Phylogenetic evaluation of systematics and biogeography of the leech family Glossiphoniidae

Mark E. Siddall[A,B], *Rebecca B. Budinoff*[A] *and Elizabeth Borda*[A]

[A]Division of Invertebrate Zoology, American Museum of Natural History,
Central Park West at 79th Street, New York, New York 10024, USA.
[B]Corresponding author. Email: siddall@amnh.org

Abstract.   The phylogenetic relationships of Glossiphoniidae, a leech family characterised by its high degree of parental care, were investigated with the combined use of morphological data and three molecular datasets. There was strong support for monophyly of most accepted genera in the group, many of which are consistent with eyespot morphology. The genera *Desserobdella* Barta & Sawyer, 1990 and *Oligobdella* Moore, 1918 are suppressed as junior synonyms of *Placobdella* Blanchard, 1893 and thus recognising each of *Placobdella picta* (Verrill, 1872) Moore, 1906, *Placobdella phalera* (Graf, 1899) Moore, 1906, and *Placobdella biannulata* (Moore, 1900), comb. nov. The species *Glossiphonia elegans* (Verrill, 1872) Castle, 1900 and *Helobdella modesta* (Verrill, 1872), comb. nov. are resurrected for the North American counterparts to European non-sanguivorous species. *Glossphonia baicalensis* (Stschegolew, 1922), comb. nov. is removed from the genus *Torix* Blanchard 1898 and *Alboglossiphonia quadrata* (Moore, 1949) Sawyer, 1986 is removed from the genus *Hemiclepsis* Vejdovsky, 1884. The biogeographic implications of the phylogenetic hypothesis are evaluated in the context of what is already known for vertebrate hosts and Tertiary continental arrangements.

Introduction

Glossiphoniidae is among the more species rich leech families in terms of described numbers of species (Sawyer 1986; Ringuelet 1985). The group is comprised principally of dorsoventrally flattened leeches normally found feeding on the blood of turtles or amphibians though some species, like those in the genera *Helobdella* Blanchard, 1896 and *Glossiphonia* Johnson, 1816, feed on haemolymph of aquatic oligochaetes and snails. The family also is characterised by a remarkable degree of parental care that is unmatched in the phylum Annelida. Glossiphoniids secrete a membranous bag to hold their eggs on their underside. Covering their eggs, adults will fan the brood until they hatch. The young leeches then will turn and attach to the venter of their parent and, when the parent finds its next host, they are carried to their first blood meal. However, Light and Siddall (1999) have already demonstrated that use of parental care to distinguish among subfamilies of glossiphoniids cannot be justified.

The last 15 years has seen a broadening of our understanding of the unique anticoagulative properties of the salivary secretions from several members of this family. The most numerous of these are the antistasin and antistasin-like inhibitors of coagulation factor Xa, which also exhibit antimetastatic properties on established tumours (Holt *et al.* 1989). These include the original antistasin, ghilanten and lefaxin from species of *Haementeria* Filippi, 1849 (Tuszynski *et al.* 1987; Blankenship *et al.* 1990; Faria *et al.*

1999) and therostatin from *Theromyzon tessulatum* (Müller, 1774) (Chopin *et al.* 2000). The anticoagulative properties of saliva from species of *Haementeria* probably have been the best studied, yielding the XIIIa inhibitor, tridegin (Seale *et al.* 1997), a collagen binding platelet adhesion inhibitor, LAPP (Connolly *et al.* 1992; Huizinga *et al.* 2001) and the fibrinogenolytic enzymes hementin and hementerin (Malinconico *et al.* 1984; Chudzinski-Tavassi *et al.* 1998). In addition to the Xa inhibitor, several other protease inhibitors (theromin, therin and tessulin) have been isolated from *Theromyzon tessulatum* (Chopin *et al.* 1998*a*, 1998*b*; Salzet *et al.* 2000). A new class of antiplatelet glycoprotein IIb-IIIa antagonists called 'ornatins' has been characterised from *Placobdella ornata* (Verrill, 1872) (Mazur *et al.* 1991) and various hyaluronidases are well known as well (Hovingh and Linker 1999).

More recently there has been renewed interest in the endosymbiotic bacteria harbored in specialised esophageal organs in three different genera of glossiphoniids. The largely North American genus *Placobdella* Blanchard, 1893 hosts a unique clade of endosymbiotic alphaproteobacteria in paired esophageal diverticuli (Siddall *et al.* 2004) whereas South American species of *Haementeria* and Old World species of *Placobdelloides* Sawyer, 1986 retain enterobacteriaceae in structurally distinct organs associated with the esophagus (Kikuchi and Fukatsu 2002).

