Population Genetic Structure of the Japanese Large-Footed Bat (*Myotis macrodactylus*) Along Three Rivers on Hokkaido Island, Northern Japan

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Population genetic structure of the Japanese large-footed bat (*Myotis macrodactylus*) along three rivers on Hokkaido Island, northern Japan

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Abstract. Rivers are habitats favored by insectivorous bats. The Japanese large-footed bat (*Myotis macrodactylus*) is one of such species strongly associated with rivers. To clarify the population structure and migration pattern of *M. macrodactylus*, mitochondrial DNA (mtDNA) phylogeography was investigated in 15 subpopulations along three rivers (Tokachi, Ishikari and Teshio Rivers) on Hokkaido Island, northern Japan. Of 267 bats examined, nine mtDNA cytochrome *b* haplotypes were identified and separated into two major genetic clades, which did not reflect geographic distributions within and among the river regions. Principal component analysis of mtDNA haplotypes showed that most subpopulations in Ishikari and Teshio of the three river regions were grouped into one genetically related group, whereas those in the Tokachi river region were remotely related to those of the other two river regions. The results could be ascribed to no geographic barriers preventing bats' migration between the former two river regions. By contrast, it is possible that the Daisetsuzan and Hidaka mountain ranges play a role as effective geographic barriers against migrations of this species between the Tokachi and Ishikari/Teshio river regions.

Key words: cytochrome *b*, Hokkaido, *Myotis macrodactylus*, population structure, river.

Bats are a diverse mammalian group that have succeeded in adaptive radiation and are found throughout the world except in polar regions and some isolated oceanic islands (Kunz and Pierson 1994; Willig et al. 2003). Because of their high adaptability, they utilize a variety of environments as both foraging (e.g., forests, riparian zones and urban areas) and roosting (e.g., tree cavities, underground sites and buildings) habitats (Kunz 1982; Altringham et al. 1998; Kunz and Lumsden 2003; Patterson et al. 2003), and their habitat preferences vary with species (e.g., Russo and Jones 2003; Davidson-Watts et al. 2006). In addition, their dispersal abilities differ among species because interspecific variations in their wing morphology affect their vagility distances (Norberg and Rayner 1987). Therefore, the genetic structures of each species or population may be influenced by various geographic features. In fact, the occurrences of mountain ranges, straits and forests affect bat genetic structures (Castella et al. 2000; Ruedi et al. 2008; Campbell et al. 2009).

A number of insectivorous bat species strongly depend on riparian habitats and aquatic environments such as rivers and lakes, which support large numbers of insects, as foraging habitats (Racey 1998; Grindal et al. 1999; Law and Chidel 2002; Fukui et al. 2006; Campbell et al. 2009). The bats also tend to utilize riparian forests and artificial structures along rivers as roosting habitats to reduce predation risk and energy cost (Speakman 1991; Sedgeley 2001). Thus riparian bats could be faithful to one river, and it is possible that their genetic structures are formed independently in each river region. However, there have been very few studies comparing their genetic

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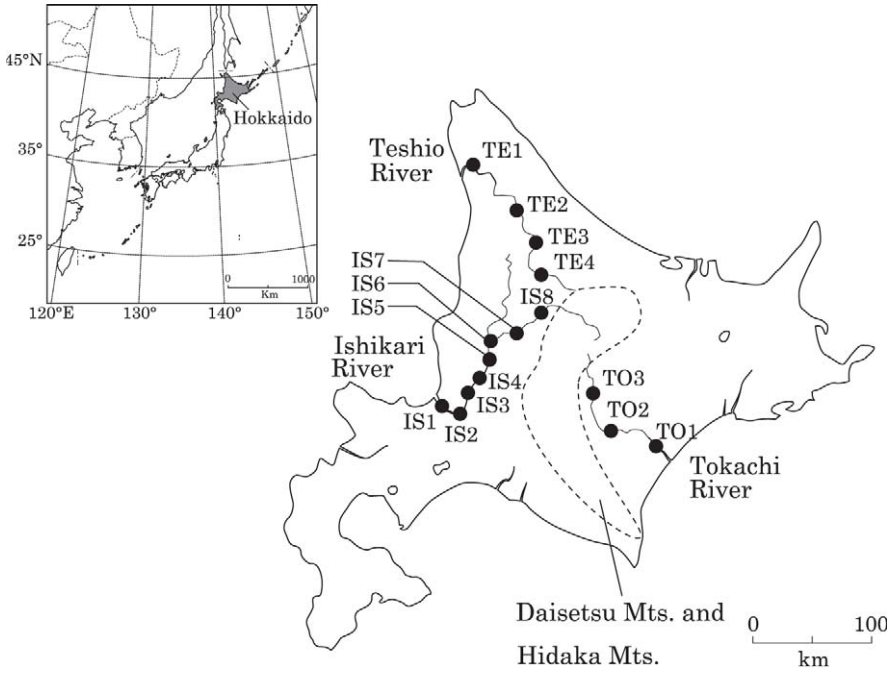

