

The occurrence of an Australian leech species (genus *Helobdella*) in German freshwater habitats as revealed by mitochondrial DNA sequences

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Abstract

The freshwater leech *Helobdella europaea* Kutschera 1987 was discovered twenty years ago in Germany and described as a new species. Here, we show that this leech is genetically identical with the Australian species *Helobdella papillornata* (CO-I-mt-DNA sequence identity of alignment positions: 98%). We conclude that *H. europaea* (syn. *H. papillornata*) represents an introduced annelid that occupies the same ecological niche as the common European leech *H. stagnalis* L.

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1. Introduction

The glossiphoniid leech *Helobdella stagnalis* L., which is characterized by a small chitinous scute on the dorsal side of its neck, is one of the most common freshwater leeches in the world that has been found on every continent except Australia. In various stagnant and running freshwater ecosystems throughout Europe, *H. stagnalis* can be regularly encountered on the underside of rotten leaves and flat stones. Detailed studies have shown that this small leech occurs most abundantly in eutrophic ponds and lakes, where it inhabits the leaves of reed beds in preference to stones (Herter, 1968). Like other glossiphoniid leeches, *H. stagnalis* has an eversible proboscis which is used to suck off the soft portions of small invertebrates such as oligochaetes (i.e., *Tubifex* sp.), insect larvae (i.e., *Chironomus* sp.), small crustaceans (i.e., *Asellus aquaticus*) and occasionally small water snails (i.e., *Physa acuta*) (Kutschera and Wirtz, 2001).

In 1982, a second *Helobdella*-species was discovered in a stream in Southern Germany and described as a new species, *H. striata* (Kutschera, 1985). Because the species name *striata* was preoccupied by a morphologically similar South American leech, *H. triserialis* var. *striata*, the German *Helobdella* sp. was alternatively renamed *Helobdella europaea* (Kutschera, 1987). The leech *H. europaea* exhibits parental care similar to *H. stagnalis* (Kutschera and Wirtz, 1986, 2001). Based on a detailed study of the polymorphic warm water species *H. triserialis*, which occurs throughout South and North America, it was concluded that *H. europaea* is not identical with this American leech (Kutschera, 1987, 1992). Two years ago, a second free-living population of *H. europaea* was discovered in an unnamed pond in Berlin-Tiergarten. In addition, in aquaria in Halle and Berlin (Germany) numerous specimens of this leech were found (Kutschera, unpublished).

To reveal the taxonomic status of the enigmatic *H. europaea*, we observed the feeding behaviour of this leech and compared it with that of *H. stagnalis*. Then, we sequenced part of the cytochrome *c* oxidase I (CO-I) gene from seven leech species (and an earthworm) and

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compared these molecular data with published results (Siddall and Borda, 2003).

2. Materials and methods

2.1. Taxa

The eight taxa (seven leech and an oligochaete species) are listed in Table 1. The species included in this investigation were chosen to represent a broad number of glossiphoniid leeches of the sub-families Haementeriinae, Glossiphoniinae, and Theromyzinae (Sawyer, 1986). One *Helobdella*-species was collected in North America (California), the other leeches were obtained from stagnant ponds or streams in Germany. The earthworms (outgroup) were collected from the underside of stones in a garden. Six to eight adult individuals of average size were placed into ethanol (95%) and stored at -20°C .

2.2. Feeding experiments

Groups of *H. europaea* ($n = 10$) from the original population (Kutschera, 1985) were maintained in the laboratory ($22 \pm 1^{\circ}\text{C}$) in 500 ml-aquaria filled with pond water. Aquatic plants (*Elodea canadensis*, *Myriophyllum demersum*) were collected from the natural habitat of the leeches and added to the aquaria. The feeding behaviours of adult *H. europaea* and *H. stagnalis*-individuals (collected from all local pond) were investigated and documented as described by Kutschera (2003).

