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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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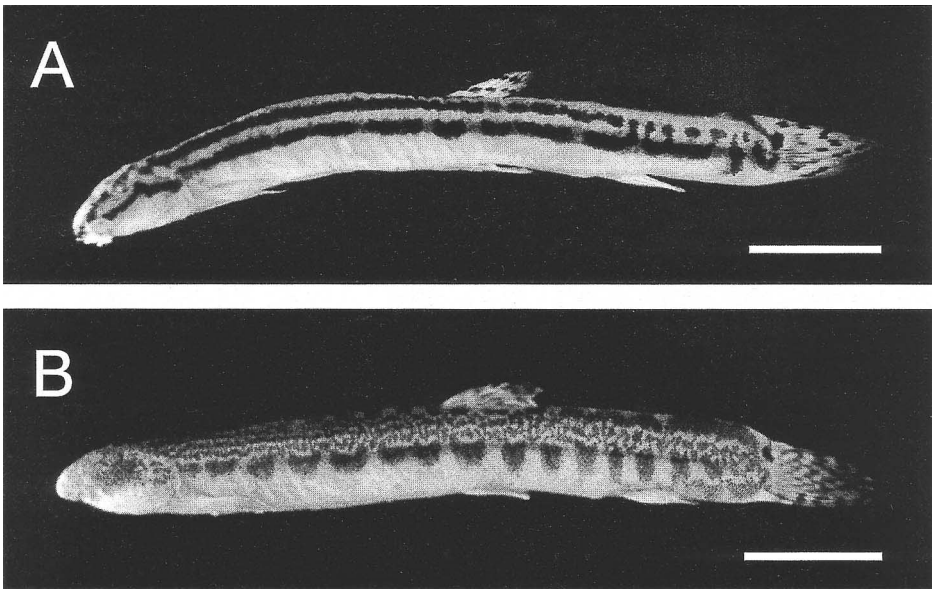

Fig. 1. A–*Cobitis takatsuensis* from Chugoku district (FRLM 24892), 56.7 mm SL; B–C. *takatsuensis* from Shikoku Island (FRLM 24912), 53.8 mm SL. Scale bars indicate 10 mm.

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

Species		Distribution	Diploid number
Cobitis takatsuensis		Western parts of Honshu and Shikoku, and northern part of Kyushu	48
C. biwae		Honshu and Shikoku	48 or 96
C. matsubarai		Kyushu and western tip of Honshu	86 or 94
C. sp. 1		Lake Biwa	98
C. sp. 2	subsp. 1	Sanyo Region	♂49, ♀50
	subsp. 2	Pacific slope of Tokai Region	50
	subsp. 3	San-in Region, and western part of Kyushu	50
	subsp. 4	Lake Biwa	50
C. sp. 3		The regions around Seto Inland Sea	50
Niwaella delicata		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 interspecific 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