Previous phylogenetic analyses of the family Glossiphoniidae have focused principally on North American taxa

(Light and Siddall 1999) or only on the relationships of one non-blood-feeding genus (Siddall and Borda 2003). In order to more completely address the evolution of sanguivory, parental care and symbioses in the group, a more global taxonomic sampling of glossiphoniid leeches in a combined morphological and molecular phylogenetic assessment was pursued here using mitochondrial cytochrome *c* oxidase subunit 1 (*COI*), mitochondrial NADH dehydrogenase subunit 1 (*ND1*) and nuclear small subunit ribosomal DNA (SSU rDNA) genes.

Materials and methods

Material examined

Fifteen of the 53 species of Glossiphonidae used in this study were newly collected in the field (accession numbers in parentheses for collected lots are denoted AMNH Annelida; those for specimens from which DNA was isolated are denoted AMNH AMCC, and those for DNA sequences are denoted GenBank (http://www.ncbi.nlm.nih.gov/ Genbank/index.html, verified June 2005)).

Placobdelloides jaegerskioeldi (Johansson, 1909), South Africa, near Nelspruit, from rectum of *Hippopotamus amphibious*, vi. 1993 (AMNH Annelida 5265; AMNH AMCC 125863; GenBank AY962450, AY962463, AY962438).

Placobdelloides siamensis (Oka, 1917), Thailand, Bankok, Wat Bovorn, from turtle, coll. Eugene Burreson, ii.1999 (AMNH Annelida 5266; AMNH AMCC 125864; GenBank AY962449, AY962462, AY962437).

Placobdelloides fimbriata (Johansson, 1909), Burkina Faso, Bobo Dioulasso, from venter of water scorpion (*Laccotrephes* sp.), coll. Jérémy Bouyer, xi.2004 (AMNH Annelida 5267; AMNH AMCC 125865; GenBank AY962452, AY962465).

Oosthuizobdella garoui (Harding, 1932), South Africa, Gauteng, Glen Austin pan, from *Pyxicephalus odsperus*, coll. Elizabeth and Lorenzo Prendini, xi.1998 (AMNH Annelida 5268; AMNH AMCC 125866; GenBank AY962460, AY962434).

Hemiclepsis quadrata (Moore, 1924), Namibia, coll. Elizabeth and Lorenzo Prendini (AMNH Annelida 5269; AMNH AMCC 125867; GenBank AY962441, AY962455, AY962412).

Batracobdelloides tricarinata (Blanchard, 1897), South Africa, near Hoedspruit, Gwalagwala tented camp, Maia's Dam, 31.xii.1998 (AMNH Annelida 5270; AMNH AMCC 125868; GenBank AY962445, AY962457, AY962430).

Alboglossiphonia weberi (Blanchard, 1897), Hawaii, Oahu, from under rocks in Kalihi Stream, i.2003 (AMNH Annelida 5271; AMNH AMCC 125869; GenBank AY962440, AY962453).

Alboglossiphonia lata (Oka, 1910), Taiwan, I-Lan County, coll. Lai Yetai (AMNH Annelida 5272; AMNH AMCC 125870; GenBank AY962454, AY962411).

Glossiphonia concolor (Apathy, 1888), Sweden, Kila River, under submerged branches, viii.1998 (AMNH Annelida 5273; AMNH AMCC 125871; GenBank AY962446, AY962458, AY962431).

Glossiphonia verrucata (Müller, 1844), Italy, Rio s'Adde, coll. Paola Magnetti and Renato Bacchetta, 1998 (AMNH Annelida 5274; AMNH AMCC 125872; GenBank AY962447, AY962459, AY962432).

Placobdella costata (Müller, 1846), Italy, coll. Paola Magnetti and Renato Bacchetta, ix.2000 (AMNH Annelida 5275; AMNH AMCC 125873; GenBank AY962448, AY962461, AY962436).

Placobdella multilineata Moore, 1953, USA, Louisiana, Maurepas Swamp, from *Alligator mississippiensis*, coll. Jeff Boundy (AMNH Annelida 5276; GenBank AY962451, AY962464, AY962439).

Helobdella cordobensis Ringuelet, 1942, Chile, Lago Neltume, under rocks, ii.2002 (AMNH Annelida 4343; AMNH AMCC 125862; GenBank AY962442).

Helobdella pichipanan Siddall, 2004, Chile, Lago Huerquehue, under rocks, ii.2002 (AMNH Annelida 4332; AMNH AMCC 125861; GenBank AY962443, AY962456, AY962429).

