

## Research Article

## Integrative taxonomy reveals the occurrence of the Asian freshwater snail *Sinotaia cf. quadrata* in inland waters of SW Europe

Andrés Arias<sup>1,\*</sup>, Irene Fernández-Rodríguez<sup>1</sup>, Omar Sánchez<sup>1</sup> and Yaisel J. Borrell<sup>2</sup>

<sup>1</sup>Department of Organisms and Systems Biology (Zoology), University of Oviedo, Oviedo 33071, Spain

<sup>2</sup>Department of Functional Biology, University of Oviedo, Oviedo 33007, Spain

Author e-mails: [ariasandres@uniovi.es](mailto:ariasandres@uniovi.es) (AA), [irefdezrguez@hotmail.com](mailto:irefdezrguez@hotmail.com) (IFR), [omarelrdd@hotmail.com](mailto:omarelrdd@hotmail.com) (OS), [borrellyaisel@uniovi.es](mailto:borrellyaisel@uniovi.es) (YJB)

\*Corresponding author

**Citation:** Arias A, Fernández-Rodríguez I, Sánchez O, Borrell YJ (2020) Integrative taxonomy reveals the occurrence of the Asian freshwater snail *Sinotaia cf. quadrata* in inland waters of SW Europe. *Aquatic Invasions* 15(4): 616–632, <https://doi.org/10.3391/ai.2020.15.4.05>

**Received:** 2 May 2020

**Accepted:** 6 July 2020

**Published:** 2 October 2020

**Handling editor:** Mikhail Son

**Thematic editor:** Ian Duggan

**Copyright:** © Arias et al.

This is an open access article distributed under terms of the Creative Commons Attribution License ([Attribution 4.0 International - CC BY 4.0](https://creativecommons.org/licenses/by/4.0/)).

## OPEN ACCESS

### Abstract

A multisource approach to assessing freshwater biodiversity reveals the first occurrence of the Asian freshwater snail *Sinotaia cf. quadrata* (Gastropoda, Viviparidae) in inland waters of the northern Iberian Peninsula (Spain) and southwestern France. We present a detailed characterization, combining morphological traits and molecular tools for a genetic identification, and illustration of the species and its ultrastructure, as well as key information regarding its reproductive biology and ecology at the new locations. We discuss the most plausible introduction pathways and vectors, its potential invasiveness and the subsequent impacts that it may generate in the receiving localities. Our preliminary results raise concerns about the threat of *Sinotaia cf. quadrata* to human health and ecosystem dynamics, since this species acts as an intermediate host for several parasite species. Finally, this study confirms once again the usefulness of an integrative taxonomical approach for shedding light on hidden biodiversity and the invasion of freshwater ecosystems.

**Key words:** alien species, bioinvasions, Viviparidae, biodiversity, Iberian Peninsula, France, freshwater ecosystems

### Introduction

Non-indigenous species (NIS) that become invasive can cause strong ecological impacts in receiving environments worldwide, including irreversible habitat alterations and damage to ecosystem functioning (Carlton and Geller 1993; Justine et al. 2014; Tricarico et al. 2016). Invasive species are an important cause of biodiversity loss and ecosystem homogenization through competition (Cowie 2005; Frederico et al. 2019) and alteration of physical or chemical properties of habitats (Katsanevakis et al. 2014a, b). Those consequences lead to loss of ecosystem services, impacts on food provision, tourism and lifecycle maintenance, often affecting human health and economic activities (Katsanevakis et al. 2014a, b; Borrell et al. 2017; Tricarico et al. 2017). During recent decades there has been an alarming increase in the number of non-indigenous species establishing populations worldwide (Simberloff 2014). Therefore, the early

detection of these non-native species is crucial for proper management and for preventing or mitigating potential threats (Arias and Torralba-Burrial 2014; Pochon et al. 2015; Tricarico et al. 2017). In this regard, molecular tools have been proven useful for species identifications, early detection and effective ecosystem monitoring when facing invaders (Ardura et al. 2017).

Freshwater ecosystems are particularly vulnerable to non-native species introductions, as they are usually closely related to human activities, and most aquatic organisms present a high dispersal rate (Tricarico et al. 2017). European freshwater environments host 756 non-native species (Nunes et al. 2015), of which 30 are molluscs (Cianfanelli et al. 2016). Aquaculture, sport fishing and aquarium and pet trade are among the most remarkable introduction pathways for non-native species in freshwater habitats (Mazza et al. 2014). To date, 18 introduced mollusc species have been reported in the Iberian freshwater environments and five of them, belonging to the Gastropoda, have developed an invasive behavior in the receiving ecosystems (i.e. *Physella acuta* (Draparnaud, 1805), *Melanooides tuberculata* (Müller, 1774), *Potamopyrgus antipodarum* (Gray, 1843), *Pomacea canaliculata* (Lamarck, 1822) and *P. insularum* (d'Orbigni, 1835)) (García-Berthou et al. 2007).

Members of the family Viviparidae are operculate freshwater snails with high intraspecific variability, occurring naturally in temperate and tropical regions worldwide (Ovando and Cuezco 2012; Hirano et al. 2019; Stelbrink et al. 2020). Two species of viviparids have been reported as invasive in North America: *Heterogen japonica* (Martens, 1861) and *Cipangopaludina chinensis* (Gray in Griffith and Pidgeon, 1833), with devastating effects on the distribution and abundance of native snails (Johnson et al. 2009; Solomon et al. 2010). Furthermore, *C. chinensis* has been also reported in Europe, from the Netherlands (Soes et al. 2011). Another species of the family, *Sinotaia cf. quadrata* (Benson, 1842), has been recorded as invasive in Argentina, South America (Ovando and Cuezco 2012; Ferreira et al. 2017) and more recently, from Central Italy (Cianfanelli et al. 2017).

During the course of a series of eco-monitoring programs aimed at investigating the freshwater fauna of rivers and ponds from Asturias (northern Spain) and Cazaux (southwest France), several specimens conchologically consistent with the diagnosis of the viviparid genus *Sinotaia* were found. These constitute the first record of this genus from the Iberian Peninsula. In this paper we present a detailed characterization, combining morphological traits and molecular tools for a genetic identification, and illustration of the species, as well as some notes on its reproductive biology and ecology at the new location. Moreover, we discuss the most plausible introduction pathways and vectors, its current status (established or invasive) and the potential impacts that it may generate in the receiving localities, including associated biosanitary risks.

## Materials and methods

### *Specimen collection, morphological analysis and taxonomic procedures*

Studied specimens (6 females, 1 male and 2 juveniles) were collected during May 2017 and June 2018 in the Nora River (43°22'N; 5°47'W, Colloto, Asturias, northern Spain). Another specimen was collected during August 2017 in the Cazaux Lake (44°29'N; 1°9'W, Gironde/Landes, Nouvelle Aquitaine, southwestern France). All collected specimens were prepared for preservation *in situ* and subsequently fixed and stored in 70% ethanol. The specimens were deposited at the Zoological Collection of the Department of Organisms and System (BOS) of the University of Oviedo (<https://bos.uniovi.es/>).

Specimens were examined under a dissecting stereomicroscope. Temporary glycerol slides of radulae and other internal structures were examined under a compound light microscope. Selected specimens were dissected to observe the internal anatomy and, in females, the developing eggs and embryos. Photographs were taken with a Canon EOS 1200D Digital SLR Camera with EF-S 18–55 mm f/3.5–5.6 III Lens; photomicrographs were taken with a Nikon Digital Sight DS-L1 camera mounted on a Nikon SMZ-U stereomicroscope. Selected juvenile specimens were prepared for scanning electron microscopy (SEM) for the study of their general morphology. Specimens were dehydrated in an ascending series of ethanol, critical point dried using acetone as the transition liquid, mounted on aluminum stubs and sputter coated with gold. Samples were then imaged using a JEOL 6610 LV scanning electron microscope. Line drawings were made with the aid of a *camera lucida* and digital photography.

Systematics and nomenclature follow MolluscaBase (2020). Terminology follows Reeve (1863), Ovando and Cuezco (2012) and Cianfanelli et al. (2017).

