*Evolutionary history of the sable (*Martes zibellina brachyura*) on Hokkaido inferred from mitochondrial* Cytb *and nuclear* Mc1r *and* Tcf25 *gene sequences*

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Volume 58 · Number 1 · January 2013

Acta Theriologica

Springer

Mammal Research Institute
Polish Academy of Sciences
Białowieża

Springer

Springer

ORIGINAL PAPER

Evolutionary history of the sable (*Martes zibellina brachyura*) on Hokkaido inferred from mitochondrial *Cytb* and nuclear *Mc1r* and *Tcf25* gene sequences

**Kotaro Ishida · Jun J. Sato · Gohta Kinoshita ·
Tetsuji Hosoda · Alexey P. Kryukov · Hitoshi Suzuki**

Abstract We examined sequence variation in mitochondrial and nuclear genes of seven species of the genus *Martes* (Mustelidae, Carnivora): *Martes americana* (American marten), *Martes flavigula* (yellow-throated marten), *Martes foina* (beech marten), *Martes martes* (pine marten), *Martes melampus* (Japanese marten), *Martes pennanti* (fisher) and *Martes zibellina* (sable), focusing on the phylogenetic history of the Hokkaido subspecies of the sable, *Martes zibellina brachyura*. Nucleotide sequence analysis of the mitochondrial cytochrome *b* gene confirmed the view that the Hokkaido sable population has lower genetic diversity. In contrast, network analysis of a nuclear gene related to coat colour, melanocortin-1 receptor (*Mc1r*), revealed two different haplogroups for this population: one shared with that of Russian sables and the other specific to this population but with a close relationship with the American and Japanese martens, implying that these endemic haplotypes are composed of uncharacterised ancestral lineages of a past population. We also examined the sequence variation in a neighbouring nuclear gene, transcription factor 25 (*Tcf25*), located about 5 kb upstream from the *Mc1r* gene, and found similar trends. The sable genome leaves the impression that Hokkaido hosted ancient marten lineages, with subsequent recent migrations from the continent. The validity of a candidate *Mc1r* mutation for the entirely yellow coat observed on Hokkaido sables was also discussed.

K. Ishida · G. Kinoshita · H. Suzuki
Laboratory of Ecology and Genetics,
Graduate School of Environmental Earth Science,
Hokkaido University,
Kita-ku,
Sapporo 060-0810, Japan

J. J. Sato (✉)
Laboratory of Animal Cell Technology,
Faculty of Life Science and Technology, Fukuyama University,
Higashimura-cho, Aza, Sanzo, 985,
Fukuyama 729-0292, Japan
e-mail: jsato@bt.fubt.fukuyama-u.ac.jp

T. Hosoda
Taikyu High School,
1985 Yuasa-cho, Arida-gun,
Wakayama 643-0004, Japan

A. P. Kryukov
Laboratory of evolutionary zoology and genetics,
Institute of Biology & Soil Science Far East Branch Russian
Academy of Sciences,
Vladivostok 690022, Russia

Present Address:
T. Hosoda
Kansai University, Internatinal Education,
1-2-20, Satake-dai,
Suita-shi, Osaka 565-0855, Japan

Introduction

The impact of the Quaternary ice ages on population genetic dynamics has been studied thoroughly for higher-latitude species in the Northern Hemisphere. Comparative phylogeographic studies have shown that Holarctic terrestrial organisms experienced spatial expansions and contractions in response to the glaciation cycles in the Pleistocene, giving rise to common features in the population histories of diverse taxa (e.g., Hewitt 1996, 1999, 2000, 2001, 2004, 2011; Soltis et al. 2006). The repeated migrations might

have been accompanied by introgressive hybridisation between genetically differentiated lineages and even between different species. Such secondary contact has been observed for terrestrial animals and plants in Europe and North America (e.g., Hewitt 1996, 1999, 2000, 2001, 2011; Swenson and Howard 2004, 2005; Rissler and Smith 2010; Swenson 2010), while it is under-represented in north-eastern Eurasia, including the eastern islands of the Japanese archipelago. Tracing the secondary admixture among diverged biotas in north-eastern Eurasia would provide additional insight into the effect of Pleistocene climatic changes on population history and speciation.

Four species in the genus *Martes* (Mustelidae, Carnivora), *Martes americana* (American marten), *Martes martes* (pine marten), *Martes melampus* (Japanese marten) and *Martes zibellina* (sable), are forest-dwelling carnivores that occur allopatrically in coniferous and deciduous forests in the Holarctic region (Nowak 1999; Wozencraft 2005). The evolutionary responses of these Holarctic martens to Pleistocene climate changes remain unclear. However, deforestation during the Pleistocene likely affected the populations of arboreal Holarctic martens, making this group suitable for inferring the effect of repeated Pleistocene climate changes on their population dynamics and secondary contacts with northern Eurasian mammals. This study focused on the phylogenetic pattern and hybridisation history of the Hokkaido subspecies of the sable, *Martes zibellina brachyura*.

Hokkaido, the northernmost large island in the Japanese archipelago, is suitable for studying secondary contacts. The islands peripheral to Eurasia have served as reservoirs for both anciently and recently migrated lineages (e.g., Kawamura 2007; Hosoda et al. 2011; Suzuki 2009; McKay 2012). The Japanese archipelago is home to two species of Holarctic marten: the sable and Japanese marten. The sable occurs only on Hokkaido, while the Japanese marten occurs on Honshu, Shikoku, Kyushu and Tsushima (Murakami 2009; Masuda 2009; Monakhov 2011). Sato et al. (2012) showed that the Japanese marten lineage arose before that of the sable. Therefore, it is reasonable to think that the Japanese marten migrated to the Japanese archipelago before the sable. Assuming that the ancestors of both species migrated to the Japanese archipelago through a northern route via Sakhalin, the two ancestral lineages likely came into contact on Hokkaido. By examining genomic components of the sables currently present on Hokkaido, it might be possible to obtain evidence of their lineage contacts on Hokkaido.

Currently, both species occur on Hokkaido because the fur industry introduced the Japanese marten there in the 1940s (Inukai 1975), and it has now spread to southern Hokkaido (Murakami and Ohtaishi 2000; Sugimoto et al. 2009). Therefore, it is possible that the two martens on

Hokkaido hybridise in the contact zone between their current distributions. With one exception (Murakami et al. 2004), genetic analyses have found no sign of hybridisations between them (Hosoda et al. 1999; Kurose et al. 1999; Sugimoto et al. 2009; Inoue et al. 2010). However, because the previous studies examined mitochondrial DNA only, further analyses with nuclear loci would shed more light on the issue of ongoing genetic flow.

Non-recombining molecular markers such as mitochondrial DNA (mtDNA) provide valuable phylogeographic information in a variety of organisms (Avise 2000). However, the uniparental inheritance of mtDNA is problematic for detecting hybridisation. Recently, it was demonstrated that an analysis of recombination among tandemly linked genetic loci can provide valuable information for assessing the extent and mode of historical hybridisations (Koopman et al. 2007; Nunome et al. 2010). Furthermore, the haplotype structure between linked markers in a chromosome region separated by a defined interval such as 10 kb has been used in rough chronological estimations of introgressive hybridisation (Nunome et al. 2010).

