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Population structure and demographic history of *Sicyopterus japonicus* (Perciformes; Gobiidae) in Taiwan inferred from mitochondrial control region sequences

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ABSTRACT. The amphidromous goby *Sicyopterus japonicus* is distributed throughout southern Taiwan and Japan. Larvae of this freshwater fish go through a long marine stage. This migratory mode influences population genetic structure. We examined the genetic diversity, population differentiation, and demographic history of *S. japonicus* based on the mitochondrial DNA control region. We

identified 102 haplotypes from 107 *S. japonicus* individuals from 22 populations collected from Taiwan and Islet Lanyu. High mean haplotype diversity (h = 0.999) versus low nucleotide diversity (θ_π = 0.008) was detected across populations. There was low correspondence between clusters identified in the neighbor-joining tree and geographical region, as also indicated by AMOVA and pairwise F_{ST} estimates. Both mismatch distribution analysis and Tajima's D test indicated that *S. japonicus* likely experienced a demographic expansion. Using a Bayesian skyline plot approach, we estimated the time of onset of the expansion of *S. japonicus* at 135 kyr (during the Pleistocene) and the time of stable effective population size at approximately 2.5 kyr (last glacial maximum). Based on these results, we suggest 1) a panmictic population at the oceanic planktonic larval stage, mediated by the Kuroshio current; 2) a long planktonic marine stage and long period of dispersal, which may have permitted efficient tracking of environmental shifts during the Pleistocene; and 3) a stable, constant population size ever since the last glacial maximum.

Key words: *Sicyopterus japonicus*; D-loop; Planktonic larvae; Population structure; Demographic history

INTRODUCTION

The life histories of marine and freshwater animals are generally very different. In most studies of the population genetics of freshwater species, particularly fishes, significant genetic structuring is usually detected among populations in highly disjunctive habitats, such as river systems and lakes separated by geographical or geological barriers and islands (Ward et al., 1994). Marine animals often have planktonic larvae that are potentially highly dispersive (Wellington, 1989). These larval dispersal capabilities have a prominent influence on the distribution of populations and their population structure (Taylor and Hellberg, 2003). Species that have long-lived planktonic larvae tend 1) to be more widespread geographically, 2) to show lower rates of endemism, and 3) to have less genetic population structuring (Lessios et al., 2001; Colgan et al., 2005).

Amphidromous species have a specific life-history with larvae hatching in freshwater, which are rapidly carried by river currents to the sea, where they begin an early planktonic life stage. In the larval stage, individuals migrate back to freshwater for maturation and reproduction (Shen and Tzen, 2002). In amphidromous gobies, dispersal and colonization in remote islands occurs only during the larval planktonic marine phase. The duration of this planktonic stage varies from 133 to 266 days for *S. lagocephalus* (Hoareau et al., 2007). It is possible that the strength and direction of marine currents and the duration of the planktonic phase together could influence dispersal ability.

Geographically, *S. japonicus*, one of the amphidromous gobies, is distributed from southern Taiwan to Fukushima Prefecture, Japan (Watanabe et al., 2006; Shen and Tzeng, 2008). Every year, at least 5-10 million transparent *S. japonicu*s postlarvae are estimated to recruit from oceans to streams in eastern Taiwan. *S. japonicu*s is one of the most abundant gobiid

species in these streams. In Taiwan, *S. japonicus* could be considered to be an indicator species of stream pollution because they only grow in streams of high water quality (Shen and Tzeng, 2008). The spawning season is from early July to September, and the larval recruitment season occurs primarily from April to May (Iida et al., 2009). Based on otolith analyses, the oceanic planktonic larval stage is estimated to last 130 to 253 days (Idle et al., 2008; Shen and Tzeng, 2008). Their population structure resembles that of strictly freshwater species, whereas their larvae have a long marine stage, thus suggesting that this species tends to have high oceanic dependency (Watanabe et al., 2006; Idle et al., 2008).

Mitochondrial markers are useful for evolutionary studies due to their high mutation rates and lack of recombination (Avise et al., 1987). In the mitochondrial genome, the D-loop control region evolving at a rate 2-5 times that of coding sequences (Aquadro and Greenberg, 1983) is a suitable marker for studying population genetics (Rosel et al., 1995). In the present study, we examined the entire D-loop region sequence (843 bp) to infer population structure, historical demography and differentiation in genetic diversity of *S. japonicus* of Taiwan and Islet Lanyu.