### *DNA extraction, PCR amplification and sequencing*

DNA was extracted from 20–50 mg of ethanol-preserved tissues from the foot of each of the eight studied specimens, using E.Z.N.A Mollusc DNA Kit (Omega Bio-tek, Omega Bio-Tek, Norcross, GA, USA) and following the manufacturer's protocol. The success of the extraction was checked through a horizontal electrophoresis (1% agarose gel), and DNA samples were stored at –20 °C. The mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified by means of polymerase chain reaction (PCR) in a total volume of 25 µl, using the universal primers LCO1490 and HCO2198 (Folmer et al. 1994). The reaction mixture contained 2.5 µL template DNA, 2.5 µM MgCl<sub>2</sub>, 1.25 µM deoxyribonucleotides triphosphate, 0.5 µM of each primer, 0.2 U Taq polymerase and the appropriate buffer at 1x final concentration. PCR conditions used were an initial denaturation step of 94 °C for 4 min, then 45 cycles of 94 °C for 30 s, 48 °C for 1 min, 72 °C for 2 min, and finally an extension of 72 °C for 7 min and 20 °C for 1 min.

A horizontal electrophoresis (2% agarose gel) with 0.05 µl/ml of SimplySafe™ (EURx Ltd. 80-297 Gdańsk Poland) was performed with the PCR products (25 µl), which were lately purified with Agarose-Out DNA Purification Kit (EURx Ltd. 80-297 Gdańsk Poland), following the manufacturer's instructions. Finally, the samples were sent for forward and reverse sequencing to MACROGEN (Amsterdam, the Netherlands), using standard Sanger sequencing method (Sanger and Coulson 1975).

### *Genetic analysis*

The consensus sequences obtained were edited and aligned using ClustalW in the freeware BIOEDIT (Hall 1999). Although all the studied individuals were sequenced, sequences obtained for two of the eight studied individuals were shorter than the others, and therefore they were excluded for the analysis. After alignment and corrections, preliminary genetic species identification was attempted using nBlast to search in BOLD ([http://boldsystems.org/index.php/IDS\\_OpenIdEngine](http://boldsystems.org/index.php/IDS_OpenIdEngine)) and GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) databases. Phylogenetic analysis was conducted using 61 COI sequences downloaded from Genbank from a wide range of Viviparidae. The taxon range includes the genus *Sinotaia* Haas, 1939 (Bellamyinae clade A from Stelbrink et al. (2020)) (Supplementary material Table S1). Other sequences already included in JCR published papers were included in the analyses (Schultheiß et al. 2014; Hirano et al. 2015, 2019; Gu et al. 2015a, b; Cianfanelli et al. 2017 and Stelbrink et al. 2020) (Table S1). The sequences were analyzed using the MEGA 7 software (Kumar et al. 2016). The Model Test software included in Mega 7 was used to predict the nucleotide substitution model showing the best BIC scores (Bayesian information criteria) (Nei and Kumar 2000). A Maximum Likelihood tree was done using the Tamura 3 parameter model (T92+G+I) of molecular evolution and 2000 bootstrap replicates. Moreover, the Network 5 program using the median-joining model (Bandelt et al. 1999; Fluxus Technology Ltd. 2020) was used for obtaining a haplotype network from representative species in the genus *Sinotaia* and including *Torotaia* as an external group.

