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Research Paper

The Golden Gate Leech *Helobdella californica* (Hirudinea: Glossiphoniidae): Occurrence and DNA-Based Taxonomy of a Species Restricted to San Francisco

key words: leeches, eutrophication, endemic species, DNA barcoding, parental care

Abstract

Leeches of the genus *Helobdella* are small brooding annelids that inhabit lakes and streams on every continent, notably in South America. The type species, *H. stagnalis* L. 1758, occurs in Europe and North America. Here I provide novel observations on the occurrence, morphology, and parental care patterns of the related *H. californica*, a taxon described in 1988, based on specimens collected in Stow Lake, Golden Gate Park, San Francisco. In 2007, the original *H. californica* population no longer existed, possibly due to eutrophication of this popular "duck pond". However, in other, cleaner lakes of the Golden Gate Park dense, stable populations of *H. californica* were discovered. Between 2007 and 2010 adult individuals were investigated in the laboratory with respect to their pigment patterns and feeding behaviour. The leeches suck the red, haemoglobin-rich haemolymph from insect larvae (*Chironomus* sp.) and other small aquatic invertebrates and feed their young attached to their ventral surface. A typical feeding episode is described and documented. In addition, a neighbour-joining analysis was performed based on a newly acquired DNA sequence of part of the mitochondrial gene cytochrome *c* oxidase subunit I (CO-I) for *H. californica*, combined with other, related leech species. These molecular data corroborate that the "Golden Gate leech" is a separate species and not a colour variant of *H. stagnalis*. Since, over the past 25 years, *H. californica* has only been found in the freshwater ecosystems of the Golden Gate Park, it is concluded that this rare leech is a species restricted to San Francisco.

1. Introduction

Twenty-five years ago, the author discovered a population of small freshwater leeches in Stow Lake, Golden Gate Park, San Francisco, California. The dark-grey coloured, ca. 10 to 18 mm long annelids of the genus *Helobdella* (BLANCHARD, 1896), family Glossiphoniidae, were attached to the underside of flat stones and fallen leaves, where these organisms subsisted on the body fluids sucked from small aquatic invertebrates, such as crustaceans, water snails, and oligochaetes. A close examination of numerous individuals revealed that these proboscis-bearing, dorso-ventrally flattened leeches were not identical with the North American variety of the European *Helobdella stagnalis* (LINNAEUS, 1758), the type species of the genus. This "two-eyed flat leech", which occurs in freshwater ecosystems on every continent, with the exception of Australia (SAWYER, 1972, 1986; NESEMANN and NEUBERT, 1999), was not found in any pond of the Golden Gate Park. However, some *H. stagnalis*

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individuals were discovered in an un-named creek on the campus of Stanford University in Palo Alto, California (KUTSCHERA, 1987, 1988).

On the basis of a detailed morphological and anatomical comparison between the "Stow Lake-leeches" and specimens of *H. stagnalis*, the *Helobdella* sp. population from the Golden Gate Park was described as a new species, *Helobdella californica* (KUTSCHERA, 1988). In a subsequent publication, the reproductive behaviour and parental care patterns of *H. californica* were documented (KUTSCHERA, 1989). As in other members of the genus *Helobdella*, breeding *H. californica* feed their young attached to the belly of the parent with captured prey organisms (KUTSCHERA and WIRTZ, 1986; 2001).

In this report the occurrence and morphology of this leech is described in more detail and new observations on its breeding behaviour are provided. In addition, the taxonomic status of *H. californica* is documented based on DNA sequence data and a neighbour joining analysis.

2. Materials and Methods

The observations and experiments reported here were carried out on two different occasions: February 1986 to September 1987, and February 2007 to September 2010. The following freshwater ecosystems in the Golden Gate Park were inspected: Stow Lake, Gunnera Creek and an un-named pond in the Botanical Gardens at Strybing Arboretum; Elk Glen Lake, Lloyd Lake, Mallard Lake, Matson Lake, and Middle Lake. The distance between these artificial freshwater ecosystems is ca. 0.5 to 2.0 km. North- and South Lake as well as Spreckels Lake were not investigated.

