



Insights into the fauna associated with *Egeria densa* at the upper section of the international Minho River estuary (NW Iberian Peninsula) 3 decades after its establishment

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Abstract

The macrophyte *Egeria densa* Planchon, 1849 is a freshwater plant native to the subtropical regions of South America. Fast vegetative reproduction and efficient dispersal allow this species to form extensive beds that produce high levels of oxygen in freshwater ecosystems, generating microhabitats that act as refuge and nursery for an array of organisms, increasing sedimentation and light availability. Despite its undisputable ecological role, it is considered invasive and is present on all continents except Antarctica with the first records in Europe occurring at the 20th century. At the international Minho River (NW Iberian Peninsula), the observation of its presence was noted in the 1990s and is now an established population in this ecosystem. This study is the first descriptive-taxonomical assessment of the associated fauna with the exotic macrophyte *E. densa*, using both morphological and molecular approaches, three decades after its establishment in the international Minho River. Results indicate the presence of a faunal assemblage, composed a total of 20 identified species, including platyhelminths, hydrozoans, bryozoans, molluscs (Gastropoda), annelids (Oligochaeta and Hirudinea), crustaceans (Ostracoda, Copepoda, Branchiopoda and Amphipoda), aquatic mites and insects. *Paludicella* aff. *articulata* (Ehrenberg, 1831), *Girardia sinensis* Chen & Wang, 2015, *Lebertia insignis* Neuman, 1880 and *Ceriodaphnia rigaudi* Richard 1894 are recorded for the first time in Portugal.

Keywords Exotic species · Limnology · Bioengineers · Invertebrate assemblages

Introduction

The species *Egeria densa* Planchon, 1849 is a freshwater perennial plant native to the subtropical regions of South America, which forms dense mats that cover extensive areas. Considered invasive is now present on all continents except Antarctica (Yarrow et al. 2009). Development of *E. densa* beds lowers water velocity causing sedimentation of suspended particles, increasing light availability (Yarrow et al. 2009). Thus this aquatic plant plays an important role on the structure and function of freshwater environments, generating microhabitats used as refuge and nursery for zooplankton, macroinvertebrates and fish (Pelicice and Agostinho 2006; Yarrow et al. 2009). Furthermore *E. densa* promotes phytoplankton biomass decline (Mazzeo et al. 2003), stimulates an increase in epiphytic macroinvertebrate biomass which subsequently increases carnivorous fish density (Diehl and Kornijów 1998). The first records in

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Europe occurred at 20th century (Yarrow et al. 2009), and the first observation at the international Minho River (NW Iberian Peninsula) occurred during the 90's (Antunes, pers. comm.). Since then *E. densa* developed populations along this freshwater system, generating dense mats on otherwise sandy intertidal environments, forming a microhabitat distinct from its native counterparts that cover mainly the subtidal section. A first attempt at a descriptive-taxonomical assessment of the fauna associated with the exotic macrophyte *E. densa* at the International Minho River, using both morphological and molecular approaches, provided 12 new records for the study area, including the exotic species *Girardia sinensis* Chen & Wang, 2015, *Menetus dilatatus* (Gould 1841) and *Helobdella europaea* (Kutschera 1985).

Methods

Study area

The estuarine area of the international Minho River, located at the northwest Spanish/Portuguese border (NW Iberian

Peninsula), has a length of approximately 40 km (Sousa et al. 2008a), with a mesotidal partially mixed system tending towards a salt wedge estuary during the high floods (Sousa et al. 2005). Sampling was performed at the upper section of the estuary, on the Portuguese margin in front of Morraiceira Island (Vila Nova de Cerveira), approximately 19 km from the river's mouth (Fig. 1a). This section of the river is a predominantly freshwater system throughout most of the year, characterized by saline intrusions during the summer season, especially during the dryer months (Pereira et al. 2022) and its margins are covered by monospecific dense mats of *Egeria densa* (Fig. 1b,c) that overlays the supratidal area, extending to the upper subtidal.

Specimen sampling, identification and preservation

Samplings were performed in the upper section of the international Minho River estuary (Fig. 1), near Morraiceira Island, Vila Nova de Cerveira, Portugal. Aerial parts of *E. densa* were sampled monthly from October 2021 to March 2022, in an intertidal zone, with each sample being collected manually in a area delimited by a square box of

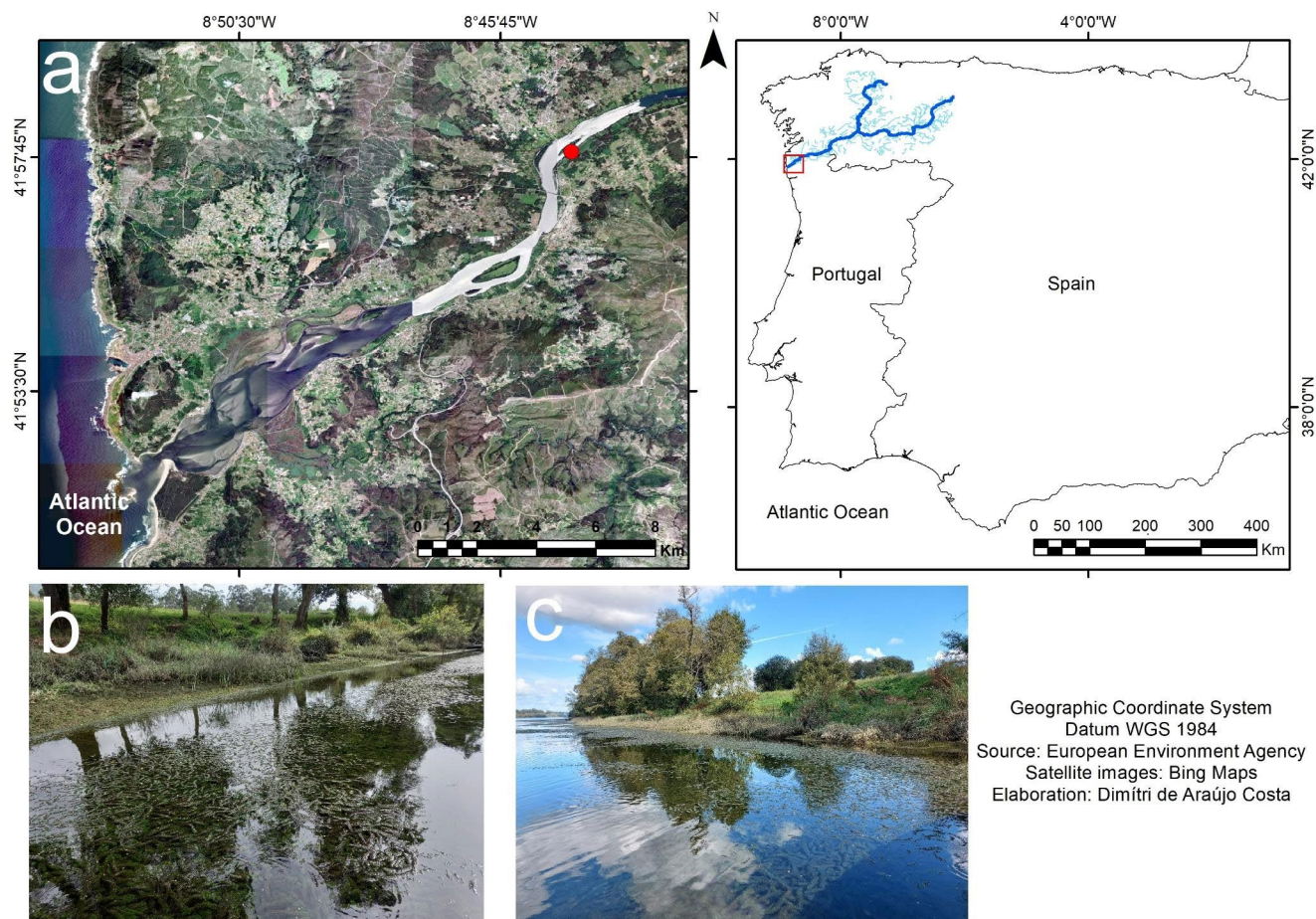


Fig. 1 Study Area: Minho River (Iberian Peninsula), with highlight on the sampling point (a) and an overview of the area (b,c), satellite images: Bing Maps

0,1 m² and bagged for posterior analysis. Species identification was performed using specialized literature (Brinkhurst 1971; Brinkhurst and Jamieson 1971; Lincoln 1979; Cornelius 1995; Alonso 1996; Tachet et al. 2000; Wood 2015; Govedich and Moser 2015; Rogers 2019; Horne et al. 2019; Lee and Lee 2019; Noreña et al. 2019; Conesa-García 2021). All specimens were photographed with a Leica EZ4W stereomicroscope, a Nikon Digital Sight D5-L1 camera using a Nikon SMZ800 stereomicroscope and a Nikon ECLIPSE 50i microscope. Specimens were preserved in 70% ethanol and deposited at the Natural History Museum of the Iberian Peninsula (NatMIP – “Museu de História Natural da Península Ibérica”), sited at Aquamuseu do Rio Minho, Vila Nova de Cerveira, North Portugal.