MATERIAL AND METHODS

Sampling and molecular methods

In total, 107 individuals of *S. japonicus* were collected from 22 locations in Taiwan. Sampling covered the entire range of the species (Figure 1, Table 1). Total genomic DNA was isolated (from muscle tissue or fins preserved in 95% ethanol) using proteinase K digestion at 55°C. DNA was purified by standard phenol:chloroform extraction and ethanol precipitation. DNA was subsequently re-suspended in 50 µL TE buffer.

The entire D-loop gene was amplified using the polymerase chain reaction (PCR) with the primers SJDL-F (5'-TCAGCGCCAGAGCGCCG(GT)CTTGTAA-3') and SJDL-R (5'-GGGCCCATCTTAACATCTTCAG-3') which are similar to the primer locations metioned in previous work (Chen et al., 2002). We amplified the target segment from a protein of transfer RNA (tRNA)-Thr to 12S rRNA partial sequence, which contained the entire D-loop sequence. Each 100 µL PCR reaction contained 10 ng template DNA, 10 µL 10X reaction buffer, 10 µL dNTP mix (8 mM), 10 pmol each primer, and 4.0 U *Taq* polymerase (Promega, Madison, WI, USA). PCR was programmed on an ABI 2720 Thermal Cycler at one cycle of denaturation at 95°C for 4 min and 30 cycles of denaturation at 94°C for 35 s, annealing at 55°C for 35 s, and extension at 72°C for 1 min 30 s, followed by 72°C extension for 10 min and 4°C for storage. PCR products were verified by electrophoresis on a 1.0% agarose gel using 1X TAE buffer. The gel was stained with ethidium bromide, and the desired DNA band was cut and eluted using the Agarose Gel Purification kit (QIAGEN, Valencia, CA, USA). Each purified PCR product was used in a cycle sequencing reaction with Applied Biosystems Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The D-loop was sequenced in both directions using the primers SJDL-F and SJDL-R. The sequencing reaction conditions consisted of an initial 50 s of denaturation at 96°C, followed by 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 10 s and extension at 60°C for 4 min and 30 s. The resulting cycle sequencing fragments were purified using Dye Ex Spin kits (QIAGEN) following the supplier instructions. Finally, products were visualized using an Applied Biosystems Prism 377 automated sequencer.

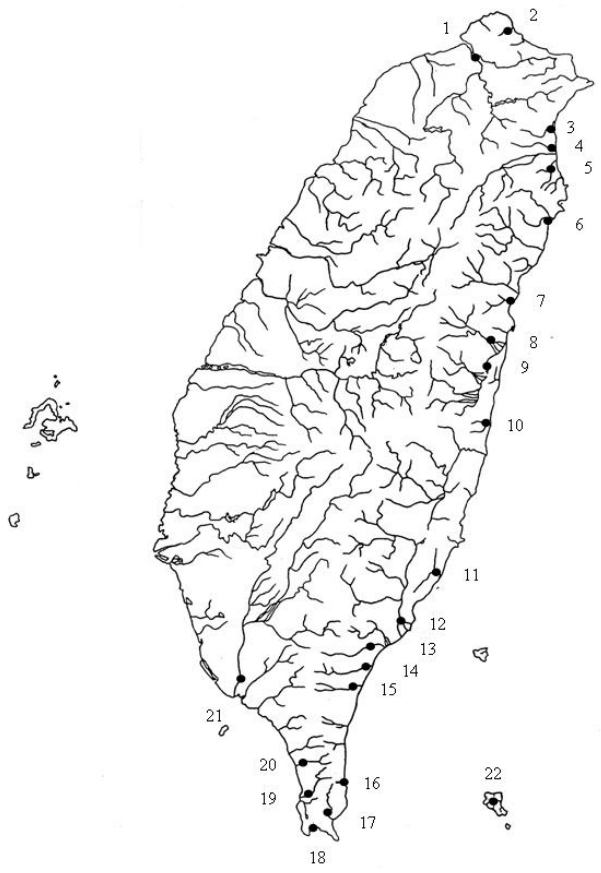

Figure 1. Sampling locations of *Sicyopterus japonicus*. Sample numbers correspond with those in Table 1.

Table 1. Source locations of *Sicyopterus japonicus* samples.

