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

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Abstract

A scourge of tropical and subtropical jungles, bloodfeeding terrestrial leeches of Haemadipsidae have long confused systematists and defied sensible biogeographic interpretation. The family Haemadipsidae usually includes problematic taxa that neither fit the typical IndoPacific distribution of the group, nor properly match diagnostic characters used to define the family. Historically, four additional families—Xerobdellidae, Diestecostomatidae Mesobdellidae and Nesophilaemonidae—have occasionally been recognized for New World and European representatives, though agreement on the composition of those families has not been consistent. Here, we expand the phylogenetic sampling of non-IndoPacific (among other) genera to include Meso American *Diestecostoma* species and *Nesophilaemon skottsbergii* from the Juan Fernandez Archipelago in order to critically assess prior hypotheses in a molecular phylogenetic analysis of arhynchobdellid leeches. The result, based on nuclear 18S rDNA and 28S rDNA and mitochondrial *COI* indicates that there are two distantly related lineages of bloodfeeding terrestrial leeches. The otherwise monophyletic family Haemadipsidae is found to exclude species of *Xerobdella*, *Mesobdella* and *Diestecostoma*. Xerobdellidae is formally resurrected to accommodate species of those three genera. Morphological characteristics corroborate the distinction of Haemadipsidae and Xerobdellidae on the basis of sexual and nephridial characters. *Idiobdella seychellensis* belongs in Haemadipsidae notwithstanding its lacking respiratory auricles. *Nesophilaemon skottsbergii* too is in Haemadipsidae notwithstanding its geographic proximity to the xerobdellid *Mesobdella gemmata*. The characters used to define haemadipsoid families are reevaluated. Feeding preferences and biogeographic patterns are also examined.
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1. 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, p. 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 are distributed throughout tropical and subtropical Indo-Pacific landmasses. There are, however, 10 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

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bloodfeeding in habit; (2) had a unique "haemadipsine" ocular arch with five pairs of eyespots and the 4th and 5th pair separated by two annuli. (Fig. 1a); (3) had lateral nephridia, with the last pair concealed beneath respiratory auricles (Fig. 1b); and (4) had a distribution on landmasses throughout the Indian Ocean and the Pacific Ocean. There were some geographic exceptions. Blanchard (1917) tentatively included the Chilean *Mesobdella gemmata* (E. Blanchard, 1849) and the European *Xerobdella lecomtei* (v. Frauenfeld, 1868) in Haemadipsidae, yet recognized that they deviated from other members of the family due to their isolated distributions, the eyespot arrangement, the ventral (rather than lateral) position of the nephridia and the lack of respiratory auricles. Not surprisingly, others proposed that 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 spite of being duognathous (two-jawed) and sharing characters with other duognathous IndoPacific haemadipsid leeches (e.g. lateral nephridia, respiratory auricles; Fig. 1b), Richardson (1971) proposed that *Nesophilaemon skottsbergii* (Johansson, 1924) shared a greater 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 1).

Table 1
Classification schemes for bloodfeeding terrestrial leeches: (A) adapted from Sawyer (1986) and (B) adapted from Ringuelet (1953[a], 1982[b]), Borda and Siddall (2004a)[c] and Richardson (1975[d], 1978[e])

A	B
Annelida	Annelida
Oligochaeta	Oligochaeta
Hirudinida	Hirudinida
Arhynchobdellida	Arhynchobdellida
Hirudiniformes	Hirudiniformes
Haemadipsidae	Diestecostomatidae[a] [1 Genus]
	Diestecostoma
Trignathous series [5 Genera]	
Haemadipsa	Xerobdellidae[c] [2 Genera]
	Diestecostoma?
Diestecostoma	*Mesobdella*
Mesobdella	*Xerobdella*
Xerobdella	Haemadipsoidea
Duognathous series [12 Genera]	
Group A	Haemadipsidae[d] [2 Genera]
	Haemadipsa
Idiobdella	Domanibdellidae[d,e] [21 Genera]
Nesophilaemon	*Chtonobdella*
	Malagabdella
Group B	Idiobdellidae[e] [2 Genera]
Chtonobdella	*Idiobdella*
Malagabdella	Nesophilaemonidae[b] [1 Genus]
	Nesophilaemon

