

Higher Level Relationships of Leeches (Annelida: Clitellata: Euhirudinea) Based on Morphology and Gene Sequences

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Received September 24, 1998; revised December 29, 1998

The evolutionary patterns of divergence of seven euhirudinean families were investigated by cladistic analysis of 33 euhirudinean species. *Oligochaetes*, *Acanthobdella peledina*, and branchiobdellidans were included as outgroup taxa. Cladistic analysis employed 1.8 kb of nuclear 18S ribosomal DNA and 651 bp of mitochondrial cytochrome *c* oxidase subunit I in addition to morphological data. The use of two molecular data sets, one nuclear gene and one mitochondrial gene, as well as morphological data combined historical information evolving under a variety of different constraints and therefore was less susceptible to the biases that could confound the use of only one type of data. Results suggest that the nuclear 18S rDNA gene yields a meaningful historical signal for determining higher level relationships. The more rapidly evolving CO-I gene was informative for recent or local areas of the evolutionary hypothesis, such as within-family relationships. Analyses combining all data from the three character sets yielded one most-parsimonious tree. Most of the higher taxa in recent leech systematics were well corroborated in the resulting topology. However, these results suggested paraphyly of the order Rhynchobdellida, which contradicts the presence of a proboscis as a synapomorphy. The medicinal leech family Hirudinidae was polyphyletic because Haemadipsidae and Haemopidae each have a hirudinid ancestor. In addition, all but one of the genera within the family Erpobdellidae must be either abandoned or renamed. Unusual findings included compelling evidence of historical plasticity in blood-feeding behavior, having been lost at least four times in the course of euhirudinean evolution. Biogeographic patterns supported a New World origin for Arhynchobdellida. © 1999 Academic Press

INTRODUCTION

Euhirudinea, or the true leeches, are clitellate annelids exhibiting a marked scope of diversity, including ectocommensalism, parasitic sanguivory, and predatory life-history strategies, as well as a variety of

reproductive behaviors. They occur in habitats that range from terrestrial to aquatic (both marine and freshwater) environments and are found on all continents. The remarkable diversity in morphology and behavior among species of leeches has been of interest to several fields of biology. Sanguivorous leeches recently have been the focus of pharmaceutical companies seeking to expand their repertoire of anticoagulants in light of their biomedically important salivary components and clinical use in microsurgeries to prevent blood clot formation (e.g., Walsmann and Markwardt, 1985; Lent, 1986; Munro *et al.*, 1992). As well as being parasites themselves, some leech species serve as vectors of blood parasites for aquatic vertebrates. Blood parasites have been thought to coevolve with their respective leech hosts as a result of long-term ecological associations (Siddall and Bureson, 1995). Leeches also are used extensively in neurobiological and developmental studies (e.g., Shankland, 1991; Aisemberg *et al.*, 1993; Ramirez *et al.*, 1995). Because nonsanguivorous species play a role in soil mineral balance as well as recycling the benthos of lakes and streams, particularly in eutrophic or polluted situations, they have been used as environmental stress indicators (Della Croze, 1955; McCall and Fisher, 1980). The systematics of leeches is poorly known for most regions of the world, and a phylogenetic system is needed to develop a database which conservation and biomedical initiatives can utilize.

Leeches are among the most poorly studied invertebrate taxa with respect to their evolutionary histories. The within-group relationships of leeches have been neglected by most annelid systematists (e.g., Apathy, 1888; Selensky, 1915; Wendrowsky, 1928; Livanow, 1931; Autrum, 1939). Recent considerations of the systematics of leeches (e.g., Sawyer, 1986; Siddall and Bureson, 1995, 1998; Light and Siddall, 1999) suggest that some widely accepted groupings may be artificial. Current species groups are not monophyletic; therefore understanding relationships among species requires resolution at higher taxonomic levels.

The taxonomic composition of this study was de-

signed to cover the full scope of higher systematic groupings of leeches. Euhirudinea consists of nine principle families that traditionally are divided into two taxonomic groups (Fig. 1). The families Glossiphoniidae, Ozobranchidae, and Piscicolidae constitute the order Rhynchobdellida, so named for their possession of a protrusible muscular proboscis with which they feed. The members of the order Arhynchobdellida are marked by the lack of a proboscis, and traditionally this taxon consists of two suborders: the unarmed nonsanguivorous Erpobdelliformes and the Hirudiniformes, which includes the medicinal leech family Hirudinidae, the terrestrial Haemadipsidae, and the predaceous Haemopidae. Relationships have been assessed at the familial level using either morphology (Siddall and Burrenson, 1995), or life history (Siddall and Burrenson, 1996), or mitochondrial sequence data (Siddall and Burrenson, 1998) on limited taxonomic subsets. Objectives of this project were to assess the monophyly of groups identified in recent taxonomic studies of leeches in a total evidence framework using multiple sources of data and a broader taxonomic scope.

MATERIALS AND METHODS

Taxa

The taxa sampled and their localities are listed in Table 1, along with GenBank accession numbers for their 18S rDNA and CO-I mtDNA sequences. Taxa were chosen to test the monophyly of leech families and of ordinal ranks as well as to represent a broad range of morphological variation among leeches. Our analysis used a global sample consisting of members representing seven euhirudinean families. Members of Salifidae and Cylicobdellidae were not available for analyses. Outgroup taxa consisted of two oligochaetes and the putatively related Branchiobdellida and monotypic Acanthobdellida. The members of Acanthobdellida and Branchiobdellida possess both oligochaete and leech morphological features (Purschke *et al.*, 1993) and are considered to be intermediate between oligochaetes and euhirudineans (Livanow, 1906, 1931; Michaelsen, 1919; Brinkhurst and Gelder, 1989; but see Holt, 1989).

