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Population genetic structure of the Japanese eel *Anguilla japonica*: panmixia at spatial and temporal scales

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ABSTRACT: Since the 1970s, the population of the Japanese eel *Anguilla japonica* has dramatically declined in East Asia. Consequently, conservation and resource management of this species are urgently required. However, the population genetic structure of this species, in temporal and spatial scales, is still poorly understood. We used 8 polymorphic microsatellite DNA loci to investigate its genetic composition. For cohort analysis, juvenile (glass) eels were collected yearly between 1986 and 2007 from the Danshui River, Taiwan; for arrival wave analysis, glass eels were collected monthly from Fulong Estuary, Taiwan; and for spatial analysis, glass eels were collected from Taiwan, China, Korea and Japan. Genetic differentiation among annual cohorts, arrival waves and spatial samples was very low; a significant difference was observed among annual cohorts and spatial samples, but not among arrival waves. However, specific temporal or spatial scale patterns were not seen in either pairwise genetic comparisons or the phylogenetic tree of all samples. Occasional genetic variations among samples occurred randomly, but a stable lasting genetic structure could not be formed. The isolation by distance (IBD) test showed no evidence of genetic structuring at the spatial scale, and the results of the isolation by time (IBT) test were insignificant among arrival waves. Genetic heterogeneity over a 21 yr time scale showed marginal significance, potentially reflecting a genetic drift in the Japanese eel. Our results suggest the existence of a single panmictic population of Japanese eel in East Asia. Therefore, the Japanese eel should be considered as a single management unit for conservation.

KEY WORDS: Genetic differentiation · Japanese eel · *Anguilla japonica* · Microsatellite DNA · Panmixia · Isolation by time · IBT · Isolation by distance · IBD

INTRODUCTION

The scales of population structure in marine species usually depend on the extent to which larvae from different populations are mixed in plankton. Larvae may drift in the plankton for weeks or months, resulting in a relatively high connectivity among populations (Shanks et al. 2003). In theory, larvae released from a given location can mix well with the larvae released from populations elsewhere by diffusing over a large area and can thus prevent genetic structuring of populations (Gaylord & Gaines 2000). However, the dynamics of larval dispersal may show high stochasticity in time and space owing to the limited mixing of larvae from distinct sources (Gaines et al. 2003, Siegel et al. 2003), patchy environmental selection in the plankton (Gaines & Bertness 1992, Ellien et al. 2004) or 'sweepstakes' reproductive success resulting from small effective population sizes (Hedgecock 1994a,b). The spatial restriction on gene flow can be described by the island, stepping-stone or isolation by distance (IBD) models (Rousset 1997). In addition to the population structure in space, many populations comprise a mixture of individuals that reproduce at different times within a reproductive season (reviewed in Hendry & Day 2005). The heritable reproductive time is thought to contribute to this temporal restriction on gene flow between early and late reproducers, thus creating a

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pattern of isolation by time (IBT) (Hendry & Day 2005). To understand the population genetic structure of some species, the spatial and temporal components across populations should be considered simultaneously.

The Japanese eel *Anguilla japonica* is a temperate catadromous fish with a long migratory loop and lengthy leptocephalus stage. The spawning area of this species is presumed to be in the western Mariana Islands near 14°–16° N, 142° E (Tsukamoto 1992, 2006), 2000 to 3500 km away from the East Asian continent. The leptocephalus larvae are born between April and November (Tsukamoto 1990, Tzeng 1990, Tsukamoto et al. 2003) and drift from their spawning area with the North Equatorial Current (NEC), followed by the Kuroshio Current (KC), for 4 to 6 mo, eventually reaching the coasts of East Asia (Cheng & Tzeng 1996). They metamorphose into juvenile (glass) eels along the continental shelf and then enter estuaries in pulses known as 'arrival waves,' mainly into Taiwan, China, Korea and Japan (Tesch 2003). The eels live in freshwater rivers for more than 4 yr before metamorphosing into silver eels (onset of sexual maturation) in autumn and winter (Han et al. 2003, 2009), after which they migrate back to their marine birthplace to spawn and eventually die (Tsukamoto 1992, 2006, Tesch 2003). The Japanese eel is a commercially important aquaculture species in East Asia. However, its recruitment has been rapidly declining in the past decades, possibly due to overfishing, habitat destruction and the effects of global climate change (Tzeng 1986, Han et al. 2009). A similar trend has been reported among European eels *A. anguilla* (Dekker 2003a,b). To develop an effective management plan for this declining species and ensure its sustainability, it is vital to gain a thorough understanding of the partitioning of its genetic structure in both spatial and temporal scales.

In the past, the concept of panmictic populations for temperate eels was accepted on the basis of evidence from mtDNA sequences such as those from the Japanese eel (Sang et al. 1994, Ishikawa et al. 2001a), European eel (De Ligny & Pantelouris 1973, Avise et al. 1986, Avise 2003) and American eel *Anguilla rostrata* (Avise et al. 1986, Avise 2003). Although mtDNA gene regions are powerful markers for resolving phylogenetic problems at the species level, these are of limited use in population genetic studies because of their limited resolving power and the presence of mitochondrial pseudogenes in the nuclear genome of many organisms (reviewed in Zhang & Hewitt 2003, Wan et al. 2004). On the other hand, although studies using allozyme markers have detected spatial heterogeneity in allele frequency within the American eel (Williams et al. 1973), European eel (De Ligny & Pantelouris 1973, Comparini et al. 1977) and Japanese eel (Chan et al. 1997), the significant clinal shift in allele frequency was putatively attributed to single-generation selection along an environmental gradient (Avise 2003). In recent studies, which used more sensitive microsatellite markers, panmixia in the European eel was challenged by the evidence of a weak but significant population structuring (Daemen et al. 2001, Wirth & Bernatchez 2001, Maes & Volckaert 2002). Wirth & Bernatchez (2001) and Maes & Volckaert (2002) also found evidences for IBD. However, Dannewitz et al. (2005) and Palm et al. (2009) suggested panmixia occurred in the European eel, and Dannewitz et al. (2005) suggested that the discrepancies among studies could be due to the fact that temporal variation was not accounted for in the previous studies. Many studies in which annual cohorts or arrival waves were compared have shown a pattern of genetic patchiness (Maes et al. 2006, Pujolar et al. 2006, 2007). Furthermore, temporal genetic variation within sites may exceed the geographical factor within sites. Because of the complexity of the oceanic environment, eel reproduction may be regarded as a sweepstake event in which only a fraction of the adult population contributes to the next generation, resulting in a large variance in the reproductive success of each cohort (Dannewitz et al. 2005, Maes et al. 2006, Pujolar et al. 2006, 2007). No apparent deviations from panmixia were observed in the American eel (Wirth & Bernatchez 2003) or in two other species, speckled longfin eel *A. reinhardtii* (Shen & Tzeng 2007a) and shortfin eel *A. australis* (Shen & Tzeng 2007b).

