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Genetic characteristics of local populations of the fluvial eight-barbel loach, *Lefua* sp., in Tottori and Okayama Prefectures

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The fluvial eight-barbel loach, *Lefua* sp., has been included in the Red Data Book as an endangered species (IB) by the Ministry of the Environment of Japan. Previous population genetic studies have clarified the existence of two genetically distinguished populations within this species (Sanyo group and Kii-Shikoku group). This species had not been discovered in Tottori Prefecture till 2003 when one individual was collected, and after that, no individual has been discovered. In 2008, the author captured six individuals in a tributary of the Sendai River system in Tottori Prefecture. In this study, the six individuals from Tottori Prefecture were genetically examined and, for comparison, individuals from Okayama Prefecture captured in 2008 were also examined. The obtained DNA sequence data were compared also with the data reported in previous studies. The result suggests that the population of *L.* sp. inhabiting the Sendai River system was a local endemic population that was genetically different from other populations found in neighboring areas.

Keywords
Fluvial eight-barbel loach, *Lefua* sp., genetic differences, local populations, Tottori Prefecture

1 Introduction

The fluvial eight-barbel loach, *Lefua* sp. (sensu Hosoya, 1993)[1] has been reported to be distributed along the coastal slopes facing the Seto Inland Sea from Wakayama through Okayama Prefecture and from Tokushima through Ehime Prefecture, along the coast of the Sea of Japan from Fukui through Hyogo Prefecture, in Aichi Prefecture, and in Shizuoka Prefecture[1]. The fluvial eight-barbel loach is considered to be a rare species. So, this species has been included in the Red Data Book[2] as an endangered species by the Ministry of the Environment of Japan. It has also been de- signated as a vulnerable or threatened status in the red list of each prefecture where its existence has been confirmed.

A population genetic study of this species was conducted by Mihara et al.[3]. It reported the existence of two genetically distinguished populations within this species (Sanyo and Kii-Shikoku).

It had not been officially confirmed whether *Lefua* sp. exists in Tottori Prefecture, until one individual of this species was discovered in the Hatto River of the Sendai River system in Tottori Prefecture in 2003 by Hara[4]. Then, in 2008, Kobayashi discovered and collected six individuals in the Minari River of the Sendai River system in Tottori Prefecture (data unpublished). This habitat was located at the westernmost margin of the distribution range along the coast of the Sea of Japan.

In this study the six individuals collected by the author were used as specimens for DNA analysis. Specimens were also collected from a tributary of the Yoshii River system in Okayama

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Prefecture, which is located opposite to the Sendai River system across the Chugoku Mountains. Inhabiting of this species in the Yoshii River system was reported by Kobayashi and Kobayashi[5] and Mihara et al. [3].

In order to understand the genetic characteristics of the Tottori population, the nucleotide sequences of control regions [displacement-loop (D-loop) regions] in the mitochondrial DNA of samples collected in the Tottori and Okayama Prefectures were determined. The obtained DNA sequence data were then compared with the data reported by Mihara et al [3].

2 Materials and methods

2.1 Materials

The samples analyzed in this study consisted of 17 specimens collected from a location in Tottori Prefecture and 3 locations in Okayama Prefecture on 22nd of March 2008 (Fig. 1, Table 1). The results were compared with the nucleotide sequence data of 13 specimens reported by Mihara et al.[3], who reported data collected from 19 specimens of this species. However, among them, six specimens, SHIRAKAGAWA, NATASYO, KASUGA, SUMOTO, NAKATSU, and IYOMISHIMA were excluded, because the nucleotide sequence data of SHIRAKAGAWA were not registered in the DNA Data Bank of Japan (DDBJ), and the other five specimens each contained some ambiguous site in their nucleotide sequence.

The small numbers of specimens captured in each population (2-6) were judged not to increase the probability of the extinction of each population because they were obtained only narrow areas in those where the fish were found. I released some of specimens to their populations, and will keep a specimen from each population in formalin after observation of the behavior.

2.2 Methods

A piece of about five millimeters by two millimeters of the caudal fin of each individual fish were excised at the sampling site to use as a sample. DNA was extracted from the sample by using the DNeasy Tissue Kit (QIAGEN, USA).

