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Full Length Research Paper

Genetic diversity in the mtDNA control region and population structure in the *Sardinella zunasi* Bleeker

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We investigated the genetic structure and phylogeographical patterns of *Sardinella zunasi* in Northwestern Pacific. The mitochondrial DNA control region was sequenced for 77 individuals of *S. zunasi* from four localities over most of the species range. A total of 215 polymorphic sites (72 parsimony informative) and 69 haplotypes were found in the 77 individuals. Three distinct lineages were detected, which might have diverged in the marginal seas during Pleistocene low sea levels. The demographic history of *S. zunasi* was examined using neutrality tests and mismatch distribution analysis. The results suggested a late Pleistocene population expansion for *S. zunasi*. Contrary to homogenization expectation, there were strong differences in the geographical distribution of the three lineages. Analyses of molecular variance and the population statistic Φ_{ST} also revealed significant genetic structure among populations of *S. zunasi*. These results indicate that gene flow in *S. zunasi* is far more spatially restricted than predicted by the potential dispersal capabilities of this species.

Key words: *Sardinella zunasi*, control region, mitochondrial DNA, genetic structure, Pleistocene.

INTRODUCTION

Assessments of intraspecific genetic diversity and population genetic structure can provide important biological and evolutionary data. From a biological perspective, the assessment can reveal whether the species is panmictic throughout its range or not, and can help to identify the potential sites of historic refugia. Population genetic analyses can provide indirect measures of connectivity among populations (Bossart and Prowell, 1998; Waples, 1998; Hellberg et al., 2002). An accurate definition of population structure is important for the management of commercial marine fishes.

Sardinella zunasi is a pelagic and subtropical fish species belonging to the family Clupeidae, which is found near shore, including semi-enclosed sea areas, on sandy mud bottom, and often forms schools in coastal waters. This species is widely distributed in Western Pacific Ocean: southern coasts of Japan to about Taiwan and forms an important fisheries resource in China and Japan (Whitehead, 1985). Mature adults spawn once a year and fecundity is high, with individual females producing several thousands of eggs. Spawning occurs offshore in marine waters and pelagic eggs hatch in 36 h at 20℃. The period of the pelagic larval stage is about 4 weeks and the early life-history characteristics of *S. zunasi* indicate that potential larval dispersal is high. A number of ecological and morphological studies have been done on the species; however, there is no research information about the population genetic structure of this species.

The population genetic structures of marine species are often influenced by the ocean currents and Pleistocene ice ages. Genetic homogeneity across large geographic scales is often related to the connections generated by sea surface currents. In the Pacific Ocean, the structure seems influenced by both the Tsugaru Current and Okhotsk Cold Current flowing from northeast to southwest along the Kuril Islands until they meet the Tsugaru Current or Kuroshio Current (Watanabe, 1964; Kawai, 1972). When the southwest summer monsoon winds prevail, the southern part of China Coastal Current is reversed and

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flows northward into the East China Sea. The West Korea Coastal Current flows southward and enters the Sea of Japan through the Tsushima Strait. The Tsushima Current, a branch of the Kuroshio Current, flows into the Sea of Japan through the Tsushima Strait (Li et al., 2000). If pelagic larvae of marine organisms travel on these currents, the connectivity among populations would be expectedly high.

Some literature has suggested that historic barriers, such as sea level changes during the Pleistocene, may have played important roles in creating isolated populations by cutting off local sea basins from the Northwestern Pacific region (Liu et al., 2006). A series of marginal seas separate Asia from the Pacific, straddling the world's largest subduction zone in the Western Pacific (Tamaki and Honza, 1991). We focus on the marginal seas in the Northwestern Pacific: East China Sea and Sea of Japan. The East China Sea is bounded by China, Korea and southern Japan. It is separated from the Pacific Ocean by the NE–SW-extending Ryukyu Islands Arc. The Sea of Japan is a semi-enclosed marginal sea, which is distinguished from the other marginal seas of the Western Pacific by its extremely shallow sills (Kitamura et al., 2001). It was almost isolated from the Pacific Ocean during glaciation events (Kitamura et al., 2001). During the Pleistocene glacial cycles, lowered sea levels would have resulted in divergent populations in these marginal ocean basins (Wang, 1999; Voris, 2000). It is important to understand the extent to which present conditions or historical events have determined the current genetic structure within a species, because genetic data are interpreted as indirect estimates of dispersal in marine organisms.

