ZOOLOGICAL SCIENCE **30**: 642–650 (2013)

Refugia in Glacial Ages Led to the Current Discontinuous Distribution Patterns of the Dark Red-backed Vole *Myodes rex* on Hokkaido, Japan

Kuniko Kawai[1,2], Frank Hailer[3,4], Anna Pauline de Guia[5], Hideo Ichikawa[1], and Takashi Saitoh[1]*

[1]*Field Science Center, Hokkaido University, Kita-11, Nishi-10, Sapporo 060-0811, Japan*
[2]*Division of Mammals, National Museum of Natural History, Smithsonian Institution, Washington, DC 20013-7012, USA*
[3]*Center for Conservation and Evolutionary Genetics, Smithsonian Conservation Biology Institute, 3001 Connecticut Ave. NW, Washington, DC 20008, USA*
[4]*Biodiversity and Climate Research Center (BiK-F), Ecological Genomics, Senckenberg Gesellschaft für Naturforschung, Senckenberganlage 25, 60325 Frankfurt, Germany*
[5]*Animal Biology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, Laguna 4031, Philippines*

The terrestrial mammalian fauna of the North Japanese island, Hokkaido, is more similar to that of Southern Siberia than to the main island of Japan, Honshu. Three species of the genus *Myodes* (Muridae, Rodentia) are found on Hokkaido, but not on Honshu. While *Myodes rufocanus* and *M. rutilus* are widely distributed across Hokkaido as well as the Eurasian continent, *M. rex*, which is endemic to Hokkaido and its adjacent islands, shows a discontinuous distribution pattern. We analyzed the phylogeographic history of *M. rex* using the mitochondrial DNA control region in order to interpret their discontinuous distribution pattern. Phylogenetic relationships among 54 distinct haplotypes showed that *M. rex* can be divided into four clades that occur on the northern, central, and southern regions of the Hokkaido mainland and on Rishiri Island, respectively. The phylogroups in the northern and central regions were largely separated in space, although several areas of sympatry were found. The phylogroup in the southern region, which was clearly separated from other phylogroups, showed markedly low genetic variability. All analyzed individuals from the population on Rishiri belonged to a separate lineage. Across a range of divergence rate estimates, we dated the basal divergence of all phylogroups to the mid to late Pleistocene, with subsequent signals of population expansion within lineages. We conclude that current phylogeographic structure in *M. rex* likely reflects Pleistocene survival in several separate refugia in situ. Past glacial ages have thus played an important role in shaping the current distribution patterns of mammalian species on Hokkaido.

Key words: *Myodes rex*, mitochondrial DNA control region, haplotype, distribution pattern, refugia

INTRODUCTION

The Japanese archipelago is a long chain of islands located on the eastern coast of Asia, and includes four major islands: Hokkaido in the north, Honshu in the central region, Shikoku, in the west, and Kyushu in the southwest. Hokkaido is separated from Honshu by the Tsugaru Strait, which is known as a major biogeographical demarcation called "Blakiston's Line." Honshu harbors many endemic mammalian species, with approximately 40% of all Japanese endemic mammals (Abe et al., 2005). In contrast, the terrestrial fauna of Hokkaido, located to the north of Blakiston's Line, is more similar to that of Southern Siberia than that of Honshu (Fujimaki, 1994). Because of the shallowness of the Mamiya and Soya Straits, Hokkaido was repeatedly connected to the Eurasian continent during periods of lower sea level in glacial ages, allowing terrestrial mammals to migrate from Siberia to Hokkaido across a land bridge (Fig. 1). On the other hand, the Tsugaru Strait is relatively deep and has separated Hokkaido from Honshu since the Late Pleistocene, although some migrations between Hokkaido and Honshu occurred in the Middle Pleistocene (Oshima, 1990). Thus, the terrestrial mammalian fauna of Hokkaido shows a rela-

* Corresponding author. Tel. : +81-11-706-2590;
Fax : +81-11-706-3450;
E-mail: tsaitoh@fsc.hokudai.ac.jp
doi:10.2108/zsj.30.642

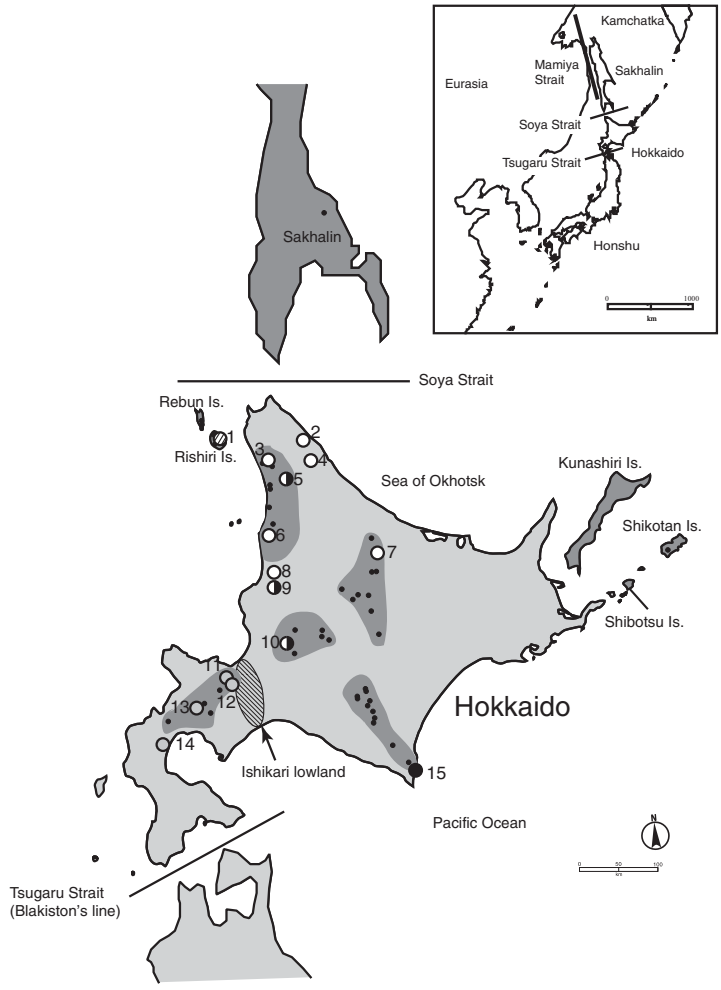

Fig. 1. Collection localities for *M. rex* on Hokkaido and adjacent islands. Circles indicate the localities collected in this study; color intensity indicates which phylogroup the specimen belongs to, corresponding to Figs. 2 and 3. Numbers with circles correspond to the locality number in Table 1. Black dots indicate localities found in previous reports (Nakata, 2000; Ichikawa, 2002; Motokawa, 2008; Abramson et al., 2009), and grey shadow indicates the distributional area by Iwasa and Kaneko (2009).

