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Divergence of mitochondrial DNA lineage of the rocky intertidal goby *Chaenogobius gulosus* around the Japanese Archipelago: reference to multiple Pleistocene isolation events in the Sea of Japan

S. Hirase · M. Ikeda

Abstract Geographic variations in the mitochondrial cytochrome *b* gene sequence (1,062 nucleotides) of the rocky intertidal goby *Chaenogobius gulosus* were examined and compared against the phylogeographic pattern of its sympatric sister species *Chaenogobius annularis*, which we studied previously. We collected *C. gulosus* specimens from 17 localities around the Japanese Archipelago and Korean Peninsula. Thirty-one haplotypes were obtained. The phylogenetic tree showed three geographical lineages, of which one was distributed along the coast of the Sea of Japan, suggesting that a Pleistocene isolation event in the Sea of Japan affected the genetic divergence of this species, similar to that which was observed in *C. annularis*. The net nucleotide divergence between the Sea of Japan and Pacific Ocean lineages in *C. gulosus* was much lower than that of *C. annularis*. Both traditional molecular clock and hierarchical approximate Bayesian computational models suggest that different divergence events affected the genetic divergences of *C. gulosus* and *C. annularis*. Our study on the sister species pair strongly supports multiple isolation events in the Sea of Japan, leading to varying levels of genetic divergence between the Pacific Ocean and Sea of Japan lineages in the Japanese coastal marine species.

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Introduction

Pleistocene glacial–interglacial cycles have strongly influenced the present-day biodiversity of many coastal marine species (Maggs et al. 2008). Indeed, several recent phylogeographic studies for coastal marine species have shown that low sea levels during the glacial maxima created land bridges that changed ocean circulation patterns and effectively divided populations of marine organisms (Avise 2000). Accumulating knowledge on the phylogeographic patterns in many coastal marine species would provide comparative insight into the effect of complex paleoenvironmental events on coastal marine biota.

Oscillatory changes in the marine environments around the Japanese Archipelago were significant in the Sea of Japan during the Pleistocene, particularly in locations between Japan and the Asian mainland (e.g., Oba et al. 1991; Tada 1994). The Sea of Japan is semi-enclosed and connected to the Pacific Ocean and other seas through shallow and narrow straits, such as the Tsushima, Tsugaru, and Soya Straits, which have sill depths of <130 m (Fig. 1). Currently, the warm Tsushima Current flows into the Sea of Japan through the southern Tsushima Strait (Fig. 1). Oba et al. (1991) reported that the Sea of Japan was largely isolated from the surrounding seas during the Pleistocene glacial periods, when the sea level was 120 m below the present sea level (Fig. 1). On the other hand, it has been suggested that the Sea of Japan opened and inflow from the Tsushima Current occurred during each interglacial period following the middle Pleistocene (Kitamura et al. 2001; Kitamura and Kimoto 2006). Therefore, it is expected that

Fig. 1 Present oceanic condition around Japanese Archipelago and sampling localities for *Chaenogobius gulosus* (*upper left*) oceanic condition during last glacial period (Crusius et al. 1999)
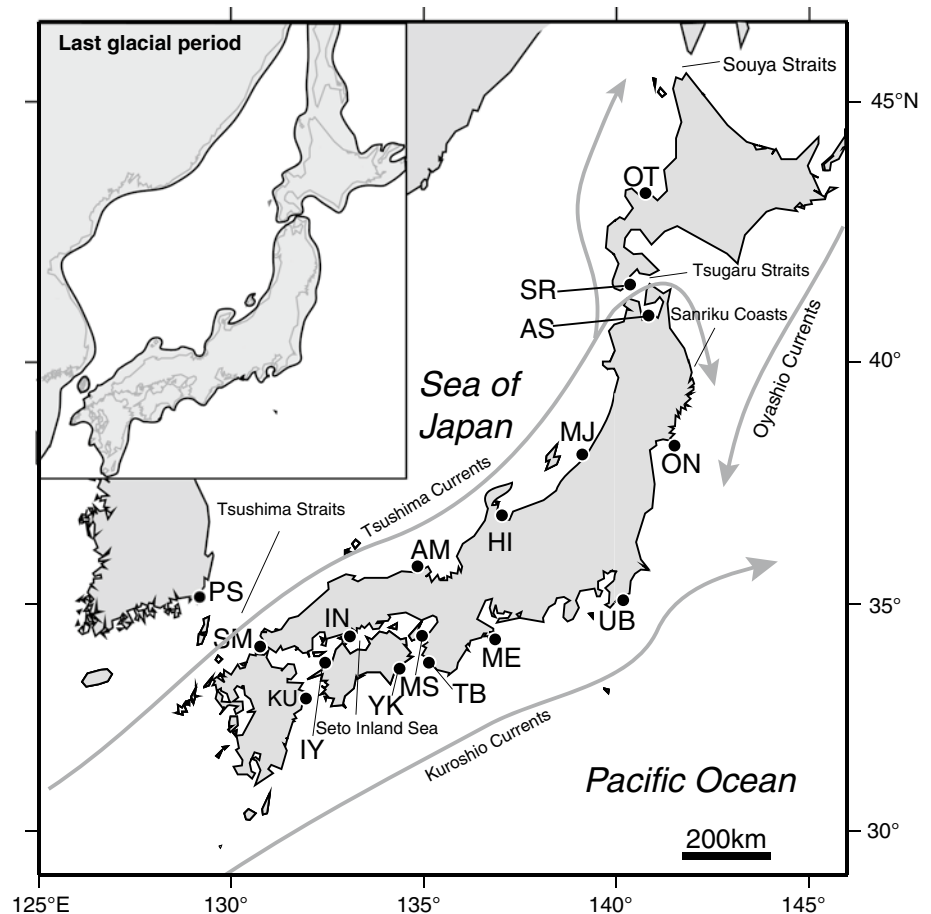
these complex isolation events of the Sea of Japan have a major impact on the historical process of the current distribution of the coastal marine species around the Japanese Archipelago.

