Journal of Water Resource and Protection, 2017, 9, 238-253
http://www.scirp.org/journal/jwarp
ISSN Online: 1945-3108
ISSN Print: 1945-3094

Intraspecific Relationships and Variation of Two *Lefua* Species (Balitoridae, Cypriniformes) in the Tokai Region, Honshu, Japan

Jun-Ichi Miyazaki[1], Seiya Hida[1], Takurou Ozaki[1], Yuichirou Tabata[1], Misaki Iwata[1], Masashi Nakazawa[1], Youki Fukasawa[2], Tomonari Asaka[3]

[1]Faculty of Education and Human Sciences, University of Yamanashi, Kofu, Japan
[2]Department of Education, Graduate School of Medical and Engineering, University of Yamanashi, Kofu, Japan
[3]Mikawa Freshwater Life Network, Toyokawa, Japan
Email: miyazaki@yamanashi.ac.jp

How to cite this paper: Miyazaki, J.-I., Hida, S., Ozaki, T., Tabata, Y., Iwata, M., Nakazawa, M., Fukasawa, Y. and Asaka, T. (2017) Intraspecific Relationships and Variation of Two *Lefua* Species (Balitoridae, Cypriniformes) in the Tokai Region, Honshu, Japan. *Journal of Water Resource and Protection*, **9**, 238-253.
https://doi.org/10.4236/jwarp.2017.92016

Received: January 10, 2017
Accepted: February 12, 2017
Published: February 15, 2017

Copyright © 2017 by authors and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).
http://creativecommons.org/licenses/by/4.0/
Open Access

Abstract

Two species *Lefua echigonia* and *Lefua* sp. 2 of the eight-barbel loach inhabit the Tokai region of Honshu, Japan. We determined sequences of the mitochondrial D-loop region to elucidate intraspecific phylogenetic relationships and variation in these two species. *Lefua* sp. 2 represented high intraspecific genetic similarity and complicated haplotype network, but three assemblages were recognized, including specimens mainly from Yahagi, Toyo, and Tenryu River systems, respectively, and named Groups 1 to 3. Divergence of Group 1 from the others was marginally supported, but Group 2 was paraphyletic to Group 3, suggesting the existence of two populations, *i.e.* Yahagi River population and Toyo-Tenryu River population. *Lefua echigonia* also represented high intraspecific genetic similarity, and two assemblages with slight genetic differentiation were discernible, including specimens from Shizuoka and southeastern Aichi prefectures and those from northwestern Aichi, Gifu, and Mie prefectures, respectively, and named Groups A and B. Star-like relationships of haplotypes suggested the dispersal origin located in eastern Aichi prefecture. The two species are threatened to extinction and thus we proposed evolutionary significant units for conservation.

Keywords

Population, Phylogeography, Mitochondrial D-Loop Region, Conservation, River Capturing

1. Introduction

Eight-barbel loaches belonging to the genus *Lefua* (Balitoridae, Cypriniformes) are primary freshwater fish less than 10 cm in total length. The evolutionary process in *Lefua* forms part of the evolutionary history of Japanese and East Asian freshwater fishes. Four species have been formally described. *Lefua nikkonis* (Jordan et Fowler, 1903) is endemic to Hokkaido, Japan, *L. echigonia* Jordan et Richardson, 1907 inhabits Tohoku to Kinki districts in Honshu, Japan, and *L. costata* (Kessler, 1876) is distributed widely in East Asia including Korea, China, and Russia, but has been introduced to restricted areas in Honshu, Japan [1]. *Lefua pleskei* (Herzenstein, 1887) is distributed in eastern Russia [2]. Another species, *Lefua* sp. was separated from *L. echigonia* based on morphological traits with a greater distance between the dorsal and ventral fins, longer snout length, lower body height, and narrower body width of *Lefua* sp. than *L. echigonia* [3] [4], and on ecological traits with habitats of relatively fast-flowing mountain streams with gravelly beds of *Lefua* sp. contrasting with habitats of relatively slow-flowing streams with muddy beds in marshlands, spring water, rice paddy irrigation channels, and the backwaters of floodplains of *L. echigonia* [5]. *Lefua* sp. is waiting for a formal scientific description. This species is distributed in western Japan including Kinki, Hokuriku, and Chugoku districts in Honshu and also in Shikoku. Additionally, we suggested recently that *Lefua* loach occurring locally in Aichi and Shizuoka prefectures of the Tokai region in Honshu, Japan is the sixth possible species [6] [7]. Although the loach had been identified as *Lefua* sp. on morphological and ecological grounds as above, our phylogenetic study by mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) sequencing showed that the loach in the Tokai region comprised a monophyletic group and was more closely related to *L. echigonia* than to *Lefua* sp. In our previous study, we designated it the *L.* sp. Tokai population, and thereafter *Lefua* sp. in western Japan was classified as *Lefua* sp. 1 and the loach in the Tokai region as *Lefua* sp. 2 in the Red List issued by the Japanese Ministry of the Environment in 2014 (see also [8]). According to the classification, we herein designate the loach in the Tokai region as *Lefua* sp. 2. Some studies showed embryological differences in *Lefua* loaches [9] [10] [11] [12]. However, we cannot perform comparative embryology among the above six species and among intraspecific populations of each species, because those studies have been done using only limited samples of two species, *Lefua* sp. 1 and *L. echigonia.*

Although the taxonomic status of *Lefua* sp. 2 remains to be specified, it has a unique evolutionary background clearly [6]. We suggested parallel morphological evolution between *Lefua* sp. 1 and *Lefua* sp. 2 [7]. Although their distributions are disjunct, both species occur in relatively fast-flowing mountain streams with gravelly beds and generally have more slender bodies for adapting to fast-flowing streams than *L. echigonia*. The slender bodies allow them to avoid fast flowing water through exploitation of spaces within the gravel bed of streams. *Lefua* sp. 2 and *L. echigonia* inhabit the Tokai region. Complex geological events

in their distribution range are likely associated with the evolutionary process. Upliftment in the whole range might have forced ancestors of *Lefua* sp. 2 to adapt to fast-flowing mountain brooks [7]. Later *L. echigonia* invaded the Tokai region during erosion of the western flanking zone (Ryoke metamorphic belt) of the Median Tectonic Line (MTL). At present, *Lefua* sp. 2 preferentially inhabits the eastern flanking zone (Sanbagawa metamorphic belt) of MLT and the zone of uplifted volcanic and marine deposit. The Ryoke metamorphic belt tends to be strongly eroded, producing relatively flat terrain suitable for development of *L. echigonia* habitat. This is not the case in the Sanbagawa metamorphic belt of a different rock composition.

