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Long-term vicariance and post-glacial expansion in the Japanese rocky intertidal goby *Chaenogobius annularis*

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ABSTRACT: Studies of mitochondrial DNA phylogeography of several Japanese coastal marine species have shown the existence of Pacific Ocean and Sea of Japan groups, which diverged because of the isolation of the Sea of Japan in the Pleistocene glacial periods. Here we performed a combined analysis of mitochondrial DNA (mtDNA) cytochrome *b* and 8 microsatellite loci among *Chaenogobius annularis* populations to reveal the levels of congruence between the 2 markers, the admixture of the 2 groups, and the post-glacial history of the Sea of Japan group. Population-level and individual-level genetic analyses based on these markers showed no or minimal admixtures between the Pacific Ocean and Sea of Japan groups, despite the temporal opening of the Sea of Japan in multiple interglacial periods. The genetic diversity within the Sea of Japan group was much lower than that within the Pacific Ocean group. However, the Shimonoseki population, sampled in southern parts of the Sea of Japan, revealed higher mitochondrial nucleotide diversity and microsatellite allelic richness, suggesting post-glacial expansion from the southern area, accompanied by genetic drift, i.e. the founder effect. Congruent patterns of mtDNA and microsatellite DNA markers suggest long-term vicariance and severe genetic drift in the Sea of Japan group since the divergence in the early Pleistocene.

KEY WORDS: Paleogeographical events · Phylogeography · Mitochodrial DNA · Microsatellite DNA · Japanese Archipelago · Sea of Japan · Glacial refugia

INTRODUCTION

Phylogeographic studies have shown that past geographic barriers associated with paleoenvironmental changes have caused genetic divergences between populations (Avise 2000). These genetic divergences may permit 'allopatric speciation,' as in the example of transisthmian sister taxa across the Isthmus of Panama (Knowlton et al. 1993). These studies have contributed to our understanding of speciation and conservation units (Avise 2000). A decade or so ago, most studies focused exclusively on the variations of single mitochondrial DNA (mtDNA), mainly because of its rapid evolution and maternal transmission without intermolecular recombination (Avise 2000). However, overdependence on mtDNA has often been criticized because the mitochondrial genome represents only a minuscule fraction of the total historical record within a sexual organismal pedigree (e.g. Degnan 1993, Palumbi & Baker 1994), and the stochastic nature of coalescent processes in mtDNA differs from that of the nuclear genome (e.g. Wilson & Veraguth 2010). This may provide a misleading picture of population-level processes, namely, situations

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in which introgressive hybridization and postglacial expansion may be involved. Therefore, assessing congruence among multi-locus genetic data is a critical issue in phylogeography (Avise 2009), and combined approaches have allowed us to resolve genetic signatures of both long-term vicariance and recent postglacial expansion of species (e.g. Aldenhoven et al. 2010).

The Sea of Japan, which is located between the Japanese Archipelago and the Asian mainland, is characterized by the multiple Pleistocene isolation events that occurred during glacial periods (e.g. Tada 1994). Several mtDNA phylogeographic studies have indicated that these events led to genetic divergences in coastal marine species between the Pacific Ocean and the Sea of Japan (Kojima et al. 1997, 2004, Akihito et al. 2008, Kokita & Nohara 2011, Hirase et al. 2012b), with a wide range of divergence times between geographic lineages (Kojima et al. 1997, 2004, Kokita & Nohara 2011, Hirase et al. 2012b). Moreover, because the paleogeographical history of the Sea of Japan has been well documented, efforts have been made to estimate accurate divergence time of 2 groups of *Chaenogobius annularis*, using molecular clocks calibrated by paleogeographical history (Hirase et al. 2012b). Therefore, pairs of allopatric groups of Japanese coastal marine species might be suitable to study the process of adaptive evolution and allopatric speciation. For example, Kokita et al. (2013) succeeded in identifying candidate genomic regions under positive selection in the Sea of Japan lineage.

However, there still remain some issues for the history of the 2 allopatric groups to be resolved. During each interglacial period following the middle Pleistocene, the Sea of Japan connected to other seas and the Tsushima Current inflow occurred (Kitamura et al. 2001, Kitamura & Kimoto 2006), and these events possibly promoted gene flow between the 2 groups. However, previous phylogeny-based studies based on mtDNA alone (e.g. Hirase et al. 2012b) were insufficient to detect the gene flow between them. Therefore, population genetic studies based on mtDNA and nuclear DNA markers, particularly polymorphic microsatellite DNA, and large sample sizes are required to verify genetic admixtures between the Pacific Ocean and the Sea of Japan groups. Such studies could provide some insights into the process of allopatric speciation promoted by historical events.

In addition, microsatellite DNA markers would contribute to understanding the demographic history of the isolated Sea of Japan group. This group expanded rapidly following inflow of the Tsushima Current in the Pleistocene interglacial periods, but underwent severe bottlenecks during glacial periods (Kojima et al. 1997, 2004, Akihito et al. 2008, Kokita & Nohara 2011, Hirase et al. 2012b). A previous study suggested that the expansion occurred from the southern area, which showed higher mtDNA diversity (Kokita & Nohara 2011). The southern area, which is the eastern shelf of the Tsushima Strait, received a small but constant inflow of fresh seawater through a portion of the Strait, even during the last glacial period (Gorbarenko & Southon 2000). Therefore, the 'southern richness and northern purity' might show that continued inflow of the Tsushima Current during glacial periods formed refugia in the southern area and enabled the expansion. However, there has been no other critical biological evidence to support the hypothesis of refugia in the southern part of the Sea of Japan. The allelic richness (El Mousadik & Petit 1996) of nuclear DNA markers is a useful estimator for identifying glacial refugia. Glacial refugia are expected to harbor higher levels of allelic richness than areas recolonized after glacial periods, because recolonizations often involve only a few individuals (Comps et al. 2001, Takahashi et al. 2005, Tsuda & Ide 2005, Aizawa et al. 2009, Hu et al. 2010). Therefore, if the expansion of the Sea of Japan groups occurred from the southern refugia, allelic richness should decrease from south to north. Thus, detailed sampling along the coast of the Sea of Japan groups is required to elucidate the genetic consequences of Pleistocene isolation events.

Chaenogobius annularis is a rocky intertidal species that is distributed throughout the coastline of Japan (Hokkaido southward to Yakushima Island) and Korea (Akihito et al. 2002). To date, we have conducted phylogeographic analyses for this species based on mtDNA (cyt *b* and ND2: 2340 bp) and revealed 2 distinct allopatric groups: the Pacific Ocean and Sea of Japan groups (Hirase et al. 2012b). In this previous study, we estimated that the Pacific Ocean and Sea of Japan mtDNA lineages diverged 1.7 million years ago (MYA) based on a likely evolutionary rate of 2.2% million yr^{-1} and the detailed demographic history of the Pacific Ocean group. Yet, this study was insufficient to assess the level of gene flow between the 2 allopatric groups and the postglacial history of the Sea of Japan group because of the limited sample size per locality (~8 specimens) and the use of only mtDNA markers. Therefore, in the present study, we analyzed mtDNA cytochrome *b* and 8 microsatellite DNA loci for the local populations of *C. annularis* sampled around the Japanese

Archipelago and compared the results of mtDNA and microsatellite DNA analyses. Our aims were (1) to evaluate the congruence between mtDNA and microsatellite DNA markers; (2) to evaluate the level of gene flow between the Pacific Ocean and Sea of Japan groups; and (3) to reconstruct the postglacial history of the Sea of Japan group associated with Pleistocene isolation events.

