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Molecular phylogeography of the Japanese weasel, *Mustela itatsi* (Carnivora: Mustelidae), endemic to the Japanese islands, revealed by mitochondrial DNA analysis

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To investigate the process of the population divergence of mammalian species endemic to continental islands, we studied the phylogeography of the Japanese weasel, *Mustela itatsi*, compared with its closely related continental species, the Siberian weasel *M. sibirica*, using mitochondrial DNA control region sequences. *Mustela itatsi* is endemic to the main Japanese islands (Honshu, Shikoku and Kyushu Islands), except Hokkaido Island, whereas *M. sibirica* is widespread in eastern Asia, southern Siberia, Taiwan and Tsushima Island. Fifty individuals of *M. itatsi* collected from 19 localities in Japan were examined. For *M. sibirica*, 27 individuals were analysed: 12 specimens from five localities within native habitats and 15 individuals (from the population introduced to Japan) from eight localities in western Japan. We identified 32 haplotypes for *M. itatsi*, which were clustered into two main clades (Honshu and Kyushu–Shikoku clades), whereas there were 11 haplotypes for *M. sibirica*, all of which were clustered into one clade. The grade of genetic differentiation within each clade of *M. itatsi* was similar to each other and to that of *M. sibirica* from samples distributed widely across northern Eurasia. The two clades in *M. itatsi* could have been established as a result of alternative zoogeographical events: geographical isolation of Honshu and Kyushu–Shikoku Islands or independent migration of the two lineages from the continent to Japan at different times. The molecular phylogeographical and demographic analyses indicated that the population of *M. itatsi* of Honshu Island expanded more recently than those of Kyushu and Shikoku Islands, which could have been refugia in the middle Pleistocene. In addition, the genetic differentiation and population expansion of *M. itatsi* on the Japanese islands could have occurred earlier than for other mammalian species endemic to Japan. However, the phylogeographical results for *M. sibirica* showed much less genetic variation through Eurasia, and that the introduced population in western Japan originated from a small founder population from the Korean Peninsula.

ADDITIONAL KEYWORDS: control region – population expansion – refugia.

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INTRODUCTION

Endemic species distributed on continental islands are a natural experiment for studies of genetic differentiation and speciation owing to dispersion and geographical isolation, compared with closely related continental species. Geographical isolation within islands could have enabled the descendant ancestral immigrants to genetically differentiate from the original populations on the continent. In Asia, the isolation process has produced a higher diversity of endemic mammalian species on the Japanese islands (Millien-Parra & Jaeger, 1999). Of 117 mammalian species, excluding orders Cetacia and Sirenia and introduced species, 49 native species (about 42%) are endemic to Japan (Motokawa, 2009). Among eight species of the family Mustelidae (order Carnivora) occurring in Japan, four species are endemic: the Japanese weasel *Mustela itatsi*, Japanese marten *Martes melampus*, Japanese badger *Meles anakuma* and Japanese otter *Lutra nippon* (almost extinct). The species most closely related to these endemic mustelids are distributed on the Asian continent. Comparative phylogeographical studies between endemic Japanese species and related continental species contribute to further our understanding of the processes of the differentiation and evolution of species.

The Japanese weasel is a medium-sized mustelid species endemic to the three main islands of Japan (Honshu, Kyushu and Shikoku) and adjacent small islands (Yakushima, Tanegashima and Ohshima), excluding Hokkaido Island (Fig. 1). This weasel was first described as a distinct species *Mustela itatsi* Temminck, 1884; however, *itatsi* was subsequently considered to be a subspecies of the Siberian weasel *Mustela sibirica* Pallas, 1773, which is widespread in eastern Asian and Siberia (Corbet & Hill, 1991; Wozencraft, 1993; Masuda & Watanabe, 2009; Sasaki, 2009) (Fig. 1). Few data on the speciation and biogeography of *M. itatsi* throughout the Japanese islands are known. From the middle and late Pleistocene layers on Honshu, fossils of *Mustela* spp. have been recorded, although it is unclear whether they are

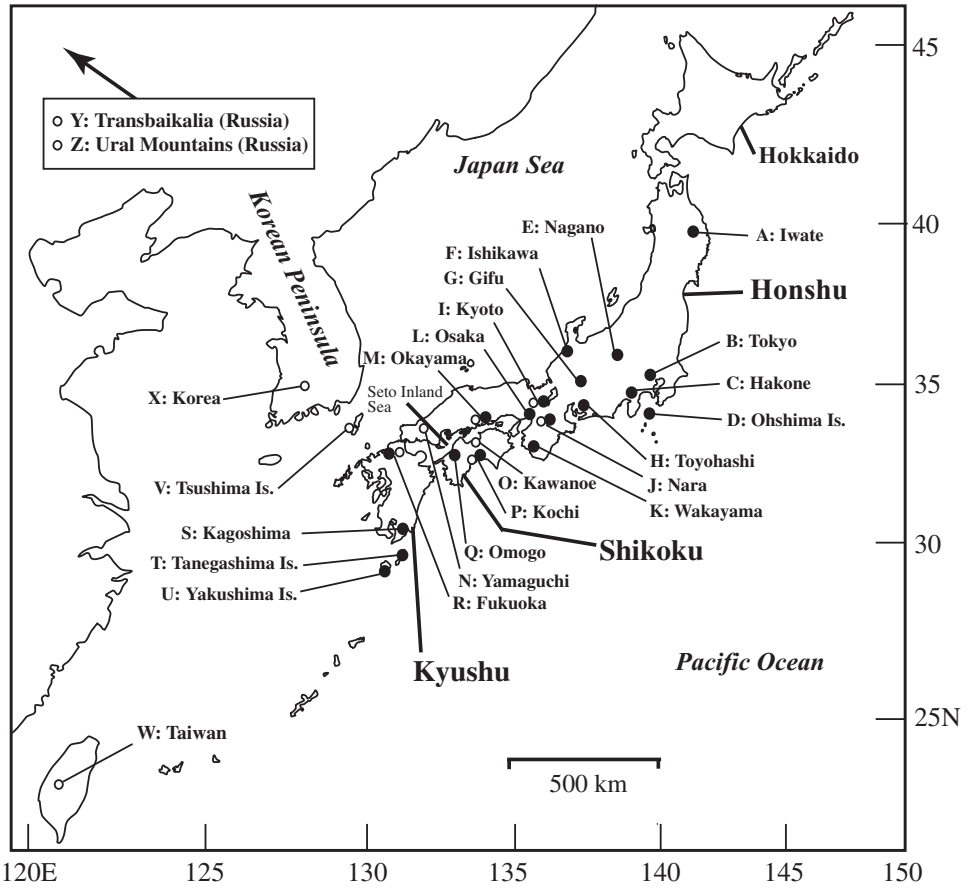

Figure 1. Sampling localities of *Mustela itatsi* and *M. sibirica* around the Japanese islands, Taiwan and the Eurasian Continent: filled circles, *M. itatsi*; open circles, *M. sibirica*.

of *itatsi*, *sibirica* or others (Kawamura, Kamei & Taruno, 1989; Masuda & Watanabe, 2009).

