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Large-scale gene rearrangements in the mitochondrial genomes of two calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus* (Crustacea), with notes on new versatile primers for the srRNA and COI genes

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Abstract

Partial sequences of the mitochondrial genomes were determined for two calanoid copepods, *Eucalanus bungii* [9530 bp (base pairs)] and *Neocalanus cristatus* (7965 bp), using an approach that employs a long polymerase chain reaction (PCR) technique and primer walking. In contrast to *Tigriopus japonicus* (Harpacticoida), in which the complete mitochondrial genome has been determined, genes are encoded on both strands of the genome. In spite of the close relationship between *E. bungii* and *N. cristatus*, the gene orders of these calanoid copepods differed greatly from each other. From the sequence we obtained, new copepod-specific versatile primers were designed for the cytochrome *c* oxidase I (COI) gene (1200 bp). These primer pairs, as well as the previously designed primer pairs for the small ribosomal RNA (srRNA) gene (500 bp), were applied for 20 species representing four orders of both calanoid and non-calanoid copepods and amplification of sequences from at least one species from each order was successful.

Keywords: Evolutionary rate; Versatile primer; Harpacticoida; Cyclopoida; Poecilostomatoida; Calanoida

1. Introduction

The Copepoda, one of the infraclasses of the phylum Crustacea, are among the most numerically abundant metazoans on the Earth. They comprise 11,500 nominal species placed in about 200 families and 1650 genera, although this figure may represent only 15% of the actual number of species (Mauchline, 1998). They are found in various aquatic habitats from freshwater to marine or hypersaline inland waters, and from subzero polar waters to hot spring temperature regimes (Huys and Boxall, 1991). They also have an immense vertical range, occurring from depths of >10,000 m in the Philippine Trench to an altitude of 5540 m in the Himalayan mountains (Huys and Boxall, 1991). For a better understanding as to how copepods acquired such an immense diversity in the aquatic realm, phylogenetic relationships need to be clarified before any evolutionary inferences are made.

Mitochondrial DNA is a circular molecule of approximately 15–17 kilobases in length, which encodes genes for 13 proteins, 22 transfer RNAs, and 2 ribosomal RNAs. Because of the lack of intermolecular recombination, its maternal inheritance, the relatively rapid evolutionary rate, and the large numbers of copies, mitochondrial DNA has been widely used for analyses of metazoan phylogenetic relationships at various taxonomic levels (Avise, 2000).

Abbreviations: ATPase6 and 8, ATPase subunits 6 and 8; bp, base pair (s); COI–III, cytochrome *c* oxidase subunits I–III; CR, control region; cyt *b*, cytochrome *b*; ND1–6 and 4L, NADH dehydrogenase subunits 1–6 and 4L; PCR, polymerase chain reaction; srRNA and lrRNA, small and large subunits ribosomal RNA; tRNA, transfer RNA.

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Recently, polymerase chain reaction (PCR)-based approaches for sequencing complete mitochondrial DNA sequences has been demonstrably useful in vertebrates such as fishes (Miya and Nishida, 1999) and birds (Sorenson et al., 1999). For example, Miya et al. (2001, 2003) have determined the complete mitochondrial DNA sequences for more than 100 teleost fish species using this approach.

Mitochondrial DNA has also been used in phylogenetic analyses of the copepods (e.g. Bucklin et al., 2003; Braga et al., 1999). Braga et al. (1999) analyzed phylogenetic relationships of the Euchaetidae using 300 bp of the mitochondrial lrRNA gene and proposed that the ancestral species for the Euchaetidae was a deep-living, vertically migrating copepod. Bucklin et al. (2003) determined the phylogenetic relationships of 34 calanoid species belonging to 10 genera and 2 families using 693 bp of the cytochrome *c* oxidase I (COI) mitochondrial gene.

So far, a complete mitochondrial DNA sequence has only been determined for *Tigriopus japonicus* (Harpacticoida) (Machida et al., 2002). In this study, we attempted to determine the complete mitochondrial DNA sequences for two calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus* that dominate in the subarctic Pacific. *E. bungii* and *N. cristatus* are major grazing zooplankters in the subarctic Pacific and various aspects of their biology have been studied, including life histories (e.g. Miller and Clemons, 1988), early development (Saito and Tsuda, 2000), feeding (e.g. Dagg, 1993), and distributions (e.g. Tsuda et al., 2000). We have employed the long PCR and the primer walking technique. Although, we were unable to determine the complete mitochondrial DNA sequence, some peculiar characteristics of calanoid mitochondrial DNA were demonstrated.