MATERIALS AND METHODS

Samples

Sampling localities are shown in Fig. 1 and Table 1. Between 2006 and 2010, *Chaenogobius annularis* were collected by hand nets from 13 localities on the Japanese coastline and from 2 localities on the coastline of the Korean Peninsula, roughly encompassing the whole distribution range of this species. These samples include the specimens used in a previous phylogeographic study; 8 and 7 localities represent the Pacific Ocean and Sea of Japan groups, repectively (Hirase et al. 2012b). Most samples were used for both mtDNA and microsatellite DNA analyses, and a subset of samples was used for one or the other analysis. All samples were identified based on morphological characteristics, i.e. many rows of black dots in the pectoral and caudal fins and a caudal fin without a white posterior margin, as described by Akihito et al. (2002). We observed no differences in these morphological characteristics between localities. Total DNA was extracted from muscle or fin by phenol/chloroform extraction (Asahida et al. 1996). The DNA samples were stored at –30°C prior to polymerase chain reaction (PCR) analysis.

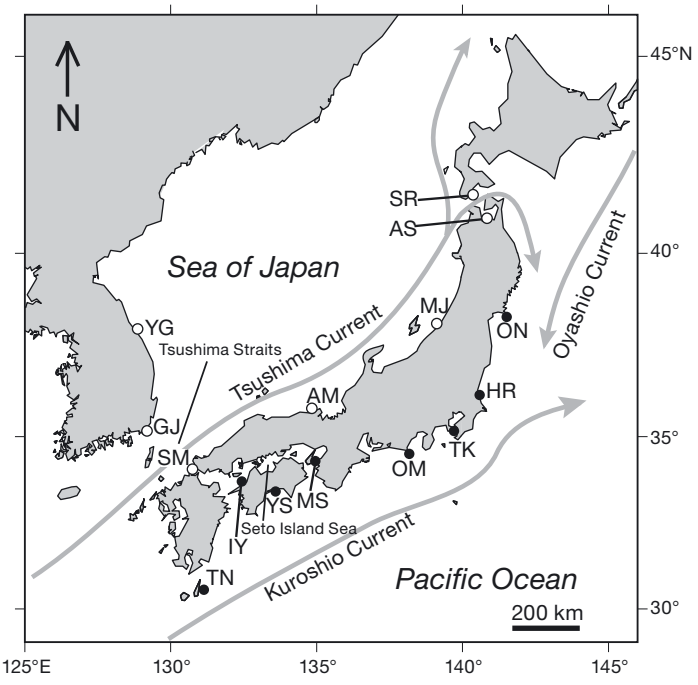

Fig. 1. *Chaenogobius annularis*. Sampling localities. Filled circles: distribution of the Pacific Ocean group; open circles: Sea of Japan group, as elucidated by our previous phylogeographic study (Hirase et al. 2012b). Locality codes as in Table 1

Table 1. *Chaenogobius annularis*. Sampling localities, latitude and longitude, and sample sizes used for mitochondrial and microsatellite DNA analyses. *h*: haplotype diversity; π: nucleotide diversity; Pref.: prefecture

Locality	Code	Date	Latitude (° N)	Longitude (° E)	Sample size		*h*	π (%)
					mtDNA	Microsatellite		
Onagawa, Miyagi Pref.	ON	May–June 2006	38° 26′	141° 27′	45	40	0.540	0.170
Hiraiso, Ibaraki Pref.	HR	June 2007	36° 21′	140° 37′	30	28	0.464	0.166
Takeoka, Chiba Pref.	TK	August 2007	35° 12′	139° 50′	35	35	0.844	0.272
Omaezaki, Shizuoka Pref.	OM	March 2008	34° 35′	138° 13′	18	20	0.386	0.459
Misaki, Osaka Pref.	MS	September 2006	34° 19′	135° 9′	30	26	0.625	0.427
Yasu, Kochi Pref.	YS	August 2008	33° 26′	133° 23′	22	22	0.805	0.589
Iyo, Ehime Pref.	IY	April 2009, August 2010	33° 42′	132° 39′	40	41	0.705	0.331
Tanegashima, Kagoshima Pref.	TN	October 2009	30° 41′	131° 4′	25	21	0.157	0.075
Shiriuchi, Hokkaido Pref.	SR	October 2007	41° 31′	140° 25′	28	28	0.516	0.148
Asamushi, Aomori Pref.	AS	March 2009	40° 53′	140° 51′	37	32	0.614	0.139
Majima, Niigata Pref.	MJ	September 2007	38° 16′	139° 26′	31	45	0.822	0.264
Amino, Kyoto Pref.	AM	September 2009	35° 41′	135° 2′	28	26	0.331	0.079
Shimonoseki, Yamaguchi Pref.	SM	July 2009	33° 57′	130° 52′	32	38	0.889	0.431
Gijang, Pusan Pref.	GJ	June 2009	35° 12′	129° 13′	39	38	0.726	0.218
Yeongok, Gangneung Pref.	YG	June 2009	37° 51′	128° 51′	24	26	0.083	0.016

DNA analysis

Mitochondrial DNA

Partial cytochrome *b* of each specimen was amplified using primers AJG15 and H5 (Akihito et al. 2000). For amplification, the following reagents were added to each microtube: 10 ng of template DNA, 0.25 U of Blend *Taq* –Plus– (TOYOBO), 1 µl of the 10× buffer, 1 µl of 2 mM dNTP, and 0.1 µl of each primer (25 µM). Enough sterile deionized H_2O was added to each sample to bring the volume up to 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 following the ethanol/EDTA method outlined in the Applied Biosystems (ABI) BigDye Terminator v3.1 Cycle Sequencing Kit (BigDye v3.1) Manual. PCR products were bi-directionally sequenced using ABI BigDye v3.1 following the manufacturer's instructions and using the forward primer used for the PCR. Reactions were purified by the ethanol/EDTA/sodium acetate method as per the manual and analyzed in an ABI 3130 capillary sequencer.

Microsatellite DNA

We employed 8 of 15 microsatellite DNA markers developed in a previous paper (Hirase et al. 2010) because the other 7 markers were not amplified in some specimens. Multiplex PCR with the primer combinations *CA*64–*CA*346 (annealing temperature: *T*a = 62°C), *CA*410–*CA*587–*CA*645*A* (*T*a = 62°C), and *CA*151–*CA*234–*CA*626 (*T*a = 53°C) was carried out using a Qiagen Multiplex PCR Kit. For amplification, the following reagents were added to each microtube: 10 ng of template DNA, 3 µl of 2× Qiagen Multiplex PCR Master Mix, and 0.2 µM of each primer. Enough sterile deionized H_2O was added to each sample to make a total of 6 µl. PCR conditions consisted of 25 cycles of denaturation at 94°C for 30 s (15 min for the first denaturation only), annealing at 53 or 62°C for 30 s, and extension at 72°C for 90 s (10 min for the last extension only). Hi-Di Formamide (10 µl; Applied Biosystems) and GeneScan™ 500LIZ™ Size Standard (0.1 µl; Applied Biosystems) were added to 1 µl of each sample after PCR amplification. Amplification products were detected using an ABI3130 Genetic Analyzer, and electropherograms were analyzed using Gene Mapper v. 4.0 (Applied Biosystems).

Data analysis

Mitochondrial DNA

The identities of sequences were confirmed by BLAST (Altschul et al. 1997) searching of GenBank (http://blast.ncbi.nlm.nih.gov/) and by comparison with published sequences. Sequences collected were aligned by CLUSTAL W (Thompson et al. 1994). Arlequin v. 3.1 (Excoffier et al. 2005) was used to assign specimens to haplotypes, these haplotypes were submitted to the DNA Data Bank of Japan (DDBJ: AB853332–AB853423; www.ddbj.nig.ac.jp). Phylogenetic analyses were carried out on all haplotypes using the neighbor-joining (NJ; Saitou & Nei 1987) and maximum likelihood (ML; Felsenstein 1981) methods in MEGA 5.0 (Tamura et al. 2011). MEGA 5.0 was run for 1000 bootstrap replicates using TN93 (Tamura & Nei 1993) + G (for NJ) and TN93 + G + I (for ML) substitution models which were selected as best-fit models (based on Akaike's information criterion; Akaike 1974) by MEGA 5.0. A sympatric sister species, *Chaenogobius gulosus* (accession: AB684974), and a related species, *Gymnogobius petschiliensis* (accession: AY525784) were used as outgroups. Phylogenetic relationships among *C. annularis* haplotypes were also estimated by the median-joining (MJ) method in NETWORK 4.5.0.1 (Bandelt et al. 1999). Haplotype diversity (*h*) and nucleotide diversity (π) of each population were calculated using Arlequin v. 3.1.