In this study, we examined the genetic diversity and population genetic structure of *S. zunasi* using sequence analysis of a portion of the mitochondrial DNA (mtDNA) control region, a genetic marker that has been shown to be particularly sensitive in detecting population genetic structures of marine fish species. We discuss the results with regard to the biological and geological factors that influence population structure. Phylogeographical structure provides an indication of the dispersal capabilities of this species. Such knowledge on the genetic consequences of the recent history of *S. zunasi* may be critical for the conservation and sustainable management of its genetic resources.

MATERIALS AND METHODS

Sampling and sequencing

Seventy seven individuals were collected from four geographic localities throughout the distribution area of *S. zunasi* during 2005–2006 (Table 1 and Figure 1). Muscle samples were preserved in 95% ethanol before DNA extraction. Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol–chloroform method. The first hypervariable segment of the mtDNA control region was amplified with fish primers L15623 and sd–2. The primer sequences are L15623, 5′–CCACCACTAGCTCCCAAAGC–3′ (forward) and sd–2, 5′–TATGCTTTGTTTAAGCTACA–3′ (reverse), which amplify a 686 bp fragment (Liu et al., 2006). A polymerase chain reaction (PCR) amplication was carried out in 50 µL volumes containing 1.25 U *Taq* DNA polymerase (Takara Co.), 10 – 100 ng template DNA, 200 nm forward and reverse primers, 200 µm of each dNTP, 10 mm Tris pH 8.3, 50 mm KCl, 1.5 mm $MgCl_2$. The PCR amplification was carried out in a Biometra thermal cycler under the following conditions: 3 min initial denaturation at 95℃, and 40 cycles of 45 s at 94℃ for denaturation, 45 s at 52℃ for annealing, and 45 s at 72℃ for extension, and a final extension at 72℃for 5 min. All sets of PCR included a negative control reaction tube in which all reagents were included except template DNA. PCR product was purified with a Gel Extraction Mini Kit (Watson BioTechnologies Inc.). The purified product was used as the template DNA for cycle sequencing reactions performed using BigDye Terminator Cycle Sequencing Kit (version 2.0, Applied Biosystems), and sequencing was conducted on an ABI PRISM 3700 (Applied Biosystems) automatic sequencer with both forward and reverse primers. The primers used for sequencing were the same as those for PCR amplification.

Data analysis

Sequences were edited and aligned using Dnastar software (DNASTAR, Inc.). Molecular diversity indices such as number of haplotypes, polymorphic sites, transitions, transversions and indels, were obtained using the program ARLEQUIN (version 2000; Schneider et al., 2000). Haplotype diversity (*h*), nucleotide diversity (π) and their corresponding variances were calculated after Nei (1987) as implemented in ARLEQUIN. The gamma distribution shape parameter for the rate heterogeneity among sites and nucleotide sequence evolution was calculated using the program Modeltest version 3.06 (Posada and Crandall, 1998). Genetic relationships among haplotypes were reconstructed using the neighbour-joining (NJ) method (Saitou and Nei, 1987) implemented in PAUP* (Swofford, 2002). We used bootstrap analysis with 1000 replicates to evaluate support for phylogenetic relationships (Felsenstein, 1985). In addition, genealogical relationships were examined by constructing haplotype networks using reduced median-network approach (Bandelt et al., 1995).

Population structure was measured with an analysis of molecular variance (AMOVA) incorporating sequence divergence between haplotypes (Excoffier et al., 1992). To test our hypotheses, we conducted AMOVA analysis with three groups representing three marginal seas. Samples from Qingdao, Zhoushan, and Aichi and Kagawa were grouped as Yellow Sea, East China Sea, and Sea of Japan, respectively. The significance of the covariance components associated with the different possible levels of genetic structure was tested using 5000 permutations. In addition, pairwise genetic divergences between populations were estimated using the fixation index Φ_{ST} (Excoffier et al., 1992), which includes information on mitochondrial haplotype frequency (Weir and Cockerham, 1984) and genetic distances. The significance of the Φ_{ST} was tested by 10000 permutations for each pairwise comparison. All the genetic structure calculations were performed in ARLEQUIN.

