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Original Article

Allozyme variation and genetic differentiation in the loach *Misgurnus anguillicaudatus*

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SUMMARY: Allozyme variation of diploid loach *Misgurnus anguillicaudatus* was examined in samples collected from 44 localities in Japan. Genetic differentiation was estimated based on allele frequencies of 12 enzyme loci. Nine loci (36.9%) were polymorphic (<0.95 in major allele frequency) and average heterozygosities were unusually high ($H_o=0.110\pm0.051$, $H_e=0.131\pm0.052$). The coefficient of gene differentiation ($G_{st}=0.774$) suggested high genetic differentiation among populations. Based on the unweighted pair-group method using arithmetic averages (UPGMA) dendrogram, loach populations were grouped into six clusters. The population of Memanbetsu, Hokkaido (group 1) was remarkably different from others ($D=0.286$). Populations distributed in Niigata, Nagano, Tochigi, Saitama, Chiba and Shizuoka Prefectures (group 6) were also genetically distinct from other loaches ($D=0.192$). These results suggest genetic differentiation among groups 1, 6, and other loaches may be inter-subspecies level. Other loaches could be separated to four clusters (groups 2–5) at $D=0.100$ – 0.065 . Genetic differentiation among them seems to be at local race level.

KEY WORDS: allozyme, genetic distance, *Misgurnus anguillicaudatus*, population genetics.

INTRODUCTION

The loach *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes) is widely distributed in Japan, Korea, Sakhalin, Taiwan and east coasts of Asian Continent from Amur River to North Vietnam.¹ Although the taxonomic relationship among these loaches has not yet been clarified, presence of taxonomically or genetically distinct loaches has been suggested. Oliva and Hensel determined, on the basis of morphological observations, that the specimens from Yang-tse-kiang, China, belong to *Misgurnus a. tungting* Nichols, but those from Hyogo and Kagawa Prefectures, Japan, belong to *M. a. anguillicaudatus* Cantor.² They also determined that the specimens from Nagasaki, Kyushu, and Kagawa Prefectures, Japan, belong to *Misgurnus mizolepis elongatus* Kimura. However, cytogenetic studies have shown that the loach *M. anguillicaudatus* from Hubei Province, China, is tetraploid with 100 chromosomes,³ but the loach in Japan is diploid with 50 chromosomes.^{4–6} The allelic

differences were observed at the two diagnostic isozyme loci LDH-1* and MDH-2* between normal diploid loach ($2n=50$) and natural tetraploid loach ($4n=100$) which had been found in fish dealers.⁷

In Japanese diploid loach, Kimura examined the genetic variation of samples from Aomori, Akita, Ibaraki, Aichi, Gifu and Shiga Prefectures by protein electrophoresis and concluded presence of two geographical races.⁸ Dong *et al.* strongly suggested sympatric distribution of the unknown loach species which has never been found as a species, together with *M. anguillicaudatus* in Torentsu river, Ibaraki Prefecture based on allozymes and morphological traits.⁹ Recently, Zhang and Arai reported 25 triploids in 1815 loaches collected from 35 localities in Japan and they also found the diploid loach, which laid unreduced diploid eggs, in the locality showing a relatively high frequency of triploids.¹⁰ They suggested that natural triploid loaches might have resulted from fertilization of such unreduced eggs. The occurrence of such unreduced diploid eggs has been reported in unisexual vertebrates with hybrid origin¹¹ and interspecific hybrids.¹² Thus, the appearance of such a unique diploid loach and natural triploid individuals suggest the presence of genetically distinct loaches and possible hybridization between them in nature.

As described above, previous genetic studies of this species are still fragmentary and inconclusive, but seem

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to be suggestive for the presence of genetically distinct loaches. In the present study, allozyme variation was surveyed among samples from 44 localities in Japan by starch-gel electrophoresis and then genetic differentiation was examined by the cluster analyses based on allele frequencies of gene loci examined.

MATERIALS AND METHODS

Fish specimens

Samples of the loach *Misgurnus anguillicaudatus* were collected from 44 different localities from Hokkaido, Honshu, Shikoku, and Kyushu Islands, Japan, from May 1996 to October 1998 (Table 1). Loach samples from Nakamura (sample 43 in Table 1) were collected from the water way of a carp farm, and those from Hirokami (sample 18) were from a fish farm. Other samples were collected from rice fields, rivers, lakes, ponds, or artificial water ways. After confirmation of diploidy of each sample by flow-cytometry for DNA content of erythrocytes,¹³ muscle and liver tissues were taken and immediately frozen at -80°C , then stored until electrophoretic analysis. In samples collected, no mud loach *M. mizolepis* was found based on their external morphology.

Electrophoresis

The enzymes analyzed, E.C. numbers, abbreviation of enzymes and the buffer systems used for horizontal starch-gel electrophoresis are shown in Table 2. Electrophoresis was conducted using amine-citrate buffer (CA 6, CA 7)¹⁴ and tris-borate-citrate-lithium hydroxide buffer (TBCL) system.¹⁵ After electrophoresis, the gel slices (about 1 mm thickness) were histochemically stained for different enzymes as described in Allendorf *et al.*¹⁶ and Aebersold *et al.*¹⁷ with some modifications.