Against such a background, the phylogenetic relationships among several Asian *Mustela* species were studied using mitochondrial DNA (mtDNA) cytochrome *b* (Masuda & Yoshida, 1994a; Kurose, Abramov & Masuda, 2000a) and 12S rRNA (Kurose, Abramov & Masuda, 2008) sequences, and it was revealed that there is a relatively large genetic distance between *M. itatsi* and *M. sibirica*, although both weasel species are still phylogenetically more closely related to each other than to other *Mustela* species. Masuda & Yoshida (1994b) calculated the divergence time to be about 1.6–1.7 million years ago (Mya) based on partial cytochrome *b* sequence differences between the two species. Sato *et al*. (2003) investigated the molecular phylogeny of many species of Mustelidae, and reported that the divergence time was 1.7 Mya from the whole sequence of cytochrome *b* data and 2.4 Mya from nuclear interphotoreceptor retinoid binding protein (*IRBP*) data. These divergence dates correspond to the beginning of or just before the Pleistocene. In addition, comparative chromosome analysis showed clear karyotypic differences between *M. itatsi* and *M. sibirica*, whereas the chromosome numbers ($2n = 38$) were identical (Kurose *et al*., 2000b).

Certain differences in external morphology between the two weasel taxa have been reported: the ratio of the tail (T) length to the head and body (HB) length (T/HB) of *sibirica* is more than 50%, whereas that of *itatsi* is around 40% (Imaizumi, 1960). The body of *itatsi* is smaller than that of *sibirica*, and the coat colour of *itatsi* is more dark brown than that of *sibirica* (Masuda & Watanabe, 2009; Sasaki, 2009). Abramov (2000a, b) studied the morphotypic characteristics of the skull, body size, coloration and baculum, and positioned *itatsi* as an independent species in the phylogeny of *Mustela*. Recently, Suzuki, Abe & Motokawa (2011) reported interspecific and sexual differences in the skulls between the two species. Thus, both morphological and genetic data strongly support the first description that *itatsi* is a distinct species.

Among the Japanese islands, *M. sibirica* is indigenous only on Tsushima Island, which is located between Japan and the Korean Peninsula (Fig. 1) (Imaizumi, 1960; Abe *et al*., 1994; Sasaki, 2009). Some other mammals from Tsushima Island are common to the Asian continent: for example, the leopard cat, *Prionailurus bengalensis*, and the Asian lesser white-toothed shrew, *Crocidura shantungensis* (Abe & Ishii, 1987). Meanwhile, *M. sibirica* has been reported to have been introduced from the Korean Peninsula to Kyushu and Honshu in two ways: escape or release from fur farms located in western Honshu in the 1930s, or direct introduction from Korea to Kyushu from ships (Miyashita, 1963, 1976). After introduction to Japan, *M. sibirica* adapted to the Japanese environment, mostly in areas around human habitations, such as villages and cities, and expanded its distribution from western to central Japan. At present, *M. sibirica* is much more dominant in Kyushu, Shikoku and western Honshu, and the westernmost limit to its distribution has been reported to be around Gifu Prefecture, central Honshu (Ando, 1989) (Fig. 1). In western Japan, native populations of *M. itatsi* are currently observed in relatively mountainous areas, possibly as a result of *M. sibirica*'s invasion to the lowland habitats of native *M. itatsi*. Because both species are phylogenetically closely related within the genus *Mustela* (Masuda & Yoshida, 1994a; Kurose *et al*., 2000a, 2008; Sato *et al*., 2003) and because they share identical chromosome numbers (Kurose *et al*., 2000b), hybridization between them is possible, as reported in combinations of other *Mustela* species (Ternovsky, 1977; Ternovsky & Ternovskaya, 1994).

In the present study, we investigated the greatest degree of genetic variation in mtDNA genes by determining sequences from the control region, from *M. itatsi* collected from the Japanese islands and from *M. sibirica* collected from its widespread geographical distribution and the introduced Japanese population, and examined the phylogeography of the two species. We discuss the divergence and migration history of *M. itatsi* on the Japanese islands, compared with the closely related continental species, including both native and introduced populations.

MATERIAL AND METHODS

Animals and DNA extraction

Specimens of the two weasel species examined in the present study were obtained from 1984 to 2001, and the sampling localities and numbers of the animals are shown in Figure 1 and Tables 1 and 2. The T/HB value of each weasel collected in Japan was calculated to separate *M. itatsi* from *M. sibirica* according to Imaizumi's (1960) classification (more than 50% for *M. sibirica* and less than 50% for *M. itatsi*), and body size and pelage coloration were also recorded.

Tissue samples, including muscle, stomach, kidney and lung, were frozen at –80 °C or preserved in 70% ethanol until use. Total DNA was extracted according to the phenol/proteinase K/sodium dodecylsulphate (SDS) method of Sambrook, Fritsch & Maniatis (1989) with some simplified modifications (Masuda & Yoshida, 1994a). In brief, a small piece (about $2 \times 2 \times 2$ mm^3) of each tissue was homogenized with 500 μL of STE buffer [0.1 M NaCl/10 mM Tris/1 mM

Table 1. Mitochondrial DNA (mtDNA) haplotypes and sampling localities of *Mustela itatsi* examined in the present study

Haplotype	No. of animals (code and locality)*	Accession no.†
IT-01	1 (A: Iwate)	AB007328
IT-02	3 (A: Iwate)	AB007326
IT-03	1 (B: Tokyo)	AB007341
IT-04	1 (B: Tokyo); 1 (C: Hakone)	AB007342
IT-05	4 (D: Ohshima Is.)	AB007339
IT-06	2 (C: Hakone)	AB010278
IT-07	1 (C: Hakone)	AB010279
IT-08	2 (E: Nagano)	AB007344
IT-09	1 (F: Ishikawa)	AB007352
IT-10	1 (F: Ishikawa)	AB007324
IT-11	1 (F: Ishikawa); 1 (G: Gifu)	AB007325
IT-12	2 (G: Gifu)	AB007331
IT-13	1 (G: Gifu)	AB007332
IT-14	1 (H: Toyohashi)	AB007345
IT-15	2 (H: Toyohashi)	AB007349
IT-16	1 (I: Kyoto)	AB007335
IT-17	1 (I: Kyoto)	AB007327
IT-18	1 (I: Kyoto); 2 (L: Osaka)	AB007330
IT-19	2 (J: Nara)	AB007329
IT-20	1 (J: Nara)	AB007336
IT-21	2 (K: Wakayama)	AB007350
IT-22	1 (M: Okayama)	AB007337
IT-23	1 (M: Okayama)	AB007338
IT-24	2 (P: Kochi)	AB007347
IT-25	1 (P: Kochi)	AB007333
IT-26	2 (Q: Omogo)	AB007353
IT-27	1 (R: Fukuoka)	AB007334
IT-28	1 (S: Kagoshima)	AB007340
IT-29	1 (S: Kagoshima)	AB007348
IT-30	1 (T: Tanegashima Is.)	AB007343
IT-31	2 (U: Yakushima Is.)	AB007351
IT-32	1 (U: Yakushima Is.)	AB007346

*Numbers before parentheses indicate numbers of weasels sharing that haplotype. Letters and localities in parentheses correspond to those in Figure 1.
†These sequence data will appear in the DDBJ/GenBank/EMBL nucleotide databases with the accession numbers.

