Spira 9 (2023) 71-78

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A new European population of *Sinotaia quadrata* (Benson, 1842) (Gastropoda: Viviparidae) located at the Segura River basin (SE Spain)

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Rebut el 30 d'octubre de 2023
Acceptat el 20 de desembre de 2023

Editat per Jordi Corbella

Keywords: invasions, freshwater, Iberian Peninsula, gastropods

Paraules clau: invasions, aigua dolça, península Ibèrica, gastròpodes

ABSTRACT

A new population of the Viviparid alien snail *Sinotaia quadrata* is reported for the Segura River basin (Southeastern Iberian Peninsula), after the finding of specimens in two consecutive seasons in the river and adjacent irrigation canals. Attribution to the species was confirmed by the use of morphological and genetic characters. The population seems established, as juvenile specimens were found. The species identity of all other known European populations of the genus is confirmed as *S. quadrata* by comparative molecular analysis.

RESUM

Es descriu una nova població del vivipàrid invasor *Sinotaia quadrata* per a la conca del riu Segura (SE península Ibèrica), després de la troballa d'espècimens per dues temporades consecutives al riu i canals d'irrigació adjacents. L'atribució de l'espècie es confirma amb l'ús de caràcters morfològics i genètics. La població sembla establerta, ja que es van trobar espècimens juvenils. La identitat específica de les altres poblacions europees del gènere es confirma com a *S. quadrata* amb anàlisis moleculars comparatives.

Introduction

Freshwater ecosystems worldwide are facing a fast colonization of non-native species, including among them a huge number of molluscs (Strayer, 2010; Oliva-Paterna *et al.*, 2021). In the south-east Iberian Peninsula, a few large non-native aquatic gastropods have been cited in recent years, including *Cipangopaludina chinensis* (Gray, 1834), *Pomacea diffusa* Blume, 1957, and *Sinotaia quadrata* (Benson, 1842), all of them in the Valencian Autonomous Community, in a restricted area in the province of Alicante (Hernández Núñez de Arenas *et al.*, 2020, 2022; Quiñonero-Salgado *et al.*, 2022). Aquarium pet trade seems the most likely way of introduction for these species.

Sinotaia quadrata is a snail belonging to the Viviparidae family, native from East Asia which has colonized multiple countries, including other Asian areas, but also Argentina in South America (Ovando *et al.*, 2012; Reyna *et al.*, 2018; Altieri *et al.*, 2021), U.S.A in North America (O'Leary, 2021), Johannesburg in South Africa (Miranda *et al.*, 2022), and is also present in Europe (Cianfanelli *et al.*, 2017; Arias *et al.*, 2020). This species seems to feed on epiphytic algae and detritus (Altieri *et al.*, 2021) but it has been reported to feed also on fish eggs. It has a relative short lifespan, high fecundity, and its high tolerance to changes in pH, conductivity, and nutrient and bacterial concentration make this species a successful invader (Cianfanelli *et al.*, 2017; Arias *et al.*, 2020).

The first reported population of *S. quadrata* in Europe was in the Arno River in Tuscany (Italy) (Cianfanelli *et al.*, 2017), which seems well established, and was likely introduced in an intentional way. Afterwards, a few specimens were recorded from NW Spain and southern France (Arias *et al.*, 2020), and more recently some specimens were also found in the locality of Gandía, in the south-east (Quiñonero-Salgado *et al.*, 2022). While in the first Iberian population a couple of juveniles were also found, no further confirmation of its establishment has been reported, thus suggesting that both Iberian populations corresponded to localized introductions, probably related to release of animals acquired through aquarium pet trade. As the snail may also be used as bait in sport fishing, accidental loss of specimens by sport fishermen may be an alternative way of introduction (Reyna *et al.*, 2018). In Italy, human consumption has been reported as the apparent reason for its introduction (Cianfanelli *et al.*, 2017).