Tseng et al. (2006) divided the genetic populations of the Japanese eel into low-latitude (South China and Taiwan) and high-latitude (Japan, Korea and North China) groups; the IBD pattern was not detected for either groups. They suggested that most progeny tend to be transported back to similar locations, as in the case of their ancestors, with low gene flow between the south and north eel groups leading to a stable genetic isolation of these 2 populations. However, one factor that cannot be excluded is that the spatial differentiation found in the Japanese eel may be due to stochastic genetic heterogeneity (genetic patchiness), as in the case of the European eel. In addition, the distribution of the Japanese glass eel in East Asia strongly depends on the KC, which flows past the eastern coast of Taiwan to the north of Japan. The distance between Taiwan and Japan ranges from 1000 to 2000 km, and the velocity of the KC averages between 77 and 116 km d^{-1} (Nitani 1972). Thus, the mean time lag for larval transportation is approximately 9 to 26 d, which matches the mean difference in the age at metamorphosis of the glass eels of Taiwan and Japan (Cheng & Tzeng 1996). Due to the short time difference in larval dispersal between Taiwan and Japan, it seems quite unlikely that the leptocephali would be transported to the same

locations as their parents by the KC with high selectivity. Thus, to obtain a better understanding of the population genetic characteristics of the Japanese eel, a more thorough investigation of the population genetic structure is required in which both spatial and temporal components are considered simultaneously.

We report the results from the most extensive genetic study to date of the Japanese eel in which 8 microsatellite loci were examined. We studied (1) small-scale genetic variation among arrival waves from Taiwan and Japan collected in the same fishing season to test whether genetic differentiation or IBT exists among arrival waves within and between sites, (2) long-term genetic variation among annual cohorts from the Danshui River, Taiwan, over a period of 21 yr and (3) the spatial population genetic structures of Japanese eels collected from 9 locations in East Asia using the IBD test. The aim of this study was to investigate whether genetic patchiness, IBD or IBT play an important role in shaping the population genetic structure of the Japanese eel.

MATERIALS AND METHODS

Sample collection. In Taiwan, the recruitment of Japanese glass eels usually begins in late October and ends in early April of the following year (Chang et al. 2007, Han et al. 2009), which is defined as an annual cohort; however, this process is usually delayed by approximately 1 mo in Japan and 1 to 3 mo in other East Asian countries (Y. S. Han et al. unpubl. data). The arrival of glass eels usually occurs in pulses, and these batches of samples are defined as arrival waves. In this study, arrival waves were collected monthly from November 2001 through April 2002 by means of a fyke net in the Fulong estuary (FL), which is located in the Shuangsi River estuary in northern Taiwan. More samples were harvested from February 2002 through April 2002 from Mikawa Bay (MB), Japan (Fig. 1, Table 1). Annual cohorts were collected from 1986 to 2007 from the Danshui River estuary (DS), northern Taiwan (Fig. 1, Table 1). Some annual cohorts were not collected. For spatial analysis, samples were also collected from Taiwan (Tungkang), China (Xiamen, Min Jiang, Qiantang Jiang and Yangtze estuaries) and Korea (Yalu Jiang) (Fig. 1, Table 1). The captured glass eels were immediately preserved in 95% ethanol. A total of 1770 specimens were randomly selected from the total collection of samples. Before microsatellite DNA analysis, the total length (TL, to the nearest 0.1 mm) of each individual was measured, and its pigmentation state was assessed (Table 1). Pigmentation of glass eels was judged by using a modification of the parameters used for the European eel (Tesch 2003). Stages VA and VB (Tesch 2003) were combined into Stage V, VIA1 and VIA2 into Stage VIA, and stages higher than VIA3 into Stage VIB.

DNA extraction. Genomic DNA of glass eels was extracted from a small piece of muscle tissue using a commercial DNA purification and extraction kit (Bioman Scientific). Ethanol was removed by evaporation before treatment. A piece of muscle weighing approximately 20 mg was digested in 200 µl lysis buffer (10 mM Tris-HCl, 2 mM EDTA, 10 mM NaCl, 1% sodium dodecyl sulfate and 10 mg ml^{-1} dithiothreitol, pH 8.0) and 20 µl Proteinase K (10 mg ml^{-1}) for 2 h at 60°C. The digested tissue was ground, and the solution was then transferred to a spin column, washed with ethanol buffer, eluted with 50 µl elution buffer (10 mM Tris-HCl, pH 8.5) and stored at –20°C before PCR analysis.

PCR and genotyping. Eight microsatellite loci were selected, 6 (GT)n loci and 2 (GA)n loci (Table 2). These loci were selected from GenBank and exhibited moderate to high polymorphism. These microsatellite DNA were amplified using PCR, as described by Han et al. (2008). Briefly, the amplification was performed in a total reaction volume of 25 µl with the following composition: 0.3 µl of DNA template, 0.3 µl of *Taq* polymerase (5 U $µl^{-1}$), 1 µl of 10 µM forward and reverse