The historical demographic pattern of *S. zunasi* was investigated by neutrality tests and mismatch distribution analysis. The Tajima's (1989a) and Fu's (1997) tests of selective neutrality were used to examine the haplotyes for the effect of selection. The pairwise frequency distribution of individuals was used to determine its population expansion. Both mismatch analysis and neutrality tests were performed in ARLEQUIN.

The molecular clock for the control region seems to vary among major taxonomic groups of marine fishes. Evolution of the control region seems to be much faster than protein coding mtDNA regions in some bony fishes. However, the control region seems to mutate

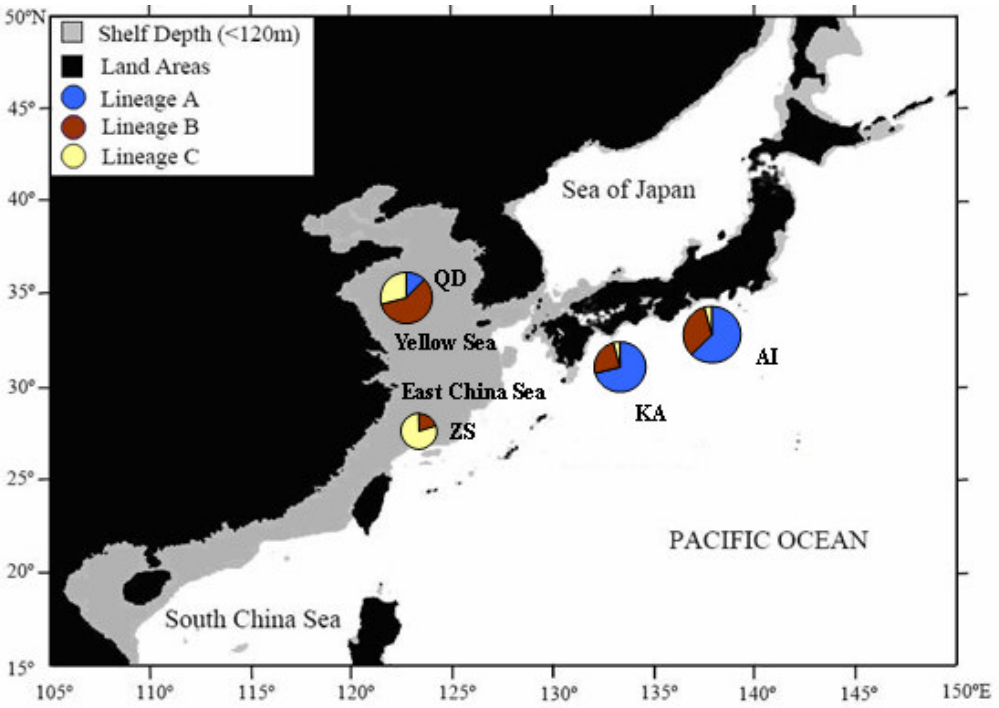

Figure 1. Haplotype frequencies for *S. zunasi* from Zhoushan (ZS) to Aichi (A). The area of the circle is proportional to sample size.

at about the same rate as protein-coding mtDNA regions in some other bony fishes (reviewed by Bowen et al., 2006). In the present study, sequence divergence rate of 3–10% Myr was applied for the control region analysis of *S. zunasi*.

RESULTS

Intraspecific sequence variation

In *S. zunasi*, a 686-bp segment at the 5' end of control region revealed 215 polymorphic sites (72 parsimony informative) with 155 transitions, 34 transversions and five indels. A total of 69 haplo- types were found in the 77 individuals, one of which was shared among the populations, and three were found in more than one individual, but only in one population. All analyses of this work were based on the 626 bp segment of the mitochondrial DNA control region.

