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Author(s)	Azuma, Noriko; Grant, William S; Templin, William D; Kunihiro, Yasushi; Mihara, Eiji; Yanagimoto, Takashi; Abe, Syuiti
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## **Molecular Phylogeny of a Red-Snow-Crab Species Complex using Mitochondrial and Nuclear DNA Markers**

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# Molecular Phylogeny of a Red-snow-crab Species Complex Using Mitochondrial and Nuclear DNA Markers

Noriko Azuma<sup>1\*</sup>, William S. Grant<sup>2,3</sup>, William D. Templin<sup>2</sup>, Yasushi Kunihiro<sup>4</sup>, Eiji Mihara<sup>5</sup>, Takashi Yanagimoto<sup>6</sup> and Syuiti Abe<sup>7</sup>

<sup>1</sup>Nodai Bioresource Institute, Tokyo University of Agriculture, 196 Yasaka, Abashiri, Hokkaido 099-2493, Japan

<sup>2</sup>Gene Conservation Laboratory, Alaska Department of Fish and Game, 333 Raspberry Road, Anchorage, Alaska 99518, USA

<sup>3</sup>Department of Biological Sciences, University of Alaska Anchorage, Anchorage, Alaska 88508, USA

<sup>4</sup>Mariculture Fisheries Research Institute, Hokkaido Research Organization Fisheries Research Department, 1-156-3 Funami-cho, Muroran, Hokkaido 051-0013, Japan

<sup>5</sup>Wakkanai Fisheries Research Institute, Hokkaido Research Organization Fisheries Research Department, 4-5-15 Suehiro, Wakkanai, Hokkaido 097-0001, Japan

<sup>6</sup>National Research Institute of Far Seas, Fisheries Research Agency, 2-12-4 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-8648, Japan

<sup>7</sup>Division of Marine Bioscience, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

Nucleotide sequence variation of mitochondrial DNA COI and nuclear rRNA gene regions was used to reconstruct phylogenetic relationships for the red-snow-crab species complex, including the red snow crab, *Chionoecetes japonicus*, its nominal subspecies, *C. japonicus pacificus*, and the triangle tanner crab, *C. angulatus*. The topologies of the Bayesian and neighbor-joining (NJ) trees of the COI and of NJ trees of rRNA sequences placed *C. japonicus* and *C. angulatus* in a single clade. The net sequence divergence between these taxa was  $d_{\text{net}} = 0.000$  in COI, and strongly suggests that these taxa represent a single species. In contrast, haplotypes in *C. j. pacificus* clustered separately from the *C. japonicus* – *C. angulatus* clade. Net sequence divergence from *C. japonicus* – *C. angulatus* to *C. j. pacificus* was  $d_{\text{net}} = 0.026$  in COI, indicating that *C. j. pacificus* should be elevated to a separate species, *C. pacificus*. A 165 bp insert appeared in the rRNA gene of *C. j. pacificus*, but was absent in the remaining species of *Chionoecetes*. This autapomorphic condition in *C. j. pacificus* adds support for an independent evolution of this taxon. Evolutionary divergences between these taxa may reflect contrasting evolutionary process influenced by ocean bathymetry.

**Key words:** molecular phylogeny, cytochrome oxidase subunit 1, speciation, rDNA, Decapoda

## INTRODUCTION

Marine animals with planktonic larvae potentially disperse long distances in ocean-currents, and generally show little genetic population structure over the species' range (Palumbi, 1994). For example, populations of sea urchins in the genera *Echinometra* and *Diadema* tend to show little divergence from one another on regional scales along a coast. However, on ocean-wide scales, these genera are subdivided into several species (McCartney et al., 2000; Lessios et al., 2001). Most models invoke divergence and

reproductive isolation in allopatry to account for marine speciation (e.g. Palumbi, 1994; Floeter et al., 2008; Rocha and Bowen, 2008). Populations can become geographically isolated for several reasons, including shoreline changes from tectonic movements, drops in sea level, and changes in current patterns that redirect larval dispersals (Palumbi, 1994).

Crabs in the genus *Chionoecetes* (Decapoda, Brachyura) are widely distributed in the cold-temperate and subpolar waters of the Northern Hemisphere. These crabs have large body sizes and relatively large populations, which support economically important fisheries in the USA, Canada, Russia, and Japan. The genus presently includes five species, divided into two groups based on vertical distribution. Shallow-water species on continental shelves include the Tanner crab, *C. bairdi* Rathbun, 1893, and snow crab, *C. opilio* Fabricius, 1788. Deep-water species on the continental slope include the red snow crab, *C. japonicus*

\* Corresponding author. Phone: +81-152-48-3903;  
Fax : +81-152-48-3897;  
E-mail: anoriko@fish.hokudai.ac.jp

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Rathbun, 1932, triangle Tanner crab, *C. angulatus* Rathbun, 1893, and grooved Tanner crab, *C. tanneri* Rathbun, 1893 (Jadamec et al., 1999).

