

A NEW SPECIES OF *HAEMOPIS* (ANNELIDA: HIRUDINEA):  
EVOLUTION OF NORTH AMERICAN TERRESTRIAL LEECHES

by

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## ABSTRACT OF THE THESIS

A New Species of *Haemopis* (Annelida: Hirudinea): Evolution of North American  
Terrestrial Leeches

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Among the relatively few terrestrial leeches known worldwide, only two (*Haemopis terrestris*, *Haemopis septagon*) are described from North America. Here we report a third terrestrial leech collected from the southern part of New Jersey, USA. Tissue samples were obtained from 14 individuals representing three populations, and morphological characters were scored after dorsal and ventral dissections. Maximum Parsimony and Bayesian Inference analyses resolved phylogenetic relationships within the genus *Haemopis* using cytochrome *c* oxidase subunit 1 (CO1), 12S ribosomal RNA (rRNA), and 28S rRNA gene fragments, establishing the monophyly of North American haemopids and terrestriality as a synapomorphy for some members of the group. Geographic isolation, morphological distinctions and combined phylogenetic analyses support the designation of a new species of terrestrial leech, *Haemopis ottae* n. sp. Phylogeographic interpretations of the haemopid clade suggest that terrestriality was derived from a northern, aquatic ancestor whose descendants were initially confined to Midwestern States and central Canada by the Appalachian Range.

More recently, the terrestrial lineage diverged near the southern extent of its range and began a northeasterly migration along coastal states giving rise to *H. septagon* and *H. ottae* n. sp., the latter of which appears to define the leading edge of a northward expansion.

## **DEDICATION**

I dedicate this thesis to my father, Dimitri Wirchansky. Although he isn't here to witness my success, I know that he would see it as another inevitable step in the journey to find my place in this world. His impact on me is immeasurable and I'm certain I wouldn't have had the fortitude to complete this task without 25 years of his insight and counsel.

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## Introduction

Leeches are a diverse order of Oligocheata comprising ~650 known species (Siddall et al., 2006); collectively, they display remarkable biodiversity thriving in every continent except Antarctica. In contrast to the common perception of blood-feeding behavior (i.e., sanguivory), many leeches have adopted a predaceous feeding method, preying on soft-bodied animals such as earthworms and snails. The majority of leeches occur in freshwater habitats—indicative of their general susceptibility to desiccation—but a small number of terrestrial leeches are known worldwide, mostly from tropical or sub-tropical rainforests. These include members of the families Haemopidae, Cylicobdellidae and Americobdellidae as well as the African genus *Semiscoloides* (Borda et al., 2008). Only two North American terrestrial leeches, *Haemopsis terrestris* (Forbes, 1890) and *Haemopsis septagon* (Sawyer and Shelley, 1976; Shelley et al., 1979), are described, occurring in drier, temperate climates compared with other terrestrial species.

Haemopids are among the largest leeches in the world (>30 cm for some species), and are grouped in the same suborder (Arhynchobdellida) as *Hirudo medicinalis* and *Hirudo verbana*, which have numerous research and medical applications (e.g., source of anti-thrombin blood thinners, oxygenating peripheral tissue in reconstructive surgeries; Markwardt, 2002; Knobloch et al., 2007). The genus *Haemopsis* spans two continents (i.e., Europe, North America) but the

majority of described species reside in North America, particularly in northern latitudes (Klemm, 1982). With the exception of *H. terrestris* and *H. septagon*, other species in the genus, namely *Haemopsis grandis* Verrill 1874, *Haemopsis marmorata* Moore 1912, *Haemopsis kingi* Mathers 1952 and *Haemopsis lateromaculata* Mathers 1963, are aquatic freshwater leeches most prevalent in Canada and the northern half of the continental United States. Maloney and Chandler (1976) explain this apparent geographical restriction by correlating higher levels of dissolved oxygen with cold water; note that species of *Haemopsis* are relatively large thus decreasing their surface area/volume ratio and increasing oxygen demand. In contrast, the two described North American terrestrial leeches occur in southern and midwestern (*H. terrestris*) or southeastern (*H. septagon*) states.

Several years ago, we received a specimen of *Haemopsis* collected near a local resident's garden in southern New Jersey that did not match the characters of other *Haemopsis* species. Subsequent fieldwork identified two additional populations in NJ. Our collective morphological and phylogenetic analyses suggest that these populations represent a third species of terrestrial leech in North America, and that terrestriality occurred only once from a northern, aquatic ancestor from which the three terrestrial *Haemopsis* lineages were derived.

## Materials and methods

### *Specimen collection and maintenance*

*Haemopsis* specimens collected throughout New Jersey were transported to Rutgers University (Camden, NJ) and maintained in separate aquaria based on collection location. Aquaria contained 1-2 cm 0.3% Instant Ocean (Aquarium Systems) and were elevated ~2 cm at one end to create a terrestrial to aquatic continuum. Leeches were fed one adult earthworm (e.g., *Eisenia fetida*) per week (found in the field or purchased from local pet stores), and typically survived 2+ years in the laboratory. In addition, 16 live specimens identified as *Haemopsis terrestris* were mailed to Rutgers University after being collected in Belton, Missouri.

### *Dissections*

Specimens were fixed in 70% ethanol. External traits of live specimens were observed under a stereomicroscope (Miji EMZ-TR, Meiji Techno Co. Ltd.). Preserved specimens were dissected dorsally and ventrally, with representative sketches of internal morphology derived directly from type specimens. The type specimen is deposited in The Smithsonian Institution collection (Washington, D.C.) and the American Museum of Natural History (New York, NY) received a paratype.

Dissections were performed primarily at Rutgers University, but several representative specimens of *Haemopsis terrestris* from MO, *Haemopsis marmorata*-like specimens collected in Camden, and the putative new species from NJ were dissected at the Smithsonian Institution with William Moser. Photographs of dissected *Haemopsis terrestris* (Fig. 1) and the new species from NJ (Fig. 2) were taken for comparison.

#### *DNA extraction*

Tissue samples from live specimens were obtained by placing the leech in a 10% ethanol sedating solution until it was unresponsive to touch. 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. Leeches were maintained in 2% streptomycin for ~ 72 h before returning to aquaria. Whenever possible, tissue from postmortem specimens was taken from the caudal sucker, in order to avoid contamination from gut contents. Genomic DNA was extracted by solubilizing tissue with Proteinase K, as described (Sambrook and Russell, 2001). To remove residual pigment (which blocked downstream applications), DNA was cleaned with the PowerClean™ DNA Clean-Up kit (MO BIO Laboratories, Inc.) according to the manufacturer's instructions.

### *Amplification of target genes*

Nuclear 12S and 28S ribosomal RNA (rRNA) and mitochondrial cytochrome c oxidase subunit 1 (COI) gene fragments were amplified from genomic DNA using the polymerase chain reaction (PCR). All 12S sequences were obtained at the American Museum of Natural History laboratory under the conditions described by Borda and Siddall (2004). For 28S rRNA, universal primers LROR (ACCCGCTGAACTTAAGC; Bunyard et. al. 1994) and LR5 (ATCCTGAGGGAACTTC; Vilgalys and Hester 1990) were incorporated into PCR reactions generating a ~1,060 bp fragment. PCR conditions were 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min, for 30 cycles with a final extension at 72°C for 10 min. For COI, universal primers LCO (GGTCAACAAATCATAAAGATATTGG) and HCO (Folmer et al., 1994) often failed with this *Haemopsis* species; consequently the leech-specific primer COI.4 (TCCTA(TC)AGGATCAAAAAAAGTAG) proximal to the HCO primer region was designed, and a ~600 bp COI fragment was successfully amplified from all individuals using an LCO/COI.4 primer set. COI PCR conditions were 94°C for 30 sec, 52°C for 1 min, and 72°C for 1 min, for 30 cycles, with final extension at 72°C for 10 min. Reactions were performed under standard conditions using Titanium Taq DNA polymerase (ClonTech), supplemented with 1.5 mM MgCl<sub>2</sub>, in a Techne TC-312 Thermal Cycler.

