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Phylogeny and cocoon production in the parasitic leech *Myzobdella lugubris* Leidy, 1851 (Hirudinidae, Piscicolidae)

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Abstract

Myzobdella lugubris is a commensal leech on crustaceans and a parasite to fishes, surviving predominantly in brackish waters throughout North America. Specimens in this study were collected within the tidal zone of the Delaware River basin (New Jersey and Pennsylvania). To compare regional *M. lugubris* specimens, defined characters were scored after dorsal and ventral dissections, and phylogenetic relationships were resolved using cytochrome *c* oxidase subunit 1 (CO1), 12S ribosomal RNA (rDNA) and 18S rDNA gene fragments. Variance between regional populations was low, suggesting recent dispersal events and/or strong evolutionary constraints. The reproductive biology of *M. lugubris* was explored by quantitative analysis of secreted cocoons. Specimens produced 32.67 ± 4.50 cocoons with fertilization ratios of 88.1% and hatching times of 48 ± 7 days at 17°C under laboratory conditions. At 22°C, 46 ± 28 cocoons were produced with fertilization ratios of 70.27% and hatching times of 28 ± 5 days. Surprisingly, each cocoon supported only one embryo, which is unusual among oligochaetes.

Keywords

Reproduction, oligochaete, channel catfish, crustacean

Introduction

Leeches in the family Piscicolidae are among the least characterized members of Hirudinae, due largely to their predominantly marine habitat, relatively small size (1–2 mm) and difficulties in sample preparation. Pisicolids are parasitic to both freshwater and marine fishes and less often to crustaceans and mollusks (Moore 1946). *Myzobdella lugubris*, a representative of the subfamily Platybdellinae is sister to a Piscicolinae/Pontibdellinae clade. Evaluation of historical patterns of freshwater and marine dispersal remains unresolved, pending more taxonomic sampling.

Myzobdella lugubris inhabits brackish water and cannot tolerate high salinities, but can survive in freshwater for several weeks. They spend most of their life on brackish water teleost species, especially white catfish, *Ictalurus catus*, flathead grey mullet, *Mugil cephalus*, flounder *Paralichthys* spp. and *Fundulus* spp., and are distributed broadly throughout North America (Becker *et al*. 1966; Sawyer 1972; Sawyer and Shelley 1976; Becker and Dauble 1979; Klemm 1972, 1985; Schramn *et al*. 1981; Williams *et al*. 1994; Font 2003) with any sympatric fish species considered a potential host (Moser *et al*. 2006); reported fish host species are listed by Meyer (1940, 1946), Sawyer (1986) and Klemm (1982, 1995). Synonymous leech species include *Illinobdella alba*, *I. elongata*, *I. richardsoni*, *I. moorei*, *Ichthyobdella rapax*, *Cystobranchus virginicus*, and *Myzobdella moorei* (Sawyer *et al*. 1975).

Most commonly, *M. lugubris* adheres to the fins and other skin surfaces of host fishes (Amin 1981; Daniels and Sawyer 1975; Sawyer *et al*. 1975). Attached specimens are described on pectoral, caudal, pelvic and dorsal fins of logperch, *Percina caprodes*, and brown bullhead, *Ictalurus nebulosus*, along beaches of Lake St. Clair, Canada (Appy and Cone 1982), and attached to fins of largemouth bass, *Micropterus salmoides*, usually on the inside of the pectoral fins or below the lower jaw (Troxel 2010). An epidemic of severe ulcerations of the tongue and buccal cavity was reported in largemouth bass, *Micropterus salmoides*, from Currituck Sound, North Carolina (Noga *et al*. 1990).

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Table I. Primers used for PCR amplification and DNA sequencing

Gene	Primer	Primer sequence	Reference
18S rDNA	C	5'- CGGTAATTCCAGCTCCAATAG -3'	Apakupakul *et al*. (1999)
	Y	5'-CAGACAAATCGCTCCACCAAC -3'	Apakupakul *et al*. (1999)
12S rDNA	12S-A	5'-AAACTAGGATTAGATACCCTATTAT-3	Palumbi, 1996
	12S-B	'5'-AAGAGCGACGGGCGATGTGT-3'	Simon *et al*., 1990
CO-I	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer *et al*. (1994)
	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	Folmer *et al*. (1994)

Interestingly, *M. lugubris* attaches to crustaceans upon reaching maturity for cocoon deposition and dispersal. Here we investigated the phylogeny and reproductive biology of *M. lugubris* specimens collected in New Jersey at the mouths of Raccoon Creek and the Delaware River, harvested from multiple channel catfish, *Ictalurus punctata*. Our data suggest that *M. lugubris* specimens are genetically homogeneous across their geographic range, and that only a single egg is deposited into each secreted cocoon.

Materials and Methods

Specimens

Leech specimens were generously provided by David Keller (Academy of Natural Sciences, Philadelphia, PA) and Mike Martinez. Leeches were transported live to Rutgers University (Camden, NJ), and maintained at room temperature in 0.2% (3g/15L) Instant Ocean© (Aquarium Systems) or non-ionic aquarium salt (1g/15L). Dissected and whole specimens were deposited into the collections of the Academy of Natural Sciences in Philadelphia, PA, under the museum numbers ANSP GI 19490, ANSP GI 19491and ANSP GI 19492.

Dissections

Specimens were fixed with 10% formaldehyde in PBS and dissected dorsally and ventrally; characters were observed and scored under a Miji (EMZ-TR, Meiji Techno Co. Ltd.) stereomicroscope. Representative sketches of internal morphology were derived directly from the type specimen.

DNA extraction

Tissue samples from live adult specimens were obtained by placing the leech in a 10% ethanol sedating solution until it was unresponsive to touch. To avoid contamination from gut contents, approximately half of the caudal sucker was removed with a scalpel, and tissue cuttings were immediately processed using the E.Z.N.A.™ Tissue DNA kit (Omega Bio-Tek) following the manufacturer's instructions. Tissue samples were also obtained from unfed juveniles. To remove residual pigment, DNA was processed with a Wizard SV Gel and PCR Clean-Up System kit (Promega, Inc.).