Loci were numbered consecutively from the anodal to the cathodal side. Thus, the most anodal one was designated '1'. Gene nomenclature followed by Shaklee *et al.*¹⁸ The electrophoretic bands corresponding to multiple alleles at each locus were alphabetically named as *a, *b, *c ---- in the order of detection.

Genetic analysis

Allele frequencies were calculated directly from observed genotypes. The distribution of observed genotypes was compared with that expected, calculated from the Hardy-Weinberg equilibrium using a χ^2 test.

When the most common (major) allele existed in a frequency less than 0.95 at a given locus, this locus was regarded as polymorphic. The mean proportions of

heterozygous loci per individual, mean proportions of polymorphic loci per population and average number of alleles per locus were calculated so as to show the extent of genetic variability.¹⁹ Expected (H_e) and observed average heterozygosity (H_o) were also calculated.²⁰

The coefficient of gene differentiation (G_{ST})²¹ was calculated in order to estimate diversity between sample lots. Genetic distance values (D)²¹ were calculated from allele frequencies for all possible pairs of sample lots. The analyses of allozyme data were performed using BIOSYS-1 version 1.7.²² Based on the D -values, a dendrogram was made using the unweighted pair-group method using arithmetic averages (UPGMA) method.²³⁻²⁵

RESULTS

Allozyme variation and genotype

The electrophoretic patterns of muscle and liver samples showed that the enzymes were controlled by 12 presumptive loci. Allele frequencies were calculated directly from observed genotypes at these loci in samples from 44 localities (Table 3).

Aspartate aminotransferase (AAT), esterase-D (EST-D), and tricerol-3-phosphate dehydrogenase (G3PDH) were dimeric enzymes and heterozygotes exhibited three banded pattern. Each enzyme was controlled by at least two loci. However, genotypes were presumed in AAT-1*, EST-D-1*, and G3PDH-1*, respectively. Creatine kinase (CK) was a monomeric enzyme controlled by at least two loci. The genotype was determined only in CK-2*.

Schematic representation of electrophoretic patterns of lactate dehydrogenase (LDH) is illustrated in Fig. 1. Lactate dehydrogenase was a tetrameric enzyme controlled by two loci: LDH-1* and LDH-2*. Three alleles were involved in LDH-1* genotypes, while there were two alleles in LDH-2*.

Malate dehydrogenase (MDH) was a dimeric enzyme controlled by four different loci: MDH-1*, MDH-2*, MDH-3*, and MDH-4* (Fig. 1). The MDH-1* locus was faintly expressed in both muscle and the liver, and reliable genotypes were difficult to read. In liver, genotypes of MDH-2* were easily read because of the suppression of the MDH-3* locus. Genotypes of MDH-3* were presumed on the electrophoretic pattern of muscle samples. MDH-4* was expressed both in the liver and muscle, and genotypes were presumed on the electrophoretic pattern.

Mannose phosphate isomerase (MPI) and phosphoglucumutase (PGM) were monomeric enzymes, controlled by single MPI* and PGM* locus, respectively. Three alleles were detected in both loci. Genotypes of PGM* locus are schematically shown in Fig. 1.

Superoxide dismutase (SOD) was a dimeric enzyme and controlled by a single SOD* locus which was strongly expressed in liver tissues. The SOD* locus was polymorphic and seven alleles were detected (Fig. 1).

Table 1 Samples of the loach *Misgurnus anguillicaudatus*

Sample	Prefecture	Locality	No. of fish	Date of collection
		Locality ¹		
1	Hokkaido	Memambetsu T. [Hongou]	20	June 98
2		Higashikawa T.	20	August 96
3		Iwamizawa C.	46	August 96
4		Ebetsu C. (Sanaebetsu R.)	14	July 98
5		Oono T.	33	September 96
6	Akita	Kakunodate C. (Hinokinai R.)	19	July 98
7	Iwate	Morioka C.	25	September 96
8		Oohasama T. (Hienuki R.)	20	October 96
9		Towa T. (Sarugaishi R.)	20	November 96
10		Kitakami C. (Kitakami R.)	20	November 96
11	Miyagi	Naruko T. (Kawatabi P.)	20	June 98
12		Shiwahime T. (Izunuma L.)	20	October 96
13	Fukushima	Tadami T.	20	October 96
14	Yamagata	Kushibiki T. (Itai R.)	36	September 96
15	Niigata	Maki T.	20	October 96
16		Yahiko V. [Murayama]	20	June 97
17		Nakanoshima T. [Nakanoshima]	20	July 97
18		Hirokami V.	12	June 96
19	Tochigi	Nasu T. (Naka R.)	40	December 97
20		Nikko C. (Naka R.)	10	August 98
21	Saitama	Hanyu C. (Kasai W.)	17	June 98
22	Chiba	Futtsu T. (Iwase R.)	20	August 98
23	Nagano	Ueda C. (Urano R.)	15	July 98
24	Shizuoka	Inasa T.	19	December 96
25	Mie	Tamaki T. [Iwade]	20	November 97
26		Ise C. (Yokowa R.)	20	December 97
27	Shiga	Makino T. (Shourai R.)	20	August 96
28		Imazu T. [Hamabun]	20	October 98
29	Fukui	Obama C. (Eko R.)	20	July 98
30	Gifu	Hashima C. [Masakicho, Oura]	20	August 98
31 ²	Kyoto	Mineyama T. (A) [Masutome]	20	June 98
32 ²		Mineyama T. (B) [Goka]	20	June 98
33	Tottori	Tomari V. [Ishiwaki]	20	June 98
34	Shimane	Izumo C.	13	November 97
35	Hiroshima	Higashi-hiroshima C.	36	October 97
36		Hiroshima C. (Misasa R.)	12	October 97
37	Yamaguchi	Toyoura T. (Kawadana R.)	37	September 97
38		Yuya T. (Asai R.)	20	June 98
39	Tokushima	Hiwasa T.	20	November 96
40 ³	Ehime	Uwa T. (A) [Kaminaru]	20	June 98
41 ³		Uwa T. (B) [Kaida]	20	June 98
42		Mima T. [Kitamasuho, Korenobi]	12	June 98
43	Kochi	Nakamura C.	12	August 96
44	Miyazaki	Saito C.	15	September 98
		Total	923	

