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Molecular Phylogenetic Analysis of *Euphausia pacifica*, *Thysanoessa longipes* and *T. inermis* (Crustacea : Euphausiacea) in the Subarctic Pacific Region, with Notes on Non-Geographical Genetic Variations for *E. pacifica*

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Abstract

Nucleotide sequences of a 1,854 base pair region of the nuclear small subunit (18S) rDNA gene and of a 475 or 476 base pair region of the mitochondrial large subunit (mt16S) rRNA gene were determined for *Euphausia pacifica* collected from the western/eastern subarctic Pacific, Okhotsk Sea and Japan Sea, and *Thysanoessa inermis* from the Okhotsk Sea *T. inermis* from the Okhotsk Sea and Bering Sea. Interspecific differences were 0.16–0.86% for 18S rDNA gene and 2.3–9.3% for 16S rRNA gene. Intraspefic differences are of special interest of *E. pacifica* from geographically distant sites, but were very small (0.0–<0.1% for 18S rDNA, and 0.0–0.4% for 16S rRNA). The lack of the regional haplotype structure in *E. pacifica* suggests significant gene flow mediated by the ocean-scale circulation system for those living in the west-east subarctic Pacific and Bering Sea. For the Japan Sea population which has been isolated from populations in the Subarctic Current originating 8,000 years ago, the time scale is considered to be not long enough to observe variations. On the basis of phylogenetic trees established, the position of the three euphausiids was briefly discussed.

Key words : *Euphausia pacifica*, *Thysanoessa inermis*, *Thysanoessa longipes*, 18S rDNA, 16S rRNA, subarctic Pacific, population genetics

Introduction

Eighty-five species of euphausiids are known in the world oceans (Mauchline and Fisher, 1969). In the epipelagic zone of the entire subarctic Pacific Ocean and its marginal seas, the three euphausiids including *Euphausia pacifica*, *Thysanoessa longipes* and *T. inermis* predominated (cf. Brinton, 1962 ; Mauchline and Fisher, 1969). These three euphausiids prey largely on phytoplankton and are predated upon various pelagic and demersal fishes, whales and seabirds (Nicol and Endo, 1997 ; Everson, 2000), thus forming a vital link between primary production and production of animals at higher trophic levels. Of the three euphausiids, a large regional variation in life history parameters (spawning season, growth pattern, age at maturity, longevity, etc) have been well documented on *E. pacifica* (cf. review of Siegel, 2000). It is unclear that presence of genetically separated populations in *E. pacifica* dependent on environmental conditions which differ from one region to the next. In contrast to *E. pacifica*, little is known about life history of *T. spinifera* and *T. inermis* in the subarctic Pacific region (Iguchi and Ikeda, 2004).

Analysis of several mitochondrial genes for euphausiids [e.g. the ND1 gene for *Euphausia superba* (Zane et al., 1998) and *Meganyctiphanes norvegica* (Zane et al., 2000 ; Papetti et al., 2005), the COI and CYB genes for *M. norvegica* (Bucklin et al., 1997), and the 16S rRNA and/or COI genes for eight species (mostly *Euphausia* spp. in the Southern Ocean)(Patarnello et al., 1996 ; Jarman et al., 2000)] reveals phylogenetic relationship among species or genetic diversity within the same species. Nuclear 18S rDNA gene has been also used for reconstructing phylogenetic tree of crustaceans (Spears et al., 1994 ; Spears and Abele, 1997 ; Bucklin et al., 2003).

In this study, we investigate nucleotide sequences of the nuclear 18S rDNA gene and the mitochondrial 16S rRNA gene of *E. pacifica*, *T. longipes* and *T. inermis*. Furthermore, sequence variabilities in these two genes were examined on *E. pacifica* collected from geographically distant regions to examine whether there exist regional populations.