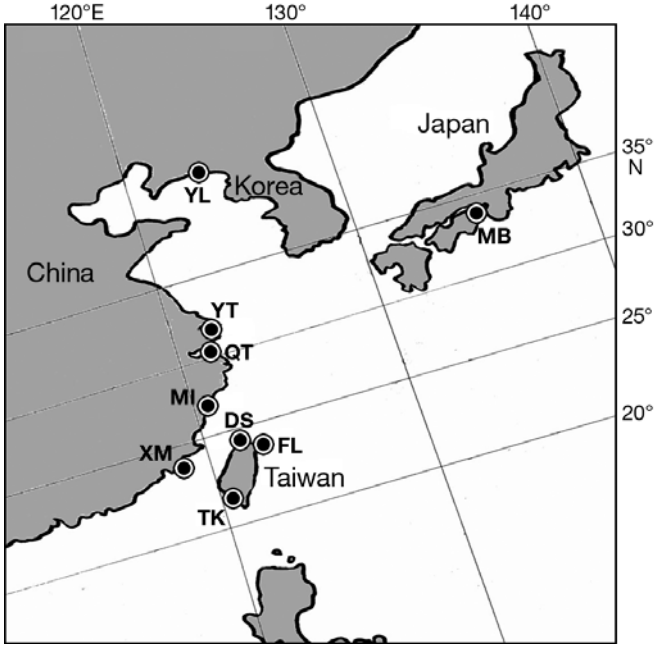

Fig. 1. *Anguilla japonica*. Locations where Japanese glass eels were collected in coastal waters of Taiwan, China, Korea and Japan. YL: Yalu Jiang estuary; MB: Mikawa Bay; YT: Yangtze River estuary; QT: Qiantang Jiang estuary; MI: Min Jiang estuary; DS: Danshui River estuary; FL: Fulong estuary; XM: Xiamen estuary; TK: Tungkang estuary

Table 1. *Anguilla japonica*. Sampling date, location, size, mean total length (TL) and genetic variation of Japanese glass eels. H_o: observed heterozygosity; H_e: expected heterozygosity; AR: allele richness

Sample identification	Collecting location	Date	n	Pigmentation stage (%) V	VIA	VIB	TL (mm) Mean ± SD	Range	Microsatellites H_o	H_e	AR
Taiwan											
TK9302	Tungkang	1993, Feb	45	100	0	0	54.8 ± 2.3	48.8–60.8	0.788	0.867	14.82
DS8612	Danshui	1986, Dec	54	95.7	4.3	0	57.5 ± 2.2	52.0–62.4	0.841	0.865	14.31
DS8812	Danshui	1988, Dec	69	100	0	0	52.2 ± 2.2	47.3–59.0	0.810	0.857	14.87
DS8912	Danshui	1989, Dec	68	92.5	6.3	1.3	55.4 ± 2.3	48.3–62.7	0.809	0.864	14.55
DS9101	Danshui	1991, Jan	62	97	3	0	55.7 ± 2.5	50.2–61.2	0.809	0.867	14.50
DS9402	Danshui	1994, Feb	55	100	0	0	53.7 ± 2.4	46.5–58.3	0.847	0.858	13.47
DS9501	Danshui	1995, Jan	33	50	37.5	12.5	55.1 ± 3.2	47.8–61.7	0.853	0.868	14.14
DS9712	Danshui	1997, Dec	68	64.2	14.3	21.5	54.4 ± 2.1	50.8–60.0	0.847	0.866	14.40
DS9901	Danshui	1999, Jan	48	100	0	0	52.6 ± 2.5	48.0–58.6	0.768	0.845	14.45
DS0002	Danshui	2000, Feb	62	80.4	17.6	2	53.5 ± 2.3	47.0–58.5	0.832	0.865	14.38
DS0012	Danshui	Dec	71	98	2	0	55.0 ± 2.5	49.5–61.5	0.823	0.865	14.87
DS0211	Danshui	2002, Nov	65	0	0	100	54.6 ± 3.3	46.6 – 66.4	0.837	0.860	14.49
DS0401	Danshui	2004, Jan	57	84	16	0	54.8 ± 2.3	49.5–60.9	0.825	0.873	15.60
DS0501	Danshui	2005, Jan	73	96.1	0	3.9	56.2 ± 1.7	52.0–60.5	0.814	0.856	14.82
DS0601	Danshui	2006, Jan	54	96	2	2	55.4 ± 2.4	49.3–60.9	0.806	0.851	14.66
DS0611	Danshui	Nov	52	0	40	60	53.3 ± 2.6	48.8–58.0	0.852	0.868	15.13
DS0712	Danshui	2007, Dec	60	82.	12.5	4.7	54.9 ± 2.3	47.9–59.6	0.814	0.863	15.09
FL0111	Fulong	2001, Nov	64	25.5	40.3	34.2	54.8 ± 2.2	47.6–49.5	0.837	0.865	14.20
FL0112	Fulong	Dec	75	62.9	13	24.1	54.4 ± 2.5	49.3–60.0	0.829	0.870	15.27
FL0201	Fulong	2002, Jan	65	97.9	2.1	0	57.0 ± 2.3	51.3–62.3	0.796	0.861	14.52
FL0202	Fulong	Feb	52	15	32.5	52.5	54.0 ± 2.2	48.9–60.3	0.802	0.877	15.13
FL0203	Fulong	Mar	59	1.7	15.5	82.8	53.3 ± 2.7	46.6–59.0	0.806	0.862	14.80
FL0204	Fulong	Apr	60	81.1	10.8	8.1	54.5 ± 3.0	48.0–62.4	0.819	0.874	15.30
China											
MJ9402	Min Jiang	1994, Feb	40	94.9	3.4	1.7	52.5 ± 1.9	47.1–56.4	0.750	0.865	14.42
QT9403	Qiantang Jiang	Mar	40	100	0	0	53.1 ± 2.0	48.8–56.6	0.832	0.863	15.05
XM0001	Xiamen	2000, Jan	56	100	0	0	52.2 ± 2.2	46.7–57.4	0.770	0.847	14.96
YT0002	Yangtze	Feb	57	96.4	3.6	0	53.1 ± 1.6	50.2–57.0	0.845	0.875	14.84
Japan											
MB0202	Mikawa Bay	2002, Feb	60	98	2	0	56.0 ± 2.1	49.9–59.8	0.790	0.861	14.65
MB0203	Mikawa Bay	Mar	45	97.6	2.4	0	54.6 ± 1.7	51.0–58.5	0.835	0.870	15.67
MB0204	Mikawa Bay	Apr	50	0	14.3	85.7	54.6 ± 2.2	50.3–59.7	0.811	0.867	14.83
Korea											
YL9404	Yalu Jiang	1994, Apr	51	0	80.8	19.2	55.2 ± 2.9	49.3–60.0	0.860	0.854	13.96

Table 2. *Anguilla japonica*. Characteristics of 8 microsatellite DNA loci in Japanese eels including repeat motif, primer sequence, annealing temperature, GenBank accession no. and fluorescence label. F: forward; R: reverse

