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Description of a new leech species from North America, *Helobdella austinensis* n. sp. (Hirudinea: Glossiphoniidae), with observations on its feeding behaviour

U. Kutschera*, [1], H. Langguth[1], D.-H. Kuo[2, 3], D. A. Weisblat[2] and M. Shankland[4]

[1] Institute of Biology, University of Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany
[2] Department of Molecular and Cell Biology, University of California, Berkeley, California 94720, USA
[3] Department of Life Science, National Taiwan University, Taipei, Taiwan
[4] Department of Cell and Developmental Biology, University of Illinois, Urbana, Illinois 61801, USA

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Abstract

Freshwater leeches of the genus *Helobdella* Blanchard 1896 are small, proboscis-bearing glossiphoniids of the family Rhynchobdellidae that have been found worldwide. Over the past three decades, annelid developmental biologists have used several *Helobdella* species as model organisms. Here we describe a variable taxon, that has been studied extensively, and was formerly labelled as *Helobdella* sp. "robusta" (Austin), as a new species, *Helobdella austinensis* n. sp. Diagnostic features of this polymorphic species, which has a body length of 13 to 17 mm and a yellow-brown colour, include a characteristic pattern of dark longitudinal stripes with black, conical papillae on the dorsal side of the body, irregularly arranged white pigment spots, and the lack of a nuchal scute. The type locality is Shoal Creek, Austin, Texas, USA. Based on the sequence of part of the mitochondrial gene cytochrome *c* oxidase subunit I, *H. austinensis* n. sp. is a defined taxon that differs genetically from other, morphologically similar *Helobdella* species by 11 to 17 %. Under laboratory conditions, hungry leeches suck body fluids from living freshwater snails, dead (frozen-thawed) *Chironomus* larvae, and crushed (wounded) crustaceans. Aquatic oligochaetes (*Tubifex* worms) and living insect larvae are ignored. This specific prey selection is a characteristic feature of *H. austinensis*, a taxon that differs, based on morphological features, from its relatives of the South American *triserialis* species complex.

Key Words

Leeches
Systematics
DNA barcoding
feeding behaviour
Helobdella

Introduction

Although blood-sucking medicinal leeches of the genus *Hirudo* Linnaeus, 1758 are the best-known members of the class Hirudinea, the inconspicuous, dorsoventrally flattened, proboscis-bearing glossiphoniids of the genus *Helobdella* Blanchard, 1896 represent the most widely distributed group of freshwater hirudineans (family Rhynchobdellidae). The type-species of this group is the grey, ca. 10 mm long "two-eyed flat leech" *Helobdella stagnalis* Linnaeus, 1758. This cosmopolitan annelid occurs in freshwater ecosystems on every continent except Australia (Castle 1900; Sawyer 1972, 1986). Leeches in this genus feed by sucking body fluids from aquatic invertebrates (insect larvae, oligochaetes etc.) and display an unusually high degree of parental care (Kutschera & Wirtz 2001; Elliott & Kutschera 2011).

Sawyer (1986) sub-divided the genus *Helobdella* into two morphologically distinct groups of species: A "stagnalis"-group characterized by a chitinous scute on the dorsal side of the head region (*H. stagnalis* L. 1758, etc.), and a "triserialis"-group, which lacks the scute but has longitudinal stripes on the dorsal surface. This second group contains at least 30 described species, with the "type species" being *Helobdella triserialis* Blanchard, 1849 from South America (Chile). Molecular phylogenies suggest that these two subdivisions

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* Corresponding author, e-mail: kut@uni-kassel.de

are monophyletic (Siddall & Borda 2003; Siddall et al. 2005; Siddall & Budinoff 2005; Lai et al. 2009; Oceguera-Figueroa et al. 2010).

Freshwater leeches from the *Helobdella triserialis* species complex have been used for developmental studies since the 1970s, and have served as model organisms for the animal super-phylum Lophotrochozoa once that taxon was recognized. Based on mitochondrial DNA sequence studies, Bely & Weisblat (2006) showed that morphologically similar leeches that were previously assembled under the name "*H. triserialis* Blanchard, 1849" represent multiple taxa. In particular, these authors pointed out that the taxon *Helobdella robusta* (Shankland et al. 1992), as reported in the literature, was composed of at least two separate species.

