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Phylogenetic Relationships and Intraspecific Variations of Loaches of the Genus *Lefua* (Balitoridae, Cypriniformes)

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ABSTRACT—Three nominal species are known in East Asian balitorid loaches of the genus *Lefua*, i.e. *L. echigonia*, *L. nikkonis*, and *L. costata*. *Lefua echigonia*, with large morphological variations was recently separated into two groups, *L. echigonia* including the holotype and *L.* sp., based on morphological and ecological traits. We performed protein and DNA analyses to elucidate phylogenetic relationships among loaches of the genus *Lefua* and to settle the taxonomic status of *L.* sp. We also investigated intraspecific variations in *L. echigonia* s. str. to shed light on the process of formation of freshwater fish fauna in Japan.

Protein analyses using two-dimensional gel electrophoresis showed that genetic distances between *L.* sp. and *L. echigonia* s. str. and between *L.* sp. and *L. nikkonis* were as large as that between *L. echigonia* s. str. and *L. nikkonis*. DNA analyses of the mitochondrial D-loop region showed that *L.* sp. and *L. echigonia* s. str. were monophyletic, respectively, while neither *L. nikkonis* nor *L. costata* was monophyletic and these species formed together a clade. The results supported the specific status of *L.* sp. and proposed reevaluation of the taxonomic status of *L. nikkonis* and *L. costata*. DNA analyses also showed that *L.* sp. was more closely related to *L. echigonia* s. str. than to the *L. nikkonis-L. costata* complex, and four local populations were distinguished in *L. echigonia* s. str. Distribution patterns of the four local populations of *L. echigonia* s. str. in Japan were approximately congruent with those of the medaka, *Oryzias latipes*, suggesting that differentiation in the two distantly related fishes have a common historical background.

Key words: intraspecific variation, two-dimensional gel electrophoresis, D-loop, mitochondria, phylogeography

INTRODUCTION

Three nominal species are known in the genus *Lefua* Herzenstein, 1888 of the family Balitoridae, Cypriniformes (Nelson, 1994). *Lefua costata* (Kessler, 1876) is distributed widely in East Asia including Korea, China, and Russia, but not in Japan. *Lefua nikkonis* (Jordan and Fowler, 1903) is naturally endemic to Hokkaido Island of Japan and has been introduced in restricted areas of Honshu Island of Japan. *Lefua echigonia* Jordan and Richardson, 1907 which was known to have large morphological variations among local populations (Fujita and Okawa, 1975), was considered to inhabit widely from the Tohoku district except for Aomori prefecture to the Kinki district in Honshu Island and also in eastern Shikoku Island. However, it was shown by recent morphological and ecological studies that conventional *L. echigonia* could be separated into two groups. The group distributed from Wakayama to Okayama prefectures in Honshu Island with an isolated distribution in Aichi and Shizuoka prefectures and also in eastern Shikoku Island was given the Japanese name “Nagare-hotoke-dojo”, assuming that it is specifically distinct from the rest (Hosoya, 1993). By principal component analysis, this group (*L.* sp.) was distinguished from the other group (i. e. *L. echigonia* including the holotype, referred to as *L. echigonia* s. str. below) by the length between the dorsal and ventral fins, the snout length, the body height, and the body width (Hosoya, 1994). The specific status of *L.* sp. was also suggested in terms of habitat segregation from *L. echigonia* s. str. (Yamashina *et al.*, 1994). *Lefua* sp. lives in relatively fast-flowing streams with gravelly beds, while *L. echigonia* s. str. inhabits relatively

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slow-flowing streams with muddy beds. However, genetic divergence between *L.* sp. and *L. echigonia* s. str. and interspecific and intraspecific relationships of loaches of the genus *Lefua* remain to be tested in order to confirm the taxonomic status of *L.* sp.

In this study, we investigated the phylogenetic relationships among loaches of the genus *Lefua* and intraspecific variations of *L. echigonia* s. str. by two different approaches, comprehensive protein analysis by two-dimensional gel electrophoresis and DNA analysis by sequencing the mitochondrial D-loop region.

Two-dimensional gel electrophoresis (2D electrophoresis) was developed by O'Farrell (1975) and improved by Hirabayashi (1981). This method separates proteins by their differences in isoelectric points and molecular weights, and allows us to compare numerous proteins simultaneously. Comparison of hundreds of proteins on 2D electrophoresis patterns can reduce the deviation caused by sampling of a small number of particular genetic characters. This technique can assess the comprehensive genetic divergence in the nuclear genome (Brown and Langley, 1979). Taking advantage of this method, earlier studies revealed the usefulness of this method for phylogenetic analysis (Aquadro and Avise, 1981; Goldman *et al.*, 1987; Miyazaki *et al.*, 1987; Miyazaki *et al.*, 1988; Spicer, 1988). The efficacy was also supported by recent studies, which showed that the results obtained by 2D electrophoresis agreed well with consensus phylogenetic relationships proposed by other techniques (Spicer, 1991; Tsubokawa and Miyazaki, 1993; Miyazaki *et al.*, 1998; Tokita *et al.*, 2002).

The analysis of the mtDNA sequence is useful for the investigation of gene flow among populations because of the maternal inheritance of mitochondria. The applicability of advanced statistical analyses to the sequence data is also an advantage of this method. The method has been applied to study geographic variations of genotypes to reconstruct the process of intraspecific diversification (Tsuda *et al.*, 1997; Miyake *et al.*, 2001; Okazaki *et al.*, 2002). The displacement-loop (D-loop), which is the non-protein-coding control region of the mitochondrial genome, is useful for phylogenetic analysis of intraspecific genetic relationships because of its high evolutional rate (Shedlock *et al.*, 1992).

