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Historical Decline in the Japanese Eel *Anguilla japonica* in Northern Taiwan Inferred from Temporal Genetic Variations

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Mei-Chen Tseng, Wann-Nian Tzeng and Sin-Che Lee (2003) Historical decline in the Japanese eel *Anguilla japonica* in northern Taiwan inferred from temporal genetic variations. *Zoological Studies* **42**(4): 556-563. The existence of temporal genetic variations was tested for in 6 polymorphic microsatellite DNA loci of 89 Japanese eel *Anguilla japonica* collected from a single location in the Tanshui River Estuary, northern Taiwan during 1997-1999. The high Nei's genetic identity coefficients (0.868-0.941) and exact test of temporal genetic structure revealed no significant differentiation ($p > 0.05$) among cohorts. Parameters of genetic diversity were examined including mean observed heterozygosity (H_o) (0.695-0.732) and change in the total number of alleles per year (*na*) (73-81). Significant deviations from Hardy-Weinberg equilibrium appeared in 5 of 6 loci, due to an insufficient number of heterozygous individuals in all cohorts. Totals of 22 private and 14 solitary missing alleles were found in all 6 loci. The decrease in numbers of total alleles and private alleles and the increased number of solitary alleles in consecutive years suggested that genetic polymorphism was gradually decreasing. By Bayesian parameters assay, we found that the effective population size was declining. The demographic decline estimated to be 3500-8000 years ago, is significant due to large-scale events such as oceangeographic changes since the most recent glacial stage. http://www.sinica.edu.tw/zool/zoolstud/42.4/556.pdf

Key words: Japanese eel, *Anguilla japonica*, Microsatellite, Genetic diversity, Population decline.

The Japanese eel *Anguilla japonica* Temminck & Schlegel is a temperate freshwater eel, distributed in rivers of the northeastern Asian countries of Taiwan, China, Japan, and Korea (Tesch 1977). As a catadromous fish, it experiences an interesting life history of spawning in the sea and growing up in rivers. Spawning grounds of this species are presumed to be in the western Mariana Islands, at a salinity front near 15°N 140°E, as evidenced by the occurrence of newly hatched larvae (leptocephali) in the area (Tsukamato 1992). Leptocephalus larvae drift from their spawning grounds with the North Equatorial Current for 4-5 mo, being conveyed by the mechanism of Ekman transport (Kimura et al. 1994) and take a further 1 month to reach the coasts of Northeast Asia by the Kuroshio Current. The leptocephali metamorphose into glass eels en route from the Kuroshio Current to coastal waters (Cheng and Tzeng 1996). The translucent glass eels become pigmented elvers during their upstream migration. Eels live in rivers for 5 to 8 yr until their gonads reach maturity in late autumn when they metamorphose into silver eels and are ready to migrate downstream to the distant sea for spawning (Tzeng 1986, Tzeng et al. 2000). Silver eels have to migrate across the strong Kuroshio Current, and it takes them nearly 9 mo to reach their spawning grounds at a distance of 2000-3000 km (Tsukamoto and Umezawa 1990).

The Japanese eel is an important fishery resource in many Asian countries. For cultivation, great numbers of elvers are caught in estuaries during the course of their upstream migration between Nov. and the following Mar. (Tzeng 1983 1985 1986) resulting in a serious problem of over-

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catching. In the rivers of Taiwan, Japanese eel populations have significantly decreased in the past decade (Tzeng et al. 1994, Tzeng 1996). Because of limited knowledge of the early life history of natural populations, some researchers have attempted to establish a complete cultivation system to ensure a continuous supply of elvers. Although this has recently been successful, but it is still difficult to achieve mass production for commercial purposes. Understanding the population structure of Japanese eel is fundamental work for the effective management of this resource.

Otolith examinations and molecular markers can provide useful information when studying life cycles and population structures of this catadromous eel. Cheng and Tzeng (1996) determined the ages at metamorphosis and at the time of arrival at estuaries depending upon daily growth of otoliths of elvers which increases ages from south to north. The concept of a panmictic population in Japanese eel derived from mtDNA sequences in the D-loop region (Sang et al. 1994, Ishikawa et al. 2001) is contradictory to that derived by microsatellite markers (Tseng et al. 2001b). The significant genetic diversity of microsatellites among localities is similar to that in the European eel, *Anguilla anguilla*, for which the population genetic structure does not support the panmictic population hypothesis (Wirth and Bernatchez 2001). Both studies (Tseng et al. 2001b, Wirth and Bernatchez 2001) show clear spatial differences in the genetic structure of eels.

