



Molecular phylogeny of selected predaceous leeches with reference to the evolution of body size and terrestriality

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Abstract

The phylogenetic relationships of erpobdellid leeches collected throughout Europe were investigated using newly obtained mitochondrial cytochrome *c* oxidase subunit I (CO-I) gene sequence data from 10 taxa. Monophyly of the five European *Erpobdella* species (sub-family Erpobdellinae) was supported, but a newly discovered leech, *E. wuttkei* Kutschera, 2004 (the smallest member of its genus, discovered in an aquarium) was only distantly related to this clade. Three members of the semiaquatic Trochetinae were included in this study. The largest European leech species discovered so far, *Trocheta haskonis* Grosser, 2000, was found to be a terrestrial predator that feeds on earthworms. The rare species *T. haskonis* is the sister taxon of *T. bykowskii* Gedroyc, 1913, a well-known amphibious leech. Based on a comparison of body sizes and a phylogenetic tree the evolution of terrestriality in the family Erpobdellidae is discussed.

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Introduction

Contrary to popular misconceptions, not all members of the Euhirudinea (true leeches) are bloodsuckers. More than 200 described species are predators that feed frequently (about every third day) and grow continuously. This is in contrast to haematophagous (blood-sucking) leeches, such as *Hirudo medicinalis*, which feed infrequently and, as a result, are characterized by saltatory growth (Sawyer 1986; Westheide and Rieger 1996).

All leeches of the family Erpobdellidae investigated so far are macrophagous predators that swallow prey organisms whole or in pieces (Kutschera and Wirtz 1986, 2001). Laboratory experiments with the type species *Erpobdella octoculata* L. 1758 have shown that this representative aquatic member of the Erpobdellidae is not only a predator that sucks off insect larvae and small oligochaetes, but also a scavenger (Kutschera 1983, 2003). Dead bodies of a variety of invertebrates (water snails, earthworms, etc.) and vertebrates (fish, amphibians) are attacked by these “worm leeches” and tissue fluids extracted with the aid of a powerful muscular (jawless) pharynx. These results show that the diet of the widely distributed predaceous (macrophagous) erpobdellid leeches is opportunistic and non-specific.

Erpobdellid leeches live in a wide range of habitats. Most *Erpobdella*-species are aquatic but several members of the closely related genus *Trocheta* have amphibious tendencies and spend part of the year under stones in damp places (Elliott and Mann 1979). In Europe, five *Erpobdella*-species have been described: *E. octoculata* Linnaeus, 1758, *E. testacea* Savigny, 1822, *E. nigricollis* Brandes, 1900, *E. monostriata* Lindenfeld, 1890, and *E. vilnensis* Liskiewics, 1925 (Nesemann and Neubert 1999). Not all of these aquatic predators of medium size (length at rest ~30–70 mm) have been characterized by GenBank-DNA-sequences. No published mitochondrial cytochrome *c* oxidase subunit I (CO-I) gene sequences are available for the following species: *E. nigricollis*, *E. monostriata* and *E. vilnensis* (Siddall and Burrenson 1998; Apakupakul et al. 1999; Trontelj et al. 1999; Siddall 2002; Borda and Siddall 2004a, b).

Recently, a new *Erpobdella*-species was described based on specimens found in a freshwater aquarium in Germany (*E. wuttkei*, Kutschera, 2004). Since it is known that leeches can escape from freshwater aquaria via aquatic plants into the wild (Pfeiffer et al. 2004) it is conceivable that free-living *E. wuttkei*-populations exist somewhere in Europe.

Five years ago, Grosser (2000) described a giant semiaquatic erpobdellid leech that was discovered in a little explored freshwater ecosystem in the eastern part of Germany. With an extended length of up to 22 cm, *Trocheta haskonis* represents the largest predaceous leech in Europe. In a preliminary report, some aspects of the biology of this new giant leech were described (Grosser and Kutschera 2004). However, the systematic position of this taxon, as well as that of *E. wuttkei*, is unknown.

The biological observations on *T. haskonis* presented here are combined with a CO-I phylogeny of ten predaceous European leech species, with particular emphasis on the evolution of body size and terrestriality.

Taxa, feeding experiments and phylogenetic methods

The 11 taxa investigated in this study (ten leech species and an earthworm) are listed in Table 1. Species used for phylogenetic analysis were selected to present all European members of the sub-family Erpobdellinae, two semiaquatic species of *Trocheta* (Trochetinae) and two recently described taxa (*E. wuttkei*, *T. haskonis*). Sampling localities, Gen Bank Accession Numbers for seven new CO-I sequences (*T. haskonis*, *T. bykowskii*, *T. pseudodina*, *E. wuttkei*, *E. monostriata*, *E. nigricollis*, *E. vilnensis*) and classification of all species (including the outgroup) are listed in Table 1. Three to five adult individuals of average size were stored in 95% ethanol at -20°C . To document body size of representative taxa, leeches were preserved in 70% ethanol.

