論文 Article

Genetic relationships of *Tribolodon hakonensis* in Japan

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日本産ウグイの遺伝的関係

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Abstract: This study examined fifty-four samples of *Tribolodon hakonensis*, collected from 43 river basins in Japan, for genetic relationships based on DNA sequences of a part (709bp) in the mitochondrial cytochrome b region. A total of 35 haplotypes were identified. The average genetic distance was found to be highest in the Tokai-Hokuriku-Kinki region and lowest in the Chugoku and Kyushu regions. Haplotype diversity was highest in the Hokkaido region and lowest in the Chugoku region. In the Shikoku region, all haplotypes were recorded only in this region. Three groups were recognized in the network: Hokkaido, Tohoku, Kanto-Koshinetsu, and Tokai-Hokuriku-Kinki; Tohoku and Kanto-Koshinetsu; and Tokai-Hokuriku-Kinki, Chugoku, Shikoku, and Kyushu. In addition, two large clades were revealed in the genetic tree with a border line connecting the Wakasa Bay in the Sea of Japan side and the Ise Bay on the Pacific Ocean side, although some haplotypes were distributed across the Itoigawa-Shizuoka tectonic line. Furthermore, deeply differentiated lineages were observed in the upper reaches of large river basins, such as the Kitakami, Kumano, and Niyodo River basins.
Keywords: Genetic relationship, Cyt*b*, *Tribolodon hakonensis*, Japan

要旨：北海道から九州に至る 43 水系で採集された 54 個体のウグイについて，ミトコンドリア・チトクロム *b* 領域の一部（709bp）の塩基配列を調べた結果，計 35 のハプロタイプがみられた。平均遺伝子距離は東海－北陸－近畿地方で最も高く，中国および九州地方で最も低く，ハプロタイプ多様度は北海道で最も高く，中国地方で最も低かった。四国地方ではすべてのハプロタイプが本地方のみで見られたものであった。ネットワーク解析では，北海道－東北－関東甲信越－東海北陸近畿・東北－関東甲信越・東海北陸近畿－中国－四国－九州の 3 群に分かれた。ハプロタイプ系統樹では若狭湾－伊勢湾を結ぶ線を境に大きく東西のグループに分かれたが，糸魚川－静岡構造線を跨ぐハプロタイプが見られた。さらに北上川・熊野川・仁淀川水系などの大河川の上流域に深く分岐した系統が見られることが明らかになった。
キーワード：遺伝的関係，チトクロム *b*，ウグイ，日本

Ⅰ. Introduction

Four species of far eastern daces belonging to genus *Tribolodon* (Cyprinidae) are distributed in Japan: *T. sachalinensis* and *T. nakamurai*, both strictly freshwater species with relatively smaller geographic ranges and *T. brandtii* and *T. hakonensis*, both wider-ranging diadromous species (Sakai ,1989). Among these, *T. hakonensis* is widely distributed in Japan and lives in a wide variety of environments including rivers, lakes and marine waters. It shows an ecological plasticity, and is divided into two groups: freshwater and diadromous populations. Nevertheless, rapid shrinking of freshwater population of *T. hakonensis* has been observed in many rivers in Japanese Archipelagoes, and the causes of this phenomenon are required to be clarified by freshwater fisherman.

Watanabe et al. (2018) examined partial mitochondrial DNA sequences especially of river basins of eastern regions of Japan and those flowing into the Sea of Japan in Sakhalin to Korean Peninsula. He recognized 6 genetic groups of this species distributed in far-east areas, among which haplotype groups TH1, TH3 and TH6 comprise Japanese, Russian and/or Korean samples and TH2, TH4 and TH5 comprise only Japanese

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samples. They also suggested the western Japan population originating from the invaders of continental populations via Korean Peninsula.

Besides, the distribution of organisms is well known to be limited by some geological factors. In Japan, 'Fossa Magna' region, particularly the western limit of this region, 'Itoigawa Shizuoka tectonic line', is a representative of this factor (Iguchi, 2018). According to Watanabe et al. (2018), a haplotype group TH4 of *T. hakonensis*, was distributed across the Fossa Magna' region. Long mountain range or chain is also a representative. Watanabe et al. (2018) reported a division of dace population into 2 large groups by Suzuka Mountains.