¹ Parentheses indicate name of river (R), lake (L), pond (P), or water way (W). Other samples were collected from rice fields or fish farm (samples 18 and 44). Bracket indicates name of a section in city (C), town (T), or village (V).

² Collected in different sections in the same town. Distance between the two places was very close (about 5 km).

³ Collected in different sections in the same town. Distance between the two places was very close (about 2 km).

The χ^2 test was made in all the cases of polymorphic loci between observed and expected genotypes, based on Hardy-Weinberg equilibrium. However, the test was not effective in many cases in which the expected values were <5.

Genetic variability

The mean proportion of heterozygous loci per individual was 27.4 ± 14.5 (mean $\pm$ SD)% from the average of 44 sample lots and ranged from 5.6% (sample 35) to 75.0%

Table 2 Enzymes examined and electrophoretic buffers used

Enzyme	Abbreviation	E.C. no.	Tissue ¹	Buffer system
Aspartate aminotransferase	AAT	2.6.1.1	Muscle	CA 6 ¹
Creatine kinase	CK	2.7.3.2	Muscle	TBCL ²
Esterase-D	EST-D	3.1.1.-	Muscle	CA 7 ³
Glycerol-3-phosphate dehydrogenase	G3PDH	1.1.1.8	Muscle	CA 7
Lactate dehydrogenase	LDH	1.1.1.27	Muscle	CA 6
Malate dehydrogenase	MDH	1.1.1.37	Muscle/liver	CA 6
Mannose phosphate isomerase	MPI	5.3.1.8	Muscle	TBCL
Phosphoglucumutase	PGM	2.7.5.1	Muscle	CA 6
Superoxide dismutase	SOD	1.51.1.1	Muscle/liver	TBCL

¹ CA 6, amine-citrate buffer (pH 6.0); ¹³ ² TBCL, Tris-borate-citrate-lithium hydroxide buffer; ¹⁴ ³ CA 7, amine-citrate buffer (pH 7.0). ¹³

Fig. 1 Schematic representation of electrophoretic patterns in lactate dehydrogenase (LDH), malate dehydrogenase (MDH), phosphoglucumutase (PGM) and superoxide dismutase (SOD) of the loach *Misgurnus anguillicaudatus*. Genotypes of given locus were shown in the bottom of each illustration. Arrows indicate alleles of given locus. Arrowheads denote the origin of electrophoresis.

Table 3 Allele frequencies at 12 presumptive loci of the loach *Misgurnus anguillicaudatus*