Feeding experiments were carried out as follows. Adult *T. haskonis* Grosser, 2000 were maintained in the laboratory ($18\text{--}22^{\circ}\text{C}$) in 50-L-aquaria that contained on one side fresh (moist) earth from the Botanical Garden of the University of Kassel; the other side was filled with pond water (depth $\sim 2\text{--}3\text{ cm}$). The terrestrial/aquatic areas were separated by flat stones so that the leeches were able to move freely. As prey organisms, earthworms (*Lumbricus terrestris*, *L. castaneus*, *Eiseniella foetida*) were added to the aquaria. Feeding behaviour was recorded with a video camera as previously described (Kutschera 2003; Grosser and Kutschera 2004). All feeding experiments were repeated at least five times with different leeches.

Most specimens were collected from the underside of stones in their natural habitat and preserved in 95% alcohol. The preservation time varied from 1 week to 2 years. Tissue samples from the caudal sucker were used in order to prevent contamination from host/prey DNA observed in the gastric and intestinal regions of some adult leeches. From these samples (about 0.2–0.5 g of fresh mass), DNA was extracted using the QIAamp Tissue Kit (Qiagen GmbH, Hilden, Germany) as described by Pfeiffer et al. (2004).

PCR amplifications of mitochondrial (mt) cytochrome *c* oxidase subunit I (CO-I) gene fragments (710 bp) was accomplished with the universal DNA primers described by Folmer et al. (1994). Amplification yielded DNA fragments of approximately 663 bp, excluding the primer sequences. Methods for amplification reactions and purification of PCR-products are described in Pfeiffer et al. (2004).

Amplification products were sequenced using ABI-Prism Dye Kit V3 (Applied Biosystems, Foster City, USA) in 10 μl volume that contained 2 μl purified PCR-product and 5 pmol of primer. Sequencing reactions were performed in a Hybaid thermocycler and electrophoresed as previously described (Pfeiffer et al. 2004).

Mitochondrial CO-I fragments of equal length (663 bp) were aligned by Multi Align Software (Corpet 1988) across all taxa investigated (Table 1). Deletions or insertions were not detected. Phylogenetic analyses were performed using the computer programs as described by Kumar et al. (2001); the software package was obtained from www.megasoftware.net (Kimura 2-Parameter UPGMA Bootstrap consensus tree).

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			
Erpobdellidae			
Erpobdellinae	<i>Erpobdella octoculata</i> (Linnaeus, 1758)	Germany, Kassel-Wehlheiden	—
	<i>Erpobdella testacea</i> (Savigny, 1822)	Germany, Kassel-Harleshausen	—
	<i>Erpobdella vilnesis</i> (Liskiewics, 1925)	Germany, Wörlitzer Park	DQ009663
	<i>Erpobdella nigricollis</i> (Brandes, 1900)	Germany, Kassel-Wehlheiden	DQ009664
	<i>Erpobdella monostriata</i> (Lindenefeld, 1890)	Germany, Magdeburg	DQ009665
	<i>Erpobdella wuttkei</i> (Kutschera, 2004)	Germany, Backnang (aquarium)	DQ009666
Trochetinae	<i>Trocheta bykowskii</i> (Gedroyc, 1913)	Austria, Mauerbach	DQ009667
	<i>Trocheta haskonis</i> (Grosser, 2000)	Germany, Wörlitzer Park	DQ009668
	<i>Trocheta pseudodina</i> (Nesemann, 1990)	Germany, Freiburg-Weingarten	DQ009669
Hirudinidae			
Haemopinae	<i>Haemopsis sanguisuga</i> (Linnaeus, 1758)	Germany, Kassel-Harleshausen	—
Oligochaeta			
Lumbricoidea			
Lumbricidae	<i>Lumbricus castaneus</i> (Savigny, 1826)	Germany, Kassel-Harleshausen	AY576009

All animals were collected in their natural habitat, with the exception of *E. wuttkei*. Taxonomy according to Kutschera and Wirtz (2001); Nesemann and Neubert (1999). CO-I = GenBank accession No. for newly acquired data.