Locus	Repeat motifs	Primer sequence (5′–3′)	Annealing temperature (°C)	Accession no.	Fluorescence (reverse)
AJMS-2	(GA)n	F: ATT TCA CGT CAT CGG ACC TGC R: GCT GGG AGC GAC GCT TTA TC	60	AJ297600	5′FAM
AJMS-3	(GT)n	F: GGT ATG AAT GCA GGC GTT TAT G R: GCA ACC GAT TTG ATC TCC AG	60	AJ297601	5′TAMRA
AJMS-5	(GT)n	F: CCT TCA GAT TGC TAG CAC R: CGG AGT CTA ATT GTC TCC TC	58	AJ297602	5′HEX
AJMS-6	(GT)n	F: ACA GAG CCA GAC AAA CAG AC R: GGT CAG CAA GCA AAA CGA AC	58	AJ297603	5′HEX
AJM-1	(GT)n	F: AGT AAA GAG TCC CAC GCA TTC R: AAG GTG GAT TTT TGC TGG CTC	60	AM062761	5′TAMRA
AJM-8	(GT)n	F: TGG CTG AAG TGA GTA TGC T R: AGA TAT GGA AGC AGG ATG GAG	60	AM062762	5′HEX
AjTR-12	(GA)n	F: AAC GTT AGT CCC TAG GTT CC R: TAA GGG TGT TAT ATG TTC AG	58	AB051084	5′FAM
AjTR-37	(GT)n	F: AGA CCT TAT GTC ACC TTA TGC T R: AAG ATG TTA AAT TCA ATT GTG C	58	AB051094	5′FAM

primers, 2.5 µl of 10× PCR buffer, 0.6 µl of 10 mM dNTPs and 19.3 µl of Milli-Q H_2O. The reverse primers contained FAM, TAMRA, or HEX fluorescent labels for genotyping (Table 2). The PCR amplification protocol was as follows: initial denaturation at 94°C for 3 min followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 58 to 60°C for 30 s (Table 2) and extension at 72°C for 30 s. A final extension was carried out at 72°C for 10 min. For genotyping, 1 µl of the PCR product was diluted with 12 µl Milli-Q H_2O, and fragment analysis was performed using a Megabase 1000 DNA analysis system (Amersham Biosciences). The data were scored with Genetic Profiler™ v. 2.0 (Amersham Biosciences), and the sizes of each allele were inspected visually.

Data analysis. One-way ANOVA was used to test the differences in the mean TL of the glass eel samples. To correct for multiple testing, we also used Tukey's HSD test. The observed numbers of alleles (n_a), observed (H_o) and expected (H_e) heterozygosities and deviations from the Hardy-Weinberg equilibrium (HWE) were independently calculated for each locus using ARLEQUIN v. 3.1 (Schneider et al. 2000). Computation of the allelic richness for the specified sample sizes was based on the rarefaction method (Hurlbert 1971) using FSTAT v. 3.9.5. Significant differences in allelic richness or heterozygosity among samples were tested using a nonparametric Friedman test followed by a pairwise Wilcoxon signed-ranks test. The allele dropout, null alleles and scoring errors for each sample were tested using Micro-Checker v. 2.2.3 (Van Oosterhout et al. 2004). Partitioning of genetic differentiation was performed by ARLEQUIN v. 3.1 using the locus-by-locus analysis of molecular variance (AMOVA) with 10 000 permutations. Pairwise Wright's fixation indices, F_{ST}, were calculated using FSTAT v. 3.9.5 (Goudet 1995), and the significance levels were adjusted by a sequential Bonferroni correction (Weir & Cockerham 1984, Rice 1989). A phylogenetic tree based on pairwise Nei's (1983) unbiased genetic distances (D_A) was constructed by a neighbor-joining phenogram using the DISPAN program with 1000 bootstrap replicates. IBT and IBD were estimated using Mantel tests (Mantel 1967) implemented in the ARLEQUIN v. 3.1 software by correlating the spatial distance (kilometers between sites) or temporal distance (days or months between samples) with the D_A.

Statistical power was analyzed with the program POWSIM (Ryman & Palm 2006). This novel simulation method was applied to assess the statistical power for detecting population heterogeneity at various true levels of divergence, with the applied set of markers and sample sizes. The program detected significant differentiation (using chi-square and Fisher's exact tests) under a specified level of population divergence given by $1 - (1 - 1/2N_e)^t$, where t is the time since divergence and N_e the effective population size. Simulations were run to detect an expected divergence of F_{ST} = 0.001 to 0.0025 for arrival waves between Taiwan and Japan, with 7 microsatellite loci for 9 groups with 530 individuals. N_e/t combinations corresponded to 500/5, 2000/10 and 4000/20 for F_{ST} = 0.0025, and 2500/5, 5000/10 and 10000/20 for F_{ST} = 0.001.

RESULTS

Morphometric data

The mean and range for TL of 31 samples of glass eel are shown in Table 1. Significant differences were observed when the mean TL of all samples (F = 50.37, $p < 0.001$) was compared; however, no specific trend was detected (Fig. 2). The mean TL was in the range of 52.2 to 57.5 mm with great variation among samples (Fig. 2). Highly significant differences were observed among samples within the sites of both the Danshui River (annual cohorts) and Fulong estuary (arrival waves). However, a nested ANOVA showed no significant differences among the sites in Taiwan when temporal samples within the site were pooled ($p > 0.05$ for all). The mean TL for samples from China was significantly lower than those from other areas ($p < 0.001$ for all). No significant differences in mean TL were found for samples from Taiwan, Japan and Korea when within-area specimens were pooled.

The pigmentation stages of all 31 samples are shown in Table 1. As indicated, most samples had Stage V pigmentation during collection. However, some samples from Yalu Jiang, Mikawa Bay, Fulong and Danshui showed >50% Stage VI pigmentation (Table 1).