Helobdella duplicata Moore, 1911, Chile, Lago Panguipuilli, under rocks, ii.2002 (AMNH Annelida 4319; AMNH AMCC 125860; GenBank AY962444).

Methods

The *COI* and *ND1* data for all other species have been used in prior published phylogenetic analyses (i.e. Light and Siddall 1999; Siddall *et al.* 2001; Siddall and Borda 2003), but some SSU rDNA data were newly acquired for this study (GenBank accession numbers AY962410 through AY962439).

Leeches were stored in 95–100% ethanol at 20°C or at ambient temperature until used for DNA extraction. The caudal sucker, or a portion thereof, was removed and utilised for DNA extraction. The caudal sucker is specifically used in order to minimize the possibility of contamination from host/prey DNA found in the gastric regions. DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA, USA) was used for tissue lysis and DNA purification. *18S* rDNA as well as mitochondrial cytochrome *c* oxidase (*COI*) and NADH dehydrogensae (*ND1*) genes were amplified using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Each 25-μL reaction contained 1 μL of 10 mM primer pair mix (primer sequences are identical to those in Apakupakul *et al.* 1999 and in Light and Siddall 1999), 1 μL of template and 23 μL of water. Reaction mixtures were heated to 94°C for 5 min, followed by 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (45 s) and then a final extension of 72°C (7 min) on Eppendorf Mastercyclers (Hamburg, Germany). The ArrayIt™ PCR Purification Kit (TeleChem International, Sunnyvale, CA, USA) was used to purify PCR products. Amplification products were sequenced in both directions. Each sequencing reaction mixture, including 1 μL BigDye™ dye terminators (Applied Biosystems, Foster City, CA, USA, Perkin-Elmer Corporation, Wellesley, MA, USA), 1 μL of 1 mM primer and 3 μL of DNA template, ran for 40 cycles of 96°C (15 s), 50°C (30 s) and 60°C (4 min). Sequences were purified by ethanol precipitation to remove unincorporated primers and dyes. Products were re-suspended in 6 μL formamide and were electrophoresed in an ABI Prism™ 3730 sequencer (Applied Biosystems).

The *COI* sequences were aligned-by-contig in GeneJockey (Biosoft, Ferguson, MO, USA) because there were no apparent insertion/deletion events (INDELs) across all taxa. Similarly *ND1* sequences exhibited a 4-amino-acid (12 nucleotide (nt)) insertion only for species of *Glossiphonia*, *Alboglossiphonia* Lukin, 1976 and for *Hemiclepsis quadrata*, and otherwise were devoid of INDELs. A section of tRNA LEU preceding the *ND1* data was aligned with CLUSTAL W resulting in seven of 42 aligned positions exhibiting INDELs, three of which were autapomorphic. The small subunit ribosomal rDNA gene sequences were aligned with CLUSTAL W; with the exception of an autapomorphic 18 nt insertion in *18S* for *Haementeria lutzi* Pinto, 1920, all other indel events either were between the ingroup and the outgroup or were single nt autapomorphic insertion events for an ingroup species. As such, alternative alignment was not deemed to be a problematic issue even for SSU rDNA sequences. Aligned blocks of sequences are available from the *Invertebrate Systematics* website in Accessory Publication – Molecular and from the Leech PEET website: http://research.amnh.org/ users/siddall/PEET/, verified June 2005.

Morphological characters (adapted from Light and Siddall 1999) included: number of pairs of eyespots; separation of gonopores; sanguivory; presence or absence of mycetomal diverticuli; mycetomal balls or oesphageal organ; number of pairs of testisacs; and presence or absence of the chitinoid scute or nuchal glands on somite VIII (available from the *Invertebrate Systematics* website in Accessory Publication – Morphology and from http://research.amnh.org/users/siddall/, verified June 2005).

Phylogenetic analyses were conducted with PAUP 4.0b10 (Swofford 2002) with 100 random-taxon-addition search sequences and TBR branch breaking in which all characters were of equal weight and non-additive. Jackknife support values were determined with five random-taxon-addition-search sequences and TBR branch breaking leaving out 37% of characters for 100 replicates.