Region	No.	Location	Abbreviation	Latitude	Longitude
Taiwan (TW)	1	Danshue river	DS	24°57'	121°32'
	2	Laomei river	LM	25°28'	121°55'
	3	Fudeken river	FD	24°50'	121°49'
	4	Tasi river	TS	24°39'	121°34'
	5	Wulaoken river	WL	24°61'	121°81'
	6	Nanao river	NA	24°44'	121°84'
	7	Mukua river	MK	23°94'	121°53'
	8	Sanzan river	SZ	23°94'	121°51'
	9	Hualien river	HL	23°73'	121°48'
	10	Siukuluan river	SK	23°28'	121°29'
	11	Mawuku river	WK	22°59'	121°17'
	12	Beinan river	BN	22°54'	121°52'
	13	Ghihben river	GP	22°41'	121°32'
	14	Taimali river	TM	22°36'	120°58'
	15	Jinlun river	JL	22°53'	120°96'
	16	Luliao river	LL	22°02'	120°53'
	17	Kankau river	KK	22°02'	120°80'
	18	Shiniu river	SN	21°57'	120°47'
	19	Sichong river	SC	22°57'	120°44'
	20	Fangshan river	FS	22°26'	120°67'
	21	Kaoping river	KP	22°36'	120°26'
	22	Lanyu	LY	22°24'	121°33'

Genetic diversity

MtDNA sequences were aligned with the program CLUSTAL X 1.81 (Thompson et al., 1997), and consensus sequences were determined for each individual based on the raw forward and reverse sequence data. Haplotype diversity (*h*) and nucleotide diversities (θ_π) and (θ_w) for each population were calculated with DnaSP v. 5.0 (Librado and Rozas, 2009).

Phylogenetic and phylogeographic analyses

Sequence data were analyzed with the neighbor-joining method using the Kimura 2-parameter distance method with MEGA 4 (Tamura et al., 2007). Neighbor-joining tree nodes and branch lengths were tested statistically using a bootstrap method of 1000 replicates (Felsenstein, 1985).

Historical demography

To infer the population demographic history of *S. japonicus*, we employed several methods, including Tajima's *D* statistics, Fu's (*F*s) test of neutrality and the frequency distribution of pairwise differences between mtDNA haplotypes (i.e., mismatch distribution). Departures from neutrality of *Fs* test and Tajima's *D* test indicate recent population expansion under assumptions of neutrality. Significance of *F*s test and Tajima's *D* values were evaluated using the coalescent algorithm implemented in DnaSP 5.0 (Librado and Rozas, 2009), in which the observed value is compared to a null distribution generated by 10,000 replicates, given an empirical population sample size and the observed number of segregating sites. The demographic history of *S. japonicus* was explored using mismatch analysis of D-loop mitochondrial sequences. This method is based on the premise that, relative to a constant population size, population growth or decline leaves a distinctive signature in DNA sequences. If the D-loop region examined here evolves neutrally and has been transmitted under equilibrium conditions, a multimodal distribution of haplotypes should then result. Alternatively, a unimodal distribution (i.e., a large number of closely related haplotypes) suggests non-equilibrium conditions, particularly population expansion. To compare the observed distributions with those expected under an expansion model, we calculated the sum of square deviation (SSD) and Harpending's raggedness index (Rg) (Harpending, 1994). Current genetic diversity is based on pairwise differences between sequences, whereas historical genetic diversity is based on the number of segregating sites among sequences. To infer demographic history, coalescence methods require an initial demographic model to be specified. Where evidence of population expansion was found, the timing of expansion in generations (t) was estimated from $\tau = 2\mu t$, where τ (tau) is a parameter of the time to expansion in units of mutations and where μ is the mutation rate per generation for the DNA sequence under study. We used the Bayesian skyline plot implemented in the BEAST version 1.4.7 program (Drummond and Rambaut, 2007) to depict the change in female *S. japonicus* effective population size (*Nfe*) since the time of the most recent common ancestor (TMRCA) of the sampled mitochondrial haplotypes. A mutation rate of 3.6% per nucleotide myr was used for D-loop as the mean rate for fish (Aboim et al., 2005). A mean value of 3.6% per myr was selected as the mutation rate to apply to the

divergence rate of the entire D-loop sequences and assumed a generation time of one year (Shen et al., 2008; Iida et al., 2008). This coalescent-based approach estimates the posterior distribution of effective population size at intervals along a phylogeny, thereby allowing one to infer population fluctuations over time. A total of 10^6 generations were run. Burn-in and plots for each analysis were visualized using Tracer v. 1.5 (Rambaut and Drummond, 2009).

Population genetic differentiation

Pairwise F_{ST} values and analysis of molecular variance (AMOVA) were used to assess the population configuration and the geographical pattern of population subdivision, as implemented by ARLEQUIN ver 3.5 (Excoffier and Lischer, 2010). Pairwise F_{ST} values among sites were calculated and assessed for significance by comparison with 10,000 permutations of data. For the hierarchical analysis, populations were grouped according to geography (Taiwan and Islet Lanyu). Statistical significance of differentiation at the three levels was quantified and tested using ARLEQUIN ver 3.5 (Excoffier and Lischer, 2010).