The geographic structure of genetic variation was assessed using hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992), by defining groups in accordance with the results of our phylogenetic analysis, and subsequent spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002). AMOVA and SAMOVA were performed based on Φ_{CT} (a measure to assess genetic differences among groups assigned *a priori*) and Φ_{ST} (a measure used to calculate genetic differences among populations), which are analogous to the *F*-statistics. For AMOVA, significance was tested by 1000 permutations of the original data set. The AMOVA was performed by Arlequin v. 3.1. SAMOVA based on a simulated annealing procedure and was used to define subgroups in SAMOVA 1.0 software (Dupanloup et al. 2002). SAMOVA iteratively seeks the composition of a user-defined *K* number of groups of geographically adjacent populations that maximizes Φ_{CT} (the proportion of variation among groups), run here with 100 simulated annealing processes for $K = 2$ to 4.

Microsatellite DNA

Genotype frequency conformity to Hardy–Weinberg equilibrium (HWE) for each locus and linkage disequilibrium (LD) for all pairs of loci in each population were tested using GENEPOP 3.3 (Raymond & Rousset 1995). Levels of statistical significance for multiple tests of HWE and LD were determined using sequential Bonferroni adjustments for simultaneous tests (Rice 1989). Number of alleles (A), allelic richness per locus (A_R) and mean expected (H_E) and observed (H_O) heterozygosities were determined using GENEPOP 3.3 and FSTAT 2.9.3.2 (Goudet 1995). The average number of private alleles per specimen (P) was calculated using GenAlex 6.5 (Peakall & Smouse 2012). Genetic structure was assessed based on an NJ tree, AMOVA, and SAMOVA. The NJ tree was constructed based on the modified Cavalli–Sforza distance (D_A) (Nei et al. 1983), implemented in POPULATIONS 1.2.30 (Langella 2007). AMOVA and SAMOVA were performed based on F_{CT} (a measure to assess genetic differences among groups assigned *a priori*) and F_{ST} (a measure used to calculate genetic differences among populations) using the same procedure as for mtDNA (see subsection above). Genetic differentiation among populations was assessed based on pairwise F_{ST} (Weir & Cockerham 1984), implemented in Arlequin v. 3.1. Genetic differentiation was also assessed using R_{ST} values (Michalakis & Excoffier 1996) calculated in SPAGEDI (Hardy & Vekemans 2002). R_{ST} accounts for the stepwise mutations that characterize microsatellite loci, and thus a comparison between F_{ST} and R_{ST} can estimate the relative importance of genetic drift and mutation. These estimators are expected to be similar when drift is most important, while R_{ST} should increase relative to F_{ST} as the contribution of stepwise mutation to differentiation increases. We then compared observed R_{ST} values to expected values (pR_{ST}) based on 1000 permutations of allele size, following Hardy et al. (2003) with SPAGEDI. Consequently, where R_{ST} is significantly larger than pR_{ST}, we can infer that stepwise mutation is contributing to differentiation (Hardy & Vekemans 2002), whereas a non-significant difference suggests the importance of genetic drift and that F_{ST} is a more suitable estimator.

The above analyses were used for the populations as the unit of comparison, whereas the Bayesian clustering analysis in STRUCTURE version 2.2 (Pritchard et al. 2000, 2007) was used for the specimen as the unit, assigning specimens into K subpopulations (clusters) based on an admixture model and a correlated allele frequencies model (hereafter, the F-model; Falush et al. 2003). The F-model assumes that all K clusters diverged from a common ancestral population at the same time, but allows the possibility that the clusters may have experienced different degrees of genetic drift since the divergence event to be considered (Falush et al. 2003). In the F-model, the amount of genetic drift for each cluster is described as 'F,' the values of which are analogous to F_{ST} values between clusters and an ancestral population. We used runs involving 20 000 Markov chain Monte Carlo iterations after a burn-in period of 10 000 iterations without prior information on the populations of origin of the sampled specimens. The analysis was run 10 times, with K ranging from 1 to 15. The optimal value of K was estimated by calculating ΔK to identify the top level in the hierarchical structure, according to Evanno et al. (2005). This analysis revealed the distinct genetic divergence between the Pacific Ocean and Sea of Japan groups (see 'Results'), and this divergence must have occurred prior to the genetic differentiation within each group. Because the F-model assumes that all K clusters diverged from a 'common' ancestral population 'at the same time,' it was therefore not appropriate to find sub-structure within the 2 groups using the whole dataset. Thus, the Bayesian clustering analysis was repeated for each group separately to determine the sub-structure. For only the Sea of Japan group, we evaluated the genetic relationship among 3 clusters assumed by the Bayesian clustering analysis within this group (see 'Results') by constructing an NJ tree based on pairwise F_{ST} values. This analysis was implemented in the computer program NEIGHBOR from the PHYLIP 3.5c phylogeny package (Felsenstein 1993).

In addition to F values from the Bayesian clustering analysis, we performed heterozygosity excess (Cornuet & Luikart 1996), mode-shift (Luikart & Cornuet 1998), and M-ratio tests (Garza & Williamson 2001) based on microsatellite DNA loci to evaluate whether the Sea of Japan group had experienced a bottleneck. Heterozygosity excess and mode-shift tests were implemented in BOTTLENECK 1.2.02 software (Piry et al. 1999), under the assumptions for both the infinite allele mutation model and the 2-phase model. While the above 2 tests detect recent bottlenecks, the M-ratio test detects ancient bottlenecks because the reduction can last for hundreds of generations if the effective population size (Ne) or mutation rate remains low (Spear et al. 2006). Moreover, this analysis can distinguish between populations that have been recently reduced and those that have been small for a long time; this occurs because rare alleles do not persist long in small populations

due to genetic drift (Garza & Williamson 2001). Therefore, if one population, which shows higher genetic variability than other populations, reveals significantly low *M*-ratio values, this population is expected to have experienced a bottleneck during its long history. The *M*-ratio was calculated for each sample population using M_P_val software (Garza & Williamson 2001). Significance was assessed by comparison between the mean *M* value across all loci and a value of $M = 0.680$, the threshold value below which a population can reasonably be assumed to have undergone a reduction in population size (Garza & Williamson 2001).

RESULTS

mtDNA analysis

We obtained 92 haplotypes from 464 specimens of *Chaenogobius annularis*. The NJ tree showed that 51 haplotypes formed a Pacific Ocean clade and 41 haplotypes formed a Sea of Japan clade with high bootstrap probabilities (83 and 100%). The 2 clades were supported by the ML tree, although the bootstrap probability of the Pacific Ocean clade was low (33%; see Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m499p217_supp.pdf). There were no populations in which the 2 lineages coexisted. The values of *h* ranged from 0.083 (study site YG; locality codes are listed in Table 1) to 0.889 (SM), and π ranged from 0.016 (YG) to 0.589 (YS; Table 1).