In this report we describe the taxon *Helobdella* sp. from Austin, Texas (USA), previously regarded as a geographical isolate of *H. robusta* (Seaver & Shankland 2000), as a nova species. In addition, we analyze the feeding behaviour and host range of this model organism, that has been used in recent developmental studies (Quigley et al. 2010; Gline et al. 2011; Kuo & Weisblat 2011), and compare it with related taxa.

Materials and methods

In 1997 the senior author (M. Shankland) collected roughly thirty leeches from Shoal Creek in Austin, Texas (USA) over the course of several months. These specimens were used to establish a laboratory breeding colony at the University of Texas at Austin, TX (Seaver & Shankland 2000); descendants were used to establish a second colony at UC Berkeley (CA) in 2001 (Weisblat & Kuo 2009). These leeches were originally assigned to the taxon *H. robusta* (Shankland et al. 1992) on the basis of morphological criteria (Seaver & Shankland 2000). But in 2006, Bely & Weisblat performed a phylogenetic analysis of a ca. 700 bp-DNA-sequence of the mitochondrial gene cytochrome *c* oxidase subunit I (CO-I), and concluded that this strain, which they renamed *H.* sp. "*robusta*" (Austin, TX) is not conspecific with *H. robusta* (Sacramento, CA) or any other previously reported taxon, and must be regarded as a distinct species.

All leeches investigated here are the descendants of the UC Berkeley lab populations, which were cultivated at 20 to 22 °C in glass bowls (diameter: 20 cm, depth: 8 cm), filled 2/3 with artificial pond water (Instant Ocean Sea salt, stock solution: 128 g per L of distilled water; dilution: 1/100). The leeches were fed every third day with water snails (*Physa* sp., *Planorbarius* sp.) or frozen-thawed insect larvae (*Chironomus* sp.).

In September 2011 and March 2012, twenty adult leeches each were collected at random from these lab populations, which consisted of up to 150 individuals per jar. The living leeches were imported to Germany, for cultivation and investigation in the Institute of Biology, University of Kassel. Thirty adult specimens were photographed to document the variability of the morphology of this undescribed species.

For anatomical studies, fixed specimens were sectioned at 10 μm on a microtome and stained with haematoxylin/eosin using standard techniques (Kutschera 1988, 1989). To study the feeding behaviour and prey selection preference, single leeches were first maintained in isolation (without food) for 7 days and thereafter offered the following potential host organisms: living water snails (*Physa* sp., *Planorbis* sp., collected in Matadero Creek, Palo Alto, CA, USA), living or frozen-thawed *Chironomus* larvae, living *Tubifex* worms, purchased from a local pet shop in Kassel (Germany), crustaceans (*Asellus aquaticus*, *Gammarus pulex*), and pieces of earthworms (*Lumbricus castaneus*). The last three host organisms were collected in local ponds in Kassel, Germany. All prey selection and feeding experiments were repeated at least three times with different leeches and potential hosts, and documented via series of photographs as described by Kutschera (2003). Specimens of *Helobdella robusta* (Shankland et al. 1992), *H. papillata* (syn. *H. triserialis* USA, Siddall & Borda 2003; Klemm et al. 2012), and *H. europaea* (Kutschera 1987) were collected in Sacramento (American River, California, USA), Palo Alto (Matadero Creek, California, USA), and in the ponds of the Tiergarten, Berlin (Germany), respectively. The leeches were cultivated in aquaria and representative individuals photographed.

DNA extractions, sequencing of fragments of the mitochondrial gene CO-I, and phylogenetic analyses, using GenBank data, were performed as described by Pfeiffer et al. (2004).

Results

Description of *Helobdella austinensis* n. sp.

Figures 1–5

Type-material. One leech of average size and morphology was selected from 30 photographed, adult living specimens, fixed in 10% formalin, and stored in 70% ethanol. This holotype, plus 10 paratypes, were deposited in the Department of Invertebrate Zoology and Geology, California Academy of Sciences, 55 Music Concourse Drive, Golden Gate Park, San Francisco, CA 94118, USA. Holotype: Ethanol-fixed specimen, CASZ No. 190366; Paratypes: Ten ethanol-fixed specimens, CASZ No. 190367.

Etymology. The specific name, *austinensis*, is for the type locality, Shoal Creek in Austin, Texas, where the first specimens were collected in 1997 (Seaver & Shankland 2000). Seven years later, additional specimens were collected by D.-H. Kuo in a fish pond at Brackenridge Field Laboratory, Austin, Texas.