Molecular Data

DNA extraction and purification. Field-collected specimens were identified and then either immediately used for DNA extraction or were preserved in 100% ethanol at ambient temperature for later extraction. Whenever possible, tissue was taken from the caudal sucker so as to prevent possible contamination by host erythrocyte DNA in the gut. Genomic DNA was extracted from specimens using the QIAamp Tissue Kit (QIAGEN Inc., Valencia, CA).

PCR amplification. Molecular characters for phylogenetic inference were obtained from the nuclear small subunit (18S) ribosomal gene and from cytochrome *c* oxidase I (CO-I) gene sequences. Mitochondrial CO-I sequences (651 bp) were amplified from purified genomic DNA using the universal primers LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO-2198: 5'-TAACTTCAGGGTGACCAAAAATCA-3' (Folmer *et al.*, 1994). A nested PCR approach was used to obtain 18S rDNA templates. The initial amplification used the primers 5'-AACCTGGTTGATCCTGCCAGT-3' and 5'-TGATCCTTCCGCAGGTTACCT-3' (primers "A" and "B", respectively; Medlin *et al.*, 1988), yielding a 1.8-kb fragment. Subsequent amplifications used internal primers (primer "L": 5'-CCAACTACGAGCTTTT-TAACTG-3', primer "C": 5'-CGGTAATTCAGCTC-CAATAG-3', primer "Y": 5'-CAGACAAATCGCTCC-ACCAAC-3', primer "O": 5'-AAGGGCACCACCAG-GAGTGGAG-3') to yield three overlapping shorter double-stranded DNA fragments (denoted AL, CY, and BO) of approximately 600 bp in length each.

Amplification reaction mixtures for CO-I contained 10 × II buffer, 2.5 mM MgCl₂, 0.25 mM of each dNTP, 1 μl of each primer, 1.25 units AmpliTaq DNA polymerase (Perkin-Elmer Corp., Foster City, CA), and template DNA in a 50-μl total volume. The reaction mixtures were heated to 94°C for 4 min and then cycled in a PTC-100 Programmable Thermal Controller for 35 cycles at 94°C for 15 s, 44°C for 20 s, and 70°C for 90 s with a final extension of 72°C for 7 min. 18S rDNA reaction mixtures were heated to 94°C for 4 min and then cycled 35 times at 94°C (20 s), 47°C (20 s), and 68°C (105 s) with a final extension of 70°C (7 min).

CLASS:	HIRUDINEA		
SUBCLASS:	EUHIRUDINEA		
ORDER:	<u>Rhynchobdellida</u>	<u>Arhynchobdellida</u>	
SUBORDER:		Erpobdelliformes	Hirudiniformes
FAMILY:	Glossiphoniidae Piscicolidae Ozobranchidae	Erpobdellidae Salifidae	Haemadipsidae Haemopidae Cylicobdellidae Hirudinidae

FIG. 1. Current taxonomy of leeches, sensu Sawyer (1986).

Amplification reactions for the BO fragment of 18S rDNA also included 10% DMSO to stabilize against secondary structure formation. The amplified DNA was purified in an agarose gel and manually excised over UV light. Further purification was performed either

TABLE 1
Taxa Used in Phylogenetic Analyses of Leeches

Taxon	Locality	GenBank accession no.	
		18S rDNA	CO-I
<i>Oligochaeta</i>			
<i>Tubifex</i>	Great Britain	U67145	U74076
<i>Lumbricus</i>	Great Britain	Z83753	U24570
<i>Acanthobdellida</i>			
<i>Acanthobdella peledina</i>	Sweden	AF115978	AF003264
<i>Branchiobdellida</i>			
<i>Xironogiton victoricensis</i>	Oregon	AF115977	AF116014
<i>Cambarincola holti</i>	Tennessee	AF115975	AF116012
<i>Cronodrilus ogygius</i>	Georgia	AF115976	AF116013
<i>Rhynchobdellida</i>			
GLOSSIPHONIIDAE			
Glossiphoniinae			
<i>Glossiphonia complanata</i>	England	AF115982	AF003277
<i>Hemiclepsis marginata</i>	France	AF115981	AF003259
<i>Placobdella parasitica</i>	Ontario	AF115990	AF003261
<i>Desserobdella picta</i>	Ontario	AF115988	AF116020
Haementeriinae			
<i>Alboglossiphonia heteroclita</i>	Michigan	AF115983	AF116016
<i>Desmobdella paransensis</i>	Uruguay	AF115987	AF116019
<i>Haementeria gracilis</i>	Uruguay	AF115984	AF003276
<i>Haementeria ghilianii</i>	Brazil	AF115985	AF116017
<i>Helobdella stagnalis</i>	France	AF115986	AF116018
<i>Marsupiobdella africana</i>	South Africa	AF115979	AF116015
<i>Oligobdella biannulata</i>	North Carolina	AF115989	AF116021
Theromizinae			
<i>Theromyzon tessulatum</i>	France	AF115980	AF003279
OZOBRANCHIDAE			
<i>Ozobranchus margo</i>	Virginia	AF115991	AF003268
PISCICOLIDAE			
Piscicolinae			
<i>Branchellion torpedinis</i>	South Carolina	AF115993	AF003265
<i>Calliobdella vivida</i>	Virginia	AF115992	AF003260
<i>Piscicola geometra</i>	France	AF115995	AF003280
Platybdellinae			
<i>Myzobdella lugubris</i>	Virginia	AF115994	AF003269
Pontobdellinae			
<i>Stibarobdella macrothela</i>	Virginia	AF115996	AF116022

