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Genetic Differentiation of Piscivorous Chub (genus *Opsariichthys*) in Japan, Korea and Russia

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ABSTRACT—A comparison of allozyme variation, restriction fragment length polymorphisms in the mitochondrial DNA and partial sequences of the ND II gene (496 bp) was made among two lacustrine populations of the piscivorous chub (*Opsariichthys uncirostris uncirostris*) in Japan, four fluvial populations in Korea, one lacustrine population in Russia and one specimen from the Amur River (*O. u. amurensis*). All analyses indicate that the Japanese populations of piscivorous chub are separable from the Asian mainland populations of Korea and Russia. The latter populations were further divided into the Korean and Russian fish. Although opinions are divided on the phylogenetic position of the population in Lake Mikata, Japan, which shows unique morphological traits intermediate between those of the population in Lake Biwa and the mainland populations, the current analysis indicates a closer relationship to the population in Lake Biwa.

Key words: *Opsariichthys*, mt-DNA, genetic differentiation, Japan, Asian mainland

INTRODUCTION

The piscivorous chub, a member of the cyprinid genus *Opsariichthys*, is widely distributed in East Asia. Its natural range extends from the Amur basin to the rivers that enter the Yellow Sea on the Korea Peninsula and from the Yangtze basin in China to the northern parts of Vietnam and Laos including Hainan Island (Berg, 1948; Uchida, 1939; Wu *et al*., 1964). The fish is also found in central Japan (Nakamura, 1969; Fig. 1). Banarescu (1968) recognized four subspecies in *Opsariichthys uncirostris* on the basis of the number of scales; *O. u. uncirostris* in Japan; *O. u. amurensis* in the Amur drainage, parts of North China and Korea; *O. u. bidens* in Central and South China; *O. u. hainanensis* in Hainan Island. Banarescu (1968) also notified that the Korean specimens are intergrades between O*. u. uncirostris* and *O. u. amurensis*.

The population in Lake Biwa is unique insofar as these fish have a larger number of scales on the lateral line than those in the other piscivorous populations (Nakamura, 1969). The fish distributed in the Amur basin, Korea and China have a small number of scales (Banarescu, 1968), while the number of scales in the population in Lake Mikata is intermediate between those in Lake Biwa and those on the Asian mainland (Tanaka, 1970). The above differences have led to some hypotheses as to the origin of the Japanese forms and their routes of dispersal from Korea. They include a sea route via the Sea of Japan and an overland route (Tsubokawa, 1988; Tomoda, 1989).

This study examines the genetic differentiation of the piscivorous chub that Banarescu (1968) suggested to be closely related. The samples were collected in Japan, Korea and Russia and examined using the data of allele-frequency, mitochondrial(mt) DNA restriction fragment length polymorphisms and mtDNA sequences, which could also elucidate the dispersal route to Japan.

MATERIALS AND METHODS

Specimens

Fish were caught with traps and hand nets in each locality from 1990 to 1996. Specific locations are shown in Fig. 1 and sample sizes are listed in Tables 2, 3 and 5. *Opsariichthys u. uncirostris* were collected in the Hasu River as it entered Lake Mikata, and *O. u. amurensis* were collected in the Somjin R., the Yongsan R., the Kum R., the Han R., the Amur R. and Lake Khanka. Fish collected respectively at three sites on Lake Biwa and at two sites on the Han River were pooled for analyses. Whole fish were frozen or preserved in ethanol immediately after collection until they were

