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Phylogeography of the eight-barbel loach *Lefua nikkonis* (Cypriniformes: Nemacheilidae): how important were straits in northern Japan as biogeographical barriers?

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Abstract Many straits in the Japanese archipelago have been proposed as biogeographical boundaries, but there is disagreement regarding their importance as historic barriers against dispersal of terrestrial and freshwater taxa. Mitochondrial DNA haplotype and phylogenetic analyses of *Lefua nikkonis*, a primary freshwater fish inhabiting northern Japan and descendent from Siberia, revealed that the species is genetically structured within its geographic range, but that two major haplotypes are widely distributed across the Ishikari Lowland of Hokkaido Island as well as across the Tsugaru Strait between Hokkaido and Honshu Islands, two well-known biogeographical boundaries of northern Japan. The two major haplotypes were separated from each other by only one mutational step, and many other haplotypes, including those endemic to the region south of these barriers, have diverged from the major haplotypes, suggesting rapid range expansion and local differentiation. Divergence-time estimates, based on vicariance of the Honshu endemic congener *L. echigonia* via uplift of the Central Highlands, demonstrated that the southward dispersal of *L. nikkonis* from Hokkaido Island to Honshu Island occurred less than 0.08–0.19 Mya, suggesting that a land bridge emerged at the Tsugaru Strait during the Riss glaciation. Given that other freshwater taxa crossed the strait earlier (during the Middle Pleistocene), it is likely that land bridges in the strait have repeatedly emerged. The fact that *L. nikkonis* invaded only the northern part of Honshu, and that many other freshwater species also have the limit of their distribution ranges in this area as well, indicates that a faunal transition zone might persist even without the Tsugaru Strait. Thus, straits and lowlands in northern Japan are likely to have been less important as dispersal barriers to freshwater taxa than is currently thought.

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Introduction

A main line of inquiry in biogeography has long been to examine why some biotas have clear distribution boundaries. Perhaps the most famous example of such biogeographical boundaries is the Wallace Line, which separates Indomalaya and Australasia (Wallace 1860; Mayr 1944). Geological barriers, such as straits and mountain chains, are thought to have frequently contributed to the formation

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of biogeographical boundaries (e.g., Johnson and Weckstein 2011; Oheimb et al. 2013). In the case of the Wallace Line, the deep Makassar and Lombok Straits have been a persistent water barrier (even during low seawater levels) that has kept the fauna and flora of Australia separated from those of Asia (Whitten et al. 2002). However, controversy remains regarding to what extent these straits and mountains may have ultimately acted as dispersal barriers to both terrestrial and freshwater taxa (e.g., Watanabe et al. 2006; Parenti and Ebach 2009).

The Japanese archipelago is composed of numerous islands, and many straits and mountains have often been proposed as biogeographical boundaries (e.g., Blakiston and Pryer 1880; Sato 1969). Hokkaido is the northernmost island of the Japanese archipelago and is presently separated from Sakhalin Island to the north by the Soya Strait, and from Honshu Island to the south by the Tsugaru Strait (Fig. 1). The terrestrial and freshwater faunas of Hokkaido have been largely shaped by colonization from Far Eastern Siberia via Sakhalin across the Soya Strait, where land bridges emerged repeatedly during the Pleistocene, including the last glacial period (the Würm glaciation: 10,000–70,000 years ago) (Fujimaki 1994; Goto 1994). However, geologists estimate that the Tsugaru Strait has not had a land bridge since 0.14–0.15 million years ago (Mya) (Ohshima 1991; Ono 1994), including the last glacial maximum (about 20,000 years ago) (Ohshima 1990; Matsui et al. 1998). This lack of a land bridge would have prevented the dispersal of terrestrial and freshwater faunas across the Tsugaru Strait for a longer duration than across the Soya Strait. This geological pattern resulted in the formation of a biogeographical boundary presently known as Blakiston's Line, separating the Siberian sub-region (Hokkaido) from the Manchurian sub-region (Honshu) (Fujimaki 1994; Goto 1994).

At least three species of primary freshwater fishes are known to have colonized Hokkaido from Siberia across the Soya Strait: the pond minnow *Rhynchocypris percnurus sachalinensis*, the Siberian stone loach *Barbatula oreas*, and the eight-barbel loach *Lefua nikkonis* (Aoyagi 1957; Goto 1982, 1994; Sakai et al. 2014). The southern limit of the native ranges of these three species is considered to be located around the present-day Ishikari Lowland of Hokkaido (Fig. 1) (Goto et al. 1978; Goto 1982, 1994). This southern limit is hypothesized to have arisen because Hokkaido Island was once separated into two or more islands by a strait(s) near this lowland during the Mindel–Riss Interglacial (0.18–0.23 Mya) and Riss–Würm Interglacial (0.07–0.13 Mya) stages (Japan Association for Quaternary Research 1987), thereby preventing their southward dispersal (Goto 1994). Therefore, the Ishikari Lowland, rather than Blakiston's Line, is considered as a prominent biogeographical boundary that limited the distributions of primary freshwater fishes from Siberia to Hokkaido (Goto 1982, 1994).

The eight-barbel loach *L. nikkonis* has nevertheless been found in the southern part of Hokkaido, beyond the lowland (Kuwahara 1996), as well as in the northern part of Honshu (Aomori Prefecture) across the Tsugaru Strait (Takeuchi and Ohta 1993; Ooyagi 2013). These populations are currently believed to have been introduced by humans, since the Ishikari Lowland is assumed to have been an ecological barrier to their southward dispersal (Goto 1982, 1994). However, the hypothesis of artificial introductions has not been tested molecularly. Given that the sea-level decline during the Riss Glacial Stage (0.14–0.18 Mya), when a land bridge(s) emerged across the Tsugaru Strait, postdates the formation of straits around the Ishikari Lowland during the Mindel–Riss Interglacial Stage (0.18–0.23 Mya) (Ono 1994), it is possible that some primary freshwater fishes had an opportunity to disperse to the south. If the *L. nikkonis* populations on southern Hokkaido Island and on Honshu Island are each native, this implies that neither the Ishikari Lowland nor Blakiston's
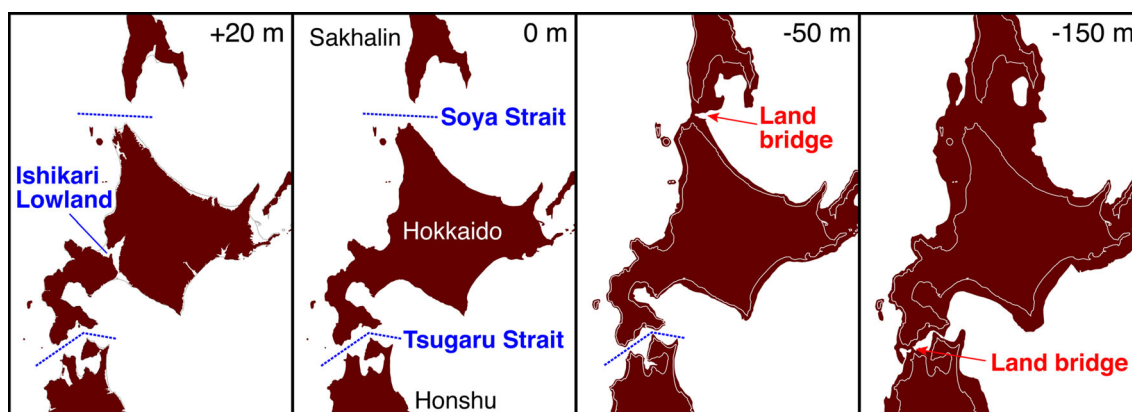

