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Evaluation of ITS2-28S as a molecular marker for identification of calanoid copepods in the subtropical western North Pacific

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We evaluated the internal transcribed spacer 2 and large subunit ribosomal DNA (ITS2-28S) region as a molecular marker for identifying calanoid copepods in the subtropical western North Pacific. The ITS2-28S sequence was successfully amplified in 232/244 individuals, a much higher rate of success than for cytochrome oxidase I amplification (77/244 individuals). A total of 194 sequences of ITS2-28S were obtained using a single primer pair. A high degree of genetic variability in ITS2-28S was observed in *Lucicutia flavicornis* ($n = 13$), *Nannocalanus minor* ($n = 9$), *Pleuromamma abdominalis* ($n = 9$) and *Spinocalanus spinosus* ($n = 2$), each showing several sequence types based on phylogenetic analysis of the ITS2-28S. A difference in the body size that corresponded with two sequence types of *P. abdominalis* was observed. The other species showed consistently lower genetic variability within species (0–0.001) than between species (≥0.005) using ITS2-28S of 810–968 bp aligned in each family. Although further morphological and genetic analysis of a larger sample size are necessary, our results suggest that ITS2-28S is a nuclear marker that does not need to be cloned and is easily amplified and sufficiently variable as a possible marker for DNA barcoding or to identify genetic groups of calanoid copepods.

KEYWORDS: calanoid copepods; subtropical ocean; DNA barcoding; ribosomal DNA

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INTRODUCTION

The Calanoida is an order of copepods representing one of the most abundant groups of marine metazoans (Humes, 1994). Calanoid copepods are a major food source for small fish and other crustaceans and therefore play a significant role in the marine food web as secondary and tertiary producers. The taxonomy of calanoid copepods using morphological characteristics is relatively well established, compared with that of the Poecilostomatoida and Cyclopoida. Calanoid copepods are highly diverse; ~1800 species have been reported in the aquatic environment, but the actual number is much higher (Mauchline, 1998).

Copepod diversity is strongly correlated with temperature and higher diversity is observed in the oligotrophic oceans of the lower latitudes (Woodd-Walker *et al.*, 2002; Rombouts *et al.*, 2010). Oceanic taxa tend to show diversity peaks between 20° and 40°N latitudes, especially along currents such as the Kuroshio Current (Rombouts *et al.*, 2009; Tittensor *et al.*, 2010). The subtropical ocean off Southeast Asia and Japan is a hotspot with a high diversity of oceanic taxa (Tittensor *et al.*, 2010). However, copepod diversity in this region has scarcely been studied (Rombouts *et al.*, 2009). One factor hampering the study of copepods in the lower latitudes is the difficulty in morphological classification. For instance, the copepod community is dominated by small species of the genera *Clausocalanus* and *Paracalanus* in the epipelagic layer of the subtropical/tropical oceans (Schnack-Schiel *et al.*, 2010). Moreover, copepods in the mesopelagic zone are also highly diverse (Mauchline, 1998; Kosobokova and Hirche, 2000), but taxonomic information is relatively limited compared with that of copepods in the epipelagic zone. Due to the large number of species, small body size and limited amount of available morphological information, morphological classification of subtropical copepods is difficult and time consuming.

Copepods have primarily been identified on the basis of morphological differences by taxonomists or ecologists with sophisticated expertise. However, molecular techniques enable the rapid identification of copepods, even without morphological knowledge (Bucklin, 2000). The use of molecular techniques can also reveal cryptic species and reproductively isolated populations of copepods, which are difficult to identify morphologically (Lee, 2000; Goetze, 2003, 2010; Dippenaar *et al.*, 2010).

Cytochrome oxidase I (COI) and the small and large subunits of mitochondrial rRNA (12S and 16S) have commonly been used as molecular markers for identifying or determining the phylogenetic relationships of copepods (Bucklin *et al.*, 1995, 2003; Böttger-Schnack and Machida, 2010). Mitochondrial DNA is inherited maternally without recombination, and a low effective population size and rapid evolution rates make COI and mitochondrial rRNA gene sequences suitable for molecular analysis (Avise *et al.*, 1987; Birky *et al.*, 1989). However, calanoid copepods have a high level of genetic diversity (Minxiao *et al.*, 2011), and it is difficult to construct a single universal primer pair to amplify their mitochondrial genes.

Two of the nuclear rRNA subunits (18S and 28S) are considered highly conserved and have been used to determine deep phylogenic relationships among families or genera of copepods (Braga *et al.*, 1999; Bucklin *et al.*, 2003; Blanco-Bercial *et al.*, 2011). The 28S rDNA sequence is known to possess a diverse region at the 5′ end (D1/D2), suggested to be useful as a diagnostic region for species-level identification (Hassouna *et al.*, 1984; Sonnenberg *et al.*, 2007).

Kiesling *et al.* (Kiesling *et al.*, 2002) identified adult and naupliar stages of copepods in Florida Bay using DNA hybridization in the 28S region. According to Llinas (Llinas, 2008), 20 of 22 copepod species in the Chukchi and Beaufort seas were successfully classified using 28S sequence analysis. The 28S sequences also helped identify copepods living in sediments in the Antarctic region (Xu *et al.*, 2011). However, some sibling species could not be fully resolved using the 28S region, e.g. *Calanus glacialis* and *Calanus marshallae* (Llinas, 2008) and *Labidocera aestiva* and *Labidocera scotti* (Kiesling *et al.*, 2002). The internal transcribed spacer 2 (ITS2) region, located adjacent to the 5′ end of 28S, is as diverse as 28S in the genus *Neocalanus* (Machida and Tsuda, 2010). It is expected that combined sequences of ITS2 and 28S would show genetic variability with species-level resolution.

The purpose of this study was to evaluate ITS2-28S as a diagnostic region for identifying calanoid copepods from epipelagic and mesopelagic waters in the subtropical regions of the Pacific. The PCR success rate of ITS2-28S was compared with that of COI, and the sequencing success rate of ITS2-28S was estimated. In addition, the level of genetic variability of ITS2-28S was investigated within and between morphological species, and a minimum value for genetic distance was used for the identification of genetic groups in individuals that could not be fully identified morphologically. The evaluation of ITS2-28S as a new molecular marker is significant, and this study provides evidence of the utility of ITS2-28S sequences for identifying calanoid copepods in subtropical regions with high diversity.