Results

Phylogenetic analysis of the combined data (3162 characters, of which 897 were informative) resulted in two equally parsimonious trees with a length of 6729 steps and a retention index of 0.4883 (strict consensus shown in Fig. 1) and disagreeing only on the relative relationships of three species of *Helobdella* in the '*triserialis*' complex. These trees corroborate several notable sets of relationships traditionally suggested by morphology. Glossiphoniid leeches with two pairs of eyespots in which the anterior pair are coalesced and are adpressed to the posterior pair and that possess a pair of mycetomal diverticuli all were in a monophyletic group with the type species of *Placobdella*, *Placobdella costata*. Glossiphoniid leeches with one pair of eyespots and two pairs of globular mycetomes all were in a monophyletic group with the type species of *Haementeria*, *Haementeria ghilianii* Filippi, 1849. Glossiphoniid leeches lacking mycetomal tissue of any sort fell into two distinct clades. Those with one pair of eyespots were in a monophyletic group with the type species of *Helobdella*, *Helobdella stagnalis* (Linnaeus, 1758). Also, leeches in this clade, which possess either a chitinoid plaque ('scute') or a nuchal gland on somite VIII, formed a monophyletic group. Glossiphoniid leeches with three pairs of eyespots were in a monophyletic group with the type species of *Glossiphonia*, *Glossiphonia complanata* (Linnaeus, 1758). However, leeches possessing esophageal organs comprised of symbiont-bearing cell clusters around the posterior portion of the esophagus did not form a monophyletic group. Two such leeches formed the first diverging glossiphoniid lineage with *Placobdelloides fimbriata* and *Marsupiobdella africana* Goddard & Malan, 1912. Another, *Batracobdelloides tricarinata*, grouped with *Hemiclepsis marginata* (Müller, 1774), which does not possess these cells. Neither of the foregoing grouped with the esophageal-organ-bearing genera *Placobdelloides* and *Oosthuizobdella* Sawyer, 1986.

Two regions of the consensus tree (Fig. 1) demonstrated generalized poor support in terms of jackknife analyses conducted on the entire dataset. Support levels for each of these were reinvestigated in isolation in order to determine whether there were obvious secondary signals underlying these values or if low jackknife scores reflected insufficient character support. Jackknife resampling of the clade comprising species of *Batracobdelloides* Oosthuizen, 1986, *Hemiclepsis* Vejdovsky, 1884, *Theromyzon* Philippi, 1867, *Oosthuizobdella* and *Placobdelloides* suggested that was considerably more internal support (e.g. 73% for a sister-group relationship of *Batracobdelloides* and *Hemiclepsis*)

than appears in the analysis of all taxa. This result was obtained using species of *Glossiphonia* and *Alboglossiphonia* with or without the addition of *Helobdella* and *Haementeria* species as an outgroup. Inclusion of *P. fimbriata* and *M. africana* in these isolated analyses suggested that there was some molecular phylogenetic signal shared between this species and the other esophageal organ bearing taxa. Nonetheless, for *P. fimbriata* and *M. africana* to group with this clade required an additional nine steps. Jackknife resampling of the clade containing species of *Placobdella* using *Haementeria* species as a functional outgroup yielded the same relationships (Fig. 1).

Discussion

The resulting phylogenetic hypothesis for the family Glossiphoniidae contains many of the same groups found to be monophyletic by Light and Siddall (1999). The principal difference afforded with this newly expanded taxonomic scope is a fundamental re-rooting of the ingroup. The prior hypothesis rooted the Glossiphoniidae between a *Haementeria*/*Helobdella* clade and the remainder of the included taxa. However, at the time the support for that rooting was admittedly low (Bremer support of only 1). Our results corroborate all of the principal clades found by Light and Siddall (1999) with the exception that *Placobdelloides fimbriata* and *Marsupiobdella africana* are placed outside of the rest of the Glossiponiidae as the first diverging lineage in the group. This placement is intriguing insofar as *M. africana* is parasitic exclusively on *Xenopus laevis* in southern Africa (Oosthuizen and Siddall 2002), a frog that itself appears be part of the earliest diverging lineage of anurans (Hoegg *et al.* 2004). Furthermore, *M. africana* is the only glossiphoniid that does not form a membranous cocoon for its eggs; this species holds its developing young in a ventral brood pouch.

Phylogenetic impact on taxonomy

The notion that monophyly is a prerequisite to the naming of taxa now has a long history and is not even problematic in the ongoing debates about phylogenetic v. Linnaean codes of nomenclature. Leech systematics already has undergone considerable revision in light of emerging phylogenetic hypotheses (Trontelj *et al.* 1996, 1999; Trontelj and Sket 2000; Siddall 2002; Siddall and Borda 2003; Borda and Siddall 2004). Similarly, here, several preconceptions about group membership, and even species-level designations, bear reconsideration in light of this phylogeny.