During February 1986 to September 1987, *ca.* 150 adult individuals of *H. californica* were collected in Stow Lake and analyzed in the laboratory. The type locality of this *nova species* is Stow Lake; Holotype and Paratypes were deposited in the collection of the California Academy of Sciences (CAS Nos. 064209 and 064210, respectively). Between February 2007 and September 2010, *ca.* 120 individuals of this species were collected in the Gunnera Creek and Mallard Lake, respectively. For comparison, 20 adult specimens of *H. stagnalis*, collected in an un-named creek on the campus of Stanford University, were investigated (species determination according to CASTLE, 1900 and SAWYER, 1972). The leeches were kept in small aquaria and fed on *Chironomus* larvae, water snails (*Physa* sp.), *Tubifex* worms, or crustaceans (*Gammarus* sp.) as described by KUTSCHERA (2003). For microscopical examination and documentation, adult leeches were fixed in their original position in 70% ethanol. Ten minutes later, the specimens were inspected in a photomicroscope (Stemi DV4, Carl Zeiss, Jena, Germany), equipped with a digital camera (Canon Power Shot G2, Canon Inc., Tokyo, Japan).

For DNA sequencing, specimens of *H. californica* were fixed in 95–100% ethanol and stored at –20 °C. The caudal sucker was removed and used for DNA extraction, which was carried out as previously described (KUTSCHERA *et al.*, 2007). The universal primers of FOLMER *et al.* (1994) were used for polymerase chain reaction (PCR) amplification of part of the protein-coding mitochondrial gene cytochrome *c* oxidase subunit I (CO-I) (length of the fragments *ca.* 660 base pairs, bp), according to the methods described by PFEIFFER *et al.* (2004). DNA-sequences of the amplification products were obtained as previously described (PFEIFFER *et al*., 2004) and alignments carried out using Mulit Align Software (CORPET, 1988). A neighbour – joining analysis was performed, using the newly acquired DNA sequence and published CO-I data, as described by KUMAR *et al*. (2001) and KUTSCHERA *et al.* (2007). The CO-I-DNA-sequence for *H. californica* was obtained four times, based on individuals collected in 2007, 2008, and 2009, respectively (identity > 99%). On February 13, 2011, the sequence was deposited in the GenBank data base (Accession No.: HQ686307).

3. Results

3.1. The Occurrence of Helobdella californica

On February 2, 1986 the undersides of stones in the flat-water regions along the shore of Stow Lake, Golden Gate Park, San Francisco were inspected and large, dense populations of small, grey leeches of the genus *Helobdella* discovered that superficially resembled the type-

species *H. stagnalis* L. 1758. In other ponds of the Golden Gate Park these enigmatic "flat leeches" were not found. Based on the morphology and anatomy of *ca.* 150 individuals that were collected on 14 different occasions between February 1986 and September 1987 from its type locality this un-identifiable species was described as *H. californica* (KUTSCHERA, 1988). As a reference work, the monograph of SAWYER (1972) was used. During a last visit on September 15, 1987, the *H. californica*-population in Stow Lake was dense and stable. Within 10 minutes, *ca.* 30 individuals were collected, adults and juveniles. Most of them had dark-coloured crop caeca, indicating that they had fed on crustaceans or other aquatic prey organisms.

On February 18, 2007 the author visited Golden Gate Park again and searched for individuals of the *Helobdella* species described in 1988. In its *locus typicus* (Stow Lake), not a single individual was found. A visit to the Botanical Gardens at Strybing Arboretum was more successful. In the Gunnera Creek of the Primitive Plants Garden, and in Mallard Lake, 12 individuals were collected that were morphologically indistinguishable from the Paratypes in the private leech collection of the author (Figs. 1 and 2). In September/October 2007, February/March and August/September of the years 2008, 2009, and 2010 the Golden Gate Park was visited again and the ponds listed above were investigated.

As in 2007, no *H. californica*-individuals were found in the Stow Lake, but dense, stable populations persisted in the other aquatic ecosystems of the Park. Hence, the large *H. californica* population investigated between February 1986 and September 1987, on which the species description is based, had disappeared over the past two decades.

