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Novel tools for early detection of a global aquatic invasive, the zebra mussel Dreissena polymorpha

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ABSTRACT

1. This study presents a species-specific DNA-based marker for detection of the zebra mussel *Dreissena polymorpha*, recognized as one of the worst invasive species worldwide.

2. The marker was developed *in silico* and experimentally tested on environmental samples. Gel and capillary electrophoreses for visualization of the PCR products were compared.

3. Marker specificity and sensitivity were assessed *in vitro* by cross-amplifications and serial dilutions, respectively. The method allows detecting at least 0.7 ng of *Dreissena* DNA per μL and cross-species amplification was not found in any case.

4. Next-generation sequencing (NGS) metabarcoding (PCR amplification and massive sequencing of a DNA barcode) was used as an independent method for verifying presence of *Dreissena* DNA molecules in environmental plankton samples collected from the south-eastern Baltic Sea.

5. The consistency between NGS results reporting presence of *Dreissena* and positive PCR amplification of the marker from the plankton samples confirmed the efficacy of this highly reproducible, fast, cheap and technically easy method.

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KEY WORDS: biosecurity; eDNA; metabarcoding; monitoring; invertebrates; alien species

INTRODUCTION

One of the current widely recognized anthropogenic threats to marine biodiversity is the cross-regional transfer of non-indigenous species (NIS) causing irreversible alterations in communities, habitats and ecosystem functioning (Carlton and Geller, 1993). Early detection of non-indigenous and particularly potentially invasive species is a prerequisite for the efficient and timely response – further spread

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prevention, eradication (if possible) and mitigation of threats to ecosystems and economy (Olenin *et al.*, 2011; Pochon *et al.,* 2015). However at the early stage of incursion the species is likely to be sparsely distributed in the ecosystem, reducing the chances of detection by routine surveillance methods. On the other hand, the dispersal life stages, such as planktonic eggs or larvae that might be relatively abundant are not identified in traditional zooplankton surveys owing to their cryptic morphology and lack of sufficient specific taxonomical expertise (Neigel *et al.,* 2007; Ardura *et al.,* 2010; Pochon *et al.*, 2013). This poses biosecurity risks, with invasive sessile organisms not being identified until they are already established and widespread and thus difficult to eradicate (Freire *et al.*, 2014; Zaiko *et al.*, 2014).

Among many registered nuisance organisms, the zebra mussel *Dreissena polymorpha* is recognized as one of the worst invasive species worldwide. For example, in 30 years since zebra mussel was introduced in North America, it has become one of the most widespread and abundant freshwater invertebrates, and has fundamentally transformed freshwater food webs and biogeochemistry. Indeed, few human impacts have been greater or more far-reaching than the arrival of this single species in North American freshwater ecosystems (Strayer, 2009). NIS-related indicators are increasingly included in national and regional legislative initiatives, such as the Marine Strategy Framework Directive (MSFD) (European Commission, 2008) and are a particular focus in various marine monitoring programmes. For example, the abundance and distribution range of the zebra mussel is proposed as a core indicator for the NIS descriptor by the Helsinki Commission (HELCOM, 2013). This urges the development of efficient and reliable methods of species detection, given that benthic zebra mussel populations tend to be temporally and spatially unstable with patchy distribution (Zaiko *et al.*, 2009; Minchin and Zaiko, 2013). Since the traditional surveillance tools seem to be inadequate to detect NIS in aquatic environments when organisms are in low abundance, alternative approaches have been investigated. Several studies have demonstrated the efficacy of environmental DNA (eDNA) coupled with next-generation sequencing of informative regions such as 16S rRNA and cytochrome oxidase I gene (COI) as a tool for eukaryote species detection in aquatic environments (Ficetola *et al.*, 2008; Dejean *et al.*, 2011; Jerde *et al.*, 2011; Taberlet *et al.*, 2012; Thomsen *et al.*, 2012; Pochon *et al.*, 2013; Wood *et al.*, 2013). Species-specific molecular markers can be PCR (polymerase chain reaction)-amplified from the eDNA, allowing detection of target organisms such as NIS from early invasion fronts (Jerde *et al.*, 2011), or threatened and indicator species (Thomsen *et al.*, 2012).

The present study focused on the development and validation of species-specific molecular markers for detection of the zebra mussel *D. polymorpha* from environmental (plankton) samples. In addition to the risk of continuing secondary spread of the species within the region there have been recent considerations of using zebra mussel cultivation/harvesting as a complementary measure to control nutrient concentrations in coastal waters (Lindahl *et al.*, 2012; Schernewski *et al.*, 2012). This has enhanced the need for operational surveillance methods aimed at zebra mussel populations and dispersion patterns of their planktonic larvae.