RESULTS

Genetic diversity

Sequences of 843 bp of the mtDNA D-loop were aligned from 107 *S. japonicus* individuals. A total of 102 haplotypes were identified. Alignment of all D-loop sequences consisted of 68 variable sites (8.06%), of which 19 were singletons and 49 (5.81%) were parsimony-informative. The nucleotide composition of the D-loop sequences was AT rich (A, 31.1%; T, 31.4%), as observed in other bony fishes (Aboim et al., 2005; Song et al., 2010). In contrast, G and C nucleotides were 15.5 and 21.9%, respectively. G was deficient, as characteristic of the mitochondrial genome (Zhang and Hewitt, 1996).

Sample size, number of haplotypes, and values of θ_π and *h* within each population are presented in Table 2. Overall, the mean *h* among the 107 samples was estimated to be 0.999, and the mean nucleotide diversity was estimated at 0.081 (Table 2). θ_π among populations varied from 0.004 (SK) to 0.013 (WL). Among populations, mean haplotype diversity was high, but nucleotide diversity was low.

Phylogenetic and genetic analyses

The neighbor-joining tree was reconstructed based on the mtDNA D-loop sequences. No long internal branches supported partitioning of specific clades, and no geographic structure could be identified among the Taiwanese populations (Figure 2).

Pairwise F_{ST} tests indicated no significant genetic differentiation among sampling locations (-0.000 to 0.511) after Bonferroni correction. However, there were shared haplotypes between FD03 and FS04, FD02 and SZ02, SC07 and DS04, FS02, LL02 and BN04. The results of the AMOVA indicated that most of the molecular variance (82.97%) was attributable to variation among populations and 22.59% to variation within populations: -5.56% of molecular variance was related to variation among groups (Table 3).

Table 2. Summary of sample size, haplotype number, haplotype diversity (*h*), and nucleotide diversity (θ_π and θ_w) for mtDNA D-loop sequences in each population.

Populations	Sample size	Haplotype numbers	Haplotype diversity (*h*)	Nucleotide diversity (θ_π)	Nucleotide diversity (θ_w)
DS	4	4	1.000	0.006	0.007
LM	5	5	1.000	0.006	0.006
FD	5	5	1.000	0.005	0.006
TS	5	5	1.000	0.009	0.010
WL	5	5	1.000	0.013	0.001
NA	5	5	1.000	0.007	0.007
SZ	5	5	1.000	0.010	0.009
MK	5	5	1.000	0.010	0.010
HL	4	4	1.000	0.007	0.008
SK	5	5	1.000	0.004	0.005
WK	5	5	1.000	0.009	0.010
BN	5	5	1.000	0.005	0.006
GP	5	5	1.000	0.008	0.010
TM	5	5	1.000	0.011	0.011
JL	5	5	1.000	0.009	0.010
LL	4	4	1.000	0.007	0.008
KK	4	4	1.000	0.007	0.006
SN	5	5	1.000	0.005	0.005
SC	8	8	1.000	0.008	0.010
FS	5	5	1.000	0.008	0.008
KP	3	3	1.000	0.007	0.007
LY	5	5	1.000	0.008	0.010
Total	107	102	0.999	0.008	0.008

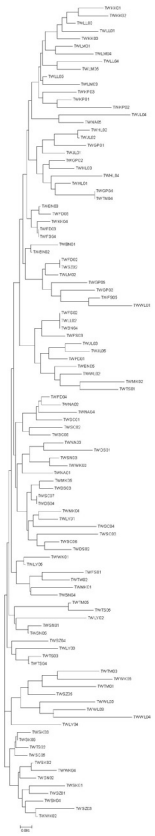

Figure 2. Neighbor-joining tree of individual sequences of the entire mtDNA D-loop region of *Sicyopterus japonicus*.

Table 3. AMOVA results for testing genetic subdivision between populations based on mtDNA D-loop variation among zoogeographic zones.

Source of variation	Variance components	Percentage of variation
Among groups	-0.21709	-5.56
Among populations within groups	0.88173***	22.59
Within populations	3.23902***	82.97

*P < 0.05, ** P < 0.01, ***P < 0.001

Demographic history

Demographic history analysis revealed marked differences among populations. A signature of recent expansion was detected in all populations as evidenced by the significant *F*s test (*Fs* = -164.284, P < 0.001), although Tajima's *D* was not significant (D = -1.44187, P > 0.05) (Table 4). Fu's test has been shown to be much more sensitive in detecting population growth than Tajima's *D*. The model of population expansion could not be rejected because of its concordance with the expectation of a historically expanding population when all samples were pooled together (SSD = 0.088, P = 0.45 for demographic expansion (data not shown). This outcome was also supported by the low Harpending's Raggedness index (r = 0.240, P = 0.55). Demographic analyses of the overall data showed evidence of population expansion. Tajima's D and Fu's tests were both significantly negative, indicating that this species experienced a demographic expansion event (under a neutral model). To further characterize the expansion pattern, a model of sudden demographic growth was fitted to the pairwise sequence mismatch distribution (Figure 3), i.e., parametric bootstrap goodness-of-fit tests failing to reject the model.