2. Materials and methods

2.1. Copepod samples and DNA extraction

Specimens of *E. bungii* and *N. cristatus* were collected from Sagami Bay (35°00′N, 139°20′E), central Japan, using a large-conical plankton net (ORI net; mouth diameter, 1.6 m) during the cruise KT-00-10 of the R/V Tansei Maru, Ocean Research Institute, University of Tokyo. Specimens were sorted out for genomic extraction immediately after collection. Total genomic DNA was extracted from whole copepods using a DNeasy kit (Qiagen) following the manufacturer's protocol.

2.2. PCR and sequencing

A partial sequence for the cytochrome *b* (cyt *b*) gene of *N. cristatus* was determined by PCR using the primer pair, L10319-CYB (CCT TGG GGK CAG ATG TCT TTT TGG G) and H10648-CYB (GAT AAA ATT TTC WGG GTC). These primers were newly designed with reference to aligned sequences from several crustacean and insect species deposited in DDBJ/EMBL/GenBank (*Artemia franciscana*, X69067; *Daphnia pulex*, AF117817; *Pagurus longicarpus*, AF150756; *Penaeus monodon*, AF217843; *T. japonicus*, AB060648). The numbers in the primer names refer to the positions of the 3′ ends of the oligonucleotide with reference to the *A. franciscana* mitochondrial DNA sequence (Valverde et al., 1994).

The PCR was done in a model 9700 thermal cycler (Applied Biosystems) and reactions carried out with 30 cycles of a 15-μl reaction volume containing 9.8 μl of sterile, distilled H_2O, 1.5 μl of 10× buffer, 1.0 μl of dNTP (2.5 mM each), 1.0 μl of each primer (5 μM), 0.1 μl of 0.25 unit Z Taq (Takara), and 1.0 μl of template. The thermal cycle profile was as follows: denaturations at 98 °C for 1 s, annealing at 45 °C for 5 s, and extension at 72 °C for 10 s. The PCR products were electrophoresed on a 1% L 03 agarose gel (Takara) and stained with ethidium bromide for band characterization using ultraviolet transillumination.

Double-stranded PCR products were purified by a Pre-Sequencing Kit (USB), and were subsequently used for direct cycle sequencing with dye-labeled terminators (Applied Biosystems). Primers used were the same as those for PCR. All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were analyzed on a Model 377 DNA sequencer (Applied Biosystems).

A portion of the mitochondrial genomes of *E. bungii* and *N. cristatus* were amplified using a long PCR technique (Machida et al., 2002). Two primers, H13842-12S (TGT GCC AGC ASC TGC GGT TAK AC) and H26-Met (TCC TAT CAA AGA GTT ATG AGC TCT), were newly designed based on the aligned sequences from *A. franciscana* (Valverde et al., 1994), and *T. japonicus* (Machida et al., 2002). Species-specific primers were also designed on the basis of the partial sequence for the cyt *b* gene for *N. cristatus* (Necr-CYB-L1; TTG GTG GTG ACT TGG TAC AGT GG) described above. Applied pairs of primers were H13842-12S × H26-Met for *E. bungii* and H13842-12S × Necr-CYB-L1 for *N. cristatus* (Fig. 1). We have used these primer pairs because they amplified the longest portion of the mitochondrial genome among the primer pairs those we have tried.

Long PCR was done in a Model 9700 thermal cycler (Applied Biosystems) and reactions carried out with 30 cycles of a 25-μl reaction volume containing 12.75 μl of sterile, distilled H_2O, 2.5 μl of 10× buffer, 4 μl of dNTP (2.5 mM each), 1.0 μl of each primer (5 μM), 0.25 μl of 1.25 unit LA Taq (Takara), and 1.0 μl of template. The thermal cycle profile was that of "shuttle PCR": denaturation at 98 °C for 10 s, and annealing and extension combined at the same temperature (68 °C) for 8–10 min depending on the size of products. Annealing temperature was progressively decreased with advancing cycles (−0.5 °C per cycle) from 68 to 60 °C during the first 16 cycles and remained at 60 °C during the subsequent 21 cycles (Lavrov et al., 2000). Long PCR products were electro-