Because of its extinction risk and unique evolutionary background, *Lefua* sp. 2 was registered as endangered species in the Red List as well as *L. echigonia*, *Lefua* sp. 1, and *L. nikkonis* [4] [13] [14]. These *Lefua* loaches have been severely threatened by habitat destruction due to human activities. We demonstrated that *L. echigonia* comprised genetically seven intraspecific populations (Hokuriku, Tohoku, Yamagata, North-Kanto, South-Kanto, Kinki, and Tokai) and *Lefua* sp. 1 comprised two intraspecific populations (Kii-Shikoku and Sanyo) [7] [15]. These populations occur in regions that are geographically well separated by mountain ranges and highlands [1] [16]. The populations should be considered evolutionary significant units (ESUs, as defined by Moritz [17]) for the protection of the endangered loaches [1]. However, intraspecific phylogenetic relationships and variation in *Lefua* sp. 2 have not been investigated thoroughly. Elucidation of cryptic ESUs is crucial for the protection of this unique loach.

In the present study, we demonstrate intraspecific phylogenetic relationships and variation in *Lefua* sp. 2 and *L. echigonia* (Tokai population) by sequencing the mitochondrial D-loop region. We present fundamental information for the protection of these endangered species, and discuss their evolutionary history in the Tokai region.

2. Materials and Methods

2.1. Materials

Collecting localities of *Lefua* sp. 2 (83 specimens) and *L. echigonia* (40 specimens) are shown in **Figure 1** and information on all samples including outgroup specimens is listed in **Table 1**. Since both species was registered as endangered species in the Red List, we used large circles on **Figure 1** to conceal detailed information of habitats for the conservation of the two endangered species. Specimens of *Lefua* sp. 1 obtained from Hidaka (Sanyo population) and *L. echigonia* obtained from Ouchi (Hokuriku population) were used as the outgroup for the *Lefua* sp. 2 phylogeny. Those of *L. echigonia* obtained from Aogaki (Kinki population) and Ouchi were used as the outgroup for the *L. echigonia* phylogeny in the Tokai region. *Lefua* sp. 2 and *L. echigonia* are sympatric in Sanagawa, Aichi Prefecture (sample Nos. 69 and 111), and occur in the close vicinity in Kosai, Shizuoka Prefecture (sample Nos. 80 and 118).

Table 1. Sample list.

No.	Species	Collection site		Accession No.
1	*Lefua* sp. 2	YahagiB4	Aichi, Yahagi R.	LC062792
2		YahagiB5	Aichi, Yahagi R.	LC062793
3		YahagiB6	Aichi, Yahagi R.	LC062794
4		YahagiB7	Aichi, Yahagi R.	LC062795
5		YahagiC2	Aichi, Yahagi R.	LC062796
6		YahagiD1	Aichi, Yahagi R.	LC062797
7		YahagiE3	Aichi, Yahagi R.	LC062798
8		YahagiF1	Aichi, Yahagi R.	LC062799
9		YahagiG0	Aichi, Yahagi R.	LC062800
10		YahagiG1	Aichi, Yahagi R.	LC062801
11		YahagiG8	Aichi, Yahagi R.	LC062802
12		YahagiG9	Aichi, Yahagi R.	LC062803
13		YahagiI1	Aichi, Yahagi R.	LC062804
14		Yahagi1	Aichi, Yahagi R.	*AB251875
15		Yahagi2	Aichi, Yahagi R.	*AB251877
16		Yahagi3	Aichi, Yahagi R.	LC062805
17		Yahagi4	Aichi, Yahagi R.	LC062806
18		Yahagi5	Aichi, Yahagi R.	LC062807
19		YahagiF0	Aichi, Yahagi R.	LC062808
20		Yahagi2012	Aichi, Yahagi R.	LC062809
21		Yahagigawa	Aichi, Yahagi R.	LC062810
22		Nishikiriyamagawa	Aichi, Yahagi R.	LC062852
23		Mitogawa	Aichi, Mito R.	LC062811
24		Nisidagawa	Aichi, Nisida R.	LC062851
25		OtowagawaA1	Aichi, Otowa R.	LC062812
26		OtowagawaA2	Aichi, Otowa R.	LC062813
27		OtowagawaA3	Aichi, Otowa R.	LC062814
28		OtowagawaA4	Aichi, Otowa R.	LC062815
29		OtowagawaA5	Aichi, Otowa R.	LC062816
30		OtowagawaB1	Aichi, Otowa R.	LC062817
31		OtowagawaB2	Aichi, Otowa R.	LC062818
32		OtowagawaB3	Aichi, Otowa R.	LC062819
33		OtowagawaB4	Aichi, Otowa R.	LC062820
34		OtowagawaB5	Aichi, Otowa R.	LC062821
35		OtowagawaC1	Aichi, Otowa R.	LC062822
36		OtowagawaC2	Aichi, Otowa R.	LC062823
37		OtowagawaC3	Aichi, Otowa R.	LC062824
38		OtowagawaC4	Aichi, Otowa R.	LC062825
39		OtowagawaC5	Aichi, Otowa R.	LC062826
40		Yamazakigawa1	Aichi, Otowa R.	LC062827

Continued

41	Yamazakigawa2	Aichi, Otowa R.	LC062828
42	Yamazakigawa3	Aichi, Otowa R.	LC062829
43	Yamazakigawa4	Aichi, Otowa R.	LC062830
44	Yamazakigawa5	Aichi, Otowa R.	LC062831
45	Shitara2	Aichi, Toyo R.	LC062832
46	Shitara6	Aichi, Toyo R.	*AB251872
47	Shitara8	Aichi, Toyo R.	*AB251873
48	Shitara21	Aichi, Toyo R.	*AB251874
49	Shitara25	Aichi, Toyo R.	LC062833
50	Shitara26	Aichi, Toyo R.	*AB251876
51	Shimoshimada	Aichi, Toyo R.	LC062834
52	Motoyagawa	Aichi, Toyo R.	*AB599766
53	Ichinosegawa	Aichi, Toyo R.	LC062839
54	Bunyagawa	Aichi, Toyo R.	LC062840
55	Takaragawa	Aichi, Toyo R.	LC062841
56	Aigou	Aichi, Toyo R.	LC062842
57	Soezawaonsen	Aichi, Toyo R.	LC062843
58	Tadamochi	Aichi, Toyo R.	LC062844
59	Tugegawa	Aichi, Toyo R.	LC062845
60	Houraiji	Aichi, Toyo R.	LC062846
61	Hourai	Aichi, Toyo R.	*AB251871
62	Marukomesawa	Aichi, Toyo R.	LC062847
63	Takise	Aichi, Toyo R.	LC062848
64	Wakamiyajinja	Aichi, Toyo R.	LC062849
65	Aderagawa	Aichi, Toyo R.	LC062850
66	Shimada	Aichi, Toyo R.	LC062853
67	Uregawa	Aichi, Toyo R.	LC062854
68	Ozawagawa	Aichi, Toyo R.	LC062855
69	Sanagawa	Aichi, Sana R.	*AB599767
70	Gotengawa	Aichi, Tenryu R.	*AB251870
71	Takihashigawa	Aichi, Tenryu R.	LC062835
72	Fukayagawa	Aichi, Tenryu R.	LC062836
73	Nanegawa	Aichi, Tenryu R.	LC062838
74	Nagaishigawa	Shizuoka, Tenryu R.	LC062837
75	Kaminobegawa	Shizuoka, Tenryu R.	LC062856
76	Mikkabichouhonmachi	Shizuoka, Miyakoda R.	LC062858
77	Oota	Shizuoka, Miyakoda R.	**AB599770
78	Santou	Shizuoka, Miyakoda R.	LC062859
79	Kougataihei	Shizuoka, Miyakoda R.	**AB599768
80	Kosai	Shizuoka, Miyakoda R.	*AB251878

