

Environmental DNA unveils deep phylogeographic structure of a freshwater fish

Tetsu Yatsuyanagi¹, Takashi Kanbe¹, Kazuya Fujii¹, Shouko Inoue¹, and Hitoshi Araki¹

¹Hokkaido University

October 20, 2023

Abstract

Phylogeography bears an important part in ecology and evolution. However, current phylogeographic studies are largely constrained by limited numbers of individual samples. Using the newly developed environmental DNA (eDNA) assay for phylogeographic analyses, this study provides detailed information regarding the history of Siberian stone loach *Barbatula toni*, a primary freshwater fish across the whole range of Hokkaido, Japan. Based on an eDNA metabarcoding on 293 river water samples, we detected eDNA from *B. toni* in 189 rivers. Among the *B. toni* eDNA-positive sample set, 51 samples were chosen to implement the *Barbatula*-specific eDNA assay on a fine scale with the goal of determining the phylogeographic pattern. As a result, two regionally restricted, genetically distinct lineages of the species were revealed. According to a molecular clock analysis, they have been genetically isolated for at least 1.5 million years, suggesting their ancient origin and colonization of Hokkaido, presumably in the glacial periods. These results demonstrate how freshwater fishes can alter their distributions over evolutionary timescales and how eDNA assay can deepen our understanding of phylogeography.

Title:

Environmental DNA unveils deep phylogeographic structure of a freshwater fish

Authors: Tetsu Yatsuyanagi¹, Takashi Kanbe², Kazuya Fujii^{1,3}, Shouko Inoue², Hitoshi Araki^{2*}

Affiliations:

¹Graduate School of Agriculture, Hokkaido University; Sapporo, 060-8589, Japan. ²Research Faculty of Agriculture, Hokkaido University; Sapporo, 060-8589, Japan. ³Fukuda Hydrologic Center; Sapporo, 001-0024, Japan. *Corresponding author: Hitoshi Araki Email: arakih@agr.hokudai.ac.jp

Abstract:

Phylogeography bears an important part in ecology and evolution. However, current phylogeographic studies are largely constrained by limited numbers of individual samples. Using the newly developed environmental DNA (eDNA) assay for phylogeographic analyses, this study provides detailed information regarding the history of Siberian stone loach *Barbatula toni*, a primary freshwater fish across the whole range of Hokkaido, Japan. Based on an eDNA metabarcoding on 293 river water samples, we detected eDNA from *B. toni* in 189 rivers. Among the *B. toni* eDNA-positive sample set, 51 samples were chosen to implement the *Barbatula* -specific eDNA assay on a fine scale with the goal of determining the phylogeographic pattern. As a result, two regionally restricted, genetically distinct lineages of the species were revealed. According to a molecular clock analysis, they have been genetically isolated for at least 1.5 million years, suggesting their ancient origin and colonization of Hokkaido, presumably in the glacial periods. These results demonstrate how freshwater fishes can alter their distributions over evolutionary timescales and how eDNA assay can deepen our understanding of phylogeography.

Short running title

eDNA-based phylogeography of loach species in Japan

Competing interests

We declare that we have no competing interests.

Keywords

phylogeography, environmental DNA, freshwater fish, haplotypes

Main Text:

Phylogeography relates the formation of species distributions, ecological shifts, and evolutionary processes to geology (1). For example, one phylogeographic study has linked multiple glacial refugia in Europe to genetic diversity in plants (2). Other phylogeographic studies have revealed the common African origins of humans and multiple origins of domesticated livestock (3–5). In addition, environmental DNA (eDNA) analysis of sediment samples has unveiled a multimillion year old ecosystem (e.g., from the Kap København Formation in Greenland) (6). Nevertheless, the current phylogeographic approaches are largely limited by less number of samples. To comprehensively understand the ecology and evolution of a target species, hundreds or thousands of individual tissue samples are required within a large-scale geographic scheme. Small or unequal sample sizes leads to errors in statistical models, while low spatial coverage of the sampling points obscures phylogeographic patterns (7–9).

Advancements in bioinformatic processing methods, especially amplicon sequence variant (ASV) methods, can distinguish biological sequence variants from erroneous ones. These technologies have substantially improved eDNA-based population genetic studies while negating the need for sampling target species in the wild (10–12). Because this technique minimizes the effort of sample collection (for instance, fish eDNA can be detected from water scooped from water bodies), it can innovatively reveal the fine-scale phylogeographic structure of a species without sampling biases.

