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Source: Zoological Science, 18(2) : 249-259

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.18.249>

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Two genetically Divergent Groups in the Japanese Spined Loach, *Cobitis takatsuensis*, and Their Phylogenetic Relationships among Japanese *Cobitis* Inferred from Mitochondrial DNA Analyses

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ABSTRACT—The Japanese spined loach, *Cobitis takatsuensis*, has some unique morphological and ecological features among Japanese *Cobitis* species. Mitochondrial DNA analyses were conducted to investigate the magnitude of intraspecific differentiation and phylogenetic relationships among Japanese congeners of *C. takatsuensis*. PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis of the ND1 region with 16 restriction enzymes was carried out for thirteen *C. takatsuensis* populations throughout the species' distributional range. Populations in each river system possessed their own haplotypes, with marked genetic differentiation between the populations from Chugoku and Kyushu (Chugoku-Kyushu group) and those from Shikoku (Shikoku group). The two allopatric groups also showed different color pattern. Subsequently, sequencing analysis of part (725 bp) of the cytochrome *b* gene was carried out for *C. takatsuensis* and six other closely-related Japanese species. The phylogenetic tree indicated the presence of two major mtDNA lineages within Japanese *Cobitis*. It was noteworthy that the Chugoku-Kyushu and Shikoku groups of *C. takatsuensis* were included in separate mtDNA major lineages, and each group was closely related to other species. It is inferred that the distinct mtDNA relationship between the two allopatric *C. takatsuensis* groups is a result of the parallel evolution or mtDNA introgression, rather than divergence by geographic isolations.

INTRODUCTION

The Japanese spined loach, *Cobitis takatsuensis* (Mizuno, 1970), is small species inhabiting only stony bottoms in mountain streams of western part of Japan (Kimizuka *et al.*, 1982; Shimizu and Mizuno, 1994). It is rare species listed as 'Threatened IB' rank in the 1997 Red List of Threatened Animals of Japan (Environment Agency of Japan, 1997). This species is morphologically characterized by thin caudal peduncle, incomplete sexual dimorphism, and unique color pattern (Mizuno 1970, 1975). These ecological and morphological features are peculiar among Japanese *Cobitis* species.

In addition, *C. takatsuensis* exhibits considerable morphological variation among local populations (Shimizu and

Mizuno, 1994; Mizuno, 1995). In particular, marked differences in color pattern exist between populations from Chugoku District (western Honshu) and Kyushu Island, and those from Shikoku Island. The former were roughly represented by striated, and the latter by spotted types (Shimizu and Mizuno, 1994; Fig. 1). Although they have been regarded as intraspecific geographical variations, their taxonomic status remains obscure.

In this study, we conducted restriction fragment length polymorphism (RFLP) and sequence analyses on some parts of the mtDNA to investigate the magnitude of genetic differentiation between the two types of *C. takatsuensis* and their phylogenetic relationships among Japanese congeners, *C. biwae*, *C. matsubarai*, and three undescribed species of *Cobitis* (*C. sp. 1*, *C. sp. 2*, and *C. sp. 3*), which taxonomically follow as Hosoya (1993) (Table 1). Recently, Saitoh *et al.* (2000) reported the presence of three distinct mtDNA lineages within *C. biwae*, corresponding to geographical proximity (east-

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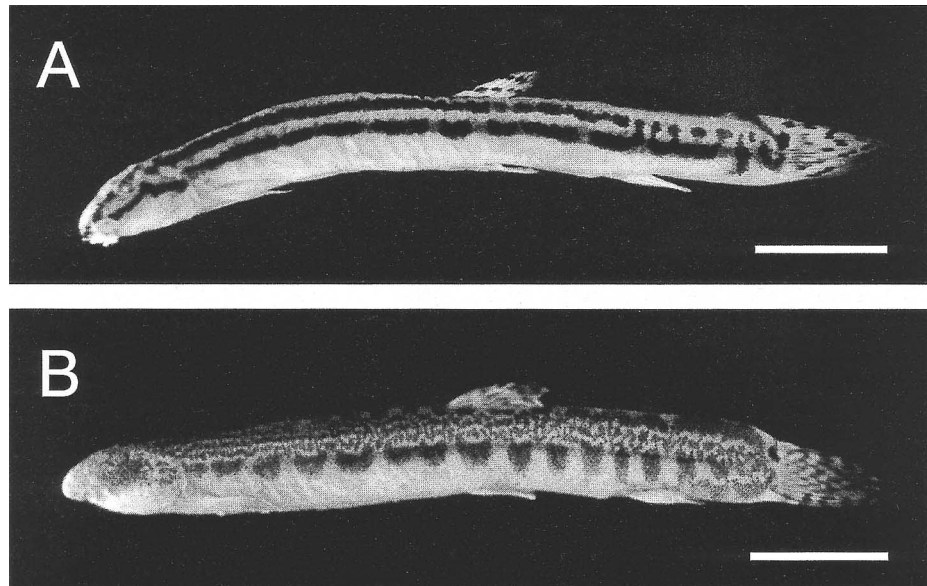


Fig. 1. A—*Cobitis takatsuensis* from Chugoku district (FRLM 24892), 56.7 mm SL; B—*C. takatsuensis* from Shikoku Island (FRLM 24912), 53.8 mm SL. Scale bars indicate 10 mm.

Table 1. Distribution and chromosome number of *Cobitis* and *Niwaella* species in Japan, according to Hosoya (1993).

Species	Distribution	Diploid number
<i>Cobitis takatsuensis</i>	Western parts of Honshu and Shikoku, and northern part of Kyushu	48
<i>C. biwae</i>	Honshu and Shikoku	48 or 96
<i>C. matsubarai</i>	Kyushu and western tip of Honshu	86 or 94
<i>C. sp. 1</i>	Lake Biwa	98
<i>C. sp. 2</i>	subsp. 1	49, 50
	subsp. 2	
	subsp. 3	50
	subsp. 4	50
<i>C. sp. 3</i>	The regions around Seto Inland Sea	50
<i>Niwaella delicata</i>	Central Honshu	50

ern part of Honshu, central-western part of Honshu, and Pacific slope of Shikoku). We considered them as distinct groups in *C. biwae* here.

