Efficient Analysis for Natural Diet of Marine Animal Larvae using Peptide Nucleic Acid (PNA)-mediated PCR Clamping

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Introduction

Nucleotide sequence analysis *via* polymerase chain reaction (PCR) may be a promising tool to investigate gut content of marine animal (Martin et al., 2006). When the gut contents could be efficiently isolated from the host tissue, subtle amount of the host genome in the sample would not substantially alter subsequent PCR amlification of non-host DNA. However, it is considerably difficult to physically separate the gut and its content in invertebrate larvae. For instance, Suzuki et al. (2006, 2008) used 18S rDNA to investigate diet of phyllosoma larvae of lobsters (palinurid and scyllarid), where they adopted restriction fragment length polymorphism (RFLP) analysis to select clones showing non-host restriction patterns. Nearly 90 % clones of 2,341 examined by Suzuki et al. (2006, 2008) were determined to be of the host lobster larvae, indicating that substantial amount of contamination from the host genome may not be obviated. PCR clamping by peptide nucleic acid (PNA) was introduced as a novel procedure to selectively amplify DNA (Ørum et al., 1993). The PNA molecule was an artificial nucleic acid, having two unique properties; 1) PNA oligomer binds tightly to the fully complementary single strand DNA and its thermal stability is greater than the corresponding DNA-DNA binds and 2) PNA oligomer has resistance against the DNA polymerase. Here, we report the results of our attempt to inhibit amplification of rDNA sequence of the Japanese spiny lobster (*Panulirus japonicus*) using the PNA-mediated-PCR clamping and to determine natural diets for the lobster larvae (Fig. 1).

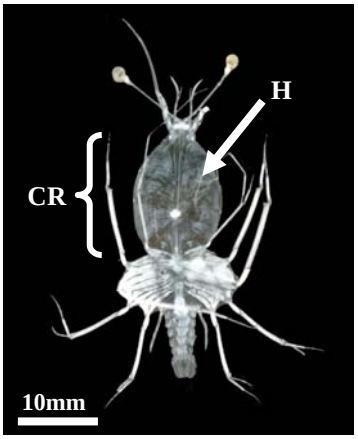

Fig. 1. A final stage phyllosoma larva of the Japanese spiny lobster (*Panulirus japonicus*). Hepatopancreas (H) is located in the cephalic region (CR), to where the diets are ingested.

Efficiency of the PNA-mediated-PCR clamping

We adopted rDNA internal transcribed spacer region 1 (ITS1) as a target DNA segment. This non-coding segment may evolve rapidly, but the ITS1 of wide variety of eukaryote taxa may be amplified using an universal primer pair designed in flanking conservative rDNA regions (18S and 5.8S). According to the ITS1 sequence data of lobster species of the genus *Panulirus* (accession numbers of AB426502 to AB426512) and wide variety of eukaryotes, an universal primer pair was designed (ITS1F1: 5'-CGGAAGGATCATTAMCG-3' and 5.8S2R: 5'-ATTTCGCTGCGTTCTTCAT-3'). Sequence of an additional reverse primer (5.8S) for semi-nested PCR was 5'-CGCTGCGTTCTTCATCG-3', which was designed at 5 bases upstream of 5.8S2R. Lobster specific PNA oligomer (SPL1F: 5'-CATTAACGTTGCTTGCAAGC-3') was designed at the boundary region between 18S rDNA and ITS1, sharing eight bases with ITS1F1. The Japanese lobster (*Panulirus japonicus*) and the Japanese eel (*Anguilla japonica*) were used as model organisms to test efficiency of the PNA-mediated-PCR clamping (PMPC). Crude DNA samples extracted from *P. japonicus* and *A. japonica* (each adjusted to 50 ng/µl) were mixed at six ratios (1:0.01, 1:0.005, 1:0.001, 1:0.0005, 1:0.0001 and 1:0.00005), and 1 µl of the mixed DNA templates were added to PCR reaction mixture (final volume 20 µl) with a pair of primer (ITS1F1 and 5.8S2R) and 0 or 2 µM final concentrations of PNA probe. Amplification condition used was; initial denaturation at 94℃ for 5 min, followed by 35 cycles consisting of denaturation at

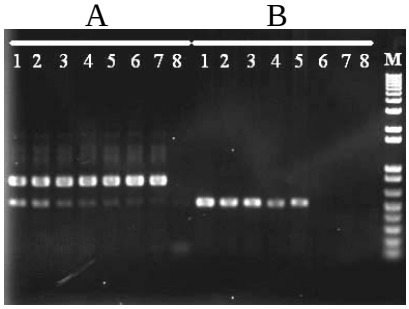

Fig. 2. Result of PCR amplification containing 0 µM (A) and 2µM (B) of the lobster specific PNA. Different mixture ratio of lobster and eel DNA were used for lanes 1 to 8 (see text).