Sample	AAT-1*				CK-2*		EST-D-1*				G3PDH-1*				LDH-1*			LDH-2*	
	*a	*b	*c	*d	*a	*b	*a	*b	*c	*d	*a	*b	*c	*d	*a	*b	*c	*a	*b
1	0.650	0.350	—	—	1.000	—	0.900	0.100	—	—	—	0.550	0.450	—	1.000	—	—	1.000	—
2	1.000	—	—	—	1.000	—	1.000	—	—	—	—	0.925	0.075	—	1.000	—	—	1.000	—
3	1.000	—	—	—	1.000	—	0.794	0.184	0.022	—	0.033	0.771	0.196	—	1.000	—	—	1.000	—
4	1.000	—	—	—	1.000	—	0.786	0.214	—	—	0.143	0.821	0.036	—	1.000	—	—	1.000	—
5	1.000	—	—	—	1.000	—	1.000	—	—	—	—	0.878	0.092	0.030	1.000	—	—	1.000	—
6	0.921	0.079	—	—	1.000	—	0.974	0.026	—	—	0.026	0.974	—	—	1.000	—	—	1.000	—
7	1.000	—	—	—	1.000	—	0.980	—	—	0.020	0.020	0.840	0.140	—	1.000	—	—	1.000	—
8	0.975	0.025	—	—	1.000	—	1.000	—	—	—	0.150	0.725	0.125	—	1.000	—	—	1.000	—
9	1.000	—	—	—	1.000	—	0.850	0.150	—	—	0.132	0.842	0.026	—	1.000	—	—	1.000	—
10	0.975	0.025	—	—	1.000	—	1.000	—	—	—	0.050	0.925	0.025	—	1.000	—	—	1.000	—
11	1.000	—	—	—	1.000	—	1.000	—	—	—	0.050	0.475	0.475	—	1.000	—	—	1.000	—
12	0.975	0.025	—	—	1.000	—	1.000	—	—	—	—	0.975	0.025	—	1.000	—	—	1.000	—
13	1.000	—	—	—	1.000	—	0.925	0.075	—	—	—	0.925	0.075	—	1.000	—	—	1.000	—
14	1.000	—	—	—	1.000	—	0.805	0.181	0.014	—	—	0.694	0.306	—	1.000	—	—	1.000	—
15	0.900	0.025	0.075	—	1.000	—	0.925	0.075	—	—	0.025	0.775	0.200	—	0.125	0.875	—	1.000	—
16	0.950	0.025	0.025	—	1.000	—	0.950	0.050	—	—	0.075	0.900	0.025	—	0.025	0.975	—	1.000	—
17	0.875	0.025	0.100	—	1.000	—	0.875	0.125	—	—	—	0.900	0.100	—	0.225	0.775	—	1.000	—
18	0.833	0.167	—	—	1.000	—	0.792	0.208	—	—	0.083	0.667	0.250	—	1.000	—	—	1.000	—
19	0.846	0.103	0.051	—	1.000	—	0.850	0.150	—	—	0.012	0.863	0.125	—	0.213	0.787	—	1.000	—
20	0.813	0.062	0.125	—	1.000	—	1.000	—	—	—	—	1.000	—	—	—	1.000	—	1.000	—
21	0.909	—	0.091	—	1.000	—	1.000	—	—	—	0.059	0.912	0.029	—	0.059	0.941	—	1.000	—
22	0.944	0.028	0.028	—	1.000	—	0.875	0.125	—	—	—	0.975	0.025	—	0.150	0.850	—	1.000	—
23	0.900	0.033	0.067	—	1.000	—	0.866	—	0.134	—	0.167	0.767	0.066	—	0.067	0.933	—	1.000	—
24	0.737	0.026	0.237	—	1.000	—	0.921	0.053	0.026	—	0.211	0.763	0.026	—	—	1.000	—	1.000	—
25	0.900	0.100	—	—	1.000	—	1.000	—	—	—	0.025	0.975	—	—	1.000	—	—	1.000	—
26	1.000	—	—	—	1.000	—	0.975	0.025	—	—	—	0.975	0.025	—	1.000	—	—	1.000	—
27	1.000	—	—	—	0.975	0.025	0.933	0.067	—	—	0.125	0.875	—	—	1.000	—	—	1.000	—
28	1.000	—	—	—	1.000	—	0.925	—	0.075	—	0.025	0.925	0.050	—	1.000	—	—	1.000	—
29	0.900	—	—	0.100	0.975	0.025	0.775	0.225	—	—	0.075	0.800	0.125	—	1.000	—	—	1.000	—
30	0.975	0.025	—	—	1.000	—	0.950	0.050	—	—	—	1.000	—	—	0.900	0.100	—	0.950	0.050
31	1.000	—	—	—	1.000	—	1.000	—	—	—	0.125	0.850	0.025	—	1.000	—	—	1.000	—
32	1.000	—	—	—	1.000	—	1.000	—	—	—	—	1.000	—	—	0.975	0.025	—	0.975	0.025
33	1.000	—	—	—	1.000	—	1.000	—	—	—	0.150	0.625	0.050	0.175	1.000	—	—	1.000	—
34	0.769	0.213	—	0.018	1.000	—	1.000	—	—	—	0.038	0.962	—	—	1.000	—	—	1.000	—
35	1.000	—	—	—	1.000	—	1.000	—	—	—	—	0.750	0.250	—	0.778	—	0.222	1.000	—
36	1.000	—	—	—	1.000	—	1.000	—	—	—	—	0.792	0.208	—	1.000	—	—	1.000	—
37	0.959	0.014	0.027	—	1.000	—	0.892	0.095	0.013	—	—	0.946	0.054	—	1.000	—	—	1.000	—
38	1.000	—	—	—	1.000	—	0.850	0.150	—	—	—	1.000	—	—	1.000	—	—	1.000	—
39	1.000	—	—	—	0.900	0.100	1.000	—	—	—	0.150	0.850	—	—	1.000	—	—	1.000	—
40	1.000	—	—	—	1.000	—	0.400	0.600	—	—	—	1.000	—	—	1.000	—	—	1.000	—
41	1.000	—	—	—	1.000	—	0.950	0.050	—	—	—	1.000	—	—	1.000	—	—	1.000	—
42	1.000	—	—	—	1.000	—	0.708	0.292	—	—	—	1.000	—	—	1.000	—	—	1.000	—
43	0.917	0.083	—	—	1.000	—	0.833	0.167	—	—	0.042	0.916	0.042	—	—	1.000	—	1.000	—
44	0.933	0.067	—	—	1.000	—	1.000	—	—	—	0.267	0.700	0.033	—	0.167	0.833	—	1.000	—