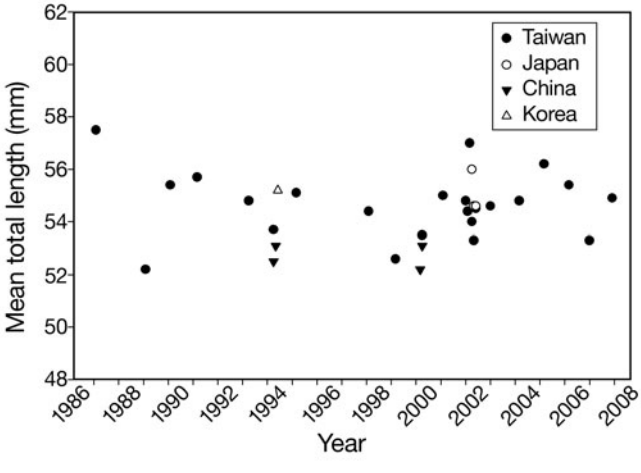

Fig. 2. *Anguilla japonica*. Mean total length distribution of all 31 Japanese eel samples from 1986 through 2007

Genetic diversity within samples

A total of 8 polymorphic loci were screened; all were moderately to highly polymorphic such that the total number of alleles ranged from 14 in locus *AJMS*-3 to 46 in locus *AJM*-8 (Table 3). The H_o and H_e of each sample ranged from 0.542 to 0.961 and from 0.515 to 0.965, respectively. The HWE test showed only a few significant deviations after Bonferroni corrections for all loci (k = 248), with the exception of the *AJMS*-2 locus (4 of 31 for *AJMS*-2 and 5 of 217 for the other 7 loci). These deviations were scattered in the *AJM*-1, *AJM*-8, and *AjTR*-12 loci without a sample- or locus-specific pattern. Null alleles were concentrated in the *AJMS*-2 locus (16 of 31), as tested by Micro-Checker. The other loci showed a scattered pattern for null alleles (24 of 217) without a sample- or locus-specific pattern. There was no evidence of allele dropout, and very few scoring errors (6 of 248) were detected for all loci. AMOVA for each locus indicated that most of the total genetic variation was within samples (>99.4%), and only <0.6% was between samples, with the exception of *AJMS*-2 (1.5% between samples) (Table 3). Owing to the poor quality of data for the *AJMS*-2 locus, it was excluded from subsequent analyses.

Table 3. *Anguilla japonica*. Locus characteristics of the genetic variation for observed (H_o) and expected (H_e) heterozygosity and F_{ST} for 8 loci

Locus	No. of alleles	H_o	H_e	F_{ST}	p-value
AJMS-2	36	0.854	0.911	0.015	<0.001
AJMS-3	14	0.684	0.646	0.004	<0.001
AJMS-5	30	0.843	0.904	0.001	0.173
AJMS-6	30	0.833	0.872	0.002	0.047
AJM-1	27	0.816	0.880	0.005	<0.001
AJM-8	46	0.865	0.954	0.002	0.006
AjTR-12	41	0.864	0.933	0.002	0.008
AjTR-37	25	0.813	0.853	0.001	0.179

The mean H_o, mean H_e, and allele richness (AR) of the 31 samples are shown in Table 1. There were no overall significant differences in AR (p = 0.486) or H_o (p = 0.141) among the samples. Pairs with significant differences in AR or H_o were scattered without a spatial- or temporal-specific pattern.

Spatial-temporal genetic structure

Overall, genetic differentiation among the 31 samples was very low, yet significant (F_{ST} = 0.003, p < 0.001, Table 4). Genetic differentiation among annual cohorts collected from a single location was studied for glass eels collected from the Danshui River, Taiwan. Low but significant genetic differentiation among 16 samples (1986 to 2007) was observed (Table 4). However, among these, pairwise F_{ST} comparisons were significant only in 2 of 120 tests (between Danshui samples [see Table 1] DS0012 and DS8612, and DS0012 and DS0401, k = 100). No significant difference was observed in genetic differentiation among arrival waves from the Fulong estuary, Taiwan, and Mikawa Bay, Japan (Table 4), or in the pairwise F_{ST} tests of recruits from these 2 locations (k = 50). When combining data from the Fulong estuary and Mikawa Bay, the 2-level hierarchical analysis also showed no significant genetic differentiation either among locations (F_{CT} = 0, p = 0.527) or among temporal recruits (F_{SC} = 0.001, p = 0.319) (Table 4).

Table 4. *Anguilla japonica*. Test for spatial and temporal genetic differentiations in Japanese eel samples by 2-level hierarchical AMOVA based on 7 loci. Analyses 1 to 3 refer to comparisons between spatial and temporal samples within given sites, analyses 4 to 6 refer to comparisons between temporal samples within given sites, and analyses 7 to 10 refer to comparisons between locations in given years and area. F_{ST}, F_{CT}, F_{SC}: tests permuting genotypes among populations and among groups, permuting whole populations among groups, and permuting genotypes among populations but withhin groups, respectively

Analysis	Item	Comparison	No. of samples	No. of ind.	Indicies	p-value
1	Spatial and temporal samples	All samples (9 sites)	31	1770	F_{ST} = 0.003	<0.001
					F_{SC} = 0.002	<0.001
					F_{CT} = 0.001	0.002
2		Taiwan and Japan (2001–2002, 2 sites)	9	530	F_{ST} = 0.001	0.322
					F_{SC} = 0.001	0.319
					F_{CT} = 0.000	0.527
3		Taiwan (3 sites)	23	1371	F_{ST} = 0.002	<0.001
					F_{SC} = 0.002	<0.001
					F_{CT} = 0.001	0.085
4	Temporal samples	Taiwan annual cohorts (Danshui)	16	951	F_{ST} = 0.004	<0.001
5		Taiwan arrival waves (Fulong)	6	375	F_{ST} = 0.001	0.375
6		Japan arrival waves (Mikawa Bay)	3	155	F_{ST} = 0.001	0.347
7	Spatial samples	Locations (1994, 4 sites)	4	186	F_{ST} = 0.006	0.002
8		Locations (2000, 3 sites)	3	175	F_{ST} = 0.006	0.001
9		China (4 sites)	4	193	F_{ST} = 0.008	<0.001
10		All locations (9 sites)	10	518	F_{ST} = 0.004	<0.001

Genetic differentiation between locations within annual cohorts was also determined for glass eels collected in 1994 (4 sites) and 2000 (3 sites). We observed a low but significant genetic differentiation among locations in samples from 1994 and 2000 (Table 4). Pairwise F_{ST} comparisons showed a significant difference between Qiantang and Yalu Jiang in 1994 and between Xiamen and Fulong in 2000. When temporal samples were pooled within sites, the overall differentiation remained significant for glass eels from Taiwan (3 sites), China (4 sites) and all locations (9 sites) (Table 4). Two-level hierarchical AMOVA analyses of the samples from Taiwan showed that genetic variations among samples within sites (F_{SC} = 0.002, p = 0) were larger than the differences among sites (F_{CT} = 0.001, p = 0.085). When testing pairwise F_{ST} for glass eels collected from all 9 locations in 1993, 1994, 2000 and 2002, only 2 of the 45 tests were found to be significant (k = 50).