In Watanabe et al, (2018), however, genetic diversity of *T. hakonensis* was not compared among the regions in Japan. Besides, genetic relationships have not been clarified yet between the populations of rivers flowing into the Sea of Japan and those flowing into the Seto Inland Sea/Pacific Ocean. Although the origin of Japanese dace has been estimated to be a population of the supposed freshwater 'Sea of Japan Lake' (Nishimura, 1980), based on the present distribution of *Tribolodon* confined to the surrounding areas of the Sea of Japan, this hypothesis has not yet been confirmed. Furthermore, the factors determining genetic differentiation level were not estimated in relation to scale of river basin or geologic events in the past.

In this study, we tried to clarify genetic relationships of this dace species, *T. hakonensis*, most widely distributed in Japan, and to compare genetic diversity and differentiation between populations of different geographical regions based on the DNA sequences of the cytochrome *b* region of mitochondrial DNA. Besides, we discussed on the factors determining the genetic relationship and diversity and on the origin of this species in Japan from the viewpoints of geographical and geological border lines.

Ⅱ. Materials and methods

1. Samples

We collected dace samples at 45 sites of a total of 41 river basins from Hokkaido to Kagoshima prefecture (Fig. 1) in March to September in 2015-2017. We

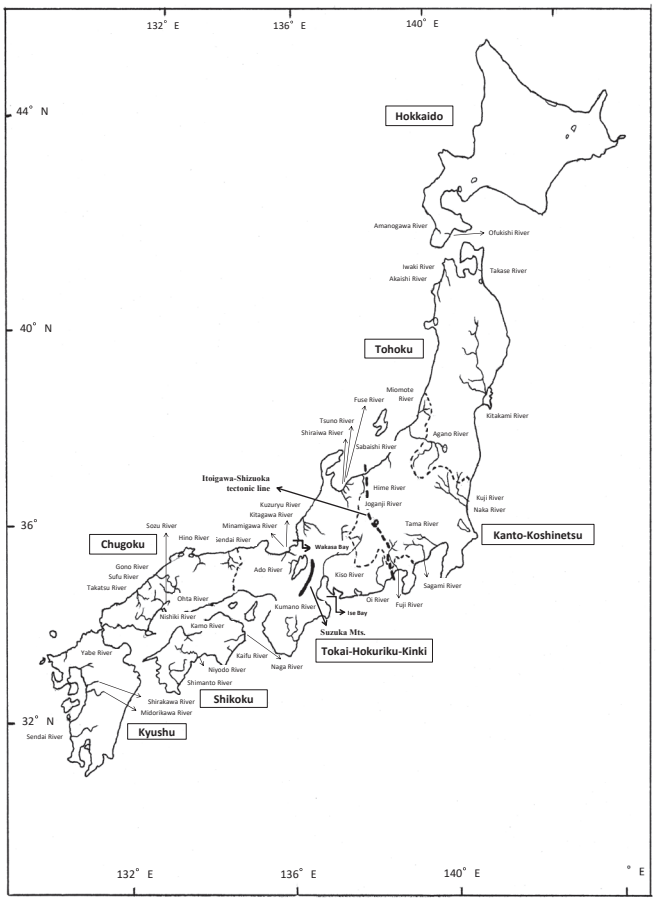

Fig. 1 Map of Japanese Archipelagoes, showing 43 river basins a geological line and a mountain chain.

identified the species of daces according to the description in Sakai (1989).

We performed a sampling by fishing using earthworm or fly larvae as baits. Samples were transported to the laboratory as a live form using a potable aeration system. After morphological identification, a fin clip was cut from the caudal fin and stored in an Eppendorf tube at -20 ℃ until use.

2. DNA extraction, PCR and sequencing

Template DNA was prepared from 1 to 3 individuals at each site using DNeasy Tissue Kit (Quiagen, Tokyo, Japan), according to the manufacturer's instruction.

The cytochrome *b* region (1141bp) of mitochondrial DNA was amplified by PCR with a mixture of a template DNA (10 ng) and primers L14690-Cb-AH (5'-GGTCATAATTCTTGCGA-3'; Mukai, 2008) and H15913-Thr-AH (5'-CCGATCTTCGGATTACAAGACCG-3'; Mukai, 2008) by using the TaKaRa PCR Amplification kit (TaKaRa, Ohtsu, Japan) in a thermal cycler (Mastercycler personal; Eppendorf, Hamburg, Germany) using the following protocol: preheating at 94℃ for 11 min, followed by 35 cycles of denaturation at 94℃ for 30 s → annealing at 52℃ for 40 s → extension at 72 ℃ for 1 min and a final extension at 72 ℃ for 10 min. PCR products was purified using NucleoSpin Gel and PCR cleanup (Takara, Ohtsu, Japan).

