

New Data on Exotic Muricid Species (Neogastropoda: Muricidae) from Spain Based on Integrative Taxonomy

Rafael Bañón ^{1,*} , Juan Fariña ² and Alejandro de Carlos ^{3,4} 

¹ Grupo de Estudos do Medio Mariño (GEMM), Edif. Club Náutico Bajo, 15960 Ribeira, Spain

² Cofradía Santiago Apóstol de Barallobre, Paseo Portuario s/n, 15528 Fene, Spain; asistenciatecnica@cofradiabarallobre.org

³ Departamento de Bioquímica, Xenética e Inmunoloxía, Facultade de Bioloxía, Universidade de Vigo, Campus Universitario Lagoas-Marcosende s/n, 36310 Vigo, Spain; adcarlos@uvigo.es

⁴ Centro de Investigación Mariña, Edificio Filomena Dato, Campus Universitario Lagoas-Marcosende s/n, 36310 Vigo, Spain

* Correspondence: anoplogaster@yahoo.es

Abstract: The occurrence of *Ocenebrellus inornatus* and *Rapana venosa*, two exotic marine gastropods of the family Muricidae originating from the northwest Pacific, is reported in Spanish waters, specifically in the Galician waters (NW Spain) in 2023. Live specimens of *O. inornatus* were found on Illa de Arousa, in the Ría de Arousa, southern Galicia, where they are already established. Two new specimens of *R. venosa* are recorded in Galicia, one of them for the first time out of the Ría de Arousa, representing a range expansion for the species. The DNA barcoding analysis confirms the previous morphological identifications. It is suspected that both species may have been introduced through importation of clam spat and middle-sized oysters from countries such as France, the United Kingdom or Italy for subsequent culture, as has been the case with other exotic species that can currently be found in Galician waters. The continuous arrival of marine exotic species strongly supports the need to establish a monitoring program in Galician waters.

Keywords: mollusca; gastropods; *Ocenebrellus inornatus*; *Rapana venosa*; invasive species



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1. Introduction

With over 1600 extant described species, the family Muricidae is one of the most species-rich and morphologically diverse families of molluscs living worldwide, from tropical to polar seas, and ranging from the intertidal zone down to more than 3000 m depth [1]. There are 52 muricid species reported in Spanish waters [2], of which 14 can be found in the waters off Galicia, including several exotic ones [3].

The introduction and spread of exotic (alien, non-native, non-indigenous) species is considered one of the main threats to biodiversity, as they displace native species, modify community structure and food webs, and alter fundamental processes such as nutrient cycling and sedimentation [4]. A total of 1369 marine alien species have been reported in European seas, including 110 cryptogenic and 139 questionable species [5]. Regarding Spanish marine waters, a total of 574 species have been identified with an alien, cryptogenic, crypto-expanding or debatable status [6]. The European Union (EU) recognises the need for strong action to control biological invasions and thus mitigate their impact on biodiversity, ecosystem services and human activities [7].

The Japanese oyster drill *Ocenebrellus inornatus* (Récluz, 1851) is a muricid gastropod mollusc native to the North Pacific, ranging from northern China, Korea and Japan to the Sakhalin and Kuril Islands in Russia [8,9]. In this native area, its distribution is largely overlapping with *Magallana gigas* (Thunberg, 1793), which is its main prey and on whose shells it tends to lay its eggs [9]. *Ocenebrellus inornatus* was first discovered outside its natural range on the west coast of the United States in 1924, linked to the importation of

M. gigas from Japan [10], spreading to all the states of the west coast of the United States and to British Columbia in Canada in a few years. In Europe, it was first reported from France in 1995 [11], probably due to the importation of *M. gigas* brood stock from British Columbia in the 1970s, as the resemblance of this species to the native *Ocenebra erinaceus* (Linnaeus, 1758) caused it to go unnoticed for years. It was subsequently reported from the coasts of the Netherlands [12], Portugal [13] and Denmark [9], usually associated with aquaculture activities of *M. gigas*.

