FULL PAPER

Morphologies and population genetic structures of the eight-barbel loach of the genus *Lefua* on southern Sakhalin

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Abstract

Coldwater, primary freshwater, fish such as the eight-barbel loach, *Lefua nikkonis*, are thought to have colonised Hokkaido from the continental Far East *via* Sakhalin Island during the Late Pleistocene. *Lefua* populations have been reported on southern Sakhalin, but detailed morphological and population structure analyses have not yet been completed. This information is important for reconstructing the colonisation history of *L. nikkonis* to Hokkaido. In this study, morphological analysis revealed that *L. nikkonis* and two continental congeners, *Lefua pleskei* and *Lefua costata*, are distinguishable from each other, and *Lefua* collected from southern Sakhalin is morphologically more similar to *L. nikkonis*. Random forest analysis, a machine learning classification method, classified all Sakhalin individuals as *L. nikkonis*. Haplotype analysis of mitochondrial DNA sequences revealed that all but one Sakhalin haplotype are shared with *L. nikkonis* and none is shared with the two continental congeners, which supports the hypothesis that Sakhalin *Lefua* are *L. nikkonis*. However, none of the Sakhalin haplotypes was distributed on northern Hokkaido. This discontinuous distribution of haplotypes across Sakhalin and Hokkaido suggests that the Sakhalin *Lefua* populations are not native. Some of the Sakhalin haplotypes were found only on Hokkaido's Ishikari River system or the Tokachi River system, suggesting that they originated from these regions. Because previous field surveys reported wild *Lefua* only from northwestern Sakhalin, we concluded that native *Lefua* on southern Sakhalin may have gone extinct after they colonised Hokkaido in the Middle Pleistocene.

Keywords Random forest analysis · MtDNA · Siberian primary freshwater fish · Alien species

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Introduction

One dispersal mechanism used by coldwater, primarily freshwater, fish to move between islands was land bridges that appeared during glacial periods (Goto and Nakano 1993; Avise 2000). In the Japanese archipelago, these fish likely moved from the continental Far East to the island of Hokkaido *via* one of the land bridges that repeatedly formed between the islands of Sakhalin and Hokkaido (Goto and Nakano 1993). Consistent with this view, coldwater, primary freshwater, fish on Hokkaido have conspecific populations, or closely related (sub)species, on both Sakhalin and the continental mainland (Lindberg 1972; Watanabe et al. 2006). Population structure analyses also support this hypothesis. For example, populations of the lake minnow, *Rhynchocypris perenurus sachalinensis*, on northern and eastern Hokkaido share mitochondrial DNA (mtDNA) haplotypes with those on southern Sakhalin (Sakai et al. 2014).

The eight-barbel loach *Lefua nikkonis* is another cold-water, primary freshwater, fish distributed throughout Hokkaido and parts of northern Honshu (the Shimokita Peninsula) (Takeuchi and Ohta 1993; Ooyagi 2013; Nakajima and Uchiyama 2017). Recently, Ooyagi et al. (2018) examined the population structures of *L. nikkonis* using mtDNA haplotypes and demonstrated that they dispersed southward, from Hokkaido to Honshu, *via* a land bridge(s) that emerged at the Tsugaru Strait during the MIS 5e (i.e. 130,000–200,000 years ago). However the origins of the Hokkaido populations of this primary freshwater species remain unclear. Although *Lefua* populations have been reported on southern Sakhalin (Shedko and Shedko 2003; Shedko et al. 2008; Pietsch et al. 2012; Labai et al. 2014; Dyldin and Orlov 2016; Nakajima and Uchiyama 2017), detailed morphological analysis and population structure analysis, using molecular markers, have not been completed. Since *L. nikkonis* has two continental congeners, *Lefua pleskei* and *Lefua costata*, (Fig. 1; Bogutskaya and Naseka 1996; Novikov et al. 2002; Bogutskaya et al. 2008; Shedko et al. 2008; Pietsch et al. 2012; Dyldin and Orlov 2016), the Sakhalin *Lefua* populations are very important

for reconstructing the colonisation history of *L. nikkonis* on Hokkaido.

In this study, we examine the morphologies of Sakhalin *Lefua* and compare them with *L. nikkonis*, collected from Hokkaido and Honshu, and with the two continental congeners. We also investigate mtDNA haplotypes within the Sakhalin populations and compared them with those of *L. nikkonis* collected throughout their geographic range. Based on the results, we discuss the origin of the Sakhalin *Lefua* populations and the colonisation history of *L. nikkonis* on Hokkaido.

