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

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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
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).
<p>| Species locations, including drainage system (see Fig.1), of bagrid catfish populations |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>No.</th>
<th>Sample location</th>
<th>Sample size</th>
<th>Drainage</th>
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<td><strong>L. ussuriensis</strong></td>
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<td>53</td>
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<td>3</td>
<td>Namhan R.</td>
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</table>
purified with a QIA quick spin column (Quiagen, Germany). 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.

PCR

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 5'RNA-Glu) was amplified by PCR using the following primer pairs: (5'-CAYATYMARCCGMAAATGRTAYT-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.

Sequencing

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 5'RNA-Glu) was amplified by PCR using the following primer pairs: (5'-TGAACCTGAARACCAAYGTTG-3') and (5'-RGRAAAKARRAA 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 (Qiagen, 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 cycling 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 El, 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 substitution 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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Fragment patterns (× 10 bp)</th>
<th>Enzyme</th>
<th>Fragment patterns (× 10 bp)</th>
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<td><strong>Hinf I</strong></td>
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<td>B</td>
<td>60, 60, 34, 25, 11</td>
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<tr>
<td>C</td>
<td>76, 68, 38, 10</td>
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</table>

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

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Species</th>
<th>Composite fragment pattern</th>
<th>N</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Pseudobagrus nudiceps</em></td>
<td>AAAAAAAAAAAAAAAAA</td>
<td>42</td>
<td>11, 12, 13(2), 14(2)<em>, 15, 16, 17, 18, 19, 20, 21, 22, 23</em>, 24, 25, 26, 27, 28, 29, 30, 31, 32</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>AAAAAAAAABAAAA</td>
<td>2</td>
<td>13(1), 14(1)</td>
</tr>
<tr>
<td>3</td>
<td><em>P. tokienis</em></td>
<td>BAAAABACBCAAC</td>
<td>14</td>
<td>1, 2, 3, 4*, 5, 6</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>BAAAAABAAABCAAC</td>
<td>7</td>
<td>7(2), 8(2), 9*</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>BAAAABABBCAAC</td>
<td>1</td>
<td>7(1)</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>BAAABAAABBCAAC</td>
<td>1</td>
<td>8(1)</td>
</tr>
<tr>
<td>7</td>
<td><em>P. aurantiacus</em></td>
<td>AAAACABADCBDBAB</td>
<td>12</td>
<td>33, 34*, 35, 36</td>
</tr>
<tr>
<td>8</td>
<td><em>P. ichikawai</em></td>
<td>DGDHACGBIEBNGAC</td>
<td>1</td>
<td>10(1)</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>DGDHACGBIEBBOGAC</td>
<td>2</td>
<td>10(2)*</td>
</tr>
<tr>
<td>10</td>
<td><em>P. koreanus</em></td>
<td>ACBFAACAEABEDAD</td>
<td>2</td>
<td>41*, 42</td>
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<tr>
<td>11</td>
<td>—</td>
<td>ACAFAAECABEED</td>
<td>2</td>
<td>40(1),</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>ACBFAACAEABFED</td>
<td>6</td>
<td>38(2)*, 39, 40(2)</td>
</tr>
<tr>
<td>13</td>
<td>—</td>
<td>ACBFAACAEABFEAB</td>
<td>1</td>
<td>38(1)</td>
</tr>
<tr>
<td>14</td>
<td><em>P. fulvidraco</em></td>
<td>EEADAFAFGDCICBA</td>
<td>11</td>
<td>43, 44(2), 45, 46*, 48*</td>
</tr>
<tr>
<td>15</td>
<td>—</td>
<td>EEADAFAFGDCJCB</td>
<td>3</td>
<td>47*</td>
</tr>
<tr>
<td>16</td>
<td>—</td>
<td>EEADAFAFGDMCBA</td>
<td>1</td>
<td>44(1)</td>
</tr>
<tr>
<td>17</td>
<td><em>P. brevicorpus</em></td>
<td>ABABAAACABAPAAC</td>
<td>2</td>
<td>37(2)*</td>
</tr>
<tr>
<td>18</td>
<td>—</td>
<td>ABABAAACJBAAPAAC</td>
<td>1</td>
<td>37(1)</td>
</tr>
<tr>
<td>19</td>
<td>Leiocassis nitidus</td>
<td>CFCEACEAHFALBAE</td>
<td>2</td>
<td>50(1), 51(1)*</td>
</tr>
<tr>
<td>20</td>
<td>—</td>
<td>CFCEACEAHFAGBAE</td>
<td>6</td>
<td>49, 50(1)*, 51(2)</td>
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<tr>
<td>21</td>
<td>—</td>
<td>CFCEACEAHFAHBAE</td>
<td>1</td>
<td>50(1)</td>
</tr>
<tr>
<td>22</td>
<td><em>L. ussuriensis</em></td>
<td>BBGADDAGFKFAC</td>
<td>6</td>
<td>52, 53*</td>
</tr>
</tbody>
</table>


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).
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, was made for the first and second positions of the root in the former trees are reasonable.

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. breviceps* 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

### 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.

<table>
<thead>
<tr>
<th>Species</th>
<th><em>Pseudobagrus</em></th>
<th><em>P. tokiensis</em></th>
<th><em>P. aurantiacus</em></th>
<th><em>P. ichikawai</em></th>
<th><em>P. koreanus</em></th>
<th><em>P. fulvidraco</em></th>
<th><em>P. breviceps</em></th>
<th><em>Leiocassis</em></th>
<th><em>L. nitidus</em></th>
<th><em>Silurus</em></th>
<th><em>Liobagrus</em></th>
<th><em>L. reini</em></th>
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</thead>
<tbody>
<tr>
<td><em>P. nudiceps</em></td>
<td>—</td>
<td>.154</td>
<td>.146</td>
<td>.317</td>
<td>.116</td>
<td>.195</td>
<td>.157</td>
<td>.304</td>
<td>.405</td>
<td>.801</td>
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<td>(.000–.021)</td>
<td>(.151–.157)</td>
<td>(.228–.238)</td>
<td>(.203–.209)</td>
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<td>.381</td>
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<td>.136</td>
<td>.223</td>
<td>.322</td>
<td>.358</td>
<td>.386</td>
<td>.796</td>
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<td>.384</td>
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<td>(.392–.399)</td>
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<td>(.217–.223)</td>
<td>(.217–.223)</td>
<td>(.217–.223)</td>
<td>(.217–.223)</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
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<td>(.217–.223)</td>
<td>(.217–.223)</td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Ranges are shown in parentheses. The differences of this tree from the NJ tree are (1) the branching order of *P. ichikawai* and *Leiocassis* species. 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.

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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.

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 phyllogenetic 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 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.

**ACKNOWLEDGMENTS**

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