Body size of aquatic and amphibious species

Adult leeches vary considerably in length, even after making allowances for the unusual extensibility of their muscularous bodies. Four representative alcohol-preserved leeches of average size are depicted in Fig. 1. In order to prevent strong contraction of the annelids, 70% ethanol (v/v) was added to the water containing live leeches, gradually increasing the concentration over a period of 20 min until movement ceased. Thereafter, the dead specimens were removed, passed between the fingers to straighten them, and then covered with 70% ethanol.

Compared with the type-species *E. octoculata* L. 1758, the recently discovered relative *E. wuttkei* Kutschera, 2004 is very small. Adult *E. wuttkei* reach a length of 15–19 mm, whereas *E. octoculata* are 45–55 mm long. Both species do not leave the water. Adult specimens of the amphibious species *T. bykowskii* Gedroyc, 1913 and *T. haskonis* Grosser, 2000 are shown on the same photograph (Fig. 1). Both semiaquatic leech species are considerably larger than their aquatic relatives; individual *T. haskonis* reach a length of 10–12 cm when contracted and are significantly longer than *T. bykowskii* (length ~8–9 cm).

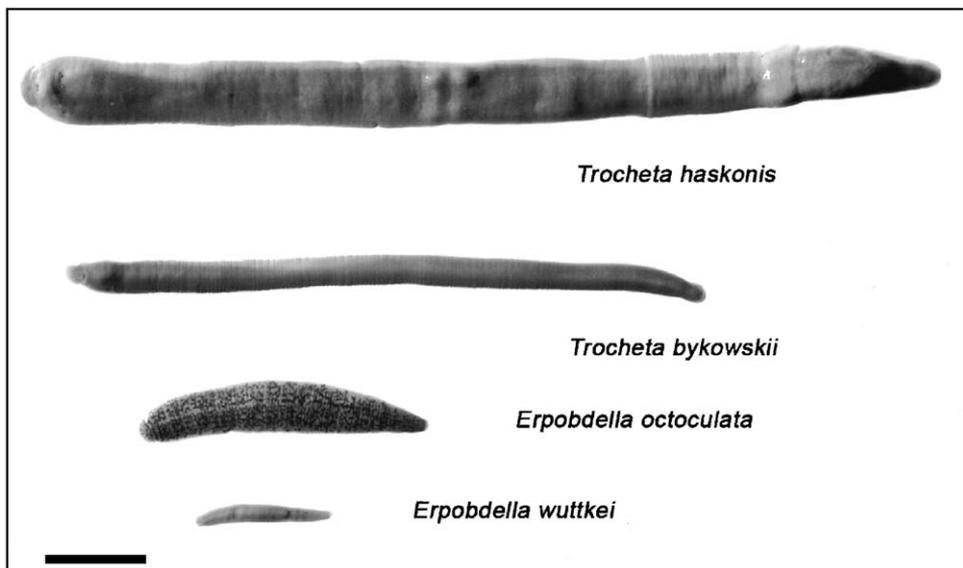


Fig. 1. Dorsal views of adult individuals of erpobdellid leeches, preserved in 70% alcohol in relaxed position (in situ). *Erpobdella wuttkei* Kutschera, 2004 is the smallest member of its genus described so far, whereas *Trocheta haskonis* Grosser, 2000 represents the largest erpobdellid leech found in Europe. Bar = 1 cm.

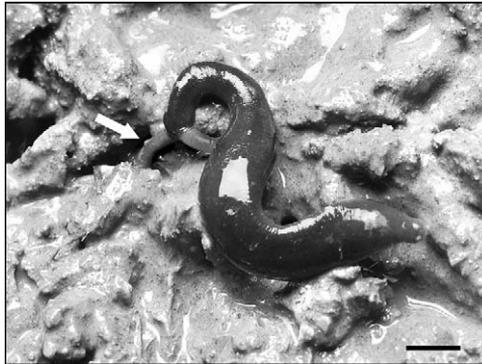


Fig. 2. Adult *Trocheta haskonis* in moist soil, in the process of feeding on an earthworm (*Eiseniella foetida*). The prey organism is swallowed whole (arrow). Bar = 1 cm.

Feeding behaviour of an amphibious predator

In a previous study the feeding behaviour of hungry *T. haskonis* was investigated on leeches kept in pond water (Grosser and Kutschera 2004). Under these artificial conditions, earthworms were attacked and swallowed by day. However, the leeches, when maintained in aqua-terraria under conditions that more closely correspond to their natural habitat, invariably foraged by night in the moist soil. Upon coming into contact with an earthworm (*Lumbricus terrestris*, *L. castaneus*, *Eiseniella foetida*), the predator seizes it with its mouth and tries to swallow its prey whole. A typical feeding episode is shown in Fig. 2. In contrast to the misnamed “horse leech” (*Haemopsis sanguisuga*) of Europe, which likewise feeds on earthworms (see Sawyer 1986), *T. haskonis* does not attach its caudal sucker to its victim. This large leech has a very restricted foraging repertoire (Grosser and Kutschera 2004). Hence, the mode of prey capture is rather primitive in this giant amphibious erpobdellid (Fig. 2) and reminiscent of that of the aquatic type species *E. octocolata* (Kutschera 1983, 2003).

