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# Genetic evidence for panmixia of Japanese eel (*Anguilla japonica*) populations in China

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**ABSTRACT.** The Japanese eel population has dramatically declined since the 1970s. In order to conserve this species, the background genetic structure affecting these populations should be well documented. Previous genetic studies of this species have produced seemingly conflicting results, ranging from no detectable heterogeneity to small, but statistically significant variance. This study investigates the population structure of Japanese glass eels collected from 10 localities in China in 2009 using a mitochondrial DNA (mtDNA) control region and 19 polymorphic microsatellite loci. Results revealed evidence of low genetic differentiation using both mtDNA ($F_{ST}$ = 0.001, P = 0.291) and microsatellite data ($F_{ST}$ = 0.003, P = 0.008). Pairwise F-statistic values generated from mtDNA and microsatellite DNA were similar, showing little evidence of significant genetic differentiation. The minimum spanning haplotype network constructed using mtDNA control regions produced no clear phylogeographic structure. The Mantel test revealed no significant correlation with distances for both mtDNA and microsatellite DNA. Therefore, our results suggest

 

a panmictic population of Japanese eels in China, which should be conserved as a single management unit.

**Key words:** Population genetics; *Anguilla japonica*; Mitochondrial DNA; Microsatellite DNA; Panmixia

## INTRODUCTION

Many marine fish larvae and juveniles are transported and dispersed across vast geographic regions via oceanic currents. Without the hindrance of the physical barriers faced by terrestrial organisms, these species are less likely to experience genetic or behavioral constraints on their reproductive processes. Marine fish populations share several general characteristics, including large effective population sizes, wide population distributions, and highly fecund populations, which all lead to the expectation of a weak or nonexistent population structure (Gyllensten, 1985; Ward et al., 1994). The study of marine fish population structure by means of molecular markers can be challenging due to the vast distributions and sizes of these populations (Palm et al., 2009). Additionally, gene flow among populations may be substantial, and effective population sizes within parental populations can limit the extent of genetic differentiation observed among subpopulations (Waples and Gaggiotti, 2006). Larval and juvenile marine fish species often exhibit high fecundity; a factor that may further impact spurious observations of genetic differentiation based on the inability to distinguish between generations and subpopulations.

The Japanese eel (*Anguilla japonica* Temminck & Schlegel) is a temperate catadromous fish with an extensive migratory loop and a complex life cycle. The freshwater distribution of the species ranges across rivers of the northeastern Asian regions of Taiwan, China, Japan, and Korea (Tsukamoto, 1992). The spawning area of this species is located in the western Mariana Islands at 14°-16° N, 142° E, approximately 2000 to 3500 km away from the East Asian continent (Tsukamoto, 1992; Tsukamoto et al., 2011).

Leptocephalus larvae are born between April and November (Tsukamoto, 1992) and then disperse from their original spawning area via the North Equatorial Current (NEC) and the Kuroshio Current (KC), reaching the coasts of East Asia within only 4 to 6 months (Cheng and Tzeng, 1996). Leptocephalus larvae subsequently undergo metamorphosis into juvenile Japanese eels, which are commonly referred to as "glass eels". They can develop in either freshwater, brackish water, or seawater for more than 4 years before metamorphosing into adult silver eels, and reach sexual maturation in autumn and winter (Han et al., 2010b). Sexually mature eels migrate back to their spawning area to engage in semelparous reproduction, resulting in the death of the reproductive individual during the spawning process (Tsukamoto, 1992).

The Japanese eel is a commercially important aquaculture species in East Asia. However, environmental changes, habitat destruction, and the threat of commercial overfishing have resulted in an overall population decline of approximately 90% from 1970 to 2003, and this downward trend continues currently (Han et al., 2010a). Because of the species' importance as a commercial product, its management is increasingly critical. Assessment of this population's genetic structure allows researchers and policy makers to develop an understanding of Japanese eel population structure and demographic history that is urgently needed for effective management and conservation planning.

 

The concept of panmictic populations for temperate eels, including the Japanese eel, was originally established through the use of genetic studies focusing on allozyme and mitochondrial DNA (mtDNA) markers (Sang et al., 1994; Ishikawa et al., 2001). Similar results were demonstrated for European eel (Lintas et al., 1998) and American eel (Avise et al., 1986) populations, with the exception of clinal allozyme variation putatively imposed by selection (Williams et al., 1973; Chan et al., 1997). Recent studies have challenged the existence of a panmixia population structure in temperate eels. For example, Wirth and Bernatchez (2001) used seven microsatellite loci to report significant spatial differences and isolation by distance (IBD) across the continental range of European eel populations. Similar results were presented in several other contemporary studies (Daemen et al., 2001; Maes and Volckaert, 2002). Based on an analysis of eight microsatellite loci, Tseng et al. (2006) found slight population genetic differentiation in Japanese eels based on the geographic subdivision of low-latitude (South China and Taiwan) and high-latitude (Japan, Korea, and North China) subpopulations with no apparent IBD pattern. Researchers theorized that the spatial differences observed between European and Japanese eel populations could be caused by non-random return of larvae to the freshwater habitat of their parents, perhaps through inherited traits or environmental cues (Wirth and Bernatchez, 2001; Maes and Volckaert, 2002; Tseng et al., 2006).