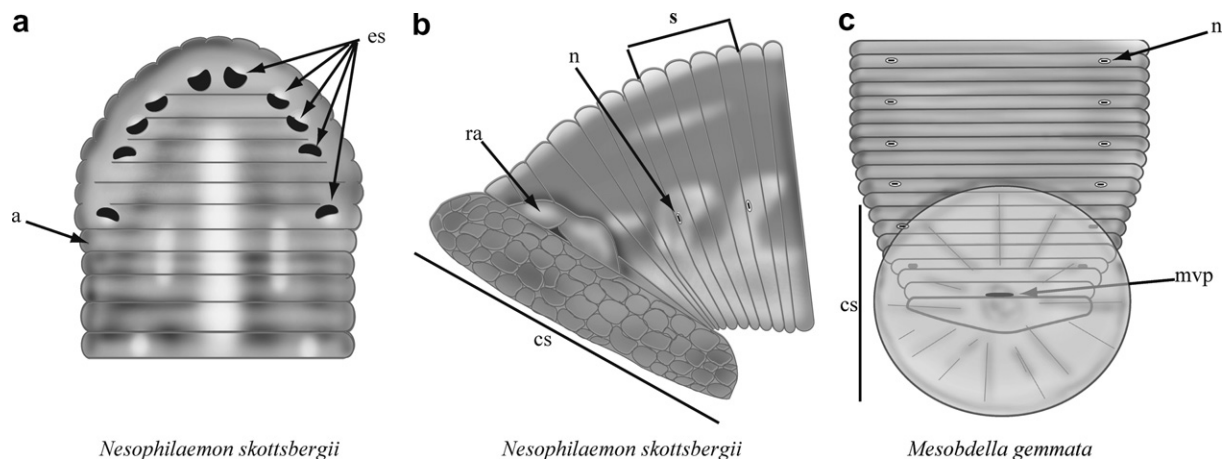Listed genera are only those included in this study.

Fig. 1. Diagnositic characters for haemadipsoid terrestrial leeches. (a) "Haemadipsine" ocular arch of *Nesophilaemon skottsbergii*, showing fourth and fifth eyespot pairs separated by two annuli (=segment); (b) lateral view of *Nesophilaemon skottsbergii* showing respiratory auricles and lateral nephridia; (c) ventral view of *Mesobdella gemmata* showing ventral nephridia and the medioventral pore (through the caudal sucker). a = annulus (annuli; pl.); cs = caudal sucker; es = eyespots; gp = gonopore; mvp = medioventral pore; *n* = nephridiopore; ra = respiratory auricles; s = somite.

1.1. Phylogenetic relationships of bloodfeeding terrestrial leeches

Trontelj et al. (1999) and Kutschera et al. (2007) showed that *Xerobdella lecomtei* was distantly related to the haemadipsid leeches based on nuclear 18S rDNA and *COI*, respectively. 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) and Kutschera et al. (2007) proposed returning the subfamily Xerobdellinae (Moore, 1946) to family status (*sensu* Harrant 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.

In this study, we have expanded the taxonomic representation of Haemadipsidae (*sensu* Sawyer, 1986; Table 1A) and also include the type species of each non-IndoPacific putative family (*sensu* Moore, 1946; Harrant and Grassé, 1959; 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.

