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Detection and characterisation of the biopollutant *Xenostrobus securis* (Lamarck 1819) Asturian population from DNA Barcoding and eBarcoding

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ABSTRACT

DNA efficiently contributes to detect and understand marine invasions. In 2014 the potential biological pollutant pygmy mussel (*Xenostrobus securis*) was observed for the first time in the Avilés estuary (Asturias, Bay of Biscay). The goal of this study was to assess the stage of invasion, based on demographic and genetic (DNA Barcoding) characteristics, and to develop a molecular tool for surveying the species in environmental DNA. A total of 130 individuals were analysed for the DNA Barcode cytochrome oxidase I gene in order to determine genetic diversity, population structure, expansion trends, and to inferring introduction hits. Reproduction was evidenced by bimodal size distributions of 1597 mussels. High population genetic variation and genetically distinct clades might suggest multiple introductions from several source populations. Finally, species-specific primers were developed within the DNA barcode for PCR amplification from water samples in order to enabling rapid detection of the species in initial expansion stages.

1. Introduction

New transport technologies and enhanced spatial connectivity have intensified the process of biological pollution worldwide (Perrings et al., 2002), giving non-indigenous species (NIS) the opportunity to expand and thrive in other regions. A minimum of 1369 NIS have been introduced in Europe (Katsanevakis et al., 2013), and the number is probably underestimated because there are still problems estimating the true identity, global distribution, introduction vectors and population demography of many invasive species (Rilov and Crooks, 2009). DNA helps to reduce taxonomical uncertainties in biota inventories and contributes to identify exotic species, for example molluscs (e.g. McDonald and Koehn (1988); Pejović et al. (2015)), arthropods (Geller et al., 2010) and many other. Many studies have successfully employed DNA for identifying genetic diversity, population structure and putative origin of biological invaders. DNA barcodes such as the cytochrome oxidase subunit I (COI) gene can provide a more detailed description about source populations than historical data (Hoos et al. (2010). Moreover, integrating genetic and non-genetic data can enhance the power of inferring the introduction vector of invasions (Geller et al., 2010). Techniques based on environmental DNA (eDNA) allow to identifying biota directly from water or sediment samples (e.g. Harvey et al. (2009); Ardura et al. (2015); Zaiko et al. (2015a, 2015b)). This facilitates rapid detection of potential marine invaders thus enabling a rapid reaction for eradication and control of potential invaders.

Mussels contain several strongly invasive species that are carried as biological pollutants by shipping. The genus *Xenostrobus* is originally from Australia and New Zealand (Wilson and Hodgkin, 1967, Colgan and da Costa, 2013), where they live in brackish waters. The black-pygmy mussel (*Xenostrobus securis*) is a biological pollutant that exhibits nowadays a global distribution. Inadvertently transported by ships, it was first observed out of its native range in Japan in 1972 (Kimura et al., 1999). Over the next decades it spread over more regions in South East Asia (Abdel-Razek et al., 1993a, Kohama et al., 2001, Iwasaki, 2006, Morton and Leung, 2015). In Europe it was probably inadvertently introduced with aquaculture imports as well (Giusti et al., 2008). The species arrived in Italy in 1991 (Sabelli and Speranza, 1994) and expanded over the Adriatic Sea (Crocetta, 2011), along the Western Mediterranean: Italy (Lazzari and Rinaldi, 1994, Giusti et al., 2008), South-France and East-Spain (Barbieri et al., 2011). Out of Mediterranean waters, in Europe it was found in Galicia, Northwest Iberia, in 1995 (Garci et al., 2007, Bañón et al., 2008), and in Bay of Biscay in 2010 (Adarraga and Martínez, 2012).

X. securis was detected in the central coast of Spanish Bay of Biscay for the first time in 2014 (Pejović et al., 2015), at low density and inside one port. It was therefore expected that these specimens were part of a recent introduction event, with low genetic population variation due to

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founder effects. The present work follows that discovery. Ecological and DNA barcoding were carried out to assess population status, detect (if any) signs of population expansion, and develop a Barcode-based tool for easy detection of the species from water samples (environmental DNA approach).

2. Methods

2.1. The species

Xenostrobus securis exhibits a salinity range from 2 to 31 PSU and can withstand temperatures from 1 °C (Iwasaki and Yamamoto, 2014) to 33 °C (Kimura and Sekiguchi, 2009). During periods of extreme salinities it can close the valves and survive without feeding up to four months (Wilson, 1968). Larvae have a planktonic phase that lasts for approximately two months (Abdel-Razek et al., 1993b). With a maximum lifespan of two years and an average size between 20 and 30 mm (maximum 47 mm) individuals can mature within their first year of survival (Wilson, 1969), generally starting from 10 mm or even smaller (Abdel-Razek et al., 1993b) and occupy different niches with its capability for adhesion to different substrates (Babarro and Lassudrie, 2011).

2.2. Study area and survey protocol

Avilés (43°34′07″N, 5°55′20″W) is a major port of Asturias located within Avilés Estuary (Flor et al., 2006). Every year it receives around 800 commercial, 200 recreational and 40 fishing vessels (Autoridad Portuaria de Avilés, 2014). The estuary contains considerable industrial and infrastructural development (coking and steel plants, between others).

