Islands for their phylogenetic work. Their results established that Moore's (1946) Xerobdellidae was valid and should be formally erected to include members of the genera *Mesobdella*, *Diestecostoma* and *Xerobdella*. Borda et al. (In Press) also determined that *Nesophilaemon skottsbergii* was unequivocally a haemadipsid leech. These relationships were not only supported by molecular data, but also were corroborated by external and reproductive morphological characteristics (Borda et al., In Press; see also Moore, 1924).

The membership of the family or families associated with bloodfeeding terrestrial leeches, particular from the IndoPacific, has been debated among workers, resulting in multiple revisions of the group (Blanchard, 1917; Moore, 1924; Caballero, 1940; Soós, 1967; Richardson, 1969, 1971; 1975, 1978; Ringuelet, 1976; 1982; Sawyer, 1986). In light of the phylogenetic reconstruction presented here, Haemadipsidae should formally be recognized for the bloodfeeding terrestrial leeches from the IndoPacific, contrary to Sawyer's (1986) definition of the family and in favor of Richardson's (1975) higher-level classification (in part). Although, the phylogeny lacks exact correspondence to Richardson's (1975) subdivisions, his revision best represents their complex biogeographic history and morphological diversity.

Haemadipsidae

Species of the trignathous genus *Haemadipsa* Tennet, 1859 are the most wide-spread and diverse (in terms of described species) members of Haemadipsidae. *Haemadipsa* species are distributed throughout (yet are limited to) the Indian subcontinent, East Asia and Southeast Asia (Southern China, Vietnam, Borneo, Burma, Cambodia, Thailand, Japan, Laos, Taiwan, Philippines and the Malay peninsula). Encounters with these much-maligned annelids have established them as pests of man throughout tropical Asia. Despite their prevalence, the paucity of external diagnostic characters has led to a poor understanding of the classification of *Haemadipsa* species. These species typically have been distinguished from other haemadipsid genera in being five-annulate, with five annuli between the male (XI b5/b6) and female gonopore (XII b5/b6), and the characteristics of the area between the eyespots (Blanchard, 1917; Moore, 1924; Soós, 1967; Keegan et al., 1968; Richardson, 1975). Moore (1924) recognized five species: *Haemadipsa zeylanica, Haemadipsa montana, Haemadipsa sylvestris, Haemadipsa ornata* and *Haemadipsa dussumieri*. Each were recognized based on the combination of: a) color pattern; b) the presence or absence of furrow pits; c) the presence or absence of a complete or partial annulus between the third and fourth eyespot pairs; and d) the presence or absence of a well-developed prehensile lobe. Unfortunately, color patterns often are lost during preservation, resulting in species designation on the basis of the remaining external characteristics, several of which overlap between taxa (Keegan et al., 1968). Moore (1924) and others (Keegan et al., 1968; Nessemann and Sharma, 1996; 2001) also recognized several subspecies of *Haemadipsa zeylanica* and one subspecies of *Haemadipsa sylvestris*, based on variations of the aforementioned characters. To complicate matters, since the late 1970's several new *Haemadipsa* species have been described from China, India and Thailand using color pattern as one of the main diagnostic characters (Song et al., 1977; Tan 1980, 1983, 1988; Liu, 1984; Bandyopadhyay and Mandal, 2006; Ngamprasertwong et al., In Press).

The lack of resolution within Clade **C** appears to represent a rapid radiation for *Haemadipsa* species, particularly for species distributed throughout Asia. The phylogeny also reveals an underestimate of the known diversity of *Haemadipsa* species. The lack of discrete morphological characters, such as external color pattern, by which to distinguish between *Haemadipsa* species confounded identification to species for several of the *Haemadipsa* specimens included in this study. For example, notwithstanding the similarities in general morphology, the specimens identified in this study as *Haemadipsa zeylanica* and *Haemadipsa sylvestris* were not monophyletic. Some species (e.g. *Haemadipsa crenata*, *Haemadipsa trimaculosa*) were identified on the basis of color patterns and were found to be genetically distinct from other *Haemadipsa* species. Until a more thorough evaluation of discrete morphological characteristics is accomplished, with special emphasis of the reproductive systems, this phylogenetic hypothesis only provides a stepping-stone by which to begin to re-evaluate the classification of *Haemadipsa* species.