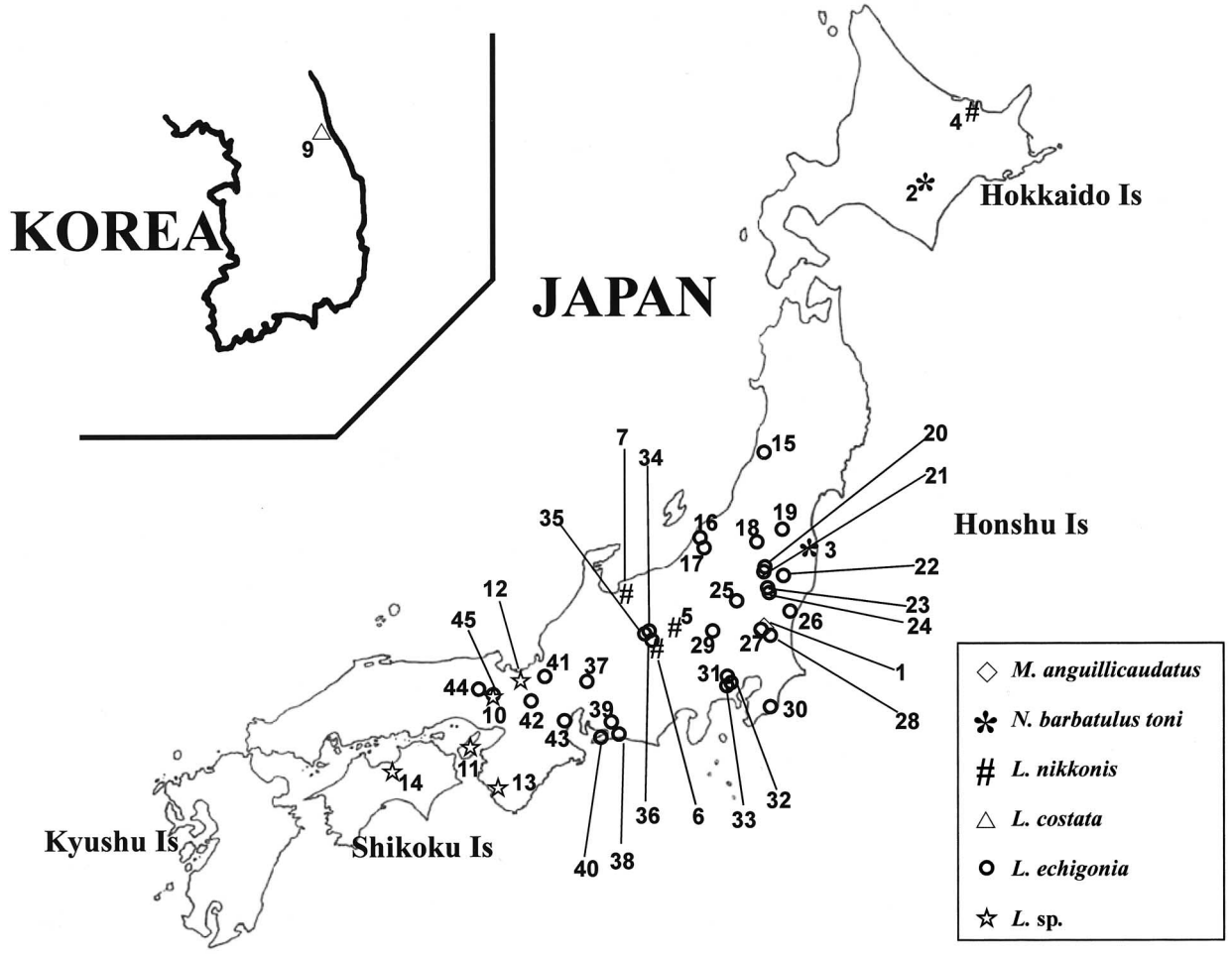


Fig. 1. Collection sites of loaches. ○, *Lefua echigonia* s. str.; ☆, *L.* sp.; #, *L. nikkonis*; △, *L. costata*; ⁎, *Noemacheilus barbatulus toni*; ◇, *Misgurnus anguillicaudatus*. *Lefua nikkonis* and *N. barbatulus toni* are naturally endemic to Hokkaido Island and have been introduced in restricted areas of Honshu Island. Refer to Table 1 for details of the collection sites and sample numbers.

Table 1. Sample list

#	species	collection site	analyses
1	*Misgurnus anguillicaudatus*	YASATO, IBARAKI	P
2	*Noemacheilus barbatulus toni*	SHIKAOI, HOKKAIDO	P
3		NAMIE, FUKUSHIMA*	N
4	*Lefua nikkonis*	MEMANBETSU, HOKKAIDO	N/P
5		UEDA, NAGANO*	N
6		MIDORIKO, YAMANASHI*	N
7		KUROBE, TOYAMA*	N
8	*L. costata*	(KOREA)**	N
9		NAMDAE(KOREA)**	N
10	*L. sp.*	KASUGA, HYOGO	N/P
11		SUMOTO, HYOGO	N
12		NADASHO, FUKUI	N
13		NAKATSU, WAKAYAMA	N
14		IYOMISHIMA, EHIME	N
15	*L. echigonia s. str*	HIRATA, YAMAGATA	N
16		TERADOMARI, NIIGATA	N/P
17		NAGAOKA, NIIGATA	N
18		SHIOKAWA, FUKUSHIMA	N
19		FUKUSHIMA, FUKUSHIMA	N
20		TEN-EI, FUKUSHIMA	N
21		DAISHIN, FUKUSHIMA	N
22		ISHIKAWA, FUKUSHIMA	N
23		NASU, TOCHIGI	N
24		KUROBANE, TOCHIGI	N/P
25		IMAICHI, TOCHIGI	N
26		HITACHI, IBARAKI	N/P
27		YASATO, IBARAKI	N/P
28		ISHIOKA, IBARAKI	N
29		YOSHII, GUNMA	N
30		ONJUKU, CHIBA	N
31		ASAKAWA, TOKYO	N
32		IKUTARYOKUCHI, KANAGAWA	N
33		ZAMA, KANAGAWA	N
34		AZUSAGAWA, NAGANO	N
35		MATSUMOTO, NAGANO	N
36		SHIOJIRI, NAGANO	N
37		GIFU, GIFU	N
38		KOSAI, SHIZUOKA	N
39		SHINSHIRO, AICHI	N
40		ATSUMI, AICHI	N/P
41		NISHIASAI, SHIGA	N
42		SHIGA, SHIGA	N
43		ISE, MIE	N
44		AOGAKI, HYOGO	N/P
45		KASUGA, HYOGO	P

#, sample number; *, introduced from Hokkaido; **, obtained from a commercial source; P, 2D gel electrophoresis ; N, sequencing of the mitochondrial D-loop region.

Our results show that *L.* sp. comprises a monophyletic group that is divergent from monophyletic *L. echigonia* s. str., while neither *L. nikkonis* nor *L. costata* is monophyletic. Our results also show that *L.* sp. is more closely related to *L. echigonia* s. str. than to *L. nikkonis* and *L. costata*, and that *L. echigonia* s. str. has high intraspecific variations and four local populations are distinguished on the basis of genetic differentiation.

MATERIALS AND METHODS

Materials

Locations where specimens of *Lefua echigonia* s. str., *L. nikkonis*, and *L.* sp. were collected are shown on the map (Fig. 1) and sample numbers are described in the sample list (Table1). *Misgurnus anguillicaudatus* of the family Cobitidae was used as an outgroup for protein analyses by 2D electrophoresis, because balitorid species except for *Lefua* spp. were not available earlier in this study. Thereafter, *Noemacheilus barbatulus toni* of the family Balitoridae was used for comparison of genetic distances obtained by protein analyses and also used as an outgroup for mtDNA analyses. Two specimens of *L. costata* were obtained from commercial sources. Both of them were collected in Korea, but the exact sampling location of one of the specimens is not known. Collection sites and sample numbers of *M. anguillicaudatus*, *N. barbatulus toni*, and *L. costata* are also shown (Fig.1 and Table 1). *L. echigonia* and *L.* sp. were sympatric only in Kasuga, Hyogo Prefecture.

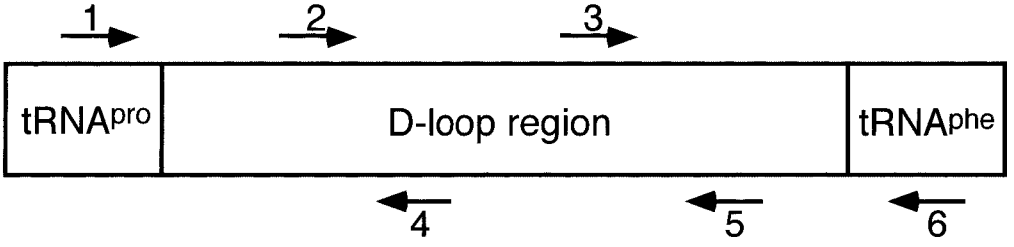
Protein analyses by 2D electrophoresis

Livers were dissected out from loaches and used for protein analyses. Two-dimensional gel electrophoresis was carried out as described previously by Hirabayashi (1981) and Oh-ishi and Hirabayashi (1988). Proteins were extracted by homogenizing the livers from 1 to 3 individuals in 20 volumes of a medium containing 5 M urea and 2 M thiourea. After centrifuging at 60,000 x g, the supernatant was subjected to isoelectric focusing of the first dimension for 13,500 V • h. SDS-polyacrylamide gel electrophoresis of the second dimension was performed using the running gel of 12% acrylamide and the stacking gel of 3% acrylamide. Proteins were stained with Coomassie brilliant blue in picrate as described by Stephano *et al.* (1986).

Electrophoretic patterns were compared visually by the triplet method (Miyazaki *et al.*, 1987), in which three patterns derived from two different samples (60 μl each) and their mixture (40+40 μl) were prepared and compared with one another to examine whether the protein spots overlapped or not.

Genetic distances were calculated according to the formula of Aquadro and Avise (1981): D=1–2Nxy/(Nx+Ny), where D is the genetic distance between specimens x and y, Nxy is the number of protein spots shared by x and y, and Nx and Ny are the numbers of protein spots scored for x and y, respectively. Based on genetic distances, two dendrograms were constructed according to the UPGMA (Sneath and Sokal, 1973) and NJ (Saitou and Nei, 1987) methods. In order to construct the NJ tree, NEIGHBOR in PHYLIP was used (Felsenstein, 1994).

