Different habitat salinity between genetically divergent groups of a worm-like goby *Luciogobius guttatus*: an indication of cryptic species

Seiya Hashimoto [1] · Itsuro Koizumi [1,2] · Kotaro Takai [1] · Seigo Higashi [1]

[1] Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060-0810, Hokkaido, Japan

[2] Creative Research Institution, Hokkaido University, Sapporo 001-0021, Hokkaido, Japan

Corresponding author: Itsuro Koizumi

Tel.: +81 11 706 9247; fax: +81 11 706 9272; email: itsuro@cris.hokudai.ac.jp

Emails of other authors

Seiya Hashimoto: seiya_hashimoto@ees.hokudai.ac.jp

Kotaro Takai: ktakai@ees.hokudai.ac.jp

Seigo Higashi: hisemato@ees.hokudai.ac.jp

Abstract

Gobies of the genus *Luciogobius* have unusual morphological adaptations to interstitial rocky coastal habitats in far eastern Asia; an elongated scale-less body, the loss the first dorsal fin, and a drastically increased number of vertebrae. Convergent evolution makes the species distinction difficult and the existence of many cryptic species has been postulated. Two divergent lineages of *L. guttatus* had been reported with the possibility of niche differentiation between marine and brackish habitats. Here, we quantitatively assessed the water salinity of the habitats used by the two lineages in Hokkaido, Japan, as well as their morphology. One lineage occurred exclusively in high-salinity habitats in intertidal zones (>25 ‰) and the other occurred mostly, but not exclusively, in low-salinity habitats near river mouths (<5 ‰). This result, together with mtDNA molecular phylogeny, suggests that the brackish type might have originated from a marine ancestor. Two lineages occurred sympatrically on some shores. No apparent difference was observed in the external morphology between the lineages, whereas the number of vertebrae was significantly different. Our results support the preposition that the divergent lineages within *L. guttatus* represent cryptic species.

Keywords Biogeography · Blakiston's line · Convergence · Distribution · Phylogeography · Mitochondrial DNA

Introduction

Gobies of the genus *Luciogobius* have unusual morphological adaptations to interstitial rocky coastal habitats in far eastern Asia (Yamada et al. 2009); the bodies are elongated and lack scales, the first dorsal fin is absent, and the vertebral column has an unusually large number of vertebrae (Akihito et al. 2000). The peculiar appearance is described by the Japanese common name “mimizu-haze” (English translation: “worm-goby”). The convergent morphological adaptation of *Luciogobius* gobies to interstitial habitats makes species distinction difficult. In fact, the existence of cryptic species of the genus has been indicated, which can be differentiated only with great difficulty using morphological criteria but can readily be identified by genetic analysis. Suzuki et al. (2004) predicted that the number of *Luciogobius* species may almost double if such cryptic species are described as separate taxa. Indeed, Yamada et al. (2009) detected at least 17 putative species among seven previously described morphological species through mtDNA (cytochrome-b region) and nuclear DNA (ITS region) sequencing. However, morphological, ecological and environmental data for putative cryptic species are almost lacking (but see Yamada et al. 2009).

Mukai and Nishida (2004) reported two divergent mtDNA lineages within *L. guttatus*, which might have been differentiated five million years ago. They also noted that one lineage was found in rocky shores and the other in river mouths, although no quantitative assessment has been done. Yamada et al. (2009) confirmed the divergent lineages of *L. guttatus* with both mitochondrial and nuclear DNA. They estimated the divergent time to be 2-3 million years ago. Therefore, the two lineages may represent cryptic species. To confirm the cryptic species, however, further ecological and morphological surveys are required.

In this study, we quantitatively assessed the habitat salinity and morphology of the