Table 4. Mismatch distribution analyses and tests of neutrality of *Sicyopterus japonicus* for mtDNA D-loop sequences in each population.

Populations	Tajima's D	*Fs*	τ	SSD	Rg
DS	-0.280	-0.324	7.100	0.1007	0.3333
LM	-0.654	-1.411	5.070	0.0779	0.1600
FD	-0.596	-1.554	4.979	0.0370	0.1000
TS	-0.801	-0.696	8.959	0.0906	0.1600
WL	-0.027	-0.213	10.432	0.1178	0.3200
NA	-0.628	-1.139	5.984	0.1270	0.4600
SZ	-0.172	-0.733	8.135	0.0494	0.1200
MK	-0.848	-0.010	7.998	0.065	0.1667
HL	-0.584	-0.253	6.312	0.069	0.1667
SK	-0.624	-1.952	3.781	0.0050	0.0400
WK	-0.801	-0.696	5.545	0.0590	0.1400
BN	-0.596	-1.554	4.812	0.0669	0.2200
GP	-0.649	-0.832	7.469	0.2065*	0.5600
TM	0.227	-0.421	8.416	0.0971	0.1600
JL	-0.076	-0.714	9.459	0.0776	0.1800
LL	-0.564	-1.011	4.539	0.0481	0.1200
KK	0.083	-0.339	5.617	0.1470	0.3333
SN	-0.198	-1.633	4.992	0.0341	0.1200
SC	-1.031	-3.185*	5.830	0.0131	0.0459
FS	-0.130	-0.918	7.750	0.0507	0.1200
KP	-	-	6.137	0.3479	1.1111
LY	-0.649	-0.832	7.020	0.0582	0.1400
Total	-9.598	-164.284***	mean 6.652		

*P < 0.05, ** P < 0.01, ***P < 0.001. The parameters of the model of sudden expansion are reported as well as goodness-of-fit test to the model; SSD = sum-of-squared deviation; Rg = raggedness indexes. Tajima's D and Fu's test values, their statistical significance are also given (τ = time parameter for a generation).

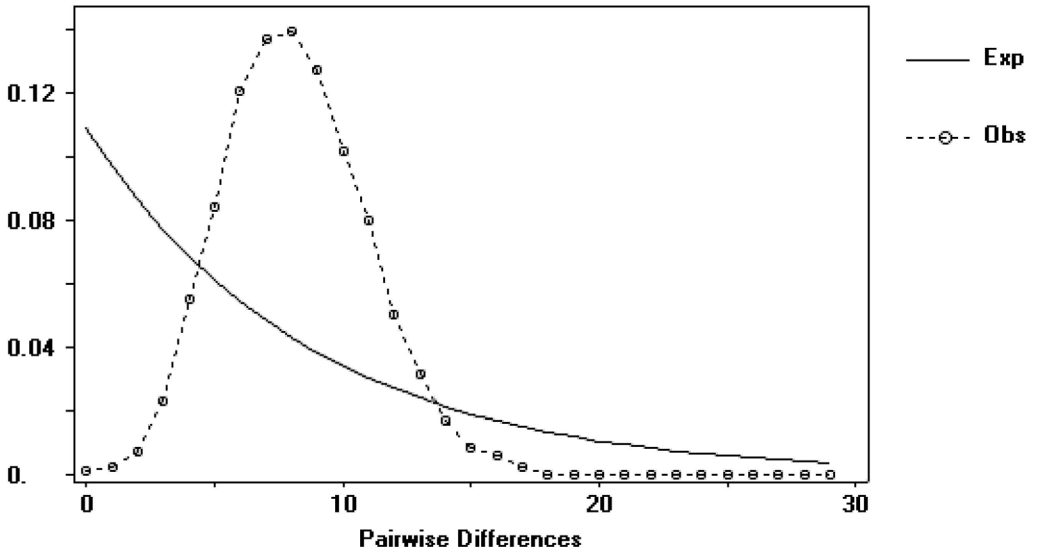

Figure 3. Mismatch-distribution analysis of *Sicyopterus japonicus* mtDNA D-loop haplotype sequences. A simulated Poisson distribution is indicated by the dotted line.