PCR amplification and DNA analysis

Nuclear 18S rDNA, mitochondrial 12S rDNA and partial cytochrome *c* oxidase subunit 1 (COI) gene fragments were amplified from genomic DNA using the polymerase chain reaction (PCR). All 12S sequences were obtained under conditions described by Borda and Siddall (2004 a, b). PCR amplification protocols of target genes were conducted as described by Wirchansky and Shain (2010) employing primers in Table 1. PCR conditions for CO1 and 12s rDNA fragments were: 94°C for 5 min followed by 30 cycles of 94°C (20sec),

Fig. 1. Locations of collected *Myzobdella lugubris* specimens; Raccoon Creek (39°48′31″N ─ 75°22′53″W) and Pier 38 (39°56′59.09″N ─ 75°8′18.86″W)

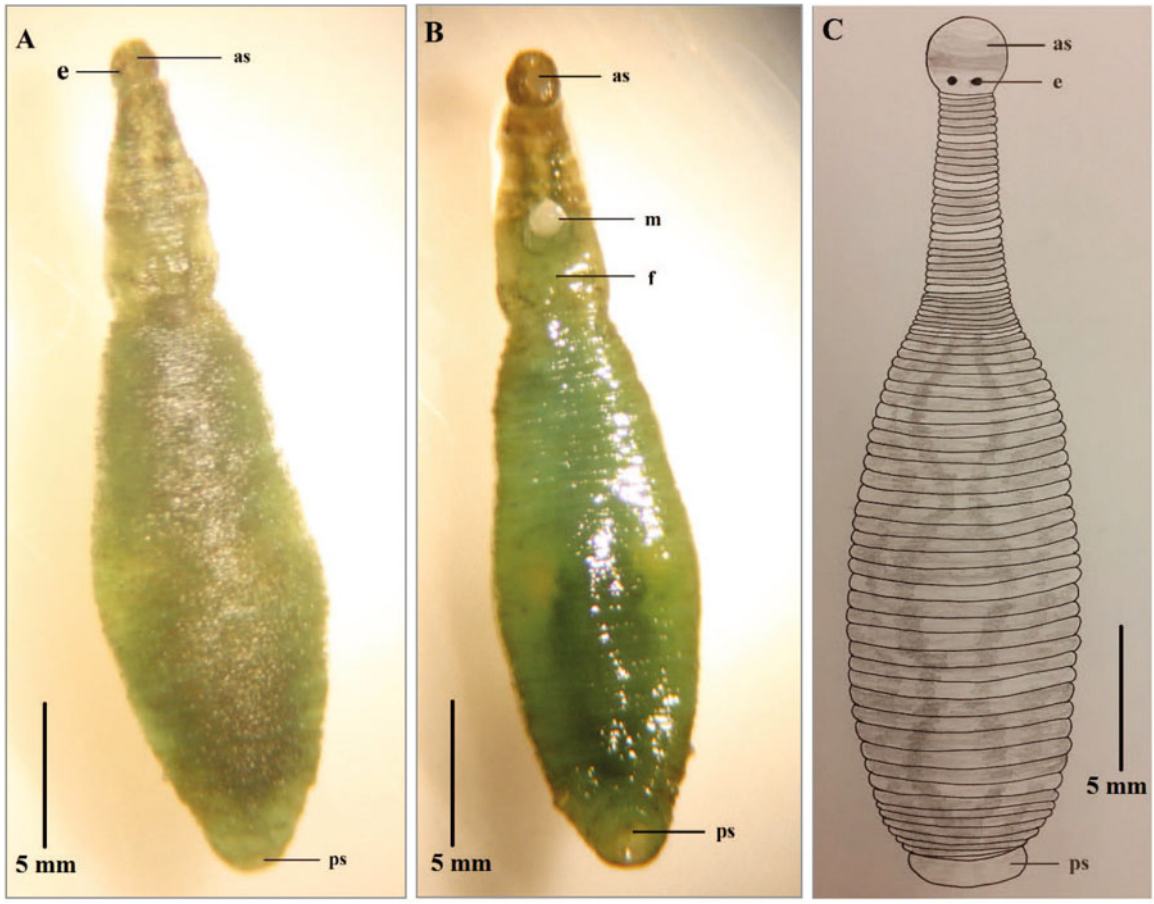

Fig. 2. View of dorsal (A) ventral (B) and dorsal drawing (C) of adult *Myzobdella lugubris*. as, anterior sucker; e, eyes; f, female gonopore; m, male gonopore; ps, posterior sucker

52°C (30 sec), 72°C (45 sec), and final extension at 72°C for 7 min. Conditions for 18S rDNA were identical, except annealing was at 55°C. All reactions were performed in 50 µl using Titanium Taq polymerase (ClonTech) according to the manufacturer's specifications, and supplemented with 25 mM $MgCl_2$. PCR products were excised from 1% agarose gels and prepared for sequencing using GeneClean (MP Biomedicals, LLC). Sequencing was conducted on both strands by GeneWiz, Inc. (South Plainfield, NJ).

Phylogeny

Sequence data of all *Myzobdella lugubris* samples were trimmed using the BioEdit programe v7.2.5 (Hall, 1999) and Chromas 2.4.3 (Technelysium), aligned in Clustal-W (Higgins *et al*. 1994; Larkin *et al*. 2007), MUSCLE (Edgar 2004) or Omega (EMBL-EBI), and overlapping sequence fragments were assembled into contigs. Maximum Parsimony (MP) heuristic searches used 100 replicates of random addition sequences and tree-bisection-reconnection (TBR) branch swapping. Bremer support and clade support using non-parametric bootstrapping with 100 replicates was determined with the Willi Henning Society edition of Tree analysis using New Technology (TNT; Goloboff *et al*. 2008). Bayesian Inference (BI) analysis was performed on the combined data set (morphology, 18S, CO1 as Nexus format) in MrBayes v. 3.2.1x64 (Ronquist and Huelsenbeck 2003; Ronquist *et al*. 2011). Data were partitioned for 12S and 18S rDNAs, and by codon position for CO1. ModelTest (Posada and Crandall 1998) via FindModel was used to determine the optimal model of evolution for each gene under the Akaike Information Criterion (AIC; Posada and Buckley 2004). The general time reversible (GTR) model with a gamma distributed rate parameter was used for CO1, 12S and 18S. Two analyses were simultaneously run with all parameter sets unlinked by partition for two million generations each, sampling every 100 generations, with a burn-in achieved by <50,000 generations. Setting the burn-in to 500,000 generations left a total of 1,012 trees sampled for assessment of posterior probabilities. Gaps were treated as missing data and default settings were used for all other parameters.

Results

Parasitic leeches, *Myzobdella lugubris* were collected from the mouth of Raccoon Creek (39°48′31″N — 75°22′53″W),

Gloucester County, NJ and Pier 38 (39°56′59.09″N — 75°8′18.86″W), Philadelphia, PA, from multiple channel catfish, *Ichtalurus punctatus* (Fig. 1). Leeches were found predominantly on the underside of the mouth and gill area.

Morphological data

Characters were adapted from Sawyer (1986), Siddall and Burreson (1995), Apakupakul *et al*. (1999), Williams and Burreson (2006), Utevsky and Trontelj (2004) and Borda and Siddall (2004a). The criteria for morphological characters and a matrix of 25 morphological characters are shown in Tables 2–3, respectively.

