

Polymorphisms of GA/GT Microsatellite Loci from *Anguilla japonica*

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Abstract: Six novel microsatellite loci, containing (GA)_{15–17} or (GT)_{10–19} perfect tandem repeats, were isolated and characterized for the catadromous eel *Anguilla japonica*. The allelic size of the 6 loci ranged from 79 to 226 bp in length. All loci were polymorphic with a mean number of 14.7 alleles per locus and a mean heterozygosity of 0.67, suggesting higher polymorphism than that of freshwater and anadromous fishes, but lower than that of marine fishes. Genotype diversity of the 6 loci ranged from 0.22 to 0.61 with a mean value of approximately 0.5. Cross-species amplification showed that 5 of the 6 microsatellite primers proved to be useful in addressing questions of population genetics for all *Anguilla* species.

Key words: microsatellite, catadromous, *Anguilla japonica*, polymorphism, genotype diversity.

INTRODUCTION

Anguilla japonica Temminck and Schlegel, 1846 is a temperate freshwater eel distributed in rivers of northeastern Asian countries including Taiwan, China, Japan, and Korea (Tesch, 1977). As a catadromous fish, it experiences an interesting life cycle of spawning in the sea and growing up in rivers. The spawning grounds were inferred to be to the west of the Mariana Islands, at 15°N, 140°E (Tsukamoto, 1992), evidently from the occurrence of freshly hatched leptocephali in the area. Taking 4 to 6 months to drift passively to the coasts of northeastern Asia by the Kuroshio Current, the leptocephali undergo a metamorphosis on the way to their destinations (Cheng and Tzeng, 1996). The metamorphosis into translucent elvers is completed before

the commencement of upstream migration. Eels live in rivers for 5 to 20 years until their gonads begin to mature in late autumn, when they are ready to migrate downstream to the ocean for spawning (Tesch, 1977).

Japanese eel is an important aquaculture species in Taiwan, China, Korea and Japan. In order to meet the requirements of eel farmers in these countries, large numbers of elvers are caught from estuaries during their upstream migration from November to March (Tzeng, 1985). Because of the limited knowledge of their life history and genetic population structure, some researchers have attempted to establish a complete cultivation system to ensure a continuous supply of elvers. However, as far as we know, this has not yet been successful. As to the population structure, the geographical clines of glass eels based on allozymes (Chan et al., 1997) and the mitochondrial DNA sequences in the D-loop regions (Sang et al., 1994) do not show interpopulational genetic variation, suggesting that it is a panmictic population with low levels of genetic structuring due to high levels of gene flow. However, the above results remain somewhat

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controversial. Other genetic markers with high polymorphism are needed. Because microsatellite markers offer many advantages over allozyme electrophoresis and mtDNA sequencing, the present study may reveal finer details of the population structure in these fishes.

Previous studies of life cycles and population structures of aquatic animals have been limited owing to the lack of proper markers. A possible solution to the questions about the Japanese eel is the use of repetitive DNA markers characterized by high variability. The consistencies of microsatellite sequences include unique DNA sequences and short tandem repeats of 1 to 5 bases in length (Beckmann and Weber, 1992). Because they tend to have higher variability, are inherited in a Mendelian fashion, and require the least amounts of tissue for assay, microsatellites have been regarded as the most appropriate genetic markers for answering many questions of biology (Wright and Bentzen, 1994; O'Reilly and Wright, 1995). For example, O'Connell et al. (1998) used 5 highly variable microsatellite loci to investigate the population structure among Alaskan Pacific herring populations. Nevertheless, the prospective value of the microsatellite is in the search for universal primers for use among closely related taxa. Recent developments in cloning and characterization of microsatellite loci in teleost fishes have enabled scientists to resolve many problems of population biology with these new markers (Crooijmans et al., 1997; Jones and Avise, 1997; Beacham and Dempson, 1998; Roques et al., 1999; Beheregaray and Sunnucks, 2000).

The objective of this paper is to describe the characterization of 6 polymorphic microsatellite loci in the Japanese eel genome. All primer sets of microsatellite loci may have a universal application to other freshwater eel species. These markers are expected to further unveil details of population structure, stock identification, and migratory behavior in life cycles of *Anguilla* species.

MATERIALS AND METHODS

Sampling

In total, 62 *A. japonica* elvers were collected alive in December 1999 and February 2000 from two locations: Sha-lung (121°25'E, 25°10'N), in the estuary of the Tanshui River, northwestern Taiwan, and Fangliao (120°38'E, 22°20'N), in southwestern Taiwan. Another 6 closely related congeneric species were collected: *A. marmorata* and *A. bicolor pacifica* from Fangliao; and *A. reinhardti*, *A. rostrata*, *A. nebulosa nebulosa*, and *A. australis australis* ob-

tained abroad. All these freshwater eels were preserved in 95% ethanol.