MATERIALS AND METHODS

Specimens

Specimens were caught by hand net during 1995 and 1999. Total of forty-seven individuals of *C. takatsuensis* were obtained from thirteen locations in twelve river systems (Fig. 2, Table 2). In addition, nine individuals of other species (*C. biwae*, *C. matsubarai*, *C. sp. 1*, *C. sp. 2* subsp. 2, *C. sp. 3*, and *Niwaella delicata*) were included for inter-specific phylogenetic analysis. Three groups of *C. biwae* (sensu Saitoh *et al.*, 2000), are herein temporarily referred to as Eastern, Western (consisted of diploid and tetraploid races (Kobayashi, 1976)), and Kochi groups, respectively. Accordingly, four individuals of *C. biwae* representing the three groups and a tetraploid race were included (Table 2). The identification of diploid or tetraploid specimens was determined from blood-smear preparations (Sezaki and Kobayashi, 1978). The remaining five individuals represented four other *Cobitis* species and a sister genus species *Niwaella delicata* as an outgroup. Saitoh *et al.* (2000) reported close genetic relationships among

subspecies within *C. sp. 2*, and also that karyotype races within *C. matsubarai*. For simplification in the analysis, a single subspecies (subsp. 2) and a race ($2n=94$, inferred from collecting locality) each represented (Table 2). Samples were frozen immediately following collection, and stored at -20°C until analysis. Voucher specimens were deposited in the collection of the Fisheries Research Laboratory of Mie University (FRLM: catalogue numbers were listed in Table 2).

Total DNA was extracted from approximately 100 mg of frozen muscle tissue, as described by Asahida *et al.* (1996).

PCR-RFLP for intraspecific analysis

Restriction analysis was conducted for all specimens of *C. takatsuensis*.

A segment of about 2.0 kbp, containing a complete NADH dehydrogenase subunit 1 (ND1) and part of the 16S ribosomal RNA (16SrRNA) gene of mtDNA, was amplified by the polymerase chain reaction (PCR). A pair of primers (forward: 5' - ACC CCG CCT GTT TAC CAA AAA CAT - 3' and reverse: 5' - GGT ATG AGC CCG ATA GCT TA - 3') described by Hall and Nawrocki (1995) was used. However, because these were not effective for fish from Shikoku, another pair of primers, modified for carp (forward: 5' - GCC TCG CCT GTT TAC CAA AAA CAT - 3' and reverse: 5' - GGT ATG GGC CCG AAA GCT TT - 3'), were used. PCR consisted of 30 cycles of 1

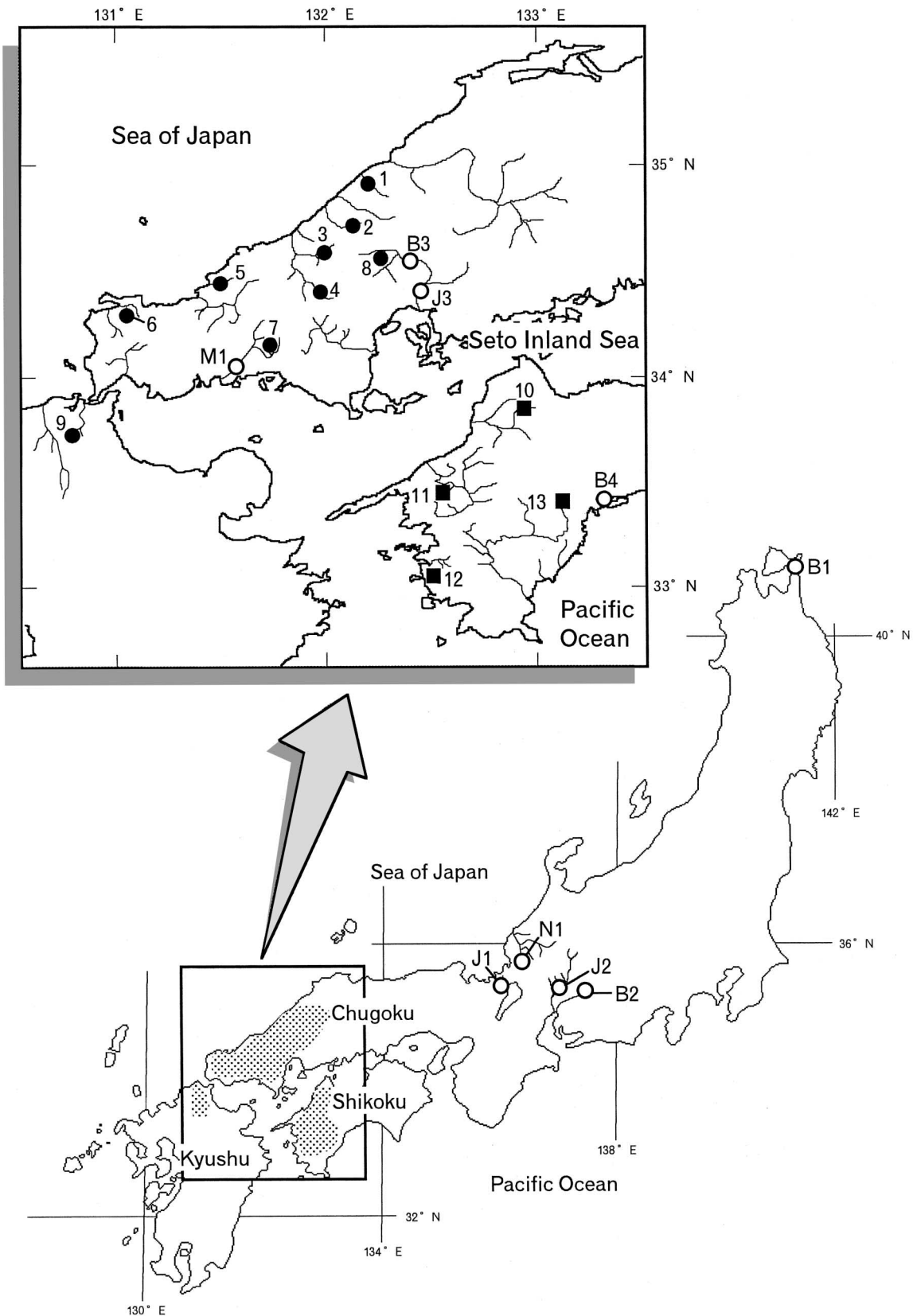


Fig. 2. Geographic distribution of *C. takatsuensis* (dotted range) and sampling localities in the present study. Only rivers inhabited by *C. takatsuensis* (Shimizu and Mizuno, 1994) are shown on the enlarged map. Locality numbers correspond to those in Table 2. ○; *C. takatsuensis* (Chugoku-Kyushu group), □; *C. takatsuensis* (Shikoku group), ●; other species.