Conversely, previously observed IBD patterns were shown to be unstable over time when temporal replicates of geographical sampling were included in one of the most comprehensive analyses of Japanese eel populations conducted to date, which included a total of 41 sampling sites containing 12 inter-annual samples (Dannewitz et al., 2005). Dannewitz et al. (2005) and Palm et al. (2009) also suggested that panmixia was likely to occur in the European eel. A comparison of annual cohort (or "arrival wave") studies focusing on European eel populations revealed evidence of genetic patchiness (Maes et al., 2006; Pujolar et al., 2006). In Japanese eel populations, Han et al. (2010a) observed slight, but distinctly significant, genetic differentiation among glass eel annual cohorts collected from a single location in the Danshui River, Taiwan.

Although the genetic structure of the Japanese eel has been widely addressed, results have remained inconclusive and conflicting, often demonstrating no detectable heterogeneity (Sang et al., 1994; Ishikawa et al., 2001; Chang et al., 2007; Han et al., 2010a,b) or latitudinal genetic variation and differentiation (Chan et al., 1997; Tseng et al., 2006). Some of these studies, including all studies cited above, were limited by research methodology, employing only one DNA marker (microsatellites) (Sang et al., 1994; Chan et al., 1997; Ishikawa et al., 2001; Tseng et al., 2006; Chang et al., 2007; Han et al., 2010a,b). Other results were skewed by the use of relatively small numbers of loci (6-8 microsatellite loci) (Tseng et al., 2006; Han et al., 2010a) or sampling bias due to specimen collection from a single-annual cohort, resulting in misleading conclusions when applied to an entire geographic population (Sang et al., 1994; Ishikawa et al., 2001; Tseng et al., 2006; Han et al., 2010a).

In the present study, the use of mtDNA control region sequences combined with an analysis of 19 distinct microsatellite loci allowed an investigation of the population genetic structure of the Japanese eel, specifically glass eel populations from the Yellow Sea, the East China Sea, and the Southern China Sea, that is significantly more comprehensive than previous studies. By isolating a single-annual cohort (1 year) from the same developmental stage (glass eel), results of this study provide a detailed understanding of: 1) Japanese glass eel population structure and variation along the coast of China, and 2) the pattern of genetic differentiation and isolation variation within the geographic distribution of Japanese glass eel subpopulations.

## MATERIAL AND METHODS

### Sampling and DNA extraction

To reduce sampling bias, only glass eels were used in this study. A total of 362 individuals were collected from 10 localities along the coastline of China between January and April of 2009 (Table 1; Figure 1). The specimens were collected using a standard set net and were preserved in 95% ethanol. Total DNA extraction was performed according to the well-documented phenol:chloroform:isoamyl alcohol method. The extracted DNA material was stored at -20°C.

**Table 1.** Summary of the collection data and sizes of Japanese glass eels used in the microsatellite and mtDNA analyses.

| Sample site | Region | Latitude (°N) | Longitude (°E) | Collected date | Number of individuals for each analysis | | CR GenBank accession No.† |
|---|---|---|---|---|---|---|---|
| | | | | | MS | CR | |
| South China Sea (SCS) | | | | | | | |
| Xinhui (XH) | Northern SCS | 22.20 | 113.09 | Jan. 2009 | 35 | 11 | JF277166-JF277176 |
| Shantou (ST) | Northern SCS | 23.34 | 116.75 | Jan. 2009 | 40 | 13 | JF277177-JF277189 |
| East China Sea (ECS) | | | | | | | |
| Xiamen (XM) | Taiwan Strait | 24.44 | 118.04 | Jan. 2009 | 34 | 12 | JF277190-JF277200 |
| Fuqing (FQ) | Taiwan Strait | 25.66 | 119.51 | Jan. 2009 | 30 | 12 | JF277201-JF277212 |
| Ningde (ND) | Southern ECS | 26.69 | 119.64 | Jan. 2009 | 35 | 12 | JF277213-JF277223 |
| Yuhuan(YH) | Southern ECS | 27.95 | 121.15 | Mar. 2009 | 41 | 12 | JF277224-JF277234 |
| Taizhou(TZ) | Southern ECS | 28.69 | 121.46 | Mar. 2009 | 34 | 12 | JF277235-JF277245 |
| Cixi (CX) | Southern ECS | 30.45 | 121.13 | Feb. 2009 | 35 | 11 | JF277246-JF277256 |
| Jiuduansha (JD) | Southern ECS | 31.18 | 121.91 | Apr. 2009 | 40 | 12 | JF277257-JF277268 |
| Yellow Sea (YS) | | | | | | | |
| Dafeng (DF) | Southern YS | 33.28 | 120.81 | Feb. 2009 | 38 | 12 | JF277269-JF277280 |
| Overall | | | | | 362 | 119 | - |

MS = microsatellite; CR = mtDNA control region; †nucleotide sequences shared between other localities were not obtained by the accession number.