Light and Siddall (1999) suggested that the genus *Placobdella* should be expanded to include species traditionally, or recently, included in other genera. Barta and Sawyer (1990) established the genus *Desserobdella* for *Clepsine picta* Verrill, 1872 in light of the species' possession of diffuse salivary tissue. This species had been placed in *Placobdella* by Moore (1906) and then later transferred to
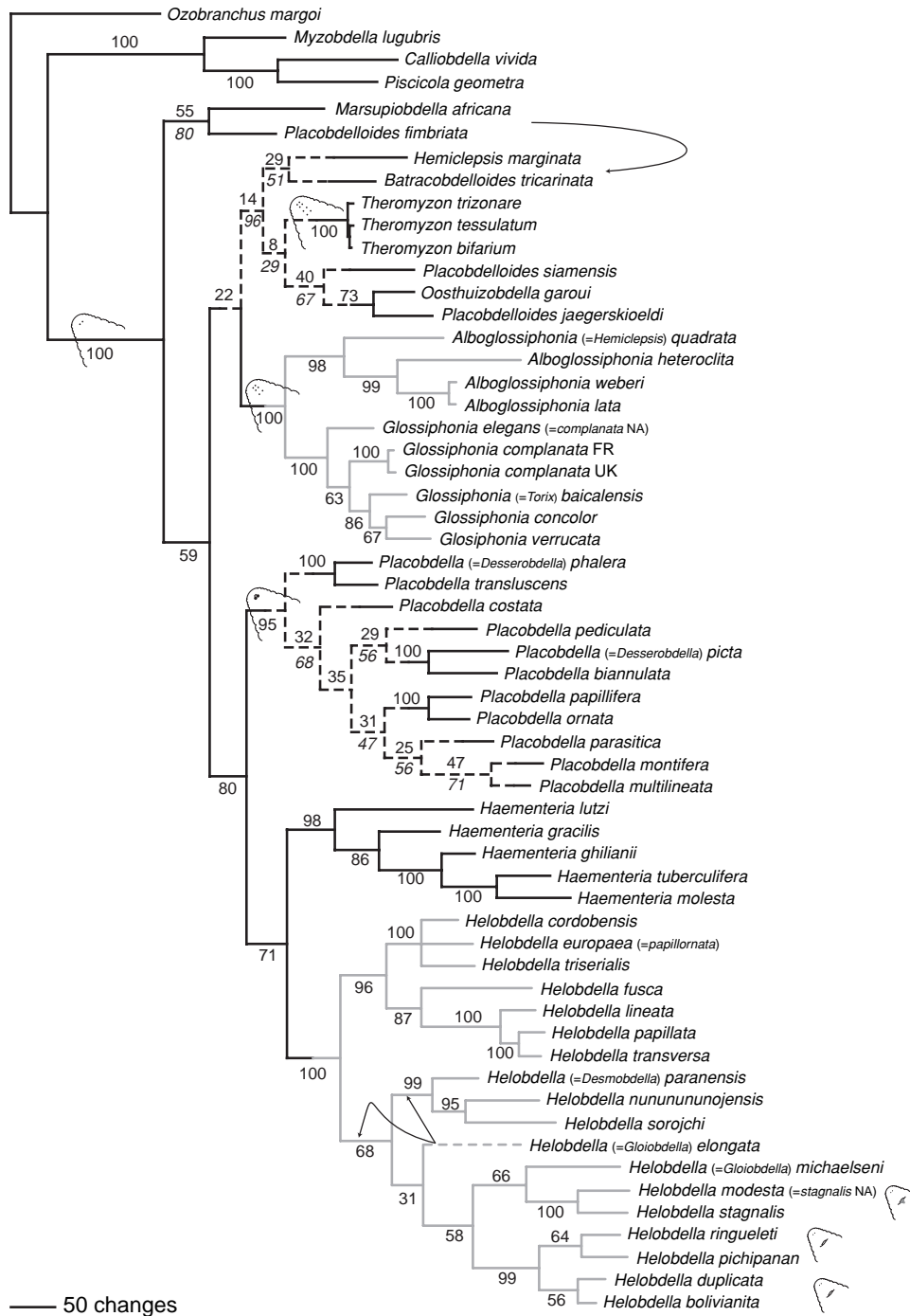
Fig. 1.   Strict consensus of two equally parsimonious trees for the phylogenetic relationships of the leech family Glossiphoniidae ($L = 6694$; $CI = 0.2958$; $RI = 0.4886$) based on combined data from *COI*, *ND1*, *18S* rDNA and morphological characters. Jackknife support values are labelled at internodes. Dashed lines indicate lineages considered poorly supported by the data and worthy of further investigation in the future. Jackknife values in italics for the clade composed of species of *Batracobdelloides*, *Hemiclepsis*, *Theromyzon*, and *Placobdelloides* result from examination of this group in isolation. Jackknife values in italics for the genus *Placobdella* result from examination of this group using only species of *Haementeria* as the functional outgroup. Arrows indicate near, but suboptimal, solutions for the placement of *Placobdelloides fimbriata* and *Marsupiobdella africana* as well as *Helobdella elongata* (Castle, 1900). Sanguivorous lineages are drawn black, non-sanguivorous lineages are drawn grey. Transformations from the plesiomorphic single pair of eyespots are illustrated at the base of the genera *Theromyzon*, *Glossiphonia*, and *Placobdella*. Presence of nuchal glands or a scute are also illustrated for six species of *Helobdella*.