Populations of *C. japonicus* extend from the central Sea of Japan and along the Pacific coast of northern Japan to the Sea of Okhotsk. Adults occur at 500–3000 m depth, with the greatest abundances at about 1000 m (Yosho and Hayashi, 1994). *Chionoecetes angulatus* inhabits deep waters extending from the Sea of Okhotsk, across the Bering Sea, and into the Northeast Pacific as far as Oregon (Jadamec et al., 1999). Although the distributions of both species overlap in the Sea of Okhotsk, these species putatively can be distinguished by carapace morphology. The spine at the intersection of the two dorsal ridges on the carapace is large in *C. angulatus*, but small in *C. japonicus* (Jadamec et al., 1999). Spine length, however, appears to vary along a continuum within species, and no quantitative data are available on spine-length variability in these species. The validity of this morphological trait for classification has been questioned by Somerton and Donaldson (1996), because of similarities between the two species in vertical distribution, size at maturity, and body shape. The nominal subspecies *C. japonicus pacificus* occurs allopatrically on seamounts, e.g. the Emperor Seamounts, in the mid North Pacific and is discriminated from the typical red snow crab, *C. japonicus japonicus*, by carapace color (Sakai, 1978).

The present study aims to clarify the phylogenetic relationships among the three taxa in the red-snow-crab species complex, as well as to reconstruct a phylogeny using both nuclear and mitochondrial (mt) DNA markers for all the species in the genus *Chionoecetes*, including the three other North Pacific species: the grooved Tanner crab, *C. tanneri*, snow crab, *C. opilio*, and Tanner crab, *C. bairdi*. *Chionoecetes tanneri* is distributed from the California-Mexico border in the Northeastern Pacific northward to the Bering Sea. The morphology of this species differs greatly from that of both the red snow and triangle Tanner crabs (Jadamec et al., 1999), indicating a distant relationship from the remaining three red-snow-crab taxa. *C. opilio* has the widest distribution, extending from the Sea of Japan, east of the Korean Peninsula, through the Sea of Okhotsk and the Bering Sea, and into the Northwest Atlantic. *Chionoecetes bairdi* occurs in the North Pacific from the southeastern Bering Sea to Oregon, and westward from the Aleutian Islands to Hokkaido, Japan.

A previous phylogenetic study using 5' region of nucleotide sequences of the cytochrome *c* oxidase subunit 1 (COI) and 16S ribosomal rRNA genes of the mtDNA (Yanagimoto, 2007a) failed to resolve relationships among *C. japonicus*, *C. opilio* and *C. bairdi*, because of small sample sizes for each species and because of inconsistent results from the two mtDNA markers. The present study included larger sample sizes of *C. japonicus*, *C. j. pacificus*, *C. opilio* and *C. tanneri*, but only a small sample of *C. bairdi*, to examine phylogenetic relationships among species. The 3' region of nucleotide sequence in the COI gene resolved the population structure of the hair crab *Erimacrus isenbeckii* around Japan (Azuma et al., 2008), and was used here to infer relationships among the taxa of *Chionoecetes*. A molecular-clock calibration was used to date the nodes in the phylogenetic tree for *Chionoecetes*. The struc-

tural variation of internal spacer regions in a nuclear rRNA gene also was examined, because this variability has been informative for phylogenetic inferences in other crab taxa (Tang et al., 2003; Yanagimoto, 2007b).

## MATERIALS AND METHODS

### Sample collection and DNA extraction

In this study, the name *C. japonicus* refers to *Chionoecetes japonicus japonicus*, but excludes *Chionoecetes japonicus pacificus*. Individuals of *C. japonicus* ( $n = 421$ ) were collected from nine localities in the Sea of Japan, the Sea of Okhotsk, and the northwestern Pacific Ocean (Table 1, Fig. 1). Individuals of *C. j. pacificus* ( $n = 48$ ) were collected from the Emperor Seamount in the mid-Pacific, and individuals of *C. angulatus* ( $n = 95$ ) were collected from the southeastern Bering Sea. Two samples of *C. tanneri* were collected in the eastern Bering Sea and the Gulf of Alaska ( $n = 96$ ), and samples of *C. opilio* ( $n = 78$ ) and *C. bairdi* ( $n = 5$ ) were collected in the northwestern Pacific off Hokkaido, Japan.