### *Primer design*

Multiple attempts to amplify the COI gene using the universal primers were made. Despite altering primer, template and MgCl<sub>2</sub> concentrations and annealing temperatures successful amplification using *Haemopsis* DNA was not achieved. Leech specific COI primers were designed by creating a global alignment that included all *Haemopsis* COI sequences deposited in GenBank and the COI sequence for *Hirudo medicinalis* (Fig.3). Two degenerate primers at the 5' end and two degenerate primers at the 3' end of the sequence were designed using regions of high sequence identity.

An experiment was performed using the four newly designed primers and two universal primers in order to choose the optimal primer set for this genus. Although all primer combinations (except the universal primers) were successful in amplifying the target gene, the LCO/COI.4 set had the best result. The sequences of all primers used in these experiments are listed in Table 1.

### *DNA sequencing and editing*

PCR products were excised from 1% agarose gels and prepared for sequencing using GeneClean (MP Biomedicals, LLC). DNA sequencing was conducted with forward and reverse primers by GeneWhiz Inc. (South Plainfield, NJ), and at the American Museum of Natural History (New York, NY) as described in Borda and Siddall (2004). Sequences were manually adjusted in ChromasPro (Technelysium, Queensland, Australia) or BioEdit (Hall, 1999) and aligned with MUSCLE (Edgar, 2004) or CLUSTALW (Higgins et al., 1994).

Accession numbers for COI, 12S and 28S sequences obtained from NCBI GenBank are listed in Table 2.

### *Phylogeny*

Maximum parsimony analyses (MP) of combined COI, 12S, and 28S data, in addition to each individual gene, were performed in PAUP 4.06b10 (Swofford, 2000). 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). PAUP was used to determine retention and consistency indices (RI, CI respectively).

Bayesian Inference (BI) analysis was performed on the combined data set in MrBayes v. 3.1 (Ronquist and Huelsenbeck, 2003). Data were partitioned for 12S and 28S, and by codon position for COI. 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 both 12S and the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> codon positions of COI, and the HKY85 model with a gamma distributed rate parameter for the 28S partition, yielding a total of five model-independent partitions. Two analyses were simultaneously run, with all parameter sets unlinked by partition for two million generations each, sampling every 200 generations, with a burn-in

achieved by <50,000 generations. Setting the burn-in to 500,000 generations left a total of 15,002 trees sampled for assessment of posterior probabilities. Gaps were treated as missing data, and default settings were used for all other parameters.

## Results

### *Habitat*

The species of *Haemopsis* comprising the focus of this study was verified in three geographically distinct locations in New Jersey (Fig. 4). Winslow Township (39°37'26.98"N, 74°53'44.29"W) was the northern-most location, Alloway (39°33'58.23"N, 75°20'6.82"W) the western-most, and Pomona (39°28'55.63"N, 74°31'58.80"W) the most southeastern. All populations were separated by at least 34 km. Ten specimens were collected in Alloway, three in Winslow, and one in Pomona. COI sequence comparisons revealed three haplotypes in total, one for each population (i.e., individuals within each population were genetically identical within the ~600 bp CO1 fragment); 12S and 28S sequences showed no sequence divergence between or within populations. Leeches were typically found in moist terrestrial environments, with cedar bogs proving the most prevalent habitat (Fig. 5), though some specimens were found several hundred meters from a water source. The most common micro-habitats were aquatic-terrestrial transition zones (e.g., under leaf litter at the edge of streams, inside partially submerged logs)(Fig. 6). Water in these areas ranged from pH 4-5.

Additionally, *Haemopsis terrestris* specimens sent from Missouri were reportedly found underneath a wood pile in a fully terrestrial environment. Specimens collected from Camden, which were morphologically similar to *Haemopsis marmorata*, were found in a swampy transition zone at the edge of the Cooper River.

### *Phylogenetic analyses*

The combined COI, 12S, and 28S analysis contained 27 terminals with 2,921 aligned characters. Maximum parsimony of the combined data set (Fig. 7) yielded five equally parsimonious trees with 1,150 steps (CI = 0.753, RI = 0.776), which differed only in the relative arrangement of individual isolates in *Haemopsis ottae* n. sp. Bremer support for the node designating *Hirudo medicinalis* sister to the *Haemopsis* genus was 20, and that separating North American aquatic leeches from terrestrial leeches (*Haemopsis terrestris* and *Haemopsis ottae* n. sp.) was seven. The node supporting the *Haemopsis ottae* n. sp. as a distinct species had a Bremer support value of 15.

Analyses of the combined data set for MP and BI generated trees bearing identical topologies with all major clades having strong support, with one exception: the most parsimonious tree had *Haemopsis lateromaculata* and *Haemopsis grandis* as a sister clade to *Haemopsis marmorata*, whereas the BI analysis put *H. marmorata* and *H. lateromaculata* as a sister clade to *H. grandis* (Fig. 7). Most parsimonious trees for COI (Fig. 8), 12S (Fig. 9), and the combined analyses (MP and BI) contained all *Haemopsis ottae* n. sp. specimens in a clade of their own, with the combined analysis tree having a bootstrap support of 100 and posterior probability of 1.00. Only the most parsimonious for 28S (Fig. 10) resulted in a polytomy with *Haemopsis terrestris*.

With the exception of the above noted topology discrepancy, all other nodes had bootstrap values  $\geq 89$  and posterior probability  $\geq 0.95$ . The genus

*Haemopsis* was clearly resolved as a monophyletic lineage, and European leeches (*Haemopsis sanguisuga* and *Haemopsis caeca*) were resolved from North American *Haemopsis* sp. *Haemopsis terrestris* was sister to *Haemopsis ottae* n. sp. with strong branch support (bootstrap value = 95, posterior probability = 1.00). Both analyses strongly supported the monophyly of *Haemopsis ottae* n. sp. as a distinct species, but its observed terrestriality as a synapomorphy with *H. terrestris*. Putative *H. marmorata* specimens collected in NJ were sister to *H. marmorata* (GenBank; AY425380, AY425423, AF003270) supported by bootstrap values of 100 and posterior probability of 1.00, suggesting a new cryptic species (Bely and Weisblat, 2006; Gustafsson et al., 2008).

*Haemopsis ottae* n. sp.

Description: Adults up to ~30 cm long and ~3 cm wide. Dorsum pigmentation medium to dark brown with variable mid-dorsal stripe, moderate to extensive black mottling (Fig. 11). With classic Hirudinid arc eyespot pattern (Sawyer, 1986), containing five pairs bilateral eyespots. White-tipped papillae distributed bilaterally, wrapping around the periphery of dorsum and ventrum, absent in medial regions. First three sets of papillae on every third annulus, beginning at annulus with the posterior-most eyespots, every fifth annulus thereafter in mid-body segments (~16 papillae were present per annulus), every other annulus in seven posterior-most annuli. Gonopores situated in furrow between annuli, separated by 7-7 1/2 annuli, female pore having a pronounced nipple-like appearance. Typical *Haemopsis* body type evident (Fig. 12): wider

posterior, firm muscular body, small caudal sucker characteristic of other terrestrial leeches (Sawyer and Shelly, 1976). Seventeen pairs of nephridiopores present in standard positions for Hirudinea (Fig. 11). Pharynx with 15 folds merging into three jaws, with ~10 distichodont teeth per jaw. Teeth color variable from translucent white to pale yellow. Male reproductive apparatus notably large and long, with thick muscular penis sheath terminating in a bulbous prostate (Fig. 13). Epididymis relatively large, more than twice size of pearlescent-sheened sperm sac (Figs. 2 & 13a). Female reproductive system relatively uncoiled tubing, lacking a distinct vagina. Oviducts slightly coiled, terminating in oblong, bi-lobed ovaries.