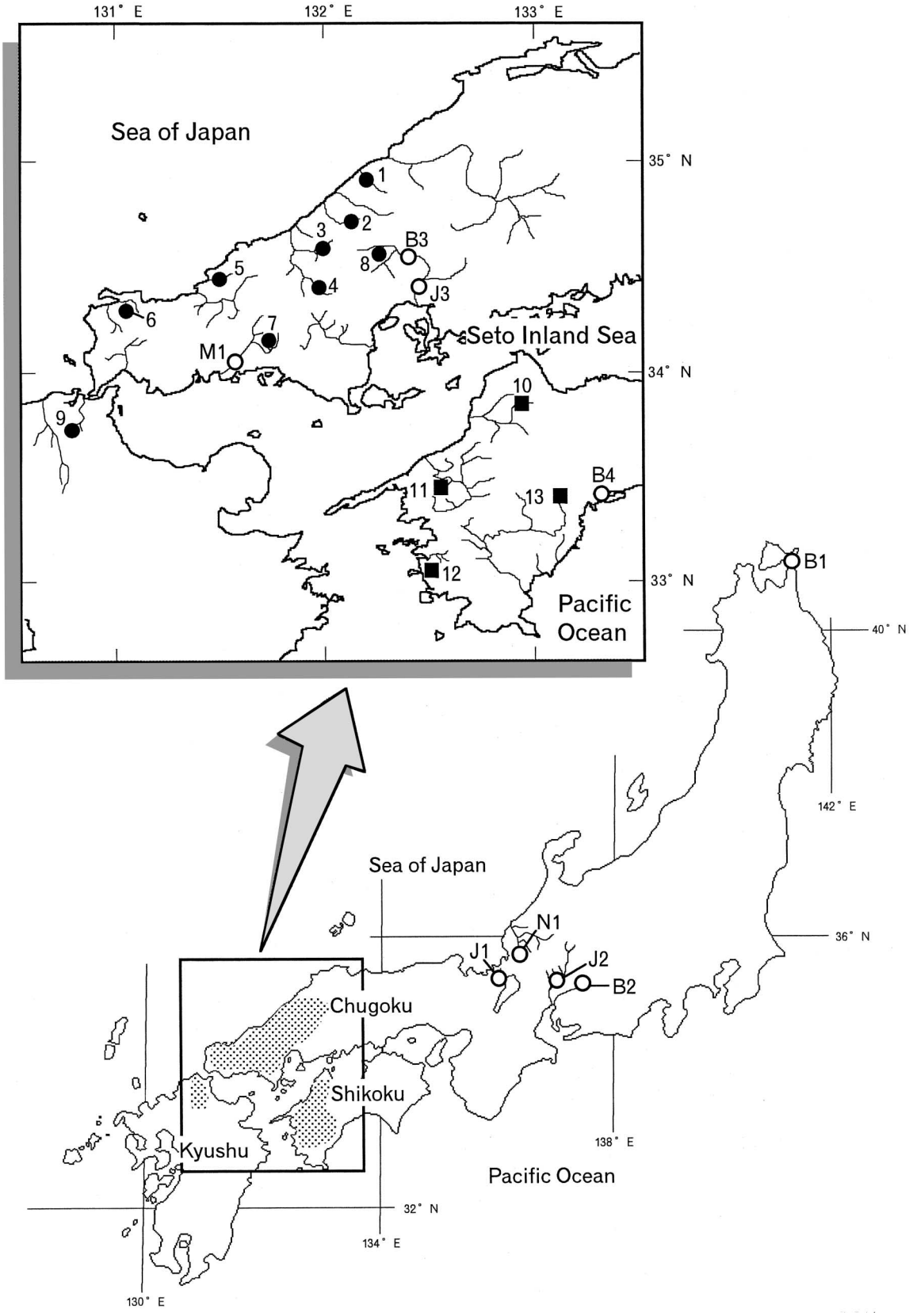

Fig. 2. Geographic distribution of *C. takatsuensis* (dotted range) and sampling localities in the present study. Only rivers inhabited by *C. takatsuensis* (Shimizu and Mizuno, 1994) are shown on the enlarged map. Locality numbers correspond to those in Table 2. ●; *C. takatsuensis* (Chugoku-Kyushu 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.
Cobitis takatsuensis	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
C. biwae (Eastern group)*	B1	Mena R. (Mutsu R. s.)	1	FRLM 24915
(Western group)*	B2	Toki R. (Shonai R. s.)	1	FRLM 24916
(Western group, tetraploid)*	B3	Ota R.	1	FRLM 24917
(Kochi group)*	B4	Uranouchi R.	1	FRLM 24918
C. matsubarai	M1	Saba R.	1	FRLM 24919
C. sp. 1	J1	Chinai R.	1	FRLM 24920
C. sp. 2 subsp. 2	J2	Otani R. (Nagara R. s.)	1	FRLM 24921
C. sp. 3	J3	Ota R.	1	FRLM 24922
Niwaella delicata	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)
Aci I	
a	510 380 330 230 225 130 90 70
b	650 380 330 230 130 90 85 70
c	510 330 270 230 225 130 110 70
d	510 330 295 270 230 130 110 90
e	510 270 230 225 180 150 130 110 90 70
f	620 490 250 230 140 100 90
g	620 490 320 250 140 100
h	620 520 320 250 140 100
Alu I	
a	550 320 280 230 180 130 80 75 70
b	420 320 280 230 180 130 130 80 75 70
c	790 350 340 200 180 80
d	790 540 350 180 80
e	790 350 240 200 180 100 80
Bfa I	
a	390 305 295 270 180 170 165 110 70
b	390 305 295 270 180 170 165 70
c	490 295 270 265 200 150 140 110
d	300 295 270 200 190 185 150 140 110 80
e	490 270 265 205 200 195 140 110 100
f	490 295 270 265 200 180 140 110
*Bst*U I	
a	1820 180
b	2000
c	1680 320
Dde I	
a	540 330 280 190 185 180 110 100
b	610 395 210 185 180 160 110 100
c	610 280 260 210 185 180 115 110
d	610 280 210 185 180 160 115 110 100
Hae III	
a	380 310 265 260 210 180 175 120 100
b	440 380 310 260 210 180 120 100
c	310 305 265 260 210 180 175 120 100 75
d	520 290 275 260 200 180 140 90
e	290 275 265 260 255 200 180 140 90
f	550 520 275 200 180 140 90
Hha I	
a	1150 380 360 110
b	910 600 380 110
Hinf I	
a	985 310 300 205 190
b	985 500 300 205
c	985 500 205 200 100
d	985 500 210 205 100
Hsp 92 II	
a	870 690 340 100
b	1210 690 100
Mbo I	
a	730 630 250 210 90 80
b	740 630 250 210 90 80
c	1370 250 210 90 80
Msp I	
a	1070 430 320 180
b	1030 430 360 180
Rsa I	
a	810 680 490
b	810 680 510
c	820 520 370 290
Sau 96 I	
a	420 290 260 240 225 185 180 170
b	420 260 240 230 225 185 180 170
c	420 260 240 230 225 180 165 110
d	420 260 240 230 225 185 180 165
*Scr*F I	
a	820 630 270 210
b	820 630 280 270
c	630 560 390 220 200
d	810 630 560
e	630 610 560 200
Taq I	
a	1470 230 190 110
b	1470 420 110
c	690 680 510 90
d	700 680 510 90
e	720 680 510 90
Tsp E I	
a	300 280 250 225 220 170 135 110 95 90
b	300 280 250 225 220 135 130 110 95 90
c	300 280 250 225 220 170 140 135 110 90
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 it's