A further study of temporal genetic variation that examines the annual stability of the population structure is important in fishery management. Despite their great significance, studies of temporal genetic diversity have often been neglected. Herein, we attempted to examine the temporal genetic stability of juvenile Japanese eels in a particular estuary we sampled, using statistical analyses to observe whether genetic variations occurred among cohorts in 3 successive yr (1997-1999). Finally, the test of population demographics and the effective population size are further discussed.

MATERIALS AND METHODS

Sampling

In total, 89 *Anguilla japonica* elvers were collected alive from the Tanshui River Estuary at Shalung (121°25'E, 25°10'N) in the winters of northern Taiwan in 1997 to 1999 (Fig. 1). Elvers were immediately preserved in 95% ethanol until DNA extraction.

Genotyping

Genomic DNA was isolated and purified from muscle tissue. DNA was obtained using a standard proteinase K extraction procedure as described in Kocher et al. (1989). Quantification of genetic variation was performed using 6 polymorphic microsatellite loci including 4 $(GT)_n$ (AJMS-1, AJMS-3, AJMS-5, and AJMS-6) and 2 $(GA)_n$ (AJMS-2 and AJMS-10) loci. These microsatellite sequences registered with EMBL have the accession numbers AJ297599-AJ297603 and AJ297605 (Tseng et al. 2001a).

Microsatellites were amplified via the polymerase chain reaction (PCR) and then electrophoresed. Forward primers were labeled with γ-^{32}P-ATP (Amersham, UK) and then PCR was performed in a volume of 5 μl including 0.15 ng genomic DNA, 1.25 pmol reverse primer, 0.125 pmol labeled forward primer, 1.125 pmol unlabeled forward primer, 5 mM dNTP, 0.05-0.1 mM $MgCl_2$,

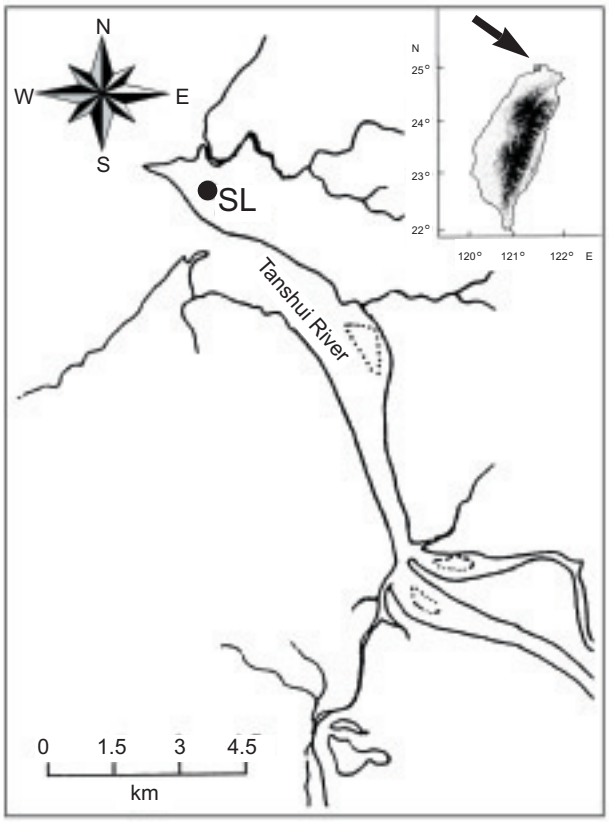

Fig. 1. Sampling locality for *Anguilla japonica*, Tanshui River Estuary at Shalung (SL) in northern Taiwan.