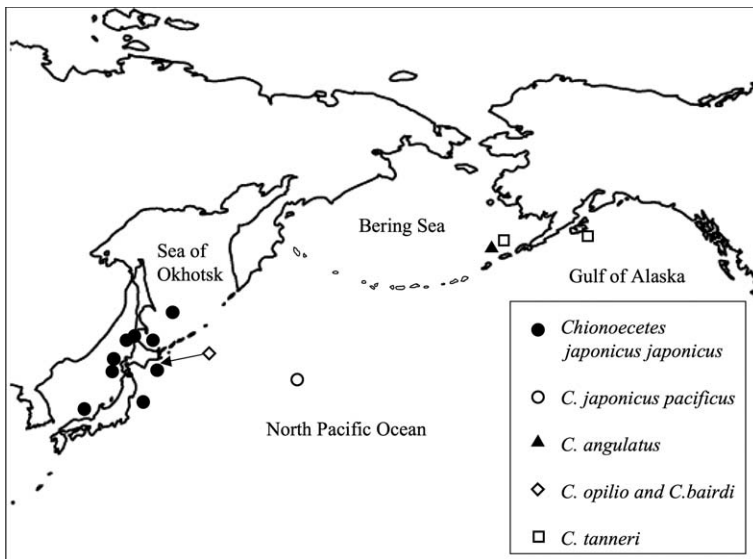
Genomic DNA was extracted from about 20 mg of leg muscle of adults with a PUREGENE™ kit (Qiagen) or DNeasy™ Blood & Tissue kit (Qiagen), following the manufacturer's protocols. Extracted DNA was dissolved in 150–250  $\mu$ L Tris-EDTA, pH 8.0.

### Mitochondrial DNA analysis

About 600 bp of the 3' end of the COI gene was amplified with the polymerase chain reaction (PCR) using the brachyuran specific primers KBCOImf (5'-ATTGTTAGTCAAGAATCCGG-3', Azuma et al., 2008) and BeCOI1 (5'-GGTAAATAATACAGAGCG-3', newly designed for the present study). The PCR solution including 100  $\mu$ M of each dNTP, 0.3  $\mu$ M of each primer, 0.5 U of *Taq* DNA polymerase (Sigma) and 10 x buffer was prepared following manufacturer's protocols, and 5–15 ng of genomic DNA was added to each 20  $\mu$ L solution as template. Thermal-cycle profiles consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 95°C for 45 s, 48°C for 30 s and 72°C for 60 s, with a post-cycle extension at 72°C for 5 min. PCR products were purified with magnetic beads (AMPure, Agencourt, Beverly, MA) and sequenced on a 3130xl genetic analyzer (Applied Biosystems) with the BigDye Terminator Sequencing Ready Reaction 3.1 (Applied Biosystems) using the forward and reverse primers noted above. Sequences were aligned and edited to 550 bp with DNASIS (Hitachi) and deposited in DDBJ (Accession nos. AB450389–AB450427, AB450658–AB450688, AB451006–AB451017, AB469203–

**Table 1.** Samples of *Chionoecetes* crabs used in this study. *N* is sample size, and year, latitude and longitude indicate the date and place of collection samples. \* purchased in market as crabs from the Sea of Okhotsk.

Taxon	N	Year	Latitude	Longitude
<i>Chionoecetes japonicus</i>	53	2006	46.4 N	146.0 E
	55	2005		no data*
	48	2005	45.9 N	140.1 E
	49	2006	45.8 N	139.8 E
	36	2005	42.4 N	139.5 E
	44	2005	41.4 N	140.0 E
	56	2008	36.4 N	134.3 E
	35	2005	42.3–42.4 N	144.0–144.3 E
	45	2007	37.1 N	134.3 E
	<i>C. japonicus pacificus</i>	48	2007	41.1 N
<i>C. angulatus</i>	95	1996	54.8 N	167.5 W
<i>C. tanneri</i>	48	1994	56.3 N	170.4 W
	48	1996	54.0 N	162.0 W
<i>C. bairdi</i>	5	2005	42.5 N	144.0 E
<i>C. opilio</i>	78	2005	42.5 N	144.0 E



**Fig. 1.** Map of the North Pacific and NW Pacific showing sample collection sites of *Chionoecetes japonicus* around Japan, *C. angulatus* in the southeastern Bering Sea and *C. japonicus pacificus* in the mid Pacific Ocean.

AB469282).

The Akaike information criterion (AIC) in MODELTEST 3.7 (Posada and Crandall, 1998) indicated that the sequences fit the Tamura-Nei substitution model (TrN; Tamura and Nei, 1993) with heterogeneous sites TrN + G model (shape parameter  $\alpha = 0.596$ ). A neighbor-joining (NJ) tree was constructed using MEGA 4.0 (Tamura et al., 2007) with the appropriate substitution model with 10,000 bootstrap replicates to assess the significance of the nodes in the tree. A Bayesian tree was constructed with BEAST 1.5 (Drummond and Rambaut, 2007) and the TrN + G substitution model and visualized with FIGTREE 1.2.2 (Rambaut, <http://tree.bio.ed.ac.uk>). Only representative divergent haplotypes from each taxon were used for Bayesian tree construction to shorten run times. For both the NJ and Bayesian trees, *E. isenbeckii* was used as an outgroup. The nodes in the Bayesian tree were dated using a molecular-clock calibration based on 2% sequence divergence per million years (Marko, 2002).

