Genetic divergence of *Argis lar* and *A. hozawai*, distinct sibling species of deep-sea crangonid shrimp from the Sea of Japan

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**Abstract:** *Argis lar* and *A. hozawai* are commercially important deep-sea crangonid shrimps of closely similar morphology. A previous study described three morphological characters to distinguish *A. lar* and *A. hozawai* was based on comparison of specimens of different size, raising the possibility that these characters are features of intraspecific differences related to body size. In the present study, mitochondrial DNA fragments encoding the cytochrome oxidase subunit I (COI) gene were sequenced in order to reassess the morphological criteria purported to separate *A. lar* and *A. hozawai*, based on specimens collected under a synchronous and sympatric sampling design. Two distinct clades were detected (p-distance 7.72%) supported by high bootstrap values, which completely correspond to *A. lar* and *A. hozawai* identified by morphological characters. Accordingly, *A. lar* and *A. hozawai* should be treated as two distinct species for appropriate fisheries management.

**Key words:** *Argis hozawai*, *Argis lar*, COI, Crangonidae, molecular taxonomy

There are 10 recognized species in the crangonid genus *Argis* which inhabit the sandy-mud bottom of the cold and deep waters of the North Pacific Ocean (Butler 1980, Komai 1997, Komai & Komatsu 2009, Hayashi 2010). Many *Argis* species are commercially important as local fisheries resources (Ito 1978, Holthuis 1980) and also play a key role in marine benthic ecosystems as highly abundant prey and predators of demersal fishes and large benthic invertebrates (Muto et al. 1992, Ikeda & Koyama 2001).

*Argis lar* (Owen, 1839) and its putative sibling *A. hozawai* (Yokoya, 1939) are sympatriquently distributed in the Sea of Japan, the Sea of Okhotsk and on the Pacific coast of northern Japan (Komai & Komatsu 2009). In the Sea of Japan, their bathymetric distributions are segregated with a small overlap: *A. hozawai*, 160–190 m; *A. lar*, 160–260 m (Uji 1994). They have long been confused because of their similar overall appearance (Komai & Amaoka 1992). By the early 1900s, “*Argis lar*” had already been divided into two morphs (Komai & Amaoka 1992), which were apparently distinguishable by the presence or absence of a tooth-like tubercle just behind the rostrum. Nevertheless, most researchers had believed that such morphs were only significant at the intraspecific level of *A. lar* (Rathbun 1904, Kobjakova 1937, 1958, Zarenkov 1960).

Komai & Amaoka (1992) recognized these two morphs as distinct species, *A. lar* and *A. hozawai*, and provided sufficient criteria to separate them: *A. hozawai* has a tooth-like tubercle just behind the rostrum (Fig. 1: #1); a more prominent median carina on the two most anterior abdominal somites (Fig. 1: #2); and a proportionately stouter palm on the first pereopod. However, their description was made on the basis of small specimens of *A. hozawai* and larger *A. lar* specimens, raising the possibility that these diagnostic characters may be related to the difference in body size. A molecular based approach was therefore used to clarify the taxonomic status of the two species described by Komai & Amaoka (1992) by comparing genetic differences with Komai & Amaoka’s (1992) morphological classification.

We adopted the synchronous and sympatric sampling strategy in order to minimize the influence of spatial-temporal variations and thereby detect the genetic differentiation adequately. *Argis lar* and *A. hozawai* were collected off the Tango Peninsula, Kyoto, the Sea of Japan (Fig. 2, Table 1)
using beam trawl during the cruises of RV Heian-Maru (Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center) in July 2010, and identified on the basis of keys presented by Komai & Amaoka (1992). Thirty specimens each of *A. laris* and *A. hozawai* were preserved in 70% ethanol prior to DNA extraction. Three specimens of *A. toyamaensis* collected from the same sampling locality were also preserved and used as the outgroup for phylogenetic analysis.

Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) from abdominal muscle tissue according to the manufacturer's protocol. The mitochondrial DNA cytochrome oxidase subunit I (COI) gene of the three species was amplified via polymerase chain reaction (PCR) with Ex Taq polymerase (Takara, Shiga, Japan) using a newly designed forward primer (COIF-Argis: 5′-CCGGTAT-AGTAGGAAACCGCCCTAGTTA-3′) and a newly designed reverse primer (COIR-Caridea: 5′-TAKACTTCTGGTGVCCRAARAAAYCA-3′). COIF-Argis was used as the internal primer to the universal forward primer "LCO1490" (Folmer et al. 1994) after initial PCR and sequencing was conducted using "LCO1490" and the universal reverse primer "HCO2198" (Folmer et al. 1994). COIR-Caridea was designed in the region corresponding to "HCO2198" by comparing four caridean shrimp complete mitochondrial sequences (Liu & Cui 2010) archived in DDBJ/EMBL/GenBank: *Exopalaemon carinicauda* (NC012566), *Halocaridina rubra* (NC008413), *Macrobrachium rosenbergii* (NC006880) and *Macrobrachium lanchesteri* (NC012217) after multiple alignment with the Clustal W (Thompson et al. 1994) program implemented by BioEdit (Hall 1999). PCR was carried out in a Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following thermocycling profile: initial denaturing step of 94°C for 5 min, 35 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 30 s (extension) and a final extension of 72°C for 7 min. A total of 3 μL of each PCR product (15.1 μL) was used for 1.0% agarose gel electrophoresis to confirm the amplification of a single band as visualized with ethidium bromide under ultraviolet light. The remainder of each PCR product was purified with ExoSAP-IT (GE Healthcare UK Ltd.) for direct sequencing following the manufacturer's protocol. Sequences were determined by the dideoxy chain-termination method with an ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems).