Genetic analysis

Genomic DNA extraction was performed using a using the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek), following manufacturer’s instructions. Amplification of the COI-5P region was carried out using the primers LoboF1 (5′-KBTCHACAAAYCAYAARGAYATHGG-3′) and LoboR1 (5′-TAAACYTCWGGRTGWCCRAARAAYCA-3′) (Lobo et al. 2013), and a pre-made PCR mix (VWR International, LLC, Pennsylvania, USA). PCR reactions were comprised of 1× PCR buffer, 1.5 mM of MgCl₂, 0.2 mM of the dNTP mixture, 1 U of DNA Taq polymerase, plus 0.5 μM of each primer, ca. 30–50 ng of genomic DNA, and sterile MilliQ-grade water to make up a total volume of 25 μL. The reactions were run on a Hybaid PxE Thermocycler (Thermo Electron Corporation, Milford, Massachusetts), following the PCR conditions in Lobo et al. (2013). PCR products were electrophoresed through a 1% agarose 1× Tris-acetate-EDTA buffer (TAE) gel stained with GreenSafe Premium (Nzytech, Lisbon, Portugal). Positive PCR products were purified using the ExoFast method, in which an enzymatic cleanup that eliminates unincorporated primers and dNTPs is performed with Exonuclease I (*Escherichia coli*) and FastAP Thermosensitive (SAP), and then sequenced directly. The sequencing reactions were performed using a BigDye Terminator v1.1 from the Applied Biosystems kit (Applied Biosystems, Carlsbad, California), and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems, Stabvida, Oeiras, Portugal). DNA sequence homology searches and species level identifications were performed using BOLD (Hebert and Ratnasingham 2007) and with BLAST (Altschul et al. 1990) in GenBank databases (Benson 2004). COI sequences were edited and manually aligned using MEGA X (Kumar et al. 2018) and checked for insertions, deletions and stop codons. For phylogenetic reconstruction, COI-5P sequences were retrieved from public databases as follows: sequences for

the species in analysis, sequences from the respective genus and sequences from a species of the same family used as outgroup. For the species *Bothrioneurum vej dovskyanum*, *Cypridopsis vidua* and *Menetus dilatatus*, which had no available sequences for congeners, only sequences belonging to members of the same family were retrieved. Specimens of *Paludicella* aff. *articulata* and *Embolocephalus* sp., had no match in BOLD or GenBank. Thus, phylogenetic reconstruction for *P.* aff. *articulata* was carried out using sequences belonging the order Ctenostomida and Cyclostomatida for outgroup. For *Embolocephalus* sp. other sequences from the genus *Embolocephalus*, as well as from the subfamily Tubificinae were included with Naidinae used as outgroup. Phylogenetic relationships were reconstructed through Maximum-Likelihood, using the substitution model GTR+I+G, as chosen by JModelTest (Posada 2008). MOTU’s were delimited using the species delineation tool Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012). Intra- and inter-specific distances were calculated on MEGA X using the K2P substitution model.

Results

Morphological analysis

A total of 3370 specimens were examined, representing 20 species belonging to 6 phyla, 16 families and 16 genera. Among these 20 species identified using morphological characters, 4 could only be identified to the family level only (Table 1).

Genetic analysis

The majority of the taxa examined formed monophyletic MOTU’s with less than 2% genetic distance namely, *Helobdella europaea*, *Lebertia insignis*, *Gammarus chevreuxi*, *Atyaephyra desmarestii*, *Ischnura elegans*, *Menetus dilatatus* and *Girardia sinensis* (Figs. 2 and 3). The specimen identified morphologically as *Paludicella articulata* did not matched any sequence available on BOLD and GenBank, and phylogenetic reconstruction through COI sequences did not reveal any reliable placing or grouping within Ctenostomatida (Fig. 4a). The asexual ostracod *Cypridopsis vidua* revealed a complex comprised of seven distinct groups (22 MOTU’s, 7–15% genetic distance), with the specimen analysed nesting within a large cosmopolitan lineage (Group 1), comprised of several distinct MOTU’s (Fig. 4b). *Psectrocladius limbatellus* may also be a large cryptic complex with 5 distinct MOTU’s (14–22% genetic distance) (Fig. 5a). *Bothrioneurum vej dovskyanum* also formed a possible cryptic complex with three distinctly monophyletic

Table 1 Morphological/Genetic identification obtained for each species examined and respective complementary analysis

Species	Morphological identification	Genetic identification	Complimentary analysis
<i>Helobdella europaea</i>	<i>Helobdella stagnalis</i>	<i>Helobdella europaea</i> (similarity score 100%)	Specimens re-identified as <i>Helobdella europaea</i> (0.1% internal distance)
<i>Bothrioneurum vej dovskyanum</i>	Naididae sp.	<i>Bothrioneurum vej dovskyanum</i> (similarity score 100% for both sequences)	Species level identification obtained with molecular data (2 lineages with 5% genetic distance)
<i>Embolocephalus</i> sp.	<i>Embolocephalus</i> cf. <i>velutinus</i>	No match with public sequences	Identification incongruent with known sequences of <i>Embolocephalus</i> sp (15% genetic distance with <i>Embolocephalus velutinus</i>)
<i>Chaetogaster</i> cf. <i>diaphanus</i>	<i>Chaetogaster</i> cf. <i>diaphanus</i>	-	Single specimen kept for further analysis
<i>Lebertia insignis</i>	Lebertiidae sp.	<i>Lebertia insignis</i> (similarity score 97%)	Species level identification obtained with molecular data (0.6% internal distance)
<i>Ceriodaphnia rigaudi</i>	<i>Ceriodaphnia rigaudi</i>	-	Specimens kept for further analysis
<i>Eurycercus lamellatus</i>	<i>Eurycercus lamellatus</i>	-	Specimens kept for further analysis
<i>Macrocylops</i> sp.	<i>Macrocylops</i> sp.	-	Single specimen kept for further analysis
<i>Gammarus chevreuxi</i>	<i>Gammarus chevreuxi</i>	<i>Gammarus chevreuxi</i> (similarity score 99%)	Identification congruent with known sequences of <i>Gammarus chevreuxi</i> (1% internal distance)
<i>Atyaephyra desmaresti</i>	<i>Atyaephyra desmaresti</i>	<i>Atyaephyra desmaresti</i> (similarity score 99%)	Identification congruent with known sequences of <i>Atyaephyra desmaresti</i> (2% internal distance)
<i>Procambarus clarkii</i>	<i>Procambarus clarkii</i>	-	Specimens kept for further analysis
<i>Cypridopsis vidua</i>	Cyprididae sp.	<i>Cypridopsis vidua</i> (similarity score 97%)	Species level identification obtained with molecular data (3% internal distance within group A)
<i>Psectrocladius limbatellus</i>	Chironomidae sp.	<i>Psectrocladius limbatellus</i> (similarity score 99%)	Species level identification obtained with molecular data (1.8% internal distance within MOTU 4)
<i>Ischnura elegans</i>	<i>Ischnura elegans</i>	<i>Ischnura elegans</i> (similarity score 100%)	Identification congruent with known sequences of <i>Ischnura elegans</i> (0.3% internal distance)
<i>Paludicella</i> aff. <i>articulata</i>	<i>Paludicella articulata</i>	No match with public sequences	<i>Paludicella</i> aff. <i>articulata</i>
<i>Sertularia cupressina</i>	<i>Sertularia cupressina</i>	COI amplification failed	-
<i>Ancylus fluviatilis</i>	<i>Ancylus fluviatilis</i>	-	Specimens kept for further analysis
<i>Menetus dilatatus</i>	<i>Gyraulus parvus</i>	<i>Menetus dilatatus</i> (similarity score 99%)	Specimens re-identified as <i>Menetus dilatatus</i> (0.1% internal distance)
<i>Physella acuta</i>	<i>Physella acuta</i>	Chimeric DNA with <i>Chaetogaster limnaei</i>	-
<i>Girardia sinensis</i>	<i>Schmidtea</i> sp.	<i>Girardia sinensis</i> (similarity score 100%)	Species level identification obtained with molecular data (1.6% internal distance)

lineages with 4–5% genetic distance, two of which occur sympatrically in the study area (Fig. 5b). *Embolocephalus* sp. (identified morphologically as *Embolocephalus* cf. *velutinus*) is revealed to be a sister taxon of *E. velutinus* (15% genetic distance) forming a distinct clade within the genus *Embolocephalus* (Fig. 5c). Exotic species, *H. europaea*, *M. dilatatus* and *G. sinensis* present shared haplotypes spanning multiple continents (Figs. 2a and 3b and c). COI amplification of the hydrozoan *Sertularia cupressina* failed, revealing a need of specific primers for this group. *Physella acuta* amplification has shown the presence of chimerical DNA between this gastropod and the epizoic oligochaete *Chaetogaster limnaei*, which was posteriorly confirmed inside *P. acuta* mantle cavity.

Integrated taxonomy (morphology and genetic analysis)

A total of 3370 specimens were examined, representing 20 species belonging to 6 phyla, 16 families and 20 genera. Twelve species constituted new records for the International Minho River (SI). Four species had species level misidentifications, using morphological characters, which could be resolved through the integrative analysis of morphological and genetic features. Another 4 species could only be assertively identified after genetic analysis (Table 1).

Phylum Annelida Lamarck, 1802.
 Class Clitellata Michaelsen, 1919.
 SubClass Hirudinea Savigny, 1822.
 Order Rhynchobdellida Blanchard, 1894.

