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Phylogeographic structure and genetic disturbance of the oily bitterling *Tanakia limbata* in central Honshu, Japan

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Abstract: We examined mitochondrial cytochrome *b* sequences (919 bp) from 261 individuals of *Tanakia limbata* collected mainly from river systems around Ise Bay in central Honshu, Japan. Seventy-one haplotypes were identified and classified into five major clades (LI1–LI5). Haplotypes detected in populations from the Ise Bay region belonged to LI5, a clade distinct from LI4, which was detected mainly in populations from the Lake Biwa basin. The distributions of these populations are separated by the Suzuka Mountains. Genetic divergence between these clades (*p*-distance = 0.017) was comparable to that reported among regional clades of other cyprinid fishes. Significant genetic differentiation among river-system populations within the Ise Bay region indicated strong drainage-based population structure. In contrast, haplotypes belonging to non-native lineages (LI1 and LI4) were detected in several river systems, suggesting the presence of artificially introduced populations. We propose the native populations of the Ise Bay region, represented by LI5, as an evolutionarily significant unit and emphasize the need for drainage-based management to prevent further genetic disturbance.

Key words: Conservation, genetic population structure, Ise Bay region

Introduction

Freshwater fishes of the Japanese Archipelago often exhibit regional population structure with genetic differentiation, primarily among river systems that have been historically isolated by geological processes such as mountain uplift and the formation of straits (Watanabe *et al*., 2017). These regional populations are often regarded as Evolutionarily Significant Units (ESUs) and should therefore be treated as fundamental units for conservation (Moritz, 1994; Frankham *et al*., 2010). In recent decades, however, artificially introduced populations have promoted interpopulation hybridization across Japan, placing many ESUs at risk of extinction through genetic disturbance (Nature Conservation Committee of the Ichthyological Society of Japan, 2013). This problem is particularly serious in bitterling fishes (Cyprinidae: Acheilognathinae), for which non-native domestic populations have been documented in 14 of the 16 native species, resulting in the widespread loss of regionally endemic populations (Hata *et al*., 2019; Tominaga *et al*., 2020; Ito *et al*., 2021, 2023). Accordingly, clarifying the genetic characteristics of regional populations and defining appropriate conservation units have become urgent tasks. The river systems draining into Ise Bay in the Tokai region—encompassing Aichi, Gifu, and Mie prefectures (hereafter, the Ise Bay region)—are located adjacent to the Kinki region, which includes Lake Biwa and the Yodo River system. These two regions were separated approximately one million years ago by the uplift of the Suzuka Mountains, and many freshwater fishes in this area are known to possess genetically distinct populations corresponding to these regions (Watanabe *et al*., 2017; Ito *et al*., 2019; Tominaga *et al*., 2020). Although the Ise Bay region is thought to have formed a single paleo-river system during the Last Glacial Maximum (Moriyama, 2004), genetic analyses based on partial mitochondrial DNA (mtDNA) sequences have revealed clear genetic differentiation within this region for many freshwater fishes (Watanabe & Mori, 2008; Ito *et al*., 2019, 2020, 2025; Ito & Koya, 2022). At the same time, many of the endemic populations in this region have been reported to experience genetic disturbance through hybridization with artificially introduced populations originating from western Japan, raising serious conservation concerns (Horikawa & Mukai, 2007; Umemura *et al*., 2012; Suzuki *et al*., 2016; Kitanishi *et al*., 2016; Kitazima *et al*., 2015; Tominaga *et al*., 2020; Ito *et al*., 2021). Therefore, it is necessary to clarify both the conservation units and the current status of genetic disturbance for each freshwater fish species

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inhabiting the Ise Bay region.

The oily bitterling *Tanakia limbata* is widely distributed naturally from western Honshu to Kyushu, Japan (Taniguchi *et al*., 2021). Phylogeographic studies based on partial mtDNA sequences of the cytochrome *b* (cyt *b*) or the NADH dehydrogenase subunit 2 (ND2) regions have shown that this species is genetically differentiated into three to four allopatric clades extending from Kyushu to the Kinki region (Hashiguchi *et al*., 2006; Uemura *et al*., 2018; Tominaga *et al*., 2020; Taniguchi *et al*., 2021). In contrast, populations from the Ise Bay region have been treated as part of the same clade (Clade 1) as those from the Kinki and Sanyo regions in the previous study based on the ND2 region (Taniguchi *et al*., 2021), and the genetic characteristics of the Ise Bay populations have not been fully evaluated. Although haplotype differences and shallow divergence between the Ise Bay and Kinki populations have been suggested (Tominaga *et al*., 2020; Taniguchi *et al*., 2021), the limited number of samples from the Ise Bay region has been insufficient to adequately assess the genetic structure of regional populations. In addition, artificially introduced populations of T. limbata have been recorded in the Yahagi River system in Nishio City, Aichi Prefecture, and in the irrigation channels in Gifu City, Gifu Prefecture, raising concerns about genetic disturbance caused by artificial introduction in this region (Mukai, 2019; Chimura, 2025).

