Mar Biotechnol
DOI 10.1007/s10126-010-9301-3

ORIGINAL ARTICLE

Investigation on Natural Diets of Larval Marine Animals Using Peptide Nucleic Acid-Directed Polymerase Chain Reaction Clamping

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Received: 6 February 2010 / Accepted: 25 May 2010

Abstract The stomach contents of the larvae of marine animals are usually very small in quantity and amorphous, especially in invertebrates, making morphological methods of identification very difficult. Nucleotide sequence analysis using polymerase chain reaction (PCR) is a likely approach, but the large quantity of larval (host) DNA present may mask subtle signals from the prey genome. We have adopted peptide nucleic acid (PNA)-directed PCR clamping to selectively inhibit amplification of host DNA for this purpose. The Japanese spiny lobster (*Panulirus japonicus*) and eel (*Anguilla japonica*) were used as model host and prey organisms, respectively. A lobster-specific PNA oligomer (20 bases) was designed to anneal to the sequence at the junction of the 18 S rDNA gene and the internal transcribed spacer 1 (ITS1) of the lobster. PCR using eukaryote universal primers for amplifying the ITS1 region used in conjunction with the lobster-specific PNA on a mixed DNA template of lobster and eel demonstrated successful inhibition of lobster ITS1 amplification while allowing efficient amplification of eel ITS1. This method was then applied to wild-caught lobster larvae of *P. japonicus* and *P. longipes bispinosus* collected around Ryukyu Archipelago, Japan. ITS1 sequences of a wide variety of animals (Ctenophora, Cnidaria, Crustacea, Teleostei, Mollusca, and Chaetognatha) were detected.

Keywords PCR-clamping · PNA · Gut content analysis · Larval diet · Spiny lobster

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Introduction

Although information on predator–prey relationships is fundamental to understanding marine food webs, difficulty in characterizing the diets of small marine animals, especially the larval forms of invertebrates, has been a major constraint to advancing our knowledge (e.g., Blankenship and Yayanos 2005). Nucleotide sequence analysis of gut contents may be a promising tool, because DNA is a relatively stable molecule and very small quantities of prey DNA may be detected using the polymerase chain reaction (PCR; e.g., Symondson 2002; King et al. 2008). Molecular detection of known target prey species in the gut or the feces of predators may not prove difficult when using species-specific primers (Asahida et al. 1997; Chen et al. 2000; Jarman et al. 2002, 2004; Rosel and Kocher 2002; Saitoh et al. 2003; Deagle et al. 2005; Casper et al. 2007). However, the detection and determination of unknown prey organisms may be much more difficult. Martin et al. (2006) adopted a PCR-denaturing gradient gel electrophoresis (DGGE) approach targeting 18 S rDNA for investigating the dietary composition in the gut and fecal pellets of Antarctic krill (*Euphausia superba*) and reported successful

Published online: 10 June 2010

detection of many diverse diet items. When the gut contents could be efficiently isolated from the host tissue, small quantities of the host genome present in the sample would not be a substantial obstacle for PCR amplification of nonhost DNA and subsequent molecular analysis. However, it is considerably more difficult to physically separate the gut and its contents in marine invertebrate larvae. Suzuki et al. (2006, 2008) used 18 S rDNA to investigate the diet of phyllosoma larvae of lobsters (palinurid and scyllarid), adopting restriction fragment length polymorphism (RFLP) analysis to select clones showing nonhost restriction patterns. Although they could detect 18 S rDNA molecules of prey candidates such as Cnidaria, Urochordata, and Teleostei, the technique was not only time consuming but also inefficient in detecting nonhost DNA in the gut. Nearly 90% of 2,341 clones examined by Suzuki et al. (2006, 2008) were determined to be of the host lobster larvae, apparently indicating that a substantial amount of contamination from the host genome occurred. If amplification from the host species was inhibited, analytical procedures to detect organisms in the gut may be considerably improved. Several methods for inhibiting the amplification of nontarget DNA to selectively enrich the amplification of target sequences are available; enzymatic restriction of nontarget DNA (Blankenship and Yayanos 2005; Dunshea 2009), using blocking primers with a 3′ modification that will not prime polymerization (Liles et al. 2003; Vestheim and Jarman 2008), and PNA clamping (Egholm et al. 1992, 1993; Ørum et al. 1993; Ørum 2000). The use of restriction enzymes is efficient but entirely dependent on the nontarget having a unique restriction site (Dunshea 2009). Blocking primers have the advantage of being relatively inexpensive because 3′ modifications such as phosphorylation (Liles et al. 2003) and C3 spacers (Vestheim and Jarman 2008) are common and readily available commercially. Also, to fine-tune the melting temperature of blocking primers, locked nucleic acids can be incorporated into the DNA oligonucleotides (Senescau et al. 2005); however, the addition of each locked nucleic acid increases the price of the primer. PCR clamping by peptide nucleic acid (PNA) is the most extensively trialed method for selectively enriching target sequences of a mixed template (Nielsen 1999; Ørum 2000; Ray and Nordén 2000; Paulasova and Pellestor 2004; Pellestor and Paulasova 2004); however, it is also the most expensive (about six times the price of a blocking primer). PNA clamping works because (1) PNA-DNA duplexes generally have greater thermal stability than the corresponding DNA–DNA duplexes, and (2) PNA oligomers are not recognized by DNA polymerases and consequently cannot serve as primers during PCR amplification (Nielsen et al. 1991). Of these available methods, we used PNA clamping based on convenience, and had it not worked, we would have moved to one of the other methods outlined above that are equally valid.