#### *Remarks*

In addition to its apparent geographic isolation, *H. ottae* n. sp. has several morphological traits separating it from other North American terrestrial leeches. For example, *H. ottae* n. sp. contains eight bilateral pairs of circumferentially positioned, white-tipped papillae on every fifth annulus; similar structures are reported on *H. marmorata* but not *H. terrestris* or *H. septagon*. Also, *H. ottae* n. sp. lacks the lateral stripes present on both *H. septagon* and *H. terrestris* (Sawyer and Shelley, 1976; Shelley et al., 1979).

Internal reproductive structures among the three *Haemopsis* terrestrial leeches are variable as well (Fig. 13). Specifically *H. ottae* n. sp. is characterized by a large epididymis more than twice the size of the sperm sac (Figs. 2 & 13a), while the same structure in *H. terrestris* (Figs 1 & 13c) and *H. septagon* is

relatively small and does not extend much past the sperm sac. The vagina in *H. ottae* n. sp. is significantly less pronounced and the vaginal duct is relatively straight when compared to *H. terrestris* or *H. septagon*. The number of teeth also varies, with *H. ottae* n. sp. having ~10 distichodont teeth, and *H. terrestris* and *H. septagon* having ~15.

## Discussion

### *Haemopsis ottae*, *habitat and behavior*

Based on morphological and genetic criteria, we formally propose the new species designation, *Haemopsis ottae*, for the leech characterized in this study—named after the Ott family who first reported this leech near Alloway, NJ. More remarkable than its status as one of the largest leeches in North America, *H. ottae* was discovered in the most densely populated state in the United States (albeit in a somewhat fragmented, rural setting). Regional COI comparisons showed that distinct populations within NJ displayed high sequence identity ( $\geq 98.8\%$ ), indicating relatively recent geographical isolation; habitat fracturing caused by recent urbanization may account for low levels of divergence between populations. *Haemopsis ottae* appears to be patchily distributed within its environment, often concentrated in only a small fraction of contiguous suitable habitat, and although northern NJ was thoroughly searched for specimens in comparable habitats, none were found or have been reported. Additional sampling throughout the east coast will help to determine whether current populations represent the leading edge of a northern or southern expansion, or whether *H. ottae* is endemic to NJ.

### *North American terrestrial leeches*

Only two other species of terrestrial leech in North America, *Haemopsis terrestris* and *Haemopsis septagon*, are described. *Haemopsis terrestris* occurs

from the Great Lakes region to the Gulf Coast states (including Florida), and as far west as Colorado (Klemm, 1982). Interestingly, no reports of *H. terrestris* have surfaced east of the Appalachian Mountains since its original description (Forbes, 1890), suggesting a significant geologic and/or climatic barrier (see below). The other known North American terrestrial leech, *H. septagon*, appears restricted to southeastern states, namely the Carolinas and the southern part of Virginia (Sawyer and Shelley, 1976; Shelley et al., 1979), and has not been sighted in over 30 years (T. Shelly, personal communication). To date, *H. ottae* has only been collected in southern NJ.

The habitat preference for these three terrestrial *Haemopsis* species is similar, undoubtedly related to their common diet of earthworms and snails. *Haemopsis terrestris*, however, is often found many kilometers from a water source; this does not appear to be the case for *H. septagon* and *H. ottae*, which are more typically observed near swampy or wetland areas. Nevertheless, the Alloway, NJ field site at which *H. ottae* was first collected contains a transient spring river bed that dries each summer, thus *H. ottae* can survive at least a few months each year in dry conditions. Under laboratory conditions, both *H. terrestris* and *H. ottae* displayed a preference for a terrestrial habitat in their sloped aquarium chambers, though they were regularly observed at the “land-water” interface and occasionally submerged in water. All three *Haemopsis* species are good swimmers suggesting a common aquatic ancestry, and all have small posterior suckers consistent with terrestriality.

### *Evolutionary considerations*

Both MP and BI analyses yielded trees with virtually identical topologies and very strong support at every branch, with the exception of the relationship between *H. marmorata*, *H. lateromaculata*, and *H. grandis* (Fig. 7). The cladogram from the combined data set of two nuclear genes (12S and 28S) and mitochondrial COI strongly corroborates *H. ottae* as a previously undescribed haemopid species, and further resolves other ambiguous relationships within the genus *Haemopis*. For example, the monophyly of *Haemopis* is strongly supported and the tree topology agrees with higher level taxonomic analyses (Borda and Siddall, 2004a; Borda and Siddall, 2004b; Apakupakul et al., 1999). Current geographical distributions of haemopids are concurrent with the tree topology, with strong branch support separating the North American and European clades, suggesting a Laurasian ancestry. High boot-strap values and posterior probabilities for the branch separating *H. terrestris* and *H. ottae* from aquatic *Haemopis* leeches (*H. grandis*, *H. lateromaculata*, *H. marmorata*, *H. kingi*) further supports terrestriality as a synapomorphy within the genus. Bremer supports for these branches reveal that an additional 14 steps would be needed to induce a polytomy, and merging *H. terrestris* with *H. ottae* would require 38 additional steps.

Interestingly, we collected *Haemopis* specimens from one field site (Camden, NJ) in our study that were morphologically indistinguishable from *H. marmorata* but are well supported as sister to that clade, indicating a new cryptic species; similar evolutionary patterns have been detected in other oligochaete

groups including *Tubifex tubifex*, *Lumbriculus variegatus*, and species of *Helobdella* and *Hirudo* (Erséus and Bergfeldt, 2007; Bely and Weisblat, 2006; Siddall et al., 2007; Gustaffson et al., 2008; Erséus and Gustaffson, 2009).

#### *Speciation based on molecular clock values*

To estimate an evolutionary time frame for the described speciation events, COI molecular clock variance values (Nagaraja, et al., 2004; Brower, 1994; Soto-Adames, 2002; Knowlton et al., 1993) were considered in the context of haemopid evolution. The observed ~10% COI sequence divergence between *H. ottae* and *H. terrestris* (Table 3) suggests that speciation occurred ~10–20 mya in the early-mid Miocene, while divergence from a putative aquatic ancestor occurred 30–40 mya. Both phylogenetic and morphological analyses argue for a single terrestrialism event among North American leeches, and subsequent divergence concomitant with continental colonization. The current distribution of aquatic haemopid leeches (Klemm, 1982) shows the greatest diversity in temperate higher latitudes, suggesting a deeply rooted ancestry in this region and the likely aquatic stock from which terrestrial haemopids arose.

#### *Evolution and dispersal of North American terrestrial leeches*

After transitioning onto land, the ancestral terrestrial lineage was likely forced south in response to multiple ice ages that occurred throughout the Quaternary. With the Appalachian Mountains acting as a natural barrier to East-West gene flow, terrestrial leeches moving southwest probably formed the *H.*

*terrestris* lineage, continuing their southern migration until reaching the Gulf Coast. The range of *H. terrestris* extends throughout the Gulf Coast states (including FL), thus allowing a putative northeastern migration up the Atlantic coast and into the Carolinas before speciating to form *H. septagon*. This lineage probably continued northward, with *H. ottae* currently representing the leading edge of a northern expansion (Fig. 14). This pattern also suggests a primarily active mechanism of dispersal (i.e., crawling) since active transport (e.g., birds; Davies et al., 1982; Edward and Bohlen, 1996) should have permitted colonization of *H. terrestris*, *H. septagon* and *H. ottae* on either side of the Appalachian range. Morphological criteria arguably favor *H. septagon* as an intermediate species between *H. terrestris* and *H. ottae*; specifically, the distance between gonopores (7-7 ½), presence of dorsal mottling, and similar size ratio of sperm sac/epididymis allies *H. septagon* with *H. ottae*, while *H. septagon* shares long, convoluted vaginal ducts and the presence of lateral stripes with *H. terrestris* (Table 4). Further, descriptions by Shelley and Sawyer (1976) note that some *H. septagon* specimens lacked lateral stripes, suggesting that trait is being lost.