2. Materials and methods

2.1. Taxa

Sampling localities and GenBank accession numbers are listed in Table 2. 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

Table 2

Collection localities and GenBank accession numbers for taxa used for the phylogenetic analyses of haemadipsoid leeches

Taxon	Locality	GenBank accession No.		
		18S	28S	COI
Ingroup				
Chtonobdella bilineata	Australia	AF116006[a]	AY425361[c]	AF003267[d]
Chtonobdella whitmani	Australia	EU100065	EU100074	EU100087
Diestecostoma magna	Mexico	EU100067	EU100076	EU100088
*Diestecostoma mexicana**	Mexico	EU100068	EU100077	EU100089
Diestecostoma trujillensis	Mexico	EU100066	EU100075	EU100090
Haemadipsa interrupta	Thailand	EU100069	EU100078	EU100091
Haemadipsa sumatrana	Borneo	AY425464[c]	AY425372[c]	AY425446[c]
Haemadipsa sylvestris	Vietnam	AF116005[a]	AY425373[c]	AF003266[d]
*Idiobdella seychellensis**	Seychelle Islands	EU100070	EU100081	EU100094
Malagabdella fallax	Madagascar	EU100071	EU100083	EU100096
*Mesobdella gemmata**	Chile	AY425472[c]	EU100084	EU100097
*Nesophilaemon skottsbergii**	Juan Fernandez Islands	EU100072	EU100085	EU100098
Xerobdella lecomtei	Slovenia	AF099947[b]	EU100086	EU100099
Outgroup				
Aliolimnatis michaelseni	Congo	AF116010[a]	AY425388[c]	AF116029[a]
Americobdella valdiviana	Chile	AY425461[c]	EU100073	AY425443[c]
Cylicobdella coccinea	Bolivia	AY425462[c]	AY425362[c]	AY425444[c]
Haemopis grandis	Manitoba	AY425465	AY425377	AY425447
Haemopis kingi	Manitoba	AY425466	AY425378	AY4254848
Haemopis terrestris	Ohio	AY786465[e]	EU100080	EU100092
Hirudo medicinalis	France	AY786464[e]	EU100079	EU100093
Limnatis nilotica	Israel	AY425470	AY425389	AY425452
Macrobdella decora	Michigan	AF116007[a]	EU100082	EU100095
Semiscolex similis	Bolivia	AY425475[c]	AY425402[c]	AY425457[c]

(*) indicates type species for proposed familes: Diestecostomatidae, Idiobdellidae, Mesobdellidae and Nesophilaemonidae.

[a] Apakupakul et al. (1999).
[b] Trontelj et al. (1999).
[c] Borda and Siddall (2004a).
[d] Siddall and Burreson (1998).
[e] Borda and Siddall (2004b).

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, 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 a ~10% ethanol solution and gradually adding 95%–100% ethanol until the leeches were relaxed (and dead). Leeches collected from the field were stored in 95–100% ethanol at −20 or at −4 °C until used for DNA extraction.

2.2. 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. Photodocumentation 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 of the reproductive systems for *Haemadipsa sylvestris* was adapted from Keegan et al. (1968).

2.3. Molecular techniques

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. The DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA) was used or phenol–chloroform extractions were performed 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 3. Amplification reaction mixtures used Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) with: 23 µl of RNase-free H_2O, 0.5 µl of each 10 µM primer, and 1 µl DNA template (total volume, 25 µl). All amplification reactions were performed in an Eppendorf® Master Cycler®. Gene fragments were amplified using the following protocol: heated to 94 °C (1 min), followed by 35 cycles of 94 °C (30 s), 48–50 °C (30 s), and 68 or 72 °C (45 s) 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 deletion events. Individual rDNA

Table 3
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	28srD5a	GGYGTTGGTTGCTTAAGACAG	Whiting (2002)
	28srD7b1	GACTTCCCTTACCTACAT	Whiting (2002)
Mitochodrial			
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)

datasets were selected for inclusion in the combined analyses based on those alignment parameters that produced optimal trees.