This study examined the genetic diversity of sables sampled from various localities in Far East Russia and Hokkaido. First, we sequenced the mitochondrial cytochrome *b* (*Cytb*) and a nuclear gene related to coat colour, melanocortin-1 receptor (*Mc1r*). Then, we sequenced another nuclear gene, transcription factor 25 (*Tcf25*), located about 5 kb upstream from the *Mc1r* gene to assess the haplotypes of the chromosome region between these two genes. Because of the highly conserved synteny between the dog and the house mouse (see Ensemble Genome Database, http://www.ensembl.org/), we assumed that the sable also has the same gene order. Phylogenetic and network analyses of these gene sequences were conducted to better understand the evolutionary history of the Holarctic martens and Hokkaido sables. We also searched for the polymorphism of the *Mc1r* gene responsible for the unusual yellow colour phenotype of Hokkaido sables.

Materials and methods

Samples and nucleotide sequences examined

The samples analysed in this study are listed in Table 1. We examined 58 *M. zibellina* collected from 17 localities in Far East Russia and Hokkaido (Fig. 1) and 1–5 individuals of each of the following congeneric species: *M. americana*, *M. foina*, *M. flavigula*, *M. martes*, *M. melampus* and *M. pennanti* (all the species in the genus *Martes* except *M. gwatkinsii*). For each sample, partial nucleotide sequences of the mitochondrial *Cytb* and nuclear *Mc1r* and *Tcf25* genes were determined and used for phylogenetic and network analyses.

Table 1 Samples examined in this study

Species	Collection locality[a]	Sample code[b]	Cytb[c]	Assigned haplotypes[d]	
				Mc1r	Tcf25
Martes americana	Maine, USA	HS990	−	14, 15 (1)	6, 6
		HS991	−	14, 14	6, 6
		HS992	−	14, 14	6, 6
		HS993	−	16, 16	6, 6
	Unknown	HS2587	−	14, 15 (1)	6, 6
Martes flavigula	Yunnan, China	HS1224	−	19, 19	−
Martes foina	Gera, Turingia, Germany	HS1752	−	18, 18	6, 6
Martes martes	Tver, Russia	HS1393	−	17, 17	7, 8 (1)
Martes melampus	Tochigi, Honshu, Japan	HS515	−	12, 12	6, 6
	Wakayama, Honshu, Japan	HS517	−	12, 13	6, 6
	Tsushima Island, Japan	HS520	−	12, 12	6, 6
	Iwanai, Hokkaido, Japan	HS813	−	12, 12	6, 6
	Eniwa, Hokkaido, Japan	HS4565	−	13, 13	6, 6
Martes pennanti	unknown	HS2588	−	20, 20	9, 9
Martes zibellina	1: Evoron lake, Khabarovsk, Russia	AK2169	+	−	−
		AK2170	−	2, 8 (2)	1, 4 (1)
	2: Bikin, Khabarovsk, Russia	HS1880	−	1, 1	−
		HS1881	−	1, 1	1, 1
		HS1882	−	1, 6 (1)	1, 1
		HS1883	−	1, 3 (1)	1, 5 (1)
		HS1884	−	1, 2 (1)	1, 1
	3: Sal'da river, Pozharsky district, Primorsky, Russia	AK1309	−	1, 1	1, 1
		AK1310	−	3, 3	1, 1
		AK1311	−	Failed[e] (3)	1, 1
		AK1312	−	1, 2 (1)	1, 1
	4: Bol'shaya Ussurka river, Mel'nichnoe, Primorsky, Russia	AK1276	−	1, 1	1, 1
		AK1277	−	1, 1	1, 5 (1)
		AK1278	−	1, 1	1, 1
		AK1279	−	1, 1	1, 5
		AK1280	−	1, 1	1, 5 (1)
		AK1281	−	1, 1	1, 1
		AK1282	−	3, 3	1, 1
		AK1283	−	2, 3 (2)	1, 1
		AK1284	−	1, 1	1, 5 (1)
		AK1292	−	1, 1	1, 1
		AK1293	−	1, 3 (1)	1, 1
	5: Sitca river, Terney, Primorsky, Russia	AK739	−	1, 2 (1)	1, 3 (1)
	6: Bear Stream, Koksharovka vil., Chuguevka, Primorsky, Russia	AK1269	+	1, 1	1, 3 (1)
		AK1285	+	1, 1	1, 1
		AK1286	−	1, 6 (1)	1, 4 (1)
		AK1287	−	1, 1	1, 1
		AK1288	−	1, 1	1, 1
		AK1210	+	1, 4 (1)	1, 1
		AK1211	−	1, 1	−
		AK1212	−	1, 4 (1)	1, 1
		AK1213	+	3, 4 (2)	−
		AK1214	−	1, 3 (1)	1, 1

Table 1 (continued)

| Species | Collection locality[a] | Sample code[b] | Cytb[c] | Assigned haplotypes[d] | |
				Mc1r	Tcf25
		AK1215	–	1, 4 (1)	1, 1
		AK1216	–	1, 8 (3)	1, 1
	Unknown, Russia	HS709	–	1, 1	1, 1
	7: Horonobe, Hokkaido, Japan	TH47 Yellow	+	5, 5	2, 2
		TH53	+	7, 7	Failed (2)
	8: Nakagawa, Hokkaido, Japan	HS4862	–	2, 2	–
		HS4864	–	2, 2	–
		HS4865	+	1, 2 (1)	–
		HS4866	–	1, 2 (1)	–
		HS4867	–	1, 2 (1)	–
	9: Otoineppu, Hokkaido, Japan	TH43	+	9, 11 (1)	2, 2
	10: Nishiokoppe, Hokkaido, Japan	HS4701	–	Failed (2)	1, 3 (1)
	11: Horokanai, Hokkaido, Japan	TH44	+	9, 9	6, 6
		TH45	+	Failed (2)	Failed (2)
		TH46	+	2, 10 (2)	–
	12: Oozora, Hokkaido, Japan	HS4715	–	2, 3 (2)	Failed (2)
	13: Kamishihoro, Hokkaido, Japan	HS3863	–	1, 9 (4)	2, 6 (1)
		HS3864	+	Failed (2)	Failed (2)
		HS3865	–	2, 9 (3)	2, 6 (2)
	14: Tsukigata, Hokkaido, Japan	HS4869	–	2, 2	1, 1
		HS4871	–	1, 2 (1)	2, 2
	15: Tobetsu, Hokkaido, Japan	HS4823 Yellow	–	failed (2)	–
		HS4855	–	2, 2	–
		HS4870	–	–	1, 1
	16: Nopporo, Hokkaido,Japan	HS4705	–	1, 2 (1)	1, 2 (1)
	17: Tomakomai, Hokkaido, Japan	HS4800	–	9, 9	6, 6
	18: Hidaka, Hokkaido, Japan	HS4890	–	1, 1	2, 2
	Unknown, Hokkaido, Japan	HS3934	–	1, 2 (1)	–