To corroborate species identity and to expand character details, here we document key morphological features of our collected specimens (deposited into the Academy of Natural Sciences, Philadelphia, PA). Body divided into the trachelosome and urosome, the former being about 1/3 the length of the latter (Fig. 2). Some midbody segments had 12 or 14 annuli. Specimen was 8.5–29.7 mm long, 1.8–5–3.2 mm wide, anterior sucker width 1.2–2.6 mm (Fig. 2A), posterior sucker width 1.8–3.1 mm (Fig. 2B). Dorsum pigmentation variably green or yellowish-brown, with brownish zig-zagged longitudinal stripes. Preserved leeches were greenish-brown and dirty white. The ventral color was mixed translucent creamy and greenish-brown. One pair of rather large, eyes amorphous ellipses clearly separated from each other on segment III. Proboscis connected with its base in segment IX (Fig. 3). Gonopores situated in furrow between annuli separated by two annuli, female and male pore in urosome area (male and female gonopores situated in furrow XI a1/a2 and XI/XII, respectively). Five pairs of testisac situated intersegmentally at XIV to XVIII. Atrial cornua and sperm duct together with S-shaped arch extended to the right and left lateral sides. Ovisacs in XII, large, connected with long and curled common oviduct. Oviduct is 1/3 the length of the ovisac, end curved and skein-shaped (Fig. 4).

Cocoon secretion

Swollen clitella of sexually mature specimens protruded ventrally (Fig. 5). Secreted cocoons were relatively small (~1 × 0.5 mm), hard-shelled, lemon-shaped with longitudinal striations, containing opercula at either end of the long axis (Fig. 6), and were cemented firmly to glass bowls. At 17°C, adult gravid specimens laid 32.67 ± 4.50 per individual with 88.1% fertilization and mean hatching time of 48 ± 7 days; at 22°C, 46 ± 28 cocoons were laid per individual, 70.27% fertilization and hatching time of 28 ± 5

Table II. Criteria for morphological characters

Character 1: pairs eyes: absent (0); one (1); two (2); three (3); four (4); five (5).
Character 2: testisacs: five pair (0); six pair (1); ten pair (2); clusters (3);
Character 3: presence of conducting (vector) tissue: absent or weakly developed (0); present (1).
Character 4: Feeding habit: Macrophagous (0); Haematophagous (1).
Character 5: Pulsatile vesicles per mid-body segment: absent (0); one pair (1); two pairs (2)
Character 6: deposition of cocoons: slipped off head (0); secreted ventrally (1).
Character 7: cocoons: cemented to a substrate (0); not cemented to a substrate (1); attached to ventral side of leech body (2).
Character 8: cocoons: without spongy covering (0); with spongy covering (1).
Character 9: eyespots: absent (0); one pair per annulus (1); at least two pairs per annulus (2).
Character 10, Testisac arrangement: Discretely arranged on vasa deferens (0); Grape-like cluster profusely arranged on vasa deferens (1).
Character 11: intestine: acaecate (0); caecate (1).
Character 12: body shape: vermiform (0); dorsoventrally flattened (1).
Character 13: |Body shape: flattened, distinctly divided into trachelosome and urosome (0); cylindrical, indistinctly divided into trachelosome and urosome (1)
Character 14: Ocelli on posterior sucker: absent (0); present (1)
Character 15: Accessory gland cells: absent (0); present (1).
Character 16: Annuli per somite: three (1); five (2); six (3); seven (4); 12 (5); 14 (6).
Character 17: Somatic sense organs: absent (0); papillae (1); tubercles (2)
Character 18: Lateral sinus: absent (0); present (1).
Character 19: cephalic eyespots: absent (0); dorsolateral (1); dorsal (2).
Character 20: Branchiae: absent (0); 31 pairs (1); 33 pairs (2).
Character 21: Segments with pulsatile vesicles: 10 (0); 11 (1).
Character 22: Mouthpore location in oral sucker: central (0); terminal (1).
Character 23: Caudal sucker attachment: subterminal (0); terminal (1).
Character 24: Crop cecae per segment: absent (0); one (1); two (2); three (3); four (4).
Character 25: Postcecae: absent (0); present (1)

Table III. A matrix of morphological data used in phylogenetic analyses of leeches

	123456789012345678901234 5
CCL (Racoon Creek)	10010000100010050020-0011
myz3 (Pier 38)	10010000100010050020-0011
Myzobdella lugubris Virginia,USA	10010000100010050020-0111
Myzobdella lugubris Hawaii, USA; freshwater, HI	10010000100010050020-0111
Myzobdella lugubris Virginia,USA; estuary,EST	10010000100010050020-0111
Myzobdella lugubris Virginia,USA; freshwater, FW	10010000100010050020-0111
Austrobdella californiana SG	00010000000000130000-0111
Branchellion lobata	00011000000000011010110011
Branchellion torpedinis	20011000100000011012210031
Calliobdella vivida	21111000100010160120110 31
Johanssonia arctica	01111000000010151100100 11
Oxytonostoma typica	01112000000010141100101 11
Piscicola geometra	21111000100011160120100 41
Piscicolaria reducta	10010000100010010120-0111
Erpobdella octoculata	430000002100-0020110-0010
Glossiphonia complanata	320001101011-0010120-0011
Haemopis marmorata	520000111000-0020110-0010
Hirudo medicinalis	520100111000-0020110-0010

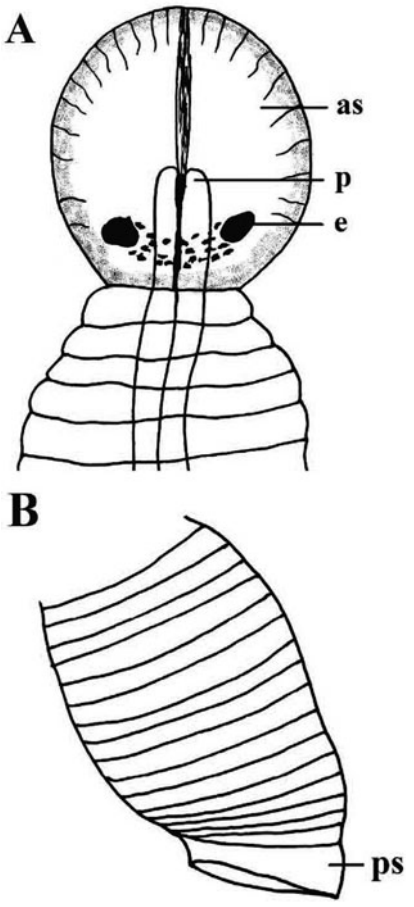

Fig. 3. View of anterior (A) and posterior (B) suckers of *Myzobdella lugubris*. as, anterior sucker; e, eyes; p, proboscis; ps, posterior sucker

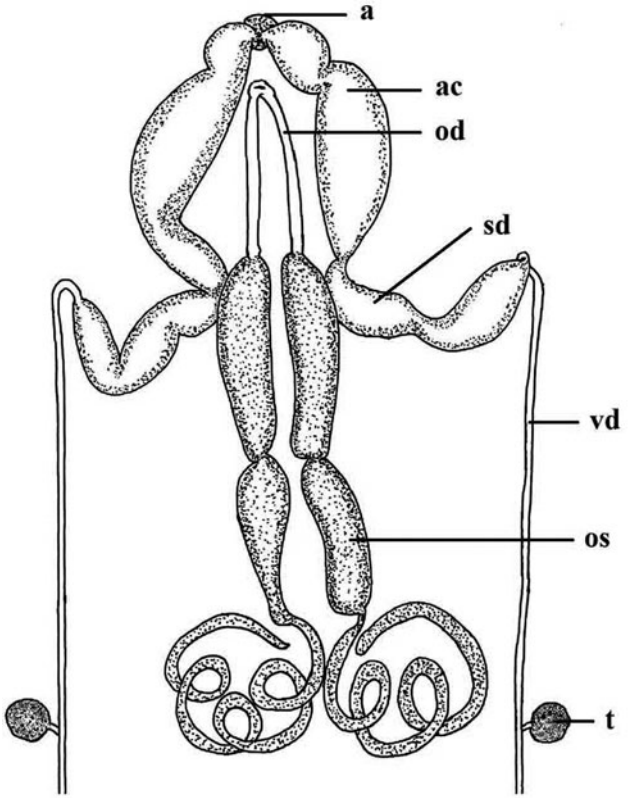

Fig. 4. Dorsal view of *Myzobdella lugubris* reproductive system. a, atrium; ac: atrial cornua; os: ovisac; od, oviduct; sd, sperm duct; t, testisac, vd. vas deferens