Fig. 1. Map showing the sampling locations of *Myotis macrodactylus* along the Tokachi (TO), Ishikari (IS) and Teshio (TE) river regions on Hokkaido Island, northern Japan. Broken-lined areas show the Daisetsuzan and Hidaka Mountain Ranges. The upper left map indicates the position of Hokkaido Island in the Japanese archipelago.

structures along river regions.

The Japanese large-footed bat (*Myotis macrodactylus*) is widely distributed in the Japanese archipelago (Sano 2009) and known as a river-depending species because of its roosting and feeding habitat use (Akasaka et al. 2007; Fukui et al. 2006, 2007). Although banding surveys have been implemented to estimate the migration pattern of this species, the low recapture rate prevents researchers from obtaining effective ecological data (Kuramoto et al. 1973). In such conditions, genetic analysis of their range could provide invaluable information to further understanding the population structure of the bats.

In the present study, we hypothesized that river-depending bat, *M. macrodactylus*, is faithful to one river and their population genetic structure have been formed independently in each river region. To test this hypothesis, we aimed to investigate the population genetic structure of *M. macrodactylus* distributed along three large rivers on Hokkaido Island (Fig. 1), based on the phylogeography of the mitochondrial DNA (mtDNA) cytochrome *b* gene (cytb). We then discuss whether the genetic structure of this bat species is influenced by three large rivers within Hokkaido.

Materials and methods

Sampling and DNA extraction

Tissue samples for DNA analysis were collected in 15 localities along three large rivers on Hokkaido Island: three sampling localities (TO1–TO3) along Tokachi River (156 km long), eight (IS1–IS8) along Ishikari River (268 km long) and four (TE1–TE4) along Teshio River (256 km long) (Fig. 1). In each locality, the bats, which were roosted in short tunnels under embankments, were captured by hand or using a hand net. The sampling periods were from June 6 to July 7, 2009 along Tokachi River and from September 19 to October 7, 2009 along Ishikari and Teshio Rivers. Bat wing membranes were punched using medical biopsy punchs (4 mm in diameter: Kai Industries Co), and then the bats were released. The membrane tissues were preserved in 99% ethanol at 4°C until use. Total DNA was extracted from the sample tissues using the DNeasy Blood & Tissue Kit (Qiagen) or QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's instructions.

PCR and PCR product purification

To obtain the entire cytb sequence (1,140 base-pair, bp), two separate segments, which partially overlapped

each other, were amplified by polymerase chain reaction (PCR) for each sample. The reaction was performed in a total volume of 50 μl containing 5 μl of 10 × PCR buffer (Takara), 4 μl of 2.5 mM dNTP mixture (Takara), 0.25 μl *Taq* DNA polymerase (5 units/μl: Takara), 0.5 μl of 25 μM each primer, 5–10 μl DNA extract, and distilled water for adjustment. The first segment (603 bp) was amplified by the primer set, BTCB1F (5'-ATGGACT TAAACCCACGACTAG-3') and MMCB1R (5'-CTAT GACCATGGCTGAGATG-3'), and the second segment (740 bp) was amplified by MMCB1F (5'-CATGAG GCCAAATATCCTTC-3') and BTCB1R (5'-TCTCTT GAGTCTTTGGGAGAA-3'). The above two primers (BTCB1F and BTCB1R) were newly designed based on the complete mtDNA sequence of the Japanese common pipistrelle (*Pipistrellus abramus*) available in the DNA Data Bank of Japan (DDBJ) (accession number AB061528), and the other two primers (MMCB1F and MMCB1R) were newly designed based on the cytb sequence of *M. macrodactylus* available in DDBJ (accession number AB085736). The PCR amplifications were carried out in a PCR thermal cycler (Takara TP600) under the following conditions: one cycle of denaturing at 96°C for 3 min; 40–45 cycles of denaturing at 96°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min; and a final extension cycle at 72°C for 10 min.

