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SHORT COMMUNICATION

DNA barcodes reveal the presence of the introduced freshwater leech *Helobdella europaea* in Spain

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Abstract

We report the finding of the freshwater leech *Helobdella europaea* in Spain for the first time. Three leech specimens were found attached to the European pond turtle *Emys orbicularis*. *Helobdella europaea* is not a blood feeder and, like all members of the genus, feeds on the hemolymph of aquatic invertebrates including snails and worms. Despite the fact that the original geographical distribution or source population of this species is unknown, the close relationship between *H. europaea* and leeches of the "*triserialis*" series (*sensu* Sawyer, 1986) suggests a New World origin. Given its ability to invade and persist in new environments, this leech has been described as a new species by local taxonomists resulting in some nomenclatural problems. The presence of this introduced organism in Spain may represent serious obstacles to the current efforts to preserve endemic fauna and the potential negative impacts of this species in European environments should be investigated.

Keywords

DNA barcoding, freshwater leech, *Helobdella europaea*, introduced species, Spain

History

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Introduction

Leeches constitute a small monophyletic group of highly specialized annelids. In freshwater ecosystems, some leeches play important roles as invertebrate predators while others are notorious for their ability to feed on vertebrate blood (Sawyer, 1986). Infestation of aquatic and semi-aquatic reptiles by blood feeding leeches can lead to anemia in these hosts and induce bacterial and fungal infections through the lesions caused by their mouth structures. In addition, leeches can be intermediate hosts of *Trypanosoma*, *Haemogregarina*, *Hepatozoon* and *Karyolysus*, all hemoparasites of reptiles (Sawyer, 1986) and have been proposed as mechanical vectors of the marine turtle herpes virus (Greenblatt et al., 2004). In addition, introduced macrophagous leeches can cause an unbalance and even disappearance of invertebrate species and may contribute to the decline of amphibian populations and their natural environments (Galán, 2011; Stead & Pope, 2010). The genus *Helobdella* Blanchard, 1896, includes species that are commonly found in freshwater ecosystems where they feed on the hemolymph of aquatic invertebrates, particularly snails and oligochaetes (Sawyer, 1986). *Helobdella* is the most speciose genus of leeches with more than 35 described species (Sket & Trontelj, 2008) and is the most diverse in South America where more than 20 species have been recorded (Sawyer, 1986). In contrast, only two European species of *Helobdella* have been described: *Helobdella stagnalis* (Linnaeus, 1775) that is considered to be a part of the native fauna and the recently described *Helobdella europaea* (Kutschera, 1985), a member of the "*triserialis*" series (*sensu* Sawyer, 1986). *Helobdella europaea* is the only *Helobdella* species described from Europe in almost 240 years since the description of *H. stagnalis* by Linnaeus in 1775.

Helobdella europaea has a short but complicated taxonomic history. It was originally described as *Helobdella lineata* Kutschera, 1985, based on the specimens collected from an aquarium and surrounding areas in Germany. Soon after that, Kutschera (1987) found that the specific epithet "*lineata*" was pre-occupied by the South American leech *Helobdella triserialis lineata* (Verrill, 1874) and assigned a replacement name for the European species: *H. europaea*. Nesemann & Neubert (1999) asserted that the German specimens were probably representatives of the South American *H. triserialis* (Blanchard, 1849) that escaped from an aquarium where it previously had been reported (Pederzani, 1980).

A second leech species with morphological characteristics that resembled members of the *Helobdella* "*triserialis*" series was described as a new taxon from an area far from the Americas: *Helobdella papillornata* Govedich & Davies, 1998, from Victoria, Australia. This species, as noted by the authors (see Govedich & Davies 1998), is the unique species of the genus from Australia, a place were species of *Alboglossiphonia*, closely related with *Helobdella*, are common. Pfeiffer et al. (2004) found that *H. europaea* from Germany and *H. papillornata* from Australia are genetically identical when comparing sequences of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*), and, in fact, the same species.

Moreover, using *COI* sequences, Siddall & Budinoff (2005) demonstrated that the European *H. europaea* and the Australian

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H. papillornata, together with unnamed samples from South Africa, actually belong to the same species. The fact that samples from different continents display almost identical *COI* sequences and similar morphology, together with the finding of *H. europaea* in aquaria and in regions modified by humans, strongly suggested that *H. europaea* is a member of the *H.* ''*triserialis*'' series that was accidentally transported to new areas. Later, Lai et al. (2009), using molecular data, added Taiwan to the list of countries where this introduced species has been collected.