Table 3 (continued)

Sample	MDH-2*				MDH-3*		MDH-4*			MPI*			PGM*			SOD*						
	*a	*b	*c	*d	*a	*b	*a	*b	*c	*a	*b	*c	*a	*b	*c	*a	*b	*c	*d	*e	*f	*g
1	—	0.925	0.075	—	1.000	—	1.000	—	—	0.675	0.325	—	0.925	0.050	0.025	0.100	0.775	—	—	—	0.125	—
2	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.350	0.450	—	0.200	—	—	—
3	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.462	0.313	0.038	0.112	0.075	—	—
4	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	0.964	—	0.036	0.571	0.357	—	0.036	0.036	—	—
5	1.000	—	—	—	1.000	—	0.984	0.016	—	1.000	—	—	0.969	0.031	—	0.625	0.375	—	—	—	—	—
6	0.975	0.025	—	—	1.000	—	0.948	0.026	0.026	0.974	0.026	—	0.921	0.079	—	0.606	0.342	—	0.026	—	—	0.026
7	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.380	0.620	—	—	—	—	—
8	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.300	0.700	—	—	—	—	—
9	0.525	0.475	—	—	1.000	—	1.000	—	—	0.650	0.350	—	0.925	0.075	—	0.375	0.575	0.050	—	—	—	—
10	0.975	0.025	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.450	0.550	—	—	—	—	—
11	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.750	0.250	—	—	—	—	—
12	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.600	0.400	—	—	—	—	—
13	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	0.950	0.050	—	0.625	0.350	—	0.025	—	—	—
14	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	0.736	0.264	—	0.444	0.556	—	—	—	—	—
15	—	1.000	—	—	1.000	—	1.000	—	—	0.725	0.275	—	0.750	0.150	0.100	0.125	0.450	0.175	0.225	—	0.025	—
16	—	1.000	—	—	0.975	0.025	1.000	—	—	0.625	0.375	—	0.750	0.100	0.150	0.125	0.700	0.125	0.050	—	—	—
17	—	1.000	—	—	0.975	0.025	1.000	—	—	0.775	0.225	—	0.750	0.075	0.175	0.150	0.350	0.325	0.150	—	0.025	—
18	0.916	0.042	—	0.042	0.958	0.042	0.958	0.042	—	0.708	0.292	—	0.625	0.375	—	—	0.917	0.042	—	0.041	—	—
19	0.050	0.950	—	—	1.000	—	1.000	—	—	0.700	0.300	—	0.825	0.062	0.113	0.125	0.563	0.150	0.050	0.112	—	—
20	—	1.000	—	—	1.000	—	1.000	—	—	0.700	0.250	0.050	0.800	0.100	0.100	0.050	0.550	0.300	0.100	—	—	—
21	—	1.000	—	—	1.000	—	1.000	—	—	0.706	0.294	—	0.692	0.039	0.269	—	0.500	0.059	0.118	0.029	0.294	—
22	0.075	0.900	0.025	—	1.000	—	1.000	—	—	0.775	0.225	—	0.825	0.025	0.150	0.075	0.475	0.100	0.175	—	0.125	0.050
23	—	1.000	—	—	1.000	—	1.000	—	—	0.667	0.333	—	0.867	0.067	0.066	—	0.533	0.167	0.067	—	0.233	—
24	0.105	0.895	—	—	1.000	—	1.000	—	—	0.700	0.300	—	0.895	0.026	0.079	—	0.974	—	0.026	—	—	—
25	0.800	0.200	—	—	1.000	—	1.000	—	—	0.775	0.225	—	1.000	—	—	0.250	0.425	0.325	—	—	—	—
26	0.800	0.200	—	—	1.000	—	1.000	—	—	0.750	0.250	—	1.000	—	—	0.075	0.650	0.225	—	—	—	0.050
27	0.625	0.375	—	—	1.000	—	0.975	0.025	—	0.725	0.275	—	0.975	0.025	—	0.250	0.150	0.600	—	—	—	—
28	1.000	—	—	—	0.975	0.025	1.000	—	—	0.550	0.450	—	0.950	—	0.050	0.175	0.100	0.725	—	—	—	—
29	0.700	0.300	—	—	1.000	—	1.000	—	—	0.650	0.350	—	1.000	—	—	0.225	—	0.750	—	0.025	—	—
30	0.775	0.225	—	—	0.975	0.025	1.000	—	—	0.850	0.150	—	0.950	—	0.050	0.225	0.575	0.075	—	0.025	0.075	0.025
31	0.650	0.350	—	—	1.000	—	1.000	—	—	0.750	0.250	—	0.975	—	0.025	0.200	0.275	0.250	0.275	—	—	—
32	0.400	0.600	—	—	1.000	—	1.000	—	—	0.775	0.225	—	1.000	—	—	0.300	—	—	0.700	—	—	—
33	0.750	0.250	—	—	1.000	—	1.000	—	—	0.500	0.500	—	1.000	—	—	—	—	—	0.550	0.450	—	—
34	0.538	0.462	—	—	1.000	—	1.000	—	—	0.500	0.500	—	0.769	—	0.231	—	—	—	1.000	—	—	—
35	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	—	1.000	—	—	—	—	—
36	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.167	0.792	—	0.041	—	—	—
37	0.662	0.338	—	—	1.000	—	1.000	—	—	0.676	0.324	—	0.918	0.041	0.041	0.054	0.162	0.054	0.730	—	—	—
38	0.900	0.100	—	—	1.000	—	0.975	0.025	—	0.600	0.400	—	1.000	—	—	—	0.025	0.100	0.825	—	0.025	0.025
39	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.700	0.300	—	—	—	—	—
40	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.300	—	—	0.700	—	—	—
41	1.000	—	—	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.575	0.350	—	0.075	—	—	—
42	0.708	0.208	0.084	—	1.000	—	1.000	—	—	1.000	—	—	1.000	—	—	0.500	0.167	—	0.167	0.166	—	—
43	0.500	0.500	—	—	1.000	—	1.000	—	—	1.000	—	—	0.792	—	0.208	0.333	0.417	0.125	0.125	—	—	—
44	0.933	0.067	—	—	1.000	—	1.000	—	—	0.367	0.633	—	0.700	0.100	0.200	—	0.500	0.200	0.233	—	0.067	—

