Title

Metabarcoding and post-sampling strategies to discover non-indigenous species: a case study in the estuaries of the central south Bay of Biscay.

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Abstract

Estuaries are highly productive habitats that generate more organic material than other areas of comparable size, such as forests, meadows or agricultural lands. They exhibit high biodiversity and host relevant species, which can be at risk because these areas are most affected by human activities. In this study, environmental DNA (eDNA) and metabarcoding analyses were performed in two estuaries within the Bay of Biscay that are important in terms of seafood production (Ría del Eo and Ría de Villaviciosa, Asturias, Northern Spain). The main goal was to assess the potential of these novel tools to detect possible introductions of non-indigenous species. Alien genera that might have been introduced through exotic shellfish cultures in these estuaries were detected despite limited sampling. Of the NIS found, *Crepidula fornicata* was the only one that was already included in the Spanish invasive species catalogue (BOE, 2013). After the initial invasive species detection through the metabarcoding study, post-NGS samplings and classical DNA barcoding were performed, and they confirmed the presence of the highly invasive species *C. fornicata* in these estuaries. Although metabarcoding still has some drawbacks, such as a lack of universal PCR primers and reference sequences for all the species in the databases as well as frequent false positives, it represents a powerful tool that can facilitate the monitoring and management of these important ecosystems, especially if it is accompanied by post-NGS samplings to confirm species occurrences.

Keywords: Cytochrome Oxidase I; Pyrosequencing; NGS; post-NGS samplings; invasive species; *Crepidula fornicata*; Asturias.

Introduction

Estuaries are amongst the most important coastal habitats on Earth; they exhibit high biological diversity and play an important role in the global production of molluscs and crustaceans. The

different levels of biodiversity (genetic diversity, species diversity, and ecosystem diversity) are sensitive indicators of the health and balance of these ecosystems, whose proximity to human settlements make them particularly vulnerable. These valued ecosystems can especially be affected by environmental and anthropogenic factors (Kennish, 2002), but there is still surprisingly low sensitivity to these conservation problems. Stakeholders, including local and regional authorities, should be especially concerned since estuaries and the adjacent coastal waters support many essential fisheries, and thus the associated human communities, around the world (Blaber *et al.*, 2000; Semeraro *et al.*, 2016).

The introduction of alien species (non-indigenous species, NIS) is a direct cause of biodiversity loss and a direct consequence of human activities such as engineering, shipping and aquaculture (Naylor, Williams, & Strong, 2011; Katsanevakis *et al.*, 2014a). The impacts of invasive alien species on ecosystem services and biodiversity mainly affect food provision, ocean nourishment, recreation and tourism, and lifecycle maintenance. In particular, NIS ecosystem engineers have the greatest impacts since they modify, create, or re-define habitats by altering their physical or chemical properties (Katsanevakis *et al.*, 2014b). Through aquaculture, 64 species (mainly macrophytes and invertebrates) have been introduced in the Mediterranean Sea, and there are several NIS hotspots, such as the Thau lagoon (Gulf of Lion, France) and the Venice lagoon (northern Adriatic, Italy) (Katsanevakis *et al.*, 2014a). There may be other, less well-known hotspots for NIS introductions worldwide, which likely include degraded or overused estuaries because they contain brackish waters and are therefore especially vulnerable. Salinity is a very important species range limiting factor and native species seem to reach a minimum species richness at intermediate salinities (e.g., Paavola, Olenin, & Leppakoski, 2005; Pejovic *et al.*, 2016). The two main estuaries in the Asturias region (the central area of the southern Bay of Biscay) are the Ría del Eo and the Ría de Villaviciosa (Figure 1), and they are the sites of almost all the shellfish harvesting and aquaculture in the region.

Some reports have found evidence of NIS expansion and establishment in these two estuaries (Semeraro *et al.*, 2016) as well as hybridization between native and introduced exotic species (e.g., Genus *Ruditapes* in Habtemariam, Arias, García-Vázquez, & Borrell (2015)). Recent barcoding analyses have revealed that approximately 35% of more than 600 marine invertebrates in eight coastal areas and ports in the Bay of Biscay (Asturias) are exotic species (Pejovic *et al*., 2016; Miralles *et al.,* 2016). At the same time, Asian exotic red seaweeds, which probably accompanied species of farmed oysters, have recently been reported in the area (Montes, Rico, García-Vázquez, & Borrell, 2016).