Sequences of *COI* have been used to distinguish between closely related species of *Helobdella* in places with relatively high diversity such as Mexico (Oceguera-Figueroa et al., 2010) in addition to being the gene fragment upon which the international barcode initiative for species identification relies (http://www.barcodeoflife.org/). For these reasons, *COI* sequences are analyzed in this study to identify the leech specimens collected in Valencia, Spain.

Materials and methods

Samples

Three adult leech specimens (one specimen with young leeches attached to the ventral surface) were collected on 5 April 2012. Leeches were removed from the carapace of three European pond turtles, *Emys orbicularis* Linnaeus, 1758, collected in a conservation reserve created in 2011 in Burriana, Castellon, Valencia, Spain (39° 52′ 52.7154″ N and 0° 03′ 12.6828″ W, 3 m a.s.l.). Collected specimens were transported to the laboratory in plastic containers. Pictures of live specimens were taken with a Leica DFC 500 digital camera adapted to a stereomicroscope Leica Z16APO. Leeches were relaxed through the gradual addition of 70% ethanol and finally fixed and preserved in 96% ethanol. Voucher specimens were deposited in the Colección de Invertebrados, at the Museo Nacional de Ciencias Naturales, Madrid, Spain. (Catalog number MNCN 16.02/122).

DNA extraction and amplification

A small portion of the posterior sucker of each of the leeches was used for total DNA extraction. This tissue was selected in order to avoid contamination from the leech gut content. Extraction of DNA was accomplished with GENOMED's JETFLEX Genomic DNA Purification Kit (GENOMED 600100) following the manufacturer's protocol. Nucleotide sequences of the mitochondrial *COI* gene were amplified using 1 μl of primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) at 10 μM, 1 μl of each of dNTP's at 2.5 μM, 0.25 μl of Taq Polymerase, 5 μl of Buffer 10×, 39.75 μl of MilliQ H_2O and 2 μl of DNA sample for a total volume of 50 μl. Reaction mixtures were heated to 94 °C for 5 min, followed by 30 cycles of 94 °C (45 s), 48 °C (45 s), and 72 °C (45 s) and a final extension at 72 °C (7 min). Purification of PCR products was done with Macherey-Nagel's NucleoFast 96 PCR plate, based on ultrafiltration and designed for rapid clean-up.

DNA sequencing

Amplification products were sequenced in both directions with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Each 10 μl sequencing reaction mixture included 0.5 μl of Terminator Ready Reaction Mix, 1.5 μl of Dye ''extender'' buffer (1 M Tris, pH 9; 25 mM $MgCl_2$), 1 μl of 1 μM primer, 2 μl of H_2O and 5 μl of amplification product. Reaction mixtures were heated to 96 °C (1 min), followed by 99 cycles of 96 °C (10 s), 50 °C (5 s) and 60 °C (4 min). Samples were sequenced on a sequencer ABI-3730 (Applied Biosystems). GenBank accession numbers are provided in Table 1.

Sequence analyses

COI gene sequences of representative species of *Helobdella* as well as of three *Haementeria* species were obtained from GenBank (Table 1). *Haementeria* species were used as outgroups because they belong to the sister group of *Helobdella* (Siddall et al., 2005). Sequences were aligned with MUSCLE (Edgar, 2004) using the default parameters. The final nucleotide sequence matrix included 91 terminals and 648 aligned characters. The Neighbor Joining (NJ) distance tree analysis was conducted in PAUP* (Swofford, 2002) using the K2P nucleotide substitution model (Kimura, 1980), as suggested by DNA barcoding practitioners (Hebert et al., 2004). In addition, a maximum parsimony analysis was performed in TNT (Goloboff et al., 2008) using the New Technology Search options with the ratchet (Nixon, 1999) and tree-fusing (Goloboff, 1999) algorithms. Bootstrap values were estimated in TNT using 100 pseudoreplicates (Felsenstein, 1985).

