Phylogeny of the leech family Erpobdellidae (Hirudinida : Oligochaeta)

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Abstract. The phylogenetic relationships of the family Erpobdellidae, a group of non-sanguivorous leeches useful as bioindicators, were investigated with the combined use of morphological characters, mitochondrial cytochrome c oxidase subunit I, mitochondrial 12S rDNA and nuclear 18S rDNA. Analyses of separate data sets and the combined data provide strong support for the contention that generic distinctions in the family are not reflective of phylogeny. The resulting hypothesis indicates the number of pairs of labial eyes as the sole morphological predictor of phylogenetic relationship. The traditionally used degree of annular subdivision is the least consistent character. In the absence of readily definable morphological synapomorphies for the resulting clades, the genera Dina, Mooreobdella, Nephelopsis and Trocheta are formally synonymised under the genus Erpobdella, the type genus of the family.

Introduction

Erpobdellid leeches are macrophagous predators of aquatic invertebrates (Young and Ironmonger 1979; Toman and Dall 1997) having abandoned the blood feeding habits of their ancestors (Siddall and Burreson 1998; Apakupakul et al. 1999). Species in this group have long been investigated as model organisms both for ecological studies of invertebrate species interactions (Anholt 1986; Seaby et al. 1995; Zerbst-Boroffka 1999) and as indicator species for freshwater toxicology (Wicklum and Davies 1996; McNicol et al. 1997; Wicklum et al. 1997; Zaranko et al. 1997). Members of the Erpobdellidae (species of Erpobdella, Dina, Mooreobdella, Trocheta and Nephelopsis as well as the recently established genera Motobdella and Croatobranchus) are characterised by their possession of multiple testisacs per somite and are most common in North America and Europe where they form assemblages that are distinct from the erpobdelliform Salifidae (e.g. species of Salifa and Barbronia) more common in Africa and Asia (Soos 1966; El Shmy 1996).

Blanchard’s (1892) establishment of the genus Dina is broadly understood to have failed to distinguish it from Erpobdella (see Moore 1912; Pawlowski 1955). As such, the generic status of erpobdellids has experienced considerable confusion particularly concerning the separation of Erpobdella and Dina. Most treatments currently consider only the number or subdivision of annuli per somite (e.g. Sawyer 1986; Neubert and Neemann 1995; Trontelj and Sket 2000). In this framework, species of Erpobdella have the plesiomorphic five-annulate condition whereas erpobdellids possessing a widened and subdivided fifth annulus are collectively placed in the genus Dina. Similarly, the more extensively subdivided eight-annulate taxa are considered to belong to Trocheta (e.g. Nesemann and Neubert 1994). Generic distinctions not based on this annulation paradigm include Mooreobdella species, which lack anteriorly directed portions of the sperm ducts (preatrial loops), and Motobdella species which possess paired postcaeca, as well as those in monotypic genera like Nephelopsis obscura, which is very large and has a pair of highly coiled cornua on the male atrium, and Croatobranchus mstorovi, a cave-dwelling leech with somatic appendages. Indeed, since Whitman (1886, 1892), annulation patterns have held an important position in leech systematics (e.g. Blanchard 1894; Moore 1900, 1939; Harant 1929; Mann 1953; Pawlowski 1955; Harant and Grassé 1959; Sawyer 1986). Johansson (1910a, 1910b) appears to have dissented from this typological view, having placed the type species of Dina (i.e. Dina lineata) in the genus Herpobdella [sic]. However, he later expressed his own confusion over the establishment of Dina (Johansson 1914) and continued thereafter to include the subgeneric term Dina in parentheses (e.g. Johansson 1929; compare also Bennike 1940 with Bennike 1943). Moore’s position on the matter is equally obscure insofar as he initially seems to have relied on male sexual anatomy (Moore 1912) adding later that the ‘most obvious distinction is not found in the enlargement and subdivision of [annulus] b6... but in the form of the male atrium’ (Moore 1930:182). However, he then proceeded to place Dina dubia only on the basis of the subdivided fifth
annulus (Moore and Meyer 1951; but see Sawyer 1986). Similarly, Mann (1959) held that both the bursal and ovarian organisations were distinctive between Erpobdella and Dina notwithstanding that he too had earlier included the type species of Dina in the genus Erpobdella (see Mann 1952).