METHOD

Sampling

Calanoid copepods were collected from three stations (St. 3, St. 7 and C3100) in the subtropical area of the western North Pacific (Fig. 1). Sampling was conducted at St. 3 (24°56.3′ N, 127°59.3′ E) and St. 7 (28°49.3′ N, 129°29.9′ E) during the KT-10-21 cruise aboard the R. V. *Tansei-Maru* of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC), on 28 and 30 September 2010, respectively. Sampling at C3100 (31°00.0′N, 137°59.9′E) was carried out during the SY-11-05 cruise aboard the R. V. *Soyo-Maru* of the National Research Institute of Fisheries Science, Fisheries Research Agency, on 9 May 2011. Only St. 7 is located in the Kuroshio Current, while the others are outside the current. Sampling was performed by vertical tows using a vertical multiple plankton sampler (VMPS; Terazaki and Tomatsu, 1997) with a 250-cm^2 mouth opening area and 100-μm mesh size. The water column was divided into sampling layers as follows: 0–200, 200–500, 500–1000 and 1000–2000 m depths at St. 3 and C3100; and 0–100, 100–200, 200–400 and 400–600 m depths at St. 7. Sampling was carried out during the daytime at St. 3 (1028–1049 GMT +9) and C3100 (0925–0956 GMT +9), while sampling at St.7 was done at night (2018–2020 GMT +9). Samples were preserved in 99% ethanol and kept at 4°C. Ethanol was replaced 24 h after sampling.

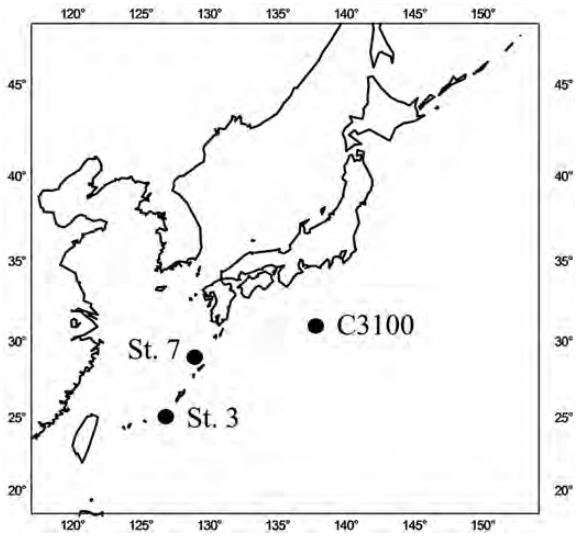

Fig. 1. Sampling locations in this study. Black dots show sampling stations.

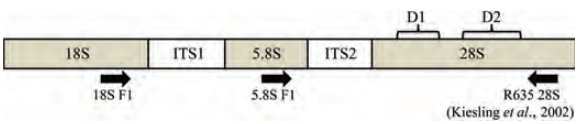

Fig. 2. Schematic illustration of primer positions (arrows) on nuclear rRNA genes.

DNA extraction and morphological classification

In this study, samples taken from St. 3 (0–200, 200–500 and 500–1000), St. 7 (0–100 m) and C3100 (0–200 and 500–1000 m) were used for further molecular and morphological study. Each sample was split into 1/8 (0–100 and 0–200 m) or 1/2 (200–500 and 500–1000 m). Adult female calanoid copepods were selected from each aliquot. Because large numbers of taxa from certain families such as Clausocalanidae and Eucalanidae were present in the samples, not all individuals were used in this study. In total, 244 adult females from various taxa were used for morphological classification and DNA analyses. Of the 244 individuals selected, 224 were classified morphologically. The remaining 20 individuals, who could not be fully identified using morphological characters, were classified to genus or family level and then by the following genetic analysis. For each individual, one of the two first antennae was used for DNA extraction and the other for morphological classification according to keys by Chihara and Murano (Chihara and Murano, 1997). In total, 20 μL of genomic DNA was extracted using the method of Böttger-Schnack and Machida (Böttger-Schnack and Machida, 2010), which was modified from Schizas *et al.* (Schizas *et al.*, 1997), and kept at −20°C.

PCR and sequencing

For amplification of ITS-28S sequence of ~1000-bp using PCR, the primers 5.8S F1 (5′-GACACTTTGA ACGCATATTGC-3′) and R635 28S (5′-GGTCCGTG TTTCAAGACGG-3′; Kiesling *et al.*, 2002) were used (Fig. 2). For species for which a clear sequence was not obtained using this primer pair, a different primer pair (18S F1: 5′-CGTCGCTACTACCGATTG-3′ and R635 28S) was used instead. The primers 5.8S F1 and 18S F1 designed for this study were based on GenBank sequences from various species of copepods (http://www.ncbi.nlm.nih.gov/genbank/index.html). For the amplification of 710 bp of COI, the metazoan universal primer pair LCO1490 (5′-GGTCAACAAATCATA AAGATATTGG-3′) and HCO2198 (5′-TAAACTTCA GGGTGACCAAAAAATCA-3′) was used (Folmer *et al.*, 1994).

PCR was carried out for 40 cycles in 15 μL reaction mixtures containing 9.95 μL distilled water, 1.50 μL

10 × buffer (Takara), 1.20 μL dNTPs (2.5 mM each), 0.60 μL of each primer (5 mM), 0.15 μL Z Taq (Takara) and 1.00 μL template DNA. The PCR cycling was performed using a Model 9700 Thermal Cycler (Applied Biosystems). PCR cycling for ITS2-28S or 18S-28S was conducted as follows: 5-s denaturation at 94°C, 5-s annealing at 60−55°C and 10-s extension at 72°C. Annealing temperature was decreased stepwise by 0.5°C from 60 to 55°C for the first 10 cycles and kept at 55°C for the following 30 cycles. An annealing temperature of 45°C was used for COI amplification over 40 cycles. The PCR protocol for COI was same as that for ITS2-28S. DNA from all 244 individuals was used for both ITS2-28S and COI amplification.

DNA amplifications were confirmed by electrophoresis on 2% L03 TBE agarose gels (Takara). "PCR success" was defined as the observation of a single band around 1000 bp (ITS2-28S) or 710 bp (COI) after staining the PCR products with ethidium bromide. The results of PCR were compared between ITS2-28S and COI amplifications. PCR products of ITS2-28S were purified with ExoSap-IT (GE Health BioScience), sequenced directly using dye-labeled terminators and analyzed on a 3130 DNA Sequencer (Applied Biosystems). Sequencing reactions were performed according to the manufacturer's protocol using the primer pair 5.8S F1 and R635 28S.

