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First record of the introduced freshwater leech *Helobdella europaea* Kutschera 1987 in Portugal (Hirudinea, Glossiphoniidae)

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Abstract

First record of the alien freshwater leech Helobdella europaea *Kutschera 1987 in Portugal (Hirudinea, Glossiphoniidae).* Recent spread of alien leech species has been reported throughout Europe, but a severe knowledge gap concerning their impact, distribution and ecology remains. Here, we document the occurrence of *Helobdella europaea* in a freshwater habitat in the north of Portugal, the first published record of this species in the country. Four specimens were found during water sampling at Ferreira River and promptly collected for study. Morphological identification was performed and tissue samples were also obtained for DNA barcode sequencing. Genetic analysis yielded a single haplotype shared by the four specimens, with very low divergence relative to other sequences deposited in public databases, confirming its identity.

Key words: Introduced species, DNA barcoding, Cytochrome *c* oxidase I (COI), Freshwater ecosystem, Biodiversity

Resumen

Primer registro de la sanguijuela exótica de agua dulce Helobdella europaea *Kutschera 1987 en Portugal (Hirudinea, Glossiphoniidae).* Se ha reportado la reciente propagación de especies de sanguijuelas exóticas en toda Europa y todavía existe una gran laguna en el conocimiento de su impacto, distribución y ecología. Aquí documentamos la presencia de *Helobdella europaea* en un hábitat de agua dulce en el norte de Portugal, que constituye el primer registro publicado para esta especie en el país. Se encontraron cuatro especímenes en un muestreo de agua en el río Ferreira que se recolectaron rápidamente para su estudio. Se realizó la identificación morfológica y se obtuvieron muestras de tejido para la secuenciación del código de barras de ADN. El análisis genético dio como resultado un solo haplotipo compartido por los cuatro especímenes y con muy poca divergencia de otras secuencias disponibles en bases de datos públicas, lo que confirma su identidad.

Palabras clave: Especies introducidas, Códigos de barras de ADN, Citocromo *c* oxidasa I (COI), Ecosistema de agua dulce, Biodiversidad

Resum

Primer registre de la sangonera exòtica d'aigua dolça Helobdella europaea *Kutschera 1987 a Portugal (Hirudinea, Glossiphoniidae).* S'ha comunicat la propagació recent d'espècies de sangoneres exòtiques a tot Europa i encara hi ha una gran llacuna pel que fa a l'impacte que puguin tenir, com també a la seva distribució i ecologia. Aquí documentem la presència d'*Helobdella europaea* en un hàbitat d'aigua dolça al nord de Portugal, que constitueix el primer registre publicat per a aquesta espècie al país. Se'n van trobar quatre espècimens en un mostreig d'aigua al riu Ferreira que van ser recol·lectats ràpidament per estudiar–los. Se'n va fer la identificació morfològica i es van obtenir mostres de teixit per a la seqüenciació del codi de barres d'ADN. L'anàlisi genètica va donar com a resultat un sol haplotip compartit pels quatre espècimens i amb molt poca divergència d'altres seqüències disponibles en bases de dades públiques, la qual cosa en confirma la identitat.

Paraules clau: Espècies introduïdes, Codis de barres d'ADN, Citocrom *c* oxidasa I (COI), Ecosistema d'aigua dolça, Biodiversitat

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Introduction

Leeches of the genus *Helobdella* Blanchard 1986 are distributed worldwide. They are characterized, in part, by a small chitinous scute on the dorsal side of the neck, a protrusible proboscis, and their brooding behaviour (Kutschera and Wirtz, 1986; Sawyer, 1986; Pfeiffer et al., 2004). In Europe, two species of this genus have been reported to date: *Helobdella stagnalis* L. 1758 and *Helobdella europaea* Kutschera 1987. The latter of the two is considered an invasive species and has a complicated taxonomic history, having been described more than once in the last 40 years.

In 1982, in a stream in the western part of Germany, Kutschera found leeches of the genus *Helobdella*, which he described and named *Helobdella striata* (Kutschera, 1985). In later years, however, Kutschera found that the specific epithet 'striata' had been used previously to describe a variety of the species *H. triserialis* and, later, as a separate species, by Ringuelet (Ringuelet, 1943; Kutschera, 1987). Consequently, the 'new' European species was renamed *H. europaea* Kutschera 1987.