It is interesting that the generic level of distinction among erpobdellids has not been resolved despite this group being the subject of more phylogenetic analyses than any other group of leeches (e.g. Trontelj et al. 1996; Govedich et al. 1998; Trontelj and Sket 2000). In part this is due to prior analyses including only European taxa (e.g. Trontelj et al. 1996; Trontelj and Sket 2000) or only North American taxa (e.g. Govedich et al. 1998). Here, European and North American species in the family Erpobdellidae are included in a combined phylogenetic analysis using nuclear, mitochondrial and morphological data.

Materials and methods

Most of the sequence information and accession numbers used in this study have been reported in prior works (Siddall and Burreson 1998; Apakupakul et al. 1999; Trontelj et al. 1999; Trontelj and Sket 2000; Siddall et al. 2001; Sket et al. 2001). Sequences new to this study include mitochondrial cytochrome c oxidase subunit I for Haemoglossus sanguisuga [AF462021] from Swart Arn, Sweden, and mitochondrial 12S rDNA for Dina duchia [AF462022] from Northern Michigan, Dina japonica [AF462023], Erpobdella punctata [AF462024], Erpobdella testacea [AF462025], Mooreobdella bucera [AF462026], Mooreobdella melanostoma [AF462027] and Nephelopsis obscura [AF462028].

Leeches were stored in 100% ethanol at −20°C or at ambient temperature until used for DNA extraction. Tissue from the caudal sucker was removed and utilised for DNA extraction. The caudal sucker is specifically used in order to minimise the possibility of contamination from prey DNA found in the gastric regions. DNeasy Tissue Kit (QIAGEN Inc. Valencia, CA) was used for tissue lysis and purification.

The universal primers, LCO1490, 5'-GGTCAACAATCATAAAGATATTGG-3' and HCO2198, 5'-TAAACTTCAGGGTGACCAAA-3' were used to amplify cytochrome c oxidase subunit I (CO-I) fragments of 665 base pair (bp) length. The 12S mitochondrial ribosomal gene was amplified with primers 12S-A 5'-AAGCCTTATCAATCAATAAA-3' and 12S-B 5'-GGGAGATGGCGTAGTGTG-3'. Amplification reactions for CO-I and for 12S rDNA contained 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer Corporation, Foster City, CA), 10xII Buffer, 2.5 mM magnesium chloride, 0.25 µM of each dNTP (1 mM total), 10 µM of primer pair mix, and template for a 25 µL total volume. Alternatively, Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) were used, for which each 25 µl reaction contains 1.5 units Taq DNA polymerase, 10 mM Tris-Hydrochloric acid (pH 10), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 mM each of the dNTP stabilizers, 10 µM of primer pair mix, template and water. In a GeneAmp PCR System 9700 (Applied Biosystems, Perkin-Elmer Corporation), reaction mixtures were heated to 94°C for 5 minutes, followed by 15 cycles at 94°C (45 sec), 46°C (45 sec) and 72°C (45 sec), then 25 cycles at 94°C (20 sec), 45°C (20 sec) and 72°C (30 sec) and a final extension at 72°C (6 min). The QIAquick PCR Purification Kit protocol (QIAGEN, Inc.) was employed to purify amplification products.

Amplification products were sequenced in both directions. Each sequencing reaction mixture, including 4 µL BigDye™ (Applied Biosystems, Perkin-Elmer Corporation), 2 µL 1 µM primer (single primer for each direction) and 5 µL of DNA template, ran for 40 cycles at 96°C (10 sec), 50°C (10 sec) and 60°C (4 min). Sequences were purified by running each reaction through Centri-Sep columns loaded with G-50 Sephadex to remove primers and unincorporated dyes. Products were electrophoresed in an ABI Prism™ 3700 sequencer (Applied Biosystems, Perkin-Elmer Corporation).