DNA Extraction and Cloning of Microsatellites

Genomic DNA was isolated and purified from muscle tissues. Five hundred micrograms of tissues with 1 ml lysis buffer were digested with 55 μ l proteinase K solution (10 mM Tris-HCl, pH 8.0, 2 mM ethylenediaminetetra-acetic acid [EDTA], 10 mM NaCl, 1% sodium dodecylsulfate [SDS], 10 mg/ml dithiothreitol [DTT], 0.5 mg/ml proteinase K). DNA extraction was carried out twice with an equal volume of phenol, twice with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl (24:1) as described in Kocher et al. (1989). DNA was precipitated once with 95% ethanol, once with 70% ethanol, and then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) following a standard procedure (Sambrook et al., 1989).

High molecular weight DNA from the genome of one adult individual was digested with *AluI*, *HaeIII*, and *RsaI* restriction enzymes (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Fragments of 200 to 800 nucleotides were gel-purified using the BIO101 GeneClean Kit (Perkin Elmer) and inserted into the *SmaI* blunt site of pUC 18 (Bayou Biolabs, Harahan, La.). Recombinants carrying the fragments were transfected in the competent *Escherichia coli* DH5 α strain. The library was plated onto 2YT medium plates, and colonies were lifted onto Whatman filters. The probes used for hybridization were oligo DNA (GT)₁₀ and (GA)₁₀, which were labeled with ³²P- γ -ATP (Amersham). Hybridization was carried out overnight at 58°C with hybridization buffer containing 5 \times SSPE, 2 \times Denhardt's, 0.5% SDS, and 100 μ g/ml RNA (Sambrook et al., 1989). After hybridization, the filter was washed twice in primary washing buffer (1 \times SSC/0.2% SDS) at room temperature, washed once in secondary washing buffer (0.5 \times SSC/0.2% SDS) at room temperature, and washed twice in a third washing buffer (0.1 \times SSC/0.2% SDS) at the hybridization temperature. The results were visualized by exposure of the filters to x-ray film for 3 hours at -70°C. Positive clones were sequenced on an Applied Biosystems (ABI, Foster City, Calif.) automated DNA sequencer 377 (version 3.3) using a Bigdye sequencing kit (Perkin-Elmer).

Screening and Analysis of Microsatellite Loci

Pair primers of 18 to 25 nucleotides in length were designed using DNASTAR Primer Select software (version 4.0) for 12 clones containing perfect repeat sequences. Polymerase

chain reaction (PCR) was performed in a volume of 5 µl including 0.1 µl genomic DNA (1–2 ng), 0.5 U *Taq* polymerase (Takara), 0.125 pmol forward primer labeled with 0.3 µCi ³²P-γ-ATP, 1.125 pmol forward primer, 1.25 pmol reverse primer, 5 mM dNTP, 0.05–0.1 mM MgCl₂, and 0.5 µl 1% Tween 20. Amplification was performed in a PC-960G Microplate Thermal Cycler (Corbett Research) scheduled under the following successive steps: 5 minutes at 94°C; 38 cycles of 30 seconds at 94°C, 30 seconds at T°C, and 30 seconds at 72°C, where T corresponds to the annealing temperatures in Table 1. In the cross-species amplification experiment, the annealing temperature of PCR was decreased to 50°C. All species were double-checked with an additional 1 to 2 individuals.

After amplification, the PCR products were subjected to electrophoresis on an 8% denatured polyacrylamide gel and visualized by exposing the dried gel to x-ray film overnight at room temperature. The sizes of each allele were checked by comparison with a M13mp18 sequencing ladder (Amersham T7 sequenase sequencing kit). Observed values of heterozygosity (*H_o*) were determined as the percentage of observed hetgerozygous individuals divided by the number of all individuals. Expected heterozygosity was estimated as $(1 - \sum Pi^2)$, where *Pi* is the frequency of the *i*th allele (DeWoody and Avise, 2000). The polymorphism of microsatellite loci was calculated as the frequency of all genotypes divided by the number of all individuals.