[a] Serial numbers of collection localities are the same as in Fig. 1

[b] Voucher numbers constitute of the following abbreviations and serial numbers for collections of DNA or tissue samples, or whole body specimen: AK, Alexey P. Kryukov's collection deposited in the Institute of Biology and Soil Science, Russian Academy of Sciences, Vladivostok, Russia; HS, Hitoshi Suzuki's collection deposited in the Laboratory of Ecology and Genetics, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan; Tetsuji Hosoda's collection deposited in the Laboratory of Ecology and Genetics, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan

[c] The mitochondrial cytochrome b gene sequences were determined in individuals marked with +

[d] Haplotypes assigned by the program PHASE. Numbers are the same as in Figs. 3 and 4. Numbers in parentheses are the number of heterozygous sites

[e] "Failed" means that we failed to assign haplotypes to each individual by the program PHASE

Based on the existing supported phylogenetic hypothesis (e.g. Wolsan and Sato 2010; Sato et al. 2012), *M. foina* (GU935936; Li et al. 2011) was used as the outgroup for the *Cytb* phylogenetic analysis of the subgenus *Martes* (*M. americana*, *M. foina*, *M. martes*, *M. melampus* and *M. zibellina*). The reported *Cytb* sequence for *M. melampus* (AB455609; Sato et al. 2009) was also used in the phylogenetic analysis.

Polymerase chain reaction amplification and sequencing strategy

Total genomic DNA was extracted from ethanol-preserved tissues using the standard phenol–chloroform method (Sambrook and Russell 2001). The DNA was amplified by polymerase chain reaction (PCR) using an automated thermal cycler. The *Cytb* gene sequences (638 bp) were

Fig. 1 Sampling localities of the sables examined in this study. The *inset* shows a wide area map, and the *square in the inset* is the area we focused on. The *numbers* for the localities correspond to those in Tables 1 and 2
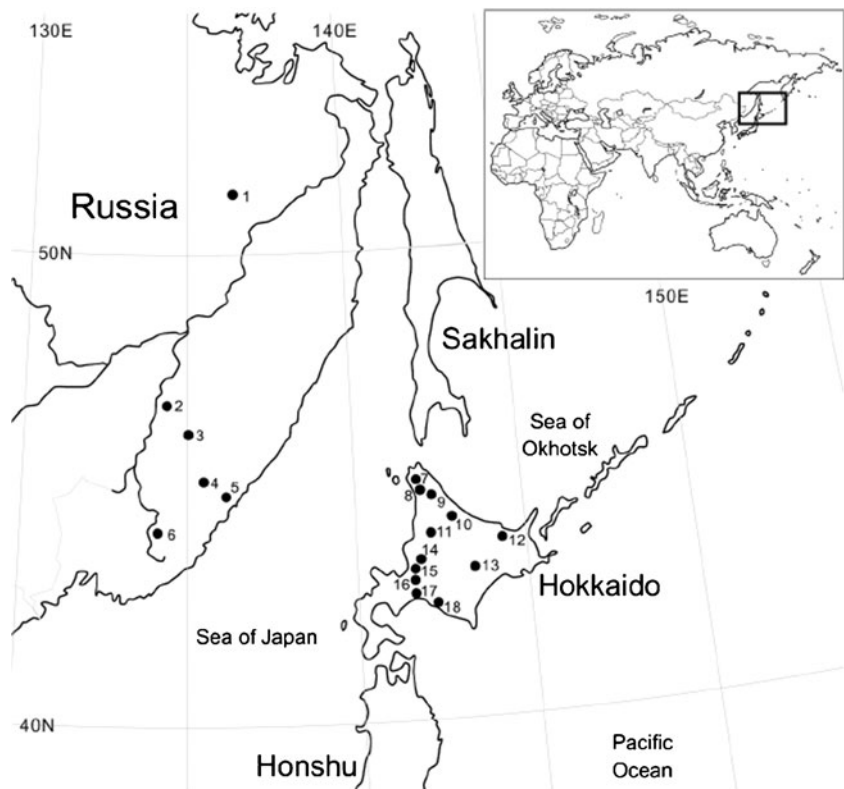
determined using the methods described in Hosoda et al. (2000). For the phylogenetic analyses, additional sequences in the DDBJ/EMBL/GenBank DNA databases were used.

A coding region of the *Mc1r* gene (about 800–900 bp) was sequenced using a seminested PCR method. The coding region was sequenced in two parts. For the first, the initial primers were 157 (5′-GATGAGCTGAGCGGGACGC CTG-3′) and 159 (5′-GGTATCGCAGCGCGTAGA AGATG-3′), and the PCR consisted of 35 cycles of 95 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min. The primers for the second PCR were 158 (5′-CTGCGAGTG AGGACCCCTTTCTG-3′) and 159, and the PCR consisted of 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. The primers for the second half of the sequence examined were 160 (5′-TGTCCAGCCTCTGCTT CCTG-3′) and 643 (5′-GAGCACAGCAGCACC TCTTGGAG-3′) for the first PCR and 162 (5′-TTCCTGGGCGCCATCGCCGTGGAC-3′) and 643 for the second PCR. In both of these reactions, the PCR consisted of 35 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. The region between the positions of primers 162 and 159 overlaps in the two parts of the sequence.

An intron of the *Tcf25* gene (308 bp) was amplified using primers 830 (5′-CTCTTCTTCCGGTCCCTGTT-3′) and 831 (5′-TGGAAGTTGGCCATCATGTC-3′) with 35 PCR cycles of 95 °C for 30 s, 48 °C for 30 s and 72 °C for 30 s.

PCR was performed using the AmpliTaq Gold® 360 Master Mix kit (ABI). In addition to the conditions described above, all of the PCR reactions involved an initial 10-min denaturation at 95 °C and a final 2-min extension at 72 °C. For more efficient amplification of the *Mc1r* and *Tcf25* gene fragments, we added 1 μL "GC enhancer" to each 20-μL reaction mixture. The PCR products were sequenced using the BigDye Terminator Cycle Sequencing kit v3.1 (ABI), followed by automated sequencing on an ABI3130 genetic analyser. Novel sequences were deposited in the DNA databases with accession numbers AB725399–AB725545.

Sequence analyses

The sequences were aligned manually. The alignments of the *Cytb* and *Tcf25* gene sequences were straightforward because there were no insertions or deletions (indels) in the sequences examined. Consequently, we used the total determined sequences of the *Cytb* (638 bp) and *Tcf25* (305 bp) genes for the phylogenetic and network analyses. The *Mc1r* gene sequences contained some indels. Because they were all multiples of three, the alignment was straightforward. As in Hosoda et al. (2005), a 45-bp deletion was detected in all of the Holarctic martens. A novel 6-bp deletion and a previously observed 18-bp deletion (28-bp deletion plus 10-bp insertion; Hosoda et al. 2005) were both observed in *M. foina*. We excluded the observed indels from all of the *Mc1r* analyses throughout this study. The final aligned length of the *Mc1r* gene sequence was 794 bp.