Fig. 1 The presumed land boundaries of Hokkaido Island. Sea levels are presumed to have changed by +20 m, 0 m, −50 m, and −150 m, estimated from contour and isobathic maps

Line was a barrier against the southward dispersal of primary freshwater fishes from Siberia. Furthermore, evidence that a faunal transition zone may persist in the absence of a strong geological barrier may provide insight into how biogeographical boundaries are otherwise formed.

In this study, we examined the population structure of *L. nikkonis* throughout its range on Hokkaido and Honshu Islands by comparing mitochondrial DNA (mtDNA) sequences. We used the data to reconstruct the phylogeny of haplotypes and estimate the divergence times among major lineages. We demonstrate that the *L. nikkonis* populations on southern Hokkaido Island and on Honshu Island are each native, and that colonization between the islands probably occurred during the Riss glaciation or later. Based on our findings, we assess how effective the Ishikari Lowland of Hokkaido and the Tsugaru Strait may have been as barriers against dispersal of freshwater taxa.

Materials and methods

Field collections. Lefua nikkonis has been categorized as endangered by the Ministry of the Environment, Government of Japan (2012). Even so, there is currently no law protecting this species from collection except from inside national parks. In this study, we obtained permission from local park offices to collect the fish inside parks and conducted non-destructive sampling whenever possible. A total of 241 individuals from 45 locations were collected throughout Hokkaido (36 locations, 194 individuals) and northern Honshu (nine locations, 47 individuals) [Fig. 2; see also Electronic Supplementary Material (ESM) Table S1]. One to 11 individuals (average 5.4 individuals) were sampled per site. In addition, five *Lefua pleskei*, a continental congener of *L. nikkonis*, were collected from five locations in Far East Russia (i.e., Komissarovka River, Vostok Bay, Lazurnaya Bay, Mramornaya River, and Tsukanovka River). Upon collection, the whole body, pectoral fin, pelvic fin, or caudal fin of each individual was preserved in 99% ethanol.

Molecular methods. DNA was extracted from fin samples using either a DNeasy Blood and Tissue Kit (Qiagen, Venlo, The Netherlands) or GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, USA), following the manufacturer's protocols. We amplified two mtDNA regions, D-loop and cytochrome *b* (cyt *b*), using polymerase chain reaction (PCR) with the primer pairs ProS and Phe AS (Miyazaki et al. 2011), and Le-L4 and Le-H4 (Saka et al. 2003), respectively. PCRs were performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) or an Applied Biosystems® 2720 Thermal Cycler (Life Technologies Japan Ltd.) using TaKaRa Ex Taq™ (Takara Bio Inc., Otsu, Japan). The PCR conditions

for the mitochondrial gene fragments were: denaturation at 94 °C for 120 s, 30 cycles of amplification (94 °C for 30 s, 60 °C for 30 s, and 74 °C for 30 s), and a final extension at 74 °C for 10 min for D-loop, or else denaturation at 95 °C for 180 s, 32 cycles of amplification (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s), and a final extension at 72 °C for 7 min for cyt *b*. The PCR products were purified using polyethylene-glycol precipitation.

The sequencing reactions were performed in one direction using the primers Phe AS and Le-L4, respectively, for D-loop and cyt *b*, and a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forester City, CA). The reaction conditions were: denaturation at 94 °C for 60 s and 40 cycles of amplification (94 °C for 10 s, 50 °C for 5 s, and 60 °C for 120 s) for D-loop, or else denaturation at 96 °C for 60 s and 25 cycles of amplification (96 °C for 10 s, 50 °C for 5 s, and 60 °C for 120 s) for cyt *b*. Sequence data were obtained using an Applied Biosystems 3500 Genetic Analyzer (Life Technologies Japan Ltd.) or from the sequencing facility of Macrogen Japan Corporation (Kyoto, Japan) equipped with an Applied Biosystems automated sequencer.

Haplotype networks. All sequences obtained from the 246 individuals (241 *L. nikkonis* and five *L. pleskei*) were aligned separately for D-loop and cyt *b* using the ClustalW option in MEGA 7 ver. 7.0.14 (Kumar et al. 2016), and the alignment was later corrected by eye. There were indels in the aligned D-loop sequences, but there was no ambiguous site there (ESM File S1). The aligned sequences of D-loop (943 bp) and cyt *b* (960 bp) were concatenated into a single 1,903 bp sequence.

Unique haplotypes were detected from the 246 concatenated sequences using DnaSP ver. 5.10.01 (Librado and Rozas 2009). Sequences of the unique haplotypes (77 and five haplotypes for *L. nikkonis* and *L. pleskei*, respectively) were deposited into DDBJ under accession numbers LC271593–LC271674. Haplotype networks were constructed from the unique haplotype data set of *L. nikkonis* with TCS ver. 1.2.1 (Clement et al. 2000), using the 99% parsimony criterion. Each gap was treated as a fifth state in the analysis, where a block of consecutive gaps was regarded as a single gap. Network loops were resolved based on the criterion of Crandall and Templeton (1993) and the haplotype distributions. The haplotype network was then hierarchically nested following the methods of Templeton and Sing (1993).