Phylogenetic relationships and habitat preferences

In order to reveal the taxonomic positions of the two newly described species (*E. wuttkei*, *T. haskonis*; Fig. 1), phylogenetic trees, based on CO-I sequence data, were constructed. One representative bootstrap consensus tree with the corresponding sequence of an earthworm as outgroup is shown in Fig. 3.

The molecular phylogeny is in accordance with the traditional classification of erpobdellid leeches (Table 1). All five members of the Erpobdellinae found in European freshwater ecosystems (*E. octocolata*, *E. testacea*, *E. vilnensis*, *E. nigricollis*, *E. monostriata*) grouped together, with the morphologically similar taxa *E. monostriata* and *E. nigricollis* as closest relatives. Our results show that

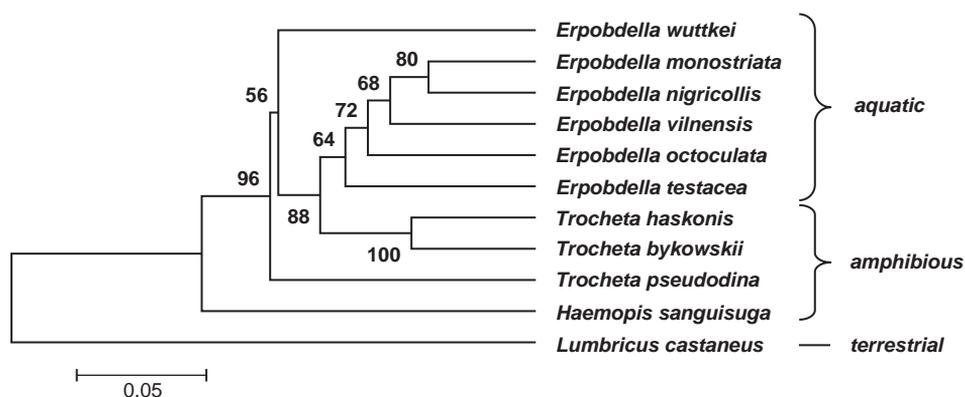


Fig. 3. Phylogenetic relationships of ten erpobdellid leech species with the earthworm *L. castaneus* as outgroup. The diagram shows a Bootstrap consensus tree (with corresponding values) obtained from our CO-I DNA sequence data and indicates the preferred habitat of the annelids (terrestrial, amphibious, aquatic).

E. wuttkei does not group close to the European *Erpobdella*-species, samples of which were all collected in German freshwater ecosystems.

Two leeches of the subfamily Trochetinae, *T. bykowskii* from Austria and *T. haskonis* found in Germany, are sister taxa, whereas the third species, *T. pseudodina* (collected in Southern Germany) is only distantly related to the former group. The leech *H. sanguisuga* was included for comparison as a second outgroup.

The habitat preferences of the annelids investigated here were written into the phylogenetic tree shown in Fig. 3. Terrestrial (earthworm), amphibious (leeches of the genera *Haemopsis*; *Trocheta*) and aquatic taxa (leeches of the genus *Erpobdella*) are grouped together. However, this classification, which displays a tendency towards terrestriality in leeches with larger body sizes (see Fig. 1), should be interpreted with caution.

Discussion

With the descriptions of *E. wuttkei* Kutschera, 2004 and *T. haskonis* Grosser, 2000 the smallest and largest members of the leech family Erpobdellidae found so far have been documented in the literature (Fig. 1). In contrast to *E. wuttkei*, which was found in a freshwater aquarium, the giant leech *T. haskonis* was discovered in the wild. This rare leech spends part of its life on land (moist habitats), but the juveniles which hatch from cocoons that are burrowed in the soil, creep into the water where they feed on small oligochaetes and insect larvae (Grosser and Kutschera 2004). The observations of leeches kept in aqua-terraria described here document that adult *T. haskonis* leave the shallow water zone by night in search for food. They creep

through the moist soil with peristaltic body movements reminiscent of those of earthworms. The predaceous leeches forage in a random manner. If the head of the leech comes into contact with a prey organism (terrestrial oligochaeta of the genera *Lumbricus* or *Eiseniella*), the predator attempts to grasp the worm and swallow it. Small earthworms are sucked in whole (Fig. 2), larger ones are separated and eaten in pieces (unpublished observations). Like its close relative *T. bykowskii*, the giant leech investigated here is an amphibious annelid. Mann (1959) reported observations on the ecology of *T. bykowskii*, a leech that was new to the British fauna at that time. He found small specimens under stones in the water, close to the shore, and larger (adult) *T. bykowskii* under stones which were resting on moist soil a little way above the water's edge. Examination of the gut contents of free-living *T. bykowskii* showed that the leeches had been feeding on earthworms (Mann 1959).

