



| | |
|------------------|--|
| Title | Rhamphocottus nagaakii (Cottoidea : Rhamphocottidae), a new species of grunt sculpin from the northwestern Pacific, with notes on the phylogeography of the genus Rhamphocottus |
| Author(s) | Munehara, Hiroyuki; Togashi, Kouji; Yamada, Sayuri; Higashimura, Takushi; Yamazaki, Aya; Suzuki, Shota; Abe, Takuzo; Awata, Satoshi; Koya, Yasunori; Tsuruoka, Osamu |
| Citation | Ichthyological research, 70, 268-285 https://doi.org/10.1007/s10228-022-00885-y |
| Issue Date | 2022-08-23 |
| Doc URL | http://hdl.handle.net/2115/90323 |
| Rights | This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature 's AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: http://dx.doi.org/10.1007/s10228-022-00885-y |
| Type | article (author version) |
| File Information | Ichthyological Research_Munehara, H., Togashi, K., Yamada, S. et al.pdf |



[Instructions for use](#)

***Rhamphocottus nagaakii* (Cottoidea: Rhamphocottidae), a new species of grunt sculpin from the northwestern Pacific, with notes on the phylogeography of the genus**

Rhamphocottus

Hiroyuki Munehara¹ · Kouji Togashi² · Sayuri Yamada² · Takushi Higashimura² · Aya Yamazaki³ · Shota Suzuki^{3,4} · Takuzo Abe⁴ · Satoshi Awata⁵ · Yasunori Koya⁶ · Osamu Tsuruoka⁷

This article was registered in the Official Registry of Zoological Nomenclature (ZooBank) as D4109B0C-FDB7-482C-8EF7-0CF7428BBD19.

(Editorial note from the managing editor: please link the above register number to:

<http://zoobank.org/urn:lsid:zoobank.org:pub:D4109B0C-FDB7-482C-8EF7-0CF7428BBD19>)

¹ Field Science Center for Northern Biosphere, Hokkaido University, 152 Usujiri, Hakodate, Hokkaido 041-1613, Japan

² Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

³ Graduate School of Environmental Science, Hokkaido University, 152 Usujiri, Hakodate, Hokkaido 041-1613, Japan

⁴ Minami-Sanriku Nature Center, Shizugawa, Minami-Sanriku cho, Motoyoshi-gun, 101 Numata, Miyagi 986-0725, Japan

⁵ Graduate School of Science, Osaka Metropolitan University, 3-3-138 Sugimoto, Sumiyoshi, Osaka 558-8585, Japan

⁶ Graduate School of Education, Gifu University, 1-1 Yanagito, Gifu, Gifu 501-1193, Japan

⁷ Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate,
Hokkaido 041-8611, Japan

✉ Hiroyuki Munehara

hm@fsc.hokudai.ac.jp

Running head: New species of *Rhamphocottus*

Full paper

Number of text pages: 31

Number of figures: 6

Number of tables: 2

Abstract A new species of grunt sculpin, *Rhamphocottus nagaakii* inhabiting the northwestern Pacific, previously identified as *Rhamphocottus richardsonii* Günther 1874, is described based on genetic evidence and morphological differences. The new species can be distinguished based on morphometric characters related to the head, including head length [45.3–54.6% of standard length (SL)], postorbital head length (18.8–25.5% SL) and the length of pectoral-fin base (15.8–20.7% SL), which are smaller than in *R. richardsonii* (53.6–60.5% SL, 26.2–31.7% SL, and 19.5–25.2% SL, respectively). Genetic differences between two species markedly exceed levels for intra-specific differences. *Rhamphocottus nagaakii* is considered to have arisen from a common ancestor of the two species, which probably inhabited somewhere the North Pacific Rim around the Aleutian Archipelago. During a period of cooling in the Pliocene or the Miocene, *R. nagaakii* and *R. richardsonii* became separated to the southern regions of the northwestern and northeastern Pacific, and subsequently underwent speciation.

Keywords Perciformes · *Rhamphocottus richardsonii* · Mitochondrial DNA · Divergence time · Discrete distribution

Introduction

The genus *Rhamphocottus* Günther 1874 is characterized by having the head as long as the body trunk; a body almost entirely covered by spike-like scales; a highly modified pelvis; an elongate sub-pelvic keel projecting forward and an antero-dorsally projecting supra-pelvic keel; four infra-orbitals; palatine without teeth; six branchiostegal rays; and all fin rays unbranched (Günther 1874; Nelson et al. 2016). This is a very distinctive sculpin with an elongated head and snout. For a long time, *Rhamphocottus richardsonii* Günther 1874, which is widely distributed from the eastern Aleutian Islands to Santa Monica Bay in southern California, was recognized as the only species in the genus *Rhamphocottus* (Hart 1973; Maslenikov et al. 2013). In 1963, a specimen was retrieved from the oral cavity of an individual of *Sebastes oblongus* Günther 1877 that was captured off Kadonohama, Iwate Prefecture, Japan (Abe 1963), and several specimens of *Rhamphocottus* have occasionally been collected in the coastal waters off Japan in the northwestern Pacific (Hayashi and Nishiyama 1980; Aonuma 1994). These specimens of *Rhamphocottus* were considered to be *R. richardsonii* that had been transported to Japan from elsewhere in the northeastern Pacific in the ballast water of ships. However, following a report of juvenile specimens of *Rhamphocottus* being caught off the coast of Ibaraki Prefecture in 1986 (Saruwatari et al. 1987), and breeding observed underwater off the coast of Miyagi Prefecture in 1999 (Munehara et al. 1999), this study was undertaken to determine whether any differences exist between specimens collected from the northeastern Pacific and northwestern Pacific. Specifically, we examined morphological differences, inferred genetic distances based on partial mitochondrial DNA sequences (mtDNA), and performed phylogeographic analysis. The findings revealed that differences between the two populations of *R. richardsonii* are so large that the population of the *Rhamphocottus* from Japan should be regarded as a new distinct species, *Rhamphocottus nagaakii*. “*Rhamphocottus nagaakii*” firstly appeared in Sato

et al. (2022), published in Jul 2022, without a designation of the holotype. Accordingly, the name appeared in Sato et al. (2022) is unavailable following Art.16.4 of ICZN (1999).

Materials and methods

Genetic analysis. A total of 14 specimens were collected by SCUBA diving in the coastal waters of the northwestern Pacific off Minami-Sanriku cho and Onagawa Bay, Miyagi Prefecture, Japan, from 2000 to 2020. In addition, 24 specimens were collected in the northeastern Pacific off Unalaska Island, Vancouver Island and Puget Sound from 2006 to 2015. Some of the specimens were released back into their former habitat after underwater collection of the tips of the right pectoral fin ($n = 3$), other specimens were collected as larvae hatching from eggs ($n = 3$), and the remainder were brought to the laboratory for tissue sampling and morphometric analysis ($n = 8$). Tissue specimens preserved in 99.5% ethanol were used for DNA extraction which was performed using a QuickGene DNA tissue kit S (Kurabo Industries Ltd.) following the manufacturer's instructions. The cytochrome oxidase *c* subunit I (COI) and the cytochrome *b* (Cyt*b*) regions of mtDNA were amplified with the primer sets FishF1: 5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3' and FishR1: 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' published by Ward et al. (2009) and Cyt*b*-L: 5'-ATG GCA AGC CTA CGA AAA A-3' and Cyt*b*-R: 5'-TCC TAA GGC CTT GTT TTC TA-3' published by Kimura et al. (2007), respectively. Polymerase chain reactions (PCR) were performed in 20 μ l volumes containing 10 μ l of Emerald Amp PCR Master Mix (Takara Bio Inc.), 8.6 μ l of H₂O, 0.5 μ l of each 0.2 μ M primer, and 1 μ l (10–50 ng) of DNA. The PCR profile consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 44 or 56°C for 30 s for COI and at 52 or

58°C for 30 s for *Cytb*, extension at 72°C for 30 s, and a final extension step of 72°C for 7 min. After the final extension step, samples were stored at 4°C. Amplification was performed using a TaKaRa PCR Thermal Cycler Dice (Takara Bio Inc.) and PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co.). PCR products were sequenced with an auto-sequencer (3130 Genetic Analyzer, Applied Biosystems) by Macrogen Japan Corporation using the same PCR primers. Unclear sequence data were excluded from the phylogenetic analyses (one COI sample from *Rhamphocottus richardsonii* and four *Cytb* samples from *R. nagaakii*). The obtained sequences were deposited in GenBank under the accession numbers shown in Table 1, with *R. nagaakii* listed as a new species. A COI sequence from one specimen (deposited as HUMZ 199383 at Hokkaido University Museum, Hakodate, Hokkaido) that was previously collected off Unalaska Island had already been registered as LC1256638, so only the *Cytb* sequence of this specimen was additionally sequenced in this study. In addition, four COI sequences were obtained for *R. richardsonii* from GenBank and used for the phylogenetic analysis in this study.

