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Genetic Relationships of Japanese and Korean Bagrid Catfishes Inferred from Mitochondrial DNA Analysis

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ABSTRACT—Partial sequences of the cytochrome *b* gene (740 bp) and restriction fragment length polymorphisms in the mitochondrial DNA were used to examine inter-and intraspecific relationships among nine species of bagrid catfishes (family Bagridae) in Japan and Korea. Several opinions have been expressed regarding the kinship among Japanese and Korean bagrid catfishes, based on external morphological similarities. Almost all of them, however, were rejected by the current data sets. For instance, it has been considered that the Korean species, *Pseudobagrus brevicorpus* and *P. fulvidraco*, were closely-related to *P. ichikawai* and *P. nudiceps*, respectively, found in Japan. Resulting trees indicated that *P. ichikawai* branched off separately from all of the remaining *Pseudobagrus* species. Similarly, *P. nudiceps* and *P. fulvidraco* were represented by distantly separated branches. The intraspecific genetic divergence of bagrid catfishes was relatively small, even among geographically distant populations.

INTRODUCTION

The siluriform family Bagridae is widely distributed from Africa to east Asia (Berra, 1981). Through past studies, it is considered to be represented in Japan by four species (*Pseudobagrus nudiceps*, *P. aurantiacus*, *P. tokiensis* and *P. ichikawai*) and in Korea by six species (*P. fulvidraco*, *P. koreanus*, *P. brevicorpus*, *Leiocassis nitidus*, *L. ussuriensis* and *L. longirostris*; Miyadi *et al*., 1976; Jeon, 1984; Lee and Kim, 1990; Watanabe and Maeda, 1995). While the Japanese species are all distributed allopatrically, more than one species have been recorded from single river systems in Korea. In recent years, *L. longirostris* has not been reported in Korea (Lee and Kim, 1990).

External morphological similarities among bagrid catfishes have hindered clarification of their systematic relationships, *P. aurantiacus* and *P. tokiensis*, which are distributed in the westernmost and northeast regions of Japan, respectively, at one time being considered a single species (e.g., Miyadi *et al*., 1976). Substantial genetic differences inferred from allozyme and karyological analysis provided the key for the recognition of two species (Ueno, 1974). Several proposal of systematic relationships among Japanese and Korean bagrid catfishes have been put forward, based on external morphological similarities. In particular, it has been considered that the Korean species, *Pseudobagrus fulvidraco* and *P. brevicorpus*, were closely-related to *P. nudiceps* and *P. ichikawai*, respectively, found in Japan (Uchida, 1939; Miyadi *et al*., 1976). In the past, *P. ichikawai* and *P. brevicorpus* were even included together in a separate genus, *Coreobagrus* (Mori, 1936; Okada and Kubota, 1957). Although an outline of the genetic relationships among Japanese bagrid catfishes has already been given based on allozyme analysis (Maeda *et al*., 1994), the systematic relationships, among Japanese and Korean bagrid catfishes has remained obscure.

This study examined the genetic relationships of bagrid catfishes in Japan and Korea, as well as *P. fulvidraco* and *L. nitidus* in Russia, based on mitochondrial(mt) DNA sequence and restriction site polymorphisms.

MATERIALS AND METHODS

Specimens

Specimens were caught with hand nets and longlines in Japan, South Korea and Russia, from 1991 to 1997. Specific locations are shown in Fig. 1 and sample sizes listed in Table 1. Examples of *L. longirostris* were not obtained. Aquarium-reared *P. ichikawai*, one of the protected animals designated by the state, having originated from the Nagara River population, were made available by Miwa Elementary School, Minokamo City, Gifu Prefecture with the permission of the Agency for Culture Affairs. Whole fish were frozen or preserved in ethanol immediately after collection until processed for analysis.

Restriction analysis

Total DNA was extracted from approximately 500 mg of white muscle tissue from each fish following Lansman *et al.* (1981). Fragments of about 1.9 kilo base pairs (kbp) in length from the cytochrome