Table 1. Sampling localities and phylogroup of *Myodes rex*.

Locality number[1]	Locality	Number of individuals	Latitude	Longitude	Phylogroup
1	Rishiri	24	45.24	141.22	D
2	Hamatonbetsu	2	45.13	142.36	A
3	Teshio	8	44.89	141.74	A
4	Utanobori	3	44.68	142.50	A
5	Nakagawa	21	44.68	142.12	A/C
6	Haboro	3	44.36	141.71	A
7	Takinoshita	18	43.88	143.18	A
8	Obira	1	44.05	141.86	A
9	Mashike	12	43.86	141.76	A/C
10	Bibai	2	43.20	141.88	A/C
11	Otarunai	1	43.07	141.10	B
12	Okujozankei	1	42.85	141.17	B
13	Niseko	9	42.80	140.69	B
14	Kunnui	6	42.47	140.25	B
15	Hidaka	11	42.09	143.27	C

[1] These locality numbers correspond with those of Fig. 1 and Table 2.

tively low degree of endemism.

Species of the genus *Myodes* (Arvicolinae, Muridae, Rodentia) are widely distributed across the Eurasian continent, including Hokkaido, but are absent from Honshu. Three species are currently recognized: the dark red-backed vole *Myodes rex* (Imaizumi, 1971), the gray-sided vole *M. rufocanus* (Sundevall, 1846), and the northern red-backed vole *M. rutilus* (Pallas, 1779). *Myodes rufocanus* and *M. rutilus* are widely distributed on Hokkaido as well as the Eurasian continent (Ohdachi et al., 2009), while *M. rex* is rare and restricted to Hokkaido and its adjacent islands (Iwasa and Kaneko, 2009). *Myodes rex* is, therefore, an important species that could help elucidate the formation and evolution of the mammalian fauna of Hokkaido. However, knowledge of the geographic distribution of *M. rex* is limited, and the phylogeography of the species has not been reported.

The distribution of *M. rex* is discontinuous in Hokkaido (Fig. 1): the northwestern (Nakata, 2000), the central such as the Taisetsu Mountains (Abe et al., 1971; Kashiwabara and Onoyama, 1988; Kaneko, 1994; Nakata, 2000), the Hidaka Mountains (Imaizumi, 1972; Haga et al., 1979; Yanagawa and Itoh, 1990; Nakata, 2000), and the southern regions (Kadosaki and Inage, 1998; Nakata, 2000; Ichikawa, 2002). No records have been reported from the northeastern and eastern regions of Hokkaido. *Myodes rex* also occurs in the adjacent northwestern islands of Rebun and Rishiri (Imaizumi, 1971; Kaneko and Sato, 1993; Nakata, 2000), Sakhalin Island (Abramson et al., 2009), probably in Shikotan (Iwasa et al., 2001; Motokawa, 2008), Kunashiri, and Shibotsu Islands (Koyasu et al., 1996).

Why is the distribution of *M. rex* discontinuous, differing from that of the other two *Myodes* species on Hokkaido? A combination of geographical and gene genealogical information can yield important insights into the phylogeographic history that have shaped the current distribution and patterns of genetic variation of extant species (Avise, 2000). For example, phylogeographic analyses have contributed to understanding the current fauna formation in Europe. Mediterranean phylogroups did not contribute to the postglacial recolonization of central and northern Europe for temperate forest mammal species, and the presence of central glacial refugia are evidenced by case studies on the bank vole (*Myodes glareolus*) (Bilton et al., 1998; Deffontaine et al., 2005; Kotlik et al., 2006). The current discontinuous distribution of *M. rex* might therefore result from the phylogeographic history, although ecological competition with the dominant species *M. rufocanus* may also play a role (Iwasa and Nakata, 2011).

In this paper, we surveyed genetic variation at the mitochondrial DNA (mtDNA) control region and analyzed the phylogeographic history of *M. rex* in order to elucidate the processes which have shaped its current discontinuous distribution.

MATERIALS AND METHODS

The Field Science Center for Northern Biosphere, Hokkaido University has been collecting specimens of the genus *Myodes*,

acquiring more than 1500 individuals in total from various areas of Hokkaido between 1998 and 2011, with support from the Hokkaido Regional Office, Forestry Agency of Japan in the period of 1998–2008. Of these samples, 112 individuals from 12 localities in Hokkaido were identified as *M. rex* (Fig. 1, Table 1). Species identification of *M. rex* was based on whether the form of upper third

Table 2. Localities and phylogroups of 54 haplotypes of *Myodes rex*.

			Locality														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Haplotype	PG[1]	n[2]	Rishiri	Hamatonbetsu	Teshio	Utanobori	Nakagawa	Haboro	Takinoshita	Obira	Mashike	Bibai	Otarunai	Okujozankei	Niseko	Kunnui	Hidaka
rex01	C	4	–	–	–	–	2	–	–	–	2	–	–	–	–	–	–
rex02	C	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
rex03	C	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2
rex04	C	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
rex05	C	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
rex06	C	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4
rex07	A	6	–	–	–	2	1	1	–	–	2	–	–	–	–	–	–
rex08	A	1	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–
rex09	A	10	–	–	2	–	6	–	1	–	1	–	–	–	–	–	–
rex10	A	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–
rex11	A	1	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–
rex12	A	2	–	–	–	–	1	–	1	–	–	–	–	–	–	–	–
rex13	A	1	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–
rex14	A	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–
rex15	A	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–
rex16	A	15	–	–	–	–	1	1	9	–	4	–	–	–	–	–	–
rex17	A	2	–	–	–	–	–	–	2	–	–	–	–	–	–	–	–
rex18	A	1	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–
rex19	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex20	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex21	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex22	D	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex23	D	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex24	D	2	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex25	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex26	D	5	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex27	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex28	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex29	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex30	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex31	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex32	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex33	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex34	B	8	–	–	–	–	–	–	–	–	–	–	–	–	4	4	–
rex35	B	4	–	–	–	–	–	–	–	–	–	–	–	1	1	2	–
rex36	B	4	–	–	–	–	–	–	–	–	–	–	1	–	3	–	–
rex37	A	6	–	1	1	1	2	–	1	–	–	–	–	–	–	–	–
rex38	A	2	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–
rex39	A	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–
rex40	A	1	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–
rex41	C	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
rex42	C	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
rex43	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex44	D	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rex45	A	2	–	–	–	–	1	–	–	–	1	–	–	–	–	–	–
rex46	A	1	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–
rex47	A	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–
rex48	C	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–
rex49	A	4	–	–	4	–	–	–	–	–	–	–	–	–	–	–	–
rex50	A	1	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
rex51	C	1	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
rex52	A	1	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–
rex53	A	2	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–
rex54	B	1	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–
Total	–	122	24	2	8	3	21	3	18	1	12	2	1	1	9	6	11

[1] Phylogroup.
[2] Total number of individuals.

molar is complex, since this character has been demonstrated to yield reliable identification of this species (Kaneko et al., 1998). The liver or pectoral muscle were sampled and preserved in 99% ethanol.