Several phylogeographic studies have indicated that these events led to the genetic divergence of coastal marine species between the Pacific Ocean and the Sea of Japan populations (Kojima et al. 1997, 2004; Akihito et al. 2008; Kokita and Nohara 2011; Hirase et al. 2012). Using evolutionary rates that were calculated in other taxa, some of these studies have shown a wide range of divergence times between geographic lineages (Kojima et al. 1997, 2004; Kokita and Nohara 2011). These results suggest that multiple isolation events of the Sea of Japan during the Pleistocene period led to multiple vicariance events that promoted the genetic divergence of coastal marine biota. A comparative phylogeographic insight across taxonomic groups would allow us to statistically differentiate between "single-event" and "multiple-event" biogeographic hypotheses (Edwards and Beerli 2000). However, the evolutionary rate calculated in closely related species should be applied to estimate the divergence time of focal species according to the idea of past studies (e.g. Thorne et al. 1998), and it is unclear whether accurate divergence times were estimated

in the aforementioned studies, and a wide range of divergence times reflects multiple vicariance events or use of unreasonable evolutionary rates. To verify the variation of divergence times and to test the hypothesis of the multiple vicariance events associated with isolation events of the Sea of Japan, it is necessary to compare the levels of genetic divergence between closely related species, which are expected to show little evolutionary rate difference.

In the present study, we focused on the two closely related species *Chaenogobius gulosus* and *Chaenogobius annularis* as suitable models to compare the levels of genetic divergence and tested the hypothesis of multiple vicariance events associated with isolation events of the Sea of Japan. *C. gulosus* and *C. annularis* are distributed throughout the rocky coastline of the Japanese Archipelago (from Hokkaido southward to the Yakushima Islands) and the Korean Peninsula (Akihito et al. 2002). They mainly inhabit tide pools and have similar ecological features (Sasaki and Hattori 1969). Previously, we found that the Pacific Ocean and Sea of Japan lineages of *C. annularis* had diverged 1.7 million years ago (MYA) (Hirase et al. 2012). Because of the similar life history of *C. gulosus* and *C. annularis*, it is possible that *C. gulosus* also has the Pacific Ocean and Sea of Japan lineages, and we can verify

whether the two lineages of two species were divided by different isolation events. Because there are few studies on the close relationship between *C. gulosus* and *C. annularis*, we first examined this relationship. Second, we confirmed the existence of the Sea of Japan and Pacific Ocean lineages within *C. gulosus* by studying the mitochondrial DNA (mtDNA) cytochrome *b* gene (cyt *b*). References of cyt *b* evolutionary rates are abundant; this rate was conserved among the related genus *Gymnogobius* (Harada et al. 2002; Sota et al. 2005). Moreover, we can apply the evolutionary rate of *C. annularis* cyt *b*, which was calibrated on the basis of paleogeographical information (Hirase et al. 2012) to estimate divergence times within *C. gulosus*. Finally, we compared divergence level within *C. gulosus* with that within *C. annularis* and tested multiple vicariance events based on traditional molecular clock and hierarchical

approximate Bayesian computation (HABC) models (Hickerson et al. 2006).

Materials and methods

Samples and DNA sequencing

Sampling localities are shown in Fig. 1 and Table 1. Samples of *C. gulosus* were collected by hand nets from 16 localities on the Japanese coastline (total of 83 specimens) and from one locality on the coastline of the Korean Peninsula (total of 8 specimens) between 2006 and 2009. Total genomic DNA was extracted from the fin of each sample, using a phenol/chloroform procedure (Asahida et al. 1996). The DNA samples were stored at −30 °C prior to

Table 1 Sampling localities of *Chaenogobius gulosus* and genetic diversity of each locality