In this study, we conducted an eDNA-based phylogeographic study of a primary freshwater fish, the Siberian stone loach *Barbatula toni*, in Hokkaido (the northernmost large island in Japan). Goto et al. proposed that *B. toni* colonized rivers in Hokkaido from Sakhalin, the northern continental Far East, via the Soya Strait, where land bridges were periodically formed during the ice age (13). Terrestrial and freshwater faunae in Hokkaido are particularly unique to the Japanese archipelago as they are segregated by a biogeographic boundary known as the Blakiston Line. In addition, the organisms on this island have been subjected to numerous large-scale volcanic and glacial events, leaving behind geological evidences, such as sedimentary layers (14, 15). *B. toni* is among the most common primary freshwater fishes inhabiting the rivers in Hokkaido. Members of this primary freshwater fish species depend exclusively on their inhabited river system throughout their life history, often resulting in strong congruence between their geographic placements and phylogenetic breaks in their populations (16). Biologists have examined the history of colonization, expansion, and diversification of primary freshwater fishes in relation to geological and/or climatic changes. For example, European freshwater fishes (genus *Sabanejewia*) expanded rapidly and became genetically homogenized during Pleistocene glaciations (17) and Mesoamerican fishes expanded and diverged from their South American origins via historical drainage connectivity (18). Thus, the intraspecific phylogeographic pattern of *B. toni* might provide insights into the paleogeological and climatic events that have shaped the distribution and diversity of living organisms on the Hokkaido island. Although eDNA analyses have captured large-scale intraspecific genetic diversity (11, 12), no studies have addressed the novel histories of freshwater fishes over evolutionary timescales using eDNA till date.

Applying the eDNA technique, we detected the intraspecific genetic variations in the mitochondrial cytochrome *b* (*cyt-b*) gene of *B. toni* and assessed their phylogeographic pattern throughout Hokkaido. After revealing the phylogeographic structures, we conducted molecular analyses of tissue-derived DNA to evaluate the potential scenarios of population expansion and vicariance of *B. toni*.

DNA sequencing

We developed the enhanced eDNA assay to detect intraspecific genetic diversity of *B. toni* by introducing unique molecular tagging systems (see Supplementary Materials). Utilizing stringent ASV methods, this assay minimizes the risk of false positives caused by index hopping and sequencing errors (19, 20). To select a river that hosts *B. toni* across Hokkaido for this study, we analyzed eDNA samples collected from 293 rivers. In this step, we prepared five dual-indexed libraries using the fish-universal MiFish primer set (21) and performed eDNA metabarcoding on a MiSeq sequencing platform (Illumina, San Diego, CA, USA). Throughout the five MiSeq runs, the eDNA of *B. toni* was detected in 189 out of 293 river samples (64.5%; fig S1). Among these valid samples, we selected 51 river samples covering the coastline of Hokkaido to implement the *Barbatula*-specific eDNA assay on a fine scale with the goal of determining the phylogeographic pattern of *B. toni* (table S1). Using newly-developed PCR primers amplifying 266 bp of the mitochondrial DNA *cyt-b* gene of *B. toni*, we prepared a dual-indexed library comprising four replicates of the 51 river samples, four field blanks, and eight PCR blanks. After trimming the adapters and primers from the result of a single MiSeq sequencing run, 9,193,270 reads were obtained. After read merging, quality filtering, denoising, and chimera removal, 5,981,225 reads and 65 haplotypes of *B. toni* were finally obtained. Furthermore, no eDNA was detected in the field and PCR blanks. A conservative decision was then made to accept 50 out of 65 haplotypes based on two criteria: 1) detection in all four sequencing replicates and 2) eDNA concentration > 1.0 copy per liter. The first criterion eliminated 15 haplotypes (one haplotype with 3 out of 4 detections, two with 2 out of 4 detections, and twelve with 1 out of 4 detections) as potential erroneous haplotypes with low detection frequencies (22). The eDNA concentrations of all remaining haplotypes exceeded the second criterion (8.3 copies/L at minimum).

To reliably infer the phylogenetic relationship among the *B. toni* in Hokkaido and Sakhalin (Russia), we obtained tissue-derived DNA sequences through Sanger sequencing (Materials and Methods-2). Using the primer set Le-L4 and Le-H4 (23), we amplified 1,055 bp of the *cyt-b* DNA sequences of five individual tissue samples collected from four river systems: Shiribeshi-Toshibetsu River, Koetoi River, Tokoro River (Nos. 46, 7, and 17 in table S1), and a river in Sakhalin.