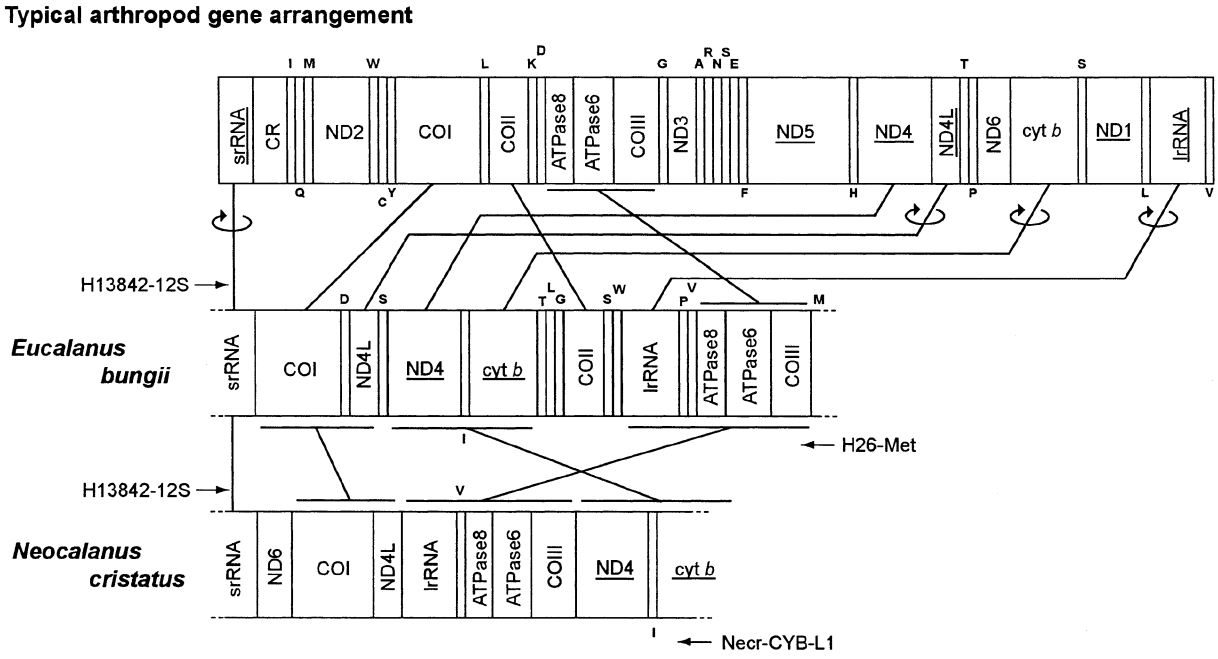

Fig. 1. Gene organization of *Eucalamus bungii*, *Neocalamus cristatus*, and typical arthropod mitochondrial DNA. Pairs of long-PCR primers amplifying portions of the mitochondrial genome are indicated by arrows. NDI-6, and 4L indicates NADH dehydrogenase subunits 1–6, and 4L, respectively; COI–III, cytochrome *c* oxidase subunits I–III; cyt *b*, cytochrome *b*; ATPase6 and 8, ATPase subunits 6 and 8. All protein and ribosomal RNA genes are transcribed from left to right except those underlined to indicate opposite orientation. Transfer RNA genes are identified by the one-letter codes for the corresponding amino acids. Transfer RNA genes indicated in the lower column are encoded on the minor-coding strand. The rearrangements that are needed to interconvert the pair of maps (disregarding transfer RNA genes) are shown.

phoresed on a 1.0% L 03 agarose gel (Takara) and later stained with ethidium bromide for band characterization using ultraviolet transillumination. Long PCR products were purified with a Pre-Sequencing Kit (USB) following the manufacturer's protocol, and were subsequently used for direct cycle sequencing with dye-labeled terminators (Applied Biosystems). Initial sequencing primers were the same as those for long PCR and those subsequently used were designed with reference to the previously determined sequences (primer walking). All sequencing reactions were performed according to the manufacturer's instructions. Labeled fragments were analyzed on a Model 377 DNA sequencer (Applied Biosystems).

2.3. Sequence analysis

The DNA sequences were analyzed using the computer software package program DNASIS version 3.5 (Hitachi Software Engineering). Locations of the protein-coding and rRNA genes were determined by sequence similarity with those of other crustaceans. A preliminary screening for the tRNA genes was done using tRNAscan-SE 1.1 (Lowe and Eddy, 1997). Those tRNA genes that could not be found by tRNAscan-SE were visually searched for through inspection of anticodon sequences and their proposed cloverleaf secondary structures (Kumazawa and Nishida, 1993). Sequence data are available from DDBJ/EMBL/GenBank under accession number AB091772 for *E. bungii* and AB091773 for *N. cristatus*. To calculate similarities of genes, inferred amino acid sequences for the COI gene from *T. japonicus*, AB060648), *A. franciscana*, X69067), *D. pulex*, AF117817), *P. monodon*, AF217843), *P. longicarpus*, AF150756), *Drosophila yakuba*, X03240), *Limulus polyphemus*, AF216203), and *Lumbricus terrestris,* U24570) were used for the alignment using CLUSTAL X (Thompson et al., 1997) with default gap penalties. Ambiguous regions were excluded manually from the alignment using MacClade 4.0 (Maddison and Maddison, 2000).