Sequencing was performed directly with the Genetic Analyzer 3130xl (Applied Biosystem, CA, USA) in the Genetic Research Center of Hiroshima University. A part (709bp) of this region was selected for analyses.

3. DNA analysis

Haplotyping, nucleotide diversity and haplotype diversity were performed by DnaSP ver. 6 (Rozas et al., 2017). Genetic distance was determined as Kimura-2 parameter using MEGA 6. Haplotype network was constructed by NETWORK 5.0.1.1. Alignment was performed by Clustal W (Thompson et al., 1994) and a genetic tree was constructed by Neighbor-Joining method using Kimura-2 parameter as a distance by MEGA 6.

Ⅲ. Results

A total of 52 samples were collected from 41 river basins. Body length was in the range of 5.7-22.3cm and body weight was in the range of 2.2-151.3g. Two open data were also used for Kiso and Kitagawa Rivers (Accession Nos. LC277218 and LC277212, respectively).

1. Haplotyping

A total of 35 haplotypes were recorded (Table 1). Among

Table 1 Haplotype composition in 43 river basins.

River (No. samples)	Branch	Haplotype
Hokkaido Region		
Amanogawa River (1)		21
Ofukishi River (1)		34
Tohoku Region		
Iwaki River (2)		24, 30
Takase River (1)		31
Akaishi River (1)		28
Kitakami River (3)	Shizukuishi River (1)	24
	Waga River (1)	32
	Eai River (1)	29
Kuji River (1)	Yamizo River	33
Agano River (1)	Aga River	27
Kanto-Koshinetsu Region		
Nakagawa River (1)		25
Tama River (1)	Akigawa River	26
Sagami River (1)	Katsura River	25
Fuji River (2)	Ashi River	23, 24
Miomote River (2)		18, 19
Sabaishi River (1)		22
Hime River (1)		20
Tokai-Hokuriku-Kinki Region		
Oigawa River (1)		15
Kiso River (1)		35
Fuse River (1)		20
Tsuno River (1)		22
Shiraiwa River (1)		23
Joganji River (1)		21
Kuzuryu River (1)	Hino River	16
Kitagawa River (1)		5
Minamigawa River (3)		17, 17, 17
Ado River (1)		2
Kumano River (1)	Shingu River	14
Chugoku Region		
Sendai River (1)	Hatto River	12
Hino River (1)	Nomoto River	11
Gono River (1)	Kannose River	10
Sufu River (1)		13
Takatsu River (2)		12, 12
Sozu River (1)		1
Ohta River (1)		6
Nishiki River (2)	Nishiki River (1)	1
	Usa River (1)	12
Shikoku Region		
Kamo River (1)		3
Naga River (1)		3
Kaifu River (1)		7
Niyodo River (1)	Omogo River	8
Shimanto River (1)	Hiromi River	9
Kyushu Region		
Yabe River (3)	Yabe River (1)	5
	Hoshino River (2)	6, 6
Shirakawa River (1)		4
Midorikawa River (1)	Makugawa River	4
Sendai River (1)		2

these, as many as 33 haplotypes were recorded at the first time in this study (Accession Nos. LC598726, LC598727, LC598728 and LC598729 for haplotypes 1-4, and Nos. LC598730, LC598731, LC598732, LC598733, LC598734, LC598735, LC598736, LC598737, LC598738, LC598739, LC598740, LC598741, LC598742, LC598743, LC598744, LC598745, LC598746, LC598747, LC598748, LC598749, LC598750, LC598751, LC598752, LC598753, LC598754, LC598755, LC598756, LC598757 and LC598758 for haplotypes 6-34, respectively). In the Hokkaido Region, 2 haplotypes were recorded, among which a haplotype, 34, was observed only in this region. Another haplotype 21 was shared by Tokai-Hokuriku-Kinki Region. In the Tohoku Region, 8 haplotypes were recorded, among which as many as 7 haplotypes were observed only in this region. Another haplotype was shared by Kanto-Koshinetsu Region. In the Kanto-Koshinetsu Region, 8 haplotypes were recorded, among which 4 haplotypes were observed only in this region. Other haplotypes were shared by Tohoku or Tokai-Hokuriku-Kinki Region. In the Tokai-Hokuriku-Kinki Region, 11 haplotypes were recorded, among which only 5 haplotypes were observed only in this region. Other haplotypes were shared by Hokkaido, Kanto-Koshinetsu or Kyushu Region. In the Chugoku Region, 6 haplotypes were recorded, among which as many as 5 haplotypes were observed only in this region. Another haplotype was shared by Kyushu Region. In the Shikoku Region, 4 haplotypes were recorded, all of which were observed only in this region. In the Kyushu Region, 4 haplotypes were recorded, among which only 1 haplotype was observed only in this region. Other haplotypes were shared by Tokai-Hokuriku-Kinki or Chugoku Region.