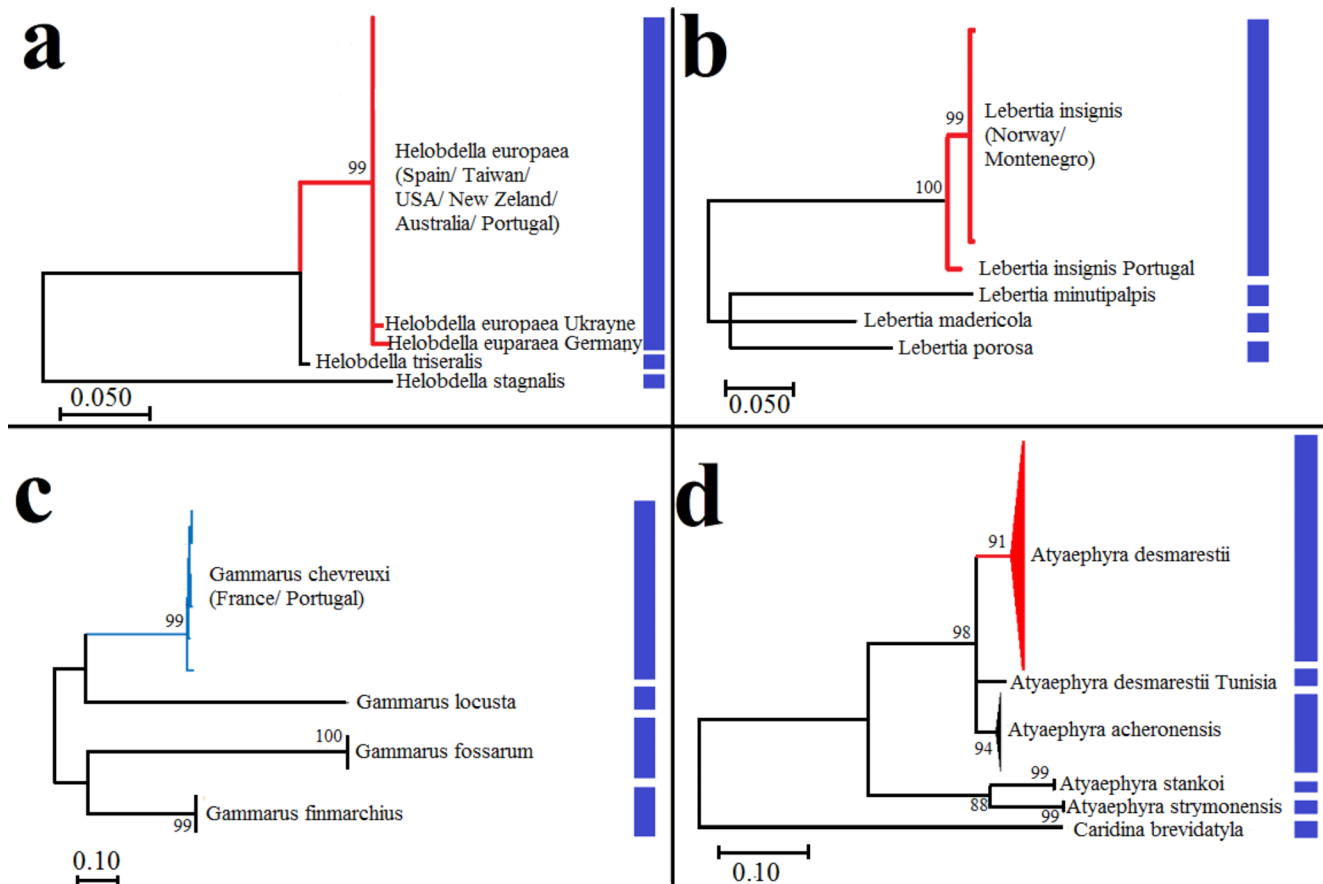


Fig. 2 Maximum-likelihood tree obtained from COI-5P sequences of *Helobdella europaea* (a); *Lebertia insignis* (b); *Gammarus chevreuxi* (c); *Atyaephyra desmarestii* (d). Clade for the species in analyses high-

lighted in red. Value at nodes corresponds to bootstrap support (values below 70 are omitted)

Family Glossiphoniidae Vaillant, 1890.

Genus *Helobdella* Blanchard, 1896.

Helobdella europaea (Kutschera 1985) (Fig. 6a).

Type material Holotype- 1 specimen collected in 1982, deposited at Zoologisches Museum Hamburg (Kutschera 1985).

Type locality Schobbach/Moosbach, Vörstatten, Germany (Kutschera 1985).

Material examined 28 specimens collected from October to December 2021, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP912901.

Geographical distribution Species with scattered records worldwide (Kutschera 2004).

Distribution in Portugal International Minho River (**this study**) and Ferreira River (Valongo, Porto) (Ferreira et al. 2022).

Ecological notes Freshwater leach, reaching up to 20 mm (Kutschera 1985); predator (Kutschera 1987); sampled in salinities ranging from 0.06 to 0.05, more abundant in October when water temperatures reached 17.75 °C.

Remarks Kutschera (2004), suggested that this species is exotic to Europe based on how the species COI sequences clustered closely to *Helobdella triserialis* (Blanchard, 1849) from South America, instead of its European counterparts; haplotypes shared between Europe, Oceania, Asia and North America is indicative of a generalized colonization event originating from a similar founding population (Fig. 3a).

SubClass Oligochaeta Grube, 1850.

Order Tubificida Jamieson, 1978.

Family Naididae Ehrenberg, 1831.

Genus *Bothrioneurum* Stolč, 1886.

Bothrioneurum vej dovskyanum Štolc 1886 (Fig. 6b).

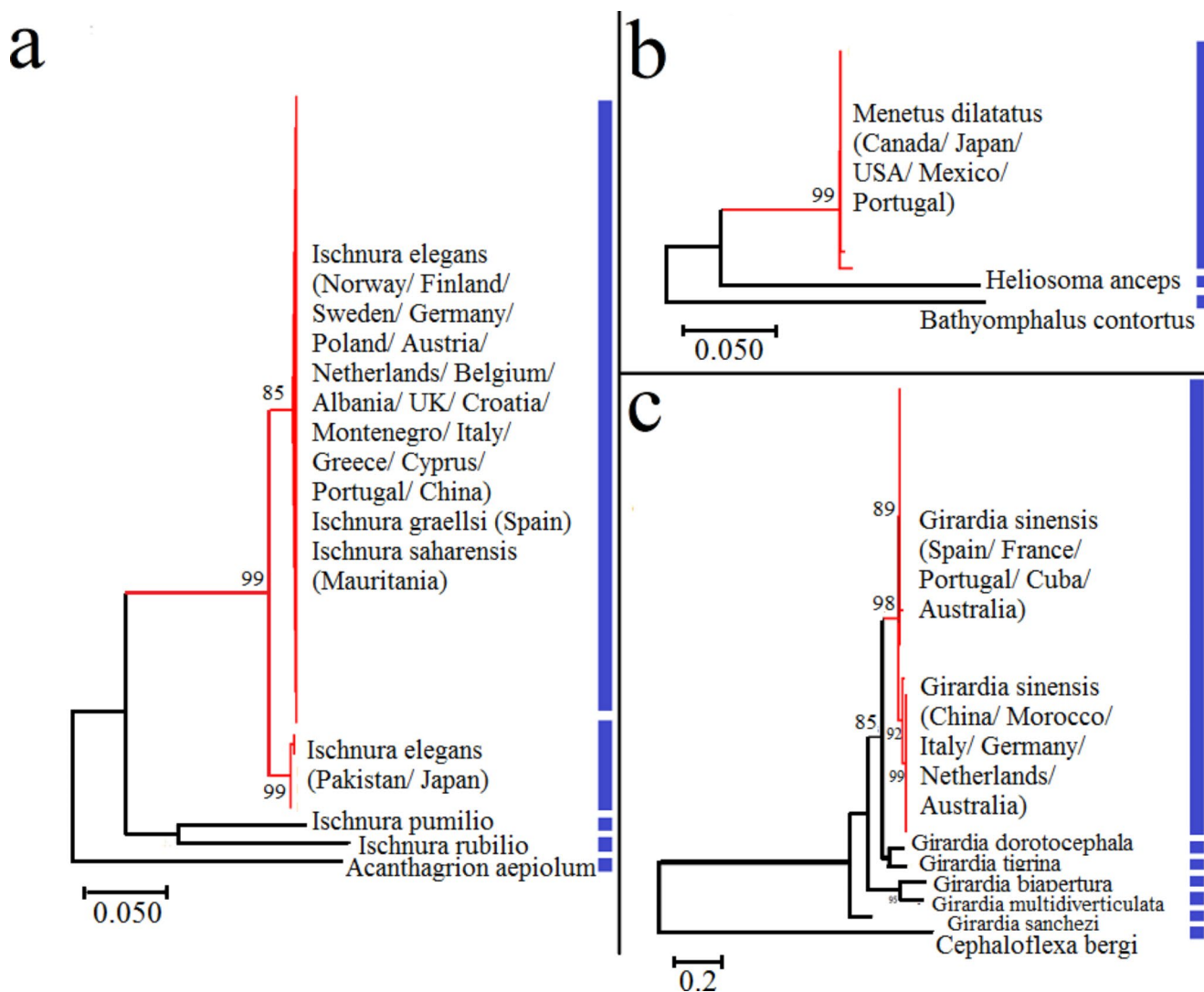


Fig. 3 Maximum-likelihood tree obtained from COI-5P sequences of *Ischnura elegans* (a); *Menetus dilatatus* (b); *Girardia sinensis* (c). Clade for the species in analyses highlighted in red. Value at nodes corresponds to bootstrap support (values below 70 are omitted)

Type material Unknown.

Type locality Štvanice island, Vltava river, Prague, Czech Republic (Štolc 1886).

Material examined 67 specimens, collected October 2021 and from January to March 2022, on *E. densa* leaves; 2 specimens with COI sequence, GenBank accession number: OP912523 and OP912987.

Geographical distribution Europe, Asia, North America and Africa (Brinkhurst and Jamieson 1971).

Distribution in Portugal International Minho River, Ria de Aveiro and Mondego River (Timm and Abarenkov 2021).

Ecological notes Common on stagnant waters rich in organic matter specially among macrophytes (Dumnicka 2007); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks Species level identification based on molecular data; detected the presence of two sympatric lineages with 5% genetic distance in the study area (Fig. 5b).

Genus *Embolocephalus* Randolph, 1892.

Embolocephalus sp. (Fig. 6c)

Material examined 2 specimens, collected March 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP918896.