Phylogenetic analyses. Phylogenetic relationships among the unique haplotypes of *L. nikkonis* and the five *L. pleskei* were estimated, as follows. First, D-loop and cyt *b* sequences of two individuals of *Lefua costata*, a congener in the Korean Peninsula, were obtained from the DNA Data Bank of Japan (DDBJ) (KT943751, DQ105257, and DQ105196). Similarly, D-loop and cyt *b* sequences from
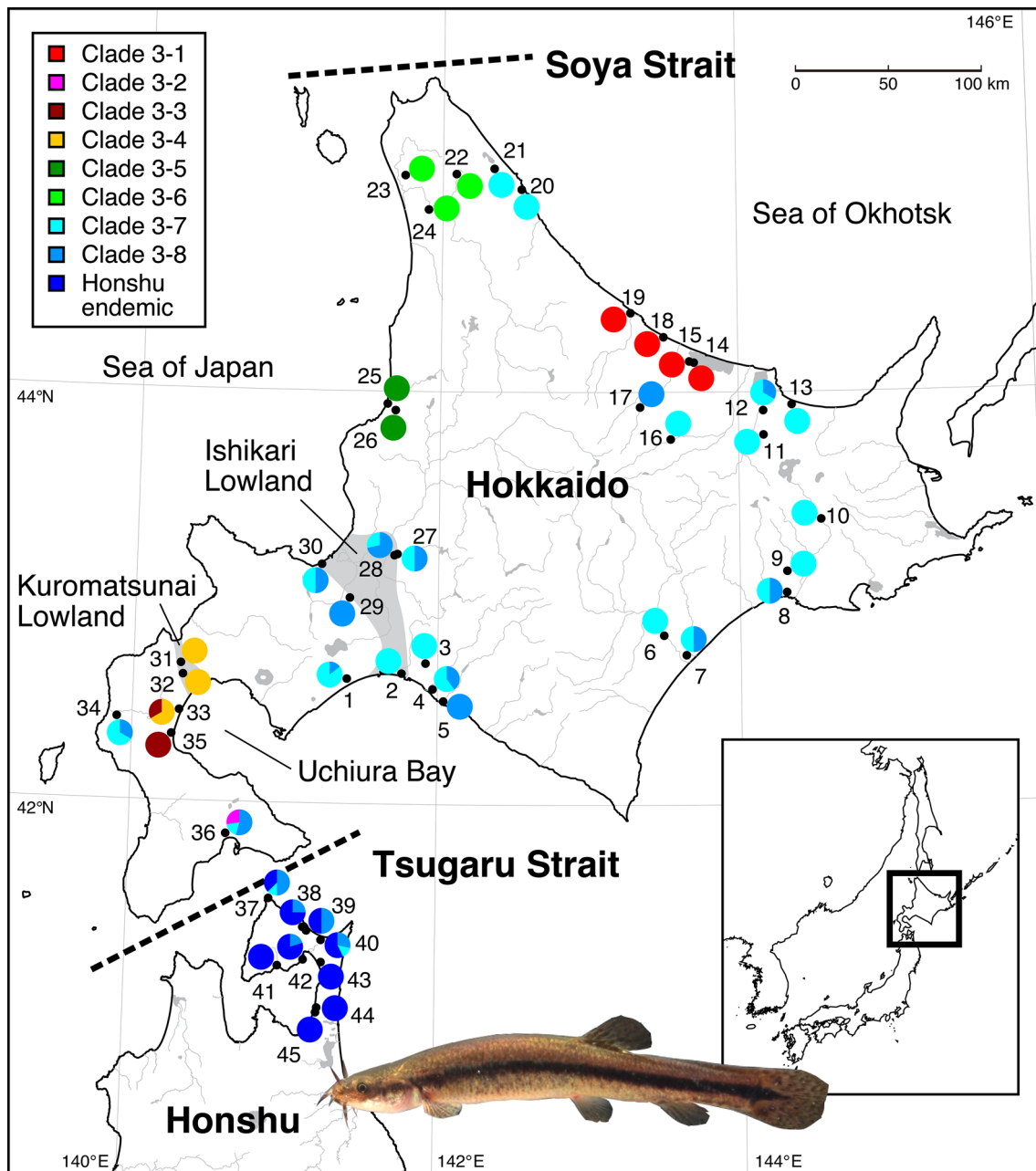
Fig. 2 Map showing the location of each collection site and the distribution of level-3 nested clades. Level-3 nested clades in the haplotype network (Fig. 3) are color coded

four populations of *Lefua echigonia*, a Honshu endemic congener, were obtained from Miyazaki et al. (2011) and Saka et al. (2003), who independently examined *L. echigonia* population structures using D-loop and cyt *b* sequences, respectively. The four *L. echigonia* populations were: Imaichi (North-Kanto Clade; AB102831 and AB080154), Zama (South-Kanto Clade; AB102839 and AB080170), Kakegawa (Tokai Clade; AB177699 and AB080177), and Aogaki (Kinki Clade; AB102850 and AB080181) (note that Kakegawa is not included in Saka et al. (2003); instead Kikugawa is included as a Tokai Clade

population. In this study, we treated Kakegawa and Kikugawa as a single population, Kakegawa, in the Tokai Clade, because their collection sites were very close to each other). The sequences of the two *L. costata* individuals and the four *L. echigonia* populations were aligned with our sequence data set [that is, sequences of 77 *L. nikkonis* (only unique haplotypes) and five *L. pleskei*], separately for the D-loop and cyt *b*, using ClustalW. Any site where a gap was found in any D-loop haplotype was removed. The aligned sequences of D-loop (854 bp) and cyt *b* (960 bp) were concatenated into a single 1,814 bp sequence.
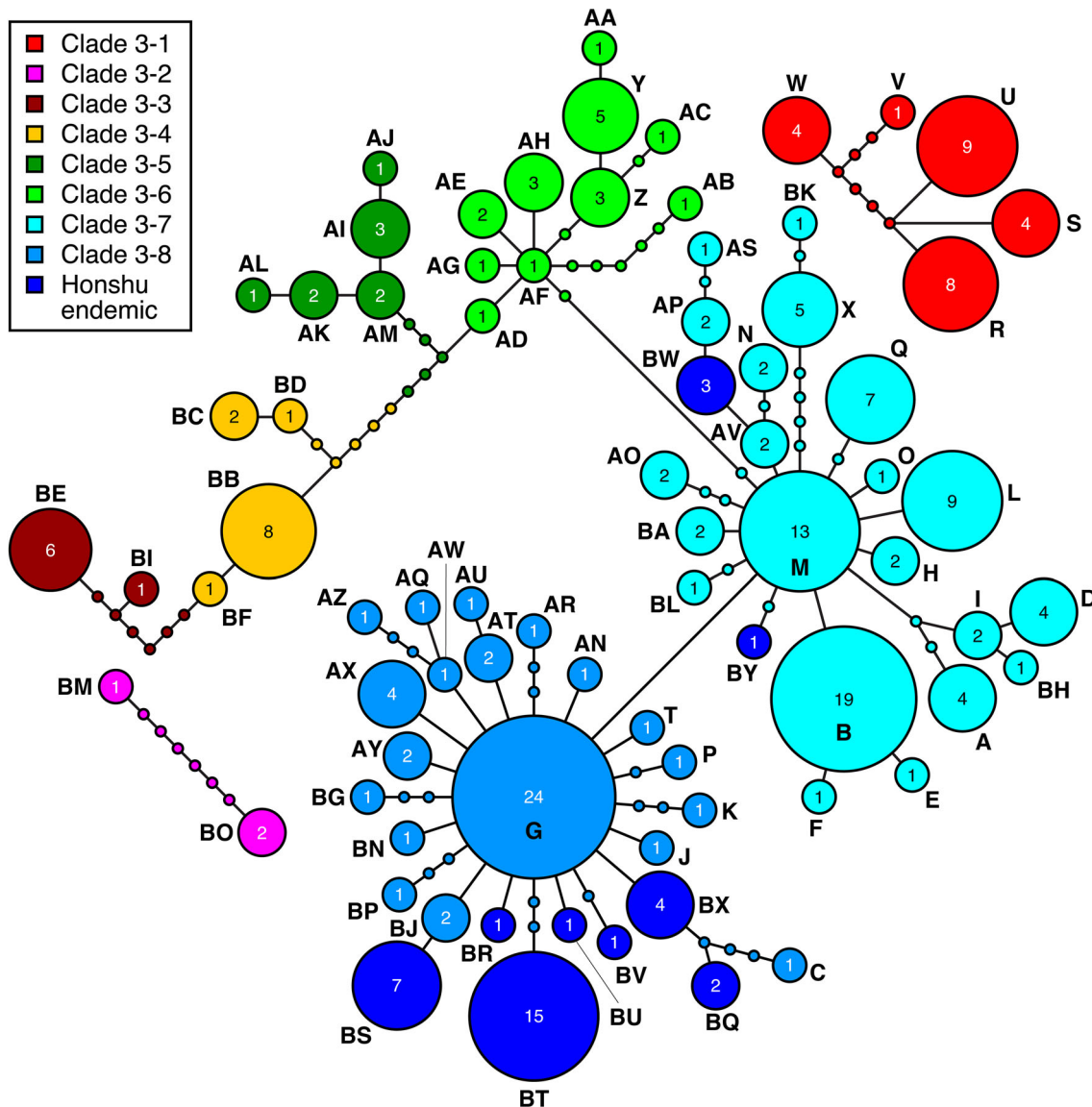
Fig. 3 Haplotype networks based on mitochondrial genome sequences of 1,903 bp (D-loop: 943 bp and cyt *b*: 960 bp). Level-3 nested clades and Honshu endemic haplotypes are color coded. *Numerals* indicate the number of individuals. *Small circles without the number of individuals in the network* represent missing haplotypes