MATERIAL AND METHODS

Development of a zebra mussel species-specific PCR marker

Total DNA was extracted employing a method based on silica gel columns (QIAmp DNA Mini kit, Qiagen) from five *Dreissena polymorpha* adult individuals collected in the Curonian Lagoon (Figure 1), following the manufacturer's instructions. The mussels were identified *de visu* by taxonomic experts. DNA samples were stored at 4 °C for immediate follow-up analysis, and aliquots were frozen at –20 °C for long-term preservation.

Another five bivalve mollusc species with a planktonic larval stage were sampled from the Lithuanian coast of the Baltic Sea: the native *Cerastoderma glaucum*, *Macoma balthica*, *Mytilus*

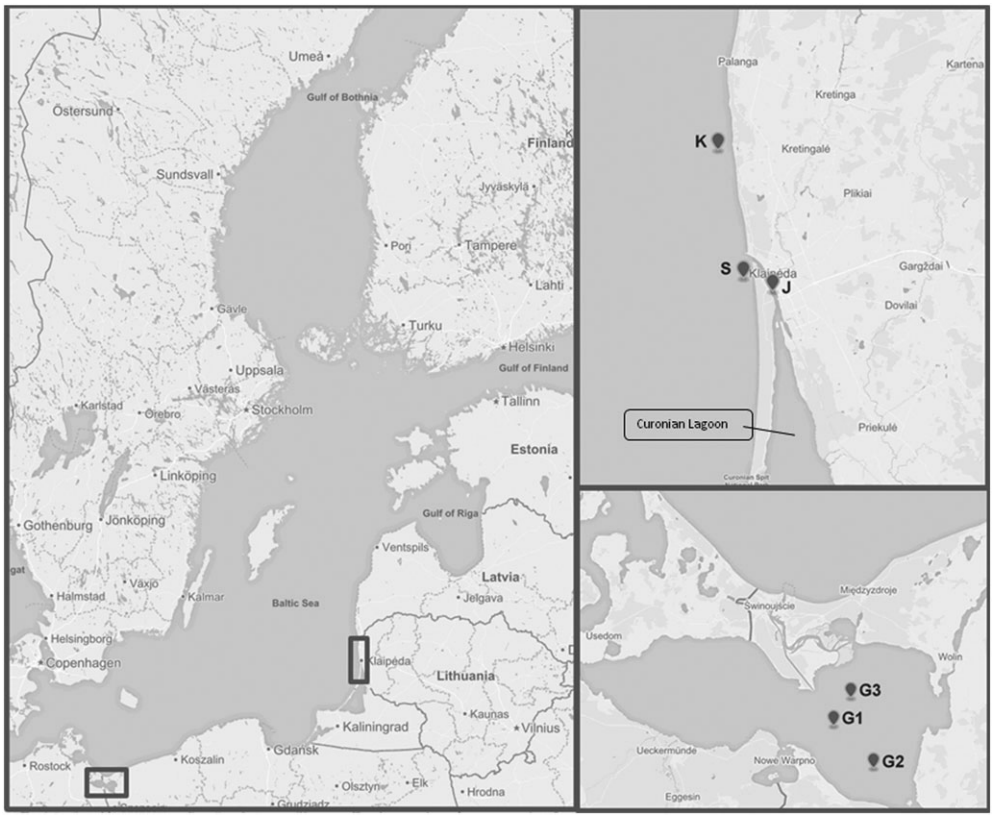

Figure 1. Map of the Baltic Sea with the sampling points analysed in this work.

trossulus; the already established invader *Rangia cuneata* and the cryptogenic *Mya arenaria* (the origin of the latter is disputed among scientists, Carlton, 1996). The DNA of these species were extracted the same way as described above. The sequences obtained (~470 bp long) were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/) where they are available with the accession numbers KP052743-KP052753, corresponding to *C. glaucum*, *D. polymorpha*, four different haplotypes of each of *M. balthica*, *M. arenaria*, *M. trossulus* and three haplotypes of *R. cuneata*. In addition to these sequences, 25 16S rDNA sequences representative of a wide range of marine taxonomic groups, including algae, invertebrates and vertebrates, were retrieved from GenBank (Table S1, Supplementary material) in order to check the specificity of the new PCR marker.