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Material and methods

Specimens of *Euphausia pacifica* were collected during 2000 at four sites in the western subarctic Pacific (WSP1 ; 42°00′N 141°30′E in June, WSP2 ; 45°30′N 145° 10′E in May, WSP3 ; 38°40′N 144°00′E in April, and WSP4 ; 38°20′N 144°00′E in April) and at two sites in the eastern subarctic Pacific (ESP1 ; 49°40′N 124°14′W in May, and ESP2 ; 47°59′N 144°58′W in July), at one site in the Japan Sea (JS ; 37°16′N 137°40′E in July) and at one site in the Okhotsk Sea (OS ; 45°30′N 145°10′E in May) (Fig. 1). *T. inermis* specimens were collected at the OS, and *T. longipes* at the OS and at an additional site in the Bering Sea (BS ; 56°00′N 166°00′E in July). All collections were made obliquely or vertically with Bongo or similar nets from <500 m depth. At each collection, specimens were sorted immediately on board the ship, preserved in 95% ethanol and stored at <10°C in the dark.

Preserved specimens were dissected and only abdomens of which exoskeleton removed were used in the following analyses. Individual abdomens were rehydrated in distilled water for overnight, and homogenized using a grinder. Genomic DNA was extracted from the homogenate using the Wizard DNA extraction kit (Promega). 18S rRNA genes were amplified using universal primer set for eukaryotes and directly sequenced as previously described (Taniguchi et al., 2004). For amplification of mt16S rRNA as DNA template, the homogenates treated with Chelex 100 (Sigma) were used (see Taniguchi et al., 2004). 16S-arL and 16S-CB primer set, latter primer is known to produce superior PCR products for wide-ranging crustaceans, was used for the amplification (Braga et al., 1999).

For phylogenetic analyses, sequences were compared against the current version of the public databases to retrieve the 100 closest relatives of each sequence. These neighboring sequences were then included in the databases of aligned sequences of this study. A set of sequences related to the Euphuasiacea could thus be selected for the present analysis in order to root the Euphausiacea clade. All sequences were manually aligned by reference to our databases of already aligned sequences. Phylogenetic trees were constructed according to three different methods [Neighbor-Joining (NJ), Maximum Likelihood (ML) and Maximum Parsimony (MP)]. For the NJ analysis, a matrix distance was calculated according to the Kimura 2 parameters correction. Bootstraps were done using 1,000 replications, bioNJ and Kimura two parameters corrections. BioNJ was according to Gascuel (1997), maximum likelihood and maximum parsimony data were from PHYLIP (Phylogeny Inference Package, version 3.573c, distributed by J. Felsenstein, Department of Genetics, UW,