An aliquot (9 μl) of the PCR product was electrophoresed on a 2% agarose gel, stained by ethidium bromide, and then DNA bands in the gel were visualized under an ultraviolet illuminator. The remaining PCR products (41 μl) were purified using the QIAquick PCR Purification Kit (Qiagen) for subsequent sequencing.

Direct sequencing

Purified PCR products (2 μl) were mixed with 2.0 μl of the 5 × Sequencing buffer (Applied Biosystems), 0.5 μl of the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and 1.6 μl of 1 μM primer (MMCB1F or MMCB1R) in a total volume of 10 μl. Thermal cycling profiles of the sequencing reaction consisted of 25–30 cycles of 10 sec at 96°C, 5 sec at 46°C and 4 min at 60°C. After purification, the reactions were run on an ABI 3730 or ABI 3130 sequencer (Applied Biosystems).

Phylogenetic analysis

Haplotypes of mtDNA and distribution of their frequencies were determined using ARLEQUIN version 3.5.1.2 (Excoffier and Lischer 2010). To infer the phylogenetic relationships among the mtDNA haplotypes, neighbor-joining (NJ) and maximum likelihood (ML) methods were implemented using MEGA version 5.0 (Tamura et al. 2011). Kimura's two-parameter model (Kimura 1980) was used for genetic distance. A homologous sequence of the Daubenton's bat (*M. daubentonii*), available in DDBJ (accession number AF376847), was used as an outgroup. The supports of internal branches were assessed with 1000 bootstrap resampling. The other settings were set by default. In addition, the maximum parsimony (MP) network was made using TCS version 1.21 (Clement et al. 2000).

Analyses of population genetic structure

For further statistical analyses, one sampling locality was treated as one subpopulation. Two subpopulations, IS1 and IS3, were eliminated from analysis of the population genetic structure due to their small population sizes. First, the levels of genetic differentiations among subpopulations within each river region and among river regions were assessed with hierarchical analysis of molecular variance (AMOVA: Excoffier et al. 1992) by computing overall and pairwise F_{ST} using ARLEQUIN. Secondly, based on the mtDNA haplotype frequencies obtained, principal component analysis was implemented to examine the relationships among subpopulations using STATISTICA version 06J (Statsoft Japan). Thirdly, to check patterns of isolation by distance (IBD) within each river region and among river regions, we plotted linearized F_{ST} values (Slatkin 1995) against the geographical distances and implemented mantel tests using ARLEQUIN. In this analysis, we used both euclidean distances (EU distance) and the distances along rivers (AR distance), which were measured using electronic map with a scale of 1 to 25,000 (Japan TOPO10M Plus, Garmin), as the geographic distances. The AR distance between subpopulations in different river regions were measured via a straight line connected between the closest positions of two rivers. Finally, to visualize the relationships among subpopulations, an NJ tree based on F_{ST} values was reconstructed using MEGA.

Results

Phylogenetic relationships among mtDNA haplotypes identified in Hokkaido

From the 267 individuals in 15 subpopulations, nine mtDNA haplotypes (H1–H9) were identified (Fig. 2 and