3. Results and discussion

3.1. Technical difficulties of copepod mitochondrial genomic studies

We have encountered several technical difficulties during the experiments. The first is the unconservative nature of gene arrangement and coding strand of genes, which caused uncertainty in directionality and matching of PCR primers that amplify intergenes. The second is technical difficulties in PCR amplification. Although long PCR products of the regions of small ribosomal RNA (srRNA)/tRNAMet for *E. bungii* and srRNA/cyt *b* for *N. cristatus* yielded clear and distinct single bands, no band was amplified for the reminder of the mitochondrial genome. We have designed several species-specific primers complementary to the end of each

mitochondrial segment and tried to amplify the reminder of the genome. However, we have not successful amplification despite repeated attempt with experimental conditions. Thus, we were unable to sequence a portion of the rest of the regions that would have included the putative control region. Stable stem-and-loop structures in the control region, which often contains stretches of specific nucleotides, may have disrupted PCR and sequencing reactions. The third is the presence of multiple haplotypes of mitochondrial DNA in individuals. Although most sequences were clear, we occasionally observed double peaks in electropherograms, which were expressed by degenerate bases in the sequence data deposited in the DDBJ/EMBL/GenBank. We are currently analyzing those double peaks in the genus *Neocalanus* by cloning those PCR products and have confirmed the presence of multiple haplotypes (Machida et al., unpublished data).

3.2. Genomic organization

We have been successful in amplifying portions of the mitochondrial genome for 9530 bp for *E. bungii* and 7965 bp for *N. cristatus* (Tables 1 and 2). The portion of the genome sequenced for *E. bungii* contained 8 protein-coding, 11 transfer RNA, and 2 ribosomal RNA genes. Although the same number of protein-coding and ribosomal RNA genes was identified in the sequence of *N. cristatus*, a much smaller number (2) of transfer RNA genes were found. Unlike the mitochondrial genome of *T. japonicus* (Machida et al., 2002) in which all genes are encoded on one strand, genes were encoded on both strands in *E. bungii* and *N. cristatus*. Length of intergenic nucleotide sequences varied greatly in both species. The longest intergenic nucleotide sequences were 97 and 115 for *E. bungii* and *N. cristatus*, respectively. Overlapping of genes was observed as in other animal mitochondrial DNA, although it occurred only twice in *E. bungii* and once in *N. cristatus*.

Table 1
Location of features in the mitochondrial genome of *E. bungii*

Features[a]	Position number		Size[b] (bp)	Codon		Intergenic nucleotides[c]
	From	To		Start	Stop	
srRNA	–	513	–			35
COI	549	2081	1533	ATC	TAA	10
$tRNA^{Asp}$	2092	2155	64			97
ND4L	2253	2540	288	ATA	TAA	−14
$tRNA^{Ser(UCN)}$	2527	2594	68			51
ND4	2646	3944	1299 (L)	ATA	TAA	2
$tRNA^{Ile}$	3947	4008	62 (L)			65
cyt *b*	4074	5210	1137 (L)	ATG	TAA	13
$tRNA^{Thr}$	5224	5285	62			60
$tRNA^{Leu(UUR)}$	5346	5410	65			68
$tRNA^{Gly}$	5479	5542	64			4
COII	5547	6254	708	ATA	TAA	4
$tRNA^{Ser(AGR)}$	6259	6311	53			2
$tRNA^{Trp}$	6314	6379	66			0
LrRNA	6380	7696	1317			0
$tRNA^{Pro}$	7697	7760	64			0
$tRNA^{Val}$	7761	7822	62			0
ATPase8	7823	7987	165	ATA	TAA	0
ATPase6	7988	8695	708	ATG	TAA	19
COIII	8715	9506	792	ATG	TAA	−1

[a] For abbreviations of genes, see Fig. 1 legend.
[b] "L" denotes that the gene is encoded on the L-strand.
[c] Numbers correspond to the nucleotides separating different genes. Negative numbers indicate nucleotides overlapping between adjacent genes.