Morphology. Size of the holotype: Body length at rest 16.2 mm, maximal width 5.1 mm; paratypes 13 to 17 mm long, with a maximum width of 4.0 to 5.5 mm (Figs 1a, b). Form and annulation: Body lanceolate, broadest in posterior half of the animal. The segments I through IV form the head region, which is in some individuals broadened. Dorsum convex, with conspicious, irregularly arranged black conical papillae; venter flat to slightly concave, without papillae. Anterior sucker oval, with a sub-terminal mouth pore. Posterior (caudal) sucker circular and concave, diameter about half of the width of posterior segments; no mid-dorsal scute in the head region of the body. One pair of triangular eyes at segments III and IV (Figs 2a, 3a).

Annulation along the axis of the body displays a constant pattern, with the segments I and II uni-, and segments III/IV biannulate (head region). Midbody segments V through XXIV triannulate, with each annulus sub-divided (a 1, a 2, a 3); XXV and XXVI biannulate, XXVII uniannulate.

Colour and pattern: In living specimens, the dorsal side of the body appears yellow-brown, due to chromatophores that are arranged in 16 to 20 faint longitudinal lines. The ventral side is grey-brown, as a result of

Figure 1. Dorsal and ventral views of the living holotype of *Helobdella austinensis* n. sp., photographed under enhanced artificial light; **a.** On the dorsal side, 8 to 10 pairs of longitudinal lines are evident, of which the paramedial pair is most prominent; a somewhat irregular and variable distribution of black conical papillae and white pigments spots is also apparent; **b.** On the ventral side, some elements of the genital system are visible. **as** – anterior sucker, **at** – atrium, **ps** – posterior sucker, **ts** – testisac.

chromatophores distributed throughout the surface (Figs 1, 2). Dorsally, one pair of solid, black paramedial lines from segment IV to XXVI, with three irregular longitudinal rows of black-tipped papillae. In addition, irregularly arranged white pigment spots are distributed throughout the dorsal side of the body (Figs 3a, b). When present in any given midbody segment, papillae and white pigment spots occur in the same annulus. On the venter of the body, papillae and white pigment spots are absent.

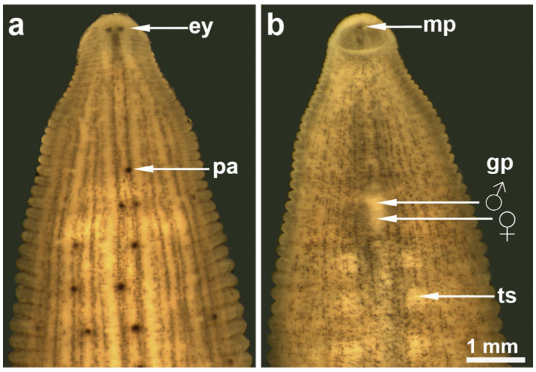

Figure 2. Alcohol-preserved holotype of *Helobdella austinensis* n. sp.; **a.** in dorsal and **b.** ventral views. Note that in this treatment, the white pigment spots are faded and the intensity of the brown pigment is enhanced relative to the live specimen. In the anterior part of the body, the eyes in the head region, the mouth pore within the sucker, and the male/female gonopores are visible. **ey** – eyes, **gp** – gonopores, **mp** – mouth pore, **pa** – papilla, **ts** – testisac.

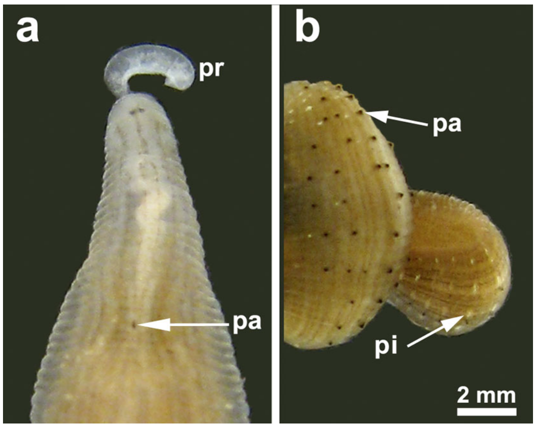

Figure 3. Living, adult paratypes of *Helobdella austinensis* n. sp., head region **a.** with the everted proboscis; **b.** with conical, black papillae, and white pigment spots. **pa** – papillae, **pr** – proboscis, **pi** – pigment spots.