We performed population genetic analyses for the 2 groups based on mtDNA data. The Pacific Ocean group was characterized by a haplotype network within which haplotypes were separated by multiple missing intermediate haplotypes (Fig. 2). Within the Pacific Ocean group, AMOVA showed that significant genetic variance (mtDNA: $\Phi_{ST} = 0.710$, $p < 0.001$) was distributed among populations (see Table S1 in the Supplement). The SAMOVA suggested that optimum partitioning was obtained when localities were divided into 3 subgroups, viz. northern (ON, HR, TK), central (OM, MS, YS), and southern (IY and TN), since Φ_{CT} reached a plateau at $K = 3$, and 1 or more groups contained a single population for $K \geq 3$, indicating loss of group structure. Significant genetic variance ($\Phi_{CT} = 0.532$, $p = 0.003$; Table S1) was distributed among and within these subgroups ($\Phi_{ST} = 0.295–0.556$, $p < 0.001$). These 3 subgroups were also

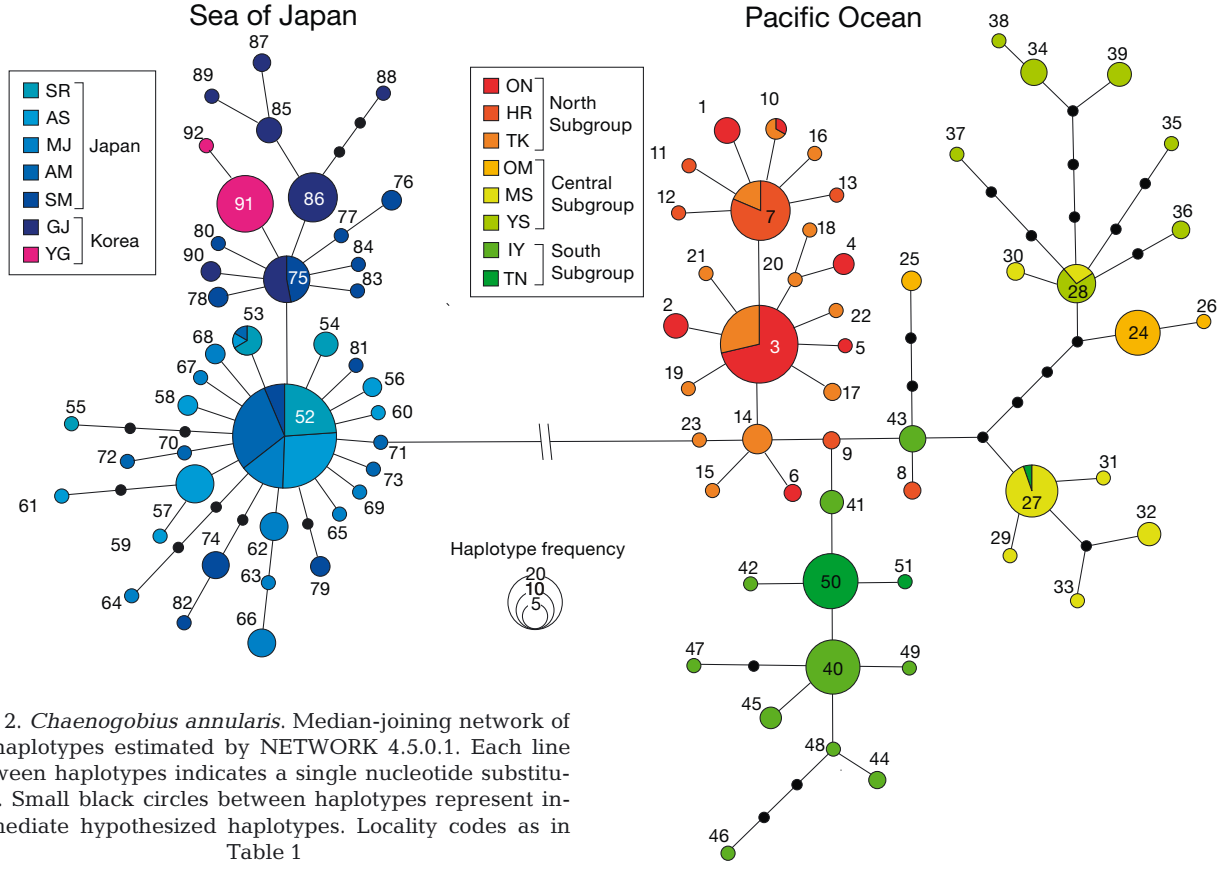

Fig. 2. *Chaenogobius annularis*. Median-joining network of 92 haplotypes estimated by NETWORK 4.5.0.1. Each line between haplotypes indicates a single nucleotide substitution. Small black circles between haplotypes represent intermediate hypothesized haplotypes. Locality codes as in Table 1

identified in a previous analysis (Hirase et al. 2012b), thus this structure seems to be robust.

The Sea of Japan group showed a star-like network with 2 central haplotypes, CYB52 and CYB75, which differed from each other by 1 change (Fig. 2). The AMOVA showed that significant genetic variance (mtDNA: Φ_{ST} = 0.478, p < 0.001, Table S1) was distributed among populations within the Sea of Japan group. Although SAMOVA did not suggest any partitions, there was a weak geographical association of haplotype distributions: the CYB52 group (CYB52 and haplotypes differing from CYB52 by 1 to 6 changes) and the CYB75 group (CYB75 and haplotypes differing from CYB75 by 1 to 3 changes); 4 Japanese populations (AS, SR, MJ, AM) belonged to the former and 2 Korean populations (YG and GJ) to the latter haplotype groups, respectively. The SM population had both haplotype groups. All haplotypes except CYB52, CYB53, and CYB75 were restricted to 1 population (Fig. 2).

Microsatellite DNA polymorphisms

In total, 466 *Chaenogobius annularis* were genotyped for the 8 microsatellite loci. Genetic variability of each locus in each population is shown in Table 2. *A* ranged from 1.6 (YG) to 6.8 (TK). Average H_E per population ranged from 0.089 (YG) to 0.636 (IY), and average H_O ranged from 0.087 (YG) to 0.636 (IY). *P* ranged from 0 (SR, AS, MJ, YG) to 0.3 (SM), and A_R ranged from 1.6 (YG) to 5.9 (TK). Significant deviations from HWE (p < 0.05) were detected for *CA*151 in IY, *CA*234 in MS, *CA*346 in SM, *CA*410 in TK, *CA*645*A* in ON, YS, and YG, and *CA*626 in YS. However, no loci showed significant departure across all populations, and no populations showed significant departure across all loci. In addition, these significant deviations were not detected after Bonferroni correction (Rice 1989) (Table 2). Therefore, the deviations in HWE were most likely caused by the existence of null alleles in particular populations and loci. These results were insufficient to reject the null hypothesis of random mating in the populations and the usability of these microsatellite markers. LDs were not detected after Bonferroni correction.

Genetic variability of the Sea of Japan populations was lower than that of the Pacific Ocean populations as a whole, but only the SM population showed the same amount of variability as the Pacific Ocean populations. There was steep geographic cline of genetic variability within the Sea of Japan, in which the SM population showed the highest variability, while northern and southern populations showed relatively low genetic variability within the Pacific Ocean (Table 2).

Population differentiation revealed by microsatellite DNA

Allele frequencies of each microsatellite DNA locus for each population are shown in Fig. 3. Allele frequencies were different between the Pacific Ocean and Sea of Japan groups, and the 2 groups shared no alleles at the *CA234* locus. Within the Sea of Japan group, there were some north–south clines in the allele distributions, whereas the Pacific Ocean group showed no clines.

The NJ tree showed that the Pacific Ocean and Sea of Japan groups formed 2 clusters (Fig. 4). An AMOVA was performed first at the level of 15 populations (F_{ST} = 0.440, p < 0.001; Table S2). Afterwards, we divided these into 2 groups (Pacific Ocean and Sea of Japan groups). Significant portions of genetic variance (F_{CT} = 0.356, p < 0.001; Table S2) were distributed between them.

The AMOVA within the Sea of Japan group showed significant genetic variance (F_{ST} = 0.401, p < 0.001; Table S2) among populations. The SAMOVA divided the samples into the Japanese subgroup (SR, AS, MJ, AM) and the Shimonoseki–Korean subgroup (SM, GJ, YG), and significant genetic variance was evident between these subgroups (F_{CT} = 0.291, p < 0.001; Table S2) and within them (F_{ST} = 0.084, 0.476, p < 0.01). Genetic differentiation within the Shimonoseki–Korean subgroup was high, and the SAMOVA partitioned the SM population into another subgroup when assuming K = 3.