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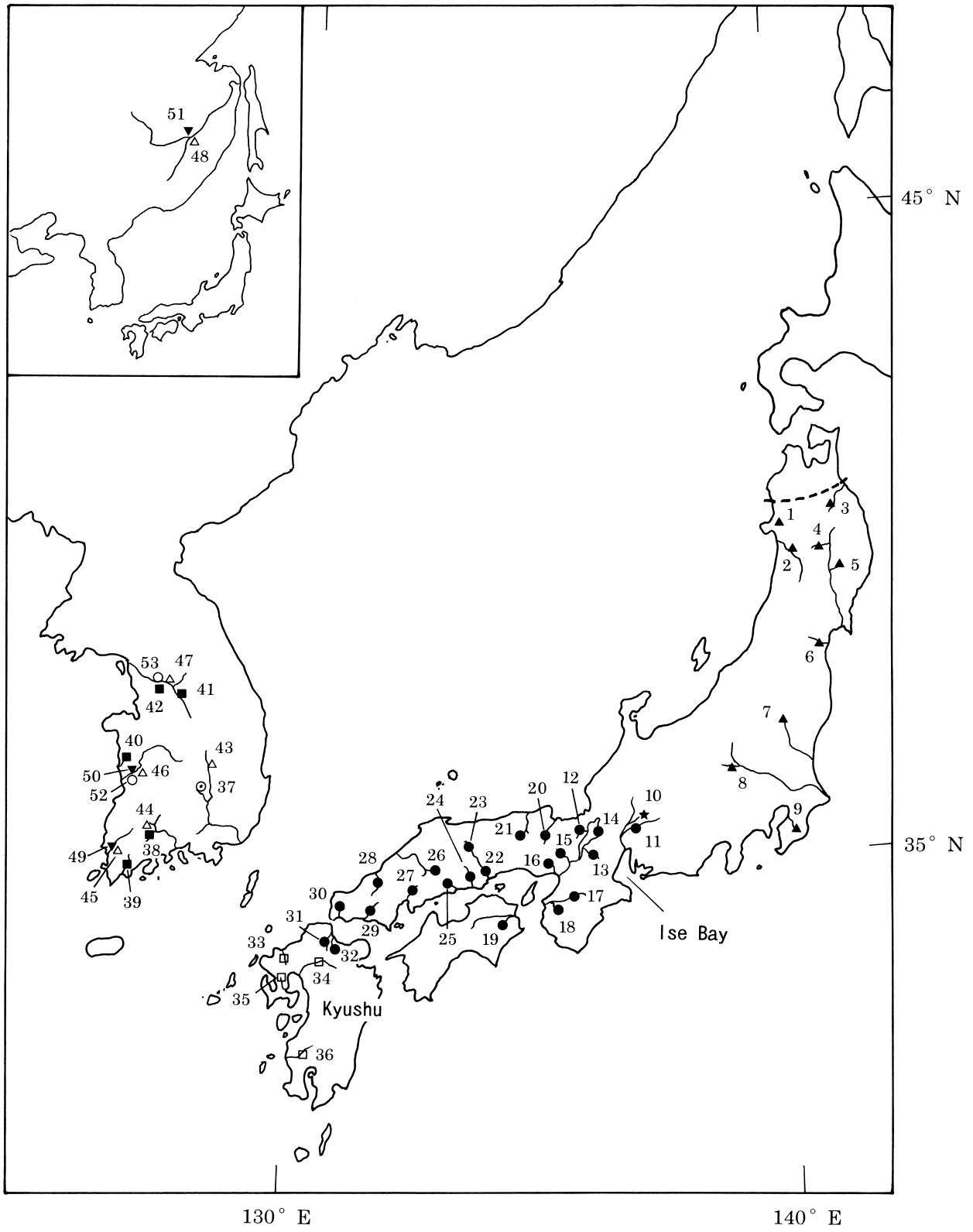

Fig. 1. Map of East Asia showing the sites where bagrid catfishes were collected. *Pseudobagrus tokiensis* (▲), *P. ichikawai* (★), *P. nudiceps* (●), *P. aurantiacus* (□), *P. brevicorpus* (⊙), *P. koreanus* (■), *P. fulvidraco* (△), *Leiocassis nitidus* (▼) and *L. ussuriensis* (○). Names of sampled populations provided in Table 1. Broken line indicates northern distribution boundary of Japanese bagrid catfishes (Sugiyama, 1985; Takeuchi *et al.*, 1985).

Table 1. Sample locations, including drainage system (see Fig.1), of bagrid catfish populations

Species	No.	Sample location	Sample size	Drainage
Pseudobagrus tokiensis				
	1	Gojyome, Akita	2	pond
	2	Yuhwa, Akita	2	Omono River
	3	Ninohe, Iwate	3	Mabechi R.
	4	Shizukuishi, Iwate	2	Shizukuishi R.(Kitakami R. system)
	5	Esashi, Iwate	2	Ide R.(Kitakami R. system)
	6	Sendai, Miyagi	3	Hirose R.
	7	Kuroiso, Tochigi	3	Naka R.
	8	Annaka, Gunma	3	Tone R.
	9	Ichihara, Chiba	3	Yourou R.
P. ichikawai				
	10	Minokamo, Gifu	3	Kawaura R. (Nagara R. system)
P. nudiceps				
	11	Kakamigahara, Gifu	1	Kiso R.
	12	Adogawa, Shiga	1	Ado R.
	13	Minakuchi, Shiga	3	Yasu R.
	14	Nagahama, Shiga	3	Lake Biwa
	15	Sonobe, Kyoto	1	Sonobe R. (Yodo R. system)
	16	Inagawa, Hyogo	3	Ina R.
	17	Yoshino, Nara	2	Kino R.
	18	Nogami, Wakayama	2	Kishi R. (Kino R. system)
	19	Kamiyama, Tokushima	3	Akui R.
	20	Fukuchiyama, Kyoto	2	Yura R.
	21	Hidaka, Hyogo	1	Maruyama R.
	22	Okayama, Okayama	2	Asahi R.
	23	Yatsuka, Okayama	2	Asahi R.
	24	Soujya, Okayama	1	Takahashi R.
	25	Mitsugi, Hiroshima	2	Ashida R.
	26	Mirasaka, Hiroshima	3	Gouno R.
	27	Hiroshima, Hiroshima	3	Ohta R.
	28	Nichihara, Shimane	1	Takatsu R.
	29	Hohfu, Yamaguchi	3	Saba R.
	30	Toyota, Yamaguchi	1	Koya R.
	31	Saigawa, Fukuoka	2	Ima R.
	32	Toyotsu, Fukuoka	2	Harai R.
P. aurantiacus				
	33	Kyuragi, Saga	3	Matsuura R.
	34	Yoshii, Fukuoka	3	Chikugo R.
	35	Isahaya, Nagasaki	3	Honmyo R.
	36	Hiwaki, Kagoshima	3	Sendai R.
P. brevicorpus				
	37	Changchon-ri, Kyongsangbuk-do	3	Naktong R.
P. koreanus				
	38	Pongjo-ri, Chollanam-do	3	Somjin R.
	39	Kaesan-ri, Chollanam-do	2	Tamjin R.
	40	Nochon-ri, Chungchongnam-do	3	Ungchon R.
	41	Pobchon-ri, Kangwon-do	1	Namhan R.
	42	Ohak-ri, Kyonggi-do	2	Namhan R.
P. fulvidraco				
	43	Wolgok-ri, Kyongsangbuk-do	2	Naktong R.
	44	Pongjo-ri, Chollanam-do	3	Somjin R.
	45	Soho-ri, Chollanam-do	3	Yongsan R.
	46	Hwangsan-dong, Chungchongnam-do	3	Kum R.
	47	Ohak-ri, Kyonggi-do	3	Namhan R.
	48	Chabarovsk, Russia	1	Amur R.
Leiocassis nitidus				
	49	Soho-ri, Chollanam-do	3	Yongsan R.
	50	Hwangsan-dong, Chungchongnam-do	3	Kum R.
	51	Chabarovsk, Russia	3	Amur R.
L. ussuriensis				
	52	Hwangsan-dong, Chungchongnam-do	3	Kum R.
	53	Ohak-ri, Kyonggi-do	3	Namhan R.

