Phylogeography of the copepod *Tigriopus japonicus* along the Northwest Pacific rim

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Copepod genus *Tigriopus* is distributed worldwide in coastal splash-pools, and is recognized as a suitable taxon for testing evolutionary and environmental hypotheses that are also relevant to water column research. Here, we present the complete DNA sequence (7709 bp) of a single ribosomal DNA (rDNA) unit of a *Tigriopus* species distributed along the coastlines of the Northwest Pacific ocean. The genomic organization of the *Tigriopus japonicus* (*T*. japonicus) rDNA was observed to be 18S (1817 bp)-ITS1 (465 bp)-5.8S (165 bp)-ITS2 (226 bp)-28S (3628 bp)-IGS (1417 bp). The relationship of populations was studied using rDNA ITS (internal transcribed spacer) sequences from different geographical areas. The phylogenetic analysis of 18S rDNAs showed a single monophyletic clade of the Copepoda with an unresolved polytomy of the Harpacticoida, Monstrilloida and Siphonostomatoida. In Harpacticoida phylogeny, three species of *Tigriopus* were monophyletic; *T*. japonicus and *Tigriopus californicus* as sister species formed a cluster with *Tigriopus brevicornis*. Furthermore, phylogeographic analysis of ITS-5.8S sequences showed that *Tigriopus* from 15 Asian locations formed four distinct clades according to their geographical origins. When ITS-5.8S of those Asian specimens was compared with the corresponding mitochondrial COI (cytochrome c oxidase subunit I) gene data, a high similarity was found in the identical genotypes. However, they were different between the localities. This suggests that four groups identified from Asian *Tigriopus* would be different (sub)species, and the pattern of Asian *Tigriopus* genetic relatedness is due to their geographical separation.

INTRODUCTION

Copepod *Tigriopus* is a widespread metazoan in the splash-pools of the upper intertidal zone of rocky shores (Edmands, 2001; Johnson, 2001; Kim et al., 2003). *Tigriopus* can be frequently found in this habitat from tropical to temperate coastal waters. Eleven species of *Tigriopus* have been described to date, distributed according to their geographical localities. For example, *Tigriopus californicus* (*T*.* californicus*) on the Pacific coast of North America, and *Tigriopus japonicus* (*T*.* japonicus*) on the Far East coast, and both *Tigriopus brevicornis* (*T*.* brevicornis*) and *Tigriopus fulvus* (*T*.* fulvus*) on the Atlantic European coast and the Mediterranean Sea. *Tigriopus* spp. are omnivorous but typically graze microflora of their splash-pool habitat. They are easy to capture from the rock pools and their culture and maintenance in the laboratory is simple. These attributes make them ideal model organisms for population genetics (Ganz and Burton, 1995; Burton, 1998; Edmands, 1999; Jung et al., 2006), ecotoxicity testing (Misitano and Schiewe, 1990; Damgaard and Davenport, 1994; Davenport et al., 1997; Forget et al., 1998; Barka et al., 2001; Ara et al., 2002; Forget et al., 2003; Marcial et al., 2003; Kwok and Leung, 2005; Lee et al., 2007a), and environmental...
toxicogenomics (Lee et al., 2005; Seo et al., 2006a, b; Lee et al., 2007b; Raisudden et al., 2007).

*Tigriopus californicus* was the first species of the genus to be used in molecular genetics studies as the cytochrome c oxidase subunit I (mtDNA COI) gene was used to analyze the molecular phylogeny of its populations (Edmands, 2001). In *T. californicus*, mtDNA revealed extreme genetic divergence, even over short geographical distances along the west coast of North America (Burton and Lee, 1994, 1998; Ganz and Burton, 1995). Recently, Burton et al. (Burton et al., 2005) found an unusual structure of the nuclear ribosomal DNA (rDNA) in *T. californicus*, lacking internal subrepeats of intergenic spacers (IGS). They reported that mtDNA is one of the key factors for speciation events in *T. californicus* (Ellison and Burton, 2008). However, other species of the genus *Tigriopus* have not been well studied genetically so far, with the exception of the mtDNA sequence analysis of *T. japonicus* by Machida et al. (Machida et al., 2002) and Jung et al. (Jung et al., 2006).

