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Molecular tools for invasion biology: a new approach for amplification of dinoflagellate nitrogen transport genes with unknown exon-intron structure

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Summary

Dinoflagellates are an important group of unicellular eukaryotes widespread in aquatic ecosystems. Many dinoflagellates are mixotrophic, toxic or potentially toxic, highly competitive and invasive, while molecular mechanisms that underpin their success in natural communities remain enigmatic. Due to peculiar features of dinoflagellate genome, little is known about the structure and expression of genes in these organisms. We analyzed the transcriptome databases of the dinoflagellate *Prorocentrum minimum* in order to identify the sequences of urea transporter (*dur3*) and nitrate transporter (*nrt2.1*) genes. Taking into account prospective exon-intron organization of dinoflagellate genome we suggested two variants of choosing the primer positions. We designed six primer pairs for amplification of the urea transporter gene fragments and three – for amplification of the nitrate transporter gene fragments. As a result of PCR, fragments of target genes were obtained. Alignment of amplicons with database transcriptome sequences showed that those sequences were identical. Primers developed in this study can be further used for examination of *P. minimum* gene expression by RT-qPCR. This approach would provide a better understanding of the influence of various nitrogen sources on physiological characteristics of these protists responsible for their effective adaptations to fluctuating environment.

Key words: dinoflagellates, invasions, nitrogen metabolism, *Prorocentrum minimum*, protists, transcriptome

Introduction

Dinoflagellates are unicellular protists widespread in marine and freshwater ecosystems. About one-half of all dinoflagellates are phototrophs and mixotrophs (Stoecker, 1999; Matantseva and Skarlato, 2013 and references therein) thus representing an important group of primary producers in the ocean. Many of them play a significant role in the formation of harmful algal blooms in marine coastal regions (Alonso-Rodríguez and Páez-Osuna, 2003; Glibert et al., 2012). Consumption of seafood which is contaminated with dinoflagellate metabolites by humans and by

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organisms in aquaculture can cause intoxication or even lethal cases (Denardou-Queneherve et al., 1999; Tango et al., 2005).

Moreover, some dinoflagellate species are successful invaders to new marine environments, and consequences of those invasions often alter ecosystems' structure, functions and biodiversity paradigms (Telesh, 2016; Skarlato and Telesh, 2017). One of the brightest examples is the potentially toxic, mixotrophic, bloom-forming planktonic dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller, or *Prorocentrum cordatum* (Ostenfeld) Dodge according to priority rule, and its invasion history in the Baltic Sea. Within two decades after this invasion, *P. minimum* had outcompeted its congeners from the complex of dominant dinoflagellate species (Telesh et al., 2016). However, fine mechanisms behind the competitive advantages and pronounced adaptive potential of dinoflagellates are not fully understood (Skarlato et al., 2017).

Research on the dinoflagellate genomes has always been challenging due to the large size of genomic DNA. There is still no complete genome sequence for these organisms, and little is known about the structure and expression of their genes. The use of such tool as reverse transcription quantitative real-time PCR (RT-qPCR) for examination of gene expression would allow a better understanding of dinoflagellate physiology determining their role in natural ecosystems, but it is hampered by the scarcity of genomic information necessary for the primer design. Expressed sequence tag (EST) projects and transcriptome databases provide an alternative way for molecular studies; however, they do not possess information about exon-intron gene structure in dinoflagellates. Recently Mendez and co-authors (Mendez et al., 2015) found out that introns of the dinoflagellate *Crypthecodinium cohnii* are flanked by specific combinations of nucleotides (AGG) at the splice sites. This allows to assume intron locations and gene structure, which can be useful for the purposes of primer design.