70 divergent lineages of *L. guttatus* on Hokkaido Island, Japan, to support the ecological differences
71 between the putative cryptic species. We also investigated to what extent, if any, they occur
72 sympatrically within the same shores. *L. guttatus* are widely distributed around the Japanese
73 archipelago, but one lineage may occur mostly in the western side of Japan (i.e., Sea of Japan)
74 whereas the other in the eastern side (Pacific Ocean) based on the limited sampling of a previous
75 study (Mukai and Nishida 2004). The present study also provides basic biogeographic information
76 on gobiidae species in the northern range margin where the faunal compositions remains largely
77 unknown. Specifically, we investigated whether the Tsugaru Strait between Hokkaido and Honshu
78 islands has been a major geographical barrier for the coastal species similar with many terrestrial
79 and freshwater animals and plants (Aizawa et al. 2007; Dobson and Kawamura 1998; Koizumi et
80 al. 2012; Sota and Hayashi 2007; Watanabe 2012).
81
82
83 **Materials and methods**
84
85 *Luciogobius* gobies and bio- (phylo-)geographic patterns of Japanese coastal species
86
87 *Luciogobius* gobies occur in far eastern Asia, including easternmost Russia and mainland
88 China, the Korean peninsula, the Japanese archipelago, Taiwan, Hainan Island, and Hong Kong
89 (Yamada et al. 2009). Sixteen *Luciogobius* species has been described so far through the
90 distribution range of the genus, and 15 are found in Japan (Arai 1981; Kanagawa et al. 2011;
91 Suzuki et al. 2004). High species richness in Japan is probably due to a great diversity of habitats
92 resulting from the wide occurrence of gravel-rich shores in the country and also from the wide
93 climatic range, spanning cool-temperate through subtropical zones. However, due to strong human

94 impacts on coastal areas in the past century, such as construction of harbors, moles, and land
95 reclamation (Washitani 2008), some *Luciogobius* gobies have been declining (e.g. Ministry of the
96 Environment 2003).
97 Most *Luciogobius* species and/or divergent lineages within species (i.e. putative cryptic
98 species) distribute in allopatry within the Japanese archipelago, although some species spread
99 widely, such as *L. guttatus*, *L. elongatus*, and *L. grandis* (Yamada et al. 2009). On Hokkaido Island,
100 the northern range margin of gobies, however, systematic studies on *Luciogobius* have rarely been
101 conducted. Fragmentary information indicates that among *Luciogobius* gobies, only *L. guttatus*
102 occurs in parts of Hokkaido (Mukai and Nishida 2004; Shiretoko Museum 2003; Yamada et al.
103 2009); in other parts of the island, there have been no reports of the genus. Only one exception is
104 an unidentified species near Hakodate at the southern tip of Hokkaido (Yamada et al. 2009).
105 It is important to investigate bio- and phylo-geography of Japanese coastal species, such as
106 gobies, because some clear patterns have been detected in terrestrial and freshwater species across
107 the Japanese archipelago. In many terrestrial animals and plants, as well as freshwater species, the
108 Tsugaru Strait has been a major historical barrier, separating the fauna and flora of Hokkaido from
109 those of the rest of Japan (Aizawa et al. 2007; Dobson and Kawamura 1998; Koizumi et al. 2012;
110 Sota and Hayashi 2007; Watanabe 2012): the biogeographic boundary delineated by the Tsugaru
111 Strait is called the Blakiston's line. Some coastal marine species, on the other hand, have diverged
112 significantly between the western and eastern sides of Honshu Island, i.e. Sea of Japan and Pacific
113 Ocean, respectively, although few samples from Hokkaido were included (Akihito et al. 2008;
114 Hirase et al. 2012; Kojima et al. 1997; Kokita and Nohara 2011). Therefore, it is important to see
115 whether the Tsugaru Strait has been a major barrier for coastal species as well and whether eastern
116 and western divergence has been recognized also within Hokkaido Island. To do so, we examined
117 the broad phylogeographic structure of *Luciogobius* gobies to draw inferences on population

118 history, using our data, together with information in the DDBJ genetic databank.

119

120 Distribution of *Luciogobius* gobies in Hokkaido

121

122 Between May 2008 and September 2009, we sampled 59 locations around the coast of Hokkaido,
123 including the surrounding islands (Fig. 1). We chose diverse habitats along river mouths and in
124 intertidal zones to include as many species as possible. Water salinity was measured with a
125 PAL-06S ATAGO meter near the sea floor, as *Luciogobius* gobies are benthic. All gobies were
126 caught in a dipnet by one person, Seiya Hashimoto; he collected fish for 1 hour at each location.
127 When *Luciogobius* was present at a location, fewer than seven individuals per morphologically
128 distinct species (based on the criteria by Akihito et al. 2000 and Maeda et al. 2008) were sacrificed
129 for subsequent analysis. We clipped a piece of the right-side pectoral fin for preservation in 95%
130 ethanol for later DNA extraction. The rest of the body was preserved in 10% formalin for
131 morphological measurements.

