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Genetic Variation of the Japanese eel *Anguilla Japonica* Based on Microsatellite DNA

Abstract

Six microsatellite loci, containing GA/GT tandem repeats were characterized and used to examine the genetic variability in spawning populations of the catadromous Japanese eel *Anguilla japonica*. The allelic size of the six loci ranges from 79 to 220 bp in length. All loci are polymorphic with a mean number of 15.3 alleles per locus and a mean observed heterozygosity (H_o) of 0.66, suggesting its having higher polymorphism than freshwater fishes, but lower than marine fishes. Since the provisional support of the Japanese eel as a panmictic species by mtDNA sequencing, this hypothesis was tested with other gene marker, i. e., microsatellite, on the samples from Taiwan and Mainland China. Our results revealed a slight genetic differentiation (Fst = 0.0314) among populations. Cross-species amplification showed that five of the six microsatellite primers are suitable for studying population genetics of all relevant *Anguilla* species.

Key words: Microsatellite, Catadromous, *Anguilla japonica*, Polymorphism, Genetic differentiation

The Japanese eel *Anguilla japonica* Temminck & Schlegel is a temperate freshwater eel, distributed in the rivers of northeastern Asian countries, Taiwan, China, Japan, and Korea[1]. 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 is presumed to be in the western Mariana Islands, at a salinity front near 15°N 140°E, evidently from the occurrence of newly hatched leptocephali in the area[2]. The leptocephalus larvae take 4-5 months to drift with the North Equatorial Current conveyed by the mechanism of Ekman transport from their spawning grounds[3] and take further 1 month to reach the coasts of Northeastern Asia by the Kuroshio Current. The leptocephali metamorphose into glass eels on the way from the Kuroshio to the coastal waters[4]. The translucent glass eels become pigmented elvers during upstream migration. Eels live in rivers for 5 to 8 years until their gonads reach to early maturity in late autumn when they are ready to migrate downstream to the far sea for spawning[5]. Silver eel has to migrate across the strong Kuroshio current taking nearly 9 months to reach the spawning ground at a distance of 2000-3000 km[6].

Japanese eel is an important fishery resource in the Northeastern Asian countries. A large amount of elver is caught in estuaries during their course of upstream migration between November and forthcoming

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March[7]. Due to the rapid expansion of the eel aquaculture industry in Taiwan, China and Japan, the supply of elvers for stocking is far insufficient to meet the demand of farmers[8]. Because of the limited knowledge of the early life history of the natural population, nowadays researchers still can not successfully establish a complete cultivation system to ensure a continuous supply of elvers.

From previous studies of life cycles and population structures of other aquatic animals, many nongenetic markers such as parasitic specificity, meristic characters, growth and gonadal indices have been used to clarify population structure and discriminate stocks[9]. Because of the handicap of these markers, which are strongly environment-dependent, the use of molecular markers for fish population study has substantially increased their importance since late 1970s[10-11]. A slight geographical cline of certain allozymes[12] and strict homogeneity of mtDNA sequences in the D-loop region suggest that the Japanese eel is a panmictic population[13-14]. We believe a final conclusion cannot be made unless other powerful gene markers such as microsatellite analysis are conducted. Microsatellite marker is considered to be more advantageous than allozyme and mtDNA markers in the discrimination of populations.

The best way to study the Japanese eel population is structure the use of repetitive DNA markers which is characterized by high variability[15]. The consistencies of microsatellite sequences include unique DNA sequences and short tandem repeats of 1-5 bases in length[16]. Because of its high variability, being inherited in a Mendelian fashion, and least amounts of tissue required for assay, microsatellites have been regarded as the most adequate genetic markers for solving problematic populations[17-18]. This is a quite successful approach used to clarify population structure of many fish species, for example, the Alaskan Pacific herring with five highly variable microsatellite loci[19]. Nevertheless, the eventual value of the microsatellite we studied is to seek universal primers for common use among closely related taxa. Recent developments in cloning and characterization of microsatellite loci in teleost fishes enable scientists to resolve many problems of population biology with these new markers[20-21].

For this purpose, we describe the characterization of six polymorphic GA /GT 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 be used further when resolving population structure (stock identification) and unveiling the mystery of migratory behavior during the life history of *Anguilla* species.