Materials and methods

Field collections. Twenty-eight and 39 *Lefua* individuals were collected from Lake Bol'shoye Vavayskoye and Lake Maloye-Chibisanskoye, respectively, on southern Sakhalin (Fig. 1; Table S1). Each individual was euthanised using clove oil (NOW Foods, USA) (Soto and Burhanuddin 1995). The right pelvic fin was cut and preserved in 99.5% ethanol for mitochondrial analysis, described below. The remaining

Fig. 1 Schema of geographic distribution and phylogenetic relationships of *Lefua nikkonis* and two related continental congeners, *Lefua pleskei* and *Lefua constata* [the phylogeny was modified from Ooyagi et al. (2018)]. Sites from which samples for morphological analysis was obtained are also shown
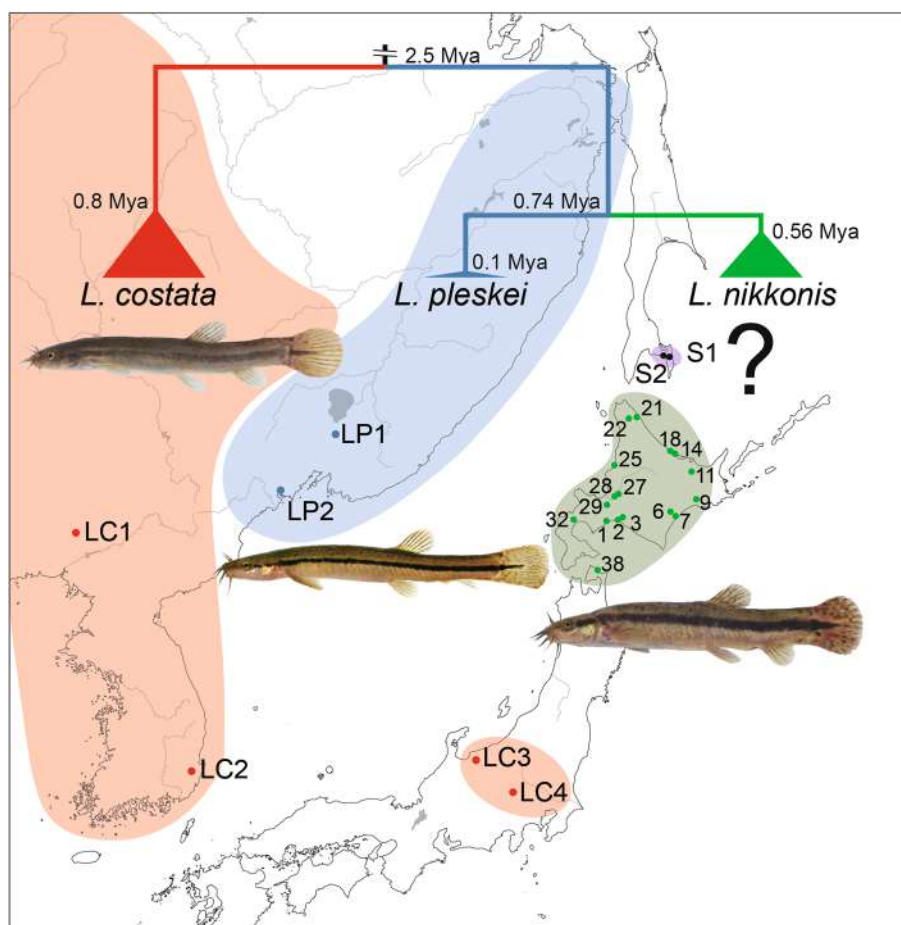
body was fixed in 10% formalin and then preserved in 70% ethanol.

Additionally, one to 10 individuals of *Lefua nikkonis* were collected from each of the 17 locations on the islands of Hokkaido and Honshu (65 individuals in total), where Ooyagi et al. (2018) examined mitochondrial haplotypes of this species (Fig. 1; Table S1). Furthermore, 10 and five individuals of *Lefua pleskei* were collected from the Illistaya River (St. LP1) and the Kraskino area (St. LP2), respectively, in Far East continental Russia (Fig. 1; Table S1). The Illistaya River is just the type locality of *L. pleskei*. Twelve *Lefua costata* individuals were also collected from Midoriko, Honshu Island (St. LC4; Fig. 1; Table S1), which is known to be an introduction site for this species from the continental Far East (Nakajima and Uchiyama 2017). All collected individuals of *L. nikkonis*, *L. pleskei* and *L. costata* were euthanised, fixed in 10% formalin and transferred to a 70% ethanol solution for preservation.

Morphological analysis. Among the Sakhalin *Lefua* individuals preserved, 10 specimens (five males and five females) taken from Lake Bol'shoye Vavayskoye and Lake Maloye-Chibisanskoye were examined to assess their morphology. Twenty-three morphometric measurements (Fig. 2; Table S2) were obtained from each individual using a dial caliper (D15F, Mitutoyo) to the nearest 0.01 mm. Vertebrae numbers without Weberian apparatus (VN) were counted using radiographs (CMB-2, Softex). Each individual was sexed based on body colouration (Hosoya 2013; Kuwahara 2015; Nakajima and Uchiyama 2017). Similarly, morphometric measurements and vertebral counts were obtained from the preserved individuals of *L. nikkonis*, *L. pleskei*, and

Honshu *L. costata*. In addition, 13 individuals of *L. costata*, which were collected from the Korean Peninsula and stored in the Natural History Museum and Institute in Chiba, Japan (CBM), and in the Hokkaido University Museum in Hakodate, Japan (HUMZ), were examined in a similar manner.