The 16S rRNA and COI barcode genes were amplified for each of the *Dreissena* samples. The 16S rRNA was amplified using the primers 16Sar and 16Sbr described by Palumbi (1996) in a total volume of 20 μL, with Promega (Madison, WI), Buffer 1×, 2.5 mmol L^{-1} $MgCl_2$, 0.25 mmol L^{-1} dNTPs, 20 pmol of each primer, approximately 20 ng of template DNA and 1 U of DNA Taq polymerase (Promega). The following PCR conditions were applied: initial denaturing at 95 °C for 5 min, 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. The COI gene fragment was amplified using the primers designed by Geller *et al.* (2013) in a total volume of 20 μL and with a PCR mix containing components and proportions as those described above. The PCR conditions were: initial denaturing at 95 °C for 5 min, 35 cycles of denaturing at 95 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min.

The PCR products were electrophoresed in a 2% agarose gel, containing SimplySafe™ (EURx) and using Promega 100 bp DNA Ladder Molecular Weight Marker for determining the size of the

amplicons. The same marker served for estimating DNA quantity, by brightness. Bands were purified using the simple protocol of Illustra Exostar 1-Step (GE Healthcare life Sciences) and were then sent to MACROGEN, Amsterdam, Netherlands for sequencing, using a standard Sanger sequencing method (Sanger and Coulson, 1975).

The *D. polymorpha* sequences of COI and 16S rRNA genes were aligned using the BioEdit program (Hall, 1999) together with reference sequences of *D. polymorpha* retrieved for each targeted gene from the GenBank. Phylogenetic trees were reconstructed using MEGA version 6 (Tamura *et al.*, 2013) by the method of Neighbour-Joining with the following settings: Tamura–Nei model (Tamura and Nei, 1993) and uniform rates. Robustness of the Neighbour-Joining topology was assessed using 1000 bootstrap replicates.

From the sequence alignments, species-specific primers were designed within each 16S rRNA and COI genes. Regions about 20 nucleotides long were searched within the alignment being invariant within species but different among the species considered. A candidate primer within the alignments was found only for the 16S rRNA gene, since nucleotide regions of the sought characteristics were not found within the COI gene alignment.

Validation of the zebra mussel species-specific PCR marker

The specificity of the new marker was first validated *in silico*, by comparing the primers' sequences with the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) database using the basic local alignment search tool (BLAST). BLAST parameters were adjusted to search for short input sequences, the highly similar output sequences were considered to decide on the possibility of cross-reacting with other species.

At the next validation stage, the sensitivity and specificity of the designed primers were experimentally verified. Successive dilutions (1, 1:5, 1:10, 1:50; 1:100) of *Dreissena* DNA in distilled water were used as templates for PCRs (sensitivity test). Templates containing 70 ng μL of DNA per each of five bivalve species together with *D. polymorpha* successive dilutions (1, 1:5, 1:10, 1:50, 1:100) were used for testing for the potential cross-amplifications with other molluscs co-occurring with *D. polymorpha* in the region (specificity test). The applied PCR conditions were as follows: initial denaturing at 95 °C for 5 min, 35 cycles of denaturing at 94 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. The PCR products were electrophoresed in a 1% agarose gel, containing SimplySafe™ (EURx) and using Promega 100 bp DNA Ladder Molecular Weight Marker for amplicon size verification and DNA quantification. If no amplifications of the targeted genes were detected by the gel electrophoresis, the PCR was repeated with adjusted conditions (annealing temperatures, number of cycles, DNA concentrations, etc.). Repeatedly unsuccessful amplifications were considered as negative.

Capillary electrophoresis was also used for estimating the amplicon sizes and additional validation tests of all PCR products obtained in the course of the study. For this, only the reverse universal primer, 16br, described by Palumbi (1996) was labelled fluorescently with VIC®. Capillary electrophoresis was performed on ABI 3100 automatic DNA Sequencer and the amplicons were visualized with the PeakScanner Software v1.0, which gives peaks measuring relative fluorescence units (RFU).

Environmental samples and environmental DNA (eDNA) extraction

For field validation of the designed marker plankton samples from the locations where *D. polymorpha* could be potentially detected were used. Samples were taken from south-eastern part of the Baltic Sea: the Odra (Szczecin) Lagoon in Poland where an abundant zebra mussel population has been reported (Piesik, 1974; Leppäkoski, 1984; Jansson, 1994; Daunys *et al.*, 2006), the Klaipeda Strait area (Lithuania), where sparse settlements of zebra mussels are observed (Zaiko *et al.*, 2007) and the open Lithuanian coast where *Dreissena* is not present but planktonic larvae can occasionally

occur, transported by currents from the stock population in the Curonian Lagoon (Figure 1). The Odra Lagoon samples were considered a natural positive control, owing to the high density of *Dreissena polymorpha* adults found there (Fenske *et al*., 2013), and therefore a high concentration of DNA molecules in the water was expected. At other sampling sites (the Klaipeda Strait and Lithuanian coast) none to low abundances of the zebra mussel DNA were expected.