*Tigriopus japonicus* is regarded as the only representative of the genus in the Far East. It has been reported from Hong Kong, Taiwan, Japan, Korea and China (Song and Chang, 1993; Chi et al., 2003; Kwok and Leung, 2005; Jung et al., 2006; Dahms et al., 2007). However, recently Jung et al. (Jung et al., 2006) showed a striking difference between *T. japonicus* from Korea and Japan based on the analysis of the mitochondrial COI gene (22.5% difference in nucleotides and 4.6% difference in amino acids). This raises the question whether populations of *Tigriopus* from the Far East belong to one or several subspecies of *T. japonicus*. Until now, there are few comparative studies focusing on the genetic differences of its geographically isolated populations. Our objective is to study the patterns of genetic diversity of several populations of *T. japonicus* using the nuclear region of the internal transcribed spacer (ITS). By reconstructing the phylogeny of the genus *Tigriopus* in this geographical region, we may elucidate the speciation history of the genus.

In eukaryotes, rDNA sequences can be found in the nucleus and in organelles. The eukaryotic nuclear rDNA is tandemly organized with copy numbers up to the order of 10,000 (Schlo¨ tterer, 1998). Each transcription repeat unit consists of the genes coding for the small subunit (i.e. 18S), large subunit (i.e. 23–28S), and the 5.8S nuclear rDNA. These coding regions are separated by the primary transcript by ITS. The rDNA coding regions have remained relatively constant within the same taxon, and the ubiquity and conservation of rDNA sequences provide a key role for these DNA regions in constructing phylogenetic relationships between taxa of different phylogenetic ranks (e.g. species, genera, families). In the case of *Tigriopus*, Oakley and Cunningham (Oakley and Cunningham, 2002) used 18S and 28S rDNAs to analyze the origin of compound eyes in arthropods. More recently, Burton et al. (Burton et al., 2005) reported unusual patterns of IGS including adjacent 18S, 28S rDNA regions from *T. californicus* with various geographically samples along the Pacific coast of North America; however, the 28S was not sequenced completely. Also, several DNA sequences of partial ITS1 and 5.8S rDNA from *T. brevicornis* are available from GenBank database. However, no phylogeographic analysis of *T. japonicus* has been performed using rDNA sequences.

In this paper, we describe the full nucleotide sequence of the single unit of tandemly repeated rDNA from *T. japonicus*. In addition, phylogenetic relationships of copepods generally, including three representatives of *Tigriopus* (i.e. *T. brevicornis*, *T. californicus* and *T. japonicus*) are inferred from 18S rDNA sequences. Finally, we investigated the molecular biogeography of Asian *Tigriopus*, using complete ITS and 5.8S rDNA sequences.

**METHOD**

**Sample collection**

The organisms used in this study were sampled from different locations as summarized in Table I. After sampling, they were concentrated by filtering onto a 100 µm mesh, and preserved in 70% EtOH. The identities of species were initially checked by stereomicroscopy and further verified by COI sequence comparison (Jung et al., 2006). Samples were stored at 4°C until use.