In this study, we conducted extensive sampling of *T. limbata* across river systems in the Tokai region and performed phylogeographic analyses based on partial mtDNA nucleotide sequences. Our aims were to elucidate the genetic characteristics of populations in each river system and to examine the presence of non-native lineages. Based on these results, we further discuss the biogeographic characteristics of *T. limbata* in the Tokai region and propose appropriate conservation units for this species.

Materials and Methods

Between 2015 and 2024, a total of 261 individuals of *Tanakia limbata* were collected from 31 sites across 14 river systems in the Ise Bay region, central Honshu, Japan (Fig. 1; Table 1). Specimen preparation generally followed the procedures described by Ito *et al*. (2024). Collected specimens were deposited in either the Gifu Prefectural Museum (GPM-Z) or the Mie Prefectural Museum (MIE-Fi), together with detailed locality information (Table 1).

Genomic DNA was extracted using the DNeasy Blood

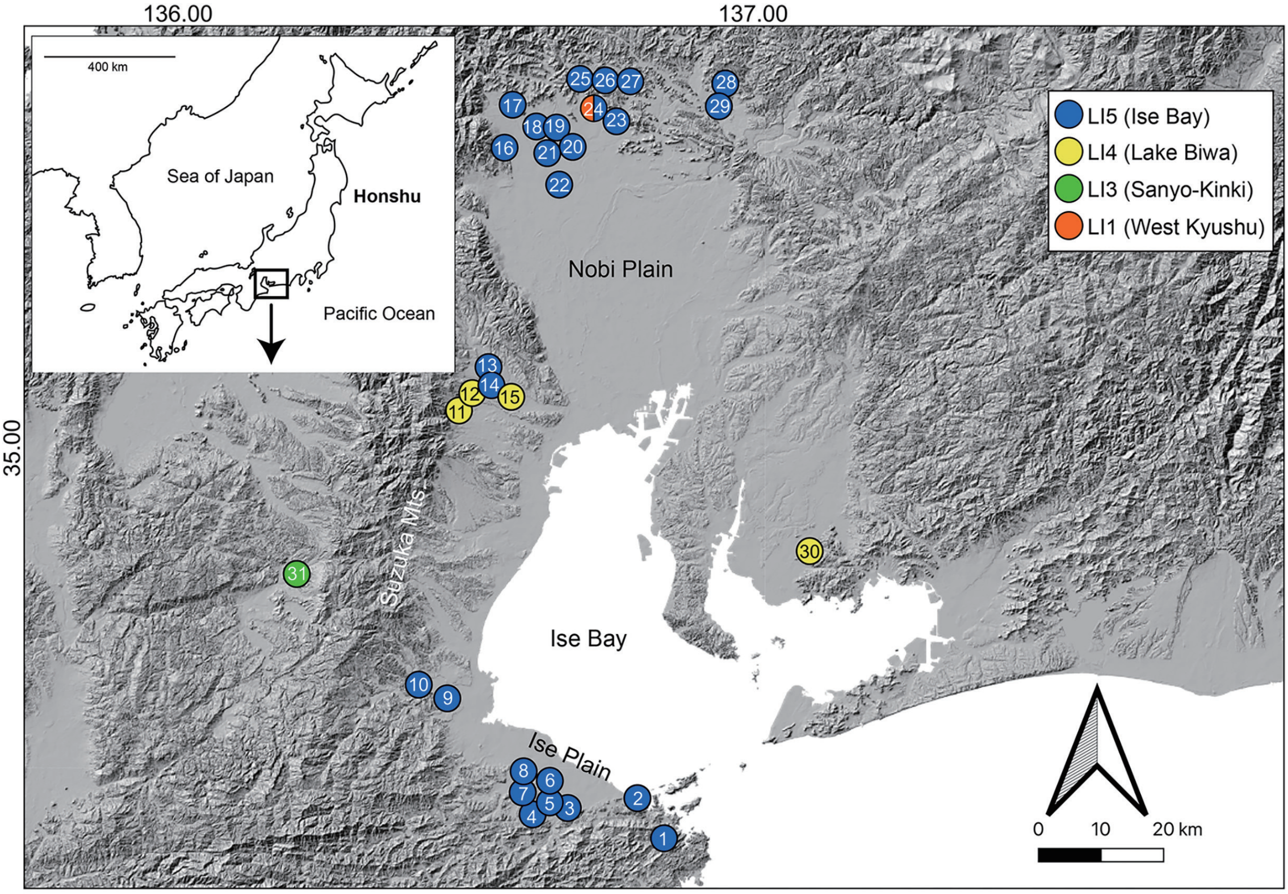

Fig. 1. Sampling localities of *Tanakia limbata* (circles). Numbers indicate site numbers. Circle colors indicate mtDNA lineages. The numbers at the top and bottom of the figure indicate longitude, and the numbers on the left and right indicate latitude.