#### Nucleotide sequence analysis of nuclear rRNA gene region

A partial sequence (ca.1600 bp) of the nuclear rRNA gene region, including the *ITS1*, 5.8S rRNA gene and *ITS2*, was amplified for four individuals in each taxon with PCR using the primers 18-28Sr-f (5'-AAAAAGCTTCCGTAGGTGAACCTGCG-3') and 18-28Sr-r (5'-ATATGCTTAAATTCAGCGGG-3') (Sajdak and Phillips, 1997). Some individuals could not be well amplified, so we used a newly designed internal primer (crab28sR700) 5'-CGTCGGGCT-GTGCCGACT-3', which is located ca. 25 bp upstream of 18-28Sr-r. Reaction solution was prepared in the same manner for COI amplifications except the volume of genomic DNA was 50–100 ng. PCR cycles included an initial denaturation at 94°C for 3 min, followed by 35 cycles of 95°C for 45 s, 55°C for 45 s and 72°C for 2 min, with a post-cycling extension at 72°C for 10 min. PCR products were purified as for COI amplifications, and direct sequencing was carried out with the primers noted above on a 3130 xl genetic analyzer (Applied Biosystems). Unique sequences were deposited in the GenBank/DBJ Database. Sequences were aligned by eye using the software of DNASIS (Hitachi).

Both nucleotide substitutions and long insertions or deletions (indels) can occur among species in the sequences of the *ITS* region. A 165 bp insertion occurred in *C. j. pacificus*, and we tested

whether this indel was taxon-specific or represented within-species variability by surveying a length polymorphism of PCR products. Based on conserved sequence regions near the insertion in all examined taxa, new PCR primers, insF1 (5'-GCAGTAGACTGTTGCGACAGAC-3') and insR1 (5'-CCAACCAAGCTCCCGGATGCTC-3'), were developed to detect the insertion. PCR was carried out using the insF1 and insR1 and genomic DNA from all individuals of *C. j. pacificus*, four each of *C. japonicus* and *C. angulatus*, and one each of *C. opilio*, *C. tanneri* and *C. bairdi*. Reaction components were basically similar to PCR for 18-28S rRNA except the primers. PCR cycles included an initial denaturation at 94°C for 3 min, followed by 35 cycles of 95°C, 60°C and 72°C (each for 30 s), and a post-cycling extension at 72°C for 10 min. Electrophoresis in a 2.0% agarose gel in TAE was used to survey for the 165 bp length polymorphism in the specimens.

Although species-specific indels may be phylogenetically informative, the occurrences of nested multiple-site polymorphisms make it difficult to assign weights to the indels in the construction of a phylogenetic tree. Therefore, only nucleotide substitutions in the *ITS* region were used to reconstruct a phylogenetic tree. An NJ tree of uncorrected *p*-distances between haplotypes was constructed with and without substitutions within the sites of indels (gap) using the software Clustal X (Thompson et al., 2007). No outgroups were used, because of the complex structure of the *ITS* region in the species of *Chionoecetes* described above, which will not allow precise sequence alignment including distant species.

## RESULTS

### Mitochondrial DNA analysis

Nucleotide polymorphisms in the 550 bp COI sequences from nine samples of *C. japonicus* from the Sea of Japan, the sea of Okhotsk and the Pacific Ocean ( $n = 421$ ) defined 38 haplotypes. Seventeen haplotypes occurred in the sample of *C. angulatus* from the southeastern Bering Sea ( $n = 95$ ), of which 11 were shared with *C. japonicus*. Five private haplotypes occurred in *C. angulatus* as singletons, and one haplotype was found in two individuals. In the pooled sample of *C. japonicus* – *C. angulatus* ( $n = 516$ ), 41 polymorphic sites defined 44 haplotypes. A total of 36 polymorphic sites defined 29 haplotypes in the samples of *C. j. pacificus* ( $n = 48$ ). Forty-six haplotypes occurred in *C. tanneri* ( $n = 96$ ), 11 in *C. opilio* ( $n = 50$ ) and two in *C. bairdi* ( $n = 5$ ). No indels were observed in these haplotypes within or among the latter three species.

Mean sequence divergences between haplotypes within species ranged from 0.0036 (*C. angulatus*) to 0.0099 (*C. j. pacificus*). Both the NJ and Bayesian trees placed the three taxa of the red-snow-crab complex into a monophyletic group with significant bootstrap support (72%), but relatively weak posterior probability (0.47) (Figs. 2 and 3). Haplotypes of *C. angulatus* were interspersed among those of *C. japonicus* in the trees, and the two taxa shared an abundant haplotype (AB450423) with a frequency of 0.79 in *C. japonicus* and 0.83 in *C. angulatus*. Net sequence divergence between *C. japonicus* and *C. angulatus* was  $d_{\text{net}} = 0.000$ . Net sequence divergence between *C. japonicus* – *C. angulatus* and *C. j. pacificus* was  $d_{\text{net}} = 0.026$  (Table 2). The remaining three species were sister taxa to the red-snow-

crab complex, showing different topologies between the Bayesian and NJ trees. The NJ tree placed *C. bairdi* outside all other species of *Chionoecetes*, while the Bayesian tree placed *C. bairdi* as a sister taxon to *C. tanneri* (posterior probability = 0.63) (Figs. 2 and 3).