Within the Pacific Ocean group, although the AMOVA showed significant genetic variance (F_{ST} = 0.167, p < 0.001; Table S2) among populations, the SAMOVA suggested no subgroups.

All pairwise F_{ST} values between the Pacific Ocean and Sea of Japan groups were significant after Bonferroni correction (p < 0.05; Table 3) and larger than those within each group. Allele size randomization tests showed that most of the pR_{ST} values were not significantly higher than the F_{ST} values (Table 3), indicating that stepwise mutation did not contribute to their genetic divergence, and F_{ST} is the more suitable estimator for microsatellite DNA. However, when all populations were pooled in each group, the pR_{ST} value was significantly higher than the F_{ST} value (p = 0.046). Within the Sea of Japan and the Pacific Ocean groups, all F_{ST} values were significant after Bonferroni correction (p < 0.05, Table 3).

Table 2. *Chaenogobius annularis*. Genetic diversity at 8 microsatellite DNA loci in 15 populations (locality codes as in Table 1). *n*: total number of analyzed specimens; H_E: expected heterozygosity; H_O: observed heterozygosity; F_{IS}: Weir & Cockerham's (1984) estimate index; *A*: number of alleles; *P*: number of private allele per specimen; A_R: allele richness. No F_{IS} indicated significant deviations from Hardy–Weinberg equilibrium after standard Bonferroni correction of the 5% threshold

Locus	Pacific Ocean group								Sea of Japan group						
	ON (n = 40)	HR (n = 28)	TK (n = 35)	OM (n = 20)	MS (n = 26)	YS (n = 22)	IY (n = 41)	TN (n = 21)	SR (n = 28)	AS (n = 32)	MJ (n = 45)	AM (n = 26)	SM (n = 38)	GJ (n = 38)	YG (n = 26)
CA64															
A	2	6	7	6	8	5	4	2	1	1	1	1	4	1	1
H_E	0.289	0.638	0.586	0.695	0.740	0.742	0.733	0.278	0.000	0.000	0.000	0.000	0.125	0.000	0.000
H_O	0.350	0.607	0.629	0.800	0.692	0.864	0.683	0.333	0.000	0.000	0.000	0.000	0.132	0.000	0.000
F_{IS}	−0.200	0.067	−0.058	−0.126	0.084	−0.142	0.080	−0.176	–	–	–	–	−0.036	–	–
CA151															
A	3	7	7	3	6	3	5	2	1	1	3	3	7	2	1
H_E	0.433	0.529	0.599	0.401	0.658	0.569	0.203	0.278	0.000	0.000	0.628	0.665	0.700	0.317	0.000
H_O	0.500	0.536	0.571	0.450	0.615	0.500	0.171	0.238	0.000	0.000	0.622	0.692	0.763	0.395	0.000
F_{IS}	−0.141	0.005	0.061	−0.096	0.084	0.144	0.173	0.167	–	–	0.021	−0.022	−0.077	−0.233	–
CA234															
A	1	2	1	1	2	1	2	1	1	1	1	2	3	2	1
H_E	0.000	0.035	0.000	0.000	0.204	0.000	0.070	0.000	0.000	0.000	0.000	0.440	0.077	0.361	0.000
H_O	0.000	0.036	0.000	0.000	0.077	0.000	0.073	0.000	0.000	0.000	0.000	0.500	0.079	0.368	0.000
F_{IS}	–	0.000	–	–	0.635	–	−0.026	–	–	–	–	−0.117	−0.018	−0.006	–
CA346															
A	5	15	14	9	8	7	11	6	8	6	11	11	12	6	4
H_E	0.693	0.908	0.865	0.716	0.756	0.776	0.742	0.372	0.675	0.698	0.802	0.873	0.783	0.606	0.305
H_O	0.725	0.893	0.886	0.700	0.731	0.864	0.659	0.429	0.786	0.750	0.689	0.923	0.789	0.605	0.346
F_{IS}	−0.034	0.035	−0.01	0.048	0.053	−0.090	0.125	−0.129	−0.146	−0.058	0.152	−0.038	0.005	0.014	−0.114
CA410															
A	7	6	8	11	10	7	13	7	4	3	3	3	7	6	1
H_E	0.725	0.766	0.809	0.880	0.876	0.777	0.833	0.672	0.568	0.574	0.511	0.208	0.782	0.501	0.000
H_O	0.625	0.857	0.657	0.800	0.923	0.773	0.927	0.714	0.679	0.563	0.511	0.231	0.684	0.605	0.000
F_{IS}	0.151	−0.101	0.201	0.116	−0.034	0.029	−0.101	−0.038	−0.177	0.035	0.011	−0.091	0.138	−0.196	–
CA587															
A	1	2	4	2	3	3	2	1	2	1	1	1	2	1	1
H_E	0.000	0.469	0.616	0.495	0.511	0.660	0.283	0.000	0.035	0.000	0.000	0.000	0.051	0.000	0.000
H_O	0.000	0.679	0.571	0.400	0.500	0.727	0.195	0.000	0.036	0.000	0.000	0.000	0.053	0.000	0.000
F_{IS}	–	−0.433	0.087	0.216	0.041	−0.079	0.322	–	0.000	–	–	–	−0.014	–	–
CA626															
A	3	8	8	6	6	7	6	3	2	2	3	5	7	7	2
H_E	0.296	0.598	0.414	0.778	0.737	0.821	0.746	0.577	0.069	0.089	0.407	0.470	0.778	0.576	0.334
H_O	0.225	0.643	0.371	0.750	0.846	0.864	0.683	0.619	0.071	0.094	0.400	0.462	0.895	0.632	0.346
F_{IS}	0.252	−0.058	0.118	0.061	−0.129	−0.028	0.097	−0.048	−0.019	−0.033	0.029	0.037	−0.136	−0.083	−0.018
CA645A															
A	6	5	5	5	2	5	3	1	3	3	3	2	5	1	2
H_E	0.768	0.628	0.607	0.509	0.488	0.745	0.356	0.000	0.531	0.647	0.597	0.204	0.557	0.000	0.074
H_O	0.650	0.643	0.629	0.550	0.385	0.500	0.293	0.000	0.500	0.750	0.511	0.231	0.526	0.000	0.075
F_{IS}	0.166	−0.006	−0.021	−0.056	0.231	0.349	0.190	–	0.077	−0.144	0.155	−0.111	0.069	–	1.000
Average															
A	3.5	6.4	6.8	5.4	5.6	4.8	5.8	2.9	2.8	2.3	3.3	3.5	5.9	3.3	1.6
P	0.05	0.18	0.20	0.05	0.08	0.09	0.07	0.00	0.00	0.00	0.00	0.04	0.26	0.08	0.00
A_R	3.3	5.8	5.9	5.4	5.4	4.7	5.1	2.9	2.6	2.2	2.8	3.4	5.0	2.8	1.6
H_E	0.401	0.571	0.562	0.559	0.621	0.636	0.496	0.272	0.235	0.251	0.368	0.357	0.482	0.295	0.089
H_O	0.384	0.612	0.539	0.556	0.596	0.636	0.460	0.292	0.259	0.270	0.342	0.380	0.490	0.326	0.087