Variability: The holotype (Figs 1, 2) represents an average individual of the two lab-populations investigated. Phenotypic variants (paratypes) are shown in Figure 4. It is apparent that the numbers of black-tipped papillae and white pigment spots are variable parameters. Hence, *H. austinensis* n. sp. is a polymorphic species of freshwater leech.

Anatomy. The internal arrangement of the organs, reconstructed on the basis of series of sections through 6 different paratypes, is shown in Figure 5. The alimentary tract consists of the proboscis (see Fig. 3a), the gastric caeca, and the intestinal caeca. The proboscis is enclosed in a membranous sheath; the base of this feeding organ is at segment XII. Diffuse salivary tissues on both sides of the region between the crop and the base of the proboscis. Six posteriorly curved crop caeca

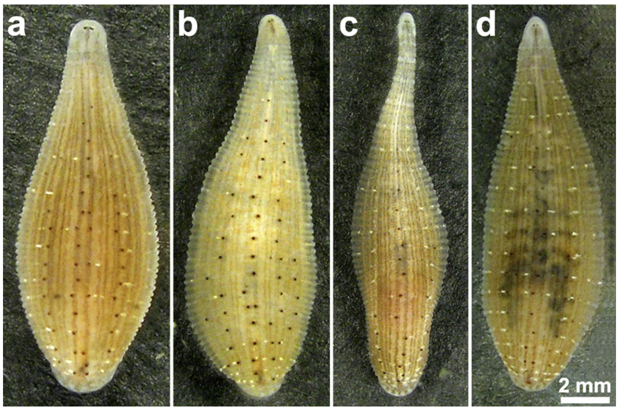

Figure 4. Phenotypic variability in a population of *Helobdella austinensis* n. sp.; **a–d.** Photographs of four adult living paratypes in dorsal views. Note that the distributions of black-tipped papillae and white pigment spots are variable, but they always occur on the central annulus of the segment in which they are found. The leech shown in (**d.**) has visible crop caeca as a result of feeding on a water snail; this is not a phenotypic variation.

(see Fig. 4d), intestine from XIX/XX through XXIV, with four lobes; anus at XXVI/XXVII.

The reproductive system consists of the atrium with the male gonopore on XII a1/a2 and the female gonopore at XII a2/a3, i.e., the gonopores are separated by one annulus (Fig. 2b). Six pairs of testisacs visible ventrally in adult, sexually mature specimens (see Figs 1b, 2b), and two tube-shaped ovisacs that extend to segment XVIII (Fig. 5).