Table 2. Mitochondrial DNA (mtDNA) haplotypes and sampling localities of *Mustela sibirica* examined in the present study

Haplotype	No. of animals (code and locality)*	Accession no.†
SB-01	3 (I: Kyoto); 1 (J: Nara); 1 (M: Okayama); 1 (N: Yamaguchi); 2 (O: Kawanoe); 2 (P: Kochi); 4 (R: Fukuoka)	AB007356
SB-02	1 (R: Fukuoka)	AB007355
SB-03	1 (V: Tsushima)	AB007357
SB-04	1 (V: Tsushima)	AB007359
SB-05	1 (V: Tsushima)	AB007360
SB-06	1 (X: Korea)	AB007354
SB-07	1 (X: Korea)	AB007358
SB-08	4 (Z: Ural Mountains, Russia)	AB113197
SB-09	1 (Y: Transbaikalia, Russia)	AB113198
SB-10	1 (Y: Transbaikalia, Russia)	AB113199
SB-11	1 (W: Taiwan)	AB113200

*Numbers before parentheses indicate numbers of weasels sharing that haplotype. Letters and localities in parentheses correspond to those in Figure 1.
†These sequence data will appear in the DDBJ/GenBank/EMBL nucleotide databases with the accession numbers.

ethylenediaminetetraacetic acid (EDTA)] containing a final concentration of 0.5% SDS and 5 μg mL^{-1} of proteinase K. After incubation at 37 °C overnight, the homogenate was extracted two or three times with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and once with chloroform/isoamyl alcohol (24 : 1). Hair roots from two individuals were washed with 70% ethanol, incubated in 5% Chelex-100 (Bio-Rad) at 56 °C overnight, and boiled for 8 min (Walsh, Metzger & Higuchi, 1991). One microlitre of the tissue extract or 10 μL of the hair extract was used as template for the following polymerase chain reaction (PCR) amplification.

PCR AMPLIFICATION AND DIRECT SEQUENCING

The entire sequence of the mtDNA control region (about 1000 base pairs, bp) was amplified using two primers, Cb-Z and D4 (Kurose, Masuda & Yoshida, 1999b). As a result of direct sequencing of the PCR product, tandem repeats of 10-bp motifs were found at the 3′ end; however, it was impossible to determine the entire repetitive regions for possible heteroplasmy. Meanwhile, because the 5′ end region of about 600 bp was found to be readable and informative for the reconstruction of the phylogenetic relationships among weasels, the sequences were used for analysis.

The PCR amplification was performed in a total volume of 50 μL of the reaction mixture. If the PCR was inhibited for some reason, 20 μg of bovine serum albumin (Roche) was added to the reaction mixture. The PCRs were repeated 35 times using a DNA thermal cycler (PJ2000, Perkin-Elmer Cetus) as follows: denaturation at 94 °C for 1 min; annealing at 57 °C for 1 min; extension at 72 °C for 2 min; and completion of the reaction at 72 °C for 10 min. To check PCR amplification, an aliquot of 10 μL from the PCR product was electrophoresed on a 2% agarose

gel, stained by ethidium bromide and visualized under an ultraviolet illuminator. The remaining 40 μL of the PCR product was purified using the QIAquick purification kit (Qiagen).

Purified PCR products were labelled using Catalyst (Perkin-Elmer Cetus) and sequenced using the ABI PrismTM 377 DNA sequencing system (Perkin-Elmer Cetus). Sequencing was performed using primers Cb-Z, DS1 and MSD (Kurose *et al*., 1999b).

Sequence analysis

Sequence alignment was performed with the computer software GeneWorks (Intelligenetics). Insertions or deletions (indels) were corrected after visual observation, and eliminated from the phylogenetic analysis. The mtDNA control region sequence of the least weasel *Mustela nivalis* (Accession no. AB006721: Kurose *et al*., 1999b) was used as an outgroup. MODELTEST 3.7 (Posada & Crandall, 1998) was employed to find the best nucleotide substitution model in PAUP 4.0 beta10 (Swofford, 2002). In MODELTEST, the Akaike information criterion selected the TIM (transition) + I + G model with the proportion of invariable sites (I) set at 0.6561 and the gamma distribution (G) at 0.7479. The maximum likelihood (ML) phylogenetic tree was constructed using this model in PAUP. In maximum parsimony (MP) analysis, all sites were treated as unordered and equally weighted, and the heuristic search option with random addition of sequences (100 replicates) and the tree bisection–reconnection (TBR) branch swapping algorithm was used for tree searching in PAUP. Neighbor-joining (NJ) (Saitou & Nei, 1987) tree construction was conducted in MEGA 5 (Tamura *et al*., 2011) using Kimura's (1980) two-parameter distances. To assess interbranching support, bootstrapping (Felsenstein, 1985) was performed with 200, 1000 and 1000 replicates in ML, MP and NJ methods, respectively. In addition, to examine the interspecific variation of *M. itatsi*, minimum spanning networks among mtDNA haplotypes were constructed using TCS 1.21 (Clement, Posada & Crandall, 2000).

Average numbers of haplotype diversity, nucleotide diversity (π: Nei, 1987) and polymorphic sites within species and populations were calculated using ARLEQUIN 3.5.1.2 (Excoffier & Lischer, 2010). To estimate divergence times between populations, raw (D_{xy}) and net (D_a) nucleotide divergences between species and between populations were calculated using DNASP 5.10.01 (Librado & Rozas, 2009). It is proposed that $D_a = 2mT$, where $2m$ is the divergence rate and T is the divergence time. The divergence rate ($2m$) of the *Mustela* control region was calculated using D_a values between *M. itatsi* and *M. sibirica* obtained in the present study, and 1.7 Mya (cytochrome *b* data: Masuda & Yoshida, 1994b; Sato *et al*., 2003) or 2.4 Mya (*IRBP* data: Sato *et al*., 2003) as the divergence time between the two species. The divergence times between populations were then estimated as $T = D_a/2m$.

Demographic history analysis

To test recent demographic expansion of *M. itatsi*, Tajima's D (Tajima, 1989), Fu's F_s (Fu, 1997) and mismatch distributions of species and populations were computed in ARLEQUIN. Population expansion shows large negative values of D and F_s under the assumption of neutrality. In mismatch distribution analysis, recent population expansion is considered to generate a unimodal distribution of pairwise sequence differences (Slatkin & Hudson, 1991; Rogers, 1995; Schneider & Excoffier, 1999). The parameter τ, calculated from mismatch distributions, is an estimate of the expansion time after major population reduction, and is expressed as $\tau = 2mlt$, where $2m$ is the divergence rate, as in the above calculation of the divergence time, l is the number of nucleotides of the analysed fragment and t is the time subsequent to expansion (Harpending *et al*., 1993; Marmi *et al*., 2006). The 95% confidence intervals for τ were calculated from 1000 bootstrap replicates in ARLEQUIN.

RESULTS

Morphological and mtDNA sequence differences between *M. itatsi* and *M. sibirica*

Weasels with > 50% T/HB values commonly had larger bodies and lighter brown coats, whereas those with < 50% T/HB values were relatively smaller with darker brown coats in both males and females. According to Imaizumi's (1960) classification, the former was classified as *M. sibirica* and the latter as *M. itatsi*. In northern Kyushu, Shikoku and western Honshu, 15 weasels were classified as *M. sibirica*, which were thought to be offspring of introduced individuals, and are called here the introduced Japanese population (Table 2, Fig. 1). As mentioned below, the two morphologically distinct taxa clearly corresponded to separate lineages in the phylogenetic tree constructed using the mtDNA control region sequences (Figs 2 and 3). There were no individuals with intermediate morphological characteristics.