## **Results**

### *Systematics and taxonomy*

Class Gastropoda Cuvier, 1795

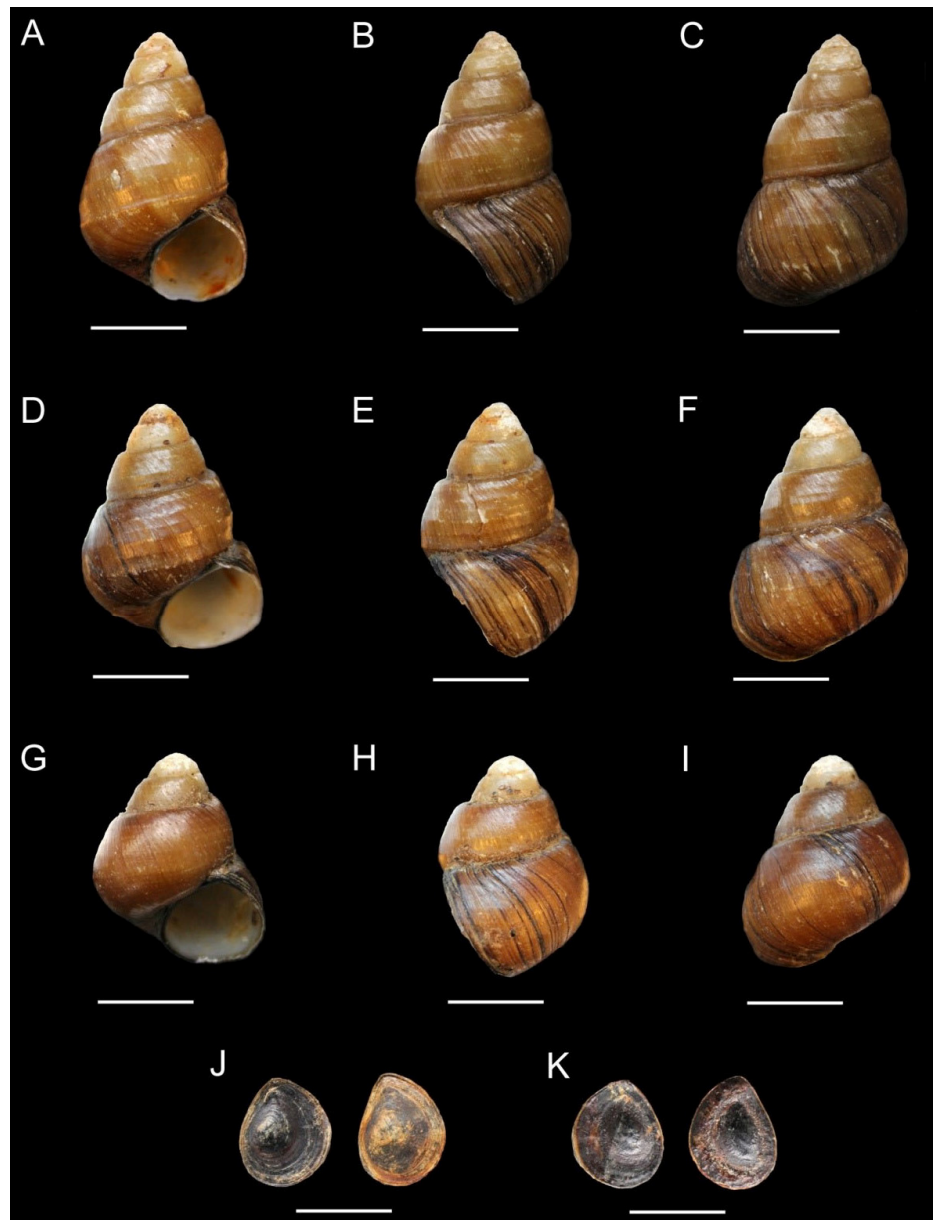
Order Architaenioglossa Haller, 1890

Family Viviparidae Gray, 1847

Genus *Sinotaia* Haas, 1939

### ***Sinotaia cf. quadrata* (Benson, 1842)**

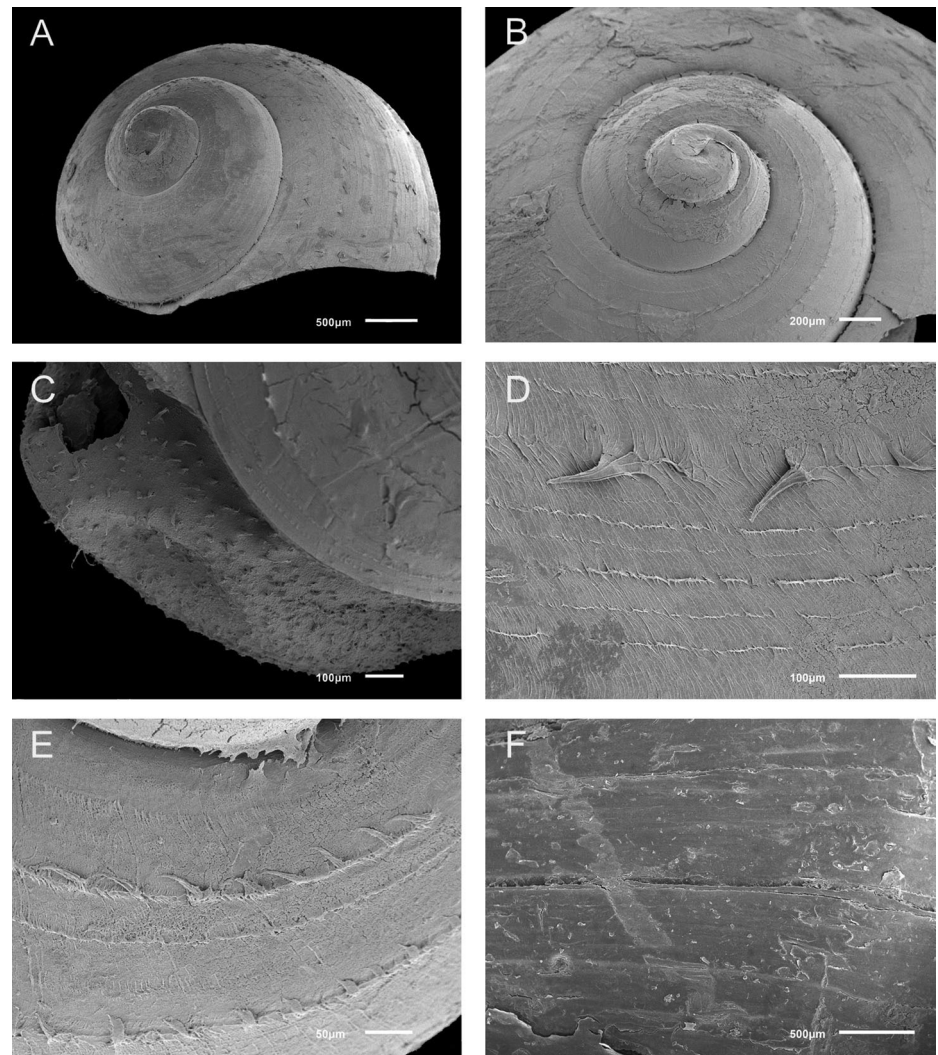
**Synonyms:** *Paludina lapillorum* Heude, 1890; *Paludina quadrata* Benson, 1842; *Paludina quadrata* var. *Heudei* Dautzenberg and H. Fischer, 1905; *Viviparus quadratus* (Benson, 1842).



**Figure 1.** *Sinotaia cf. quadrata*, adult specimens collected in the Nora river (northern Spain). A–C and D–F, different views of the shell of female individuals; G–I, views of the shell of a male individual; J–K, operculae (inner and outer views). All scale bars 10 mm. Photo by the authors.

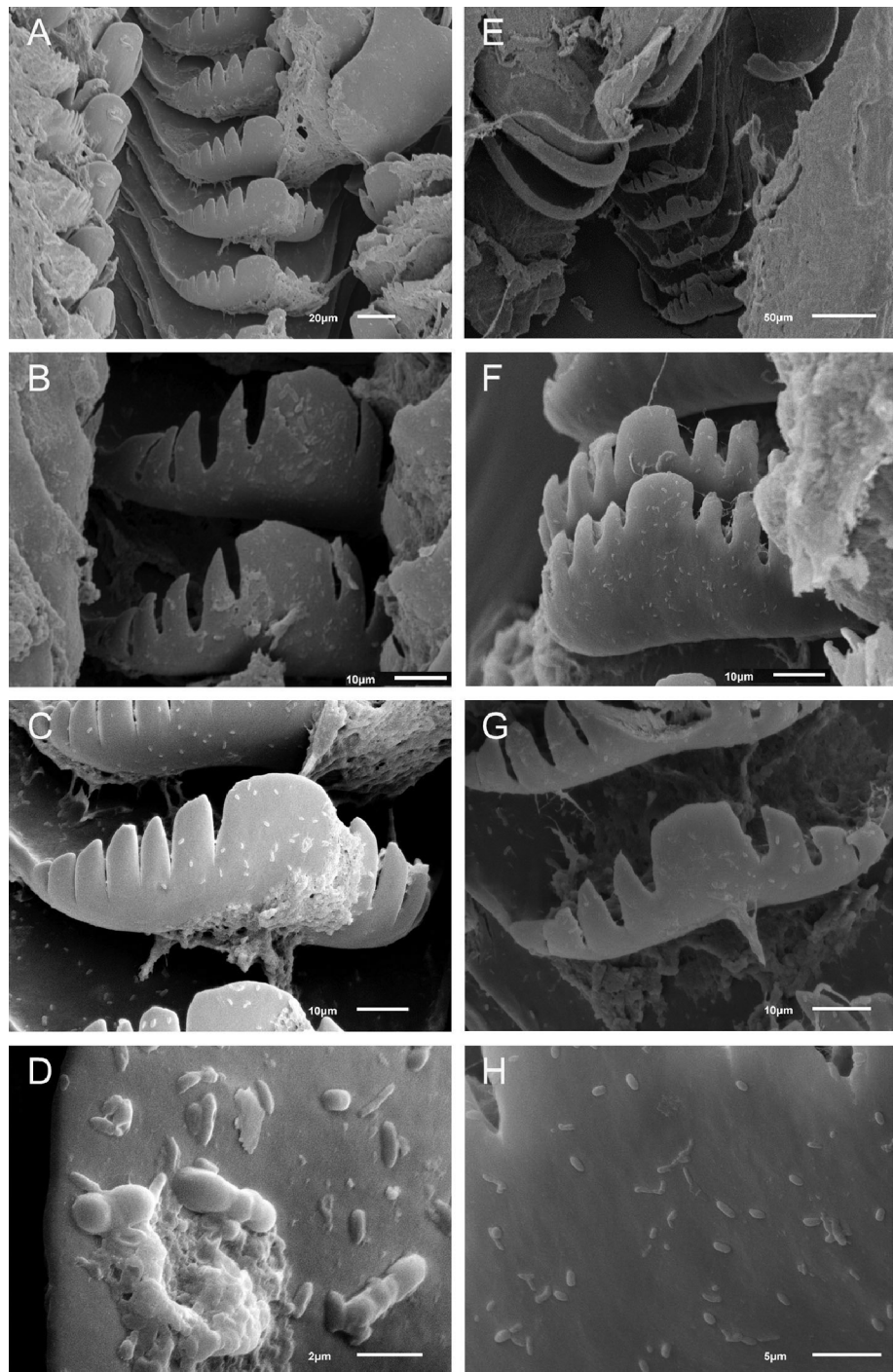
**Material examined:** 7 specimens from Nora River (Asturias, northern Spain), May 2017; 2 specimens from Nora River (Oviedo, Asturias, northern Spain), June 2018; 1 specimen from Cazaux Lake (Arcachon, Aquitania, southwestern France), August 2017.

**Description:** Thick, elongated and conical shell, 18–27 mm width and 25–36 mm height, yellowish-green or brown-olive, outer margin of aperture blackish (Figure 1A–I). Aperture pear-circular (Figure 1A, D, G), blackish axial growth lines usually present on first whorls, low spiral carina. Protoconch with 2–4 spiral raised lines with thin and triangular hairs in juveniles (Figure 2); often ground down in adults. Six to seven whorls slightly convex, separated by deep sutures, with a thin brownish band below suture on spire. Carinated whorls with 2–3 spiral keels, sometimes



**Figure 2.** Scanning Electron Photomicrographs of *Sinotaia cf. quadrata*. A, General view of juvenile shell; B, Detailed view of the micro-sculpture of the protoconch in juvenile specimen; C, detailed view of juvenile body surface; D–E, detailed view of the first whorl of juvenile shell showing the triangular periostracal hairs; F, view of the first whorl of the shell in an adult specimen. Photo by the authors.