RESULTS AND DISCUSSION

Among 1362 clones screened, 52 had positive signals. After sequencing, only 38 different microsatellite sequences could be retained for use in this study. They include 4 false-positive clones, 7 GA, and 19 GT microsatellites. The remainder contained both GA and GT repeats. In fishes, GT repeats are more frequent than GA repeats (Estoup et al., 1993), as was found in this study. Primers were designed for only 12 of the 38 microsatellite sequences. The remaining microsatellites sequenced were not used owing to the occurrence of a repeated site in one of the flanking regions, the short sequences (limited by the restriction site), and the imperfect and compound repeat sequences. Primer sets of 12 loci did not produce good amplifications which were probably affected by different molecular structures.

We tested different concentrations of MgCl₂, annealing temperatures, and other PCR conditions. After resolution on 4% agarose gels, the 5 loci gave PCR products of a nonspecific amplification and an inaccurate size. However,

Table 1. PCR Conditions, Sample Size, Number and Size of Alleles per Locus, Observed Heterozygosity (*H_o*), Expected Heterozygosity (*H_e*), and Polymorphism of *A. japonica* Microsatellite Loci Detected by PCR Amplification

Locus	Repeat motifs	Primer sequence (5'-3')	Annealing temp. (°C)	MgCl ₂ (mM)	No. of samples	No. of alleles	Range of allele sizes (bp)	Heterozygosity		Polymorphism	EMBL accession no.
								(<i>H_o</i>)	(<i>H_e</i>)		
AJMS-1	(GT) ₁₆	F: TCGAGACACCAGATAGTCAC R: ACATCTTAGGCTCAGACC	58	0.5	35	13	194–226	0.63	0.89	0.611	AJ297599
AJMS-2	(GA) ₁₅	F: ATTTACAGTCATCGACCTGC R: GCTGGAGCGGCGCTTTATC	56	0.5	41	18	103–137	0.67	0.92	0.609	AJ297600
AJMS-3	(GT) ₁₀	F: GGTATGAATGCAGCGTTTATG R: GCAACCGATTTGATCTCCAG	56	0.5	59	7	79–91	0.58	0.72	0.220	AJ297601
AJMS-5	(GT) ₁₅	F: CCTTCAGATTGGTAGCAC R: OGGAGTCTAAATTTGTTCTCTC	58	0.5	28	17	117–153	0.89	0.96	0.525	AJ297602
AJMS-6	(GT) ₁₉	F: ACAGAGCCAGACAAACAGAC R: GGTTCAGCAAGCAAAACGAAC	56	1.0	52	13	85–111	0.54	0.84	0.442	AJ297603
AJMS-10	(GA) ₁₇	F: TGTCTAACACTAAGAAAAGGAGAGG R: GGCTGCCAGTATCTTCTCAAAG	58	1.0	40	20	137–175	0.70	0.97	0.549	AJ297605
Mean						14.7		0.67	0.88	0.50	