Titetrabdella scandens Moore 1938 is the only four-annulate, trignathous leech overlapping in range with East Asian and Southeast Asian *Haemadipsa* species. The phylogenetic hypothesis suggests that this trignathous species is not closely related to *Haemadipsa* species. Instead *Titetrabdella scandens* was sister to the only other non-*Haemadipsa* trignathous representative, a previously undescribed five-annulate Cave Leech n. sp. from Northern Laos. *Titetrabdella scandens* and the Cave Leech n. sp. can be distinguished from *Haemadipsa* species on the basis of a reduced number of annuli between the gonopores (3 1/2 and 2 annuli, respectively) and by their unique reproductive morphologies that deviate from the typical haemadipsoid form in having thick sperm ducts and a short oviducal glandular sac. This form was also seen in *Nesophilaemon skottsbergii* (Borda et al., In Press). In order to fully assess the relationships between these trignathous species, further study is still needed with the incorporation of a thorough evaluation of morphological characters.

The IndoPacific Haemadipsidae was inferred to be monophyletic, in contrast to Richardson's hypothesis. Specifically, the trignathous Indian and Asian species he placed in Haemadipsidae, *Haemadipsa* species and *Titetrabdella* species) were paraphyletic. It would, therefore, seem reasonable to re-establish a subfamily Haemadipsinae for *Haemadipsa* species, and to erect a new subfamily Titetrabdellinae for other trignathous bloodfeeding terrestrial leeches. Titetrabdellinae was strongly supported as sister to the duognathous leeches, which emphasizes the paraphyly of trignathous leeches and that duognathous leeches are likely derived from a three-jawed ancestral stock. This is contrary to Richardson (1975) who proposed that haemadipsids were derived from a two-jawed macrophagous terrestrial ancestor and the third jaw was the result of the modification of the dorsomedial pad.

Domanibdellidae and Idiobdellidae

Richardson (1975, 1978) established Domanibdellidae for 21 duognathous genera and placed the species from the Seychelles Islands (i.e. *Idiobdella*) in Idiobdellidae. Richardson (1975, 1978) further subdivided Domanibdellidae into: Domanibdellinae Richardson 1975, Chtonobdellinae Richardson 1975, Philaemoninae Richardson 1975, Leiobdellinae Richardson 1975 and Malagabdellinae Richardson 1978, based on geography and characteristics of the anterior and posterior annulation series. The phylogeny inferred Domanibdellidae as paraphyletic, with Idiobdellidae nested within the domanibdellid Clade **F**. With the exception of Malagabdellinae, Richardson's subfamilies were not found to be monophyletic.

Borda et al. (In Press) showed that the four-annulate *Nesophilaemon skottsbergii* belonged to Haemadipsidae and was nested among the duognathous leeches in a clade with *Malagabdella fallax* from Madagascar and *Idiobdella seychellensis* from the Seychelles Islands. Johansson (1924) proposed that *Nesophilaemon skottsbergii* was allied to members of the Australian, four-annulate genus *Philaemon*. In this study, *Philaemon* species were included for the first time to examine the relationship between *Nesophilaemon* and *Philaemon*. Despite the inclusion of *Philaemon* species in the analyses, the phylogeny corroborated the relationships found by Borda et al. (In Press). That is, that *Nesophilaemon skottsbergii* is not related to Australian species, nor to *Philaemon* species. Instead, *Nesophilaemon skottsbergii* was again found in Clade **F** among duognathous species from the Philippines, Papua New Guinea, Madagascar and the Seychelles Islands.