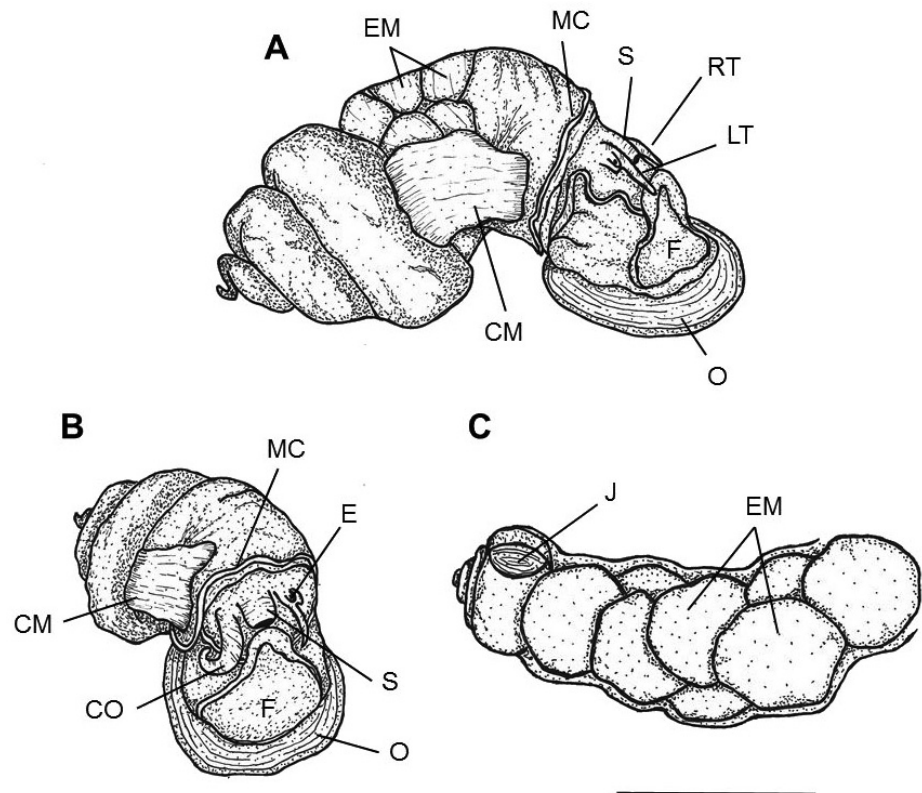
with spiral lines between keels that may carry triangular lamellae often absent in adult stages (Figure 2). Juveniles present a protoconch with 2–4 raised lines with long periostracal thin and triangular hairs (Figure 2A, B). The first part of the foot present dark spots in juveniles. Circular, horny operculum, smaller than the aperture, with concentric growth lines, occupying dorsal foot surface (Figure 1K, J). Taenioglossate radula, narrow and no longer than 2 mm (Figure 3A–G). Rachidian tooth with rectangular central cusp followed by 4–6 triangular cusps on each side (Figure 3A–G). Two lateral teeth per row, curved and multicuspid, similar to the central teeth. Body with width cylindrical snout with an aperture in the anterior margin (Figure 4A, B). Two large tentacles (double length of the snout) with a short ommatophore in the outer surface (Figure 2A). Males with modified right tentacle that serves as a copulatory organ (Figure 4B). Males and female specimens differing in the right margin of the mantle cavity, which



**Figure 3.** Scanning Electron Photomicrographs of *Sinotaia cf. quadrata* radulae. Radula of female (A–D) and male (E–H) specimens. A, Overall view of female radula; B, Detailed view of central rachidian teeth of the same; C, Female rachidian tooth showing rectangular central cusp and six triangular cusps on each side; D, Detailed view of high bacterial concentration on rachidian tooth; E, Overall view of male radula; F, Detailed view of central rachidian teeth; G, Male rachidian tooth with rectangular central cusp and four triangular lateral cusps; D–H, Detailed view of bacterial colonies on rachidian tooth. Photo by the authors.

is filled by the oviduct in females (Figure 4C). This oviduct works as a brood pouch filled by capsules, covered by a thin membrane, with different stages of developing oocytes, embryos and juvenile snails (Figure 4C).

**Remarks:** Studied *S. cf. quadrata* specimens displayed high radular variability, confirming previous observations by Ferreira et al. (2017) from



**Figure 4.** Line drawings of *Sinotaia cf. quadrata* soft body morphology. A, Lateral view of female specimen; B, Frontal view of male specimen showing the copulatory organ, i.e. modified right tentacle; C, Section of the oviduct with embryos. CM – columellar muscle; CO – copulatory organ; E – eye; Em – embryos; F – foot; J – juveniles; LT – left tentacle; MC – mantle collar; O – operculum; RT – right tentacle; S – snout. Scale bar: 10 mm.

the introduced populations in Argentina. Dissected females presented 5 to 6 triangular cusps on each side of the rachidian tooth (Figure 3A–C). Otherwise, the dissected male possessed only 4 cusps (Figure 3E–G). Studied specimens were  $25.9 \pm 2.5$  mm height and  $16.2 \pm 1.9$  mm width (mean  $\pm$  SD). Six out of 7 individuals were females (from Nora Rver), all of them carrying developing embryos and juveniles. Males were smaller than females (Height<sub>males</sub> =  $22.45 \pm 1.5$  mm; Height<sub>females</sub> =  $27 \pm 1.3$  mm), and sutures between whorls were deeper in males. The two specimens found in 2018 were juveniles of 9–10 mm height and 7–8 mm width.

#### *Biology and ecology*

*Sinotaia cf. quadrata* is an ovoviviparous species (commonly referred only as “viviparous”), in which the eggs develop within the female body and are birthed live. Studied specimens presented gonadal asynchrony with a continuous development of germ cells. Fertilized and unfertilized eggs and embryos in different stages of development were found along the female gonad (Figure 4A–C). Mature oocytes were subspherical to slightly trapezoidal in shape (Figure 4C) with a mean diameter of 2.07 mm (N = 15; SD = 0.11). Brooded juveniles ranged in shell height from 2 to 5 mm, with a mean of 3.4 mm (N = 20; SD = 0.86).



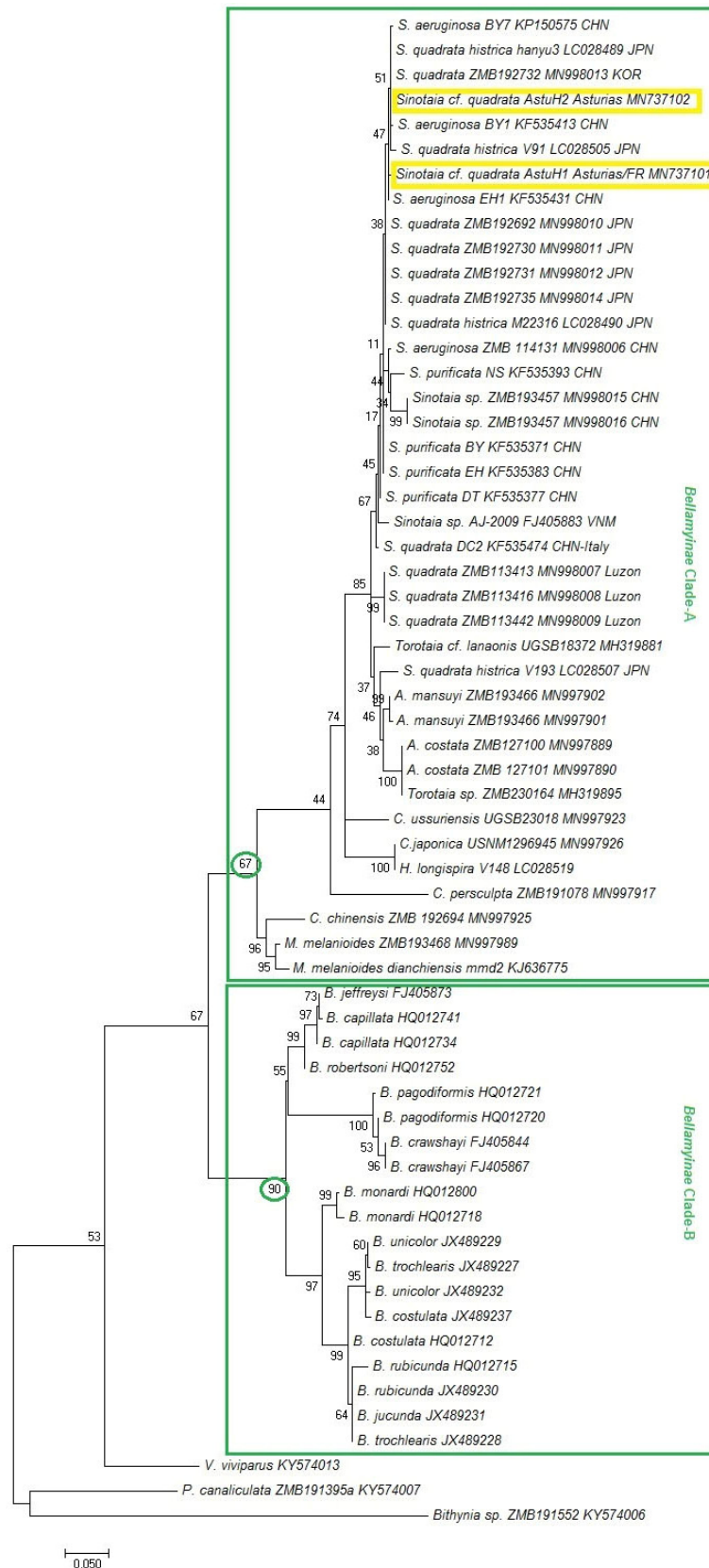
When the snails were prepared for the morphological study and placed in small Petri dishes (containing clean water) under illuminated light, two trematode cercariae were observed leaving the snails. The cercariae presented the following combination of morphological features: non-forked tail longer than its body and without finger-like processes; body entirely anterior to the tail and oral and ventral suckers present. Furthermore, colonies of cocci- and bacilli-shaped bacteria were found on the shell surface and the external body parts of the snails, as well as on the radula (Figure 3C–H).