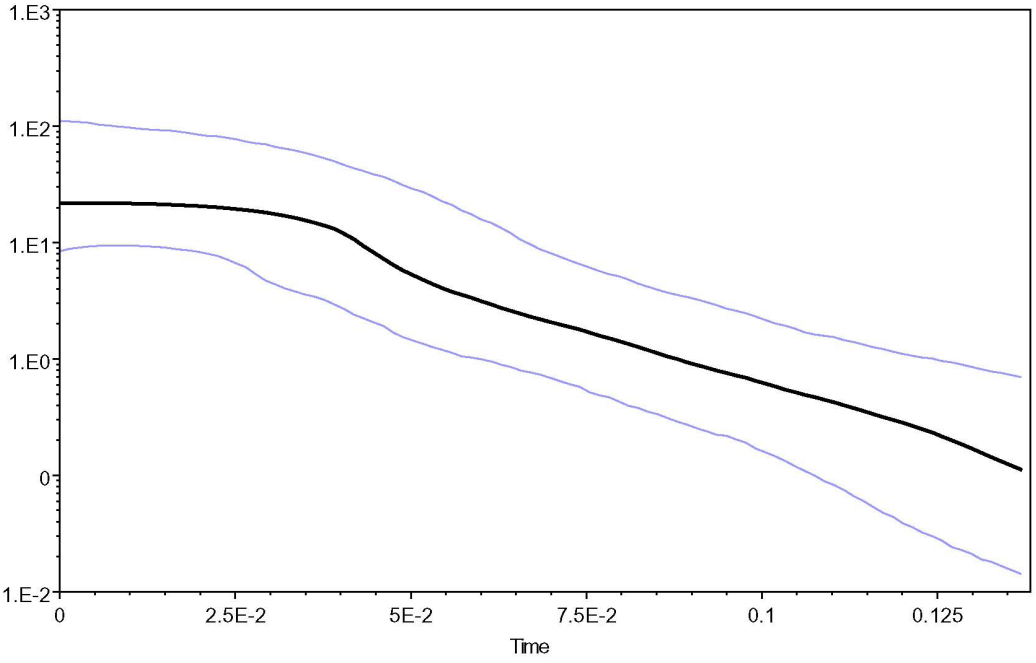

Figure 4. Bayesian skyline plot of effective population size through time for *Sicyopterus japonicus*.

Bayesian skyline plots revealed a complex demographic history. The effective sample size for each of the Bayesian skyline analyses was greater than 200, suggesting that 10 million generations were sufficient to determine the demographic history of *S. japonicus*. Bayesian skyline plots for *S. japonicus* indicated that recent population expansion occurred 135 kyr ago and that the population reached a stable effective population size approximately 2.5 kyr ago (Figure 4). ARLEQUIN calculated the mean value of τ as 6.652, and the time since population expansion was estimated to be approximately 110 kyr. These estimates of expansion time are similar to those inferred from both Bayesian skyline plots and ARLEQUIN coalescent statistics.

DISCUSSION

Genetic variation within *S. japonicus*

Nucleotide and haplotype diversities provide information on the history that *S. japonicus* populations experienced. Grant and Bowen (1998) interpreted four basic scenarios for population history based on haplotype and nucleotide diversities. The present results revealed a pattern of high haplotype diversity *vs* low-to-moderate nucleotide diversity in all populations (Table 2). Genetic variability in the mtDNA D-loop has also been reported in other fish species (Aboim et al., 2005; Liu et al., 2006; Han et al., 2008). Several scenarios have been proposed to explain the maintenance of high haplotypic diversity within populations, including large population size, environmental heterogeneity, and life history traits that favor rapid population increase. *S. japonicus* inhabits ecosystems with high environmental heterogeneities, experiences specific life-history in freshwater and marine habitats, and has a widely distributed population ranging from Taiwan to Japan, altogether likely accounting for the high levels of haplotypic diversity observed in this study. Low nucleotide diversity in the mtDNA D-loop region instead implies that recent population expansion occurred via bottlenecked populations.

Population differentiation

Genetic structure and differences resulting from drainage isolation have often been detected in freshwater fishes, such as *Zacco platypus* and *Opsariichthys bidens* (Perdices and Coelho, 2006) and *Glyptothorax* (Chen et al., 2007), mostly due to their isolated habitats and limited dispersal capacity. *S. japonicus* is a freshwater fish species; however, our results showed an absence of significant population differentiation and a lack of phylogeographic structure.