In this study, we introduce the PNA-directed PCR clamping method to efficiently inhibit amplification of rDNA sequence of the Japanese spiny lobster (*Panulirus japonicus*). Using PCR clamping, we also report the results of our attempt to determine natural diets for the lobster larvae for which other research methods have previously provided very limited information.

Materials and Methods

Sample Information and DNA Extraction

The collection date, location, and developmental stage of lobster phyllosoma larvae collected by RV Shunyo-Maru (Fisheries Research Agency, Japan) are shown in Table 1 (see also Chow et al. 2006a, b). The larval body surface was rinsed well with sterilized sea water and fixed with 70% ethanol on board the vessel, stored in refrigerator (4°C), and transferred to the laboratory where they were kept at 4°C till use. The 39 larvae of the Japanese spiny lobster (*P. japonicus*) (PJ) and eight of the long legged spiny lobster (*P. longipes bispinosus*) (PL) used in this study were mid to final stages ranging from 11.8 to 34.7 mm in body length. The body surface of the larvae was washed well with sterile distilled water to eliminate contaminants from the body surface. DNA was extracted from the cephalic region (containing hepatopancreas = gut) and thoracic region using a standard phenol–chloroform method as described in Suzuki et al. (2006, 2008) and was stored at 4°C. All lobster larvae were identified using nucleotide sequence analysis of the mitochondrial cytochrome oxidase subunit I (COI) gene (Chow et al. 2006a, b). Ethanol preserved muscle tissue of the Japanese eel *Anguilla japonica*, as a model prey species, was donated from Dr. H. Tanaka, National Research Institute of Aquaculture, from which we extracted crude DNA using the technique described above.

Designing PNA Probe and Primers for PCR

In this study, we utilized the nuclear rDNA internal transcribed spacer 1 (ITS1) region. The ITS1 sequences may vary considerably even between closely related species (Chow et al. 2006c, 2009), which makes designing a species or group specific PNA probe straightforward. Positions, sequences, and melting temperatures (T_m) of PCR primers and PNA are shown in Fig. 1 and Table 2. Nucleotide sequences of the ITS1 region of five spiny lobster species (*P. japonicus*, *P. longipes bispinosus*, *P. l. longipes*, *P. femoristriga*, and *P. brunneiflagellum*) collected in the Northwest Pacific were adopted from Chow et al. (2009), and those from two individuals each

Table 1 Collection information and stage of phyllosoma larvae used in this study

No. larvae used[a]		Date	Latitude	Longitude
P. japonicus	*P. l. bispinosus*			
13 (8–10)	–	May 2003	21°30′–24°30′N	123°25′–126°12′E
5 (9, 10)	–	May 2004	26°07′–27°45′N	129°07′–129°50′E
4 (7)	2 (9)	November 2004	21°50′–24°50′N	123°25′–126°12′E
3 (7)	4 (8–10)	January–February 2007	26°00′–31°04′N	142°50′–143°07′
14 (9, 10)	2 (7, 10)	May 2007	23°00′–25°10′N	123°54′–125°30′E

[a] Number in parenthesis represents the larval developmental stage

from *Panulirus cygnus* and *Jasus edwardsii* collected in distant locales (Indian Ocean and Southwest Pacific, respectively) were determined in this study (GenBank accession numbers of AB492043–AB492051). We observed highly conserved sequence at the 3′ region of 18 S rDNA among these lobster species. Six species of the genus *Panulirus* used were observed to share conserved sequence at the 5′ region of ITS1 sequences but distinct from that of *J. edwardsii*. Thus, the PNA oligomer (SPL1F) designed may be the genus *Panulirus* specific. The forward primer (ITS1F1) was designed to anneal at the 3′ end of the 18 S rDNA gene, sharing eight nucleotides with the PNA probe. Two universal reverse primers (5.8 S and SP-1–3 for seminested PCR) were designed to anneal near the 5′ end of the 5.8 S rDNA gene and were adopted from Chu et al. (2001) and Vilgalys and Hester (1990), respectively.