**Long-range and typical polymerase chain reactions and DNA sequencing**

For long-range polymerase chain reactions (long-PCR), a small piece of tissue was isolated from single individuals of *T. japonicus*, and used as a template for long-PCR without further genomic DNA extraction. In brief, individuals of *T. japonicus* were washed six times with 1x TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) using a capillary pipette under a stereomicroscope, and were transferred into 200 µL thin-walled PCR tubes containing 5 µL of TE buffer. The tubes were maintained at 95°C for 10 min with an iCycler thermoblock (Bio-Rad, CA, USA), and were subsequently cooled to 4°C for the following PCR amplification. Long-PCR with one primer set (TJ-rDNA-long-F, 5’-AGC TGC GAA CGG CTC ATT AAA TCA CAC CTC-3’; TJ-rDNA-long-R, 5’-TCT CAA AAG ATC GCA ATG TGG TTG CTC-3’ was used to get the complete transcription repeat unit (18S rDNA-ITS1-5.8S rDNA-ITS2-28S).
rDNA-IGS) of *T. japonicus* rDNA. Long-PCR was carried out in 50 μL reaction mixtures containing 30.5 μL sterile distilled water, 5 μL 10× LA PCR buffer II (TaKaRa, Japan), 8 μL dNTP (4 mM), 5 μL of each primer (5 μM), 0.5 μL LA *Taq* polymerase (2.5 U) and 1 μL of the previously mentioned crude lysate. PCR cycling was performed in an iCycler (Bio-Rad) with 35 cycles of 98 °C for 25 s and 68 °C for 12 min. The resulting PCR products were electrophoresed in a 1.0% agarose gel, stained with ethidium bromide and visualized by ultraviolet transillumination. A long-PCR product was sequenced directly using the previously mentioned long-PCR primers with subsequent primer walking. Briefly, long-PCR amplicons were purified with QIAquick PCR purification kit (Qiagen GmbH, Germany). DNA sequencing reactions were performed in an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, CA, USA) using the PCR products (2 μL) as the template. Labeled DNA fragments were analyzed on a Model 373S sequencer (Applied Biosystems Inc., CA, USA).

Table I: Characteristics of Tigriopus species, including other Harpacticoida, used in this study and DNA sequence GenBank accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Isolation locality</th>
<th>Nr. rDNA</th>
<th>COI</th>
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</table>

*DNA sequences revealed in the present study.

 ITS-5.8S rDNA sequences from geographical samples

For the ITS-5.8S amplification from different geographical samples, we directly used a single individual of a
Tigriopus specimen as a DNA template without genomic DNA extraction, as previously (Ki et al., 2004). The region was amplified with the primers (TJ-rDNA-18E 5'-GCT ACT ACC GAT TGA ACG TTT TAG-3', TJ-rDNA-28R, 5'-CAT TCG CCA TTA CTA GGG GCA TC-3'). PCR thermocycling was as follows: 95°C for 5 min; 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. PCR products from 15 geographical samples were subcloned into a pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA), and all the subcloned DNAs were sequenced according to the manufacturer's suggested protocol with commercial primers (e.g. T7 and M13R). In addition, we investigated some intra-individual variations in the rDNA ITS, by sequences from five colonies from a plate growing the transformed Escherichia coli cells, which contained ITS fragments from a Tigriopus individual. For inter-individual genetic variations, we sequenced the ITS region of at least three individuals isolated from each locality sample using the TJ-rDNA-18F and TJ-rDNA-28R primers. In this case, PCR amplicons were directly sequenced using identical PCR and internal primers (e.g. TJ-58F1, 5'-TGA AGA ACG CAG GTA ACT GC-3' and TJ-58R1, 5'-ACG GTC CGC AA TA TG CGT TC -3').

Editing and contig assembly of the rDNA sequences were carried out with Sequencher 4.1.4 (Gene Codes, MI, USA). All sequences have been deposited at GenBank (Accession Numbers EU054307, EU057568–EU057580).

Sequence analyses
For genetic variation in T. japonicus, complete ITS-5.8S and COI sequences (see 11 isolates in Table I) were used for similarity and parsimony analyses. The complete ITS and partial COI sequences (Jung et al., 2006) were aligned in ClustalW ver.1.8 (Thompson et al., 1994). Pairwise genetic distances were estimated with the Kimura two-parameter model (Kimura, 1980) between 11 pairs of Asian Tigriopus using both COI and ITS-5.8S sequences. The corrected pairwise (p) distance was calculated with MEGA 4.0 (Kumar et al., 2001). Repeat sequence patterns in the rDNA IGS sequences were analyzed using Genetyx ver.7.0 program (Hitachi Software Engineering Co. Ltd., Japan). Dot-plot analyses were carried out using the MegAlign 5.01 software (DNAstar Inc., Madison, WI, USA).