Sequences of complimentary strands were edited and reconciled using Sequence Navigator (Applied Biosystems, Perkin-Elmer Corporation). Alignment of CO-I fragments was done by eye across all taxa because there were no insertions or deletions. Alignment of 18S rDNA and 12S rDNA was accomplished with Clustal W (in MacVector, Oxford Molecular Group).

Morphological characters that could be scored across all of the included taxa were those that had traditionally been used to distinguish among various erpobdellid taxa:

1. Number of annuli between gonopores: (0) two; (1) two and a half; (2) four or more.
2. Number of labial eyes: (0) two (i.e. one pair); (1) four (i.e. two pairs).
3. Preatrial loops: (0) present; (1) absent.
4. Ovisacs: (0) elongate along nerve cord; (1) convoluted or folded back to gonopore.
5. Fifth annulus: (0) not subdivided; (1) subdivided.

All data and alignments are available at http://research.amnh.org/∼siddall/.

Phylogenetic analyses were performed using PAUP* (Swofford 2000). Heuristic searches used 20 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All characters were left non-additive and of equal weight. Bremer support (b) indices (Bremer 1988) were obtained using TreeRot (Sorenson 1999) and parsimony jackknife (jac) values were obtained with 200 replicates of five random additions of branch swapping with XAC (Farris 1999). Retention indices and bootstrap values were calculated with PAUP* (Swofford 2000).

Results

Mitochondrial cytochrome c oxidase subunit I exhibited approximately equal variation among the Erpobdellidae (between 13% and 17%), but only the monophyly of Erpobdella punctata with the genus Mooreobdella resulted from parsimony jackknife analysis of this gene alone. The nuclear 18S rDNA gene showed the least variation across the ingroup (≤5% divergence) whereas the mitochondrial 12S rDNA showed substantial variation across the Erpobdellidae (between 5% and 27%). Both of these data partitions yielded well-supported groups with parsimony jackknifing that were congruent with those obtained from combining all data in analyses (below).

Parsimony analysis of the combined nuclear, mitochondrial and morphological data (2941 characters) yielded one optimal tree with 1350 steps and a retention index of 0.69 (Fig. 1). In that tree, the only non-monotypic erpobdellid genus that was monophyletic was Trocheta. There are two major clades in the ingroup, both of which include nominal species of Erpobdella and Dina. The North American and Asian Dina species group with the European Erpobdella species (and thus the type of that genus) whereas the North American Erpobdella and Mooreobdella species group with Croatobranchus mestrovii and the European Dina
species (and thus the type of that genus). The morphological character with the worst fit (retention index = 0.50) on the resulting hypothesis of phylogenetic relationships was the presence or absence of a subdivided 5th annulus. The character with the best fit to the tree was the number of labial eye spots (retention index = 1.00), transforming from two (i.e. one pair) to four (i.e. two pairs) once on the tree as a synapomorphy for the monophyletic group that contains, among other species, *Trocheta* spp. and *Erpobdella octoculata* (the type species of that genus). The character with the next-best fit (retention index = 0.83) was the apomorphic condition of having folded-back ovaries (occurring once as a synapomorphy for the clade containing *Dina punctata, Erpobdella punctata, Mooreobdella spp.* and *Croatobranchus*; but reversing within that for *Dina lineata*, and changing convergently for *Nephelopsis obscura*).

**Discussion**

The results of the phylogenetic analysis, including 13 ingroup taxa from the family Erpobdellidae with a salifid and a hirudinid as outgroups and combining data from mitochondrial CO-I, mitochondrial 12S rDNA, nuclear 18S rDNA and the five morphological characters, strongly corroborate the contention of Apakupakul et al. (1999) that erpobdellid systematics is in need of radical revision. Trontelj and Sket (2000) have already noted that the degree of subdivision of annuli is an unreliable character to distinguish between species of *Dina* and *Trocheta*. Because *Trocheta krasense* grouped with *Dina punctata* and *Dina lineata*, they formally suppressed the subfamily Trochetinae (see Trontelj and Sket 2000) although they retained *Dina* and *Erpobdella*, notwithstanding their determination that this would render the former paraphyletic with respect to the European species they included. Inclusion of the North American erpobdellids here indicates that retention of all of these groups would be unwise because it would render *Mooreobdella* paraphyletic and *Dina* and *Erpobdella* polyphyletic.