Table 1. List of sampling localities for *Tanakia limbata*

Site No.	Sampling sites	River system	n	Haplotypes (n)	Museum specimen number
Ise Bay region					
1	Channel, Toba, Mie	Kamo R.	7	TB59 (1), TB60 (6)	MIE-Fi4773
2	Channel, Ise, Mie	Isuzu R.	17	TB39 (9), TB43 (8)	MIE-Fi3666-3669, 4836
3	Shirutani R., Ise, Mie	Miya R.	10	TB19 (5), TB39 (1), TB60 (4)	MIE-Fi3659-3663, 4835
4	Channel, Tamaki, Mie	Tokida R.	10	TB20 (4), TB59 (1), TB60 (5)	MIE-Fi3644-3648, 4837
5	Sougo R., Ise, Mie	Tokida R.	9	TB39 (5), TB61 (4)	MIE-Fi3651-3655, 4834
6	Channel, Meiwa, Mie	Ohori R.	10	TB39 (7), TB41 (1), TB42 (2)	MIE-Fi3637-3643, 4833
7	Channel, Meiwa, Mie	Sasabue R.	10	TB39 (10)	MIE-Fi3672-3678, 4839
8	Channel, Meiwa, Mie	Kushida R.	3	TB38 (1), TB39 (2)	MIE-Fi4849
9	Nakamura R., Matsusaka, Mie	Kumozu R.	10	TB6 (1), TB7 (1), TB8 (1), TB9 (2), TB10 (3), TB11 (1), TB12 (1)	MIE-Fi4844
10	Channel, Tsu, Mie	Kumozu R.	10	TB10 (3), TB11 (3), TB12 (4)	MIE-Fi4298-4307
11	Channel, Komono, Mie	Asake R.	4	TB65 (1), TB66 (3)	MIE-Fi2760-2763
12	Channel, Komono, Mie	Asake R.	8	TB63 (1), TB65 (6), TB66 (1)	MIE-Fi3972
13	Channel, Inabe, Mie	Inabe R.	6	TB16 (6)	MIE-Fi3923
14	Channel, Inabe, Mie	Inabe R.	9	TB40 (9)	MIE-Fi4827
15	Channel, Toin, Mie	Inabe R.	10	TB62 (10)	MIE-Fi4372-4384
16	Kuise R., Ikeda, Gifu	Ibi R.	10	TB2 (6), TB3 (2), TB5 (2)	MIE-Fi4511-4520
17	Shiraishi R., Ibigawa, Gifu	Ibi R.	13	TB15 (11), TB18 (1), TB47 (1)	MIE-Fi4496-4508
18	Mimizu R., Ono, Gifu	Nagara R.	7	TB2 (1), TB47 (1), TB49 (1), TB53 (2), TB54 (1), TB58 (1)	MIE-Fi4697
19	Sai R., Motosu, Gifu	Nagara R.	12	TB13 (1), TB14 (1), TB15 (3), TB26 (3), TB31 (1), TB37 (1), TB46 (1), TB57 (1)	MIE-Fi4802
20	Goroku R., Motosu, Gifu	Nagara R.	10	TB24 (1), TB25 (1), TB26 (1), TB47 (2), TB50 (1), TB51 (1), TB52 (1), TB53 (1), TB56 (1)	MIE-Fi4801
21	Chougoji R., Mizuho, Gifu	Nagara R.	9	TB4 (1), TB27 (2), TB31 (1), TB48 (1), TB49 (1), TB50 (1), TB56 (2)	MIE-Fi4886
22	Hoe R., Anpachi, Gifu	Nagara R.	6	TB15 (2), TB34 (1), TB48 (2), TB55 (1)	MIE-Fi3853, 4733
23	Toishi R., Gifu, Gifu	Nagara R.	6	TB2 (4), TB28 (1), TB37 (1)	MIE-Fi4805
24	Murayama R., Gifu, Gifu	Nagara R.	6	TB31 (1), TB47 (1), TB69 (2), TB70 (1), TB71 (1)	MIE-Fi4669-4674
25	Itaya R., Gifu, Gifu	Nagara R.	1	TB31 (1)	MIE-Fi4690
26	Channel, Yamagata, Gifu	Nagara R.	12	TB2 (1), TB29 (3), TB31 (4), TB32 (1), TB33 (1), TB36 (1), TB56 (1)	MIE-Fi4866
27	Toba R., Yamagata, Gifu	Nagara R.	1	TB2 (1)	MIE-Fi4795
28	Shizuno R., Seki, Gifu	Nagara R.	5	TB1 (2), TB21 (2), TB35 (1)	MIE-Fi4806
29	Tsubo R., Seki, Gifu	Nagara R.	16	TB17 (2), TB21 (7), TB22 (1), TB23 (1), TB24 (1), TB30 (1), TB44 (2), TB45 (1)	GPM-Z-21845
30	Sumi R., Nishio, Aichi	Yahagi R.	11	TB64 (7), TB67 (4)	MIE-Fi4858
Seto Inland Sea region					
31	Tsuge R., Iga, Mie	Yodo R.	3	TB68 (3)	MIE-Fi4920

& Tissue Kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification and sequencing of the mitochondrial cyt *b* region followed the protocols described in Ito *et al*. (2025). DNA sequencing was conducted by commercial sequencing services provided by Takara Bio or Eurofins Genomics using premixed sequencing reagents. The resulting 919 bp cyt *b* sequences were deposited in the DDBJ/EMBL/GenBank databases under accession numbers LC912863–LC912933.