Fig. 1. Morphological differences (black arrows) between *Argis laris* and *Argis hozawai* (photo by J. Fujita). *A. hozawai* is distinguished by the presence of a tooth-like tubercle just behind the rostrum (compare #1) and a more distinct median carina on the anterior two abdominal somites (compare #2).
The primer used for sequencing reactions was the same forward primer as those for PCR amplification. The sequences were aligned using the program BioEdit.

A phylogenetic tree was constructed under the neighbor-joining (NJ) algorithm with best-fit nucleotide substitution model implemented with the MEGA 5 beta version (Tamura et al. 2007). The substitution model selected under the Akaike information criterion (AIC) was the Tamura and Nei substitution model (Tamura & Nei 1993) with a proportion of invariant sites (TrN+I, I=0.73) and therefore the genetic distances among haplotypes were calculated as TrN+I distance. Net nucleotide divergence was based on the most parsimonious connections of haplotypes (Lefebure et al. 2006), net nucleotide divergence was calculated from p-distance, Kimura 2-parameter (K2P) distance and maximum likelihood (ML) distance. A network based on the most parsimonious connections of haplotypes was obtained with the program TCS version 1.21 (Clement et al. 2000).

Direct sequencing of the COI mtDNA gene yielded 571 base pairs (bp) of unambiguous sequence, corresponding to position 131 to 701 of the atyid shrimp Halocaridina rubra (NC008413) and the palaemonid prawn Macrobrachium rosenbergii (AY659990) mitochondrial genome without insertions or deletions. Of the 571 bp analyzed, 85 sites (14.9%) exhibited variation and 80 sites (14.0%) were parsimony informative. The estimated transition (T) to transversion (Tv) bias under the K2P parameter model was 3.60, and empirical base frequencies of the fragment were A=25.1%, T=34.7%, G=18.3%, and C=21.9%.

The resulting molecular phylogeny revealed two distinct clades supported by high bootstrap values, which corresponded exactly with the specimens of A. lar and A. hozawai identified by morphological characters (Fig. 3). Therefore, the genetic differences between sympatric A. lar and A. hozawai suggest that the two species are reproductively isolated and supports morphological criteria to separate them as demonstrated by Komai & Amaoka (1992). The dataset contained ten haplotypes from the A. lar clade, and only one haplotype each from the A. hozawai clade and A. toyamaensis (used as the outgroup). For the A. lar clade, haplotype (h) and nucleotide (π) diversities were 0.517 and 0.152%, respectively. The nucleotide sequence for each haplotype has been deposited in DDBJ (accession nos. AB640849–AB640860).

The COI sequence divergences between the two clades (p-distance: 7.72%) were slightly lower than the pairwise values with species A. toyamaensis (p-distance between A. lar and A. toyamaensis 9.46%; p-distance between A. hozawai and A. toyamaensis 11.91%), but much greater than the intra-clade values (p-distance within the A. lar clade 0.15%; p-distance within the A. hozawai clade 0.00%). Furthermore, COI sequence divergences between the A. lar and A. hozawai clades (K2P distance 8.30%; ML distance 8.33%) are lower than the universal crustacean threshold to help gauge species delimitation (K2P distance 16%; Lefebure et al. 2006), and slightly higher than nucleotide divergences for intraspecific phylogroup pairs of Crangon crangon (ML distance 2.62–6.19%; Lutikhuizen et al. 2008). These results suggest that A. lar and A. hozawai only recently diverged to form distinct species.

The haplotype network for the A. lar clade displays a typical star phylogeny (Fig. 3), suggesting that the population experienced a drastic bottleneck followed by an explosion of population size (Avise 2000). The A. hozawai clade showed only one haplotype and therefore a low level of genetic diver-

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**Table 1. List of sampling locations**

| Species            | Area                  | Location                    | Depth (m) |
|--------------------|-----------------------|-----------------------------|-----------|
| Argis hozawai      | off the Tango Peninsula | 35°52.30’N, 135°25.20’E     | 180       |
| Argis lar          | off the Tango Peninsula | 35°55.85’N, 134°55.07’E     | 249       |
| Argis toyamaensis  | off the Tango Peninsula | 35°55.85’N, 134°55.07’E     | 249       |
Argis lar (n = 30)

Argis hozawai (n = 30)

Argis toyamaensis (n = 3)

Fig. 3. Neighbor-joining tree under the TrN+I nucleotide substitution model illustrating the relationships of mtDNA COI haplotypes of 30 Argis lar and 30 Argis hozawai from the sympatric sampling station in the Sea of Japan. Numbers at tree nodes indicate bootstrap values (only values >70% are shown). Scale bar: 0.01 of TrN+I distance. The haplotype network of Argis lar is illustrated to the right. Each black-filled circle represents a unique haplotype with its frequency as indicated. Open circles indicate the presumed ancestral haplotypes.

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