2.4. 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 jacknife (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* (five partition; 5p); and (2) partitioned by gene, 18S, 28S and *COI* (three partition; 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 18,002 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).
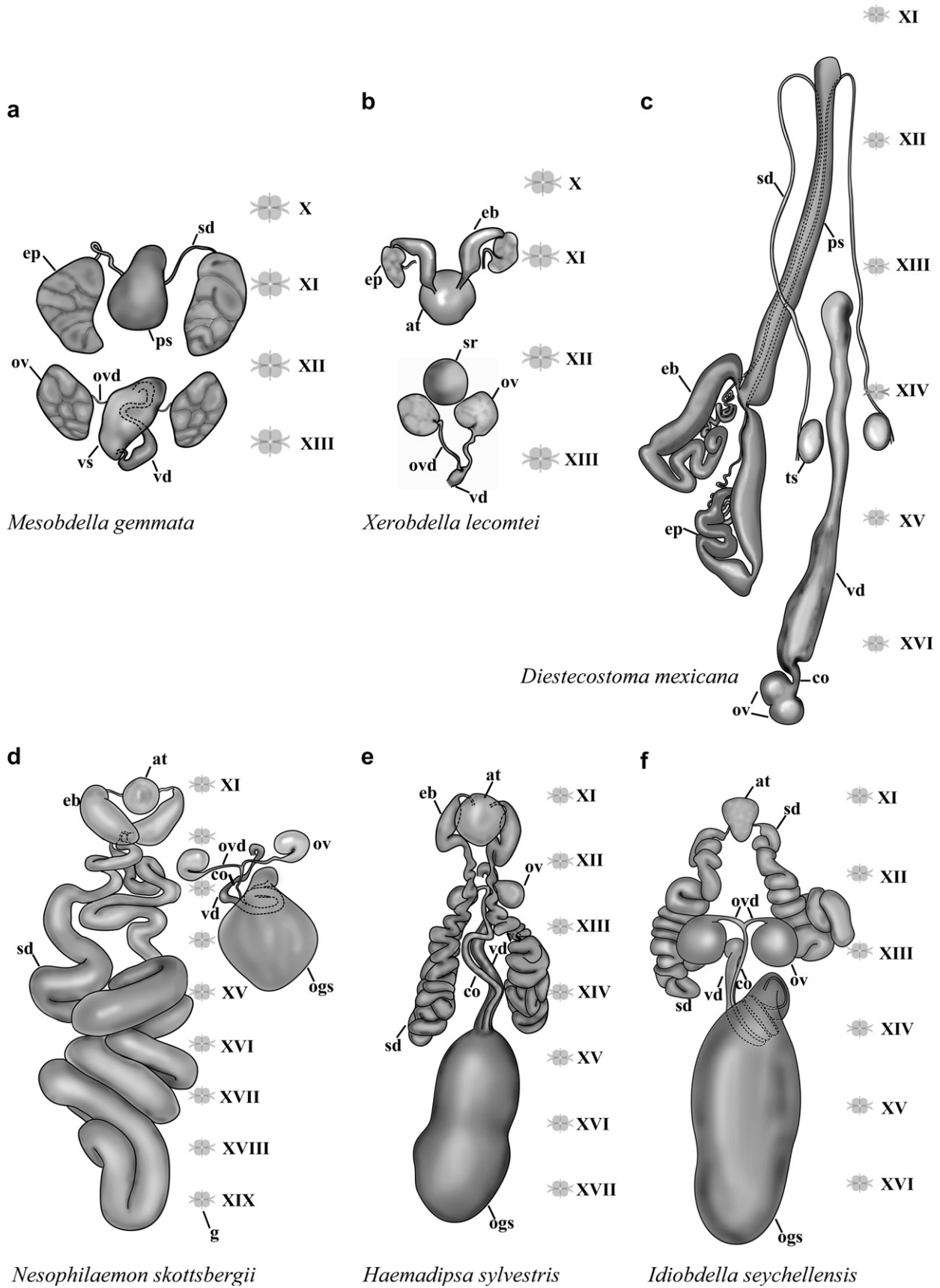
3. Results

3.1. Morphology of terrestrial leeches

With respect to the general classification of land leeches, the external and reproductive morphologies of select taxa were evaluated (Figs. 1 and 2). 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. 1c). Annulation patterns of mid-body somites across these genera were variable: *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) (e.g. Fig. 1a).