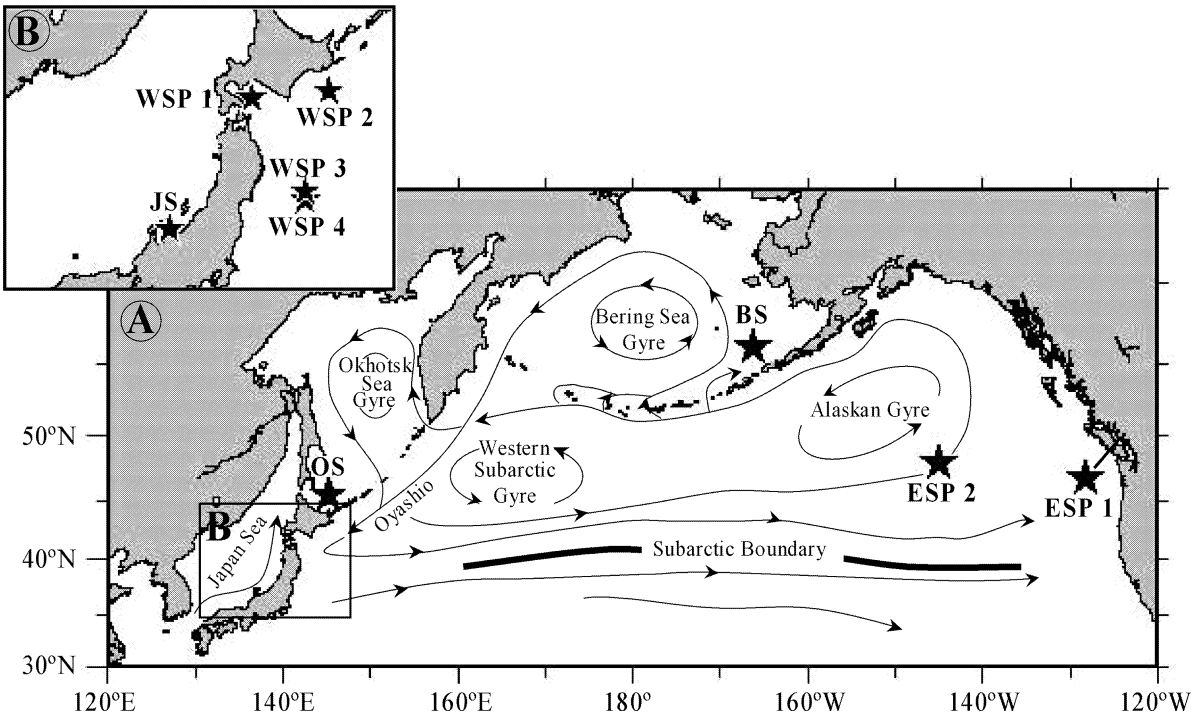

Fig. 1. Map showing collection sites (asterisks) of *Euphausia pacifica* in the western (WSP1 to 4) and eastern subarctic Pacific (ESP 1 and 2), Japan Sea (JS), and Okhotsk Sea (OS). *Thysanoessa longipes* was collected at OS, and *T. inermis* at OS and Bering Sea (BS). Schematic diagram of current systems is superimposed (redrawn from Favorite et al. 1976).

Seattle, WA, USA). The phylogenetic trees were drawn using NJPLOT (Perriere and Gouy, 1996) and Clarisdraw softwares for Apple Macintosh. Domains used to construct phylogenetic trees were regions available for all sequences and excluding positions likely to show homoplasy (Taniguchi et al., 2004).

Results

The data of 18S rDNA sequences (1854bp) for *Euphausia pacifica*, *Thysanoessa longipes* and *T. inermis* obtained in the present study were deposited in the GenBank/EMBL/DDBJ database under accession number AY141010 to AY141012.

A consensus nucleotide sequence of 18S rDNA was determined by comparing 13 sequences obtained from individual specimen of *Euphausia pacifica*. The variations in the 18S rDNA sequences between *E. pacifica* and *Thysanoessa* spp. were 15 nucleotides (0.81%), and those between *T. longipes* and *T. inermis* were 3 nucleotides (0.16%) (Table 1A). Thus, overall interspecific differences were less than 0.86%. Out of 13 specimens of *E. pacifica* analyzed in this study, intraspecific variations were observed in four specimens (JS2, JS3, OS, and WSP3) at three nucleotide sites [240 (JS2 and JS3), 449 (OS), and 1130 (WSP3) at *E. pacifica* 18S rDNA position], respectively. The phylogenetic analysis based on 18S rDNA sequences identified euphausiids (*Euphausia* and *Thysanoessa*) and *Thysanoessa* (*T. longipes* and *T. inermis*) as a robust clade supported by all phylogenetic analysis (NJ, MP, and ML) with more than 90% bootstrap value (Fig. 2); however, many relationships within this group could not be resolved, largely because of current paucity of the data of this gene on other euphausiid species at present.

Partial mt16S rRNA sequences of *E. pacifica* (475bp), *T. intermis* (475bp) and *T. longipes* (476bp) were determined. These sequences were deposited to GenBank/EMBL/DDBJ databases under accession numbers AY688435 to AY688461. Haplotypic sequences observed were 0.0–0.4% (0–2 sites difference in 474bp) for each batch of the three specimens of *E. pacifica* from eight geographically distant regions, 0.2–1.1% (1–5 sites in 475) for the three specimens of *T. longipes* from the Bering Sea (site101), and 0.0–0.2% (0–1 site in 474bp) for the three specimens of *T. inermis* from the Okhotsk Sea (site 56) (Table 1C). Within the three euphausiid species analyzed, similarities of sequences were 91.7% between *E. pacifica* and *T. longipes* (39 differences), 91.1% between *E. pacifica* and *T. inermis* (42 differences), and 97.3% between *T. longipes* and *T. inermis* (13 differences). Thus, phylogenetic affiliation based on mt16S rRNA sequence demonstrated monophylies of the species *E. pacifica*, *T. longipes* and *T. inermis* (Fig. 3).