PCR Clamping by PNA and Nucleotide Sequence Analysis

PCR was performed in a 20-μL reaction mixture containing 10 μL of 2×GC buffer I, 7.5 pmol of each primer, each deoxynucleoside triphosphate at a concentration of 0.4 mM, 1.25 U of LA Taq polymerase (TaKaRa Bio) and template DNA, in addition to 0, 2 or 4 μM of the PNA probe. The same amplification conditions were used for both first and second rounds of PCR; initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing/elongation step at 53 to 60°C for 3 min, and a final elongation step consisting of 72°C for 7 min. A 1-μL aliquot of amplification product from the first PCR using ITS1F1 and SP-1–3 primer pair was used as template for the second semi-nested PCR using ITS1F1 and 5.8S primer pair. PCR products were subjected to electrophoresis through a 1.5%–2.0 % agarose gel to confirm amplification.

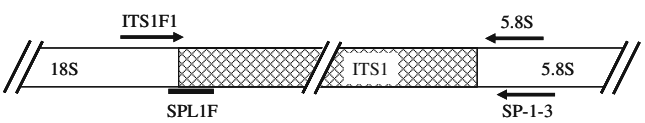

Fig. 1 Positions of PCR primers and PNA used in this study

Amplicons from the second PCR were directly subjected to cloning using pGEM T-Easy cloning kit (Promega, Madison, WI, USA). Plasmid DNA was extracted by QIAprep Spin Miniprep Kit (Qiagen, Ontario, Canada), and the nucleotide sequences of the PCR products inserts were determined by automated sequencer (model 310)(Applied Biosystems, Foster City, CA, USA) with M13 universal primer. If a sequence was observed to have a conserved 5.8 S rDNA-specific sequence (GGCTC or GGTTC) seven nucleotides upstream from the priming position, together with a semi-conserved 5′ end (ACAACT or its modified form) of the 5.8 S rDNA gene, the sequence was determined to be ITS1. Sequences showing 95% or more homology to one another were pooled in one phylogroup (see Chow et al. 2009), and one representative sequence arbitrarily chosen from each phylogroup was subjected to a homology search using the FASTA algorithm in DDBJ (DNA Data Base of Japan). Where the search failed to find ITS1 sequences with homology greater than 70%, the query sequence was determined to be "unidentified."

Results

PCR-Clamping Using Model DNA Mixture

Using mixed templates of DNAs extracted from the thoracic region of the PJ larvae and from the Japanese eel, PCR clamping with 4 μM PNA was attempted at several annealing/elongation temperatures (Fig. 2). The concentration of template DNA was adjusted to 100 pmol for the lobster and 1 pmol for the eel. Strong amplification of the PJ ITS1 (ca. 700 bp) and weak amplification of eel ITS1 (ca. 500 bp) were observed at all annealing/extension temperatures (53, 55, 58, and 60°C) without PNA, while no amplification of PJ ITS1 and greater amplification of eel ITS1 were observed with PNA. We adopted 53°C for annealing/extension temperature hereafter, since better amplification of eel ITS1 was observed at this temperature.

Table 2 Nucleotide sequence and melting temperature (T_m) of primers and PNA

Primer and PNA	Sequence	T_m (°C)
ITS1F1[a]	5′-CGGAAGGATCATTAMCG-3′	50.7–53.1
SPL1F[a]	NH_2-CATTAACGTTGCTTGCAAGC-$CONH_2$	73.7
SP-1–3[b]	5′-ATTTAGCTGCGGTCTTCATC-3′	51.7
5.8S[c]	5′-CGCTGCGTTCTTCATCG-3′	55.5

[a] This study
[b] Chu et al. (2001)
[c] Vilgalys & Hester (1990)

The PCR clamping was further investigated using different concentrations of DNA templates and PNA (Fig. 3). Seven sets of DNA template were tested, where the ratios of the PJ and eel DNAs were adjusted to 100 pmol is to 1–0.016 pmol. Three sets of PNA concentrations (0, 2, and 4 μM) were tested. Amplification of both PJ and eel ITS1 was observed without PNA, but that of eel ITS1 was weak and observed to decline as the DNA concentration decreased (Fig. 3a). Amplification of the PJ ITS1 was successfully inhibited at both 2 and 4 μM PNA concentrations, while amplification of the eel ITS1 was considerably enhanced (Fig. 3b, c). We hereafter used 4 μM PNA for wild phyllosoma samples.