Haplotype (*h*) and nucleotide (π) diversities were assessed using DnaSP ver. 5.0 (Librado and Rozas 2009). A phylogenetic tree was inferred using the neighbour-joining method (NJ; Saitoh and Nei 1987) implemented in the program PAUP* ver. 4.0b10 (Swofford 2002) based on the aligned 638-bp sequences of the *Cytb* gene. Pairwise distances were calculated with the HKY substitution model selected under the Akaike information criterion in Modeltest ver. 3.7 (Posada and Crandall 1998; see also Posada and Buckley 2004). The level of support for inferred relationships was evaluated using nonparametric bootstrap proportions (Felsenstein 1985). For the nuclear *Mc1r* and *Tcf25* gene analyses, we first separated the individual sequences of sables with more than two heterozygosities into two haplotypes using the program PHASE version 2.1 (Stephens et al. 2001; Stephens and Sheet 2005). To infer the relationships of the obtained haplotypes, a median-joining network was reconstructed using the program Network ver. 4.6 (Bandelt et al. 1999).

Results

Cytb sequence variation

We determined the mitochondrial *Cytb* gene sequences (638 bp) for 13 sables from Far East Russia (*n*=5) and Hokkaido (*n*=8). Together with previously determined
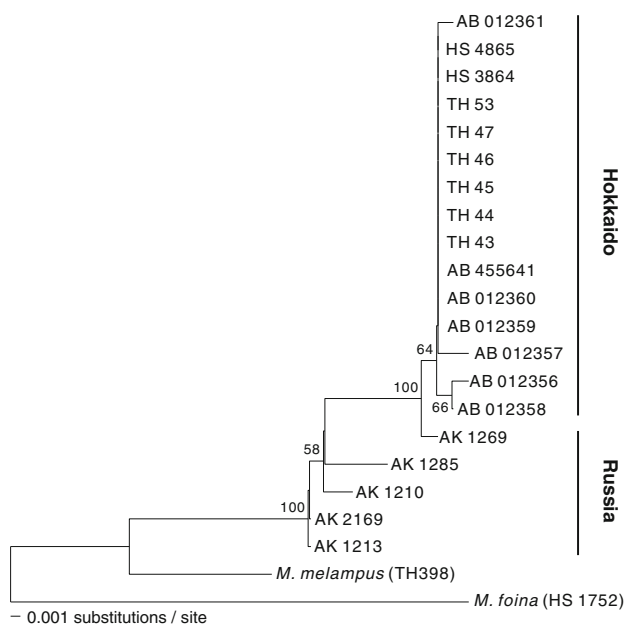
Fig. 2 An NJ tree of the mitochondrial cytochrome *b* gene sequences (638 bp) of *M. zibellina* from Far East Russia and Hokkaido, using *M. foina* as the outgroup. The *numbers at nodes* are bootstrap values (1,000 replicates). The geographic origin of each individual is represented by the *vertical line on the right side* of the phylogenetic tree

homologous gene sequences (Kurose et al. 1999), four and five haplotypes were detected for the sables in Far East Russia and Hokkaido, respectively. The genetic diversity of the Hokkaido population was lower than that of the Far East Russia population (*h*=0.476 vs. 0.900 and π=0.00122 vs. 0.00909, for Hokkaido and Russian sables, respectively). The NJ tree indicated that the Hokkaido sables are monophyletic (Fig. 2).

Mc1r sequence variation

Previously, we sequenced half of the *Mc1r* gene (498 bp) of two individuals from Hokkaido (Hosoda et al. 2005). Here, we extend the examined region to 794 bp, covering almost the entire coding region of the *Mc1r* gene. In total, 59 sables from Russia (*n*=35) and Hokkaido (*n*=24) were analysed. Thirteen variable sites were found. Of these, ten involved nonsynonymous changes and three involved synonymous changes. An individual with a yellow coat (HS4823) was heterozygous for the mutation (G104T) thought to be responsible for the yellow phenotype of the Hokkaido sable. Heterozygous sites were found in 16 of 35 Russian sables (46 %) and 15 of 24 Hokkaido sables (63 %). More than two heterozygous sites were observed in 13 sables (Table 1). Using the program PHASE, we separated the genotype sequences into 11 haplotypes (1–11 in the *Mc1r* column of Table 1) and assigned 2 of them to each individual. Only those reliably assigned 11 haplotypes were considered in the subsequent analyses. We also detected nine additional haplotypes in the sequences of 14 individuals representing six other *Martes* species: *M. americana*, *M. flavigula*, *M. foina*, *M. martes*, *M. melampus*, and *M. pennanti* (12–20 in the *Mc1r* column of Table 1).

The median-joining network analysis revealed two clusters (hereafter, haplogroups I and II), which were separated by two mutations (Fig. 3). Notably, the haplotypes from the Hokkaido population were divided into both groups, while those from the Russian population were confined to haplogroup I. The haplotypes in haplogroup II were closely related to different Holarctic martens (*M. americana* and *M. melampus*), differing by a single mutation at the minimum. Focusing on haplogroup I, the haplotype frequencies of each Russian and Hokkaido population differed from each other. Although both populations shared haplotypes 1, 2, and 3, haplotypes 1 and 3 were mostly observed on the continent, while haplotype 2 was more frequent on Hokkaido (Fig. 3). Furthermore, haplotypes 4, 6, and 8 were specific to Russia, while haplotypes 5 and 7 were found only on Hokkaido.

Tcf25 sequence variation

We sequenced the *Tcf25* gene (305 bp) for 48 sables from Far East Russia (*n*=32) and Hokkaido (*n*=16). There were

Fig. 3 A median-joining network with the melanocortin-1 receptor (*Mc1r*) gene sequences (794 bp) of *M. zibellina* from Far East Russia (*gray*) and Hokkaido (*white*). *M. americana*, *M. flavigula*, *M. foina*, *M. martes*, *M. melampus*, and *M. pennanti* are shown in *black*. The shortest branch length corresponds to one mutation and the branch length is proportional to the number of mutations, except the branches leading to *M. flavigula*, *M. foina*, and *M. pennanti*, marked by *double slashes*. The size of the circle is proportional to the number of haplotypes. The *smallest black circles* represent median vectors
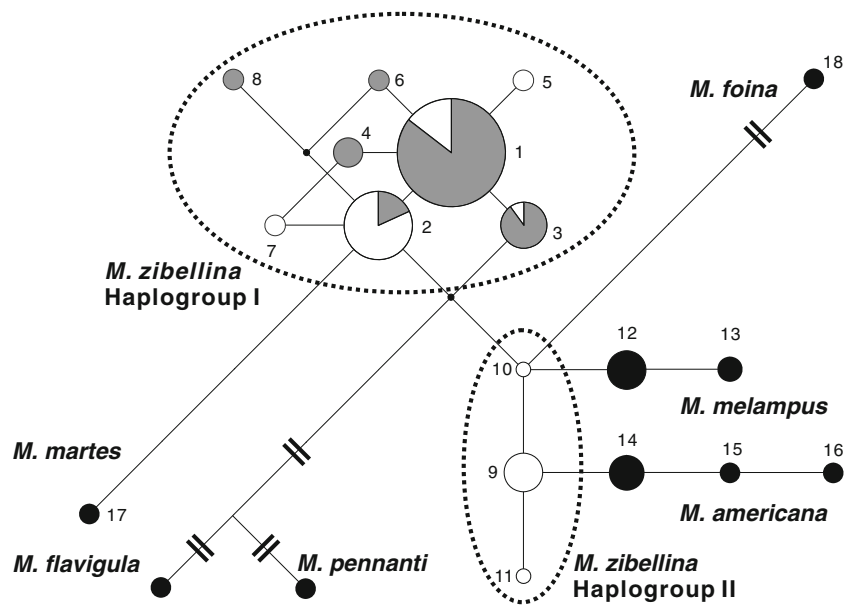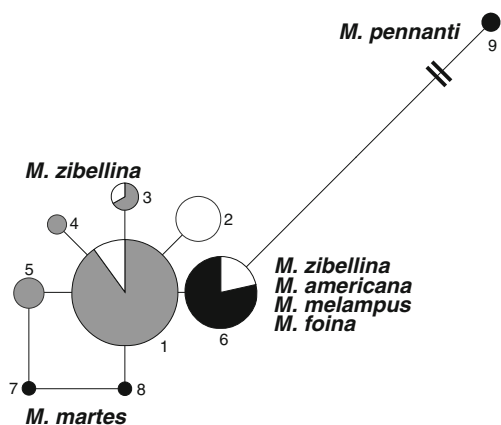