The taxonomy of the genus *Sinotaia* F. Haas, 1939 has been the subject of debate, since some species have high morphological plasticity and variability, resulting in an apparent chaos when molecular works have used misidentified specimens (Arias *et al.*, 2020). For this reason, a number of publications have cautiously

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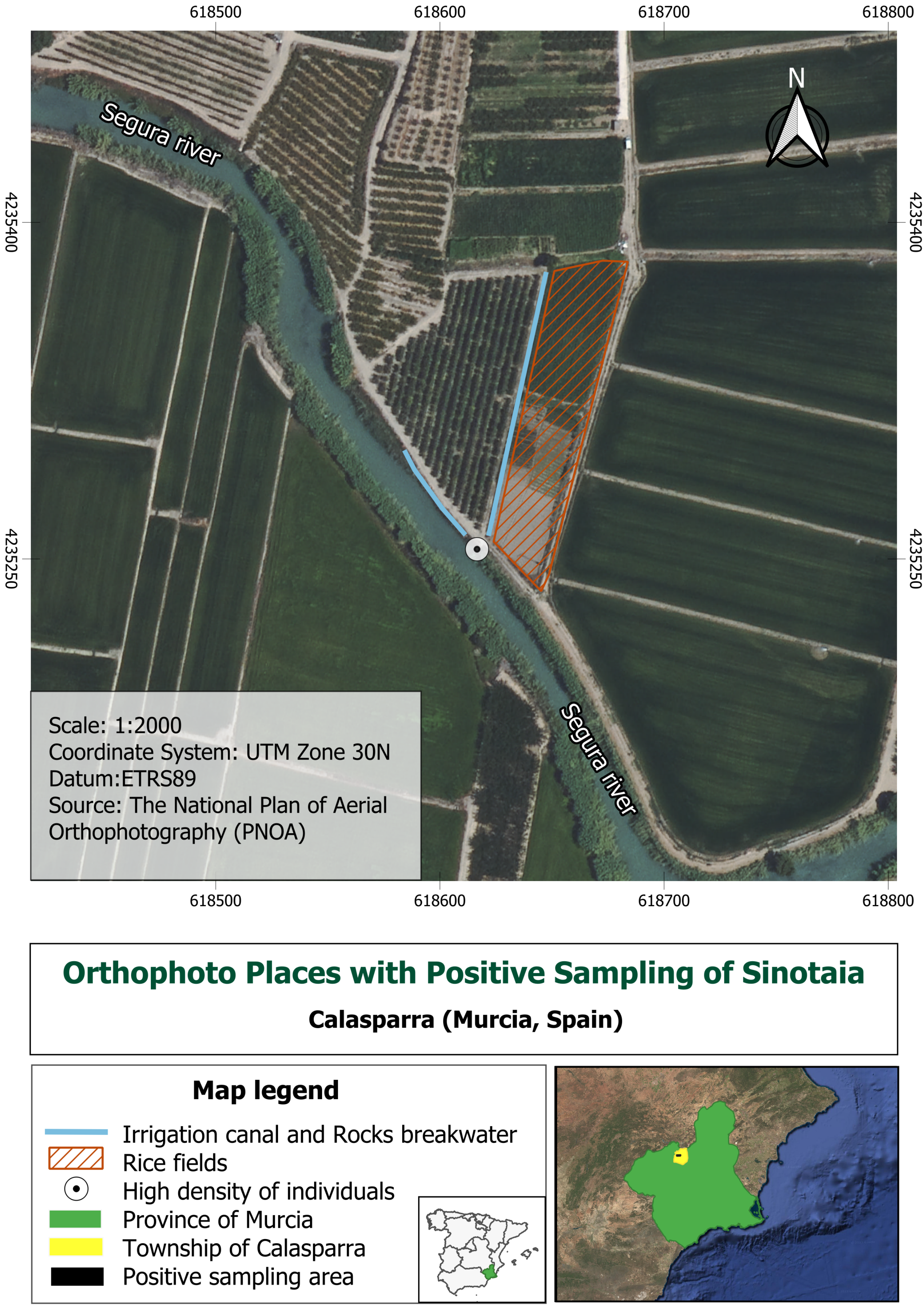

Figure 1. Location and detailed orthophoto of positive sampling sites for *Sinotaia quadrata*.

used the terminology "cf. *quadrata*" for the assignation of their specimens, given the uncertainty on this genus. The recent paper by Ye *et al.* (2021) seems to have settled the problem quite convincingly, showing that shell morphology is not enough for a correct species identification, given the high degree of plasticity in the genus. In their multidisciplinary work, genetic and morphological methodologies were combined to delimit the taxonomy of the genus *Sinotaia*, reaching the conclusion that the studied species do not present sufficient morphological and genetic differences to allow differentiation into species, therefore considering *S. lapillorum* (Heude, 1890), *S. lapidea* (Heude, 1889), *S. aeruginosa* (Reeve, 1863), *S. purificata* (Heude, 1890), *S. angularis* (O.F. Müller, 1774) and *S. turrita* (Yen, 1939) morphotypes and synonyms of *S. quadrata*. Indeed, minor variations in shell shape are not indicative of distinct *Sinotaia* species, and its variation shows no clear separations and seems rather related to certain environmental plasticity (Ye *et al.*, 2021).