Results

The three adult leech specimens removed from the carapace of the European pond turtle in Burriana, Valencia, Spain, have the suite of morphological characters of the species of *Helobdella* of the ''*triserialis*'' series (*sensu* Sawyer, 1986) and in particular with those described for *H. europaea* and *H. papillornata*, including a single pair of well-separated eyespots in the anterior region, the dorsal surface with multiple longitudinal pigmented lines and 3–5 longitudinal rows of brown or dark-brown papillae arranged in metameric order in a2 of each complete somite (Figure 1). Based exclusively on morphological data, we determine our specimens to be *H. europaea*.

The resulting NJ tree (Figure 2) places the three samples of the leech specimens collected in Spain in a single cluster, with all other samples of *H. europaea* from a variety of localities. The genetic distance between the samples using the Kimura two-parameter algorithm was approximately 0.001%, disregarding their sampling locality. The parsimony analysis resulted in four most parsimonious trees with 1688 steps. The strict consensus of those trees is shown in Figure 3. The three samples of *Helobdella* collected in Spain form a monophyletic and well-supported clade with samples of *H. europaea* from several localities. It is worth noting that in both analyses, the group formed by the different samples of *H. europaea* appeared next to samples of *H. socimulcensis* from Mexico.

Discussion

The presence of the introduced freshwater leech *H. europaea* in ecosystems of Spain represents a major finding given the negative impact that this organism might have in the native fauna from areas far from its original distribution. Species of *Helobdella* are well known because of their voracious feeding to the detriment of populations of freshwater invertebrates, such as mollusks, tubificid worms, insect larvae and freshwater oligochaetes (Paez et al., 2004; Sawyer, 1986). They also exhibit a high rate of reproduction, including parental care strategies that seem to be characteristic of species of the family Glossiphoniidae and unique among annelids (Siddall et al., 2006). The fact that stable populations of *H. europaea* have been found on at least four continents might suggest that this reproductive behavior is an effective strategy to invading and persisting in new ecosystems.

Low genetic variation of introduced species seems to be common (Tsutsui et al., 2000) and it is evident in the case

Table 1. Taxa, localities and GenBank accession numbers for leeches used in the analysis of the relationships of the species of *Helobdella*.