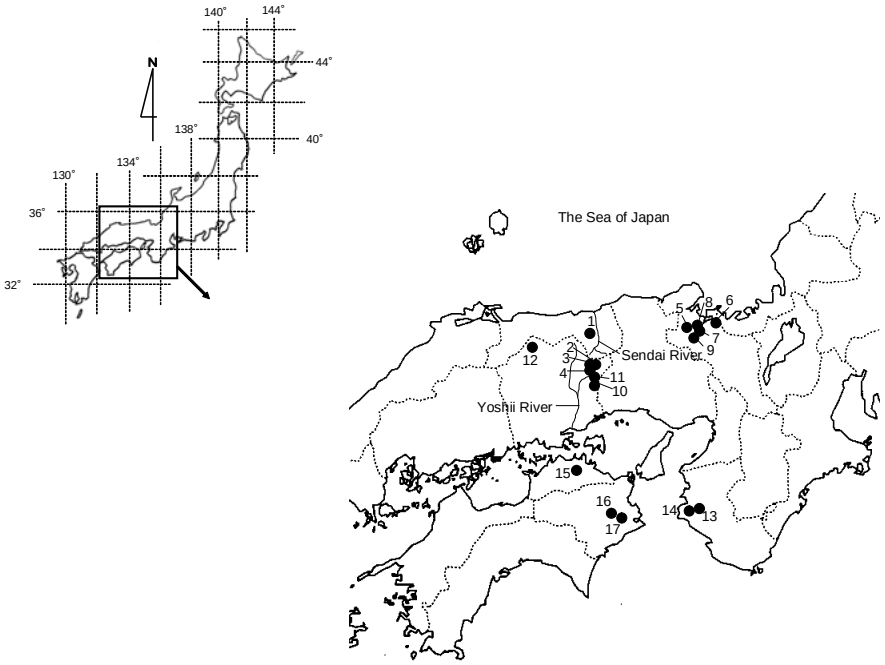

Fig. 1 Collection sites of *L.* sp.
Sites 1-4 are the collection sites of specimens analyzed in this study, and sites 5-17 are the collection sites of specimens used in Mihara et al. (2005)[3].

Table 1 Sample list.

No.	Collection site	River of collection site	Number of specimens
1	Mochigase, Tottori	Minari River of Sendai River system	6
2	Ute, Mimasaka, Okayama #1	Ute River of Yoshii River system	6
3	Ute, Mimasaka, Okayama #2	Ute River of Yoshii River system	3
4	Higashidani, Mimasaka, Okayama	Higashidani River of Yoshii River	2
5	Ooe, Fukuchiyama, Kyoto		1
6	Matsuo, Maizuru, Kyoto		1
7	Kumida, Kyoto		1
8	Aoi, Maizuru, Kyoto		1
9	Nishikata, Ayabe, Kyoto		1
10	Enohara, Mimasaka, Okayama		1
11	Ohara, Mimasaka, Okayama		1
12	Hiruzen, Maniwa, Okayama		1
13	Hidaka, Hidaka, Wakayama		1
14	Arita, Aritagawa, Wakayama		1
15	Shioe, Takamatsu, Kagawa		1
16	Miyame, Tokushima		1
17	Sanakouchi, Tokushima		1

Specimens of No. 1-4 were examined in the present study, and the data of No. 5-17 were from Mihara et al. (2005)[3].

Table 2 Nucleotide substitution site of each haplotype.

Haplotype	Position of nucleotides													
	91	109	132	134	137	164	178	207	365	373	542	791	848	879
Lsp 01	C	A	T	T	G	G	G	—	T	A	T	A	A	G
Lsp 02	·	·	·	·	·	·	A	·	·	·	C	·	·	A
Lsp 03	·	·	·	·	·	·	·	·	·	·	C	·	·	A
Lsp 04	T	·	·	·	·	·	·	T	·	·	·	·	·	·
Lsp 05	·	G	C	A	·	A	·	T	C	G	C	G	G	A
Lsp 06	·	G	C	C	T	·	·	T	C	·	·	G	G	·

Dot (·) shows the nucleotide identical to that of Lsp 01.
Hyhen (-) shows nucleotide insertion/deletion.