Genetic distances

Consensus sequences of ITS2-28S were generated for each individual after assembly of forward and reverse sequences using Geneious version 5.3.4 (Biomatters Ltd; Drummond *et al.*, 2010). We sequenced both the forward and reverse strands to confirm the sequences. The level of genetic variability of ITS2-28S sequences was evaluated within each family. Multiple sequences in each family were aligned using Clustal X (Thompson *et al.*, 1997), and the aligned sequences were then edited in SeaView version 4 (Gouy *et al.*, 2010). Internal variations in ITS and 28S have been observed among rDNA gene repeats (Long and Dawid, 1980). ITS and 28S are multi-copy nuclear genes, and clear double-base calls were observed in one individual. In this study, we defined equivalent double peaks and double peaks, which indicate intra-species dissimilarity as ambiguous bases, and sites containing such ambiguous bases were removed from the sequence analysis to detect genetic groups which were reproductively isolated in natural condition. In total, 194 reliable ITS2-28S sequences (GenBank accession number: AB753519-AB753715) were obtained for further analysis (Table I). Mismatches caused by gaps (indels) and ambiguous bases were removed for calculating genetic distances and phylogenetic analyses. All ambiguous sites for each species were counted (Supplementary data).

Genetic distances within and between species were calculated for ITS2-28S in each family using Kimura's two-parameter (K2P) model (Kimura, 1980), the model generally used to calculate genetic distance. Aligned sequence length ranged from 810 to 968 bp after removing indels and ambiguous sites (Table II). A threshold value of 0.005 for genetic distance was used to identify genetic groups in individuals that could not be identified to species level by morphological analysis. Both morphological species and genetic groups were used in calculating genetic distances and the following phylogenetic analyses.

Phylogenetic analyses

Phylogenetic analyses were performed using maximum likelihood (ML) and Bayesian inference (BI) using GTR + G + I, which was selected as the best-fit nucleotide substitute model based on the Akaike Information Criterion in MrModeltest 2.3 (Nylander, 2004). Only *Acartia negligens* was excluded from the phylogenetic analysis, owing to its excessively high variance. Only 28S sequences (587 bp after the removal of indels and ambiguous sites) were used for this phylogenetic analysis because of the frequent occurrence of insertions and deletions in ITS2 when comparing sequences among various families. The ML analysis was performed using 1000 bootstrap replications for nodal support in MEGA version 5.0 (Tamura *et al.*, 2011). The Bayesian analysis was performed in MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). The Markov Chain Monte Carlo chains were run for 10^6 generations and trees were sampled every 100 generations. The first 25% of the generated trees were eliminated as burn-in. Three non-calanoid copepods (*Oithona* sp., *Oncaea* sp. and *Corycaeus* sp.) were used as the outgroup.

Four species (*Nannocalanus minor*, *Lucicutia flavicornis*, *P. abdominalis* and *Spinocalanus spinosus*) showed relatively high genetic variability within species. Phylogenetic analyses (ML and BI) were performed for these species using ITS2-28S sequences, following the procedure described above. The best-fit substitution models were GTR + G + I for Calanidae, HKY + G for Metridiniidae and GTR + G for Spinocalanidae and Lucicutiidae. *Mecynocera clausi* was used as the outgroup for Calanoida, *Haloptilus chirchiae* for Metrididae and Lucicutiidae, and *Euchaeta indica* for Spinocalanidae. In addition, each of the four species was further analyzed to detect the morphological variation within species. Body sizes were compared among samples collected at

Table I: List of species collected in this study

Taxon	Species	Sampling site (depth)	Total	PCR success		ITS2-28S sequence	Ambiguous base
				ITS2-28S	COI		
Augaptiloidea							
Augaptilidae	*Haloptilus chierchiae*	St. 3 (500–1000)	1	1	0	1	0
Metridinidae	*Metridia brevicauda*	St. 3 (500–1000)	3	2	0	2	0
	Metridia princeps	C3100 (500–1000)	1	1	0	1	1
	Metridia venusta	C3100 (500–1000)	4	4	0	1	0
	Pleuromamma abdominalis	St. 3 (200–500; 500–1000), C3100 (500–1000)	14	14	5	9	0
	Pleuromamma gracilis	St. 3 (200–500)	10	9	3	3	1
	Pleuromamma xiphias	St. 3 (200–500; 500–1000)	6	6	4	4	1
Lucicutiidae	*Lucicutia flavicornis*	St. 3 (0–200; 200–500), C3100 (0–200)	23	21	7	13	2
	Lucicutia curta	St. 3 (500–1000)	1	0	0	x	x
Centropagoidea							
Acartiidae	*Acartia danae*	St. 3 (0–200)	1	0	0	x	x
	Acartia negligens	St. 3 (0–200), C3100 (0–200)	2	2	1	2	5
Candaciidae	*Candacia curta*	St. 3 (0–200), St.7 (0–100)	3	3	3	3	1
	Paracandacia bispinosa	C3100 (0–200)	1	1	1	x	x
	Paracandacia simplex	St. 3 (0–200)	1	1	1	x	x
	Paracandacia truncata	St. 7 (0–100)	1	1	0	x	x
Centropagidae	*Centropages calaninus*	St. 7 (0–100)	1	0	0	x	x
	Centropages gracilis	St. 7 (0–100)	2	1	0	1	0
Pontellidae	*Labidocera detrancata*	St.7 (0–100)	1	1	1	1	0
	Pontellina plumata	St.7 (0–100)	2	2	1	1	2
Temoridae	*Temora discaudata*	St. 3 (0–200), C3100 (0–200)	2	2	0	1	1
	Temora turbinata	C3100 (0–200)	2	2	1	2	0
Clausocalanoidea							
Aetideidae	*Aetideus acutus*	St. 3 (0–200), C3100 (0–200)	3	3	1	2	1
	Euchirella curticauda	St. 3 (500–1000)	2	2	2	2	1
	Euchirella messinensis	St. 7 (0–100), C3100 (500–1000)	2	2	1	2	0
	Euchirella pulchra	St. 3 (500–1000)	1	1	1	1	1
	Gaetanus armiger	St. 3 (500–1000)	1	1	0	1	1
	Gaetanus minor	C3100 (500–1000)	1	1	0	1	0
	Undeuchaeta major	St. 7 (0–100), C3100 (500–1000)	2	2	1	2	1
	Undeuchaeta plumosa	St. 3 (500–1000), C3100 (500–1000)	5	5	5	5	1
Clausocalanidae	*Clausocalanus farrani*	St. 7 (0–100)	1	1	0	1	0
	Clausocalanus lividus	St. 3 (0–200), St.7 (0–100), C3100 (0–200)	8	8	0	7	3
	Clausocalanus minor	St. 3 (0–200), St.7 (0–100)	10	10	0	8	0
	Ctenocalanus vanus	St. 3 (0–200), C3100 (0–200)	19	19	0	17	2
Euchaetidae	*Euchaeta indica*	St. 3 (0–200)	1	1	0	1	0
	Euchaeta rimana	C3100 (0–200)	2	2	0	2	0
	Paraeuchaeta media	St. 3 (200–500)	4	3	0	3	0
Phaeniidae	*Phaenna spinifera*	St.7 (0–100)	1	1	0	1	0
Scolecitrichidae	*Scaphocalanus echinatus*	St. 3 (500–1000)	1	1	0	1	0
	Scolecithrix danae	St.7 (0–100)	4	4	0	3	1
	Scottocalanus securifrons	C3100 (500–1000)	1	1	0	1	0
	Scaphocalanus sp.[a]	St. 3 (500–1000)	1	1	0	1	0
	Scolecitrichidae G1[a]	St. 3 (500–1000)	1	1	1	1	0
	Scolecitrichidae G2[a]	St. 3 (500–1000)	1	1	1	1	0
	Scolecitrichidae G3[a]	St. 3 (200–500)	1	1	1	1	0
	Scolecitrichidae G4[a]	St. 3 (200–500)	1	1	1	1	0
	Scolecitrichidae G5[a]	St. 3 (200–500)	1	1	1	1	0
Eucalanoidea							
Eucalanidae	*Eucalanus californicus*	St. 3 (200–500; 500–1000)	7	7	6	7	1
	Eucalanus sp.	C3100 (500–1000)	1	1	0	1	1
	Pareucalanus parki	St. 3 (500–1000), C3100 (0–200; 500–1000)	4	4	3	4	0
	Pareucalanus sp.	St. 3 (0–200), C3100 (0–200)	2	2	0	2	0
	Rhincalanus rostrifrons	C3100 (500–1000)	1	1	0	1	0
	Rhincalanus nasutus	St. 3 (500–1000), C3100 (500–1000)	5	5	5	5	1
	Subeucalanus subtenuis	St. 3 (0–200), C3100 (0–200)	4	4	0	4	0
Megacalanoidea							
Calanidae	*Cosmocalanus darwini*	St. 7 (0–100)	6	5	5	5	3
	Nannocalanus minor	St. 3 (0–200), St.7 (0–100), C3100 (0–200)	9	9	6	9	1