The family Idiobdellidae was represented by a single species, but is clearly nested within a duognathous clade **F**. As such, continued acceptance of the family status for the Seychelles taxon is not valid. The BI topology inferred the placement of *Idiobdella seychellensis* as sister to the leeches from Madagascar, corroborating Borda et al. (In Press), however, the MP analysis found the non-auriculate *Elocobdella novabrittaniae* was sister to the non-auriculate *Idiobdella seychellensis* in the MP analysis; however, the relationship from each analysis were poorly supported. This is likely an artifact due to insufficient data for *Idiobdella seychellensis*, which was represented here only by partial sequences for COI and 28S.

Clade **F** included the most diverse representation of duognathous species and genera, including the seven annulate *Elocobdella*, the six annulate *Phytobdella*, the five annulate *Domanibdella*, *Malagabdella* and *Idiobdella* and the four annulate *Nesophilaemon.* The placement of the new species of *Domanibdella* from Papua New Guinea was not stable. *Domanibdella* n. sp. was found either to be sister to all dougnathous leeches or sister to Clade **F**. Additionally, the basal relationships within this clade were not well supported. Therefore, the current available sampling and data for these species causes some difficultly in explaining the relationships within Clade F.

Within the monophyletic Australian Clade **G**, relationships were not well supported. The MP and BI topologies differed with respect to the basal relationships within this clade. The placements of *Xenobdella mediarubra, Philaemon pungens, Castrabdella nymboidae* and *Philaemon c.f. grandis* were well supported in the BI analyses, though not by MP. Surprisingly, the five-annulate *Chtonobdella* and *Amicibdella* were paraphyletic with respect to *Philaemon* n. sp. The current taxonomic sampling suggests that Richardson's (1975) monotypic genera (e.g. *Amicibdella, Castrabdella, Elocobdella, Micobdella, Xenobdella*) represented here may be in need of revision. Therefore, Sawyer's (1986) revision (and synonymization) of Richardson's (1975) haemadipsid genera may have precedence. Based on the placement of *Philaemon* n. sp., a new four-annulate genus may need to be established, however without a better understanding of the relationships among four-annulate species in general, the taxonomic status of this species remains uncertain.

Biogeography and Evolution of Haemadipsidae

The origin and mode of diversification of haemadipsids throughout the IndoPacific has been cause for much speculation among workers. Moore (1924) proposed that bloodfeeding terrestrial leeches evolved from a freshwater sanguivorous, likely amphibious, leech (e.g. *Hirudinaria*) found in Northern India during the Tertiary period, followed by dispersal to other IndoPacific regions on birds. Richardson (1975) proposed that haemadipsids were derived from a duognathous ancestor found on a former landmass that has since fragmented into New Guinea and the islands of Oceana. Based on the current distribution of Haemadipsidae, one might postulate that they were Gondwanan in origin, with speciation via vicariance associated with the breakup of Gondwanan landmasses. The phylogeny, however, only provides some insights towards understanding the biogeography and species diversity for Haemadipsidae.

Haemadipsid leeches are well supported as having a single origin; however, the diversification of Haemadipsidae remains unresolved. Though haemadipsids are widespread throughout the IndoPacific, the phylogeny reveals several localized speciation events. Based on the phylogenetic estimate and the known distribution of trignathous haemadipsid leeches, it appears as if haemadipsids originated in Asia, or India. *Haemadipsa* species appear to be Indian in origin, based on the phylogeny that is reminiscent of diversification associated with India's collision with Eurasia followed by an 'out of India' dispersal event. The lack of resolution among *Haemadipsa* species might represent a rapid radiation associated with this event. The placement of the Asian Titetrabdellinae as the sister group to duognathous leeches points towards an Asian origin for Clade **B** (Titetrabdellinae + Duognathous leeches) and Clade **E** (i.e. duognathous leeches). This is inconsistent with a Gondwanan origin for Haemadipsidae. If haemadipsids originated in Asia or India, the presence of haemadipsids on landmasses that have been isolated for greater than 65 million years (e.g. Madagascar, Seychelles Islands, Australia) or on volcanic islands that are estimated to be less than 2 million years old (e.g. Juan Fernandez Islands) cannot be easily explained by vicariance, but rather by long distance dispersal mechanisms (e.g. rafting, birds). This would require that duognathous species were able to cross oceanic barriers to arrive at their current distribution (e.g. *Nesophilaemon skottsbergii*, and *Malagabdella* species).