Table 3 Frequency of haplotypes in each population.

Population	Haplotype						Total number of specimens
	Lsp 01	Lsp 02	Lsp 03	Lsp 04	Lsp 05	Lsp 06	
Mochigase, Tottori	2	3	1				6
Ute, Mimasaka, Okayama #1					6		6
Ute, Mimasaka, Okayama#2				1	2		3
Higashidani, Mimasaka, Okayama						2	2

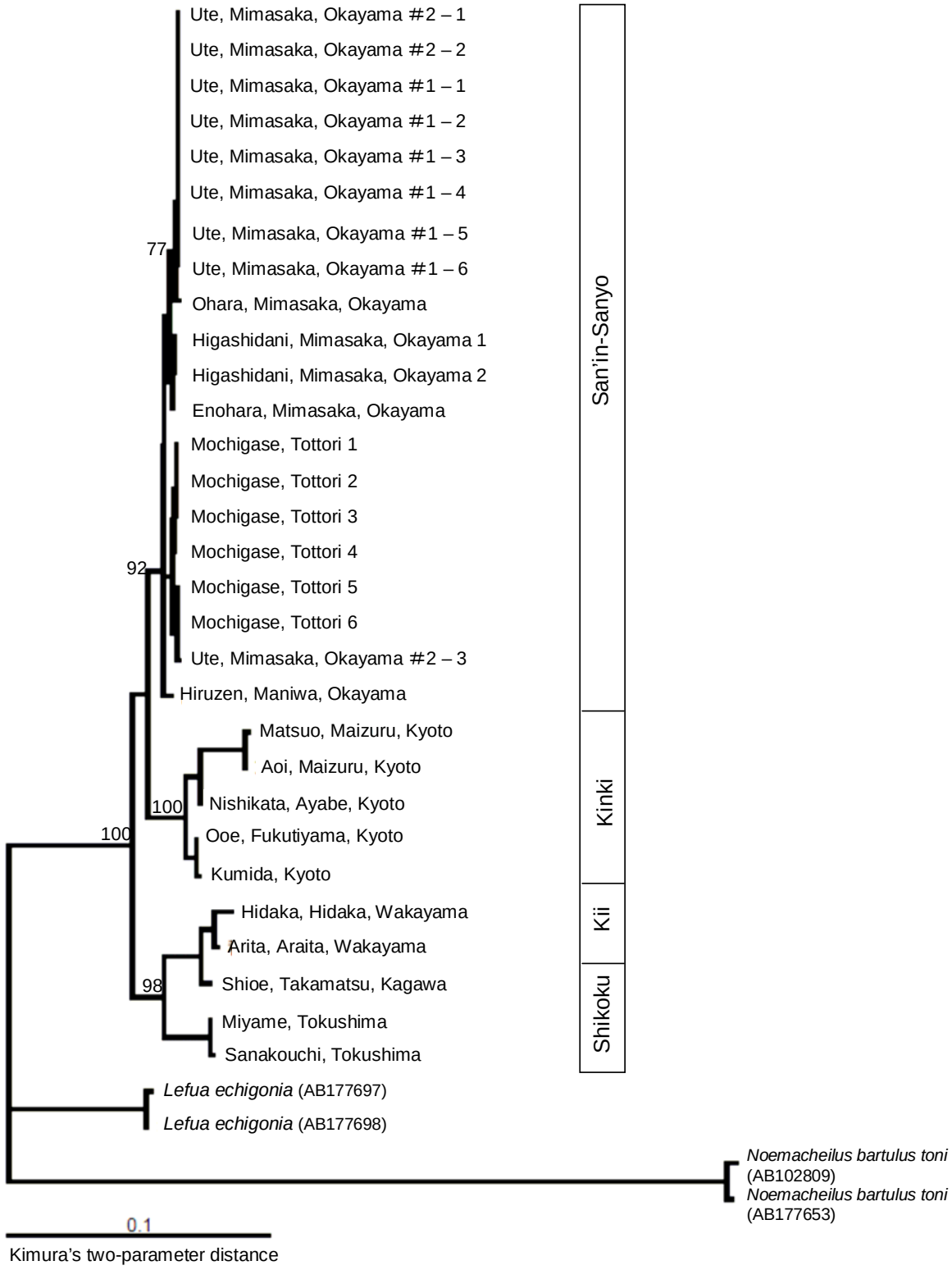

Fig. 2 Molecular phylogenetic tree for mt DNA haplotypes constructed by the neighbor-joining method.
Numbers on the branches are bootstrap probabilities (%). Numbers in parentheses