Both *M. itatsi* and *M. sibirica* were found to have tandem repeats of 10-bp motifs (microsatellite-like sequences) at the 3′ end of the mtDNA control region (data not shown). The entire sequence of tandem repeats was not readable for possible heteroplasmy,

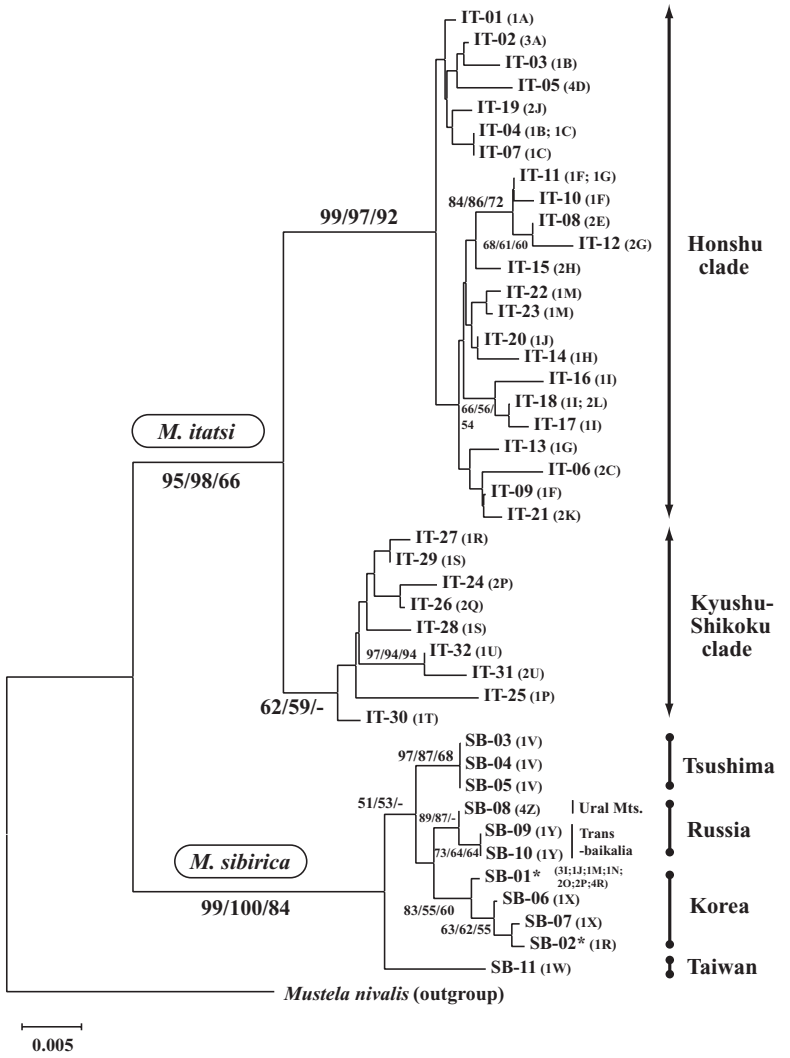

Figure 2. The neighbor-joining (NJ) phylogenetic tree (as a representative) of mitochondrial DNA (mtDNA) control region haplotypes of *Mustela itatsi* (IT-01 to IT-32) and *M. sibirica* (SB-01 to SB-11) using Kimura's two-parameter model. Numbers around internal branches are the bootstrap values (%) of NJ, maximum parsimony (MP) and maximum likelihood (ML) methods in order. Bootstrap values of less than 50% are shown by dashes around the main branches, or not shown around minor branches. Haplotype names are shown, together with the numbers of animals and locality codes in parentheses, referring to those in Figure 1 and Tables 1 and 2. Asterisks indicate haplotypes (SB-01 and SB-02) identified from the introduced Japanese populations of *M. sibirica*. The homologous part of the *M. nivalis* mtDNA control region (Accession no. AB006721) was used as an outgroup.

similar to the composition of the mtDNA control region sequences of other *Mustela* species, such as *M. nivalis* and the ermine *M. ermine*, as reported by Kurose *et al.*(1999b) and Kurose, Abramov & Masuda (2005). Hoelzel *et al.* (1994) reported that such tandem repeats are found commonly in the mtDNA control regions of several carnivoran species. In contrast, because the 5′ end sequences of about 600 bp were clearly determined and phylogenetically informative, they were used for the following sequence analysis.

Genetic variation and divergence within *M. itatsi*

The sequence alignment of *M. itatsi* showed that 53 sites were polymorphic among 596-bp sequences when including indels (Table 3). From the sample of 50 individuals of *M. itatsi*, 32 mtDNA haplotypes (IT-01 to IT-32) were identified. Nucleotide diversity and haplotype diversity within *M. itatsi* were 0.019 ± 0.010 (standard deviation, SD) and 0.980 ± 0.008, respectively (Table 3).

