J. Fish. Sci. Technol. 10(4), 179-185, 2007

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

PCR of Gut Contents for a Food Web Study of a Marine Ecosystem

Nack Keun Kim, Kyoung Sun Kim and Hyun-Woo Kim*

Department of Marine Biology, Pukyong National University Busan 608-737, Korea

Understanding dietary habits is one of the most important factors in studying food webs and other ecological processes. Here we designed universal primers to amplify portions of the 18S and 28S rDNA sequences to examine gut contents using PCR techniques. The gut contents of sailfin sandfish (*Arctoscopus japonicus*) and pacific squid (*Todarodes pacificus*) were examined. In total, 11 families of prey were identified with 18S and 28S rDNA using the universal primers. The DNA sequence data indicated that the primer sets successfully amplified a wide spectrum of species and represented gut contents in a relatively convenient way. We found that information in the NCBI database was not yet sufficient to discriminate the species we isolated. In addition, technology for the separation of heterogeneous PCR products and better resolution and quantification protocols would help increase data accuracy.

Key words: PCR, Marine ecosystem, Gut contents, rDNA, *Arctoscopus japonicus, Todarodes pacificus*

Introduction

The study of the dietary behaviors of marine animals is one of the most important factors in understanding ecological process, including food webs, energetics, or environmental effects on target species. One of the most widely used methods for diet analyses involves the direct observation of feeding by dissecting specimens. Although this method has been used to study gut contents, there are potential problems that can result in misleading results. First, digestion time varies among species. Animals with soft tissue, such as mollusks or cnidarians, are generally digested much faster than crustaceans or the remnants of bones, nails, otoliths, or beaks from ingested animals. Accordingly, crustaceans have been studied more frequently than other species. Second, many marine invertebrates cannot be identified solely by morphology. Even for well-trained taxonomists, identification of gut contents, which are mixtures of various partially digested prey, is onerous. In addition, it takes several years to train new taxonomists. Third, direct observation is a time-consuming task; several days are usually required to glean statistically meaningful data, costing researchers considerable time and money. To overcome these problems, PCR techniques have been introduced for the analysis of gut contents. DNA from gut contents can be amplified by PCR, as long as the consumed organisms are not completely digested (Zaidi et al. 1999). Target sequences usually include mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) because they represent evolutionary distance and relationships (Aguinaldo et al., 1997; Adoutte et al., 1999). From the explosion of nucleotide sequence information, nucleotides themselves can provide good markers for distinguishing taxa, and mitochondrial cytochrome oxidase I (COI) genes are used for 'barcoding' individual species (Moritz and Cicero, 2004). Many species-specific or group-specific primers designed to analyze gut contents are very effective (Agusti et al., 2003; Jarman et al., 2004; Deagle et al., 2005). In addition, a few attempts have been made to amplify material from a broad array of taxa from various environmental samples (Blankenship and Yayanos, 2005; Markmann and Tautz, 2005). Here we designed universal primer sets for 18S and 28S rDNA sequences to provide insight into the gut contents of marine predators. Seawater and gut contents from two marine species, sailfin sandfish, *Arctoscopus japonicus,* and pacific squid, *Todarodes pacificus*, revealed that PCR is a powerful tool for understanding many ecological phenomena and is a good alternative method to support traditional ecological studies.

*Corresponding author: kimhw@pknu.ac.kr

Materials and Methods

Materials

Seawater samples were collected from Young-Il Bay, Korea, and centrifuged for 20 min at 8,000×*g*. After discarding the supernatant, the precipitants were stored at -20°C before use. Live squid were caught through a jigging fishery in the littoral area of Busan, Korea. They were stored on ice in buckets (approximately -1°C) and dissected the next day. Sailfin sandfish were collected in the nearshore area of the East Sea, around Pohang, Korea, with specimens treated the same as squid samples.

