



A new species of *Clione* distinguished from sympatric *C. limacina* (Gastropoda: Gymnosomata) in the southern Okhotsk Sea, Japan, with remarks on the taxonomy of the genus

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ABSTRACT

A new species of the genus *Clione* (Clionidae) was collected in shallow water from coastal eastern Hokkaido, in the southern Okhotsk Sea, Japan, and characterized by an integrated study of morphology, predatory behaviour and genetic evidence. A key taxonomic feature of Clionidae is the number of buccal cones; species with three pairs of buccal cones are classified in the genus *Clione*. This generic classification of the new species is supported by other anatomical features and by its predatory behaviour. Diagnostic characters of the new *Clione* include: (1) very short buccal cones; (2) three ciliated rings (anterior, mid-body and near tail) retained in adult stage; (3) large orange-red visceral mass; (4) no space between head tentacles and mouth; (5) presence of search behaviour; (6) buccal cones not used during predation; (7) mtDNA COI genetic barcode (distinguished from sympatric *C. limacina* and allopatric *C. antarctica*). On the basis of a phylogenetic analysis of COI sequences in this and previous studies, we suggest that *C. 'limacina'* populations in the North Atlantic and North Pacific Oceans are distinct species, for which the valid names are *C. limacina* (Phipps, 1774) and *C. elegantissima* Dall, 1871, respectively.

INTRODUCTION

The Pteropoda are one of several groups of planktic gastropods, including the Thecosomata (shelled pteropods) and Gymnosomata (naked pteropods) (Klussmann-Kolb & Dinapoli, 2006), which are common in all marine environments from the poles to the equator and from the ocean surface to bathypelagic depths (Pelseneer, 1888; Tesch, 1950; Van der Spoel & Dadón, 1999; Gofas, 2004). In addition to the fact that pteropods possess a wing-shaped foot used in swimming, several other features of their anatomy and physiology have been suggested to be associated with a planktic lifestyle: adept planktic predation is facilitated by their sensitive eyes; the swimming behaviour of some gymnosomes aids their predation on thecosomes (Lalli, 1970; Lalli & Gilmer, 1989; Harbison & Gilmer, 1992) and antipredation compounds produced by some thecosomes reduce their palatability (Bryan *et al.*, 1995; McClintock & Baker, 1998).

In the Pacific Ocean, 34 species of Thecosomata and 69 species of Gymnosomata have been recorded (Richter & Seapy, 1999; Van der Spoel & Dadón, 1999), including two thecosomes and three gymnosomes from the southern Okhotsk Sea (Hamaoka, 2002; Suzuki, 2005).

The Okhotsk Sea, located in the northwestern corner of the North Pacific Ocean, is one of the largest semi-enclosed seas in the world (Fig. 1). Whereas sea ice is typically present in Antarctic and Arctic waters during the winter, when it covers an area of up

to 7% of the earth's surface (Comiso *et al.*, 1990), in the North Pacific Ocean sea ice occurs during only a part of the winter (Ruth & Patrick, 1998). From spring to autumn, *Clione limacina* (Phipps, 1774) lives at a depth of *c.* 200 m in the Okhotsk Sea, while in winter this species migrates toward the coast of the southern Okhotsk Sea with drift ice (Hamaoka, 2002).

Despite being important members of zooplankton communities, the Gymnosomata are in general poorly understood (Lalli & Gilmer, 1989; Sromek *et al.*, 2015). Although their anatomy and ecology have been well studied (Martens, 1675; Wanger, 1885; Smith, 1902; Massy, 1932; Morton, 1958; Mileikovsky, 1970; Lalli & Conover, 1976; Van der Spoel, 1976; Lalli & Gilmer, 1989; Gilmer & Lalli, 1990; McClintock & Janssen, 1990; Richter & Seapy, 1999; Van der Spoel & Dadón, 1999; Suprenand, Jones & Torres, 2015), the genetics of these pteropods have received little attention (Jennings *et al.*, 2010; Sromek *et al.*, 2015). The characteristics of the family Clionidae are a thin integument and the presence of anterior tentacles, which are mostly transparent and typically very small. The defining character of the genus *Clione* is three pairs of buccal cones (Van der Spoel, 1976; Lalli & Gilmer, 1989). Members of the genus have been called 'sea angels' owing to their characteristic swimming behaviour (Wrobel & Mills, 1998). *Clione* individuals are an important component of pelagic food webs in temperate and polar waters, providing a food source for baleen whales, salmon and other fish species (Martens, 1675;