Nine environmental samples were collected covering the presumed spawning season of the zebra mussel between May and August (Sprung, 1993; Garton *et al.,* 2013; and unpublished data from the local surveys). Three samples were obtained from the Odra Lagoon (Stations G1-G3). In the Lithuanian coastal zone, two samples were taken from the Klaipeda Strait area (Station J), three samples from the entrance to the strait from the seaward side (Station S), and one on the open coast, north to the Curonian Lagoon (Station K, Figure 1).

For each sample, a plankton net (55 cm diameter, 80 μm mesh size) was towed vertically 3–10 m, depending on the depth. The concentrated samples (approximately 10 mL) were kept on ice until delivered to the laboratory (1–4 h), then filtered through 0.12 μm NucleporeTM membrane, which was thereafter preserved with 96% ethanol for future bulk DNA extraction. Total genomic DNA of each sample was extracted using QIAamp® DNA Mini Kit (Qiagen, Germany). DNA extraction was conducted in sterile conditions, inside a laminar air flow chamber disinfected by UV light and absolute ethanol to prevent contamination.

The eDNA samples obtained were used to amplify the 16S rRNA region with the designed species-specific primer pairs. Two methods were applied for testing the new primers: (i) unlabelled species-specific primers within the 16S rRNA gene visualized in gel, and (ii) fluorescently labelled specific primers visualized by capillary electrophoresis. For an independent validation of *Dreissena* detection by species-specific marker, eDNA aliquots from the same samples were analysed using next-generation sequencing (NGS) and a metabarcoding approach.

NGS analysis

NGS metabarcoding (using cytochrome oxidase gene, COI, as DNA barcode) was employed as an independent method for confirming the presence of the *D. polymorpha* DNA in the environmental samples. The modified universal COI primers (Geller *et al*., 2013) were used for PCR amplification from the eDNA samples. PCR reactions were undertaken by Macrogen Korea based on the original protocol described by Geller *et al*. (2013) using blank amplifications as controls for discarding possible contaminations. Library constructions included control steps for sizes (Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip) and quantity (Roche's Rapid library standard Quantification solution and calculator). Roche's 454 pyrosequencing technology was employed. Later on, the sequence reads were assigned to samples based on their nucleotide barcode (demultiplexing). The software used barcode sequences to segregate the reads from each sample, by matching the initial and final bases of the reads to the known tag sequences used in the preparation of the libraries. Zero base errors were allowed in this sorting by tag step. Quality filtering was also performed at this step, removing any low quality or ambiguous reads.

Pre-processing and clustering was performed by CD-HIT-OTU (Wu *et al*., 2011). Short reads ($<$100 nucleotides) were filtered out and primers were trimmed. Filtered reads were aligned and clustered at 100% identity using CD-HIT-DUP. Chimeric reads were identified and eliminated. Secondary clusters were recruited into primary clusters. Sequences of 150 nucleotides or less were removed. Remaining representative reads from non-chimeric sequences were clustered using a greedy algorithm into OTUs at a 97% cutoff (i.e. at a species level). The OTUs were BLASTed against the NCBI database, with e-value threshold of 0.01, $\geq$97% sequence homology and $>$90% sequence coverage for accepting hits. The OTUs taxonomically assigned to *D. polymorpha* were retained and used as evidence for the presence of the mollusk and for comparison with the new method of detection developed in this work (species-specific marker).

RESULTS

Marker design and validation

The design of species-specific primers was based on the phylogenetic trees obtained for the region with the least intra-specific and most inter-specific variation, in this case in the 16S rRNA gene (Figure 2).

The sequence of the newly designed forward primer is:

DP-16Sar: 5′- TTAAGAGAAATAGCTTAGAA -3′.

Employing the primer 16Sbr described by Palumbi (1996) as a reverse primer together with the new specific forward primer designed, the pair flanks a region of 258 base pairs (bp) (Figure 3).

In silico validation of the new marker resulted in a perfect match of the input sequences (100% of query coverage and 100% of identity) with *D. polymorpha* reference sequences only. The next best hits (with much higher thus less significant e-values) corresponded to reference sequences for *Ovis canadensis*, *Cucumis melo* and *Solanum lycopersicum*. These three species are the bighorn sheep, the melon and the potato, evidently not aquatic species and thus not expected to co-occur with *Dreissena*, therefore they are not relevant for the purpose of identifying *Dreissena* from the water samples and can be disregarded.

