Arhynchobdellida (Annelida: Oligochaeta: Hirudinida): phylogenetic relationships and evolution

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Abstract

A remarkable diversity of life history strategies, geographic distributions, and morphological characters provide a rich substrate for investigating the evolutionary relationships of arhynchobdellid leeches. The phylogenetic relationships, using parsimony analysis, of the order Arhynchobdellida were investigated using nuclear 18S and 28S rDNA, mitochondrial 12S rDNA, and cytochrome *c* oxidase subunit I sequence data, as well as 24 morphological characters. Thirty-nine arhynchobdellid species were selected to represent the seven currently recognized families. Sixteen rhynchobdellid leeches from the families Glossiphoniidae and Piscicolidae were included as outgroup taxa. Analysis of all available data resolved a single most-parsimonious tree. The cladogram conflicted with most of the traditional classification schemes of the Arhynchobdellida. Monophyly of the Erpobdelliformes and Hirudiniformes was supported, whereas the families Haemadipsidae, Haemopidae, and Hirudinidae, as well as the genera *Hirudo* or *Aliolimnatis*, were found not to be monophyletic. The results provide insight on the taxonomic positions for the taxonomically problematic families Americobdellidae and Cylicobdellidae, the genera *Semiscolex*, *Patagoniobdella*, and *Mesobdella*, as well as genera traditionally classified under Hirudinidae. The evolution of dietary and habitat preferences is examined.
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1. 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 AD (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 (Sawyer, 1986; Shipley, 1927), rendered wild populations either threatened or endangered in over 15 European countries (Elliott and Tullett, 1984, 1992; Council of Europe, 1998; IUCN, 1993; Wells et al., 1983). This traditional use led to medical and biochemical uses for post-operative haematoma treatment (Lent, 1986; Walsmann and Markwardt, 1985), as well as to the discovery of anti-thrombin and anti-platelet activities in their salivary secretions (Baskova et al., 1987; Hong et al., 1999; Munro et al., 1992a,b; Vindigni et al., 1994; Yang et al., 1997).

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 entails temperate terrestrial leeches and even 'eyeless' cave-dwelling species. Exhibiting a marked

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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 (Aisemberg et al., 2001; Burrell et al., 2003; Munro et al., 1992b), phenology (Demirsoy et al., 2001), ecology (Dall, 1987; Schalk et al., 2002), environmental science (Shapkarev and Vagner, 1989), and conservation (Council of Europe, 1998; Kasparek et al., 2000; Sawyer, 1979, 1981).

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 (Apakupakul et al., 1999; Sawyer, 1986; Siddall and Burreson, 1995, 1998; Trontelj et al., 1999).

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

2. Materials and methods

2.1. Taxa

Thirty-nine species included for phylogenetic analyses were chosen to represent the seven arhynchobdellid families (Table 1): Americobdellidae, Cylicobdellidae, Erpobdellidae, Haemopidae, Haemadipsidae, Hirudinidae, and Salifidae. Collectively these cover an extensive global distribution (Table 2). Species not included in previous phylogenetic studies are *Aliolimnatis africana*, *Americobdella valdiviana*, *Cylicobdella coccinea*, *Erpobdella costata*, *Haemadipsa picta*, *Haemadipsa sumatrana*, *Haemopis kingi*, *Haemopis grandis*, *Hirudinaria manillensis*, *Hirudo nipponia*, *Limnatis nilotica*, *Macrobdella ditetra*, *Mesobdella gemmata*, *Oxyptychus braziliensis*, *Oxyptychus striatus*, *Semiscolex similis*, *Patagoniobdella variabilis*, and *Patagoniobdella fraterna*. Outgroup taxa were selected based on prior phylogenetic work (Apakupakul et al., 1999; Light and Siddall, 1999; Siddall and Burreson, 1995, 1996, 1998; Siddall et al., 2001) and included 16 species of rhynchobdellid leeches from two families: 11 species from the Glossiphoniidae and five from the Piscicolidae. All taxa included in this study, their sampling localities and

Table 1
Current classification (Sawyer, 1986; Siddall, 2002), number of arhynchobdellid families and genera, and list of arhynchobdellid genera included in this study

Subphylum: Clitellata
Class: Hirudinea
Subclass: Euhirudinea
Order: Rhynchobdellida
Family: Glossiphoniidae
Piscicolidae
Order: Arhynchobdellida
Suborder: Hirudiniformes [5 Families]
Family: Americobdellidae [1 Genus]
Americobdella
Cylicobdellidae [6 Genera]
Cylicobdella
Haemopidae [6 Genera]
Haemopis
Patagoniobdella
Semiscolex
Hirudinidae [22 Genera]
Aliolimnatis
Hirudinaria
Hirudo
Limnatis
Macrobdella
Oxyptychus
Haemadipsidae [17 Genera]
Chtonobdella
Haemadipsa
Mesobdella
Xerobdella
Suborder: Erpobdelliformes [2 Families]
Family: Erpobdellidae [3 Genera]
Erpobdella
Salifidae [4 Genera]
Barbronia

GenBank Accession Numbers for new sequences, as well as sequences reported in other work are listed in Table 2.