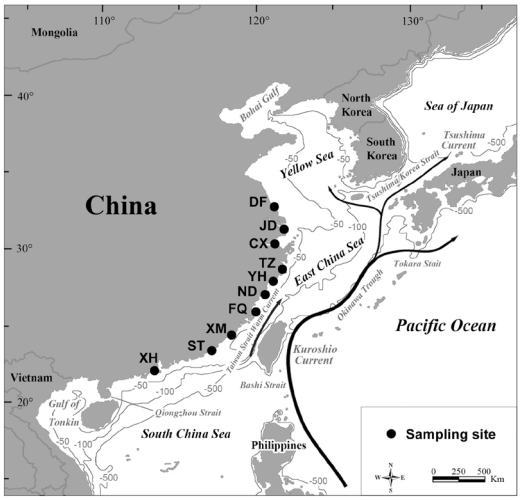


**Figure 1.** Sampling localities of *Anguilla japonica*. The sampling localities of Japanese glass eels (*A. japonica*) are shown with black circles. Detailed information about the samples was in Table 1.

## Mitochondrial DNA sequencing

The whole sequence of the mtDNA control region was amplified using the following primers: CR-F (5'-GCATCGGTTTTGTAATCCG-3') and CR-R (5'-GGGGATATAGGGCATT CTCA-3'). Polymerase chain reaction (PCR) was performed in the Mastercycler Eppendorf Gradient system (Eppendorf, Germany) with a total reaction volume of 50 µL composed of 2 µL genomic DNA (20 ng/µL), 25 µL 2X Taq PCR Master Mix (0.1 U/µL Taq DNA Polymerase, 0.4 mM of each dNTPs, 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$) (TianGen), 2.5 µL (10 µM) of each primer, and 18 µL distilled water. The PCR amplification condition was 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s after heating at 94°C for 5 min. The PCR product was sequenced using CR-F and CR-F2 (5'-GGCATAAAATGAACTATTAC-3') with an ABI 3730 automated sequencer, and these sequences were aligned and edited manually using SEQUENCHER 4.8 (Gene Codes Corporation). The 1258-bp mtDNA fragment was truncated to a 980-bp whole control region fragment after primer sequences and ambiguous sequences were removed. Before manual alignment, all sequences were automatically aligned using ClustalX (Thompson et al., 1997).

## Microsatellite genotyping

Nineteen microsatellite loci, which were obtained from previous studies (Wirth and Bernatchez, 2001; Gong et al., 2008) and NCBI (http://www.ncbi.nlm.nih.gov), were also used in the present study in order to obtain more comprehensive results. PCR was performed in a 12-µL reaction volume composed of 0.8 µL genomic DNA (20 ng/µL), 6 µL 2X Taq PCR Master Mix (0.1 U/µL Taq DNA Polymerase, 0.4 mM of each dNTPs, 20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM $MgCl_2$) (TianGen), 0.6 µL (10 µM) of each primer, and 4.6 µL distilled water. The reaction was carried out at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing temperature for 30 s (Table 2), and 72°C for 30 s, followed by a final 72°C extension for 10 min. The PCR product was visualized using the QIAxcel multicapillary gel electrophoresis system (Qiagen, Germany) and separated with the OM1000 method in a 12-channel gel cartridge QIAxcel DNA High-Resolution Kit. Fragment sizes were scored using the Biocalculator software (Qiagen).

Prior to statistical analysis, MicroChecker version 2.2.3 was used to test for null alleles, stutter miscalls, and allelic dropout. Departures from Hardy-Weinberg equilibrium and linkage equilibrium were tested using GENEPOP 4.0 (Rousset, 2008). Null alleles, large allele dropout, and scoring errors due to stutter peaks were also tested using MICROCHECKER 2.2.0.3 (Van et al., 2004).

## Statistical power of microsatellites

The statistical power of microsatellites was analyzed with the POWSIM software (Ryman and Palm, 2006). POWSIM is a simulation-based computer program that estimates statistical power when testing the null hypothesis of genetic homogeneity for different combinations of sample quantities, sample sizes, locus quantities, allele quantities, and allele frequencies for any hypothetical degree of true differentiation (quantified as $F_{ST}$). Significant differentiation (using chi-squared and Fisher exact tests) was detected under a specified level of population divergence given by the formula: $1 - (1 - 1 / 2Ne)^t$, where $t$ is the time since divergence and $Ne$ is the

