African Journal of Biotechnology Vol. 4 (4), pp. 318-325, April 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Genetic variation of Japanese loach inferred from restriction fragment length polymorphism analysis of mitochondrial DNA

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Accepted 12 January, 2005

Genetic analysis using restriction fragment length polymorphisms (RFLPs) of D-loop region in the mitochondrial DNA including the part of 12s RNA and cytochrome *b* genes was made to clarify genetic variations and relations and relationships among eleven populations of Japanese loach *Misgurnus anguillicaudatus.* Ten haplotypes were detected using seven restriction enzymes (*Alu* I, *Hinc* II, *Msp* I, *EcoR* I, *Hin*f I, *Hae* III and *Taq* I). The differences between each pair of the populations were significant (p<0,0001~p<0.05), except for the test involving populations Saito and Nikko, and Ueda and Futtsu (p=1). The haplotypic and nucleotide diversities within populations ranged from 0 to 0.889 and 0 to 22.222%, respectively. The average nucleotide diversity (π_{xy}) among 11 populations ranged from 0 to 15.255%, with a mean of 6.272 ±0.004% and net nucleotide divergence (δ) ranged from 0 to 15.255%, with a mean of 5.312±0.004%. The net nucleotide divergence between the two northern populations (Memanbetsu and Naruko) were high (12.521%, ranged from 5.083 to 15.256%), in contrast, the net nucleotide divergence among southern populations were relatively low (3.190%, ranged from 0 to 8.478%). The bootstrapped UPGMA dendrogram and NJ tree, which were constructed based on the net nucleotide divergences, showed that loach diverged into two groups. The northern group comprised two populations (Memanbetsu, Hokkaido Prefecture and Naruko, Miyagi P.) and the southern group contained nine other populations. In the southern group, the Hashima (Gifu P.), Izumo (Shimane P.) and Tomari (Tottori P.) populations clustered into one subgroup whereas the other six populations, Yuya (Yamaguchi P.), Saito (Miyazaki P.), Nikko (Tochigi P.), Ueda (Nagano P.), Futtsu (Chiba P.), and Nasu (Tochigi P.), categorized into another subgroup. The homogeneity test and AMOVA indicated in the consensus tree reconstructed by NJ method, the two genetically groups were considerably differentiated (0.0001<p<0.05) to each other. However, the NJ tree and UPGMA dendrogram based on the genetic distances, also showed that despite the pronounced genetic differences among some populations, four populations form the southern group, i.e. Saito, Nikko, Ueda and Futtsu, were closely related.

Key words: RFLP, mtDNA, D-loop, *misgurnus anguillicaudatus*.

INTRODUCTION

The loach *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes) is widely distributed in Japan. The electrophoresis studies using protein polymorphisms suggested the presence of genetically distinct populations in Japanese loaches (Kimura, 1987; Dong et al., 1999), but the results were fragmentary and inconclusive due to the small number of localities examined and specimens used. Recently, Khan and Arai (Khan and Arai, 2000) conducted allozyme analyses of 12 presumptive loci in total 923 individual collected from

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44 populations all over Japan and then determined six (1-6) groups on the basis of cluster analysis. Genetic differentiation among the group 1 (Memanbetsu, Hokkaido), 6 (central area of Honshu island from Shin-etsu, Kanto, to Tokai district) and others was likely to be inter-subspecies level, whereas divergence in other groups (2, 3, 4 and 5) seemed to be a local-race level. However, many geographically distant populations frequently showed genetically close relationship. Such inconsistencies between genetic and geographical relationships might be due to insufficient number of samples and/or less sensitivities of analytical method.

As described above, the genetic relationship among loach populations are still unsolved and a more sensitive genetic analysis is required to acquit them. Direct examination of genetic variation at the DNA has enhancing analytical power. Mitochondrial DNA does not recombine, maternally inherits, and has a relatively fast evolutionary rate, compared to a typical single copy nuclear DNA (Brown et al., 1979; Wilson et al., 1985). Thus, a great deal of attention has been conferred to mtDNA analyses to determine genetic differentiation among populations and/or species.

The objective of the present work was to study genetic diversities of loach populations using restriction fragment length polymorphisms (RFLPs) of mtDNA D-loop region sample collected from 11 selected populations.