TABLE 1—Continued

Taxon	Locality	GenBank accession no.	
		18S rDNA	CO-I
ARHYNCHOBDELLIDA			
ERPOBDELLIFORMES			
ERPOBDELLIDAE			
<i>Dina dubia</i>	Michigan	AF115997	AF116023
<i>Dina japonica</i>	Korea	AF116000	AF116026
<i>Erpobdella punctata</i>	Ontario	AF116002	AF003275
<i>Erpobdella octoculata</i>	France	AF116001	AF003274
<i>Erpobdella testacea</i>	France	AF116003	AF116027
<i>Mooreobdella melanostoma</i>	Michigan	AF115999	AF116025
<i>Mooreobdella buccera</i>	Michigan	AF115998	AF116024
<i>Nephelopsis obscura</i>	Ontario	AF116004	AF003273
HIRUDINIFORMES			
HAEMADIPSIDAE			
<i>Chtonobdella bilineata</i>	Australia	AF116006	AF003267
<i>Haemadipsa sylvestris</i>	Vietnam	AF116005	AF003266
HAEMOPIIDAE			
Haemopiinae			
<i>Haemopsis lateromaculata</i>	Michigan	AF116009	AF116028
<i>Haemopsis marmorata</i>	Michigan	AF116008	AF003270
HIRUDINIDAE			
Hirudininae			
<i>Hirudo medicinalis</i>	France	AF116011	AF003272
<i>Limnatis michaelsoni</i>	Congo	AF116010	AF116029
Macrobdellinae			
<i>Macrobdella decora</i>	Michigan	AF116007	AF003271

according to the Qiaquick PCR Purification kit protocol (QIAGEN) or by centrifugation through Sephadex G-50 beads (SIGMA Chemical Co., St. Louis, MO) in Centri-sep columns (Princeton Separations, Adelphia, NJ).

DNA sequencing. Sequencing reactions contained 1 µl primer, 2.5 µl purified amplification product, and 2 µl Big Dye (Applied Biosystems, Perkin-Elmer Corp.) and were cycled 35 times at 96°C (70 s), 44°C (5 s), and 60°C (4 min). Unincorporated dyes were removed from sequencing reaction products with Centri-sep columns (Princeton Separations) loaded with G-50 Sephadex (Sigma). Sequencing products were electrophoresed in a 4% polyacrylamide gel in an ABI Prism 377 Sequencer (Applied Biosystems). 18S rDNA was sequenced in three fragments of approximately 600 bp each in both directions. As well, the light and heavy strands of CO-I mtDNA were sequenced in both directions.

Alignment. Complementary strands for all sequences were reconciled using Sequence Navigator (Perkin-Elmer). Alignments of CO-I sequences were trivial in that there were no insertions or deletions. Alignments of 18S rDNA sequences were accomplished with Clustal in Gene Jockey (Taylor, 1994) or with MALIGN (Wheeler and Gladstein, 1994) using the following parameters: internal 1, changecost 1, alignaddswap, alignswap, alignrrootnode, treeswap, treeaddswap, keeptrees 20, keepaligns 20. Clustal alignments were examined and edited a posteriori for order-

dependent illogical placement of gaps. MALIGN alignments were examined a posteriori as well but editing was not required.

Morphology

Characters and character states used are adapted from Siddall and Bureson (1995). The character matrix is shown in Table 2.

Character 1: setae: present (0); absent (1).

Character 2: testisac arrangement: one pair (0); grape-

like clusters (1); four pair (4); five pair (5); six pair (6); ten pair (9).

Character 3: coelomic organization: open with complete septa (0); reduced to lacunae without complete septa (1).

Character 4: conducting (vector) tissue: present (0); absent (1).

Character 5: nephridia: complete in genital somites (0); suppressed in genital somites (1).

Character 6: pharynx: not protrusible (0); modified into protrusible proboscis (1).

Character 7: intestine: acaecate (0); caecate (1).

Character 8: cephalic eyespots: dorsolateral (0); dorsal (1).

Character 9: coelomic architecture: internal to the circular muscle (0); external to the circular muscle (1).

Character 10: intestinal blood sinus: absent (0); present (1).

Character 11: body shape: not dorsoventrally flattened (0); dorsoventrally flattened (1).

Character 12: surface covering of cocoons: proteinaceous (0); membranous (1).

Character 13: deposition of cocoons: slipped off head (0); secreted ventrally (1).

Character 14: arrangement of salivary tissue: diffuse (0); discrete glands (1).

Character 15: male bursa: bilobed (0); single bulb (1).

Character 16: eyespots: one pair per annulus (0); at least two pairs per annulus (1).

Character 17: myognaths: armed (0); unarmed (1).

Character 18: testisacs: discretely arranged on vasa deferentia (0); hundreds of sacs profusely arranged (1).

Character 19: ovisacs: tubular (0); spheroid (1).

Character 20: nephridia: single funnel (0); multiple funnels located in ciliated organ (1).

Character 21: urinary bladder: absent (0); present (1).

Character 22: cocoons: cemented to a substrate (0); not cemented to a substrate (1).

Character 23: female median reproductive apparatus: simple pocket (0); modified into vaginal tube (1).

Character 24: respiratory auricles: absent (0); present (1).

Character 25: epididymes: loosely arranged (0); tightly coiled mass (1).

Character 26: cocoons: without spongy covering (0); with spongy covering (1).

Character 27: pairs eyes: zero (0); one (1); two (2); three (3); four (4); five (5).

Character 28: annuli per somite: one (1); two (2); three (3); five (5); six (6); >10 (9).

Character 29: location of male gonopore: on ring (0); in furrow (1).

Character 30: salivary papillae: absent (0); present (1).