132

133

134 Morphological measurement

135

136 We followed Akihito et al. (2000) and Maeda et al. (2008) in the selection of morphometric traits:
137 standard length (SL), pre-dorsal fin length (PDL), pre-anal length (PAL), vent to anal fin length
138 (VAFL), head length (HL), snout length (SnL), eye diameter (ED), body depth (BD), pectoral fin
139 length (P1L), pelvic fin length (P2L), caudal fin length (CL), number of dorsal fin rays (D), and
140 number of anal fin rays (A). The number of vertebrae (V) was also counted from radiographs
141 taken by a multi soft X-ray film shooting apparatus (SOFTEX TYPE EMB CMB-2, SOFTEX CO.,

LTD, Kanagawa, Japan) with 2.5 mA and 30 kVp for 1-2 seconds. We standardized these traits by fish length (SD) except for numerical counts (i.e., D, A and V). We used Welch's *t*- test in the R 2.10.1 software package (Team 2009) to compare morphological traits between species or divergent lineages. Wilcoxon's rank sum test was also performed in the same program to compare habitat salinity between species or lineages.

Mitochondrial DNA phylogeny and phylogeography

We used a total of 76 fish from 25 Hokkaido locations for DNA analysis; we also included five individuals of *L. guttatus* provided by the Wakayama Prefectural Museum of Natural History (Table 1). We analyzed the same mtDNA regions (12S rRNA and 16S rRNA for 568bp and 482bp, respectively) where Mukai and Nishida (2004) constructed a phylogenetic tree of *Luciogobius* gobies, including *L. guttatus*, *L. pallidus*, *L. elongatus*, and *L. grandis*. We combined our data with those from DDBJ to investigate phylogeny and phylogeography (Table 1). Because only *L. guttatus* was detected among 76 fish collected in Hokkaido (see Results), we used the following *L. guttatus* samples from DDBJ; one individual from each of the collections at Yamagata (AB1231994, AB1231995), Kanagawa (AB121986, AB121987), Ehime (AB121996, AB121997), Kagoshima (AB108559, AB108569), and Iriomote Island (AB121988, AB121989), and two individuals from each of the collections at Wakayama (AB121998, AB122000, AB121999, AB122001) and Okinawa Island (AB121990, AB121992, AB121991, AB121993). The DNA sequence of *Luciogobius pallidus* in DDBJ (AB121984, AB121985) was used as an outgroup for constructing rooted phylogenetic trees.

Total genomic DNA was extracted with a PureGene DNA isolation kit (Applied

Biosystems) following the manufacturer's instructions. DNA was dissolved in TE buffer (10 mM Tris-HCI and 1 mM EDTA; pH 7.5). We used the following PCR primers for 12S rRNA: OMT16SF (5'- TGC CAG CCA CCG CGG TTA TAC G -3'), tRNA02 (5'- GGA TGT CTT CTC GGT GTA AG -3') and for 16S rRNA: L2510 (5'-CGC CTG TTT ATC AAA AAC AT-3') and H3080 (5'-CCG GTC TGA ACT CAG ATC ACG T-3') (Mukai et al. 2003). All reactions were performed in 10-µl volumes using Takara PCR Thermal Cycler SP. The reaction mixture contained 0.5 U Taq polymerase (Ampli Taq Gold, Applied Biosystems), 0.5 µM of each primer, 0.2 mM dNTP, 50 mM KCl, 15 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, and about 50 ng of genomic DNA as a template. The PCR profile was 10 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The PCR products were purified by PEG precipitation. Purified products were cycle-sequenced using an ABI BigDye terminator version1.1 Cycle Sequencing kit (Applied Biosystems) and run on an ABI PRISM 3130 genetic analyzer (Applied Biosystems).

We used MrBayes 3.1.2 and PAUP 4.0b10 software for phylogenetic analyses by Bayesian phylogenetic analysis and maximum parsimony, respectively. Using the hierarchical likelihood-ratio test in MrModeltest 2.3 software, we determined that the GTR+G model was appropriate for the nucleotide evolution in our system. We ran MrBayes MCMC through 100,000 generations, of which the first 2,500 were discarded as burn in.