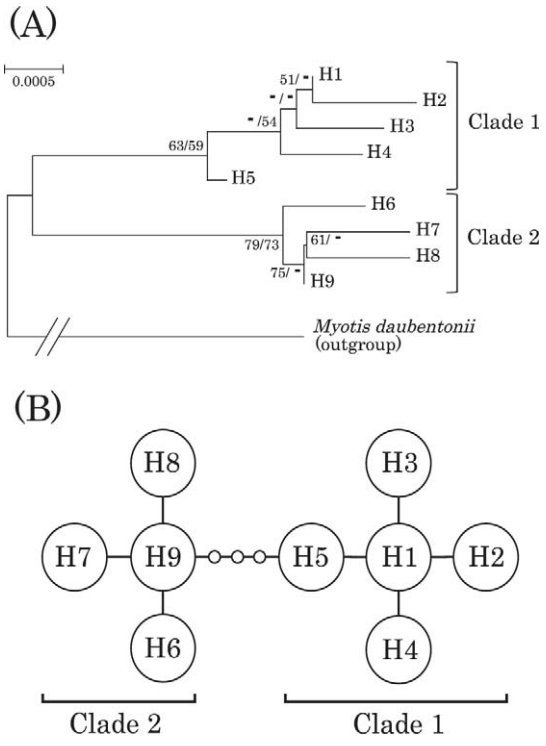

Fig. 2. (A) The neighbor-joining (NJ) relationships among nine mtDNA haplotypes identified from *Myotis macrodactylus* in Hokkaido. The values around internal branches refer to bootstrap values (%), which are shown for NJ and ML methods in order, and dashes indicate < 50%. Two major clades are supported by 59–79% bootstrap values. A homologous sequence of *Myotis daubentonii* was used as an outgroup. (B) A network tree of mtDNA haplotypes based on the maximum parsimony (MP) method. Circles show mtDNA haplotypes, and open small circles indicate presumed haplotypes. One bar between two circles shows one nucleotide substitution.

Table 1). Of the 1,140 nucleotide sites, 11 nucleotide sites were polymorphic, and all of the substitutions were transitions without any alignment gaps. Haplotype H1 referred to a sequence previously registered in DDBJ (accession number AB085736), whereas the other eight haplotypes were newly found in the present study and deposited in DDBJ with accession numbers: AB621574–AB621581. Haplotype H1 was the most common in Hokkaido (67.79%: 181/267 bats) and was found in all subpopulations except one subpopulation in the Ishikari river region (IS1) (Table 1). Two haplotypes (H7 and H8) were distributed in both the Ishikari and Teshio river regions. The other six haplotypes were not shared among the three river regions.

Because NJ and ML analyses yielded similar topologies, which consisted of two major clades, the NJ tree is shown in Fig. 2A as a representative of the phylogenetic trees. The two clades were supported by more than 59% bootstrap values in both NJ and ML methods. TCS analyses generated MP networks, and the phylogeny was in concordance with the two main clades identified by both the NJ and ML methods (Fig. 2B). However, the phylogenetic relationships among mtDNA haplotypes did not reflect the geographic relationships among sampling points, within each river region and among river regions.

Population genetic structure

AMOVA showed that the levels of mtDNA genetic divergence among river regions and among subpopulations were not high. Most variations (63.8%) were explained by the 'within subpopulation' factor, whereas 'among regions' variations and 'among subpopulations' variations accounted for 13.1 % and 23.1%, respectively. Nevertheless, overall fixation indices were still significant ($P < 0.001$). Pairwise F_{ST} values in 46 out of 78 comparisons were not significant, whereas two subpopulations in the Ishikari river region (IS5 and IS6) were significantly ($P < 0.05$) differentiated from all of the other subpopulations (Table 2).

Principal component analysis of haplotype frequencies showed that the 15 subpopulations were separated into three main groups (Fig. 3). The three subpopulations (TO1, TO2 and TO3) of the Tokachi river region formed one group, and the four subpopulations of the Teshio river region (TE1, TE2, TE3 and TE4) were closely related to each other. By contrast, although four of six subpopulations from the Ishikari river region were closely related to each other and to the Teshio group, the other two (IS5 and IS6) in the Ishikari river region were remotely related to the other subpopulations in Hokkaido. The relationships among subpopulations based on F_{ST} values also showed that IS5 and IS6 were segregated from the other subpopulations (Fig. 4).

Mantel tests within each river showed no significant correlations between genetic and two geographic distances. In addition, there are no significant correlations between genetic and two geographic distances among populations in the different river regions. Thus mtDNA gene flow on two geographic scales (within each river region and among river regions) did not follow a simple pattern of IBD.