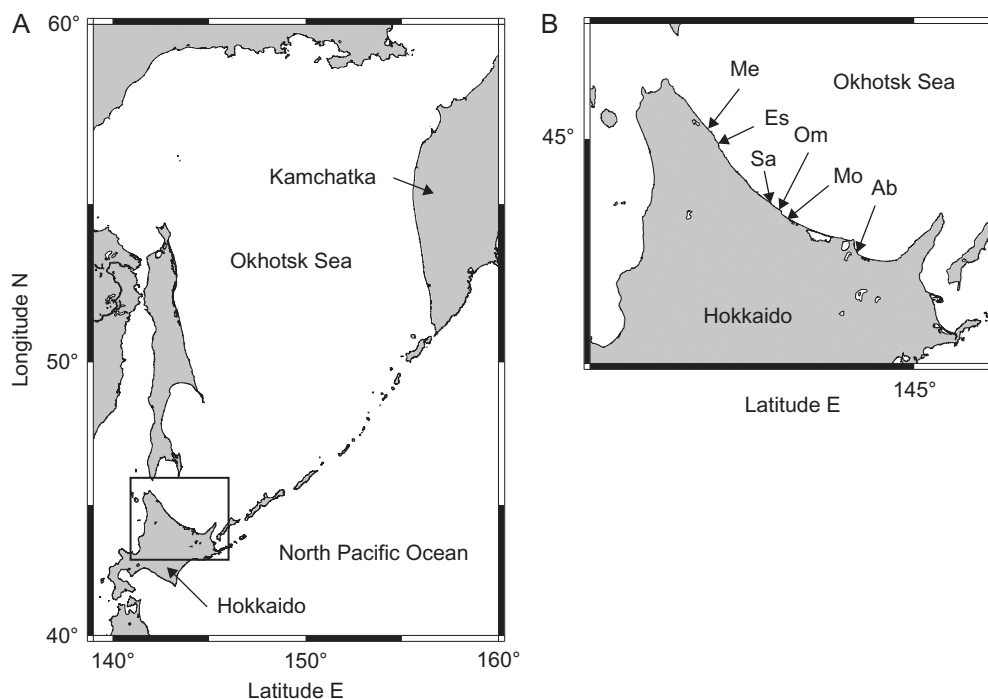


Figure 1. Map of sampling localities and adjacent waters. **A.** Okhotsk Sea. **B.** Sampling localities in eastern Hokkaido. See Supplementary Material Table S1 for details of sampling locations and dates. Abbreviations: Me, Menashidomari; Es, Esashi; Sa, Sawaki; Om, Omusaro; Mo, Mombetsu; Ab, Abashiri.

Phipps, 1774; Lalli & Gilmer, 1989; Azuma, 1995; Davis *et al.*, 2009).

The two previously recognized *Clione* species, *C. antarctica* Smith, 1902 and *C. limacina*, are distributed in the southern and northern hemispheres, respectively. These species have been distinguished using morphological (Eliot, 1907; Gilmer & Lalli, 1990), physiological (Rosenthal *et al.*, 2009; Dymowska *et al.*, 2012) and genetic evidence (Sromek *et al.*, 2015).

We observed live individuals of both the new *Clione* and sympatric *C. limacina* at locations from Menashidomari to Abashiri, in the southern Okhotsk Sea (Fig. 1). This paper will discuss the systematic implications of their comparative morphology, predatory behaviour and genetic barcodes.

MATERIAL AND METHODS

Sampling

Sampling was conducted from 2015 to 2016 (Supplementary Material Table S1) in shallow waters of the southern Okhotsk Sea, along a stretch of coast from Menashidomari to Abashiri, Japan (Fig. 1). We collected more than 500 specimens of the new species and more than 8,000 specimens of the sympatric species *Clione limacina* for comparison. In addition, we collected 17 specimens of *Limacina helicina* (shell length range 1–2 mm) as food material for the predation experiment. The depth range at which the specimens were captured was 0.3–1.5 m below the surface. Larvae of *C. limacina* for morphological comparison were collected in April and May 2010 from locations 1 km off Mombetsu at depths ranging from the surface to 9.5 m (Supplementary Material Table S1). All material was collected using a North Pacific Standard (NPAC) plankton net developed by Motoda *et al.* (1957). The NPAC plankton net has a 40-cm mouth diameter, a conical net length of 120 cm and mesh size of 335 μm (NMG52).

Reference materials (including types) have been deposited in the Shellfish Museum of Rankoshi, Japan (SMRZ), Okhotsk Sea

Ice Museum of Hokkaido (OKIMZ) and the National Museum of Nature and Science, Tokyo (NSMT).