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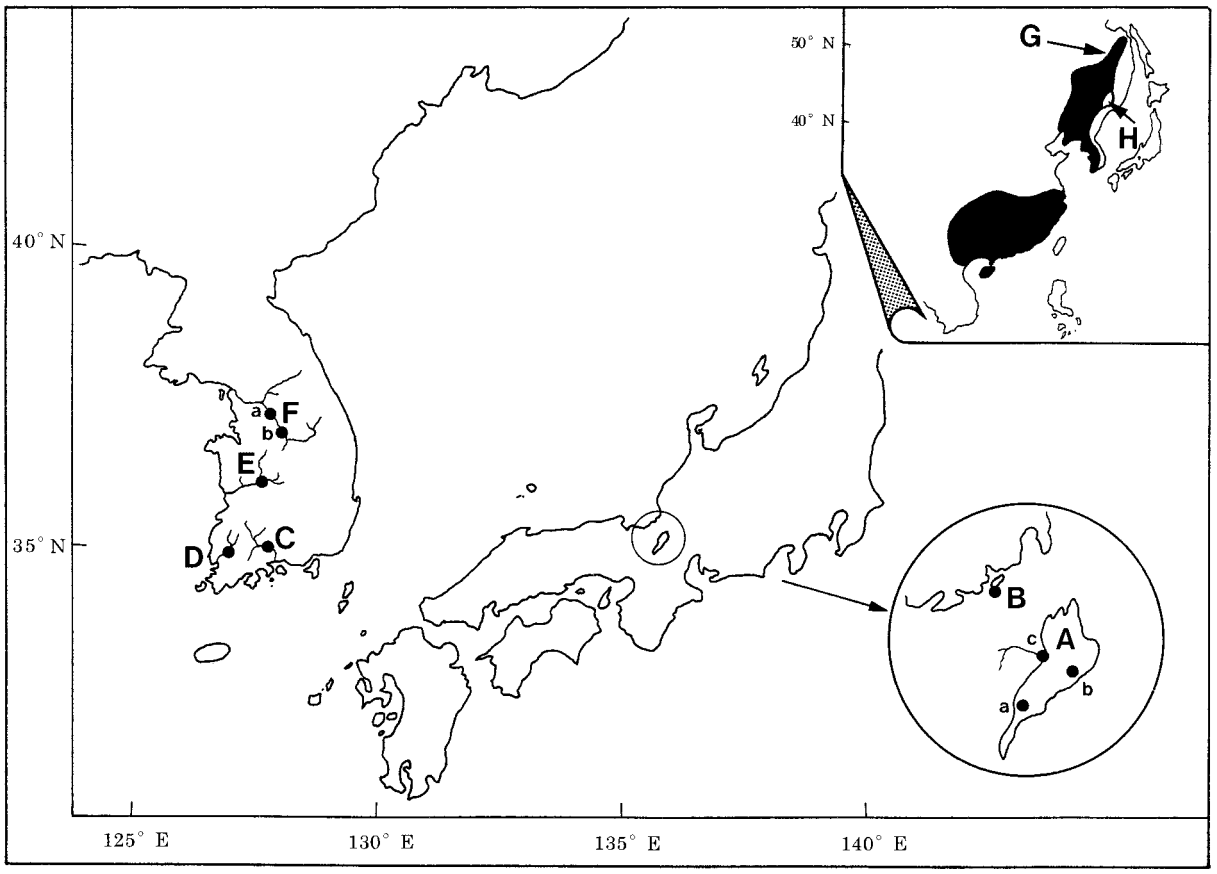


Fig. 1. Map of East Asia showing the sites where piscivorous chub were collected and the endemic range of this fish (shaded; Berg, 1948; Uchida, 1939; Wu *et al*., 1964; Nakamura, 1969). A) Lake Biwa (a;Mano, b;Sugoshi, c;Ado) ; B) L. Mikata; C) Somjin River (Yongdu-ri); D)Yongsan R. (Kumsong-ri); E) Kum R. (Changwi-ri); F) Han R.(a;Sewol-ri, b;Kahung-ri) ; G) Amur R (Troickoje).; H) L. Khanka.

processed for analysis. Only analysis of mtDNA was performed in the case of the specimens from the Amur River and Lake Khanka, which were preserved in ethanol.

The dark chub, *Zacco temmincki* (collected in the Onga River, Fukuoka Prefecture) and the venus fish, *Aphyocypris chinensis* (collected in the Chikugo River, Fukuoka Prefecture), comprising subfamily Danioninae along with the piscivorous chub (Hosoya, 1993), were included as an outgroup for restriction and sequence analyses.

Number of lateral scales were counted from the first scale in contact with the pectoral girdle to the structural base of the caudal fin.

Allozyme analysis

Analysis on horizontal starch gels was performed as described by May *et al*. (1979) and Harris and Hopkinson (1976). Liver, skeletal muscle, eye ball and heart tissues were used, and thirty protein systems were examined (Table 1). The nomenclature of loci and genes followed that of Shaklee *et al*. (1990). If multiple loci code for a protein, the loci are numbered starting with the one which encodes the proteins with the least anodal migration. The most common allele at a given locus of fish collected in Lake Biwa was designated *100, and other alleles were assigned numbers according to the mobility of their homomeric protein products relative to that of the *100 allele.

The genetic basis of banding variants was inferred by application of the following criteria: (1) the banding patterns had to be consistent with the known molecular structure of the protein; and (2) when a gene was expressed in more than one tissue, variant phenotypes had to be parallel among tissues (Grant *et al*., 1983).

Restriction analysis

Total DNA was extracted from approximately 500 mg of the white muscle tissue of each fish by the method of Asahida *et al*. (1996). Fragments of about 1.5 kilo base pairs (kbp) in length from the mitochondrial gene for $tRNA^{Pro}$ to that for 12S rRNA, including the control region, were amplified by PCR (Saiki *et al*., 1988) from whole genomic DNA using primers PRO-L(CTACCTCCAACTCCCAAAGC) and 12SAR-H (ATAGTGGGGTATCTAATCCCAGTT; Palumbi *et al*., 1991). PCR was conducted for 30 cycles in a PJ-9600 apparatus (Perkin Elmer Cetus, CA) at 94°C for 0.5 min., 55°C for 0.5 min., and 72°C for 2.0 min. All products of PCR were confirmed to be of equal length by electrophoresis and were subjected directly to digestion with restriction endonucleases.

The products of PCR were digested according to recommendations of the manufacturer of enzymes. We used enzymes that recognize four or five nucleotides (Table 4; from New England Biolabs, Beverly, MA; and Takara Shuzo, Kyoto). Restriction fragments were separated by electrophoresis on horizontal 3.0% agarose gels. The bands of DNA were visualized for photography by staining with ethidium bromide and exposure to UV light. The restriction analysis was conducted to supplement sequence analysis.