**Table 2.** Primer sequences and PCR parameters for *Anguilla japonica* microsatellite loci.

| Locus | Repeat motifs | Primer sequence (5'-3') | Annealing temperature (°C) | Accession No. | k | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|
| AM1-01 | $(GT)_n$ | R: CCAACGAAGAAAAGGGTGTG<br>F: AAAACGGCTGCAGAAGAGAA | 58 | EU003898 | 23 | 0.914 | 0.930 |
| AM1-07 | $(GT)_n$ | R: CGTGTCGAAGTGTCCTTGAG<br>F: GCTGCAGGTTGTCAGCAAT | 58 | EU003900 | 14 | 0.783 | 0.855 |
| AM5-06 | $(CA)_n$ | R: TCACACCATCTGTGGGAAGA<br>F: GGAGCCTTGTGGATGAAAAA | 58 | EU003908 | 17 | 0.856 | 0.826 |
| AM7-06 | $(CA)_n$ | R: AGGGATTTGCACAAAGAGGA<br>F: CTCATTCCATTGAAGGCACA | 58 | EU003912 | 16 | 0.677 | 0.681 |
| AM7-07 | $(GT)_n$ | R: GCTCCGCAAGGTAAGTGCAG<br>F: GTACCGTCGACGATCAGCTCT | 58 | EU003913 | 25 | 0.856 | 0.913 |
| AM8-15 | $(CA)_n$ | R: TAACCAAGAGAACCCCGTGA<br>F: TGCATGCGAGTGTGTGG | 58 | EU003914 | 11 | 0.586 | 0.574 |
| AM9-03 | $(CA)_n$ | R: GACGCATGCTTCTGGAGTCT<br>F: GTCCTGTGGTCCTCCTGTGT | 58 | EU003915 | 36 | 0.925 | 0.949 |
| M07-08 | $(AC)_n$ | R: CCTTCAGATTGCTAGCAC<br>F: CGGAGTCTAATTGTCTCCTC | 56 | AJI297602 | 20 | 0.895 | 0.901 |
| M25-26 | $(AC)_n$ | R: CCAGGCTGATCAAAGATGG<br>F: GTCCAGTACAACACAGTCAG | 58 | AB233977 | 17 | 0.873 | 0.904 |
| M27-28 | $(TG)_n$ | R: AGCGGGCGTTCACACTTCTG<br>F: GTCCGGTCTTCATGCACACCT | 58 | AB233976 | 24 | 0.818 | 0.839 |
| M37-38 | $(CA)_n$ | R: GAAAACAATCGGGTACCACAG<br>F: ACAGTCAGTCACAATGAGCC | 56 | AF237900 | 17 | 0.791 | 0.884 |
| M61-62 | $(TG)_n$ | R: TGCATGTGTGTTTGAGTGC<br>F: TGTAGCGATCGGTCAATCAA | 55 | AB233960 | 24 | 0.741 | 0.828 |
| HML12 | $(AC)_n$ | R: CCCAGCCTGCATGAGAGAGA<br>F: TGACCACTCAGGTGAGCCCTAA | 59 | FJ461373 | 27 | 0.870 | 0.924 |
| HML21 | $(GT)_n$ | R: CCAGAGCGTACAGCAGTTTGTGA<br>F: GAAATGCAGAGGGCGCTGTT | 59 | FJ461382 | 12 | 0.460 | 0.536 |
| HML43 | $(AC)_n$ | R: GCGTGTGTGCATGAGAGAGGA<br>F: GGGCACACGGACAGATTCTC | 61 | FJ461404 | 23 | 0.880 | 0.926 |
| HML48 | $(AC)_n$ | R: CCACACTGGGCAGCACATTT<br>F: AGGGCTGTGGGCAGCCAGTAT | 61 | FJ461409 | 15 | 0.831 | 0.861 |
| HML49 | $(AC)_n$ | R: CCAGCTGCGGGTCAGTGTTA<br>F: TGAACACTCATGCCCACCCTTA | 58 | FJ461410 | 22 | 0.833 | 0.911 |
| HML54 | $(GT)_n$ | R: CCCCATACCAGCTTGGGGTA<br>F: TGCTCCAGAACACAGCAGGTG | 61 | FJ461415 | 14 | 0.731 | 0.817 |
| HML59 | $(CA)_n$ | R: AAGCACCGAAGGACCACTGAA<br>F: GCCCGTGGACAGATTGAAGA | 61 | FJ461420 | 19 | 0.459 | 0.491 |

$k$ = total number of alleles found; $H_O$ = observed heterozygosity; $H_E$ = expected heterozygosity.

effective population size. Simulations (1000 replications) were conducted to detect an expected divergence of $F_{ST}$ = 0.001 to 0.0025 for *A. japonica* population locations, with 19 microsatellite loci for 10 locations with 362 individuals. *Ne*/*t* combinations corresponded to 2500/5, 5000/10, and 10,000/20 for $F_{ST}$ = 0.001, and 1000/5, 2000/10, and 4000/20 for $F_{ST}$ = 0.0025.

## Genetic diversity

For mtDNA data, the haplotype diversity ($h$) and the nucleotide diversity ($\pi$) were calculated using ARLEQUIN version 3.5 (Excoffier et al., 2005). To allow for significant differences in mtDNA haplotype and nucleotide diversities between sample locations, Welch's *t*-tests were applied for pairwise comparison of locations because of inequality of variances.

For microsatellite analysis, FSTAT 2.9.3.2 (Goudet, 1995) and ARLEQUIN version 3.11 (Excoffier et al., 2005) were used to calculate observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), allelic richness ($A_R$), and total number of alleles ($K$). Welch's *t*-tests were applied in order to assess differences in microsatellite allelic richness between population locations.