The diversification of duognathous leeches is similarly complex. Found in two major clades, duognathous leeches were associated with an exclusive Australian radiation (Clade **G**), while the second, Clade **F**, includes species found in isolation on islands throughout the IndoPacific. The close relationships between species found in Madagascar, the Seychelles Islands, Papua New Guinea, the Philippines, and even more bizarrely, the Juan Fernandez Islands are completely anomalous. Although, long distance dispersal on birds is one plausible explanation for the distribution of these species, this hypothesis is problematic given the feeding behavior of haemadipsid leeches. Typically, after a leech has obtained a blood meal, they voluntarily release from their host after about an hour. Neither do all of these taxa presently feed on migratory birds.

CONCLUSIONS

The incongruence between BI and MP analyses and the lack of unambiguous biogeographic patterns within some clades (i.e. Clade **C**; Clade **F**) may be due to insufficient sampling from some major geographic areas (e.g. mainland India, Indonesia) and limitations of current available data (i.e. molecular data alone). The current sampling and phylogenetic estimation for Haemadipsidae impede a definitive assessment of dispersal events for this group, though some patterns were suggested. Although the phylogeny suggests that Haemadipsidae may have an origin in a trignathous, sanguivorous stock from Asia or India, the shared ancestry of haemadipsid leeches remains unresolved without a better understanding of the placement of Haemadipsidae among Hirudiniformes (Borda and Siddall, 2004).

Future work should include additional sampling from missing geographic areas (mainland India, Indonesia, islands of Oceana), the exploration of new genes with variable rates of evolution to help resolve the relationships between species, as well as the incorporation of morphological data. The current phylogenetic hypothesis establishes a foundation of continuing work and the framework by which to begin addressing the taxonomic and biogeographic complexities of what still remains a poorly understood annelid group.

CHAPTER 7

Summary

Before the initiation of this study, major advances towards our knowledge and understanding of the evolutionary history of leeches had already been made, establishing the foundation for future phylogenetic work (Siddall and Burreson, 1995, 1996, 1998; Apakupakul et al., 1999; Light and Siddall, 1999; Trontelj et al., 1999). Although previous work had provided a general overview of the evolution of leeches at the subordinal and family levels, a comprehensive examination of the phylogenetic relationships of Arhynchobdellida was still lacking. Apakupakul et al. (1999) provided the first phylogenetic hypothesis for the major groups of leeches based on combined molecular (18S rDNA and COI) and morphological data. However, their analyses lacked representation of most arhynchobdellid families, particularly for Hirudiniformes. Arhynchobdellid leeches are remarkably diverse in life history strategies, geographic distributions and morphological characters, providing a rich substrate for investigating the evolutionary relationships this group. The major goals of this study were two-fold: 1) to estimate a phylogenetic hypothesis for this group and to increase the representation of all of the traditionally recognized arhynchobdellid families based on the most recent classification by Sawyer (1986); and 2) to examine the phylogenetic relationships of bloodfeeding terrestrial leeches of the arhynchobdellid family Haemadipsidae (sensu Sawyer, 1986).

This study has provided the broadest taxonomic sampling, to date, of globally distributed arhynchobdellid leeches. In continuation with previous phylogenetic work, the relationships of arhynchobdellid leeches also were examined using molecular data and morphological data (Borda and Siddall, 2004a, b). The hypothesis conflicted with most traditional classification schemes for Arhynchobdellida (sensu Sawyer, 1986).