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

2.3. Nuclear and mitochondrial DNA sequence amplification

PCR amplification of nuclear 18S and 28S rDNA, and mitochondrial 12S rDNA and cytochrome *c* oxidase subunit I (CO-I) 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)

Table 2
Taxa used for phylogenetic analyses of arhynchobdellid leeches

Taxon	Locality	GenBank Accession Number			
		12S	18S	28S	CO-I
Ingroup					
Aliolimnatis africana	Ctr. African Rep.	AY425428	AY425469	AY425387	AY425451
Aliolimnatis michaelseni	Congo	AY425429	AF116010[e]	AY425388	AF116029[e]
Americobdella valdiviana	Chile	AY425407	AY425461	AY425358	AY425443
Barbronia weberi	Austria	–	AF099951[f]	–	–
Chtonobdella bilineata	Australia	AY425410	AF116006[e]	AY425361	AF003267[g]
Cylicobdella coccinea	Bolivia	AY425411	AY425462	AY425362	AY425444
Erpobdella lineata	Denmark	AF099952[f]	AF099950[f]	AY425367	–
Erpobdella bucera	Michigan	AF462026[a]	AF115998[e]	AY425394	AF116024[e]
Erpobdella costata	Georgia	AY425442	AY425478	AY425406	AY425460
Erpobdella dubia	Michigan	AF462022[a]	AF115997[e]	AY425365	AF116023[e]
Erpobdella japonica	Korea	AF462023[a]	AF116000[e]	AY425366	AF116026[e]
Erpobdella melanostoma	Michigan	AF462027[a]	AF115999[e]	AY425395	AF116025[e]
Erpobdella mestrovi	Croatia	–	AF272842[d]	–	–
Erpobdella obscura	Ontario	AF462028[a]	AF116004[e]	AY425396	AF003273[g]
Erpobdella octoculata	France	AF099954[f]	AF116001[e]	AY425368	AF003274[g]
Erpobdella punctata	Ontario	AF462024[a]	AF116002[e]	AY425369	AF003275[g]
Erpobdella testacea	France	AF462025[a]	AF116003[e]	AY425370	AF116027[e]
Haemadipsa picta	Borneo	–	AY425463	–	AY425445
Haemadipsa sumatrana	Borneo	AY425415	AY425464	AY425372	AY425446
Haemadipsa sylvestris	Vietnam	AY425416	AF116005[e]	AY425373	AF003266[g]
Haemopis caeca	Romania	AY425419	AY040687[c]	AY425376	AY040702[c]
Haemopis grandis	Manitoba	AY425420	AY425465	AY425377	AY425447
Haemopis kingi	Manitoba	AY425421	AY425466	AY425378	AY425448
Haemopis lateromaculata	Michigan	AY425422	AF116009[e]	AY425379	AF116028[e]
Haemopis marmorata	Michigan	AY425423	AF116008[e]	AY425380	AF003270[g]
Haemopis sanguisuga	Sweden	AF099960[f]	AF099941[f]	AY425381	AF462021[a]
Hirudinaria manillenis	Puerto Rico	AY425426	AY425467	AY425384	AY425449
Hirudo medicinalis	BioPharm, UK	AF099961[f]	AF116011[e]	AY425385	AF003272[g]
Hirudo nipponia	Korea	AY425427	AY425468	AY425386	AY425450
Limnatis nilotica	Israel	AY425430	AY425470	AY425389	AY425452
Macrobdella decora	Michigan	AY425431	AF116007[e]	AY425390	AF003271[g]
Macrobdella ditetra	Georgia	AY425432	AY425471	AY425391	AY425453
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
Xerobdella lecomtei	Slovenia	–	AF099947[f]	–	–
Outgroup					
Branchellion torpedinis	South Carolina	AY425408	AF115993[e]	AY425359	AF003265[g]
Calliobdella vivida	Virginia	AY425409	AF115992[e]	AY425360	AF003260[g]
Desserobdella picta	Ontario	AY425413	AF115988[e]	AY425364	AF116020[e]
Glossiphonia complanata	United Kingdom	AY425414	AF115982[e]	AY425371	AF003277[g]
Haementeria ghilianii	French Guyana	AY425417	AF115985[e]	AY425374	AF329035[b]
Haementeria gracilis	Uruguay	AY425418	AF115984[e]	AY425375	AF329034[b]
Helobdella paranensis	Uruguay	AY425412	AF115987[e]	AY425363	AF329037[b]
Helobdella stagnalis	France	AY425424	AF115986[e]	AY425382	AF116018[e]
Hemiclepsis marginata	France	AY425425	AF115981[e]	AY425383	AF003259[g]
Marsupiobdella africana	South Africa	AY425433	AF115979[e]	AY425392	AF116015[e]
Oligobdella biannulata	North Carolina	AY425435	AF115989[e]	AY425397	AF116021[e]
Piscicola geometra	France	AY425437	AF115995[e]	AY425400	AF003280[g]
Placobdella parasitica	Ontario	AY425438	AF115990[e]	AY425401	AF003261[g]
Pontobdella muricata	Slovenia	AF099958[f]	AF099945[f]	–	–
Stibarobdella macrothela	Virginia	AY425440	AF115996[e]	AY425403	AF116022[e]
Theromyzon tessulatum	France	AF099957[f]	AF115980[e]	AY425404	–

[a] Siddall, 2002.
[b] Siddall and Borda, 2003.
[c] Siddall et al., 2001.
[d] Sket et al., 2001.
[e] Apakupakul et al., 1999.
[f] Trontelj et al., 1999.
[g] Siddall and Burreson, 1998.