Genomic DNA was extracted from the tissue using a DNeasy Blood & Tissue Kit (Qiagen, Germany). The mtDNA control region was amplified using primers of Lpro (5′-TCAGCACCCAAAGCTGATATTCTACTT-3′) and Hphe (5′-ATCTAAGGCATTTTCAGTGCTTTGCTT-3′), which were designed based on the *M. rufocanus* sequence. PCR amplification was carried out using a GeneAmp PCR System 9700 (Applied Biosystems, USA) in 20 μl reaction mixtures containing 0.2 mM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.25 μM of each primer, and 0.5 units of AmpliTaq Gold (Applied Biosystems). 0.5 μl solution containing 10–100 ng genomic DNA was used for each reaction. After incubation at 95°C for 10 minutes, cycling was performed for 30 cycles of 20 s at 93°C, 20 s at 50°C, and 60 s at 72°C, with a postcycling extension at 72°C for 7 minutes. After removing excess primers and dNTP, the PCR products were sequenced using the primer Lpro and BigDye Terminator v3.1 Cycle Sequencing chemistry (Applied Biosystems). Cycle sequencing products were run on an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

Alignment of the sequences was done with the Clustal W 1.82 program within the Geneious Pro 4.7.5 (Biomatters Ltd., USA) software, with further manual corrections. To infer the distinct haplotypes and to calculate haplotype (*H*) and nucleotide diversity (π, based on uncorrected genetic distances p) using all of the data, the DNASP Ver. 5.10.01 (Rozas et al*.,* 2003) and ARLEQUIN Ver. 3.5.1 (Excoffier et al., 2010) programs were used.

Haplotype trees were constructed using two methods: Maximum Likelihood (ML) analysis was implemented in the RAxML 7.0.4 (Stamatakis, 2006); Bayesian analysis in the MrBayes v.3.2.1 (Ronquist et al., 2012). Both analyses were conducted with GTR+G+I model. For the Bayesian, MCMC settings were as follows; chain length 6,400,000, subsampling frequency 1,000, number of heated chain 4, burn-in length 1,600,000 and temperature of heated chain 0.2. The haplotype trees were drawn as an unrooted tree. This is because lengths of the control region among genus *Myodes* species were diverse (Matson and Baker, 2001), and we could not align the sequences of *M. rex* with those of the other *Myodes* species.

A statistical parsimony network (Templeton et al., 1992) of the nucleotide sequences was constructed using the TCS 1.21 program (Clement et al., 2000) with the default setting of 95% parsimony connection limit.

We calculated mean genetic distances between phylogroups based on the number of base substitutions per site using MEGA5 software (Tamura et al., 2011). Standard error was obtained by a bootstrap procedure with 1,000 replicates. The analyses were conducted using the Tamura-Nei model of sequence evolution (Tamura and Nei, 1993), modeling the rate variation among sites with a gamma distribution.

To investigate the demographic history of *M. rex* within phylogroups, Tajima's *D* (Tajima, 1989) and Fu's F_S (Fu, 1997) values were calculated based on the total number of mutations in the alignment. We tested for signals of sudden population expansion using mismatch distribution approach (Rogers and Harpending, 1992) implemented in the ARLEQUIN software. The 95% confidence interval of τ was calculated with parametric bootstrapping. The expansion event was dated using the formula $t = \tau / 2u$, where $u = \mu$ (mutation rate per site and year) × k (sequence length). To obtain a time estimate, we used two different rate calibrations for the divergence of the mtDNA control region of arvicoline rodents: μ = 3.6% per bp and million years (Myr) (Matson and Baker, 2001) and 17% per bp and Myr (Fedorov and Stenseth, 2001).

RESULTS

Distribution and Genetic diversity

In total, 112 *M. rex* samples were collected at 15 localities (Table 1, Fig. 1). Although most localities of our collections (circles in Fig. 1) overlapped with those of previous records (black dots in Fig. 1), we found new records for five localities in the southern, central, and northern regions of Hokkaido, where *M. rex* had not been recorded previously: two individuals found at Locality 2 and three at Locality 4 (both in the northeastern region); one at Locality 8, and nine at Locality 9 (in the central region); six at Locality 14 repre-