Specifically, the families Haemadipsidae, Haemopidae and Hirudinidae were not found to be monophyletic, Erpobdelliformes and Hirudiniformes were each found to be monophyletic, however the placement of the hirudiniform monotypic family Americobdellidae remains unresolved. Therefore, these results have provided a tool in order to re-evaluate traditional classification schemes in light of phylogeny. In addition, with a better understanding of the phylogenetic relationships of arhynchobdellid leeches, and Hirudinida in general, we can now infer that leeches likely have a common origin from an ectoparasite of vertebrate blood, with several convergent losses of sanguivory for a carnivorous way of life throughout their evolutionary history. There also may have been a secondary return to sanguivory for Hirudiniformes coincident with the development of armed jaws. Leeches appear to have evolved from a freshwater ancestor, with a movement into marine environments from an ancestor of the piscicolid leeches and onto land for the ancestor of hirudiniform leeches. The latter is further corroborated by mode of copulation and cocoon deposition in the group.

With respect to the bloodfeeding terrestrial leeches, the Haemadipsidae have long confused leech systematists and seem to defy sensible biogeographic interpretation. The family Haemadipsidae has consisted of problematic taxa that neither followed the typical IndoPacific distribution for the group, nor coincided with diagnostic characters used to define the family. Therefore, it was surprising when bloodfeeding terrestrial leeches were found in two clades essentially separated geographically and upon closer inspection, morphologically (Trontelj et al., 1999; Borda and Siddall, 2004; Borda et al., In Press). Borda and Siddall (2004) proposed the resurrection of the subfamily Xerobdellinae and raising it to family to accommodate non-IndoPacific leeches, while the IndoPacific species should be restricted to Haemadipsidae. At the time, the lack of a broader sampling of IndoPacific and New World species impeded a formal revision. Therefore, in order to critically assess prior hypotheses of bloodfeeding terrestrial leeches (Trontelj et al. 1999; Borda and Siddall, 2004), an increased sampling of non-IndoPacific (among other) genera was expanded to include Meso American *Diestecostoma* species and *Nesophilaemon skottsbergii* from the Juan Fernandez Archipelago. The results showed that a monophyletic family Haemadipsidae must exclude species of *Xerobdella* (Europe), *Mesobdella* (Chile) and *Diestecostoma* (MesoAmerica). Based on these results and corroborating morphology (i.e. reproductive and nephridial characters), Xerobdellidae could be formally resurrected for the species of those three genera. Additionally, this study also revealed that *Idiobdella seychellensis* from the Seychelles Islands and *Nesophilaemon skottsbergii* belong in Haemadipsidae, allied to the Malagasy *Malagabdella* species, notwithstanding the lack of respiratory auricles in the former and the geographic proximity to the Chilean *Mesobdella gemmata* in the latter.

Our current knowledge of the evolutionary history of bloodfeeding terrestrial leeches from the IndoPacific indicates that there was a single origin for this group, but the geographic center of origin remains unclear. The distribution of Haemadipsidae coincides with a Gondwanan origin; however the phylogenetic estimates deviate from expected Gondwanan relationships explained only by vicariance. Though the phylogeny is limited by the current taxonomic sampling, haemadipsid leeches are seen to have a more complex biogeographic history than previously thought. The hypothesis also supported the notion that duognathous leeches are derived within Haemadipsidae and share a common ancestry with a trignathous ancestor. This is consistent with the plesiomorphic trignathous condition across hirudiniform leeches.

Although, the results from this study leave many questions left unanswered, it provides a rich framework for further study. The currently available data have already 1) revealed the genetic and morphological diversity across this group, as well as newly identified biodiversity; 2) provided a foundation for phylogeographic work, particularly for *Haemadipsa* species; 3) provided a means to begin examining the ecological niche requirements of a highly specialized tropical annelid group; and 4) provided some insights in order to more critically evaluate the systematics of Haemadipsidae that may arrive to a more stable classification for this group.

As we continue to increase the taxonomic sampling from key biogeographic areas, better our understanding the morphological diversity exhibited by haemadipsid leeches, my hope is to one day unveil a clearer picture of the evolutionary history of bloodfeeding terrestrial leeches

DISCLAIMER

New species and new family names presented in Chapter 6 are not intended to formally establish new names.

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AUTOBIOGRAPHICAL STATEMENT

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