MATERIALS AND METHODS

Specimens

A total of 82 loach individuals were selected from 11 populations, which were already examined for allozyme analysis (Table 1). After the confirmation of diploidy of each sample by flow-cytometry for DNA content of erythrocytic cells (Zhang and Arai, 1996), muscle specimen was taken and immediately frozen at -80°C, then stored until mtDNA analysis.

mtDNA preparation

Approximately 40 mg of muscle tissue from each fish was placed in 400 µL TNES-urea buffer (Asahida et al., 1996) and digested with 40 µL of proteinase K (0.01 g/l) at 37°C for overnight. The DNA was extracted following to the method of Maniatis et al. (1989). Fragments approximately 2.0 kilo base pairs (kbp) in length from the cytochrome *b* to the 12S rRNA genes, including the entire D-loop, were amplified by PCR from whole genomic DNA, using the following universal primer pairs: L-15560 (5′→3′, 23 mer, CAT ATT AAA CCC GAA TGA TAT TT), H-1067 (5′→3′, 25 mer, ATA ATA GGG TAT CTA ATC CTA GTT T) (Martin et al., 1992). PCR was conducted for 30 cycles using an ASTEC PC-800 thermal cycler at 94 °C for 1.0 min denaturation, 45.5°C for 1.0 min annealing and 72°C for 2.0 min extension. All products from the PCR amplification were confirmed as being of equal length by electrophoresis and were subjected directly to digestion with restriction endonucleases. The mtDNA solutions were stored at - 20°C until used.

Restriction endonuclease digestion

The PCR products were digested following the recommendations of the manufactures. Seven enzymes (*Alu*I, *Hinc*II, *Msp* I, *Eco*RI, *Hin*f I, *Hae*III and *Taq* I) recognizing four to six nucleotides (bases) were used. Incubating under at 37°C for 4-6 h with appropriate amounts of the enzymes under conditions described digested the mtDNA. Agarose gel (3%) electrophoresis was carried out in the standard TAE buffer (0.04 M Tris, 0.002 M EDTA, 0.02 M sodium acetate, pH 7.8). Staining with ethidium bromide (0.1 µg/ml) and exposure to UV light visualized the mtDNA fragments. The I Kb DNA ladder (Life technologies TECGH-LINE ^{TM}) was used as molecular weight marker and the size of mtDNA fragments were measured by using software, Diversity Database^{TM} (PDI Inc., ver. 1.0, 1996). A, B, C, etc., in the order of detection designated the cleavage patterns by each enzyme.

Data analysis and dendrogram

Presence or absence of restriction sites in the control region was inferred for each of the seven enzymes from series of restriction fragment patterns that differed by a single site. The site codes across the control region for a restriction enzyme were concatenated and each fish was assigned a 7-letter code that described its composite, multi-enzyme haplotype. A binary character state matrix consisting of presence or absence of all restriction sites in composite haplotypes was produced using the GENERATE program in REAP (McElroy et al., 1991). Haplotype and nucleotide diversities within populations were estimated according to Nei (1987). The average nucleotide diversity (π_{xy}) and net nucleotide divergence (δ) among populations were estimated according to Nei and Tajima (1981) using REAP. Phylogenetic relationship among the haplotypes was estimated by the neighbor-joining (NJ) method (Saitou and Nei, 1987) and UPGMA dendrogram (Sneath and Sokal, 1973) based on genetic distance estimated by Kimura's two-parameter method (Kimura, 1980).

The homogeneity of the haplotype frequencies within and between geographic regions was evaluated using the contingency χ^2 test (Roff and Bentzen, 1989), with 1000 Monte Carlo simulations generated by the CHIRXC program (Zaykin and Pudovkin, 1993). In order to assess the extent of genetic differentiation at the different level of geographic hierarchy, the overall molecular variance was partitioned into component corresponding to the population divergence within and among regions using the analysis of molecular variance model (AMOVA) (Excoffier et al., 1997), with Arlequin version 1.1 program package (Schneider et al., 1997). For AMOVA, populations were grouped geographically referring to the tree obtained by the above NJ algorism, in which the topology was tested by a bootstrap analysis with 1,000 pseudo- replicate trees. Significance of the variance components and F_{ST} values was tested with permutation method.