Early detection of invasive species is a crucial step for successful post-introduction management (Pochon, Zaiko, Hopkins, Banks, & Wood, 2015). Genetic analysis has been demonstrated to be a useful tool for inventorying biodiversity (Thorpe, Solé-Cava, & Watts, 2000; Feral, 2002) and for studying marine biological invasions (Dlugosch & Parker, 2008; Estoup & Guillemaud, 2010; Zaiko *et al.*, 2015; Semeraro *et al.*, 2016; Ardura *et al.*, 2016; Devloo-Delva *et al.*, 2016), and the advent of next generation sequencing technologies (NGS) and the possibility of directly analysing DNA from water and sediments (environmental DNA, eDNA) has opened new possibilities for early NIS detection in marine ecosystems, where invasions might remain unnoticed for extended periods (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012; Freire, Genzano, Neumann-Leitao, & Perez, 2014; Zaiko *et al.*, 2015). Furthermore, detecting short, species-specific eDNA fragments from aquatic environments is theoretically more sensitive than traditional survey methods, which can be both time-consuming and costly (Ardura, Linde, Moreira, & Garcia-Vazquez, 2010; Pochon, Bott, Smith, & Wood, 2013). There have been several platforms available for NGS such as the Ion Torrent PGM, the Roche/454 Life Sciences and the Illumina MiSeq systems (Quail *et al.*, 2012; Pochon *et al.,* 2013). Specifically, the Roche/454 pyrosequencing technique, which generates longer reads, have been applied for species identification by using barcodes with diagnostic sequence variations

between species in conjunction with sequence databases such as GenBank or BOLD (Barcoding of Life Data System) (De Battisti *et al.*, 2014). Therefore, eDNA and NGS analyses are increasingly employed to detect rare or invasive species (e.g., Frischer, Kelly & Nierzwicki-Bauer, 2012; Mahon *et al.*, 2013; Rees, Maddison, Middleditch, Patmore, & Gough, 2014; Ardura *et al.*, 2016) even though uncertainties about positive and negative results can remain due to several factors (Ficetola *et al.,* 2015; Goldberg *et al.,* 2016).

In this study, environmental DNA and metabarcoding were performed in the two estuaries that are important for shellfish production (Ría del Eo and Ría de Villaviciosa, Asturias, Northern Spain, Bay of Biscay). The aim was to explore the usefulness of these novel tools for the detection of possible alien species introductions as a consequence of anthropogenic activities (i.e., mollusc and crustacean aquaculture) in these valuable ecosystems. Post-NGS conventional samplings and individual barcoding were used to confirm the occurrence of the non-indigenous species detected by NGS metabarcoding.

Materials and methods

Sampling and DNA extraction

Water samples (5x0.5 L per sampling point) were collected from different sampling points in the two study estuaries in February 2014 (Figure 1). A total of 7.5 L of water were collected from three sampling points in the Villaviciosa estuary: VI) the interpretation centre, VC) the chapel, and VF) near a cider factory. Another 7.5 L of water were collected from three sampling points in the Eo estuary: EO) near the oyster park, EP) at Figueras Port, and EU) in the upstream of the Ría del Eo (Figure 1). Samples were collected approximately 10-20 cm below the water surface during low tide and stored at -20ºC until DNA extraction, for which 2x0.5 L from each location were employed using a commercial kit (PowerWater® DNA isolation kit) from MO-BIO Laboratories, Inc. (Carlsbad,

California, USA) and following the manufacturer's recommendations. DNA extractions were performed in a sterile room in a dedicated building using negative controls, and extractions were conducted for different samples on different days. Measures were employed to ensure sterility, including the use of a laminar air-flow chamber that was continuously disinfected by UV light as well as cleanings with absolute ethanol and a 10% bleach solution to prevent contamination. All DNA obtained from the two replicates of the same sample were pooled in one Eppendorf tube under the same conditions.

Polymerase chain reaction (PCR), massive sequencing and bioinformatics analyses

Barcoding primer pairs coupled with barcode sequences and the key tracts for massive sequencing with the Roche/454 platform were used for PCR amplification of a fragment (658 bp) of the mitochondrial cytochrome oxidase I (COI) gene; the primer pairs used for COI amplifications were those from Geller, Meyer, Parker, & Hawk (2013) (jgLCO1490 and jgHCO2198). The PCR reactions were performed by Macrogen Korea using negative controls to monitor possible contamination as well as Roche FastStart™ High Fidelity Taq DNA Polymerase and the protocols described in the Amplicon Library Preparation Manual (Roche 2010; GS FLX Titanium Series). Thermocycling conditions were 1x: 94°C for 3 min; 35x: 94°C for 15 sec, 55°C for 45 sec and 72°C for 1 min; and finally, 1x: 72°C for 8 min and 4°C on hold. Library constructions included quality controls for size (Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip) and quantity (Roche's Rapid library-standard quantification solution and calculator). The bands of expected size (800 bp) were sequenced in the 1/8 plate GS-FLX run (Roche/454 Life Sciences, Branford, USA).