To evaluate the genetic divergence between *L. echigonia* s. str. and *L.* sp. and also intraspecific genetic variations in *L. echigonia* s. str., genetic distances at the familial, generic, specific, and intraspecific levels were obtained. For this purpose, specimens of *L. echigonia* s. str. from Aogaki were used as standard counterparts for comparisons. *M. anguillicaudatus, N. barbatulus toni,* and *L. nikkonis* were used for comparisons at the familial, generic, and specific levels, respectively. Specimens of *L. echigonia* s. str. obtained at 7 sites were used for comparisons at the intraspecific



#	Primer	sequence
1.	Pro S	GCATCGGTCTTGTAATCCGAAGAT
2.	296 S	ATATATTAATGTAGTAAGAAACCACCAACCAG
3.	651 S	TCAACACATCCTTATACTATATGCC
4.	334AS	ATATATCACCTTCCACTTATGTCCC
5.	194AS	ACATTAATAAAACTCGTTAATTTTATTGCGCTC
6.	Phe AS	GGACCAAGCCTTTGTGCATGCGGAG

Fig. 2. Primers for sequencing the mitochondrial D-loop region. Three forward (Pro S, 296 S, and 651 S) and three reverse (334 AS, 194 AS, and Phe AS) primers were constructed. The Pro S and Phe AS primers were designed according to sequences of tRNA ^{pro} and tRNA ^{phe} of the loach (*Crossostoma lacustre*) and the carp (*Cyprinus carpio*). The others were designed according to the consensus sequence determined in this study for some specimens of *Lefua echigonia* s. str.

level (Table 1).

DNA analyses by sequencing of the mitochondrial D-loop region

Back muscles were cut off from the loaches. The muscle from each individual was homogenized in 250 μl of a proteinase solution (0.1 mg/ml proteinase K, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 0.45% NP-40, 0.45% Tween 20, and 10 mM Tris-HCl • pH 8.0) and placed at 37°C overnight or at 55°C for 2 hr. The resulting solution was extracted with phenol, phenol/chloroform, and chloroform. The supernatant was precipitated with the same volume of 4 M ammonium acetate and 4 volumes of 95% ethanol. The pellet was washed with 70% ethanol, dried *in vacuo,* and dissolved in 100 μl of TE buffer.

For amplification of fragments containing the D-loop region, PCR was carried out in 100 μl of a solution containing KOD dash (TOYOBO) with 30 cycles of 30 sec denaturation at 94°C, 5 sec annealing at 62°C, and 30 sec extension at 74°C using total DNA as a template. Six primers (Fig. 2) were designed according to mtDNA sequences of the carp (*Cyprinus carpio*) and the loach (*Crossostoma lacustre*). The amplified fragment was purified by phenol/chloroform extraction and ethanol precipitation as described above or by filtration through a QIA quick column (QIAGEN). Direct sequencing of the purified double-strand PCR product was per-

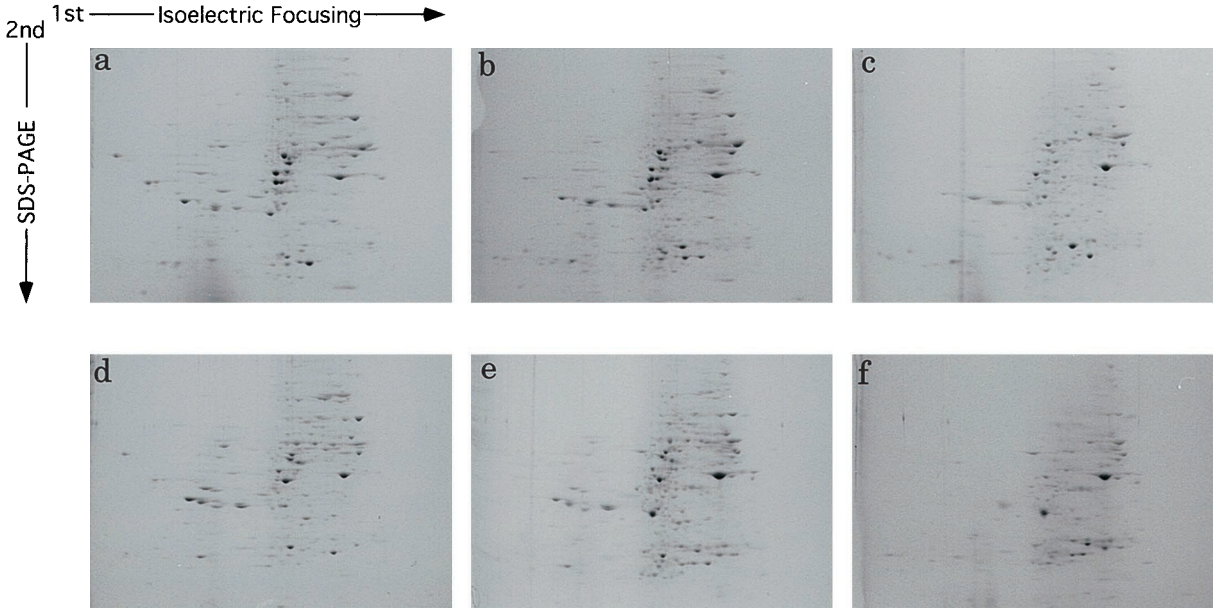


Fig. 3. Representative two-dimensional gel electrophoresis patterns. Liver protein constituents were compared by two-dimensional gel electrophoresis. The triplet method was used for comparisons of electrophoretic patterns. For example, *Lefua nikkonis* (a) was compared with *L.* sp. (c) through a mixture pattern from both specimens (b). *Lefua echigonia* s. str. (d) was compared with *Misgurnus anguillicaudatus* (f) through a mixture pattern from both specimens (e).

Table 2. Genetic distances among loaches obtained by 2D electrophoresis

	Lec(Ao)	Lec(Ka)	Lec(At)	Lec(Ya)	Lsp	Lni	Nto	Man	Lec(Ku)	Lec(Hi)	Lec(Te)
Lec(Ao)	–	0.089	0.166	0.226	0.239	0.224	0.335	0.368			
Lec(Ka)	598	–									
Lec(At)	529		–	0.190							
Lec(Ya)	386			0.050					0.081	0.158	0.252
			489	558							
Lsp	407				–	0.207					
Lni	552				455	–					
Nto	385						–				
Man	370							–			
Lec(Ku)				519					–		
Lec(Hi)				475						–	
Lec(Te)				737							–

Genetic distances (above the diagonal) were calculated according to the formula of Aquadro and Avise (1981). The total numbers of protein spots for comparisons of specified pairs are given below the diagonal. Lec, *Lefua echigonia* s. str.; Lsp, *L.* sp.; Lni, *L. nikkonis*; Nto, *Noemacheilus barbatulus toni*; Man, *Misgurnus anguillicaudatus*. Collection sites of *L. echigonia* s. str. are denoted in parentheses; Ao, Aogaki; Ka, Kasuga; At, Atsumi; Ya, Yasato; Ku, Kurobane; Hi, Hitachi; Te, Teradomari. Genetic distances in *Lefua echigonia* s. str. are underlined.

formed using an ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on a Model 377 DNA sequencer (Applied Biosystems) according to the manufacturer's directions.

DNA sequence data were edited with DNASIS (Hitachi Software Engineering), aligned using DNASIS or CLUSTAL W, and corrected by visual inspection for phylogenetic analyses. Dendrograms were constructed based on genetic distance (NJ tree) and character-state (MP tree) matrices. Genetic distances were computed by Kimura's two-parameter method (Kimura, 1980) and the NJ tree was depicted with CLUSTAL W. The MP tree was depicted with PAUP (Swofford, 1993), and the majority-rule consensus tree based on 1000 bootstrap replicates was produced.

DNA sequence data showed nucleotide deletions especially in 5' and 3' portions of the mitochondrial D-loop region. Deletion patterns were also used to construct the dendrogram. First, we searched for more than four missing contiguous bases shared by at least two specimens. Next, we searched for 2 or 3 base deletions in the segments where deletions of more than 4 bases were found at the first step. Deletion patterns were compiled and the majority-rule consensus MP tree based on 1000 bootstrap replicates was produced.