## Population structure

The minimum spanning haplotype network was constructed using ARLEQUIN version 3.11 to effectively investigate the population structure of Japanese glass eels from 10 localities (Excoffier et al., 2005). Resultant data were interpreted and hand-drawn with Adobe Illustrator (CS5), which is an appropriate method for viewing the evolutionary relationship between closely related haplotypes where ancestry may not be strictly bifurcating, avoiding the limitation of constructing bifurcating phylogenetic trees (Posada and Crandall, 2001). The fixation index ($F_{ST}$) of haplotype frequencies between localities for all pairwise comparisons among 10 localities was computed, and the permutation test (10,000 permutations) was performed for each comparison using ARLEQUIN version 3.11 (Excoffier et al., 2005).

For microsatellite data, locus-by-locus analysis of molecular variance (AMOVA) was performed using ARLEQUIN version 3.11 (Excoffier et al., 2005). The permutation tests for each comparison were conducted using 10,000 permutations. Due to some indications of null alleles revealed by MICROCHECKER, the program FREENA (Chapuis and Estoup, 2007) was employed to estimate frequencies of putative null alleles using the EM algorithm, and to further calculate the unbiased $F_{ST}$ estimates adjusted for the presence of null alleles by the ENA method (Chapuis and Estoup, 2007). The relationship between coastal geographical distance and genetic isolation were investigated using ARLEQUIN version 3.11 (Excoffier et al., 2005). Correlations between genetic distinction of the F-statistics calculated for mtDNA, microsatellites, and geographic distance were assessed with a Mantel test (10,000 permutations). In addition, the genetic distance of ($F_{ST}$ / 1 - $F_{ST}$) correlating the spatial distances were estimated using Mantel tests.

# RESULTS

## Microsatellite quality control and statistical power of microsatellites

Of 190 (19 loci x 10 localities) tests for Hardy-Weinberg equilibrium across all loci, only two deviations remained significant following application of the Bonferroni correction

(Rice, 1989). These deviations were observed in the *M37-38* locus in the YH and CX localities. The microsatellite data revealed no evidence for linkage equilibrium in any of the pairwise tests ($P < 0.05$). Loci showed a scattered pattern for null alleles (17 of 190) without a sample or locus-specific pattern, demonstrating no evidence of scoring error or large allele dropout due to stutter peaks.

Based on the present locus quantity, average allele frequencies, and sample sizes, the POWSIM analysis of statistical power revealed that microsatellite data could provide an average 80% probability of detecting an $F_{ST}$ of 0.001 in a chi-squared test and a 73% probability in a Fisher test. Moreover, statistical power could provide 100% probability for both chi-squared and Fisher tests when the expected divergence of $F_{ST}$ in simulations was set to 0.0025. Although the likelihood of failure to detect some specific genetic differentiation cannot be excluded, the overall degree of genetic differentiation among the studied locations was demonstrated to be minor.

## Genetic diversity

For mtDNA, 980 bp of the control region were unambiguously aligned. Haplotypes from these sequences were deposited in GenBank (Accession Nos. JF277166-JF277280). In the 119 individuals, the control region sequences exhibited 148 polymorphic sites (including 30 insertion/deletion sites) resulting in 115 haplotypes. Single haplotypes were shared between three different pairs of localities: XH and ND, XH and TZ, and XM and YH. For microsatellites, a total of 376 alleles were observed in all populations. The mean number of alleles per sample was 13.29, and $A_R$ ranged from 11.77 to 12.44. The $H_O$ ranged from 0.76 to 0.79 and the $H_E$ ranged from 0.80 to 0.83 (Table 3). Overall, there were no significant differences in genetic diversity indices, expressed as $h$, $\pi$, $H_O$, and $A_R$, among the samples (Table 3).

**Table 3.** Mitochondrial DNA (mtDNA) control region and microsatellite diversity statistics for *Anguilla japonica* at each location.

| Sample site | mtDNA | | | | | Microsatellites | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $N_H$ | $h$ | $\pi$ (%) | $D$ | $F_S$ | $k$ | $A_R$ | $H_O$ | $H_E$ |
| Xinhui (XH) | 11 | 1.00 | 1.02 | -1.31$^{NS}$ | -3.63* | 13.47 | 12.41 | 0.76 | 0.80 |
| Shantou (ST) | 13 | 1.00 | 1.03 | -1.46$^{NS}$ | -5.53** | 13.58 | 12.23 | 0.79 | 0.83 |
| Xiamen (XM) | 12 | 0.98 | 0.75 | -1.50$^{NS}$ | -3.50$^{NS}$ | 13.42 | 12.44 | 0.76 | 0.81 |
| Fuqing (FQ) | 12 | 1.00 | 1.16 | -1.51$^{NS}$ | -4.46* | 12.68 | 12.17 | 0.78 | 0.80 |
| Ningde (ND) | 12 | 1.00 | 1.09 | -1.59* | -4.14* | 13.21 | 12.15 | 0.78 | 0.82 |
| Yuhuan (YH) | 12 | 1.00 | 0.71 | -1.58* | -5.73* | 14.00 | 12.31 | 0.77 | 0.81 |
| Taizhou (TZ) | 12 | 1.00 | 0.82 | -1.46* | -5.74** | 12.74 | 11.90 | 0.78 | 0.81 |
| Cixi (CX) | 11 | 1.00 | 0.88 | -1.11$^{NS}$ | -4.34* | 12.74 | 11.77 | 0.79 | 0.82 |
| Jiuduansha (JD) | 12 | 1.00 | 0.68 | -1.58* | -6.19** | 13.42 | 12.16 | 0.78 | 0.81 |
| Dafeng (DF) | 12 | 1.00 | 1.09 | -1.77* | -4.44* | 13.63 | 12.30 | 0.79 | 0.83 |
| Mean | 119 | 1.00 | 0.94 | -2.18*** | -24.40*** | 13.29 | 12.18 | 0.78 | 0.81 |