Purification of genomic DNA

Gut contents were collected and weighed. To eliminate unwanted cells from the examination, the samples were rinsed with distilled water and carefully drained using Pasteur pipettes. Gut contents were then centrifuged for 20 min at 8,000×*g* and collected by discarding the supernatant. Equal volumes of distilled water were added and the samples were homogenized with a PT3100 homogenizer (Polytron Inc, USA). In accordance with the capacity of the genomic DNA extraction kit, 100 μL of the homogenized mixture was used to purify the genomic DNA. Nuclear genomic DNA was isolated using a DNeasy Blood & Tissue Kit (Qiagen), with minor modifycations. Briefly, 180 μL of ATL solution and 20 μL of DNase were added to the 100 μL sub-sample and incubated at 56°C until all tissue was lysed and the mixture became clear. All other procedures followed the manufacturer's instructions. The isolated genomic DNA was then quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, USA) and stored at -20°C until used.

PCR study from gut contents

In total, 114 18S and 72 28S rDNA sequences were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) and aligned using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html) to find a conserved nucleotide sequence region from Porifera to Chordata. Degenerated primers for amplifying each region were designed as in Fig. 1 and were synthesized by the Bioneer company. One hundred μg of genomic DNA were used for each reaction. PCR conditions were as follows: 94°C for 1 min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min 20 sec (35 cycles); 72°C for 2 min. Amplified PCR reactants were identified by 2% agarose gel electrophoresis as previously described (Kim et al. 2005). Each PCR band was cut using a clean razor blade and was purified using a QIAquick Gel Extraction Kit (Qiagen). Extracted PCR bands were then concentrated by evaporation at 70°C and ligated using a TOPO TA Cloning Kit (Invitrogen). After transformation, positive clones were identified by PCR using vector M13 forward and reverse primers. Clones of different sizes were selected, and their sequences were determined using an ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems).

Sequence analysis and annotation

The DNA sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) and classified as described in Fig. 2. Briefly, if a nucleotide sequence was a 100% match, the sequence was considered to be from the same species. If the identity of the nucleotide sequence was below 98% it was classi-

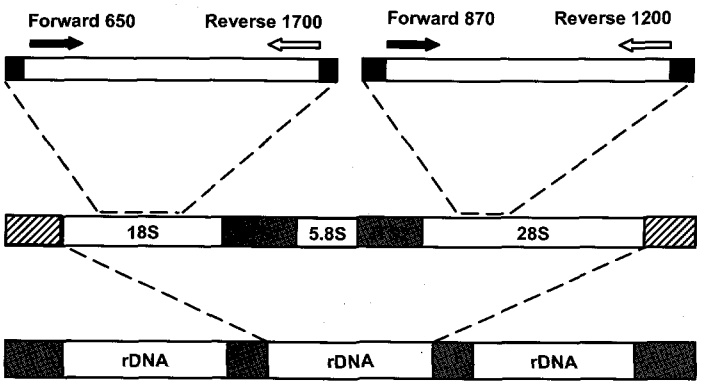

Fig. 1. Universal primer sets for nuclear ribosomal rDNA amplification. Primers were designed not to comprise IS region from the nuclear rDNA concatamers. Forward 650 and reverse 1,200 for 18S and forward 870 and reverse 1200 for 28S were made to comprise all possible species from chordata to porifera. (18S forward 650: AAGTCTGGTGCCAGCASCCGCGGT; 18S reverse 1700: AAGGGCATYACAGAC CTGTTATTG; 28S forward 870: CCCGTCTTGAAACACGGACCA; 28S reverse 1200: TTCGATTAGTCTTTCGCCCCTAT).