RESULTS

RFLPs by seven enzymes in mtDNA D-loop region of 82 individuals examined are summarized in Table 2. The number of cleavage patterns produced by site variation was two (A, B) in *Eco* RI, three (A,B and C) in *Alu*I and *Msp*I, and four (A, B, C and D) in *Hin*fI and *Hae*III (Table 2). *Hinc*II and *Taq*I yielded monomorphic pattern in all the individuals examined. The geographic distribution of ten different haplotypes with their frequencies in 11

Table 1. Total 44 population of the loach were studied by allozyme marker and 6 cluster groups were observed. Among them 11 populations were used in the present study, populations were selected atleast one from each cluster groups.

Population no.[1]	No of group[2]	Prefecture	Locality[3]	No. of fish	Date of collection
1 [1]	1	Hokkaido	Memanbetsu T. [Hongou]	5	Jun. 98
2 [30]	2	Gifu	Hashima C. [Masakicho, Ooura]	3	Aug. 98
3 [34]	3	Shimane	Izumo C.	7	Nov. 97
4 [33]	3	Tottori	Tomari V. [Ishiwaki]	10	Jun. 98
5 [38]	3	Yamaguchi	Yuya T. (Asai R.)	6	Jun. 98
6 [11]	4	Miyagi	Naruko T. (Kawatabi P.)	5	Jun. 98
7 [44]	5	Mizaki	Saito C.	10	Sep. 98
8 [20]	6	Tochigi	Nikko C. (Naka R.)	10	Aug. 98
9 [23]	6	Nagano	Ueda C. (Urano R.)	9	Jul. 98
10 [22]	6	Chiba	Futtsu T. (Iwasa R.)	9	Aug. 98
11 [19]	6	Tochigi	Nasu T. (Naka R.)	8	Dec. 97
		Total		82	

[1]Parenthesis shows that population nos. were the same to those used in Khan and Arai (2000).
[2]Groups (1-6) were determined by a cluster analysis from allozyme data (Khan and Arai, 2000)
[3]Parenthesis indicates the name of the river ® and pond (P). Bracket indicates name of a section in city ©, town (T) or village (V).

Table 2. Restriction fragement patterns of Japanese loach mtDNA obtained by seven endonuclease digestion. The number of cleavage patterns produced by site variation was three (A, B, and C) in *Alu* I and *Msp* I, two (A and B) in *EcoR* I, and four (A, B, C, and D) in *Hinf* I and *Hae* III. *Hinc* II and *Taq* I produced monomorphic pattern of cleavage.

Endo-nucleases	*Alu* I			*Hinc* II	*Msp* I			*EcoR* I		*Hinf* I				*Hae* III				*Taq* I
type	A	B	C	N	A	B	C	A	B	A	B	C	D	A	B	C	D	N
Fragment size	1141	782	905	1358	998	1627	905	2002	1166	–	998	905	–	–	–	1608	–	2002
bp	371	371	371	669	669	–	669	–	577	715	–	715	715	1358	1358	–	–	–
	–	299	–	–	220	220	220	–	299	–	669	–	669	–	–	–	1256	–
	–	–	220	–	–	172	172	–	–	371	–	–	–	–	–	–	344	–
	–	–	203	–	144	–	–	–	–	–	299	299	299	–	259	259	–	–
	–	172	–	–	–	–	–	–	–	288	–	–	–	–	220	–	–	–
	150	–	150	–	–	–	–	–	–	–	–	–	259	203	203	–	203	–
	–	144	–	–	–	–	–	–	–	230	–	–	–	198	–	–	–	–
	137	137	–	–	–	–	–	–	–	203	–	–	–	172	–	172	172	–
	130	–	130	–	–	–	–	–	–	155	–	–	–	–	–	–	–	–
Total length (bp)	1929	1905	1979	2027	2031	2019	1966	2002	2042	1952	1966	1919	1942	1931	2040	2039	1975	2002
No. of fragment	5	6	6	2	4	3	4	1	3	6	3	3	4	4	4	3	4	1

N: monomorphic restriction types.

populations is shown in Table 3. A total of ten haplotypes were observed in the 11 populations. They were designated by roman numerals I-X. Each haplotype had 17-20 cleavage sites. The occurrence of haplotypes was mostly associated with populations, although haplotypes VIII and IX were shared in some populations. In addition,

Table 3. Distribution of loach mtDNA D-loop haplotypes and their frequencies in 11 populations of loach. Ten haplotypes were observed in the 11 populations, among them seven haplotypes were population specific and multiple haplotypes were found only in three populations, Hashima, Futtsu and Nasu.