Materials and Methods

Sample collection

A total of 68 *A. japonica* elvers were collected alive during December 1999 and April 2000 from the following four locations: the Tanshui River estuary at Salun (121°25'E, 25°10'N) on NW Taiwan, Fangliao (120°38'E, 22°20'N) on SW Taiwan, Shantou (116°30'E, 23° 30'N) on SE China and the Yalu River estuary (124°30'E, 40°00'N) on NE China (Fig 1). The exact dates of collection and sample sizes are listed in Table 1. For comparison five other closely related congeneric species were also collected: *A. bicolor pacifica* from Fangliao; and *A. reinhardti*, *A. rostrata*, *A. nebulosa nebulosa*, and *A. australis australis* obtained abroad. All these freshwater eels were preserved in 95% ethanol.

DNA extraction

Genomic DNA was isolated and purified from muscle tissue. Five hundred micrograms of tissue with 1 m*l* lysis buffer was digested with 55 μ*l* proteinase K solution (10 mM Tris-HCl, pH 8.0, 2 mM ethylene diamine tetra-acetic acid (EDTA), 10 mM NaCl, 1% sodium dodecyl sulphate (SDS), 10 mg/m*l* dithiothreitol

(DTT), 0.5 mg/m*l* 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.[22]. 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[22].

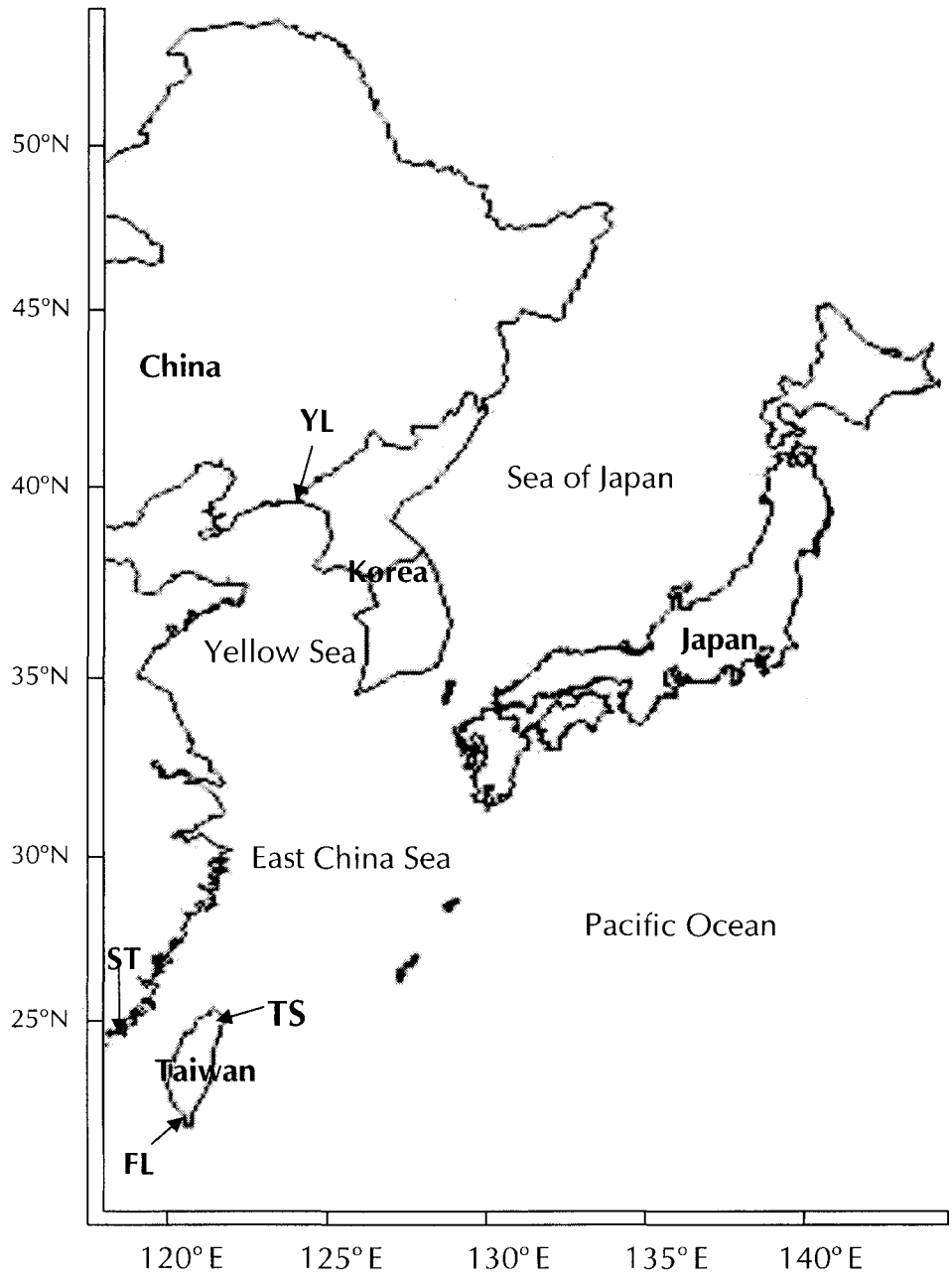

Fig. 1. Location of elver sampling sites designated in Table 1.

Table 1. Sampling locations, date and sample size of the Japanese eel elvers.

Location	*Date*	*Sample size*	*Sample designation*
Tanshui River estuary (NW Taiwan)	Dec. 6, 1999	18	TS
Fangliao (SW Taiwan)	Feb. 3, 2000	19	FL
Yalu River estuary (NE Mainland China)	Apr. 26, 2000	12	YL
Shantou (SE Mainland China)	Feb. 28, 2000	19	ST

Cloning of microsatellites

High-molecular-weight DNA from the genome of one adult individual was digested with *Alu* I, *Hae* III, and *Rsa* I restriction enzymes (Stratagene) according to the manufacturer's instructions. Fragments of 200-800 nucleotides (nt) were purified on gel using the BIO101 Geneclean Kit (PE) and inserted into the *Sma* I blunt end of pUC 18 (Bayou Biolabs). 