Continued

81		Fumagawa	Shizuoka, Oota R.	LC062857
82		Kanzagawa	Shizuoka, Oota R.	LC062860
83		Morimachiichimiya	Shizuoka, Oota R.	**AB599769
84	*Lefua echigonia*	Atsumi	Aichi	*AB102846
85		Iwafuzigawa	Aichi	LC062861
86		Usukogawa	Aichi	LC062862
87		Ooshiro	Aichi	LC062863
88		Kairikegawa	Aichi	LC062864
89		Shirotorigawa	Aichi	LC062865
90		Shinshiro	Aichi	*AB102845
91		Damine-1A	Aichi	LC062867
92		Damine-1B	Aichi	LC062868
93		Damine-2	Aichi	LC062869
94		Donodagawa	Aichi	LC062870
95		Tomoegawa	Aichi	LC062871
96		Harakawa	Aichi	LC062872
97		Miyashitagawa	Aichi	LC062874
98		Yanamisawa	Aichi	LC062875
99		Sizenkansatsunomori	Aichi	LC062876
100		Norisadagawa	Aichi	LC062877
101		Hirayabugawa	Aichi	LC062878
102		Torakumagawa	Aichi	LC062879
103		Ikedagawa	Aichi	LC062880
104		Otowagawa	Aichi	LC062881
105		Hagurigawa	Aichi	LC062882
106		Terashimogawa	Aichi	LC062883
107		Kazikawa	Aichi	LC062884
108		Mitogawa	Aichi	LC062885
109		Miyagawa	Aichi	LC062873
110		Suyahara	Aichi	LC062866
111		Sanagawa	Aichi	LC062886
112		Shouzinngawa	Aichi	LC062891
113		Shizuoka	Shizuoka	*AB599746
114		Sagara	Shizuoka	*AB251866
115		Ishioka	Shizuoka	LC062887
116		Ono	Shizuoka	LC062888
117		Kakegawa	Shizuoka	*AB177699
118		Kosai	Shizuoka	*AB102844
119		Seirigawa	Shizuoka	LC062889
120		Morimachiookubo	Shizuoka	LC062890
121		Gifu	Gifu	*AB102843
122		Seki	Gifu	LC062892
123		Ise	Mie	*AB102849
124	*Lefua* sp.1	Hidaka	Wakayama	*AB177672
125	*Lefua echigonia*	Aogaki	Hyogo	*AB102850
126	*Lefua echigonia*	Ouchi	Akita	*AB177677

*Deposited previously; **Deposited previously and revised in this study.

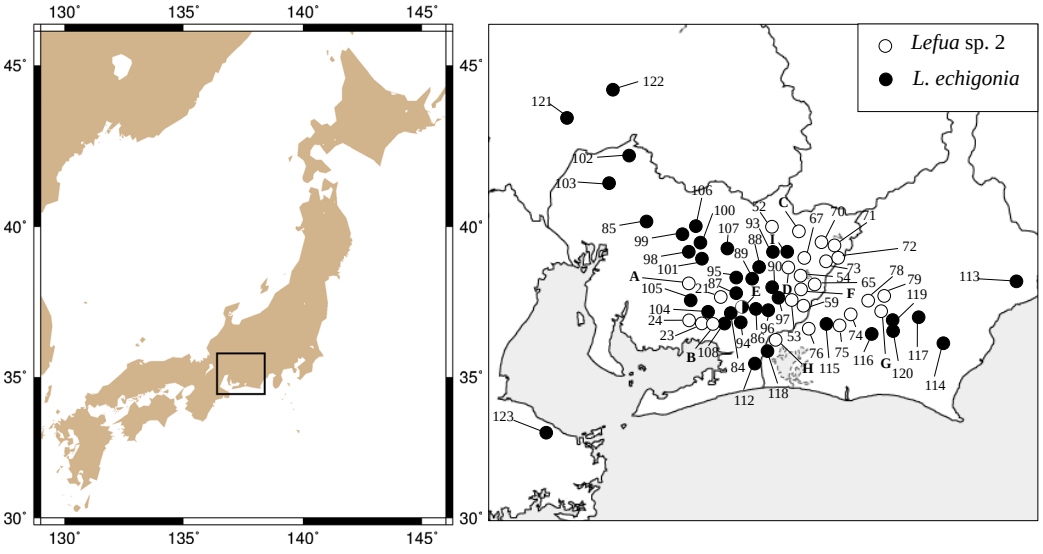

Figure 1. Locations in the Tokai region where specimens of *Lefua* sp. 2 and *L. echigonia* were collected Refer to **Table 1** for details of the collection sites and sample numbers. We used large white circles for sample localities of *Lefua* sp. 2 and large black circles for those of *L. echigonia*. As a result, each circle does not always represent a single locality and in some cases integrated pleural localities. The numbers accompanied with circles denote the sample numbers in **Table 1**. A to I accompanied with circles include the following samples; A, 1-20; B, 22, 25-44; C, 45-50, 55, 57, 63; D, 51, 56, 58, 61, 66, 68; E, 69, 109, 110, 111; F, 60, 62, 64; G, 80, 82; H, 77, 81, 83; I, 91, 92.