The multiplexed reads were assigned to samples while accounting for their nucleotide barcodes (demultiplexing). Zero base errors were allowed in this sorting by tag step. CD-HIT-OTU (Wu, Zhu, Fu, Niu, & Li, 2011) was used to filter out erroneous and chimeric reads by combined sequence clustering and statistical simulations. Quality filters based on the characteristics of each sequence

were applied to remove short (<100 bp) and low-quality reads (<20 Phred values) as well as extra-long tails. Filtered reads were aligned and clustered at 100% identity using CD-HIT-DUP, and chimeric reads were identified and eliminated from the duplicate clusters (CD-HIT-OTU User's Guide (http://weizhong-lab.ucsd.edu/cd-hit-otu)). Secondary clusters were then recruited into the primary clusters, and the remaining representative reads from the non-chimeric clusters were grouped into OTUs using a greedy algorithm with a 97% cut-off (e.g., at a species level following Stackebrandt & Goebel (1994)). This is used to avoid false OTUs because of PCR error, sequencing error and other technical errors.

QIIME (Caporaso *et al.* 2010) was used for cytochrome oxidase I (COI)-focused metabarcoding analysis. A reference taxonomical database was created by downloading all available mitochondrial cytochrome oxidase I sequences from the NCBI taxonomic databases following Galal-Khallaf, Osman, Carleos, Garcia-Vazquez & Borrell (2016), and the OTUs were identified using the "*de novo* OTU picking and diversity analyses using 454 data" (qiime.org/tutorials/tutorial.html) QIIME protocol. Taxonomy was inferred through a BLAST search against our reference gene database using 90% identity and an *E*-value threshold of < 1e-100 as cut-offs (QIIME procedures and protocol). Next, the sequences were clustered into operational taxonomic units (OTUs) with their consensus lineage; only OTUs represented by >10 sequences were considered. We focused on the composition of marine invertebrates in this study since the primer pairs were specifically designed for COI amplifications in this taxa (Geller *et al*., 2013), which can help avoid Type-I (false positives) and Type-II (false negatives) errors in species screenings (Ficetola *et al.,* 2015; Galal-Khallaf *et al.,* 2016).

Post-NGS samplings of Crepidula spp. and classical barcoding genetic analyses.

The species *C. fornicata* is native to the western Atlantic Ocean, specifically the Eastern coast of North America, and it is currently listed among the 100 most invasive species by the Invasive Species Specialist Group (ISSG) and has been incorporated into the Spanish Invasive species catalogue (BOE,

2013). A sampling protocol was then implemented to find *Crepidula* spp. following Pejovic *et al*. (2016) and inspired by the rapid assessment survey (RAS) approach from Minchin (2007). Rocky substrates and walls closer to two of the study locations (Ría de Villaviciosa - VC (the chapel) and Ría del Eo – EO (oyster park)) were sampled (Figure 1). Animals that were detected visually and those attached to the shells of oysters were collected. The individuals were then transported to the Genetic Laboratory of Natural Resources (University of Oviedo) and preserved in ethanol for species identification and molecular analyses. The taxonomic nomenclature was verified against the World Register of Marine Species (WoRMS) (2016).

DNA was extracted from approximately 10 mg of tissue from the ethanol-preserved individuals using the EZNA Mollusk DNA Kit (Omega Bio-Tek Inc., USA). The mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified using the universal primers designed by Geller *et al.* (2013) and the conditions described therein. PCR products were examined on 2% agarose gel stained with SimplySafe™ (EURx, Poland), and positive amplicons (evidenced by a clear single band of the right size) were sequenced by Macrogen Inc. (The Netherlands) with ABI3730xl DNA sequencer (Applied Biosystems).

The obtained DNA sequences were visually inspected and edited with BioEdit v7.2.5 (Hall, 1999), and all sequences were compared with online public databases using BLASTn in NCBI (www.ncbi.nlm.nih.gov/) and BOLD Systems (www.boldsystems.org/). Genetic identification was accepted for nucleotide identity scores higher than 97% and E-values < 1e-100. Sequences were aligned with the ClustalW (Thompson, Higgins, & Gibson, 1994) application in BioEdit, and haplotypes were determined with DnaSP software v5.10 (Librado & Rozas, 2009). A phylogenetic tree was constructed with MEGA v7 (Kumar, Stecher, & Tamura, 2016) using COI haplotype and reference sequences obtained from voucher specimens (of known geographical origin) that were

downloaded from NCBI. The maximum likelihood method was employed with best-model Tamura 3 parameters (+G) (Tamura, 1992) and 500 bootstrap replicates.

Results

DNA extraction from water samples was successful and yielded DNA concentration values between 3.07 ng/uL (sample EU) and 4.51 ng/uL (sample VI) (Table 1). The NGS Library quality controls after the PCRs showed positive results, obtaining fragments of approximately 800 bp and concentrations from $1.74x10^9$ molecules/uL (sample VF) to $4.91x10^9$ molecules/uL (sample VC) (Table 1). Massive sequencings of the PCR products obtained from the samples using the COI primer pairs resulted in a total of 92,839 raw reads, 62,041,108 bases and an average read length of 668 bp. After the quality control and clustering steps, taxonomy was inferred through a BLAST search against our reference gene database using *E*-values and identity criteria. A total of 55,866 hits were found after the assignments. Mollusc and crustacean community compositions were the focus of this study. Assignments to the phyla Arthropoda and Mollusca were isolated from the original OTU table to create a new table with only these taxa summarizing 4,229 hits. A total of 32 mollusc and crustacean genera were obtained (Figure 2).