five variable sites in the aligned sequences. A total of 8 of 32 Russian (25 %) and 8 of 16 Hokkaido (50 %) sables possessed heterozygous sites. More than two heterozygous sites were detected in five individuals (Table 1). Using the program PHASE, we found six haplotypes (1–6 in the *Tcf25* column of Table 1) and assigned two of them to each individual. As in the *Mc1r* gene analyses, we only used confidently assigned six haplotypes for further analyses. We also detected four haplotypes in the sequences of 13 individuals of five other *Martes* species: *M. americana*, *M. foina*, *M. martes*, *M. melampus*, and *M. pennanti* (6–9 in the *Tcf25* column of Table 1).

We constructed a median-joining network based on the *Tcf25* gene sequences (Fig. 4). The Hokkaido sables possessed four haplotypes (1–3 and 6), as did the Russian sables (1 and 3–5). Haplotype 1 was the most frequent

Fig. 4 A median joining network with the transcription factor 25 (*Tcf25*) gene sequences (305 bp) of *M. zibellina* from Far East Russia (*grey*) and Hokkaido (*white*). *M. americana*, *M. foina*, *M. martes*, *M. melampus*, and *M. pennanti* are shown in *black*. The branch length and circle size are as in Fig. 3

haplotype and was shared by both Russian and Hokkaido sables. Haplotype 3 was also shared by both sable populations. Haplotypes 4 and 5 were specific to Russia, while haplotypes 2 and 6 were specific to Hokkaido. Haplotype 6 observed on Hokkaido was shared with different species (*M. americana*, *M. foina*, and *M. melampus*), as was haplogroup II of the *Mc1r* gene.

Haplotype structures between the two linked genes

The association between the *Mc1r* and *Tcf25* gene polymorphisms was determined with the program PHASE (Table 2). The *Mc1r* and *Tcf25* haplotypes were assigned successfully for 10 individuals (20 haplotype blocks) from Hokkaido. Almost all of *Mc1r* haplotype 1 was associated with *Tcf25* haplotype 2 (Table 2). Hereafter, we refer to the haplotype structure composed of *Mc1r* haplogroup I and *Tcf25* haplotypes 1–5 as haploblock I. The *Mc1r* haplotype 9 specific to Hokkaido was associated mostly with *Tcf25* haplotype 6 (Table 2). Hereafter, we refer to the haplotype structure consisting of *Mc1r* haplogroup II and *Tcf25* haplotype 6 as haploblock II. The result suggested linkage disequilibrium between these two genetic loci, which are physically separated by about 5 kb. On the other hand, we found two recombinant haplotype between haploblocks I and II (Table 2), in which the individual TH 43 from Otoineppu, Hokkaido, has haplotypes composed of haplotype 9 or 11 in *Mc1r* and haplotype 2 in *Tcf25* genes.

Discussion

Our molecular phylogenetic analyses revealed that the Hokkaido sable population possesses two differentiated

Table 2 Haplotype associations between *Mc1r* and *Tcf25* for sables in Hokkaido

Collection locality	Sample code	Assigned haplotypes[a]	
		Mc1r	*Tcf25*
7: Horonobe, Hokkaido, Japan	TH47	5	2
		5	2
9: Otoineppu, Hokkaido, Japan	TH43	*9*	2
		11	2
11: Horokanai, Hokkaido, Japan	TH44	*9*	*6*
		9	*6*
13: Kamishihoro, Hokkaido, Japan	HS3863	1	2
		9	*6*
	HS3865	2	2
		9	*6*
14: Tsukigata, Hokkaido, Japan	HS4869	2	1
		2	1
	HS4871	1	2
		2	2
16: Nopporo, Hokkaido,Japan	HS4705	1	1
		2	2
17: Tomakomai, Hokkaido, Japan	HS4800	*9*	*6*
		9	*6*
18: Hidaka, Hokkaido, Japan	HS4890	1	2
		1	2

[a] Values in italics show haplotypes closely related to different species

haplogroups of *Mc1r* gene sequences, one of which is closely related to different species in the genus *Martes*: *M. americana* and *M. melampus* (Fig. 3). This genetic diversity is also reflected in the large numbers of heterozygous individuals in this population. A similar trend was observed for the *Tcf25* gene sequences (Fig. 4), and linkage disequilibrium was suggested (Table 2). These observations markedly contrast with the lower level of genetic diversity in the mitochondrial genes of the Hokkaido sable population compared to the continental population (*Cytb*, this study; D loop, Inoue et al. 2010; *Nd2*, Sato et al. 2011). The results for two nuclear genes allowed us to draw an unprecedented picture of the evolutionary history of the Holarctic martens and the origin of the sable on Hokkaido.

Evolutionary history of the Holarctic martens

Anderson (1994) considered the Holarctic marten a single circumboreal species based on the similarity in morphological characteristics among the four Holarctic martens. Genetic studies have suggested hybridisation between different species or geographically differentiated subspecies, as in the hybrids between *M. martes* and *M. zibellina* (called "kidus" or "kidas"; Rozhnov et al. 2010; Monakhov 2011), *M. americana* and *M. caurina* (Small et al. 2003) and *M.*

melampus and *M. zibellina* (Murakami et al. 2004; but see Sugimoto et al. 2009). These observations imply that the reproductive isolation of the Holarctic martens is incomplete. Consequently, genetic exchange likely occurred during the differentiation of the Holarctic martens. The lineage sorting of genetic polymorphisms in an ancestral population may have been incomplete in different "species", leaving ancestral haplotypes in the current population. In this study, we discovered unexpected haplotypes in the Hokkaido sable population, showing close affinity with two different Holarctic martens: *M. americana* and *M. melampus* [haplotypes 9–11 in *Mc1r* (Fig. 3) and haplotype 6 in *Tcf25* (Fig. 4)]. Those haplotypes might be relicts of an ancestral population phylogenetically linking *M. americana* and *M. melampus*.