Results

MtDNA haplotype genealogy

Comparing our mtDNA haplotypes (12S rRNA and 16S rRNA) with those in DDBJ databank, all 76 individuals collected in Hokkaido were classified into *L. guttatus*. Together with additional 14 samples from other parts of Japan (Mukai and Nishida 2004), a total of 35 haplotypes was detected in *L. guttatus* (Table 1). In the Bayesian tree using the GTR+G model, these haplotypes were clustered in two divergent lineages with high posterior probabilities (Fig. 2a). Sub-group was also found within one lineage, consistent with previous findings (Mukai and Nishida 2004; Yamada et al. 2009). The maximum-parsimony (MP) tree almost exactly matched the Bayesian tree (Fig. 2b). Inclusion of our Hokkaido samples did not significantly alter the phylogenetic trees previously reported (Fig. 3): general patterns are as follows. Both divergent lineages (clade I and II) were found widely around the Japanese archipelago, although clade I tended to distribute in the Sea of Japan and clade II in the Pacific Ocean. Clade II was further divided into two sub-groups where clade IIA was found mostly in northern Japan and clade IIB in southern Japan. The distribution of clade I, on the other hand, was rather homogenous without prominent genetic clustering. For example, haplotype Hok22 occurred in southern Japan (Wakayama, Kanagawa) and on the northernmost island (Akaiwa on Rebun Island).

Distribution and habitat use of *Luciogobius guttatus*

As mentioned above, we found only *Luciogobius guttatus* around the coast of Hokkaido: other *Luciogobius* species was not detected. They occurred all around the coast, but not at some sites (24 of 59, Fig. 1) where substrata were fine-grained, e.g., sand and mud. Most of the specimens belonged to clade II. Clade I was found only from four sites but from both the Sea of Japan and Pacific Ocean. Of the four sites, clade I occurred sympatrically in three sites. Habitat

salinity between the locations each clade was detected was significantly different (Fig. 4, median = 33 and 1 for clade I and clade II, respectively, $W = 6.5$, $P = 0.004$). Clade I was found exclusively in high-salinity habitats (>25 ‰) in intertidal zones, whereas clade II occurred mostly in low-salinity habitats (< 5 ‰) near river mouths, with a few individuals occurring in higher-salinity habitats (>25 ‰)(Fig. 2a). The few individuals found in high salinity habitats in clade II should not result from temporary wash out via river current because some locations were not near river mouths or individuals were not caught during snow meting season. Hereafter, we designate the divergent lineage from low-salinity habitats as brackish type (clade II), and the lineage from high-salinity habitats as marine type (clade I).

Morphological differences between brackish and marine types

Across all 13 external characteristics we measured, there were no significant differences between brackish and marine types (Table 2). When we standardized traits by SL, three of ten proportional characters differed significantly between types. The BD, P1L, and P2L of the marine type were greater than measurements on the brackish type, although ranges widely overlapped. The number of vertebrae, however, was significantly different between the brackish and marine types.

Discussion

We quantitatively confirmed the previous note (Mukai and Nishida 2004) that one lineage within *L. guttatus* occur high salinity habitats and the other low salinity ones. Importantly, the

238 marine type was restricted to fully saline waters, whereas the brackish type occurred in both high-
239 and low-salinity habitats. This is reasonable considering that most *Luciogobius* gobies are marine
240 species (Yamada et al. 2009) and it is hypothesized that the brackish type might have originated
241 from a marine ancestor, most probably from the marine type of *L. guttatus*.

242 External morphology was indistinguishable between marine and brackish types of *L.*
243 *guttatus*. They inhabit similar interstitial habitats and, therefore, morphological convergence may
244 maintain the similarity. However, size-standardized body depth, and pectoral and pelvic fin
245 lengths were statistically different between the marine and brackish types, although the range
246 overlapped widely. Interestingly, the shape of pelvic fins is markedly different among putative
247 cryptic species in *Luciogobius elongatus* (Yamada et al. 2009). In addition, different *Luciogobius*
248 species inhabit shores with different gravel sizes (Yamada et al. 2009). Therefore, the observed
249 morphological difference might represent their microhabitat preferences to the gravel size. The
250 number of vertebrae, on the other hand, was clearly different between the lineages. *Luciogobius*
251 gobies have evolved to increase the number of vertebrae and this morphometry should be a good
252 indicator of species identification (Yamada et al. 2009). Thus, our results add pieces of evidence
253 that the marine and brackish types represent distinct species.

254 No significant genetic divergence was observed between Hokkaido and Honshu islands for
255 both marine and brackish types of *L. guttatus*, contrast with many terrestrial and freshwater
256 species. Similarly, no divergence was observed between samples from western and eastern sides of
257 Hokkaido, although the sampling location is limited for marine types. These gobies should have
258 the potential of long-distance dispersal ability perhaps due to ocean currents because they have
259 pelagic larval stage (Dôtu 1957). This is especially plausible for marine types considering the
260 widespread haplotype and no prominent genetic structure within the Japanese archipelago. On the
261 other hand, west-east comparison in Honshu Island was difficult due to limited sampling locations.