Haplotype frequencies

The NJ tree (Figure 2) constructed using the complete data set of 69 haplotypes identified three distinct lineages (labelled A, B and C). Net average genetic distances (Tamura and Nei with gamma correction) between lineages (± SE) were A/B: 2.9%; A/C: 2.5%; B/C: 1.7%. Applying the control region sequence divergence rate (3–10% Myr), the divergence of lineage B from lineage C may have occurred about 170–567 kyr before present (bp). The genetic distance between lineage A and lineage B corresponds to 290–967 kyr bp. The divergence of lineage A from lineage C may date back to 250–833 kyr bp. The average pairwise divergence between individuals within each of the lineages (± SE) were 0.41% (± 0.08), 1.98% (± 0.35) and 2.36% (± 0.40) for lineages A, B and C, respectively.

There were strong geographical differences in haplo- type frequencies of the three lineages (Table 2). A larger number of haplotypes and private haplotypes were found in all the populations (Table 3). Network of lineage A was star-like with a dominant haplotype (80%) shared by the two Japan Sea locations (Figure 3). And the frequency was very high with Kagawa (70.8%) and Aichi (62.5%). Lineage A was also found in the Yellow Sea population (12.5%), which indicate A from the Japan ocean. Lineage B was sympatric with lineage A in the East China Sea populations. Lineage B dominates the Yellow Sea. And lineage B was also found in the Aichi (27.6%) and Kagawa (20.7%). Lineage C dominates the Yellow Sea (53.8%) and the East China Sea populations (30.8%).

Genetic diversity and genetic structuring

According to the AMOVA analysis, the haplotype diversity was very high in the three populations with the Japan Sea population having the least (0.97) (Table 2). The nucleo-

Table 1. Sampling information, number and proportion of individuals and number of haplotypes and private haplotypes for the phylogenetic lineages A, B and C in different populations.

ID	Population	Sample size	Date of collection	Lineage A		Lineage B		Lineage C	
				Number of individuals (proportion, %)	Number of haplotypes (private)	Number of individuals (proportion, %)	Number of haplotypes (private)	Number of individuals (proportion, %)	Number of haplotypes (private)
AI	Aichi	23	May 2005	15(62.5)	13(12)	8(33.3)	8(8)	1(4.2)	1(1)
KA	Kagawa	24	June 2005	17(70.8)	14(13)	6(25)	6(6)	1(4.2)	1(1)
QD	Qingdao	24	April 2006	3(12.5)	3(3)	14(58.3)	13(13)	7(29.2)	7(7)
ZS	Zhoushan	5	April 2006	0	0	1(20)	1(1)	4(80)	3(3)
				35(45.5)	30(28)	29(37.7)	28(28)	13(16.8)	12(12)

Table 2. Summary of molecular diversity for *S. zunasi*.

Groups	n	No. of haplotype	*k*	S	*h*	*π*	Tajima's *D*		Fu's F_S		Mismatch distribution		
							D	*P*	F_S	*P*	τ(95%CI)	θ_0	θ_1
All	77	69	31.4±13.86	215	0.994±0.005	0.0502±0.0245	—	—	—	—	—	—	—
Lineage A	35	30	4.09±2.09	54	0.97±0.02	0.0066±0.0037	-2.53	0.000	-5.79	0.00	2.62 (1.83,3.4)	0.00	99999
Lineage B	29	28	13.12±6.08	100	0.998±0.01	0.0211±0.0109	-2.04	0.006	-6.08	0.00	3.79 (1.85, 16.26)	10.37	99999
Lineage C	13	12	17.47±8.31	42	0.987±0.04	0.0281± 0.0150	-1.86	0.017	-1.82	0.15	16.22 (6.97, 21.27)	0.00	19.9

Number of individuals (n), number of haplotype, average pairwise differences among individuals (*k*), number of segregating sites (*S*), haplotype diversity (*h* ± standard deviation), nucleotide diversity (*π* ± standard deviation) for each grouping of samples. Tajima's *D* and Fu's F_S, corresponding *P*–value, and mismatch distribution parameter estimates for each lineage were also indicated.

tide diversity was highly different among groups, while the Japan Sea was least (0.0066) and the East China Sea was largest (0.0281). The AMOVA analysis showed that 35.12% of the genetic diversity was found among the three marginal seas ($P < 0.01$). Genetic subdivision was highly significant among groups ($\Phi_{CT} = 0.351$; $P < 0.01$) and among populations within groups ($\Phi_{SC} = -0.0277$; $P < 0.01$) (Table 4).