Maximum likelihood (ML) and Bayesian inference (BI) methods were used for the estimation of phylogenies, treating the *L. echigonia* populations as outgroups. ML analyses were performed by raxmlGUI ver. 1.31 (Silvestro and Michalak 2012). A rapid bootstrap analysis of 10000 replicates was conducted using a GTR+I+G model, separating the mitochondrial regions (i.e., D-loop vs. cyt *b*) and the codon positions for cyt *b*, as selected by jModelTest ver. 2.1.7 (Darriba et al. 2012) based on Akaike's information criterion. BI analyses were conducted with MrBayes ver. 3.2.4 (Ronquist et al. 2011), using a codon-nonspecific HKY + I + G model for D-loop, and using GTR + G, GTR, and GTR + I models for the first,

second, and third codon positions of cyt *b*, respectively, as selected by jModelTest. Analyses were run using the following settings: ngen = 3000000, samplefreq = 100, and burnin = 750000.

Divergence time estimation. For many freshwater taxa native to the Japanese archipelago, the differentiation of a sister species or subspecies across the center of Honshu is often hypothesized as resulting from vicariance associated with uplift of the Central Highlands 3–7 Mya (Machida et al. 2006; Watanabe et al. 2006; Tominaga et al. 2016). Therefore, we estimated the divergence times between major clades within *L. nikkonis* by assuming that the phylogeny of *L. echigonia* populations reflects that

vicariance, wherein we regarded Imaichi (North-Kanto Clade) and Zama (South-Kanto Clade) as representative populations east of the Central Highlands, and Kakegawa (Tokai Clade) and Aogaki (Kinki Clade) as representative populations west of the Central Highlands.

Lognormal relaxed clock analyses were performed on the 1,814 bp data sets (854 bp of D-loop, and 960 bp of cyt *b*) using the program BEAST ver. 1.8.1 (Drummond and Rambaut 2007). We employed the uplift age of the Central Highlands (ca. 3–7 Mya), for the node between the east and west *L. echigonia* populations as the calibration point; a lognormal prior distribution with mean = 5 Mya and Log(Stdev) = 0.25 (5% and 95% quantiles were 3.2 and 7.3 Mya, respectively) was set. The analyses were run using a HKY + I + G (codon non-partitioned) model for D-loop, and a GTR + I + G (codon partitioned) model for cyt *b*. The other settings were as follows: coalescent (constant size), ngen = 300000000, samplefreq = 1000, and burnin = 75000000.

Results

Haplotype distribution. Based on the mitochondrial sequence data, 77 unique haplotypes were detected among the 241 individuals of *Lefua nikkonis* collected from 45 sites on Hokkaido and Honshu Islands (Table 1). Of the 77 haplotypes, 61 (79%) were unique to single sites, and ten (13%) were found at only two sites (Table 1). Fifteen haplotypes (BB–BP) were unique to Hokkaido Island south of the Ishikari Lowland (Table 1; Fig. 2). Similarly, nine haplotypes (BQ–BY) were unique to Honshu Island. Haplotypes G and M were shared among 13 (29%) and 7 (16%) of the 45 sites, respectively, and occurred on both Hokkaido and Honshu Islands (Table 1). On the other hand, all of the five individuals of *Lefua pleskei* carried unique haplotypes.

Haplotype network. Three distinct parsimony haplotype networks were evident under a 99% confidence interval cutoff threshold: five haplotypes (R, S, U, V, and W) from four populations on the Sea of Okhotsk coast (Sites 14, 15, 18 and 19) (Fig. 2) and haplotypes BM and BO from the southern tip of Hokkaido (Site 36) were isolated from the remaining 70 haplotypes (Fig. 3). Within those 70 haplotypes, two star-shaped networks with many minor haplotypes were exhibited: one radiating from the major Haplotype G and the other radiating from Haplotype M. Most minor haplotypes, including the haplotypes unique to Honshu Island, were separated from Haplotypes G or M by only one or a few mutational steps. Haplotypes G and M themselves were also separated from each other by only one mutational step. Haplotypes Y–AM from the northern end (i.e., Sites 22–24) and the Sea of Japan coast of Hokkaido (i.e., Sites 25 and 26) were separated by 3–10

mutational steps from Haplotype M. Deeper divergence was detected in Haplotypes BB–BF and BI (12–20 steps from Haplotype B), which were found from populations around the Kuromatsunai Lowland of southwestern Hokkaido (i.e., Sites 31–33, and 35).