Haplotype no.	Haplotypes	Populations										
		Memanbetsu	Hashima	Izumo	Tomari	Yuya	Naruko	Saito	Nikko	Ueda	Futtsu	Nasu
I	ANAAAAN	5 (1.000)										
II	BNABBAN		1 (0.333)									
III	CNABBAN		2 (0.667)									
IV	BNABCCN			7 (1.000)								
V	BNBBBAN				10 (1.000)							
VI	BNCBBBN					6 (1.000)						
VII	BNAAABN						5 (1.000)					
VIII	BNABBBN							10 (1.000)	10 (1.000)	9 (1.000)	8 (0.889)	6 (0.750)
IX	BNAACDN										1 (0.111)	1 (0.125)
X	BNAADCN											1 (0.125)

Note : composite haplotypes reflect digestions with the following restriction endonucleases (left to right): *Alu* I, *Hinc* II, *Msp* I, *EcoR* I, *Hinf* I, *Hae* III and *Taq* I (see also Table 2). Haplotypes are numbered by ranging in alphabetical order the digestion types of each restriction endonuclease.

Table 4. Genetic variability such as the rate of haplotypes, haplotypic and nucleotide diversities among 11 populations of Japanese loach were 0.1 to 0.667, 0.395 to 0.889 and 2.476 to 22.222%, respectively.

Parameter	Populations										
	1	2	3	4	5	6	7	8	9	10	11
Sample size analyzed	5	3	7	10	6	5	10	10	9	9	8
No. of haplotypes[1]	1	2	1	1	1	1	1	1	1	2	3
Rate of haplotype[2]	0.200	0.667	0.143	0.100	0.167	0.200	0.100	0.100	0.111	0.222	0.380
Haplotypic diversity	0	0.889	0	0	0	0	0	0	0	0.395	0.610
Necleotide diversity (%)	0	22.222	0	0	0	0	0	0	0	2.476	2.633

[1]Number of haplotype occurrence.
[2]Rate of haplotype occurrence; Number of haplotype occurrence/sample size analyzed.

multiple haplotypes were observed in only three populations, Hashima, Futtsu and Nasu.

Genetic variabilities among 11 populations of loach are shown in Table 4. Haplotypes occurred at a rate of 0.1 to 0.667 in the 11 populations. Haplotypic and nucleotide diversities were observed in three populations, Hashima, Futtsu and Nasu, and ranged from 0.395 to 0.889 and 2.476 to 22.222%, respectively. These diversities in each of other populations were zero. Results of the homogeneity test among 11 populations of loach haplotypes are shown in Table 5. The differences between each pair of the populations were (p<0.0001~p<0.05), except for the test involving populations Saito and Nikko, and populations Ueda and Futtsu. However, the neighbor-joining dendrogram, based on the genetic distances of Saitou and Nei (1987)

Table 5. The probabilities of homogeneity test among the populations of Japanese loach ranged from <0.0001 to <0.05 except for the test involving populations Saito and Nikko, and populations Ueda and Futtsu.

Populations	2	3	4	5	6	7	8	9	10	11
1	$p<0.05$	$p<0.005$	$p<0.0001$	$p<0.005$	$p<0.01$	$p<0.0001$	$p<0.0001$	$p<0.001$	$p<0.005$	$p<0.001$
2		$p<0.05$	$p<0.01$	$p<0.05$	$p<0.05$	$p<0.01$	$p<0.05$	$p<0.05$	$p<0.1$	$p<0.05$
3			$p<0.0001$	$p<0.001$	$p<0.005$	$p<0.0001$	$p<0.0001$	$p<0.001$	$p<0.001$	$p<0.005$
4				$p<0.0001$	$p<0.0005$	$p=0$	$p=0$	$p<0.001$	$p<0.001$	$p<0.001$
5					$p<0.005$	$p<0.0001$	$p<0.0001$	$p<0.001$	$p<0.001$	$p<0.001$
6						$p<0.0005$	$p<0.0005$	$p<0.001$	$p<0.001$	$p<0.001$
7							$p=1$	$p>0.1$	$p>0.1$	$p>0.1$
8								$p>0.1$	$p>0.5$	$p>0.1$
9									$p=1$	$p>0.1$
10										$p>0.8$
11										

Probabilities calculated after Roff and Bentzen (1990).