Genetic relationships

As expected from the star-like networks, the mismatch distributions for lineage A were unimodal (Figure 4) closely fitted to the expected distributions under the sudden expansion model. For lineage B and C, the mismatch distribution was clearly bimodal, one mode corresponded to the number of differences among sublineages, and the other to differences among individuals within sublineages. The Fu's *F*s tests and Tajima's *D* tests of lineage A and B were negative and highly significant ($P = 0.00$), which indicated population expansion. For lineage C, the *D* statistic was negative (–1.86) and highly significant ($P = 0.017$). However, the F_S test for lineage C was negative (–1.82) and insignificant ($P = 0.148$). The tau value (τ), which reflects the location of the

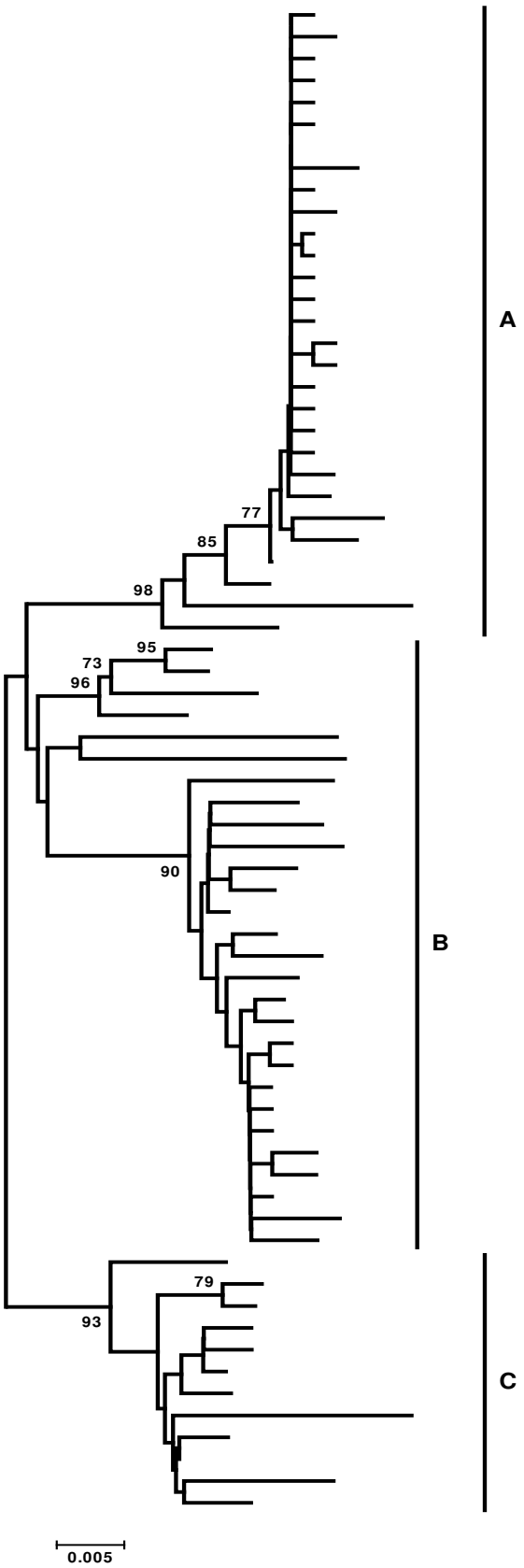

Figure 2. Unrooted neighbour-joining tree constructed model with invariable sites for 69 haplotypes of *S. zunasi*.