Table 2. Sampling localities, sample sizes, and catalogue numbers. Locality numbers correspond to those in Fig. 2.

Species	Locality No.	River (River systems)	N	Catalogue No.
<i>Cobitis takatsuensis</i>	Chugoku District (western Honshu)			
	1	Hamada River	5	FRLM 24868-872
	2	Misumi R.	5	FRLM 24873-877
	3	Hikimi R. (Takatsu R. s.)	3	FRLM 24878-880
	4	Takatsu R.	2	FRLM 24881-882
	5	Go R.	5	FRLM 24883-887
	6	Daibo R. (Kakefuchi R. s.)	4	FRLM 24888-891
	7	Suginokouchi R. (Saba R. s.)	5	FRLM 24892-896
	8	Tsutsuga R. (Ota R. s.)	1	FRLM 24897
	Kyushu Island			
	9	Murasaki R.	1	FRLM 24898
	Shikoku Island			
	10	Shigenobu R.	5	FRLM 24899-903
11	Yako R. (Hiji R. s.)	5	FRLM 24904-908	
12	Hoba R. (Iwamatsu R. s.)	3	FRLM 24909-911	
13	Shimanto R.	3	FRLM 24912-914	
<i>C. biwae</i> (Eastern group)* (Western group)* (Western group, tetraploid)* (Kochi group)*	B1	Mena R. (Mutsu R. s.)	1	FRLM 24915
	B2	Toki R. (Shonai R. s.)	1	FRLM 24916
	B3	Ota R.	1	FRLM 24917
	B4	Uranouchi R.	1	FRLM 24918
<i>C. matsubarai</i>	M1	Saba R.	1	FRLM 24919
<i>C. sp. 1</i>	J1	Chinai R.	1	FRLM 24920
<i>C. sp. 2</i> subsp. 2	J2	Otani R. (Nagara R. s.)	1	FRLM 24921
<i>C. sp. 3</i>	J3	Ota R.	1	FRLM 24922
<i>Niwaella delicata</i>	N1	Hino R. (Kuzuryu R. s.)	1	FRLM 24923

* See text for the explanation.

min. at 94 °C, 1 min. at 50°C and 2 min. at 72°C. Subsequently, PCR products were digested with sixteen restriction enzymes that specifically recognize four or five base nucleotides (Table 3; from New England Biolabs, Beverly, MA; and Takara Shuzo, Kyoto), following the protocols recommended by the manufacturers. The restricted fragments were separated by horizontal electrophoresis in 3% agarose gel. Digested fragments were visualized and photographed on an ultraviolet transilluminator after ethidium bromide staining. Nucleotide sequence divergences between mtDNA haplotypes were estimated according to Nei and Li (1979), using the D program from REAP (MacElroy *et al.*, 1992). Phylogenetic trees were generated by the UPGMA method using the Neighbor program from PHYLIP ver. 3.572 (Felsenstein, 1996).

Sequencing for interspecific phylogenetic analysis

Sequence analysis was performed on mtDNA fragments comprising part of the cytochrome *b* gene. Seven individuals of *C. takatsuensis*, representing each RFLP cluster, and the nine individuals of closely-related species (Table 2) were sequenced. A pair of primers (forward: 5' - TGA CTT GAA RAA CCA YCG TTG - 3' and reverse: 5' - RGC RAA KAR RAA RTA YCA TTC - 3') described by Palumbi *et al.*, (1991) were used. PCR conditions for the sequencing analysis were similar to those for RFLP analysis, with the annealing temperature set at 54°C. Amplified DNA was purified with a QIA quick spin column (Qiagen, Germany). Direct sequencing of purified products was achieved using the Ready Reaction Dye Terminator Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's protocol. Primers used were the same as those for PCR. Sequences were analyzed on an Applied Biosystems Model 377 automated DNA sequencer. Each DNA strand was determined the sequence two or three times. DNA sequence data were edited using DNASIS programs

(Hitachi Software Engineer. Co. Ltd.). The nucleotide sequence data reported here are available from DDBJ, EMBL and GeneBank accession numbers AB039337-AB039352.

Three different methods were conducted to infer the phylogenetic relationships. A distance matrix was calculated based on Kimura's two-parameter method (Kimura, 1980) and clustered by the neighbor-joining method (Saitou and Nei, 1987) using MEGA ver. 1.0 (Kumar *et al.*, 1993), and a maximum likelihood analysis performed using DNAML in PHYLIP ver. 3.572 (Felsenstein, 1996). A maximum parsimony analysis was performed with the Heuristic Search program from PAUP ver. 3.1.1 (Swofford, 1993). The robustness of each phylogeny was assessed by bootstrap analyses consisting of 1,000 replicates (Felsenstein, 1985).

RESULTS

PCR-RFLP for intraspecific analysis

The digested mtDNA fragment patterns are listed alphabetically for each of the 16 enzymes (Table 3). The total length of the digested DNA fragments varied in some cases, since the detection of small-sized fragments (< 70 bp) was not possible with the method used. The composite genotypes (haplotypes) detected among *C. takatsuensis* populations and the location of each are given in Table 4. All 16 enzymes produced variable restriction patterns. Restriction patterns detected in the populations from Chugoku and Kyushu were so different from those from Shikoku as to have hardly any fragments in common. Therefore, length-difference method

Table 3. Fragment patterns and approximate molecular size (in base pairs; bp) generated by sixteen restriction enzymes.