A molecular clock calibration of 2% divergence per million years indicated a separation between the red-snow-crab species complex and the remaining three species of *Chionoecetes* of about 4.6 million years ago (mya). However, the posterior probability and bootstrap value of this node was only 0.47 and 72%, indicating the node is not supported by high probability. The node separating *C. japonicus* – *C. angulatus* and *C. j. pacificus* was suggested to be about 3.4 mya. The root of the *C. japonicus* and *C. j. pacificus* haplotypes was dated to about 2.1 mya and 2.3 mya, respectively, near the end of the Pliocene.

### rRNA gene sequence variability

PCR for the rRNA region was not successful in some individuals, probably due to sequence polymorphisms in the priming sites. We obtained PCR products from four individuals in each of *C. japonicus*, *C. angulatus*, *C. j. pacificus*, *C. tanneri* and *C. opilio* and two in *C. bairdi*, and sequenced in three of *C. japonicus*, one each of *C. angulatus* and *C. j. pacificus*, two each of *C. tanneri* and *C. opilio*, but none of *C. bairdi*. The heterozygous rRNA gene region, i.e. occurrence of different haplotypes within individuals, probably caused the failure of sequencing in some PCR products even after successful amplification. Sequences from *C. japonicus* and one from *C. angulatus* were identical to the haplotype CJ1 of *C. japonicus* retrieved from GenBank (accession number: AB193504). Sequence of *C. opilio* matched the nucleotide sites 133–1531 of the GenBank haplotype CO2 (AB193500). Novel sequences of *C. j. pacificus* and *C. tanneri* were found in the present study and deposited in the DDBJ/Genbank with accession numbers AB5021212 and AB546598.

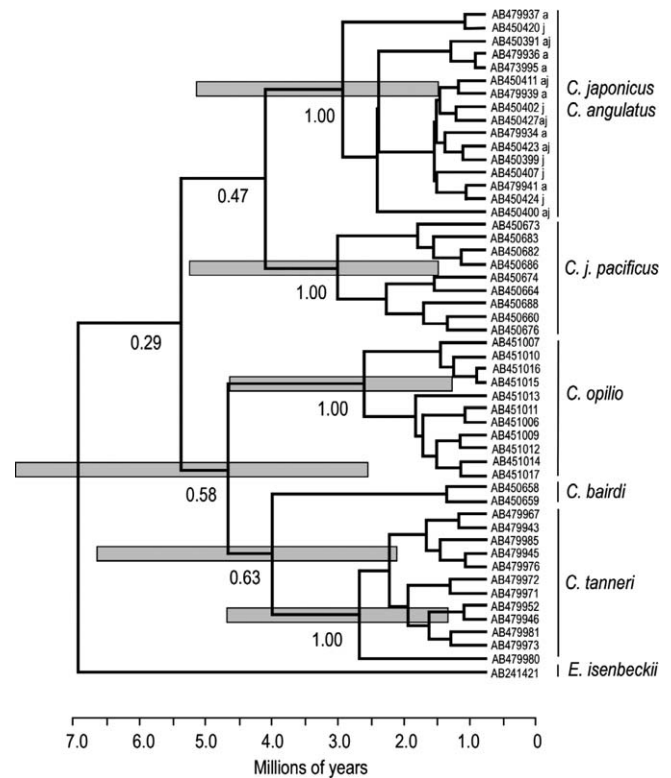
Additional sequences of the same region were retrieved from the GenBank for *C. japonicus* (haplotypes CJ2 and CJ3; AB193505 and AB193506), *C. opilio* (CO1; AB193499), and *C. bairdi* (CB1 and CB2; AB193501 and AB193502). A total of nine sequences, each spanning 1593 bp with indels, were aligned, in which 329 polymorphic sites were found, including 278 indels and 62 nucleotide substitutions (Fig. 4 and Supplemental Table S1 online). Eleven single nucleotide polymorphisms occurred in the indels. The transversion/transition ratio was 0.931, indicating almost equal frequency of the two types of substitutions. The longest indel was 165 bp in the aforementioned *ITS1* region of

**Table 2.** Net sequence divergence (TrN) between species of *Chionoecetes* based on 3'-end sequences of the mtDNA COI gene.

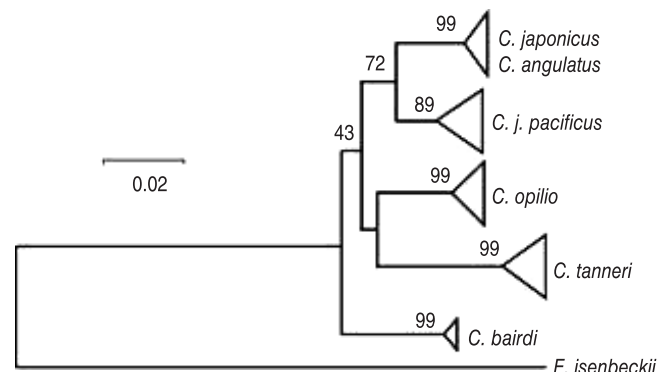
	1.	2.	3.	4.	5.
1. <i>Chionoecetes japonicus</i>					
2. <i>C. angulatus</i>	0.0000				
3. <i>C. japonicus pacificus</i>	0.0203	0.0319			
4. <i>C. tanneri</i>	0.0534	0.0534	0.0349		
5. <i>C. bairdi</i>	0.0628	0.0630	0.0570	0.0517	
6. <i>C. opilio</i>	0.0600	0.0597	0.0432	0.0548	0.0660

*C. j. pacificus*.