In the Hawaiian Islands, which extend approximately 600 km, no phylogeographic structure has been found among five discrete island populations of four amphidromous gobiid fishes (*Awaous guamensis*, *Stenogobius hawaiiensis*, *Lentipes concolor*, and *S. stimpsoni*). Studies based on otolith growth estimate the following durations of the oceanic planktonic larval stage: 150 to 169 days for *A. guamensis*, 119 to 151 days for *S. hawaiiensis* and 63 to 106 days for *L. concolor* (Radtke et al., 1988). *S. lagocephalus* has a planktonic larval stage of 133 to 266 days (Hoareau et al., 2007), allowing its larvae to connect with remote populations and thereby maintain gene flow throughout the 18,000-km wide Indo-Pacific area. These species appear to have high oceanic dependency (Watanabe et al., 2006; Idle et al., 2008). The data here suggest that populations of amphidromous species are genetically structured at a scale similar to marine species.

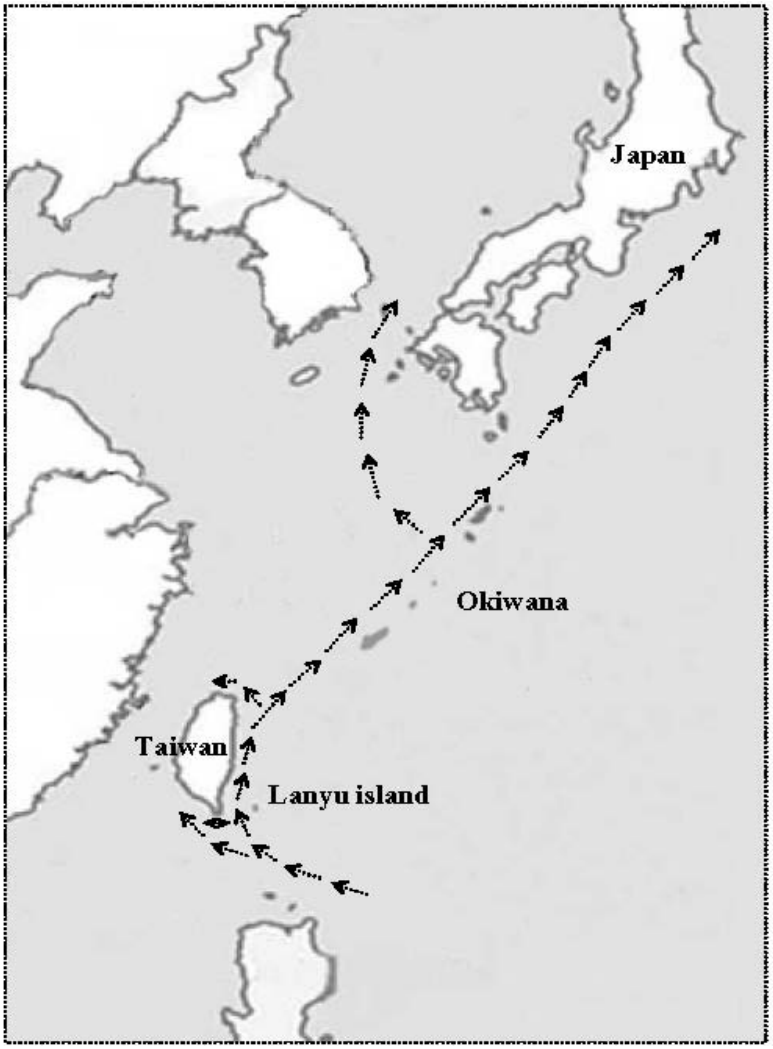

Figure 5. Map showing the Kuroshio current. Ocean currents are indicated by the dashed markers.

The amphidromous *S. japonicus* has a long larval marine phase, achieving the oceanic planktonic larvae stage at approximately 5-8 months (as estimated by analysis of otolith growth; Shen and Tzeng, 2008). The pattern of genetic structure observed in *S. japonicus* may be influenced by ocean currents. The Kuroshio Current is a strong, warm water mass flowing steadily northward along the east coast of Taiwan and beyond, past Okiwana, Japan. Along the west coast, there is also a persistent northward flow through the Taiwan Strait (Figure 5). Along the coastline of Taiwan (spanning approximately 400 km), the Kuroshio Current flows from south to north at an average rate of 25 cm/s and can carry fish larvae over 3000 km. Therefore, it appears that both the life history of oceanic planktonic larvae and ocean currents are the most important factors facilitating the gene flow of *S. japonicus* in Taiwan.