Phylogenetic analysis inferred from 18S rDNA
Recently, Huys et al. (Huys et al., 2006, 2007) reported on the phylogenetic relationships of the orders Cyclopoida, Monstrilloida, Siphonostomatoida within the copepod lineage using complete 18S rDNA sequences. Our phylogenetic analysis was focusing on the Calanoida, Harpacticoidea and Pocillostomatoida including certain copepod species studied earlier by Huys et al. (Huys et al., 2006, 2007). In order to select an appropriate outgroup, we tested three Maxilopoda species such as Argulus nobilis proviral (Branchiura, M27187); Denochelocaris typicus (Mystacocarida, L81937), and Semibalanus balanoides (Thecostraca, AM497882). These taxa belong to the Maxilopoda of the Crustacea. In preliminary phylogenetic analysis, we found that the above selected outgroups, including Squilla empusa (Stomatopoda, L81946) used by Huys et al. (Huys et al., 2006), could substantially affect the branching pattern, possibly due to a long branch attraction caused by very unequal rates of evolution. Therefore, the phylogenetic relationships of the previously mentioned taxa were studied without outgroups. For a phylogenetic reconstruction of the Copepoda, a total of 35 18S rDNA sequences, including the genus Tigriopus, were aligned using ClustalW. Various regions were further aligned manually, and ambiguous regions that could not be aligned were excluded from the analysis. Thus, only unambiguous positions (i.e. 1730 out of 1878 alignment positions) of the nearly complete 18S rDNA alignment were used in the analysis. Modeltest 3.07 (Posada and Crandall, 1998) identified the optimal model of DNA substitution for the maximum likelihood (ML) construction. As the best-fit model for this data set according to the Akaike information criterion (AIC) was the general time reversible plus invariant sites plus gamma distributed model (GTR + I + G) with lnL = 13 472.3. An ML tree of the data set was constructed with the GTR + I + G model in PAUP* 4.0b10 (Swofford, 2002). Bootstrap analyses with 100 replicates were conducted to determine the robustness of the clades. All phylogenetic trees were visualized with TreeView ver.1.6.6 (Page, 1996).

Bayesian analysis of the data set mentioned earlier was implemented with MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001), using the GTR + I + G model. The Markov Chain Monte Carlo process was set to two chains, and 1 000 000 generations were conducted. The sampling frequency was assigned at every 100 generations. The first 2000 trees were discarded as burn-in process and a consensus tree was constructed. Bayesian posterior probabilities (PP) > 0.50 were indicated at each branch node.

Phylogenetic analysis of the Harpacticoidea (six families, eight species) was carried out with 25 partial DNA sequences using the previously described method. In this case, 822 out of the 930 alignment positions for
the 5’ end side of 18S rDNA alignment were used. A Bayesian tree of the data set was constructed with the F81 model in MrBayes ver. 3.1.2. The cyclopoid Pachos sp. (GenBank No. AY627014) was used as outgroup. Further ML analysis was carried out with the equal-frequency Transition Model (TVM + I) and the best-fit model for the ITS-5.8S data set by AIC. An ML substitution rates AC = 0.5289, CG = 0.6759, CT = 3.3337; and gamma distribution shape parameter = 0.5289. A published sequence from T. Californicus (GenBank No. AY599492) was used as outgroup.

Phylogeography of T. japonicus inferred from ITS-5.8S rDNA

The phylogeography of Asian Tigriopus was studied with complete ITS-5.8S rDNA sequences. A total of 15 DNA sequences were analyzed following the method described earlier. In this analysis, 940 out of the 947 nucleotides from the complete ITS-5.8S alignment were used. The Modeltest 3.07 was used to find out the optimal model of DNA substitution for ML construction which suggested the Transversion model plus gamma (TVM + G) model with \(-\ln L = 3244.2\) as the best-fit model for the ITS-5.8S data set by AIC. An ML tree of T. japonicus was constructed with the selected TVM + G model in PAUP* 4.0b10, using the following likelihood settings: base frequencies = equal; base substitution rates AC = 1.0000, AG = 1.2265, AT = 0.6759, CG = 0.6759, CT = 3.3337; and gamma distribution shape parameter = 0.5289. A published sequence from T. Californicus (GenBank No. AY599492) was used as outgroup.