Gut Content Analysis for Phyllosoma Larvae

Using 4 μM PNA and 53°C for annealing/extension temperature, PCR clamping was attempted for DNA templates extracted from the cephalic region of lobster phyllosoma larvae. A total of 39 PJ and 8 PL larvae were used. In all samples, no apparent amplification of the lobster ITS1 was observed in the first and second PCR. Fragment amplification was observed in 10 PJ and 8 PL larvae in the second PCR. Reason for the heterogeneous amplification efficiency between species is unknown, since we observed no apparent relationships between larval collection year and size and the amplification efficiency. The second PCR products were subsequently subjected to cloning. Inserts shorter than 100 bp were not analyzed further, since most marine animals are observed to have ITS1 sequences longer than 100 bp (Chow et al. 2009). Nucleotide sequences determined from a total of 353 clones were categorized into 61 phylogroups. Eleven sequences from 23 clones of PJ larvae were not ITS1. One representative sequence each from the remaining 50 phylogroups (PAN1–50) was deposited in DDBJ, EMBL, and GenBank with accession numbers of AB490718 to AB490767. The results of homology searches for the 50 query sequences and their occurrence in the larvae analyzed are shown in Table 3. Fungal ITS1 was the most frequently observed sequence, with 12 sequences (PAN1–12) detected in 9 larvae (6 PJ and 3 PL). Since it was not determined whether the fungi were symbiotic, parasitic, or from laboratory contamination, we excluded the fungal sequences from subsequent analysis. Among metazoan ITS1 detected, those of Ctenophora, Cnidaria, and Crustacea were relatively common. Ctenophora sequences (PAN13–15) were observed in 4 PJ and 1 PL larvae. Cnidaria sequences (PAN16–18) were observed in 3 PJ and 1 PL larvae. A sequence (PAN19) nearly identical to scyllarid lobster (*Scyllarus cultrifer*) was observed in 2 PJ and 2 PL larvae. Two sequences (PAN20–21) detected in one of each PJ and PL larvae were nearly identical to a flying fish (*Cheilopogon pinnatibarbatus*) and Japanese anchovy (*Engraulis japonicus*), respectively. A sequence (PAN22) detected in one PJ larva was almost identical to an abalone (*Haliotis discus*). The closest match to a sequence (PAN23) detected from a PJ larva was an arrow worm (*Sagitta* sp.: Chaetognatha). The ITS1 sequence data of the arrow worm in the database were only partial, which may be responsible for the relatively low homology (75%). One sequence (PAN24) obtained in 24 clones from 3 PJ larvae appeared to be of the host larvae, suggesting that some host amplification escaped the PNA clamping. Although preliminary phylogenetic investigation of the remaining 26 sequences (PAN25–50) indicated that some of them may be related to fungi (PAN50) or crustaceans (PAN35), the long branches aspects in the phylogenetic tree prevented identification of these sequences to any known taxon.

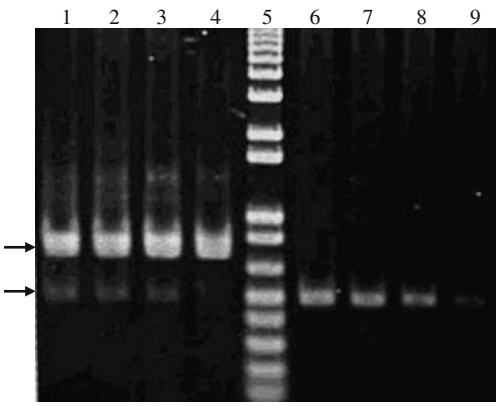

Fig. 2 PCR amplification using mixed DNA template of lobster (*P. japonicus*) and eel (*A. japonica*) at different temperatures without PNA (lanes 1–4) and with PNA (lanes 6–9). Temperatures tested are 53°C (lanes 1 and 6), 55°C (lanes 2 and 7), 58°C (lanes 3 and 8), and 60°C (lanes 4 and 9). Lane 5 is molecular marker (100-bp ladder). Amplified fragments of lobster and eel are ca. 650 and 500 bp, respectively (*arrows*)

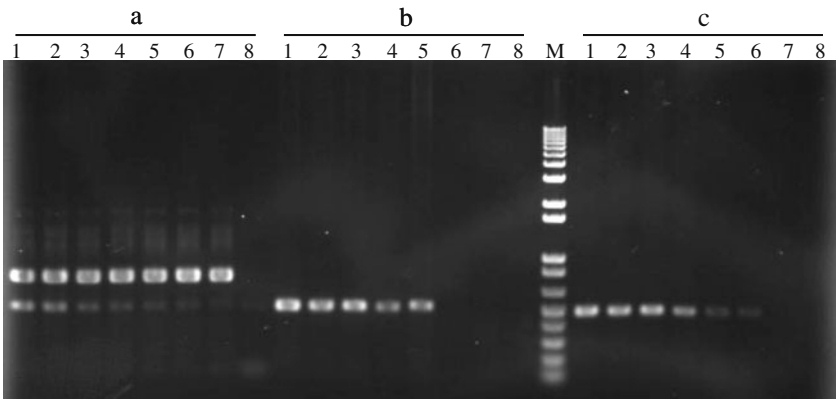

Fig. 3 Results of PCR clamping using different concentration of DNA templates and PNA. PNA concentrations are 0 μM (**a**), 2 μM (**b**), and 4 μM (**c**). Lane 8 is negative control (no DNA). The same amount of lobster DNA (100 pmol) was used to all (1–7), while diluted eel DNAs were added (lane 1: 1 pmol, lane 2: 0.5 pmol, lane 3: 0.25 pmol, ;lane 4: 0.125 pmol, lane 5: 0.062 pmol, lane 6: 0.031 pmol, and lane 7: 0.016 pmol). *M* molecular marker (100-bp ladder)