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 *Hin*f 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.

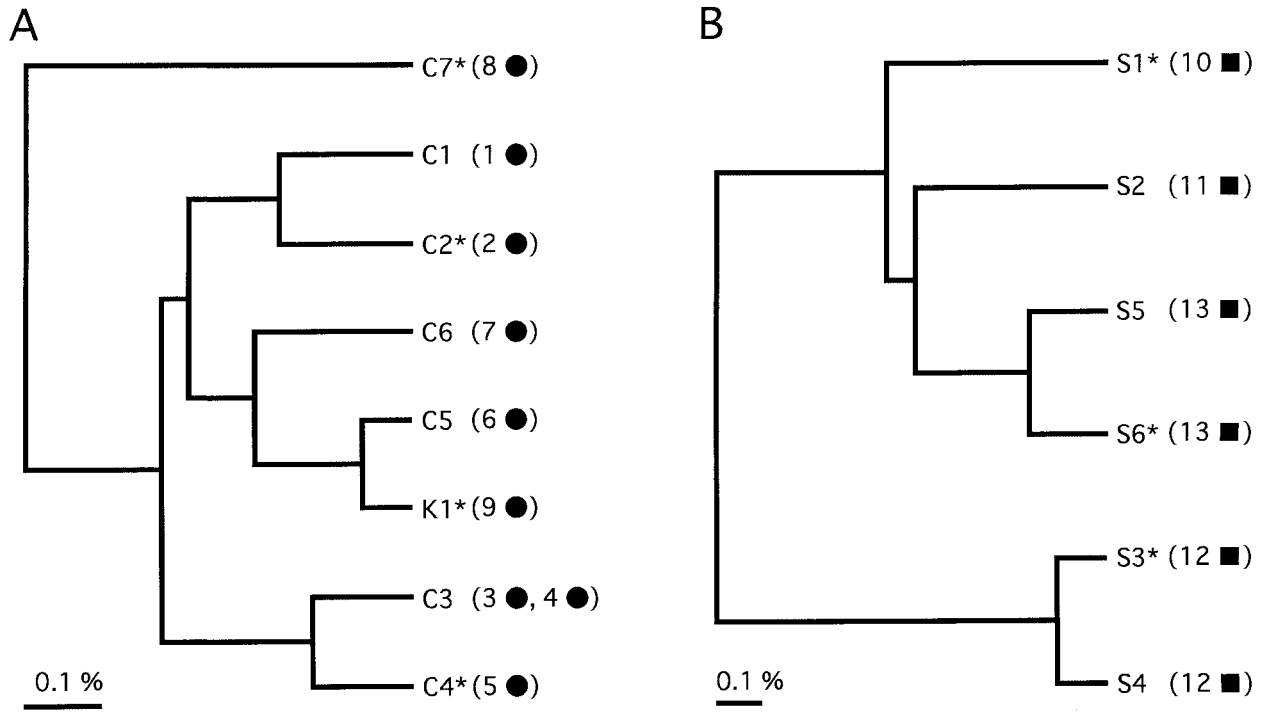

Fig. 3. UPGMA tree for ND1 haplotypes from the Chugoku-Kyushu (A), and Shikoku (B) groups of *C. takatsuensis*, based on nucleotide sequence divergence (Nei and Li, 1979). Numbers and symbols in parentheses correspond to those in Fig. 2. Asterisks (*) indicate individuals used for sequence analysis.

