

DNA-barcoding evidence for widespread introductions of a leech from the South American *Helobdella triserialis* complex.

Mark E. Siddall* and Rebecca B. Budinoff

Division of Invertebrate Zoology, American Museum of Natural History

Abstract

Morphological examination and a molecular phylogenetic analysis of leeches from Australia, New Zealand, South Africa and Hawaii show that these specimens are members of a species in the South American *Helobdella triserialis* species complex. Though it has been seen before, this leech was not recognized as an invasive species. Rather, it was first described as *Helobdella striata* from Germany later renamed *Helobdella europaea* and then independently described as *Helobdella papillornata* from Australia. Because the appropriate name for this leech from its South American endemic locale, *Helobdella (triserialis) lineata*, is preoccupied by a North American species, we formally recognize *Helobdella europaea* as the valid taxon name. Although this invader is not a bloodfeeder it may be expected to have an impact on native annelid and mollusc faunas.

Introduction

There is a substantial role for systematics and taxonomy in a variety of conservation related issues. With respect to invasive species in particular, identification of taxa, their ultimate geographic origins and identity with source populations has had considerable impact. Pathogens first identified from oysters in the Chesapeake Bay have proven to have been introduced along with non-native oyster introductions from Asia (Burreson et al., 2000). McGlynn (1999)

determined that 35% of invasive ant species have a non-native island habitat as their type-locality (the locality where the species was first collected by the systematist who first encountered and described it). These findings are not trivial in light of the fact that most new species descriptions for invertebrates compare and contrast only to other related taxa in the geographic region in which the putative new species was found. So-called DNA 'barcoding' (Hebert et al. 2004a), provides a rapid means by which one can assess species identities at the molecular level. Though it is not without pitfalls (Moritz and Cicero, 2004), it does offer the opportunity to scrutinize, at the molecular level, taxonomic identifications of morphologically similar specimens.

Leeches of the genus *Helobdella* Blanchard 1896 have been found worldwide. Species of this genus are proboscis-bearing, brood their young and subsist on a diet of aquatic invertebrates instead of vertebrate blood (Soos 1969; Sawyer 1986; Davies 1991). Although the prey preferences of most species in the genus are not known, oligochaetes and molluscs appear to be favored (Young & Ironmonger 1980; Young & Proctor 1985). As such, the introduction of these predaceous leeches to a freshwater environment could affect resident mollusc and annelid communities, in turn disrupting trophic interactions (Govedich et al. 2002)

Accidental introduction of leeches is not unknown, two examples being the widespread Asian *Barbronia weberi* (e.g., AquaSense 2003; Govedich et al. 2002, 2003; Rutter & Klemm 2001; Pamplin & Rocha 2000; Mason 1976; Moore 1946) and the introduction to Asia of the North American *Placobdella multilineata* Moore 1953 (Yang & Davies 1985). *Helobdella* species that appear to be native to South America, have been found in Europe and are suspected to have been introduced via aquatic plants or snails (Pfeiffer et al. In review; Bennike 1943). Recent collections have suggested that leeches in the genus *Helobdella* may have been

introduced via similar accidental transport. Leeches that clearly belong to the genus *Helobdella* and are distinct from previously known endemics have been found in collections from New Zealand, South Africa, Australia and Hawaii. Here, we present morphological observations and a molecular phylogenetic analysis identifying them all as *Helobdella (triserialis) lineata* sensu Ringuelet 1943. Understanding the dynamics of this and other possible species introductions could help us improve our knowledge not only of the freshwater distribution of leeches, but also of the effects of these predators on oligochaete and mollusc densities.

Materials and Methods

Leeches were obtained via primary collections and by receipt from colleagues. In February 2001, three *Helobdella cordobensis* specimens were collected from Lago Calafquen, (39.29°26.2'S, 72.08°50.1'W, altitude 207m) Chile [AMNH Annelida 4342]. In September of 2001, three leeches were collected in Gwalagwala (24.27°S, 31.06°E), a tented-camp near Hoedspruit South Africa [AMNH Annelida 5248]. In September 1998, ten leeches from Magill Creek in Queensland, Australia, were collected by Stephen Cameron (AMNH Annelida 5247). In May 2003, two leeches from Five Mile Bay in Wellington New Zealand (38.74°S, 176.05°E) were collected by Geoff Read [AMNH Annelida 5245]. In March 2003, three leeches from Oahu, Hawaii, were collected by Ron Englund [AMNH Annelida 5246]. Six leeches from Aura Vale Lake, Australia, type locality of *Helobdella papillornata* Govedich and Davies, 1998 were received from Fred Govedich [AMNH Annelida 5244].