Another plausible evolutionary scenario involves a split between eastern and western *Haemopis* lineages north of the Appalachian range, with the western branch defining *H. terrestris* and the eastern branch giving rise to *H. ottae* and *H. septagon*, the latter defining the leading edge of a putative southern expansion. Our genetic data cannot rule out this evolutionary pattern, but the consideration of morphological traits proves more problematic (e.g., lateral stripes, reproductive

structures ally *H. septagon* with *H. terrestris*). Alternatively, a single passive dispersal event across the mid-Appalachian range could have given rise to a terrestrial *Haemopsis* population closely related to *H. terrestris*, either *H. ottae* or *H. septagon*, but available evidence cannot distinguish this scenario from those proposed above. Clearly, the collection and analysis of additional *Haemopsis* specimens throughout Canada and the continental United States will refine the details of the phylogeographic patterns that led to the current distribution of terrestrial leeches in North America.

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Table 1. Primer sequences used in phylogenetic analyses of haemopid leeches.

Gene	Primer Name	Primer Sequence (5' → 3')	Reference
<i>Nuclear</i>			
12s			
	A1	AACTAGGATTAGATACCCTATTAT	
	B1	AAGAGCGACGGGCGATGTGT	
28S			
	LROR	ACCCGCTGAACTTAAGC	Bunyard et al., 1994
	LR5	ATCCTGAGGGAACTTC	Vilgalys and Hester 1990
<i>Mitochondrial</i>			
COI			
	HCO	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994
	LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
	COI.1	GC(TC)TGATCAGCTATA(TGC)TAGG	
	COI.2	ATTGAG(TC)(TC)(AG)(GT)C(CTA)CA(AG)CCTGG	
	COI.3	AAAAAAAGTAGTATTTAAT(CTA)CGATC	
	COI.4	TCCTA(TC)AGGATCAAAAAAAGTAG	

Table 2. Accession numbers used in phylogenetic analyses of haemopid leeches.

Taxon	GenBank accession No.		
	28S	12S	COI
Ingroup			
<i>Haemopsis sanguisuga</i>	AY425381	AF099960	AF462021
<i>Haemopsis ceaca</i>	AY425376	AY425419	AY040702
<i>Haemopsis kingi</i>	AY425378	AY425421	AY425448
<i>Haemopsis marmorata</i>	AY425380	AY425423	AF003270
<i>Haemopsis lateromaculata</i>	AY425379	AY425422	AF116028
<i>Haemopsis grandis</i>	AY425377	AY425420	AY425447
<i>Haemopsis terrestris</i>	EU100080.1	AY786446.1	AY786459.1
<i>Haemopsis terrestris</i> (MO)	FJ897505	N/A	FJ897514
<i>Haemopsis marmorata</i> -like (Camden, NJ)	FJ897504	FJ897509	FJ897515
<i>Haemopsis ottae</i> (Alloway, NJ)	FJ897511	FJ897507	FJ897510
<i>Haemopsis ottae</i> (Winslow, NJ)	FJ897506	FJ897508	FJ897512
<i>Haemopsis ottae</i> (Pomona, NJ)	N/A	N/A	FJ897513
Outgroup			
<i>Mesobdella gemmata</i>	EU100084.1	AY425434.1	EU100097.1
<i>Aliolimnatis michaelsoni</i>	AY425388.1	AY425429.1	AF116029.1
<i>Hirudo medicinalis</i>	EU100079.1	DQ097197.1	EU100093.1

Table 3. Percent similarity of cytochrome *c* oxidase subunit 1 gene fragment based on pairwise alignment using MUSCLE.

	Mesobdella gemmata	Aliolimnatis michaelseni	Hirdo medicinalis	Haemopsis sanguisuga	Haemopsis ceaca	Haemopsis kingi	Haemopsis marmorata NJ
Mesobdella gemmata	~	76.8%	76.6%	78.9%	79.1%	76.8%	74.9%
Aliolimnatis michaelseni	76.8%	~	81.2%	81.4%	80.5%	80.3%	78.0%
Hirdo medicinalis	76.6%	81.2%	~	82.7%	82.8%	81.9%	78.9%
Haemopsis sanguisuga	78.9%	81.4%	82.7%	~	98.5%	87.5%	82.5%
Haemopsis ceaca	79.1%	80.5%	82.8%	98.5%	~	87.3%	81.6%
Haemopsis kingi	76.8%	80.3%	81.9%	87.5%	87.3%	~	84.3%
Haemopsis marmorata NJ	74.9%	78.0%	78.9%	82.5%	81.6%	84.3%	~
Haemopsis marmorata	75.0%	77.3%	77.7%	82.3%	81.2%	82.3%	91.8%
Haemopsis lateromaculata	74.5%	77.1%	77.1%	82.1%	81.1%	83.2%	91.6%
Haemopsis grandis	75.0%	77.0%	77.3%	82.5%	81.4%	82.7%	92.1%
Haemopsis terrestris	77.7%	80.2%	80.7%	88.2%	87.5%	87.3%	82.3%
Haemopsis terrestris MO	77.3%	79.5%	81.1%	87.3%	86.6%	88.0%	82.7%
Haemopsis ottae-pomona	77.1%	81.2%	82.1%	88.4%	87.8%	87.8%	83.2%
Haemopsis ottae-winslow	77.5%	81.9%	82.5%	88.7%	88.5%	87.8%	83.7%
Haemopsis ottae-alloway	77.3%	81.8%	82.5%	88.9%	88.7%	87.8%	83.7%

Table 3 continued

	Haemopsis marmorata	Haemopsis lateromaculata	Haemopsis grandis	Haemopsis terrestris	Haemopsis terrestris MO	Haemopsis ottae- pomona	Haemopsis ottae- winslow	Haemopsis ottae- alloway
Mesobdella gemmata	75.0%	74.5%	75.0%	77.7%	77.3%	77.1%	77.5%	77.3%
Aliolimnatis michaelseni	77.3%	77.1%	77.0%	80.2%	79.5%	81.2%	81.9%	81.8%
Hirdo medicinalis	77.7%	77.1%	77.3%	80.7%	81.1%	82.1%	82.5%	82.5%
Haemopsis sanguisuga	82.3%	82.1%	82.5%	88.2%	87.3%	88.4%	88.7%	88.9%
Haemopsis ceaca	81.2%	81.1%	81.4%	87.5%	86.6%	87.8%	88.5%	88.7%
Haemopsis kingi	82.3%	83.2%	82.7%	87.3%	88.0%	87.8%	87.8%	87.8%
Haemopsis marmorata NJ	91.8%	91.6%	92.1%	82.3%	82.7%	83.2%	83.7%	83.7%
Haemopsis marmorata	~	96.7%	96.7%	80.9%	81.6%	81.9%	81.9%	81.8%
Haemopsis lateromaculata	96.7%	~	97.8%	80.7%	81.4%	82.5%	82.1%	81.9%
Haemopsis grandis	96.7%	97.8%	~	81.2%	81.4%	82.3%	81.9%	81.8%
Haemopsis terrestris	80.9%	80.7%	81.2%	~	97.8%	90.5%	90.3%	90.1%
Haemopsis terrestris MO	81.6%	81.4%	81.4%	97.8%	~	90.3%	90.1%	90.0%
Haemopsis ottae-pomona	81.9%	82.5%	82.3%	90.5%	90.3%	~	98.3%	98.2%
Haemopsis ottae-winslow	81.9%	82.1%	81.9%	90.3%	90.1%	98.3%	~	99.8%
Haemopsis ottae-alloway	81.8%	81.9%	81.8%	90.1%	90.0%	98.2%	99.8%	~

Table 4. Comparison of morphological traits for the three North American terrestrial leeches. Green shading, traits that ally *H. terrestris* and *H. septagon*; yellow shading, traits that ally *H. ottae* and *H. septagon*; pink shading, traits unique to *H. ottae*.