RESULTS

Protein analyses by 2D electrophoresis

Representative 2D electrophoresis patterns are shown for comparisons between *Lefua nikkonis* and *L.* sp. (Fig. 3a–c) and between *L. echigonia* s. str. and *Misgurnus anguillicaudatus* (Fig. 3d–f). The triplet method was used to compare the electrophoretic patterns of the loach livers. In addition to patterns of each sample (a, c, d, and f in Fig. 3), a mixture pattern of samples from two specimens to be compared (b and e in Fig. 3) was indispensable for precise comparison. Thirteen sets of triplet patterns were prepared and the average of 497 (370 to 737) protein spots on those patterns was compared (Table 2).

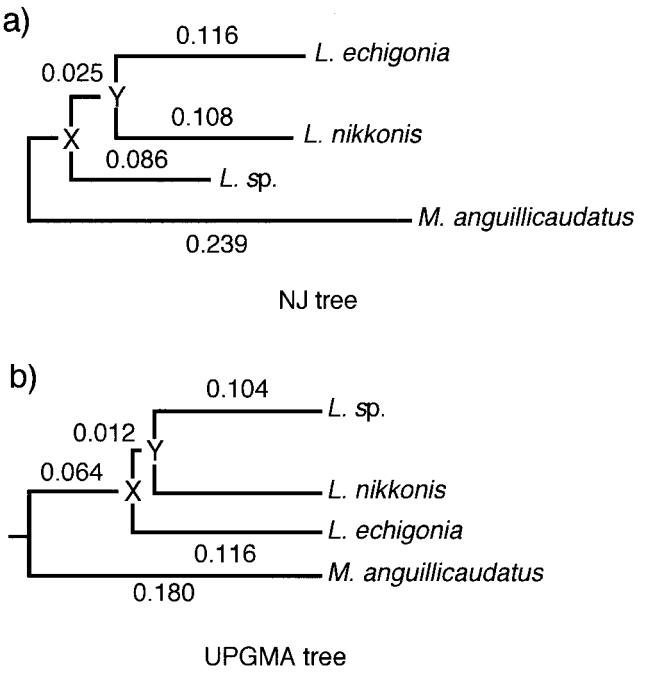


Fig. 4. Phylogenetic relationships of loaches of the genus *Lefua* inferred by protein analyses. Two dendrograms were generated by NJ (a) and UPGMA (b) methods using *Misgurnus anguillicaudatus* as an outgroup. X, branching point leading to the ancestor of the most closely related species and also to the other *Lefua* species; Y, branching point leading to two species most closely related.

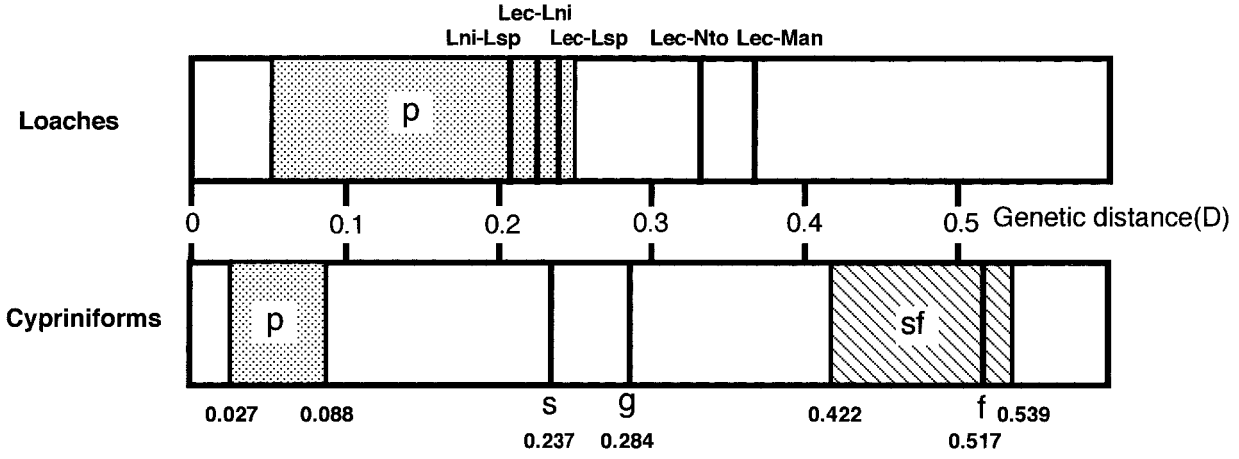


Fig. 5. Large genetic variations in *Lefua echigonia* s. str. Genetic distances at the specific, generic, and familial levels were compared by two-dimensional gel electrophoresis, using *Lefua echigonia* s. str. from Aogaki and *Acheilognathus tabira* as the standard counterparts in loaches (upper) and cypriniform fishes (lower), respectively. Intraspecific genetic distances in *L. echigonia* s. str. (p in the upper scheme) presented extraordinarily high intraspecific genetic variations (0.050–0.252). Those in *A. tabira* (p in the lower scheme) were 0.027 to 0.088 and usually below 0.11 in various animals. Genetic distances at the specific level were 0.207 to 0.239 (including that between *L. nikkonis* and *L.* sp.) in loaches, which were close to 0.237 between *A. tabira* and *A. rhombeus* (s in the lower scheme). Genetic distances increased at the generic (0.335) and familial (0.368) levels in loaches as previously shown between *A. tabira* and *Rhodeus ocellatus ocellatus* (0.284, g in the lower scheme) and between cyprinid *A. tabira* and cobitid *Misgurnus anguillicaudatus* (0.517, f in the lower scheme). Those at the subfamilial level ranged from 0.422 to 0.539 in cyprinid fishes (sf in the lower scheme). Lni-Lsp, *L. nikkonis* vs *L.* sp.; Lec-Lni, *L. echigonia* s. str. vs *L. nikkonis*; Lec-Lsp, *L. echigonia* s. str. vs *L.* sp.; Lec-Nto, *L. echigonia* s. str. vs *Noemacheilus barbatulus toni*; Lec-Man, balitorid *L. echigonia* s. str. vs cobitid *M. anguillicaudatus*.