3.2. Morphology and Parental Care

In the original species description and the associated paper on the reproductive behaviour of *H. californica* only a brief account of the morphology of this leech was provided (KUTSCHERA, 1988; 1989). Based on specimens collected in 2008 and 2009, more detailed

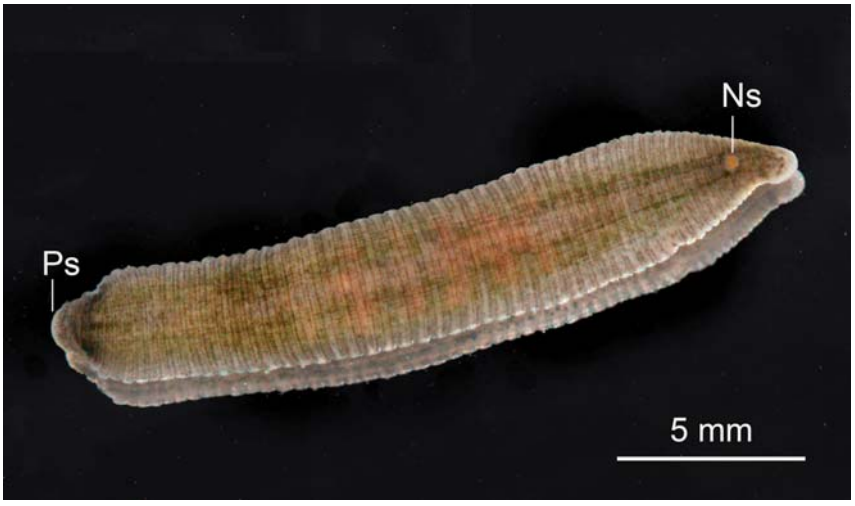

Figure 1. The "Golden Gate leech" *Helobdella californica* KUTSCHERA 1988, adult living specimen, dorsal view. The leech was collected in February 2007 in the Gunnera creek of the Primitive Plants Garden, Golden Gate Park, San Francisco, California, and the photograph taken in the laboratory. Ns = nuchal scute in the neck region of the annelid, Ps = posterior sucker.

Figure 2. Photograph of adult living specimens of *Helobdella californica*, collected in Mallard Lake, Golden Gate Park, San Francisco, and *Helobdella stagnalis*, collected in the Matadero Creek, Palo Alto, California (September 2009). Note the nuchal scute (black dot) in the neck region of the largely un-pigmented *H. stagnalis*.

morphological data are summarized here. The species-specific pigmentation on the dorsal and ventral sides of the body, respectively, are shown in Figure 3A, B. Specimens, fixed in ethanol, were photographed *ca.* 10 min after preservation. It should be noted that the posterior sucker of *H. californica* is characterized by a ring of dark spots, a feature that has not been detected in any individual of the morphologically similar *H. stagnalis* (Fig. 2). Adult *H. californica* (length: 10–15 mm) that die in the aquarium usually eject their proboscis during the last hours of life. Two 10 mm long specimens, fixed in ethanol immediately after their death, are shown in Figure 4A, B. These photographs document that the proboscis of *H. californica* has a size of *ca.* 0.7 × 0.2 mm, corresponding to ca. 5% of the body length of the leech.

During earlier investigations of *H. californica*-individuals kept in aquaria, three aquatic invertebrates collected in Stow Lake were offered as host organisms: crustaceans (*Gammarus* sp.), water snails (*Physa gyrina*), and oligochaetes (*Tubifex* sp.) (KUTSCHERA, 1989). In the habitats of *H. californica* investigated systematically between 2007 and 2010 (Gunnera Creek/Bot. Garden and Mallard Lake), red insect larvae (*Chironomus* sp.) were regularly found on the underside of stones. The presence of free haemoglobin in their body fluid (haemolymph) gives them their red colour. During the 1980s, these conspicuous larvae were not found in Stow Lake and therefore were not offered to hungry leeches. Therefore, a series of corresponding feeding experiments was carried out on adult, brooding *H. californica*. One representative feeding episode, using a *Chironomus* larvae as prey organism, is shown in Figure 5A, B. In this experiment, the adult and the attached young booth took up haemolymph simultaneously (duration of the feeding process *ca.* 20 min.). Five days later, both the adult and the attached juveniles were hungry again and took up haemolymph from another added *Chironomus* larvae as described above.