Trait	<i>H. terrestris</i>	<i>H. septagon</i>	<i>H. ottae</i>	Usable traits
Coloring (dorsal)	Gray to black; no mottling/spots	Dark olive green; numerous small irregularly scattered black flecks	Dark brown; heavy black mottling	No
Coloring (ventral)	Lighter gray	Lighter olive green; no flecks	Dark brown; no mottling	No
Coloring other	Dark dorsal mid-line; paler buff marginal stripes; dark ventral sub-marginal stripe	Dark strip along dorsal mid-line; yellow marginal stripes	Distinct black dorsal mid-line	No
Papillae	None	None mentioned	8 present every 5 <sup>th</sup> annulus; white tipped	Yes
Size	7 inches long; 0.75 inches wide 80 – 150mm long	69 mm long; 12mm wide 165 mm long; 25mm wide	248mm long; 28mm wide	Yes
Distance between gonopores	5 -5.5 annuli; on annulus	6.5 -7 annuli; male pore in furrow	7 -7.5 annuli; in furrow	No
Teeth (type)	Bicuspid	distichodont	distichodont; blunt	No
Teeth (#)	12-15	15	20	No
Eye spots	5 pair – typical hirudinid position	5 pair – typical hirudinid position	5 pair – typical hirudinid position	No
# testisacs	10 pair	11 pair	8 – 12 pair	No
Sperm sac/epididymis	Sperm sac smaller compared to epididymis; epididymis does not extend past ss; ss narrow and curved	= to 1.5x; ss straight; epididymis extends beyond ss	More than 2xs the size of the epididymis; epididymis extends far past the ss	Yes
Ovaries	Moderately sized; bi-lobed	Small paired; bi-lobed	Small; bi-lobed	Yes
Vaginal duct	Long & convoluted	Long & convoluted	Long & relatively straight	Yes
vagina	Large and wide; tubular	Large & tubular	Small & tubular	Yes

Figure 1. Photograph of dissected *Haemopsis terrestris* specimen. Magnified view of reproductive systems and pharynx (A). Dissection showing ventral cut and full size of specimen, including lateral stripe indicative of *H. terrestris* (B). Scale bar is 1.5 cm.



Figure 2. Photograph of dissected *Haemopsis ottae* specimen. Magnified view of reproductive systems and pharynx (A). Dissection showing ventral cut and full size of specimen (B). Scale bar is 1.5 cm.



Figure 3. Alignment used to design *Haemopsis* specific COI primers. Circled regions designate sequence used for primer design. Blue, COI.1; Red, COI.2; Yellow, COI.3; Green, COI.4.





	COI.3	COI.4	
Haemopsis terrestris	TTAAATA C T A C T T T T T T T	G A T C C T A T A G G A	GGTGGAGATCCAGTATTGTT 650
Haemopsis elegans	TTAAATA C T A C T T T T T T T	G A T C C T A T A G G A	GGAGGAGATCCAGTTTTATT 583
Hirudo medicinalis	TTAAATA C T A C T T T T T T T	G A T C C A A T T G G A	GGAGGGGATCCAGTCTTATT 650
Haemopsis caeca	TTAAATA C T A C T T T T T T T	G A T C C T A T A G G A	GGTGGTGACCCAGTTTTATT 644
Haemopsis sanguisuga	TTAAATA C T A C T T T T T T T	G A T C C T A T A G G A	GGTGGTGACCCAGTTTTATT 642
Haemopsis kingi	TTAAATA C T A C T T T T C T T T	G A T C C T G T A G G A	GGAGGAGATCCAGTTTTATT 640
Haemopsis grandis	TTAAATA C T A C T T T T T T T	G A T C C T G T A G G A	GGGGGGGACCNTATTTTATT 643
Haemopsis lateromaculata	TTAAATA C T A C T T T T T T T	G A T C C T G T A G G A	GGGGGAGATCCTATTTTATT 644
Haemopsis marmorata	TTAAATA C T A C T T T T T T T	G A T C C T G T A G G A	GGGGGAGACCCTATTTTATT 644
	*****	***** * ***	** * * * * * * * * * * *
Haemopsis terrestris	TCAACACTTATTTTGATTTT	TGGTCATCCAGAAGTATATATTTGATTT	700
Haemopsis elegans	TCAGCACTTATTTTGATTTT	-----	603
Hirudo medicinalis	TCAACATCTATTTTGTTCTTTGGTCATCCAGAGTTTATATTTAATTT		700
Haemopsis caeca	TCAGC-----		649
Haemopsis sanguisuga	TCAAC-----		647
Haemopsis kingi	TCAGC-----		645
Haemopsis grandis	TCAAC-----		648
Haemopsis lateromaculaa	TCAACAC-----		651
Haemopsis marmorata	TCAACTT-----		651
	*** *		

Figure 4. Distribution of New Jersey *Haemopsis* populations. Shaded area represents land designated as New Jersey Pinelands. Distance between field sites indicated in km.



Figure 5. Photographs of the Winslow field site. Pictures are representative of the cedar bog habitat where *Haemopsis ottae* is most commonly found. These areas have a high water table and are prone to flooding from adjacent streams (A). There is little undergrowth and habitat often contains *Sphangum* sp. moss, which only grows in low pH environments (B).



Figure 6. Fallen log at Winslow field site from which a *Haemopsis ottae* specimen was collected. Although the log is saturated with water, it is not submerged. Arrow identifies an earth worm that was proximal to the collected leech.



Figure 7. Phylogenetic tree of the combined data set resolving relationships within the genus *Haemopsis*. Maximum Parsimony and Bayesian Inference analyses were performed on the combined data set (COI, 12S, 28S); posterior probabilities indicated above, and bootstrap values below branches.