The leech *H. europaea* remained the second European species of the genus for several years. However, in 1999, the species nativeness to Europe was questioned, as Neubert and Nesemann postulated that, given the absence of new species in Europe since 1758, *H. europaea* were merely escapees of *H. triserialis* from German aquaria (Neubert and Nesemann, 1999) (it was later found that this species had been discovered, years prior to

its description, in German aquaria [Pederzani, 1980] but this was unknown to the authors). Almost simultaneously, a new species, *Helobdella papillornata*, was described in Australia, the first of this genus for the country (Govedich and Davies, 1998). In 2004, however, through the use of mitochondrial DNA and morphological analysis, not only was *H. europaea* proven to be an independent species, but it has also been shown that *H. europaea* and *H. papillornata* (syn.) are identical to each other (despite some dietary and ecological differences) and separate from the South American *H. triserialis* species (Kutschera, 2004; Pfeiffer et al., 2004). Given that a year earlier *H. papillornata* (= *H. europaea*) was shown to be the sister taxa to the South American *H. triserialis* (Siddall and Borda, 2003), all evidence suggested that *H. europaea* was imported from that subcontinent.

Conclusively, in 2005, again through the use of DNA barcoding and morphology, Siddall and Budinoff determined that the German species had originated in South America and was identical to another variety of *H. triserialis* described by Ringuelet in 1943, *H. (triserialis) lineata.* Because the name *H. lineata* (Verrill, 1874) was already taken, *H. europaea* is the valid name of the species, which is not originally European (Siddall and Budinoff, 2005). The comprehensive phylogenetic analysis performed by Morhun et al. (2021) implied that populations of an undescribed species closely related to *H. europaea* occur in Arizona (USA). The further related *H. socimulcensis* Caballero, 1931 occurs in Mexico.

In summary, *H. europaea* is a North American species with broad dietary preferences. It has been introduced at several times in many parts of the world, most likely through the international trade of plants and animals for aquaria. Over the years, the species has been reported from New Zealand, South Africa, Hawaii and California, the Netherlands, Taiwan, Spain, Hungary and the Ukraine (Kutschera, 1985; Govedich and Davies, 1998; Haaren et al., 2004; Siddall and Budinoff, 2005; Bely and Weisblat, 2006; Lai et al., 2009; Reyes–Prieto et al., 2013; Málnás et al., 2016; Morhun et al., 2021).

In *Helobdella,* DNA barcoding has been repeatedly used to identify specimens (Siddall and Budinoff, 2005; Oceguera–Figueroa et al., 2010; Morhun et al., 2021). In fact, not only has it been extremely important in the understanding of the true origins of the species, but it has also revealed the presence of *H. europaea* in several countries (Siddall and Budinoff, 2005; Morhun et al., 2021).

In this work, we report the presence of a glossiphoniid leech species in northern Portugal. The captured specimens were morphologically and genetically identified, confirming their identity as *Helobdella europaea*, an invasive species.

Material and methods

In 2019, L. P. da Silva and S. Ferreira were sampling streams and rivers in the north of Portugal within the context of the project FUNAQUA and using standardized methods. Sampling consisted of collecting water and sediments from a section of the waterbody with 40–50 cm depth. On the return from sample collection at Rio Ferreira in Couce (Valongo–Porto District), specimens of leeches were observed on the legs of L. P. da Silva and collected for further study. Specimens were morphologically identified by S. Utevsky based on Kutschera (1987), and using a stereomicroscope Konus Crystal–45. These specimens were deposited in IBI–InBIO Barcoding Initiative reference collection at CIBIO–InBIO (Vairão–Portugal). A tissue sample of four specimens was processed for DNA barcoding. Genomic DNA was extracted using EasySpin Genomic DNA Tissue Kit (Citomed, Lisboa, Portugal) following the manufacturer's protocol, except for the lysis period which was extended to enhance extraction success. The cytochrome *c* oxidase I (COI) barcoding fragment was amplified using the LCO1490 and HC02198 primers (Folmer et al., 1994). PCR reactions had 10 µL of final volume, containing 5 µL of Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.4 µM of each primer, and 1–2 µL of DNA. PCR amplification was carried out on a T100