Morphology

Individuals were maintained in a 1-tonne aquarium at 4 °C at the OKIMZ. The external morphology of the living animals was observed *in situ* in the aquarium and under a stereomicroscope. The radulae and hooks of eight specimens were extracted from the buccal mass, cleaned in 1N-NaOH solution and observed with a stereomicroscope and a scanning electron microscope (SEM; JSM-5600) at the Mutsu Institute for Oceanography, Japan Agency for Marine-Earth Science and Technology. The buccal mass was dissected under a stereomicroscope to observe buccal cones, hooks and hook sacs.

Predation on *Limacina helicina*

The predatory behaviour of the new species and *C. limacina* was observed in an aquarium and recorded using a Hi-vision video camera (SANYO Xacti DMX-CA100) in February 2015. On the basis of direct observation and stomach analyses, *Clione* species are known to be specialized predators of *L. helicina* (Boas, 1886; Meisenheimer, 1905; Lebour, 1931; Manteufel, 1937; McGowan, 1968; Lalli, 1970; Conover & Lalli, 1972, 1974). The experiments were conducted in a 2-l seawater aquarium at 4 °C, as follows: (1) one live *Clione* (new species or *C. limacina*) was added to the aquarium using a 10-ml low-density polyethylene (LDPE) dropper; (2) one live *L. helicina* was added as the food source using a 20-ml LDPE dropper. The tips of the two droppers were rounded using sandpaper (220 gr). The assays were repeated 15 times for each pairing.

Culture experiment for observation of ciliated rings

From January 2015 to January 2016, 280 specimens of the new species were cultured in three 120-l seawater aquaria at 4 °C. No

reproduction occurred, but ciliated rings of individuals were observed under a stereomicroscope 1 year after the start of the experiment and compared with those of *C. limacina* larvae and adults.

Molecular analysis

Twenty-four specimens were used for DNA extraction, amplification and sequencing: 10 specimens of the new species and 14 of *C. limacina*. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). The mtDNA COI gene was amplified using the universal DNA primers LCO-1490 (5'-GGTC AACAAATCATAAAGATATTGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994). PCRs were performed using a GeneAmp PCR System 9600 (Applied Biosystems). Reactions were cycled under the following protocol: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min; final extension at 72 °C for 5 min (Jennings *et al.*, 2010). PCR products were purified using the Wizard SV Gel and PCR Clean Up System (Promega). Sequencing reactions were performed using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) in a GeneAmp PCR System 9600 (Applied Biosystems). Reaction products were purified using EDTA, sodium acetate and ethanol precipitation, and then electrophoresed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were aligned and edited using MEGA7 (Kumar, Stecher & Tamura, 2016). There were 480 positions in the final dataset.

New sequences were deposited in the DNA Data Bank of Japan (accession numbers: LC144837–LC144860). Ten COI sequences of *C. limacina* already available in GenBank were included in the calculation of genetic distance. Four COI sequences for gymnosome and pseudothecosome pteropods were downloaded from GenBank and used as an outgroup to root the tree (see Fig. 6 for accession numbers). The analysis involved 47 nucleotide sequences.

Molecular phylogenetic analysis was conducted using the Maximum Likelihood (ML) method based on the Kimura 2-parameter (K2P) model (Kimura, 1980), implemented in MEGA7 (Kumar *et al.*, 2016). Initial tree(s) for the heuristic search were obtained by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the ML programme, and then selecting the topology with superior log likelihood values. A bootstrap consensus tree was calculated from 10,000 replicates and branches found in less than 50% of the bootstrap replicates were collapsed.

RESULTS

Morphology

The shell is lacking and gills are absent in *Clione* species. In the new species, the body is more barrel shaped than in other species of *Clione* and the length is only up to 8 mm. Three ciliated rings are visible, on the anterior, the mid-body and near the tail (Fig. 2A). The visceral mass occupies a high proportion of the anterior body and is visible owing to its bright orange-red colour. There is no space between the head tentacles and mouth (Fig. 2A). The surface of each buccal cone is studded with clusters or rosettes of capitulate papillae. There are three pairs of very short buccal cones; the middle pair is slightly larger than the ventral one (Fig. 4B). The paired hook sacs are of medium size and each contains *c.* 36 curved ventricular chitinous hooks of unequal size (Fig. 3B). The radula formula is 7-1-7; the lateral teeth are *c.* 250 µm in length (Fig. 3A, C) and the central tooth is present throughout and is broad with many small cusps (Fig. 3C, D).