Due to this provision of food, the juvenile leeches grow within a breeding period of 3 to 4 weeks from about 1 to *ca.* 5 mm in length. When they have reached a body size of *ca.* 25% of that of their parent, the young leave the adult leech, and feed, often together with other juveniles, on small invertebrates (oligochaetes, insect larvae *etc.*).

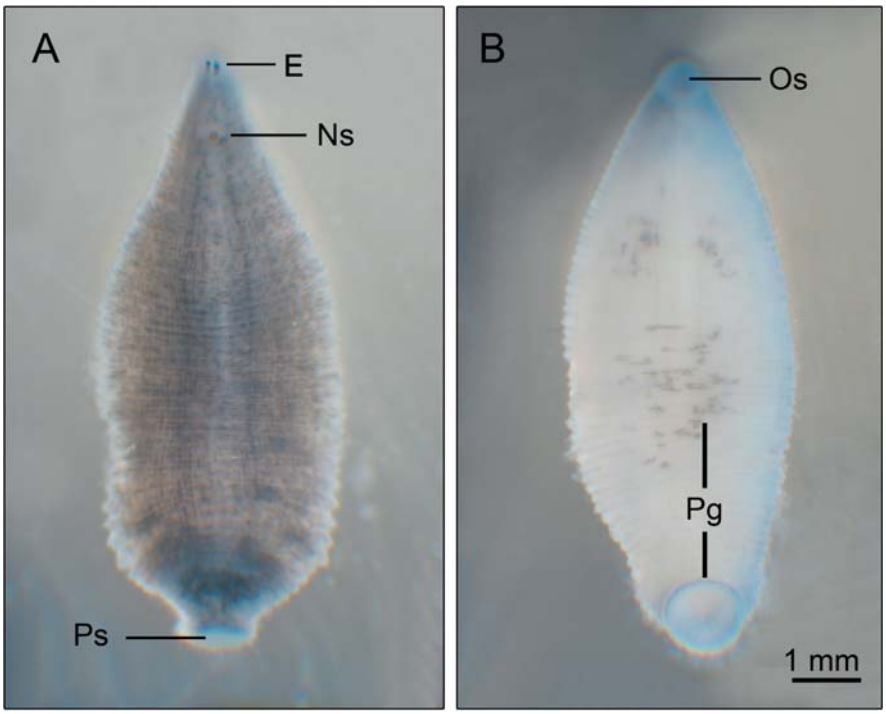

Figure 3. Morphology of *Helobdella californica*, alcohol-preserved specimen, in dorsal (A) and ventral (B) view, respectively. E = eyes, Ns = nuchal scute, Os = oral sucker, Pg = pigment spots, Ps = posterior sucker.

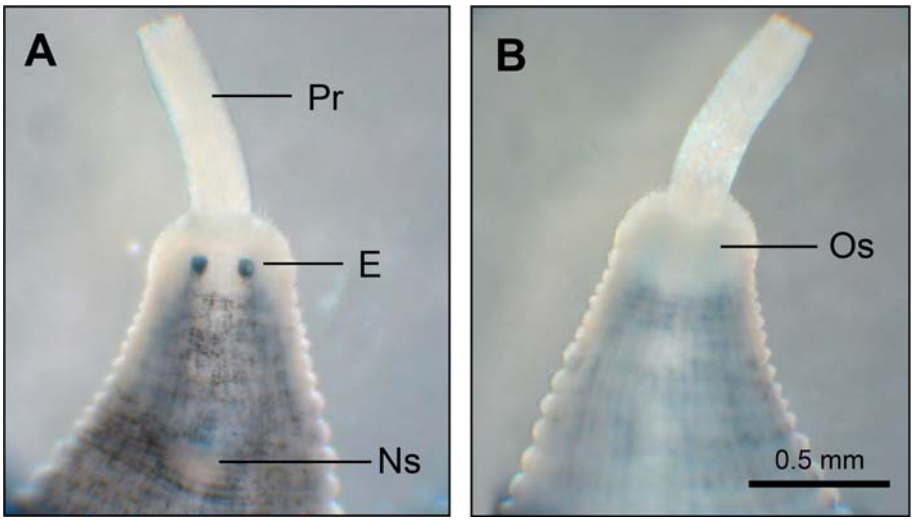

Figure 4. Morphology of the head region of *Helobdella californica*, alcohol-preserved specimens in dorsal (A) and ventral (B) view, respectively. E = eyes, Ns = nuchal scute, Os = oral sucker, Pr = proboscis.

