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Development and validation of eDNA markers for the detection of *Crepidula fornicata* in environmental samples

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ABSTRACT

The invasive *Crepidula fornicata* caused major problems along the European Atlantic coast, especially in France and Netherlands where high densities leads on changes in the habitat, disturb native marine wildlife as well as it originates competition for space and food. Despite its dangerous invasive nature, regular monitoring to alert about its presence in risk areas, like the south Bay of Biscay (Spain and south France), is not done yet. Here, we developed a species-specific marker to detect the presence of *C. fornicata* in environmental samples (eDNA) of seawater. The novel *C. fornicata* specific primers amplified a region of 239 bp within the COI gen. We employed this tool to check its presence in 6 estuaries of the Cantabrian Sea, an area comprised between the Spanish and French limits of the previously reported presence of this limpet in the south Bay of Biscay. The presence of *C. fornicata* was confirmed in A Coruña (Galicia, Spain), Eo and Villaviciosa estuaries (Asturias, Spain) while it was not detected in Santander, Bilbao (Spain), and Bayonne (France). This new method to detect *C. fornicata* could be easily implemented in regular monitoring to prevent and manage future invasions of this species.

1. Introduction

The common Atlantic slipper limpet *Crepidula fornicata* is an invasive species along the European Atlantic coast, with extreme densities in the intertidal and subtidal zones of France and Netherlands (Blanchard, 1995, 1997, 2009; Ehrhold et al., 1998; Thieltges et al., 2004; Sauriau et al., 2006). This gastropod was first introduced accidentally into England (McMillan, 1938), then into several European countries during 1930s due to oyster trade, especially into the north of France (Blanchard, 2009). This slipper limpet is strongly resistant to environmental variations, particularly temperature and salinity (Blanchard, 1995, 1997; Diederich and Pechenik, 2013) what helped in its spread in all Europe, from Norway to Spain, and other regions, like Japan or the west coast of United States (Blanchard, 1997). Also, it is likely to have high phenotypic plasticity and resilience to physico-chemical variations, which may have determined its success as an invader (Noisette et al., 2015). Furthermore, *C. fornicata* cause significant impacts on biodiversity and ecosystem functioning in the areas where it has been established (de Montaudouin and Sauriau, 1999; Decottignies et al., 2007a, 2007b; Martin et al., 2007). Nowadays, the Brittany coast of France supports some especially large populations of *C. fornicata* (sediment with up to several thousand individuals per square meter — Blanchard, 1997, 2009; Ehrhold et al., 1998; Thieltges et al., 2004). Such high densities heavily impact the colonized habitat, irreversibly modifying the nature and structure of the bottom (Ehrhold et al., 1998; Grall and Hall-Spencer, 2003), creating local competition for resources and space with suspension-feeders of commercial interest (like oysters and scallops — Blanchard, 1997; Beninger et al., 2007; Decottignies et al., 2007a, 2007b) and disturbing both oyster aquaculture and commercial fisheries relying on dredging (Blanchard, 1997, 2009), together with other important ecological effects like, for example, affecting biodeposition production, altering the turbidity of surrounding areas or causing a marked depletion of phytoplankton (Valdizan et al., 2009; Henry and Lyons, 2016). Despite its dangerous invasive nature in this area, its presence along the coast of the Bay of Biscay (France and Spain) was not monitored yet, even when several focus of infection were already described in previous local studies in areas like Galicia and Asturias in Spain (South west Bay of Biscay) (Bañón et al., 2008; Bañón, 2012; Borrell et al., 2017a, 2017b) and across the French coast just down to Marennes-Oléron (East Bay of Biscay; de Montaudouin and Sauriau, 1999). In order to detect possible invasion events, we have developed a new species-specific molecular marker to trace the