The veined rapa whelk *Rapana venosa* Valenciennes 1846 is native to the Sea of Japan, the Yellow Sea, the Bohai Sea and the East China Sea to Taiwan [14]. This species was first discovered outside its native biogeographic range in the Black Sea in 1947 and later its range was extended to the Aegean and Adriatic seas, South America (Argentina and Uruguay), North America (eastern USA), France (Brittany coast) and the North Sea [15].

The aim of this paper is to report new data on the occurrence of two species of exotic muricid mollusc from Spanish Galician waters based on morphology and DNA barcoding.

2. Materials and Methods

2.1. Study Area

Galicia is an autonomous region of Spain located in the northwestern corner of the Iberian Peninsula, between the river Eo ($43^{\circ}32' N$, $7^{\circ}01' W$) and the river Miño ($41^{\circ}50' N$, $9^{\circ}40' W$) (Figure 1). The Galician coast has a length of 1498 km and is characterised by the presence of the Rías, which are tectonic estuaries penetrating the coast almost perpendicular to the coastline. The Ria of Arousa is the largest of the eighteen Galician Rias; it is located in the southern part of the Galician coast, belonging to the Rías Baixas group. It has an area of 230 km² and the maximum depth is 69 m at its mouth, which is open to the Atlantic Ocean along a northeast–southwest axis. Along the Ria, there are numerous commercial and fishing ports with important fishing and shellfishing activity.

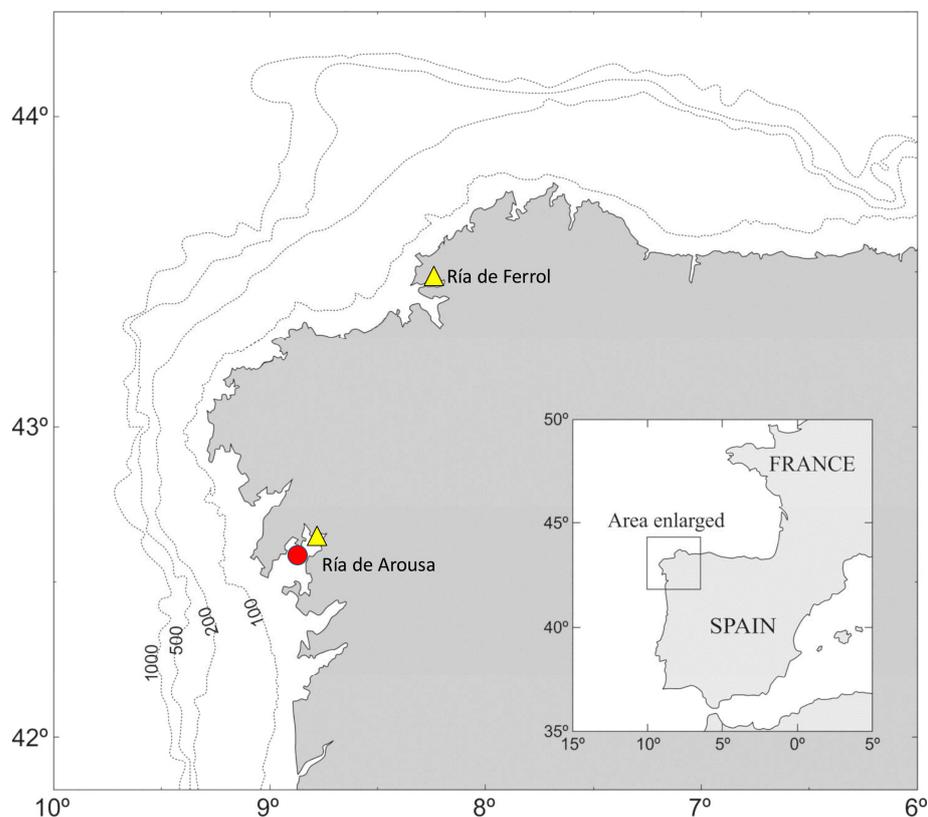


Figure 1. Map showing the location of the recorded specimens of *Ocinebrellus inornatus* (red circle) and *Rapana venosa* (yellow triangles).