Sequence alignment of haplotypes was performed using ClustalW implemented in MEGA-X software (Kumar et al. 2018), after selection of the nucleotide substitution model for each loci using Kakusan4 (Tanabe 2011). Molecular phylogenetic relationships were inferred by the maximum likelihood (ML) method after selection of the best model using MEGA-X software (Kumar et al. 2018). *Marukawichthys ambulator* Sakamoto 1931 (GenBank Accession No. AP006787), which has been closely to the genus *Rhamphocottus* (see Smith and Busby 2014; Nelson et al. 2016), was used as the out-group. The robustness for construction of phylogenetic tree was tested using the bootstrap method with 1,000 replicates (Felsenstein 1985). Nucleotide divergence among individuals was computed based on the Kimura 2-parameter (K2P) model by MEGA-X software (Kimura 1980; Kumar et al. 2018).

The divergence time of the two species was estimated based on the observed base substitution rates, assuming 1.5–2.5% base substitution rate/million years that is the generally accepted molecular clock for *Cytb* in fishes (Meyer et al. 1990) and 0.8–3.0% (calculated from 0.36–0.70 base substitution rate and 0.23–0.45 million years) for *Cytb* in the genus *Myoxocephalus* (see Podlesnykh and Moreva 2014). The Markov chain Monte Carlo (MCMC) method implemented in BEAST ver. 2.5 (Bouckaert et al. 2019) was subsequently run for 100 million steps with sampling every 1,000 steps, and the first 10% discarded as burn-in. Tracer v.1.7.2 (Rambaut et al. 2018) to ensure that the effective sample size (ESS) of each statistic was at least 200. The resulting tree and log files were combined using the computer program LogCombiner v.2.6.6 in BEAST. The common ancestor of these two species was inferred to have inhabited somewhere the North Pacific Rim between the western most distribution of *R. richardsonii* and the eastern most distribution of *R. nagaakii*. The Bering Sea off the Aleutian Islands was a warm period when the Bering Strait was opened by glacial melting 5.5–3.1 million years ago (Ma) (Marincovich and Gladenkov 2001). The ancestor was assumed to have moved to southward of both sides during a period of glacial cooling.

Morphological analysis. A total of 26 specimens from the northwestern Pacific and 8 specimens from the northeastern Pacific that were collected by SCUBA diving, as well as an additional 15 specimens from the northeastern Pacific held at the University of Washington Fish Collection (UWFC; Seattle, USA), were subjected to morphometric analysis. Three of the 26 specimens from the northwestern Pacific and the 8 specimens from the northeastern Pacific collected by SCUBA were also used for genetic analysis (Table 1). Counts, measurements, and descriptive terminology followed Miller and Lea (1976) and Kai and Nakabo (2009). Terminology of head spines followed Mecklenburg et al. (2002). The last vertebra with rib were included in the abdominal vertebrae, and the caudal vertebrae were counted from the first vertebrae without rib. All fin ray elements were counted, except those

of the caudal fin. Significant differences between the two species were determined using the *T*-test for counts and the ANCOVA for each measurement, with $p < 0.05$ considered to indicate statistical significance.

Results

Genetic analyses. Of the 42 specimens subjected to genetic analysis (including sequence data from GenBank), unambiguous nucleotide sequences in a 606 base pair (bp) region of COI and a 625 bp region of *Cytb* were obtained for 41 and 35 specimens, respectively. A total of 17 haplotypes were identified for the COI region: 7 haplotypes from 13 specimens from the northwestern Pacific and 10 haplotypes from 28 specimens from the northeastern Pacific. A total of 12 haplotypes were identified for the *Cytb* region; all 10 sequences from the northwestern Pacific showed the same haplotype (Hap 12), with the remaining 11 haplotypes distributed among the 25 specimens from the northeastern Pacific. At both loci, no haplotypes were shared between the two populations, as shown by the phylogenetic trees from the selected best model, the K2 + G (COI) and HKY + G (*Cytb*) (Fig. 1). Clustering of specimens from the northwestern Pacific and northeastern Pacific corresponded to *Rhamphocottus nagaakii* and *R. richardsonii*, respectively. In all of the specimens sequenced, a total of 49 and 48 polymorphic sites were identified in the COI and *Cytb* regions, respectively. The genetic differences (K2P distance) between species in the COI and *Cytb* regions were 0.063–0.079 (mean = 0.066) and 0.068–0.073 (mean = 0.071), respectively, and those rates within species were 0.002–0.008% for both loci. The number of mutations at each locus was less than 3 mutation steps within a species, and we found no genetic differentiation in *Cytb* was observed among 10 specimens of *R. nagaakii*. In contrast, between-species haplotypes were located more than ten steps apart. In terms of the K2P distance of both loci, the values

between species markedly exceeded the value within species, which is greater than the value of 0.01 that is adequate for separating between species (Ward et al. 2009).

Assuming the base substitution rate for *Cytb* estimated in this study, these two species were estimated to have diverged from approximately 2.8–4.7 Ma (1.5–2.5% per million years) and 2.3–8.8 Ma (0.8–3.0% per million years). Analysis with BEAST software program for only *Cytb* was the ESS < 200 due to short sequence data. Adding COI data set, species divergence was inferred to have occurred during 5.5–24.9 Ma selected as the largest ESS value.

Counts and measurements. Proportional measurements and counts are given of Table 2. The ranges in all counts examined overlapped between the two species, but there were slight differences in modes. In counts, there was significant difference for only vertebrae number (larger in *R. richardsonii*).

Since the body size of *R. nagaakii* was significantly smaller than that of *R. richardsonii*, comparisons of morphometric characters between species were conducted after the measured values were converted to % SL were calculated allometrically relative to SL. Significant differences between species were observed in head length, postorbital head length, and pectoral-fin base (Table 2). These characters markedly different, with mostly almost no overlapping between the two species, were smaller in *R. nagaakii* than in *R. richardsonii* (Fig. 2).

***Rhamphocottus nagaakii* Munehara, Yamazaki and Tsuruoka, sp. nov.**

(Standard Japanese name: Kuchibashi-kajika; New English name: Nihon grunt sculpin; Figs. 3, 4, 5; Table 2)

Rhamphocottus nagaakii: unavailable name, Sato et al. 2022: 311, 314.

Rhamphocottus richardsonii (not of Günther 1874): Abe 1963: 51, pl. XII, fig.14 (Iwate Prefecture, Japan); Fujita and Kamei 1984: 39, fig. 1 (Tokyo Bay, Japan); Saruwatari et al. 1987: 388, fig. 2 (Ibaraki Prefecture, Japan); Aonuma 1994: preface (Iwate Prefecture, Japan); Munehara et al. 1999: 2, fig. 1 (Miyagi Prefecture, Japan).

Holotype. HUMZ 232510, 38.2 mm SL, 38°39'N, 141°29'E, off Minami-Sanriku cho, Miyagi Prefecture, Japan, 15 m depth, 12 Mar. 2018, hand net, collected by T. Abe.