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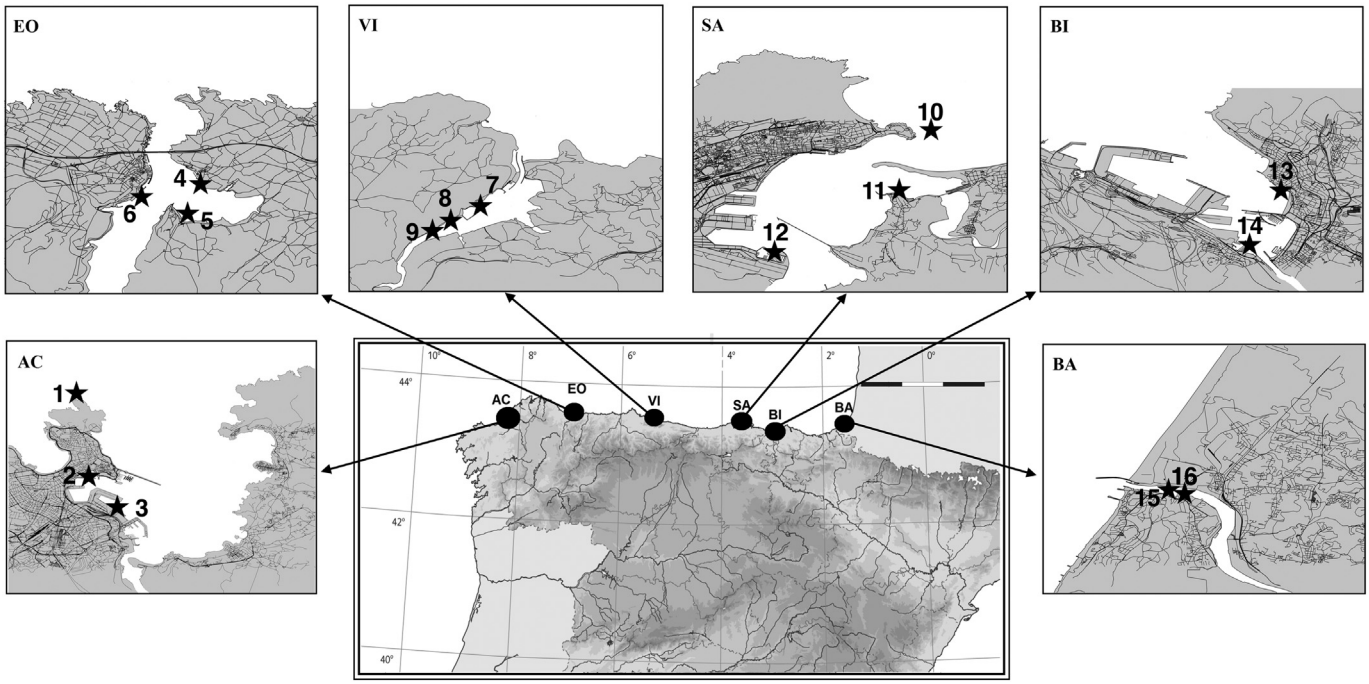

Fig. 1. Maps and details of the Bay of Biscay seawater samplings. AC: A Coruña (Galicia, Spain); EO: river Eo estuary (Asturias, Spain); VI: Villaviciosa estuary (Asturias, Spain); SA: Santander bay (Cantabria, Spain); BI: Bilbao estuary (Pais Vasco, Spain); BA: Bayonne estuary (Anglet, France). Numbers correspond to different sampling points (1–16).

presence of *C. fornicata* in environmental DNA (eDNA) samples. Finally, we tested this new marker in different seawater samples from estuaries from the Cantabrian Sea in the South Bay of Biscay.

2. Material and methods

2.1. Sample acquisition and processing

The sampling area was located in the Cantabrian Sea, in the South Bay of Biscay. A selection of the main estuaries in the area was done since the specie was detected in the north of Spain only in estuaries of Galicia (Rolán and Trigo, 2007; Bañón et al., 2008; Besteiro et al., 2009; Bañón, 2012) and Asturias (Borrell et al., 2017b). Six different Spanish and French estuaries with a total of 16 different points were sampled (Fig. 1) following the protocol from Borrell et al. (2017a, 2017b). The number of sampling points within each estuary varies from 2 (Bayonne and Bilbao) to 3 (A Coruña, Eo, Villaviciosa and Santander) depending on the extension of the sampling area and the risk of invasion according to previous reports. Samples correspond to 1 L of seawater and were taken during winter, from January to March 2017. Seawater samples (1 L) were immediately filtered and DNA extracted with MOBIO Power Water (now Qiagen DNAeasy Power Water) extraction kit also following Borrell et al. (2017a, 2017b). The seawater filtration process and eDNA extractions were done under strict sterile conditions, in an isolate eDNA laboratory unit to avoid any possible contamination. Blanks containing only distillate water were included during the whole process at different stages (sampling, filtration and DNA extraction) and used as negative controls to confirm that contamination did not occur in the process.