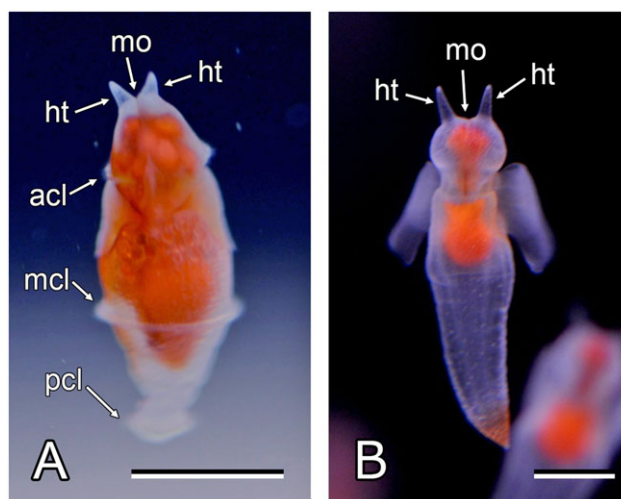


Figure 2. Dorsal views of *Clione* species from the southern Okhotsk Sea. **A.** *C. okhotensis* new species. Holotype (SMRZ M8). **B.** *C. limacina*. Scale bars = 2 mm. Abbreviations: mo, mouth; ht, head tentacle; acl, anterior ciliated ring; mcl, middle ciliated ring; pcl, posterior ciliated ring.

In contrast, the sympatric *C. limacina* is more slender in shape and much larger, with a body length of up to 30 mm. There is a gap between the head tentacles where the mouth is situated, ciliated rings are absent in the adult and the visceral mass occupies a smaller proportion of the body (Fig. 2B). The buccal cones are longer and the pairs are of unequal length (Fig. 5B, C). These characters are distinctly and diagnostically different between the two species.

Predation on *Limacina helicina*

The predatory behaviour of the new species was initiated in the presence of prey and occurs in the following sequence: (1) searching behaviour: swimming with the body stretched out and elongated, and excretion of a sticky fluid (visible trailing from the wings in Fig. 4A); (2) attempt behaviour: the hooks and/or the six buccal cones are everted and retracted (Fig. 4B, C); (3) prey-manipulation behaviour: the prey is rapidly manipulated with the hooks so that its shell aperture is pressed against the mouth of the predator (Fig. 4D). Thereafter, the chitinous hooks are evaginated from the hook sacs and inserted into the soft tissues of the prey. When the *Clione* has a secure hold on the prey, the hooks and radula are used to pull the body from its shell before the prey is swallowed whole. Predation behaviours (1) and (2) were observed in all bioassays; however, the entire behavioural sequence was only observed twice.

In contrast, no searching behaviour or excretion of sticky fluid (1) were observed in the predatory behaviour of *C. limacina* in any bioassay; otherwise, the sequence of predation behaviours (2) to (3) was similar to that seen in the new species (Fig. 5).

Observations of ciliated rings in culture

After 1 year in culture, 212 of the original 280 specimens of the new species survived. No reproduction occurred and body length did not increase noticeably, but three ciliated rings were still present in all specimens, as at the start of the experiment. Three ciliated rings were present in the larvae of *C. limacina*, but not in the adults.

Molecular analysis

The ML analysis generated a tree with a very similar topology to that of the bootstrap consensus tree obtained from the ML