In vitro assays also confirmed the specificity of the marker. *Dreissena*-specific primers yielded positive PCR amplifications with one single apparent band (approx. 250 bp) only when *D. polymorpha* was present in a sample, and never from DNA of other molluscs using the PCR conditions described above. Cross-amplification was thus not found in any case (Figure 3(A)). PCR amplifications from different mixtures of DNA of different molluscs were successful with the new primer only when *D. polymorpha* DNA occurred in the mixture.

The sensitivity test of the marker, performed on different dilutions of *D. polymorpha* DNA, resulted in successful PCR amplifications with clearly visible bands of the expected amplicon size

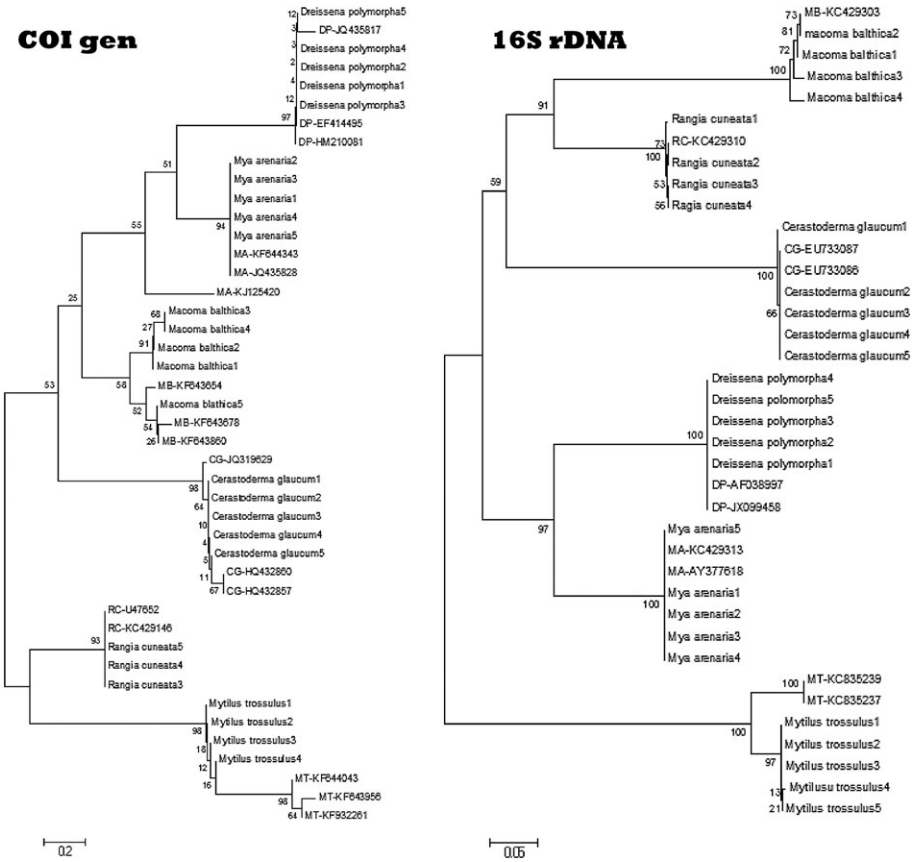

Figure 2. Phylogenetic trees containing the reference and *D. polymorpha* sequences obtained in this work, for COI and 16S rRNA genes, respectively.