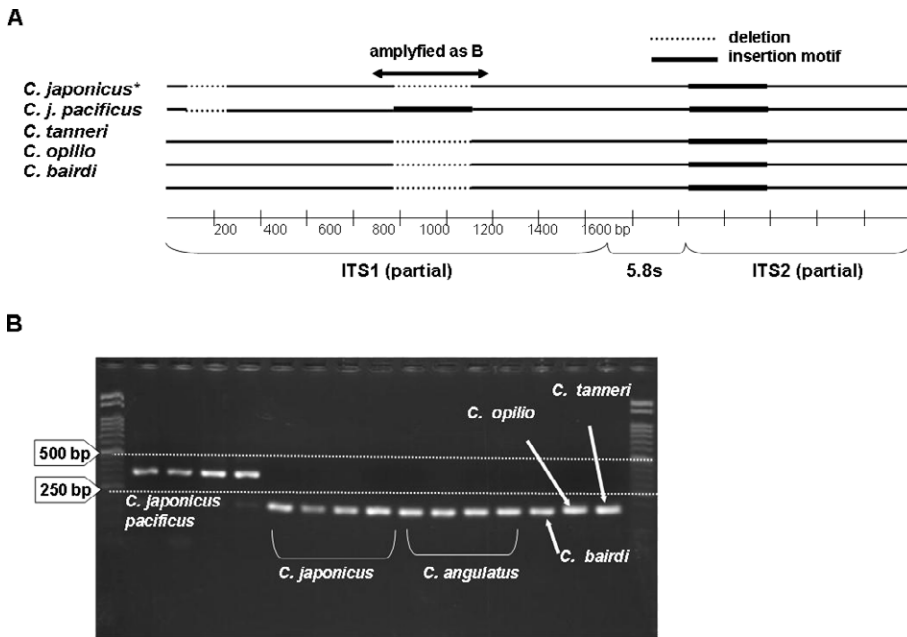
A schematic presentation of the characteristic sequences from each taxon is given in Fig. 4A. A homologous sequence of the longest insert occurred in the *ITS2* region of all the examined taxa, including *C. j. pacificus*. The second longest insert of 67 bp occurred in *Chionoecetes*



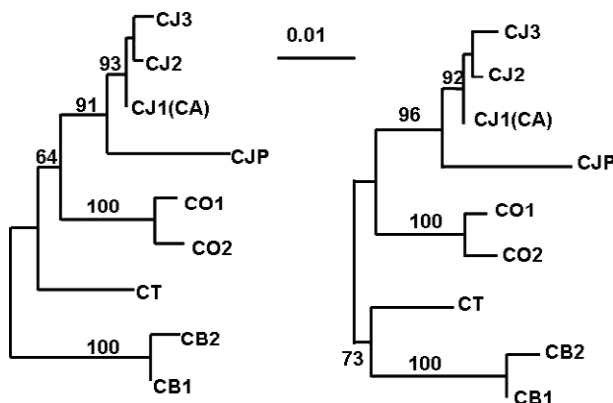
**Fig. 2.** Bayesian tree of 3' region of COI based on Tamura-Nei distance showing relationships among *Chionoecetes* crab haplotypes. A common haplotype in *Erimacrus isenbeckii* was used as an out-group. Bayesian posterior probabilities are shown at the nodes, and 95% highest probability densities for positions of nodes are indicated by gray bars. The letters 'a' and 'j', placed after GenBank accession numbers in the *C. japonicus* – *C. angulatus* cluster, indicate haplotypes appearing in samples of *C. japonicus* and *C. angulatus*.



**Fig. 3.** Neighbor-joining tree of the 3' region sequences of COI based on Tamura-Nei distance. Numbers at nodes represent bootstrap support with 10000 bootstrap replicates. The scale bar indicates sequence divergence.



**Fig. 4.** (A) Structure of rRNA region examined in the present study. The horizontal line indicates a haplotype. \*Sequence in *C. angulatus* was completely identical to one in *C. japonicus*. A 165 bp insert in *ITS1* occurred only in *C. japonicus pacificus*. (B) Electrophoretic separation of PCR amplifications was carried out in 2.0% agarose gel in TAE buffer. PCR fragments were ca. 350 bp in *C. j. pacificus*, but ca. 180 bp in the other species examined.