TABLE 2

Character and State Matrix of Morphological Data Used in Phylogenetic Analyses of Leeches

Taxon	Characters
	11111111112222222223
	123456789012345678901234567890
<i>Tubifex tubifex</i>	0001100?000000??---000?00001-0
<i>Lumbricus terrestris</i>	0001000?000000??---000?00001-0
<i>Cambarincola holti</i>	10010000000000??---000?00001-0
<i>Cronodrilus ogygius</i>	10010000000000??---000?00001-0
<i>Xironogiton victoriensis</i>	10010000000000??---000?00011-0
<i>Acanthobdella peledina</i>	00010000000000??---000000011-0
<i>Marsupiobdella africana</i>	1611011101111000-0000100002300
<i>Theromyzon tessulatum</i>	1611011101111000-0000100004300
<i>Hemiclepsis marginata</i>	1?11011101111000-0000100004300
<i>Glossiphonia complanata</i>	1911011101111000-0000100003300
<i>Alboglossiphonia heteroclita</i>	1911011101111000-0000100003300
<i>Haementeria gracilis</i>	1511011101111100-0000100002300
<i>Haementeria ghilianii</i>	1511011101111100-0000100002300
<i>Helobdella stagnalis</i>	1611011101111000-0000100002300
<i>Desmobiobdella paranensis</i>	1611011101111000-0000100002300
<i>Desserobdella picta</i>	1611011101111000-0000100002300
<i>Oligobdella biannulata</i>	1611011101111000-0000100002200
<i>Placobdella parasitica</i>	1611011101111100-0000100002300
<i>Ozobranchus margoi</i>	1410010000000000-0000000002300
<i>Calliobdella vivida</i>	1610010110000000-0000000002600
<i>Branchellion torpedinis</i>	1511010110000000-0000000002300
<i>Myzobdella lugubris</i>	1511010100000000-0000000002900
<i>Piscicola geometra</i>	1610010110000000-0000000002900
<i>Stibarobdella macrothela</i>	1610010110000000-0000000002300
<i>Dina dubia</i>	111110000000000111000000004500
<i>Mooreobdella bucera</i>	111110000000000111000000003500
<i>Mooreobdella melanostoma</i>	111110000000000111000000003510
<i>Dina japonica</i>	111110000000000111000000003500
<i>Erpobdella octoculata</i>	111110000000000111000000003500
<i>Erpobdella punctata</i>	111110000000000111000000003510
<i>Erpobdella testacea</i>	111110000000000111000000003510
<i>Nephelopsis obscura</i>	111110000000000111000000004610
<i>Haemadipsa sylvestris</i>	191110000000001000111111115511
<i>Chthonobdella bilineata</i>	191110000000001000111111115511
<i>Macrobdella decora</i>	191110000000001000111110115500
<i>Haemopsis marmorata</i>	191110000000001000111110115510
<i>Haemopsis lateromaculata</i>	191110000000001000111110115510
<i>Limnatis michaelsoni</i>	191110000000001000111110115511
<i>Hirudo medicinalis</i>	191110000000001000111110115510

Phylogenetic Analysis

All characters were unordered. Indels were treated as missing data. Parsimony analyses with unweighted,

unordered characters were conducted with PAUP* (Swofford, 1998) in Macintosh and DOS environments with 20 random sequence additions of taxa and tree bisection reconnection (TBR) branch swapping. The incongruence length difference (ILD) test (Farris *et al.*, 1994) was conducted across the three data partitions in PAUP*. AutoDecay (Eriksson and Wikström, 1996) was used to calculate Bremer support values (Bremer, 1988).

RESULTS

Parsimony analysis of 18S rDNA alone (2023 characters) resulted in 69 equally most-parsimonious trees, each of which had 1444 steps and a retention index (RI) of 0.802. The strict consensus of these trees (Fig. 2A) resolved 27 clades out of a total 37 possible for the 39 taxa. Analysis of CO-I alone (651 characters) resulted in one most-parsimonious tree with 2911 steps and an RI of 0.411 (Fig. 2B). The CO-I tree and the 18S rDNA tree have 16 clades in common. Use of CO-I alone resulted in an unexpected placement of Branchiobdellida well within Euhirudinea. When molecular data sets were combined, heuristic searches yielded one most-parsimonious tree (length = 4383; RI = 0.5904). The three data sets were not significantly incongruent (frequency of $ILD_{\text{random}} \geq ILD_{\text{observed}} = 0.80$).

Use of all of the available data (the two molecular data sets in addition to 30 morphological characters) in parsimony analysis also resolved one most parsimonious tree (tree length = 4443; RI = 0.6034) identical to that found with the combined molecular data (Fig. 3). In this hypothesis, the following groups were recognized as monophyletic: the order Arhynchobdellida (combining Erpobdelliformes and Hirudiniformes); the suborders Erpobdelliformes and Hirudiniformes; the families Glossiphoniidae, Piscicolidae, Haemopidae, and Haemadipsidae; as well as the subfamilies Piscicolinae and Hirudininae. Polyphyly was indicated for the family Hirudinidae (*Hirudo*, *Limnatis*, *Macrobodella*), the subfamilies Haementeriinae and Glossiphoniinae, and the genera *Dina* and *Erpobdella*. Rhynchobdellida and *Mooreobdella* each were paraphyletic. The sister-group relationship of piscicolids to Arhynchobdellida was supported by a Bremer support index of only three. Six additional steps were needed to make Rhynchobdellida (Glossiphoniidae + Ozobranchidae + Piscicolidae) a monophyletic group.

Within Glossiphoniidae, neither of the subfamilies Haementeriinae nor Glossiphoniinae were monophyletic. Thirty-two additional steps were required for a monophyletic Haementeriinae and 34 for a monophyletic Glossiphoniinae as these subfamilies are presently constituted (Sawyer, 1986). Within the Piscicolidae, the subfamily Piscicolinae (*Branchellion*, *Calliobdella*, *Piscicola*) was monophyletic (Bremer support index = 4). *Stibarobdella macrothela*, a pontobdel-