Enzyme	Fragment patterns (bp)										Enzyme	Fragment patterns (bp)										
<i>Acl</i> I											<i>Hinf</i> I											
a	510	380	330	230	225	130	90	70			a	985	310	300	205	190						
b	650	380	330	230	130	90	85	70			b	985	500	300	205							
c	510	330	270	230	225	130	110	70			c	985	500	205	200	100						
d	510	330	295	270	230	130	110	90			d	985	500	210	205	100						
e	510	270	230	225	180	150	130	110	90	70	<i>Hsp</i> 92 II											
f	620	490	250	230	140	100	90				a	870	690	340	100							
g	620	490	320	250	140	100					b	1210	690	100								
h	620	520	320	250	140	100					<i>Mbo</i> I											
<i>Alu</i> I											a	730	630	250	210	90	80					
a	550	320	280	230	180	130	80	75	70		b	740	630	250	210	90	80					
b	420	320	280	230	180	130	130	80	75	70	c	1370	250	210	90	80						
c	790	350	340	200	180	80					<i>Msp</i> I											
d	790	540	350	180	80						a	1070	430	320	180							
e	790	350	240	200	180	100	80				b	1030	430	360	180							
<i>Bfa</i> I											<i>Rsa</i> I											
a	390	305	295	270	180	170	165	110	70		a	810	680	490								
b	390	305	295	270	180	170	165	70			b	810	680	510								
c	490	295	270	265	200	150	140	110			c	820	520	370	290							
d	300	295	270	200	190	185	150	140	110	80	<i>Sau</i> 96 I											
e	490	270	265	205	200	195	140	110	100		a	420	290	260	240	225	185	180	170			
f	490	295	270	265	200	180	140	110			b	420	260	240	230	225	185	180	170			
<i>Bst</i> U I											c	420	260	240	230	225	180	165	110			
a	1820	180									d	420	260	240	230	225	185	180	165			
b	2000										<i>Scr</i> F I											
c	1680	320									a	820	630	270	210							
<i>Dde</i> I											b	820	630	280	270							
a	540	330	280	190	185	180	110	100			c	630	560	390	220	200						
b	610	395	210	185	180	160	110	100			d	810	630	560								
c	610	280	260	210	185	180	115	110			e	630	610	560	200							
d	610	280	210	185	180	160	115	110	100		<i>Taq</i> I											
<i>Hae</i> III											a	1470	230	190	110							
a	380	310	265	260	210	180	175	120	100		b	1470	420	110								
b	440	380	310	260	210	180	120	100			c	690	680	510	90							
c	310	305	265	260	210	180	175	120	100	75	d	700	680	510	90							
d	520	290	275	260	200	180	140	90			e	720	680	510	90							
e	290	275	265	260	255	200	180	140	90		<i>Tsp</i> E I											
f	550	520	275	200	180	140	90				a	300	280	250	225	220	170	135	110	95	90	
<i>Hha</i> I											b	300	280	250	225	220	135	130	110	95	90	
a	1150	380	360	110							c	300	280	250	225	220	170	140	135	110	90	
b	910	600	380	110							d	300	280	250	230	225	220	170	135	110	80	
											e	520	380	170	165	160	140	130	110	90	70	
											f	385	380	170	165	160	140	130	110	100	90	70
											g	520	380	180	170	160	140	130	110	70		

(Nei and Li, 1979) cannot be applied for the analysis between them (Nei, 1987). Accordingly, we referred to them as Chugoku-Kyushu and Shikoku groups, respectively. The sequence divergences of the two groups were calculated separately.

Within the Chugoku-Kyushu group, ten of the sixteen enzymes were polymorphic. A total of eight haplotypes (C1-7 and K1) were observed among the 31 fish analyzed (Table 4). Populations in each river system were fixed for their own

haplotype (Table 4). The resulted UPGMA tree (Fig. 3. A) revealed some sub-clusters corresponding to geographical proximity. The estimated range of pairwise sequence divergence between haplotypes varied from 0.13 to 1.33%.

Within the Shikoku group, twelve of the sixteen enzymes were polymorphic. A total of six haplotypes (S1-6) were observed among the 16 fish analyzed (Table 4). The estimated range of pairwise sequence divergence among all haplotypes varied from 0.24 to 2.14%. Each population possessed its

Table 4. Composite PCR-RFLP haplotypes for 13 populations of *C. takatsuensis*.

Haplotype	Composite fragment pattern*	Locality
C1	a a a a a a a a a a a a a a a a	1 (5)
C2	b a a a a a a a a a a b a a a b	2 (5)
C3	c b b a a a a a a a a b a b a c	3 (3), 4 (2)
C4	c a b a a b a a a a a b a b a c	5 (5)
C5	d a a a a a a a a a a b a a a d	6 (4)
C6	c a b a a c a a a a a b a a a a	7 (5)
C7	e b b a a a a b a a a b b b b c	8 (1)
K1	d a a a a c a a a a a b a a a d	9 (1)
S1	f c c b b d b c b b b c c c c e	10 (5)
S2	g d d b c e b c b b b c c c d e	11 (5)
S3	h e e b d f b d b c b c d d e f	12 (1)
S4	h e e b d f b d b c b c c e e f	12 (2)
S5	h c c c b e b c b b b c c c d g	13 (2)
S6	g c f c d e b c b b b c c c d g	13 (1)

* Letters, from left to right, refer to digestion profiles produced the enzyme listed in order *Aci* I, *Alu* I, *Bfa* I, *Bst* U I, *Dde* I, *Hae* III, *Hha* I, *Hinf* I, *Hsp* 92 II, *Mbo* I, *Msp* I, *Rsa* I, *Sau* 96 I, *Scr* F I, *Taq* I and *Tsp* E I. Numbers in parentheses indicate number of individuals.