Locality	Code	Latitude, longitude	No. of samples	*h*	*π*(%)
Japan					
Ubara, Chiba Pref.	UB	35°12′N, 139°50′E	3	0.667	0.063
Anrakujima, Mie Pref.	ME	34°27′N, 136°52′E	1	–	–
Tanabe, Wakayama Pref.	TB	33°43′N, 135°21′E	8	0.429	0.040
Misaki, Osaka Pref.	MS	34°19′N, 135°9′E	8	0.714	0.081
Yuki, Tokushima Pref.	YK	33°46′N, 134°35′E	2	1.000	0.094
Innoshima, Hiroshima Pref.	IN	34°21′N, 133°10′E	1	–	–
Iyo, Ehime Pref.	IY	33°42′N, 132°39′E	8	0.679	0.104
Kamiura, Oita Pref.	KU	33°2′N, 131°56′E	5	0.600	0.057
Onagawa, Miyagi Pref.	ON	38°26′N, 141°27′E	6	0.000	0.000
Otaru, Hokkaido Pref.	OT	43°14′N, 141°0′E	8	0.250	0.047
Shiriuchi, Hokkaido Pref.	SR	41°31′N, 140°25′E	7	0.810	0.099
Asamushi, Aomori Pref.	AS	40°53′N, 140°51′E	5	0.900	0.132
Majima, Niigata Pref.	MJ	38°16′N, 139°26′E	8	0.250	0.024
Himi, Toyama Pref.	HI	36°48′N, 137°2′E	3	0.667	0.063
Amino, Kyoto Pref.	AM	35°41′N, 135°2′E	3	0.667	0.126
Shimonoseki, Yamaguchi Pref.	SM	33°57′N, 130°52′E	7	0.810	0.314
Korea					
Gijang, Pusan Pref.	PS	35°12′N, 129°13′E	8	0.786	0.188

h haplotype diversity, *π* nucleotide diversity

polymerase chain reaction (PCR) analysis. We amplified the cyt *b* genes using primers AJG15 and H5 (Akihito et al. 2000). For amplification, the following reagents were added to each microtube: 1 μL (10 ng/μl) of template DNA, 0.25 U of Blend *Taq* Plus (TOYOBO), 1 μL of 10 × buffer, 1 μL of dNTPs (2 mM), and 0.1 μL of each primer (25 μM). A sufficient amount of sterile deionized H₂O was added to each sample to obtain 10 μL. PCR conditions consisted of 30 cycles of denaturation at 94 °C for 30 s (1 min for the first denaturation only), annealing at 50 °C for 30 s, and extension at 72 °C for 1 min (5 min for the last extension only). The PCR products were purified by the ethanol/ethylenediaminetetraacetic acid (EDTA) method of the Applied Biosystems (ABI) BigDye Terminator v3.1 Cycle Sequencing Kit Manual. PCR products were bidirectionally sequenced using the ABI BigDye Terminator Cycle Sequencing Kit v3.1, following the manufacturer's instructions and using the same primers used for the amplification. Samples were purified by the ethanol/EDTA/sodium acetate method of the Kit Manual and analyzed in an ABI 3130 capillary sequencer.

mtDNA analysis

The identities of sequences were confirmed by BLAST analysis (Altschul et al. 1997) of GenBank (http://blast.ncbi.nlm.nih.gov/) and by comparison with published sequences. Sequences were then aligned using ClustalW (Thompson et al. 1994). Arlequin ver. 3.1 (Excoffier et al. 2005) was used to assign individuals to the unique haplotypes. Haplotypes obtained in the study were submitted to the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp; AB775375–AB775405).

Phylogenetic analyses were carried out in two stages: confirmation of the sister taxa relationship between *C. annularis* and *C. gulosus* and detection of lineages within *C. gulosus*. BLAST analysis suggested the similarity of cyt *b* sequences between *Chaenogobius* and *Gymnogobius* genera. Thus, we performed phylogenetic analysis on cyt *b* sequences of *Gymnogobius* species (AB073934–AB073953, Harada et al. 2002; AY525784, Kim et al. 2004), *C. annularis* (AB684846–AB684973, Hirase et al. 2012), and *C. gulosus* haplotypes obtained in the study to confirm the sister taxa relationship between *C. annularis* and *C. gulosus* using the maximum likelihood (ML) approach in MEGA 5.0 (Tamura et al. 2011). In this analysis, all sequence lengths were cut into 639 bp of *Gymnogobius* species. MEGA 5.0 was run for 300 bootstrap replicates using TNr (Tamura and Nei 1993) + I + G, which was selected as a best-fit substitution model by MEGA 5.0. The sister taxa relationship between *C. annularis* and *C. gulosus* was confirmed (see "Results" section); we further performed phylogenetic analysis on

C. gulosus haplotypes (1,062 bp) to detect geographic lineages using the ML method in MEGA 5.0. The ML method was run for 300 bootstrap replicates using the TNr + G + I substitution model. The phylogenetic pattern based on the ML method was confirmed by neighbor-joining (NJ) and Bayesian methods. The NJ method was performed with the same substitution model used in the ML method, and 1,000 bootstrapping was implemented in PAUP* 4.0b10 (Swofford 2002). The Bayesian method was performed using the GTR + G + I model and relied on MrBayes 3.2.1 (Huelsenbeck and Ronquist 2001). Markov chain Monte Carlo (MCMC) runs extended for one million generations, with trees sampled every 100th generation. The sequences of *C. annularis* used in the phylogenetic analysis, as well as those of *Gymnogobius petschiliensis and Gymnogobius urotaenia* (DDBJ accession number: AY525784 and AB560892), were also used. Phylogenetic relationships between *C. gulosus* haplotypes were also estimated by constructing the median-joining network, as implemented in NET-WORK 4.610 (Bandelt et al. 1999). Haplotype diversity (*h*) and nucleotide diversity (*π*) for *C. gulosus* were calculated using Arlequin ver. 3.1. To compare the levels of genetic divergence between the Pacific Ocean and Sea of Japan lineages in *C. annularis* and *C. gulosus*, we simply estimated net sequence divergences between the Pacific Ocean and Sea of Japan lineages implemented in Arlequin ver. 3.1.