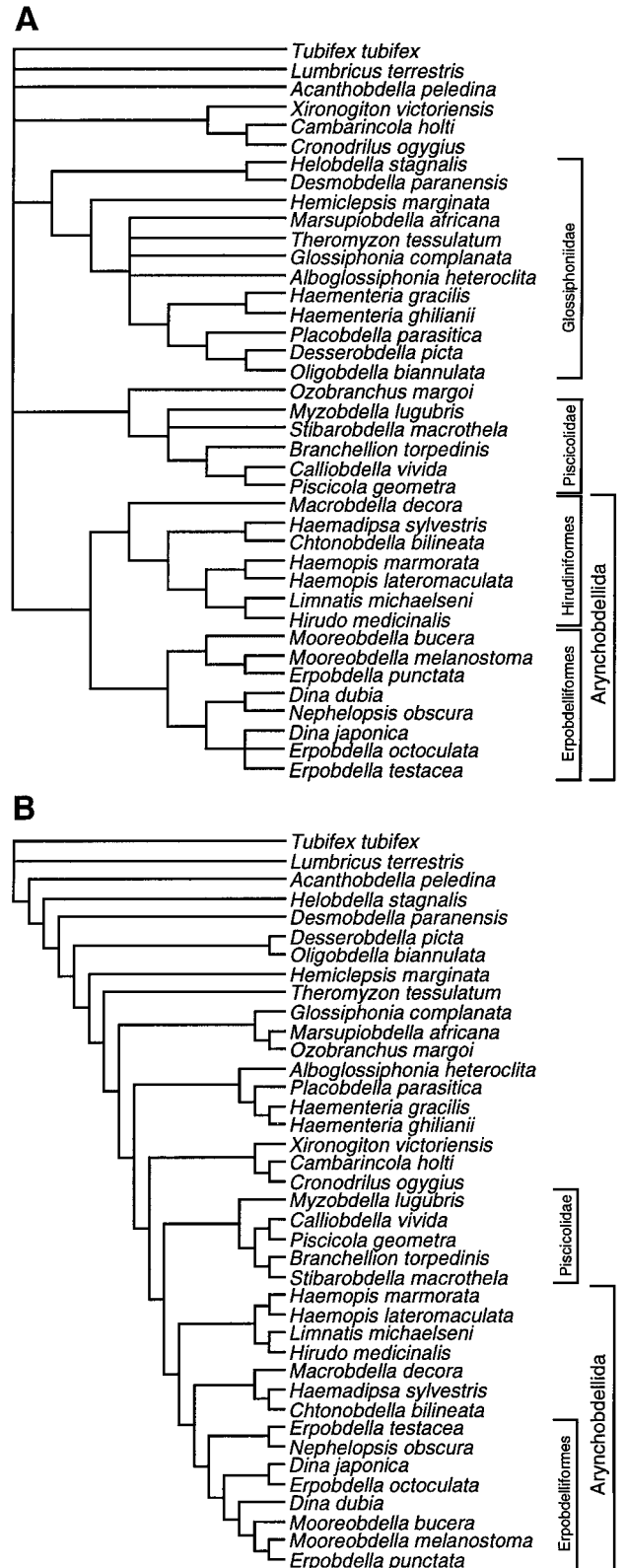


FIG. 2. Strict consensus (A) of 48 equally parsimonious trees obtained from 18S rDNA and (B) of 2 equally parsimonious trees obtained from CO-I.

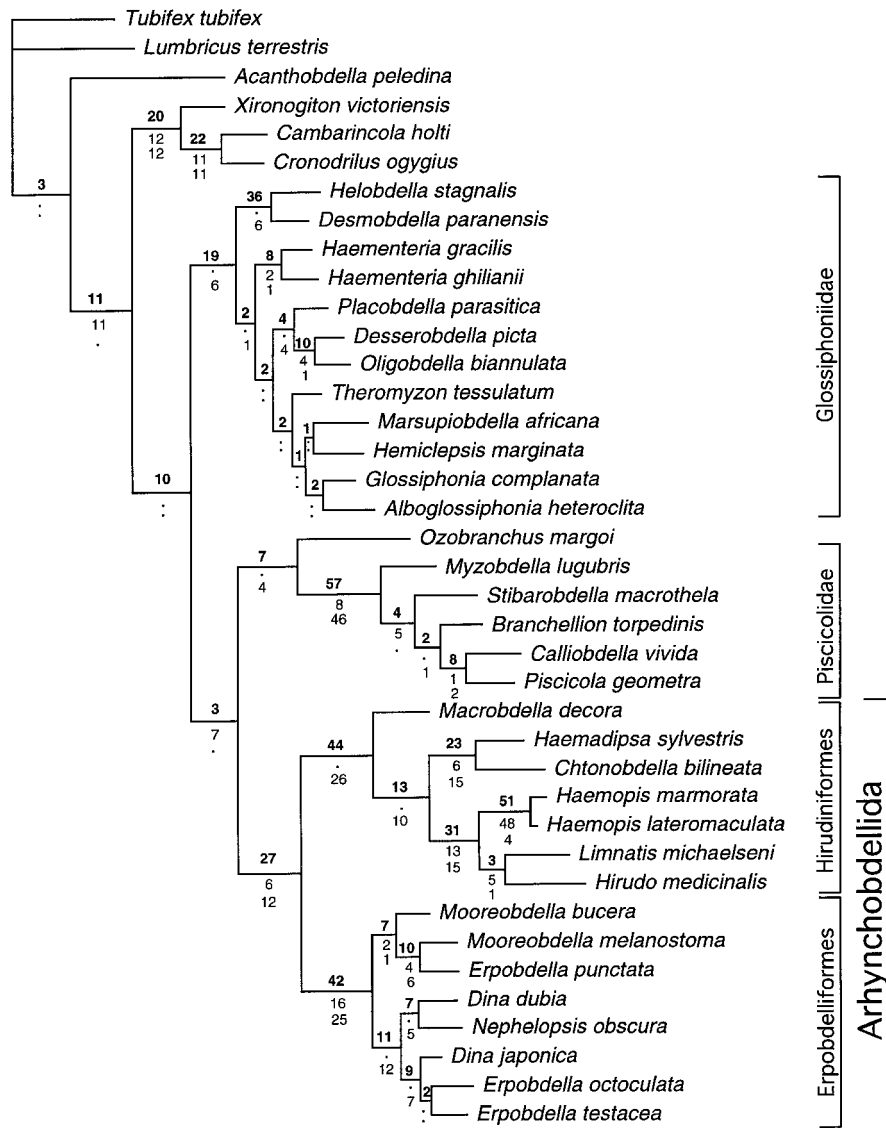


FIG. 3. Most-parsimonious hypothesis resulting from the combination of morphological, 18S rDNA, and CO-I data. Numbers above internodes indicate the Bremer support for that branch based on the combined data set. Upper and lower values below internodes indicate Bremer support based on CO-I and 18S rDNA alone, respectively.

lid, was sister to Piscicolinae. *Ozobranchus margo*, the sole included representative of the family Ozobranchidae, was corroborated as sister to the Piscicolidae.