(sample 18) (Table 4). The mean proportion of polymorphic loci per population was $36.9 \pm 17.4\%$ from the average of 44 sample lots and ranged from 8.3% (sample 12) to 75.0% (sample 18). Mean number of alleles per locus was 1.6 ± 0.3 in average and ranged from 1.2

(sample 35) to 2.3 (sample 22). The observed heterozygosity (H_o) was 0.110 ± 0.051 in average and ranged from 0.025 (sample 8) to 0.225 (sample 17). The average heterozygosity (H_e) was 0.131 ± 0.053 and ranged from 0.049 (sample 12) to 0.219 (sample 44).

Table 4 Genetic variabilities at 12 loci in 44 sample lots of the loach *Misgurnus anguillicaudatus*

Sample	Mean proportion of polymorphic loci per population (%)	Mean proportion of heterozygous loci per individual (%)	Mean no. alleles per locus	Heterozygosity	
				H_o	H_e
1	58.3	35.0	1.8	0.170	0.190
2	16.7	10.0	1.3	0.083	0.066
3	25.0	6.5	1.7	0.096	0.115
4	25.0	28.6	1.6	0.107	0.108
5	16.7	12.1	1.4	0.035	0.066
6	33.3	42.1	1.9	0.088	0.094
7	16.7	12.0	1.3	0.030	0.066
8	16.7	15.0	1.3	0.025	0.077
9	50.0	30.0	1.7	0.096	0.183
10	16.7	20.0	1.4	0.045	0.062
11	16.7	10.0	1.3	0.071	0.078
12	8.3	15.0	1.3	0.041	0.049
13	33.3	20.0	1.4	0.054	0.073
14	33.3	11.1	1.4	0.081	0.137
15	58.3	35.0	2.1	0.166	0.205
16	50.0	40.0	2.1	0.121	0.155
17	58.3	40.0	2.1	0.225	0.213
18	75.0	75.0	2.0	0.181	0.213
19	66.7	20.0	2.2	0.137	0.215
20	33.3	40.0	1.8	0.131	0.149
21	50.0	35.3	1.9	0.166	0.167
22	58.3	40.0	2.3	0.113	0.185
23	58.3	46.7	2.0	0.194	0.192
24	50.0	36.8	1.9	0.140	0.151
25	41.7	25.0	1.5	0.142	0.132
26	25.0	25.0	1.6	0.088	0.112
27	41.7	40.0	1.8	0.125	0.163
28	41.7	30.0	1.7	0.100	0.115
29	50.0	35.0	1.8	0.192	0.186
30	58.3	45.0	2.1	0.154	0.151
31	33.3	25.0	1.7	0.095	0.161
32	25.0	25.0	1.4	0.104	0.115
33	33.3	20.0	1.5	0.150	0.164
34	33.3	38.5	1.5	0.154	0.155
35	16.7	5.6	1.2	0.051	0.060
36	16.7	16.7	1.3	0.042	0.058
37	58.3	18.9	1.3	0.115	0.156
38	33.3	25.0	2.0	0.079	0.108
39	25.0	15.0	1.7	0.050	0.073
40	16.7	10.0	1.2	0.075	0.076
41	16.7	10.0	1.3	0.063	0.054
42	25.0	25.0	1.5	0.104	0.133
43	50.0	50.0	1.8	0.167	0.182
44	58.3	46.7	1.9	0.194	0.219
Average	36.9	27.4	1.6	0.110	0.131
Standard deviation	17.4	14.5	0.3	0.051	0.053

Genetic differentiation

The coefficient of gene differentiation (G_{ST})²¹ was estimated to be 0.774, showing high genetic differentiation among populations. The genetic distance (D)²¹ varied from 0.001 to 0.399. The minimum D -value (0.001) was observed between Oono (sample 5) and Morioka (sample 7). The maximum value (0.399) was observed between Memanbetsu (sample 1) and Saito (sample 44). The average D -values between below-mentioned clustering groups and those within each group are shown in Table 5.