In this paper, we report a new population of *Sinotaia quadrata* in Spain, and confirm its identity by molecular markers, showing the species to be identical to the other European populations known to date.

Material & methods

Sampling

The Segura River Basin covers a great variety of freshwater ecosystems with both natural and artificial water bodies. For water management purposes these freshwaters bodies are highly connected. Annually, the Segura River Basin Authority (Confederación Hidrográfica del Segura) undertakes studies for the monitoring of the ecological status of surface freshwater. During the execution of the sampling campaigns, valuable information on the presence and dispersion of invasive species in the basin is obtained.

After the finding of some *S. quadrata* specimens in the Segura River in the locality of Calasparra during the spring of 2022 (Figure 1), a more exhaustive sampling campaign was carried out in 2023 to preliminarily evaluate the extent of dispersion of the species. For this, ten sites located in the middle and lower course of the Segura River were selected to visually detect the species (Table 1). To maximize the chance of finding *S. quadrata*, the habitats sampled within a site were selected by its suitability for hosting communities of the species (breakwaters, rice fields and irrigation canals) (Figures 1 & 2).

Specimens of three European known populations were analysed genetically: Arno in Italy, and Gandía (Valencia province) in Spain, altogether with the recent discovered population in the Segura River (Murcia province). A total of 32 specimens of the new population were measured for total shell height.

DNA extraction, PCR amplification and sequencing

DNA was extracted from 20–50 mg of ethanol-preserved tissue from the foot, using E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, Norcross, GA, USA) and following the manufacturer's instructions. DNA integrity was checked by a horizontal electrophoresis (1% agarose gel), and DNA samples were stored at −20 ℃. The mitochondrial cytochrome c oxidase subunit I (COI) gene fragment was amplified by means of polymerase chain reaction (PCR) in a total volume of 40 µL, using the universal primers LCO1490 and

Figure 2. Main habitats where *Sinotaia quadrata* was located. A) Overall view of the Segura River at Calasparra (Murcia province); B) Detail of a submerged breakwater along the riverbed; C) A specimen photographed in the mud in the river; D) Irrigation canal along a rice field.

Table 1. Sampling localities in this study, including municipality, type of habitat, coordinates, and date of sampling.

Sample ID	Municipality	Habitat	Coordinates (Datum: ETRS89)		Sampling date
			Start of section	End of sectiom	
1	Hellín (Albacete)	Rice field. Mundo-Segura river confluence	30S 616685 4241755	30S 616585 4241887	10/04/2023
2	Calasparra (Murcia)	Rice field. Argos-Segura river confluence	30S 615284 4233757	30S 615252 4233818	27/04/2023
3	Calasparra (Murcia)	Rice field. Segura River	30S 618073 4235160	30S 618133 4235336	26/04/2023
4	Calasparra (Murcia)	Breakwater. Segura River	30S 618189 4235189	30S 618238 4235209	26/04/2023
5	Calasparra (Murcia)	Irrigation canal. Segura River	30S 618225 4235450	30S 618288 4235267	26/04/2023
6	Calasparra (Murcia)	Breakwater. Segura River	30S 618586 4235293	30S 618620 4235250	26/04/2023
7	Calasparra (Murcia)	Rice field and irrigation canal (right bank). Segura River	30S 618648 4235379	30S 618620 4235258	26/04/2023
8	Calasparra (Murcia)	Rice field Segura River	30S 618683 4235377	30S 618644 4235241	26/04/2023
9	Blanca (Murcia)	Breakwater. Segura River	30S 642007 4227042	30S 642057 4226882	11/04/2023
10	Guardamar del Segura (Alicante)	Sediment. Segura River	30S 704847 4218499	30S 704939 4218561	11/04/2023
11	Guardamar del Segura (Alicante)	Breakwater and sediments (river mouth). Segura River	30S 706629 4220895	30S 706811 4220940	11/04/2023

HCO2198 (Folmer *et al.*, 1994). The reaction mixture contained 2.5 µL template DNA, 2.5 µM $MgCl_2$, 1.25 µM deoxyribonucleotide 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 ℃ for 5 min, then 35 cycles of 94 ℃ for 45 s, 48 ℃ for 45 s, and 72 ℃ for 30 s, and finally an extension of 72 ℃ for 7 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, 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 & Coulson, 1975).