### *Genetic results*

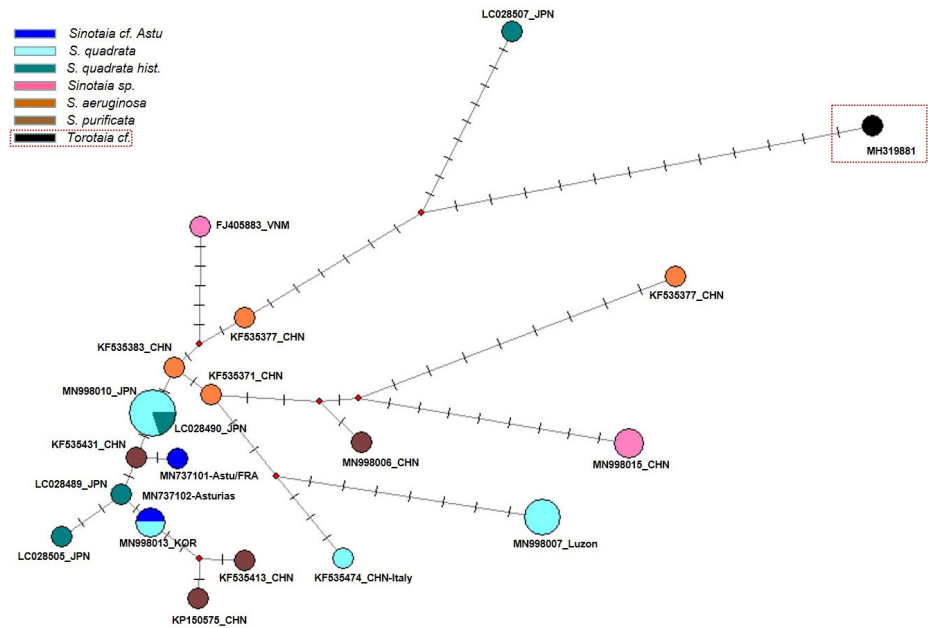
Fragments of 574-bp COI were successfully obtained from six studied specimens, and a total of two different haplotypes (*Sinotaia* AstuH1 and *Sinotaia* AstuH2) were obtained and deposited in GenBank under accession numbers MN737101 and MN737102 respectively. The NCBI Blast procedures conducted for genetic identifications revealed that more than 95 (*Sinotaia* AstuH1) and more than 100 (*Sinotaia* AstuH2) deposited sequences in Genbank coming from more than 8 putatively different species (*B. aeruginosa*, *B. purificata*, *B. quadrata*, *B. dispiralis*, *B. angularis*, *B. lapillorum*, *S. quadrata historica*, *B. turritus*) showed all 100% query coverage, E value = 0 and more than 99% of identity with the Asturian haplotypes.

Phylogenetic relationships using COI sequences from a wide range of Viviparidae were assessed through Maximum Likelihood estimations (Figure 5). The analysis revealed a similar topology and clades previously reported by Stelbrink et al. (2020). Our specimens showed two different entities (haplotypes) and were located within the Bellamyinae Clade A species complex, closely related to *S. quadrata*, *S. quadrata historica*, *S. purificata* and *S. aeruginosa*, all of them with Asian origin (Figure 5). In this clade we found low bootstrapping values (i.e. 67%) (Figure 5). The African species complex (Bellamyinae Clade B) showed higher support as evidenced by bootstrapping values (i.e. 90%).

The haplotypes analyses using Network revealed two different origins for the new reported haplotypes *Sinotaia* AstuH1 and *Sinotaia* AstuH2 haplotypes (Figure 6). The *Sinotaia* AstuH1 (MN737101) haplotype found in France and Asturias was connected to one named *S. purificata* haplotype (KF535431) found in China by Gu et al. (2015a, b) (Figure 6). The second Asturias haplotype (MN737102) coincided with a *S. quadrata* haplotype (MN998013) found in Korea (Stelbrink et al. 2020) and were connected to a haplotype named *S. quadrata historica* in Japan (LCO28489) (Hirano et al. 2015) (Figure 6). Few mutational changes connected most haplotypes from the reported species *S. quadrata*, *S. quadrata historica*, *S. purificata* and *S. aeruginosa*. Despite this, haplotypes representing those species and also *Sinotaia* sp. specimens reported by Stelbrink et al. (2020) are shown slightly further apart (in terms of a high number of mutational changes) from the



**Figure 5.** Molecular Phylogenetic analysis by Maximum Likelihood method based on COI sequences in Viviparidae using the T92+G+I model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 61 nucleotide sequences and a total of 424 positions in the final dataset. The clades previously reported by Stelbrink et al. (2020) and their bootstrapping values are indicated. In the genus *Sinotaia*, the Genbank numbers together region/locality data are also indicated.



**Figure 6.** The mitochondrial haplotypes COI network from representative species in the genus *Sinotaia* with *Torotaia* as external group. The legend shows species names and the Genbank numbers and region/locality where the haplotypes have been found are indicated. Node sizes are proportional to the number of samples in which the haplotype was observed. Colour portions refer to the proportion of species in which the haplotype was present. Bars indicate the number of mutations needed to get from one haplotype to another and red rhombuses represent hypothetical nodes.

cluster of haplotypes/species where the Asturian haplotypes were localized (Figure 6). These results support the morphological identification of our specimens, which undoubtedly belong to the Asian genus *Sinotaia*. Moreover, they seem to be part of a highly genetically diverse single species (probably *S. quadrata*) as suggested by morphology. Both statements justified the use of “cf.” (short for the Latin: confer/conferatur), evidencing the uncertainty or the provisional taxonomic status of *S. quadrata* (Ferreira et al. 2017).

### Discussion

The herein reported occurrence of *S. quadrata* from northern Spain represents the second record of the family Viviparidae in the Iberian Peninsula. The only previously reported species of the family is *V. viviparus*, which was recorded from three localities of Portugal and northern Spain (Morelet 1845). However, the current occurrence of *V. viviparus* in Iberia is considered unlikely, since the original record was only based on a few empty shells collected from Portugal (Morelet 1845) and new records of this species were absent in more recent malacological studies from Portugal and northern Spain (Castillejo 1982; Holyoak et al. 2019). *Sinotaia cf. quadrata* is an Asian viviparid snail, native to China, Korea and Taiwan (Lee 2009), which has subsequently been introduced to Japan, Thailand and Philippines (Ovando and Cuzzo 2012).