Table 3
Primers used for PCR amplification and sequencing

Gene	Primer name	Primer sequence
Nuclear		
18S rDNA		
	A	5′-AACCTGGTTGATCCTGCCAGT-3′
	L	5′-CCAACTACGAGCTTTT- 3′
	C	5′-CGGTAATTCCAGCTC-3′
	Y	5′-CAGACAAATCGCTCC-3′
	B	5′-TGATCCTTCCGCAGGTTCACCT-3′
	O	5′-AAGGGCACCACCAG-3′
28S rDNA	28S-A	5′-GACCCGTCTTGAAGCACG-3′
	28S-B	5′-TCGGAAGGAACAGCTACTA-3′
Mitochodrial		
12S rDNA	12S-AI	5′-AAACTAGGATTAGATACCCTATTAT-3′
	12S-BI	5′-AAGAGCGACGGGCGATGTGT -3′
CO-I	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′
	HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′

each in length (Apakupakul et al., 1999). Amplifications of 28S, 12S, and CO-I 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 min, followed by 35 cycles of 94 °C (15 s), 44 °C (20 s), and 70 °C (90 s) and a final extension at 72 °C (7 min); 28S—heated to 94 °C for 5 min, 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 min, 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 CO-I—heated to 94 °C for 5 min followed by 15 cycles of 94 °C (45 s), 47 °C (45 s), and 72 °C (45 s), then 25 cycles of 94 °C (20 s), 45 °C (20 s), and 72 °C (30 s) and a final extension at 72 °C (6 min). The QIAquick PCR Purification Kit protocol (Qiagen) was employed to purify amplification products.

2.4. DNA sequencing

Amplification products were sequenced in both directions. Each 8 μl sequencing reaction mixture included 2 μl BigDye (Applied Biosystems, Perkin–Elmer), 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 s), 50 °C (10 s) 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).

2.5. DNA sequence alignment

Sequences of complimentary strands were edited and reconciled with Sequence Navigator (Applied Biosystems). Alignments of the two nuclear and the mt12S genes were accomplished using Clustal W in Macvector 6.53 (Oxford Molecular Group) and alignment of CO-I fragments was done by eye across all taxa because there were no insertions or deletions. Only 385 positions in rDNA were deemed to be alignment ambiguous (regions of many equally-parsimonious alignments): 193 in 18S, 133 in 12S, and 22 in 28S.

2.6. Morphological data

All taxa were evaluated for 24 morphological characters (Table 4). The characters were principally adapted from Siddall and Burreson (1995) and Apakupakul et al. (1999) with the inclusion of additional arhynchobdellid specific characters:

Character 1, Muscular jaws: (0) Absent, (1) Present
Character 2, Toothed jaw: (0) Monostichodont, (1) Distichodont, (2) Astichodont, (3) With stylets
Character 3, Number of jaws: (0) Agnathous, (1) Duognathous, (2) Trignathous
Character 4, Feeding habit: (0) Macrophagous, (1) Haematophagous
Character 5, Salivary papillae: (0) Absent, (1) Present
Character 6, Number of annuli with eyespots: (0) None, (1) One, (2) Two, (3) Three, (4) Four, (5) Five
Character 7, Eyespots per annulus: (0) One pair, (1) Two or more pairs