Sequence analysis

Nine individuals representing each haplotype resulting from restriction fragment length polymorphism (RFLP) analysis, were selected for sequence analysis. A partial region of the mitochondrial

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Table 1. Electrophoretic analysis of allozymes from piscivorous chub collected in Japan and Korea

Enzyme	Enzyme number	Locus	Tissue[a]	Buffer[b]
Aspartate aminotransferase	2.6.1.1	AAT-1	E,L,M	3,4
		AAT-2	E,H,L,M	3,4
Acid phosphatase	3.1.3.2	ACP	L	3,4
Adenosine deaminase	3.5.4.4	ADA	E	2
Alcohol dehydrogenase	1.1.1.1	ADH	L	3
Aconitate hydratase	4.2.1.3	AH-1	H,L	3
		AH-2	H,L	3
Adenylate kinase	2.7.4.3	AK	M	3
Alanine aminotransferase	2.6.1.2	ALAT	M	1
Creatine kinase	2.7.3.2	CK	M	1
Fructose-bisphosphate aldolase	4.1.2.13	FBALD-1	M	3
		FBALD-2	E	3
Fumarate hydratase	4.2.1.2	FH	M	3
beta-Galactosidase	3.2.1.23	bGAL	L	1
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	GAPDH-1	M	3
		GAPDH-2	M	3
Glycerol-3-phosphate dehydrogenase	1.1.1.8	G3PDH	M	3
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6PDH	E	2
Glucose-6-phosphate isomerase	5.3.1.9	GPI-1	M	1,3
		GPI-2	M	1,3
Glutathione reductase	1.6.4.2	GR	L	1
Isocitrate dehydrogenase($NADP^+$)	1.1.1.42	IDHP-1	M	3,4
		IDHP-2	L	3,4
		IDHP-3	L	3,4
L-Lactate dehydrogenase	1.1.1.27	LDH-1	L	3,4
		LDH-2	E,H,M	3,4
		LDH-3	E,H,M	3,4
Malate dehydrogenase	1.1.1.37	mMDH-1	H,M,L	3
		mMDH-2	H,M,L	3
		sMDH-3	E,H,L	3
		sMDH-4	E,H,M	3
Malic enzyme($NADP^+$)	1.1.1.40	MEP	M	4
Mannose-6-phosphate isomerase	5.3.1.8	MPI	E,H	3
Dipeptidase	3.4. –. –	PEPA	E,M	1,2
Tripeptide aminopeptidase	3.4. –. –	PEPB	E,M	1,2
Peptidase-C	3.4. –. –	PEPC	E,M	1,2
Proline dipeptidase	3.4.13.9	PEPD	M	1,2
Peptidase-S	3.4. –. –	PEPS	E,M	1,2
Phosphogluconate dehydrogenase	1.1.1.44	PGDH	M	3
Phosphoglucomutase[c]	5.4.2.2	PGM-1	M	4
		PGM-2	M	4
Superoxide dismutase	1.15.1.1	SOD	L	2,3,4
Triose-phosphate isomerase	5.3.1.1	TPI-1	E	3
		TPI-2	E	3

[a] E, eye; H, heart; L, liver; M, muscle.

[b] 1=Discontinuous Tris-citric acid (gel, pH 8.5), lithium hydroxide-boric acid (tray, pH 8.5) buffer system, as described by Ridgway *et al.* (1970) .
2=Tris-boric acid-EDTA buffer (pH 8.5), as described by Markert & Faulhaber (1965).
3=Amine [N-(3-aminopropyl)-morpholine] citrate buffer (pH 6.5), as described by Clayton & Tretiak (1972).
4=Amine [N-(3-aminopropyl)-diethanolamine] citrate buffer (pH 7.0), as described by Numachi *et al.* (1979) with slight modifications based on the work of Clayton and Tretiac (1972) .

[c] Note that the PGM-1 locus was not routinely scored because of poor resolution.

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ND II gene (496 bp following $tRNA^{Met}$) which is considered one of the most variable region among protein coding genes in mtDNA (Meyer, 1994) was amplified by PCR using the following primer pairs: (5'-TAAGCTATCGGGCTCATACC-3') and (5'-TTTGAAGGC-TYTTGGTCTAA-3'). These were designed based on the reported primer (Hall and Nawrocki, 1995) and the sequences of mitochondrial DNA from carp (Chang *et al*., 1994) and human (Anderson *et al*., 1981). PCR was conducted for 30 cycles in the abovementioned apparatus at 94°C for 1.0 min., 52°C for 1.0 min., and 72°C for 2.0 min. Amplified DNA was resolved by electrophoresis on 2% agarose gels, stained with ethidium bromide and purified with a QIA quick spin column (Qiagen, Germany).

Sequence data were obtained using the dye-primer Taq cycle-sequencing reaction and an automated DNA sequencer (Applied Biosystem 377A). Sequences were determined three times. The nucleotide sequence data reported in this paper are available from DDBJ, EMBL and GenBank accession numbers AB050615-050619.

Data analysis

Nei's unbiased genetic distance (Nei, 1978) values were estimated from allele-frequency data and a phenogram was generated by the unweighted pair-group method (UPGMA; Sneath and Sokal, 1973). Since an outgroup was not included in the analysis, we used this procedure.