Leeches were stored in 95-100% ethanol at -20 C or at ambient temperature until used for DNA extraction. The caudal sucker was removed and utilized for DNA extraction. The caudal sucker is specifically used in order to minimize the possibility of contamination from

host/prey DNA found in the gastric regions. DNeasy Tissue Kit (QIAGEN Inc. Valencia, California) was used for tissue lysis and DNA purification. We were unable to extract amplifiable DNA from our Hawaiian specimens.

PCR amplification and sequences of two mitochondrial gene regions were used for molecular phylogenetic analysis. The universal primers, LCO1490, 5' GGTCACAAATCATAAAGATATTGG 3' and HCO2198, 5' TAAACTTCAGGGTGACCAAAAATCA 3', were used to amplify cytochrome *c* oxidase subunit I (CO-I) fragments of 665 base pair (bp) length. Nicotinamide adenine dinucleotide dehydrogenase subunit I (ND-I) fragments (654 bp) were amplified using the primer pairs, LND300, 5' TGGCAGAGTAGTGCATTAGG 3' and HND1932 5' CCTCAGCAAATCAAATGG 3' (Light and Siddall, 1999). Amplification reactions for CO-I and for ND-I contained 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer Corporation, Foster City, California), 10X II Buffer, 2.5 mM magnesium chloride, 0.25 mM of each dNTP (1mM total), 10 mM of each primer, and template for a 25 μ l total volume. Alternatively, Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) were used; each 25 μ l reaction contains 1.5 units DNA polymerase, 10 mM Tris-hydrochloric acid (pH 10), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 mM of each dNTP, stabilizers, 10 mM of primer pair mix, template and water. In a GeneAmp PCR System 9700 (P E Applied Biosystems), reaction mixtures were heated to 94 C for 5 minutes, followed by 15 cycles of 94 C (45 sec), 46 C (45 sec), and 72 C (45 sec), then 25 cycles of 94 C (20 sec), 45 C (20 sec) and 72 C (30 sec) and a final extension at 72 C (6 min). The ArrayIt™ PCR Purification Kit was used to clean PCR products.

Amplification products were sequenced in both directions. Each sequencing reaction mixture, including 2 µl BigDye™ (Applied Biosystems, Perkin-Elmer Corporation), 2 µl of 1 µM primer (single primer for each direction), and 5 µl of DNA template, ran for 40 cycles of 96 C (10 sec), 50 C (10 sec) and 60 C (4 min). Sequences were purified by ethanol precipitation to get rid of primers and unincorporated dyes. Products were re-suspended in 6 µl formamide and were electrophoresed in an ABI Prism™ 3730 sequencer (Applied Biosystems).

Sequences of complimentary strands were edited and reconciled using Sequence Navigator (Applied Biosystems). Sequences for 14 other species of *Helobdella* and 5 species of *Haementeria* were obtained from Siddall and Borda (2003) and from Pfeiffer et al., (In review). Alignment of CO-I fragments was done by eye across all taxa because there were no insertions or deletions. ND-I fragments were aligned according to inferred amino acid sequences. Phylogenetic analyses were performed using PAUP* (Swofford, 2003). Heuristic searches used 100 replicates of random taxon addition and tree-bisection-reconnection branch swapping. All characters were left non-additive.