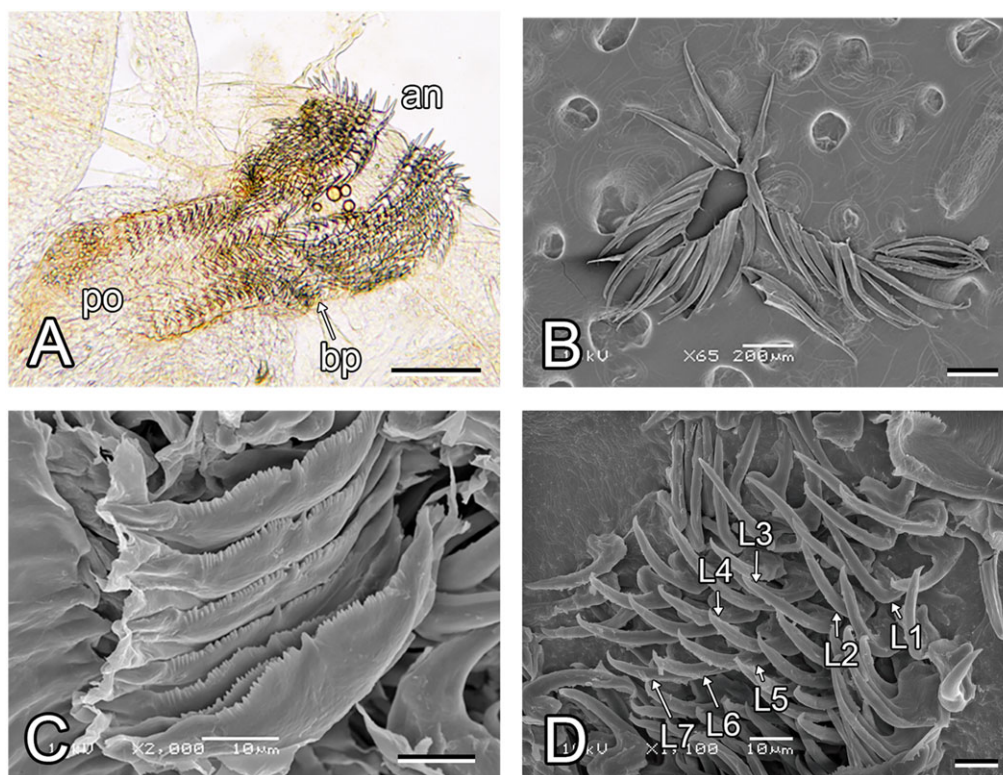


Figure 3. *Clione okhotensis* new species. **A.** Entire radula under light microscope. **B.** Hooks from a single hook sac under SEM. **C.** Central teeth of radula under SEM. **D.** Lateral teeth under SEM. Scale bars: **A** = 50 µm; **B** = 200 µm; **C** = 10 µm; **D** = 10 µm. Abbreviations: an, anterior; bc, buccal cone; L1 (inside)–L7 (outside), first to seventh lateral teeth; po, posterior; bp, bending point; hk, hooks; hs, hook sac; m, mouth.

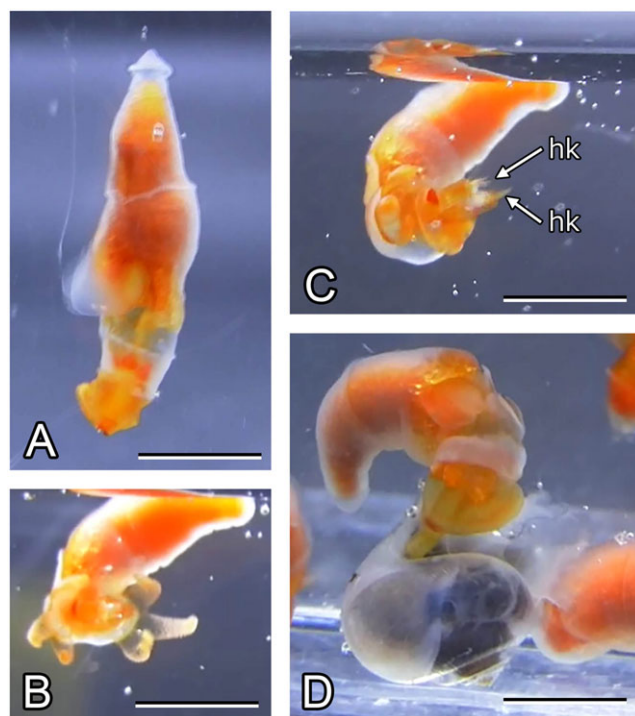


Figure 4. Predatory behaviour of *Clione okhotensis* new species captured by a Hi-vision video camera. **A.** Searching behaviour. **B.** Attempt behaviour, showing retracted and extended buccal cones. **C.** Attempt behaviour, showing retracted and extended hooks (hk). **D.** Prey manipulation behaviour with *Limacina helicina* prey. Scale bars = 1 mm.