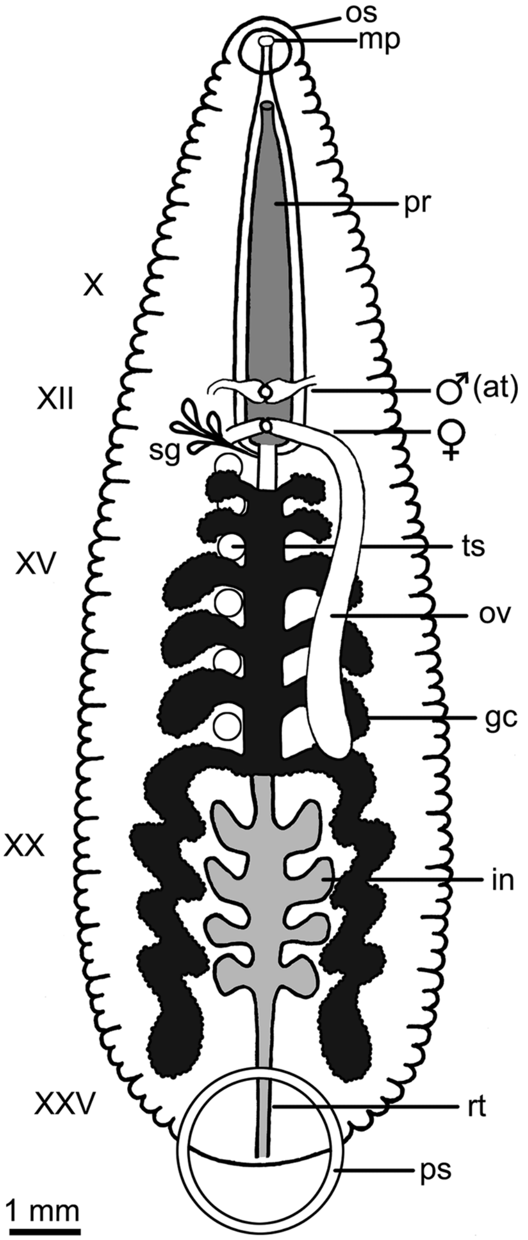

Figure 5. Diagram of the basic anatomy of *Helobdella austinensis* n. sp., illustrating the position and shape of the proboscis (**pr**), the salivary glands (**sg**) (shown on the left side), the atrium (**at**), the male/female gonopores, the 6 pairs of testisacs (**ts**) (shown on the left side), the single pair of ovasacs (**ov**) (shown on the right side), gastric (= crop) caeca (**gc**), intestinal caeca (**in**), and rectum (**rt**). X to XXV – body segments No. 10 to 25. The posterior (caudal) sucker (**ps**) is disk-shaped; the oral sucker (**os**) contains the mouth pore (**mp**).

Phylogenetic analysis

After DNA extraction from paratypes, a fragment of the mitochondrial gene CO-I (669 bp) was sequenced. This newly aquired CO-I DNA-sequence for *H. austinensis* n. sp. was deposited in the GenBank (Accession-No. KC812736), and found to be nearly identical with GenBank Acc.-Nos. DQ995310 and DQ995307, respectively (Table 1). According to Bely & Weisblat (2006), the first Acc.-No. (Lab identifier TXAU-5) is *H. robusta* (Austin), collected 2004 by D.-H. Kuo in Shoal Creek, Austin, TX (type locality), and the second Acc.-No. (TXAU-2) corresponds to *H. robusta* (Austin), collected 2004 by D.-H. Kuo in a fish pond at Brackenridge Field Laboratory (Austin, TX). These data show that the free-living leech populations that existed in 2004 are genetically indistinguishable from the descendants of the original lab-cultures established in 1997, maintained over many years, which were investigated here (2011/12) and used for the species description (Figs 1–5). Hence, the phylogenetic trees of Bely & Weisblat (2006) for *H. robusta* (Austin), based on CO-I sequences, are valid for paratypes of *H. austinensis* n. sp. described in this report, i.e., this taxon represents a nova species.

The CO-I sequence identities of *H. austinensis* n. sp. (GenBank Acc.-No. KC812736) (= 100 %), compared to morphologically related species (Fig. 8), are 89,2 % for *H. robusta* Shankland et al., 1992 (GenBank Acc. No. DQ995299), 82,7 % for *H. papillata* (syn. *H. triserialis* USA, Siddall & Borda, 2003; Klemm et al., 2012) (DQ995303), and 82.9 % for *H. europaea* Kutschera, 1987 (AY576008) (Table 1).

Prey selection experiments

Adult individuals of *H. austinensis* n. sp. were kept without food for 7 days and thereafter exposed to different potential prey organisms. Water snails (*Physa* sp., *Planorbis* sp., with red hemoglobin) were rapidly attacked by hungry leeches. The rhynchobdellids attached to the shell, intruded their anterior ends between the shell and the foot of the prey and sucked the body fluids with their inserted proboscis. After 1 to 3 h, the empty shell and ca. 5 % of the soft parts of the snail (white gelatinous material) are left over (Figs 6a–c). When the leeches feed on *Planorbis* sp., the red, hemoglobin-containing body fluids of the host organism leach into the water and the crop caeca of the parasites are finally filled with red fluid (Fig. 6d).

Hungry leeches also feed on dead (frozen-thawed) insect larvae, such as "blood worms" (*Chironomus* sp., with red hemoglobin) (Figs 7a, b). However, living *Chironomus*-individuals, which display rapid, vigorous body movements, are not attacked. Crustaceans (*Asellus aquaticus*, *Gammarus pulex*) that were wounded by crushing the organism between the tips of a forceps are also accepted as host organism. The hirudineans sucked body fluids from the soft parts of these immobilized

Table 1. Mitochondrial DNA-sequence identities between *Helobdella austinensis* n. sp. and the corresponding region in the mt-genome of five morphologically similar *Helobdella* species from North America (USA) and Europe (EU) (see Fig. 8), inclusive of the GenBank Accession Numbers for the mt-gene cytochrome *c* oxidase subunit I (CO-I).