0.5 μl 1% Tween 20, 10X buffer and 0.2 U *Taq* polymerase (Takara, Japan). Amplification was performed in a PC-960G microplate thermal cycler (Corbett Research, Australian) programed with the following schedule: initial denaturing at 95°C for 4 min; 38 cycles of 30 s at successive thermal regimes of 94°C, 56-58°C, and 72°C. Five microliters of loading dye (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to products of each PCR, which were then denatured at 90°C for 5 min. Eight microliters of product was then subjected to gel electrophoresis on an 8% denaturing polyacrylamide sequencing gel and visualized by exposing the dried gel to x-ray film overnight at room temperature. The sizes of alleles were determined by alignment with a co-migrating M13mp18 sequencing ladder (Amersham).

Data analysis

All computations were performed using the POPGENE (Yang and Yeh 1993), Arlequin vers. 2.000 (Schneider et al. 2000) and Bayesian (Beaumont 1999) program packages. Allele number (*na*), allele frequencies, effective number of alleles (*ne*), and observed (H_o) and expected (H_e) heterozygosities were calculated independently for each locus. Multilocus estimates for each of the 3 cohorts were calculated as well. Linkage disequilibrium among all pairs of loci in overall samples was tested using Burrows' composite measure and χ^2 value (Ohta 1982). To detect whether there was a significant departure from Hardy-Weinberg equilibrium (HWE), a modified version of the Markov-Chain random walk algorithm (Guo and Thompson 1992) was adopted. Tests for allelic frequency differences between cohorts were conducted using Fisher's exact test (Raymond and Rousset 1995, Goudet et al. 1996). Analysis of population genetic identity (*I*) was carried out using Nei's (1978) unbiased measures and cohort pairwise F_{ST} and R_{ST} were based on the distance method (Weir and Cockerham 1984, Tamura and Nei 1993, Raymond and Rousset 1995). Bayesian demographic parameters of a sample were estimated using Markov chain Monte Carlo (MCMC) simulations based on microsatellite data (Beaumont 1999). The main demographic parameters were quantified: (i) θ, which is defined as 2 N_0 *Mu*, where N_0 is the current effective number of chromosomes and *Mu* was calculated based on a mutation rate of 5 x 10^{-4} per generation (Angers and Bernatchez 1998); (ii) t_f, equals to t_a/N_0 , where t_a denotes the number of generations that have elapsed since the decline or expansion began; and (iii) *r*, equals N_0/N_1, where N_1 is the number of chromosome at same previous point at time t_f . These analyses supplied all exponential results which were performed using multilocus data in the present report.

RESULTS

In total, 108 alleles were scored from 6 microsatellite loci including 19 at AJMS-1, 24 at AJMS-2, 7 at AJMS-3, 13 at AJMS-5, 15 at AJMS-6 and 20 at AJMS-10. Temporal variation in allelic frequencies over the 3 yr (1997-1999) were observed at all loci analyzed (Table 1). Although totals of 22 private and 14 solitary missing alleles were found in all 6 loci, most of them were commonly shared by all 3 cohorts. High polymorphism resulted from the absence of identical genotypes among all temporal cohorts. Pairwise comparisons of allele diversity among cohorts revealed a decrease in the number of total alleles and mean number of effective alleles per locus in recent years. Consequently, the number of private alleles was found to have decreased with increasing numbers of solitary missing alleles indicated in the past 3 successive years.

Observed heterozygosities (H_o) ranged from 51.7%-64.3% at locus AJMS-3 to 71.8%-82.1% at locus AJMS-2 (Table 2). Expected heterozygosities (H_e) ranged from 62.2%-67.1% at locus AJMS-3 to 92.9%-94.2% at locus AJMS-10. Within the 18 probability tests, a significant departure from Hardy-Weinberg equilibrium was occurred at loci AJMS-2, AJMS-5, and AJMS-10 in the 3 cohorts, due to an insufficiency of heterozygous individuals sampled. Few or no significant deviations were observed at the loci AJMS-1, AJMS-3, and AJMS-6. As a result, we were able to reject the null hypothesis of random mating in samples of Japanese eel. Both observed and expected mean heterozygosities showed slight temporal fluctuation during 1997 and 1999, with H_o ranging from 0.695 (± 0.091) to 0.732 (± 0.067), and H_e from 0.848 (± 0.114) to 0.865 (± 0.106).

Burrows' composite measure for linkage disequilibrium (LD) among the 6 loci for all samples revealed slight disequilibrium for the entire data set. The total variance of allelic disequilibrium at interlocus was estimated to be $D_{IT}^2 = 0.0125$.