Genetic analysis

The forward and reverse sequences obtained by Sanger sequencing were edited using Geneious Prime 2022.2.2 (https://www.geneious.com) for quality trimming, primer removal and manual correction to check any possible wrong base calling. Then they were aligned using ClustalW under default parameters. After alignment and corrections, a consensus sequence was generated with the default parameters.

A preliminary genetic species identification was attempted using nBlast implemented in Geneious Prime using the default values to search in GenBank databases. A phylogenetic analysis was conducted using 7 new sequences of *Sinotaia quadrata* and 48 sequences from Genbank of *Sinotaia* and *Bellamya* Jousseaume, 1886 species plus one sequence from *Viviparus viviparus* (Linnaeus, 1758) used as outgroup. The test software included in MEGA 11 (Tamura *et al.*, 2021) was used to predict the nucleotide substitution model showing the best AIC scores. The sequences were analyzed using RaxML 8.2.11 software (Stamatakis, 2014) implemented in Geneious Prime 2022.2.2. A Maximum Likelihood tree was done using Rapid Bootstraping Algorithm and a search was conducted for the best scoring tree using the General Time Reversible model (GTR+G) of molecular evolution and 10,000 bootstrap replicates. A consensus tree was generated with a 10% of burning and a 50% node support threshold value for bootstrapping but interpreted as significant nodes from a minimum value of 70%. Moreover, the PopART 1.7 program using the median-joining model (Bandelt *et al.*, 1999) was used for obtaining a haplotype network from representative sequences of European, African, and American introduced populations of *S. quadrata.*

Results

Molecular assignment

As depicted in the tree (Figure 3), all specimens clearly belong to *S. quadrata* group species as described by Ye *et al.* (2021). Sequences were deposited in GenBank with the accession numbers shown in Table 2.

The Blast identification engine identified the sequences of the specimen as *S. quadrata* or any of its synonyms suggested by Ye *et al.* (2021). Better Blast hits show that the sequences from Segura River have a 99.7% of pairwise identity with different sequences of *Bellamya* sp. (*Sinotaia* sp. acording to Ye *et al.*, 2021) and *B. purificata* (Heude, 1890) (now *S. quadrata* according to Ye *et al.*, 2021) from Liangzi Lake in China (KF535532.1; KF535534.1; KP966854.1) and with the recent population found in the Nora River (Asturias province, NW Spain) (MN737102.1). The sequence from Gandía River presents 99.5% of pairwise identity with a sequence of *B.*

Table 2. Better hits of BLAST for the assembly sequences. Names marked with (*) refer to names that have been updated according to the current accepted taxonomy.

Accesion number	Sample location	Similarity (%)	Query Coverage	GenBank Accession of Better hit Blasts	Organism	Location
OR500102	Segura River	99.7	99.9	KF535532.1 KF535534.1 KP966854.1 MN737102.1 NC_039097	*Sinotaia* sp. * *Sinotaia* sp. * *S. quadrata* * *S. quadrata* *S. quadrata* *	Liangzi Lake, China Liangzi Lake, China Liangzi Lake, China Nora River, Spain Unknown
OR500103	Segura River	99.7	100	NC_039097 KF535534.1	*S. quadrata* * *Sinotaia* sp. *	Unknown Liangzi Lake, China
OR500104 OR500105	Gandía	99.5	100	NC_039097 KF535534.1	*S. quadrata* * *Sinotaia* sp. *	Unknown Liangzi Lake, China
OR500106 OR500108	Arno River	100	100	KF535474.1	*S. quadrata* *	Dianchi Lake, China
OR500107	Arno River	100	100	KP966874.1	*S. quadrata* *	Taihu Lake, China