2. Average genetic distance, nucleotide diversity and haplotype diversity

Average genetic distance and nucleotide diversity were the highest in the Tokai-Hokuriku-Kinki Region followed by Tohoku Region (Table 2). It was the lowest in the Chugoku and Kyushu Regions and very low in the Shikoku Region. Haplotype diversity was the highest in the Kokkaido Region and the lowest in the Chugoku Region.

3. Haplotype network

In the network analysis, the structure of 'torso' was not observed (Fig. 2). Three large haplotype groups: Hokkaido, Tohoku, Kanto-Koshinetsu and Tokai-Hokuriku-Kinki (G1), Tohoku and Kanto-Koshinetsu (G2) and Tokai-Hokuriku-Kinki, Chugoku, Shikoku and Kyushu (G3), were made. Besides, G1 was divided into 2 subgroups: G1-1 (Hokkaido, Tohoku, Kanto-

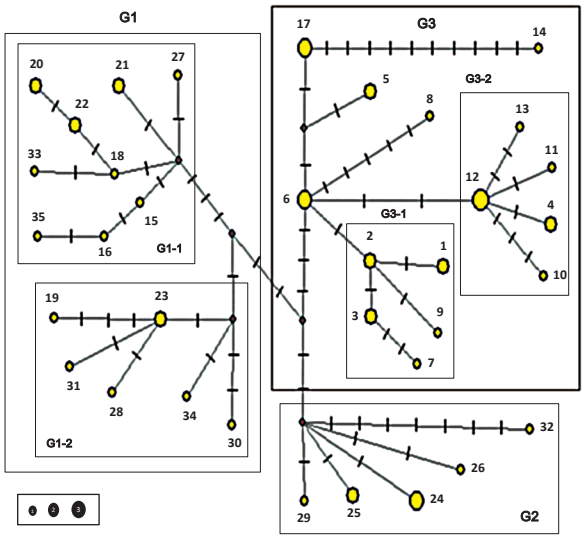

Fig. 2 A network of 35 haplotypes. Size of circle is proportional to number of samples. Vertical bars on connection lines show mutated positions. Red dots represent missing haplotypes not detected in this study. G1, haplotype group 1; G2, haplotype group 2; G3, haplotype group 3.

Table 2 Average genetic distance, nucleotide diversity and haplotype diversity in 7 regions.

Region (No. samples)	Average genetic distance	Nucleotide diversity	Haplotype diversity
Hokkaido (2)	0.009	0.009	1
Tohoku (9)	0.012	0.012	0.972
Kanto-Koshinetsu (9)	0.011	0.011	0.972
Tokai-Hokuriku-Kinki (13)	0.014	0.014	0.962
Chugoku (10)	0.004	0.004	0.844
Shikoku (5)	0.005	0.005	0.9
Kyushu (6)	0.004	0.004	0.867

Koshinetsu and Tokai-Hokuriku-Kinki) and G1-2 (Hokkaido, Tohoku, Kanto-Koshinetsu and Tokai-Hokuriku-Kinki). G3 comprised two groups: G3-1 (Tokai-Hokuriku-Kinki, Chugoku, Shikoku and Kyushu) and G3-2 (Chugoku and Kyushu). Some haplotypes: 8 (the Omogo River of the Niyodo River Basin), 14 (the Shingu River of the Kumano River Basin) and 32 (the Waga River of the Kitakami River Basin), were deeply differentiated.