b to the 12S rRNA genes, including the entire D-loop, were amplified by PCR (Saiki *et al.*, 1988) from whole genomic DNA, using the following primer pairs: (5'-CAYATYMARCCMGAATGRTAYTT-3') and (5'-ATARTRGGGTATCTAATCCYAGTT-3') (Palumbi *et al.*, 1991). PCR was conducted for 30 cycles in a PJ-9600 apparatus (Perkin Elmer Cetus, CA) at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2.0 min. All products of PCR were confirmed as being of equal length by electrophoresis and were subjected directly to digestion with restriction endonucleases.

The products of PCR were digested following the recommendations of the enzyme manufacturers. Sixteen enzymes recognizing four or five nucleotides were used (Table 2; 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 mtDNA were visualized for photography by staining with ethidium bromide and exposure to UV light.

Sequence analysis

Fifteen individuals representing haplotypes of each species and regional populations resulting from restriction fragment length polymorphism (RFLP) analysis, were selected for sequence analysis. A partial region of the mitochondrial cytochrome *b* gene (740 bp following tRNA-Glu) was amplified by PCR using the following primer pairs: (5'-TGACTTGAARAACCAYCGTTG-3') and (5'-RGCRAAKARRAA RTAYCATTC-3') (Palumbi *et al.*, 1991). 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 (Quiagen, Germany). *Liobagrus reini* (Amblycipitidae; collected in the Shinano River, Niigata Prefecture) and *Silurus asotus* (Siluridae; collected in Lake Biwa), comprising order Siluriformes along with family Bagridae, were included as an outgroup (Hosoya and Yamada, 1993).

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 for both DNA strands. The nucleotide sequence data reported in this paper are available from DDBJ, EMBL and GenBank accession numbers AB015986–AB015995.

Data analyses

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. Phenograms were generated by the unweighted pair-group method (UPGMA; Sneath and Sokal, 1973) and neighbor-joining analysis (NJ; Saitou and Nei, 1987).

DNA sequence data were processed using DNASIS programs (Hitachi Software Engineer. Co. Ltd.). Genetic distances were obtained using Kimura's two parameter method (transitions/transversions [ts/tv] ratio = 5; Kimura, 1980). Two different methods were employed to infer the relationships among taxa; the PAUP 3.1.1. computer program (Swofford, 1993) for maximum parsimony analysis (MP), and the PHYLIP 3.572 computer package (Felsenstein, 1996) for NJ analysis (Saitou and Nei, 1987). The reliability of each interior branch was tested by 1,000 bootstrap replications.

RESULTS

Restriction analysis

Each of the 16 restriction endonucleases yielded multiple banding patterns (restriction morphs; Table 2). However, the use of *Tsp EI*, which gave ambiguous banding patterns in a preliminary study, was discontinued. Each species was characterized by combinations of different morphs produced by 14 of the 15 remaining restriction enzymes. *Bst UI* was monomorphic and common to all of the species examined.

Table 3 shows composite genotypes (haplotypes) detected among bagrid catfishes and their locations appeared. Regarding river populations within a single species, all morphs produced were identical in both *P. aurantiacus* and *L. ussuriensis*. Similarly, most populations of *L. nitidus* were identical, except for morphs produced by *Nla III*. Specimens of *P. tokiensis* collected in rivers north of the Naka River yielded unique morphs following digestion by *Hinf I*. The morph "*Sau96 I* D" appeared solely in the Namhan River population of *P. koreanus* and likewise the morph "*Nla III* J" in that of *P. fulvidraco*. Only a few variant individuals were found among *P. nudiceps* populations through the range of the species. Of *P. brevicorpus* and *P. ichikawai*, both represented in the present study by a single population only, variant morphs following digestion by *Hinf I* and *Nla III* were observed, respectively.

Among the 126 bagrid catfishes examined, 22 haplotypes were identified (Table 3), all species having their own haplotypes. The estimated pairwise sequence divergence among haplotypes ranged from 0.18 to 6.61 %. Both phenograms by UPGMA and NJ (Fig. 2), based on the above, clearly indicate that the structuring of haplotypes conformed to each species. It is noteworthy that the both phenograms show considerable deviation of *P. ichikawai* from the remaining species of *Pseudobagrus* although the phenograms did not show complete congruity. Furthermore the structuring of the remaining species did not conform to their geographic locations, namely Japan and South Korea. *L. ussuriensis* and *L. nitidus* formed a single cluster, which deviated from *Pseudobagrus* species, except *P. ichikawai*. *P. tokiensis* and *P. aurantiacus*, at one time being considered a single species, were clearly distinguishable each other.

Sequence analysis

Following the RFLP analysis, 15 individuals (Table 3), representing haplotypes of each species and differing geographic locations, were selected for sequence analysis to elucidate their possible phylogenetic relationships. The aligned sequences of a 740 bp segment of those individuals are shown in Fig. 3.