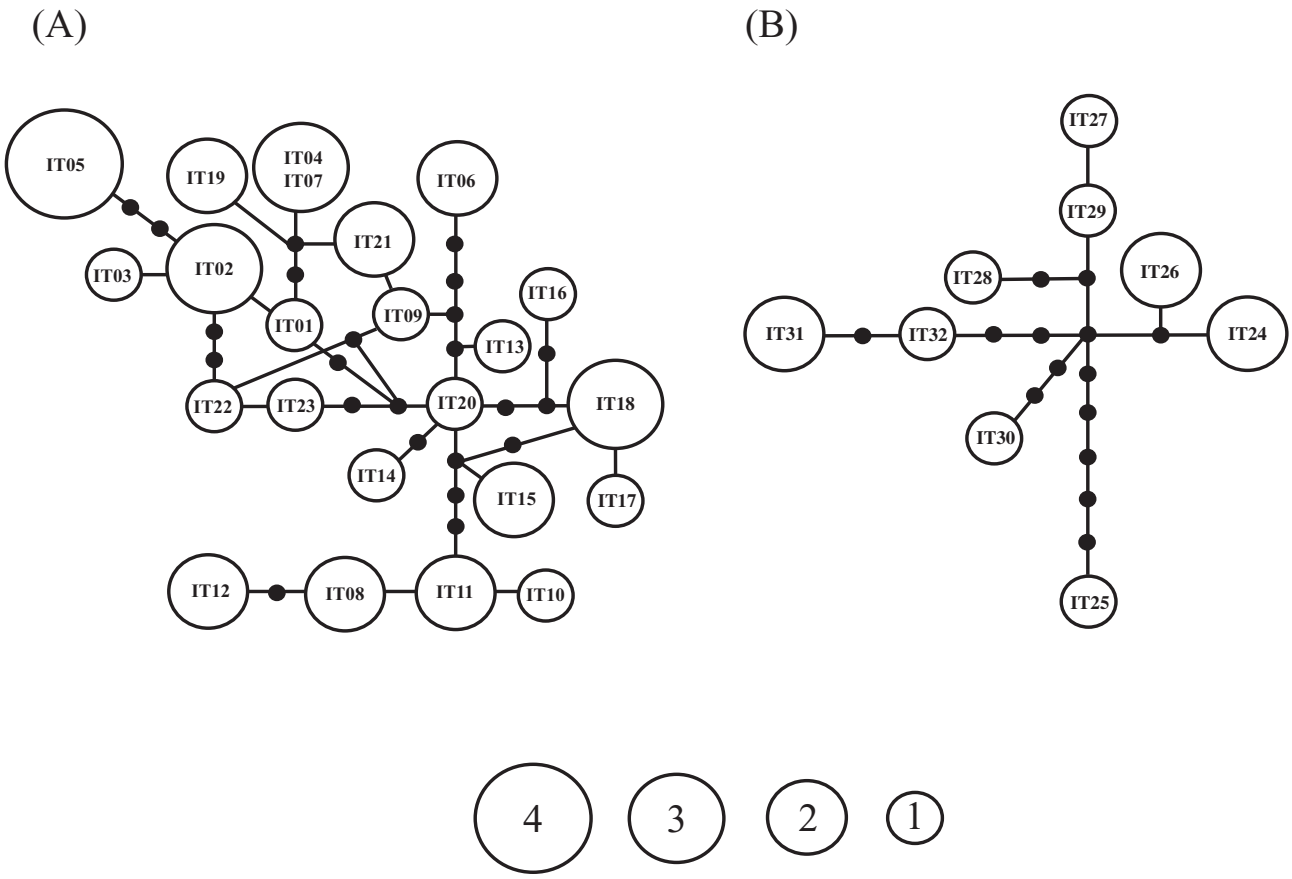

Figure 3. Minimum spanning networks among mitochondrial DNA (mtDNA) control region haplotypes of the Honshu population (A) and the Kyushu–Shikoku population (B) of *M. itatsi*. Numbers in circles show haplotype names. Circle sizes and numerals below indicate the numbers of animals. One bar between two circles represents one nucleotide substitution. Filled circles indicate missing haplotypes. Because the sequence of IT-04 is the same as that of IT-07 when indel sites are eliminated, both haplotypes are described in the common circle.

Table 3. Molecular diversity indices for the 596-bp mitochondrial DNA (mtDNA) control region fragment of *Mustela itatsi* and *M. sibirica*

Species	Population	Sample size	No. of haplotypes	Haplotype diversity (SD)	No. of polymorphic sites	Nucleotide diversity (SD)
M. itatsi	All	50	32	0.980 (0.008)	53	0.019 (0.010)
	Honshu	38	23	0.970 (0.012)	34	0.010 (0.006)
	Kyushu–Shikoku	12	9	0.955 (0.047)	26	0.010 (0.006)
M. sibirica	Native all	12	9	0.909 (0.080)	28	0.013 (0.007)
	Japan-introduced	15	2	0.133 (0.112)	2	0.001 (0.001)

SD, standard deviation.

Because the clustering of the main clades in phylogenetic trees was very similar among NJ, MP and ML methods, Figure 2 shows the NJ tree as a representative and bootstrap values around internal branches for NJ, MP and ML methods. All individuals of *M. itatsi* were clustered with 95/98/66% bootstrap values (Fig. 2). The cluster of *M. itatsi* was further divided into two lineages: the Honshu clade (including Oshima Island) with 99/97/92% bootstrap values, and the Kyushu–Shikoku clade (including Yakushima

Table 4. Mitochondrial DNA (mtDNA) divergences and estimated divergence times within *Mustela itatsi* populations and between *M. itatsi* and *M. sibirica*

	All *itatsi*	Honshu *itatsi*	Kyushu–Shikoku *itatsi*	Native *sibirica*
All *itatsi*		–	–	1.70–2.40
Honshu *itatsi*	–		0.83–1.17	2.01–2.84
Kyushu–Shikoku *itatsi*	–	0.0319		1.60–2.26
		0.0223		
Native *sibirica*	0.0577	0.0626	0.0513	
	0.0455	0.0539	0.0429	

Above the diagonal: estimated divergence times (Mya) based on the divergence rate of 0.0190–0.0268/nucleotide site/million years. Below the diagonal: raw (D_{xy}, top value) and net (D_a, bottom value) nucleotide divergences as substitutions/nucleotide site/million years.

Island and Tanegashima Island, which represent the southernmost part of the geographical distribution of *M. itatsi*, see Fig. 1) with 62/59/< 50% bootstrap values. Of the 50 specimens sharing 32 haplotypes (IT-01 to IT-32), 38 individuals with 23 haplotypes (IT-01 to IT-23) comprised the Honshu clade: the average values of nucleotide divergence and haplotype divergence within the Honshu clade were 0.010 ± 0.006 (SD) and 0.970 ± 0.012 (SD), respectively (Table 3). Meanwhile, the Kyushu–Shikoku clade was formed by 12 individuals with nine haplotypes (IT-24 to IT-32): the average values of nucleotide divergence and haplotype divergence within the Kyushu–Shikoku clade were 0.010 ± 0.006 (SD) and 0.955 ± 0.047 (SD), respectively (Table 3). These results indicate that the molecular diversity within the Honshu population is very similar to that within the Kyushu–Shikoku population.

Within each clade, haplotypes identified from geographically neighbouring localities were not always more phylogenetically closely related, and were clustered with low bootstrap values (Table 1, Fig. 2). In addition, minimum spanning networks of haplotypes (Fig. 3) showed a topology similar to that of Figure 2. The numbers of missing haplotypes between identified haplotypes were larger in the Kyushu–Shikoku population (Fig. 3B) than in the Honshu population (Fig. 3A). In the Honshu clade, some haplotypes from northern areas were closely related to those from western areas (Figs 2 and 3A). In the Kyushu–Shikoku clade, although Kyushu is an island separated from Shikoku, genetic distances did not always reflect geographical distances between sampling localities from the two islands (Figs 2 and 3B). Haplotypes from Yakushima Island (IT-31 and IT-32) and Tanegashima Island (IT-30), both of which are located south of Kyushu, were distantly related to others in the Kyushu–Shikoku clade (Fig. 3B).

Table 4 shows the nucleotide divergences between the two species and between populations of *M. itatsi*. The net nucleotide divergence (D_a) between *M. itatsi* and *M. sibirica* was 0.0455 substitutions/site. When the divergence times between the two species were set at 1.7 Mya (Masuda & Yoshida, 1994b; Sato *et al*., 2003) and 2.4 Mya (Sato *et al*., 2003), the divergence rates ($2m$) were calculated as 0.0268 and 0.0190 substititions/site/million years, respectively. Using these divergence rates and the D_a value between the Honshu and Kyushu–Shikoku populations (0.0223 substitutions/site), the divergence time between the two clades of *M. itatsi* was estimated as 0.83–1.17 Mya (Table 4).

Genetic variation within *M. sibirica*

Sequence alignment indicated that 19 sites were polymorphic among haplotypes (596 bp) of native *M. sibirica* (Table 3). From all 27 individuals of *M. sibirica*, 11 haplotypes (SB-01 to SB-11) were identified. Of the 11 haplotypes, nine were shared by 12 native individuals: the average values of nucleotide diversity and haplotype diversity were 0.013 ± 0.007 (SD) and 0.909 ± 0.080 (SD), respectively (Table 3).