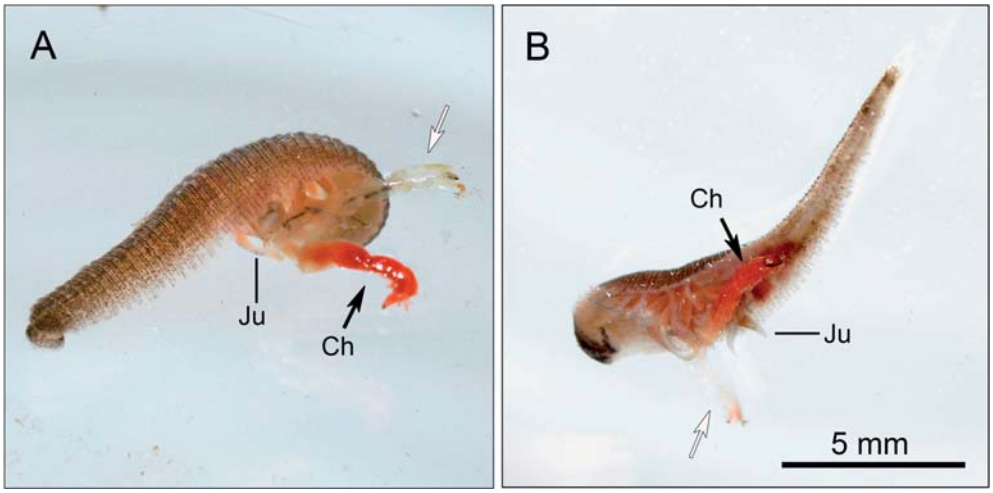

Figure 5. Photographs of an adult brooding *Helobdella californica* with attached young in the process of feeding on a captured *Chironomus* larva (A). After *ca.* 15 minutes, both the adult and the juveniles have dark, blood-filled crop caeca, due to the uptake of haemolymph from their host organism (B). Ch = *Chironomus* larva. Dark arrow: living (*i.e.*, haemolymph-filled) part, white arrow: empty skin of the larva. Ju = juvenile leeches attached to the ventral side of the adult.

3.3. The taxonomic Status of Helobdella californica *Based on DNA-Sequence Data*

In the original description of *H. californica* (KUTSCHERA, 1988) it was suggested that this leech from the Golden Gate Park may be a close relative of the cosmopolitan type species *H. stagnalis* (LINNAEUS, 1758). Both leech species have a dorsal scute, which is interpreted as the remnant of an embryonic attachment organ (CASTLE, 1900; SAWYER, 1972, 1986), but differ with respect to their pigmentation (Fig. 2) and anatomical features. To further analyze the taxonomic status of *H. californica*, the so-called 'DNA barcoding'-technique was employed (BELY and WEISBLAT, 2006). After DNA extraction from parts of the isolated posterior (caudal) sucker of alcohol-preserved specimens, fragments of the mitochondrial gene cytochrome *c* oxidase subunit I (CO-I) were amplified and sequenced.

A representative neighbour – joining analysis based on our newly acquired CO-I sequence for *H. californica* (GenBank Acc.-No. HQ686307), combined with published molecular data (PFEIFFER *et al.*, 2004) for three *Helobdella* species (*H. stagnalis*, *H. triserialis*, and *H. europaea*), two related taxa from the family Glossiphoniidae (the snail- and duck leech, *Glossiphonia complanata* and *Theromyzon tessulatum*, respectively), and two Erpobdellidae as outgroup, is shown in Figure 6. As in previous studies (KUTSCHERA, 2004; PFEIFFER *et al.*, 2004), *H. triserialis*, collected in the ponds of the Golden Gate Park, and the widely distributed species *H. europaea* were sister taxa, and the snail- and duck leech grouped together. In this molecular analysis, *H. californica* represents a separate taxon that is sister to the three other *Helobdella* species and is only distantly related to other members of the Glossiphoniidae. Hence, the neighbour joining analysis (Fig. 6) confirms the earlier morphology-based suggestion that the "Golden Gate leech" is a separate species and not a colour variant of the widely distributed *H. stagnalis* (KUTSCHERA, 1988; 1989).