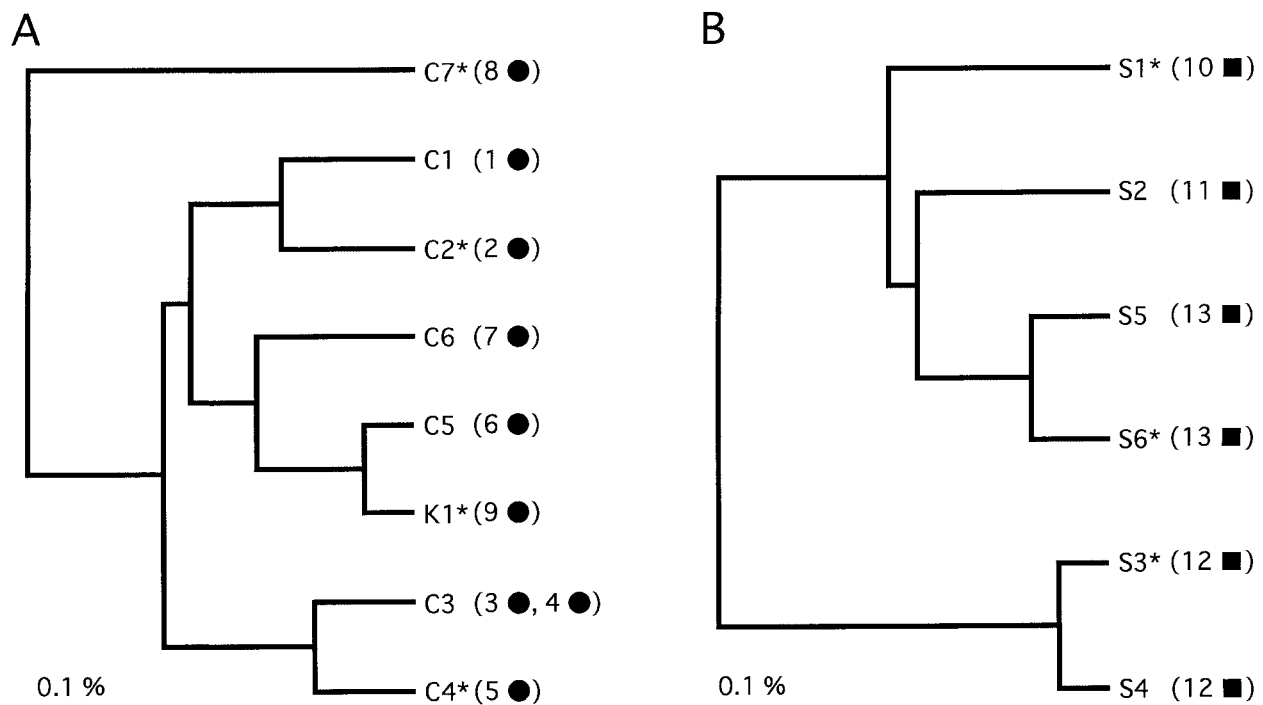


Fig. 3. UPGMA tree for ND1 haplotypes from the Chugoku-Kyushu (A), and Shikoku (B) groups of *C. takatsuensis*, based on nucleotide sequence divergence (Nei and Li, 1979). Numbers and symbols in parentheses correspond to those in Fig. 2. Asterisks (*) indicate individuals used for sequence analysis.

own haplotypes (Table 4). In the UPGMA tree (Fig. 3. B), haplotypes detected in the same populations (S3 and S4, S5 and S6) were clustered.

Sequencing for interspecific phylogenetic analysis

A 725 base pair region of the cytochrome *b* gene was

successfully sequenced for all specimens. No insertions or deletions were observed.

The NJ tree (Fig. 4) constructed from all three codon positions (725 bp) on the cytochrome *b* sequence indicated two major lineages of *Cobitis* supported by high bootstrap value (98% and 99%, respectively). Nucleotide divergence between