Paratypes. HUMZ 231930–231937, 33.0–47.0 mm SL, 38°39'N, 141°29'E, off Minami-Sanriku cho, Miyagi Prefecture, Japan, 8–18 m depth, 1 Dec. 2007, hand net, collected by Na. Satoh and H. Munehara, collected with the holotype; HUMZ 231938–231943, 33.0–38.0 mm SL, 38°39'N, 141°29'E, off Minami-Sanriku cho, Miyagi Prefecture, Japan, 8–18 m depth, 16–17 Dec. 2008, hand net, collected by Na. Satoh; HUMZ 231944, 231945, 36.9–37.1 mm SL, 38°39'N, 141°29'E, off Minami-Sanriku cho, Miyagi Prefecture, Japan, 8–18 m depth, 28 May 2015, hand net, collected by T. Abe; HUMZ 231946–231954, 36.3–46.6 mm SL, 38°39'N, 141°29'E, off Minami-Sanriku cho, Miyagi Prefecture, Japan, 8–18 m depth, 29 June–1 July, 2010, hand net, collected by Na. Satoh, H. Munehara, M. Kimura and No. Satoh.

Diagnosis. Distinguished from *Rhamphocottus richardsonii* by having the following combination of characters: head small, length 45.3–54.6% SL (vs. 53.6–60.5% SL in *R. richardsonii*); orbit large, length 10.8–14.0% SL (vs. 9.1–11.8% SL); postorbital head short, length 18.8–24.5% SL (vs. 26.2–31.7% SL); and pectoral fin base short, length 15.8–20.7% SL (vs. 19.5–25.2% SL). This species is also distinguished from *R. richardsonii* by unique haplotypes at the mitochondrial *Cytb* and *COI* loci.

Description. Data range of all specimens are shown first, followed by the values for the holotype value in parentheses.

Body short, compressed, body depth at origin of first dorsal fin 1.8–2.6 (2.3) times body width, body depth at origin of anal fin 3.0–4.7 (3.1) times body width. Head large, compressed, depth 1.5–1.8 (1.8) times head width, dorsal profile moderately rounded from nape to orbit (Fig. 3). Trunk tapering suddenly posteriorly. Caudal peduncle firm, compressed, depth 82.8–135.0% (91.7%) in length. Snout slender, protruding, upper profile moderately steep, length 32.4–39.1% (36.0%) of head length.

Nasal spine short, sharp, directed posterodorsally. Anterior nostril on outer base of nasal spine, forming short tube. A single sharp postocular spine over each eye. Posterior nostril on anterior margin of orbit, with low rim. Mouth small; upper jaw (premaxilla) extending forward slightly beyond lower jaw (dentary). Maxilla extending to level of anterior margin of orbit. Small conical teeth on jaws and vomer, forming villiform bands. No teeth on palatine. Gill opening small confined to region above pectoral fin base; gill membranes completely united to pectoral girdle and isthmus. Eye large, in center of head length; length of orbit 21.0–28.5% (21.0%) of head length. Interorbital space narrow, deeply concave, width 20.6–25.0% (25.0%) of head length. Occipital region concave; two massive fronto-parietal ridges extending to origin of first dorsal fin. Nuchal spine blunt, united with fronto-parietal ridge. Single preopercular spine on each side sharp, stout, straight, and long. Supra-cleithral spine sharp and stout. Cleithral spine on clavicle behind gill opening, sharp, stout, and long.

Numerous prickles with 1–4 minute hooks at their tips scattered on head and body; prickles on head much smaller than on body. No cirri. Dermal flap on snout tip, firm but tiny.

First dorsal fin originating at level of posterior margin of opercular flap; dorsal spines very flexible; multi-hooked prickles covering base of dorsal fins. First and second dorsal fin separated by small space. Basal length of first dorsal fin 49.6–77.1% (60.0%) of base of second dorsal fin. Second dorsal fin originating at upper center of trunk; posterior tip of second dorsal fin reaching base of caudal fin. Anal fin originating directly ventral to fifth to seventh (seventh) ray of second dorsal fin. Pectoral fins large, with pointed distal margin;

base of uppermost ray ventral to point of nuchal spine; lower six rays entirely free from membrane, slightly thickened. Pelvic fin slender, located midway between ventral base of pectoral fin and anus; consisting of one spine and three rays; middle ray longest, reaching near origin of anal fin; spine and outermost soft ray closely united. Caudal fin with rounded margin. All fin rays unbranched. Anus located ventral to third to seventh (seventh) spine of second dorsal fin.

Vertebrae arc dorsally above abdomen, curving downward to caudal; all ossified.

Males with no external urogenital papilla.

Live coloration (Fig. 4). Body creamy yellow or orange, with conspicuous irregular dark stripes, partially dotted curved lines running obliquely across body, edged with black; similar stripes comprising small spots radiating from eye in all directions, one stripe to end of snout, one extending downward forming a triangle, one extending backward and downward to middle of preopercle, then turning upward and extending almost to nuchal spine, and two or three short stripes above; deep orange band extending across caudal peduncle; dark brown spots near base of dorsal fin, pectoral fin and anal fin rays.

Color in alcohol (Fig. 3). Head and entire body almost dull white, with conspicuous irregular gray stripes, partially dotted curved lines running obliquely across body, small spots radiating from eye in all directions, one stripe to end of snout, one extending downward forming a triangle, one extending backward and downward to middle of preopercle; dull white and gray bands across caudal peduncle; all fins almost whitish.

Distribution. Known from Iwate Prefecture, Miyagi Prefecture, Ibaraki Prefecture to Tokyo Bay along the northwestern Pacific Ocean in Japan (Fig. 5). Distributional records in off Kuril Islands and/or eastern Kamchatka (e.g., Nakabo and Kai 2013; Parin et al. 2014) is not confirmed as far our knowledge.

Etymology. The new species is named for Mr. Nagaaki Satoh, a professional diving instructor, who first observed the reproductive behavior of this species underwater and

informed us of his observations. The English name “Nihon” refers to the Japanese name for Japan, emphasizing that the new species is distributed around Japan and is distinct from the congeneric species *R. richardsonii*.

Remarks. Only two species are recognized in the genus *Rhamphocottus*.

Rhamphocottus nagaakii with a maximum length probably less than 47.0 mm SL is smaller than the only other the member of the genus *Rhamphocottus*. In the coastal waters off Minami-Sanriku cho, Miyagi Prefecture, adults inhabit rock crevices or empty shells of large barnacles at depths of 30 m. The following information on the reproductive ecology were cited from Munehara et al. (1999), and the mature size and recent breeding situation were ascertained through collection of the present specimens of *R. nagaakii*. The breeding season is restricted to the winter (December to February). Both sexes are mature at more than 35 mm SL and above. A female deposits a single clutch containing 150–400 eggs (2.3–2.6 mm in diameter) per season, and males exhibit parental care and protect the eggs from predators until they hatch (Fig. 6). The reproductive mode of *R. nagaakii* is external fertilization without copulatory behavior. There is no distinct morphological sexual dimorphism. Body coloration varies among individuals, irrespective of sex. The entire area inhabited by *R. nagaakii* was severely damaged by the Great East Japan Earthquake in 2011, and breeding of this species was not observed until 2015. The population is considered to be recovering gradually.

Comparisons. In the original description of *R. richardsonii*, the head is as long as the body without caudal fin rest, the narrow pointed snout is twice as long as the eye, the diameter of which is contained five times and a half in the head length, and the standard length is 2.5 inches (Günther 1874). In comparing with the two species of *Rhamphocottus* examined in this study, the head length relative to the standard length (50% SL) is included within the range (45.3–54.6% SL) of specimens from the northwestern Pacific not the range (53.6–60.5% SL) from the northeastern Pacific, but the value is plotted near median between the regression lines of these species (Fig. 2a). The length of orbit (9.1% SL) is included

within the range (9.1–11.0% SL) of the specimens from the northeastern Pacific but out of the range (10.8–14.0% SL) from northwestern Pacific. The standard length (63.5 cm) is within the size range (28.2–66.4 cm) of the specimens from the northeastern Pacific but more than the largest size (47.4 mm) of the northwestern Pacific. Consequently, the specimen described by Günther (1874) and the present specimens from the northwestern Pacific (*R. nagaakii*) are regarded not in identical species.