Clearly annulation typology has not served the systematics of leeches very well. As subdivision of the fifth
annulus is the least consistent character on the tree, its use should simply be abandoned (see also Trontelj and Sket 2000). Similarly, though the lack of pretrarial loops has traditionally been used to define the North American genus *Mooreobdella*, the resulting phylogeny supports the view that this has reversed in *Erpobdella punctata*, also North American.

In the face of such a disparity between phylogeny and systematics there are various options available for the future of erpobdellid leech taxonomy. One option is to retain subdivisions in spite of the lack of sufficient morphological characters to distinguish taxa phylogenetically (Trontelj and Sket 2000); that is, retain existing delimitations of *Dina, Trocheta, Erpobdella, Mooreobdella* and *Nephelopsis* on the basis of typological character delimitations (see for example, Cantino et al. 1999, Table II). This is unsatisfactory in that it does not allow the names applied to taxa to reflect any new understanding of relationships or of the conservation of morphological characters. A simple alternative to that aphylogenetic approach would be to synonymise all erpobdellid genera under the oldest generic name for the group (i.e. *Erpobdella*) on the grounds, for example, that the European *Erpobdella octoculata* and North American *Erpobdella punctata* belong to distinctly separate parts of the tree. A third alternative would be to find something predictive from character transformations and alter taxon names to reflect that new understanding. Clearly the type species of *Dina* and the type species of *Erpobdella* fall into separate clades (Fig. 1). However, it might be unwise, and is certainly impractical, to require generic determination on the basis of DNA sequence data and grouping in a phylogenetic tree. One striking feature of the hypothesis (Fig. 1) is the fact that *Erpobdella octoculata* falls into an exclusively eight-eyed clade (two pairs of labial eyes) and *Dina lineata* falls into a six-eyed clade (one pair of labial eyes, the only exception is the blind, cave-dwelling *Croatobranchus*). Redefining the limits of *Erpobdella* and *Dina* might be thought to be simple. *Erpobdella* species could be those with the typical erpobdellid multiple clusters of testisacs per somite and lack of pharyngeal styles that possess the apomorphic condition of two labial doublets of eye spots where present (and then usually four more eyespots posteriorly in two pairs). *Dina* would then be redefined as those species with the typical erpobdellid multiple clusters of testisacs per somite and lack of pharyngeal styles possessing the plesiomorphic condition of one labial pair of eye spots where present (and then usually four more eyespots posteriorly in two pairs). Because the *Trocheta*-like annulation pattern has already been shown to be convergently acquired, and in light of annulation in general not being consistent with phylogeny, it would be appropriate to simply recognise the octoculate *Trocheta* as species of *Erpobdella*. However, the *Dina* condition of possessing only two (one pair of) labial eyes is plesiomorphic for the group—being present in *Salifa* and *Barbromia* (Nesemann 1995; El Shiny 1996)—and should not be relied on to predict a monophyletic group. Unfortunately no other morphological character is sufficiently consistent to define a monophyletic group. Moreover *Croatobranchus*, like other cave-dwelling leeches, are eyeless (Manoleli et al. 1998; Kerovec et al. 1999), which would preclude their generic determination at all. Thus, each of *Dina, Mooreobdella, Nephelopsis* and *Trocheta* are suppressed as subjective junior synonyms of *Erpobdella* Blainville, 1818. This does, however, raise the problem of homonymy for the resulting North American *Erpobdella punctata* (Leidy, 1870) and European (previously *Dina*) *Erpobdella punctata* (Johansson, 1927). Johansson (1927) described the latter originally as *Dina lineata punctata*, but Nesemann’s (1990) action validated the species. Thus, a new name with obvious etymology, *Erpobdella johanssoni* is proposed here for *Dina punctata* (Johansson, 1927) Nesemann, 1990. The genus *Erpobdella* Blainville, 1818 then includes the following 37 nominal species:

*Erpobdella absoloni* (Johansson, 1913), comb. nov.
*Erpobdella anoculata* (Moore, 1898)
*Erpobdella apathyi* (Gedroyc, 1916)
*Erpobdella bucera* (Moore, 1953), comb. nov.
*Erpobdella bykowski* (Gedroyc, 1913), comb. nov.
*Erpobdella concolor* Annandale, 1913
*Erpobdella costalis* Sawyer & Shelley, 1976
*Erpobdella dubia* (Moore & Meyer, 1951)
*Erpobdella eturpschem* (Sket, 1989), comb. nov.
*Erpobdella fervida* (Verrill, 1874), comb. nov.
*Erpobdella japonica* (Pawlowski, 1952), comb. nov.
*Erpobdella johanssoni* (Johansson, 1927), nom. nov.
*Erpobdella krasense* (Skei, 1968)
*Erpobdella krilata* (Sket, 1989), comb. nov.
*Erpobdella lahontana* Hovingh & Klemm, 2000
*Erpobdella latestriata* (Neubert & Nesemann, 1995), comb. nov.
*Erpobdella lineata* (Müller, 1774)
*Erpobdella maoriana* (Mason, 1976), comb. nov.
*Erpobdella mauchi* Nesemann, 1995
*Erpobdella melanostoma* (Sawyer & Shelley, 1976), comb. nov.
*Erpobdella mestrovi* (Kerovec et al., 1999), comb. nov.
*Erpobdella microstoma* (Moore, 1901), comb. nov.
*Erpobdella monostritia* (Gedroyc, 1916)
*Erpobdella nigricolis* (Brandeis, 1900)
*Erpobdella obscura* (Verrill, 1872), comb. nov.
*Erpobdella octoculata* (Linnaeus, 1758). *Type species. Erpobdella parva* (Moore, 1912), comb. nov.
*Erpobdella punctata* (Leidy, 1870)
*Erpobdella quaternaria* (Moore, 1930), comb. nov.
*Erpobdella rathschaensis* (Kobakhidze, 1958), comb. nov.
*Erpobdella stschegelewi* Lukin & Epshtein, 1950
*Erpobdella subviridis* (Dutrochet, 1817), comb. nov.
Erpobdella sviüestra (Sket, 1989), comb. nov.
Erpobdella testacea (Savigny, 1820)
Erpobdella tetragon (Sawyer & Shelley, 1976), comb. nov.
Erpobdella triannulata Moore, 1908
Erpobdella xiangjiangensis (Yang, 1983), comb. nov.

The above approach of summary synonymisation might seem nihilistic to some. However, it does serve to clarify the taxonomic status of the monotypic genera. Admittedly, Erpobdella obscura, comb. nov. is an unusually large erpobdellid (Verril 1872; Moore 1912). Beyond that, though, its anatomy is unremarkable and there seems to be no additional reason to exclude it from the genus Erpobdella. In contrast, Erpobdella mestrovi, comb. nov. does possess a remarkably altered morphology with somatic appendages (Kerovec et al. 1999). Nonetheless, its inclusion in the genus Erpobdella underscores from whence this strange cave-dwelling adaptation arose—ultimately from a common ancestor with Erpobdella lineata, comb. nov. and Erpobdella johnassoni, comb. nov. in Europe.

It is possible that there are other valid erpobdellid genera. The genus Motobdella was recently established for two species with an otherwise unknown synapomorphy of paired postcaeca and the group is supported as monophyletic (Govedich et al. 1998). Their possession of the plesiomorphic single pair of labial eyes suggests that Motobdella may be sister to the remaining Erpobdellidae. Expansion of taxonomic samples to include these, Fadejewosbodella, Archaeobdella, the Asian and African salifids as well as the unusual South American genera, Americobdella and Lumbricobdella, should serve to further clarify our understanding of the evolutionary history of these important non-blood-feeding leeches.

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