2.2. Sampling Data

A total of 113 live specimens of *O. inornatus* were randomly collected from Xastelas beach, in the Illa de Arousa, in 2023 and transported to the laboratory to be measured. Two specimens of *R. venosa* were caught in 2023 by small-scale fishing vessels that regularly fish along the Galician coast. The identification was initially made by examination of morphological characters following molluscan literature. Total shell height and shell width were measured to 0.01 mm using a digital calliper (TesaCallIP65) and specimens were weighed with a digital scale (Mettler Toledo) to 0.1 g.

A subsample of 11 specimens of *O. inornatus* and 4 of *O. erinaceus*, used as comparative material, was reserved frozen for the purpose of molecular identification. Finally, 16 specimens (11 *O. inornatus*, 4 *O. erinaceus* and 1 *R. venosa*) were deposited at the Museo de Historia Natural da Universidade de Santiago de Compostela (MHN USC, Santiago de Compostela, Spain) under reference codes 25217-1 to 25217-11 for *O. inornatus*, MHN USC 25218-1 to 25218-4 for *O. erinaceus* and MHNUSC 25214 for *R. venosa*.

2.3. DNA Extraction, PCR, and Sequencing

DNA was extracted from a tissue sample from each individual using the E.Z.N.A Tissue DNA Kit. The 5' region of the mitochondrial gene coding for subunit I of the cytochrome c oxidase enzyme (*COI*-5P) was amplified using the universal primers LCO1490 and HCO2198 [16]. The enzymatic reaction was carried out using the Horse Power Green Taq DNA Polymerase mix (Canvax, Valladolid, Spain) with a classical PCR thermal regime [17]. The resulting amplicons were sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit, in both directions using the same primers as for amplification. The sequences were 658 nucleotides long, contained no insertions or deletions, and encoded a polypeptide of 219 amino acids. All information regarding the specimens as well as their barcodes, images, places of capture and other complementary data are available in the project "Marine Invertebrates" (code INVMA) at the Barcode of Life Data System (BOLD Systems, www.boldsystems.org, accessed on 30 August 2023). The sequences were also submitted to GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) having the accession numbers OR524177–OR524179, OR524182–OR524185 and OR524187–OR524190 for *O. inornatus*; OR524176, OR524180, OR524186 and OR524191 for *O. erinaceus* and OR524181 for *R. venosa*.

2.4. Sequences Alignment

Sequences were aligned with each other and with other sequences obtained from the BOLD database. The number of differences among sequences of *O. inornatus* and *O. erinaceus* samples was calculated using uncorrected p-distances [18] in order to obtain a phylogenetic tree to be used as a taxonomic dendrogram by the neighbor joining (NJ) method [19], including resampling values (1000) in bootstrap form [20]. Prior to phylogenetic analysis, the sequences were collapsed into haplotypes using FaBox 1.61 [21]. The analysis included 11 nucleotide sequences with 658 positions in the final dataset and was conducted in MEGA11 [22].

The *COI* sequence from *R. venosa* was used to: (a) double check the morphological identification of the voucher specimen via DNA barcoding and (b) to assess possible genetic structure across individuals from different sampling sites. For this, a median joining network [23] was constructed with PopART 1.7 [24], with $\epsilon = 0$, including all sequences available in BOLD whose geographic origin was specified.

3. Results

3.1. Systematics

Class Gastropoda Cuvier, 1795
Order Neogastropoda
Family Muricidae Rafinesque, 1815
Genus *Ocinebrellus* Jousseaume, 1880
Ocinebrellus inornatus (Récluz, 1851) (Figure 2).