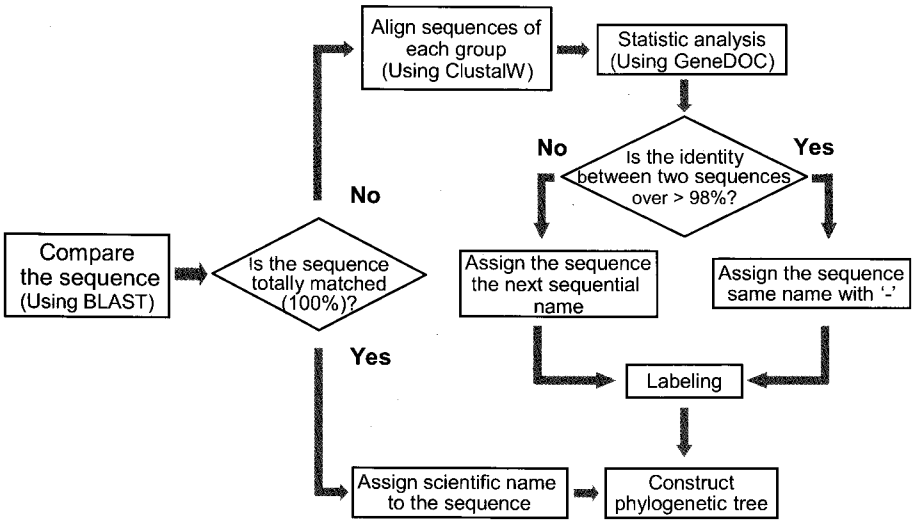

Fig. 2. Flow diagram of rDNA sequence analysis.

fied as a sequence from a different species. The differrent species sequences were named according to the scientific name of the examined species, the amplified rDNA region, and the sample number. For example, TP18S1 indicates that the sample was the first unidentified 18S rDNA sequence from *Todarodes pacificus*. If another unidentified sequence showed more than 98% identity with TP18S1, it was represented as TP18S1-1. A phylogenetic tree was constructed using ClustalW and was annotated graphically using the program Treeview.

Results and Discussion

PCR of DNA from seawater

We performed PCR analysis on DNA found in seawater from the Young-Il gulf area to test the efficiency of the universal primer sets. PCR products were cloned into TOPO vectors, and positive clones were selected and analyzed for their nucleotide sequence (Fig. 4). Six clones contained one genus of urochordate, two *Podocorynes,* and three copepods. Except for one clone, which contained *Centropages hamatus*, the other five sequences did not show 100% identity with the NCBI database. *Podocorynes* sp.-like sequences found in the five unidentified clones appeared to be *Aurelia aurita* because the samples were collected in March, coinciding with its reproductive cycle when many planktonic ephyra would have been present. Unfortunately, there was no rDNA sequence information for *Aurelia aurita* in the NCBI database; the closest species within the same class was retrieved. In addition, two jellyfish were different enough to be classified as different species. The blooming of moon jellyfish is problematic for fisheries in the Pacific Asian region, and genomic studies are necessary to understand their life cycle and to control their spread. Although only six samples were tested, the universal primer set successfully amplified urochordates, arthropods, and cnidarians. From these results, we conclude that the universal primer set was efficient for amplifying various marine animal phyla, from parazoans to chordates.

PCR of gut content DNA

We chose two species, sailfin sandfish (*Arctoscopus japonicus*) and pacific squid (*Todarodes pacificus*) for gut content analysis. From *T. pacificus*, 30 clones were analyzed; three families each were identified from 18S rDNA (Table 1) and 28S rDNA (Table 2), including Pleuronectidae, Pectinidae, Ommastrephidae, Anisakidae, and Onychoteuthidae. The nucleotide sequence similarities for positive clones from 18S rDNA and 28S rDNA were shown on distance-based phylogenetic trees (Figs. 3 and 4, respectively). From these results, we suggest that pacific squid preys mainly on benthic animals. A previous study reported that most juvenile squid prey on small crustaceans, gradually switching to pelagic fish and other cephalopods as they grow (O'Dor and Wells, 1987). In fact, we could not find any crustaceans in our samples; instead, we identified relatively more bivalve sequences. These differing results may be a result of differences in sample size or species-specific feeding habits. According to previous direct observations of *T. pacificus*, mollusks and fish made up about 90% of the gut contents (Song et al., 2006), which agrees strongly with our results. In addition, a relatively high frequency of bivalve sequences was identified compared to previous direct observations.