2.3. DNA extraction

To avoid contamination with the gut contents of the annelids muscular caudal sucker tissue (in the case of the earthworms, the posterior 3 mm) was excised from two ethanol-fixed specimens. From this material (about 0.1–

0.5 g per sample), DNA was extracted using the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturers' handbook. Isolated DNA was diluted in 50 μl HPLC- H_2O and used for further analyses. DNA extractions (and the experiments described below) were repeated three times with a different set of specimens each.

2.4. Mitochondrial DNA sequence amplification

Fragments (710 bp) of the protein-coding mitochondrial (mt) gene cytochrome *c* oxidase submit I (CO-I) were amplified using the universal DNA primers described by Folmer et al. (1994):

LCO 1490: 5'-GGTCAACAAATCATAAAGATA TTGG-3' and

HCO 2198: 5'-TAAACTTCAGGGTGACCAAAA AATCA-3'

Amplification reactions for CO-I included 10 \times PCR buffer (15 mM MgCl_2 , pH 8.3) and Q-solution, 100 μM for each dNTP, with 1 M *Taq* polymerase and 10 pmol of each primer and 4 μl of the DNA-extract, for a total volume of 20 μl . The amplification was carried out with initial denaturation at 95°C for 10 min, followed by 35 cycles of one denaturation step at 94°C for 40 s, primer annealing at 52°C for 40 s and primer extension at 72°C for 45 s in a Hybaid thermocycler. PCR-products were purified using the QIAEX II Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.5. mt-DNA sequencing

Sequencing was performed using ABI-Prism Dye Kit V3 (Applied Biosystems) in a 10 μl volume containing 2 μl purified PCR-product and 5 pmol of primer. Sequencing reactions underwent 27 cycles of 30 s at 94°C , 30 s at 50°C and 3 min at 60°C in a Techne thermocycler. The dye terminators were removed by sephadex-G45

Table 1

Classification and origin of the annelid species (leeches, earthworm) used for DNA extraction and sequencing in this study

Classification	Species	Origin	CO-I
Hirudinea			
Rhynchobdellida			
Glossiphoniidae			
Haementeriinae	<i>Helobdella stagnalis</i> (Linnaeus, 1758)	Germany, Kassel-Harleshausen	—
	<i>Helobdella triserialis</i> (Blanchard, 1849)	North America, California, Stanford	—
	<i>Helobdella europaea</i> (Kutschera, 1987)	Germany, Freiburg-Vörstetten	AY 576008
	<i>Alboglossiphonia heteroclita</i> (Linnaeus, 1761)	Germany, Kassel-Wehlheiden	—
Glossiphoniinae	<i>Glossiphonia complanata</i> (Linnaeus, 1758)	Germany, Kassel-Wehlheiden	—
	<i>Hemiclepsis marginata</i> (O.F. Müller, 1774)	Germany, Kassel-Wehlheiden	—
Theromyzinae	<i>Theromyzon tessulatum</i> (O.F. Müller, 1774)	Germany, Kassel-Wilhelmshöhe	—
Oligochaeta			
Lumbricoidea			
Lumbricidae	<i>Lumbricus castaneus</i> (Savigny, 1826)	Germany, Kassel-Harleshausen	AY576009

Taxonomy according to Kutschera and Wirtz (2001). CO-I = GenBank accession no. for newly acquired data (*H. europaea*, *L. castaneus*).

column purification (Millipore). Sequencing reactions were electrophoresed for 2 h on an ABI Prism 3100 genetic analyzer (Applied Biosystems) according to the manufacturers instructions.

2.6. Sequence alignment and phylogenetic tree

Mitochondrial DNA-sequences for light and heavy strands were examined and reconciled using BioEdit Version 5.0.9. Sequence Navigator (Hall, 1999). CO-I fragments of equal length (663 bp, excluding primer regions) were aligned by MultiAlign Software (Corpet, 1988) across all of the eight taxa and we did not detect any deletions or insertions. The sequence of *H. europaea* (GenBank Accession No. AY576008) was aligned with CO-I data in Siddall and Borda (2003). To construct phylogenetic trees, we used the computer programs as described by Kumar et al. (2001). The software package (Molecular Evolutionary Genetics Analysis version 2 for exploring and analysing aligned DNA sequences from an evolutionary perspective) was obtained from www.megasoftware.net (Kumar et al., 2001). The method of phylogeny reconstruction was Kimura 2-Parameter UPGMA Bootstrap consensus tree.