RESULTS AND DISCUSSION

Complete sequence and organization of T. japonicus rDNA

The total length of a single rDNA transcription repeat unit from T. japonicus (at Tongyoung) was 7709 bp (GenBank No. EU054307). It was organized in the typical eukaryotic fashion of rDNA, i.e., 5′-ETS (external transcribed spacers)-18S-ITS-5.8S-ITS2-28S-3′-ETS-NTS (non-transcribed spacers). The organization of the gene is shown in Fig. 1. The DNA sequence of the T. japonicus rDNA gene was 1817 bp (18S rDNA), 463 bp (ITS1), 165 bp (5.8S rDNA), 226 bp (ITS2), 3628 bp (28S rDNA) and 1417 bp (IGS). Each rDNA transcription repeat unit was separated by non-coding regions (IGS) and the arrangement was the same as that of T. Californicus rDNA (Burton et al., 2005). In the present study, we reported the complete rDNA of T. japonicus. Earlier, Burton et al. (Burton et al., 2005) reported an incomplete sequence of rDNA of T. Californicus with an unusual structure of the rDNA IGS sequences lacking internal subrepeats.

The 18S rDNA of T. japonicus contained 1817 nucleotides. It is of similar size to those of other harpacticoids (e.g. Cancrincola plumipes (C. plumipes) [LB1938], 1831 bp, 51.3%; Bryocamptus pygmaeus (B. pygmaeus) [AY627015], 1809 bp, 50.2%; Bradya sp. [AY627016], 1789 bp, 49.3%), and the GC content of the 18S rDNA was 49.6%. Interestingly, the GC content in harpacticoids was relatively higher than those of other copepods; e.g. Calanus pacificus showed 47.7%, Lepeophtheirus salmonis (L. salmonis) 46.6% and Chondracanthus lophii (C. lophii) 47.3%. On the other hand, the GC content of the entire 28S rDNA of T. japonicus was 54.1%.

The rDNA IGS of T. japonicus contained 1417 nucleotides in length, and its GC content was measured as 47.4% (Fig. 1A). Interestingly, T. japonicus had significantly shorter sequences in the rDNA IGS, when compared with other eukaryotic rDNA IGS. For example, the rDNA IGS varies in length from \(\sim 2\) kb in the yeast (Saccharomyces cerevisiae) to \(\sim 21\) kb in mammals (Moss and Stefanovsky, 1995), and it contains significant regions of internal repeats with very few exceptions. The rDNA IGS sequences of the close relatives T. brevicornis and T. californicus were recorded to be 1928 bp and 3360 bp in length, respectively (Burton et al., 2005). We determined whether there was any sequence homology through parallel sequence alignments by dot-matrix analysis of the rDNA IGS sequences of T. japonicus with T. brevicornis and T. californicus (Fig. 2). The graphical analyses showed that T. japonicus has quite different nucleotide sequences in the rDNA IGS, when compared with two other relatives. Thus, no homology between T. californicus and T. japonicus was observed across the rDNA IGS sequences. Both species are considered close relatives as far as morphology and molecular phylogeny of the 18S rDNA is concerned (discussed later, see Burton et al., 2005).

Regarding the typical rDNA IGS organization, T. japonicus rDNA consists of the following three components: 3′-ETS, NTS and 5′-ETS. In this study, we identified an internal bi-repeat of the following sequence: 5′-CAA A/G/C/G GGA AAT AAA CGT (G/A)CT AAA GGC-3′ positioned with 737–763 and 787–813 in the rDNA IGS. This kind of bi-repeat motif would be one key component in a proximal promoter domain and an upstream control element motif (Marilley and Pasero, 1996; Chen et al., 2000). Considering these motifs as promoters, transcription probably begins 20–30 bp downstream of the second motif. The 5′-ETS region is \(\sim 200\) bp long in T. japonicus. We found a poly-(dT) tract located at positions 323–340 from the end side nucleotide of 5′ rDNA IGS.
which is considered as a signal for the termination of the rDNA transcript (Lang et al., 1994; Lang and Reeder, 1995; Jeong et al., 1996; Mason et al., 1997). Therefore, the 5'-ETS is 340 bp long. The remaining nucleotide sequence belonged to the NTS, which is 480 bp long. In the putative 3'-ETS region, we identified microsatellite-like sequences such as ‘GA’ and ‘TC’ repeats (Fig. 1B).