Phylogenetic relationship and geographic structure

A Bayesian phylogenetic analysis classified the eDNA haplotypes into two distinct lineages (Fig. 1; Clade-A and Clade-B), each containing three genetic groups. The network analysis also confirmed their phylogenetic relationships (Fig. 2). Groups 1 and 6 revealed a large genetic divergence between the two distinct lineages, with the dominant haplotypes BTW-00 and BTO-00 occupying the centers of each group. Additionally, the haplotypes in other groups were connected in a star-like manner to Groups 1 and 6. Moreover, the distributions of these phylogenetic groups were regionally restricted in Hokkaido (Fig. 3). Clade-A and Clade-B were separately distributed in southern and northern Hokkaido, respectively. While the eDNA concentrations largely varied among rivers, the detected haplotype richness was not significantly correlated with eDNA concentration (copy/L) ($R^2 = 0.04$, $p = 0.18$; figs. S2, S3, and S4).

The phylogenetic analysis using Sanger-sequenced *cyt-b* sequences (868 bp) supported the phylogenetic relationship constructed using the eDNA-based analysis (fig. S5). In the haplotype derived from tissue-derived DNA of the Sakhalin specimen, a 221-bp sequence of the region targeted by the eDNA assay was identical to BTO-00 of Group-6, the major haplotype found in northern Hokkaido. While the reference sequences of *B. toni* and a closely-related species *B. nuda* collected from the continent (northern China and Amur Basin) were positioned at the ancestral outgroup, the *B. toni* haplotype obtained from the Sakhalin specimen was included within the northern lineage (Clade-B) in Hokkaido.

A molecular clock analysis (2.5%–2.8%/Myr (23–25)) informed that the two clades diverged in the early Pleistocene (1.59–1.78 Mya, table S2). During the early Pleistocene, the glacial and interglacial periods repeated in cycles and land bridges emerged at the Soya Strait due to sea-level drops during glaciation (26). Therefore, it is suggested that the ancestors of Clade-A first colonized Hokkaido from Sakhalin during a low-sea-level period in the early-Pleistocene glaciation. During an interglacial period, they were long-

term isolated by the opening of the Soya Strait. In contrast, Clade-B includes the northern Hokkaido and Sakhalin populations, which diverged circa 0.16–0.18 Mya. This estimate indicates that the ancestors of Clade-B dispersed southward to Hokkaido from Sakhalin via land bridges formed around that period.

Spatial demography and population expansion

To examine the process of regional demography and the potential occurrence of genetic bottlenecks, we performed a mismatch distribution analysis using the spatial expansion model in Arlequin ver. 3.5.2.2 (27, 28). Within each group except Group 1, the models did not reject the expected spatial expansion scenario with multimodal distributions of pairwise haplotypic differences (fig. S6 and table S3). In Group 1, the mismatch distribution exhibited a dominant frequency peak at one pairwise difference, indicating that the populations in this group had experienced a genetic bottleneck. The τ value, indicating the relative time span since a population spatially expanded through a region, was high in Group 2 (Clade-A) and also in Group 4 (Clade-B).

The southwestern part of Hokkaido, in which Group 1 is distributed with low genetic variation, had unique geological characteristics. For instance, the Ishikari Lowland (Fig. 3b) was submerged in seawater during the Mindel–Riss (0.18–0.23 Mya) and Riss–Würm (0.07–0.13 Mya) Interglacial periods (14). Moreover, results of the MiFish metabarcoding (fig. S1) also suggested the existence of many rivers in the west of the Ishikari Lowland where *B. toni* could not be detected using eDNA. This finding is consistent with the conventional view that the Ishikari Lowland prevented the southward dispersal of primary freshwater fishes from Siberia (13). In addition, the Shikotsu–Toya volcanic field have many active volcanoes, where large caldera-forming eruptions have occurred since approx. 0.10 Mya (15). Repeated seawater transgressions and large eruptions in this region have probably isolated the *B. toni* populations in southwestern Hokkaido, causing their genetic bottleneck. Several previous studies, mainly on terrestrial animals, have reported similar phylogeographies in this region. For example, the phylogeographic vicariance of the brown bear *Ursus arctos*, red fox *Vulpes vulpes*, and ezo salamander *Hynobius retardatus* in the Ishikari Lowland (29–31) and low genetic variability among the southwestern populations of the dark red-backed vole *Myodes rex* (32) support the hypothesis that geological events affected the historical expansion and demography of terrestrial and freshwater organisms in southwestern Hokkaido.