Table 6. Nucleotide diversity (%) and net nucleotide divergence (%) among the populations of Japanese loach.

Populations	no.	1	2	3	4	5	6	7	8	9	10	11
Memanbetsu	1	–	10.523	13.761	13.401	15.255	5.083	13.793	13.793	13.793	13.631	13.648
Hashima	2	9.046	–	7.650	4.988	7.049	13.943	5.546	5.546	5.546	5.850	6.422
Izumo	3	13.761	6.174	–	5.868	5.070	10.204	3.845	3.845	3.845	3.864	3.784
Tomari	4	13.401	3.512	5.868	–	2.664	12.470	3.889	3.889	3.889	4.187	4.741
Yuya	5	15.256	5.572	5.070	2.664	–	9.447	0.986	0.986	0.986	1.664	2.520
Naruko	6	5.083	12.466	10.204	12.470	9.445	–	8.475	8.475	8.475	8.651	8.950
Saito	7	13.793	4.070	3.845	3.889	0.986	8.475	–	0	0	0.641	1.457
Nikko	8	13.793	4.070	3.845	3.889	0.986	8.475	0	–	0	0.641	1.457
Ueda	9	13.793	4.070	3.845	3.889	0.986	8.475	0	0	–	0.641	1.457
Futtsu	10	12.990	3.731	3.226	3.546	1.023	8.010	0	0	0	–	1.846
Nasu	11	12.346	3.606	2.446	3.402	1.181	7.611	0.118	0.118	0.118	0	–
	Nucleotide diversity						Net nucleotide divergence					
Average	6.272±0.004						5.312±0.004					
Minimum	0						0					
Maximum	15.255						15.255					

Nucleotide diversity is given above the diagonal and net nucleotide divergence is given below.

also showed that despite the pronounced genetic differences among some populations, four populations i.e. Saito, Nikko, Ueda, and Futtsu, were closely related.

The nucleotide diversity (%) and the net nucleotide divergences (%) among 11 populations of loach are shown in Table 6. The nucleotide diversity (π_{xy}) ranged from 0 to 15.255, with a mean of 6.272±0.004% (Table 6; above diagonal) and net nucleotide divergences (δ) ranged from 0 to 15.255, with a mean of 5.312±0.004% (Table 6; below diagonal). The average nucleotide diversities between the two northern populations (Memanbetsu and Naruko) were high (12.822%, ranged from 5.083 to 15.255%), in contrast, diversities among southern populations were relatively low (3.909%, ranged from 0 to 8.950%). Similarly, the net nucleotide divergence between the two northern populations (Memanbetsu and Naruko) were high (12.521%, ranged from 5.083 to 15.256%), in contrast, the net nucleotide divergence among southern populations were relatively low (3.190%, ranged from 0 to 8.475%).

The dendrogram by bootstrap NJ method showed two distinct clusters of Japanese loach, a northern group containing two populations and a southern group containing nine populations (Figure 1). The northern group included Memanbetsu (Hokkaido) and Naruko (Miyagi) populations whereas the southern group