94 ºC for 1 min, annealing/elongation step at 53 ºC for 3 min, and a final elongation step consisting of 53 ºC for 7 min. The results of the PMPC are shown in Fig. 2. Strong amplification of the lobster ITS1 (700 bp) and weak amplification of eel ITS1 (520 bp) were observed without PNA (Fig. 2A), and positive relationship between the amplification efficiency for eel ITS1 and concentration of eel DNA template. On the other hand, no amplification of lobster ITS1 and greater amplification of eel ITS1 were observed under presence of PNA oligomer (Fig. 2B). Thus, it was demonstrated that 2 µM final concentration of the lobster specific PNA probe in the PCR reaction could efficiently inhibit amplification of the lobster ITS1.

Gut content analysis for phyllosoma larvae

Crude DNAs were extracted from the cephalic region (Fig. 1 CR) of the Japanese spiny lobster (*P. japonicus*) and the long legged cray (*P. l. bispinosus*) larvae collected around Ryukyu Archipelago, which were subjected to the PMPC with excess amount of PNA probe (4 µM) using a primer set of ITS1F1 and 5.8S2R for the first PCR and ITS1F1 and 5.8S for the second semi-nested PCR. Of 40 *P. japonicus* and 9 *P. l. bispinosus* larvae used, apparent fragment amplification was observed in 11 *P. japonicus* and 8 *P. l. bispinosus* larvae, which were subjected to TA cloning. Colony direct PCR was performed to select positive clones. A total of 301 clones obtained and sequenced were categorized into 65 phylogroups. One representative sequence from each phylogroup was subjected to homology search using FASTA, and the results are summarized in Table 1. Two sequences having no conservative 3' end of 18S and 5' end of 5.8S rDNAs were determined not to be ITS1. Among 63 sequences remained, 39 sequences of 146 clones showing low homology scores (<70 %) were determined unidentified. Of 24 sequences, fungi were the most frequently detected eukaryote, where 12 phylogroups belonging to 10 genera were observed with high homology score (>90 %). All these fungi were observed to have short ITS1 (<270 bp). Three cnidarian sequences were detected from 4 larvae, and 3 ctenophore sequences from 5 larvae. ITS1 of bony fish was observed in 2 larvae. ITS1 of Crustacea detected in five of four larvae had high homology score with that of a slipper lobster (*Scyllarus cultrifer*), and one sequence was determined to be of the host larva. Mollusc (*Haliotis*) and Chaetognath (*Sagitta*) ITS1 were detected in one larva each. Thus, the PMPC analysis appeared to be powerful tool to investigate stomach content in small samples such as marine animal larvae. The present study demonstrated that the phyllosoma larvae of spiny lobster appeared to be a opportunistic carnivore, in which the diet comprised of wide variety of animal taxa. However, more than half of clones could not be determined because of the poor background database. As marine animal ITS1 database is gradually expanding (Chow et al., in press), affiliation of the large number of unidentifined sequences obtained in this study may be clarified.

References

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Table 1. Number of clones of eukaryote taxa detected in DNA extracted from hepatopancreas of the Japanese lobster *Panulirus japonicus* (PJ) and long legged cray *P. longipes bispinosus* (PL).

	PJ1	PJ2	PJ3	PJ4	PJ5	PJ6	PJ7	PJ8	PJ9	PJ10	PJ11	PL1	PL2	PL3	PL4	PL5	PL6	PL7	PL8
No. clones	3	23	9	19	21	16	43	6	22	24	5	10	11	10	12	8	11	25	23
Fungi		1	6			4		6	1	10		3			4				8
Cnidaria				7		5			1				3						
Ctenophora	1	1							8		3			4					
Fish	1															3			
Crustacea	1	12					1*						5	1					
Mollusca				3															
Chaetognata									1										
UN[§]		1	2	1	15	7	27		11	7	1	7	3	5	8		11	25	15

[§]Unidentified, *host clone.