The loci AJMS-2 and AJMS-10 had higher effective numbers of alleles (*ne*) (13.04 and 14.93,

Table 1. Allelic sizes and frequencies on locus basis observed from elvers sampled from the Tanshui Estuary during 1997-1999

Locus	Allele size (*bp*)	Allele frequencies by year		
		Tanshui 1997 *n* = 28	Tanshui 1998 *n* = 29	Tanshui 1999 *n* = 32
AJMS-1				
	188	0.0179		
	190			
	192	0.0179		
	194	0.0179	0.0172	0.0312
	196	0.0179	0.0345	0.0156
	198	0.0536	0.0172	0.0156
	200		0.0862	0.0469
	202	0.1071	0.1897	0.1875
	204	0.0357	0.1207	0.0938
	206	0.2679	0.0517	0.2188
	208	0.1786	0.1897	0.0625
	210	0.1429	0.0690	0.2031
	212	0.0893	0.1034	
	214	0.0179	0.0345	0.0781
	216		0.0345	
	218	0.0179	0.0172	0.0156
	220	0.0179		0.0312
	224		0.0172	
	230		0.0172	
AJMS-2				
	102	0.0357		
	104	0.0179		
	106	0.0536		0.0156
	108	0.0357		0.0469
	110	0.0179	0.0690	0.0625
	112	0.0357	0.0517	0.0312
	114	0.0536	0.0172	0.0781
	116	0.0714	0.0172	0.0156
	118	0.0179	0.0517	0.0469
	120	0.0536	0.1034	0.0938
	122	0.1964	0.1034	0.1406
	124	0.0893	0.0517	0.2031
	126	0.0357	0.1207	0.1094
	128	0.0536	0.1379	0.0312
	130	0.0357	0.1552	0.0625
	132	0.0893		
	134	0.0357	0.0172	0.0312
	136		0.0172	
	138			0.0312
	140	0.0357	0.0345	
	142	0.0179		
	144	0.0179	0.0172	
	150		0.0172	
	152		0.0172	
AJMS-3				
	79			0.0156
	81	0.0179		
	83	0.0179		
	85	0.3214	0.2586	0.2656
	87	0.4643	0.4828	0.5469
	89	0.1429	0.2414	0.1250
	91	0.0357	0.0172	0.0469
AJMS-5				
	115		0.0517	
	117	0.1071	0.0517	
	119	0.0893	0.0517	0.0156
	121	0.0357	0.0345	0.0312
	123	0.1071	0.0862	0.1094
	125	0.1786	0.0690	0.0625
	127	0.1429	0.1379	0.2188
	129	0.0714	0.0862	0.0781
	131	0.1250	0.1034	0.1250
	133	0.0893	0.0517	0.1719
	135	0.0179	0.1552	0.0625
	137	0.0179	0.0862	0.0781
	139	0.0179	0.0345	0.0156
AJMS-6				
	85	0.0357		
	87	0.0179		0.0625
	89	0.0179	0.0862	0.0312
	91	0.0536	0.1552	0.0156
	93	0.1429	0.2069	0.1875
	95	0.1429	0.2069	0.1875
	97	0.2321	0.1379	0.1562
	99	0.2321	0.0345	0.2031
	101	0.0357	0.0517	0.0625
	103	0.0536	0.0345	0.0312
	105	0.0357	0.0172	
	107		0.0345	0.0156
	109		0.0172	0.0156
	111		0.0172	
	117			0.0312
AJMS-10				
	140		0.0172	
	142	0.0893	0.0172	0.0625
	144	0.0179	0.0690	0.0625
	146	0.0714	0.0690	0.1250
	148	0.0536	0.0862	0.1094
	150	0.0893	0.1034	0.0781
	152	0.0357	0.1552	0.0312
	154	0.0714	0.1379	0.1094
	156	0.1071	0.0172	0.0938
	158	0.1250	0.0517	0.0469
	160	0.0714	0.0690	0.0156
	162	0.0536	0.0690	0.0312
	164	0.0714	0.0172	0.0469
	166	0.0357	0.0172	0.0312
	168	0.0179	0.0172	0.0312
	170	0.0179	0.0172	
	172	0.0179	0.0345	0.0312
	174	0.0179		0.0312
	178			0.0312
	180	0.0357	0.0345	0.0312