2.2. DNA Sequencing

Total DNA from fin or muscle of each fish was prepared using a DNeasy® Blood & Tissue Kit (QIAGEN GmbH, Hilden) according to the manufacturer's protocol. To amplify mtDNA containing the D-loop region, PCR was performed in a reaction solution (50 µl) containing template DNA (2 µl) and KOD dash (Toyobo Co., Ltd., Osaka) with an initial denaturation at 94˚C for 2 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 10 sec, and extension at 74˚C for 30 sec, and with final extension at 74˚C for 7 min. Sequences of the primers used for amplification are shown in **Table 2** [7]. The amplified DNA fragment was purified using a QIAquick Purification Kit (QIAGEN GmbH, Hilden). Direct sequencing of the double-stranded PCR product was performed using an ABI PRISM BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems Inc., California) and the primers used for PCR on Model 377 and 377XL DNA sequencers (Applied Biosystems) according to the manufacturer's directions. Alternatively, direct sequencing was performed using a Genome Lab™ DTCS-Quick Start Kit on a CEQ™ 2000XL DNA Analysis system (Beckman Coulter Inc., California) according to the manufacturer's directions.

New sequence data were deposited in the DDBJ database under accession numbers **LC062792-062892**. We had previously determined mtDNA sequences used in this study (AB102843-102846, AB102849, AB102850, AB177672, AB177677, AB177699, AB251866, AB251870-251878, AB599746, AB599766-599770 [1] [6] [7] [16]).

Table 2. Primers used in this study.

Sense	ProS	5'GCATCGGTCTTGTAATCCGAAGAT3'
	296S	5'ATATATTAATGTAGTAAGAAACCACCAACCAG3'
	651S	5'TCAACACATCCTTATACTATATGC3'
Antisense	334AS	5'ATATATCACCTTCCACTTATGTCCC3'
	194AS	5'ACATTAATACTCGTTAATTTTATTGCGCTC3'
	PheAS	5'GGACCAAGCCTTTGTGCATGCGGAG3'

2.3. Phylogenetic Analysis

DNA sequences of the mitochondrial D-loop region were edited and aligned using DNASIS (Hitachi Software Engineering Co., Ltd., Tokyo) and MEGA 6.0 [18], and the alignments were corrected by visual inspection. We used 825 bp for the *Lefua* sp. 2 phylogeny and 867 bp for the *L. echigonia* phylogeny in the Tokai region, excluding indels and ambiguous sites. Neighbor-joining (NJ) and maximum parsimony (MP) trees were constructed using MEGA 6.0 and PAUP*4.0 beta10 [19], respectively. Genetic distances were computed by Kimura's two-parameter model [20]. Tree reliability was evaluated by generating 1,000 bootstrap replicates. The majority-rule consensus MP tree was constructed by conducting a heuristic search based on the 1,000 bootstrap replicates with an unweighted ts/tv ratio. The Bayesian (BA) tree was constructed using MrBayes version 3.1.2 [21] based on the model evaluated by the MrModeltest 2.3 [22]. The best models were GTR + I + G for the *Lefua* sp. 2 phylogeny and HKY + G for the *L. echigonia* phylogeny. The Monte Carlo Markov chain (MCMC) length was 5×10^6 generations, and we sampled the chain every 100 generations. MCMC convergence was assessed by calculating the potential scale reduction factor, and the first 1×10^4 generations were discarded. The minimum spanning tree representing relationships among haplotypes was constructed using MEGA 6.0 and haplotype and nucleotide diversities and Genetic differentiation (Fst) were calculated using Arlequin 3.5.1.2 [23]. The statistical significance of Fst was evaluated by calculating 1×10^4 values.

3. Results

1) Phylogenic relationships in *Lefua* sp. 2 and *L. echigonia* from the Tokai region

To determine phylogenetic relationships of 83 specimens in *Lefua* sp. 2, the NJ tree was constructed based on 825-bp sequences using *Lefua* sp. 1 from Hidaka and *L. echigonia* from Ouchi as the outgroup (**Figure 2**). There were 58 variable sites and 29 parsimony informative sites. Most branches were very short, indicating high sequence similarities. The monophyly of *Lefua* sp. 2 was well supported (NJ, 100; MP, 100; BA, 1.00), but other groupings within *Lefua* sp. 2 were generally not well supported because of high sequence similarities. We arbitrarily designated assemblies including specimens mainly from Yahagi, Toyo, and Tenryu River systems as Groups 1 to 3, respectively. Group 1 comprised specimens collected in the major Yahagi and neighboring minor Mito, Nishida, and