Table 4
Morphological character and state data matrix

Taxon	Characters																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Aliolimnatis africana	1	0	2	1	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Aliolimnatis michaelseni	1	0	2	1	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Americobdella valdiviana	1	2	0	0	0	0	–	0	–	0	–	0	–	0	1	0	1	1	0	?	0	?	0	0
Barbronia weberi	1	3	0	0	0	2	1	0	–	0	–	0	–	0	0	1	0	1	2	1	0	0	0	0
Chtonobdella bilineata	1	0	1	1	1	5	0	1	1	1	1	0	0	0	1	0	0	1	0	2	0	1	1	1
Cylicobdella coccinea	1	2	0	0	0	0	–	0	–	0	–	0	–	0	0	0	0	1	0	2	0	?	0	0
Erpobdella bucera	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella costata	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella dubia	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella japonica	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella lineata	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella melanostoma	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella mestrovi	1	2	0	0	0	0	–	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella obscura	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella octoculata	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella punctata	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Erpobdella testacea	1	2	0	0	0	2	1	0	–	0	–	0	–	0	0	0	0	0	–	1	0	0	0	0
Haemadipsa picta	1	0	2	1	1	5	0	1	1	1	1	0	0	0	1	0	0	1	0	2	0	1	1	1
Haemadipsa sumatrana	1	0	2	1	1	5	0	1	1	1	1	0	0	0	1	0	0	1	0	2	0	1	1	1
Haemadipsa sylvestris	1	0	2	1	1	5	0	1	1	1	1	0	0	0	1	0	0	1	0	2	0	1	1	1
Haemopis caeca	1	2	2	0	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Haemopis grandis	1	2	2	0	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Haemopis kingi	1	1	2	0	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Haemopis lateromaculata	1	1	2	0	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Haemopis marmorata	1	1	2	0	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Haemopis sanguisuga	1	1	2	0	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Hirudinaria manillensis	1	0	2	1	1	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Hirudo medicinalis	1	0	2	1	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Hirudo nipponia	1	0	2	1	0	5	0	1	1	1	1	1	1	1	1	0	0	1	0	2	0	1	0	0
Limnatis nilotica	1	0	2	1	1	5	0	1	1	1	1	0	0	0	1	0	0	1	0	2	0	1	0	0
Macrobdella decora	1	0	2	1	0	5	0	1	0	1	1	0	0	0	1	1	0	1	0	2	0	1	0	0
Macrobdella ditetra	1	0	2	1	0	5	0	1	0	1	1	0	0	0	1	1	0	1	0	2	0	1	0	0
Mesobdella gemmata	1	0	2	1	0	5	0	1	1	1	0	0	0	0	1	0	0	1	0	2	1	1	1	0
Oxyptychus braziliensis	1	0	2	1	0	5	0	1	0	1	1	0	0	0	1	0	0	1	0	2	0	1	0	0
Oxyptychus striatus	1	0	2	1	0	5	0	1	0	1	1	0	0	0	1	0	0	1	0	2	0	1	0	0
Patagoniobdella fraterna	1	2	0	0	0	5	0	1	0	1	1	1	0	0	1	0	0	1	1	2	0	1	0	0
Patagoniobdella variabilis	1	2	0	0	0	5	0	1	0	1	1	1	0	0	1	0	0	1	1	2	0	1	0	0
Semiscolex similis	1	2	0	0	0	5	0	1	0	1	1	1	0	0	1	0	0	1	0	2	0	1	0	0
Xerobdella lecomtei	1	0	2	1	?	5	0	1	1	1	0	0	0	0	1	0	0	1	0	2	1	1	1	0
Branchellion torpedinis	0	–	–	1	0	2	0	0	–	0	–	0	–	0	0	0	1	1	0	1	1	0	0	0
Calliobdella vivida	0	–	–	1	0	2	0	0	–	0	–	0	–	0	0	0	1	1	0	1	1	0	0	0
Desmobdella paranensis	0	–	–	0	0	1	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Desserobdella picta	0	–	–	1	0	1	1	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Glossiphonia complanata	0	–	–	0	0	3	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Haementeria ghilianii	0	–	–	1	0	1	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Haementeria gracilis	0	–	–	1	0	1	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Helobdella stagnalis	0	–	–	0	0	1	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Hemiclepsis marginata	0	–	–	1	0	2	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Marsupiobdella africana	0	–	–	1	0	1	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Oligobdella biannulata	0	–	–	1	0	1	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Piscicola geometra	0	–	–	1	0	2	0	0	–	0	–	0	–	0	0	0	1	1	0	1	1	0	0	0
Placobdella parasitica	0	–	–	1	0	1	1	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0
Pontobdella muricata	0	–	–	1	0	1	0	0	–	0	–	0	–	0	0	0	1	1	0	1	1	0	0	0
Stibarobdella macrothela	0	–	–	1	0	1	0	0	–	0	–	0	–	0	0	0	1	1	0	1	1	0	0	0
Theromyzon tessulatum	0	–	–	1	0	4	0	0	–	0	–	0	–	0	0	0	0	1	0	0	0	0	0	0

(–), not applicable; (?), unknown.

Character 8, Vaginal tube: (0) Absent, (1) Present
Character 9, Vaginal caecum: (0) Absent, (1) Present
Character 10, Ovisac shape: (0) Tubular, (1) Spheroid
Character 11, Common oviduct: (0) Absent, (1) Present
Character 12, Male atrium extended into elongated penis and sheath: (0) Absent, (1) Present
Character 13, Penis shape: (0) Straight, (1) Recurved
Character 14, Ejaculatory ducts: (0) U-shaped, (1) S-shaped
Character 15, Atria: (0) Bilobed, (1) Fused
Character 16, Copulatory glands: (0) Absent, (1) Present
Character 17, Intergonadal conducting tissue: (0) Absent, (1) Present
Character 18, Testisac arrangement: (0) Grape-like cluster profusely arranged on vasa deferentia, (1) Discretely arranged on vasa deferentia
Character 19, Testisacs per body somite: (0) One pair, (1) Two pairs, (2) Four pairs
Character 20, Cocoons: (0) Brooded, (1) Cemented, (2) Spongy and deposited on land
Character 21, Mid-body nephropores: (0) Ventromedial, (1) Ventrolateral
Character 22, Nephridia: (0) Single funnel apparatus, (1) Multiple funnels in a ciliated organ
Character 23, Friction rays on caudal sucker: (0) Absent, (1) Present
Character 24, Respiratory auricles: (0) Absent, (1) Present

2.7. 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 100 pseudoreplicates and subtree-pruning-regrafting branch swapping with 37% deletion (Farris, 1999). TreeRot.v2b (Sorenson, 1999) was used to calculate support values (b, see Bremer, 1988). Retention indices (RI) and consistency indices (CI) were calculated with PAUP* (Swofford, 2000).

3. Results

Parsimony analysis of 24 morphological characters resulted in 181 most-parsimonious trees with a tree length (L) of 46 steps, an RI of 0.958 and CI of 0.717. The strict consensus of all morphological trees did not fully resolve higher taxonomic groups (Fig. 1). There was some resolution with respect to the Hirudiniformes, Americobdellidae, Cylicobdellidae, and Haemadipsidae. Members of the Hirudinidae and Haemopidae each were polyphyletic.