Each morphometric measurement was divided by SL and log-transformed, and morphometric variations among the samples were described using principal component analysis (PCA). Because sexual differences in fin lengths were evident (Fig. S1, see also Results), the PCAs were conducted separately for fin length measurements (five total) and body-shape measurements (17 total).

We also classified the Sakhalin *Lefua* into one of the three *Lefua* species using random forest analysis (Breiman 2001; Cutler et al. 2007). The morphometric measurements and VN of *L. nikkonis* from Hokkaido and Honshu, *L. pleskei* and *L. costata* were used as 'training data'. Random forest analysis is a machine learning classification method, which 1) can be used when the number of variables is larger than the number of samples; 2) does not overfit; 3) performs well, even if many variables do not contribute to the results; 4) incorporates variable interactions and 5) estimates each variable's importance (Díaz-Uriarte and De Andrés 2006). In this study, we constructed 10,000 decision tree, using the 23 morphometric measurements (each divided by SL and log-transformed), VN (log-transformed) and the sex of each individual as input variables. For each decision tree, one optimal input variable was chosen at each node from five randomly selected input variables based on the Gini coefficient. To detect the input variables that did not contribute to the decision-tree construction, we removed

Fig. 2 The positions measured in morphological analysis. See Table S2 for definitions of abbreviations
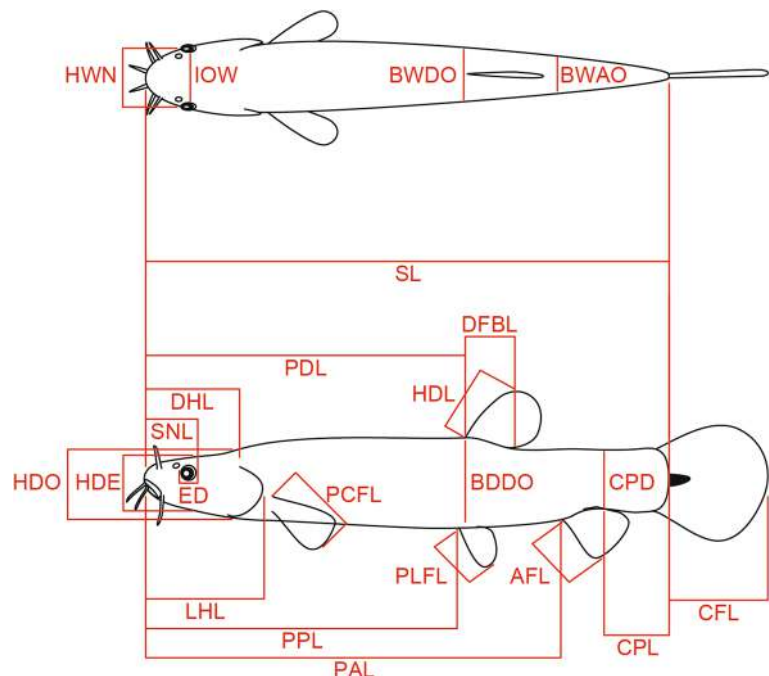
input variables, one by one, in an ascending order of mean decrease in the Gini coefficient, until the out-of-bag data error rate increased. After removing less-contributory input variables, we re-constructed 10,000 decision trees in the same manner as described above. Using these decision trees, the importance of each variable was estimated by the mean decrease in the Gini coefficient, and partial dependence plots were obtained for the four most important variables. The partial dependence plots predict that individuals having a certain phenotypic value in the focal variable are one of the three species when all other variables are average. A discrimination prediction model was also obtained from the 10,000 decision trees, Each of the Sakhalin individuals was classified into one of this three species using the prediction model. The analyses were conducted using the R (ver. 3.4.3) statistical packages randomForest (ver. 4.6-14) and edarf (ver. 1.1.1).

Mitochondrial analysis. DNA was extracted from each of the preserved fins collected from the 67 Sakhalin *Lefua* specimens (28 and 39 individuals from Lake Bol'shoye Vavayskoye and Lake Maloye-Chibisanskoye, respectively) using a DNeasy Blood and Tissue Kit (Qiagen, Venlo, the Netherlands), following the manufacturer's protocols. An mtDNA region, including a partial sequence (960 bp) of cytochrome *b* (cyt *b*), was amplified and sequenced, using the methods and primers described by Ooyagi et al. (2018). The sequence was obtained successfully for 66 of the 67 individuals examined.