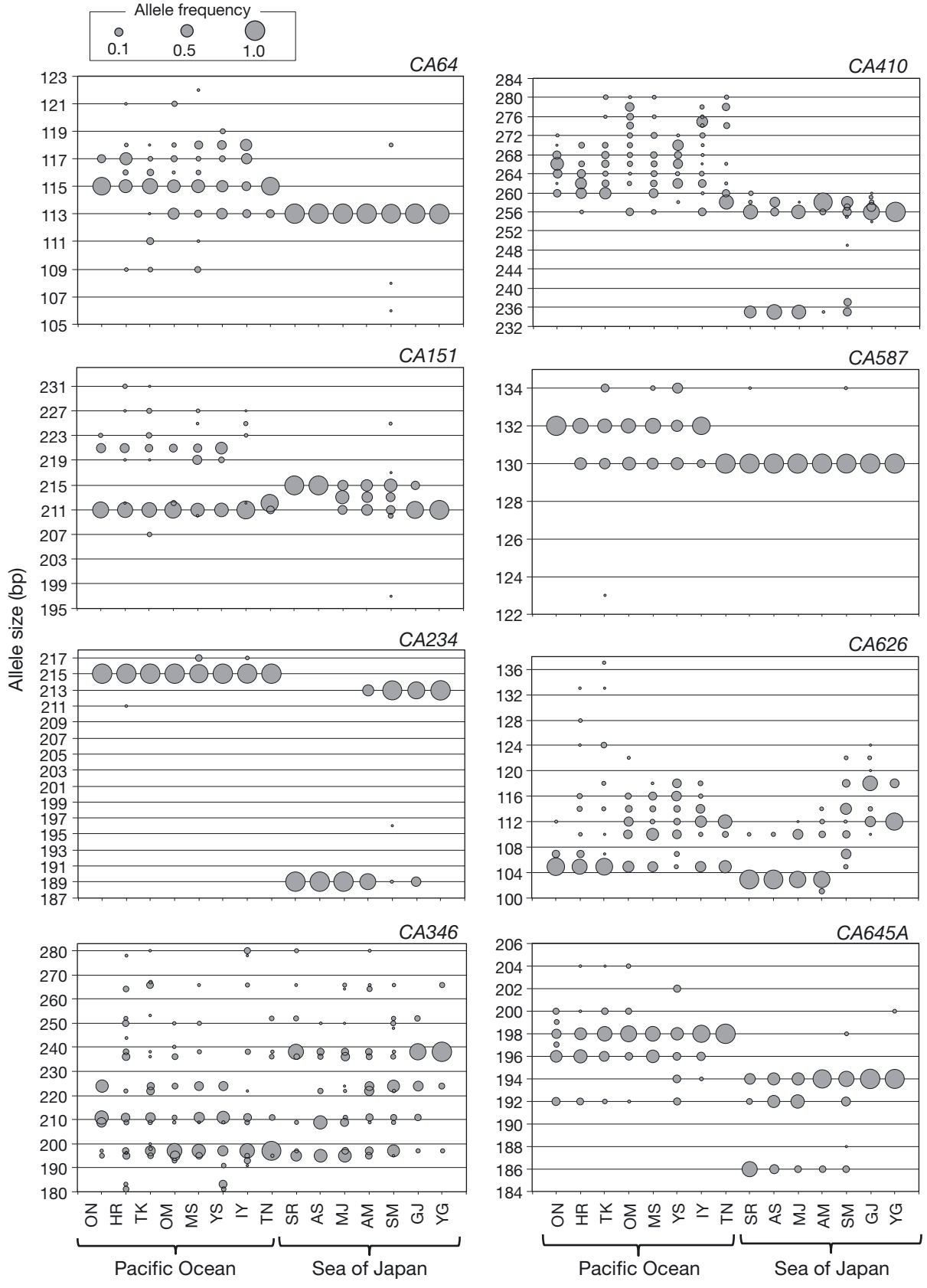

Fig. 3. *Chaenogobius annularis*. Allele frequencies and size distributions at 8 microsatellite loci in 15 populations. Locality codes as in Table 1

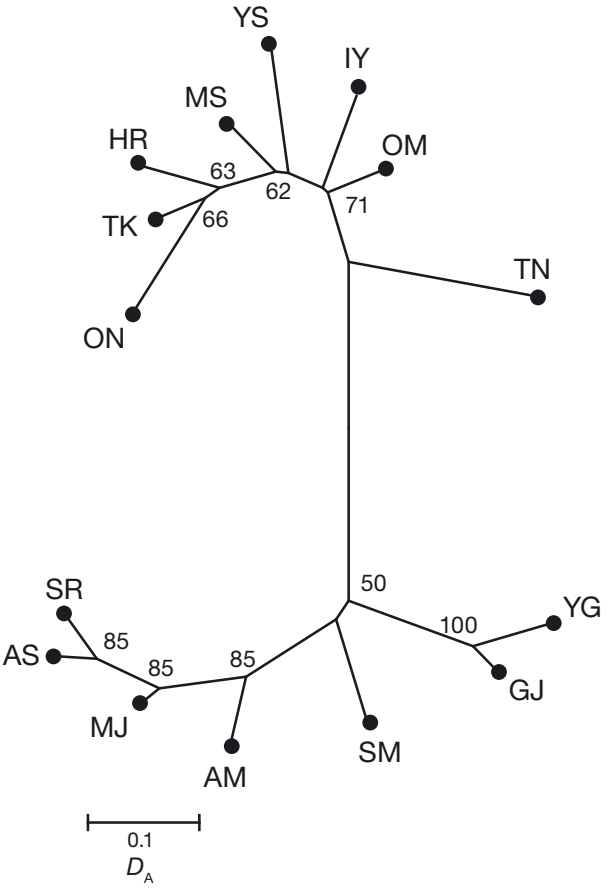

Fig. 4. *Chaenogobius annularis*. Unrooted neighbor-joining tree of 15 populations based on Nei's D_A calculated from 8 microsatellite DNA data. The values shown are percentages of 1000 bootstrap replicates supporting the respective nodes (only values exceeding 50 are shown). Locality codes as in Table 1

In the Bayesian clustering analysis, the ΔK at $K = 2$ was considerably higher than for other K values (Fig. 5); thus, 2 was considered the optimal number of genetic clusters. The membership proportions in this analysis revealed significant geographic patterns. The Cluster 1 membership coefficients (q values) were high ($q > 0.9$) for the genomes of each examined specimen in the Pacific Ocean group, while those of Cluster 2 were high ($q > 0.9$) for specimens in the Sea of Japan group. A total of 5 specimens in YS, YG, and SM populations showed signs of genetic admixture ($0.1 < q < 0.9$); however, we could not conclude that these specimens show actual genetic admixtures or homoplasy/genotyping mistakes because of the minimal admixture. F values of Clusters 1 and 2 were 0.110 and 0.369, respectively.

For the Sea of Japan group, we found the maximum value of ΔK at $K = 2$, with a secondary peak at $K = 3$ (Fig. 5). Assuming $K = 2$, we obtained Cluster 1 consisting of specimens from 3 northern populations (SR, AS, MJ) and Cluster 2 consisting of specimens from 3 southern populations (SM, YG, GJ), which corresponds to the partitioning by the SAMOVA. Genetic admixtures between the 2 clusters occurred in the AM population. Assuming $K = 3$, we obtained Cluster 1 consisting of specimens from 3 northern populations (SR, AS, MJ), Cluster 2 consisting of specimens from 2 southern populations (YG and GJ), and Cluster 3 consisting of specimens from the SM population. Genetic admixtures between Clusters of 1 and 3 occurred in the AM population. Because the SM population showed unique genetic composition, as described below, we concluded that $K = 3$ was the

Table 3. *Chaenogobius annularis*. Pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) between 15 populations based on microsatellite DNA. All F_{ST} values were significant ($p < 0.05$) after Bonferroni correlation. Asterisks in the upper matrix indicate significant ($^*p < 0.05$) values of $R_{ST} > F_{ST}$. Locality codes as in Table 1