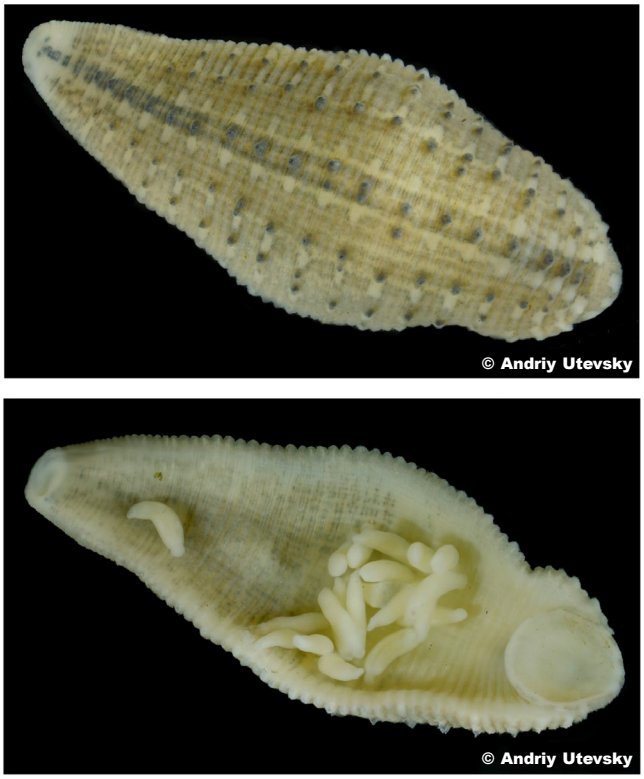

Fig. 1. Dorsal and ventral photographs of one of the Portuguese specimens of *Helobdella europaea* Kutschera 1987. Note the characteristic longitudinal rows of tubercles on the dorsum (left) and the young leeches attached to the ventral side of the maternal individual.

Fig. 1. Fotografía dorsal y ventral de un especimen de Helobdella europaea *Kutschera 1987 recolectado en Portugal. Destacan las características filas longitudinales de tubérculos en el dorso (izquierda) y las sanguijuelas jóvenes unidas a la cara ventral de la madre.*

Thermal Cycler (BioRad, Hercules, CA, USA) using the following conditions: initial denaturation at 95 ºC for 15 min; 5 cycles at 95 ºC for 30 s, 47 ºC for 45 s, 72 ºC for 45 s; then 40 cycles at 95 ºC for 30 s, 51 ºC for 45 s, 72 ºC for 45 s; and a final elongation step at 60 ºC for 10 min. Amplified DNA fragments were purified and sequenced (most with both primers) using the same primers as in the PCR, following ABI PRISM BigDye Terminator protocols. Sequences were visualized on a 310 Applied Biosystem DNA Sequencing Apparatus. All sequences were submitted to BOLD and GenBank databases.

Geneious v.6.1.5 (http://www.geneious.com/) was used for sequence assembly and curation. The sequence obtained was blasted against GenBank and BOLD databases. The average divergence (uncorrected p–distance) between the sequence of Portuguese specimens and sequences available in GenBank and BOLD was calculated in MEGA v.5.2.1 (Tamura et al., 2011).

Results and discussion

Species diagnosis and description of Portuguese specimens

All four specimens were morphologically identified as *Helobdella europaea* (fig. 1). DNA barcoding results included a single haplotype common to the four specimens (BOLD Accession numbers: IBIAH005–20, IBIAH006–20, IBIAH007–20, IBIAH008–20; BOLD BIN code: IBOLD: AAC5160) and equal to sequences of publicly available specimens from Australia, Hungary, Spain, South Africa, Taiwan, USA and Ukraine. The Portuguese sequences differ by 0.06 % to the single sequence available from Germany where the species was described but later considered not native, and by 0.05 % from New Zealand sequences.

Invasive potential and possible impacts

Early dietary assessments of *H. europaea* concluded that it predated several aquatic animals, namely aquatic Oligochaeta, insect larva, crustaceans and snails. Nonetheless, since then it has been found attached to larger vertebrates, most notably freshwater turtles (Reyes–Prieto et al., 2013; Perera et al., 2019). While no large populations have been found in Portugal to date, the proliferation of such a generalist species of leech may contribute to displacement of native species and it may harm already vulnerable populations of aquatic animals. Although the parasitism of *H. europaea* on vertebrate hosts was hypothesized (Perera et al., 2019), this hypothesis should be substantiated through revealing the respective evidence of feeding behaviour (Iwama et al., 2019). Since the current distribution of this invasive species is unknown, given its potential for a vast impact on ecosystems, it is imperative to screen and monitor freshwater bodies for this and similar species to determine which measures, if any, must be undertaken to control its spread. For this assessment, DNA barcoding may be a vital tool for fast and accurate identification of this species.

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