2.2. Crepidula fornicata specific marker design and validation

The *C. fornicata* species-specific marker was designed using Prise2 (Huang et al., 2014). The primer pairs was first tested *in silico* using Primer-BLAST website and the BLAST tool of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to discard all possible cross amplification within any known sequence described on universal databases. In other words, to confirm that they only aligned significantly with the target species. Then, it was tested *in vitro* with DNA of six taxonomically related and different molluscs species that could be found in the same type of ecosystems worldwide (*Crepipatella dilatata, Magallana gigas*, *Ostrea stentina*, *Patella vulgata*, *Patella depressa* and *Acantochitona crinita*) to discard any cross amplifications. It may occur that unspecific band could appear in this step, since databases were not complete and only a few species has its complete genome sequenced. The sensitivity of the specific marker was determined *in vitro* with serial dilutions from 1 to 10 million of *C. fornicata* DNA from a known concentration (33.3 ng/μL). To confirm that all tissue and water samples employed in the study had good DNA quality for amplification, the COI gene was amplified following Geller et al. (2013) to discard false negatives in the PCR.

PCR with the specific primers was performed in a final volume of 20 μL, including GoTaq®Buffer 1×, 2.5 mM $MgCl_2$, 0.25 mM dNTPS, 1 μM of each primer, 8 μL of template DNA, and 0.65 U of DNA Taq polymerase (Promega). To avoid possible PCR inhibitions 200 ng/μL of BSA (bovine serum albumin) were included in the mix. PCR conditions were an initial denaturation at 95 °C for 5 min followed by 45 cycles of denaturation at 95 °C for 25 s., annealing at 60 °C for 25 s., extension at 72 °C for 25 s. and a final extension step at 72° for 7 min. All the PCR products were visualized in 2% agarose gels with 2.5 μL of SimplySafe™. All eDNA PCRs (all water samples, including replicates) were repeated three times to confirm all the results. Finally, positive species-specific amplicon of expected size were sequenced by Macrogen services.

3. Results and discussion

In this study, we have developed the first species-specific marker designed to detect the invasive *Crepidula fornicata* in environmental samples. The amplicon obtained with this molecular marker is located within the cytochrome oxidase subunit I (COI) gen using the primers sequences: Forward 5′-GATGATCAACTATACAATGTA-3′ and Reverse 5′- TAAACCGTTCAACCGG-3′.

A region of 239 nucleotides was amplified and no cross-

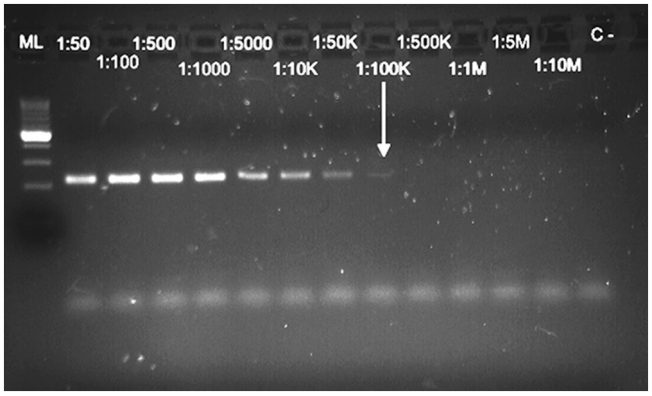

Fig. 2. The *Crepidula fornicata* detection threshold for PCR-visualization in agarose gels using the new specific primer pairs. Serial dilutions and positive amplifications until 1:50.000 can be observed in the fig. C-: PCR negative control. ML: Size ladder (Perfect 100–1000 bp DNA Ladder from EurX).

amplification was detected *in silico,* neither *in vitro*. The detection threshold for PCR-visualization in agarose gel was 0.33 ng/L, since a weak but visible band was detected in the dilution 1:100000 from a sample with a concentration of 33.3 ng/μL (Fig. 2). It is a very sensitive marker compared with other invasive molluscs specific markers. For example, the detection limit for the first *Dreissena polymorpha* specific markers was set at 700000 ng/L (Ardura et al., 2017) or *Melanoides tuberculata* set at 3000 ng/L (Clusa et al., 2017). Its extremely sensitive detection threshold allows detecting the presence of *C. fornicata* in water samples at low densities. Apart from accurate, the method is very fast and easy to repeat; the PCR takes less than 1 h and results can be directly visualized in agarose gels. Those particularly characteristics enhanced its utility for early detection, since a regular monitoring with this genetic tool could help to detect this invasive species in early stages and preventing future invasions in areas with a potential risk. Actually, this potential risk becomes a real fact in areas currently surrendered by *C. fornicata* invasive processes, like the Cantabrian Sea in the Bay of Biscay.