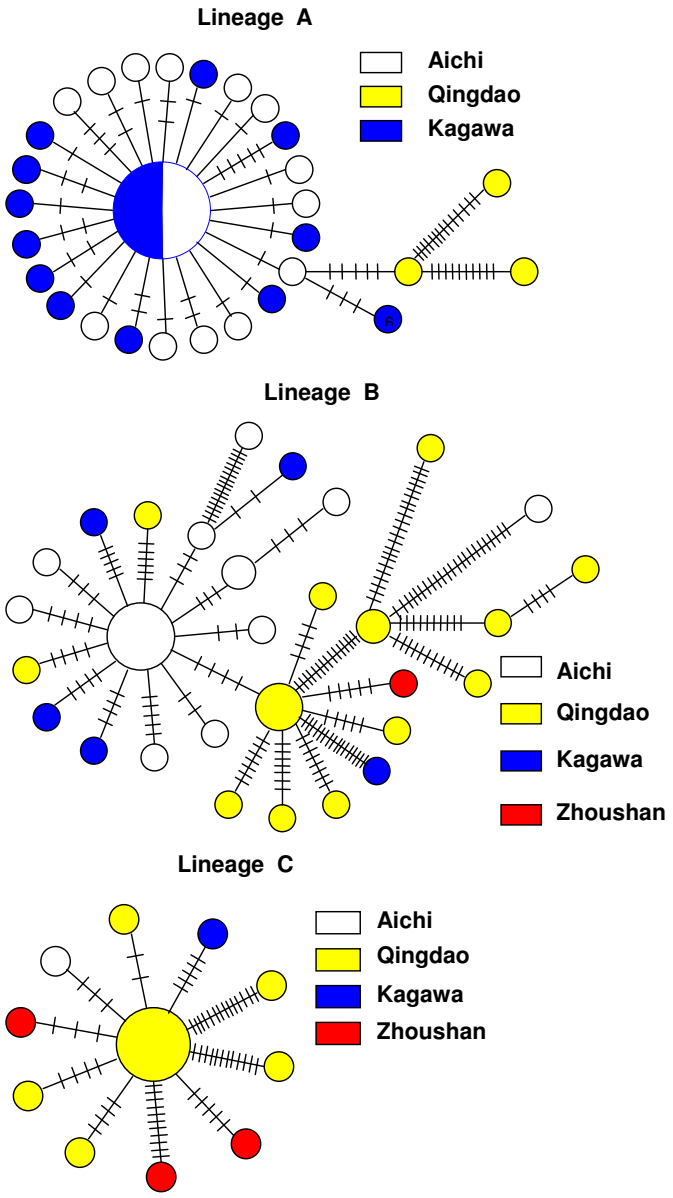

Figure 3. Reduced median-networks showing genetic relationship among control region haplotypes in lineage A, B and C. The sizes of circles are proportional to haplotype frequency. Haplotypes are marked by names that correspond to Table 3. Perpendicular tick marks on the lines joining haplotypes represent the number of nucleotide substitutions.

mismatch distribution crest, provides a rough estimate of the time when rapid population expansion started. The observed values of the age expansion parameter (τ) were 2.62 and 3.79 of mutational time for lineage A and lineage B, respectively. The tau value of lineage C (16.22) was much larger than those of lineage A and lineage B. The estimate of time of expansion for lineage A, based on the rates mentioned above for control region, was 42–138 kyr ago. For lineage B, this estimate was 61–202 kyr ago. In contrast, the estimated for lineage C was much higher,