On the other hand, because principal component analysis showed that all subpopulations within Ishikari and Teshio, except IS5 and IS6, were considered one closely related genetic group, mantel tests within this genetic group was implemented in the same way. Con-

Table 1. Distribution and frequencies of nine mtDNA haplotypes identified from 267 bats in 15 subpopulations in three rivers. Sample sizes of subpopulations are shown in parentheses

Subpopulation (*n*)	Haplotypes								
	H1	H2	H3	H4	H5	H6	H7	H8	H9
TO1 (20)	14			2		4			
TO2 (20)	16			1		3			
TO3 (19)	11			1		7			
Tokachi River (59)	**41**			**4**		**14**			
IS1 (5)							5		
IS2 (20)	12						4	1	3
IS3 (12)	5						5	2	
IS4 (20)	16						4		
IS5 (20)	1						15	4	
IS6 (20)	6				1		7	6	
IS7 (20)	16						2	2	
IS8 (17)	14		2					1	
Ishikari River (134)	**70**		**2**		**1**		**42**	**16**	**3**
TE1 (21)	20							1	
TE2 (19)	19								
TE3 (18)	17	1							
TE4 (16)	14						1	1	
Teshio River (74)	**70**	**1**					**1**	**2**	
Total (267)	**181**	**1**	**2**	**4**	**1**	**14**	**43**	**18**	**3**
% frequency	**67.79**	**0.37**	**0.75**	**1.5**	**0.37**	**5.24**	**16.1**	**6.74**	**1.12**

The total number of haplotypes identified in each river region is shown in bold.

Table 2. Pairwise F_{ST} values between subpopulations of *Myotis macrodactylus*

River	Subpopulation	TO1	TO2	TO3	IS2	IS4	IS5	IS6	IS7	IS8	TE1	TE2	TE3	TE4
Tokachi	TO1	–												
	TO2	0.000	–											
	TO3	0.014	0.065	–										
Ishikari	IS2	0.059	0.101	0.010	–									
	IS4	0.000	0.000	0.070	0.030	–								
	IS5	0.663***	0.718***	0.523***	0.452***	0.654***	–							
	IS6	0.302***	0.362***	0.163*	0.079*	0.282***	0.175**	–						
	IS7	0.000	0.000	0.063	0.033	0.000	0.658***	0.278***	–					
	IS8	0.046	0.008	0.202*	0.205**	0.047	0.805***	0.461***	0.026	–				
Teshio	TE1	0.069*	0.021	0.245***	0.249**	0.070	0.838***	0.510***	0.048	0.000	–			
	TE2	0.145	0.095	0.326**	0.334**	0.153	0.889***	0.578***	0.135	0.023	0.000	–		
	TE3	0.134	0.086	0.311**	0.320***	0.141	0.879***	0.563***	0.125	0.017	0.000	0.003	–	
	TE4	0.000	0.000	0.118	0.105	0.000	0.744***	0.369***	0.000	0.000	0.000	0.071	0.060	–

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

sequently, a significant positive correlation between genetic distances and two geographic ones (EU distances: correlation coefficient = 0.647, $P < 0.01$, AR distances: correlation coefficient = 0.555, $P < 0.05$) was detected in this group, indicating a simple distribution pattern of mtDNA haplotypes by IBD.

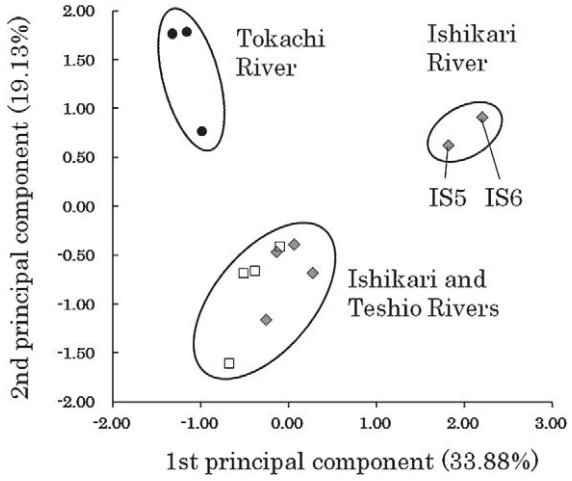

Fig. 3. Factor map of the two main axes of principal component analysis implemented with mtDNA haplotype frequencies. Both subpopulations IS5 and IS6 are in the Ishikari river region (Fig. 1). Circles, subpopulations in the Tokachi river region; diamonds, those in the Ishikari river region; squares, those in the Teshio river region.