All representatives of the non-IndoPacific genera that were examined shared the position of the male and female gonopores within somites XI (male) and XIII (female), regardless of number of annuli separating the gonopores. *Xerobdella lecomtei* also possessed an accessory pore in XII associated with the seminal receptacle. Both *Mesobdella gemmata* and *Xerobdella lecomtei* had micromorphic male and female apparati (Fig. 2a and 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 ducts that inserted into sac-like epidydimes, whereas the *Xerobdella lecomtei* possessed a spherical atrium, with defined ejaculatory bulbs that insert into small epidydimes. 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. In contrast, *Xerobdella lecomtei* possessed a short vaginal duct, with paired globular ovaries that inserted independently into the vaginal duct; vaginal sac absent. A spherical seminal receptacle was found anterior to the female reproductive system in XII. The reproductive systems of *Diestecostoma mexicana* was robust and elongated (Fig. 2c). 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 epidydimes that terminated into thin sperm ducts that traveled anteriorly beneath the penis sheath before turning posteriorly into gonopore at XI. The vaginal duct of *Diestecostoma mexicana* was also long, extending from XIII to XVI, and slightly widened into an elongated sac. At the base was a pair of fused ovisacs.

In contrast to the other non-IndoPacific species, the four-annulate *Nesophilaemon skottsbergii* possessed a lateral position of the paired nephridia, respiratory auricles (Fig. 1b) and had a "haemadipsine" ocular arch

E. Borda et al. / Molecular Phylogenetics and Evolution xxx (2007) xxx–xxx

Mesobdella gemmata

Xerobdella lecomtei

Diestecostoma mexicana

Nesophilaemon skottsbergii

Haemadipsa sylvestris

Idiobdella seychellensis

(Fig. 1a). The male system of *Nesophilaemon skottsbergii* (Fig. 2d) 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 inserting into the gonopore 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, via oviducts, into a common oviduct that coiled along with the vaginal duct beneath the oviducal glandular sac. The reproductive systems of the five-annulate *Haemadipsa sylvestris* (Fig. 2e) and *Idiobdella seychellensis* (Fig. 2f) 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 included an elongated vaginal duct that either inserted directly (*Haemadipsa sylvestris*), or coiled beneath before inserting (*Idiobdella seychellensis*) into the oviducal glandular sac. The female gonopore was found in XII (*Haemadipsa sylvestris*) and XIII (*Idiobdella seychellensis*). Like *Nesophilaemon skottsbergii*, both haemadipsid species also possessed globular ovisacs that inserted independently, via oviducts, 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 lateral position of the nephridia and the "haemadipsine" ocular arch.

3.2. 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 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 3p GTR $+ I + \Gamma$ model. The results from BI analyses are based on the topology and posterior probabilities of the 5p GTR $+ I + \Gamma$ model (Fig. 3).

The analyses of the combined 18S, 28S and *COI* data yielded identical topologies with all major clades strongly

supported, regardless of optimality criterion used for analyses. 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 seven 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* (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).

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) consisted of the Malaysian *Haemadipsa interrupta* + the Borneoan *Haemadipsa sumatrana* (jac = 96; $b = 7$; pp = 0.98), which together 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 = 90; $b = 7$; pp = 1.00).