Fig. 4(A) shows the NJ tree based on Kimura's two-parameter model (ts/tv = 5) generated for the third position data sets (Table 4). Since many nucleotide substitutions at the third position of codon are silent and do not change amino acids, the rate of nucleotide substiution is much higher at the third position than at the first and second positions. In this analysis we used the data sets of the third nucleotide position of codons alone to eliminate the effect of the variance of substitutions of the first and second positions according to Kimura (1980). This tree indicates that *P. ichikawai* is separated from a major clade comprising the remaining species of *Pseudobagrus*. A clade comprising *L. ussuriensis* and *L. nitidus* is also indicated. The above is congruent with that resulting from the RFLP analysis. Within the major *Pseudobagrus* clade, *P. tokiensis*

Table 2. Restriction enzymes used to examine bagrid catfish mt-DNA and their resulting fragment patterns

Enzyme	Fragment patterns (× 10 bp)
Aci I	
A	110, 28, 19, 19, 16
B	138, 19, 19, 16
C	154, 19, 19
D	125, 28, 19, 16
E	102, 28, 19, 19, 16, 8
Afa I	
A	108, 41, 27, 10
B	108, 43, 27, 10
C	108, 27, 26, 15, 10
D	108, 33, 30, 30, 10, 7
E	94, 43, 27, 14, 10
F	108, 33, 21, 10, 7, 7
G	108, 51, 27,
Alu I	
A	94, 36, 29, 10, 10
B	75, 36, 29, 19, 10, 10
C	75, 36, 20, 14, 14, 10, 10
D	61, 36, 29, 19, 14, 10, 10
Bfa I	
A	46, 33, 29, 18, 18, 17, 16, 12
B	62, 33, 29, 18, 18, 17, 12
C	86, 29, 29, 17, 16, 12
D	70, 29, 29, 20, 20, 17
E	70, 29, 29, 20, 20, 13
F	62, 29, 29, 20, 17, 17, 13
G	54, 29, 29, 18, 18, 17, 16, 8
H	92, 29, 29, 17, 16, 8
Bst UI	
A	125, 48, 19
Dde I	
A	93, 37, 33, 13, 8
B	64, 37, 33, 29, 13, 8
C	88, 33, 18, 18, 13, 8, 6
D	61, 37, 33, 26, 13, 8, 6
Hae III	
A	48, 38, 37, 31, 22, 13
B	48, 38, 37, 22, 22, 13, 9
C	75, 48, 31, 22, 13
D	61, 41, 38, 31, 22
E	48, 41, 38, 31, 22, 13
F	72, 48, 38, 22, 13
G	107, 36, 22, 13, 11
Hha I	
A	86, 68, 38
B	86, 38, 34, 34
C	76, 68, 38, 10
Hinf I	
A	47, 38, 28, 26, 22, 12, 11, 8
B	64, 47, 28, 22, 12, 11, 8
C	47, 38, 34, 28, 26, 11, 8
D	64, 47, 28, 19, 17, 17
E	42, 38, 38, 25, 22, 11, 8
F	64, 47, 46, 34
G	47, 38, 26, 25, 22, 15, 11, 8
H	47, 46, 28, 26, 26, 10, 7
I	60, 46, 25, 15, 15, 13, 8, 8
J	47, 38, 38, 26, 22, 12, 8,
Mbo I	
A	105, 76, 12
B	76, 65, 40, 12
C	103, 70, 12, 8
D	193
E	76, 40, 35, 30, 12
F	160, 33
G	77, 76, 40
H	105, 60, 16, 12
Msp I	
A	100, 37, 33, 22
B	133, 37, 22
C	100, 37, 23, 22, 10
Nla III	
A	50, 40, 33, 28, 23, 10
B	50, 40, 33, 28, 16, 10, 7
C	74, 42, 40, 28
D	74, 40, 28, 26, 16
E	50, 42, 40, 28, 26
F	54, 50, 42, 40
G	50, 50, 40, 33, 10, 7
H	55, 50, 40, 33, 12
I	50, 40, 28, 23, 23, 19, 7
J	50, 40, 33, 23, 19, 19, 7
K	58, 40, 33, 33, 16, 10
L	50, 50, 42, 40, 7
M	50, 42, 40, 28, 23, 7
N	50, 42, 40, 33, 16, 7
O	50, 40, 39, 37, 16, 7
P	50, 42, 40, 28, 23, 7
Sau 96 I	
A	92, 33, 22, 22, 17, 16
B	92, 39, 22, 22, 17
C	92, 22, 22, 21, 18, 17
D	92, 38, 22, 22, 18
E	92, 56, 22, 22
F	83, 22, 22, 21, 18, 17, 9
G	83, 33, 22, 22, 17, 15
Scr FI	
A	96, 96, 60
B	60, 60, 34, 25, 11
Taq I	
A	170, 22
B	192
C	152, 40
D	132, 60
E	152, 22, 18
F	184, 8

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

Table 3. Composite haplotypes for restriction enzyme polymorphisms detected among bagrid catfishes, showing numbers and locations of fish with each haplotype