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 P^{32}-γ ATP (Amersham). Hybridization was carried out overnight at 58 ℃ with hybridization buffer containing 5x SSPE, 2x Denhardts, 0.5% SDS, and 100 μg/m*l* RNA[23]. After hybridization, the filter was washed twice in primary washing buffer (1x SSC/0.2% SDS) at room temperature, washed once in secondary washing buffer (0.5x SSC/0.2% SDS) at room temperature and washed twice in third washing buffer (0.1x SSC/0.2% SDS) at the hybridization temperature. The results were visualized by exposing the filters to X-ray film for 3 hours at –70 ℃. Positive clones were sequenced on an Applied Biosystems (ABI) automated DNA sequencer 377 (version 3.3) using a Bigdye sequencing kit (PE).

PCR amplification and genotype score of microsatellite loci

Pair primers of 18-25 nt in length were designed using DNASTAR Primer Select software (version 4.0) and subsequently visualized with the naked eye for 12 clones containing perfect repeat sequences. PCR reactions were performed in a volume of 5 μ*l* including 0.1μ*l* genomic DNA (1~2 ng), 0.5 unit *Taq* polymerase (Takara), 0.125 pmole forward primer labeled with 0.3μ Ci P^{32}-γ ATP, 1.125 pmol forward primer, 1.25 pmol reverse primer, 5 mM dNTP, 0.5~1.0 mM $MgCl_2$, and 0.5 μ*l* 1% Tween 20. Amplification was performed in a PC-960G Microplate Thermal Cycler (Corbett Research) scheduled under the following successive steps: 5 min at 94 ℃; 38 cycles of 30 s at 94 ℃, 30 s at Y ℃, and 30 s at 72 ℃, where Y corresponds to the annealing temperatures as indicated in Table 2. 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 M13 mp18 sequencing ladder (Amersham T7 sequenase sequencing kit). In the

cross-species amplification experiment, the annealing temperature of PCR was decreased to 50 ℃. All species were double-checked with an additional 1~2 individuals.

Table 2. PCR conditions and range of alleles size per locus.

Locus	*Annealing temp. (°C)*	*$MgCl_2$ (mM)*	*Range of allele sizes (bp)*
MS-1	58	0.5	190-220
MS-2	56	0.5	104-136
MS-3	56	0.5	79-91
MS-5	58	0.5	117-145
MS-6	56	1.0	83-109
MS-10	58	1.0	135-179

Statistical analyses

All statistical treatments and calculations were performed by the POPGENE version 1.32 software. Proportion of heterozygosity under random mating was estimated by Nei[24]. Pairwise F_{ST} estimates were calculated using the F-statistics for a sampling hierarchy by Weir[25]. The expected genotypic frequencies were computed under random mating using the algorithm[26]. The estimation of genetic distance was made by Nei's and the subsequent dendrogram constructed based on Nei's genetic distances using UPGMA[27-28].