Taxon	Locality	GenBank Acc.-No. CO-I	Identity (%)
H. austinensis n.sp.	UC Berkeley, Lab population, CA, USA	KC812736	100
H. robusta (Austin) TXAU-5	Shoal Creek, Austin, TX, USA	DQ995310	99.4
H. robusta (Austin) TXAU-2	Fish pond, Brackenridge, Austin, TX, USA	DQ995307	99.4
H. robusta Shankland et al. 1992	American River, Sacramento, CA, USA	DQ995299	89.2
H. papillata Siddall & Borda 2003	Golden Gate Park, San Francisco, CA, USA	DQ995303	82.7
H. europaea Kutschera 1987	Schobbach, Freiburg, Germany, EU	AY576008	82.9

aquatic arthropods until the empty carapace is left over (Fig. 7c). Usually, several leeches sucked on one host organism (communal feeding, see Figs 6b, d). The leeches ignore *Tubifex* worms (living and dead specimens) as potential prey organisms (Fig. 7d). However, hungry individuals suck body fluids from the wounded regions of cut pieces of earthworms (*Lumbricus castaneus*) (not shown).

Comparison to other *Helobdella* species

Despite the variable morphology among individual *H. austinensis* n. sp. (Fig. 4), it was possible to define criteria by which to distinguish this taxon from other species of the genus *Helobdella*. The species *H. robusta* appears to be very similar (Fig. 8a), with a typical distribution of longitudinal brown stripes and irregularly distributed papillae and white pigment spots. However, in *H. robusta*, the white pigment spots may occur on every annulus (instead of just the central annulus as in *H. austinensis* n. sp.) and are concentrated in the regions between the stripes. Distinguishing *H. austinensis* n. sp. from either *H. papillata* (Fig. 8b) or *H. europaea* (Fig. 8c) is more straightforward. In contrast to the variable mix of white pigment spots and black-tipped papillae in *H. austinensis* n. sp., the dorsal surface of *H. papillata* features an orthologal grid of white pigment spots, with no papillae, while *H. europaea* displays a grid of papillae with few or no pigment spots.

Discussion

Maximum parsimony and -likelihood trees of *Helobdella* species, based on CO-I sequences, have shown that the non-scutiferous glossiphoniid leech "*H. robusta* TXAU-5" (= *H. austinensis* n. sp.) is a separate taxon (Bely & Weisblat 2006). Here, we have described *H. austinensis* n. sp. and shown that this new species can easily be distinguished from related species of *He-*