4. A genetic tree of 35 haplotypes

Two large clades: an eastern clade of Hokkaido, Tohoku, Kanto-Koshinetsu and Tokai-Hokuriku-Kinki Regions and a western clade of Tokai-Hokuriku-Kinki, Chugoku, Shikoku and Kyushu Regions, were recognized (Fig. 3). In the eastern clade, haplotype 16 (the Hino River of the Kuzuryu River) and haplotype 35 (the Kiso River) were the westernmost in the Sea of Japan side and in the Pacific Ocean side, respectively. In the western clade, haplotype 5 (the Kitagawa River) and haplotype 14 (the Shingu River of the Kumano River) were the easternmost in the Sea of Japan side and in the Pacific Ocean side, respectively. The eastern clade was much more deeply differentiated than the western clade.

In the Sea of Japan side, haplotype 22, collected in the Sabaishi River of the Kanto-Koshinetsu Region, situated eastern to the Itoigawa-Sizuoka tectonic line, and haplotype 20, collected in the Hime River of the same region, situated on the Itoigawa-Sizuoka tectonic line, were both the members of the eastern clade. In the

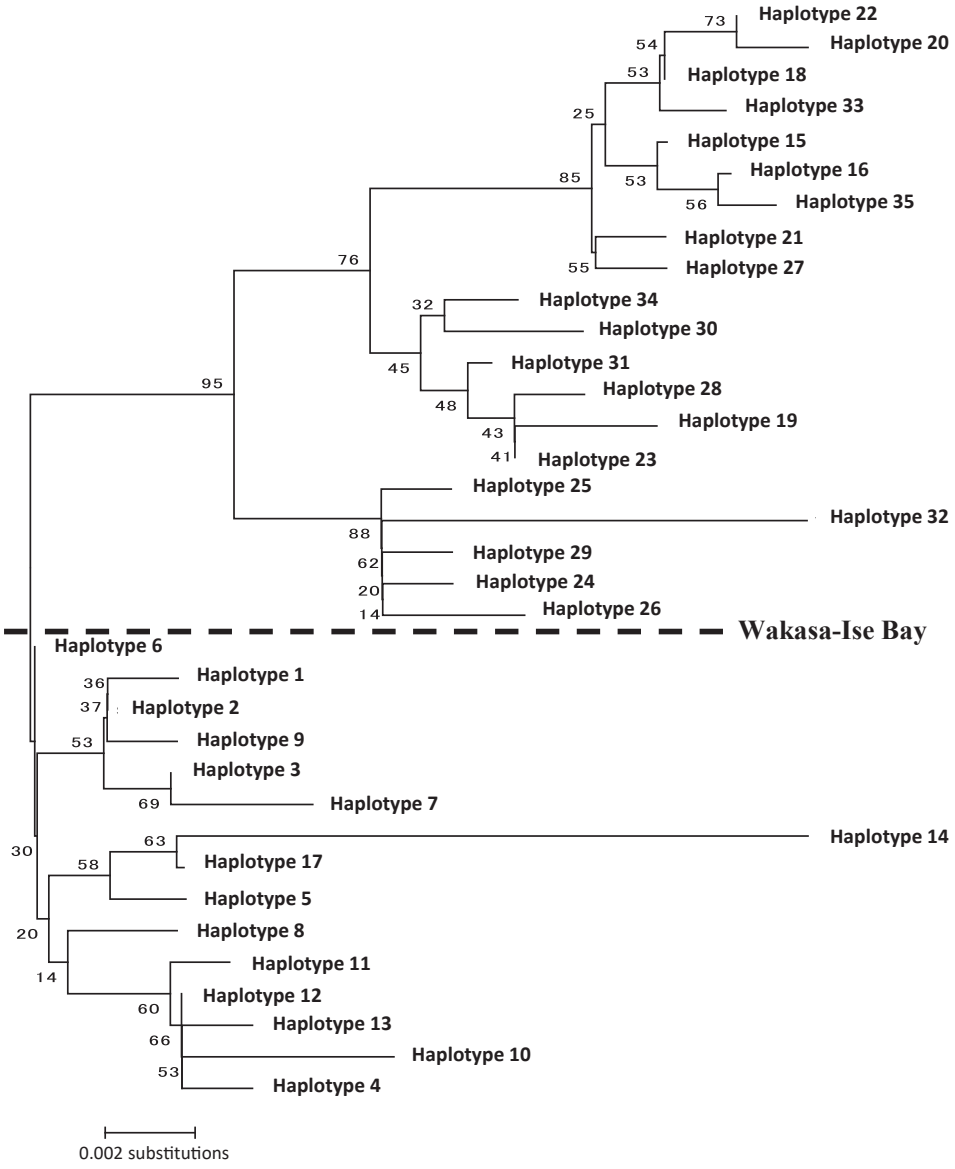

Fig. 3 A genetic tree of 35 haplotypes. Each number at a node shows a bootstrap value.