Since strong genetic divergence has been observed between western and eastern lineages in several coastal marine species in Honshu Island (Akihito et al. 2008; Hirase et al. 2012; Kojima et al. 1997; Kokita and Nohara 2011), further assessment is certainly required.

Habitat differentiation (i.e., intertidal rocky shores vs. river mouth) may be a primary factor contributing to reproductive isolation between two types. However, marine and brackish types occurred sympatrically on some shores. Such salinity-differentiated cryptic species also occur in *Pomatoschistus* gobies in France, but they reproduce viable offspring in sympatry (Berrebi et al. 2005; Rigal et al. 2008). Since no hybrid have been observed so far between the two lineages of *L. guttatus* and they might have diverged several million years ago (Yamada et al. 2009), reproductive isolation might have had been completed in our gobies. Experimental approaches, such as artificial breeding and salt-water challenge test, would clarify the mechanisms of reproductive isolation for the marine and brackish types of *L. guttatus*. Further investigation is needed to better understand the biology of *Luciogobius* gobies since they are unique coastal species around the Japanese archipelago and could provide an interesting model system for adaptive radiation (Yamada et al. 2009).

Acknowledgements

We appreciate useful comments by two anonymous reviewers. We also thank Mr. K. Hirashima of Wakayama Prefectural Museum of Natural History for providing samples. Sampling carried out in this study complies with the current laws in Japan.

References

Aizawa M, et al. (2007) Phylogeography of a northeast Asian spruce, *Picea jezoensis*, inferred from genetic variation observed in organelle DNA markers. Mol Ecol 16:3393-3405 doi:10.1111/j.1365-294X.2007.03391.x

Akihito, et al. (2008) Evolution of Pacific Ocean and the Sea of Japan populations of the gobiid species, *Pterogobius elapoides* and *Pterogobius zonoleucus*, based on molecular and morphological analyses. Gene 427:7-18 doi:10.1016/j.gene.2008.09.026

Akihito, Sakamoto K, Ikeda Y, Iwata A (2000) Gobioidei. In: Nakabo T (ed) Fishes of Japan with Pictoral Key to the Species. Tokai University Press, Tokyo, p 1139-1310 (in Japanese)

Arai R (1981) Fishes of *Luciogobius* and *Inu* (Gobiidae) from the Izu Peninsula, Central Japan. Mem Natl Sci Mus Tokyo 14:151-166 (in Japanese)

Berrebi P, Rodriguez P, Tomasini JA, Cattaneo-Berrebi G, Crivelli AJ (2005) Differential distribution of the two cryptic species, *Pomatoschistus microps* and *P. marmoratus*, in the lagoons of Southern France, with an emphasis on the genetic organisation of *P. microps*. Estuar Coast Shelf S 65:708-716 doi:10.1016/j.ecss.2005.07.008

Dobson M, Kawamura Y (1998) Origin of the Japanese land mammal fauna: Allocation of extant

305 species to historically-based categories. Daiyonki Kenkyu (The Quaternary Research)
306 37:385-395
307 Dôtu Y (1957) The life history of the goby, *Luciogobius guttatus* Gill. Sci Bull Fac Agric Kyusyu
308 Univ 16:93-100 (in Japanese with English abstruct)
309 Hirase S, Ikeda M, Kanno M, Kijima A (2012) Phylogeography of the intertidal goby
310 *Chaenogobius annularis* associated with paleoenvironmental changes around the Japanese
311 Archipelago. Mar Ecol Prog Ser 450:167-179 doi:Doi 10.3354/Meps09584
312 Kanagawa N, Itai T, Senou H (2011) The new species of freshwater gobies of the genus
313 *Luciogobius* (Perciformes: Gobiidae) from Japan. Bull Kanagawa Prefect Mus 40:67-74
314 Koizumi I, Usio N, Kawai T, Azuma N, Masuda R (2012) Loss of genetic diversity means loss of
315 geological information: the endangered Japanese crayfish possessed remarkable historical
316 footprints. PLoS ONE 7:e33986 doi:10.1371/journal.pone.0033986
317 Kojima S, Segawa R, Hayashi I (1997) Genetic differentiation among populations of the Japanese
318 turban shell *Turbo* (*Batillus*) *cornutus* corresponding to warm currents. Mar Ecol Prog Ser
319 150:149-155 doi:Doi 10.3354/Meps150149
320 Kokita T, Nohara K (2011) Phylogeography and historical demography of the anadromous fish
321 *Leucopsarion petersii* in relation to geological history and oceanography around the
322 Japanese Archipelago. Mol Ecol 20(1):143-164 doi:DOI