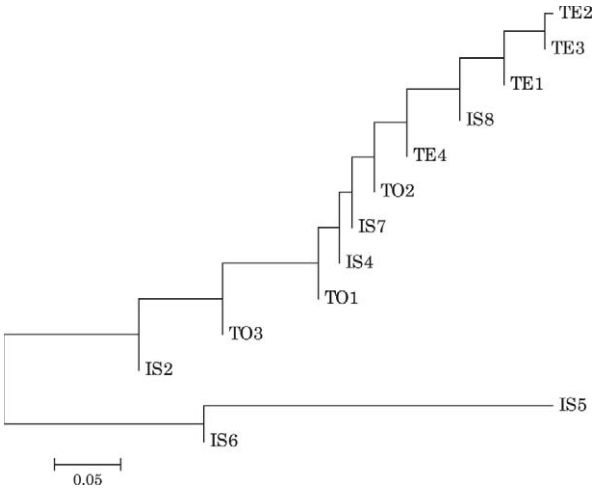

Fig. 4. Neighbor-joining (NJ) relationships among subpopulations based on pairwise F_{ST} values.

Discussion

Nine unique mtDNA haplotypes identified in the present study were divided into two major clades, although the molecular phylogeny was not in congruence with the geographic locations of haplotypes within and among the river regions in Hokkaido (Fig. 2). The result indicates that each clade has not evolved uniquely within the present river regions in Hokkaido. Although the history of evolution and migration of *M. macrodactylus* living in Hokkaido has not been well clarified, the results of present study suggest that the mtDNA clades were divided before the expansion of the populations into each river region in Hokkaido. In addition, the occurrence of private haplotypes in each river region indicates loss of some haplotypes due to genetic drift during bat expansion. In other words, the populations in each river region have lost some haplotypes by chance, and the specific haplotypes remained in single river regions.

Principal component analysis revealed that Teshio and Ishikari subpopulations except IS5 and IS6 were grouped to one genetically related group (Fig. 3). This findings show gene flow between the Ishikari and Teshio river regions, resulting from migration between them. Although we expected population genetic structure of the river-depending bat have been formed independently among river regions, the present study rejected the hypothesis. In addition, it is indicated that genetic distance correlated more strongly with EU distances than AR distances in IBD analysis. Therefore, it is suggested that this bat species migrates not only along river but also through land areas such as forests. On the other hand, Tokachi is genetically isolated from the other two river regions in principal component analysis. This might be due to geographic features and flight ability of *M. macrodactylus*. The Daisetsuzan and Hidaka Mountain Ranges (altitudes of about 1,500–2,200 m) located between Tokachi and the other two river regions in central Hokkaido (Fig. 1) could have played a role as effective geographic barriers against migration of this bat species. In general, migration distances of bats are influenced by their flight ability, which is strongly correlated with wing morphology (Norberg and Rayner 1987). Fukui et al. (2011) predicted a relatively low flight ability of *M. macrodactylus* from the wing morphology compared to long-migrating species (e.g., genera *Nyctalus* and *Vespertilio*). Kuramoto et al. (1973) reported that the migration distance is about 30 km. Although *M. macrodactylus* can migrate between different rivers, it might be difficult that the species migrates through areas such as mountain ranges without favored habitats and foraging sites due to the flight ability. Therefore, their migration between Tokachi and the other two rivers might not be as frequent as that between Teshio and Ishikari rivers.

The migration pattern discussed above is for females, because the data were obtained from maternally inherited mtDNA. Although high natal philopatry rate of female has been reported for many bat species (Wilkinson 1987; Wilkinson and Chapman 1991; Burland et al. 1999; Kerth et al. 2000; Castella et al. 2001; Ruedi et al.

2008), the present study indicates that females of *M. macrodactylus* are not philopatric to their natal colonies along rivers in Hokkaido. This is concordant with low philopatry (≤ 35%) found by an ecological survey of *M. macrodactylus* at Akiyoshi-dai Plateau on western Honshu, the main island of Japan (Kuramoto et al. 1978).