It is known that nitrogen availability limits phytoplankton growth in many marine ecosystems (Vitousek and Howarth, 1991; Falkowski, 1997; Dyhrman and Anderson, 2003; Howarth and Marino, 2006). However, at the present time anthropogenic eutrophication leads to elevated concentration of nitrogenous compounds, such as urea, in coastal habitats (Galloway et al., 2004; Glibert et al., 2005, 2016). Under eutrophication conditions, dinoflagellates often demonstrate competitive advantages over the other phytoplankton groups (Glibert et al., 2012; Telesh et al., 2016), since these organisms exploit diverse nutrition strategies and thus can effectively satisfy their nitrogen requirements. It was suggested that high concentrations of urea in seawater may promote harmful dinoflagellate blooms (Anderson et al., 2002; Heisler et al., 2008). Dinoflagellates readily use this organic compound as a nitrogen source, often in preference to nitrate (Glibert et al., 2006; Solomon and Glibert, 2008). Moreover, it was demonstrated that the presence of urea in the medium has an inhibitory effect on nitrate uptake by dinoflagellates *P. minimum* (Matantseva et al., 2016a, 2016b).

Presumably, nitrogen uptake can be regulated at the level of transcription; however, information about molecular aspects of nitrogen metabolism of dinoflagellates is still scarce. Our preliminary analysis of incorporation of H^3-uridine by dinoflagellate cells revealed that addition of diverse nitrogen sources to the culture medium resulted in different RNA synthesis rates (Pechkovskaya et al., 2016). We therefore hypothesized that the observed differences in the RNA synthesis rate may be related to the transcriptional regulation of synthesis of proteins involved in nutrient transport.

Proteins NRT2 and DUR3 are known to be responsible for the transport of nitrate and urea, correspondingly. A protein homologous to NRT2.1, a high-affinity nitrate transporter of the major facilitator family (MFS), has been identified as the most likely candidate to be involved in nitrate transport in the dinoflagellate *Lingulodinium polyedrum* (Bellefeuille and Morse, 2016). Transporter DUR3 is a high-affinity secondary active urea transporter which is present in a wide range of organisms from microalgae to higher plants (Wang et al., 2008; Witte, 2011). Homologs of both NRT2.1 and DUR3 have been found in the *P. minimum* transcriptomes (Matantseva et al., 2016b). Expression levels of genes encoding these transporters are assumed to vary depending on available nitrogen sources; further studies using such methods as RT-qPCR could reveal important features of the nitrogen transport regulation in *P. minimum*. This approach requires internal standards such as housekeeping genes used for normalization of gene expression. The research of Guo and Ki (2012) provides references for internal controls suitable for examination of gene expression in *P. minimum*.

The goal of this study was to develop a primer design strategy based on the transcriptomic sequences of dinoflagellates and obtain primers for amplification of *P. minimum dur3* and *nrt2.1* genes suitable for further RT-qPCR analysis.

Material and methods

Strain and culturing conditions

A monoculture of dinoflagellates *Prorocentrum minimum* from The Culture Collection of Algae and Protozoa, Scottish Marine Institute, Oban, UK (CCAP clone 1136/16) was used. The culture was grown at salinity 25 in f/2 medium without silicate based on artificial seawater (Guillard and Ryther, 1962). The culture was exposed to a 12 h light: 12 h dark cycle at 21-23 °C and photon flux density 50 μmol photons m^{-2} s^{-1}.

Transcriptome analysis

The sequences of *dur3* and *nrt2.1* for *P. minimum* were obtained from the transcriptome database Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP, Keeling et al., 2014) (Table 1). Dinoflagellate (*Symbiodinium* sp.) and coccolithophyceae (*Emiliania huxleyi*) gene sequences from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/protein) were used as queries in local BLAST search. Local BLAST search was performed by means of the BioEdit 7.2.5 software (Hall, 1999) with BLOSUM62 matrix (E-value < 10^{-10}).