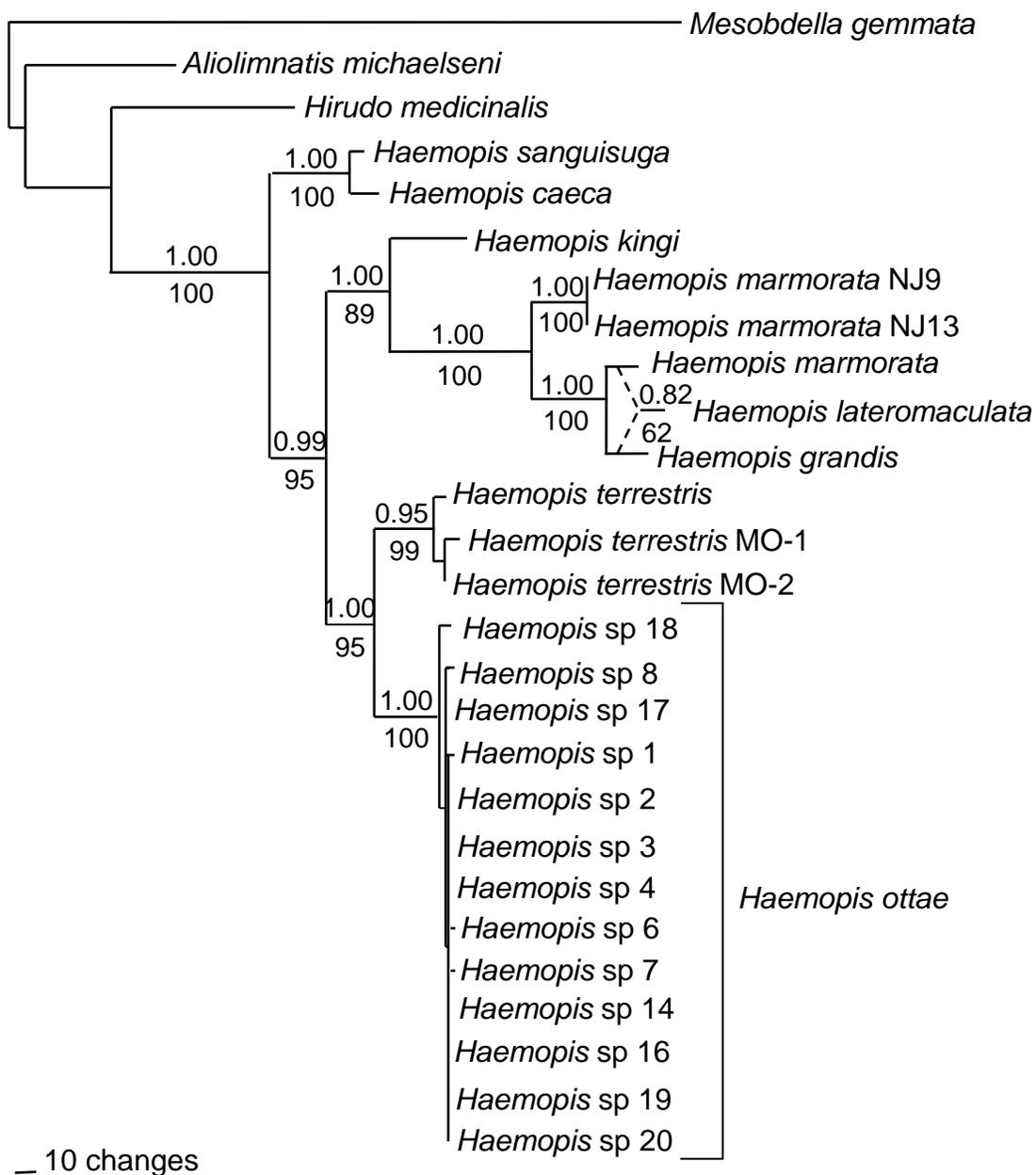


Figure 8. Most parsimonious tree obtained from cytochrome *c* oxidase subunit 1 mtDNA, for the genus *Haemopsis*. All *Haemopsis ottae* samples are a clade sister to *Haemopsis terrestris*.