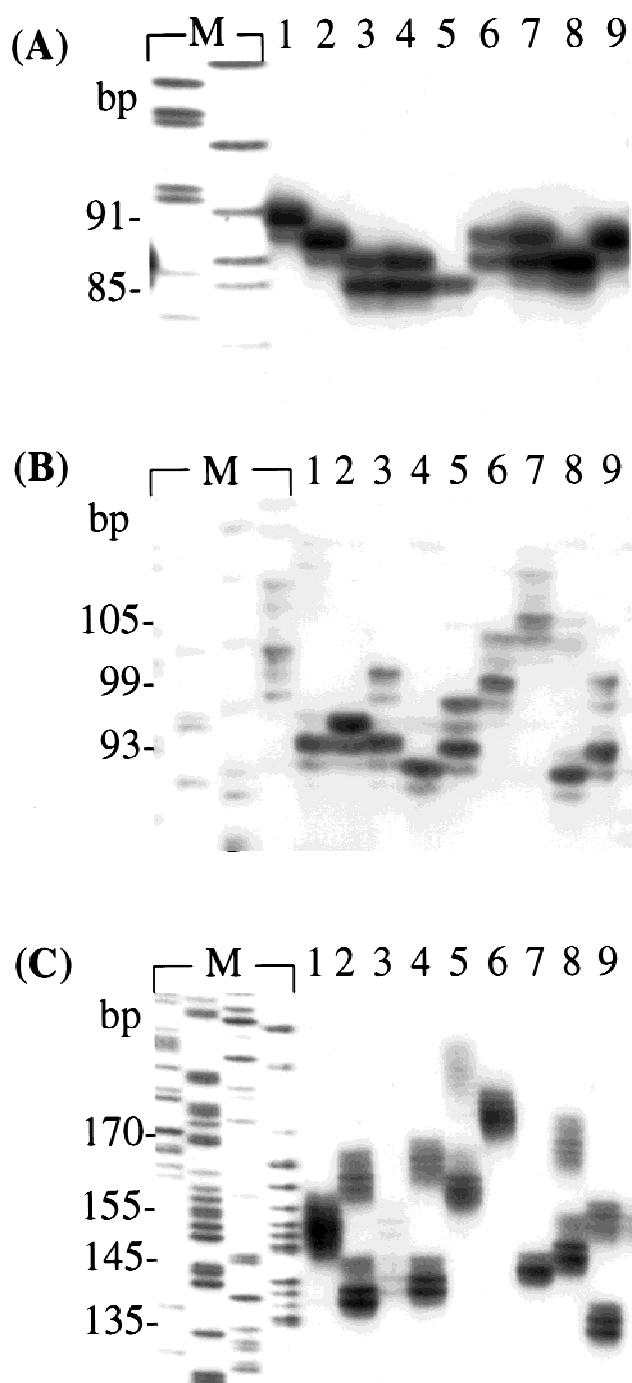


Figure 1. Genetic variability for microsatellite loci AJMS-3 (A), AJMS-6 (B), and AJMS-10 (C). The right lanes numbered 1–9 indicate each Japanese eel sample; the left lanes marked with M represent the M13 marker.

we succeeded in testing for amplification on 7 loci that yielded a consistent PCR product corresponding to a single locus of the expected size. One of the 7 loci (AJMS-8) was monomorphic in the individuals screened ($n = 20$); the

remaining 6 loci were highly variable. Primer sequences and PCR conditions of the above 6 polymorphic loci that were correctly amplified are given in Table 1. All 6 loci are variable, with the number of alleles ranging from 7 in AJMS-3 to 20 in AJMS-10. The sizes of the alleles in the 6 loci ranged from 79 bp in AJMS-3 to 226 bp in AJMS-1; AJMS-3 was the only locus to have an allele size shorter than 100 bp (Figure 1).

The sequence of identified microsatellites included three categories: perfect repeats, imperfect repeats, and compound repeats (Weber, 1990). These are represented in $(GT)_n$ and $(GA)_n$ microsatellites cloned from the genome of the Japanese eel. The microsatellites consist mainly of imperfect repeat sequences (20 of 34 microsatellite sequences, or approximately 59%). The total number of repeated motifs of the core sequence ranges from 12 to 83 for $(GT)_n$ microsatellites and from 11 to 70 for $(GA)_n$ microsatellites. In total, 6 amplified loci are $(GA)_n$ or $(GT)_n$ perfect repeats. The mutations at these 6 microsatellite loci are due to the change of copy number in the 2-bp repeat unit (Figure 2). A high rate of mutation in the microsatellite loci is probably affected by the slip-strand mispairing (SSM) molecular mechanism. This model infers that the instability of the microsatellite is caused by errors derived from the elevated rate of SSM during DNA replication (Levinson and Gutman, 1987; Goldstein and Schlotterer, 1999).

The frequency percentages of the major genotype across the above 6 loci screened ranged from 34% (AJMS-1 and AJMS-2) to 64% (AJMS-3). The mean number of alleles per locus in the catadromous Japanese eel was estimated to be 14.7, which is higher than that of other freshwater fishes (9.1) and other anadromous fishes (10.8), but lower than that of marine fishes (19.9) on average (DeWoody and Avise, 2000).

Observed heterozygosity (H_o) ranged from 0.54 (AJMS-6) to 0.89 (AJMS-5), with a mean value of 0.67, which is smaller than the expected heterozygosities (H_E) of 0.72 (AJMS-3) to 0.97 (AJMS-10), with an average of 0.88. The result shows strong heterozygote deficiencies when tested with Hardy-Weinberg equilibrium. By contrast, the significantly small mean heterozygosity (0.54) assayed in freshwater fishes and a high value of 0.77 in marine fishes (DeWoody and Avise, 2000) reveals that congeneric *Anquilla* species are intermediate with respect to mean levels of microsatellite variation. Polymorphism ranged from 0.22 (AJMS-3) to 0.61 (AJMS-1) (mean, 0.50). Six loci display high polymorphism despite only 7 alleles being found at the microsatellite AJMS-3 locus alone.