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

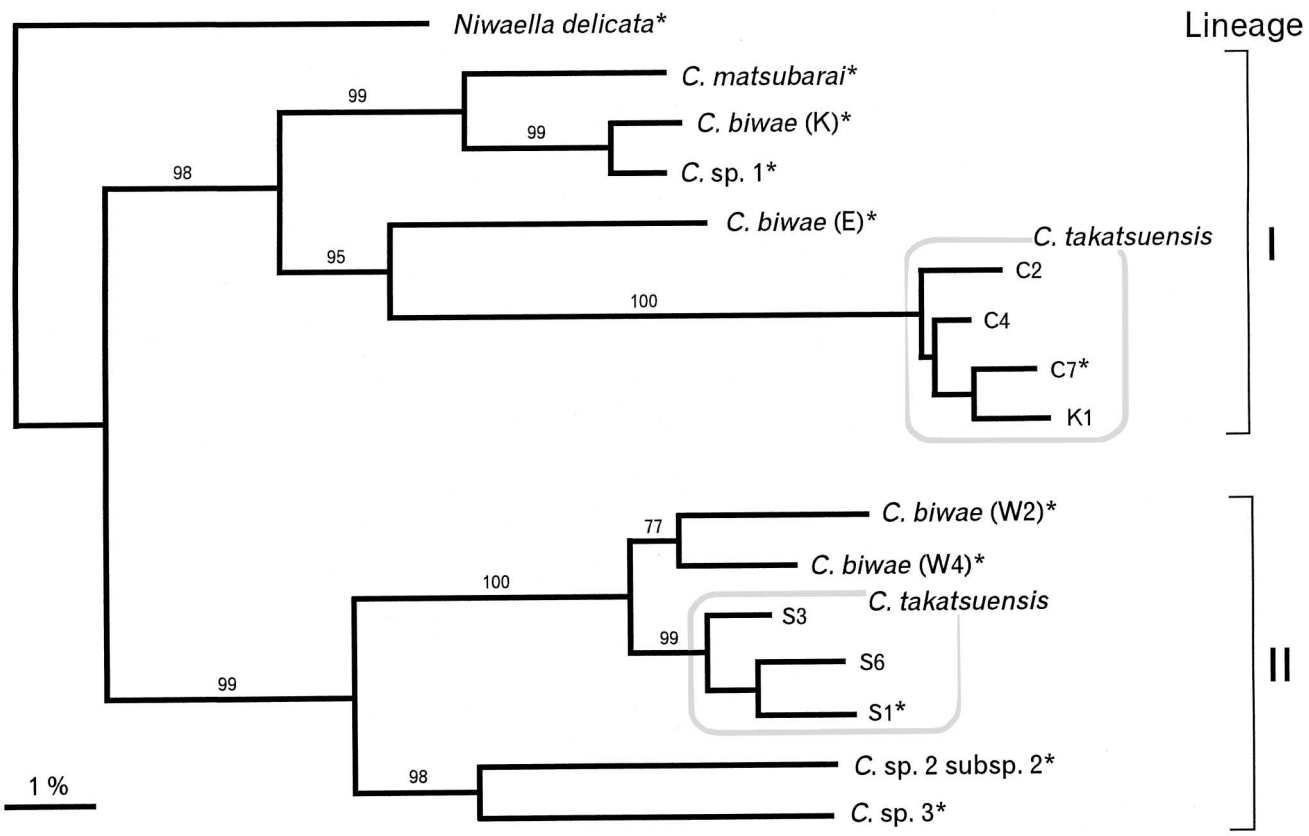

Fig. 4. Neighbor-joining network for Japanese *Cobitis* and *Niwaella* species based on nucleotide sequence divergence (Kimura, 1980). Bootstrap probabilities (%) with 1,000 replications are shown for each cluster. Asterisks (*) indicate individuals used for maximum likelihood and maximum parsimony analysis.