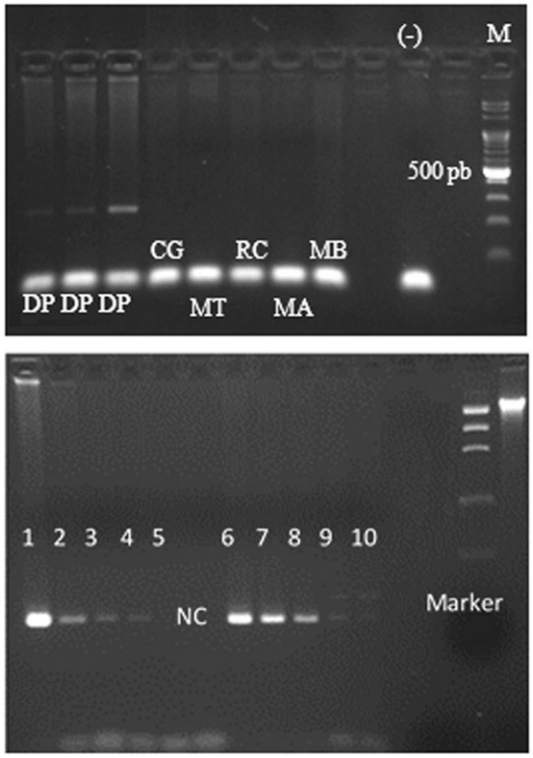

Figure 3. A, above: Agarose gel of PCR products with *Dreissena*-specific primers from *D. polymorpha* (DP), *C. glaucum* (CG), *M. trossulus* (MT), *R. cuneata* (RC), *M. arenaria* (MA), *M. balthica* (MB), and NC: negative control. Marker: DNA size marker 100 bp ladder. B, below: Agarose gel of PCR products obtained with *Dreissena*-specific primers. Left: serial dilutions 1:5 to 1:100 of *D. polymorpha* in distilled water (1–5). Center: NC, negative control. Right: serial dilutions 1:5 to 1:100 of *D. polymorpha* together with 70 ng μL^{-1} of each of the other five mollusks (6–10). Marker: low DNA mass ladder. DP: *D. polymorpha* DNA extraction.

for the dilutions assayed down to 1:100 (Figure 3(B)). Hence, the species detection limit could be set at a DNA concentration of 0.7 ng μL^{-1}. In experimental DNA mixtures, *Dreissena*-specific primers exhibited the same sensitivity as for *Dreissena* DNA alone (1:100). The amplicon was observed at the expected size (258 bp) (Figure 3(B)). It can be concluded that there is no interference when DNA from other co-occurring molluscs is present.

NGS results and species-specific primer validation on environmental samples

DNA extractions from the nine environmental samples yielded good quality DNA, indicated by nanophotometer absorbance ratios 260/280 ranging between 1.7 and 2.1. The concentration of extracted DNA ranged from 6.3 to 65.7 ng μL^{-1}. The positive PCR amplicons produced a total of 99 113 sequences assigned (≥97% homology and >90% bp coverage) to a species or a genus level. Since the objective of this study was *D. polymorpha* and not the entire biodiversity, only a shallow taxonomic overview will be presented here. Excluding Prokaryotes and Archaeas, 89 681 Eukaryote sequences were obtained. The principal taxonomic groups represented by >100 sequence reads were Arthropods (64.6%), Rotifera (22.9%), Chordata (fish; 7.3%). Bacillariophyceae (2.2%), Annelida (1.2%), Streptophyta and Porifera (0.5% each), Mollusca (0.4%) and Ascomycota (0.2%). A total of 110 sequence reads from that dataset were assigned to *D. polymorpha*, and appeared in five samples (Table 1): at all three locations within the Odra Lagoon (61, 19, 24 reads at G1, G2, G3, respectively) and in the samples from the Klaipeda Strait area (three reads at both J2 and J3).

The universal primers (Palumbi, 1996) were used as positive control for DNA quality in the environmental samples, clear and solid bands were obtained demonstrating successful PCR amplification from the nine eDNA samples (Table 1). PCR amplification products obtained with the newly designed specific primers for *D. polymorpha* on eDNA samples were visualized in agarose gel only for the two samples from Odra Lagoon (G1 and G2) (Table 1, Figure 4). For labelled species-specific primers, PCR amplicons of the expected size (258 bp) were clearly visualized in PeakScanner chromatograms (more than 3000 RFU) for the three Odra Lagoon samples (G1, G2 and G3) but not for any of the other samples (Table 1, Figure 5).

DISCUSSION

This study presents a newly-designed robust species-specific marker that serves for detecting the presence of the global invasive species *D. polymorpha* from environmental samples, even at very low concentrations of the species' DNA. The marker can be fully genotyped in a few hours, requires only minimal technical knowledge, especially if it is visualized in agarose gels (Table 2). Although it may depend on the country,

Table 1. Results of NGS (number of *D. polymorpha* sequence reads detected with stringent quality filters), PCR amplification with universal primers described by Palumbi (1996) used here as positive amplification controls for eDNA, *Dreissena*-specific primers described in this article in agarose gel and in automatic DNA sequencer. +: positive and -: negative. In grey shade are the water samples where *D. polymorpha* DNA was detected by all the methods

	LithuaniaNorth to Curonian Lagoon	LithuaniaEntrance to the strait (sea side)			LithuaniaKlaipeda strait		PolandOdra Lagoon		
	K_1	S_1	S_2	S_3	J_2	J_3	G_1	G_2	G_3
Number of reads of *Dreissena polymorpha* NGS sequences (Proportion of sequence reads assigned to *Dreissena,* % of the total number of sequence reads in sample)	0 (0)	0 (0)	0 (0)	0 (0)	3 (0.119)	3 (0.048)	61 (0.813)	19 (1.225)	24 (0.159)
PCR amplification with Palumbi primers (Palumbi, 1996)	+	+	+	+	+	+	+	+	+
PCR amplification with *Dreissena*-specific primers visualized in agarose gel	-	-	-	-	-	-	+	+	-
PCR amplification with labelled *Dreissena*-specific primers as visualized in automatic DNA sequencer	-	-	-	-	-	-	+	+	+

laboratory and/or company, at present (2015) the estimated cost would be 5–10 € per sample, or less if it is automated. The process is the same for tissue and water samples, with the only difference of an extra step of water filtration in the latter case.