In morphological comparing the two species, *R. nagaakii* can be distinguished based on morphometric characters related to the head, including head length, postorbital head length and pectoral-fin base, which smaller than in *R. richardsonii*. There is less differences in the other measurements, and the ranges of counts are overlapped between the two species. Body color variants are overlapped between the two species excepting for band extending across caudal peduncle in live specimens; deep orange in *R. nagaakii* contrast to bright cherry red in *R. richardsonii* (Fig. 4).

Discussion

Evidence of notable genetic divergence, well defined morphological differences and geographic isolation by discrete distribution showed that the species of *Rhamphocottus* inhabiting the northwestern Pacific differs from *Rhamphocottus richardsonii* and should therefore be regarded as a new species, *R. nagaakii*.

Rhamphocottus richardsonii, which is distributed from the Gulf of Alaska to Santa Monica Bay in southern California (Hart 1973) and report has not been observed in the Bering Sea (Mecklenburg et al. 2002). Maslenikov et al. (2013) reported that two specimens of *R. richardsonii* are collected off Unalaska Islands in the eastern Aleutian Islands. As well

as these specimens, the record of a specimen (HUMZ 199383) examined in this study are considered to represent the northernmost and westernmost record of *R. richardsonii*.

On the other hand, *R. nagaakii* is only known to inhabit a narrow area of the Pacific coast of Tohoku region of Japan. Thus, while these species of *Rhamphocottus* have been collected on both sides of the Pacific Rim, no specimens have been collected from the region extending from the central Bering Sea to the Okhotsk Sea and the Japan Sea. The discrete distribution of these closely related sister species, which are considered to have diverged from a common ancestor, is interpreted within the phylogeographic context of a molecular clock (Avice 2000). In this study, the common ancestor of these species was hypothesized to have inhabited somewhere the North Pacific Rim around the Aleutian Archipelago during glacial melting 5.5–3.1 million years ago (Ma) (Marincovich and Gladenkov 2001). During a period of glacial cooling, populations of the ancestral species may have moved southward and acclimated to a temperate environment resulting in the formation of separate species with discrete distributions. The divergence time estimated from the BEAST software program was 5.5–24.9 Ma, although the assumption of a glacial period after the opening of the Bering Strait. The divergence time estimated from base substitution rates was 2.3–8.8 Ma, which corresponds to the Pliocene (2.5–5.3 Ma) or the Miocene (5.3–23 Ma). It was implied that the speciation had already initiated during a cooling period before the glacial melting. Based on the genetic and morphological differences observed between the two species examined in this study, it is unlikely that *R. nagaakii* was introduced into Japanese waters in the ballast water of ships; rather, it is considered that *R. nagaakii* is a valid endemic species that arose by typical biogeographical processes.

Acknowledgments We would like to thank Katherine Pearson Maslenikov (UWFC) for the loan of specimens; Mamoru Yabe for valuable advice regarding morphological analyses; Fumihito Tashiro for taking X-ray photographs; Noriyoshi Satoh, Motoko Kimura-

Kawaguchi, and Phillip Bruecker for assistance with field collections; Theodore W. Pietsch, Jeffery B. Marliave, Jimmer McDonald and Alyssa McDonald for their assistance with research logistics in North America. We are grateful to Yoshiaki Kai for heartfelt comments for acceptance of the manuscript. This study was supported by Grants-in-Aid from JSPS KAKENHI (Grant/Award Number: 17405019, 25304011, 26292098).

References

- Abe T (1963) New, rare or uncommon fishes from Japanese waters. VIII. A record of *Rhamphocottus richardsoni*. Japan J Ichthyol 10:51–52
- Aonuma Y (1994) *Rhamphocottus richardsonii* captured at Sanriku coast. Aquabiology 16:preface (In Japanese)
- Avice JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge
- Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, Heled J, Jones G, Kühnert D, Maio ND, Matschiner M, Mendes FK, Müller NF, Ogilvie HA, du Plessis L, Poppinga A, Rambaut A, Rasmussen D, Siveroni I, Suchard MA, Wu CH, Xie D, Zhang C, Stadler T, Drummond AD (2019) BEAST 2.5: an advanced software platform for Bayesian evolutionary analysis. PLoS Comput Biol 15(4):e1006650
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
- Fujita K, Kamei M (1984) Records of *Rhamphocottus* off Tokyo and *Aldrovandia* from Sagami Bay, Japan. J Tokyo Univ Fish 71:39–44
- Günther A (1874) Descriptions of new species of fishes in the British Museum. Ann Mag Nat Hist (Ser 4) 14:368–371

- Günther A (1877) Preliminary notes on new fishes collected in Japan during the expedition of H. M. S. 'Challenger'. *Ann Mag Nat Hist (Ser 4)* 20:433–446
- Hart JL (1973) Pacific fishes of Canada. Bulletin 180. Fisheries Research Board of Canada, Ottawa
- Hayashi M, Nishiyama K (1980) Fishes collected by the stationary net in the west coast of Sagami Bay. The catalogue of fish collections of Sagami Bay. *Nat Hist Rep Kanagawa* 1:15–27
- ICZN (1999) International code of zoological nomenclature, 4th edn. International Trust of Zoological Nomenclature, London
- Kai Y, Nakabo T (2009) Taxonomic review of the genus *Cottiusculus* (Cottoidei: Cottidae) with description of a new species from the Sea of Japan. *Ichthyol Res* 56:213–226
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Kimura M, Yanagimoto T, Munehara H (2007) Maternal identification of hybrid eggs in *Hexagrammos* spp. by means of multiplex amplified product length polymorphism of mitochondrial DNA. *Aquat Biol* 1:187–194
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35:1547–1549
- Marincovich L Jr, Gladenkov AY (2001) New evidence for the age of Bering Strait. *Quat Sci Rev* 20:329–335
- Maslenikov K, Orr JW, Stevenson DE (2013) Range extensions and significant distributional records for eighty-two species of fishes in Alaskan marine waters. *Northwest Nat* 94:1–21
- Mecklenburg CW, Mecklenburg TA, Thorsteinson LK (2002) Fishes of Alaska. American Fisheries Society, Maryland

- Meyer A, Kocher TD, Basasibwaki P, Wilson AC (1990) Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial-DNA sequences. *Nature* 347:550–553
- Miller DJ, Lea RN (1976) Guide to the coastal marine fishes of California. *Fish Bull* 157:1–249
- Munehara H, Satoh N, Watanabe S (1999) The reproductive biology of grunt sculpin, *Rhamphocottus richardsonii*. *IOP Diving News* 10:2–3
- Nakabo T, Kai Y (2013) *Rhamphocottus richardsonii*. In: Nakabo T (ed) *Fishes of Japan with pictorial keys to the species*. Third edition. Tokai University Press, Hadano, pp 1158, 2061
- Nelson JS, Grande TC, Wilson MVH (2016) *Fishes of the World*, 5th Edition. John Wiley & Sons Inc, Hoboken
- Parin NV, Evseenko SA, Vasil'eva ED (2014) *Fishes of Russian Seas: annotated catalogue*. KMK Scientific Press, Moscow
- Podlesnykh AV, Moreva IN (2014) Variability and relationships of the Far Eastern species of sculpins *Myoxocephalus* and *Megalocottus* (Cottidae) based on mtDNA markers and karyological data. *Russ J Genet* 509:949–956
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol* 67:901–904
- Sakamoto K (1931) Type of a new family of mailed-cheek fish from the Japan Sea, *Marukawichth ambulator* n. g. n. sp. *J Imp Fish Inst* 26:53–56
- Saruwatari T, Betsui K, Okiyama M (1987) Occurrence of the grunt sculpin (*Rhamphocottus richardsoni*) larvae from northern central Japan. *Ichthyol Res* 34:387–392
- Sato N, Seki K, Munehara H (2022) *An underwater guide to the coastal fishes of northern Japan*. Hokkaido Univ Press, Sapporo

- Smith WL, Busby MS (2014) Phylogeny and taxonomy of sculpins, sandfishes, and snailfishes (Perciformes: Cottoidei) with comments on the phylogenetic significance of their early-life-history specializations. *Mol Phylogenet Evol* 79:332–352
- Tanabe AS (2011) Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. *Mol Ecol Resour* 11:914–921
- Ward RD, Hanner R, Hebert PDN (2009) The campaign to DNA barcode all fishes, FISH-BOL. *J Fish Biol* 74:329–356