Three haplotypes (SB-08, SE-09 and SE-10) identified from *M. sibirica* from Russia (Transbaikalia and the Ural Mountains) were clustered in NJ/MP/ML methods with bootstrap values of 89/87/< 50%, respectively (Fig. 2). Moreover, SB-09 and SB-10 from two individuals from Transbaikalia were closely related to each other, but remote from SB-08 in the Ural Mountains (Fig. 3).

Three haplotypes (SB-03, SE-04 and SE-05) identified from three weasels native to Tsushima Island were grouped with bootstrap values of 97/87/68%. Meanwhile, one haplotype (SB-11) from Taiwan was phylogenetically remote from the other haplotypes of *M. sibirica* (Fig. 2). These results indicate that indi-

viduals from Tsushima and Taiwan could be genetically differentiated from the continental populations of the Korean Peninsula and Russia. These data are concordant with the level of intraspecific morphological variation in *M. sibirica* reported by Abramov (2006).

Two haplotypes (SB-01 and SB-02) were found in 15 individuals of the introduced Japanese population of *M. sibirica*: SB-01 was shared by 14 individuals from various areas, including northern Kyushu, Shikoku and western Honshu, and SB-02 was obtained from one individual in northern Kyushu (Table 2, Fig. 1). Average values of nucleotide diversity and haplotype diversity in the introduced Japanese population were 0.001 ± 0.001 (SD) and 0.133 ± 0.112 (SD), respectively (Table 3). These results indicate a lack of molecular divergence in the introduced Japanese population of *M. sibirica*. In addition, the two haplotypes (SB-01 and SB-02) from the introduced Japanese population were clustered with SB-06 and SB-07, both of which originated from native Korean *M. sibirica*, showing bootstrap values of 83/55/60%. This close phylogenetic relationship indicates that the introduced Japanese population originated from Korean populations of *M. sibirica*.

Demographic history of *M. itatsi*

To test the recent demographic expansion in the Honshu and Shikoku–Kyushu populations of *M. itatsi*, Tajima's *D* and Fu's F_s were calculated (Table 5). In the Honshu population of *M. itatsi*, both *D* and F_s values were negative, and the latter ($F_s = -6.70$) was significant ($P = 0.01$). The Kyushu–Shikoku population showed negative values of *D* and F_s, both of which were not significant. Overall, in *M. itatsi*, F_s was negative and significant (Table 5).

The pattern of the mismatch distribution of pairwise sequence differences in the Honshu population of *M. itatsi* was nearly unimodal, although it included minor peaks (Fig. 4B). This suggests the recent expansion model. By contrast, the pattern in the Kyushu–Shikoku population of *M. itatsi* was not unimodal, but multimodal (Fig. 4C), indicating a rejection of the recent expansion model. Overall, *M. itatsi* showed a bimodal distribution (Fig. 4A), because it comprised two genetically different clades: Honshu and Kyushu–Shikoku populations.

In addition, the numbers of missing haplotypes between identified haplotypes were fewer in the Honshu population (1–4 types: Fig. 3A) than in the Kyushu–Shikoku population (1–8 types: Fig. 3B), supporting the more recent establishment of the population in Honshu. This might be attributed to the relatively smaller number of individuals sampled from the Kyushu–Shikoku population.

Based on τ parameters obtained from the mismatch distribution and the mutation rates of the mtDNA control region calculated in the present study (0.0190 and 0.0268 substitutions/site/million years), expansion times were estimated (Table 5). The expansion time of the Honshu population of *M. itatsi* was 0.54 or 0.77 Mya (range, 0.31–0.90 Mya), and corresponded to the middle Pleistocene. For the Kyushu–Shikoku population of *M. itatsi*, the expansion time was older than that of the Honshu population, 0.59 or 0.83 Mya (range, 0.38–1.07 Mya), although no clear evidence for recent expansion was shown by *D* and F_s values or mismatch distribution analysis in the Kyushu–Shikoku population.

Table 5. τ parameter obtained from mismatch distribution, estimated expansion times and values of Tajima's *D* and Fu's F_s on populations of *Mustela itatsi*

		τ			Tajima's *D*		Fu's F_s	
Population	Sample size	Estimate	95% confidence interval	Expansion times (Mya)*	*D*	*P*	F_s	*P*
All	50	5.25	2.15–21.55	0.46 (0.19–1.90) 0.33 (0.13–1.35)	0.15	0.66	–7.91	0.02†
Honshu	38	8.67	5.07–10.16	0.77 (0.44–0.90) 0.54 (0.31–0.64)	–0.33	0.41	–6.70	0.01†
Kyushu–Shikoku	12	9.42	6.03–12.16	0.83 (0.53–1.07) 0.59 (0.38–0.76)	–0.91	0.17	–1.13	0.26

*Expansion times (t) for τ parameters and the 95% confidence intervals in parentheses were calculated using the formula $\tau = mlt$, where l is the mtDNA fragment length (596 bp) and m is the mutation rate (0.0190 substitutions/site/million years for top value; 0.0268 substitutions/site/million years for bottom value).
†Statistically significant.

