

## Eukaryotes from the hepatopancreas of lobster phyllosoma larvae

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**Abstract** In order to investigate unknown prey organisms for lobster phyllosoma larvae, molecular approach was developed and adopted using the laboratory-reared and wild phyllosoma samples. The central domain of 18S rDNA was amplified via nested PCR using crude DNAs extracted from the hepatopancrea of the larvae and cloned into plasmid. Clones having 18S rDNA different from the hosts were screened by RFLP analysis, and the nucleotide sequences determined were subjected to FASTA homology search and phylogenetic analysis to identify the source of eukaryotic organisms. Feasibility of this method was confirmed in the laboratory-reared larvae of the Japanese spiny lobster (*Panulirus japonicus*) fed exclusively on either gonad of common mussel (*Mytilus edulis*) or *Artemia* nauplii. From the two wild-caught larvae (*Scyllarus* sp. and *P. japonicus*), 178 and 168 clones were isolated, of which 132 and 4 clones had different restriction profiles from the hosts, respectively. DNAs of Cnidaria and Urochordata were observed from the scyllarid larva, while those of fungi and host variant were observed in *P. japonicus* larva.

**Key words:** prey organisms, phyllosoma larvae, *Panulirus*, scyllarid, 18SrDNA

Lobsters are very important fishery resources. Their larvae called as phyllosoma have the long planktonic period extending from several months to more than a year (Chittleborough and Thomas, 1969; Lesser, 1978; Kittaka, 1988). Although predator-prey interaction may be very important information to understanding larval ecology under dynamics of food web, the prey organisms of the phyllosoma in natural environment are not well known. In scyllarid phyllosoma larvae, a unique behavior as riding on jelly fish, called "piggyback riding", has been observed and expected as a means of transport with meals (Shojima, 1963; Thomas, 1963). Laboratory experiments offering a variety of planktons indicated that phyllosoma larvae of *Panulirus interruptus* and *Jasus edwardsii* preferred medusae, ctenophores, chaetognaths and other soft-bodied animals (Mitchell, 1971; Kittaka, 1994). However, the phyllosoma larvae may change prey organisms as

they grow and disperse into very wide range during the long larval period. Unlike the other Decapod crustacean larvae, laboratory observation indicated that phyllosoma larvae may be a sucking predator (Murakami, personal communication). Observations on developments in the mouthpart suggested that early-stage phyllosoma of *J. edwardsii* would benefit from a diet comprising soft gelatinous zooplankton (Johnston and Ritar, 2001).

Although rearing the phyllosoma larvae has been considerably difficult, successful metamorphoses have been achieved in several Palinurid species by feeding *Artemia* nauplii supplemented with gonad of common mussel (*Mytilus edulis*) (Kittaka, 1994; Kittaka, 1997; Kittaka *et al.*, 1997). Yet, the survival rate is still very low. Thus, the information of natural diets for phyllosoma larvae may be very important to prepare their food for aquaculture.

Recently, DNA-based approaches to reveal prey

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organisms have been applied to the gut contents of several marine organisms (Asahida *et al.*, 1997; Jarman *et al.*, 2002; Rosel and Kocher, 2002; Saitoh *et al.*, 2003; Jarman *et al.*, 2004; Blankenship and Yayanos, 2005). These studies, however, dealt with adult predator and/or used PCR primers designed for suspicious or target prey organisms. In order to investigate unknown prey organisms of the lobster phyllosoma larvae, we have designed universal primers for amplifying relatively variable central domain of 18S rDNA, and attempted to develop detection technique of foreign DNAs from an alimentary canal. Here, we introduce the technique and report the preliminary results of our attempts.

### Materials and Methods

#### Phyllosoma specimens and DNA extraction

Six phyllosoma larvae (five of palinurid and one of scyllarid lobsters) were used in this study (Table 1). The developmental stages of these phyllosoma larvae were determined according to Inoue (1981), Matsuda and Yamakawa (2000), and Johnson (1971a, b). Four larvae were of laboratory-reared Japanese spiny lobster, *P. japonicus*, hatched on July 2002 and kept at the Minamiizu Station, National Center for Stock Enhancement, Japan. The phyllosoma larvae were divided into two feeding conditions; one exclusively fed common mussel (*Mytilus edulis*) gonads (Myt) and the other fed *Artemia* nauplii (Art). Two wild-caught phyllosoma larvae were from the Pacific (Pac-1 and -2) through research cruises in 2002 and 2003. The specimens were frozen immediately after sampling, transferred to the laboratory and preserved in ethanol. Prior nucleotide sequence analysis on the mitochondrial COI gene

(data not shown) indicated that one (Pac-2) was of *P. japonicus* but the other (Pac-1) showed poor identity for the scyllarid species because of the small number of reference sequences in the DNA data base. Consequently, this larva was morphologically assigned to *Scyllarus* sp. according to Johnson (1971a, b).