Genus *Rapana* H. C. F. Schumacher, 1817
Rapana venosa (Valenciennes, 1846)

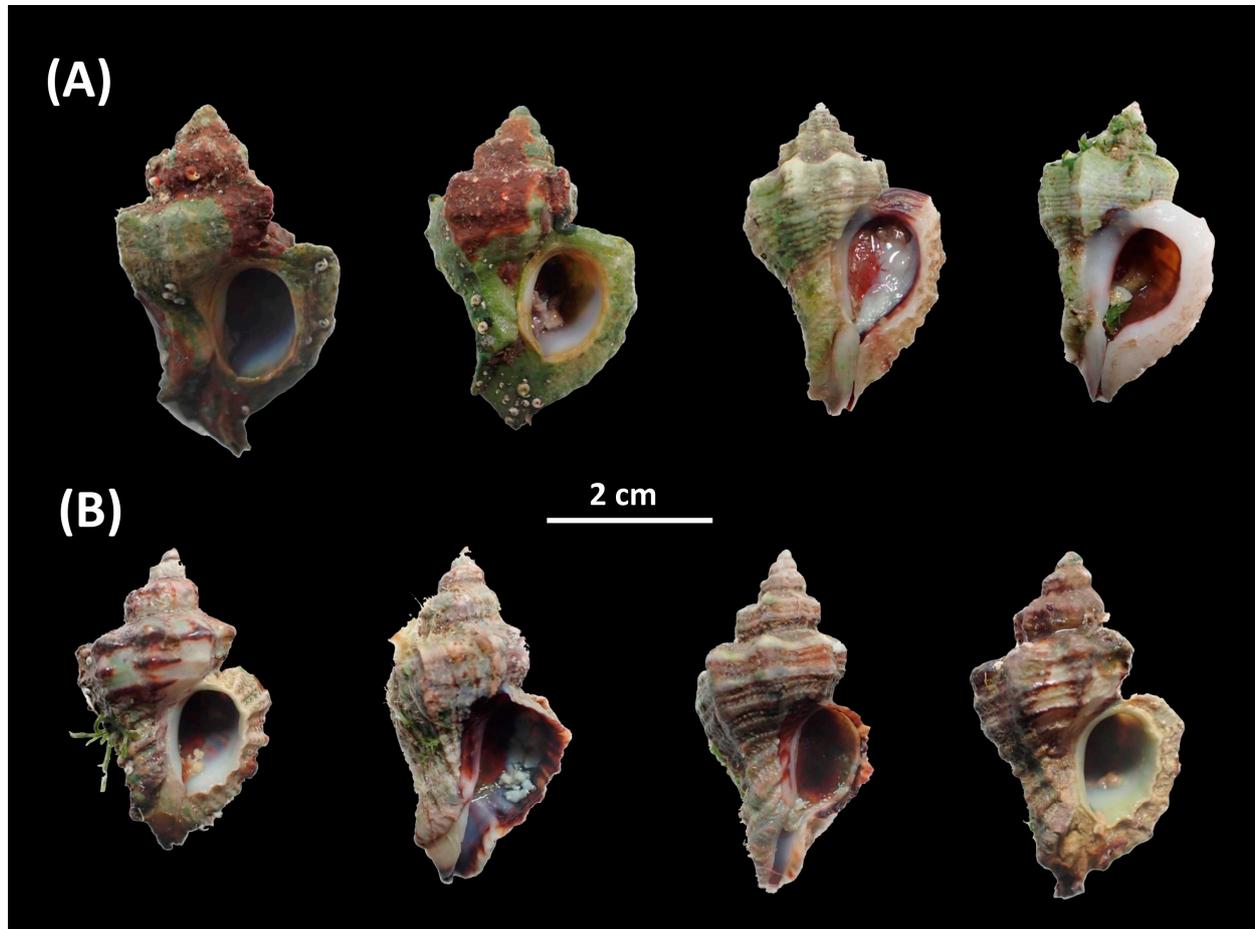


Figure 2. Specimens of exotic *Ocinebrellus inornatus* (A) and native *Ocenebra erinaceus* (B), collected from the Illa de Arousa, Galicia (NW Spain).