**Homogeneity of tandem rDNA ITS, IGS**

Intra-individual variations in the rDNA sequences, particularly in rDNA ITS and IGS, of *T. japonicus* were investigated by sequencing and PCR assay. In this study, we sequenced five random colonies after cloning to pCR2.1 TA vector, which contained rDNA ITS fragments from a *T. japonicus* (at Tongyoung, Korea), and found all the sequences were identical to the GenBank No. EU054307 (Table I). In addition, we sequenced 3 colonies containing rDNA ITS fragments from a *Tigriopus* individual (Thailand: Bangkok), and found all rDNA ITS sequences were identical to EU520441. These results indicate that variations in rDNA sequences within an individual of *T. japonicus* are minor because the 18S–28S rDNA is a multigene family and is subject to concerted evolution (Elder and Turner, 1995). In addition, we confirmed the homogeneity of rDNA IGS lengths in *T. japonicus* by PCR assay.

**Fig. 1.** Schematic representation of the single transcription repeat unit of rDNA complex (A) and nucleotide sequence (B) of *T. japonicus*. Solid boxes indicate the ribosomal genes, and thin lines represent ITS or IGS. Nucleotide sequences in length and GC composition of each locus are represented on/under the line by calculation from a single unit of rDNA from a Tongyoung specimen. Position and orientation of primers described in Methods are indicated by arrows. In the rDNA IGS sequence, bi-repeats are indicated by asterisks, and microsatellite-like nucleotides are marked in dotted lines. A putative termination signal (poly-'T' track) is represented in bold line.
However, the rDNA IGS regions are relatively difficult to amplify due to their substantial length (up to 30 kb), poly 'T' and 'A' nucleotide sequences, or microsatellites (e.g. AC, AT) (Fig. 1B). In some organisms, the NTS region, which is located between the 5'0- and 3'0-ETS region, can be directly amplified using a long-PCR with universal primers that bind to conserved 18S and 28S sequences, since the NTS regions vary in length (3–30 kb) among plants, fungi and animals (Baldwin and Markos, 1998). In cases where the NTS is short (<3 kb), conventional PCR may be sufficient for the isolation of all regions. In case of T. japonicus, conventional PCR was successful in amplifying the rDNA IGS of T. japonicus because of a relatively short sequence length (i.e. 1417 bp). Based on our data, we designed two primers, viz., TJ-IGSF1 (5'-GAT TTG TTC TTG TCA GCT AGA C-3') and TJ-IGSR1 (5'-GAC TAC TGG CAG GAT CAA CG-3') from near the 3' end sequences of 28S rDNA, and from the 5' end of the 18S rDNA sequence of Tigriopus, respectively and used them for the amplifications of the entire rDNA IGS region. All individuals of the same species shared identical length of the rDNA IGS. The single amplicon provided evidence to confirm the identical length (single type) of the rDNA IGS rather than multiple lengths (i.e. 28S-IGS-18S) within the individuals of same species, while it may vary considerably among the Tigriopus spp. (Burton et al., 2005).