The 648 bp region of the COI gene forms the primary barcode sequence for members of the animal kingdom and almost all results show that more than 95% of species in test assemblages of varied animal groups have been shown to possess distinctive COI sequences (Ratnasingham and Hebert 2007, 2013). Comparison of 5' COI sequences from 13,000 pairs of congeneric species has showed a mean divergence of 11.3% (Hebert et al. 2003a). Ninety-eight percent of the species pairs under study by Hebert et al. (2003a, b) exhibited greater than 2% COI divergence (i.e., 10 substitutions per 500 bp). Cases of incomplete resolution involve species that are closely allied or taxonomically understudied groups (Ratnasingham and Hebert 2007, 2013). It seems clear that the subfamily Bellamyinae and the genus *Sinotaia* in particular can be one of these cases mentioned above. Recently, Kagawa et al. (2019) reported that shell morphologies of *Sinotaia* spp. in Japan easily change under the influence of the environment and advised that high flexibility in shell morphology should be carefully considered when managing or studying these freshwater molluscs. Gu et al. (2019) reported that the *Bellamya* monophyletic Chinese group showed very little genetic differentiation among species due to reversal of the river courses and the influence of intermingling of different lineages in China (Gu et al. 2019). In river snails, due to the lack of comprehensive taxonomic and genetic data sets, the phylogenetic relationships and their global diversification dynamics are still poorly understood (Stelbrink et al. 2020). These authors claimed that the genetic dataset from recent phylogenetic studies (i.e. Gu et al. 2019; Hirano et al. 2019) is taxonomically very incomplete, including misplaced species and/or using inadequate mutation rates (Stelbrink et al. 2020).

The genetic study conducted here demonstrates the inefficacy of blast assignments for species identifications in the genus *Sinotaia* due to the huge amount of misidentified sequences that are currently available in the Bold and Genbank databases. Moreover, even using only published sequences with voucher IDs for phylogenetic and network haplotypes analyses, our results suggest the occurrence of a single species (i.e. *S. cf. quadrata*) with a confused nomenclature probably due to a high phenotypic plasticity both in adults and juvenile stages. Studied specimens of *S. cf. quadrata* (Spain and France), *S. aeruginosa* from China (Gu et al. 2015a), *S. purificata* from China (Gu et al. 2015b), *S. quadrata histrica* from Japan (Hirano et al. 2015), *S. quadrata* from China from Gu et al. (2015a, b) and Stelbrink et al. (2020), and a recent haplotype found in Italy (Cianfanelli et al. 2017), showed all to have very low levels of COI genetic divergence among what are supposedly considered as different species. It is probable that the use of more barcodes from other ribosomal or nuclear genes could help elucidate the discernment of *Sinotaia* spp. nomenclature. Stelbrink et al. (2020), using analyses based on the genes 28S rRNA, COI, and H3, found very high levels of support for their phylogenetic trees. It is obvious that the

taxonomy of the genus *Sinotaia* requires revision, but this is outside of the scope of the present study. Even so, it is clear from the morphological observations, and the genetic results obtained here, that our studied specimens belong to the genus *Sinotaia*, and fit correctly with the *S. quadrata* species complex. Furthermore, the haplotypes network analysis may suggest multiple introduction events and may be from different origins. Multiple introduction episodes and adaptation of introduced populations constitute a serious risk and conditions for invasive outbreaks in all receiving ecosystems (Frankham 2005).

The population found at Nora River can be considered as established based on the finding of adult and reproductive-active females bearing embryos in 2017, and the presence of juveniles in 2018. Different studies have stated that *S. quadrata* is tolerant of a wide range of environmental variables, including pH and conductivity (Ovando and Cuezco 2012; Ferreira et al. 2017), which could be an advantage for successful establishment. Further, the viviparous strategy ensures fecundity and short lifespan, favoring successful reproduction (Ferreira et al. 2017). Another feature that may enhance their establishment is the presence of a strong and thick shell that may protect them from potential predators (Ovando and Cuezco 2012). Even so, future samplings are required in the area of study to confirm the persistence of this species in Nora River. A common introduction pathway of non-native species in aquatic environments is the aquarium trade; many gastropod species, including *Sinotaia* spp., have been introduced unintentionally in association with ornamental plants or fishes (Arias and Torralba-Burrial 2014; Ng et al. 2016; Patoka et al. 2017). The common water hyacinth (*Eichhornia crassipes* (Mart.) Solms, 1883) was found in the study area during our samplings, although this was removed later (authors *pers. obs.*). *Sinotaia* cf. *quadrata* may have been introduced in different juvenile stages with this ornamental plant. Another possible pathway could be an intentional introduction for human consumption, since these snails constitute a common food resource in Asia (Qian et al. 2014). This mechanism has been proposed as the most plausible introduction method to some regions of Italy, with presence of resident Asian communities (Cianfanelli et al. 2017). Another possible way of introduction was discussed by Ferreira et al. (2017) from Argentine populations. These authors consider that, since *Sinotaia* spp. are commonly used in aquaculture as a food source of carp and allied species, *S. cf. quadrata* may be accidentally introduced with fishes to artificial ponds in Argentina. Subsequently, the occasional floods could have connected the artificial ponds with natural water bodies, favoring the dispersion of the snail in the wild (Ferreira et al. 2017).

*Sinotaia* cf. *quadrata* may compete with native species for space or resources, or even change physicochemical parameters, turbidity or organic matter concentration of the water. Further, they may alter plant and algae

biomass, thereby affecting the native communities (Ovando and Cuezco 2012). *Sinotaia chinensis*, a species similar to *S. quadrata*, was introduced to North America and competed with other freshwater snails (e.g. *Physa* sp. and *Lymnaea* sp.), decreasing their abundances (Johnson et al. 2009). Moreover, *S. quadrata* also feeds on eggs and larval stages of some freshwater fishes such as the bluegill (*Lepomis macrochirus* Rafinesque, 1819), even producing a significant decrease in their abundance (Nakao et al. 2006). In the Nora River, the demersal eggs and embryos of two common fish species, minnows (*Phoxinus phoxinus* Kottelat, 2007) and brown trout (*Salmo trutta* Linnaeus, 1758), are similar to those of bluegills and unlike the bluegill, these species do not provide parental care to their offspring, making their predation by *S. cf. quadrata* even easier.

The trematode cercariae found leaving the body of one of the *S. cf. quadrata* studied specimens, are morphologically consistent with the diagnosis of the echinostome-like cercaria (Schell 1970). Several members of the genus *Echinostoma* Rudolphi, 1809 and close-related genera can infect humans as well as other mammals. In humans, these trematodes cause the symptomatology called Echinostomiasis, an intestinal infection commonly evidenced by a minor affliction, but in certain situations, the echinostomes can cause severe infections with ulceration of the intestinal mucosa and subsequent abdominal pain, diarrhea, anemia, and/or edema (Schell 1970). Previous studies revealed that *S. quadrata* populations from Taiwan and Japan might host metacercariae of echinostomid trematodes (Gracick and Fried 1998). Echinostomid metacercaria may affect humans by eating undercooked infected snails (Gracick and Fried 1998). Furthermore, populations of *S. quadrata* from Japan may also host the parasitic nematode *Angiostrongylus cantonensis* (Chen, 1935) (Lu et al. 2018). This nematode can cause Angiostrongyliasis in humans, damaging the brain and even the lungs, and causing meningitis (Lu et al. 2018). Consequently, the introduction of this species in the Nora River, northern Spain, implies a biosanitary risk, as they may infect the human population nearby and spread these diseases. Besides, our individuals presented bacteria on the radula and shell surface (Figure 3C, D, H) in densities high enough to make difficult the observation of the radular structures. Specimens of *S. quadrata* in some areas of Italy (where this species had been released for food consumption) presented a rate of bacterial content 234 times higher than the rate suitable for human consumption and thus, their harvestmen were forbidden by the local government in order to avoid a serious sanitary risk (Empoli 2019).