Multiple sequence alignment was performed using MAFFT version 7 (Katoh *et al*., 2019). Phylogenetic relationships were inferred using the maximum-likelihood (ML) method implemented in IQ-TREE version 2.4.0 (Minh *et al*., 2020). The best-fitting nucleotide substitution model was selected

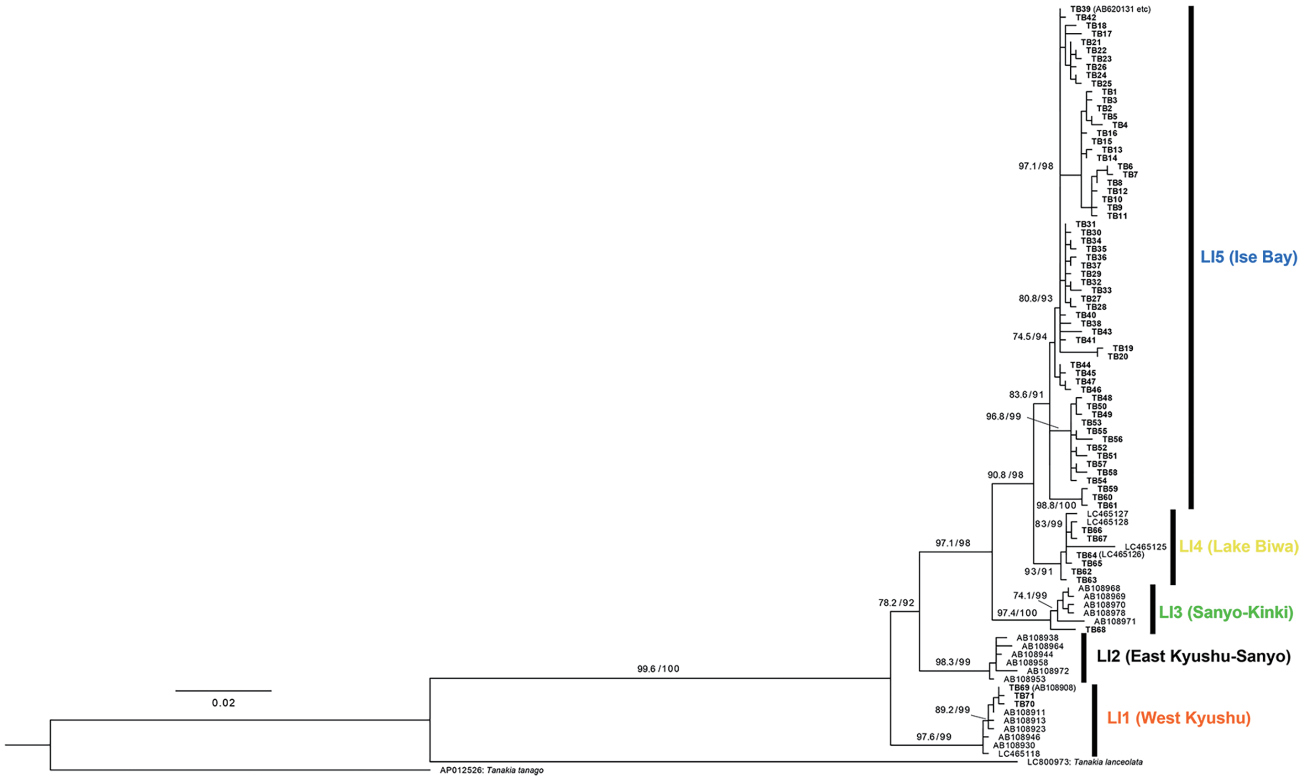

Fig. 2. Maximum likelihood (ML) tree of the 919 bp cytochrome *b* gene sequences of *Tanakia limbata*. Numbers at nodes indicate Shimodaira-Hasegawa-like approximate likelihood ratio test values (left), and ultrafast bootstrap values (middle) in the ML tree. Each value is indicated when it exceeds 70% and 90%. The parentheses after each clade name indicate the natural distribution area. Haplotypes identified in this study are indicated in bold.

using ModelFinder (Kalyaanamoorthy *et al*., 2017) based on the Bayesian Information Criterion (BIC), and the TN+F+R2 model was applied. Branch support was assessed using 1,000 replicates of the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; Guindon *et al*., 2010) and ultrafast bootstrap (UFBoot; Hoang *et al*., 2018), with values ≥ 80% for SH-aLRT and ≥ 95% for UFBoot considered to indicate strong support.

In addition to sequences obtained in this study, homologous cyt *b* sequences of *T. limbata* reported by Hashiguchi *et al*. (2006), Kawamura *et al*. (2014), and Tominaga *et al*. (2020) were included in the phylogenetic analyses (Fig. 2). Sequences of *Tanakia lanceolata* and *Tanakia tanago* were used as outgroups (Fig. 2). Genetic distances among the major clades identified in the phylogenetic tree were calculated as *p*-distances using MEGA 12.1 (Stecher *et al*., 2025).