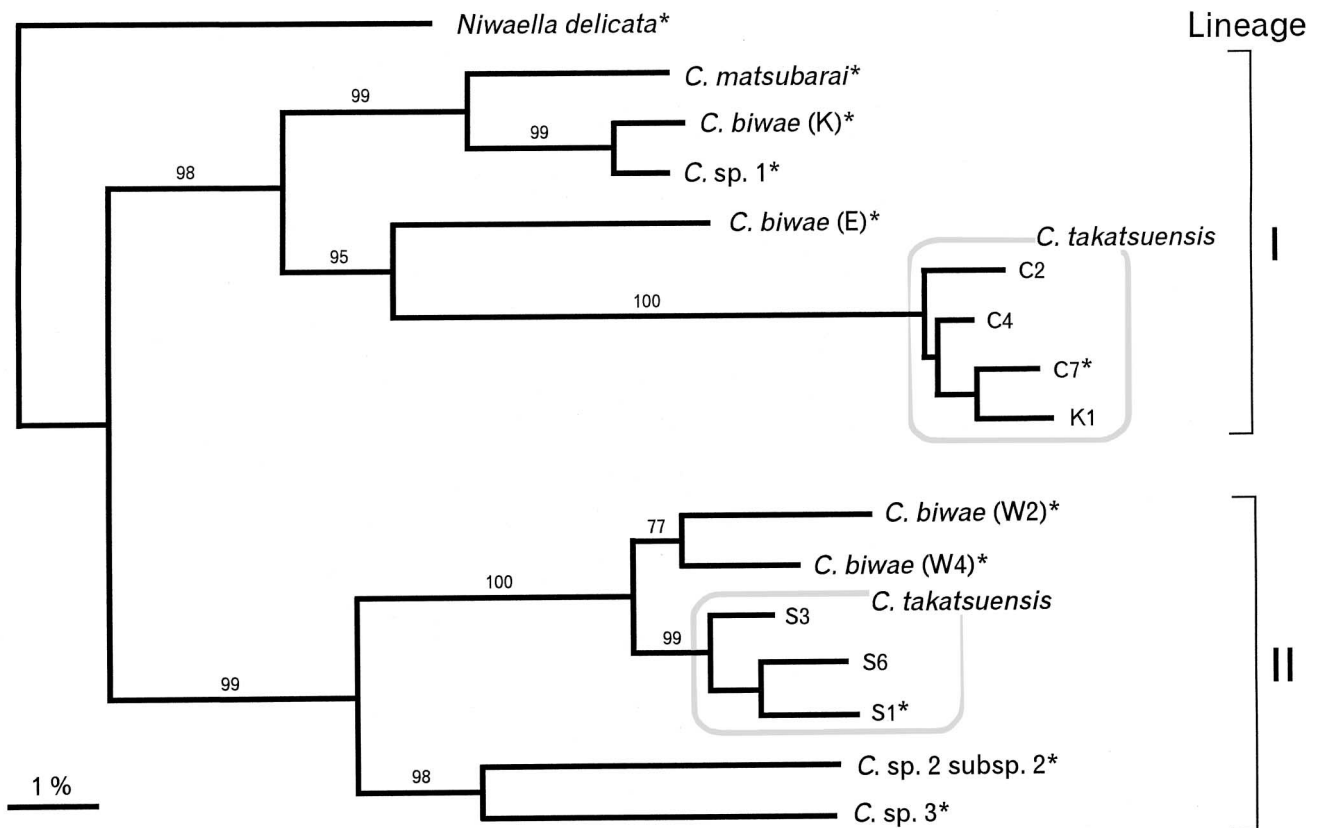


Fig. 4. Neighbor-joining network for Japanese *Cobitis* and *Niwaella* species based on nucleotide sequence divergence (Kimura, 1980). Bootstrap probabilities (%) with 1,000 replications are shown for each cluster. Asterisks (*) indicate individuals used for maximum likelihood and maximum parsimony analysis.

the two major lineages averaged 17.21%. It was noteworthy that the haplotypes of *C. takatsuensis* from the Chugoku-Kyushu (C2, C4, C7, and K1) and Shikoku (S1, S3, and S6) groups were included in separate major lineages. Similarly, haplotypes of *C. biwae* from the Western group (W2 and W4) were included in different major lineages to those from the Eastern (E) and Kochi (K) groups. *Cobitis takatsuensis* from the Shikoku group (S1, S3, and S6) were clustered very closely with *C. biwae* from the Western group (W2 and W4). On the other hand, *C. takatsuensis* from the Chugoku-Kyushu group (C2, C4, C7, and K1) was associated with *C. biwae* from the Eastern group (E) with a high bootstrap value (95%).

Maximum likelihood analysis was conducted for the two individuals of *C. takatsuensis* (C7 and S1) representing each group, and the nine individuals of other species which were analyzed by the NJ analysis. The transition / transversion ratio was set at 4.6, following the method of Oohara *et al.* (1999). Although a change in the transition / transversion ratio value to accommodate a wide range (from 1 to 10) was attempted, the topology of the tree was not entirely changed. The topology of the ML tree (Fig. 5. A) was concordant with that constructed by the NJ method (Fig. 4). The two major lineages of *Cobitis* were completely (100%) supported by bootstrap replications. The each close relationship between *C. takatsuensis* (S1) and *C. biwae* (W2 and W4), and *C. taka-*

tsuensis (C7) and *C. biwae* (E) were also strongly supported (100% and 96%, respectively).

The nucleotide substitutions at the third position of codon between each taxon were counted from 10 to 106 of the 242 sites, indicating saturation between distantly-related taxa. There was a possibility that multiple substitutions could not be adequately corrected in maximum parsimony analysis (Nei, 1987). Therefore, only first and second codon positions data set was used for MP analysis. The latter produced a topology concordant with those obtained by the NJ (Fig. 4) and ML (Fig. 5. A) methods, with the exception of an ambiguity in the branching order deep within the lineage I (Fig. 5. B).

DISCUSSION

The present result indicated the marked mtDNA divergence between *C. takatsuensis* populations from Chugoku-Kyushu and those from Shikoku, corresponding to color pattern differentiation proposed by Shimizu and Mizuno (1994) (Fig. 1). RFLP haplotype divergences within the Chugoku-Kyushu and Shikoku groups were 0.13–1.33 % and 0.24–2.14%, respectively. Since present RFLP data were estimated from a part of mtDNA, we could not directly compare to other population studies that have used whole mtDNA. Nevertheless, these values seem to be relatively high compared with