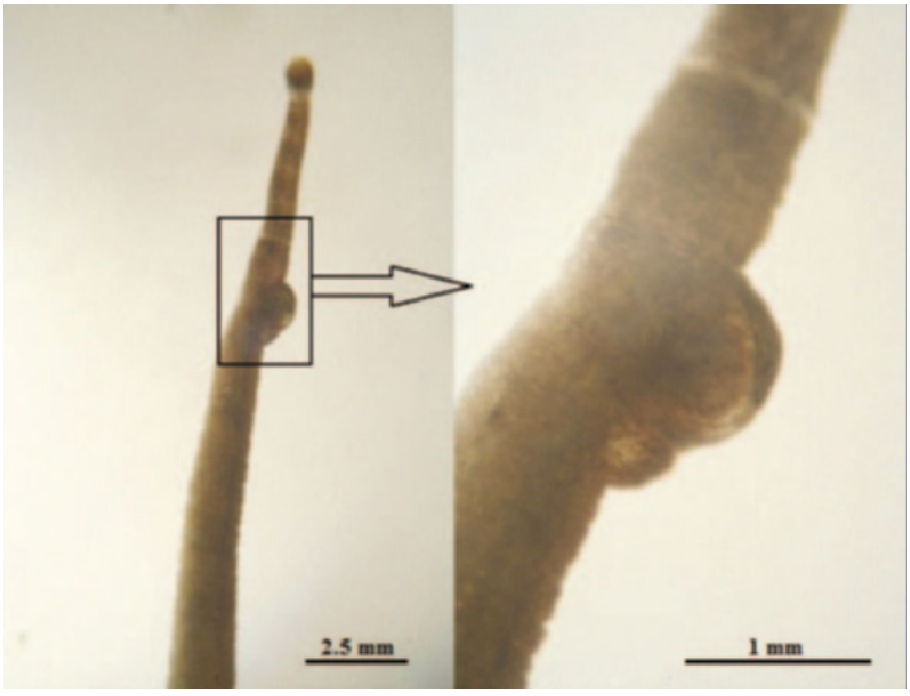

Fig. 5. Swollen clitellum protruded ventrally in a gravid *Myzobdella lugubris* specimen

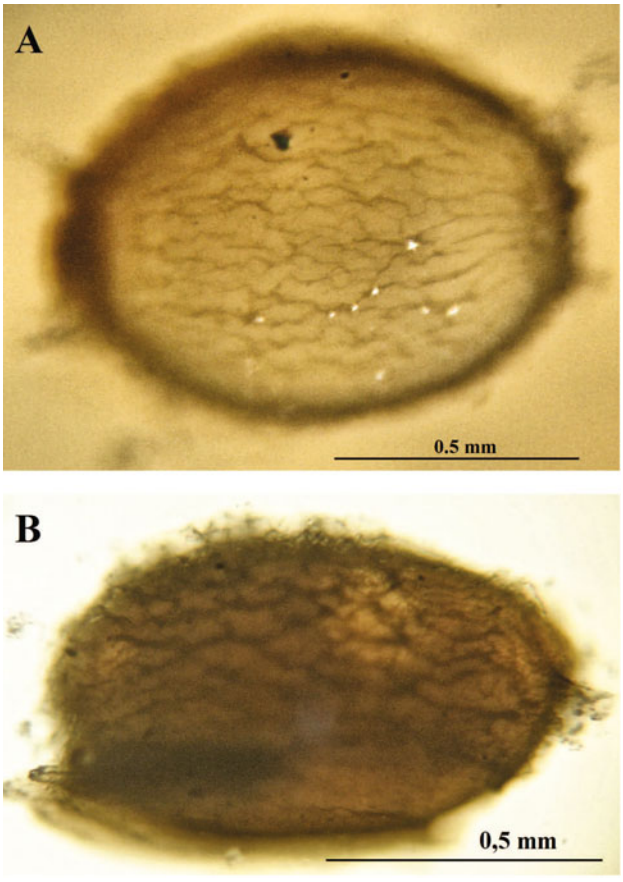

Fig. 6. Dorsal (A) and lateral (B) view of a *Myzobdella lugubris* cocoon

(Table 4). Only one juvenile (~2.5 mm) hatched from each cocoon at either temperature (Fig. 7A-D).

Phylogenetic analyses

Combined morphology (25 characters), CO1 (645 bp), and 18S (642 bp) analyses included 1,312 aligned characters with 33 informative positions. Maximum Parsimony (MP) of the combined data set yielded five equally parsimonious trees with 1,002 steps. Tree topologies revealed shallow divergences among regional *M. lugubris* specimens, and likely misclassification of *Piscicolaria reducta* (GenBank DQ414294) into the *Myzobdella* clade (Fig. 8). Concordant trees resulted from CO1 data alone (Fig. 9). GenBank accession numbers for all CO1, 12S and 18S sequences are listed in Table 5. Population structure was shallow among the collected specimens (<1.6% divergence at CO1).

Discussion

Relatively little scientific data has been documented on the parasitic leech, *Myzobdella lugubris*, since its description by Meyer (1940, 1946). Our results complete a morphological documentation with standard characters, and also identify features associated with reproduction and cocoon secretion (Figs. 2–7). Most notably, specimens in our study secreted an atypically high number of cocoons per individual (up to 67 at 22˚C; see Table 4), and each cocoon sup-

ported only one egg/embryo. This observation, also described in *Piscicola geometra* (Kutschera 2016), is unusual among Oligochaeta; specifically, embryo: cocoon ratios are typically high such that many embryos are deposited into relatively few cocoons. In the glossiphoniid leech, *Theromyzon tessulatum*, for example, several hundred eggs are deposited into 5–8 cocoons (Wilkialis and Davies 1980; Sayers *et al*., 2009; Mason *et al*. 2004) and Erpobdellids normally deposit 5–10 eggs per cocoon (Wrona *et al*. 1987; Kutschera and Wirtz 2001).