Primer design

Nine PCR primer pairs targeting nitrate (NRT2.1) and urea (DUR3) transporter genes were designed. Each primer was evaluated for possible formation of secondary structures, self- and hetero-dimers using OligoAnalyzer 3.1 tool (Integrated DNA Technologies, Inc.; https://eu.idtdna.com/calc/analyzer). Specificity of primers was tested *in silico* using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI database and *P. minimum* transcriptome. PCR primer oligonucleotides were synthesized by Beagle Co. Ltd. (St. Petersburg, Russia).

Table 1. Sequences of the nitrate and urea transporter genes revealed in the transcriptome of *Prorocentrum minimum* CCMP1329.

Gene	Accession number in the transcriptome CCMP1329
dur3	263164_1
nrt2.1	47143_1

DNA extraction

P. minimum cells were lysed by freezing at −80 °C for 10-15 min, thawed at room temperature and centrifuged at 10,000 g for 5 minutes. Total DNA was isolated using a DNA extraction kit (BioSilica Ltd., Russia) according to the manufacturer's instructions.

PCR amplification, electrophoresis and sequencing

The optimal annealing temperature for each primer pair was determined by means of temperature gradient PCR. PCR reactions were carried out in a 10 μl mixture containing 5 μl 2X DreamTaq MasterMix (Thermo Fisher Scientific, USA), 1 μl of forward and reverse primers (0.5 μM), 1 μl genomic DNA template (10.6 ng/μl) and 2 μl PCR grade water. Amplification was performed as follows: pre-denaturation step at 94 °C for 3 min followed by 39 cycles of denaturation at 94 °C for 30 s, primer annealing at 55–60 °C for 30 s (Table 2), and elongation at 70 °C for 1 min. Cycling was completed by a final elongation step at 70 °C for 7 min. We determined the optimal annealing temperature that resulted in the best product specificity and yield. Unspecific amplification was observed at lower annealing temperatures. Tubulin-specific primers TUAF and TUAR were used as a positive control (Guo and Ki, 2012).

PCR products were separated in a 2 % agarose gel in 1× TAE buffer, and amplicon sizes were estimated using GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific, USA). Gel was further stained by ethidium bromide and amplified fragments were visualized under UV light. The PCR products were then extracted from agarose gel with BioSilica gel extraction kit (BioSilica Ltd., Russia) according to the manufacturer's instructions. DNA sequencing was performed by Beagle Co. Ltd.

Fig. 1. A schematic representation of the primer location. A – Primers flanking the exon region between two AGG triplets; B – primers flanking the AGG triplet and putative intron.

Results

In this study, we propose an approach for development of an effective primer design strategy using transcriptomic sequences of dinoflagellates and plausible exon-intron structure of the target genes based on the findings of Mendez et al. (2015). Two variants of choosing primer positions were suggested: (1) on the exon region between the two AGG triplets (Fig. 1A), or (2) flanking the short region including the AGG triplet (Fig. 1B). The second option allows recognizing possible presence of intron within the target gene fragment.

We developed a set of primers for specific amplification of the fragments of *P. minimum* genes. Six primer pairs were designed for amplification of fragments of the urea transporter *dur3* gene and three – for amplification of fragments of the nitrate transporter *nrt2.1* gene (Table 3). Putative exon regions between the AGG triplets were targeted by primer pairs DUR3F1/DUR3R1, DUR3F2/DUR3R2, DUR3F3/DUR3R3 designed for the *dur3* gene and NRT2.1F1/NRT2.1R1 designed for the *nrt2.1* gene. The AGG triplet and a putative intron within the target fragment were targeted by primer pairs DUR3F4/DUR3R4, DUR3F5/DUR3R5, DUR3F6/DUR3R6 designed for the *dur3* gene and NRT2.1F5/NRT2.1R5 and NRT2.1F6/NRT2.1R6 designed for the *nrt2.1* gene.