after *Lefua echigonia* and *Noemacheilus barbatulus toni* are accession numbers of DDBJ.

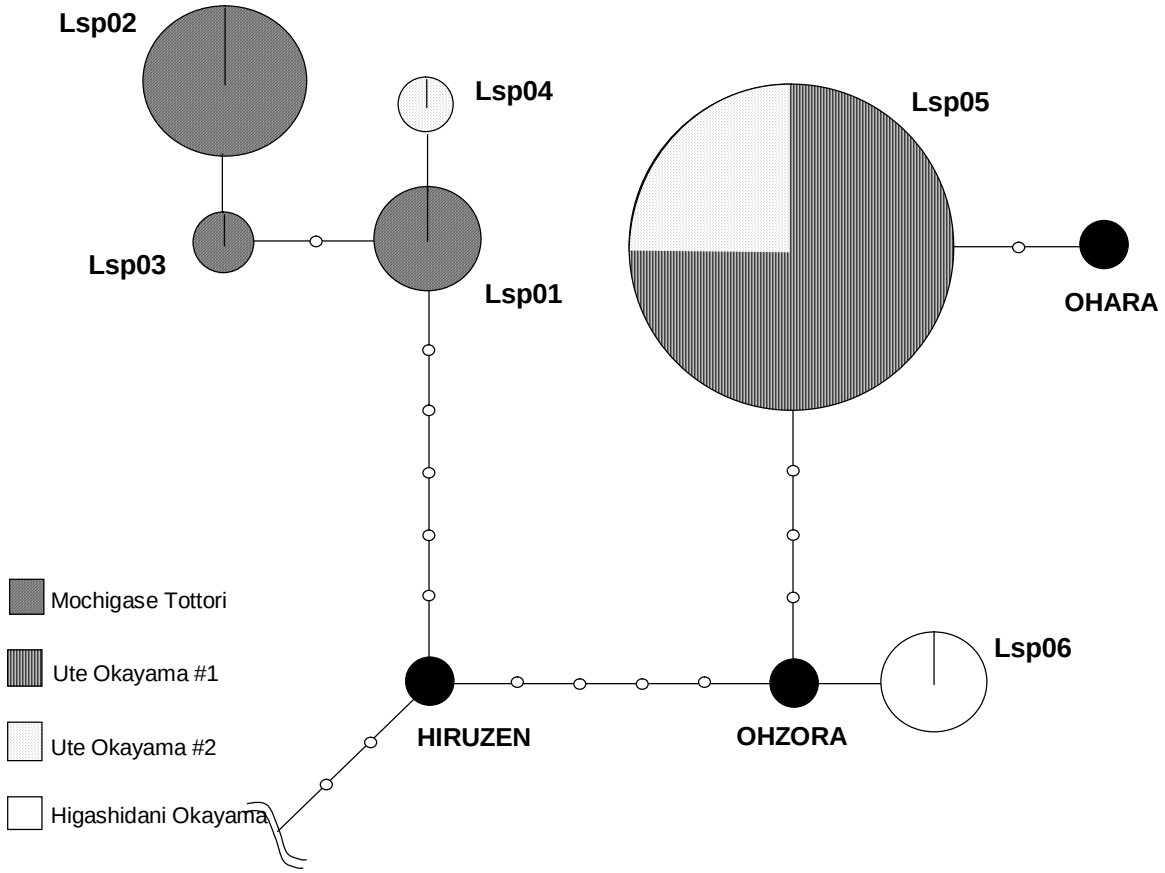

Fig. 3 Network of mt DNA haplotypes detected from a population in Tottori Prefecture and three populations of Okayama Prefecture.
Black circles mean haplotypes found in three populations reported in Mihara et al.(2005)[3]. Size of each circle is proportional to number of individuals. One division (a part between two small white dots) in the branch shows one substitution.