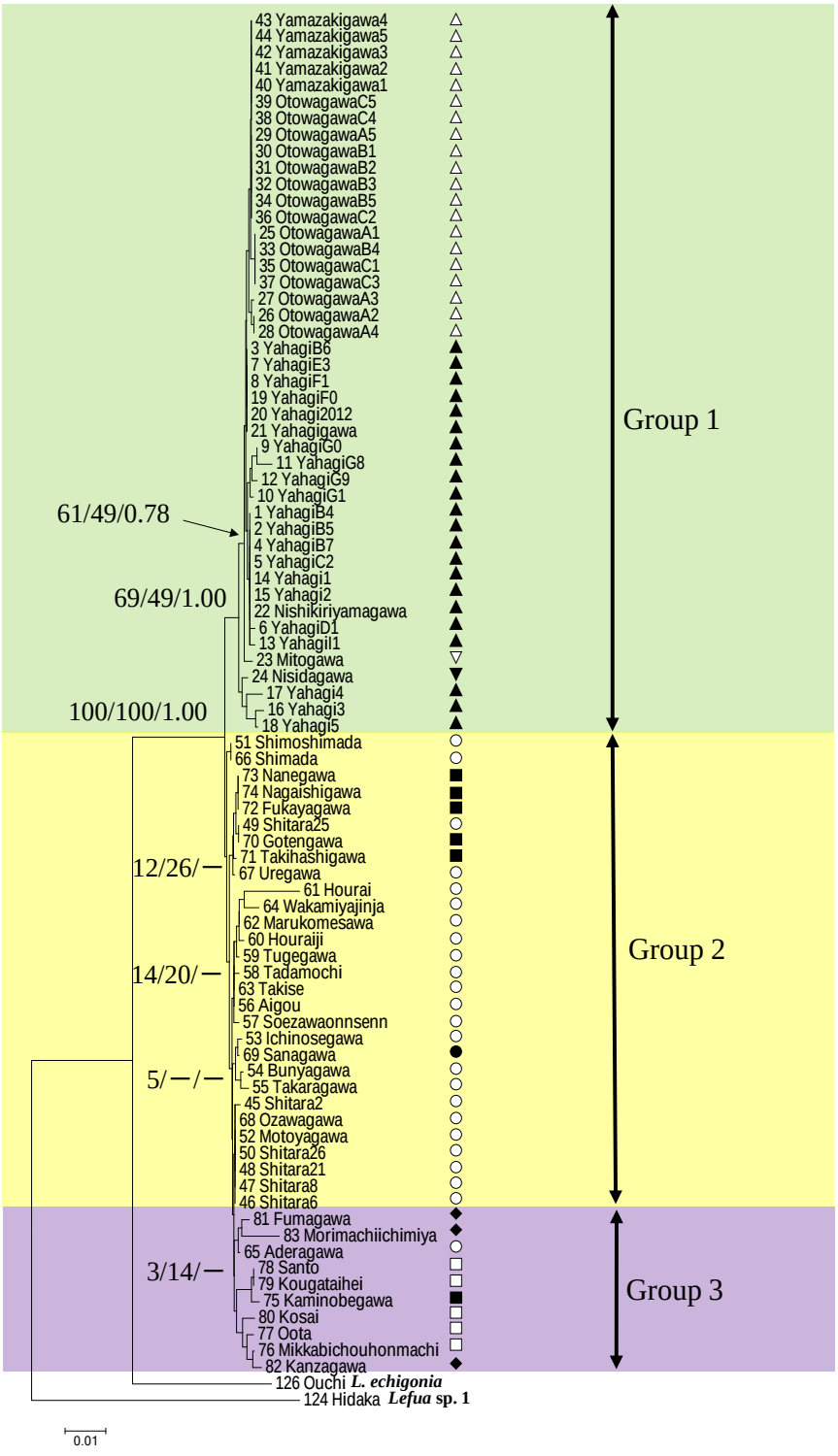

Figure 2. Phylogenetic relationships in *Lefua* sp. 2 based on the 825-bp mitochondrial D-loop sequences. The NJ tree was constructed based on genetic distances calculated with Kimura's two-parameter model using a total of 85 samples including *Lefua* sp. 1 from Hidaka and *L. echigonia* from Ouchi as the outgroup. The scale bar indicates 0.01 substitutions per site. Statistical supports of tree topologies are specified with NJ (left) and MP (middle) bootstrap values and BA posterior probabilities (right) in the vicinity of corresponding nodes. Simbols denote river systems: ▲, Yahagi; ▽, Mito; ▼, Nishida; △, Otowa; ○, Toyo; ●, Sana; ■, Tenryu; □, Miyakoda; ◆, Oota.

Otowa River systems (hereafter abbreviated as the Yahagi River system), and was marginally supported (NJ, 69; MP, 49; BA, 1.00). Group 2 was consisted of specimens collected in the major Toyo and neighboring minor Sana River systems (hereafter abbreviated as the Toyo River system), and was paraphyletic to Group 3. Group 3 comprised specimens collected in the major Tenryu and neighboring minor Miyakoda and Oota River systems (hereafter abbreviated as the Tenryu River system), and was poorly supported (NJ, 3; MP, 14; BA, -). Group 2 + Group 3 is monophyletic, albeit poorly supported. Group 2 included exceptionally five specimens collected in the Tenryu River system. As described in the Discussion, localities of those specimens from the Tenryu River system, Gotengawa, Takihashigawa, Fukayagawa, Nanegawa, and Nagaishigawa (sample Nos. 70-74 in Table 1), are very close to tributaries of the Toyo River system. Group 3 included an exceptional specimen from the Toyo River system (Aderagawa, sample No. 65).

The p-distance and *F*st were low between Groups 2 and 3, although those between Groups 1 and 2 and between Groups 1 and 3 were relatively higher (Table 3), suggesting that *Lefua* sp. 2 in the Yahagi River system was moderately differentiated from that in the other river systems. MP and BA trees demonstrated similar basal divergences, but splitting at the tip was not conservatively recovered (data not shown).

To determine phylogenetic relationships of 40 specimens in *L. echigonia*, the NJ tree was constructed based on 867-bp sequences using *L. echigonia* from Aogaki and Ouchi as the outgroup (Figure 3). There were 105 variable sites and 26 parsimony informative sites. The monophyly of specimens from the Tokai region, *i.e.* the Tokai population of *L. echigonia,* was well supported (NJ, 95; MP, 97; BA, 0.99). We arbitrarily designated assemblies as Groups A and B. Group A comprised specimens from Shizuoka and southeastern Aichi prefectures (NJ, 41; MP, -; BA, 0.90) and Group B from northwestern Aichi, Gifu, and Mie prefectures (NJ, 47; MP, 56; BA, 0.61). The p-distance and *F*st between Groups A and B were low, but their p-distance was higher than those within each group (Table 4). MP and BA trees demonstrated similar basal divergences, but splitting at the tip was not conservatively recovered (data not shown).

2) Haplotype relationships in *Lefua* sp. 2 and *L. echigonia* from the Tokai region

Forty-five haplotypes were detected in *Lefua* sp. 2, and the minimum spanning tree was constructed to represent their relationships (Figure 4). The tree showed a complicated network of haplotypes, but haplotypes represented by

Table 3. Genetic divergence in *Lefua* sp. 2.

	Group 1	Group 2	Group 3
Group 1	0.00314	0.64271*	0.65775*
Group 2	0.01046	0.00408	0.22491*
Group 3	0.01412	0.00767	0.00854

Above diagonal, *F*st; on diagonal, intragroup p-distance; below diagonal, intergroup p-distance. *statistically significant ($P < 0.05$).