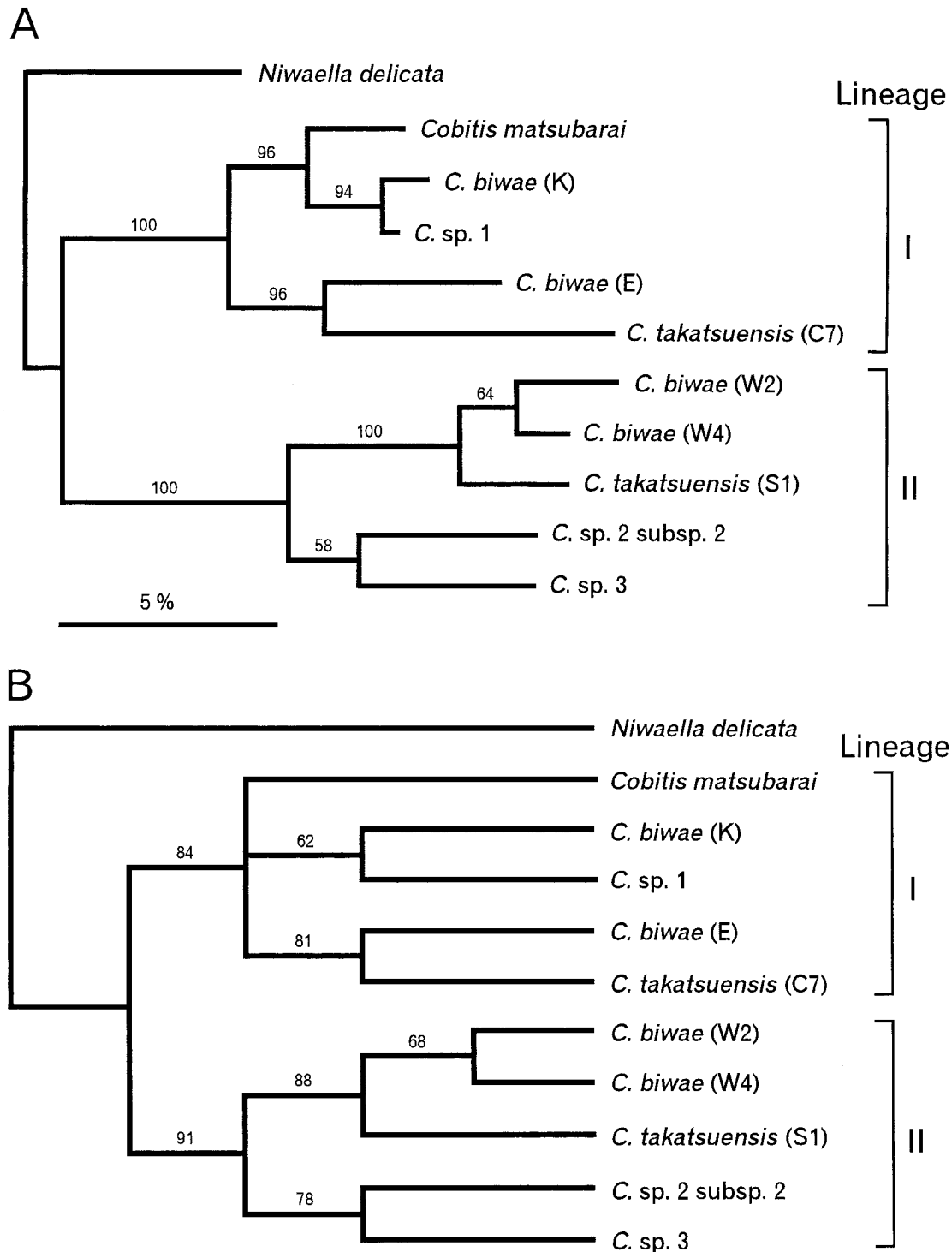


Fig. 5. Maximum likelihood (Ln likelihood = -2834.324) (A), and maximum parsimony trees (50% majority rule consensus) (B) for Japanese *Cobitis* species. Bootstrap probabilities (%) with 1,000 replications are shown for each cluster.

other intraspecific divergence in freshwater species summarized by Bernatchez and Wilson (1998) (the maximum intraspecific divergence value < 1%, in the half of 42 species). Moreover, no shared haplotypes among river systems were observed. Within the each group, such considerable differentiations among populations must be attributed to the

isolations for their exclusive mountain stream habitats (Kimizuka *et al.*, 1982; Shimizu and Mizuno, 1994). On the other hand, it is unlikely that the differentiation between the two groups (19.24% sequence divergence in cytochrome *b* gene sequence) has resulted from such local isolations. If a conventional 2% divergence per Myr (Brown *et al.* 1979) is

applied to this data, the two groups of *C. takatsuensis* could have diverged about 9–10 million years ago. This time estimation implies that the divergence occurred much long before the geographical isolation among western Chugoku, northern Kyushu and Shikoku Regions (the isolation time is shorter than 20,000 years, after Kaizuka, *et al.* 1995).

The present phylogenetic results provided some hints for distinct mtDNA compositions between the two allopatric groups of *C. takatsuensis*. The phylogenetic trees revealed the presence of two major mtDNA lineages within Japanese *Cobitis*. It is noteworthy that the two divergent groups within *C. takatsuensis* (Chugoku-Kyushu and Shikoku) were included