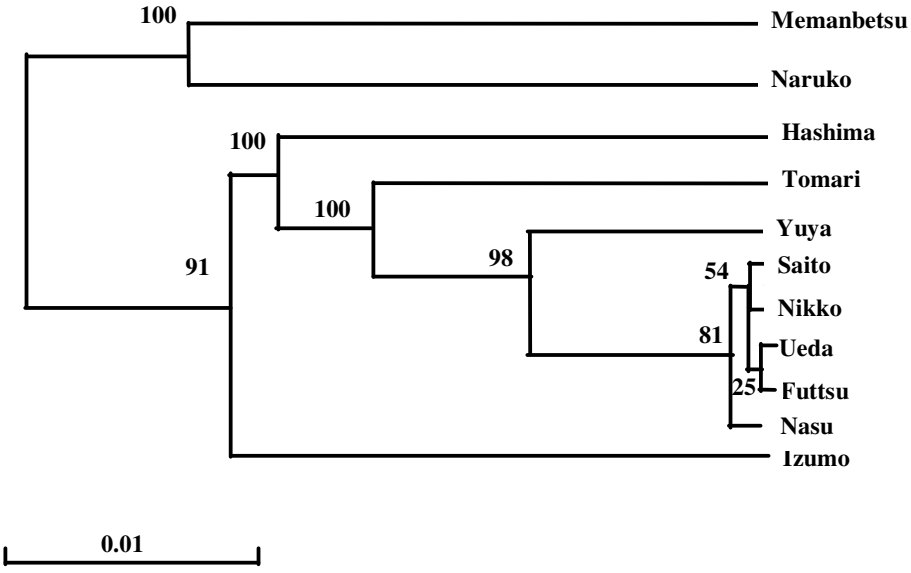

Figure 1. Neighbor-Joining (NJ) clustering of composite loach haplotypes based on nucleotide divergence (Saitou and Nei, 1987). Numbers at forks indicate bootstrap values (1,000 replicates). Three population clusters, northern (Memanbetsu and Naruko), southern 1 and 2, were obtained on the consensus tree, with more than 90% of bootstrap support for the clusters of northern and southern 1, respectively. The southern 2 separated from each other two clusters, but with less than 70% of the nodal value.

contained two subgroups; one of which contained the Hashima (Gifu), Izumo (Shimane) and Tomari (Tttori), and the other one contained the Yuya (Yamaguchi), Saito (Miyazaki), Nikko (Tochigi), Ueda (Nagano), Futtsu (Chiba) and Nasu (Tochigi) populations. The dendrogram by the UPGMA method also gave essentially same topology, showing two different geographical groups (not shown).

The geographic structure of loach was tested by AMOVA and the statistical differences among and within geographical groups and populations were calculated.

The contingency chai-square test revealed a highly significant heterogeneity in the haplotype frequencies for the entire sets of the populations (p<0.0001), northern (p<0.0001), and all southern populations (p<0.0001), (Table 5). Such significant heterogeneity was also observed for the sets of southern subgroup I (Hashima, Izumo and Tomari), and subgroup 2 (Yuya, Saito, Nikko, Ueda, Futtsu and Nasu) populations (p=0), although no significant heterogeneity was shown for the closely related (Saito-Nikko and Ueda-Futtsu) populations (p=1) (Table 5).

Using the NJ method, three population clusters, northern (Memanbetsu and Naruko), southern 1 and 2, were obtained on the consensus tree, with more than 90% of bootstrap support for the clusters of northern and southern 1, respectively (Figure 1). Early and late runs populations from the southern 2 separated from each other, but with less than 70% of the nodal value, and the heterogeneity in their haplotype was not significant by the chai-square test (p<0.1). Including these observations and the results of the above contingency chai-square tests, grouping of two geographic regions, i.e. northern and southern (subgroup 1 and 2) was considered to be most appropriate for AMOVA. The AMOVA analysis indicated that the magnitude of variance among populations within groups was moderate but significant (29.16%, p≥0.001). However, the variance within populations was very low (8.37%, p≥ 0.01) whereas most of the variation were occurred among groups (62.47%, p≥0.0001) (Table 5).

DISCUSSION

Mitochondrial DNA variation has been extensively surveyed in a number of animal species at the intraspecific level (Avise et al., 1987; Avise 1994). According to these studies in 21 species including invertebrates, fishes, amphibians, reptiles, birds and mammals, intraspecific variation of mtDNA ranged between 0.1 and 8.7% and average was 2.8%. The intra-population mtDNA variabilities were observed in four populations of Japanese loach, Hashima, Ueda, Futtsu, and Nasu. The nucleotide diversities within each of these populations were estimated to be 2.388% to 2.954%, whereas those within the other seven populations were zero. In mammals, Avise and Lansman (1983) reported that intra-populational variability of pocket gophers was less than 1%, and that of loach is larger than or equivalent to this range. The inter-populational nucleotide divergences among local populations to species level observed in fishes and amphibian are summarized in Table 7. The present study revealed that

Table 7. Nucleotide sequence divergence estimate of mtDNA species to local populations level using RFLP analysis.