The most abundant genus in all zones of the Eo samples was the copepod *Copilia* with a relative abundance of 37.4% for the EP, 26.4% for the EO and 24.0% for the EU samples (Figure 2, Supp. Table A.1). Other genera such as *Solenocera* and *Barnea* were also represented in the three estuary zones, reaching 15.4% in the oyster park (EO) and 22.4% upstream (EU), and possible alien genera were also found in all these Eo areas (*Crepidula, Macrobrachium, Scapharca, Strombus, Hydatina*) (WoRMS, 2016) (Figure 2, Suppl. Table A.1). Approximately 54.5% of all sequences belonging to the invasive *Crepidula* genus (27 out of 47 sequences in total) were found in Eo (the oyster park sample) (Figure 2). In the Villaviciosa samples, some genera exhibited high relative abundances, such as

Eurythenes (17.5%) and *Copilia (*25.6%), which are native copepods from European coasts (Figure 2, Suppl. Table A.1). Other genera were found in these samples that are not considered less common to Cantabrian coasts, such as *Barnea* (7.5%) and *Scopelocheirus* (8.1%) (WoRMS, 2016) (Figure 2). The monospecific genus *Coelomactra* has been recorded as native from the Pacific coasts (WoRMS, 2016) and appeared with an abundance of 19.4% in the VF sample (Suppl. Table A.1). Other invasive genera were also present in all Villaviciosa areas (*Macrobrachium* and *Hydatina*) (WoRMS, 2016) (Figure 2). Again, the occurrence of the exotic genus *Crepidula* in the chapel samples (VC) was indeed interesting since this finding had never been reported before (Figure 2).

The current Spanish invasive species catalogue (BOE, 2013) includes only one (*Crepidula*) of the exotic genera found in these estuaries using NGS and metabarcoding. This motivated a specific post-sampling strategy for detecting *Crepidula* spp.in Asturias with more intense scrutiny of the Villaviciosa estuary, where *C. fornicata* had not been previously described (Miralles *et al*., 2016). This approach confirmed the physical presence of *C. fornicata* in this estuary (Figure 3), and the analysis of the COI haplotype obtained from *Crepidula* specimens from Asturian estuaries (GenBank ID KU697654) resulted in conclusive species assignments. A BLASTn procedure indicated a 100% identity score and the highest similarity scores to the SERCINVERT0690 *C. fornicata* voucher sample from the Lower Chesapeake Bay, Cape Henry (GenBank ID KU906061). Additionally, molecular phylogenetic analyses of the COI gene sequences obtained from the metabarcoding study of the water samples (NGS consensus sequence AS-I6V) and from classical barcoding of the *C. fornicata* individuals from Asturian estuaries (GenBank ID KU697654) confirmed that they were clustering together (Figure 3). The approximate cost of the NGS procedure and the bioinformatics analysis in this study was 203€ per sample.

Discussion

Human activities have deteriorated the health of estuaries, making them one of the most threatened ecosystems on Earth (Kennish, 2002). Biodiversity monitoring to discover new invasive species, measures for the environmental recovery of these ecosystems and strict controls to avoid an increase in harvest pressure, among other measures, have been recommended to improve the management of these important ecosystems (Blaber *et al*., 2000; Kennish, 2002; Semeraro *et al.,* 2016). These valuable habitats must be regularly managed to keep them as healthy as possible to assure environmental quality (Kenchington, Heino, & Nielsen, 2003).

New tools (metabarcoding) based on genetic techniques, such as NGS, are now available and allow genetic identification of community components to be performed rapidly and effectively (Frischer *et al.,* 2012, Mahon *et al.,* 2013). However, many factors can lead to incorrect conclusions about species presence/absence, including the lack of technical and sample replicates (Jerde & Mahon, 2015; Ficetola *et al*., 2015). Moreover, interpreting eDNA detection in biological systems is not simple (Shogren *et al.,* 2016; Furlan *et al.,* 2016) as the eDNA of a given species could just be a consequence of its presence nearby or at some distance upstream or its occurrence in the past. Moreover, there is also the possibility of false negatives (Type–II errors) due to the lacks of universality of the primers (Clarke et al., 2014; Deagle et al., 2014). Thus, species detection based on eDNA may just be a useful tool to augment conventional and targeted sampling efforts to confirm species presence or to inform and guide intensive sampling near a positive eDNA detection (Shogren *et al*., 2016). In this study, three different samples from two estuaries (limited sampling) were analysed, and the results were used to guide more intensive sampling for physical evidence of invasive species.