When employing this new tool, the species *C. fornicata* has been detected in environmental samples of 7 points (and its replicates) from the south Bay of Biscay (samples 2, 3, 4, 5, 7, 8, 9 - Fig. 3). Artefact amplifications out of the expected sizes for the new marker did not influence qualitative detections (*e.g.* Clusa et al., 2017). The sequences of the positive amplicons on expected size had the accession numbers LC387550-LC38551 in the DNA Data Bank of Japan, DDBJ Center (Fig. 3). These results confirmed previous reports about the presence of this invasive mollusc in A Coruña (Galicia, Spain) (Bañón et al., 2008) and also in the Eo (Asturias, Spain) and Villaviciosa estuaries (Asturias, Spain) (Borrell et al., 2017b). Noteworthy, the reported density for *C. fornicata* in Asturias was very low and the presence of individuals seems to be scarce (Borrell et al., 2017b). Our results revealed that there are still some sampling points within A Coruña (Galicia) and Eo estuary (Asturias) in which *Crepidula fornicata* was no detected (samples 1 and 6). In the case of A Coruña (1) it might be explained because this sampling point is located outside the estuary, in a more wave-exposed and climatologically adverse area with higher salinity and the species might prefer a more shelter area or the species is not dense enough to colonize it yet. Despite this, it had been reported that this species is strongly resistant to environmental variations, particularly temperature and salinity (*e.g.* Diederich and Pechenik, 2013) so regular monitoring of this area would be highly recommended for controlling the expansion of already detected populations. On the other hand, the presence of *C. fornicata* has been related with oyster farms from the beginning of its European invasion (Blanchard, 1997). In the case of Eo estuary in Asturias, samples 4 and 5 (with positive results) were very close to the oyster farm operating in this estuary. However, sample 6 (negative) was distant and located on the other side of the Eo river. Again, regular monitoring on this area is recommended to prevent possible expansion of the NIS. The presence of *C. fornicata* was not detected in other estuaries from the south Bay of Biscay (Santander and Bilbao in Spain and Bayonne in France). There was not positive amplification in any of its environmental samples (numbers 10–15 and its replicates; Fig. 3), neither previous references about its presence.

Finally, considering the potentially dangerous presence of the invasive *C. fornicata* in European coasts, we recommend the use of this novel molecular tool to monitor the invasion of the slipper limpet. This species-specific marker can detect initial states of invasions due to its sensitivity, it is cheap, easy to replicate and fast. Moreover, early detection of invasive species is indispensable to prevent the establishment of new biological invasions (Mehta et al., 2007) and a crucial step for successful post-introduction management (Pochon et al., 2015). Thus, annual eDNA samplings during winter (*i.e.* between reproduction peaks) by employing this new molecular marker in areas under risk of introduction could be useful and would help to monitor, control and minimize the possibility of a new *C. fornicata* invasion.

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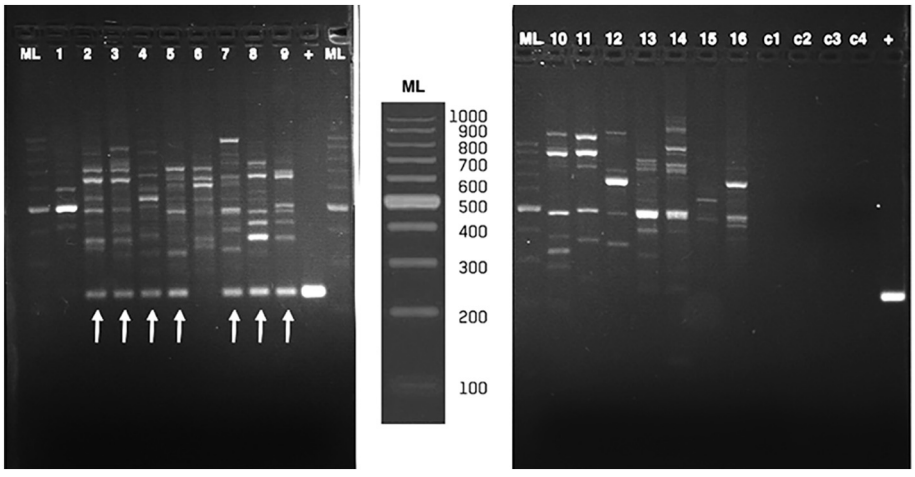

Fig. 3. Qualitative detection of *Crepidula fornicata* in agarose gels using the new specific primer pairs on eDNA from Bay of Biscay samples: 1–3 A Coruña (Galicia, Spain); 4–6 Eo estuary (Asturias, Spain); 7–9 Villaviciosa estuary (Asturias, Spain); 10–12 Santander (Cantabria, Spain); 13–14 Bilbao (Pais Vasco, Spain); 15–16 Bayona (Anglet, France) (see Fig. 1 for details). C1: Negative control of sampling; C2: Negative control of filtration, C3: Negative control of DNA extraction; C4: PCR negative control; +: positive control of *Crepidula fornicata* dilution 1:100; ML: Size ladder (Perfect 100–1000 bp DNA Ladder from EurX).

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