3.2. Material Examined

A total of 113 live specimens of *O. inornatus* between 28.3 and 45.7 mm shell height (Table 1, Figure 2) were caught on 5 June 2023 in Xastelas beach, 42.531 N, -8.867 W, Ría de Arousa, Galicia, NW Spain, at a depth of 0–0.5 m. Two specimens of *R. venosa* were captured in the south and north of the Galician coast (Figure 3). The first specimen, of 140.7 mm shell height and 104.2 mm shell width, was caught on 6 June 2023 by local fishermen using fyke nets, near to Illa de Cortegada, Ria de Arousa, south of Galicia, 42.607 N, -8.778 W, at 5 m depth. The second specimen, of 160.4 mm shell height and 130.1 mm shell width, was caught on 14 June 2023 by local fishermen using a dredge for molluscs, in the Ría de Ferrol, north of Galicia, 43.465 N, -8.241 W, at 10 m depth.

Table 1. Morphometric data of *Ocinebrellus inornatus* collected from the northern coast of Spain. Size is expressed in millimetres and weight in grams.

Character	Number	Minimum	Maximum	Mean	SD
Shell height	113	28.3	45.7	37.1	3.8
Shell width	113	15.6	28.8	22	2.7
Weight	113	2	11.2	5.8	1.8

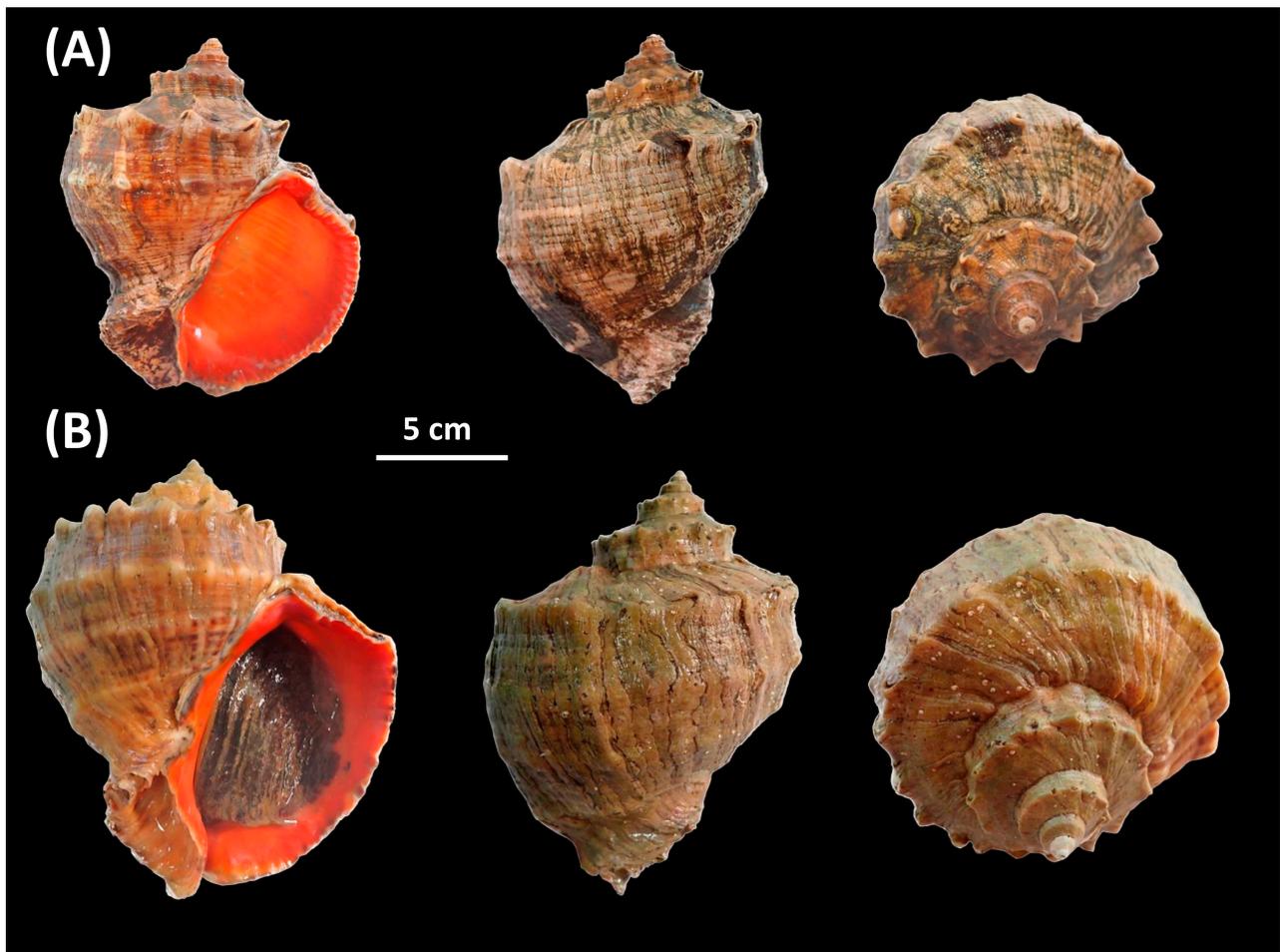


Figure 3. Specimens of *Rapana venosa* 140.7 mm shell height (A) and MHNUSC 25214 160.4 mm shell height (B), collected from Galicia (NW Spain).

3.3. Remarks