Batrac(*h*)*obdella* Viguier, 1879 for similar reasons (Richardson 1949; Mann 1962). Jones and Woo (1990) concurred with Barta and Sawyer (1990) in their placing *Clepsine phalera* Graff, 1899 in the new genus *Desserobdella*. Species of *Desserobdella*, in addition to *Placobdella pediculata* Hemingway, 1908 and *Oligobdella biannulata* (Moore, 1900) Moore, 1918, have the typical placobdellid ocular morphology (two pairs with coalescence of the anterior eyespots) notwithstanding their having diffuse salivary tissue not organised into discrete glands more typical for the genus *Placobdella*. Light and Siddall (1999) were not able to formally recommend inclusion of these species into the genus *Placobdella* for lack of the type species of the genus, *Placobdella costata*. That species now is included in these analyses. All glossiphoniids with the placobdellid ocular pattern form a monophyletic group with *Placobdella costata*. Nominal species of *Desserobdella* are not even monophyletic themselves. Furthermore, *Oligobdella biannulata*, which had been placed in its own genus because of its possession of two annuli per somite, also is a member of this clade and does not group with the other biannulate leech in the analysis '*Torix*' *baicalensis*. Neither is the possession of compact salivary glands a unique characteristic of species of *Placobdella*; it occurs in all species of *Haementeria* and even some species of *Helobdella*. There seems to be no remaining justification for recognition of the genera *Desserobdella* or *Oligobdella*. *Oligobdella* Moore, 1918 thus is a subjective junior synonym of *Placobdella* rendering the new combination *Placobdella biannulata*. *Clepsine phalera* Graf, 1899 reverts to *Placobdella phalera* (Graf, 1899) Moore, 1906. *Clepsine picta* Verrill, 1872 reverts to *Placobdella picta* (Verrill, 1872) Moore, 1906. *Placobdella transluscens* Sawyer and Shelley, 1976 remains in the genus also implying a new combination *Placobdella michiganensis* for the closely related *Batracobdella michiganensis* Sawyer, 1972.

Insofar as biannulation of midbody somites does not appear to be a phylogenetically consistent morphological character, it is not surprising to find that the Baikalan leech that specialises in feeding on amphipods, *Torix baicalensis* (Stschegolew, 1922), is found with other non-sanguivorous leeches in the genus *Glossiphonia*. *Glossphonia baicalensis*, comb. nov., forms a clade with the other European species in the genus, *Glossiphonia complanata*, *Glossiphonia concolor* and *Glossiphonia verrucata*, to the exclusion of the North American taxon typically referred to as *Glossiphonia complanata* (L.) as well. The fact that North American representatives show considerable genetic distinction, and are not monophyletic with their European counterparts, is ample justification to resurrect Verrill's (1872) specific epithet, *Glossiphonia elegans* (Verrill, 1872) Castle, 1900 in spite of the general morphological similarities for these geographically widely separated species. For similar reasons we also resurrect Verrill's (1872) specific epithet, *Helobdella*

modesta (Verrill, 1872), comb. nov. for the North American counterpart of the European *Helobdella stagnalis* (L.). Lukin (1976) predicted that the genus *Glossiphonia* would require subdivision and established as a provisional subgenus *Alboglossiphonia* comprising those species of *Glossiphonia* for which the anterior-most pair of eyes were close together and which lacked substantial somatic pigmentation. Even though it poses a problem for long preserved museum specimens bleached of all recognisable pigments, Lukin's taxon has universally achieved the status of genus rank. Indeed, nominal species of *Alboglossiphonia* were found to be monophyletic here and we recognise the taxon as valid. *Alboglossiphonia quadrata* (Moore, 1949) Sawyer 1986 had been transferred to *Hemiclepsis* in light of its discoid anterior morphology (Oosthuizen 1991): a characteristic of species of *Hemiclepsis*, and of *Helobdella michaelseni* Blanchard, 1900.