Analysis of all molecular data combined (3620 characters total, of which 1219 were parsimony-informative) yielded one most-parsimonious tree with 7757 steps (RI = 0.635; CI = 0.370). Incorporating all data (24 morphological characters and four molecular data sets) also resulted in a single most-parsimonious tree (Fig. 2) ($L = 7812$; RI = 0.641; CI = 0.372), which was the same as that obtained from the molecular data alone. Use of mitochondrial DNA data alone failed to resolve relationships among most families and the use of nuclear DNA data alone failed to distinguish among most hirudinid genera.

The resulting cladogram from the combined data supports monophyly of Arhynchobdellida with a basal split of the erpobdelliforms and hirudiniforms. Within the erpobdelliforms, Erpobdellidae was found to be monophyletic with Salifidae, represented only by *Barbronia weberi*, as its sister group ($b = 28$; jac = 100). Americobdellidae was included in the erpobdelliform clade and in a position basal to Salifidae and Erpobdellidae ($b = 10$; jac = 100). Constraining *Americobdella valdiviana* to group with the Hirudiniformes, as traditionally classified (Table 1), required an additional 15 steps.

Relationships among the Hirudiniformes are much more complex. Cylicobdellidae was sister to the rest of the Hirudiniformes ($b = 11$; jac = 98). In terms of traditional classification (Table 1), the families Haemadipsidae, Haemopidae, and Hirudinidae, and the genera *Hirudo* and *Aliolimnatis* were found to be polyphyletic. Within the Haemadipsidae, the tropical terrestrial *Chtonobdella bilineata* from Australia and the Asian *Haemadipsa* spp. formed a clade ($b = 28$; jac = 100), to the exclusion of the terrestrial *Mesobdella gemmata* and *Xerobdella lecomtei* (sister to the hirudinid/haemopid clade). Forcing these into a monophyletic Haemadipsidae increases the tree length by five steps. The medicinal leeches of the Hirudinidae are roughly divided into New World and Old World clades. The former clade ($b = 23$; jac = 100) includes the South American 'haemopids' (*Semiscolex* and *Patagoniobdella*) together in a monophyletic group with the North American *Macrobdella* spp. and South American *Oxyptychus* spp., with the African *Limnatis nilotica* sister to the remainder. The Old World hirudinids 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. For example, exclusion of alignment ambiguous sites ($n = 358$) caused the Haemadipsidae to switch position with the mostly New World hirudinids at the node with a Bremer support index of 1 (jac = 62). A monophyletic Hirudinidae would require an additional 108 steps. Finally, Haemopidae also was polyphyletic, with the South American 'haemopids' sister to the New World macrobdellids and the remaining *Haemopis* spp. ($b = 19$; jac = 100) sister to the Old World hirudinids
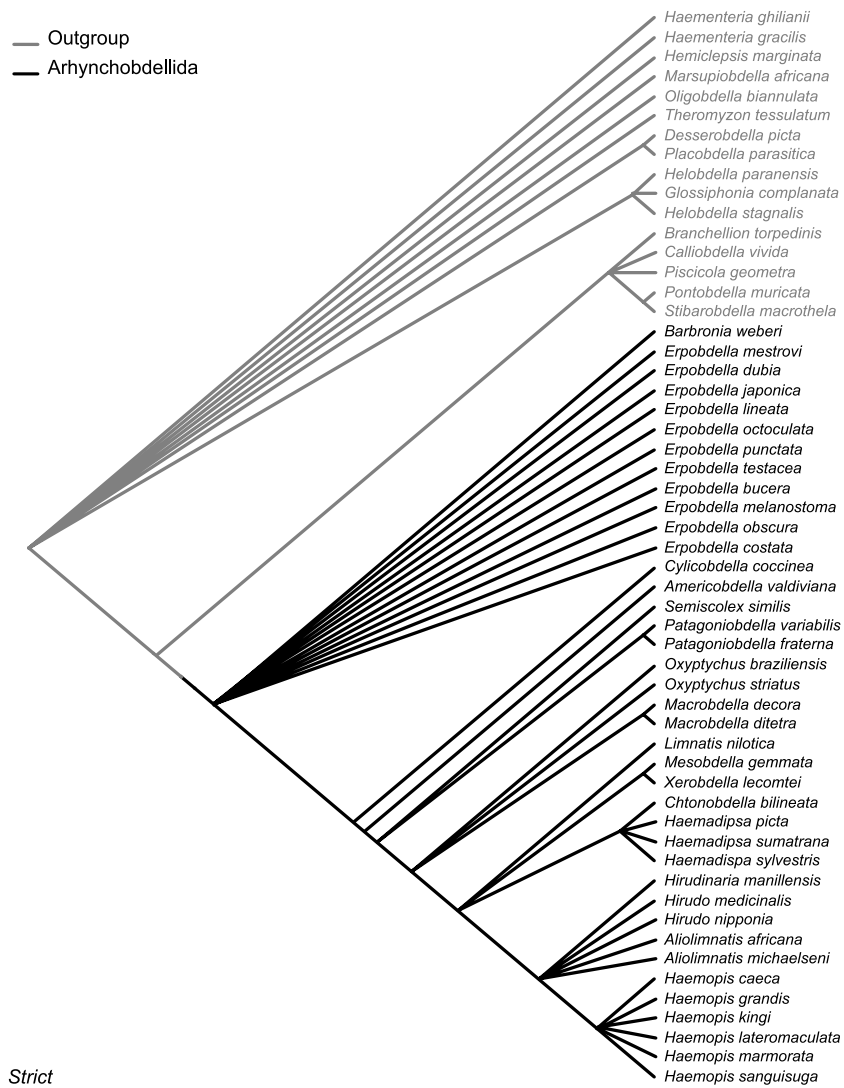
Fig. 1. Strict consensus of 181 equally parsimonious trees obtained from 24 morphological characters.