4. Discussion

Our results corroborate previous findings (Trontelj et al., 1999; Borda and Siddall, 2004a; Kutschera et al., 2007) 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 Xerobdellidae, as well as on the placement of proposed associated genera. The expanded sampling of all

Fig. 2. Comparison of the reproductive morphology of haemadipsoid leeches. Xerobdellidae: (a) *Mesobdella gemmata*, (b) *Xerobdella lecomtei*, (c) *Diestecostoma mexicana*. Haemadipsidae: (d) *Nesophilaemon skottsbergii* (female reproductive system moved to the side to show male system), (e) *Haemadipsa sylvestris*, (f) *Idiobdella seychellensis*. at = atrium; eb = ejaculatory bulb; ep = epididymes; g = ganglion; ov = ovaries; ovd = oviduct; co = common oviduct; ogs = oviducal glandular sac; ps = penis sheath; sd = sperm ducts; sr = female seminal receptacle; ts = testisac; v = vagina; vd = vaginal duct; vs = vaginal sac.
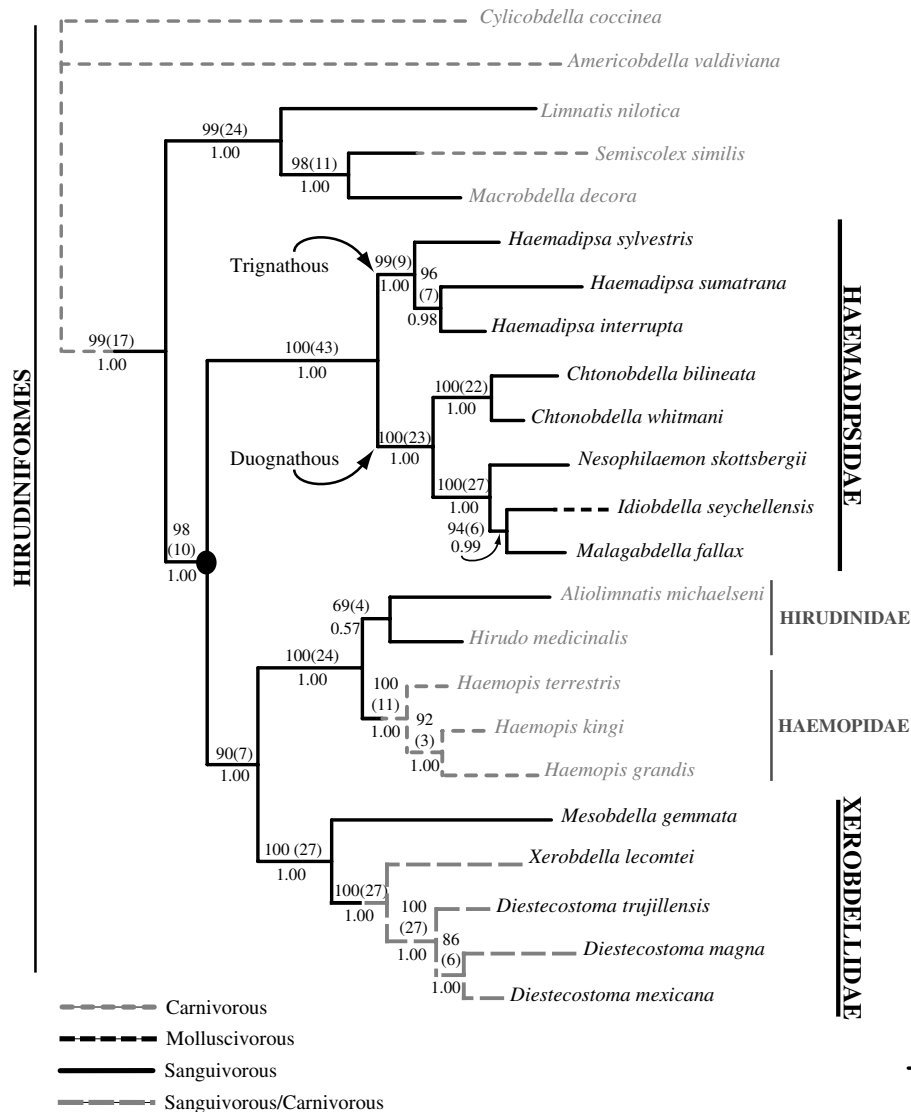
Fig. 3. Bayesian Inference (BI) tree topology based on combined data analysis for 18S rDNA, 28S rDNA and *COI* (5p). Maximum parsimony (MP) analysis resulted in a single most parsimonious tree ($L = 4136$; CI $= 0.556$; RI $= 0.513$). Numbers above the nodes refer to MP jackknife values and Bremer support, in parentheses. BI posterior probabilities are indicated below the nodes. Dotted lines denote non-bloodfeeding lineages.