The included species of *Oosthuizobdella*, a genus that is distinguished from *Placobdelloides* only on the basis of accessory anterior pigmentation (Sawyer 1986), predictably was found clustered with two species of *Placobdelloides*. However, in light of the poor support in the phylogenetic tree for this group and its relation to the other esophageal organ bearing genera like *Batracobdelloides* and *Marsupiobdella* Goddard & Malan, 1912, and in light of our not having included the type species for the genus, *Placobdelloides multistriata* (Johansson, 1909), we reserve judgement on any taxonomic changes. Presumably additional species from this Old World assemblage will aid in resolving relationships.

Biogeography

Some components of the resulting phylogenetic hypothesis entail compelling biogeographic corollaries, though only one appears to be consistent with a vicariant pattern of species diversification (Fig. 2). The (*Albo*)*Glossiphonia* clade of non-blood feeding leeches, which typically are predators of freshwater snails, exhibit a pattern of cladogenesis that is consistent with the breakup of the Northern part of Pangea. Specifically with respect to species of *Alboglossiphonia*, there is the Laurasian/African sister-group relationship reflected in a variety of taxonomic groups corresponding to the opening of the Tethys (~175 Mya). This is followed by vicariance of the North American and European representatives of the genus *Alboglossiphonia* (~100 Mya) and eventual dispersal to Hawaii of *A. weberi*. The remainder of the *A. weberi* species-group (including the East Asian *A. lata*) is widespread throughout south-central Asia. Separation of the North American and Eurasian plates also is reflected in the sister-group, *Glossiphonia* spp.

The genus *Placobdella* is a wholly North American clade with the exception of the European *Placobdella costata* nested well within the group. Although this position for *P. costata* may at first appear to be odd, it is completely consistent with patterns of host association. In Europe,
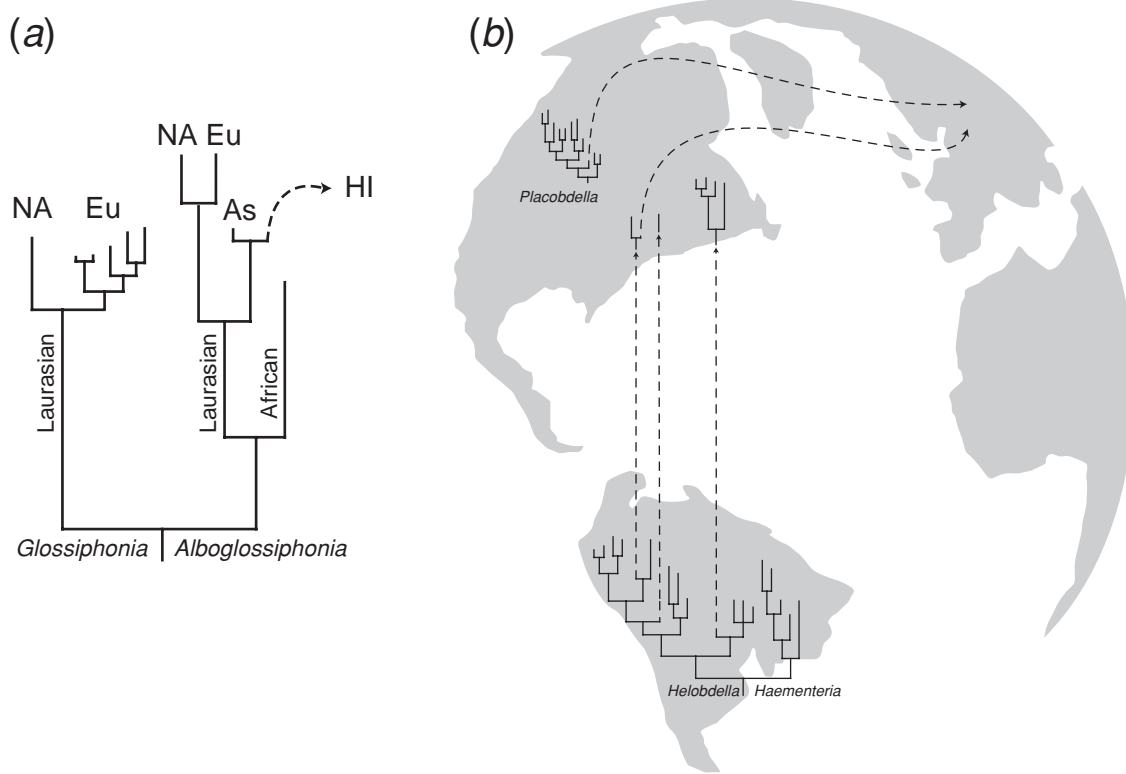
Fig. 2. Biogeographic correlates to portions of the phylogenetic hypothesis for the Glossphoniidae. (*a*) Relationships among species of *Glossiphonia* and *Alboglossiphonia* appear to correspond to patterns expected for freshwater taxa occupying Northern Pangea. (*b*) Approximate patterns for continents during the Tertiary and probable dispersal events for species of *Placobdella* and *Helobdella* (arrows). NA = North America, Eu = Europe, HI = Hawaii, As = Asia.