Several rivers in our study were found to co-host populations of different lineages. In the Tokoro River system (No. 17 in Fig. 3b), phylogenetically distant genetic groups have seemingly and uniquely experienced secondary contact. The presence of genetically distinct individuals in this river was further confirmed through Sanger sequencing of DNA from captured individual fish samples (fig. S5). Examination of the evolutionary mechanism of this coexistence, such as reproductive isolation of the lineages, might reveal cryptic diversity in *B. toni*. Co-occurrences of phylogenetically distant groups were also found in other rivers, but whether such coexistence results from natural distribution or artificial translocations cannot be discerned. Although *B. toni* is not a target for aquaculture, human transmission through Hokkaido is possible through mixing with other fish resources such as salmon.

Comparative phylogeographic inference

The eDNA extracted from water scoops revealed the large-scale phylogeographic structure of *B. toni*. Two distinct lineages were found in the northern and southern regions of Hokkaido, which were not separated by obvious geographic barriers. The observed pattern differed from that of other aquatic species reported in Hokkaido. Besides *B. toni*, two other species of primary freshwater fishes are also known to have colonized in Hokkaido from Sakhalin across the Soya Strait: the pond minnow *Rhynchocypris percunurus sachalinensis* and eight-barbel loach *Lefua nikkonis* (13, 33). The lineages of *R. p. sachalinensis* are clearly differentiated on the east and west sides of the Hidaka Mountains at the center of Hokkaido (34). The Japanese crayfish *Cambaroides japonicus* (35) also exhibits the similar phylogeographic pattern. The earliest-branched lineage of *L. nikkonis* (belongs to the Nemacheilidae family, like *B. toni*) is distributed along the Sea of Okhotsk (northeastern Hokkaido), while the late-branched lineage has wide-range distribution without either north–south or east–west divided structure (33). No prior study on aquatic organisms has demonstrated clear

north–south phylogeographic vicariance in Hokkaido without current geographic barriers.

Based on the estimated divergence times, we concluded that *B. toni* immigrated multiple times to Hokkaido via land bridges during the ice age. The north–south phylogeographic structure of this species possibly arose from the long time lag (over one million years) between the independent colonization of Hokkaido by the ancestral populations of the two clades. The phylogeography of this species is a potentially important indicator of the historical footprints in regions of Hokkaido and the past connection between Hokkaido and Sakhalin. Through future studies on this species in Sakhalin and other Far East regions, we could understand the distribution expansion of *B. toni* and other species from the continent to the islands. Our findings highlight that *B. toni*, which has been overlooked as a common species, is a valuable source of biogeographic information on past geology and environments.

Conclusion

With eDNA assay, this study revealed the fine-grained and deep phylogeographic structure of a primary freshwater fish, *B. toni*, over its large distribution area. Combining the eDNA assay with conventional approaches and a molecular clock analysis, novel phylogeographic events originating from the glacial period over one million years ago were uncovered. Conventional surveys in phylogeographic studies are prone to sampling biases due to the difficulty of directly collecting many individuals from different sites. The eDNA-based method provides an unbiased understanding of population genetic structures over a wide geographic range. When combined with a sufficient number of appropriate capture-based analyses, eDNA-based analyses can considerably reduce the effort and time required for acquiring high-resolution phylogeographic information. In fact, approximately 300 river samples were collected for this study, covering the entire Hokkaido region with high geographic density in 32 days. One of the advantages of this approach is that the same eDNA samples can be reused for detecting many other species in the same river systems, facilitating comparative phylogeographic studies. At present, extensive eDNA projects are being executed on national and global scales. For example, the All Nippon eDNA Monitoring Network includes more than 800 monitoring sites across the Japanese archipelago (<https://db.anemone.bio/>), whereas the eDNA expedition project of UNESCO World Heritage marine sites covers more than 15 countries (<https://www.unesco.org/en/edna-expeditions>). The integration of broad-scale eDNA biomonitoring with phylogeographic analyses has the potential to largely advance our understanding of global biodiversity. Our findings demonstrate the huge advantages of the eDNA technique as an innovative population genetic method that can rapidly and extensively detect biodiversity patterns.