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, 2004a; Kutschera et al., 2007). Borda and Siddall (2004a) proposed the resurrection of Xerobdellidae (*sensu* Moore, 1946 and Harrant 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 Haemadipsi-

nae (=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 that the Meso American *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 (e.g. Fig. 1c), 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, p. 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 and from the examination of the reproductive morphologies 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, 1944, 1953, 1954) 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) 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 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 (Ringuelet, 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 Indo-Pacific 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 and another for those found in the Seychelles (Idiobdellidae).

IndoPacific Haemadipsidae (*sensu stricto*) was supported as a monophyletic group, however, our hypothesis was in conflict with Sawyer's (1986) revision of Haemadipsidae (see Table 1A). Sawyer's (1986) classification did not consider the zoogeography for haemadipsoid leeches, nor ignored Moore's (1946) and Richardson's (1975) taxonomic recommendations. 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, p. 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, p. 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). The name Xerobdellinae (Moore, 1946) also satisfies the International Code of Zoological Nomenclature descriptive requirements in which the family group name is to include species in genera that are the same in "the position of the nephridiopore, the first pair buccal, the last median beneath the sucker, and the remaining pairs ventral, in the absence of auricles, and the structure of the caudal sucker" (Moore, 1946, p. 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.

4.1. 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, 1927; 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), which possess ventral nephridia.

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*, although there are some exceptions (e.g., *Idiobdella*). Contrasting 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. 2a and b). An accessory female seminal receptacle is exclusive to *Xerobdella* species (Minelli, 1971, 1979) 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, the medioventral pore is unique to Xerobdellidae. The considerable internal morphological variation between the genera of Xerobdellidae might be best explained by long-term isolation from each other.

In contrast to Xerobdellidae, the reproductive systems of the haemadipsids in this study appear to share a basic form with a micromorphic male atrium, globular ovaries that insert independently into a common oviduct, and a long vaginal duct (either straight or coiled) that inserts into an oviducal glandular sac (Fig. 2d–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 (e.g. Fig. 2d–f).

The availability of fresh samples of *Nesophilaemon skottsbergii* facilitated the reexamination of its reproductive morphologies. The only 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.

4.2. Feeding habits of land leeches

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; Kutschera and Wirtz, 2001; Utevsky and Trontelj, 2004; Borda and Siddall, 2004a,b; Williams and Burreson, 2006). 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 (Fig. 3).

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; Kutschera et al., 2007). Its closest relatives, *Diestecostoma* species similarly have also been found on salamanders (e.g. *Ambystoma* species), as well as preying 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).

4.3. Biogeographic patterns

The diversification of Annelida has been attributed to the Cambrian explosion (Ayala et al., 1998; Rousset et al., 2007) with the oldest known fossils dating back to the Silurian (>400 Mya; Eriksson et al., 2004). 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 Fig. 3 appear to be consistent with geological events that might have contributed to the diversification of xerobdellid and haemadipsid leeches (Fig. 4).