Table 1. Clones identified using 18S rDNA primer set

Name	Species	Family	Sequence identity	Frequency
AJ18S1	*Euphausia pacifica*	Euphausiidae	875/889 (98%)	1
AJ18S2-1	*Euphausia pacifica*	Euphausiidae	881/885 (99%)	1
AJ18S2-2	*Euphausia pacifica*	Euphausiidae	881/882 (99%)	1
AJ18S2-3	*Euphausia pacifica*	Euphausiidae	878/882 (99%)	1
AJ18S2-4	*Euphausia superba*	Euphausiidae	881/882 (99%)	1
AJ18S3	*Euphausia superba*	Euphausiidae	815/829 (98%)	1
AJ18S4	*Euphausia eximia*	Euphausiidae	821/833 (98%)	1
AJ18S5	*Parathemisto gaudichaudi*	Hyperiidae	816/827 (98%)	1
Euphausia pacifica		Euphausiidae		3
TP18S1-1	*Hippoglossoides dubius*	Pleuronectidae	888/890 (99%)	1
TP18S1-2	*Hippoglossoides dubius*	Pleuronectidae	888/890 (99%)	1
TP18S1-3	*Hippoglossoides dubius*	Pleuronectidae	889/890 (99%)	1
TP18S2-1	*Mimachlamys varia*	Pectinidae	882/883 (99%)	1
TP18S2-2	*Mimachlamys varia*	Pectinidae	880/883 (99%)	1
TP18S2-3	*Mimachlamys varia*	Pectinidae	882/883 (99%)	1
TP18S2-4	*Mimachlamys varia*	Pectinidae	880/883 (99%)	1
TP18S2-5	*Mimachlamys varia*	Pectinidae	878/883 (99%)	1
TP18S3	*Illex coindeti*	Ommastrephidae	1097/1194 (91%)	1
TP18S4	*Illex coindeti*	Ommastrephidae	1098/1193 (92%)	1
TP18S5	*Illex coindeti*	Ommastrephidae	1100/1198 (91%)	1
TP18S6	*Illex coindeti*	Ommastrephidae	1105/1201 (92%)	1
TP18S7-1	*Illex coindeti*	Ommastrephidae	1104/1200 (92%)	1
TP18S7-2	*Illex coindeti*	Ommastrephidae	1102/1202 (91%)	1
Hippoglossoides dubius		Pleuronectidae		1
Mimachlamys varia		Pectinidae		3

Table 2. Clones identified using 28S rDNA primer set

Name	Species	Family	Sequence identity	Frequency
AJ28S1-1	*Euphausia superba*	Euphausiidae	241/242 (99%)	1
AJ28S1-2	*Euphausia superba*	Euphausiidae	240/242 (99%)	1
AJ28S1-3	*Euphausia superba*	Euphausiidae	241/242 (99%)	1
AJ28S2-1	*Euphausia eximia*	Euphausiidae	309/310 (99%)	1
AJ28S2-2	*Euphausia eximia*	Euphausiidae	308/310 (99%)	1
AJ28S3	*Ichthyophonus hoferi*	Ichthyophonida	291/304 (95%)	5
AJ28S4-1	*Themisto gaudichaudii*	Hyperiidae	340/345 (98%)	1
AJ28S4-2	*Themisto gaudichaudii*	Hyperiidae	344/347 (99%)	4
AJ28S5	*Themisto gaudichaudii*	Hyperiidae	343/347 (98%)	1
Euphausia eximia		Euphausiidae		5
TP28S1	*Mizuhopecten yessoensis*	Pectinidae	315/319 (98%)	1
TP28S2	*Raphidascaris acus*	Anisakidae	308/317 (97%)	1
TP28S3	*Moroteuthis knipovitchi*	Onychoteuthidae	541/582 (92%)	1
TP28S4-1	*Moroteuthis knipovitchi*	Onychoteuthidae	537/584 (91%)	1
TP28S4-2	*Moroteuthis knipovitchi*	Onychoteuthidae	532/581 (91%)	1
TP28S4-3	*Moroteuthis knipovitchi*	Onychoteuthidae	532/581 (91%)	2
Mizuhopecten yessoensis		Pectinidae		4
YB28S1	Oikopleura sp.	Oikopleuridae	290/308 (94%)	1
YB28S2	Podocoryne carnea	Hydractiniidae	293/318 (92%)	1
YB28S3	Podocoryne carnea	Hydractiniidae	308/317 (97%)	1
YB28S4	Centropages hamatus	Calanidae	282/289 (97%)	1
YB28S5	Calanus hyperboreus	Calanidae	273/289 (94%)	1
Centropages hamatus		Calanidae		1