Discussion

Nuclear 18S rDNA is known to occur in the genomes of all living organisms, but it evolves at a relatively slow rate owing to its functional role in ribosome formation and protein synthesis (cf. Spears and Abele, 1997). For this reason, the 18S rDNA has been used for broad analyses such as the entire cirriped phylogeney (Spears et al., 1994), higher-order crustacean phylogeny (Spears and Abele, 1997) and evolutionary history from invertebrates to vertebrates (Wada and Saitoh, 1994). In contrast with nuclear DNAs which experience recombination at each generation, mitochondrial DNA (including 16S rRNA gene) is considered to be inherited from the maternal parent without recombination in almost organisms (cf. Avise et al., 1987). Mitochondrial DNA exhibits greater evolutionary rate than nuclear DNAs (Birky et al., 1989), thus allowing greater population differentiations. The present results suggest that nuclear 18S rDNA is useful to analyze phylogenetic relationships above the genus level, but that it is of limited use to analyze the specific differentiations of euphausiids, as was shown to oceanic copepods in our previous study (Taniguchi et al., 2004).

The nuclear 18S rDNA gene in euphausiids has not been studied until the present study. Then, the present results on both genes are compared with those of marine planktonic copepods. A divergence in the range of $<0.2\%$ for between-species of *Neocalanus* spp. and 1.3 to 1.4% between the genera of *Neocalanus* and *Calanus* has been reported in 1802bp regions of 18S rDNA (Taniguchi et al., 2004), both of which are near comparable to the present results (0.2% between *Thysanoessa* spp. and 0.8–1.1% between *Euphausia* and *Thysanoessa*, cf. Table 1A). The higher divergence in the order of 1.8 to 24.3% in ca. 1600–1800 base pair regions of the 18S rDNA has also been documented for cirriped crustaceans belonging to different suborders or orders (Spears et al., 1994).

The nucleotide sequences of the mitochondrial 16S rRNA of eight euphausiids has been analyzed by Partarnello et al. (1996) and Jarman et al. (2000) but they gave no information about interspecific and intraspecific variations of the sequence. In the same study of Taniguchi et al. (2004) mentioned above, the within-species (*Neocalanus* spp.) and between-species (*Neocalanus* vs. *Calanus*) divergence was 4.1–6.1 and 16.9–21.7%, respectively, for a 410bp region of the 16S rRNA, which is much greater than 2.3–2.7% for *Thysanoessa* spp. for the former and 8.7–9.3% for *Euphausia* vs. *Thysanoessa* for the latter in the present

Table 1 Similarity matrixes of 18S rDNA sequence (1,854 sites) (A) and mt16S rRNA (475 or 476 sites (B) of *Euphausia pacifica* (EP) from western subarctic Pacific (WSP), eastern subarctic Pacific (ESP), Japan Sea (JS) and Okhotsk Sea (OS), *Thysanoessa longipes* (TL) from OS, and *T. inermis* (TI) from OS and Bering Sea (BS), and (C) intraspecific variations in these genes collected at the same sites.

(A)