References

1. J. C. Avise, J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb, N. C. Saunders, INTRASPECIFIC PHYLOGEOGRAPHY: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. *Annu. Rev. Ecol. Syst.* **18**, 489–522 (1987).
2. R. J. Petit, I. Aguinagalde, J. L. De Beaulieu, C. Bittkau, S. Brewer, R. Cheddadi, R. Ennos, S. Fineschi, D. Grivet, M. Lascoux, A. Mohanty, G. Müller-Starck, B. Demesure-Musch, A. Palmé, J. P. Martín, S. Rendell, G. G. Vendramin, Glacial refugia: Hotspots but not melting pots of genetic diversity. *Science* **300**, 1563–1565 (2003).
3. E. K. F. Chan, A. Timmermann, B. F. Baldi, A. E. Moore, R. J. Lyons, S. S. Lee, A. M. F. Kalsbeek, D. C. Petersen, H. Rautenbach, H. E. A. Förtsch, M. S. R. Bornman, V. M. Hayes, Human origins in a southern African palaeo-wetland and first migrations. *Nature* **575**, 185–189 (2019).
4. G. Larson, K. Dobney, U. Albarella, M. Fang, E. Matisoo-Smith, J. Robins, S. Lowden, H. Finlayson, T. Brand, E. Willerslev, F. Rowley-Conwy, L. Andersson, A. Cooper, Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. *Science* **307**, 1618–1621 (2005).
5. A. R. Templeton, Out of Africa again and again. *Nature* **416**, 45–51 (2002).
6. K. H. Kjaer, M. W. Pedersen, B. De Sanctis, B. De Cahsan, C. S. Michelsen, K. K. Sand, S. Jelavić, A.

- H. Ruter, A. M. Z Bonde, K. K. Kjeldsen, A. S. Tesakov, I. Snowball, J. C. Gosse, I. G. Alsos, Y. Wang, C. Dockter, M. Rasmussen, B. Skadhauge, A. Prohaska, J. Å Kristensen, M. Bjerager, E. Allentoft, E. Coissac, P. Consortium, A. Rouillard, A. Simakova, A. Fernandez-Guerra, C. Bowler, M. Macias-Fauria, J. J. Welch, A. J. Hidy, M. Sikora, M. J. Collins, R. Durbin, N. K. Larsen, E. Willerslev, A 2-Million-year-old ecosystem in Greenland uncovered by environmental DNA. *Nature* . **612** (2022).
7. D. M. Leigh, C. B. van Rees, K. L. Millette, M. F. Breed, C. Schmidt, L. D. Bertola, B. K. Hand, M. E. Hunter, E. L. Jensen, F. Kershaw, L. Liggins, G. Luikart, S. Manel, J. Mergeay, J. M. Miller, G. Segelbacher, S. Hoban, I. Paz-Vinas, Opportunities and challenges of macrogenetic studies. *Nat. Rev. Genet.* **22** , 791–807 (2021).
8. X. P. Liu, G. A. Duffy, W. S. Pearman, L. R. Pertierra, C. I. Fraser, Meta-analysis of Antarctic phylogeography reveals strong sampling bias and critical knowledge gaps. *Ecography*. e06312 (2022).
9. J. D. Phillips, R. A. Gwiazdowski, D. Ashlock, R. Hanner, An exploration of sufficient sampling effort to describe intraspecific DNA barcode haplotype diversity: examples from the ray-finned fishes (Chordata: Actinopterygii). *DNA Barcodes* . **3** , 66–73 (2016).
10. C. I. M. Adams, C. Hepburn, G.-J. Jeunen, H. Cross, H. R. Taylor, N. J. Gemmell, M. Bunce, M. Knapp, Environmental DNA reflects common haplotypic variation. *Environ. DNA* . (2022).
11. S. Tsuji, N. Shibata, R. Inui, R. Nakao, Y. Akamatsu, K. Watanabe, Environmental DNA phylogeography: Successful reconstruction of phylogeographic patterns of multiple fish species from cups of water. *Mol. Ecol. Resour.* (2023).
12. K. Weitemier, B. E. Penaluna, L. L. Hauck, L. J. Longway, T. Garcia, R. Cronn, Estimating the genetic diversity of Pacific salmon and trout using multigene eDNA metabarcoding. *Mol. Ecol.* **30** , 4970–4990 (2021).
13. A. Goto, T. Nakanishi, H. Utoh, K. Hamada, A preliminary study of the freshwater fish fauna of rivers in southern Hokkaido. *Bull. Fish. Sci. Hokkaido Univ.* **29** , 118–130 (1978).
14. Japan Association for Quaternary Research, *Quaternary Maps of Japan (with explanatory text)*. Univ. Tokyo Press (1987).
15. M. Nakagawa, M. Amma-Miyasaka, D. Miura, S. Uesawa, Tephrostratigraphy in Ishikari Lowland, Southwestern Hokkaido: Eruption history of the Shikotsu-Toya volcanic field. *Jour. Geol. Soc. Japan* . **124** , 473–489 (2018).
16. E. Bermingham, J. C. Avise, Molecular zoogeography of freshwater fishes in the southeastern United States. *Genetics* . **113** , 939–965 (1986).
17. A. Perdices, I. Doadrio, P. S. Economidis, J. Bohlen, P. Bănărescu, Pleistocene effects on the European freshwater fish fauna: Double origin of the cobitid genus *Sabanejewia* in the Danube basin (Osteichthyes: Cobitidae). *Mol. Phylogenet. Evol.* **26** , 289–299 (2003).
18. R. G. Reeves, E. Bermingham, Colonization, population expansion, and lineage turnover: Phylogeography of Mesoamerican characiform fish. *Biol. J. Linn. Soc.* **88** , 235–255 (2006).
19. L. E. MacConaill, R. T. Burns, A. Nag, H. A. Coleman, M. K. Slevin, K. Giorda, M. Light, K. Lai, M. Jarosz, M. S. McNeill, M. D. Ducar, M. Meyerson, A. R. Thorner, Unique, dual-indexed sequencing adapters with UMIs effectively eliminate index cross-talk and significantly improve sensitivity of massively parallel sequencing. *BMC Genomics* . **19** , 30 (2018).
20. D. W. Fadrosh, P. G. Bing Ma, N. Sengamalay, S. Ott, R. M. Brotman, J. Ravel, An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* . **2** , (2014).