($b = 6$; jac $= 70$). Northern Hemisphere *Haemopis* spp. had distinct monophyletic groups for each of Europe and North America. The temperate terrestrial Chilean *Mesobdella gemmata* and European *Xerobdella lecomtei* ($b = 4$; jac $= 87$) formed a clade sister to the Old World Hirudinidae and *Haemopis* species.

4. Discussion

The resulting cladogram from the combined, total-evidence approach, analyzing two nuclear genes, two mitochondrial genes and morphology, yielded a hypothesis which conflicts with most 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). This

phylogenetic analysis suggests a 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 (Blanchard, 1849; Johansson, 1913; Richardson, 1969; Ringuelet, 1954; Sawyer, 1986) where groups are distinct and conveniently classified in either with Erpobdelliformes or with Hirudiniformes. The discovery of South American species deviating from that strict classification and exhibiting both ''erpobdellid'' and ''hirudinid'' characters confounded taxonomists (Moore, 1946; Richardson, 1969; Ringuelet, 1954; Soos, 1966). 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*,
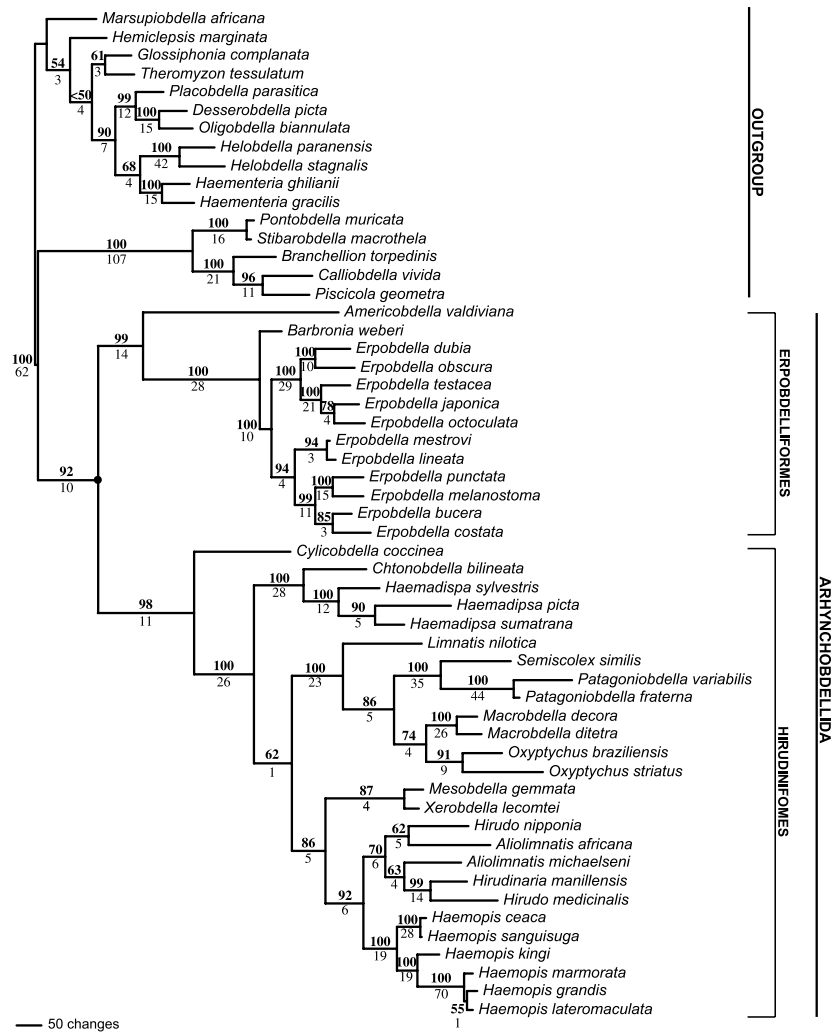
Fig. 2. Most-parsimonious hypothesis from the combined analysis of morphology, 18S rDNA, 28S rDNA, 12S rDNA, and COI. Numbers above and below nodes indicate Jackknife (jac) and Bremer (b) support values respectively.

Mesobdella, and others traditionally classified under Hirudinidae.