Haplotype	Species	Composite fragment pattern	N	Location
1	*Pseudobagrus nudiceps*	AAAAAAAAAAAAAAA	42	11, 12, 13(2), 14(2)*, 15, 16, 17, 18, 19, 20, 21, 22, 23*, 24, 25, 26, 27, 28, 29, 30, 31, 32
2	—	AAAAAAAAAAABAAA	2	13(1), 14(1)
3	*P. tokiensis*	BAAAABAACBBCAAC	14	1, 2, 3, 4*, 5, 6
4	—	BAAAABAAABBCAAC	7	7(2), 8(2), 9*
5	—	BAAAABAABBBCAAC	1	7(1)
6	—	BAABABAAABBCAAC	1	8(1)
7	*P. aurantiacus*	AAACAABADCBDBAB	12	33, 34*, 35, 36
8	*P. ichikawai*	DGDHACGBIEBNGAC	1	10(1)
9	—	DGDHACGBIEBOGAC	2	10(2)*
10	*P. koreanus*	ACBFAACAEABEDAD	2	41*, 42
11	—	ACAFAACAEABEEAD	2	40(1),
12	—	ACBFAACAEABFEAD	6	38(2)*, 39, 40(2)
13	—	ACBFAACAEABFEAB	1	38(1)
14	*P. fulvidraco*	EEADAAFAGDCICBA	11	43, 44(2), 45, 46*, 48*
15	—	EEADAAFAGDCJCBA	3	47*
16	—	EEADAAFAGDCMCBA	1	44(1)
17	*P. brevicorpus*	ABABAAACABAPAAC	2	37(2)*
18	—	ABABAAACJBAPAAC	1	37(1)
19	*Leiocassis nitidus*	CFCEACEAHFALBAE	2	50(1), 51(1)*
20	—	CFCEACEAHFAGBAE	6	49, 50(1)*, 51(2)
21	—	CFCEACEAHFAHBAE	1	50(1)
22	*L. ussuriensis*	BDBGADDAFGAKFAC	6	52, 53*

Note: Variable enzymes from Table 2 listed in order (*Aci* I, *Afa* I, *Alu* I, *Bfa* I, *Bst* UI, *Dde* I, *Hae* III, *Hha* I, *Hinf* I, *Mbo* I, *Msp* I, *Nla* III, *Sau*96 I, *Scr* FI, *Taq* I). Names for sample locations given in Table 1.
Numbers in parentheses indicate number of individuals observed; numbers not followed by parentheses indicate all individuals identical. Asterisks indicate individuals used for sequence analysis.

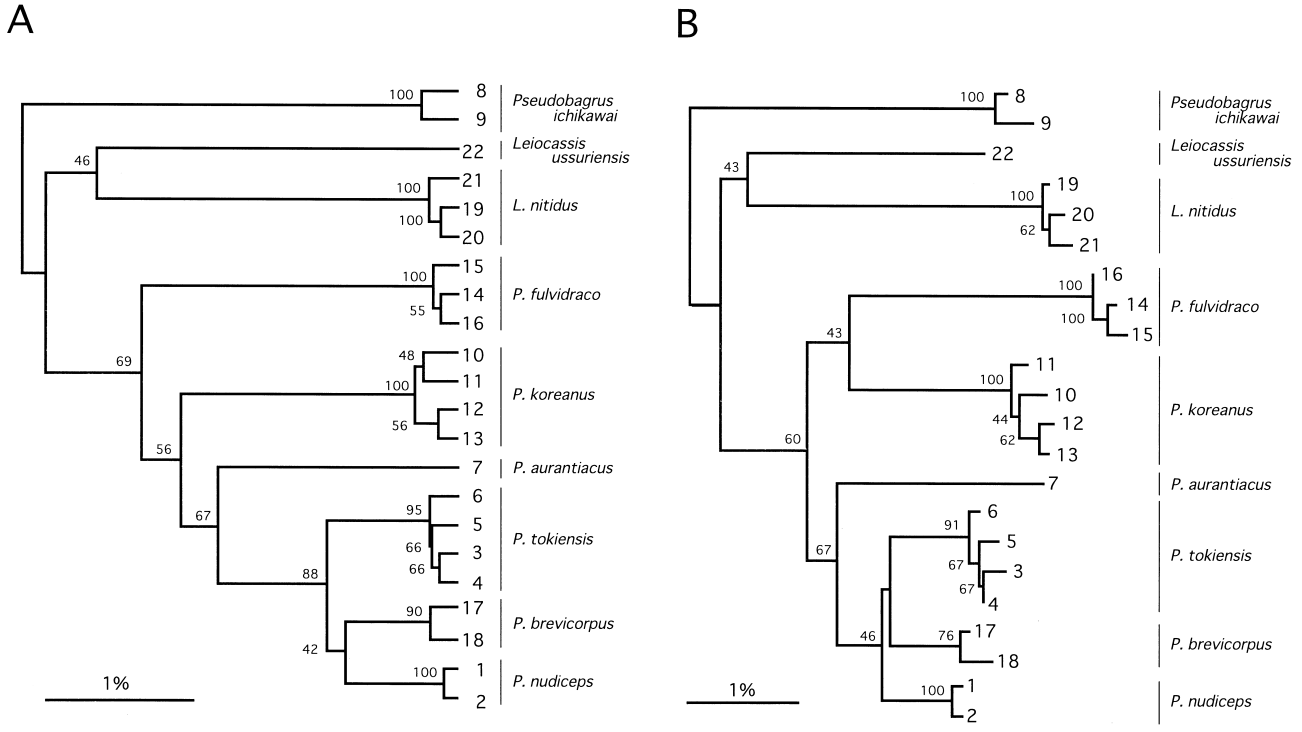

Fig. 2. UPGMA (A) and NJ (B) clusterings of composite bagrid catfish haplotypes based on nucleotide sequence divergence (Nei and Li, 1979). Numbers at forks indicate bootstrap values (1,000 replicates). Numbers at end of tree forks indicate haplotypes, defined in Table 3.