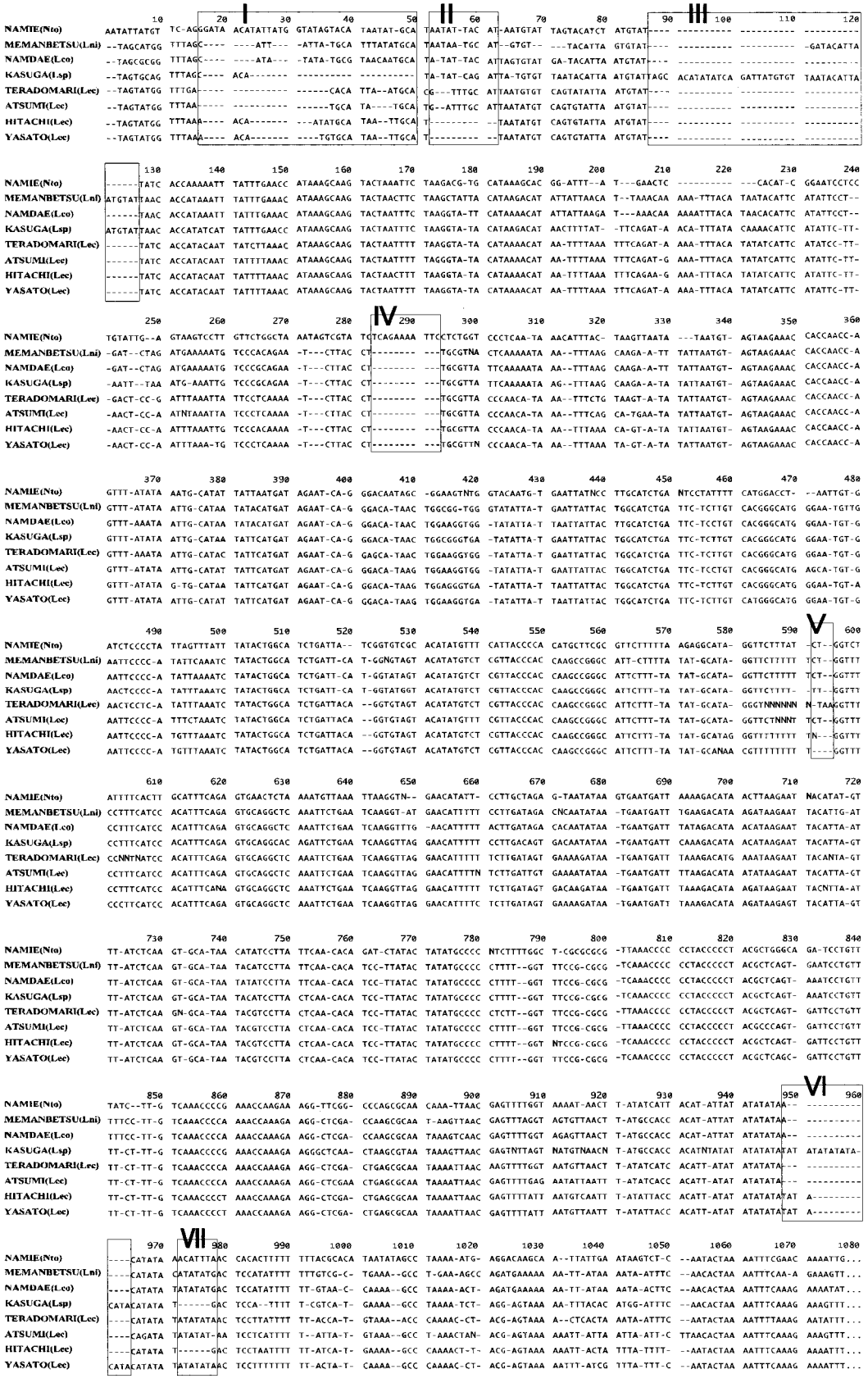


Fig. 6. Representative DNA sequences of the mitochondrial D-loop region. Four representative sequences for *L. echigonia* s. str. and one each of sequences for the remaining species are shown. The segments including the deletions are indicated (see Table 4).

Based on the comparisons of 2D electrophoresis patterns, genetic distances (Aquadro and Avise, 1981) were calculated. NJ and UPGMA trees were constructed using *M. anguillicaudatus* as an outgroup (Fig. 4). In the NJ tree (Fig. 4a), *L. nikkonis* was more closely related to *L. echigonia* than to *L.* sp. In the UPGMA tree, *L. nikkonis* was more closely related to *L.* sp. rather than to *L. echigonia*. The topologies of the trees depicted by the two different methods were inconsistent, failing to define the unequivocal branching order of three species of the genus *Lefua*. However, the trees indicated the trichotomous nature in diversification of the three species, because the node (Y in Fig. 4) leading to the most closely related species was positioned very closely to the node (X) leading to a presumptive ancestor of the two species and to the other species in either tree. The results support the specific status of *L.* sp.

In the process of this study, we realized that there were large genetic variations of *L. echigonia* s. str. and thus compared intraspecific genetic distances in *L. echigonia* s. str. with genetic distances at the specific, generic, and familial levels by 2D electrophoresis. Specimens of *L. echigonia* s. str. obtained from 7 different localities, two other species of the genus *Lefua* (*L.* sp. and *L. nikkonis*), *Noemacheilus barbatulus toni* (Balitoridae), and *M. anguillicaudatus* (Cobitidae) were used for comparisons (Fig. 5 and Table 2). The genetic distances were 0.207-0.239, 0.335, and 0.368 at the specific, generic, and familial levels, respectively. Thus, they increased according to the taxonomic levels as shown by the previous study on cypriniform fishes (Miyazaki *et al.*, 1998). On the other hand, the genetic distances at the intraspecific level ranged from 0.050 to 0.252 in comparisons of 8 pairs of *L. echigonia* s. str. specimens. Some of those values were extraordinarily high (normally below 0.11 in various animals at the intraspecific level) and exceeded those at the specific level. The results highlight the need for more extensive surveys including *L. echigonia* s. str. specimens from many different localities in order to elucidate the phylogenetic relationships of loaches of the genus *Lefua.*

DNA analyses by sequencing of the mitochondrial D-loop region

To investigate further the phylogenetic relationships of loaches of the genus *Lefua* and also intraspecific variations of *L. echigonia* s. str., we compared sequences of the mitochondrial D-loop region, because DNA sequencing is more suitable than 2D electrophoresis to analyze a large number of specimens from different localities. Sequences were determined for 30 specimens of *L. echigonia* s. str., 5 spec-

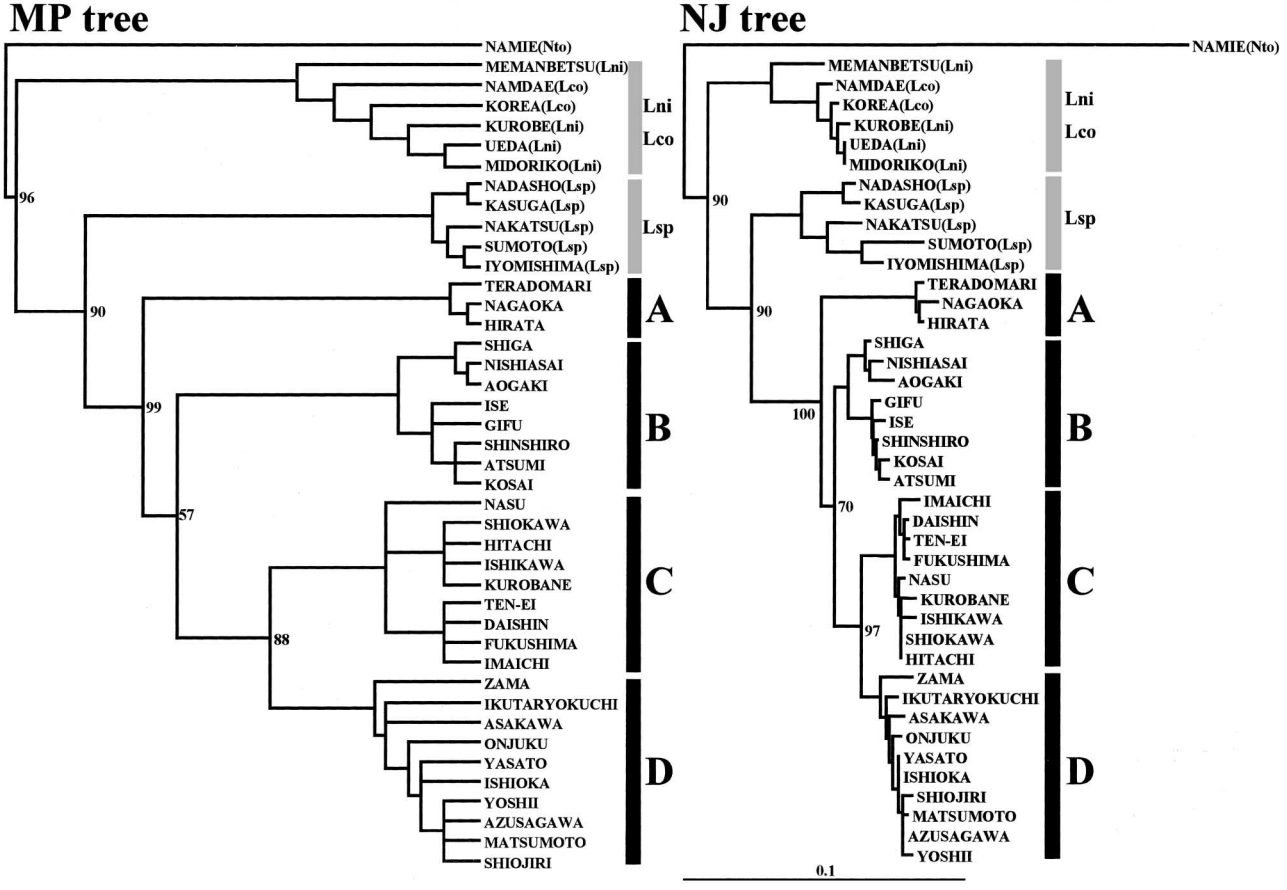


Fig. 7. Phylogenetic relationships of loaches of the genus *Lefua* inferred by DNA sequence analyses. Two dendrograms were generated by MP and NJ methods using *Noemacheilus barbatulus toni* as an outgroup. In both dendrograms, 1000 bootstrap replicates were computed and probabilities (when exceeded 50%) are denoted at the major branching points.