This conclusion is further supported by a comparative quantitative analysis of published CO-I sequence data (Table 1). With respect to *H. californica* as a reference (= 100%), DNA sequence identities (*i.e.*, base pair matches) between 82 and 85% were calculated for the

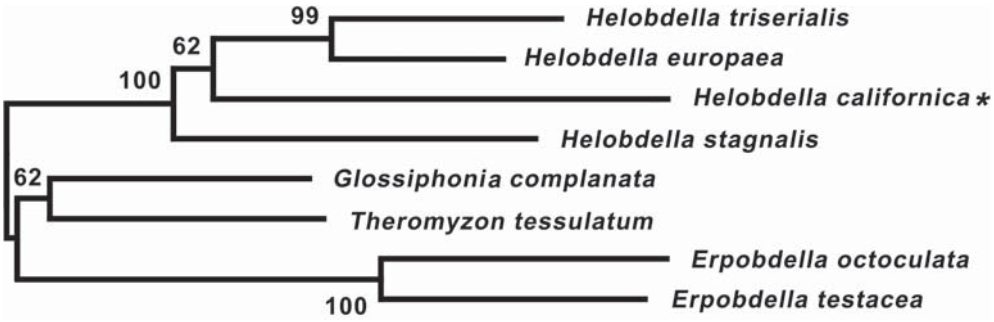

Figure 6. Phylogenetic relationships of six selected leech species of the family Glossiphoniidae (genera *Helobdella*, *Glossiphonia*, and *Theromyzon*), with the Erpobdellidae as outgroup (*Erpobdella octoculata* and *E. testacea*). The diagram shows a neighbour-joining analysis obtained from the newly acquired CO-I DNA sequence for *H. californica* (asterisk) and published GenBank data. The branch support values were added.

Table 1. Mitochondrial DNA-sequence identities between *Helobdella californica* (KUTSCHERA, 1988) and the corresponding region in the mt-genome of five morphologically similar *Helobdella* species from North America (USA), Europe (EU), South America (SA) and Australia (AU), 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. californica	Golden Gate Park, California, USA	HQ686307	100
H. europaea	Schobbach Freiburg, Germany, EU	AY576008	85
H. europaea	Aura Vale Lake, Australia, AU	AF329052	85
H. papillornata	Magill Creek, Brisbane, AU	AY856047	84
H. triserialis	Golden Gate Park, California, USA	DQ995303	84
H. triserialis	Laguna Volcán, Bolivia, SA	AF329054	84
H. stagnalis	Lake Cotsworlds, England, EU	AF329041	83
H. stagnalis	Wild Goose Lake, Michigan, USA	AF329038	82
H. robusta	Amer. River, Sacramento, California, USA	DQ995301	82

five other selected *Helobdella* species, inclusive of *H. stagnalis*. It should be noted that "*H. papillornata*" from Australia listed in Table 1 is identical with *H. europaea* collected in Germany, Australia, South Africa, Hawaii and North America (SIDDALL and BUDINOFF, 2005; BELY and WEISBLAT, 2006).

4. Discussion

In this article it is documented that the "Golden Gate leech" *H. californica*, described in 1988 and characterized by only one type locality (Stow Lake) was, two decades later, no longer present in its original freshwater habitat (*locus typicus*). The reason for this decline and final extinction of the leech population is obvious. In 1986/87, the water of Stow Lake