Results and Discussion

Characterization of microsatellite loci in Japanese eel

The screening of the DNA library revealed 52 positive clones from 1362 recombinant clones. The average size of the cloned inserts determined after digestion and electrophoresis on 1.2% agarose gel was 280 bp. 38 different microsatellites were sequenced in full length. These could be retained for use in this study. Sequence analysis revealed 4 false-positive clones, 7 GA, and 9 GT microsatellites. The remainder contained both GA and GT repeats. The copy number of repeats from 12 to 83 for GT microsatellites and from 11 to 70 for GA microsatellites. Primers designed were confined from 12 out of the 34 microsatellites sequenced. The remaining microsatellites sequenced were not used due to the occurrence of repeated region in one of the flanking regions, the short sequences (limited by the restriction site), and the imperfect repeat sequences. Primer sets of 12 loci did not produce good amplifications which were probably affected by special molecular structures. When testing with different $MgCl_2$ concentrations, annealing temperatures, and PCR conditions, we succeeded in amplification of six loci which yielded a consistent PCR product corresponding to a single locus of the expected size. All these six loci are polymorphic, with 7- 23 alleles in range and respective 79 bp- 220 bp in size.

Allele frequency distribution

All of the above 6 loci found were common alleles shared by the samples from different localities (Table 3). The number of alleles per locus per location ranged 4-23, with their sizes at each locus not exceeding 44 base pairs. Despite the lowest number of alleles (7) that occurred at the locus MS-3, the overall six loci sequenced generally displayed high polymorphism which showed significant allelic variation with the locations where the fish were collected. The average observed heterozygosity (H_o) taken from six microsatellite loci ranged 0.51- 0.81

(mean 0.66), which is smaller than the expected heterozygosities (H_E) (0.67 - 0.94, averaged 0.86). Level of mean heterozygosity may imply osmoregulatory adaptability of the fish, as in the case of the marine fishes which have higher mean heterozygosity (H_o = 0.77) than that of freshwater fishes (0.54)[29]. Mean levels of microsatellite variation in *Anguilla* revealed from our result is intermediate.

Table 3. Allele frequencies, mean heterozygosity and mean number of alleles per locus in the samples screened. Standard errors of mean heterozygosity and mean number of alleles per locus are given in parentheses.

Locus	Allele size	Allele frequencies			
		ST n=19	YL n=12	TS n=18	FL n=19
MS-1	190		0.0833		
	192		0.1667		
	194			0.0556	0.0263
	196	0.0263	0.1667	0.0278	
	198	0.1053	0.0417		0.0789
	200	0.0789		0.0278	0.2105
	202	0.0789	0.1667	0.0833	0.0789
	204	0.0789	0.2083	0.1111	0.2105
	206	0.1579	0.0833	0.2778	0.2105
	208	0.2895	0.0417	0.0833	0.0526
	210	0.1316	0.0417	0.2778	0.0789
	212	0.0526			
	214			0.0278	
	216				0.0263
	218				0.0263
	220			0.0278	
MS-2	104	0.1053		0.0263	0.0368
	106	0.0263		0.0263	0.0147
	108		0.0556	0.0789	0.0588
	110		0.0278	0.1579	0.0515
	112	0.0789	0.0278		0.0368
	114		0.1111		0.0294
	116	0.0526		0.0526	0.0368
	118	0.0789	0.0278	0.1053	0.0588
	120	0.3158	0.1389	0.0789	0.1691
	122	0.1053	0.1111	0.1053	0.1176
	124	0.0263	0.1944	0.1053	0.1176
	126		0.1389	0.0526	0.0662
	128	0.0789	0.0556	0.0526	0.0662
	130	0.0789	0.1111	0.0789	0.0809
	132	0.0263		0.0789	0.0294
	134				0.0147
	136				0.0074
	140	0.0263			0.0074
MS-3	79		0.0417		
	81				0.0526
	83	0.3421	0.2917	0.2500	0.3421
	85	0.4211	0.5000	0.5833	0.3421
	87	0.1842	0.1250	0.1111	0.2368
	89	0.0263	0.0417	0.0556	0.0263
	91	0.0263			

Table 3. Continued.