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. takatsuensis* (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

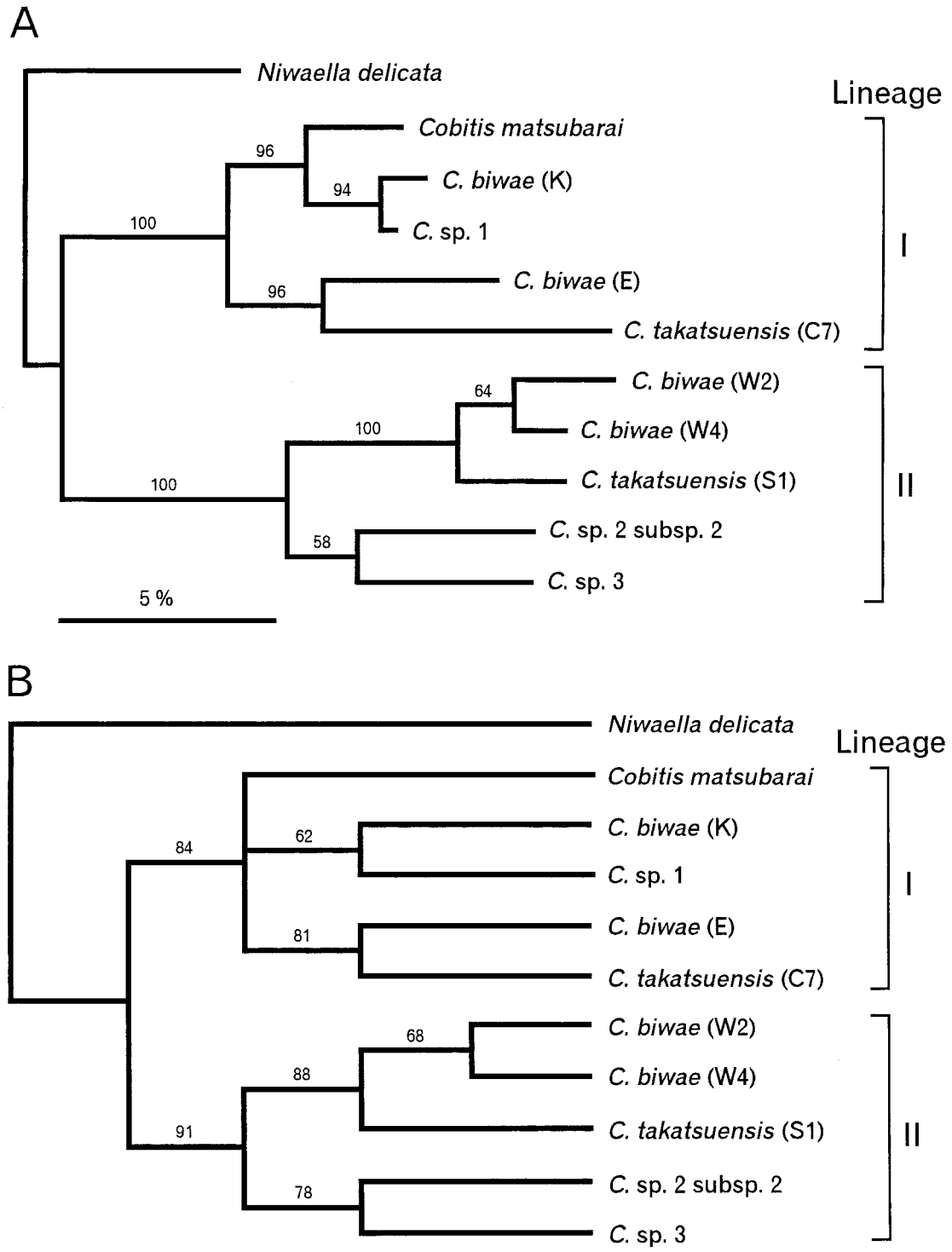

Fig. 5. Maximum likelihood (Ln likelihood = –2834.324) (A), and maximum parsimony trees (50% majority rule consensus) (B) for Japanese *Cobitis* species. Bootstrap probabilities (%) with 1,000 replications are shown for each cluster.