Table 2. Levels of genetic variation observed at 6 microsatellite loci within 3 Japanese eel samples: sample size (*n*); effective number of alleles (*ne*) and observed number of alleles (*na*) at each locus, observed (H_o) and expected (H_e) heterozygosities within samples, number of private alleles in each sample, and number of solitary missing alleles in each sample

Locus	Tanshui 1997 (*n* = 28)	Tanshui 1998 (*n* = 29)	Tanshui 1999 (*n* = 32)
AJMS-1			
H.-W. test	***	***	n.s.
na	14	15	12
ne	6.673	8.538	6.759
H_o	0.679	0.724	0.750
H_e	0.866	0.898	0.866
AJMS-2			
H.-W. test	***	***	***
na	20	17	15
ne	12.250	10.512	9.570
H_o	0.821	0.759	0.718
H_e	0.935	0.921	0.910
AJMS-3			
H.-W. test	n.s.	n.s.	n.s.
na	6	4	5
ne	2.931	2.789	2.579
H_o	0.643	0.517	0.531
H_e	0.671	0.653	0.622
AJMS-5			
H.-W. test	***	***	***
na	12	13	11
ne	8.759	10.646	7.847
H_o	0.786	0.724	0.718
H_e	0.902	0.922	0.886
AJMS-6			
H.-W. test	***	n.s.	***
na	11	12	12
ne	6.297	6.979	6.781
H_o	0.714	0.759	0.687
H_e	0.856	0.865	0.866
AJMS-10			
H.-W. test	***	***	***
na	18	18	18
ne	13.402	11.442	13.386
H_o	0.756	0.690	0.781
H_e	0.942	0.929	0.940
Total no. of alleles	81	79	73
Mean na	13.500 (± 5.050)	13.167 (± 5.036)	12.333 (± 4.320)
Mean ne	9.385 (± 3.932)	8.434 (± 3.263)	7.797 (± 3.639)
H_o	0.732 (± 0.067)	0.695 (± 0.091)	0.698 (± 0.088)
H_e	0.862 (± 0.100)	0.865 (± 0.106)	0.848 (± 0.114)
No. of private alleles	9	9	4
No. of solitary missing alleles	3	5	6

H.-W.: Hardy-Weinberg.

***Significant at the 1% level; n.s., not significant.

respectively) and the greatest total number of alleles (24 and 20, respectively) compared to other loci. They were the main critical loci that influenced the temporal genetic diversity of the Japanese eel. Exact tests of global genetic differentiation among samples were examined using 10 000 steps in a Markov chain. However, it showed no any significant temporal genetic differentiation ($p > 0.05$). The stability of the genetic composition among these samples was reflected in low F_{ST} and R_{ST} estimates and high genetic identity. Values of population genetic identity (I), pairwise F_{ST} and R_{ST} were $I = 0.868$, $F_{ST} = 0.002$, and $R_{ST} = 0.011$ between 1997 and 1998; $I = 0.941$, $F_{ST} = 0.013$, and $R_{ST} = 0.017$ between 1997 and 1999; and $I = 0.893$, $F_{ST} = 0.013$, and $R_{ST} = 0.022$ between 1998 and 1999.

In order to detect population declines and expansions, we followed the procedure of Beaumont (1999). The estimated Bayesian parameters are scaled in terms of current population size and main demographic parameters. The numbers of mutations per locus ranged from 134.64 (AJMS-3) to 939.14 (AJMS-2) with an average of 671.60. The current effective population size (N_0), which ranged 4127-6057 individuals, was estimated according to the log (θ) values (range, 0.615-0.782; mean, 0.704). The obtained log (r) value of -2.05 was an exponential transformation of N_0/N_1, which indicates a declining population. The r value is a measure of change in the population size, i.e., $r < 1$ for a declining population, $r = 1$ for a stable population, and $r > 1$ for an expanding population. These results clearly indicate a significant decrease in the effective population size ($r < 1$). Judging from the log (t_f) value (-0.775) and sexual maturity at an age of 5-8 yr (Tzeng 1986), the onset of demographic decline was estimated to have begun 693-1018 generations (t_a) ago, which corresponds roughly to 3500-8000 calendar years.