Locus	Allele size	Allele frequencies			
		ST	YL	TS	FL
MS-5	117			0.0278	
	119	0.0263		0.0556	
	121	0.0789	0.0417	0.0833	0.0526
	123	0.1053		0.1667	0.1053
	125	0.1316	0.2083	0.2222	0.2895
	127	0.0526		0.1389	0.1842
	129	0.1579	0.0417	0.0556	0.1316
	131	0.1842	0.1250	0.0556	0.0789
	133	0.1579	0.2500	0.1111	0.0526
	135	0.0263	0.1667	0.0278	0.0526
	137	0.0526	0.1250		0.0263
	139	0.0263	0.0417		0.0263
	143			0.0278	
	145			0.0278	
MS-6	83	0.0526			
	85				0.0526
	87	0.0526		0.1111	
	89	0.0526			
	91	0.0263	0.0833	0.0278	0.1053
	93	0.0789	0.2500	0.1667	0.1579
	95	0.3158	0.2500	0.2500	0.3947
	97	0.1842	0.1250	0.0833	0.1316
	99	0.1579	0.2500	0.1944	0.1316
	101	0.0263		0.0556	0.0263
	103		0.0417	0.0556	
	105	0.0263			
	107	0.0263		0.0278	
	109			0.0278	
MS-10	135	0.0789			0.0221
	137	0.0263			0.0074
	139	0.0263			0.0074
	141	0.0263	0.0417	0.1111	0.0515
	143	0.0263	0.1250	0.0833	0.0809
	145	0.1053	0.0833	0.1944	0.1324
	147	0.0263	0.0417	0.1389	0.0882
	149	0.1316	0.0417	0.0833	0.1029
	151	0.0526	0.0833	0.0278	0.0735
	153	0.1053	0.1250	0.0833	0.0882
	155	0.0789	0.1250		0.0515
	157	0.0263	0.0417	0.0278	0.0221
	159	0.0263	0.0833		0.0294
	161	0.0526	0.0417	0.0278	0.0515
	163	0.0263		0.0278	0.0294
	165	0.0526	0.0417	0.0278	0.0294
	167	0.0263	0.0833		0.0221
	169	0.0263	0.0417		0.0147
	171	0.0263		0.0278	0.0221
	173	0.0526		0.0278	0.0368
	175				0.0074
	177			0.0556	0.0147
	179			0.0556	0.0147
Mean observed Heterozygosity (H_o)		0. 7281 (±0.0517)	0. 6111 (±0.1255)	0. 5648 (±0.1700)	0. 7281 (±0.2193)
Mean expected Heterozygosity (H_E)		0. 8535 (±0.0892)	0. 8551 (±0.0996)	0. 8378 (±0.1212)	0. 8502 (±0.0798)
Mean no. Alleles per locus		11.3333 (±4.9261)	8.8333 (±3.3116)	10.3333 (±3.6148)	9.8333 (±3.4303)

Sample sites : ST, YL, TS and FL refer to Table 1.

Genetic population structure of Japanese eel

Genetic population subdivision among *A. japonica* samples was determined from *Fst* estimation. The corresponding value for each pairwise comparison among the overall loci averaged 0.0314. Comparisons of *Fst* among six microsatellite loci, only MS-1 locus (*Fst* = 0.0548) revealed significant differences suggesting a slight genetic differentiation among populations. Allelic frequencies of six microsatellite loci when making pairwise comparisons between different geographic localities were used to calculate the intersample genetic distances. The greatest genetic distance (d = 0.1747) was observed between samples from Yalu River (40 °00'N) and Fangliao (22°20'N) while the smallest values (d = 0.1258) was observed between samples from Tanshui River (25°10'N) and Fangliao (22°20'N) (Table 4). A neighbor-joining dendrogram modified from Nei's[27] showed closer clustering of three low latitude geographic populations than to that of high latitude (Fig 2). This seems to fit the model of isolation by distance resulting from non-random mating among eels from different locations. We infer several different temporal or spatial spawning groups of Japanese eel existing on the north Pacific Ocean.

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Population	*ST*	*YL*	*TS*	*FL*
ST	--	0.8449	0.8623	0.8733
YL	0.1685	--	0.8646	0.8397
TS	0.1482	0.1455	--	0.8818
FL	0.1355	0.1747	0.1258	--

Sample sites: ST, YL, TS and FL refer to Table 1.