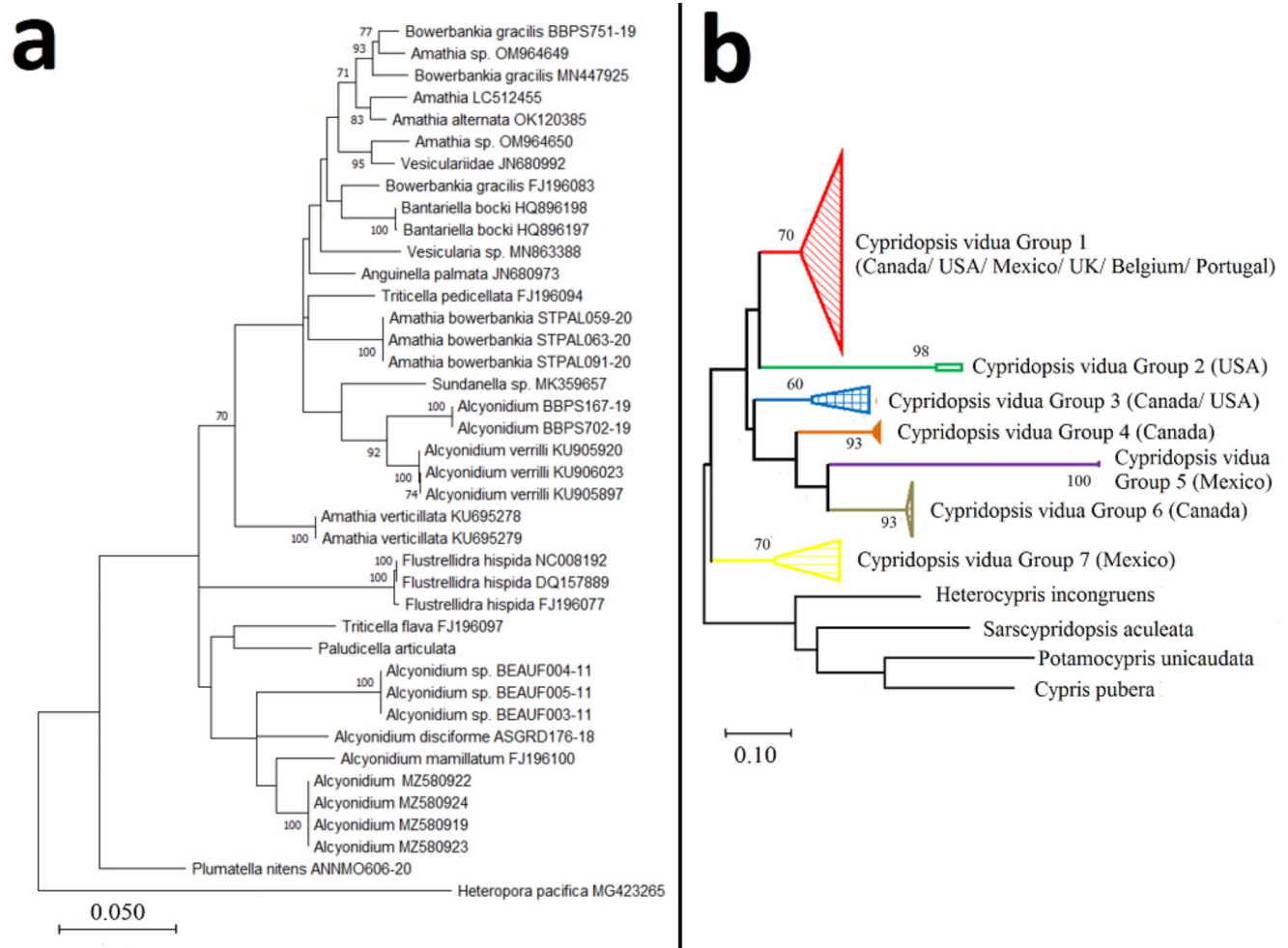


Fig. 4 Maximum-likelihood tree obtained from COI-5P sequences of *Paludicella* aff. *articulata* (**a**); *Cypridopsis vidua* species complex (**b**). Value at nodes corresponds to bootstrap support (values below 70 are omitted)

Ecological notes Sampled in salinity of 0.04, water temperature 10.74 °C.

Remarks Genetic analysis shows this specimen as a sister taxon to *Embolecephalus velutinus* with a 15% distance between the two species, thus forming a distinct clade within the genus *Embolecephalus*, indicating the need for a revision of this genus in western Europe (Fig. 5c).

Genus *Chaetogaster* von Baer, 1827.

Chaetogaster cf. *diaphanus* (Gruithuisen, 1828) (Fig. 6d).

Material examined 1 specimen, collected March 2022, on *E. densa* leaves.

Geographical distribution Species recorded in Europe, Africa, North America and Asia (Brinkhurst and Jamieson 1971; Jongwoo 2011).

Distribution in Portugal Leixões (Azevedo et al. 2020).

Ecological notes Fresh and brackish water species; maximum size up to 25 mm (Brinkhurst 1971); feeds on algae and zooplankton (Jongwoo 2011); sampled in salinity of 0.04, water temperatures 10.74 °C, inside a tube formed by detritus.

Remarks This specimen requires further examination to clarify its identification. Nonetheless this is the first record of the genus *Chaetogaster* for the study area.

Phylum Arthropoda Siebold, 1848.

SubPhylum Chelicerata Heymons, 1901.

Class Arachnida Cuvier, 1812.

Order Trombidiformes Reuter, 1909.

Family Lebertiidae Thor, 1900.

Genus *Lebertia* Neuman, 1880.

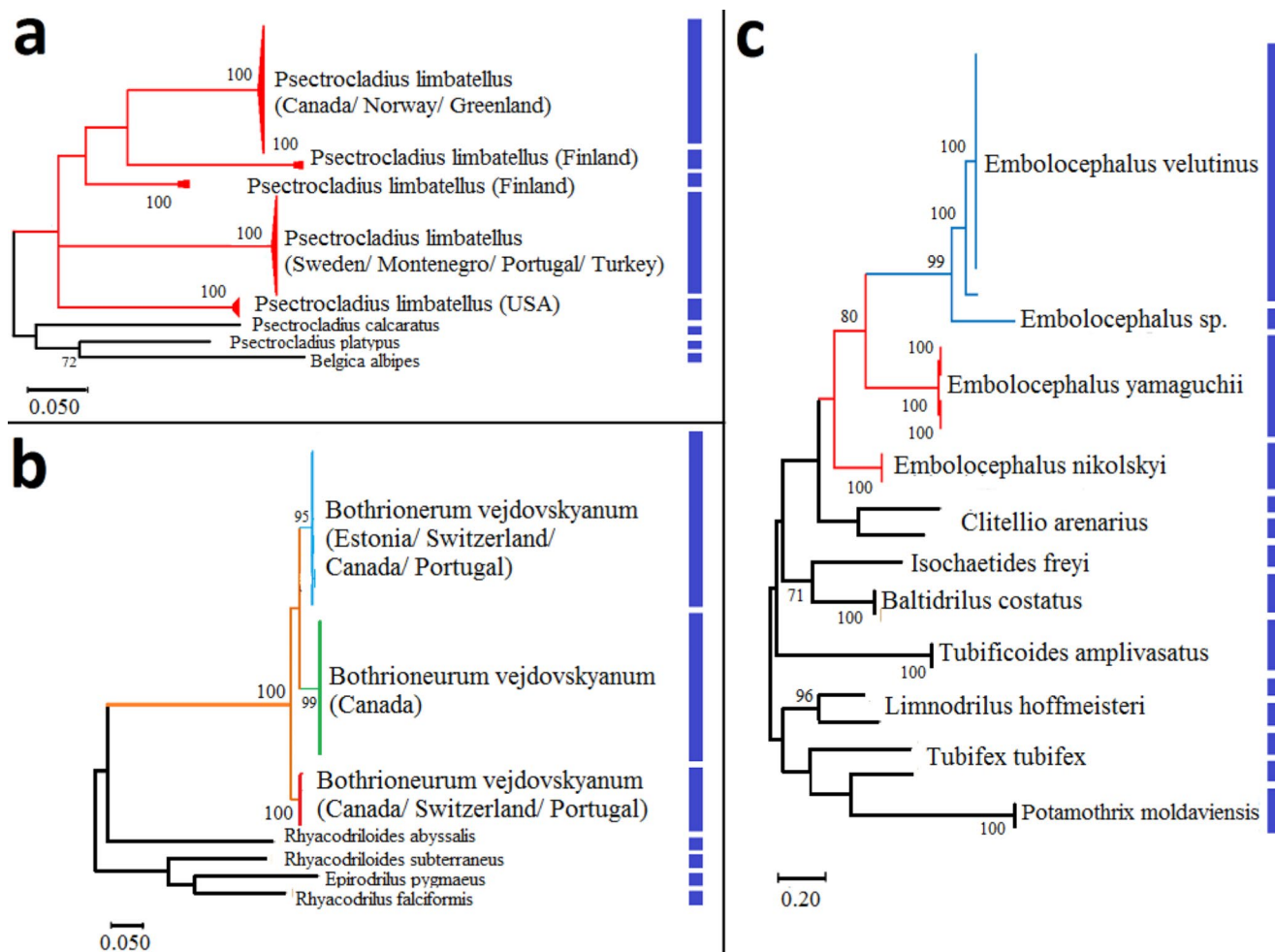


Fig. 5 Maximum-likelihood tree obtained from COI-5P sequences of *Psectrocladius limbatellus* species complex (clade highlighted in red) (a); *Bothrioneurum vej dovskyanum* species complex (different MOTU's highlighted by blue, green a red square) (b); *Embolocephalus*

lus sp. (clade for the genus *Embolocephalus* highlighted in red and *E. velutinus* and *Embolocephalus* sp. in blue) (c). Value at nodes corresponds to bootstrap support (values below 70 are omitted)

Lebertia insignis Neuman, 1880 (Fig. 7a).

Type material Holotype missing (Gerecke 2009); Paratypes: Preserved specimens deposited at Forschungsinstitut und Natur-Museum Senckenberg, catalogue numbers 44901-2001-VIE/169, 44902-2005-VIE/169 and 44903-2006-VIE/169 (Senckenberg 2022).

Type locality Sweden (Gerecke 2009).

Material examined 35 specimens, collected from October 2021 to March 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP912882.

Geographic distribution Central and North Europe with a few records on Southern Europe (Gerecke 2009).

Distribution in Portugal International Minho River (this study).

Ecological notes Sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C; found on pools of streams (Sabatino et al. 2010).

Remarks Species level identification based on molecular data; although the specimen formed a MOTU within the 2% threshold with DNA barcodes of *Lebertia insignis* from Norway and Montenegro, it forms a clearly distinct lineage (Fig. 2b): the relationship of these lineages may be solved with the analysis of specimens from Southwestern Europe.

SubPhylum Crustacea Brünnich, 1772.
Class Branchiopoda Latreille, 1817.