Species/region	EP/WSP1	EP/WSP2	EP/WSP3	EP/WSP4	EP/ESP1	EP/ESP2	EP/OS	EP/JS (1)	EP/JS (2)	EP/JS (3)	EP/JS (4)	EP/JS (5)	EP/JS (6)	TL/OS	TI/OS
EP/WSP1	100														
EP/WSP2	100	100													
EP/WSP3	99.9	99.9	100												
EP/WSP4	100	100	99.9	100											
EP/ESP1	100	100	99.9	100	100										
EP/ESP2	100	100	99.9	100	100	100									
EP/OS	99.9	99.9	99.9	99.9	99.9	99.9	100								
EP/JS(1)	100	100	99.9	100	100	100	99.9	100							
EP/JS(2)	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100						
EP/JS(3)	99.9	99.9	99.9	99.9	99.9	99.9	99.9	99.9	100	100					
EP/JS(4)	100	100	99.9	100	100	100	99.9	100	99.9	99.9	100				
EP/JS(5)	100	100	99.9	100	100	100	99.9	100	99.9	99.9	100	100			
EP/JS(6)	100	100	99.9	100	100	100	99.9	100	99.9	99.9	100	100	100		
TL/OS	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.2	99.2	100	
TI/OS	99.2	99.2	99.1	99.2	99.2	99.2	99.1	99.2	99.1	99.1	99.2	99.2	99.2	99.8	100

(B)

Species/region	EP/WSP1 (1)	EP/WSP1 (2)	EP/WSP1 (3)	EP/WSP2 (1)	EP/WSP2 (2)	EP/WSP2 (3)	EP/WSP3 (1)	EP/WSP3 (2)	EP/WSP3 (3)	EP/WSP4 (1)	EP/WSP4 (2)	EP/WSP4 (3)	EP/ESP1 (1)	EP/ESP1 (2)	EP/ESP1 (3)	EP/ESP2 (1)	EP/ESP2 (2)	EP/ESP2 (3)	EP/OS (1)	EP/OS (2)	EP/OS (3)	EP/JS (1)	EP/JS (2)	EP/JS (3)
EP/WSP1(1)	100																							
EP/WSP1(2)	100	100																						
EP/WSP1(3)	100	100	100																					
EP/WSP2(1)	100	100	100	100																				
EP/WSP2(2)	100	100	100	100	100																			
EP/WSP2(3)	100	100	100	100	100	100																		
EP/WSP3(1)	100	100	100	100	100	100	100																	
EP/WSP3(2)	100	100	100	100	100	100	100	100																
EP/WSP3(3)	100	100	100	100	100	100	100	100	100															
EP/WSP4(1)	100	100	100	100	100	100	100	100	100	100														
EP/WSP4(2)	100	100	100	100	100	100	100	100	100	100	100													
EP/WSP4(3)	100	100	100	100	100	100	100	100	100	100	100	100												
EP/ESP1(1)	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	100											
EP/ESP1(2)	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	100	100										
EP/ESP1(3)	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	100	100									
EP/ESP2(1)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100								
EP/ESP2(2)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100	100							
EP/ESP2(3)	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	100						
EP/OS(1)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100	100	99.8	100					
EP/OS(2)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100	100	99.8	100	100				
EP/OS(3)	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.6	99.6	99.6	99.8	99.8	99.6	99.8	99.8	100			
EP/JS(1)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100	100	99.8	100	100	99.8	100		
EP/JS(2)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100	100	99.8	100	100	99.8	100	100	
EP/JS(3)	100	100	100	100	100	100	100	100	100	100	100	100	99.8	99.8	99.8	100	100	99.8	100	100	99.8	100	100	100

(B)-continued

	TL/OS (1)	TL/OS (2)	TL/OS (3)	TI/BS (1)	TI/BS (2)	TI/BS (3)	EP*
TL/OS(1)	100						
TL/OS(2)	99	100					
TL/OS(3)	99.2	99.8	100				
TI/BS(1)	97.3	97.3	97.5	100			
TI/BS(2)	97.3	97.3	97.5	100	100		
TI/BS(3)	97.5	97.5	97.7	99.8	99.8	100	
EP*	91.3	90.7	90.9	90.9	90.9	90.9	100

*most frequent sequence

(C)

	Sequence heterogeneity (%)/site differed	
18S rDNA	EP/JS (n=6)	0.05/1
16S rRNA	EP/WSP1 (n=3)	0/0
	EP/WSP2 (n=3)	0/0
	EP/WSP3 (n=3)	0/0
	EP/WSP4 (n=3)	0/0
	EP/ESP1 (n=3)	0.2/1
	EP/ESP2 (n=3)	0/0
	EP/OS (n=3)	0.2/1
	EP/JS (n=3)	0/0
	TL/JS (n=3)	0.6/3
	TI/BS (n=3)	0/0