This may be a result of mastication; squid do not ingest bivalve shells, resulting in a lack of identifycation in observational studies. Another interesting finding is that the pacific squids were also cannibalistic, i.e., they preyed on each other. Cannibalism is particularly important during large scale migrations for many ommnastrephids (Markaida, 2006), and our results reconfirm those findings. Many previous studies

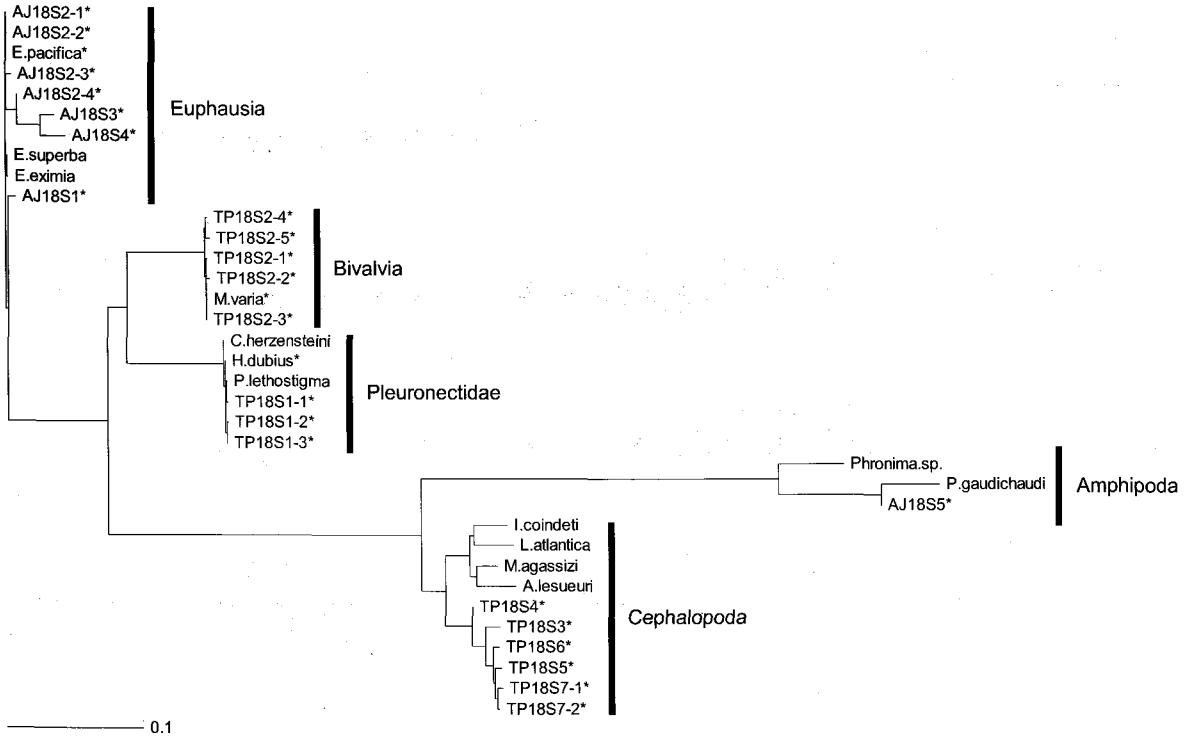

Fig. 3. Distance-based phylogenetic tree of 18S rDNA sequences from the gut of *Todarodes pacificus* (a) and *Arctoscopus japonicus* (b). The phylogenetic tree was generated using the neighbor-joining method with the GeneDoc program. Asterisks indicate the sequences from the gut contents and others eukaryotic sequences were form the GeneBank which have the highest nucleotide sequence similarity to those asterisked sequences. Assigned names are composed of two letter of acronyms, 18S, species number, and subspecies (if available) numbers.