10.1111/j.1365-294X.2010.04920.x

Maeda K, Yamasaki N, Kondo M, Tachihara K (2008) Occurrence and morphology of larvae and juveniles of six *Luciogobius* species from Aritsu Beach, Okinawa Island. Ichthyol Res 55:162-174 doi:10.1007/s10228-007-0018-x

Ministry of the Environment (2003). Threatened Wildlife of Japan – Red Data Book, 2nd edn, Vol. 4, Pisces – Brackish and Freshwater Fishes. Japan Wildlife Research Center, Tokyo, Japan. 230 pp (in Japanese)

Mukai T, Nishida M (2004) Intraspecific mitochondrial DNA phylogeny of a Japanese brackish water goby, *Luciogobius guttatus*. Jpn J Ichthyol 51:157-161 (in Japanese with English abstruct)

Mukai T, Suzuki T, Nishida M (2003) Genetic differentiation of the brackish water goby, *Eutaeniichthys gilli* (Perciformes, Gobiidae) between the Japanese and the Ryukyu Archipelagos. Biogeography 5:49-53

Shiretoko Museum (2003) Fishes in Shiretoko. Hokkaido Shimbum Press (in Japanese)

Rigal F, et al. (2008) Osmoregulation as a potential factor for the differential distribution of two cryptic gobiid species, *Pomatoschistus microps* and *P-marmoratus* in French Mediterranean lagoons. Sci Mar 72:469-476

Sota T, Hayashi M (2007) Comparative historical biogeography of *Plateumaris* leaf beetles

(Coleoptera: Chrysomelidae) in Japan: interplay between fossil and molecular data. J Biogeogr 34:977-993 doi:10.1111/j.1365-2699.2006.01672.x

Suzuki T, Shibukawa K, Yano K (2004) A Photographic Guide to the Gobioid fishes of Japan. Heibonsha, Tokyo (in Japanese)

Team RDC (2009) R: A language and environment for statistical computing. R Foundation for Statistical Computing ISBN 3-900051-07-0, URL http://www.R-project.org

Washitani I (ed)(2008) Ecosystem Crisis in Japan. Koseisha Koseikaku, Tokyo (in Japanese)

Watanabe K (2012) Faunal structure of Japanese freshwater fishes and its artificial disturbance. Environ Biol Fish 94:553-547 doi:DOI 10.1007/s10641-010-9601-5

Yamada T, Sugiyama T, Tamaki N, Kawakita A, Kato M (2009) Adaptive radiation of gobies in the interstitial habitats of gravel beaches accompanied by body elongation and excessive vertebral segmentation. BMC Evol Biol 9:145 doi:1471-2148-9-145

357 **Figure legends**

358

359 Fig. 1. Sampling locations around the island of Hokkaido. Black (*N* = 35) and white (*N* = 24)
360 circles represent presence and absence of *Luciogobius* gobies, respectively. Numbers in the figure
361 (i.e. 1-24) denote sample location ID (see Table 1).

362

363

364 Fig. 2. Bayesian tree (a) and Maximum-parsimony tree (b) representation of the molecular
365 phylogeny of *Luciogobius guttatus* (12S and 16S rRNA; 1050 bp). *L. pallidus* is the out-group.
366 Open circles in figure 2a are the haplotypes detected in the locations where water salinity is 20 or
367 less, whereas filled squares are those detected in the locations where water salinity is more than
368 25.

369

370

371 Fig. 3. Distribution of mtDNA haplotypes of marine (left panel) and brackish (right panel) types of
372 *Luciogobius guttatus*. In the right panel, open and closed circles represent clades IIA and IIB,
373 respectively. Numbers and characters in the figure denote sample location ID (see Table 1).
374 M1-M12 are the locations examined in Mukai and Nishida (2004).

375

376

377 Fig. 4. Frequency distributions of water salinities (‰) in habitats where brackish (white bar) and
378 marine (shaded bar) types of goby were collected.

379

380