Figure legends

Fig. 1 Molecular phylogenetic tree estimated by the ML method (using 1,000 bootstrap replications) representing mitochondrial DNA phylogeny among species of *Rhamphocottus* based on **a** COI (606 bp) and **b** Cytb (625 bp) datasets. The scale bars indicate genetic distance expressed as units of expected nucleotide substitutions per site, and values greater than 0.01 are indicated near the branches. The bootstrap values more than 50 (Italic type) are shown on the branches. Specimens are represented by accession number, locality, and haplotype. Localities are abbreviated as (A) Anacortes, (T) Tofino, (S) Seattle, (WS) Washington State, (PS) Puget Sound, (Ala) Alaska, (U) Unalaska Island, (M) Monterey Bay, (J) Japan

Fig. 2 Plots of measurements showing significant differences between *Rhamphocottus richardsonii* (blue squares) and *R. nagaakii* (orange circles) among morphometric characters. **a** Head length; **b** postorbital head length; **c** length of pectoral-fin base

Fig. 3 *Rhamphocottus nagaakii*, new species, HUMZ 232510, 38.2 mm SL, holotype, Minami-Sanriku cho, Miyagi Prefecture, Japan, 38°39'N, 141°29'E, 10 m depth

Fig. 4 *Rhamphocottus nagaakii* (left photograph taken by Na. Satoh, in Minami-Sanriku cho, Miyagi Prefecture, Japan, 26 Mar. 2007; specimens not collected) and *Rhamphocottus richardsonii* (right photograph collected in Tofino, Vancouver Island, Canada, 9 Jul. 2015, HUMZ 231958, 56.5 mm SL)

Fig. 5 Distribution area of *Rhamphocottus richardsonii* (faint blue) and *R. nagaakii* (blue) based on the specimens examined here and literature records

Fig. 6 *Rhamphocottus nagaakii* with eggs (specimens not collected). The male (left) successfully mated with a mature female, and after several hours she deposited her eggs near a previously deposited egg mass and then the male released sperm on the newly spawned egg mass (right; photograph by Na. Satoh, in Minami-Sanriku cho, Miyagi Prefecture, Japan, 17 Dec. 2003)

Table 1 List of specimens examined in this study

(a) For morphological analysis

| Species | Collection site | Collection date | Collectors | Standard length (mm) | Type category | Collection number |
|-------------------------------|--|-----------------|------------|----------------------|---------------|-------------------|
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 12 Mar 2018* | T A | 38.2 | Holotype | HUMZ 232510 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 46.7 | Paratype | HUMZ 231930 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 46.7 | Paratype | HUMZ 231931 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 47.4 | Paratype | HUMZ 231932 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 47.0 | Paratype | HUMZ 231933 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 43.4 | Paratype | HUMZ 231934 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 41.8 | Paratype | HUMZ 231935 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 33.0 | Paratype | HUMZ 231936 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 34.0 | Paratype | HUMZ 231937 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 31.7 | Paratype | HUMZ 231938 |

| | | | | | | |
|-------------------------------|--|-------------------|----------------------|------|----------|----------------|
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 31.7 | Paratype | HUMZ 231939 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 35.2 | Paratype | HUMZ 231940 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 33.0 | Paratype | HUMZ 231941 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 38.0 | Paratype | HUMZ 231942 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 37.2 | Paratype | HUMZ 231943 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 28 May 2015 | T A | 37.1 | Paratype | HUMZ 231944 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 28 May 2015 | T A | 36.8 | Paratype | HUMZ 231945 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 39.9 | Paratype | HUMZ 231946 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 43.3 | Paratype | HUMZ 231947 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 44.4 | Paratype | HUMZ 231948 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 37.0 | Paratype | HUMZ 231949 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 36.9 | Paratype | HUMZ 231950 |

| | | | | | | |
|-----------------------------------|--|-------------------|----------------------|------|----------|-------------|
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 38.7 | Paratype | HUMZ 231951 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 46.6 | Paratype | HUMZ 231952 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 42.3 | Paratype | HUMZ 231953 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 29 Jun–1 Jul 2010 | Na S, H M, M K, No S | 36.3 | Paratype | HUMZ 231954 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 59.6 | Non-type | HUMZ 199315 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 66.4 | Non-type | HUMZ 199316 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 63.0 | Non-type | HUMZ 199317 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 20 Mar 2006 | Na S, H M, O T | 66.4 | Non-type | HUMZ 199348 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 20 Mar 2006 | Na S, H M, O T | 65.9 | Non-type | HUMZ 199349 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 20 Mar 2006 | Na S, H M, O T | 52.0 | Non-type | HUMZ 199350 |
| <i>Rhamphocottus richardsonii</i> | USA: Unalska Island, Dutch harbor (53°52'N, 166°33'W) | 27 Jun 2006 | Na S, H M, O T, S A | 54.4 | Non-type | HUMZ 199383 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 9 Jul 2015 | Na S, H M, O T, S A | 56.5 | Non-type | HUMZ 231958 |

| | | | | | | |
|-----------------------------------|---|-------------|---------------------------|------|----------|----------|
| <i>Rhamphocottus richardsonii</i> | USA: San Jual Island, West shore of San Juan Channel | 1 Jul 1932 | W.M. CHAPMAN, A.C. DELACY | 39.1 | Non-type | UW002145 |
| <i>Rhamphocottus richardsonii</i> | USA: San Jual Island, West shore of San Juan Channel | 1 Jul 1932 | W.M. CHAPMAN, A.C. DELACY | 36.2 | Non-type | UW002145 |
| <i>Rhamphocottus richardsonii</i> | USA: San Jual Island, West shore of San Juan Channel | 1 Jul 1932 | W.M. CHAPMAN, A.C. DELACY | 32.9 | Non-type | UW002145 |
| <i>Rhamphocottus richardsonii</i> | USA: San Jual Island, West shore of San Juan Channel | 1 Jul 1932 | W.M. CHAPMAN, A.C. DELACY | 30.9 | Non-type | UW002145 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Indianola | 6 Nov 1948 | unknown | 32.3 | Non-type | UW006128 |
| <i>Rhamphocottus richardsonii</i> | USA: Griffin Bay (between San Juan and Lopez Islands) | 23 Jul 1931 | J.E. GUBERLET | 49.1 | Non-type | UW001744 |
| <i>Rhamphocottus richardsonii</i> | USA: San Juan Archiperago, off Sucia Island | 25 Jul 1931 | J.E. GUBERLET | 43.2 | Non-type | UW001745 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Elliot Bay, Seattle | unknown | unknown | 46.4 | Non-type | UW010216 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Elliot Bay, Seattle | unknown | unknown | 35.0 | Non-type | UW010216 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Elliot Bay, Seattle | unknown | unknown | 32.0 | Non-type | UW010216 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, off Golden Gardens | unknown | unknown | 31.4 | Non-type | UW014327 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, off Golden Gardens | unknown | ANONYMO US | 30.6 | Non-type | UW014327 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Point Defiance | unknown | unknown | 44.5 | Non-type | UW016390 |

| | | | | | | |
|-----------------------------------|--|-------------|------------------|------|----------|----------|
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Agate Passage | 24 Sep 1948 | VAN CLEVE | 28.2 | Non-type | UW014340 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, North Beach, Seattle | 5 Apr 1938 | WELANDE R, CLASS | 34.5 | Non-type | UW004685 |