Using the ClustalW option in MEGA7 ver. 7.0.14 (Kumar et al. 2016), the cyt *b* sequences of the 66 Sakhalin *Lefua* were aligned with those of the 241 *L. nikkonis* individuals from Hokkaido and northern Honshu, the five *L. pleskei* individuals sequenced by Ooyagi et al. (2018) and the two cyt *b* sequences of *L. costata* (KT943751 and DQ105196) retrieved from the DNA Data Bank of Japan (DDBJ). Based on the 960-bp sequences obtained by this alignment, unique haplotypes were detected from 288 sequences (i.e. 66 Sakhalin *Lefua*, 241 *L. nikkonis*, five *L. pleskei*, and two *L. costata* individuals) using the DnaSP ver. 5.10.01 (Librado and Rozas 2009). One haplotype endemic to Sakhalin had been deposited at the DDBJ.

Results

Morphological analysis. The PCA revealed that 34.2% and 13.9% of the variances in body shape were explained by the first (PC1) and second (PC2), respectively. PC1 and PC2 reflected overall head size (e.g. HDO and HDE) and body width (e.g. BWAO and BWDO), respectively (Table S3). A scatter plot of the PC1 and PC2 scores showed that the three *Lefua* species tended to be separate from each other (Fig. 3): *Lefua nikkonis* exhibited a lower PC1 (i.e. larger head) and a lower PC2 (i.e. wider body), *Lefua costata* a higher PC1 and a lower PC2 (i.e. smaller head and wider body), and *Lefua pleskei*, a higher PC2 (i.e. slender body) (see also

Fig. 3 Scatterplots of the first two axes of the principal component analysis based on 17 body shape measurements of the three *Lefua* species. See Fig. 1 and Table S1 for site numbers for *Lefua nikkonis*
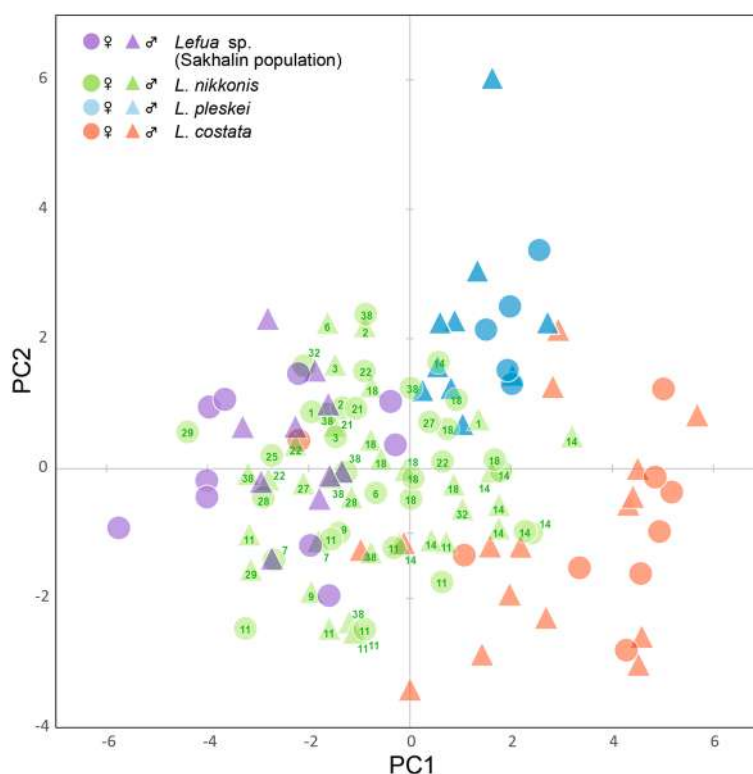
Table S4; Fig. S1). Individuals from the Sakhalin *Lefua* were clustered with the *L. nikkonis* individuals (Fig. 3); no or only a little overlap with *L. pleskei* and *L. costata* was found. The PCA on fin lengths revealed clear sexual dimorphism (Fig. S2): males tended to have longer fins (except for the caudal fin; see Fig. S1). No interspecific difference in fin lengths was found. *L. nikkonis* tended to have fewer vertebrae (median = 33, range = 32–35) than *L. pleskei* (median = 36, range = 35–37) and *L. costata* (median = 35, range = 34–37) (Table 1). The number of vertebrae in Sakhalin *Lefua* (median = 34, range = 33–34) was within the range of *L. nikkonis*.

Fifteen morphometric characters and sex were judged to be less contributory variables in the random forest analysis for discrimination of *L. nikkonis*, *L. pleskei* and *L. costata*. Using the remaining eight characters, the best prediction model was constructed, with a high accuracy of 94.29%. Among the eight characters, interorbital width (IOW), head depth at the end of the opercle (HDO), vertebrae numbers (VN) and the head depth at the eyes (HDE) were the four most important traits for classifying the three species (Fig. S3). Partial dependence plots revealed that *L. nikkonis* is distinguishable from *L. pleskei* and *L. costata* using these

four characters. In particular, individuals with IOW/SL of less than 0.075 were predicted to be *L. costata*, whereas *L. nikkonis* was the most likely species when IOW/SL was more than 0.080 (Fig. 4). Individuals with higher HDO/SL, fewer vertebrae and higher HDE/SL were also predicted more likely to be *L. nikkonis* (Fig. 4). Discrimination, by the optimal model, revealed that all 20 specimens from Sakhalin were designated with a high probability as *L. nikkonis* (Table 2).