Continued

Table I: Continued

Taxon	Species	Sampling site (depth)	Total	PCR success		ITS2-28S sequence	Ambiguous base
				ITS2-28S	COI		
	Calanus sinicus	St. 3 (0–200), C3100 (0–200)	12	12	0	11	6
	Neocalanus gracilis	St. 3 (0–200; 500–1000)	4	4	4	4	0
	Neocalanus robustior	C3100 (0–200)	1	1	0	1	0
	Mesocalanus lighti	St. 3 (0–200)	1	1	0	1	0
Calocalanidae	*Calocalanus* G1[a]	C3100 (0–200)	1	1	0	1	0
	Calocalanus G2[a]	St. 3 (0–200)	1	1	0	1	0
	Calocalanus G3[a]	C3100 (0–200)	1	1	0	1	0
	Calocalanus G4[a]	C3100 (0–200)	1	1	0	1	0
Mecynoceridae	*Mecynocera clausi*	St. 3 (0–200), St.7 (0–100), C3100 (0–200)	7	7	4	7	0
Paracalanidae	*Paracalanus denudatus*	St. 3 (0–200)	2	2	0	2	0
	Paracalanus parvus	St. 3 (0–200), St.7 (0–100)	3	2	0	2	3
	Paracalanus sp.[a]	St. 3 (0–200), C3100 (0–200)	2	2	0	2	0
	Paracalanidae G1[a]	St. 3 (0–200)	2	2	0	2	0
	Paracalanidae G2[a]	St. 3 (0–200)	1	1	0	1	0
	Paracalanidae G3[a]	C3100 (0–200)	1	1	0	1	0
	Paracalanidae[b]	St. 3 (0–200)	1	0	0	x	x
Spinocalanoidea							
Spinocalanidae	*Spinocalanus angusticeps*	St. 3 (500–1000)	1	1	0	1	0
	Spinocalanus longicornis	St. 3 (500–1000)	1	1	0	1	0
	Spinocalanus spinosus	St. 3 (500–1000)	2	2	0	2	0
	Spinocalanidae G1[a]	St. 3 (500–1000)	1	1	0	1	0
	Spinocalanidae G2[a]	St. 3 (500–1000)	1	1	0	1	0
	Spinocalanidae[b]	St. 3 (500–1000)	1	1	0	0	0

Numbers of individuals analyzed, PCR success of ITS2-28S, PCR success of COI, obtained ITS2-28S sequences, and observed ambiguous bases are shown for both morphological species and genetic groups.
x, No clear sequence obtained.
[a]Genetic groups separated using the calculated minimum K2P genetic distance (0.005).
[b]Unidentified individuals, both morphologically and molecularly.

the same site to avoid the effects of temperature or food availability.

RESULTS

PCR and sequencing

Individual Calanoid copepods (244), representing 19 families, 35 genera and 59 species based on morphology were collected (Table I). Twenty individuals could not be identified to species level, mainly owing to the poor condition of the samples. ITS2-28S sequences (~1000 bp) were successfully amplified for 232 of the 244 samples (95.1% success rate). The samples for which ITS2-28S could not be amplified did not all belong to a single calanoid family. COI sequences (~700 bp) were successfully amplified for 77 of the 244 samples (31.6% success rate). COI could not be amplified for any of the samples from some families, such as Clausocalanidae, despite the large numbers of samples analyzed.

In total, 194 ITS2-28S sequences were obtained, with a success rate of 79.5%. In *Lucicutia curta*, *Acartia danae*, *Paracandacia bispinosa*, *Paracandacia simplex*, *Paracandacia truncata* and *Centropages calaninus*, no clear sequences of ITS2-28S were obtained, despite at least three sequencing trials per sample (Table I).