imens of *L.* sp., 4 specimens of *L. nikkonis*, 2 specimens of *L. costata*, and one specimen of *N. barbatulus toni*. Four representative sequences for *L. echigonia* s. str. and one each of sequences for the remaining species were aligned (Fig. 6). The sequence data is deposited in DDBJ, EMBL, and GenBank databases under accession numbers ABI102809-102850. The length of the sequences varied among species and even within species due to several deletions (or insertions), and thus 618 nucleotides excluding deletions, gaps, and ambiguous sites were used for constructing NJ and MP dendrograms derived from genetic distance and character-state matrices, respectively. Out of 618 sites, 252 were variable. Variations were localized mainly in the 5' and 3' portions of the sequences and the central portion was relatively conserved as reported previously (Lee *et al.*, 1995; Shedlock *et al.,* 1992). The NJ and MP trees using *N. barbatulus toni* as an outgroup presented fundamentally the same topologies (Fig. 7). There were three major clusters; the first including *L. nikkonis* and *L. costata*, the second including all the specimens of *L.* sp., and the third including

Table 3. Intraspecific and interspecific genetic distances of loaches of the genus *Lefua*

	Lni-Lco	Lsp	Lec	Lni-Lco vs Lsp	Lni-Lco vs Lec	Lsp vs Lec				
Average	0.021	0.044	0.038	0.100	0.130	0.107				
Range	0.000–0.047	0.010–0.070	0.000–0.081	0.078–0.125	0.109–0.143	0.089–0.151				
	A	B	C	D	A vs B	Avs C	A vs D	B vs C	B vs D	C vs D
Average	0.008	0.017	0.008	0.010	0.058	0.073	0.071	0.045	0.043	0.033
Range	0.005–0.010	0.005–0.033	0.000–0.016	0.000–0.023	0.050–0.066	0.068–0.081	0.064–0.081	0.038–0.054	0.032–0.051	0.026–0.042

Genetic distances were calculated by Kimura's two-parameter method. A to D denote subclusters in *L. echigonia* s. str. Other abbreviations are as in Table 2.

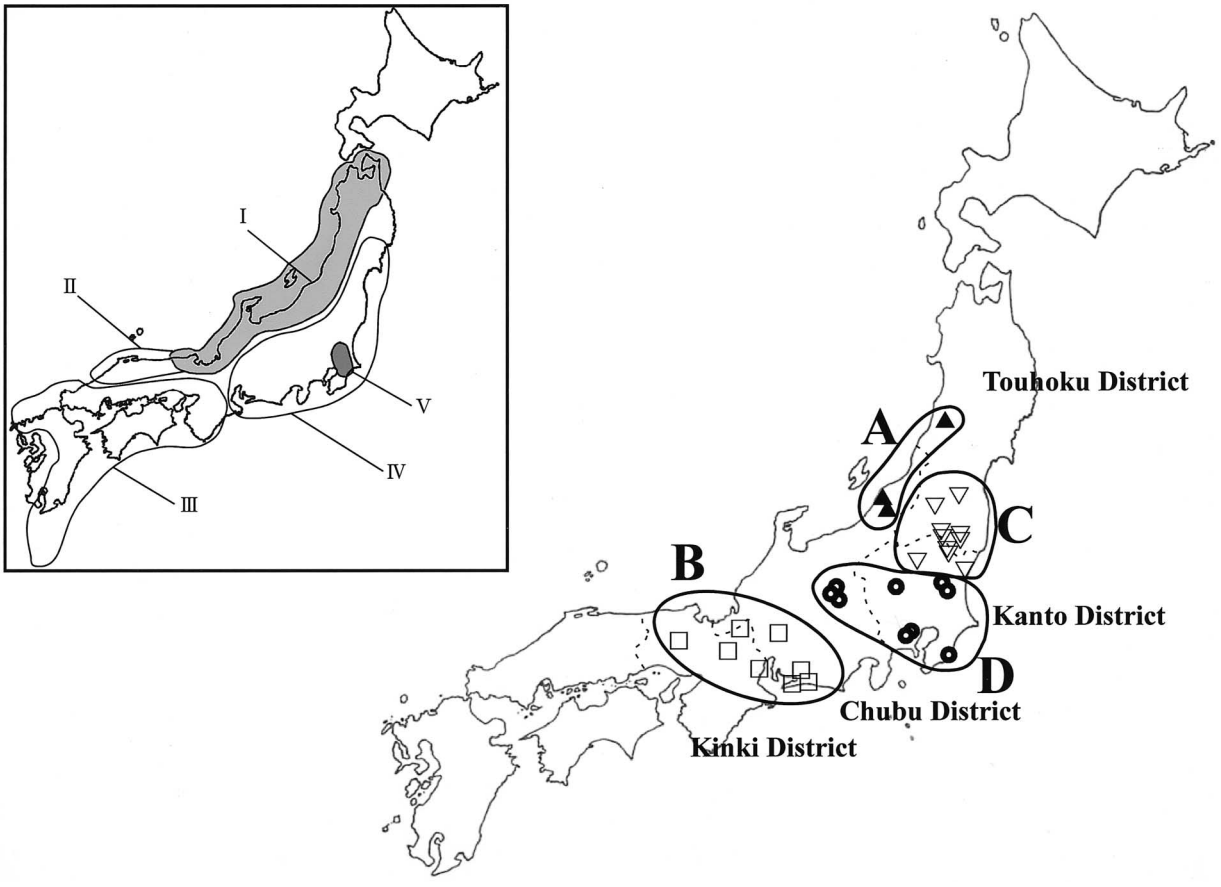


Fig. 8. Distributions of specimens included in subclusters A to D of *Lefua echigonia* s. str. ▲, Subcluster A; □, subcluster B; ▽, subcluster C; ○, subcluster D. Inset shows distributions of populations of beloniform freshwater fish, *Oryzias latipes*. Two populations, Northern Japan population and Southern Japan population, were identified by genetic studies (Sakaizumi, 1986; Matsuda *et al.*, 1997). The latter was further divided into several subpopulations. I, Northern Japan population; II, Sanin subpopulation; III, Setouchi subpopulation; IV, Eastern Japan subpopulation; V, Mouka subpopulation.

all the specimens of *L. echigonia* s. str. Surprisingly, the trees showed that neither *L. nikkonis* nor *L. costata* was monophyletic and these species together comprised a clade. The trees also showed that *L.* sp. was more closely related to *L. echigonia* s. str. than to the clade consisting of *L. nikkonis* and *L. costata*. Intraspecific and interspecific genetic distances calculated by Kimura's two-parameter method were 0.000–0.047 in the *L. nikkonis-L. costata* complex, 0.010–0.070 in *L.* sp, 0.000–0.081 in *L. echigonia* s. str., 0.078–0.125 between the *L. nikkonis-L. costata* complex and *L.* sp., 0.109–0.143 between the *L. nikkonis-L. costata* complex and *L. echigonia* s. str., and 0.089-0.151 between *L.* sp. and *L. echigonia* s. str. (Table 3).

In the cluster of *L. echigonia* s. str., MP and NJ trees gave four subclusters, which were designated as subclusters A to D (Fig. 7). The subcluster A included specimens from 3 localities (Teradomari, Hirata, and Nagaoka), the subcluster B specimens from 8 localities (Aogaki, Shiga, Nishiasai, Ise, Gifu, Shinshiro, Atsumi, and Kosai), and the subcluster C specimens from 9 localities (Ten-ei, Daishin,