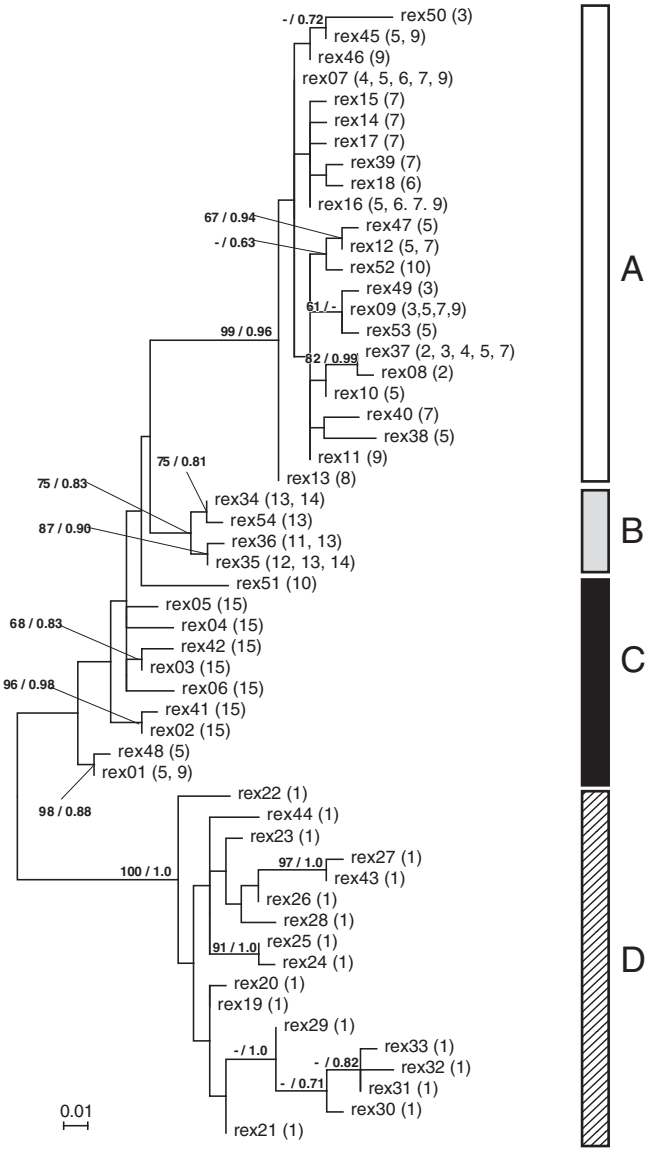

Fig. 2. Unrooted Maximum Likelihood tree for 54 haplotypes of *M. rex* mitochondorial DNA control region*.* Maximum likelihood probabilities values > 50% and Bayesian probabilities > 0.5 are shown at branches on the tree. The scale indicates the number of substitutions per site. A figure in parentheses indicates a locality at which that haplotype was found. Vertical lines indicate the four phylogroups A, B, C and D by color intensity, corresponding to Figs. 1 and 3.

sent the southernmost known occurrence of *M. rex*. These results suggest that the distribution of *M. rex* in Hokkaido is wider than previously thought.

Using 720-bp sequences of the partial mtDNA control region from the 112 *M. rex* specimens, 71 segregating sites that defined 54 distinct mtDNA haplotypes were found (Table 2). The sequences have been deposited in Genbank (Accession numbers: AB732022–AB732075). No insertions or deletions were observed. Based on sequences from all specimens, haplotype diversity (*H*) was 0.965, the average number of nucleotide differences (*K*) was 12.5, and nucleotide diversity (π) was 0.017.

While 44 haplotypes were restricted to one locality, 10 haplotypes were shared among 2 to 5 localities. Haplotype Rex16 was the most frequent in the total data set, occurring in 15 individuals from 4 localities in the central-northern region of Hokkaido (Table 2). None of the 17 haplotypes found in the specimens from Rishiri Island were shared any other localities.

Phylogenetic relationships among haplotypes

To elucidate phylogenetic relationships among the 54 haplotypes, we constructed unrooted haplotype trees and a statistical parsimony network. The ML tree was supported with the highest log likelihood (–1759.2492). The tree (Fig. 2) and network (Fig. 3) both showed that *M. rex* haplotypes were divided into four phylogroups, namely A, B, C, and D. While the Phylogroups A, B, and D were supported with high probability in the tree, the Phylogroup C was not, but it could be separated from the other three in the network. The haplotype network was divided into two unconnected networks at the 95% probability connection limit in the TCS. All haplotypes from Rishiri (phylogroup D) constituted one network, and those from the remaining region (phylogroups A, B, and C) clustered in another network. Phylogroup A was separated from B/C by at least eight mutational steps. The four phylogroups reflected the underlying geographic distribution of our sampling: phylogroup A consisted of haplotypes from specimens in the central-northern region of the Hokkaido mainland; B in the southern region of the mainland; C in the central region of the mainland; D in Rishiri Island. While specimens from most localities (12 out of 15) had haplotypes from only one phylogroup (either A, B, C, or D), specimens from the other three localities had haplotypes related to phylogroups A and C, namely Nakagawa (5 in Fig. 1), Mashike (9 in Fig. 1), and Bibai (10 in Fig. 1).

Estimates of haplotype diversity (*H*) and nucleotide diver-

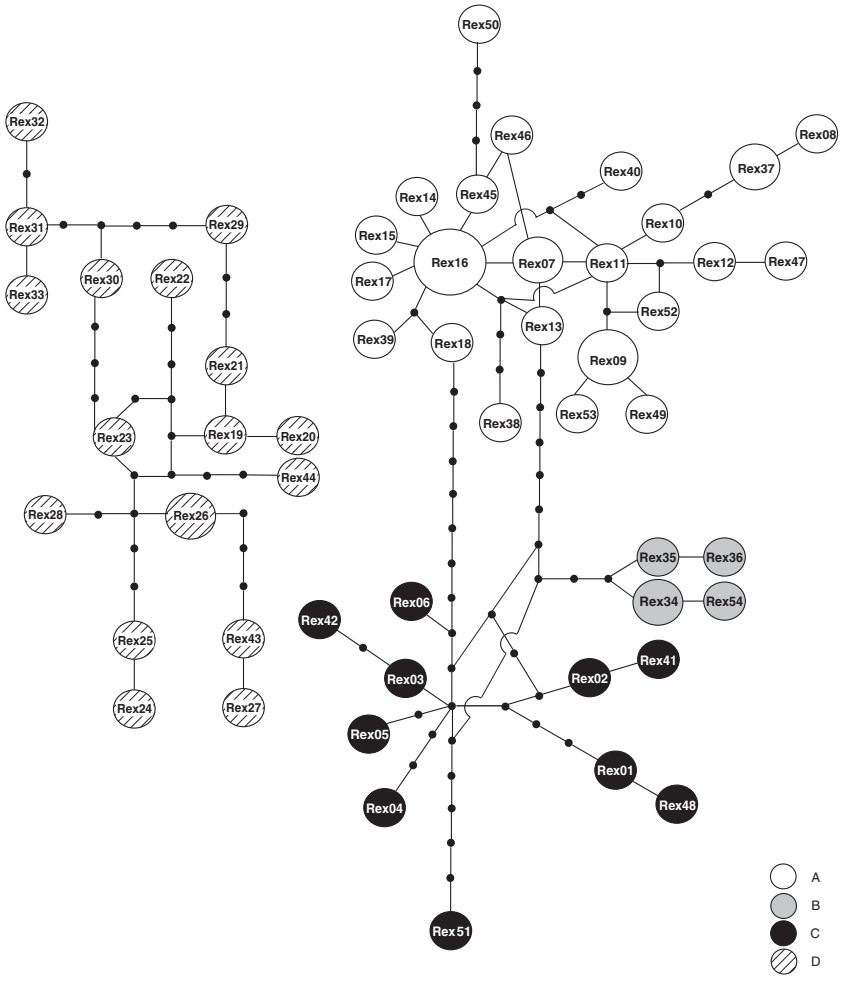

Fig. 3. Haplotype network of 54 haplotypes of *M. rex* mitochondrial DNA control region. The size of each circle is related to frequency of haplotype. Four phylogroups (A–D) are shown by color intensity, corresponding to Figs. 1 and 2. Dots between haplotypes are inferred intermediate variants that were not found in the data set.