(b) For genetic analysis

| Species | Collection site | Collection date | Collectors | COI/Cytb Haplotypes | Sample information | Accession No GenBank (COI) | Accession No GenBank (Cytb) |
|--|--|-----------------|---------------------------|---------------------|--|----------------------------|-----------------------------|
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Onagawa Bay (38°26'N, 141°27'E) | 26 Jun 2004 | Na S, H M | 13/- | Non-type (use up) | LC627534 | --- |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Onagawa Bay (38°26'N, 141°27'E) | late Feb 2005 | Na S, H M, M K, No S, O T | 8/- | Non-type (use up) | LC627509 | --- |
| <i>Rhamphocottus nagaakii</i> (embryo) | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 26 Feb 2006 | Na S, H M | 8/12 | Non-type (use up) | LC627533 | LC627794 |
| <i>Rhamphocottus nagaakii</i> (embryo) | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 26 Feb 2006 | Na S, H M | 9/12 | Non-type (use up) | LC627529 | LC627790 |
| <i>Rhamphocottus nagaakii</i> (embryo) | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 26 Feb 2006 | Na S, H M | 10/12 | Non-type (use up) | LC627526 | LC627785 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 8/12 | Non-type (Release after collecting tissue) | LC627539 | LC627811 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 10/12 | Non-type (Release after collecting tissue) | LC627537 | LC627809 |

| | | | | | | | |
|-----------------------------------|--|----------------|---------------------|-------|--|----------|----------|
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 1 Dec 2007 | Na S, H M | 8/12 | Non-type (Release after collecting tissue) | LC627538 | LC627808 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | -/12 | Non-type (use up) | --- | LC627542 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 16–17 Dec 2008 | Na S | 11/- | HUMZ 231943, paratype | LC627532 | --- |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 28 May 2015 | T A | 12/- | HUMZ 231944, paratype | LC627518 | --- |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 28 May 2015 | T A | 13/12 | HUMZ 231945, paratype | LC627516 | LC627802 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 5 Oct 2020 | T A | 13/12 | Non-type (DNA kept in Usujiri lab) | LC627535 | LC627813 |
| <i>Rhamphocottus nagaakii</i> | Japan: Miyagi Prefecture, Minami-Sanriku cho (38°39'N, 141°29'E) | 20 Oct 2020 | T A | 14/12 | Non-type (DNA kept in Usujiri lab) | LC627536 | LC627814 |
| <i>Rhamphocottus richardsonii</i> | USA: Unalaska Island, Dutch harbor (53°52'N, 166°33'W) | 27 Jun 2006 | Na S, H M, O T, S A | 1/1 | HUMZ 199383 | LC125638 | LC627544 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 1/1 | HUMZ 199315 | LC627513 | LC627786 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 1/2 | HUMZ 199316 | LC627512 | LC627545 |

| | | | | | | | |
|-----------------------------------|--|----------------|--------------------------|------|---------------------------|----------|----------|
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 1/1 | HUMZ 199317 | LC627511 | LC627806 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 1/1 | (DNA kept in Usujiri lab) | LC627510 | LC627803 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 2/2 | (DNA kept in Usujiri lab) | LC627508 | LC627800 |
| <i>Rhamphocottus richardsonii</i> | USA: Puget Sound, Anarcortes (48°30'N, 122°49'W) | 9–10 Mar 2006 | Na S, H M, O T | 2/2 | (DNA kept in Usujiri lab) | LC627507 | LC627796 |
| <i>Rhamphocottus richardsonii</i> | USA: Washington State, off Seattle | -- ---- 2000 | K D C | 6/10 | (from Aquarium) | LC627540 | LC627812 |
| <i>Rhamphocottus richardsonii</i> | USA: Washington State, off Seattle | -- ---- 2000 | K D C | 1/11 | (from Aquarium) | LC627541 | LC627810 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 20 Mar 2006 | Na S, H M, O T | 3/3 | HUMZ 199348 | LC627517 | LC627793 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 20 Mar 2006 | Na S, H M, O T | 4/4 | HUMZ 199349 | LC627515 | LC627791 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 20 Mar 2006 | Na S, H M, O T | 2/2 | HUMZ 199350 | LC627514 | LC627788 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 9 Jul 2015 | Na S, H M, S A, A Y, Y O | 1/2 | HUMZ 231958 | LC627528 | LC627804 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 7 Jul 2015 | Na S, H M, S A, A Y, Y O | 1/2 | (DNA kept in Usujiri lab) | LC627530 | LC627807 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 10–11 Jul 2005 | Na S, H M, S A, A Y, Y O | 1/5 | (DNA kept in Usujiri lab) | LC627525 | LC627799 |

| | | | | | | | |
|-----------------------------------|---|----------------|--------------------------|------|---------------------------|----------|----------|
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 10–11 Jul 2005 | Na S, H M, S A, A Y, Y O | 1/2 | (DNA kept in Usujiri lab) | LC627523 | LC627797 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 10–11 Jul 2005 | Na S, H M, S A, A Y, Y O | 1/2 | (DNA kept in Usujiri lab) | LC627522 | LC627792 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 10–11 Jul 2005 | Na S, H M, S A, A Y, Y O | 1/2 | (DNA kept in Usujiri lab) | LC627521 | LC627789 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | 10–11 Jul 2005 | Na S, H M, S A, A Y, Y O | 1/2 | (DNA kept in Usujiri lab) | LC627520 | LC627787 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | late Feb 2017 | S A, T I | 5/6 | (DNA kept in Usujiri lab) | LC627519 | LC627543 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | late Feb 2017 | S A, T I | 6/7 | (DNA kept in Usujiri lab) | LC627531 | LC627805 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | late Feb 2017 | S A, T I | 7/8 | (DNA kept in Usujiri lab) | LC627527 | LC627801 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | late Feb 2017 | S A, T I | 1/1 | (DNA kept in Usujiri lab) | LC627524 | LC627798 |
| <i>Rhamphocottus richardsonii</i> | Canada: Vancouver Island, Tofino (49°09'N, 125°59'W) | late Feb 2017 | S A, T I | -/9 | (use up) | --- | LC627795 |
| <i>Rhamphocottus richardsonii</i> | USA: Washington State, eastern North Pacific, Puget Sound | (from Genbank) | | 15/- | (from Genbank) | KY570361 | --- |
| <i>Rhamphocottus richardsonii</i> | USA: California State, Monterey Co., off Point Sur | (from Genbank) | | 1/- | (from Genbank) | GU44050 | --- |
| <i>Rhamphocottus richardsonii</i> | USA: Alaska State, Alaska Sea Life Center | (from Genbank) | | 16/- | (from Genbank) | KP827343 | --- |

| | | | | | | |
|-----------------------------------|--|----------------|-----------|----------------|----------|----------|
| <i>Rhamphocottus richardsonii</i> | USA: Washington State, eastern North Pacific | (from Genbank) | 17/- | (from Genbank) | JQ354323 | --- |
| <i>Marukawichthys ambulator</i> | no information | (from Genbank) | out-group | (from Genbank) | AP006787 | AP006787 |

In collectors: *Na S* Nagaaki Satoh, *H M* Hiroyuki Munehara, *T A* Takuzou Abe, *M K* Motoko Kimura, *No S* Noriyoshi Satoh, *O T* Osamu Tsuruoka, *S A* Satoshi Awata, *K D C* Karen D. Crow, *A Y* Aya Yamazaki, *Y O* Yohei Ootomo, *T I* Takeshi Itoh