$N_H$ = number of haplotypes; $h$ = haplotype diversity; $\pi$ = nucleotide diversity with standard deviation (SD) in bracket; $D$ = Tajima's $D$; $F_S$ = Fu's $F_S$. *P < 0.05; **P < 0.01; ***P < 0.001; NS = not significant.

## Population structure

The minimum spanning haplotype network (MSHN) (Figure 2) from the mtDNA control region revealed no obvious phylogeographic structure, indicating that the Japanese eel

population most likely possesses low levels of genetic differentiation. Genetic differentiation among the 10 samples was low when using both mtDNA ($F_{ST}$ = 0.001, P = 0.291) and microsatellite data ($F_{ST}$ = 0.0026, P = 0.008). Pairwise F-statistic values were also similar for mtDNA, although analysis of microsatellites showed no significant genetic differentiation (Table 4). Pairs of localities with statistical differences (P < 0.05) between different regions for mtDNA control regions exhibited $F_{ST}$ values ranging from -0.008 to 0.008, while microsatellite DNA $F_{ST}$ values ranged from -0.003 to 0.005. Based on the microsatellite DNA, locus-by-locus AMOVA indicated that the vast majority of total genetic variation was located within samples (99.38%), with only 0.49% of the variation distributed among samples.

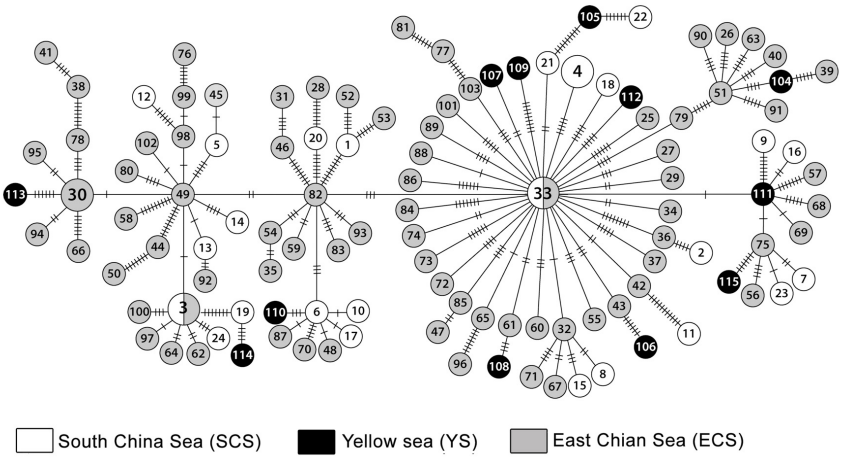


**Figure 2.** Minimum spanning haplotype network (MSHN) for Japanese eels (*Anguilla japonica*). The minimum spanning haplotype network for 119 control region sequences of *A. japonica* was constructed using ARLEQUIN version 3.11. The size of the circles is representative of the number of individuals possessing that haplotype. The smallest circles represent a haplotype frequency of one. Each perpendicular line represents one mutation step between haplotypes.

**Table 4.** Pairwise $\Phi_{ST}$ values and $F_{ST}$ values (adjusted for presence of nulls by ENA method) among 10 populations of *Anguilla japonica* based on mitochondrial sequences of control region (lower diagonal) and 19 microsatellite loci (upper diagonal).

| | nSCS | | Taiwan strait | | sECS | | | | | sYS |
|---|---|---|---|---|---|---|---|---|---|---|
| | XH | ST | XM | FQ | ND | YH | TZ | CX | JD | DF |
| 1 XH | - | 0.001 | 0.001 | 0.005 | 0.003 | 0.002 | 0.004 | 0.002 | 0.003 | 0.003 |
| 2 ST | 0.000 | - | -0.001 | 0.001 | 0.000 | 0.001 | 0.000 | -0.001 | 0.000 | -0.003 |
| 3 XM | 0.008 | 0.008 | - | 0.000 | -0.001 | -0.003 | -0.002 | -0.002 | -0.001 | -0.001 |
| 4 FQ | 0.000 | 0.000 | 0.008 | - | 0.004 | -0.001 | 0.002 | 0.004 | 0.000 | 0.004 |
| 5 ND | -0.008 | 0.000 | 0.008 | 0.000 | - | -0.002 | 0.000 | 0.001 | -0.002 | -0.001 |
| 6 YH | 0.000 | 0.000 | 0.001 | 0.000 | 0.000 | - | -0.001 | -0.001 | -0.002 | 0.000 |
| 7 TZ | -0.008 | 0.000 | 0.008 | 0.000 | 0.000 | 0.000 | - | -0.001 | -0.002 | -0.001 |
| 8 CX | 0.000 | 0.000 | 0.008 | 0.000 | 0.000 | 0.000 | 0.000 | - | -0.002 | -0.001 |
| 9 JD | 0.000 | 0.000 | 0.008 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | - | 0.002 |
| 10DF | 0.000 | 0.000 | 0.008 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | - |

nSCS = northern South China Sea; sECS = southern East China Sea; sYS = southern Yellow Sea. For population abbreviations, see Table 1.