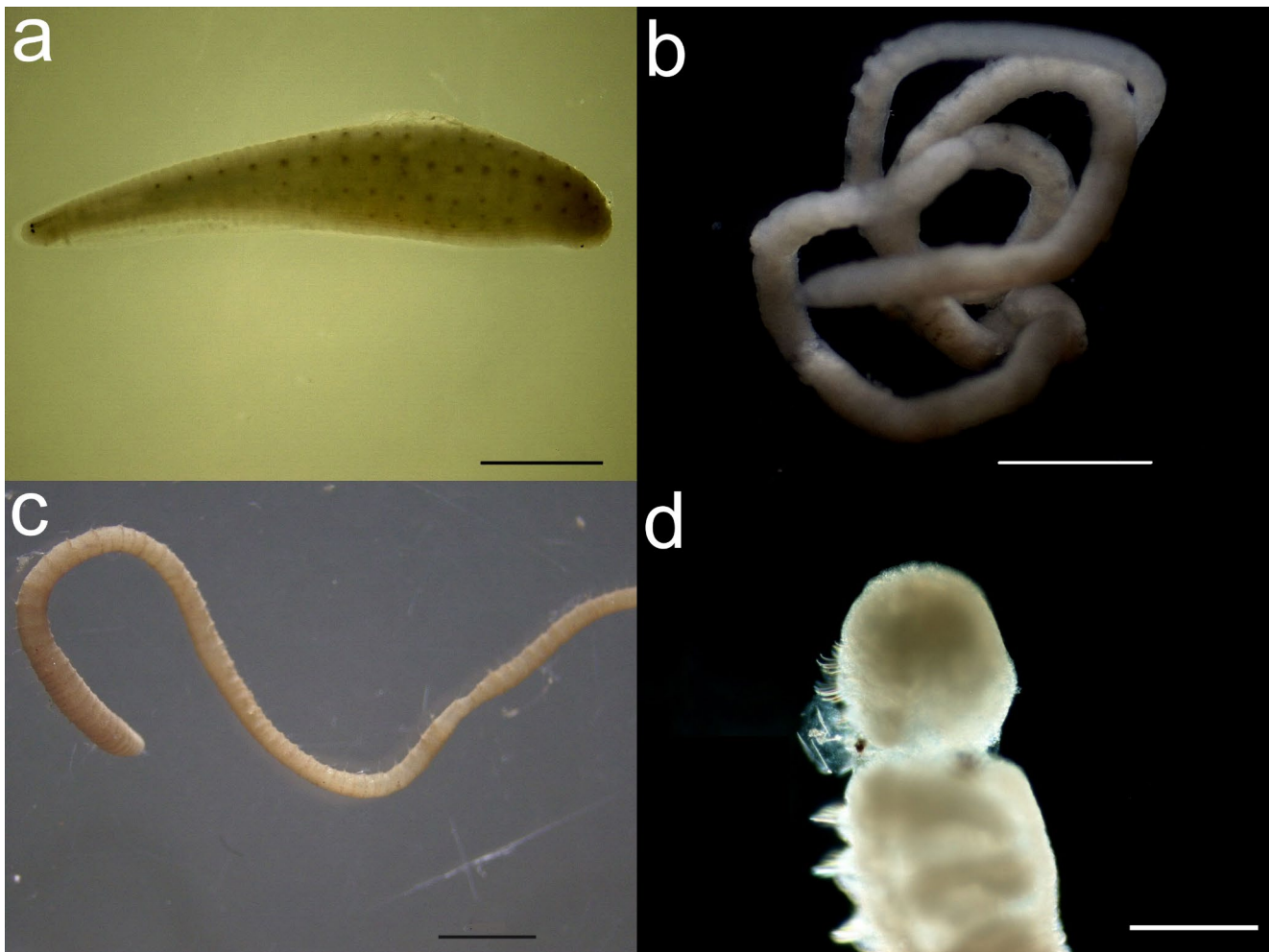


Fig. 6 *Helobdella europaea* (Kutschera 1985) dorsal view (a); *Bothrioneurum vej dovskyanum* Štolc 1886 (b), *Embolocephalus* sp. (c); *Chaetogaster* cf. *diaphanus* (Gruithuisen, 1828) (d). Scale bars: a,c: 2 mm; b: 1 mm; d: 0.25 mm

Order Anomopoda Sars, 1865.
Family Daphniidae Straus, 1820.
Genus *Ceriodaphnia* Dana, 1853.

Ceriodaphnia rigaudi Richard 1894 (Fig. 7b).

Type material Unknown.

Type locality Lao-Kay, Vietnam (Richard 1894).

Material examined 3 specimens, collected October 2021, on *E. densa* leaves.

Geographical distribution Commonly distributed in tropical and subtropical regions, more rarely in temperate regions (Alonso 1996).

Distribution in Portugal International Minho River (**this study**).

Ecological notes Maximum body size up to 0.4 mm (Alonso 1996); filter feeder (Alonso 1996); sampled in salinity of 0.06, water temperature 17.75 °C.

Family Euryceridae Kurz, 1875.
Genus *Eurycerus* Baird, 1843.

Eurycerus lamellatus (Müller 1776) (Fig. 7c).

Type material Holotype- Preserved specimen deposited at Natural History Museum of Denmark, catalogue number NHMD-84,865 (Eibye-Jacobsen et al. 2019).

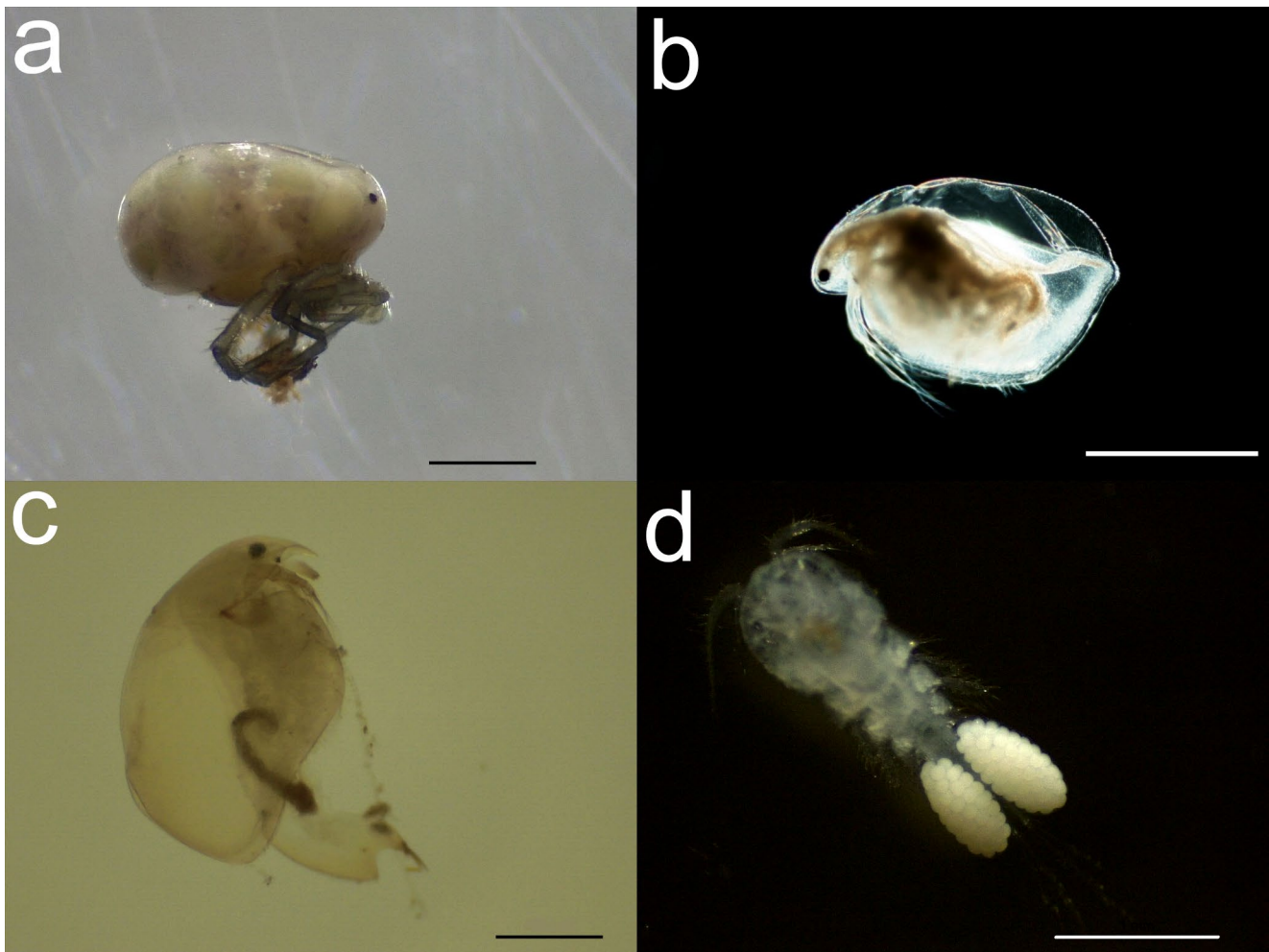


Fig. 7 *Lebertia insignis* Neuman, 1880 (**a**); *Ceriodaphnia rigaudi* Richard 1894 (**b**); *Eurycerus lamellatus* (Müller 1776) (**c**); *Macrocylops* sp. (**d**). Scale bars: a-c: 0,5 mm; d: 1 mm

Type locality Denmark (Müller 1776).

Material examined 6 specimens, collected from January to February 2022, on *E. densa* leaves.

Geographical distribution Palaearctic (Alonso 1996).

Distribution in Portugal International Minho River (**this study**) and Coimbra (Frey 1971), however details for this second record are lacking.

Ecological notes Maximum body size up to 3.3 mm (Alonso 1996); filter feeder (Alonso 1996); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 9.62 to 9.45 °C.

Class Hexanauplia Oakley, Wolfe, Lindgren & Zaharof, 2013.

SubClass Copepoda Milne Edwards, 1840.

Order Cyclopoida Burmeister, 1834.

Family Cyclopidae Rafinesque, 1815.

Genus *Macrocylops* Claus, 1893.