We attempted to verify whether multiple vicariance events were associated with the genetic divergence of the two species based on the phylogenetic time tree. Recently, relaxed-clock methods (Drummond et al. 2006) with multiple calibration points have been used to estimate divergence times more widely than global clock methods (Zuckerkandl and Pauling 1965). However, calibration points available for *Chaenogobius* are restricted, and multiple calibration points are necessary to estimate accurate divergence times by relaxed-clock methods (Warnock et al. 2012). On the other hand, the global clock method provides relatively accurate estimates close to those obtained through the relaxed-clock method with multiple calibration points (Weir and Schluter 2008). Moreover, there are many reference evolutionary rates for fish cyt *b*. Thus, we attempted to apply the global clock method. First, we generated a clocklike phylogenetic tree, including *C. annularis* and *C. gulosus*, based on the Bayesian coalescent approach using BEAST v1.7.4 (Drummond and Rambaut 2007). Model parameters consisted of the TNr + I + G substitution model with a strict molecular clock of *C. annularis* (1.5–3.4 %/MY; Hirase et al. 2012) and Yule (speciation) tree prior. The MCMC was run for 1×10^8 steps, logging data every 1,000 steps. Other settings were default. Chain mixing was assessed using Tracer v1.5 (Drummond and

Rambaut 2007), which indicated that chains reached stationarity after 1×10^8 steps for the datasets (ESS >300 for all parameters). Second, we performed the likelihood ratio test (Felsenstein 1981) for strict- and relaxed-clock models and examined the evolutionary rate difference between the two species. We also conducted a two-cluster test and a branch length test to evaluate the evolutionary rates using LINTRE (Takezaki et al. 1995). *G. petschiliensis* (AY525784) was used as outgroup for this analysis.

In addition to the traditional molecular clock methods, we employed MTML-msBayes using hierarchical approximate Bayesian computation (Huang et al. 2011). This approach can test multiple isolation events without being affected by variations across taxa in the stochasticity of the coalescent process, demographic history, and evolutionary rate (Hickerson et al. 2006). Because *C. gulosus* has two major Pacific Ocean lineages (PA1 and PA2 clades; see "Results" section) and we could not evaluate which lineage is closely related to the Sea of Japan lineage (SJ clade) by any probabilities, we used two-type datasets including the *C. annularis* two-lineage pair and PA01-SJ or PA02-SJ pairs for MTML-msBayes. We utilized default bounds for prior distributions of all parameters as generated by the program and evaluated the posterior probabilities of models with one or two divergence times for the population pairs of these species.

A previous phylogeographic study of *C. annularis* revealed the rapid expansion of the Sea of Japan lineage and estimated that this expansion was caused by the inflow of the Tsushima Current into the Sea of Japan in the Pleistocene interglacial period (Hirase et al. 2012). To examine whether the putative Sea of Japan lineages

of *C. gulosus* also underwent a similar population expansion, we performed mismatch distribution analysis (Rogers and Harpending 1992) implemented in Arlequin ver. 3.1. We compared the observed frequency distribution of pairwise nucleotide difference with their expected distribution in a simulated population expansion. For these, goodness of fit was evaluated using the estimates of the raggedness statistic and the sum of square deviations (*SSD*). In addition, to provide estimates of change in effective population size (*N*e), Tajima's *D* test (Tajima 1989) was performed. Results of these tests were compared with the expected empirical distribution under the neutral model as generated by 1,000 simulated resamplings.

Results

A total of 1,062 bases of the mtDNA cyt *b* gene were obtained from 91 specimens of *C. gulosus*. Across individuals, there were 50 polymorphic sites comprising 46 transitions and six transversions, which constituted 31 haplotypes (DDBJ accession number: AB755375–AB755405). Phylogenetic relationships between the obtained sequences of *C. gulosus*, *C. annularis*, and *Gymnogobius* species were analyzed using ML methods based on the aligned 639-bp fragment. The ML phylogenetic tree supports a sister–taxon relationship between *C. gulosus* and *C. annularis* at 100 % bootstrap value (Fig. 2).