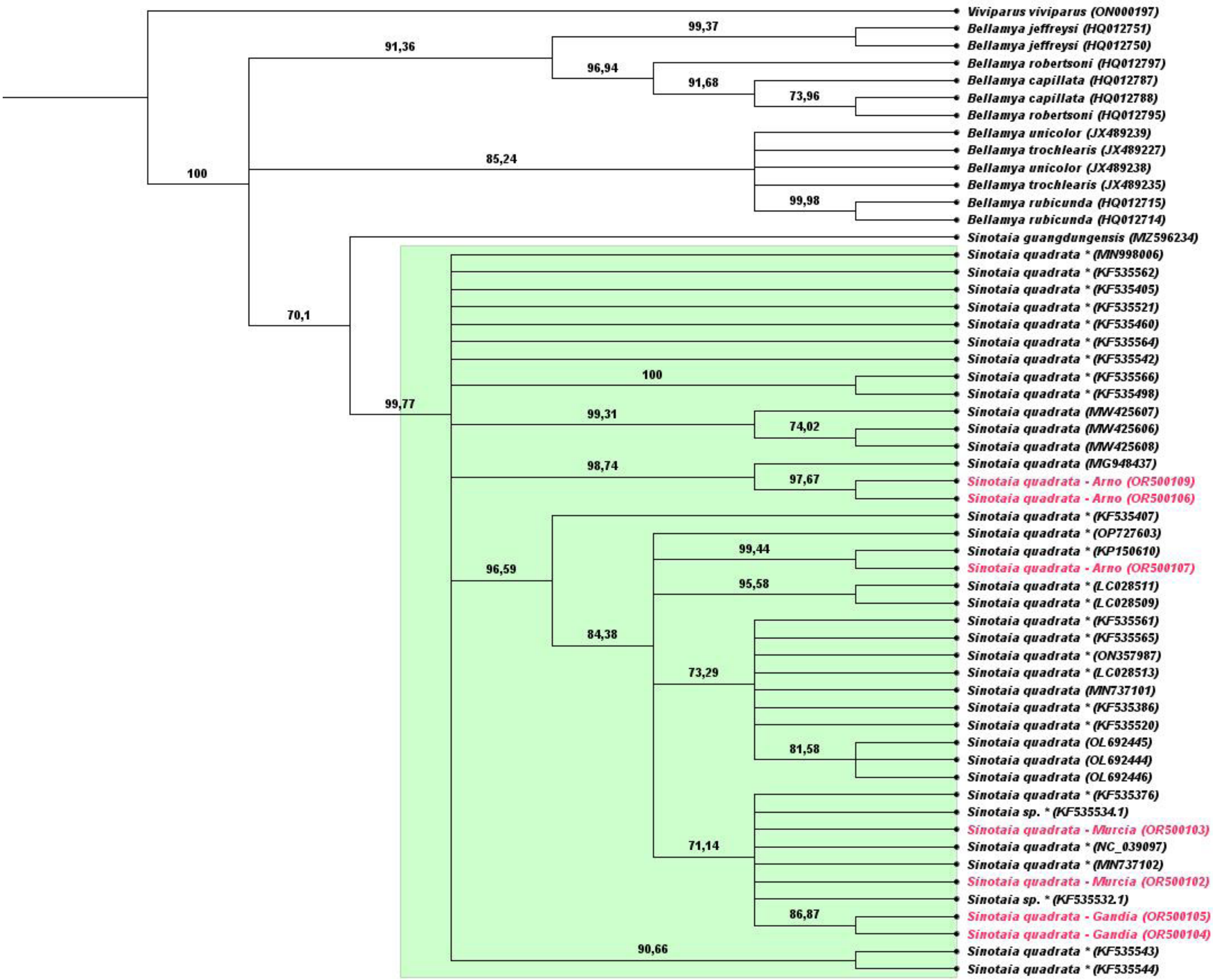

Figure 3. Cladogram of the genus *Sinotaia* and related species. Maximum Likelihood tree generated using 10,000 bootstrap replicates with a 10% of burning interpreted as significant nodes from a minimum value of 70%. *Sinotaia quadrata* clade is shown in green and the new sequences analysed in this work are marked in red. Names marked with (*) refer to names that have been updated according to the current accepted taxonomy.