Dur3 gene amplification with primer pairs DUR3F1/DUR3R1, DU3F2/DUR3R2, DUR3F3/DUR3R3, DU3F4/DUR3R4 and DUR3F5/DUR3R5 resulted in the amplificons of expected size. No amplification products were obtained with DU3F6/DUR3R6 primers (Fig. 2). *Nrt2.1* gene amplification using primer pairs NRT2.1F1/NRT2.1R1, NRT2.1F5/NRT2.1R5 and NRT2.1F6/NRT2.1R6 also resulted in the products of expected size. However, unspecific amplification was observed when the primer pair NRT2.1F6/NRT2.1R6 was applied (Fig. 3). It is worth noting that none of the PCR products obtained using primer pairs of the second type (flanking the short region including the AGG triplet, putative splice sites) contained an intron, which makes them possible to use.

Table 2. Optimal annealing temperature for amplification.

Primer pairs	Optimal T_A, °C
NRT2.1F1/NRT2.1R1	56
NRT2.1F5/NRT2.1R5 NRT2.1F6/NRT2.1R6	57
DUR3F1/DUR3R1 DU3F2/DUR3R2 DUR3F3/DUR3R3 DUR3F4/DUR3R4	58
DUR3F5/DUR3R5 DUR3F6/DUR3R6	60

Table 3. Primers for specific amplification of *dur3* and *nrt2.1* genes.

Primer name	Primer sequence (5′-3′)	Target gene	Expected product size, bp
DUR3F1 DUR3R1	CCTTCATCTTCTTCTGCCTGC ACAGGATCACGCCCCAC	*dur3*	138
DUR3F2 DUR3R2	GCAACTGGCGATCAGATTCTC GTTCCACAGGGGCACCAC	*dur3*	165
DUR3F3 DUR3R3	TCGGCGAGATCACGGTG CAGACTGGTCGTTCTCCATC	*dur3*	171
DUR3F4 DUR3R4	GTGTACTTGGACAAGACCGC CACTGCGACACAATGACCGAC	*dur3*	110
DUR3F5 DUR3R5	CACCGTGTTCGTGGACCAGTC GAACCACACCAAGCCGCCC	*dur3*	97
DUR3F6 DUR3R6	CAGCAAAACGCCTCGTCCATAC CCGTCCCCAGGAGGAGTAG	*dur3*	105
NRT2.1F1 NRT2.1R1	CGAAGTCCTCCCTAAGTGCG GCACAAAGATCGTGATGCCGAC	*nrt2.1*	97
NRT2.1F5 NRT2.1R5	CATTGGGCGGCATCTTCAGC CATGGCTGCGAGTTCTCCAC	*nrt2.1*	136
NRT2.1F6 NRT2.1R6	CGTTTGGGACGAGAGTTGTG GATCGTGATGCCGACCGAC	*nrt2.1*	199

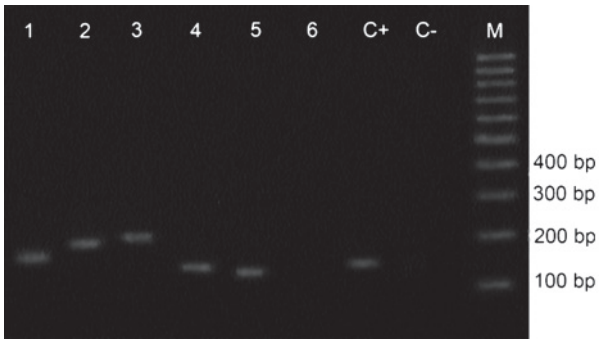

Fig. 2. Gel electrophoresis of urea transporter *dur3* gene amplification products. PCR performed with primers: 1 – DUR3F1/DU3R1, 2 – DUR3F2/DUR3R2, 3 – DUR3F3/DUR3R3, 4 – DUR3F4/DUR3R4, 5 – DUR3F5/DUR3R5, 6 – DUR3F6/DUR3R6, C+ – positive control, C- – negative control, M – molecular weight marker.