Table 2
Location of features in the mitochondrial genome of *N. cristatus*

Features[a]	Position number		Size[b] (bp)	Codon		Intergenic nucleotides[c]
	From	To		Start	Stop	
srRNA	–	502	–			15
ND6	518	991	474	ATA	TAA	19
COI	1011	2543	1533	GTT	TAA	54
ND4L	2598	2882	285	ATT	TAG	0
lrRNA	2883	4110	1228			0
$tRNA^{Val}$	4111	4173	63			7
ATPase8	4181	4339	159	ATT	TAA	0
ATPase6	4340	5047	708	ATG	TAA	−1
COIII	5047	5838	792	ATG	TAA	115
ND4	5954	7255	1302 (L)	ATA	TAA	3
$tRNA^{Ile}$	7259	7322	64 (L)			10
cyt *b*	7333	–	– (L)		TAA	–

[a] For abbreviations of genes, see Fig. 1 legend.
[b] "L" denotes that the gene is encoded on the L-strand.
[c] Numbers correspond to the nucleotides separating different genes. Negative numbers indicate nucleotides overlapping between adjacent genes.

3.3. Base composition

The A + T contents of the strands that contain the majority of the open reading frames in *E. bungii* (A = 30.0%, C = 20.2%, G = 20.1%, T = 29.6%) and *N. cristatus* (A = 32.9%, C = 14.5%, G = 16.8%, T = 35.5%) were 59.6% and 68.4%, respectively (Table 3). Although the complete mitochondrial genome sequences for *E. bungii* and *N. cristatus* have not yet been determined, we compared their base composition with those of previously determined mitochondrial genomes to look for general trends in calanoid copepods. The overall

Table 3
Mitochondrial genomic information for the calanoid copepods, *E. bungii* and *N. cristatus* compared with five other crustaceans (*A. franciscana, D. pulex, P. monodon*, *P. longicarpus,* and *T. japonicus*)[a]

Taxon	Length (bp)	A + T%	GC-skew	AT-skew
Eucalanus bungii	9530	59.6	−0.003	0.006
Neocalanus cristatus	7965	68.4	0.072	−0.038
Tigriopus japonicus	14,628	60.4	0.268	−0.171
Artemia franciscana	15,770	64.4	0.005	−0.039
Daphnia pulex	15,333	62.2	−0.116	0.011
Penaeus monodon	15,984	70.6	−0.137	−0.001
Pagurus longicarpus	15,630	71.2	−0.213	0.029

[a] Accession nos.: *A. franciscana* (X69067), *D. pulex* (AF117817), *P. longicarpus* (AF150756), *P. monodon* (AF217843), *T. japonicus* (AB060648).

A + T content of *E. bungii* (59.6%) was lower than *P. longicarpus* (71.2%) and *P. monodon* (70.6%), but similar to that of *T. japonicus* (60.4%) and branchiopod crustaceans (64.4% in *A. franciscana*, 62.2% in *D. pulex*). On the contrary, much higher value was observed in *N. cristatus* (68.4%).

The base compositional differences between the two strands were calculated as GC- and AT-skews (Perna and Kocher, 1995). For *E. bungii* and *N. cristatus*, GC-skew values were −0.003 and 0.072, and AT-skew values were 0.006 and −0.038, respectively. The absolute values for both GC- and AT-skews were low in *E. bungii* but moderate in *N. cristatus* (Table 3). This is in contrast to *T. japonicus*, in which the highest values for arthropods were observed both in GC- and AT-skews.

3.4. Gene order

Gene arrangements in the *E. bungii* and *N. cristatus* mitochondrial genomes differed greatly from those in other metazoan animals (Fig. 1). The positions of tRNA genes in different mitochondrial genomes are much more variable than those of other genes (Wolstenholme, 1992), so even if the tRNA genes are excluded from the comparison, only the order and transcriptional direction of ATPase8/ATPase6/COIII were comparable to typical arthropod mitochondrial genomes. In addition, gene arrangements of cyt *b*/ND4 were also shared with the *T. japonicus* mitochondrial genome. *E. bungii* and *N. cristatus*, which belong to different superfamilies, shared only two arrangements, lrRNA/ATPase8 and COI/ND4 in addition to the above-mentioned arrangements (Fig. 1).