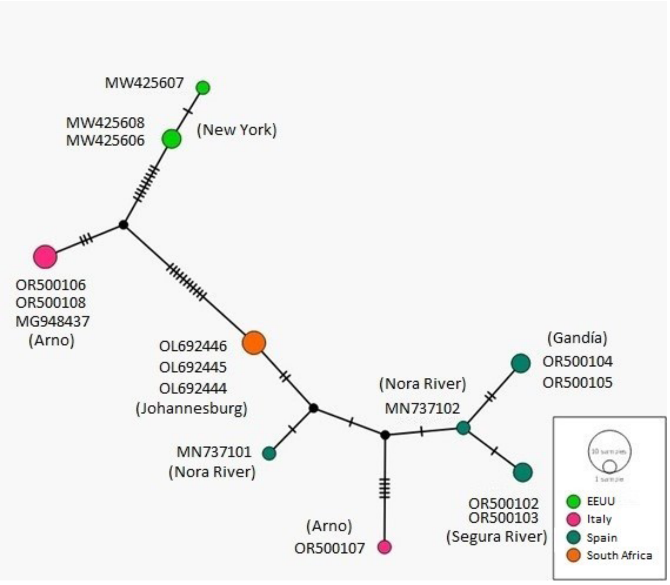

Figure 4. The mitochondrial haplotypes COI network from representative sequences of introduced populations of *Sinotaia quadrata* out of the Asian continent. The legend shows the country where *Sinotaia quadrata* sequences was introduced (Genbank accession numbers are indicated). Node sizes are proportional to the number of sequences in which the haplotype was observed. Bars indicate the number of mutations needed to get from one haplotype to another, and black circles represent hypothetical nodes.

purificata (now *S. quadrata* according to Ye *et al.*, 2021) from Liangzi Lake in China (KF535534.1). The sequences from Arno River show total similarity with two different sequences, one from Taihu Lake, China (KP966874.1) and another from Dianchi Lake, China (KF535474.1) as reported in Cianfanelli *et al.* (2017).

Phylogenetic relationships using COI sequences from different species of Bellamyinae Rohrbach, 1937 subfamily was assessed through Maximum Likelihood estimations (Figure 3). The analysis revealed a similar topology and clades previously reported by Arias *et al.* (2020) and Ye *et al.* (2021). Our specimens were located within the *Sinotaia quadrata* Group with a high bootstrap support (i.e. 99.77%) (Figure 3). This cluster was composed of all suggested synonyms of *S. quadrata* by Ye *et al.* (2021), all of them with Asian native origin (Figure 3).

The haplotype analyses using PopART revealed different origins for the new reported populations of *S. quadrata* (Figure 4). The haplotype found in Gandía (Spain) (OR500104; OR500105) and the one found in Segura River (Spain) (OR500102; OR500103) were connected to one sequence named *S.* cf. *quadrata* haplotype (MN737102) found in NW Spain by Arias *et al.* (2020). The new Italian haplotype (OR500107) was closely connected to a Spanish node more than from haplotypes known from this locality (Figure 4).

Few mutational changes connected most haplotypes from the reported introduced populations of *S. quadrata* of Spain and South Africa. However, North American and Italian populations are shown slightly further apart (in terms of a high number of mutational changes) from the cluster of haplotypes from Spain and South Africa (Figure 4).

Shell characteristics

Shells from the new population are conical with a relative low spire, with a marked carina with 2-3 spiral keels, and some axial growth lines, 5-7 whorls with strong sutures, yellowish-green or olive-brown in colour, and showing a black peristome, slightly reflected on the columellar side, as previously reported elsewhere (Cianfanelli *et al.*, 2017; Arias *et al.*, 2020) (Figure 5). The operculum is horny, with an eccentric nucleus. Shell morphology perfectly matches with that described for other European populations (Cianfanelli *et al.*, 2017; Arias *et al.*, 2020; Quiñonero-Salgado *et al.*, 2022).

A total of 32 individuals were measured with a height ranging from 12 to 26 mm, the 20-25 mm size class being the most numerous, slightly bigger in average than Italian specimens, where the main size class was 15-20 mm (Cianfanelli *et al.*, 2017).

According to Ye *et al.* (2021), snails living in rice fields and water canals seemed to have a lower spire and smaller shell height, although no significant differences were observed among individuals collected in the irrigation canal or the rice fields compared to specimens found in the Segura River.

Habitat and status of the population

The new population was found in the Segura River downstream of Calasparra (Murcia province). There the river flows through a wide floodplain occupied by farmlands (mainly fruit trees and rice fields), interconnected by a network of irrigation canals. The riparian forest has been cleared and the riverbanks are now formed by dispersal trees and accumulations of helophytes (*Phragmites australis* and the invasive *Arundo donax*).

During the first sampling season (spring of 2022) the specimens were found on semi-submerged rocks forming a lateral breakwater on the left side. The maximum density of individuals (2-5 in each rock) was in the mouth of a small irrigation canal running perpendicular from the adjacent fields, from where the density was decreasing along the breakwater.