Twenty-five haplotypes were unique, and six haplotypes were shared between/among localities. Haplotype CG13 was shared among seven localities. The ML

Fig. 2 Maximum likelihood (ML) tree of 31 haplotypes of *Chaenogobius gulosus* and haplotypes of other related species estimated by MEGA 5.0 using the TNr + I + G model of sequence evolution. Branch lengths are proportional to the estimated number of nucleotide substitutions per site. *Numbers* above the branches indicate bootstrap probabilities (values <50 % are not shown)
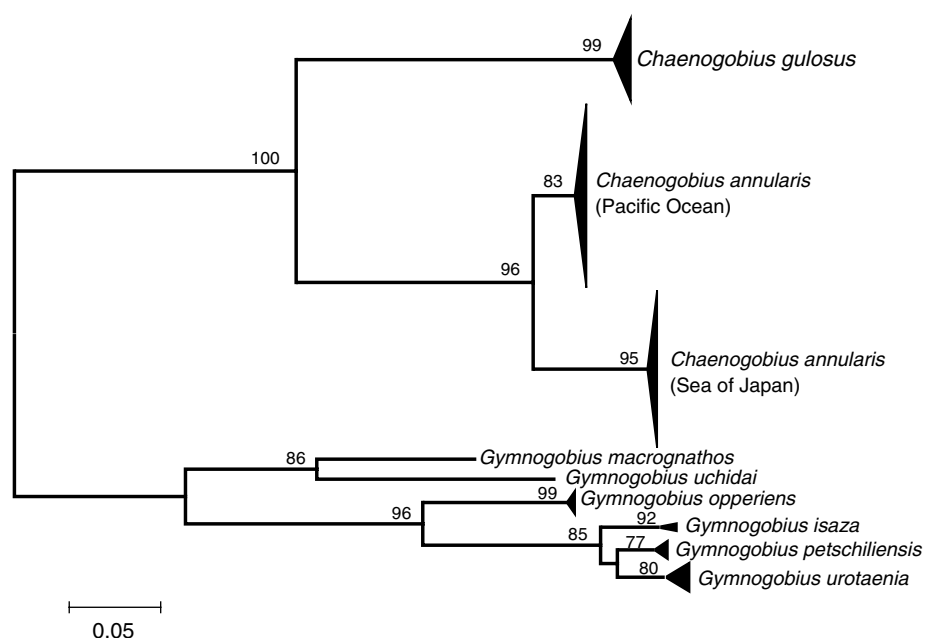
Fig. 3 Maximum likelihood (ML) tree of the 31 haplotypes of *Chaenogobius gulosus* and those of *Chaenogobius annularis* estimated by MEGA 5.0 using the TNr + I + G model of sequence evolution. Branch lengths are proportional to the estimated number of nucleotide substitutions per site. Numbers of branches indicate ML, NJ bootstrap (values <50 % are not shown) and Bayesian posterior (values <95 % are not shown) probabilities from above, respectively
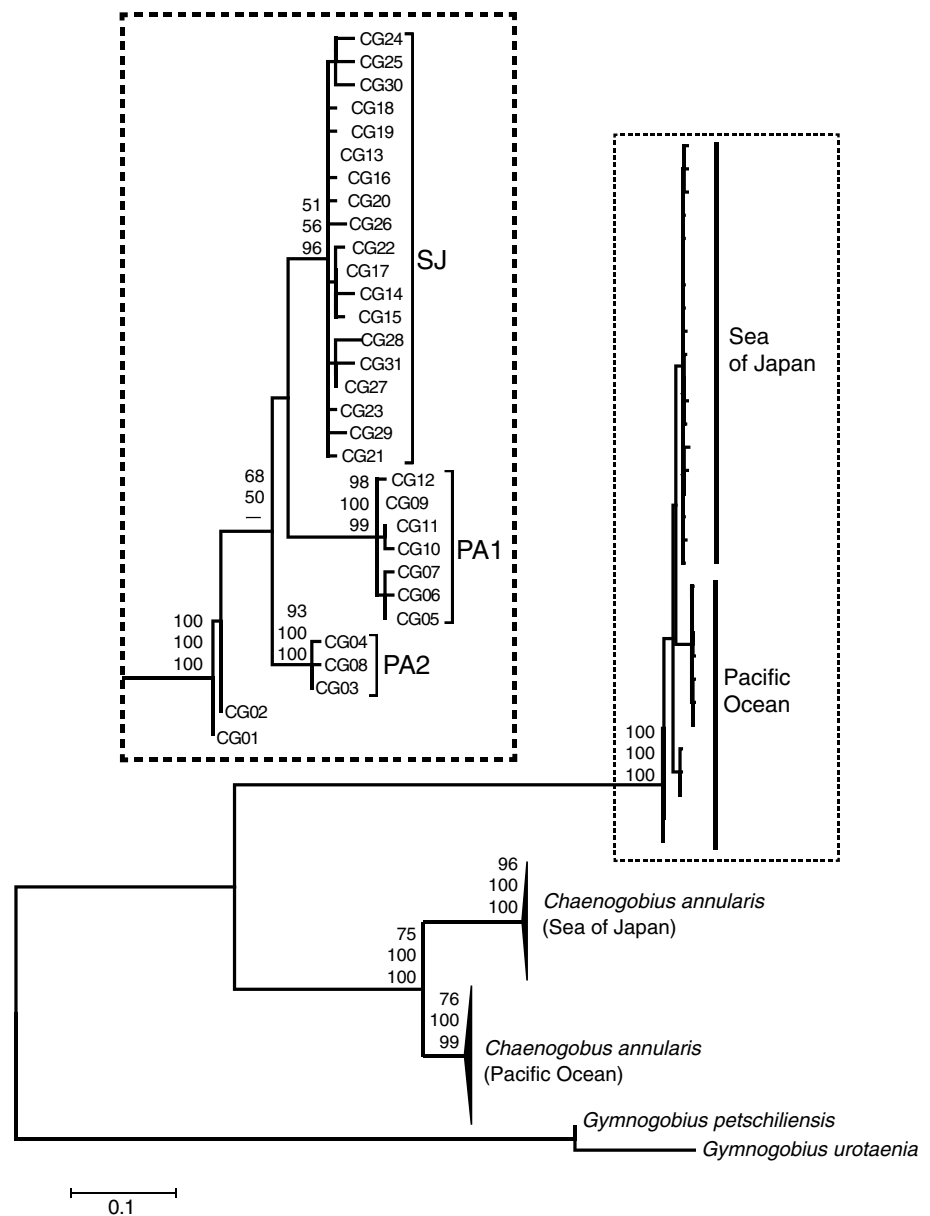
phylogenetic tree using full sequences of *C. gulosus* shows that the individual haplotypes are grouped into three genetically and geographically divergent clades (SJ, PA1, and PA2; Figs. 3, 4). Each clade is supported by 51–100 % bootstrap probability (Fig. 3). These clades are also supported by 50 % > NJ bootstrapping and 95 % > Bayesian posterior probabilities. The SJ clade is distributed along the Sea of Japan coast and the northern Pacific Ocean coast. Other clades are distributed along the central and southern parts of the Pacific Ocean coastline (Fig. 5). There were no locations where the members of these major clades occurred sympatrically. The haplotype diversity (*h*) and nucleotide diversity (*π*) of each locality are shown in Table 1. When all populations were pooled, the haplotype diversity (*h* ± SD) and nucleotide diversity