21. M. Miya, Y. Sato, T. Fukunaga, T. Sado, J. Y. Poulsen, K. Sato, T. Minamoto, S. Yamamoto, H. Yamanaka, H. Araki, M. Kondoh, W. Iwasaki, MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. *R. Soc. Open Sci.* **2** , 150088 (2015),
22. S. Tsuji, M. Miya, M. Ushio, H. Sato, T. Minamoto, H. Yamanaka, Evaluating intraspecific genetic diversity using environmental DNA and denoising approach: A case study using tank water. *Environ. DNA* **2** , 42–52 (2020).
23. R. Saka, Y. Takehana, N. Suguro, M. Sakaizumi, Genetic population structure of *Lefua echigonia* inferred from allozymic and mitochondrial cytochrome *b* variations. *Ichthyol. Res.* **50** , 301–309 (2003).
24. D. M. Irwin, T. D. Kocher, A. C. Wilson, Evolution of the cytochrome *b* gene of mammals. *J. Mol. Evol.* **32** , 128–144 (1991).
25. G. Orti, M. A. Bell, T. E. Reimchen, A. Meyer, Global survey of mitochondrial DNA sequences in the threespine stickleback: Evidence for recent migrations. *Evolution.* **48** , 608–622 (1994).
26. Y. Ono, The Northern Landbridge of Japan. *Quat. Res.* **29** , 183–192 (1990).
27. L. Excoffier, Patterns of DNA sequence diversity and genetic structure after a range expansion: Lessons from the infinite-island model. *Mol. Ecol.* **13** , 853–864 (2004).
28. L. Excoffier, H. E. L. Lischer, Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Mol. Ecol. Resour.* **10** , 564–567 (2010).
29. N. Azuma, J. I. Hangui, M. Wakahara, H. Michimae, Population structure of the salamander *hynobius retardatus* inferred from a partial sequence of the mitochondrial DNA control region. *Zoolog. Sci.* **30** , 7–14 (2013).
30. T. Matsubashi, R. Masuda, T. Mano, M. C. Yoshida, Microevolution of the mitochondrial DNA control region in the Japanese brown bear (*Ursus arctos*) population. *Mol. Biol. Evol.* **16** , 676–684 (1999).
31. T. Oishi, K. Uruguchi, K. Takahashi, R. Masuda, Population structures of the red fox (*Vulpes vulpes*) on the hokkaido Island, Japan, revealed by microsatellite analysis. *J. Hered.* **102** , 38–46 (2011).
32. K. Kawai, F. Hailer, A. P. De Guia, H. Ichikawa, T. Saitoh, Refugia in glacial ages led to the current discontinuous distribution patterns of the dark red-backed vole *Myodes rex* on Hokkaido, Japan. *Zoolog. Sci.* **30** , 642–650 (2013).
33. A. Ooyagi, D. F. Mokodongan, J. Montenegro, I. F. Mandagi, N. Koizumi, Y. Machida, N. Inomata, S. V. Shedko, A. A. Hutama, R. K. Hadiaty, K. Yamahira, Phylogeography of the eight-barbel loach *Lefua nikkonis* (Cypriniformes: Nemacheilidae): how important were straits in northern Japan as biogeographical barriers? *Ichthyol. Res.* **65** , 115–126 (2018).
34. H. Sakai, T. Ueda, R. Yokoyama, Genetic structure and phylogeography of northern Far Eastern pond minnows, *Rhynchocypris perenurus sachalinensis* and *R. p. mantschuricus* (Pisces, Cyprinidae), inferred from mitochondrial DNA sequences. *Biogeography* **16** , 87–109 (2014).
35. I. Koizumi, N. Usio, T. Kawai, N. Azuma, R. Masuda, Loss of genetic diversity means loss of geological information: The endangered Japanese crayfish exhibits remarkable historical footprints. *PLoS One* **7** , e33986 (2012)
36. T. Yatsuyanagi, H. Araki, Understanding seasonal migration of Shishamo smelt in coastal regions using environmental DNA. *PLoS One* **15** , e0239912 (2020).
37. T. Minamoto, M. Miya, T. Sado, S. Seino, H. Doi, M. Kondoh, K. Nakamura, T. Takahara, S. Yamamoto, H. Yamanaka, H. Araki, W. Iwasaki, A. Kasai, R. Masuda, K. Uchii, An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols. *Environ. DNA* **3** , 8–13 (2021).