Discussion

PCR Clamping

The model experiment performed in this study using a mixed template of lobster and eel DNAs demonstrated the remarkable efficiency of PNA clamping to inhibit amplification of lobster ITS1. With the lobster phyllosomas, we also demonstrated the efficiency of inhibition, where we detected only 24 host ITS1 clones out of 353 examined for 18 lobster phyllosoma larvae. This is a significant improvement over the results obtained by Suzuki et al. (2006, 2008) using a standard PCR approach, where they detected 2,062 host 18 S rDNA clones out of 2,341 examined from 16 phyllosoma larvae. However, it appears that some amplification of host DNA escaped the PNA inhibition. The universal primer may have annealed to the lobster rDNA before the PNA, permitting extension by the DNA polymerase. Furthermore, the occurrence of nonspecific PCR clamping cannot be ruled out. Five nucleotides (CATTA) of the PNA oligomer are at the 3′ end of 18 S rDNA and universal. The T_m of this portion of the PNA probe is 39°C, far below that of the universal forward primer (50.7–53.1°C). When the relatively conserved three nucleotides (ACG or CCG) at the 5′ end of ITS1 are taken into account, the T_m of the eight nucleotide portion (CATTAACG) of the PNA would be 48.1°C, comparable with that of the forward primer. Thus, the lobster-specific PNA designed and used in this study may have potential to clamp some other eukaryotes. Although we expected the arrangement of the universal primer and PNA to be efficient for PNA clamping, further investigation on the positional relationships between PNA and PCR primers and effects of the nucleotide composition of these oligomers for annealing appears to be necessary for developing more efficient PCR clamping and for avoiding nonspecific PCR clamping.

ITS1 as a Target Molecule

Choice of genes may be important for molecular analysis of predation. Multicopy DNAs such as mitochondrial DNA (mtDNA) genes and nuclear ribosomal DNAs (nuclear rDNA) have been the preferred target for molecular gut content analysis (King et al. 2008), as some copies of these DNAs are expected to survive for a longer period of time than single copy DNA when subjected to digestive processes. According to the summary by King et al. (2008), mtDNA cytochrome oxidase subunit I (COI) and rDNAs (12 S and 16 S) followed by the small and large subunits of nuclear rDNAs (18 S and 28 S) have been frequently utilized. King et al. (2008) suggested that the ITS region is probably best avoided because of the intraindividual and intraspecific variation. In fact, intra-individual and intraspecific variation in ITS region may not be rare as observed in a wide variety of eukaryotes (Tang et al. 1996; Hsueh et al. 2001; Chow et al. 2006c; Rosselló et al. 2007; Chow et al. 2009). However, the preference toward the mtDNA genes and nuclear rDNA subunits is primarily due to the much larger size of the database than for ITS and not due to the intraindividual and intraspecific variation. Furthermore, intraspecific variation in the nuclear rDNA subunits (18 S and 28 S) is not typically investigated. ITS does have a limited public database specifically for marine animals (Chow et al. 2009), and consequently, this may be responsible for the incorrect species determination for more than half of phylotypes obtained in this study (PAN25-50). In contrast, almost all 18 S rDNA clones could be assigned to known taxa in the previous molecular diet analysis for the lobster larvae using homology search and phylogenetic analysis (Suzuki et al. 2006, 2008). Because of the large number of nucleotide substitutions even between closely related species, Chow et al. (2009)

Table 3 Number of sequences detected from 18 lobster phyllosoma larvae and the results of homology search for the sequences