Results

All leeches from localities in Australia, New Zealand, South Africa and Hawaii had 5 dorsal rows of papillae on annulus a2 of the mid-body and posterior somites. Each exhibited 12 to 14 dorsal stripes of pigment with interruptions in this longitudinal pigmentation in the cephalic region (Fig. 1 inset). Internally each was equipped with 4 pairs of gastric caeca plus another pair of gastric postcaeca. Testisacs could not be reliably observed for most dissected specimens. Ovisacs extended to about the junction of somites XIV and XV. Moreover, there was no difference in COI or NDI sequences obtained from these leeches save for one pyrimidine transition difference in COI for the leech from the type locality of *Helobdella papillornata*. Of

the 144 nucleotides available for COI from *Helobdella europaea* (Pfeiffer et al. In review), only 2 sites differed by a purine transition.

Phylogenetic analysis resulted in 102 equally parsimonious trees each with 2239 steps and a retention index of 0.52. The only lack of resolution in the strict consensus was an inability to distinguish any resolution for the leeches from localities in Australia, New Zealand, South Africa and Germany (including *Helobdella papillornata* and *Helobdella europaea*). The remainder of the tree was fully resolved. Sister to the *europaea/papillornata* cluster was *Helobdella triserialis* from Bolivia and sister to those was *Helobdella cordobensis* from Chile.

Discussion

The identity of the widespread leech matching the description of *Helobdella (triserialis) lineata* Ringuelet 1943 is complicated by its having apparently been described twice and renamed once. Moreover, it is likely that neither of the type localities for the descriptions is truly the naturally endemic point of origin for the species. *Helobdella europaea* was originally described from Germany as *Helobdella striata* by Kutschera (1985). However, the species name *striata* was preoccupied by a very similar South American leech that was originally a subspecies, *Helobdella (triserialis) striata* as detailed by Ringuelet (1943). Thus, Kutschera (1987) renamed the German leech *Helobdella europaea*. However, Neubert and Neseemann (1999) asserted that the German specimens were probably just the South American *Helobdella triserialis* escaped from aquaria where it previously had been reported (Pederzani 1980). Adding some credence to this hypothesis is the fact that the leeches were found in urban areas and that there had not been a new species of *Helobdella* found in Europe since Linnaeus' (1758) description of *Helobdella stagnalis*. Likewise, Australia was thought to be without any representative species of

Helobdella until Govedich and Davies (1998) discovered and described *Helobdella papillornata* from Southeastern Australia. Because they are genetically identical, and both are described as having 5 pairs of testisacs and 5 pairs of gastric caeca, *Helobdella papillornata* Govedich and Davies 1998 should be considered a junior synonym of *Helobdella europaea* Kutschera, 1987 which has already replaced the preoccupied *Helobdella striata* Kutschera, 1985.

Apparently contradicting Neubert and Neemann (1999), *Helobdella europaea* is not genetically identical to the Bolivian representative of *Helobdella triserialis*. Nonetheless, they are sister taxa in our analysis and in turn have *Helobdella cordobensis* as their closest relative. This is not surprising; *Helobdella cordobensis* also was described by Ringuélet (1943) as a variant of *Helobdella triserialis*. Predictably, all of the foregoing group together in a *Helobdella triserialis* species complex (Fig. 2), members of which have only 5 pairs of gastric caeca. The Bolivian leech matches the description of the species *sensu stricto* (Siddall 2001), but in addition to *triserialis*, *cordobensis* and *striata* already mentioned above, Ringuélet (1943) described *Helodeblla triserialis* variants *lineata*, *nigricans*, and *unilineata*. *Helobdella europaea* matches the *Helobdella (triserialis) lineata* variant (Ringuélet 1943). Nonetheless, because the North American *Helobdella lineata* (Verrill 1874) preoccupies that name, the unfortunately named *Helobdella europaea* is valid.

The ultimate origin of the now widespread *Helobdella europaea* would seem to be somewhere in South America and introductions could easily have been coincident with accidental introductions of common aquatic invasive plant species. Introductions of *Pistia stratiotes* and *Salvinia molesta*, for example, are known for each of Germany, Australia, New Zealand and Hawaii. Perhaps the first suggestion of an introduced species of leech was a specimen from Chile that appeared to be identical to the European duck leech, *Theromyzon*