Taxon	Locality	GenBank Accession number
Haementeria ghilianii	Biopharm (French Guiana)	AF329035
Haementeria gracilis	Arroyo Espinas, Uruguay	AF329034
Haementeria lutzi	Rio Pastaza, Ecuador	AF329033
Helobdella aff. *robusta*	New York, Valhalla College, USA	DQ995305
Helobdella aff. *stagnalis*	Temixco, Morelos, Mexico	HQ179861
Helobdella aff. *stagnalis*	Temixco, Morelos, Mexico	HQ179862
Helobdella atli	Aljojuca, Puebla, Mexico	HQ179850
Helobdella atli	Totolcingo, Tlaxcala, Mexico	HQ179851
Helobdella atli	Xochimilco, D. F. Mexico	HQ179852
Helobdella bolivianita	Laguna Volcán, Bolivia	AF329053
Helobdella californica	California, USA	HQ686307
Helobdella elongata	Jalisco, Mexico	HQ179863
Helobdella elongata	Michigan, USA	AF329045
Helobdella europaea	Australia	AF329052
Helobdella europaea	Berkeley, California, USA	DQ995304
Helobdella europaea	Galt, California, USA	DQ995297
Helobdella europaea	Galt, California, USA	DQ995298
Helobdella europaea	New Zealand	AY856049
Helobdella europaea	South Africa	AY856047
Helobdella europaea	South Africa	AY856048
Helobdella europaea	Taipei, Taiwan	FJ000349
Helobdella europaea	Taipei, Taiwan	FJ000350
Helobdella europaea	Taipei, Taiwan	FJ000351
Helobdella europaea	Taipei, Taiwan	FJ000352
Helobdella europaea **7**	**Castellon, Valencia, Spain**	**KC904241**
Helobdella europaea **10**	**Castellon, Valencia, Spain**	**KC904242**
Helobdella europaea **14**	**Castellon, Valencia, Spain**	**KC904243**
Helobdella fusca	Wild Goose Lake, Michigan, USA	AF329038
Helobdella lineata	Michigan, USA	AF329039
Helobdella melananus	Taipei, Taiwan	FJ000353
Helobdella melananus	Taipei, Taiwan	FJ000354
Helobdella melananus	Taipei, Taiwan	FJ000355
Helobdella michaelseni	Lago Calafquen, Chile	AF536824
Helobdella modesta	Columbus, Ohio, USA	AF329040
Helobdella modesta	New Haven, Connecticut, USA	JF319988
Helobdella modesta	New Haven, Connecticut, USA	JF319989
Helobdella modesta	New Haven, Connecticut, USA	JF319990
Helobdella modesta	New Haven, Connecticut, USA	JF319991
Helobdella modesta	New Haven, Connecticut, USA	JF319992
Helobdella modesta	New Haven, Connecticut, USA	JF319993
Helobdella modesta	New Haven, Connecticut, USA	JF319994
Helobdella modesta	New Haven, Connecticut, USA	JF319995
Helobdella modesta	New Haven, Connecticut, USA	JF319996
Helobdella modesta	Washington, USA	HQ179853
Helobdella modesta	Washington, USA	HQ179854
Helobdella nununununojensis	Madidi, Bolivia	AF329048
Helobdella nununununojensis	Tojoloque, Bolivia	AF329047
Helobdella octatestisaca	Ameca, Jalisco	HQ179856
Helobdella octatestisaca	Guanajuato, Mexico	HQ179858
Helobdella octatestisaca	Hidalgo, Mexico	HQ179857
Helobdella octatestisaca	Querétaro, Mexico	HQ179855
Helobdella octatestisaca	South Africa	HQ179860
Helobdella octatestisaca	Tabasco, Mexico	HQ179859
Helobdella octatestisaca	Taipei, Taiwan	FJ000342
Helobdella octatestisaca	Taipei, Taiwan	FJ000343
Helobdella octatestisaca	Taipei, Taiwan	FJ000344
Helobdella octatestisaca	Taipei, Taiwan	FJ000345
Helobdella octatestisaca	Taipei, Taiwan	FJ000346
Helobdella octatestisaca	Taipei, Taiwan	FJ000347
Helobdella octatestisaca	Taipei, Taiwan	FJ000348
Helobdella papillata	Michigan, USA	AF329042
Helobdella papillata	Michigan, USA	AF329043
Helobdella papillata	Virginia, USA	AF329046
Helobdella paranensis	Arroyo Espinas, Uruguay	AF329037
Helobdella pichipanan	Lago Chico, Chile	AY962456
Helobdella ringueleti	Madidi, Bolivia	AF329051
Helobdella robusta	Austin, Texas, USA	DQ995306
Helobdella robusta	Austin, Texas, USA	DQ995307
Helobdella robusta	Austin, Texas, USA	DQ995308

(*continued*)

Table 1. Continued

Taxon	Locality	GenBank Accession number
Helobdella robusta	Austin, Texas, USA	DQ995309
Helobdella robusta	Austin, Texas, USA	DQ995310
Helobdella robusta	Sacramento, California, USA	DQ995299
Helobdella robusta	Sacramento, California, USA	DQ995300
Helobdella robusta	Sacramento, California, USA	DQ995301
Helobdella robusta	Sacramento, California, USA	DQ995302
Helobdella socimulcensis	Cuautla, Morelos, Mexico	HQ179872
Helobdella socimulcensis	Cuautla, Morelos, México	HQ179871
Helobdella socimulcensis	Guanajuato, Mexico	HQ179870
Helobdella socimulcensis	Guanajuato, México	HQ179867
Helobdella socimulcensis	Hidalgo, México	HQ179869
Helobdella socimulcensis	Jalisco, Mexico	HQ179866
Helobdella socimulcensis	Querétaro, Mexico	HQ179868
Helobdella socimulcensis	Xochimilco, D. F. México	DQ995311
Helobdella sorojchi	Madidi, Bolivia	AF329049
Helobdella sorojchi	Madidi, Bolivia	AF329050
Helobdella sp.	California, USA	DQ995303
Helobdella sp.	San Luis Potosi, Mexico	HQ179865
Helobdella stagnalis	Costwolds, UK	AF329041
Helobdella transversa	Michigan, USA	AF329044
Helobdella triserialis	Laguna Volcán, Bolivia	AF329054
Helobdella virginiae	Veracruz, Mexico	HQ1798641