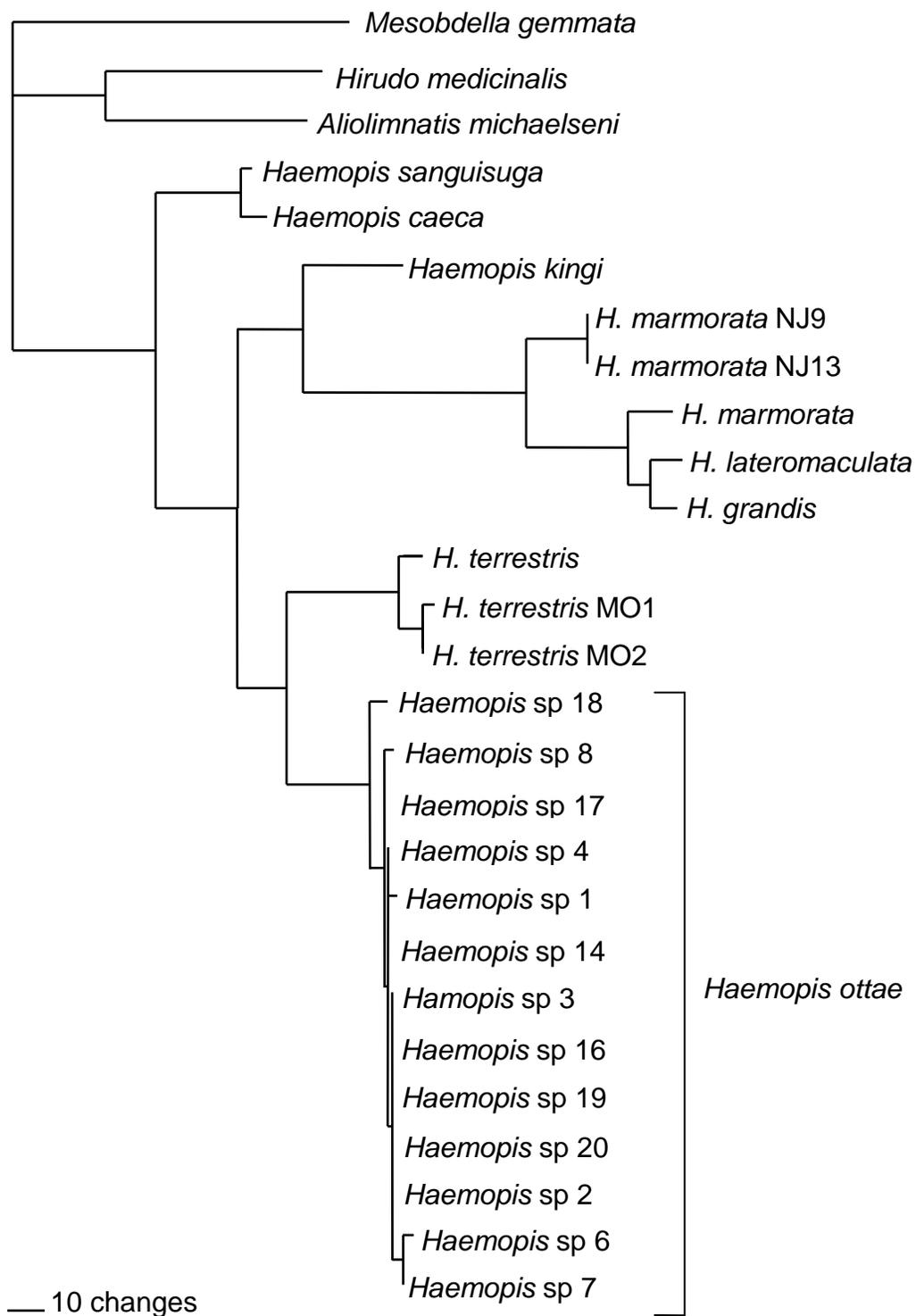
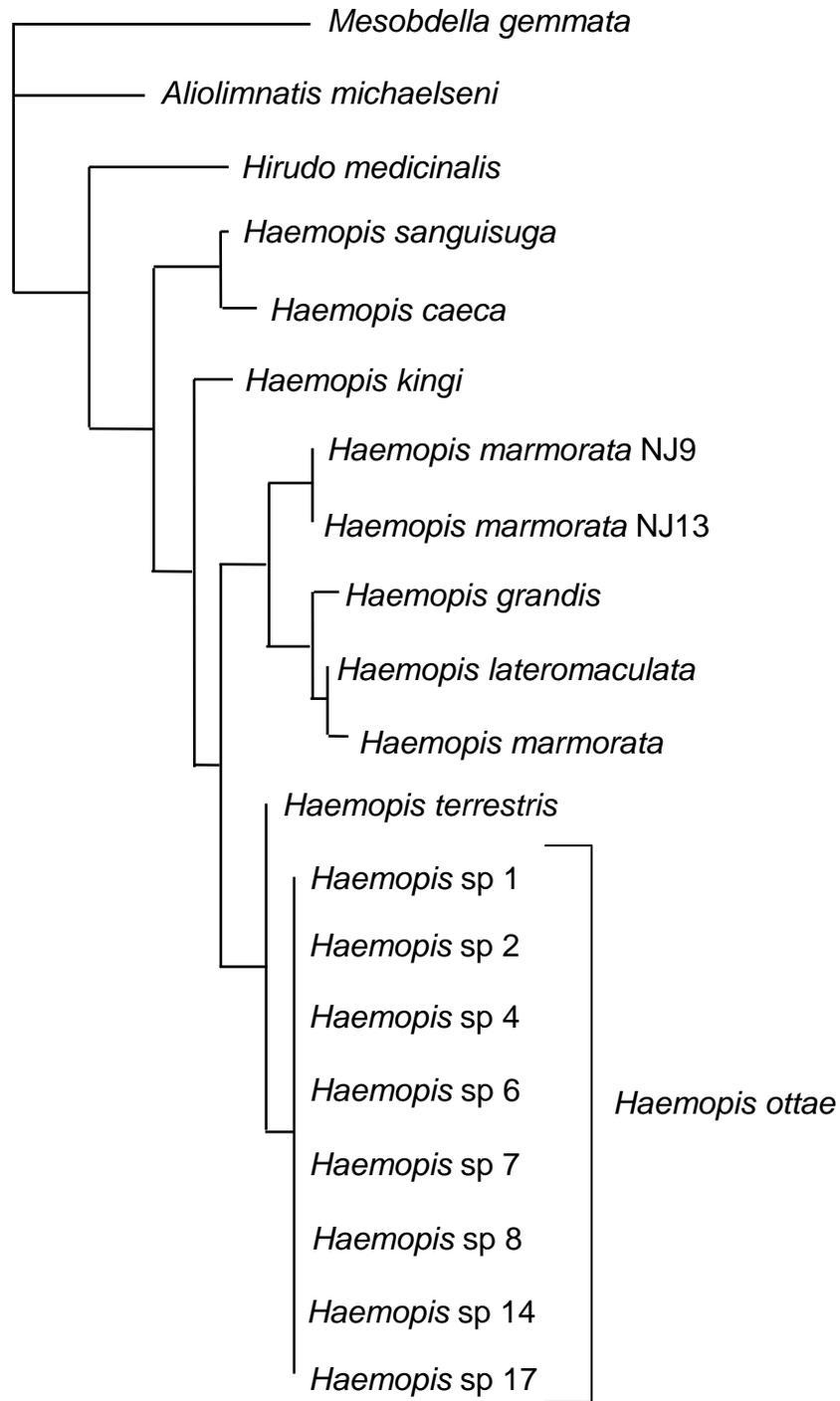
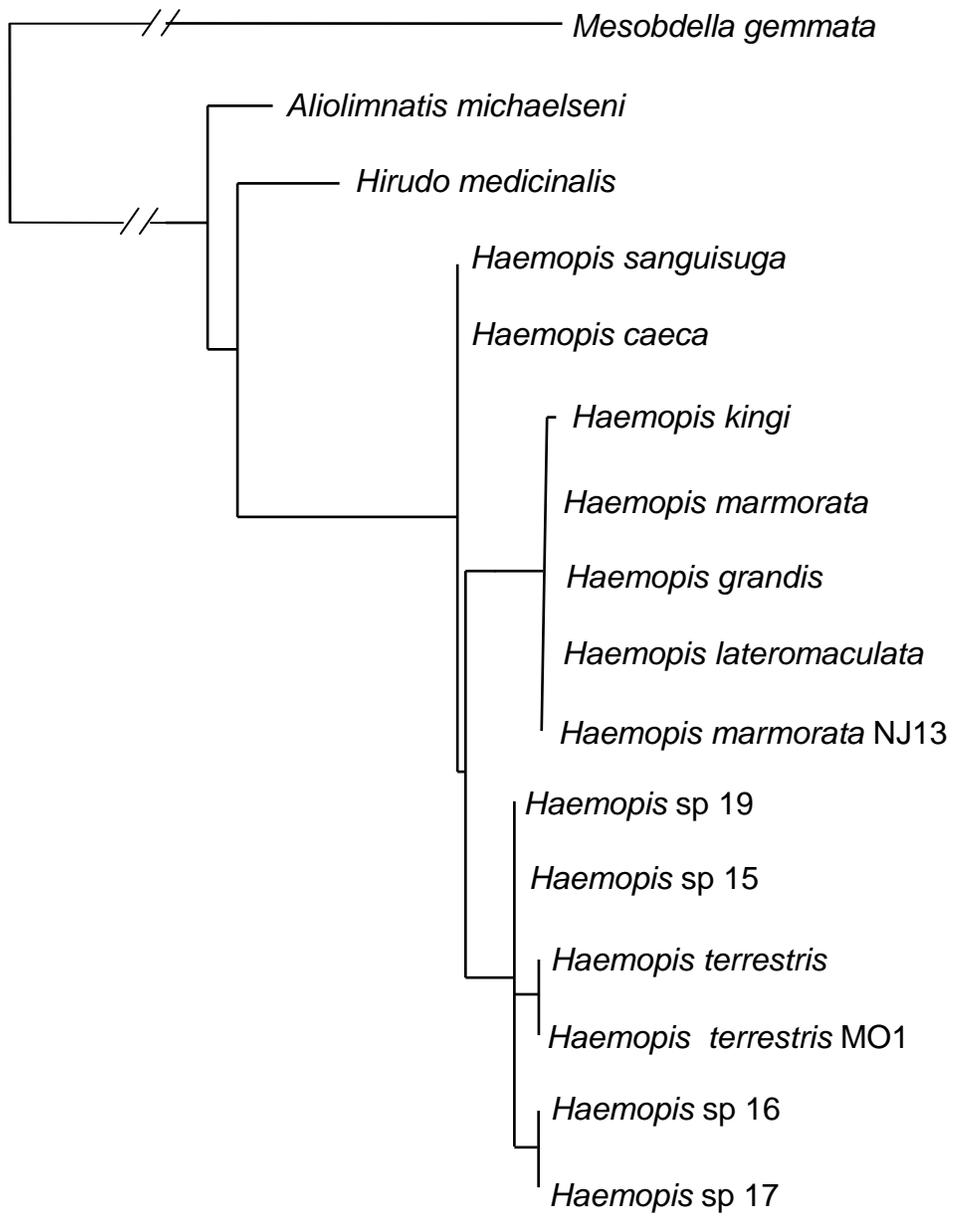


Figure 9. Most parsimonious tree obtained from 12S rDNA, for the genus *Haemopis*. All *Haemopis ottae* samples are a clade sister to *Haemopis terrestris*.



— 10 changes

Figure 10. Most parsimonious tree obtained from 28S rDNA, for the genus *Haemopsis*. Multiple polytomies present indicating insufficient data to resolve relationships between closely related species.



— 5 changes

Figure 11. Sketches from dorsal and ventral views of adult *Haemopsis ottae* type specimen from Alloway, NJ, viewed under enhanced artificial light. Insets are magnifications of anterior dorsum (A), and ventral gonopores (B). Faint, often broken midline and moderate to heavy black mottling present on dorsum. Seven annuli were present between gonopores, with male and female pores located in the furrow. White-tipped papillae (~ 16 per annuli) present every fifth annulus; five pairs of eyespots in a classic Hirudinid arc. cs, caudal sucker; es, eyespot; n, nephridiopore; p, papillae.