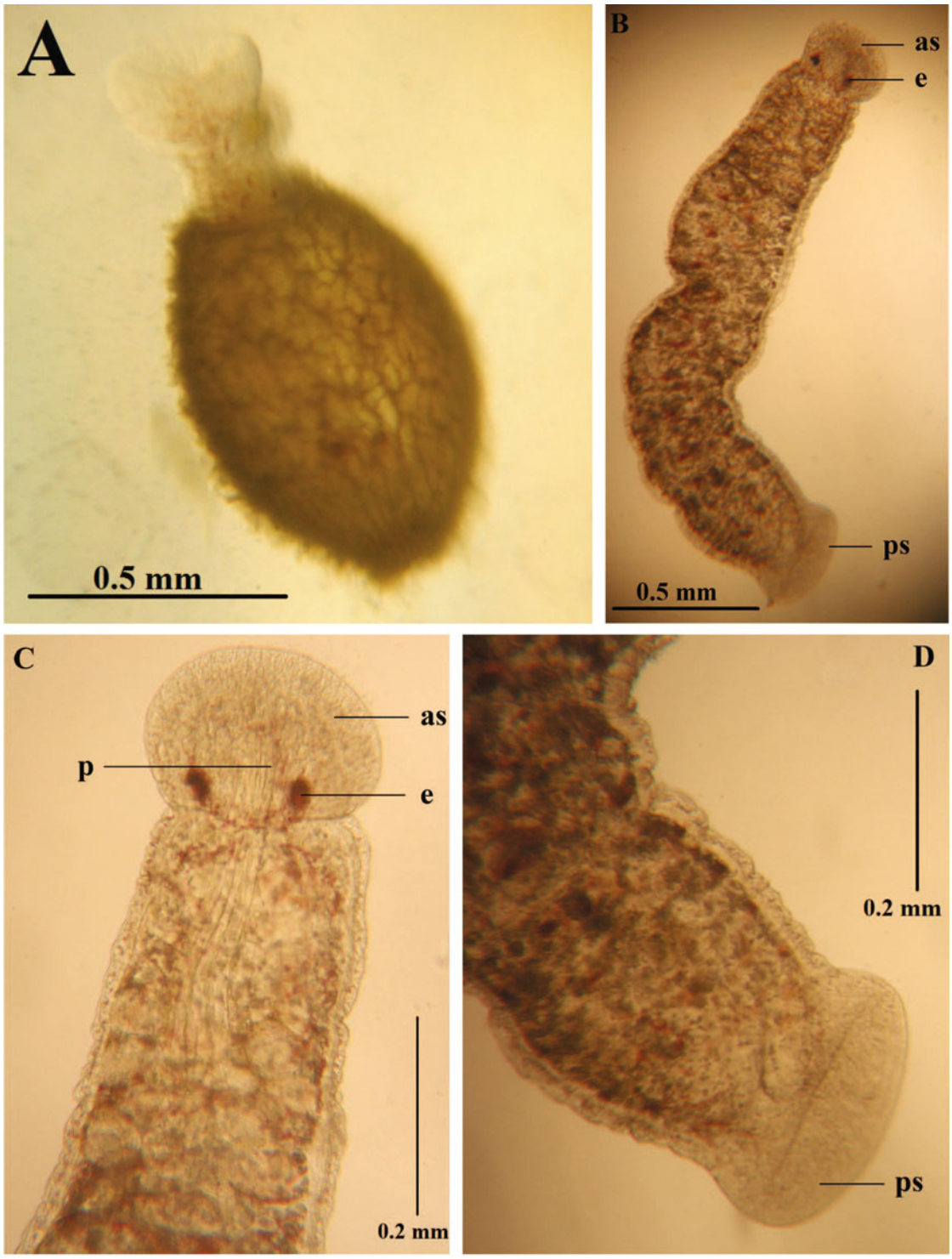

Fig. 7. View of Juvenile *Myzobdella lugubris* specimen. A) juvenile exiting cocoon. B) Juvenile. C) Anterior sucker of juvenile. D) Posterior sucker of juvenile. as, anterior sucker; e, eyespot; p, proboscis; ps, posterior sucker.

Table IV. Data of cocoon secretion for *Myzobdella lugubris* in the laboratory condition

Parameter	Condition 1	Condition 2
Hatching temperature	17°C	22°C
Size of cocoons	~1 × 0.5 mm	~1 × 0.5 mm
The mean number of cocoons per leech ± SE (range)	32.67 ± 4.50 (27–38)	46.00 ± 28.00 (18–67)
The mean hatching time ± SE (range) (Days)	48 ± 7 (41–55)	28 ± 5 (23–33)
Fertilization ratios of cocoons	88.10%	70.27%
Juvenile hatched from each cocoon	1	1
Length of hatched juvenile	~2.5 mm	~2.5 mm