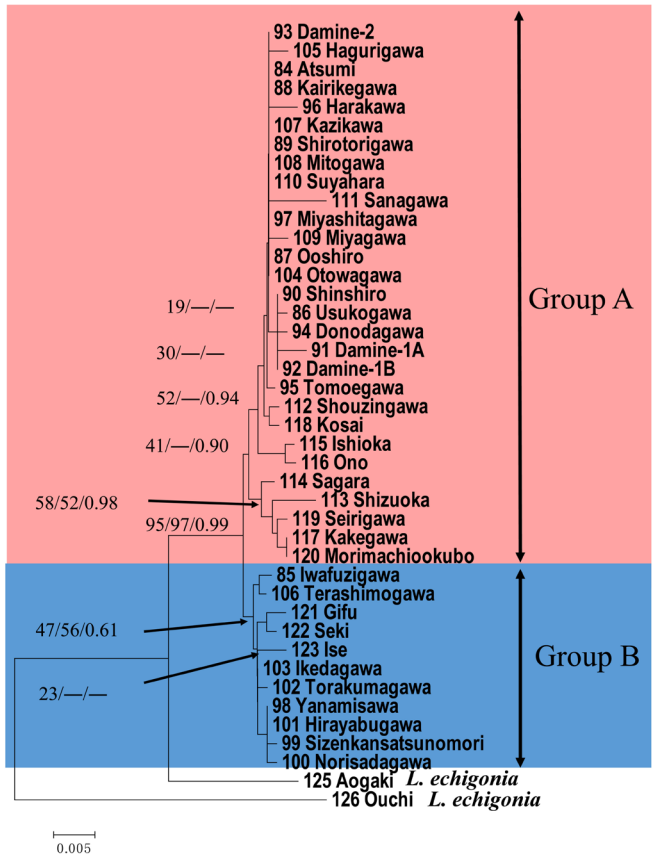

Figure 3. Phylogenetic relationships in *L. echigonia* based on the 867-bp mitochondrial D-loop sequences The NJ tree was constructed based on genetic distances calculated with Kimura's two-parameter model using a total of 42 samples including *L. echigonia* from Aogaki and Ouchi as the outgroup. The scale bar indicates 0.005 substitutions per site. Statistical supports of tree topologies are specified with NJ (left) and MP (middle) bootstrap values and BA posterior probabilities (right) in the vicinity of corresponding nodes.

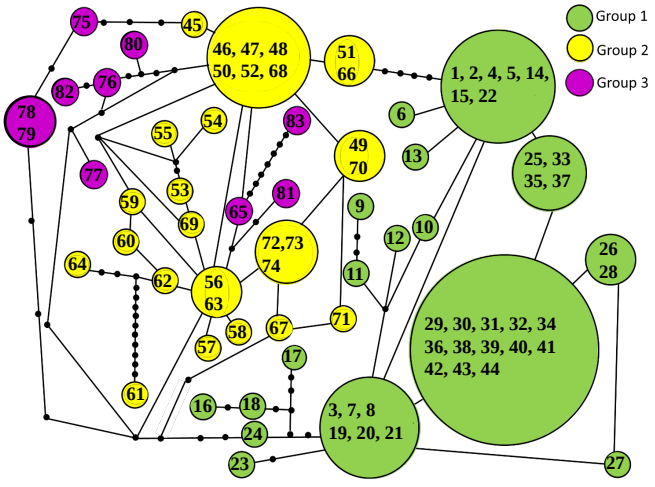

Figure 4. Minimum spanning tree of 45 haplotypes in *Lefua* sp. 2 Circles indicate haplotypes and their dimensions are proportional to the number of individuals possessing the same haplotype. Black circles on lines indicate hypothetical haplotypes not found in this study. The numbers denote the sample numbers in Table 1. Dark grey (purple in the color version), Group 3; pale grey (yellow), Group 2; intermediate grey (green), Group 1.

Table 4. Genetic divergence in *L. echigonia*.

	Group A	Group B
Group A	0.005068	0.44564*
Group B	0.008262	0.003649

Above diagonal, *F*st; on diagonal, intragroup p-distance; below diagonal, intergroup p-distance; *, statistically significant ($P < 0.05$).

specimens belonging to the three groups were roughly separated from one another. The haplotypes of those belonging to the Yahagi River system were relatively well separated from the others, supporting that *Lefua* sp. 2 in the Yahagi River system was genetically differentiated from the others. The haplotypes of Gotengawa, Takihashigawa, Fukayagawa, Nanegawa, and Nagaishigawa from the Tenryu River system were included in the assembly of haplotypes from the Toyo River system, while those of Aderagawa, Fumagawa (sample No. 81), and Morimachiichimiya (83) from Group 3 were included in the assembly of haplotypes from Group 2. Therefore, *Lefua* sp. 2 in the Toyo River system was not genetically distinguishable well from that in the Tenryu River system.

Twenty-eight haplotypes were detected in *L. echigonia*, and the minimum spanning tree was constructed to represent their relationships (**Figure 5**). A star-like structure of haplotype relationships was represented with the haplotype of the greatest majority shared by 10 specimens from eastern Aichi prefecture.

3) Haplotype and nucleotide diversities in *Lefua* sp. 2 and *L. echigonia* from the Tokai region

Values of the haplotype diversity in *Lefua* sp.2 were relatively high and similar between groups (**Table 5**), but values of the nucleotide diversity were very low and those in Groups 1 and 2 (Yahagi and Toyo River systems) were lower than that in Group 3 (Tenryu River system). High haplotype and low nucleotide diversities in *L. echigonia* were similar between Groups A and B.

4. Discussion

Our results showed that *Lefua* sp. 2 was an established entity in the *Lefua* loaches (see also [7] [15]), and that there were not well-defined genetic structures discernible within *Lefua* sp. 2. Taking the D-loop as the fastest evolving region in mitochondrial DNA into account, *Lefua* sp. 2 dispersed quite recently, or it has been maintained as a single population for long time. *Lefua* sp. 2 is more closely related to *L. echigonia* than to *Lefua* sp. 1. Divergence of *Lefua* sp. 2 and *L. echigonia* was estimated at 1.4 - 1.5 million years ago, whereas that of the Tokai and Kinki populations of *L. echigonia* at 0.7 million years ago [1] [7]. Despite its long history, *Lefua* sp. 2 represented low intraspecific genetic distances (**Table 3**) and low nucleotide diversity, although haplotype diversity was relatively high because a number of haplotypes with small nucleotide changes were detected (**Table 5**). Therefore, *Lefua* sp. 2 might have once reduced its population size and dispersed recently from the relic area(s), which is not the case when its

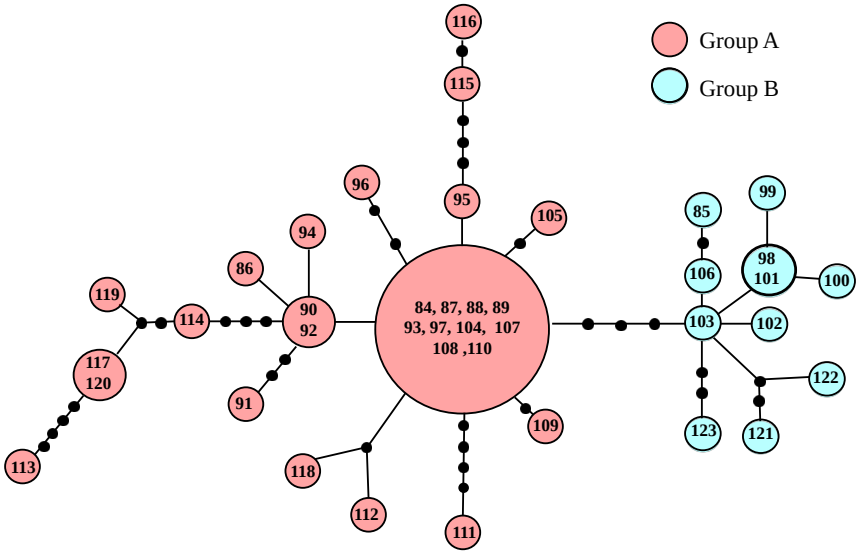

Figure 5. Minimum spanning tree of 28 haplotypes in *L. echigonia* Circles indicate haplotypes and their dimensions are proportional to the number of individuals possessing the same haplotype. Black circles on lines indicate hypothetical haplotypes not found in this study. The numbers denote the sample numbers in Table 1. Dark grey (pink in the color version), Group A; pale grey (blue), Group B.