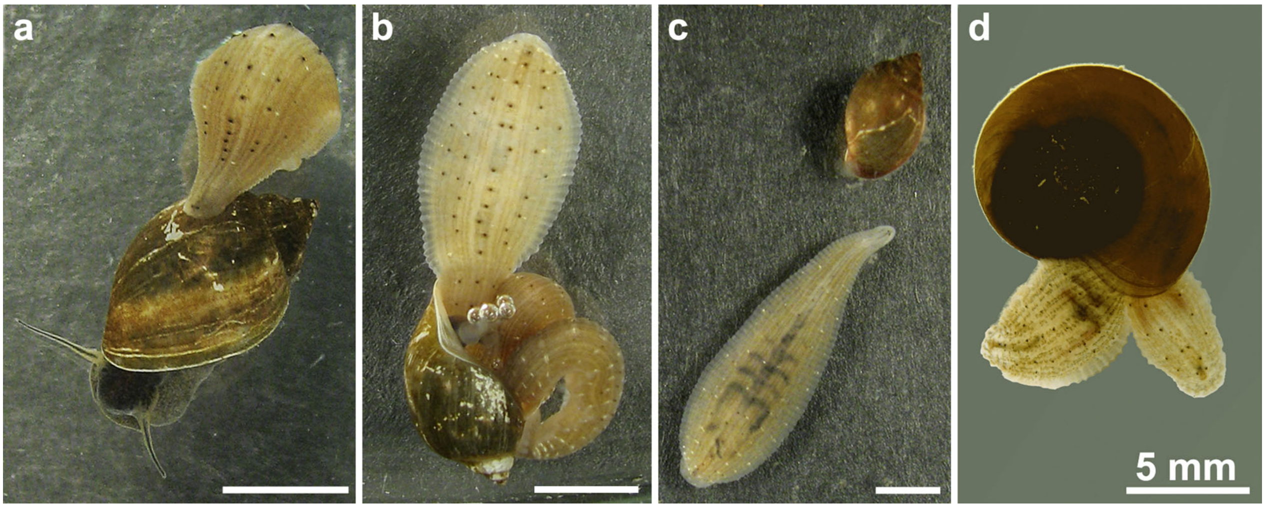

Figure 6. Foraging and feeding behaviour of adult *Helobdella austinensis* n. sp. Prey organisms: water snails; **a–c.** *Physa* sp.; **d.** *Planorbis* sp.; **a.** Upon coming into contact with a *Physa* sp., the leech rapidly attaches to the shell with its oral sucker, inserts the anterior portion of its body between the shell and foot, and then thrusts its proboscis through the soft parts of its host and sucks body fluids; **b.** The sucking leech is frequently joined by conspecifics; **c.** When the crop caeca are filled with hemolymph, the leech rests in the vicinity of the empty shell; **d.** Two leeches in the process of feeding on a *Planorbis* sp.

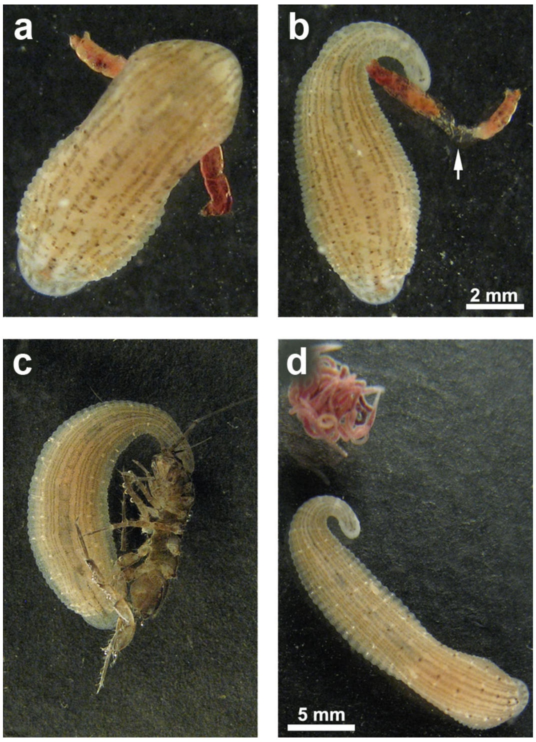

Figure 7. Feeding behaviour of an adult *Helobdella austinensis* n. sp.; **a, b.** The hungry leech was offered a dead (frozen-thawed) insect larva (*Chironomus* sp.). The annelid curls its anterior body region ventrally about the larva, inserts its proboscis, and feeds on the larva (a) until part of the host organism is left over as an empty skin (arrow) (b). **c, d.** Prey selection in adult *Helobdella austinensis* n. sp. Wounded crustaceans (*Asellus aquaticus*) (c), or living oligochaetes (*Tubifex* sp.) (d) were offered. Crushed, immobile (living) crustaceans are attacked and drained (c), whereas *Tubifex* worms are ignored as prey organism (d).

lobdella on morphological grounds. In addition, our CO-I-sequence alignment studies revealed that *H. austinensis* n. sp. differs from *H. robusta*, *H. papillata*, and *H. europaea* by ca. 11 and 17 %, respectively. These data suggest that the nova species described here diverged from its sister taxa at least 10 million years ago (Wirchansky & Shain 2010).

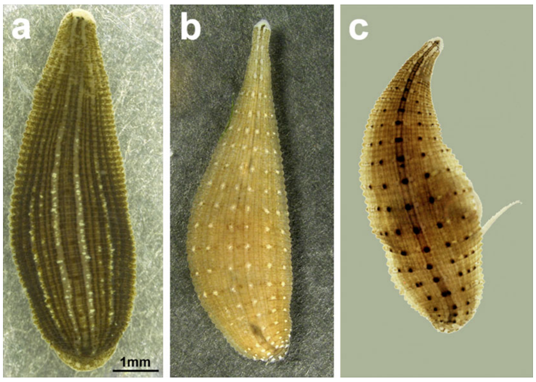

Figure 8. Living adult specimens of **a.** *Helobdella robusta*, collected from its type locality in Sacramento, California, **b.** *H. papillata* (syn. *H. triserialis* USA) from Palo Alto, California, and **c.** *H. europaea* from Berlin, Germany. The leeches were photographed in dorsal views.