Several mechanisms have been proposed for mitochondrial gene rearrangements (e.g. Boore, 2000). One of the most widely accepted mechanisms for mitochondrial gene rearrangement is tandem duplication of gene regions as a result of slipped-strand mispairing followed by deletion of genes. This mechanism, however, does not account for the copepod mitochondrial gene arrangement, because the observed rearrangements have involved inversion of the coding polarity in the copepod mitochondrial genomes. Transposition of genes seems unlikely because terminal repeats, characteristics of most transposable elements (Calos and Miller, 1980), are lacking in the copepod mitochondrial genomes. DNA recombination, which had been thought to be improbable in animal mitochondria, is now known to occur in nematodes (Lunt and Hyman, 1997). Following recombination breakage, reincorporation of the minifragment in the reverse orientation results in inversion of genes (Dowton and Austin, 1999), although it remains to be demonstrated whether or not such intramitochondiral recombination is a general phenomenon.

From our results, it is not clear which mechanism could explain the gene rearrangement observed in the copepods mitochondrial genomes.

3.5. Protein-coding genes

Seven protein-coding genes (ATPase6, ATPase8, COI, COIII, cyt *b*, ND4, and ND4L) were found both in *E. bungii* and *N. cristatus*. Additionally, COII and ND6 genes were found in *E. bungii* and *N. cristatus*, respectively (Tables 1 and 2, Fig. 1). Most of the protein-coding genes started with one of the initiation codons, ATG, ATT, or ATA which are commonly found in crustaceans. COI genes of both *E. bungii* and *N. cristatus* started with ATC and GTT, which has been reported to be the initiation codons in metazoan animals (Wolstenholme, 1992). All but one of the protein-coding genes had the termination codon TAA. Only one protein-coding gene, ND4L in *N. cristatus*, had a TAG termination codon. No incomplete termination codons were observed either in *E. bungii* or *N. cristatus*.

In Table 4, amino acid sequence similarities of the COI gene are compared between seven species of crustaceans plus insects and also two outgroup animals, *L. polyphemus* and *L. terrestris*. It should be noted that the sequence similarities of the copepods, *E. bungii*, *N. cristatus*, and *T. japonicus*, were the lowest compared to the outgroups (72.8–75.3% in *L. polyphemus*, 71.8–73.4% in *L. terrestris*), suggesting that the substitution rate for the amino acid sequence has been accelerated not only in the lineage of *T. japonicus* but also in these calanoid copepods (Machida et al., 2002).

Table 4
Pairwise percentage similarities of the amino acid sequences of cytochrome *c* oxidase subunit I gene among representative arthropods and their outgroups[a,b]

	N. cristatus	*T. japonicus*	*A. franciscana*	*D. pulex*	*P. monodon*	*P. longicarpus*	*D. yakuba*	*L. polyphemus*	*L. terrestris*
E. bungii	92.8	77.7	76.9	77.5	78.1	77.7	76.9	75.3	71.8
N. cristatus		76.7	76.9	78.3	77.1	76.3	76.3	74.6	72.6
T. japonicus			72.6	75.1	74.2	74.6	73.4	72.8	73.4
A. franciscana				84.3	82.0	81.8	81.0	80.4	74.0
D. pulex					86.7	85.3	84.0	83.8	76.3
P. monodon						92.0	85.5	84.0	73.6
P. longicarpus							83.0	82.0	74.9
D. yakuba								81.2	74.9
L. polyphemus									78.1

[a] Total of 489 sites were used for the calculations.

[b] Accession nos.: *E. bungii* (AB091772), *N. cristatus* (AB091773), *T. japonicus* (AB060648), *A. franciscana* (X69067), *Daphnia pulex* (AF117817), *P. longicarpus* (AF150756), *P. monodon* (AF217843), *Drosophila yakuba* (X03240), *L. polyphemus* (AF216203), *L. terrestris* (U24570).