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

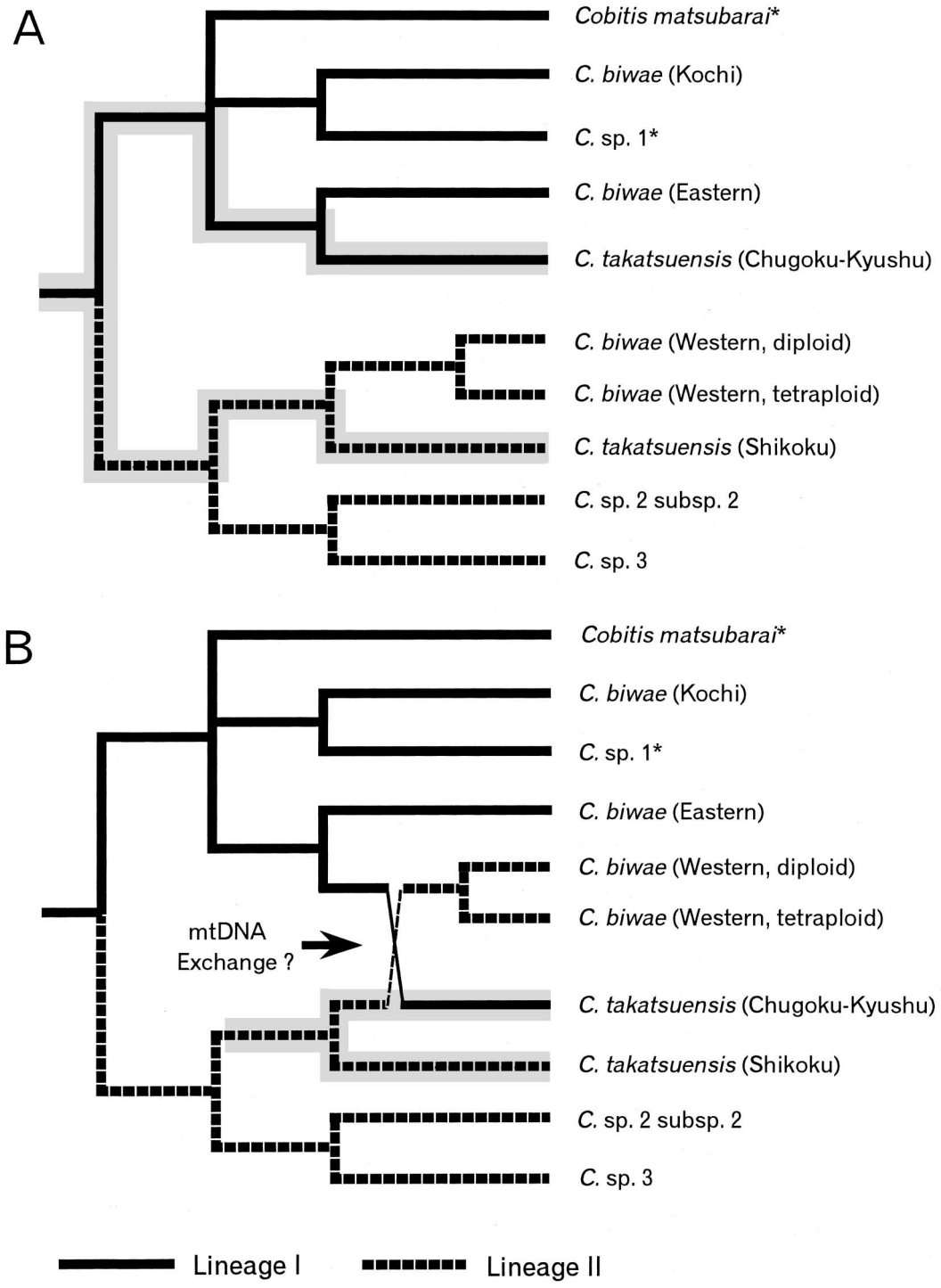

Fig. 6. Illustration of parallel evolution scenario (A), and the most parsimonious scenario under the mtDNA introgression hypothesis (B). Fine solid and broken lines indicate the mtDNA phylogeny determined in the present study. Dotted portions show putative lineages of *Cobitis takatsuensis*. Asterisks (*) 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	
C. takatsuensis	Chugoku-Kyushu	48	12	18	18	Kimizuka et al., 1982
	Shikoku	48	12	18	18	Kimizuka *et al*., 1982
		48*	12	16	20	Kimizuka *et al*., 1982
C. biwae	Eastern	48	20	24	4	Ueno, 1981
	Western	48	20	22	6	Ueno *et al*., 1980
		48	16	22	10	Ueno *et al*., 1980
		96 (tetraploid)	32	54	10	Ueno *et al*., 1980
	Kochi	48	16	24	8	Ueno *et al*., 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 (Avise, 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 (Avise 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.

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