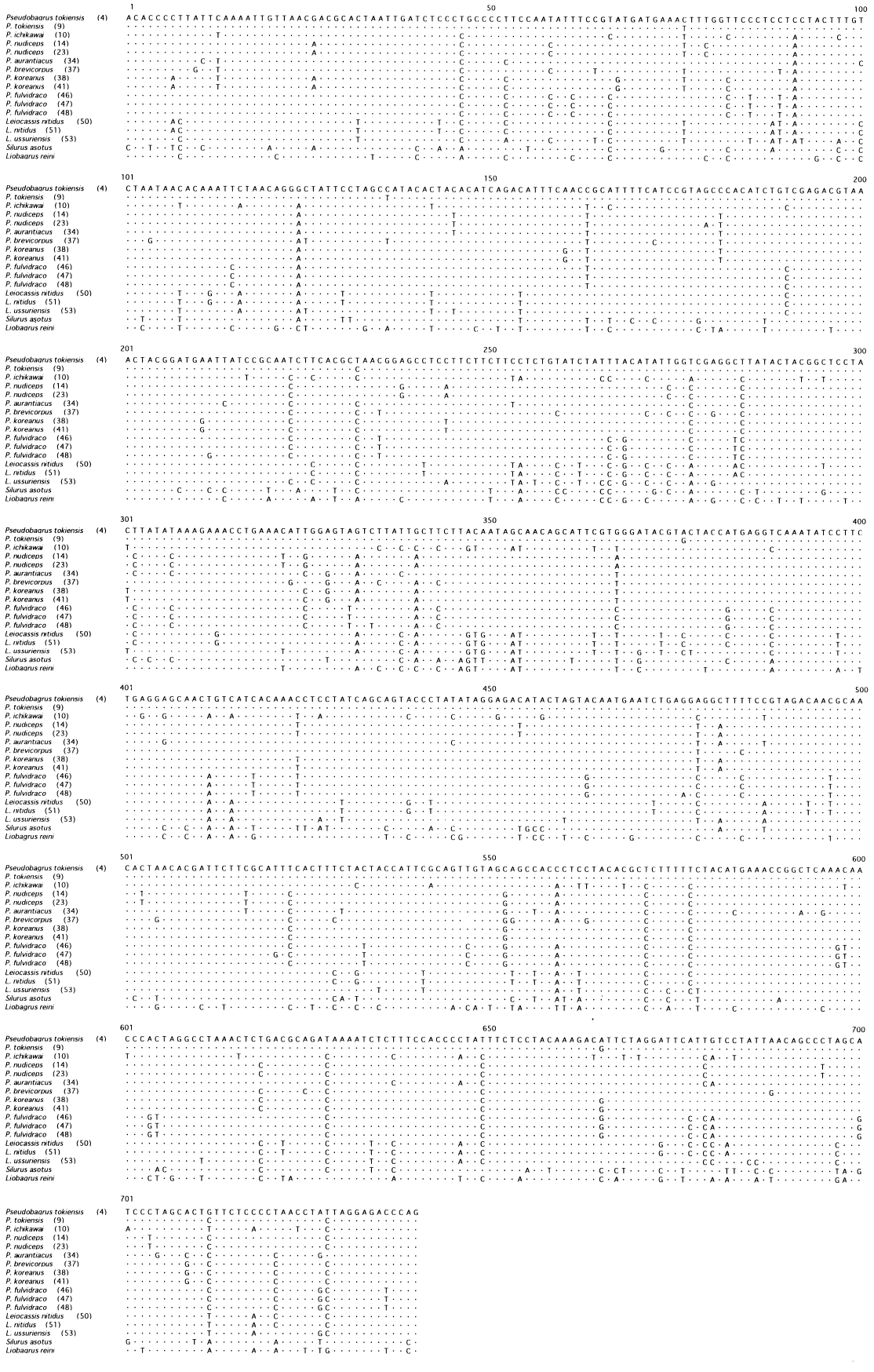

Fig. 3. Aligned sequences of a 740 bp segment of the cytochrome *b* gene. Dots indicate identical to sequence of *Pseudobagrus tokiensis* (4). Numbers in parentheses indicate specimens used (shown in Table 3).

Table 4. Average estimates of the number of nucleotide substitutions per site between part of the cytochrome *b* gene of the bagrid catfishes, *Silurus asotus* and *Liobagrus reini* for the third nucleotide position of codons based on Kimura's two-parameter model (ts/tv = 5). Ranges are shown in parentheses.

Species	*Pseudobagrus nudiceps*	*P.tokiensis*	*P.aurantiacus*	*P.ichikawai*	*P.koreanus*	*P.fulvidraco*	*P.brevicorpus*	*Leiocassis ussuriensis*	*L.nitidus*	*Silurus asotus*	*Liobagrus reini*
Pseudobagrus nudiceps	—	.154 (.152–.157)	.146	.317	.116	.195 (.194–.199)	.157	.304	.405 (.402–.409)	.801	1.275
P. tokiensis		.011 (.000–.021)	.199 (.196–.201)	.305 (.304–.306)	.154 (.151–.157)	.232 (.228–.238)	.192 (.184–.201)	.382 (.381–.383)	.381 (.373–.389)	.724 (.706–.741)	1.049 (1.021–1.077)
P. aurantiacus			—	.288	.136	.223 (.221–.227)	.200	.358	.386 (.383–.390)	.786	1.275
P. ichikawai				—	.290	.319 (.317–.324)	.342	.322	.356 (.353–.360)	.858	1.025
P. koreanus					—	.195 (.193–.198)	.136	.368	.384 (.381–.388)	.806	1.236
P. fulvidraco						.002 (.000–.004)	.222 (.220–.226)	.412 (.409–.417)	.406 (.400–.414)	.884	1.064 (1.058–1.077)
P. brevicorpus							—	.372	.396 (.392–.399)	.806	1.162
Leiocassis ussuriensis								—	.220 (.217–.223)	.967	1.373
L. nitidus									.001 (.000–.004)	.850	1.279 (1.268–1.29)
Silurus asotus										—	.985
Liobagrus reini											—

forms an independent subclade.