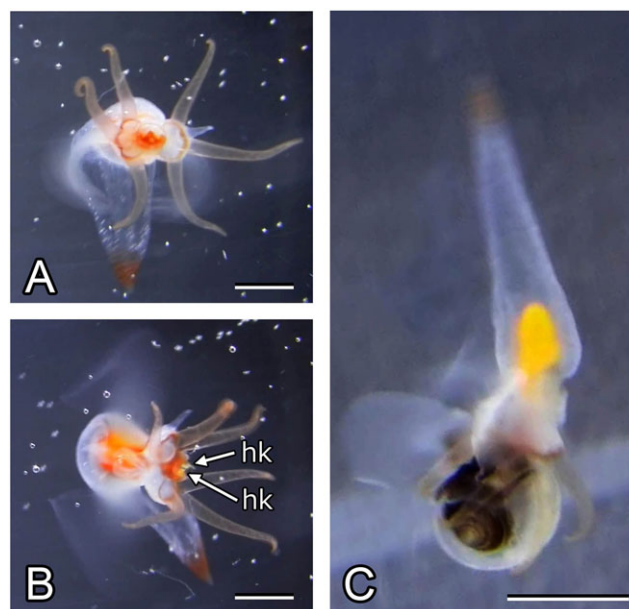


Figure 5. Predatory behaviour of *Clione limacina* captured by a Hi-vision video camera. **A.** Attempt behaviour, showing retracted and extended buccal cones. **B.** Attempt behaviour, showing retracted and extended hooks (hk). **C.** Prey manipulation behaviour with *Limacina helicina* prey. Scale bars = 1 mm.

program analysis (Fig. 6). The monophyly of the ingroup (genus *Clione*) and the sister relationship between the new species and *C. limacina* were strongly supported (bootstrap, BS = 98% and 100%, respectively). Within *C. limacina*, the Arctic and northern

NEW SPECIES OF *CLIONE* IN JAPAN

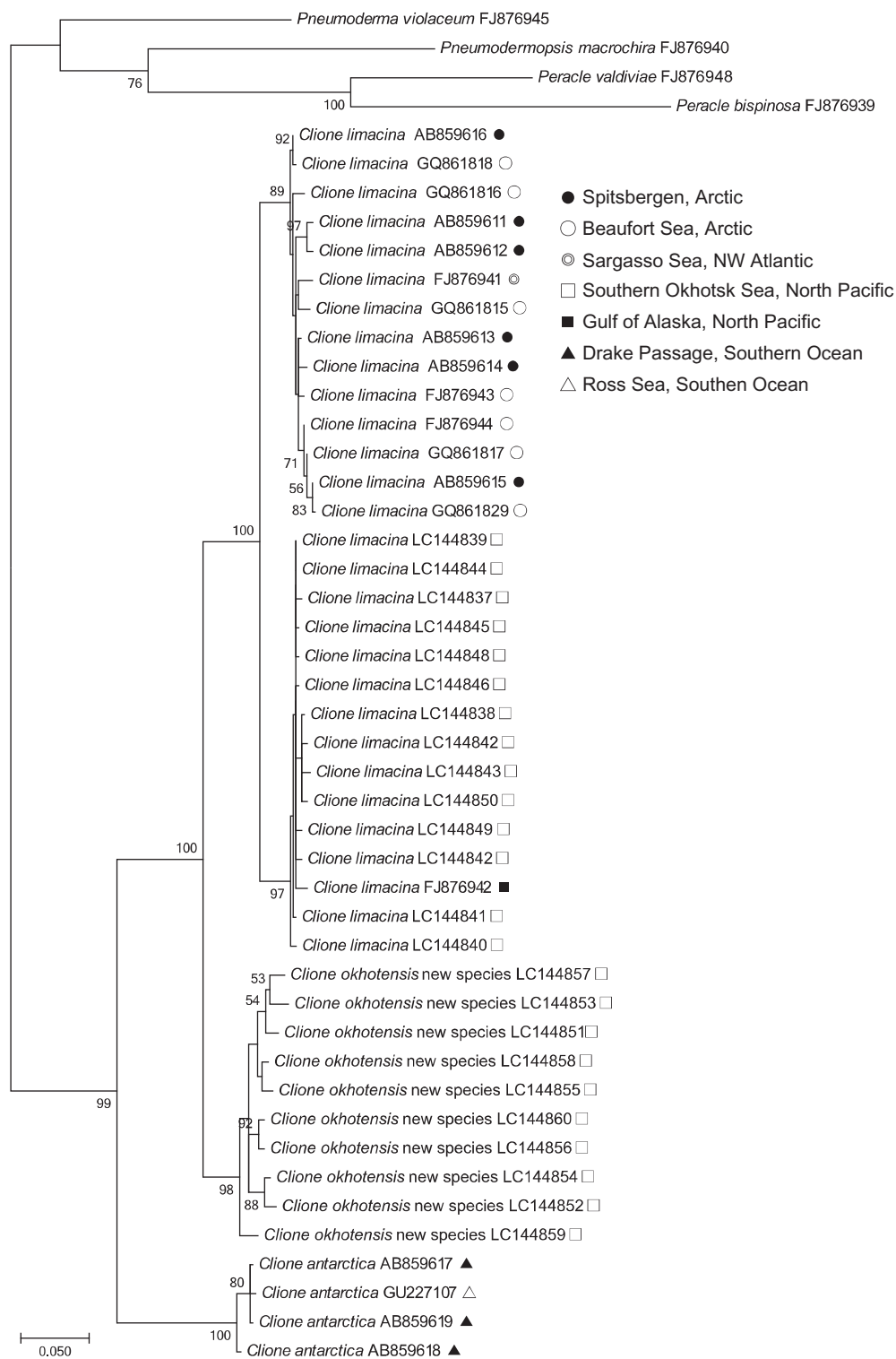


Figure 6. ML phylogenetic tree of *Clione* species, based on mtDNA COI sequences. Numbers at nodes represent bootstrap support.