Nucleotide sequence divergence was estimated by comparing the electrophoretic patterns of the two DNA sequences in question (length-difference method; Nei and Li, 1979) digested by a restriction endonuclease. DNA sequence data were processed using Higgins parameter in DNASIS programs (Hitachi Software Engineer. Co. Ltd. Japan). Genetic distances for sequence analysis were obtained using Kimura's two parameter method (Kimura, 1980). Phenograms were generated by neighbor-joining analysis (NJ; Saitou and Nei, 1987) and the reliability of each interior branch was tested by 1,000 bootstrap replications both using the MEGA 1.02 computer package (Kumar *et al*., 1993)

RESULT

Numbers of lateral scales

The distribution of the number of scales on the various specimens is shown in Table 2. The range for fish from Lake Biwa did not overlap that of fish from the Asian mainland. The average number of scales on fish from Lake Mikata was intermediate between that on fish from Lake Biwa and that from the Asian mainland, as described by Tanaka (1970). Significant differences (Welch's t test; P<0.01) were observed between Lake Mikata population and Lake Biwa population and between Lake Mikata and Asian mainland populations.

Allozyme analysis

Among 44 presumable loci surveyed, 14 loci seemed polymorphic in the piscivorous chub populations when we applied the criterion that a locus should be considered polymorphic in populations in which the frequency of the most common allele is less than 0.99. Though the *PGM-1* locus was highly polymorphic, it was not routinely scored because of poor resolution. At all polymorphic loci, deviation of the observed number from the expected number was not significant (χ^2 test on the assumption of a Hardy-Weinberg equilibrium).

Allelic frequencies at polymorphic and diagnostic loci for the collected specimens are listed in Table 3. The allelic frequencies of the Korean populations are taken from a previous report (Okazaki *et al*., 1993). The alleles in Japanese fish were replaced by alleles different from those in the Korean samples at three loci. By contrast, the genetic diversity within the Japanese populations was quite small. A significant difference in frequencies of alleles was barely observed at the *Ldh-1* locus (G test , P<0.01; Sokal and Rohlf, 1995) and the *Ah-2* locus (G test, P<0.05) when we compared populations from Lake Biwa and Lake Mikata. Two-allele polymorphism at the *Ldh-1* locus was only observed in the population from Lake Biwa. In case of the Korean fish some characteristic traits were observed in the examined populations. The population from the Kum River was characterized by the unique allele *Ah-2-160* which was found at high frequency. Populations from the Somjin and Yongsan Rivers were polymorphic for the *Ada* locus but no variation was seen in the remaining populations. The estimates of expected average heterozygosity (revised by reference to sample size; Nei, 1978) ranged from 1.2% to 3.2% (Table 3).

A phenogram (UPGMA), generated on the basis of Nei's unbiased genetic distances (Fig. 2), indicates that the Japanese populations of piscivorous chub are separable from the Korean populations.

Table 2. Distribution of the number of scales on the lateral line for each population of the piscivorous chub

Locality	Sample size	Number of scales																				Average
		43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	
L. Biwa	23										5	4	4	2	2	2	3				1	54.78
L. Mikata	25					1	8	5	5	2	4											49.44
Somjin R.	16		2		4	6	4															46.63
Yongsan R.	16		2	9	3	2																45.31
Kum R.	16			8	5	3																45.69
Han R.	16	2	1	7	2	3	1															45.38
Amur R.	1				1																	46.00
L. Khanka	4			1	3																	45.75

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Table 3. Observed allelic frequencies at polymorphic and diagnostic loci for populations of piscivorous chub from Japan and Korea

		Lake Biwa	L. Mikata	Somjin River[1)]	Yongsan R.[1)]	Kum R.[1)]	Han R.[1)]
*ACP**	**100*	.958	1.000	1.000	1.000	.969	.938
	**90*	.010	.000	.000	.000	.031	.031
	**110*	.031	.000	.000	.000	.000	.031
*ADA**	**100*	1.000	1.000	.000	.000	.000	.000
	**60*	.000	.000	.844	.875	1.000	1.000
	**15*	.000	.000	.156	.125	.000	.000
*ADH**	**100*	1.000	1.000	1.000	1.000	.969	1.000
	**400*	.000	.000	.000	.000	.031	.000
*AH-1**	**100*	1.000	1.000	.000	.000	.000	.000
	**120*	.000	.000	1.000	.969	1.000	.906
	**30*	.000	.000	.000	.031	.000	.094
*AH-2**	**100*	.500	.663	.000	.000	.000	.000
	**80*	.032	.000	.000	.000	.000	.000
	**115*	.043	.000	.000	.000	.000	.000
	**140*	.426	.336	1.000	1.000	.531	1.000
	**160*	.000	.000	.000	.000	.469	.000
*CK**	**100*	.990	1.000	1.000	1.000	1.000	1.000
	**80*	.010	.000	.000	.000	.000	.000
*FBALD-2**	**100*	1.000	.990	1.000	1.000	1.000	1.000
	**95*	.000	.010	.000	.000	.000	.000
*GAPDH-2**	**100*	1.000	1.000	.531	.469	.593	.406
	**150*	.000	.000	.469	.531	.406	.593
*G3PDH**	*–100*	1.000	1.000	1.000	1.000	.969	1.000
	–150	.000	.000	.000	.000	.031	.000
*GPI-2**	**100*	.990	1.000	1.000	1.000	.938	1.000
	**140*	.000	.000	.000	.000	.063	.000
	**160*	.010	.000	.000	.000	.000	.000
*LDH-1**	*–100*	.688	1.000	1.000	1.000	1.000	1.000
	–65	.313	.000	.000	.000	.000	.000
*sMDH-3**	**100*	1.000	1.000	1.000	.969	.969	1.000
	**80*	.000	.000	.000	.000	.031	.000
	**70*	.000	.000	.000	.031	.000	.000
*PEPC**	**100*	1.000	1.000	.000	.000	.000	.000
	**110*	.000	.000	1.000	1.000	1.000	1.000
*PEPS**	**100*	1.000	.980	1.000	.906	1.000	1.000
	**125*	.000	.020	.000	.094	.000	.000
*PGM-2**	**100*	.990	1.000	1.000	1.000	1.000	1.000
	**75*	.010	.000	.000	.000	.000	.000
Sample size		49	50	16	16	16	16
Average heterozygosity		0.027	0.012	0.019	0.024	0.032	0.019