**Fig. 5.** Neighbor-joining trees of ca. 1600 bp of partial rRNA sequence in *Chionoecetes* crabs based on *p*-distance including (left) and excluding (right) the nucleotide substitutions in gapped sites. Haplotype names of CJ, CJP, CO, CT and CB indicate the origin of each haplotype, *C. japonicus*, *C. japonicus pacificus*, *C. opilio*, *C. tanneri* and *C. bairdi*, respectively. We found that the haplotype CJ1 was shared in *C. japonicus* and *C. angulatus*. Haplotypes of CJ1, CO2, CJP and CT were observed in the present study. CJ1 and CO2 were identical to the sequences previously reported, and CJP and CT were newly founded in the present study. CJ2, CJ3, CO1, CB1 and CB2 were cited from GenBank. The same haplotype names were shown in Supplemental Table S1 online. The sequences of all haplotypes were shown in Supplemental Table S1 online. The number above each branch indicates the bootstrap values for each node.

species other than *C. japonicus* – *C. angulatus* and *C. j. pacificus*. The 67 bp region was considered to be a deletion in an ancestral lineage of the red-snow-crab species com-

plex, rather than an insertion in the other taxa, supporting the monophyly of the red-snow-crab species complex. The sequence in *C. j. pacificus* differed from the haplotype CJ1 by 11 substitutions and a 166 bp insert.

PCR amplification of the neighboring region of the longest insertion using insF1 and insR1 was successful in all examined specimens. Fig. 4B shows the electrophoretic separation of the PCR products. A comparison of the sequences indicated that the lengths of the PCR products were expected to be ca. 349 bp, 177 or 176 bp, 182 bp, 183 bp and 182 bp for *C. j. pacificus*, *C. japonicus* – *C. angulatus*, *C. tanneri*, *C. opilio*, and *C. bairdi*, respectively. The obtained PCR products of *C. j. pacificus* were distinguishable from those of other species on the electrophoretic gel, although such discrimination was not possible among *C. japonicus* – *C. angulatus*, *C. tanneri*, *C. opilio*, and *C. bairdi*. The

longer PCR product of *C. j. pacificus* than that of the other taxa indicated the presence of the 165 bp insert, which was absent in the others, including *C. japonicus* and *C. angulatus*. PCR products from all individuals of *C. j. pacificus* were about 350 bp in length, indicating that the long insert in *C. j. pacificus* did not appear to reflect intra-specific variation but was specific to this taxon. Thus, the 165 bp insert in the *ITS1* region provides a discriminative marker for *C. j. pacificus*.

The NJ trees of ca. 1600 bp of partial rRNA sequence in *Chionoecetes* crabs based on *p*-distance with and without the nucleotide substitutions in the gapped sites supported the monophyly of *C. japonicus*, *C. angulatus* and *C. j. pacificus*, and significant bootstrap support suggested differentiation between *C. japonicus* and *C. j. pacificus* (Fig. 5). Additionally, *C. japonicus* – *C. angulatus*, *C. j. pacificus* and *C. opilio* were grouped together, but this group was not supported with a significant bootstrap value in the trees. The position of *C. bairdi* differed between the two trees. This species clustered with *C. tanneri* in the tree made without indels but was most divergent from the other taxa in the tree including indels. *C. bairdi* differed from the other taxa of *Chionoecetes* by species-specific substitution at 18 sites. However, seven of these substitutions were at positions 49, 50, 51, 59, 79, 98, and 103, where some taxa had deletions (see Supplemental Table S1 online). Therefore, the seven substitutions were neglected in the analysis excluding indels, which led to different topologies in the trees constructed with and without indels.

## DISCUSSION

The present study of mtDNA *COI* and nuclear rRNA gene sequence variation among species of *Chionoecetes*

showed a close relationship between *C. japonicus* and *C. angulatus*, but a substantial amount of divergence between *C. j. pacificus* and the former two taxa. The molecular phylogenetic results of this study indicate that the taxonomies of the red-snow crab species complex should be revised.

#### Taxonomies of *C. japonicus* and *C. angulatus*

The results of the COI sequence analysis indicated a lack of species-level, or even subspecies-level divergence between the red snow crab *C. japonicus* and triangle tanner crab *C. angulatus*. First, the two taxa shared a common abundant haplotype. Second, haplotypes of these taxa were interspersed in the NJ tree based on the full population dataset (not shown) and the Bayesian tree with a reduced number of haplotypes (Fig. 2). Third, both the NJ (99%) and Bayesian (100%) trees provided a large amount of statistical support for the monophyly of haplotypes in the two taxa (Figs. 2 and 3). These results strongly suggest that *C. japonicus* and *C. angulatus* represent a single species and that these taxa should be synonymized.