3. Results

Adult individuals of the species *H. stagnalis* (obtained from a local pond) and *H. europaea* are depicted in Fig. 1A. Both *Helobdella* species are 12–15 mm long and can be distinguished by a list of characters (Kutschera, 1985). As mentioned before, *H. europaea* and *H. stagnalis* rapidly capture prey organisms such as oligochaeta and suck off the body fluids with the aid of a proboscis

(Kutschera and Wirtz, 2001). In this set of experiments we offered insect larvae (*Chironomus* sp.) to hungry leeches. The first step of feeding behaviour is shown in Fig. 1B for *H. europaea* and the type species *H. stagnalis*. Upon coming into contact with an insect larva the leeches coil the anterior part of the body ventrally, thereby holding the prey organism and sucking body fluids (not shown). In two previous publications on the biology of *H. europaea* the following prey organisms were identified: water snails, oligochaeta (*Tubifex* sp.) and *A. aquaticus* (Kutschera, 1985, 1987). Fig. 1B shows that insect larvae (*Chironomus* sp.) are also accepted as prey organism by both *Helobdella* species investigated here.

For a systematic analysis of the DNA taxonomy of the *Helobdella* species found in freshwater habitats of Germany we selected the type species *H. stagnalis* as a reference point. Since this widely distributed leech inhabits ponds, lakes and streams of both hard and soft waters, we decided to collect a sample from the presumed locus typicus: a stagnant, eutrophic pond (Table 1). Our mt-DNA sequence was 97% identical with the corresponding CO-I data (GenBank reference AF329041, *H. stagnalis* from England, Siddall and Borda, 2003). Our *H. stagnalis* sequence was aligned with the common North American species *H. triserialis*, *H. europaea* (GenBank Accession No. AY 576008) and related glossiphoniid leeches. The earthworm *Lumbricus castaneus* was sequenced (GenBank Accession No. AY 576009) and included in our study for comparison.

A comparative analysis of published CO-I data (Siddall and Borda, 2003; Table 2) revealed that *H. europaea* is genetically very similar to *H. papillornata* from Australia (GenBank Accession No. AF329052; 98% identity of alignment positions). A sequence identity of 92% was

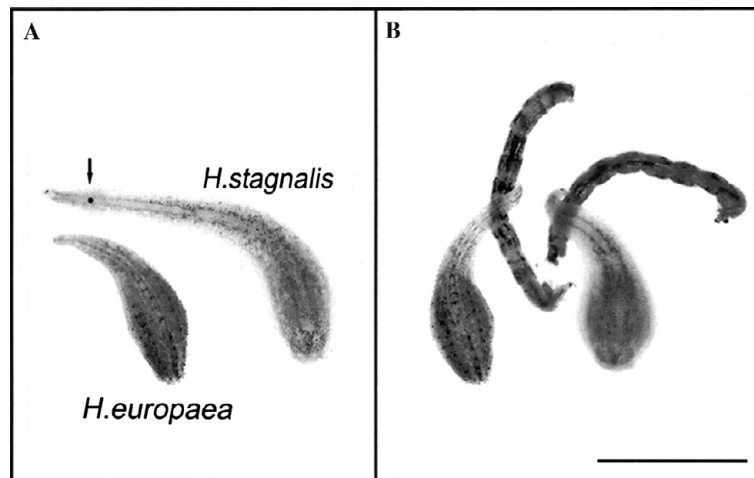


Fig. 1. Dorsal views of adult individuals of two-eyed flat leeches: *Helobdella europaea* (left) and *Helobdella stagnalis* (right). The latter species is characterized by a dorsal chitinous plate (arrow), the remnant of an embryonic attachment gland (A). Capture of a prey organism (*Chironomus* larvae): the leech (*H. europaea*, left; *H. stagnalis*, right) quickly attaches to it with its oral sucker (B), then coils the anterior part of its body ventrally and sucks the soft part of its victim (not shown).