was still relatively clean, so that numerous crustaceans (*Gammarus* sp.) could live on the underside of stones and rotten leaves in the flatwater areas of the lake. Twenty years later, considerable eutrophication was apparent in this popular "duck pond", which had caused the growth of numerous planktonic algae with the result that the water is now dark-green in colour. Both the leeches and one of their prey organisms (*Gammarus* sp.) were no longer found. It is suggested that extensive feeding of the ducks and other water birds by visitors was the reason for the intense eutrophication of Stow Lake, but quantitative data to support this hypothesis are lacking. However, between 2007 and 2010 dense populations of *H. californica* were discovered in other, clean aquatic ecosystems of the Golden Gate Park (notably Mallard Lake), indicating that this species is not threatened. In addition, several specimens of the duck leech (*Theromyzon tessulatum*) were observed on the underside of flat stones. This parasite was found before in these aquatic habitats of the Park (MASON *et al.*, 2005). It should be mentioned that in all of these ponds and lakes, where *H. californica* occurs, no *H. stagnalis* were detected. However, on the campus of Stanford University and in a small creek in Palo Alto (distance from the Golden Gate Park in San Francisco ca. 45 km), numerous specimens of *H. stagnalis*, plus some individuals of *H. triserialis*, were collected (KUTSCHERA, 1992; SHANKLAND *et al.*, 1992). In none of these aquatic habitats outside of San Francisco a single individual of *H. californica* was discovered. To my knowledge, over the past 25 years, no *H. californica*-individuals have been found in any freshwater ecosystem outside the Golden Gate Park, indicating that this taxon may be a rare, endemic leech species.

This conclusion is supported by the following observations. SIDDALL (2001a, b) conducted faunistic surveys in the high Andean portion of Bolivia (South America) and participated in an excursion for aquatic invertebrates in the lowland regions. During these searches, he discovered several new species of *Helobdella*. Moreover, SIDDALL (2001a, b) noted that, with the exception of the widely distributed *H. nununununojensis*, each *Helobdella* species he found in the Apolobamba range occurred only in a single valley. Hence, according to this author, the Andes appear to exhibit a high rate of endemism for the species of the genus *Helobdella*. It is conceivable that *H. californica* likewise represents a geographically isolated leech species that is restricted to the freshwater ecosystems of the Golden Gate Park in San Francisco. However, since this unique Park was established only *ca.* 140 years ago, and many aquatic plants from foreign countries may have been imported at that time, it is conceivable that *H. californica* is an introduced leech species. It is well known that the genus *Helobdella* has its centre of distribution in South America (SIDDALL and BORDA, 2003; KUTSCHERA, 2004; BORDA and SIDDALL, 2004a, b; SIDDALL *et al.*, 2005; SIDDALL and BUDINOFF, 2005). Hence, it is possible that the "Golden Gate Leech" originated somewhere in South America millions of years ago. During the 1850s, a sub-population may have been introduced into these North American freshwater ecosystems, notably Stow Lake.

The CO-I sequence divergence data compiled in Table 1 shed light on the evolutionary time interval for the speciation event that separated *H. californica* from its sister taxa. According to WIRCHANSKY and SHAIN (2010), a *ca*. 10% CO-I DNA sequence divergence value between two leech species suggests that allopatric speciation (*i.e*., geographic isolation of the populations) occurred about 10 to 20 million years ago. Based on the CO-I sequence divergences of 15 to 18% between *H. californica* and its relatives reported here, one can estimate that the earliest populations of the "Golden Gate leech" separated from a common ancestor at least 15 million years ago. Although we currently do not know whether *H. californica* represents a species that originated in North America or is an imported taxon from South America, the results of the present report unequivocally document that *H. californica* is a "true" leech species and not a colour variant of the European "two-eyed flat leech" *H. stagnalis* (LINNAEUS, 1758) that also occurs in North America (CASTLE, 1900; SAWYER 1972, 1986).

In this report it is shown that insect larvae of the genus *Chironomus*, the most heavily utilized prey organisms of the type species *H. stagnalis* and related taxa (SAWYER, 1971, 1972,

1986; NESEMANN and NEUBERT, 1999), are captured by brooding "parents". These leeches feed their young via the provision of wounded or killed prey organisms. It is obvious that, due to this behaviour of the adult leech, the chances of survival of the juveniles are enhanced considerably when they finally leave their "parent" at a body size of about 25% of their adult "care provider" (KUTSCHERA and WIRTZ, 1986, 2001).

In summary, the results of this report document that the "Golden Gate leech" *H. californica* is, 25 years after its discovery, still present in the aquatic ecosystems of this large Park. It is concluded that this rare brooding annelid is a unique invertebrate restricted to the freshwater habitats in the city area of San Francisco.

5. Acknowledgements

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