These characteristics would suggest the use of this marker for early detection of *D. polymorpha* and further surveillance of the established populations. The tools used in the early detection and monitoring of invasive species need to be rapidly deployable, cost-effective, technically accessible, and accurate (Darling and Blum, 2007). In the traditional approaches these criteria are not well met because taxonomists with expertise in multiple taxa are needed for identification of complex communities, thus significantly increasing the costs of surveillance (Lawton *et al.*, 1998). In addition, some taxa or particular life stages can be very difficult to detect and unambiguously identify. In the case with *D. polymorpha*, it has a pelagic larval stage easily confounded with other mollusc larvae. The marker developed here can aid detection of the species from water samples and allows instant results retrieved within one working day at a reasonable price.

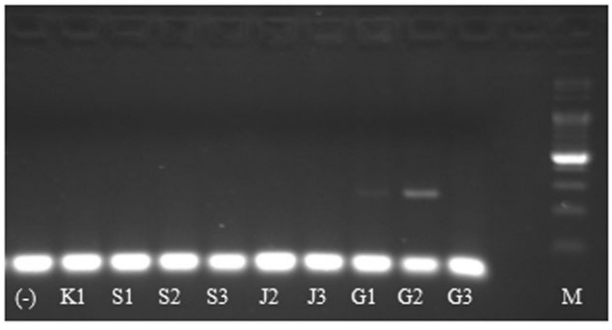

Figure 4. Agarose gel with PCR products obtained with *Dreissena*-specific primers from Baltic water samples, as K_1 (Lithuanian coast); S_1, S_2, S_3 (entrance to the Klaipeda Strait from the sea side); J_2, J_3 (Klaipeda Strait).

Similar approaches (application of the species-specific molecular markers on eDNA samples) can be adapted for detecting other species of interest (invasive, indicator, threatened, etc.). There are, however, some considerations to be taken into account before suggesting such tools for routine surveillance of the target organisms. First, the DNA region of choice must anneal on every individual of the target species. In this work, although the NGS validation was performed with cytochrome oxidase I (COI) since comprehensive collection of the reference sequences is available in databases for this marker (Hebert *et al.*, 2003), the specific primer was designed within the 16S rRNA region because the intra-specific variation is lower in this gene (Figure 2).

Second, the marker must be species-specific to ensure consistent species detection (Diaz-Ferguson *et al.*, 2014). In the present study, no cross-