Query sequence[a]	Best score organism			Homology (%)	*P. japonicus*										*P. longipes bispinosus*							
	Taxon	Species	Accession no.		PJ1 7[b] JF[c]	PJ2 7 JF	PJ3 8 M	PJ4 9 M	PJ5 9 M	PJ6 9 M	PJ7 9 M	PJ8 9 M	PJ9 10 M	PJ10 10 M	PL1 7 M	PL2 8 JF	PL3 8 JF	PL4 9 N	PL5 9 N	PL6 10 JF	PL7 8 JF	PL8 9 JF
PAN1	Fungi	*Phanerochaete laevis*	AY219348	74								1			1							
PAN2	Fungi	*Aspergillus tubingensis*	EF634380	99						3												
PAN3	Fungi	*Aspergillus vitricola*	EF652046	95						1					1			4				8
PAN4	Fungi	*Aspergillus versicolor*	AM883156	100										2								
PAN5	Fungi	*Candida tropicalis*	EU288196	99		1	2						1	6								
PAN6	Fungi	*Cercozoa* sp.	EU567255	81																		
PAN7	Fungi	*Cryptococcus albidus*	AF444340	99			1					2										
PAN8	Fungi	*Hyphodontia alutaria*	DQ873603	82										1								
PAN9	Fungi	*Leptosphaerulina chartarum*	EU272492	98								2										
PAN10	Fungi	*Pichia guilliermondii*	AM160625	98								1										
PAN11	Fungi	*Sagenomella chlamydospora*	AJ519984	88											1							
PAN12	Fungi	*Trametes hirsuta*	EU326211	100			3															
PAN13	Ctenophora	*Beroe* sp.	AB377602	91										3								
PAN14	Ctenophora	*Cestum veneris*	AB377606	100									13									
PAN15	Ctenophora	*Hormiphora plumosa*	AF293676	89	1	1											6					
PAN16	Cnidaria	*Vogtia serrata*	AB377577	81				1														
PAN17	Cnidaria	*Nausithoe* sp.	AB377543	85				6		5			1									
PAN18	Cnidaria	Cnidaria sp.	AB377540	99												3						
PAN19	Arthropoda	*Scyllarus cultrifer*	AB426501	99	1	37										5	3					
PAN20	Teleostei	*Cheilopogon pinnatibarbatus*	AB375584	99	1																	
PAN21	Teleostei	*Engraulis japonicus*	AB375608	99															3			
PAN22	Mollusca	*Haliotis discus*	AY146404	99				6														
PAN23	Chaetognatha	*Sagitta elegans*	AF342799	75									1									
PAN24	Arthropoda	*Panulius japonicus*	AB426508	93				4			14		6									
PAN25	–	–	–	–				1														
PAN26	–	–	–	–						7			1									
PAN27	–	–	–	–									1									
PAN28	–	–	–	–		25																
PAN29	–	–	–	–					1				1	2								
PAN30	–	–	–	–													2					
PAN31	–	–	–	–				8					4									
PAN32	–	–	–	–									6									
PAN33	–	–	–	–									1									
PAN34	–	–	–	–					1													
PAN35	–	–	–	–							14											

PAN36	–	–	–	–			1									
PAN37	–	–	–	–		13										
PAN38	–	–	–	–	1											
PAN39	–	–	–	–							3					
PAN40	–	–	–	–										1		
PAN41	–	–	–	–						3						
PAN42	–	–	–	–									3			
PAN43	–	–	–	–					1							
PAN44	–	–	–	–					4							
PAN45	–	–	–	–									1			
PAN46	–	–	–	–								3	1			
PAN47	–	–	–	–								5			7	
PAN48	–	–	–	–										8	18	15
PAN49	–	–	–	–										2		
PAN50	–	–	–	–				2								

[a] Fifty nucleotide sequences were deposited to DNA database (AB490718 to AB490767). Querying database with 26 sequences (PAN25–50) was failed, since no similar sequence was presented from the database

[b] Larval stage and [c] month (January–February, May, November) the larvae collected

tentatively determined 30% nucleotide difference may be the lower limit for obtaining confident sequence alignments in ITS1. Many comparisons between marine animal species of different genera within family were observed to exceed this level (Chow et al. 2009), indicating apparent limits of ITS1 data for phylogenetic applications among distant taxa. Yet, it is promising for phonetic (barcoding) applications such as diet studies and affiliation for unidentified sequences obtained in the present study may be attained as the database for ITS sequence expands. Use of ITS1 as a target for molecular gut content analysis has several advantages. Large nucleotide sequence difference even between closely related species would allow us to design species-specific PNA probes. Suzuki et al. (2006, 2008) using 18 S rDNA observed chimera molecules comprising different eukaryotes such as host and fungi. The chimera molecules may be de novo synthesized during PCR amplification process via highly conserved sequence regions observed even between considerably distant taxa. Such conserved regions between distant taxa or even between species in the same family do not exist in ITS1 (Chow et al. 2009), which may avoid synthesis of chimera molecules. ITS is also characterized by large length differences between taxa (von der Schulenburg et al. 2001; Chow et al. 2009), which is an advantage because sensitive fragment separation systems such as temperature or denaturing gradient gel electrophoresis is often not necessary. However, the length difference in ITS1 is a double-edged sword, as there may be different amplification efficiency and susceptibility for digestion between shorter and longer fragments (Wattier et al. 1998; Zaidi et al. 1999; Hoogendoorn and Heimpel 2001). For example, fish larvae may be very credible as prey for phyllosomas (Suzuki et al. 2008), but it is likely that fish ITS1 have been underrepresented in our dataset because of the relatively long length of the ITS1 of bony fishes (318 bp to 1,518 bp; Chow et al. 2009).