Some haplotypes previously reported from the Kinki region were based on shorter sequence lengths than those used in the present study (Uemura *et al*., 2018). To evaluate the identity of a specific haplotype (TB68), an additional phylogenetic analysis was conducted using homologous sequences trimmed to 887 bp, following the same analytical procedures described above.

In addition, a median-joining haplotype network (Fig. 3) was constructed using PopART version 1.7 (Leigh & Bryant, 2015). Analysis of Molecular Variance (AMOVA) was performed using Arlequin version 3.5 (Excoffier & Lischer, 2010) to assess genetic structure among and within river systems. Furthermore, we estimated genetic differentiation coefficient (Φ_{ST}; Excoffier *et al*., 1992) between river systems using Arlequin version 3.5. Critical significance levels for multiple testing were adjusted using the sequential Bonferroni procedure (Rice, 1989). For these analyses, 11 river systems were included after excluding the localities where non-native lineages were detected.

Results

A total of 919 bp of the mitochondrial cyt *b* region was sequenced from 261 individuals of *Tanakia limbata* collected mainly from the Ise Bay region. Seventy-one haplotypes (TB1–TB71) were identified (Table 1). The ML phylogenetic tree inferred from these haplotypes revealed five major clades (LI1–LI5; Fig. 2). The monophyly of clades LI1–LI3 was strongly supported by high support values (SH-aLRT > 97%, UFBoot > 99%). In contrast, clades LI4 and LI5 showed relatively high SH-aLRT support (> 83%) but slightly lower UFBoot values (91%).

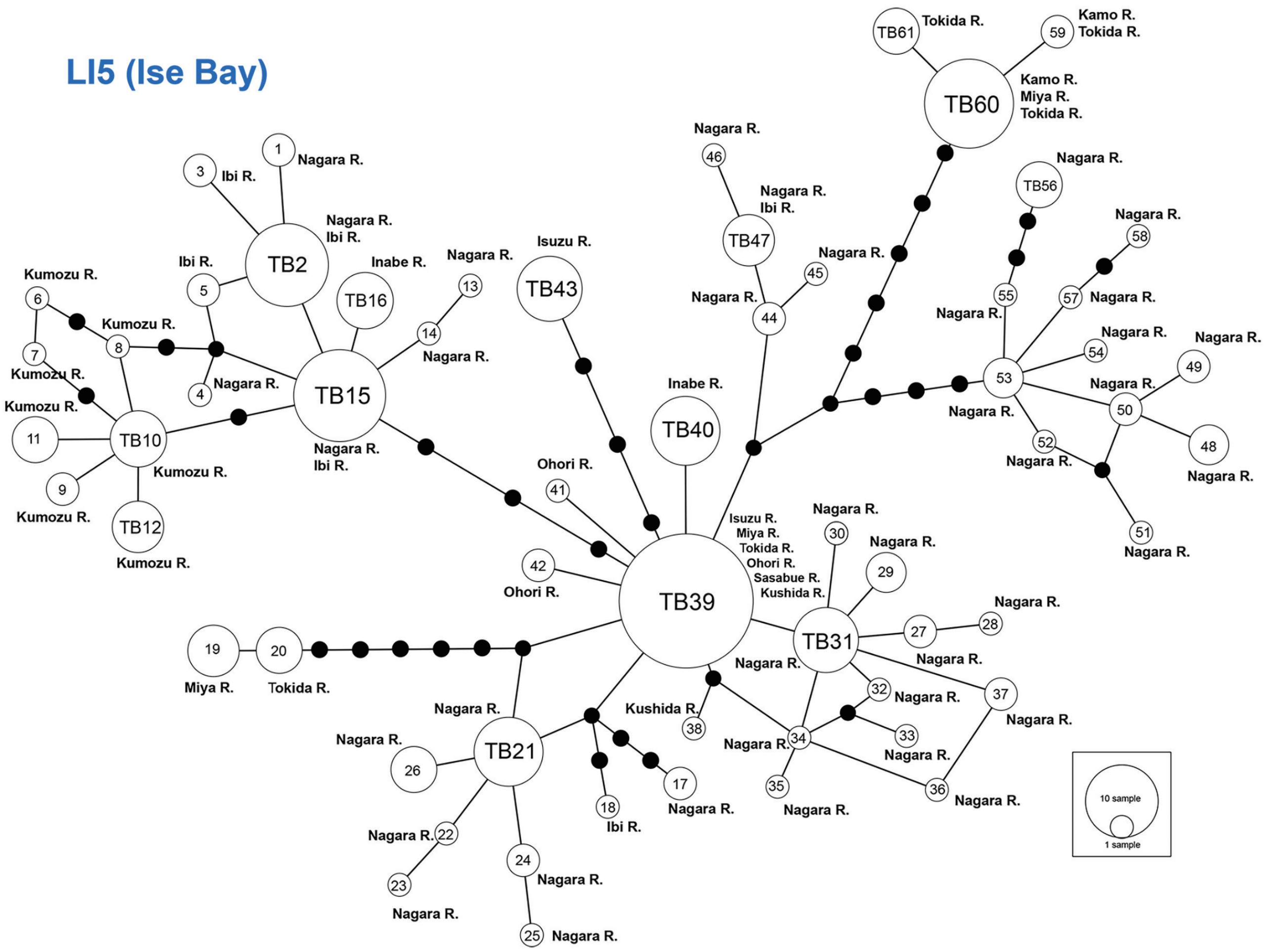

Fig. 3. The median joining network of the 919 bp cytochrome *b* gene sequences of *Tanakia limbata.*