Prior to DNA extraction, the surface of the phyllosoma larvae was washed well with sterile distilled water to remove possible contaminations from the surface. Hepatopancreas tissues with the contents were pipetted out from small incision made in the cephalic region. This "hepatopancreas content" and a part of pereopod were homogenized using tephlon pestle followed by crude DNA extraction using standard proteinase K digestion /phenol-chloroform extraction. The washed water from the wild phyllosoma larvae was recovered, ethanol-precipitated and used as a negative control.

#### PCR primers to amplify 18S rDNA

Two sets of universal primers were designed to amplify the central domain of 18S rDNA for nested PCR. Nucleotide sequences of the primers are available by connecting with the present authors. The priming regions were highly conserved among eukaryotic organisms to the extent that both primer combinations could amplify homologous regions of diatom (*Chaetoceros* sp.), lobster (*P. japonicus*), eel (*Anguilla japonica*) and human (*Homo sapiense*) (data not shown). The PCR reaction mixture contained 0.2 U Taq DNA polymerase (Applied Biosystems Inc.), 0.2 mM of each dNTP, 1.0  $\mu$ l 10 x buffer with 15 mM MgCl<sub>2</sub> (Applied Biosystems Inc.), 10 pmol of each primer, and 10 to 50 ng template DNA. The final volume of the reaction mixture was

**Table 1.** Samples of lobster phyllosoma larvae used in this study

Source or locality	Family	Species	N	BL (mm)	stage	ID
Laboratory-reared						
<i>Aremia</i> feeding	Palinuridae	<i>Panulirus japonicus</i>	2	9.4, 10.6	VII	Art
<i>Mytilus</i> feeding	Palinuridae	<i>Panulirus japonicus</i>	2	9.0, 9.4	VII	Myt
Wild-caught						
Pacific	Scyllaridae	<i>Scyllarus</i> sp.	1	19.3	VII?	Pac-1
	Palinuridae	<i>Panulirus japonicus</i>	1	11.5	VII	Pac-2

N : number of specimens, BL : body length

made up to 10  $\mu$ l with sterile distilled water. PCR was carried out with an initial denaturation at 95 °C for 2 min, followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min). The first and second PCR products were electrophoresed through a 1.5-2.0 % agarose gel in TBE buffer (50 mM Tris, 1 mM EDTA, and 48.5 mM boric acid) to confirm amplification.

#### Cloning, screening and sequencing of amplified fragments

Amplicons from the second PCR were directly subjected to TA cloning without further purification. Cloning was carried out using pGEM<sup>®</sup>-T Easy Vector System I (Promega). Transformed clones were first screened by colony-direct PCR with a primer set used for the second PCR. Since preliminary restriction assay indicated three endonucleases (*Alu* I, *Hpy*188I and *Mse* I) to have multiple restriction sites in the phyllosoma larvae, the PCR products were digested with these enzymes. Digested samples were electrophoresed on an agarose gel to select clones having restriction profiles distinct from those of the hosts. One or a few PCR product from each restriction type was selected arbitrarily and purified using ExoSAP-IT (Amersham). Sequencing reaction was carried out using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems Inc.) with the forward primer for the second, and nucleotide sequences were determined on an automated DNA sequencer (ABI 310, Applied Biosystems Inc.).

#### Homology search and sequence analysis

DNA sequences obtained were subjected to homology search using FASTA algorithm through DDBJ (DNA Data Bank of Japan) to investigate sequence similarities among organisms. Highly identical sequences nominated by FASTA (basically >95 % identity score) were selected and incorporated into phylogenetic analysis. Alignments of DNA sequences were performed with ClustalW algorithm (Thompson *et al.*, 1994) followed by manual editing. The aligned sequences were imported into PAUP\* (Swofford, 2000) to infer positions of the cloned DNAs on neighbor-joining (NJ) tree. Genetic distances among sequences were calculated under Kimura's two parameter (K2P) substitution model with complete deletion option. Bootstrap probabilities with 1,000 replications were calculated to assess reliability on each node of NJ tree.