One limitation of this conclusion is that only a single sample ( $n = 95$ ) of *C. angulatus* from one location was included in the study. This sample was collected in the SE Bering Sea at the putative center of the distribution of *C. angulatus* and was separated by several thousand kilometers from the closest sample of *C. japonicus* used in this study. The large geographical distance from the nearest populations of *C. japonicus* makes it unlikely that the genetic similarity between these taxa was due to contemporary introgression or hybridization. Additional sequences in GenBank of *C. angulatus* from British Columbia, Canada extend the geographical scope of this conclusion. Sequence divergences between COI sequences at the 5' end of the gene (597 bp; accession numbers AB211160 and AB211161 for *C. japonicus*, and DQ 882045 for *C. angulatus*) were small. Net divergences were  $d_{\text{net}} = 0.0018$  between the two sequences of *C. japonicus* and  $d_{\text{net}} = 0.0060$  between these two sequences and one of *C. angulatus* from British Columbia. These small sequence divergences imply that there is little genetic differentiation among populations of *C. angulatus*, further supporting the synonymy of *C. japonicus* and *C. angulatus*.

The lack of nuclear rRNA sequence divergence between *C. japonicus* and *C. angulatus* also supports the synonymy of these taxa. An rRNA sequence in *C. japonicus* was identical to a homologous rRNA sequence in *C. angulatus* over a region spanning more than 1 kbp (Fig. 4A). The NJ trees constructed from rRNA sequences with only nucleotide substitutions, but without indels, showed an identity of *C. japonicus* and *C. angulatus* and separation between *C. japonicus* and *C. japonicus pacificus* (Fig. 5). Thus both sequence datasets indicate a lack of divergence between *C. japonicus* and *C. angulatus*.

#### Divergence of *C. j. pacificus* from *C. japonicus* – *C. angulatus*

The trees based on COI sequences indicated a substantial degree of divergence between the red snow crab and the nominal subspecies, *C. j. pacificus*. Phylogenetic reconstructions suggest that the COI haplotypes of *C. j. pacificus* are monophyletic with a Bayesian probability of

100% (Fig. 2). The net sequence divergence between *C. japonicus* – *C. angulatus* and *C. j. pacificus* was substantial ( $d = 0.026$ ), and is typical of divergences between well-differentiated species (Johns and Avise, 1998). The rRNA sequences also indicated a substantial amount of divergence between *C. japonicus* – *C. angulatus* and *C. j. pacificus*. The NJ trees constructed from substitutions showed a large amount of differentiation between *C. japonicus* and *C. j. pacificus* (Fig. 5). In addition, the 165 bp insert in the rRNA *ITS1* region of *C. j. pacificus* was absent, not only in red snow crabs, but also in the other three species of *Chionoecetes*. From the viewpoint of parsimonious evolution, this insertion should be considered to have occurred once, in only one lineage of *C. j. pacificus* among *Chionoecetes* taxa, suggesting that this species is independent from the other *Chionoecetes*. These results also support the elevation of *C. j. pacificus* to full species status.

#### Molecular clock estimation and speciation scenario

Sequence divergences of the COI between the *C. japonicus* – *C. angulatus* and *C. j. pacificus* and their geographical distributions give some insight into the timing and origin of the latter. Unlike most seamount clusters, one end of the Emperor Seamounts lies close to the continental shelf, so that dispersal from continental slope populations might have easily occurred given the duration of the planktonic larval stage and the obvious ability of continental slope populations to disperse across the rim of the North Pacific during warm interglacial periods. The Bayesian tree indicated a divergence between *C. japonicus* and *C. j. pacificus* about 3.4 mya. This estimate is close to the onset of a northern hemisphere ice age, when strong ocean-climate shift in temperature influenced the rim of the North Pacific. These temperature changes may have forced populations of the red snow crab to move south along the edge of Asian continental shelf and ancestral populations of *C. j. pacificus* may have migrated farther down the seamount chain, thereby isolating the two groups and promoting independent paths of adaptive divergence. Even though populations of these taxa may have been relatively close to one another during brief interglacial periods, they have been well isolated from each other by inhospitable conditions in the northern hemisphere most of the Pleistocene.

Northern cold-waters experienced considerable temperature fluctuations during the Quaternary, in addition to large-scale shoreline changes (Takahashi, 1998). In the case of *C. japonicus* – *C. angulatus*, these fluctuations might have led to severe range contraction, followed by recovery and range expansion around the rim of the North Pacific, thereby resulting in wide distribution of this taxon. They may have expanded rapidly on the continuous continental slope which is highly suitable habitat. On the other hand, the geographical discontinuity between continental slope area and mid-ocean seamounts may have led to the isolation and speciation of *C. j. pacificus*.

#### CONCLUSION

Our analyses strongly suggested that *C. japonicus* and *C. angulatus* are a single species and their nomenclature should be synonymized, and that *C. j. pacificus* is an evolutionarily independent species that should be given full spe-



cies recognition as *C. pacificus*. We used the rRNA gene region, which includes insertion/deletion of nucleotides, for species identification and phylogenetic inference. The present study is part of a series of studies in our laboratory on the population genetics of the red snow crab–triangle tanner crab complex. Additional studies of population structure can benefit from the use of high-resolution markers, such as microsatellite DNA.

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