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 north of 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 Seychelle 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 (>150 Mya; 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
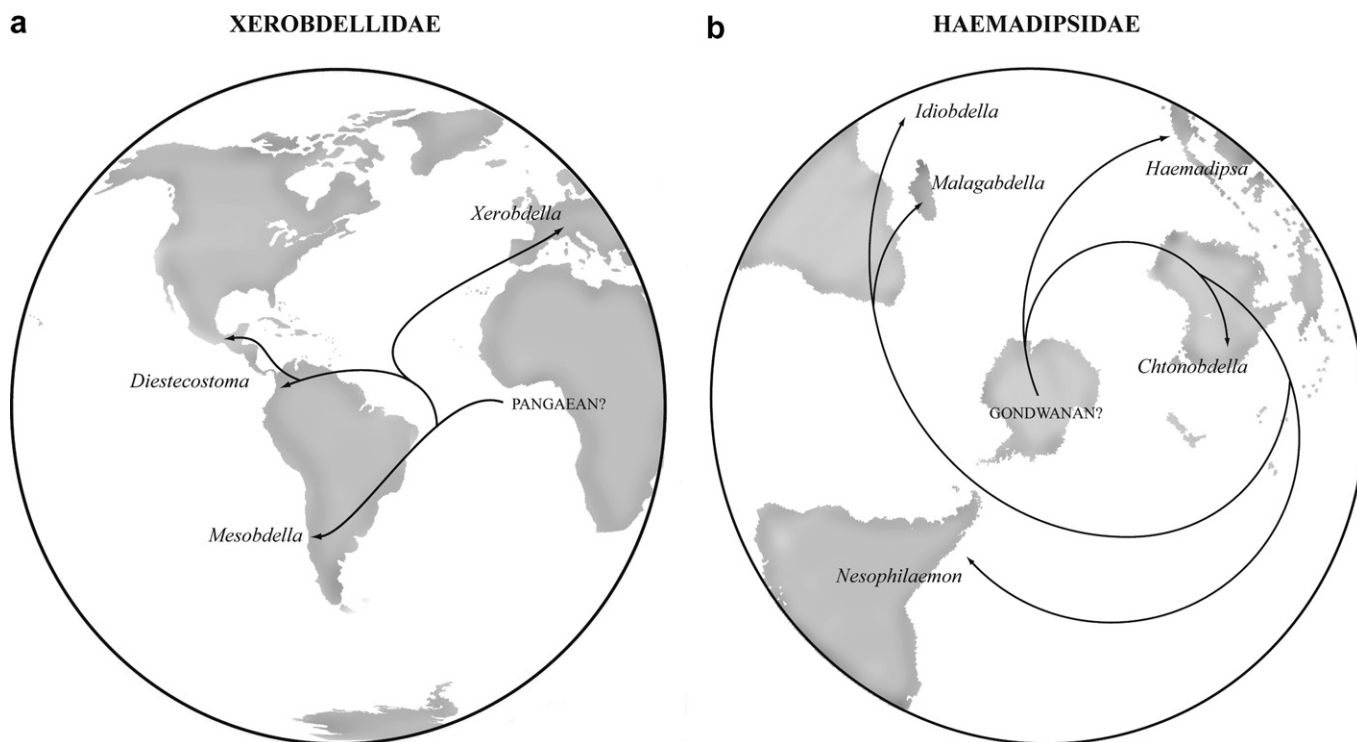

Fig. 4. Biogeographic correlates to portions of the phylogenetic hypothesis for Haemadipsidae and Xerobdellidae. (a) Relationships among xerobdellid leeches appear to correspond to patterns expected for terrestrial taxa initially occupying Southern Pangaea, with eventual dispersal northward, then following 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).

ancestor was at least present on the IndoMalagasy landmass. After the separation from Madagascar (~85 Mya; Storey et al., 1995), the Indian landmass left behind remnants, which later became known as the Seychelle Islands (~65 Mya; Briggs, 2003) as India moved northward towards Asia (Scotese, 2004). 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 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 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 *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 softbodied invertebrates that leave no fossil trace. Our robust phylogeny for the terrestrial bloodfeeding leeches, leaving perhaps as many questions unanswered as answered, beckons more extensive consideration of these notorious annelids.

Acknowledgments

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