### Results and Discussion

#### Detection of dietary DNA using laboratory-reared phyllosomas

The target region of 18S rDNA was well amplified in all laboratory-reared larvae by the nested PCR. Results of the experiment are summarized in Table 2. Individuals in each feeding condition were considered together. Transformation efficiency was high for both samples (>80 %). Of 125 white colonies in Art and 108 in Myt, subjected to colony direct PCR using the second PCR primers, amplicons were obtained in 116 and 91 colonies, respectively.

In the sample fed on *Artemia* nauplii (Art), a total of five clones had restriction profiles inconsistent from the host. FASTA search indicated that the

**Table 2.** Results of feed DNA detection using laboratory - reared phyllosoma larvae

Sample ID	No. of amplification/PCR	RFLP profile		No. of types	Identified eukaryote (no. of clones)
		host	non-host		
Art	116/125	111	5	4	<i>Artemia</i> (1)
					unidentified (3)
					<i>Panulirus</i> * (1)
Myt	91/108	55	36	8	<i>Mytilus</i> (14)
					<i>Panulirus</i> * (5)
					<i>Cryptomeria</i> (15)
					unidentified (2)

\*Supposed to be host variants

insert of one clone appeared to be of *Artemia salina* (99 % nucleotide identity). Three clones were unidentified because of the low nucleotide identity (<85 %). The last clone had similar 18S rDNA sequence with *Panulirus* species with high identity (>95 %) and determined as a host variant.

In the sample fed on gonad of common muscle (Myt), 36 clones with 8 restriction types were observed to have restriction profiles inconsistent from the host. A total of 14 clones were determined to be *Mytilus* 18S rDNA, whereas 15 clones were apparent contaminants of terrestrial plant, *Cryptomeria japonica* (Japanese cedar), five being host variants. The other two clones were unidentified because of the low identity (80 %).

The results obtained in the laboratory-reared samples clearly indicate that the experimental procedure presented in this study is powerful enough to detect DNAs of fed organisms in the hepatopancreas of lobster phyllosoma larvae. The detecting ability for dietary DNA may depend on the amount of tissues consumed (Wallace, 2004; Deagle *et al.*, 2005) and also on a degree of digestion. Better detection observed in the Myt sample could be caused by the difference in amount of consumed either *Mytilus* gonad or *Artemia* nauplii. Actually, cephalic region of phyllosoma fed on *Mytilus* gonad was yellowish, while that of *Artemia* was transparent. Validation of detecting ability for quality in consumed food will be expected in future.

#### Analysis of wild-caught phyllosoma larvae

Results of experiment using hepatopancreas of wild-caught phyllosoma larvae are summarized in Table 3. Little amplification was observed from washing water samples, and hence, indicated

that cross contamination from the body surface of phyllosoma larvae was very little. From the hepatopancreas contents each of Pac-1 and Pac-2, 178 and 168 clones were obtained, respectively. Of all the clones derived from the Pac-1, 132 clones (74 %) showed restriction profiles distinct from the host, in contrast to only four clones (2.4 %) doing in the Pac-2. Based on the RFLP analysis, eight and two restriction types were distinguished from the host in Pac-1 and Pac-2, respectively.