(*π* ± SD) were 0.896 ± 0.026 and 0.816 ± 0.421 (%), respectively.

Net nucleotide divergence between SJ and PA1 or PA2 was 1.2 and 0.9 %, respectively, whereas that between the Pacific Ocean and the Sea of Japan lineages in *C. annularis* was 6.3 %. The likelihood ratio test for strict (likelihood value = −5,016.41) and relaxed-clock (likelihood score = −4,950.72) models by BEAST failed to reject the molecular clock hypothesis. The two-cluster test also indicated that the evolutionary rate of cyt *b* did not differ significantly between and within *C. annularis* and *C. gulosus*. Evaluation of the divergence times with msBayes revealed a high degree of discordance (Ω) as the variance of population divergence time *τ* (Fig. 6) and higher posterior probabilities for a model of two timing of separation for these

Fig. 4 A median-joining network of the 31 mitochondrial DNA haplotypes. Each *line* between haplotypes indicates a single nucleotide substitution. Small *white circles* between haplotypes represent intermediate hypothesized haplotypes
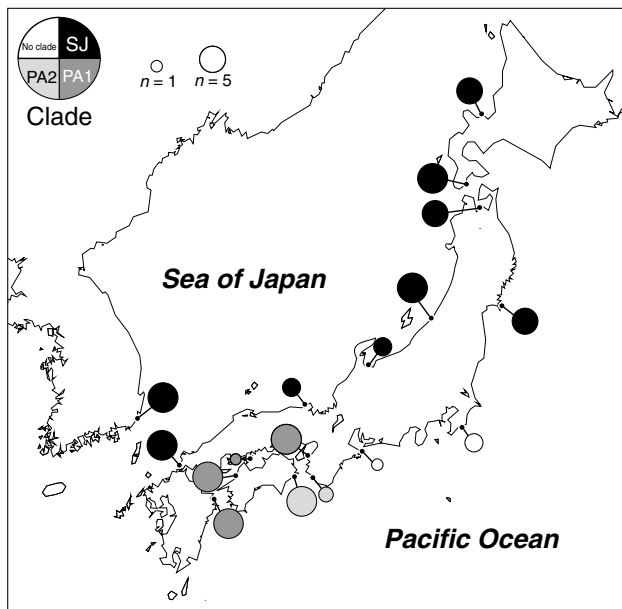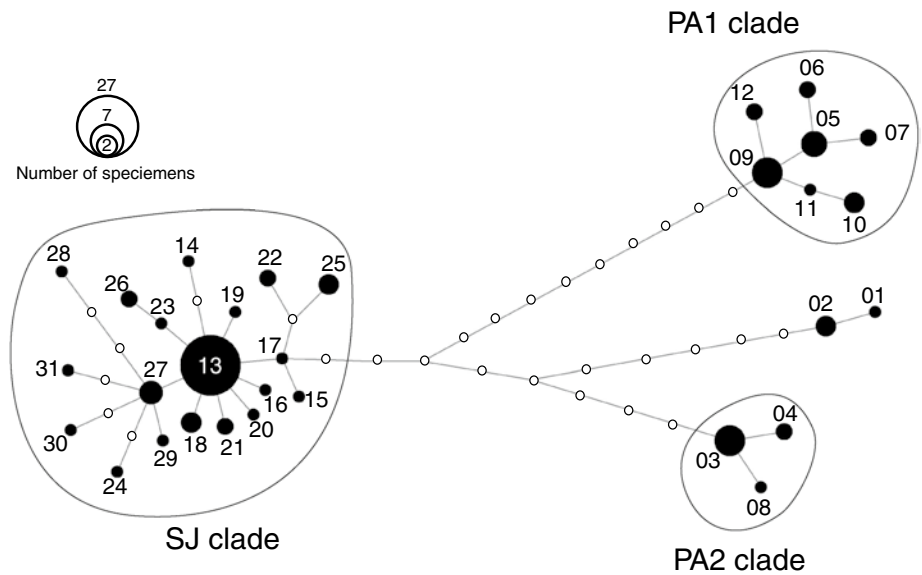