A dendrogram was drawn by the UPGMA method as shown in Fig. 2. When a vertical line was drawn across the dendrogram as an attempt to delimitate a group

having $D=0.06$, six groups can be recognized (Fig. 2). The sampled localities of each group are shown in Fig. 3. Group 1 comprised only of the Memanbetsu population (sample 1) was clearly separated from others ($D=0.286$). Average genetic distances between group 1 and each of the other groups were large (0.246–0.385) as seen in Table 5. Group 6 was then separated from other four groups at the D -value of 0.192 (Fig. 2). Average genetic distances between group 6 and each of the other four groups were relatively large (0.216–0.089) as seen in Table 5. This group included loaches distributed in the central area of Honshu island from Shin-etsu, Kanto to Tokai districts, comprising Niigata (except for Hirokami population), Nagano, Tochigi, Saitama, Chiba, and Shizuoka Prefectures. (Fig. 3).

Table 5 Average D -values ($\pm$ SD) between groups and those within each group of the loach on the basis of 12 allozyme loci

	Group 2	Group 3	Group 4	Group 5	Group 6
Within group	0.016 $\pm$ 0.017 (0.001–0.073)	0.027 $\pm$ 0.012 (0.009–0.038)	0.013 $\pm$ 0.012 (0.002–0.026)	0.041	0.013 $\pm$ 0.008 (0.004–0.026)
Group 1 ¹	0.285 $\pm$ 0.050 (0.188–0.384)	0.246 $\pm$ 0.057 (0.181–0.324)	0.371 $\pm$ 0.007 (0.364–0.378)	0.385 $\pm$ 0.020 (0.371–0.399)	0.258 $\pm$ 0.021 (0.227–0.285)
Group 2		0.069 $\pm$ 0.027 (0.021–0.157)	0.075 $\pm$ 0.020 (0.044–0.121)	0.122 $\pm$ 0.030 (0.077–0.178)	0.197 $\pm$ 0.037 (0.116–0.292)
Group 3			0.119 $\pm$ 0.027 (0.075–0.169)	0.148 $\pm$ 0.030 (0.105–0.209)	0.216 $\pm$ 0.051 (0.104–0.303)
Group 4				0.065 $\pm$ 0.019 (0.044–0.099)	0.172 $\pm$ 0.013 (0.141–0.191)
Group 5					0.089 $\pm$ 0.028 (0.048–0.134)

¹ Group 1 consisted of a single sample lot (Memanbetu, Hokkaido). Groups 1–6 are as in the dendrogram shown in Fig. 2. Parentheses show range of D -value.

Fig. 2 UPGMA dendrogram for 44 sample lots of the loach *Misgurnus anguillicaudatus*.

In other loaches from 34 localities, the cluster comprising groups 2 and 3 can be further separated from that comprising groups 4 and 5 at the $D=0.100$ (Fig. 2). The genetic distance between groups 2 and 3 was 0.069 and that between groups 4 and 5 was 0.065 (Table 5). Loaches of group 2 were discontinuously distributed in Japan (Fig. 3). Based on the geographical distribution pattern, they can be subgrouped into three: (i) Hokkaido–Northern Tohoku (Iwate and Akita Prefecture) district; (ii) Chubu (Gifu and Mie Prefectures)–Kinki (Shiga, Fukui, and Kyoto Prefectures) district; and (iii) Sanyo (Hiroshima and Yamaguchi Prefectures)–Shikoku (Ehime Prefecture) district. Only the Hirokami sample lot (18) was geographically isolated from the other subgroups. These subgroups cannot be classified by the genetic distances because geographically distant samples were often arranged as genetically close relationship as seen in Fig. 2.

Figure 3 also shows that loaches of group 3 distributed in Kinki (Kyoto Prefecture), San-in (Tottori and Shimane Prefectures), Sanyo (Hiroshima and Yamaguchi

Prefectures), and Shikoku (Ehime and Tokushima Prefectures) districts. Sample 32 was included with group 3 but sample 31 was collected very close to group 2. Similar situation was seen between samples 40 and 41. Group 4 included samples from Southern Tohoku district comprising Yamagata, Miyagi, and Fukushima Prefectures. While genetically close, group 5 included loaches from Kochi Prefecture, Shikoku island and Miyazaki Prefecture, Kyushu island.

DISCUSSION

The proportion of polymorphic loci per population was $36.9 (\text{mean}) \pm 17.4 (\text{SD})\%$ in the loach. This value is much higher than the reported average ($19.4 \pm 2.3\%$) based on allozyme studies in 41 marine teleost species.²⁶ When compared with freshwater species, the value is relatively similar to that for fluvial dace (22%)²⁷ but higher than fluvial sculpin (3%),²⁸ and land-locked stickleback (4%).²⁹ Thus, genetic polymorphism is maintained in the

Fig. 3 Geographical distribution of six genetically distinct groups, including 44 sample lots of the loach *Misgurnus anguillicaudatus*.

loach. This conclusion is also suggested by the mean proportion of heterozygous loci ($27.4 \pm 14.5\%$) and the mean number of alleles per locus (1.6 ± 0.3) in the loach.