Genetic distance between species

In all families, genetic distance was observed between species (Table II), and the amount of divergence between species varied in each family. The least genetic distance (0.005) was observed between *P. abdominalis* and *Pleuromamma xiphias*. Therefore, a value of 0.005 for genetic distance was set as the threshold for discriminating between genetic groups in this study. On the basis of this criterion, 20 morphologically unidentified individuals were found to belong to 16 genetic groups. These genetic groups are shown in Table I (e.g. Paracalanidae G1). Individuals of *Eucalanus* sp. and *Pareucalanus* sp. were distinguished based on the morphology, using specimens in the same family, and treated as morphologically identified species. ITS2-28S sequences were obtained for 53 morphological species and 16 genetic groups.

According to the phylogenetic analysis of 28S, most species and genetic groups were grouped into clades

Table II: Comparisons of genetic distances between/within species in each family based on ITS2-28S sequences

Family	No. of species and groups	No. of samples	Length (bp)	Genetic distance	
				Within species and group	Between species and group
Acartiidae	1	2	918	0	No data
Aetideidae	8	16	860	0	0.008–0.097
Augaptilidae	1	1	890	No data	No data
Calanidae	6	31	883	0 (0.01–0.021)[a]1	0.016–0.114
Calocalanidae	4	4	856	No data	0.106–0.139
Candaciidae	1	3	969	0	No data
Centropagidae	1	1	882	No data	No data
Clausocalanidae	4	33	889	0–0.001	0.010–0.061
Eucalanidae	7	24	863	0	0.018–0.179
Euchaetidae	3	6	917	0	0.035–0.062
Lucicutiidae	1	13	841	(0.005–0.014)[a]2	No data
Mecynoceridae	1	7	903	0	No data
Metridinidae	6	20	874	0 (0.002)[a]3	0.005–0.049
Paracalanidae	6	10	810	0–0.001	0.005–0.118
Pheannidae	1	1	910	No data	No data
Pontellidae	2	2	903	No data	0.22
Scolecitrichidae	9	11	848	0	0.009–0.094
Spinocalanidae	5	6	835	(0.004)[a]4	0.009–0.146
Temoridae	2	3	864	0	0.142

Length (bp) is the number of base pairs after alignment and removal of sites containing indels and ambiguous bases in each family.
[a]Species with large genetic distances within species (1, *Nannocalanus minor*; 2, *Lucicutia flavicornis*; 3, *Pleuromamma abdominalis*; 4, *Spinocalanus spinosus*). Genetic distances within these four species are shown in parentheses.

with high bootstrap and posterior probability values that followed the morphological classification (Fig. 3). The families Aetideidae, Calanidae and Pontellidae were not supported with sufficient bootstrap and posterior probability values. Each family was also grouped into six superfamilies with high bootstrap and posterior probability values, although the relationship between Spinocalanoidea and Clausocalanoidea was not fully resolved. Families in the Centropagoidea, including Acartiidae, were highly divergent from other taxa. *Acartia negligens* was positioned outside the outgroups *Corycaeus* sp., *Oncaea* sp. (Poecilostomatoida) and *Oithona* sp. (Cyclopoida) and was therefore eliminated from the phylogenetic tree.

Genetic distance within species

Species in Clausocalanidae and Paracalanidae displayed genetic variability of only 0.001; no variability was observed within most species (Table II). However, sample sizes were not sufficient to obtain an exact measurement of the genetic variability within species. The number of ambiguous sites ranged from 0 to 6 (Table I). Maximum ambiguous sites (six) were observed in *Calanus sinicus*, followed by *A. negligens* (five ambiguous sites). Most species and genetic groups showed 0–3 ambiguous sites, though this number depended on sample size. Genetic distances between species ($\geq$0.005) were larger than within species, except for *N. minor* and *L. flavicornis* (Fig. 4 and Table II). Greater genetic distances were observed in *N. minor* (0.010–0.021), *L. flavicornis* (0.005–0.014) and *S. spinosus* (0.004) than in *P. abdominalis* (0.002), which showed morphological differences between sequence types. Samples of *N. minor* could be divided into three types according to the ITS2-28S phylogenetic tree for the family Calanidae (Fig. 5a). Three types of *N. minor* and *Cosmocalanus darwini* formed monophyletic clade with high bootstrap values and posterior probability values (82 and 100% each), although their relationships were not fully resolved in this study. There was no genetic variability within each sequence type. Samples of *P. abdominalis* were classified into two types (Fig. 5b). These two groups were within a clade with 77% bootstrap and 99% posterior probability support. The mean prosomal lengths were 19.6 $\pm$ 0.5 mm (mean $\pm$ S.D.) in *P. abdominalis* 1 ($n = 5$) and 21.3 $\pm$ 1.2 mm in *P. abdominalis* 2 ($n = 3$). *Pleuromamma abdominalis* 2 was significantly larger than *P. abdominalis* 1 at St. 3 ($P < 0.05$; *t*-test). Samples of *L. flavicornis* could also be divided into four types separated by genetic distances of 0.005–0.014 (Fig. 5c) by ITS2-28S. A genetic distance of 0.004 was calculated between 2 *S. spinosus* individuals, which formed a monophyletic clade supported by 99% bootstrap and posterior probability values (Fig. 5d).

