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

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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 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 \( d_{net} = 0.000 \) 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 \( d_{net} = 0.026 \) 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 (McCarterney 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*...

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 (Yoshio 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 potentially 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* (Yanagimoto, 2007a) failed to resolve relationships among 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 structural 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 DNAeasy™ Blood & Tissue kit (Qiagen), following the manufacturer’s protocols. Extracted DNA was dissolved in 150–250 μL 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′-ATTGTTAGTCGAAGATCGCG-3′, Azuma et al., 2008) and BeCOIr1 (5′-GGTGAAATATACAGGCG-3′, newly designed for the present study). The PCR solution including 100 μM of each dNTP, 0.3 μM of each primer, 0.5 μL 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 μL 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, AB450656–AB450688, AB451006–AB451017, AB469203–AB469208).

### 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.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>N</th>
<th>Year</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chionoecetes japonicus</em></td>
<td>53</td>
<td>2006</td>
<td>46.4 N</td>
<td>146.0 E</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>2005</td>
<td>no data*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2005</td>
<td>45.9 N</td>
<td>140.1 E</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>2006</td>
<td>45.8 N</td>
<td>139.8 E</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>2005</td>
<td>42.4 N</td>
<td>139.5 E</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>2005</td>
<td>41.4 N</td>
<td>140.0 E</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>2008</td>
<td>36.4 N</td>
<td>134.3 E</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>2005</td>
<td>42.3–42.4 N</td>
<td>144.0–144.3 E</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>2007</td>
<td>37.1 N</td>
<td>134.3 E</td>
</tr>
<tr>
<td><em>C. japonicus pacificus</em></td>
<td>48</td>
<td>2007</td>
<td>41.1 N</td>
<td>170.4 E</td>
</tr>
<tr>
<td><em>C. angulatus</em></td>
<td>95</td>
<td>1996</td>
<td>54.8 N</td>
<td>167.5 W</td>
</tr>
<tr>
<td><em>C. tanneri</em></td>
<td>48</td>
<td>1994</td>
<td>56.3 N</td>
<td>170.4 W</td>
</tr>
<tr>
<td><em>C. bairdi</em></td>
<td>48</td>
<td>1996</td>
<td>54.0 N</td>
<td>162.0 W</td>
</tr>
<tr>
<td><em>C. opilio</em></td>
<td>5</td>
<td>2005</td>
<td>42.5 N</td>
<td>144.0 E</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>2005</td>
<td>42.5 N</td>
<td>144.0 E</td>
</tr>
</tbody>
</table>
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 (Posada and Crandall, 1998) indicated that the sequences fit the AB469282).

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 18-28S-r (5′-GCACTGCAGACACTTGCGTGG-3′) and 18-28S-r (5′-ATATGCTAAATTCTACGGG-3′) (Sajdak and Phillips, 1997). Some individuals could not be well amplified, so we used a newly designed internal primer (crab28sR700) 5′-GCTCGGCGTGAGCGACT-3′, which is located ca. 25 bp upstream of 18-28Sr. 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 for COI amplifications, and direct sequencing was carried out with the primers noted above on a 3130xl genetic analyzer (Applied Biosystems). Unique sequences were deposited in the GenBank/DDBJ 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′-GGCGTTAGGCTTGCGACAGAC-3′) and insR1 (5′-CCAAACAAAGCTCCCGAGTGGCT-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 was 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 dNet = 0.000. Net sequence divergence between C. japonicus – C. angulatus and C. j. pacificus was dNet = 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 *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*

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

<table>
<thead>
<tr>
<th></th>
<th>1.</th>
<th>2.</th>
<th>3.</th>
<th>4.</th>
<th>5.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Chionoecetes japonicus</em></td>
<td>0.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. <em>C. angulatus</em></td>
<td>0.0203</td>
<td>0.0319</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. <em>C. japonicus pacificus</em></td>
<td>0.0534</td>
<td>0.0534</td>
<td>0.0349</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. <em>C. tanneri</em></td>
<td>0.0628</td>
<td>0.0630</td>
<td>0.0570</td>
<td>0.0517</td>
<td></td>
</tr>
<tr>
<td>5. <em>C. bairdi</em></td>
<td>0.0600</td>
<td>0.0597</td>
<td>0.0432</td>
<td>0.0548</td>
<td>0.0660</td>
</tr>
<tr>
<td>6. <em>C. opilio</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 complex, 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 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.

**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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