The haplotypes were separated into eight level-3 nested clades (Fig. 3), which showed a geographic pattern in their distribution. Clade 3-1 was composed of Haplotypes R, S, U, V, and W from the four Okhotsk populations, and Clade 3-2 contained Haplotypes BM and BO found only from the southern end of Hokkaido (Site 36). Clades 3-3 (Haplotypes BE and BI) and 3-4 (Haplotypes BB–BD and BF) were composed of haplotypes from the Kuromatsunai Lowland of Hokkaido. Clade 3-5 was composed of Haplotypes AI–AM from along the Sea of Japan coast, while Clade 3-6 contained Haplotypes Y–AH from the northern part of Hokkaido. Clades 3-7 and 3-8 were represented by Haplotypes M and G, respectively, and were widely distributed throughout Hokkaido Island and on Honshu Island (Figs. 2 and 3).

Molecular phylogeny. The molecular phylogenies based on ML and BI analyses revealed that the 77 unique haplotypes in *L. nikkonis* indicated monophyly, which was supported by values of the ML bootstrap (MLB) = 51% and Bayesian posterior probability (BPP) = 0.94. The ML and BI phylogenies also demonstrated several major lineages within *L. nikkonis* (Fig. 4). The lineage composed of the haplotypes from the four Okhotsk populations (Fig. 2), which belonged to Clade 3-1 in the haplotype network (Fig. 3), branched off at the most basal position, and the monophyly of this clade was supported by the values of MLB = 99% and BPP = 1. Thereafter, two lineages, composed of the haplotypes found from the southern tip of Hokkaido (Clade 3-2 in Fig. 3) and the Kuromatsunai Lowland (Clades 3-3 and 3-4 in Fig. 3), branched off, both of which were supported by values of MLB ≥95% and BPP = 1. The haplotypes found in the western and northern part of Hokkaido (Clades 3-5 and 3-6 in Fig. 3) branched off next. The remaining haplotypes, which belonged to Clades 3-7 and 3-8 in the haplotype network, formed a monophyletic clade at the most terminal position supported by values of MLB = 41% and BPP = 0.61, whereby all Honshu-endemic haplotypes were included.

Divergence-time estimates. The time to the most recent common ancestor of *L. nikkonis* and *L. pleskei* was estimated to be about 0.74 Mya (Fig. 5, node 1; see also ESM Fig. S1). The split of the Okhotsk haplotypes (Clade 3-1 in the haplotype network of Fig. 3) occurred about 0.56 Mya (Fig. 5, node 2). The clades of haplotypes representing southern Hokkaido (Clade 3-2, Fig. 3) and the Kuromatsunai Lowland (Clades 3-3 and 3-4, Fig. 3) split off about 0.37 Mya (node 3 of Fig. 5) and 0.23 Mya (node 4 of Fig. 5), respectively. Thereafter, the haplotypes found in

Table 1 Sampling sites, sample size, and haplotype frequency of *Lefua nikkonis*

Site number	Site	Island	*n*	Haplotype
1	Shiraoi	Hokkaido	7	A(4), B(2), C(1)
2	Yufutsu	Hokkaido	5	B(2), D(1), E(1), F(1)
3	Atsuma	Hokkaido	4	B(1), D(3)
4	Mukawa	Hokkaido	5	G(1), H(2), I(1), J(1)
5	Hidaka	Hokkaido	1	K(1)
6	Ikusota	Hokkaido	6	L(6)
7	Urahoro	Hokkaido	6	G(3), L(3)
8	Kushiro	Hokkaido	2	G(1), M(1)
9	Tsurui	Hokkaido	3	M(1), N(2)
10	Shibecha	Hokkaido	4	B(4)
11	Bihoro	Hokkaido	3	M(2), O(1)
12	Memanbetsu	Hokkaido	3	M(2), P(1)
13	Tofutsu	Hokkaido	7	Q(7)
14	Baro	Hokkaido	10	R(6), S(4)
15	Yubetsu	Hokkaido	2	R(2)
16	Rubeshibe	Hokkaido	5	M(5)
17	Engaru	Hokkaido	4	G(3), T(1)
18	Pon	Hokkaido	10	U(9), V(1)
19	Monbetsu	Hokkaido	4	W(4)
20	Esashi	Hokkaido	10	B(10)
21	Hamatonbetsu	Hokkaido	5	X(5)
22	Toikanbetsu	Hokkaido	7	Y(5), Z(1), AA(1)
23	Sarobetsu	Hokkaido	5	Z(2), AB(1), AC(1), AD(1)
24	Teshio	Hokkaido	7	AE(2), AF(1), AG(1), AH(3)
25	Rumoi-1	Hokkaido	8	AI(3), AJ(1), AK(2), AL(1), AM(1)
26	Rumoi-2	Hokkaido	1	AM(1)
27	Nishikawa	Hokkaido	8	G(1), AN(1), AO(2), AP(1), AQ(1), AR(1), AS(1)
28	Kamihoromui	Hokkaido	7	G(1), AT(2), AU(1), AV(2), AW(1)
29	Toyohira	Hokkaido	6	AX(4), AY(2)
30	Tarunai	Hokkaido	6	G(2), AP(1), AZ(1), BA(2)
31	Kuromatsunai	Hokkaido	8	BB(8)
32	Osyamanbe	Hokkaido	2	BC(2)
33	Kunnui	Hokkaido	3	BD(1), BE(1), BF(1)
34	Shiribeshitoshibetsu	Hokkaido	3	I(1), BG(1), BH(1)
35	Yakumo	Hokkaido	6	BE(5), BI(1)
36	Hokuto	Hokkaido	11	G(2), BJ(2), BK(1), BL(1), BM(1), BN(1), BO(2), BP(1)
37	Oma	Honshu	8	G(4), M(1), BQ(1), BR(1), BS(1)
38	Ohata	Honshu	8	G(2), BS(4), BT(2)
39	Dedogawa	Honshu	2	G(1), BT(1)
40	Kadosawa	Honshu	7	G(2), M(1), BQ(1), BU(1), BV(1), BW(1)
41	Kawauchi	Honshu	4	BS(2), BW(2)
42	Ashizaki	Honshu	5	G(1), BX(4)
43	Okunai	Honshu	1	BY(1)
44	Fukkoshi	Honshu	8	BT(8)
45	Hibaritai	Honshu	4	BT(4)