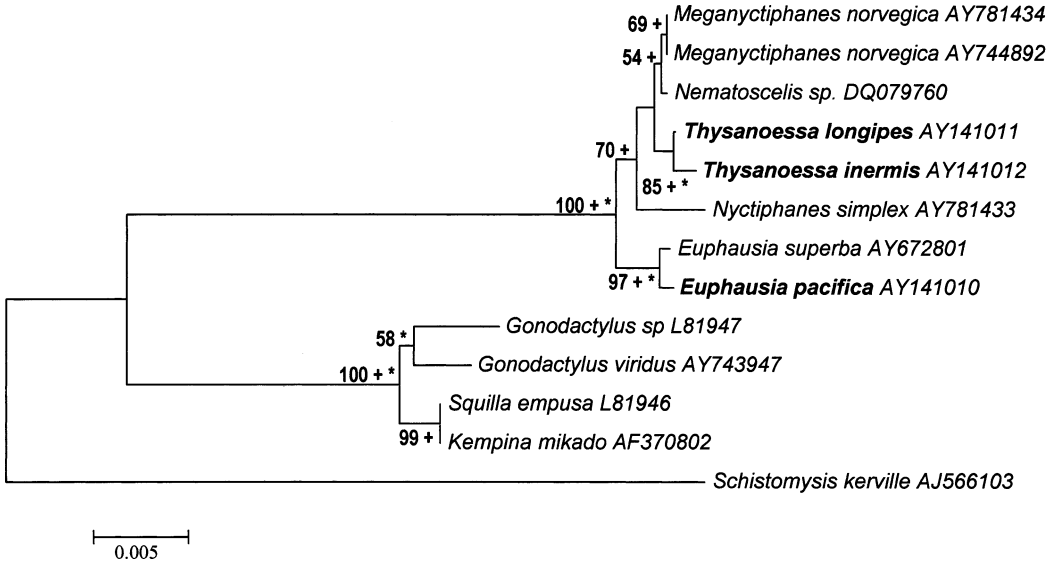

Fig. 2. Unrooted neighbor-joining tree of euphausiid species belonging to the genera *Euphausia* and *Thysanoessa* based on nuclear 18S rDNA gene sequences (the present data are in bold face). Codes denote accession numbers deposited in GenBank/EMBL/DDJB database. Numbers at nodes are bootstrap proportions (%) based on 100 replicates. Nodes supported by Maximum Parsimony method (+) or Maximum Likelihood method at $p<0.01$(*) are shown. Scale : accumulated changes per nucleotide.

study (Table 1B). Euphausiids appear to exhibit less within- and between-species variations in the 16S rRNA nucleotide sequences, since the divergence as high as 7 to 24% for between-species of *Calanus* spp. and 44 to 56% for between the genera *Calanus* and *Metridia* has been evaluated in 387bp regions of this gene (Bucklin et al., 1995). The present results showed that intraspecific variations of the 18S rDNA gene in the six specimens of *E. pacifica* from the Japan Sea (<0.4%), and the 16S rRNA among the specimens of *E. pacifica* from eight geographically distant locations (0.2%) are very small (Table 1).