After the initial finding of the species in 2022, a more comprehensive approach was undertaken in 2023 to establish the extension of the population. The species was located in only two of the ten sites sampled (numbers 6 & 7, in the municipality of Calasparra, Murcia province), corresponding to the same breakwater rocks as well as the perpendicular irrigation canal (a section of 100 m) and the adjacent rice field. Furthermore, the sampling included nearby locations and sites located in the middle and lower course of the Segura River (from the joining of the Segura and Mundo rivers to the mouth in the Mediterranean Sea), with negative findings.

In this second campaign the river flow, the current, and the water turbidity were higher due to water release from a dam situated upstream (the rocks were almost completely submerged), preventing more exhaustive sampling. Thus, although it was not possible to obtain an accurate estimation of densities inside the river, the point with the maximum density was again the joining of the canal into the river. At this point there is a small shallower area in the river with higher water temperature and less current, with mud above the rocks, maybe optimum for the species.

The density along the irrigation canal was lower, with dispersed specimens. The rice field was flooded, making impossible the sampling (specimens were collected only from their sides) although the presence was apparently very low.

The presence of both adults and juveniles, and males and females, was confirmed. Gravid females were captured, indicative of reproductive behaviour and success. The species was found together with another very common invasive bivalve, *Corbicula fluminea* (O.F. Müller, 1774), and the also invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852).

Discussion

While Arias *et al.* (2020) reported the presence of a few juvenile specimens in the Nora River population in NW Spain, suggestive of an established population, its status seems dubious, as no further specimens have been found since then (OS & AA, personal observations). Similarly, the small number of specimens in the Gandía population, located in an isolated small pond and canal system without juveniles, suggests also that there is no evidence of an established population here. Nonetheless, further sampling is needed to confirm this point in both populations.