1) taken from Okazaki *et al*, 1993

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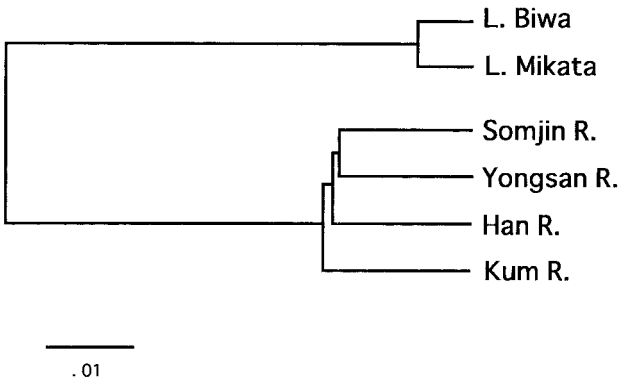


Fig. 2. UPGMA phenogram for the populations of the piscivorous chub of Japan and Korea based on genetic distances (Nei, 1978) using 43 loci.

Restriction analysis

Seven out of 14 restriction endonucleases yielded multiple banding patterns (restriction morphs; Table 4). Samples from Japan and the Asian mainland were characterized by different morphs produced by *Alu* I, *Hae* III, *Scr*F I and *Taq* I. The specimens from the Amur River and Lake Khanka, both consisting a single river system, yielded unique morphs upon digestion by *Hinf* I, *Scr*F I and *Taq* I.

Table 5 shows 9 composite genotypes (haplotypes) detected among 53 piscivorous chub examined and their localities. The populations of Lake Biwa, Lake Mikata, the Kum River and the Hun River had their own haplotypes. The specimens examined from the Amur River and Lake Khanka shared a same haplotype. A phenogram drawn by the NJ procedure (Fig. 3-A) revealed two definite clusters, from Japan and the Asian mainland, as was also found after the analysis of allozymes (Fig. 2). The mainland cluster is clearly divided into the Korean samples and the specimens from the Amur River and Lake Khanka.

Sequence analyses

Four hundred and ninety-six base pairs of the nucleotide sequences following $tRNA^{Met}$ were determined from the 9 specimens of the piscivorous chub representing each RFLP haplotype and two specimens of the outgroup species. Fig. 4 summarizes the variable nucleotide positions.

A constructed phenogram by the NJ procedure (Fig. 3-B) as well as those obtained in the allozyme and in the restriction analyses revealed that the Japanese populations are separable from the Asian mainland populations of Korea and Russia. This phenogram also indicates that the Amur River specimen is separated from the Korean samples. The above is supported by high bootstrap values. The difference was observed in the branching order among haplotype-6, -7 and -8 between the restriction and sequence analyses. The probability of these relationships, however, did not receive high bootstrap support. In spite of the slight difference observed in the branching order among the Korean fish, the topology of UPGMA phenogram was almost same

Table 4. Restriction enzymes used to examine piscivorous chub mt-DNA and their resulting fragment patterns