Fig. 5 Geographic distributions of the three clades of *Chaenogobius gulosus*

Fig. 6 Results of the hierarchical approximate Bayesian computation analyses using msBayes. Posterior probability densities for Ω (Var(τ)/E(τ)) among lineage pairs of *C. gulosus* (PA1–SJ or PA2–SJ) and *C. annularis*. Prior (*dashed lines*) and posterior (*solid lines*) distributions

lineages (PA1 vs. SJ: 0.994, PA2 vs. SJ: 0.998) than for a model of one divergence time based on simple rejection (0.504, 0.499).

We then estimated the time to the most recent common ancestor (TMRCA, Rosenberg and Feldman 2002) between the Pacific Ocean and the Sea of Japan clades of *C. gulosus* based on the BEAST clocklike tree. Because the PA1 and SJ clades are closely related but not supported by high bootstrap values, we estimated the TMRCA of all clades that corresponds to the lower bound of the divergence time between the Pacific Ocean and the Sea of Japan lineages.
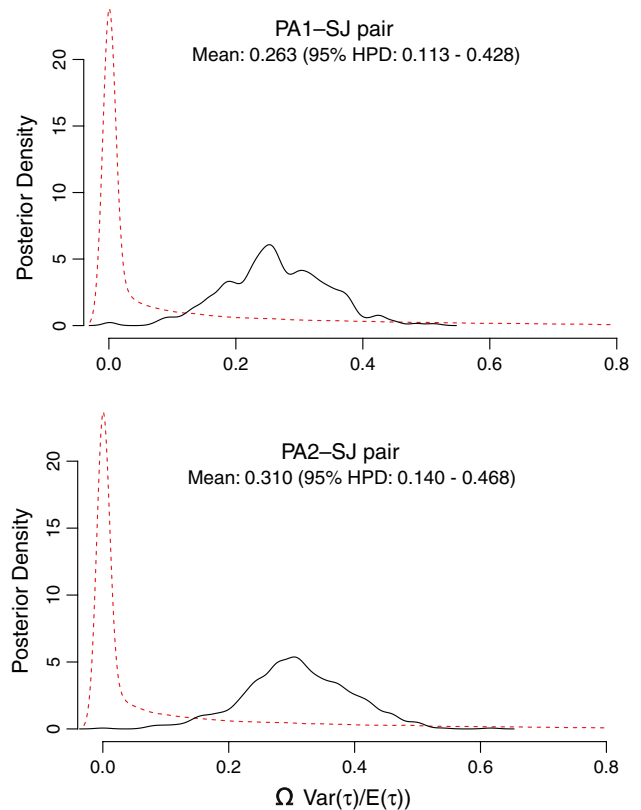
The TMRCA among all clades of *C. gulosus* was estimated to be 0.28 (95 % HPD 0.19–0.38; 3.4 % rate) to 0.65 (95 % HPD 0.45–0.88; 1.5 % rate) MYA. For *C. annularis*, the

TMRCA between the Pacific Ocean and the Sea of Japan lineages was estimated to be 1.24 (95 % HPD 0.96–1.54; 3.4 % rate) to 2.83 (95 % HPD 2.20–3.50; 1.5 % rate) MYA. These estimates do not overlap. The TMRCA of the SJ clade of *C. gulosus* was estimated to be 0.05 (95 % HPD 0.07–0.17) to 0.12 (95 % HPD 0.17–0.38) MYA.

Demographic history of the SJ clade was analyzed with mismatch distribution and Tajima's *D* test. Mismatch distributions of the clade were unimodal. We observed nonsignificant ($P > 0.05$) *SSD* or raggedness indices, which suggests recent sudden expansion of the clade (data not shown). The sudden expansion was further supported by the negative value of Tajima's *D* (-2.07; $P < 0.01$).