Previous studies failed to clearly resolve the interrelationships among the lineages of *M. martes*–*M. zibellina*, *M. americana* and *M. melampus* (e.g. Koepfli et al. 2008; Sato et al. 2012). Our result supports a close affinity between *M. americana* and *M. melampus* and suggests a following evolutionary history for the Holarctic martens (Fig. 5). The ancestral Holarctic martens might have once been separated into two geographic lineages: western and eastern Holarctic martens (stage I). Following stage I, geographic expansion of the eastern marten into North America

(NA) and Honshu in the Japanese archipelago occurred (stage II), leading to *M. americana* and *M. melampus*, respectively (stages III and IV). On the other hand, the western martens separated into two lineages and differentiated into *M. martes* and *M. zibellina* (stages III and IV).

Fig. 5 Hypothesised evolutionary history of the Holarctic martens. The *dotted outer circle* represents the population. *Roman numerals in circles* mean two haploblocks I and II. *Arrows* are symbols for population dispersals. *NA* North America. See the text for further explanation of the evolutionary events in stages I–IV

Finally, *M. zibellina* likely dispersed to eastern Eurasia and replaced the mitochondrial genome of the eastern martens on Hokkaido (Fig. 5, stage IV). The hypotheses described above explain why ancestral haplotypes are present on Hokkaido, although they should be tested rigorously by analysing more samples of Holarctic martens.

Origin of the sable on Hokkaido

Hosoda et al. (1999, 2000) and Sato et al. (2011) found that the mitochondrial *Cytb* and *Nd2* gene sequences, respectively, supported the monophyly of Hokkaido sables, while Inoue et al. (2010) found two independent lineages on Hokkaido with D loop sequences and concluded that the Hokkaido population has not differentiated from continental populations sufficiently. Using *Cytb* gene sequences, Malyarchuk et al. (2010) also reported non-monophyly of the Hokkaido sable with two Magadan individuals included in a clade composed of mostly Hokkaido individuals. Our mitochondrial *Cytb* gene analysis supports monophyly, while the nuclear *Mc1r* and *Tcf25* gene analyses indicate multiple origins of the Hokkaido sable. However, as described above, the divergent haplotypes from Hokkaido (haploblock II) could be descendants maintained from an ancestral population that was not technically a sable. Therefore, the available data suggest that the Hokkaido sable originated via one historical event and that the highly variable D loop polymorphisms have not been sorted completely. One of the Magadan individuals in Malyarchuk et al. (2010) had a sequence identical to the Hokkaido sable. Consequently, anthropogenic introduction might have also caused the non-monophyly of the sables on Hokkaido. The haplotype compositions of the sable, i.e. nuclear *Mc1r* haplotypes 1–8 (haplogroup I) and *Tcf25* haplotypes 1–5, differed between Russian and Hokkaido populations, supporting some endemism of the Hokkaido sable population. It seems likely that the endemism has largely been influenced by genetic drift on the isolated island.

When did the sable originate on Hokkaido? Using the genetic recombination rate, it is possible to estimate when hybridisation occurred between divergent haplotype blocks. Usually, a haplotype block in the genome collapses via recombination every generation in the same population (Koopman et al. 2007). Consequently, the number of recombinations is correlated with the elapsed number of generations. Therefore, the recombination rate between the *Mc1r* and *Tcf25* genes would provide chronological information on the hybridisation between the sable and eastern marten lineages on Hokkaido. We calculated the time of hybridisation using the method of Stephens et al. (1998) and Koopman et al. (2007) with the formula $P=(1-r)^{G}$, where P is the proportion of unchanged haplotype blocks (non-recombinants), r is the recombination rate inferred from the
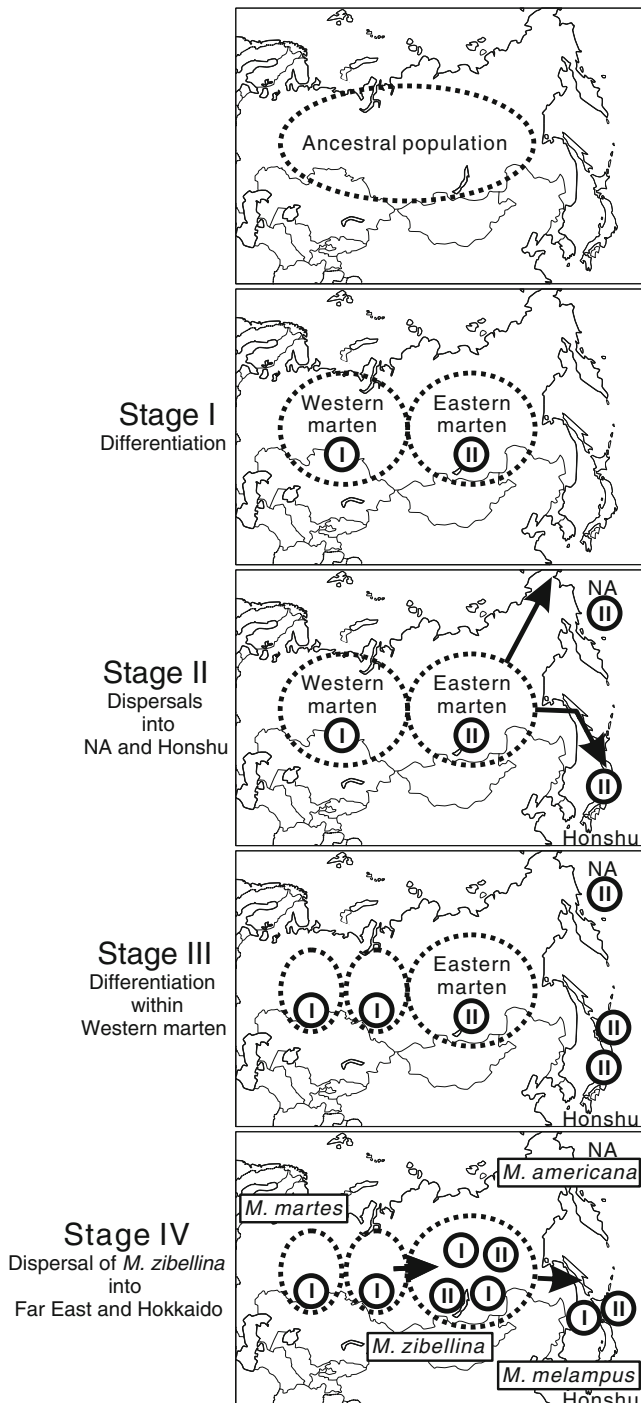
physical length between genomic regions (the recombination rate was assumed to be the same across generations) and G is the generation time. Of eight chromosomes assigned haplogroup II in the *Mc1r* gene, six have *Tcf25* haplotype 6, constituting haploblock II (Table 2). The remaining two haplotypes observed in individual TH43 were recombinants between haploblocks I and II (Table 2). Therefore, we used $P=0.75$ (6/8). The recombination rate is correlated with the physical distance in the genome. However, no linkage map has been reported for *M. zibellina*. Thus, we extrapolated the value of r (cM/100) using the overall recombination rate reported for humans, mice, and rats (about 0.5–1.2 cM/Mb; Jensen-Seaman et al. 2004). We applied values of r ranging from 0.000025 [=0.005 (cM/100)×5,000 (bp)/1,000,000 (bp)] to 0.00006 (=0.012× 5,000/1,000,000) based on the physical distance between the *Mc1r* and *Tcf25* genes (about 5 kb). Incorporating those values into the above formula, we obtained $G=4,795$– 11,507. The generation time of the sable is difficult to estimate. Mead (1994) and Monakhov (2011) suggested that the approximate first mating of the sable was at 2–4 years of age, which we assumed as the generation time. Therefore, we calculated that hybridisation between the sable and eastern marten occurred 9,589–46,028 years before present. The estimated age implies that the hybridisation was caused by the migration of sable from the continent during the last glacial period (about 10,000–70,000 years before present). However, the age is not consistent with the migration time inferred in other studies, e.g., about 100,000 years ago in Sato et al. (2011) and about 80,000 years ago in McKay (2011). Therefore, migration events might have occurred repeatedly in the late Pleistocene, which concurs with the idea that multiple colonisation events formed the Hokkaido fauna, as predicted from studies of the brown bear (Matsuhashi et al. 1999, 2001) and red fox (Inoue et al. 2007). An alternative explanation of the difference in those estimates is that the sable generation time is longer than 2– 4 years. There are some records of reproduction by individuals of *M. americana* more than 10 years old (Nowak 1999; Mead 1994). The last possibility is that recombination might have been suppressed under selective pressures favouring haploblock II on Hokkaido. Further analyses with more samples and genomic regions are necessary to trace the origin of the sable on Hokkaido. Such studies will contribute to our understanding of the faunal development on Hokkaido.