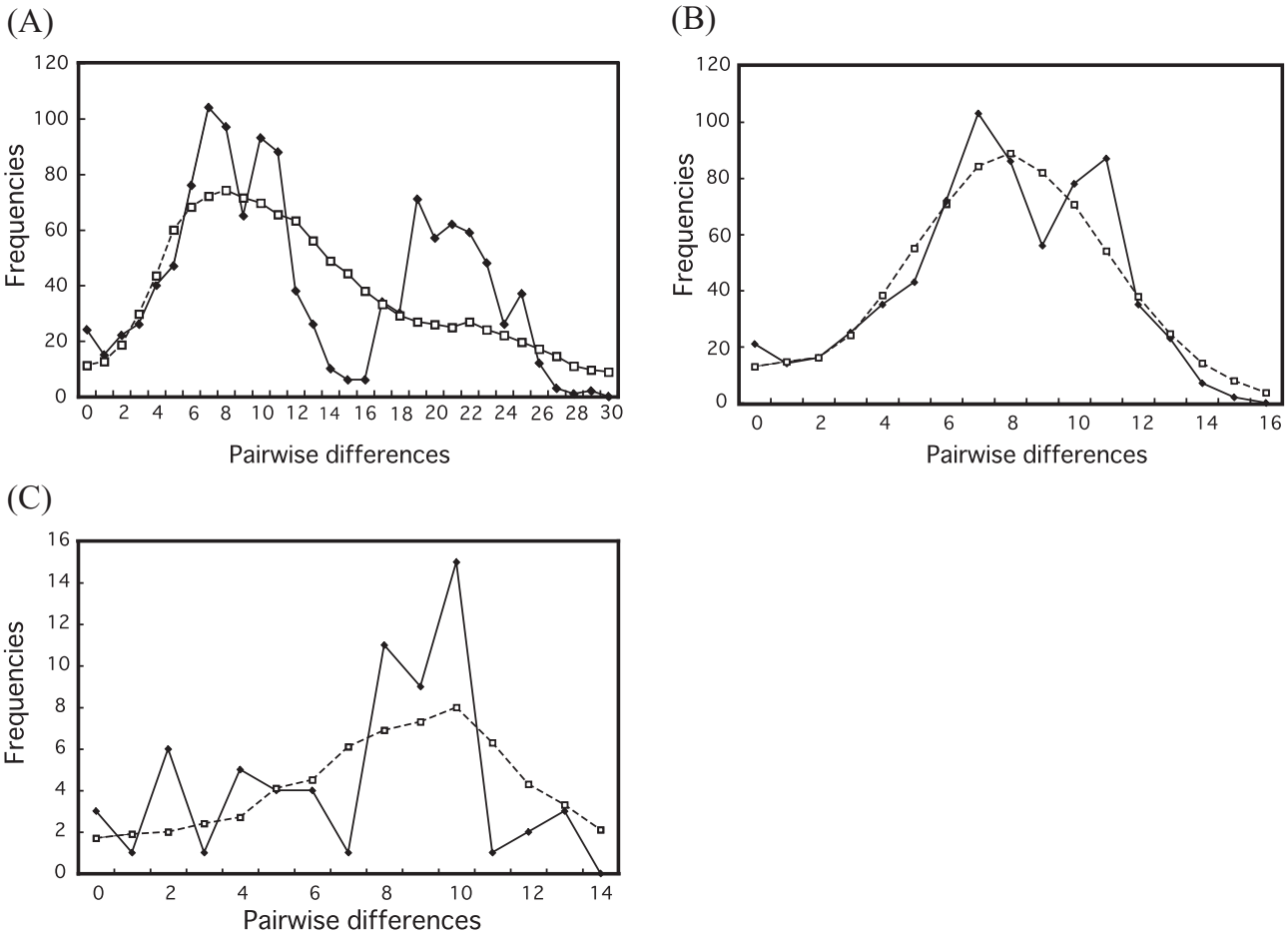

Figure 4. Mismatch distributions of pairwise differences of *Mustela itatsi* mitochondrial DNA (mtDNA) control region sequences from all individuals (A), the Honshu population (B) and the Kyushu–Shikoku population (C). Full lines and filled diamonds show the observed frequencies, and broken lines and open squares indicate the expected frequencies.

DISCUSSION

Phylogeography and migration history of *M. itatsi* as an endemic species to the Japanese islands

The present study demonstrates the phylogeographical characteristics and population structure of *M. itatsi*, which is endemic to the Japanese islands, compared with those of *M. sibirica* from throughout Tsushima Island, Taiwan, the Korean Peninsula, and Russia, including Transbaikalia and the Ural Mountains. In addition to previous studies on morphological and genetic characteristics, the present study clearly shows that *M. itatsi* is a distinct species and phylogenetically different from *M. sibirica.* Generally, there are two opinions regarding the evolutionary and migration histories of *M. itatsi*. One is that *M. itatsi* evolved in the Asian continent from an ancestor common to *M. sibirica*, and the continental populations of *M. itatsi* became extinct after some had migrated to the Japanese islands over land bridges, and the Japanese populations have survived until now. The other is that one population of *M. sibirica* was isolated on the Japanese islands and evolved as *M. itatsi* within the islands. Kawamura *et al.*(1989) reported that bone remains of *M. sibirica* or *M. itatsi* were excavated from middle and late Pleistocene layers in Japan, although the species was not clearly identified. Masuda & Yoshida (1994b) calculated the divergence time between *M. sibirica* and Honshu *M. itatsi* to be about 1.6–1.7 Mya, using the molecular clock based on cytochrome *b*. In addition, Sato *et al*. (2003) reported that the divergence time between the two weasel species was 1.7 Mya using cytochrome *b* data and 2.4 Mya using *IRBP* data from many mustelid species. Using these divergence times and the nucleotide divergences obtained in the present study, the divergence rates of the mtDNA control region for the two species were calculated as 0.0190 or 0.0268 substitutions/site/million years. Marmi *et al*. (2006)

calculated a very similar divergence rate (0.0192 substitutions/site/million years) for the *Mustela* mtDNA control region, using the sequence data of *M. nivalis* and *M. erminea* reported by Kurose *et al*. (2005) and the fossil record in Europe; therefore, it is reasonable to use the divergence rates calculated in the present study, and the above divergence time between the two species. Although no fossil records of *M. itatsi* have been reported in the Asian continent, the above divergence times suggest that the ancestor of *M. itatsi* and *M. sibirica* evolved in the late Pliocene or early Pleistocene. Between then and the present time, the Japanese islands have been connected with the Asian continent many times. Speciation of *M. itatsi* on the continent is more plausible, because the Japanese islands were connected with the Korean Peninsula via land bridges until at least about 150 000 years ago (Ohshima, 1990, 1991, 2000). Of course, it cannot be neglected that *M. itatsi* might have remained in Japan since its speciation there. In that case, *M. sibirica* may have been unable to migrate to Japan owing to competitive exclusion by *M. itatsi*, which was already distributed in Japan.

In the present study, we identified two mtDNA lineages in *M. itatsi*: Honshu and Kyushu–Shikoku clades (Fig. 2). Molecular diversity was almost identical between the Honshu and Kyushu–Shikoku populations (Table 3). The divergence time between the two clades of *M. itatsi* was calculated to be 0.83–1.17 Mya in the present study (Table 4), and indicates that, during the Pleistocene, there were at least two waves of colonization of *M. itatsi* within the Japanese islands at different times and/or through different routes. A similar phylogeographical pattern of two mtDNA lineages has been reported for the Japanese sika deer *Cervus nippon*, which is endemic to the Japanese islands and eastern Asia: the northern and southern Japanese lineages diverged about 0.3 Mya and colonized the Japanese islands separately through southern (via the Korean Peninsula) and northern (via Sakhalin Island) routes from the Asian continent and/or at different times (Nagata *et al*., 1999). In addition, Iwasa & Abe (2006) examined the phylogeography based on cytochrome *b* of the Japanese water shrew (*Chimarrogale platycephala*), which is endemic to Honshu and Kyushu (probably extinct in Shikoku), and reported that the Kyushu lineage diverged at least 0.39 Mya from the Honshu lineage. Around the Japanese islands, *M. itatsi*, *Cervus nippon* and *Chimarrogale platycephala* could have experienced similar colonization routes in the Pleistocene. Moreover, the earlier divergence of the Kyushu lineage within each species is also a characteristic in the evolution of *M. itatsi*.