Groupings within the suborder Erpobdelliformes appeared to be geographically arranged but not taxonomically consistent. For example, the European *Erpobdella octoculata* (type species for the genus and for the family Erpobdellidae) and *Erpobdella testacea* formed a group sister to *Dina japonica*, obtained from Korea (Bremer support index = 9). Twenty-seven additional steps were required to group *Dina japonica* with the North American *Dina dubia*. The North American representative of *Erpobdella*, however, grouped with *Mooreobdella* species (Bremer support index = 7). Ten additional steps were required for a monophyletic *Mooreobdella* clade,

while grouping *Erpobdella punctata* with the other two species of *Erpobdella* would require 65 extra steps.

Although the families Haemopidae (Bremer support index = 51) and Haemadipsidae (Bremer support index = 23) each had only two representatives in this analysis, both were monophyletic groups within the suborder Hirudiniformes. Hirudinidae, however, was rendered polyphyletic because *Macrobdella decora* did not group with *Hirudo medicinalis* and *Limnatis michaelsoni*. The haemopids were sister to the hirudinids (Bremer support index = 31). Grouping *M. decora* with the hirudinids would require 48 additional steps.

A total of 160 additional steps was needed when constraints based on current systematic taxonomic groupings (Sawyer, 1986) were placed on the analysis.

A tree with a monophyletic bloodfeeding clade required 208 extra steps while a tree with a monophyletic nonbloodfeeding clade required 200 extra steps. *Acanthobdella peledina* was not the sister group to Euhirudinea in this analysis but rather was sister to a leech + branchiobdellidan clade. These differences were highly significant in nonparametric Wilcoxon signed-ranks tests ($N = 279$; $z = 10.0874$; $P < 0.0001$).

DISCUSSION

The resulting cladogram from the combined data indicates that current leech systematics for the most part has been reasonably accurate. We find current higher taxonomic categories of leeches to be consistent with monophyletic groups identified in our analysis. The order Arhynchobdellida, the suborders Erpobdelliformes and Hirudiniformes, the families Glossiphoniidae, Piscicolidae, Erpobdellidae, Haemopidae, and Haemadipsidae, and the subfamily Piscicolinae each are groups that are well supported. The most-parsimonious tree, however, does not support all of the traditionally recognized groups. The orders Rhynchobdellida and Arhynchobdellida are distinguished by the possession of a protrusible muscular proboscis in the former and its absence in the latter. Usually, it is the lack of a character that denotes the plesiomorphic state and is indicative of paraphyly (Eldredge and Cracraft, 1980), such that a priori one would expect to find a paraphyletic Arhynchobdellida. Relationships depicted in Fig. 3, however, show Arhynchobdellida as a derived, monophyletic group having lost the proboscis. Rhynchobdellida is paraphyletic in that Ozobranchidae and Piscicolidae form the sister group to Arhynchobdellida to the exclusion of Glossiphoniidae. This further corroborates Siddall and Burreson's (1998) hypothesis based only on CO-I that the presence of a proboscis is not a synapomorphy for Rhynchobdellida. Because only six extra steps are needed to have a monophyletic Rhynchobdellida and because there is a Bremer support index of three for the Ozobranchidae + Piscicolidae + Arhynchobdellida clade, formal revision of ordinal taxonomy is still premature. Moreover, although Trontelj (1997) also found a paraphyletic Rhynchobdellida, his analyses placed glossiphoniids as sister to Arhynchobdellida, which differs from our results.

Nuclear genes such as 18S rDNA have a slow rate of change appropriate for resolving deep branching patterns and therefore higher level relationships (Hillis and Dixon, 1991). If evolutionary rates are too slow, however, there is not sufficient change within lineages to provide resolution in local areas of the emergent phylogenetic hypothesis. The use of 18S rDNA data alone incompletely resolved within-family relationships of leeches, as is evident from the Bremer support indices in Fig. 3. 18S rDNA is a non-protein-coding structural gene that folds on itself and thus may

involve compensatory changes as a possible source of error in phylogenetic analysis (Wheeler and Honeycutt, 1988). These nucleotide sequences are also subject to insertions and deletions. Notably, clustal alignment and two equally optimal alignments from MALIGN returned trees of different length for 18S rDNA. However, all of these trees were topologically identical, suggesting that the total evidence hypothesis is robust to insertion/deletion events.

Mitochondrial genes such as the protein-coding CO-I gene usually evolve more rapidly and can provide for resolution of more recent relationships. It has been argued that if rates of change are too fast, phylogenetic signal can be swamped by noise and could yield spurious deeper groupings (Miyamoto and Boyle, 1989; Hillis and Moritz, 1990; Swofford and Olsen, 1990; Cracraft and Helm-Bychowski, 1991). Because analysis of CO-I (Fig. 2B) alone roots the ingroup within the glossiphoniids, it yields unexpectedly paraphyletic groupings for most major leech groups. CO-I has no insertions or deletions and is also very AT rich, which may introduce unique biases. The use of two independent molecular data sets in addition to morphological data combines historical information evolving under a variety of different constraints (nuclear and mitochondrial; coding and noncoding; fast rate of change and slow) and should be less susceptible to the biases that can confound the use of only one type of data (Wheeler *et al.*, 1993). Where these data offer mutually corroborating support should be due to some extrinsic commonality; that is, history (Eernisse and Kluge, 1993).