Ocinebrellus inornatus is very similar to the native species *O. erinaceus* from which it is very difficult to distinguish due to the morphological variability of both species [3]. The main differences are a light brown operculum in *O. inornatus* versus brown in *O. erinaceus* and the shoulders of whorls, which are more prominent and angulated giving a more pronounced turreted effect in *O. inornatus* [25].

3.4. Molecular Taxonomy

All sequences obtained from *O. inornatus* specimens collected in Illa de Arousa showed the same haplotype of the barcoding region of the *COI* gene. In contrast, the four sequences of *O. erinaceus* represent three different haplotypes. The NJ dendrogram shows three clades, with two of them grouping sequences named as *O. inornatus*, separated by a 16.41% distance between the closest sequences, the one representing the individual collected in Illa de Arousa OR524179, and a sequence from Korea HM180493. It should be noted that the latter contains a sequence named *Neptunea cumingi* Crosse, 1862 (Figure 4). The distance between the sequence of *O. inornatus* from the Illa de Arousa and its nearest neighbour in the *O. erinaceus* clade is 12.46%, to a sequence of a specimen collected at the same site, OR524176. Mean distances within each clade ranged from 0.33% to 0.43%.

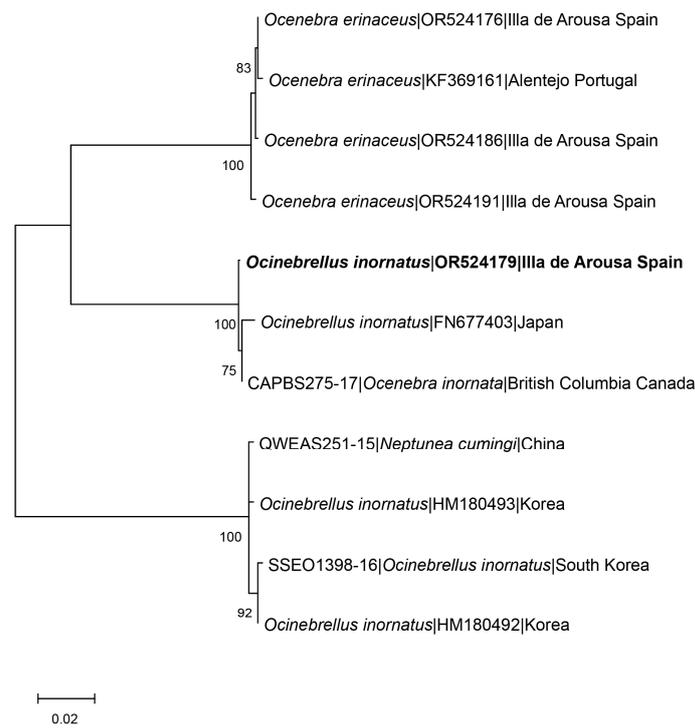


Figure 4. Uncorrected p-distance neighbor joining analysis of *COI*-5P barcode haplotypes of *Ocinebrellus inornatus* and *Ocenebra erinaceus*, including the one from specimens found in the Illa de Arousa, Spain (bolded).