Finally, our results confirm once again, the usefulness of an integrative taxonomical approach to shedding light on hidden biodiversity and the invasion process of freshwater ecosystems.

## Acknowledgements

We thank Nuria Anadón (University of Oviedo) and Qian-Hong Gu (Henan Normal University) for their help and advice during the preparation of the ms. We are also grateful to Alfredo J. Quintana (SCTs, University of Oviedo) for assistance with SEM microscopy. We also thank two anonymous referees for their careful reviews and helpful suggestions. This is a contribution of the Asturias University Institute of Biotechnology (IUBA) and the Marine Observatory of Asturias (OMA).

## Funding declaration

This work was funded by the Project PAPI-19-EMERG-26 (University of Oviedo) and a grant from the Asturias Government to Research Groups FC-GRUPIN-IDI/2018/000201. Irene Fernández-Rodríguez is supported by a Severo Ochoa fellowship from the Principality of Asturias (BP16192).

## References

- Ardura A, Zaiko A, Borrell YJ, Samuiloviene A, García-Vázquez E (2017) Novel tools for early detection of a global aquatic invasive, the zebra mussel *Dreissena polymorpha*. *Aquatic Conservation: Marine and Freshwater Ecosystems* 27: 165–176, <https://doi.org/10.1002/aqc.2655>
- Arias A, Torralba-Burrial A (2014) First European record of the giant ramshorn snail *Marisa cornuarietis* (Linnaeus, 1758) (Gastropoda: Ampullariidae) from northern Spain. *Limnetica* 33: 65–72
- Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48, <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Borrell Y, Miralles L, Martínez-Marqués A, Semeraro A, Arias A, Carleos CE, García-Vázquez E (2017) Metabarcoding and post-sampling strategies to discover non-indigenous species: A case study in the estuaries of the central south Bay of Biscay. *Journal of Nature Conservation* 42: 67–74, <https://doi.org/10.1016/j.jnc.2017.07.002>
- Carlton JT, Geller JB (1993) Ecological roulette: the global transport of non-indigenous marine organisms. *Science* 261: 78–82, <https://doi.org/10.1126/science.261.5117.78>
- Castillejo J (1982) Los moluscos terrestres de Galicia (Subclass Pulmonata). Resúmen Tesis Doctoral, Universidad de Santiago
- Cianfanelli S, Talenti E, Bodon M (2016) *Mieniplotia scabra* (Müller, 1774), another gastropod invasive species in Europe and the status of freshwater allochthonous molluscs in Greece and Europe. *Mediterranean Marine Science* 17: 253–263, <https://doi.org/10.12681/mms.1331>
- Cianfanelli S, Stasolla G, Inghilesi AF, Tricarico E, Goti E, Strangi A, Bodon M (2017) First European record of *Sinotaia cf. quadrata* (Benson, 1842), an alien invasive freshwater species: accidental or voluntary introduction? (Caenogastropoda: Viviparidae). *Bollettino Malacologico* 53: 150–160
- Cowie RH (2005) Alien non-marine mollusks in the islands of the tropical and subtropical Pacific: a review. *American Malacological Bulletin* 20: 95–103
- Empoli S (2019) *Sinotaia quadrata* nell'Arno, la Lega Empoli: “Vietare la raccolta della lumaca d'acqua”. <https://www.gonews.it/2019/08/29/sinotaia-quadrata-nellarno-la-lega-empoli-vietare-la-raccolta-della-lumaca-dacqua/> (accessed June 2020)
- Ferreira AC, Paz LE, Rumi MA, Ocon CS, Altieri PD, Rodrigues-Capítulo A (2017) Ecology of the non-native snail *Sinotaia cf. quadrata* (Caenogastropoda: Viviparidae). A study in a lowland stream of South America with different water qualities. *Anais da Academia Brasileira de Ciências* 89: 1059–1072, <https://doi.org/10.1590/0001-3765201720160624>
- Fluxus Technology Ltd. (2020) Network 10.1.0.0 User Guide. Fluxus Technology Ltd., London, 55 p (accessed December 2020)
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3(5): 294–299
- Frankham R (2005) Resolving the genetic paradox in invasive species. *Heredity* 94: 385–385, <https://doi.org/10.1038/sj.hdy.6800634>
- Frederico RG, Salvador GN, Andrade A, Rosa GR, Torquato GV (2019) Freshwater ecosystem vulnerability: Is native climatic niche good enough to predict invasion events? *Aquatic Conservation: Marine and Freshwater Ecosystems* 29: 1890–1896, <https://doi.org/10.1002/aqc.3223>
- García-Berthou E, Boix D, Clavero M (2007) Non-indigenous animal species naturalized in Iberian inland waters. *Biological Invaders in Inland Waters: Profiles, Distribution, and Threats* 2: 123–140, [https://doi.org/10.1007/978-1-4020-6029-8\\_6](https://doi.org/10.1007/978-1-4020-6029-8_6)
- Gracick T, Fried B (1998) Echinostomiasis: a common but forgotten food-borne disease. *American Journal of Tropical Medicine and Hygiene* 58: 501–504, <https://doi.org/10.4269/ajtmh.1998.58.501>

- Gu QH, Husemann M, Ding B, Luo Z, Xiong BX (2015a) Population genetic structure of *Bellamya aeruginosa* (Mollusca: Gastropoda: Viviparidae) in China: weak divergence across large geographic distances. *Ecology and Evolution* 5: 4906–4919, <https://doi.org/10.1002/ece3.1673>
- Gu QH, Zhou CJ, Cheng QQ, Li XJ, Zhu GR, Zhang M, Gao YN, Dong J (2015b) The perplexing population genetic structure of *Bellamya purificata* (Gastropoda: Viviparidae): low genetic differentiation despite low dispersal ability. *Journal of Molluscan Studies* 81: 466–475, <https://doi.org/10.1093/mollus/eyv017>
- Gu QH, Husemann M, Wu HH, Dong J, Zhou CJ, Wang XF, Nie GX (2019) Phylogeography of *Bellamya* (Mollusca: Gastropoda: Viviparidae) snails on different continents: Contrasting patterns of diversification in China and East Africa. *BMC Evolutionary Biology* 19: 82, <https://doi.org/10.1186/s12862-019-1397-0>
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41: 95–98
- Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, Series B* 270: 313–322, <https://doi.org/10.1098/rspb.2002.2218>
- Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London, Series B (Suppl.)* 270: S96S99, <https://doi.org/10.1098/rsbl.2003.0025>
- Hirano T, Saito T, Chiba S (2015) Phylogeny of freshwater viviparid snails in Japan. *Journal of Molluscan Studies* 81: 435–441, <https://doi.org/10.1093/mollus/eyv019>
- Hirano T, Saito T, Tsunamoto Y, Koseki J, Prozorova L, Do VT, Matsuoka K, Nakai K, Suyama Y, Chiba S (2019) Role of ancient lakes in genetic and phenotypic diversification of freshwater snails. *Molecular Ecology* 28: 5032–5051, <https://doi.org/10.1111/mec.15272>
- Holyoak DT, Holyoak GA, da Costa Mendes RM (2019) A revised check-list of the land and freshwater Mollusca (Gastropoda and Bivalvia) of mainland Portugal. *Iberus* 37(1): 113–168
- Johnson PTJ, Olden JD, Solomon CT, Vander-Zanden MJ (2009) Interactions among invaders: community and ecosystem effects of multiple invasive species in an experimental aquatic system. *Oecologia* 159: 161–170, <https://doi.org/10.1007/s00442-008-1176-x>
- Justine JL, Winsor L, Gey D, Gros P, Thévenot J (2014) The invasive New Guinea flatworm *Platydemus manokwari* in France, the first record for Europe: time for action is now. *PeerJ* 2: e297, <https://doi.org/10.7717/peerj.297>
- Kagawa O, Saito T, Uchida S, Chiba S (2019) Phenotypic divergence in viviparid snails in a recently converted freshwater lagoon. *Plankton and Benthos Research* 14: 189–196, <https://doi.org/10.3800/pbr.14.189>
- Katsanevakis S, Coll M, Piroddi C, Steenbeek J, Rais B, Lasram F, Zenetos A, Cardoso AC (2014a) Invading the Mediterranean Sea: Biodiversity patterns shaped by human activities. *Frontiers in Marine Science* 1: 32, <https://doi.org/10.3389/fmars.2014.00032>
- Katsanevakis S, Wallentinus I, Zenetos A, Leppäkoski E, Çinar ME, Öztürk B, Grabowski M, Golani D, Cardoso AC (2014b) Impacts of invasive alien marine species on ecosystem services and biodiversity: A pan-European review. *Aquatic Invasions* 9: 391–423, <https://doi.org/10.3391/ai.2014.9.4.01>
- Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. *Molecular Biology and Evolution* 33: 1870–1874, <https://doi.org/10.1093/molbev/msw054>
- Lee JS (2009) Rediscovery of *Sinotaia cf. quadrata* (Architaenioglossa: Viviparidae) of Kumpung Reservoir in the Jellabuk-do, Korea. *The Korean Journal of Malacology* 25(3): 243–245
- Lu X-T, Gu Q-Y, Limpanont Y, Song LG, Wu Z-D, Okanurak K, Lv ZY (2018) Snail-borne parasitic diseases: an update on global epidemiological distribution, transmission interruption and control methods. *Infectious Diseases of Poverty* 7: 28, <https://doi.org/10.1186/s40249-018-0414-7>
- Mazza G, Tricarico E, Genovesi P, Gherardi F (2014) Biological invaders are threats to human health: an overview. *Ethology, Ecology and Evolution* 26: 112–129, <https://doi.org/10.1080/03949370.2013.863225>
- MolluscaBase (eds) (2020) MolluscaBase. <http://www.molluscabase.org> (accessed 27 February 2020)
- Morelet A (1845) Description des mollusques terrestres et fluviatiles du Portugal. J. B. Bailliere, Paris, 116 pp, <https://doi.org/10.5962/bhl.title.12994>
- Nakao H, Kawabata T, Fujita K, Nakai K, Sawada H (2006) Predation on bluegill (*Lepomis macrochirus*) broods by native snails. *Japanese Journal of Ichthyology* 53: 167–173, <https://doi.org/10.11369/jji1950.53.167>
- Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University Press, New York, 333 pp
- Ng TH, Tan SK, Wong WH, Meier R, Chan SY, Tan HH, Yeo DCJ (2016) Molluscs for sale: assessment of freshwater gastropods and bivalves in the ornamental pet trade. *PLoS ONE* 11: e0161130, <https://doi.org/10.1371/journal.pone.0161130>
- Nunes AL, Tricarico E, Panov V, Katsanevakis S, Cardoso AC (2015) Pathways and gateways of freshwater invasions in Europe. *Aquatic Invasions* 10: 359–370, <https://doi.org/10.3391/ai.2015.10.4.01>



