Note

An effective method for detecting prey DNA from marine dinoflagellates belonging to the genera Dinophysis and Phalacroma using a combination of PCR and restriction digestion techniques

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Received 27 November 2017; Accepted 20 January 2018  Responsible Editor: Akihiro Tuji

Abstract: The marine dinoflagellates species belonging to the genera Dinophysis and Phalacroma have been reported as mixotrophic or heterotrophic actively feeding on planktonic ciliates. However, limited information is available on the identification of ciliate species preyed on by Phalacroma mitra. An effective method is introduced for detecting prey DNA of dinoflagellates belonging to these two genera. Three cells of P. mitra possessing food vacuoles were isolated from natural seawater, and the prey DNA within the vacuoles was analyzed. After the polymerase chain reaction (PCR)-based amplification, a restriction enzyme specific to the DNA of Dinophysis and Phalacroma was used to concentrate the prey DNA. Gene cloning revealed that the undigested PCR products contained DNA of ciliate species (Euplotes sp., Mesodinium rubrum, Spirostrombidium sp., etc.), as well as that of dinoflagellates, cryptophytes, and radiolarians. These results imply that the prey diversity of P. mitra can be traced. Furthermore, this method can provide useful data to reveal novel insights into food webs in the planktonic ecosystem.

Key words: Dinophysis, Phalacroma, prey, diversity, restriction enzyme

Due to handling difficulty in feeding experiments, most studies focusing on predator-prey interactions in the plankton community have utilized polymerase chain reaction (PCR)-based amplification of genomic DNA with universal primers for 18S rDNA. However, mixed information from not only prey DNA but also predator DNA is obtained through this method. The extracted DNA comprises a larger quantity of predator DNA, probably owing to digestion of prey DNA within the predator cell, thereby resulting in preferential amplification of predator DNA over prey DNA (Polz & Cavanaugh 1998). Therefore, a method to eliminate or inhibit the amplification of predator DNA is required when using a universal primer set.

In this study, the target organism for prey analysis was Phalacroma mitra Schütt (previously named Dinophysis mitra), a diarrhetic shellfish toxin-producing dinoflagellate species (Lee et al. 1989). The genus Phalacroma had been considered as a synonym of Dinophysis, but recent molecular analyses of a large group of Dinophysiales supports the view that Phalacroma is a separate genus (Handy et al. 2009, Jensen & Daugbjerg 2009). Dinophysis and Phalacroma species use a feeding tube, called the "peduncle," to absorb the cellular contents of their ciliate prey (Hansen 1991). Mixotrophic species in Dinophysis have plastids (chloroplasts) of cryptophyte origin for the ingestion of the ciliate Mesodinium rubrum (Lohmann), while P. mitra contains plastids originating from wide taxonomic groups: Bacillariophyceae, Bolidophyceae, Chrysophyceae, Dictyochophyceae, Haptophyceae, and Pelagophyceae (Koike et al. 2005, Nishitani et al. 2012); suggesting the difference in ciliate prey species between Dinophysis and Phalacroma.

A total of six Dinophysis species have now been cultured, and they all prey on M. rubrum (see Reguera et al. 2012). However, no studies have succeeded in culturing P. mitra; its prey remains unknown, and its feeding habits little un-
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understood. Clarifying the prey species of *P. mitra* is a critical prerequisite for establishing a systematic culture, thereby enabling further studies. In this study, we used a method combining PCR with a universal primer set and restriction digestion for the enrichment of prey DNA to analyze the prey of *P. mitra*. This technique has already been used for analyzing the prey of juvenile crustaceans (Blankenship & Yayanos 2005) and bivalve larvae (Maloy et al. 2013). Here, we demonstrate that this approach is also valid for prey analysis of naturally occurring cells of *P. mitra*.

Four cells of *P. mitra* (Figs. 1A, B, C, and D) were isolated from natural sea water collected from Mutsu Bay, Aomori Prefecture, northern part of Japan, on August 8, 2016. Three isolated cells (Figs. 1B, C, and D) possessed numerous food vacuoles, indicating that prey cells were well preserved in the vacuoles; one cell (Fig. 1A) did not have any food vacuoles and was hence used as a negative control. These cells were isolated under a nearly axenic environment to prevent contamination from air and were washed at least three times with filtered seawater (0.1-μm pore size mesh). After washing each cell and placing them in individual PCR tubes of 0.2 ml containing 10 μL of 10% Chelex® suspension (Bio-Rad, Hercules, CA), DNA was extracted in accordance with the method of Richlen & Barber (2005). Single cell PCR for nuclear 18S rDNA was conducted using a universal primer set for many taxonomic groups of marine eukaryotes: forward primer, 18S-F1298 (5′-AGT GAT TTG TCW GGT TWA TTC CG-3′) (newly designed for this study); reverse primer, 18S-R1772 (5′-TCA CCT ACG GAA ACC TTG TTA CG-3′) (Nishitani et al. 2012). This primer set generates an approximately 490-bp single fragment from *Dinophysis* and *Phalacroma* DNA; however, when a *Dinophysis* or *Phalacroma* cell contains *Mesodinium* DNA, two bands may appear in the gel owing to the presence of short sequences (ca. 420 bp) of *Mesodinium* species. PCR was performed using a Veriti thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA) in a reaction mixture (20 μL) containing 1.0 μL template DNA, 0.2 mM of each dNTP, 1X PCR buffer, 1.5 mM Mg²⁺, 1.0 U KOD -Plus-ver.2 (TOYOBO, Osaka, Japan) that has intensive 3′→5′ exonuclease activity, and 0.3 μM of each primer. The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min; 38 cycles at 98°C for 10 sec, 56°C for 30 sec, and 68°C for 30 sec. These are general PCR methods, and the specific protocol we adopted is described below. After the PCR, restriction digestion was conducted immediately. We used the restriction enzyme, *Psh*BI (TAKARA BIO, Shiga, Japan), which recognizes the sequence ATT AAT located in the amplicons of *Dinophysis* and *Phalacroma*. The enzyme selectively cleaves the sequences of these dinoflagellates, but does not influence those of marine ciliates (Fig. 2). Therefore, when the ciliates are contained inside the cells of *Dinophysis* or *Phalacroma*, the DNA of ciliates remains at its original length, while most of the PCR amplicons of dinoflagellates are cleaved, becoming two short fragments (ca. 220 and 270 bp). The undigested PCR bands were then eluted from the agarose gel (1.5%) using a FastGene Gel/PCR Extraction