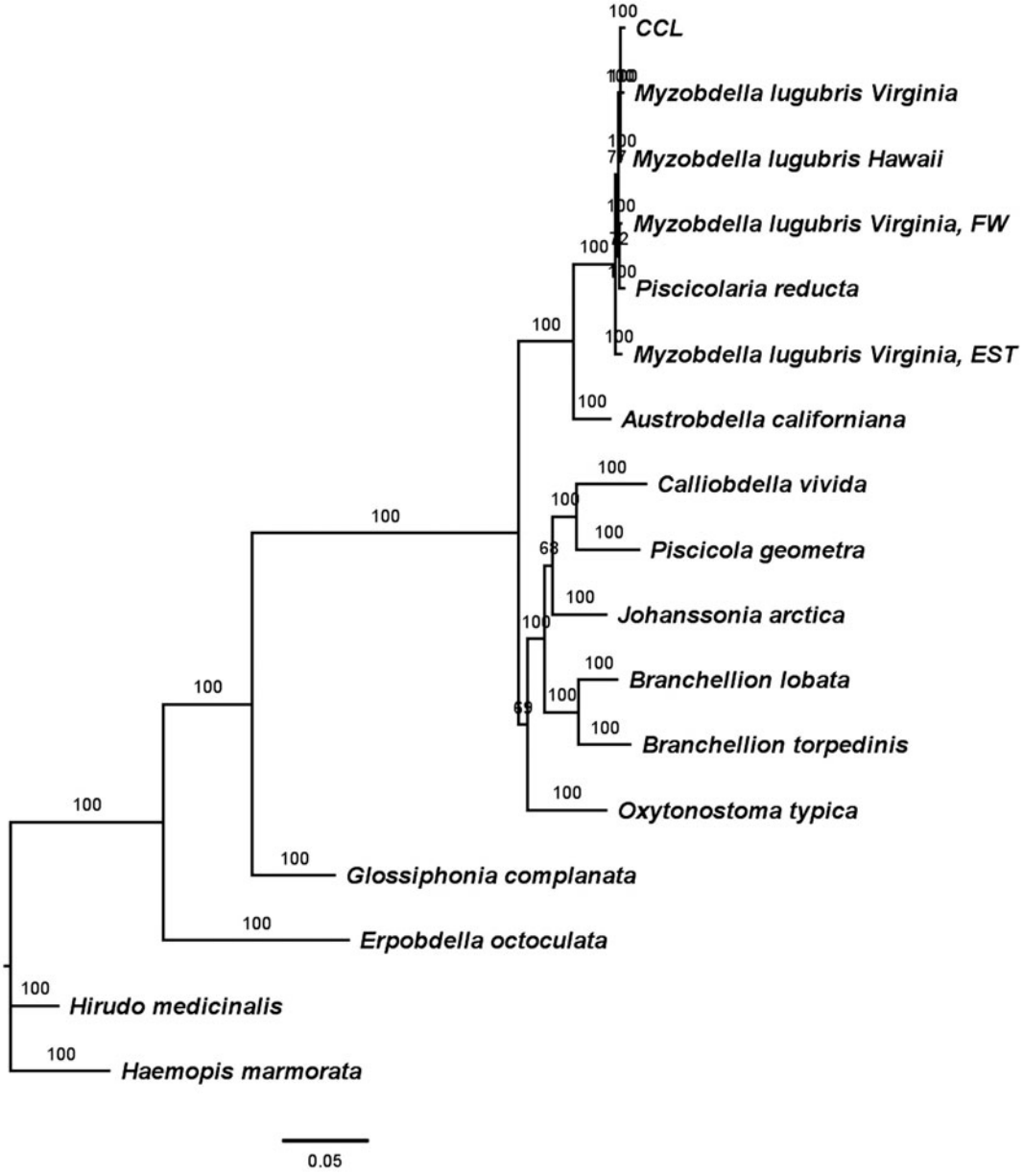

Fig. 8. Consensus tree from Maximum Parsimony and Bayesian Inference analyses resolving relationships within *Myzobdella* and related genera. Analyses performed on the combined data set (morphology, 18S rDNA and COI); branch support values indicated. See Table 5 for GenBank accession numbers

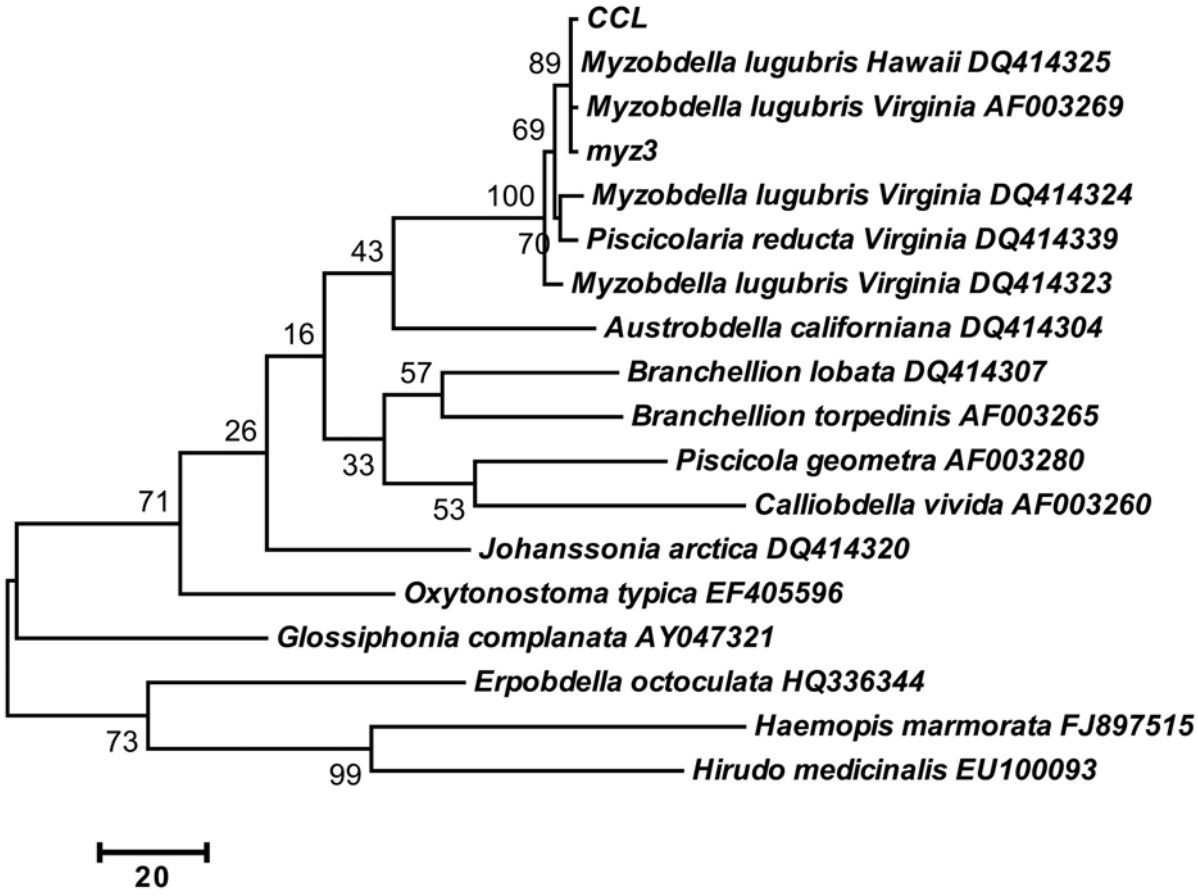

Fig. 9. Phylogenetic tree obtained from CO1 mtDNA resolving *Myzobdella* and related genera. CCL (Racoon Creek) and myz3 (Pier 38) specimens were collected in the current study. Branch support values are indicated.

The process of cocoon secretion is energetically expensive, requiring the coordinated synthesis of up to 10^7 proteinaceous micro-granules within the clitellum that collectively build the cocoon wall and opercula, which seal either end of the cocoon (Rossi *et al*. 2013, 2016). From a materials perspective, it is more efficient to house many embryos in a small number of cocoons; thus, the reproductive strategy of *M. lugubris*, which secretes unusually high numbers of cocoons each containing only a single egg, is perplexing. Most likely, the reproductive fecundity of the species hinges on the relatively low probability that a secreted cocoon remains adhered to its crustacean host, and that hatched juveniles transfer successfully to their alternate fish host. We note that although cocoons were tightly cemented to glass bowls in the laboratory, a crustacean exoskeleton is likely a better substrate for cocoon deposition compared with the mucous-covered surface of a fish. Further, surface area:volume ratios favor stronger adherence for a smaller cocoon, which may explain why only a single egg—which develops into a juvenile significantly larger that the cocoon's long axis prior to hatching—is deposited into each cocoon. Curiously, segments anterior to the clitellum in *M. lugubris* are elongated compared with other midbody segments (see Fig. 2), which appears morphologically well-suited for the construction of a relatively small cocoon that accommodates only one developing embryo.

Phylogenetic analyses using combined (i.e., CO1, 18S) or individual sequences yielded concordant tree topologies, with all *M. lugubris* haplotypes grouping into a shallow clade, distinct from other species considered (with the exception of *Piscicolaria reducta*, which was likely a misclassified *M. lugubris* specimen). Interestingly, *M. lugubris* specimens collected from distant geographic locations (e.g., New Jersey, Hawaii) were closely related (i.e., 0.2% at CO1), suggesting a robust dispersal mechanism, either natural (e.g., avian; Trauger and Bartonek 1977) or anthropogenic (e.g., shipment of crayfish or fish for commercial purposes). Possibly the passive dispersal of juveniles or adult leeches by either host species (i.e., crustacean, fish) results from the hosts' natural dispersal or the predaceous feeding of shore birds (Friend 1999).