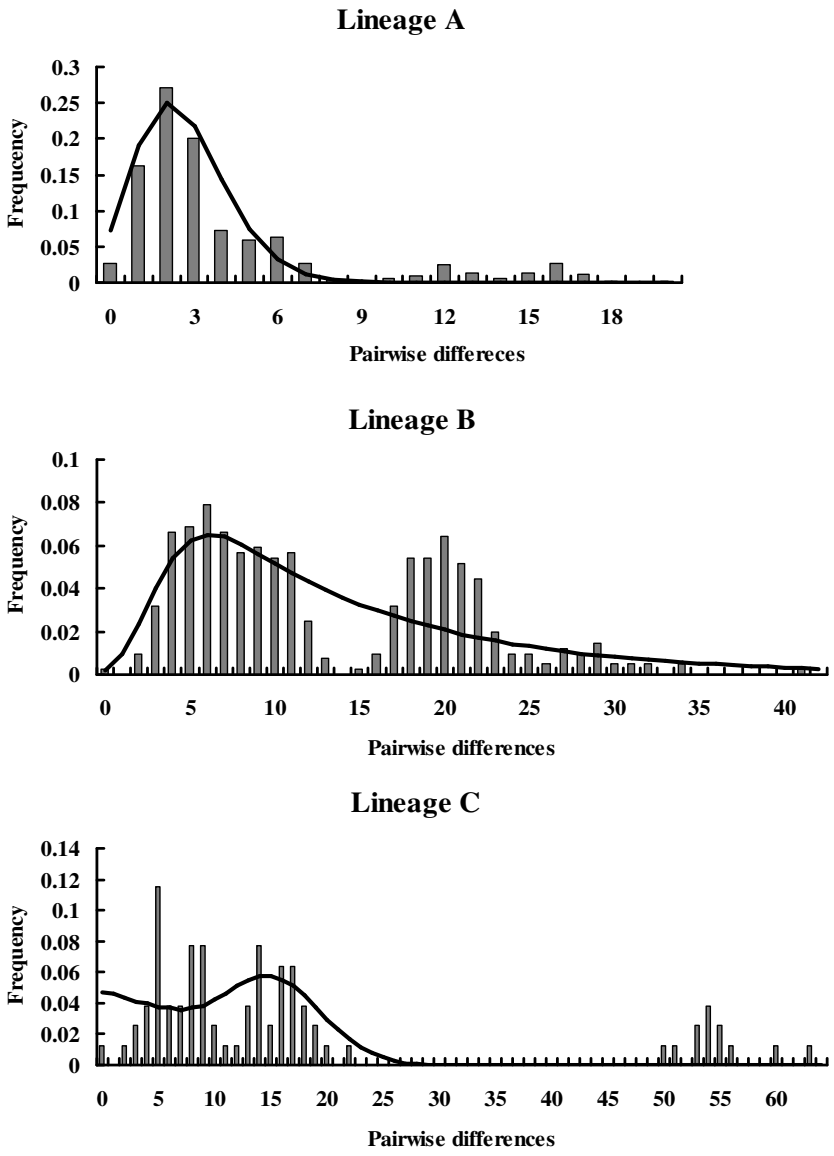

Figure 4. The observed pairwise differences (bars) and the expected mismatch distributions under the sudden expansion model (solid line) for the control region haplotypes in lineage A, B and C.

about 259–864 kyr ago. Estimated effective female population size after expansion (θ_1) was 9643 times higher than before expansion (θ_0) for lineage B. This estimate (θ_1/θ_0) was infinite for lineage A and C (Table 2).

DISCUSSION

Three distinct lineages were found in *S. zunasi*, likely reflecting isolation of the marginal seas of the Northwestern Pacific during Pleistocene low sea-level stands. The genetic differentiation may be correlated to geographical isolation and sea environment such as water temperature or currents during glacial periods in Pleistocene. Analysis of microsatellite DNA at 6 loci revealed shallow but significant population differentiation between two geographical populations of Japan- ese anchovy near Taiwan island (Yu et al., 2002). A similar result was found in European eel, *Anguilla anguilla*, for which microsatellite DNA analyses revealed shallow global genetic differentiation (Wirth and Bernatchez, 2001).

The frequency distribution of lineage A indicated a possible origin in the Sea of Japan. Lineage C dominated the East China Sea locations, which may indicate that this lineage was isolated and diverged in the East China Sea. Lineage B dominates the Yellow Sea locations. The provisional molecular clock yields an estimate of middle to late Pleistocene divergence among the three lineages.

Table 3. Distribution of haplotypes among localities.

Lineage A population						Lineage B population						Lineage C population					
Hap.*	AI	KA	QD	ZS	Tot. n*	Hap.*	AI	KA	QD	ZS	Tot.n*	Hap.*	AI	KA	QD	ZS	Tot. n*
A01	3	3			6	B01	1				1	C01			1		1
A02	1				1	B02	1				1	C02			1		1
A03	1				1	B03		1			1	C03			1		1
A04	1				1	B04	1				1	C04				1	1
A05	1				1	B05	1				1	C05			1		1
A06	1				1	B06	1				1	C06			1		1
A07	1				1	B07	1				1	C07				2	2
A08	1				1	B08			1		1	C08		1			1
A09	1				1	B09		1			1	C09				1	1
A10	1				1	B10		1			1	C10			1		1
A11		2			2	B11			1		1	C11			1		1
A12		1			1	B12		1			1						
A13		1			1	B13			2		2						
A14		1			1	B14	1				1						
A15		1			1	B15		1			1						
A16		1			1	B16	1				1						
A17	1				1	B17			1		1						
A18	1				1	B18			1		1						
A19	1				1	B19				1	1						
A20		1			1	B20			1		1						
A21		1			1	B21			1		1						
A22		1			1	B22			1		1						
A23		1			1	B23		1			1						
A24		1			1	B24			1		1						
A25		1			1	B25			1		1						
A26			1		1	B26			1		1						
A27		1			1	B27			1		1						
A28			1		1	B28			1		1						
A29			1		1	B29	1				1						