4.1. Americobdellidae

The taxonomic status of the large and anatomically unusual *Americobdella valdiviana* has eluded systematists since its discovery (Moore, 1924; Philippi, 1872; Pinto, 1923). 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, *A. valdiviana* has a pair of ducts connecting the male atrium and the female ovarian ducts more typical of piscicolid leeches (Caballero, 1940; Moore, 1924; Siddall and Burreson, 1995). Reflecting this mixture of traits, *A. valdiviana* has

been classified as a member of the Erpobdellidae (= Herpobdellidae) (Blanchard, 1917; Harant, 1929; Weber, 1915), a subfamily within the 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 *A. valdiviana* was sister to Arhynchobdellida in its own lineage corroborating Ringuelet's (1954; p. 11) belief that *A. valdiviana* "se ha originado de los antiguos Arincobdelos antes de la evolucion de las otras familias." Our analysis further substantiates the notion that *A. valdiviana* originated from an ancient stock of arhynchobdellid leeches, however, allied to the Erpobdelliformes—not to the Hirudiniformes as previously thought (Moore, 1924; Ringuelet, 1976; Sawyer, 1986).

4.2. 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, 1972a,b). Cylicobdellid species were first classified as erpobdellids (Blanchard, 1896; Cordero, 1937; Harant, 1929; Ringuelet, 1944, 1954), but later were afforded their own family as predaceous hirudinids allied to the Erpobdelliformes (Ringuelet, 1972a,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, *C. 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.

4.3. Haemadipsidae

Blanchard (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 nephripores (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 subcontinent, 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 Blanchard (1849), *M. 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; Caballero, 1940; Richardson, 1971; Ringuelet, 1943). The presence of caudal (respiratory) auricles is a synapomorphy for haemadipsid leeches (Siddall and Burreson, 1996); however, *M. 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 *X. 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 *M. gemmata* does not group with the other Gondwanan terrestrial tropical leeches, but with the temperate Palearctic *X. lecomtei*, together forming a sister group to the Old World hirudinids and *Haemopis* species. Several workers had anticipated this relationship separate from haemadipsids (Moore, 1946; Richardson, 1971; Ringuelet, 1943, 1972b) and it seems reasonable, therefore, to recognize the family Xerobdellidae, elevated from the subfamilial rank described by Frauenfeld (1868). Further analysis of this group should include the other New World non-auriclate sanguivorous terrestrial leech genus *Diestecostoma*, for which greater morphological affinities with the genera *Mesobdella* and *Xerobdella* have been suggested (Moore, 1946; Richardson, 1971; Ringuelet, 1954).

4.4. Hirudinidae and Haemopidae

Because the terrestrial bloodfeeders are not each others 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*. Our broader taxonomic sampling further substantiates this grouping with the associated finding that transformations between sanguivory and carnivory are homoplastic for aquatic hirudiniforms.

Blanchard (1896) characterized leeches in the 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 Blanchard, 1896), *Semiscolex* and *Patagoniobdella* are exclusively macrophagous in habit, but are monophyletic with the macrobdellids (i.e., *Macrobdella* and *Oxyptychus* spp.). 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, 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 (Blanchard, 1896; Scriban and Autrum, 1934) as its sister taxon. However, the basal position of *Limnatis nilotica* complicates its inclusion in either family. Morphologically, *L. nilotica* grouped near haemadipsid leeches (Fig. 1)—only molecular synapomorphies place this species 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 precedent 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. 2) appears to corroborate this distinction, but neither *Aliolimnatis* nor *Hirudo* appears to be a monophyletic group.

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.

4.5. Evolution of bloodfeeding

The most prominent aspect of leech behavior is the sanguivory of certain species. There have been several hypotheses speculating on the evolutionary patterns of bloodfeeding in leeches. Sawyer (1986) suggested that the ancestor of arhynchobdellid leeches was a predator of invertebrates (i.e., *Haemopis*), following a continuum from macrophagy to omnivory to sanguivory, with independent origins of bloodfeeding in Rhynchobdellida and Arhynchobdellida. Siddall and Burreson's (1996) findings corroborated a macrophagous ancestor with independent origins of bloodfeeding for the hirudinids and rhynchobdellid leeches. Apakupakul et al. (1999) challenged both hypotheses with their own findings proposing an ancestral sanguivorous hirudinid for Arhynchobdellida, and with convergent loss of bloodfeeding for the Erpobdelliformes and *Haemopis* species.

An optimized reconstruction of bloodfeeding in leeches is shown in Fig. 3A. It appears that leeches have a common origin in an ancestral sanguivorous leech as suggested by Apakupakul et al. (1999). The ancestor of the Arhynchobdellida may or may not have been carnivorous (Apakupakul et al., 1999; Sawyer, 1986; Siddall and Burreson, 1996; Trontelj et al., 1999), but like the Erpobdelliformes and Cylicobdellidae, none of which are bloodfeeders, the ancestral arhynchobdellid seems to have lacked a proboscis and armed jaws. However, because of the phylogenetic position of *Cylicobdella coccinea*, it is not possible to determine the ancestral feeding preference for Arhynchobdellida. Either the ancestral arhynchobdellid was a bloodfeeder with at least four transformations to predation, or the ancestor had already become predaceous, with sanguivory being re-acquired later in the hirudiniforms.