In summary, the unusual reproductive strategy of *M. lugubris* (e.g., large number of cocoons containing only a single egg) appears to have co-evolved with its complex life cycle between two hosts (i.e., crustaceans, fishes), leading to the species' robust dispersal (natural and/or anthropogenic) and propagation throughout North America.

Table V. Accession numbers used in phylogenetic analyses

Taxon	GenBank Accession No.		
	CO1 mtDNA	12s rDNA	18s rDNA
CCL (Racoon Creek)	KY440058	KY440057	KY440056
myz3 (Pier 38)	KY440059	–	–
Myzobdella lugubris Virginia,USA	AF003269	–	AF115994
Myzobdella lugubris Hawaii, USA; freshwater, HI	DQ414325	–	DQ414279
Myzobdella lugubris Virginia,USA; estuary,EST	DQ414323	–	DQ414277
Myzobdella lugubris Virginia,USA; freshwater, FW	DQ414324	–	DQ414278
Austrobdella californiana SG	DQ414304	–	DQ414258
Branchellion lobata	DQ414307	–	DQ414261
Branchellion torpedinis	AF003265	AY425408	AF115993
Calliobdella vivida	AF003260	AY425409	AF115992
Johanssonia arctica	DQ414320	AY336036	DQ414274
Oxytonostoma typica	EF405596	EF405565	DQ414288
Piscicola geometra	AF003280	AF099959	AF115995
Piscicolaria reducta	DQ414339	–	DQ414294
Erpobdella octoculata	HQ336344	AF099954	AF099949
Glossiphonia complanata	AY047321	AY425414	JQ821519
Haemopis marmorata	FJ897515	FJ897509	AF116008
Hirudo medicinalis	EU100093	JN118993	AY786464

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References

Amin O.M. 1981. Leeches (Hirudinea) from Wisconsin, and the description of the spermatophore of *Placobdella ornate*. *Transactions of the American Microscopical Society*, *100*, 42–51. DOI; 10.2307/3225782

Anisimova M., Gascuel O. 2006. Approximate likelihood ratio test for branchs: A fast, accurate and powerful alternative. *Systematic Biology*, 55, 539–52. DOI: 10.1080/10635150600755453

Apakupakul, K., Siddall, M.E., Burreson, E.M. 1999. Higher level relationships of leeches (Annelida : Clitellata : Euhirudinea) based on morphology and gene sequences. *Molecular Phylogenetics and Evolution*, 12**,** 350–359. DOI: 10.1006/mpev.1999.0639

Appy R.G., Cone D.K. 1982. Attachment of *Myzobdella lugubris* (Hirudinea: Piscicolidae) to Logperch, *Percina caprodes*, and Brown Bullhead, *Ichtalurus nebulosus*. *Transactions of the American Microscopical Society*, 101, 135–141. DOI: 10.2307/3225766

Becker D.A., Heard R.G., Holmes P.D. 1966. A preimpoundment survey of the helminth and copepod parasites of Micropterus spp. of Beaver Reservoir in northwest Arkansas. *Transactions of the American Fisheries Society*, 95, 23–34. DOI: 10.1577/1548-8659(1966)95[23:APSOTH]2.0.CO;2

Becker C.D., Dauble D.D. 1979. Records of Piscivorus Leeches (Hirudinea) from The Central Columbia River, Washington State. *Fishery Bulletin*, 76, 926–931

Borda E., Siddall M.E. 2004a. Arhynchobdellida (Annelida: Oligochaeta: Hirudinida) phylogenetic relationships and evolution. *Molecular Phylogenetics and Evolution*, *30*, 213–225. DOI 10.1016/j.ympev.2003.09.002

Borda E., Siddall M.E. 2004b. Review of the evolution of life history strategies and phylogeny of the Hirudinida (Annelida: Oligochaeta). *Lauterbornia*, 52, 5–25

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Phylogenetics and Evolution*, 17, 540–552. DOI: 10.1093/oxfordjournals.molbev.a026334

Chevenet F., Brun C., BanulsAL., Jacq, B., Chisten R. 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics*, 7, 439. DOI: 10.1186/1471-2105-7-439

Daniels B.A., Sawyer R.T. 1975. The biology of the leech *Myzobdella lugubris* infesting blue crabs and catfish. *Biological Bulletin*, 148, 193–198. DOI: 10.2307/1540542

Edgar R.C. 2004. *MUSCLE:* multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, 19, 1792–1797

Font W.F. 2003.The Global Spread of Parasites: What Do Hawaiian Streams Tell Us? *BioScience*, 53, 1061–1067. DOI: 10.1641/0006-3568(2003)053[1061:TGSOPW]2.0.CO;2

Friend M. 1999. Nasal Leeches. Chapter 34. In: Friend, M. & Franson, J.C. (Eds). Field manual of wildlife diseases: general field procedures and diseases of birds. Madison, WI: US Geol. Survey, National Wildlife Health Center. pp. 245–92

Goloboff P.A., Farris J.S., Nixon K.C. 2008. TNT, a free program for phylogenetic analysis. *Cladistics*, 24, 774–786. DOI: 10.1111/j.1096-0031.2008.00217.x

Guindon S., Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, 52, 696–704. DOI: 10.1080/10635150390235520

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98

Higgins D., Thompson J., Gibson T., ThompsonJ.D., Higgins, D.G., Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680. DOI: 10.1093/nar/22.22.4673

Klemm D. J. 1972. Biota of Freshwater Ecosystems Identification Manual No 8. The leeches (Annelida: Hirudinea) of North America. Environmental Protection Agency Project No 18050ELD. The University of Michigan, Ann Arbor, Michigan. pp. 53

Klemm D.J. 1982. The leeches (Annelida: Hirudinea) of North America. Cincinnati, Aquatic Biology Section, Environmental Monitoring and Support Laboratory, United States Environmental Protection Agency

Klemm D.J. 1985. Freshwater leeches (Annelida: Hirudinea). In Klemm DJ, editor. A guide to freshwater Annelida (Polychaeta, naidid and tubificid Oligochaeta, and Hirudinea) of North America. Dubuque: Kendall Hunt Publishing Co. pp. 198

Klemm D.J. 1995. Identification guide to the freshwater leeches (Annelida: Hirudinea) of Florida and other southern states. Tallahassee: Florida Department of Environmental Protection. pp. 82

Kutschera U., Wirtz P. 2001. The evolution of parental care in freshwater leeches. *Theory in Biosciences*, *120*, 115–137. DOI: 10.1007/s12064-001-0012-1

Kutschera U. 2016. Sex versus gender in sea urchins and leeches two centuries after Lamarck 1816. *Journal of Marine Science: Research and Development*, 6/5:1–3