The Mantel test revealed no significant correlation with coastal geographical distances for both mtDNA and microsatellites for each type of genetic differentiation index (Figure 3). In the case of mtDNA, there was a negative correlation between coastal distance and $F_{ST}$, which was not statistically significant (r = -0.088, P > 0.05). In the case of microsatellites, there was a positive, but insignificant, correlation between coastal distance and $F_{ST}$ (r = 0.267, P > 0.05). Similar results were obtained when the genetic distance $F_{ST}$ / (1 - $F_{ST}$) was used instead of the F-statistic. The Mantel test for geographic distance and genetic differentiation remained insignificant: mtDNA, r = -0.087, P > 0.05; microsatellite DNA, r = 0.267, P > 0.05 (Figure 3).

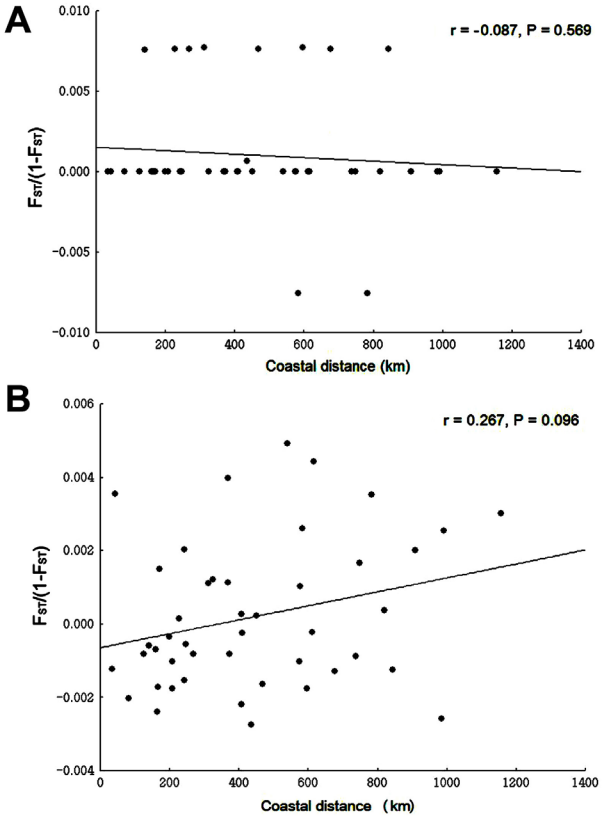


**Figure 3.** Partial Mantel tests. Correlation between genetic differentiation and coastal distance between sites. **A.** Based on mtDNA for $F_{ST}$ / (1 - $F_{ST}$). **B.** Based on 19 microsatellite loci for $F_{ST}$ / (1 - $F_{ST}$).

## DISCUSSION

No genetic differences were found between Japanese glass eels sampled from 10 localities along the coastline of China based on the analysis of mtDNA and 19 distinct microsatellites, which indicates a panmictic population. Overall, pairwise F-statistic values, using both mtDNA and microsatellite data, revealed no significant signs of genetic differentiation. The MSHN for mtDNA control regions revealed no clear phylogeographic structure to indi-

cate selective mating restrictions. The Mantel test revealed no significant correlation between coastal geographical distances and genetic distance, providing supporting evidence of panmixia across Japanese eel populations.

## Sources of genetic variation

For the microsatellite DNA, AMOVA for each locus indicated that the majority of total genetic variation was located within samples (99.38%) and a mere 0.49% of the variation was found between samples, which is similar to results found by Han et al. (2010a). AMOVA for each locus indicated significant genetic variation within samples (>99.4%), which is much higher than the previously reported levels of 95.73% documented by Tseng et al. (2006). This variation can likely be accounted for due to the use of variant microsatellite markers across these studies. The AJMS-2 locus analysis reported by Tseng et al. (2006) demonstrated deviations from Hardy-Weinberg equilibrium, but also revealed abundant null alleles. Significant levels of null alleles can cause errors in estimation of population differentiation, $F_{ST}$, and genetic distance values (Slatkin, 1995).