Table 2. Genetic distances among the major clades of *Tanakia limbata*

	LI1	LI2	LI3	LI4
LI1 (West Kyushu)				
LI2 (East Kyushu-Sanyo)	0.039 ± 0.006			
LI3 (Sanyo-Kinki)	0.044 ± 0.006	0.042 ± 0.006		
LI4 (Lake Biwa)	0.051 ± 0.007	0.039 ± 0.006	0.030 ± 0.005	
LI5 (Tokai)	0.053 ± 0.007	0.041 ± 0.006	0.031 ± 0.005	0.017 ± 0.003

Data shown as average ± SD (*p*-distance)

Most of the haplotypes obtained in this study (TB1–TB61) were assigned to clade LI5. Among them, TB39 was identical to three sequences previously reported from the Kushida River system (AB239406, AB630131, LC465124). Of the 61 haplotypes included in clade LI5, 55 haplotypes were detected in a single river system, whereas six were shared among adjacent river systems (Table 1).

Six haplotypes (TB62–TB67) detected in the Inabe, Asake, and Yahagi River systems were assigned to clade LI4 and were identical or closely related to four haplotypes previously reported from the Lake Biwa basin. Haplotype TB68 was assigned to clade LI3. Comparison with previously reported haplotypes based on shorter sequence lengths showed that TB68 formed a monophyletic group with haplotypes detected in populations from Sanyo Region (Okayama Prefecture) and the Yodo River system, and was most closely related to haplotypes from the Yodo River system (Fig. 4). In addition, three haplotypes (TB69–TB71) were assigned to clade LI1 and were identical or closely related to haplotypes previously reported from populations in the West-Kyushu region.

Genetic distances among the major clades were calculated based on *p*-distances. Clade LI5 showed a mean genetic distance of 0.031 ± 0.005 from clade LI3 and 0.017 ± 0.003 from clade LI4 (Table 2).

The median-joining haplotype network revealed a structure characterized by numerous missing haplotypes centered on TB39. In addition, several shallowly diverged haplotypes formed sub-networks radiating from central haplotypes such as TB10, TB21, and TB53 (Fig. 3).

AMOVA conducted for 11 river systems after excluding the localities where non-native lineages were detected showed that 30.46% of the total genetic variation was attributable to differences among populations ($P < 0.01$), 22.65% to among population within groups ($P < 0.01$), and 46.89% to variation within population ($P < 0.01$) (Table 3).

Global Φ_{ST} was 0.508 ($P < 0.01$), indicating genetic differentiation overall. Pairwise Φ_{ST} values among the 11 river systems ranged from -0.033 to 0.986, and 34 of the 55 comparisons remained significant after sequential Bonferroni correction. In particular, the Kumozu and Ibi river systems showed significant differentiation from all other river systems. In addition, the Kamo, Isuzu, Inabe, and Nagara river systems each differed significantly from seven other river systems. In contrast, significant differentiation was detected for only two, three, and four other river systems in the Kushida, Sasabue, and Ohori river systems, respectively (Table 4).

Discussion

Genetic distinctiveness of the Ise Bay populations. We reconstructed the phylogenetic tree of *T. limbata* using sequences obtained mainly from samples collected in the Ise Bay region, together with sequences from previous studies. As a result, haplotypes detected in populations in the Ise Bay region formed a monophyletic group corresponding to clade LI5, whereas haplotypes from the Lake Biwa basin formed another monophyletic group corresponding to clade LI4. Although UFBoot support for the monophyly of the clades were slightly lower (91%), SH-aLRT support values were relatively high (> 83%), indicating that the monophyly of each clade was reasonably supported.

The distributions of populations belonging to clades

Table 3. Hierarchical analysis of molecular variance (AMOVA) among regions of *Tanakia limbata*

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	10	307.748	1.171	30.46**
Among population within groups	14	123.405	0.870	22.65**
Within population	194	349.733	1.803	46.89**