Table 3. Genetic variability of four phylogroups of *Myodes rex*.

Phylogroup	Number of individuals	Number of Haplotypes	No. of transions	No. of transversions	Haplotype diversity (± SD)	Nucleotide diversity (± SD)
A	64	23	17	6	0.905 ± 0.0220	0.0048 ± 0.00030
B	17	4	4	0	0.706 ± 0.0075	0.0022 ± 0.00025
C	17	10	21	4	0.904 ± 0.0500	0.0076 ± 0.00074
D	24	17	20	5	0.953 ± 0.0310	0.0088 ± 0.00070

Table 4. Mean genetic distance between phylogroups of *Myodes rex*. Pairwise distance based on the Tamura-Nei model is shown below the diagonal. Standard error estimates are above the diagonal.

Phylogroup	A	B	C	D
A	–	0.005	0.005	0.007
B	0.019	–	0.003	0.006
C	0.021	0.012	–	0.006
D	0.035	0.026	0.029	–

Table 5. Demographic values in nine groupings of *Myodes rex*.

Groupings of phylogroup	Neutrality test			Mismatch analysis									
						Confidential intervals[2] of the τ		Estimated population expansion time (years ago) based on 3.6%/Myr[3]			Estimated population expansion time (years ago) based on 17%/Myr[3]		
	Tajima's *D*	Fu's F_S		τ[1]	P-value	Low	High		Low	High		Low	High
A/B/C/D	–0.17112	–15.62795	***	14.910	0.450	4.914	97.910	575,231	189,583	3,777,392	121,814	40,147	799,918
A/B/C	–0.42202	–9.33682	*	2.750	0.130	0.041	87.750	106,096	1,582	3,385,417	22,467	335	716,912
A	–0.91263	–10.45783	***	4.504	0.165	1.678	7.277	173,762	64,738	280,748	36,797	13,709	59,453
B	0.97159	0.85812		2.688	0.382	0.000	5.432	103,684	0	209,568	21,957	0	44,379
C	–1.03157	–1.20123		6.531	0.066	2.689	9.848	251,977	103,742	379,938	53,360	21,969	80,458
D	–0.21284	–5.91989	**	6.930	0.389	3.871	8.873	267,349	149,344	342,323	56,615	31,626	72,492
A/B	0.08056	–5.32277		2.086	0.192	0.000	16.158	80,476	0	623,380	17,042	0	132,010
A/C	–0.63494	–10.02966	**	2.293	0.245	0.002	20.592	88,463	77	794,444	18,733	16	168,235
B/C	–0.795	–1.06534		8.742	0.060	4.182	12.723	337,269	161,343	490,856	71,422	34,167	103,946

* $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$

[1] Deviation from the sudden expansion model was assessed by parametric bootstrapping in the ARLEQUIN.

[2] Confidential intervals were obtained by the percentile method (alpha = 0.050) based on 1000 replicates.

[3] Population expansion time was calculated based on the mutation rate of 3.6% or 17% per million years (Myr).

sity (π; per site) were similarly high for the phylogroup A, C, and D, while phylogroup B exhibited lower variability (Table 3).

Consistent with the results from the phylogenetic analyses (Figs. 1, 2), average genetic distances among the phylogroups indicated that phylogroup D was clearly differentiated from the other phylogroups, while phylogroups B and C were closely related to each other (Table 4).

Demographic history

Phylogroup A showed a star-like pattern in the haplotype network (Fig. 3), in which the haplotypes radiated from the haplotype Rex16 by a single or double mutational changes. The phylogroup C also showed a star-like pattern, but no core haplotype for the radiation was found. These network shapes suggested that *M. rex* populations of the northern and central regions of the mainland (phylogroups A and C) were long isolated and have experienced recent demographic expansions. On the other hand, phylogroup D from Risihri did not show a star-like shape, and the network shape for phylogroup B was unclear, possibly due to the small number of observed haplotypes.

To substantiate the above inferences regarding population growth and/or range expansion, we calculated Tajima's *D* and Fu's F_S. These tests were conducted for nine demographic groupings, as follows (Table 5): All four phylogroups (A/B/C/D); all of the three phylogroups of the mainland (A/B/C); each phylogroup separately (A, B, C, D); conceivable groupings of two phylogroups on the mainland (A/B, A/C, B/C). Phylogroup B and A/B showed positive values of Tajima's *D*, while the others were negative, but all of them were not significantly different from zero, consistent with effectively neutral evolution of the sequences. Fu's F_S was significantly negative for A/B/C/D, A/B/C, A, D, and A/C, indicating that most populations/groupings underwent a demographic expansion in the past. Results for the phylogroup B remained less conclusive (see above).

The validity of the sudden expansion model was tested by the sum of square deviations between the observed and the expected mismatch distributions. No significant differences were found between these distributions for all the nine groupings (Table 5; Fig. 4), supporting that the sudden expansion model was valid. The observed distribution pattern of A/B/C/D showed multimodal, supporting that the *M. rex* populations comprise at least three clades. The observed distribution patterns of A/B/C, A/B, and A/C were also bimodal. The observed distribution pattern of each phylogroup on its own was unimodal. The phylogroups C and D showed a higher value of τ among the groups, suggesting that they are older than the other groups (Table 5). The distribution pattern of phylogroup B was biased towards to zero, suggesting that this population underwent a recent bottleneck.

The expansion time was estimated based on τ in the mismatch distribution analysis (Table 5). Irrespective of the rate calibration employed (3.6% or 17%/Myr), the divergence of all phylogroups was placed in the mid to late Pleistocene (0.12 to 0.58 million years ago, Mya). Based on the 3.6% rate, estimated expansion time for the individual phylogroups was between 0.10–0.27 Mya, or between 0.022–0.057 Mya based on the 17% rate (see Table 5 for details and 95% confidence intervals).