Three years later, Hartley (1962) reported similar observations on the related species *T. subviridis*: mature leeches spend much of their time during the autumn and winter away from the stream. Mann (1959) noted that another species of leech, *H. sanguisuga*, inhabits moist regions along streams and ponds. Like *T. haskonis*, this amphibious annelid deposits its cocoons on land and feeds on earthworms. Competition between *H. sanguisuga* and *T. haskonis* (or *T. bykowskii*) is likely, but there is a lack of direct evidence (Elliott and Mann 1979).

The amphibious tendencies of the third *Trocheta* species analysed here, *T. pseudodina*, are less clear. Kutschera (1986) studied a large population of this leech in Southern Germany and only occasionally observed single adult specimens out of the water. More than 95% of all individuals collected in the wild were attached to the underside of flat stones in a fast running stream (unpublished results). According to Nesemann (1990), *T. pseudodina* is a semiaquatic leech that inhabits the shore of running waters. Our phylogenetic tree (Fig. 3) shows that *T. haskonis* and *T. bykowskii* are sister taxa, whereas *T. pseudodina* appears to be more closely related to the *Erpobdella* clade. This result is in accordance with a study of Trontelj and Sket (2000), who concluded that the sub-family Trochetinae (Table 1) is problematic and should be abandoned. Siddall (2002) recommended recognising the (octoculate) *Trocheta* as species of *Erpobdella*. This proposal is incompatible with the fact that at least three *Trocheta*-species (*T. haskonis*, *T. bykowskii*, *T. subviridis*) are large amphibious annelids that feed on earthworms, whereas all European *Erpobdella* species are aquatic (Fig. 3) and preferentially feed on *Chironomus* larvae and *Tubifex* worms (Kutschera 1983, 2003; Kutschera and Wirtz 1986, 2001; Sawyer 1986).

The results of this systematic analysis confirm that all aquatic European leeches of the genus *Erpobdella* are small annelids (length ca. 1.5–5.0 cm, Fig. 1), a conclusion that is in accordance with data from the literature (Elliott and Mann 1979; Nesemann and Neubert 1999). All amphibious species (*T. bykowskii*, *T. subviridis*, *T. haskonis*, *H. sanguisuga*) are considerably larger (length ca. 7–12 cm at rest). This observation may shed light on the evolution of body size in predaceous leeches. In birds and mammals, families and orders contain more small-bodied than large-bodied species (see Bokma 2004 and references cited therein). This rule also applies to the leech taxa studied here. The evolutionary forces that have selected for larger

body size in leeches obviously are related to the adaptation to life on land (moist soil), associated with the ability to hunt for and feed on earthworms. However, as [Blanckenhorn \(2000\)](#) has pointed out, the major selective pressures that affect the phylogenetic development of body size in multicellular organisms are still a matter of debate, notably in annelids and other invertebrates ([Ashton 2002](#); [Kutschera and Niklas 2004](#)).

Conclusions

According to [Borda and Siddall \(2004a, b\)](#) the ancestral leech was a relative of extant erpobdellids (or piscicolids) that was adapted to a freshwater environment. This aquatic “proto-leech” deposited hardened protective cocoons onto a suitable solid substrate (smooth stones, etc.), a behaviour that is apparent in the aquatic erpobdellids and is exhibited in the “primitive leech” *Acanthobdella peledina* ([Kutschera and Wirtz 1986, 2001](#); [Sawyer 1986](#)). The giant semiaquatic leech *T. haskonis* is the only known member of the Erpobdelliformes that deposits its cocoons on land (damp soil, see [Grosser and Kutschera 2004](#)). Hence, clues to the evolutionary origin of terrestriality in predaceous leeches may lie with the rare species *T. haskonis*, although the relictual taxon *Americobdella valdiviana* from South America may be of equal importance ([Borda and Siddall 2004b](#)). Like *T. haskonis*, this amphibious leech is very large (up to 20 cm) and feeds on earthworms. The exact taxonomic status and reproductive biology of *A. valdiviana* are largely unknown, i.e., more work is required to further elucidate the phylogenetic development of terrestriality in the Euhirudinea.

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