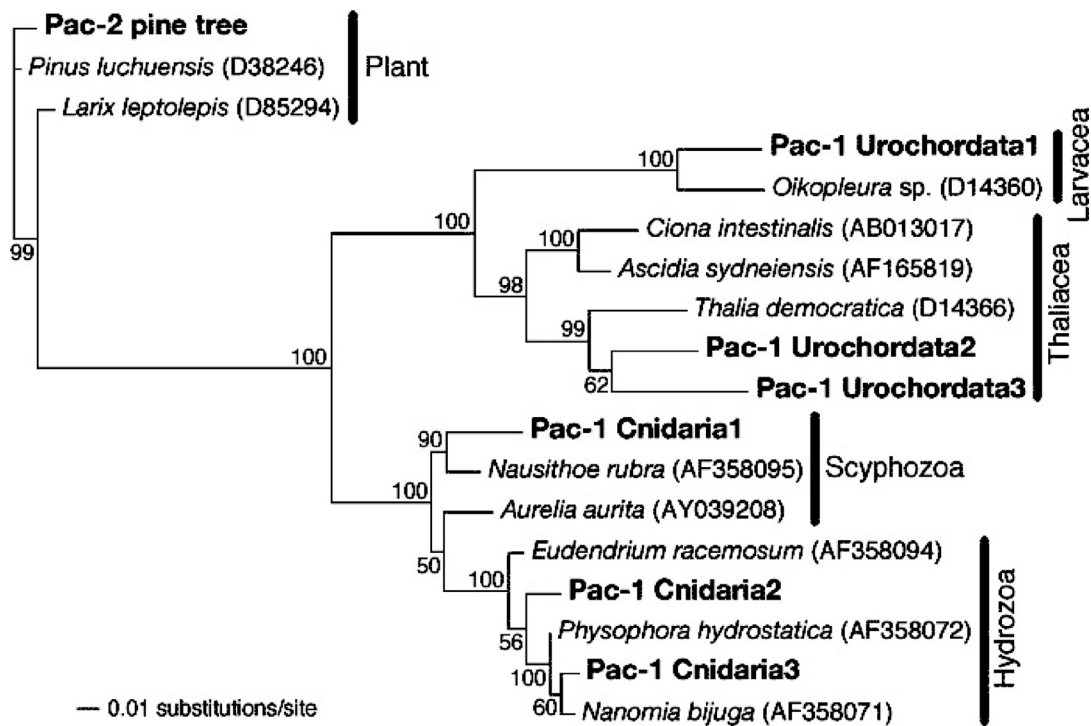
The nucleotide sequence alignment among hepatopancreas-derived and FASTA-nominated sequences revealed eight clones in Pac-1 and three in Pac-2 to be chimeric artifacts between partial sequences of fungus and host 18S rDNA. These chimera sequences were excluded from the phylogenetic analysis. One restriction type in Pac-1, including 10 clones, was not identified because of difficulty for sequencing.

The FASTA searches and phylogenetic analysis arranged six sequences from Pac-1 and one from Pac-2 on the unrooted NJ tree among possible organism nominated in FASTA (Fig. 1). Two animal taxa (phylum Cnidaria and subphylum Urochordata) and one plant family (Pinaceae) were clearly assembled with 100 % bootstrap probabilities. All clonal sequences obtained from Pac-1 were fallen into the animal clade, whereas one sequence from Pac-2 was into the opposite plant clade, suggested to be Ryukyu island pine by FASTA. This pine DNA may be introduced into the laboratory environment from airborne pollen. Such is the case with the Japanese cedar observed in the laboratory-reared phyllosoma larvae. Of the sequences fallen into the cnidarian clade, one (Pac-1 Cnidaria1) was assigned to Scyphozoa and the other two (Pac-1 Cnidaria2

**Table 3.** Results of eukaryotes DNA detection using wild-caught phyllosoma larvae

Specimen ID	No. of amplicons/PCR	RFLP profile		No. of types*	Identified eukaryote <sup>No. of types</sup>	
		host	non-host		(No. of clones)	
Pac-1	178/192	46	132	8	Urochordata <sup>3</sup>	(55)
					Cnidaria <sup>3</sup>	(59)
					chimera <sup>1</sup>	(8)
					unidentified <sup>1</sup>	(10)
Pac-2	168/184	164	4	2	pine tree <sup>1</sup>	(1)
					chimera <sup>1</sup>	(3)

\*Host type excluded



**Fig. 1.** Neighbor-joining tree for affiliating 18S rDNA sequences obtained from the wild phyllosoma hepatopancreas. Sequences from the hepatopancreas were shown in bold host ID and eukaryote 18S rDNAs nominated by FASTA search were in italic with the accession No. in parenthesis. Numbers on branches indicate bootstrap probabilities with 1,000 replications.

and 3) to Hydrozoa. In the Urochordata clade, one sequence (Pac-1 Urochordata1) was assigned to Larvacea and the other two (Pac-1 Urochordata2 and 3) to Thaliacea.

In the present analysis for wild-caught phyllosoma larvae, Cnidaria and Urochordata are likely to be prey organisms at least for the scyllarid phyllosoma Pac-1. This corresponds to the observation for nematocysts in the fecal mass of a scyllarid phyllosoma (Sims and Brown, 1968). Alternatively, no clones derived from possible prey were detected from the Japanese spiny lobster larva Pac-2. This discordance might also be caused by the difference in the amount of consumed food and/or degree of digestion like as the case of laboratory-reared larvae. In the reared phyllosoma sample fed *Artemia* nauplii (Art), only one clone of *Artemia* feed could be found in a total of 116 amplicons through colony-direct PCR, in spite of sufficient feeding and immediate

sampling after meal. More clones with much experimental effort might have produce detection for wild-caught larvae. It should be consider how effort detects effectively foreign DNAs derived from possible prey organisms.

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