In Japan, the three main islands (Honshu, Kyushu and Shikoku) are separated by narrow and shallow straits. The Seto Inland Sea (see Fig. 1) finally separated Honshu, Shikoku and Kyushu Islands 7000–5000 years ago, and the separation was completed by the formation of the Kanmon Strait located between Honshu and Kyushu (Ohshima, 1990, 1991, 2000). By that time, ancient populations of *M. itatsi* from Kyushu could have migrated to Shikoku via land bridges. In the present study, mismatch distribution analysis, Tajima's D and Fu's F_s values and minimum spanning networks of haplotypes indicate an experience of recent expansion in the Honshu population of *M. itatsi*, but no clear evidence of recent expansion in the Kyushu–Shikoku population. These results suggest that the southern areas (around the Seto Inland Sea) in Japan were refugia for *M. itatsi* during the Pleistocene, and that the ancestors of the present Honshu lineage expanded finally throughout Honshu Island (located north of Kyushu and Shikoku Islands) as the climate warmed during the Pleistocene. This expansion pattern in Honshu is in congruence with the mtDNA phylogeography of other mustelids endemic to Japan, such as the Japanese marten *Martes melampus* (Kurose *et al*., 1999a), Japanese badger *Meles anakuma* (Kurose *et al*., 2001; Tashima *et al*., 2011) and other endemic mammalian species, such as the flying squirrel *Petaurista leucogenys* (Oshida, Masuda & Ikeda, 2009), Japanese macaque *Macaca fuscata* (Kawamoto *et al*., 2007) and *Chimarrogale platycephala* (Iwasa & Abe, 2006). In particular, the present study indicates that the phylogeographical pattern of the Kyushu–Shikoku population of *M. itatsi* was established slightly earlier than the Honshu population. Most of these endemic mammals currently inhabit broad-leaved forests in the temperate zone on the Japanese islands, except Hokkaido. On the Japanese islands, at the beginning of the Holocene (after the last glacial period: called the 'Jomon Period' in Japanese archaeological history), broad-leaved forests and mixed deciduous forests expanded northwards in Honshu according to the temperature of the environment; and boreal coniferous forests spread further north (Dobson, 1994). In addition, similar to the pattern seen in mtDNA of endemic Japanese mammals, the phylogeographical pattern of the Japanese beech *Fangus crenata* mtDNA shows that, in the past, expansion occurred to northern Japan from refugia in western Japan, including Kyushu and Shikoku, where higher divergence of mtDNA haplotypes was found (Koike *et al*., 1998; Tomaru *et al*., 1998). Mammalian fauna, including endemic species, could have followed the northward expansion of these broad-leaved and mixed deciduous forests.

However, the present study shows that the expansion of the Honshu population of *M. itatsi* occurred at 0.31–0.90 Mya (Table 5), and corresponded to the

middle Pleistocene, and that the expansion of the Kyushu–Shikoku population was slightly earlier (0.38–1.07 Mya: Table 5), but still corresponded to the middle Pleistocene. The results suggest that the colonization of *M. itatsi* on the Japanese islands started earlier than that of other mammalian species endemic to Japan, and that the expansion and retreat in distribution were repeated owing to climate changes during repeated glacial/interglacial periods, until the last glacial period. In addition, because there are many mountains on the Japanese islands, including elevations of more than 2000 m, the refugia could have been located not only in southern Japan but also at lower altitudes within Honshu. Such a complex biogeographical history within Honshu could have led to the phylogeographical pattern of *M. itatsi*.

The Kyushu–Shikoku clade comprises populations from Kyushu, Shikoku and the neighbouring small islands (Tanegashima and Yakushima), all of which are isolated by narrow and shallow straits (see Fig. 1). Haplotypes of *M. itatsi* from Tanegashima and Yakushima Islands are included in the Kyushu–Shikoku clade (Figs 2 and 3). Similar to this phylogeny, mtDNA control region haplotypes of *Macaca fuscata* from Yakushima Island have been reported to cluster with those from Kyushu and Shikoku (Kawamoto *et al*., 2007). The Osumi Strait, located between Kyushu and Yakushima and Tanegashima Islands, was formed between 100 000 and 150 000 years ago (Ohshima, 1990, 1991, 2000). Around Kyushu, land bridges formed over such straits several times during the Pleistocene, so that the small island populations are not so fully differentiated genetically from those of the main island of Kyushu. This could explain why genetic distances are not always in concordance with the geographical distances between sampling localities, and why the genetic structures of populations among these small islands in southern Japan, as well as between Kyushu and Shikoku, are complicated. In addition, in another closely related species, *M. nivalis*, in central Europe, mtDNA phylogeography has been reported to have been influenced by climatic changes in the last glacial period, resulting in an advantage of a lineage originating from one refugia and the formation of the suture zone in present-day Poland (Mcdevitt *et al*., 2012).

Phylogeography of native *M. sibirica* and founder effects in the introduced populations

Mustela sibirica is distributed widely in northern Eurasia, from the Ural Mountains in the west, through southern Siberia, to the Far East. In the present study, the cluster of *M. sibirica* was subdivided into four lineages with high bootstrap values: the Korean group including the introduced Japanese population, the Tsushima group, the Russian group and the Taiwanese group (Fig. 2). Molecular diversity (Table 3) indicated that the level of genetic differentiation within the *M. sibirica* clade is smaller than that within *M. itatsi*, although samples of *M. sibirica* were collected across the vastness of northern Eurasia (Table 2, Fig. 1). For instance, the direct geographical distance between the Ural Mountains (western limit of *M. sibirica* distribution) and Transbaikalia in Russia is about 3000 km, but the genetic distance between SB-08 (from the Ural Mountains) and SB-09 and SB-10 (from Transbaikalia) is similar to that within Korean haplotypes (Fig. 2). This suggests that northern continental populations in Russia could have occupied their habitats for a shorter time, possibly after the last glacial period. Southern regions of Eurasia, such as the Korean Peninsula, Tsushima and Taiwan, could have been refugia for *M. sibirica* during glacial periods. Kurose *et al*. (2005) examined the wide-ranging phylogeography of *M. nivalis*, which is distributed in northern Eurasia, and reported that populations in the southern periphery of its current distribution show specific (private) mtDNA lineages and that these regions could have been refugia in the last glacial period.

Until now, no intermediate forms or any evidence of hybridization between *M. sibirica* and *M. itatsi* have been found in Japan, even though Masuda & Yoshida (1994a) and Sato *et al*. (2003) reported the very close relationship between these two species among *Mustela* species. Interestingly, the present study clearly shows that mtDNA haplotypes of the introduced Japanese population are among the Korean group (Fig. 2). This indicates that the introduced Japanese population currently distributed in western Japan is genetically closely related to some Korean populations, which is in concordance with the historical record of their artificial introduction from Korea to Japan (Miyashita, 1963, 1976). The Tsushima population is positioned outside both the introduced Japanese and the native Korean populations (Fig. 2), indicating that the population on Tsushima Island was not involved in the artificial introduction of *M. sibirica* to the Japanese main islands, although Tsushima Island is located between the Korean Peninsula and the main Japanese islands. Moreover, only two haplotypes (SB-01 and SB-02) were identified from 15 animals in the introduced Japanese population, one (SB-01) of which was shared by 14 animals sampled from various localities in western Japan (Table 2, Figs 1 and 2). Such low genetic diversity indicates a founder effect resulting from the small founder population size of *M. sibirica* introduced from Korea. A similar low variation and distribution pattern of mtDNA haplotypes as a founder effect has

been reported in the Japanese population of the masked palm civet (*Paguma larvata*: Viverridae, Carnivora), which was artificially introduced at least from Taiwan and is currently expanding in Honshu and Shikoku in Japan (Masuda *et al*., 2008, 2010).

Based on the present study and previous records, we suggest the route of the artificial introduction and expansion of *M. sibirica* from the Korean Peninsula to western Japan. Since the report by Ando (1989), no information has been available on the expansion of the most eastern limit of the distribution of *M. sibirica* in Honshu. In addition, two haplotypes (SB-06 and SB-07) were identified from two individuals in Korea in the present study (Table 2). Further investigations of the phylogeography and diversity of native *M. sibirica* in the Korean Peninsula will provide further insights into our understanding of the origin of the introduced Japanese populations.

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