**P < 0.01

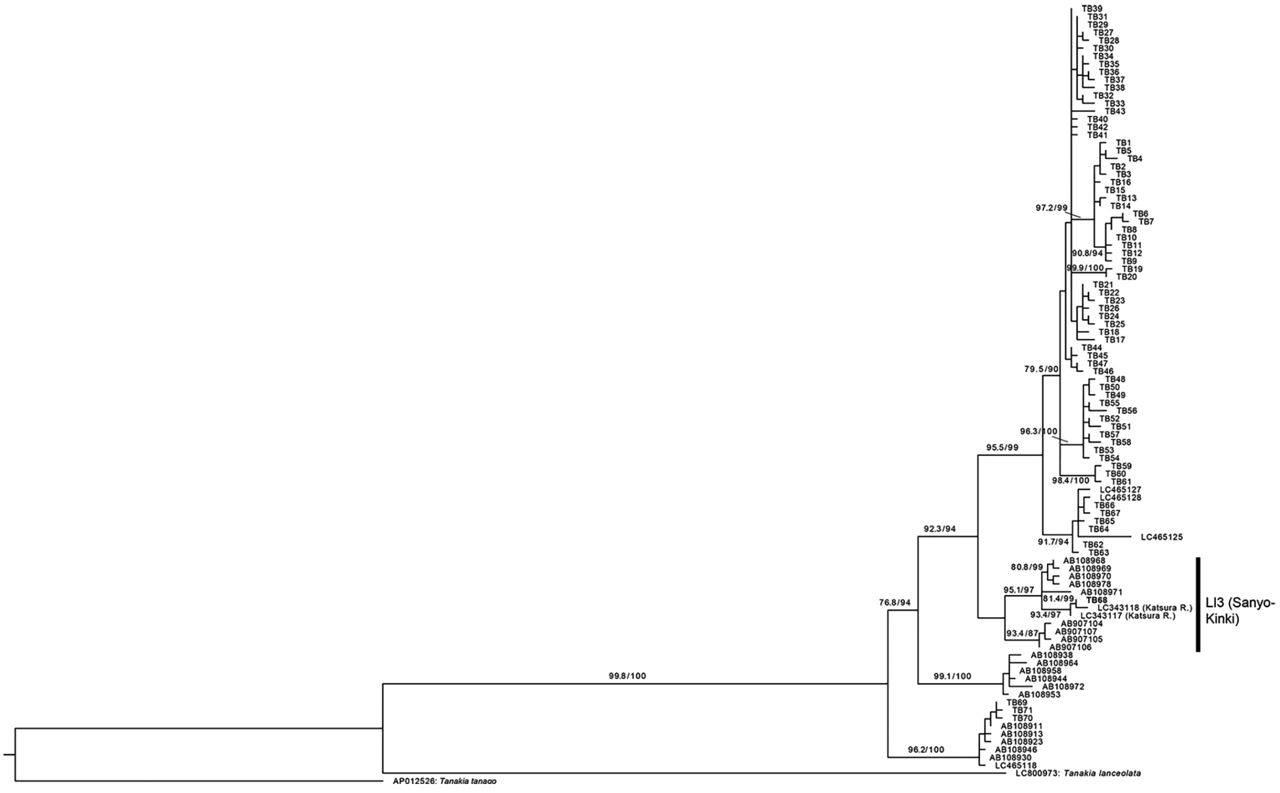

Fig. 4. Maximum likelihood (ML) tree of the 887 bp cytochrome *b* gene sequences of *Tanakia limbata*. Numbers at nodes indicate Shimodaira-Hasegawa-like approximate likelihood ratio test values (left), and ultrafast bootstrap values (middle) in the ML tree. Each value is indicated when it exceeds 70% and 90%. Bold indicates the haplotype (TB68) evaluated for identity.

Table 4. Pairwise Φ_{ST} values among populations of river systems.

Population	Kamo	Isuzu	Miya	Tokida	Ohori	Sasabue	Kushida	Kumozu	Inabe	Ibi	Nagara
Kamo											
Isuzu	0.844*										
Miya	0.443	0.459*									
Tokida	0.231	0.401*	0.053								
Ohori	0.947*	0.343	0.382*	0.313							
Sasabue	0.986*	0.365	0.395	0.318	0.074						
Kushida	0.937	0.259	0.205	0.190	0.148	0.412					
Kumozu	0.921*	0.796*	0.706*	0.667*	0.837*	0.857*	0.786*				
Inabe	0.799*	0.423*	0.431*	0.389*	0.331	0.348	0.220	0.647*			
Ibi	0.882*	0.691*	0.631*	0.584*	0.706*	0.727*	0.658*	0.563*	0.421*		
Nagara	0.524*	0.188*	0.289*	0.259*	0.068	0.062	-0.033	0.454*	0.139*	0.305*	

*Significant after sequential Bonferroni correction.

LI4 and LI5 are geographically separated by the Suzuka Mountains, and similar divergences associated with this geographic barrier have been reported in many freshwater fishes in Japan (Watanabe *et al*., 2017; Ito *et al*., 2019; Tominaga *et al*., 2020). The genetic distance between LI4 and LI5 was estimated to be 0.017 ± 0.003, which is comparable to the levels of divergence reported between regional clades of other cyprinid fishes, such as *Opsariichthys platypus* (0.019, single estimate) and *Hemigrammocypris neglecta* (0.016 ± 0.002) (Watanabe *et al*., 2014; Kitanishi *et al*., 2016). These results suggest that *Tanakia limbata* populations in the Ise Bay region represent a regionally endemic population that is genetically distinguishable from populations in other regions, including the Lake Biwa basin.

Haplotype TB68, detected in Iga City, Mie Prefecture, was assigned to clade LI3. In this study, haplotypes belonging to clade LI3 were detected from Okayama Prefecture to the Yodo River system, indicating that Clade 1 proposed by Taniguchi *et al*. (2021) can be subdivided into three clades (LI3–LI5). Furthermore, haplotype TB68 was closely related to haplotypes from the Katsura River in the Yodo River system (LC343117, LC343118). This result suggests that the population in Iga City represents a native population naturally distributed within the Yodo River system.