	Pacific Ocean group								Sea of Japan group						
	ON	HR	TK	OM	MS	YS	IY	TN	SR	AS	MJ	AM	SM	GJ	YG
ON	–	0.060	0.033	0.257	0.086	0.325*	0.034	0.263	0.644	0.717	0.607	0.442	0.331	0.603	0.750
HR	0.115	–	−0.009	0.191	0.088	0.200	0.051	0.193	0.405	0.500	0.441*	0.210	0.125	0.232	0.270
TK	0.102	0.041	–	0.148	0.052	0.165	0.026	0.164	0.449	0.527	0.470	0.243	0.166	0.281	0.329
OM	0.209	0.090	0.079	–	0.035	0.035	0.025	0.043	0.628*	0.666*	0.586*	0.462	0.394	0.626	0.737
MS	0.165	0.065	0.061	0.041	–	0.044	−0.002	0.075	0.579	0.626*	0.550*	0.389	0.293	0.523	0.629
YS	0.220	0.105	0.111	0.073	0.056	–	0.049	0.051	0.636	0.665	0.580	0.481*	0.359	0.633*	0.759*
IY	0.235	0.127	0.138	0.061	0.093	0.140	–	0.059	0.480	0.512	0.473	0.282	0.221	0.354	0.402
TN	0.491	0.349	0.298	0.259	0.289	0.317	0.357	–	0.610	0.625	0.543	0.442	0.329	0.624	0.738
SR	0.666	0.563	0.564	0.559	0.535	0.524	0.588	0.697	–	0.228	0.139	0.123	0.375	0.406	0.514
AS	0.657	0.560	0.562	0.557	0.536	0.518	0.590	0.683	0.152	–	0.009	0.274	0.433	0.630	0.740
MJ	0.596	0.491	0.500	0.464	0.460	0.438	0.520	0.596	0.219	0.174	–	0.180	0.360	0.509	0.611
AM	0.594	0.482	0.485	0.458	0.445	0.413	0.509	0.579	0.363	0.315	0.250	–	0.233	0.293	0.436
SM	0.528	0.417	0.423	0.378	0.374	0.339	0.443	0.494	0.421	0.401	0.332	0.224	–	0.246	0.339
GJ	0.614	0.511	0.515	0.483	0.483	0.443	0.511	0.641	0.531	0.554	0.428	0.363	0.229	–	0.106
YG	0.699	0.603	0.599	0.591	0.578	0.559	0.583	0.773	0.720	0.733	0.576	0.572	0.392	0.186	–

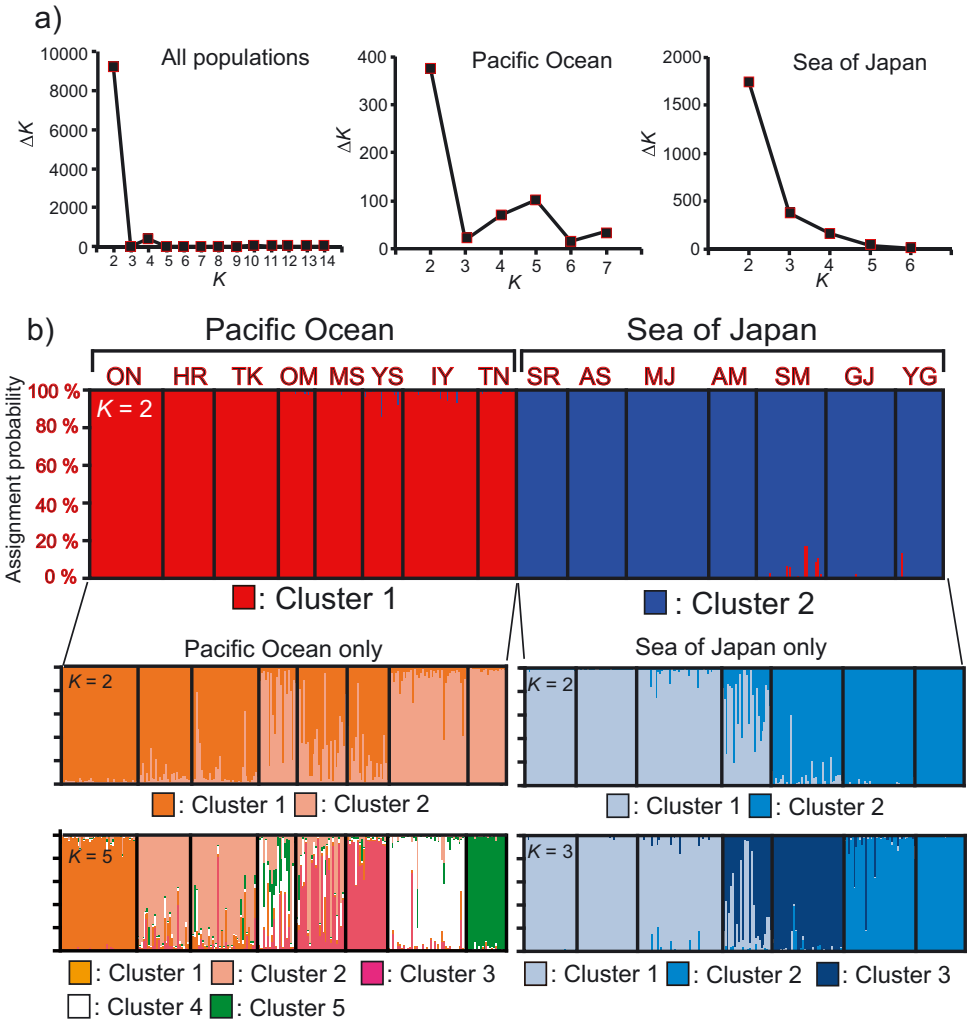

Fig. 5. *Chaenogobius annularis*. Individual assignment based on 8 microsatellite DNA loci using Bayesian clustering analysis: (a) estimation of ΔK following Evanno et al. (2005); and (b) assignment plots showing individual genotype membership to K clusters (each cluster is represented by a different color, and each vertical bar represents a specimen). Bayesian clustering analysis was initially run with all populations ($K = 2$ shown), followed by subsequent individual runs for the Pacific Ocean group ($K = 2$ and 5 shown) and the Sea of Japan group ($K = 2$ and 3 shown). Locality codes as in Table 1

most likely K. The F values of Clusters 1, 2, and 3 were 0.407, 0.434, and 0.045, respectively. The NJ tree for the 3 clusters showed ancestral features of Cluster 3 (Fig. S2 in the Supplement).

For the Pacific Ocean group, we found a maximum value of ΔK at $K = 2$, with a secondary peak at $K = 5$ (Fig. 5). Assuming $K = 2$, we obtained Cluster 1, consisting of specimens from 3 northern populations (ON, HR, TK) and Cluster 2, consisting of specimens from the OM population and 2 southern populations (IY and TN). Genetic admixtures between the 2 clusters occurred in populations MS and YS. The F values of Clusters 1 and 2 were 0.063 and 0.147, respectively. Assuming $K = 5$, we obtained Cluster 1, consisting of specimens from ON; Cluster 2, consisting of specimens from HR and TK; Cluster 3, consisting of specimens from YS; Cluster 4, consisting of specimens from IY; and Cluster 5, consisting of specimens from TN. Genetic admixtures among Clusters 2, 3, and 5 occurred in OM and MS populations. The F values of Clusters 1, 2, 3, 4, and 5 were 0.043, 0.514, 0.173, 0.398, and 0.113, respectively.

Bottleneck tests for the Sea of Japan group

Heterozygosity excess tests detected a bottleneck signal ($p < 0.05$) only in the MJ population under the infinite allele mutation model, whereas mode-shift tests indicated no signals. M-ratio tests suggested that

bottlenecks occurred in the northern and southern portions in the Sea of Japan, in populations SR (0.66), AM (0.60), and SM (0.54; Fig. S3 in the Supplement).

DISCUSSION

Genetic admixture between the Pacific Ocean and the Sea of Japan groups

Congruence between the genetic structures revealed by mtDNA and microsatellite DNA markers strongly showed 2 distinct allopatric groups, the Pacific Ocean and Sea of Japan groups. Moreover, these groups did not share the 2 mtDNA lineages and microsatellite DNA alleles in the *CA234* locus, and the Bayesian clustering analysis indicated no or minimal genetic admixtures between them. Allozyme analysis also showed distinct genetic divergence at the *Mdh-1* locus (Hirase et al. 2012a). We identified all samples as *Chaenogobius annularis* based on clear morphological characteristics, and phylogenetic trees with sympatric sister species revealed the monophyly of mtDNA from all samples; misidentification of species is therefore not likely the case in this study. Taken together, these results suggest long-term vicariance since the early Pleistocene divergence (Hirase et al. 2012b).