Atlantic Ocean population and the northern Pacific Ocean population each formed a well-supported clade (BS = 89% and 97%, respectively).

The highest difference in K2P distance between the COI sequences of the new species and *C. limacina* was 12.44% (average, AV = 10.4%; standard deviation, SD = 0.68), whereas the highest

difference between the new species and *C. antarctica* was 20.79% (AV = 19.5%, SD = 0.78). Furthermore, we detected a difference of 7.36% (AV = 6.12%, SD = 0.49) in the COI sequences of *C. limacina* collected from the Arctic and northern Atlantic Ocean population and the northern Pacific Ocean population (Supplementary Material Table S2).

SYSTEMATIC DESCRIPTION

Family CLIONIDAE Rafinesque, 1815
Genus *Clione* Pallas, 1774*Clione okhotensis* new species

(Figs 2–4)

Type material: Holotype: 4.3 × 1.7 mm, SMRZ M8 (Fig. 2, alive), in 99.9% ethanol. Paratypes 1–10 SMRZ M9–18, in 99.9% ethanol. Paratypes 11–20 OKIMZ M1–10, in ice at –20 °C. Paratypes 21–23 NSMT-Mo 78967, in 99.9% ethanol. Coastal waters off Mombetsu, Hokkaido, Japan, southern Okhotsk Sea, 0.3 to 1.5 m depth; 44°19'46.0''N, 143°22'35.0''E.

ZooBank registration: F8C32AB3-A079-4958-945B-6A6FFED68521.

Etymology: In reference to type locality.

Diagnosis: Body length up to 8 mm, barrel-shaped. Parapodia small. Buccal cones short. Three ciliated rings on anterior, mid-body and near tail. No space between head tentacles and mouth. Visceral mass orange-red, occupying most of the mid-body.

Distribution: The only known localities are in the southern Okhotsk Sea (Supplementary Material Table S1).

Remarks: The new species most closely resembles *C. limacina*. The WoRMS database lists 14 synonyms of that species (Gofas, 2004); all original descriptions were checked to establish whether any names were available for the new species.

The new species resembles its two congeners *C. limacina* and *C. antarctica* in having the three pairs of buccal cones that are diagnostic of the genus.

It differs from the sympatric *C. limacina* in having three ciliated rings on the body (anterior, mid-body and near the tail), which are absent in adult *C. limacina*, and in the considerably larger orange-red visceral mass, the smaller parapodium wings, shorter buccal cones and more barrel-shaped body. The radula is similar in both species with only slight differences in the curvature of cusps and in the bases of the lateral teeth.

The allopatric species *C. antarctica* differs from *C. okhotensis* with respect to the radula. In *C. antarctica*, the central teeth on the radula are present in the first rows (rows 1–2), whereas in the posterior rows, central teeth are absent (Eliot, 1907). In contrast, in *C. okhotensis* the central teeth are present throughout (Fig. 3A). In addition, there are differences in the structure of the chitinous hooks, morphology of the radula, maximal size, tentacles, space between tentacles and mouth, and proportion of the visceral mass (Gilmer & Lalli, 1990).

The adults of the new species (Fig. 2A) share with the larvae of *Clione limacina* the presence of three ciliated rings; however, other characters, i.e. body morphology and size differ between them. In the larvae, which are less than 3.5 mm in length, the wings, head tentacles and buccal cones are immature and not yet developed. Although three ciliary bands are present in clionid larvae, these are usually lost in the adult (Gilmer & Lalli, 1990). *Paedoclione doliformis* (Danforth, 1907), from the northwestern Atlantic, retains three ciliary bands even when mature (Danforth, 1907), but is distinguished from *C. okhotensis* by possession of only two pairs of buccal cones.

Predatory behaviour in *C. okhotensis* is characteristic, particularly with respect to its searching behaviour (Fig. 4A), during which the animal swims with body extended and excretes a sticky fluid; this is not observed in *C. limacina*.