Macrocylops sp. (Fig. 7d)

Material examined 1 specimen collected January 2022, on *E. densa* leaves.

Ecological notes Predator (Abdullahi 1992); sampled in salinity of 0.06, water temperature 9.62 °C.

Remarks Single specimen kept for further morphological analysis. Three species of this genus are reported for Europe, *Macrocylops albidus* (Jurine, 1820), *Macrocylops distinctus* Richard, 1887 and *Macrocylops fuscus* (Jurine, 1820).

Class Malacostraca Latreille, 1802.

Order Amphipoda Latreille, 1816.

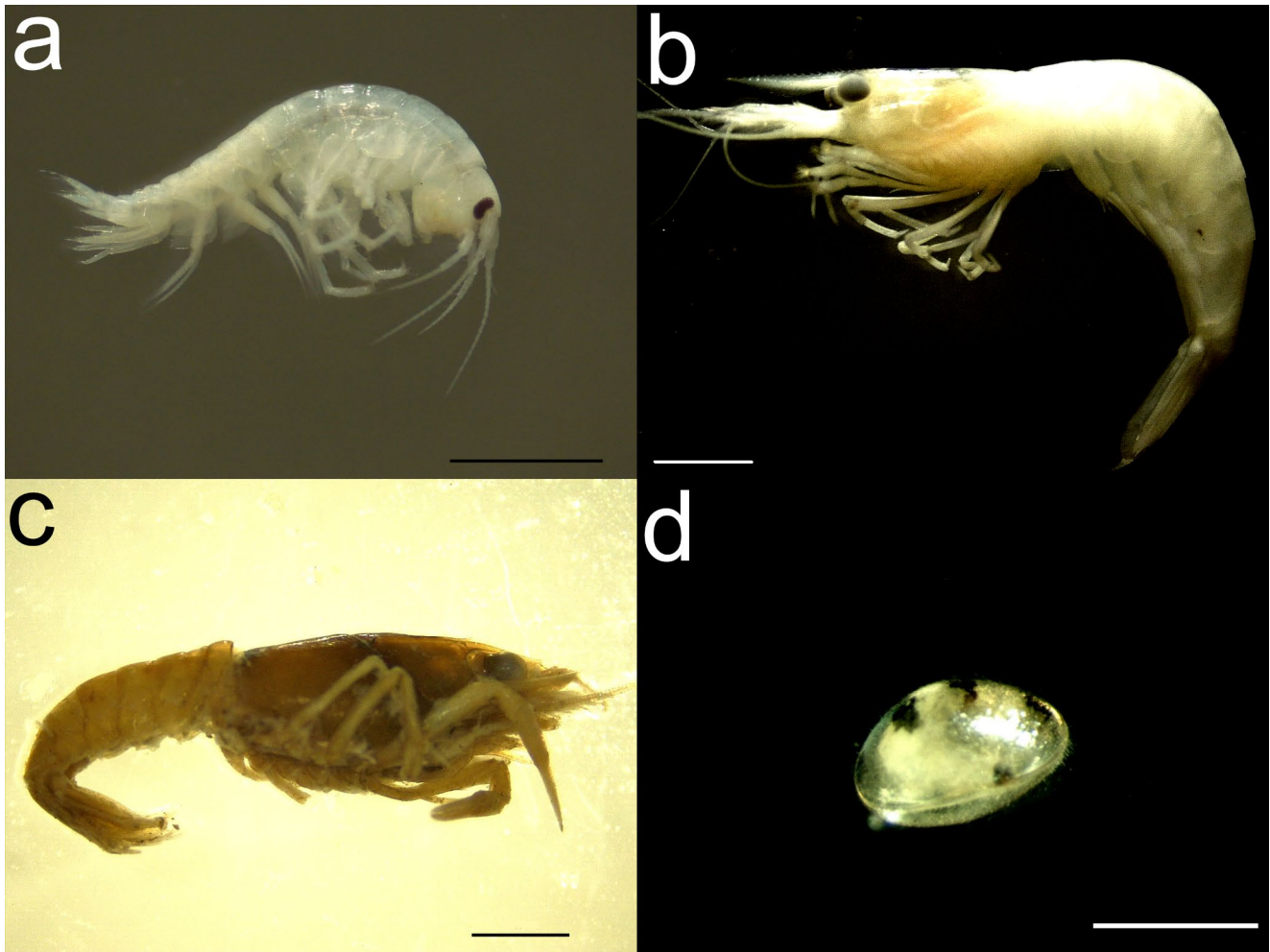


Fig. 8 *Gammarus chevreuxi* Sexton, 1913 (a); *Atyaephyra desmarestii* (Millet, 1831) (b); *Procambarus clarkii* (Girard, 1852) (c); *Cypridopsis vidua* (Müller 1776) (d). Scale bars: a-c: 2 mm; d: 0.5 mm

Family Gammaridae Leach, 1814.
Genus *Gammarus* Fabricius, 1775.

Gammarus chevreuxi Sexton, 1913 (Fig. 8a).

Material examined 77 specimens, collected from October 2021 to March 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP913220.

Ecological notes Sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks For synonymy details, geographical distribution and distribution in Portugal see Gomes et al. (2022a).

Order Decapoda Latreille, 1802.
Family Atyidae De Haan, 1849.
Genus *Atyaephyra* de Brito Capello, 1866.

Atyaephyra desmarestii (Millet, 1831) (Fig. 8b).

Material examined 24 specimens, size range 19 to 36 mm, collected from October 2021 to February 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP913236.

Ecological notes Sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks For synonymy details, geographical distribution and distribution in Portugal see Gomes et al. (2022b).

Family Cambaridae Hobbs, 1942.
Genus *Procambarus* Ortmann, 1905.

Procambarus clarkii (Girard, 1852) (Fig. 8c).

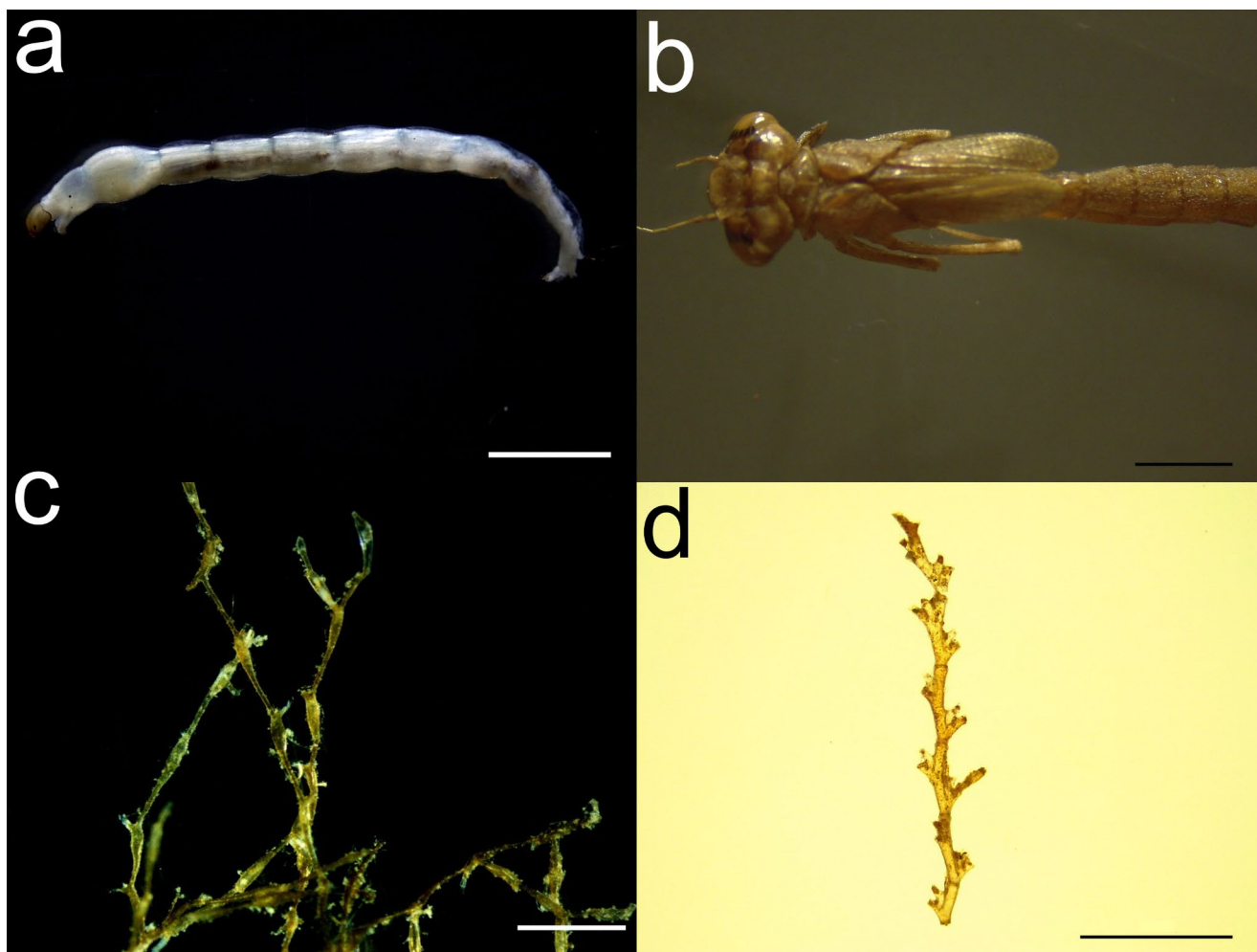


Fig. 9 *Psectrocladius limbatellus* (Holmgren 1869) (a); *Ischnura elegans* (Vander Linden 1820) (b); *Paludicella* aff. *articulata* (Ehrenberg, 1831) (c); *Sertularia cupressina* Linnaeus 1758 (d). Scale bars: a-d: 2 mm

Material examined 2 juvenile specimens, size range 10 to 13 mm, October 2021, on *E. densa* leaves.

Ecological notes Sampled in salinity of 0.06, water temperature 17.75 °C.