Since the ts/tv ratio for the third position for each pair of the species among *Pseudobagrus* stood at about 5 in the current study based on Kimura (1980), we used this value for the analysis. Although we tried to find the trees using seven parameters (ts/tv = 2, 3, 4, 5, 6, 7 and 8), the NJ trees using 2 to 6 hardly affected the topologies excepting the slight change in the branching order of *P. ichikawai* and *Leiocassis* species. However, the branching order of *P. tokiensis* and *Leiocassis* species was converted when parameters 7 and 8 were used.

Parsimony analysis (MP), generated for all three codon positions data sets using equal weighting for all substitutions, resulted in two equally-shortest trees, such being identical except for the branching order of *P. ichikawai* and *Leiocassis* species. A consensus tree of 1,000 bootstrap replicates is shown in Fig. 4(B). This tree also indicates that *P. ichikawai* is separated from all of the remaining *Pseudobagrus* species. The differences of this tree from the NJ tree are (1) the branching order of *P. ichikawai* and *Leiocassis* species, (2) the branching order of *P. fulvidraco* and *P. tokiensis* and (3) the position of *P. brevicorpus*. The probability of these relationships, however, received low bootstrap support.

Since the out group species used in the current analysis genetically deviated from the bagrid catfishes examined, there is a possibility of multiple substitutions not adequately corrected. Therefore, the MP analysis, using equal weighting for all substitutions, was made for the first and second positions data sets of one individual of each species to determine the root. A consensus tree (50% majority-rule consensus) of 1,000 bootstrap replicates shown in Fig. 4(C) indicates that the positions of the root in the former trees are reasonable.

DISCUSSION

The NJ (Fig. 4 (A)) and MP (Fig. 4 (B, C)) analyses were generally congruent in defining relationships among the Japanese and Korean bagrid catfishes. Although several opinions have been previously proposed regarding the above bagrid relationships, almost all were rejected by the current data sets.

Although *P. ichikawai* in Japan has been considered most closely related to *P. brevicorpus* in Korea, the trees derived here indicated that *P. ichikawai* branched off separately from all of the remaining *Pseudobagrus* species. Similarly, *P. nudiceps* and *P. fulvidraco*, which have been suggested to comprise a single species (Uchida, 1939), are represented by distantly separated branches. *P. tokiensis* and *P. aurantiacus*, also considered at one time to represent a single species (Miyadi *et al.*, 1976), were subsequently found to have substantial genetic and morphological differences (Ueno, 1974; Juso, 1979; Hosoya and Yamada, 1993; Watanabe and Maeda, 1995). The present analysis indicated that the two species not only occupy separate branches, but also that they do not comprise a sister group. Although a kin relationship between *P. koreanus* and *P. aurantiacus* has already been suggested from their karyological coincidence (Ueno, 1985), the current analysis did not assure their closest affinities.

The above indicates that past external morphological studies have lead to misunderstandings of the above species' systematic relationships, and that karyological analyses alone cannot resolve them.

Because bagrid catfishes are also widely distributed in east Asia, with a greater number of species being distributed in mainland China than in Korea and Japan (Mo, 1991), the low likelihood the Korean and Japanese representatives together being monophyletic, must be noted. There is a limit to resolving true systematic relationships based solely upon