DISCUSSION

The Hokkaido-endemic species *Myodes rex* is classified as "Near Threatened" in the Japanese Red List (Iwasa and Kaneko, 2009), due to its discontinuous distribution and limited records. Fragmentation of distribution ranges generally leads to a reduction of population size, and subsequently to a decrease of genetic divergence via genetic drift. The low genetic divergence of *M. rex* in Hokkaido on the mtDNA cytochrome *b* (*Cyt b*) gene has been understood in this context (Iwasa and Nakata, 2011). In this study, we revised the distribution range of *M. rex*, suggesting that the species is more widely distributed than previously thought, and revealed that *M. rex* on Hokkaido harbors considerable genetic variability at the mtDNA control region. Since sequences of the mtDNA control region evolve more rapidly than those of the *Cyt b* gene (Matson and Baker, 2001), our data from the mtDNA control region can provide a more detailed understanding of the evolutionary history of *M. rex.*

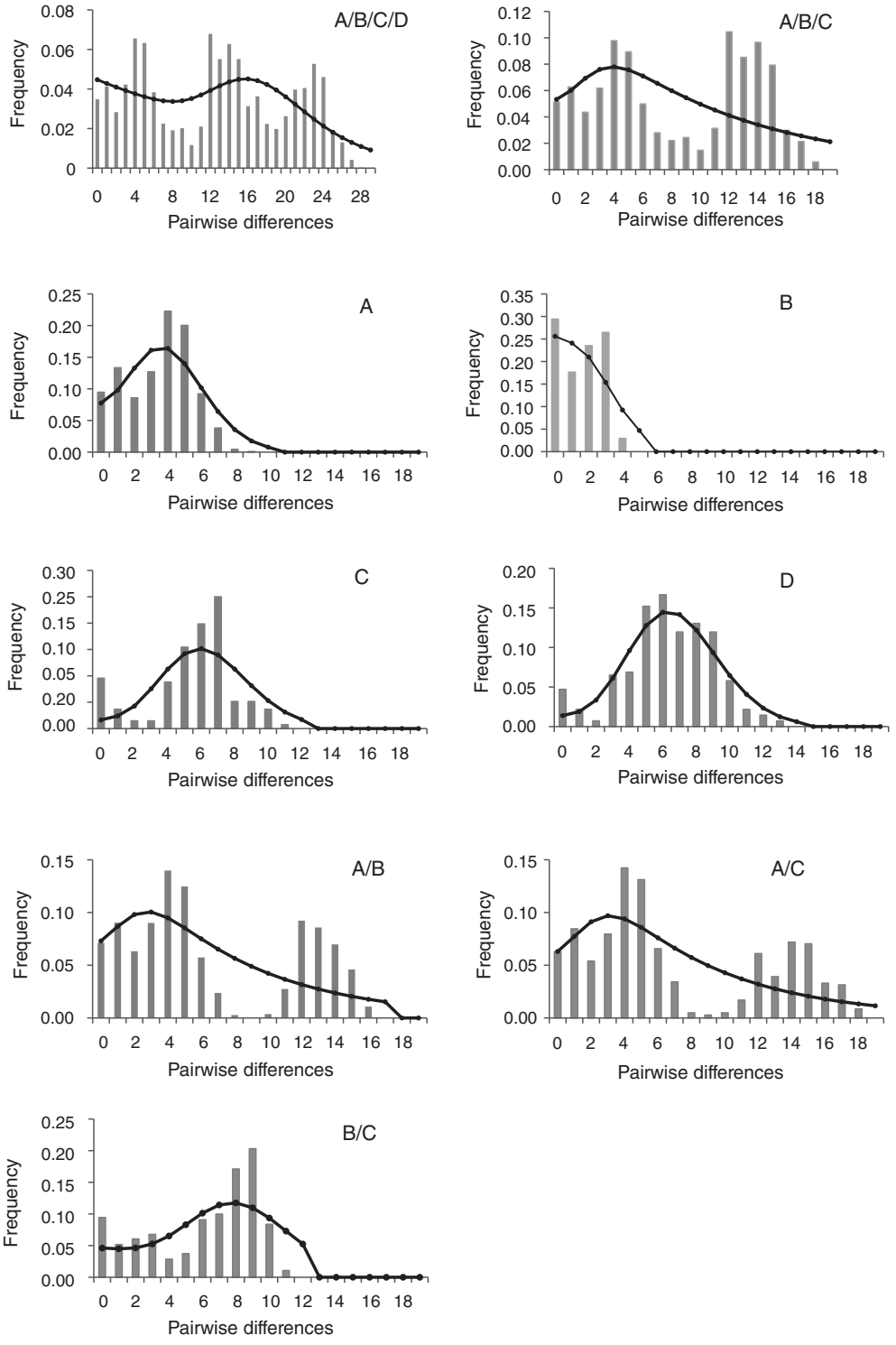

Fig. 4. Mismatch distribution for nine groupings of *M. rex*. On each grouping, bars indicate observed frequency, and a line indicates expected frequency, which was based on a population expansion model. A, phylogroup in the northern region of the mainland Hokkaido. B, in the southern region of the mainland. C, in the central region of the mainland. D, in Rishiri.

Our results suggest that *M. rex* includes four phylogroups corresponding to regional populations found in the northern, central, and southern regions of the mainland Hokkaido, as well as in Rishiri. The haplotype tree (Fig. 2), haplotype network (Fig. 3), and mismatch distribution analysis (Fig. 4) we generated all support the idea that the four distinct haplogroups reflect *M. rex* populations in these four regions.

Our estimates of the timing of population divergence and demographic expansions remain vague due to limitations in the *Myodes* fossil record and uncertainty about the true evolutionary rate of the mtDNA control region. Nevertheless, our estimates indicate that *M. rex* has been present on Hokkaido since the mid- or late Pleistocene (0.12–0.58 Mya). Wakana et al. (1996) estimated that *M. rufocanus* colonized on Hokkaido since the last glacial maximum, ca. 0.01–0.02 Mya. McKay (2012) shows that five divergence events occurred in terrestrial mammals of Hokkaido at 0.048 Mya (0–0.192, the 95% highest posterior densities), 0.104 Mya (0–0.456), 0.256 Mya (0–0.896), 0.896 Mya (0.384–1.416), and 1.360 Mya (0.736–1.608). This suggests that *M. rufocanus* may have colonized Hokkaido from the Eurasian continent at the latest divergence event, while *M. rex* likely arrived as the result of an earlier event. Iwasa and Nakata (2011) explain that the discontinuous distribution pattern of *M. rex* may result from ecological competition with dominant species of *M. rufocanus*. They implicitly assumed that *M. rex* was continuously distributed in Hokkaido before the arrival of *M. rufocanus*. Our results, however, indicate that *M. rex* genetically differentiated into the four populations prior to the arrival of *M. rufocanus* (Table 5). Therefore, the current distributional pattern of *M. rex* may have been shaped by past demographic history rather than by competition with *M. rufocanus*.