* Reared for two months before preservation

Table 2 Counts and proportional morphometric characters of *Rhamphocottus nagaakii* and *R. richardsonii*

| | <i>Rhamphocottus nagaakii</i> | | | <i>Rhamphocottus richardsonii</i> | | Significances |
|-------------------------------------|-------------------------------|-------------------------------------|-------------------|-----------------------------------|-------------------|----------------|
| | Holotype | Paratype specimens (<i>n</i> = 25) | | <i>(n</i> = 23) | | |
| Total length (TL; mm) | 45.5 | 35.2–59.1 | | 35.3–81.1 | | |
| Standard length (SL; mm) | 38.2 | 31.7–47.4 | | 28.2–66.4 | | |
| Counts | Data | Range | Mode | Range | Mode | <i>P</i> value |
| Dorsal-fin spines | VII | VI–VIII | VII | VII–IX | VIII | 0.003 |
| Dorsal-fin rays | 11 | 11–13 | 12 | 12–14 | 12 | 0.006 |
| Anal-fin rays | 6 | 6–7 | 7 | 6–8 | 7 | 0.012 |
| Pectoral-fin rays | 16 | 13–16 | 15 | 15–16 | 16 | 0.001 |
| Pelvic-fin rays | I, 3 | I, 3 | I, 3 | I, 3 | I, 3 | - |
| <i>Vertebrae</i> | <i>12+14</i> | <i>(12–13) + (13–14)</i> | <i>12 + 14</i> | <i>(12–*13) + (14–15)</i> | <i>12 + 15</i> | <i>0.953</i> |
| Proportion in SL (%) | Data | Range | Mean ± <i>SD</i> | Range | Mean ± <i>SD</i> | <i>P</i> value |
| <i>Head length</i> | <i>52.4</i> | <i>45.3–54.6</i> | <i>50.4 ± 2.1</i> | <i>53.6–60.5</i> | <i>56.6 ± 2.1</i> | <i>0.165</i> |
| Predorsal-fin length | 59.4 | 53.9–71.4 | 61.2 ± 4.1 | 59.1–68.8 | 64.0 ± 2.3 | 0.030 |
| Body depth at 1st dorsal fin origin | 44.5 | 41.3–51.9 | 46.7 ± 2.6 | 43.0–50.6 | 47.5 ± 2.5 | 0.000 |
| Body width at 1st dorsal fin origin | 19.6 | 19.6–25.4 | 22.3 ± 1.7 | 18.3–28.1 | 21.8 ± 2.4 | 0.000 |
| Body depth at anal fin origin | 30.4 | 27.1–34.5 | 30.4 ± 1.9 | 29.4–36.5 | 32.6 ± 1.8 | 0.000 |

| | | | | | | |
|-------------------------------------|------|-----------|------------|-----------|------------|-------|
| Body width at anal fin origin | 10.0 | 6.2–10.0 | 8.5 ± 1.0 | 8.3–12.4 | 10.3 ± 1.3 | 0.000 |
| Length of 1st dorsal fin base | 14.9 | 13.2–17.7 | 15.6 ± 1.1 | 12.5–18.6 | 16.0 ± 1.5 | 0.000 |
| Length of 2nd dorsal fin base | 24.9 | 20.0–28.8 | 24.2 ± 1.8 | 22.0–28.7 | 25.7 ± 1.7 | 0.005 |
| Prepelvic-fin length | 47.6 | 45.0–54.4 | 50.2 ± 2.4 | 43.9–56.6 | 50.3 ± 3.3 | 0.000 |
| Preanal-fin length | 80.4 | 77.2–88.5 | 82.5 ± 3.0 | 73.7–82.0 | 78.3 ± 2.0 | 0.000 |
| Length from anus to anal-fin origin | 5.2 | 4.3–12.4 | 8.1 ± 2.2 | 5.1–7.1 | 6.1 ± 0.6 | 0.000 |
| Length of anal-fin base | 12.3 | 10.2–13.5 | 12.2 ± 0.9 | 11.7–16.0 | 13.3 ± 1.0 | 0.000 |
| Length of caudal peduncle | 12.6 | 7.9–12.8 | 10.2 ± 1.5 | 9.0–13.9 | 11.1 ± 1.2 | 0.000 |
| Depth of caudal peduncle | 11.5 | 9.6–12.2 | 10.8 ± 0.6 | 10.2–12.2 | 10.9 ± 0.6 | 0.005 |
| Body width at caudal peduncle | 6.5 | 4.3–6.5 | 5.5 ± 0.6 | 4.8–6.9 | 5.7 ± 0.7 | 0.000 |
| Head depth | 44.5 | 38.2–49.7 | 43.0 ± 2.9 | 39.4–48.9 | 45.2 ± 2.0 | 0.000 |
| Head width | 25.1 | 23.9–29.6 | 26.5 ± 1.4 | 23.2–29.9 | 26.3 ± 1.6 | 0.000 |
| Snout length | 18.9 | 15.0–20.1 | 18.4 ± 1.2 | 17.7–21.9 | 19.1 ± 0.9 | 0.000 |
| Length of orbit | 11.0 | 10.8–14.0 | 12.1 ± 1.0 | 9.1–11.8 | 10.6 ± 0.8 | 0.013 |
| <i>Postorbital head length</i> | 23.3 | 18.8–25.5 | 21.4 ± 1.6 | 26.2–31.7 | 29.3 ± 1.5 | 0.064 |
| Interorbital width | 13.1 | 10.2–13.1 | 11.4 ± 0.7 | 10.0–14.2 | 12.7 ± 1.1 | 0.000 |
| Length of upper jaw | 13.1 | 11.9–15.3 | 13.3 ± 1.0 | 13.7–17.0 | 14.7 ± 0.9 | 0.000 |
| Length of lower jaw | 10.5 | 8.3–10.5 | 9.5 ± 0.7 | 9.0–11.8 | 10.0 ± 0.7 | 0.000 |
| Length of pectoral fin | 40.8 | 25.1–48.4 | 38.4 ± 6.3 | 40.5–50.1 | 44.5 ± 2.6 | 0.000 |
| <i>Length of pectoral-fin base</i> | 20.7 | 15.8–20.7 | 18.7 ± 1.1 | 19.5–25.2 | 21.6 ± 1.5 | 0.263 |
| Length of pelvic fin | 26.4 | 16.6–38.0 | 25.8 ± 5.2 | 21.3–32.8 | 27.9 ± 2.2 | 0.000 |

Morphometric data are given in percent standard length and presented as the range, mean, and standard deviation

Significances refers to that determined from T-test for counts and ANCOVA for measurements
In vertebrae, total number was compared between two species
Italic type indicates significant differences between two species (see Fig. 2)