Pacific Ocean side, similarly, haplotype 24, collected in the Fuji River of the Kanto-Koshinetsu Region, situated eastern to the Itoigawa-Sizuoka tectonic line, and haplotype 15, collected in the Oigawa River of the Tokai-Hokuriku-Kinki Region, situated western to the Itoigawa-Sizuoka tectonic line, were both the members of the eastern clade. In contrast, haplotypes 16, 20, 21, 22 and 23, collected in the Fuse to Kuzuryu Rivers in the Tokai-Hokuriku-Kinki Region, were the members of the eastern clade whereas haplotypes 5 and 17, collected in the Kitagawa and Minamigawa Rivers in this region, were the members of the western clade. In addition, there were many clades constructed by both haplotypes distributed in the rivers flowing into the Sea of Japan and those distributed in the rivers flowing into the Seto Inland Sea or Pacific Ocean (clades of haplotypes 18, 20 22 and 33; haplotypes 15, 16 and 35; haplotypes 19, 23, 28 and 31; haplotypes 24, 25, 26, 29 and 32; haplotypes 5, 14 and 17). There were a few small clades constructed only by haplotypes distributed in the rivers flowing into the Sea of Japan or by those distributed in the rivers flowing into the Pacific Ocean (clades of haplotypes 21 and 27; haplotypes 3 and 7).

Ⅳ. Discussion

In this study, a total of 35 haplotypes were recorded from Hokkiado to Kagoshima prefecture. In the Tohoku Region, 8 haplotypes were recorded, among which 7 haplotypes were observed only in this region. In the Chugoku and Shikoku Regions, similarly, as many as 9 haplotypes out of 10 haplotypes were observed only in this region. Watanabe et al. (2018) also detected 10 haplotypes in the Chugoku-Shikoku Regions, among which 9 were specific to this region. Besides, 6 haplotypes out of these 9 were collected in the Shimanto River of the Shikoku Region. In the Tokai-Hokuriku-Kinki Region, in contrast, only 5 haplotypes out of 11 haplotypes were observed only in this region. This drastic difference in regional haplotype specificity might imply some differences in reproductive isolation among regions. On the other hand, average genetic distance and nucleotide diversity were the highest in the Tokai-Hokuriku-Kinki Region whereas they were the lowest in the Chigoku and Kyushu Regions in this study, although the number of samples examined was relatively high in the Chigoku Region. In addition, haplotype diversity was also high in the Tokai-Hokuriku-Kinki Region whereas it was the lowest in the Chugoku Region. This may suggest a low genetic exchange among the dace populations in the Chugoku Region, although estuarine or marine dace populations were also observed in this region by our preliminary studies. Further studies are necessary including life cycles in freshwater to estuarine areas in relation to genetic connection of exactly freshwater and diadromous populations.

In the network analysis, in this study, the structure of 'torso' was not observed. This is in accordance with no 'torso' in any of 6 haplotype groups by Watanabe et al. (2018). The absence of 'torso' might suggest a radiative derivation process of all the haplotypes from a single genetic type, although the small number of samples used in this study might also be a cause of no 'torso'. However, many missing haplotypes connecting 3 groups in this study suggest a promotion of differentiation after reproductive isolation possibly caused by low level of genetic exchange between the freshwater populations. That is, as a result of decrease in genetic diversity by 'bottle neck' effects for some reasons, a few of selected haplotypes expand their populations and then differentiation might be promoted during a long isolation period. In Watanabe et al. (2018), there were also many missing haplotypes particularly in haplotype groups TH4, distributed in the central Japan, TH5, distributed only in the western Japan, and TH6, distributed only in the coastal region of the Sea of Japan, including Russia, Korea and Japan.