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., Higgins D.G. 2007. ClustalW and ClustalX, version 2. *Bioinformatics*, *23* 2947–2948. DOI: 10.1093/bioinformatics/btm404

Mason T.A., McIlroy P.J., Shain D.H. 2004. A cysteine-rich protein in the *Theromyzon* (Annelida: Hirudinea) cocoon membrane. *Febs Letters*, 561, 167–172. DOI: 10.1016/S0014-5793(04)00167-X

Mason T.A., Sayers C.W., Paulson T.L., Coleman J., Shain D.H. 2005. Cocoon deposition and hatching in the aquatic leech, *Theromyzon tessulatum* (Annelida, Hirudinea, Glossiphoniidae). *American Midland Naturalist*, 154, 78–87. DOI: 10.1674/0003-0031(2005)154[0078:Cdahit]2.0.Co;2

Meyer M.C. 1940. A revision of the leeches (Piscicolidae) living on fresh-water fishes in North America. *Transactions of the American Microscopical Society* 59, 354–376. DOI: 10.2307/3222552

Meyer M.C. 1946. Further notes on the leeches (Piscicolidae) living on fresh-water fishes of North America. *Transactions of the American Microscopical Society* 65, 237–249. DOI: 10.2307/3223419

Moore J.P. 1946. The anatomy and systematic position of *Myzobdella lugubris* Leidy (Hirudinea). *Notulae naturae of the Academy of Natural Sciences of Philadelphia*, 184, 1–12

Moser W.E., Klemm D.J., Richardson D.J., Wheeler B.A., Trauth S.E., Daniels B.A. 2006. Leeches (Annelida: Hirudinida) of Northern Arkansas. *Journal of the Arkansas Academy of Science*, 60, 84–95

Noga E.J., Bullis R.A., Miller G.C. 1990. Epidemic oral ulceration in largemouth bass (*Micropterus salmoides*) associated with the leech *Myzobdella lugubris*. *Journal Wildlife Diseases*, 26, 132–134. DOI: 10.7589/0090-3558-26.1.132

Posada D., Buckley T.R. 2004. Model selection and model averaging in phylogenetics: advantages of Akaike Information Criterion and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, 53, 793–808. DOI: 10.1080/10635150490522304

Posada D., Crandall K.A. 1998. Modeltest: testing the modelof DNA substitution. *Bioinformatics*, 14, 817–818. DOI: 10.1093/bioinformatics/14.9.817

Rambaut A. 2016. FigTree v1.4.2; Molecular evolution, phylogenetics and epidemiology. http://tree.bio.ed.ac.uk/software/figtree/

Ronquist F., Huelsenbeck J. and Teslenko M. 2011. Draft MrBayes version 3.2 Manual: Tutorials and Model Summaries. pp. 172

Ronquist F., Huelsenbeck J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572–1574. DOI: 10.1093/bioinformatics/btg180

Rossi A.M., Saidel W.M., Marotta R., Saglam N., Shain D.H. 2013. Operculum ultrastructure in leech cocoons. *Journal Morphology*, 274, 940–946. DOI: 10.1002/jmor.20150

Rossi A.M, Saidel W.M., Gravante C., Sayers C.W., Shain D.H. 2016. Mechanics of cocoon secretion in a segmented worm (Annelida: Hirudinidae). *Micron*, 86, 30–35. DOI: 10.1016/j.micron.2016.04.004

Sambrook J., Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sawyer R.T. 1972. North American freshwater leeches, exclusive of the Piscicolidae, with a key to all Species. University of Illinois Press, Urbana, Chicago and New York

Sawyer R.T. 1986. Leech Biology and Behavior. Vol. 1–3. Oxford University Press, New York

Sawyer R.T., Lawler A.R., Overstreet R. M. 1975. Marine leeches of the eastern United States and the Gulf of Mexico with a key to the species. *Journal of Natural History*, 9, 633–667. DOI: 10.1080/00222937500770531

Sawyer R.T., Shelley R.M. 1976. New records and species of leeches (Annelida: Hirudinea) from North and South Carolina. *Journal of Natural History*, 10, 65–97. DOI: 10.1080/00222937600770061

Sayers C.W., Coleman J., Shain D.H. 2009. Cell dynamics during cocoon secretion in the aquatic leech, *Theromyzon tessulatum* (Annelida: Clitellata: Glossiphoniidae). *Tissue Cell*, 41, 35–42. DOI: 10.1016/j.tice.2008.05.003

Schramm J.C., Hardman C.H., Tarter D.C. 1981. The occurrence of *Myzobdella lugubris* and *Piscicolaria reducta* (Hirudinea: Piscicolidae) on fishes from West Virginia. *Transactions of the American Microscopical Society*, 100, 427–428. DOI: 10.2307/3226158

Siddall M. E., Burreson E.M. 1995. Phylogeny of the Euhirudinea: Independent evolution of blood feeding by leeches? *Canadian Journal of Zoology*, 73, 1048–1064. DOI: 10.1139/z95-125

Trauger D.L., Bartonek J.C. 1977. Leech parasitism of waterfowl in North America: *Wildfowl*, 28, 142–152

Troxel, D.J., 2010. Parasıtes of Largemouth Bass (*Mıcropterus salmoıdes*) in Northern California. A Thesis Presented to The Faculty of Humboldt State University In Partial Fulfillment of The Requirements for the Degree Masters of Science Natural Resources, Fisheries, pp.59

Utevsky S., Trontelj P. 2004. Phylogenetic relationships offish leeches (Hirudinea, Piscicolidae) based on mitochondrial DNA sequences and morphological data. *Zoologica Scripta* 33, 375–385. DOI: 10.1111/j.0300-3256.2004.00156.x

Wilkialis J., Davies R.W. (1980) The Reproductive-Biology of *Theromyzon tessulatum* (Glossiphoniidae-Hirudinoidea), with Comments on *Theromyzon rude*. *Journal Zoology*, 192, 421–429. DOI: 10.1111/j.1469-7998.1980.tb04241.x

Williams J. I., Burreson E.M. (2006). Phylogeny of the fish leeches (Oligochaeta, Hirudinida, Piscicolidae) based on nuclear and mitochondrial genes and morphology. *Zoologica Scripta*, 35, 627–639. DOI: 10.1111/j.1463-6409.2006.00246.x

Williams Jr., E.H., Bunkley-Williams L., Burreson E.M. (1994) Some New Records of Marine and Freshwater Leeches from

Caribbean, Southeastern U.S.A., Eastern Pacific, and Okinawan Animals. *Journal of The Helminthological Society of Washington*, 61, 133–138

Wirchansky B.A., Shain D.H. 2010. A new species of *Haemopis* (Annelida: Hirudinea): Evolution of North American terrestrial leeches. *Molecular Phylogenetics and Evolution*, 54, 226–234. DOI: 10.1016/j.ympev.2009.07.039

Wrona F.J., Linton L.R., Davies R.W. 1987 Reproductive success and growth of two species of Erpobdellidae: the effect of water temperature. *Canadian Journal of Zoology*, 65, 1253–1256. DOI: 10.1139/z87-195

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