Table 4. Deletion patterns in the mitochondrial D-loop regio*n*

segment #	I										II			III	IV	V				VI				VII	
deletion #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
nucleotide #	16-36	17-20	17-24	24-33	24-34	24-50	28-31	43-46	44-45	44-46	52-62	53-54	53-55	87-126	283-293	592-593	592-595	593-595	594-595	948-964	949-964	952-960	952-964	972-977	972-978
NAMIE(Nto)														○					○		○				
MEMANBETSU(Lnj)			○				○								○				○		○				
UEDA(Lni)			○				○							○	○				○		○				
MIDORIKO(Lni)			○				○							○	○				○		○				
KUROBE(Lni)			○				○							○	○				○		○				
KOREA(Lco)			○				○							○	○				○		○				
NAMDAE(Lco)			○				○							○	○				○		○				
KASUGA(Lsp)		○				○									○				○					○	
SUMOTO(Lsp)		○				○								○	○				○						○
NADASHO(Lsp)		○				○									○				○					○	
NAKATSU(Lsp)		○				○								○	○				○						○
IYOMISHIMA(Lsp)		○				○								○	○				○						○
HIRATA	○								○				○	○	○	○				○					
TERADOMARI	○								○				○	○	○					○					
NAGAOKA	○								○				○	○	○	○				○					
GIFU	○							○				○		○	○			○		○					
KOSAI	○									○		○		○	○				○	○					
SHINSHIRO	○									○		○		○	○				○	○					
ATSUMI	○							○				○		○	○				○	○					
NISHIASAI	○									○		○		○	○				○	○					
SHIGA	○									○		○		○					○	○					
ISE	○							○				○		○	○				○	○					
AOGAKI	○									○		○		○	○				○	○					
SHIOKAWA		○		○					○		○			○	○			○					○	○	
FUKUSHIMA		○		○					○		○			○	○			○					○	○	
TEN-EI		○		○					○		○			○	○			○					○	○	
DAISHIN		○		○					○		○			○	○			○					○	○	
ISHIKAWA		○		○					○		○			○	○			○					○	○	
NASU		○		○					○		○			○	○			○					○	○	
KUROBANE		○		○					○		○			○	○			○					○	○	
IMAICHI		○			○				○		○			○	○			○					○	○	
HITACHI		○		○					○		○			○	○			○					○	○	
ASAKAWA		○			○				○		○			○	○		○					○			
YASATO		○			○				○		○			○	○		○					○			
ISHIOKA		○			○				○		○			○	○		○					○			
YOSHII		○			○				○		○			○	○		○								
ONJUKU		○			○				○		○			○	○		○					○			
IKUTARYOKUCHI		○			○				○		○			○	○			○				○			
ZAWA		○			○				○		○			○	○			○				○			
AZUSAGAWA		○			○				○		○			○	○		○					○			
MATSUMOTO		○			○				○		○			○	○		○					○			
SHIOJIRI		○			○				○		○			○	○		○					○			

Circles indicates the presense of deletions in the specified nucleotide positions. Abbreviations are as in Table 2.

Fukushima, Imaichi, Nasu, Kurobane, Ishikawa, Shiokawa, and Hitachi). The subcluster D consisted of specimens from the remaining 10 localities. The subclusters C and D comprised a clade supported by high bootstrap probabilities, and the clade was linked to the subcluster B with support of low bootstrap values. Genetic distances within and among the subclusters are shown in Table 3. The subclusters of *L. echigonia* s. str. were well separated geographically from one another, when the areas encompassing the collection sites in the respective subclusters were depicted on the map (Fig. 8).

Nucleotide deletions were found especially in 5' and 3' portions of the mitochondrial D-loop region (Fig. 6). We considered that those deletions were also phylogenetically informative and compiled the nucleotide positions and possession by loaches of the deletions. Herein, we concerned ourselves with deletions of more than 4 contiguous bases shared by at least two specimens and 2 or 3 base deletions in the segments where 4 base deletions existed (Table 4). The deletion patterns were well consistent with the division of clusters and subclusters described in Fig. 7. Deletions of Nos. 3 and 7 in Table 4 were shared exclusively by specimens of *L. nikkonis* and *L. costata*. Deletions of No.6 were also found exclusively in specimens of *L.* sp. Deletions of Nos. 13 and 12 were found exclusively in specimens of subclusters A and B of *L. echigonia* s. str., respectively. Deletions of No. 23 and No. 4 with one exception (a specimen from Imaichi) were found in specimens of the subcluster C. Deletion Nos. 5 and 22 were shared by specimens of the subcluster D, although each had one exception (an additional specimen from Imaichi in the subcluster C and a specimen from Yoshii, respectively). Deletions of No. 11 were

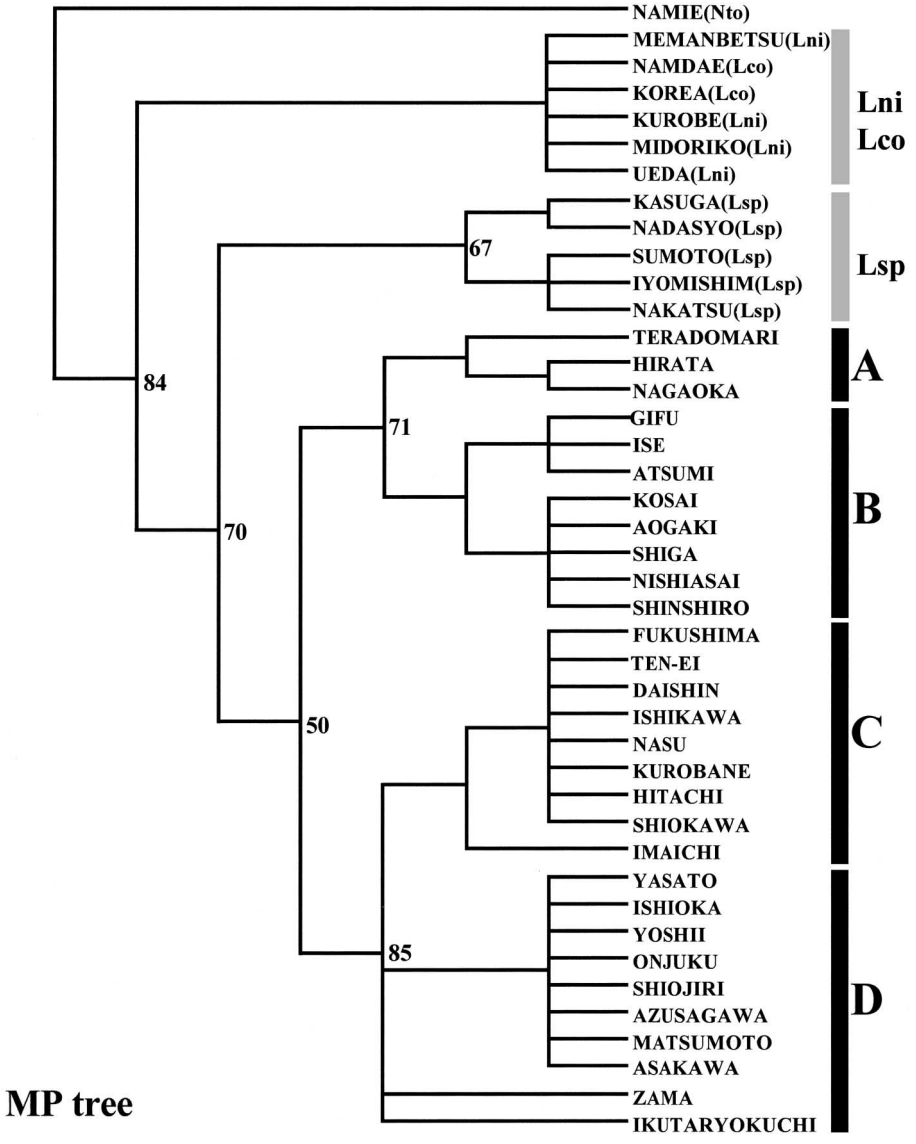


Fig. 9. Phylogenetic relationships of loaches of the genus *Lefua* inferred by deletion patterns in the mitochondrial D-loop region. Deletions in the D-loop region were shared by loaches and used to construct the MP dendrogram. *Noemacheilus barbatulus toni* was used as an outgroup. Possessions of deletions by loaches are summarized in Table 4.

shared by specimens of subclusters C and D. Therefore, the deletion patterns can be used for diagnoses of clusters and subclusters.

When the MP tree was constructed based on deletion patterns using *N. barbatulus toni* as an outgroup, there were four groups consisting of the *L. nikkonis-L. costata* complex, *L.* sp., subclusters A and B of *L. echigonia* s. str., and subclusters C and D of *L. echigonia* s. str. (Fig. 9). The topology of the tree was fundamentally consistent with those of MP and NJ trees (Fig. 7) with two exceptions; linking of the subcluster B to the subcluster A rather than to the clade of the subclusters C and D and exclusion of Ikutaryokuchi and Zama specimens from the subcluster D. The relationships based on deletion patterns correlated well with those based on mtDNA sequences.