have pointed out the possibility of DNA contamination from unwanted samples (Blankenship and Yayanos, 2005; Deagle et al., 2005), but we could not find any evidence of foreign gene contamination. For sailfin sandfish, 32 clones were analyzed, and two and four families were identified with 18S rDNAs (Table 1) and 28S rDNA sequences (Table 2), respectively. These families include Euphausiidae, Hyperiidae, and Ichthyophonidae. Interestingly, most of the identified clones from sailfin sandfish were crustacean sequences, whereas no crustacean sequence was found in squid. This result provides strong evidence that our primer sets were efficient at detecting different species from gut contents. We designed primer sets to amplify 11 families using ribosomal rDNA and provided evidence that the primers provided an effective and convenient method for studying the gut contents of marine animals. However, we also found that several factors could increase the data accuracy. First, PCR, by its nature, will preferentially amplify abundant copies, suppressing DNA sequences that have relatively lower copy numbers, which also suffer lower transformation efficiencies. If the sizes of amplified PCR products were too similar to separate, the sample would have a lower chance of being analyzed. Multiple competitive PCR followed by denaturing gradient gel electrophoresis (DGGE) is now widely used for both qualification and quantification of microbes from mixed samples (Nubel et al., 1999; Pintado et al., 2003). However, the application of the method to eukaryotic animal systems has not been successful because of the complexity and redundancy of rDNA.

Second, a lack of nuclear ribosomal DNA sequence databases also hinders accurate analysis. Scientists have mainly focused on the mitochondrial COI gene. The 5′ region of the COI gene was selected as the basis for a DNA barcoding system, because of the availability of primers for a broad range of taxa, accuracy from a single run of sequencing reactions, and nucleotide sequence variation that is sufficient for species classifications (Hebert et al., 2003). However, there is variation in the nucleotides on either side of the primer region and in the sequence orientation, which has forced scientists to design different primers to amplify the same region. Besides the COI