The phylogenetic trees based on the 18S rDNA and 16S rRNA indicated that *Euphausia pacifica*, *Thysanoessa longipes* and *T. inermis* are a clade (Figs. 2 and 3). The 18S rDNA tree shows closer lineage of the three euphausiids with those of the superorder Hoplocarida rather than the other members of the same Eucarida, making Hoplocarida as a paraphylic taxon. Because of the paucity of available data of the former, the results (Fig. 3) from the latter are more informative to discuss geographical distribution and speciation of the genera *Euphausia* and *Thysanoessa*. Jarman et al. (2000), using the 16S rRNA/CO1 genes, investigated phylogenetic relationships among eight *Euphausia* spp. (mostly Southern Ocean species with only a North Pacific species *E. pacifica*), and suggested vicariant speciation of Southern Ocean *Euphausia* spp. in the north (sub-Antarctic) and south (Antarctic) of the Antarctic Convergence since it established ca. 15 million years ago. Thus, the seven *Euphausia* spp. are separated into Antarctic and sub-Antarctic clades, with only *E. pacifica* being outside the two clades. Allopatric speciation resulting from population subdivision has been considered as one of the major factors for causing the genetic differentiation that leads to speciation (Ovenden, 1990). The phylogenetic tree for the 16 euphausiid species established in this study (Fig. 3) is somewhat different from Jarman et al's results ; typical Antarctic euphausiids (*E. superba* and *E. crystallorophias*) and *E. pacifica* are the closest species among the other Antarctic euphausiids [*E. triacantha* and *E. longirostris*, cf. Fig. 1 in Jarman et al. (2000)].

16S rRNA sequence data of *Thysanoessa* spp. other than *T. longipes* and *T. inermis* of this study are limited to those of *T. macrura* in the Southern Ocean. The phylogenetic tree for the three species supports strongly that the three species are monophyletic lineage (Fig. 3). Within this clade, one specimen of *T. longipes* (JS1) diverged from the other two (JS2 and JS3). The sequence difference (4 nucleotids or 1% of the sequence) of this order of magnitude has been observed among subspecies of a copepod *Calanus pacificas* (Bucklin et al., 1995). Our careful examination of *T. longipes* samples from the Japan Sea revealed admixture of two morph types known in this species (spined and spineless forms, cf. Brinton, 1962) and we did not separated them in the present analysis.

From the analysis of ND1 gene, the existence of genetically and geogaphycally distinct local populations has been reported for *Meganyctiphanes norvegica* in the northeastern Atlantic Ocean and Mediterranean Sea