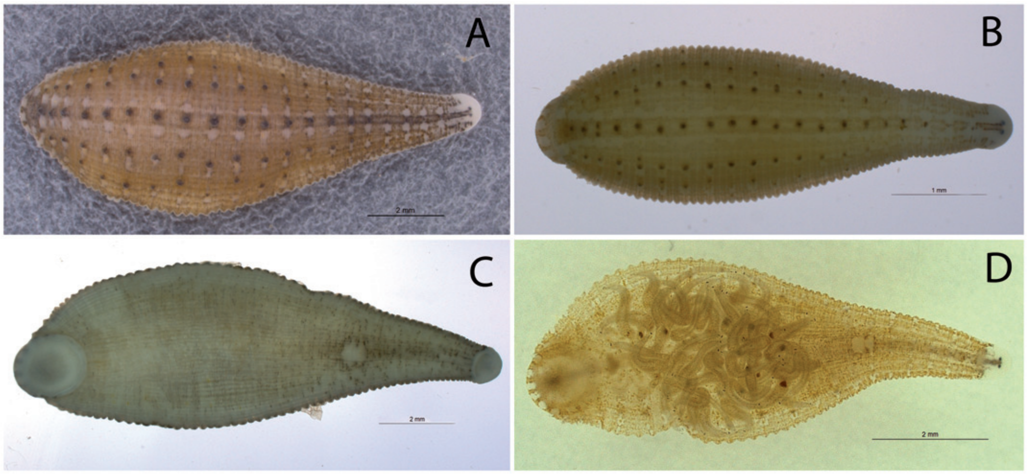


Figure 1. Dorsal view of *Helobdella europaea* showing variations of coloration pattern (a and b). Ventral view of *Helobdella europaea* (c). Specimen showing young leeches attached to the ventral surface (d).

presented here. Samples of *H. europaea* show approximately 0.001% *COI* divergence notwithstanding their transcontinental collection locality, contrasting enormously with the high genetic variation documented for other leeches in their natural environment. For example, the genetic variation within *Salifa perspicax* (Blanchard, 1897) samples from Rwanda alone reaches 2.4% (Oceguera-Figueroa et al., 2011) and more dramatically *Erpobdella Mexicana* (Dugès, 1876), that reaches 11.9% (Oceguera-Figueroa et al., 2005). These findings strongly suggest that samples of *H. europaea*, now widespread through Europe, Australia, Taiwan and South Africa, all came from a single population. However, given the poor molecular characterization of the leech species of South and Central America, the exact geographical location of the source population has yet to be determined.

The presence of this leech in Spain and Germany suggests that this species might have a broad distribution across the European continent. In contrast with the record from Germany from an aquarium, the specimens from Spain were collected in a biological reserve. The reserve is a highly modified environment where, in addition to the introduced leech reported here, other exotic elements have been recorded, including the Florida turtle *Trachemys scripta* (Schoepff, 1972), the American red crab *Procambarus clarkii* (Girard, 1852), the goldfish *Carassius auratus* (Linnaeus, 1758) and the American Perch *Micropterus salmoides* (Lacépède, 1802) (see Barba et al., 2012).

In addition to *H. europaea*, another three leech taxa have been found transcontinentally: *Barbronia weberi* (Arhynchobdellida: Salifidae) that has been recorded in all five continents and has an unknown original distribution (Oceguera-Figueroa et al., 2011), *Helobdella octatestisaca* known from Taiwan (Lai et al., 2009) but apparently native of Central Mexico (Oceguera-Figueroa et al., 2010) and *Hirudinaria manillensis* (Arhynchobdellida: Haemadipsidae) native of South East Asia but also recorded in Puerto Rico and the Dominican Republic (Phillips, 2012). The dispersal of *H. europaea* seems to be accidental. It has been related with the trade of aquatic plants or fish for aquaria, however other explanations can be proposed. A more intensive sampling in freshwater ecosystems from Europe, and particularly from Spain, is needed in order to define the current geographical distribution of *H. europaea*. This information is required in order to propose