tessulatum Müller 1974. Blanchard (1892) wondered whether it was transported on migratory birds, was introduced via domesticated ducks or via moist soils on aquatic plants. Another non-bloodfeeding leech known to be accidentally introduced on a global scale is the south Asian *Barbronia weberi* (e.g., AquaSense 2003; Govedich et al. 2002, 2003; Rutter & Klemm 2001; Pamplin & Rocha 2000; Mason 1976; Moore 1946), which feeds predominantly on chironomid larvae, unlike the aquatic oligochaete and mollusc dietary preferences for species of *Helobdella*. *Placobdella multilineata*, a North American native sanguivore, has been introduced to Asia by way of its host, *Alligator mississippiensis* (Yang & Davies 1985). *Hirudinaria manillensis* Lesson 1842, endemic to the Philippines and Malaysia, was probably introduced to the Caribbean where it too was described as a new species (Moore 1901). In each case the means of introduction is different and the impact that these leeches may have on their new habitats is yet unstudied.

The use of DNA 'barcoding' for identification of species is not uncomplicated. Here we have successfully used the technique to demonstrate the existence of a globally disseminated invasive leech species. We are confident in the method's utility in this regard. However there is an important distinction to be made between use of mitochondrial loci to corroborate the identity of specimens with described species (as in this study), and the use of such loci for recognition and establishment of new species (e.g., Hebert et al 2004b). The former demonstrates genetic continuity across vast intercontinental distances. The latter relies genetic discontinuity, which may reflect species differences, but may also simply reflect patchy or non-random sampling effort of a single species.

AquaSense (2003) Critter Alert: *Barbronia weberi* (Blanchard 1897), een nieuwe soort