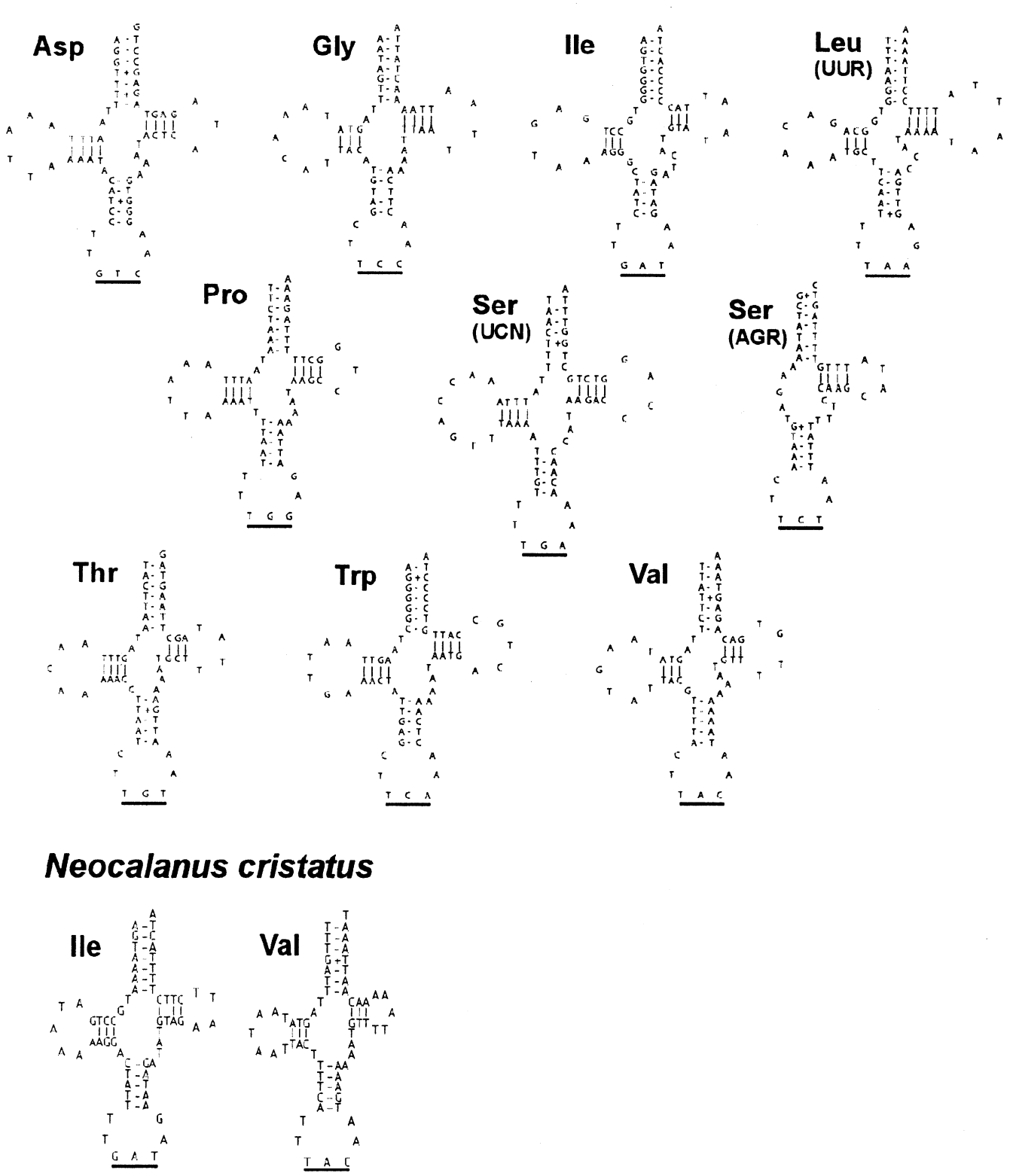

Fig. 2. Putative secondary structures of *E. bungii* and *N. cristatus* mitochondrial transfer RNA genes. The anticodon sequences are indicated by underlining. Watson-Crick and T-G bonds are indicated by dashes and crosses, respectively.

3.6. Transfer RNA genes

A total of 11 and 2 tRNA genes, respectively, were identified in the sequenced portions of the *E. bungii* and *N. cristatus* mitochondrial genomes. Transfer RNA genes ranged in size from 53 to 68 nucleotides (Tables 1 and 2), most of which were large enough for the encoded tRNA to fold into the cloverleaf secondary structures characteristic of tRNA genes (Fig. 2). The tRNASer(AGR) gene of *E. bungii* had lost the D arm, which has been observed not only for other arthropods (Machida et al., 2002), but also in other metazoan animals such as nematodes (Wolstenholme, 1992), brachiopods (Noguchi et al., 2000), and molluscs (Yamazaki et al., 1997). The anticodon nucleotides were identical to those commonly found for the corresponding tRNA genes in other mitochondrial DNAs.