The *H. triserialis*-species series, to which *H. austinensis* n. sp. belongs, has its centre of distribution in South America (the type locality of *H. triserialis* Blanchard, 1849 is in Chile) (Sawyer 1972, 1986; Siddall & Borda 2003; Borda & Siddall 2004a, b; Lai et al. 2009; Oceguera-Figueroa et al. 2010). Therefore, it is conceivable that the species discovered in Austin, Texas, is an imported leech from South America. Circumstantial evidence suggests that the taxa *H. europaea* and *H. californica*, which are distributed in aquatic freshwater ecosystems of Europe, Australia, New Zealand, South Africa, Hawaii, Thailand, and California, respectively, are likewise imported species from South America (Kutschera 2004, 2011). On the other hand, phylogenetic analyses document that *H. austinensis* (i.e., "*H. robusta* TX AU1") resides in a monophyletic clade of North American *Helobdella* species (Oceguera-Figueroa et al. 2010). These data strongly suggest that the leeches from Texas originated in North America, but more work is required to further corroborate this conclusion.

In accordance with observations on the North American glossiphoniids *H. papillata* (syn. *H. triserialis* USA, Siddall & Borda 2003; Klemm et al. 2012) and *H. californica* (Kutschera 1988, 1989, 2011), living freshwater snails appear to be the preferred host organism of *H. austinensis* n. sp. Our prey selection studies revealed that *H. austinensis* n. sp. sucks the body fluids (hemolymph) from prey organisms such as water snails, insect larvae, crustaceans and cut pieces of earthworms. In our experiments, the leeches did not attach to living (agile) *Chironomus* larvae, and to the body of crustaceans that move around, but dead or wounded (immobilized) host organisms were rapidly attacked. The feeding behavior of *H. austinensis* n. sp. is similar to that of *H. papillata* from North America (Kutschera 1987, 1992) in that it ignores *Tubifex* worms, and is unable to capture living *Chironomus* larvae.

This is in contrast to the well-established feeding preference of the type species *H. stagnalis*. Under laboratory conditions, *H. stagnalis* feeds preferentially on living aquatic oligochaetes, such as *Tubifex* worms, and insect larvae (*Chironomus* sp.); other potential host organisms (water snails, crustaceans) are only accepted when these "worm-shaped" prey organisms are not available (Sawyer 1972, 1986; Kutschera & Wirtz 2001). Under the same laboratory conditions, the invasive leech *H. europaea* and the North American *H. californica* fed on living *Chironomus* larvae and agile crustaceans, as did the type species *H. stagnalis* (Kutschera 1989, 2004, 2011).

According to Sawyer (1972, 1986), *H. stagnalis* and related glossiphoniids are liquidosomatophagous predators that feed exclusively upon small benthic invertebrates, primarily *Tubifex* worms, chironomid larvae, and molluscs. Liquidosomatophagous feeding is a specialized type of predation of some leeches, whereby a proboscis sucks up body fluids and soft parts of an invertebrate prey organism. According to this definition, leeches of the genus *Helobdella* would be predators, not parasites. However, this classical interpretation is incompatible with more recent studies, which have shown that *H. stagnalis* and *H. europaea* are not only liquidosomatophagous predators, but also ectoparasites. The leeches attach to relatively large organisms (amphibians etc.), and suck body fluids, but do not kill their host (Kutschera 2004; Kutschera et al. 2010; Tiberti & Gentillie 2010).

Our observation that *H. austinensi*s n. sp. sucks body fluids from the soft parts of wounded (living) crustaceans, such as *Asellus aquaticus*, documents that this species is not only a predator, but can also obtain food as an ectoparasite on the body of a relatively large host organism. However, more work is required to answer the question whether or not leeches of the genus *Helobdella* behave, in the wild, as haemolymph-sucking ectoparasitic annelids, analogous to members of the genus *Hirudo* (Sket & Trontelj 2008; Elliott & Kutschera 2011).

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