Mitochondrial analysis. A total of 62 haplotypes were detected from the cyt *b* sequences of the 241 individuals from Hokkaido and Honshu that were obtained by Ooyagi et al. (2018) (Table S5), while only five haplotypes were detected from the 66 specimens collected from Sakhalin. Among these five haplotypes, four haplotypes (Hap-07, Hap-11, Hap-12 and Hap-42) were also distributed either on Hokkaido or on both Hokkaido and Honshu (Table S5; Fig. S4). Among these four, Hap-42 was most dominant on Sakhalin (44 individuals) but was only found at Kamihoromui in the Ishikari River basin on Hokkaido (Fig. 5; Table S5). Hap-07 was found in 13 individuals on Sakhalin, while Hap-12 was found in six individuals on Sakhalin. These haplotypes were widely distributed on Hokkaido, including the eastern

Table 1 Frequency distribution of vertebrae numbers without Weberian apparatus of the three *Lefua* species

Species	Site number	Vertebrae numbers without Weberian apparatus					
		32	33	34	35	36	37
Lefua sp.	S1		5	5			
	S2		4	6			
L. nikkonis	1			2			
	2		2				
	3		2				
	6		1		1		
	7		2				
	9			1	1		
	11	2	4	4			
	14			7	3		
	18	1	2	7			
	21	1	1				
	22		3	1			
	25		1				
	27		2				
	28		2				
	29	1	1				
	32			1	1		
	38	1	6	1			
L. pleskei	LP1				5	4	1
	LP2				1	3	1
L. costata	LC1				1	1	
	LC2			3	4		
	LC3			1	3		
	LC4			2	7	2	1

Fig. 4 Partial dependence plot of the first four most important characters for classifying the three *Lefua* species, estimated by random forest analysis using the training data. The total prediction values of the three species sum up to 1
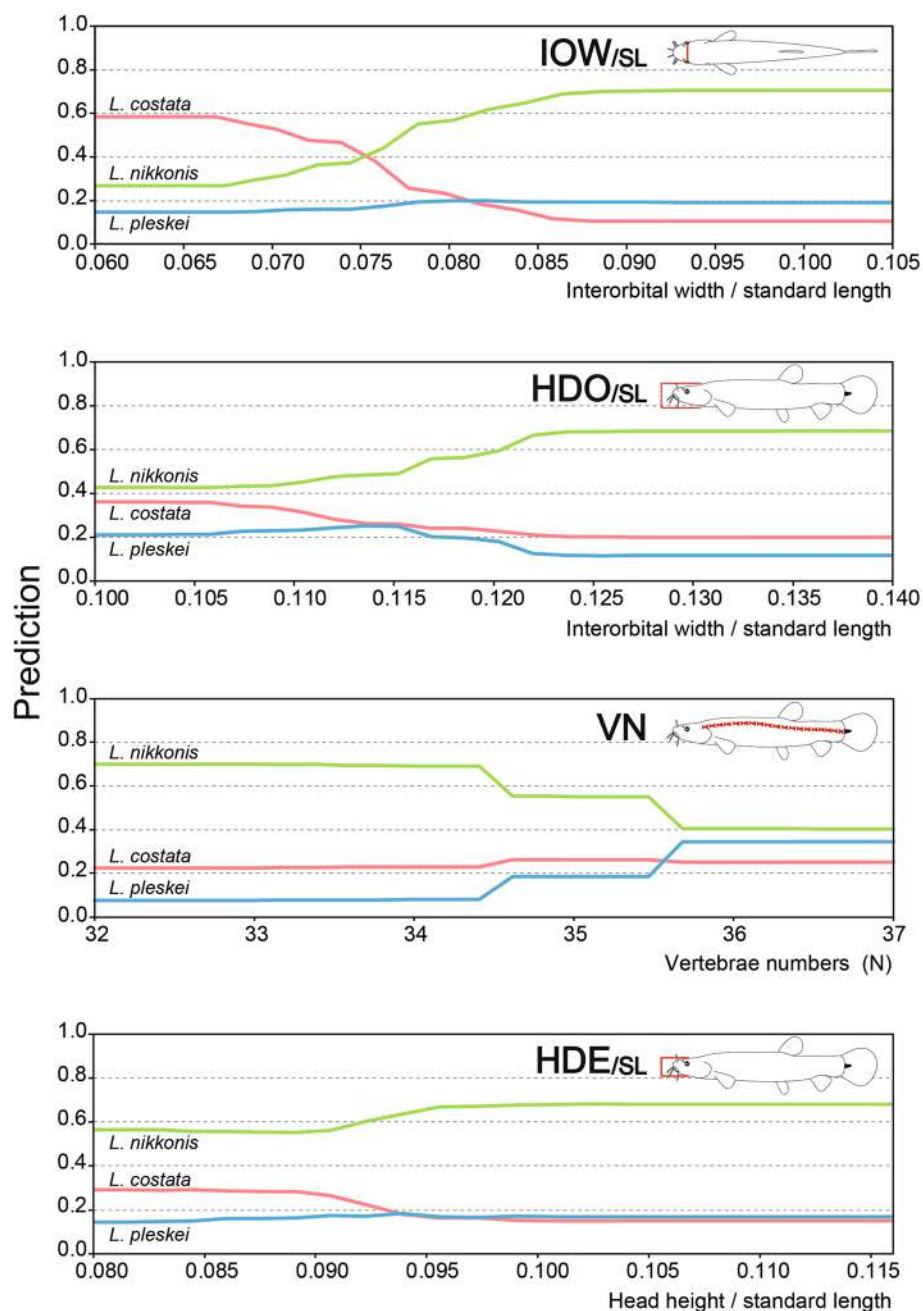
part of the island, the Ishikari area, the Iburi area, the southern part of the Oshima Peninsula and Honshu's Shimokita Peninsula (Fig. 5; Table S5). Hap-11 was detected only in a single Sakhalin specimen. This haplotype was found only in the Tokachi area on Hokkaido (in the Ikusota and Urahoro areas). No haplotypes were shared between Sakhalin and northern Hokkaido (Fig. 5).