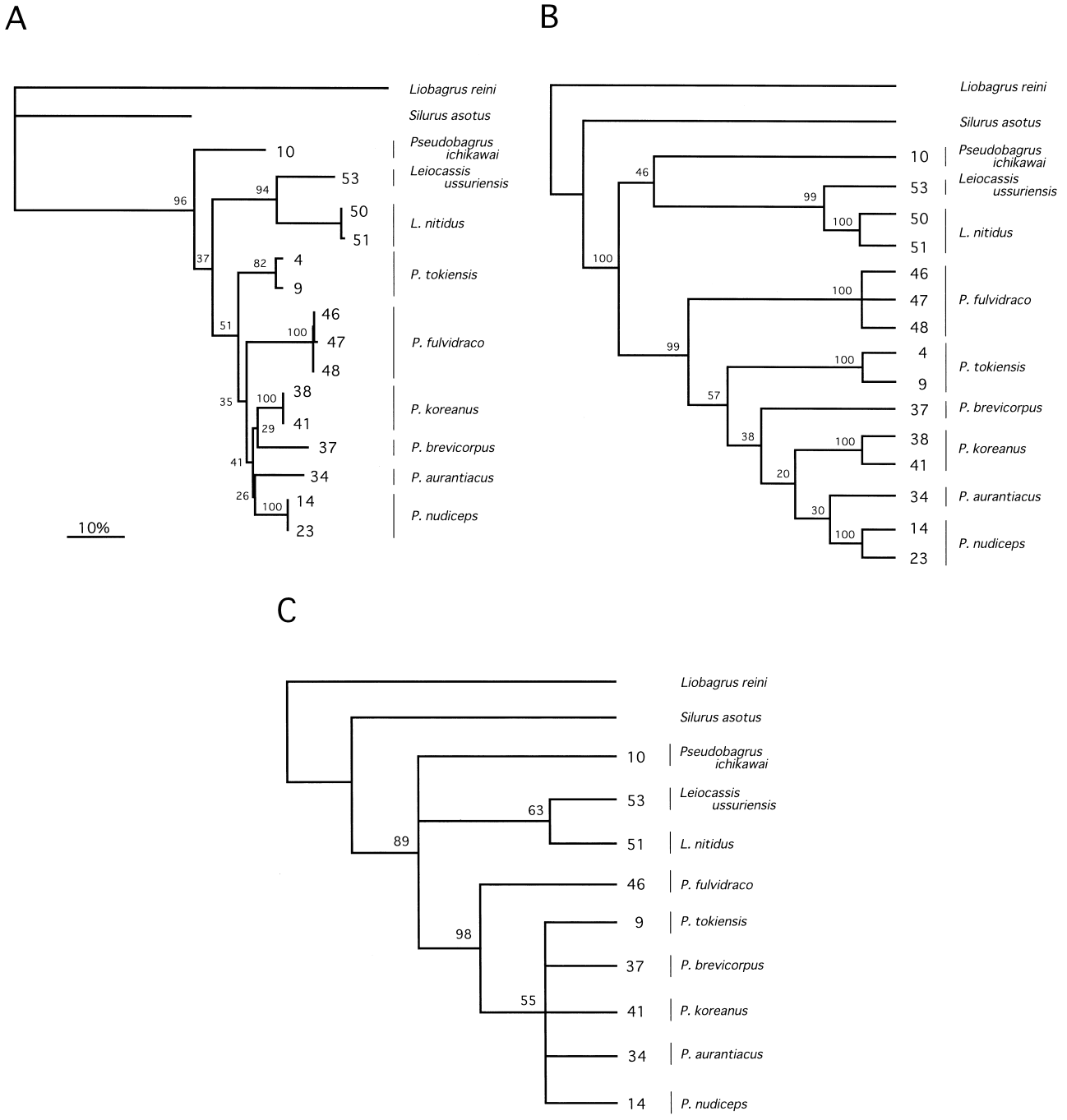

Fig. 4. Phylogenetic trees reconstructed from the sequence of part of the cytochrome *b* gene of bagrid catfishes. Numbers at end of tree forks indicate specimens used (shown in Table 3) and numbers at forks indicate bootstrap values (1,000 replicates). (**A**) NJ tree generated for the third position data sets; (**B**) Consensus tree generated from MP analysis for all the three codon positions data sets; (**C**) Consensus tree (50% majority rule consensus) generated from MP analysis for the first and second positions data sets.

specimens from the latter regions.

The stand-alone position of *P. ichikawai* on the trees determined in this study, is notable. The endemic range of *P. ichikawai* is confined to the rivers entering the Bay of Ise, such being very unusual compared with other Japanese freshwater fishes. The current study rejected the past suppositions that this species was closely-related to *P. brevicorpus* in Korea and indicated an absence of any kin relationship between the two species, with *P. ichikawai* representing an early offshoot of the species examined. It is, therefore, difficult to resolve its origin based solely upon the current data sets. A similar zoogeographical pattern, associating central Japan with the southern tip of the Korean Peninsula, is also seen in the loach genus *Niwaella* (Sawada and Kim, 1977), although lack of genetic information precludes further clarification.

Most organisms inhabiting Japan seem to have kin rela-

tionship to those on the Asian Continent. Freshwater fishes are believed to have penetrated Japan when that country and the Asian mainland were joined by land bridges during various glacial periods (Aoyagi, 1957; Mizuno, 1987). Freshwater fishes in Japan often belong to the same species with those inhabiting on the Asian Continent. This is most frequently observed between freshwater fishes in western Japan and those in Korea (Lindberg, 1972). If we simply apply the hypothesis that Japanese bagrid catfishes were also derived from the Asian Continent we can envisage the following scenario. *P. ichikawai* is the earliest of the bagrid catfishes to penetrate Japan. After *P. ichikawai*, *P. tokiensis* branched amongst the Japanese bagrid catfishes. This, as well as its northernmost endemic range, suggests *P. tokiensis* as being the next bagrid to enter Japan. Subsequently, *P. nudiceps* penetrated Japan, followed by *P. aurantiacus*, according to the evidence of both the phylogenetic trees and their endemic ranges. We should also consider the possibility of Japanese bagrid catfishes having speciated independently in the Japanese Archipelago. It requires to be accumulated further data sets for various freshwater fishes to draw a complete picture.

Although miscellaneous rates of clocks are proposed to date for variable taxa (Martin and Palumbi, 1993). Japanese and Korean bagrid catfishes are suggested as having diverged since the Miocene based on the about threefold rate of the widely-used "vertebrate clock" (2% per million years; Brown *et al.*, 1979) as only the third positions were used for the current analysis (Table 4; Kimura, 1980). It is noteworthy that the genetic divergence apparent within individual species is relatively small, even between geographically distant populations. Between the Amur River and Korean populations of *L. nitidus* and *P. fulvidraco*, the RFLP analysis, which included the rapidly evolving D-loop region, produced no different morphs (Table 3). The genetic distance values obtained from the sequencing of the cytochrome *b* gene of these species were both 0.41%. Similarly, variations were very few throughout the entire range of *P. nudiceps*. The genetic distance value, for example, between the individuals collected in Lake Biwa(St. 14) and the Asahi River (St. 23), having the same haplotype in the RFLP analysis, was 0% (Table 4). Since this may suggest the existence of slower mt DNA clocks in the bagrid catfishes which is also presumed in some other fish species (Martin and Palumbi, 1993), further investigation is required to find their time of divergence.

Recently, *P. nudiceps* has been found in rivers outside its presumed endemic range, apparently having accompanied the transplantations of Ayu (*Plecoglossus altivelis*) from Lake Biwa. It is believed that the Kiso River specimen (St. 11) examined in this study originated from such a transplantation. Regarding the recent known distribution in northeastern Kyushu (Hosoya and Yamada, 1993), Mizoiri *et al.* (1997) suggested that they represented native populations, owing to their continuous geographical distribution. However, the present results can not provide any support for this, since any unique haplotypes which distinguish them from other populations, including the populations of Lake Biwa, were not observed.

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