Statistical power

The POWSIM analysis of statistical power revealed that 7 microsatellite loci were sufficient to provide a >80% probability of detecting an F_{ST} of 0.001 for a chi-square test when analyzing a total of 530 specimens distributed over 9 groups. Although the likelihood of not detecting genetic differentiation could not be excluded, the true degree of genetic differentiation among the studied arrival waves must, nevertheless, have been very small.

IBD, IBT, and cluster analysis

The neighbor-joining phenogram based on the D_A among all 31 samples did not show any clustering of temporal samples within sites or locations within regions (Fig. 3). The most robust node was supported in only 48% of the bootstrap replicates.

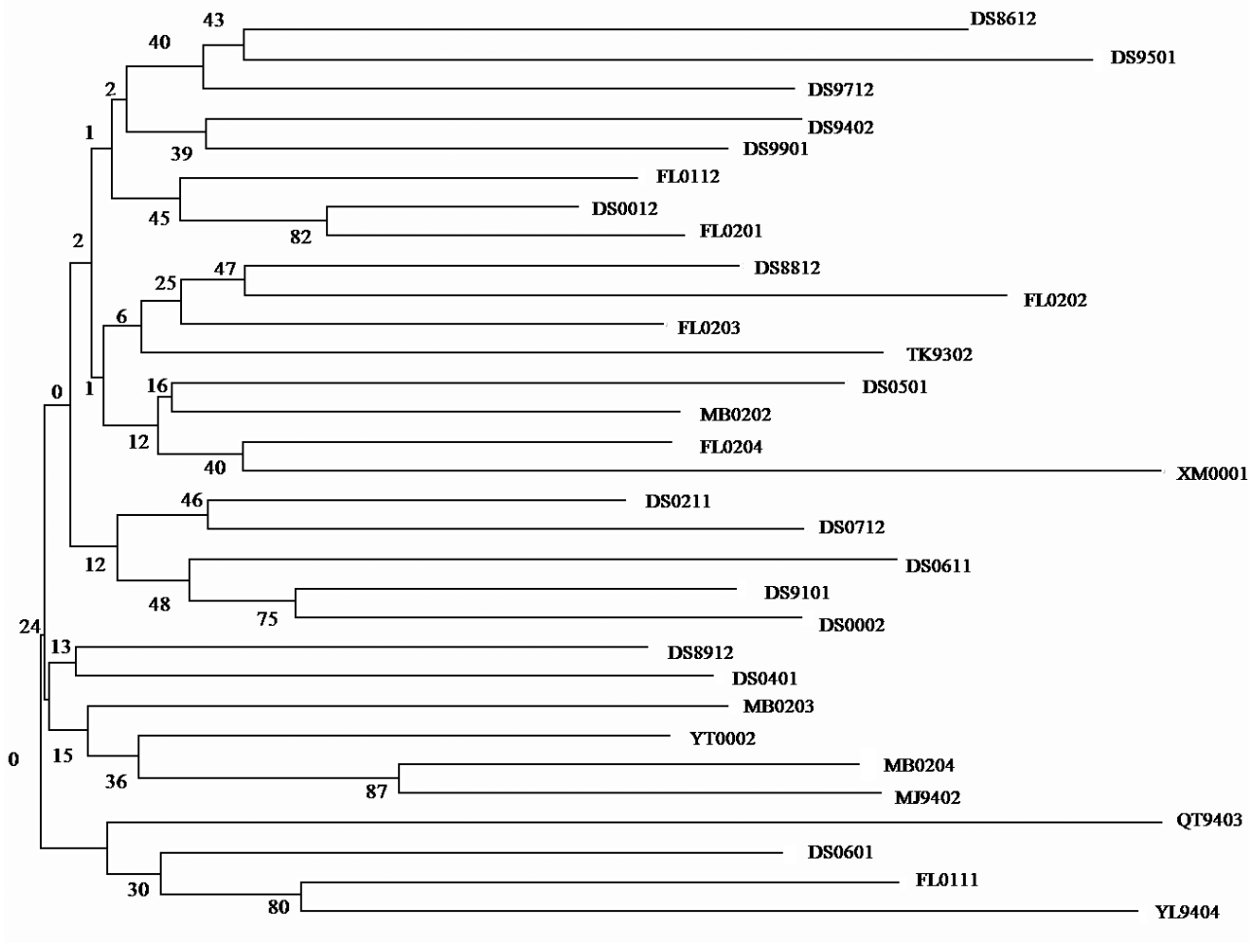

Fig. 3. *Anguilla japonica*. Neighbor-joining phenogram based on Nei's (1983) unbiased genetic distance among 31 samples of the Japanese eel. Bootstrap values at each node were calculated using 1000 replicates over loci. The designation marks refer to the samples listed in Table 1

The Mantel test revealed no significant correlation between differences in the days of recruitment and D_A among arrival waves (Fulong) (r = 0.190, p = 0.497) (Fig. 4a). If the genetic distance $F_{ST}/(1 - F_{ST})$ was used instead of D_A, the IBT test for arrival waves remained insignificant (data not shown). There was also no significant correlation between differences in the months of recruitment and D_A among annual cohorts (Danshui) (r = 0.146, p = 0.112) (Fig. 4b). However, if the genetic distance $F_{ST}/(1 - F_{ST})$ was used, the correlation showed marginal significance (r = 0.094, p = 0.027). We also found no correlation between D_A and the nearest sea distance by using pairwise comparisons of 9 locations (r = 0.094, p = 0.541) (Fig. 4c). If the genetic distance $F_{ST}/(1 - F_{ST})$ was used, IBD test for all locations remained insignificant (data not shown).