Enzyme	Fragment patterns (×10bp)	Enzyme	Fragment patterns (×10bp)
Aci I		*Hin f* I	
A	6, 15, 21, 21, 86	A	17, 19, 23, 92
B	5, 6, 15, 21, 102	B	23, 36, 92
		C	8, 23, 36, 84
Afa I		D	8, 17, 19, 23, 84
A	8, 22, 42, 70	E	23, 30, 36, 62
B	8, 42, 92	F	17, 19, 23, 42, 50
C	8, 18, 24, 92	G	23, 33, 36, 59
D	6, 12, 32, 92		
		Mbo I	
Alu I		A	150
A	9, 16, 28, 36, 55		
B	9, 16, 16, 20, 28, 55	*Msp* I	
C	9, 36, 99	A	13, 22, 116
D	14, 16, 16, 36, 62	B	13, 22, 49, 67
		C	13, 22, 25, 91
Bfa I		D	5, 8, 22, 116
A	10, 16, 42, 82		
B	10, 14, 18, 42, 66	*Nla* III	
C	9, 9, 35, 97	A	16, 17, 35, 38, 41
		B	16, 16, 17, 41, 57
Bst U I			
A	12, 20, 45, 72	*Sau* 96 I	
B	20, 56, 73	A	8, 12, 21, 105
Hae III		*Scr* F I	
A	9, 11, 125	A	19, 23, 23, 32, 55
B	9, 11, 18, 107	B	12, 32, 42, 66
C	9, 9, 30, 95	C	32, 42, 78
D	9, 11, 30, 95	D	23, 32, 42, 55
E	9, 11, 19, 106	E	19, 23, 32, 78
		F	23, 32, 97
Hha I			
A	73, 77	*Taq* I	
B	17, 33, 43, 57	A	9, 30, 54, 55
		B	9, 30, 54, 60
		C	67, 81
		D	26, 55, 69
		E	29, 52, 69

Note: Letter designations for polymorphic enzymes indicate separate fragment patterns.

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Table 5. Composite haplotypes for restriction enzyme polymorphisms detected in piscivorous chub, *Zacco temmincki* and *Aphyocypris chinensis*, showing numbers and locales of fish with each haplotype

Haplotype	Composite mtDNA restriction morphs	Collection locale ★	Number of fish
1	AAAAAAAAAAAAAA	L. Biwa(A)	6
2	AAAAABAAAAAAAA	L. Biwa(A)	2
3	AAAAAAABAAAAAA	L. Mikata(B)	8
4	ABBAACACABAABB	Somjin R. (C), Yongsan R. (D)	8, 1
5	ABBAACACAAAACB	Yongsan R. (D)	7
6	ABBAADADAAAACB	Kum R. (E)	8
7	ABBAADACAAAACB	Han R. (F)	5
8	ABBAADABAAAACB	Han R. (F)	3
9	AABAADAEAAAADC	Amur R. (G), L. Khanka (H)	1, 4
	Zacco temmincki	Onga R.	2
10	ACCBAAAFACAAED		
	Aphyocypris chinensis	Chikugo R.	2
11	BDDCBEBGADBAFE		

Note: Variable enzymes from Table 3 listed in order(*Aci* I, *Afa* I, *Alu* I, *Bfa* I, *Bst* U I, *Hae* III, *Hha* I, *Hin* f I, *Mbo* I, *Msp* I, *Nla* III, *Sau* 96 I, *Scr* F I, *Taq* I).
★Alphabets in parentheses correspond to those in Fig. 1.

with that of NJ phenogram (data are not shown).

DISCUSSION

Differentiation between fish from Japan and the Asian mainland

Results obtained by analysis of both allozymes and mtDNA indicated that the Japanese populations of piscivorous chub are separable from the Asian mainland populations of Korea and Russia. The latter populations were further divided into the Russian and Korean fish. Although the topology of the Korean fish differed slightly among results of the three analyses, the phenograms that we obtained were almost identical (Figs. 2 and 3).

It is noteworthy that the mtDNA restriction analysis revealed that almost all populations examined had their own haplotypes. This result indicates that analysis of restriction site polymorphism, combining PCR and digestion of DNA, is a powerful tool for discrimination among river populations of this fish.

Originality of the Lake Mikata fish

In spite of the unique feature in the number of scales, the current analysis indicates a genetic similarity between *Opsariichthys u. uncirostris* (Banarescu, 1968) in Lake Mikata and that in Lake Biwa. Until recently, the endemic range of the piscivorous chub in Japan was confined to Lake Biwa, Lake Mikata and their adjoining rivers (Nakamura, 1969). Nowadays, however, this species is found in many other rivers and lakes as a consequence of transplantations from Lake Biwa incidental to transplantation of the Ayu fish, *Plecoglossus altivelis*, from Lake Biwa (Sunaga, 1980). Based on the above, Tomoda (1989) supposed that the piscivorous chub in Lake Mikata population was originated in Lake Biwa. However, the unique haplotypes observed in this lake (Table 5), which are clearly distinguishable from those observed in Lake Biwa, allows us to eliminate the possibility that the Lake Mikata population was derived from Lake Biwa population by recent transplantation. This is also supported by the absence of the *Ldh-1-(-65)* allele in the Lake Mikata population, which appeared exclusively in the Lake Biwa population at high frequency (Table 3).

Dispersal of piscivorous chub to Japan

It is considered that some organisms that are now native to Japan have close relationship to those on the Asian mainland (Lindberg, 1972). Freshwater fishes are believed to have penetrated Japan when Japan and the Asian Continent were joined by a land bridge during certain glacial periods (Lindberg, 1972). Since the Japanese freshwater fishes with the closest affinity to those on the Korean Peninsula are generally distributed in western Japan, it is presumed that the most of them came from the Korean Peninsula (Aoyagi, 1957; Mizuno, 1987).