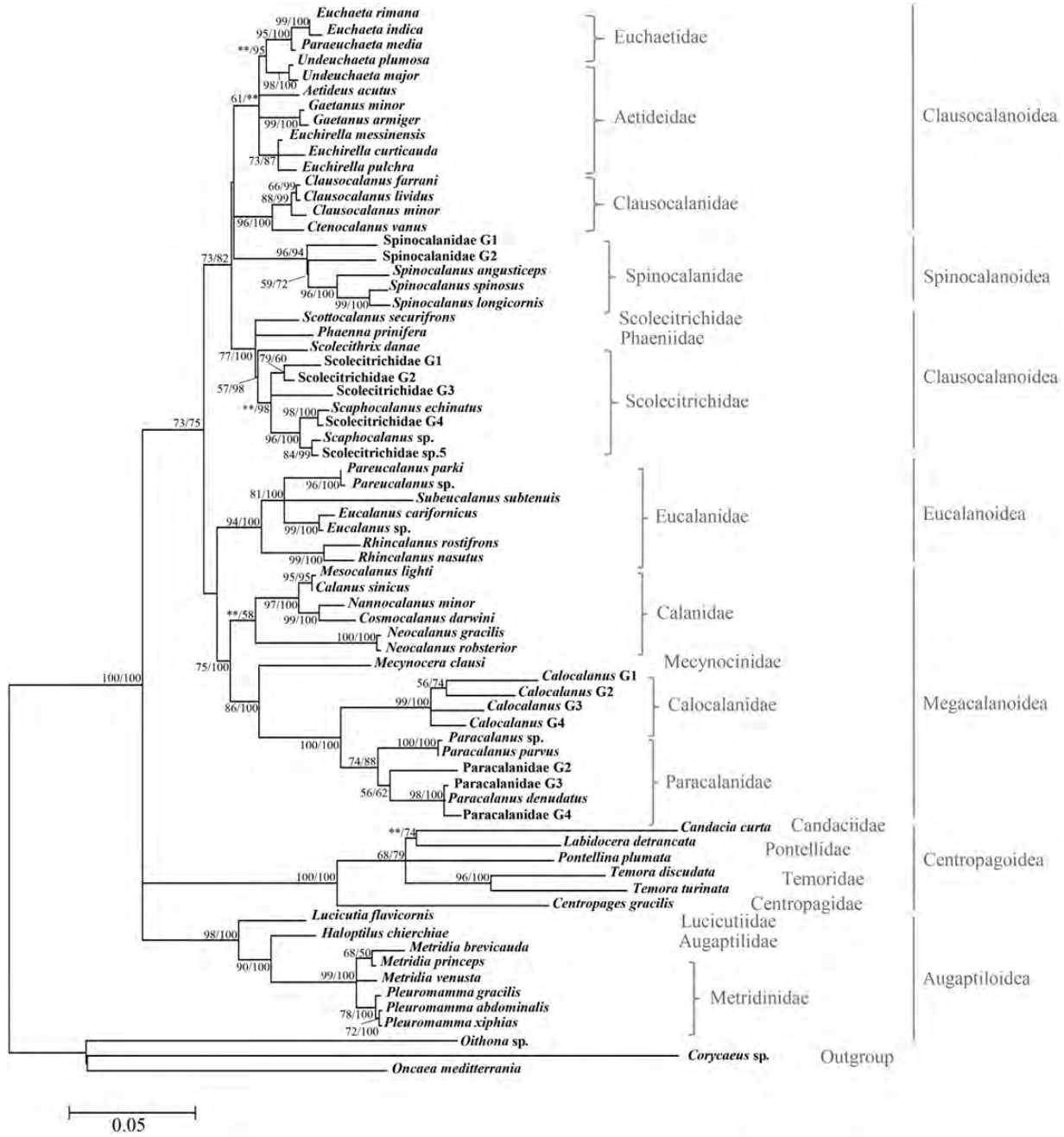

Fig. 3. ML 50% majority-rule consensus tree based on 28S sequences of 52 species and 16 genetic groups of calanoid copepods (587 bp). Scale bar indicates genetic distances. Numbers at the nodes indicate the bootstrap values for ML (left) and posterior probabilities for Bayesian analyses (right) >50%. Asterisks indicate the values <50%. The sequence of *A. negligens* was too divergent and was removed from this analysis.

DISCUSSION

Suitability of ITS2-28S as a molecular marker

Our method using ITS2-28S sequences achieved high rates of PCR and sequencing success. ITS2-28S was found to be sufficiently divergent at the species level and can be used as a molecular maker for DNA barcoding of calanoid copepods in the subtropical western North Pacific. The variability of ITS2-28S was also helpful in identifying genetic groups. ITS2-28S had a much higher PCR success rate than the COI region. The ITS2-28S

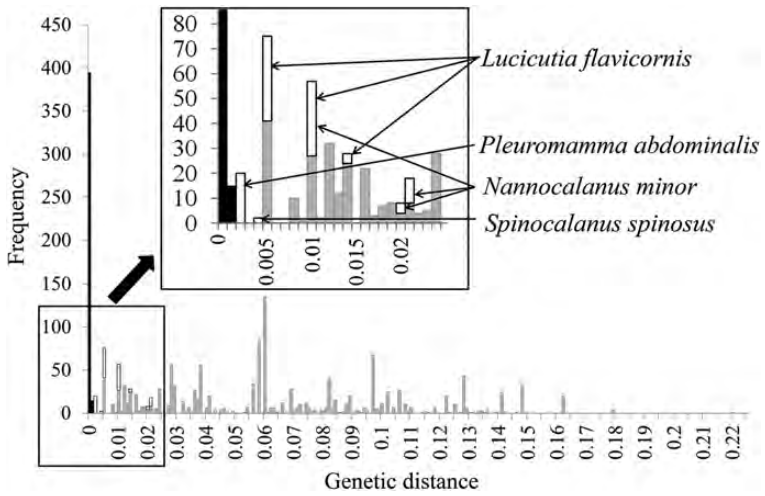

Fig. 4. Pairwise K2P genetic distances of calanoid copepods within and between species. K2P genetic distances within species are shown by black bars and those between species by gray bars. White bars represent species with high genetic variability (*N. minor*, *L. flavicornis*, *P. abdominalis* and *S. spinosus*).