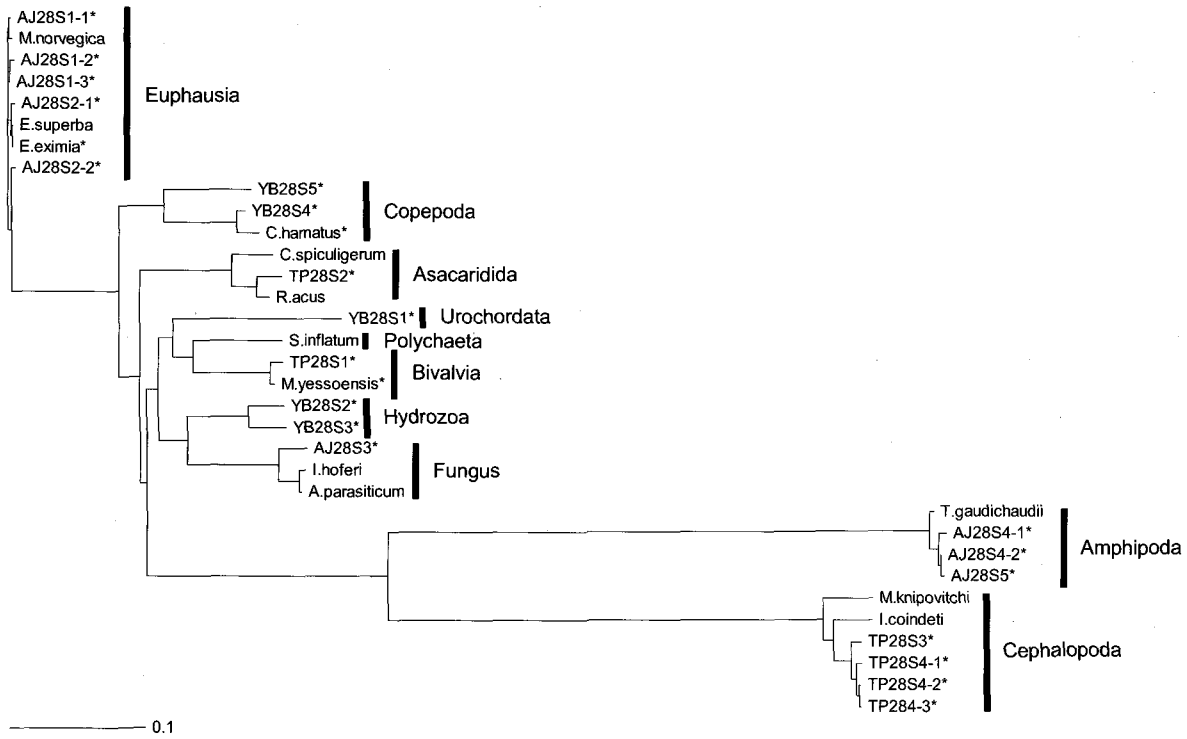

Fig. 4. Distance-based phylogenetic tree of 28S rDNA sequences from the gut of *Todarodes pacificus* (a) and *Arctoscopus japonicus* (b). The phylogenetic tree was generated using the neighbor-joining method with the GeneDoc program. Asterisks indicate the sequences from the gut contents and others eukaryotic sequences were form the GeneBank which have the highest nucleotide sequence similarity to those asterisked sequences. Assigned names are composed of two letter of acronyms, 28S, species number, and subspecies (if available) numbers.

gene, nuclear 18S and 28S rDNA sequences have also been adapted for classifying species (Halanych et al., 1995; Aguinaldo et al., 1997). Those two sequences have usually been used together for accurate analysis because the degree of contribution for constructing phylogenetic trees differs among cases (Mallatt et al., 2004; Passamaneck et al., 2004; Mallatt and Giribet, 2006). One major drawback of nuclear ribosomal DNA sequences is the size of the PCR product when using universal primer sets for discrimination, because of the relatively high degree of sequence conservation. More precise nucleotide databases should be constructed for accurate species identification.

Finally, precise quantification methods are needed. There are two commonly used concepts for the quantification of conventional direct observations. 'Occurrence' indicates the frequency of a species in the total number of individuals, and 'Dry weight' is considered to estimate the spatial contribution of individual species. To our knowledge, no reports have evaluated a method for quantifying each species in gut contents using PCR techniques. One main obstacle for quantification is that species differ in the number of rDNA copies per cell. In most eukaryotes, rDNA consists of tandemly repeated arrays of three or four genes, and the gene multiplicity varies widely (Long and Dawid, 1980). Although there appears to be a positive correlation between rDNA copy number and genome size, there is considerable variation in this relationship among taxonomic units (Prokopowich et al., 2003). Since there is no scientifically accepted equation for calculating copy number from PCR results, we prefer alternative methods at this time. One possible method is to calculate the relative copy number for each species from the total number of rDNA copies. Although this method is theoretically possible, it would not be easy to calculate each copy number from heterogeneous multiple PCR products. Separation techniques for isolating amplified species-specific bands and optimization of PCR conditions would be key factors for the successful analysis of complex gut contents using PCR techniques. Here we designed universal primer sets for studying gut contents of marine animals and successfully amplified a wide spectrum of species. Although

there are a few technical drawbacks at the present time, PCR could provide a convenient and accurate technique for studying marine food webs.

Acknowledgments

The research was supported by the Human Resources Development Center for Marine Bio Nutraceuticals 2007. Nack Keun Kim was supported by NURI.

References

Adoutte, A., G. Balavoine, N. Lartillot and R. de Rosa. 1999. Animal evolution. The end of the intermediate taxa? Trends Genet., 15, 104-108.

Aguinaldo, A.M., J.M. Turbeville, L.S. Linford, M.C. Rivera, J.R. Garey, R.A. Raff and J.A. Lake. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. Nature, 387, 489-493.

Agusti, N., S.P. Shayler, J.D. Harwood, I.P. Vaughan, K.D. Sunderland and W.O. Symondson. 2003. Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. Mol. Ecol., 12, 3467-3475.