Significant global genetic differentiation was found in microsatellite data extracted from Japanese glass eel samples ($F_{ST}$ = 0.0026, P = 0.008), and these results were similar to those of other studies using one or more of these markers ($F_{ST}$ = 0.003, P < 0.001) (Han et al., 2010a). Nonetheless, the conclusion that the population genetic structure for Japanese eels shows spatial genetic differentiation is not easily drawn based on the present analysis. Although the genetic composition of the Japanese eel in a single location was found to be generally stable, global genetic differentiation for samples taken from cohorts over multiple years was significant ($F_{ST}$ = 0.002, P = 0.002) (Han et al., 2008). The seemingly conflicting results among previous studies of genetic structure in European and Japanese eel populations, ranging from no detectable heterogeneity to small but statistically significant differences, can be explained through isolation by distance or temporal patterns among eel specimen samples distributed across vast continental regions (Daemen et al., 2001; Wirth and Bernatchez, 2001; Maes and Volckaert, 2002; Dannewitz et al., 2005; Andrello et al., 2006; Maes et al., 2006; Pujola et al., 2006; Tseng et al., 2006; Chang et al., 2007; Palm et al., 2009; Han et al., 2010a). These differences may also be accounted for by variations in sampling design and choice of molecular markers, combined with a lack of power estimates or complex methods for effectively comparing existing results that may exhibit various margins of error (Palm et al., 2009). The presence of a spatial genetic structure in the Japanese eel cannot be excluded by our findings; however, the results suggest that if global genetic differentiation does exist, it must be extremely small. For purposes of resource management and conservation practices and planning, these results suggest that, in practice, the population structure of Japanese eels should be considered to be panmictic.

## Comparison with previous studies

Based on an analysis using six microsatellite DNA loci, Tseng et al. (2006) suggested that the Japanese eel was composed of two genetically distinct groups: low-latitude (southeastern China and Taiwan) and high-latitude (Japan, Korea and northeastern China). The authors invoked a non-random return of larvae hypothesis to account for the genetic segregation of the Japanese eel population. Specifically, they hypothesized that leptocephali of Japanese

eel tend to be transported back to specific latitude ranges, often within the native range of their parents, with little gene flow occurring between low-latitude and high-latitude groups.

The current study selected CX, JD, and DF specimen samples from the high-latitude group, while the remaining samples were collected from the low-latitude group, as previously defined by Tseng et al. (2006). The MSHN of mtDNA revealed no clear phylogeographic structure distinguishing the two groups. In conjunction with these findings, the Mantel test revealed no significant correlation between coastal geographical distances and genetic distance (i.e., no evidence of IBD). Although the overall $F_{ST}$ showed a significant difference in spatial recruits, only a few pairwise $F_{ST}$ tests showed significant genetic differentiation. Han et al. (2010a) reported similar results.

Temporal genetic variation can result in misinterpretation of spatial genetic variation if the temporal component is taken into consideration over multi-year sampling (Tseng et al., 2006). Because the current study spanned only a single year, this potential error can be considered as negligible or nonexistent. A previous study of changes in the genetic composition of Japanese eel populations over two decades found slight year-to-year variation in the genetic composition of temporal samples, possibly due to random genetic drift (Han et al., 2010a). When a given pattern of spatial variation is inconsistent over time, inference of the genetic structure of the population may be inaccurate (Waples, 1998). Furthermore, such temporal variation among samples may also be misinterpreted as geographical isolation, as is the case in several studies of the European eel (Dannewitz et al., 2005; Maes et al., 2006).

### Larval mixing

Genetic differentiation studies in marine environments suggest that larval dispersion can be expected to play a large role in the dispersal of individuals within a population, and therefore influences gene flow between populations (Hellberg, 2009). High levels of larval mixing results in populations containing low genetic differentiation, particularly for marine animals. Japanese eel populations have only one spawning ground (Tsukamoto, 1992), possess a long leptocephali dispersal time (4 to 6 months), and sexually mature adults have a protracted spawning season (Cheng and Tzeng, 1996). These factors, combined with the complex hydrographic characteristics of the Pacific Ocean, result in passive transport by the NEC and KC, both of which exhibit considerable changes in speed, eddy structure, and route at daily, monthly, and even annual scales (Aoyama, 2009). Spawning of the Japanese eel is synchronized monthly according to the lunar cycle (Tsukamoto et al., 2011). These factors cumulatively suggest high larval mixing in Japanese eel populations that may hinder genetic differentiation in population structure.

The population structures of Japanese eels have long been considered to be panmictic, fitting with their life history characteristics. During sexual maturation, European and Japanese eel adults migrate and spawn at the same site, and their larvae are passively transported back to their native habitats by oceanic currents, making population genetic structuring difficult (Tsukamoto, 1992; Aoyama, 2009).

## CONCLUSION

Japanese eel populations have been rapidly declining, resulting in the loss of a major commercial resource. Many factors are involved in this decline, such as long-term oceanic

and climatic changes (Knights, 2003), commercial overfishing (Han et al., 2008), environmental pollution and alterations, development and migration of disease, and over-exploitation of freshwater habitats. Therefore, development of a global management strategy is urgently required for Eastern Asian countries, as is further evidenced by the results of the present study. As previous studies using neutral microsatellite markers have failed to detect adaptive variation between populations by selection, future studies of the population structure of Japanese eel require analyses using putative non-neutral markers, such as expressed sequence tag-linked microsatellite loci (Pujolar et al., 2006) or large scans of single nucleotide polymorphisms (Morin et al., 2004).

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