Discussion

The phylogenetic tree of *C. gulosus* shows several distinct geographical clades, suggesting the existence of past dispersal barriers within this species. Because the SJ clade was distributed along the coast of the Sea of Japan, while the other geographical clades were located along the Pacific Ocean coastline, we suggest that the isolation events of the Sea of Japan restricted gene flow between the Pacific Ocean and the Sea of Japan coasts, similar to *C. annularis* (Hirase et al. 2012). The Sea of Japan specimens formed a monophyletic group and showed a derivative feature in the phylogenetic tree; thus, it is possible that the parts of the Pacific Ocean lineages dispersed to the Sea of Japan coasts and became isolated.

Net nucleotide divergence between the Sea of Japan and Pacific Ocean lineages in *C. gulosus* is approximately six times larger than that within *C. annularis.* Likelihood ratio and relative rate tests did not show evolutionary rate variations between *C. annularis* and *C. gulosus*, and the lower bound of TMRCA between the Pacific Ocean and Sea of Japan lineage of *C. gulosus* did not overlap with that of *C. annularis* in the 95 % HPD interval. Moreover, msBayes based on the HABC model suggests that two divergence events formed the Pacific Ocean and Sea of Japan lineages of the two species. Therefore, the heterogeneity of the evolutionary rate and demographic history is probably not attributed to the differing amounts of mtDNA divergence within the two species. Thus, it is reasonable that more recent events of the Sea of Japan isolated the partial population of *C. gulosus*, which resulted in mtDNA divergence smaller than that of *C. annularis*. The TMRCA within *C. gulosus* was estimated to be during the late Pleistocene. Because the Sea of Japan underwent repeated opening and closing after the middle Pleistocene (Kitamura et al. 2001; Kitamura and Kimoto 2006), *C. gulosus* dispersed to the Sea of Japan during these periods and subsequently became isolated following one of these episodes. Alternatively, the

primitive lineages of *C. gulosus*, which were isolated in the Sea of Japan at the same time as that of *C. annularis*, might have become extinct because of the severe environment of this coastal area (Oba et al. 1991; Tada 1994).

Unimodal mismatch distributions, "star-like" haplotype network centering on CG13, and significant negative Tajima's *D* value of the Sea of Japan lineage are shown. Similar patterns of genetic diversity and branching pattern of the Sea of Japan lineages have also been described for *C. annularis* and other coastal marine species (Kojima et al. 2004; Akihito et al. 2008; Kokita and Nohara 2011; Hirase et al. 2012). These patterns of the mtDNA are suggestive of a sudden increase in the probability of survival of mtDNA lineages due to a burst of population growth (Avise et al. 1984; Di Rienzo and Wilson 1991; Grant and Bowen 1998). Hirase et al. (2012) assumed that the sudden expansion of the Sea of Japan lineage of *C. annularis* was caused by a sea-level rise and subsequent inflow of the Tsushima Current during the Mindel–Riss interglacial periods. The TMRCA of the SJ clade, which is considered as the lower bound for the expansion, was estimated at 0.12–0.27 MYA; it may correspond to the Riss–Würm or Mindel–Riss interglacial periods. Thus, the Sea of Japan lineage of *C. gulosus* might have also expanded suddenly because of the inflow of Tsushima Currents during these interglacial periods. The Sea of Japan lineage of *C. gulosus* is distributed along the Japanese and Korean coasts (facing the Sea of Japan), and Sanriku Coast up to Onagawa in the Pacific Ocean. Because this distribution area corresponds to the flow of the Tsushima Current, we believe that the distribution area was likely to have been formed by the inflow of the Tsushima Current after the expansion of the Sea of Japan lineage.

The Sea of Japan lineages of Japanese coastal marine species show similar distributions corresponding to the inflow of the Tsushima Currents, but there is minimal difference (Kojima et al. 2004; Akihito et al. 2008; Kokita and Nohara 2011; Hirase et al. 2012). The difference in distributions between *C. annularis* and *C. gulosus* was also observed: The lineage of *C. annularis* is distributed along the Sanriku Coast up to Hachinohe (200 km northward from Onagawa; Hirase et al. 2012). This suggests that the Sea of Japan lineage of *C. gulosus* expanded further southward than *C. annularis* in the Pacific Ocean. Larvae of *C. gulosus* have been observed in the inner bays where rocky coasts and *C. annularis* have not been observed (Sakai et al. 2007; Murase et al. 2007). Although it is unclear whether *C. gulosus* reproduce in these areas, these observations suggest more flexible habitat preferences or broader dispersal ability of *C. gulosus* than those of *C. annularis*, or both. The difference in these ecological features might have affected the broader expansion of the Sea of Japan lineage of *C. gulosus*.

Comparative phylogeography across a large number of loci and taxa as a way to infer biogeographic events is expected to become common (Emerson et al. 2011). On the basis of the traditional molecular clock and HABC model, we showed a lower genetic divergence in terms of million years between the Pacific Ocean and Sea of Japan lineages within *C. gulosus* than that of its sister species *C. annularis*. Our results strongly support that multiple Pleistocene isolation events in the Sea of Japan led to varying levels of genetic divergence. Because it can be assumed that the glacial–interglacial cycles have led to multiple vicariance events in many areas of the world, our results show the importance of comparative phylogeography between closely related species and that it can reveal complex biogeographic events.

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