Figure 5 shows a haplotype network of 43 sequences, 614 nucleotides long, of the mitochondrial *COI* marker of *R. venosa*. Individuals collected from China, Japan and Korea showed a wide variety of haplotypes, 12 out of a total of 13, whereas all non-Asian sequences clustered into a single haplotype H4.

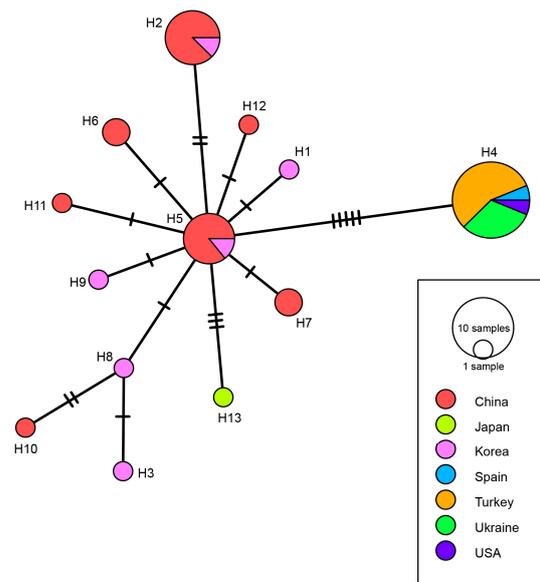


Figure 5. Haplotype network of *COI* sequences of *Rapana venosa* (n = 43). Median joining network ($\epsilon = 0$) created in PopART v1.7. Each circle represents a haplotype; the size of circles corresponds to the number of individuals with the haplotype. Colours indicate sampling sites. Bars indicate the number of mutations between two haplotypes.

4. Discussion

The morphological features of the specimens of *O. inornatus* and *R. venosa* are in agreement with previous descriptions [14,15,26] and the taxonomic identification is confirmed by the similarity of the nucleotide sequences obtained, with others of the same nominal species present in the BOLD and GenBank databases.

The presence of *O. inornatus* in Galicia is not surprising given that it was introduced a long time ago into European waters, and it is widely distributed to the north and south of the reported area. Previously, this species had been recorded in Galician waters based on dead specimens [27]. *Ocenebrellus inornatus* has been transported around the world together with *M. gigas* transplants. The latter species was introduced into the southern Galician waters in the 1980s, and has been cultivated in the vicinity of the Illa de Arousa since the 1990s, from spat continuously imported from France [28], where *O. inornatus* is largely introduced [8,29].

In colonised areas in different parts of America and Europe, *O. inornatus* prefers the substrates of oyster beds naturally found between the intertidal zone and 5–6 m depth, of species such as *M. gigas*, *Ostrea lurida* P. P. Carpenter, 1864 and *O. edulis* [30]. However, there are no natural populations of *O. edulis* in the Ría de Arousa. Naturalised populations of *M. gigas* only exist on the north coast of Galicia, in the Rías altas [31]. Therefore, in the absence of oysters in the Galician occupied area, *O. inornatus* opts for a different diet, mainly other commercial and abundant bivalves present in Xastelas beach such as *Ruditapes philippinarum* (Adams and Reeve, 1850) and *Ruditapes decussatus* (Linnaeus, 1758), with the consequent economic loss for the shellfish sector. *Ocenebrellus inornatus* has 15 times higher reproductive effort and a better average growth rate than *O. erinaceus* which may explain the fast rapid invasive establishment of this species [32].