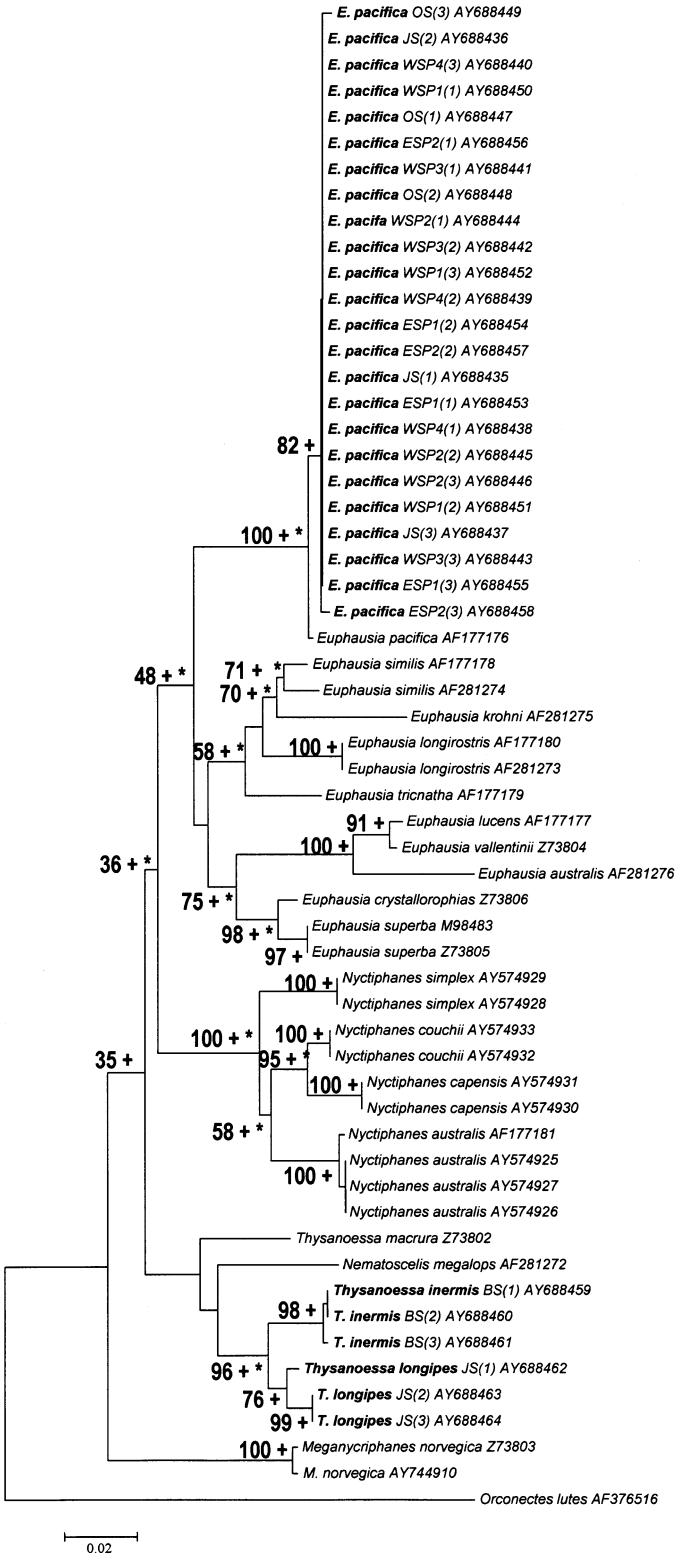

Fig. 3. Unrooted neighbor–joining tree based on mitochondrial 16S rDNA sequences for *Euphausia* spp., *Thysanoessa* spp. and others (the present data are in bold face). Codes denote accession numbers deposited in GenBank/EMBL/DDJB database. For *Euphausia* spp. and *Thysanoessa* spp., extra capital letters and numbers in parentheses between species names and accession numbers are collection sites (WSP : western subarctic Pacific, ESP : eastern subarctic Pacific, JS : Japan Sea, OS : Okhotsk Sea, and BS : Bering Sea, cf. Figure 1), and individual number analyzed, respectively. Numbers at nodes are bootstrap proportions (%) based on 1,000 replicates. Nodes supported by Maximum Likelifood method (+) or Maximum Parsimony method at $p<0.01$(*) are also shown. Scale : accumulated changes per nucleotide.

(Zane et al., 2000 ; Papetti et al., 2005). Despite documentation of a marked regional difference in life history parameters of *Euphausia pacifica* across West-East subarctic Pacific and its adjacent seas (cf. Siegel, 2000), the present results of both 18S rDNA and 16S rRNA genes of the specimens from the four sites in the western subarctic Pacific, two sites in the eastern subarctic Pacific, and the single sites in the Japan Sea and Okhotsk Sea (Fig. 1) indicate extremely low divergence (<0.4%, Table 1A, B). Considering geographical distances among these four sampling sites of *E. pacifica* [maximum ; ca. 8,000 km between the JS (in the Japan Sea) and ESP1 (in the eastern subarctic Pacific) sites] and possible topographical barriers of Japan Islands for the Japan Sea population and Kuril Islands for the Okhotsk Sea population) (see Fig. 1), the lack of genetic structure of this euphausiid is rather surprising. However, such the lack of genetic structure has already been noted on a copepod *Neocalanus cristatus* of which geographical distribution overlaps *E. pacifica* (Taniguchi et al., 2004). As interpreted by Taniguchi et al. (2004) for *N. cristatus*, the lack of regional gene structure of *E. pacifica* may be due to a large oceanic circulation system aiding their gene dispersal throughout the entire subarctic Pacific and Okhotsk Sea (Fig. 1).

This oceanic current-aided gene flow explanation cannot be applied to the *Euphausia pacifica* population in the Japan Sea, where the effect of the subarctic circulation is interrupted by Japan Islands (Fig. 1). Paleooceanological evidences suggest that the Japan Sea has been isolated from the effect of subarctic water since 8,000 yr ago (Oba et al., 1991). However, the time scale of this order (8,000 yr) appears to be too short to induce any detectable nucleotide divergence in mitochondrial 16S rRNA gene if one assumes the evolution rates of the gene to be 0.69% (±0.12) per million years (cf. Jarman et al., 2000).

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