On the other hand, there are multiple clades constructed by both haplotypes distributed in the rivers flowing into the Sea of Japan and those flowing into the Seto Inland Sea or Pacific Ocean in contrast to only one clade constructed only by haplotypes distributed in the rivers flowing into the Pacific Ocean. In contrast, there are a few small clades constructed only by haplotypes distributed in the rivers flowing into the Sea of Japan or by those distributed in the rivers flowing into the Pacific Ocean. Such a fact of genetic similarity between the populations of the Sea of Japan side and the Pacific Ocean side was revealed firstly by the present study, and this might be explainable by a hypothesis in Nishimura (1980) of range expansion and differentiation history of Japanese *Tribolodon* from the Sea of Japan side into the Pacific Ocean side. Indeed, Kikko et al. (2008) suggested

white-spotted char dispersion into the northern inlet rivers of Lake Biwa from adjacent inlet rivers of the Sea of Japan by watershed exchanges in the glacial periods of the Pleistocene.

In this study, some haplotypes: 8 (the Omogo River of the Niyodo River), 14 (the Shingu River of the Kumano River) and 32 (the Waga River of the Kitakami River), were prominently deeply differentiated in a network. These were all collected at the upper reaches of a long-reach tributary of the rivers with huge catchment areas, suggesting a promotion of genetic differentiation during a long history of reproductive isolation.

The Itoigawa-Sizuoka tectonic line has been recognized as a biogeographical border line (Iguchi, 2018) and there are drastic differences in geological features between the eastern and western areas to this line. Besides, active volcanic upheavals have occurred in the northern regions along this line during this 1Myr. In this study, however, haplotype 22, collected in the Sabaishi River of the Kanto-Koshinetsu Region, situated eastern to the Itoigawa-Sizuoka tectonic line, and haplotype 20, collected in the Hime River of the Tokai-Hokuriku-Kinki Region, situated on this line, were both the members of the eastern clade. Similarly, haplotype 24, collected in the Fuji River of the Kanto-Koshinetsu Region, situated eastern to this line, and haplotype 15, collected in the Oigawa River of the Tokai-Hokuriku-Kinki Region, situated western to this line, were both the members of the eastern clade. These results show no populational division of daces in association with this tectonic line, suggesting a low significance of the Itoigawa-Sizuoka tectonic line for daces. This supports the results in Watanabe et al. (2018) showing a haplotype group TH4, distributed across this tectonic line.

In contrast, in the eastern clade in this study, the Hino River of the Kuzuryu River, and the Kiso River were recognized to be the westernmost in the Sea of Japan side and in the Pacific Ocean side, respectively. In the western clade, similarly, the Kitagawa River, and the Shingu River of the Kumano River were recognized to be the easternmost in the Sea of Japan side and in the Pacific Ocean side, respectively. Collectively, there were no haplotypes showing the distribution across the Suzuka Mountains. As a result, a border line connecting the Wakasa Bay and the Ise Bay might be seen. This mountain chain was estimated to be uplifted about 1.0-1.5 Myr (Yokoyama, 1988). This result is a complete accordance with those in Watanabe et al. (2018). Haplotype group TH3-TH4 was separated by this mountain chain from TH5. However, some doubts may still remain after consideration of the presence of diadromous population in this dace species.

The western clade was only shallowly differentiated as compared with a deeply differentiated eastern clade. Besides, a haplotype group TH5, distributed only in the western Japan, in Watanabe et al. (2018) was reported to be closely related to TH6, distributed only in the coastal region of the Sea of Japan, including Russia, Korea and Japan. These results suggest an eastward invasion of ancestral population to Japanese *T. hakonensis* via Korean Penninsula. However, Watanabe et al. (2018) also showed the presence of TH1 and TH3, both comprising a coastal region facing to the Sea of Japan in Russia, Sakhalin and the eastern Japan populations, suggesting some possibility of southward invasion via Sakhalin. It is necessary to examine the genetic relationships of much more samples from the rivers surrounding the Sea of Japan, including chronological studies in order to clarify the origin of Japanese *T. hakonensis*.

In this study, only a small number of samples were examined based on a part of DNA sequences of mitochondrial cytochrome *b* region by financial restriction. Considering rapid shrinking of freshwater dace population particularly in western Japan, much more studies seem to be necessary to maintain or restore healthy freshwater ecosystems by new and more effective methods incorporating microsatellite analysis, concatenated sequence data set and the whole-genome sequencing.

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（2020 年 8 月 31 日受付）
（2021 年 11 月 6 日受理）