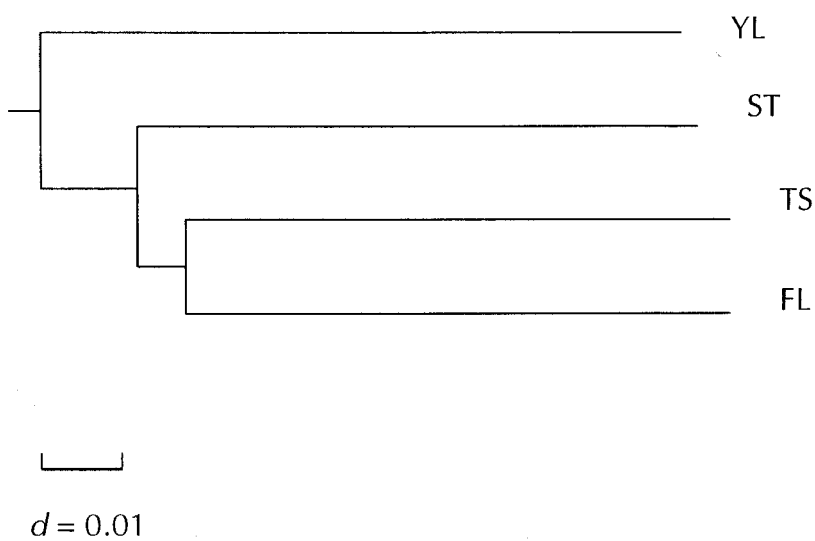

Fig. 2. A dendrogram based on genetic distance showed close clustering of 3 populations on low latitude (sampling sites: YL, ST, TS and FL refer to Table 1).

Chan et al.[12] inferred the geographical substructuring of *A. japonica*, based on the genetic geographic clines at certain isozyme loci. Sang et al.[13] and Ishikawa et al.[14] on their studies of Japanese eel population structure with the control region of mitochondrial DNA, eventually can not support the existence of genetic heterogeneity which is consideredly but a single panmictic population. It is probable that the present mt DNA analysis is not sensitive enough to further examine genetic differentiation. However, the present study of microsatellite DNA on Japanese eel shows weak genetic differentiation (*Fst* = 0.0314) among geographic populations.

Application of microsatellite

Previous studies of microsatellite primers proved to be of universal use for a wide taxonomic range for instance, those isolated from *Carassius auratus* may well work across most of the genera in the Cyprinidae[30]. With the exception of MS-1 locus, the other five microsatellite primers identified in *A. japonica* have been amplified for broad use in other congeneric species including *A. reinhardti*, *A. rostrata*, *A. nebulosa nebulosa*, *A. bicolor pacifica*, and *A. australis australis* (Table 5). The above-mentioned five microsatellite loci are universally conserved in all *Anguilla* species. The amplification of the MS-1 primers did not fit in well with *A. rostrata* and *A. bicolor pacifica* since the unique DNA of the microsatellite loci were deleted or degenerated by point mutations.

Table 5. Cross-species amplification with six pairs of Japanese eel (*A. japonica*) microsatellite primers. +, Indicates the success amplification; – , indicates the failure of amplification (determined by agarose gel electrophoresis using ethidium bromide staining). The table is modified from Tseng[15].

Species	Locus					
	MS-1	MS-2	MS-3	MS-5	MS-6	MS-10
Anguilla japonica	+	+	+	+	+	+
A. reinhardti	+	+	+	+	+	+
A. rostrata	-	+	+	+	+	+
A. nebulosa nebulosa	+	+	+	+	+	+
※ *A. bicolor pacifica*	-	+	+	+	+	+
※ *A. australis australis*	+	+	+	+	+	+

※, Indicates short fin eels; the others are long fin eels.

In the long-term pursuit of excellence in microsatellite study of *Anguilla* eels, we provide a preliminary description of the characteristics of six novel microsatellite loci in the Japanese eel genome and their advantage as genetic 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, catadromous, and marine fishes. We are also investigating the potential value of microsatellite primers for extensive use in related taxa.

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from China and other anonymous persons who kindly provided eel samples from abroad. We are indebted to the technicians in the central laboratory for handling sequencing procedures. This study was financially supported by the National Science Council of Taiwan for a grant to SCL (NSC89-2311-B-001-221).

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