P. costata is found most often parasitizing the pond turtle *Emys orbicularis,* which in turn is the sole European derivative of the New World Emydidae (Feldman and Parham 2002). Fossil and molecular data suggest that the dispersal of *E. orbicularis* from North America to Europe occurred in the early Miocene (Lenk *et al*. 1999; Fritz 1998). It appears they may have arrived with leeches attached (Fig. 2*b*).

Paralleling the dominance of *Placobdella* in North America is the preponderance of species of *Helobdella* in South America. With the exception of the Mexican *Haementeria officinalis* Filippi, 1849, the sister-group too is exclusively South American, which points unequivocally to a South American origin for both clades (Fig. 2*b*). Assuming the 1.2 million years since the closing of the Panamanian isthmus is insufficient time to explain the diversification of *Helobdella fusca* (Castle, 1900) and allies in North America, the phylogeny suggests at least three northward passive dispersal events for species of *Helobdella*. Migratory birds have been traversing the pan-American corridor since the two continents drifted into proximity in the Tertiary (Mayr 1946; Böhning-Gaese *et al*. 1998) and could have spread species of *Helobdella* along with their molluscan prey. At this time we are at a loss to explain the exclusively northern directionality to this phenomenon.

Symbiotic associations with sanguivory

Reichenow (1921, 1922) first recognised the presence of bacterial endosymbionts within the specialised esophageal organs of *Placobdella costata*. These esophageal organs, called mycetomes, are paired caecate diverticuli situated posterior to the proboscis, each opening into the esophageal lumen on either side of the esophagus. Transmission electron microscopy (TEM) and fluorescent *in situ* hybridization (FISH) have demonstrated that the resident bacteria are gram-negative rods situated intracellularly in the mycetomal epithelial cells (Siddall *et al*. 2004). Deoxyribonucleic acid sequencing and phylogenetic analysis revealed the bacteria to be a new genus of alphaproteobacteria within or sister to the Rhizobiaceae. Interestingly, juvenile leeches removed off the parent's venter (before their first bloodmeal), also harbor the endosymbionts, suggesting vertical transmission from parent to offspring (Siddall *et al*. 2004).

Placobdella, largely North American, is not the only genus with esophageal organs and bacterial endosymbionts. Several other genera, including Mesoamerican and South American *Haementeria* species as well as Old World species of *Placobdelloides*, *Batracobdelloides* and *Marsupiobdella*, each possess esophageal organs with very different morph-

ologies. Leeches of the genus *Haementeria* have two pairs of globular structures connected to the esophagus via thin tubules whereas other genera have an esophageal organ consisting of a cluster of cells closely surrounding the esophagus where it connects to the gastric tissue (Van Der Lande and Tinsley 1976). In both of these lineages, the bacteria are irregular in shape and DNA sequencing reveals them to be enterobacteria in the gammaproteobacteria, closely related to the symbionts of insects such as *Buchnera* and *Wrigglesworthia* (Kikuchi and Fukatsu 2002).

From the phylogeny in Fig. 1 we can infer that the ancestral condition for the family was a possession of the *Placobdelloides*/*Marsupiobdella* esophageal organ structure, presumably with enterobacteria. Thereafter, there would have to have been at least two independent changes to account for the second enterobacterial lineage in the globular mycetomes of *Haemanteria* species and the alpha-proteobacterial lineage hosted by the cecate mycetomes of *Placobdella* species. The precise nature of the symbiotic relationship is not presently known, but it is important to note that non-sanguivorous leeches in the family Glossiphoniidae do not possess esophageal organs of any sort and do not apparently harbor endosymbiotic bacteria. Endosymbionts of other monophagous organisms and obligate bloodfeeders can be a source of nutrients lacking in this limited diet (Auden 1974; Hill *et al*. 1976; Hypsa and Askoy 1997; Noda *et al*. 1997).

Acknowledgments

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