How were the four phylogroups of *M. rex* formed? Since the distribution range of *M. rex* is restricted to Hokkaido and its adjacent islands (Iwasa and Kaneko, 2009), these phylo-

groups likely evolved in situ.

Phylogroup D occurs only on Rishiri Island, thus indicating that isolation by sea causes the evolution of this group. The haplotype network showed that phylogroup D was divided from the others at the 95% probability connection limit. The genetic distances between phylogroup D and the other groups are relatively high (Table 4). Previous molecular phylogeny of *M. rex* based on *Cyt b* also suggested that the *M. rex* on Rishiri Island were relatively independent of those on the Hokkaido mainland and ancestral (Abramson et al., 2009; Iwasa and Nakata, 2011). In the present study, we could not determine which phylogroup is ancestral by the analysis shown in Fig. 2, because it was an unrooted tree. However, it is more likely that phylogroup D is basal, since the onset of population expansion for this group in Rishiri was estimated at 0.057–0.267 Mya, which predates the separation of Rishiri Island from the Hokkaido mainland (0.013 Mya; Naitoh and Ohdachi, 2006). The fact that phylogroup D did not show a star-like shape could be explained by higher demographic stability in that region, by subsequent population reductions and a loss of diversity, or by more complex demographic scenarios.

To explain the evolution of other phylogroups, we speculate that several refugia existed in Hokkaido mainland during the glacial age of the mid Pleistocene. The phylogeographic pattern of the bank vole (*M. glareolus*) observed in central Europe is explained by the presence of glacial refugia, but not by the postglacial recolonization from Mediterranean phylogroups (Bilton et al., 1998; Deffontaine et al., 2005; Kotlik et al., 2006). Since large parts of Europe were covered by ice during the Last Glacial Maximum (LGM), forests as preferred glacial refugia are thought to have been located in the river systems near the Alps or in the Carpathian mountains and the Hungarian plain (Deffontaine et al., 2005). The present study revealed that *M. rex* immigrated into Hokkaido by the Middle Pleistocene, meaning that *M. rex* must have survived several glacial periods. The current phylogeographic pattern of *M. rex* could be therefore have been formed by the refugia through the past glacial periods, as in the case of the bank vole. Fragmented refugia that harbored *M. rex* populations may have been located in the river systems in the western and central regions of Hokkaido, and those fragmented populations may have evolved independently each other.

Populations of phylogroup B did not combine with those of the phylogroups A and C, even in the interglacial age. The Ishikari lowland is thought to have been under the sea by the Middle Pleistocene marine transgression (0.8–0.4 Mya; Fig. 1), and the western part of the Ishikari lowland, which contained the populations of phylogroup B, was isolated from other regions of the Hokkaido mainland (Akamatsu, 1988). The Ishikari lowland has been repeatedly influenced by marine transgressions thereafter, and thus the connectivity of the western part with other regions of Hokkaido is weak. A similar pattern of the *M. rex* phylogroup B is found for red foxes (Oishi et al., 2011) and for hares (Kinoshita et al., 2012) in Hokkaido.

Phylogroups A and C in the northern and central in the mainland showed clear genetic differentiation each other, while they were sympatrically distributed at Locality 5, 9, and 10. The two phylogroups may have been geographically isolated and evolved independently in allopatric regions. If that is the case, the two populations may have extended their distribution range and exhibited sympatric distribution at some localities, raising two possible scenarios: In the first, both populations of phylogroups A and C may have evolved at allopatric refugia in the Hokkaido island during a glacial period; alternatively, either population of phylogroups A or C came from Sakhalin or other adjustment islands. Abramson et al. (2009) report *M. rex* in Sakhalin was more closely related to those in Takinoue (near Locality 7) than in Teshio (near Locality 3 or 5), which seems to support the second scenario. However, their sample sizes are small; just one specimen for each sampling point, and information on this species in Sakhalin is still insufficient. Further studies focusing on Sakhalin and other adjustment islands, such as Shibotsu, Kunashiri, and Shikotan, are needed to reach a conclusion.

Refugia utilized by boreo-temperate adapted species during past glacial ages have played an important role in shaping the current phylogeographic structure of species in Eurasia and North America (reviewed by Hewitt, 2000). In addition to these effects, migration from other areas is also important in shaping the distribution patterns and phylogeographic structure of species on islands. Therefore, endemic species to Hokkaido may provide us with useful examples for understanding the combined effects of past refugia and migration on the current distribution patterns and phylogeographic structure.

ACKNOWLEDGMENTS

We are indebted to Keisuke Nakata and the Forestry Agency of Japanese Government for providing the material analyzed in this paper. We thank members of Field Science Centre, Hokkaido University who helped to collect samples in the field. We are grateful to Dr. Kristofer M. Helgen and Dr. Koji Fujimura for valuable comments and supporting, and Dr. Takahiro Segawa for supporting to construct haplotype trees. This work was supported by a JSPS Grant-in-Aid for Scientific Research (22370006) to TS. FH was supported by the Smithsonian Restricted Endowment Funds, the Smithsonian Center for Conservation and Evolutionary Genetics, and "LOEWE – Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz" of Hesse's Ministry of Higher Education, Research, and the Arts. Specimens collected in Niseko are deposited in Hokkaido University Natural History Museum under the following ID number; HUNHM46316, HUNHM48103, HUNHM48257, HUNHM48282, HUNHM 48283, HUNHM48284, HUNHM 48445, HUNHM 48446, and HUNHM 48777.

REFERENCES

Abe H, Ishii N, Ito T, Kaneko Y, Maeda K, Miura S, Yoneda M (2005) A guide to the mammals of Japan. Tokai University Press, Kanagawa, Japan (in Japanese)

Abe H, Kobayashi T, Maeda K, Hayata I (1971) Result of the small mammal survey on the Daisetsu area. Annual Report of JIBP/CT-S for the Fiscal Year of 1970, pp 13–22

Abramson NI, Abramov AV, Baranova GI (2009) New species of Red-Backed Vole (Mammalia: Rodentia: Cricetidae) in fauna of Russia: molecular and morphological evidences. Proc Zool Inst RAS 313: 3–9

Akamatsu M (1988) Middle Pleistocene marine transgression and its significance in Hokkaido. J Geol Soc Japan 94: 173–186 (in Japanese with English abstract)

Avise JC (2000) Phylogeography. The history and formation of species. Harvard University Press, Cambridge, MA