DISCUSSION

Systematic position

The observation of three pairs of buccal cones confirmed that the new species belongs to the genus *Clione* in the family Clionidae. The number of buccal cones is an important taxonomic characters of clionid genera: three pairs in *Clione*, two pairs in *Paraclione*, *Paedoclione* and *Thalassopterus*, and one pair in *Fowlerina* (Van der Spoel, 1976).

The genus *Clione* currently consists of two recognized species, *C. antarctica* and *C. limacina*, which are distributed in the southern and northern hemispheres, respectively. These species have been characterized using morphological (Eliot, 1907; Gilmer & Lalli, 1990), physiological (Rosenthal *et al.*, 2009; Dymowska *et al.*, 2012) and molecular approaches (Sromek *et al.*, 2015). Comparison of morphology, predatory behaviour and COI sequences clearly support recognition of *C. antarctica* as an additional species in the genus. With regard to K2P distances in gymnosomes, Jennings *et al.* (2010) reported intraspecific differences of 0–11.7% and differences between species within genera of 1.5–60.8%. Although use of the ‘barcoding gap’ in species delimitation is controversial (e.g. Meier *et al.*, 2008), the 12.44% K2P distance between *C. limacina* and *C. okhotensis*, together with their well-supported reciprocal monophyly and sympatric occurrence, provide strong evidence these are distinct phylogenetic and biological species.

Larva or adult

The three ciliated rings observed in the new species resemble those of pteropod larvae (Gilmer & Lalli, 1990; Van der Spoel & Dadón, 1999). However, head tentacles, buccal cones and parapodial wings are immature in the larvae of Clionidae (Lebour, 1931). The interpretation of the specimens of the new species as adults is supported by the fact that their morphology did not alter during a year of culturing and by the presence of tentacles, wings and buccal cones. The retention of juvenile characters in the adult, or pedomorphosis, is a common developmental phenomenon. The generic name *Paedoclione* implies that the presence of three ciliated rings in the adults of members of this genus has been interpreted as pedomorphic and a similar explanation may apply to *C. okhotensis*. Further developmental and phylogenetic studies are required to test this hypothesis and to confirm that the specimens described here are indeed reproductively adult.

Taxonomic status of Clione limacina populations in the North Pacific and North Atlantic Oceans

Specimens of *C. limacina* from the North Pacific and North Atlantic are anatomically similar, but differ markedly in body size. Adults attain a maximal body length of *c.* 30 mm in the North Pacific Ocean, but 70 to 85 mm (the largest size of any gymnosome) in Arctic and subarctic waters of the North Atlantic Ocean (Gilmer & Lalli, 1990). These populations also differ with respect to the number and size of hooks: *c.* 30 hooks of 0.7 mm in the North Pacific and *c.* 60 hooks of 1.7 mm in the North Atlantic. We calculated a maximal K2P distance of 7.36% (AV = 6.12%, SD = 0.49) between the COI sequences of *C. limacina* from these two populations and weak to high bootstrap support (89% and 97%) for their reciprocal monophyly (Fig. 6, Supplementary Material Table S2). This difference is comparable with both intra-specific and intrageneric distances in pteropods (Jennings *et al.*, 2010). However, the combination of morphological and genetic evidence suggests that these allopatric populations should be considered as distinct species.

Clione limacina was described by Phipps (1774) from Spitsbergen in the far northern Atlantic, and therefore this name should be

applied to the population in the Arctic and subarctic waters of the North Atlantic. Two nominal taxa of *Clione* have been described from the North Pacific Ocean. The first to be described was *C. elegantissima* Dall, 1871 from eastern Kamchatka, Russia. Pelseneer (1887) examined a specimen of *C. elegantissima* received from W.H. Dall, reporting that this specimen “does not differ at all from *C. limacina* of the same length; the hook-sacs, the radula, the buccal cones, the foot, & c., are quite similar” (Pelseneer, 1887: 47). The specimen was therefore considered to be a young individual of *C. limacina* by Pelseneer (1887). The second described taxon was *C. dalli* (Krause, 1885) from north of Akutan Pass (Bering Strait), Aleutian Islands, Alaska; Dall (1908) considered it to be an immature individual of *C. limacina*. Indeed, the figure accompanying the original description of *C. dalli* shows an absence of tentacles and presence of ciliated rings. These opinions have been accepted by recent authors (e.g. Van der Spoel, 1976; Gofas, 2004). *Clione elegantissima* and *C. dalli* are clearly taxa based on the North Pacific Ocean population of *C. limacina* s. l., and we suggest that the earlier name *C. elegantissima* should be applied to this population.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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