The average heterozygosity ($H_o = 0.110 \pm 0.051$; $H_e = 0.131 \pm 0.053$) estimated in the loach is unusually higher than the average values (0.059 ± 0.007) from 41 marine fishes,²⁴ and the values reported in fluvial or land-locked species such as dace (0.04),²⁷ sculpin (0.01),²⁸ stickleback (0.01),²⁹ freshwater goby (0.04),³⁰ medaka (0.004–0.111).³¹ Thus, the loach is maintaining much higher heterozygosity than other fluvial fishes.

The G_{ST} value (0.774) of the total population is nearly equivalent to those estimated in other Japanese fluvial fishes such as freshwater goby (0.698)³⁰ and southern group of brook lamprey (0.629).³² This suggests a large proportion of genetic variation in the loach which may be due to differences among populations from different localities.

Loaches can be grouped into six as shown in the dendrogram. Group 1 (Memanbetsu, Hokkaido) was a highly divergent population which was separated from others at $D = 0.286$. Genetic differences between Memanbetsu population (group 1) and other populations of groups 2–6 were in the range of D -values approximately between 0.4 and 0.2. The group 6 was separated from others at $D = 0.192$. Average genetic distances between group 6 and others were in the range approximately between 0.2 and 0.1. These D -values are lower than those between different species of freshwater teleosts in the same genus reported in *Lepomis* ($D = 0.627$),^{33,34} *Menidia* ($D = 0.421$),³⁵ *Oncorhynchus* ($D = 0.392$),³⁶ *Salmo* ($D = 0.496$),³⁶ *Salvelinus* ($D = 1.169$),³⁶ *Zacco* ($D = 0.640$),³⁷ and *Rhinogobius* ($D = 0.35$ – 1.77),³⁸ but nearly equivalent to those between subspecies in the same species reported in *ayu*³⁹ ($D = 0.242$). Shimizu *et al.*³⁰ reported that D -values among the five groups of *Rhinogobius* ranged from 0.07 to 0.23 and suggested that the highest genetic differentiation among them was the species or subspecies level. Nei found that in a variety of animals, D is approximately 1.0 for between-species comparisons, around 0.1 for subspecies, and 0.01 for local races.²⁴ Ayala reported that the D -value between subspecies is approximately 0.20.⁴⁰ Judging from the above criteria, genetic differentiation among group 1, group 6, and others (groups 2–5) of the Japanese diploid loach is likely to be inter-subspecies level. Genetically divergent groups 1 and 6 are geographically separated from others and distribute in the northern Hokkaido and the central area of Honshu Island, respectively.

In the near future, mechanisms of reproductive isolation among them should be elucidated by experimental hybridizations between them and subsequent observations of survival and reproductive potential of the resultant hybrids. In the case of Japanese frog species, *Rana japonicus*, distinct genetic differentiation was observed between the eastern and western geographical groups at the mean D -value of 0.156.⁴¹ In reciprocal hybrids

between these two races, a remarkable preponderance of males was observed.⁴² In interspecific hybridization of medaka species, female hybrids produced unreduced diploid eggs, while male hybrids were sterile.¹² In the loach, Zhang and Arai have found relatively high frequencies of natural triploids in Ichinomiya town, Aichi Prefecture and Hirokami village, Niigata Prefecture.¹⁰ Relatively high frequency of natural triploids was also observed in Memanbetsu, Hokkaido (S. Horie and K. Arai, unpubl. data, 1999). These localities seem to be in zones between genetically distinct groups shown in the present study. An accidental hybridization between genetically distinct loaches may give rise to the occurrence of sterile progeny or semi-fertile progeny which are able to produce unreduced eggs. In Hirokami, the occurrence of unreduced diploid eggs was found in diploid loach.¹⁰

In other populations, the cluster including groups 2 and 3 was separated from that including groups 4 and 5 at the D -value of 0.100. The loaches of group 2 distributed all over Japan except for the central part (Shin-etsu, Kanto, and Tokai district) and Kyushu district. Group 3 was genetically close to group 2 and its distribution overlapped with group 2 in Chugoku and Shikoku district. Some samples, such as 31 and 32, and 40 and 41, were geographically very close, but they were separated into different groups (2 and 3). Group 4, distributed in Southern Tohoku district, was genetically close to group 5, distributed in Shikoku and Kyushu district. Genetic differentiation among these populations seems to be a local race level, judging from the aforementioned examples.²⁴

Although group 2 showed discontinuous distribution, these samples are likely to be categorized into three geographically independent subgroups: Hokkaido–Northern Tohoku, Chubu–Kinki and Western Chugoku–Shikoku, with the exception of Hirokami samples in Niigata Prefecture. These populations were not clearly separated as independent clusters by the present genetic studies using 12 allozyme loci. The analyses suggest that the loach has a tendency to split to a number of local populations, although the question of whether each population was wild or a mixture due to artificial factors such as implantation remains. Observed inconsistencies between genetic and geographical distances in several populations might be due to an insufficient number of samples and genetic loci examined. These problems must be clarified by a series of further studies on more samples using additional gene markers such as mtDNA and microsatellites.

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