Among the five Sakhalin haplotypes, Hap-63 was endemic to Sakhalin; this haplotype was carried only by two individuals. Hap-63 differed from Hap-07 only by a single mutational step (Fig. S4). Additionally, the sequence of Hap-63 was considerably different from any of the five *L.*

pleskei haplotypes sequenced by Ooyagi et al. (2018). It was also completely different from the two *L. costata* haplotypes.

Discussion

Morphological and genetic characteristics of the Sakhalin *Lefua*. We found that *Lefua nikkonis*, *Lefua pleskei* and *Lefua costata* are morphologically distinct from one another. In particular, the number of vertebrae was an important diagnostic character to distinguish *L. nikkonis* from *L. pleskei*. This finding is consistent with that of Shedko et al. (2008)

Table 2 Prediction (%) of species for each of the 20 Sakhalin individuals estimated by random forest analysis

Individual ID	*L. nikkonis*	*L. pleskei*	*L. costata*
Sak-01	99.54	0.07	0.39
Sak-02	67.77	11.41	20.82
Sak-03	75.27	13.13	11.60
Sak-04	88.78	9.79	1.43
Sak-05	83.05	6.67	10.28
Sak-06	100.00	0.00	0.00
Sak-07	100.00	0.00	0.00
Sak-08	95.68	0.00	4.32
Sak-09	99.26	0.26	0.48
Sak-10	100.00	0.00	0.00
Sak-11	99.99	0.01	0.00
Sak-12	77.28	7.93	14.79
Sak-13	99.99	0.01	0.00
Sak-14	97.73	1.79	0.48
Sak-15	94.22	3.37	2.41
Sak-16	76.20	7.66	16.14
Sak-17	96.18	0.00	3.82
Sak-18	100.00	0.00	0.00
Sak-19	99.80	0.11	0.09
Sak-20	100.00	0.00	0.00

who reported that *L. nikkonis* and *L. pleskei* differ in several meristic characteristics, including the number of vertebrae. We also found several morphometric differences among the three species. Generally, *L. nikkonis* had larger heads than the other two species, as shown by clear differences in head depth and interorbital width measurements. Nakajima and Uchiyama (2017) indicated that *L. nikkonis* can be distinguished from *L. costata* by body height. We also found that *L. pleskei* has more slender bodies than *L. nikkonis* and *L. costata*. Naseka and Bogutskaya (2004) reported that *L. pleskei* and *L. costata* are distinguishable by eye size and pre-dorsal length. Thus, these three species are distinguishable from one other by a combination of several morphological characters. Interestingly, however, *L. nikkonis* in some Okhotsk populations (St. 14 and St. 18), known to carry unique haplotypes that are closer to those of *L. pleskei*, tended to be morphologically closer to the continental species (Fig. 3). Further morphological analyses, especially those covering wider geographic range of the continental *Lefua* may be necessary to demonstrate whether they are valid species.