Aquaculture may significantly affect the natural biodiversity of estuaries, including the deliberate introduction or release of exotic species (Naylor *et al.,* 2011; Arias and Anadón, 2012). This is the case for American slipper limpet (*C. fornicata*), whose invasion processes and impacts related to

the cultivation of oysters and mussels have been extensively described (e.g., Blanchard, 1997; Grant & Hall-Spencer, 2003). The metabarcoding results in this study revealed the presence of this genus in the samples taken near the chapel in Villaviciosa (VC) and the oyster park in Eo (EO). Both locations have a common characteristic: current oyster aquaculture in Eo and past aquaculture in Villaviciosa (Semeraro *et al.,* 2016). These results pointed to culture of exotic species (like *Crassostrea* spp.) as the introduction vehicle in these estuaries.

Among other effects, *Crepidula* spp. increases trophic and spatial competition that can reduce the growth of commercial bivalves (Blanchard, 2009) and produces negative impacts on the distribution and abundance of young-of-the-year sole fish (*Solea solea*) in coastal nursery areas in the Bay of Biscay (Le Pape *et al.,* 2004). The species was first described in Aldán, Galicia, in the Cantabrian Sea in the second half of the 1970s (Rolán, 1983), and it was then observed in the Ría del Vigo and the Ría del Arousa by the end of the 1980s (Otero & Trigo, 1987). From 1998 to 2009, it was found in the four Rías Baixas and in the Ría del Ferrol in Galicia (Rolán & Trigo, 2007; Besteiro, Urgorri, Moreira, & Díaz-Agras, 2009). In Asturias, there was one previous report, without any genetic assessment, of *C. fornicata* in the Eo estuary (Arias, Richter & Anadón, 2012) and one piece of genetic evidence of the species in Figueras Port in the same estuary in 2016 (Miralles *et al.,* 2016). Currently, the American slipper limpet' expansion seems to be continuing along the coast of Cantabria with the culture of the Japanese oyster *Crassostrea gigas* (Rolán, 1983; Blanchard, 1997) as well as the shared aquacultural practices of the fishermen of the region (e.g., Borrell *et al*., 2014). The post-sampling strategy in this study confirmed, and genetically demonstrated, the presence of *C. fornicata* in Villaviciosa, which demonstrates the value of metabarcoding as an exploratory method to detect new non-indigenous species (which is extremely relevant in the issue of biological invasions) that can be later confirmed by in situ field sampling.

Another case of alien detection using metabarcoding (this work) and physical confirmation by monitoring was the case of the non-indigenous genus *Anadara* (as *Scapharca* in Figure 2). It has been recently found in Ría de Villaviciosa and Ría del Eo estuaries by other authors, with presence of two species: *A. kagoshimensis* in Eo estuary and *A. transversa* in both Villaviciosa and Eo estuaries (Fernández-Rodríguez et al., 2016). We also detected the presence of other potentially invasive species using metabarcoding e.g.: *Macrobrachium* (De Kock & Wolmarans, 2008; FAO, 2014; 2016). *Macrobrachium* is the most diverse genus among palaemonid decapods, distributed worldwide in tropical and subtropical streams, rivers and estuaries. These shrimps, commonly known as prawns, have economic interest being the subject of important fisheries and aquaculture in Asia and Latin America (Chong-Carrillo *et al.,* 2016). Two southeastern Asian species, *M. nipponense* and *M. rosenbergii* have been previously reported as alien species in Europe and America, respectively. Their introduction vectors were most likely the aquaculture escapes and their accidental introduction with ornamental goldfishes and carps from southeastern Asia (De Grave & Ghane, 2006; Mohammed *et al.,* 2011). Other genera found raise interesting questions: e.g.: the subtropical-tropical gastropod genera *Cantharidus*, *Hydatina* and *Strombus* comprise species with circumtropical distribution worldwide and have not been reported at these latitudes to date. However, physical evidences were not found for any of them in monitoring and thus its actual presence in northern Spain cannot be confirmed. In our opinion, metabarcoding findings along are not enough to raise NIS alerts. It should be confirmed by more than one metabarcoding procedure (e.g: the use of different genetic markers (18S, COI) in studies giving coincident results) but mostly by "*in situ*" physical confirmations. This can really help to avoid Type-I errors (false positives) and also can help to minimize Type-II ones (false negatives).

Conclusions

The results obtained in this study confirm that DNA metabarcoding can be a useful tool to monitor the biodiversity of estuaries even with limited sampling, especially for the early detection of invasive species. Indeed, post-NGS sampling and classical DNA barcoding confirmed the presence of the highly invasive species *C. fornicata* in Villaviciosa, where the first indication of this invasive species came from metabarcoding. Although some of the problems with metabarcoding remain unsolved, such as cost (Bott *et al*., 2010; Galal-Khallaf *et al.* 2016), the lack of universal PCR primers (Clarke, Soubrier, Weyrich, & Cooper, 2014; Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014), the availability of reference sequences for all species in the databases (Kwong, Srivathsan, Meier, 2012), and sensitivity (Ficetola *et al.,* 2015; Furlan *et al*., 2016), it represents a powerful tool for the management of crucial ecosystems such as estuaries.