Bilton DT, Mirol PM, Mascheretti S, Fredga K, Zima J, Searle JB (1998) Mediterranean Europe as an area of endemism for small mammals rather than a source for northwards postglacial colonization. Proc R Soc Lond B Biol Sci 265: 1219–1226

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1660

Deffontaine V, Libois R, Kotlik P, Sommer R, Nieberding C, Paradis E, et al. (2005) Beyond the Mediterranean peninsulas: evidence of central European glacial refugia for a temperate forest mammal species, the bank vole (*Clethrionomys glareolus*). Mol Ecol 14: 1727–1739

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10: 564–567

Fedorov VB, Stenseth NC (2001) Glacial survival of the Norwegian lemming (*Lemmus lemmus*) in Scandinavia: inference from mitochondrial DNA variation. Proc R Soc Lond B Biol Sci 268: 809–814

Fu Y-X (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147: 915–925

Fujimaki Y (1994) Over the straits—mixture of faunas. In "Formation History of Nature of Hokkaido" Ed by K Ishigaki, M Fukuta, Hokkaido University Press, Sapporo, Japan, pp 167–179 (in Japanese)

Haga R, Fujimaki Y, Onoyama K (1979) Mammals. In "Research Report on Natural Ecosystem of the Hidaka Mountains" Hokkaido Government, Sapporo, Japan, pp 3–56 (in Japanese)

Hewitt GM (2000) The genetic legacy of the Quaternary ice ages. Nature 405: 907–913

Ichikawa H (2002) Occurrence of *Myodes rex* in Niseko and its habitat. Bulletin of Botanic Garden, Hokkaido University 2: 66–68 (in Japanese)

Imaizumi Y (1971) A new vole of the *Clethrionomys rufocanus* group from Rishiri Island, Japan. Journal of the Mammalogical Society of Japan 5: 99–103

Imaizumi Y (1972) Land mammals of the Hidaka Mountains, Hokkaido, Japan, with special references to the origin of an endemic species of the genus *Clethrionomys*. Memoirs of the National Science Museum 5: 131–149 (in Japanese with an English summary)

Iwasa MA, Kaneko Y (2009) Muridae. In "The Wild Mammals of Japan" Ed by SD Ohdachi, Y Ishibashi, MA Iwasa, T Saitoh, Shoukadoh, Kyoto, Japan, pp 144–179

Iwasa MA, Serizawa K, Sato M (2001) Taxonomic problems of the dark red-backed vole, *Clethrionomys rex*. Rishiri Studies 20: 43–53 (in Japanese with English abstract)

Iwasa MA, Nakata K (2011) A note on the genetic status of the dark red-backed vole, *Myodes rex*, in Hokkaido, Japan. Mammal Study 36: 99–103

Kadosaki M, Inage M (1998) An individual of *Clethrionomys rex* captured at Shiraigawa, Jozankei. Forest Protection 266: 31–32 (in Japanese)

Kaneko Y (1994) Muridae. In "A Prictorial Guide to the Mammals of Japan" Ed by H Abe, Tokai University Press, Tokyo, Japan, pp 90–109, 168–183 (in Japanese)

Kaneko Y, Sato M (1993) Identification and distribution of the red-backed voles from Is. Rishiri, Hokkaido (Preliminary study). Annual Report of the Rishiri Museum 12: 37–47 (in Japanese)

Kaneko Y, Nakata K, Saitoh T, Stenseth NC, Bjørnstad ON (1998) The biology of the vole *Clethrionomys rufocanus*: A review. Res Popul Ecol 40: 21–37

Kashiwabara S, Onoyama K (1988) Karyotypes and G-banding patterns of the red-backed voles, *Clethrionomys montanus* and *C. rufocanus bedfordiae* (Rodentia, Microtinae). Journal of the Mammalogical Society of Japan 13: 33–41

Kinoshita G, Nunome M, Han SH, Hirakawa H, Suzuki H (2012) Ancient colonization and within-island vicariance revealed by mitochondrial DNA phylogeography of the Mountain hare (*Lepus timidus*) in Hokkaido, Japan. Zool Sci 29: 776–85

Kotlik P, Deffontaine V, Mascheretti S, Zima J, Michaux JR, Searle JB (2006) A northern glacial refugium for bank voles (*Clethrionomys glareolus*). P Natl Acad Sci USA 103: 14860–14864

Koyasu K, Zholnerovskaya EI, Oda S (1996) Mammals from the Kuril Islands and the Sakhalin Islands in the Zoological Museum in Siberia. J Growth 35: 125–129

Matson CW, Baker RJ (2001) DNA sequence variation in the mitochondrial control region of red-backed voles (*Clethrionomys*). Mol Biol Evol 18: 1494–1501

McKay BD (2012) A new timeframe for the diversification of Japan's mammals. J Biogeogr 39: 1134–1143

Motokawa M (2008) Taxonomic status of *Neoaschizomys sikotanensis* Tokuda, 1935 (Rodentia, Muridae) after re-examination of type specimens. Mammal Study 33: 71–75

Naitoh Y, Ohdachi SD (2006) Population genetic structure of *Sorex unguiculatus* and *Sorex caecutiens* (Soricidae, Mammalia) in Hokkaido, based on microsatellite DNA polymorphism. Ecol Res 21: 586–596

Nakata K (2000) Distribution and habitat of the dark red-backed vole *Clethrionomys rex* in Japan. Mammal Study 25: 87–94

Ohdachi SD, Ishibashi Y, Iwasa MA, Saitoh T (2009) The wild mammals of Japan. Shoukadoh, Kyoto, Japan

Ohshima K (1990) The history of straits around the Japanese islands in the late- Quaternary. The Quaternary Research 29: 193–208 (in Japanese with English abstract)

Oishi T, Uraguchi K, Takahashi K, Masuda R (2011) Population structures of the red fox (*Vulpes vulpes*) on the Hokkaido Island, Japan, revealed by microsatellite analysis. J Heredity 102: 38–46

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9: 552–569

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61: 539–342

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496–2497

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 9: 512–526

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28: 2731–2739

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132: 619–633

Yanagawa H, Itoh H (1990) Vertical distribution of small mammals on Mt. Petegari, the Hidaka Mountains, Hokkaido. Research Bulletin of Obihiro University, Natural Science 17: 69–75

Wakana S, Sakaizumi M, Tsuchiya K, Asakawa M, Han S-H, Nakata K, Suzuki H (1996) Phylogenetic implications of variations in rDNA and mtDNA in red-backed voles collected in Hokkaido, Japan, and in Korea. Mammal Study 21: 15–25

(Received August 22, 2012 / Accepted March 25, 2013)