Diet of Lobster Phyllosoma Larvae

It is unlikely that large numbers of prey organisms can be detected in a single lobster larva. Excluding fungi and host sequences, the number of different sequences detected in a single specimen of phyllosoma larva ranged from 0 (PJ3) to 10 (PJ9) (Table 3). PJ9 appears to be exceptional, since the number of diet candidates for all other specimens was a maximum of four. Secondary predation may partially be responsible, as DNA of a vital predator such as slipper lobster larvae (*S. cultrifer*) is frequently detected along with cnidarian and ctenophora DNAs (PJ1, PJ2, PL2, and PL3). The size of ITS1 in most marine animals is expected to be larger than 200 bp (Chow et al. 2009). Half of the ITS1 sequences unidentified in this study (PAN25–31, 40–43,

and 50) were short (105–248 bp) and therefore may be from microorganisms. Laboratory experiments offering a variety of plankton species as prey have indicated that the soft-bodied zooplankton and fish larvae may be major prey items for lobster phyllosoma larvae (Mitchell 1971; Kittaka 1994; Suzuki et al. 2008), corresponding to the results of our molecular diet analysis as well as those of previous surveys (Suzuki et al. 2006, 2008). Further diet items such as a slipper lobster (*S. cultrifer*), gastropod (*H. discus*), and arrow warm (*Sagitta* sp.) were identified in the present study (Table 3), corroborating the assumption that the phyllosoma larvae are opportunistic carnivores capable of capturing and digesting a wide variety of zooplankton prey (Jeffs et al. 2004; Johnston et al. 2004; Suzuki et al. 2008).

Acknowledgments We thank T. Yoshimura, Seikai National Fisheries Research Institute; K. Satoh and M. Sakai, National Research Institute of Far Seas Fisheries; and the members of RV Shunyo-Maru and Kaiyo-Maru, for assistance with the sample collection. We also thank T. Kawashima and C. Takahashi for their technical assistance in DNA analysis. This study was supported in part by grants from the Ministry of Agriculture, Forestry, and Fisheries of Japan and Japan Society for the Promotion Science (JSPS) and Foundation for Research, Science and Technology (FRST) under the Japan–New Zealand Research Cooperative Program.

References

Asahida T, Yamashita Y, Kobayashi T (1997) Identification of consumed stone flounder, *Kareius bicolonatus* (Basilewsky), from the stomach contents of sand shrimp, *Crangon affinis* (De Haan) using mitochondrial DNA analysis. J Exp Mar Biol Ecol 217:153–163

Blankenship LE, Yayanos AA (2005) Universal primers and PCR of gut contents to study marine invertebrate diets. Mol Ecol 14:891–899

Casper RM, Jarman SN, Bruce E, Deagle BE, Gales NJ, Hindell MA (2007) Detecting prey from DNA in predator scats: a comparison with morphological analysis, using *Arctocephalus* seals fed a known diet. J Exp Mar Biol Ecol 347:144–154

Chen Y, Giles KL, Payton ME, Greenstone MH (2000) Identifying key cereal aphid predators by molecular gut analysis. Mol Ecol 9:1887–1898

Chow S, Yamada H, Suzuki N (2006a) Identification of mid to final stage phyllosoma larvae of the genus *Panulirus* White, 1847 collected in the Ryukyu Archipelago. Crustaceana 79:745–764

Chow S, Suzuki N, Imai H, Yoshimura T (2006b) Molecular species identification of spiny lobster phyllosoma larvae of the genus *Panulirus* from the Northwestern Pacific. Mar Biotechnol 8:260–267

Chow S, Nakagawa T, Suzuki N, Takeyama H, Matsunaga T (2006c) Phylogenetic relationships among *Thunnus* tuna species inferred from rDNA ITS1 sequence. J Fish Biol 68:24–35

Chow S, Ueno Y, Toyokawa M, Oohara I, Takeyama H (2009) Preliminary analysis of length and GC content variation in the ribosomal first internal transcribed spacer (ITS1) of marine animals. Mar Biotechnol 11:301–306

Chu KH, Li CP, Ho HY (2001) The first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in crustacean. Mar Biotechnol 3:355–361

Deagle BE, Tollit DJ, Jarman SN, Hindell MA, Trites AW, Gales NJ (2005) Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Mol Ecol 14:1831–1842

Dunshea G (2009) DNA-based diet analysis for any predator. PLoS ONE 4(4):e5252

Egholm M, Buchardt O, Nielsen PE, Berg RH (1992) Peptide nucleic acids (PNA). Oligonucleotide analogues with an achiral peptide backbone. J Am Chem Soc 114:1895–1897

Egholm M, Buchardt O, Christensen L, Behrens C, Frier SM, Driver DA, Berg RH, Kim SK, Norde´n B, Nielsen PE (1993) PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen bonding rules. Nature 365:566–568

Hoogendoorn M, Heimpel GE (2001) PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. Mol Ecol 10:2059–2067

Hsueh JYC, JR BRP, Didier PJ, Tang X, Lasbury ME, Li B, Jin S, Bertlett MS, Smith JW, Lee C-H (2001) Internal transcribed spacer regions of rRNA genes of *Pneumocystis carinii* from monkeys. Clin Diagn Lab Immunol 8:503–508