For surveying *X. securis* in the Avilés Estuary in January 2015, the sampling protocol was adapted from the Bernice P. Bishop Museum (BPBM) protocols (Bishop and Hutchings, 2011). Seven places (AV01 to AV07) with three random replicates from each one were chosen within the estuary. The site AV02 is the site where the species was found in 2014 by Pejović et al. (2015). All the mussels within a 100 cm^2 quadrat were collected, measured, counted and stored in absolute ethanol. Morphometric features (maximum length, width and height) were obtained with a Vernier calliper (error ± 1 mm).

2.3. Individual DNA Barcoding

From each site 15–25 *X. securis* individuals were randomly picked for DNA Barcoding. DNA was extracted from the adductor muscle (±3 mm^3) with Chelex method (Estoup et al., 1996). The Barcode COI gene was PCR amplified with universal primers (Geller et al., 2013) in a Veriti Blue Thermal Cycler, Applied Biosystems following the protocol described by Geller et al. (2013). PCR products were examined on a 2% agarose gel stained with SimplySafe™. PCR products were sent to Macrogen (Amsterdam, The Netherlands) for sequencing in ABI3730xl DNA sequencer (Applied Biosystems).

DNA Barcoding was also applied to other molluscs present in site AV07 employing the same methodology.

2.4. Development of Xenostrobus securis specific eBarcoding

2.4.1. In silico design of species-specific primers

For designing species-specific primers for *Xenostrobus securis* we followed the approach described by Ardura et al. (2015) for *Rangia cuneata* but using the cytochrome oxidase I (COI) gene as a DNA Barcode instead of 16S rDNA. Briefly, COI gene sequences of a wide taxonomic range of mollusks were downloaded from public databases: GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and BOLD (http://www.boldsystems.org/). They were aligned using ClustalW (Thompson et al., 1994) application on BioEdit. Species-specific primers were developed within the COI region after aligning all the *X. securis* sequences retrieved from the databases, those generated in the present study, and a set of sequences from closely related bivalves; then a region with maximum identity with the target species and minimum with the rest was visually selected. Primer characteristics were checked with the IDT SciTools Oligo Analyzer v3.0. (Coralville, USA). For in silico validation of the new primers, GenBank database was searched for highly similar sequences (MEGABLAST) with parameters adjusted for short input sequences, to confirm the primers only hybridize with *X. securis* sequences.

2.4.2. Marker validation with fresh tissue

The primers were first assayed in DNA extracted from fresh tissue of *X. securis* as explained above (see Section 2.3). PCRs were conducted on Veriti Blue Thermal Cycler. For a final volume of 20 μl 1× Taq buffer, 1.5 mM MgCl2, 0.25 mM dNTPs, 1 μM of each primer, 200 ng/μl Bovine Serum Albumin (BSA), 0.0325 U/μl Taq polymerase (Promega) and 4 μl of target DNA (±50 ng) were used. Conditions were set to an initial denaturation at 95 °C for 5′; then 45 cycles of denaturation of 30″ at 95 °C, annealing at 62 °C for 30″, extension at 72 °C for 30″ and a final extension at 72 °C for 7′ with a final step of 20 °C for 1′. The result was visualized on a 2% agarose gel stained with SimplySafe™.

2.4.3. Aquarium experiments for marker validation

Marine water was obtained from a coastal site nearby (S. Juan de Nieva rocky beach, corresponding to the sampling point AV07) without any visible individual or previous record of *X. securis* and high density of other mollusks: *Crassostrea gigas*, *Littorina littorea*, *Mytilus edulis*, *Mytilus galloprovincialis*, *Ostrea edulis*, *Ostrea stentina*, *Patella depressa*, *Patella vulgata*. Small (1 L) experimental aquarium tanks were prepared adding none, one or five living *X. securis* individuals (two replicates for each density). The individuals were left in the water for 48 h, then removed, sacrificed, and the body (without the shell) dry-weighted. The water was immediately vacuum-filtered through Supor® 200 PES Membrane Disc Filters (Pall Corporation, Life Sciences, USA) with 0.2 μm pore size and 47 mm diameter.

2.4.4. In situ water samples for marker validation

Sea water was obtained from areas with or without detected presence of *Xenostrobus securis* (positive or negative controls respectively). As a negative control we used the site AV07, and as positive control the sites AV01, AV02 and AV05. Water samples of 1 L were directly taken in plastic bottles just below the surface, approx. at 1 m distance from the mussel bed, during low tide. They were stored in cold ice during the transport to the laboratory, where they were immediately frozen until filtering as explained above (Section 2.4.2).

2.4.5. DNA extraction and PCR amplification from water samples

After unfreezing the water at room temperature (or directly from aquarium experiments) DNA was extracted from the filters. Total genomic DNA was extracted using the PowerWater® DNA Isolation Kit (MOBIO Laboratories, USA) which yields high quality DNA for DNA barcoding or meta-barcoding applications. Manufacturer's instructions were followed.

PCR amplification from that DNA was performed using the primers developed here and the same conditions explained above (see Section 2.4.2). PCR products were run on 2% agarose gels stained with SimplySafe™, using EURX perfect 100–1000 bp DNA ladder for band size determination.

2.5. Data analysis

Correlations of mussel densities and morphometric features, and between *Xenostrobus* and *Mytilus* densities, were assessed with STATISTICA v8.0 (StatSoft, 2007). DNA sequences were visually inspected on BioEdit v7.2.5 (Hall, 1999). Every sequence was blasted against the GenBank Nucleotide database and a minimum of 98%