In the total evidence analysis, the subfamilies Glossiphoniinae and Haementeriinae were found to be polyphyletic within a monophyletic Glossiphoniidae. Traditionally, the diagnostic character in the two subfamilies has been the mode of cocoon deposition: members of Glossiphoniinae attach cocoons directly onto a substrate whereas those of Haementeriinae attach cocoons onto the venter of the parent (Sawyer, 1971). Our findings corroborate those originally found by Light and Siddall (1999) based on CO-I and ND-I and continue to show that these are unnatural groupings. This suggests that these characters either may have arisen independently or are poorly characterized for the group.

Ozobranchus margo, the sole included representative of the family Ozobranchidae, was supported as sister to the Piscicolidae. The ozobranchids are ectoparasitic on marine turtles and are distinguished by the presence of lateral digitiform branchiae (MacCallum and MacCallum, 1918). Traditionally, the family Piscicolidae is divided into three subfamilies: Piscicolinae, Pontobdellinae, and Platybdellinae. The shark leech *Stibarobdella macrothela*, a pontobdellid, was found to be sister to the subfamily Piscicolinae, which is consistent with the possession of external circulatory vessels or pulsatile vesicles. These appendages may have been

a recent adaptation to marine environments, and it has been speculated that they serve an osmoregulatory or circulatory function (Herter, 1936). *Myzobdella lugubris*, the only representative of the subfamily Platybdellinae, falls as sister to a Piscicolinae + Pontibdellinae clade. Although *Myzobdella lugubris* also occurs in marine environments, platybdellines do not have pulsatile vesicles. Evaluation of the historical patterns of freshwater and marine immigration awaits further data from a more extensive taxonomic sample of these subfamilies.

Members in the suborder Erpobdelliformes appear to be descended from a common ancestor. However, most generic groupings within this clade were found to be unnatural. *Erpobdella* is usually defined by having a preatrial loop on the paired seminal ducts and by body somites being five-annulate, with each annulus of approximately equal size (Sawyer, 1986). *Erpobdella octoculata*, the type species of this genus and of the family Erpobdellidae, appears to be more closely related both to *Nephelopsis* and to *Dina* species than to its North American congener *Erpobdella punctata*. The genus *Dina* also is characterized by being five-annulate but differs from *Erpobdella* species in that every fifth annulus is distinctly widened and subdivided (Sawyer, 1986). Although the type species of *Dina* (the European *Dina lineata*) was not included, *D. japonica* often has been mistaken for it (Sawyer, 1986), and the failure of the nearctic *D. dubia* to group with palearctic *D. japonica* suggests that this genus also is in need of revision. The genus *Mooreobdella* distinguished by a lack of preatrial loops, was not found to be monophyletic unless it included *Erpobdella punctata*. Without specifying his rationale, Sawyer (1986) placed *Mooreobdella* species, all of which are North American, in the genus *Erpobdella*. The most parsimonious tree however suggests either the placement of *Erpobdella punctata* in a genus (i.e., *Mooreobdella*) separate from the European erpobdellids or the expansion of the genus *Erpobdella* to include all of the Erpobdellidae. The lack of specimens from Salifidae (see Nesemann, 1995) and the South American family Cylicobdellidae, members of which have both erpobdellid- and hirudinid-like characteristics, presently precludes a comprehensive revision of that group.

Of the three families within the Hirudiniformes that were analyzed, the families Haemadipsidae and Haemopidae were found to be monophyletic. While Siddall and Burreson (1995, 1998) could not corroborate a monophyletic Haemopidae, here it was supported. The basal placement of *Macrobodella decora* renders the medicinal leech family Hirudinidae polyphyletic (Fig. 3). The arrangement corroborates the notion of two separate medicinal leech families, the Old World Hirudinidae and the New World Macrobodellidae, as previously suggested by Richardson (1969).

Evolution of Bloodfeeding

Because leeches are best known for their bloodfeeding habits, it is perhaps not widely acknowledged that several common species of leeches do not bloodfeed and instead prey on invertebrates. Members of the freshwater family Erpobdellidae, such as the popular bait leech *Nephelopsis obscura*, are carnivorous on other oligochaetes (Klemm, 1972). *Haemopsis* species, closely related to the sanguivorous medicinal leeches, also are predaceous and many have large teeth for shredding prey as they are ingested. Sawyer (1986) reasoned that the evolution of feeding behavior in the hirudiniformes originated with macrophageous feeding by haemopids and culminated with sanguivory in the hirudinids and haemadipsids. Neither of these hypotheses is corroborated in the most-parsimonious tree. Rather, it appears that the ancestral hirudiniform (and the ancestral leech more generally) was sanguivorous and that sanguivory has been lost at least four times in the course of leech evolution (Fig. 4). Bloodfeeding was lost twice within the Glossiphoniidae by the ancestor of the *Glossiphonia complanata* + *Alboglossiphonia heteroclita* clade and that of the *Helobdella stagnalis* + *Desmobdella paranensis* clade (Fig. 4). As well, a carnivorous mode of nutrition has been adopted independently over sanguivory by the erpobdellids and the haemopids. This corroborates the notion previously raised by Siddall and Burreson (1996) that bloodfeeding is a plastic character easily lost by leeches. Because of the omission of certain taxa, our findings are likely to underestimate the number of times sanguivory has