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Figures legends.

Figure 1. The two main estuaries in Asturias (Northern Spain, Bay of Biscay): (1) Ría del Eo and (2) Ría de Villaviciosa. Sampling points are localized as EU (Upstream within Ría del Eo), EO (Oyster Park), EP (Port of Figueras), VF (near Cider Factory El Gaitero), VI (Centro de Interpretación), and VC (The Chapel).

Figure 2. The OTU heatmap per sample for molluscs and crustaceans in the two main estuaries in Asturias (Northern Spain, Bay of Biscay): Ría del Eo and Ría de Villaviciosa. The higher the relative abundance of an OTU in a sample, the more intense the color at the corresponding position in the heatmap. Data has been log-transformed. Sampling points are indicated as EU (Upstream within Ría del Eo), EO (Oyster Park), EP (Port of Figueras), VF (near Cider Factory El Gaitero), VI (Centro de Interpretación), and VC (The Chapel).

Figure 3. Findings related with the detection of the *C. fornicata* species in Asturian estuaries. a) Molecular Phylogenetic analysis based on the Tamura 3-parameter model (+G=0,4092) by Maximum Likelihood method of COI gene sequences from *Crepidula* spp. including NGS consensus sequence from this work (AS-I6V) and COI haplotype sequence from *Crepidula* specimens (KU697654) found in Asturias, Spain. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 9 nucleotide sequences and there were a total of 204 positions in the final dataset. b and c) Pictures showing ventral and distal views of the *C. fornicata* specimens found in Asturian estuaries.

Figure 1.

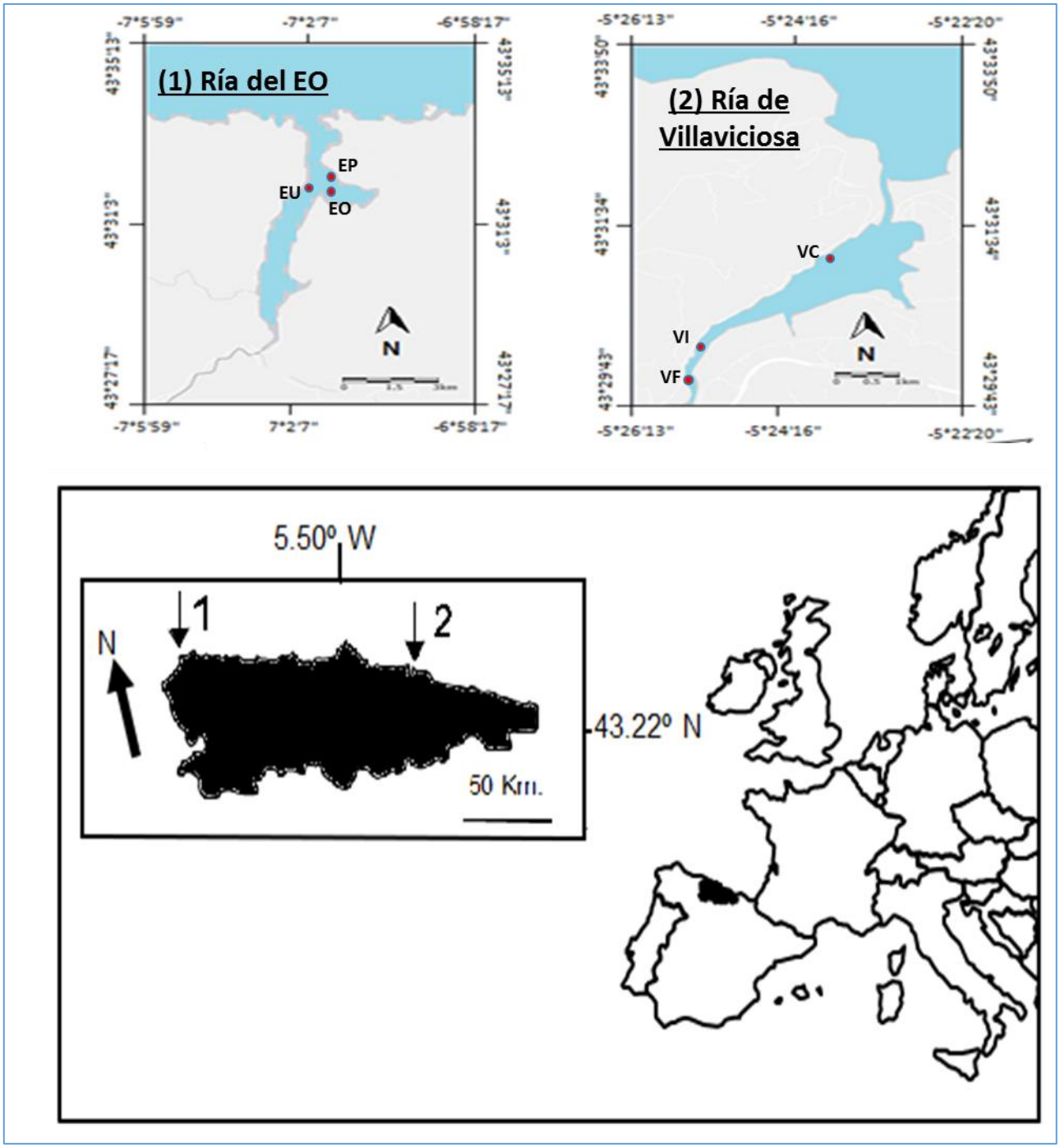


Figure 2.