Remarks Common species for the study area; possibly introduced between the end of 80's and the beginning of the 90's (Sousa et al. 2013), replacing the native crayfish *Austropotamobius pallipes* (Sousa et al. 2008b). For synonymy details, geographical distribution and distribution in Portugal see Gomes et al. (2022b).

Class Ostracoda Latreille, 1802.
Order Podocopida Sars, 1866.
Family Cyprididae Baird, 1845.
Genus *Cypridopsis* Brady, 1867.

Cypridopsis vidua (Müller 1776) (Fig. 8d).

Type material Holotype- Preserved specimen deposited at Natural History Museum of Denmark, catalogue number NHMD-86,667 (Eibye-Jacobsen et al. 2019).

Type locality Denmark (Müller 1776).

Material examined 28 specimens collected from October 2021 to January 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP912986.

Geographical distribution Holarctic (Hunt et al. 2007).

Distribution in Portugal International Minho River (**this study**); common species in Portuguese freshwater environments (Fernandes Martins et al. 2010).

Ecological notes Maximum body size up to 500 µm (**this study**); epifaunal (Horne et al. 2019); nektobenthic, usually on macrophytes (Hunt et al. 2007); deposit feeder (Smith et

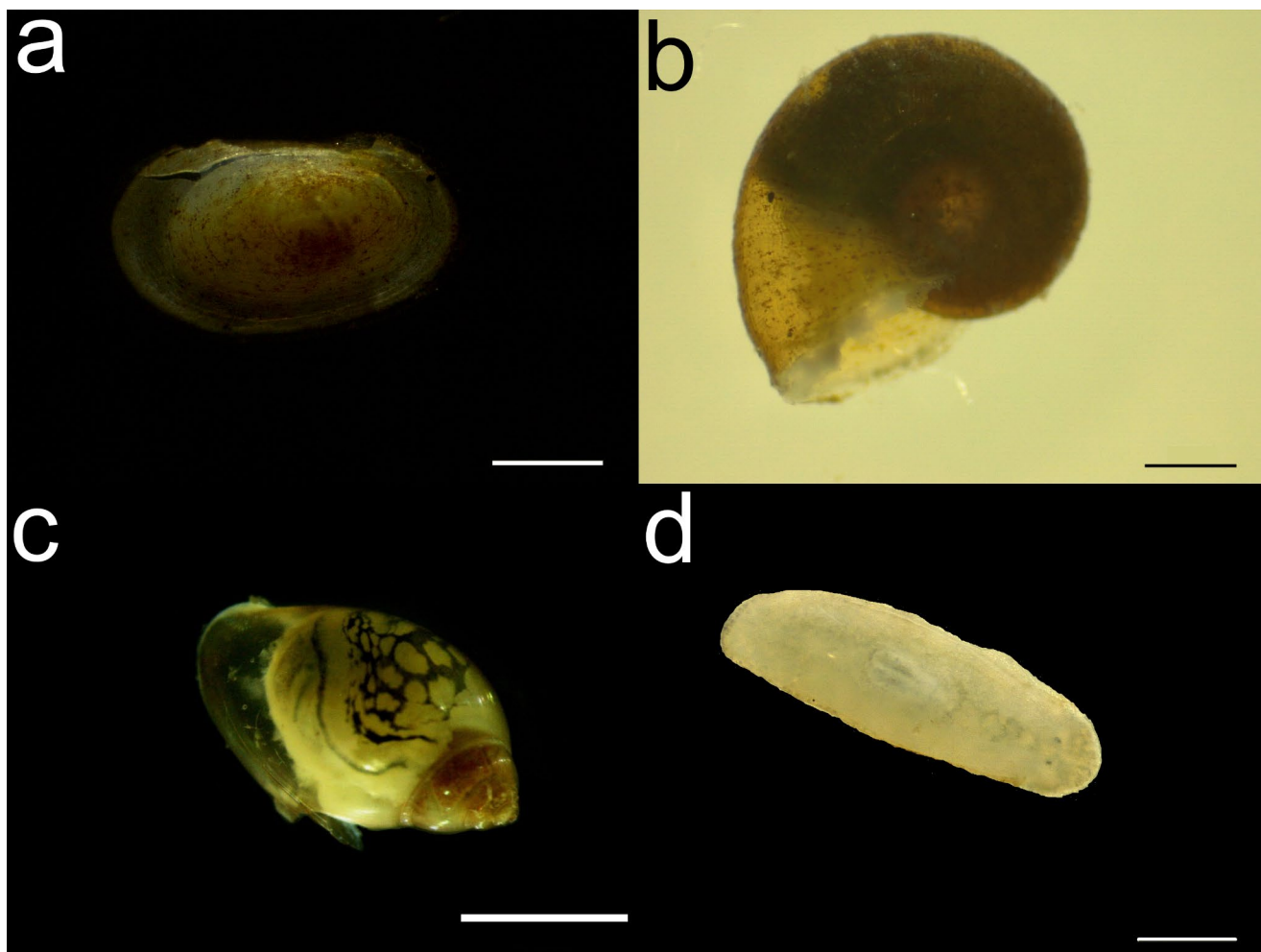


Fig. 10 *Ancyclus fluviatilis* Müller 1774 (a); *Menetus dilatatus* (Gould 1841) (b); *Physella acuta* (Draparnaud 1805) (c); *Girardia sinensis* Chen & Wang, 2015 (d). Scale bars: a-b: 0.5 mm; c: 2 mm; d: 1 mm

al. 2015); sampled in salinities ranging from 0.06 to 0.05, water temperatures ranging from 17.75 to 7.69 °C.

Remarks Species level identification based on molecular data; obtained DNA sequence formed a singleton MOTU, nested within group 1 of *Cypridopsis vidua* complex (Fig. 4b), comprised of several MOTU's. This unusual complex is further described in Cywinska and Hebert (2002).

Subphylum Hexapoda Latreille, 1825.

Order Diptera Linnaeus 1758.

Family Chironomidae Newman, 1834.

Genus *Psectrocladius* Kieffer, 1906.

Psectrocladius limbatellus (Holmgren 1869) (Fig. 9a).

Type material Lectotype- Preserved adult specimen deposited at Swedish Museum of Natural History, catalogue number NHRS-GULI000066396 (Holston 2022).

Type locality Svalbard, Norway (Holmgren 1869).

Material examined 660 specimens (larvae) collected from October 2021 to March 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP913217.

Geographical distribution Holarctic (Stur and Ekrem 2020).

Distribution in Portugal International Minho River (**this study**), species recorded between Bragança and Chaves, in River Divor, on Arraiolos (Cobo et al. 2001) and at the Azores archipelago (Murray et al. 2004).

Ecological notes Sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks Species level identification based on molecular data; genetic analysis revealed the presence of 5 different MOTU's; the sequence obtained nested within a MOTU comprised of exclusively of European sequences (Fig. 5a); minimum and maximum K2P distances between lineages are 14 and 22% respectively.

Order Odonata Fabricius, 1793.
Family Coenagrionidae Kirby, 1890.
Genus *Ischnura* Charpentier, 1840.

Ischnura elegans (Vander Linden 1820) (Fig. 9b).

Type material Unknown.

Type locality Bologna, Italy (Vander Linden 1820).

Material examined 85 specimens (larvae), collected from October 2021 to February 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP913230.

Geographical distribution Europe and Asia, from Iberian Peninsula to Japan (Boudot and Salamun 2015).

Distribution in Portugal Species recorded in the rivers Minho (**this study**), Mondêgo (Silva-Santos et al. 2004).

Ecological notes (larvae) Maximum body size up to 21 mm (Conesa-García 2021); predator (Thompson 1978); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks Low genetic distance between *Ischnura elegans*, *Ischnura graelsii* and *Ischnura saharensis* further discussed in Sánchez-Guillén et al. (2014).

Phylum Bryozoa Ehrenberg, 1831.
Order Ctenostomatida Busk, 1852.
Family Paludicellidae Allman, 1885.
Genus *Paludicella* Gervais, 1836.

Paludicella aff. *articulata* (Ehrenberg, 1831) (Fig. 9c).

Type material Unknown.

Type locality Berlin, Germany (Allman 1856).

Material examined 20 colonies, collected from September 2021 to February 2022, on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number OP913190.

Geographical distribution Cosmopolitan (Rogick and Schalie 1950; Massard and Geimer 2008).

Distribution in Portugal International Minho River (**this study**).

Ecological notes Maximum body size up to 8 cm (Smith et al. 2005); sessile (Loppens 1906; Rogick and Schalie 1950); filter feeder (Riisgård et al. 2010); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C; colonies on *E. densa* leaf surface, mostly on senescent individuals.

Remarks Phylogenetic reconstruction through COI sequences did not reveal any reliable placing or grouping within Ctenostomatida (Fig. 4a).

Phylum Cnidaria Hatschek, 1888.
Class Hydrozoa Owen, 1843.
Order Leptothecata Cornelius, 1992.
Family Sertulariidae Lamouroux, 1812.
Genus *Sertularia* Linnaeus 1758.

Sertularia cupressina Linnaeus 1758 (Fig. 9d).

Type material Specimen deposited at the Linnaean Collections, from The Linnean Society of London, catalogue number LINN 1298.5, 1298.6 (Cornelius 1979).

Type locality “Oceano” (Linnaeus 1758).

Material examined 1 fragmented specimen, collected November 2021, on *E. densa* leaves.

Geographical distribution North Atlantic, Baltic Sea to Portugal (MarLIN 2006 BIOTIC; Cornelius 1995).

Distribution in Portugal International Minho River (**this study**).

Ecological notes Maximum body size up to 50 cm; sessile; predator (MarLIN 2006 BIOTIC); Sampled in salinity of 0.06, water temperatures 7.69 °C.

Remarks Considering that this species is usually found in brackish habitats, its presence at the upper section of the estuary (freshwater) may be explained by upstream transportation during high flood tides.

Phylum Mollusca Linnaeus 1758.
Class Gastropoda Cuvier, 1795.
Family Planorbidae Rafinesque, 1815.
Genus *Ancylus* Müller, 1773.

Ancylus fluviatilis Müller 1774 (Fig. 10a).