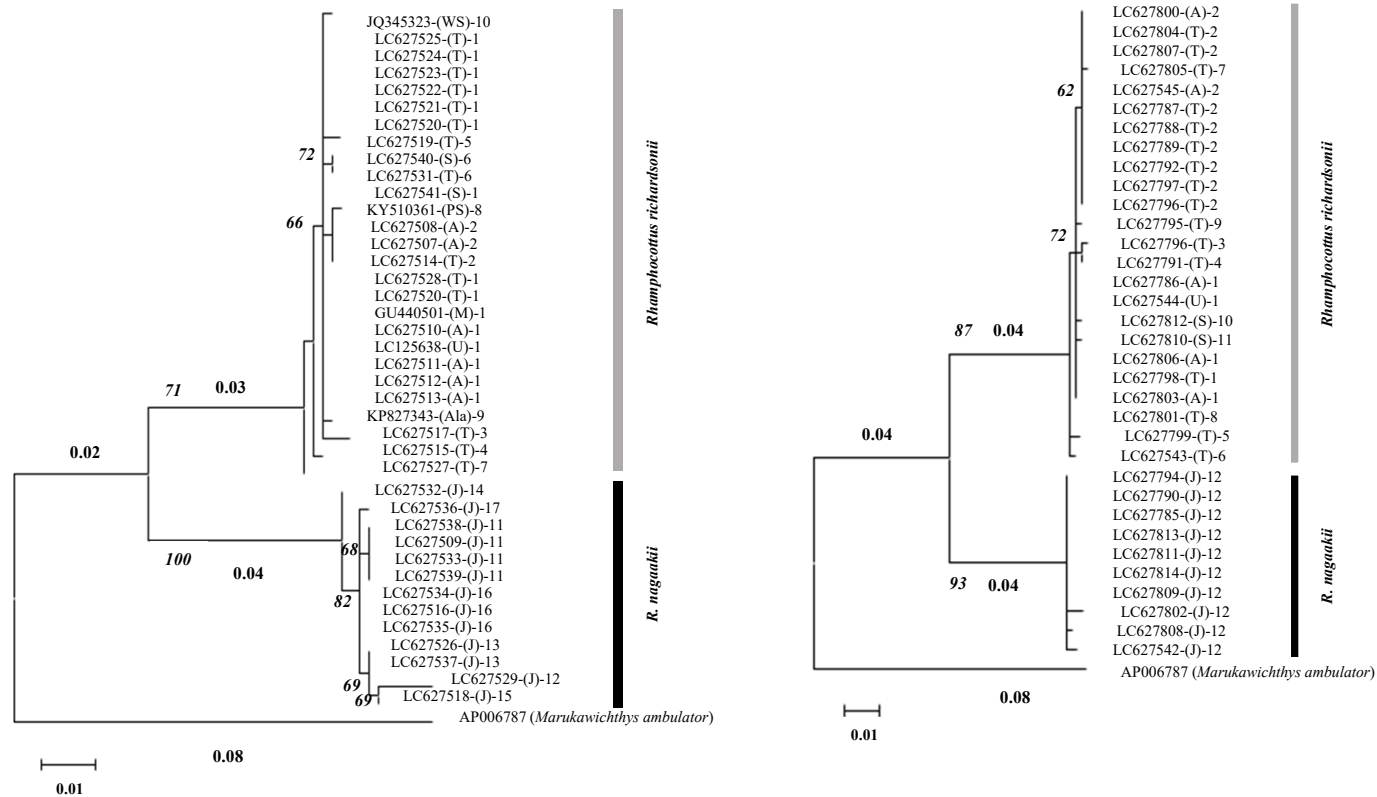


Fig. 1. Molecular phylogenetic tree estimated by the ML method (using 1000 bootstrap replications) representing mitochondrial DNA phylogeny among *Rhamphocottus* species based on (a) COI (606bp) and (b) Cytb (625bp) datasets. The scale bars indicate genetic distance expressed as units of expected nucleotide substitutions per site, and values greater than 0.01 are indicated near the branches. The bootstrap value more than 50 (Italic type) is shown on the branch. Specimens are represented by accession number, locality, and haplotype. Localities are abbreviated as (A): Anacortes, (T): Tofino, (S): Seattle, (WS): Washington State, (PS): Puget Sound, (Ala): Alaska, (U): Unalaska Island, (M): Monterey Bay, and (J)-: Japan.

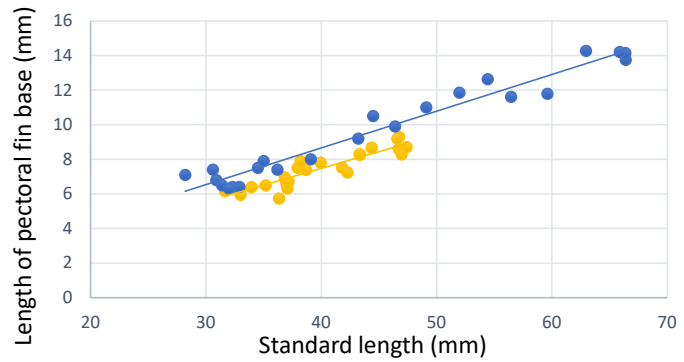
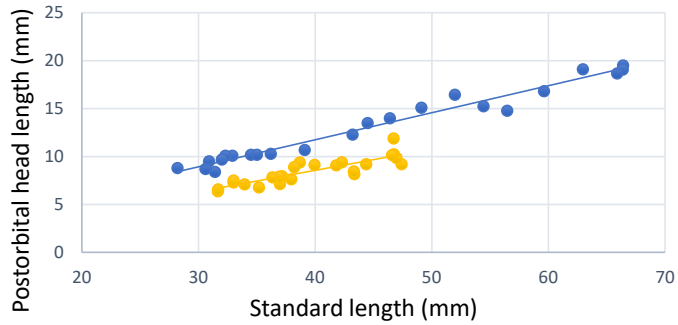
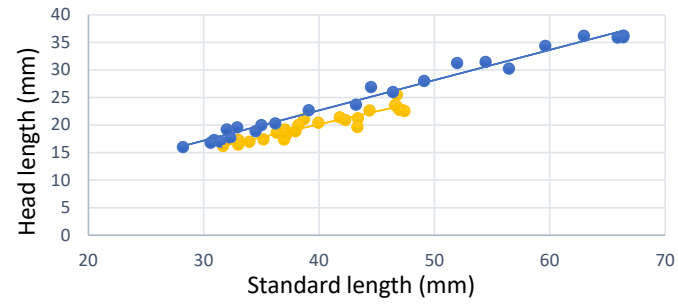


Fig. 2. Plots of measurements showing significant differences between *Rhamphocottus richardsonii* (blue squares) and *R. nagasaki* (orange circles) among morphometric characters. (a) Head length; (b) Postorbital head length; (c) Length of pectoral-fin base.



Fig. 3. *Rhamphocottus nagaakii*, new species, HUMZ232510, 38.2 mmSL, holotype, Minami-sanrikucho, 39° 39'N, 141° 29'E, 10 m depth.



Fig. 4. *Rhamphocottus nagaakii* (left photograph taken by Na. Satoh, in Minami-Sanriku-cho, Miyagi Prefecture, Japan, 26 March 2007; non-type) and *Rhamphocottus richardsonii* (right photograph collected in Tofino, Vancouver Island, Canada, 9 July 2015, HUMZ231958, 56.5 mm SL).

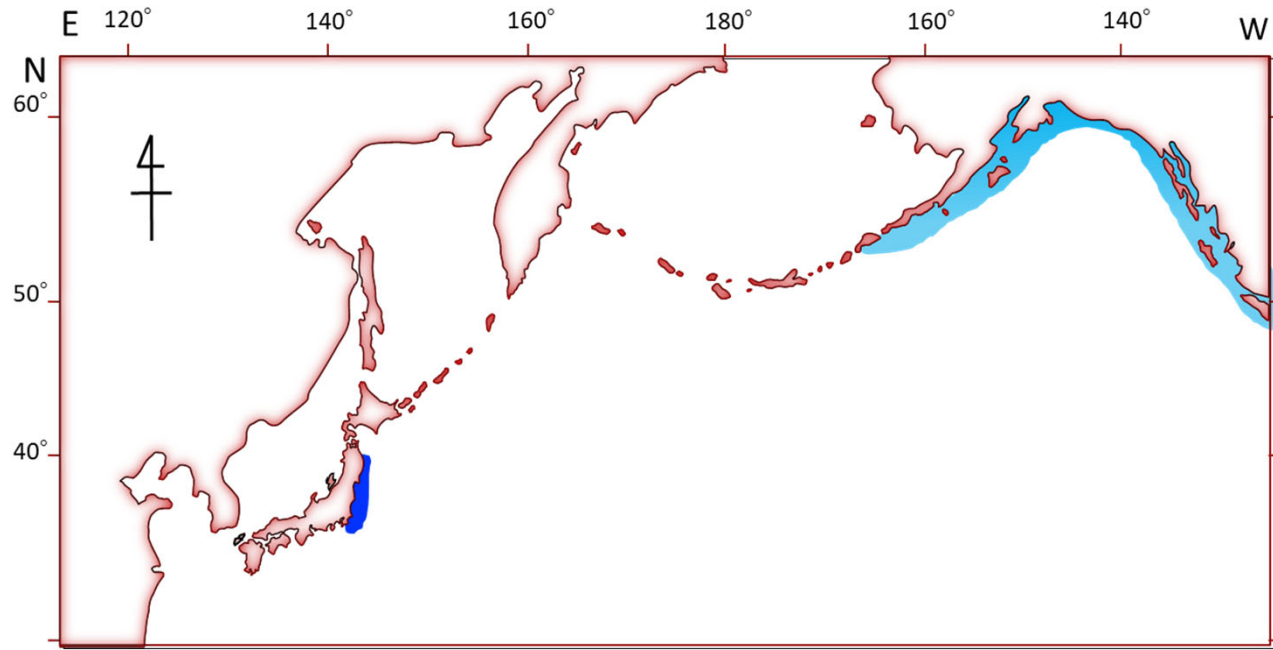


Fig. 5. Distribution area of *Rhamphocottus richardsonii* (faint blue) and *R. nagakii* (blue).



Fig. 6. This is a photograph of *Rhamphocottus nagaakii* with eggs. The male (left) successfully paired with a mature female, and after several hours she deposited her eggs near a previously deposited egg mass and then the male released sperm on the newly spawned egg mass (right; photograph by Na. Satoh, in Minami-Sanriku-cho, Miyagi Prefecture, Japan, 17 December 2003).