Jarman SN, Gales NJ, Tierney M, Gill PC, Elliott NG (2002) A DNA based method for identification of krill species and its application to analysing the diet of marine vertebrate predators. Mol Ecol 11:2679–2690

Jarman SN, Deagle BE, Gales NJ (2004) Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. Mol Ecol 13:1313–1322

Jeffs AG, Nichols PD, Mooney BD, Phillips KL, Phleger CF (2004) Identifying potential prey of the pelagic larvae of the spiny lobster *Jasus edwardsii* using lipid signatures. Comp Biochem Physiol Part B 137:487–507

Johnston D, Ritar A, Thomas C, Jeffs A (2004) Digestive enzyme profiles of spiny lobster *Jasus edwardsii* phyllosoma larvae. Mar Ecol Prog Ser 275:219–230

King RA, Read DS, Traugott M, Symondson WOC (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. Mol Ecol 17:947–963

Kittaka J (1994) Culture of phyllosomas of spiny lobster and its application to studies of larval recruitment and aquaculture. Crustaceana 66:258–270

Liles MR, Manske BF, Bintrim SB, Handelsman J, Goodman RM (2003) A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Appl Environ Microbiol 69:2684–2691

Martin DL, Robin M, Ross RM, Quetin LB, Murray AE (2006) Molecular approach (PCR-DGGE) to diet analysis in young Antarctic krill *Euphausia superba*. Mar Ecol Prog Ser 319:155–165

Mitchell JR (1971) Food preferences, feeding mechanisms and related behavior in phyllosoma larvae of the California spiny lobster, *Panulirus interruptus* (Randall). MS Thesis, San Diego State College, San Diego, CA

Nielsen PE (1999) Applications of peptide nucleic acids. Curr Opin Biotechnol 10:71–75

Nielsen PE, Egholm M, Berg RH, Buchardt O (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 254:1497–1500

Ørum H (2000) PCR clamping. Curr Issue Mol Biol 2:27–30

Ørum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley C (1993) Single base pair mutation analysis by PNA directed PCR clamping. Nuc Acid Res 21:5332–5336

Paulasova P, Pellestor F (2004) The peptide nucleic acids (PNAs): a new generation of probes for genetic and cytogenetic analyses. Ann Génét 47:349–358

Pellestor F, Paulasova P (2004) The peptide nucleic acids (PNAs): introduction to a new class of probes for chromosomal investigation. Chromosoma 112:375–380

Ray A, Nordén B (2000) Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J 14:1041–1060

Rosel PE, Kocher TD (2002) DNA-based identification of larval cod in stomach contents of predatory fishes. J Exp Mar Biol Ecol 267:75–88

Rosselló JA, Lázaro A, Cosín R, Molins A (2007) A phylogeographic split in *Buxus balearica* (Buxaceae) as evidenced by nuclear ribosomal markers: when ITS paralogues are welcome. J Mol Evol 64:143–157

Saitoh K, Takagaki M, Yamashita Y (2003) Detection of Japanese flounder-specific DNA from gut contents of potential predators in the field. Fish Sci 69:473–477

Senescau A, Berry A, Benoit-Vical F, Landt O, Fabre R, Lelievre J, Cassaing S, Magnaval J (2005) Use of a locked-nucleic-acid oligomer in the clamped-probe assay for detection of a minority Pfcrt K76T mutant population of *Plasmodium falciparum*. J Clin Microbiol 43:3304–3308

Suzuki N, Murakami K, Takeyama H, Chow S (2006) Molecular attempt to identify prey organisms of lobster phyllosoma larvae. Fish Sci 72:342–349

Suzuki N, Hoshino K, Murakami K, Takeyama H, Chow S (2008) Molecular diet analysis of phyllosoma larvae of the Japanese spiny lobster *Panulirus japonicus* (Decapoda: Crustacea). Mar Biotechnol 9:49–54

Symondson WOC (2002) Molecular identification of prey in predator diets. Mol Ecol 11:627–641

Tang J, Toe L, Back C, Unnasch TR (1996) Intra-specific heterogeneity of the rDNA internal transcribed spacer in the *Simulium damnosum* (Diptera: Simuliidae) complex. Mol Biol Evol 13:244–252

Vestheim H, Jarman SN (2008) Blocking primers to enhance PCR amplification of rare sequences in mixed samples—a case study on prey DNA in Antarctic krill stomachs. Front Zool 5:12

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4238–4246

von der Schulenburg JHG, Hancock JM, Pagnamenta A, Sloggett JJ, Majerus MEN, Hurst GDD (2001) Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Mol Biol Evol 18:648–660

Wattier R, Engel CR, Saumitou-Laprade P, Valero M (1998) Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental evidence at the dinucleotide locus Gv1CT in *Gracilaria gracilis* (Rhodophyta). Mol Ecol 7:1569–1573

Zaidi RH, Jaal Z, Hawkes NJ, Hemingway J, Symondson WOC (1999) Can multiple copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Mol Ecol 8:2081–2087