Type material Syntype- Preserved specimen deposited at Natural History Museum of Denmark, catalogue number NHMD-90,989 (Eibye-Jacobsen et al. 2019).

Type locality Ilm river, Germany (Müller 1774).

Material examined 4 specimens, size 1.5 mm, collected November 2021 and February 2022 on *E. densa* leaves.

Geographical distribution Western Palaearctic (Pfenninger et al. 2003).

Distribution in Portugal Species recorded in the rivers Minho (Sousa et al. 2005, 2007), Febros (Pinto et al. 2010), Mondêgo (Silva-Santos et al. 2004) and its tributaries (Calapez et al. 2014), Guadiana (Pérez-Quintero 2007) and on Madeira island (Hughes 1995).

Ecological notes Maximum body size up to 12 mm (Rolán and Otero-Schmitt 1996); grazer (Calow 1973); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 9.45 to 7.69 °C.

Remarks Pfenninger et al. (2003) suggested the existence of four cryptic species of *Ancylus fluviatilis*, three of which occur in Portugal (one at the north region, one at the south and another occurring along the coastal line). (Holyoak et al. 2019) further suggested the revision of the three species described by (Morelet 1845), *Ancylus vitraceus* Morelet 1845, *Ancylus strictus* Morelet 1845 and *Ancylus obtusus* Morelet 1845 as base to formally solve these cryptic lineages.

Genus *Menetus* Adams & Adams, 1855.

Menetus dilatatus (Gould 1841) (Fig. 10b).

Type material Syntype- Preserved specimens deposited at Smithsonian Institution, National Museum of Natural History, catalogue number USNM 121,002 (Orrell 2022).

Type locality USA, Massachusetts, Nantucket island (Gould 1841).

Material examined 1499 specimens collected from October 2021 to March 2022 on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OP913162.

Geographical distribution Native to North America and introduced in Europe (Czyż et al. 2016).

Distribution in Portugal Species recorded in the rivers Minho (**this study**), Mondego and at Praia de Mira (Holyoak et al. 2019).

Ecological notes Sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks Shared haplotypes between Europe, Asia and North and Central America suggests a generalized colonization event originating from a same founding population (Fig. 3b).

Family Physidae Fitzinger, 1833.

Genus *Physella* Haldeman, 1842.

Physella acuta (Draparnaud 1805) (Fig. 10c).

Type material Unknown.

Type locality Garonne River, France (Draparnaud 1805).

Material examined 326 specimens collected from October 2021 to March 2022 on *E. densa* leaves.

Geographical distribution Cosmopolitan, native to North America (Lydeard et al. 2016).

Distribution in Portugal Species recorded in the rivers Minho (Sousa et al. 2005, 2007), Febros (Pinto et al. 2010), Mondêgo (Silva-Santos et al. 2004), Guadiana (Pérez-Quintero 2007), and on Vila Nova de Milfontes (Tornero et al. 2014) (on temporary ponds), at Tavira (França 1921) (unspecified collection data), and on Madeira island (Hughes 1995).

Ecological notes Maximum body size up to 13 mm (Spyra et al. 2019); grazer (Bernot et al. 2005); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C; specimens with epizoic oligochaete *Chaetogaster limnaei* inside the mantle cavity.

Remarks As this species first description is based on specimens collected in France (Draparnaud 1805), its introduction in Europe probably occurred before the 19th century, with the first record from the Iberian Peninsula dating from 1845 (García-Berthou et al. 2007) and from 1872 at Macaronesian archipelagos (Taylor 2003).

Phylum Platyhelminthes Minot, 1876.

Order Tricladida Lang, 1884.

Family Dugesidae Ball, 1974.

Genus *Girardia* Ball, 1974.

Girardia sinensis Chen & Wang, 2015 (Fig. 10d).

Type material Specimens deposited at Institute of Zoology, Chinese Academy of Sciences, Beijing, China (Chen et al. 2015).

Type locality Xinghu Lake, Zhaoqing, Guangdong Province, China (Chen et al. 2015).

Material examined 521 specimens collected from October 2021 to March 2022 on *E. densa* leaves; 1 specimen with COI sequence, GenBank accession number: OQ185384.

Geographical distribution Scattered records worldwide.

Distribution in Portugal International Minho River (**this study**).

Ecological notes Maximum body size up to 15.2 mm (Chen et al. 2015); sampled in salinities ranging from 0.06 to 0.04, water temperatures ranging from 17.75 to 7.69 °C.

Remarks Species level identification based on molecular data; shared haplotypes between Europe, Oceania, Asia, North Africa, North and Central America suggests a generalized colonization event originating from small founding populations (Fig. 3c). Considering that the genus *Girardia* is known for its distribution from North to South America only (Sluys et al. 2005), and based on the phylogenetic placement as a sister taxa to the North American *Girardia tigrina* and *Girardia dorocephala*, *G. sinensis* is most likely of North American ancestry.

Discussion

Of the 20 species examined, 6 were previously recorded at the Minho River estuary, namely *Gammarus chevreuxi* (Sousa et al. 2008a), *Atyaephyra desmaresti*, *Procambarus clarkii* (Gomes et al. 2022b), *Bothrioneurum veydovskyanum* (Timm and Abarenkov 2021), *Physella acuta* and *Ancylus fluviatilis* (Sousa et al. 2005, 2007). The remaining 12 are reported here for the first time from this geographic location, representing new records of *Helobdella europaea*, *Chaetogaster* cf. *diaphanus*, *Emboloccephalus* sp., *Paludicella* aff. *articulata*, *Girardia sinensis*, *Menetus dilatatus*, *Eurycerus lamellatus*, *Cypridopsis vidua*, *Ceriodaphnia rigaudi*, *Lebertia insignis*, *Psectrocladius limbatellus* and *Ischnura elegans*. Our record of *P.* aff. *articulata*, *G. sinensis*, *L. insignis* and *C. rigaudi* further constitute the first known occurrence of these species in Portuguese territory.

Crustacea was the most represented group, accounting for 7 species, while Gastropoda was the most abundant group with 1829 individuals from 3 species examined. A single colony fragment of the hydrozoan species *Sertularella cupressina* was found on *E. densa* leaves, however, given that this species is mostly distributed on brackish waters, its record in this study may be the result of upstream transport during strong high flood tides. Rare species, such as (*A. fluviatilis*, *C. rigaudi*, *E. lamellatus* and *Macrocyclops* sp. may have been underestimated due to their smaller size, as the methodology used was designed to collect larger macroinvertebrates. Exotic species established in the study area include *P. clarkii* and *P. acuta*, (previously recorded at the Minho River estuary), and the novel records of *M. dilatatus*, *G. sinensis* and *H. europaea*. These last three species reveal shared haplotypes among different continents (Figs. 2a and 3b and c), possibly resulting from globalized colonization events from similar founding populations (Bodt et al. 2020). Species level identification of Oligochaeta remains rather challenging for non-specialists (Table 1), even with highly detailed taxonomic guides, all specimens collected required further morphological and genetic analysis. The specimens identified as *E.* cf. *velutinus* have shown an incongruent identification with known COI-5P sequences for this species (15% genetic distance) which indicates the need for a revision of this genus in western Europe (Fig. 5c). Phylogenetic analysis of (*B. veydovskyanum*) revealed a possible species complex forming three marked monophyletic clades with 4–5% genetic distance, with two of those lineages occurring sympatrically in the study area (Fig. 5b). In turn the sequence of *P.* aff. *articulata*, did not retrieve significant percentage of similarity to any other sequence available on BOLD and GenBank. A placement within the order Ctenostomatida was attempted, however COI phylogenetic signal did not produce significant results, with low bootstrap support (Fig. 4a) and erratic topologies, which can be explained by an incipient state of Ctenostomida DNA reference libraries.

Species identification is becoming a highly dynamic process with the addition of DNA barcoding methodologies, forcing the re-evaluation of identifications based on morphological characters in a constant feedback loop. The absence of dichotomous keys for recently introduced exotic species (such as *G. sinensis*, *M. dilatatus* and *H. europaea*) in identification guides clearly hampered our study, as did the lack of historic data regarding macroinvertebrate assemblages before and after the introduction of *E. densa* in the study area. Furthermore, without the performed genetic analysis, misidentifications (*M. dilatatus* identified as *Gyraulus parvus*, *G. sinensis* identified as *Schmidtea* sp. and *H. europaea* identified as *Helobdella stagnalis*) and incomplete identifications (Table 1), could had further propagate the error on to

other efforts developed in the study area. This highlights the necessity of well-prepared DNA reference libraries that can provide points of comparison between species identifications, reduce errors of interpretation and incomplete information provided by most identification guides. Comparing our faunal composition, associated with *E. densa*, with results obtained by Pedroza-Ramos et al. (2016) on Lake Tota, Colombia, reveals a similar composition at the family level and overlapping genera such as *Helobdella*, *Eurycercus*, *Cypridopsis*, *Macrocyclus*, *Ischnura*, *Girardia* and *Physella*. Since most studies related to *E. densa* associated fauna have been performed in freshwater environments (Collier et al. 1999; Pedroza-Ramos et al. 2016), this study is one of the first attempts to characterize taxonomically its interactions on estuarine habitats, and so it is interesting that we found such similarities, especially regarding the American ancestry of some of this genera.

Conclusions

This study represents the first taxonomic characterization of the fauna associated with the macrophyte *Egeria densa* at the upper section of the international Minho River. Twenty species were recorded, 12 of which constituted new occurrences in the study area, and 4 were exotic species, with the first records for Portugal of the species *Paludicella* aff. *articulata* (Ehrenberg, 1831), *Girardia sinensis* Chen & Wang, 2015, *Lebertia insignis* Neuman, 1880 and *Ceriodaphnia rigaudi* Richard 1894. Local fauna catalogues remain largely unfinished, or outdated, and rarely account for the dynamic shifts of macroinvertebrate assemblages related to established bioengineer exotic species, such as *E. densa*. However, it is important to know this associated biodiversity for a better cause-effect assessment of species introductions and understand its impact on local ecosystems in order to optimise environmental management.

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Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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