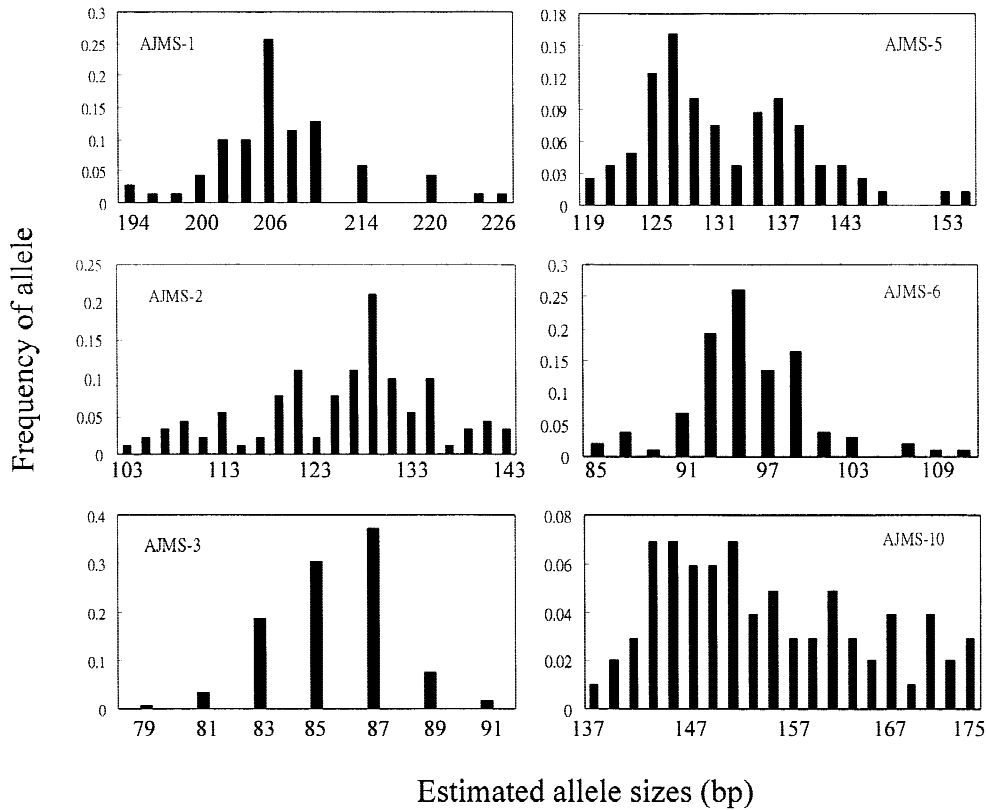


Figure 2. Observed frequency distribution of alleles at loci AJMS-1, AJMS-2, AJMS-3, AJMS-5, AJMS-6, and AJMS-10 for all Japanese eel samples.

Table 2. Cross-Species Amplification with six Pairs of Japanese Eel (*A. japonica*) Microsatellite Primers*

Species	Locus					
	AJMS-1	AJMS-2	AJMS-3	AJMS-5	AJMS-6	AJMS-10
<i>Anguilla japonica</i>	+	+	+	+	+	+
<i>A. marmorata</i>	+	+	+	+	+	+
<i>A. reinhardti</i>	+	+	+	+	+	+
<i>A. rostrata</i>	-	+	+	+	+	+
<i>A. nebulosa nebulosa</i>	+	+	+	+	+	+
<i>A. bicolor pacifica</i> †	-	+	+	+	+	+
<i>A. australis australis</i> †	+	+	+	+	+	+

*Plus (+) indicates the amplification was successful; minus (-) indicates the failure of amplification (determined by agarose gel electrophoresis using ethidium bromide staining).

†Short fin eels; others are long fin eels.

Previous studies of microsatellite primers proved that they have a wide taxonomic range; for instance, those isolated from *Carassius auratus* may well work across a wide range of a portion of the genera in the Cyprinidae (Zheng et al., 1995). With the exception of AJMS-1, the other 5 microsatellite primers identified in *A. japonica* have been amplified for broad use in other congeneric species such as *A. marmorata*, *A. reinhardti*, *A. rostrata*, *A. nebulosa nebulosa*, *A. bicolor pacifica*, and *A. australis australis* (Table 2).

The above-mentioned 5 microsatellite loci are universally conserved in all *Anguilla* species. The amplification of the AJMS-1 primers did not fit in well with *A. rostrata* and *A. bicolor pacifica* since the unique DNAs of the microsatellite loci were deleted or degenerated by point mutations.

In the long-term pursuit of excellence in microsatellite study of *Anguilla* eels, we provide a preliminary description of the characteristics of 6 novel microsatellite loci in the Japanese eel genome and their advantage as markers to

study genetic variation. The subsequent approach for us is to compare the polymorphism of microsatellite variations in several fish species with respect to a tendency of changing genetic variability in the progression among freshwater, anadromous, catadromous, and marine fishes. We have also investigated the potential value of microsatellite primers for extensive use in related taxa. The results confirm that these primers are proper genetic markers for further addressing questions of population genetics and life cycles of *Anguilla* species.

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