Table 5. Haplotype and nucleotide diversities in *Lefua* sp. 2 and *L. echigonia*.

	n	Haplotype diversity	Nucleotide diversity
Group 1	44	0.8721 ± 0.0340	0.003135 ± 0.001901
Group 2	25	0.9444 ± 0.0295	0.004082 ± 0.002401
Group 3	14	0.9778 ± 0.0540	0.008539 ± 0.004956
Group A	29	0.8783 ± 0.0595	0.005178 ± 0.002926
Group B	11	0.9818 ± 0.0463	0.003649 ± 0.002305

n, number of samples.

evolutionary rate has been extremely slow. However, specimens from the Yahagi River system were moderately differentiated from those from the Toyo and Tenryu River systems, although it is difficult to distinguish definitely specimens between the Toyo and Tenryu River systems. Thus, we can consider that *Lefua* sp. 2 consists of two natural populations, that is, the Yahagi River population and the Toyo-Tenryu River population.

Star-like relationships of haplotypes in *L. echigonia* suggested the dispersal origin located in eastern Aichi prefecture. This does not look consistent with our assumption that *L. echigonia* invaded the Tokai region from the western habitat, near the boundary between the distributional ranges of the Tokai and Kinki populations. The invasion was caused by erosion of the western flanking zone of MLT and the development of relatively flat terrain more suitable for *L. echigonia* than for *Lefua* sp. 2. However, it is possible that the dispersal origin of the former western habitat has already been lost, possibly because of destruction by human activities.

In *Lefua* sp. 2, the specimens from Gotengawa, Takihashigawa, Fukayagawa, Nanegawa, and Nagaishigawa in the northwestern Tenryu River system were more closely related to those in the Toyo River system than to those in the southeastern Tenryu River system. The distance between tributaries of the northwestern Tenryu River and northeastern Toyo River is less than 1.5 km. Therefore, we assumed that *Lefua* sp. 2 was introduced from the Toyo River to Tenryu River by river capturing. This provides a biological clue in resolving whether river capturing between the Tenryu and Toyo Rivers occurred previously. River capturing has been suggested based on river trajectories, while Ikeda [24] insisted from research of riverine sediments that there was no river capturing between the two rivers. The present study indicated that at least a northeastern part of the Toyo River was captured by a northwestern part of the Tenryu River. The specimen from Aderagawa in the Toyo River system was exceptionally included in Group 3. The collection site of Aderagawa is very close to the tributary of Tenryu River. This also suggests river capturing between the Toyo and Tenryu Rivers, although we cannot eliminate the possibility of artificial transplantation of *Lefua* sp. 2 from the Tenryu River to Toyo River. Nevertheless, the present results suggest that the Toyo River and Tenryu River systems form a single population through gene flow facilitated by river capturing. Population genetic studies of various taxa can present more solid information of geological settings such as river capturing in the Tokai region.

The two populations should be considered as evolutionary significant units (ESUs) for the conservation of *Lefua* sp. 2. The translocation of fishes is severely prohibited by a guideline issued by the Ichthyological Society of Japan; however, there are some problems to follow this guideline. One of them is how to define ESUs. We consider it important to regard a natural population as an ESU. Many fish habitats have been fragmented by anthropogenic activities. Especially for fishes inhabiting montane streams, such as *Lefua* sp. 2, dam and weirs construction is critical by preventing fishes from returning upstream to their original habitats when they are displaced downstream by strong currents after heavy rains. Individuals are often isolated from each other and divided into small assemblages, and thus a natural population is destroyed by the obstruction of gene flow. Small assemblages are susceptible to bottlenecking, and haplotypes included in an original population can be fixed in different manners in different tributaries. When assemblages are investigated using genetic makers, they can be recognized as genetically distinct and thus reasoned to be assigned to different ESUs. However, they are small anthropogenic populations, but not natural populations. In this case, translocation is prohibited based on anthropogenic but not natural grounds. Next is how finely ESUs should be defined. We still have genetic markers separating organisms up to the individual level, and thus we can define ESUs more finely using faster-evolving genetic markers. However, it is very difficult or impossible and even impractical to conserve so many finely defined ESUs. We have to resolve these problems to adopt the best procedure for the fish conservation. Specimens of *L. echigonia* from the Tokai region showed lower genetic dif-

ferences between Group A and B (0.008 in Table 4) than those of *Lefua* sp. 2 between Group 1 and Group 2 or 3 (over 0.010 in Table 3). Presently we cannot say whether two groups of *L. echigonia* can be regarded as ESUs.

Many Japanese freshwater fishes are facing to extinction. Urgent measures appropriate to each fish species are indispensable to protect them and guide lines are needed to be further refined. The present study contributes to protection of endangered eight-barbel loaches by giving fundamental genetic information and defining new ESUs in *Lefua* sp. 2. Studies such as the present study are crucial and will together lead to comprehensive conservation of Japanese freshwater fishes in future.

Acknowledgements

We express our sincere thanks to Mr. Michikazu Matsuda, Mr. Masayoshi Sugiura, Dr Seiichi Mori, Dr. Kazumi Hosoya, Mr. Tesuo Kamei, Mrs. Yumiko Yamashina, Mrs. Keiko Muraoka, Mr. Osamu Inaba, Mr. Koichi Kirihara, and Mr. Sakae Nakamura for their assistance in collecting loaches and for providing useful information on the biology of *Lefua* loaches.