38. T. A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41** , 95–98 (1999).
39. J. D. Thompson, D. G. Higgins, T. J. Gibson, CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22** , 4673–4680 (1994).
40. S. Tsuji, N. Shibata, H. Sawada, M. Ushio, Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. *Mol. Ecol. Resour.* **20** , 1323–1332 (2020).
41. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet* . **17** , 10–12 (2011).
42. R. C. Edgar, Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* . **26** , 2460–2461 (2010).
43. X. Turon, A. Antich, C. Palacín, K. Præbel, O. S. Wangenstein, From metabarcoding to metaphylogeography: separating the wheat from the chaff. *Ecol. Appl.* **30** , 1–19 (2020).
44. J. P. Huelsenbeck, F. Ronquist, MRBAYES: Bayesian inference of phylogenetic trees. *Bioinforma. Appl. Note* . **17** , 754–755 (2001).
45. S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **35** , 1547–1549 (2018).
46. J. W. Leigh, D. Bryant, POPART: full-feature software for haplotype network construction. *Methods Ecol. Evol.* **6** , 1110–1116 (2015).
47. S. Harada, S. Jeon, I. Kinoshita, M. Tanaka, M. Nishida, Phylogenetic relationships of four species of floating gobies (*Gymnogobius*) as inferred from partial mitochondrial cytochrome *b* gene sequences. *Ichthyol. Res.* **49** , 324–332 (2002).

Acknowledgments:

We thank Hokkaido Aquaculture Promotion Corporation, Michio Fukushima and Hiroki Mizumoto for providing a part of the water samples, Fumito Tashiro for providing a tissue sample, Ryukoku University Center for Biodiversity Science for analyzing a part of the eDNA metabarcoding samples, and Kazusa DNA Research Institute for DNA extraction of a part of the eDNA samples. We also thank Akira Goto and the members in Animal Ecology lab in Faculty of Agriculture, Hokkaido University for many suggestions in the development of this study. We used the DNA Sequencing Facility of Research Faculty of Agriculture, Hokkaido University.