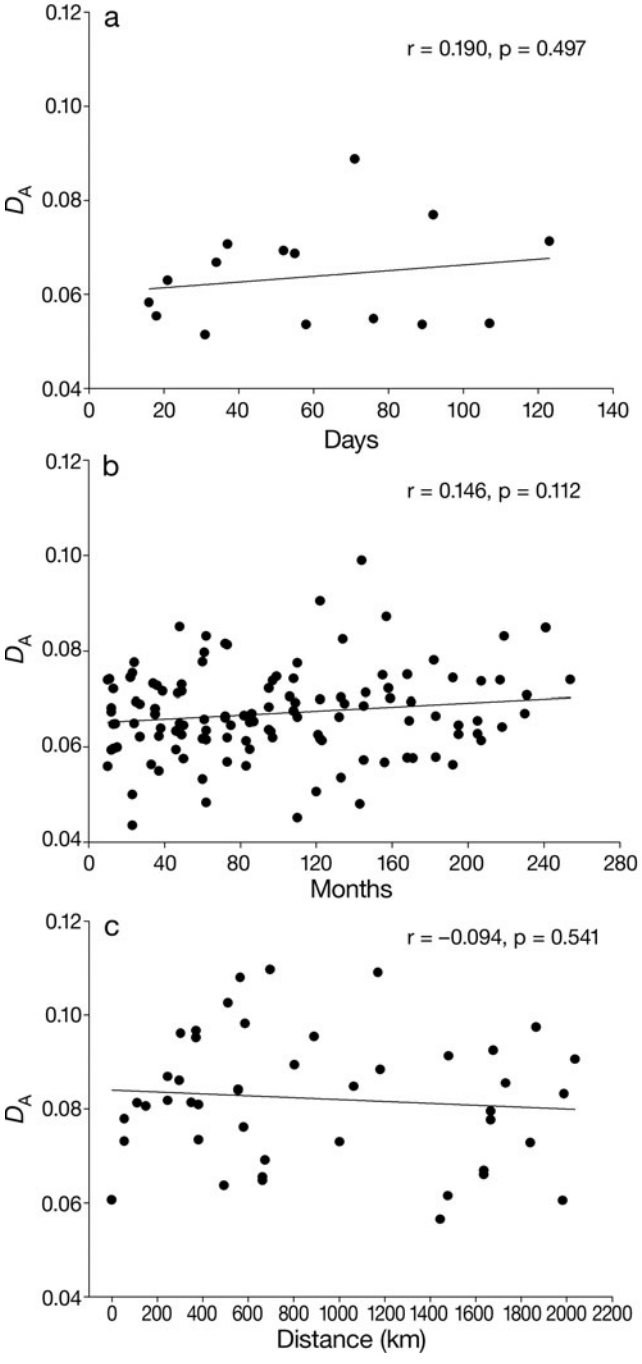

Fig. 4. *Anguilla japonica*. Regression of genetic differentiation (D_A) at microsatellites on temporal distance (a) in days among arrival waves of Fulong, (b) in months among annual cohorts of Danshui and (c) on spatial distance in kilometers from the 9 sites in the present study. Pearson's correlation coefficient r and p-values were obtained from the Mantel test

DISCUSSION

Morphometric heterogeneity

Our study shows a significant heterogeneity in size among all samples. One possible explanation for the morphometric heterogeneity is the seasonal or interannual changes in feeding conditions, with larvae that migrated across the ocean under different primary production reaching different sizes (Tzeng 1990, Desaunay & Guerault 1997, Reveillac et al. 2008). Nevertheless, no specific trend was observed for the overall mean TL among annual cohorts over the last 2 decades. In the European (Desaunay & Guerault 1997) and American eels (Haro & Krueger 1988, Castonguay et al. 1994), however, the biometrics of glass eel appear to exhibit a decrease since the early 1980s. Since we have no samples from before the 1980s, no comparison is currently available.

Eel recruits from China were generally smaller than those from other areas. In the European eel, Boëtius & Boëtius (1989) supposed that the metamorphosed glass eel might not immediately enter the estuary but stay offshore and starve. Thus, a different dispersal route might account for the differences in the sizes of recruits; that is, the Japanese glass eel might have to move a long distance across the continental shelf, away from the KC, to reach the estuary in mainland China. This may account for the smaller size of the samples from China in comparison with those from Taiwan or Japan. However, the sizes of the specimens from Yalu Jiang were equivalent to those of the specimens from Taiwan and Japan; this is probably because of the advanced development stage of these specimens when they were caught, as indicated by their pigmentation stage.

For the 6 arrival waves of Taiwan (Fulong), some specimens exhibited an advanced pigmentation stage (VIA or VIB), indicating a potential mixing between neighboring arrival waves with specimens born in the same period. Nevertheless, the age difference between Stage V and VI individuals was usually less than

1 mo (Tzeng 1990, Tzeng & Tsai 1992). Thus, it is quite impossible for these samples to hinder the IBT test with a 6 mo interval.

Evidence of panmixia in the Japanese eel

The results of the present study suggest that the Japanese eel population has no specific pattern of genetic differentiation in both spatial and temporal scales. First, the overall genetic variations among the arrival waves from both Taiwan and/or Japan were not significant. Second, although the overall F_{ST} showed significance for spatial recruits within the indicated years or areas, pairwise F_{ST} tests showed few significant genetic differentiations. Third, the phylogenetic tree showed no spatial- or temporal-specific patterns. Taken together, these results clearly suggest that the Japanese eel probably constitutes only a single population both spatially and temporally.

The population structures of anguillid eels have long been considered panmictic. This is because sexually mature stocks migrate and spawn in a single site, and their larvae are passively transported back to their growth habitats by oceanic currents with a long larval duration, making population genetic structuring quite impossible (Schmidt 1925, Tsukamoto 1992, Avise 1994, Tesch 2003, Aoyama 2009). However, Tseng et al. (2006) suggested the presence of 2 genetically different groups in an indicated year, i.e. low-latitude (southeastern China and Taiwan) and high-latitude (Japan, Korea and northeastern China) groups based on 6 microsatellite DNA loci analyses. The authors presumed that the leptocephali of the Japanese eel tend to be transported back to certain latitude ranges where their parents were located, with little gene flow between the north and south eel groups. In the present study, however, the neighbor-joining phenogram did not show any clustering of temporal samples within sites or locations within regions. When the samples were analyzed in greater detail, the overall F_{ST} showed a significant difference in spatial recruits within the indicated years or areas. However, only a few pairwise F_{ST} tests showed significant genetic differentiations, and these genetic variations were not consistent over time when the temporal component was taken into consideration. In the European eel, temporal variation among samples may be misinterpreted as geographical isolation (Dannewitz et al. 2005, Maes et al. 2006). Therefore, one possible explanation for the genetic partitioning of spatial samples within an indicated year in Tseng et al.'s (2006) study may be 'chaotic genetic patchiness' in which random variations in parental contributions to reproductive activity, incomplete mixing of larvae and kin aggregation may act in concert to produce offspring with genetic heterogeneity (Hedgecock 1994a,b, Selkoe et al. 2006). This sporadic occurrence of genetic differentiation in space would mask the true population genetic structure of the Japanese eel when there is a lack of replicates over time. Alternatively, another possible explanation for the discrepancy between Tseng et al.'s (2006) results and those obtained in the present study might be that the *AJMS*-2 locus was incorporated in the former study (locus *MS*-2 in Tseng et al. 2006). This locus showed some deviations from HWE and had abundant null alleles in comparison with other loci, as observed in this study. When the *AJMS*-2 locus was included in the analyses, the degree of genetic differentiation among samples became more pronounced, although spatial- or temporal-specific patterns could still not be detected.