Yellow coat colour phenotype observed on Hokkaido

A small proportion of Hokkaido sables have an entirely bright yellow coat in winter, while others have a light yellow or dark grey coat (Hosoda et al. 2005). Hosoda et al. (2005) examined half of the *Mc1r* gene and suggested that a mutation at site 104 (G104T) was associated with the completely yellowish pelage. In this study, we examined the same single nucleotide polymorphism (SNP) for individuals collected on Hokkaido. In addition to the yellow specimen (TH47) used in Hosoda et al. (2005), we examined a specimen with a similar yellow pelage (HS4823) and other non-yellow sables. The G104T mutation was detected in HS4823 and not in the individuals with non-yellow coats. Therefore, it is very probable that this mutation is associated with the unusual yellow phenotype of the sable on Hokkaido. Here, we assume that the observed SNP is a variant within the sable, rather than the ancestral population that persisted on Hokkaido. This is because haplotype 5 retained by individual TH47 was included in haplogroup I (Fig. 3) as were the four haplotypes theoretically possible in individual HS4823 (data not shown). Because individual HS4823 has the mutation G104T in a heterozygous state, it likely functions as a dominant gene. This result is consistent with Hosoda et al. (2005), although it is based on only two individuals. Therefore, it might be premature to discuss any evolutionary meaning of this phenotype. The pattern of *Mc1r* polymorphisms was also remarkable, as most of the detected mutations were non-synonymous. Sables might have experienced special evolutionary forces acting on this gene related to coat colour on Hokkaido.

Conclusion

The origin of the sable on Hokkaido is complex. The sable genome contains an impression of both ancient and recent migrations of lineages from the continent. Such migrations would have exploited land bridges that formed during the Pleistocene glacial–interglacial cycles. This led to inter- or intra-specific hybridisation on Hokkaido. Ultimately old and young genealogies have been maintained in the sable population on Hokkaido. Further studies would shed more light on the concealed, uncharacterised evolutionary history of Hokkaido sables, probably associated with Quaternary global climatic and sea level changes.

References

Anderson E (1994) Evolution, prehistoric distribution, and systematics of *Martes*. In: Burkirk SW, Harestad AS, Raphael MG, Powell

RA (eds) Martens, sables, and fishers: biology and conservation. Cornell University Press, Ithaca, pp 13–25

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

Bandelt H-J, Foster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc 58:247–276

Hewitt GM (1999) Post-glacial re-colonization of European biota. Biol J Linn Soc 68:87–112

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

Hewitt GM (2001) Speciation, hybrid zones and phylogeography—or seeing genes in space and time. Mol Ecol 10:537–549

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. Phil Trans R Soc Lond B 359:183–195

Hewitt GM (2011) Quaternary phylogeography: the roots of hybrid zones. Genetica 139:617–638

Hosoda T, Suzuki H, Iwasa MA, Hayashida M, Watanabe S, Tatara M, Tsushiya K (1999) Genetic relationships within and between the Japanese marten *Martes melampus* and the sable *Martes zibellina*, based on variation of mitochondrial DNA and nuclear ribosomal DNA. Mamm Stud 24:25–33

Hosoda T, Suzuki H, Harada M, Tsuchiya K, Han S-H, Zhang Y-P, Kryukov AP, Lin L-K (2000) Evolutionary trends of the mitochondrial lineage differentiation in species of genera *Martes* and *Mustela*. Genes Genet Syst 75:233–243

Hosoda T, Sato JJ, Shimada T, Campbell KL, Suzuki H (2005) Independent non-frameshift deletions in the MC1R gene are not associated with melanistic coat coloration in three mustelid lineages. J Hered 95:607–623

Hosoda T, Sato JJ, Lin L-K, Chen Y-J, Harada M, Suzuki H (2011) Phylogenetic history of mustelid fauna in Taiwan inferred from mitochondrial genetic loci. Can J Zool 89:559–569

Inoue T, Nonaka N, Mizuno A, Morishima Y, Sato H, Katakura K, Oku Y (2007) Mitochondrial DNA phylogeography of the red fox (*Vulpes vulpes*) in northern Japan. Zool Sci 24:1178–1186

Inoue T, Murakami T, Abramov AV, Masuda R (2010) Mitochondrial DNA control region variations in the sable *Martes zibellina* of Hokkaido Island and the Eurasian continent, compared with the Japanese marten *M. melampus*. Mamm Stud 35:145–155

Inukai T (1975) Animals in Northernmost Japan (Hoppou Doubutsushi). Hokuensha, Japan, in Japanese

Jensen-Seaman M, Furey T, Payseur B, Lu Y, Roskin KM, Chen CF, Thomas MA, Haussler D, Jacob HJ (2004) Comparative recombination rates in the rat, mouse and human genomes. Genome Res 14:528–538

Kawamura Y (2007) Last glacial and Holocene land mammals of the Japanese islands: their fauna, extinction and immigration. Quat Res 46:171–177

Koepfli K-P, Deere KA, Slater GJ, Begg C, Begg K, Grassman L, Lucherini M, Veron G, Wayne RK (2008) Multigene phylogeny of the Mustelidae: resolving relationships, tempo and biogeographic history of a mammalian adaptive radiation. BMC Biol 6:1–22