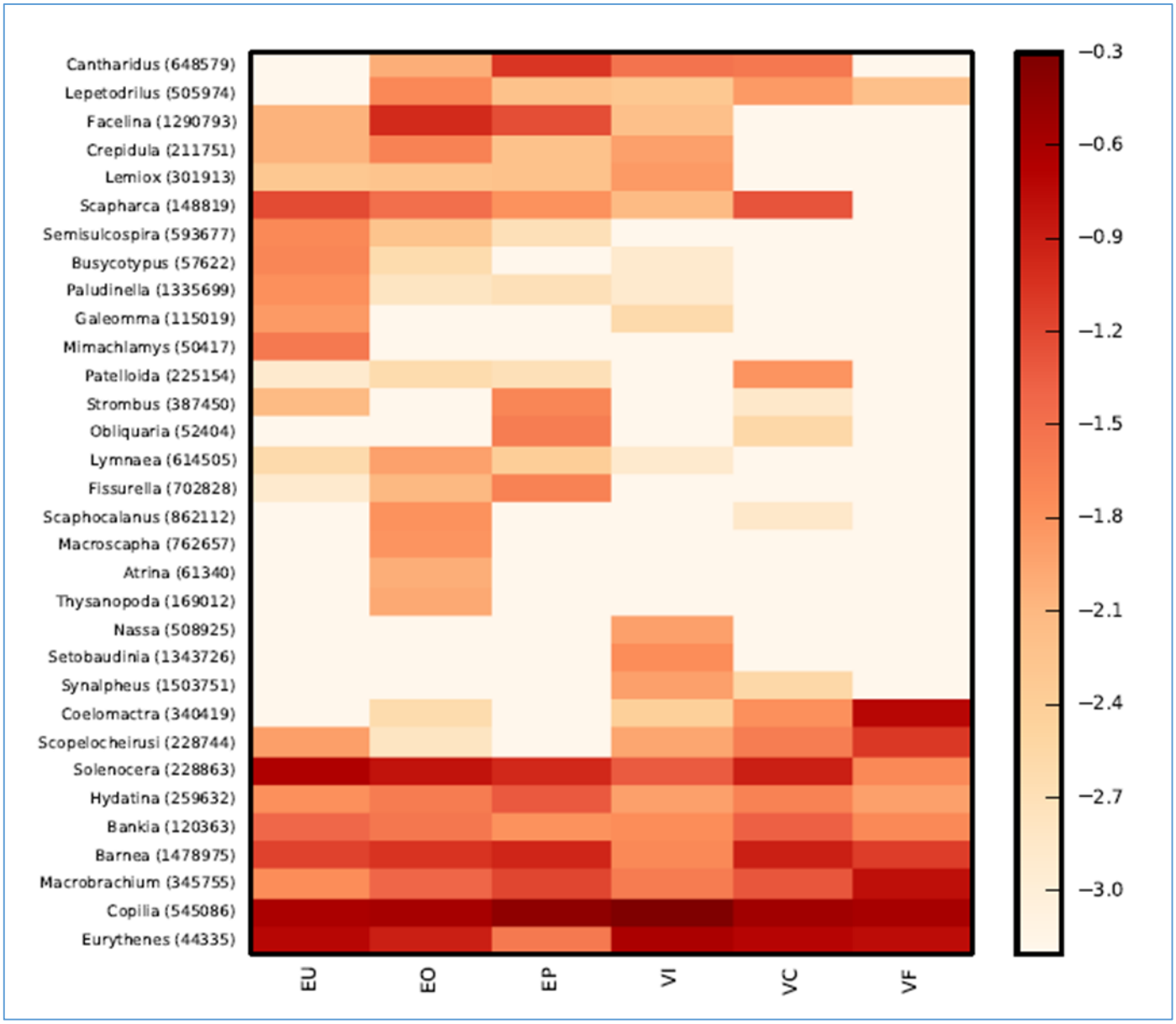
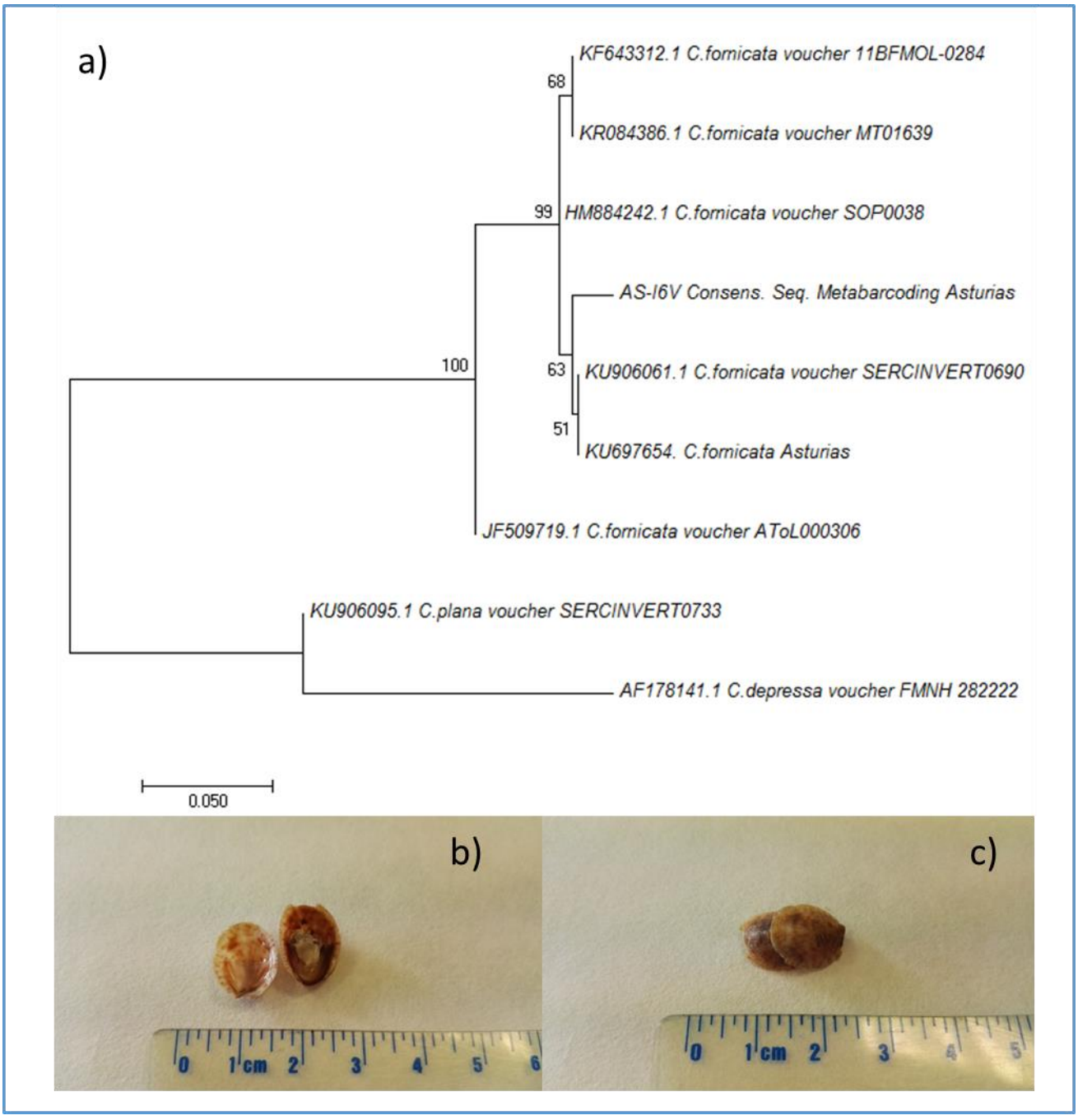