The settlement of *R. venosa* in Galicia has been comparatively slower than the other exotic muricid species. *Rapana venosa* was first introduced in the Ría de Arousa in 2003 [33]. Subsequently, only two specimens have been reported, one in 2005 [34] and one in 2007 [35], both also in the Ría de Arousa. *Rapana venosa* has a very showy and colourful shell, so empty shells are often kept as decorative elements, which have made it possible to backtrack the catches in time [33].

As for the two new reported specimens of *R. venosa*, although the first one also comes from the Ría de Arousa, the other was captured, for the first time, outside this ría, in the north of Galicia, extending the range of the invaded areas.

In the marine environment, the introduction of exotic species is mainly the result of aquaculture activities, especially shellfish farming, which represents a major cause of introduction, intentional or not, of exogenous species [36]. The most likely vector for the introduction of the exotic muricids, and other invasive molluscan species in Galician waters is also the unintentional co-transport of these species in commercial bivalve cultures, mainly clams and oysters, imported from Mediterranean or Atlantic areas, where they are already well established [35,37,38]. However, in the case of *R. venosa* the ship transport of egg cases or adults as biofouling is also plausible, especially in the case of the northern specimen, given the proximity to the site of capture of a major port of a liquefied natural gas company, with significant vessel movements.

Unlike the other exotic muricids already established in Galician waters [38], only six specimens of *R. venosa* have been reported in the last 20 years. Several factors may condition the low occurrence rate of this species, such as non-viable egg clutches or a low egg survival rate. The fact that the specimens are not accessible to different fishing gears, or that the pelagic larval stage causes high dispersion, may also play a role [33].

The DNA barcoding technique is a useful tool for marine species identification, including exotic molluscs [39,40]. Molecular taxonomy showed discordant results, with two distant groups containing sequences of *O. inornatus*. Although DNA barcoding is predominantly a tool for species identification, profound genetic divergences, when found, could be due to misidentification of specimens or involve cryptic or unrecognised speciation events [41].

The haplotype of *O. inornatus* from the Illa de Arousa grouped with others of the same species from Canada and China, showing a typical mean intraspecific distance value, and the distance to its nearest neighbour of *O. erinaceus* was within typical interspecific values, showing a classical barcoding gap. Surprisingly, the other clade also has haplotypes of *O. inornatus* from China and Korea, where this species also occurs. However, sequences of this clade contain one haplotype named as *N. cumingi*, a morphologically similar species, which would point to a misidentification of the *O. inornatus* specimens of this clade. Gaps and inconsistencies in reference DNA databases can make it difficult to accurately identify taxa to the species level, suggesting the need to strengthen the DNA barcoding reference datasets [42]. The comparison of newly generated barcodes with published data may help to detect misidentifications, taxonomic uncertainties or real cases of haplotype sharing among species [43].

In the case of *R. venosa*, collections from the native range, the far East, showed high levels of genetic variation, while collections from all introduced populations showed a total lack of genetic diversity; a single haplotype was common to all introduced individuals in the Mediterranean Sea and Atlantic Ocean. This finding has been observed before, and is consistent with the hypothesis of the introduction and establishment of a population in the Black Sea that would later serve as a source of secondary invasions mediated by vectors such as ballast water transport [44]. Although non-native *R. venosa* populations currently appear to be thriving in their new environments, the lack of genetic variability raises questions regarding their evolutionary persistence.

Galicia, but especially the Ría de Arousa, is a very important area of introduction for exotic marine organisms in Spain [35,38,39], and both *O. inornatus* and *R. venosa* are carnivorous gastropods whose main diet consists of a variety of native molluscs (shellfish and other bivalves), which could lead to important ecological and economic losses [32,33,35]. The aim of this report is to alert interested parties to the importance of establishing a monitoring program to assess new observations of alien species, in order to detect the increasing expansion of their range and to predict the impacts they may cause.

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Conflicts of Interest: The authors declare no conflict of interest.

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