4.6. Evolution of terrestrialism

An intriguing conclusion that can be drawn from our results, notwithstanding the overwhelming preponderance of freshwater taxa, is that the ancestral hirudiniform unequivocally was terrestrial (Fig. 3B). However, because the basal-most lineages in Erpobdelliformes are amphibious (Americobdellidae and Salifidae) and those basal in the Hirudiniformes are terrestrial (Cylicobdellidae and Haemadipsidae) the arhynchobdellid ancestor cannot be determined. Counterintuitively, several aspects of aquatic hirudinid sexual biology would seem to corroborate a terrestrial ancestry. Unlike most rhynchobdellid and erpobdellid leeches that mate by way of traumatic insemination (hypodermic implantation of a membrane-bound spermatophore that injects sperm in response to an osmotic pressure change), the haemadipsids and other hirudiniforms are characterized by internal fertilization (gonopore to gonopore copulation with a protrusible penis and a compensatory vagina). Moreover, even though the hirudinids and macrobdellids are aquatic as adults, their cocoons are deposited on land (e.g., moist shorelines) and hatchling leeches must find their way to nearby water when they emerge.

5. Conclusion

Inasmuch as this study points to several difficulties associated with hirudiniform systematics, like the need for separation of the so-called Haemadipsidae and Hirudinidae into two and three groups, respectively, revision on the basis of the currently included taxa might be premature. There are, for example, dozens of terrestrial leech species known, with doubtless many more yet to be collected, discovered and described (Table 1). The phylogenetic hypothesis outlined here should provide a framework for pursuit of a classification scheme for the bloodfeeding leeches that will prove more stable than have previous taxonomic groupings.
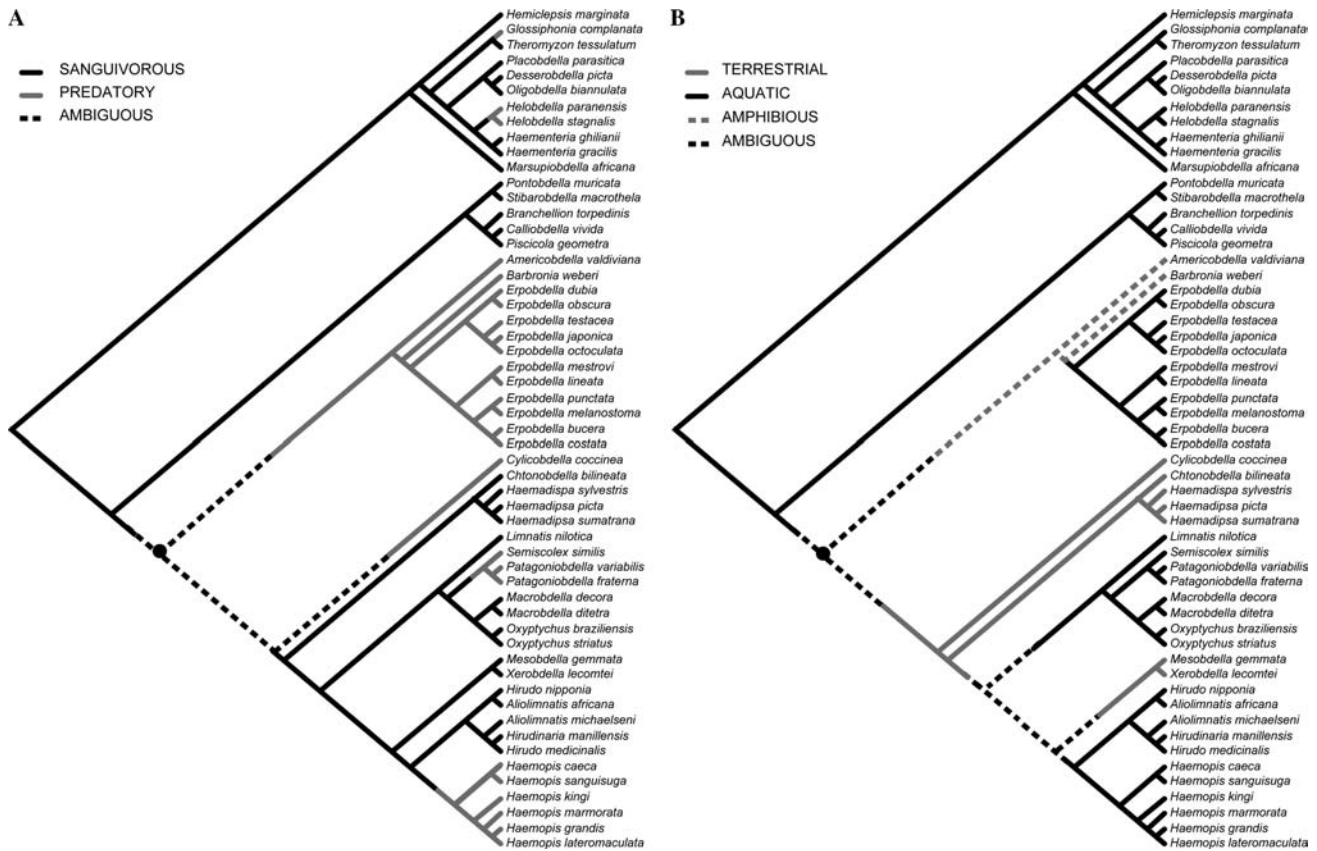
Fig. 3. Evolution of life-history strategies in Arhynchobdellida: (A) most-parsimonious reconstruction of the evolution of bloodfeeding; (B) most-parsimonious reconstruction of the evolution of terrestrialism.

Acknowledgments

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