Islet Lanyu is located approximately 65 km east of Taiwan. The Kuroshio Current passes Taiwan and Islet Lanyu at a maximum flow rate of approximately 100 cm/s. However, this study detected no genetic differentiation between Taiwan and Islet Lanyu populations, suggesting that the Kuroshio Current does not act as a barrier to gene flow. It is likely that gene flow between these regions is facilitated by typhoons (mature tropical cyclones). Typhoons are common during the summer and autumn in the West Pacific. Each year, several typhoons move from the Pacific westward, directly hitting Taiwan, Okinawa, Japan and nearby seas. Most follow the Kuroshio Current northward or continue further east to the Pacific (including Islet Lanyu). Weaker typhoons affect surface currents over an area of approximately 300-400 km in radius; for stronger typhoons, this area is approximately 800 km in radius. The maximum observed current speed in the Taiwan current has been 1.7 m/s, and 2 m/s in the Pacific. These current speeds suggest that typhoons induce strong surface flows in Taiwan and Pacific waters (Chang et al., 2010). The fluid mechanics of marine and terrestrial systems are surprisingly similar on spatial and temporal scales. Not surprisingly, the dispersal of organisms is influenced by the fluid environment of seawater (Dawson and Hommer, 2008). For example, the Indian rice frog (Toda et al., 1998) and the freshwater crab *Geothelphusa tawu* (Shih et al., 2004) in Islet Lanyu originally came from eastern Taiwan via similar modes of dispersal. These patterns of current flow suggest that the dispersal of *S. japonicus* populations between Taiwan and Islet Lanyu by typhoon activity may induce atypical flow in the surface layer of the Kuroshio current, thereby maintaining gene flow in *S. japonicus* populations.

Several factors likely promote a high dispersal capability of *S. japonicu*s, including the flow of the Kuroshio current from Taiwan to Japan, annual typhoon activity, an extensive spawning season from July to September (Iida et al., 2009) and a long planktonic marine phase. The distance between south Taiwan to Fukushima Prefecture, Japan is approximately 2500 km (Watanabe et al., 2006). If larvae travel with the currents, gene flow among geographic populations must be high, suggesting that *S. japonicus* shares a common gene pool between the Taiwan and Japan populations.

Demographic history

To date, little is known regarding the demographic history of *Sicyopterus*. Results from the unimodal mismatch distribution of pairwise differences between *S. japonicus* haplotypes suggest that this species fits a model of population expansion. This finding is corroborated by the negative Tajima's D and Fu's statistics, and by the high haplotype diversity and low nucleotide diversity. Using a Bayesian skyline plot approach, this study estimated the timing of onset of population expansion of *S. japonicus* at approximately 135 kyr ago and the population reaching a stable effective population size approximately 2.5 kyr ago. The estimated time period of population increase is concurrent with the Pleistocene. The higher haplotype and lower nucleotide diversity are attributed to population expansion following a period of low effective population size. Many species belonging to this category are believed to have originated in the Pliocene or early Pleistocene (Grant and Bowen, 1998). During environmental changes of the Pleistocene, including sea level changes associated with glacial cycles (Haq et al., 1987), genetic signatures of population expansion have been detected in other marine organisms. Examples include the crab *Callinectes bellicosus* (population expansion estimated at 67 kyr ago; Pferler et al., 2005), gastropods *Nerita scabricosta* (population

expansion estimated at approximately 50 to 70 kyr ago) and *Nerita funiculate (*population expansion estimated at 150 to 200 kyr ago; Hurdato et al., 2007), and fish *Albula* sp. (expansion estimated at 130 kyr ago; Pfeiler et al., 2008). Although these marine species differ in several aspects from *S. japonicus*, they all share a planktonic larval stage in their life history. It seems likely that a planktonic larval stage played an important role in responding to environmental changes during the Pleistocene (Pfeiler et al., 2008). The amphidromous goby *S. japonicus* has evolved in response to oceanic and continental habitat requirements. *S. japonicus* has a long planktonic marine phase and should have long distance dispersal ability. Thus, the long planktonic marine stage and extensive dispersal capability of *S. japonicus* may have sustained gene flow in the Pleistocene during periods of sea level fluctuations, which in turn may have favored increases in effective population size. Alternatively, *S. japonicus* may have exhibited demographic stability during the last glacial maximum (LGM), approximately 18-23 kyr ago (Cunha et al., 2011), following a drop in sea level and the emergence of more continental rivers. Freshwater habitats on the small island would have restricted population expansion (Xu et al., 2009). Consequently, *S. japonicus* population expansion may have been limited and reached saturation upon the emergence of continental freshwater habitats, with the population maintaining its demographic stability during LGM.

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