The pattern of distribution of the piscivorous chub is quite unique, in that their natural distribution does not include western Japan (Nakamura, 1969), although populations have been established after they were transplanted to these areas (Sunaga, 1980). Tsubokawa (1988) and Tomoda (1989) hypothesized that the piscivorous chub never inhabited western Japan in the past and penetrated the areas around Lake Mikata directly from Korea through the Sea of Japan when it was a putative freshwater lake. However, geographic and paleontologic data indicate that the Sea of Japan was never a freshwater lake at least after the late Miocene (about 15 million years (MY) ago; Koizumi,

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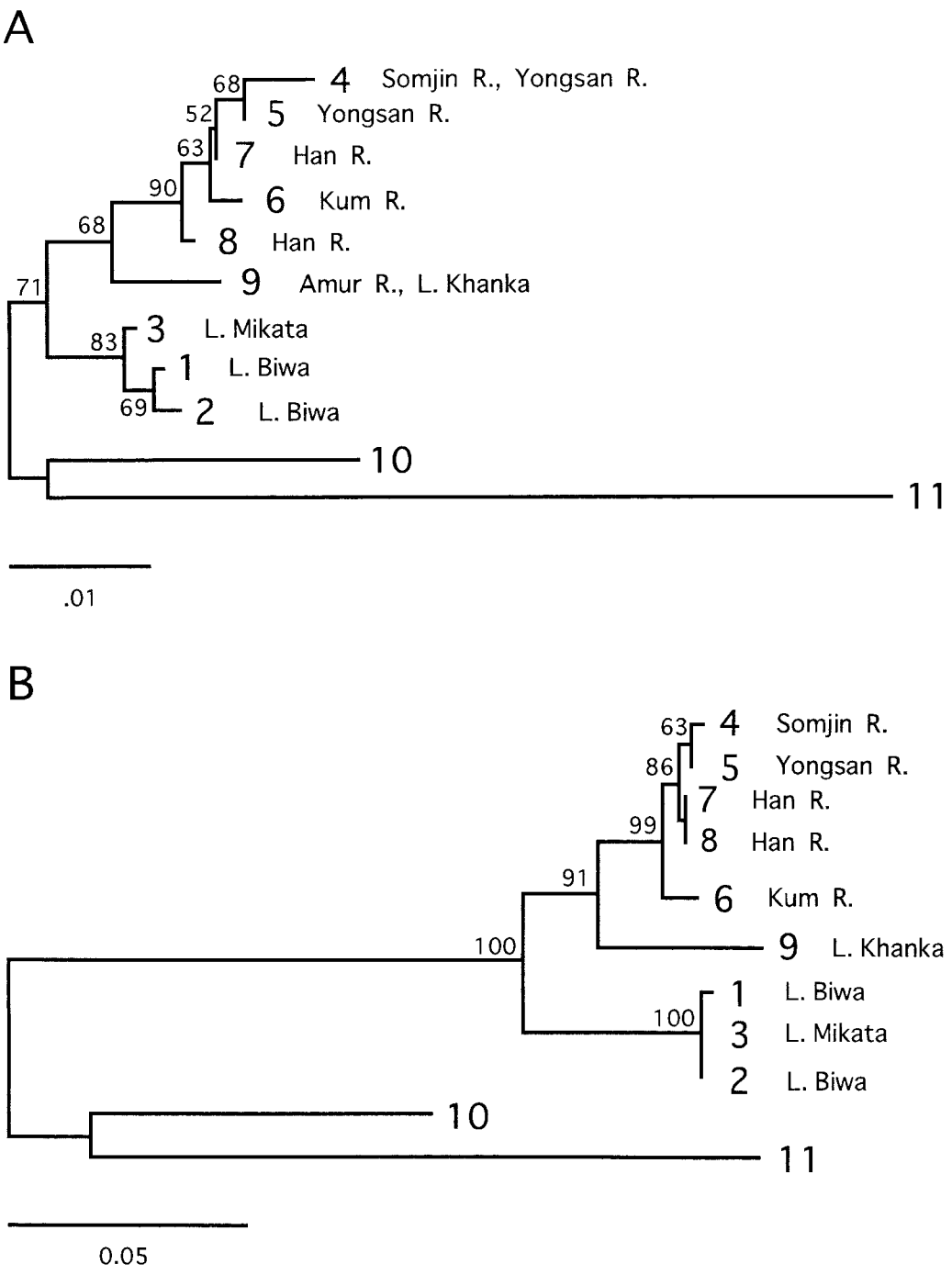


Fig. 3. NJ clustering based on restriction analysis (A) and NJ phenogram based on genetic distances estimated from the partial mitochondrial ND II gene sequences (B) of nine composite haplotypes of the piscivorous chub of Japan, Korea and Russia. Numbers at end of tree forks indicate haplotype number (shown in Table 5) and numbers at forks indicate bootstrap values (1,000 replicates).