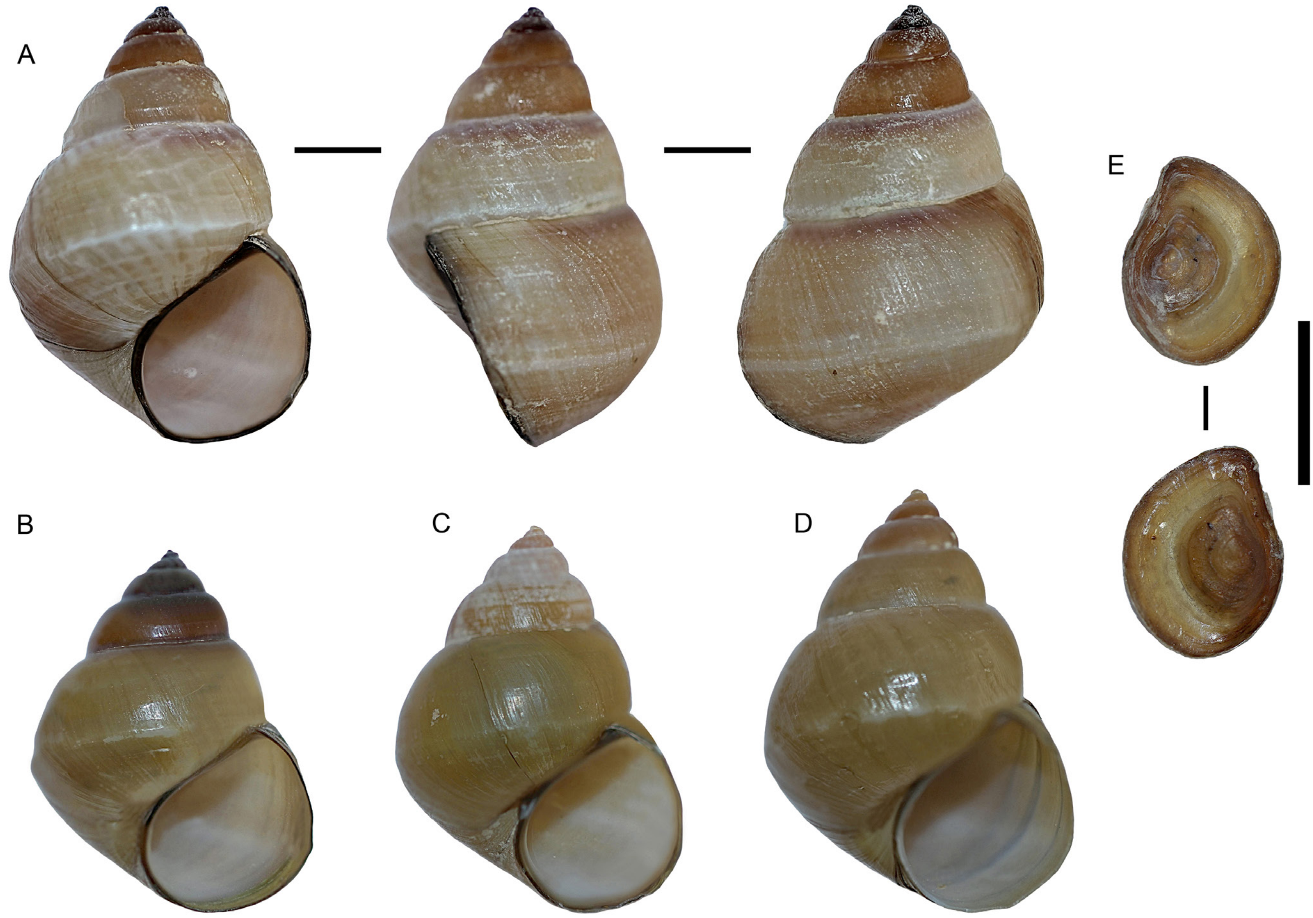

Figure 5. Pictures of some shells of *Sinotaia quadrata* from Calasparra (Murcia). A) Different views of the same specimen; B-D) Different adult specimens; E) Outer and inner view of the operculum. Scale: 1 cm.

To date, the Segura River is the biggest *S. quadrata* population reported in Spain. It is present in habitats (a large river connected by irrigation canals to rice fields) that can also favour its establishment and dispersal in the coming years in this area or the whole river basin. So far, both adults and juvenile/subadults have been found at two consecutive years, in numbers that support a likely established population, in addition to a number of gravid females bearing hatchlings, showing success in reproductive effort. However, the high river flow during samplings has not allowed carrying surveys in potential habitats in the riverbed and other adjacent water bodies, so it is expected that the actual number and densities of specimens are far larger than those reported in this study. Ongoing work by the authors is oriented to confirm this issue, all along the Segura River basin, in the course of new sampling campaigns.

Aquarium trade seems the most likely origin for this new population, as it was suggested for the specimens in Gandía and Nora River, probably as stowaways with other organisms such as aquatic plants (Arias *et al.*, 2020; Hernández Núñez de Arenas *et al.*, 2022). A direct involvement of sport fishing in its origin and/or dispersal cannot be ruled out, since this activity is highly developed in this area, particularly in the Quípar reservoir. Given their different haplotypes, the Segura and Gandía specimens seem not to come from a direct importation from Italy in a secondary spread episode.

In conclusion, this work confirms the identity of all known European populations of the genus as *S. quadrata.* However, the presence of different haplotypes between populations (Italian versus Iberian) suggests they may have at least two different origins and may actually derive from different introduction episodes coming directly from the native countries, probably all related to aquarium trade. One single introduction episode could be common, however, for the three Iberian populations. So far, the population of the Segura River seems the only established population in Spain, and the second one in Europe after the Italian one in the Arno basin. Future samplings should determine the extent of this population, at present apparently restricted to a small portion of the river basin, close to paddy fields and canals that constitute an ideal habitat for the species, with mud bottoms and relatively little water flow. These findings are in consonance with the continuous arrival of non-indigenous species to freshwater ecosystems in western Europe. In some specific areas, such as the SE of the Iberian Peninsula, over the last decades numerous exotic molluscs and other animals have been introduced.

Acknowledgements

Our acknowledgement to Han Raven and an anonymous reviewer for comments that have contributed to improve the manuscript. Special thanks to Jesús Hernández Núñez de Arenas and Dr. Simone Cianfanelli for their generous gift of samples from Gandía and Arno River, respectively. To all members of Cimera who have collaborated in the samplings on the Segura River and other survey campaigns. The Confederación Hidrográfica del Segura supported this study through the project "Desarrollo del Programa de Seguimiento para determinar el Estado de las Aguas Continentales y el Control de las Zonas Protegidas en la Demarcación Hidrográfica del Segura, Expediente 07.834-0011/0411 (Confederación Hidrográfica del Segura, Ministerio para la Transición Ecológica y el Reto Demográfico)". JLS and SQS are members of MINVACAT (Mol·luscs Invasors de Catalunya) Project.

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