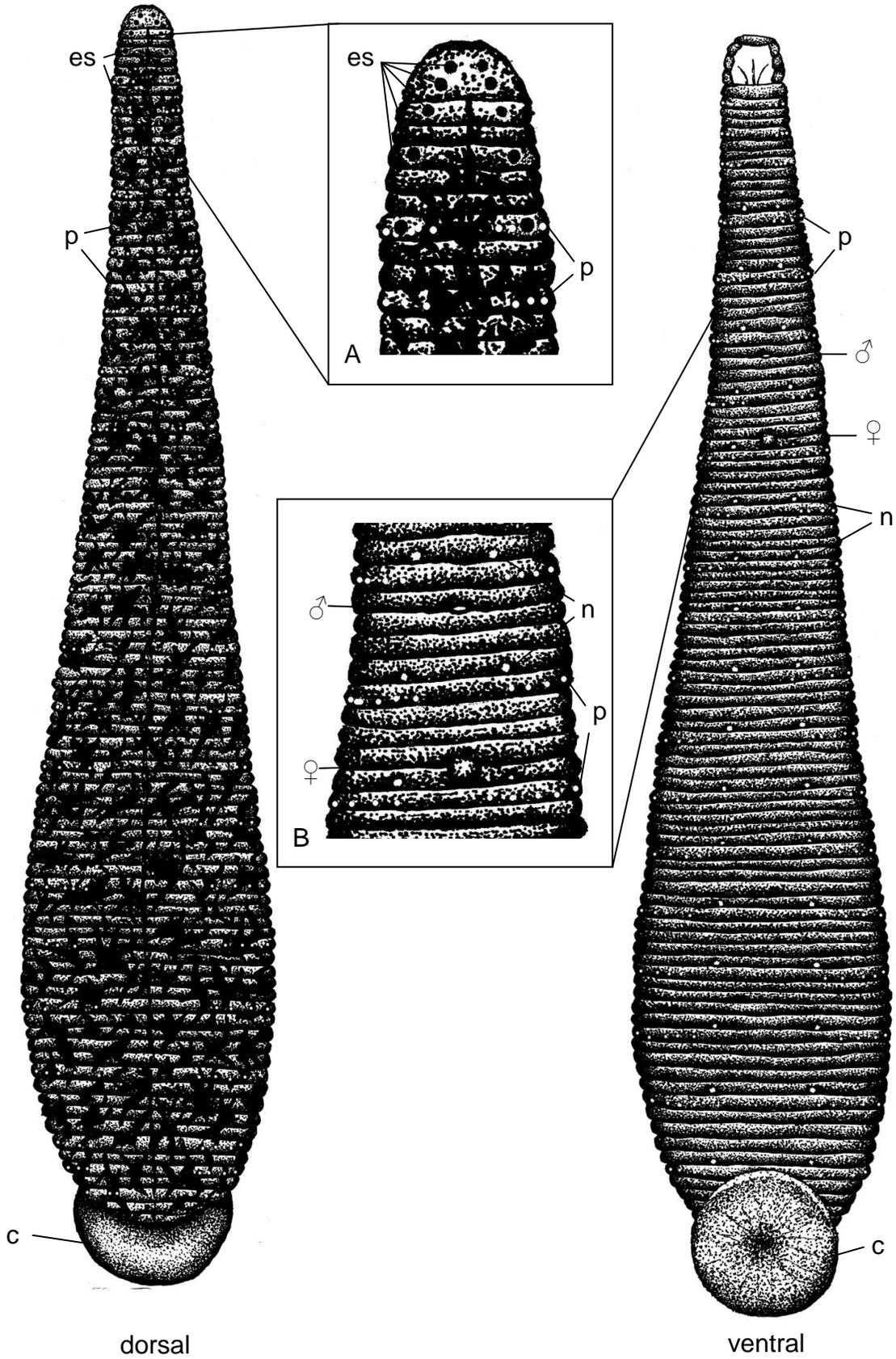
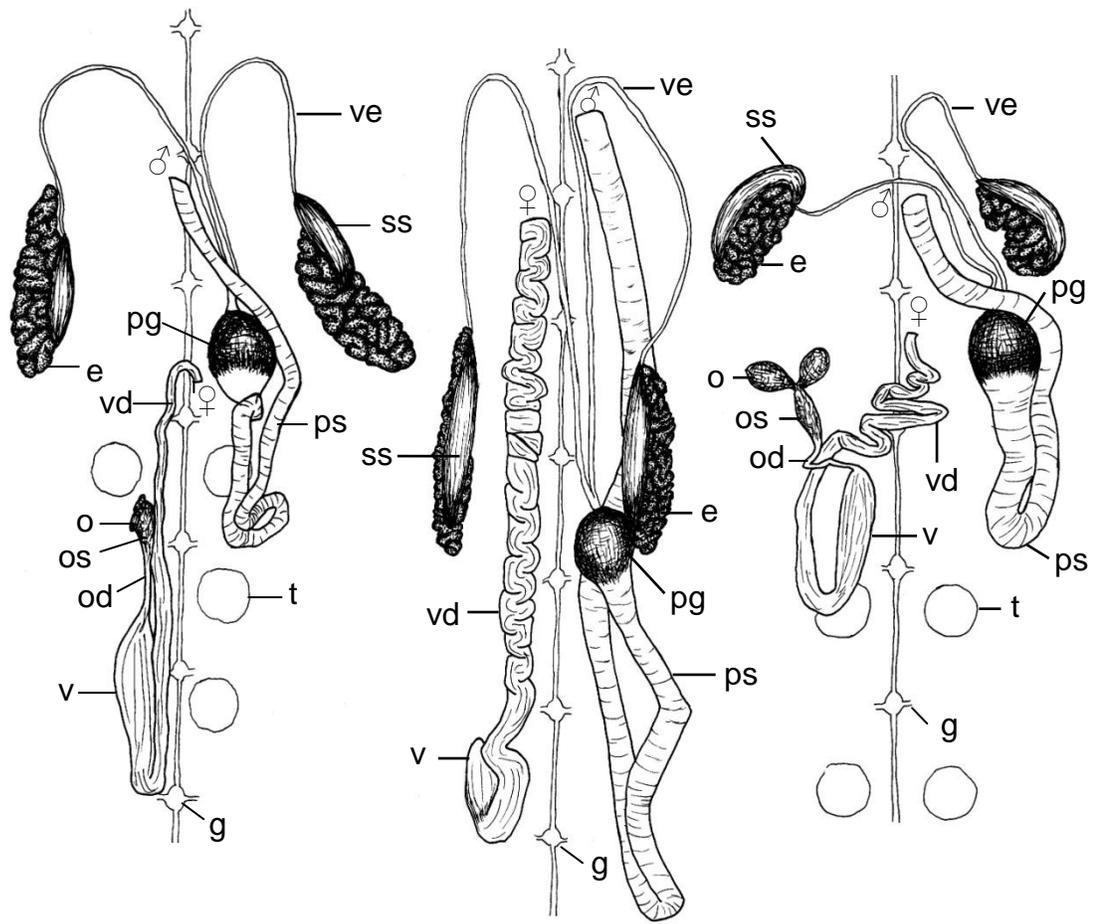


Figure 11. Photograph of live *Haemopsis ottae*. Specimen collected at the Winslow field site from inside a decaying log. Photograph taken by Avi Steinhardt, Courier Post.



Figure 9. Sketches made from dorsal view of reproductive structures. (A) *Haemopsis ottae*. (B) *Haemopsis marmorata*-like specimen found in Camden, NJ. (C) *Haemopsis terrestris* collected from Belton, Missouri. *H. ottae* epididymis was notably large, extending far past the sperm sac; vaginal duct and oviduct were relatively straight and uncoiled, ovaries were significantly small, vagina was narrow and less pronounced. e, epididymis; g, ganglion; o, ovary; od, oviduct; os, ovisac; ps, penis sheath; pg, prostate gland; ss, sperm sac; t, testis; v, vagina; vd, vaginal duct; ve, vas eferens.



A

B

C

Figure 13. Map depicting the putative evolutionary history of terrestrial leeches in North America. Distributions of *Haemopsis terrestris* and *Haemopsis septagon* are from Klemm (1986) and Sawyer and Shelly (1976), respectively. Shaded area represents the Appalachian Range. ● *Haemopsis ottae*; ■ *Haemopsis terrestris*; ▲ *Haemopsis septagon*.