AI = Aichi, KA = Kagawa, QD = Qingdao, and ZS = Zhoushan.

Table 4. Pairwise Φ_{ST} (below diagonal) and associated *P* values (above diagonal) among *S. zunasi* populations. The analysis incorporates fish from all lineages.

Population	Aichi	Kagawa	Qingdao	Zhoushan
Aichi	0.9891 ± 0.0173	0.0126	0.0000	0.0000
Kagawa	-0.0195	0.9855 ± 0.0179	0.0000	0.0000
Qingdao	0.2019	0.2033	0.9964 ± 0.0133	0.0046
Zhoushan	0.6791	0.6438	0.2267	0.9 ± 0.161

Geological events during this interval evidently created barriers among populations in the Sea of Japan and the East China Sea. The Sea of Japan was almost isolated from the Pacific Ocean and the East China Sea during Pleistocene glaciation events due to the shallow sills (< 135 m) (Wang, 1999; Kitamura et al., 2001). A large land bridge extending from eastern China to Taiwan, the Ryukyus and probably to the main islands of Japan formed in the late Pleistocene (Kimura, 1996). Populations of *S. zunasi* could have become isolated in the Sea of Japan and the East China Sea with two refuges, resulting in the three lineages observed in the mitochondrial genome. It is, therefore, reasonable to assume that clade B and C originated in the same refugia, in which *S. zunasi* was possibly restricted during glacial periods.

Both the neutrality tests and the mismatch dis- tribution analysis indicated population expansion in *S. zunasi*. Estimate of population expansion time for clade C (259–864 kyr ago) in *S. zunasi* was much older than clade A and clade B. Similarly, the estimate for clade B (61–202 kyr ago) was much older than clade A (42–138 kyr ago). During the same period of time, series of glacial events took place. Pleistocene climatic oscillations produced changes in temperatures, current patterns, upwelling intensity, and the displacement, or eradication of coastal habitats (Bond et al., 1997; Kennett and Ingram, 1995; Kotilainen and Shackleton, 1995; Lambeck et al., 2002; Petit et al., 1999). Climatic oscillations induced events had before caused population fluctuations. Fluctuations in populations of *S. zunasi* must have occurred when sea levels lowered (120–140 m below present sea level) during glacial maxima, which may be the reason for the population expansion. Similar conclusions have been reached for other marine fishes. Historical population expansions were detected in mackerel *Scomber scombrus* and chub mackerel *Scomber japonicus* (Zardoya et al., 2004) and *Chelon haematocheilus* (Liu et al., 2007), and Pleistocene population expansions were also detected in northern anchovy *Engraulis mordax* and Pacific sardine *Sardinops sagax* (Lecomte et al., 2004).

It has been inferred that the main lineages seemed to be generated by population expansion and isolation with change of sea level and/or sea environment such as water temperature or currents during glacial periods in Pleistocene in many marine invertebrates and vertebrates (Palumbi, 1994; Lessios, et al., 2001, 2003; Uthicke and Benzie, 2003). In such a case, a strait, which was once closed as a land bridge or isthmus in glacial periods, likely remained as a border between distinct lineages. Subsequently, once climatic conditions were restored, contact between the differentiated populations was resumed, leaving a genetic signature in the non-recombinant genes of the mtDNA. So three distinct lineages were found in *S. zunasi*, likely reflecting isolation of the marginal seas of the Northwestern Pacific during Pleistocene low sea-level stands.

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