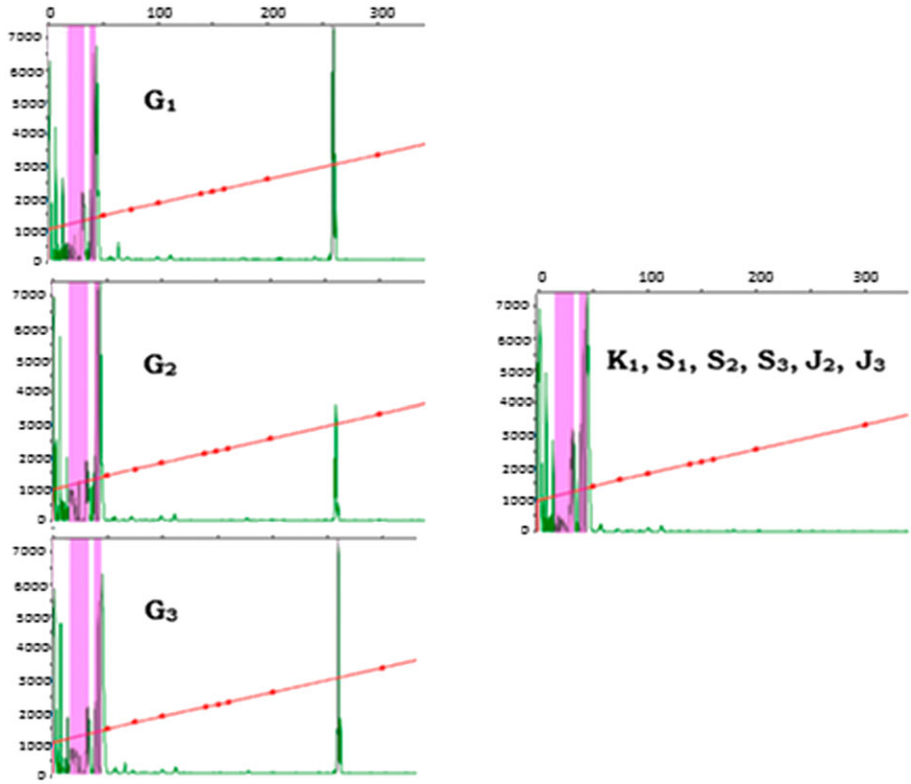

Figure 5. Visualization of fluorescently labelled amplicons with PeakScanner. Clear positive amplicons were observed for the three samples obtained from Odra Lagoon (G_1, G_2 and G_3) and none for the rest of the sampling points (K_1 (Lithuanian Coast); S_1, S_2, S_3 (entrance to the Klaipeda Strait from the sea side); J_2, J_3 (Klaipeda Strait)).

amplification with other aquatic species that could occur in the same environment was detected (Figure 3(a)), as also confirmed by the *in silico* tests. However, before applying the same markers in other ecosystems the cross-amplification tests should be conducted involving the local closely related organisms.

Third, the threshold of detection is very important, particularly if the species-specific primers are considered for early detection of NIS. At the early stage of incursion non-indigenous organisms are expected to be at very low densities and sparsely distributed. Here the marker yielded PCR amplification with visible amplicons at low DNA concentrations (Table 1, Figure 5) thus allowing species' detection from a few cells in a sample. Indeed, NGS-based methodology is also capable of species detection and is highly sensitive,

Table 2. Time and technical difficulty of the proposed *Dreissena*-specific marker

	Activity	Equipment required	Technical difficulty	Time
Step 1	DNA extraction	Basic (extraction kit, pipettes, centrifuge)	Easy	1–2 h
Step 2	PCR amplification	Medium (thermal cycler)	Easy	2–4 h (depending on cycles)
Step 3-a	Separation by electrophoresis in agarose gel	Basic (electrophoresis cuvettes, electricity supply)	Easy	20 min
Step 3-b	Separation by capillary electrophoresis	Medium (automated sequencer such as ABI Prism or similar)	Medium (can be externalized)	Depending on system - <1 h
Step 4-a	Visualization by UV	Basic (UV lamp)	Easy	5 min
Step 4-b	Visualization onscreen (chromatograms)	Medium (computer with adequate software)	Easy	5 min

but the cost of the sequencing is significantly higher and substantial analytical effort (bioinformatics) is needed to ensure efficient exploration of sequence data obtained from the multi-species communities (Blanchet, 2012). In this work, consistent results in detection of *Dreissena* were obtained using NGS and the newly developed specific marker from water samples from the Odra Lagoon (samples G). In water samples from the Klaipeda Strait (J2 and J3), the number of reads detected by NGS was really low (=3) and would be generally eliminated from the assigned species list after applying more stringent OTU-picking filters (Bokulich *et al.*, 2013). The use of capillary electrophoresis for detecting amplicons of the newly developed marker could be recommended for more precise detection of *D. polymorpha* from environmental samples. This is supported by the higher consistency observed between results obtained by this method and those obtained by NGS. On the other hand, NGS might underestimate the presence of the species as well, owing to the relatively reduced performance of primers employed here, since in standard assays they registered only 54% success in Bivalvia (Geller *et al.*, 2013). Notwithstanding this, the comparison between locations is still valid because the bias, if occurring, would be the same in all samples.

Another advantage of the new marker is that the target fragment is very short (258 bp) and can be PCR amplified even in partly degraded DNA with further visualization in agarose gel. This is very important for species detection from environmental samples because short DNA fragments can persist in the environment for a longer time than the large fragments and thus report the 'recent biodiversity' rather than the actual one. For instance, it was proven that DNA fragments of approximately 400 bp may persist for up to one week at 18 °C in lake water (Matsui *et al.*, 2001). This is particularly important for target species detection from plankton samples because of the dynamic and unstable nature of the plankton communities.

Since environmental samples can contain cells or tissues of the species suspended in the water column (Minamoto *et al.*, 2012; Diaz-Ferguson *et al.*, 2014), use of eDNA for species detection appears a promising tool and can be incorporated into surveillance and management programmes related to conservation and early detection/rapid response actions (Diaz-Ferguson *et al.*, 2014). For the zebra mussel cultivation-related studies, the markers can be used for the rapid screening of spatial-temporal patterns of larvae dispersal and assigning suitable areas and/or timing for deployment of cultivation facilities. In summary, the molecular tools presented here are technically accessible and will facilitate the monitoring of zebra mussel populations at a minimal cost.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site.

Table S1. 16S rDNA sequences representative of a wide range of marine taxonomic groups, including algae, invertebrates and vertebrates retrieved from GenBank