**Fig. 1.** Micrographs of natural cells of *Phalacroma mitra* used in this study. A: Cell without food vacuole but possessing transparent vacuoles (negative control); B–D: Cells with food vacuoles (arrows). Arrows indicate examples of food vacuoles in the cells. Scale bars represent 20 μm.
Low DNA yield may affect the subsequent gene cloning step; therefore, if the undigested PCR band is thin, the DNA concentration should be increased by reducing the final yield of the purification kit, alternatively, PCR and restriction enzyme digestion should be performed in triplicate, and the resulting DNA should be pooled in one tube. Clone libraries were prepared from purified PCR products with a cloning kit in accordance with the method of Nishitani et al. (2012). Partial sequences of 18S rDNA were aligned, and an unrooted phylogenetic tree was generated by MEGA version 7 software (Kumar et al. 2016) using the maximum-likelihood (ML) method with the default settings. The topology of the phylogenetic tree was evaluated using the bootstrap method with 100 replicates. Sequences thus obtained for the 18S rDNA have been deposited in DDBJ/EMBL/GenBank databases under accession numbers LC318552-LC318560.

Further, as we expected, restriction digestion of these PCR products resulted in a thin band of undigested DNA and two thick bands of digested DNA (Fig. 3B; b, c, d). In the negative control (Fig. 3B; a), no undigested DNA was detected, confirming that this host cell did not contain prey DNA.

To validate the effectiveness of this restriction digestion method, we first conducted gene cloning using untreated PCR products (bands of a–d in Fig. 3A). Although a total of 96 sequences were determined, only host DNA was detected (data not shown).

The three undigested thin bands obtained from each cell of *P. mitra*, B, C, and D, (Fig. 3B; b–d) were cloned, and 30–34 sequences were determined from each clone library. In cell B (n=30), 7 sequences (23%) were of host origin, while the remaining 23 (77%) were of non-host origin (Fig. 4; B). Of the remaining sequences, 4 and 19 were of ciliate and dino-
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flagellate origin, respectively. The recovered sequences from cell C (n=32) constituted 71% of host DNA, indicating that restriction digestion of the host DNA was insufficient. Ciliate and radiolarian sequences were also recovered from cell C (29%, Fig. 4; C). Of 34 sequences recovered from cell D, only 2 (6%) were of host origin and the remaining 32 (94%) were of ciliate and/or cryptophyte origins (Fig. 4; D). The detection rate of the host sequences in this study thus varied from 6% to 71% among cells B, C, and D. This difference may depend on the copy number of 18S rDNA in a cell of \textit{P. mitra} and the degree of prey DNA digestion in the food vacuoles. However, unless such a restriction digestion method is employed, all of the sequences would be host DNA. Therefore, this method can be considered to be valid for the detection of prey DNA. Although the approach combining restriction digestion and a predator-specific blocking primer enhances detection rate of prey DNA (Maloy et al. 2013), we could not design a suitable blocking primer in this study.

Sequences, other than those of the host, recovered from the three cells of \textit{P. mitra} were assembled in five topological clusters within the phylogenetic tree (Fig. 5). We discerned at least eight unique sequences in total from the cells of \textit{P. mitra}, and the number might increase further through addition of recovered clones or isolated cells. Ciliate sequences were detected in all three cells of \textit{P. mitra}, five of which were of ciliate and/or cryptophyte origins (Fig. 4; D). The detection rate of the host sequences in this study thus varied from 6% to 71% among cells B, C, and D. This difference may depend on the copy number of 18S rDNA in a cell of \textit{P. mitra} and the degree of prey DNA digestion in the food vacuoles. However, unless such a restriction digestion method is employed, all of the sequences would be host DNA. Therefore, this method can be considered to be valid for the detection of prey DNA. Although the approach combining restriction digestion and a predator-specific blocking primer enhances detection rate of prey DNA (Maloy et al. 2013), we could not design a suitable blocking primer in this study.

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Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (JSPS) (KAKENHI; No. 17K07886 and No. 25450256).

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