Generally, stepwise mutations should contribute to genetic differentiation in microsatellite DNA loci between populations that diverged for a sufficiently long time (Hardy et al. 2003). However, the comparisons among pairwise F_{ST}, R_{ST}, and pR_{ST} showed little contribution of stepwise mutation to the genetic differentiation between the Pacific Ocean and Sea of Japan populations. On the other hand, a significantly higher R_{ST} value was obtained when all populations within each group were pooled. This suggests that genetic drift, which is described in more detail later, reduced genetic variability of the Sea of Japan group and neutralized the effect of stepwise mutations between this group and the Pacific Ocean group. On the basis of previous paleoenvironmental studies (e.g. Tada 1994, Gorbarenko & Southon 2000), the Sea of Japan was expected to be a severe environment for temperate coastal species in the Pleistocene glacial periods, which promoted lineage-specific adaptive evolution (Kokita et al. 2013). Therefore, the combination of selection and genetic drift might have promoted population isolation and resulted in the restricted gene flow found between the 2 *Chaenogobius annularis* groups, as theorized by Wills (1977). Moreover, because the 2 allopatric groups of *C. annularis* diverged concurrently with those of the Japanese threespine stickleback, which has 2 reproductively isolated groups (1.5–2 MYA; Kitano et al. 2007), *C. annularis* would be a suitable target for finding evidence of allopatric speciation associated with past geographic events. A detailed survey of boundary zones would be required to understand the evolutionary consequence of Pleistocene isolation events in the Sea of Japan.

To date, mtDNA phylogeographic studies have demonstrated the existence of Pacific Ocean and Sea of Japan groups in Japanese coastal marine species, including fishes, and gastropods (Kojima et al. 1997, 2004, Akihito et al. 2008, Kokita & Nohara 2011, Hirase et al. 2012b). Avise (2000) termed such spatial agreement in phylogenetic partitions across taxa 'aspect III,' showing that these geographical groups are evolutionarily significant units (ESUs, Moritz 1994). In addition, Avise (2000) pointed out that the proper identification of the ESUs within any species should depend on a detectable amount of differentiation, no admixtures, and genealogical concordance between independent genetic markers. However, such genetic divergence was shown only in *Leucopsarion petersii* (Kokita & Nohara 2011). In the present study, we confirmed the congruence between mtDNA and microsatellite DNA markers in the 2 groups of *Chaenogobius annularis*. Consequently, our results, together with the previous discussion, strongly support the 2 allopatric groups being ESUs, and highlight the need for establishing the coastal areas of the Pacific Ocean and Sea of Japan as separate biosphere reserves to protect genetic diversity of coastal marine species.

Expansion history of the Sea of Japan group

Because both DNA analyses showed lower genetic diversity and heterozygosity excess, and mode-shift tests could not detect recent genetic drift in the Sea of Japan group, this low genetic variability may be attributable to the rapid expansion after severe bottlenecks in the glacial periods as described by Hirase et al. (2012b). The MJ network suggested that haplotype compositions differed between Japanese and Korean populations, and the SAMOVA and the Bayesian clustering analyses based on microsatellite DNA also showed the existence of Japanese and Korean subgroups. Thus, the Tsushima Strait, which opened in each Pleistocene interglacial period (Kitamura & Kimoto 2006), may have restricted historical gene flow between the Japanese and Korean coasts.

As an exception, the SAMOVA and the Bayesian clustering analyses based on microsatellite DNA posi-

tioned the SM population into the Korean subgroup. On the other hand, this population had both mtDNA haplotypes characteristic of the 2 subgroups (HB52, HB75 and haplotypes derived from the 2 major haplotypes), and had higher microsatellite DNA allelic richness and a larger number of private alleles than the other Sea of Japan populations. This 'southern richness' possibly involves 2 scenarios: admixture between the Japanese and Korean subgroups during each glacial period when the Tsushima Strait was narrow or closed, and the formation of glacial refugia. One key feature that differentiates between these 2 scenarios is that the refugia tend to harbor high numbers of private alleles not found elsewhere and which presumably have not participated in the recolonization process (Provan & Bennett 2008). Therefore, the larger number of private alleles in the SM population strongly supports the notion of glacial refugia around Shimonoseki, and that the Sea of Japan group may have expanded from this area via genetic drift, i.e. the founder effect, as described in the Introduction. The founder effect during rapid expansion is additionally supported by the high *F*-value of clusters corresponding to the putative recolonized populations. The expansion routes from the southern area were also supported by the NJ tree, by the relatedness among the 3 clusters inferred by the Bayesian clustering analysis, and by the steep geographical cline of genetic variability, in which the SM population was at the center.

Conversely, the *M*-ratio test detected a bottleneck in the SM population, but not in the putative recolonized populations. The *M*-ratio test can distinguish between populations that have been reduced in size and those that have been small for a long time (Garza & Williamson 2001). Thus, the low *M*-ratio of the SM population implies that this population has a long history and has undergone a size reduction. On the other hand, the relatively high *M*-ratios of the other populations may suggest that these populations were formed by limited numbers of founders that have low microsatellite DNA variation.

Genetic uniqueness of glacial refugia areas contributes to total genetic diversity within species, and there is a consensus that refugia areas should be maintained for long-term conservation purposes (Petit et al. 2003). Thus, further investigations focusing on other suitable species are required to confirm the existence of glacial refugia in the Sea of Japan. Although the Sea of Japan group of *Chaenogobius annularis* showed low microsatellite DNA variability, that of *Leucopsarion petersii*, which had probably undergone rapid expansion, revealed similar microsatellite DNA variability with its Pacific Ocean group (Kokita et al. 2013). Kokita et al. (2013) considered that this species expanded before the last glacial period, and that sufficient time had passed for the microsatellite alleles to lose the molecular footprint of past demographic events and thus restore variability. Our results may imply that the Sea of Japan groups of a subset of coastal marine species have only limited genetic variability, highlighting the necessity for conserving their genetic background.

Genetic structure of the Pacific Ocean group

Microsatellite DNA analysis could not detect the 3 distinct (northern, central, and southern) subgroups confirmed by mtDNA analysis in this and previous studies (Hirase et al. 2012b). This conflict might be due to the mtDNA effective population size (*N*e) being 4 times smaller; therefore, the mtDNA marker would be more susceptible to the effects of genetic drift and genetic differentiations than would the nuclear DNA markers (Birky et al. 1989). Alternatively, it is possible that sex-biased dispersal caused this discrepancy (Pardini et al. 2001).

Microsatellite DNA analysis showed that the southern TN and the northern ON populations had lower genetic variability than other populations; however, mtDNA revealed low nucleotide diversity in 3 northern populations (ON, HR, TK) and 2 southern populations (IY and TN). This discrepancy suggests that recent expansions of northern and southern subgroups (Hirase et al. 2012b) are not likely to affect the lower microsatellite DNA variability in the 2 populations. Alternatively, the lower *N*e at the margins of the range than at the center, together with the lower rate of gene flow, may be associated with the low microsatellite DNA variability (Eckert et al. 2008).

In conclusion, mtDNA and microsatellite DNA markers indicate the distinct genetic divergence between the Pacific Ocean and Sea of Japan groups with no or minimal gene flow. Since the early Pleistocene divergence, the Sea of Japan group was estimated to have expanded rapidly from the southern area and to have undergone severe genetic drift during this process. These results lead to the hypothesis that the combination of long-term vicariance and genetic drift promoted adaptive divergence and reproductive isolation between the 2 geographic groups of *Chaenogobius annularis*. This study constitutes a first step toward investigating the allopatric groups of *C. annularis* as a model for understanding the evolutionary consequences of Pleistocene isolation events of the Sea of Japan.

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