**Phylogenetic position of T. japonicus among the Harpacticoida as inferred from 18S rDNA**

Nuclear rDNA has been useful in molecular systematics to estimate divergence and phylogenetic relationships between taxa (Kellogg, 1998). Due to the high level of conservation among these sequences, nuclear rDNA has particularly been applied to delineating higher taxonomic levels, e.g. families and orders. In contrast, ITS regions are considered to be useful in defining intra-specific differences, because they are less subject to functional constraints and would evolve more rapidly. Therefore, we investigated copepod phylogeny that was inferred from complete 18S rDNA of representatives belonging to different orders. We reconstructed phylogenetic trees of the Copepoda with the addition of the three free-living orders Calanoida, Harpacticoida and Pycnogonida in order to reveal the place of the harpacticoid Tigriopus within the Harpacticoida. We also used some copepod species that were earlier studied by Huys et al. (Huys et al., 2006, 2007). A Bayesian tree showed distinct clades that separate the various orders (Fig. 3). Additional ML analysis of the same data set generated nearly identical branch patterns when compared with those of the Bayesian prediction mentioned earlier. Therefore, bootstrap values from the ML analysis were incorporated into the Bayesian tree to indicate the robustness of each branch. Bayesian analysis of 18S rDNAs showed that copepods were separated into two clades: the subclass Gymnochaeta (where the primarily planktonic Calanoida belong), and the subclass Podoplea, including five of the orders mentioned earlier (Fig. 3A and B). In addition, the six copepod orders (e.g. Calanoida, Cyclopoida, Harpacticoida, Pycnogonida, Pycnogonida and Siphonostomatoida) cluster according to their taxonomic belonging at order level (Fig. 3A). However, in the Podoplea lineage, the
Fig. 3. Phylogenetic relationships of six copepod orders (A, unrooted Bayesian tree) and five harpacticoid families (C, rooted Bayesian tree) according to Bayesian analyses, including ML bootstrap values. The tree of (B) was generated from the tree of (A) with unscaled branches. The copepod tree was inferred from near complete 18S rDNA sequences (a total of 35) of copepod members, including Calanoida, Cyclopoida, Harpacticoida, Monstrilloida, Pocelostomatoida and Siphonostomatoida. In the phylogeny of Harpacticoida, 17 18S sequences from various T. californicus isolates are collapsed into a triangle named T. californicus, and the cyclopoid Pachos sp. (GenBank No. AY627014) was used as outgroup. The numbers (top) at the nodes are PP in which PP > 0.50 are indicated at each node; the numbers (bottom) are bootstrap values (%) for
relationship between Harpacticoida, Monstrilloida, and Siphonostomatoida could not be resolved. The Podoplea clade was separated from that of the Cyclopoida, supported by 0.88 of PP and 100% of bootstrap value. This finding is in accordance with the morphological classification of the Copepoda, as well as with previous molecular phylogenetic studies (Huys et al., 2006, 2007). However, a wider phylogenetic analysis of the Harpacticoida with complete 18S sequences is restricted due to a lack of known sequences from this copepod group. Hence, we investigated the molecular relationship due to a lack of functional constraints, while addressing variation caused by a lack of functional constraints. ITS is useful for population level studies due to an elevated branch of the ML trees as computed by PAUP* 4.0b10 in which bootstrap values >50% are indicated at each node. A total of 69 copepod 18S rDNA sequences included here were as follows: 39 Harpacticoida (listed in Table I), 12 Cyclopoida (Calanus finmarchicus, EU054307). In addition, we compared the sequences of those 18S rDNA ITSs in T. japonicus with a wide geographical range. We investigated ITS sequences of 15 Asian Tigriopus populations collected from areas as geographically disjunct as Thailand, Hong Kong, Taiwan, Korea, China and Japan (Table I). Tigriopus was found

Geographical distribution of T. japonicus inferred from ITS and 5.8S sequences

ITS is useful for population level studies due to an elevated variation caused by a lack of functional constraints, since it only serves as a spacer between 18S and 28S. In the present study, we focused on genetic variations of rDNA ITSs in T. japonicus with a wide geographical range. We investigated ITS sequences of 15 Asian Tigriopus populations collected from areas as geographically disjunct as Thailand, Hong Kong, Taiwan, Korea, China and Japan (Table I). Tigriopus was found

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<th>Isolate</th>
<th>Similarity (%)</th>
<th>Distance (%)</th>
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<td>0.3</td>
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<td>BK</td>
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*The number of individuals tested. SD, standard deviation.
China, Korea and Japan, and each predicted clade was according to their geographical origin (Fig. 4), such as *Tigriopus* californicus. The ITS1 lengths are quite dissimilar among various geographical samples. Among them, the ITS1 of T. japonicus is considerably different from those of *T. californicus* (e.g. 61.9% similarity, 0.4114 genetic distance). In addition, the ITS1 lengths are quite dissimilar among various geographical samples of *T. japonicus*, ranging from 412 bp (SC) to 463 bp (BK). Overall, the ITS1 of *T. californicus* is longest (480 bp, AY599492), whereas *T. brevicornis* has a short ITS1 (412 bp; AM083336). Besides, the ITS2, and 5.8S sequence lengths were nearly identical to those of *T. californicus*. However, the GC composition was different among the three *Tigriopus* species compared here, and it is also different among the populations of *T. japonicus*.