Within the Ishikari river region, the two subpopulations (IS5 and IS6) were genetically isolated from the other subpopulations. However, because no mtDNA distribution pattern of IBD nor obvious geographic barriers among subpopulations were detected, these two subpopulations might not have been isolated. These results are incomprehensible. One possible explanation for these results is sampling bias due to small sample size. In addition, because sequence differences among haplotypes were small, the substitution rate of this marker might be so low that the F_{ST} values did not reflect the real genetic structure of their subpopulations. Another possibility is likely that IS5 and IS6 are admixed subpopulations consisting of bats that originated from different maternal lineages of different local populations, leading to the collapse of original mtDNA haplotype frequency. In autumn when we collected samples, temperate bats generally move to swarming, mating or hibernating roosts. At the roosts in the IS5 and IS6, the number of roosting *M. macrodactylus* increased from the late summer to autumn (E. Kojima, personal communication).

In the present study, there is no genetic difference between subpopulations in Teshio and Ishikari Rivers, although genetic structure in Tokachi River was differentiated from other rivers. This suggests that *M. macrodactylus* migrates relatively frequently between different river regions without huge barriers such as mountains. Therefore, the hypothesis that this bat species is faithful to rivers and their population genetic structures have been formed independently in each river regions was not supported. The present study, however, provides new insights to studying the migration pattern and genetic structure of this species. To further understand the distribution of this riparian bat species, it is necessary to perform population genetic studies of gene flow using nuclear markers such as biparental microsatellites and paternal genes. In addition, it is also useful to use the methods based on other disciplines, such as behavioral research by biotelemetry and analysis of the riparian habitats by geographic information systems (GIS).

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Appendix 1.

Information on samples in the Tokachi population

Subpopulation	Site	Male	Female	Composition of species
TO1 (20)	TO-site1	10	0	mixed with *M. petax*
	TO-site2	4	0	mixed with *M. petax*
	TO-site3	1	0	single
	TO-site4	3	0	mixed with *M. petax*
	TO-site5	1	0	mixed with *M. petax*
	TO-site6	1	0	single
		20	**0**	
TO2 (20)	TO-site7	1	0	single
	TO-site8	4	3	mixed with *M. petax*
	TO-site9	2	0	mixed with *M. petax*
	TO-site10	0	1	single
	TO-site11	1	0	single
	TO-site12	7	1	single
		15	**5**	
TO3 (19)	TO-site13	12	7	single
		12	**7**	
Tokachi River (59)		**47**	**12**	

Appendix 3.

Information on samples in the Teshio population

Subpopulation	Site	Male	Female	Composition of species
TE1 (21)	TE-site1	2	0	single
	TE-site2	4	0	single
	TE-site3	6	0	single
	TE-site4	9	0	single
		21	**0**	
TE2 (19)	TE-site5	2	0	single
	TE-site6	8	1	single
	TE-site7	3	1	single
	TE-site8	1	1	single
	TE-site9	1	0	single
	TE-site10	1	0	single
		16	**3**	
TE3 (18)	TE-site11	10	7	single
	TE-site12	0	1	single
		10	**8**	
TE4 (16)	TE-site13	8	8	single
		8	**8**	
Teshio River (74)		**55**	**19**	

Appendix 2.

Information on samples in the Ishikari population

Subpopulation	Site	Male	Female	Composition of species
IS1 (5)	IS-site1	0	1	single
	IS-site2	0	1	single
	IS-site3	2	1	mixed with *M. petax*
		2	**3**	
IS2 (20)	IS-site4	13	2	single
	IS-site5	0	1	single
	IS-site6	0	1	single
	IS-site7	1	1	single
	IS-site8	0	1	single
		14	**6**	
IS3 (12)	IS-site9	0	1	single
	IS-site10	1	0	single
	IS-site11	3	2	single
	IS-site12	1	1	single
	IS-site13	0	1	single
	IS-site14	0	2	single
		5	**7**	
IS4 (20)	IS-site15	18	2	single
		18	**2**	
IS5 (20)	IS-site16	19	1	single
		19	**1**	
IS6 (20)	IS-site17	17	3	single
		17	**3**	
IS7 (20)	IS-site18	19	1	single
		19	**1**	
IS8 (17)	IS-site19	0	1	single
	IS-site20	1	0	single
	IS-site21	5	0	single
	IS-site22	2	0	single
	IS-site23	0	1	single
	IS-site24	2	0	single
	IS-site25	0	1	single
	IS-site26	4	0	single
		14	**3**	
Ishikari River (134)		**108**	**26**	