n sample size

The number of haplotypes is in parentheses

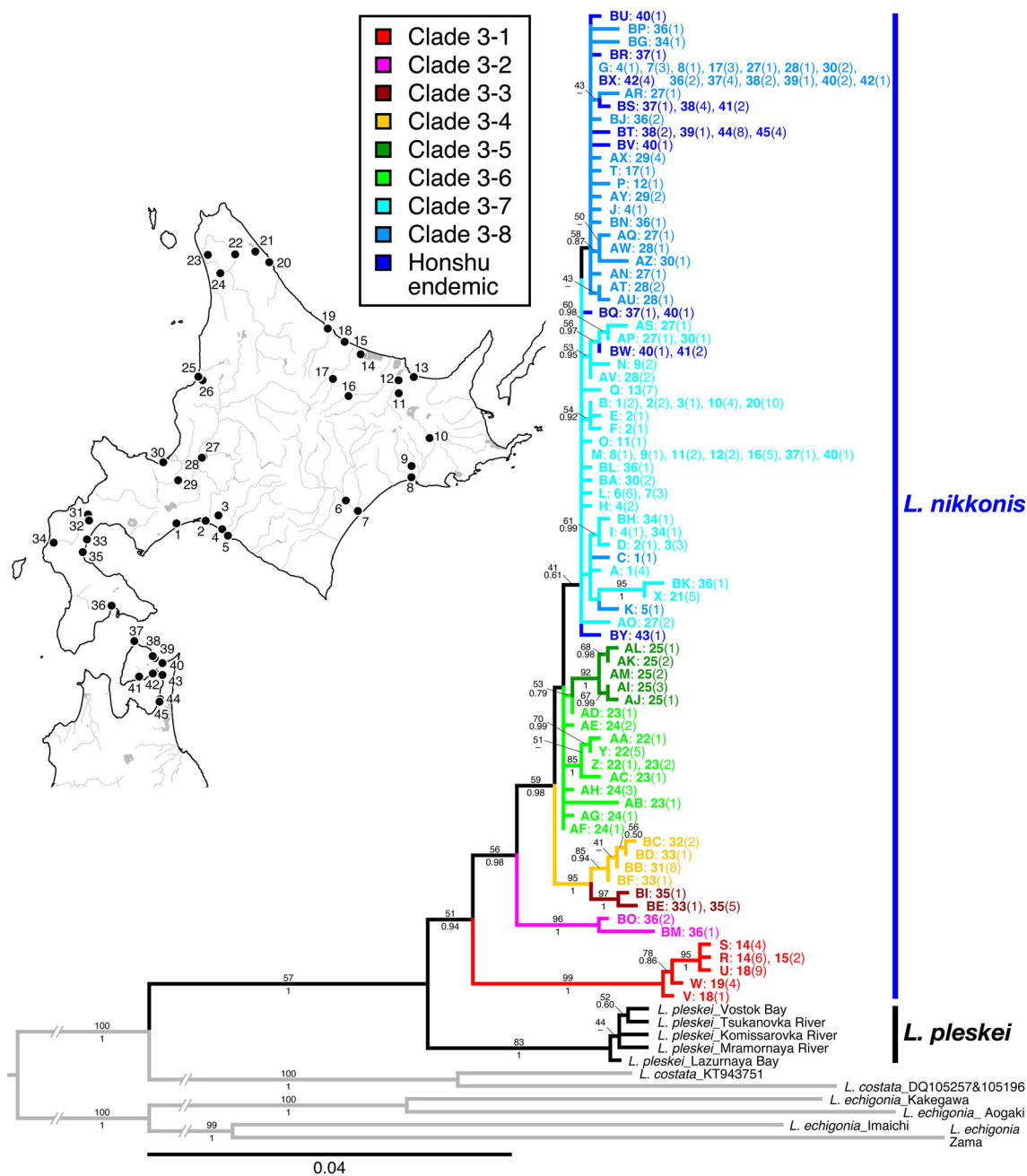Site numbers coincide with those in the map (Fig. 2)

Fig. 4 Maximum likelihood phylogeny among haplotypes based on mitochondrial genome sequences of 1,814 bp (D-loop: 854 bp and cyt *b*: 960 bp). *Lettered characters, numbers, and numbers in parentheses* indicate haplotypes, site numbers, and number of individuals, respectively. *Numbers on branches* are maximum likelihood bootstrap values (top) and Bayesian posterior probabilities (bottom). *The scale bar* indicates the number of substitutions per site. Level-3 nested clades in the haplotype network (Fig. 3) are color coded

the western and northern parts of Hokkaido (Clades 3-5 and 3-6, Fig. 3) split off about 0.18 Mya (node 5 of Fig. 5). The time to the most recent common ancestor of Clades 3-7 and 3-8 in the haplotype network was estimated to be 0.13 Mya (95% HPD = 0.08–0.19 Mya) (node 6 of Fig. 5). The mean of uncorrelated lognormal relaxed clock was estimated to be 0.0154 and 0.0247 substitution/site/Myr for D-loop and cyt *b*, respectively.