Figure 3.



Tables

Table 1. Environmental DNA (eDNA) samples details from the two main estuaries in Asturias (Northern Spain, Bay of Biscay): Ría del Eo and Ría de Villaviciosa. Sampling points are indicated as EU (Upstream within Ría del Eo), EO (Oyster Park), EP (Port of Figueras), VF (near Cider Factory El Gaitero), VI (Centro de Interpretación), and VC (The Chapel).

Estuary	Sample	eDNA Conc. (ng/ul)	Amplicon Library fragm 800bp-Conc. (molecules/ul)
Villaviciosa	VF	3.426	1.74 X 10 ^ 9
	VI	4.506	3.33 X 10 ^ 9
	VC	4.236	4.91 X 10 ^ 9
Eo	EU	3.064	4.50 X 10 ^ 9
	EO	4.366	4.29 X 10 ^ 9
	EP	3.251	4.46 X 10 ^ 9

Supplementary Tables.

Table A.1. The OTU counts per sample for mollusks and crustaceans in the two main estuaries in Asturias (Northern Spain, Bay of Biscay): Ría del Eo and Ría de Villaviciosa. Sampling points are indicated as EU (Upstream within Ria del Eo), EO (Oyster Park), EP (Port of Figueras), VF (near Cider Factory El Gaitero), VI (Centro de Interpretación), and VC (The Chapel).