Table 2

Sequence identity between *Helobdella europaea* (GenBank Accession No. AY 576008) and the corresponding region in the mt-genome of five morphologically similar *Helobdella* species from America and Australia

Taxon	Locality	GenBank Accession No. CO-I	Identity (<i>H. europaea</i> = 100%)
<i>H. triserialis</i> black tipped	Round Lake, Michigan, USA	AF329043	85
<i>H. triserialis</i> colourless	Lake Huron, Michigan, USA	AF329042	85
<i>H. triserialis sensu strictu</i>	Laguna Volcan, Bolivia, South America	AF329054	92
<i>H. ringueleti</i>	Madidi, Bolivia, South America	AF329051	83
<i>H. papillornata</i>	Magil Creek, Brisbane, Australia	AF329052	98

This analysis is based on the data of Siddall and Borda (2003).

obtained when *H. europaea* was compared with *Helobdella triserialis sensu strictu* from South America. With the other *Helobdella* species listed in Table 2 (*H. triserialis*, *H. ringueleti*) the identity in our selected CO-I mt-DNA region was considerably lower (83–85%).

The aligned mitochondrial CO-I DNA sequences were used to construct phylogenetic trees from an evolutionary perspective (Kumar et al., 2001). One representative bootstrap consensus tree is depicted in Fig. 2. The tree has been rooted using the sequence of an earthworm as outgroup. Our results agree with the traditional (non-molecular) classification of glossiphoniid leeches (Table 1). All three members of the sub-family Haementeriinae (genus *Helobdella*) grouped with each other, with the exception of the controversial taxon *Alboglossiphonia heteroclita* (Sawyer, 1986). The Glossiphoniinae (*Glossiphonia complanata*, *Hemiclepsis marginata*) formed one clade and the only member of the Theromyzinae (the duck leech *Theromyzon tessulatum*) grouped with the morphologically similar snail leech *G. complanata*. Within the *Helobdella*-clade the following results were obtained: *H. europaea* was not identical with the morphologically similar *H. triserialis* from North America, and *H. stagnalis* is the sister taxon to these closely related leech species.

4. Discussion

Until the 1980s only one *Helobdella* species, the pale-gray *H. stagnalis*, had been found (Herter, 1968; Elliott

and Mann, 1979). The discovery and description of a second *Helobdella* species in 1982 (*H. europaea*, Kutschera, 1987) prompted the question whether this leech represents an introduced annelid or must be classified as a European *nova species*. Because *H. europaea* has similar colour patterns to the widely distributed warm-water species *H. triserialis* it was first suggested that these taxa be indistinguishable (Kutschera, 1987). However, a detailed comparative investigation of the anatomy and feeding behaviour of *H. europaea* versus *H. triserialis* (from North America) led to the conclusion that these species are not identical (Kutschera, 1987, 1992).

The results of this report show that although both *H. stagnalis* and *H. europaea* display a similar feeding behaviour (Fig. 1B) and occupy essentially the same ecological niche these taxa represent two well-separated species. However, the phylogenetic tree (Fig. 2) shows that *H. europaea* is a relative of the North American *H. triserialis*.

The validity of our results is corroborated by the fact that the lower part of our phylogenetic tree (Fig. 2) is largely identical with molecular phylogenies reported by other investigators. For instance, the relationships between the duck-, snail- and fish leech (*T. tessulatum*, *G. complanata*, *H. marginata*, respectively) corroborates the findings in previous work (Apakupakul et al., 1999; Light and Siddall, 1999; Siddall and Burreson, 1998). Moreover, the phylogenetic relationships between the species *T. tessulatum*, *G. complanata* and *A. heteroclita* are identical to those reported by Trontelj et al. (1999), based on combined 18S rDNA and mt12S rDNA sequence data. This confirmation of known relationships by our CO-I sequence analysis of different glossiphoniids provides evidence for the correctness of our conclusions concerning the *Helobdella*-clade.