Discussion

Origin and geographic structure of *Lefua nikkonis*. Our phylogeny and divergence-time estimation determined that *Lefua nikkonis* branched off from continental congeners in the Middle Pleistocene (around 0.74 Mya; 0.47–1.05 Mya). However, it is unclear whether this vicariance event occurred at Soya Strait, which separates
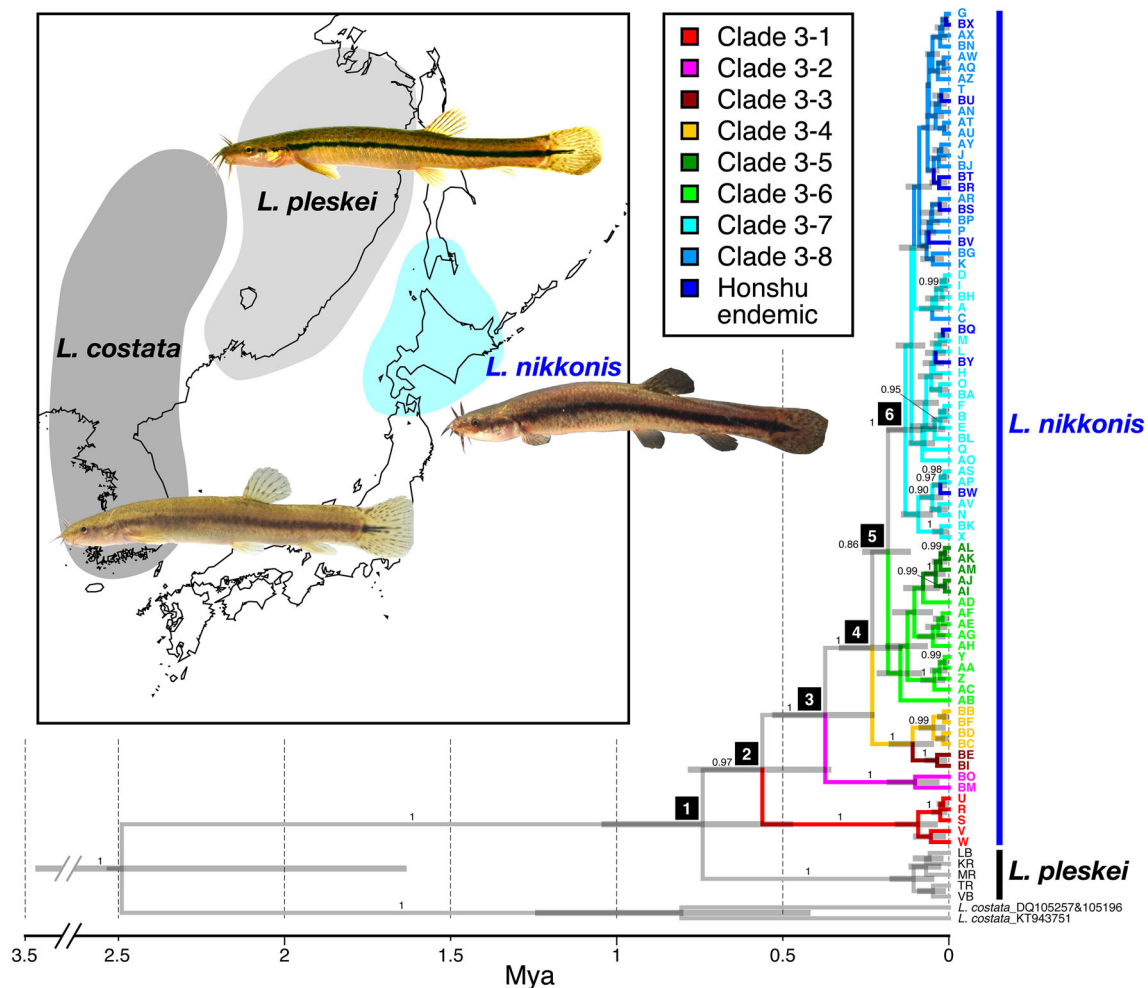
Fig. 5 Bayesian chronogram of haplotypes based on mitochondrial genome sequences of 1,814 bp (D-loop: 854 bp and cyt *b*: 960 bp). Only the clade of *Lefua nikkonis*, *L. pleskei*, and *L. costata* was enlarged (see Fig. S1 for the overall view). *Numbers on branches* are Bayesian posterior probabilities, and *bars* represent 95% high posterior density. Level-3 nested clades in the haplotype network (Fig. 3) are color coded. The pictures of *L. costata* were provided by Mizoguchi G

present-day Hokkaido from Sakhalin, or at Mamiya Strait (Strait of Tatary), which separates Sakhalin from mainland Asia. Because species of *Lefua* is also distributed in southern Sakhalin (Shedko et al. 2008), the phylogenetic relationship of the Sakhalin populations must be estimated to answer this question.

We demonstrated that the *L. nikkonis* populations (Clade 3-1) along the Sea of Okhotsk coast branched off earliest. These populations are estimated to have been isolated for more than a half million years. However, there is presently no obvious barrier that might confine them to that region, and therefore it is unclear how they came to be isolated there. Another unique geographic structure was evident in the southern part of Hokkaido: some early-branching lineages were distributed in this region. Indeed, the second most basal haplotypes (Clade 3-2) occurred from the southern tip of the island. Considering that Clade 3-2 branched off 0.37 Mya (node 3 in Fig. 5), the common

ancestor of *L. nikkonis*, which colonized Hokkaido from Sakhalin, dispersed southward and probably reached the southern end of the island by that time.

In contrast, haplotypes widely distributed in the central and eastern parts of Hokkaido appear phylogenetically more late branched and less genetically divergent. This phylogeographic pattern is in contrast to the pattern reported for the pond minnow *Rhynchocypris percnurus sachalinensis* (see Sakai et al. 2014) and Japanese crayfish *Cambaroides japonicus* (see Koizumi et al. 2012), whose respective populations are both clearly differentiated on the east and west sides of the Hidaka Mountains. Especially, Haplotypes G and M, positioned at the center of the haplotype network, appear widely distributed not only on Hokkaido but also on Honshu, suggesting rapid and recent geographic range expansion. Many other of the haplotypes sequenced differed from Haplotypes G or M by only a few mutational steps, indicating local genetic differentiation

after rapid range expansion. Further examination is needed to uncover details of the species' historical range expansion.

The importance of straits in northern Japan as biogeographical barriers. Many of the *L. nikkonis* haplotypes found south of the Ishikari Lowland of Hokkaido appear to be quite old and endemic to that region, indicating that the populations of southern Hokkaido Island are composed of native haplotypes. In contrast, the absence of old haplotypes on Honshu Island and a resultant discontinuous distribution of the late-branching haplotypes across the southern Hokkaido indicate that the Honshu populations might have been artificially introduced. However, we propose that only as a less likely scenario, based on the following reasons. First, the presence of Honshu-endemic haplotypes suggests that populations on Honshu Island are native. Indeed, more than 74% (35/47) of the Honshu haplotypes were unique to Honshu Island. Moreover, the Hokkaido and Honshu populations shared only two haplotypes, i.e., Haplotypes G and M, both of which were positioned at the center of the haplotype networks. These findings suggest that the Honshu-endemic haplotypes were locally differentiated from Haplotypes G and M, which expanded into Honshu. All of the Honshu-endemic haplotypes differed from these widespread haplotypes by only a few mutational steps, supporting this view (though the two internal haplotypes, Haplotypes BW and BX, might reflect the possible uncertainty in network construction). Theoretically, such a combination of unique haplotypes and widespread haplotypes would also be expected for introduced populations from unsampled populations, because many local populations in Hokkaido possessed site-specific haplotypes in high frequency (it is theoretically possible that Haplotypes BW and BX originated from artificial introductions of undiscovered haplotypes on Hokkaido Island). Further samplings are desired to solve this problem.

Second, the genetic diversity of the Honshu populations is relatively high, were it assumed that they originated from artificial introduction. *Lefua nikkonis* itself has no commercial importance, and it is believed that they could have been accidentally introduced to Honshu from Hokkaido stock via releases of the weather loach *Misgurnus anguillicaudatus* (based on a hearing investigation by Takeuchi and Ohta 1993). Accidental introductions, if any ever occurred, would lead to the loss of genetic diversity of a population due to founder effects. However, as many as 12 haplotypes were found among the 47 Honshu individuals that were collected from the relatively small geographic range. Of course, if the weather loach was frequently introduced from Hokkaido for a long time, many *L. nikkonis* could be brought with it. However, the weather loach for shipping is usually collected from irrigation canals, which are not a typical habitat of *L. nikkonis*; they commonly inhabit marshes and little streams in wetlands. Moreover, *L. nikkonis* are distributed in many river systems on Honshu Island. These facts are inconsistent with an accidental introduction scenario. Furthermore, the endemism of Honshu populations has been previously suggested following analyses of several morphological traits, including the morphology of the caudal part of the body and the pattern of blotches on the top of the head (Ooyagi 2013).