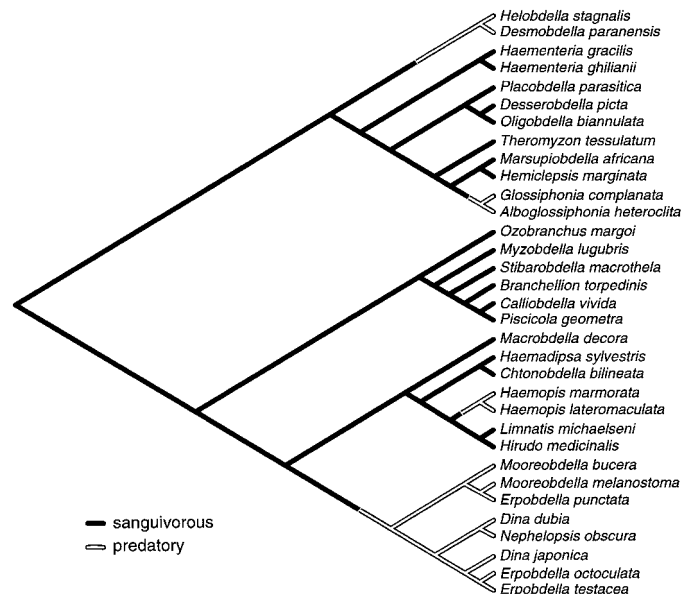


FIG. 4. Most-parsimonious reconstruction of the evolution of sanguivory in leeches indicates a common origin for the trait in the ancestral leech and four independent changes to a carnivorous mode of nutrition.

been lost. For example, *Mysidobdella borealis*, a piscicolid which is known to parasitize mysid shrimp (Burreson and Allen, 1978), was not included in the analysis and yet we predict it to group with other piscicolids. If so, every major group of leeches would then have taxa indicative of independent losses of this behavior for which leeches are so well known.

Cocoons

Within the Euhirudinea there is a diversity in cocoon types and the manner in which cocoons are produced and deposited. Like the rest of the Clitellata, most leeches slip the secreted cocoon off of the head, which then hardens and darkens to form a proteinaceous covering. Hirudiniform cocoons, which have a spongy covering unlike the cocoons of other leeches, are deposited out of water and abandoned. The cocoons of piscicolids and of the Erpobdelliformes normally are attached to a submerged substrate, whether it be an inanimate object or a crustacean. Glossiphoniids have the distinction of being the only clitellate annelids that exhibit parental care characterized by a protective brooding behavior. As opposed to a hardened protective cocoon, these leeches secrete a thin membranous cocoon in which fertilized eggs are deposited either on a substrate or on the venter side of the parent (Sawyer, 1971, 1986). Even after hatching, glossiphoniids remain with their young, a behavior that corresponds historically to the loss of the protective proteinaceous covering of those species that abandon their cocoons (Siddall and Burreson, 1996). Mann (1962) suggested that membranous cocoons are plesiomorphic to hardened cocoons and therefore that glossiphoniids are primitive to other leeches. This notion is unwarranted because *Acanthobdella peledina* and branchiobdellidans deposit proteinaceous cocoons (Siddall and Burreson, 1995). Moreover, the most-parsimonious hypothesis indicates that brooding behavior and membranous cocoons are not primitive states but rather are unreversed synapomorphies for the monophyletic Glossiphoniidae. As well, uncemented spongy cocoons that are abandoned appear to be synapomorphies for the Hirudiniformes.

Biogeography

The sampling of taxa in our study includes representatives from six continents and from a diversity of environments. As discussed previously, the revision of generic-level groupings is recommended to better characterize some groups. Some of these revisions stem from observations that where traditional leech systematics fail, many species group geographically (e.g., erpobdellid species, hirudinid species). Indeed, we found that in many cases, North American species are more closely related to other North American species than they are to their European counterparts.

The terrestrial Haemadipsidae, represented here by *Chtonobdella bilineata* and *Haemadipsa sylvestris*, are

only known to occur in Australia, the Wallacean archipelago, Southeast Asia, India, and Madagascar. Because they do not also occur in Africa, this distribution appears to postdate the initial breakup of Gondwanaland. Alternatively, this particular distribution may be attributable to recent dispersal via Indonesia and not to vicariance biogeography. Additional haemadipsid taxa could provide more information that may determine which of these hypotheses is accurate.

This study, the first to combine molecular data in addition to morphology, depicts the most complete phylogenetic higher-level analysis of the Euhirudinea to date. The results establish a foundation for more in-depth phylogenetic determination of species relationships and form a basis for investigating the nature of historical ecological associations. Although this analysis includes representatives from all continents except Antarctica, the inclusion of additional taxa is desirable in order to further clarify relationships. Of particular interest would be to see where members of the unrepresented families Salifidae and Cylicobdellidae would fall. The Salifidae traditionally has been grouped as an erpobdelliform family, and morphological data suggest a basal paraphyletic relationship to the family Erpobdellidae. Members of the South American family Cylicobdellidae traditionally are considered to be Hirudiniformes in light of having typical hirudiniform eye arrangement; however, their median male reproductive apparatus is more erpobdellid-like. As well, expansion of analyses to include more oligochaetes and some polychaetous outgroups should eventually lead to an understanding of which oligochaete family is most closely related to leeches, branchiobdellidans, and *Acanthobdella peledina*.

ACKNOWLEDGMENTS

We thank Soraya Bartol, BioPharm (UK), Gustavo Calvo, Kathryn Coates, Pierre Delaporte, Louis de Preez, Stuart Gelder, Richard Kraus, Jessica E. Light, Brad Moon, Bob Murphy, Cynthia Sims Parr, and Peter Rothlisberg for their assistance in obtaining specimens. The critical comments of Kathryn Coates, Gonzalo Giribet, Sharon Jansa, Arnold Kluge, and Jessica E. Light on earlier drafts of the manuscript are greatly appreciated. This research was supported by funds from the University of Michigan Museum of Zoology and Horace H. Rackham School of Graduate Studies and grants from the University of Michigan Department of Biology and the National Science Foundation (BIO/DEB-9615211). Aligned data are available at <http://limnatis.ummz.lsa.umich.edu/>.

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