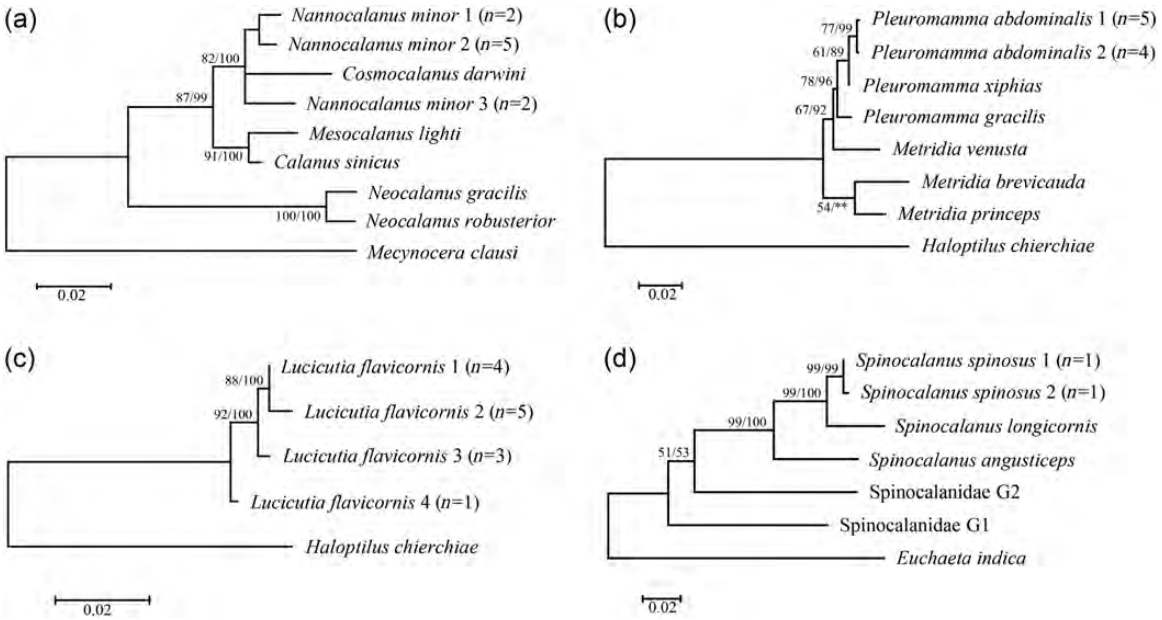

Fig. 5. ML 50% majority-rule consensus trees of family (**a**) Calanidae, (**b**) Metridinidae, (**c**) Lucicutiidae and (**d**) Spinocalanidae. Numbers at the nodes indicate the bootstrap values for ML (left) and posterior probabilities for Bayesian analyses (right) which are >50%. Asterisks indicate values <50%. Scale bars indicate genetic distances. The number of analyzed individuals (*n*) has been added to each sequence type. No genetic variation was observed within each group. *Mecynocera clausi* is the outgroup for Calanoidae, *H. chirchiae* for Metrididae and Lucicutiidae and *Euchaeta indica* for Spinocalanidae.

primer pair used in this study was designed to amplify the highly conserved regions of 5.8S and 28S rDNA, resulting in a high PCR success rate. COI was shown to be much more divergent than ITS or 28S in the genus *Neocalanus* (Machida and Tsuda, 2010). In this study, COI could only be amplified for 77 individuals, and the PCR results were inconsistent even within a single species. It may be difficult to construct universal primers for amplifying diverse mitochondrial genes (including COI), especially in the highly diverse subtropical calanoid copepods. Another study of copepods showed that COI cannot to be amplified consistently using a universal primer pair (Cepeda *et al.*, 2012). Blanco-Bercial and Álvarez-Marqués (Blanco-Bercial and Álvarez-Marqués, 2007) used primers specific to *Clausocalanus* spp. to amplify COI sequences. The family Clausocalanidae

could not be amplified in our study, suggesting it would be necessary to construct various COI primer pairs for PCR of subtropical copepods with high species diversity.

ITS2-28S sequences were not obtained for six of the species sampled. According to Lindeque *et al.* (Lindeque *et al.*, 2006), the amplification rate is low when samples are not well preserved in ethanol. In our study, DNA was extracted from all samples within 1 year, and DNA quality should not have been a significant problem. However, both PCR success and failure were observed for the same species, which might indicate that the DNA extraction procedure affected our PCR results. Primer mismatch in specific taxa is a more likely cause of PCR failure, because sequencing failures were observed mainly in the species belonging to the superfamily Centropagoidea. According to a 28S phylogenetic analysis of Centropagoidea, the species are highly genetically divergent within the superfamily and different from other superfamilies; this finding has also been reported in studies using multiple-gene analyses (18S, 28S, COI and Cyt b) of the calanoid copepods (Blanco-Bercial *et al.*, 2011). The primer pair used in this study was designed for all calanoid copepods, and the high level of divergence in the Centropagoidea may have led to the low sequencing success rate.

A total of 194 ITS2-28S sequences from the 244 individuals were used for sequencing analysis (79.5% success rate). In a similar study, an 85% success rate (>1550 specimens from 1800 individuals) was achieved by cloning and sequencing the 28S region of copepod nauplii, including non-calanoid copepods in the Arctic Ocean (Llinas, 2008). However, the number of species of copepods was much higher in this study than in the report by Llinas (Llinas, 2008), and our success rate could be improved by designing specific primers for the Centropagoidea.

28S phylogeny

A 28S phylogenetic analysis of 52 species and 16 genetic groups (excluding *A. negligens*) of calanoid copepods from the subtropical ocean showed a high degree of similarity to classification based on morphological characters. A similar result was reported using 19 species of calanoid copepods from Florida Bay (Kiesling *et al.*, 2002). Therefore, 28S sequences reflected the phylogenetic relationships of calanoid copepods in this study. Blanco-Bercial *et al.* (Blanco-Bercial *et al.*, 2011) conducted phylogenetic analyses of calanoid copepods using multiple genes (18S, 28S, COI and Cyt *b*) and obtained higher bootstrap support values than our study, suggesting multiple-gene analysis could be used to gain a deeper understanding of the phylogenetic relationships of the calanoid copepods in this study. Although ITS2 was highly divergent and suitable for differentiating between species, the alignment contained many indels, indicating its unsuitability for phylogenetic analysis of multiple families.

Patterns of genetic variability vary in each taxon, e.g. genetic distance between species was lower in Metridinidae than in other families. Calocalanidae and Spinocalanidae showed relatively high genetic variability. There was high genetic variability, in particular, in families in the Centropagoidae, as discussed above. *Acartia negligens* was positioned outside the outgroup in the 28S phylogenetic tree. A similar analysis conducted using 18S sequence data from GenBank showed the same results as 28S, and in this analysis, some *Acartia* species were even positioned outside of *Euphausia pacifica* (Malacostraca). According to Kiesling *et al.* (Kiesling *et al.*, 2002), three *Acartia* species (*Acartia longiremis*, *Acartia tonsa* and *Acartia* sp.) were included in the order Calanoida based on 28S sequences, even though high levels of genetic variability were observed and phylogenic relationships were not resolved. The genus *Acartia* may have a more rapid rate of evolution in the 28S region than other copepods (Kiesling *et al.*, 2002), and further investigation is required. The high level of divergence of the Centropagoidae is considered to reflect the complex evolutionary history of this taxon (Adamowicz *et al.*, 2010)

Genetic variability between species

The variability of ITS2-28S was sufficient to identify all calanoid copepod species in this study. Calanoid copepod species (157) were reported in epipelagic and mesopelagic waters in Sagami Bay (Shimode *et al.*, 2006) and 86 species in the epipelagic water in the Taiwan Strait (Lee *et al.*, 2009), where sampling stations are close to those used in this study. Although not all calanoid copepods were included in this study, our results show that ITS2-28S could be used for species identification of calanoid copepods in various genera or families. This finding suggests that the ITS2-28S region should be variable in other calanoid copepods. In the subarctic ocean, three closely related species of *Neocalanus* (*Neocalanus cristatus*, *Neocalanus flemingeri* and *Neocalanus plumchrus*) were shown to be identified by ITS or 28S sequences by both Llinas (Llinas, 2008) and Machida and Tsuda (Machida and Tsuda, 2010).