Biogeographically, Bayesian inference showed that the Asian *Tigriopus* populations included here clustered according to their geographical origin (Fig. 4), such as China, Korea and Japan, and each predicted clade was supported by high PP values (1.00). In addition, eight Korean *Tigriopus* populations were divided into two clades, with high PP values. A clade of south and southwest Korean *Tigriopus* populations showed a sister relationship with a clade of south Chinese populations. On the other hand, two *T. japonicus* populations from eastern coastal waters of Korea formed a separate cluster, a polytomy with *T. californicus* and the other *T. japonicus* populations. Two populations of *T. japonicus* from Japan formed a clade, and were separated from the other populations.

Of the 15 Asian *Tigriopus* populations, we numerically compared 11 full ITS-5.8S rDNA sequences, including the corresponding mitochondrial COI sequences (Jung et al., 2006), and found that similarities and genetic distances calculated between the ITS-5.8S pairs were generally similar to those of the COI gene sequences (Table III). Quantitatively, mean β-distance corrected with the Kimura two-parameter model was recorded as higher at the mitochondrial COI gene (0.219) than at the nuclear non-coding ITSs (0.106). This suggests that the ITS region in *T. japonicus* is relatively more conserved against the partial COI gene (~2.07 times in evolutionary rate) over evolutionary history. This is in accordance with a previous report (Schizas et al., 1999) that showed that the ITS region was more conserved than the mitochondrial cytochrome b in the
harpacticoid copepod Microarthridion littorale. This population genetic analysis demonstrated that *T. japonicus* is comprised of distinct populations of increasing genetic distances along the coastal areas of the Northwest Pacific rim in the Far East (Fig. 4). Considering these genetic characteristics (Table III), the tree topology (Fig. 4), as well as their geographical origins, we separated 15 Asian *Tigriopus* populations into four groups of genotypes, designated as a South Chinese group (including specimens from Thailand, Hong Kong and Taiwan), Southwest Korean, East Korean and Japanese group. Similarities within the same types (Table III) were considerably high (>94% similarity in the Southwest Korean group; >95% in the South Chinese group); however, similarities between the four groups (e.g. Korea vs. China, Korea vs. Japan) were significantly lower (<78% similarity). These data suggest that four groups identified from Asian *Tigriopus* populations may represent four different (sub)species. Their morphological differences are much less clear. However, Chullasorn *et al.* (Chullasorn *et al.*, unpublished results) recently observed *Tigriopus* specimens from Thailand that seemed to be morphologically distinct.

**CONCLUSIONS**

Copepod genus *Tigriopus* is widely distributed along the coastlines of East Asian countries such as China, Korea, Japan and Taiwan. As noted previously, all their identities have been considered as a single species of *T. japonicus* to date. The present study, however, showed that the Asian *Tigriopus* could be subdivided into four different genetic lineages, as judged by the divergence of nuclear rDNA ITS genes. Phylogenetic analysis separated the *Tigriopus* populations along the coastline of the Northwest Pacific rim into four distinct clades. These were concordant with their geographical locations, such as China, Japan and Korea (including two Korean groups). At present, we did not address why they have genetically diversified and how we can discriminate to four different species. In East Asia, the warm Kuroshio Current and the Tsushima Current from the south China Sea provide a mild climate even in winter along the Northwest Pacific coastline of the Far East. These oceanographic features probably inhibit gene flow between geographically separated populations of Asian *Tigriopus*. Once there is geographical isolation, *T. japonicus* populations may diverge as a consequence of genetic
drift and adaptation to different environments. The population genetics of *T. japonicus* should expand by including more geographical locations and the phylogenetic relationships of taxa could be revised, considering evidence from traditional structural data sets (morphology), molecular characters and by crossbreeding experiments that would prove or disprove their status as biological species. In this aspect, the full sequence of *T. japonicus* nuclear rDNA including the ITS and IGS regions can be used as a molecular marker of copepod nuclear rDNA as well as population history for closely related species.

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