The extracted DNA was used as a template for the polymerase chain reaction (PCR) technique, and the entire D-loop region of the mitochondrial DNA was amplified. The nucleotide sequence was then determined by the direct sequencing method, in which the amplified PCR product was used as a direct template. The primer set for the PCR and sequencing techniques used in this study was the same as that used by Sakai et al.[6].

The nucleotide sequence data of the 17 specimens were analyzed in comparison with the nucleotide sequence data of the 13 specimens reported by Mihara et al.[3]. An alignment was performed using the ClustalW software (DDBJ version)[7]. After the alignment, polymorphism sites were arranged by executing SITES [8].

Moreover, using the Arlequin software (ver. 2.0)[9], the fixation index Fst (a measure of the genetic difference between populations) and the nucleotide diversity were calculated. Furthermore, using the Phylogeny Inference Package (PHYLIP, ver. 3.57)[10], the genetic distance was calculated by Kimura's two-parameter method, and a molecular phylogenetic tree was constructed by the neighbor-joining method. In order to construct the molecular phylogenetic tree, the nucleotide sequence data of *L. echigonia* and *Noemacheilus barbatulus toni* were also used as outgroup data[3].

3 Results and Discussion

The nucleotide sequences of the entire D-loop regions of the mitochondrial DNA of all the 17 specimens were determined. The nucleotide sequences were found to be 909-910 bp long. On the basis of the nucleotide sequence alignment results, a nucleotide insertion/deletion site and 13 nucleotide substitution sites were recognized (Table 2).

Six haplotypes from Lsp01 to Lsp06 were detected (Tables 2, 3; DDBJ access numbers AB468988-469004.). In the population "Ute, Mimasaka, Okayama #1", all the six individuals had the same haplotype Lsp05, and this haplotype was also found in the adjacent population "Ute, Mimasaka, Okayama #2". Each of the other haplotypes was endemic to one population.

The high Fst value (0.9095) was shown between two populations "Mochigase, Tottori" and "Ute, Mimasaka, Okayama #1" ($p<0.01$).

As shown in the molecular phylogenetic tree and network of the haplotypes (Fig. 2, 3), all the samples collected in the Chugoku area belonged to the same cluster.

Moreover, except for one individual from the population "Ute, Mimasaka, Okayama #2", all the samples collected from the same river system tended to form a cluster. This tendency was supported by a high bootstrap probability (Fig. 2).

These results suggest that the population of *Lefua* sp. inhabiting the Sendai River system was a local endemic population that was genetically different from other populations found in neighboring areas.

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鳥取県と岡山県における ナガレホトケドジョウの遺伝的特性

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ナガレホトケドジョウ *Lefua* sp. は，環境省の絶滅危惧種（IB 指定）としてレッドデータブックにも記載されている．本種に関するこれまでの個体群遺伝学的な研究は，本種が遺伝的に二つのグループ（山陽群と紀伊－四国群）に分かれることを明らかにしてきた．本種は，鳥取県では，2003 年に 1 個体が捕獲されるまで発見の報告がなく，それ以後，1 個体も発見されてこなかった．筆者は 2007 年に鳥取県の千代川の，ある支流で本種 6 個体を捕獲した．本論文では，鳥取県で捕獲されたこれら 6 個体と，比較のために岡山県で捕獲された 11 個体を遺伝的に調べた．得られた DNA 分析の結果は，これまでの研究で報告されている DNA 分析データとも比較された．今回の研究結果は，鳥取県の千代川水系に生息している本種の個体群は，近隣県の個体群とは遺伝的に異なった地域固有群であることを示すものであった．

キーワード ナガレホトケドジョウ，*Lefua* sp.，遺伝的差異，鳥取県

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