- Ovando XMC, Cuzzo MG (2012) Discovery of an established population of a non-native species of Viviparidae (Caenogastropoda) in Argentina. *Molluscan Research* 32(3): 121–131
- Patoka J, Bláha M, Kalous L, Kouba A (2017) Irresponsible vendors: Non-native, invasive and threatened animals offered for garden pond stocking. *Aquatic Conservation: Marine and Freshwater Ecosystems* 27: 692–697, <https://doi.org/10.1002/aqc.2719>
- Pochon X, Atalah J, Wood SA, Hopkins GA, Watts A, Boedeker C (2015) *Cladophora ruchingeri* (C. Agardh) Kützing, 1845 (Cladophorales, Chlorophyta): a new biofouling pest of green-lipped mussel *Perna canaliculus* (Gmelin, 1791) farms in New Zealand. *Aquatic Invasions* 10: 123–133, <https://doi.org/10.3391/ai.2015.10.2.01>
- Qian ZX, Fang YF, He J (2014) A conchological review of Bellamyinae (Gastropoda: Viviparidae) of China. *Shell Discoveries* 1(3): 3–12
- Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System ([www.barcodinglife.org](http://www.barcodinglife.org)). *Molecular Ecology Notes* 7: 355–364, <https://doi.org/10.1111/j.1471-8286.2007.01678.x>
- Ratnasingham S, Hebert PDN (2013) A DNA-Based Registry for All Animal Species: The Barcode Index Number (BIN) System. *PLoS ONE* 8: e66213, <https://doi.org/10.1371/journal.pone.0066213>
- Reeve LA (1863) Monograph of the genus *Paludina*. *Monograph of the genus Paludina* 14: 11–15
- Sanger F, Coulson AR (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology* 94: 441–448, [https://doi.org/10.1016/0022-2836\(75\)90213-2](https://doi.org/10.1016/0022-2836(75)90213-2)
- Schell SC (1970) The Trematodes. WMC Brown Company Publishers, US, 355 pp
- Schultheiß R, Van Bocxlaer B, Riedel F, von Rintelen T, Albrecht C (2014) Disjunct distributions of freshwater snails testify to a central role of the Congo system in shaping biogeographical patterns in Africa. *Evolutionary Biology* 14: 42, <https://doi.org/10.1186/1471-2148-14-42>
- Simberloff D (2014) Biological invasions: What's worth fighting and what can be won? *Ecological Engineering* 65: 112–121, <https://doi.org/10.1016/j.ecoleng.2013.08.004>
- Soes DM, Majoor GD, Keulen SMA (2011) *Bellamyia chinensis* (Gray, 1834) (Gastropoda: Viviparidae), a new alien snail species for the European fauna. *Aquatic Invasions* 6: 97–102, <https://doi.org/10.3391/ai.2011.6.1.12>
- Solomon CT, Olden JD, Johnson PTJ, Dillon RT, Vander-Zanden MJ (2010) Distribution and community-level effects of the Chinese mystery snail (*Bellamyia chinensis*) in northern Wisconsin lakes. *Biological Invasions* 12: 1591–1605, <https://doi.org/10.1007/s10530-009-9572-7>
- Stelbrink B, Richter R, Köhler F, Riedel F, Strong EE, Bocxlaer BV, Albrecht CH, Hauffe T, Page TJ, Aldridge DC, Bogan AE, Du L, Marivene R, Manuel-Santos RM, Marwoto RM, Shirokaya AA, Von Rintelen T (2020) Global Diversification Dynamics Since the Jurassic: Low Dispersal and Habitat-Dependent Evolution Explain Hotspots of Diversity and Shell Disparity in River Snails (Viviparidae). *Systematic Biology* (in press), <https://doi.org/10.1093/sysbio/syaa011>
- Tricarico E, Junqueira AO, Dudgeon D (2016) Alien species in aquatic environments: a selective comparison of coastal and inland waters in tropical and temperate latitudes. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26: 872–891, <https://doi.org/10.1002/aqc.2711>
- Tricarico E, Borrell YJ, García-Vázquez E, Rico JM, Rech S, Scapini F, Johović I, Rodríguez-Ezpeleta N, Basurko OC, Rey A, Gough P, Aquiloni L, Sposimo P, Inghilesi AF, Haubrock P, Delgado JF, Skukan R, Hall D, Marsh-Smith S, Kilbey D, Monteoliva AP, Muha TP, Rodríguez-Rey M, Rolla M, Rehwald HK, Garcia de Leaniz C, Consuegra S (2017) Developing innovative methods to face aquatic invasions in Europe: the Aquainvad-ED Project. *Management of Biological Invasions* 8: 403–408, <https://doi.org/10.3391/mbi.2017.8.3.13>

### Supplementary material

The following supplementary material is available for this article:

**Table S1.** GenBank accession numbers of the sequences of the studied specimens and of the other specimens used for the genetic analysis, with their collection area.

**Appendix 1.** References for Table S1.

This material is available as part of online article from:

[http://www.reabic.net/aquaticinvasions/2020/Supplements/AI\\_2020\\_Arias\\_etal\\_SupplementaryMaterials.xlsx](http://www.reabic.net/aquaticinvasions/2020/Supplements/AI_2020_Arias_etal_SupplementaryMaterials.xlsx)