Genus or species	No. of Species or subspecies surveyed	No. of restriction surveyed	Nucleotide divergence (%) (average)
Pagrus major	4 populations	6	0.82 to 1.05 (0.97) (Avise and Lansman, 1983)
Tribolodon hakonensis	3 populations	10	0.77 to 0.94 (Tabata and Nagayama, 1999)
Spirinchus lanceolatus	4 populations	9	0.27 to 0.54 (Hanzawa et al, 1987)
Paralichthys olivaceus	8 populations	11	0.83 to 1.32 (0.99) (Suzuki et al, 2000)
Rana japonica	2 subspecies	8	2.30 to 7.70 (4.8) (Okazaki et al, 1999)
Pseudobagrus	9 species	15	0.18 to 6.61 (Tabata and Taniguchi, 2000)
Pagrus	2 species	6	0.402 to 1.625 (Ashida et al, 1998)

the net nucleotide divergences between the southern and northern groups of loach (3.190-12.521%, 7,855% on average) were higher than those observed among populations of red seabream (0.82-1.05% 0.97% on average) (Tabata and Nagayama, 1999), dace (0.77-0.94%) (Hanzawa et al., 1987), smelt (0.27- 0.54%) (Suzuki et al., 2000), flatfish (0.83-1.32%, 0.99% on average) (Asahida et al., 1998), *Pagrus* (0.402-1.625%) (Tabata and Taniguchi, 2000) and bagrid catfishes (0.18-6.61%) (Okazaki et al., 1999). Thus, the results suggested that there is a big difference (probably species level) between the northern and southern groups of populations.

Whereas the northern group included the Memanbetsu and the Naruko populations, the southern group comprised two subgrups, the Hashima population and the other southern populations. The net nucleotide divergence between the Hashima and other eight southern populations (5.252%) was greater than the net nucleotide divergence among the eight populations (3.558%) whereas such pair-wise net nucleotide divergence between the northern and southern populations was 7.855% (see Table 6). The Hashima population also showed multiple haplotyes (haplotypes II and III) including uncommon cleavage sites which were not found in the other populations (Table 2). These results suggested that the Hashima population might have derived from mixing of different population. In the heterogeneity test, the variance within populations was very low (8.37%, $p \geq 0.01$). This fact suggests that the populations within groups are genetically indistinguishable. The most of the variation occurred among groups (62.47%, $p \geq 0.0001$). The results of AMOVA indicate that the two geographic groups of populations are genetically differentiated from each other.

In the allozyme analysis of loaches, Khan and Arai (2000) determined genetically different six groups on the basis of cluster analysis. The allozyme group 1 (Memanbetsu, Hokkaido), 6 (central area of Honshu island from Shin-etsu, Kanto, to Tokai district) and others were separated and diversities among them were likely to be inter-subspecific (Khan and Arai, 2000). Whereas, differentiation among other groups (group 2, 3, 4 and 5) seemed to be inter-localracial (Khan and Arai, 2000). The northern group identified here must include allozyme group 1 (Memanbetsu, Hokkaido) and group 4 (Naruko, Miyagi). On the other hand, the southern mtDNA group may comprise allozyme group 6 distributed in central area of Honshu, because four populations (Nasu and Nikko, Tochigi; Futtsu, Chiba; Ueda, Nagano) were classified to be group 6 previously. Tomari (Tottori), Izumo (Shimane), and Yuya (Yamaguchi) populations, which were the allozyme group 3, were also categorized to the southern group. In addition, Saito (Miyazaki) population of allozyme group 5 was also included in the southern group. These results suggest that the southern group should contain populations of allozyme groups 3, 5 and 6.

However, the populations of allozyme group 2 were not conclusively classified in the present study. Although populations of the group 2 discontinuously distribute in Hokkaido-Northern Tohoku, Chubu-Kinki and Western Chugoku-Shikoku area (Khan and Arai, 2000), only Hashima population was examined in the present study. Thus further RFLP analyses of mtDNA D-loop region should be made in more populations, especially belonging to the allozyme group 2.

ACKNOWLEDGMENTS

We wish to thank Dr. M. Sumida, Laboratory of Amphibian Biology, Graduate school of Science, Hiroshima University, for his advice and encouragement during the data analysis, and Dr. O. Gotoh, Department

of Biochemistry, Saitama Cancer Center Research Institute, Saitama for supplying the program software for calculating the nucleotide sequences divergences among haplotypes. We are also very grateful to Dr. S. Abe, Chromosome Research Unit, Faculty of Science, Hokkaido University, for his critical reading of our manuscript.

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