Koopman WJM, Li Y, Coart E, van de Weg WE, Vosman B, Roldán-Ruiz I, Smulders MJ (2007) Linked vs. unlinked markers: multilocus microsatellite haplotype-sharing as a tool to estimate gene flow and introgression. Mol Ecol 16:243–256

Kurose N, Masuda R, Siriaroonrat B, Yoshida MC (1999) Intraspecific variation of mitochondrial cytochrome *b* gene sequences of the Japanese marten *Martes melampus* and the Sable *Martes zibellina* (Mustelidae, Carnivora, Mammalia) in Japan. Zool Sci 16:693–700

Li B, Xu YC, Ma Y, Elmeros M, Lan TM, Bai SY (2011) A PCR-RFLP based method to distinguish Sable (*Martes zibellina*) and Pine marten (*M. martes*). Acta Theriol 56:283–288

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Malyarchuk BA, Petrovskaya AV, Derenko MV (2010) Intraspecific structure of sable *Martes zibellina* L. inferred from nucleotide variation of the mitochondrial DNA cytochrome *b* gene. Russ J Genet 46:64–68

Masuda R (2009) *Martes melampus* (Wagner, 1840). In: Ohdachi SD, Ishibashi Y, Iwasa MA, Saitoh T (eds) The wild mammals of Japan. Shokadoh, Kyoto, pp 250–251

Matsuhashi T, Masuda R, Mano T, Yoshida MC (1999) Microevolution of the mitochondrial DNA control region in the Japanese brown bear (*Ursus arctos*) population. Mol Biol Evol 16:676–684

Matsuhashi T, Masuda R, Mano T, Murata K, Aiurzaniin A (2001) Phylogenetic relationships among worldwide populations of the brown bear *Ursus arctos*. Zool Sci 18:1137–1143

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

Mead RA (1994) Reproduction in *Martes*. In: Buskirk SW, Harestad AS, Raphael MG, Powell RA (eds) Martens, sables, and fishers: Biology and conservation. Cornell University Press, Ithaca, pp 404–422

Monakhov VG (2011) *Martes zibellina* (Carnivora: Mustelidae). Mamm Species 43:75–86

Murakami T (2009) *Martes zibellina* (Linnaeus, 1758). In: Ohdachi SD, Ishibashi Y, Iwasa MA, Saitoh T (eds) The wild mammals of Japan. Shokadoh, Kyoto, pp 196–197

Murakami T, Ohtaishi N (2000) Current distribution of the endemic sable and introduced Japanese marten in Hokkaido. Mamm Stud 25:149–152

Murakami T, Asano M, Ohtaishi N (2004) Mitochondrial DNA variation in the Japanese marten *Martes melampus* and Japanese sable *Martes zibellina*. Jpn J Vet Res 51:135–142

Nowak RM (1999) Walker's mammals of the world, vol 2, 6th edn. The Johns Hopkins University Press, Baltimore

Nunome M, Ishimori C, Aplin KP, Tsuchiya K, Yonekawa H, Moriwaki K, Suzuki H (2010) Detection of recombinant haplotypes in wild mice (*Mus musculus*) provides new insights into the origin of Japanese mice. Mol Ecol 19:2474–2489

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol 53:793–808

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Rissler LJ, Smith WH (2010) Mapping amphibian contact zones and phylogeographical break hotspots across the United States. Mol Ecol 19:5404–5416

Rozhnov VV, Meschersky IG, Pishchulina SL, Simakin LV (2010) Genetic analysis of sable (*Martes zibellina*) and pine marten (*M. martes*) populations in sympatric part of distribution area in the northern Urals. Russ J Genet 46:488–492

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Hapbor

Sato JJ, Yasuda SP, Hosoda T (2009) Genetic diversity of the Japanese marten (*Martes melampus*) and its implications for the conservation unit. Zool Sci 26:457–466

Sato JJ, Hosoda T, Kryukov AP, Kartavtseva IV, Suzuki H (2011) Genetic diversity of the sable (*Martes zibellina*, Mustelidae) in Russian Far East and Hokkaido. Mamm Stud 36:209–222

Sato JJ, Wolsan M, Prevosti FJ, Delia G, Begg C, Begg K, Hosoda T, Campbell KL, Suzuki H (2012) Evolutionary and Biogeographic

history of weasel-like carnivorans (Musteloidea). Mol Phylogenet Evol 63:745–757

Saitoh N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–25

Small MP, Stone KD, Cook JA (2003) American marten (*Martes americana*) in the Pacific Northwest: population differentiation across a landscape fragmented in time and space. Mol Ecol 12:89–103

Soltis DE, Morris AB, McLachlan JS, Nanos PS, Soltis PS (2006) Comparative phylogeography of unglaciated eastern North America. Mol Ecol 15:4261–4293

Stephens M, Scheet P (2005) Accounting for decay of linkage disequilibrium in haplotype inference and missing-data imputation. Am J Hum Genet 76:449–462

Stephens JC, Reich DE, Goldstein DB, Shin HD, Smith MW, Carrington M, Winkler C, Huttley GA, Allikmets R, Schriml L, Gerrard B, Malasky M, Ramos MD, Morlot S, Tzetis M, Oddoux C, di Giovine FS, Nasioulas G, Chandler D, Aseev M, Hanson M, Kalaydjieva L, Glavac D, Gasparini P, Kanavakis E, Claustres M, Kambouris M, Ostrer H, Duff G, Baranov V, Sibul H, Metspalu A, Goldman D, Martin N, Duffy D, Schmidtke J, Estivill Z, O'Brien SJ, Dean M (1998) Dating the origin of the *CCR5-Δ32* AIDS-resistance allele by the coalescence of haplotypes. Am J Hum Genet 62:1507–1515

Stephens M, Smith N, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68:978–989

Sugimoto T, Miyoshi K, Sakata D, Nomoto K, Higashi S (2009) Fecal DNA-based discrimination between indigenous *Martes zibellina* and non-indigenous *Martes melampus* in Hokkaido, Japan. Mamm Stud 34:155–159

Suzuki H (2009) A molecular phylogenetic view of mammals in the "three-story museum" of Hokkaido, Honshu, and Ryukyu Islands, Japan. In: Ohdachi SD, Ishibashi Y, Iwasa MA, Saitoh T (eds) The wild mammals of Japan. Shokadoh, Kyoto, pp 261–263

Swenson NG (2010) Mapping the suturing of a continental biota. Mol Ecol 19:5324–5327

Swenson NG, Howard DJ (2004) Do suture zones exist? Evolution 58:2391–2397

Swenson NG, Howard DJ (2005) Clustering of contact zones, hybrid zones, and phylogeographic breaks in North America. Am Nat 166:581–591

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland

Wolsan M, Sato JJ (2010) Effects of data incompleteness on the relative performance of parsimony and Bayesian approaches in a supermatrix phylogenetic reconstruction of Mustelidae and Procyonidae (Carnivora). Cladistics 26:168–194

Wozencraft WC (2005) Order Carnivora. In: Wilson DE, Reeder DM (eds) Mammal species of the world: a taxonomic and geographic reference, vol 1, 3rd edn. Johns Hopkins University Press, Baltimore, pp 532–628