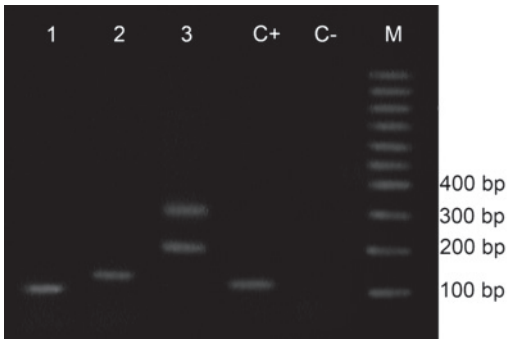

Fig. 3. Gel electrophoresis of nitrate transporter *nrt2.1* gene amplification products. PCR performed with primers: 1 – NRT2.1F1/NRT2.1R1, 2 – NRT2.1F5/NRT2.1R5, 3 – NRT2.1F6/NRT2.1R6, C+ – positive control, C- – negative control, M – molecular weight marker.

Alignment of the sequenced amplicons with the respective transcriptomic sequences showed that they were identical, which proves specificity of gene amplification with the designed primers (Fig. 4).

Discussion

Several previous attempts to design gene-specific PCR primers based on *P. minimum* sequences retrieved from the transcriptomic database of the MMETSP project were not successful (Pechkovskaya, Matantseva et al., unpublished data). Presumably, amplification was hampered by the presence of introns in the target sequences. Recent investigations shed light on the exon-intron organization of dinoflagellate genes exemplified by *Crypthecodinium cohnii*. According to Mendez et al. (2015), dinoflagellate genome sequences possess identical repeated intron boundary sequence that always contain a GG (or often an AGG) at the 3' splice site and always have an AGG at the 5' splice site, which gives an opportunity to take intron localization into consideration if primer design is based on transcriptomic sequences. However, it is still not clear whether this feature of splicing sites is universal for all dinoflagellates.

The primer pairs developed in this study can be successfully applied to amplify 100–200 bp fragments of the *dur3* and *nrt2.1* genes of the dinoflagellate *P. minimum*. These primers can be further used for RT-qPCR analysis of the urea transporter and nitrate transporter gene expression in *P. minimum*. Investigation of the expression level of genes involved in nitrogen transport is necessary for a clearer understanding of the influence of various nitrogen sources on physiological parameters of dinoflagellates, which benefits will be manifold for a number of disciplines, including aquatic ecology and invasion biology.

Specifically, as shown recently in the laboratory experiments with *P. minimum*, high viability of dinoflagellate cells in brackish waters with non-optimal salinity may be explained (at least in part) by speed up of their metabolic activity under stress (Skarlato et al., 2017). This cellular response may be considered as an effective mechanism which allows the bloom-forming dinoflagellates to dominate in hash environment for the extended periods of time (Skarlato and Telesh, 2017). In particular, the recently demonstrated invasion success of *P. minimum* in the Baltic Sea (Telesh et al., 2016) was assumed to be largely due to diverse feeding strategies and high intra-population variability of cellular responses to external stress (Matantseva et al., 2016b).

These and other specific traits of dinoflagellates that still need to be investigated, e.g. expression of nitrogen-transporting genes and nitrogen metabolism in general, most likely back up the remarkable environmental plasticity of *P. minimum* and allow this invasive species to conquer new environments, including the plankton species-rich coastal ecosystems subject to elevated nutrient loads (Telesh, 2016; Skarlato et al., 2017). Considering high rates of anthropogenic eutrophication worldwide and rapid

Fig. 4. Fragments of alignments of amplicons with database sequences of urea transporter DUR3 (accession number 263164_1 in the transcriptome CCMP1329) and nitrate transporter NRT2.1 (accession number 47143_1 in the transcriptome CCMP1329) genes.

range expansion of the bloom-forming dinoflagellate species, the development of gene-specific primers, e.g. targeting urea and nitrate transporter genes of dinoflagellates, opens new perspectives for studies of metabolic regulation of these highly competitive, potentially toxic, invasive protists aiming at prognostic modeling of their ecosystem impacts under global environmental change.

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