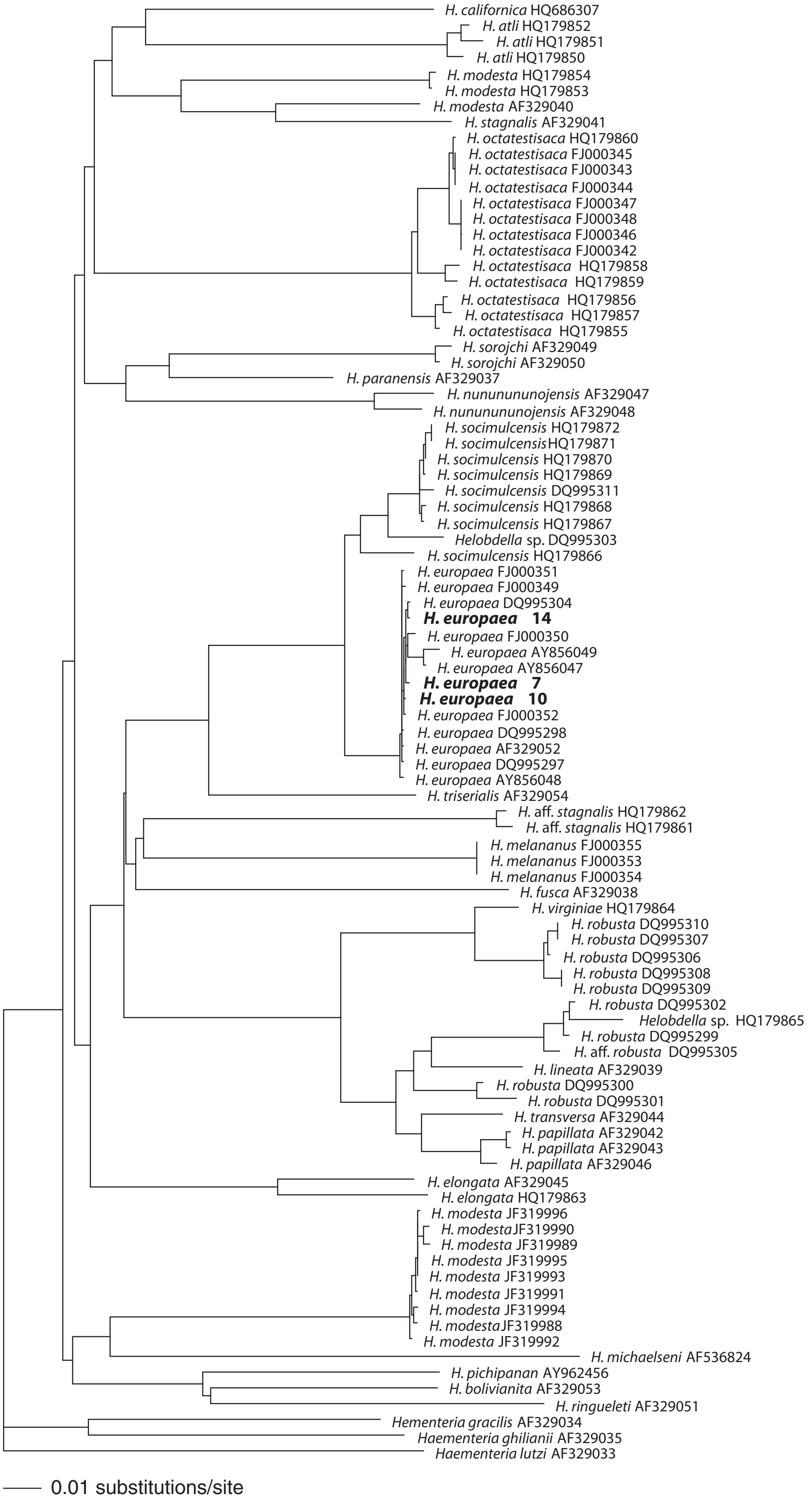
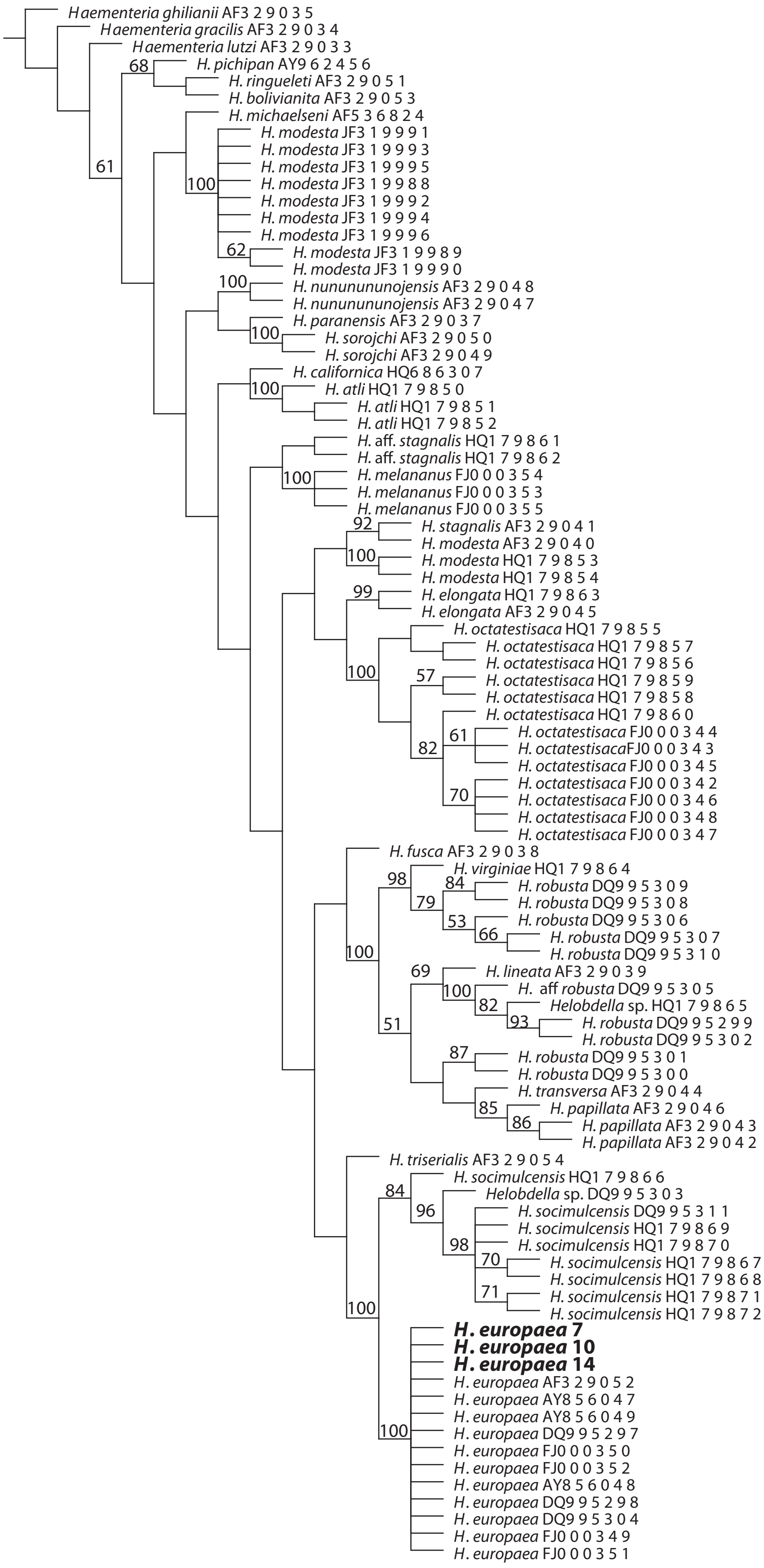