The discontinuous distribution of the late-branching lineages, Clades 3-7 and 3-8, across southern Hokkaido was probably formed by southward dispersal bypassing this region. For instance, Uchiura Bay (Funka Bay) might have housed relic populations. The mouth of Uchiura Bay is shallower than the interior of the bay (Ohtani and Kido 1980); hence, the bay may have once been closed off and desalted during periods of low sea level. The distinct haplotypes in this region (Clades 3-3 and 3-4) could be the outcome of isolation in this inland pool of water, and the late-branching haplotypes could disperse southward along the east coast bypassing the inland pool. Interestingly, the late-branching haplotypes are mixed with the early-branching haplotypes at the southern tip of Hokkaido, where the land bridge emerged across the Tsugaru Strait. This fact supports our view that Haplotypes G and M, which are the central haplotypes in the late-branching haplotypes, crossed here onto Honshu.

These interpretations contradict the conventional view that the Ishikari Lowland of Hokkaido has been a barrier for the southward dispersal of primary freshwater fishes from Siberia (Goto et al. 1978; Goto 1982). On the contrary, our results suggest that *L. nikkonis* even dispersed southward across the Tsugaru Strait. A subsequent question of interest is thus when did the species cross the strait? Our estimation of the time to the most recent common ancestor (TMRCA) of Clades 3-7 and 3-8, to which all the Honshu-endemic haplotypes belonged, is 0.08–0.19 Mya (mean = 0.13 Mya). Because the estimated mean clock rate of cyt *b*, i.e., 4.9% per Myr, may be a little higher than the rates previously reported (although that of D-loop, 3.1% per Myr, is quite possible) (Watanabe and Takahashi 2010), the TMRCA of Clades 3-7 and 3-8 might be a little underestimated. Even if so, however, the TMRCA would be still after the Mindel glaciation (0.23–0.30 Mya). In any case, the TMRCA is just the maximum limit of population divergence, indicating that southward dispersal across the strait may have occurred any time after that. However, the presence of many Honshu-endemic haplotypes suggests that the population divergence was not a recent event. Given that the Tsugaru Strait did not have a land bridge during the last glacial maximum (about 20,000 years ago) (Ohshima 1991; Ono 1994), it is probable that *L. nikkonis*

dispersed from Hokkaido to Honshu during the Riss glaciation (0.14–0.18 Mya). The sea level during this glaciation was 130–140 m lower than present, which resulted in a land bridge across the Tsugaru Strait (Fig. 1) (Ono 1994). Some mammals are also thought to have crossed the strait via that land bridge. For example, the ermine *Mustela erminea* exhibits a cross-strait distribution, and genetic divergence between Hokkaido and Honshu ermine populations occurred approximately 0.15 Mya (Kurose et al. 1999). Considering that mammals might be able to swim the strait, evidence from a primary freshwater fish that can disperse only by land adds strength to the hypothesis that a land bridge existed across the Tsugaru Strait during this glacial period.

Freshwater taxa in general are believed to have crossed the strait much earlier than the dates estimated in our analyses of *L. nikkonis*. For example, phylogeographic analyses of the Japanese crayfish *Cambaroides japonicus* estimated that its colonization from Hokkaido to Honshu occurred during the Middle Pleistocene (0.9-1.3 Mya) (Koizumi et al. 2012). Similarly, the landlocked sculpin *Cottus nozawae* is estimated to have crossed the strait approximately 1.5 Mya (Yokoyama and Goto 2002). In contrast, no apparent genetic difference between populations on the two islands was reported in the landlocked lamprey *Lethenteron* sp. N, suggesting that dispersal of this species to its present distribution occurred recently (Yamazaki et al. 2003). However, caution is needed in interpreting the genetic patterns uncovered among the sculpin and lamprey, since landlocked species may have diadromous ancestors. The Japanese silver crucian carp *Carassius auratus langsdorfii*, which is a temperate primary freshwater fish of Chinese origin, is believed to have dispersed from Honshu northward to Hokkaido (Goto 1994), although no molecular dating of the dispersal has been attempted. To our knowledge, this is the first study that demonstrates a Late Pleistocene dispersal across the Tsugaru Strait by a freshwater taxon. For more robust estimations of the divergence time between Hokkaido and Honshu populations, simulation-based approaches using multiple nuclear markers should be performed in the future.

Given that other freshwater taxa crossed the strait much earlier (during the Middle Pleistocene), it is likely that land bridges have emerged repeatedly on this strait. If so, the Tsugaru Strait and the Ishikari Lowland of Hokkaido may be less important as barriers to the dispersal of freshwater taxa than is currently thought. However, the boundary between northern Honshu Island and southern Hokkaido Island is a well-known biogeographical transition zone between the Manchurian and Siberian sub-regions of the Palearctic Region. In the case of primary freshwater fishes, many species have the limit of their range in this area. As mentioned, the pond minnow *R. p. sachalinensis* and the

Siberian stone loach *Barbatula oreas*, both primary freshwater fishes descendent from Siberia, as is *L. nikkonis*, are not found on Honshu Island, and several fish species of Chinese origin, such as *Cobitis* spp. of loach and the small cyprinid *Tanakia lanceolata*, have northern limits at the northern end of Honshu Island (Masuda et al. 1988). Of course, the success of a species in dispersing from one major island to another depends on the dispersal ability of that species. However, it is also possible that distributional limits might persist even in the absence of a strait. The fact that *L. nikkonis* and *Cottus nozawae*, both of which dispersed southward from Hokkaido to Honshu, remain only in the northern part of Honshu suggests that they are unable to disperse further southward (although one undescribed Siberia-descendent species of *Misgurnus* may occur as far as central Honshu; Nakajima 2017). Similarly, the distribution of the crayfish *Cambaroides japonicus* on Honshu Island is limited to the northern part. The silver crucian carp *Carassius auratus langsdorfii*, which colonized Hokkaido from Honshu, is not currently found at Sakhalin, although land bridges have existed across the Soya Strait for longer durations than across the Tsugaru Strait (Ohshima 1991; Ono 1994). These observations suggest that the faunal transition zone would persist around northern Honshu and southern Hokkaido even if the Tsugaru Strait did not exist. We think that the biogeographical boundary here instead reflects an ecological transition from cold-water stenothermal fauna to warm-water eurythermal fauna, which may be set even without climatic boundary. Additional exploration of the distribution limits of various trans-strait species may provide insight into the importance of straits versus the intrinsic physiological characteristics of a species in the delimitation of biogeographical boundaries.

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