Helobdella europaea was found only twice in German freshwater ecosystems: 1982 in a stream in Freiburg i. Br. and 2000 in an unnamed pond in Berlin/Tiergarten. Does this taxon represent a European leech species or is it an imported animal? Pederzani (1980) reported that a glossiphoniid leech that resembles the American warm-water species *H. triserialis*, later identified by Kutschera as *H. europaea*, occurred in aquaria in Berlin. This *Helobdella* species feeds on *Chironomus* larvae and

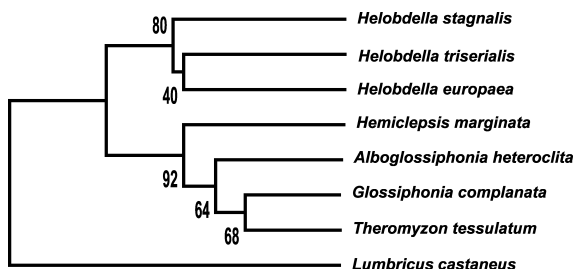


Fig. 2. Phylogenetic relationships of seven glossiphoniid leech species with the earthworm *L. castaneus* as outgroup. Bootstrap consensus tree (with corresponding values) obtained from our CO-I sequence data.

displays a similar parental care pattern as *H. europaea* (Kutschera, 1985; Kutschera and Wirtz, 1986, 2001). In an aquarium shop in Halle (Germany), a large *H. europaea*-population is maintained in one of the containers for aquatic plants (Kutschera, unpublished). In outdoor and freshwater aquaria as well as a pond in Israel, an American *Helobdella* species that exclusively fed upon water snails was found (Mienis, 1986). These reports demonstrate that *Helobdella* species, presumably from South America, are imported to Europe via freshwater plants or snails.

Our CO-I sequence data demonstrate that *H. europaea* is genetically identical with the species *H. papillornata* from Australia (Table 2). With respect to its external morphology, the careful description by Govedich and Davies (1998) and the photograph in Siddall and Borda (2003) showed that there are no external morphological characters available to distinguish *H. europaea* from *H. papillornata*. However, the Australian *Helobdella* species feeds exclusively on gastropod snails, but does not attack other invertebrate prey organism (Govedich and Davies, 1998). In contrast, *H. europaea* feeds on *Chironomus* larvae (Fig. 1B), oligochaeta and *A. aquaticus* (Kutschera, 1985). This much broader range of potential prey organisms is compatible with the suggestion that *H. europaea* may be considered as a close relative (or subspecies) of *H. papillornata*. A divergence in the feeding strategies may have occurred as a result of geographical separation of these taxa.

Based on our findings, we suggest that *H. europaea* may represent an imported *Helobdella* species from Australia that reached Germany as a by-product and escaped from aquaria into the wild. Alternatively, it is conceivable that both *H. europaea* and *H. papillornata* are imported from somewhere else, presumably South America. However, *H. europaea* is not morphologically identical with the American species *H. triserialis* (which feeds exclusively on water snails), as suggested by Neseemann and Neubert (1999) in the most recent monograph on European clitellata. In addition, *H. europaea* is not genetically identical to *H. triserialis sensu strictu* or *H. papillata* (formally a North American *H. triserialis*-like leech) (Siddall and Borda, 2003).

According to Williamson (1996) most biological invasions fail, i.e., only a limited number of taxa spread in their new environment. The question whether or not the leech *H. europaea* (syn.: *H. papillornata*) will successfully compete with *H. stagnalis* is unanswered. However, it is likely that other *H. europaea* populations exist in European freshwater ecosystems that have not yet been detected. Siddall and Borda (2003) suggest that *H. papillornata* is an introduced species from a genetic stock of another continent, possibly South America. Evidence in support of this idea is currently lacking.

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