Figure 2. Neighbor-joining tree based on the Kimura two-parameter substitution model of *COI* of selected species of *Helobdella* showing the samples from Valencia (in solid caps) well nested in a cluster of *Helobdella europaea* with a low genetic intraspecific variation.

Figure 3. Parsimony tree based on *COI* sequences of selected specimens of *Helobdella*. Samples of *Helobdella* from Valencia (in solid caps) form a monophyletic and well-supported clade with samples of *Helobdella europaea*. Numbers above the nodes indicate bootstrap values.



strategies to control or eliminate wild populations of the introduced leech.

Conclusions

The presence of the South American introduced leech *Helobdella europaea* in Spain, as revealed by morphological and molecular (*COI* gene sequences) analysis, is a major finding given the negative impact that this organism might have on the native fauna. The extremely low genetic variation between samples of *H. europaea* notwithstanding their collection locality including Europe, Taiwan, South Africa and Australia, is in contrast with the high genetic variation of other leech taxa in their natural environment and strongly supports the hypothesis that this introduced species came from a single, undetermined population from South or Central America. The correct taxonomic identification of introduced species using traditional morphology data in combination with new approaches, such as the Barcode of Life platform, provides important information for both taxonomy and conservation biology, and represents the starting point for biodiversity inventories and conservation/restoration projects.

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Declaration of interest

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