Japanese eels appear to spawn in a restricted area, and their larvae are passively transported by the NEC and KC, both of which exhibit considerable changes in speed, eddy structure and route at daily, monthly and even yearly levels (reviewed in Aoyama 2009). If there were actually 2 genetic populations of Japanese eels, they must have been a consequence of a nonrandom return of larvae to the place where their parents formerly resided. One possibility might be the heritable differences in larval durations between southern and northern groups. Since the newly arrived recruits are quite similar in TL (Tsukamoto 1990, Umezawa & Tsukamoto 1990, Cheng & Tzeng 1996, Kawakami et al. 1999, Chang et al. 2007) indicating a size-dependent metamorphosis of the leptocephali in the Japanese eel, it is likely that the fast-growing leptocephali have a heritable short larval duration and mostly metamorphose in the south, while the slow-growing ones with a heritable long larval duration may mostly drift north along with the KC. We tested this possibility by examining genetic differentiation between eel groups with different larval durations. However, no significant genetic differentiations were observed between samples with short or long larval durations (Y. S. Han et al. unpubl. data).

In summary, on the basis of our present knowledge of oceanographic variations and the larval migration of the Japanese eel, we cannot hypothesize any mechanism by which this species can be grouped into multiple populations.

No IBT patterns for arrival waves

Japanese eels appear to spawn in synchrony each month around the new moon periods (Ishikawa et al. 2001b, Tsukamoto et al. 2003) indicating the existence of multiple 'spawning stocks.' Thus, glass eels in estuaries usually occur in pulses and last for a few months (Chang et al. 2007, Han et al. 2009). Based on back-

calculations of the otolith daily growth rings, it has been proven that arrival waves within sites consist of individuals spawned in different months (Tzeng 1990, Reveillac et al. 2008). If the spawning time of the Japanese eel is heritable, genetic differentiation might have occurred within arrival waves on account of the temporal isolation of reproductive activity. In a previous study, Chang et al. (2007) found that glass eels of arrival waves recruited to the Danshui estuary in Taiwan exhibited subtle genetic patchiness without overall significant temporal genetic variations. In the present study, we found no overall genetic differentiations among arrival waves either from the Fulong estuary in Taiwan or from Mikawa Bay in Japan. IBT tests using the genetic distance $F_{ST}/(1 - F_{ST})$ or D_A also showed no significance, suggesting a panmixia for the Japanese eel on a temporal scale.

The low genetic differentiations among arrival waves might indicate a potential larval mixing. The long dispersal time for the leptocephali (4 to 6 mo) and protracted spawning season of the reproducers provide conditions required for larval mixing between monthly arrival waves. In fact, the birth dates of previous arrival waves caught in Taiwanese estuaries partially overlapped with those of the next arrival wave (Tzeng 1990). Shinoda (2004) analyzed glass eels collected from 9 sites in East Asia and reported that the mean larval duration of the Japanese eel is 156 d with a large variation range of 98 to 227 d. Therefore, the larval mixing might buffer genetic heterogeneities between arrival waves to some extent. Alternatively, if the spawning time between early and late arrival waves is heritable, a temporal Wahlund effect may occur owing to the mixing of recruits from groups with distinct spawning times (Nielsen et al. 2003); such circumstances were not found in the present study. Since spawning stocks may start to migrate from distant locations at different times, and even by different routes and swimming speeds, the times required for each individual to reach the spawning area must differ. Thus, it is not possible for a heritable spawning time to be established in the Japanese eel.

Slight genetic shift for annual cohorts over 21 yr

In our previous study (Han et al. 2008), we investigated changes in the genetic composition of Japanese eel recruits over the last 2 decades using 6 polymorphic microsatellite DNA loci. Our data showed that although the overall genetic differentiation among all samples was significant, only 2 of 120 pairwise F_{ST} tests were significant. The Mantel test using $F_{ST}/(1 - F_{ST})$ showed an insignificant correlation ($r = 0.173$, $p = 0.0504$). In the present study, we used mostly overlapped samples with 7 microsatellite DNA loci for reanalyses and similar results were obtained. However, the Mantel test for annual cohorts using $F_{ST}/(1 - F_{ST})$ became marginally significant ($p = 0.027$), suggesting a slight year-to-year variation in the genetic composition of temporal samples, possibly due to a random genetic drift. It is likely that yearly variations in reproductive success led to random slight allele frequency shifts among annual cohorts and that cumulative genetic variation may be observed after many generations. For the Japanese eel, >50% of individuals had a life cycle between 5 or 6 yr (Han et al. 2009). Thus, an average of 4 generations was included in the samples encompassing 21 yr. This may also explain why overall genetic differences were significant among annual cohorts, but not among arrival waves of a single site when samples were collected and analyzed in detail. To trace long-term changes in the genetic composition of the Japanese eel, sample collection over a longer duration is needed. However, these data are currently not available.

In conclusion, the patterns of genetic structure in the Japanese eel can be explained on the basis of the following: (1) a panmixia of the Japanese eel in spatial and temporal scales, as evidenced by a chaotic phylogenetic tree and a lack of both IBT and IBD patterns, (2) a slight genetic drift of the genetic composition for annual cohorts within a single site over the last 2 decades, as evidenced by a generally stable AR and H_o but a marginally significant Mantel test result, and (3) sporadic genetic variations among samples, which occurred on a random scale and could not have created a stable genetic structure that would last more than a year. Thus, our findings have important implications for implementing a reasonable management program for the Japanese eel. A panmictic population of the Japanese eel in East Asia indicates that it is a single management unit and regulations must involve all East Asian countries to prevent further decline of this valuable eel resource.

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