DISCUSSION

Although there were high genetic identity and negligible genetic differentiation detected among samples (exact test, $p > 0.05$) collected during 1997 to 1999, there were some private alleles and fewer solitary missing alleles which existed in these 3 cohorts. The private alleles may be deduced from new genealogical mutations, slipped strand mispairing during DNA duplication, or crossover during DNA recombination (Goldstein and Schlötterer 1999). Solitary missing alleles occurring within different cohorts is a result of genotype change, loss or sampling error. All cohorts containing deficiencies of observed heterozygosity (H_o) may be caused by inbreeding, the Wahlund effect, and null alleles.

Although the observed heterozygosity (H_o) almost did not change, the number of total alleles did decrease or otherwise the number of solitary missing alleles increased during 1997 to 1999, suggesting a steady decrease in genetic diversity. Aside from the influence of climate change on oceanic currents, other factors possibly responsible for the fluctuation in genetic diversity may include over-fishing of elvers and destruction of adult eel habitat. These may result in a decreasing effective population size and drop in eel catches.

A wider range of spatial genetic variability than temporal genetic variability when expressed in observed heterozygosity (H_o) (0.565-0.728 v.s. 0.695-0.732), and the estimation in the present study are similar to those for Nei's genetic distance (0.126-0.175 v.s. 0.060-0.142)(Tseng et al. 2001b). Significant genetic differentiation is indicated among geographic populations ($F_{ST} = 0.031$, $p < 0.01$); however, there was no temporal genetic subdivision among the 3 cohorts. The population structure of Japanese eel elvers appearing in northern Taiwan remained temporally constant during 1997 to 1999.

Temporal stability in the same geographic region between progenies and ancestors is due to their inherited migration behavior. Silver eels from different localities mate when attaining maturity in their spawning grounds at different times (Chan et al. 1997). Once the hatched leptocephali drift toward the continental shelf, they then metamorphose into glass eels with further growth as elvers while entering estuaries. The high genetic identity and stable temporal genetic structure of the Japanese eel may be explained by member-vagrant migration model (Iles and Sinclair 1982, Fortier and Gangé 1990), i.e., most progeny tend to reside in a similar locality as did their ancestors.

Major decisions concerning the management of Japanese eel populations require information on such parameters as effective population size and temporal demographic changes which are still largely unknown. Following the procedure of Bayesian (Beaumont 1999) to quantify genetic data and to detect declines or expansions in Japanese eel populations, the effective population size (N_o= 4127-6057) observed for the Japanese eel is close to that of the European eel (N_o= 4410-

5388), but lower than that of American eel (N_o = 9236) (Wirth and Bernatchez 2003). The low value of the Bayesian parameter, *r*, observed in the Japanese eel clearly indicates a significant decline in its effective population size. Similar results were found in both the European and the American eels (Wirth and Bernatchez 2003). Such dramatic declines might be caused by large-scale events such as oceanographic changes. Assuming 5-8 yr for 1 generation time over 850 generations (t_a), the onset of population decline in the Japanese eel largely coincides with the early to middle Holocene (6 to 9 ka) during a time when the climate was warming (Thompson et al. 1997). Changes in oceanic circulation via climatic change may have affected the reproductive success ocurring in the far sea spawning grounds (Tzeng 1996, Wirth and Bernatchez 2003). This emphasizes that the demographic of the Japanese eel have been adversely affected since the last glacial stage.

However, it is appropriate to draw the conclusion from this study, that temporal genetic diversity in a single population of *A. japonica* is less significant than that among geographically discrete samples (Tseng et al. 2001b). These results confirm that the temporal genetic identity is an indication of population stability for the Japanese eel observed during 1997 to 1999. The gradually decreasing in allelic diversity appears to be correlated to overfishing of elvers, to destruction of adult eels habitats, and partially to long-term climatic fluctuations since the last glaciation (Tzeng 1996, Wang et al. 2001, Hauser et al. 2002). For conservation measures of eel resources, it is very important to census populations in consecutive years to ensure a high quality of environmental conditions favorable for the life of eels.

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