1977; Ujiie and Ichikura, 1977). The presumed timing of the divergence of the piscivorous chub between Japan and Korea, as deduced from the present sequence analysis (6.9–7.3% between them), is the first half of the Pliocene (4 MY ago) if we simply apply the vertebrate mtDNA clock (2% per MY (Brown *et al.*, 1979). If we apply the figure of 0.76% per MY (Zardoya and Doadrio, 1999), which is the cytochrome *b* region of mtDNA clock examined for a number of cyprinid fishes, the divergence dates back to 10 MY ago. Considering a much faster molecular clock in ND II region used in this study compared to the cytochrome *b* region (Meyer, 1994), the timing of divergence may not go back as early as the period when the Sea of Japan was a putative freshwater lake though some errors are unavoidable in this method. The above indicates little possibility of a dispersal route from Korea through the Sea of Japan.

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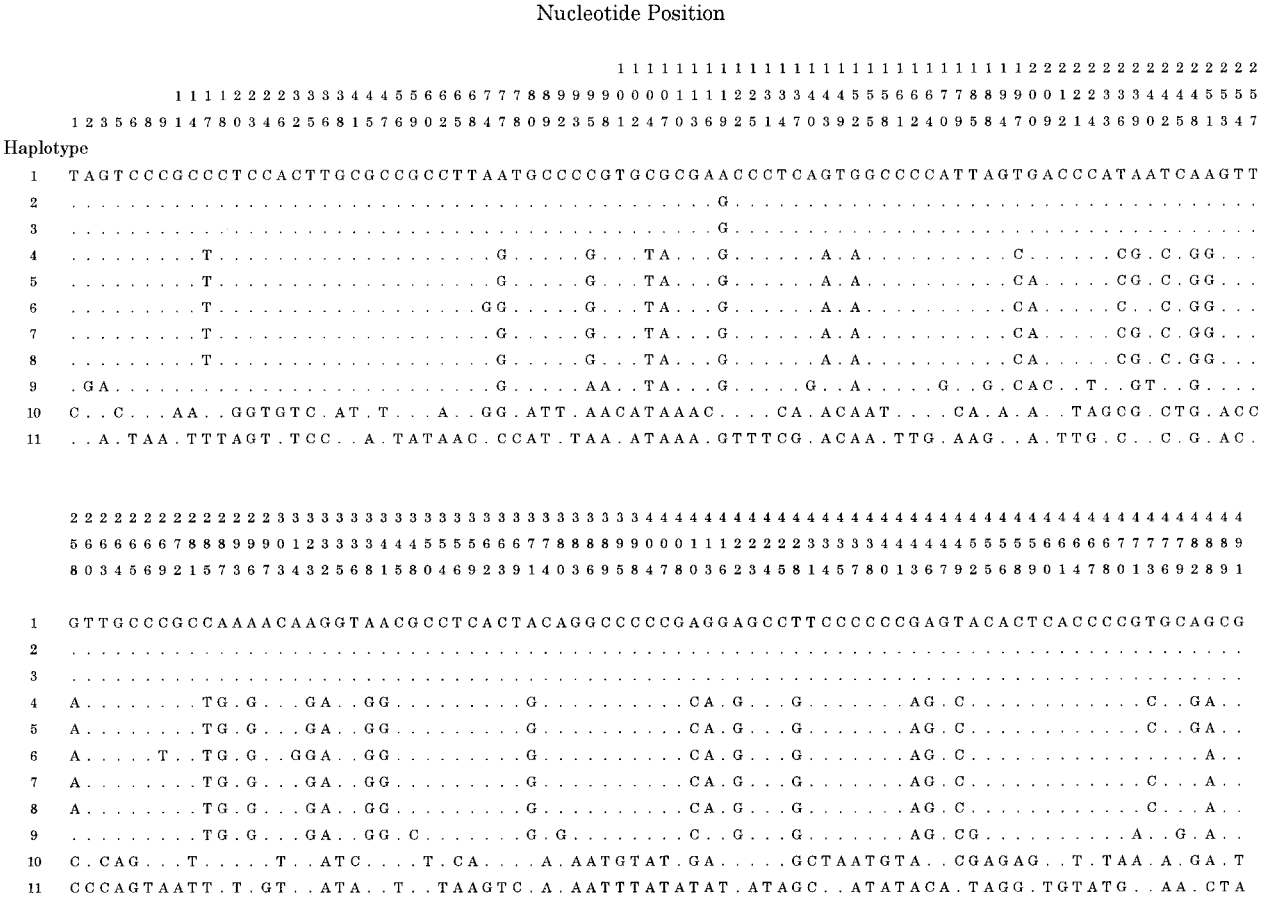


Fig. 4. Variable nucleotide sites in haplotypes (shown in Table 5) from the aligned 496 bp nucleotide sequence of the ND II gene of the piscivorous chub, *Zacco temmincki* and *Aphyocypris chinensis*. Identity with the first sequence denoted by dots.

In tracing the dispersal route of the piscivorous chub, it is probably more reasonable to apply the accepted routes of other cyprinid fishes than to set up a specific hypothesis for this particular fish (Mizuno, 1987; Tomoda, 1989). It is possible to consider the piscivorous chub that penetrated western Japan survived in Lake Biwa and Lake Mikata alone through the sea level fluctuation. Similar supposition was proposed for some freshwater fishes in western Japan by Lindberg (1972).

Complete picture will emerge as *O. u. hainanensis* and *O. u. bidens* are included for further analysis in the future.

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