3.7. Ribosomal RNA genes

As in all other metazoan animals, the *E. bungii* and *N. cristatus* mitochondrial genomes contained genes for small and large ribosomal RNAs (srRNA and lrRNA). Coding polarity of these two genes were identical in both species (Fig. 1). Although the complete gene sequences were obtained for the lrRNA both in *E. bungii* and *N. cristatus*, only partial sequences were obtained for the srRNA in both species. If we assume that the rRNA genes occupied all of the available space between adjacent genes, the lengths of the lrRNA genes can be estimated as 1317 and 1228 bp for *E. bungii* and *N. cristatus*, respectively (Tables 1 and 2). These values are moderate among the crustaceans, although much higher than in *T. japonicus* in which a very short lrRNA gene (1034 bp; Machida et al., 2002) has been reported.

```
L1384-COI
                    5′-GGTCATGTAATCATAAAGATATTGG-3′
A. franciscana        AT..TACG.....C..G........
D. yakuba             T......A.....T..A........
T. japonicus          GGG.G..T.....T..A........
N. cristatus          GG...TGT.....T..A........
E. bungii             GG..CTGT..C..T..A..C..C..

H2612-COI
                    5′-AGGCCTAGGAAATGTATMGGGAAA-3′
A. franciscana        ..A....A.......TGA......
D. yakuba             .AT....AA......TGG......
T. japonicus          .....C.AA.....C..A......
N. cristatus          .AA....A.........A......
E. bungii             ................C......

L13337-12S
                    5′-YCTACTWTGYTACGACTTATCTC-3′
A. franciscana        C.....T..T.............
D. yakuba             T.....A..T............T
T. japonicus          GT....T..C.............
N. cristatus          TT....T..C.............
E. bungii             T.....T..C.............

H13845-12S
                    5′-GTGCCAGCAGCTGCGGTTA-3′
A. franciscana        .........T.C.....C.
D. yakuba             ..........TC.......
T. japonicus          .........C.C.......
```

Fig. 3. Primers compared to homologous sequences of *A. franciscana* (X69067), *D. yakuba* (X03240), *T. japonicus* (AB060648), *E. bungii* (AB091772) and *N. cristatus* (AB091773), mitochondrial DNAs. L and H numbers refer to the strand and position of the 3′ base in the *A. franciscana* (Valverde et al., 1994) sequence.

Table 5
Amplification results using copepod versatile primers[a]

Order / Species	COI	srRNA
Harpacticoida		
Tigriopus japonicus	A	A
Calanoida		
Eucalanus bungii	A	A
Neocalanus cristatus	A	A
Heterorhabdus vipera	A	A
Metridia pacifica	A	A
Paraeuchaeta elongata	C	C
Scottocalanus securifrons	C	A
Cyclopoida		
Lernaea cyprinacea	C	C
Oithona nana	C	A
Oithona similis	B	A
Oithona decipiens	B	A
Oithona fallax	B	A
Oithona plumifera	A	A
Oithona longispina	A	A
Oithona rigida	A	A
Oithona setigera	A	A
Oithona vivida	A	C
Oithona simplex	A	A
Paroithona sp.	C	C
Poecilostomatoida		
Sapphirina opalina	A	A

[a] A; good amplification, B; good amplification with second PCR, C; no amplification. All of the PCR reactions were carried out with same condition as mentioned in the text.

3.8. Versatile primers

We designed a set of versatile primers for the COI genes based on highly conservative mitochondrial DNA regions [L1384-COI (GGT CAT GTA ATC ATA AAG ATA TTG G) × H2612-COI (AGG CCT AGG AAA TGT ATM GGG AAA), Fig. 3]. Indeed, one of the primers for the COI gene (L1384-COI) is almost identical to the LCO1490 sequence that was reported by Folmer et al. (1994), with some modifications at the 5′ end. This primer pair and the previously described primer pairs for the srRNA gene [Machida et al., 2002; L13337-12S (YCT ACT WTG YTA CGA CTT ATC TC) × H13845-12S

(GTG CCA GCA GCT GCG GTT A), Fig. 3] were employed for PCR amplification of sequences from 6 species of calanoid copepods and 14 species of non-calanoid copepods (Table 5).

Good amplifications were observed in at least one species from each Order, both in the COI and srRNA genes. A total of 12 of the 20 species and 16 of the 20 species showed good amplification for the COI and srRNA genes, respectively. Although primers for the COI gene were designed based on the two species of calanoid copepods, some of the calanoid copepods, such as *Paraeuchaeta elongata* and *Scottocalanus securifrons*, yielded no product (Table 5). In the Oithonidae, 9 of the 11 species were amplified by both primers for the COI and srRNA genes (Table 5).

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