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A new species of *Haemopis* (Annelida: Hirudinea): Evolution of North American terrestrial leeches

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ABSTRACT

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 terrestrialism as a synapomorphy for some members of the group. Morphological distinctions and geographic isolation support the designation of a new species of terrestrial leech, *Haemopis ottorum*. Phylogeographic interpretations of the haemopid clade suggest that terrestrialism was derived from a northern, aquatic ancestor whose descendents were initially confined to Midwestern States and central Canada by the Appalachian Range. More recently, the terrestrial lineage (i.e., *Haemopis terrestris*) diverged near the southern extent of its range and began a northeasterly migration along coastal states giving rise to *Haemopis septagon* and *Haemopis ottorum*, the latter of which appears to define the leading edge of a northward expansion.

1. 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 aquatic 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. Examples 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, *Haemopis terrestris* (Forbes, 1890), and *Haemopis septagon* Sawyer and Shelley, 1976, 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* Linnaeus, 1758, and *Hirudo verbana* Carena, 1820, 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 *Haemopis* 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 *Haemopis terrestris* and *Haemopis septagon*, other species in the genus, namely *Haemopis grandis* Verrill, 1874, *Haemopis marmorata* Moore, 1912, *Haemopis kingi* Mathers, 1952, and *Haemopis 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 *Haemopis* 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 (*Haemopis terrestris*) or southeastern (*Haemopis septagon*) states.

Several years ago, we received a specimen of *Haemopis* collected near a local resident's garden in southern New Jersey that did not match the characters of other *Haemopis* species. Subsequent fieldwork identified two additional populations in NJ. Our collective morphologic, geographic and phylogenetic data suggest that these populations represent a third species of terrestrial leech in North

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America, and that terrestrialism occurred only once from a northern, aquatic ancestor from which the three terrestrial *Haemopis* lineages were derived.

2. Materials and methods

2.1. Specimen collection and maintenance

Haemopis specimens collected in New Jersey were transported to Rutgers University (Camden, NJ) and maintained in separate aquaria based on collection location. *Haemopis terrestris* specimens were collected in Belton, Missouri, a generous gift from Karl and Kathleen Young. No new specimens of *Haemopis septagon* specimens were collected in this study. Aquaria contained 1–2 cm 0.03% 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.

2.2. Dissections

Specimens were fixed in 70% ethanol. External traits of live specimens were observed under a stereomicroscope (Meiji EMZ-TR, Meiji Techno Co., Ltd.). Preserved specimens were dissected dorsally and ventrally, with representative sketches of internal morphology derived directly from the type specimen. The type specimen, collected from the Winslow field site, is deposited in the Smithsonian Institution collection (Washington, DC; Catalog No. USNM 1125241).

2.3. DNA extraction

Tissue samples from live specimens were obtained by placing the leech in a 10% ethanol sedating solution until 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.

2.4. 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 (2004a,b). For 28S rRNA, universal primers LROR (ACCCGCTGAACTTAAGC; Bunyard et al., 1994) and LR5 (ATCCTGAGGGAAACTTC; Vilgalys and Hester, 1990) were incorporated into PCRs generating a ~1060 bp fragment. PCR conditions were 94 °C for 30 s, 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 *Haemopis* 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 the LCO/COI.4 primer set. COI PCR conditions were 94 °C for 30 s, 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 $MgCl_2$, in a Techne TC-312 Thermal Cycler.

2.5. 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 (2004a,b). Sequences were manually adjusted in ChromasPro (Technelysium, Queensland, Australia) or BioEdit (Hall, 1999) and aligned with MUSCLE (Edgar, 2004) or CLUSTAL W (Higgins et al., 1994; Larkin et al., 2007). Accession numbers for COI, 12S and 28S sequences obtained from NCBI GenBank are listed in Table 1.

2.6. 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 repli-

Table 1
Accession numbers used in phylogenetic analyses of haemopid leeches.

Taxon	GenBank Accession No.		
	28S	12S	COI
Ingroup			
Haemopis sanguisuga Linnaeus, 1758	AY425381	AF099960	AF462021
Haemopis caeca Manoleli, Klemm and Sarbu, 1998	AY425376	AY425419	AY040702
Haemopis kingi Mathers, 1954	AY425378	AY425421	AY425448
Haemopis marmorata Moore, 1912	AY425380	AY425423	AF003270
Haemopis lateromaculata Mathers, 1963	AY425379	AY425422	AF116028
Haemopis grandis Verrill, 1874	AY425377	AY425420	AY425447
Haemopis terrestris Forbes, 1891	EU100080.1	AY786446.1	AY786459.1
Haemopis terrestris (MO)	FJ897505	N/A	FJ897514
Haemopis marmorata-like (Camden, NJ)	FJ897504	FJ897509	FJ897515
Haemopis ottorum (Alloway, NJ)	FJ897511	FJ897507	FJ897510
Haemopis ottorum (Winslow, NJ)	FJ897506	FJ897508	FJ897512
Haemopis ottorum (Pomona, NJ)	N/A	N/A	FJ897513
Outgroup			
Mesobdella gemmata Blanchard, 1849	EU100084.1	AY425434.1	EU100097.1
Aliolimnatis michaelseni Augener, 1936	AY425388.1	AY425429.1	AF116029.1|
Hirudo medicinalis Linnaeus, 1758	EU100079.1	DQ097197.1	EU100093.1