Blankenship, L.E. and A.A. Yayanos. 2005. Universal primers and PCR of gut contents to study marine invertebrate diets. Mol. Ecol., 14, 891-899.

Deagle, B.E., S.N. Jarman, D. Pemberton and N.J. Gales. 2005. Genetic screening for prey in the gut contents from a giant squid (*Architeuthis* sp.). J. Hered., 96, 417-423.

Halanych, K.M., J.D. Bacheller, A.M. Aguinaldo, S.M. Liva, D.M. Hillis and J.A. Lake. 1995. Evidence from 18S ribosomal DNA that the lophophorates are protostome animals. Science, 267, 1641-1643.

Hebert, P.D., A. Cywinska, S.L. Ball and J.R. deWaard. 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci., 270, 313-321.

Jarman, S.N., B.E. Deagle and N.J. Gales. 2004. Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. Mol. Ecol., 13, 1313-1322.

Kim, H.W., E.S. Chang and D.L. Mykles. 2005. Three calpains and ecdysone receptor in the land crab *Gecarcinus lateralis*: sequences, expression and effects of elevated ecdysteroid induced by eyestalk ablation. J. Exp. Biol., 208, 3177-3197.

Long, E.O. and I.B. Dawid. 1980. Repeated genes in eukaryotes. Annu. Rev. Biochem., 49, 727-764.

Mallatt, J. and G. Giribet. 2006. Further use of nearly complete 28S and 18S rRNA genes to classify Ecdysozoa: 37 more arthropods and a kinorhynch. Mol. Phylogenet. Evol., 40, 772-794.

Mallatt, J.M., J.R. Garey and J.W. Shultz. 2004. Ecdysozoan phylogeny and Bayesian inference: first use of nearly complete 28S and 18S rRNA gene sequences to classify the arthropods and their kin. Mol. Phylogenet. Evol., 31, 178-191.

Markaida, U. 2006. Food and feeding of jumbo squid *Dosidicus gigas* in the Gulf of California and adjacent waters after the 1997-98 E lNiño event. Fish. Res., 79, 16-27.

Markmann, M. and D. Tautz. 2005. Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. Philos. Trans. Roy. Soc. Lond., B. Biol. Sci., 360, 1917-1924.

Moritz, C. and C. Cicero. 2004. DNA barcoding: promise and pitfalls. Plos. Biol., 2, e354.

Nubel, U., F. Garcia-Pichel, M. Kuhl and G. Muyzer. 1999. Quantifying microbial diversity: morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. Appl. Environ. Microbiol., 65, 422-430.

O'Dor, R.K. and M.J. Wells. 1987. Energy and nutrient flow in cephalopods. In: Celphalopod Life Cycles. Vol. II. Boyle, P.R. ed., Academic Press, London, UK, 109-133.

Passamaneck, Y.J., C. Schander and K.M. Halanych. 2004. Investigation of molluscan phylogeny using large-subunit and small-subunit nuclear rRNA sequences. Mol. Phylogenet. Evol., 32, 25-38.

Pintado, J., J.P. Guyot and F. Ampe. 2003. Multiple competitive PCR-DGGE as a tool for quantifying and profiling defined mixed cultures of lactic acid bacteria during production of probiotics from complex polysaccharides. J. Appl. Microbiol., 95, 921-933.

Prokopowich, C.D., T.R. Gregory and T.J. Crease. 2003. The correlation between rDNA copy number and genome size in eukaryotes. Genome, 46, 48-50.

Song, H.J., G.W. Baeck, S.A. Kim and S.H. Huh. 2006. Feeding habits of *Todarodes pacificus* (Cephalopods: Ommnastrephidae) in the coastal waters of Busan, Korea. J. Kor. Fish. Soc., 39, 42-48.

Zaidi, R.H., Z. Jaal, N.J. Hawkes, J. Hemingway and W.O. Symondson. 1999. Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Mol. Ecol., 8, 2081-2087.

(Received October 2007, Accepted December 2007)