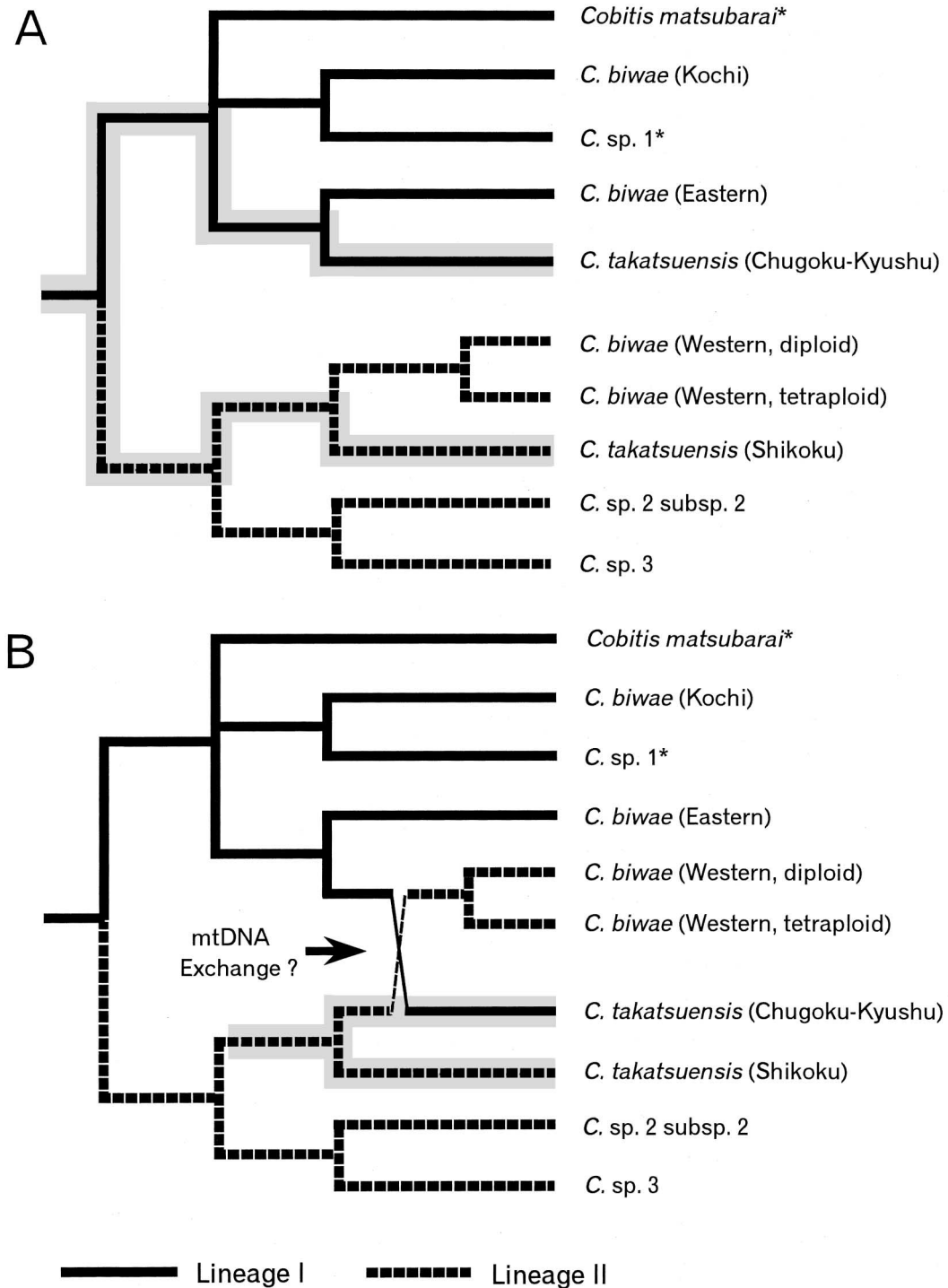


Fig. 6. Illustration of parallel evolution scenario (A), and the most parsimonious scenario under the mtDNA introgression hypothesis (B). Fine solid and broken lines indicate the mtDNA phylogeny determined in the present study. Dotted portions show putative lineages of *Cobitis takatsuensis*. Asterisks (*) indicate allotetraploid species whose maternal origins are *C. biwae* (Saitoh *et al.* 2000).

Table 5. Brief comparison of published data of karyotypes of *C. takatsuensis* and *C. biwae*.

	Group	Diploid number	Karyotype			Reference
			m	sm, st	a	
<i>C. takatsuensis</i>	Chugoku-Kyushu	48	12	18	18	Kimizuka <i>et al.</i> , 1982
	Shikoku	48	12	18	18	Kimizuka <i>et al.</i> , 1982
		48*	12	16	20	Kimizuka <i>et al.</i> , 1982
<i>C. biwae</i>	Eastern	48	20	24	4	Ueno, 1981
	Western	48	20	22	6	Ueno <i>et al.</i> , 1980
		48	16	22	10	Ueno <i>et al.</i> , 1980
		96 (tetraploid)	32	54	10	Ueno <i>et al.</i> , 1980
	Kochi	48	16	24	8	Ueno <i>et al.</i> , 1980

m, metacentric; sm, submetacentric; st, subtelocentric; a, acrocentric chromosomes.

* Unusual type detected in one individual from Shigenobu River system.

in separate major *Cobitis* lineages, as well as three distinct groups of *C. biwae* (Eastern, Western, and Kochi groups). A simple, general explanation for the distinctive mtDNA composition within one species is possible random lineage sorting of ancestral polymorphic mtDNA (Billington and Herbert, 1991). In the present case, however, the divergence time of the two lineages (approximately 8–10 million years ago) calculated from the conventional vertebrate mtDNA clock (2% divergence per Myr; Brown *et al.*, 1979) is much too long to have survived ancestral mtDNA polymorphisms (Avice, 1994). Accordingly, this explanation should be rejected.

Two alternative possible hypotheses exist satisfying the distinct mtDNA composition between the two groups. The first is that the two *C. takatsuensis* groups may have evolved independently from distinct lineages (Fig. 6. A). If this is correct, the two groups of *C. takatsuensis* must be regarded as two distinct species.

The second is that mtDNA introgression from another species to one of the *C. takatsuensis* groups may have occurred. The most parsimonious scenario under the introgression hypothesis is that mtDNA exchange between ancestors of Chugoku-Kyushu group *C. takatsuensis* and Western group *C. biwae* (W2 and W4) may have occurred (Fig. 6. B). In this scenario, two groups of *C. takatsuensis* can be considered as having evolved monophyletically. On the other hand, since *C. matsubarai* and *C. sp. 1* were inferred as allotetraploid species whose maternal origins are *C. biwae* (Saitoh *et al.*, 2000), it is reasonable that *C. biwae* is also considered as a monophyletic group. In fish species, interspecific mtDNA introgression, including a reciprocal case as in the present hypothesis, have been reported in several species (Avice and Saunders, 1984; Billington and Herbert, 1991; Dowling and Hoeh, 1991; Dowling and DeMarais, 1993; Bernatchez *et al.*, 1995, etc.). Moreover, evolutions originated from interspecific hybridization, being highly suggestive the possibility of mtDNA introgressions, had reported among several *Cobitis* species (Vasil'ev *et al.*, 1989; Kim and Lee, 1990; Sezaki *et al.*, 1994; Ráb and Slavík 1996; Saitoh *et al.*, 2000). Published karyological data (Table 5) indicates that *C. takatsuensis* exhibits little karyotypic variations even between the two divergent groups (Kimizuka *et al.*, 1982). Although *C. biwae* shows some

variations, their karyotypes are characterized by unusual compositions among *Cobitis* species (high proportion of metacentric chromosomes; Ueno *et al.*, 1980; Ueno, 1981). The above seems to support the monophyletic evolutions of *C. takatsuensis* and *C. biwae* lineages, respectively.

However, it is impossible to distinguish whether parallel evolution or mtDNA introgression (or a combination of both) is responsible for the distinct mtDNA compositions of the two groups of *C. takatsuensis*, based solely upon mtDNA data. The two above hypotheses are presently the subject of ongoing nucleic DNA analyses.

ACKNOWLEDGMENTS

We thank I. Oohara (National Research Institute of Fisheries Science), K. Kawamura (National Research Institute of Aquaculture), S. Kimura (Fisheries Research Laboratory of Mie University), and K. Saitoh (Tohoku National Fisheries Research Institute) for variable advice and assistance. We are grateful to G. S. Hardy (Thames, New Zealand) and two anonymous reviewers for their critically reading and improving the manuscript. We also thank Y. Fujioka (Shiga Prefectural Fisheries Experimental Station), I. Tanaka (Masuda City, Shimane Prefecture), D. Inoue (Kitakyushu High School) and N. Uechi (Mie University) for providing specimens used in the present study.

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(Received March 3, 2000 / Accepted November 9, 2000)