DISCUSSION

Phylogenetic relationships of loaches of the genus *Lefua* and the taxonomic status of *L.* sp.

MP and NJ dendrograms based on mtDNA sequences (Fig. 7) consistently revealed three clusters, two of which were composed of all the specimens of *L. echigonia* s. str. and *L.* sp. In the other cluster, specimens of *L. nikkonis* and *L. costata* were included sporadically, showing that neither species was monophyletic. The preliminary osteological study showed a sister group relationship of *L. nikkonis* and *L. costata* and a lack of definite autapomorphy which distinguished between them (Tsuchiya, 1996). This suggests that *L. nikkonis* is a synonym of *L. costata*. However, we have to be careful to conclude the synonymy of the two species, because only two specimens from Korea were examined in this study. It is necessary to analyze more specimens of *L. nikkonis* and *L. costata* from China and Russia as well as Korea and Japan in order to evaluate the specific status of the two species.

The MP and NJ trees of mtDNA sequences (Fig. 7) also showed that *L.* sp. was more closely related to *L. echigonia* s. str. than to the *L. nikkonis-L. costata* complex, while protein analyses by 2D electrophoresis provided NJ and UPGMA trees having different topologies (Fig. 4). The discrepancy between DNA and protein trees could be due to several possible factors. It may be due to distinct evolutionary constraints exerted on the regions analyzed by two approaches. Protein data are derived from the coding region mainly in genomic DNA, but DNA data are from the mitochondrial non-coding region. These regions are possibly under different evolutionary constraints (e.g. by selective pressure). The discrepancy may be attributable to actual trichotomous splitting of loaches of the genus *Lefua* from their common ancestor. It is very difficult to resolve the branching order when splitting of the closely related species occurred sequentially over very short geological time. However, it seems most likely that the extraordinarily large intraspecific variations in *L. echigonia* s. str. led to the discrepancy. It is conceivable that loaches of the genus *Lefua* can be readily isolated and accumulate genetic variations, because of their specific and restricted habitats (springs and rivulets between hills and on mountains). In this context, protein analyses by 2D electrophoresis did not sufficiently take into account of intraspecific variations of *L. echigonia* s. str.

Originally, *L.* sp. was distinguished from *L. echigonia* s. str. by examining the morphological characters of about 800 specimens collected over wide areas in Japan (Hosoya, 1994). Differences between them were also reported in terms of their habitats (Yamashina *et al*., 1994). *Lefua* sp. inhabits relatively fast-flowing streams with gravelly beds, while *L. echigonia* s. str. prefers relatively slow-flowing streams with muddy beds. Dendrograms based on mtDNA sequences (Fig. 7) showed that *L.* sp. and *L. echigonia* s. str. were monophyletic groups and their bifurcation from a common ancestor was supported with high bootstrap values (90 both in the MP and NJ trees). The sympatry of *L.* sp. and *L. echigonia* s. str. in Kasuga (Hyogo pref.) strongly suggests reproductive isolation between them (Hosoya, 1994). These indicate *L.* sp. to be a biological species and warrant *L.* sp. being taxonomically described as a species in its own right.

The specific status of *L.* sp. is also supported by protein analyses. Both NJ and UPGMA dendrograms (Fig. 4) showed the trichotomous nature of splitting of *L.* sp., *L. echigonia* s. str., and *L. nikkonis*. The genetic distances (Table 2) between *L.* sp. and *L. echigonia* s. str. (0.239) and between *L.* sp. and *L. nikkonis* (0.207) were comparable to that between *L. echigonia* s. str. and *L. nikkonis* of nominally described species (0.224). Inspecting the results obtained so far by 2D electrophoresis, we realized an empirical tendency in the distribution of genetic distances (Miyazaki, 1989). Intraspecific genetic distances are usually lower than 0.11. The genetic distances among *L.* sp. and congeners were definitely larger than this value, and very close to that for congeneric species of Cypriniformes (0.237 in Fig. 5). The specific status of *L.* sp. can be firmly confirmed by further comparative studies on morphological and genetic variations of loaches of the genus *Lefua*.

Phylogeography of *L. echigonia* s. str.

The protein analyses by 2D electrophoresis of specimens from 7 collection sites suggested high genetic variations in *L. echigonia* s. str. (Table 2). To investigate extensively the intraspecific variations in *L. echigonia* s. str., the mitochondrial D-loop region of specimens from 30 collection sites was sequenced. The maximum genetic distance within *L. echigonia* s. str. (0.081) was very close to the minimum genetic distance between *L. echigonia* s. str. and *L.* sp. (0.089) and exceeded that between *L.* sp and the *L. nikkonis–L. costata* complex (0.078), showing again high genetic variations in *L. echigonia* s. str. (Table 3). The specimens were grouped into subclusters A to D in MP and NJ trees of mtDNA sequences (Fig.7). The subcluster A consisted of specimens from the northern Chubu (i. e. Hokuriku) district (# 16 and 17 in Table 1) and the Tohoku (# 15) dis-

trict, and the subcluster B from the Kinki district (# 41 to 44) and the southern Chubu district (# 37 to 40). The subcluster C consisted of specimens from the northern Kanto district (# 23 to 26) and the Tohoku district (# 18 to 22), and the subcluster D from the southern Kanto district (# 27 to 33) and the central Chubu district (# 34 to 36). The subclusters C and D were combined with high bootstrap values (88 in MP and 97 in NJ). We tentatively designated subclusters A to D as Hokuriku, Kinki, Northern Kanto, and Southern Kanto populations, respectively.

Deletions in the D-loop region are normally disregarded, because alignment of portions including the deletions is sometimes difficult. However, the present results showed that deletion patterns provided useful criteria to identify the four populations of *L. echigonia* s. str. (Table 4). The MP tree based on deletion patterns (Fig. 9) gave a grouping corresponding to subclusters A to D with exceptional specimens from Ikutaryokuchi and Zama (Kanagawa pref.). The exclusion of the specimens from the subcluster D may be due to deletion Nos. 17 and 18 in the segment V (Table 4), where the specimens presented different deletion patterns from those of other specimens in the subcluster D. In the MP tree, the subcluster B was linked to the subcluster A rather than to the clade consisting of the subclusters C and D. However, the linkage was not supported with the high bootstrap value (71), and in the MP and NJ trees based on sequence data, the subcluster B was linked to the clade of the subclusters C and D with relatively low bootstrap values (57 in MP and 70 in NJ). Therefore, the assignment of the subcluster B is not conclusive at present. Nevertheless, deletions in the D-loop region were phylogenetically valuable and fundamentally supported the results based on sequence data.

Interestingly, distributions of the populations of *L. echigonia* s. str. are approximately consistent with those of Beloniform freshwater fish, *Oryzias latipes* (Sakaizumi, 1986; Matsuda *et al*., 1997), although the latter has broader distributions in Japan Islands than the former (Fig. 8). It is likely that the Hokuriku and Kinki populations of *L. echigonia* s. str. correspond to the Northern Japan population and the Setouchi subpopulation (in the Southern Japan population) of *O. latipes*, respectively. The Southern and Northern Kanto populations of *L. echigonia* s. str. seemingly correspond to the Eastern Japan subpopulation (in the Southern Japan population) of *O. latipes*. The precise outlines of distributions do not perfectly match between the two species, possibly because of differences in their migration and adaptation abilities. However, it is reasonable to assume that the approximate overlap of distributions in fishes of highly divergent groups (Cypriniformes and Beloniformes) reflects historical events in the process of formation of current freshwater fish fauna in Japan Islands. Although our results are not sufficient at present to speculate profoundly about the events, the diversification of the Hokuriku populations from the remaining populations is probably due to the mountain range running from north to south in the center of Honshu Island causing an obstacle. The boundary of the Kinki population from the Northern and Southern Kanto populations is closely related to the west margin of fossa magna, which is a large-scale subsiding zone in central Honshu Island. The drastic change in freshwater fish fauna across the fossa magna zone was revealed by studies of distributions of diverse freshwater fishes (Lindberg, 1972; Watanabe, 1998).

Lefua echigonia and *L.* sp. were assigned by the Environmental Ministry of Japan to species threatened with extinction in 2000 (category EN endangered). The present study provides fundamental genetic information useful to conserve those endangered species.

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