The 5′ end of the 28S region (D1/D2) is both divergent and suitable for species identification in various taxa (Sonnenberg *et al.*, 2007); however, ITS2 sequences were more diverse than 28S sequences in most calanoid copepods in this study. This result corresponds with the

results of Machida and Tsuda (Machida and Tsuda, 2010), which revealed higher genetic variability in ITS2 than in 28S in the genus *Neocalanus*. Goetze (Goetze, 2003) mentioned the utility of ITS2 as an informative site for species identification in copepods. ITS regions have recently been used for species identification or phylogenetic analysis of copepods in specific families or genera (Nonomura *et al.*, 2008; Malgorzata *et al.*, 2009; Wyngaard *et al.*, 2010). However, no clear phylogenetic relationship was reflected in the ITS2 phylogenetic tree when the phylogeny included various families, because of frequent occurrences of insertions and deletions in ITS2. 28S sequences can be used to infer the phylogenetic relationships in copepods, as shown in this and other studies (Kiesling *et al.*, 2002; Song *et al.* 2008). The combination of the ITS2 and 28S is considered to be well suited to species-level analysis and determination of phylogenetic relationships.

Genetic variation within species

In this study, families with a small body size and short generation time (Clausocalanidae and Paracalanidae) showed only low levels of genetic diversity within species (0.001), and other larger species with a longer generation time tended to have much lower variability (0% in this study). Higher levels of intraspecific genetic variation have also been reported in small copepods with shorter generation times than in large copepods (Llinas, 2008). In our study, no intraspecific genetic variability was detected in most species, but other studies have shown some genetic variability in ITS2 or 28S within species (Llinas, 2008; Machida and Tsuda, 2010). This discrepancy in results may be due to our method of eliminating ambiguous bases derived from variation among rDNA repeats. According to Sonnenberg *et al.* (Sonnenberg *et al.*, 2007), intra-individual genetic variation in the 28S D1/D2 regions is $<0.1\%$ on average in most metazoan taxa. However, Llinas (Llinas, 2008) reported the level of intraspecific variability in 28S D1/D2 was mostly lower than 0.5% in copepods. The method used in Llinas (Llinas, 2008) is different from the one used in this study; the differing results for intraspecific genetic variability might be due to the dissimilarity within rDNA genes, owing to tandem repeats in the genome. The least genetic distance between species in this study was 0.005 (between *P. abdominalis* and *P. xiphias*), which was higher than the within-species genetic variability. In addition, the hundreds of copies of ribosomal genes in a genome typically evolve in concert (Elder and Turner, 1995). The numbers of observed ambiguous sites were not large in this study, suggesting low genetic variability within a single species. The genetic distance of 0.005 was used as a threshold to discriminate between genetic groups of copepods which suggested a possibility of biological species. However, our sample sizes were insufficient to assess exact intraspecific and interspecific variability, and greater sampling effort is required for further understanding of genetic variability within species. In addition, some specimens may show levels of genetic variability between 0.001 and 0.005, such as *P. abdominalis* and *S. spinosus* in this study. For these specimens, further molecular and morphological study would be suggested.

In this study, no geographically based genetic variation within morphological species was observed between different sampling sites. Distances among our sampling sites were not large, and there may be no reproductive isolation. According to Goetze (Goetze, 2003), both mitochondrial and nuclear molecular markers showed geographically based genetic variation in the family Eucalanidae among different ocean basins, suggesting geological isolation of pelagic copepods. ITS2-28S is a highly variable nuclear marker and may be suitable for detecting geographically isolated genetic groups within species. Our sampling was restricted in the subtropical western North Pacific off Japan. To study geographical differences, greater sampling effort covering a wide area is required.

ITS2-28S revealed that the genetic difference (0.002) between samples of *P. abdominalis* was clearly coincident with body size. Two nucleotide differences were observed in 28S between the large and small forms, and there was no difference in ITS2 in this study. A similar study conducted in *P. xiphias* using COI and ITS2 showed no genetic variability in ITS2; *P. xiphias* was grouped into four clades by COI, and body size was related to the teo major clades (Goetze, 2011). In *L. flavicornis*, our results showed four sequence types of ITS2-28S sequence with relatively large genetic distances (0.005–0.014), greater than the minimum genetic distance between morphological species in this study, suggesting that the groups are reproductively isolated. In *N. minor*, three sequence types with large genetic distances, higher than between *Calanus sinicus* and *Mesocalanus lighti*, were observed. In the genus *Nannocalanus*, *Nannocalanus elegans* was reported from the Southeastern Pacific (Andronov, 2001). Our specimens of *N. minor* were not clearly classified into *N. elegans*. Two forms of *N. minor*, i.e. *major* and *minor*, were reported on the basis of body size (Toda, 1986). However, body size could not be successfully compared among our genetic groups because of the lack of a sufficient number of

samples. Similarly, Bucklin *et al*. (Bucklin *et al*., 1996) reported two genetically different types of *N. minor* using 16S rDNA sequences and showed high levels of genetic variation between them. In addition, two major haplotypes were observed in samples of the large form by Bucklin *et al*. (Bucklin *et al*., 1996). ITS2-28S was shown to be variable between species in our study, suggesting *N. minor* may be composed of at least three reproductively isolated populations in the subtropical western North Pacific.

The taxonomy of the family Spinocalanidae is not well established; morphological classification of this family is difficult because the individuals have fragile bodies. Genetic variation within *S. spinosus* was observed only in ITS2, not in 28S, which is a similar pattern to *Neocalanus flemingeri*, which shows the genetic variation in ITS between the large and small forms (Machida and Tsuda, 2010). For four morphological species (*P. abdominalis*, *L. flavicornis*, *N. minor* and *S. spinosus*), further morphological and molecular study using independent mitochondrial molecular markers is needed to reveal a possibility of cryptic species.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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