References

[1] Mihara, M., Sakai, T., Nakao, K., Martins, L. de O., Hosoya, K. and Miyazaki, J.-I. (2005) Phylogeography of Loaches of the Genus *Lefua* (Balitoridae, Cypriniformes) Inferred from Mitochondrial DNA Sequences. *Zoological Science*, **22**, 157-168. https://doi.org/10.2108/zsj.22.157

[2] Shedko, S.V., Miroshnichenko, I.L. and Nemkova, G.A. (2008) On the Systematics and Phylogeography of Eight-Barbel Loaches of the Genus *Lefua* (Cobitoidea: Nemacheilidae) mtDNA Typing of *L. pleskei*. *Russian Journal of Genetics*, **44**, 817-825. https://doi.org/10.1134/S1022795408070090

[3] Hosoya, K. (1994) *Lefua echigonia*. In: The Fisheries Agency, Ed., *Basic Data on Threatened Wild Aquatic Organism in Japan-I*, The Fisheries Agency, Tokyo, 386-391. (In Japanese)

[4] Hosoya, K. (2014) *Lefua echigonia* and *Lefua* sp. 1. In: Ministry of the Environment, Ed., *Red Data Book* 2014-*Threatened Wildlife of Japan*-, Gyosei Corporation, Tokyo, 196-199. (In Japanese)

[5] Yamashina, Y., Kamei, T. and Hosoya, K. (1994) Preliminary Report on Two *Lefua* Species Obtained from Hikami District. *Hyogo Freshwater Biology*, **45**, 5-11. (In Japanese)

[6] Miyazaki, J.-I., Nakao, K., Mihara, M., Sakai, T., Gunji, Y., Tojo, K., Muraoka, K. and Hosoya, K. (2007) Incongruence between mtDNA Phylogeny and Morphological and Ecological Characters in Loaches of the Genus *Lefua* (Balitoridae, Cypriniformes). *Zoological Science*, **24**, 666-675. https://doi.org/10.2108/zsj.24.666

[7] Miyazaki, J.-I., Dobashi, M., Tamura, T., Beppu, S., Sakai, T., Mihara, M. and Hosoya, K. (2011) Parallel Evolution in Eight-Barbel Loaches of the Genus *Lefua* (Balitoridae, Cypriniformes) Revealed by Mitochondrial and Nuclear DNA Phylogenies. *Molecular Phylogenetics and Evolution*, **60**, 416-427. https://doi.org/10.1016/j.ympev.2011.05.005

[8] Hosoya, K. (2013) Cobitidae. In: Nakabo, T., Ed., *Fishes of Japan with Pictorial Keys to the Species*, 3rd Edition, Tokai University Press, Hadano, 328-334, 1819-1822. (In

Japanese)

[9] Okada, Y. and Seiishi, R. (1938) Studies on the Early Life History of Nine Species of Fresh-Water Fishes. *Bulletin of the Biogeographical Society of Japan*, **8**, 223-253.

[10] Miyadi, D., Kawanabe, H. and Mizuno, N. (1976) *Lefua echigonia*. In: *Colored Illustrations of the Freshwater Fishes of Japan*, Hoikusha, Osaka, 254-256. (In Japanese)

[11] Hosoya, K. (1988) *Lefua echigonia* Jordan et Richardson. In: Okiyama, M., Ed., *An Atlas of the Early Stage Fishes in Japan*, Tokai University Press, Tokyo, 272-276.

[12] Aoyama, S. and Doi, T. (2011) Morphological Comparison of Early Stages of Two Japanese Species of Eight-Barbel Loaches: *Lefua echigonia* and *Lefua* sp. (Nemacheilidae). *Folia Zoologica*, **60**, 355-361.

[13] Kuwahara, T. (2014) *L. nikkonis*. In: Ministry of the Environment, Ed., *Red Data Book* 2014-*Threatened Wildlife of Japan*-, Gyosei Corporation, Tokyo, 194-195. (In Japanese)

[14] Miyazaki, J.-I. (2014) *Lefua* sp. 2. In: Ministry of the Environment, Ed., *Red Data Book* 2014-*Threatened Wildlife of Japan*-, Gyosei Corporation, Tokyo, 200-201. (In Japanese)

[15] Sano, I., Kirikane, M., Ogata, K., Kobayshi, K., Tojo, K. and Miyazaki, J.-I. (2016) Phylogeny in Eight-Barbel Loaches of the Genus *Lefua* (Balitoridae, Cypriniformes) Based on Nucleotide Sequences of the Mitochondrial Cytochrome *b* Gene. *Bulletin of the Faculty of Education* (*University Yamanashi*), **1**, 183-192. (In Japanese)

[16] Sakai, T., Mihara, M., Shitara, H., Yonekawa, H., Hosoya, K. and Miyazaki, J.-I. (2003) Phylogenetic Relationships and Intraspecific Variations of Loaches of the Genus *Lefua* (Balitoridae, Cypriniformes). *Zoological Science*, **20**, 501-514. https://doi.org/10.2108/zsj.20.501

[17] Moritz, C. (1994) Defining "Evolutionarily Significant Units" for Conservation. *Trends in Ecology & Evolution*, **9**, 373-375. https://doi.org/10.1016/0169-5347(94)90057-4

[18] Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, **30**, 2725-2729. https://doi.org/10.1093/molbev/mst197

[19] Swofford, D.L. (2002) PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4.0 Beta 10. Sinauer Associates, Sunderland.

[20] Kimura, M. (1980) A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide Sequences. *Journal of Molecular Evolution*, **16**, 111-120. https://doi.org/10.1007/BF01731581

[21] Huelsenbeck, J.P., Ronquist, F., Nielsen, R. and Bollback, J.P. (2003) Bayesian Inference of Phylogeny and Its Impact on Evolutionary Biology. *Science*, **294**, 2310-2314. https://doi.org/10.1126/science.1065889

[22] Posada, D. and Buckley, T.R. (2004) Model Selection and Model Averaging in Phylogenetics: Advantages of the AIC and Bayesian Approaches over Likelihood Ratio Tests. *Systematic Biology*, **53**, 793-808. https://doi.org/10.1080/10635150490522304

[23] Excoffier, L. and Lischer, H.E.L. (2010) Arlequin Suite Ver 3.5: A New Series of Programs to Perform Population Genetics Analyses under Linux and Windows. *Molecular Ecology Resources*, **10**, 564-567. https://doi.org/10.1111/j.1755-0998.2010.02847.x

[24] Ikeda, Y. (1971) Kasen No Soudatu To Teni. *Kenkyu Kiyou of Aichi Prefectural Education Center*, **45**, 107-136. (In Japanese)

Scientific Research Publishing

Submit or recommend next manuscript to SCIRP and we will provide best service for you:

Accepting pre-submission inquiries through Email, Facebook, LinkedIn, Twitter, etc.
A wide selection of journals (inclusive of 9 subjects, more than 200 journals)
Providing 24-hour high-quality service
User-friendly online submission system
Fair and swift peer-review system
Efficient typesetting and proofreading procedure
Display of the result of downloads and visits, as well as the number of cited articles
Maximum dissemination of your research work

Submit your manuscript at: http://papersubmission.scirp.org/
Or contact jwarp@scirp.org