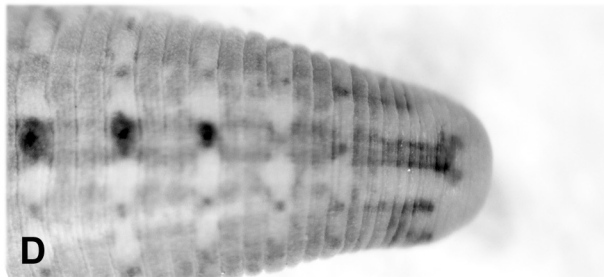
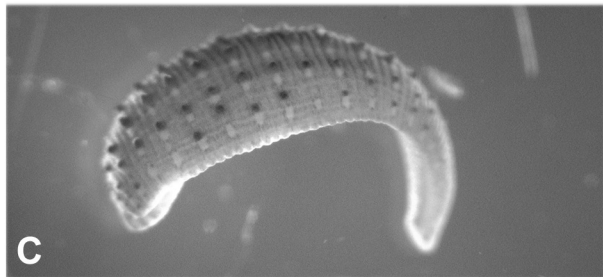
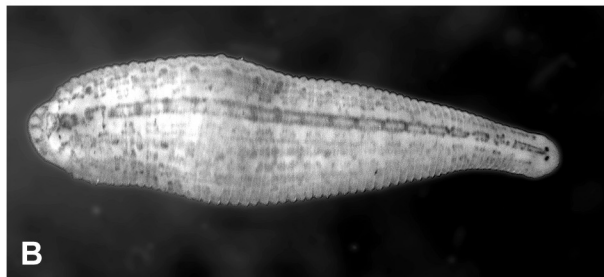
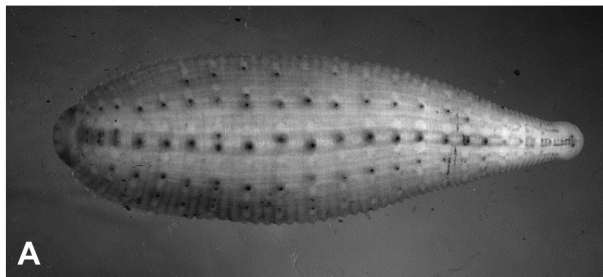
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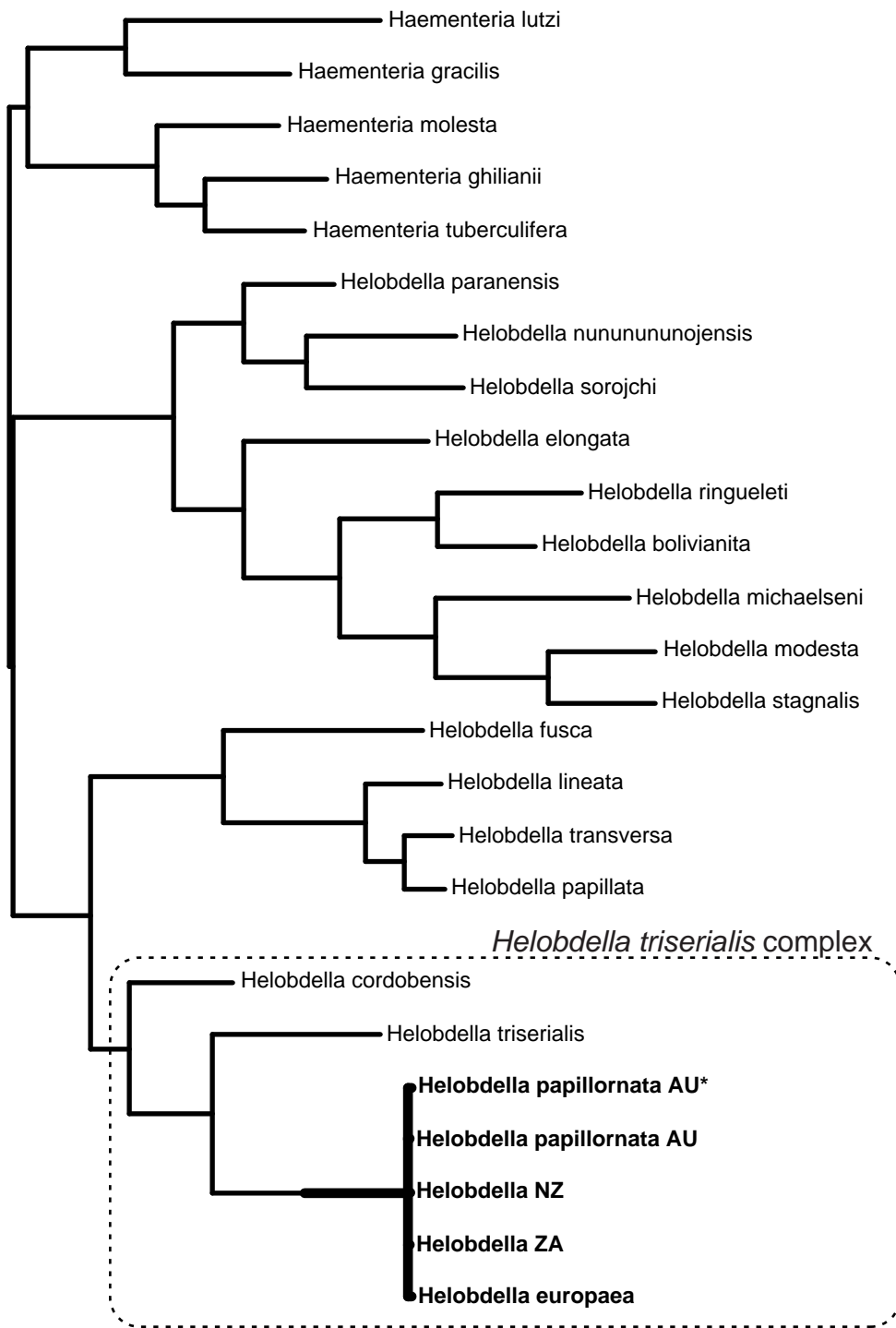
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Fig. 1: External dorsal pigmentation patterns of A) *Helobdella* "papillornata" from 5246 Hawaii, B) *Helobdella triserialis* from Bolivia, C) *Helobdella europaea* from South Africa, and D) *Helobdella europaea* from New Zealand.

Fig. 2. Strict consensus of the most parsimonious trees resulting from a phylogenetic analysis of COI and ND1 combined. There was insufficient genetic distinction among the invasive leeches (bold) to resolve any relationship among them. The rest of the genus *Helobdella* was fully resolved confirming that *Helobdella europaea* belongs to the South American *Helobdella triserialis* species complex





— 50 changes