Apart from the three species' taxonomic status, we found that the Sakhalin *Lefua* is morphologically more similar to *L. nikkonis* on Hokkaido and Honshu. The random forest analysis also classified all of the specimens collected from Sakhalin as *L. nikkonis*. In the random forest analysis, several characters representing head sizes (such as interorbital width

and head depths) and vertebral numbers were determined to be important diagnostic characters. All Sakhalin *Lefua* had fewer than 35 vertebrae and had relatively larger heads than the two continental congeners (Table 1; Table S4; Fig. S1), which contributed to their classification as *L. nikkonis*. Although Labai et al. (2014) reported an introduced population of *L. costata* in southern Sakhalin, we hypothesise that it is actually *L. nikkonis*. The haplotype analysis of mitochondrial DNA supports this view: all Sakhalin haplotypes, except for one, were shared by *L. nikkonis* on Hokkaido and Honshu. Although one Sakhalin haplotype (Hap-63) was unique, it was not found in either *L. costata* or *L. pleskei*. Indeed, this private haplotype was different from the other non-private haplotype (Hap-07) by only one mutational step, indicating that it is also a haplotype of *L. nikkonis*

Artificially introduced *L. nikkonis* from Japan to Sakhalin. We found that two out of the five haplotypes found on Sakhalin *Lefua* (Hap-07 and Hap-12) were also widely distributed on the islands of Hokkaido and Honshu. However, none of the Sakhalin haplotypes, including the two widespread haplotypes, were found on northern Hokkaido. This discontinuous distribution of haplotypes across Sakhalin and Hokkaido suggests that the Sakhalin *Lefua* populations are not native. As mentioned above, although one unique haplotype (Hap-63) was detected, it differed from the other non-private haplotype (Hap-07) only by one mutational step, suggesting that Hap-63 was not detected by Ooyagi et al. (2018). Thus, we conclude that the Sakhalin *Lefua* is *L. nikkonis*, which was probably artificially introduced.

The fact that some of the Sakhalin haplotypes exhibit very limited distributions on Hokkaido provides insight into possible source populations for their introduction. The major Sakhalin haplotype (Hap-42) was found only in the Ishikari River system and another Sakhalin haplotype (Hap-11) was found only in the Tokachi River system. This strongly suggests that Sakhalin *Lefua* originated from the Ishikari and Tokachi regions. The wide distribution of the non-private haplotype (Hap-07) around the Ishikari area supports this view. The other non-private haplotype (Hap-12) was not distributed in the Ishikari and Tokachi areas, but it would probably have originated from the Tokachi areas also, given that it was discovered in the nearby Kushiro area. Further analyses of mitochondrial haplotypes and/or genome-wide nuclear markers such as single nuclear polymorphisms throughout *L. nikkonis*'s geographic range are required to determine the origins of the Sakhalin *L. nikkonis* populations.

It has been reported that the distribution of *Lefua* on southern Sakhalin is limited only to Lake Vavayskoye and its surroundings (Pietsch et al. 2012; Dyldin and Orlov 2016), despite there being plenty of potential habitats throughout southern Sakhalin (Y. Machida, personal observations). In the field collections for this study, *L. nikkonis* were indeed only caught around Lake Vavayskoye. This limited