Genetic population structure within the Ise Bay region. AMOVA revealed that 30.46% of the total genetic variation was significantly attributable to differences among river systems, and the global Φ_{ST} value was also significant, indicating that *T. limbata* is genetically structured primarily at the river-system level within the Ise Bay region. Moreover, most haplotypes belonging to clade LI5 were restricted to single river systems. The pairwise Φ_{ST} results also supported this pattern, with significant genetic differentiation detected among many river-system pairs. The Ise Bay region is thought to have formed a single paleo-river system during the Last Glacial Maximum, approximately 20,000 years ago (Moriyama, 2004), and in several other freshwater fishes, including its close relative *T. lanceolata*, identical haplotypes have been reported on opposite sides of Ise Bay, suggesting relatively recent genetic exchange via paleo-river connections (Watanabe & Mori, 2008; Ito *et al*., 2020, 2025). In *T. limbata*, however, these results suggest that extensive dispersal through the paleo-river system was limited even during that period. *T. limbata* is known to occur more frequently than *T. lanceolata* in small channels (Mukai, 2019). Such ecological characteristics may have been a factor limiting interdrainage movement in this species.

In contrast, the results suggest that partial genetic exchange occurred among some river systems. In the pairwise Φ_{ST} analysis, the Kushida, Sasabue, and Ohori river systems showed significant differentiation from relatively few other river systems, indicating that the degree of genetic differentiation was relatively low among these river systems on the Ise Plain. In addition, some haplotypes, such as TB39 and TB60, were shared among river systems on the Ise Plain. Because these river systems are adjacent to one another and in some areas have unclear watershed boundaries, they may have been more prone than other areas to local genetic exchange through temporary interdrainage connections associated with flooding or river capture. Similar genetic exchange among river systems on the Ise Plain has also been suggested for *T. lanceolata* and *Cobitis minamorii tokaiensis* (Ito *et al*., 2020, 2025).

In addition, the high proportion of genetic variation within sampling sites (46.89%) and the presence of multiple shallowly diverged sub-networks in the haplotype network suggest fine-scale genetic structuring within river systems. However, this pattern may also reflect differences in demographic history, including effective population size, rather than genetic differentiation within river systems alone. Future genome-wide analyses based on nuclear DNA will enable a more detailed evaluation of genetic differentiation both within and among river systems.

Genetic disturbance in the Ise Bay region. In this study, haplotypes belonging to clade LI1, which has been detected in populations from western Kyushu, were detected at one locality in the Nagara River system. In addition, haplotypes belonging to clade LI4, which is naturally distributed in populations from the Lake Biwa basin, were detected at multiple localities in the Asake, Inabe, and Yahagi River systems. As discussed above, populations belonging to clade LI5 are considered the only population naturally distributed in the Ise Bay region. In addition, haplotypes belonging to LI1 have also been detected in an introduced population in the Kunichika River, Ehime Prefecture (Hashiguchi *et al*., 2006). Therefore, the occurrence of haplotypes belonging to LI1 and LI4 in this region is most likely attributable to the presence of artificially introduced populations.

Numerous freshwater fishes in the Tokai region have been reported to include introduced populations originating from the Lake Biwa basin (Horikawa & Mukai, 2007; Suzuki *et al*., 2016; Kitanishi *et al*., 2016). Moreover, *T. limbata* is a popular species for ornamental purposes and fishing, and records indicate that it has been introduced into at least 14 prefectures, making it one of the most frequently introduced bitterling species in Japan (Ito *et al*., 2023). Taken together, these lines of evidence strongly suggest that haplotypes belonging to clade LI4 in the Ise Bay region were introduced via artificially introduction from the Lake Biwa basin.

The detection of non-native lineages in the Nagara and Yahagi River systems is consistent with previous reports by Mukai (2019) and Chimura (2025). In contrast, the presence of non-native lineages in the Asake and Inabe River systems is reported here for the first time. In particular, in the Inabe River system, native and non-native populations occur in close proximity, raising concerns about future genetic admixture. Genome-wide analyses will be necessary to assess potential hybridization and to develop appropriate conservation strategies for native populations.

Conservation units and management implications. The present study demonstrates that *T. limbata* populations in the Ise Bay region are genetically distinct from populations in other regions and are characterized by the clade LI5. Although the genetic distinctiveness of this regional population has remained unclear, our results support recognizing these native populations an ESU (Moritz, 1994). Furthermore, the pronounced genetic differentiation observed among river systems within the Ise Bay region indicates that at least each river system should be treated as a Management Unit (MU; Moritz, 1994; Frankham *et al*., 2010). At the same time, the substantial genetic variation within river systems suggests that the release of individuals within the same river should also be approached with caution. By comprehensively analyzing *T. limbata* populations throughout the Tokai region, this study has clarified the spatial extent of genetic disturbance in the region. In particular, the close proximity of native and non-native populations in the Inabe River system underscores the urgent need to develop concrete management measures that prioritize the conservation of native populations.

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