Funding:

Environment Research and Technology Development Fund of the Environmental Restoration and Conservation Agency Provided by the Ministry of Environment of Japan grant no. JPMEERF20204004 (HA)

JSPS KAKENHI grant no. 20H03005 (HA)

JSPS KAKENHI grant no. 23H00329 (HA)

Grant-in-Aid for JSPS Fellow grant no. JP21J12688 (TY)

Author contributions: Conceptualization: TY, TK, HA Methodology: TY, TK, SI Investigation: TY, KF, HA Visualization: TY Funding acquisition: TY, HA Project administration: TY, HA Supervision: HA Writing – original draft: TY, TK, KF, SI, HA Writing – review & editing: TY, TK, KF, SI, HA **Data availability:**

Raw sequence reads are deposited in DDBJ Sequence Read Archive (DRA). For the MiFish sequence data, accession numbers are DRR440535–DRR440728 and DRR488036–DRR488183. For *Barbatula toni* 's cyt-*b* sequence data, accession numbers are DRR490086–DRR490140. Full details of the methods, results, supporting tables and figures are available in Supplementary Information.

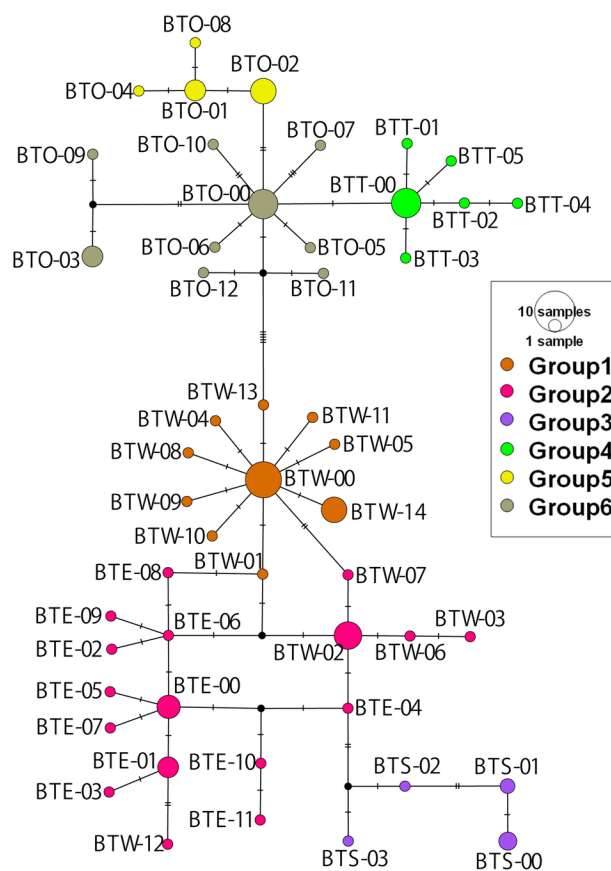


Fig. 2. Minimum-spanning network of eDNA haplotypes of *Barbatula toni*: The colors of the circles correspond to the phylogenetic groups in Fig. 1. Each crossed line on the network represents one mutation between the connected haplotypes. The black dots represent missing haplotypes. The size of each circle is related to its haplotype frequency.

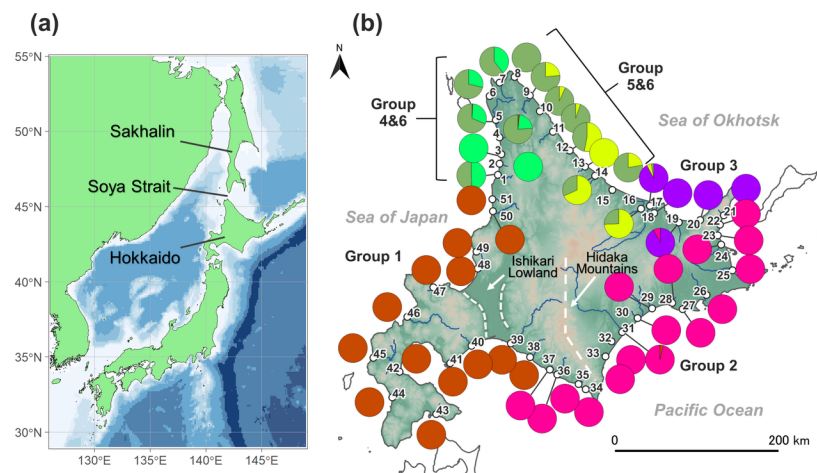


Fig. 3. Maps and distributions of eDNA-based phylogenetic groups of *Barbatula toni*: (a) map of the Japanese archipelago and Sakhalin; (b) distributions of eDNA-based phylogenetic groups of *B. toni* in Hokkaido. The different colors are described in the captions of Figs. 1 and 2 and proportions in the two-color circles indicate the haplotype frequencies in each phylogenetic group. The numbers are the IDs of the rivers from which the groups were extracted.