Fig. 5 Map showing the haplo-type distributions found from the Sakhalin populations
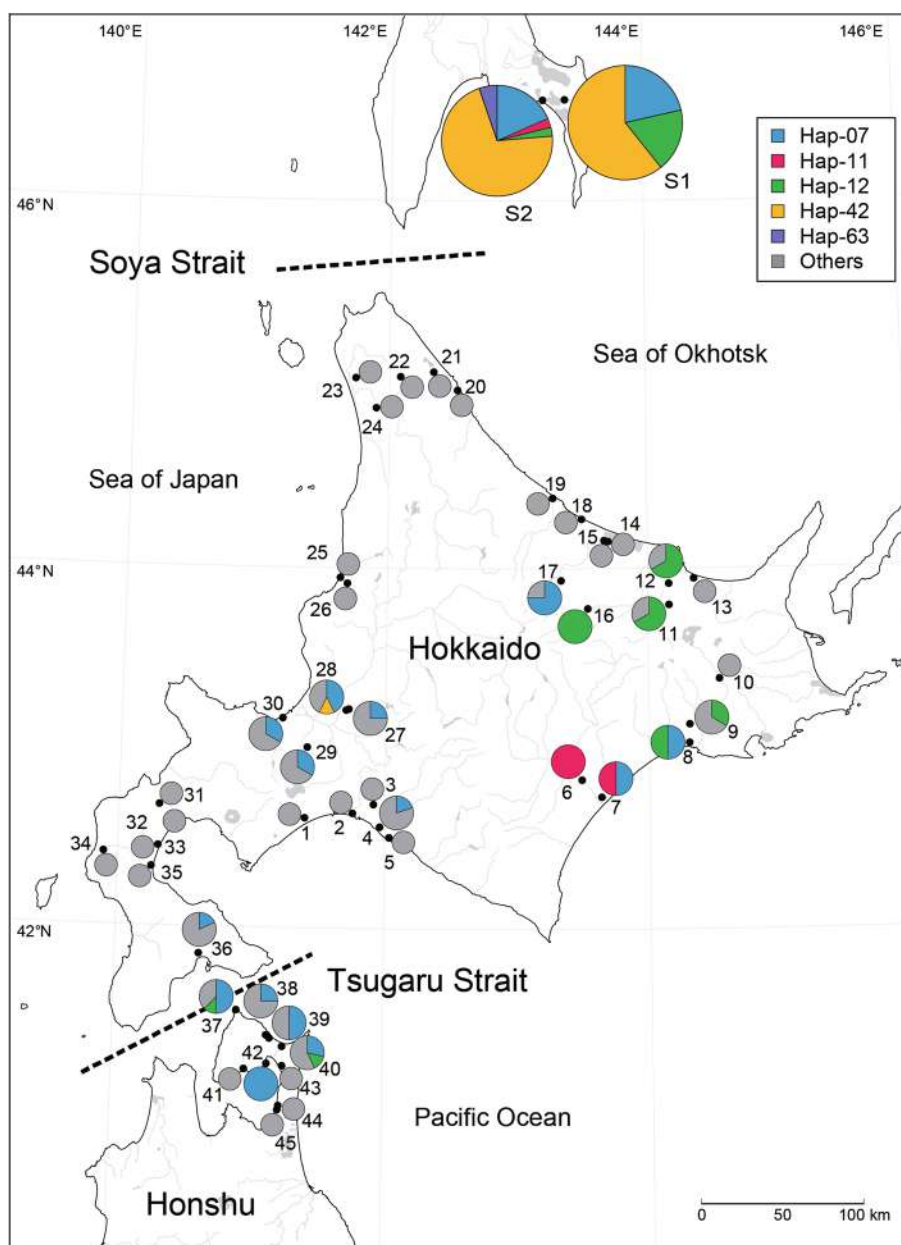
distribution also supports the artificial introduction hypothesis. In comparison, there are several records claiming that carp (*Cyprinus* sp.) and crucian carp (*Carassius* sp.) were introduced to the Lake Vavayskoye areas from Japan during the Japanese occupation periods (Karafutoteichi Gyogyousuisan Kumiai 1931; Karafutocho 1973; Zenkoku Karafuto Renmei 1978; Nikoforov et al. 1994). Since most colonists came from Hokkaido (Karafutocho 1973), it is likely that the carps also originated from Hokkaido. It is, therefore, likely that *L. nikkonis* was introduced accidentally at the same time.

There are several reports that the eggs of freshwater fish can be dispersed into remote habitats by waterfowl (e.g. Hirsch et al. 2018). However, this is unlikely to be the case

for *L. nikkonis* because its reproduction period peaks from May to June (Kuwahara 2015), by which time most waterfowl have departed from Hokkaido (Ministry of the Environment 2019). Furthermore, some waterfowl on Hokkaido, such as the mallard (*Anas platyrhynchos*), migrate to the continent without passing through Sakhalin (Higuchi 2012). It is also known that the embryos of *L. nikkonis* are not very sticky (Sawada 2005) and are unable to develop normally in saltwater, even at very low salt concentrations (Shimizu 2011), which would prevent dispersal by waterfowl that sometimes rest on the sea during their migration.

Colonisation history of *Lefua* from the continent to Hokkaido. *Lefua nikkonis* is considered to have colonised Hokkaido from the continental Far East *via* Sakhalin.

Ooyagi et al. ([2018](#)) estimated that *L. nikkonis* branched off from continental congeners in the Middle Pleistocene (around 0.74 Mya; 0.47–1.05 Mya). The southern Sakhalin populations were expected to elucidate whether this vicariance event occurred at Soya Strait, which separates present-day Hokkaido from Sakhalin, or at Mamiya Strait (Strait of Tatary), which separates Sakhalin from mainland Asia. However, it was confirmed in this study that they are not native. This implies that wild *Lefua* is maybe exclusively distributed only on northwestern Sakhalin (Safronov and Nikiforov [2003](#); Pietsch et al. [2012](#); Dyldin and Orlov [2016](#)). Because some of the previous surveys, such as by Pietsch et al. ([2012](#)), were very large (covering an area of about 70% of the island of Sakhalin) and time-consuming (3 years' duration), it could be safely concluded that no *Lefua* is distributed on the southern part of the island.

This discontinuous distribution on Sakhalin suggests that *Lefua* became virtually extinct on the island, except in the northwest. It was suggested that the Japanese crayfish *Cambaroides japonicus* might have experienced a similar large-scale extinction event on eastern Hokkaido, which was subject to discontinuous permafrost during some glacial periods (Koizumi et al. [2012](#)). If most freshwater systems had also been severely frozen in Sakhalin, several freshwater species, especially wetland species such as *Lefua*, might not have survived. To successfully reconstruct the colonisation history of *Lefua* to Hokkaido, not only the phylogeography of extant populations but also possible large-scale extinctions may have to be taken into account.

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